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Funcionalização e Modificação de Superfície de Nanomateriais para aplicações em Biomedicina e Biotecnologia

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I. Introdução

A presente tese de concurso de livre docência resume em 3 principais linhas as pesquisas realizadas pelo grupo do proponente. Cada capítulo apresenta uma breve introdução sobre os principais conceitos e os desafios para as pesquisas relacionadas. No final de cada capítulo as principais soluções e contribuições são apresentadas na forma de artigos científicos publicados e/ou aceitos recentemente para publicação.

O Capítulo I apresenta as contribuições nos estudos de nanomateriais magnéticos. Devido as suas propriedades magnéticas, nanopartículas de óxido de ferro são poderosas plataformas para aplicações em biomedicina ou em biotecnologia. Essas aplicações, basicamente residem na capacidade de interação dos momentos magnéticos dessas nanopartículas com um campo magnético externo. Assim, o uso de estímulo externo, campo magnético, confere estratégias interessantes para as mais diferentes soluções nas quais nanopartículas magnéticas podem atuar. Por serem magnéticas, podem ser guiadas pela ação de um campo magnético. Desta propriedade advêm várias aplicações em biomedicina como em sistemas de liberação controlada de fármacos ou no desenvolvimento de kits de separação de organelas, proteínas ou marcadores biológicos de interesse em testes de diagnóstico. Além disso, quando o campo magnético externo aplicado for alternado, a interação dos momentos magnéticos das nanopartículas com o campo alternado poderá gerar calor por diferentes mecanismos. Este calor provocado pelo estímulo externo pode ser usado para aquecer alvos específicos no corpo humano, provocando o tratamento por hipertermia magnética. Este aquecimento das nanopartículas na presença de um campo magnético alternado pode também servir como estímulo externo para transformações em polímeros termosensíveis presentes na superfície das NP, conforme será abordado no capítulo 2.

No capítulo 2 estão descritos os principais resultados das pesquisas desenvolvidas com materiais poliméricos. A linha de pesquisa consiste na síntese de copolímeros a partir de seus monômeros, sempre com vistas as aplicações em biomedicina. Os materiais poliméricos apresentam diversas características e propriedades que os tornam importantes para aplicações como em liberação controlada de fármaco, aonde atuam como plataforma carreadora. Esses polímeros podem liberar fármacos contidos inter-cadeias por diferentes mecanismos. Além disso, a possibilidade de construção de cadeias anfifílicas de copolímeros permite o carregamento de fármacos hidrofílicos ou hidrofóbicos. A liberação destes medicamentos pode ocorrer por hidrólise das cadeias poliméricas ou por modificações estruturais causadas por estímulos externos como aquecimento ou internos como variação de pH. Mais especificamente no caso de estímulo externo por aquecimento, a estratégia consiste em recobrir a superfície de nanopartículas magnéticas com polímeros termosensíveis contendo algum fármaco dissolvido em moléculas de água inter-cadeia. Assim, através da aplicação de um campo magnético alternado, o núcleo

magnético do nanodispositivo irá aquecer e consequentemente aquecerá a camada polimérica contendo o fármaco. Esse aquecimento provoca o colapso das estruturas poliméricas através de uma transição de fase hidrofílica-hidrofóbica, promovendo a liberação controlada do medicamento em questão. Para essas aplicações, dependendo do fármaco e do meio aonde ele deverá ser entregue há diferentes polímeros com possibilidade de sintonizar o intervalo de pH ou temperatura em que as transições ocorrerão através da síntese de copolímeros adequados.

No Capítulo 3 é abordado o uso de nanopartículas magnéticas como suportes para enzimas em diferentes aplicações biotecnológicas. Geralmente, as enzimas são um dos principais custos nos processos industriais biotecnológicos, além da infraestrutura demandada. Isso se deve ao fato de que essas enzimas são solúveis nos meios em que biocatalisam alguma transformação. Assim, nos processos de purificação e obtenção dos produtos desejados, geralmente essas enzimas acabam sendo perdidas. Existem várias soluções tecnológicas propostas para permitir a recuperação das enzimas e sua reutilização em novos ciclos de processo produtivo. Dentre as alternativas encontra-se a ligação dessas enzimas em suportes sólidos como esferas de argaroses que podem ser recuperadas ao final do ciclo através de processos de centrifugação. Outro método consiste em fazer ligações cruzadas entre as próprias enzimas de forma a obter um “novelo” de enzimas que pode ser recuperado por centrifugação, conhecidas pela sigla em inglês CLEA (*Crosslinking Enzyme Aggregates*). Uma estratégia proposta por alguns grupos de pesquisa consiste na elaboração de um CLEA magnético (MCLEA) através da obtenção de agregados de enzimas com nanopartículas através de ligações cruzadas. Esses MCLEAS podem ser recuperados do meio reacional através do uso de separação magnética, facilitando o processo de recuperação. Além disso, outra vantagem destes MCLEAs é o fato de permitirem que as enzimas mantenham suas atividades, e em algumas situações, passem a apresentar atividade ótima em pH e temperaturas diferentes daquelas das enzimas livres. Essas mudanças de parâmetros de síntese podem gerar economias para as indústrias quando os processos passam a acontecer em temperaturas e pH mais brandos.

II. Nanopartículas magnéticas: Síntese, funcionalização e modificação de superfície

II.1. Contribuições na síntese de nanopartículas magnéticas

II.1.1 Síntese sonoquímica

As características das NP magnéticas tais como forma, tamanho e distribuição de tamanho de partícula são fatores decisivos para o bom funcionamento nas diferentes nanotecnologias, assim alguns critérios para a síntese das partículas para aplicações *in vivo*, as NP devem possuir propriedades superparamagnéticas. Materiais superparamagnético são facilmente magnetizados, mas não apresentam magnetização remanente, ou seja, eles são livres de “memória magnética”. Quando um campo é aplicado nas partículas superparamagnéticas, elas se tornam magnetizadas e sofrem aglomeração devido a interação entre as partículas. Entretanto, quando o campo magnético (ímã) é retirado, a ausência de “memória magnética” auxilia na rápida redispersão das partículas, possibilitando a distribuição das NP na solução, bem como a liberação de espécies que não sejam o alvo e que podem ter sido capturadas fisicamente entre as partículas aglomeradas magneticamente.

Sonoquímica é uma área da química que estuda as reações químicas de moléculas e íons na presença de uma forte radiação ultrassom (20 kHz a 10 MHz) [1]. O fenômeno físico responsável pelo processo sonoquímico é a cavitação acústica. Este método foi inicialmente proposto na síntese de nanopartículas de ferro [2] e atualmente é utilizado para sintetizar diferentes materiais. Além de permitir o controle do tamanho de partículas do produto através da variação da concentração dos precursores [3], uma das vantagens deste método é a sua simplicidade e operacionalidade em condições ambientes.

Durante a cavitação, a quantidade de gás (ou vapor) que difunde para a cavidade depende da área de superfície da bolha. A cavidade cresce durante cada expansão [4] e será sempre maior do que no processo de compressão. Assim, vários ciclos acústicos podem levar o crescimento da bolha até um tamanho crítico onde a energia proveniente da irradiação ultrassônica pode ser eficientemente absorvida. Este tamanho crítico, também conhecido por tamanho ressonante, depende do líquido e da frequência do som; por exemplo, a 20 kHz este tamanho é da ordem de 170 μm . Neste ponto, a cavidade pode crescer rapidamente durante um único ciclo de som. A partir deste instante a cavidade não consegue mais absorver a energia ultrassônica eficientemente, e sem a absorção de energia a cavidade não consegue mais se auto sustentar. Neste ponto ocorre a implosão da cavidade, criando um ambiente único para as reações químicas.

Diferentes teorias foram desenvolvidas para explicar como uma radiação sônica de 20 kHz é capaz de quebrar ligações químicas. O mecanismo de calor localizado (*hot spot*) é uma das teorias mais aceitas e explica a quebra de ligações durante o colapso das bolhas. Altas temperaturas são obtidas durante o colapso das bolhas na cavitação [1]. Uma vez que este colapso ocorre em menos de 1 nanosegundo, altas taxas de resfriamento (10^{11} K s^{-1}) são também obtidas. Altas taxas de resfriamento e aquecimento impedem a organização e cristalização dos produtos. Assim, a rápida cinética de aquecimento e resfriamento não permite o crescimento de núcleos e

em cada bolha que colapsa poucos centros de nucleação são formados e o crescimento deles é limitado pelo tempo curto de duração do colapso.

Reações químicas de polimerização, oxidação, redução, dissolução e decomposição podem ser causadas por uma intensa onda de ultrassom produzindo cavitações em um líquido. Durante o processo sonoquímico em meio aquoso, três diferentes regiões são formadas: (1) uma região interna de colapso de bolhas (fase gasosa) onde elevadas temperatura (5000 °C) e pressão (2000 atm) são produzidos e causam a vaporização da água e sua pirolise em radicais $H\bullet$ e $OH\bullet$; (2) a região de interface líquida entre a cavitação de bolhas e o corpo da solução, a temperatura nesta região é menor que no interior das bolhas, porém ainda é alta o suficiente para permitir a decomposição térmica de solutos e (3) o corpo da solução onde em temperatura ambiente as reações entre as moléculas (ou íons) de precursores reagem com radicais $H\bullet$ ou $OH\bullet$. Porém, as reações sonoquímica acontecem dentro da região de interface [5].

II.1.2. Síntese por spray pirolise

O processo de síntese por spray pirólise se baseia na formação de aerossol. Uma fonte ultrassônica intensa próxima à superfície do líquido produzirá um "gêiser", resultando em uma formação de aerossol devido aos efeitos de cavitações na superfície do líquido. O diâmetro médio da gota pode ser calculado de acordo com a equação 1 de acordo com Lang [6], e dependerá da natureza do líquido (tensão e densidade da superfície) e da frequência do ultrassom gerado.

$$D_d = 0.34 \left(\frac{8\pi\gamma}{\rho f^2} \right)^{1/3} \quad \text{Equação 1}$$

onde D_d é o diâmetro da gota, γ é a tensão superficial da solução, ρ é a densidade da solução e f é a frequência ultrassônica.

O diâmetro médio das partículas formadas (D_p) pode ser calculado pela equação de Nedeljkovic [7], a seguir:

$$D_p = D_d \left(\frac{C_{pr} M_{prod}}{\rho_{prod} M_{pr}} \right)^{1/3} \quad \text{Equação 2}$$

onde D_p é o diâmetro médio das partículas obtidas, C_{pr} é a concentração do precursor, M_{prod} é a massa molecular do produto, M_{pr} é a massa molecular do precursor e ρ_{prod} é a densidade teórica do produto.

Uma solução de nitrato de ferro é aspergida usando um gerador ultrassônico operando em uma frequência de atomização de 2,4 MHz. As gotículas assim formadas são conduzidas por um gás de arraste (ar atmosférico) através de uma zona seca (100-200 ° C) e, em seguida, uma zona de aquecimento (400-700 ° C). Após o segundo forno, o produto é coletado por uma câmara eletrostática trabalhando a 10 kV. Um esquema do aparelho de pirólise por spray usado pelo proponente é apresentado na figura 1.

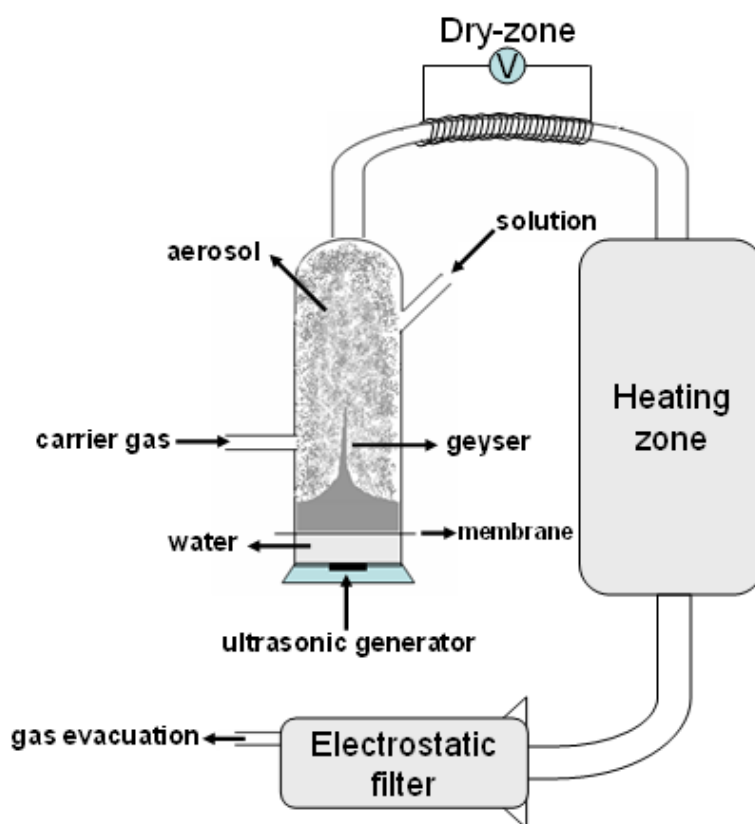


Figura 1: Esquema do equipamento de síntese por spray de pirólise.

II.1.3. Nanopartículas magnéticas obtidas por eletroprecipitação

Foi desenvolvido um método eletroquímico para a formação de nanopartículas de magnetita (Fe_3O_4). Dois cilindros de carbono, 15,0 cm de comprimento e 0,5 cm de diâmetro,

serviram como um par de eletrodos em uma cela eletroquímica de 100 mL. Durante o experimento, a solução foi mantida sob agitação magnética. A corrente elétrica foi fornecida por uma fonte de alimentação DC ISO-Tech, modelo IPS-1630D, operando em modo de tensão constante. Como precursor do ferro, utilizou-se 90 mL de solução de etanol 0,01 mol.L⁻¹ Fe(NO₃)₃.9H₂O e a área do eletrodo imersa foi igual a 11,77 cm². A densidade de corrente foi inicialmente ajustada para J = 4,75 mA/cm² e tensão, para V = 60V.

A eletrossíntese utilizada é baseada em uma reação eletroquímica que aumenta o pH nas proximidades do cátodo, produzindo íons hidroxilas resultantes da redução de nitrato e água [8-10]. À medida que o pH aumenta, o Fe³⁺ é precipitado na superfície do cátodo para formar Fe(OH)₃. Finalmente, o hidróxido de ferro (III) é reduzido a magnetita (Fe₃O₄). O H⁺ consumido no processo provém da oxidação do etanol no ânodo. O produto é coletado da superfície e lavado várias vezes com etanol. A redução do nitrato (NO₃⁻) foi confirmada pela formação de nitrito (NO₂⁻), verificada por um teste analítico qualitativo simples. O processo geral e as reações envolvidas estão esquematizados na figura 2.

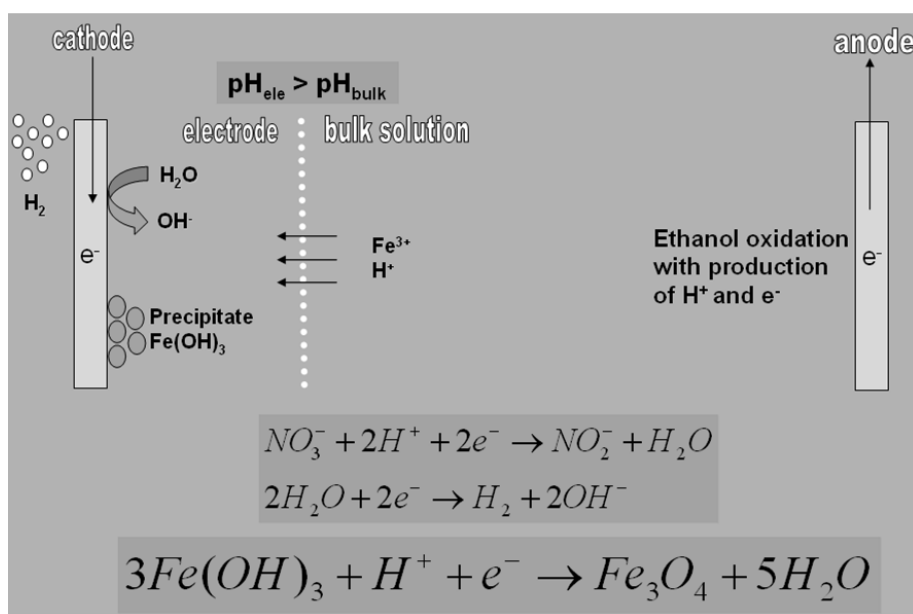


Figura 2: Processo geral e reações de formação de magnetita por eletroprecipitação.

Magnetic Nanoparticles Obtained by Homogeneous Coprecipitation Sonochemically Assisted

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The present work demonstrates preparation of magnetic nanoparticles by a novel method. Magnetic nanoparticles with potential for biomedical and environmental applications were obtained by homogeneous coprecipitation method sonochemically assisted. The effect of ultrasonic cavitation on changing the reaction environment was studied. The chemical reaction media used here was very similar to those used for Sono-Fenton process. The ultrasonic energy has driven the growth of particles; smaller diameter nanoparticles were obtained by applying a higher power. For the first time, it was demonstrated the iron oxide nanoparticles synthesis at pH lower than 6.

Keywords: *magnetic nanoparticles, homogeneous coprecipitation, sonochemistry*

1. Introduction

Nowadays nanotechnology is very important for the advancement of science, since it makes use of the manipulation of matter on a scale in which materials show different characteristics than those displayed in the micro and macro scale¹. These properties changes are attributed to the large increase in surface area in relation to the volume². In this context, magnetic nanomaterials, such as iron oxide, particularly magnetite (Fe₃O₄) have been applied in various fields such as drug carriers and contrast agents in magnetic resonance imaging³.

For this application, certain parameters must be controlled during the synthesis, such as the size and shape of the nanoparticles⁴. The control of the size, as well as size distribution, is necessary because allows the control of the material's properties, such as superparamagnetism and hyperthermia⁵.

Depending on its size, iron oxides particles present different behaviors when an external magnetic field is applied. It is known that abrupt changes in magnetic properties occur when the particle size is reduced of micrometer scale to nanometer. In nanoscale phenomena of finite size and surface effects start to dominate the magnetic behavior of individual nanoparticles⁶. Frenkel & Dorfman³ were the first to suggest that particles of ferromagnetic material below a critical particle size (less than 15 nm for common materials) would consist of magnetic monodomains, presenting a uniform magnetization state at any field.

The magnetic behavior of these particles above a certain temperature, the blocking temperature (T_B), is the same of the paramagnetic particles, except that a large magnetic moment and, consequently, susceptibility are presents. For biomedical applications, nanoparticles that exhibit superparamagnetic behavior at body temperature (T_B under the human's body temperature) are the most studied because of the absence of magnetic remanence and present a fast change in the magnetic state in the presence of an external magnetic field⁷.

Concerning the particles shape, ellipsoid-shaped nanoparticles (elongated) are more cytotoxic than those with spherical shape. The production of several inflammatory cytokines by human monocytes can be induced when ellipsoid-like nanoparticles are present inside the body. Moreover, the spherical nanoparticles are also more appropriate for drug transport and delivery into specific targets than other forms, such as hexagonal and cubic^{7,8}.

There are different methods for iron oxide nanoparticle syntheses described in the literature such as microemulsion; sol gel process and co-precipitation. Darbandi et al.⁹ described a method to synthesize iron oxide nanoparticles from a water/oil microemulsion, at room temperature. In this synthesis, the surfactant stabilizes the system and form a layer around the final nanoparticles. Roth et al.¹⁰ have studied the synthesis of nanoparticles by precipitation of iron salt precursors in alkaline medium, without using any surfactant agent as stabilizer. However, these methods do not allow a strict control of particle size. In this context, synthesis have been sonochemically assisted, since using ultrasound it is

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possible to change the reaction medium, allowing to achieve higher energy and pressure values in a lower time interval (acoustic cavitation)^{11,12}. Another wet chemical route is the homogenous coprecipitation by using urea decomposition, at 80°C, which results in a gradual rise in pH, keeping the alkaline medium and thus allowing co-precipitation at higher temperatures¹³.

The aims of this work were to prepare magnetic iron oxide nanoparticles by homogeneous coprecipitation method sonochemically assisted and to evaluate the influence of ultrasound power during synthesis. The main scientific contribution of this work was to obtain iron oxide nanoparticles at pH around 6.

2. Material and Methods

The synthesis by homogeneous coprecipitation was performed by mixing a 0.01 mol.L⁻¹ ferrous chloride precursor solution with 2,0 mol.L⁻¹ urea, using different ultrasound amplitudes in which sample. Three samples were made: C_50, with 50% of amplitude; C_60, with 60% of amplitude; and C_70, with 70% of amplitude.

During the synthesis, the reaction medium was heated and stirred at 80°C, which correspond to the urea decomposition temperature. When temperature reached 80°C, the ultrasonic power was instantly turned on and the nucleation process has started, as observed by the changes in the color of the solution (Figure 1). After one hour, the ultrasound was turn off and the precipitated was washed several times with distilled water. All samples were dried in ovens and analyzed by X-ray Powder Diffraction (XRD) and Scanning Electron Microscopy (SEM) in order to evaluate its crystalline phases and morphology, respectively.

3. Results and Discussions

Figure 2 present the graph of pH changes in functions of the temperature for samples C_70, C_60 and C_50. As it can be observed, as temperatures increase the pH decrease until a inflection point at 67°C, 72°C and 75°C, for samples C_70, C_60 and C_50, respectively; after that the pH sharply increased up to 6.2. When water is irradiated with ionizing radiation, the initial radical species generated are hydrated electrons, hydrogen atoms and hydroxyl radicals, and the initial molecular species formed are hydrogen and hydrogen peroxide¹³. The H₂O₂ formed will cause the decrease in pH and its concentration will depends on the ultrasound power. As observed in figure 1, the higher the amplitude, shorter the

pH before curve inflection. It can also be observed that for short amplitude the pH start increasing near 80°C, where the urea decompositions take place. As it will be discussed latter, the short ultrasound amplitude allows the syntheses of pure magnetite. The pH curve presents an abrupt decrease from 80°C to 82°C, corresponding to the Magnetite precipitation.

Figure 3 presents the X-ray powder diffraction for samples. As observed from de Bragg Peak Position of the standard PDF file, pure magnetite phase was formed when using ferrous chloride in the urea homogenous precipitation assisted with lower energy ultrasound, suggesting different reaction mechanisms with the counterions and free radicals in solution produced by the cavitation effect. Using XRD data and Scherrer's equation the crystallite sizes was calculated for samples obtained from ferrous chloride precipitation (Table 1).

The difference in the crystallite size may be occurring due to a possible growth of goethite at surface of oxidized magnetite or as isolated goethite particles, as it will be seen later in the scanning electron microscopy figures.

Fe²⁺ + The chemical reaction media used here to synthesizes iron oxide magnetic nanoparticles, has propitiated an environment very similar to those used for Sono-Fenton process¹⁴. Fenton process, consist of a mixture of ferrous ion and hydrogen peroxide generating hydroxyl radicals (OH•) at room temperature as described by Walling et al.¹⁵. When Fenton reagents, Fe²⁺/H₂O₂, are coupled with ultrasound, active hydroxyl radicals will be generated¹⁶ followings the sonochemistry reactions described below, which will to produce the intermediate complex (Fe-O₂H²⁺):

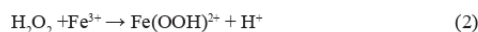


Table 1. Results of the Scherrer's Equation.

Samples	B (°)	2θ _B (°)	D (nm)
C_50	0.27	35.59	36.171
C_60	0.26	35.29	37.422
C_70	0.28	35.62	34.89



Figure 1. Changes in the solution color during ultrasonic cavitation: nucleation and homogeneous coprecipitation.

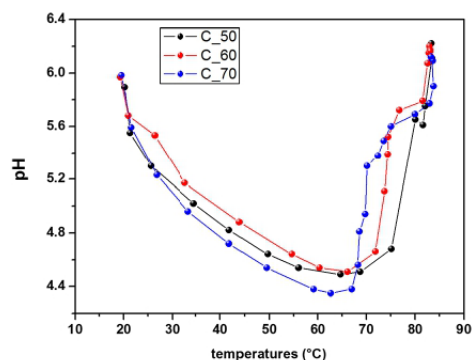
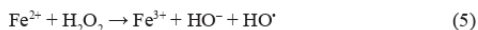
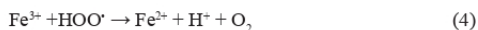


Figure 2. pH variation as a function of the temperature for samples C_50, C_60 and C_70.

In Fenton process, the rate of reaction between H_2O_2 and Fe^{2+} increases with an increase in temperature leading to formation of more number of hydroxyl radicals, which in turn results in enhanced degradation efficiency¹⁷. However, for higher amplitude ultrasound at $\text{pH} > 4$, the formation of Fe (II) complexes reduces the generation of free radicals and also regeneration of ferrous ion will get inhibited by the precipitation of ferric oxyhydroxides¹⁸. Since iron ions can oxidize in the presence of oxygen, it may have formed goethite around the magnetite (creating a nucleus of magnetite involved in a goethite layer), which helps the generation of larger nanoparticles than it was expected. It can be considered that the reaction medium became more oxidant in the presence of the species produced in sonolysis, causing the formation of goethite.

Figure 4 presents the SEM morphology images analysis of the samples C_50 (a, d); C_60 (b, e) and C_70 (c, f). As it can be observed, sample C_50 (Figure 4d) present rounded-like shaped particles with lower aggregation than those particles obtained using higher ultrasound amplitude (Figure 4e, f). Samples obtained using higher amplitudes, as observed in XRD, present goethite and magnetite phases. The elongated particles observed in samples C_60 and C_70 are characteristic of goethite.

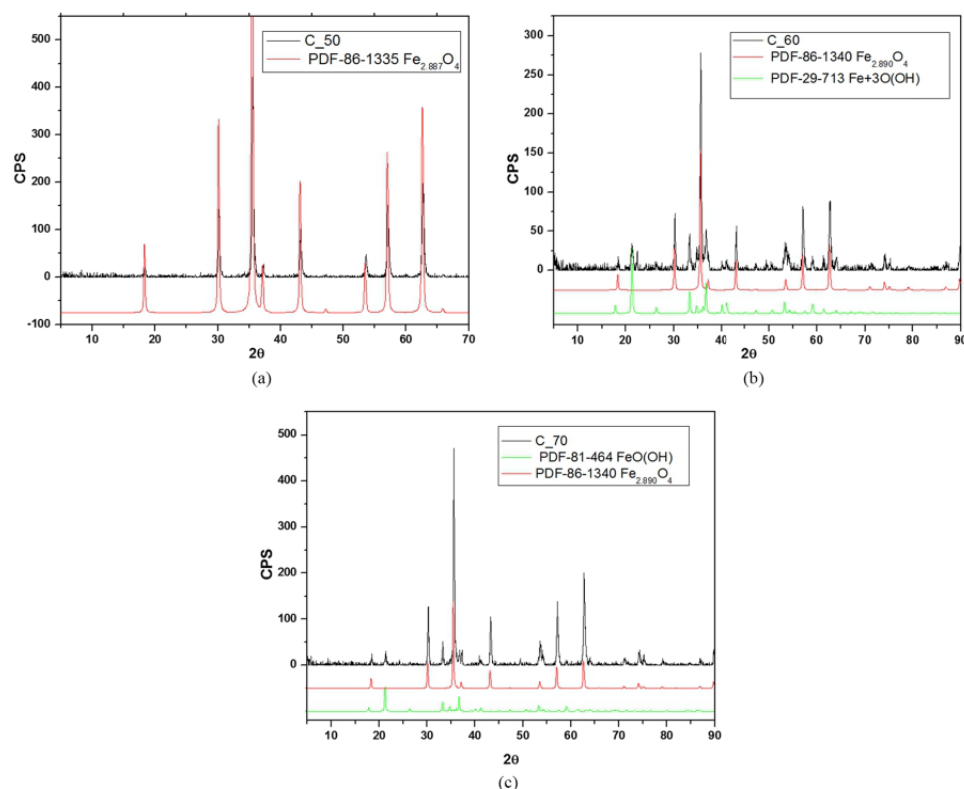


Figure 3. XRD pattern of the samples: (a) C_50; (b) C_60 and (c) C_70.

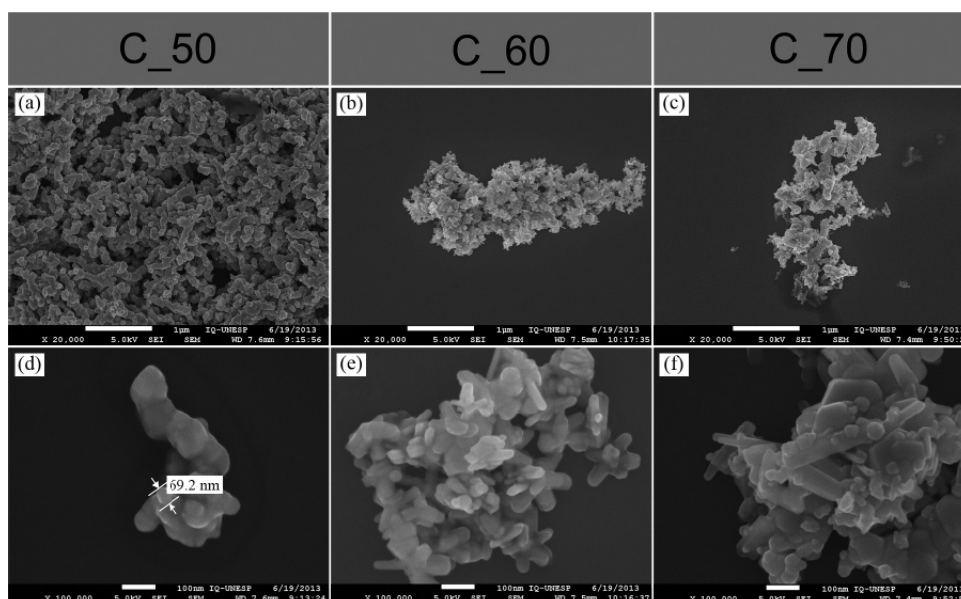


Figure 4. SEM images of the samples: C_50 (a, d); C_60 (b, e) and C_70 (c, f). Scale Bars: (a), (b) and (c) is 1 µm; (d), (e) and (f) is 100 nm.

4. Conclusions

Iron oxide nanoparticles were obtained by sonochemically assisted homogeneous coprecipitation. Samples obtained from iron chloride(II) solutions showed a magnetite phase with crystallite size inversely proportional to the percentage of amplitude applied. The higher the energy dissipated in the reaction medium higher is the number of nuclei formed, leading to formation of precipitates with a smaller particle size. This behavior suggests that the ultrasonic energy accelerates the reactions. It is expected that this acceleration can interrupt the growth of particles, thus creating smaller diameter nanoparticles by applying a higher

power. Sono-Fenton process can be addressed to explain the pH variation during the reaction, creating a chemical environment for nanoparticle synthesis. Nevertheless, the sharply rise in pH due to homogenous coprecipitation through urea decomposition at ~80°C, allow its magnetite synthesis at pH ~6,2. Usually, magnetic iron oxides are formed at pH higher than 9.

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Nanocomposites Materials Generated from a Spray.

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The Spray Pyrolysis (SP) process has been employed at moderate temperature (470-970 K) for the synthesis of micro- and nano-particles of hydrated metal oxides and of related nanocomposites. It has been applied to iron nitrate solutions, without or with the addition of sodium chloride as a flux. After removal of the flux, nanocomposites of ferrihydrite ($5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$) / hematite ($\alpha\text{-Fe}_2\text{O}_3$) / and an amorphous Fe_2O_3 are obtained, with surface area $140 \text{ m}^2/\text{g}$. It has been applied also to a sol of Aluminum-tri-sec-butoxide, without or with activation by europium or terbium ions. Boehmite ($\gamma\text{-AlOOH}$) powders are synthesized at 470 K, with surface area equal to $180 \text{ m}^2/\text{g}$. Transition alumina ($\gamma\text{-Al}_2\text{O}_3$) are synthesized at 970 K. Nanocomposites made by reaction of the boehmite or $\gamma\text{-Al}_2\text{O}_3$ particles with the amino acid asparagin (ASN) have been prepared and characterized. The luminescence of the Eu^{3+} or Tb^{3+} doped nanocomposites has been investigated: they can be considered as bio-compatible luminescent nanoparticles.

Keywords: Spray-pyrolysis, Nano-powders, Ferrihydrite, Hematite, Boehmite, Alumina.

I. INTRODUCTION

Spray Pyrolysis (SP) is an aerosol process commonly used to form a wide variety of materials in powder form including metals, metal oxides, ceramics, superconductors, etc ... One of the major advantages of SP is the flexibility: pyrolysis may be conducted at high temperatures (1270 K or more) so that high temperature phases may be achieved in one step. Alternatively, the whole process may also be conducted at lower temperature: it may then be described as a rapid drying of the spray. In the past years, we intended to take benefit of this flexibility in order to synthesize sub-micrometric or nanometric particles in a wide range of chemical compositions and considering a wide range of possible applications [1–6]. Sub-micronic phosphor powders for plasma display applications were synthesized and post-annealed [1, 2], nanometric powders of zincite ZnO for cosmetic applications were synthesized by SP of mixtures of zinc and lithium (or sodium) nitrates [3]; sub-micronic particles of boehmite (AlOOH) were obtained by spray-drying of an aluminium alcoolate [4], these particles spontaneously dispersed in water to form stable suspensions of boehmite nanoparticles that could be doped with photo luminescent lanthanide ions, then surface-modified to become bio-compatible [5, 6]. The purpose of the present paper is to show how SP, conducted at moderate temperature, may be used for the synthesis of highly divided, highly reactive metal (here Fe or Al) hydroxides and oxides powders.

Iron oxyhydroxides and oxides are of technological importance as catalytic materials, sorbents, pigments, flocculents, coatings, gas sensor and ion exchanger [7]. Among them, ferrihydrite is a natural material present in many near surface environments [8, 9]. Due to its high surface area and reactivity, ferrihydrite plays an important role in the sequestration of contaminants from groundwater. Adsorption on ferrihydrite is commonly used to arsenate sequestration from soils,

groundwater and surface water system [10]. Ferrihydrite is the first phase to precipitate during hydrolysis of Fe^{3+} ions in solution. Its rough formula is $5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$. Its exact formula is still being debated, as is controversial also its exact crystal structure (see for instance [11, 12]). This is not really surprising since the synthesis conditions have a great impact on the average crystal size and on the reactivity towards water of these nanocrystalline materials. Among the huge literature dealing with structural characterizations, we selected the multiple structural phases model described by Jansen et al. [13] and revisited by Y. Cudennec et al. [14]. Ferrihydrite is metastable and generally considered as goethite $\alpha\text{-FeO(OH)}$ or hematite $\alpha\text{-Fe}_2\text{O}_3$ precursor, depending on pH and/or temperature. Hematite is the end-product of thermal decomposition of ferrihydrite in oxidative atmosphere [15]. Hematite has been demonstrated as a potential catalyst for many environmental application including oxidative removal of carbon monoxide or methane combustion. Currently in the market, NANOCAT superfine Fe_2O_3 nanoparticles (supplied by Mach-I Inc.) are composed of a mixture of amorphous and crystalline structures. The higher catalytic activity of NANOCAT than others commercially available Fe_2O_3 (hematite) is ascribed to its amorphous constituents.

The aluminium oxyhydroxide boehmite $\gamma\text{-AlOOH}$ is the most important precursor of the transition aluminas. Transition aluminas are widely used in the industry of adsorbents and catalysts [16, 17]. $\gamma\text{-AlOOH}$ is traditionally prepared by i) solid state decomposition of gibbsite, ii) precipitation from acidic or basic aluminium aqueous solutions, iii) sol gel procedures from aluminium alcoholates. The third method allows the preparation of very pure boehmite, although from more expensive chemicals. A very common procedure was first described in [18, 19]. It consists of aluminium alkoxide hydrolysis followed by peptization to a clear sol, the gel formation, and then the gel drying to get hydrated boehmite. Further pyrolysis at higher temperature gives porous alumina. The control of the drying conditions is necessary to control the properties of the boehmite powder. The names pseudoboehmite or nano crystalline boehmite are used in con-

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trast to microcrystalline or well crystallized boehmite. The nanocrystalline boehmites exhibit crystallite size less than 10 nm, defects and numerous adsorbed water molecules: these features strongly depend on the preparation conditions [20]. The so-called transition aluminas, refer to the group of partially dehydrated aluminium hydroxides other than anhydrous α -alumina (corundum). The diffuse characteristic of the powder patterns reflects a high degree of structural disorder in the transition aluminas. The transition aluminas are widely used in industry as adsorbents, catalysts or catalyst carriers, coatings and soft abrasives because of their fine particle size, high surface area and the catalytic activities of their surfaces. γ - Al_2O_3 is the first transition alumina obtained by thermal decomposition of the boehmite [21, 22].

One aim of this work is to investigate the possibility to obtain by SP α - Fe_2O_3 /ferrihydrite spherical and mesoporous-like structures. Consequently, we propose an alternative material to commercially available NANOCAT product. Nanocomposites are generated from a precursor solution containing $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ and NaCl. Herein are investigated the influence of NaCl in the iron nitrate precursor solution and of other operating conditions on the nature, the structure, the morphology and the crystallinity of the powders. Another aim is to synthesize by SP boehmite and transition aluminas as highly divided nanometric powders. The nanometric and/or sub-micronic hydrated aluminas are highly reactive, especially towards amino acids (AA). The nanocomposite hybrids made of boehmite or alumina with the asparagin (ASN) have been studied and characterized as described herein. One possible application of these nanoparticles is luminescent bio-labelling after their activation by an emissive lanthanide ion (Eu^{3+} , Tb^{3+}). Data concerning their optical spectroscopy are described and discussed.

II. EXPERIMENTAL

Samples are prepared by the spray pyrolysis process which is based on an aerosol formation. An intense ultrasonic source close to the liquid surface will produce a geyser, resulting in an aerosol formation due to cavitations effects on the liquid surface. The aerosol, formed by the precursor droplets in an air stream, is conducted through a dry zone at 370-1390 K, then a decomposition-densification zone which can be maintained in the range 570-1470 K. Collection of the dried powders is achieved by a home made electrostatic collector. The ultrasonic generator operates at 2.4 MHz. The adjustable operating parameters are the flow rate (by which the residence time in the furnace is controlled), the temperature in decomposition zone T_{syn} , and of course the nature of the precursor solution. Spheres composed by α - Fe_2O_3 /ferrihydrite nanoparticles are obtained through spray pyrolysis of a precursor solution containing $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ and NaCl as a flux. The idea is to obtain spheres constituted of two phases: the iron oxide phase and a water soluble phase (NaCl). The as-prepared composite spheres are washed several times in water to remove NaCl and the iron oxide is recovered, as schematized in Figure 1. T_{syn} is fixed at 660 or 820 K. For the alumina-based compositions, the precursor solution is prepared following the methodology established by B. Yoldas [18, 19]. A stable sol of Aluminum-tri-sec-butoxide is diluted

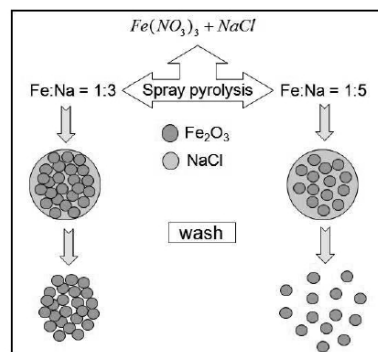


FIG. 1: schematic representation of iron oxide nanoparticles synthesized by SP using a soluble flux (NaCl).

in water to concentration 0.2 mol/L of Al^{3+} , and poured into the vessel over the piezoelectric pellet. T_{syn} is fixed at 470 or 970 K. Activation by optically active ions (Eu^{3+} or Tb^{3+}) is achieved by addition of 2% ([Eu] or [Tb])/[Al] to the distilled water before addition of the aluminum-tri-sec-butoxide. For all SP syntheses, the air flow is 0.3 m^3/h , the residence time in the drying zone is 3-4 seconds and about 6-8 sec in the decomposition-densification zone. Amino-Acids - aluminosilicates nanocomposites have been obtained by dispersing the aluminium oxyhydroxide or oxide particle from SP in asparagines water solutions, under agitation for 12 hours. Adsorption/desorption isotherms have been performed by using a Belsorp-mini (BEL Japan Inc. apparatus), between 0 and 99 P/P₀ of nitrogen and at 77 K after a pre-treatment at 353 K under vacuum during 2 hours. Thermal analyses have been performed in a Labsys TG/DTA (SETARAM) analyser in an alumina crucible at 5 K/min, under oxygen. The size, the morphology and the crystalline structure of the particles have been studied by transmission electron microscopy (TEM) on a Philips CM12 microscope. The crystalline structures of samples have been investigated by X ray powder diffraction (Seifert XRD 3000 diffractometer) and the quantitative phases analysis obtained by Rietveld refinements [23] using the program GSAS [24]. In order to obtain an internal standard without microstrain and with a high average crystallite size, the 99.99 % cerium oxide is fired at 1570 K for 48 h. Room temperature luminescence excitation and emission spectra have been measured by a Hitachi F1100 spectrofluorimeter at 2.5 nm spectral resolution.

III. RESULTS AND DISCUSSION

A. Iron oxyhydroxides and oxides.

Sample FE1 has been prepared without NaCl in the precursor solution. As expected in spray pyrolysis synthesis under the selected operating conditions, the particles are spherical and their diameters fall in the range 100 to 2000 nm. One TEM image of sample FE1 is displayed in figure 2a. All

sample	[Fe]	[Na]	Fe:Na	T _{syn}	XRD
FE1	0.15	—	—	820	HEM
FE2	1.0	—	—	820	HEM
FE3	1.5	—	—	820	HEM
FENA1	0.15	0.45	1:3	820	HEM+FEH+NaCl
FENA2	0.15	0.45	1:3	660	HEM+FEH+NaCl+NaNO ₃
FENA3	0.10	0.50	1:5	820	HEM+FEH+NaCl

TABLE I: experimental synthesis conditions for iron-based samples: [Fe] and [Na] in mol.L⁻¹, T_{syn} in K, XRD: crystalline phases identified (HEM= α -Fe₂O₃, FEH=ferrhydrite)

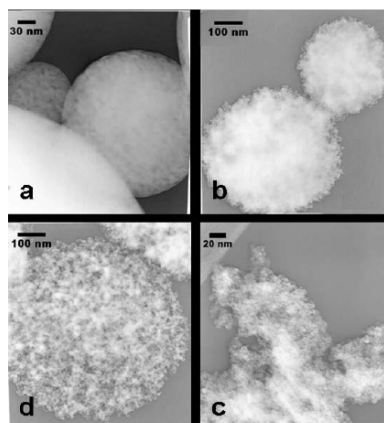


FIG. 2: TEM images of samples FE1(a); FeNa2(b); FENA2-washed (c), and FENA1-washed (d)

XRD peaks observed for this sample may be indexed with hematite (α -Fe₂O₃ - PDF no 89-2810), as shown in figure 3. The XRD patterns of as obtained samples FENA 1-3 display well defined narrow peaks superposed on broad components. Hematite is detected, together with NaCl for the three samples and with NaNO₃ in sample FENA2 synthesized at 660 K. This results from the reaction of nitrate species freed by the iron precursor with sodium ions. Nitrate species are evolved when the synthesis is conducted at higher temperature (820 K). These observations prove the complexity of the SP synthesis during which occur in a limited range of time the solute precipitation, the solvent evaporation and chemical reactions between the constituents in each droplet [25]. After water washing, XRD patterns of soluble phases NaCl and NaNO₃ are not observed, the diffraction diagrams exhibit the peaks of α -Fe₂O₃ and broad features assigned to hydrated species (ferrhydrites) as discussed below.

The particles of samples FENA1-3 are spherical and sub-micrometric. The spheres observed are nanocomposites made of the iron salts and the sodium salts (Figure 2b). For sample FENA3, obtained with the Fe:Na ratio 1:5, some NaCl cubic-like aggregates are also observed. These cubic aggregates are no more observed after washing the sample in water. After wash, highly porous aggregates, retaining the spherical shape of the nanocomposite particles are observed by TEM for FENA1 (Figure 2d), but hardly seen for FENA2,

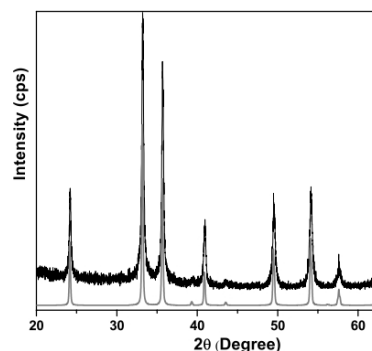


FIG. 3: XRD analysis. Sample FE1 and calculated diagram for hematite α -Fe₂O₃.

washed (Figure 2c), same for FENA3 washed (not shown). These observations suggest first that at higher T_{syn}, (820 for SPNA1 versus 660 K for SPNA2), the individual nanoparticles start to coalesce during the spray pyrolysis, and the formation of necks prevents desagglomeration of the spheres during washing. Another important parameter is the Fe:Na ratio in the precursor: despite of being obtained at T_{syn} = 820 K, aggregates of nanoparticles in FENA3 after wash are no more spherical, suggesting that the high NaCl concentration has not allowed enough contact among iron-based nanoparticles so that necks could not be formed prior to dissolution of the NaCl flux. Therefore, by carefully adjusting Fe:Na ratio and furnace temperature, sub-micronic spheres of iron oxides nanoparticles can be obtained through spray pyrolysis. The N₂ adsorption-desorption isotherms of samples FENA1 (washed) have been measured: it comes that the surface area is 140 ± 15 m²/g. Compared to sample FE1, the XRD pattern recorded for sample FENA1 after wash exhibits significantly broadened peaks which can be assigned to poorly crystalline ferrhydrite, superimposed to the narrower diffraction peaks of hematite. Hematite is the end-product of thermal decomposition of the complex ferrhydrite. At same T_{syn} (820 K), the reaction is then less completed following the flux-assisted SP process than following the more conventional one phase SP. A more precise and quantitative description of the phases present in the SPNA1, after wash, has been achieved. A precisely weighted standard (CeO₂ at 11.4 % w/w) has been added to the sample in order to normalize the different fractions calculated by Rietveld analysis routine. In this model (from references [13, 14]), the structure of ferrhydrite is considered as being a superposition of two components: a defect-free phase of trigonal space group (P-31c) and a defective phase with a trigonal P3 space group. Rietveld analysis results are presented in Table II and in Figure 4: besides 27 weight % of hematite and 32 weight % of ferrhydrite, it is concluded that 41 weight % of the sample are amorphous. Taking the general formula 5Fe₂O₃·9H₂O for the ferrhydrite, it comes from the Rietveld quantitative analysis that 5.4 weight % of the sample FENA1(washed) is H₂O. Experimentally, the total weight loss of the sample measured by

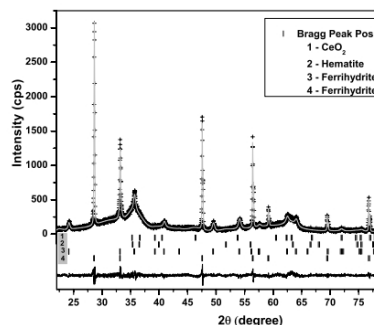


FIG. 4: XRD analysis. Sample FENA1 washed + internal standard CeO_2 ; Rietveld simulation.

thermal analysis between 300 and 920 K is 7 ± 0.14 weight %, which is consistent with the previous estimation. In turn, it proves that the composition of the amorphous phase is anhydrous Fe_2O_3 .

phase	amorphous	HEM	FEH(P3)	FEH (P-31c)
weight%	41	27	11	21
H_2O weight%	—	—	1.85	3.55

TABLE II: sample FENA1 (washed): Refined phases weight% HEM = α - Fe_2O_3 , FEH=ferritohydrate

B. Aluminium oxyhydroxides and oxides.

The synthesis method selected to elaborate by SP the aluminium based samples is in fact the fast drying of a colloidal solution of boehmite, in order to stop the sol-gel process prior to the long range ordering of the solid phase. The elaboration conditions, the crystal phases identified and some typical properties of the samples are gathered in table III. As for the previous case, the SP synthesis produces NPs agglomerated in sub-micronic spheres. The measured surface area is high: $180 \pm 15 \text{ m}^2/\text{g}$ (sample AL200). The sample elaborated at the lower temperature (AL200) exhibits the XRD pattern of nanocrystalline boehmite γ - AlOOH (Figure 5). It may be spontaneously dispersed in water. In ref.[5] we have shown that the rapid drying (a few seconds) of the sol stops the development of the aluminium hydroxide network at the level of a few crystal cells. Half the hydroxyl groups are in fact at the external surface of the crystallites. This resulted in a very large number of surface hydroxyls free for water adsorption, actually the chemical composition of SP nano crystallized boehmite is $\text{AlOOH} \cdot (1.18 \pm 0.24) \text{H}_2\text{O}$. The very hydrophilic nature of the surface of SP-boehmite crystallites is at the origin of their ability to form stable suspensions in water. When the synthesis is performed at higher temperature (AL700), the XRD peaks are still broader and fit well with what is reported in literature as the transition alumina γ - Al_2O_3 . AL700 powder precipitates in water. Nanocomposites obtained by reac-

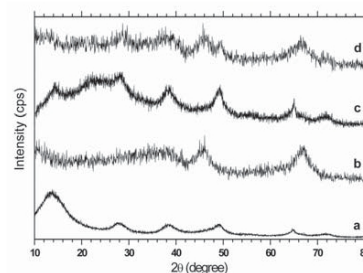


FIG. 5: XRD patterns of samples AL200 (a), AL700 (b), AL200-ASN (c), AL700-ASN (d).

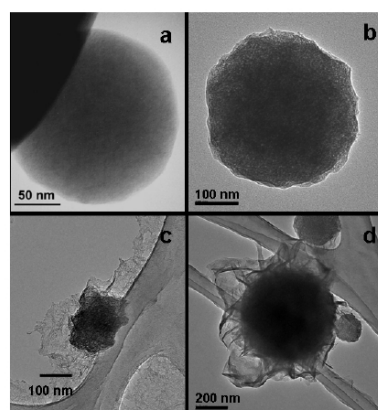


FIG. 6: TEM images of samples AL200 (a), AL700 (b), AL200-ASN (c), AL700-ASN (d).

tion with ASN behave the same way with respect to dispersion. The interactions between the ASN and the particles surfaces are imaged in Figure 6. The AL200 (boehmite) spheres have a smooth surface (Figure 6a), whereas the AL700 (γ - Al_2O_3) are less regular, and denser (Figure 6b). After reaction with ASN at molar ratio $\text{ASN}:\text{Al} = 0.3:1$ and drying, most of the boehmite spheres have disaggregated and the sample looks like a film in which are dispersed nanoparticles. Aluminium is identified by local analysis (EDXS in TEM) for all parts of the film, and the XRD is that of boehmite. The TEM image selected here (Figure 6c) shows the composite film and one mineral bigger particle not completely destroyed. For the transition aluminas AL700-ASN (Figure 6d), the mixing of the ASN and of the mineral part is less intimate: one observes an organic film wrapping the sub micronic γ - Al_2O_3 particles.

The peculiar emission of Eu^{3+} in AL200:Eu (atomic ratio $\text{Eu}:\text{Al} = 0.02:1$) is very well preserved in the composite AL200:Eu-ASN ($\text{Eu}:\text{Al} = 0.02:1$, $\text{ASN}:\text{Al} = 0.3:1$) displayed in Figure 7a. In the same way, the emission spectra of Eu^{3+} in AL700:Eu and in AL700:Eu-ASN samples are very similar (the last one displayed in Figure 7b). One recognizes easily

sample	XRD	T _{syn}	morphology	water dispersion
AL200	BOEH	470	spheres, sub- μ m	stable
AL700	TA	970	spheres, sub- μ m	precipitated
AL200-ASN	BOEH	—	organic film + BOEH NPs	stable
AL700-ASN	TA	—	spheres + film at surface	precipitated

TABLE III: Aluminium-based samples : synthesis temperature T_{syn} (K), crystalline phases BOEH= γ -AlOOH, TA= γ -Al₂O₃, morphology and stability in water.

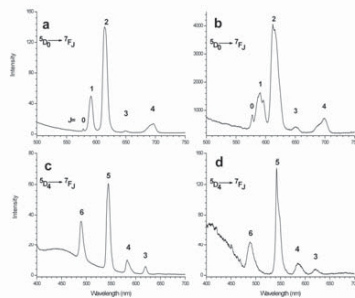


FIG. 7: Photoluminescence emission spectra recorded at room T, spectral resolution 2.5nm. Excitation at 394nm, samples AL200:Eu-ASN (a) and AL700:Eu-ASN (b); excitation at 250nm, samples AL200:Tb-ASN(c) and AL700:Tb-ASN (d)

the well known $^5D_0 \rightarrow ^7F_{0-4}$ emissions in the visible wavelength range for both samples, the changes observed in the details of the spectra exemplify the possibility to make use of Eu^{3+} as a local structural probe. Eu^{3+} in γ -Al₂O₃ exhibits larger splitting than in γ -AlOOH for all the transitions (particularly visible for $^5D_0 \rightarrow ^7F_1$), which traduces that the average crystal field acting on Eu^{3+} is higher in the case of γ -Al₂O₃. The Eu^{3+} excitation spectra (not shown here) exhibit the intra-4f⁶ narrow excitation lines and a broad absorption to the Eu—O charge transfer state (CTS). In γ -Al₂O₃, as it is observed also in most of the oxide matrices, excitation in CTS is more powerful than intra-4f⁶ excitation, but it is remarkably inefficient in γ -AlOOH. It is not clear if the band shifts at lower wavenumbers for the boehmite samples or if absorption to the CTS is simply less intense, in any case this effect traduces that the oxygen to metal interaction is weaker in doped boehmite than it is in doped γ -Al₂O₃. The third point is that the $^5D_0 \rightarrow ^7F_{0-4}$ emissions are in-homogeneously broadened in transition alumina, much more than in boehmite, showing that the Eu^{3+} ions are in numerous different environments in the highly porous γ -Al₂O₃.

These spectroscopic characteristics have been interpreted in [4, 5] as follows: partly hydrated europium ions are di-

rectly bonded to OH groups at the boehmite surface, and are well maintained at the NPs surface in the nanocomposites. In the transition alumina, Eu^{3+} ions enter (at least partly) the structure and are linked via Al—O—Eu bonds. The interaction with ASN does not modify the spectra. Terbium (Tb^{3+}) is also well known to be a good photoluminescent emitter in the green range (540 nm). We have synthesized and investigated the analogous nanocomposites with this ion: AL200:Tb-ASN ($Tb:Al = 0.02:1$, $ASN:Al = 0.3:1$) and AL700:Tb-ASN. The corresponding emission spectra are recorded in Figure 7 c, and d, respectively. There is no difference between the two spectra because Tb^{3+} is not really a good structural probe at the low spectral resolution employed here. Due to the similarities of chemical properties along the lanthanides series, it seems reasonable to assume that Tb^{3+} ions behave the same way that Eu^{3+} ions as for their interaction with the aluminium oxide matrix and with the ASN. Further work is in progress because in fact it appears that the physico-chemical interactions in the ternary systems aluminate (boehmite or transition alumina) - lanthanide - ASN are not exactly the same for the two lanthanides Eu and Tb. Nevertheless it may be noticed that luminescent nanocomposites emitting in the green range and stable in water may be obtained from SP synthesis.

IV. CONCLUSION

We have shown how Spray Pyrolysis conducted at moderate temperature (from 470 to 970 K in the decomposition-densification area) may be employed for the synthesis of highly divided, highly reactive metal (here Fe or Al) hydroxides and oxides powders. Two different synthesis strategies have been explored. In the case of iron-based oxides, a soluble flux (NaCl) has been added to the precursor ($Fe(NO_3)_3$) in water solution. Under controlled synthesis conditions, after removal of the flux, nanocomposites of ferrihydrite ($5Fe_2O_3 \cdot 9H_2O$) / hematite α -Fe₂O₃ / and an amorphous Fe₂O₃ are obtained, with surface area 140 m²/g. In the case of aluminium-based oxides, an Al-alcoholate precursor has been preferred, in order to get only the boehmite γ -AlOOH (at the lower temperature) or the transition alumina γ -Al₂O₃. Boehmite powders are obtained with surface area equal to 140 m²/g that can be easily dispersed in water. Nanocomposites made by reaction of the boehmite or γ -Al₂O₃ particles with the amino acid asparagin (ASN) have been investigated. These nanocomposites have been doped with a lanthanide ion (Eu^{3+} or Tb^{3+}), and their luminescence investigated since they can be considered as bio-compatible luminescent nanoparticles.

Acknowledgments

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Mössbauer spectroscopy study of iron oxide nanoparticles obtained by spray pyrolysis

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Abstract Mössbauer spectroscopy was used in this study to investigate magnetite nanoparticles, obtained by spray pyrolysis and thermal treatment under H₂ reduction atmosphere. Room temperature XRD data indicate the formation of magnetite phase and a second phase (metallic iron) which amount increases as the time of reduction under H₂ is increased. While room temperature Mössbauer data confirm the formation of the cubic phase of magnetite and the occurrence of metallic iron phase, the more complex features of 77 K-Mössbauer spectra suggest the occurrence of electronic localization favored by the different crystalline phase of magnetite at low temperatures which transition to the lower symmetry structure should occur at T ~120 K (Verwey transition).

Keywords Magnetite · Hematite · Spray pyrolysis · Mössbauer spectroscopy

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1 Introduction

As the interest in magnetic nanoparticle (NP) for biomedical applications has been dramatically increased lately, their precise characterizations in terms of magnetic properties, crystal structure, particle size, and bio-compatibility are being urgently required to support the technical specifications [1–3]. Among the biomedical applications of magnetic NP *in vitro* diagnostic by magnetic separation and selection appear to be an important research field. The process of isolation and separation of specific molecules are used in almost all areas of biosciences and biotechnology. Magnetic NP can also be used in immunoassays for detection of viruses or assays of various hormones [4, 5]. This concept involves a heterogeneous suspension of functionalized magnetic spheres with the blood to be analyzed. Surface modified magnetic NPs will bind to the desired target (cells, nucleic acid, proteins or other biomolecules), taking into account the nature of the surface coating species [6]. Then, the resulting target–NP complex can be removed out from the suspension using a magnet.

In the present study Mössbauer spectroscopy was used to characterize spherical magnetite particles. In order to obtain magnetic spheres for cell separation applications, hematite samples obtained by spray pyrolysis were reduced under H₂ atmosphere to form magnetite particles (Fe₃O₄).

2 Experimental

Samples were prepared by the spray pyrolysis process which is based on an aerosol formation. An intense ultrasonic source close to the liquid surface will produce a “geyser”, resulting in an aerosol formation due to cavitations effects on the liquid surface. The aerosol formed is dried at 110°C and then decomposed by a second furnace operating at high temperature. For hematite preparation, an iron nitrate solution was sprayed out using an ultrasonic generator operating in an atomizing frequency of 2.4 MHz. The so-formed droplets were conducted by a carrier gas (atmospheric air) through a dry-zone (100–200°C) and then, to a heating-zone (400–700°C). After the furnace, the product was collected by an electrostatic chamber working at 10 kV. The as-obtained hematite (sample SP) was reduced under H₂ atmosphere to form magnetite (Fe₃O₄). Samples were thermally treated under H₂ at 390°C/1 h (sample 1H) and 390°C/6 h (sample 2H).

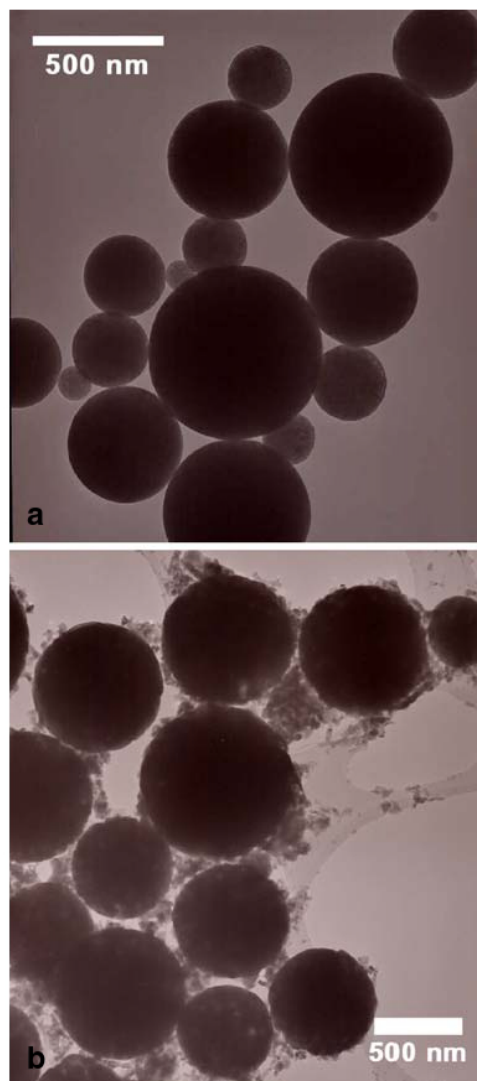
The morphology and size of obtained nanoparticles were characterized by a Philips CM12 transmission electron microscope (TEM) operating at 120 kV. The crystalline structures of products were investigated by X-ray diffraction (XRD) using a commercial system (Seifert XRD 3000 TT diffractometer).

Transmission Mössbauer spectra were recorded at 77 K and room temperature using a conventional spectrometer with a γ -radiation source of 50 mCi ⁵⁷Co/Rh. The spectra were least square fitted using crystalline sites or magnetic distributions.

3 Results and discussion

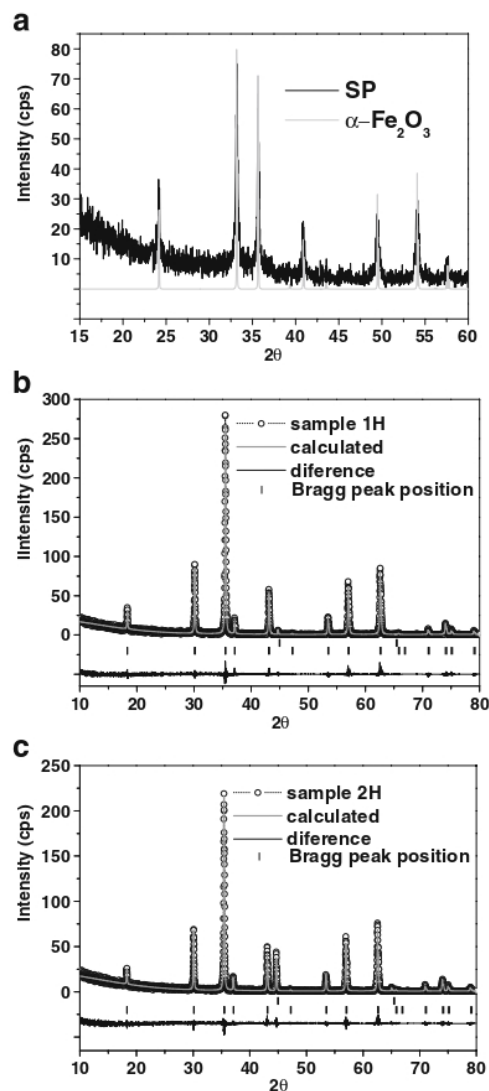
The TEM images of spherical hematite (α -Fe₂O₃) prepared by spray pyrolysis (sample SP) are displayed in Fig. 1a. Dense spherical particles were formed, though

Fig. 1 TEM images of: **a** hematite ($\alpha\text{-Fe}_2\text{O}_3$) prepared by spray pyrolysis (sample SP), **b** magnetite obtained through reduction of sample SP under H_2 at $390^\circ\text{C}/1\text{ h}$ (sample 1H) and **c** under H_2 at $390^\circ\text{C}/6\text{ h}$ (sample 2H)



revealing a wide particle size distribution. As far as the particle size profile is concerned size dispersity is still a problem to overcome in powder synthesis by the spray pyrolysis technique. The TEM images of sample 1H obtained through reduction of hematite (sample SP) under H_2 at 390°C for 1 h is displayed in Fig. 1b. The shape and size of spherical particles has not changed after the reduction chemical reaction. However some nanoparticles appear to become loose from the spheres. This behavior can be ascribed to the cell volume variation due to phase transformation from hematite to magnetite and also metallic iron. The volume cell of hematite is 1.89 times smaller than that of magnetite and 12.41 times bigger than that of metallic iron. Thus, one concludes that the volume variation during phase transformation of

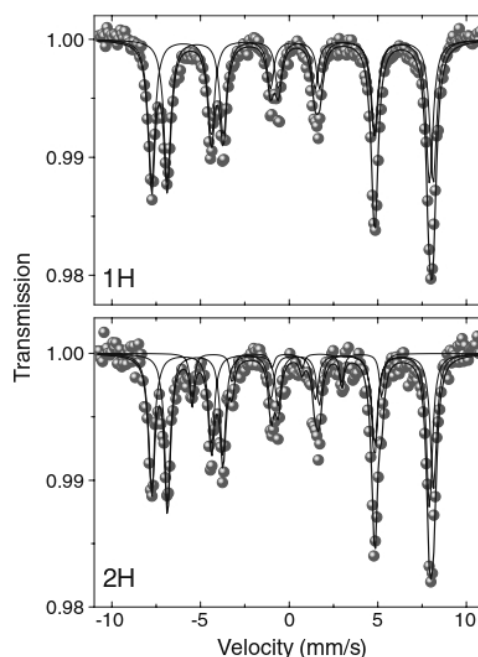
Fig. 2 X-ray patterns of samples 1H and 2H. Observed (—○—) and calculated (—) curves together Bragg peak positions of contributing phases (vertical bars) and the difference between observed and calculated patterns (bottom curve) are shown



crystallites from hematite to magnetite and also metallic iron will cause a sudden volume change and, therefore, some nanoparticles will be loosed from the sphere.

Figure 2a shows the XRD patterns of spherical hematite prepared by spray pyrolysis (sample SP) and the hematite ($\alpha\text{-Fe}_2\text{O}_3$) standard XRD patterns. As observed, single iron oxide hematite phase was obtained. Figure 2b and c present the XRD patterns and their Rietveld refinements for samples 1H and 2H, respectively. As will be confirmed by Mössbauer measurements, the magnetite phase formation was confirmed in samples 1H and 2H, though an additional phase identified as metallic iron (Fe) was also found after reduction. As the thermal treatment time increases so will the amount of metallic Fe. In order to quantify the metallic iron

Fig. 3 Room-temperature Mössbauer spectra of 1H and 2H samples fitted with two and three magnetic sextets (solid lines), respectively



content in our samples the magnetite and metallic iron (strongest diffraction peaks at $2\theta \sim 45^\circ; 65^\circ$) phases were considered in the quantitative phase analyses calculated by the Hill and Madsen formalism implemented in the GSAS program [5]. Results indicate that sample 1H presents 99.5% of magnetite and 0.5% of metallic iron. On the other hand, sample 2H is composed by 91.8% of magnetite and 8.2% of metallic iron. This finding indicates that care must be taken during the reduction process in order to avoid the metallic iron formation, which is not interesting for biomedical applications due to metallic iron toxicity.

Figures 3 and 4 show typical Mössbauer (MS) spectra recorded at 297 and 77 K, respectively. The room-temperature spectra for both samples were well fitted considering six-line patterns (magnetic subspectra). No evidence of superparamagnetic relaxation was determined at room temperature for both samples, which is in agreement with the large size of the particles determined from XRD analysis (above 100 nm). Values of the hyperfine parameters obtained at room temperature are collected in Table 1. As observed in Fig. 3 the spectrum for sample 1H is well resolved by two six-line patterns. Values of hyperfine parameters of both samples (Table 1) are consistent with the two crystallographic sites of iron ions in the cubic spinel ($Fd3m$) structure of magnetite [7]. The site with the hyperfine field $B_{\text{hf}} \sim 49$ T was assigned to iron ions occupying the tetrahedral site (A) whereas the site with $B_{\text{hf}} \sim 46$ T was associated to the octahedral site (B). While small values of quadrupole splitting (QS) for both sites suggest negligible structural distortions affecting both sites, different isomer shifts (IS) for both sites suggest the presence of mainly Fe^{3+} in site A and both Fe^{3+} and Fe^{2+} in site B, as it is commonly expected. However, the spectral area ratio is found to be $\sim 1:1$ for both samples, which is quite different

Fig. 4 77-K Mössbauer spectra of 1H and 2H samples fitted with three and four magnetic sextets (solid lines), respectively

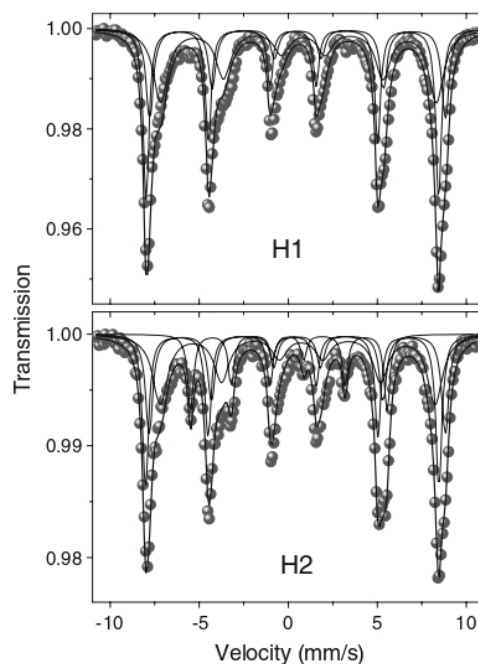


Table 1 Hyperfine parameters obtained from the fit of the Mössbauer spectra carried out at room temperature and 77 K

<i>T</i> (K)	Sample		Γ (mm/s)	IS (mm/s)	QS (mm/s)	B_{hf} (T)	<i>S</i> (%)
297	1H	A	0.46(1)	0.305(3)	−0.020(7)	49.2(1)	50(1)
		B	0.47(1)	0.654(3)	−0.038(5)	45.9(1)	50(1)
	2H	A	0.43(2)	0.320(4)	−0.030(5)	49.3(1)	44(2)
		B	0.40(1)	0.646(4)	−0.015(10)	45.8(1)	46(2)
		Fe- α	0.30	−0.011(7)	−0.001(10)	33.1(1)	10(1)
77	1H	Fe ³⁺ (A)	0.39(1)	0.362(2)	−0.037(2)	50.9(1)	36(1)
		Fe ³⁺ (B)	0.51(2)	0.639(5)	−0.014(6)	51.5(1)	24(2)
		Fe ²⁺ (B)	1.02(2)	0.820(8)	−0.266(13)	48.0(1)	40(2)
	2H	Fe ³⁺ (A)	0.43(2)	0.349(5)	−0.049(6)	51.0(1)	30(3)
		Fe ³⁺ (B)	0.50(2)	0.600(9)	0.012(9)	51.5(1)	22(3)
		Fe ²⁺ (B)	1.06(3)	0.777(12)	−0.185(17)	48.2(1)	35(2)
		Fe- α	0.40	0.123(2)	0.040(7)	34.2(1)	13(1)

The isomer shift values are relative to the iron metal absorber. Error bars are indicated in parentheses

from the expected ratio (2:1 for free-defect crystals). This finding can be explained by arguing that the recoil-free fractions are different for both sites. Mössbauer reports on bulk magnetite indicate almost similar recoil-free fractions for both sites (A and B) [8]. Assuming similar recoil-free fractions for both subspectra in our samples the almost similar iron population in both sites, determined from the spectral area, strongly suggests the occurrence of cation vacancies mainly in the B site. In sample

2H (sample obtained at longer H₂-reduction atmosphere), besides the magnetite sextets, an additional sextet was determined. This extra pattern, not resolved in sample 1H, was associated to metallic iron due to its hyperfine parameters (see Table 1). The amount of this extra phase (~10%) is in good agreement with the XRD data analysis.

At 77 K the MS spectrum of sample 1H is well fitted using three sextets, whereas one extra sextet (four sextets) is required to get a good fit for the sample 2H, as shown in Fig. 4. That extra sextet corresponds to the metallic iron in agreement with the results obtained at room temperature. The hyperfine parameters, linewidths and spectral areas as listed in Table 1. The component with the largest isomer shift ($\text{IS} \sim 0.8 \text{ mm/s}$) and $B_{\text{hf}} \sim 48 \text{ T}$ was assigned to Fe^{2+} located in the octahedral (B) site, whereas the components with $B_{\text{hf}} \sim 51 \text{ T}$ and $\sim 52 \text{ T}$ were assigned to Fe^{3+} located in the tetrahedral (A) and octahedral (B) sites, respectively. This assignment is in agreement with previous reports [7]. The distinctive feature observed at 77 K when compared to the room-temperature patterns must be associated to the change of lattice symmetry from cubic to triclinic phase, which is expected to happen at $T_V \sim 125 \text{ K}$ (the Verwey transition) in bulk magnetite system. Low-field magnetization data carried out varying the temperature in these samples (not shown here) show a clear anomaly at $T \sim 120 \text{ K}$, confirming the occurrence of the Verwey transition. The three magnetic components associated with the magnetite in the low temperature phase reflects the occurrence of electronic localization in B sites due to the absence of the electron hopping process which governs the transport and magnetic properties of magnetite in its high temperature phase [9].

In summary, spherical magnetite (Fe_3O_4) particles of relatively large size (above 100 nm) were successfully obtained from hematite ($\alpha\text{-Fe}_2\text{O}_3$) particles by reduction in H₂-atmosphere. Room-temperature XRD data indicate the formation of magnetite phase with spinel structure and the formation of metallic iron which amount increases as the time of treatment in H₂-reduction atmosphere is increased. Room-temperature Mössbauer data confirm the formation of magnetite phase and the occurrence of metallic iron for the sample 2H. The almost equal population of iron ions in both sites (A and B) suggests the occurrence of cation vacancies, mainly in site B. The more complex features of MS spectra observed at 77 K is associated to the electronic localization favored by the different crystalline phase of magnetite stabilized at temperatures below $T_V \sim 120 \text{ K}$.

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Electro-precipitation of Fe₃O₄ nanoparticles in ethanol

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ABSTRACT

The preparation of superparamagnetic magnetite (Fe₃O₄) nanoparticles by electro-precipitation in ethanol is proposed. Particle average size can be set from 4.4 to 9 nm with a standard deviation around 20%. Combination of wide-angle X-ray scattering (WAXS), Electron energy loss spectroscopy (EELS) and Mössbauer spectroscopy characterizations clearly identifies the particles as magnetite single-crystals (Fe₃O₄).

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1. Introduction

The prospect of a new generation of materials and devices based on nanoparticles (NPs) is a major driving force in the rapidly emerging field of nanoscale research. Magnetic NPs, and more particularly magnetite (Fe₃O₄) and maghemite (γ-Fe₂O₃), have been widely used for biomedical applications such as cell targeting, cell separation, drug delivery, hyperthermia [1,2], or in environmental sciences, for metal separation from wastewater [3]. Due to their magnetic moment, magnetic NPs can be driven by an applied magnetic field into specific regions of the human body for *in vivo* applications. For *in vitro* diagnosis or for metal separation, magnetic separation and selection can be done. For these applications, magnetic NPs have to become magnetized at low magnetic field. However, in order to avoid any agglomeration phenomenon, the magnetic NPs must not present magnetic remanence, i.e. must have a zero magnetization in the absence of an applied magnetic field. This particular behavior is achieved with superparamagnetic NPs. Typically, magnetite NPs become superparamagnetic at sizes below ~15 nm.

Chemical synthesis of colloidal magnetite has been known for a long time: aqueous mixture of ferric and ferrous salts is mixed with an alkali in order to induce the precipitation of magnetite

particles (maghemite can then be obtained by soft oxidation of magnetite) [4]. The average diameter of particles can be tuned between 5–100 nm by varying experimental conditions (concentration, temperature, nature of alkali, ionic strength, agitation, etc.) but the system is always polydispersed in size [5,6] due to the Oswald ripening mechanism (the large particles will grow at the cost of the small ones) [7]. Organized assemblies or complex structures have been used as nanoreactors (microemulsion, vesicle, polymer matrix media synthesis) in order to obtain nearly monodispersed ultrafine iron oxide NPs. Readers interested by these methods can have a look on the excellent review written recently by Gupta and Gupta [8].

In practice, even if better control can actually be done over the size and the size distribution of NPs, progress in the use of superparamagnetic NPs depends on the improvement of synthetic methods. In this paper we propose a completely new process based on electro-precipitation of magnetite in ethanol media, which yields NPs with a controlled distribution avoiding the use of nanoreactors.

2. Experimental part

This electrochemical method used for magnetite (Fe₃O₄) NPs precipitation needs two carbon cylinders (5.0 cm long and 0.5 cm of diameter) which are used as a pair of electrodes in an electrochemical bath of 100 ml. During the experiment, the solution was stirred using a magnetic bar. Electrical current was

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Table 1
Synthesis conditions, and average size of Fe₃O₄ nanoparticles

Sample	Fe(NO ₃) ₃ ·9H ₂ O (mol/l)	Current density (mA/cm ²)	Synth. time (h)	Average size (nm)	Standard deviation (nm/%)
S1	10⁻²	5	12	4.4	0.7/16
S2	2 × 10⁻²	30	12	6.15	1.15/18
S4	4 × 10⁻²	80	12	8.1	1.44/18
S8	8 × 10⁻²	110	12	8.5	1.5/18
S8b	8 × 10 ⁻²	30	12	7.3	1.9/26
S8c	8 × 10 ⁻²	110	2	8.9	1.9/21
S16	1.6 × 10⁻¹	220	12	?	> 100%

Bold characters highlight the parameters modified between successive syntheses.

provided by an ISO-Tech Laboratory DC Power Supply model IPS-1630D. A 90 ml of 0.01–0.16 mol/l Fe(NO₃)₃·9H₂O ethanol solutions were used as iron precursor and the electrode area immersed was equal to 4 cm² (for each electrode). During the procedure the voltage between electrodes was kept constant at 62 V whereas the current could fluctuate slightly (≈ 10%) especially during the first hour. Current densities reported in Table 1 are values noted after 1 h of electrolysis. Magnetite particles where collected on the cathode as black magnetic macroscopic platelets and washed with ethanol before to be dried under air at room temperature.

The size, the morphology and the crystalline structure of the particles were studied by transmission electron microscopy (TEM) on a Philips CM12 microscope at 120 kV (0.3 nm resolution). Particle size was determined by measuring at least 200 NPs taken on five different photos. Electron energy loss spectroscopy (EELS) chemical analysis was performed on a 200 kV Tecnai microscope using a GIF tridiem post-column energy filter.

Mössbauer measurements were obtained on a constant-acceleration conventional spectrometer with a 25 mCi source of ⁵⁷C (Rh matrix). The absorber was a powdered sample enclosed in a 20 mm diameter cylindrical, plastic sample-holder. Variable temperature spectra were obtained in the 4.4–300 K range, by using a MD 306 Oxford cryostat, the thermal scanning being monitored by an ITC4 Oxford servocontrol device (0.1 K accuracy).

The magnetic ac susceptibility was recorded at increasing temperatures and selected frequencies in the 1–852 Hz range, after an initial cooling from RT down to 4.5 K in the absence of the field (ZFC procedure); on a superconducting quantum interference device (SQUID) magnetometer model MPMS2.

Wide angle X-ray scattering (WAXS) has been commonly applied by several authors [9] and may be one of the most powerful techniques to study the medium range order (0–4 nm) in nanometric materials. The experimental reduced radial distribution function (RDF), which reveals the distribution of the interatomic distances, was calculated similarly as given in another paper [9a]. Theoretical models were built up from the crystallographic growth of one bulk unit cell, with eventual parameters adjustment. The theoretical reduced RDF was calculated for these structural models as reported elsewhere [9a].

3. Results and discussion

Synthesis conditions and average size of Fe₃O₄ NPs are regrouped in Table 1 whereas Fig. 1 shows the TEM images.

For low Fe(NO₃)₃·9H₂O concentration (< 4 × 10⁻² mol/l) the solution remains clear during the electrolysis and magnetite particles can be collected on the cathode or from the bath as black magnetic macroscopic platelets. For higher iron (and also water content) concentration the solution becomes rapidly turbid during the electrolysis (probably due to a co-precipitation of an

amorphous iron hydroxide) and pure black magnetite sample can be collected only on the cathode. For S16 sample (0.16 mol/l of Fe(NO₃)₃·9H₂O), even on the cathode, the sample shows a black-brownish color indicating that magnetite is polluted. By the same way, TEM picture shows several big spherical magnetite particles (> 10 nm) embedded inside an undefined amorphous matrix. Similarly, an addition of 1 ml of water in the S1 bath causes the same effect: pure magnetic particles are not formed. Consequently, we can suggest that for collecting pure magnetite particles we need a relatively water-free bath. However, the role played by water is not only negative. Indeed, as we can see on Table 1, iron nitrate increase, and consequently water increase, allowed to reach a high current density.

The electro-precipitation process leading to Fe₃O₄ NPs is not yet totally understood. However, we can suggest that the local increase of the pH at the vicinity of cathode causes Fe(OH)₃ precipitation. The pH increase probably comes from hydroxide ions production resulting from the nitrate and water reduction: NO₃⁻ + H₂O + 2e⁻ → NO₂⁻ + 2OH⁻ and 2H₂O + 2e⁻ → H₂ + 2OH⁻ [10,11]. The reduction of nitrate (NO₃⁻) is confirmed by nitrite (NO₂⁻) formation, verified by a simple qualitative analytical test (colorimetric test) whereas hydrogen production is clearly observed on the cathode. Finally, the iron (III) hydroxide is reduced to magnetite (Fe₃O₄) following the global reaction: 3Fe(OH)₃ + 2OH⁻ + e⁻ → Fe₃O₄ + 4H₂O. Note that this last reaction regenerates the water consumed in nitrate and water reduction. The H⁺ consumed in the process should come from the ethanol oxidation at the anode (CH₃CH₂OH + $\frac{1}{2}$ O₂ → 2H⁺ + 2e⁻ + CH₃COOH). Fig. 2 summarizes this general scheme of the magnetite electro-precipitation process.

The analysis of Table 1 shows that particle size can be adjusted from 4.4 nm to almost 9 nm, by increasing the Fe(NO₃)₃·9H₂O concentration from 10⁻² to 8 × 10⁻² mol/l and the current density from 5 to 110 mA/cm². Probably higher concentration and current density leads to larger particles, however, those particles are contaminated rendering size measurements impossible (Fig. 1(f)).

Because iron, water content, and current density are correlated parameters, we have synthesized sample S8b where only the current density decreases compared to S8 sample. This experiment indicates that a smaller current density leads to slightly smaller particles. On the contrary, electrolysis time (S8c sample) does not have a significant effect.

X-ray diffraction is the method of reference for the structural study of crystalline materials. However, as the size of crystallites decreases, diffraction peaks get broader, making structure interpretation in the reciprocal space ambiguous. WAXS analysis, i.e. recording of the complete scattering pattern followed by Fourier transform, allows retrieval of the remaining structural information and its representation in the simpler real space as a RDF. Fig. 3 shows the RDF obtained from S1 Fe₃O₄ NPs. It very well matches the RDF computed from the spinel structure. However, since this spinel structure occurs in both magnetite and

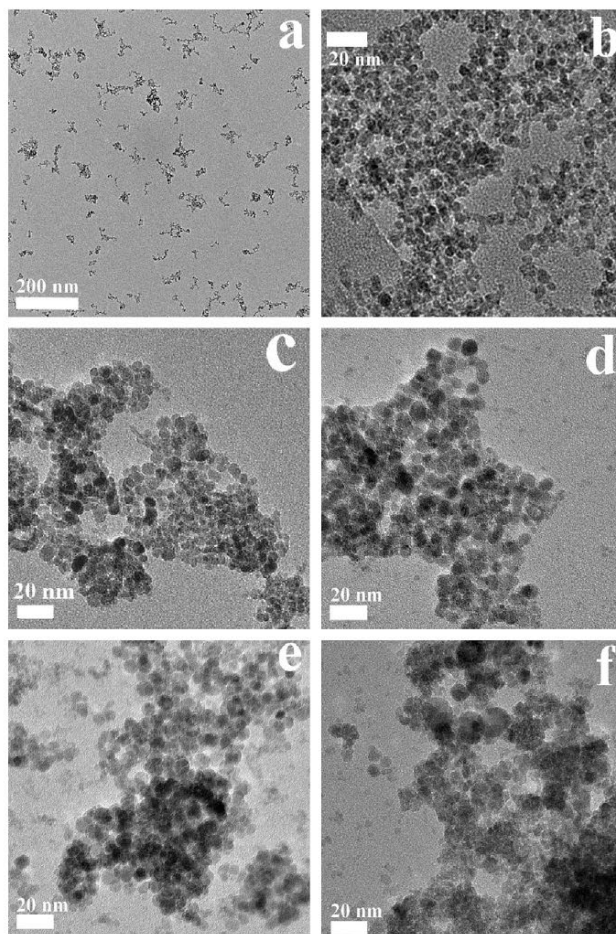


Fig. 1. TEM images for: (a) and (b) S1 sample ($\phi = 4.4$ nm); (c) S2 ($\phi = 6.15$ nm); (d) S4 ($\phi = 8.1$ nm); (e) S8 ($\phi = 8.5$ nm); (f) S16 ($\phi =$ unknown). S1: $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O} = 10^{-2}$ mol/l and 5 mA/cm^2 ; S2 = 2×10^{-2} mol/l and 30 mA/cm^2 ; S4 = 4×10^{-2} mol/l and 80 mA/cm^2 ; S8 = 8×10^{-2} mol/l and 110 mA/cm^2 ; S16 = 16×10^{-2} mol/l and 220 mA/cm^2 .

maghemite, WAXS alone cannot completely assert the real nature of the particles. We also observe that the NPs are mostly monocrystalline, since the complete damping of the peaks in the RDF occurs beyond 4 nm, thus in a good agreement with the size of the particles measured by TEM.

Mössbauer spectroscopy shown on Fig. 4 reveals that S1 NPs are magnetite and not maghemite. Indeed, the magnetic spectrum recorded at 5 K (in the ferrimagnetic state) clearly shows two sextets (sextet 1: 53%; sextet 2: 47%). The first sextet (493 kOe) is related to Fe^{2+} and Fe^{3+} ions in octahedral sites whereas the second one (513 kOe) is only due to Fe^{3+} ions in the tetrahedral sites of the inverse spinel structure of magnetite. Because $\gamma\text{-Fe}_2\text{O}_3$ contains only Fe^{3+} ions, Mössbauer spectroscopy cannot resolve the two substructures in maghemite and the corresponding spectrum presents only one sextet [12]. At 5 K the hyperfine

magnetic fields of the two sextets are, respectively: sextet 1 = 489 kOe, 53% and sextet 2 = 521 kOe, which is very close to the values reported by Szabo and all [13] for $\phi \approx 9$ nm Fe_3O_4 NPs recorded at 80 K (also in the ferrimagnetic state, sextet 1 = 493 kOe, sextet 2 = 513 kOe). At 293 K the Mössbauer spectrum of our NPs is purely superparamagnetic without any ferrimagnetic contribution. EELS chemical analysis shows a Fe/O ratio equal to 0.74 ± 0.01 , closely to Fe_3O_4 ($\text{Fe/O} = 0.75$) confirming Mössbauer results.

The thermal variation of the in phase and out of phase components of the ac susceptibility (χ' and χ'' , respectively) of sample the S1 (Fig. 5) has the characteristic features expected for superparamagnetic NPs: below $T_F = 200$ K χ' depends on the field frequency f and χ'' is non-zero, while for $T_F > 200$ K χ' is not frequency dependent and χ'' is close to zero. This behavior can be

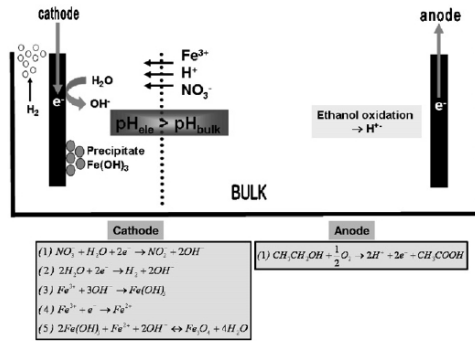


Fig. 2. General scheme of the magnetite electro-precipitation process.

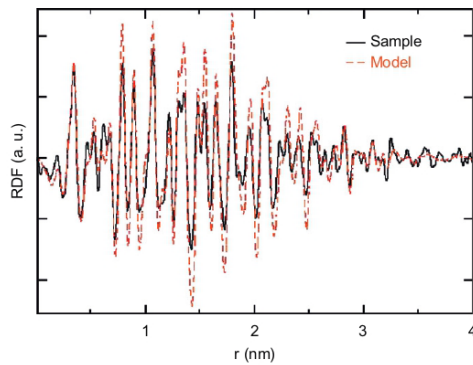


Fig. 3. WAXS analysis: Comparison between experimental RDF function for S1 sample ($\phi = 4.4$ nm, plain line) with theoretical one calculated for Fe_3O_4 spinel model ($\phi = 4$ nm, dotted line).

regarded as follows: at low temperature, the NPs cannot cross the energy barrier E to reverse the magnetization within the characteristic time of measurement. As the temperature increases, particles with lower energy start to cross the barrier, until $T = T_F$ where all particles can follow the field. In first approximation, E is proportional to the NPs volume ($E = K_{\text{eff}}V$, where K_{eff} is the effective anisotropy constant) and can be estimated from the $\tau = 1/f$ dependence of the maximum of χ' , the blocking temperature T_B , expected to follow the Néel–Arrhenius relation [14]:

$$\tau = \tau_0 \exp\left(\frac{E}{k_B T_B}\right) \quad (1)$$

where τ_0 is a microscopic characteristic time and k_B is the Boltzmann constant. Using Eq. (1) to describe $T_B(\tau)$ of sample S1 yields $\tau_0 \sim 10^{-36}$ s. This value is too low and does not have a physical meaning. Actually, typical values for magnetite or maghemite NPs are of the order of 10^{-9} – 10^{-12} s [15,16]. This implies that the canonic superparamagnetic NPs model does not adequately describe the present system, probably due to the existence of dipolar interactions. In fact, interactions are likely to occur, since the system is composed of rather close NPs. Dipolar interactions can be modeled by the super spin-glass description [17,18]. In this framework, we have fitted the variation of τ with T_B

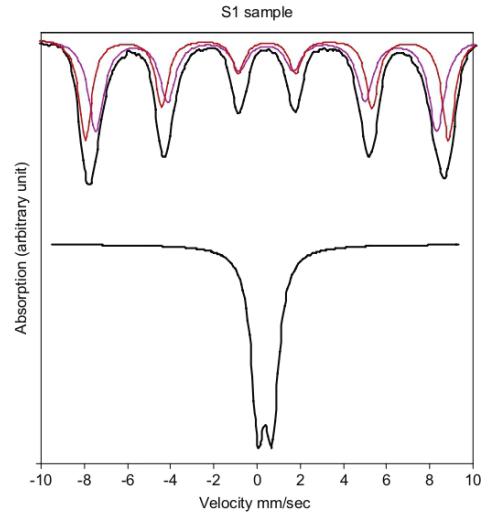


Fig. 4. Mössbauer spectra for S1 NPs ($\phi = 4.4$ nm) at, respectively, 5 K (up), 293 K (down).

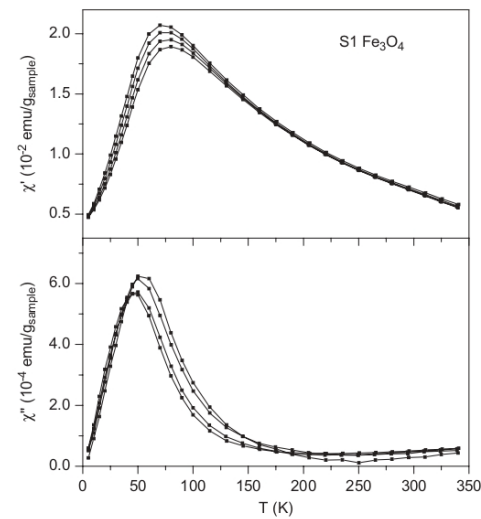


Fig. 5. Thermal variation at four different frequencies of the in phase and out of phase components of the ac susceptibility (χ' and χ'') of the S1 Fe_3O_4 sample ($\phi = 4.4$ nm).

to the Fulcher law:

$$\tau = \tau_0 \exp\left(\frac{E}{k_B(T - T_0)}\right) \quad (2)$$

As noted in Ref. [15], several sets of value yield fits of similar quality. Considering $\tau_0 = 10^{-12}$ s as a typical value for magnetite

NPs, we obtain $E = 598 \pm 7$ K and $T_0 = 51 \pm 1$ K for the S1 sample. Taking into account the average size of the magnetite NPs determined by TEM, 4.4 nm, K_{eff} is estimated to be of the order of 10^6 erg/cm³. This is an order of magnitude higher than expected for bulk magnetite (1.9×10^5 erg/cm³) (see, for instance, Ref. [19]) and might indicate that K_{eff} has an important surface contribution [20], as it has been already reported for 4 nm magnetic NPs [21].

4. Conclusions

The new method proposed here allows the production of superparamagnetic magnetite NPs with controlled size and size dispersion. Why are NPs produced during the electro-precipitation process almost monodispersed? Indeed, this phenomenon is quite surprising since no stabilizing agent or micelles were used to stop cluster growth, as it is usually the case in many chemical routes. It can be supposed that monodispersed NPs are produced because the precipitation phenomenon occurs in a very restricted region (inside the electrochemical double layer) during a very short time. Thus, only nucleation phenomenon has time to occur, whereas Oswald ripening is strongly reduced. Clusters growth is probably inhibited because outside the double layer area, magnetite NPs are perfectly insoluble in the ethanol media, making the necessary re-dissolution step for Oswald ripening difficult.

Acknowledgments

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II.2. Contribuições na funcionalização de superfície de nanopartículas magnéticas via hidrólise e condensação de alcoxissilanos

As diferentes aplicações das NP dependem de uma engenharia de superfície. Esta engenharia envolve várias reações de funcionalização e modificações de superfícies. As reações de funcionalização geralmente envolvem estratégias que consistem em disponibilizar grupos reativos na superfície das NP. Estes grupos reativos servirão como pontos para reações com as espécies que se pretende modificar a superfície. Por exemplo, para a modificação de superfície de NP magnéticas com ácido fólico, é preciso considerar qual parte da molécula poderá ser utilizada para se fixar à superfície sem que isso ocasione a perda da atividade desejada. Como não é possível a ligação direta do ácido fólico à superfície, uma estratégia para a funcionalização de superfície de partículas consiste na hidrólise e condensação de organosilanos que possuam o grupo terminal reativo a ser disponibilizado na superfície. Assim, uma estratégia consiste em funcionalizar a superfície com grupos amina, provenientes de um alcoxissilano, e na sequência reagir esses grupo com grupos ácido carboxílicos provenientes do ácido fólico, formando uma ligação peptídica.

Ester silicatos são compostos que contém 4 ligações de oxigênio entre o átomo de silício e um grupo orgânico, ou seja, Si-OR. Antes os organosilanos eram nomeados como derivados do ácido ortosilícico ($\text{Si}(\text{OH})_4$), tal como o tetraetil ortosilicato ($\text{Si}(\text{OC}_2\text{H}_5)_4$), porém, com o advento dos compostos de silício contendo ligações Si-C, a nomenclatura foi modificada e estes compostos passaram a se chamar alcoxi derivados, por exemplo ($\text{Si}(\text{OC}_2\text{H}_5)_4$) recebeu a nomenclatura de tetraetoxissilano [11].

A grande variedade de aplicações dos alcoxissilanos relaciona-se a bifuncionalidade destes compostos, pois um dos grupos hidrolisáveis pode ser substituído por um grupo não hidrolisável. Assim, alcoxissilanos disponíveis comercialmente, apresentam a característica de poderem se ligar em superfícies dielétricas, tais como óxido de ferro, através das reações de hidrólise e condensação de seus grupos alcóxidos e ainda possuírem um grupo terminal não hidrolisável. Este grupo, geralmente é utilizado para ancorar drogas, modificar superfícies, formar ligação cruzada (cross-link) entre substratos inorgânicos e polímeros orgânicos, etc. A tabela I lista três destes alcóxidos e seus respectivos grupos terminais.

Tabela I: Agentes de silanização e os respectivos grupos terminais.

Agente de silanização	Grupo terminal
(3-Mercaptopropyl)trimethoxysilane (MPTS)	-SH
(3-aminopropyl)trimethoxysilane (APTS)	-NH ₂
n-propyltrimethoxysilane (NPTS)	-CH ₃

Basicamente, a estrutura de um alcoxissilano mono-substituído pode ser representada como na figura 3:

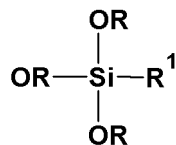


Figura 3: Estrutura de um alcoxissilano mono-substituído

onde R é, geralmente, um grupo metil ou etil, formando junto com a ligação -Si-O- um grupo metoxido ou etoxido, respectivamente; e R₁ o grupo não hidrolisável que irá funcionalizar a superfície de NP's, tal como o grupo amina (-NH₂) no alcoxissilano (3-aminopropyl)trimethoxysilane (APTS).

Quimicamente o processo sol-gel de funcionalização de superfícies pode ser representado pelos seguintes equilíbrios:

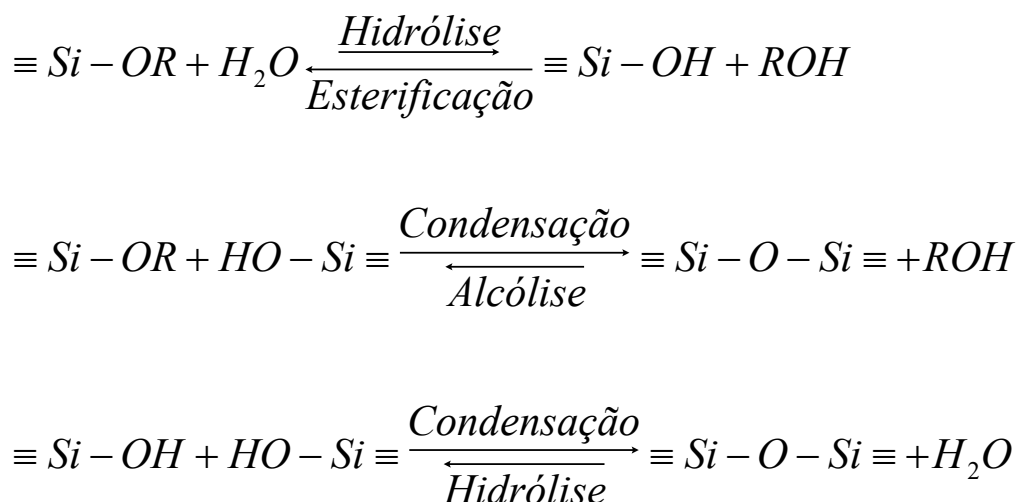


Figura 4: Equilíbrios químicos na hidrólise e condensação de alcoxissilanos.

A hidrólise de alcoxissilanos leva a formação de silanois e condensação de silanois com outro alcoxissilano leva a formação de siloxanos. Porém, dependendo das condições experimentais, as reações inversas podem ocorrer e a alcoolise de silanois e quebra de ligações siloxanos por álcool ou água pode desestabilizar o sistema.

II.2.1. Hidrólise de alcoxissilanos:

A velocidade de hidrólise é mínima em pH neutro e aumenta com o aumento da concentração de H⁺ ou OH⁻ [12]. O modelo de hidrólise de alcoxissilanos baseia-se em um mecanismo de substituição nucleofílica bimolecular (S_N2-Si) via um estado de transição penta-

coordenado [13]. Na catálise ácida, uma molécula de água interage com o átomo de silício, conforme mecanismo abaixo:

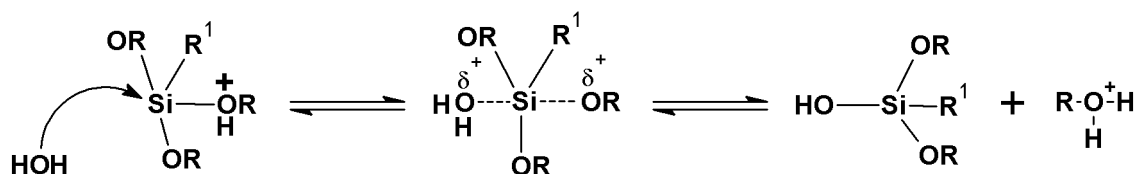


Figura 5: Mecanismo de hidrólise em meio ácido

Na catálise básica o ânion hidroxila quem ataca o átomo de silício conforme mecanismo abaixo:

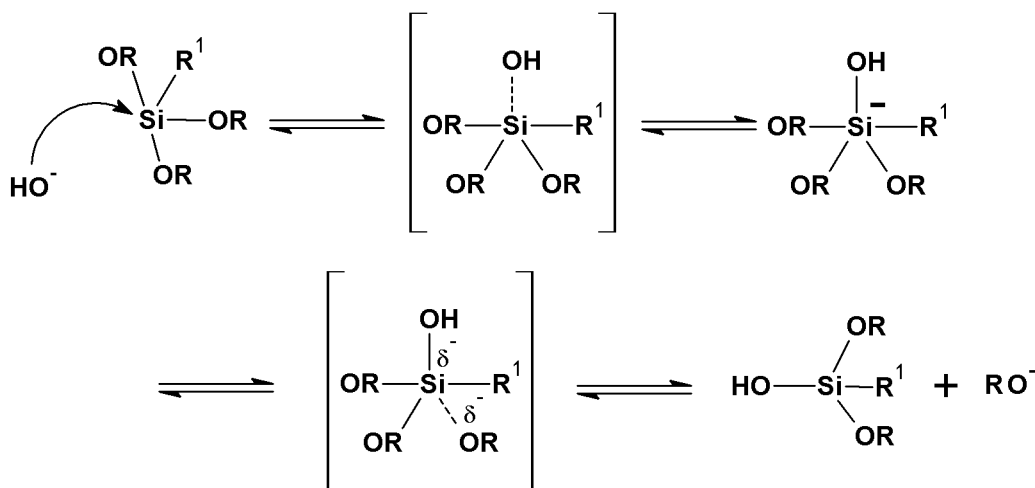


Figura 6: Mecanismo de hidrólise em meio básico

II.2.2. Condensação de alcoxissilanos:

Da mesma forma que a hidrólise, a condensação também é catalisada por ácido ou por base. Assim, após a hidrólise, a condensação catalisada por ácido inicia pela protonação dos grupos silanois, conforme mecanismo abaixo:

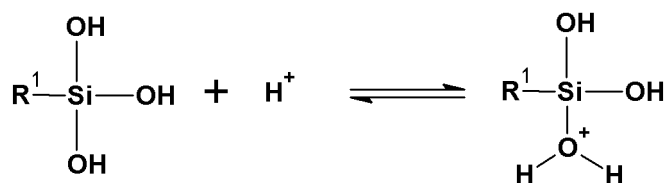


Figura 7: Mecanismo de condensação em meio ácido

O caráter eletrofílico da molécula de silanol é maior no estado protonado. Portanto, a molécula protonada é mais susceptível ao ataque nucleofílico por outra molécula de silanol. Quanto maior o caráter básico da molécula de silanol, mais fácil será a protonação. Considerando que a basicidade de moléculas de silanol diminuem na seguinte ordem $\text{Si}(\text{OH})_4 > \text{RSi}(\text{OH})_3 > \text{R}_2\text{Si}(\text{OH})_2 > \text{R}_3\text{Si}(\text{OH})$, a reação de condensação predominantemente ocorre entre moléculas neutras e grupos silanois de monômeros ou grupos hidroxilas localizados no final da cadeia de siloxano. Assim, diferente da hidrólise, o aumento da concentração de H^+ não causa um aumento na velocidade de condensação. [12]

Assim como em meio ácido, a condensação em meio básico se dá através de um mecanismo $\text{S}_{\text{N}}2\text{-Si}$ via um estado de transição penta-coordenado [13]:

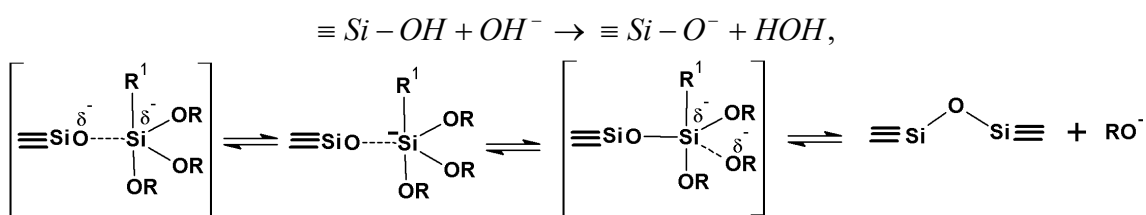


Figura 8: Mecanismo de condensação em meio básico

Neste caso, a substituição de grupos básicos OH^- (ou OR^-) por grupos $-\text{OSi}$ causa uma diminuição na densidade de elétrons no átomo de silício e consequentemente um aumento no caráter ácido de prótons nos grupos silanois remanescentes [14]. Portanto, a reação de condensação irá acontecer entre clusters grandes de moléculas condensadas e clusters menores com moléculas fracamente ligadas. Assim, a policondensação de compostos formados pode ocorrer dependendo de fatores como material inicial [15, 16], temperatura [17, 18], catalisador [19, 20] e concentração de água [21]. Porém, o pH do meio é o principal fator responsável pelos mecanismos nestes processos [22, 23].

II.2.3. Problemas com a policondensação

Na prática, porém, a rápida hidrólise da ligação (metoxi)etoxi-silano e a criação de grupos hidroxilas no átomo central de Si, podem levar a oligomerização entre os alcoxissilanos. Silanois são unidades metaestáveis que tendem a sofrer condensação para formar siloxanos termodinamicamente mais estáveis através da eliminação de água. Conforme discutido anteriormente, a formação de estruturas oligoméricas formadas pela condensação de silanois é influenciada pelo tipo de grupo funcional no constituinte orgânico e pelas condições da reação de hidrólise (pH, temperatura, concentração e catalisador) [24]. Dependendo das condições experimentais, a formação de monosilanol, silanodiol ou silanotriol pode ocorrer na hidrólise do

(3-aminopropyl)triethoxysilane (APTS), conforme esquema ilustrado na figura 9. A condensação destes silanois pode levar a formação de estrutura M, D e T, dependendo das condições experimentais.

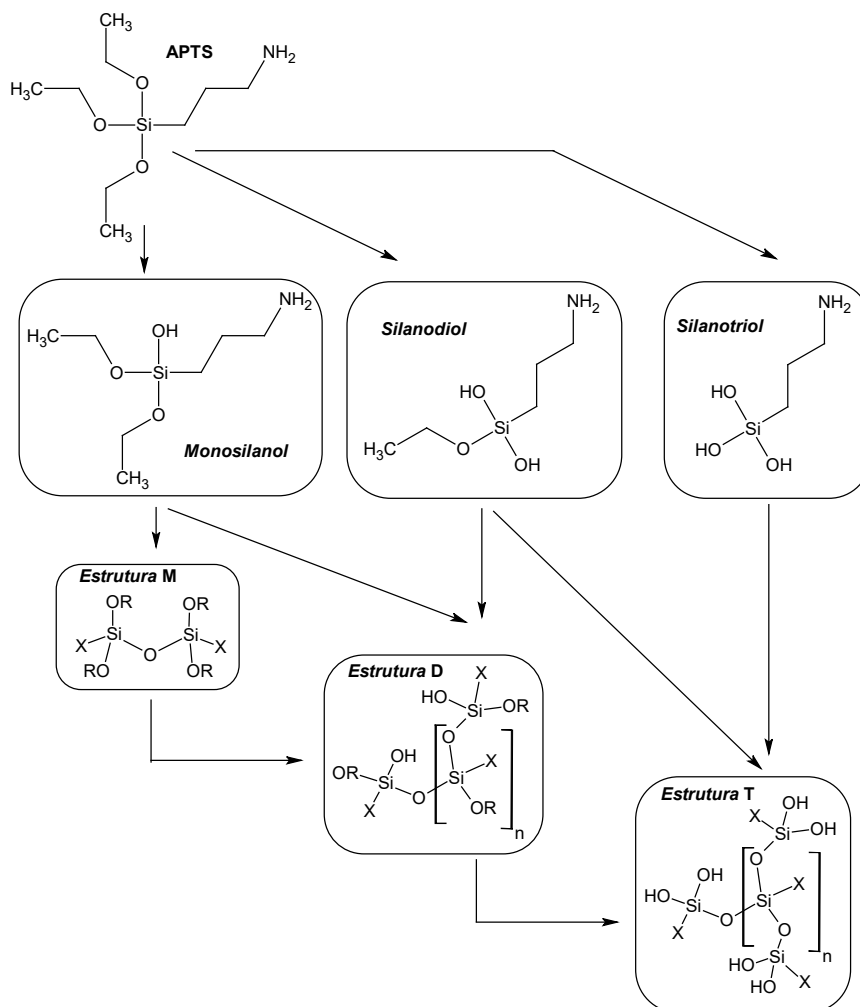


Figura 9: Reações de hidrólise e condensação do (3-aminopropyl)triethoxysilane (APTS) [44]

Assim, funcionalizar a superfície de NP magnéticas com APTS pode levar a policondensação dos silanois em solução para formar ligações Si-O-Si estáveis antes da formação de ligações Si-O-Fe na superfície das NP, conforme esquema ilustrado na figura 10. O desafio na funcionalização de NP magnéticas com APTS reside no caráter básico que o grupo amina confere a molécula do APTS. Conforme discutido anteriormente, em pH básico as reações de policondensação de alcóxissilanos são mais significativas e o crescimento de clusters envolvendo as NPs magnéticas em uma matriz de alcóxissilano pode levar a sistemas agregados que não apresentam as características necessárias para a aplicação das NPs em separação biomagnética.

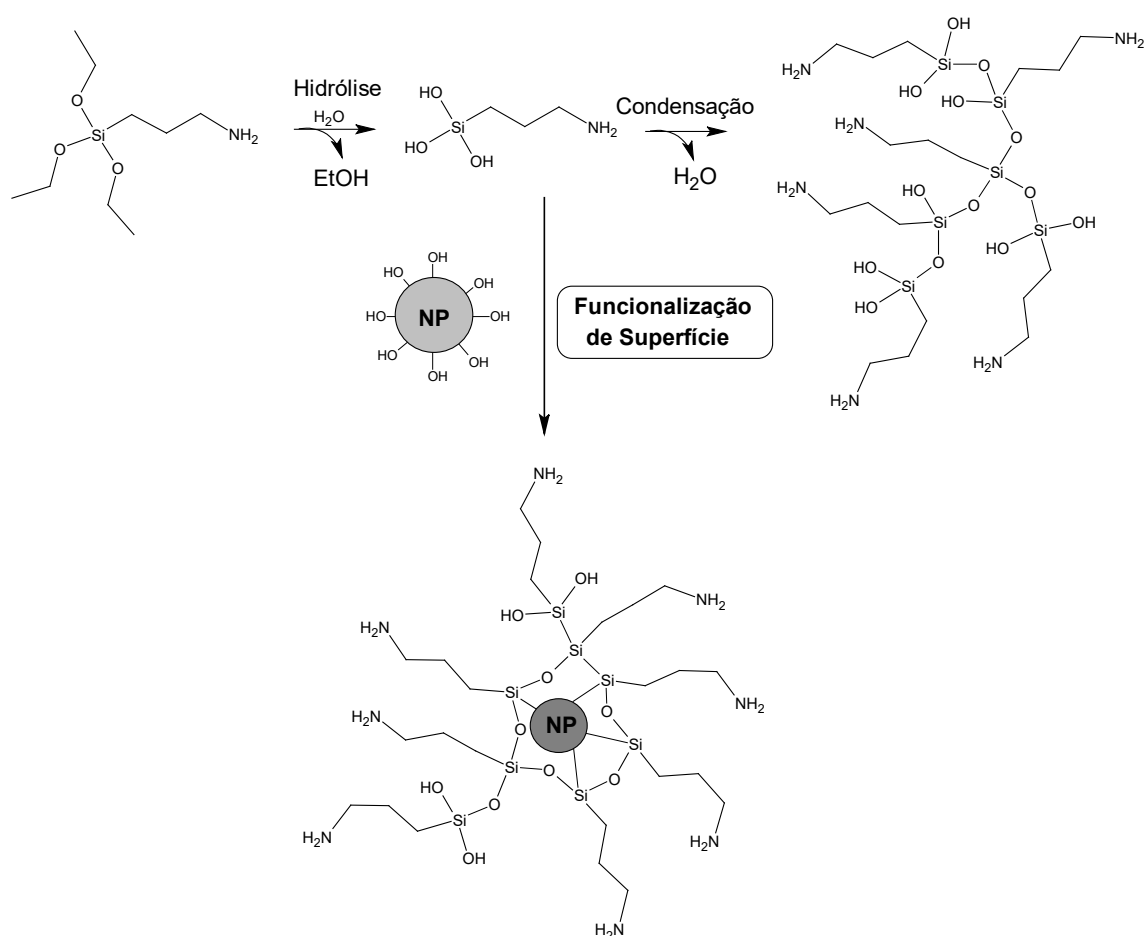


Figura 10: Funcionalização de superfície de NP com (3-aminopropyl)trimethoxysilane (APTS)



Synthesis and functionalization of magnetite nanoparticles with different amino-functional alkoxysilanes

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ABSTRACT

Superparamagnetic iron oxide (SPIO) nanoparticles show great promise for many biotechnological applications. This paper addresses the synthesis and characterization of SPIO nanoparticles grafted with three different alkoxysilanes: 3-aminopropyl-triethoxysilane (APTES), 3-aminopropyl-ethyl-diethoxysilane (APDES) and 3-aminopropyl-diethyl-ethoxysilane (APES). SPIO nanoparticles with an average particle diameter of 10 nm were prepared by chemical sonoprecipitation. As confirmed by Fourier transform infrared (FTIR) spectroscopy, silylation of these nanoparticles occurs through a two-step process. Decreasing the number of alkoxide groups reduced the concentration of free amino groups on the SPIO surface ($[SPIO-NH_2]: APTES > APDES > APES$). This phenomenon results from steric contributions and the formation of H-bonded amines provided by the ethyl groups present in the APDES and APES molecules. A simulation of SPIO nanoparticles in a saline physiologic solution shows that the ethyl groups impart larger steric stability onto the ferrofluids, which reduces aggregation. The magnetization (M) versus magnetic field (H) curves show that the synthesized iron oxide nanoparticles display superparamagnetic behavior. The zero-field cooling (ZFC) and field cooling (FC) curves show that the changes in the blocking temperature depend on the alkoxysilane-functionalized particle surface.

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1. Introduction

Nanoscale magnetic materials have generated significant interest in recent years. Investigations of iron oxides (Fe_3O_4 and $\gamma-Fe_2O_3$), synthesized by several methods and functionalized for different applications, are being increasingly reported in the literature. This increased interest is partly due to the simplicity of preparing nanoparticles and their stable storage in the colloidal form. Colloidal suspensions of stabilized magnetic nanoparticles are called ferrofluids. These ferrofluids can interact with a magnetic field [1]. The development and the understanding of superparamagnetic iron oxide (SPIO) nanoparticles dispersions are becoming more important for biomedical applications. SPIO is constituted of single magnetic domains, in which all of the atomic magnetic moments are magnetized in the same direction. Single-domain particles do not have domain walls, so in the superparamagnetic (SPM) state, the entire magnetic moment of each particle is oriented in the same direction. This phenomenon is characterized by the absence of hysteresis cycles (remnant

magnetization and the coercive field are null) [2]. Because of this magnetic property, SPIO nanoparticles do not interact with each other, minimizing aggregation after the field is removed. This behavior is important for *in vivo* (drug delivery, magnetic resonance imaging and magnetic hyperthermia) and *in vitro* (bioseparation, biosensing and magnetofection) biomedical applications [3–6].

The surface modification of SPIO can be carried out with alkoxysilanes via silylation reaction, in which reagents with non-hydrolyzable amino groups are commonly used. 3-Aminopropyl-triethoxysilane (APTES) is the most commonly used alkoxysilane due to its terminal amino group. The silylation process is very complex and does not involve a single mechanism. Thus, many different intermediates are possible [6–9]. Several papers have reported on the functionalization of SPIO nanoparticles with bioactive molecules through interface-containing amino groups (via APTES). The surface concentration of the bioactive molecules is correlated to the free amino group concentration, so the control of grafted APTES molecules becomes an important step in this system [7].

SPIO nanoparticles grafted with silica and alkoxysilanes to be biocompatible and to have hydrophilic properties are promising reagents in nanobiotechnology. The huge diversity of alkoxysilanes allows for different types of nanoparticle surface functionalization,

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i.e., introduces charges on the system that can prevent the aggregation of the particles in liquids and improve their chemical stability through surface charge control [10–12]. In addition to studying the size, structure and composition of the colloidal particles, their colloidal stability must be evaluated. Controlling the stability of the ferrofluids against aggregation is important because the formation of aggregates alters their specific surface area and dispersibility as well as their efficiency as a drug vehicle [13]. Aggregation of the nanoparticles can cause venous emboli.

In the present work, the influence of the number of alkoxide groups (hydrolyzable) in the alkoxysilanes containing the amino groups was systematically studied and correlated with the colloidal stability. The control of the free amino group density on the SPIO surface was investigated, and electrokinetic measurements in different saline concentrations were performed. The results suggest that surface charge can be controlled by grafting SPIO nanoparticles with different alkoxysilanes.

2. Materials and methods

To synthesize the iron nanoparticles, the following reactive substances were used: the iron bromine (II) and (III) (FeBr_2 and FeBr_3) (Aldrich). These chemicals were grade reactive and were used as received. For the silylation process with the iron particles, the following alkoxysilanes were used: 3-aminopropyl-triethoxysilane (APTES), 3-aminopropyl-ethyl-diethoxysilane (APDES) and 3-aminopropyl-diethyl-ethoxysilane (APES) (Aldrich). The dispersion medium was deionized (DI) water and alcohol (Aldrich). The magnetite nanoparticles were synthesized in a two-step procedure. First, nanosized magnetite (Fe_3O_4) was synthesized by a new sonoprecipitation method [14]. Second, the surface-functionalized SPIO samples were produced via hydrolysis and condensation of the organosilane agents. The nanosized magnetite was silylated using silane coupling agents in an aqueous-alcohol solution. The native nanosized magnetite sample was added to the 3-aminopropyl-triethoxysilane (APTES) aqueous-alcohol solution and refluxed at 125°C for 2 h. The samples were then washed several times with deionized water. The sample was dried at 60°C in a stove vacuum. The same procedure was also carried out for the alkoxysilanes 3-aminopropyl-ethyl-diethoxysilane (APDES) and 3-aminopropyl-diethyl-ethoxysilane (APES) (Fig. 1).

The electron micrographs were taken with a Philips CM 120 transmission electron microscope (TEM). Dry samples of the magnetite complexes were dispersed in isopropyl alcohol and cast onto amorphous carbon-coated copper grids for analysis. Great care was taken to ensure that both the eucentric height and focus were set consistently for each sample to reduce the uncertainty in the digital image analysis. The X-ray diffraction (XRD) pattern was recorded in a Philips powder diffractometer PW 3040/60 with $\text{Cu K}\alpha$ radiation. Infrared spectra were recorded in the $4000\text{--}400\text{ cm}^{-1}$ range on a Fourier transform infrared spectrometer (FTIR, Nicolet Instruments Corporation). To determine the surface concentration of the amino groups on the

sample, the UV spectroscopy method proposed by Moon et al. [15] and adapted to nanoparticles by Marques et al. [14] was used. Dynamic light scattering (DLS) measurements were conducted with a Malvern Zetasizer Nano ZS particle analyzer (Malvern Instruments Ltd., Malvern) at a wavelength of 633 nm with a solid-state He–Ne laser at a scattering angle of 173° at 25°C . The intensity, volume and number average diameters were calculated with the Zetasizer Nano 4.2 software utilizing an algorithm based upon the Mie theory, which transforms time-varying intensities to particle diameters [16]. For the DLS analysis, each sample was diluted to approximately 0.1 mg mL^{-1} after the solution was filtered through a Whatman Anotop alumina membrane with a pore diameter of 220 nm . The experiments were carried out at NaCl concentrations of 1×10^{-3} and 0.15 mol L^{-1} at pH 7. Each sample was analyzed directly following filtration and measured every 3 min over a 1 h period. Electrokinetic measurements were also performed with a Zetasizer ZS at 25°C , in which the measured particle velocity is due to an applied electric field that is related to the surface charge [17]. The electrokinetic measurements were taken by dispersing the sonicated samples at a concentration of 0.1 mg mL^{-1} . The ionic strength was adjusted to $1 \times 10^{-3}\text{ mol L}^{-1}$ NaCl. The pH was adjusted to 3.0 using 0.1 mol L^{-1} hydrochloric acid. The electrokinetic mobility was measured by increasing the pH to 12 using 0.1 mol L^{-1} sodium hydroxide. Hydrochloric acid and sodium hydroxide were determined to be in different electrolytes, and they did not greatly affect the surface charge density of the magnetite. The field-dependence magnetization (M versus H) and the temperature-dependence magnetization under zero-field cooled (ZFC) or field-cooled (FC) conditions and an applied field of 500 Oe were measured with a Quantum Design Physical Properties Measuring System (PPMS). The experimental data were normalized by the mass of the samples used in the measurements.

3. Results and discussion

Fig. 2 shows the results of the X-ray diffraction patterns analysis of the uncoated and amino-functionalized Fe_3O_4 samples. Fig. 2(a) shows the uncoated sample. The magnetite and maghemite patterns from the JCPDS database [18] were included for comparison. Magnetite can be observed as the dominant phase; however, some maghemite may have formed during the sonoprecipitation method. The process to obtain the particles was carried out in an aqueous medium. Magnetite nanoparticles are very sensitive to oxygen. In the presence of air, some of the particles might undergo oxidation to $\text{Fe}(\text{OH})_3$ or to the maghemite ($\gamma\text{-Fe}_2\text{O}_3$) phases [19]. The XRD pattern showed the presence of the inverse spinel structure, corresponding to either magnetite phase having a spinel structure. The presence of broad and intense peaks confirmed the formation of crystalline nanoparticles. Fig. 2(b) shows the experimental measurements of the (i) APES, (ii) APDES and (iii) APTES samples. The alkoxysilanes did not structurally change the samples. Fig. 3(a), (b) and (c) shows the TEM images of the APES, APDES and APTES samples, respectively. The electron micrographs show the nanoparticles have a nearly spherical shape with a diameter of approximately 10 nm . The average particle size was also determined from the broadening of the diffraction peaks from Scherrer's equation [20]. The size was calculated to be approximately 11.6 nm , which agrees with the size estimated from the TEM images.

To confirm the grafting of the magnetite surface through the silylation reaction, an FTIR spectrum of the functionalized-magnetite was obtained (Fig. 4). The characteristic absorption bands of the Fe–O bond of the grafted SPIO shifted to higher wavenumbers, 628.7 and 577.9 cm^{-1} , compared to the uncoated SPIO

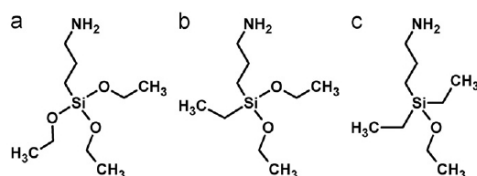


Fig. 1. Structural formulas of the alkoxysilanes used in this work. (a) APTES, (b) APDES and (c) APES.

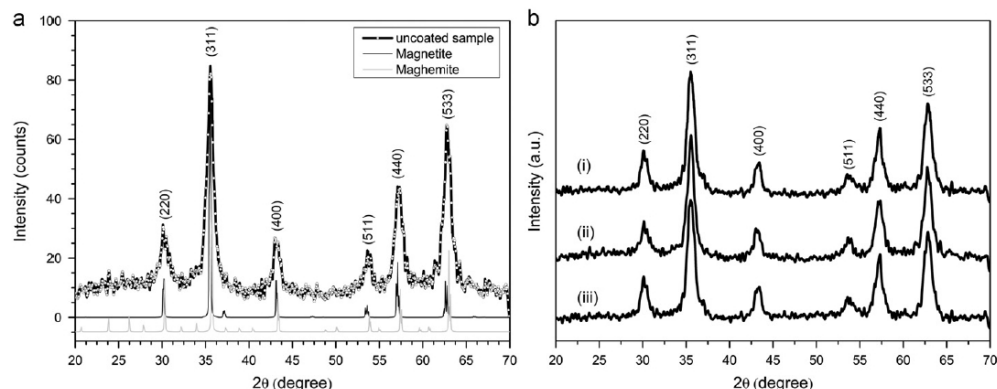


Fig. 2. X-ray powder diffraction patterns of the uncoated sample with the magnetite and maghemite patterns from the JCPDS database (a) and the APES (i), APDES (ii) and APTES (iii) samples (b).

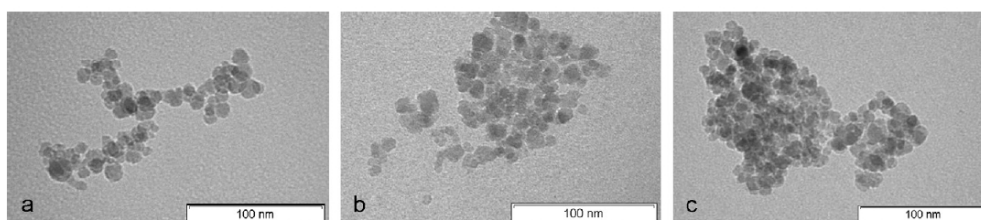


Fig. 3. TEM micrographs of the amino-functionalized Fe_3O_4 nanoparticles treated with APES (a), APDES (b) and APTES (c).

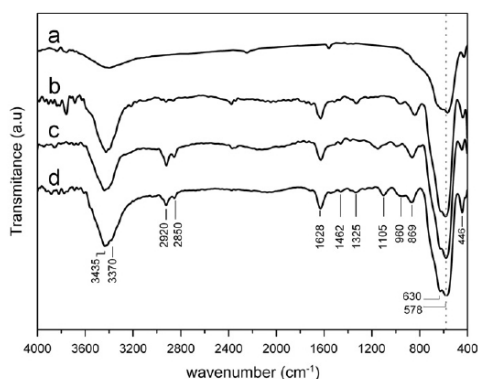


Fig. 4. The FTIR spectra of the uncoated (a), APES (b), APDES (c) and APTES (d) samples.

(in 621.4 and 574.2 cm^{-1}). This phenomenon can be explained by the formation of Fe–O–Si bonds. The Fe–O–H groups on the SPIO surface are replaced by Fe–O–Si(O–) $_2$ –R as shown in Fig. 5. The greater electronegativity of –Si(O–) $_2$ compared to H leads to an enhancement of the bond forces for the Fe–O bonds, shifting the absorption bands to high wavenumbers [21,22].

Surface modification by alkoxysilanes is a complex process because it does not involve a single mechanism and several experimental parameters influence the system. The FTIR spectra show the absorption bands for the silanol (Si–O–H) and siloxane (Si–O–Si) groups. Si–O stretching and Si–O–H bending vibrations at 960 and 869 cm^{-1} were observed for all of the samples. For the APTES- and APDES-coated nanoparticles, the stretching Si–O–Si was verified at 1150–1100 cm^{-1} [23]; however, this stretching was not observed for the APES sample, which was expected due to the presence of only one alkoxy group. The presence of amino groups associated with the 3-aminopropyl of the alkoxysilanes was observed. The absorption bands at 1325 and 2920–2850 cm^{-1} are ascribed to the C–N and C–H stretching vibrations, respectively. A broad band at 1628 cm^{-1} can be ascribed to the N–H stretching vibration, which is indicative of the free amino group. A small shoulder at 3370 cm^{-1} corresponds to the N–H asymmetric stretching of the amine H-bonds, indicating a possible NH_2 interaction toward the SPIO surface [23].

Table 1 shows the amino group density results. The APTES sample had the highest amino group density, and a decrease in the amino group density was observed for the APDES and APES samples. The same alkoxysilane concentration was used in each synthesis, and similar values for the amino group density on the Fe_3O_4 surface were expected, indicating the influence of the ethoxy group (–OC $_2$ H $_5$) on the alkoxysilane and the presence of ethyl groups on the condensation process. Fig. 5 shows an illustration of the suggested models of alkoxysilane condensation on the SPIO surface. The polycondensation process was outstanding for the APTES molecule due to its three ethoxy groups, which

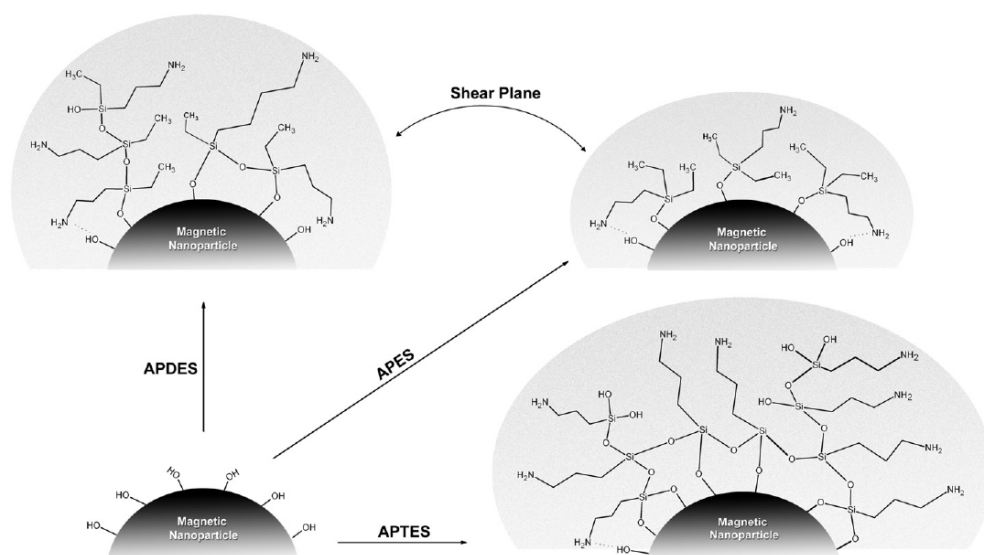


Fig. 5. Possible SPIO surfaces after coating with APTES, APDES or APES.

Table 1
Amino group density and zeta potentials of the samples.

Samples	Amino group density (nmol g ⁻¹)	Zeta potential (mV) at pH=7	
		1 × 10 ⁻³ mol L ⁻¹ NaCl	0.15 mol L ⁻¹ NaCl
Uncoated	–	5.85 ± 0.21	–2.72 ± 0.25
APES	2.28 ± 0.11	12.90 ± 0.18	2.50 ± 0.21
APDES	3.20 ± 0.09	16.76 ± 0.20	3.45 ± 0.23
APTES	8.72 ± 0.07	25.05 ± 0.16	9.36 ± 0.19

contributed to the large amino group density. The APDES and APES molecules have fewer ethoxy groups. However, the ethyl groups on the APDES and APES structures cause steric hindrances, which consequently slow the condensation process and reduce the amino group density. A decrease in the amino group density may also be related to hydrogen bonding, as indicated by the FTIR spectra. The formation of H-bonded amino groups is more favored for APDES and APES than for APTES due to the smaller number of condensing groups. Thus, the free amino group can react with the surface, and intermolecular hydrogen bonds can be formed, as schematically shown in Fig. 5.

Fig. 6(a) shows the zeta potential as a function of pH for a suspension of particles in a 1 × 10⁻³ mol L⁻¹ NaCl solution. The zeta potential curve for the synthesized nanoparticles suggests a “passive surface for the uncoated iron oxide, with the zeta potential ranging from positive values (+20 mV) at low pH values to negative values (–48 mV) at high pH values and an isoelectric point (IEP) at pH 7.30. The zeta potential of the amino-functionalized samples varied considerably with pH. At lower pH values, it was verified that the ζ-potential increased with the increase in amino group density, which correlates well with the surface concentration of the alkoxy silanes bonded to the nanoparticles, as measured by UV spectroscopy. This increase in the positive

surface charge provided by the surface amino groups also results in a shift of the isoelectric point to higher pH values. APTES has an IEP at pH 10.05, which agrees with previous data [7–9]. The APDES and APES samples showed a shift to low pH with different IEP values (Fig. 6).

Fig. 6(b) and (c) shows the hydrodynamic diameters of the functionalized Fe₃O₄ samples measured by dynamic light scattering in 0.0010 and 0.15 mol L⁻¹ NaCl at pH 7. Upon increasing the electrolyte concentration, the slope of the hydrodynamic diameter versus time curve increases. The uncoated sample showed lower stability than the coated samples for both NaCl concentrations. By comparing Fig. 6(b) and (c), the increased electrolyte concentration induced the formation of larger aggregates by suppressing the double-layer repulsion between the particles. According to the Derjaguin and Landau, Verwey and Overbeek (DLVO) theory, the electrical double-layer thickness (1/κ) repulsion decreases with increasing salt concentrations [24,25]. In this work, the hydrodynamic diameters (i.e., the inverse of the Debye–Hückel parameter (1/κ) [24]) of the samples measured in 0.0010 and 0.15 mol L⁻¹ NaCl at 298 K were 13.6 and 1.1 nm, respectively. These 1/κ values agree with the zeta potential values in Table 1, and they help to explain the different behaviors in Fig. 6(b) and (c).

Fig. 6(b) shows that the APTES coating resulted in fewer aggregates because of the large charge density on the surface, as shown in Table 1. However, APES (ζ = +12.90 mV) had smaller aggregates than the APDES sample (ζ = +16.76 mV). This finding was not expected due to the difference in the surface potentials. This behavior was also observed in the samples measured in 0.15 mol L⁻¹ NaCl. Fig. 6(c) shows that the nanoparticles treated with APES (ζ = +2.50 mV) had smaller aggregates than the other amino-functionalized samples with greater zeta potentials, such as APTES (ζ = +9.36 mV). These samples have low surface potentials, and the aggregation could be a spontaneous process in the system. In addition to hydrodynamic diameter measurements,

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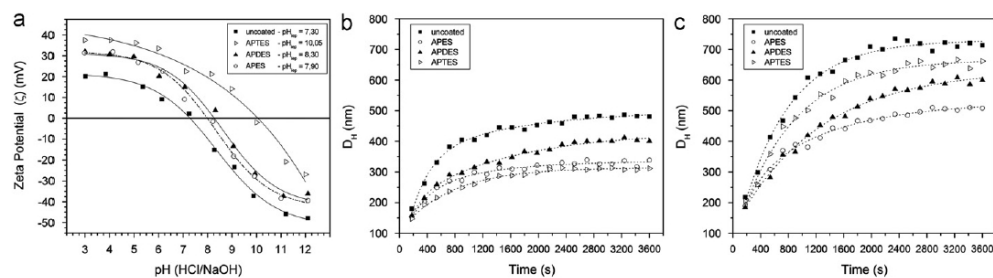


Fig. 6. (a) The zeta potential of the amino-functionalized Fe_3O_4 particles in the 1×10^{-3} mol L $^{-1}$ NaCl solution. The hydrodynamic diameters of the samples measured by dynamic light scattering plotted versus time for 1×10^{-3} and 0.15 mol L $^{-1}$ NaCl at pH 7, respectively (b) and (c).

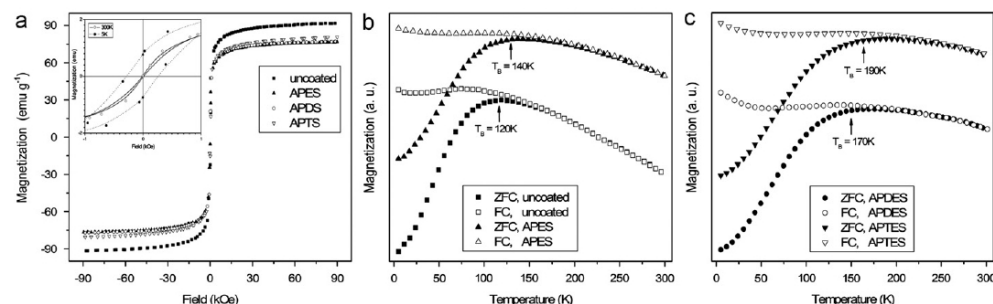


Fig. 7. (a) M versus H plot of the uncoated and amino-functionalized Fe_3O_4 samples at 300 K. Inset M versus H plot of the uncoated sample at the low-field region showing coercivity. The unit emu g^{-1} represents the magnetization, which was normalized by the mass of the samples in the measurements. The ZFC–FC plots showing the blocking temperature (b) and (c).

DLS can be used to determine the polydispersity index (PDI) of a dispersion. Higher PDI values were obtained for the uncoated sample: 0.3054 and 0.4286 in 0.0010 and 0.15 mol L $^{-1}$ NaCl at pH 7, respectively. For the amino-functionalized samples, the polydispersity values agree with the hydrodynamic diameters, slopes and the zeta potentials. For the APTES sample, the observed PDI values were 0.1765 and 0.3934 for 0.0010 and 0.15 mol L $^{-1}$ NaCl, respectively. For the APDES samples, the PDI values were 0.2629 and 0.3538 for 0.0010 and 0.15 mol L $^{-1}$ NaCl, respectively. For the APES samples, the PDI values were 0.1835 and 0.3249 for 0.0010 and 0.15 mol L $^{-1}$ NaCl, respectively. The influence of the ethyl groups of the APDES and APES molecules is evident. The ethyl groups provide steric stabilization, generating an electrosteric system that confers proportionally larger stability as compared to the electrostatic stabilization by APTES.

Fig. 7 shows the magnetic measurements of the uncoated and functionalized Fe_3O_4 samples. At 300 K, SPM behavior was registered. The samples did not exhibit hysteresis, and neither coercivity nor remanence was detected (Fig. 7(a)). Ferromagnetic behavior with a coercivity of about 280 Oe is observed at 5 K for the uncoated sample (inset of Fig. 7a). This behavior was also observed for the functionalized Fe_3O_4 samples. The saturation magnetizations of samples were found to be 80.45, 65.05, 65.45 and 68.82 emu g^{-1} for the uncoated, APES, APDES and APTES samples, respectively.

The temperature dependence of the magnetization $M(T)$ was taken in ZFC and FC conditions (Fig. 7b and c). The maximum in the ZFC curve defines the blocking temperatures, at which the thermal energy becomes comparable to the anisotropy energy barrier [26]. The blocking temperatures (T_B) for the uncoated, APES, APDES and

APTES samples were verified to be approximately 120, 140, 170 and 190 K, respectively. The blocking temperatures changed due to the different alkoxy silanes on the surface. The blocking temperatures of the SPIO nanoparticles depend on the surface state, interparticle interactions, size and size distribution [26]. The contributions to the anisotropy energy can originate from the intrinsic anisotropies of the particles (shape, magnetocrystallinity or stress anisotropies) or interparticle interactions (dipolar or exchange). Separating these effects is difficult [27]. The same procedure was carried out for all of the samples. Thus, any increases in T_B could be ascribed to the addition of the alkoxy silanes into the system. The FTIR, zeta potential and amino group density analyses indicate that ($-\text{OC}_2\text{H}_5$) condensation was more effective for APTES. The increase in this condensation can increase the interparticle aggregation, leading to the formation of larger aggregates particles. The ZFC/FC measurements are sensitive to changes in the size distribution of the system (Fig. 7(b) and (c)). The formation of larger aggregates also can be verified by the y-shaped curves. Below the T_B , the magnetization decreases for the ZFC, while for FC, the magnetization increases. This behavior is normally ascribed to interparticle interactions with some degree of agglomeration [28].

4. Conclusions

Superparamagnetic iron oxide particles coated with alkoxy silanes were successfully synthesized. A decrease in the number of alkoxydes ($-\text{OC}_2\text{H}_5$) on the surface caused a decrease in the concentration of free amino groups on the SPIO surface. The electrokinetic

measurements, which showed that the IEP shifted for smaller values of pH, provided evidence concerning the effect of the surface amino group. The DLS measurements in 0.15 mol L^{-1} NaCl showed that APDES and APES, with smaller amino group densities and zeta potentials, impart greater stability than APTES, which has a larger surface potential. The main difference among the samples was the alkoxy group number, relative to APTES. APDES has the insertion of an ethyl group, while APES has two ethyl groups. Therefore, the decrease in the concentration of the free amino group and zeta potential shifts can be related to the reactivity of the molecules. The shifts in the blocking temperatures show that the concentration of alkoxides ($-\text{OC}_2\text{H}_5$) on the functionalized molecules affects interparticle aggregation. The differences in the colloidal stability are due to steric contributions provided by the ethyl groups. This work examined the influence of alkoxy groups on the colloidal stability of surface-modified SPIO nanoparticles. Future studies will have to be conducted. The magnetic properties and colloidal stability of the nanoparticles in a physiological saline solution were correlated with the interparticle interactions due to the different shell configurations on the SPIO surface. Colloidal stability is an extremely important parameter for *in vivo* SPIO biomedical applications and helps to inhibit capillary emboli when carrying biological molecules or drugs.

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Surface functionalization of magnetite nanoparticle: A new approach using condensation of alkoxysilanes



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ABSTRACT

In this study we report on successful production of two samples (BR15 and BR16) comprising magnetite (Fe₃O₄) nanoparticles (~10 nm) surface-functionalized via hydrolysis and condensation of alkoxysilane agents, namely 3-aminopropyl-trimethoxysilane (APTS) and N-propyl-trimethoxysilane (NPTS). The as-produced samples were characterized using transmission electron microscopy (TEM), x-ray diffraction (XRD), magnetization measurements (5 K and 300 K hysteresis cycles and zero field-cooled/field-cooled measurements), and Mössbauer spectroscopy (77 and 297 K). The Mössbauer data supported the model picture of a core-shell magnetite-based system. This material system shows shell properties influenced by the surface-coating design, either APTS-coated (BR15) or APTS+NPTS-coated (sample BR16). Analyses of the Mössbauer spectra indicates that the APTS-coated sample presents Fe(III)-rich core and Fe(II)-rich shell with strong hyperfine field; whereas, the APTS+NPTS-coated sample leads to a mixture of two main nanostructures, one essentially surface-terminated with APTS whereas the other surface-terminated with NPTS, both presenting weak hyperfine fields compared with the single surface-coated sample. Magnetization measurements support the core-shell picture built from the analyses of the Mössbauer data. Our findings emphasize the capability of the Mössbauer spectroscopy in assessing subtle differences in surface-functionalized iron-based core-shell nanostructures.

1. Introduction

Nowadays magnetic nanoparticles (MNP) are essential building blocks in nanotechnology, supporting a wide variety of industrial and biomedical applications [1]. As far as the biomedical applications are concerned superparamagnetism is unique while allowing remote manipulation (under gradient of magnetic field) and heating (under alternate magnetic field) of nanomaterials [2]. Besides, magnetic nanomaterials, such as magnetic nanoparticles and molecular magnets, are very much interesting from the basic point of view [3]. Surface functionalization of magnetic iron oxide nanoparticles using bioactive moieties supports different technologies, such as magnetic drug delivering, magnetohyperthermia and contrast agent for magnetic resonance imaging [4,5]. In recent years, surface functionalization of metal-oxide magnetic nanoparticles has been receiving much attention with major impact while developing new nanobiocompatible products [6–8]. For human imaging purpose, superparamagnetic iron oxide

(SPIO) particles need to be biocompatible while providing a safe route of elimination [9]. Also, SPIO particles, like cubic ferrites, can be surface functionalized and therefore tailored to bind to a wide variety of drugs, proteins, enzymes, antibodies, and cell targets [10].

On the other hand, SPIO particles show very interesting basic phenomena such as quantum confinement of carriers and magnetic quantum tunneling effect [11,12]. These phenomena are connected with quantum size effect and superparamagnetic behavior, which support surface charge modulation from negative to positive (thus allowing surface functionalization with different moieties) and external manipulation (magnetic pliers) using gradients of magnetic field. Besides, single nanoparticles core-shell structures, with a reactive molecular shell (RMS) around the metal-oxide core, provide extra opportunity for surface functionalization of the core magnetic nanoparticle [13]. In searching for RMS candidates, we have finding those that can offer reactive amino groups facing outside the shell, which may provide important breakthroughs as far as the biological and medical

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applications are concerned. In this regard hydrolysis and condensation of alkoxyisilane agents onto metal-oxide surfaces represent an excellent approach.

Hence, comprehensive characterization of the magnetic behavior of the end nanostructure is of major interest. This can be addressed by several and well-established experimental techniques, such as the superconducting quantum interference device magnetometry and Mössbauer spectroscopy. In this study, we reported on the successful preparation and magnetic characterization of surface amino-modulated nanosized magnetite particles. Discussion of the Mössbauer data revealed new insights regarding the effect of surface coating species in the iron oxide-based core's properties and will be supported by transmission electron microscopy (TEM) data, x-ray diffraction (XRD) patterns, and magnetometry.

2. Experimental section

Iron (II) and iron (III) chlorides (Aldrich 98%), 3-aminopropyl-triethoxysilane (Aldrich 97%), N-propyl-triethoxysilane (Aldrich 97%) and ethanol absolute (Panreac) were used as received. Distilled water was used throughout all preparation procedures. Surface functionalized magnetite nanoparticles were preparing using a two-step protocol, as shortly described herein. Firstly, nanosized magnetite (Fe_3O_4) had been synthesizing by a new sonoprecipitation method developed by Marques et al. [14]. Secondly, surface functionalized magnetite-based samples, labeled BR15 and BR16, had been preparing via hydrolysis and condensation of two organosilane agents, namely 3-aminopropyl-triethoxysilane (APTS) and N-propyl-triethoxysilane (NPTS), schematically represented in Fig. 1. The as-synthesized magnetite nanoparticle was silylating using the silane-based coupling agents in aqueous-alcohol solution. Two aqueous-alcohol solutions containing the silane-based agents were preparing by adding 0.3 mL of APTS (for sample BR15) or 0.15 mL of APTS plus 0.15 mL of NPTS (for sample BR16) into 10 mL of 95% ethanol-5% water solution. The native magnetite nanoparticle were adding to the APTS or APTS and NPTS solutions and the resultant solution was refluxing at 85 °C for 2 h. The as-produced samples (BR15 and BR16) were washing several times with ethanol.

➡ For the absolute determination of the amine density on the nanoparticles surface of samples prepared in this work the methodology proposed by Moon J.H. et al. [15–17] was used. The methodology consists of the reaction between an aldehyde and amine groups on the surface of NP forming imines. Imines are unstable in aqueous medium and are readily hydrolyzed to form the aldehyde and the amine again. Thus, the colorimetric determination of the aldehydes released after the hydrolysis of the imines allows the indirect determination of the concentration of amines on the surface of the NPs.

The shape and size of the surface functionalized magnetite nanoparticles were examining by a Philips CM 120 transmission electron

microscope (TEM) operating at 120 kV. Briefly, dried samples of the as-produced nanoparticles were dispersing in isopropyl alcohol and cast onto amorphous carbon-coated copper grids for imaging. The crystalline structure of the samples was investigating by x-ray diffraction (XRD) using a Philips powder diffractometer PW 3040/60 operating with Cu-K α radiation. X-ray fluorescence spectroscopy measurements had been carrying out using a Shimadzu spectrometer EDX-72. The relative amount of Fe and Si were determining to be respectively 97% and 3% (sample BR16) and 80% and 20% (sample BR15). ^{57}Fe Mössbauer spectra were recording at 77 and 297 K using a conventional constant acceleration transmission spectrometer with a $^{57}\text{Co(Rh)}$ source and a bath cryostat. The recorded spectra had been fitting using the MOSSWIN program whereas $\alpha\text{-Fe}$ foil had been using for calibration. Values of the isomer shift (δ) had quoted with respect to $\alpha\text{-Fe}$ at room temperature. DC magnetic measurements were carrying out using a MPMS3 (Quantum Design, Inc) Superconducting Quantum Interference Device (SQUID) magnetometer. Applied magnetic field varied from 0 up to ± 70 kOe whereas the sample temperature scanned in the temperature range from 5 to 300 K.

3. Results and discussion

Typical TEM micrographs (see Fig. 2) of sample BR15 (BR16) revealed spherically shaped magnetite nanoparticle. Those images had been using to count 340 and 210 nanoparticles for samples BR15 and BR16, respectively, in order to mount the histograms showed in Fig. 2. The histograms have been modeling using the lognormal distribution function [18]. The fit of data provides the average diameter (D_{TEM}) of 9.9 (10.5) nm for sample BR15 (BR16) and diameter dispersion (σ_{TEM}) of 0.23 (0.22) for sample BR15 (BR16). The XRD data (see Fig. 3) of samples BR15 and BR16 presented broad x-ray diffraction peaks, characteristic of nanoparticulated materials. The Bragg peak positions identified in both samples (BR15 and BR16) are in accordance with the standard values of the magnetite phase (JCPDS card No. 19–0629). The (3 1 1) XRD peak (see Fig. 3) of both samples (BR15 and BR16) have been using to estimate the crystallite size (D_{XRD}) employing the Scherrer's approximation (see data in Table 1). Diameter values estimated from the XRD data (D_{XRD}) are in good agreement with those obtained from the TEM image analyses (D_{TEM}). The larger values find for D_{TEM} (9.9 and 10.5 nm) while compared to D_{XRD} (7.9 nm) is likely due to the lack of good crystallinity at the surface layer, as already reported in the literature [19]. Table 1 also collected the values we find for the lattice parameter (a) and the (3 1 1) interplanar spacing (d_{311}) of samples BR15 and BR16, which are in good agreement with the standard values reported for magnetite.

➡ The amino group density for samples BR15 and BR16 was 18.47 mMol/mg and 31.5 mMol/mg of sample, respectively. The inversely proportional dependence of the amine groups density on

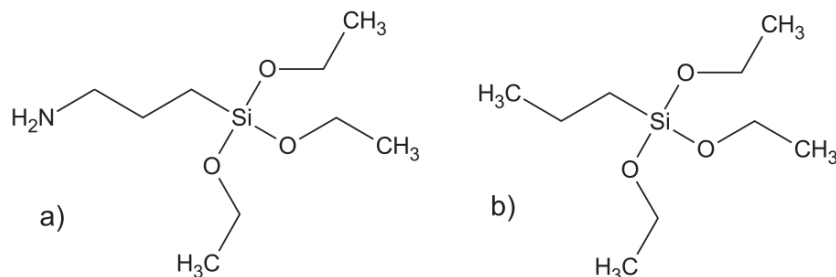


Fig. 1. (a) Chemical structure of APTS alkoxyisilane and (b) Chemical structure of NPTS alkoxyisilane.

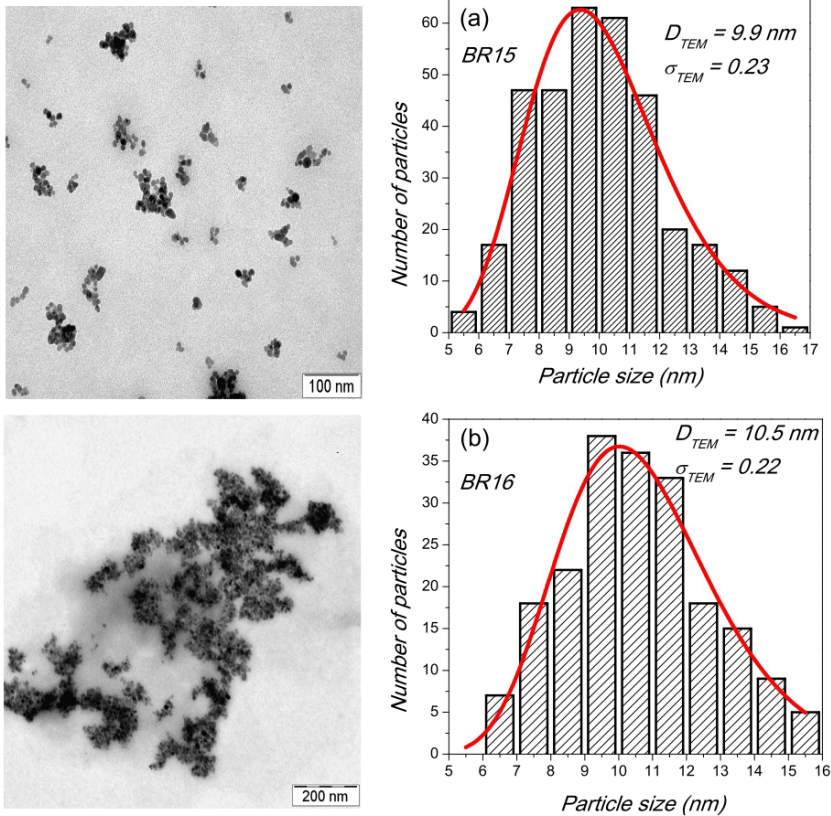


Fig. 2. TEM micrographs and corresponding particle size histograms of samples (a) BR15 and (b) BR16.

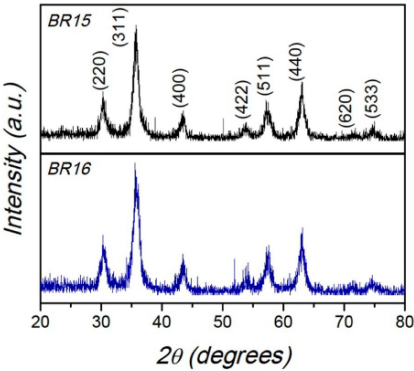


Fig. 3. X-ray diffraction patterns of samples BR15 and BR16.

Table 1
Lattice parameter (a), distance between 311 planes (d_{311}), and crystallite diameter (D_{XRD}).

Sample	a (Å)	d_{311} (Å)	D_{XRD} (nm)
BR15	8.34	2.51	7.9
BR16	8.35	2.52	7.9

the samples surface functionalized with APTS-NPTS can be explained in terms of the hydrolysis rate difference of the APTS and NPTS. In addition to the hydrolysis rate of the alkoxysilanes, the oscillatory mechanism of APTS adsorption on iron oxide surfaces must be considered. In 1990, H. Linde [20] suggested a dynamic model for the interaction of APTS with silica surfaces. The author postulated that the terminal amine group of the APTS molecule initially formed a salt with the hydroxyl groups on the surface and subsequently this orientation is reversed and the silane groups at the other end of the molecule will bound to the surface via siloxane bonds. In 1996, I. George et al. [21], demonstrated this mechanism through XPS (X-ray

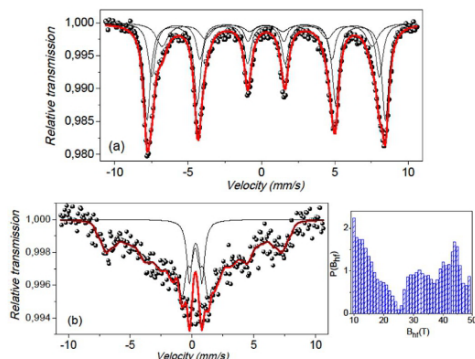


Fig. 4. (a) 77 K Mössbauer spectra of sample BR15 and (b) Room-temperature Mössbauer spectra of sample BR15 with the corresponding distribution of hyperfine field on the right hand-side.

photoelectron spectroscopy) analysis of the interface of APTS / silica films exposed to the ambient atmosphere and at different temperatures. On the other hand, studies of the adsorption of NPTS on iron oxide surfaces indicated that, unlike the APTS result, the oscillatory mechanism is not observed [22]. In contrast, the adsorption profile of NPTS in iron oxide exhibited behavior related to the Langmuir adsorption isotherm [23], indicating that the NPTS is adsorbed individually on the surface of the iron oxide until all the sites are occupied, saturating the surface. Thus, based on the observations and results of other similar systems investigated and reported in the literature, the mechanism of hydrolysis of the APTS-NPTS mixture can be inferred through a competitive adsorption model between APTS and NPTS before condensation. The hypothesis for increasing the amine density on the surface of the NP should consider the larger surface area of the NP functionalized with the APTS-NPTS mixture, due to the presence of a layer of NPTS covering the NP before the APTS condense on it.

Low-temperature (77 K) Mössbauer spectra of samples BR15 and BR16 (see upper panel in Figs. 4 and 5) are well resolved using three sextets (black solid lines); two of them describing iron ions in tetrahedral (A) and octahedral (B) sites in the core magnetite nano-

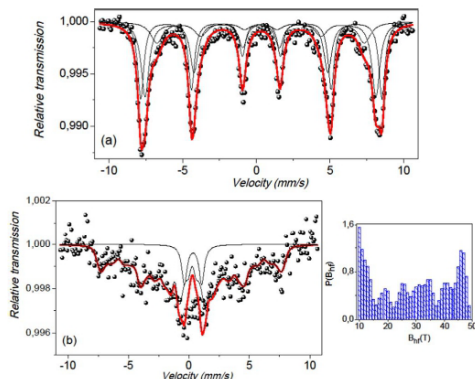


Fig. 5. (a) 77 K Mössbauer spectra of sample BR16 and (b) Room-temperature Mössbauer spectra of sample BR16 with the corresponding distribution of hyperfine field on the right hand-side.

particle. The third component however, showing the weakest hyperfine field (B_{hf}) and the broadest linewidth (Γ), has been assigning to iron ions located in defective sites, likely at the nanoparticle surface of the magnetite core where the density of defects (such as vacancies) is enhanced and the iron ions present a reduced coordination number due to the lack of translational symmetry. At room temperature (297 K), the Mössbauer spectra of samples BR15 and BR16 (see lower panel in Figs. 4 and 5) were curve-fitted using a doublet and a distribution of sextets, which represents a distribution of hyperfine fields and indicates the occurrence of magnetic collapse due to thermal relaxation of the particle's magnetic moment. The distributions of hyperfine fields obtained from the fittings of the room temperature Mössbauer spectra (samples BR15 and BR16) were also showed in Figs. 4 and 5. The room temperature Mössbauer doublet, with different values of isomer shift (IS) and quadrupole splitting (QS) for samples BR15 and BR16, represents the already relaxed particles and is included in the central part of the spectra [24]. Mössbauer parameters obtained from the fittings were collecting in Table 2.

► Differences observed in the Mössbauer data (see Table 2), particularly in regard to the weakest hyperfine field component at 77 K (B_{hf} and IS values), which has been ascribing to the shell magnetite nanoparticle, indicate major differences in the surface coating of both samples (BR15 and BR16). Additionally, the hyperfine field distributions obtained from the room temperature Mössbauer spectra (see Figs. 4 and 5) are quite different and may reflect the samples' surface coating differences as well. Note that the low temperature (77 K) Mössbauer parameters associated to the core magnetite nanoparticle (the two strongest hyperfine field components) show negligible differences for both samples. Therefore, the explanation for the differences observed in the room temperature Mössbauer spectra is associated to the shell magnetite nanoparticle and it is more likely correlated with the use of just one surface coating species to produce sample BR15 as opposed to two surface coating species while producing sample BR16. Moreover, sample BR15 is supposed to present one main nanostructure ($Fe_3O_4@ATPS$) whereas sample BR16 may present two main nanostructures ($Fe_3O_4@ATPS/NPTS$ and $Fe_3O_4@NPTS/ATPS$), which are different because they are either dominantly terminated with NPTS or APTS. In the BR15 sample the free electron pair of the APTS's amino group likely coordinates to iron ions at the nanoparticle surface, polarizing the shell magnetite nanoparticle and shifting the surface's character towards Fe(II)-rich, thus revealing a higher isomer shift of 0.47 mm/s and 0.39 mm/s associated to the Mössbauer sextets at 77 K and 297 K, respectively. Meanwhile, in the BR15 sample, the core magnetite nanoparticle may transfer electrons outwards to the shell magnetite nanoparticle, shifting the core's character towards Fe(III)-rich, thus presenting a lower isomer shift of 0.41 mm/s at 297 K. On the other hand, in the BR16 sample, the possibility of a double molecular coating layer (ATPS plus NPTS) may produce a final sample composed of two main nanostructures, as mentioned above ($Fe_3O_4@ATPS/NPTS$ and $Fe_3O_4@NPTS/ATPS$). It is reasonable to expect differences in IS values associated to the Mössbauer sextets while examining the two nanostructures $Fe_3O_4@ATPS$ (inner-shell)/ $NPTS$ (outer-shell) and $Fe_3O_4@NPTS$ (inner-shell)/ $ATPS$ (outer-shell). However, the effective IS values associated to the Mössbauer sextets of the end sample may change slightly, depending upon the relative fraction of the two possible nanostructures, as observed in the data presented in Table 2. Then, the reduction of the IS (0.41 mm/s and 0.37 mm/s) of sample BR16 (77 K and 297 K) with respect to the IS (0.47 mm/s and 0.39 mm/s) of sample BR15 (77 K and 297 K) more likely reflect the influence of the $Fe_3O_4@NPTS$ (inner-shell)/ $ATPS$ (outer-shell) nanostructure. The lower room temperature QS value associated to the doublet of sample BR15 (1.02 mm/s) with respect to sample BR16 (1.43 mm/s) supports the model picture of a Fe(III)-richer core magnetite nanoparticle for the sample BR15. Additionally, the broadening of the Mössbauer linewidth (77 K) associated to the shell magnetite sextet from sample BR15 (0.97 mm/s) to sample BR16 (1.01 mm/s) represents an extra support to the model picture of the presence of two nanostructures, more likely $Fe_3O_4@ATPS$ (inner-shell)/ $NPTS$ (outer-shell) and $Fe_3O_4@$

Table 2

Hyperfine parameters obtained from samples BR15 and BR16. Estimated uncertainties are ± 0.02 mm/s and ± 0.1 T. Error of the spectral areas is estimated at a maximum of 5%.

Sample	T (K)	Sextet					Doublet			
		IS (mm/s)	QS (mm/s)	B_{hf} (T)	Γ (mm/s)	A (%)	IS (mm/s)	QS (mm/s)	Γ (mm/s)	A (%)
BR15	297	0.39	−0.13	—	—	76	0.41	1.02	0.5	14
	77	0.45	−0.02	50.5	0.56	53	—	—	—	—
		0.40	−0.02	48.2	0.59	28	—	—	—	—
		0.47	0.06	44.5	0.97	19	—	—	—	—
BR16	297	0.37	−0.12	—	—	90	0.46	1.43	0.5	10
	77	0.45	0.00	50.8	0.49	39	—	—	—	—
		0.41	−0.04	48.6	0.64	42	—	—	—	—
		0.41	−0.02	43.7	1.01	19	—	—	—	—

NPTS(inner-shell)/ATPS(outer-shell). Even more visibly, the hyperfine field distributions assessed from the room temperature Mössbauer spectra (see lower panel, right hand-side plots, Figs. 4 and 5) also supports our model picture. Notice that the hyperfine field distribution associated to sample BR15 shows three main features; one lower field distribution correlated to the shell magnetite and two higher field distributions correlated to the core magnetite (site-A and site-B). However, the hyperfine field distribution associated to the BR16 sample shows a clear replica of the three main features as compared to the hyperfine field distribution of sample BR15, being more likely due to the presence of the two above-mentioned nanostructures, namely Fe_3O_4 @ATPS(inner-shell)/NPTS(outer-shell) and Fe_3O_4 @NPTS(inner-shell)/ATPS(outer-shell). In the BR16 sample we can see one duplicated (replica) lower field distribution assigned to the shell magnetite nanoparticle and two duplicated (replica) higher field distributions assigned to the core magnetite nanoparticle (site-A and site-B). Finally, the 77 K hyperfine field value of the shell magnetite nanoparticle of BR15 sample (44.5 T) is slightly stronger than the hyperfine field value of the shell magnetite nanoparticle of BR16 sample (43.7 T). This is more likely due the influence of the free electron pair of the ATPS's amino group, which increases the interaction between the surface molecular coating and the magnetite shell layer, leading to the enhancement of the exchange interaction within the nanoparticle shell layer. This finding also supports our model picture for the presence and influence of the two nanostructures in the BR16 sample as opposed to one single nanostructure in the BR15 sample. Indeed, while compared to sample BR16 the higher Si/Fe content ratio (0.25) found in sample BR15 indicates the strongest interaction of the Si-based moiety (ATPS) with the shell magnetite, likely due to the presence of the amino group.

The magnetic measurements (in the range of ± 70 kOe) were carrying out for both samples (BR15 and BR16) at low (5 K) and high (300 K) temperatures. Magnetization *M* versus magnetic field (*H*) curves recorded at 300 K (see upper panel in Fig. 6) showed no hysteresis, which is consistent with the superparamagnetic behavior of magnetite nanoparticles with average diameter around 10 nm. However, at low temperature (5 K), both samples showed hysteretic *M* vs *H* curves, meaning that both samples are in the blocked state (see lower panel in Fig. 6). At 5 K, coercive fields of 209 Oe and 198 Oe were determining for samples BR15 and BR16, respectively. Moreover, at 5 K the relative remanent magnetization (*M_r/M_s*) of 0.16 and 0.15 had been estimating for samples BR15 and BR16, respectively (see Table 3). These findings, namely higher coercive field and higher relative remanent magnetization observed in sample BR15, are in agreement with the picture of the nanoparticle's shell layer built from the analyses of the Mössbauer data, i.e. the enhancement of the exchange interaction within the nanoparticle's shell layer in sample BR15 with respect to sample BR16, which is credited to the stronger interaction of the ATPS's amino group and the nanoparticle's surface. Another interesting feature is the value of the rate *M* vs *H* in the high field region (above 40 kOe) of the *M* vs *H* curve. In the saturation region, sample BR16 shows a rate of 8×10^{-5} emu/gOe, which is almost twice the one determined for sample BR15. This means that a stronger magnetic disorder sets in the nanoparticle's shell layer in sample BR16, which is

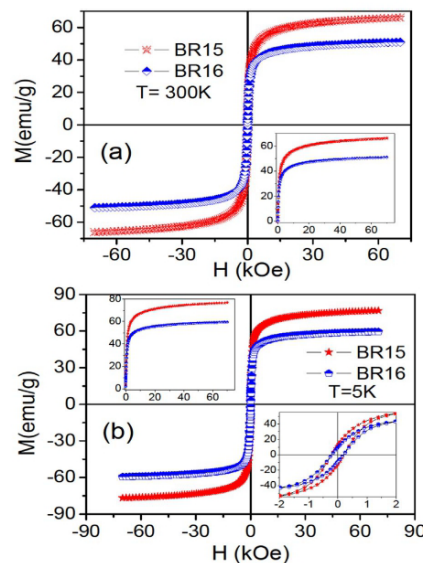


Fig. 6. Magnetization vs magnetic field curves of samples BR15 and BR16 at (a) 300 K and (b) 5 K.

Table 3

Magnetic parameters of samples BR15 and BR16.

Samples	<i>T</i> (K)	<i>M_s</i> (emu/g)	<i>M_r</i> (emu/g)	<i>M_r/M_s</i>	<i>H_c</i> (Oe)	<i>d_m</i> (nm)
B15	300	66.0	2.7	0.04	−0	4.08
	5	76.7	12.6	0.16	209	—
B16	300	50.9	1.6	0.03	−0	5.42
	5	59.4	9.2	0.15	198	—

probably related to the presence of the double molecular coating layer (ATPS plus NPTS), as opposed to the single molecular coating layer (ATPS only) in sample BR15. This finding is in very good agreement with the analysis carried out from the Mössbauer data, as supported by the lower (higher) value of the hyperfine field (linewidth) associated to the sextet assigned to the nanoparticle's shell layer in sample BR16 while compared with the values obtained from sample BR15. The initial magnetization rate of the curves at high temperatures, where the samples are expected to show a superparamagnetic behavior, can be used to estimate the magnetic size of the nanoparticles via the following relation [25]:

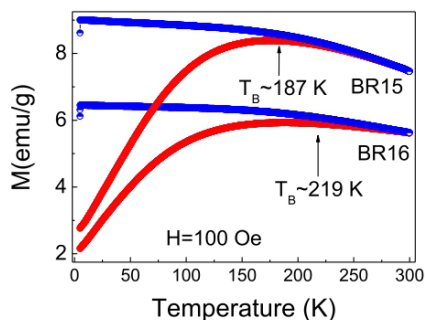


Fig. 7. Zero field-cooled (ZFC) and field-cooled (FC) curves of samples BR15 and BR16.

$$d_m = \left[\frac{18kT \left(\frac{dM}{dT} \right)_{H=0}}{\pi \rho M_s^2} \right]^{1/3}$$

where d_m is the magnetic diameter, k the Boltzmann constant, T the temperature (300 K), dM/dT the slope at near zero-field for samples BR15 (0.0645 emu/gOe) and BR16 (0.0901 emu/gOe), and ρ the density of magnetite (5.18 g/cm³). The calculation leads to $d_m = 4.08$ nm and $d_m = 5.42$ nm for samples BR15 and BR16, respectively. The values we find for the magnetic diameters are smaller than the values calculated from the TEM micrographs. This result strongly suggests the presence of a magnetic disordered shell layer on the nanoparticle's surface as widely reported in the literature [25,26]. Zero field-cooled (ZFC) and field-cooled (FC) curves (see Fig. 7) show features consistent with thermal relaxation of magnetic moments. The ZFC curves show a maximum at 187 K for the sample BR15 and 219 K for the sample BR16. Assuming these values as representatives of the average value of the blocking temperature it means that the particle-particle interaction in the sample BR16 is stronger than the particle-particle interaction taking place in the sample BR15. Once more, the picture of the nanoparticle's shell layer built from the analyses of the Mössbauer data helps to explain the apparent paradox. While in the sample BR15 the amino group of the surface coating (ATPS) faces inward binding to the nanoparticle's surface in the sample BR16 a fraction of the nanoparticles presents the Fe₃O₄@ATPS(inner-shell)/NPTS(outer-shell) structure. In the latter case, more likely the surface molecular layer interface shares the common ethoxy groups (CH₃-O-), leaving outwards the propyl group (CH₃-CH₂-CH₂-), which extends out of the nanoparticle's surface as a tail-like structure. The tail-like structure in the surface of the nanoparticles comprising the sample BR16 may favor inter-particle interaction via van der Waals' attraction more efficiently than the smoother nanoparticle surface (dressed only with ethoxy groups) of the nanoparticles comprising the sample BR15. Indeed, the stronger van der Waals inter-particle attraction among nanoparticles in the sample BR16 favors the magnetic dipole-dipole interaction more efficiently than in the case of nanoparticles in the sample BR15, likely leading to differences in the maximum observed in the ZFC curves, i.e. lower for sample BR15 (187 K) in comparison with sample BR16 (219 K).

4. Conclusions

The synthesis of magnetite nanoparticles via sonoprecipitation was successfully obtained. In addition, surface-functionalization of the as-produced nanosized magnetite via hydrolysis and condensation of alkoxyisilane agents (3-aminopropyltrimethoxysilane and propyltrimethoxysilane) was successfully demonstrated. Transmission electron microscopy of the surface-functionalized nanoparticles revealed spherically-shaped magnetite with an average diameter in agreement with the size estimated from x-ray diffraction data. Mössbauer data recorded at

77 K confirmed the magnetite phase at the core of the particle in both samples BR15 and BR16; meanwhile, in the shell region of the core magnetite, the influence of the type of surface molecular coating is more effective than the number of coating layers. Mössbauer data analyses revealed features consistent with unblocked particles and effects related to the free electron pair of the ATPS's amino group, which drives to differences in the hyperfine parameters between the one surface-coating agent (sample BR15) and two surface-coating agents (sample BR16). Magnetization data analysis indicated a strong influence of the surface-coating on the magnetic properties of the as-prepared materials. Once superparamagnetic iron oxide particles with appropriate surface coating are widely used in numerous in vivo applications, as for instance in MRI (magnetic resonance imaging) contrast enhancement, the success of attaching alkoxyisilane-coating agents onto SPIO particles is potentially interesting for MRI contrast agent and MPI technologies.

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II.4. Contribuições na modificação de superfície de nanopartículas magnéticas com ácido fólico para tratamento de câncer via hipertermia magnética

II.4.1. Hipertermia

*“Quae medicamenta non sanat; ferrum sanat.
Quae ferrum non sanat; ignis sanat.
Quae vero ignis non sanat; insanabilia reportari oportet”
(HIPOCRATES – [32])*

*Doenças cujos medicamentos não curam; a faca cura.
Doenças cuja faca não cura; o fogo cura.
Doenças cujo fogo não cura; devem ser reconhecidas como incuráveis.*

O uso de hipertermia em tratamentos de tumores é uma prática antiga na medicina. O pai da medicina, Hipócrates (460-370 ac), propôs que tumores superficiais poderiam ser curados através da cauterização com o uso de “ferro quente”. Atualmente, a comunidade científica concentra esforços no desenvolvimento da técnica de hipertermia magneticamente induzida. Nesta técnica, partículas magnéticas são acumuladas na superfície ou interior de células cancerígenas e depois submetidas a ação de um campo magnético alternado. Campos magnéticos não são absorvidos pelos tecidos vivos e podem atingir regiões profundas do organismo. Quando partículas magnéticas estão sob influência de um campo magnético alternado ocorre o aquecimento das partículas por perda de calor devido à reorientação da magnetização por histerese magnética [25]. A quantidade de calor gerado dependerá da natureza do material magnético, tais como tamanho e forma das partículas, e dos parâmetros do campo magnético tais como frequência de oscilação e força. Partículas nanométricas (monodomínios), ao contrário de partículas macrométricas (multidomínio), são mais estudadas para aplicação em hipertermia por absorverem mais energia em campos magnéticos toleráveis [26-28]. Assim, o potencial de aquecimento das NPs dependerá da distribuição de tamanho e forma das partículas, portanto, o desenvolvimento de rotas sintética de matérias com estreita distribuição de tamanho e forma uniforme é fundamental para a transferência da técnica de hipertermia da esfera acadêmica para aplicações propriamente clínicas.

Células cancerígenas são destruídas em temperaturas em torno de 43°C enquanto células normais podem sobreviver em temperaturas maiores. Assim, o tratamento por hipertermia causa a morte de tecidos cancerígenos sem prejuízo às células vizinhas ao tumor. Para tanto, as

pesquisas concentram-se na otimização da homogeneidade térmica em temperaturas moderadas (42-43°C) e com boa resolução volumétrica na área tratada [26,29]. A estratégia de hipertermia intracelular, primeiramente sugerida por Gordon et al. [30] e depois por outros autores [31], consiste em acumular NP magnéticas no tecido cancerígeno através do sistema de distribuição de drogas (DDS), conforme esquema apresentado na figura 11. Assim, as NP acumuladas podem ser usadas como ferramenta no diagnóstico do câncer através de imagem por ressonância magnética (MRI) ou por sensores de magneto impedância (MI). Após a localização do tumor, hipertermia poderá ser induzida através da aplicação de um campo magnético alternado na região sobre o tumor. Desta forma, NP magnéticas podem ser usadas na terapia de câncer ao mesmo tempo em que são usadas para diagnosticar a doença.

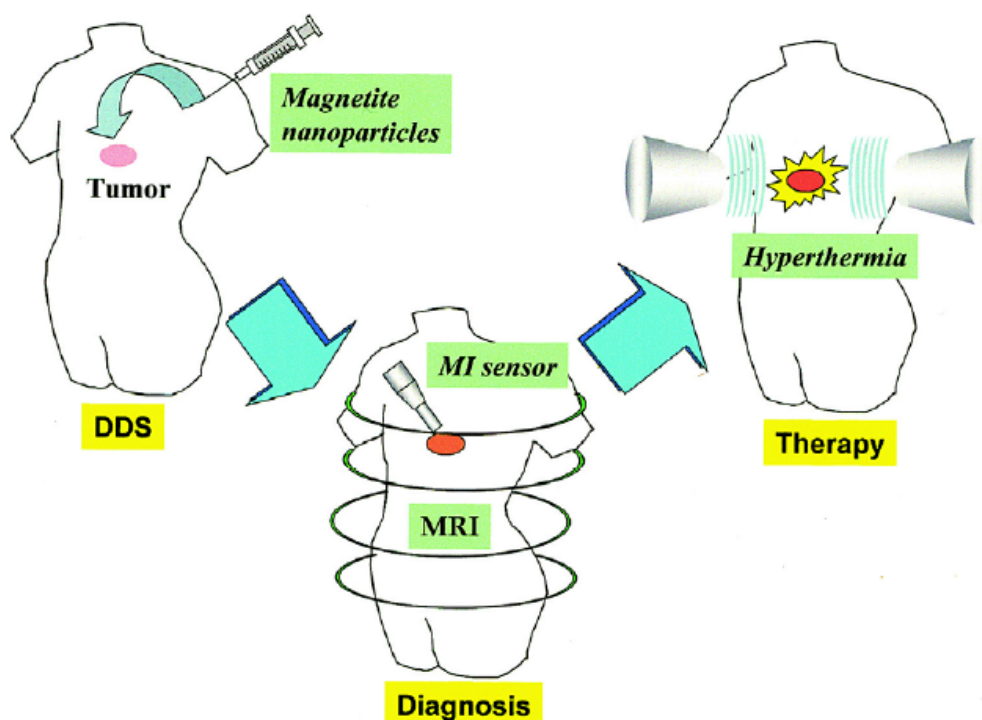


Figura 11: Ilustração esquemática da estratégia terapêutica usando NP magnéticas para diagnosticar e tratar tumores por hipertermia [32]

Para possibilitar a internalização das NP magnéticas nos tecidos cancerígenos, a superfície das NP deve ser funcionalizada e modificada de forma a permitir um tempo de circulação na corrente sanguínea suficiente para que o tecido em questão seja atingido. Esta internalização das NPs pode se dar por endocitose da fase líquida [33], endocitose mediada por receptores ou por fagocitose [34]. Para a modificação de superfície deve-se levar em conta a seletividade requerida, pois embora estudos demonstrem que certos recobrimentos com polímeros, tal como polietileno glicol (PEG) [35], aumentem a estabilidade e tempo de circulação das NP no organismo, este tipo de recobrimento permite que as NPs sejam capturados tanto pelos

tecidos saudáveis quanto pelos carcinomas, o que não é interessante pois a indução de hipertermia nestes casos causaria também a morte de tecidos saudáveis. No caso de tratamento de células cancerígenas, a modificação da superfície de nanopartículas pelo recobrimento com ácido fólico permite que as nanopartículas transitem através da membrana de células cancerígenas devido ao receptor foliato (RF) ser frequentemente mais ativo na superfície destas células [36,37]. Alta atividade do receptor foliato, geralmente ausente na maioria dos tecidos humanos, tem sido observada em diferentes tipos de células cancerígenas humanas [38]. Assim, o receptor foliato torna-se uma porta de entrada para a internalização de drogas ou agentes de contraste, quando estes apresentarem em suas superfícies moléculas de ácido fólico (figura 12) [39]. A origem do receptor foliato relaciona-se à presença da proteína glicosilfosfatidilinositol (GPI) na membrana glicoproteica, porém sua função na membrana celular ainda não é bem compreendida.

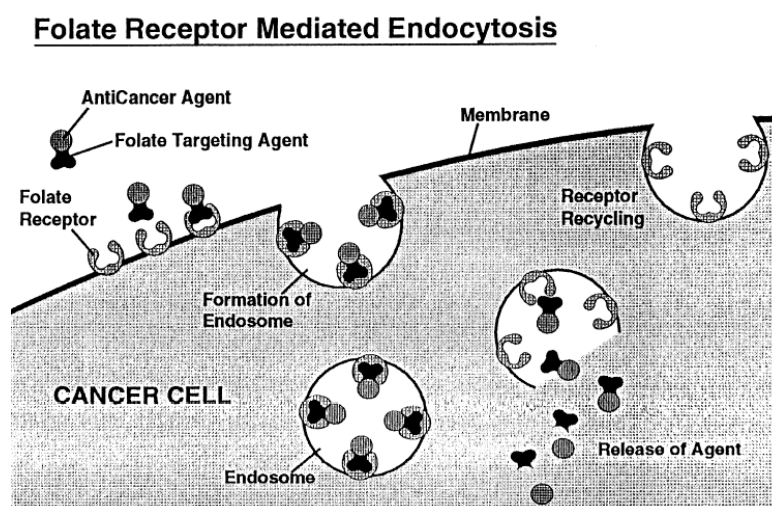


Figura 12: Representação esquemática da internalização de NP em células cancerígenas via endocitose mediada por receptor foliato na superfície das células [39].

II.4.2. Ligação peptídica como estratégia para ligar ácido fólico em superfície de nanopartículas magnéticas funcionalizadas com grupos aminas

Conforme discutido anteriormente, NP com ácido fólico na superfície são facilmente internalizadas por células cancerígenas devido ao receptor foliato ser frequentemente mais ativo na superfície de células cancerígenas. Assim, a modificação de NP com ácido fólico permitirá a internalização das NP superparamagnéticas em carcinomas para posterior tratamento via hipertermia. Porém, devido à dificuldade em ligar o ácido fólico diretamente sobre superfície de óxidos magnéticos, a superfície das NP deve ser funcionalizada antes com alcossilanos,

disponibilizando grupos amina na superfície das NP para posterior modificação com ácido fólico, conforme esquema ilustrado na figura 13. Sabe-se que alta afinidade do receptor foliato em relação à molécula de ácido fólico é mantida quando o ácido fólico é imobilizado em NP através de seu grupo carboxílico terminal γ (figura 13) [40]. Portanto, o ácido fólico deverá ser ligado aos grupos aminos na superfície das NP-APTS através do grupo ácido carboxílico γ ; com relação ao grupo carboxílico α não há necessidade em proteger este grupo visto que o impedimento estérico favorece a ligação com o grupo γ .

A ligação entre um grupo ácido carboxílico e uma amina acontece pela formação de uma amida. A funcionalidade da amida é observada nas rotas sintética de moléculas pequenas ou complexas na natureza. Porém, sínteses de proteínas envolvendo reações de acoplamento entre sequências de peptídeos (formação de ligação de amida entre α -aminoácidos ou peptídeos) são complexas, provavelmente para proteger e guardar a sequência de aminoácidos única e precisamente definida para cada proteína. Na natureza essa dificuldade é superada pela ativação dos aminoácidos através de catálise enzimática onde o aminoácido em questão é transformado em um amino-ester intermediário. Este intermediário é então envolvido em um processo bioquímico com centenas de macromoléculas (mRNAs, tRNAs, enzimas, fatores proteicos, ribossomos, etc.) para a formação da proteína [41].

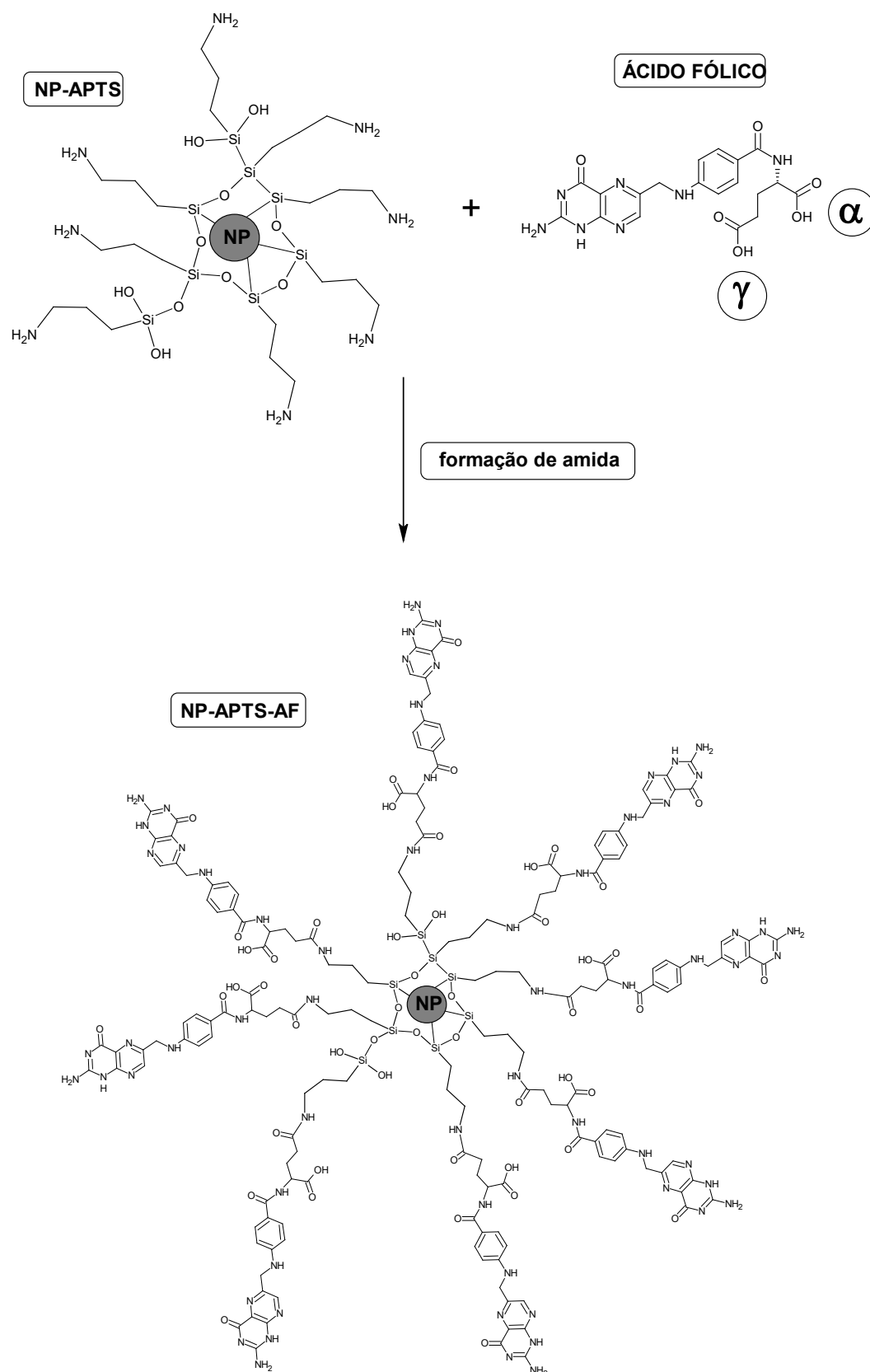


Figura 13: Esquema de modificação de superfície de SPION-APTS com ácido fólico.

Nanotechnology

PAPER

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Synthesis, characterization and applications of maghemite beads functionalized with rabbit antibodies

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Abstract

Magnetic nanoparticles (NPs) have attracted great attention owing to their applications in the biomedical field. In the present work, maghemite ($\gamma\text{Fe}_2\text{O}_3$) NPs of 6.5 nm were prepared using a sonochemical method and used to prepare magnetic beads through silanization with 3-aminopropyltrimethoxysilane (APTS). Subsequently, amino groups in the resulting APTS- $\gamma\text{Fe}_2\text{O}_3$ beads were converted to carboxylic acid (CARB- $\gamma\text{Fe}_2\text{O}_3$) through the succinic anhydride reaction, as confirmed by transmission electron microscopy (TEM), Fourier transform infrared spectroscopy and dynamic light scattering (DLS) measurements. The size of these beads was measured as 12 nm and their hydrodynamic diameter as 490 nm, using TEM analysis and DLS, respectively. The CARB- $\gamma\text{Fe}_2\text{O}_3$ beads were further functionalized by immobilizing rabbit antibodies on their surfaces; the immobilization was confirmed by flow cytometry and ionic strength. The samples were further characterized by Mössbauer spectroscopy and DC magnetization measurements. Studies on magnetic relaxivities showed that magnetic beads present great potential for application in MR imaging.

Keywords: magnetic nanobeads, flow cytometry, MRI, mössbauer spectroscopy, XRD, rabbit antibody

(Some figures may appear in colour only in the online journal)

1. Introduction

During the last few decades, researchers have been developing methods for the immobilization of proteins and enzymes on magnetic nanoparticles (NPs) [1], useful for magnetic resonance imaging (MRI) [2]. Various methods have been employed to synthesize iron oxide magnetic NPs, among the

most common being co-precipitation [3], sol-gel [4], solid state [5], sonochemical [6], microwave assisted [7] and thermal decomposition [8]. These solution based methods allow the production of NPs with controllable size, morphology and crystallinity, and variable surface chemistry. Their biocompatibility and appropriate functionalization are key conditions for their use in biomedicine [9]. Magnetic iron oxide NPs show superparamagnetic behavior, very low coercivity and high magnetic moment per gram of sample. To

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provide stability in water, iron oxide NPs are usually capped with a variety of functional groups such as amino (-NH₂) and carboxylic groups (-COOH). Magnetic beads are small particles consisting of a non-magnetic matrix—e.g. silica or polystyrene—with embedded ferromagnetic NPs [10]. This allows the manipulation of beads through magnetic forces, but avoids agglomeration of the beads since no permanent magnetic moment remains after removing an external magnetic field.

A wide selection of silica-coated magnetic NPs can be used as substrates for the attachment, through polar interactions, of a broad range of biomolecules. However, proteins do not normally bind effectively to silica beads; thus, a simple treatment to have more reactive surface groups, such as carboxylic groups (-COOH) [11] will greatly enhance protein binding.

The large surface-to-volume ratio of magnetic beads provides a further use for chemical bonding of target macromolecules [12]. Magnetic beads are well established for manipulation of biomolecules, such as proteins, cells, or bacteria [13], and can be applied in magnetic bioseparations [14]. Magnetic beads may also serve as contrast enhancing agents for MRI, for releasing heat upon absorption of radio frequencies, or they may be used for magnetic drug targeting [15, 16].

In this work, we will summarize a chemical route for the synthesis of magnetic beads using APTS and maghemite. Modification of the bead's surface was performed by succinic anhydride reaction. We have used rabbit antibodies to prove the ability of magnetic beads to couple proteins on their surface. The physical and chemical properties of magnetic beads were studied using Fourier transform IR spectroscopy (FTIR), zeta potential, transmission electron microscopy (TEM), x-ray diffraction analysis (XRD), dynamic light scattering (DLS), DC magnetization, Mössbauer spectroscopy and flow cytometry. Finally, we will apply the beads in various aqueous concentrations for MRI and in high-resolution molecular imaging.

2. Experimental

2.1. Materials

All chemicals were used as received from suppliers:

Iron (II) chloride tetrahydrate (97%), succinic anhydride (99.0%) and 3-aminopropyltrimethoxysilane (APTS) (98.0%) were purchased from Sigma-Aldrich Brazil. Dimethylformamide (DMF, 99.9%) was acquired from Panreac. Sodium hydroxide (NaOH, 97%) and ammonium hydroxide (NH₄OH, 30% (v/v)) were purchased from Synth. Iron (III) chloride hexahydrate and sodium chloride (99.0%) were purchased from Mallinckrodt Chemicals.

2.2. Synthesis of magnetic NPs (γ -Fe₂O₃) and beads with amine groups (APTS- γ -Fe₂O₃)

Magnetite was synthesized by sonoprecipitation method [17]. The NPs were obtained by dissolving 1.019 g of FeCl₂ · 4H₂O and 2.773 g of FeCl₃ · 6H₂O (in a molar ratio 1:2) in 80 ml of

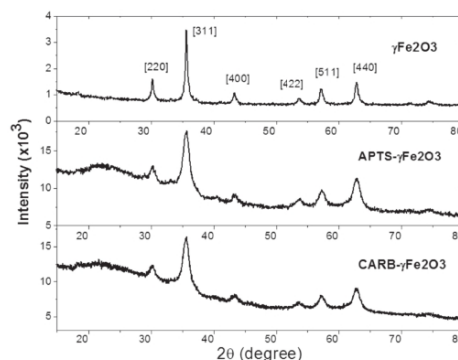


Figure 1. Diffraction patterns of bare iron oxide γ -Fe₂O₃, APTS- γ -Fe₂O₃ and CARB- γ -Fe₂O₃ beads. Numbers are the Miller indices.

distilled water. An aqueous solution at 80 °C was prepared with sodium hydroxide and 1.0 ml aliquots of H₂O added until the solution reached pH 12. Then 1.6 ml of the iron solution was added to the above alkaline solution. This solution was agitated with an ultrasonic tip probe for one hour, with 3 s of pulse on and 3 s of pulse off. Transformation of magnetite to maghemite occurred after exposing the aqueous suspension of NPs to air at room temperature for one week.

To prepare magnetic beads, we added 200 μ l of APTS to the above maghemite suspension. The as-produced beads were labeled as APTS- γ -Fe₂O₃.

2.3. Synthesis of beads with carboxylic groups (CARB- γ -Fe₂O₃)

In a second step, 200 mg of APTS- γ -Fe₂O₃ beads was dispersed in 30 ml of dimethylformamide (DMF). A solution of 0.3 g of succinic anhydride and 20 ml of DMF was prepared. These solutions were mixed and placed in a distillation flask with bubbling argon at room temperature and for 8 h [18].

2.4. Functionalization of magnetic beads with rabbit antibodies

The functionalization of magnetic beads with rabbit antibodies was done by using the methodology reported by Greg [19]—briefly, 20 mg of maghemite beads were washed three times with phosphate buffer (0.01 M) and re-suspended in 1 ml of ultrapure water. Then, 1 ml of rabbit antibody at various concentrations (10, 8, 5, 2.5, 2, 1 and 0.5 μ g ml⁻¹), 400 μ l (0.54 mg ml⁻¹) EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride) and 5 mM NHS (N-hydroxysuccinimide) were added to the magnetite dispersion. The beads were washed again, and the blocking buffer (1 M glycine at pH 8.0) was added and mixed for 1 h. Then, fluorescent secondary antibody (Anti-rabbit Alexa 488) was added at the dilution of 1/200, incubated for 30 min, and washed twice with PBS. Finally, the magnetic beads were measured by flow cytometry (FACS Canto II).

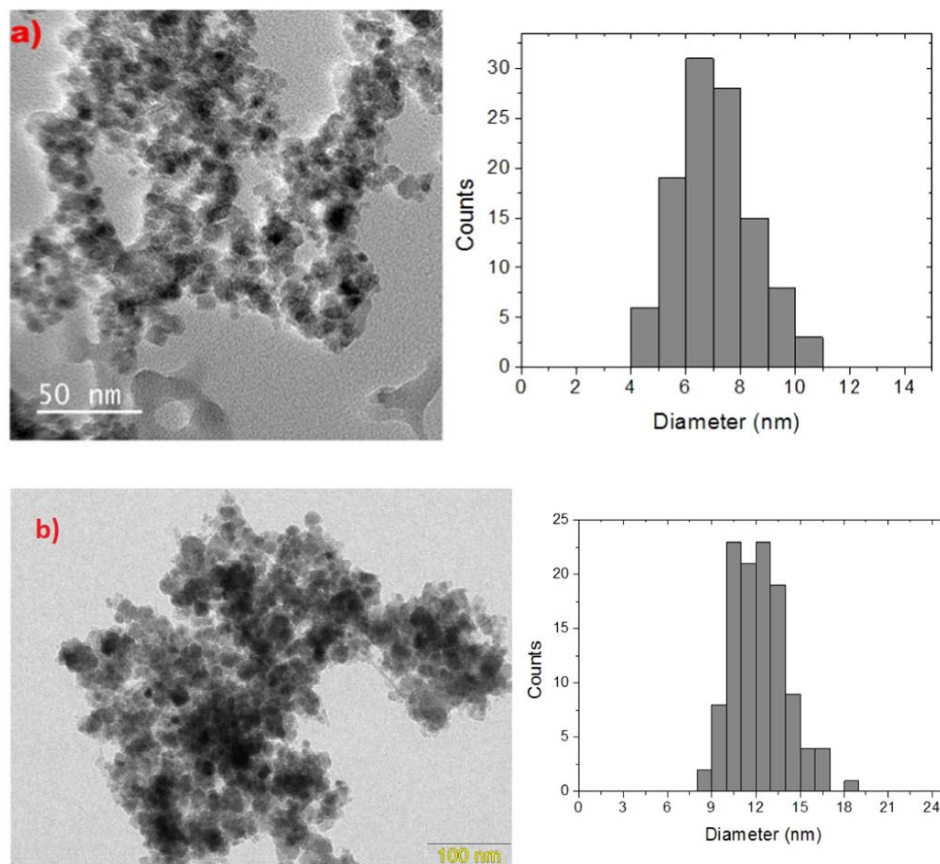


Figure 2. (a) TEM micrograph of bare $\gamma\text{-Fe}_2\text{O}_3$ NPs, and their size distribution. (b) TEM micrograph of APTS- $\gamma\text{-Fe}_2\text{O}_3$ beads, and their size distribution.

2.5. XRD measurements

Structural characterization of samples was carried out with XRD using a MiniFlexII Rigaku diffractometer equipped with a $\text{CuK}\alpha$ radiation source. XRD data were collected in the 2θ range between 10° and 85° with a scan rate of $5^\circ/\text{min}$ and steps of 0.02° .

2.6. TEM

A TEM micrograph was obtained on a Philips CM120 microscope. The beads were dispersed in isopropanol and one drop of the dispersion was deposited on a carbon covered copper grid.

2.7. Infrared spectroscopy (FTIR)

FTIR spectra were recorded in the reflectance mode on a Perkin–Elmer Frontier spectrometer to identify the composition of the NPs and the lipase content. The samples were milled, dispersed in KBr and pressed into pellets. Spectra were recorded in the range of $4000\text{--}400\text{ cm}^{-1}$ with a nominal resolution of 4.0 cm^{-1} and through 64 scans.

2.8. Zeta potential and DLS measurements

Zeta potential and the particles' hydrodynamic size were measured using a Zetasizer Nanoseries NanoZS, from Malvern Instruments. For Zeta potential measurements, the samples were previously suspended in $\text{NaCl } 1\text{ mmol L}^{-1}$.

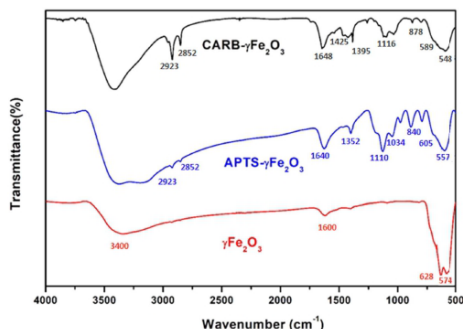


Figure 3. Infrared spectroscopy of $\gamma\text{Fe}_2\text{O}_3$, APTS- $\gamma\text{Fe}_2\text{O}_3$ and CARB- $\gamma\text{Fe}_2\text{O}_3$ samples.

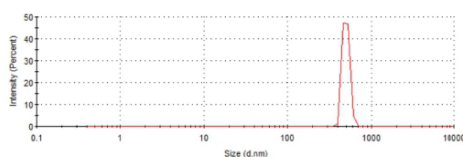


Figure 4. DLS of APTS- $\gamma\text{Fe}_2\text{O}_3$ NPs dispersed in water.

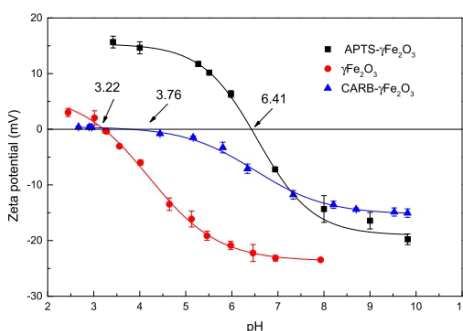


Figure 5. pH dependence of Zeta potential of $\gamma\text{Fe}_2\text{O}_3$, APTS- $\gamma\text{Fe}_2\text{O}_3$ and CARB- $\gamma\text{Fe}_2\text{O}_3$ samples.

solution. The Zeta potential was evaluated in the pH range of 2–10 and the samples were titrated with NaOH and HCL 0.1 mol L⁻¹ solution.

2.9. Flow cytometry

The samples were analyzed by FSC (Forward Scatter) size, SSC (Side Scatter) granularity and fluorescence in a flow cytometer (FACS Canto®II), and the results were analyzed using FlowJo® software version 9.3.2 of the Tree Star © with the flow cytometry platform at Fiocruz-ILMD.

2.10. Mössbauer spectroscopy

⁵⁷Fe Mossbauer spectra were recorded in the transmission mode at 300 and 12 K by using a spectrometer from SEECo equipped with a helium closed cycle cryostat from Janis; the gamma ray source had an activity of 25 μCi . The spectra were fitted by using Normos90 software; the reported isomer shifts are related to $\alpha\text{-Fe}$ metal at room temperature.

2.11. Magnetic characterization

DC magnetization measurements were made in a physical properties measurement system, (PPMS-Dynacool from Quantum Design) equipped with a vibrating sample magnetometer (VSM). The magnetization as a function of temperature was determined in the zero field cooled (Mzfc) and field cooled (Mfc) modes under a magnetic field of 70 Oe; both measurements were made in the warming mode.

2.12. MRI

The transverse (T_2) and longitudinal (T_1) relaxation times and relaxivities (r_2 , r_1) of as-prepared NPs were performed on a 0.55 T MRI instrument (Shanghai Niumai Corporation Ration NM120-Analyst). Dilutions of iron oxide NPs samples in water with various concentrations (0.08, 0.18, 0.36, 0.72 and 1.43 mM) were used for transverse and longitudinal relaxation times and T_2 imaging with additional concentrations (2.84 and 5.68 mM) as compared to water. Relaxation times T_2 and T_1 measurements were performed using TE (echo time) = 20 ms and TR (repetition time) = 4000 ms. T_2 -weighted MR images were performed on running a SE (spin-echo) sequence with TR = 1600 ms and TE = 20 ms.

3. Results and discussion

3.1. Structural characterization

3.1.1. XRD. The patterns of bare maghemite particles and beads are shown in figure 1. The diffractograms show peaks relative to the inverse spinel structure due to maghemite, no second phase like hematite or ferrihydrite was found. By using the Scherrer equation and the full width half maximum of the peak [311], [220], [511] and [440] we have determined the $\gamma\text{Fe}_2\text{O}_3$ particle size of (6.9 ± 0.9) nm, the samples APTS- $\gamma\text{Fe}_2\text{O}_3$ and CARB- $\gamma\text{Fe}_2\text{O}_3$ presented similar crystallite sizes to the bare $\gamma\text{Fe}_2\text{O}_3$ NPs, revealing that the procedure of coating with succinic anhydride and 3-aminopropyltrimethoxysilane do not affect the magnetic core. In the APTS- $\gamma\text{Fe}_2\text{O}_3$ and CARB- $\gamma\text{Fe}_2\text{O}_3$ patterns, the broad peak at about 22° is related to the amorphous SiO_2 . The diffraction peaks in figure 1 are consistent with the standard structure of maghemite (JCPDS card No. 39-1346).

3.1.2. TEM analyses. A TEM image of bare $\gamma\text{Fe}_2\text{O}_3$ NPs is presented in figure 2(a) along with their size distribution; the NPs have a spherical morphology and are not aggregated. The size distribution has a mean diameter of 6.5 nm. This result is

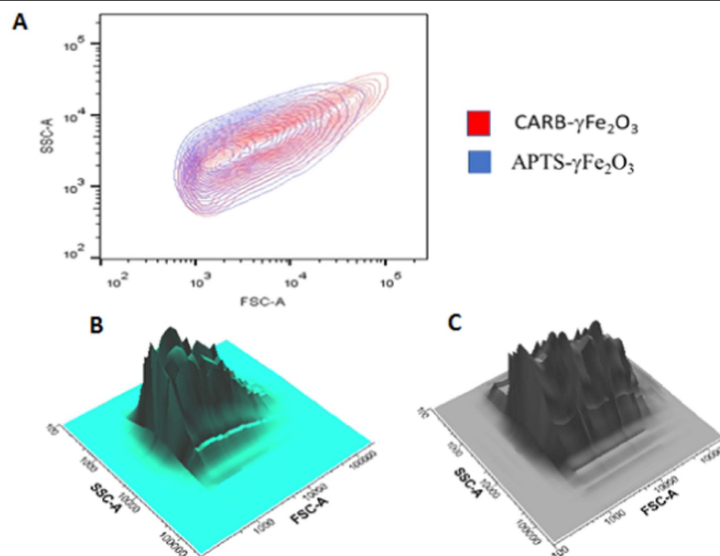


Figure 6. Overlap of APTS- $\gamma\text{Fe}_2\text{O}_3$ and CARB- $\gamma\text{Fe}_2\text{O}_3$ beads by FSC size and complexity SSC (A), surface chart of the correlation between FSC, SSC and the amount of NPs demonstrated by the APTS- $\gamma\text{Fe}_2\text{O}_3$ beads (B) and CARB- $\gamma\text{Fe}_2\text{O}_3$ beads (C).

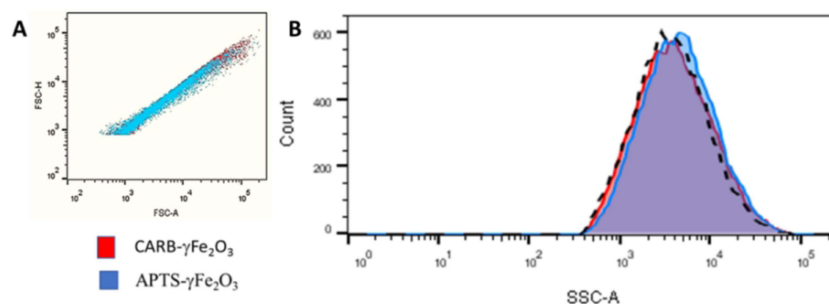


Figure 7. Evaluation of the uniformity of the samples correlating the size of the FSC-A area by the FSC-H height.

in close agreement with the crystallite size obtained in the XRD analysis.

A TEM image of APTS- $\gamma\text{Fe}_2\text{O}_3$ beads is presented in figure 2(b) along with their size distribution; the beads have a rounded morphology and form small agglomerates. The size distribution of the sample has a mean diameter of 12 nm. This result confirms the SiO_2 coating of the $\gamma\text{Fe}_2\text{O}_3$ NPs.

3.2. Vibrational characterization of magnetic beads

3.2.1. Infrared spectroscopy. The FTIR spectrum presented in figure 3 shows bands at 628 and 574 cm^{-1} which are assigned to the Fe–O stretching vibration in $\gamma\text{Fe}_2\text{O}_3$, these bands appear

slightly shifted in the spectra of the APTS- $\gamma\text{Fe}_2\text{O}_3$ and CARB- $\gamma\text{Fe}_2\text{O}_3$ samples. The bands at 3400 and 1600 cm^{-1} correspond to the O–H stretching and bending vibrations of adsorbed water, respectively [20]. The spectrum of the APTS- $\gamma\text{Fe}_2\text{O}_3$ sample has absorption bands at 1034 cm^{-1} and 1110 cm^{-1} which correspond to Si–O stretchings, confirming the silanization reaction. The amine group shows N–H out-of-plane bending at 870 cm^{-1} and C–N stretchings at 1352 cm^{-1} . The asymmetric and symmetric absorption bands at 1648 and 1425 cm^{-1} correspond to carboxylate stretching vibrations, and confirm the functionalization with succinic anhydride. The bands located in between 1425–1380 cm^{-1} are a combination of C–O stretchings and O–H deformation vibration. The band at

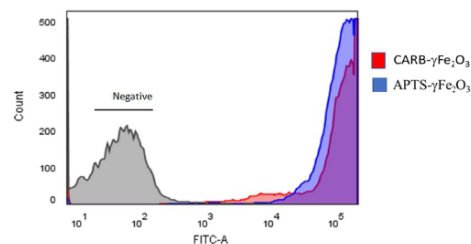


Figure 8. Overlays of the histograms showing the fluorescence of the secondary Alexa 488 Anti-Rabbit in the APTS- $\gamma\text{Fe}_2\text{O}_3$ and CARB- $\gamma\text{Fe}_2\text{O}_3$ beads conjugated in $10\ \mu\text{g}$ concentrations of primary rabbit.

$1264\ \text{cm}^{-1}$ indicates the presence of carboxylic acid dimer [20]. Furthermore, the signals at $2923\ \text{cm}^{-1}$ and $2852\ \text{cm}^{-1}$, correspond to asymmetric and symmetric C–H vibrations, these bands are also observed in CARB- $\gamma\text{Fe}_2\text{O}_3$ and APTS- $\gamma\text{Fe}_2\text{O}_3$ samples.

3.2.2. DLS and zeta potential. Figure 4 shows the DLS measurement of APTS- $\gamma\text{Fe}_2\text{O}_3$ particles in water. It shows beads with hydrodynamic mean diameter of $490\ \text{nm}$. Comparison of bead size from DLS and TEM techniques reveal aggregation of this sample.

In addition, the magnetic beads were evaluated by Zeta potential measurements. The isoelectric point (IEP) corresponds to the regime where the net charge on the NPs is zero. The zeta potentials as a function of pH curves are shown in figure 5. The IEP for $\gamma\text{Fe}_2\text{O}_3$ NPs is located at $\text{pH} = 3.22$. It can be seen that the polycondensation with APTS shifted the IEP to 6.41, which corresponds to the APTS pKa value. Therefore, the APTS- $\gamma\text{Fe}_2\text{O}_3$ beads have a positive electrical potential at low pH (<6.41) and negative potential at high pH [21, 22]. Similar behavior is observed after functionalization of APTS- $\gamma\text{Fe}_2\text{O}_3$ beads with succinic anhydride. The pKa value of succinic acid is 4.2, while the IEP for CARB- $\gamma\text{Fe}_2\text{O}_3$ is at 3.76; this result confirms the functionalization of APTS- $\gamma\text{Fe}_2\text{O}_3$ with carboxylic groups.

3.3. Morphometric analysis of beads (size and complexity)

The morphometric characteristics of the NPs were analyzed by flow cytometry via the fluorescence detector parameters that measure the degree of direct scattering (FSC, Y-axis), and the degree of lateral dispersion or complexity—lateral dispersion (SSC, X-axis), and plotted on a logarithmic scale (figure 6(A)). The results show that the APTS- $\gamma\text{Fe}_2\text{O}_3$ beads were smaller in size than the CARB- $\gamma\text{Fe}_2\text{O}_3$ beads observed in 3D-size plots by complexity (figures 6(B), (C)).

3.3.1. Morphometric analysis of beads (uniformity). Although the initial morphometric characteristics showed that the particles had different sizes, it was verified that there was aggregation between the particles during the treatment; the

singlet was analyzed by checking the particle uniformity in the parameters of lateral dispersion intensity (SSC-A) and uniformity (FSC-A) and (FSC-H). The results show that there is no difference between mean lateral dispersion intensity in both particle types compared to the untreated control (figure 7(B)) and that the particles follow a uniformity pattern (figure 7(A)) of 98.7% for APTS- $\gamma\text{Fe}_2\text{O}_3$ and 97.4% for CARB- $\gamma\text{Fe}_2\text{O}_3$.

Thus, we have performed several experiments to characterize the morphometric parameters of the beads essential to applications in biotechnology; the purity analysis can facilitate the identification of beads by flow cytometry.

3.3.2. Evaluation of fluorescence coupling capacity in beads.

The uniformity of the samples correlating the size of the FSC-A area by the FSC-H height (see figure 8) was also evaluated, showing that both samples have approximately uniform sizes comprising 92.2% and 92.9% within the analysis range, respectively.

Two subpopulations of coupled NPs were analyzed, in order to determine the percentage of fluorescence coupling according to the types of pretreatment; these results are presented in table 1.

The percentage of coupling is calculated by the initial analysis of the percentage of events within the gate determined by size and complexity (figure 6); after determining the area of analysis of the beads, the percentage of fluorescence is determined in relation to the beads without coupling or cut off. The fluorescence above the cut line determines the percentage of fluorescence (figure 8) calculated through the Flowjo program or by multiplying the fluorescence gate (fluorescence) mean by the gate (count) of size and complexity.

In the fluorescence analysis, the histograms show that both samples have about 95% coupling; this is confirmed in figure 8 by the overlapping histogram graph, that shows two populations of magnetic NPs: low coupling and high coupling (see table 1). APTS- $\gamma\text{Fe}_2\text{O}_3$ magnetic beads exhibited larger populations with high coupling percentage (93.3%) when compared to CARB- $\gamma\text{Fe}_2\text{O}_3$ magnetic beads (85%) at either of the $10\ \mu\text{g}$ or $0.5\ \mu\text{g}$ of rabbit antibody concentrations shown in table 1. However, no significant variation of fluorescence between dilutions of the antibody in the coupling was observed, indicating that a test with smaller antibody values would be required in order to determine the best concentration for coupling and the limiting dilution.

3.4. Magnetic studies

Figure 9 shows the magnetization as a function of magnetic field of CARB- $\gamma\text{Fe}_2\text{O}_3$ and APTS- $\gamma\text{Fe}_2\text{O}_3$ beads. At 300 K, the CARB- $\gamma\text{Fe}_2\text{O}_3$ and APTS- $\gamma\text{Fe}_2\text{O}_3$ samples showed saturation magnetizations of $27.4\ \text{emu g}^{-1}$ and $26.4\ \text{emu g}^{-1}$, respectively. At 5 K, their saturation magnetizations were of $35.5\ \text{emu g}^{-1}$ and $33.9\ \text{emu g}^{-1}$ for CARB- $\gamma\text{Fe}_2\text{O}_3$ and APTS- $\gamma\text{Fe}_2\text{O}_3$ beads, respectively. The low saturation magnetization is due to the SiO_2 contribution to the sample mass—as shown in figure 2, there is a thick layer of SiO_2 on the surfaces of $\gamma\text{Fe}_2\text{O}_3$ NPs—and to the

Table 1. Percentage of APTS- γ -Fe₂O₃ and CARB- γ -Fe₂O₃ beads coupled with antibodies separated into high coupling and low coupling.

Sample	High %		Low %	
	APTS- γ -Fe ₂ O ₃	CARB- γ -Fe ₂ O ₃	APTS- γ -Fe ₂ O ₃	CARB- γ -Fe ₂ O ₃
CN	0	0.052	0.37	2.57
10 μ g	95.2	88.1	4.62	10.4
8 μ g	88.9	76.2	11	2.7
5 μ g	93.1	87	6.8	10.6
2.5 μ g	95.9	82.4	3.92	15.9
2 μ g	90.9	89	8.94	9.32
1 μ g	96.6	81.1	3.29	14.2
0.5 μ g	94.8	91	4.89	7.86
Mean	93.3	85	6.21	12.7
SD	2.83	5.24	2.85	4.49

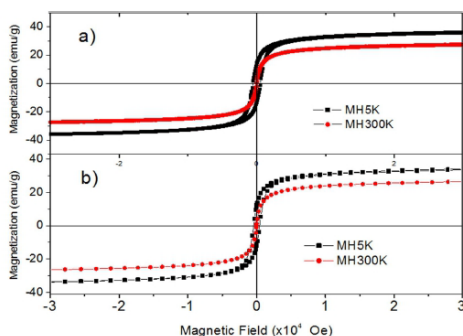


Figure 9. M–H curves at 5 K and 300 K for (a) Carboxylic acid (CARB- γ -Fe₂O₃) beads and (b) APTS (APTS- γ -Fe₂O₃) beads.

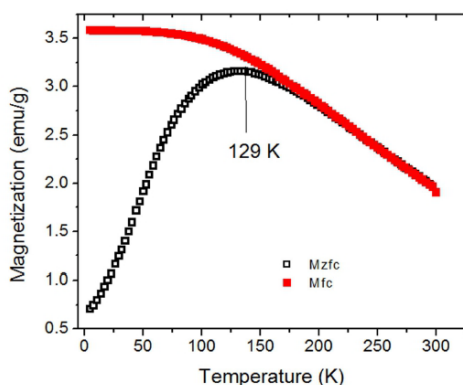


Figure 10. Mzfc and Mfc measurement under a field of 70 Oe for APTS- γ -Fe₂O₃ beads.

reduced γ -Fe₂O₃ particle size, which enhances the total surface of the sample; in this case, the outer Fe layers have broken bonds and their Fe moments may experience a canting effect and therefore show a lower saturation magnetization [23].

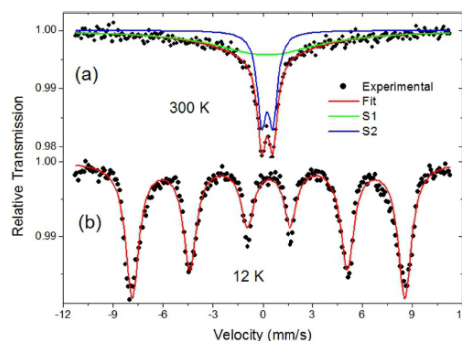


Figure 11. Mössbauer spectroscopy measurements performed at (a) 300 K and (b) 12 K for CARB- γ -Fe₂O₃ beads.

At room temperature the coercivity field for CARB- γ -Fe₂O₃ and APTS- γ -Fe₂O₃ samples were smaller than 50 Oe. Although the NPs are below the critical size to be superparamagnetic at room temperature, the low coercivity field suggest that there is some agglomeration of NPs and there are dipolar interactions between them.

Figure 10 shows the Mzfc and Mfc measurements performed for the APTS- γ -Fe₂O₃ sample. The cusp observed at $T_B^{VSM} = 129$ K indicates the thermal blocking temperature for the APTS- γ -Fe₂O₃ NPs, this temperature may be larger than expected for a system of NPs without interaction [24]. The magnetic dipolar interaction between NPs can be studied through long-range dipolar forces in terms of the interacting superparamagnetic model and the Vogel–Fulcher law [24]. Since the CARB- γ -Fe₂O₃ sample was obtained by functionalization of the APTS- γ -Fe₂O₃ sample with succinic acid, it is reasonable to conclude that both samples should have similar blocking temperatures.

The ⁵⁷Fe Mössbauer spectra for the CARB- γ -Fe₂O₃ sample is presented in figure 11. The spectrum recorded at room temperature, figure 11(a), is analyzed by taking into account a broad spectrum (S1) due to slow relaxing Fe magnetic moments, and a doublet (S2) related to fast relaxing Fe magnetic moments. The doublet has isomer shift and

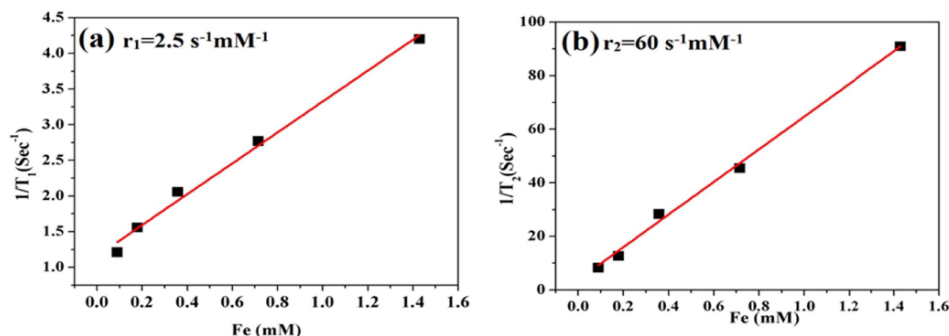


Figure 12. MRI relaxation time of the as-prepared beads CARB- $\gamma\text{Fe}_2\text{O}_3$ at various concentrations of Fe (0.08, 0.18, 0.36, 0.72 and 1.43 mM).

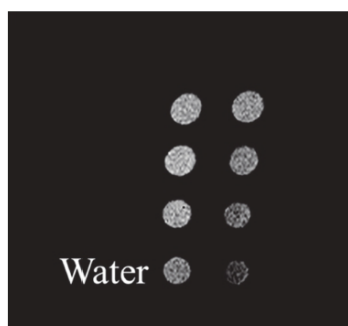


Figure 13. T2 MR imaging of pure water and CARB- $\gamma\text{Fe}_2\text{O}_3$ beads at various concentrations of Fe (0.08, 0.18, 0.36, 0.72, 1.43, 2.14 and 2.85 mM).

quadrupole splitting in agreement with Fe^{3+} . The relative absorption spectral areas (RAA) are of 63% and 37% for the S1 and S2 Fe components. Since $\text{RAA}(\text{S1})/\text{RAA}(\text{S2}) > 1$, we can conclude that this sample is thermally blocked at 300 K. This result does not contradict the M–H analyses because the Mössbauer spectroscopy measuring time (of $\sim 10^{-7}$ s) is smaller than the magnetometry measuring time (~ 10 s); this fact will lead to a higher Mössbauer blocking temperature.

^{57}Fe Mössbauer spectroscopy is an element-specific technique which provides local information on the spin relaxation and charge of iron ions, and allows an easy identification of iron oxides through their hyperfine magnetic field, isomer shift and quadrupole splitting [25].

The measurement recorded at 12 K is shown in figure 11(b). This spectrum exhibits a single sextet with hyperfine magnetic field of 51 T and isomer shift of 0.38 mm s^{-1} ; both parameters are in close agreement with the formation of maghemite [25]. No other second phase containing Fe was observed.

3.5. Magnetic resonance studies

Interest in the use of nanometric particles has increased over recent years, and has been applied in several studies. Many studies have sought to characterize and investigate applications, even proposing the use of the tool as a diagnostic test material due to its easy applicability. Flow cytometry has been used as a tool to evaluate the morphometric characteristics [26] and coupling capacity [27] of these compounds, as well as the interaction which these NPs can have with human cells, increasing the applicability of these compounds in the field of immunology [27–30]. In our study, beads prepared with APTS- $\gamma\text{Fe}_2\text{O}_3$ and CARB- $\gamma\text{Fe}_2\text{O}_3$ showed similar morphologies (figure 6) as well as sample uniformity (figure 7). The ability to investigate these morphological parameters and the coupling capacity of these NPs analyzed by flow cytometry may be fundamental for the development of a future tool for applications in nanobiotechnology [31–33].

In order to examine the features of as-synthesized beads CARB- $\gamma\text{Fe}_2\text{O}_3$ as an MR contrast agent, magnetic relaxation times and T_2 magnetic resonance images were measured on 0.55 T scanner.

The longitudinal and transverse relaxation times (r_2 , r_1) of CARB- $\gamma\text{Fe}_2\text{O}_3$ beads are shown in figures 12(a), (b). The r_1 value is $2.5 \text{ s}^{-1}\text{mM}^{-1}$ and the r_2 value $60 \text{ s}^{-1}\text{mM}^{-1}$, giving an r_2/r_1 ratio of 24—demonstrating that the fabricated beads are an efficient T_2 weighted contrast agent.

Furthermore, to prove the relation time in the favor of T_2 contrast agent, MR imaging was acquired. Figure 13 shows the T_2 -weighted MRI of beads and water. It is clearly seen that the MRI signals of NPs becomes brighter at initial low concentration because of the size of the NPs and then become darkened at high concentration as compared to the MRI image of pure water. The MRI shows that imaging intensity increases with the increase of Fe concentration. The MR relaxation data and MRI results showed the potential of as-synthesized NPs being used as T_2 MRI contrast agent.

4. Conclusions

Magnetic $\gamma\text{-Fe}_2\text{O}_3$ NPs with mean size 6.5 nm were successfully synthesized by a sonochemical method. Polycondensation of APTS on $\gamma\text{-Fe}_2\text{O}_3$ NPs led to the formation of APTS- $\gamma\text{-Fe}_2\text{O}_3$ beads. These beads were modified with succinic anhydride to obtain CARB- $\gamma\text{-Fe}_2\text{O}_3$ beads. The size of the beads was evaluated by TEM, which returned a mean size of 12 nm. The DLS results showed that the beads were aggregated and have a hydrodynamic size of 490 nm. The CARB- $\gamma\text{-Fe}_2\text{O}_3$ and APTS- $\gamma\text{-Fe}_2\text{O}_3$ beads had isoelectric points at pH of 3.76 and 6.41, respectively. The FTIR studies showed characteristic bands which demonstrated the presence of the amino and silane groups, at 870 cm^{-1} and 1352 cm^{-1} , respectively, as well as carboxylate groups, at 1648 cm^{-1} and 1425 cm^{-1} . To prove the ability of magnetic beads to couple proteins, rabbit antibody was covalently immobilized on these chemically modified beads. Mössbauer spectroscopy and VSM measurements showed that a large fraction of the beads have superparamagnetic behavior within the VSM measuring time. The XRD and Mössbauer results showed that capping with APTS did not induce any phase change in the maghemite phase. Immobilization time of rabbit antibodies was 4 h at a concentration of 120 mg per gram of NPs. The MRI results suggest that magnetic beads indeed constitute an excellent T_2 contrast agent candidate.

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EDTA-functionalized Fe₃O₄ nanoparticles



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ABSTRACT

This study analyzes the synthesis and characterization of functionalized Fe₃O₄ nanoparticles by ethylenediaminetetraacetic acid (EDTA). The syntheses were performed using the co-precipitation method under different experimental conditions: nitrogen atmosphere versus ambient atmospheric conditions, and temperatures of 25 °C versus 90 °C. X-ray diffraction techniques, Fourier transform infrared (FTIR) spectroscopy, zeta potential, and transmission electron microscopy (TEM) were used to characterize these nanoparticles. The co-precipitation method produced high homogeneity in nanostructure shape and size. The functionalization of the magnetite surface was confirmed by the FTIR analyses and the development of new bands associated with EDTA as well as by zeta potential change. The addition of EDTA was also found to change the mechanism of nucleation and nanostructure growth; EDTA was found to favor nucleation, thus decreasing nanoparticle size.

1. Introduction

Iron oxide nanoparticles are the most commonly used today types of nanostructured system, which exhibit magnetic properties. Iron oxide nanoparticles have also been used in hyperthermia and theranostic devices, a usage which has increased substantially in the last ten years [1–3].

The most frequently studied nanoparticles are iron-based nanoparticles, which are known as nanoscaled zero valent iron (nZVI), maghemite nanoparticles (γ -Fe₂O₃) and magnetite nanoparticles (Fe₃O₄) due to their proven biocompatibilities [1,2,4–6]. These iron-based nanoparticles exhibit unique chemical properties resulting from different iron oxidation states. They are widely used as catalysts in environmental remediation, and as drug delivery vehicles in biomedicine [7]. Interest in nanoparticles has increased in part because they are simple to prepare and are chemically stable when stored in colloidal suspension form. Colloidal suspensions of stabilized magnetic nanoparticles are referred to as ferrofluids and may interact with an external magnetic field [8]. Fe₃O₄ nanoparticles possess the ability to adsorb heavy metals in an aqueous medium through physical and chemical interactions with these heavy metals [7]. However, the major mechanism of contaminant removal from an aqueous medium that employs γ -Fe₂O₃

nanoparticles is physical adsorption [7].

One inevitable problem associated with these particles in this size range is their long-term instability. These particles tend to pursue agglomerates to reduce the energy associated with the increased surface-area-to-volume ratio of nanoparticles. Furthermore, the nanoparticles are chemically very active and easily oxidizes in the air, which generally results in low magnetic properties and dispersibility. For many applications, it is crucial to develop chemical and colloidal protection strategies to chemically stabilize magnetic nanoparticles against degradation during and after synthesis as well as colloidal stability through electrostatic and/or steric stabilization [9,10]. These strategies include a coverage graft with organic molecules (surfactants or polymers) or coverage with an inorganic film (silica or carbon). It is important to note that, in many cases of protection, the shell does not only stabilize the nanoparticles: it may also be used for further functionalities (such as with other nanoparticles or different ligands, depending on the desired application) [1,11]. Along this vein, recent studies [12–14] have demonstrated the high affinity of ethylenediaminetetraacetic acid (EDTA) for iron oxide nanoparticles, which may improve colloidal dispersibility, decrease high surface energy, and consequently decrease the nanoparticles agglomeration.

The chelating potential of EDTA may be used to synthesize EDTA-

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Table 1
Nomenclature of samples.

Sample	Name	Sample	Name
Fe ₃ O ₄ 25 °C; AMB.	NP25AMB	Fe ₃ O ₄ -EDTA-0.002	NP-L0.002M25AMB
Fe ₃ O ₄ 25 °C; N ₂	NP25N2	M 25 °C; AMB.	
		Fe ₃ O ₄ -EDTA-0.002	NP-L0.002M25N2
Fe ₃ O ₄ 90 °C; AMB.	NP90AMB	M 25 °C; N ₂	
		Fe ₃ O ₄ -EDTA-0.002	NP-L0.002M90AMB
Fe ₃ O ₄ 90 °C; N ₂	NP90N2	M 90 °C; AMB.	
		Fe ₃ O ₄ -EDTA-0.002	NP-L0.002M90N2
		M 90 °C; N ₂	NP-L0.02M90N2
		Fe ₃ O ₄ -EDTA 0.02	NP-L0.2M90N2
		M90 °C; N ₂	
		Fe ₃ O ₄ -EDTA 0.2	
		M 90 °C; N ₂	

functionalized Fe₃O₄ nanoparticles, which may be proved to be promising for studies on the adsorption of heavy metals due to the EDTA chelating cations, thus forming complexes with their structures [12,13].

The present contribution presents a study in the synthesis conditions and characterization of EDTA-functionalized Fe₃O₄ nanoparticles using the co-precipitation method of synthesis and changing the experimental conditions in order to carefully determine the importance of the experimental parameters in the synthesis of these compounds. The considered parameters were synthesis temperature (25 °C versus 90 °C), atmospheric conditions (presence versus absence of N₂), and EDTA concentration (0.2, 0.02, and 0.002 mol L⁻¹).

2. Experimental

The chemical reagents used for the synthesis of EDTA-functionalized Fe₃O₄ nanoparticles were iron (II) chloride tetrahydrate (Merck), iron (III) chloride hexahydrate (Aldrich), ammonium hydroxide ~ 28% (Synth), and EDTA (Synth).

The nanomaterials were synthesized by the co-precipitation method with some modifications [1,8,10,12,13]. Four syntheses were performed in the case of the Fe₃O₄ nanoparticles and another four ones were performed in the case of the Fe₃O₄-EDTA particles under the following four experimental conditions: (1) controlled synthesis temperature at 25 °C without inert atmosphere; (2) controlled synthesis temperature at 25 °C under nitrogen atmosphere; (3) controlled synthesis temperature at 90 °C under reflux and without inert atmosphere; and (4) controlled synthesis

temperature at 90 °C under reflux and nitrogen atmosphere.

In the first step, Fe₃O₄ nanoparticles were synthesized using 100 mL of precursor solution containing 0.030 mol iron (III) chloride hexahydrate and 0.015 mol iron (II) chloride tetrahydrate, to which it was added 10 mL of ammonium hydroxide. This mixture was left under constant agitation for 1 h at the experimental temperature and atmospheric conditions in (1) to (4). The final precipitate was separated using a magnet and washed many times with distilled water and acetone.

In the second step, the EDTA-functionalized Fe₃O₄ nanoparticles were synthesized by using the same procedure used for the synthesis of the Fe₃O₄ nanoparticles. Then, it was added of 50 mL of a 0.002 mol L⁻¹ EDTA solution soon after the ammonium hydroxide was added. Two additional Fe₃O₄-EDTA nanoparticle syntheses were performed using the experimental conditions in (4) in a similar procedure, but changing the EDTA concentration. In these changes, 50 mL solutions with 0.02 mol L⁻¹ of EDTA or 0.2 mol L⁻¹ of EDTA were used.

In Table 1 are presented the different synthesis prepared with the parameters evaluated. As an example, the sample NP25N2 and NP-L2mM25AMB represents NP – Fe₃O₄ nanoparticles; L – EDTA ligand; 0.002 mol L⁻¹, 0.02 mol L⁻¹ or 0.2 mol L⁻¹ – EDTA concentration; 25 or 90 – synthesis temperature in °C, and N₂ or AMB – atmospheric conditions (nitrogen atmosphere or without inert atmosphere conditions, respectively).

Nanoparticles samples were characterized based on the X-ray diffractometer patterns obtained from a Rigaku-Rint 2000 diffractometer with CuK_α radiation. Transmission electron microscopy (TEM) was performed using a Philips CM-200 instrument with a super twin a-lens. The FTIR spectrum was measured using a Vertex 70 spectrometer (Bruker instruments). The Zetasizer Nano ZS system (Malvern Instruments) was also used in the zeta potential measurements. Variations in pH were controlled using HCl/NaOH.

3. Results and discussion

Fig. 1 shows the X-ray diffractograms from different synthesis parameters for the Fe₃O₄ nanoparticles (Fig. 1-a) and the Fe₃O₄-EDTA nanoparticles (Fig. 1-b). These X-ray diffractograms show the crystallographic planes representing the diffraction of magnetite in all of the systems studied. The syntheses under ambient atmospheric conditions (NP25AMB, NP90AMB, and NP-L0.002M25AMB) produced a crystallographic plane representing magnetite (Fe₃O₄) and maghemite (γ-Fe₂O₃), a finding that indicates oxidation during synthesis. According to Gupta et al. [1], organic or inorganic molecules are typically used during the precipitation process to inhibit the oxidation of nanoparticles. The

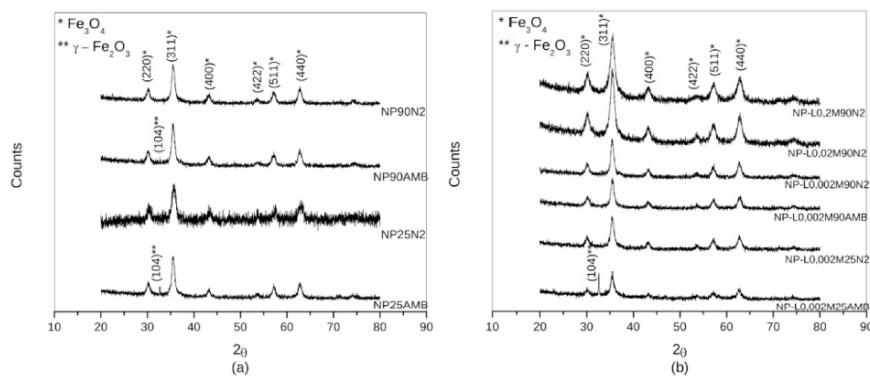


Fig. 1. X-ray diffraction patterns obtained for the samples. The magnetite (JCPDS card no. 89-0688) and maghemite (JCPDS card no. 89-0597) patterns from the JCPDS database.

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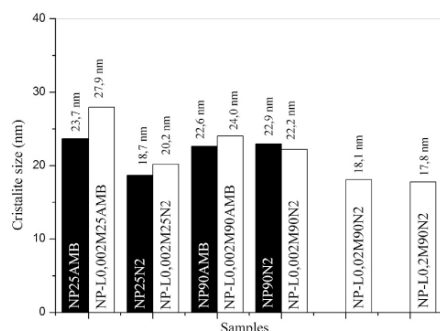


Fig. 2. Crystallite size obtained from the Scherrer equation to the samples.

magnetite (JCPDS card no. 89-0688) and maghemite (JCPDS card no. 89-0597) patterns from the JCPDS database for were included for comparison.

Even so, the oxidation of NP-L0.002M25AMB was found to occur in this experiment. However, in the case of NP-L0.002M90AMB and the other nanoparticles, the interaction was made by chemical binding between magnetite and EDTA molecules during synthesis. The EDTA molecules are attached in the nanoparticles. The EDTA-functionalized nanoparticles inhibits oxidation process of magnetite (Fe_3O_4) leading to maghemite ($\gamma\text{-Fe}_2\text{O}_3$). Kinetically controlled oxidation of ferrous species may be decreased through the use of a system to remove oxygen from the reaction medium. This may be obtained from the gas nitrogen streaming during the synthesis. The ideal molar reaction for the synthesis of

magnetite is 2:1 ratio to $\text{Fe}^{3+}:\text{Fe}^{2+}$. To guarantee this ratio, inert atmosphere is used in the reaction medium. This control is very important: it may modify the surface properties of these materials, as well as the magnetic properties, which tend to decrease with oxidation.

As can be seen, temperature does not seem to significantly influence the stability of the phases. Iron oxide nanoparticles exhibited greater crystallinity in the samples synthesized at 90 °C than in those performed at 25 °C. Taking into account that for these systems, nucleation must occur before the growth of pre-formed nucleus, the temperature should favor the growth stage of the samples synthesized at 25 °C, thus increasing the diffusion of iron atoms to the nucleus that is formed. At this point, the dominant stage begins to occur as a growth mechanism, meaning that the speed of iron atom aggregation in the nanoparticles comes to depend on the quantity of these atoms around the nucleus.

The graph in Fig. 2 shows an estimate of the crystalline sizes obtained using the Scherrer equation [15]. In this work, the Scherrer's equation was used as the first approximation to determine the particle size, taking in consideration the uncertainty associated to this equation, as previously discussed by Tomaszewski [16]. Further up, the particles size were determined using the images obtained by TEM (Figs. 3-b, 4-b and 5), which is more appropriate to this purpose. The result indicates that all of the synthesized materials exhibit a nanometric particle size between 17.8 and 27.9 nm. The EDTA-functionalized Fe_3O_4 samples also exhibited smaller particle sizes. The use of nitrogen atmosphere prevented the oxidation process and aided in the control of particle size. All of the samples to which nitrogen gas streaming was applied were smaller than those in which ambient atmospheric conditions were used. This effect was found to be more pronounced at 25 °C. Because the growth process is favored at 90 °C, the crystallite sizes are very similar, a result that indicates that the expected findings involving temperature are consistent with the experimental data.

Fig. 2 also shows that the samples NP-L0.002M90N2, NP-

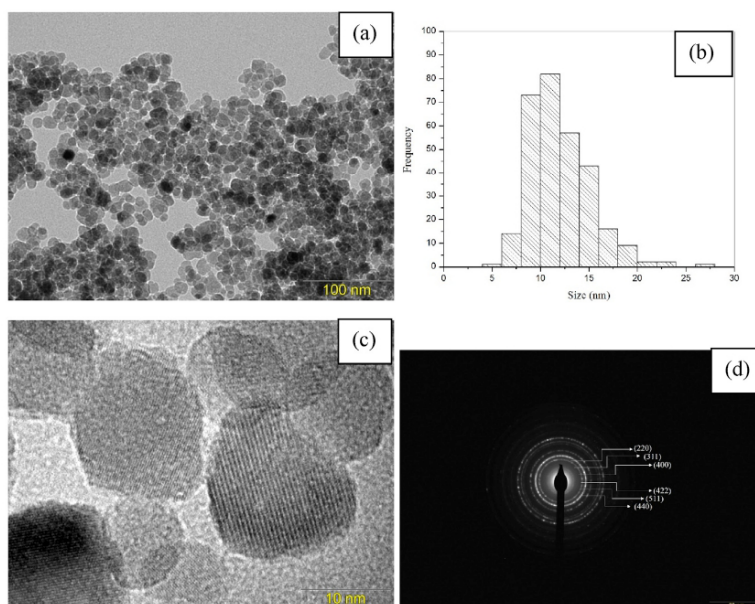


Fig. 3. Transmission electron microscopy for the nanoparticle referred to as NP90N2.

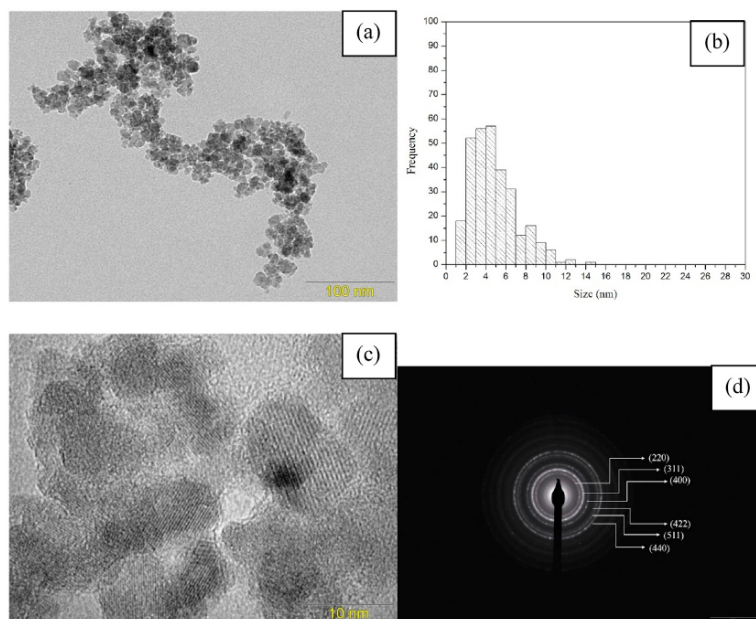


Fig. 4. Transmission electron microscopy for the nanoparticle referred to as NP-L0.2M90N2.

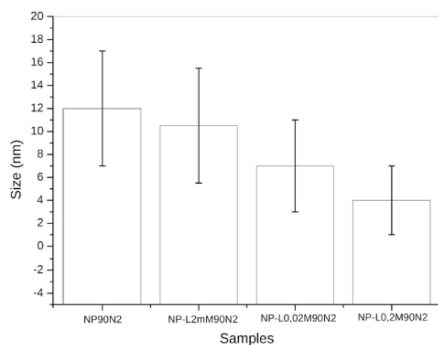


Fig. 5. Average size of some of the synthesized nanostructures.

L0.02M90N2, and NP-L0.2M90N2 nanostructures were found to be smaller in size than all of the other structures studied. As its concentrations increase, EDTA likely changes the mechanism of nanoparticle formation, which in turn, favors more nucleation than in the growth stage. In this case, EDTA is likely functioning as a barrier to the nuclei that are formed, resulting in decreased nanoparticle size.

The electron transmission micrographs in Figs. 3 and 4 shows that the nanostructures are highly homogeneous in shape and size. The micrographs (Fig. 3 and c) show that the average nanoparticle size was found to be approximately 12 nm in the case of NP90N2 nanoparticle, as verified in the histogram in Fig. 3-b. Similarly, the micrographs in Fig. 4a

and c reflect the average size of 4 nm for the case of the NP-L0.2M90N2 nanoparticle, as presented in the histogram in Fig. 4c.

The transmission electron micrographs clearly show the crystallographic planes present in the nanostructures in Figs. 3c and 4c, and Figs. 3d and 4d represent the electron diffraction in these nanostructures. These results are consistent with the crystallographic planes presented in the X-ray diffraction patterns shown in Fig. 1.

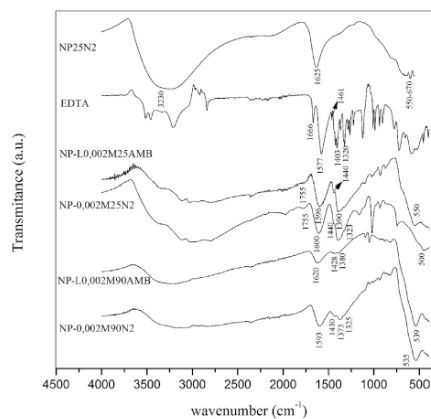


Fig. 6. Infrared spectra from investigated functionalized iron oxide nanoparticles. The main bands are featured.

Table 2
Description of the FTIR bands assignment of the spectra shown in Fig. 6.

NP25N2	EDTA	NP-L0.002M25AMB	NP-L0.002M25N2	NP-L0.002M90AMB	NP-L0.002M90N2
(550-670) ν Fe—O	—	(550) ν Fe—O	(500) ν Fe—O	(539) ν Fe—O	(535) ν Fe—O
—	(900-960) ν (C—C)	(870-930) ν (C—C)	(933) ν (C—C)	(880) ν (C—C)	(870-930) ν (C—C)
—	(1006) ν [N (—CH ₂ —) ₃]	(1020,1006 and 972) ν [N (—CH ₂ —) ₃]	(1018 and 975) ν [N (—CH ₂ —) ₃]	(1091,1048 and 975) ν [N (—CH ₂ —) ₃]	(1044-1002) ν [N (—CH ₂ —) ₃]
—	(1320) δ (NH ⁺)	(1325) δ (NH ⁺)	(1323) δ (NH ⁺)	—	(1325) δ (NH ⁺)
—	(1403) ν (C—O) COOH	(1390) ν (C—O) COOH	(1390) ν (C—O) COOH	(1382) ν (C—O) COOH	(1373) ν (C—O) COOH
—	(1461) ν_{as} (COO ⁻)	(1440) ν_{as} (COO ⁻)	(1440) ν_{as} (COO ⁻)	(1428) ν_{as} (COO ⁻)	(1430) ν_{as} (COO ⁻)
—	(1577) ν_{as} (COO ⁻)	(1596) ν_{as} (COO ⁻)	(1600) ν_{as} (COO ⁻)	(1620) ν_{as} (COO ⁻)	(1593) ν_{as} (COO ⁻)
(1625) ν (H ₂ O)	—	—	—	—	—
—	(1666) ν (C=O) COOH	(1755) ν (C=O) COOH	(1774) ν (C=O) COOH	—	—
—	(2837-3210) ν (C—H)	(2800-3112) ν (C—H)	(2792-3119) ν (C—H)	—	(2844-3042) ν (C—H)
(3230) ν (O—H)	(3458-3512) ν (O—H)	(3340) ν (O—H)	(3362) ν (O—H)	(3221) ν (O—H)	(3200) ν (O—H)

The XRD and TEM results are in agreement with the reported data by Refs. [10,17]. Because in the homogeneous nucleation process there should be supersaturation of the precursors, that should be sufficient for the formation of the critical radius for the occurrence of the precipitate. Furthermore, the nanoparticles precipitate obtained shows high surface energy and elevated surface charge and tend to form agglomerates to minimize this surface energy, thus increasing the average size of the nanoparticles. To minimize the high values of surface energy, it commonly adds organic and/or inorganic molecules, which will interact with the nanoparticles to avoid the agglomerate and consequently allows the kinetic control of growth stage.

The graph in Fig. 5 shows the average sizes of the NP90N2, NP-L0.002 M 90N2, NP-L0.02M90N2, and NP-L0.2M90N2 nanostructures. The nanoparticle sizes were determined based on the histograms from the TEM analyses. The results confirm the proposal that the addition of EDTA will favor the nucleation stage of the nanoparticles. The graph in Fig. 5 also shows the influence of EDTA concentration on particle size: the results reflect the decrease in nanoparticle size as EDTA concentration increased, which is consistent with the results provided by the use of the X-ray diffraction technique.

In order to confirm the functionalization of EDTA on the surface of the nanoparticles, infrared spectroscopy analyses were performed and the resulting graphs are shown in Fig. 6.

In the infrared spectrum of magnetite (NP25N2) shown in Fig. 6, three bands are visible: the first one is found between 560 and 670 cm^{-1} and is assigned to the binding of iron to oxygen, a property required for the formation of Fe_3O_4 ; the second band is found near 1625 cm^{-1} and is the result of the absorption of water molecules; and the third band, found near 3230 cm^{-1} , is associated with stretching ν (O—H). The latter band

proves the formation of hydroxyl groups on the surface of magnetite. Fig. 6 also allows for an analysis of the pure EDTA spectrum, on which a series of bands were found. Bands were found at 900-960 cm^{-1} due to the ν (C—C) axial deformations, at 1006 cm^{-1} due to the asymmetrical axial deformations in the group ν [N (—CH₂—)₃] of the tertiary amines, at 1320 cm^{-1} , which is attributed to angular deformation δ (NH⁺), near 1461 and 1577 cm^{-1} , which is attributed to symmetrical and asymmetrical axial deformation ν (COO⁻), and between 1403 and 1666 cm^{-1} , which corresponds to vibrational motions ν (C—O) and ν (C=O) of the —COOH group. There were also many bands between 2837 and 3210 cm^{-1} , which were associated with the ν (C—H) axial deformities of the CH₂ groups. Finally, the bands between 3458 and 3512 cm^{-1} are attributed to stretching ν (O—H) resulting from water and hydration in this compound. Table 2 contains all of the bands found for each of the samples analyzed.

Infrared spectra attributed to the functionalization of the EDTA on the surface of Fe_3O_4 nanoparticles are the in Fig. 6 (NP-L2m25AMB, NP-L2m25N2, NP-L2m90AMB, and NP-L2m90N2). FTIR spectra for the NP-L0.02M90N2 and NP-L0.2M90N2 samples are very similar to those of the NP-L0.002M90N2 nanoparticles. The appearance of new FTIR bands for pure magnetite can also be seen and they are attributed to the functionalized EDTA molecule on the magnetic nanoparticles surface. Another remarked aspect of these graphs is the decrease in band intensity associated to the stretching ν (O—H) to approximately 3230 cm^{-1} . This result indicates that, in cases of functionalization with EDTA, these groups are recovered and/or are participating in chemical reactions. Another finding is that magnetite may bind with the carboxylates of EDTA, since the carboxylates exhibit considerable distortion in the ν (C—O) and stretching ν_{as} (COO⁻) bands of pure EDTA, a result which

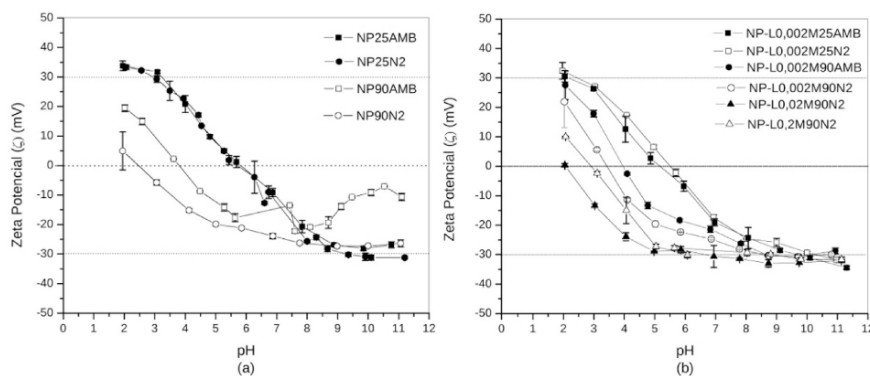


Fig. 7. Zeta potential measurements as a function of pH for (a) Fe_3O_4 nanoparticles and (b) EDTA-functionalized Fe_3O_4 nanoparticles.

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indicates that the EDTA binds on the surface of nanoparticles at these sites [11,12,14].

The FTIR results showed that occurred the EDTA functionalization in all samples of magnetite. Already Yang et al. [13] synthesized the Fe_3O_4 -EDTA nanoparticles by the coprecipitation method keeping the product at 90 °C for 30 min. The EDTA functionalization shows that the EDTA exhibits high chemical affinity for Fe_3O_4 nanoparticles, even at room temperature. Moreover, the EDTA addition should be a cheap and satisfactory alternative for protection and functionalization of nanostructures. This system shows high potential to metal [12] and anions [13] adsorption. The FTIR spectra also show that the interaction between EDTA and magnetite occurs by the carboxylate anion. This result enables future functionalizations, since there is another carboxylate group in addition to tertiary nitrogens.

Fig. 7 shows the zeta potential curves (ζ) as a function of pH for the Fe_3O_4 nanoparticles (Fig. 7a) and for the Fe_3O_4 -EDTA nanoparticles (Fig. 7b). In the case of the naked Fe_3O_4 nanoparticles, the zeta potential was found to be more influenced by the synthesis temperature than by atmospheric conditions. This result is reflected in the graph showing crystallite size as evaluated by the Scherrer equation (Fig. 2), in which the nanostructures exposed to the higher temperature (90 °C in the nitrogen atmosphere) increased slightly in size. This increase led to greater colloidal instability in these samples, and changes in the isoelectric point to lower pH values were found in the samples synthesized at 90 °C. In the case of the EDTA-functionalized Fe_3O_4 nanoparticles, behavior similar to that which was observed in the Fe_3O_4 nanoparticles was seen in the samples synthesized at 25 °C. Increased isoelectric points were found among the samples synthesized at 90 °C.

These results show that the charge density associated with the EDTA carboxyl group is predominant, and they are consistent with the results indicated by the IR spectroscopy. The analysis involving the concentration of EDTA (NP-L0.2M90N2 and NP-L0.02M90N2) found low isoelectric points due to high surface energy caused by the reduction in size, which changed the distribution of charges on the surface of the nanoparticles. These results are very promising for environmental remediation applications in the adsorption of heavy metals on the nanoparticles surface.

4. Conclusions

The results show that, in synthesis, the inert atmosphere associated with the co-precipitation method is fundamental for preventing the oxidation of magnetite. Furthermore, the X-ray diffraction analyses determined that temperature influences crystalline size, since the growth stage is favored at higher temperatures. The results reflect high homogeneity in nanoparticle shape and size in the samples studied. The addition of EDTA was also found to act as a barrier to the growth of nanoparticle nuclei, thus decreasing nanoparticle size. The

functionalization of the surface of magnetite was confirmed by the Fourier transform infrared (FTIR) spectroscopy analyses and the development of new bands associated with EDTA. The zeta potential measurements showed that the distribution of charges on the surface of the samples is affected by synthesis temperatures and EDTA concentrations.

Acknowledgements

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III. Polímeros para aplicações em biomedicina

III.1 Mecanismos de transporte de nanoplateformas no organismo

A administração de fármacos via oral é o modo mais conveniente e o mais aceitável pelos pacientes, uma vez que é um método indolor e de fácil administração [41]. No entanto, a obtenção de fármacos para uso oral apresenta diversos obstáculos. Estas barreiras são provenientes tanto do fármaco quanto do organismo. Quanto ao fármaco, está relacionada a baixa solubilidade em meio aquoso, ocasionando baixa dissolução e consequentemente, biodisponibilidade e eficiência reduzidas [42]. Quanto ao organismo, há as condições extremas de pH e a alta atividade enzimática no sistema gastrointestinal que promovem a degradação dos fármacos [41]. Para manter a atividade biológica do fármaco a fim de aumentar a biodisponibilidade, acarretando maior eficiência, faz-se uso de plataformas de liberação, tais como nanopartículas. Portanto, é necessário a caracterização da plataforma utilizada já que a absorção pelos órgãos e tecidos é altamente dependente do tamanho da partícula, carga de superfície (potencial zeta) e tipo de revestimento (hidrofobicidade) [42].

A absorção intestinal de nanopartículas ocorre através da incorporação intercelular no epitélio intestinal e no tecido linfático [43,44]. A **Figura 16** apresenta as principais rotas de absorção de sistemas nanoparticulados através da membrana celular [42-44]. As rotas são distintas entre si e culminam na transferência da nanopartícula para a circulação sistêmica, com exceção da rota com a presença da célula M: **(a)** uso de carreadores funcionalizados os quais são transportados para o interior da célula através de receptores; **(b)** ocorre o transporte dos carreadores através de transportadores; **(c)** a internalização dos carreadores através do transporte paracelular que ocorre entre as células; **(d)** a absorção pode ocorrer por transporte transcelular, onde a superfície da célula forma invaginações que englobam os carreadores; **(e)** fagocitose através de células M [43]. O uso de carreadores funcionalizados permite maior seletividade durante o tratamento neoplásico.

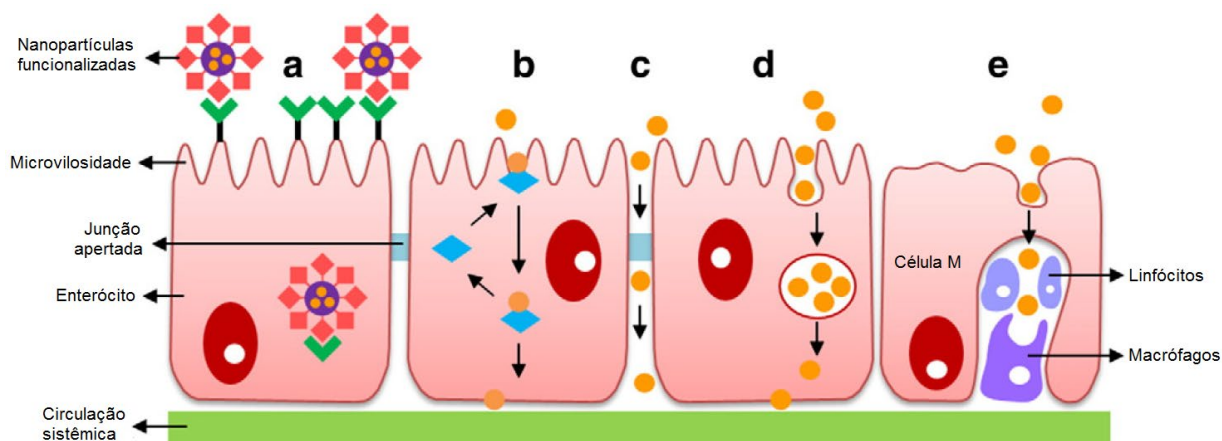


Figura 16 - Resumo esquemático dos mecanismos de transporte. (A) transporte mediado por receptores; (B) transporte mediado por transmissor; (C) transporte paracelular; (D) transporte transcelular e (E) transporte mediado por célula M – fagocitose por células M. Adaptado de Yun, Y [43].

Após o sistema nanoparticulado atingir o sistema circulatório através da mucosa intestinal, outras barreiras devem ser superadas a fim de tornar o carreador eficiente. Ao atingir a corrente sanguínea, o carreador é bombeado pelo coração para o pulmão. Os menores vasos sanguíneos do corpo se localizam nos capilares presentes no pulmão e estes possuem diâmetro compreendido entre 2 e 13 μm , formando a primeira barreira dimensional à passagem de partículas. Devido a deformações dos vasos, partículas menores que 3 μm retornam à circulação [45]. Após retornarem ao coração, as partículas são bombeadas aos demais órgãos e, portanto, novos obstáculos. As partículas passam por processos semelhantes àquele existente na membrana intestinal para que possam adentrar nos órgãos. Há uma seleção no diâmetro das partículas a serem absorvidas baseado no tipo de órgão, por exemplo: os rins restringem a entrada de partículas superiores a 60-80 nm, enquanto o fígado e o baço são capazes de filtrar partículas compreendidas entre 100-150 nm e 200 nm, respectivamente, de acordo com a espécie animal em questão. Além disso, há também o processo de fagocitose no qual as partículas, identificadas como corpos estranhos, são englobadas e digeridas pelos fagócitos (tipo de glóbulos brancos oriundos da medula óssea e presentes no sistema circulatório e linfático). A fagocitose consegue eliminar corpos compreendidos entre 100 e 200 nm de diâmetro [45-47]. Outro fator que culmina na redução do número de partículas que atingem um determinado órgão é sua estabilidade coloidal. Sistemas instáveis tendem a aglomerar a fim de reduzir a energia de superfície existente. Dessa forma, esses aglomerados de partículas são eliminados pelas barreiras mencionadas anteriormente. A compreensão dos sistemas biológicos permite a obtenção de partículas que superem as barreiras naturais impostas pelo corpo, otimizando a ação terapêutica do tratamento.

III.2 Metotrexato: um análogo do ácido fólico no combate ao câncer

Metotrexato (MTX) é amplamente utilizado no tratamento de doenças malignas, incluindo leucemia linfoblástica aguda em crianças, osteoartrite, câncer de cabeça e pescoço, câncer do pulmão, câncer de mama, psoríase entre outros [48]. Devido sua similaridade à molécula de ácido fólico, o MTX possui propriedades antimetabólicas e antifolato [49]. As estruturas das moléculas ácido fólico e MTX são apresentadas na **Figura 17**.

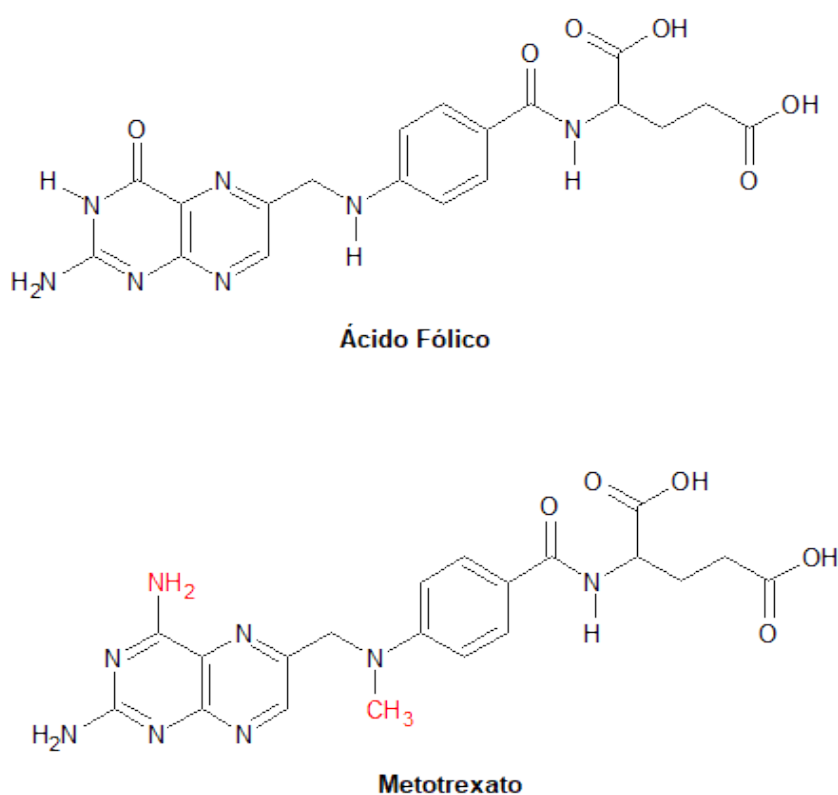


Figura 17 - Estruturas do ácido fólico e metotrexato.

As neoplasias são caracterizadas pelo crescimento descontrolado das células. A multiplicação dessas células demanda maior quantidade de nutrientes do que uma célula sadia a fim de manter a reprodução celular. Logo, estas células tendem a expressarem um aumento no número de receptores desses nutrientes em sua superfície. As vitaminas fazem parte dos nutrientes necessários e especificamente o ácido fólico (vitamina B9) é essencial na manutenção da proliferação celular. Dessa forma, a funcionalização das nanoplateformas com ácido fólico possibilitará o direcionamento delas para regiões do corpo em que há o desenvolvimento do

câncer enquanto a presença do MTX no interior da nanoplataforma irá atuar na inibição do metabolismo após o processo de internalização, tornando a plataforma um “cavalo de Tróia”.

O mecanismo de ação do MTX é baseado na inibição do metabolismo do ácido fólico através da competição irreversível aos sítios ativos da dihidrofolato redutase (DHFR) [50]. Esta enzima catalisa a conversão do dihidrofolato em tetrahydrofolato (formas reduzidas do ácido fólico) e o MTX impede esta etapa [51]. Com esta interrupção, não há as reações de transmetilação nem a formação de poliaminas. As reações de transmetilação são necessárias na síntese das bases nitrogenadas purínicas e pirimidínicas que originariam o DNA e o RNA. Portanto, MTX interfere na multiplicação de células, principalmente as que possuem altas taxas de multiplicação, tais como células cancerosas, células de medula óssea, pele, mucosas, entre outras [51].

Contudo, o uso de doses elevadas de MTX empregadas em quimioterapias e se exposto por períodos longos no corpo humano levam à efeitos colaterais severos podendo causar desde lesão renal aguda até falência de órgãos em casos extremos [52]. Dessa forma, a utilização de um dispositivo de liberação controlada de fármacos reduzirá os efeitos colaterais e permitirá a aplicação de MTX em tratamentos anti-neoplásicos.

III.3 Copolímeros de poli(caprolactona) (PCL) e poli(etilenoglicol) (PEG)

A utilização de sistemas poliméricos na liberação de fármacos hidrofóbicos no corpo humano, tais como o MTX, tem sido alvo de estudos nas últimas décadas devido a sua eficiência terapêutica em atingir locais específicos. A estrutura responsável pelo transporte do fármaco precisa ser biocompatível e, preferencialmente, biodegradável já que sua utilização tende a reduzir efeitos colaterais dos fármacos, uma vez que a quantidade empregada é diminuída [53,54].

Uma das estruturas mais citadas são polímeros unidos em blocos [53-57]. Estes compreendem duas ou mais subunidades de homopolímeros ligados por ligações covalentes sendo que diblocos e triblocos são exemplos de copolímeros em blocos constituídos de dois ou três homopolímeros, respectivamente [55]. As vantagens da união em blocos de polímeros estão no ganho de propriedades distintas como, por exemplo, a característica de interagir com moléculas polares e apolares [55,56].

Dentre os sistemas mais estudados encontram-se o polímero dibloco constituído por dois poliésteres a poli(caprolactona) (PCL) e o poli(etilenoglicol) (PEG). Inicialmente, a molécula de PCL não se encontra na forma monomérica, na qual recebe o nome ϵ -caprolactona. Essa passa por uma polimerização de abertura de anel, assumindo posteriormente a forma de monômero desejada para futura copolimerização com o PEG [57].

O copolímero PEG-co-PCL em meio aquoso, devido suas características anfífilas, apresenta repulsão da parte hidrofóbica da cadeia que começam a se posicionar na superfície da solução, enquanto a parte hidrofílica interage com o solvente. Uma vez que a concentração atingir um limite (concentração micelar crítica) inicia-se a formação de micelas, ou seja, existe uma capacidade de formação de nanoestruturas com núcleos apolares (PCL) e extremidades polares (PEG) [57]. Fatores como a composição do copolímero, a concentração das cadeias dos polímeros e agentes de segmentação influenciam diretamente na formação da micela.

As micelas apresentam determinada importância em liberação controlada de fármacos devido à capacidade de carregar substâncias apolares em sistema aquoso como o sangue humano. A **figura 18** representa a formação de uma micela em solução aquosa seguida da encapsulação de uma substância.

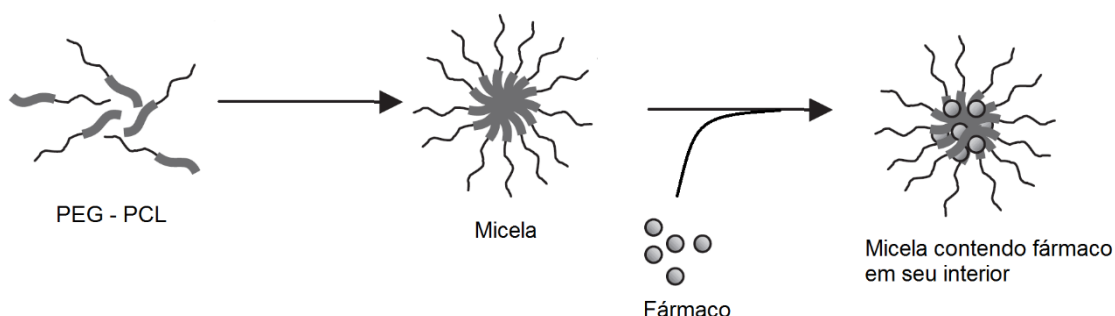


Figura 18: Esquema da formação de micelas poliméricas. Adaptado de Gaucher, G. et al.[58]

Ainda são necessários estudos que estabeleçam a melhor relação entre os blocos dos polímeros e como essa composição influencia na liberação de fármacos que combatem o câncer como metotrexato. Estes desafios estão sendo investigados no grupo de Materiais Magnéticos e Coloides do Instituto de Química, UNESP.

III.3.1 Copolímero PEG–S–S–PCL para liberação controlada de metotrexato através de gatilho químico via reação entre dissulfeto e glutathione

Em adição ao uso de PEG-PCL e do ácido fólico como direcionador através do corpo humano, há a aplicação de estímulos variados que podem ser utilizados como formas de intensificar a eficiência da liberação controlada [59]. As formas mais comuns de estímulos são: degradação ativada por pH, liberação ativada por redução, termo-sensibilidade e resposta à glicose [60]. O copolímero PEG-PCL pode ser estimulado através da mudança nos valores de pH. Quando em valores de pH menores que 7 (solução acidificada), ocorre a degradação hidrolítica, aumentando a liberação do fármaco incorporado [61]. Outro tipo de estímulo que pode ser incorporado ao sistema enriquecendo sua versatilidade é a degradação por redução [62]. Mais especificamente o emprego de ligações dissulfeto entre a união dos blocos que compõe o

copolímero [63,64]. Estas ligações são caracterizadas por serem prontamente e rapidamente reversíveis, possuindo um papel importante na manutenção das funções biológicas em células vivas [62]. Diversos fatores no corpo podem ser utilizados para estimular o rompimento desta ligação, sendo alguns deles: **(i)** glutathione reduzida (GSH) encontrada nas mitocôndrias, citoplasma, núcleo e no espaço extra celular [62]; **(ii)** NADPH (fosfato de dinucleotídeo de adenina e nicotinamida) presente em maior concentração no citoplasma [65]; **(iii)** ácido ascórbico (vitamina C) atua como antioxidante natural na redução das ligações dissulfeto [66] e **(iv)** região no corpo com valores baixos de pH, geralmente em regiões periféricas de onde se localiza o tumor [59].

O interior celular possui caráter redutor devido, principalmente, ao elevado nível de GSH presente nas mitocôndrias, citoplasma, núcleo e no espaço extracelular, sendo rapidamente degradada através de enzimas presentes no plasma humano. Estudos apontam para maiores níveis de GSH em células tumorais quando comparadas aos níveis apresentados por células saudáveis. Logo, a presença de grupos químicos susceptíveis às reações de oxirredução com a GSH, especificamente ligações dissulfeto, tornou possível desenvolver novas nanoplateformas de liberação controlada [62]. Ao reagir com a enzima, a ligação dissulfeto é rompida em dois grupos terminais sulfidril, enquanto a GSH é oxidada a GSSG.

O conhecimento gerado a partir das pesquisas envolvendo o copolímero PEG-PCL permitiu o desenvolvimento de uma nanoplateforma capaz de liberar MTX mediante estímulo intracelular. A estrutura química da nanoplateforma híbrida (NP@MPTS@HS-PCL-co-PEG-NH-NH@Ácido Fólico) sintetizada é apresentada na **Figura 19** enquanto o modelo tridimensional está ilustrado na **Figura 20**.

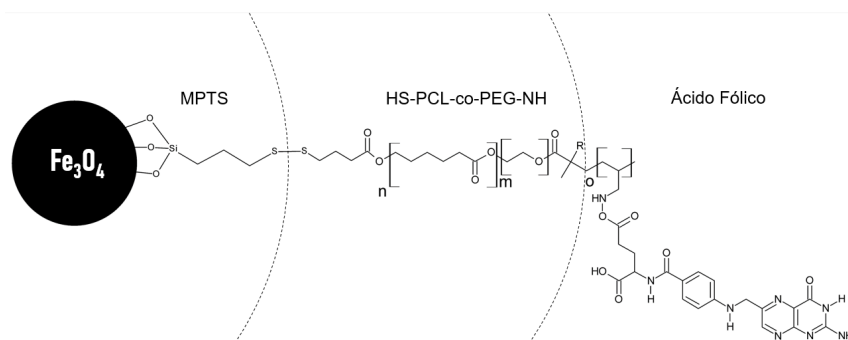


Figura 19 - Estrutura química da nanopartícula híbrida desenvolvida.

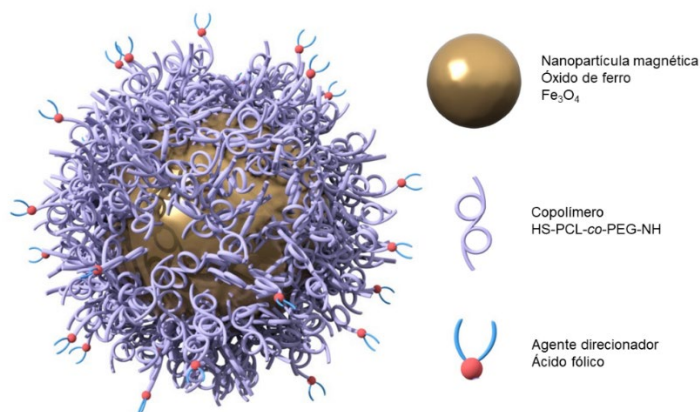


Figura 20 - Modelo tridimensional da nanopartícula híbrida desenvolvida.

III.4. Copolímeros termosensíveis: N-isopropilacrilamida (PNIPAAm) e N-N dimetilacrilamida (DMAAm)

A modificação da superfície das nanopartículas com polímeros, pode ser explorada para ajustar recursos tais como, a proteção das nanopartículas, aumento da sua estabilidade e tempo de permanência na corrente sanguínea e a especificidade das nanopartículas para células tumorais [67,68].

Polímeros inteligentes são polímeros capazes de modificar sua estrutura, mediante variações externas do ambiente em que se encontram, como temperatura, pH, força iônica, campo magnético[69]. Essas modificações permitem seu uso em diversas aplicações por exemplo, na liberação controlada de fármacos, genes e engenharia de tecidos [68-70].

Dentre os polímeros inteligentes, os polímeros termosensíveis que apresentam baixa temperatura crítica de solução (LCST), são os mais estudados para a liberação controlada de fármaco, uma vez que são capazes de liberar o fármaco com uma taxa controlada e em uma região específica, mediante o aumento da temperatura [71].

O polímero N-isopropilacrilamida (PNIPAAm), destaca-se entre os polímeros termosensíveis, pois é potencialmente viável para aplicações na área de biomateriais devido a sua insolubilidade em água quando aquecido, podendo assim ser utilizado no carreamento de fármacos [69,72]. O PNIPAAm é solúvel em água abaixo da sua LCST entre 30-35 °C (próximo a temperatura do corpo 37 °C), com predominância de interações hidrofílicas provenientes das ligações de hidrogênio do polímero com as moléculas de água intracadeia e nas vizinhanças [72]. Em temperaturas abaixo da LCST, as ligações de hidrogênio dos grupos hidrofílicos das cadeias do polímero com as moléculas de água são dominantes, resultando assim, em um polímero solúvel. Com o aumento da temperatura, as interações hidrofóbicas tornam-se mais intensas, enquanto que as ligações de hidrogênio entre o polímero e o solvente passam a ser

termodinamicamente instáveis [71-73]. Como resultado, as cadeias do polímero colapsam abruptamente para um glóbulo hidrofóbico, expulsando moléculas de água de sua estrutura [69,73,74]. Esta transição de fases hidrofílica para hidrofóbica é utilizada como estratégia para liberação de fármacos. Esse comportamento pode ser observado na Figura 21.

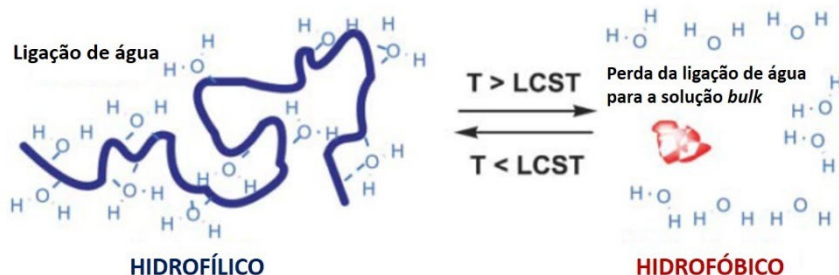


Figura 1: Representação esquemática de polímero termossensível.^{36-adaptado} (36).

Através da copolimerização do PNIPAAm com o monômero hidrofílico N-N dimetilacrilamida (DMAAm), a LCST pode ser ajustada para uma temperatura superior à temperatura do corpo (37 °C) afim de torná-lo atrativo para a liberação controlada de fármacos [70]. O aumento da LCST é desejado, pois é necessário que o fármaco não seja liberado na temperatura fisiológica do corpo durante seu transporte até a célula-alvo, sendo sua liberação realizada mediante à aquecimento externo do polímero [75].

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REVIEW



Multifunctional Redox and Temperature-Sensitive Drug Delivery Devices

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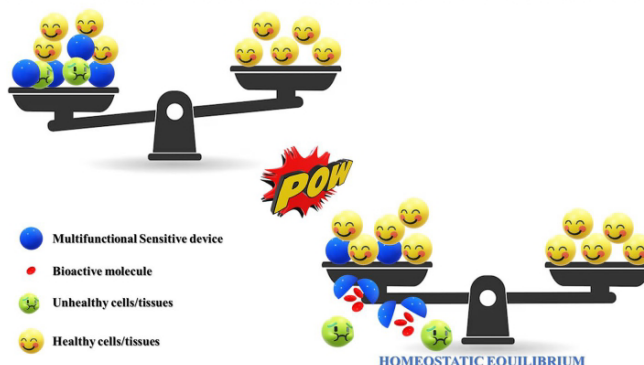
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Abstract

Despite recent advances in drug delivery systems and tissue engineering, several challenges still need to be overcome for these new technologies to reach patients. The number of new cancer cases is increasing yearly, and the future projection is frightening. Another major health concern is the rise of antibiotic-resistant bacteria. The uncontrolled and excessive use of antibiotics has allowed bacteria to undergo mutation processes, decreasing the efficiency of this sort of drug. Therefore, the development of new medical devices is a battle against time to prevent projections on the advancement of diseases from being reached. Given this scenario, redox-sensitive and temperature-sensitive drug delivery platforms show promising results in the release of bioactive molecules. This review covers the most recent advances involving devices obtained from inorganic and polymeric matrices and their structuring as scaffolds and 3D printing, focusing on their potentiality of redox and temperature sensitivity for biomedical applications.

Graphical Abstract

STIMULI-RESPONSIVE DEVICES TO RESTORE HOMEOSTATIC CONDITIONS



Keywords Redox-sensitive platforms · Temperature devices · Cancer therapy · Reactive oxygen species · Glutathione · Drug delivery

Abbreviations

AMF Alternating magnetic field
DOX Doxorubicin

FRET Fluorescence resonance energy transfer
GSH Glutathione
LCST Lower critical solubility temperature
MFH Fluid hyperthermia

Extended author information available on the last page of the article

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MOFs	Metal–organic frameworks
MRI	Magnetic resonance imaging
MWCNTs	Multi-walled carbon nanotubes
NIR	Near-infrared
NPs	Nanoparticles
PCL	Polycaprolactam
PDT	Photodynamic therapy
PEG	Polyethyleneglycol
PNIPAAm	Poly(isopropylacrylamide)
PTT	Photothermal therapy
PVCL	Polyvinyl caprolactam
QDs	Quantum dots
ROS	Reactive oxygen species
SPION	Superparamagnetic iron oxide nanoparticles

Introduction

Drug delivery systems can find applications for several diseases [1–4]; however, platforms for cancer and bacterial resistance diseases should get special attention in the next decades. According to World Health Organization [5], cancer is the second cause of death after cardiovascular diseases worldwide. Besides mortality, the economic impact has been increasing year by year due to higher medical and hospitalization costs. In 2019, the cost associated with cancer was estimated at US\$ 21.1 billion in the US [6]. Besides cancer, antibiotic resistance is one of the biggest health threats currently faced by humankind. In a frightening prospect for the European Union, it has been projected that 25,000 deaths every year from drug-resistant strains of common bacterial infections, which represent an estimated €1.5 billion in healthcare costs and productivity losses [7]. The emergency reaction against indiscriminate antibiotic prescription in human health, veterinary, and agriculture has instigated the search for other possibilities to fight illness. Since antibiotics are still the most critical medicines to treat infectious diseases, microbial resistance, as well as anti-tumoral devices, can be reduced by controlled and directed delivery of drugs based on stimuli-responsive platforms [8].

The studies on drug delivery systems have aimed to overcome the usual drawbacks of traditional formulations. The issues associated with conventional formulations consist of continuous drug administration, a lack of selectivity, and difficulty in crossing biological barriers in the body [9, 10]. In addition, the low solubility of some drugs usually is suppressed with high dosages, which is often related to the presence of side effects [11]. Thus, drug delivery devices are a powerful strategy to improve the biodistribution and bioavailability and reduce the toxicity associated with drug administration [12].

The concept of stimuli-responsive drug delivery systems comes from the physiological homeostatic simulation. This

means that under healthy conditions, no drug is released. However, when the homeostatic equilibrium is changed due to any disease, the stimuli-responsive platform releases drugs to restore healthy homeostatic conditions [13]. In other words, responsive systems try to mimic natural features discovered in live systems as an inspiration for the development of new biotechnological products [14]. According to stimuli-responsive system definition, endogenous or/and exogenous stimuli should lead to matrix composition or conformation changes responsible for triggering the release of active biomolecules [15]. Examples of main observed physicochemical changes are related to decomposition, cleavage, morphology, aggregation, isomerization, and phase changes of the matrix. The exogenous stimulus corresponds to the external source of drug triggering, such as magnetic field, electric field, light, pressure, ultrasound, and temperature. Contrarily, endogenous stimuli are those that drive drug release in the microenvironment of cells and tissues as a result of pathological conditions, such as changes in pH, ionic concentration, temperature, or the difference in redox potential [16].

At the beginning of the twenty-first century, scientific articles focused on the physical–chemistry characterization of stimulus-controlled release platforms and the adjustment of releasing kinetic properties of them, mainly due to the diffusion of nanotechnology [10, 17, 18]. The issues related to the biological environment's interactions were thin discussions. Over the years, in vivo and in vitro tests became more frequent in publications, as well as combining two or more stimuli in a multifunctional platform [19–21] and using combined systems to release more than one bioactive molecule [22–24]. From our point of view, future research on stimulus-controlled release systems is in line with personalized therapies, even though they are based on the same materials researched at the beginning of the century.

Many reviews available in the literature discuss the mechanisms involved in each sort of stimulus [13, 25–27]. Recent advances regarding temperature- and redox-responsive platforms will be highlighted in the present manuscript, emphasizing their application to cancer and bacterial infectious diseases. The potentiality of redox-sensitive devices derives from the imbalance of antioxidant molecules observed in several diseases, since inflammatory cascade to cancer microenvironment. In the same way, the influence of temperature may be used to design several devices for biomedical applications.

The Role of ROS and GSH in Redox-Stimuli Devices and Related Diseases

One of the most important factors that influence homeostasis is the equilibria of oxidative and reductive species, which is able to signal and differentiate cellular damage or even

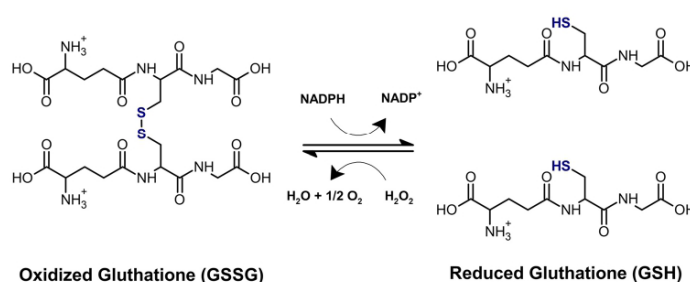
death. Reactive oxygen species (ROS) are radical derivatives from O_2 with importance in the cellular process. The most ROS found in biological processes is the singlet oxygen (1O_2), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), superoxide ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), and peroxynitrite ($ONOO^-$) [28]. ROS activity is beneficial up to a certain concentration, which induces the proliferation of healthy and cancer cells. Close to $10 \mu mol L^{-1}$, the presence of ROS may cause cell differentiation, while at concentrations of 10 to $30 \mu mol L^{-1}$, it causes DNA, RNA, and proteins to undergo mutation. In this sense, ROS would trigger oxidative stress in cells, increasing apoptosis and toxicity effects on normal and cancer cells. However, unexpected viability has been observed in cancer cells during high oxidative stress, which became resistant to several drugs and, consequently, apoptosis [29]. Cell hemostasis can be restored through the reductive molecule expression to maintain a regular metabolic pathway. Glutathione (GSH) is a tripeptide molecule (G-L-glutamyl-L-cysteinyl-L-glycine) that is synthesized by cells and mainly stored in the cytosol, although it is also found in the endoplasmic reticulum, mitochondria, and nucleus at a lower content [30]. GSH has antioxidant properties and acts as a scavenger of ROS and modulates the redox balance in cells. The concentration of reduced GSH species is 10 to 100-fold higher than oxidized ones in physiological conditions [31, 32].

Higher ROS levels are observed in the intracellular microenvironment of cancer cells compared with healthy ones. Cancer is a disease characterized by an uncontrollable growth of abnormal cells with the capacity to spread to other tissues in the body [33]. Therefore, in homeostasis imbalance, the concentration of GSH is insufficient to regulate the proportion of oxygen peroxide present in cells, triggering the formation of radical species capable of damaging DNA and other biomolecules. Oxidative stress caused by ROS is found around all cancer stages. As mentioned above, the initial step of cancer development corresponds to DNA damage and mutations, followed by increased apoptosis and, consequently, an increase in

disorder cell proliferation. In the last stage, mutagenesis continues to damage cell populations due to oxidative stress [34]. ROS may also be used as a potential strategy to fight bacterial infections. Neutrophils and macrophages are cells involved in the innate defense of immune systems and, first phagocyte viruses or bacteria [35]. The ROS cascade is activated after these cells' recognition to kill the foreign species; however, the presence of antioxidants reduces the capacity of bacterial elimination, as well as the own bacterial defense systems established for colony survival [36]. Given these circumstances, the creation of ROS-based drug delivery devices presents the fundamental features to fight diseases through the control of biological mechanisms triggered by homeostatic imbalance during the early stages of illness.

A potential redox-responsive drug delivery system can be achieved by exploiting the imbalance of molecules to treat illnesses. Disulfide bonds have been studied as a strategy for triggering bioactive molecules from drug delivery systems according to GSH levels. When exposed to a high level of reduced GSH, the disulfide bond could be broken into sulfhydryl moieties, as shown in Fig. 1, destructuring the initial morphology of the drug delivery system and facilitating drug release. Medical devices might be designed with a matrix that has a disulfide group, by grafting small molecules containing the disulfide groups via crosslinking reaction or making thiol groups react by themselves to bond [37]. Other elements from the chalcogens group can also be used as an alternative to disulfide bonds to responsive platforms. For example, diselenide bonds (Se–Se) show bond energy close to $172 kJ mol^{-1}$, while the disulfide has S–S bonds in order of $268 kJ mol^{-1}$, increasing the responsivity at the reductive medium [38]. On the other hand, the lower bond energy of diselenide makes them responsive to oxidant media. Thus, introducing diselenide crosslinking into drug delivery platforms could be an alternative strategy for a powerful device that allows a dual redox response regarding the reductive or oxidative milieu [39].

Fig. 1 Structure of oxidized and reduced GSH in biochemical equilibria



Temperature-Stimuli Devices and Related Diseases

Issues related to body temperature are older than the creation of thermometers. Since then, the ancients have been able to distinguish a healthy body from those afflicted with the disease based on temperature. For example, the Chinese prescribed warming or cooling body temperature for arthritis and cancer [40]. Hippocrates, in the Greek civilization, used heat to treat inflammation and often prescribed natural products for dropping fever, which was later discovered to be based on salicylic acid, which is present in aspirin nowadays [41]. Thermal regulation is observed in all vertebrates. In mammals, the internal temperature is regulated to be above the environmental temperature, whose heat source comes from the metabolism itself. Furthermore, the heat dissipation to the surface is tuned to ensure the physiological balance under healthy conditions [42]. Additionally, fever has long been known to accompany cases of infections. The increase in body temperature features of fever unfolds in complex changes in the body's functions concerning the production and dissipation of heat. Generally, in a febrile state, the temperature rises between 1 and 5 °C above average body temperature. The main hypothesis of thermal medicine is that if this temperature variation has lasted throughout the evolution of the species, temperature control, whether by an endogenous or exogenous source, would be beneficial for several sorts of treatments [43].

Temperature-sensitive controlled release systems can be obtained either from internal stimuli, in which temperature-sensitive materials act as a driving force to trigger the release of molecules, or from external sources. According to thermodynamics, thermosensitive polymers exhibit temperature-dependent water solubility. Above certain temperatures, known as lower critical solubility temperatures (LCST), these polymers' morphology typically changes from coil to globular, reducing their contact with the solvent, i.e., these polymers must aggregate [44]. The aggregation process is influenced by polymer-solvent and polymer-polymer interactions and depends on the system's entropy. As the temperature rise, the entropic term becomes negative and favors spontaneous aggregation due to the reduction of Gibbs free energy [45]. This means changing the polymer structure conformation and triggering drug delivery by temperature shift. Among biodegradable polymers, some N-alkylamides such as poly(isopropylacrylamide) (PNIPAAm) have received the most attention in medical devices. The PNIPAAm LCST is around 32 °C, and it is soluble at body temperature. To become sensitive to the human body, PNIPAAm must be grafted with hydrophilic groups to achieve LCST values

above 37 °C [46]. Polyvinyl caprolactam (PVCL) shows LCST similar to PNIPAAm and has been employed as an alternative in polymeric NPs since acidic hydrolysis products of PVCL have less cytotoxicity than PNIPAAm [47]. The chemical structure of PNIPAAm and PVCL is shown in Fig. 2.

Heat can also be generated by external sources, such as magnetic hyperthermia or photothermal therapy (PTT). In magnetic hyperthermia treatment, magnetic materials may release heat when submitted to an alternating magnetic field (AMF). This treatment may trigger the denaturation of proteins and lysis of cells which cause cell death. Magnetic energy is converted into heat energy according to the frequency and amplitude of the AMF employed. Two relaxation mechanisms are involved in heat generation when the poles of external magnetic fields are inverted at high rates: Néel and Brownian relaxations. The Néel relaxation corresponds to the heat energy dissipation from the movement of magnetic domain walls, according to their reorientation to the external magnetic field. The friction between particles' rotation with solvent media also results in heat production, named Brownian relaxation [48, 49].

PTT is another sort of treatment with low invasive and toxicity. The PTT is based on delivering a photosensitizer to damaged tissue and, by electromagnetic radiation, such as radio frequency, microwaves, near-infrared (NIR), or visible light, converting the energy into heat [50]. The general mechanism of heating consists of the use of metallic NPs that, when exposed to electromagnetic radiation, oscillate electrons from their conduction band. The resonance energy of these electrons is converted to heat and might employ on cellular damage [51].

Medical Devices Based on Redox Stimulus

Inorganic Materials

Inorganic materials stand out for being easy to obtain and, for many, endogenous to the human body. They are made up

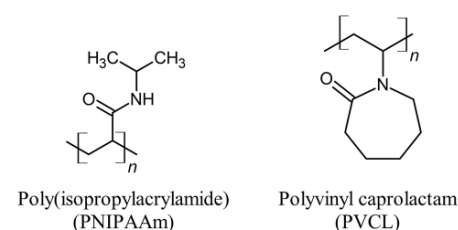


Fig. 2 Chemical structure of temperature-responsive polymers

of metals, metal oxides, quantum dots (QDs), and inorganic carbon derivatives such as graphene, carbon nanotubes, nanodiamonds, and carbon QDs. In general, these materials can also be characterized according to their morphologies. As for the nanotubes, the internal and external surfaces can be modified, increasing the drug adsorption capacity or performing multi-functionalities in these nanostructures. Similarly, hollow NPs have excelled in retaining drugs in the inner parts isolated from the environment, thus reducing drug degradation, which is very interesting since most drugs are hydrophobic and can be protected in the hollow structure of these nanomaterials.

Many inorganic platforms based on metal nanomaterials have been used as drug carriers. Among them, colloidal gold and silver NPs stand out for being easy to obtain and functional and having high chemical and colloidal stability [52, 53]. Metallic nanomaterials present surface effects due to plasmonic resonance that allow potentiating the use of these materials for heat release through the photothermal effect or even imaging [52–58]. Gold nanocages are promising nanostructures in cancer therapy, as they combine the properties of gold and allow incorporation into their hollow interior, protecting them from the external environment [58–60]. Metallic silver and copper are known for their antibacterial properties and found applications to solve issues related to disinfections, super-drug-resistant bacteria, wound healing, and reducing inflammation [54, 61–66].

Among the oxide NPs, iron oxides make up the majority of inorganic nanodrugs approved by the Food and Drug Administration [67]. Nanometre-sized iron oxides have superparamagnetic properties and therefore have shown great potential as drug delivery vehicles, contrast agents, and hyperthermia [68–70]. Zhao and collaborators studied a core@shell Fe_3O_4 @MMSN (mesoporous silica) nanoparticle-based platform that was developed as a multipurpose nanopatform for doxorubicin (DOX) delivery and magnetothermal cancer treatment simultaneously. For this, NPs were modified with chitosan linked to folic acid to obtain MMSN-SS-FA using disulfide bonds to avoid premature drug release in MMSN mesopores. The results indicated that the platform is sensitive to different stimuli, such as redox, pH, and AMF; when exposed to the target HeLa cells, overexpressed by the folate receptor, the platform showed the potent tumorgrowing inhibitory effect of 84% [71].

Demirel and Bayrak obtained a system containing a similar platform sensitive to ultrasound, redox, and pH. The system is based on a mesoporous silica-coated magnetic core (Fe_3O_4 @ SiO_2 @ mSiO_2) and pH/redox-responsive polymer layer chitosan conjugated with lipoic acid. The platform showed an efficient release of DOX in a cellular pH environment, and the pH/redox-responsive drug environment showed a rapid release profile in the presence of 10 mM GSH at pH 5.5 and 37 °C in 24 h. The platform is also

sensitive to ultrasound showing a controlled release by this stimulus, and in vitro tests showed cytotoxicity and cell mortality at pH 5.5, which makes the platform promising as a multifunctional drug releaser [72].

Even more interesting, Li and collaborators developed a system mediated by magnetic NPs responsive to the tumor microenvironment based on Fe_3O_4 NPs doubly decorated with cell-penetrating peptide (mPEG@TAT@ Fe_3O_4) cleavable (mPEG) and transcriptional transactivator, which allowed magnetic resonance imaging (MRI) and light heat-mediated immune stimulation. The platform showed sensitivity to the tumor-reducing environment by shedding the mPEG shell and strongly inhibited the proliferation of tumor cells with exposure to an AMF, reaching an inhibition rate of 86% [73].

Many other oxides have received significant attention for their characteristic properties that can be used in different therapeutic strategies. Among them, the promising biocompatible platforms are silica nanomaterials due to their low cost, biocompatibility, high stability and dispersibility in polar media, ease of obtaining, and high surface area [74]. The silica size can be controlled to allow it to overcome the body's natural barriers [75]. Furthermore, the pore size can be adjusted using suitable surface agents. These structures can be easily combined with other nanomaterials for redox-responsive drug delivery, using ligands such as disulfides or organic molecules and biomolecules. Hard coatings such as QDs can also be used [76–78], gold [79, 80], silver [81, 82], and magnetic oxides [83] providing multifunctional properties and sensitivity to external stimuli for cancer cell recognition and controlled release.

Lai and collaborators reported a nanocarrier based on mesoporous silica beads of type MCM41 for controlled release applied to cover pores as caps to prevent leakage of drugs. The drugs were rapidly released after exposure to disulfide-linked reducing agents [84]. Lai and collaborators developed a real-time monitoring system based on fluorescence resonance energy transfer (FRET), in which mesoporous silica NPs are adsorbed with cysteine and coumarin. Using the fluorescein isothiocyanate- β -cyclodextrin as a redox-responsive molecular valve to block the pores, with the reduction of the disulfide bond, the fluorescein isothiocyanate- β -cyclodextrin is removed, and the release of drugs entrapped in the pores is monitored by the FRET signal [85].

Lin and collaborators developed a dual nanocarrier, responsive to pH and redox, made with mesoporous silica NPs modified with hyaluronic acid. Herein, DOX was conjugated to silica through hydrazone bonds, which are sensitive to acidic conditions. An outer shell encapsulated the hyaluronic acid on the surface of the nanoparticle using a disulfide crosslinker. The study evaluated the release triggering under different pH and GSH conditions. It demonstrated that the

nanocarrier might be a promising drug delivery system targeted at the cytosol of cancer cells [86]. Kundu and collaborators evaluated the *in vivo* therapeutic efficacy and toxicity profile of a synthetic anticancer using mesoporous silica NPs as a nanocarrier. After drug loading, the pores were closed with gold NPs to obtain a controlled delivery of the responsive drug redox. A more significant therapeutic effect was observed when a nanocarrier was used due to increased cellular internalization and mitochondria-dependent apoptosis in MCF7 cells by oxidative stress. The nanoformulation significantly inhibited solid tumor development compared to untargeted and free nanoformulations [87].

QDs are semiconductor NPs with size and shape-dependent optoelectronic properties capable of absorbing broad-spectrum light and emitting signals of a specific wavelength. QDs are promising for real-time tissue imaging (bioimaging) and theranostics as well [88, 89]. Their tiny dimensions allow them to overcome many biological barriers making them excellent candidates for intracellular tracking, diagnosis, *in vivo* imaging, and therapies [90]. Shivaji and collaborators obtained CdS QDs with a size of 2 to 5 nm from tea leaf extract. It was observed that the material showed antibacterial activity and exhibited cytotoxicity to cancer cells [91]. Zhang and collaborators developed 12 nm dopamine-redox-sensitive dopamine-functionalized CdSe/ZnS to detect the cancer biomarker alpha-fetoprotein in human serum samples. Tyrosinase was used to catalyze the oxidation of dopamine on the surface of the nanocarriers, leading to fluorescence quenching in the presence of the alpha-fetoprotein analyte, reaching detect concentrations of 10 pM, proving the potential of the platform for detection [92].

Another important category of nanocarriers is carbon derivatives. They have attracted attention in the biomedical field for properties such as durability, high biological distribution rate, electrical, and optical properties. Ma and collaborators built a graphene oxide-based nanocarrier decorated with amphiphilic copolymers Polyethyleneglycol-polycaprolactam(PEG-PCL) and DOX via a redox-sensitive disulfide bond; the *in vitro* results proved the effective release of the anticancer drug at the tumor site [93]. Wang and collaborators developed a redox-sensitive modified graphene oxide-responsive drug carrier containing a disulfide bond between polyethylenimine and conjugating to DOX and loaded with silver NPs whose release can be accompanied by surface-enhanced Raman scattering [94]. Hou and collaborators developed a redox-sensitive system constructed from single-walled carbon nanotubes, hyaluronic acid, DOX, and gadolinium. The conjugation of DOX with hyaluronic acid by disulfide bonds is redox-sensitive, allowing drug release *in vitro*, whereas the charge of Gd^{3+} ions occurred through the sidewall defects of SWCNTs to act as MRI contrast agents. It was observed that the platform effectively accumulated in tumor cells (*in vivo*) and showed synergistic

antitumor efficacy, especially under 808 nm laser irradiation [95].

Jiao and collaborators obtained a nanocarrier capable of performing controlled redox-responsive drug delivery and real-time *in vivo* bioimaging, for which a platform based on mesoporous silica conjugated with fluorescent carbon through disulfide bonds was developed (MSNs-SS-CDPAA). Poly(acrylic acid) was used to add carboxyl groups to the carbon dots structure, after which they were anchored on the surface of the silica NPs to be used as gatekeepers to trap the drugs inside the pores. The results show that the platform was redox responsive in different media, and it was also possible to track the NPs to varying wavelengths by confocal microscopy, and an efficient therapeutic effect on cancer cells was observed by the MTT assay [96].

Metal-organic frameworks (MOFs) are classified as a subclass of coordination polymers, with the unique feature of being often porous. Therefore, MOFs are hybrid materials containing metals (metallic ions or agglomerates) and bridging organic ligands, forming one-, two-, or three-dimensional structures [97]. These structures can have up to 90% free volume and may have surface areas close to the BET limit volume for solids. These materials also make it possible to modulate host-guest interactions by functionalizing the building blocks with chemical groups, which allows the possibility of controlling the kinetic release of a therapeutic agent. Recently, Li and collaborators obtained a trojan horse nanocarrier of pH-labile zeolitic imidazolate (ZIF-8) with a disulfide-linked amphiphilic polymer-drug conjugate core; at acidic pH, there is a collapse of ZIF-8 that can induce the release of tris(3-hydroxypropyl)phosphine (THPP) which further cleaves the disulfide bond and release the drug increasing the kinetics of drug release in the target cells, causing tumor growth inhibition [98]. Wang and collaborators studied an azide-functionalized MIL-101- N_3 (Fe) MOF (TTMOF)-based system to promote drug delivery to cancer cells. The proposed platform was able to load 13.4% by weight of DOX; then, this material was modified with β -cyclodextrin and functionalized with bicyclononine(β -CD-SS-BCN), which made the platform multifunctional and dual stimuli-responsive to pH (benzoic imine bond) and cellular redox potential. Accelerated kinetics was observed caused by TTMOF that promoted the breakage of disulfide bonds greater than 140 h. Cell viability tests showed pH sensitivity caused by increased apoptosis at acidic pH (viability was 62% and 29% for pH 7.4 and 5.0, respectively). The tests performed *in vivo* showed that TMOF-DOX was able to significantly inhibit tumor growth, while its pure components (TMOF and DOX) did not show tumor inhibition [99].

Xia and collaborators combined nitric oxide therapies with photodynamic therapy (PDT) to simultaneously reduce GSH content and alleviate hypoxia in cancerous cells using

a MOF structure as a drug delivery vehicle. They developed a GSH-responsive, nitric oxide, thereby increasing the production of ROS. Nic-MOF@HA NPs were evaluated in vitro and in vivo, proving to be a multifunctional therapeutic platform with a high potential for drug loading and hyaluronic acid-sensitive drug release. After reaching the target, the coating hyaluronic acid is degraded, promoting the release of Nicorandil drug, through redox reactions with GSH, and producing nitric oxide species that increase the oxygen supply to the target, promoting an improvement in PDT in vivo [100].

Polymeric Materials

In the medical field, polymeric matrices have some advantages. In general, polymers have a number of functional groups that enable surface functionalization and derivatization, as well as the ability to lessen cytotoxicity and raise the period that medications are able to circulate within live organisms. Tang and collaborators synthesized a multi-responsive polymeric nanoparticle by enzymatic polymerization of PEG-poly (ω -pentadecalactone-*co*-*N*-methyl-diethylenamine-*co*-3,3'-dithiodipropionate-*co*-3,3'-thiodipropionate) copolymers (PEG-PPMDT). This drug delivery system was employed as an artemether carrier in chemo-sonodynamic antitumor therapy and can release drugs under the synergistic effect of acidic endoplasmic pH and high intracellular GSH/ROS levels to inhibit cancer cell growth. After chemo-sonodynamic therapy, all xenotransplants of HepG2 in nude mice shrank significantly. At the end of anticancer therapy (day 22), the mean tumor volumes of free artemether and PEG-PPMDT NPs with ultrasound irradiation treatment (1 MHz, 2 W cm⁻², 3 min) were 1400 and 621 mm³, respectively [101].

Wang and co-workers report a self-destructive polymeric carrier (PEG-PBC-TKDOX) loaded with photosensitizer chlorin e6 to be used in chemo-PDT against oral epidermoid carcinoma in mice [102]. Light employed in PDT excites chlorin e6 to produce ROS and activate a cascade reaction to release the loaded drug. Thus, the polymeric carrier depolymerizes its backbone to facilitate drug release upon ROS stimulus in PDT. There is no significant difference in intracellular uptake between the free DOX and PEG-PBC-TKDOX. However, PEG-PBC-TKDOX significantly enhanced tumor inhibition. While 40% of mice died from 2 mg kg⁻¹ of free DOX doses, no significant animal deaths were found in the polymeric carrier group with or without light irradiation. Besides that, PEG-PBC-TKDOX associated with light employment (600 nm laser, 40 J cm⁻²) suppressed tumor growth within days. Although free DOX also suppresses tumor growth, severe cardiac side effects have been observed in pathology examination.

A micellar type-DOX carrier from P(MSPA-*a*-EG) that showed ROS stimuli-responsive DOX release was designed by Wang-Guanchun and collaborators [103]. With 4.9% drug loading content and 9.81% drug loading efficiency, the micellar carrier can release DOX rapidly triggered when H₂O₂ concentration was increased from 5 to 20 mM. With 0.5 and 20 mM of H₂O₂, P(MSPA-*a*-EG) can release close to 20, 30, and 60% of the total DOX loaded, respectively, which can be attributed to micelles disassembled because hydrophobic thioether groups are transformed to hydrophilic sulfoxide groups in MSPA units.

More interestingly, Zhang and co-workers produced a biocompatible polymeric prodrug micelle with pH/redox dual-responsive based on disulfide and 4-carboxybenzaldehyde functionalized PEG for DOX delivery [104]. In cytotoxicity assay, polymeric prodrug micelle shows IC₅₀ as 7.71 μ g mL⁻¹, a value higher than those found to free DOX (IC₅₀ 0.37 μ g mL⁻¹), which can be attributed to slow internalizations and intracellular dissociation of the polymeric prodrug micelle. However, the pharmacokinetic study showed that polymeric prodrug micelle increased by 1.45 the half-life DOX when compared to the free drug. Besides that, the use of dual-responsive polymeric prodrug micelle greatly reduced tumor volumes and weights when compared with free DOX, which demonstrates that PPM can achieve long-term circulation in vivo, avoid rapid elimination, and enhance the drug accumulation in tumor tissues, improving cancer therapy.

3D Scaffolds/Bioprinting

Concerning productivity improvements and the creation of personalized products, additive manufacturing can be regarded as the most significant recent advancement in biotechnology. It finds extensive use in tissue engineering and drug delivery, which could become the primary method of getting scaffolds. It is found in early works, presented by Razzacki et al., that microfabricated systems to be used as a diagnostic device through the application of metal-oxide-semiconductor (CMOS), microneedles, bio-capsules, and the lithography technique for drug delivery [105].

Using selective laser sintering additive manufacturing, Leong and collaborators produced a 3D scaffold, using PCL and poly(-L)lactic acid. With different printing parameters, it was possible to produce drug delivery devices with varied porosities (0.333 until 0.784 g cm⁻³) that could improve the diffusion rate of active molecules/cells [106].

Ryu and collaborators used a micro-electromechanical system based on poly(lactic-*co*-glycolic acid) polymer for the development of a 3D device. The thickness of the diffusion layer in the devices varied from 50, 150, and 200 μ m and it was observed differences in the release and diffusion

of tetracycline, used as a model drug. However, structural information about the prepared 3D scaffolds and new synthetic biochemical methods for the development of potential therapeutic compounds was a challenge at the time, limiting the advancement of the additive manufacturing technique for biological applications [107].

Moving forward almost two decades of research and discoveries in this area, the 3D/4D printing brings an innovative approach corresponding to the stimuli-responsive properties which materials show when exposed to a specific stimulus. These materials found widely spread new redox devices that can be used for the development of biostructures. Li Junjie and collaborators [108], using GSH associated with terminal carboxyl groups of carboxyl-capped aniline pentamer and gelatin, developed a scaffold (Gel/AP-GSH), as shown in Fig. 3, and its properties were tested in an oxygenated microenvironment. This scaffold showed a higher capacity of water retention, where its weight increased 8352% after 72 h, confirming an advantage for better transport of metabolites and nutrients. A similar Young's modulus was

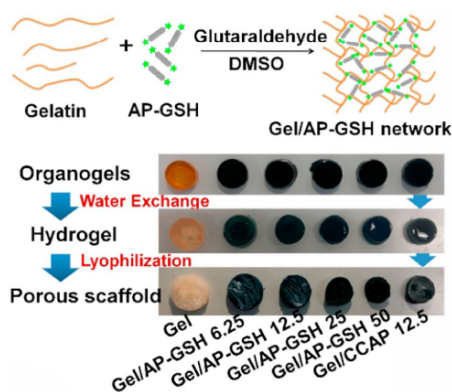


Fig. 3 Schematic illustration of Gel/AP-GSH organogel preparation [108]

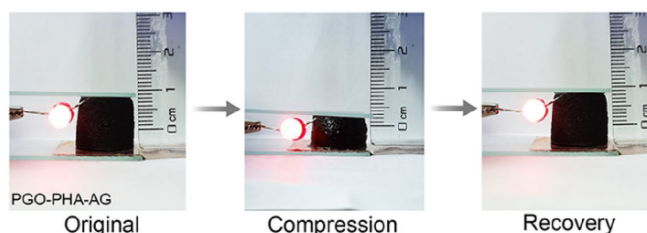
also observed, comparing the scaffold with the native tissue modulus (54–240 kPa). Measuring the radical scavenging confirmed that the increase of AP-GSH amount in the gelatin also increased the antioxidant activity caused by the presence of more H-atom donor groups. With this, these Gel/AP-GSH scaffolds improved the physical–chemistry properties to be used as biomaterials and the electric microenvironment, adhesion, and proliferation of the interested stem cells.

To control a similar oxidative environment, Yazhen Li and collaborators [109], developed a gelatin/alginate scaffold with polydopamine-mediated graphene oxide (PGO) and hydroxyapatite nanoparticles (PHA) (PGO-PHA-AG). As presented in Fig. 4, while PGO allows conductivity and transference of endogenous electrical signals to cells, which activated Ca^{2+} channels, the PHA improves bone regeneration. The antioxidative properties were evaluated by cyclic voltammetry, which curve shows the pair of redox peaks at 0.38 and 0.08 V, corresponding to the transition of catechol to quinone groups in a reverse reaction. During 120 min, scavenging of oxidative species reaches 93%, confirming an efficient method to remove the main cause of periodontal inflammation.

The issues related to oxidative stress in cells were investigated by Cam-Hoa Mac and collaborators [110], through mineralized collagen fiber scaffolds incorporated whit nanosilicon and calcium-deficient hydroxyapatite (CDHA). The scaffolds were evaluated in physiological and inflammatory environments (pH 7.4 and 6.5, respectively), resulting in the continuous evolution of H_2 and facilitating the oxidative species scavenging. Chemically, nanosilicon oxidates to silica species in aqueous solutions. After 14 days, $40 \mu\text{g mL}^{-1}$ of $\text{Si}(\text{OH})_4$ was produced and this amount is lower than the toxic level for the human body ($200 \mu\text{g mL}^{-1}$). Besides, the CDHA components promote osteogenic differentiation as well.

Another biomaterial, using the drug rapamycin associated with Poloxamer 407® and hyaluronic acid was developed by Bo Chen and collaborators [111] for intra-articular injection and thermos-response. This compound showed effective biocompatibility with chondrocytes and fibroblasts, as well as possessed sustained rapamycin being released of

Fig. 4 Structure of PGO-PHA-AG scaffold with an illuminated LED, compressed and recovered [109]



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90.1% during 14 days under 37 °C. Due to the hydrophobic interaction present in this copolymer, the combination of mechanical strength and retention time was also improved. The quantitative cell survival rates were 93.2% after 7 days and confirming a suitable biomaterial for an environment for fibroblast proliferation. Table 1 summarizes the structure, materials, and therapies/drugs involved in the manufacture of redox-sensitive devices.

Temperature Devices

Inorganic Materials

External temperature control is the primary benefit of employing inorganic materials in temperature-sensitive devices. Xia and collaborators developed a platform based on gold NPs in liposomes for use in mild temperature PTT, aiming to precisely control the temperature to favor the expression of heat shock proteins. The proposed platform showed excellent photothermal conversion efficiency with thermal fluctuations below 1 °C. Different temperatures were studied, and it was observed that at 47 °C, inhibition of the tumor tissue was observed and that this would be a new strategy for photothermal therapy in cancerous tissues [112].

More interestingly, Depciuch and collaborators used gold NPs as photosensitizers in PTT and evaluated the influence

of light energy on the size of NPs in the colon cell lines SW480 and SW620. The results showed that irradiation with a shorter wavelength laser caused higher mortality. In addition, it was observed that photothermal efficiency is inversely proportional to the size of the NPs, and these results are correlated with the absorption/extinction ratios calculated based on the Mie scattering theory [113].

Many other examples of platforms based on metallic NPs for thermal applications in therapies and environment disinfection are found in the literature, such as the work by Li and collaborators, which developed nanosize rod-shaped silver present in the pores of mesoporous silica, thus allowing that plasmonic absorption occurs from the visible to NIR. This material was dispersed in gelatin hydrogel with excellent efficiency in the photothermal conversion, capable of causing *E. coli* death superior to 99% [114]. Kim and collaborators performed a study with bovine serum albumin-coated NPs evaluating the therapeutic activity for the treatment of skin cancer, and the NPs showed cytotoxic effects on B16F10 melanoma cells caused by oxidative stress, which demonstrated the efficiency of the material to be applied in PTT [115]. Wang and collaborators obtained a hydrogel composite containing copper NPs in polyvinyl alcohol that eliminated more than 99% of bacterial *E. coli* after laser irradiation for 10 min [116].

Mohammed and collaborators synthesized different multi-walled carbon nanotubes (MWCNTs) for use in PTT;

Table 1 Summary of devices based on redox stimulus

Device structuring	Material	Therapy/drug	References
Nanoparticles	PEG-PBC-TKDOX	PDT	[102]
	PEG-PPMDT	Chemo-sonodynamic antitumor therapy	[101]
	Silica/PAA	Bioimaging	[96]
	Iron Oxide/Chitosan	DOX	[71]
	Iron Oxide/Silica	DOX	[72]
	Mesoporous silica	Cysteine/coumarin	[85]
	Mesoporous silica/Gold	Ij	[87]
	Silica/Hyaluronic acid	DOX	[86]
Qds	CdS	Bactericidal properties	[91]
	CdSe/ZnS	Biomarker	[92]
Carbon	Graphene/PEG/PCL	DOX	[93]
	Graphene oxide	DOX	[94]
	Nanotubes	DOX	[95]
MOF	Nic-MOF/Hyaluronic acid	PDT/ Nicorandil	[100]
	ZIF-8	Tris(3-hydroxypropyl)phosphine	[98]
	MIL-101-N ₃ (Fe)	DOX	[99]
Micelles	4-carboxybenzaldehyde functionalized PEG	DOX	[104]
	P(MSPA- <i>g</i> -EG)	DOX	[103]
Hydrogel/scaffold	Poloxamer 407@hyaluronic acid	Rapamycin	[111]
	Collagen fiber/calcium-deficient hydroxyapatite	Oxidative species scavenging	[110]
	Gelatin/alginate/hydroxyapatite	Bone regeneration	[109]

it was observed that the treatment for the materials was a function of the concentration of the materials and the time [117]. Naief and collaborators recently obtained a similar result using MWCNTs decorated with platinum NPs in addition, and the materials got cytotoxicity of up to 79.6% [118]. In addition, Yan and collaborators presented a work based on inspired chemodynamic therapy using inorganic carbon dots functionalized with polyethylene glycol (PEG) with NIR emission that showed a potent-responsive effect to PTT with NIR laser of 808 nm and also a bactericidal effect [119].

Another class that stands out is oxide-based. In this context, our research group has recently published several works using platforms containing iron oxides for fluid hyperthermia (MFH) applications or controlled drug delivery [47, 120, 121]. Rodriguez and collaborators functionalized Fe_3O_4 NPs with different amino acids. They observed that the NPs showed antiproliferative activity in the concentration range of 10^{-7} – 10^{-2} mg mL $^{-1}$ and showed specific absorption rate values between 64 and 81 W g $^{-1}$, which demonstrated that the synthesized NPs have great potential for use as antitumor, antiparasitic, and clinical applications [48]. Even more interesting, Piazza and collaborators carried out a study with superparamagnetic iron oxide nanoparticles (SPION) for MFH applications, and the SPION (12 nm diameters) was PEGylated conjugated with folate by polycondensation reactions. The colloidal study showed the stability of the material in PBS, and the magnetic study indicated the saturation magnetization of 73.1 emu g $^{-1}$ at 300 K. The AMF study revealed that the NPs were able to increase the temperature up to the temperature range, indicating the potential of the material to use in MFH [48].

Polymeric Materials

Temperature-responsive polymers belong to the most studied class of nanomaterials employed as drug carriers due to biocompatibility, adjustable solubility, and their high loading capacity besides offering other clinical advantages such as eliminating the need for invasive surgical implantation and the ability to bypass physiological barriers, allowing delivery to hard-to-access locations within the body [122].

In this context, Razavi and co-workers report a temperature-responsive block copolymer self-assembled to micellar morphologies based on spiropyran-terminated PNIPAAm chains for DOX release [123]. After isomerization of the spiropyran, PNIPAAm has its LCST value increased from 26.5 to 37.5 °C. The micellar structure SP-PNIPAAm was loaded with DOX (0.2120 mg mg $^{-1}$) and achieved close to 90% DOX released at temperatures above LCST at physiologic pH.

A temperature-responsive hydroxypropyl methylcellulose (HPMC)-grafted N-isopropylacrylamide (NIPAM) aerogels

for the 5-fluorouracil carrier was reported by Liu and co-workers [123]. Polymeric aerogels HPMC-NIPAM can load 5-fluorouracil via physical embedment and hydrogel bonding interactions, achieving a drug entrapment efficiency of 76% and, finally, temperatures increase from 25 to 37 °C improving 5-fluorouracil cumulative release from 70 to 90% at pH 7.4.

Simulating a tumor tissue environment, Kou et al. developed a polymer with pH/temperature-responsive targeting. The authors obtained a responsive carrier, by chemically grafting pH/temperature-sensitive groups onto cassava starch backbone [124]. A folic acid-coupled aminated hydrophobic starch with a micellar structure reached a maximum curcumin loading of 78.5%. Besides that, a cumulative release of 82.5% was achieved at high temperatures (38 °C) and low pH (5.5). Environment conditions were also found in tumor tissue, while only 3.1% were observed at 25 °C and pH 5.5.

An interesting approach consists of combining organic polymers and inorganic NPs to design intelligent nanocarriers. Long et al. produced a dual drug delivery platform based on thermoresponsive polymeric micelle (F127-PCL), which acts as a “gatekeeper” to attach on the surface of silica NPs (MSN) through disulfide bonds [125]. DOX was encapsulated in both carriers and this micellar drug delivery system, DOX@MSN-S2-F127-PCL@DOX, showed excellent temperature stimuli-responsive performance for cancer therapy. At 40 °C, the micelles collapse from MSN after GSH stimulus, and shrinkage of the F127-PCL micelle caused a fast release of DOX from the device, increasing the DOX concentration immediately. Another combination of organic polymer and inorganic NPs was described by Jahanban-Esfahlan and co-workers that produced SiO $_2$ hollow nanocapsules (HNCaps) using the grafting of a thiol-capped PNIPAAm-b-PAA by thiol-ene click reaction onto PMMA HNCaps [126]. Synthesized HNCaps showed LCST at 38–40 °C and a DOX loading efficiency of 62%. The stimuli-responsive DOX release behavior was evaluated in vitro conditions. At 37 °C and physiological pH (7.4), HNCaps showed the lowest DOX release value (20%), while increasing the medium temperature to 41 °C improved the drug release efficiency twice. The platform's cytotoxicity was investigated against MCF7 cells, and HNCaps proved to be a satisfactory device for cancer chemotherapy. DOX loaded in HNCaps has better performance than free molecules after 48 h and drug concentration comprising between 50 and 100 µg mL $^{-1}$, showing cell viability below 40%.

3D Scaffolds/Bioprinting

The stimulus that offers the most range of uses for creating scaffolds or 3D-printed items through additive manufacturing is temperature. This is because gels can be organized into scaffolds through sol/gel transitions that depend on

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temperature or by using shape-memory materials that can be printed for applications that call for the deformation of their structural components, besides the controlled drug release.

Xing Li and collaborators [127] observed the external stimulus in a scaffold based on 4-biphenylacetic acid with phenylalanine-phenylalanine bricks. The gelation and self-assembly of these scaffolds were evaluated by temperature and pH stimuli. At a temperature range of 95 °C to 25 °C, the scaffolds were composed of nanotubes and nanoribbons with regular widths of inner 2.5 nm and outer 10 nm, and for pH switch (3 to 7.4), it exhibited hydrogel structures composed of nanofibers with a width of 8 nm. This structural platform shows cell viability higher than 80% after 72 h for L929 cell culture, confirming great potential application for cartilage tissue engineering, as shown in Fig. 5. Víctor Santos-Rosales and collaborators [128] manufactured a scaffold based on PCL loaded with bioactive vancomycin molecules. This scaffold shows great bone regeneration properties besides sterilization efficiency for prophylaxis of infections. The scaffolds were treated with a continuous CO₂ flow regime to improve the sterilization efficiency against dry spores of *B. stearothermophilus* and *B. atrophaeus* and after 1 h, it was observed that *B. atrophaeus* was completely inactive. After 14 days, 50% of vancomycin was released and this scaffold showed a higher degree of vascularization compared to pure PCL, as shown in Fig. 6.

A hydrogel scaffold based on chitosan, gelatin, and β -sodium glycerophosphate, (CS/b-GP/gelatin), was studied by Xiaowei Xu and collaborators [129] and presented in Fig. 7. This hydrogel finds applications in bone regeneration as well as anti-inflammatory. Aspirin and erythropoietin (EPO) were loaded into the scaffold matrix and released after the sol–gel transition at 37 °C. In 8 days, 94.2% of aspirin and 83.4% of EPO were released. In vivo periodontal test reveals a complete regeneration of the



Fig. 5 In situ injection of the scaffold based on 4-biphenylacetic acid (BPAA) with phenylalanine-phenylalanine bricks (FF) [127]

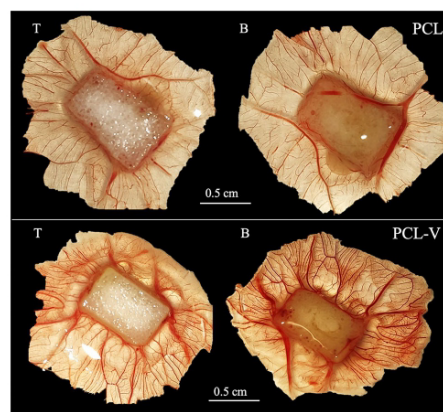


Fig. 6 Representative results at early day 14 of the embryonic development using PCL-based scaffolds [128]

affected area and an increase of 10.3% of mineral bone density when compared to pure hydrogel. Still circumventing the problems related to bone regeneration and anti-inflammatory action [130], incorporating DOX and/or lipoxin A4 molecules into thermo-reversible hydrogel polyisocyanopeptide. The hydrogel was applied in the sol state at the dog's maxillofacial region and gelation in different injection ways (Fig. 8) and it was observed immediately after the hydrogel reaches body temperature. This scaffold had a total release of A4 and DOX after 4 days and can easily be removed from the patient by applying cold water.

Furthermore, a review from Yang and collaborators shows more about the influence of thermoresponsive devices, such as the soybean oil epoxidized acrylate to fabricate a 3D structure, that could fully recover its initial state from a temporary shape at 37 °C [131]. It also exposed the use of nanofibrillated cellulose associated with acrylamide matrix for the obtation of aligned dynamic 3D structure thermoresponsive to mimetic topologic native tissues. Temperature stimuli also can be seen in the reported work from Wang and collaborators, who presented a 3D material using PCL and PNIPAM layer. While submitted to temperature reduction, its shape allows the incorporation of yeast cells and releases them when the temperature increases [132]. The same autonomous drug release control based on PNIPAM polymers is also reported by Zu and collaborators [133], showing a reduction of the drug release with the increase of shell thickness used. This behavior enables easy and efficient manipulation of the active molecules incorporated. Table 2 summarizes the structure, materials, and therapies/

Fig. 7 Photograph of the hydrogel scaffold based on chitosan, gelatin, and β -sodium glycerophosphate at room temperature (a) and in 5 min at 37 °C (b and c) [129]

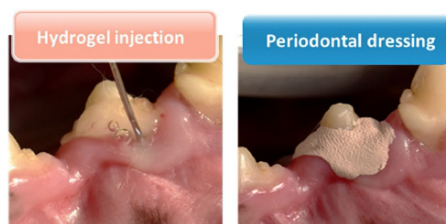
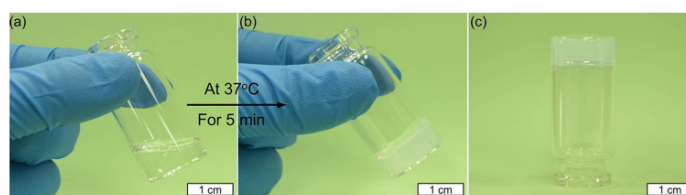


Fig. 8 Polyisocyanopeptide biomaterial implantation as hydrogel injections and periodontal dressing. [130]

drugs involved in the manufacture of temperature-sensitive devices.

Challenges and Perspectives

Though it is a bad situation, technological advancements have helped in the search for a cure since the number of new cancer cases is concerning for the approaching decades. Although medical devices are scarce on the market, potential treatment strategies based on stimulus-sensitive

delivery systems are already under development in order to reduce side effects and ensure a specific treatment in future. Issues related to surface charge and its interactions with the biological environment are bottlenecks to be overcome. The tumor microenvironment is a region that needs further investigation, so new discoveries may be helpful in adapting current technologies and developing new strategies to control the release of bioactive molecules. Recent advances in immunotherapy, a complementary therapy for cancer, have increased its popularity. The use of bacterial toxins as active molecules in immunotherapy, a form of alternative cancer treatment, has lately gained popularity. The main drawback observed in this segment is the safe use of toxins to avoid septic shock, in which stimulus-sensitive delivery systems may present potential opportunities for translating medical devices. These accomplishments will undoubtedly advance in the direction of more effective treatments, improving global well-being and raising standards of living.

Conclusions

This review summarizes recent advances in controlled release systems that are sensitive to redox and temperature stimuli. The full work of living organisms is related

Table 2 Summary of devices based on temperature stimulus

Device structuring	Material	Therapy/Drug	References
Nanoparticles	Copper/polyvinyl alcohol	Bactericidal properties	[116]
	Silver	Disinfection	[115]
	Gold	PTT	[112, 113]
	Iron oxide/PEG	MFH	[48]
	Iron oxide/amino acids	antiplasmodial activity/ MFH	[121]
	PNIPAAm-b-PAA	DOX	[123]
Carbon	Carbon dots/PEG/ MWCNTs	PTT	[117–119]
Aerogel	hydroxypropyl methylcellulose/ PNIPAAm	5-fluorouracil	[123]
Micelles	PNIPAAm/Sp	DOX	[124]
	F127-PCL	DOX	[123]
Hydrogel/scaffold	chitosan, gelatin, and β -sodium glycerophosphate	Aspirin and erythropoietin	[126]
	polyisocyanopeptide	DOX and/or lipoxin	[127]
	PCL	vancomycin	[128]

to the balance between reductive and oxidative species and significant variation in this equilibria might cause damage and cell death. Therefore, research has concentrated efforts on the development of platforms that will deliver bioactive molecules to specific sites and only when there is a real need to release them, always mimicking the behavior of the human body. The use of temperature as an internal or external stimulus also aims to achieve an increasingly personalized treatment for the proper functioning of tissues and cells. Although different strategies have been addressed, the biological effects of inorganic nanocarriers still need to be better explored, since they can release ions that can generate side effects and pose considerable challenges for clinical applications. Polymeric devices can improve bioavailability after crossing biological barriers; however, more studies related to polymer's degradation inside the cells and the behavior of prolonged use of these platforms are still necessary for these materials to reach the medical market. The current results indicate that these materials have been able to precisely release drugs in specific places and present relevant clinical results.

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Declarations

Conflict of interest Authors have no conflict of interest.

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PEGlatyon-SPION surface functionalization with folic acid for magnetic hyperthermia applications

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Abstract

Superparamagnetic iron oxide nanoparticles (SPION) are of great interest for application in magnetic fluid hyperthermia (MFH) due to their heat generation capability in an external alternating magnetic field, besides biocompatibility, and surface properties. MFH has emerged as a promisor therapeutic approach for cancer treatment and is based in controlled heating tumor tissue through the accumulation of SPIONs within cancer cells. This work describes a new route for the preparation of folate-conjugated PEGylated SPIONs, which involves the attachment of such molecules at the surface through polycondensation reactions, without the need for coupling agents or prior modification on the species involved. The size of iron oxide cores obtained by transmission electron microscopy was about 12 nm. The conjugation of folate onto SPIONs was confirmed by FTIR spectroscopy. The folate conjugated nanoparticles were colloidal stable in PBS, presenting a hydrodynamic diameter of 109 ± 1 nm and PDI 0.148. The obtained folate-targeted PEGylated SPIONs showed superparamagnetic behavior with a saturation magnetization of $73.1 \text{ emu} \cdot \text{g}^{-1}$ at 300 K. Their specific absorption rate (SAR) ranged from 32.8 to 15.0 W g^{-1} in an alternating magnetic field of $10\text{--}16 \text{ kA m}^{-1}$ and frequency of $420\text{--}203 \text{ kHz}$. The heat generated was sufficient to raise the sample temperature to the therapeutic range used in MFH establishing this system as promising candidates for use in MFH treatment.

1. Introduction

The biomedical interest in superparamagnetic iron oxide nanoparticles (SPION) is due to their biocompatibility, surface engineering capability and enhanced magnetic properties [1–3]. This material has been applied in drug delivery systems, magnetic fluid hyperthermia (MFH) [4, 5] and contrast agent for magnetic resonance imaging [6]. Theranostics systems, whose diagnosis and treatment are carried out on a single platform, has also been investigating [7, 8].

Magnetic hyperthermia has emerged as a promising non-invasive therapeutic approach for thermal cancer treatment [9]. SPION release heat when exposed to an alternated magnetic field of appropriated frequency and amplitude, by converting magnetic energy into thermal energy [3, 10]. This therapeutic platform is based on controlled heating of tumor tissue through the accumulation of SPION within cancer cells followed by exposure to an external alternating magnetic field (AMF). The SPION act as nano-heaters by increasing the local temperature in the range of $41\text{--}46^\circ\text{C}$ [11, 12], which triggers cell death mechanisms (apoptosis or/and necrosis) while altering the functionality of the protein causing high sensitivity of the cancer cells to traditional treatments (e.g. radiotherapy and chemotherapy) [13]. Sans *et al* [14] compared the temperature effect on neuroblastoma

SH-SY5Y cells in response to the application of exogenous heating sources with MFH sources. Their findings show that MFH requires 6 °C lower temperatures than exogenous heating sources to induce similar cytotoxic effect.

The ability of SPION to be driven and accumulated in the desired tissue is highly dependent on its surface and magnetic properties. Size and morphology should be finely tuned during the synthesis to increase the heating efficiency of the platform, avoiding the super dosage of magnetic fluid to reach the treatment condition [15–17]. In addition, to the success of MFH treatment, SPION surface must be functionalized to anchor specific ligands to targeting damage cancer cells and improve colloidal stability [9, 18].

In order to ensure the colloidal stability and biodistribution on physiological medium, SPION is frequently functionalized with biocompatible polymers, from a natural source (polysaccharides, such as dextran, chitosan, alginate, etc [19]) to synthetic ones (poly(lactide-co-glycolide) (PLGA) [20], polycaprolactone (PCL) [21] and polyethylene glycol (PEG) [22, 23]). PEG shows high water solubility and low cytotoxicity properties. When grafted on SPION surfaces, PEG extends their systemic circulation time reducing the binding with plasma proteins and macrophage recognition.

Concerning to the selectivity, folate receptors are overexpressed during the disordered growth of tumor cells for the internalization of nutrients required for cell division, such as folic acid [22, 24–26]. Chen *et al* [27] have described the structural basis of recognition of folic acid by folates receptors. They found that one moiety of the folic acid molecule shows strong bind affinity by the receptors, while another moiety sticks out of the pocket entrance, allowing it to be functionalized with drugs or nanoparticles, without adversely affecting the receptor binding. Bonvin *et al* [28] report a direct attachment of folic acid molecule to iron oxide surface, which folic acid acts either as coating and targeting agent. They also report the efficiency of specific uptake of composite by prostate cancer cells.

This study reports on the preparation of folate-conjugated PEGylated SPION by polycondensation reaction and the investigation of the structural, morphological, magnetic, and magneto-thermal properties. In contrast to other methodologies for PEGylation and the attachment of folate groups on nanoparticle surface, this methodology is based on de polycondensation reaction of the folate through their carboxylic acid groups with the hydroxyl groups from PEG [23], resulting in a simpler and cleaner route due to the absence of coupling agents or reactants that increase the complexity of chemical routes related in the literature [29, 30].

2. Experimental

2.1. Materials

All chemicals were used as received. Iron(III) chloride hexahydrate (AR, ACS) and sodium hydroxide (AR, ACS) were purchased from Mallinckrodt Chemicals. Iron(II) chloride tetrahydrate (99%, Aldrich), triglyme (99%, Sigma-Aldrich), poly(ethylene glycol) (average M_w 950–1050, Aldrich), citric acid monohydrate, phosphate buffer saline (PBS) (10 mmol L⁻¹ phosphate salts, 0.138 mol L⁻¹ NaCl and 2.7 mmol L⁻¹ KCl) and folic acid (97%, Sigma) were purchased from Sigma-Aldrich (Brazil).

2.2. Synthesis of folate-conjugated PEGylated SPION

Magnetite nanoparticles were synthesized by the chemical co-precipitation method described by Massart [31] with modifications. The PEGylation of the as-obtained SPION was performed following the route described previously by Viali *et al* [23]. Briefly, 250 ml of Fe³⁺ (0.30 mol L⁻¹) and Fe²⁺ (0.15 mol L⁻¹) aqueous solutions were mixed and purged with argon gas. Then, 100 ml of ammonium hydroxide (25%) aqueous solution was added to the Fe³⁺ and Fe²⁺ solution under vigorous stirring and allowed to react for 30 min. Next, the reactional suspension was heated to boiling for 1 h. The as-synthesised black precipitate (bare-Mag) was collected by magnetic decantation and washed several times with deionized water. For SPION functionalization, the obtained suspension was mixed with citric acid in the proportion of 0.37 mmol of acid per gram of iron oxide at pH 3 and kept under magnetic stirring for 12 h. The SPION functionalized with citrate were separated by magnetic decantation, washed three times with acetone, and dispersed in 120 ml of triglyme. For the PEGylation step, 30 ml of the triglyme-SPION dispersion was added to a 250 ml three-neck round-bottom flask and mixed with 30 ml of PEG. The mixture was sparged with Argon gas and heated up to 120 °C for 3 h. Then, the temperature was raised to 150 °C and kept for 21 h. Afterward, the SPION were precipitated with the addition of acetone, separated with the use of a permanent magnet and washed three times with acetone. The obtained PEGylated SPION (Mag-PEG) were dispersed in 60 ml of triglyme and added to a reaction system as described above. To this dispersion was added 100 mg of folic acid under constant stirring and the system was heated up to 120 °C for 24 h. The folate-conjugated PEGylated SPION (Mag-PEG-FA) were precipitated with acetone, separated by magnetic decantation, and washed several times with deionized water for removal of excess of folic acid. The two resulting PEGylated nanoparticles (Mag-PEG and Mag-PEG-FA) were stored in acetone.

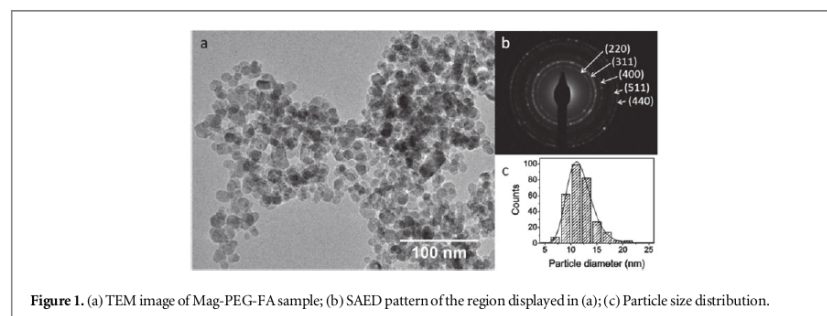


Figure 1. (a) TEM image of Mag-PEG-FA sample; (b) SAED pattern of the region displayed in (a); (c) Particle size distribution.

2.3. Characterization

X-ray diffraction (XRD) of the Mag-PEG powdered sample was recorded using the Siemens D5005 system equipped with Cu K α radiation source in the 2θ range of 20–90°. The size and morphology investigation of the SPION was performed by transmission electron microscopy analysis (TEM) using the Philips CM200 microscope operating at 200 kV. The Fourier Transform Infrared (FTIR) spectroscopy measurements were carried out with samples dispersed in KBr pellets using a PerkinElmer Frontier FT-IR spectrometer; the system resolution was set at 4 cm⁻¹ while performing 32 scans in the range of 4000 to 400 cm⁻¹. Thermogravimetric analysis (TGA) were performed from room temperature up to 800 °C under 50 ml min⁻¹ airflow, and heating rate of 10 °C min⁻¹ using the SDT 2960 system DTA-TGA from TA Instruments. The hydrodynamic diameters of the PEGylated SPION and folate-conjugated SPION were obtained by dynamic light scattering (DLS) measurements using the Zetasizer Nanoseries ZSNano ZEN3600 from Malvern Instruments. DLS measurements were performed with samples dispersed in PBS solution at 25 °C. Hysteresis loops (M-H curves) were recorded in the range of –90 to 90 kOe, at temperatures of 5 and 300 K using the commercial physical property measurement system (PPMS) model 6000 platform with the vibrating sample magnetometer (VSM) module from Quantum Design. The zero-field-cooled (ZFC) and field-cooled (FC) magnetization curves (M–T curves) were recorded in the range of 5–400 K while warming up the samples previously cooled down under zero-field and 50 Oe condition, respectively. The specific absorption rate (SAR) and the intrinsic loss power (ILP) values were obtained by exposing the samples to an AMF in the DM1000 series system, from nanoScale Biomagnetics. Field strength settings ranging from 10.0–16.0 kA m⁻¹ were used in the experiments, while the AMF frequency was varied in the range of 420.0–203.0 kHz. In a typical experiment, the sample was dispersed in a cylindrical vial vessel with 1 ml of SPION in suspension (concentration of 13 mg ml⁻¹). The vessel was placed at the center of AMF coil and the temperature was measured using a fiber optic temperature probe immersed within the suspension at its middle. The temperature versus time slope was determined by Box-Lucas equation parameters.

3. Results and discussion

3.1. Nanoparticle synthesis and characterization

The preparation of folate-conjugated PEGylated SPION followed a three-step approach, comprising the synthesis of the bare SPION, PEGylation of them, and finally their conjugation with folic acid.

The TEM image (figure 1(a)) shows nanoparticles agglomerated nearly spherical in shape. The corresponding selected area diffraction pattern (figure 1(b)) reveals the crystalline iron oxide phase which shows the characteristic d-spacing indexed as (220), (311), (400), (511), and (440) in agreement with cubic ferrite structure. The particle size histogram obtained from the TEM micrographs was curve-fitted using a log-normal distribution function (figure 1(c)). The obtained average particle size (D_{TEM}) was 12 $\pm$ 3 nm, with a polydispersity index equals to 0.2.

The XRD pattern of the Mag-PEG sample (figure 2) reveals the cubic ferrite structure of the magnetite phase, in agreement with reported protocols used to produce SPION based on the co-precipitation method. The characteristic XRD peaks marked by their Bragg indices (220), (311), (400), (422), (511), (440) and (533) are in correspondence with Fe₃O₄ phase (JCPDF Card N° 19–0629). The average x-ray crystallite diameter (D_{XRD}), calculated using the Scherrer's equation [32] was 12 nm. The observed similarity between the D_{XRD} and D_{TEM} is more likely due to the crystallinity improvement of the iron oxide nanoparticles during the PEGylation step [23].

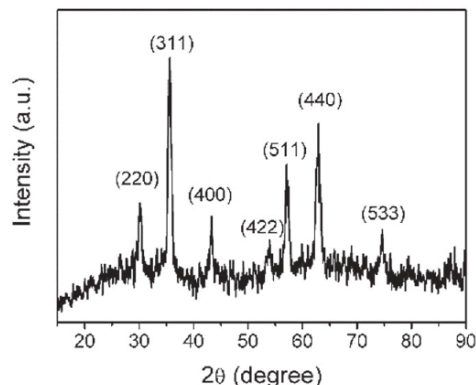


Figure 2. X-ray diffraction pattern of powdered Mag-PEGFA sample.

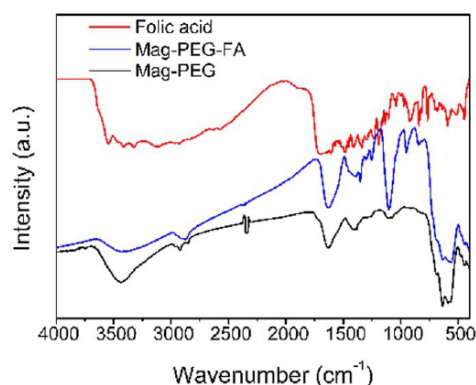


Figure 3. FTIR spectra of folic acid, Mag-PEG and Mag-PEG-FA samples.

Figure 3 shows the FTIR spectra of the folic acid, Mag-PEG-FA, and Mag-PEG samples. The IR features shown in the Mag-PEG-FA FTIR spectrum ($1700\text{--}780\text{ cm}^{-1}$ range) confirm the conjugation with folic acid. The bands in the range $1650\text{--}1450\text{ cm}^{-1}$ and $1250\text{--}780\text{ cm}^{-1}$ were attributed to ring stretching and C-H deformation vibrations, respectively. Bands due to the carboxylate stretching modes were assigned to asymmetrical (1630 cm^{-1}) and symmetrical (1390 cm^{-1}) vibrations. The strong IR band around 1100 cm^{-1} was assigned to the C–O stretching of the PEG moiety whilst the bands at 640 , 550 , and 440 cm^{-1} were attributed to Fe–O vibrations [33, 34].

TGA analysis was performed to access information regarding the amount of polymer and folic acid present on the samples Mag-PEG and Mag-PEG-FA. The TGA curves (figure 4) show that mass losses occurred in the temperature range from 50 to $455\text{ }^{\circ}\text{C}$. The first step of mass loss occurring up to $75\text{ }^{\circ}\text{C}$ and equals to 4.2% and 25.2% for Mag-PEG and Mag-PEG-FA, respectively, was associated with loss of adsorbed acetone and water. The second step, initiated at $200\text{ }^{\circ}\text{C}$ and ended around $455\text{ }^{\circ}\text{C}$ for both samples, was related to the decomposition of the organic coverage (citrate, PEG and folic acid) attached to the nanoparticle's surface. The mass loss related to organic amount was 15.0% for Mag-PEG and 26.8% for Mag-PEG-FA, which reveals an increase of 11.8% in organic coating after reaction with folic acid, thus suggesting its conjugation onto the nanoparticle's surface [33, 35].

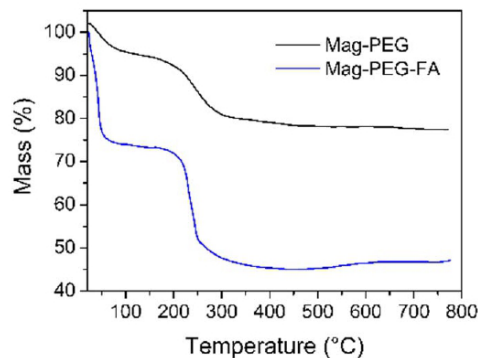


Figure 4. TGA curves for the samples Mag-PEG and Mag-PEG-FA.

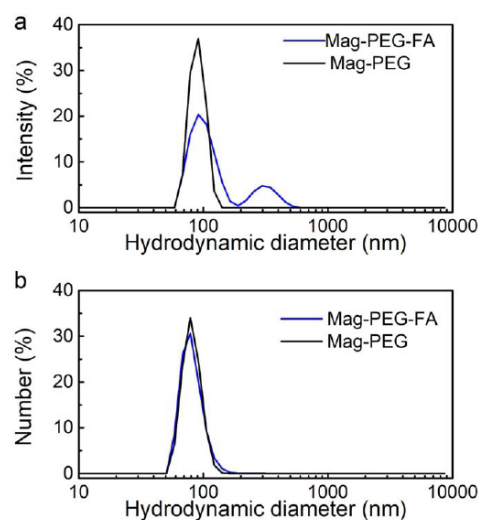


Figure 5. Hydrodynamic diameter distribution curves obtained by DLS measurements for Mag-PEG and Mag-PEG-FA samples dispersed in PBS solution (0.15 mmol L⁻¹ of salt concentration and pH 7.4). (a) Intensity and (b) Number distribution.

The colloidal stability of Mag-PEG and Mag-PEG-FA samples was evaluated through DLS measurements. The nanoparticle dispersions in PBS solution at pH 7.4 presented Z-average hydrodynamic diameters (and polydispersity indexes) equal to 94.2 ± 0.2 nm (0.14) for Mag-PEG and 109.7 ± 0.9 nm (0.15) for Mag-PEG-FA. The close values of the hydrodynamic diameters and polydispersity indexes for both samples indicate that the conjugation with folic acid did not affect the colloidal stability of the PEGylated nanoparticles. Figure 5(a) shows the hydrodynamic diameter distribution curves obtained by DLS. For sample Mag-PEG it is observed a hydrodynamic size population centered around 100 nm, which agrees with the Z-average result. However, for the sample Mag-PEG-FA this population appears broadened and it is followed by a second, less intense, particle size population centered at 290 nm. The second population does not correspond to a significant percentage of sample, being not representative when the hydrodynamic diameter is shown by number distribution (figure 5(b)). This bimodal hydrodynamic size distribution contributes to the greater Z-average hydrodynamic diameter for Mag-PEG-FA. In addition, it is important to note from the hydrodynamic size distribution curves

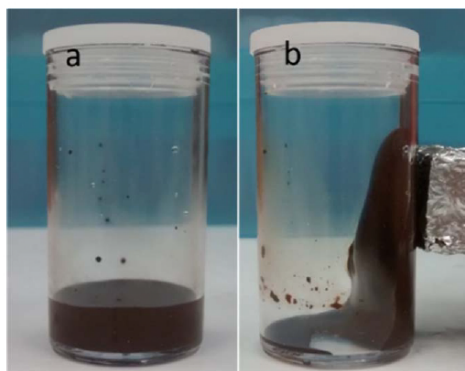


Figure 6. Mag-PEG-FA dispersion in PBS solution in the absence (a) and in the presence of an external magnetic field (b).

the absence of aggregates greater than 300 nm for both samples, which is indicative of good colloidal stability. Figure 6 shows the colloidal and magnetic responsiveness of Mag-PEG-FA dispersed in PBS solution. These SPION presented high dispersibility in this media generating a stable brown dispersion (figure 6(a)). When exposed to an external magnetic field the dispersion moves towards it due to the magnetic attraction of the SPION, without present any phase separation (figure 6(b)). These results demonstrate the Mag-PEG-FA SPION present properties that allow its use in protocols of MFH [36].

3.2. Magnetic characterization

Magnetic characteristics of the as-produced SPION were evaluated using magnetic hysteresis loops. The magnetization versus magnetic field curves (figure 7) shows that both samples, Mag-PEG and Mag-PEG-FA present similar magnetic saturation (M_S) values at 90 kOe, indicating that the folic acid surface-functionalization step did not cause a significant change in the SPION magnetism. The room-temperature M_S values obtained after normalizing the data with respect to the SPION core (using TGA data) were, 73.1 and 69.2 emu g^{-1} for the Mag-PEG-FA and Mag-PEG samples, respectively. At 5 K the normalized M_S values were 79.8 and 77.1 emu g^{-1} , respectively. The reduction of the M_S values of samples Mag-PEG and Mag-PEG-FA (normalized core) with respect to M_S value of bulk magnetite (92 emu g^{-1}) [37] is consistent with the literature and it is often attributed to the surface contribution of spin canting, surface disorder, stoichiometric deviation, cation distribution and adsorbed species, the latter supported by the TGA data [38, 39]. Moreover, the obtained results suggest that part of the saturation magnetization reduction ($\sim 4\%$) is restored by the folic acid surface-functionalization. The magnetic hysteresis loop data reveal a superparamagnetic behavior at 300 K (figure 7(a)). However, small remanence (M_R) and coercivity (H_C) were found at 5 K (figure 7(b)), indicating a thermally blocked state in both samples. The larger coercive field determined for the sample Mag-PEG-FA ($H_C = 381 \text{ Oe}$) with respect to sample Mag-PEG ($H_C = 222 \text{ Oe}$) seems to be more likely related to the stronger magnetic dipolar interaction among the SPIONs in sample Mag-PEG-FA [40]. On the other hand, ZFC/FC curves of both samples show features of blocked states at low temperature and relaxed states at room temperature are consistent with results determined from hysteresis loops. ZFC/FC curves of sample Mag-PEG show the irreversibility at around 275 K; meanwhile, sample Mag-PEG-FA (figure 7(c)) show the characteristic splitting at around 300 K, indicating that the dipolar particle-particle interactions within agglomerates are stronger in sample Mag-PEG-FA, in agreement with the larger coercive field determined for the latter sample. That scenario can be enhanced by likely the contribution of a small fraction of larger SPION, as observed in TEM micrographs [41].

The heating effect of SPION, when exposed to an AMF, is originated by the conversion of magnetic energy into thermal energy due to delay in relaxation of the magnetic moment [42–44]. The heat generation from hysteresis losses is negligible due to the superparamagnetic behavior of both samples at ambient temperature. Thus, the loss process is governed by the sum of rotational motion (Brownian relaxations) and the internal diffusion magnetic moment (Néel relaxations) [15]. The power dissipation is quantified by measuring the SAR, according to equation (1).

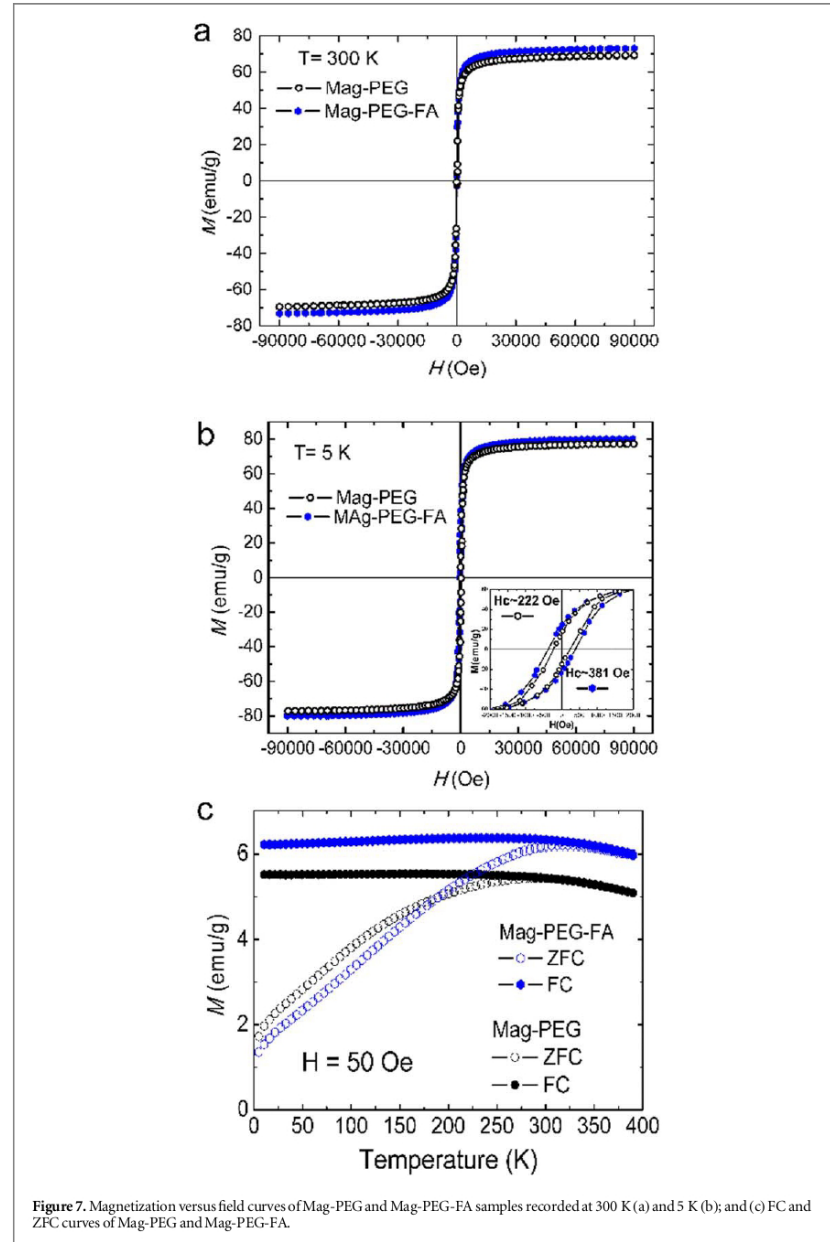


Figure 7. Magnetization versus field curves of Mag-PEG and Mag-PEG-FA samples recorded at 300 K (a) and 5 K (b); and (c) FC and ZFC curves of Mag-PEG and Mag-PEG-FA.

$$SAR = \frac{(c_{Liq} m_{Liq}) \Delta T}{m_{MNP} \Delta t} \quad (1)$$

where c_{Liq} and m_{Liq} is the heat capacity and mass of solvent, respectively; m_{SPION} is the iron oxide content, per gram, in suspension and $\Delta T/\Delta t$ is the initial slope of the time-dependent curve. The heat capacity of SPION is negligible due to the low concentration in fluid, thus the water's heat capacity (4.18 J K^{-1}) was considered [45].

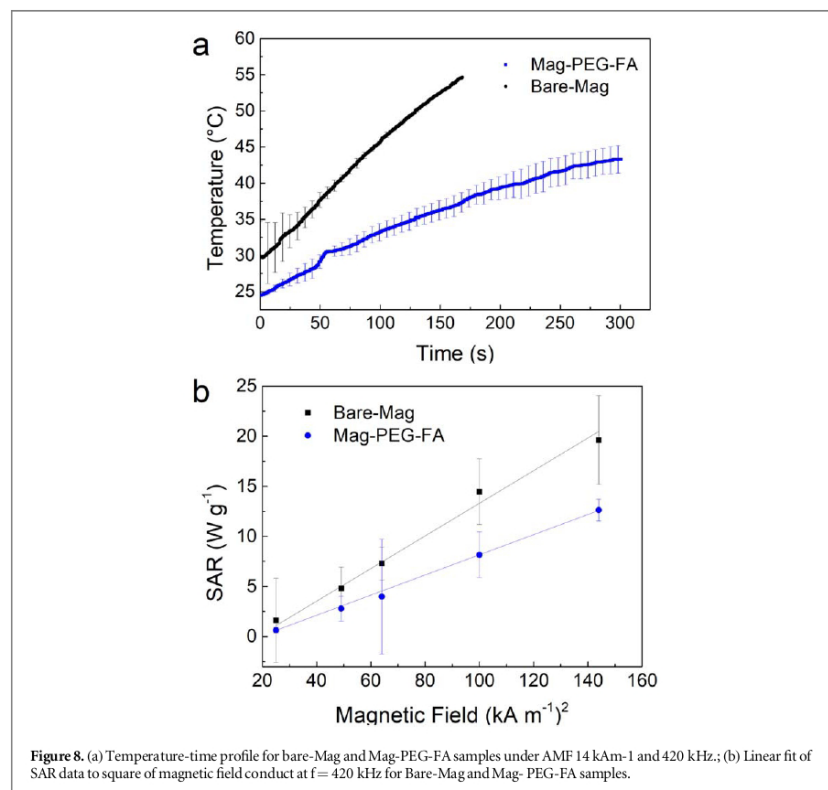


Figure 8. (a) Temperature-time profile for bare-Mag and Mag-PEG-FA samples under AMF 14 kAm-1 and 420 kHz.; (b) Linear fit of SAR data to square of magnetic field conduct at $f = 420$ kHz for Bare-Mag and Mag-PEG-FA samples.

Table 1. SAR and ILP values for magnetic heating at fixed field strength and frequencies.

Sample	f^A (kHz)	H^B (kAm ⁻¹)	SAR (W g ⁻¹)	ILP (nHm ² Kg ⁻¹)	R ²
Bare-Mag	203	16	14.9 ± 0 + 5	0.39	0.993
	280	15	22.5 ± 2.4		
	420	14	32.8 ± 7.1		
	420	10	10.3 ± 3.3		
Mag-PEG-FA	203	16	13.8 ± 0.7	0.24	0.999
	280	15	11.7 ± 2.0		
	420	14	21.6 ± 9.9		
	420	10	10.3 ± 5.5		

The heating efficiency was evaluated for uncoated (bare-Mag) and coated (Mag-PEG-FA) samples under different magnetic field strengths and frequencies. Table 1 summarized the SAR value obtained and figure 8(a) shows the temperature versus time curve for samples with the highest SAR value, 32.8 W g⁻¹ and 21.6 W g⁻¹ for bare-Mag and Mag-PEG-FA, respectively. The bare-Mag sample reaches the temperature of 42 °C in 80 s, whereas Mag-PEG-FA achieves the same temperature after 222 s after the AMF be turned on. This difference could be attributed to the organic layer comprised of citric acid, PEG and folic acid species that behave as an insulating shell around the SPION's surface [16, 36]. The SAR parameter is changed if different magnetic strengths and frequencies are carried out to evaluate the heating of the same sample. The heat dissipation shows a linear relationship with the square of magnetic field strength when the frequency is held constant. Frequency can also be used to tune the heat generation. Thus, heat generated by SPION can be optimized through the adjustment of properties of the applied magnetic field and frequency in order to obtain the highest values of SAR [46]. To allow direct comparison between different equipment and overcome problems related to standards

during the measurement a new parameter is obtained, in which SAR value is normalizing by frequency and square field amplitude, resulting in ILP parameter [4, 46], according to equation 2.

$$ILP = \frac{SAR}{fH^2} \quad (2)$$

The linearity of SAR in the function of the square magnetic field was confirmed, as showed in figure 8(b). The correlation coefficient obtained for the Bare-Mag curve fit was 0.98 and for Mag-PEG-FA was 0.99. The ILP values obtained by the slope of SAR versus H^2 curve were $0.34 \text{ nHm}^2 \cdot \text{Kg}^{-1}$ and $0.24 \text{ nHm}^2 \cdot \text{Kg}^{-1}$ for Bare-Mag and Mag-PEG-FA, respectively. This material is an interesting candidate for MFH treatment once achieve in a few minutes the temperature whose cancer cells are more sensitive.

4. Conclusions

The Folate-conjugated PEGylated SPION synthesized in this work proved to be interesting candidates for application in MFH protocols. The SPION dispersion was colloidal stable in PBS and caused a temperature increase in the range of sensitization of cancer cells in a short time interval and at low mass concentration. The presence of PEG and folate species were confirmed by FTIR and TGA analyses, evidencing the efficiency of the synthetic route adopts. The PEG layer can increase the systemic circulation and limits the aggregation minimizing the interaction of the particles avoiding a reduction on SAR values, while the folate moiety is aimed to increase the targeting of SPION for cancer cells.

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Magnetic Nanoparticles Surface Modified with Biodegradable Polymers for Controlled Methotrexate Delivery in Cancer Therapy

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Magnetite nanoparticles were surface modified with poly(ethyleneglycol)methyl-ether-poly(ϵ -caprolactone) (mPEG-PCL) copolymers in order to be dispersible in water for the efficient methotrexate release. Also its cytotoxic potential in human osteosarcoma cells was evaluated. The copolymer was synthesized by a ring-opening polymerization of ϵ -caprolactone using mPEG as a macro initiator in the presence of stannous octanoate catalyst. The samples were characterized by Dynamic Light Scattering (DLS), Fourier Transform Infrared Spectroscopy (FTIR), Transmission Electron Microscopy (TEM) and UV-Vis Spectroscopy. Magnetic nanoparticles have been successfully surface modified with copolymer mPEG-PCL forming NP-mPEG-PCL complex, in which methotrexate entrapment was obtained. At 0.5% w/v copolymer:NP ratio the system presented best behavior in terms of dispersibility and stability in aqueous medium. The major drug release was observed at temperature near 42 °C. Human osteosarcoma cultured cells were inactivated *in vitro* when 10 μ g/mL NP-mPEG-PCL-MTX complex was present in culture medium.

KEYWORDS: Cancer Therapy, Drug Delivery, Methotrexate, Magnetite Nanoparticles.

INTRODUCTION

The area of drug delivery has gained attention of the pharmaceutical industry because it offers an opportunity to expand current drug markets through new delivery technologies that will make repackaging of classic drugs possible, and also will allow the usage of drugs abandoned due to its high side effects. Delivery nanodevices can provide a more effective drug usage than those of the past by reducing recovery time of patients and extending the life cycle of the product; consequently decreasing healthcare costs. The challenge in chemotherapy is the delivery of an effective dose of a given cytotoxic agent directly to the tumor site, while at the same time minimizing unwanted side effects. Thus, it will improve the pharmacological properties of traditional chemotherapeutics by optimizing drug pharmacokinetics and biodistribution.¹ Therefore, the

development of techniques that could selectively deliver drugs to the pathological sites is currently one of the most important areas of drug research.²

Nanoscale particles have been elected as new drug delivery platforms. The dimensions of these nanoparticles (NPs) make them ideal candidates for surface engineering to obtain multifunctional nanostructures. Such modifications of NPs facilitate their use for targeted drug delivery in tumor therapy.³ NPs are envisioned as the future of drug delivery technology as they present potential to become not only useful therapeutic but also a diagnostic tools in the cancer therapy.^{2,4} Since the 80's, several drug delivery systems were developed to improve the efficiency of drugs and minimize toxic side effects.⁵ For this purpose, the amphiphilic block copolymers consisting of hydrophilic and hydrophobic segments have attracted much attention because of their potential applications as hydrophobic drug carrier systems in aqueous media.⁶ For these applications, magnetic nanoparticles should be surface modified by an amphiphilic biocompatible and biodegradable polymer.⁷

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Polymeric nanoparticles micelles can be assembled from amphiphilic copolymers segments onto magnetic nanoparticles surface, allowing drug entrapment. The hydrophobic segment creates the inner core of the micelle, while the hydrophilic segment creates the outer shell in an aqueous media.⁸ Polymeric micelles can be used as a drug delivery device by either physically entrapping the drug or by chemically conjugating the drug to the hydrophobic block prior to micelle formation.^{9,10} The advantages of using polymeric micelles as drug delivery systems are those of extending the blood circulation time of drug; passively targeting tumor; avoiding capilar emboli by small particle size aggregation; solubilization of hydrophobic drugs; reduction of the anticancer drugs adverse effects; bioactive agents preservation within the micellar core for long durations.^{6,11} Furthermore, polymeric nanoparticles are ideal candidates to cancer therapy, vaccine delivery, contraceptives, and antibiotic delivery.^{7,12} In viewpoint of chemistry, the challenge is to create new copolymers matching hydrophilic and lipophilic properties to control drug delivery.¹² Poly- ϵ -caprolactone (PCL) shows high degree of crystallinity, hydrophobicity, slow degradation and biocompatibility with tissues. Therefore, hydrophobic drugs, as methotrexate (MTX), can be dissolved in PCL chain. Due to poly(ethyleneglycol)methylether (mPEG) hydrophilicity, non-toxicity, antigenicity and immunogenicity it can be copolymerized with PCL to enhance the hydrophilicity, extending its application.¹³ It is well known that block copolymerization allows combination of the chemical properties of the main components, and the physical properties of the resulted copolymers can be tailored by adjusting the molecular weights and the composition of the building block.¹⁴ Block copolymers can be effective drug delivery vehicles because they spontaneously self-assemble in solution to form micelles with sizes ranging from tens of nanometers to several hundred nanometers. Micelles have been demonstrated to improve the apparent water solubility of hydrophobic anticancer drugs, allowing a more effective transport and biodistribution to the desired location through the bloodstream, which consists mostly of water.¹⁵

Magnetic nanoparticles (MNPs) with typical diameter <20 nm exhibit stable magnetic properties and low cytotoxicity, and can potentially be applied in bio-separation, cell targeting, biosensing,¹⁶ magnetic resonance imaging (MRI),¹⁶⁻¹⁸ drug and gene delivery,^{9,16,19} and hyperthermia cancer treatment.^{16,20} Coating these nanoparticles with amphiphilic copolymers offer new opportunities for magnetic field guided drug delivery,²¹ overcoming limitations associated with systemic distribution of conventional chemotherapies, while simultaneously reducing dosage and the deleterious side effects associated with nonspecific uptake of cytotoxic drugs by healthy tissue.²²

In order to evaluate the nanodevices obtained in this work, methotrexate was chosen as drug probe.

Methotrexate, a folate antimetabolite, is widely used in the treatment of malignancies including osteosarcoma, childhood acute lymphocytic leukemia, non-Hodgkin's lymphoma, Hodgkin's disease, head and neck cancer, lung cancer, breast cancer, psoriasis, choriocarcinoma and related trophoblastic tumors.¹¹ As an analogue of folic acid (FA), MTX can be utilized not only as a drug but also as a potential targeting agent. However, the clinical use of MTX is limited due to its toxic side effects,^{16,23} short halftime in the blood stream and the cellular efflux phenomenon of this drug. Nevertheless, the MTX entrapped onto polymeric nanoparticles of mPEG-PCL copolymer have the potential to provide a novel type of MTX-delivery carrier. Such novel types of polymeric drug delivery systems would decrease the adverse side effects of MTX and increase delivery efficiency to tumors.¹¹

The goal of this work was to prepare and to characterize mPEG-PCL copolymers, composed by hydrophilic PEG chains, which allows its dispersion in blood, and by the hydrophobic PCL chains to facilitate hydrophobic MTX dissolution. mPEG has been successfully used as a micro initiator for ring-opening polymerization of ϵ -caprolactone. Magnetite nanoparticles dispersible in water were surface coated with the copolymer and the entrapment of the MTX were successfully obtained. Cytotoxic effect in osteosarcoma human cells were evaluated.

MATERIALS AND METHODS

Materials

Poly(ethyleneglycol) mono methylether mPEG with M_n 5000 g/mol, monomer ϵ -caprolactone (99%), stannous octanoate (95%), oleic acid, methotrexate, sodium hydroxide, Minimum Essential Medium Eagle, Alpha Modification (α -MEM, Invitrogen), chloroform and hexane.

Methods

Synthesis of mPEG-PCL

mPEG-PCL diblock copolymer having 5000 g/mol mPEG and 5000 g/mol PCL was prepared by a modified method adapted from Rutnakornpituk et al.²⁴ through a ring-opening polymerization of ϵ -CL using an mPEG macro initiator in the presence of stannous octanoate catalyst. Dried mPEG (5 g), ϵ -CL (5 mL) and stannous octanoate (1.7 mL) were mixed into a 100 mL two neck round-bottom flask. The reaction proceeded at 120 °C for 3 h with magnetic stirring under N_2 atmosphere. The mixture was dissolved in chloroform and precipitated in cold hexane. Copolymer purification procedure was repeated twice to remove unreacted species and it was then dried at 40 °C under reduced pressure.

Synthesis of mPEG-PCL-MTX

All procedure was carried out in soft illumination and all the recipients were covered with aluminum foil due

to MTX degradation in presence of light. A 0.2 mg/mL water solution of mPEG-PCL copolymer was heated until it reaches 45 °C, then mixed with the 30 µg/mL solution of methotrexate in 0.1 mol·L⁻¹ NaOH and left under magnetic stirring for 2 hours. The resulting solution was cooled until 0 °C and then was inserted into a dialysis membrane and maintained by 24 h, under stirring with periodically water change.

Preparation of the Copolymer-Stabilized Aqueous-Based Magnetite Nanoparticles

The fixation of copolymer onto magnetite nanoparticle was done in aqueous medium. Dried magnetite was introduced in oleic acid hexaneous solution under stirring. To prepare the copolymer-stabilized nanoparticles, 20 mL of the hexane magnetite dispersion was introduced into 20 mL aqueous solutions of 0.1%, 0.3%, 0.5%, 1%, 3% and 5% w/v of copolymer, respectively, resulting two layers systems. The mixtures were then sonicated for 1–4 h to transfer nanoparticles from hexane top layer to aqueous bottom layer. Hexane was evaporated further by rigorous stirring at room temperature. The copolymer-magnetite was dialyzed against deionized water and refreshed twice at 24 h interval to remove excess copolymers from dispersion. Figure 1 schematically represents the formation of the nanoparticle-polymeric micelles, NP-mPEG-PCL.

Characterization

Characterization of mPEG-PCL Copolymer

mPEG-PCL copolymer samples were characterized by Differential Scanning Calorimetry (DSC). Experiments were carried out on a Perkin Elmer Pyris 1 Instrument. Samples were heated from -40 °C to 100 °C (first heating run), held for 2 min to erase the thermal history, then cooled to -30 °C at 10 °C/min, and finally heated again to 100 °C (second heating run) at 10 °C/min. Samples were also characterized by Fourier Transformed Infra-Red

Spectroscopy (FTIR). FTIR was performed on a Perkin-Elmer Model 1600 Series FTIR Spectrophotometer. The data was collected using KBr disc. Simultaneous TG-DTA curves were obtained with a thermal analysis system, model SDT 2960, from TA Instruments. The purge gas was air, with a flow of 100 mL min⁻¹, a heating rate of 10 °C min⁻¹ and samples weighing about 7 mg. Alumina crucibles were used for recording the TG-DTA curves. Zeta potential of mPEG-PCL copolymer samples were measured using a Zetasizer Nanoseries ZSNano ZEN3600 from Malvern Instruments.

Methotrexate Entrapment and Loading Efficiency

Aliquots of water were withdrawn during dialysis of mPEG-PCL-MTX samples at room temperature to evaluate how much drug remained absorbed in the copolymeric micelles. MTX chemical analysis was made by UV absorbance in a spectrophotometer (Cary 5000, Varian, Australia) at 303 nm. Concentrations were calculated according to standard curve of the MTX varying drug content from 0.01 µg/mL to 30 µg/mL, at room temperature.

Release of MTX Loaded Into mPEG-PCL

After dialysis, the dispersion containing mPEG-PCL-MTX was gradually heated from 20 to 42 °C. Aliquots of 3 mL supernatant were withdrawn at 20, 35, 37, 40 and 42 °C. The amount of MTX released from micelles was determined according to the UV absorbance at 303 nm by interpolation with standard curve of the MTX.

Magnetic Nanoparticle Surface

Modifications with Copolymer

Surface modifications with copolymer were evaluated by FTIR spectrometry. The average particle sizes and the size distributions were determined using dynamic light scattering (DLS 90 Plus Brookhaven Instrument Corp.).

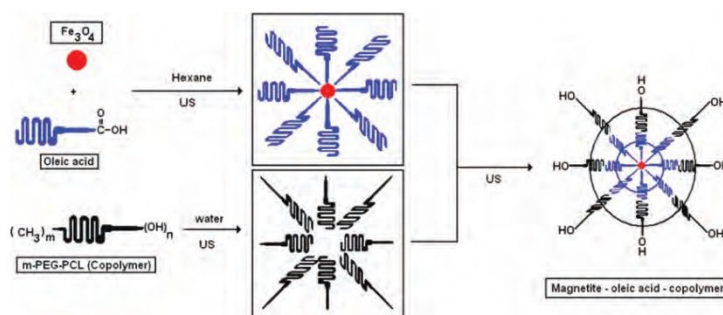


Figure 1. Schematic representation of the complex NP-mPEG-PCL formation.

The particle size and shape were studied by transmission electron microscopy (TEM). Images were taken on a Philips CM 120 kV Microscopy.

Biological Assay

The *in vitro* complex NP-mPEG-PCL-MTX (0.5% copolymer) cytotoxicity was evaluated using MTT assay on MG63 human osteosarcoma cells. Viable cells have the ability to reduce MTT from a yellow water-soluble dye to a dark blue insoluble formazan product.²⁵ Formazan crystals were dissolved in DMSO and quantified by measuring its absorbance at 570 nm, and the amount of living cells was correlated to unreduced Formazan. Cells were seeded in 96 well plates in Minimum Essential Medium Eagle, Alpha Modification (α -MEM, Invitrogen) without serum, supplemented by 100 U/mL penicillin/streptomycin (Invitrogen), at 37 °C in humidified atmosphere containing 5% CO₂. After overnight attachment, the cells were exposed to samples (1 μ g/mL and 10 μ g/mL) or its vehicle for additional 24 h and methyl tetrazolium (MTT) assay was performed as previously described.²⁶ Briefly, the extracts were replaced by 90 μ L of α -MEM and 10 μ L of MTT solution (5 mg/mL), remaining in the incubator for 4 h. Thereafter, the media was replaced by acidified isopropanol (0.04 mol·L⁻¹ HCl) to dissolve the formazan crystals. The absorbance was measured by microplate reader (Tp Reader; Thermoplate, Nanshan District, Shenzhen, China) at 570 nm.

RESULTS AND DISCUSSION

Characterization of mPEG-PCL Copolymers

Figure 2 illustrate the crystallization and melting behaviors of mPEG-PCL copolymer. All the characteristic peaks of

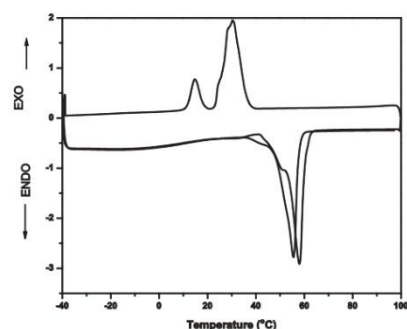


Figure 2. Thermal properties of the mPEG-PCL copolymer: crystallization peaks at 30.4 and 14.7 °C; melting peaks were observed at 58.1 °C in the first heating and 55.3 and 46.3 °C in the second heating.

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the mPEG-PCL copolymer confirmed that there was success in the synthesis of the mPEG-PCL copolymer via ring opening polymerization of the ϵ -caprolactone monomer using mPEG as macro initiator of the reaction. Furthermore, FTIR analysis of the mPEG-PCL copolymer, presented in Figure 3, showed a band at 3442 cm⁻¹ attributed to water and hydroxyl group of PEG. Bands at 2946 cm⁻¹ and 2888 cm⁻¹ are assigned to organic groups (CH and CH₂) of the aliphatic chain from PEG and PCL. The ester linkage ($-\text{OC}=\text{O}$) was observed through band at 1725 cm⁻¹, attributed to ester carbonyl stretching. This result confirms the success of the mPEG-PCL copolymer synthesis.

Investigation of Methotrexate Entrapment and Loading Efficiency

The potential application of the nanodevices as drug delivery system was evaluated by its drug release profile. The entrapped drug concentration was measured. The device has entrapped 29.57 μ g/mL of MTX, which corresponds to 98.6% of the initial drug concentration in solution. The heating and subsequent cooling procedures of mPEG-PCL solution allowed methotrexate fixation into the copolymer. Also, from dialysis, self-assembly of copolymers took place and entrapped the drug into the micelle, at same time, dialysis allowed the removal of unloaded drug and/or copolymer. Hydrophobic interactions may be responsible for the drug entrapment in the copolymer, thus hydrophobic drug like methotrexate can be bonded to PCL copolymer chains through hydrogen bonds.²⁷ Hydrophobic core composed of PCL segment has shown good drug permeability as indicated by Zhang et al.,²⁸ supporting the hydrophobic interaction of drug entrapment. The Figure 4 shows MTX release profiles from mPEG-PCL-MTX at five different temperatures (20, 35, 37, 40 and 42 °C). As illustrated in Figure 4, the amount of drug released

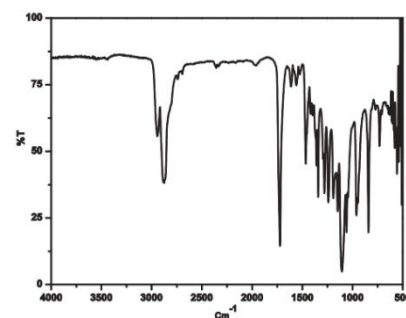


Figure 3. FT-IR of mPEG-PCL copolymer: bands at 3442 cm⁻¹ (OH) of PEG, 2946 cm⁻¹ and 2888 cm⁻¹ (CH and CH₂) of PEG and PCL and 1725 cm⁻¹ ($-\text{OC}=\text{O}$) of PCL.

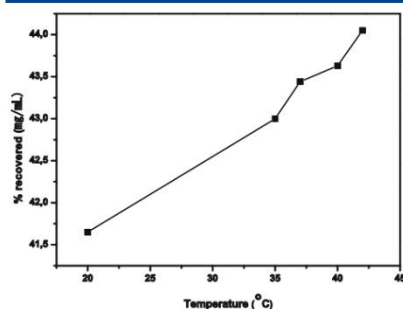


Figure 4. Release curves of the sample at 20, 35, 37, 40 and 42 °C.

has increased when temperature rose. When the temperature reaches 42 °C, it was observed a major rise of the drug released reaching 44% (13.2 µg/mL) of 98.6% (29.57 µg/mL) MTX entrapped into the micelles.

Magnetic Nanoparticle Surface Modifications with Copolymer

After fixation of the copolymer onto magnetite nanoparticles surface, IR analysis was performed to confirm if copolymer was onto magnetite nanoparticles. Figures 5 and 6 exhibit FTIR absorption bands of magnetic nanoparticles with and without mPEG-PCL copolymer in different w/v percentages (0.1%; 0.3%; 0.5%; 1%; 3% and 5%). As seen in Figures 5 and 6, when copolymers were added to magnetite nanoparticles, it was observed a change in band from 1500 to 700 cm⁻¹ compared to band of copolymer without nanoparticle. In this region, there is a more intensive band (1063 cm⁻¹) indicating that copolymer was linked

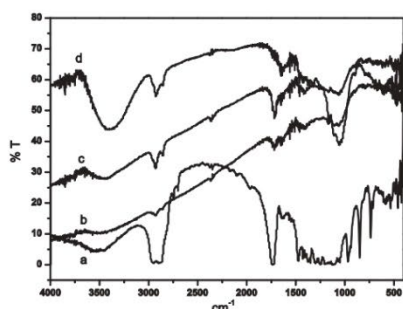


Figure 5. FTIR band (a) mPEG-PCL without NP; (b) complex 0.1% NP-mPEG-PCL; (c) complex 0.3% NP-mPEG-PCL; (d) complex 0.5% NP-mPEG-PCL.

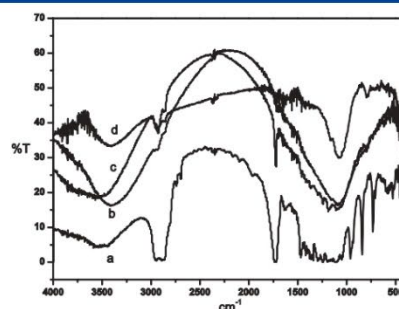


Figure 6. FTIR band (a) mPEG-PCL without NP; (b) complex 1% NP-mPEG-PCL; (c) complex 3% NP-mPEG-PCL; (d) complex 5% NP-mPEG-PCL.

to magnetite nanoparticle surface regardless variation of the copolymer concentration. Moreover, the characteristic vibrations of the Fe-O bond are observed at 590 cm⁻¹ which indicates high concentration of Fe on the sample.

Hydrodynamic diameter data for nanoparticles surface modified with copolymer are on Table I. Figure 7 shows magnetite nanoparticles dispersed in water with copolymer in different w/v percentages (0.1%, 0.3%, 0.5%, 1%, 3% and 5%). As seen in the Figure 7, NP-mPEG-PCL (0.5% copolymer) sample presents a low tendency of nanoparticles agglomeration, suggesting the best behavior in terms of dispersibility in aqueous medium compared with other samples. Moreover, it was observed that for concentration lower than 0.5% copolymer, the system shows a tendency to agglomerate with particles aggregation as well as particles adhesion on the tube. When low copolymer content is used, the unbounded oleic acid remains on nanoparticle surfaces without forming micelles with copolymer, resulting in a hydrophobic interaction with the plastic vial tube (Fig. 7). The inset in the Figure 7 presents the magnetic recovery of sample NP-mPEG-PCL-MTX (0.5% copolymer), indicating that even surface

Table I. Hydrodynamic diameter of the samples measured by DLS in different concentrations of copolymers.

Complex NP + Oleic. Ac + % mPEG-PCLCopolymer	
Copolymer concentration (%)	Hydrodynamic diameter (nm)
0	224
0.1	880
0.3	630
0.5	353
1	236
3	217
5	959

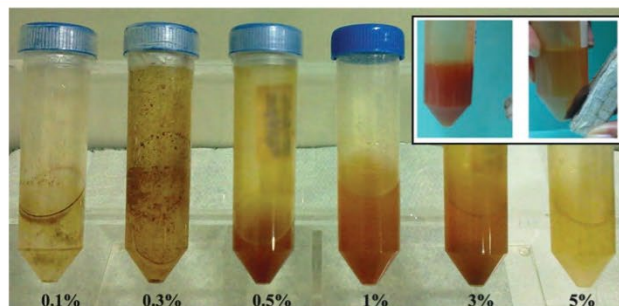


Figure 7. Magnetite nanoparticles dispersed in water in different concentrations of mPEG-PCL copolymer (0.1%; 0.3%; 0.5%; 1%; 3%; 5%). The inset presents the magnetic recovery of sample NP-mPEG-PCL-MTX (0.5% copolymer).

modified the magnetic nanoparticle can be magnetically driven. The efficiency of surface modification with polymer was checked out by thermogravimetric analysis. Thermogravimetric (TG) and differential thermoanalysis (DTA) curves for samples NP-mPEG-PCL-MTX (0.5% copolymer) and NP-mPEG-PCL-MTX (3.0% copolymer) are presented in Figure 8. The percentage weight residues were 42.7775% and 22.4473%, respectively. This result agrees with different mass ratio of polymer to nanoparticles, as it was expected that NP-mPEG-PCL-MTX (3.0% copolymer) sample has a bigger weight loss due to its highest polymeric shell.

Figure 9 shows the zeta (ζ) potential of uncoated magnetic nanoparticles (NP) and NP-mPEG-PCL (0.5% copolymer) in function of pH by suspending the particles in 1×10^{-3} mol L⁻¹ NaCl solution. The experiments were realized in triplicates and the error bars are the standard deviation between measurements. The experimental

data were fitted by sigmoidal curves. The isoelectric point (IEP) found for uncoated magnetic nanoparticles and NP-mPEG-PCL (0.5% copolymer) were $\sim$ pH 6.3 and $\sim$ 4.3, respectively. This decrease in IEP can be ascribed to the presence of PEG on nanoparticles surface, as reported in literatures.²⁹ This results show that nanoparticle surface modification with mPEG-PCL copolymers were successful obtained with this hydrophilic chains (PEG) in the shell, which allows its good dispersion in water media as blood.

Figure 10 shows the microstructure of complex NP-mPEG-PCL-MTX (0.5% copolymer), chosen to TEM examination due to its visually good dispersibility (Fig. 7). The TEM analysis presented at Figure 10 indicates a predominance of magnetite particles dispersed and some agglomerates. The presence of agglomerates can have occurred due to insufficient ultrasonication, which does not guaranteed total efficiency on the separation of magnetite nanoparticles.

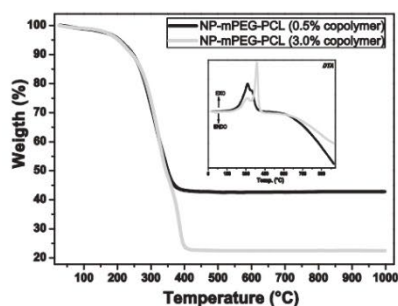


Figure 8. Thermogravimetric (TG) and differential thermoanalysis (DTA) curves for samples NP-mPEG-PCL-MTX (0.5% copolymer) and NP-mPEG-PCL-MTX (3.0% copolymer).

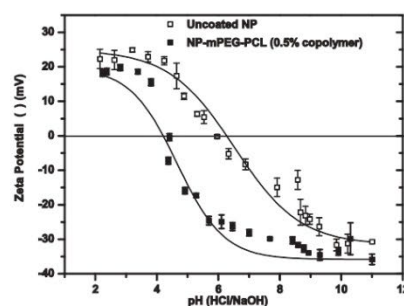


Figure 9. Zeta (ζ) potential of uncoated magnetic nanoparticles (NP) and NP-mPEG-PCL (0.5% copolymer) in function of pH.



Figure 10. TEM of complex NP-mPEG-PCL-MTX (0.5% copolymer).

Cytotoxicity Test on Human Osteosarcoma

Figure 11 shows a dose-dependent reduction in MTT absorbance in MG63 human osteosarcoma cells treated with 1 and 10 $\mu\text{g}/\text{mL}$ of NP-mPEG-PCL-MTX (0.5% copolymer) for 24 h. Cells treated with 1 and 10 $\mu\text{g}/\text{mL}$ of NP-mPEG-PCL-MTX showed 92.58% and 8.93% of viability, respectively; proving the compatibility of the nanocarriers compared with control and magnetic nanoparticle groups. The test done just with magnetic nanoparticle (NP) in both concentrations (1 and 10 $\mu\text{g}/\text{mL}$), showed that there was no cytotoxicity to osteosarcoma human, proving the biocompatibility of the magnetite nanoparticles to biology systems. The MTT test also proved the efficiency of the drug delivery of NP-mPEG-PCL-MTX. In the temperature in which the test was done (37 $^{\circ}\text{C}$), the system already has released some amount of MTX (according to the curve of drug release), enough to cause cell death.

The cytotoxicity results on the human osteosarcoma cells showed that drug released by the micelle was efficient. The samples with MTX caused loss in the viability

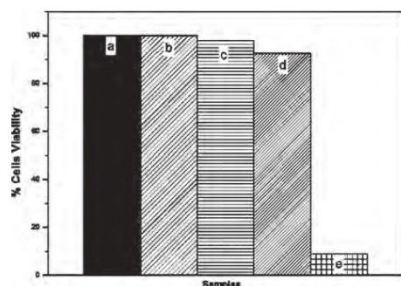


Figure 11. MTT assay showing viability of MG63 human osteosarcoma after 24 h upon various treatments: (a) control group, (b) uncoated magnetic nanoparticle 1 $\mu\text{g}/\text{mL}$, (c) uncoated magnetic nanoparticle 10 $\mu\text{g}/\text{mL}$, (d) mPEG-PCL-MTX-NP 1 $\mu\text{g}/\text{mL}$ and (e) mPEG-PCL-MTX-NP 10 $\mu\text{g}/\text{mL}$.

of tumor cells when compared to the control group cells. It can be also observed a loss viability dose-dependent of MTX, when 10 $\mu\text{g}/\text{mL}$ was used there was a decrease in cell number. Dose-dependent effect of MTX was also observed by others authors.^{11,30} Thus, when high doses are delivered, MTX causes the toxic build-up of cellular, reducing cellular viability and consequently causing cellular mortality.

CONCLUSION

Copolymer mPEG-PCL was successfully obtained via ring opening polymerization of the ϵ -caprolactone. Magnetic nanoparticles were surface modified with mPEG-PCL and subsequently MTX was loaded into the system. NP-mPEG-PCL-MTX samples showed a great cytotoxic potential in osteosarcoma human cells. Major drug release was observed at 42 $^{\circ}\text{C}$. The best NP-mPEG-PCL-MTX concentration obtained for human osteosarcoma death was 10 $\mu\text{g}/\text{mL}$.

Conflict of Interest Disclosure

Authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including of the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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mPEG-co-PCL nanoparticles: The influence of hydrophobic segment on methotrexate drug delivery



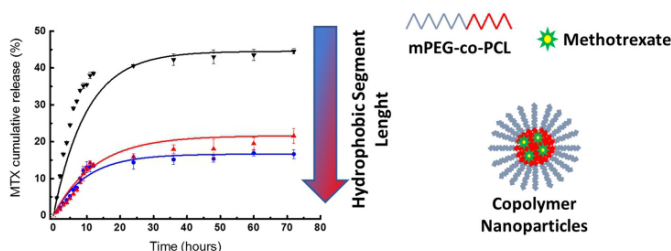
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GRAPHICAL ABSTRACT



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ABSTRACT

The use of drug delivery system based on polymeric nanoparticles (PNP) has been investigated due to its capacity to carrier hydrophobic drugs across natural barriers and to access new sites in the human body to release drugs at different tissues or cells. In the present work, polyethylene glycol monomethyl ether – co – polycaprolactone copolymer (mPEG-co-PCL) was synthesized by ring opening polymerization of the ϵ -caprolactone in the presence of polyethylene glycol monomethyl ether (mPEG) as a macroinitiator and tin(II) 2-ethylhexanoate ($\text{Sn}(\text{Oct})_2$), as a catalyst. On this step, three syntheses were realized to increase the polycaprolactone (PCL) hydrophobic segment of the copolymer. PNP of mPEG-co-PCL were obtained by emulsion – solvent evaporation method, which the surfactant concentration (Tween80[®]), cosurfactant (dodecane) and water/oil ratio were studied to control the size of nanoparticles. The PNP ability to loading and releasing drugs was evaluated with methotrexate (MTX), a drug commonly used for cancer treatment. The copolymers were synthesized with different chain length, increasing PCL hydrophobic segment, resulting in molecular weight between 2,000 to 13,000 g mol⁻¹. The lowest hydrodynamic diameter (79.5 nm) of PNP was obtained with a solution of 1×10^{-4} mol L⁻¹ Tween80[®] solution and 500 μL of dodecane. The water/oil ratio showed insignificant influence on hydrodynamic diameter, according to factorial design results. The copolymers showed a loading efficiency of MTX higher than 80.0%. The MTX releases from mPEG-co-PCL platform had occurred by anomalous transport

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mechanism in the first hours. After 16 h the release rate of MTX decrease drastically and its behavior was governed by PCL degradation process. As PCL ratio is increased on copolymer, the drug release decrease, suggesting an interaction of MTX with PNP hydrophobic segment. Thus, the MTX release could be controlled by copolymer chain length through hydrophilic and hydrophobic segment composition ratio, being a good candidate for hydrophobic drug delivery systems.

1. Introduction

Increasing the solubility of drugs is one of the challenges for pharmaceutical industry. Poorly water-soluble drugs have low bioavailability and erroneous absorption in the clinical settings. The outstanding strategy to overcome the effect of the low solubility of bioactive molecules is the use of nanoplateforms, such as polymer nanoparticles [1,2] or micelles [3–5], in which poorly soluble drugs are incorporated into hydrophobic reservoirs, increasing its biodistribution into the organism [6,7].

Besides increase the bioavailability of hydrophobic drugs, nanoplateforms allows the control of physicochemical properties (size, shape and surface charge) by surface functionalization, offering other advantages such as the reduction of drugs toxicities and degradation before it reaches the target tissue, avoiding uptake and clearance of drugs [8,9]. The performance of a tiny well designed nanoplateform is correlated to its ability to pass through capillary vessels to overcome physiological barriers and penetrate in tumors [10,11]. Irregular blood vessels are created in the tumor microenvironment and the discontinuous epithelium result in fenestras with reduced size, which assist the uptake of the drug by enhanced permeation and retention effect (EPR effect). These are the main characteristics of passive accumulation targeting therapy [11–13].

The chemistry of PCL has been investigated in the last decades for biomedical applications due to its good compatibility and low immunogenicity, as well as being approved by the Food and Drugs Administration (FDA). PCL is a semi-crystalline, straight-chain synthetic hydrophobic polyester. The degradation product of PCL is the 6-hydroxyhexanoic acid, which is a natural metabolite in the human body. The main advantage of PCL compared to other aliphatic polyesters, such as polylactic acid (PLA) and polyglycolic acid (PGA), is its high permeability to drugs, especially the hydrophobic ones [14–17]. To enhance the functionality of PCL, the synthesis of block copolymers has shown to be a promising method capable of change mechanical, physical and chemical properties by adjusting the hydrophobic-hydrophilic ratio. This new arrangement gives rise to the amphiphilic character of the copolymer molecule, whose ratio determines its hydrophilic-lipophilic balance (HLB). The HLB is an empirical scale that can express the amphiphilic nature of surfactants, such also the copolymers that show the ability to accumulate drugs with hydrophobic surfaces and interact with biological membranes by the hydrophilic segment [18]. Block copolymers composed of poly (ethylene glycol) PEG and PCL have attracted attention in recent decades [19–21]. PEG is widely used as outer shell due to high water solubility and low cytotoxicity properties [22]. In drug delivery systems, the PCL segment interacts with hydrophobic drugs, while hydrophilic PEG decreases the drug uptake by the liver and kidneys, increasing biodistribution throughout the body [17,23].

MTX is an antimetabolite and antifolate agent with antineoplastic and immunosuppressant activities widely used in cancer treatment. Due to the structural similarity to folic acid, MTX binds to folic acid receptor to be internalized to cells [24]. MTX acts on enzyme dihydrofolate reductase, resulting in inhibition of purine nucleotide and thymidylate synthesis and, subsequently, killing cells by blocking the synthesis of DNA and RNA [25]. Thus, passive targeting is preferred by mPEG-co-PCL nanoparticles for MTX release. The passive accumulation of nanoplateforms exhibits an increased circulation lifetime, resulting in enhanced accumulation at the tumor target, due to the stealth property of

the PEG shell that helps to keep the particle "invisible" to phagocytic cells, increasing its permeation in the cancer environment [26]. Once delivered into the site of the tumor due to the large spaces between the endothelial cells, the MTX is internalized by folate receptors and act as a drug on cancer treatment.

In the current work, it was investigated the influence of hydrophilic/hydrophobic ratio to cumulative MTX drug delivery profile. mPEG-co-PCL nanoplateform was synthesized by increasing the PCL ratio of the copolymer length and factorial design was applied to obtain the smallest PNP through emulsion/solvent evaporation method. The non-cytotoxicity of 1:10 mPEG-co-PLG on murine fibroblast cells line (NIH3T3) by mitochondrial viability MTT test indicates a biological compatibility of PNP, suggesting it potential as MTX drug delivery platform.

2. Experimental section

2.1. Materials

All chemicals were used as received from suppliers. Poly(ethylene glycol) monomethyl ether (mPEG, M_n 550 g mol⁻¹), ϵ -caprolactone (97.0%), stannous 2-ethylhexanoate (Sn(Oct)₂, 96.5%), methotrexate MTX, phosphate buffered saline (0.01 mol L⁻¹ phosphate, 0.135 mol L⁻¹ NaCl and 0.002 mol L⁻¹ KCl) buffer solution (PBS), Tween80[®] and dodecane (99.0%) were purchase from Sigma-Aldrich Brazil. Acetone, hexane (98.5%) and chloroform (99.8%) were purchase from Synth. Tetrahydrofuran (THF, 99.8%) was purchase from JT Baker.

2.2. Synthesis of mPEG-co-PCL

The copolymer mPEG-co-PCL synthesis was adapted from Souza, M. A. et al [27] and consist in a ring opening polymerization of ϵ -caprolactone monomer with mPEG as macroinitiator and (Sn(Oct)₂) as a reaction catalyst. In a two-neck round bottom flask, joint to a Schlenk line, were add 1.05 $\times 10^{-3}$ mol of (Sn(Oct)₂), mPEG and ϵ -caprolactone in molar ratio of 1:1; 1:10 and 1:30. The system was kept under magnetic stirring and argon atmosphere for 03 h at 120.0 °C. After cooling, the copolymer was dissolved in chloroform and precipitated in cold hexane three times to remove the monomers and catalyst.

2.3. mPEG-co-PCL nanoparticles: a factorial design

Copolymers nanoparticles were obtained through emulsion solvent evaporation method [28] by dissolving 50.0 mg of copolymer and dodecane (cosurfactant) in acetone and added to Tween80[®] solution. The emulsion was dispersed in ultrasonic probe (Sonics Vibracell 550 W) at 30.0% of amplitude and 03 s on/off pulsed mode, for 20 min, in an ice bath. A factorial design was performed to study the effect of Tween80[®] concentration, dodecane concentration and oil/water ratio on the nanoparticles size. The factor and levels for an 2³ experiments are showed in Table 1.

2.4. MTX loading study

MTX loaded copolymer nanoparticles were also prepared by emulsion solvent evaporation method under conditions that result in smallest nanoparticles (Tween80[®] (+) and Dodecane (-)). The amount of 0.20, 0.60 and 1.00 mg of MTX was dissolved in organic phase prior to

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Table 1
Factors and levels for factorial experimental design of mPEG-co-PCL NP.

Factors		Lowest level		Highest level	
		Values	Coded	Values	Coded
A	Tween80 [®]	1×10^{-5} mol L ⁻¹	(-)	1×10^{-4} mol L ⁻¹	(+)
B	Dodecane	50 uL	(-)	500 uL	(+)
C	o/w	5%	(-)	10%	(+)

dispersion procedure. After that, the nanoparticles were centrifuged and the supernatant was used to evaluate the concentration of non-incorporated MTX by UV-vis spectrophotometry. The MTX UV-vis spectra and calibration curve obtained at 303 nm, correspond to Figure S1 and S2, respectively, on supplementary information. The encapsulation efficiency (EE) and drug loading (DL) were determined by the following equations [20].

$$EE(\%) = \left(1 - \frac{w_{MTX}}{w_{total MTX}} \right) \times 100$$

$$DL(\%) = \left(\frac{w_{MTX}}{w_{MTX} + w_{NPs}} \right) \times 100$$

with w_{MTX} equals to the amount of MTX incorporated in nanoparticles, $w_{total MTX}$ is the initial amount of MTX in the system and w_{NPs} is the amount of nanoparticles after freeze-drying.

2.5. MTX releasing experiment

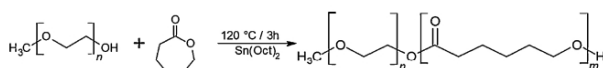
To study the kinetics and mechanism of MTX release, 10.0 mg of MTX loaded mPEG-co-PCL nanoparticles were dispersed in 0.5 mL of PBS buffer (pH 7.4) and quantitatively transferred to a dialysis bag (cut off 6 kDa). Then, the dialysis bag was placed into 15.0 mL of PBS and the system was kept at constant temperature of 37.0 °C and 200 rpm stirring. At regular time intervals, 0.75 mL of the release medium was removed and replaced with the same volume of fresh PBS and the MTX concentration was measured by spectrophotometry. The cumulative MTX release was determined by the equations [20]:

$$Cumulative MTX release (\%) = \frac{V_t \sum_{i=1}^{n-1} C_i + V_0 C_n}{w_{MTX}} \times 100$$

with V_0 equals to the total volume of release media, V_t is the volume of replaced media, C_n is the concentration of MTX in the sample.

2.6. Cell cultures

The murine fibroblast cells line (NIH3T3) were acquired commercially from American Type Culture Collection (ATCC-CRL-1658, Manassas VA) and cultivated in Dulbecco's Modified Eagle Medium (DMEM-Gibco BRL, USA). Flasks were incubated at 37 °C in 5% CO₂. All the cell lines were supplemented with glutamine 2 mmol L⁻¹, penicillin 100 U mL⁻¹, streptomycin 0.1 mg mL⁻¹ and 10% fetal bovine serum (FBS; all from Sigma-Aldrich Co. USA). Cell count and micrographs were obtained using a Carl Zeiss microscope Axiovert 40-CFL coupled with a digital high-resolution camera model AxioCam MRC. Cell total numbers were calculated from Neubauer chamber after trypsinization and labeling with trypan blue.



2.7. MTT cell viability assay

Initially the 1:1, 1:10 and 1:30 mPEG-co-PLG samples were prepared as described in Section 2.2 and sterilized via the gamma (-ray) radiation sterilization process. Samples of each formulation were incubated after successive dilutions in DMEM 3% SFB culture medium at concentrations of 3.12; 6.25; 12.5; 25.0 and 50 g mL⁻¹ for 4 h at 5×10^6 cells density. Incubation media were completely removed and each well was washed with Hank's buffer twice.

In the sequence, the MTT (Sigma-Aldrich Co., USA) test ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]) cell proliferation assay [29] was performed to assess cell viability after conducting the cytotoxic studies. Briefly, 80-μL aliquots of the MTT solution (5 mg mL⁻¹) combined with 420 μL of medium without phenol red were added to each well. Cells were then incubated at 37 °C under 5% CO₂ atmosphere for 4 h to allow the formazan-forming reaction to occur. Following incubation, the medium containing the MTT solution was removed, and formazan crystals were dissolved in 2-propanol. Optical density was measured at 570 and 690 nm using a Safire² microplate reader (TECAN Group Co.). Results were presented as percentage cell survival relative to control (untreated cells).

2.8. Statistical analysis

Statistical analyses of the cell viability assays – MTT were generated within the GraphPad Prism 6.0 (GraphPad Software, Inc.) platform. Differences between control and treated groups were evaluated using analysis of variance (ANOVA) followed by Tukey test (significance was set at $P < 0.05$).

2.9. Samples characterization

Nuclear magnetic resonance spectroscopy (¹H-NMR) was carried out on a Bruker Fourier 300 Hz spectrometer measuring samples dissolved in chloroform-d (CDCl₃) to elucidate the structural composition of copolymer. The molecular weight and molecular weight distribution of copolymers were evaluated by gel permeation chromatography (GPC) from Viscotek range, Malvern Instruments. Three thermostated (35 °C) Shodex KF-806 M series columns (SHOWA Denko, Taokyo, Japan) were used for separation and Viscotek RI VE3580 and Viscotek UV as detector. Degassed THF was used as mobile phase, with a flow rate of 1 mL min⁻¹. The injection volume was 100 uL of standard (polystyrene) and samples. The hydrodynamic diameter of nanoparticle was measured using a Zetasizer Nanoseries ZSNano from Malvern Instruments. The morphology of PNP were investigated by Scanning Electron Microscopy (SEM) in a JEOL microscope, model JSM-7500 F. An electron beam of 2 kV was used. The software ImageJ (1.45 s version) was used for images treatment. For SEM analysis, the copolymer samples were dispersed in isopropanol and deposited in a silicon grid. The images were obtained in secondary electrons. The samples were dispersed in deionized water at 25 °C. To study the loading and release of MTX an UV-vis spectrophotometer Lambda 465, from Perkin Elmer, was used at 303 nm wavelength.

3. Results and discussion

3.1. Synthesis of mPEG-co-PCL copolymer

The mPEG-co-PCL copolymer was synthesized by ring open polymerization of ε-caprolactone monomer, according to Fig. 1. The carbonyl group of ε-caprolactone is susceptible to nucleophilic attack by

Fig. 1. Synthetic route of mPEG-co-PCL.

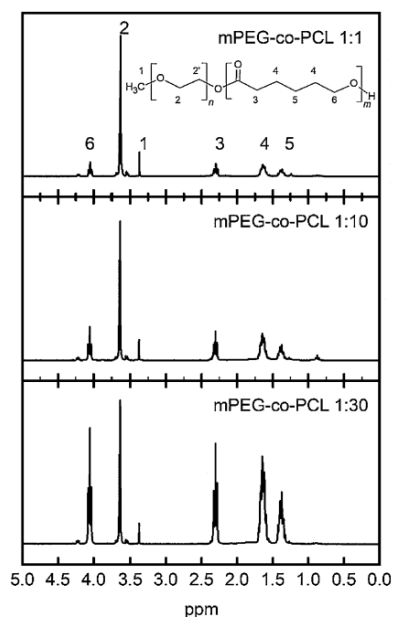


Fig. 2. ^1H NMR spectra of copolymer samples synthesizing by varying the hydrophilic/hydrophobic ratio.

hydroxyl end-group of m-PEG, acting as macroinitiator of reaction. The structure of samples was evaluated by ^1H -NMR spectroscopy and the results are displayed in Fig. 2. The peaks of methoxy and methylene protons of mPEG segment were observed at 3.37 ppm and 3.67 ppm, respectively. From hydrophobic PCL segment, the peaks at 2.29 ppm correspond to methylene proton closer carbonyl group. The other methylene protons from PCL were showed at 4.04 ppm, 1.63 ppm and 1.38 ppm. Increasing the PCL molar ratio, the mPEG signals decrease in relation to the other PCL peaks, suggesting an increase in hydrophobic segment of the copolymer.

Through GPC results, the weight (M_w), number (M_n) average molecular weights and molecular dispersity (D) were verified and summarized in Table 2. The chromatographic profile (Fig. 3) shows different retention times for each copolymer sample, whose molecular weight ranged from 2600 g mol^{-1} to 8500 g mol^{-1} . It is observed the presence of two populations for each sample: the main population correspond to the copolymer formation and the traces above 32 min should be relative to the reactants. The D value were calculated by the M_n/M_w ratio for each main peak of mPEG-co-PCL, which resulted in monomodal molecular weight distribution samples ($D > 1.2$), as bulk polymerization shows no control over the length of the polymer chain [30].

Table 2
The molecular weight of synthesized copolymers.

Sample	M_n (g mol^{-1})	M_w (g mol^{-1})	D
mPEG-co-PCL 1:1	2600	3350	1.29
mPEG-co-PCL 1:10	3980	6440	1.62
mPEG-co-PCL 1:30	8450	10960	1.46

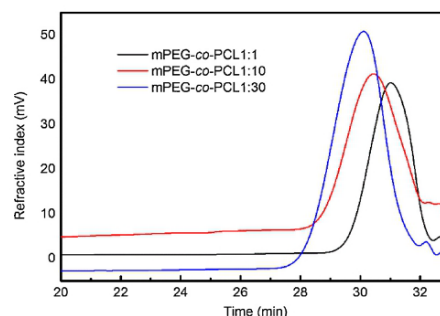


Fig. 3. Chromatographic profile of copolymer samples as a function of hydrophilic/hydrophobic ratio.

3.2. Preparation of mPEG-co-PCL nanoparticles

Obtaining nanoparticles by the solvent evaporation emulsion method can be understood by a sequence of phenomena. In the first step, an emulsion is obtained, forming droplets containing the copolymer dissolved in the dispersed phase. As the organic solvent is evaporated, the concentration of copolymer increases within the drop to a critical point at which the concentration of the polymer overcomes its solubility in the aqueous phase, occurring to the precipitation of nanoparticles in the final step [28,31].

The solvent evaporation emulsion method allows controlling the size and stabilization of the nanoparticles by adjusting the conditions during the emulsion step to avoid the coalescence and diffusional degradation of these nanoparticles. Coalescence can be avoided by adding of surfactant, which increase the surface layer and promote the steric stabilization of the polymer nanoparticles. The diffusional degradation is suppressed by the addition of cosurfactant which is insoluble in the dispersed phase, thus increasing the osmotic pressure within the droplet [30,32,33]. Thus, factorial design is a powerful tool to reduce the number of experiment and to elucidate which variables are most relevant and if there is an interaction between these variables in response to hydrodynamic diameter of nanoparticles [34]. Table 3 summarize de factorial matrix of the factors as a hydrodynamic diameter response.

The variation of the surfactant concentration indicates a decrease of 113.2% in the hydrodynamic diameter when the concentration changes from its lowest level to the highest one. The decrease in nanoparticle size is expected with increasing surfactant concentration, as more surfactant is available to cover and stabilize the nanoparticle surface [34,35]. For the increase of the cosurfactant concentration was observed opposite behavior, occurring an increase of 74.6% in the hydrodynamic diameter. The positive influence of the cosurfactant on the size of the nanoparticle is due to the encapsulation of the cosurfactant inside the nanoparticle, that is, the more cosurfactant is used in the

Table 3
Design matrix and the responses for factors (A) Tween80[®], (B) Dodecane and (C) o/w ratio.

Experiments	A	B	C	Dh (nm)
1	(-)	(-)	(-)	227.7
2	(+)	(-)	(-)	79.5
3	(-)	(+)	(-)	285.8
4	(+)	(+)	(-)	146.2
5	(-)	(-)	(+)	233.4
6	(+)	(-)	(+)	167.5
7	(-)	(+)	(+)	336.7
8	(+)	(+)	(+)	237.7

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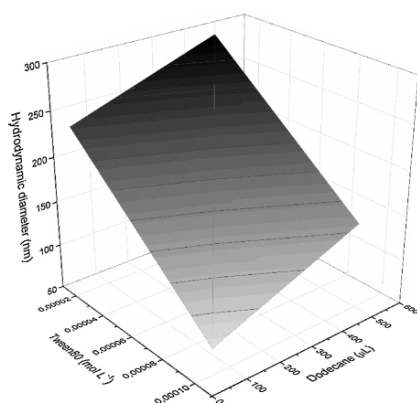


Fig. 4. 3D model to predict the hydrodynamic diameter of copolymer nanoparticles as a function of surfactant and cosurfactant.

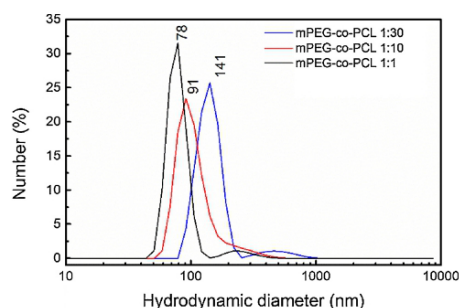


Fig. 5. Hydrodynamic diameter distribution profile of copolymers nanoparticles with different molecular weight.

synthesis the larger the nanoparticles obtained [36]. The hydrodynamic diameter is not influenced by w/o ratio. The factor interactions do not show any significance on particle size. After simplification of the non-significant factor, the response surface was showed in Fig. 4 and the mathematical model of linear regression representing this behavior was:

$$D_h = -1.59 \times 10^4 A + 0.139 B + 58.73$$

which D_h correspond to PNP hydrodynamic diameter obtained when a Tween80[®] concentration (parameter A) and a volume of dodecane (parameter B) were added during the solvent evaporation emulsion step.

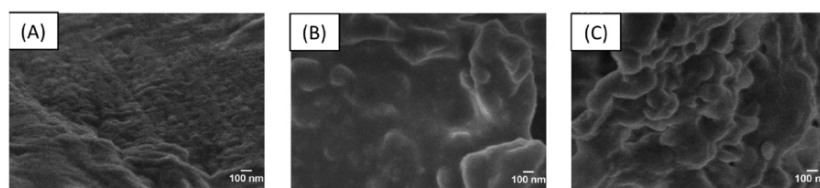


Fig. 6. SEM images of copolymers nanoparticles samples. (A) mPEG-co-PCL 1:1, (B) mPEG-co-PCL 1:10 e (C) mPEG-co-PCL 1:30.

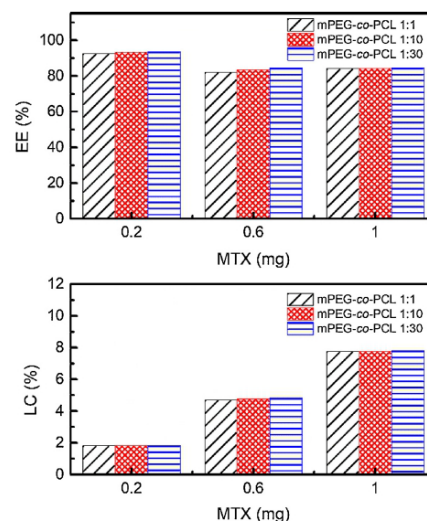


Fig. 7. Effect of hydrophilic/hydrophobic ratio on the encapsulation efficiency (upper) and loading content (lower) of MTX on copolymers nanoparticles samples.

This equation showed R^2 value equal 0.9992 and fit well the factorial design described above. The samples were submitted to the same procedure to obtain particles with smaller hydrodynamic diameter. Due to the different polymer length, hydrodynamic diameter value is near to the predicted value in linear regression. With the increase of the hydrophobic segment in the copolymer the hydrodynamic diameter increased from 78.82 nm to 141.8 nm, as can be observed in Fig. 5.

The SEM images (Fig. 6) show the formation of aggregates based on PNP with spherical morphology. The quality of PNP image have been improved with the increase of hydrophobic segment, where is possible to observe more clearly the spherical morphology. The aggregates formation occurs during drying process and it's not observed by DLS technique.

3.3. MTX loading

MTX was used as a chemotherapeutic agent to evaluate the loading properties of mPEG-co-PCL nanoparticles. Fig. 7 shows that efficiency encapsulation did not increase with increasing the amount of MTX on dispersion, reaching a plateau close to 84% using 0.6 mg of the drug. However, the value of loading content increased with the addition of MTX, reaching the maximum value of 8% at 1.0 mg of drug. The polymer chain length did not show importance both for efficiency

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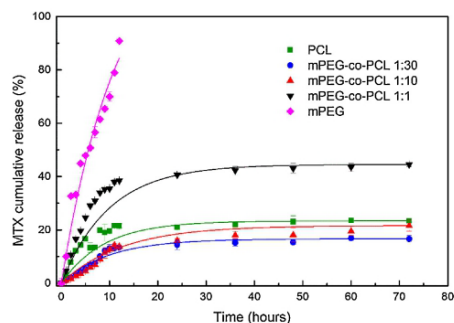


Fig. 8. MTX cumulative release of copolymers nanoparticles at 37.5 °C and pH 7.4.

encapsulation to loading content values. For the release study, the samples with the highest MTX content were considered.

3.4. Release of MTX

Fig. 8 shows the cumulative release profile of the copolymer samples, as well as the individual precursor polymers. In this Figure, the mPEG precursor sample displays a rapid release reaching 91% after 12 h, while the PCL precursor shows a low percentage (20%) after 8 h. For the copolymer samples, similar behavior was observed. The mPEG-co-PCL 1:1 sample, which showed the lowest hydrophobic segment, resulted in the release of 43% MTX in 16 h, whereas the mPEG-co-PCL 1:10 and mPEG-co-PCL 1:30 released, respectively, 16% and 14% in the same period. The literature reports that the addition of hydrophilic components to the PCL chain allows to adjust the rate of drug release in the polymer blends systems [37]. It is observed that the behavior after 24 h of release is quite different from the initial hours of analysis. The interpretation of this behavior may be related to the different interactions of MTX with the segments of the copolymer. Due to hydrophobicity, the interaction of MTX with the hydrophobic segment of the copolymer (PCL) is stronger, resulting in a slow release. Thus, the release of MTX is expected to occur as the polymer is hydrolytically and/or enzymatically degraded into the body [14,38,39]. Regarding the first few hours of the experiment, MTX released may be derived from weak interactions with mPEG.

The Peppas model was used to elucidate the mechanism in the initial stage of MTX release, allowing the determination of the release exponent (n) and the kinetic constant (k). The plot of the log release

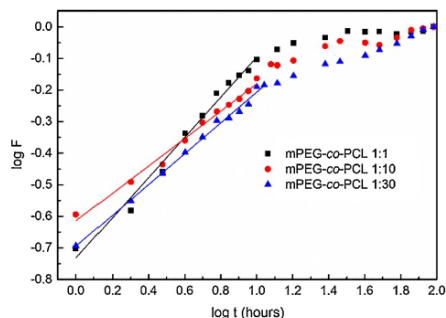


Fig. 9. Fitting the copolymer nanoparticles release data to Peppas model.

Table 4

Release kinetic parameters according to the linearization of Peppas equation.

Sample	n	k (h ⁻¹)	R ²
mPEG-co-PCL 1:1	0.63	0.185	0.993
mPEG-co-PCL 1:10	0.43	0.243	0.994
mPEG-co-PCL 1:30	0.48	0.202	0.997

fraction as a function of log time can be observed in Fig. 9 and the parameters determined in this curve summarized in Table 4.

Linearization was performed for the initial stage of monitoring, in which the Peppas model is valid and the correlation coefficient values R^2 higher than 0.99. The values of the release exponent obtained by the linearization of the curves are between 0.43 and 0.63 for the nanoparticle samples, indicating an anomalous transport (non-Fickian) [40]. This result corroborates the release profile, since the slow release of MTX is related to the interactions with the hydrophobic segment of the nanoparticle, requiring relaxation and/or degradation of the nanoparticle chains to release MTX.

3.5. Cell viability

The results presented in Fig. 10 demonstrate the correlation of cell viability as a function of the different concentrations of polymer nanoparticles obtained after the colorimetric test of MTT mitochondrial activity. In Fig. 10A we have a pronounced effect of reducing cell viability at the concentrations C3, C4 and C5 ($> 12.5 \text{ g mL}^{-1}$) incubated with 1:1 mPEG-co-PLG samples. This cell killing effect is also observed in Fig. 10C with reduced cell viability at the C4 and C5 concentrations ($> 25 \text{ g mL}^{-1}$) after incubation with 1:30 mPEG-co-PLG samples, as shown by statistical differences. However, this cytotoxicity effect is not observed for mPEG-co-PLG 1:10 sample as shown in Fig. 10B. These results suggest a specific biologically compatible effect for each nanomaterial according to its chain size, which may indicate a direct influence of the structural copolymer chain length of the particle to the highest concentrations in vitro. Cell death effects are accentuated for the samples with higher hydrophilicity and hydrophobicity (Fig. 10A and C respectively). In this context, we can verify a greater biological compatibility of the nanostructure for the sample with hydrophilic-lipophilic balance (HLB), as shown in Fig. 10B.

4. Conclusions

Different polymer chains lengths of mPEG-co-PCL copolymer were obtained from the ring-opening polymerization reaction of the ϵ -caprolactone monomer. The factorial design study allowed us to conclude that the increase of the surfactant concentration and the decrease of the cosurfactant concentration are the favorable conditions to obtain polymer nanoparticles of the smallest hydrodynamic diameter through the emulsion/solvent evaporation method. MTX was used as a reference hydrophobic drug in the mPEG-co-PCL copolymer nanoparticles and resulted in an incorporation degree higher than 80%. The release of MTX occurs by distinct mechanisms depending on the difference in the drug interaction with the hydrophilic and hydrophobic segment of the mPEG-co-PCL copolymer. In the initial stage, it was observed that the mechanism of drug release corresponds to an anomalous transport that does not obey the Fick's law of diffusion due to the interaction of MTX with the hydrophilic segment, whereas in the final stage, the drug release slows down and it will only be completed as a function of the PCL chain biodegradation. The cytotoxicity of mPEG-co-PLC on murine fibroblast cells line (NIH3T3) were evaluated and the sample mPEG-co-PCL 1:10 showed no cell kill after incubation. Cell death effects are accentuated for the samples with higher hydrophilicity and hydrophobic segments. These results suggest that sample mPEG-co-PCL 1:10 is a good candidate for MTX release in cancer treatment.

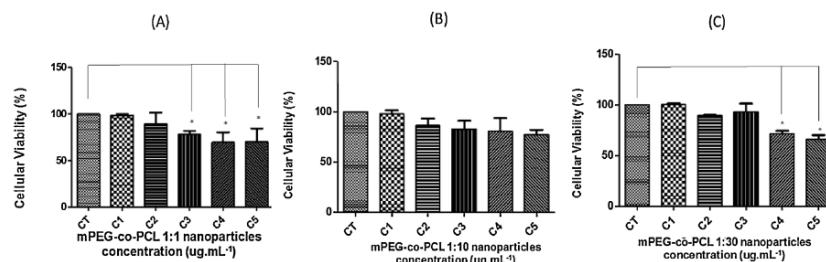


Fig. 10. Cytotoxicity of mPEG-co-PCL on murine fibroblast cells line (NIH3T3) by mitochondrial viability test MTT. Cells were incubated with different polymer proportion (1:1; 1:10 and 1:30) in concentrations C1 = 3.12; C2 = 6.25; C3 = 12.5; C4 = 25.0 and C5 = 50 µg mL⁻¹ for 4 h at 5 × 10⁶ cells density. Data are presented as mean ± SEM of three independent experiments (CT = control, *p* < 0.05).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.colsurfa.2018.06.076>.

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Synthesis and colloidal characterization of folic acid-modified PEG-*b*-PCL Micelles for methotrexate delivery



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ABSTRACT

Hydrophobic drugs, such as methotrexate, are not easily delivered into the human body. Therefore, the use of amphiphilic nanoplateforms to the transport of these drugs through the bloodstream is a challenge. While the hydrophobic region interacts with the drug, the hydrophilic outer layer enhances its bioavailability and circulation time. Poly (ethylene glycol)-*block*-poly(ϵ -caprolactone) PEG-*b*-PCL micelles are biodegradable and biocompatible, allowing its use as a nanocarrier for drug delivery systems. The stealth property of PEG that composes the outer layer of nanoplateforms, makes the micelle unperceivable to phagocytic cells, increasing the circulation time in the human body. In addition, folic acid functionalization enables micelle selectively targeting to cancer cells, improving treatment efficiency and reducing side effects. In this work, PEG-*b*-PCL copolymer was synthesized by ring opening polymerization (ROP) of the ϵ -caprolactone with Poly(ethylene glycol) as a macroinitiator and tin(II) 2-ethyl hexanoate as a catalyst. Functionalization of such micelles with folic acid occurred through the modification of the PEG terminal group. The surface modification of the copolymer micelles resulted in higher critical micellar concentration (CMC), increasing approximately 100 times. The synthesis of the copolymers resulted in molecular weight around 3000 g mol⁻¹ with low polydispersity. The polymer micelles have a hydrodynamic diameter in the range of 100–200 nm and the functionalized sample doesn't show aggregation in the considered pH range. High incorporation efficiency was obtained with a minimum percentage of 85%. The drug release profile and linearization from the Peppas model confirmed the interaction of methotrexate with the hydrophobic segment of the copolymer and its release mechanism by relaxation and/or degradation of the chains, making PEG-*b*-PCL micelles suitable candidates for hydrophobic drug delivery systems.

1. Introduction

The finding of new molecules with biological activity has been increasing in the last decades. However, most of these molecules present low solubility in hydrophilic environments such as the human body, making its use as drug unfeasible [1]. The low solubility alters the bioavailability in the body, reducing the efficiency of the treatment. A strategy to overcome these challenges caused by the low solubility is to develop controlled release nanoplateforms that act as carriers of these drugs to a specific target of the organism [2]. Among the various platforms available [2], the use of copolymer micelles has the

advantage of interacting with hydrophobic and hydrophilic drugs [3].

After the drug is internalized by the micelle, the biodistribution of the active molecule is dependent on the properties of the carrier (size, shape and surface charge). Thus, the use of carriers provides advantages in the transport of the drug, such as the reduction of its toxicity due to lower concentrations, enzymatic degradation, avoiding uptake and clearances of the drugs [4]. In order to nanoplateform be functional, it's needed to overcome the physiological barriers to the tissue's penetration [5]. To facilitate the interaction with specific receptors in the cells it's necessary the use of ligands on the carrier surface. This is the principle of the ligand-mediated targeting [6].

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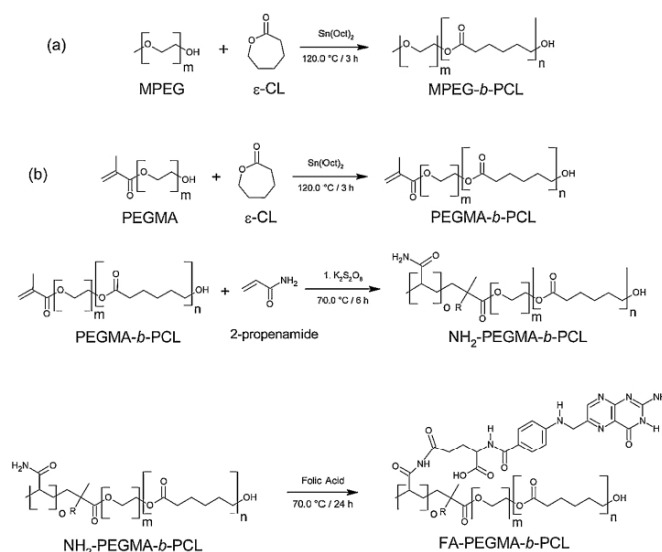


Fig. 1. Synthetic route of PEG-*b*-PCL copolymers. (a) MPEG-*b*-PCL and (b) FA-PEGMA-*b*-PCL.

The biocompatibility and low immunogenicity of polycaprolactone (PCL) have made it the target of various studies nowadays. PCL is a linear chain polyester which has hydrophobic characteristics and it is approved by the Foods and Drugs Administration (FDA). It has the advantage of not being degraded by human body's enzymes and possess a high permeability to hydrophobic drugs, allowing it to be used in controlled release systems [7,8]. However, its lipophilic characteristics make it unfeasible to be applied alone, making necessary the bonding with hydrophilic molecules to allow the balance of the hydrophilic-hydrophobic ratio [9]. After the interaction between PCL and hydrophilic molecules there is the appearance of the amphiphilic feature to the carrier. Among many options of hydrophilic molecules, the linkage of poly (ethylene glycol) PEG to PCL has attracted attention [10]. PEG is highly soluble in water, has low cytotoxicity [11] and, generally used as the outermost layer of nanoplatforms. In PEG-*b*-PCL copolymer, specifically in micelles, the PCL segment interacts with hydrophobic drugs while the PEG segment increases bioavailability and make them invisible to phagocytic cells by enhancing its action in the cancer treatment [12,13].

Neoplasia is the unbridled and disordered growth of tissue cells. To keep the multiplicative process, cancer cells absorb more nutrients than healthy cells, one of these key nutrients is the vitamin folic acid. Therefore, cancer cells tend to express on its surface more folate receptors than healthy cells. The functionalized micelle with folic acid leads to the rapid internalization of drug molecules inside the cells at higher levels than non-functionalized micelle [6,14]. Once cell uptake, the micelle promotes the controlled release of the drug, acting on cancer treatment. Methotrexate is a chemotherapeutic agent and an immune system suppressant due to antimetabolite and antifolate characteristics. It is a molecule analogous to folic acid and competitively bond to folate receptor to be internalized by the cells [15]. Methotrexate promotes cellular death by blocking the synthesis of DNA and RNA through its action on enzyme dihydrofolate reductase, resulting in inhibition of purine and pyrimidine bases and thymidylates synthesis [16].

In this work, it was investigated the influence of folic acid on the surface of PEG-*b*-PCL micelles to cumulative methotrexate release profile. In the background, the improvement of the colloidal characteristics of the micelles by functionalization was evaluated, analyzing changes in polydispersion and agglomeration of the micelles. Determination of the critical micelle concentration (CMC) through the pendant droplet method allowed to evaluate the hydrophilicity of the functionalized micelles. The non-cytotoxicity of PEG-*b*-PCL micelles on murine fibroblast cells line (NIH3T3) by mitochondrial viability MTT test indicates a biological compatibility of micelles. The overall improvement of the micelles after surface modification allow the potential use as a nanoplatform for the controlled targeted release of methotrexate.

2. Experimental section

2.1. Materials

All chemicals were used as received from suppliers. Poly (ethylene glycol) methyl ether (MPEG) with $M_n = 550 \text{ g mol}^{-1}$, Poly (ethylene glycol) methacrylate (PEGMA) with $M_n = 500 \text{ g mol}^{-1}$, ϵ -caprolactone 97.0%, folic acid 97.0%, 2-propenamide 98.0%, potassium persulfate 99.0%, phosphate buffered saline (0.01 mol L^{-1} phosphate, 0.135 mol L^{-1} NaCl and 0.002 mol L^{-1} KCl) solution (PBS 0.01 mol L^{-1} , pH 7.4), tin(II) 2-ethyl hexanoate ($\text{Sn}(\text{Oct})_2$) 92.5–100.0%, and MTT reagent ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]) were purchased from Sigma-Aldrich Co. USA. Hexane (98.5%), Acetone, Dimethyl sulfoxide (97.0%), and chloroform (99.8%) were purchased from Synth. Argonium gas commercial grade were used as inert atmosphere.

2.2. Synthesis of PEG-*b*-PCL copolymer

The synthetic routes of both copolymers are shown in Fig. 1. Both copolymers PEG-*b*-PCL were synthesized by ring opening

polymerization (ROP) of ϵ -caprolactone monomer, MPEG and PEGMA acted as macroinitiators and $\text{Sn}(\text{Oct})_2$ as the reaction catalyst, forming MPEG-*b*-PCL and PEGMA-*b*-PCL, respectively. The synthesis was adapted according to previous work [17,18]. In a round bottom flask was added 0.34 mL of $\text{Sn}(\text{Oct})_2$, 1.0 mL of PEG and 1.0 mL of ϵ -caprolactone. The reaction was carried out under an inert atmosphere and magnetic stirring for 3 h at 120 °C. The copolymer was dissolved in chloroform and washed with ice-cold hexane several times to remove unreacted monomers and catalyst remains.

2.3. Synthesis of folic acid-modified PEG-*b*-PCL copolymer

The folic acid-functionalized PEG-*b*-PCL copolymer was obtained in two steps. Initially, a radical polymerization reaction was performed between the copolymer PEGMA-*b*-PCL and 2-propenamide in the presence of the persulfate initiator. The copolymer and the amide were mixed in Erlenmeyer flask with water, and the dispersion was degassed with argon gas before adding initiator. A 10:1 M ratio of the amide to the copolymer mixture was used and the amount of the initiator was 10% relative to the mass of the copolymer. The system was kept for 6 h at 70 °C [19,20]. The material was dialyzed to remove 2-propenamide residues, freeze-dried and labeled NH_2 -PEGMA-*b*-PCL.

Finally, a condensation reaction between a folic acid carboxyl group and an amino group of the NH_2 -PEGMA-*b*-PCL copolymer was performed. Both the folic acid and the copolymer were added to an Erlenmeyer flask in a molar ratio of 10:1 (folic acid:copolymer), dispersed in DMSO, degassed and the reaction was carried at 70 °C for 24 h. Then, the sample was once again dialyzed, freeze-dried, and labeled FA-PEGMA-*b*-PCL.

2.4. Methotrexate incorporation efficiency

The methotrexate uptake by copolymer micelles occurred by solution evaporation method from 1.0 mg of methotrexate and 30.0 mg of the copolymer in acetone, then water was added to provide methotrexate incorporation in the hydrophobic region of copolymer micelles. The micelles were centrifuged and the amount of unincorporated drug was determined by UV/Vis spectroscopy. The methotrexate UV-vis spectrum and analytical curve obtained at 303 nm are shown in Figs. S1 and S2, respectively, in the supplementary information. After the incorporation, the sample was freeze-dried. The incorporation efficiency (IE) and incorporation coefficient (IC) percentage were calculated by the Eqs. (1) and (2) [21]:

$$IE(\%) = \left(1 - \frac{w_{\text{MTX}}}{w_{\text{total MTX}}} \right) \times 100 \quad (1)$$

$$IC(\%) = \left(\frac{w_{\text{MTX}}}{w_{\text{MTX}} + w_{\text{NPs}}} \right) \times 100 \quad (2)$$

with w_{MTX} equals the amount of methotrexate incorporated in micelles, $w_{\text{total MTX}}$ is the initial amount of methotrexate in solution and w_{NPs} is the amount of micelles after freeze-drying.

2.5. Methotrexate releasing experiment

To evaluate the methotrexate release profile and its kinetic mechanism, 20.0 mg of drug loaded PEG-*b*-PCL micelles were dispersed in 0.5 mL of PBS 0.01 mol L⁻¹ (pH 7.4) and transferred to a dialysis membrane (MWCO 2000). The dialysis membrane was then placed into 15.0 mL of the same buffer in a test tube and the system was kept at a constant 500 RPM stirring at 37.0 °C. From time to time, 0.75 mL of the release medium was collected for UV-vis spectroscopy analysis and the same volume of PBS buffer was replaced. The cumulative methotrexate release percentage was determined by the Eq. (3) [21]:

$$\text{Cumulative methotrexate release } (\%) = \frac{V_i \sum_{i=1}^{n-1} C_i + V_0 C_n}{w_{\text{MTX}}} \times 100 \quad (3)$$

with V_0 equals the total volume of release medium, V_i is the volume of replaced medium, C_n is the methotrexate concentration in the sample.

2.6. Cell cultures

Dulbecco's Modified Eagle Medium (DMEM-Gibco BRL, USA) was used for the cultivation of murine fibroblast cells lines (NIH3T3) acquired commercially from American Type Culture Collection (ATCC-CRL-1658, Manassas VA). Incubation occurred at 37 °C in 5% CO₂. Glutamine 2 mmol L⁻¹, penicillin 100 U mL⁻¹, streptomycin 0.1 mg mL⁻¹ and 10% fetal bovine serum (FBS; all from Sigma-Aldrich Co. USA) were used as supplement. Micrographs and cell count were obtained using a Carl Zeiss microscope Axiovert 40-CFL coupled with a digital high-resolution camera model AxioCam MRC. Neubauer chamber was used to calculate the cell total numbers after trypsinization and labeling with trypan blue.

2.7. MTT cell viability assay

The PEG-*b*-PCL samples were sterilized via laminar flow cabinet sterilization process using alcohol 70%, UV lamp, and sterile water. Each sample were incubated after successive dilutions in DMEM 3% SFB culture medium in the following concentrations: 3.12, 6.25, 12.5, 25.0 and 50.0 µg mL⁻¹, for 6 h at 37 °C and density of 5×10^6 per well. Hank's buffer was used twice in the well's washing after the incubation media was completely removed. The cultures without treatment were used as control group.

The MTT cell viability assay was performed to evaluate cell multiplication after conducting the cytotoxic studies. Briefly, was added to each well 80 µL of the MTT solution (5 mg mL⁻¹) and 420 µL of medium without phenol red. Cell's incubation occurred at 37 °C under 5% CO₂ atmosphere for 6 h, allowing the formazan-forming reaction to proceed. Then, the medium containing the MTT solution was removed, and formazan crystals were dissolved in isopropanol. Optical density was measured at 570 and 690 nm using a Safire² microplate reader (TECAN Group Co.). Results were presented as percentage cell survival relative to control (untreated cells).

2.8. Statistical analysis

Statistical analyses of the MTT cell viability assays were obtained within the GraphPad Prism 6.0 (GraphPad Software, Inc.) software. Deviations between control and treated groups were evaluated by means of one-way analysis of variance (ANOVA) followed by Tukey post-test (significance set at $P < 0.05$).

3. Characterization

Nuclear magnetic resonance spectroscopy (¹H-NMR) was performed in a Bruker Fourier 300 Hz spectrometer. Samples were dissolved in chloroform-*d* (CDCl₃) and dimethyl sulfoxide (DMSO-*d*₆) for enlightening the chemical structure of the copolymer. Gel permeation chromatography was used to determine the M_w , M_n and molecular weight distribution of the copolymers. A Viscotek GPC/SEK equipment from Malvern Instruments using degassed THF as eluent at a rate of 1 mL min⁻¹ and polystyrene as the standard calibration was used. Three thermostated (35 °C) Shodex KF-806 M series columns (SHOWA Denko, Tokyo, Japan) were used for separation and Viscotek RI VE3580 and Viscotek UV as the detector. The morphology and size of the self-assembled micelles was performed on a JEM-2100-JEOL LaB₆ transmission electron microscopy using an electron beam of 200 kV. The samples were dispersed in deionized water at 25 °C and deposited in

formvar carbon coated copper grids with 400 mesh. The colloidal properties (hydrodynamic diameter and zeta potential) of the micelles were evaluated using the Zetasizer Nano ZS from Malvern Instruments. Information on drug incorporation efficiency and the controlled release profile were obtained from UV–vis spectroscopy using a PerkinElmer equipment, model LAMBDA 465. The methotrexate quantification was measured at 303 nm wavelength.

3.1. Critical micellar concentration

The Critical Micellar Concentration (CMC) was determined using an OCA 15EC equipment (DataPhysics). The surface tension was measured indirectly using the optical pendant droplet method [22,23]. The dispersion of the copolymer is added to a vertical syringe and a droplet is released. A camera attached to the device allows photographing the droplet on the eminence of falling. The numerical fit of the theoretical droplet shape to the shape recorded by the camera was based on the droplet-air contrast (contour analysis system). After fitting, the surface tension was determined by using the Young-Laplace mathematical model [24]. The Eq. (4) expresses the model:

$$\Delta P = (P_{int} - P_{ext}) = \sigma \left(\frac{1}{R_1} + \frac{1}{R_2} \right) \quad (4)$$

with ΔP equals to the pressure difference between the inner and the outer pressures in the drop, σ is the surface tension of the drop and both R_x ($x = 1$ and 2) are the principal radius of curvature.

4. Results and discussion

4.1. Synthesis of PEG-b-PCL copolymers

The ^1H NMR spectra containing the information on the PEG-b-PCL copolymer samples structure is shown in Fig. 2. The peak of methylene protons of both PEGs (MPEG and PEGMA) was observed at 3.65 ppm. The peaks of methoxy protons end-group in MPEG and of methacrylate protons end-group in PEGMA were observed at 3.40 ppm, 6.05 ppm, and 5.56 ppm, respectively. The methylene protons from the PCL segment were showed at 1.35, 1.63, 2.30, and 4.03 ppm [25]. The radical polymerization of the methacrylate group was confirmed with the absence of the peaks at 6.05 ppm and 5.56 ppm which is attributed to the double bond protons. The coupling reaction between the 2-propenamide amino group and the folic acid carboxylate group is confirmed with the appearance of peaks at 2.68 and 2.84 ppm due to methylene protons between both carboxylate groups, 4.38 ppm assigned to amide

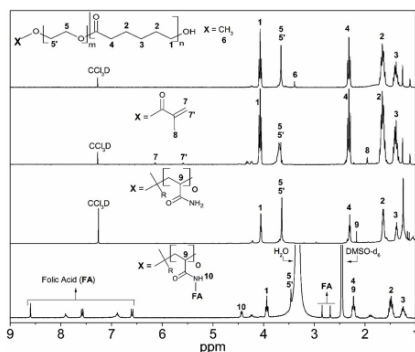


Fig. 2. ^1H NMR spectrum of PEG-b-PCL copolymers. R = Initiator = Potassium persulfate and X = substituent group.

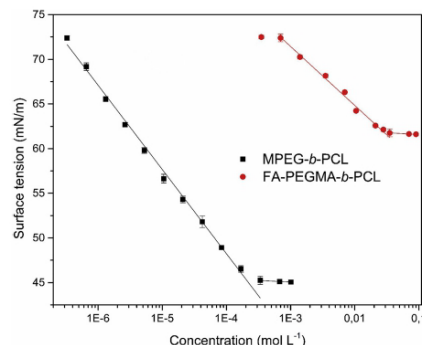


Fig. 3. Surface tension measurements of PEG-b-PCL copolymers in different concentrations values.

bond proton, 6.62 and 7.74 ppm correspond to the aromatic ring protons, and 7.63 and 8.65 ppm is characteristic of secondary amino protons [26,27].

4.2. Critical micellar concentration of the PEG-b-PCL micelles

The surface tension as a function of the log concentration of the copolymer graph is shown in Fig. 3. The amphiphilic copolymers self-assemble into micelles in an aqueous medium, the PEG segment due to its hydrophilicity will compose the shell of the micelles while the PCL segment will remain in the core, minimizing the repulsion between its carbon chain and the water molecules [28]. The modification of the PEGMA methacrylate group, culminating in the addition of acrylamide and folic acid molecules on the surface of the PEG-b-PCL micelles, is responsible for the increased hydrophilicity of the copolymer. The acrylamide molecule has amide groups that allows the formation of hydrogen bonds with the water molecules, compensating the hydrophobicity of folic acid [29]. The chemical interaction between acrylamide and water molecules causes the increase in CMC, which is in the order of about 100 times plus compared to that of MPEG-b-PCL micelle. The CMCs of the MPEG-b-PCL and FA-PEGMA-b-PCL samples are $2.04 \times 10^{-4} \text{ mol L}^{-1}$ and $2.90 \times 10^{-2} \text{ mol L}^{-1}$, respectively.

4.3. Colloidal characterization

The evaluation of the colloidal characteristics of the PEG-b-PCL samples was performed by measuring the zeta potential (ζ) and the hydrodynamic diameter (D_h) of the micelles. Both properties are directly linked to the surface of the micelles and therefore its modification will result in different dispersions. Micelles TEM images are shown in Fig. 4 where it is possible to observe spherical morphology in deionized water. The size of the micelles obtained by microscopy is in agreement with the DLS result. Table 1 shows the hydrodynamic diameter of PEG-b-PCL micelles and respective molecular weights with low polydispersity. Functionalization of micelles with folic acid promoted an approximately 50% increase in the hydrodynamic diameter and a slight increase in the molar mass of the copolymer. This occurs due to the polymerization of the methacrylate group with 2-propenamide and subsequent modification with folic acid. The high polydispersion index (PDI) demonstrate that there are distinct sizes of micelles agglomerates, possibly due to low molecular weight structure of PEG [30] and the presence of folic acid. PEG-b-PCL micelles zeta potential and hydrodynamic diameter in different pH is showed in Fig. 5. In Fig. 5A, the MPEG-b-PCL sample has isoelectric point (IEP) at 3.84, i.e., pH value at which the net surface charge of the micelle is zero. The FA-PEGMA-b-

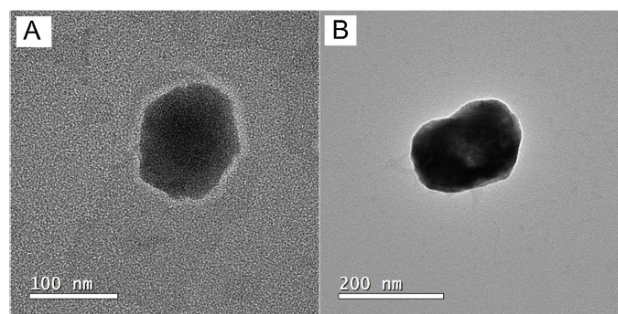


Fig. 4. TEM images of copolymer micelles samples. (A) MPEG-b-PCL and (B) FA-PEGMA-b-PCL.

Table 1

The Hydrodynamic diameter of PEG-b-PCL micelles obtained by Dynamic Light Scattering (DLS) and respective molecular weights obtained by Gel Permeation Chromatography (GPC).

Sample	D_h /nm	PDI	M_n /g mol ⁻¹	M_w /g mol ⁻¹	M_w/M_n
MPEG-b-PCL	122 ± 11	0.42 ± 0.04	2976	3375	1.134
FA-PEGMA-b-PCL	190 ± 7	0.37 ± 0.04	3430	3710	1.082

PCL sample did not show IEP and presents negative values of zeta potential in the investigated pH range [31]. None of the samples has zeta potential higher than +30 mV and lower than -30 mV, where they have sufficient electrostatic repulsion to provide colloidal stability to the micelles [32,33]. Also, it is possible to identify a distinguishable surface electric property in FA-PEGMA-b-PCL compared to MPEG-b-PCL as the groups of folic acid added to the structure sustain the negative charge until lower pH values [34]. In Fig. 5B, the nonfunctionalized sample tends to aggregate at pH close to 4 and the hydrodynamic diameter increases, while the FA-PEGMA-b-PCL did not show the same behavior. In addition, folic acid in the end of the polymer chain increased the steric stability of the micelles.

4.4. Methotrexate incorporation efficiency

Methotrexate was used as chemotherapeutic agent and was incorporated in PEG-b-PCL micelles. Table S1, in the supplementary

information, shows the incorporation efficiency and incorporation coefficient values for both samples, achieving at least 85% incorporation efficiency. The high-efficiency evidence that the incorporation process depends mainly on the hydrophobic segment of the copolymer. The small difference between the values can be attributed to the functionalization of the micelles, since both the targeting agent and the drug are analogs, part of the methotrexate is on the surface of the micelle interacting with the folic acid through weak interactions (dipole-dipole, van der Waals). For the drug release measurement, the samples with the highest methotrexate content were used.

4.5. Methotrexate release profile and pharmacokinetics analysis

The cumulative release profile of the PEG-b-PCL micelles is shown in Fig. 6A. It was observed that the FA-PEGMA-b-PCL sample shows the maximum release (55%) after 30 h of release and, later, there is a drop in the percentage released, suggesting the dilution of the medium. The MPEG-b-PCL sample reached a maximum release level (42%) after 80 h of release. This release behavior might be due to the hydrophobic barrier of the micelles core blocking the entrance of solvent molecules, avoiding diffusion of methotrexate into the hydrophilic shell. Since the two samples have the same major copolymer structure, the difference in the release may be attributed to the interactions of methotrexate with folic acid due to the similarity between these molecules. Besides the difference in the amount of methotrexate released and the time for that take place, the profile of the curves from both samples are different. While the functionalized folic acid sample initiates the release process almost immediately, the non-functionalized sample shows a delay to

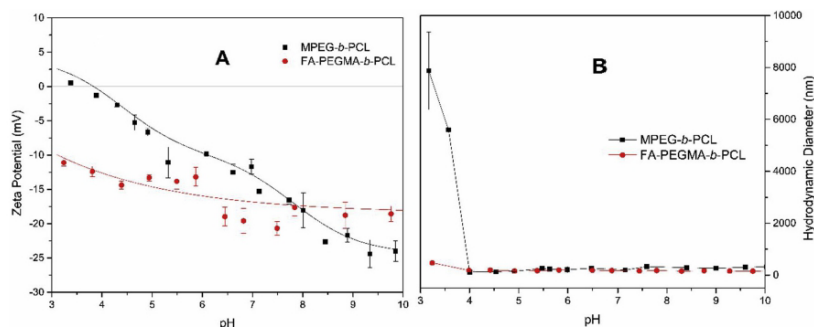


Fig. 5. A) Zeta potential and B) Hydrodynamic diameter variation in different pH values of PEG-b-PCL micelles.

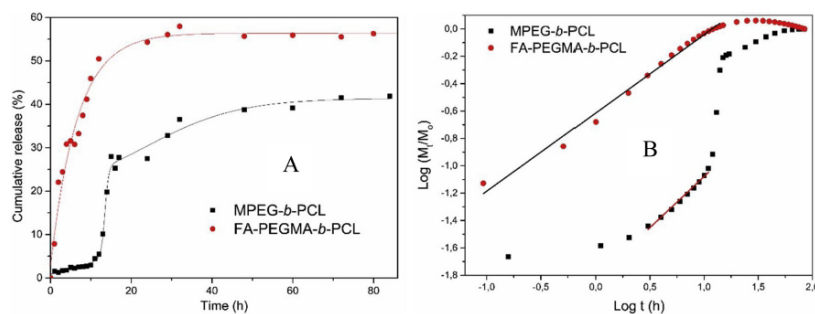


Fig. 6. A) The cumulative release profile of methotrexate by PEG-b-PCL micelles in PBS 0.01 M solution at 37 °C and B) Methotrexate release pharmacokinetic profile of PEG-b-PCL micelles and fitting accord to Peppas model.

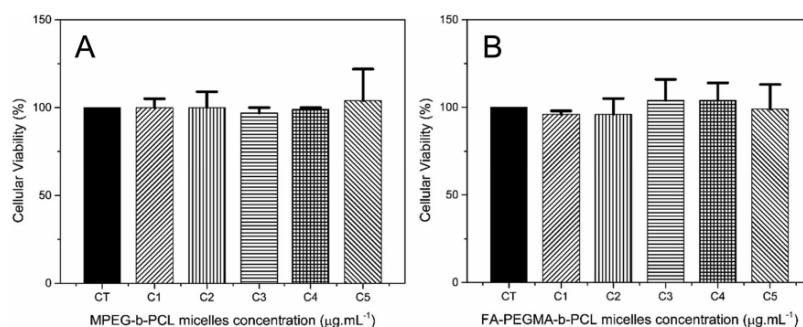


Fig. 7. Cytotoxicity of A) MPEG-b-PCL and B) FA-PEGMA-b-PCL on murine fibroblast cells line (NIH3T3) by mitochondrial viability test MTT. $C_1 = 3.12$, $C_2 = 6.25$, $C_3 = 12.5$, $C_4 = 25.0$ and $C_5 = 50.0 \mu\text{g mL}^{-1}$. Data are presented as mean $\pm$ SEM of three independent experiments. Deviations between control and treated groups were evaluated by means of one-way analysis of variance (ANOVA) followed by Tukey post-test (significance set at $P < 0.05$).

the onset of the release and, after the first 12 h, it shows a burst phenomenon, dramatically increasing the percentage of drug released [35]. These factors show that the functionalization of micelles with folic acid has altered the release mechanism.

The use of the Peppas model made it possible to clarify the mechanism involved in the first methotrexate release phase, in which the release exponent (n) and the kinetic constant (k) were determined. The PEG-b-PCL micelles release pharmacokinetic profile plot is shown in Fig. 6B and the parameters obtained from the curves summarized in table S2 in the supplementary information.

The Peppas model is valid to linearize the data obtained in the initial stages of release, confirmed by the high values of the correlation coefficient ($R^2 > 0.98$). The FA-PEGMA-b-PCL sample showed the release exponent (n) equal to 0.57, indicating an anomalous (non-Fickian) transport. The MPEG-b-PCL sample showed a burst phenomenon and the region of analysis was prior to this (indicated by the fitting in the graph of Fig. 6B). The region prior to the burst effect has n equal to 0.75 indicating also an anomalous transport, confirming that the surface modification of the micelle does not interfere with the initial mechanism (in this case, the first 12 h) of release. [36,37]. The burst phenomenon can be attributed to several factors, as presented by Kamaly, N. et al. [35]. The main factor, in this case, is the modification of micelles with folic acid, increasing its hydrophilicity and consequently, causing the absence of the burst phenomenon. The data validated the release profile, indicating the interaction of methotrexate to a greater extent, but not solely, with the hydrophobic segment (PCL) of the

copolymer, requiring relaxation and/or degradation of the polymer chain for drug release.

4.6. Cell viability

The results presented in Fig. 7 demonstrate the correlation of cell viability as a function of the micelles concentration obtained after the colorimetric test of MTT mitochondrial activity. Cell viability after 6 h incubation with both samples in the range of concentrations evaluated doesn't show significant changes when compared to untreated cells. The presence of folate and acrylamide on the surface of micelles also did not influence cell viability. These observed aspects are shown by statistical differences. Therefore, the functionalization of the PEG-b-PCL micelles did not compromise their biological application due to the lack of apparent cytotoxicity.

5. Conclusion

The structure of PEG-b-PCL copolymer obtained by the ROP reaction of ϵ -caprolactone and folic acid functionalization were confirmed by proton NMR spectroscopy. Gel permeation chromatography confirmed the molecular mass distribution around 3000 g mol^{-1} with low polydispersity. The measurement of the surface tension as a function of the concentration allowed to evaluate the amphiphilic behavior of the copolymers in water and the formation of micelles (CMC). The CMC of the MPEG-b-PCL sample is $2.04 \times 10^{-4} \text{ mol L}^{-1}$ while the FA-PEGMA-b-PCL

sample is $2.90 \times 10^{-2} \text{ mol L}^{-1}$, showing an approximately 100-fold increase in CMC value after functionalization and, consequently, more hydrophilic. The micelles of MPEG-*b*-PCL presented hydrodynamic diameter 122 nm and after the functionalization, there was a 50% increase in diameter, reaching 190 nm for the FA-PEGMA-*b*-PCL sample. The surface modification of the micelles prevents aggregation due to steric repulsion provided by folic acid. Methotrexate was used as a hydrophobic drug to be incorporated by the micelles and both samples showed incorporation efficiency above 85%. The value of the correlation coefficient above 0.98 allowed the use of the Peppas model to analyze the samples behavior. The functionalized sample presented phase II characteristic release, presenting an anomalous transport during the release. The nonfunctionalized sample presented triphasic release, having a burst effect after the first 12 h, which made it possible to analyze the transport before the burst. Before burst, anomalous transport was observed, like the functionalized sample. The biologic effect of PEG-*b*-PCL micelles on murine fibroblast cells line (NIH3T3) were evaluated and both samples does not exhibit apparent cytotoxicity. These results make PEG-*b*-PCL micelles suitable candidates for the release of methotrexate in the cancer treatment as it is possible to modify micelles properties adding bioactive molecules like folic acid.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.colsurfb.2019.02.008>.

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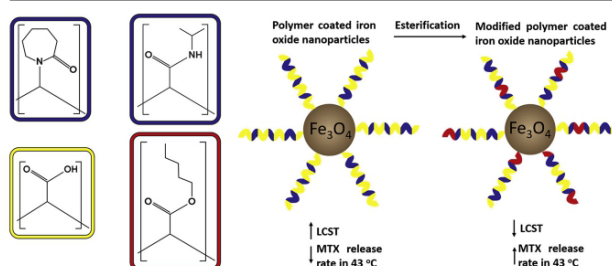
Esterification influence in thermosensitive behavior of copolymers PNIPAm-co-PAA and PNVCL-co-PAA in magnetic nanoparticles surface

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GRAPHICAL ABSTRACT



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ABSTRACT

The ability to generate heat from magnetic nanoparticle and the response of thermosensitive polymers poly(n-isopropylacrylamide) (PNIPAm) and poly(n-vinyl caprolactam) (PNVCL) are properties that have promoted several studies in controlled drug release systems. As the lower critical solution temperatures (LCST) of PNIPAm and PNVCL are between 32–35 °C and close to the temperature of the human body these polymers are adequate for biomedical applications. The LCST could be increased by copolymerizing the thermoresponsive polymer with a monomer with greater water affinity as poly(acrylic acid) (PAA), which was studied copolymerized with PNIPAm and PNVCL in aqueous solution. Cancer treatment is possible using the ability to generate heat from magnetic nanoparticles. Magnetic hyperthermia can be combined with controlled drug release and improve the treatment. In this work iron oxide nanoparticles were surface modified with a copolymer of acrylic acid with PNIPAm or PNVCL and then modified by esterification. Infrared spectroscopy indicated the presence of the polymer in the magnetic nanoparticles and showed the bound of carboxylic groups to the magnetite surface. The temperature-time experiments showed that magnetic nanoparticles coated with thermoresponsive polymers can heat from 20 °C to 40 °C, in a suspension of 15 mg of magnetite in 1 ml of water under an alternating magnetic field of 14 kA m^{-1} and frequency of 420 kHz. The polymer onto magnetic nanoparticle surface has a high release rate of methotrexate at 43 °C, but low at 37 °C. PNIPAm-co-PAA or PNVCL-co-PAA showed different behavior in methotrexate (MTX) release, as the esterified copolymer of PNIPAm showed an increase in MTX release in 43 °C and PNVCL esterification decreases the release rate. The LCST of PNIPAm-co-PAA and PNVCL-co-PAA decreased with the esterification since the reaction added hydrophilic groups in the polymer chain. These results suggest

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that the platform developed in this work can be used for drug delivery in cancer treatment, since its LCST can be tuned in function of esterification and composition to temperatures well above the human body.

1. Introduction

Nanostructured magnetic nanoparticles have many biomedical applications. Iron oxide, for example, in the form of magnetite or maghemite, has biocompatibility in the mammalian bloodstream. The most common applications are contrast media in magnetic resonance imaging or cancer treatment such as thermochemotherapy [1,2]. An even better result can be obtained by combining two distinct modes of treatment into a single material. It is possible to combine the ability to generate heat from magnetic nanoparticles with controlled release systems and improve animal survival [3]. So, polymer coatings have gained focus in biomedical application studies because thermo-responsive polymers can carry drugs and change properties by external stimulus [1].

Iron oxide nanoparticles, in cell applications, can generate heat by the relaxation of Néel. The relaxation of Néel involves the reorientation of the magnetic moment (of the particle) in the same direction of the oscillating magnetic field [1]. It is dependent on particle size since smaller particles require less energy to change their magnetic field [4]. The other way of producing heat, simultaneous to Néel relaxation, is the Brownian relaxation that will produce heat as a function of the rotation of the particle in a continuous phase. It usually occurs with larger size particles and has less influence than the relaxation of Néel [1–4].

Due to the size distribution of several nanoparticles produced industrially or in scientific studies, it is very common to use the specific absorption rate (SAR) to compare the "power" of generating heat from the magnetic nanoparticles [5]. The SAR value can be obtained by Eq. (1):

$$SAR = \frac{\Delta T}{\Delta t} \frac{C}{m_{Fe}} \quad (1)$$

Whereas T is the temperature, t the time, C is the specific heat capacity of the sample and m_{Fe} is the mass of iron oxide per mass unit of fluid.

Systems involving the controlled release of drugs has gained a great interest in stimulus responsive polymer coatings. The property of the "smart" behavior of the thermosensitive polymers is due to the volume phase transition dependent on a critical temperature, which rapidly produces changes in solubility [6]. When heated, some polymers become insoluble in aqueous solution at a temperature called the lower critical solution temperature (LCST) [1,7–9].

The most studied temperature sensitive synthetic polymer is poly (N-isopropylacrylamide) (PNIPAm) and its copolymers with hydrophilic groups like acrylic acid (PNIPAm-co-PAA). PNIPAm based copolymers chains have repeating units based on hydrophobic (isopropyl) and hydrophilic (amide) groups and their minimum critical solution temperature is near the temperature of the human body, $\sim 32^\circ\text{C}$ [10].

Below that critical temperature, the polymer forms a homogeneous water-soluble mixture. However, by heating, it becomes hydrophobic and water immiscible since interchains water molecules became thermodynamically unstable. One strategy for increasing the LCST temperature is to add a hydrophilic monomer to copolymerize PNIPAm. Recent studies showed that PNIPAm-co-PAA in a well-defined proportion can increase the LCST to 42°C [11,12]. An alternative to the n-isopropylacrylamide monomer would be n-vinylcaprolactam. The polymer composed of n-vinylcaprolactam (PVCL) presents temperature close to the human body like PNIPAm and its LCST is easily modified by the chain size or the concentration in aqueous solution. Some studies report a greater biocompatibility of PVCL, because acid hydrolysis produces carboxylic groups in contrast to PNIPAm that under acidic hydrolysis produces highly cytotoxic monomers. So PVCL has a great potential as PNIPAm substitute [13,14].

The main idea of using a thermosensitive polymer with a magnetic nanoparticle is to produce a copolymer shell charged with drug on the magnetic nanoparticle surface and use an alternated magnetic field to increase the temperature, the heat shrinks the polymer shell and the water is expelled from the polymers chain carrying a specific drug. For iron oxide nanoparticles surface modified with copolymers, it is possible to apply changes in the polymer chain by esterification method that can tune the LCST to a desired value, improving the efficiency of drug release.

In this work, we surface modified magnetic nanoparticles with the copolymer PNIPAm-co-PAA or PNVCL-co-PAA and we verified how the esterification with 1-pentanol affected the release rate of methotrexate in aqueous solution. By inserting a long chain alcohol into thermo-responsive polymers was possible to demonstrate a new methodology to change the hydrophilicity of copolymers anchored to the surface of magnetic particles, allowing its usage in drug delivery.

2. Experimental

2.1. Materials

$\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ 98% (Sigma-Aldrich), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 98% (Sigma-Aldrich), NH_4OH 28,0–30,0% (Alfa-Aesar), N-isopropylacrylamide 97% (Sigma-Aldrich), N-vinylcaprolactam 98% (Sigma-Aldrich), Acrylic Acid 90% (Alfa Aesar), dialysis tubing cellulose membrane 33 mm (Sigma-Aldrich), $\text{K}_2\text{S}_2\text{O}_8$ (Sigma-Aldrich), NaHSO_3 (Mallinckrodt), 1-pentanol 99% (Sigma-Aldrich), methotrexate (batch: PHR1396 – Sigma-Aldrich), NaCl (Sigma-Aldrich).

2.2. Magnetic nanoparticle synthesis

The synthesis used for the preparation of magnetite nanoparticles

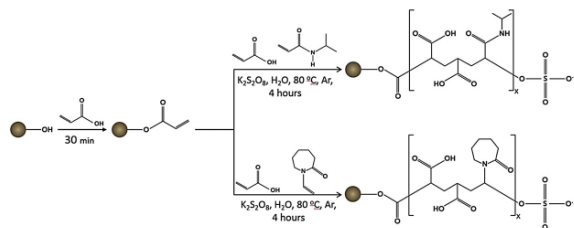


Fig. 1. Synthetic route of polymer coated magnetite.

was the coprecipitation method using iron chloride II and iron chloride III in ammonium hydroxide solution. For the synthesis, the solution of ammonium hydroxide (100 ml) was added to a solution (200 ml) of iron chloride II and iron chloride III (mole ratio $\text{Fe}^{3+}/\text{Fe}^{2+} = 2$) in a 500 ml flask under argon bubbling and constant stirring. Then the system was stirred for another 8 h at 80 °C. Finally, the nanoparticles were washed with water through magnetic separation until pH 7.0 and then the sample was stored for further analysis or reactions.

2.3. Surface modification and polymerization

For surface functionalization, 30 ml of a suspension of magnetite 5 g mL^{-1} were added to a solution of N-isopropylacrylamide (NIPAm)/acrylic acid (AA) (molar ratio NIPAm/AA = 0,5) or N-vinylcaprolactam (NVCL)/acrylic acid (molar ratio NVCL/AA = 0,5) and stayed for 30 min in magnetic stirring in 4,5 pH solution. Then potassium persulfate and potassium bisulfite were added as catalyst to start the radical polymerization, the flask was sealed in an argon atmosphere and heated to 80 °C for 4 h. After the nanoparticles were washed with distilled water through magnetic separation until pH 7.0 and subjected to dialysis for 3 days. (Fig. 1)

2.4. Polymer esterification

This method was adapted from Komura, K. et al. and used to modify the polymer in magnetic nanoparticles surface [15]. Surface modified magnetic nanoparticles (20 mg) were suspended in 20 ml of 1-pentanol. The nanoparticles 1-pentanol solution were sealed in a 50 ml reaction flask with iron chloride III as the catalyst. The reaction flask stayed at 130 °C for 24 h. Then the samples were washed with acetone and the nanoparticles were resuspended in water. (Fig. 2)

The materials were separated in five samples according Table 1.

2.5. Transmission electron microscopy (TEM)

The size of magnetic nanoparticles was evaluated by transmission electron microscopy (TEM). For the preparation of the samples the dried nanoparticles (approximately 5 mg) were suspended in isopropanol. Copper grids Formvar-coated were used to immobilize the particles suspended in isopropanol. The grids were kept in a vacuum desiccator for 2 days and sent for analysis. Particle size distribution histograms were constructed based on the count of approximately 150 particles in a total of 4 micrographs per sample. The software *ImageJ* was used for counting of the particles. The equipment used was a JEOL JEM2100 LaB6 (USP, São Carlos, Brazil) microscope at an accelerating voltage of 200 kV.

2.6. Specific absorption rate (SAR)

For this measurement, 15 mg of samples were dispersed in 1 ml of

water. The flask containing 1 ml of polymer iron oxide suspension in water was hermetically closed in a vacuum chamber for heating experiments. The frequency chosen was $f = 420 \text{ kHz}$ and the magnetic field was $H = 14 \text{ kA m}^{-1}$ in a DM100 Series (Nanoscale Biomagnetics). The temperature time dependence obtained was an exponential curve and was fitted in Box-Lucas model like Kallumadil, M. et al. [5] (Eq. (2)):

$$T(t) = A (1 - e^{-Bt}) \quad (2)$$

Whereas T is the temperature, t is the time, A is the saturation temperature and B is a parameter related to the curvature of the heating curve.

The SAR calculation was done using SAR Eq. (1) (17,18) considering $\Delta T/\Delta t$ equals the product of the parameters $A \times B$ in $t = 0$ in Box-Lucas equation (17,18). The heat capacity for magnetite C was negligible by its low contents in the samples, therefore it was used the specific heat capacity for water $C_{\text{water}} = 4.18 \text{ J g}^{-1} \text{ K}^{-1}$ as the sample's specific heat capacity.

2.7. Fourier transform infrared spectroscopy (FTIR)

All the samples were dried and dispersed in KBr and pressed into pellets in the ratio of approximately 1 mg of sample to 100 mg of potassium bromide. A FT-IR Spectrometer Frontier (Perkin Elmer) was used in transmission mode set at 4 cm^{-1} resolution while performing 28 scans in the region 4000 cm^{-1} to 400 cm^{-1} .

2.8. Dynamic light scattering (DLS) and zeta potential measurement

In order to understand the shrinking process of the polymer in the nanoparticle surface, dynamic light scattering technique was used to analyses the hydrodynamic diameter behavior in function of temperature. The samples of 0,01 mg/ml nanoparticles suspensions were filtered through a $0.45 \mu\text{m}$ cellulose acetate filter. The measurement was performed in polystyrene cuvettes and performed in triplicates at a temperature range from 20 °C to 60 °C.

For Zeta Potential measurement the samples of 0,01 mg/ml nanoparticles were kept during 12 h in a $0,01 \text{ mmol L}^{-1}$ NaCl solution and then placed in the electrophoretic cell for the experiment. The samples were titrated in the range of 2–10 pH measured with 1.0 pH increments measured in triplicate at 25 °C. The pH was modified using a solution of NaOH $0,01 \text{ mol L}^{-1}$ or a solution of HCl $0,01 \text{ mol L}^{-1}$.

For both analyses, the data was obtained using a Zetasizer Nano Series System (Malvern Instruments).

2.9. UV-vis spectroscopy

Firstly, 10 mg of polymer coated magnetic nanoparticles were placed in 10 ml solution of 1.5 g mL^{-1} of methotrexate and phosphate buffer pH 7.4 for 24 h in 10 °C to guarantee incorporation of

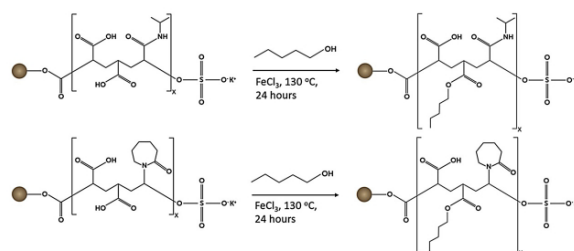


Fig. 2. Esterification of the polymer in nanoparticles surface.

Table 1
Materials and nomenclatures.

Name	Description
Mag	Magnetic nanoparticles
Mag@PNIPAm-co-PAA	Magnetic nanoparticles coated with the copolymer PNIPAm-co-PAA
Mag@PNVCL-co-PAA	Magnetic nanoparticles coated with the copolymer PNVCL-co-PAA
Mag@PNIPAm-co-PAAmod	Magnetic nanoparticles coated with the copolymer PNIPAm-co-PAA after esterification.
Mag@PNVCL-co-PAAmod	Magnetic nanoparticles coated with the copolymer PNVCL-co-PAA after esterification.

methotrexate into the polymer chain (the polymer chains are not collapsed in 10 °C). The nanoparticles were separated by centrifugation and washed with cold ultrapure water. Then, 2 mg of nanoparticles were placed in a dialysis tubing cellulose membrane 33 mm for the release test. The dialysis bag was inserted into a test tube with exactly 20 ml phosphate buffer pH 7.4 and kept at 25 °C or 43 °C. Aliquots of 1 ml were collected at different times and each aliquot was analyzed by absorption spectroscopy in the UV–vis region. After each aliquot were taken, 1 ml of phosphate buffer was added to keep solution volume constant.

The analytical calibration curve was prepared with methotrexate (batch: PHR1396 – Sigma-Aldrich) and the band at 303 nm was used for the absorbance calculation. A PDA/UV-VIS Lambda 465 (Perkin Elmer) was used in absorbance mode.

2.10. Gel permeation chromatography (GPC)

Molecular weights (Mn and Mz) and polydispersity index (PDI) of PNIPAm-co-PAA and PNVCL-co-PAA were characterized by a Malvern Instruments gel permeation chromatography (GPC) analysis system, using two conjoined chromatography columns (SHODEX - KD801 and KD803) where DMF was used as the eluent and polystyrene (Malvern Instruments) as the calibration standard. The retention volume was determined by a refractive index detector. Fig. 3 shows the calibration curve of polystyrene in DMF (Fig. 3).

2.11. Carbon, nitrogen, hydrogen elemental analysis

Atomic composition of PNIPAm-co-PAA and PNVCL-co-PAA was determined by elemental analysis (Carbon, nitrogen and hydrogen - CNH) by a Perkin Elmer Elemental Analyzer (CNH). The equipment makes the determination of the elements based on the method modified Pregl-Dumas. The samples were combusted, and the released gases composition were determined by a thermal conductivity detector.

3. Results and discussion

3.1. Size characterization

The average size of nanoparticles was determined by electron transmission microscopy (TEM). The purpose of such analysis was to characterize the functionalized nanoparticles by checking the size distribution and applying the data to a Gaussian curve to obtain the mean size (Fig. 4).

It can be observed in Fig. 4 that the average size of all samples is very close considering the deviations found. All samples nanoparticles were expected to have similar size distributions 9.4 ± 2.5 nm, since they all come from the same material. It was possible to verify that no further modification (polymerization or esterification) altered the particle size or spherical shape. With average size close to 9.5 nm the magnetic nanoparticles can be classified as nanomaterial according to expected for the intended biomedical application [1].

The data in Fig. 5 are relevant to determine that the method of synthesis of nanoparticles with ammonium hydroxide agrees with the production of magnetic nanoparticles capable of generating heat. Samples with polymer coating PNIPAm-co-PAA and PNVCL-co-PAA

showed a longer time for heating over uncoated magnetite. The presence of another material on the surface of the magnetic nanoparticles acts as a barrier that limits the diffusion of the heat generated by the nanoparticles. The greater the functionalization barrier thickness, the greater the decrease in measured SAR value [16]. The esterified material underwent a 24 h synthesis process at the temperature of 130 °C. This synthesis probably caused oxidation of the magnetite to maghemite form and probably decreases its potential for heat generation [17,18]. Besides that, the esterification with a long chain alcohol molecule cause a magnetic dissolution since at same 15 mg sample we have different mass contents of magnetic phase. Although there is a decrease in the heat generation capacity of the samples due to the surface modification process, it is still possible to detect that the nanoparticles have the capacity to generate heat under an alternating magnetic field and this is in accordance with the proposal of the work, enabling human body application (Fig. 5).

3.2. FT-IR and Zeta Potential measurement

The polymer modified iron oxide nanoparticles samples were subjected to FT-IR to verify the functionalization efficiency with the copolymers of PNIPAm-co-PAA and PNVCL-co-PAA. As can be seen from Fig. 6 for both polymers a distinct spectrum can be perceived between modified samples. For the spectra of Fig. 6 it can be observed common bands near 3500 cm^{-1} , 3500 and 1630 cm^{-1} , related to the absorbed H_2O molecules or OH^- on the surface of Fe_3O_4 (22), 2930 cm^{-1} corresponding to the asymmetric stretch of CH_2 group and 630 cm^{-1} and 580 cm^{-1} corresponding to the stretching of the Fe-O bond of iron oxide [19] (Fig. 6).

The difference found in the spectra of materials with and without functionalization is the increase in the intensity of the band near 2930 cm^{-1} that is an evidence of introduction of carbon groups (CH_2 groups). The appearance of the band at 1460 cm^{-1} that concerns the presence of carboxylated groups on the surface indicate the carboxylic group was bounded to the iron particles [20]. The carboxylic groups have a great affinity to bond to iron oxide surface due to an acid base

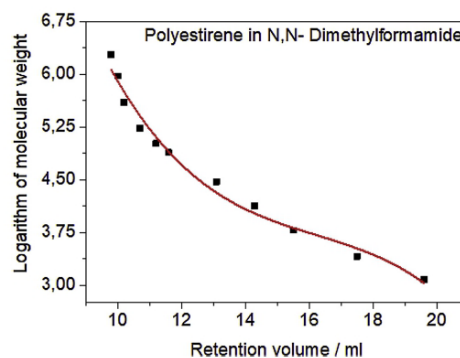


Fig. 3. Calibration curve of polystyrene in *N,N*-Dimethylformamide.

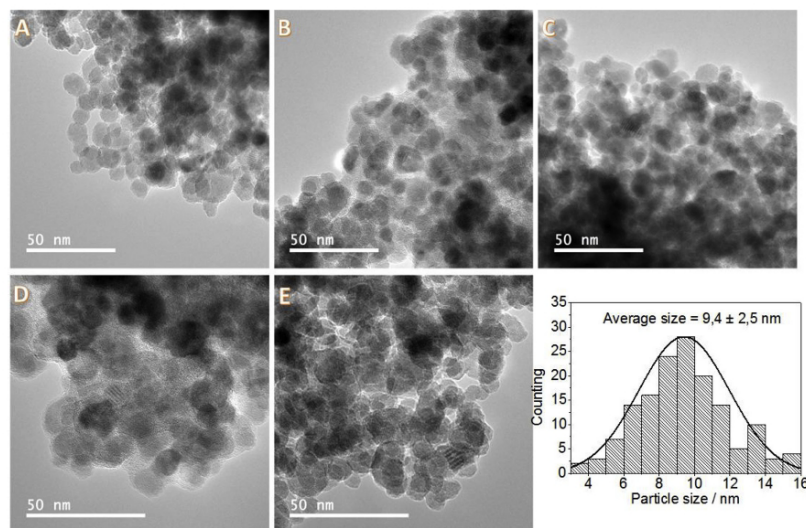


Fig. 4. Transmission electron micrographs of the magnetic nanoparticles before and after polymerization / esterification and their histogram for: (A) Mag; (B) Mag@PNIPAm-co-PAA; (C) Mag@PNIPAm-co-PAAmod; (D) Mag@PNVCL-co-PAA and (E) Mag@PNVCL-co-PAAmod.

reaction [20]. For the spectra of the polymer-functionalized samples, there is a set of bands between 1800 cm^{-1} and 1200 cm^{-1} . The observed bands indicate the presence of the polymer on the surface of the iron oxide nanoparticles. Among them it is possible to observe carboxylic acid groups ($\text{C}=\text{O}$ stretch) at 1725 cm^{-1} corresponding to the presence of poly (acrylic acid) and amide groups ($\text{C}=\text{O}$ stretch) at 1650 cm^{-1} and 1550 cm^{-1} corresponding to the groups poly(N-isopropylacrylamide) and poly(N-vinylcaprolactone). It is also possible to observe bands close to 1455 cm^{-1} corresponding to the C–N stretch [6,9,11,21].

To evaluate the surface charge of functionalized iron oxide particle and help FT-IR measurements to prove the existence of polymer shell in the surface, the zeta potential experiments were performed. Fig. 7 shows that the iron oxide nanoparticles have an isoelectric point close to pH 4.9. The surface charge in the oxide is possible due to the presence of hydroxyl groups in the surface ($\text{Fe}-\text{OH}$), as FT-IR showed [22].

This hydroxyl groups change its charge due to dissociation process ($\text{Fe}-\text{O}^-$) in higher pH values and protonation process ($\text{Fe}-\text{OH}_2^+$) in lower pH values. In intermediary values of pH there is an isoelectric point that the surface has charge near value 0 and the particle has a tendency to agglomerate [22]. As shown in Fig. 7 the functionalized samples with modified copolymers present a decrease in the isoelectric point values for smaller values than that of uncoated magnetite [4,9]. This reduction occurs exclusively due to the presence of the copolymers in the surface. Carboxylic groups of acrylic acid by having pKa close to 4.5, induces, at pH above that value, the formation of deprotonated carboxylate groups giving a negative charge to the polymeric coating [20,22]. The esterification process did not cause major changes in the isoelectric points of the samples, but as can be observed for Mag@NIPAm-co-PAA / Mag@NIPAm-co-PAAmod (red and pink) and Mag@PNVCL-co-PAA / Mag@PNVCL-co-PAAmod (blue and green) there was a reduction in surface charge at pH values higher than 5.0 after

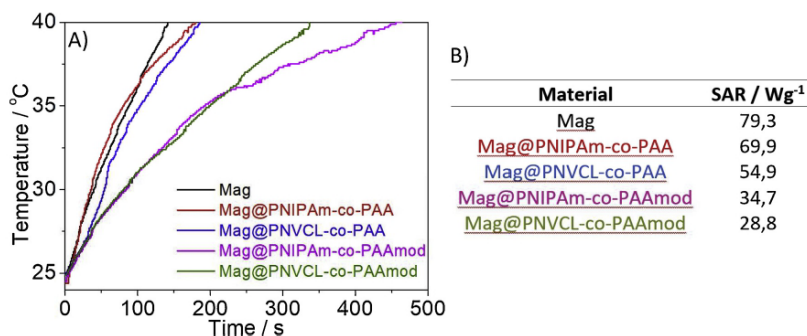


Fig. 5. A) temperature time dependence curve of samples heating in an alternating magnetic field $H = 14\text{ kA m}^{-1}$ and frequency $f = 420\text{ kHz}$. B) Absorption Rate calculated from temperature time dependence curve.

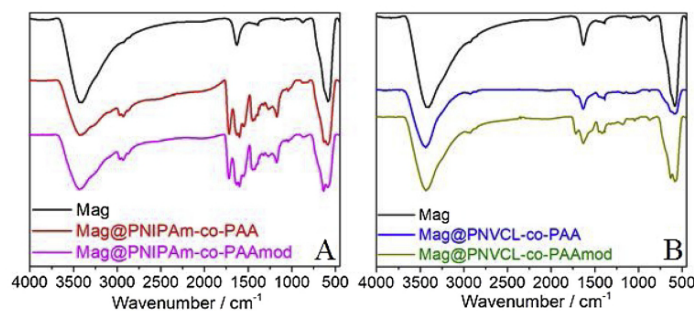


Fig. 6. FT-IR spectra of the iron oxide nanoparticles with and without the copolymer shell. A) Samples coated with copolymer PNIPAm-co-PAA. B) Samples coated with copolymer PNVCL-co-PAA.

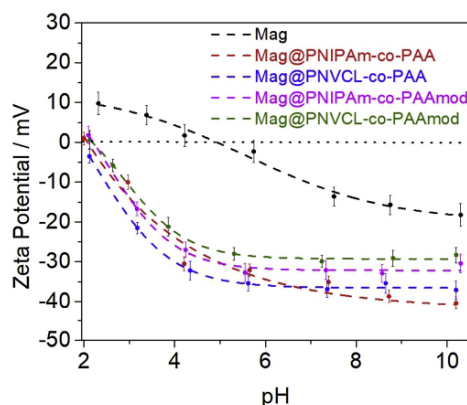


Fig. 7. Zeta potential of iron oxide nanoparticles with and without copolymers coating in function of pH value.

esterification. Such reduction was expected since after esterification the carboxylic groups of acrylic acid would be forming esters and then decreasing the formation of carboxylate anions in polymer chain as the pH value is increased. Although the esterification reaction has decreased the surface charge, there is still negative charge in the surface due the persulfate groups in the end of the polymer chain (Fig. 7).

3.3. Polymer composition and molecular weight

Polymers prepared in the molar ratio of 2:1 mol of acrylic acid to thermosensitive polymer monomer (N-isopropylacrylamide or N-vinylcaprolactam) were subjected to elemental analysis (Table 2) of carbon, nitrogen and hydrogen to verify the proportion. The composition obtained in the CHN elemental analysis for all polymers are very close to the theoretical composition of the repeating unit. This result indicate that the copolymer is in the molar ratio established in the experimental procedure. It can also be noticed that only 50% of carboxylic groups were esterified with 1-pentanol. The yield of the esterification was approximately 50% and basically there are still available carboxylic acid groups. This measure agrees with Zeta Potential analysis suggesting that negative charges of deprotonated carboxylic groups still in the polymer chain.

Fig. 8 shows PNIPAm-co-PAA and PNIPAm-co-PAA have 3 major

molar mass distribution considering peaks a, b, c, d, e and f. Table 3 shows that the esterification processes of PNIPAm-co-PAA has increased peaks molecular weight and polydisperse index compared PNIPAm-co-PAAmod. It was not possible to calculate molar mass in peak d due to calibration curve extrapolation. This data is a very strong evidence that the acrylic acid carboxyl group was esterified. For PNVCL-co-PAA and PNVCL-co-PAAmod the results are also similar (Fig. 8) (Table 3).

As can be seen from Table 3 the PNIPAm-co-PAA copolymer has 3 groups of peaks identifying 3 distinct molecular weight sizes (a - 240000 Da, b - 41060 Da and c - 8230 Da). After the esterification, the PNIPAm-co-PAA mod copolymer showed increase of the molecular mass at all peaks (d-out of the range of the calibration curve, e - 207900 Da and f - 16040) indicating that the 1-pentanol insert was in fact possible. An interesting factor to note is that the insertion of 1-pentanol into the PNIPAm-co-PAA chains resulted in increased polydispersity of the polymer chains. For the PNVCL-co-PAA copolymer the original peaks (g - 434450 Da and h - 149500 Da) showed increase after esterification, so the same conclusion leads for PNVCL-co-PAA and PNVCL-co-PAAmod.

3.4. DLS and temperature influence

The thermosensitive capacity of the functionalized magnetic

Table 2

Comparison of theoretical chemical composition and composition by elemental analysis. The modified copolymers PNIPAm-co-PAAmod and PNVCL-co-PAAmod were calculated whereas 1-pentanol molecule was esterified in the repeat unit.

	Chemical Element	Theoretical composition / %	CHN Elemental analysis / %
PNIPAm-co-PAA 1:2 ($H_{20}C_{12}O_6N$)	Carbon	52,57	50,02
	Nitrogen	5,11	6,56
	Hydrogen	7,30	6,63
PNIPAm-co-PAAmod 1:2 ($H_{20}C_{12}O_6N$) *Only one molecule of 1-pentanol in the polymer chain repetition	Carbon	59,32	57,15
	Nitrogen	4,07	5,26
	Hydrogen	8,72	7,89
PNVCL-co-PAA 1:2 ($H_{19}C_{14}O_6N$)	Carbon	56,58	53,98
	Nitrogen	4,71	5,24
	Hydrogen	6,40	6,01
PNVCL-co-PAAmod 1:2 ($H_{20}C_{14}O_6N$) *Only one molecule of 1-pentanol in the polymer chain repetition	Carbon	62,14	59,22
	Nitrogen	3,81	4,44
	Hydrogen	7,90	7,12

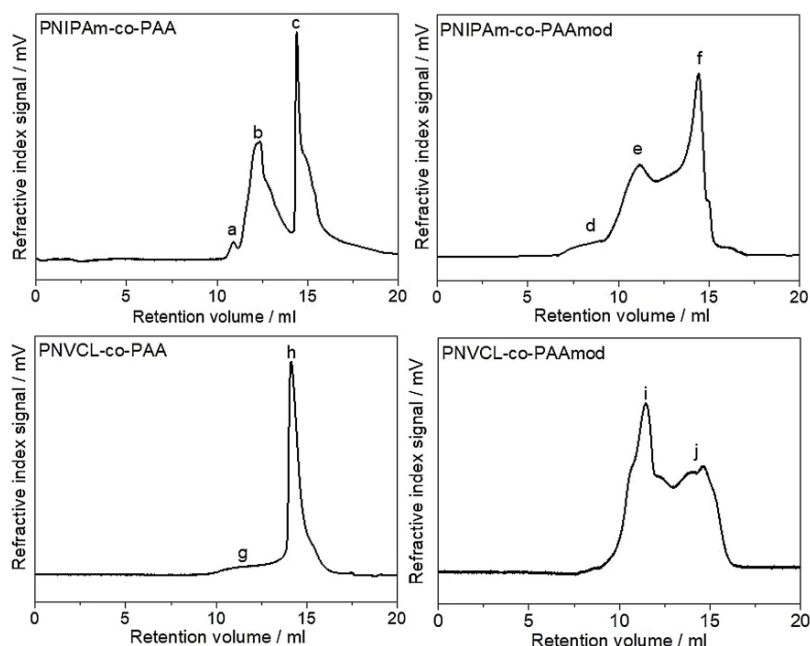


Fig. 8. Chromatogram of the polymers PNIPAm-co-PAA, PNIPAm-co-PAAmod, PNVCL-co-PAA, PNVCL-co-PAAmod. Eluent N,N – Dimethylformamide. Columns temperature 60 °C. The peaks a to j are analyzed in Table 3.

Table 3

Molar mass determination for each peak of Fig. 8. Software used to peak analysis: Omini SEC v. (5.1).

Copolymer	Peak	Mn / Da	Mw / Da	PDI
PNIPAm-co-PAA	a	218700	240000	1,10
	b	30400	41060	1,35
	c	7800	8230	1,06
PNIPAm-co-PAAmod	d	–	–	–
	e	93230	207900	2,23
	f	14350	16040	1,12
PNVCL-co-PAA	g	145680	434450	2,98
	h	13250	14950	1,13
PNVCL-co-PAAmod	i	30480	41050	1,35
	j	13900	26360	1,90

nanoparticles was analyzed by dynamic light scattering as a function of temperature. As hydrodynamic diameter change, it is possible to determine the LCST of copolymers shells and evaluate if the esterification modifies this property. Fig. 9 shows the variation of the hydrodynamic diameter of the particles as a function of temperature. (Fig. 9)

Fig. 9 shows that PNIPAm-co-PAA presents the lower critical solution temperature (LCST) close to 42 °C and it agrees with the purpose of this work that would reach 40 °C considering a higher amount of acrylic acid in the polymer composition. The 1-pentanol esterification modification of PNIPAm-co-PAA decreased the transition temperature to 37 °C. This result was expected, since hydrophobic groups (pentane) were added to the polymer chain, decreasing the hydrophilic character, decreasing interactions with the solvent (water). Although LCST of the copolymer PNIPAm-co-PAA mod has decreased compared to PNIPAm-co-PAA it is still valid for future applications. The PNVCL-co-PAA

copolymer did not show a defined transition but a transition interval close to 36 °C. For PNVCL-co-PAAmod the transition temperature interval decreased to 27 °C and it agree with the addition of hydrophobic groups in polymer structure. Comparing PNIPAm-co-PAA and PNIPAm-co-PAAmod the hydrodynamic diameter decreased nearly to the same values after the LCST, showing that esterification in the polymer chain with 1-pentanol do not changes the size of polymer shell. The shrinking effect of polymer shell for PNIPAm-co-PAA and PNIPAm-co-PAAmod is near 45 nm and for PNVCL-co-PAA and PNVCL-co-PAA is near 114 nm.

3.5. UV-VIS and drug release

Table 4 clearly shows that the samples exhibit different behavior since functionalized samples with PNIPAm-co-PAA after esterification are less able to incorporate methotrexate and PNVCL-co-PAA after esterification there is a decrease in the drug loading. A hypothesis for such behavior can be justified by the structures and hydrophobicity since PNIPAm-co-PAA has a major hydrophilic structure and allows the solution to diffuse easily between its chains, but when the esterified carboxylic groups are replaced by pentane groups and becomes an amphiphilic system. Such an amphiphilic system may hinder solvent diffusion and therefore decrease drug uptake.

For the PNVCL-co-PAAmod the case is very similar to the PNIPAm-co-PAAmod, since the non-polar groups of PNVCL interfere in the diffusion of the solvent. This is probably due to the formation of structures that protect the hydrophobic groups forming hydrophilic barriers that prevent entry of methotrexate into the structure since it is in water. With PNVCL-co-PAAmod the incorporation of MTX decreased due to the formation of a major non-polar structure is responsible for preventing the entry of methotrexate solution.

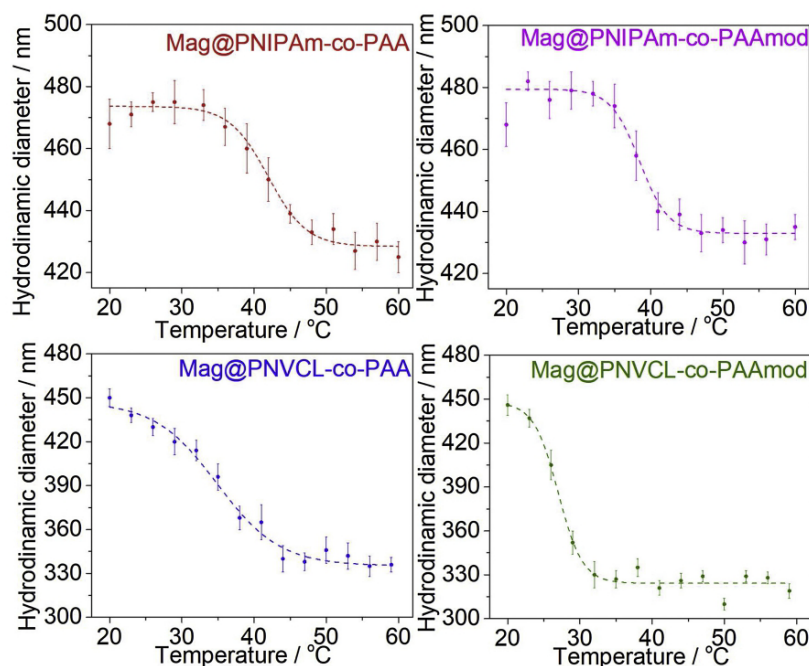


Fig. 9. Temperature dependence of polymer-coated iron oxide nanoparticles hydrodynamic diameter suspended in water measured by DLS.

To identify the released capacity of methotrexate, the samples were subjected to drug release test as a function of time. Fig. 10 represents the percent methotrexate release as a function of time for two different temperatures in phosphate buffer solution pH 7.4 (Fig. 10).

As can be seen from Fig. 10, the samples have a higher release percentage at 43 °C compared to 37 °C and fully agree with the purpose of the work which would be to promote distinct release rates at different temperatures. The effect of shrinkage of the structure is primarily responsible for the high release capacity at 43 °C, since the fully collapsed structure of the polymers expels much of the solvent (water) and simultaneously the drug (methotrexate). Another important characteristic is that the amphiphilic structure PNIPAm-co-PAAmod presented a higher release rate in 43 °C when compared to major hydrophilic PNIPAm-co-PAA. This characteristic come from the hydrodynamic diameter decrease since in 43 °C PNIPAm-co-PAAmod is almost fully collapsed with a lower hydrodynamic diameter compared to PNIPAm-co-PAA and possibly the most part of the solvent with MTX was released. For PNCVL-co-PAA it is possible to see that the modification slightly decreased the release of MTX, but both PNVCL-co-PAA and PNVCL-co-PAAmod in 43 °C are fully collapsed and there is no distinction in the hydrodynamic diameter for both samples. In that case

the main reason must be the capability of MTX diffusion in the collapsed structure, since a major non-polar structure like PNVCL-co-PAAmod probably hinder the MTX release in water.

4. Conclusion

The esterification of copolymers presents an alternative form to modify thermoresponsive polymers anchored in the surface of magnetic nanoparticles. The magnetic nanoparticles surface modified with polymer can heat until desirable temperature for further biomedical application, through alternating magnetic field (hyperthermia). The Zeta potential measurements showed a minimal modification in the surface charge of these polymers evidencing an incomplete esterification of the carboxyl groups of the copolymers PNIPAm-co-PAAmod and PVCL-co-PAAmod. There was a decrease in the LCST of the copolymers in the surface of the nanoparticles after esterification. The shrinking process of the nanoparticle act as main processes in methotrexate release for nanoparticles covered with PNIPAm-co-PAA e PNIPAm-co-PAAmod 43 °C. The same effect was not showed in PNVCL-co-PAA and PNVCL-co-PAAmod since in 43 °C both structures are completely collapsed. As confirmed it is possible to modify copolymers in the surface

Table 4

Incorporated methotrexate in iron oxide nanoparticles with copolymers shell after 24 h of incubation in 10 °C.

Materials	MTX mass incorporated / mg	Encapsulation efficiency ($m_{\text{incorporated}}/m_{\text{total}}$)*100	Incorporation coefficient ($m_{\text{incorporated}}/m_{\text{iron oxide}}$)*100
Mag@PNIPAm-co-PAA	3,72	41,3	37,2
Mag@PNVCL-co-PAA	3,32	36,9	33,2
Mag@PNIPAm-co-PAAmod	2,83	31,5	28,3
Mag@PNVCL-co-PAAmod	2,18	24,3	21,8

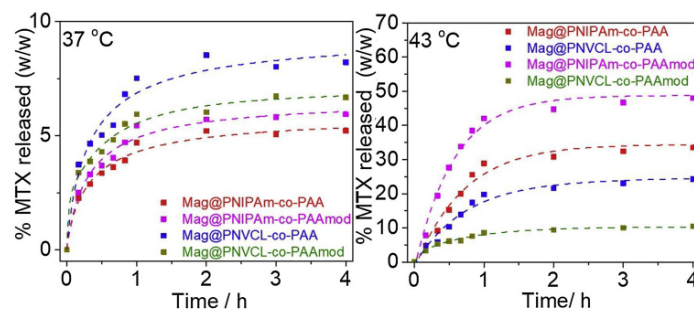


Fig. 10. Methotrexate release (MTX) for the nanoparticles modified in 37 °C and 43 °C.

of magnetic nanoparticles and the results showed good improve in the release capability of the drug methotrexate.

Acknowledgments

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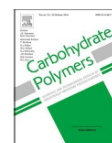
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Research Paper

Magnetic nanohydrogel obtained by miniemulsion polymerization of poly (acrylic acid) grafted onto derivatized dextran



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ABSTRACT

This study describes the synthesis of magnetic nanohydrogels by miniemulsion polymerization technique. Dextran was derivatized by the glycidyl methacrylate to anchor vinyl groups on polysaccharides backbone, allowing its use as a macromonomer for miniemulsion polymerization, as confirmed by proton nuclear magnetic resonance spectroscopy (¹H NMR). Magnetite nanoparticles were synthesized by coprecipitation, followed by air oxidation to maghemite. The results of X-ray diffractometry (XRD), Raman and transmission electron microscopy (TEM) analysis showed that maghemite nanoparticles were obtained with a diameter of 5.27 nm. The entrapment of iron oxide nanoparticles in a dextran nanohydrogel matrix was confirmed by thermogravimetric analysis (TGA), scanning transmission electron microscopy (STEM) and Zeta potential data. The magnetic nanohydrogels presented superparamagnetic behavior and were colloidal stable in physiological during 30 days. Our findings suggest that the synthesized magnetic nanohydrogel are potential candidates for use in drug delivery systems due to its physicochemical and magnetic properties.

1. Introduction

In the last decade, the use of polymers nanoparticles (Biswas, Kumari, Lakhani, & Ghosh, 2015; Karami, Sadighian, Rostamizadeh, Parsa, & Rezaee, 2016; Lu & Park, 2013; Mandal et al., 2013; Masood, 2015; Ta, Convertine, Reyes, Stayton, & Porter, 2010) (Easo & Mohanan, 2013; Hervault & Thanh, 2014; Laurent et al., 2008; Pankhurst, Thanh, Jones, & Dobson, 2009) as platform for bioactive molecules have attracted attention due to their potential in targeting tumor tissues through passive delivery via enhanced permeability retention (EPR) effect (Bertrand, Wu, Xu, Kamaly, & Farokhzad, 2014). Polymer nanoparticles show high colloidal stability in addition to its versatility to retains bioactive molecules and delivery it when stimulated, increasing the biodistribution and avoiding premature drug delivery. (Ganguly, Chaturvedi, More, Nadagouda, & Aminabhavi, 2014; Hoare & Kohane, 2008; Peppas, 1997).

Polysaccharides show some advantages over synthetic polymers once they are abundant and obtained from renewable sources (Coviello, Matricardi, Marianecci, & Alhaique, 2007). Polysaccharides are

biocompatible, biodegradable, non-toxic and have free functional groups that can be used to modify their structure and/or anchor bioactive molecules, such as proteins, antibody and drugs (Dias, Hussain, Marcos, & Roque, 2011; Liu, Jiao, Wang, Zhou, & Zhang, 2008). Dextran is a suitable polysaccharide to prepare nanohydrogels and consists, predominantly, of α -1,6-glucosidic linkage, with some degree of branching in 1,3-linkage. The dextran-based hydrogel is obtained by derivatization of its structure with vinyl groups, which can be polymerized with acrylic acid to control the crosslinking degree and pH-responsive behaviour (Medeiros, Santos, Fessi, & Elaissari, 2011). The use of nanohydrogels as drug carrier allows a greater drug load by the circulatory system, avoiding the chemical and enzymatic degradation before drug reach the targeted tissue (Ganguly et al., 2014; Iyer, Singh, Ganta, & Amiji, 2013; Liu et al., 2008; Wang et al., 2017). In addition to being capable to retain drug for a long circulation period, its desirable to guide the platform direct to the targeted site in the body, improving the therapy efficacy (Wassel, Grady, Kopke, & Dormer, 2007). To achieve this aim, nanohydrogels were supported onto

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magnetic iron oxide nanoparticles, allowing it to be driven by an external magnetic field to specific site.

Iron oxide nanoparticles are also of great interest in biomedical applications such as hyperthermia, magnetic resonance imaging and drug delivery, due to their properties such as superparamagnetism, high surface to volume area, biocompatibility, and nontoxicity. In order to avoid particle agglomeration towards physiological conditions during use in the afore mentioned applications, the surface of superparamagnetic iron oxide nanoparticles (SPION) should be functionalized with suitable molecules such as carboxylic acids (Lattuada & Hatton, 2007; Petri-Fink, Chastellain, Juillerat-Jeanneret, Ferrari, & Hofmann, 2005; Turcheniuk, Tarasevych, Kukhar, Boukherroub, & Szunerits, 2013), aminoacids (Durmus et al., 2011; Gholami, Rasoul-amini, Ebrahiminezhad, Seradj, & Ghasemi, 2015), and polymers, (e.g., dextran, chitosan, poly(ethylene glycol), etc.) (Arruebo et al., 2007; Durmus et al., 2011).

The main contribution of this investigation was the magnetic nanohydrogels synthesis by miniemulsion polymerization. This polymerization method shows particular features during the nucleation process, which results in nanoreactors formed by droplets with a limited volume of reaction (Luo, Dai, & Chiu, 2009; Mittal, 2011). The size of the resultant magnetic nanohydrogels should be comprised between 50 and 500 nm (Mittal, 2011), being suitable to passively targeting to the tumor cells (Bertrand et al., 2014). The maghemite nanoparticle was surface functionalized with acrylic acid for further encapsulation with a polymeric matrix composed of derivatized dextran and acrylic acid. The magnetic nanohydrogels were obtained through vinyl polymerization with different amounts of functionalized iron oxide. To the best of our knowledge, this is the first work describing the use of magnetic nanoparticles surface modified with derivatized dextran to be cross-linked in nanohydrogels using the miniemulsion polymerization instead of classical macroscopic hydrogels.

2. Materials and methods

2.1. Materials

All chemicals were used as received. Iron(III) chloride hexahydrate (97%), Heptane (98.5%), and ammonium persulfate (APS) were purchased from Mallinckrodt Chemicals. Dimethyl sulfoxide (DMSO, 99%) and 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS) were purchased from MERCK. Sodium hydroxide (NaOH, 97%) was purchased from Synth. Acrylic acid (AA, 97%) and 4-(dimethylamine) pyridine (DMAP, 99%) were purchased from Alfa Aesar. Iron(II) chloride tetrahydrate (99%), dextran (MW 40 kDa), glycidyl methacrylate (GMA, 97%), *N,N'*-methylenebisacrylamide (99%), sorbitan monooleate (Span® 80), phosphate buffered saline (0.01 mol L⁻¹ phosphate, 0.135 mol L⁻¹ NaCl and 0.002 mol L⁻¹ KCl) buffer solution (PBS) were purchased from Sigma-Aldrich Brazil.

2.2. Dextran derivatization

The dextran was modified through reaction with glycidyl methacrylate as described by van Dijk-Wolthuis et al. (Hoof & Hennink, 1997; Steenberg, Bosch, & Hennink, 1995). The used molar ratio of dextran:GMA:DMAP was 1:0.5:0.25 with respect to 1.0 mol of glycosidic unit. Dextran (1.5 g) and DMAP (2.4 mmol) were dissolved in 30.0 mL of DMSO in a three-neck round bottom flask under N₂ atmosphere and magnetic stirring. After complete dissolution, the system was heated up to 45 °C, then 4.8 mmol of GMA was injected into the flask, the temperature and stirring were kept for 24 h. The reaction was stopped by adding an equimolar amount of HCl to neutralize DMAP. The dextran modified polymer (Dex-GMA) was precipitate with acetone and dialyzed at 8 °C. The product was freeze-dried and a white powder was obtained.

2.3. Synthesis of SPION and functionalization with acrylic acid

The synthesis of iron oxide nanoparticles was performed by coprecipitation of Fe²⁺ and Fe³⁺. A solution containing 0.04 mol of FeCl₂·4H₂O and 0.08 mol of FeCl₃·6H₂O dissolved in 500 mL of deionized water was added drop-wise into 500 mL of 1.5 mol L⁻¹ NaOH solution under mechanical stirring (2000 rpm) and constant bubbling on N₂ gas at room temperature. A black precipitate formed instantly and after 20 min of reaction the solid was magnetically decanted and washed three times with deionized water. The SPION were suspended in water and the pH was adjusted to 3.5 with 1.0 mol L⁻¹ HCl solution. The suspension was heated in a boiling water bath under constant magnetic stirring and air bubbling during 3 h. The reddish brown dispersion was dialyzed against water for 7 days and stored for further use (Massart, 1981; Viali et al., 2010). The SPION concentration of 54.5 g L⁻¹ was obtained.

The surface functionalization of maghemite nanoparticles with acrylic acid was performed by adding 2.0 mL of 0.10 mol L⁻¹ acrylic acid solution in 3.0 mL of SPION suspension, at pH 4.0 (Nunes, Lemos, & Carneiro, 2013). The acrylic acid adsorption was performed under continuous stirring for 48 h at room temperature and then the free acrylic acid was removed by dialysis. The resulting dispersion was labeled as Magh-AA and used in further experiments.

2.4. Synthesis of magnetic nanohydrogels

The magnetic nanohydrogels were synthesized by inverse miniemulsion polymerization by using acrylic acid and derivatized dextran as monomers, in the ratio of 35% and 65% (w/w), respectively. A Span® 80 solution in *n*-heptane (4% (w/w), 40 mL) and a solution containing Magh-AA (different amounts of 50, 100, and 150 mg), AA (100.0 mg), Dex-GMA macromonomer (200.0 mg), NaOH (1.12 mmol), MBA (0.097 mmol) and APS (0.22 mmol) dissolved in 3.0 mL of deionized water was homogenized in a Turrax stirrer at 20000 rpm for 2 min and then submitted to an ultrasound probe for 30 min, an ice bath was used to avoid initiation of polymerization. Afterward, 16 mg (0.15 mmol) of sodium bisulphite were added under ultra-sonication. Sodium bisulphite and ammonium persulfate act as pair redox to decrease the temperature of thermodecomposition of initiators (Pohl & Rodriguez, 1981). The miniemulsion was transferred to a three-neck flask under magnetic stirring, purged for 15 min with Argon flux, and then heated up to 50 °C for 4 h. After cooling to room temperature, the magnetic nanohydrogels were removed by centrifugation at 10000 rpm and washed thrice with hexane. The particles were dispersed in a 0.5% Tween® 80 aqueous solution and dialyzed against water for 5 days.

2.5. Samples characterization

XRD powder diffraction of the samples was recorded in the 2θ range of 10–80° using the Siemens D5005 system equipped with a Cu Kα radiation source. The XRD diffractograms were used to check the crystalline phase of the SPION-based material as well as to estimate the average crystallite size, the latter performed by using the Scherrer's equation (Cullity, 1978). Raman scattering spectra were recorded at room temperature in a frequency range of 200–1000 cm⁻¹ from a HORIBA Jobin Yvon model LabRAM HR micro Raman apparatus equipped with a 632.8 nm laser delivering 0.6 mW power. The size and morphology of maghemite nanoparticles were investigated by transmission electron microscopy (TEM). Low magnification was obtained using a JEOL 3010 TEM-HR operating at 300 kV. For TEM measurement, a drop of the sample dispersed in isopropanol was deposited on a copper grid covered with carbon film. The morphology and size of nanohydrogels were investigated by transmission scanning electron microscopy (STEM) in a FEI Inspect F50 microscope. For STEM analysis, the samples were dispersed in isopropanol and deposited in a

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carbon coated copper grid. The images were obtained in secondary electrons, bright field and high-angle annular dark field modes (HAADF). The FT-IR measurements were carried out using a Bruker VERTEX 70 FT-IR spectrometer equipped with a diffuse reflectance infrared Fourier transform (DRIFT) collector accessory, using the system resolution set at 2 cm^{-1} , while performing 256 scans. ^1H NMR spectroscopy was carried out on a Varian INOVA 300 spectrometer measuring samples dissolved in deuterium oxide. Solid state ^{13}C NMR measurements were carried out on Bruker Avance III HD 400WB spectrometer. The powders were packed into 4 mm rotors a spun at speeds of 10000 Hz, at fixed contact time of 2 ms. The deconvoluted spectral components were obtained using Voigt profile. Thermogravimetric analyses (TGA) were carried out in STA 409C/CD system DTA-TGA from NETZSCH Instruments. Samples (15 mg) were analyzed from room temperature up to $800\text{ }^\circ\text{C}$ under 50 mL min^{-1} air flow, using a heating rate of $10\text{ }^\circ\text{C min}^{-1}$ to estimate the net weight of the SPION in the magnetic nanohydrogel. Hydrodynamic diameter and zeta potential of nanoparticle samples were measured using a Zetasizer Nanoseries ZSNano ZEN3600 from Malvern Instruments. Hydrodynamic diameter was measured by dynamic light scattering (DLS), which samples were dispersed in water to size distribution, in NaCl 1 mmol L^{-1} for pH dependence curve and in phosphate-buffered saline (PBS) or Tris buffer solution (pH 7.4) to colloidal stability measurements. For zeta potential measurements the samples were previously dispersed in NaCl 1 mmol L^{-1} solution. Magnetization measurements were performed in powder form, using a commercial Physical Property Measurement System (PPMS) model 6000 platform with the vibrating sample magnetometer (VSM) module from Quantum Design. Hysteresis loops (M-H curves) were recorded in the range of -20 to 20 kOe , at temperatures of 300 K . Zero-field-cooled (ZFC) and field-cooled (FC) curves were carried out in temperature range from $-268\text{ }^\circ\text{C}$ to $27\text{ }^\circ\text{C}$ and applying a DC magnetic field of 30 Oe .

3. Results and discussion

3.1. Characterization of dextran macromonomer

The polysaccharide dextran chains were modified by grafting methacrylate groups, through the reaction with GMA. The DMAP act as a Lewis base and induces polarization of hydroxyl groups of dextran, allowing grafting of methacrylate groups in the polymer backbone (Lo & Jiang, 2010). The dextran derivatization can occur by two mechanisms: epoxy ring opening or transesterification, as show in Fig. S1 on supplementary information. In epoxy ring opening mechanism, the methylene carbon of the GMA undergoes a nucleophilic attack by hydroxyl groups of dextran, while the transesterification results in the attack at carbonyl ester of GMA. DMSO, an aprotic polar solvent, was used in reaction environment to avoid reactions of GMA with water (Hoof & Hennink, 1997; Steenbergen et al., 1995). Both mechanisms results in the methacrylate groups grafted to dextran chain. The derivatization was evaluated by the ^1H NMR spectroscopy (Fig. 1A) and solid state ^{13}C NMR spectroscopy (Fig. 1B). From ^1H NMR results, the signals between 3.10 ppm and 5.20 ppm corresponding to the dextran chain protons. The anomeric proton of the glucopyranosyl ring has the signal shifted from the others protons at 4.91 ppm . The peak at 5.20 ppm corresponds to α -1,4 linkage among dextran units. The presence of methacrylate groups was confirmed for Dex_GMA sample. The double doublet signals in 6.19 ppm and 5.70 ppm correspond to protons of vinyl group. The single peak of methyl protons is observed at 1.90 ppm . The degree of substitution (DS) of GMA on dextran can be calculated through ^1H NMR spectrum, applying the equation $\text{DS} = 100x/y$, which x corresponds to the average integral of the vinyl doublets and y is the integral of anomeric proton plus 4% of α -1,4 linkages (Steenbergen et al., 1995). The derivatization reaction presented a yield of 80.0% in mass of polymer and DS of 28.5% . Derivatized dextran act as macromonomer, allowing further polymerization process.

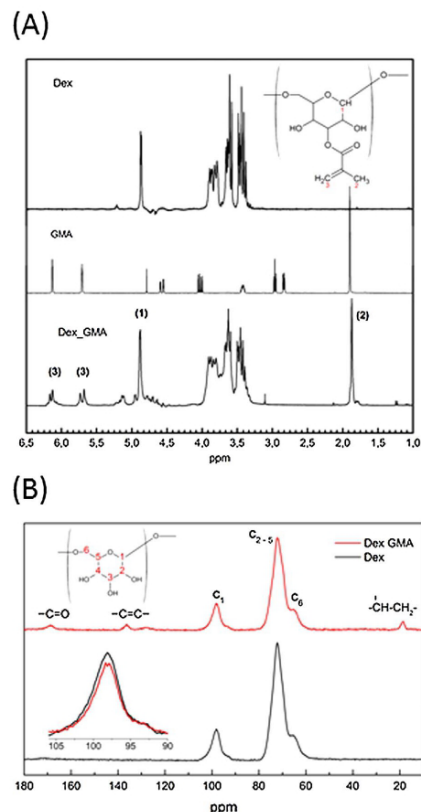


Fig. 1. (A) ^1H NMR spectra of dextran, glycidyl methacrylate (GMA) and dextran derivatized. (B) Solid state ^{13}C NMR spectra of dextran and dextran derivatized.

The ^{13}C NMR spectra of dextran sample is show in Fig. 1B. The peak at 98.09 ppm correspond to anomeric carbon (C_1), while the carbons ($\text{C}_2\text{-C}_5$) connected to hydroxyl groups show a signal at 72.15 ppm . The carbon (C_6) from $-\text{CH}_2$ group of glycoside unit is assign to 65.46 ppm (Seymour, Knapp, & Bishop, 1976). The derivatized sample show additional peaks besides from pure dextran, which correspond to carbon atom from carbonyl group at 168.72 ppm . The peaks at 136.28 and 128.26 are attributed to the carbons from vinyl groups, respectively, while the signal at 18.58 is due to $\text{CH}_2\text{-CH}$ group. Moreover, it is possible noted two peaks at 97.88 and 98.38 ppm on derivatized sample near to anomeric carbon instead of only one signal. This shift indicated that the hydroxyl groups of C_1 participated in the derivatization reaction (Zhang et al., 2014). The peak at $74\text{-}65\text{ ppm}$ was deconvoluted to Dex and Dex GMA samples, as can be seen in Fig. S2, which result in shift of peaks positions, indicating that the derivatization reaction also occurs by the hydroxyls bound to these carbons.

3.2. Characterization of SPION functionalized with acrylic acid

The black precipitate of magnetite was obtained through the addition of sodium hydroxide to a solution of ferric and ferrous chloride, in a molar ratio of $2:1$. The aqueous suspension of the magnetite was

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directly oxidized by aeration to form a brownish suspension of maghemite.

XRD analysis (Fig. S3(A)) of iron oxide sample Magh was indexed in the inverse spinel structure (Fd3m), in agreement with the protocols used to produce the superparamagnetic iron oxide SPION-based materials. The identification of structure of Magh sample was based on the Bragg Peak Position of (1 1 1), (2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1) and (4 4 0) indices. According to the International Centre for Diffraction Data (JCPDS Card N° 39-1346), the features exhibited by Magh sample can be assigned to the maghemite structure. Broadening of the X-rays diffraction peaks is an indicative of the nanocrystalline nature of the synthesized powder (Cullity, 1978). The average X-rays crystallite diameter (D_{XRD}) calculated by Scherrer's equation was 6.2 nm and is in good agreement with electron microscopy data.

The Raman spectrum of SPION samples is shown in Fig. S3(B) from 200 to 1000 cm^{-1} which vibrational modes are associated with maghemite crystal structure. Typical Raman spectrum of maghemite is characterized by three main broad features, while magnetite shows only one feature broad structure, attributed to the A_{1g} vibrational mode (Jubb & Allen, 2010). The Magh spectrum showed three bands at 345 cm^{-1} (E_g), 501 cm^{-1} (T_{2g}), and 671 cm^{-1} (A_{1g}) assigned to modes associated with tetrahedral iron sites, and one at 718 cm^{-1} assigned to the octahedral iron sites (Soler et al., 2011).

Transmission electron microscopy was performed in order to access the Magh sample average particle size and morphology. It can be observed in Fig. 2(A) the TEM image of synthesized SPIONs with nearly spherical shape. The Fig. 2A inset shows particle size histogram of sample Magh obtained from the TEM micrographs. This data was fitted to a log-normal distribution and results in average particle diameter (D_{TEM}) of 5.27 ± 0.05 nm and polydispersity index (PDI) of 0.21 ± 0.01 . The size distribution was also measurement by dynamic light scattering for Magh bare sample, which result in average hydrodynamic diameter of 48.50 ± 40 nm and PDI of 0.23 ± 0.01 , as show in Fig. 2(B). This value is higher than shown in TEM images due to electric double layer, which is involved during DLS measurement (Easo & Mohanan, 2013).

The DRIFT analysis was used to confirm the functionalization of Magh nanoparticles by acrylic acid. As show in Fig. S4, the DRIFT spectrum illustrates the characteristics infrared absorption bands of Magh bare and Magh-AA. The bands at 580 and 430 cm^{-1} correspond to Fe–O stretching vibration modes. The 3430 and 1600 cm^{-1} bands at the Magh bare can be assigned to –O–H stretching and bending vibrations, respectively, due to surface hydroxyl groups and water molecules adsorbed on the SPION surface (Cornell & Schwertmann, 2003; Nakamoto, 1970). The surface modification through acrylic acid addition was confirmed by the bands present at 1630 cm^{-1} assigned to carboxylate asymmetric and at 1434 cm^{-1} to symmetric stretching vibrations. The weak band at 2911 cm^{-1} correspond to methine stretching of acrylic acid. It was not possible to assign the C=C stretching of the vinyl group because it shows weak absorption at 1670–1640 cm^{-1} , the same region for hydroxyl groups of SPION surface (Pavia, Lampman, & Kriz, 2001).

3.3. Characterization of magnetic nanohydrogels

The magnetic nanohydrogels were obtained through inverse miniemulsion polymerization. In miniemulsion polymerization the droplet nucleation is the dominant mechanism of particle formation, which the monomer droplet being considered as a template for nanohydrogel formation, i.e. the size of nanohydrogel should be similar to the initial monomer droplet size (Asua, 2002; Gyergyek, Makovec, Mertelj, Huskić, & Drofenik, 2010; Luo et al., 2009). The dynamic light scattering technique was used to evaluate the nanohydrogels size distribution. The z-average sizes are summarized in Table S1 (size distribution profile is showed in Fig. S5). The hydrodynamic diameter distribution of nanohydrogels is in the range between 100 and 400 nm.

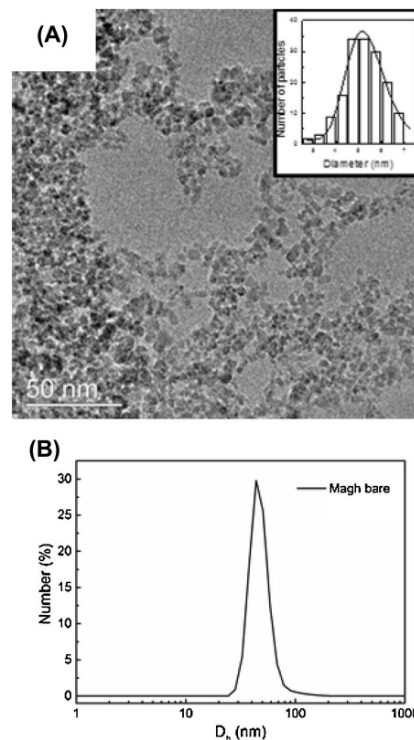


Fig. 2. (A) TEM micrograph of Magh bare sample. The insert show particle size histogram, where vertical bars represent the experimental data whereas the solid line results from the curve fitting of the data using the log-normal distribution function. (B) Average hydrodynamic diameter distribution of Magh bare sample from DLS measurements.

It can be seen that size distribution of monomer droplet, before polymerization step, was comparable to polymer nanohydrogels sizes. These results suggest that nanohydrogels are formed in a miniemulsion polymerization process.

According to FTIR spectra showed in Fig. 3(A), the stretching vibrations of Fe–O bond were observed in the same wavenumber of Magh bare sample. Added to SPION absorptions bands, the characteristics of nanohydrogel correspond to the asymmetric and symmetric stretching at 2923 cm^{-1} and 2852 cm^{-1} , respectively, that are assigned to C–H vibrations mode of dextran. The bands at 1463 cm^{-1} and 1353 cm^{-1} were attributed to methylene and methyl bending absorptions. The polysaccharides feature of C–O–C correspond to 1100 cm^{-1} stretching. The band exhibited at 1739 cm^{-1} is attributed to carbonyl group of derivatized dextran and PAA (Pavia et al., 2001).

Fig. 3(B) shows the XRD pattern of magnetic nanohydrogels. The presence of main diffraction peaks of magnetite, according to JCPDS Card N° 39-1346, confirm that any structural change occurs during polymerization step, excluding the formation of other types of iron oxides. The width of diffraction peaks was broadened for magnetic nanohydrogels samples if compared with Magh bare sample in Fig. S4(A).

Fig. 4 shows the macroscopic and scanning transmission electron microscopy (STEM) images of the magnetic nanohydrogels. Column (A)

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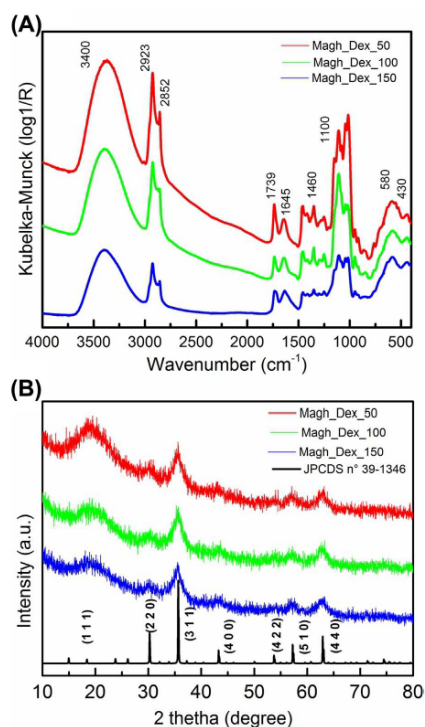


Fig. 3. (A) DRIFT spectra and (B) XRD patterns of magnetic nanohydrogel.

Table 1
Summary of TGA data analysis on evaluation of SPION encapsulation.

SPION	Weight loss (%)		SPION residue (%)	SPION/polymer
	Step I	Step II		
Magh-bare	9.23	–	87.04	–
Magh_Dex_50	11.39	83.9	4.71	0.056
Magh_Dex_100	9.14	81.8	9.06	0.110
Magh_Dex_150	9.84	81.0	9.16	0.113

show the suspended magnetic nanohydrogels in water in the absence of magnetic field, while in column (B) the samples were attracted to the magnet. The secondary electron image (column C) shows that the nanohydrogels particles have aggregated in a globules form. These aggregates were formed during drying process of nanohydrogels suspension. The encapsulation and distribution of iron oxide inside dextran-based nanohydrogels was evidenced by high-angle annular dark-field (HAADF) and bright field images (Column D and E). The magnetic nanoparticles correspond to the black areas in the bright field image and to the bright areas in the HAADF image.

The amount of SPION encapsulated by nanohydrogels was evaluated by TGA, in the range of 25–800 °C, as showed in Fig. S6. The Magh-bare sample shows only one step of 9.23% weight loss assigned to adsorbed water up to 130 °C. For magnetic nanohydrogels samples, two steps of weight loss were observed. The first step was associated with adsorbed water weight loss, in agreement with the Magh-bare sample. The second step, which starts at 165 °C and ends at 340 °C, was due to the decomposition of polymeric chains (Carp et al., 2009; Juríková, Csach, Koneracká, Kubov, & Kop, 2012). Table 1 shows the weight loss attributed to each TGA event, the iron oxide residue and the mass ratio of SPION per polymer content. The weight ratio among magnetic core content and nanohydrogels increased from 0.056 (sample Magh_Dex_50) to 0.113 (sample Magh_Dex_150). The results show that the sample Magh_Dex_100 reached the limit of encapsulation of SPION in dextran by inverse miniemulsion polymerization technique.

The magnetic properties of the samples were measured using magnetic hysteresis loop curves in the ± 20000 Oe window, at 27 °C, as showed in Fig. 5(A). The saturation magnetization values were

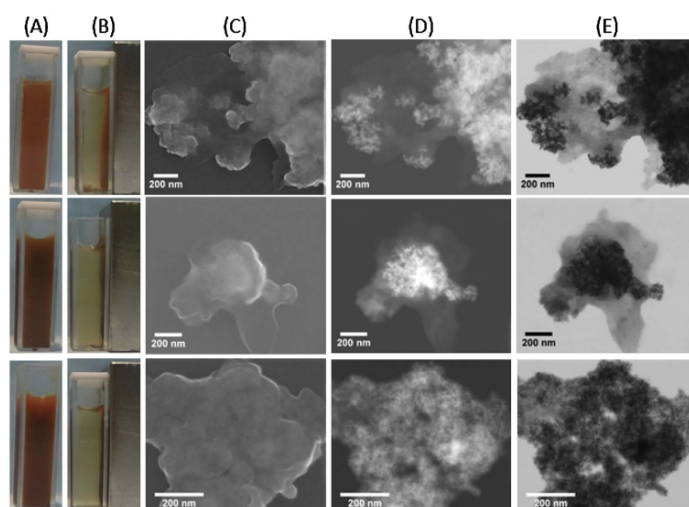


Fig. 4. Digital and STEM images of sample Magh_Dex_50 (upper) and Magh_Dex_150 (bottom): (A) Suspended magnetic nanohydrogel in absence of magnetic field, (B) in presence of magnetic field, (C) secondary electron image, (D) HAADF and (E) bright field images.

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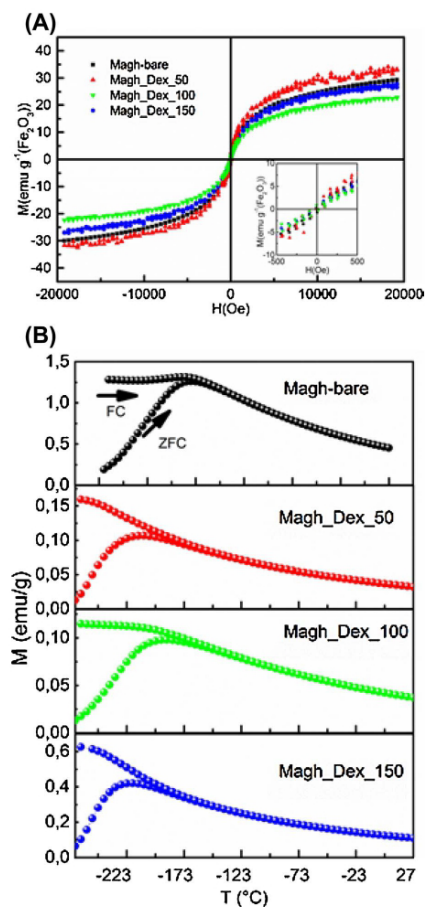


Fig. 5. (A) Magnetization versus applied field curves for Magh bare nanoparticles and magnetic nanohydrogels. The experimental data were normalized with respect to the iron oxide mass. The insert shows hysteresis loops near zero. (B) Zero-field-cooled (ZFC) and field-cooled (FC) curves as function of the temperature obtained with a DC magnetic field of $H = 30$ Oe.

normalized to the mass of iron oxide using TGA data (Medford et al., 2014). According to literature, the values of saturation magnetization for maghemite bulk were 83.5 emu g^{-1} (Cullity and Graham, 2009), while the Magh-bare sample this value decreased to 28.9 emu g^{-1} . Decrease in saturation magnetization values of SPION with respect to the saturation magnetization of bulk counterparts is often observed in nanoparticles and is attributed to the surface contribution of spin canting, surface disorder, stoichiometric deviation, cation distribution (Kodama, 1999) and adsorbed layer species (Zhang, Su, Wen, & Li, 2008). For Magh_Dex_50, Magh_Dex_100 and Magh_Dex_150 samples the values of saturation magnetization were 32.6 emu g^{-1} , 23.9 emu g^{-1} and 28.9 emu g^{-1} , respectively. Magnetic nanohydrogel based on P(NIPAm-co-AAc) synthesized by (Chou, Shih, Tsai, Chiu, & Lue, 2012) and (Fan, Li, Wu, Li, & Wu, 2011) showed different

values of saturation magnetization, whether compared with our nanoparticles, mainly due to the size of SPION which influence the magnetic properties (Iida, Takayanagi, Nakanishi, & Osaka, 2007). However, the SPION content is increased using miniemulsion polymerization method, which result in increased the sample magnetization.

The superparamagnetic behavior is a desired magnetic property to use magnetic nanoparticle as drug delivery device. On insert of Fig. 5(A), it can be seen that samples show no coercivity and remanence values when the applied magnetic field was removed, suggesting that the nanoparticles showed superparamagnetic behaviour at 27.0°C (Chou et al., 2012; Dou, Zhang, Jian, & Gu, 2010). To confirm that, zero field cooled (ZFC) and field cooled (FC) curves were obtained for all samples, as shown in Fig. 5(B). As observed, all samples show features consistent with a superparamagnetic behavior; i. e., a maximum in the ZFC trace and irreversible behavior between both traces below that maximum. Moreover, the position of the maximum (T_m) shows a dependence with the amount of coating. For the Magh bare sample the maximum is located at -178°C and that maximum is shifted to lower temperature for the magnetic nanohydrogel samples. This result strongly suggests that the particle-particle magnetic interactions are relatively stronger for the bare sample and those interactions become weaker as the amount of coating is increased.

3.4. Colloidal stability

Nanohydrogels were evaluated through zeta potential measurements in pH-dependence curve. The Magh-bare and magnetic nanohydrogels samples were measured in the constant ionic strength of 1 mmol L^{-1} NaCl, as showed in Fig. 6 (upper). The amphoteric features of SPION are due to ionization of surface hydroxyl groups. Thus, the adsorption or desorption of protons have a pH dependence. In acid medium, the surface is protonated and the zeta potential value is positive. On the other hand, the zeta potential is negative when the surface is deprotonated in basic solution. The isoelectric point (IEP) for Magh-bare was 8.2, which is in accordance with literature reports (Cornell & Schwertmann, 2003; Hajdú et al., 2012). The IEP for Magh AA sample was shift to 6.0. The magnetic nanohydrogels samples

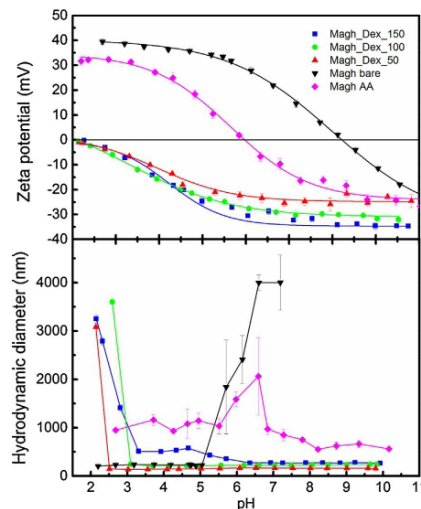


Fig. 6. pH dependence of zeta potential (upper) and pH dependence of hydrodynamic diameter (bottom) for Magh bare, Magh AA nanoparticles and magnetic nanohydrogels.

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Table 2
Magnetic nanohydrogels average hydrodynamic diameter/polydispersity index (PDI) measured in different buffers.

Sample	PBS (pH 7.4)		Tris.HCl (pH 7.4)	
	D _h (nm)/PDI t = 0 days	D _h (nm)/PDI t = 30 days	D _h (nm)/PDI t = 0 days	D _h (nm)/PDI t = 30 days
Magh_Dex50	259.2/0.230	273.6/0.220	212.0/0.256	234.5/0.244
Magh_Dex100	237.0/0.381	246.9/0.121	241.0/0.199	160.9/0.204
Magh_Dex150	527.3/0.295	218.5/0.205	611.5/0.428	248.9/0.424

Magh_Dex 50, Magh_Dex 100, and Magh_Dex 150 do not reach the IEP along of the pH range studied and show minimum zeta potential values at pH 2.0 of -0.16 mV, -0.47 mV and -1.04 mV, respectively. This behavior can be attributed to the carboxylic acid groups arising from the acrylic acid moieties present in the polymeric matrix and the sulfate groups from APS shear layer (Eissa et al., 2013). The zeta potential values were constants for magnetic nanohydrogels samples above pH 6.5.

Fig. 6 (bottom) shows the hydrodynamic diameter in the pH range from 2.0 to 10.0, measured at a constant ionic strength of 1 mmol L⁻¹ NaCl. The hydrodynamic diameter of Magh bare sample increase above pH 5.0. Although the potential zeta value indicate stability at this pH, no electrostatic or steric repulsion were predicted to this sample. Moreover, attractive dipolar magnetic force act in this sample, hindering the aggregates dispersion, until the instrument reached the limit of measurement (5 μ m). To Magh AA sample, an increase in hydrodynamic diameter was observed on isoelectric region, however, due to steric stabilization promoted by acrylic acid, the aggregation is not strong and the hydrodynamic diameter is restored. The magnetic nanohydrogels samples show an increase in the average size near pH 3.0, which causes particle coagulation due to decrease in the electrostatic repulsion between nanohydrogels caused by protonation of the carboxylate groups from the polymeric chains, as indicated in zeta potential curve (Fig. 6 upper). In fact, the magnetic nanohydrogels exhibit good colloidal stability and was observed no variation in hydrodynamic diameter over a range of pH between 6.5–10.0 where the zeta potential reaches constant values above -25.0 mV. In this pH range, the electrostatic repulsion is maximized due to complete deprotonation of the carboxylate groups.

The colloidal stability of the magnetic nanohydrogels was evaluated in buffer solution of tris.HCl and PBS, in pH 7.4. The hydrodynamic diameters were measured during a 30 days period, as showed in Table 2. The magnetic nanohydrogels were stabilized by steric repulsion promoted by the polymeric chains composed of dextran and the polyacrylate moieties. Thus, the magnetic nanohydrogels were stable over 30 days, once the hydrodynamic diameters did not show any significant increase over this period, except the sample Magh_Dex 150, in which the hydrodynamic diameter has increased to above 500 nm for both buffers solutions after the sample dispersion. This result may be related to the formation of aggregates after dispersion in buffer solution. After a period of 8 days, the aggregates sedimented and hydrodynamic diameter of the remained dispersed nanoparticles reach a steady state to 248.9 nm for tris.HCl and 218.5 nm for PBS.

4. Conclusions

Magnetic nanohydrogels based on grafting polymerization of acrylic acid and derivatized dextran were obtained by encapsulating SPION through inverse miniemulsion technique. XRD, Raman and TEM results corroborate with the formation of maghemite by coprecipitation method. These magnetic nanoparticles show spherical morphology, with diameter is 5.27 ± 0.05 nm. Dextran macromonomer was derivatized by grafting with methacrylate groups on polysaccharide backbone as confirmed by ¹H NMR and solid state ¹³C NMR. Magnetic

nanoparticles with desirable superparamagnetic behavior for biomedical applications were encapsulated by dextran nanohydrogels. The samples Magh_Dex 50 and Magh_Dex 100 resulted in stable dispersion in buffer solutions at physiological pH indicating its colloidal stability. The synergy between iron oxide nanoparticles and dextran nanohydrogel make this composite a good candidate for drug delivery systems

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.carbpol.2017.09.019>.

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The influence of pH, hydrolysis and degree of substitution on the temperature-sensitive properties of polyaspartamides

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Abstract

Temperature-sensitive polyaspartamide obtained by the aminolysis reaction of polysuccinimide with isopropylamine has been proposed as a good material for biomedical applications. The polymer is susceptible to hydrolysis producing carboxyl groups; thus the temperature-sensitive behavior of the polymer depends on pH. A set of polyaspartamides was synthesized with different degrees of hydrolysis (DH) and substitution (DS). The influence of DH, DS and pH on the lower critical solution temperature (LCST) of the polyaspartamides was studied. The LCST of the polyaspartamides increases as the pH increases and at the same pH the polymer with higher DH presents a higher LCST. Also, at the same pH an increment in the DS produces a decrease in the LCST. These tendencies are explained by the presence of a carboxylate group on the polyaspartamide structure. The behaviors can be tailored as functions of DS and DH enabling the use of temperature-sensitive polyaspartamide in different biomedical applications where pH and/or temperature changes will trigger a drug delivery.

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Keywords: temperature-sensitive polymer; pH-sensitive polymer; polyaspartamide; polysuccinimide; dual-sensitive polymer; stimuli-sensitive polymer

INTRODUCTION

In recent years, dual-sensitive polymers have received much interest due to their outstanding scientific and technological features mainly in multiple biomedical applications such as drug delivery, gene delivery, tissue engineering etc.^{1–4} The combination of pH- and temperature-sensitive properties in a unique platform allows physicochemical changes in polymers in biological media, such as variation in conformational structure and solubility, resulting in a suitable drug delivery system for oral administration once the drug loss through the gastrointestinal tract can be controlled by these properties.

In aqueous solution, temperature-sensitive polymers exhibit a volume phase transition at a critical temperature which causes an abrupt change in the solvated chains. This temperature is known as the lower critical solution temperature (LCST) and corresponds to the temperature at which the polymers become insoluble upon heating. Also, some polymers present an upper critical solution temperature above which the polymers become soluble upon heating. The LCST of a polymer can be finely tuned by copolymerization with a second monomer, where the incorporation of monomers with hydrophilic or hydrophobic domains will result in an increase or decrease respectively of the LCST value. According to the ionic features of the grafted monomers, the polymer can be tuned to respond to pH variation. Thus, charged monomers modulate the LCST. Besides, the presence of dissolved molecules (cyclodextrins, surfactants etc.) and salts in the solution modify the LCST. Usually, an increase in the solute concentration produces a decrease in the LCST. Although the synthesis of various temperature-sensitive polymers

has been reported in the literature, the most intensively used are poly(*N*-isopropylacrylamide) (PNIPAAm) and its copolymers. PNIPAAm attracts great attention due to its LCST near to body temperature, uncomplicated synthesis, sharp phase transition and facile copolymerization with other monomers.^{4–8} However, some articles report a considerable toxicity of PNIPAAm, which limits its suitable applications in biomedicine.⁹ Consequently, increased efforts have been made to find a substitute for PNIPAAm with similar characteristics and lower toxicity. Also, for internalization of the polymer in the human body biodegradability of the material is desirable.⁹

Polysuccinimide (PSI) is a biodegradable polymer with low toxicity and an easy synthesis process. The repeating unit of PSI is succinimide, a five-membered imide ring, which is susceptible to a nucleophilic ring-opening reaction that allows chemical modification to introduce new functionalities. This characteristic was employed to produce a set of versatile PSI derivatives with diverse physicochemical properties, which usually conserve nontoxicity, biocompatibility and biodegradability.^{10–12} Polyaspartamides, PSI derivatives which are produced by aminolysis,

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have been widely proposed for biomedical applications in drug delivery, gene delivery, gel scaffolds among others.^{10,13–19} Previous studies have reported the preparation of temperature-sensitive polyaspartamides.^{20–27} The reaction of PSI with isopropylamine produces a temperature-sensitive polyaspartamide with a repeating unit similar to PNIPAAm, which makes it an interesting substitute for PNIPAAm.^{22,23} However, not all the succinimides react with isopropylamine due to the steric hindrance of the isopropyl pendant and the neighboring group effect; the maximum degree of substitution (DS) is approximately 80%. Gu *et al.*²² have studied the effect of the pendant groups in the temperature-sensitive behavior by substituting the remaining succinimide groups using linear amines which react efficiently because they do not present steric hindrance. Tanimoto *et al.*²³ proposed the reaction of PSI with isopropylamine and recognized the remaining succinimides. However, they did not consider that the hydrolysis of the remaining succinimides occurs even at pH 7 producing carboxyl groups. Due to the presence of carboxyl groups, the LCST of the polymer will be influenced by pH.^{6,28,29} No previous work has investigated the effect of pH on the LCST of this polyaspartamide. In this work, we synthesized a set of temperature-sensitive polyaspartamides through the aminolysis reaction of PSI with isopropylamine. The influence of pH, the degree of hydrolysis (DH) and the DS on the LCST of the polymers was evaluated. To the best of our knowledge, this is the first time that the influence of these parameters has been evaluated together in temperature-sensitive polyaspartamides. We found that the LCST of the polyaspartamides could be finely tuned by adjusting these parameters appropriately.

EXPERIMENTAL

Materials

N,N-dimethylformamide (DMF, 99.8%), L-aspartic acid (98%), isopropylamine (99.5%) and cellulose membrane bags (cut off 14 kDa) were purchased from Sigma-Aldrich (São Paulo, Brazil). Sodium hydroxide (NaOH, 97.0%) and phosphoric acid (H₃PO₄, 85%) were obtained from Synth. Diethyl ether (98.0%) was purchased from Quemis.

Synthesis of polysuccinimide (PSI)

PSI was produced from L-aspartic acid by thermal polycondensation according to previous work.³⁰ A mixture of L-aspartic acid and phosphoric acid (10:1) was charged into a round-bottomed glass flask equipped with condenser and Dean–Stark trap and heated at 200 °C under an argon flow for 24 h. The polymer obtained was washed with methanol and deionized water several times; then it was dried at 60 °C for 24 h.

Synthesis of temperature-sensitive polysuccinimide (PSI-TS)

PSI (0.2 g) was dissolved in DMF (2.4 mL) at 70 °C for 3 h. After cooling at 30 °C, isopropylamine (1.4 mL) was added to the solution and maintained under magnetic stirring for 36 h. Cold diethyl ether was added to the solution to promote precipitation of the polymer. The precipitate was washed four times with cold diethyl ether and dried at 50 °C for 24 h.

Synthesis of temperature-sensitive poly(aspartic acid) (PAA-TS) and poly(aspartic acid) (PAA)

PSI-TS was dissolved in 1 mol L^{−1} NaOH aqueous solution and stirred for 3 h at room temperature to obtain PAA-TS. Afterwards, the solution was dialyzed in deionized water for 24 h. The dried polymer was obtained by lyophilization. The same procedure was used to obtain PAA from the PSI polymer precursor.

Synthesis of temperature-sensitive poly(aspartic acid) with different degrees of substitution (PAA-TS)

Samples of PAA-TS were prepared with different DSs. PSI (1.75 g) was dissolved in DMF (21 mL) at 70 °C for 3 h. After cooling at 30 °C, isopropylamine (12.25 mL) was added to the solution and maintained under magnetic stirring. Aliquots of 5 mL were taken from the reaction solution at 10, 12, 16, 20, 26, 34 and 48 h. Then 2 mL of 1 mol L^{−1} NaOH was added to each aliquot and magnetically stirred for 3 h at room temperature. Next each solution was dialyzed in deionized water for 24 h. The dried polymer was obtained by lyophilization. The samples were named PAA-TS followed by the time, in hours, at which the aliquots were taken out of synthesis.

A schematic synthesis route for PSI and its derivatives can be seen in Fig. 1.

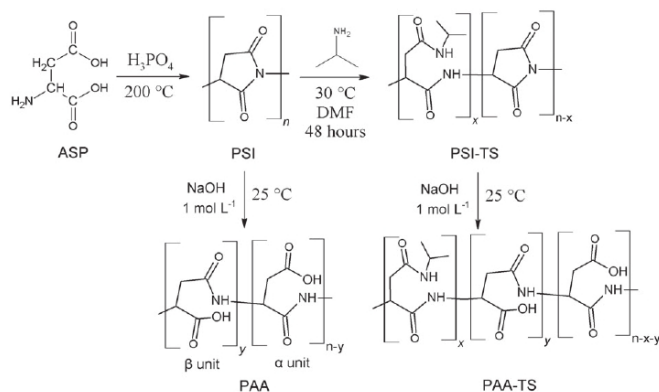


Figure 1. Synthesis route of PSI and its derivatives: ASP, L-aspartic acid; PSI, polysuccinimide; PSI-TS, temperature-sensitive polysuccinimide; PAA-TS, temperature-sensitive poly(aspartic acid); PAA, poly(aspartic acid).

Sample characterization

Fourier transform infrared (FTIR) spectra were recorded on a Perkin Elmer Frontier Dual Range FTIR spectrometer. Dried samples were milled with KBr and the mixtures were compressed into a pellet. Spectra were recorded in the range $4000\text{--}400\text{ cm}^{-1}$ with a nominal resolution of 4.0 cm^{-1} and 64 scans.

^1H NMR spectra were recorded on a Bruker Fourier 300 spectrometer. PSI and PSI-TS samples were dissolved in deuterated dimethyl sulfoxide ($\text{DMSO-}d_6$). The other samples were dissolved in D_2O .

The molecular weight and molecular weight distribution of polymer PAA were evaluated by gel permeation chromatography (GPC) using a Viscotek RImax (Malvern Instruments) equipped with three thermostatted ($35\text{ }^\circ\text{C}$) Shodex SB-806 HQ series columns (SHOWA Denko, Tokyo, Japan). Degassed 0.1 mol L^{-1} NaNO_3 solution in Milli Q water was used as the mobile phase, with a flow rate of 1 mL min^{-1} . The injection volume was $100\text{ }\mu\text{L}$ of stock solution. The polymers were characterized by the relative elution time of polyethylene oxide standards in the range $224\,000\text{--}195\text{ Da}$ (Varian, Palo Alto, CA, USA) using the calibration curve acquired before measurements.

The temperature-sensitive behavior of the polymer aqueous solution ($0.5\text{ wt\%}$) was estimated by turbidity measurements at 500 nm using a Lambda 465 UV–visible spectrophotometer equipped with a temperature-controllable cell. Transmittance was measured at different temperatures and samples were equilibrated for 4 min before each measurement. The LCST of the polymer was defined as the temperature corresponding to a 50% reduction in the transmittance of the solution at $20\text{ }^\circ\text{C}$.³¹ The LCST of the PSI-TS and PAA-TS samples was determined on varying the pH between 3.2 and 3.8 . The polymers were first dissolved in pH 3.2 water. After the measurements, the pH was increased with 0.1 mol L^{-1} NaOH . The LCST of PAA-TS samples with different DSs was measured at pH 3.4 .

RESULTS AND DISCUSSION

Polymer characterization

The GPC result showed that the molecular weight of PAA was 40 kDa for the number-average molecular weight (M_n) and 52.4 kDa for the weight-average molecular weight (M_w). The molecular weight distribution showed medium polydispersity behavior ($\text{PDI} = 1.31$), as can be seen in Fig. 2.

Figure 3 shows the FTIR spectra of the PSI, PSI-TS, PAA and PAA-TS samples. In the PSI spectrum the bands at 1796 and 1712 cm^{-1} are related to the symmetric and antisymmetric stretching modes respectively of the five-membered imide ring. This confirmed the presence of the succinimide repeating unit.³²

The PAA spectrum presents a broad band at 1580 cm^{-1} assigned to the superposition of amide I, amide II, carboxyl and carboxylate asymmetric stretching vibration modes. The band at 1396 cm^{-1} is assigned to the carboxylate symmetric stretching vibration mode. After alkaline hydrolysis of PSI, total conversion of the repeating units was confirmed by the absence of typical bands of PSI and the appearance of bands related to amide and carboxyl groups of the PAA sample.^{15,33}

The PSI-TS spectrum presents bands at 1636 and 1530 cm^{-1} which are related to the amide I and amide II vibration modes, respectively. The amide groups are formed as a consequence of the reaction between the imide ring in PSI with the isopropylamine. Also, PSI-TS does not show the bands related to the five-membered imide ring indicating that the majority of the imide ring was opened by the reaction with isopropylamine.^{17,33} The PAA-TS and

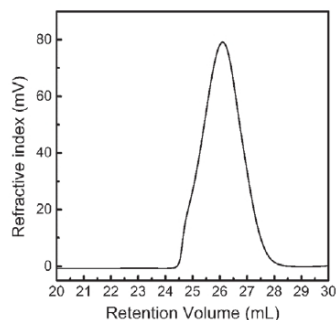


Figure 2. Chromatogram of a PAA sample.

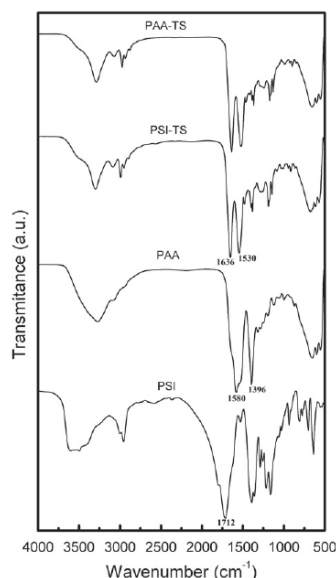


Figure 3. FTIR spectra of PAA, PAA-TS, PSI and PSI-TS samples.

PSI-TS spectra are very similar and the different details in the structure were evaluated by ^1H NMR spectroscopy.

Figure 4 displays the ^1H NMR spectra of PSI, PSI-TS, PAA and PAA-TS. Figure 4(a) shows the PSI spectrum, in which the peak at 5.3 ppm is related to methine proton ($-\text{CH}-$), the signals at 3.2 ppm and 2.7 ppm are related to methylene protons ($-\text{CH}_2-$) and the peak at 2.5 ppm is assigned to the DMSO signal.^{34–36} The PSI-TS spectrum (Fig. 4(b)) presents a broad peak at $4.3\text{--}4.6\text{ ppm}$ assigned to the superposition of succinimide unit methine protons and succinimide unit methine protons after the ring opening. The signal at 3.8 ppm corresponds to the methine proton in the isopropyl pendant group. The peak at 3.2 ppm is assigned to the succinimide unit methylene protons. The broad peak at $2.1\text{--}2.3\text{ ppm}$

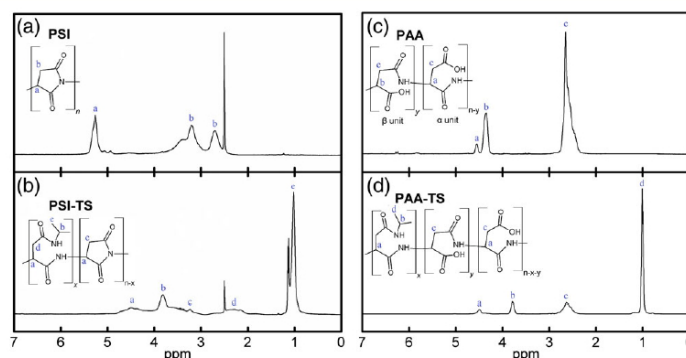


Figure 4. ^1H NMR spectra of (a) PSI and (b) PSI-TS in $\text{DMSO}-d_6$ and (c) PAA and (d) PAA-TS in D_2O .

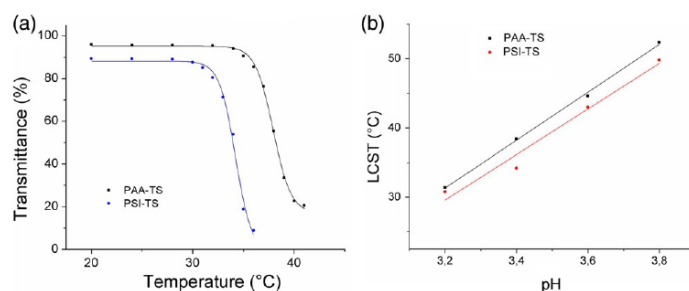


Figure 5. (a) Transmittance as a function of temperature for 0.5 wt% PAA-TS and PSI-TS aqueous solution at pH 3.4. (b) Influence of pH on the LCST of 0.5 wt% PAA-TS and PSI-TS solution.

is assigned to the succinimide unit methylene protons after the ring opening. The peak at 1.0 ppm is attributed to the six methyl protons ($-\text{CH}_3$) of the isopropyl group. The presence of peaks at 1.12 ppm and 1.14 ppm is related to the unreacted isopropylamine in the sample.^{11,15,22,37}

Deuterium oxide was used as a solvent for the PAA and PAA-TS samples and the signal was suppressed in the spectra. The PAA spectrum (Fig. 4(c)) shows signals at 4.7 ppm and 4.5 ppm which are related to methine protons of the PAA sample obtained by α - and β -opening, respectively. The relation between the areas expresses the molar proportion between the isomers; the molar ratio β/α was 3, which agrees with PAA synthesized in previous articles.^{38,39} The broad signal at 2.6–2.8 ppm is attributed to the methylene protons.³⁸ The PAA-TS spectrum (Fig. 4(d)) shows a signal at 4.6 ppm and a very low signal at 4.5 ppm related to methine protons. In this case, and contrary to the hydrolysis, the aminolysis was carried out almost exclusively via α -opening. The peak at 3.9 ppm is assigned to the methine proton in the isopropyl pendant. The broad peak between 2.9 ppm and 2.5 ppm is assigned to the methylene protons while the 1.1 ppm signal is assigned to the methyl protons of the isopropyl group.^{22,30}

As can be seen in Fig. 4 there are some differences in the molecular structure of the PAA-TS and PSI-TS samples. These differences are due to the presence of the imide ring in sample PSI-TS and the

absence of this group in the PAA-TS sample; then some difference in their temperature-sensitive behavior is expected.

The pH influence on LCST

The temperature-sensitive behavior of PSI-TS and PAA-TS solutions was evaluated by transmittance measurements as a function of temperature (Fig. 5(a)). A decrease in the transmittance is observed as the temperature of the solution increases. Although both samples present a similar curve, the decrease of the transmittance in PAA-TS takes place at a higher temperature in comparison to PSI-TS. The LCST values of PSI-TS and PAA-TS are 34.2 °C and 38.4 °C, respectively. The difference in the LCST value between PAA-TS and PSI-TS is due to the presence of carboxyl groups in the PAA-TS sample, which increases the affinity of this polymer to water.⁶

In the presence of water, the carboxyl group is susceptible to ionization to produce carboxylate anions. As the fraction of carboxylate groups increases as the pH of the solution increases, it was expected that the temperature-sensitive behavior of the PSI-TS and PAA-TS samples could be affected by the pH of the solution. Figure 5(b) displays the LCST of PSI-TS and PAA-TS samples in the pH range between 3.2 and 3.8. Below pH 3.0 the LCST of the samples is lower than room temperature and above pH 4.0 the LCST is higher than 50 °C, which is inappropriate for biomedical applications. The LCST of PAA-TS is higher than that of PSI-TS in all the

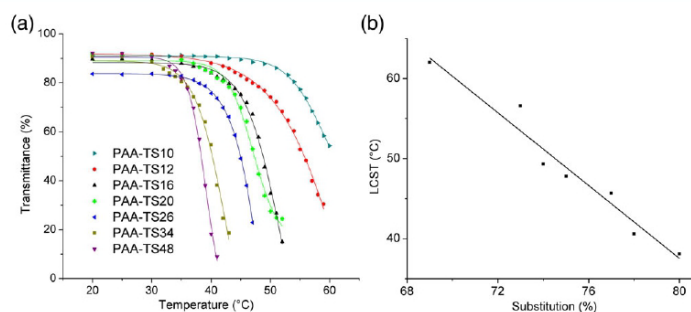


Figure 6. (a) Transmittance as a function of temperature for 0.5 wt% of each PAA-TS sample solution at pH 3.4. (b) Effect of the DS of PAA-TS samples on the LCST at pH 3.4.

measured pH values. An increment in pH increases the LCST of both solutions. As the pH increases, the proportion of carboxylate increases in the copolymers, and because carboxylate is more strongly solvated by water than the carboxyl group the LCST of the copolymers increases. This behavior could also be explained by considering that the carboxylate anion generates electrostatic repulsion between the copolymer chains so more thermal energy is necessary to overcome the electrostatic repulsion when the proportion of carboxylate increases.^{33,40}

As the LCST of sample PSI-TS is influenced by pH, there are carboxyl groups in the PSI-TS molecular structure. However, as was mentioned previously, PAA-TS presents a higher concentration of carboxyl groups due to the total hydrolysis of succinimide groups. Thus, PSI-TS is a copolymer in which there is partial hydrolysis of the succinimide. Although PSI-TS was first dissolved in a solution of pH 3.2 to avoid the hydrolysis of succinimide, carboxyl groups are present in very low quantity in the structure of PSI as end groups and open-ring structures. These results demonstrate the influence of the DH in the temperature-sensitive behavior of polyaspartamides.^{11,41}

Considering the pH working range (3.2–3.8), these polymers could be used in oral drug delivery systems for transporting drugs to the intestine avoiding drug degradation by gastric fluid in the stomach. In the acidic environment of the stomach, the polymer would be in the collapsed state protecting the drug, while in the intestinal neutral pH the polymer would dissolve and release the drug.^{34,42,43} Also, dissolved salts in the physiological fluids could interact with the negative charges of carboxyl groups, leading to a decrease in the electrostatic repulsion which could increase the pH working range above 3.8.⁴⁴

The DS influence on the LCST

The DS is defined as the mole ratio of *N*-isopropyl aspartamide units to the original succinimide units in PSI.¹⁰ The DS was evaluated from the ¹H NMR spectrum of each sample through integration of the signals at 3.9 ppm (isopropyl pendant group) and 2.9–2.5 ppm (polymer backbone). The DS of the PAA-TS samples is shown in Table 1.

As the PAA-TS samples present different DSs it was expected that they would also present different LCST values. Figure 6(a) shows the transmittance as a function of temperature of each PAA-TS sample solution at pH 3.4. The LCST of each sample was obtained from Fig. 6(a) and was related to the DS (Table 1). Figure 6(b) shows

Table 1. Degree of substitution (DS) of PAA-TS samples as a function of reaction time

Sample	Reaction time (h)	DS (%)
PAA-TS10	10	69
PAA-TS12	12	73
PAA-TS16	16	74
PAA-TS20	20	75
PAA-TS26	26	77
PAA-TS34	34	78
PAA-TS48	48	80

the influence of the DS of PAA-TS samples on the LCST at pH 3.4. It is observed that an increment in the DS produces a decrease in the LCST value. After aminolysis, the non-substituted repeating unit produces carboxyl groups due to the alkaline hydrolysis. Thus, copolymers with higher DSs also have a lower concentration of carboxyl groups. As was explained previously, an increment in the concentration of the carboxyl groups increases the LCST of the copolymer.

CONCLUSIONS

Temperature- and pH-sensitive polyaspartamides with different DH values and DS values were successfully synthesized. The LCST of the polyaspartamides rises as the pH of the solution rises. At the same pH, the LCST of PAA-TS is greater than the LCST of PSI-TS. Also, at a fixed pH, the LCST of PAA-TS polymers increases when the DS decreases. An increment in the pH or the DH increases the quantity of negatively charged carboxylate groups in the polymers. The carboxylate groups have a high affinity for water molecules and an increase of these groups in the temperature-sensitive polymers increases the LCST. The LCST of the polyaspartamides can be finely tuned by appropriately adjusting the pH, DH and DS.

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pH-responsive poly(aspartic acid) hydrogel-coated magnetite nanoparticles for biomedical applications

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ABSTRACT

A novel multifunctional nanosystem formed by magnetite nanoparticles coated with pH-responsive poly(aspartic acid) hydrogel was developed. Magnetite nanoparticles (Fe_3O_4) have been intensively investigated for biomedical applications due to their magnetic properties and dimensions similar to the biostructures. Poly(aspartic acid) is a water-soluble, biodegradable and biocompatible polymer, which features makes it a potential candidate for biomedical applications. The nanoparticles surface modification was carried out by crosslinking polysuccinimide on the magnetite nanoparticles surface and hydrolyzing the succinimide units in mild alkaline medium to obtain the magnetic poly(aspartic acid) hydrogel. The surface modification in each step was confirmed by DRIFTS, TEM and zeta potential measurements. The hydrodynamic diameter of the nanosystems decreases as the pH value decreases. The nanosystems showed high colloidal stability in water and no cytotoxicity was detected, which make these nanosystems suitable for biomedical applications.

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1. Introduction

Stimuli-responsive polymers change their properties (solubility, shape, surface characteristics, etc.) in response to a variation of the environmental conditions such as temperature, pH, electric or magnetic field, light, ultrasound, salt concentration, among others. Due to their unique properties, stimuli-responsive polymers offer many opportunities to introduce functionalities in nanostructures and enable the manufacture of various intelligent systems for application in biomedical field as imaging, disease detection, controlled drug delivery and bioseparation [1–3]. Among the so called stimuli-responsive polymers, pH-responsive polymers usually contain weakly acidic groups or weakly basic groups that will release protons or accept protons, respectively, in response to the pH variation, causing a macromolecular conformational change due to the repulsion of generated charges. This behavior has been harnessed as a mechanism for controlled drug release [4].

Poly(aspartic acid) (PASP) is a hydrophilic, biocompatible and biodegradable polymer that has received considerable attention in medicine, cosmetic and food industry [5]. PASP is commonly obtained through mild alkaline hydrolysis of polysuccinimide (PSI). This later is normally synthesized by L-aspartic acid thermal polycondensation. The repeat unit of PSI is a five-member imide ring (succinimide)

which is susceptible to nucleophilic attack, allowing its crosslinking using diamines to produce PSI gel. PASP hydrogel is obtained by mild alkaline hydrolysis of the PSI gel. Water absorption capacity of PASP bulk hydrogel has been showed to depend on pH and its variation can produce a shrinking or swelling of the hydrogel, conferring its hydrogel pH-sensitive behavior [6–9].

Nanosystems composed of magnetite nanoparticles coated with pH-responsive polymers have been proposed for multiple applications in the biomedical field, among them contrast agent in magnetic resonance imaging (MRI), delivery systems (drugs, genes, proteins), magnetic hyperthermia, bioanalysis and immunoassays, cell labeling, enzyme immobilization, etc. [1,10–12]. These applications require colloidal stability of nanoparticles in water at the biological conditions. A current approach is based on coating magnetite nanoparticles with a hydrophilic biocompatible hydrogel [13–17].

Previous works of Aurich et al. [18] and Yang et al. [19] reported the preparation of non-toxic and colloidal stable PASP coated magnetite nanoparticles. Also, poly(aspartic acid)-poly(ethylene glycol) copolymers coated iron oxide nanoparticles were reported with suitable features for biomedical applications [20–22]. Particularly Kumagai et al. [23] showed the potential utility of these type of nanoparticles as tumor-selective MRI contrast agents. Yang et al. [24] described the preparation of iron oxide nanoparticles coated by octadecyl grafted poly(aspartic acid) loaded with doxorubicin. Their results suggested the simultaneously potential uses in cancer diagnosis by MRI and

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Table 1
Concentration of the reactants to produce Fe_3O_4 -PSI.

Samples	$\text{Fe}_3\text{O}_4\text{-NH}_2$ (mg/mL)	PSI (mg/mL)	DAB ($\mu\text{L/mL}$)
$\text{Fe}_3\text{O}_4\text{-PSI}_0$	2.50	10	0
$\text{Fe}_3\text{O}_4\text{-PSI}_1$	2.50	10	1.0
$\text{Fe}_3\text{O}_4\text{-PSI}_2$	3.75	10	1.0
$\text{Fe}_3\text{O}_4\text{-PSI}_3$	2.50	10	1.3

therapy. These works were focused on nanoparticles surface coating by PASP, it does not attempt to the pH responsive possibilities since PASP used was not crosslinked.

Although some authors have studied bulk pH-responsive behavior, to the best of our knowledge, there is no report on literature concerning the preparation of magnetite nanoparticle coated by PASP pH-responsive hydrogel. In this article, we report the development of novel multifunctional magnetic nanoparticle surface modified with poly(aspartic acid) hydrogel, with magnetic- and pH-responsive features. In addition, the nanosystems show no cytotoxicity and the surface hydrogel confers it colloidal stability in water.

2. Experimental section

2.1. Materials

All chemicals were reagent grade and used without purification. Ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 98%), ferrous chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 99%), 3-aminopropyl trimethoxysilane (APTS, 99%), *N,N*-dimethylformamide (DMF, 99.8%), L-aspartic acid (98%), 1,4-diaminobutane (DAB, 99%) were purchased from Sigma-Aldrich. Ammonia solution (NH_4OH , 30%) was purchased from Alfa Aesar. Sodium hydroxide (NaOH , 97%) and phosphoric acid (H_3PO_4 , 85%) were obtained from Synth.

2.2. Synthesis of magnetite nanoparticle (Fe_3O_4)

Magnetite nanoparticles were prepared by the coprecipitation method. In a typical synthesis, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ were dissolved in 570 mL of deionized water at a concentration of $0.050 \text{ mol} \cdot \text{L}^{-1}$ and $0.028 \text{ mol} \cdot \text{L}^{-1}$ respectively. Ferrous ions were in slight excess of magnetite stoichiometric molar ratio ($\text{Fe}^{3+}/\text{Fe}^{2+} = 2:1$) in order to account for the oxidation of ferrous. After purging with argon, 30 mL of ammonia solution was added with vigorous mechanical stirring at 20,000 rpm, the resulted black suspension was stirred for 30 min. The nanoparticles were magnetically separated from the supernatant and washed with water five times. The obtained magnetite nanoparticles were dispersed by sonication in deionized water.

2.3. Preparation of amino-functionalized magnetite nanoparticles ($\text{Fe}_3\text{O}_4\text{-NH}_2$)

The surface modification of the nanoparticles was carried out through silanization process using APTS, in order to introduce amino groups at the nanoparticles surface [25]. In a three-necked round bottom flask equipped with a reflux condenser and inert atmosphere (Argon), 0.2 g of Fe_3O_4 nanoparticles were dispersed by sonication in 65 mL of water-ethanol solution (1:1 v/v). The pH of the suspension was adjusted to 9.0 with the addition of ammonia solution. Under magnetic stirring, 93.3 μL of APTS was added to the suspension and allowed to react for 1 h at 40°C . The nanoparticles were magnetically collected from suspension with an external magnetic field and washed four times with ethanol. The resulting nanoparticles sample was dispersed by sonication in deionized water.

2.4. Synthesis of polysuccinimide (PSI)

PSI was synthesized from the L-aspartic acid by acid-catalyzed thermal polycondensation [26].

A mixture of 3.0 g of L-aspartic acid and 0.3 g of phosphoric acid were charged into a three-necked round-bottomed glass flask equipped with condenser and Dean-Stark trap for removing water, then heated at 200°C in an oil bath under argon flow for 24 h. The obtained powder was washed with deionized water and methanol several times and dried at 60°C for 24 h.

2.5. Preparation of magnetite coated with crosslinked polysuccinimide ($\text{Fe}_3\text{O}_4\text{-PSI}$)

PSI was dissolved in DMF before adding $\text{Fe}_3\text{O}_4\text{-NH}_2$ nanoparticles into the solution. The suspension was maintained at 70°C for 24 h under magnetic stirring. After cooling to room temperature, it was added 1,4-diaminobutane (DAB) to the suspension and maintained for 3 h under magnetic stirring. The obtained nanoparticles were separated by an external magnetic field and washed with ethanol and water, prior to suspending in deionized water. Four samples were prepared from different concentration of the reactants according to Table 1.

2.6. Preparation of magnetite coated with poly(aspartic acid) hydrogel ($\text{Fe}_3\text{O}_4\text{-PASP}$)

Samples $\text{Fe}_3\text{O}_4\text{-PSI}_1$, $\text{Fe}_3\text{O}_4\text{-PSI}_2$ and $\text{Fe}_3\text{O}_4\text{-PSI}_3$ were suspended in $1 \text{ mol} \cdot \text{L}^{-1}$ NaOH solution and magnetically stirred for 3 h at room temperature. Afterwards, suspensions were dialyzed against deionized water for 24 h to obtain samples $\text{Fe}_3\text{O}_4\text{-PASP}_1$, $\text{Fe}_3\text{O}_4\text{-PASP}_2$ and $\text{Fe}_3\text{O}_4\text{-PASP}_3$, respectively.

2.7. Samples characterization

The crystalline structure of the samples was identified by X-ray powder diffraction (XRD) patterns recorded in the 2θ range $10\text{--}80^\circ$ on a Rigaku Geiger-Flex X-ray diffractometer using $\text{Cu K}\alpha$ radiation ($\lambda = 1.5418 \text{ \AA}$).

The identification of the functional groups in the samples was performed by Diffuse Reflectance Infrared Fourier transform spectroscopy (DRIFTS). DRIFTS spectra were recorded on an FTIR spectrometer Vertex 70 (Bruker, Germany) using the Pike EasiDiff diffuse reflectance accessory. Dried samples were diluted in KBr and recorded with 128 scans in the range of $4000\text{--}400 \text{ cm}^{-1}$ with a nominal resolution of 4.0 cm^{-1} .

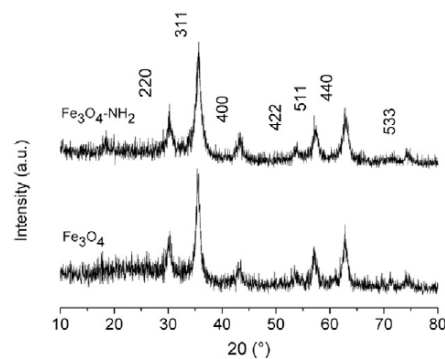


Fig. 1. XRD pattern of Fe_3O_4 and $\text{Fe}_3\text{O}_4\text{-NH}_2$.

Size and morphology of the nanoparticles were determined by Transmission Electron Microscopy (TEM). The TEM images of Fe_3O_4 were obtained in a Philips CM-120 microscope operating at 120 kV. The TEM images of $\text{Fe}_3\text{O}_4\text{-NH}_2$ were obtained in a Philips CM-200 microscope operating at 200 kV. The HAADF-STEM images of $\text{Fe}_3\text{O}_4\text{-PSI}_3$ and $\text{Fe}_3\text{O}_4\text{-PASP}_3$ were acquired using FEI Tecnai G2 F20 microscope operating at 200 kV.

Dilute isopropyl alcohol suspension of every specimen was dropped onto a carbon-coated copper grid and dried prior to analysis. It was measured the size of at least 100 nanoparticles using the software ImageJ, the average diameter and the standard deviation were calculated assuming a log-normal distribution.

The surface charge of the nanoparticles was determined by measurements of zeta potential using Zetasizer Nano ZS equipment

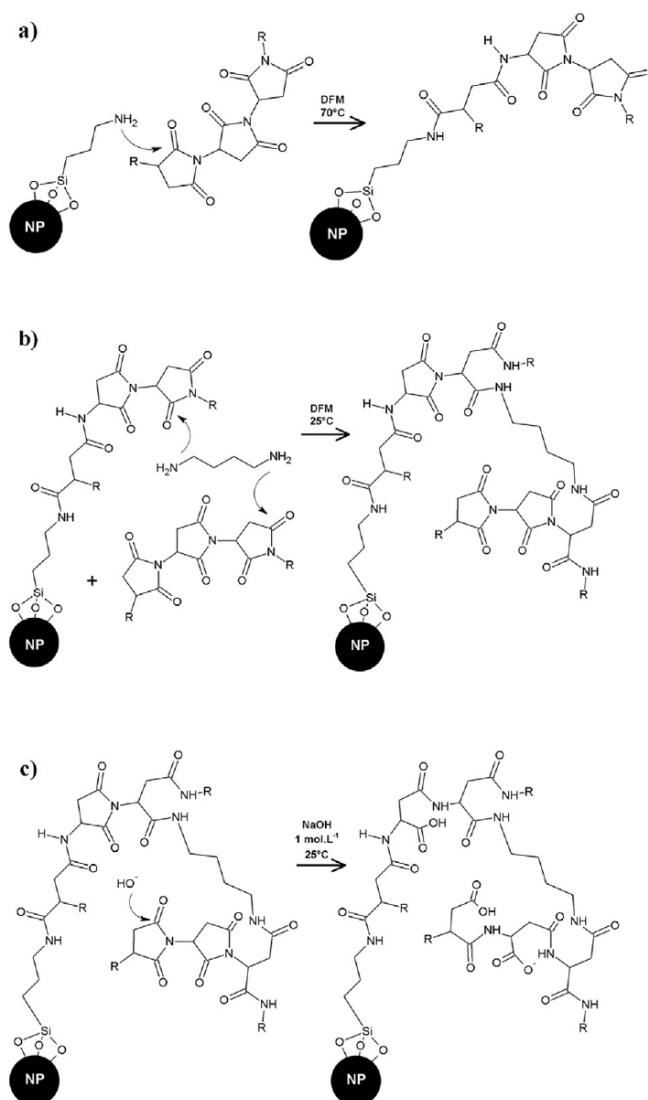


Fig. 2. Synthetic route of the multifunctional nanosystems.

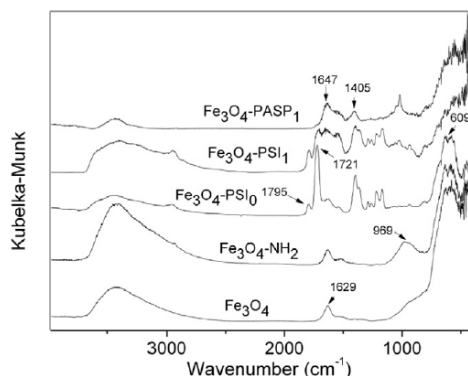


Fig. 3. DRIFTS spectra of samples Fe_3O_4 , $\text{Fe}_3\text{O}_4\text{-NH}_2$, $\text{Fe}_3\text{O}_4\text{-PSI}_0$, $\text{Fe}_3\text{O}_4\text{-PSI}_1$ and $\text{Fe}_3\text{O}_4\text{-PASP}_1$.

(Malvern Instruments). The nanoparticles were dispersed at the concentration of $3.9 \times 10^{-2} \text{ mg}\cdot\text{mL}^{-1}$ in $5 \times 10^{-3} \text{ mol}\cdot\text{L}^{-1}$ NaCl solution. The zeta potential was measured in the pH range 2–11. The pH was adjusted with MPT-2 Multi Purpose Titrator (Malvern Instruments) using $0.1 \text{ mol}\cdot\text{L}^{-1}$ NaOH and $0.1 \text{ mol}\cdot\text{L}^{-1}$ HCl solutions. All measurements were carried out in triplicate at 25°C .

The hydrodynamic diameter (D_h) of the nanoparticles dispersed in water were determined by dynamic light scattering (DLS) in a Zetasizer Nano ZS (Malvern Instruments) with a fixed scattering angle of 173° using a He-Ne laser ($\lambda = 632.8 \text{ nm}$) as the light source. The nanoparticles were dispersed by sonication in deionized water. The pH of the suspension was adjusted employing $0.25 \text{ mol}\cdot\text{L}^{-1}$ HCl solution, all measurements were carried out at least in triplicate at 25°C .

The cytotoxicity effect was evaluated on L929 cells (Instituto Adolfo Lutz, São Paulo, SP, Brazil) grown in Dulbecco's Modified Eagle Medium (Gibco), supplemented with antibiotic-antimycotic solution (Sigma-Aldrich) and 10% v/v fetal bovine serum (Gibco/inocrogen). The culture was maintained at 37°C in an atmosphere of 5% CO_2 in 95% air. (Thermo Scientific, USA). Cells were cultured until reaching confluence (90%) and removed with trypsin (0.05%)/EDTA (0.02%) (Gibco) in $1 \times \text{PBS}$. The trypsin was inactivated by the addition of culture medium, and the cells were then subjected to centrifugation, resuspended and replated. The medium was changed two to three times per week. Total viable cell counts were made in a Neubauer chamber

(New Optics), a suspension containing 1.0×10^4 cells/mL was placed in 96 well plates (TPP) and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 24 h.

After the incubation period, the culture medium was disposed. Then $200 \mu\text{L}$ of fresh culture medium containing the following concentrations of $\text{Fe}_3\text{O}_4\text{-PASP}_1$ was added to each orifice plate: 1000; 100; 10; 1; 0.1; 0.01; and $0.001 \mu\text{g}/\text{mL}$. Negative control experiments were carried out using the growth culture media without the nanoparticles. In addition, $20 \mu\text{L}$ of the Alamar Blue solution were added in each orifice plate to evaluate the cell metabolism by using the Alamar Blue® assay. The plates were maintained at 37°C in an atmosphere of 5% CO_2 for 24 h. Six wells were used for each experimental group.

The reading for the fluorescence of the samples was measured using the Fluoroskan Ascent FL fluorometer (LabSystems, Helsinki, Finland) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. All experiments were performed three times ($n = 18$).

Cell viability in each case was expressed as the percentage of fluorescence compared to negative control. In addition, the morphology of cell attachment was observed and photographed by inverted light microscope (Leica Microsystems Wetzlar GmbH; Leica DMI3000B).

3. Results and discussion

XRD diffractograms of uncoated Fe_3O_4 and $\text{Fe}_3\text{O}_4\text{-NH}_2$ are shown in Fig. 1, peaks are matched in position and in relative intensity to the characteristic pattern corresponding to inverse spinel structure of magnetite Fe_3O_4 (JCPDS – International Centre for Diffraction Data No. 33-0664), confirming the structured iron oxide in the samples and proved that the surface modification with APTS did not affect the magnetite structure of the iron oxide.

Whereas the carbonyl group of the imide ring is susceptible to nucleophilic attack, the reaction mechanism by which magnetic nanoparticles surface is coated by crosslinked PAA is proposed according to the following steps.

The amine groups on the $\text{Fe}_3\text{O}_4\text{-NH}_2$ nanoparticles attack the carbonyl groups of PSI to form a covalent bond between the nanoparticle surface and PSI, thus the nanoparticle is coated by PSI ($\text{Fe}_3\text{O}_4\text{-PSI}_0$), this process is shown in Fig. 2a.

When DAB is added, each amine group performs a nucleophilic attack on a carbonyl group of PSI, then the PSI is crosslinked on the surface of the nanoparticles ($\text{Fe}_3\text{O}_4\text{-PSI}_1$), this process is shown in Fig. 2b.

Alkaline hydrolysis of the remaining imide rings on $\text{Fe}_3\text{O}_4\text{-PSI}_1$ produces magnetic nanoparticles coated by crosslinked PAA ($\text{Fe}_3\text{O}_4\text{-PASP}_1$), this process is shown in Fig. 2c.

The surface modification of the nanoparticles in each step was examined by TEM, DRIFTS and zeta potential measurements. The

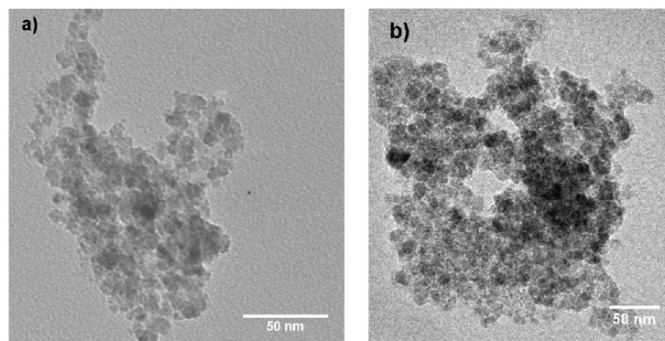


Fig. 4. TEM images: a) Fe_3O_4 b) $\text{Fe}_3\text{O}_4\text{-NH}_2$.

DRIFTS spectra of samples Fe_3O_4 , $\text{Fe}_3\text{O}_4\text{-NH}_2$, $\text{Fe}_3\text{O}_4\text{-PSI}_0$, $\text{Fe}_3\text{O}_4\text{-PSI}_1$ and $\text{Fe}_3\text{O}_4\text{-PASP}_1$ are depicted in Fig. 3.

Fe_3O_4 and $\text{Fe}_3\text{O}_4\text{-NH}_2$ exhibit three characteristic bands related to iron oxide: a band centered at about 609 cm^{-1} assigned to the Fe–O stretching vibration and the bands centered at 3420 cm^{-1} and 1629 cm^{-1} are assigned to stretching and bending vibrations of hydroxyl on the nanoparticles surface [27]. The sample $\text{Fe}_3\text{O}_4\text{-NH}_2$ presents an additional band at about 969 cm^{-1} related to Si–O stretching vibration, confirming the silanization of the magnetite nanoparticles surface [28].

The bands at 1721 and 1795 cm^{-1} in $\text{Fe}_3\text{O}_4\text{-PSI}_0$ are characteristics of the PSI repeat unit and are related to the antisymmetric and symmetric stretching modes of five-membered imide ring, respectively, demonstrating that PSI polymeric chains were anchored to the surface of the nanoparticles [29–30].

When the DAB crosslinked the PSI polymeric chains, some of the imide rings are opened and consequently the quantity of the rings decreased and amide groups are produced [30]. In sample $\text{Fe}_3\text{O}_4\text{-PSI}_1$, the intensity of the band at 1721 cm^{-1} is lower than in sample $\text{Fe}_3\text{O}_4\text{-PSI}_0$ due to the decrease of the imide rings. The presence of amide was verified by the bands centered at 1647 and 1546 cm^{-1} , which are assigned to the amide I and amide II vibration modes, respectively [31], these results prove the presence of crosslinked PSI in $\text{Fe}_3\text{O}_4\text{-PSI}_1$.

The characteristics bands of imide rings are missing in the DRIFTS spectrum of $\text{Fe}_3\text{O}_4\text{-PASP}_1$, however, it presents a broad band at 1600 cm^{-1} due to the superposition of carboxyl, amide I, amide II and carboxylate asymmetric stretching vibration mode. Also presents a band at 1405 cm^{-1} related to the carboxylate symmetric stretching vibration mode [32]. These results indicate that the remained imide rings of the PSI were totally opened by the hydrolysis process to form amide and carboxyl groups, therefore, the sample $\text{Fe}_3\text{O}_4\text{-PASP}_1$ presents crosslinked poly(aspartic acid).

Fig. 4 presents the TEM images of the samples Fe_3O_4 and $\text{Fe}_3\text{O}_4\text{-NH}_2$, in which nanoparticles are nearly spherical in shape and present some degree of agglomeration. After the silanization, the average particle size increased from $6.0 \pm 1.2\text{ nm}$ to $7.1 \pm 1.6\text{ nm}$ evidencing the existence of a silica layer over the magnetite nanoparticles in the samples $\text{Fe}_3\text{O}_4\text{-NH}_2$.

The signal in HAADF-STEM is proportional to the atomic number, appearing brighter the areas with a higher atomic number (Z).

HAADF-STEM images of $\text{Fe}_3\text{O}_4\text{-PSI}_3$ and $\text{Fe}_3\text{O}_4\text{-PASP}_3$ are presented in Fig. 5, these show the nanosystems formed by agglomerated magnetite nanoparticles (brighter region with higher Z) surrounded by polymeric phase (gray region). The polymeric phase is more extended in $\text{Fe}_3\text{O}_4\text{-PASP}_3$ than in $\text{Fe}_3\text{O}_4\text{-PSI}_3$. This is due, as will be seen later, to the higher presence of carboxylate anions in the PASP hydrogel than in PSI gel.

The zeta potential as a function of pH can be observed in Fig. 6, from which the isoelectric point (IEP) was determined as the value of pH where the zeta potential equals zero [33].

In aqueous suspension, the surface of the nanoparticles generates electric charges due to the adsorption of ions and the ionization of the functional groups on the nanoparticles surface [33]. If the functional groups have acid-base characteristics, the degree of ionization depends on the suspension pH. The value of the zeta potential also allows evaluating the colloidal stability. Nanoparticles with zeta potential higher than $+30\text{ mV}$ or lesser than -30 mV have colloidal stability due to the electrostatic repulsion, avoiding the agglomeration of the nanoparticles [34].

The IEP of Fe_3O_4 is 6.1 (Fig. 6), which was the expected value considering the presence of amphoteric hydroxyl groups on the surface of the magnetite nanoparticles.

In $\text{Fe}_3\text{O}_4\text{-NH}_2$, the presence of the amine groups (with the capacity of being protonated and generates positive charges) on the nanoparticles surface is reflected in the increase of the IEP to 8.5, this value agrees with previous data [35–36].

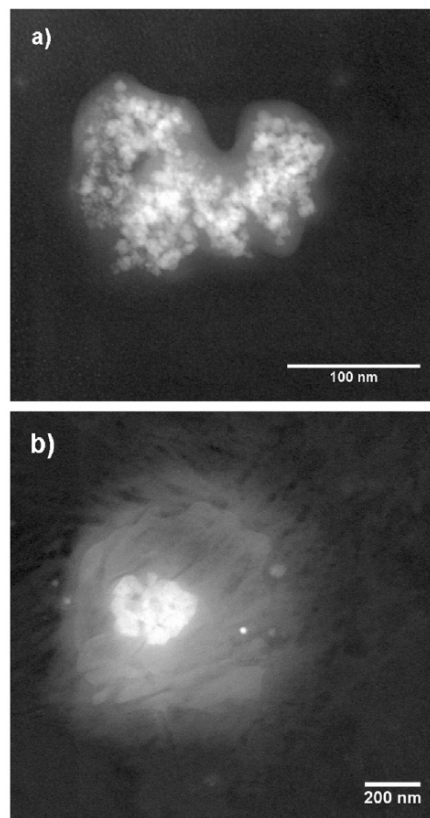


Fig. 5. HAADF-STEM images: a) $\text{Fe}_3\text{O}_4\text{-PSI}_3$ b) $\text{Fe}_3\text{O}_4\text{-PASP}_3$.

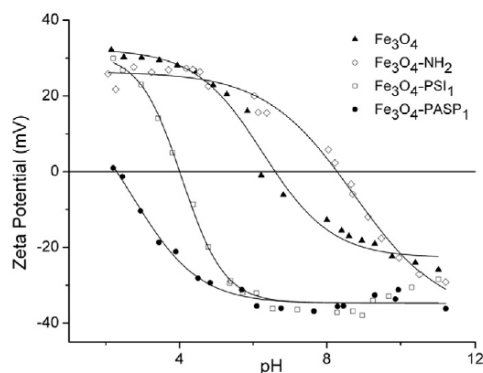


Fig. 6. Influence of the pH on the zeta potential of Fe_3O_4 (▲), $\text{Fe}_3\text{O}_4\text{-NH}_2$ (◇), $\text{Fe}_3\text{O}_4\text{-PSI}_1$ (□) and $\text{Fe}_3\text{O}_4\text{-PASP}_1$ (●).

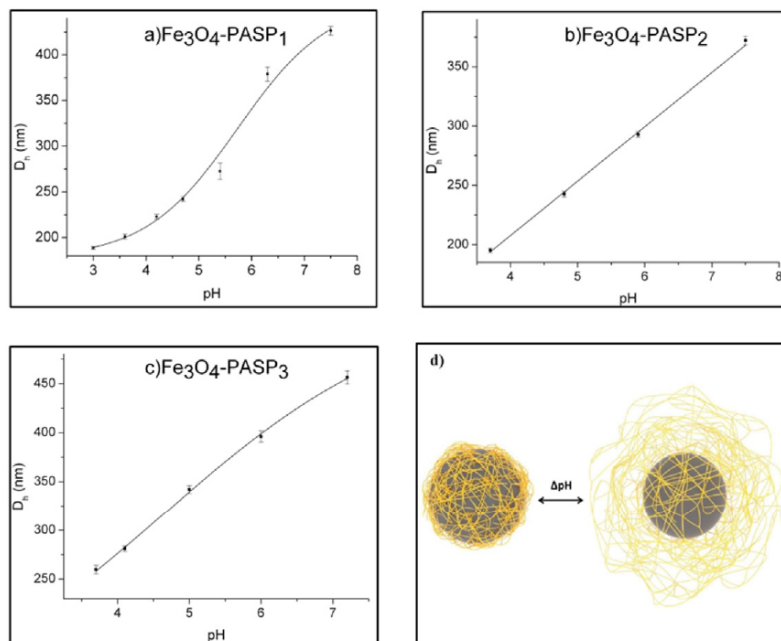


Fig. 7. a–c) Hydrodynamic diameter (D_h) of the iron oxide nanoparticles coated with poly(aspartic acid) hydrogel in function of pH, d) schematic illustration of the pH-responsive behavior of the nanosystems.

$\text{Fe}_3\text{O}_4\text{-PSI}_1$ displays an IEP of 4.0, indicating the presence of functional groups able to generate negative charges. In aqueous suspension, some of the imide rings in the PSI are opened by the hydrolysis process even at $\text{pH} = 7$, producing carboxyl groups that provide a negative charge to the system [37].

When the remained imide rings were opened through the basic hydrolysis, more carboxyl groups were formed; this was confirmed by the IEP of $\text{Fe}_3\text{O}_4\text{-PASP}_1$ which has a value of 2.3.

Applications in biomedicine require magnetic nanoparticles with colloidal stability in the environment pH. The pH in normal tissues and blood is about 7.4, however, some environments are more acidic, such as cancer tissues (pH 6.5–7.2), endosomes (pH 5.0–6.5) and lysosomes (pH 4.5–5.0) [2,15].

The zeta potential of $\text{Fe}_3\text{O}_4\text{-PASP}_1$ is less than -30 mV when the pH of the suspension is higher than 4.5, therefore the magnetite nanoparticles surface modified by poly(aspartic acid) hydrogel have colloidal stability in the biomedical applications pH range due to the negative charges in the poly(aspartic acid) hydrogel, which also acts as physical barrier providing steric stabilization.

The effect of the pH in the D_h of magnetite nanoparticles surface modified by poly(aspartic acid) hydrogel ($\text{Fe}_3\text{O}_4\text{-PASP}$) in aqueous media is shown in Fig. 7, in which the D_h decrease with decreasing the pH value, indicating that the nanosystems display pH-responsive characteristics. At neutral environment, the presence of negatively charged carboxylate groups is considerable in PASP chains, producing electrostatic repulsion between the polymeric chains. The hydrogel store

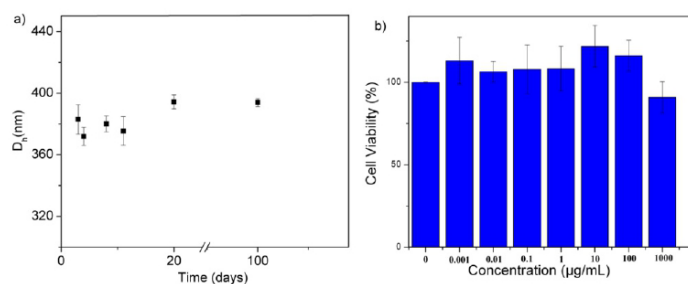


Fig. 8. a) Hydrodynamic diameter (D_h) of $\text{Fe}_3\text{O}_4\text{-PASP}_1$ in function of the days at 25 °C and pH 7, b) cell viability for $\text{Fe}_3\text{O}_4\text{-PASP}_1$ at various concentrations.

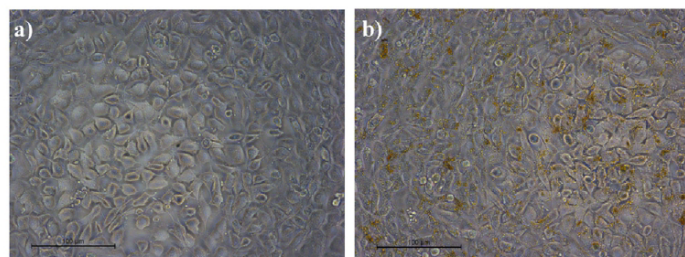


Fig. 9. Photomicrographs of L929 cells in presence of $\text{Fe}_3\text{O}_4\text{-PASP}_1$ at concentrations: a) 0 $\mu\text{g/mL}$ – Control, b) 10 $\mu\text{g/mL}$.

water molecules for stabilizing the electrical charges and separate the polymeric chains. As the pH of the suspension decreases, the amount of carboxylate anions in the hydrogel decreases owing to the protonation of these groups, which reduces the electrostatic repulsion. Consequently, the hydrogel shrinks and the volume of the nanosystem is reduced. Then, the volume of the $\text{Fe}_3\text{O}_4\text{-PASP}$ nanosystems is a function of the ionization degree of the PASP hydrogel [6,8]. Below of approximately pH 3.0 the nanosystems misplaced the colloidal stability and agglomeration took place.

The capacity to change the volume of the hydrogel in response to pH variation is influenced by the degree of crosslinking of the hydrogel, which is proportional to the number of linkages between the polymeric chains in the hydrogel. A high degree of crosslinking improves the rigidity of the hydrogel, restricting the movement of polymeric chains and also limiting the variation of the hydrogel volume [38–40].

The $\text{Fe}_3\text{O}_4\text{-PASP}_1$ and $\text{Fe}_3\text{O}_4\text{-PASP}_2$ samples showed a reduction in D_h until reaching a value of approximately 180 nm, whereas the D_h of $\text{Fe}_3\text{O}_4\text{-PASP}_3$ reached a value of about 260 nm. During the synthesis process, for a given PSI quantity, an increase in the crosslinker agent, rise the degree of crosslinking of hydrogel [39].

The $\text{Fe}_3\text{O}_4\text{-PASP}_3$ sample was prepared with a concentration of 1,4-diaminobutane (crosslinker molecule) larger than the other samples (Table 1), then the degree of crosslinking was higher in $\text{Fe}_3\text{O}_4\text{-PASP}_3$, limiting the shrinkage of the hydrogel.

Fig. 8a shown the D_h of $\text{Fe}_3\text{O}_4\text{-PASP}_1$ in function of the days, it can be seen no significant change in the D_h after 100 days of storage. The agglomeration process is avoided; therefore the nanosystems have high colloidal stability.

Fig. 8b displays the cell viability evaluated by the Alamar Blue test. The results, in accordance with ISO standard 10993-5, show that the nanoparticles at the tested concentrations are not cytotoxic with cellular viability $\geq 90\%$, when compared with the negative control group. The morphology of L929 fibroblasts cultured on the control group and with nanoparticles at the tested concentrations was observed by inverted light microscopy, as shown in Figs. 9 and S1 (Supplementary material). It can be seen that the cells cultivated with nanoparticles had preserved morphology without signs of cell death at the all tested concentrations.

It is worth to mention that the method of coating with poly(aspartic acid) hydrogel could be extended to another types of nanoparticles, such as gold or quantum dots if the surface of these nanoparticles is functionalized by amine groups.

4. Conclusions

Novel biocompatible and multifunctional nanosystems $\text{Fe}_3\text{O}_4\text{-PASP}$ hydrogel have been successfully prepared as nanocomposites responsive to magnetic field and pH. The presence of ionizable carboxyl groups in the PASP hydrogel confers its pH responsive behavior. The hydrodynamic diameter of the nanosystems decreased as pH decreased. The hydrogel provides colloidal stability through electrostatic and steric

stabilization. The nanosystems exhibited no cytotoxicity, indicating that the developed multifunctional nanosystems could be used in a wide range of biomedical applications, such as magnetic drug delivery systems, in which the changes in the pH could release the loaded drug.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.msec.2017.03.244>.

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Synthesis of core@shell nanoparticles functionalized with folic acid-modified PCL-co-PEGMA copolymer for methotrexate delivery

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ABSTRACT

Cancer is responsible for many fatalities and it is considered a public health problem. The side effects caused by conventional treatment are aggressive and painful to patients and can be reduced using nanomaterials that allows action in specific sites, making the cancer treatment more efficient, improving patient life's quality. Many kinds of nanoparticles that can be used, among these the block copolymer functionalized iron oxide nanoparticles stand out due to their simultaneous interaction with hydrophobic and hydrophilic drugs. The evaluation of these platforms properties allows optimizing their action in the human body, enhancing its biodistribution and targeting a specific region in the organism. The encapsulation efficiency and the controlled release profile is strictly dependent on the size, morphology, and interactions of the copolymer blocks. In this work, it was studied the synthesis of a hybrid nanopatform composed of an inorganic core (iron oxide) and a polymeric shell (PCL-co-PEGMA block copolymer modified with folic acid). FTIR and ^1H NMR allowed the confirmation of the nanopatform synthesis. Particles around 180 nm stable at physiological pH were obtained, allowing its application in different regions of the human body. The encapsulation efficiency of methotrexate was approximately 95%. The drug delivery assays indicated that the nanopatform is less active at pH 2; the presence of reduced glutathione enhanced the methotrexate release, reaching almost 50% methotrexate release after 96 h of analysis. The release efficiency of the nanopatform allowed to identify its potential as a controlled drug delivery system.

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1. Introduction

The estimated growth related to the emergence of cancer cases in the coming years has led to massive investments in the discovery of new molecules with biological activity. Due to the complexity of the human body, new drugs developed show low solubility in hydrophilic media, reducing its bioavailability [1]. Controlled release nanopatforms allow the targeting of drugs to specific regions in the human body, compensating for the low solubility of the new drugs [2]. Hybrid core@shell nanoparticles stand out among the available platforms due to the advantages union of the constituents that compose it.

The nanopatform physicochemical properties (size, shape, and surface charge) play a significant role in the transport of the drug and, consequently, its biodistribution. Multiple advantages arise from the use of these nanopatform, such as reduction

of drug toxicity, enzymatic degradation, avoiding uptake and clearances of the drugs [3]. Its functional application is related to the physiological barriers overcome, passing through capillary vessels and penetrating tumors [4]. To favor the drug uptake, ligands are functionalized onto the nanopatform surface in which they will interact with specific receptors [5]. This is the main characteristic of ligand-mediated targeting therapy [5,6].

Hybrid core@shell nanoparticles are nanomaterials that have at least one physical dimension below 100 nm composed of an inorganic and an organic region. In this work, it was synthesized iron oxide nanoparticles (Fe_3O_4) and functionalized with the amphiphilic copolymer PCL-co-PEGMA modified with folic acid. The inorganic core was obtained by the co-precipitation method of iron(II) and (III) salts in a basic medium [7,8] while the copolymer shell was polymerized through the ring opening of the ϵ -caprolactone [9]. The Fe_3O_4 nanoparticles have a wide range of applications, such as cellular therapy, tissue repair, drug delivery, magnetic resonance imaging, and hyperthermia. These assignments are possible by adjusting their physical and

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chemical properties, ideal for the development of nanomaterials [10]. Both polymers used are biocompatible and approved by Foods and Drugs Administration (FDA) for biomedical applications. Polycaprolactone (PCL) is a linear chain polyester, shows hydrophobic characteristics, low immunogenicity, and has the advantage of not being enzymatically degraded by the human body [9]. Poly(ethylene glycol) methacrylate, in turn, interacts with polar molecules such as water, presents low cytotoxicity and makes the nanoparticle invisible to phagocytic cells, increasing its circulation time and enhancing its activity in the cancer treatment [11]. The combination of the properties presented in the synthesized core@shell nanoparticle allowed to unite the advantages of each component in obtaining a potential candidate for a controlled release system.

Cancer is responsible for a high mortality rate. It can be characterized as a set of diseases that have in common the disordered growth of the cells that invade tissues and organs and can spread to other regions of the human body [12]. The multiplication of tumor cells requires more nutrients than a healthy cell. Thus, these cells tend to overexpress receptors of these nutrients on their surface [13]. Vitamins are part of the necessary nutrients and, specifically, folic acid is essential in the synthesis of DNA and RNA molecules [14]. Thus, the functionalization of the nanoparticles with folic acid will make it possible to target them to specific regions of the body in which there is tumor development, acting as a Trojan horse [13]. Methotrexate (MTX) is a hydrophobic drug widely used in cancer treatment due to antineoplastic activity acting as an antimetabolite and antifolate agent. MTX competitively binds to folate receptor to be internalized by cells due to its structural similarity to the folic acid molecule. Blocking the active site of the enzyme dihydrofolate reductase results in inhibition of the synthesis of thymidylate and the nucleotide purine and consequent death of the cells by preventing the synthesis of DNA and RNA [15].

In the current work, it was investigated the influence of surface functionalization of iron oxide nanoparticles with folic acid-modified PCL-co-PEGMA amphiphilic copolymer and the presence of disulfide bond to cumulative methotrexate release. It was evaluated the presence of the copolymer contribution to the nanoparticle's colloidal stability, especially at physiological pH and its influence in the reduction of the agglomeration process. The difference in pH of the medium and the presence of reduced glutathione made it possible to simulate different MTX release conditions. The results expressed suggest the potential application of the synthesized nanoparticles in the MTX controlled delivery.

2. Experimental section

2.1. Materials

The reagents were used as received from suppliers. Poly (ethylene glycol) methacrylate (PEGMA) with $M_n = 500 \text{ g mol}^{-1}$, ϵ -caprolactone 97.0%, folic acid 97.0%, iron(II) chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$) 98.0%, iron(III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) 97.0%, sodium hydroxide 98.0%, (3-mercaptopropyl) trimethoxysilane (MPTS) 95.0%, 3-mercaptopropionic acid (MPA) 99.0%, 2-propenamine 98.0%, potassium persulfate 99.0%, triethylamine 99.0%, N,N' -Dicyclohexylcarbodiimide (DCC) 99.0%, phosphate buffer solution (0.01 mol L^{-1} phosphate, 0.135 mol L^{-1} NaCl and 0.002 mol L^{-1} KCl) solution (PBS 0.01 mol L^{-1} , pH 7.4), tin(II) 2-ethyl hexanoate ($\text{Sn}(\text{Oct})_2$) 92.5–100.0% were purchased from Sigma-Aldrich Co. USA. Hexane 98.5%, Acetone, Dimethyl sulfoxide (DMSO) 97.0%, N,N -Dimethylformamide (DMF) 99.8%, and chloroform 99.8% were purchased from Labsynth Ltda. Commercial grade Argon gas was used to attain inert atmosphere.

2.2. Synthesis of Fe_3O_4 @MPTS nanoparticles

The iron oxide nanoparticles (Fe_3O_4) were synthesized by the co-precipitation method of a solution containing a 1:2 molar proportion mixture of Fe^{2+} and Fe^{3+} salts, respectively, and added to a sodium hydroxide solution [7,8]. 0.01 mol of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and 0.02 mol of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were solubilized in 0.25 L deionized water and dropped into 0.5 L of $\text{NaOH } 1.5 \text{ mol L}^{-1}$ solution. The system was kept under mechanic stirring and inert atmosphere at 80°C for 16 h . An aliquot was taken from the material and named NP. After the reaction first hour, $1 \times 10^{-3} \text{ mol}$ of MPTS was added to the mixture, providing thiol groups to the surface of the nanoparticles [16]. The black suspension was washed with deionized water until reaches pH approximately to 7.0. The sample was named NP@MPTS.

2.3. Synthesis of HS-PCL-co-PEGMA-folic acid copolymer

Fig. 1 shows the copolymer synthetic route by the ring opening polymerization (ROP) reaction of ϵ -caprolactone monomer, which PEGMA acted as macroinitiator and $\text{Sn}(\text{Oct})_2$ as the reaction catalyst, forming PCL-co-PEGMA. The synthesis was performed according to previous work [17,18]. In a round bottom flask, it was added 0.34 mL of $\text{Sn}(\text{Oct})_2$, 1.0 mL of PEGMA and 1.0 mL of ϵ -caprolactone. The reaction was carried out under Argon gas atmosphere and magnetic stirring for 3 h at 120°C . The copolymer was solubilized in chloroform and washed with ice-cold hexane several times to remove unreacted monomers and catalyst residue.

The folic acid-functionalized HS-PCL-co-PEGMA copolymer was obtained in three steps. Initially, a coupling reaction was performed between the PCL hydroxyl end group and MPA in a DMF solution containing triethylamine and DCC. A 1:1:1 molar ratio (copolymer:MPA:triethylamine:DCC) of the components was used. The system was kept for 24 h at room temperature (25°C), inert atmosphere and constant stirring [19]. The material was dialyzed, freeze-dried, and labeled HS-PCL-co-PEGMA.

A radical polymerization reaction was performed between the PEGMA methacrylate end group and 2-propenamine in the presence of the potassium persulfate initiator. The synthesis was performed according to previous work [17]. The system was kept for 48 h at 60°C , inert atmosphere, and constant stirring. The material was dialyzed, freeze-dried and labeled HS-PCL-co-PEGMA- NH_2 .

A coupling reaction between a carboxyl group from folic acid and an amino group of the HS-PCL-co-PEGMA- NH_2 copolymer was performed. The copolymer, the folic acid, and DCC were added to an Erlenmeyer flask in a molar ratio of 1:1.3:1.7 respectively, dispersed in DMSO, degassed and the reaction was carried at room temperature for 48 h [20]. The sample was dialyzed, freeze-dried, and labeled HS-PCL-co-PEGMA-NH-FA.

2.4. Synthesis of Core@Shell nanoparticles

The disulfide bond formation reaction between the iron oxide nanoparticles and the amphiphilic copolymer was performed in ambient atmosphere and at physiological pH. A 5:1 mass proportion of copolymer to nanoparticles was dispersed in PBS 0.01 mol L^{-1} (pH 7.4) and kept for 24 h at 37°C [21,22]. The ambient oxygen promoted the oxidizing medium, which aided in the formation of the disulfide bond. The sample was washed with deionized water and freeze-dried. The sample functionalized with folic acid in the surface was labeled NP@MPTS@POL-FA, while in the folic acid absent sample was labeled NP@MPTS@POL.

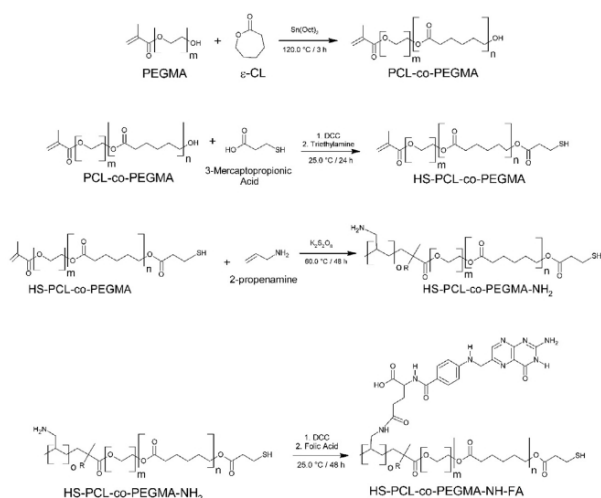


Fig. 1. Synthetic route of the PCL-co-PEGMA copolymer and its modifications.

2.5. Methotrexate encapsulation efficiency

The solvent evaporation method was applied to evaluate the methotrexate uptake efficiency by the nanoparticles. Different mass ratios of methotrexate to the nanoparticles were dispersed in acetone, then water was added to provide methotrexate encapsulation in the hydrophobic region of the copolymer shell. Three mass ratios of the drug to the platform were analyzed: 3%, 20%, and 50%. The uptake experiment details are presented in previous work [17]. The encapsulation efficiency (EE) and drug loading (DL) percentage were calculated by Eqs. (1) and (2) [17,23]:

$$EE(\%) = \left(1 - \frac{w_{MTX}}{w_{total\ MTX}}\right) \times 100 \quad (1)$$

$$DL(\%) = \left(\frac{w_{MTX}}{w_{MTX} + w_{NPs}}\right) \times 100 \quad (2)$$

with w_{MTX} - the amount of methotrexate encapsulated in nanoparticles, $w_{total\ MTX}$ - initial amount of methotrexate in solution and w_{NPs} - the amount of nanoparticles after freeze-drying.

2.6. Methotrexate releasing experiment

To evaluate the methotrexate release profile, 30.0 mg of drug-loaded nanoparticles were dispersed in two different buffers - PBS 0.01 mol L⁻¹ (pH 7.4) and Simulated Gastric Fluid (pH 2.0) - and transferred to a dialysis membrane (MWCO 2000). In addition to the pH difference of the release medium, the influence of reduced glutathione (GSH) was also evaluated on the disruption of the disulfide bond present between the nanoparticle and the copolymer. The release experiment was performed according to previous work [17]. The cumulative methotrexate release percentage was determined by Eq. (3) [23]:

$$Cumulative\ methotrexate\ release\ (\%) = \frac{V_i \sum_{j=1}^{n-1} C_j + V_0 C_n}{w_{MTX}} \times 100 \quad (3)$$

with V_0 - total volume of release medium, V_i - the volume of replaced medium, C_n - total methotrexate concentration in the sample, C_i - methotrexate concentration in the sample at time i .

3. Characterization

Nuclear magnetic resonance spectroscopy (¹H-NMR) was performed in a Bruker Fourier 600 Hz spectrometer. Chloroform-d (CDCl₃) and dimethyl sulfoxide-d₆ (DMSO-d₆) were used as solvents to elucidate the chemical structure of copolymer. Successive steps of functionalization of nanoparticles were analyzed by Fourier-Transform Infrared Spectroscopy using a Bruker equipment, model Vertex 70 through the attenuated total reflection (ATR) technique and diamond crystal. The thermogravimetric analysis was performed in a Mettler Toledo equipment, model TGA/DSC 1. Thermal degradation was evaluated in a temperature range between 30° and 800 °C, with a heating rate of 10 °C min⁻¹ under a nitrogen atmosphere (100 mL min⁻¹). The size and morphology of the core@shell nanoparticles were evaluated on a FEI Tecnai G² F20 HRTEM transmission electron microscopy using an electron beam of 200 kV. The samples were dispersed in deionized water at 25 °C and deposited in formvar carbon coated copper grids with 400 mesh. The software *ImageJ* (1.45 s version) was used for images treatment. The colloidal properties (hydrodynamic diameter and zeta potential) of the particles were evaluated using the Zetasizer Nano ZS from Malvern Instruments. Information on drug encapsulation efficiency and the controlled release profile were obtained from UV-Vis spectroscopy using a PerkinElmer equipment, model LAMBDA 465. The methotrexate quantification was measured at 303 nm wavelength.

4. Results and discussion

4.1. Synthesis of HS-PCL-co-PEGMA-folic acid copolymer

The PCL-co-PEGMA structure and its modifications are shown in the ¹H NMR spectra presented in Fig. 2. The peak of methylene hydrogens of PEGMA segment was observed at 3.68 ppm, while

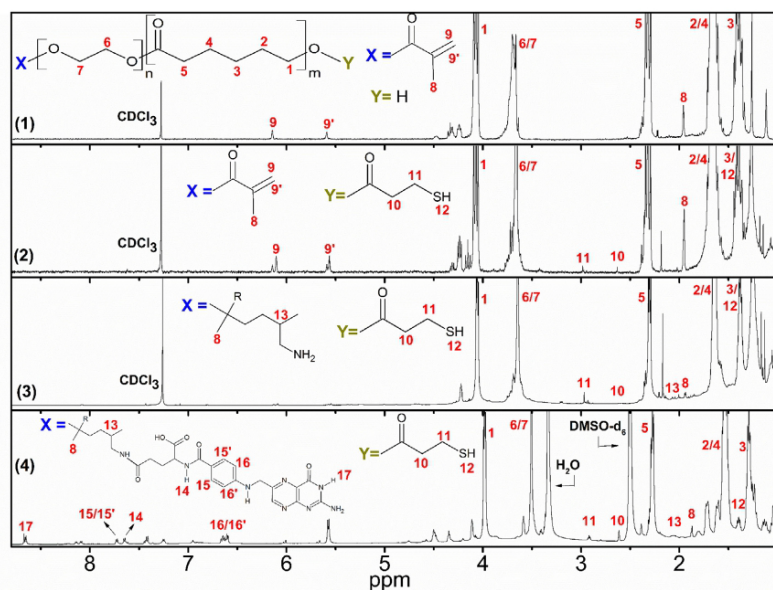


Fig. 2. ^1H NMR spectra of (1) PCL-co-PEGMA, (2) HS-PCL-co-PEGMA, (3) HS-PCL-co-PEGMA- NH_2 e (4) HS-PCL-co-PEGMA-NH-FA, respectively. R = Initiator = Potassium persulfate. X and Y = substituent group.

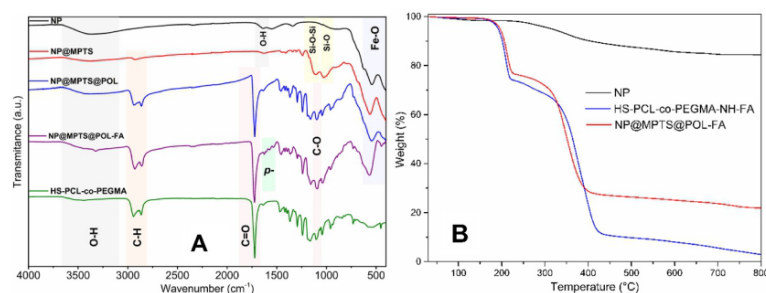


Fig. 3. (A) FTIR spectra and (B) TGA curves of the copolymer sample, the NP sample and subsequent functionalization with the folic acid-modified PCL-co-PEGMA copolymer.

the peaks of methacrylate hydrogens end-group were observed at 6.13, 5.58 and 1.95 ppm. The methylene hydrogens from the PCL segment were showed at 1.40, 1.65, 2.33, and 4.07 ppm [24]. The esterification with MPA gives rise to three peaks, two related to the hydrogens of methylene groups at 2.63 and 2.98 ppm, and one to the hydrogen of the thiol group at 1.42 ppm. Methacrylate group radical polymerization was confirmed with the disappearance of the peaks at 6.13 and 5.58 ppm, both attributed to the double bond hydrogens. The folic acid molecule bonded to the copolymer structure is confirmed due to the presence of peaks at 6.62 and 7.74 ppm that correspond to the aromatic ring hydrogens and at 7.63 and 8.65 ppm characteristic of secondary amino hydrogens [17,25]. The observed shifts between the peaks in the

spectra are due to change in the deuterated solvent used in the solubilization of the samples.

4.2. Core@shell nanoparticles synthesis and thermal analysis

The iron oxide nanoparticles functionalization with folic acid-modified PCL-co-PEGMA copolymer was confirmed through FTIR and TGA. Fig. 3A shows the FTIR spectra while Fig. 3B shows the TGA curves of the NP sample and subsequent surface modifications. The NP sample spectrum has three main bands: two related to the adsorbed water molecules on the nanoparticles surface located near 3400 and 1600 cm^{-1} ; the third double band is related to the Fe-O bond that composes the iron oxide located near 550 and 415 cm^{-1} [26]. The presence of MPTS leads to the appearance

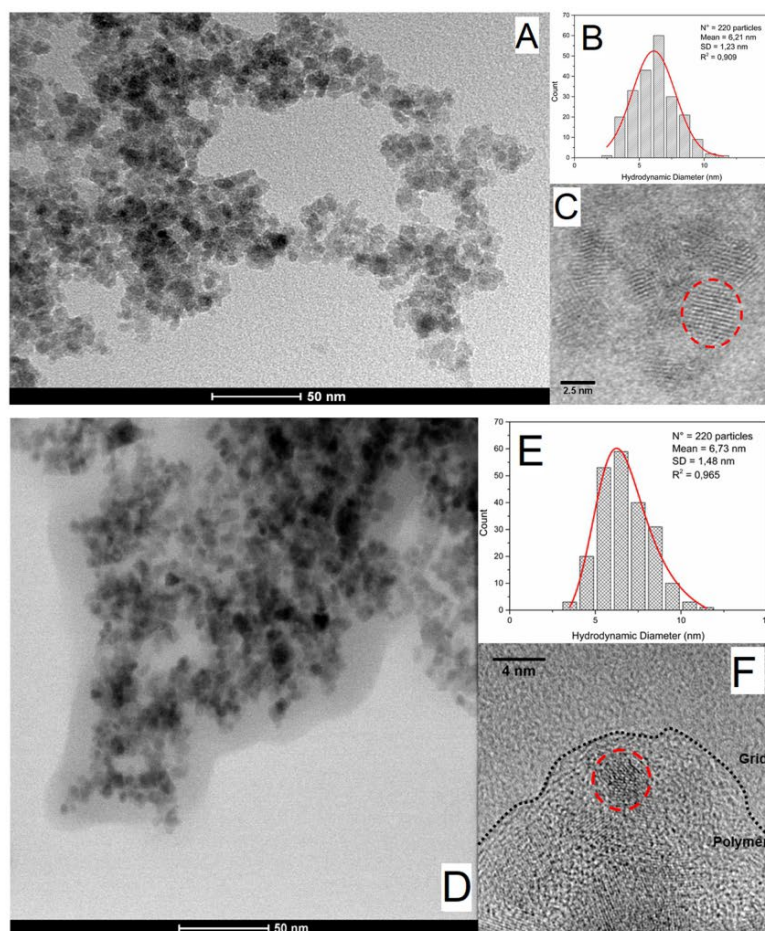


Fig. 4. (A) The TEM micrograph of NP sample. (B) The average size histogram of the NP sample. (C) High-Resolution micrograph of NP sample. (D) The TEM micrograph of NP@MPTS@POL-FA sample. (E) The average size histogram of the NP@MPTS@POL-FA sample. (F) High-Resolution micrograph of NP@MPTS@POL-FA sample.

of bands related to the carbon chain (C–H bond in 2925 cm^{-1}) and the silica region (double bands Si–O and Si–O–Si bonds in 1116 and 1027 cm^{-1} , respectively) of the siloxane [16,26]. The sample HS-PCL-co-PEGMA has a set of bands between 750 and 1500 cm^{-1} referring to the changes in the conformation of the copolymer main structure. In this sample, there is also a band near 1100 cm^{-1} and 1720 cm^{-1} , related to the C–O and C=O bonds, respectively, that composes the ethers and esters groups and a 2940 cm^{-1} and 2852 cm^{-1} double band related to the carbonic bonds present throughout the copolymer linear chain [17]. The NP@MPTS@POL sample has bands referring to the copolymer and the nanoparticles, confirming the functionalization step. The addition of the folic acid molecule causes the appearance of a set of low intensity bands (*p*-band) between 1605 and 1530 cm^{-1} for the presence of specifically *para*-substituted aromatic rings [27].

It can be seen in Fig. 3B a single event related to a mass loss of approximately 15% for the NP sample at 250°C , referring to the desorption of water molecules from the surface of the nanoparticles. The copolymer sample shows two events in the evaluated temperature range: one near 220°C , related to the desorption of the water molecules that interact with the hydrophilic segment of the copolymer, and another near 420°C , related to the copolymer chain degradation. Both events result in the loss of 97% of the sample's initial mass. The curve of the sample NP@MPTS@POL-FA behaves similarly to the HS-PCL-co-PEGMA-NH-FA sample. The desorption of water molecules occurs at a temperature close to that mentioned above (approx. 230°C), however, the degradation starts at a lower temperature, near 390°C [20,27,28]. This difference of 30°C can be attributed to the performance of the nanoparticles as a thermal conductor during the sample

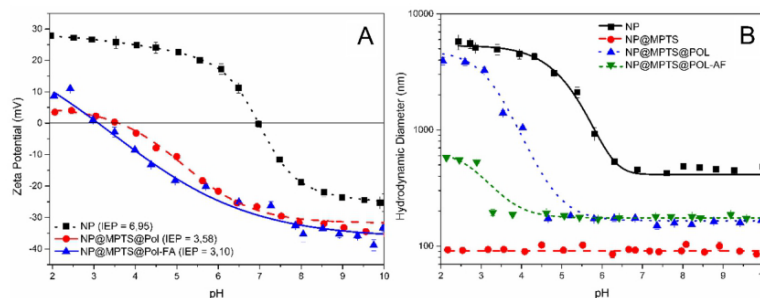


Fig. 5. (A) Zeta potential and (B) Hydrodynamic diameter variation in different pH values of the nanoparticles samples.

degradation [29]. Through the thermal analysis, it was possible to identify the composition of the core@shell nanoparticle: 20% corresponds to the iron oxide core, 60% to the copolymer shell and 20% to the presence of adsorbed water.

4.3. TEM characterization

The TEM micrographs of the samples were performed to identify the morphology and calculate the average diameter size of the samples prior and after functionalized with the copolymer. A roughly spherical shape is observed in both nanoparticles' samples TEM images presented in Fig. 4A and D. Comparing the size histogram distribution in Fig. 4B and E, it is observed that the size of the nanoparticles does not change significantly after the functionalization, ranging from about 6.1 ± 1.7 nm to 6.7 ± 2.3 nm [28,30]. The fitting curves for both samples size histogram distribution was obtained using a log-normal distribution function with a $R^2 > 0.90$. The observed growth can be attributed to the presence of MPTS added to the surface of the nanoparticles, forming a thin layer of silica in the order of angstroms. In the NP@MPTS@POL-FA sample, the presence of the copolymer onto the surface of the nanoparticles can be observed in both normal and high-resolution micrographs. Fig. 4A and C show the normal and high-resolution micrographs for the NP sample, respectively. It is possible to observe the presence of the iron oxide crystalline planes and its monocrystalline characteristic [26]. Fig. 4D and E show the continuous morphology in part of the sample and the presence of a copolymer shell around an agglomerate of NP, indicating the occurrence of the functionalization process. The copolymer does not alter the crystallinity of the nanoparticles since it is still possible to visualize the crystalline planes in the nanoparticles [26].

4.4. Colloidal characterization

The colloidal properties of the nanoparticles were analyzed using the Zetasizer Nano ZS equipment, allowing to measure its hydrodynamic diameter (D_h) and zeta potential (ζ). The size of the nanoparticles obtained by microscopy differs from the DLS result. This technique provides information about the hydrodynamic diameter of the nanoparticles, that is, the total diameter that contains the particles and the adsorbed water molecules that make up the double electric layer. Table 1 shows the hydrodynamic diameter, the polydispersity index, and the zeta potential of the nanoparticle's samples at 25 °C and pH 7.4. The value of the hydrodynamic diameter observed for the NP sample is related to two main factors: high area/volume ratio, consequently high surface energy of the particles and the absence of steric

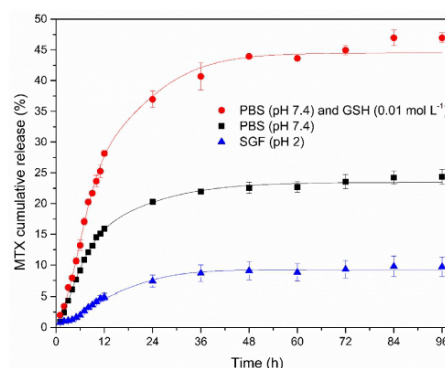


Fig. 6. (A) The cumulative release profile of methotrexate by folic acid modified core@shell nanoparticles in different media at 37 °C. PBS = Phosphate Buffer Solution, SGF = Simulated Gastric Fluid and GSH = Reduced Glutathione.

and electrostatic repulsion between particles at the measured pH value. It is possible to observe that the hydrodynamic diameter increases after the functionalization of the particles, while the polydispersity index and the zeta potential decrease [26,30]. The increase in diameter is characterized by the expansion of particle size due to the long linear chains composing the copolymer, increasing from 28.9 ± 11.4 nm to 179.7 ± 5.7 nm. However, the polydispersity index reduces with surface modifications, decreasing from 0.53 ± 0.07 to 0.24 ± 0.03 , indicating the diminution in the number of agglomerates of varied sizes and, indirectly, the increase in the colloidal stability of the synthesized system. This colloidal stability is corroborated by analyzing the zeta potential values of the particles. It is found that upon addition of the copolymer, the zeta potential is approximate -30 mV at physiological pH, which gives the particles an electrostatic stability [31]. Fig. 5A shows the zeta potential titration of the NP sample and the influence of functionalization on the curve aspect. The NP sample presents isoelectric point (IEP) close to 7, the presence of the copolymer reduces this value to near 3.6 and the addition of folic acid reduces again to 3.1 [20]. The observed IEP value for the NP sample is within the range of values identified in the literature. The presence of oxygenated groups, especially the ester groups that are protonated, causes the reduction of the IEP. The reduction of the IEP caused by folic acid is due to the presence of a

Table 1

The hydrodynamic diameter and zeta potential of core@shell nanoparticles obtained by Dynamic Light Scattering (DLS) at 25°C and pH 7.4. Data are presented as mean $\pm$ SD of three independent experiments.

Sample	D _h /nm	PDI	Zeta potential/mV
NP	28.9 $\pm$ 11.4	0.53 $\pm$ 0.07	-10.21 $\pm$ 0.03
NP@MPTS	66.6 $\pm$ 14.2	0.34 $\pm$ 0.06	n.a. ^a
NP@MPTS@POL	136.6 $\pm$ 18.6	0.36 $\pm$ 0.03	-29.27 $\pm$ 0.06
NP@MPTS@POL-FA	179.7 $\pm$ 5.7	0.24 $\pm$ 0.03	-30.84 $\pm$ 0.04

^an.a.: non applicable.

secondary carboxylic acid group which remained intact after the coupling reaction [32]. Fig. 5B shows the hydrodynamic diameter variation in different pH values. It is possible to observe through the curve's profile of the samples that these tend to aggregate next to IEP, whose no electrostatic repulsion between particles occurs, resulting in their aggregation. It was not possible to titrate the zeta potential for the NP@MPTS sample due to the presence of thiol groups on the surface of the particles. These groups could interact with the gold electrodes present in the cuvettes and, thus, compromising the integrity of the analysis [33].

4.5. MTX encapsulation efficiency

MTX is a chemotherapeutic agent and was used as a hydrophobic drug model to evaluate the encapsulation process in the core@shell nanoparticles. Table S1, in the supplementary information, shows the encapsulation efficiency and drug loading values in which different mass ratios of MTX to nanoparticles were used. The mass ratio of the drug to the platform was evaluated and three different proportions values was used: 3%, 20%, and 50%. The lowest proportion reached 71% encapsulation efficiency, while the higher achieved about 96%. In addition to the increase in efficiency, there is also an increase in the MTX content, reaching approximately 36% in the ratio 1:1 (MTX:nanoparticle). The high efficiency can be attributed to the presence of weak intermolecular interactions (dipole-dipole, van der Waals) between the MTX and the hydrophobic segment (PCL) of the copolymer [17]. Due to the structural similarity between the folic acid and the MTX, part of the methotrexate is on the surface of the nanoparticle interacting with the folic acid, contributing to the high efficiency achieved [17]. For the drug release measurement, the samples with the highest methotrexate content were used.

4.6. Methotrexate release profile

MTX cumulative release profile is shown in Fig. 6 in which different pH medium (PBS and SGF) and the presence of reduced glutathione (GSH) was evaluated [34,35]. A maximum release of approximately 10% was observed at pH 2.0, simulating gastric fluid. This release percentage was reached after 36 h of analysis and remained stable until the end of the analysis period (96 h). The assay performed at physiological pH (7.4) reached 25%, releasing 2.5 times more than in acid medium. The difference between the curves obtained can be attributed to the change in the conformation of the copolymer chains [36]. In pH 2, close to the IEP of the sample, the particle's stability is compromised and that culminates in the agglomeration of the same. Thus, the MTX is entrapped in the polymer layer present on the surface of the nanoparticles which results in the observed release percentage (10%). The low release percentage at pH values similar to those found in the human stomach enables the absorption process without significant losses of the encapsulated drug. The GSH presence enhanced the release of MTX, reaching approximately 47% after 96 h of analysis. Thus, it is possible to state that glutathione permeated along the chains of the copolymer and specifically acted on the disulfide bond, breaking it [37].

5. Conclusion

The PCL-co-PEGMA copolymer structure and its subsequent modifications, including the functionalization with folic acid were confirmed by ¹H NMR spectroscopy. FTIR spectroscopy, thermogravimetric analysis, and transmission electron microscopy proved the achievement of the core@shell system. FTIR spectroscopy corroborated the functionalization with folic acid shown at ¹H NMR spectroscopy. TGA allowed to observe that the presence of the iron oxide nanoparticles reduced the degradation temperature of the polymer layer and that the composition of the core@shell system consists of 20% of the inorganic core, 60% of the polymeric shell and 20% of adsorbed water molecules. The functionalized nanoparticles presented a hydrodynamic diameter of approximately 180 nm measured by DLS, while it was possible to identify that the functionalization did not significantly alter the diameter of the nanoparticles (core), as shown in the TEM micrographs. The surface modification reduced the agglomeration process due to the presence of a steric and an electrostatic contribution in the repulsion between particles, contributing to the colloidal stability of the core@shell system. MTX was used as a hydrophobic drug model to be encapsulated into the particles. The increase in the mass ratio of the drug to the particles caused an increase in the encapsulation efficiency, reaching an efficiency of approximately 95%. The release profile allowed identifying that the nanoparticle has a slow MTX delivery at pH 2, while the presence of reduced glutathione increased the release content, reaching approximately 50% MTX release after the analysis time. These results suggest that core@shell nanoparticles are suitable candidates for the release of MTX in cancer treatment as it is possible to modify particles properties adding bioactive molecules like folic acid.

CRediT authorship contribution statement

João Victor Brandt: Conceptualization, Investigation, Formal analysis, Methodology and Data interpretation, Visualization, Roles/writing - original draft and review & editing. **Rodolfo Debone Piazza:** Investigation, Methodology, Data interpretation, Review & editing. **Caio Carvalho dos Santos:** Investigation, Methodology, Data interpretation, Review & editing. **Jaime Vega-Chacón:** Data interpretation, Review & editing. **Bruno Estevam Amantéa:** Data interpretation, Review & editing. **Gabriel Cardoso Pinto:** Methodology, Review & editing. **Miguel Jafelici Junior:** Conceptualization, Methodology, Resource/funding acquisition. **Rodrigo Fernando Costa Marques:** Conceptualization, Methodology, Resource/funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at <https://doi.org/10.1016/j.nanoso.2021.100675>.

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IV. Nanopartículas magnéticas como suporte para enzimas em aplicações biotecnológicas

IV.1 Suportes para enzimas aplicadas na produção de etanol de segunda geração

A biomassa lignocelulósica é um material altamente heterogêneo composto por lignina, hemicelulose e celulose em uma estrutura complexa. A capacidade para converter os componentes da celulose deste material em açúcares fermentáveis para a produção de biocombustíveis depende da ação cooperativa das três enzimas do complexo enzimático celulase: a endoglucanase, exoglucanase e β -glicosidase [76]. As celulasas têm sido isoladas a partir de uma grande variedade de micro-organismos e apresentam uma ampla faixa de pH e temperatura ótimos. O custo da enzima na produção de biocombustíveis lignocelulósicos, como o etanol obtido a partir da palha e bagaço da cana-de-açúcar, conhecido como etanol de segunda geração, depende de muitos fatores como: o tipo de matéria-prima, o carregamento da enzima e o rendimento global do processo [77].

Para a obtenção de biocombustíveis por via enzimática com preços mais competitivo torna-se necessário reduzir os custos de produção, desenvolvendo métodos que permitam a reutilização da celulase por vários ciclos catalíticos, sem perda da atividade. A imobilização de enzimas é uma excelente alternativa e está sendo aplicada eficientemente em diferentes segmentos, tais como, biocatálise em alimentos, em processos químicos e na indústria farmacêutica [78].

Outro fator importante é a viabilidade econômica ao se utilizar a imobilização de enzimas. É necessário considerar que o tipo de suporte utilizado e o método selecionado influenciam na atividade e reutilização da enzima em processos biocatalíticos [79,80]. Na literatura há muitas maneiras que possibilitam a imobilização de enzimas, com o objetivo de prolongar a atividade enzimática em diversos ciclos, principalmente em processos industriais. Alguns métodos de imobilização incluem ligação covalente ou por adsorção física [81]. Uma alternativa promissora proposta em nosso grupo de pesquisa, é o uso de nanopartículas magnéticas para imobilização de enzimas por ligação covalente, havendo grande interesse na síntese de suporte magnético, que devido a presença de momentos magnéticos possibilita a interação com um campo magnético externo, conferindo a capacidade de separação magnética [82,83].

As vantagens das enzimas imobilizadas em relação às enzimas livres surgem da sua maior estabilidade e facilidade de separação, o que acarreta economia significativa no custo global do processo, desde que o procedimento de imobilização não seja muito caro, permita boa recuperação da atividade enzimática e que a estabilidade operacional da enzima imobilizada seja suficientemente longa. As vantagens da utilização de enzimas imobilizadas com nanopartículas magnéticas são: retenção do biocatalisador no reator, elevada concentração de catalisador no reator permitindo intensificar o processo, controle do microambiente da enzima e facilidade de recuperação e reutilização do catalisador, o que reduz os custos das enzimas além da possibilidade de ser utilizado em sistemas contínuos.

IV.2 Contribuições para o uso de nanopartículas magnéticas como suporte enzimático



Evaluation of the alternating magnetic field (AMF) influence in catalytic activities of enzymes immobilized into magnetic graphene oxide: A new approach

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Alternating magnetic field

ABSTRACT

The conventional methods of production of biofuels generate hazardous by-products, one way to reduce them, by using reusable enzymatic process that requires insoluble enzyme carriers. This investigation allowed the obtention of magnetic graphene oxide (MGO) carrier containing aminosilane functional groups with and without the crosslinker glutaraldehyde. Thus, it was possible to immobilize cellulases and lipases enzymes and evaluate its enzymatic activity in normal conditions, as well as the novelty that was under alternating magnetic field (AMF) conditions. Through enzymatic essays it was determined the optimal working activities for both enzymes using these two magnetic carriers, being pH 4 and 60°C for cellulases and pH 5 and 50°C for lipase. Different frequencies were used during AMF test (20, 30, 50 and 70 kHz) and it was confirmed for both groups that with the increase in frequency during the reaction, the less pronounced will be the activity decrease at pH values different from the optimal condition. Due to magnetic properties from the carrier and the medium reaction used the recycle test was satisfactory for the two immobilized groups of the cellulase enzyme, being recycled 9 times without losses greater than 50 % of relative activity. As for the two groups of immobilized lipases, drastic decreases in activity were observed, possibly caused by the strong enzyme/substrate interaction. For both groups of immobilized enzymes, an inhibitory behavior of the glutaraldehyde reagent, widely used as an enzyme/carrier cross-linker, was also observed. Nevertheless, the immobilization of cellulase and lipases enzymes into MGO derivatives shows a promising and suitable strategy for the conversion of cellulose and long-chain acyl esters into value-added inputs.

1. Introduction

Some countries have stood out for the strong investment and use of renewable energy sources [1,2]. Among these sources are biodiesel and ethanol, South and Central America are reported to be responsible for over 27 % of the total global of these biofuels production [3]. One way to further increase the production of these biofuels and reduce

environmental impacts due to the burning of fossil fuels is the use of enzymes during the production stage [4–6].

The enzymes cellulase and lipase can hydrolyze raw materials such as cellulose and long-chain acyl esters that allows the production of value-added inputs such as second-generation ethanol and biodiesel, respectively [4,7]. The advantages of its use lead to the reduction of hazardous chemical reagents and the generation of by-products when

Abbreviations: AMF, Alternating magnetic field; APTES, 3-aminopropyltriethoxysilane; GO, Graphene oxide; GLU, Glutaraldehyde; MGO, Magnetic graphene oxide; NP, Nanoparticles; LIP, *Candida rugosa* lipase; CEL, Celic Cetec cellulase.

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compared to conventional methods of obtaining different kind of bio-fuels [8,9].

Although the properties present in cellulases and lipases are promising for use in the production of glucose and biodiesel, there are some challenges to be overcome, related to stability, cost and reuse [2,10,11]. The first challenge encountered during an enzymatic process is the loss of the enzymes 3D conformation due to minimal pH and temperature changes and the need for a large number of enzymes to have a high hydrolysis yield during the biofuel obtention. Thus, reducing inhibition, improving the stability of enzymes and facilitating their separation from the reaction medium are aimed strategies for greater biofuel production with use of immobilized enzymes [12,13].

At the same time, the enzyme immobilization process into carriers is effective for the improvement of enzyme stability and reuse [14,15]. Among the materials that can be used as carrier, is graphene oxide (GO), which has a high surface area, superior mechanical, electrical and optical properties, biocompatibility and low synthesis cost. GO can also be associated with magnetic nanoparticles, such as magnetite (Fe_3O_4), which is a cheap metal oxide and promote the magnetic character of the carrier, allowing the separation of different immobilized enzymes from the reaction medium using an external magnetic field [16,17].

Therefore, the use of immobilized enzymes in magnetic graphene oxide can demonstrate to be a promising way for the hydrolysis of cellulose and triglycerides [18–20]. Such a strategy can lead to an increase the stability and efficiency of different enzymes, in addition to facilitating their recovery from the medium, increasing the energy and financial efficiency of the process [21–23]. Furthermore, the magnetic properties from magnetic graphene oxide (MGO) carrier also can be activated under alternating magnetic field (AMF) by Néel and Brown relaxation, allowing the immobilized system to rotate and collide in different ways during the catalytic activity [24–26].

In this way, AMF can cause a speed up into the normal enzymatic kinetic by increasing the collision and diffusion rate between the interested carrier and the enzymes, as already presented in previous work [27]. In this work and other among literature, it is reported an improvement of the enzymatic activity from lipases and cellulases immobilized, however into common iron oxide magnetic nanoparticles [28–30]. Nevertheless, the investigation of the AMF influence in MGO carriers and using different frequencies and amplitudes are still little addressed by the literature (commonly based on tests using 500–600 Hz and 1–1.2mT) [31,32]. In this approach, this paper reports the immobilization of lipases and cellulases into MGO for hydrolysis of triacylglycerols and celluloses in different conditions of AMF to investigate their catalytic activities.

2. Methodology

Commercial olive oil, all the followed reagents were purchased from Sigma-Aldrich®: iron (II) and (III) chloride (99 %) ($\text{FeCl}_2/\text{FeCl}_3$), 3-aminopropyltriethoxysilane (APTES) (97 %), 3,5-dinitrosalicylic acid, arabic gum from acacia tree, sodium carboxymethyl cellulose, alcohol, acetone, phosphate-citrate buffer, sodium hydroxide (NaOH), potassium hydroxide (KOH), hydrochloric acid (HCl), glutaraldehyde (25 %), Bradford reagent, bovine serum albumin (BSA), *Candida rugosa* Lipase, polyethylene glycol 3000 and cellulase Cellic Cetec®.

2.1. Synthesis of graphitic structures

For the preparation of graphene oxide and the magnetic graphene oxide with APTES was used, as the methodology applied in previous work [15]. In a 3-neck glass flask (250 mL) were added 1 g of graphite powder and 23 mL of sulfuric acid, stirring for 24 h to improve the oxidation process. After this period, the system was placed in an ice bath and 3 g of potassium permanganate were slowly added. Then the reaction mixture was stirred for 8 h and heated at 40 °C for 30 min. After this time, an aliquot of 50 mL of distilled H_2O was added and the

temperature was raised to 75 °C for 1 h under constant stirring. Finally, upon reaching room temperature, 150 mL of distilled H_2O and 5.0 mL of 30 % (v/v) H_2O_2 were added. The filtration process of this solution was made by a filter paper and using ultrasound as a final step the graphite powder became the purified graphene oxide (GO).

In a beaker, 30.0 mg of GO and 9.0 mL of 0.01 mol L^{-1} NH_4OH were added. An aqueous solution of iron was prepared by mixing two salts with a concentration of 0.1 mol L^{-1} of iron (1:2 of $\text{FeCl}_2/\text{FeCl}_3$). The GO in basic medium was slowly dripped onto the iron solution forming iron oxide nanoparticles on the surface of the GO. Then 1 mL of APTES was added in order to functionalize the surface of MGO with an amino group. To remove unreacted salts and alkoxysilanes, MGO was washed three times with distilled water and separated with a magnet.

2.2. Immobilization of *Candida rugosa* lipase on the magnetic carrier

A 5 mL aliquot of functionalized MGO-APTES was taken and added to a falcon tube containing 18 mL of pH 7.4 phosphate buffer. To evaluate the influence of distance between the lipases and the magnetic carrier for the enzymatic activity, two types of carriers were used, one containing only MGO-APTES on the surface and the other containing MGO-APTES and 2 mL of glutaraldehyde (25 %) named MGO-APTES-GLU.

The solutions were stirred on a vortex and then left to rest for 1 h at room temperature. They were washed with alcohol, water and buffer solution (twice with each solvent) to ensure removal of residual materials. In each immobilization, 50 mg of lipase (LIP) and 50 μL of polyethylene glycol were used. Both were added to the solution under vortex agitation for 5 min. The solution remained at rest for 16 h in a refrigerated environment. Using a magnet to separate the sample from the aqueous medium, the solution was washed 5 times with phosphate buffer (pH 7.4) to remove free lipases. These two groups of immobilized samples were classified as LIP/MGO-APTES and LIP/MGO-APTES-GLU.

2.3. Cellulase Cellic Cetec® Immobilization

To evaluate the influence of distance between Cellic Cetec® cellulase (CEL) and the magnetic carrier for the enzymatic activity, the same conditions as in the previous immobilization step mentioned in Section 2.2 were used. These samples were prepared and classified as CEL/MGO-APTES and CEL/MGO-APTES-GLU.

2.4. Characterization

To identify crystalline phases present in iron oxide nanoparticles on the surface of graphene oxide sheets, a SIEMENS® X-ray diffractometer, model D5005, Cu K α Radiation ($\alpha = 1.5418 \text{ \AA}$) was used. Measurements were made in a 2 θ . The samples were recorded from 5° to 80° of 2 θ , with a step of 0.01° per second. In order to establish information about the structural, molecular and electronic characteristics of carbonic materials, the RAMAN Bruker MultiRAM equipment was used, which has a He-Ne cooled air laser at a wavelength of 632.81 nm and excitation power of 0.17 mW. The spectra were obtained from 1000 to 1800 cm^{-1} , with an integration time of 60 s. To determine the calibration curve and quantify the presence of glucose in the reaction medium containing the immobilized cellulase, the UV-VIS equipment - UVVis Spectrophotometer GBC Cintra 303 was used. The imaging was performed using a scanning electron microscope, JEOL JSM 7500 F, with an accelerating voltage of 2.0 kV. The samples were dispersed and diluted in alcohol using an ultrasound tip, followed by a drop of 5.0 μL of the sample onto the silicon oxide substrate (SiO_2/Si). To evaluate the hysteresis and magnetic moments of iron oxide and magnetic graphene oxide nanoparticles, the MPS (Magnetic Particle Spectra) equipment (sold by Pure Devices®) was used. Different frequencies (10, 14, 20 and 30 kHz) and different amplitudes (5–30 mT) were used. The error bar present in the graphics, represent the 95 % confidence intervals and when not covered

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show a statistic significance at the $P = 0,05$ level.

2.5. Enzymatic activity of immobilized and free lipase

It was mixed, 2.5 mL of olive oil and water (1:1) and 2 mL of 0.1 mol L^{-1} Phosphate-buffered saline (PBS), with a pH range of 3–10. The range from 30° to 70 °C was used to determine the ideal temperature. The reaction system was maintained under stirring in a thermostatic bath. Then, 2 mL of the immobilized derivative were added, keeping the system under stirring at 37 °C for 5 min. After the incubation period, 10 mL of a mixture of ethanol and acetone (1:1) and 10 mL of a previously standardized KOH solution (0.05 mol L^{-1}) were added. Excess KOH was titrated with standardized HCl (0.05 mol L^{-1}). To calculate the relative enzymatic activity, Eq. 1 was used [15]:

$$A = \frac{(V_b - V_a) \times M \times x \times 10^6}{t \times m} \quad (1)$$

Where, A= Enzyme activity (U g^{-1}), Vb= Blank volume (L), Va= Sample volume (L), M=Molarity of the HCl solution (mol L^{-1}), t = Incubation time (minutes) and m=mass of added biocatalyst (g). Once the point of greatest enzymatic activity of the analyzed enzymes was determined, these values were considered as 100 % and the other values normalized by these maximum activities to obtain a graph of relative activity.

2.6. Hydrolytic activity of immobilized and free Celic Cetec © cellulase

The activity of the immobilized cellulases was determined by the microcrystalline cellulose method, being calculated from the amount of glucose resulting from the hydrolysis of the cellulosic substrate [33]. Initially, 0.5 mL of a suspension containing Celic Cetec © cellulase immobilized in one of the two types of carriers was added to 50 mg of carboxymethylcellulose and 1 mL of 0.05 mol L^{-1} citrate buffer pH 5. The resulting mixture was incubated for 1 h. The pH of the buffer and the incubation temperature were evaluated using a central composite experimental design, with five levels for each variable, and a central point in triplicate [34]. After incubation, 3 mL of 3,5-dinitrosalicylic acid was added to the mixture to stop the reaction. The samples were heated in a water bath at 100 °C for 5 min to allow color formation. Then the samples were cooled to room temperature and centrifuged for 5 min. The concentration of reducing sugars was measured by UV-VIS spectrophotometry ($\lambda = 540$ nm). A series of blanks, one with free cellulases and the other with immobilized cellulases, were measured in the same way to compensate for the presence of interferents. Enzymatic activity was measured by the amount of glucose produced (μ mol) per milligram of enzyme used for the duration of the assay, expressed in IU mg^{-1} . The relative activity was expressed as the ratio between the activity of each sample and the maximum activity presented among all samples.

2.7. Determination of enzyme loading efficiency

Enzyme loading efficiency was determined by the method based on protein loss in the solution before and after immobilization using Bradford assay [35]. Enzyme loading efficiency was calculated based on the following Eq. 2:

$$\text{Enzyme loading efficiency (\%)} = \frac{C_0 V_0 - C_s V_s}{C_0 V_0} \times 100 \quad (2)$$

where C_0 is the initial protein concentration (mg mL^{-1}), V_0 the initial volume of protein (mL), C_s the protein concentration in the total supernatant (mg mL^{-1}) and V_s is the total volume of supernatant (mL).

2.8. Influence of the alternating magnetic field (AMF) by an AC magnetic field generator

The magnetic field treatment with several amplitudes and frequencies were carried out in a homemade magnetic heating source, as

shown in Fig. 1, consisting of a signal generator combining a high-power amplifier and a matching transformer connected to resistor, capacitor and the inductor (RCL) circuit. The magnetic field was produced in between the gap of a ferrite nucleus with Litz wires windings. The field intensity was held constant at 40 mT during the measurements, and the frequency ranged from 20 to 70 kHz. This process was followed to confirm the variation of the enzymatic activity according to the rotation frequency of the magnetic nanoparticles present on the graphene oxide surface.

2.9. Operational stability

Using the optimal conditions of pH and temperature, as well as the minimum amounts of enzymes necessary for an effective magnetic separation, archived during the essays, the recycle test was evaluated in consecutive reuses in the same conditions as presented in steps 2.5 and 2.6. The four groups of immobilized enzymes were separated from the reaction medium using an external magnetic field. Samples were washed twice with phosphate buffer solution and used again in a new reaction mixture to study reuse in multiple cycles. The AMF system was not used for the operational stability due to the low heat produced magnetically, that was not enough to achieve the optimal temperature for enzymes tested.

3. Discussion

3.1. Synthesis of magnetic graphene oxide

In the X-ray diffractograms shown in Fig. 2, the sample of MGO-APTES and MGO-APTES-GLU are found and compared with the X-ray diffraction pattern of magnetite, from the crystalline structure database. The diffraction peaks of the synthesized magnetic carrier can be attributed to the Fe_3O_4 crystalline planes, confirming that the crystalline phase present on the surface of the magnetic graphene oxide is magnetite [15].

In order to confirm the presence of the carbonaceous structure from MGO, MGO-APTES and MGO-APTES-GLU, Raman spectroscopy was used to characterize conjugated carbon bonds and carbon-carbon double bonds. In the spectra of Fig. 3, two peaks were observed, D ($1200-1400$ cm^{-1}), associated with carbon-carbon vibrations that have sp^3 hybridization and G ($1500-1600$ cm^{-1}) referring to carbon-carbon vibrations with sp^2 hybridization [36]. Despite of the presence of magnetic properties from magnetic iron oxide nanoparticles added to the carrier together with aminosilane agents and with and without glutaraldehyde reagent, it was observed that the modified graphene oxide sheets did not lose their structural conformations.

The morphology of the GO sheets presenting the carbonaceous



Fig. 1. Alternating magnetic field setup.

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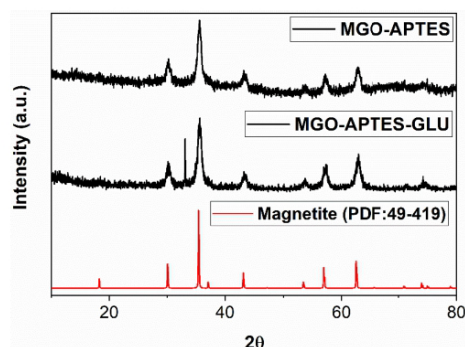


Fig. 2. XRD of the samples MGO and MGO-APTES compared with magnetite pattern.

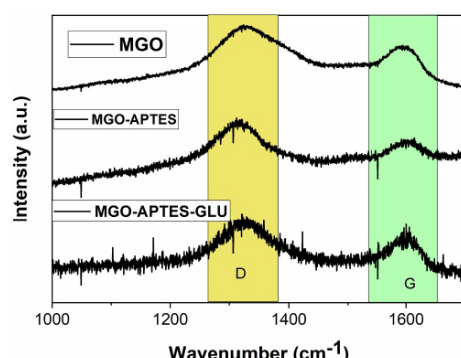


Fig. 3. RAMAN spectra of MGO, MGO-APTES and MGO-APTES-GLU samples.

structures acquired are observed in Fig. 4 A and B. These effects occur due to defects in the structure and are directly related to the degree of oxidation [37]. With the presence of oxygenated functional groups, there is an increase in interplanar spacing, making the 2D layers more distinct. The presence of iron oxide nanoparticles on the graphene surface is also found (Fig. 4 (C), (D) and (E)), although the material has undergone after many washes during the purification process, this conformation was maintained, thus determining a strong interaction between the graphene oxide sheets and the iron oxide nanoparticles cores.

A count of 100 nanoparticles present in the magnetic graphene oxide sheets was made using the ImageJ © software and the nanoparticle size distribution graph was made, Fig. 5. Through this graph it was possible to determine that the core of the particles found in the scanning electron microscopy have a diameter of $20 \pm 5,3$ nm. This demonstrates the efficiency of using nanoparticle synthesis through a simple method, such as coprecipitation, which can be used under ambient conditions and yields satisfactory results.

The magnetic moment of pure magnetite and magnetic graphene oxide samples were determined by the MPS equipment, making it possible to observe the difference in magnetic saturation of these two groups. The dynamic loops of the synthesized magnetite sample are expressed in Fig. 6 and, as found in the literature, magnetic losses due to

variations in the amplitude of the field used were shown [38]. As observed by the areas of the loops obtained, the synthesized magnetites seem to be suitable for magnetic heating, however it is also seen that the area of these loops is rapidly reduced with decreasing field amplitudes. It was also determined that the use of frequency of 20 kHz resulted in largest loop areas (when in amplitude 30mT), which confirms that the system will heat more under AMF with frequencies bigger than 20 kHz. Furthermore, although the biggest loop area is obtained at 20 kHz, which does not indicate that the system will heat the most because the heat is a product of frequency and loop area. Fig. 7 shows the calculated Néel, Brownian and effective relaxation times, indicating that the used magnetite NPs with size around 20 nm, heat by both Néel and Brownian relaxations.

Fig. 8 shows the results related to the dynamic loops of the magnetic graphene oxide samples. Although it presented the same profile as the pure magnetite samples, the MGO sample shows, some differences between the two groups of samples. Firstly, it was seen that the magnetic saturation/dissipation response of MGO is lower than synthesized magnetite, possibly due to a different magnetite concentration when compared to the pure sample. Its magnetic moment follows the employed magnetic field very well (no delay). This may occur due to the reduction of the magnetic property of magnetite, caused by the presence of diamagnetic materials such as APTES and GO sheets. It is concluded that the applied field is not strong enough to overcome the effects of the local magnetic anisotropies of the nanoparticles present on the surface of GO sheets [38].

After the magnetite NPs are attached to the graphene oxide, the Brownian relaxation of magnetite NPs under AMF is blocked due to the giant size of the graphene oxide with respect to the magnetite NPs. In this case, the heating power is reduced, which operates only with Néel relaxation. By comparing Figs. 6 and 8, it noted that in both cases using 20 kHz has the biggest loop areas, confirming that the MGO system heats more by using AMF with frequencies bigger than 20 kHz.

By comparing the hysteresis loops of magnetite NPs and MGO samples, the hysteresis loops areas of MGO samples are smaller than those of magnetite NPs samples. This is because the Brownian relaxation is blocked after magnetite NPs being attached to the surface of graphene oxide, which has higher order of size than the magnetite NPs. So, there is mainly heating effect of the MGO to the enzyme activity, whereas the mechanical effect is negligible. The heating trend of magnetite NPs at different frequencies and amplitudes are shown below in Fig. 9, which are calculated from the MPS results, and indicates that the heating power of magnetite NPs under magnetic field with higher frequencies is bigger. MGO samples heat mainly by Néel relaxation under AMF, which is more efficient using high frequencies. The heating could be used to adjust the local temperature of the enzymes, which can further affect the activity. The local movements of a big amount of magnetite NPs can transposed to the graphene oxide and then to the enzyme through the linker, which offers a new way to manipulate the enzymes.

3.2. Enzymatic loading efficiency

In Table 1 is presented the immobilization efficiency in the 4 immobilization processes used. For both enzymes used (cellulase and lipase), it was observed an enzymatic loading efficiency above 40 %, using the two magnetic carrier developed (with and without glutaraldehyde). Although the carrier enzyme efficiency is an important attribute for the immobilization process, other aspects such as reuse, enzymatic stability under pH, temperature and AMF must also be considered for an efficient performance. Besides that, even though widely used for this purpose, the Bradford protein assay, it is reported some limitations in many enzymes [39,40].

3.3. Activity of Celic Cetec © cellulase

Fig. 10 shows the relative hydrolytic activity values of the free Celic

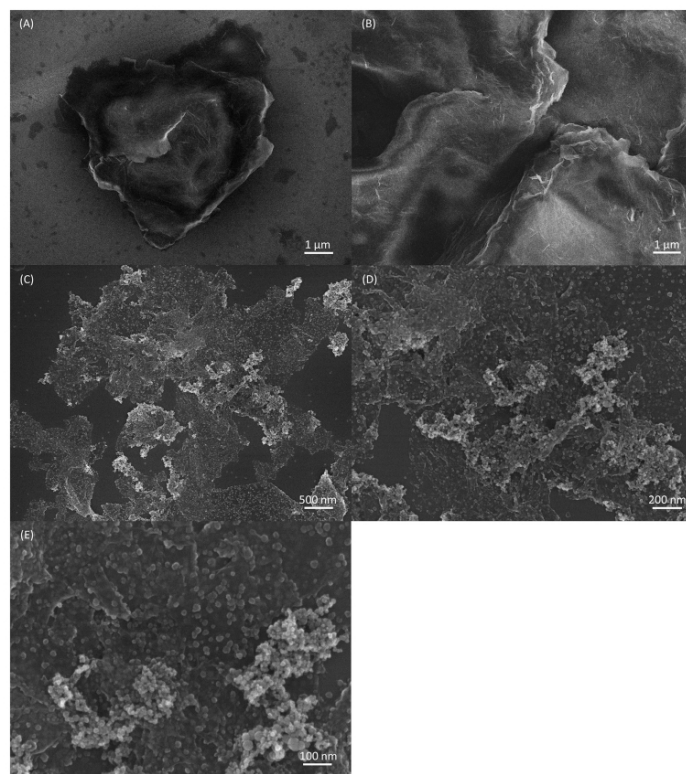


Fig. 4. Scanning electron microscopy of GO (A and B) and MGO (C, D and E) samples.

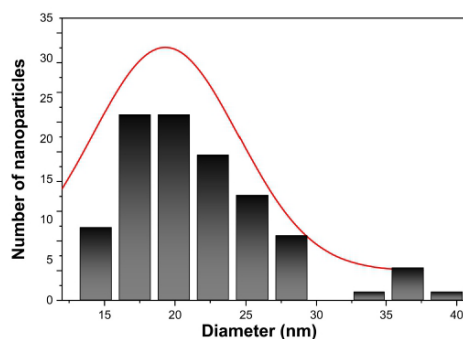


Fig. 5. Diameter of iron oxide nanoparticles present on the surface of GO sheets.

Cetec® cellulase, CEL/MGO-APTES and CEL/MGO-APTES-GLU in function of pH and temperature. It was observed for all pH conditions that the free commercial enzyme had greater activity, a phenomenon

that occurred due to the greater degree of freedom that free enzymes have, since during the immobilization process there is the possibility of enzyme/carrier binding points that can block the active site, thus reducing the activity in the reaction medium [41]. It was observed that the optimal relative activity shifted from 3 (optimal for the free) to 4 (ideal for the two immobilized groups). Between the groups CEL/MGO-APTES and CEL/MGO-APTES-GLU, a similar activity loss profiles are already observed with increasing pH value. The difference in the relative activity values obtained when compared to the free enzymes used are related to the complexity of the immobilization process. It should be considered that the magnetic carrier/enzyme interaction changes according to the immobilization method selected [33,42]. Furthermore, the confidence intervals between the immobilized cellulases are overlapping, concluding no statistical difference among them. Thus, even with the absence of the glutaraldehyde reagent, fundamental for numerous immobilization processes, does not prevent the interaction between enzyme with the developed magnetic carrier. This possibly was caused by the presence of many oxygen functional groups on the surface of graphene oxide [7,43,44]. It was observed that the optimal activity to be worked on for the 3 groups of enzymes was in 60°C and values above and below this temperature, the free cellulase group still remains superior when compared to the immobilized groups. Among the immobilized groups, a similar behavior of drastic decrease in activity was seen not only at high temperatures (above 70°C), where there is enzymatic

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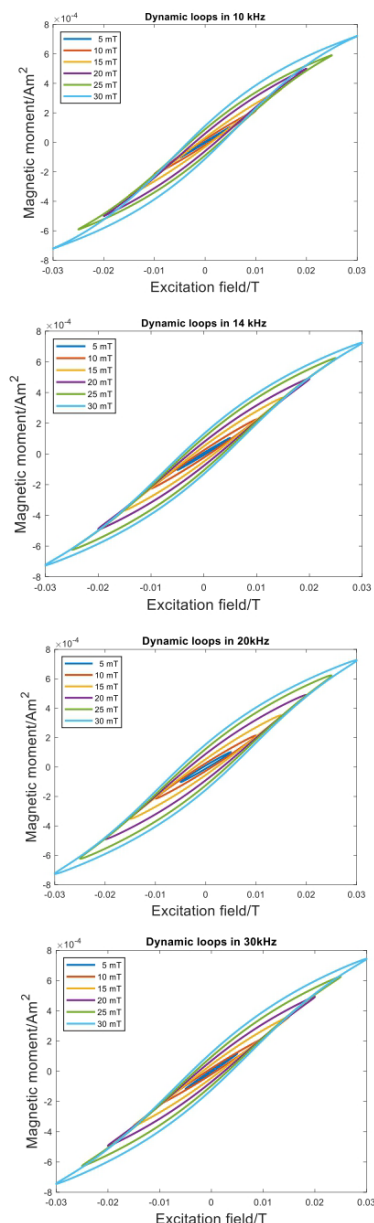


Fig. 6. Magnetic moment and hysteresis of pure magnetite samples.

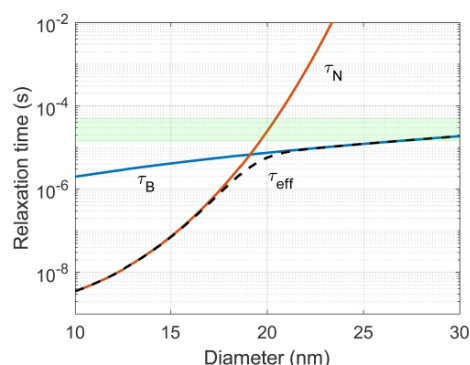


Fig. 7. Calculated Néel and Brownian effective relaxation.

denaturation, but also at low temperatures (30°C). Statistically it was seen that in 70°C the CEL/MGO-APTES presented a higher activity when compared to the CEL/MGO-APTES-GLU. Once again, this is an indication that the immobilization of cellulase on the magnetic carrier can occur without intermediate reagents, making the immobilization process even cleaner and more economical.

Figs. 11 and 12 show the hydrolytic activities of CEL/MGO-APTES and CEL/MGO-APTES-GLU as a function of the alternating magnetic field. For the three cellulase groups it was seen a similar loss of hydrolytic activity when increasing pH values. However, it was observed that with the increase of frequency used in the reaction medium, there was also a less accentuated drop-in activity, which is an indication of the need for higher frequencies that can allow enough energy to overcome the anisotropic barrier. Even at high frequencies (70 kHz) no significant temperature increases were observed in the monitored system, reaching a maximum of 28°C during the reaction. It was caused because Brown relaxation is blocked, due to the interaction of the iron oxide nanoparticles in the surface of the GO, allowing only Néel relaxation [31]. Thus, it was concluded that to achieve optimal conditions for maximum cellulase activity, it would be necessary to use even higher frequencies.

With the comparison of activity from the immobilized groups, it was observed that higher frequencies affect CEL/MGO-APTES-GLU at different monitored pH values, allowing a better enzymatic activity even in pH 8, where it should be low, due to the denaturation process. The pH value considered as the optimal activity condition was not altered in any of the frequencies used, when compared to the normal reaction conditions. Considering that the application of glutaraldehyde also serves to improve the cross-link distance between enzyme and carrier, the enzymes containing the cross-linker had greater mobility during the reaction process. With the application of the alternating magnetic field, this mobility was increased even more, consequently making it possible for the hydrolysis of the substrate in the active site to occur more easily. The CEL/MGO-APTES group had a closer enzyme/carrier interaction, resulting possible in a spatial impediment between the active site and substrate, having consequently a lower hydrolytic activity [32].

In the experimental conditions defined as optimal (pH 4 and temperature 60°C), the recycling study was carried out for Celic Cetec © cellulase immobilized with and without glutaraldehyde, as shown in Fig. 13. Being an operational challenge, although free cellulase presents greater relative enzymatic activity, its recycling is naturally not possible, which is why its comparison is not found together with the two groups immobilized into MGO. It was observed during this step that both groups could recycle up to 9 times without significant losses (up to 50 %), resulting from the immobilization of cellulase on the insoluble carrier of magnetic graphene oxide, which could be removed from the reaction

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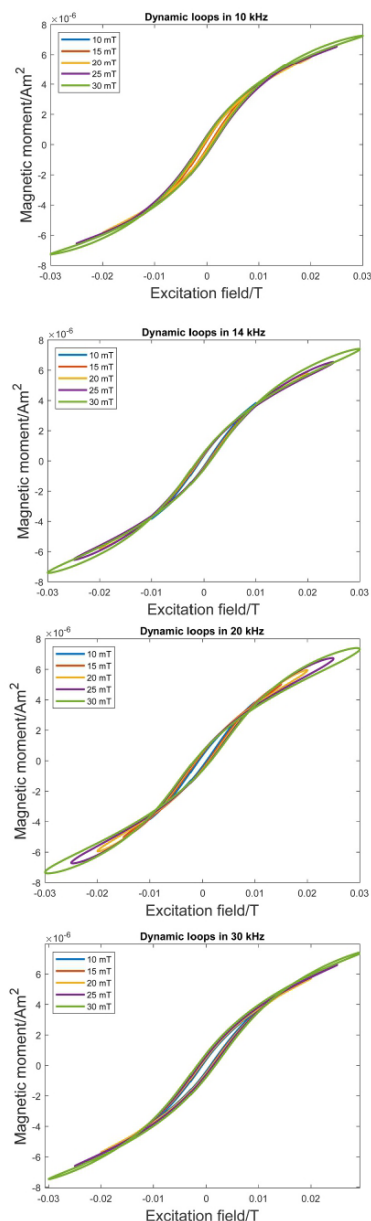


Fig. 8. Magnetic moment and hysteresis of MGO samples under different frequencies.

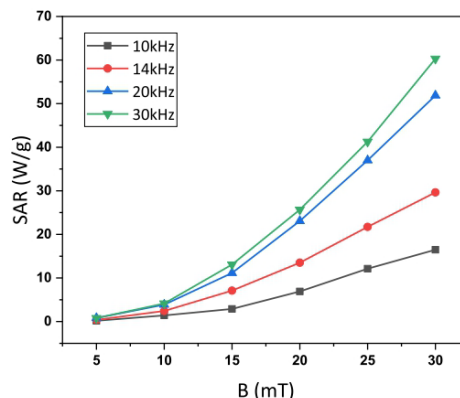


Fig. 9. Heating trend of magnetite NPs.

Table 1

Cellulase and Lipase loading efficiency by Bradford protein assay.

Magnetic carrier	Enzymatic loading efficiency (%)
CEL/MGO-APTES	44.93 %
CEL/MGO-APTES-GLU	47.27 %
LIP/MGO-APTES	40.51 %
LIP/MGO-APTES-GLU	42.46 %

medium with the use of a magnet and then used in a new cellulose hydrolysis process. By the confidence interval it was evaluated that CEL/MGO-APTES-GLU had a much greater loss of activity with increasing reuse, when compared to CEL/MGO-APTES, that showed higher activities in several points of the reuse essay.

In the literature, studies confirm, although it is a widely used reagent in the immobilization process by the cross-link method in various types of enzymes, glutaraldehyde is a toxic compound [45,46]. This reagent used for the immobilization was added without prior treatment, as describe in other works [47,48], however, the sample CEL/MGO-APTES-GLU did not prove an effective synergism during the reuse attempt. Thus, it was confirmed that the interaction formed between enzyme/carrier from CEL/MGO-APTES samples, occurred through electrostatic interactions, as well as, with the oxygenated functional groups present in the MGO sheets (hydroxyl, epoxy, carboxyl).

3.4. Activity of *Candida rugosa* lipase

Fig. 14 shows the graph of relative activity as a function of pH and temperature, of free *Candida rugosa* Lipase, LIP/MGO-APTES and LIP/MGO-APTES-GLU. The ideal condition for the 3 enzymes was at pH 6, and above this value there is a drastic loss of activity for all, confirming the sensitivity of this lipase of fungal origin. At values lower than pH 6, a decrease in activity is also observed, but much less pronounced and reaching a difference of up to 60 % of activity decrease (for free enzyme) and close to 35 % in the groups immobilized with and without glutaraldehyde. No statistical differences were observed between the immobilized groups. Besides that, the presence of aminosilane groups from APTES, which are slightly basic, helped to prevent negative conformational changes in acidic media, differently from the higher drop of free lipase sample [49,50]. Similarly, to the behavior observed between comparisons of free and immobilized cellulase activity, is seen for the lipases, the interaction carriers/lipase can decrease the activity,

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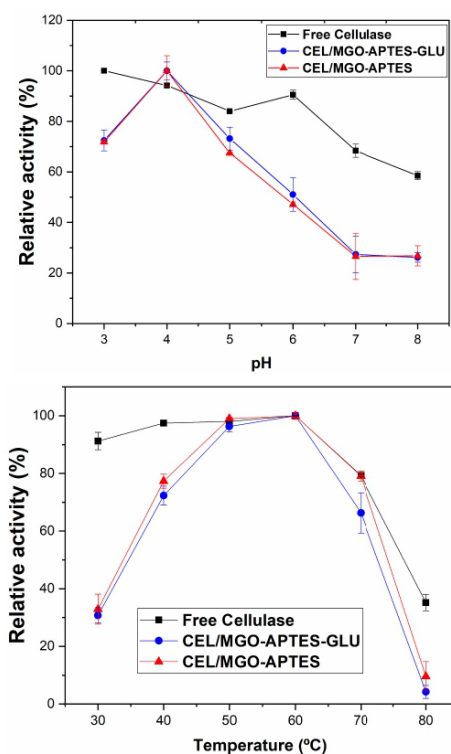


Fig. 10. Enzymatic activity of free cellulase, CEL/MGO-APTES and CEL/MGO-APTES-GLU in function of pH and temperature.

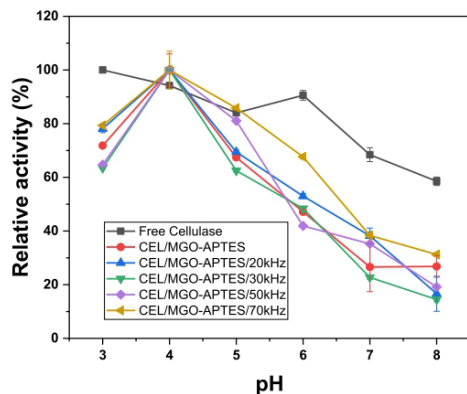


Fig. 11. Hydrolytic activities of CEL/MGO-APTES and Free cellulase in function of the AMF.

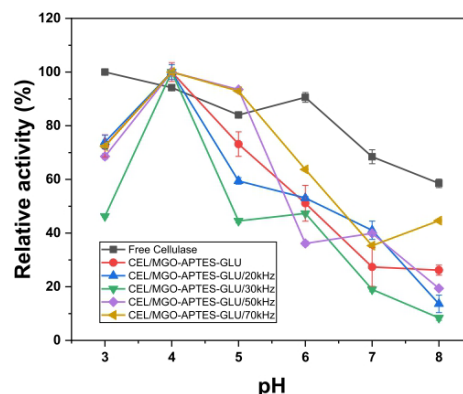


Fig. 12. Hydrolytic activities of CEL/MGO-APTES-GLU and free cellulase in function of the AMF.

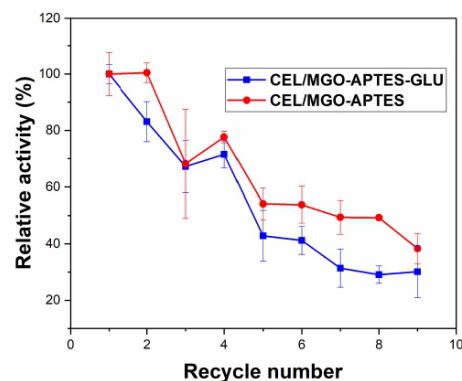


Fig. 13. Recycle test of CEL/MGO-APTES-GLU and CEL/MGO-APTES samples.

consequently caused by the occlusion of the active site in multiple points of the enzymatic structure, but on the other hand, it can bring benefits such as magnetic recovery [33,42].

In the 3 groups it was observed that the temperature of 50°C was the optimal condition to be worked on and the free enzyme had greater activity in the entire range of temperatures used. The LIP/MGO-APTES-GLU in this case also had a higher activity in 60°C, when compared to LIP/MGO-APTES. Values above 70°C completely denature the 3 groups.

The graphs in Figs. 15 and 16 are showed the hydrolytic activities of free *Candida rugosa* Lipase, LIP/MGO-APTES and LIP/MGO-APTES-GLU in function of the alternating magnetic field. The observed activities followed the same trend of loss of activity, when they deviate from the optimal condition found for the pH (pH 5), however it was seen that also for higher frequencies (70 kHz) the loss of activity is smaller. A carrier with only iron oxide nanoparticles, minor frequencies (500 – 600 Hz) are enough to improve the catalytic condition for immobilized lipases, but due to the large surface area from GO, the magnetic field applied in this work had to be bigger [51,52]. For the LIP/MGO-APTES, it is also possible to say that frequencies, such as, 70 kHz the relative activity is statistically higher at different pH values (pH 3, 4 and the optimal

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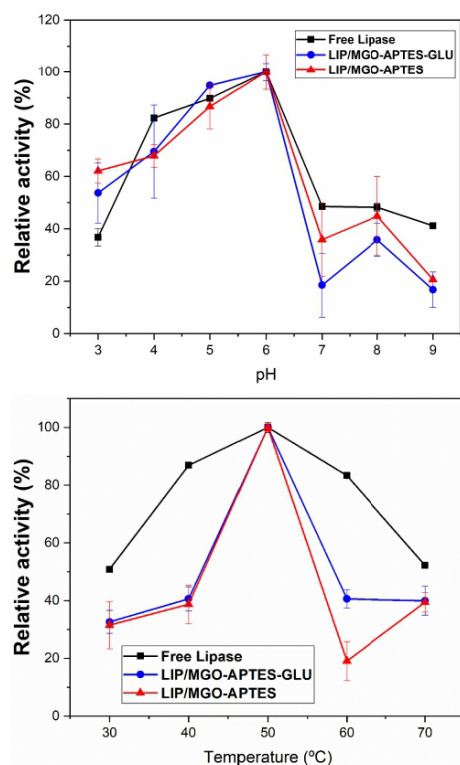


Fig. 14. Hydrolytic activities of LIP/MGO-APTES, LIP/MGO-APTES-GLU AND free lipases in function of pH and temperature.

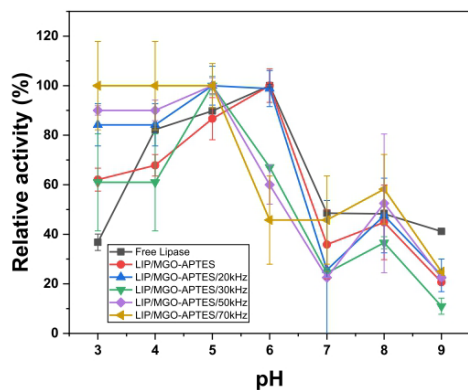


Fig. 15. Hydrolytic activities of LIP/MGO-APTES and free lipase in function of the AMF.

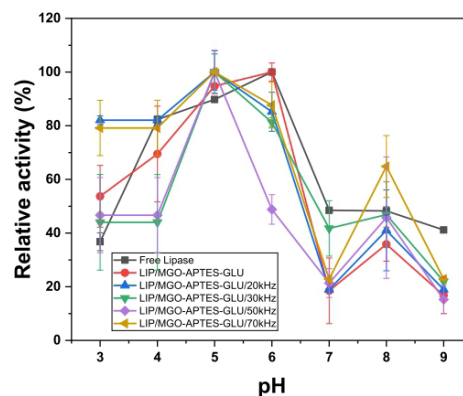


Fig. 16. Hydrolytic activities of LIP/MGO-APTES-GLU and free lipase in function of the AMF.

condition at pH 5), caused by the easier diffusion in the reaction catalyzed. Deviations were seen in both immobilized groups that statistically make the results at each pH similar, occasionally caused by the medium used. Lipases activities takes place at the water/oil interface, a more complex reaction medium when compared to that used for the cellulase enzyme, which was only aqueous.

Using the experimental conditions defined as optimal (pH 5 and 50°C), the recycling study was also carried out for LIP/MGO-APTES and LIP/MGO-APTES-GLU, as shown in Fig. 17. After removing the immobilized enzymes from the reaction medium and adding in new medium, a drastic drop-in activity for this lipase (with and without glutaraldehyde) was observed, with a yield of less than 40 %, when compared to the activity in cycle 1. It was confirmed a higher enzymatic activity for LIP/MGO-APTES, testifying the capability of an effective immobilization of lipases without glutaraldehyde. Unlike our previous work with immobilization of lipase with magnetic graphene oxide, the magnetic recycle technique in this situation was not satisfactory [15]. One of the hypotheses raised for this type of result may be the regioselectivity of the lipase used, with *Candida rugosa* lipase being a non-specific enzyme, that is, it hydrolyzes the esters present in triacylglycerol in any position.

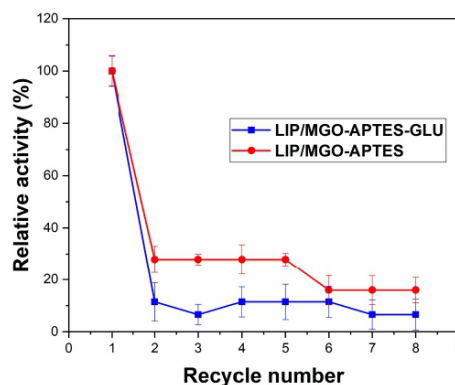


Fig. 17. Recycle test of LIP/MGO-APTES-GLU and LIP/MGO-APTES samples.

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This makes its interaction with the substrate to be greater, thus making it difficult an effective separation between the immobilized enzyme and the reaction medium.

4. Conclusions

It was concluded that an efficient synthesis of the magnetic graphene oxide carrier is possible, with the addition of graphene oxide nanoparticles through the coprecipitation method. Physical-chemical characterizations such as Raman and XRD confirmed not only the presence of the nanoparticles of interest, but also the carbonaceous structure in the synthesized graphene sheets. It was possible to immobilize lipases and cellulase on the proposed magnetic carrier with and without the glutaraldehyde cross-linker. Through enzymatic activity tests it was possible to determine the optimal working conditions for both enzymes, being for cellulase pH 4 and 60°C and for lipase pH 5 and 50°C.

The external magnetic field was used to assess differences in activity and for both groups of enzymes it was confirmed that with the increase in frequency during the reaction, the less pronounced will be the activity decrease at pH values different from the optimal condition (70 kHz being the frequency maximum used for this work). The recycle test was satisfactory for the two immobilized groups of the cellulase enzyme, being recycled 9 times without losses greater than 50 % of relative activity. As for the two groups of immobilized lipases, drastic decreases in activity were observed, possibly caused by the strong enzyme/substrate interaction. For both groups of immobilized enzymes, an inhibitory behavior of the glutaraldehyde reagent, widely used as an enzyme/carrier cross-linker, was also observed. It is therefore concluded that the magnetic graphene oxide carrier is promising for the immobilization of different enzymes and to make this method cheaper and more efficient.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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Magnetic Graphene Oxide as a Carrier for Lipases Immobilization: An Approach for Hydrolysis of Olive Oil Emulsion

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The use of lipases in industrial processes has increased due to its ability to catalyze total or partial hydrolysis reactions of fatty acids. However, due to the difficulty of isolation and purification of lipases, as well as its separation from the reaction medium, it becomes an expensive input. In this study, graphene oxide was used as carrier for enzymes. The magnetic properties of the carrier were obtained through decoration with magnetic iron oxide nanoparticles on graphene oxide surface. XRD was used to elucidate the structure and morphology of the compounds obtained. By measuring hydrolytic activity, it was possible to determine the optimum pH and temperature for three different types of immobilized lipases, being 37 °C and pH 8 (porcine pancreatic lipase), 50 °C and pH 6 (*Candida rugosa* lipase), and 40 °C and pH 9 (*Aspergillus niger* lipase). The enzymatic recovery test, during five minutes each, showed the immobilized porcine pancreatic lipase can be recycled six times without significant loss of catalytic activity. It was confirmed the efficiency of the magnetic graphene oxide as a carrier for different lipases allowing its use for transesterification process in mild temperature and pH conditions.

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Enzymes are highly specific and selective natural biocatalysts suitable for industrial applications.^{1,2} However, some enzymes have low yield extraction and difficult purification procedures, which compromises their stability and catalytic activity.^{3,4} In this way, to improve these properties, different materials have been studied as a carrier for specific enzymatic immobilization.⁵

To enable an efficient carrier/enzyme interaction, the choice of a suitable immobilization process would improve the enzymatic activity, the thermal and storage stability, high amount of carried enzyme and its reuse.^{6,7} Through an activating agent (or crosslinker) it is possible to promote a covalent bond between carrier/enzyme, avoiding the loss of the immobilized enzyme throughout the recycling process (that can happen by filtration, centrifugation or magnetic separation).⁸

For the effectiveness of the immobilization process, the selected carrier must have adequate properties such as high surface area for the efficiency of immobilization. Among these materials, it is possible to mention the widely used carriers based on graphite derivatives, for example graphene oxide, associated with nanoparticles, which applications have increased in sectors such as water waste⁹ and heterogeneous catalysis.¹⁰ Graphene oxide (GO) is a two-dimensional material composed of hexagonally arranged carbons that, due to the exceptional thermal, mechanical, electrical and high surface area characteristics, has resulted in many applications.^{11,12} The structure of the GO shows reactive functional groups, such as hydroxyl, carboxylic and epoxy groups, which presence makes feasible interactions with other materials that have different properties, such as magnetic nanoparticles.^{13,14}

The magnetic properties of iron oxide nanoparticles (MNP) are reported in several investigations,^{15–17} resulting in a lower cost synthesis when compared to other metal oxides.^{16,18,19} Decoration of the GO sheets with MNP had also been studied in order to obtain magnetic graphene oxide (MGO).^{20–22} The GO characteristics such as low toxicity, thermal stability and hydrophilic property, when

combined with magnetic properties from MNP on its surface, become a potential composite for relevant studies and new applications.^{23–25}

Related to new technologies in industrial processes, there is the use of lipase enzymes as biocatalysts, being used due to its ability to break triglycerides bonds into free fatty acids in reactions that can produce specific or non specific esters, such as transesterification, esterification and interesterification.²⁶ Their use allows reactions in reduced temperatures and pressure and can be applied for treatment of fatty wastes, food industry, ester synthesis, biodiesel production, however the high solubility of lipases limits their recovery in reaction processes.^{27,28}

With this, the use of MGO as carrier for enzymes is reported to be advantageous for ensuring their reuse (up to 8 recycles for Horseradish peroxidase with 68% of its initial activity²⁹) and also improving their catalytic efficiency in different conditions (Chloroperoxidase with more than 60% of its initial activity in 50 °C³⁰) if compared to the free enzymes. However, studies involving the use of MGO with lipases are limited and generally only associated with porcine pancreatic lipase,^{31,32} thus restricting the possibilities of applications to other sources of lipases, which are also widely applied in chemical, food and pharmaceutical sectors.⁴

Thereby, this work describes the novelty of the use of graphene oxide decorated with MNP as a carrier for the immobilization of different types of lipase to confirm its easy and advantageous interaction for the hydrolysis of olive oil emulsion. The catalytic performance of three immobilized lipases (Porcine pancreatic, *Candida rugosa* lipase and *Aspergillus niger*) were studied and compared to the free ones to access ideal pH and temperature conditions for transesterification reactions. Furthermore, the enzyme reuse was also evaluated to exploit the efficiency of magnetic separation of the immobilized enzyme onto MGO carrier.

Experimental section

Materials.—Without none previous treatment the following reagents were used: ferric chloride hexahydrate (FeCl₃·6H₂O,

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Figure 1. Digital photo of the magnetic graphene oxide synthesized dispersed in water. (A) absence and (B) the presence of a magnetic field.

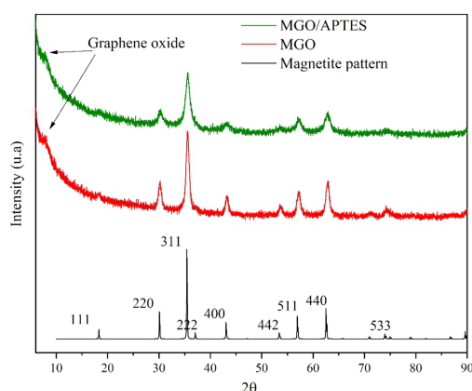


Figure 2. X-ray diffractogram comparing the magnetite pattern with magnetic graphene oxide and functionalized magnetic graphene oxide.

99%), ferrous chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 99%), Polyethylene glycol 1000 (PEG 1000, $\sim 1000 \text{ g mol}^{-1}$), 3 (amino-propyl) triethoxysilane (APTES, 97%), graphite powder +100 mesh ($\geq 75\% \text{ min}$), potassium permanganate (KMnO_4 99%), sulfuric acid (98%, Sigma), hydrogen peroxide (H_2O_2 30% v/v), absolute ethanol, acetone (99.9%), glutaraldehyde (GA) (25% in H_2O) and Porcine pancreas lipase (PPL) (triacylglycerol acyl hydrolase), lipases *Aspergillus niger* (ANL), *Candida rugosa* (CRL) were purchased from Sigma-Aldrich. Commercial soybean oil was obtained from a local grocery store.

Preparation of graphene oxide.—Graphene oxide (GO) was prepared according to the modified Hummers method.³³ Prepared by mixing, 1 g of graphite powder with 23 ml of sulfuric acid and 3 g of potassium permanganate, under constant stirring (150 rpm) and 150 ml of deionized water and 5.0 ml of H_2O_2 (30% v/v) were added. The filtration process was performed by a typical filter paper and using ultrasound.

Synthesis of magnetic graphene oxide (MGO).—30 mg of GO was dispersed in 9.0 ml of $0.01 \text{ mol l}^{-1} \text{ NH}_4\text{OH}$. After 20 ml of an aqueous solution was prepared with ratio 1:2 $\text{FeCl}_2/\text{FeCl}_3$ with a concentration of 0.1 mol l^{-1} . The solution containing GO and NH_4OH was slowly added to the iron salts solution, allowing iron oxide precipitation on the GO surface.^{34,35} Then, 1 ml of APTES was added and the sample heated at 55°C for 15 min to functionalize the MGO surface.³⁶ To remove all the reacted composite

functionalized with APTES the sample was washed twice with DI water using a magnet.

Immobilization of lipases.—A 5 ml aliquot of MGO/APTS was added to 18 ml of phosphate buffer pH 7.4. Subsequently, 2 ml glutaraldehyde (25%) was added; the solution was stirred and then rested for 1 hour at room temperature. After the sample was washed with ethanol, water, and a buffer solution (twice with each solvent) to ensure removal of glutaraldehyde. After 50 mg of PPL and 50 μl of polyethylene glycol were weighed, the same process was made with CRL and ANL. Each of the lipases was added to the solutions and then stirred for 5 min. The samples were kept for 16 h in a refrigerated environment (10°C). Using a magneto, the solution was washed 5 times with phosphate buffer (pH 7.4) for removal of free Lipase.

Enzymatic activity assay.—The free and immobilized lipase hydrolytic activities were determined using olive oil hydrolysis method.³⁷ Each lipase was mixed with 2.5 ml of olive oil and water (1:1) and 2 ml of phosphate buffer 0.1 mol l^{-1} . The reaction system was kept under stirring in a thermostatic bath (varying to 37 until 70°C) for 5 min. After the incubation period, 10 ml of a mixture of ethanol and acetone (1:1) was added to stop the reaction. 10 ml of KOH solution (0.05 mol l^{-1}) was added dropwise for the titration of free fatty acids, which were released by lipase catalysis. The excess of KOH was titrated with HCl (0.05 mol l^{-1}). It was investigated the influence of pH (3 to 10) and temperature (37 to 70°C) under the enzymatic activity of the immobilized lipase and compared with the free lipase at the same conditions. To calculate the relative enzymatic activity the Eq. 1 was used.³⁷

$$A = \frac{(V_b - V_a) \times M \times 10^6}{t \times m} \quad [1]$$

Where, A is the enzyme activity (U/g), V_b is the Blank volume (L), V_a is the sample volume (L), M is the molar solution of HCl (mol l^{-1}), t is the incubation time (in minutes) and m is the weight of the added biocatalyst (g).

Reuse.—At optimum pH and temperature conditions determined by step 2.5, as well as the minimum amounts of lipase necessary for effective magnetic separation, was assessed in consecutive reuses. The immobilized lipase was separated from the emulsion using an external magnetic field. The samples were washed twice with phosphate buffer solution and used again in a new reaction mixture, during 5 min, to study reuse in multiple cycles.

Characterizations of the composite.—Fourier transform infrared (FT-IR) spectra were measured on a VERTEX 70 spectrometer from BRUKER with ATR module (Mid-infrared absorption spectroscopy by attenuated total reflection (diamond crystal), 64 scans were performed and a resolution of 4 cm^{-1} in the spectral range of $4000\text{--}400 \text{ cm}^{-1}$. TGA was carried out in a METTLER TOLEDO SCSTARe System, with a heating rate of $10^\circ\text{C min}^{-1}$, in the temperature range from 25 to 800°C in synthetic air atmosphere. The XRD measurements were obtained by the Diffractometer SIEMENS model D5005, Cu $K\alpha$ radiation ($\lambda = 1.5418 \text{ \AA}$) and each sample was scanned from 5° to 80° , with a step size of 0.01° per second.

Results and Discussion

Synthesis and characterization of MGO.—All the characterization of the GO precursor can be found in the supplementary data (available online at stacks.iop.org/JSS/10/065008/mmedia). Figure 1 shows the macroscopy behavior of magnetic graphene oxide sample in the presence and absence of an external magnetic field. With the application of an external magnetic field (magnet), the magnetic graphene oxide was separated from aqueous media, confirming the

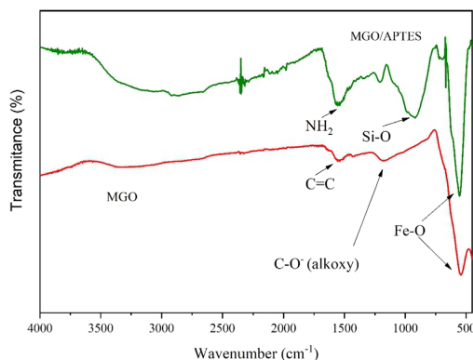


Figure 3. Spectra in the Infrared region of the MGO and MGO/APTES samples.

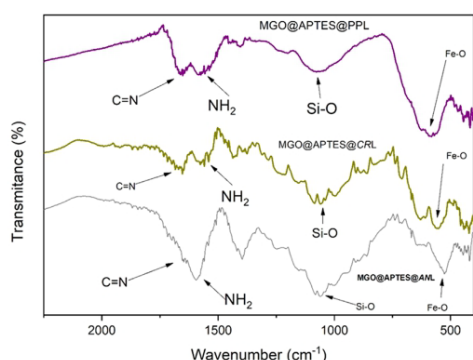


Figure 4. Spectra in the Infrared region of the immobilized PPL (A), CRL (B) and ANL (C).

presence of magnetic material adhered to the graphene oxide sheets. The acquired magnetic characteristic is attributed to the modification of the carrier surface with the addition, by the coprecipitation method, of magnetic iron oxide nanoparticles.³²

The crystalline phase of the iron oxide nanoparticles modifying the sheets of GO was evaluated by XRD. Figure 2 shows the diffractograms of magnetic graphene oxide and magnetic graphene oxide with APTES samples, as well as the X-ray diffraction pattern of magnetite, PDF N° 65-3107. The diffraction peaks of samples, found at 18.24°, 30.14°, 35.42°, 42.99°, 53.50°, 56.93°, 62.44° and 74°, can be respectively attributed to the crystalline planes of Fe₃O₄ (111), (220), (311), (400), (442), (511), (440), (533), that is in accordance with the magnetite pattern and confirming the presence of iron oxide nanoparticles.³⁸ In both samples, diffraction peaks at 8.07° are also observed, it is characteristic of graphene oxide sheets and related to the graphitic structure, present in the carrier. It is also confirmed that even though functionalization procedure with APTES is required for a covalent bond with lipase, the added aminosilane agents do not change the Fe₃O₄ crystal structure.

The main functional groups present in the MGO and MGO/APTES samples were evaluated by infrared spectroscopy, as shown in Fig. 3. The sample containing MGO has a 1620 cm⁻¹ band attributed to C=C present in the structure graphene sheets, the band at 1224 cm⁻¹ associated with C-O-alkoxy group, a ~ 560 cm⁻¹

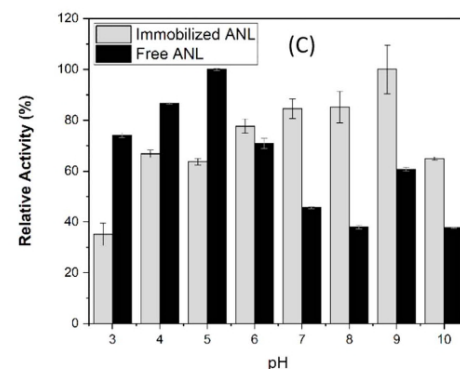
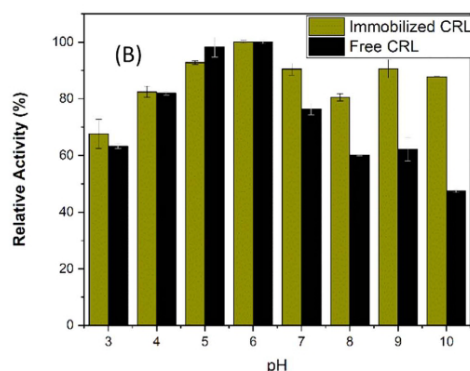
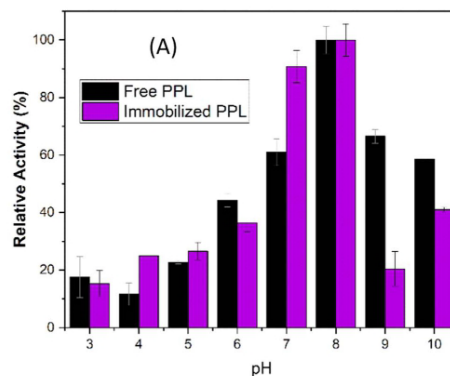


Figure 5. Influence of pH in the enzymatic activity of PPL (A), CRL (B) and ANL (C).

band is assigned to the Fe-O bonds of the magnetic nanoparticles. The MGO/APTES sample has a band at 552 cm⁻¹ also assigned to the Fe-O bond from nanoparticles; differing from the non-functionalized sample due to the presence of a band at 1000 cm⁻¹ attributed

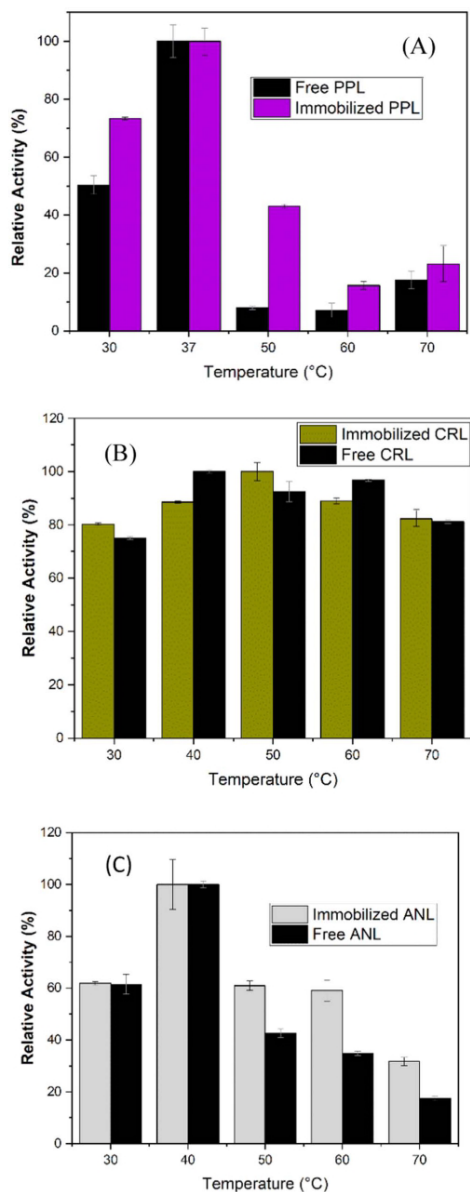


Figure 6. Influence of temperature in the enzymatic activity of PPL (A), CRL (B) and ANL (C).

to the Si-O vibration and another band at 1545 cm^{-1} which is associated with the NH_2 group. These bands are due to the presence of the aminosilane molecule.³⁹ Therefore, the presence of these

bands confirms that the surface of the graphene oxide sheets was coated with iron oxide nanoparticles and APTES.

Characterization of immobilized lipase.—To confirm the presence of the covalent bond in the studied lipases (PPL, CRL and ANL), on the magnetic carrier, a spectroscopy was performed in the infrared region, shown in Fig. 4. In the spectra of these samples bands are found close to 550 cm^{-1} (Fe-O bonds of the nanoparticles); bands were also observed in the regions of $1690\text{--}1640\text{ cm}^{-1}$, of imine bonds, functional group containing the carbon-nitrogen double bond, product of the reaction of primary amines, present in the lipases, with the carbonyl group of the aldehyde found in the crosslinking agent glutaraldehyde.⁴⁰

Effect of pH on enzymatic activity.—In order to verify the potential of magnetic graphene oxide as a carrier for lipases immobilization, it was used to immobilize different types of lipases: PPL (animal source, sn1,3-specific) and the CRL and ANL (fungal source, non specific and sn1,3-specific, respectively).⁴¹ Free lipases in general show enzymatic activity in the pH range 4 to 11 and the optimum hydrolytic activity for free PPL occurs in alkaline medium, having an attractive industrial application due to its accessibility (of cost and suppliers).⁴² Fungals lipases, CRL and ANL, present optimum hydrolytic activity in acids and neutral mediums, being used in various applications, such as, textile, biosensors, esters synthesis and widely used in detergents industry.⁴³

Figure 5 shows the relative hydrolytic activity values in function of pH for free and immobilized PPL, CRL and ANL. The tests were performed at a fixed temperature of $37\text{ }^\circ\text{C}$. The immobilized PPL (Fig. 5A) showed similar relative activity with its free lipase tests, proving that even with the immobilization process, the enzymatic behavior is kept close to the biocatalyst conditions in the free form. It is observed that for the two samples with PPL pH 8 is ideal for a more effective activity. The influence of the carrier can be seemed in pH 7, where for free PPL the hydrolytic activity decays dramatically and for the carried PPL there is only a 10% loss of activity, thus increasing the pH range to work with PPL, when immobilized.

In CRL essays (Fig. 5B) the enzymatic behavior of the immobilized CRL is, in most of the pH values, superior when compared to the free enzyme. Both enzymes showed excellent hydrolytic activity at pH 6. Free CRL at lower pH showed hydrolytic activity very closed to immobilized lipase, due to the amino groups of its structure, which are slightly basic in nature and prevent conformational changes in acidic medium. In an alkaline medium, free lipase quickly loses its activity because it is no longer protonate.⁴⁴ The immobilized CRL showed great hydrolytic activity even with pH variation, promoted by the covalent bonds with the carrier, which ensures protection in alkaline medium, thereby improving enzymatic behavior at higher pH values.^{32,44}

The third pH-relative hydrolytic activity assay was performed on free and immobilized ANL, as shown in Fig. 5C. The immobilized enzymes showed optimal catalysis conditions at pH 9, while the free enzymes at pH 5. It is observed that immobilization once again retarded the denaturing effect of ANL with increasing pH value. This feature is also analyzed in other ANL immobilization studies.⁴⁵ The best enzymatic behaviors of free lipases in acidic media can similarly be compared to the other lipases studied, being attributed to the natural protection from the amino groups present in their structure and being lost with the increase of the alkalinity of the medium. This higher resistance at lower pH values also occurs due to the enzyme origin, with lipases from *Aspergillus niger* fungus having better catalytic activity from pH 3 to 6.⁴⁶ On the other hand, the immobilized enzymes showed a good hydrolytic activity regarding the variation of the pH, even higher than free enzyme, maintaining at least 60% of relative activity from pH 4 to 10.

Effect of temperature on enzymatic activity.—Due to its specificity and for being mesophilic enzymes, free PPL and ANL are lipases that allow the formation of products with different

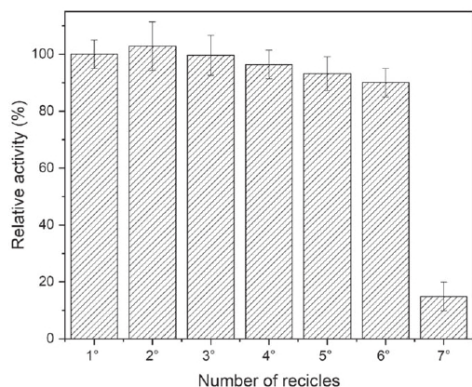


Figure 7. Reuse of the immobilized PPL.

compositions than those obtained by chemical interesterification methods and showing optimum temperature near 40 °C, having great potential in food industry for the development of new structured lipids.^{42,43} The non specificity and thermophilic characteristics of CRL enables the formation of the same products of the chemical methods, but with an advantage of a mildest reaction temperature (compared to the same reaction in ideal chemical conditions), thus being a suitable enzyme for biodiesel production applications.⁷⁷

In Fig. 6, the relative enzymatic activities of immobilized and free lipases are compared as a function of temperature and these essays occurred in their optimum pH condition. It is observed in Fig. 6A an optimum temperature for both PPL enzymes at 37 °C, as determined in other studies using the same type of porcine lipase.⁴⁸ The great loss of catalytic activity is observed increasing the temperature. Being the PPL a protein material from mammals, its optimum temperature is comprising between 35 and 40 °C, thus being more sensitive to values above this range. At high temperatures, the disruption of the three-dimensional structure of lipase reduces the enzyme activity.^{48,49}

Figure 6B contains CRL assays, performed at a fixed pH of 6, which was the optimal pH for this immobilized enzyme (as well as the free enzyme in this situation). For the free CRL, the highest hydrolytic activity temperature was 40 °C, while for immobilized CRL the best activity was obtained at 50 °C.

To ANL sample (Fig. 6C), the optimum relative activity condition was given for both enzymes at 40 °C. It is observed that the temperature increase drastically decreases the activity of free ANL, due to the mesophilic characteristics of the fungus *Aspergillus niger*. It was noted that the immobilized lipases showed superior enzymatic behavior, having in all studied temperature range (30 to 70 °C) relative activity above 30%.

Evaluation of recyclability of immobilized lipases.—Even though each immobilized lipase studied showed satisfactory catalytic activity under specific pH and temperature conditions, PPL was chosen as a model for the reuse test due to its low cost compared to the other lipases and the advantage of catalyzing reactions that form structured lipids, a product with high added value in the food industry, as already mentioned. In general, it should take into consideration that the transesterification process, common in the pharmaceutical industry, treatment of fatty waste or new food production, for example, requires chemical procedure using very acidic or basic reagents for the desired reactions to take place.⁵⁰ With a strategy to improve this situation, milder reaction media conditions, enabled by immobilized PPL and having high hydrolytic activity in neutral media, are advantageous.

In this way, using its optimum pH and temperature, as well the time and reaction medium conditions mentioned in section 2.6, the reuse was studied for the PPL. This process was repeated until the significant loss of activity, as shown in Fig. 7, which is a normalized graph using the initial activity as the 100%. In six cycles, the activity of the immobilized lipase gradually decreases, but it is still a high activity (90,1%), after that, there was a drastic loss of enzymatic activity due to the denaturation of the lipase and its loss of surface agents of the carrier through depletion mechanisms.⁵¹

The high solubility of lipases is the main challenge encountered for their recovery from the reaction medium, so the immobilization of these enzymes in materials that facilitate their separation is fundamental to promote their use in numerous production processes.³² The tests performed on the enzyme PPL immobilized on magnetic graphene oxide confirmed not only its increase in thermal stability compared to free PPL, but also enabled their recycling.

Conclusion

In this work was shown the use of MGO as a carrier to immobilize PPL, CRL and ANL. The characterizations would confirmed each proposed stage of this study as the oxidation and exfoliation of the graphite powder. The functionalization of the graphene oxide sheets with iron magnetic nanoparticles cores was also confirmed using XRD, FTIR techniques. Hydrolytic tests of the different lipases (LPP, LCR and LAN) immobilized on the magnetic carrier synthesized through covalent bonds, indicated the optimum temperature and pH conditions for the catalytic activity of each studied lipase, these being 37 °C and pH 8 for the LPP, 50 °C and pH 6 for LCR and 40 °C and pH 9 for LAN. The enzymatic behavior of the carried enzymes was satisfactory, thus presenting the advantage for using the biocatalyst in alkaline media, due to the protection guaranteed by oxidated graphitic structure of the carrier. The use of an external magnetic field showed a suitable strategy to separate the immobilized PPL from the reactor along the recycling study, reaching at least 6 recycles steps with a nonsignificant loss of activity. The use of lipase onto MGO composite might be a sustainable strategy for structured lipids production.

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Research articles

Synthesis and characterization of magnetic cross-linked enzyme aggregate and its evaluation of the alternating magnetic field (AMF) effects in the catalytic activity



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ABSTRACT

A magnetic cross-linked enzyme aggregate (MCLEA) of cellulases and a non-magnetic analogue (CLEA) were synthesized and further investigated. The obtained MCLEA and CLEA showed a high enzyme loading efficiency of 90% and 88%, respectively. However, the MCLEA presented an activity 33% higher than the CLEA one, suggesting a catalytic enhancement effect as a result of magnetic nanoparticle presence in cross-linked cellulase aggregates structure. In addition, MCLEA shows an unusual behavior with highest enzymatic activity at low temperatures near to 37 °C. Lastly, a full factorial design (2x2) with two variables at two levels, frequency (203–420 kHz) and amplitude (3–6 kA m⁻¹), was used in order to better investigate the effects of an alternating magnetic field (AMF) on MCLEA activity. AMF application decreased MCLEA activity, particularly in the highest values of frequency and amplitude, when compared to condition field free. In addition, frequency implied in more significant effects on MCLEA activity when compared to AMF amplitude.

1. Introduction

Enzymes are biocatalyst that speed up biochemical reactions used in the wide range of biotechnological interest areas, such as energy production, food science, biomedical, pharmaceutical, environment and so on [1,2,3,4]. Applications as diverse as these occur due to inherent characteristic of enzymes as high substrate specificity, low waste generation and mild operational conditions, which together, foster a more efficient and sustainable enzyme process [5,6]. Although the use of enzyme has brought to more sustainable industrial processes, the high production and purification cost, structural instability caused by changes in pH, temperature and ionic strength, inhibition, activity loss and separation difficulty, have become challenges in many applications [7]. In order to overcome these challenges, the generation of cross-linked enzyme aggregate (CLEA) has emerged as essential tool. CLEAs consist of physical aggregates of the enzyme, obtained as a result of the action of precipitation agents (e.g., salts, solvents or polymers), and

cross-linked among themselves by reticulation agents (e.g., glutaraldehyde) [8]. The main advantages of CLEAs include maintenance of the native enzyme structure, enhancing structural stability, easy preparation, decreasing in activity loss and improvement in recovery capacity [9,10]. In addition, when precipitation is carried out on solid support surface and the CLEAs are covalently bonded to it by cross-linked agent, it is possible to produce new biocatalysts that gather advantages from CLEA to intrinsic properties of the support.

With the development of nanotechnology many nanomaterials have been studied as support for enzyme immobilization, among which, highlight the magnetic nanoparticles (MNP) as magnetite (Fe₃O₄). Magnetite consists of Fe²⁺ and Fe³⁺ (1:2) iron oxide with reverse spinel structure, and when in nanometric scale, it shows properties as high surface area, adsorption capacity, diffusivity and biocompatibility, making it an ideal support for enzyme immobilization [11,12]. In addition, the easy support surface modification can increase its enzyme loading capacity, and the intrinsic magnetic properties, allows it to be

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magnetically recovered from different media by an external magnetic field, leading to decreased process cost [13,14,15]. In this context, many studies have used MNP as support for CLEAs immobilization of different enzymes. In summary, these magnetic cross-linked enzyme aggregates (MCLEAs) have shown increases in enzyme stability face to changes pH and temperature, decreases in inhibition, high activity and, mainly, an exceptional recovery capacity, making it an essential strategy in the improvement of enzymatic processes [16,17,18].

Lastly, even though positive results have been observed, advances are still necessary when thought in the use of MNP as enzyme support, mainly in concern to better understanding of the magnetic properties effect on enzymatic activity. It is worth mentioning that the use of magnetic properties of MNP is not restricted to magnetic recovery. Under an alternating magnetic field (AMF), as a result of the both Néel and Brown relaxation, MNP rotate and collide among themselves producing heat, a phenomenon known as magnetic hyperthermia [19]. An AMF can disturb enzymatic kinetic by increase the collision rate between biocatalyst and substrate, change MCLEA aggregation state, trigger conformational changes on enzymatic or increase diffusion rate, leading to improvements of MCLEA activity [20,21]. Although magnetic hyperthermia has been extensively used in biomedical application as cancer treatment and drug delivery, it remains almost unexplored in enzymatic catalysis, especially those based on MCLEAs. Specifically, Liu and co-workers have reported the improvement in the activity of lipase MCLEA under AMF when compared to the free enzyme in the resolution of (*R,S*)-2-octanol. According to the authors, frequency played a more dominant role than the field intensity [22]. However, advances regarding to different enzymes and AMF with different amplitudes and frequencies need to be carefully investigated.

Faced it, here is reported the synthesis of a cellulases MCLEA samples and a further investigation regarding to effects of an AMF in its catalytic activity. Cellulases hydrolyze β -1,4 glycosidic bonds in cellulose chain and were chosen as model enzyme because they are an important industrial enzymatic complex used in conversion processes of cellulose to biofuels, prebiotics cello-oligosaccharides, and polymers, as well as, in cellulose fibers processing in textile industries [23,24]. In order to AMF effects evaluation, two levels of frequency (203–420 kHz) and amplitude (3–6 kA m⁻¹) of magnetic field were used in a full factorial design (2x2) and, in such conditions, was observed that frequency implies in more significant effects on MCLEA activity when compared to AMF amplitude.

2. Experimental section

2.1. Materials and measurements

For syntheses, cellulase from *Aspergillus niger*, iron(II) chloride tetrahydrate ($\geq 99\%$), iron(III) chloride hexahydrate (97%), 3-amino-propyl-triethoxysilane (APTS, 99%), phosphate buffer saline (PBS) and glutaraldehyde, were purchased from Sigma-Aldrich; ammonium sulfate ($> 99.6\%$) and sodium hydroxide were obtained from Neon. In activity and loading efficiency assays, were used 3,5-Dinitrosalicylic acid (DNS, 98%) and sodium phosphate dibasic purchased from Neon, while citric acid and Bradford reagent were obtained from Sigma-Aldrich. All the starting chemicals and solvents were used without further purification. X-ray powder diffraction (XRD) patterns were obtained using a Rigaku model RINT 2000 diffractometer, in the 2θ range 5–70°, with Cu K α radiation ($\lambda = 1.5418 \text{ \AA}$), scan speed of 1 s/step, and step size of 0.02°. For magnetite, the PXRD diffractogram was simulated using Mercury 3.0 software, Cu K α radiation ($\lambda = 1.5418 \text{ \AA}$) and magnetite CIF file AMCSD-2400, crystal system cubic and space group Fd3m. Fourier transform infrared (FTIR) spectra were acquired in the range 4000–400 cm⁻¹, with resolution of 4 cm⁻¹ and 32 scanning, using KBr pellets and a PerkinElmer FT-IR Frontier spectrophotometer. Scanning electron microscopy (SEM) images were acquired with a Topcon SM-300 field emission gun (FEG) instrument, using carbon as a

sample support. For spectrophotometric measurements, a Perkin Elmer Lambda 465 spectrophotometer equipped with a UV-Vis photodiode array detector was used. The magnetic hyperthermia measurements were performed on the DM100 Series of the brand Nanoscale Biomagnetics.

2.2. Synthesis of APTS coated magnetite nanoparticles (MNP-APTS)

Magnetite nanoparticles (MNP) were prepared by the conventional coprecipitation method [25] and coated with APTS in a one-step. In a three-neck round-bottom flask equipped with a reflux condenser and argon atmosphere, were added 3.3 mL of Fe²⁺·Fe³⁺ (1:2) solution in a 3 mol L⁻¹ sodium hydroxide aqueous solution, being the mixture kept under 360 rpm stirring at 75 °C for 30 min. Then, 5 mL of APTS were added and the suspension maintained at the same conditions for more 40 min. Lastly, MNP-APTS were recovered by using a permanent magnet, washed several times with water and stored in ultrapure water until characterization and MCLEA synthesis.

2.3. Syntheses of magnetic cross-linked cellulase aggregate (MCLEA)

In a falcon tube containing 10 mL of 0.01 mol L⁻¹ PBS buffer pH 7, 100 mg of cellulase enzyme and a suspension of functionalized magnetic nanoparticles with 200 mg of MNP-APTS were incubated at room temperature, under shaking of 125 rpm, for 2 h. Then, 9 mL of chilled precipitant agent ammonium sulfate were added to the tube for precipitating cellulase on MNP-APTS surface, keeping rest for 30 min, after which, 1 mL of 3% glutaraldehyde aqueous solution was added to cross-link reaction. The system was incubated for 7 h at room temperature under shaking of 125 rpm. The MCLEA obtained was washed 3 times with 0.05 mol L⁻¹ acetate buffer pH 5 and kept in acetate buffer at 5 °C until activity assay and characterization. Non-magnetic cross-linked cellulase aggregate (CLEA) synthesis was carried out in the same way but without MNP.

The enzyme loading efficiency was determined measuring the amount of protein in the supernatant before and after immobilization by the Bradford protein assay [26] and using the Eq. (1) below:

$$\text{Enzyme loading efficiency} / \% = \frac{C_0 V_0 - C_s V_s}{C_0 V_0} \times 100 \quad (1)$$

where C_0 is the initial protein concentration (mg mL⁻¹), V_0 the initial volume of protein (mL), C_s the protein concentration in the total supernatant (mg mL⁻¹) and V_s is the total volume of supernatant (mL).

2.4. Activity assay: Central composite design (CCD)

Cellulases include three main enzymes: β -glucosidase, exoglucanase and endoglucanase (which was used as a probe to investigate both CLEA and MCLEA performance). Enzymatic activity assay was determined by measuring reducing sugar production after the reaction of immobilized cellulase with carboxymethylcellulose (CMC) sodium salt [27]. The reaction mixture contained 0.9 mL of 0.44% CMC solution in citrate-phosphate buffer and 0.1 mL of MCLEA or CLEA. The CMC hydrolysis reaction was carried out for 1 h under heating, after which 1.5 mL of DNS reagent was added to stop the reaction. Samples were heated in boiling water for 5 min to allow suitable color formation. After cooled to room temperature, the reducing sugar concentration was measured at 540 nm on a UV-Vis spectrophotometer. The amount of enzyme needed to catalyze the release of 1 μ mol of reducing sugar equivalent per min was defined as one unit (1 U) of enzymatic activity. For calculus purposes, enzymatic activity was expressed as relative activity, i.e., the ratio between the activity on each set of values of pH and temperature and the activity in the optimal condition.

A CCD was employed for enzymatic activity optimization of the MCLEA and CLEA under different pH and temperature conditions. Both independent variables were studied in five levels, pH (3.6, 4.0, 5.0, 6.0

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Table 1
Experimental conditions (real and coded) of the independent variables used in CCD for enzymatic activity optimization of the CLEA and MCLEA.

Experiment	pH	T / °C
1	4 (-1)	29 (-1)
2	4 (-1)	45 (1)
3	6 (1)	29 (-1)
4	6 (1)	45 (1)
5	3.6 (-1.41)	37 (0)
6	6.4 (1.41)	37 (0)
7	5 (0)	25.7 (-1.41)
8	5 (0)	48.3 (1.41)
9	5 (0)	37 (0)
10	5 (0)	37 (0)
11	5 (0)	37 (0)

and 6.4) and temperature (25.7, 29.0, 37.0, 45.0 and 48.3 °C). A total of 11 experiments were employed in this work, including 4 cube points, 3 replications at the central point and 4 axial points. For statistical analysis, the independent variables (X) pH and temperature were coded as x_i according to the following Eq. (2):

$$x_i = \frac{X_i - X_0}{\delta X} \quad (2)$$

where, X_i is the real valor of X to be codified, X_0 is the real valor of X at the central point, and δX presents the step-change [28]. The experimental conditions of pH and temperature for the 11 experiments carried out in the CCD are shown in Table 1.

The statistical significance of the full quadratic models predicted was evaluated by the analysis of variance (ANOVA) and least squares techniques using Statistica v.8 software.

2.5. Hyperthermia assay of the MCLEA

Evaluation of the effect of the AMF on MCLEA activity was carried out in the frequency (f) and amplitude (B) range of 203–420 kHz and 3–6 kA m⁻¹, respectively, and in the optimal condition determined in section 2.4. For this purpose, a factorial design (2x2) was performed with 2 levels for both variables and their respective levels are shown in Table 2. Enzymatic activity was measured as stated in previous section, however, with experimental vial inserted in an AMF sample holder. Analysis of the significance of the effects of AMF frequency and amplitude on MCLEA activity was carried out via analysis of variance (ANOVA) using Statistica v.8 software.

3. Results and discussion

3.1. Synthesis and characterization of MCLEA

Fig. 1 shows the XRD patterns of MNP-APTS and MCLEA. The diffraction peaks obtained for MNP-APTS and MCLEA agreed with the simulated diffractogram for magnetite (Fe₃O₄), showing the formation of this iron oxide phase and that further modification of MNP-APTS surface with cellulase enzyme maintained its crystalline structure.

The nanometric scale of MNP-APTS and MCLEA synthesized was confirmed by representative SEM images as well as their corresponding particle-size distributions shown in Fig. 2. In the SEM images spherical

Table 2
Experimental ranges and levels (coded and real) of the independent variables in the factorial design for optimization of magnetic hyperthermia assay of MCLEA.

Independent variables	Ranges and levels	
	-1	+1
f / kHz	203	420
B / kA m ⁻¹	3	6

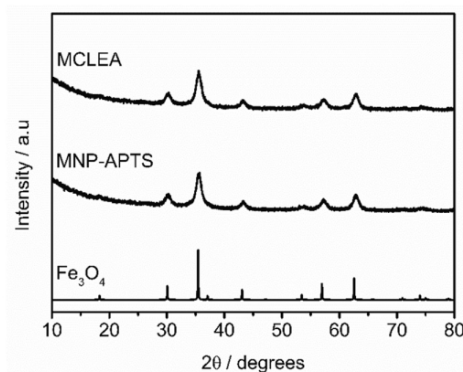


Fig. 1. XRD patterns simulated of magnetite (Fe₃O₄) and as synthesized MNP-APTS and MCLEA.

nanoparticles are observed with an average diameter of 15 and 26 nm for MNP-APTS and MCLEA samples, respectively.

Fig. 3 shows the FTIR spectra of the samples MNP-APTS, MCLEA and free cellulase enzyme. In the MNP-APTS and MCLEA FTIR spectra, the bands assigned to the νFe–O stretching mode appear at 600 and 443 cm⁻¹. Due to finite size of nanoparticles there is a split of the energy levels of the quantized nanoparticles and the band near to 600 cm⁻¹ shows split into two peaks at 635 and 582 cm⁻¹ [29]. APTS coating on magnetic NP surface was confirmed in the MNP-APTS FTIR spectrum with the band centered at 1010 cm⁻¹ assigned to νSi–O bond stretching mode [30]. In addition, the band in the region of 1630–1590 cm⁻¹ is ascribed to bending modes from δC–NH₂ bond [31]. Lastly, immobilization of cellulase on MNP-APTS surface was observed with bands centered at 1570 cm⁻¹ and 1408 cm⁻¹, assigned to νC–O bond stretching mode and δN–H bending mode, respectively [32].

3.2. Cellulase immobilization and activity assay optimized by CCD

Quantitative analysis of cellulase immobilization in the CLEA and MCLEA was performed by assaying the amount of cellulase (measured as protein) in the supernatant both before and after immobilization using Bradford protein assay. A high enzyme loading efficiency of the 88.9% and 90.7% was observed for CLEA and MCLEA, respectively. The formation of cross-linked enzyme aggregates has been successfully recognized as a highly efficient and eco-friendly immobilization method mainly due to its high stability combined with high loading capacity [9,10], which, in this work, did not suffered changes due to MNP as support.

To investigate the relative activity of the CLEA and MCLEA due to changes in pH and temperature, a CCD was used. The statistical significance of each regression model was evaluated via ANOVA using the *F*-test are shown in Table 3. The model predicts for MCLEA relative activity shows statistically significant at 95% of significance level, i.e., presents *F*-value of the regression (8.5611) is higher than *F*-critical (5.0503). Besides, the *F*-value of lack of fit (8.8099) was lower than the *F*-critical value (19.1642), at 95% confidence level, confirming that the regression did not show a lack of fit. On the other hand, the model predicts for CLEA relative activity did not show statistically at 95% confidence level, because *F*-value of the regression is lower than *F*-critical (2.1356 < 5.0503). Moreover, the model shows a high lack of fit, i.e., *F*-value of the lack of fit higher than *F*-critical (248.3161 > 19.16442).

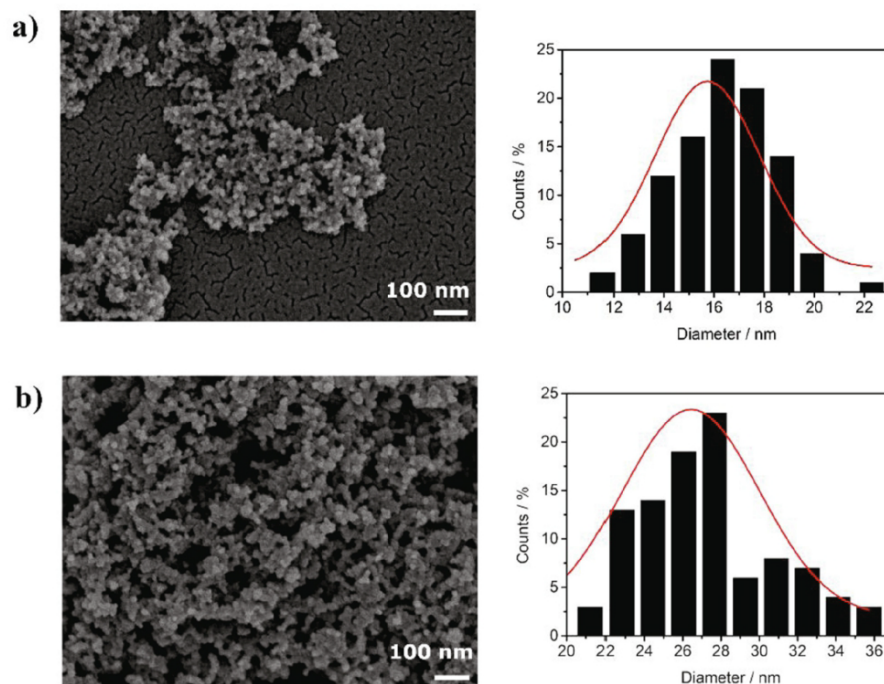


Fig. 2. FEG-SEM images of MNP-APTS (a) and MCLEA (b).

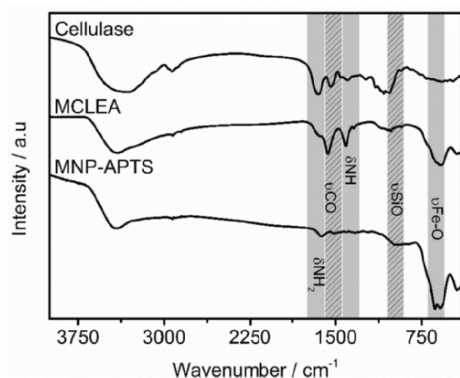


Fig. 3. FTIR spectra of the MNP-APTS, MCLEA and free cellulase enzyme.

Regression analysis was carried out to determine the coefficients of the significant effects on MCLEA relative activity and the results are presented in Table 4.

For MCLEA, only quadratic terms of pH and temperature show statistically at 95% confidence level, i.e., show *F*-value (90.6858 and 198.114, respectively) higher than *F*-critical (18.5128). The quadratic

model obtained for MCLEA relative activity as a response to pH and temperature changes can be represented by both responses and contour quadratic surface plotted in Fig. 4, which is described by the following Eq. (3).

$$\text{Relative activity} / \% = 100.02 - 19.51pH^2 - 28.84T^2 \quad (3)$$

From Fig. 4b it can be observed the high stability of relative activity of the MCLEA around central point. In terms of pH, MCLEA shows an expressive stability in the pH range 4 to 6. It is well known in the literature [33,34] that free cellulases enzymes show their highest catalyst activity at pH 5 and, usually, after immobilization procedures have been observed either acid-shift [35] or alkaline-shift [36] in its optimal pH. These shifts in the optimum reaction pH may be assigned to the presence of charges in support surface, which changed the enzymatic microenvironment after immobilization [37,38].

An unusual behavior was observed regarding temperature effects in enzymatic activity of the MCLEA. Optimal reaction temperature condition differ between free and immobilized cellulases depending on the type of support and, mainly, the immobilization method which can leads to significant changes in enzyme molecular structure [37]. While free cellulases enzymes act mainly at 50 °C and after its immobilization have been reported changes to highest temperature values [39], MCLEA synthesized in this work show a stable and atypical ideal catalytic behavior at temperatures below 45 °C. Although nearly all cellulase applications have been carried out at 50 °C or higher temperature, this MCLEA emerges as tool for modern approaches of second-generation ethanol (2GE) production from lignocellulosic biomass, e.g., simultaneous saccharification and fermentation (SSF) process. Briefly, in SSF

Table 3
ANOVA for the quadratic models predicted for the relative activity of the CLEA and MCLEA.

Source	Sample	Degrees of freedom	Sum of squares	Mean of squares	F-value	F-critical	p-value
Model	CLEA	5	1188.3989	237.6798	2.1356	5.0503	0.2123
	MCLEA	5	5725.7825	1145.1565	8.5611	5.0503	0.0171
Residual	CLEA	5	556.4591	111.2918			
	MCLEA	5	668.8070	133.7614			
Pure error	CLEA	2	1.490	0.7450			
	MCLEA	2	47.04944	23.5247			
Lack of fit	CLEA	3	554.969	184.9897	248.3161	19.16442	0.0040
	MCLEA	3	621.7576	207.2525	8.8099	19.1642	0.1036

Table 4
Significance of coefficients of the quadratic regression of MCLEA relative activity.

Coefficient	Value	Degrees of freedom	Sum of squares	Mean of squares	F-value	F-critical	p-value
pH	1.9544	1	30.469	30.4685	1.2951	18.5128	0.3730
pH ²	-19.5150	1	2133.360	2133.36	90.6858	18.5128	0.01084
T	-1.6515	1	21.757	21.7566	0.92484	18.5128	0.4376
T ²	-28.8441	1	4660.588	4660.588	198.114	18.5128	0.0050
pH × T	8.0201	1	257.288	257.2883	10.9369	18.5128	0.0805

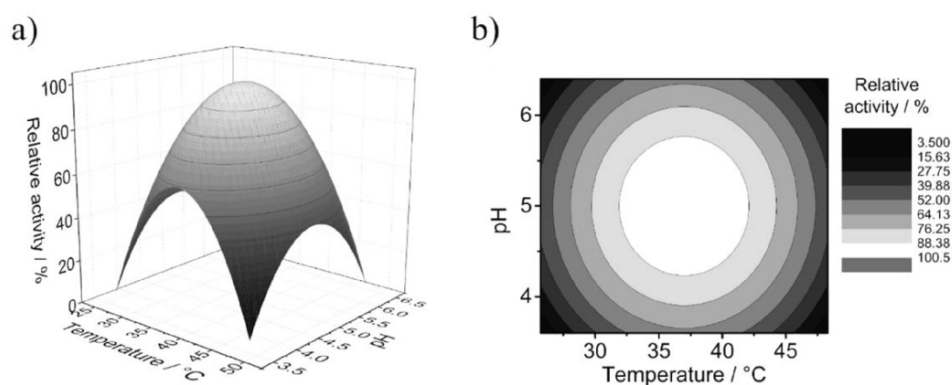


Fig. 4. Quadratic response surface (a) and contour surface (b) for MCLEA relative activity as a response to pH and temperature changes.

process the fermentation step is simultaneously carried out to saccharification (hydrolysis) of cellulose, whose main aim consists in decrease the cost of 2GE production and to avoid cellulase inhibition by cellulose hydrolysis products [40]. However, this strategy meets obstacles in the fact that saccharification and hydrolysis to be performed at very different temperatures, 37 °C and 50 °C, respectively [41]. Thus, the possibility to conduct cellulose hydrolysis in temperatures near to 37 °C shows as promising in the development of new sustainable processes for the obtention of value-added products from lignocellulosic biomass.

The highest value of relative activity for MCLEA observed in the region along to central point in CCD assay, i.e., 100% at pH 5 and 37 °C, has a real value corresponding to 79.37 U g⁻¹. Although the proposed model for CLEA relative activity has shown high lack of fit, during CCD assay, its highest enzymatic activity was observed at pH 4 and 45 °C, whose real value corresponding to only 59.25 U g⁻¹.

Although similar results were observed in enzyme loading efficiency, CLEA and MCLEA showed different enzymatic activity measured using CMC as substrate. Similar result was also observed by Kopp and co-workers immobilizing penicillin G acylase in amino-coated superparamagnetic nanoparticles using different precipitation agents

(e.g., dimethoxyethane; *tert*-butanol and polyethylene glycol 600) [42]. In the synthesis, several factors, such as protein concentration, precipitant agent, ionic strength, cross-linked agent, pH and so on can affect the catalytic properties of aggregates [43,44]. The higher activity observed for MCLEA in comparison with CLEA suggests a significant effect of MNP in the cellulase cross-linked aggregate structure. In enzyme immobilization procedures, the support determines features as enzyme stability and orientation end, consequently, the catalytic activity [45]. Since that the same conditions were employed for CLEA and MCLEA synthesis, the higher enzymatic activity observed for MCLEA can be assigned, e.g., to improvements in chemical structure stability and proper spatial orientation of enzyme established by MNP presence of MCLEA structure, as well as, unblock of its respective active site.

Many positive effects have been attributed to the use of MNP as support for enzymes immobilization [46], among which highlight some observed in this work, such as high loading efficiency, activity and stability faced to changes in the pH and temperature, besides the recovery capacity due to its intrinsic magnetic properties. However, not restrict to it, their benefits need to be further studied, mainly concern to the use of magnetic hyperthermia.

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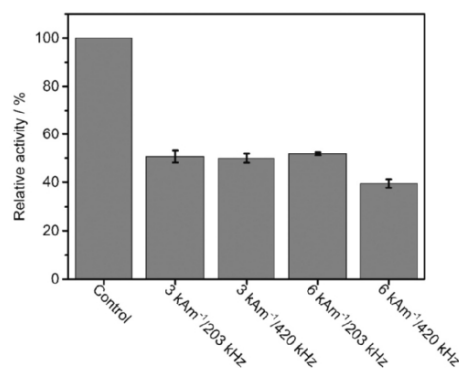


Fig. 5. Effects of AMF frequency and amplitude in MCLEA activity compared to control experiment.

3.3. Magnetic hyperthermia assay

In order to investigate the effects of an AMF on MCLEA activity a factorial design (2x2) with two variables (frequency and amplitude) at two levels (-1 and + 1) was used. Fig. 5 shows a comparison between the MCLEA activity observed at optimal condition of pH 5 and 37 °C (control) and the observed in the hyperthermia assay with different states of frequency and amplitude. AMF application decreased the MCLEA activity to around 50% when at least one variable was set at -1 level of the factorial design and to 40% when both frequency and amplitude were set at + 1 level. These results diverge from those reported by Liu and co-workers [22] and Xia and co-workers [47]. The firsts observed an increase of almost 25% in the lipase activity immobilized on MNP under application of a AMF of 0.8 kA m⁻¹ at 600 Hz, while the second observed a 16% activity increase of laccase immobilized on polymer-coated MNP at the same AMF conditions. However, it is worth mentioning that these authors made use of AMF frequency with an order of magnitude at least 300 times lower than the applied in this work. In ferrofluids at low frequency, the rotation arise as result of a small permanent magnetic moment in MNP, while at high frequency (kHz-MHz), the phase lag between the applied AMF and the induced magnetic moment results in a torque, which implies that in the high frequency range, the magnetization is not able to follow the magnetic field instantaneously [48]. In such conditions, have been shown that the rotation of the magnetic moment or physical rotation of MNP depends on AMF amplitude [49]. In addition, the rapid change of magnetic field direction at high AMF frequency can prevent the MNP from aligning with the field direction [47], which can lead to a decrease in both MNP diffusion rate and their collision with the substrate and, consequently, in the enzymatic activity results observed in this work.

Fig. 6 shows an effect size plot for AMF frequency and amplitude on MCLEA activity. As remarked, frequency plays a higher effect when compared to amplitude and its respective interactions. Similar results were reported by Liu and co-workers studying the AMF effects on lipase immobilized MNP applied in the resolution of (R,S)-2-octanol [22]. The frequency level-shift from -1 until + 1 decreased MCLEA activity by 6.5%, while the same level-shift of amplitude and interaction factor decrease by 4.7% and 5.81%, respectively. Thus, because of full factorial design is desired that further investigations should started in conditions of lower AMF frequency.

Lastly, an ANOVA was carried out in order to determine the significance of the effects of AMF frequency and amplitude on MCLEA relative activity and the results are presented in Table 5. Both variables and their respective interaction show effects statistically significant on

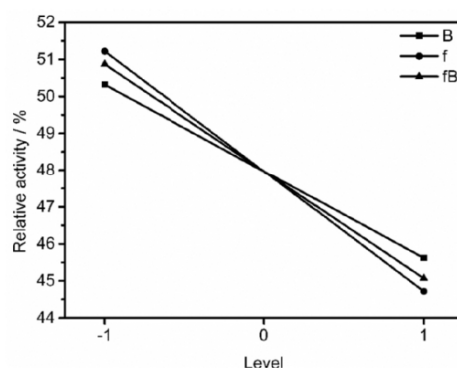


Fig. 6. Effects size plot of the AMF frequency (f), amplitude (B) and interaction factor (fB) on MCLEA activity.

Table 5

Significance of effects of AMF frequency (f), amplitude (B) and its interaction (B × f) in MCLEA relative activity.

Variable	Degrees of freedom	Sum of squares	Mean of squares	F-value	F-critical	p-value
B	1	66.10	66.10	19.8498	5.3176	0.00212
f	1	127.07	127.07	38.1591	5.3176	0.0002
fB	1	101.17	101.17	30.3813	5.3176	0.0005
Error	8	26.64	3.33			

MCLEA activity ($F\text{-value} > F\text{-critical}$). In addition, the highest $F\text{-values}$ of regression confirm that frequency plays a more significant effect when compared to amplitude and interaction factor between the variables on MCLEA activity.

4. Conclusions

In this work was reported synthesis and characterization of MCLEA of cellulases with unusual highest and stable catalytic activity at low temperatures near to 37 °C. Such behavior make this MCLEA a promising hybrid material to be employed in the conversion process of the lignocellulosic biomass, mainly those that need carried out in low temperatures as simultaneous saccharification and fermentation process. The MCLEA shows a high enzyme loading efficiency of 90%, which result is as high as the demonstrated by its non-magnetic analogue. However, its higher enzymatic activity suggests a catalytic enhancement effect as a result of the magnetic nanoparticle presence in cellulase cross-linked aggregate structure.

Applying AMF on MCLEA led to a substantial decrease in its catalytic activity, particularly in the highest values of frequency and amplitude. In addition, as result of full factorial design was observed that frequency implied in more significant effects on MCLEA activity when compared to AMF amplitude.

CRedit authorship contribution statement

Guilherme N. Lucena: Conceptualization, Methodology, Investigation, Writing - original draft. **Caio C. dos Santos:** Methodology, Formal analysis, Writing - review & editing. **Gabriel C. Pinto:** Formal analysis, Writing - review & editing. **Rodolfo D. Piazza:** Methodology, Writing - review & editing. **Wesley N. Guedes:** Writing - review & editing. **Miguel Jafelicci Júnior:** Resources, Project administration. **Ariela V. de Paula:** Resources, Supervision. **Rodrigo**

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Fernando C. Marques: Funding acquisition, Project administration, Writing - original draft, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Magnetic cross-linked enzyme aggregates (MCLEAs) applied to biomass conversion



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ABSTRACT

The increasing energy and sustainable materials demand requires the development of renewable production technologies to meet current global demands. Certainly, the progress for a sustainable development goes through the use of renewable and versatile raw materials, as the lignocellulosic biomass, in terms of application in energy, materials and environment sciences. The key role in the use of lignocellulosic biomass as renewable raw material has been its conversion to C₆ and C₅ sugars, which can be converted in different materials by several biological and chemical routes. Although its recalcitrance nature and high cost of the enzyme that promotes such conversions, obtaining a magnetic cross-linked enzyme aggregates (MCLEAs) has emerged as a green and sustainable technology to overcome these problems. This class of nanohybrids materials can significantly improve enzyme stability, activity and its recovery, decreasing cost of the process and adverse consequence of use of fossil raw materials. So, this review intends to shows the potential and recently advances of MCLEAs applied to biomass conversion to value-added chemicals, fuels and materials.

1. Introduction: a look over biomass

The well-being of our society and of the next generations is unimaginable without the recent and futures advances in the fields of energy, environment and materials science. However, it requires a paradigm shift from traditional concepts of industrial process to development of new sustainable technologies that includes aspects as the use of renewable energy and materials, avoiding hazardous chemicals and reducing or eliminating waste. This point of view is directly linked to green chemistry, basically defined as the prevention of pollution by efficient use of renewable resources of energy and materials, waste minimization and the avoidance of toxic and hazardous substances in the production and application of chemical products [1,2]. Thus, one of the main challenges of society is, therefore, make progress in discovery of new green and sustainable technologies which can be used to attend the sustainable development requirement based on green chemistry principles [3,4].

Certainly, to make any progress in the sustainable development goes through use of renewable raw materials and versatile in terms of application in energy, environment and materials science. Thus, the lignocellulosic, the most abundant and bio-renewable biomass on

earth, come as a solution [5]. Biomass is basically a stored source of solar energy initially collected by plants during the photosynthesis process whereby carbon dioxide is captured and converted to plant materials mainly in the form of cellulose, hemicellulose and lignin. The biomass materials includes principally crop residues, forest and wood process residues, animal wastes including human sewage and municipal solid waste food processing wastes, among others [6].

The biomass has a positive impact on Carbon Footprint and has been accepted as a new renewable source for global sustainable development. Basically, the use of biomass started with energy crises brooked out in 70 decade and, more recently, with a wave of interest in new energy sources because of high oil prices [7,8]. A study from European Observatory on Renewable Energy, in 2013 showed that 46% of renewable energy in the European Union comes from solid biomass, and it was accounted for 3% of electricity and 15% of the heat produced in industrial units [9]. According to U.S. Department of Energy [10], 10% of energy in the U.S. is derived from renewable energy sources. Biomass, a renewable and bio-degradable energy resource, provides 50% of renewable energy needs in the U.S. However, on global scale, biomass represents only 10% of the produced renewable energy [11,12]. Consequently, it seems that biomass plays a key role in the energy industry.

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Besides that, the researches on energy generation has been growing on biomass applied in the production of cleaner energy, such as second generation ethanol [13–18], hydrogen [19–25] and biogas [26–30], as substitute of fossil fuels to decrease the greenhouse gas emissions. Beyond energy production, biomass can replace all fossil fuels used to produce materials and chemicals [31–36].

The key in the use of biomass as renewable raw material is the use of their derivatives C_6 and C_5 sugar (e.g., glucose and xylose), which can be converted in new materials by several biological and chemical routes [37]. However, to convert lignocellulosic biomass to value-added materials remains a challenge due to its degradation resistance, energy and cost of process. Firstly, it is necessary a delignification pretreatment to eliminate the lignin and expose the cellulose and hemicellulose to the attack of hydrolytic enzymes as cellulase and xylanase, respectively. Then, the cellulose and hemicellulose should be hydrolyzed to C_6 and C_5 sugars, respectively [38]. Lastly, C_6 and C_5 sugars can be converted to a range of chemicals and materials.

Others technical difficulties have been reported to biomass conversion. Among its, cellulase enzymes are strongly inhibited by hydrolysis product [39]; enzymes are easily denatured by changes in the pH and temperature [40]; and finally, beyond of the difficult recovery from reaction medium, to enhanced hydrolysis yield, a large amount of enzyme is consumed, what has become a bottleneck process from financial point of view [41,42]. So, extensive research is currently being undertaken all over the world to address these problems.

With the advance of nanotechnology, the use of nanohybrids materials as Cross-Linked Enzyme Aggregates (CLEAs), obtained by enzyme immobilization on supports, is a technological solution when it is desired to increase the enzymatic stability, efficiency and reusability by recovering from reaction medium [43–46]. Among the supports, magnetite nanoparticles (Fe_3O_4) has caught attention due to magnetic properties, which allow it selective recovering from reaction by applying an external magnetic field.

Therefore, allied to environment benefits of the use of biomass as renewable resource for chemicals, fuels and materials, these magnetic nanohybrids materials, called Magnetic Cross-Linked Enzyme Aggregates (MCLEAs), shows as a promising green and sustainable technology when it thought in the biomass conversion. So, this article reviews the recent advances and the challenges of the biomass and their derived conversion using the MICLEAs nanohybrids materials.

2. Biomass: structure, enzymatic conversion and products

2.1. Structure

The Fig. 1 shows the typical structure of lignocellulosic biomass. The content of biomass varies depending on the biomass type. Usually, lignin, hemicellulose and cellulose constitute 10–40%, 20–40% and 40–50% wt% of the plant material respectively, which has also a small amount of esters, alcohols, steroids, sulphates, oxalates, carbonates, silicates, inorganic materials, among other, called extractives [47,48]. Cellulose is a polymer of glucose molecules in which individual glucose units are connected via 1–4 linked β -D-anhydroglucopyranose units. Hemicellulose is intrinsically linked to cellulose, having branched structure and built by a combination of C_5 or C_6 sugars, including a number of polysaccharides, which xylans are the main constituent. Lignin is the highly branched polymer of the biomass. With a heterogenic nature and formed by phenyl propane units, lignin has been found to serve as the matrix support material along with hemicellulose to embed cellulose fibers [49–51]. These components are strongly intermeshed and bonded through covalent and non-covalent bonds forming the lignocellulosic complex. This typical structure is responsible by the strong native recalcitrance to enzymes and relatively low digestibility of raw biomass materials [52].

2.2. Enzymatic conversion

The main enzymes in the biomass conversion processes are cellulase and xylanase. The cellulases are enzymes that hydrolyze β -1,4 linkages in cellulose chains. The cellulose hydrolysis occurs by a combination of three main types of cellulases [54]: endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and glucosidases (EC 3.2.1.21). Endoglucanases hydrolyze glycosidic bonds at the amorphous regions of the cellulose generating long chain oligomers for the action of exoglucanases or cellobiohydrolases, which cleave the long chain oligosaccharides generated by the action of endoglucanases into short chain oligosaccharides. There are two types of exoglucanases, acting unidirectionally on the long chain oligomers either from the reducing or nonreducing ends liberating cellobiose, which is further hydrolyzed to glucose by β -glucosidases [55]. Synergy between cellulases have been identified between different cellobiohydrolases (with specificity for reducing and non-reducing ends); between endo and exo-glucanases; between endo-glucanases; between cellobiohydrolases, endoglucanases and β -glucosidases [56–61].

The xylanases (EC 3.2.1.8) are among the xylanolytic enzyme system that include endoxylanase, β -xylosidase, α -glucuronidase, α -arabinofuranosidase, and acetylxytan esterase [55]. Xylanases are a group of glycoside hydrolase enzymes that degrade the linear polysaccharide xylan into xylose by catalyzing the hydrolysis of the glycosidic linkage (β -1,4) of xylosides. Xylanases have been classified in at least three ways: based on the molecular weight and isoelectric point [62], the crystal structure [63] and kinetic properties, or the substrate specificity and product profile [64]. The favorable system for the classification of xylanases is based on the primary structure and comparison of the catalytic domains. Among all xylanases, endoxylanases are the most important due to their direct involvement in cleaving the glycosidic bonds and in liberating short xylooligosaccharides [65,66].

2.3. Products from C_6 and C_5 sugars

The Fig. 2 shows that after cellulose and hemicellulose hydrolysis, the C_6 and C_5 sugars produced can be converted into a whole range of industrial raw materials, such as 3-hydroxy propionic acid (3-HPA), 5-hydroxymethyl furfural (5-HMF), glycerol, succinic acid, lactic acid, gluconic acid and furfural. These new biomass-derived compounds from C_6 and C_5 sugars can be used as raw materials to several important chemicals for industry [37,67]. Enzymatic processes are employed in many of these conversions, which open up a whole range of MICLEAs applications, making these nanohybrids materials an important tool for a sustainable development based on biomass conversion.

2.4. Magnetite nanoparticles as enzyme supports

Enzyme immobilization has been recognized as an effective technology to enhance its stability and reusability in industrial process. However, it is highly challenging the maintenance of their structural stability. Consequently, various materials as hydrophobic supports beads [105], macroporous zirconia [106], silica [107], polymer nanofibers [108], zeolite [109], metal-organic frameworks [110] and so forth, have been employed to enzyme immobilization. Advancements in the fields of nanoscience and nanotechnology have resulted in several possibilities for nanomaterials applications in biological and chemical fields. Among the various nanomaterials, the use of magnetic nanoparticles has received considerable attention as new supports for enzyme immobilization [111]. Magnetite (Fe_3O_4) is one the most commonly used magnetic particles due to low toxicity, biocompatibility, high surface area and affinity to proteins, possibility of surface functionalization with reactive functional groups for chemical modifications, easy and convenient isolation from reaction mixture and

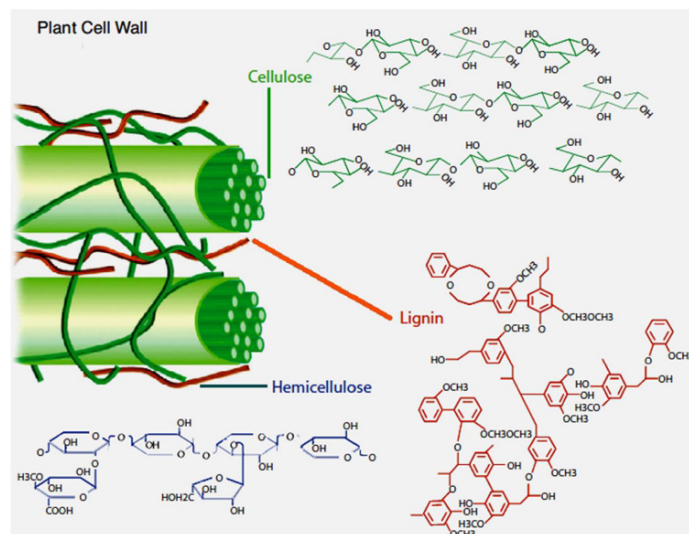


Fig. 1. Typical structure of lignocellulosic biomass. Reprinted with permission from Ref. [53]. Copyright 2018, Elsevier.

recycling for reuse [112,113]. However, due the anisotropic dipole attraction, hydrophobic nature and high surface energy, uncoated magnetite nanoparticles are generally unstable and tend to aggregate easily, beyond of to be highly susceptible to oxidation in the presence of oxygen, loosen their specific properties [114,115]. So, a suitable surface functionalization with molecules as chitosan [116], amino-silanes [117] or polymers [118], is necessary to overcome such limitations. Besides that, the enzyme stability and immobilization efficiency can be increased by cross-linking it with magnetic nanoparticles, creating the so called Magnetic Cross-Linked Enzyme Aggregate (MCLEAs). This approach allows clear advantages such as high enzyme activity; easy to be produced by inexpensive and effective methods; readily to be reused

exhibiting satisfactory stability and performance for selected application. So, aiming to further improve sustainability of process, beyond MCLEAs synthesis, different immobilization methods in magnetic supports have been studied and implemented.

2.5. Enzyme immobilization in magnetic supports and MCLEAs synthesis

Magnetic nanoparticles (MNPs) are well known for their high surface area [119,120] and the possibility of separation from media by using an external magnetic field [121], allowing it to be applied in many areas [122–124]. The use of iron oxide magnetic nanoparticles

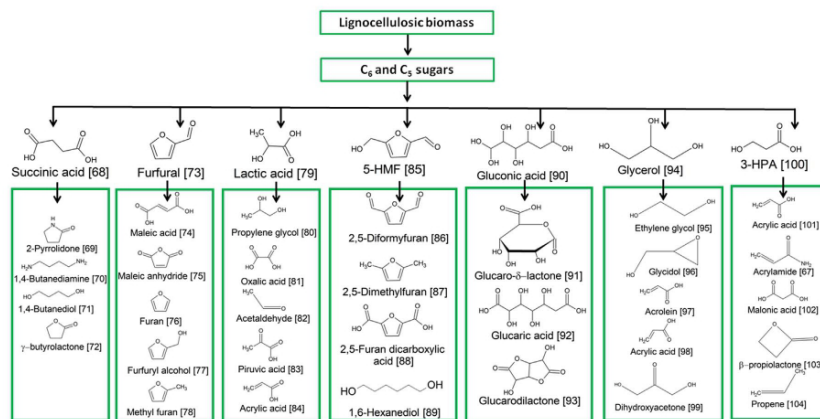


Fig. 2. Some value-added chemicals from lignocellulosic biomass [68–104].

associated with other materials to produce new composite materials is also known to promote the magnetic character to a material with defined attributes [125]. Among them, graphene oxide (GO), a monolayer of sp^2 carbon atoms hexagonally arranged with many oxygen functional groups on its surface is one of materials that MNPs can interact. Because of the decoration of MNPs on the sheets of GO it becomes the magnetic graphene oxide (MGO) which has a high thermal, electric and mechanical characteristic with the addition of the magnetic properties [126–128]. For the same purpose the decoration of polymer matrices with magnetic nanoparticles is also used [129].

To add new appropriate functional groups on the surface of synthesized nanomaterials the functionalization process is necessary. These groups e.g. carboxyl, epoxy and amino allow high colloidal stability [130] and facilitate the interaction of the nanomaterials in biological applications such as enzyme immobilization. After deciding which nanomaterials well-functionalized will be used as support, it is necessary to choose the best synthetic route for the immobilization process, being the crucial moment for support/enzyme interaction that results in an effective interaction [131], enabling properties such as: good enzymatic activity, high thermal and storage stability, high amount of enzyme loaded, and the reuse.

As shown in Fig. 3, among the enzyme immobilization methods are the physical and chemical methods, varying according to the proposed study [132,133]. The physical adsorption is the oldest method and the enzymes are absorbed through the surface of the support with no further treatment. This proceeding consists in a weaker interaction, it improves a high initial enzymatic activity, being an easier process and can also be used on a large scale, but on the other hand its catalytic activity can drastically decrease over time [134].

The entrapment and the encapsulation are other important physical methods that can be used for immobilization. Enzymes immobilized by entrapment get “trapped” in a limited space inside a polymer matrix and this microenvironment can improve the enzymatic stability [135]. The disadvantage of this process is that substrates with high molecular weight cannot access the entrapped enzyme due the impediment caused by the matrix [136]. The encapsulation is also a method to trap enzymes but, in this case, it is within a semi-permeable shell or membrane, such as hydro-gels, enabling in a high load of enzymes and a great operational stability, however the risks of rupture are high if there is no diffusion of the formed products [137].

In the chemical procedure, the covalent binding method involves the formation of covalent bonds between the enzyme and the support. The groups usually used in the covalent binding are thiol and amine

groups of enzyme [138]. However, it has been shown that the enzyme might lose its activity during the immobilization procedure if the attachment occurs between support matrix and the active center amino acids of the enzyme [139].

A way to solve these problems is to synthesize a cross-link enzyme aggregate using magnetic nanoparticle support, MCLEAs. This procedure involves a precipitation of enzyme and a magnetic nanoparticle support and the subsequent formation of the enzymatic aggregate with a bifunctional cross-linker agent [140]. A MCLEA has the advantage of its low cost, easy preparation, high specificity, high enzymatic activity, and stability over a wide pH range [141–144]. The most common cross-linkers agents are bifunctional, which have two similar reactive functional end groups, such as two amines, two thiols or two aldehydes which will interact between identical targets on the enzyme and the support [145]. Among the cross-linker agents, glutaraldehyde (GA) is the most common due to its easy interaction with the amino groups present in the enzymes, the low cost and to be easily handled [146–148]. Table 1 summarizes some data about MCLEAs synthesis applied to biomass conversion and its support, enzyme source, cross-linker used and advantages of the MCLEA compared to free enzymes, as well as number of recycles studied.

2.6. MCLEAs characterization

The structural characterization becomes the state of the art in the enzyme area because it allows obtaining information necessary for understanding the changes in the enzymatic environment and the interactions between the support and the enzyme. Thus, it is possible to evaluate and understand the interactions of protein with the support, and to produce materials more efficient for the use in industrial processes. For this purpose, several techniques can be used to characterize these materials in order to obtain structural, morphological, qualitative and quantitative information to understand the interaction of the enzyme with the support and to evaluate the efficiency of the immobilization and of the biocatalyst produced. Although the system is complex and presents many difficulties, we summarize the main techniques currently used for the characterization and understanding of the properties of the biomaterial.

2.6.1. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

As shown in Fig. 4, these microscopes can be used to study the surface morphology of the enzyme (topography), the support and the composite and to evaluate the size distribution of the particles. In the

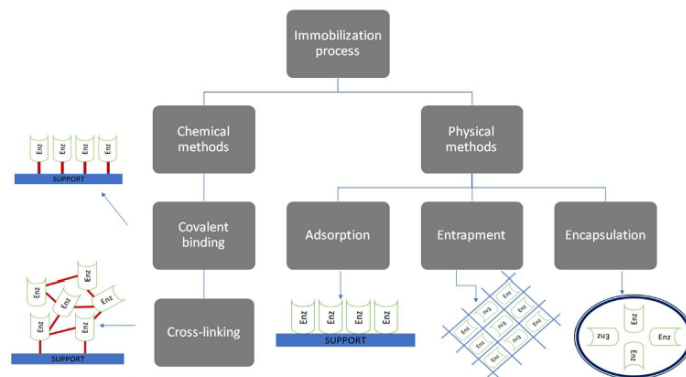


Fig. 3. Main enzyme immobilization methods.

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Table 1
Some data common about MCLEAs synthesis.

Xylanases					
Support/functionalization	Enzyme source	Cross-linker	Advantages	Number of reuses	Refs.
MNP@HPG	<i>Thermomyces lanuginosus</i>	TDI/Citric Acid	High storage stability, high initial activity, high protein loaded	10	[149]
MNP@SiO ₂ /3-PPA	<i>Thermomyces lanuginosus</i>	EDC	High pH and temperature stability	10	[150]
MNP@SiO ₂ /16-PHDA					
MNP/Chitosan	<i>Aspergillus niger</i>	Glutaraldehyde	High stability, high protein loaded	7	[146]
MNP@GO	<i>Thermomyces lanuginosus</i>	trichlorotriazine	High pH, temperature and storage stability	10	[151]
Cellulases					
MNP@MgO	cellulase cocktail (commercial β -glucosidase and cellulase from <i>Trichoderma reesei</i>)	Xylan aldehyde	High pH, temperature stability	7	[152]
MNP	Cellulase complex	EDC	Good temperature stability	6	[153]
MNP	<i>Acinetobacter</i>	Glutaraldehyde	High pH, temperature and storage stability	15	[154]
MNP/Chitosan	Not mentioned	Glutaraldehyde	High initial activity	10	[155]
MNP/MSN	<i>Trichoderma reesei</i>	APTMS	Easy separation and storage	5	[156]
MNP/APTES	Not mentioned	Glutaraldehyde	Good recovery and high temperature stability	8	[157]
MNP@SiO ₂	Not mentioned	–	Good temperature stability, easy separation	10	[158]
MNP/APTS	Not mentioned	Glutaraldehyde	High enzymatic activity, high stability	20	[159]
AF-CoFe ₂ O ₄	<i>Trichoderma reesei</i>	EDC, NHS	Easy separation	6	[160]
MNP@SiO ₂ /APTES	<i>Acremonium cellulolyticus</i>	Glutaraldehyde	High temperature stability and protein loaded	10	[161]
MNP@ β -CD/APTES	<i>Aspergillus niger</i>	Glutaraldehyde	Easy separation and NP shape control	16	[162]
MNP	<i>Trichoderma reesei</i>	Glutaraldehyde	High storage and pH stability	10	[163]
MNP@MPS	<i>Aspergillus</i>	MBA	High pH, temperature and storage stability	6	[164]
MNP	<i>Aspergillus niger</i> (β -glucosidase)	Glutaraldehyde	High storage stability	16	[165]
AMNP@ECH	<i>Sweet almond</i> (β -glucosidase)	IDA-Co ²⁺	High initial enzymatic activity and temperature stability	16	[166]
MNP/Polymer/MPS	<i>Coptotermes formosanus Shiraki</i> (β -glucosidase)	IDA-Ni ²⁺	High pH, temperature and storage stability	11	[167]

Notes: HPG – Hyperbranched polyglycerol; TDI – 2,4-toluene diisocyanate; 3-PPA – 3-phosphonopropionic acid; 16-PHDA – 16-phosphonohexadecanoic acid; EDC – 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; MSN – Mesoporous Silica nanoparticles; APTMS – 3-aminopropyltrimethoxysilane; APTES – 3-aminopropyltriethoxysilane; NHS – N-hydroxysuccinimide; CD – β -cyclodextrin; MPS – 3-(trimethoxysilyl)propylmethacrylate; MBA – methylenebis(acrylamide); AMNP – Embedding of MNPs by agarose; ECH – epichlorohydrin; IDA – iminodiacetic acid.
MPS – γ -Methacryloxypropyltrimethoxy-silane.

case of SEM, a field emission scanning electron microscopy has the advantage of obtaining higher resolution images. Another way of analyzing the samples may be by the technique known as "environmental SEM" in which it is possible to analyze moist samples, however, in this mode, the resolution is decreased. The Cryo-SEM mode has been used and is shown as a promising tool by allowing analysis of the samples close to the native conditions since the samples can be frozen fast which can avoid denaturation and deformations due to agglomeration caused by drying the sample. In addition, Cryo-SEM allows the tomography of the sample which can be a very interesting tool to reveal

the actual conformation of the enzyme on the substrate. In general, SEM has been mainly used to prove the enzymatic functionalization on the support, through the photomicrographs of the pure support after the formation of the composite [168–171].

In TEM analysis, the electron beam passes through a thin sample, the greater the electron density of the elements present in the sample the fewer electrons cross sample forming dark contrasts from denser and clearer regions to less dense ones. By using TEM analysis it is possible to elucidate the crystalline structure of a particle by using electron diffraction. In this mode an electron diffraction pattern is

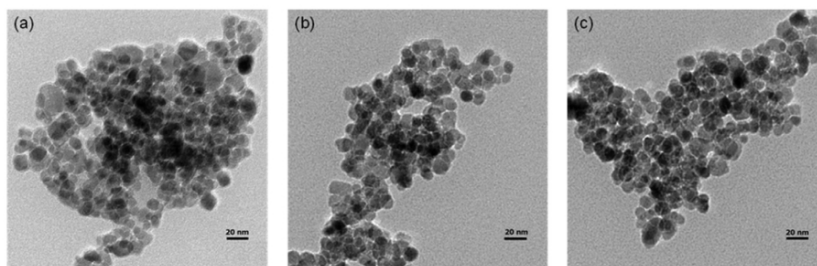


Fig. 4. TEM images of a) pure Fe₃O₄; b) Fe₃O₄-chitosan; c) cellulase MCLEA. Reprinted with permission from Ref. [155]. Copyright American Chemical Society (2014).

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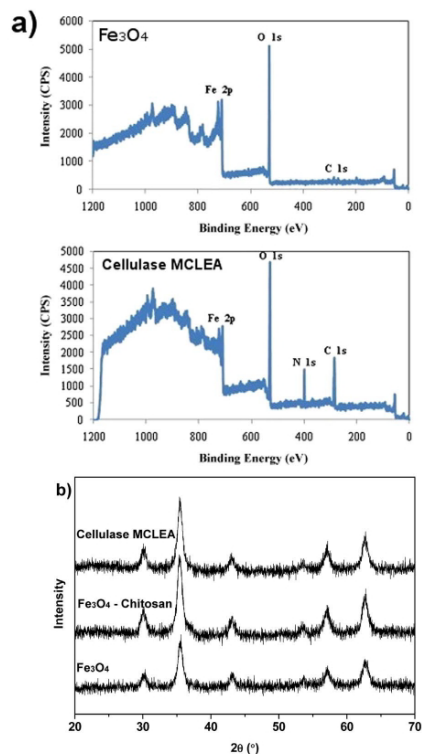


Fig. 5. a) XPS spectra of pure Fe_3O_4 and cellulose MCLEA; b) XRD patterns of, Fe_3O_4 , Fe_3O_4 -chitosan and cellulose MCLEA. Reprinted with permission from Refs. [153,155], respectively. Copyright Elsevier (2011) and American Chemical Society (2014).

generated. As in SEM in general TEM is more used to show the presence of the enzymes on the substrate by focusing the morphology and size of the substrate particles [172–175].

2.6.2. X-ray photoelectron spectroscopy (XPS)

Another powerful technique for the analysis of enzymatic biocomposites is the surface analysis known as X-ray photoelectron spectroscopy (XPS). The Fig. 5a shows that the XPS spectra give an elementary analysis of the sample based on the photoelectric effect allowing the characterization of the sample in a depth of up to 10 nm. Characteristic peaks at region of 398 and 248 eV on MCLEA XPS spectrum indicate the presence of nitrogen and carbon, respectively, suggesting the attachment of enzyme to support surface. With the exception of the lighter atoms (hydrogen and helium), all the elements in the sample can be identified by the XPS spectra. The sample is bombarded with a low-energy X-ray beam that causes a photoelectrons emission through the excitation of electrons, which are captured by a device capable of solving them in function of their kinetic energies characteristic of the atom of origin. The counting of the electrons as a function of the kinetic energy of these generates the spectrum of XPS that allows analyzing the surface of the sample, in a quantitative way being able to give information referring to the valence and the chemical bonds present in the sample [176].

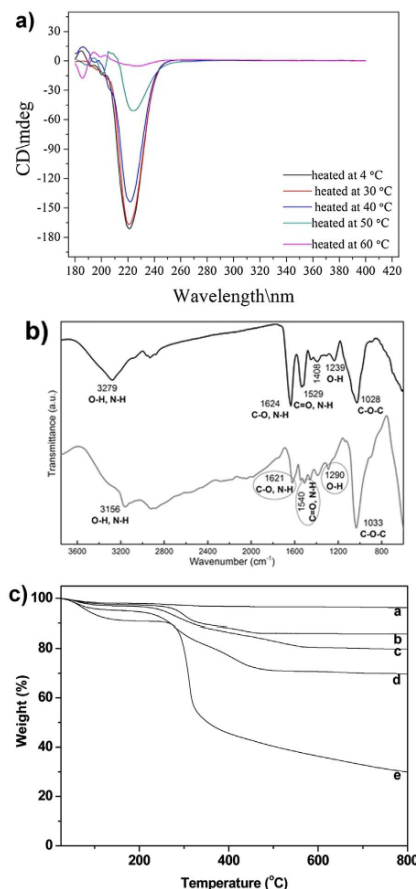


Fig. 6. a) CDS spectra of amine dehydrogenases enzyme heated at different temperatures; b) FTIR spectra of cellulase MCLEA (bottom gray line) and free cellulase (top black line); c) TGA curves of Fe_3O_4 (a), Fe_3O_4 -chitosan (b), Fe_3O_4 -chitosan-glutaraldehyde (c), cellulase MCLEA (d) and chitosan (e). Reprinted with permission from Refs. [148,181,155], respectively. Copyright Elsevier (2017), Springer Nature (2016) and American Chemical Society (2014).

2.6.3. X-ray diffraction (XRD)

The current trend of the use of nanomaterials as support for immobilized enzymes makes it necessary to characterize this support by different techniques among XRD is commonly used to characterize the crystalline structure of the support. In general, as shown in Fig. 5b, recent researches have used XRD for the characterization of the support before and after immobilization of the enzymes [177–179]. It is a powerful technique to verify if there was any change in the crystalline structure of the support after modification with enzyme.

2.6.4. Circular dichroism spectroscopy (CDS)

The circular dichroism spectroscopy (CSD) technique is a powerful technique widely used in the characterization of proteins. The CSD is a spectroscopic method based on the differential absorption of light

circularly polarized by optically active molecules and allows to determine its absolute configurations. As light passes through a stereogenic center present in the sample, there will be differential absorbance of the two circulating components to the right or to the left [180]. The technique is non-destructive, easy to operate, fast and requires low sample quantities and can be used in room temperature conditions [181]. As shown in Fig. 6a, effects of temperature in the conformational changes of the secondary structure can be studied using CDS measurements. The characteristic peak at 220 nm in CDS spectra is attributed to α -helix secondary structure of enzymes and normally has been used to investigate conformational changes. It allows quantification of the secondary structure of proteins in terms of the α -helical, β -strand and unordered structure using the distant UV range of the electromagnetic spectrum (260–170 nm). The range between (260–300 nm) is used to record the aromatic residues of the proteins. Absorption of the radiation occurs with the promotion of the ground state electrons to an excited state, in protein molecules, the peptide bond exhibits two transitions in the far UV $n-\pi^*$ at ~ 220 nm and a $\pi-\pi^*$ transition at ~ 190 nm. Being the characteristic signal influenced secondary structure of the protein, in the signal spectra, are results of the sum of all the signals present throughout the structure of the sample. In general the technique presents great potential in the characterization of immobilized enzymes allowing the investigation of structural changes occurred during the immobilization of the samples on the support, however, their use in these cases must be interpreted with caution due to the appearance of artifacts since the enzymes after immobilization have a high scattering factor and lower colloidal stability that may interfere during the measurement [182].

2.6.5. Fourier-transform infrared spectroscopy (FTIR)

FTIR is a very powerful tool for the detection and characterization of the enzymatic composites, allowing the investigation of the molecules because it allows identifying the chemical bonds vibration mode present in the sample [183–187]. The technique is based on the absorption or transmission of infrared radiation by the investigated sample. As shown in Fig. 6b, many bands near at 1540 and 1620 cm^{-1} are characteristic of amide groups of enzyme and have been used to confirm enzyme immobilization. FTIR is also important to identify if the functional group is on the surface of support before modification with enzyme.

2.6.6. Thermal analysis

Thermogravimetric studies are used to evaluate the immobilization and to study the thermal stability of conjugate. It can also be used to calculate the amount of enzyme immobilized in the support [188–190]. In this type of analysis, a mass of the sample is submitted to a temperature cycle while its properties are recorded. In a thermogravimetric analysis (TGA) the heat is used to promote reactions, decomposition and structural physical processes while the mass of the sample is monitored as a function of temperature or time as it is subjected to a controlled atmosphere-temperature program. Based on this, thermogravimetric curves allow the characterization of different physicochemical properties of the analyzed samples, such as sample decomposition, thermal stability and kinetic parameters for chemical reactions in the sample. Zang et al. [155] used TGA measurement to estimate the weight percentage of cellulase in a MCLEA based in Fe_3O_4 coated with chitosan. The amount of enzyme immobilized in the support was 10.26% (114.8 mg cellulase/g support), value consistent with the results determined by colorimetric Bradford assay (Fig. 6c), which show the importance and utility of thermal analyses.

3. MCLEAs applied to biomass conversion

Biomass can be converted to a wide range of products and raw materials and currently the studies about new technologies, such as MCLEAs applied to biomass conversion, is growing. Here we sum-

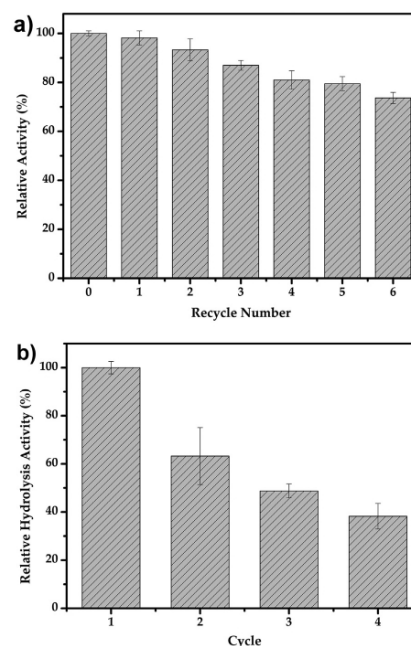


Fig. 7. a) Reuse assay of cellulase MCLEA using carboxymethyl cellulose as model substrate; b) Reuse assay of cellulase MCLEA using bamboo biomass as substrate. Reprinted with permission from Ref. [196]. MDPI License CC BY 4.0. Open access.

marizes some recent results of the MCLEAs applications to cellulose and hemicellulose hydrolysis and, in the next section, applications in conversions to important chemicals from C_6 and C_5 sugars, as well as others biomass-derived.

3.1. Cellulose conversion

The utilization of cellulosic biomass resources to produces value-added chemical by enzymatic processes is a beneficial green alternative facing fossil fuels. In this context, the use of MCLEA applied to hydrolysis of different cellulosic materials is growing [191–195]. Thus, aiming the bamboo biomass hydrolysis, Jia et al. [196] obtained a MCLEA based on cellulase glutaraldehyde cross-linked to amino-coated Fe_3O_4 . As shown in Fig. 7a, after three and six cycles, this cellulase MCLEA retained 87% and 74% of its initial activity when carboxymethyl cellulose was used as model substrate. A decrease in the recycle efficiency was observed when the authors applied the MCLEA in the bamboo biomass conversion (Fig. 7b). The hydrolysis activity of immobilized cellulase for two and four cycles was determined to be 63% and 38%, respectively. Besides that, while yield hydrolysis of bamboo biomass for free cellulase reached 44%, the MCLEA reached 21%. These decreases in the activity and recycle efficiency may be related to compact super-molecular feature of MCLEAs, which could lead to increasing diffusion limitations when the substrate were macromolecules [197]. Better results, however, were obtained by Sánchez-Ramírez et al. [148] using a cellulase MCLEA based on Fe_3O_4 coated with chitosan in the hydrolysis of *Agave atrovirens* leaves pre-treated. In this case, the yield of biomass conversion was showed to be 60.64% and 56.34% for free enzyme

and cellulase **MCLEA**, respectively. Already Abraham et al. [198] improve a hydrolysis yield of the hemp hurd biomass from 89% to 93% with a cellulase **MCLEA** based on zinc ferrite magnetic nanoparticles.

The catalysis of rice straw was studied with a **MCLEA** obtained by the combination of cellulase and β -cyclodextrin- Fe_3O_4 nanoparticles via 3-aminopropyltriethoxysilane and glutaraldehyde [199]. Although after immobilization, the cellulase activity was decreased by 10% compared to free cellulase, with the maintenance of 80% of the initial activity after 16 reaction cycles, these **MCLEA** showed to be able to produce 208.92 g L^{-1} of sugar against only 10.5 g L^{-1} of the free cellulase. This satisfactory result may be related to improve of bond between enzyme and support. Many factors can influence the activity losses after consecutive cycles reaction, among which are: enzyme activity losses associated to bond nanoparticles; enzyme deactivation during the reaction and release of enzyme from support. For example, Jordan and Theegala [200], measuring the enzyme mass in medium reaction for the each recycle, observed that after six cycle, only 35.24% of enzyme remaining attached to magnetite support and 64.76% of enzyme was released from support, attributing to this detachment, the loss in activity. This shows the importance of effective attachment of enzyme to support during **MCLEA** synthesis. Another excellent result was showed by Zhang et al. [159] in the hydrolysis of pretreated corncob. A cellulase **MCLEA** was successfully prepared through the cellulase immobilization onto amino-coated Fe_3O_4 nanoparticles via glutaraldehyde cross-linker. The immobilization method was powerful in to recovery 99.1% of initial activity of the free enzyme activity and, as showed in Fig. 8a, at an initial corncob concentration of 2.0 mg mL^{-1} , 61.94% of corncob hydrolysis yield was obtained. Due to increasing in diffusion limitations, it is observed a corncob decomposition rate

decreases when the corncob concentration was gradually increased. A satisfactory reuse capacity with recovery of 91.1% of initial activity after 16 cycles was found using corncob at 2 mg mL^{-1} with a time reaction of the 5 h (Fig. 8b).

A new strategy that has been employed is a co-immobilization of two, or more, enzymes at the same magnetic support. In this context, Chen et al. [201] synthesized a **MCLEA** by co-immobilization of cellulase and lysozyme on amino-coated Fe_3O_4 nanoparticles, using glutaraldehyde as cross-linker, for extraction of lipids from microalgae, which have been recognized as excellent biomass resource for the third generation biodiesels [202]. Although competition during immobilization, a satisfactory activity recovery was showed, 78.9% and 69.6% for cellulase and lysozyme, respectively. For this **MCLEA**, 60% initial activity was retained after six cycles with 54.07% of yield of the lipids recovery from microalgae *Nannochloropsis* sp.

Driven by actual depletion of fossil fuels, the more prominent application of the biomass conversion is, certainly, the biofuels production. The first and second generation ethanol has mainly been produced from food crops and lignocellulosic materials due to its availability and low cost [203]. Thus, a comparative evaluation between of free and cellulase **MCLEA** for enzymatic hydrolysis of sugarcane bagasse was studied by Ingle et al. [204]. Free cellulase showed a bagasse hydrolysis yield of 78%, whereas in case of **MCLEA** a yield of 72% was found. However, with magnetic recovery, **MCLEA** can be reused in subsequent reactions, maintained 52% of yield in the third cycle. In the case ethanol production from glucose obtained by sugarcane bagasse hydrolysis, similar yields were obtained by free (93%) and cellulase immobilized (89%).

3.2. Hemicellulose conversion

In recent years, the biodegradation of hemicellulose to xylooligosaccharides has attracted attention in the industry of biofuels production and value-added chemicals, enhancing the efficiency of delignification and bleaching process in the pulp industry [205,206]. So, xylanases **MCLEAs** for hemicellulose hydrolysis have been obtaining and evaluated in different applications.

In this context, Purohit et al. [207] developed a xylanase **MCLEA** by cross-linking crude xylanase and Fe_3O_4 nanoparticles with glutaraldehyde for produce xylopentose and xylohexose from agro biomass. The xylanase **MCLEA** improved the xylooligosaccharides production and enzyme reusability. Free xylanase and xylanase **MCLEA** could convert respectively more than 45% and 60% of the powdered rice straw and corn cob into xylooligosaccharides. According to the authors, the process is environmentally friendly and the predominant xylopentose and xylohexose production could find unique applications in food industry and prebiotics. Liu et al. [208], supporting xylanase on Fe_3O_4 -coated chitosan magnetic nanoparticles, also studied the production of xylooligosaccharides. The xylanase **MCLEA** synthesized by these authors can be recycled easily and exhibited improved stability, beyond released high concentration of xylooligosaccharides from birchwood xylan. Xylotriose (1.782 mg mL^{-1}) and xylobiose (2.023 mg mL^{-1}) were the main products released from birchwood xylan. The xylanase **MCLEA** recovery activity 56.5% for xylanase and the residual activity was 87.5%, even after seven recycling processes. Satisfactory results was also obtained by Shahrestani et al. [209] when they studied a hemicellulose hydrolysis aiming the clarification of fruit juices using a xylanase **MCLEA**. After 10 reaction cycles in the enrichment of the juices clarification it could still retain about 55% of the initial activity. Soozanipour et al. [210] have studied a covalent attachment of xylanase on silica-coated magnetite nanoparticles using cyanuric chloride as cross-linker agent. This unusual strategy produced a xylanase **MCLEA** able to keep as high as 80% of activity of free xylanase. Notably, this **MCLEA** showed a quite impressive stability, even after 9 reaction cycles, maintaining almost 65% of the initial activity.

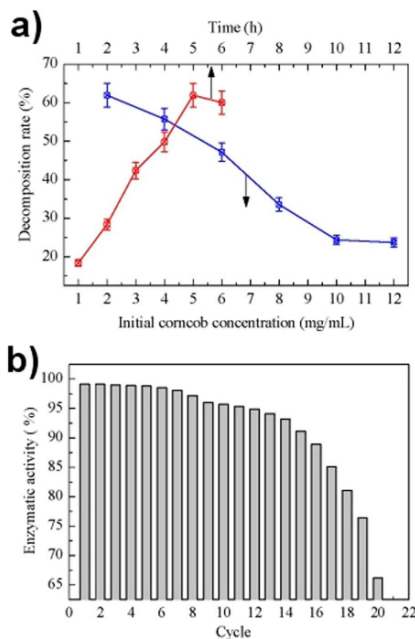


Fig. 8. a) Variation in decomposition rate of corncob as a function of initial corncob concentration and reaction time; b) Recovery and recycling stability using corncob at 2 mg mL^{-1} and time reaction of 5 h. Both experiments were carried out at pH 6 and 40°C . Reprinted with permission from Ref. [159]. Copyright Elsevier (2016).

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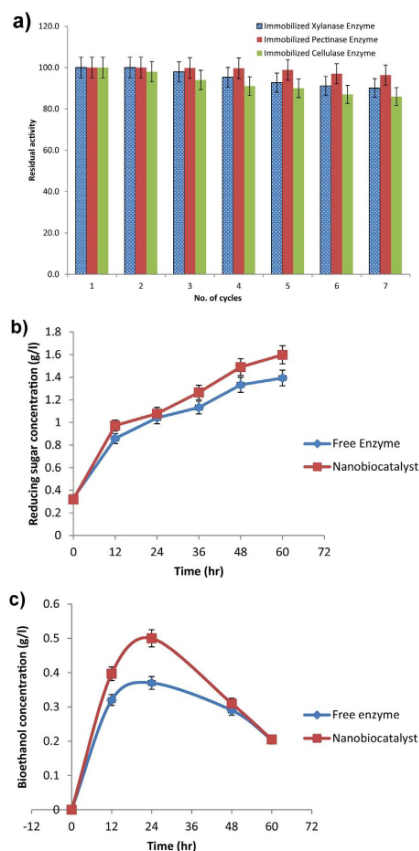


Fig. 9. a) Reusability assay of Pectinex 3xL MCLEA; b) Reducing sugar produced from wheat straw using free and Pectinex 3xL MCLEA; c) Ethanol concentration produced by hydrolysate obtained from free and Pectinex 3xL MCLEA. Reprinted with permission from Ref. [211]. Copyright Elsevier (2017).

In addition to the above mentioned examples, the multiple enzyme immobilizations have been tested recently also to xylanase. Perwez et al. [211] studied this new approach and prepared a reusable multipurpose magnetic nanobiocatalyst for industrial applications.

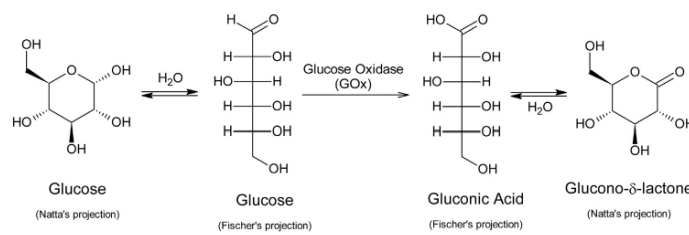


Fig. 10. Glucose to gluconic acid conversion.

The authors synthesized a **MCLEA** by conjugating Pectinex 3xL (a commercial enzyme complex containing xylanase, cellulase and pectinase) on 3-aminopropyltriethoxysilane coated Fe_3O_4 nanoparticles. This multipurpose **MCLEA** retained 69% of xylanase, 58% of cellulase and 87% of pectinase activity after conjugation on coated nanoparticles as compared to their soluble counterparts, and was reused by 7 cycles maintaining more than 80% initial activity of all immobilized enzymes (Fig. 9a). The **MCLEA** was used for production of bioethanol from pretreated wheat straw. The Fig. 9b shows that the hydrolysis of pretreated wheat straw produced 1.39 g L^{-1} and 1.59 g L^{-1} after treatment with free Pectinex 3xL and **MCLEA**, respectively, and the bioethanol production also increases, from 0.37 to 0.5 g L^{-1} , with the use of **MCLEA** (Fig. 9c), showing that the approach is an ideal candidate for biotechnology industry applications.

4. MCLEAs applied to another biomass-derived conversion

Indeed, the main applications of **MCLEAs** in biomass conversion have been targeted toward to hydrolysis of cellulosic and hemicellulosic biomass, aiming biofuels production. However, it is known that many other conversions of the biomass-derived are activated by enzymatic reactions, which open several opportunities to **MCLEAs** application.

Sugar-based biomaterials have the advantage of being cheap, biocompatible, biodegradable and sustainable [212]. Among them, glucose is the best-known sugar and has a wide range of application in materials [213–215]. However, the most commonly found linkages between glucose residues are readily hydrolysable, making it necessary to modify the base structure to obtain materials with specific characteristics. One of the possible modifications in the glucose is to confer on it the lactone function glucaro- δ -lactone, enabling a new set of materials [216].

As shown in Fig. 10, the glucose molecule, when in the acyclic form, has an anomeric terminal carbon which makes up an aldehyde group. This group is oxidized and gives rise to a carboxylate group. The conversion occurs enzymatically allowing the specific oxidation of the aldehyde group while the remaining hydroxyls remain unchanged. The enzyme glucose oxidase (GOx) promotes the simultaneous conversion of glucose into gluconic acid [217] and molecular oxygen into hydrogen peroxide [218]. The obtained gluconic acid remains in equilibrium with the lactonic form in the reaction medium [219,220], and has been an important chemical used as chelating agent, food additive, cleanser and versatile intermediate in the pharmaceutical, polymer and cosmetics industries [221].

Abbasi et al. [222] immobilized glucose oxidase (GOx) on the silica-coated magnetic nanoparticles. The synthesized GOx **MCLEA** allow the enzyme to acquire the conformational and structural arrangement for a better activity and stability, and suggests that binding of enzyme onto magnetic nanoparticles was efficient for creating biomaterial composite that can be used at wider range of pH (from 6 to 10) and temperature (from 25 to 60°C) for a variety of applications in health and food safety. As shown in Fig. 11a, in whole range of pH and temperature studied the GOx **MCLEA** showed better activity than the

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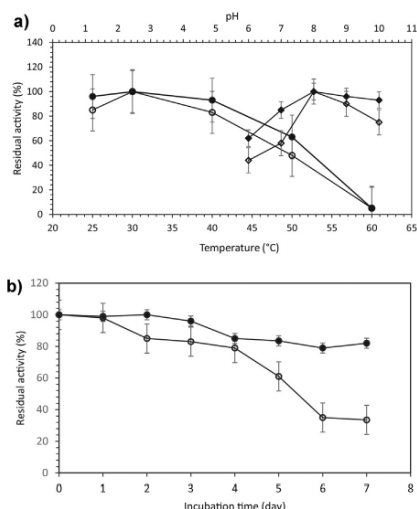


Fig. 11. a) Enzyme pH dependence (square) and thermostability (circle) of GOx MCLEA (filled symbol) and free GOx (opened symbol); b) Storage stability of GOx MCLEA (filled circle) and free GOx (opened circle). Reprinted with permission from Ref. [222]. Copyright Elsevier (2016).

free enzyme. Besides that, Fig. 11b shows that the residual activity in assessing storage stability decreases for both free and immobilized GOx over time, but the loss of activity is more than 60% after 7 days for free enzyme while it is only 20% for immobilized enzyme.

As showed in the item 2.3, glycerol can be also obtained from biomass hydrolysis. The conversion of glycerol into products of general interest, specifically biomaterials, has attracted significant effort from academics community and industry [223]. The simple conversion of glycerol to dihydroxyacetone (DHA) is one of many solutions for the endeavor employed. DHA can be produced from the selective oxidation of glycerol through of the enzyme glycerol dehydrogenase (GlyDH). The enzyme acts on the secondary hydroxyl of the polyol, converting it into a ketone group [224]. The enzymatic conversion is presented as a sustainable, cheap option and allows industrial expansion.

Zheng and Zhang [225] immobilized GlyDH on magnetic silica nanoparticles containing free amine groups on the surface via glutaraldehyde cross-linker. The Fig. 12a shows that the immobilization did not significantly alter the optimum temperature and pH of the enzyme's maximum activity but made it less sensitive to changes in pH and temperature over the range studied. When pH or temperature deviated from the optimal conditions, better activity was retained for the GlyDH MCLEA compared to the free enzyme. The increase in tolerance to variations in pH and temperature was possible due to the chemical support-enzyme binding, maintaining the original configuration of the enzyme. As shown in Fig. 12b, the enzyme activity recovery of 81.1% under the optimized immobilization condition and the immobilized GlyDH retained 91% of its original activity after 10 cycles.

Regarding the possible applications for DHA, its use as a hydrophilic polymer has attracted interest from several researchers. Zelikin et al. [226] have used DHA as the monomer in the synthesis of a biomaterial whose surface can be easily functionalized in one-step-manner and has the strength compared to a human bone. Weiser et al. [227] has combined DHA with lactic acid, both biomass monomers, for the synthesis of a copolymer that can be applied to drug delivery, tissue engineering and surgeries. Similar to the previous example, Henderson

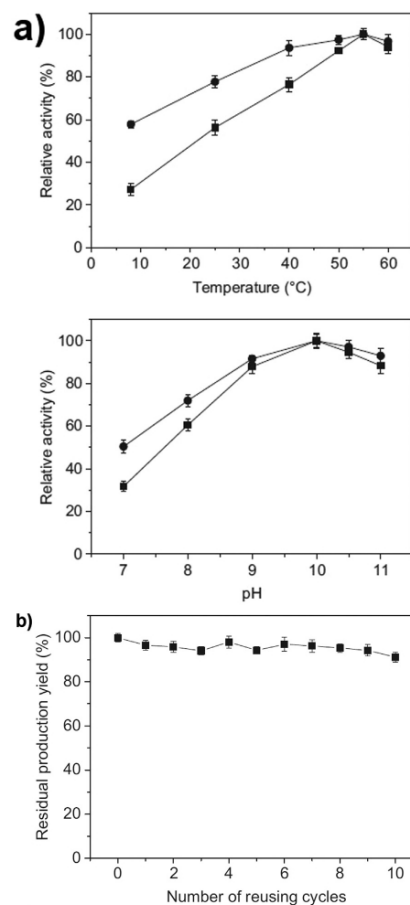


Fig. 12. a) The optimal temperature and pH of enzymatic activity for free (square) and GlyDH MCLEA (circle); b) Activity loss over 10 cycles of GlyDH MCLEA. Reprinted with permission from Ref. [225]. Copyright Taylor & Francis (2011).

et al. [228] have combined the qualities of DHA and polyethylene glycol in the synthesis of an effective and rapidly resorbable hemostatic agent which can find broad hemostatic application in a wide range of surgical procedures.

Lastly, MCLEAs have been also tested in the conversion of raw lignocellulosic biomass by lignin hydrolysis. As previously stated, lignin is a three-dimensional polymer of phenyl propane units and serves as a support for vascular plants [229,230]. Several phenolic compounds are used as cross-linking agents between the three-dimensional structure of lignin and the chains of hemicellulose [37]. Among these, is the ferulic acid (FA), which acts as a bridge between the arabinose residues of arabinosylan belonging to hemicellulose and non-core lignin [231,232]. The enzyme ferulic acid esterase (FAE) can selectively hydrolysis the ester linkage of ferulic acid and xylan fragment, so, FA can be produced from different biomass substrates and used as precursor of several chemicals including the flavouring agent vanillin [233].

Thus, Gadalkar and Rathod [234] immobilized ferulic acid esterase on magnetic nanoparticles aiming to enhance of ferulic acid from defatted rice bran in ultrasound presence. This FAE **MCLEA** was obtained via attachment of FAE on amino-coated magnetite nanoparticles using glutaraldehyde as cross-linker agent. The optimization of the synthesis parameters (enzyme loading, solvent/solute ratio, temperature and sonication time) improved yield and reduced process time through a predictive model. An immobilization yield of about 77% was obtained after 1 h, while by the conventional procedure the yield was close to 65% after 4 h. After 5 reaction cycles, the FAE **MCLEA** allowed a retained activity up to 82%, showing that this strategy can be also applied in the value-added chemicals extraction from biomass.

5. Conclusions and outlooks

The current global demand for renewable raw materials as an alternative to fossil sources has driven the search by development of new green technologies. The lignocellulosic biomass shows promising due to its abundance and wide range of possible applications. Besides that, the enzyme immobilization and the use of magnetic cross-linked enzyme aggregate (**MCLEAs**) has spurred interest in the biomass conversion processes. Many studies described in this review showed increases in the activity and enzymatic stability after enzyme immobilization in magnetic supports, as well satisfactory recovery and improves in the reactions yield to obtaining a wide range of value-added chemicals of particular interest to fuels, materials, environment and food science. Moreover, because the enzymes can be applied in several chemical reactions, the **MCLEA** applications do not is limited to biomass conversions. These show that in future the **MCLEAs** can be used in important fields as medicines production, biodiesel, textile industry, biosensors, cosmetics and bioremediation. Despite of recent advance in the production of these new nanohybrids materials, it is still desired more studies about optimization of synthesis and employ of new supports materials combined with magnetic nanoparticles to increase the efficiency of **MCLEAs** in lignocellulosic conversion and recovery. Certainly, when this objective is attained, a real step for the sustainable development will be given, once such improves can help to overcome some challenges as replace same applications of fossil sources as raw materials in a global scale.

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Easily handling penicillin G acylase magnetic cross-linked enzymes aggregates: Catalytic and morphological studies



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ABSTRACT

Biomolecules labeled with superparamagnetic nanoparticles can be selectively removed from complex reaction mixtures using an external magnetic field. Amino-functionalized superparamagnetic iron oxide nanoparticles (amino-SPION) were co-aggregated with penicillin G acylase and then cross-linked, generating magnetic cross-linked enzymes aggregates (M-CLEAs) that were quickly and efficiently recovered from the reaction medium by applying an external magnetic field. M-CLEAs and cross-linked enzymes aggregates (CLEAs) prepared under the same reaction conditions were characterized and compared. The best recovered activities were obtained for M-CLEAs prepared using polyethylene glycol 600 as precipitant and the most stable M-CLEA were obtained using tert-butanol. Successive penicillin G hydrolysis reactions were carried out using the same M-CLEA in a 50 mL reactor (3 reaction cycles), after the reactions the derivate was magnetically recovered without loss of activity demonstrating a total magnetic recovery. Line-scan energy dispersive X-ray spectroscopy showed that the amino-SPIONs were homogeneously dispersed within the structure of the M-CLEA.

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1. Introduction

Proteins aggregation is a common and well-known method for their purification and/or concentration that has been applied as a starting point for enzymes immobilization [1]. In this methodology, enzymes physically aggregated are chemically cross-linked using a bifunctional agent or a reactive polymer forming insoluble aggregates named “cross-linked enzymes aggregates” (CLEAs) that retain, completely or partially, the enzymatic activity. Despite of the many advantages of CLEA methodology over other enzyme immobilization methods [2], CLEAs particles have a drawback: they tend to form stable suspensions that are difficult to recover by conventional separation methods, such as centrifugation and filtration, which can make its application at industrial scale difficult in some cases [3,4]. To overcome the problems with CLEAs recovery and handling the co-aggregation between proteins and superparamagnetic nanoparticles has been recently used [5].

Superparamagnetic nanoparticles are attracted to a magnetic field but, ideally, they retain no residual magnetism after the magnetic field is completely removed [6]. Thus, suspended superparamagnetic nanoparticles in solution can be removed from a reaction mixture using an external magnetic field, and after the removal of the magnetic field they do not agglomerate. Superparamagnetic iron oxide nanoparticles (or SPIONs) are the most widely studied class of magnetic nanoparticles for biotechnological and biomedical applications. The removal of particles from a solution by using magnetic fields is selective [7] and can be applied to separations that would be impossible or impractical to be achieved by other techniques [8]. Although the application of superparamagnetic nanoparticles in enzymes immobilization and purification have been extensively studied in recent years [8–12] the magnetic recovery of the biocatalyst is not really efficient because of the nanometer size of the magnetic particles.

In this context, the preparation of magnetic cross-linked co-aggregates between penicillin G acylase (PGA) and amino-SPION (called M-CLEAs) was tested. PGA has been the most studied enzyme in CLEAs preparation [1,13–19]. So, it was selected as a model enzyme, to be submitted to co-aggregation and cross-linking with amino-SPIONs under different conditions. The amino-SPION was used as a magnetic tag, with the main objective of providing the

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aggregates with magnetic properties. The catalytic and morphological properties of PGA M-CLEAs and CLEAs, prepared using similar methodologies, were extensively investigated. Finally, M-CLEAs magnetic recovery efficiency was evaluated and its performance was tested in the hydrolysis of penicillin G.

2. Materials and methods

2.1. Materials

For the amino-SPION synthesis and functionalization the following reagents were used: iron bromine (II) and (III), 3-aminopropyl-triethoxysilane (APTES), and methanol, all from Aldrich. *Escherichia coli* PGA used in this research was a product from Antibioticos S.A. (Leon, Spain), kindly donated by Prof. José M. Guisán (Instituto de Catálisis y Petroleoquímica, Madrid, Spain), and contained 120IU and 26 mg of protein per mL of solution. For the M-CLEAs and CLEAs syntheses and for the evaluation of their properties, dimethoxyethane (DME) (Fluka), tert-butanol (TBA) (Vetec, Brazil), polyethylene glycol 600 (PEG 600) (Synth, Brazil), glutaraldehyde 25% (w/w) aqueous solution (Vetec, Brazil), sodium borohydride (Vetec, Brazil), 6-nitro-3-(phenylacetamido) benzoic acid (NIPAB) (Sigma), penicillin G (PG) potassium salt (donated by Produtos Laboratório Farmacêutico Ltda, Brazil), p-dimethylaminobenzaldehyde (PDAB) (Sigma), bovine serum albumin fraction V (BSA) (Sigma) and methanol (J.T. Baker) were used.

2.2. Amino-SPION synthesis and functionalization

The SPION synthesis and functionalization was done following the methodology presented by Marques et al. [20] and refined by Bini et al. [21]. The SPION was synthesized by a sonoprecipitation method using iron bromine (II) and (III). The SPION functionalization was carried out using hydrolysis and condensation of 3-aminopropyl-triethoxysilane (APTES). The uncoated SPION (1 g) was added to 30 mL of an APTES ethanol solution containing 1 mM of APTES and refluxed for 2 h. The samples were then washed several times with deionized water and resuspended in phosphate buffer (100 mM, pH 7.0).

2.3. Amino-SPION characterization

Electron micrographs were taken with a Philips CM 120 transmission electron microscope (TEM). Dry samples of the magnetite complexes were dispersed in isopropyl alcohol and cast onto amorphous carbon-coated copper grids. The X-ray diffraction (XRD) pattern was recorded in a Philips powder diffractometer PW 3040/60 with CuK α radiation. Infrared spectra were recorded in the 4000–400 cm⁻¹ range using a Fourier transform infrared spectrometer (FT-IR, Nicolet Instruments Corporation).

2.4. M-CLEAs synthesis

M-CLEAs were prepared adding 16 mL of a precipitant (DME, TBA or PEG 600) to a 8 mL solution containing PGA (40 mg, approximately 184 IU of activity) and amino-SPION (dry mass = 40 mg) in 100 mM sodium phosphate buffer pH 7.0. Immediately after the addition of the precipitant, a glutaraldehyde solution (25%, v/v) was added to final glutaraldehyde concentrations of 25 mM, 75 mM or 150 mM. The reaction was performed in a jacketed reactor under mechanical stirring at 2 °C. After 3 h, the pH was increased by adding 24 mL of 200 mM sodium bicarbonate buffer pH 10.0. After, kept under stirring by 30 min, 25 mg of sodium borohydride were added and allowed to react during 30 min. Finally the M-CLEAs particles were recovered from the reaction medium using an external magnetic field, and washed three times with 100 mM sodium phosphate buffer, pH 7.0.

2.5. CLEAs synthesis

Reference CLEAs were prepared under the same reaction conditions used for the M-CLEAs. For the CLEAs preparation, 16 mL of precipitant were added to 8 mL of a PGA solution (80 mg of protein, approximately 370 IU of activity) in 100 mM sodium phosphate buffer, pH 7.0; a glutaraldehyde solution (25%, v/v) was immediately added to a final glutaraldehyde concentration of 25 mM. The CLEAs particles were kept under stirring at 2 °C for 3 h, then the reaction volume was duplicated by adding 200 mM sodium bicarbonate buffer at pH 10.0. After 30 min, 25 mg of sodium borohydride were added and allowed to react for another 30 min. CLEAs particles were recovered by filtration using Whatman paper filter grade 5 and washed three times with 100 mM sodium phosphate buffer, pH 7.0.

2.6. PGA activity

The PGA hydrolytic activity, for the soluble enzyme, M-CLEAs and CLEAs, was measured by calculating the initial rate of hydrolysis reactions for both, the synthetic substrate NIPAB and the natural substrate penicillin G, catalyzed by a known amount of PGA. One NIPAB activity unit (U_{NIPAB}) is the amount of enzyme that hydrolyze 1 μ mol of NIPAB to 5-amino-2-nitrobenzoic acid (NABA) per minute at pH 7.5 and

25 °C. NABA formation was measured at 405 nm (NABA molar extinction coefficient is 9.09 mM⁻¹ cm⁻¹). One international activity unit (IU) corresponds to amount of enzyme that hydrolyze 1 μ mol of PG (4%, w/v) per minute at pH 8.0 (phosphate buffer 0.1 M) and 37 °C. The PG hydrolysis was followed by measuring, at 415 nm, the increase of absorbance of the PG hydrolysis product, 6-aminopenicillanic acid (6-APA), after reaction with p-aminobenzaldehyde (PDAB) [22]. The PG hydrolysis was performed in a jacketed reactor with mechanical stirring.

2.7. Protein concentration

Bradford method [23] was used to measure protein concentration in the enzyme preparations. The protein content (mg) was spectrophotometrically quantified at 595 nm using bovine serum albumin (BSA) as protein standard.

2.8. Thermal stability

Solutions containing M-CLEAs, CLEAs or free enzyme (approximately 0.3 IU per mL) were incubated at 55 °C in 100 mM phosphate buffer pH 7.0, aliquots were withdrawn and the residual activity (RA%) was measured at different incubation times using the standard NIPAB method. Half-life was defined as the time, in hours, required for the residual enzymatic activity in the sample reaches one half of its initial value.

2.9. Stability in methanol

M-CLEAs, CLEAs or the free enzyme (approximately 0.3 IU per mL) were incubated in a solution containing phosphate buffer 300 mM pH 6.5 and methanol in a 1:1 ratio (v/v). Aliquots were withdrawn and the RA% was measured at different incubation times using the standard NIPAB method.

2.10. Scanning electron microscopy (SEM) analysis and energy dispersive X-ray spectroscopy (EDS) line-scan profile

The morphology of M-CLEAs and CLEAs was investigated by using a Philips XL30 FEG operating at 25 kV; the SEM images were obtained at distinct magnifications. The particle diameter distribution (Feret's diameter) was calculated by image analysis using the ImageJ software. The EDS line-scan analysis was performed in the same equipment using an Oxford Tetra Link detector.

2.11. Magnetic recovery

A glass bottle containing 10 mL of a 100 mg/mL (dry mass) M-CLEA-TBA-G150 solution was placed on a N35 NdFeB magnet (22 mm diameter x 20 mm thickness). The M-CLEA-TBA-G150 recovery time course was monitored over time. Samples were withdrawn using a micropipette at two distinct points: 2.0 cm and 1.0 cm from the magnet surface, and PGA activity was measured using the standard NIPAB method. The calculated magnetic field strength at the bottle center was approximately 0.11 T at 2.0 cm from the magnet surface and 0.51 T at 1.0 cm from the magnet surface. The catalyst recovery time course was expressed in terms of residual activity (RA%). A reference flask was prepared in the same conditions and placed far from the magnetic field.

2.12. Penicillin (PG) hydrolysis

In a mechanically stirred jacketed reactor containing 7 mL of 5% PG solution (w/v), 1.6 IU of the M-CLEA-TBA-G150 were added. The reaction was conducted at 37 °C by 600 min and the pH was continuously adjusted to 8.0 by addition of 1 M NaOH solution aliquots using a pHstat Titrimo 718 (Metrohm AG). The PG hydrolysis rate was monitored by the titration of the H⁺ released during the reaction. After the PG hydrolysis, the M-CLEA-TBA-G150 was washed one time with distilled water and one time with 5% PG solution and the reaction was reinitiated. For comparison PG hydrolysis using free PGA (1.6 IU) was carried out at the same reaction conditions employed for M-CLEA-TBA-G150.

3. Results and discussion

3.1. Amino-SPION synthesis and functionalization

Amino modified superparamagnetic iron oxide nanoparticles (amino-SPION), the magnetic tags, were prepared using a method developed by Marques et al. [23]. Fig. S1 (available in the Supporting Information-SI) shows the TEM image of the amino-SPION. It is possible to verify that amino-SPION particles present nearly spherical shape with diameter of approximately 10 nm. Fig. S2 (available in the SI) shows the X-ray powder diffraction patterns of the uncoated SPION (black curve), and Bragg peak positions (gray curve, included for comparison) for the magnetite phase (Fe₃O₄). The presence

of broad and intense peaks confirms the formation of crystalline nanoparticles. The average particle size was determined from the broadening of the diffraction peaks applying Scherer's equation, and the calculated size was approximately 11.6 nm, agreeing with the estimated size from TEM images.

The FT-IR spectrum of the amino-SPION is shown in Fig. S3. The characteristic Fe–O bands of Fe_3O_4 bulk materials, corresponding to ν_1 and ν_2 frequencies, are located at 570 cm^{-1} and 375 cm^{-1} , respectively. When the particles size becomes nanometric, these frequencies will be shifted to a higher wave number [24]. Thus, the two bands around 632 cm^{-1} and 580 cm^{-1} result from split of the ν_1 band and the band at 447 cm^{-1} from the shift of ν_2 band. The strong absorption bands at 1100 cm^{-1} and 1035 cm^{-1} are ascribed to Si–O–Si stretching frequencies of siloxanes [24]. The bands at 935 cm^{-1} and 864 cm^{-1} involve the Si–O stretch from the Si–O–OH group. The bands at 2921 cm^{-1} and 2860 cm^{-1} are assigned to the C–H stretching mode of the propyl group present in the APTS molecule. The band at 1330 cm^{-1} is ascribed to the C–N stretching from the primary amine present in the APTS molecule [24]. The FT-IR data confirm the SPION APTS coating.

The sonoprecipitation and APTS coating method employed in this research to produce amino-SPION magnetic tags leads to magnetic nanoparticles with superparamagnetic properties. The $M \times H$ plot of amino-SPION at 300K exhibits a typical superparamagnetic behavior (hysteresis, coercivity or remanence could not be detected) and a high saturation magnetization (68 emu g^{-1}) [21]. In addition to its excellent magnetic properties, the amino-SPION size is in the same range of proteins ($\sim 11\text{ nm}$). A high surface amino group density (8.7 nmol/g) was attained with a pK_a of 10.05 (results obtained by zeta potential measurements) [21], the same pK_a of lysine amino groups. These characteristics make this material an ideal candidate for co-aggregation with proteins.

3.2. M-CLEAs synthesis

M-CLEAs were obtained using three different precipitants, 1,2-dimethoxyethane (DME), tert-butyl alcohol (TBA) and polyethylene glycol 600 (PEG). Aggregation between PGA and amino-SPION in the absence of a precipitant was not observed. Reference CLEAs (in the absence of amino-SPIONs) were prepared using the same reaction conditions. The M-CLEAs obtained in all employed reaction conditions were easily and quickly recovered by the application of an external magnetic field. After the removal of the magnetic field, the M-CLEAs were readily dispersed, forming stable suspensions. The CLEAs, in turn, formed unstable suspensions, like the ones observed by Pchelintsev et al. [3] for PGA CLEAs, which were extremely difficult to separate from the reaction medium. In the present work, centrifugation at $\sim 10,000 \times g$ was tried for this task using an Eppendorf centrifuge (model 5810R), but small aggregates were still not removed after 25 min of centrifugation, what could be confirmed by measuring PGA activity in the supernatant. Thus, filtration was the method selected for the recovery of CLEAs. Filtration allowed a complete recovery (it was not possible to measure enzymatic activity in the filtrate) but after the filtration process the aggregates were joined together, forming large clusters. The large CLEAs clusters were readily precipitated in solution, producing unstable suspensions.

Table 1 shows the recovered activity and the activity by mass unit for M-CLEAs and CLEAs obtained under the same reaction conditions. PGA activity was measured using two distinct methods, as previously mentioned: hydrolysis of the natural substrate PG and hydrolysis of the synthetic substrate NIPAB. Interestingly enough, while similar results were observed for CLEAs and M-CLEAs using the PG hydrolysis method, lower values of recovered and activity by mass unit were obtained for CLEAs using the NIPAB method. The NIPAB assay was carried out in a cuvette with a stirring

system and temperature control and the product, NABA, was measured on line by the increase of the absorbance at 405 nm. The fast and readily measurable hydrolysis of NIPAB, combined with an imperfect stirring within the apparatus turned mass-transport limitations more evident when this method was used. A similar behavior was observed for the hydrolysis of the synthetic substrate p-nitrophenyl propionate by CalB CLEAs with large clusters [2]. In this way, it is possible to infer that the increase in particle size caused by the filtration processes, observed for all PGA CLEAs, affects considerably their performance for NIPAB hydrolysis.

To verify this assumption, the CLEAs were ball-milled at 2°C for 2 h and the NIPAB activity was measured once again. After this procedure, an increase in NIPAB activity was observed, of 3.4 times for CLEA-DME, 1.8 times for CLEA-TBA and 3.0 times for CLEA-PEG, thus confirming that the large CLEAs particle size caused mass-transport limitations. It is extensively reported in the literature that decreased in size of porous carries may minimize diffusion problems, however, small carriers are most difficult to recover than the large ones [4].

In order to make a better evaluation of the mass transfer effects, NIPAB activities of M-CLEA-PEG, CLEA-PEG (before and after disrupting the cluster) and of the free enzyme were measured at 6 and at 25°C . The obtained results are reported in the support information section (Table S1). NIPAB activity of the free enzyme at 25°C was 2.15 fold higher than at 6°C , while M-CLEA-PEG activity was 1.91 fold. CLEA-PEG cluster's activities were only 1.12 fold higher at 25°C than at 6°C and the ball-milled CLEA-PEG ratio of activities was 1.77. The ratio between enzyme activities for M-CLEA-PEG (1.91) is close to the free enzyme's one (2.15), indicating a smaller mass transfer effect for this derivative, CLEA-PEG, in turn, presents a much lower ratio (1.12), an indication that severe mass transfer limitations are present. The ball-milling of CLEA-PEG, on the other hand, was able to reduce mass transport effects. These results illustrate the importance of a rapid, easy and gentle method for recovering the aggregates. For this purpose, co-aggregation between enzymes and amino-SPIONs is a good alternative.

Using the PG hydrolysis method, the observed recovered activity for M-CLEAs and CLEAs was quite similar, and the best results were obtained for M-CLEA-PEG and for M-CLEA-TBA; the CLEA-DME showed the worst result for this parameter. Similar results were obtained for recovered activity in PGA CLEAs prepared by Cao et al. [13] using TBA and PEG as precipitants, Shah et al. [17] using DME and TBA as precipitants and by Mateo et al. [14] using DME as precipitant. Takelar et al. [5] observed an increase in the recovered activity of α -amylase CLEAs containing amino-SPIONs, when compared with non-magnetic CLEAs prepared in similar reaction conditions. This unexpected behavior was attributed to an increase in the number of amino groups available for cross-linking, coming from the amino-SPIONs. The 100%-recovered activity indicates that no mass transfer limitations were present in the CLEAs containing amino-SPIONs, despite the fact that the α -amylase substrate is a macromolecule (starch).

During the preparation of M-CLEAs, 50% of the protein mass was replaced by amino-SPION superparamagnetic tags, causing a 50%-decrease in the derivative activity by mass unit (Table 1). The replacement of protein by amino-SPION was done to evaluate the co-aggregation process in terms of morphology and amino-SPION distribution in the M-CLEAs structure. Although the activity by mass unit of M-CLEAs has decreased when compared to CLEAs, it still remained within the same order of magnitude. The similar recovered activity and activity by mass unit (taking into account the dilution caused by the presence of amino-SPION particles) for M-CLEAs and CLEAs (measured in IU) show that the co-aggregation followed by glutaraldehyde cross-linking did not alter significantly the M-CLEAs hydrolytic activity when compared with CLEAs obtained under the same conditions.

Table 1

M-CLEAs and CLEAs prepared with different methodologies. RA% is the recovered activity for NIPAB and for PG hydrolysis (PDAB subscript); U/g = activity by mass unit using NIPAB hydrolysis and IU/g = activity by mass unit using PG hydrolysis. The experiments were performed all least in triplicate.

Sample	RA _{NIPAB} (%)	RA _{PDAB} (%)	U/g	IU/g
CLEA-DME ^a	9.0 (±1.0)	32.9 (±4.1)	561 (±73)	8468 (±504)
M-CLEA-DME ^a	35.2 (±2.8)	34.5 (±1.6)	1382 (±82)	3153 (±258)
CLEA-TBA ^a	8.0 (±2.4)	41.7 (±2.7)	410 (±72)	8253 (±164)
M-CLEA-TBA ^a	43.5 (±4.6)	47.9 (±3.1)	1441 (±161)	3910 (±259)
CLEA-PEG ^a	7.1 (±3.3)	45.8 (±5.0)	696 (±154)	6651 (±777)
M-CLEA-PEG ^a	42.4 (±2.0)	49.1 (±3.2)	1488 (±107)	4782 (±287)
M-CLEA-TBA-G75	38.3 (±0.3)	38.5 (±0.9)	1444 (±47)	3776 (±52)
M-CLEA-TBA-G150	31.4 (±0.9)	34.0 (±0.7)	1268 (±80)	3728 (±109)

^a Samples cross-linked with glutaraldehyde concentration of 25 mM.

Stable suspensions of M-CLEAs and CLEAs were prepared for the thermal and methanol-50% stability experiments. CLEAs formed clusters during the filtration process, which were carefully disrupted at 2 °C for 2 h before starting the experiments, in order to avoid the mass transfer limitations that could mask their NIPAB activities.

Fig. 1(a) shows the thermal stability results. M-CLEAs and CLEAs obtained employing the same precipitant showed similar behavior with respect to thermal stability (at 55 °C and pH 7.0). M-CLEAs and CLEAs obtained using DME and PEG 600 as precipitant presented a higher thermal inactivation rate than the free enzyme at the beginning of the experiment, but all other derivatives had a better performance. The best results were achieved for the aggregates prepared using TBA as precipitant. CLEA-TBA and M-CLEA-TBA presented half-lives 180% higher than the free enzyme (Table S2 in supporting information).

Activity and stability in organic solvents are important characteristics in enzymatic organic synthesis. For instance, co-solvents such as ethylene glycol and methanol may improve the selectivity toward product formation in the kinetically-controlled synthesis of β -lactam antibiotics [25]. Fig. 1(b) shows the stability of M-CLEAs and CLEAs in methanol 50% (v/v) (pH 6.5 and 25 °C). The free enzyme deactivated very quickly, and no activity was observed after 45 min. Precipitation of free enzyme in the presence of methanol, which could interfere in the residual activity measurement, was not observed. All M-CLEAs and CLEAs had a better performance than the observed for the free enzyme and once again similar behaviors were observed for M-CLEAs and CLEAs prepared using the same precipitant. The half-lives of these catalysts are in Table S2, the best results were obtained using TBA as precipitant. CLEA-TBA and M-CLEA-TBA had half-lives 1690% and 1580% higher than the free enzyme in methanol 50% (v/v), respectively. Although β -lactam antibiotics synthesis using methanol as co-solvent has been reported in previous works [26–28], PGA stability in the presence of this solvent was not systematically studied.

The increase of M-CLEAs and CLEAs stabilities in methanol 50% can be due to the reinforcement in the structural rigidity of the enzyme macromolecule, caused by glutaraldehyde cross-linking [4]. Microenvironmental effects due to the formation of aggregates may be another cause. The hydrophilic protein molecules (and the amino-SPION in the M-CLEAs) are close together in the aggregate structure, and consequently can better preserve the PGA hydration layer, and reduce negative effects caused by the methanol and other organic solvents [29].

3.3. Effect of glutaraldehyde concentration in M-CLEAs preparation

The M-CLEAs obtained using TBA as precipitant were selected to verify the effect of the glutaraldehyde concentration during the

cross-linking on the M-CLEAs properties. The RA% for M-CLEAs prepared using glutaraldehyde 75 and 150 mM (M-CLEA-TBA-G75 and -G150) are in Table 1. RA% decreased when glutaraldehyde concentration increased, a similar tendency was observed for PGA CLEAs prepared using TBA as precipitant [16] and *P. cepacia* lipase CLEAs using acetone as precipitant [17].

The M-CLEAs-TBA thermal stability was improved with the increase of glutaraldehyde concentrations, as shown in Fig. 2(a) and Table S2. This is an expected result, since a more intense cross-linking can cause a more intense rigidification of the structure of the enzyme, thus hindering conformational changes [30]. Majumder et

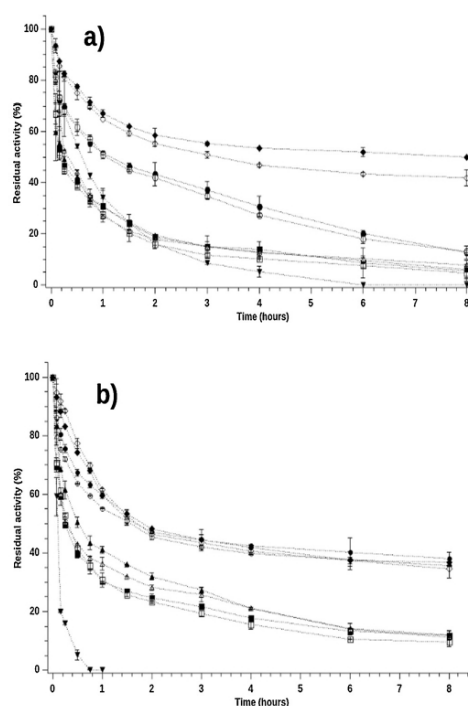


Fig. 1. (a) M-CLEAs and CLEAs thermal stability and (b) M-CLEAs and CLEAs stability in aqueous methanol 50% (v/v). Free enzyme (▼); CLEA-DME (■); M-CLEA-DME (□); CLEA-TBA (●); M-CLEA-TBA (○); CLEA-PEG (▲); M-CLEA-PEG (△); M-CLEA-TBA-G75 (◇); M-CLEA-TBA-G150 (◆). The experiments were performed in duplicate.

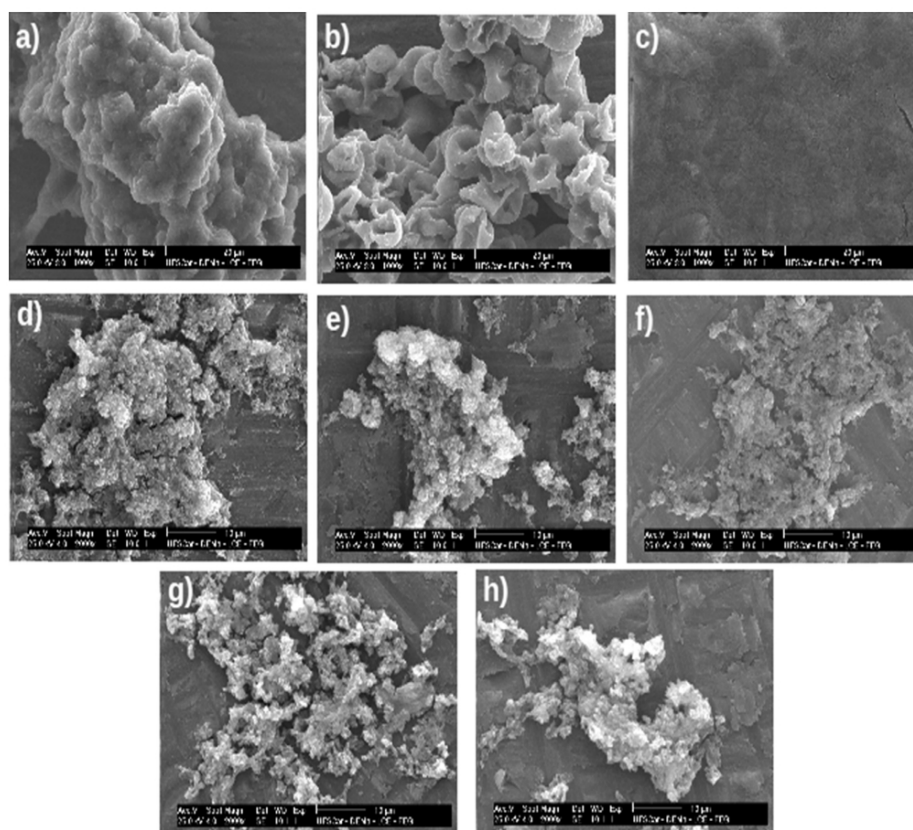


Fig. 2. SEM images to M-CLEAs and CLEAs: (a) CLEA-DME, (b) CLEA-TBA, (c) CLEA-PEG, (d) M-CLEA-DME, (e) M-CLEA-TBA, (f) M-CLEA-PEG, (g) M-CLEA-TBA-G75 and (h) M-CLEA-TBA-G150.

al. [31] also observed a significant increase in the thermal stability of lipase CLEAs with the increase of glutaraldehyde concentrations during the cross-linking step. The M-CLEAs and CLEAs PGA half-lives in this work were smaller than the ones observed by Shah et al. [17] and by Wilson et al. [19]. However, in these studies thermal stability was assessed at 50 °C, whereas in our study the stabilities were estimated at 55 °C. Thus, a direct comparison with these results would be unfavorably biased against our data. In addition, although the CLEAs preparation is a simple methodology these small differences in recovered activity and stability are expected because the structure, size, morphology and catalytic properties of CLEAs are dependent on several variables [4].

The stability of M-CLEA-TBA-G75 and M-CLEA-TBA-G150 in methanol 50% did not improve when compared with M-CLEA-TBA-G25. This fact suggests that the main factor for M-CLEAs stability in methanol is the effect of the microenvironment, rather than the increase in structural rigidity of the enzyme by glutaraldehyde cross-linking. The more hydrophilic environment in the M-CLEA structure can cause a partition effect that promotes the expulsion of methanol from the structure of the aggregate [29,30].

3.4. M-CLEAs and CLEAs structure

The fact that enzymes aggregates, such as M-CLEAs and CLEAs, are amorphous does not imply that they do not possess a certain level of organization. In reality it is expected that the enzymes aggregates present a considerable level of organization, since they are formed by proteins with a well-defined structure. In this way, enzymes aggregates are superstructures formed by supramolecular interactions. This superstructure organization is “frozen” by the action of bifunctional or polyfunctional agents.

The final structure of the aggregate and its catalytic properties are dependent on several factors: enzyme concentration, protein concentration (contaminant proteins and enzyme), physical and chemical characteristics of the proteins present in the solution and their relative concentration, precipitant agent used in the aggregation step, pH of the reaction medium, ionic strength of the reaction medium, presence of additives and their amount, nature and concentration of the cross-linking agent, among many other variables. Taking into account that the three-dimensional structure of enzymes in the aggregates is “frozen” under the influence

of many variables, unique characteristics can be achieved by the control of the aggregation conditions. Even contaminant proteins may be an important factor in defining the aggregates final properties, hence, a direct correlation between structural and catalytic properties becomes difficult.

Fig. 2 shows SEM images for all samples prepared in this study. It is possible to notice that they differ significantly with respect to their structure. Aggregates obtained using DME and TBA as precipitant apparently exhibits a less well-defined structure than aggregates obtained using TBA. CLEA-PEG was formed by large particles that covered almost the entire sampler. The aggregates obtained using TBA showed a more regular structure than the others. In Fig. S4 (supporting information), it is possible see the difference between the CLEAs and the M-CLEAs prepared using TBA. It is evident that CLEA-TBA had a more well-defined structure than the M-CLEAs obtained using TBA, but is possible to verify certain structural patterns in common between the CLEA-TBA and M-CLEA-TBA. It is possible that the higher structural organization observed in TBA aggregates caused their superior performance with respect to the recovered activity, thermal stability and methanol 50% stability, but the relationship between structure and performance is not easy to account for. Studies by Pchelintsev et al. [3] demonstrated the existence of a correlation between morphology, the catalytic properties of PGA CLEAs and the time elapsed between the precipitation and the cross-linking steps. Takelar et al. [5] have reported that SEM images of α -amylase CLEAs with and without amino-SPION present spherical appearance.

The lower recovered activity and activity by mass unit observed for CLEAs, measured using the NIPAB method, in comparison with M-CLEAs suggest a significant increase in mass-transport limitation for the CLEAs. Subsequently it was observed that disruption of CLEAs aggregates increased their NIPAB activity. To elucidate this question, the mean particle diameter for all samples was determined from their SEM images. The Feret's statistical geometric diameter was calculated from SEM images using the ImageJ software. The particle size distribution histogram (step size 20) can be seen in Fig. 3. The measured particle diameter was very similar for M-CLEAs samples, independently of the precipitant agent, the majority of particles having less than 20 μm . There were not observable particles with more than 90 μm for M-CLEAs-DME and M-CLEAs-PEG, and with more than 70 μm for M-CLEAs-TBA. In contrast, CLEAs aggregates have a wider particle size distribution, it was possible to observe particles larger than 600 μm for CLEA-DME, 650 μm for CLEA-TBA and 1800 μm for CLEA-PEG. Fig. 3d shows the particle size distribution for M-CLEA-TBA cross-linked with different glutaraldehyde concentrations. No dependence between M-CLEA-TBA particle sizes and glutaraldehyde concentration was observed. Results for *C. rugosa* lipase CLEAs [32] showed a different trend, with considerable increase in the particle size with the glutaraldehyde concentration during the cross-linking step.

3.5. EDS line scan profiles

Energy dispersive X-ray spectroscopy (EDS) line scan profile analysis of M-CLEAs samples may provide information about how the amino-SPIONs and proteins are organized in the aggregate superstructure. In this technique the SEM electron beam scans a pre-selected line across the sample. The interaction between the electron beam and the sample causes X-ray emissions detected in discrete positions along that line. Analysis of the X-ray energy spectrum at each position provides plots of the relative elemental concentration along the line. Thus it is possible to correlate the SEM image with the elemental profile.

Fig. 4 shows the line scan profile for M-CLEA-TBA-G75. The white line in Fig. 4(a) is the sample region where the scan was

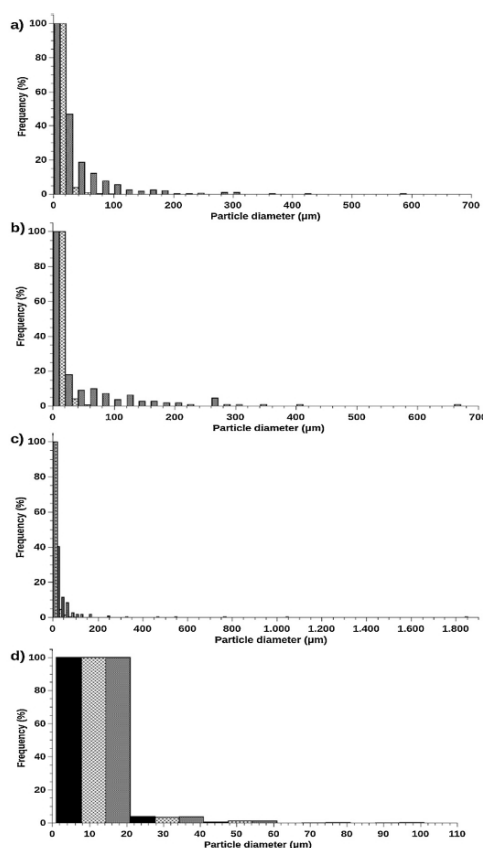


Fig. 3. Particle size distribution for M-CLEAs and CLEAs. (a) CLEA-DME (gray) and M-CLEA-DME (diagonal lines); (b) CLEA-TBA (gray) and M-CLEA-TBA (diagonal lines); (c) CLEA-PEG (gray) and M-CLEA-PEG (diagonal lines); (d) M-CLEA-TBA (black), M-CLEA-TBA-G75 (diagonal lines); and M-CLEA-TBA-G150 (gray). The particle diameter was obtained from SEM images using the ImageJ software, the frequency count was performed using a step size of 20 μm .

performed. Fig. 4(b) shows the topographical data of the sample (secondary electron mode – SE) and Fig. 4(c) shows the elemental data for iron distribution along the line. Comparing the SE plot with the iron distribution plot it is possible to clearly identify the sample region where iron is located. The similar pattern observed for these plots indicate that iron, and consequently the amino-SPION, is homogeneously dispersed throughout the M-CLEA-TBA-G150 structure. Similar results were observed for all others M-CLEAs samples (Figs. S5–S8 in the supporting information). It is reasonable that the high amino group density in the amino-SPION surface, its small size and its globular shape cause this material to behave as a protein during the aggregation process. The amino-SPION thus interacts with the proteins at a molecular level. The homogeneous distribution of amino-SPION over M-CLEAs particles in conjunction with the similar recovered activities, thermal stabilities and methanol stabilities obtained for M-CLEAs and CLEAs

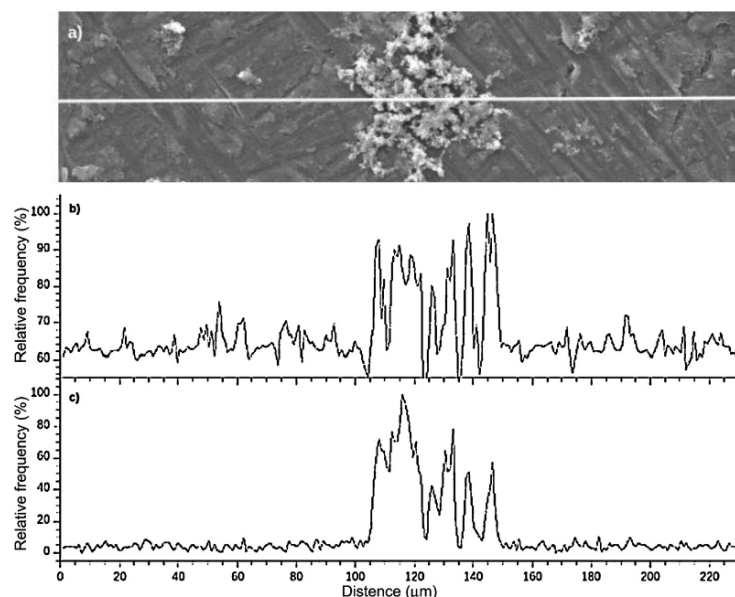


Fig. 4. M-CLEA-TBA-G75 EDS line-scan profile. (a) M-CLEA-TBA-G75 SEM; (b) SE plot (topographical data) and (c) iron presence (elemental data). The white line is the sample region where EDS analysis was performed.

prepared using the same reaction conditions suggest that the co-aggregation did not alter significantly the M-CLEAs properties in comparison with CLEAs. Thus, it would be possible to prepare M-CLEAs using the amino-SPION magnetic tag for virtually all enzyme preparation.

3.6. Magnetic recovery experiments

Despite the number of publications about immobilization of enzymes on magnetic materials, only a few report magnetic recovery experiments. To illustrate the efficiency of the M-CLEAs magnetic recovery a simple experiment was carried on using a 10 mL glass bottle containing M-CLEA-TBA-G150 (100 mg/mL), which was placed on a N35 NdFeB magnet (22 mm diameter $\times$ 20 mm thickness). Catalyst recovery time course was monitored measuring the PGA activity in the suspension at 2.0 and 1.0 cm from the magnet surface. In Fig. 5(a) it is possible to observe that the M-CLEA-TBA-G150 recovery was fast and efficient. At 2.0 cm from the magnet surface 50% of the particles were recovered after 17 s and at 1.0 cm only 25 s were required to recover 50% of the particles. A video of this experiment is available in the supporting information (video one). A reference flask containing M-CLEA-TBA-G150 (100 mg/mL) was prepared and positioned away from the magnetic field to evaluate the gravity effect under the M-CLEAs particles. The M-CLEA-TBA-G150 particles formed an extremely stable suspension in the absence of the magnetic field, after two minutes the measured activity was 98% at 2.0 cm and 100% at 1.0 cm from the flask base. The fast and efficient magnetic recovery observed in this experiment demonstrates that the co-aggregation between enzymes and magnetic tags is a valid strategy to provide magnetic properties to enzymes aggregates.

3.7. Penicillin G hydrolysis

To test the magnetic recovery efficiency of PGA M-CLEAs under more realistic process conditions, PG hydrolysis was run in a 50 mL jacketed reactor with mechanical stirring using the M-CLEA-TBA-G150. The reaction was conducted at 37 °C and the pH was kept at 8.0 using NaOH. Three successive batches were run using the same derivative. The results were compared with a typical run using the same enzymatic load (0.26 IU/mL), but with the enzyme in soluble form. Fig. 5(b) shows these results. In the first reaction cycle, after 600 min 570 μmol of PG were hydrolyzed, corresponding to approximately 61% of conversion. At this time, the reaction was stopped and the derivative was magnetically recovered; only 1.5 min were needed to recover all the derivative in the 50 mL reactor. The derivative was washed one time with water and one time with 5% (w/w) PG aqueous solution, for total removal of the formed products (phenylacetic acid and 6-APA). Washing with a small volume of PG 5% solution was more efficient than washing using buffer for removal of AFA, a PGA inhibitor. The magnetically recovered derivative was subsequently used in two other cycles, with 63% of conversion in both cases (the obtained conversion was about the same in the three consecutive batches). The reproducibility of the three cycles indicates that all derivative was magnetically recovered after the reaction/washing process. Once again, it is evident that M-CLEAs magnetic recovery is rapid, mild, and efficient. The PG hydrolysis using free PGA showed a worse performance, reaching only 48% (457 μmol) of conversion after 600 min. This was certainly because of the loss of activity caused by the process conditions (temperature, pH, and NaOH addition). So, in this experiment also evidences the high operational stability of M-CLEA-TBA-G150.

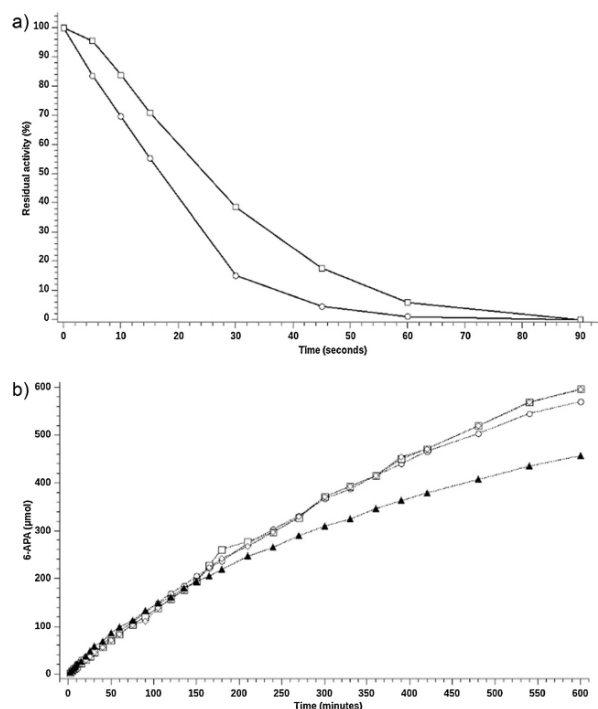


Fig. 5. (a) Catalyst recovery time course. Residual PGA activity (NIPAB method) in the suspension, at 2.0 cm (○) and 1.0 cm (□) from the surface of the magnet. The experiments were performed in triplicate; error bars were smaller than the symbols. (b) PG hydrolysis batch, pH 8.0, 37 °C, 5.0% of PG at the beginning of the reaction (w/w). (▲) free enzyme; and using M-CLEA-TBA-G150: (○) cycle 1; (□) cycle 2; (◇) cycle 3.

4. Conclusions

Co-aggregation between amino-SPIONs and the model enzyme PGA to obtain magnetic-CLEAs was investigated. The M-CLEAs presented similar recovered activity and stability as reference CLEAs, and could be quickly and efficiently recovered by applying an external magnetic field. The M-CLEAs particles were smaller than CLEAs particles, obtained under similar reaction conditions, and they present fewer problems with mass transfer limitations. The similar recovered activities, thermal stabilities and methanol stabilities obtained for M-CLEAs and CLEAs suggest that the co-aggregation with amino-SPION did not significantly alter the structure of the derivatives. The EDS line scan profiles suggest that amino-SPION particles were homogeneously distributed in M-CLEAs structure. The M-CLEAs were tested in the hydrolysis of penicillin G in a 50 mL reactor by three reaction cycles and the magnetic recovery was perfect. The morphological and catalytic studies of M-CLEAs suggest that co-aggregation between amino-SPIONs and proteins can be virtually applied to any enzyme. M-CLEAs can present high volumetric activity that, in conjunction with the capacity to be attracted by external magnetic fields, may be used in industrial processes where the biocatalyst recovery is very difficult or impracticable.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.procbio.2013.09.024>.

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Review

Organophosphate-degrading metallohydrolases: Structure and function of potent catalysts for applications in bioremediation



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ABSTRACT

Organophosphate compounds (OPs) have been employed in the agricultural industry as pesticides and insecticides for several decades. Many of the methods used currently for the detoxification of OPs are harmful and possess serious environmental consequences. Therefore, utilizing enzymes for the detection and decontamination of OPs is gaining increasing attention as an efficient and clean bioremediation strategy. Microbial enzymes, such as OP hydrolases, OP acid anhydrolases or methyl parathion hydrolase (MPH), are potent agents for OP decontamination. Their biochemical properties and biotechnological applications are discussed in this review, including a discussion on methods that may be employed to immobilize such enzymes, and essential steps to generate reusable and affordable biocatalytic systems for use in bioremediation and bioremediation.

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Abbreviations: BChE, butyrylcholinesterase; CB, carbon black; CBD, cellulose-binding domain; CNT, carbon nanotube; DFP, diisopropyl fluorophosphate; GpdQ, glycerophosphodiesterase; HFB, hollow fiber bioreactor; MBL, metallo- β -lactamase; MPH, methyl parathion hydrolase; OP, organophosphate; OPAA, OP acid anhydrolase; OPH, OP-degrading hydrolase; PEG, polyethylene glycol; PNP, p-nitrophenol; PON, paraoxonase; PTE, phosphotriesterase; QD, quantum dots.

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1. Introduction

Organophosphate² pesticides (OPs) are important in agriculture, accounting for approximately 40% of the total global pesticide usage (Fig. 1) [1–3]. OPs are also produced as chemical warfare agents (CWA); 200,000 tons of these agents are thought to be

² The term organophosphate is often used to denote a class of pesticides or nerve agents that are triesters of phosphoric, phosphonic and phosphinic acid and sulfur-containing compounds. While there are formal nomenclature conventions for these compounds they are usually referred to by their common names in the literature and this convention is followed here.

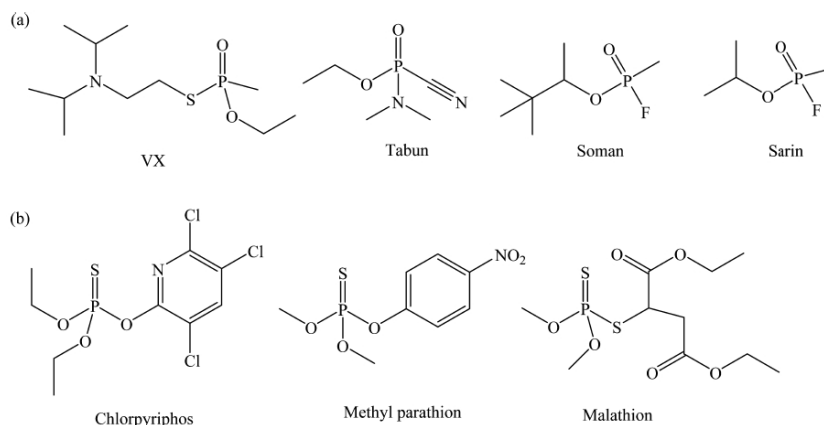


Fig. 1. Structures of OPs representing nerve agents and pesticides.

stored throughout the world [4,5]. These compounds are generally toxic, primarily due to their ability to irreversibly inhibit acetylcholinesterase [6]. OPs are partially water soluble and can easily enter the soil and ground water and, being mutagenic, they have been linked to a number of nervous and immune system-related diseases [7–23]. In both developing and developed countries OPs are also a common cause of poisoning by occupational exposure or deliberate self-harm and environmental contamination [1,2,8–16,24–39].

Disposing of OPs can be problematic. Stockpiles dumped in landfills can leach into the surrounding soil while chemical degradation can result in the production of toxic by-products [40]. Incineration of OPs is an approved but costly process that can also result in toxic emissions [41–43]. An efficient “green and clean” approach to degrade OPs is thus much desired, and enzymes expressed in some soil-dwelling microorganisms may be an ideal tool for this purpose. The history of OP-degrading enzymes dates back to 1946 when hydrolysis of diisopropylfluorophosphate (DFP) was observed in mammalian tissue extracts [44]. In 1973 it was discovered that the soil bacterium *Flavobacterium sp.* (strain ATCC 27551) is able to thrive in an environment where the OP diazinon is the sole carbon source [45]. Several other microorganisms, including *Agrobacterium radiobacter*, *Enterobacter aerogenes* and *Pseudomonas diminuta* have since been shown to possess the enzymatic machinery to degrade OPs [43,46–48]. Common to these organisms is that they have evolved an enzymatic activity that is very effective in hydrolyzing the OP triesters into di- and monoesters, and ultimately phosphate: the products of these hydrolytic reactions are generally far less harmful than their parent OP compound.

In this review several OP-hydrolyzing enzymes will be discussed with a view to describing their general physicochemical features and how they can be utilized in the context of bioremediation, i.e. the degradation of harmful OP compounds into less or non-toxic products. We realize that we have been selective in our focus and apologize in advance to authors whose significant contributions to the field may not have been covered in detail. For instance, insects are one of the main targets of OP pesticides and it is thus not surprising that they have developed some resistance, involving in particular a family of esterases [49]. However, since these enzymes do not require metal ions for their function they will not be discussed further here. Also, we like to stress that the purpose of the article is to describe enzymatic systems in context of their

potential for applications in bioremediation, not their mechanistic details (which are outlined in a series of primary literature as indicated in relevant sections).

2. Paraoxonase (PON) and OP acid anhydrolase (OPAA)

In 1946 Mazur, studying rabbit tissue samples, discovered the first example of an enzyme capable of degrading an OP, DFP [44]. Due to its ability to hydrolyze the OP paraoxon (Fig. 1) this OP acid anhydrolase (OPAA) was given the name paraoxonase (PON) [50–52]. However, it was subsequently found that this enzyme is rather promiscuous acting on a broad spectrum of diverse substrates, including a range of OPs and lactones [53,54]. While this broad substrate range complicates the assessment of the precise biological function(s) of PON, there is strong evidence that links the enzyme to atherosclerosis [55–57].

Based on sequence homology PON enzymes are divided into three groups, PON1–PON3 [58]. PON1 is the best studied group in this family of enzymes and has been identified in a variety of higher organisms, including human, rabbit, bovine, dogs, cats and horses [59]. PON1 appears to be the only family member with efficient OP hydrolase activity [53]; the enzyme is likely to act as a lactonase *in vivo* [54] but is capable of degrading a variety of OPs including the oxon metabolites of parathion, diazinon and chlorpyrifos, as well as the nerve agents Sarin and Soman [52]. In directed evolution experiments it was demonstrated that the substrate preference of PON1 can easily be modified, underlining this enzyme's potential in the detoxification of OPs, in particular for victims suffering from poisoning [60]. The enzyme consists of about 350 amino acids. The crystal structure of rabbit PON1 was solved to 2.2 Å resolution (Fig. 2), showing a six-bladed β -propeller overall structure [60]. Importantly, the active site contains two Ca^{2+} ions, one with a structural role, the second required for catalysis [58]. In the proposed model for the reaction mechanism the substrate coordinates to the catalytic calcium ion (labeled Ca1) prior to a nucleophilic attack by a hydroxide ion that does not directly coordinate to this Ca^{2+} but is activated *via* hydrogen bonding interactions involving two conserved histidine residues in the active site (Fig. 2) [60].

OPAAAs are frequently categorized in two classes, “squid-type” and “Mazur-type” [61,62]. The former prefers DFP to Soman as substrate and requires Ca^{2+} ions for activity [63]. The Ca^{2+} -dependent PON1 described in the previous section therefore classifies as a

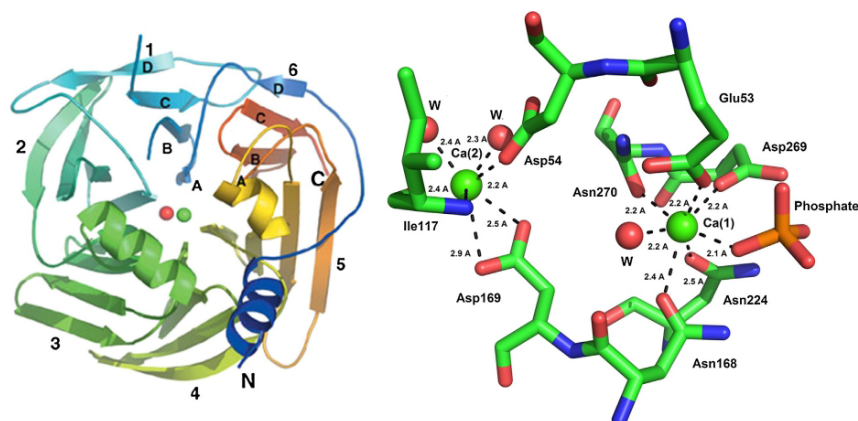


Fig. 2. Overall (left) and active site (right) structure of PON1. The two Ca^{2+} ions in the active site are shown as green spheres. Two histidine residues in the vicinity of $\text{Ca}(1)$ are proposed to activate a non-coordinating hydroxide for a nucleophilic attack on the phosphorous atom of the $\text{Ca}(1)$ -bound substrate (illustrated by a phosphate group in the structure). W represents water molecules. The figure was modified from [60].

"squid-type" OPAA, the name referring to the initial discovery of a Ca^{2+} -dependent OPAA in the squid *Loligo pealei* in 1966 [62,64]. In contrast, the "Mazur-type" OPAAs are characterized by being more efficient catalysts for the hydrolysis of Soman than DFP, and their activity is stimulated by Mn^{2+} ions. Paradigm examples are OPAAs purified from a number of *Alteromonas* species, initially strain JD6.5, and subsequently also in *A. undina*, *A. haloplanktis* and *A. macleodii* [63,65–69]. These OPAAs are characterized by their broad substrate specificity toward a wide variety of OPs, especially the highly toxic G-type nerve agents Sarin, Soman and Tabun. Specifically, they are highly active against P-F bonds, but far less so against P-O or P-C bonds [63]. Crystal structures for the enzymes from Strain JD6.5 and *A. macleodii* were recently determined to 2.3 Å and 1.8 Å, respectively [68,69]. The two enzymes share more than 60% sequence identity and also display a high degree of structural similarity with an rmsd of 0.8 Å for 421 aligned carbon backbone atoms [68]. While the OPAA from Strain JD6.5 is monomeric the *A. macleodii* enzyme is dimeric in solution [68,69]. Despite these apparent differences in their oligomeric structure the active site geometry in these two enzymes is virtually identical. Their active sites contain two closely spaced Mn^{2+} ions, separated by ~ 3.3 Å (Fig. 3). The two metal ions are bridged bidentately by the carboxyl groups of an aspartate and a glutamate side chain. In addition, an oxygen atom from either a water molecule (in *A. macleodii* OPAA) or presumably a glycolate (present in the crystallization mixture of Strain JD6.5 OPAA) links the two Mn^{2+} ions. It is interesting to point out that the active site geometry of OPAA is very similar to that of the enzyme aminopeptidase P (AMPP) from *Escherichia coli* [70,71]; the ligands of the Mn^{2+} ions are invariant. AMPP, and hence OPAA, belongs to the family of prolidases, a group of enzymes that also includes methionyl aminopeptidase (MetAP) [72,73] as well as numerous bacterial, archaeal and human prolidases, Mn^{2+} -dependent enzymes that hydrolyze specific peptide bonds [74,75]. Apart from the conservation of their active sites these enzymes also share considerable similarity in their overall structures, which are composed of two domains, an N- and C-terminal one. The C-terminal domain exhibits a "pita bread" fold that contains the dinuclear metal ion center (Fig. 3). In mammals prolidases are involved in the collagen metabolism; their role in bacteria and archaea is still unknown as the natural substrate(s) has not yet been identified. Similarly, the

natural substrates of OPAAs are still unknown but these enzymes were demonstrated to have significant *in vitro* prolidase activity [63,76]. It is thus not surprising that variants of prolidases have been isolated from hyperthermophilic bacteria and demonstrated

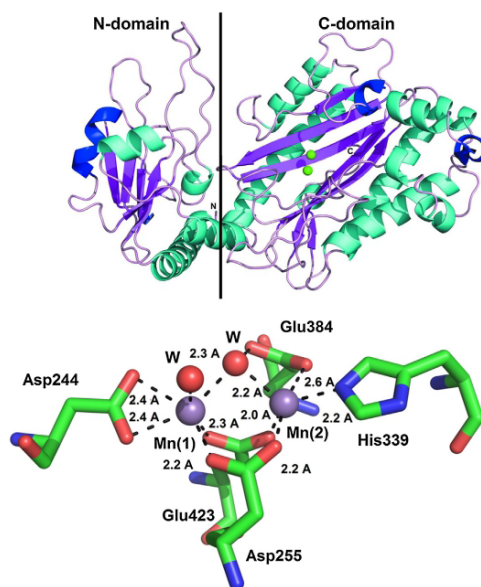


Fig. 3. Overall (top) and active site (bottom) structure of OPAA. The monomer contains two distinct domains. The active site is located in the C-terminal domain and contains two bound Mn^{2+} ions (shown as purple spheres). The metal ions are bridged via two carboxyl groups and a water molecule (red sphere). The figure was modified from [68].

to have low levels of OP-degrading activity that could be improved using directed evolution [77]. For example, the prolidase from *Pseudomonas horikoshii* (Phprol) has been the subject of *in vitro* evolution experiments in which a Phprol gene library was constructed and screened for mutants that show increased prolidase activity at 30 °C. Four mutants were found with improved catalytic parameters at various temperatures for the hydrolysis of several OP nerve agents, including p-nitrophenol Soman [41,78].

3. OP hydrolases (OPHs)

“Squid-type” and “Mazur-type” OPAAAs are not the only enzymes capable of hydrolyzing OP pesticides and nerve agents. The introduction of pesticides for agricultural applications triggered the evolution of OP-degrading hydrolases (OPHs) in a number of soil-dwelling microorganisms [46–48,79,80]. One of the best characterized of these enzymes is the phosphotriesterase (PTE) that was initially identified (in 1973) in *Flavobacterium sp* ATCC 27551 and then *P. diminuta* [45,81]. The gene encoding PTE is localized on extrachromosomal plasmids and may thus be rapidly transferred among different microorganism (*i.e.* via a horizontal gene transfer reminiscent of that associated with the rapid spread of the antibiotic-degrading dinuclear metallo- β -hydrolases) [80]. To a large extent PTE has a substrate specificity that resembles that of OPAA, being able to hydrolyze compounds such as Sarin, Soman, Paraoxon and their derivatives [82,83]. However, in contrast to OPAA, PTE is also capable of hydrolyzing VX [79]. Genetic engineering has been used to produce variants of PTE with improved catalytic properties toward selected OPs. As an example, a variant in which the histidine at position 254 was replaced by an arginine exhibits a four-fold increase in the rate of hydrolysis of Demeton-S, but 14- and ~180-fold decreases for Paraoxon and DFP, respectively [84]. Directed evolution has also been utilized to increase the catalytic efficiency toward poor substrates such as Chlorpyrifos (Fig. 1) [85].

Several crystal structures of PTE (in the presence of various substrate analogs, products, etc.) have been reported [46,84,86–88]. A representative structure is shown in Fig. 4. The active site accommodates two closely spaced divalent metal ions (~3.5–4 Å apart) but unlike in OPAAAs or prolidases the *in vivo* metal ion content is subject to debate; catalytic activity can be reconstituted with a range of metal ions including Zn^{2+} , Mn^{2+} , Cd^{2+} and Co^{2+} [89,90]. In

contrast to OPAA only one amino acid links the two metal centers, a carboxylated lysine residue. However, a bridging water/hydroxide molecule is again ideally located to initiate a nucleophilic attack on the bound substrate. A structural comparison of the active sites of PTE and OPAA indeed demonstrated that despite a lack of discernable similarity in overall structure and amino acid sequence the catalytic centers of the two enzymes are geometrically similar, leading to the proposal that they are also employing a conserved mechanistic strategy [69].

Very closely related to PTE is the OP-degrading enzyme from *Agrobacterium radiobacter* (OpdA), with ~90% sequence identity shared between the two enzymes [47,90,91]. These enzymes have similar substrate specificities with OpdA displaying a preference for methyl-substituted while PTE prefers ethyl-substituted (*e.g.* methyl- vs ethyl-paraaxon) [47]. The major difference between PTE and OpdA is their respective stability, with the former losing activity as a consequence of losing metal ions [92]. This difference in stability is also reflected in the effect of pH on their catalytic activities. PTE generally reaches optimal activity at pH values above 6.0 while OpdA is still efficient at pH values as low as 4.0 [90,93]. A recent study demonstrated that these functional variations between PTE and OpdA are likely to be associated with amino acid substitution in the second coordination sphere; while both enzymes have nearly identical metal ion binding sites (the identities and locations of the metal ion ligands are invariant; Fig. 4 [94]) the substrate binding pocket of OpdA only is characterized by the presence of a hydrogen bonding network that connects the metal ion and substrate binding sites [90]. This study also demonstrated that a single mutation in the second coordination sphere can alter the substrate specificity of OpdA, thus making this enzyme a good (and flexible) candidate for the development of a biocatalyst with a desired preference for particular OP substrates. Similar to PTE the native metal ion composition of OpdA is unclear. While atomic absorption and anomalous scattering measurements are consistent with the presence of an Fe^{2+} - Zn^{2+} center [95], *in vitro* studies demonstrated that the di- Co^{2+} , di- Mn^{2+} and di- Cd^{2+} derivatives are significantly more reactive and efficient than the heterodinuclear combination [90,93]. These studies also indicated that in particular the di- Co^{2+} derivative of OpdA is of interest for practical applications as it not only maintains a high activity over a broad pH range, but it reaches a maximum at pH 11. Spectroscopic and kinetic studies revealed that this derivative of OpdA displays

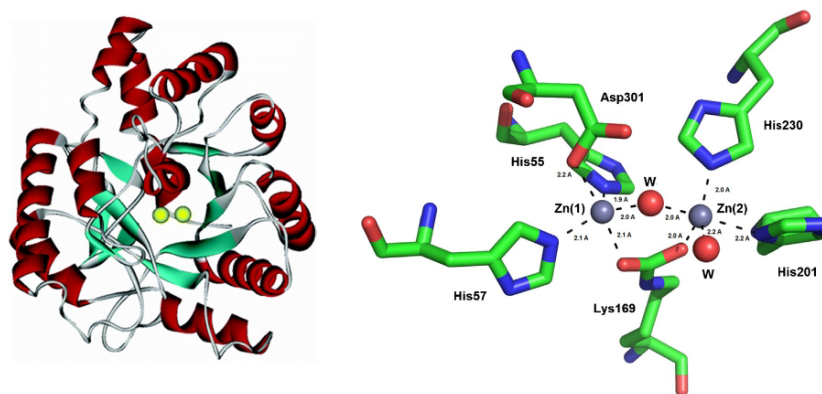


Fig. 4. Overall (left) and active site (right) structure of PTE. The two divalent metal ions in the active site (Zn^{2+} in this case, but the *in vivo* metal ion composition is subject to debate) are shown as gray spheres and are linked via a carboxylated lysine residue and a water molecule. The figure was modified from [86].

a mechanistic flexibility whereby a metal ion-bridging hydroxide acts as hydrolysis-initiating nucleophile (as is the case for most OP-degrading enzymes, as discussed in this review) at lower pH values, whereas a hydroxide bound to one metal ion only fulfills this role at higher pH [90,93,96].

OP-degrading enzymes such as PTE and OpdA have great promise in bioremediation applications, in part due to their high catalytic efficiencies and enzymatic stability, but and in particular for OpdA, also due to their functional plasticity. As pointed out in the previous section the metal ion composition may influence the reactivity and mechanism of the enzymes, while a single amino acid substitution may alter the substrate preference [93]. In an attempt to develop desired variants of OpdA directed evolution experiments were carried whereby cell growth of *E. coli* cells that recombinantly express OpdA was coupled to OP-degrading enzyme activity using the pesticide Methyl Paraoxon as sole phosphorus source [97–99]. Using this approach it was not only possible to alter the catalytic properties of OpdA but it also demonstrated how remote “outer shell” amino acids may affect metal ion binding and thus enzyme stability [92,99]. Similarly, saturation mutagenesis at specific codons has been utilized to improve the catalytic efficiency of PTE toward poorly hydrolyzed P-S-bonds containing OPs such as Demeton-S, Malathion and VX [100]. Furthermore, a highly thermostable OPH has also been identified in the extremophile *Deinococcus radiodurans*, and which is stable at temperatures up to 70 °C [101]. Site-directed- and random mutagenesis-based approaches were used to enhance the catalytic activity of this enzyme toward Ethyl and Methyl Paraoxon by 126- and 322-fold, respectively [101]. In view of the significant potential of OPHs in bioremediation the recent discovery of two “PTE-like” enzymes from *Sphingomonas* sp. strains TDK1 (i.e. Sm-PTE) and *Sphingobium* sp. strain TCM1 (i.e. Sb-PTE) is of interest [102]. These enzymes are particularly reactive toward a group of OP triesters that are frequently used as plasticizers, durable plastics, foams and flame retardants. These compounds, in contrast to OPs used as pesticides or nerve agents, do not have a labile ester bond and are not substrates for enzymes such as PTE or OpdA. Sb-PTE, however, is effective in hydrolyzing both inert and labile OPs, the latter albeit with an efficiency that is about two orders of magnitude smaller than that of PTE [103]. Nonetheless, using directed evolution approaches it may be possible to develop a stable enzymatic

catalyst with a desired substrate preference. The structure of Sb-PTE is not yet known but there is no sequence homology to PTE or OpdA, and homology modeling suggested that its active site may contain a single divalent metal ion, thus resembling rather OPAAAs such as PON1.

4. Methyl parathion hydrolase (MPH)

Methyl parathion (Fig. 1) is an OP pesticide extensively used in agriculture [104]. A genetic analysis of *Plesiomonas* sp. strain M6 and *Pseudomonas* sp. WBC-3 microbes that were found in methyl parathion-contaminated soil, has led to the discovery of an enzyme that effectively degrades this pesticide [105–107]. This enzyme was thus aptly named methyl parathion hydrolase (MPH). The enzyme from *Plesiomonas* sp. strain M6 (M6-MPH) and *Pseudomonas* sp. WBC-3 (WBC3-MPH) have identical protein and near-identical DNA sequences. Several MPH variants have since also been identified in other microorganisms, including *Ochrobactum* sp. and *Burkholderia* sp. [107–112]. MPH does not share any sequence similarity to OPHs such as PTE or OpdA.

The crystal structure of the homodimeric WBC3-MPH was solved to 2.4 Å resolution (Fig. 5) [113,114]. Similar to “Mazur type” OPAAAs, PTE or OpdA the active site accommodates two metal ions, separated by 3.5 Å. However, while in OPAAAs two bidentate carboxyl groups and in PTE/OpdA a bidentate, carboxylated lysine (all in μ -1,3 coordination; Figs. 3 and 4) connect the two bound metal ions, in MPH an aspartate residue forms a μ -1,1 link (Fig. 5). Insofar, the active site geometry of MPH is reminiscent of that of functionally unrelated dinuclear metallohydrolases such as purple acid phosphatases, Ser/Thr protein phosphatases or 5'-nucleotidases [48,115,116]. However, the highest degree of overall structural similarity is observed between MPH and another group of dinuclear metallohydrolases, the antibiotic-degrading metallo- β -lactamases (MBLs). Apart from the aspartate connecting the two metal ions all metal ion-binding residues in the active sites of MPH and MBLs are conserved [113,114]. MBLs generally require at least one Zn^{2+} ion for activity although the majority of these enzymes form dinuclear Zn^{2+} centers where the metal ion-bridging hydroxide acts as the initiator for the cleavage of the β -lactam bond [73,117]. MPH is also likely to contain dinuclear Zn^{2+} centers in its active site although in the crystal structure one of the Zn^{2+} ions was replaced by Cd^{2+} ,

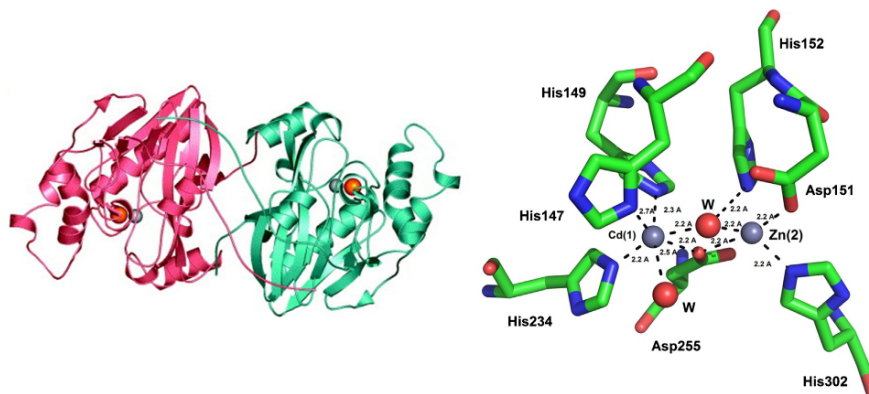


Fig. 5. Overall (left) and active site (right) structure of MPH. Both subunits of the homodimer are shown on the left, with the metal ions indicated as spheres. Although the *in vivo* metal ion composition is believed to be Zn^{2+} – Zn^{2+} one Zn^{2+} ion is replaced by Cd^{2+} in the structure, possibly an artifact of crystallization. The metals are linked via a water bridge in addition to monodentate aspartate side chain. The figure was modified from [114].

present in the crystallization solution, and the relevant catalytic mechanism is proposed to be similar to that of PTE, OpdA or MBLs [113,114]. Furthermore, despite the lack of apparent sequence similarity between PTE/OpdA and MPH both groups of OPHs may have evolved from bacterial lactonase ancestor proteins, evidenced by the considerable lactonase activity measured for both PTE and the MPH-related MBLs MIM-1 and MIM-2 [118,119].

Importantly, since the MPH-fold appears to be capable of accommodating a diverse range of substrates ranging from OPs to β -lactams and lactones MPH may be a useful agent to be developed into a potent and versatile bioremediator. Toward this aim, site-directed and random mutagenesis, as well as a semi-rational approaches using computer-aided methods and site saturation mutagenesis have all been used to improve the thermal and acid stability of MPH from *Ochrobactrum* sp M231 [120–122]. Several groups have also been successful in increasing the activity of MPH toward less preferred substrates. Jeong and colleagues designed a high-throughput screening (HTS) system that utilizes a phenolic-responsive transcription activator for directed evolution of MPH and found a mutant with 100-fold improvement in k_{cat}/K_m for the reaction using *p*-nitrophenyl diphenylphosphate as substrate [123]. Xie and co-workers subjected MPH from *P. stutzeri* strain ZK-5 to one round of error-prone PCR and found a mutant with a five-fold increase in k_{cat} for the hydrolysis of Chlorpyrifos [124], while saturation mutagenesis and DNA shuffling experiments with WBC3-MPH demonstrated the plasticity of the active site of this enzyme since a modest number of mutations was required to fine-tune the substrate preference toward Ethyl Paraoxon [125].

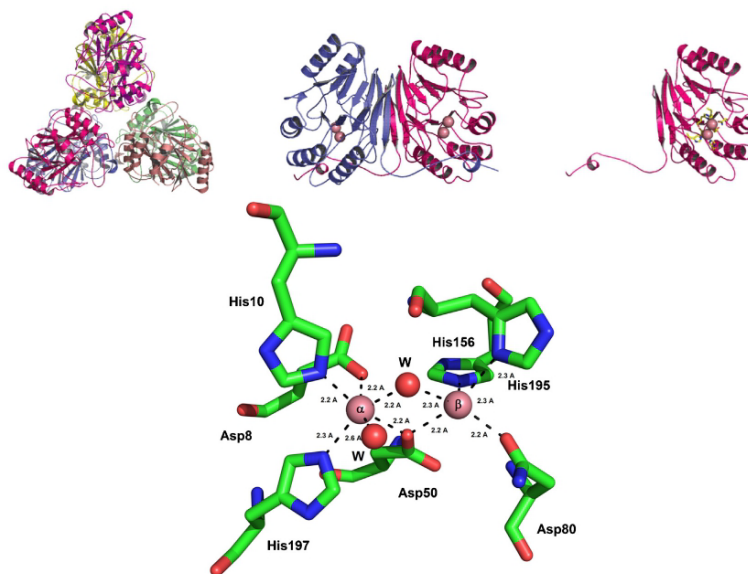
Another OPH, OPHC₂, was isolated from *P. pseudoalcaligenes* [110]. This enzyme also belongs to the MBL superfamily and shares ~45% sequence identity with MPH. Its structure was recently solved, revealing an overall fold and active site geometry

virtually identical to that of WBC3-MPH (Fig. 5) [126,127]. However, while OPHC₂ is catalytically less efficient than MPH it is more thermostable with a T_m of 97.8 °C, probably due to the presence of a disulfide bond that is absent in MPH. Thus, OPHC₂ is an interesting candidate for future OP decontamination [127,128].

5. Glycerophosphodiesterase from *Enterobacter aerogenes* (GpdQ)

GpdQ from *E. aerogenes* is one of the most promiscuous binuclear metallohydrolases, catalyzing the hydrolysis of all three classes of phosphate ester substrates, i.e. mono-, di- and triesters [129–132]. GpdQ has potential as an enzymatic bioremediator since it is active toward a range of organophosphate pesticides (triesters) and is capable of degrading EA2192, the highly toxic product of the hydrolysis of VX [133]. GpdQ is active over a very large pH range (from pH 3.0 to 11) [130,131] and is also highly promiscuous with respect to metal ions that can be used to reconstitute catalytic activity [130,131,134–137]. While similar to PTE or OpdA it is unclear what the native metal ion composition of GpdQ may be, catalytic activity is highest for the dinuclear Cd²⁺ and Mn²⁺ derivatives, while activity can be reconstituted with the apoenzyme using a broad range of divalent transition metal ions [137]. In terms of sequence homology GpdQ is not related to any of the other known OP-degrading enzymes. Its closest relative is the diesterase Rv0805 from *Mycobacterium tuberculosis*, which hydrolyzes the diester bond in cyclic nucleotides and thus may play an essential role in cell signaling [129,138].

Several crystal structures of GpdQ have been reported [135,139,140], demonstrating an intricate homo-hexameric arrangements whereby six identical subunits form a trimer of dimers (Fig. 6). Each subunit contains an active site that can



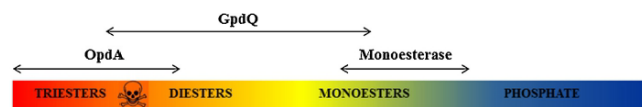


Fig. 7. Overlapping substrate preferences for OpdA and GpdQ. Their combined activities can degrade highly toxic OP triester pesticides or nerve agents into largely harmless monoester products. These monoesters can easily be further metabolized into inorganic phosphate using a plethora of monoesterases.

accommodate two metal ions (~ 3.5 Å separation; Fig. 6). A series of spectroscopic and kinetic studies indicated that the dinuclear center is composed of a tightly bound, six-coordinate metal ion located in the α site and a more loosely bound, five-coordinate metal ion in the β site [130,131,139]. Interestingly, despite having an identical coordination environment (with all seven metal ion-coordinating amino acids conserved) the diesterase Rv0805 binds both metal ions with high affinity, demonstrating the significance of outer sphere interactions in regulating the catalytically relevant metal ion center in these enzymes [138].

Indeed, GpdQ employs a regulatory mechanism to control its activity; in the resting state the enzyme is predominantly in an inactive mononuclear state (only one metal bound to the α site), but upon the addition of a substrate the enzyme is activated by the formation of a dinuclear metal center in the active site ("substrate-promoted" activation) [130,139]. The intricate regulatory mechanism employed by GpdQ complicates the enzyme's use for applications in bioremediation, for which a continuously active catalyst is of benefit. Mutations in the first and second coordination spheres were introduced to increase the affinity of the metal ion in the β site in the absence of substrate molecules. In particular, mutations in the second coordination sphere (i.e. the replacement of His81, Ser127 and His217 by alanine) led to considerable improvements in metal ion binding affinities without affecting the catalytic performance of the enzyme [137,141]. Furthermore, directed evolution experiments have revealed that the catalytic efficiency (i.e. k_{cat}/K_m) toward model substrates such as bis(*para*-nitrophenol) phosphate can be improved over 500-fold by the introduction of as few as three mutations, highlighting the strong potential of GpdQ in the development of a useful agent for bioremediation [142].

6. Enzyme immobilization and biotechnological applications

In general, enzymes may adopt a variety of roles in the environment, either as agents (in isolated, cell-bound or immobilized form) catalyzing the transformation and/or degradation of compounds polluting the environment, or as reliable and sensitive tools to detect and measure the amount and concentration of pollutants before, during and after the restoration process. There is a growing list of examples of enzymes employed (i) in biological remediation, (ii) in the transformation of pollutants to low-molecular weight additives, (iii) as biosensors used for monitoring levels of environmental pollution, and (iv) as bioindicators of water and soil quality [143]. Specifically, the previous sections highlighted the potential of a series of metal ion-dependent hydrolases in the detection and decontamination of various OP neurotoxins, including a range of pesticides and nerve agents (Fig. 1). A potentiometry-based biosensor for direct detection of OP nerve agents was developed by modifying a pH electrode with an immobilized layer of *E. coli* cells expressing PTE on their surface [144]. Since the PTE-catalyzed hydrolysis of OPs releases protons [89], the amount of hydrolyzed substrate can be easily measured. This biosensor can be stored long-term and is stable for multiple use [144]. PTE-based detection is fast and selective but not very sensitive. The detection sensitivity has been improved by using a carbon nanotube (CNT) as electrode

material [145–149]. It has also been found that by using mesoporous carbon (MC) and carbon black (CB) along with PTE as an anodic layer, the sensitivity of the sensor for *p*-nitrophenol (PNP), a product of the PTE-catalyzed reaction, is greatly enhanced [150]. In comparison to the CNT-modified electrode the MC-CB modification results in an enhanced amperometric response because it promotes the electron transfer from the enzymatically generated phenolic compound (i.e. *p*-nitrophenol). Under optimal experimental conditions, the biosensor has a detection limit of 0.12 μM (36 ppb) and a sensitivity of 198 nA/ μM for paraoxon, which is suitable for biosensing applications [150]. An alternative biosensor includes the use of CdS quantum dots [151], where PTE and (CdSe)ZnS core-shell quantum dots (QDs) form a bioconjugate. Coupling of PTE to the (CdSe)ZnS core-shell QD was achieved through electrostatic interaction between the negatively charged QD surfaces and the positively charged protein side chain, and circular dichroism spectroscopy indicated that the activity of PTE was preserved. When applied to the monitoring of paraoxon levels the detection limit was determined to be about 10^{-8} M [151].

Various current methods used for decontamination of OP compounds are environmentally challenging and include bleach treatment or *ex situ* incineration [152–157]. The successful incorporation of active PTE into aqueous fire-fighting foams was a breakthrough in the use of an enzymatic agent for surface decontamination; the method is reproducible, environmentally friendly and facilitates the decontamination of a larger area when compared to other methods [158]. Overlapping substrate preferences of several OP hydrolases may be exploited to degrade highly toxic OP triesters into less or non-toxic compounds. As illustrated in Fig. 7 OpdA and GpdQ are capable of converting toxic triesters into largely harmless monoesters; indeed an *E. coli* strain co-expressing both enzymes thrives in a medium where the OP paraoxon is the sole source for phosphorous [110]. GpdQ itself has low activity toward monoester substrates but there are numerous specialized monoesterases (e.g. purple acid phosphatases [48,115,116]) that can then convert the monoester substrates of the combined OpdA/GpdQ activity into inorganic phosphate. A current endeavor is thus to establish systems that employ both enzymes, preferably in an immobilized state to facilitate continuous use. Preliminary attempts to immobilize the OpdA-related enzyme OPH and GpdQ using mesoporous materials and magnetic nanoparticles, respectively, were encouraging since the process of immobilization led to increased stability and reactivity for both [141,159,160].

Considering the great significance of enzyme immobilization for the effective application of these catalysts for applications such as environmental remediation and restoration it is informative to review some of the recent developments in this area. Nanostructures are attractive for enzymatic immobilization processes, since they possess ideal characteristics to optimize factors that determine the efficiency of the biocatalysts, including adaptable surface area, mass transfer resistance and effective enzyme loading. Enzyme supports include silica, chitosan, gold, diamond, metal ions and metal oxides, such as graphene and zirconium [161–171]. As mentioned in the previous paragraph advances have been made in immobilization processes that lead to improved enzyme stability, enhanced activity, but also the minimization of inhibition by

reaction products and better selectivity toward desired substrates [164,172]. Another concrete example is the covalent attachment of cyclodextrin glycosyltransferase to magnetite (which was silanized with 3-aminopropyltrimethoxysilane and activated with glutaraldehyde), resulting in an adduct with increased thermal stability (i.e. the optimal temperature for activity was raised from 55 °C to 70 °C) [168]. Other methods that may prove useful in developing a reusable device for OP degradation include self-immobilization by the enzymes (e.g. cross-linked enzyme aggregates – CLEA), as well as encapsulation or entrapment using dendrispheres or PEI microspheres [169]. Enzyme immobilization on magnetic particles with functionalized surfaces has also recently been advanced to environmental applications, from enzymatic bioremediation to the synthesis of sustainable and clean energy through novel green processes [169]. The main advantages for using enzymes in such processes are their efficient catalytic properties, their biodegradability, as well as their high chemo-, regio and stereo-selectivity, resulting in low by-product formation. Furthermore, biomolecules linked to superparamagnetic nanoparticles can be selectively removed from complex reaction mixtures using an external magnetic field. For example, amino-functionalized superparamagnetic iron oxide nanoparticles (amino-SPION) were co-aggregated with penicillin G acylase and then cross-linked, generating magnetic CLEAs (M-CLEAs) that were quickly and efficiently recovered from the reaction medium by applying an external magnetic field [166]. Another approach to exploit a magnetic field for separation was developed by adsorption of enzymes in superparamagnetic hierarchically ordered mesocellular mesoporous silica (M-HMMS) with subsequent enzyme crosslinking. Specifically, enzymes such as α -chymotrypsin or lipase were adsorbed into M-HMMS and treated with glutaraldehyde, which resulted in a crosslinked enzyme aggregate with sharply enhanced (i.e. >30-fold) activity when compared to the unlinked M-HMMS [173]. Also of interest is a method that facilitates a thermally-controlled switch for enzyme activity, an approach that is based on the site-directed conjugation of temperature-responsive polymers to a unique cysteine (Cys) residue positioned near the active site of the enzyme of interest. As an example, the reversible temperature-induced collapse of *N,N*-dimethylacrylamide (DMA)/*N*-4-phenylazo-phenylacrylamide (AZAAm) copolymers (DMAAm) was used as a molecular switch to control the catalytic activity of endoglucanase [167]. Alternative approaches to enhance enzyme stability and activity for decontaminating OP neurotoxins are based on interactions between the hydrophobic polypropylene oxide (PPO) block of amphiphilic Pluronics and enzymes such as PTE. Although a detailed mechanism of how the PPO block may enhance the catalytic activity of OP-degrading enzymes has not yet been elucidated, it has been demonstrated that the aggregate has also increased resistance to inhibition by methanol, enhanced thermal stability, an extended shelf life, as well as an expanded substrate range [174]. Hydrogel-forming PTE variants have also been generated by genetically fusing this enzyme to alpha-helical leucine zipper domains (H), unstructured soluble linker domains (S) and polyhistidine purification tags, resulting in the formation of hydrogels, which proved to be effective catalytic biomaterials [175]. Furthermore, in an attempt to improve long term stability and increase the half-life of such systems, PTE was immobilized on biocompatible gelatin pads [176]. In particular the use of glutaraldehyde spacers dramatically improves enzyme stability. These PTE-gelatin pads have proven effective to eliminate the OP-insecticide methyl parathion and the nerve agent sarin [176].

The development of a cell-based biosensor for OP detection (*vide supra*) demonstrates that whole cells of genetically engineered *E. coli* are an attractive alternative to using immobilized enzymes. An *E. coli* strain that displayed PTE on the cell surface has been used successfully (i) to detoxify contaminated wastewaters

[177] and (ii) for the biotransformation of a widely used insecticide, coumaphos [178]. An *E. coli* strain that expressed a bacterial hemoglobin (VHb) in addition to PTE is considerably more potent in degrading OPs such as paraoxon than the strain expressing PTE alone [176]. A genetically engineered *E. coli* strain expressing both a cellulose-binding domain (CBD) and PTE on its cell surface has been shown to promote the detoxification of OPs [110]. Subsequently, this *E. coli* strain was immobilized in a cellulose-coated hollow fiber bioreactor (HFB) and shown to be effective in the biotransformation of a model OP pesticide, paraoxon [179]. It is envisioned that a combination of enzyme engineering (to improve their catalytic performance and structural stability) and immobilization, either using the enzymes alone or express them on the surface of carrier organisms such as *E. coli* may pave the way for very efficient and clean strategies to remove toxic compounds from environmentally challenged/polluted habitats.

Lastly, OP-degrading enzymes find increasing applications in medicine. OPs are estimated to cause the death of hundreds of thousands of people each year [1,24–27]. Human butyrylcholinesterase (hBChE) is a natural bioscavenger but the amount of native enzyme is insufficient to achieve reasonable protection. Of significance for medical applications is that intravenous pretreatment of patients with PTE prevents the inhibition of AChE activity after exposure to OPs and increases the recovery of this activity after poisoning [180]. PTE is thus a good candidate for use as catalytic bioscavenger for OP pesticides and nerve agents [181,182]. Recent trials with animals have shown that OPdA can also quickly clear most of the pesticides in the blood of poisoned rats [183]. Current limitations are the stability of the enzyme but attempts to overcome these limitations are currently being made using directed evolution methods. In addition, it could be shown that the covalent linking of enzymes such as PTE to polyethylene glycol (PEG) polymers increased the residence time of the foreign enzyme in the blood stream of guinea pigs and also reduced the production of anti-PTE antibodies [180]. Furthermore, it could also be demonstrated that the antidotal effects of OPAA from *Alteromonas* strain JD6.5 are significantly enhanced when the enzyme was encapsulated in liposomes [180]. Bioremediation by OP-degrading enzymes has the potential to become a major contributor to solving pesticide contaminations that lead to environmental pollution and human poisoning.

7. Concluding remarks

As the global population is rapidly increasing OP-based compounds are likely to remain an essential part for agriculture to meet the challenge of an ever growing demand for food. Consequently, the strain on the environment will also grow, making the development of efficient tools for bioremediation tools a high priority task. OP-degrading enzymes form the most promising avenue available for this endeavor. The challenge is to optimize such enzymes to withstand the pressures of the environment (i.e. temperature, pH), and to be sufficiently versatile to operate on a range of commonly used OP compounds. Significant advances toward these aims have been made using various mutagenesis- and evolution-type methods, but the process is ongoing. Similarly, optimal methods for the immobilization of these enzymes are still being investigated – the aim is to develop catalysts with desired catalytic properties that can be easily and continuously handled in an applied setting. In summary, this review highlighted the promise enzymes have in this area, and how they can be manipulated, but it also points out the current limitations in terms of their biotechnological applications. It is hoped that the motivated reader will draw inspiration and encouragement from this article to further develop these enzyme systems into versatile bioremedial agents.

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IV.3 Artigos em elaboração

Synthesis and characterization of magnetic cross-linked enzyme aggregates of cellulases for the potential application in producing cello-oligosaccharides

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ABSTRACT

Formation of magnetic cross-linked enzyme aggregates (MCLEAs) is an interesting enzyme immobilization approach. In this study, MCLEAs of cellulases were prepared and further studied. Firstly, the MCLEAs were prepared in the presence of magnetic nanoparticles with three different precipitation agents (ammonium sulfate, ethanol and PEG 600). After cellulases immobilization, highest values of enzymatic activity (193.42 U g^{-1}) and activity recovery (43.53 %) were observed with the use of ethanol as precipitation agent, while that PEG led to MCLEAs with the highest reusability capacity (80% of initial relative activity after five cycles of reaction). For the first time, the colloidal properties of MCLEAs were characterized by dynamic light scattering (DLS) and zeta potential analyses, which were correlated with the activity of MCLEAs. The results showed that the increased enzyme activity and thermostability of the MCLEAs were attributed to improved colloidal stability compared to free cellulase. Finally, the MCLEAs were assessed for the hydrolysis of cellulose-derived from sugarcane bagasse and straw. Despite the low hydrolysis efficiency, the use of MCLEAs led to the production of cello-oligosaccharides (COS) with degrees of polymerization (DP) of 3, 6 and 12 compared to the DP of 3 and 6 with free cellulase. The increase in DP may lead to the production of high-value COS prebiotics from lignocellulosic biomass compared to low DP COS.

Keywords: magnetic nanoparticles, enzyme immobilization, glucose polymers, biomass waste, enzymatic catalyst

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1. INTRODUCTION

Cellulases are a complex of enzymes consist of endoglucanases, exoglucanases and β -glucosidase [1]. These three enzymes act synergistically to produce glucose from cellulose, the most abundant low-cost carbohydrate polymer in nature [2]. Briefly, endoglucanases hydrolyze 1,4-glycosidic bonds at amorphous regions of the cellulose fiber and produce long-chain oligomers while exoglucanases act on the ends of cellulose oligomers with the production of cello-oligosaccharides (COS) [3]. Furthermore, COS are broken to glucose by β -glucosidases [4]. Enzymatic hydrolysis of cellulose to produce glucose has been extensively studied for second generation ethanol production [5], [6], [7]. However, the high enzyme cost is one of the significant cost barriers towards commercialization of cellulosic ethanol production [8]. In order to reduce the enzyme cost, recovery and recycling of cellulases have been extensively studied [9], [10], [11], [12]. Among various recovery and recycling methods, cellulase immobilization is often investigated [13], [14], [15], [16].

Cross-linked enzyme aggregates (CLEAs) is a robust immobilization technology because maintenance of pre-organized superstructures in the formation of CLEAs leads to good mechanical stability and retaining high enzyme activity [17]. CLEAs consist in physical aggregates of enzyme molecules obtained by use of precipitation agents (e.g., solvents, salts or polymers), which maintain their native tertiary structure, interlinked with each other through of cross-linked agents as glutaraldehyde [17]. When precipitation is carried out on solid support surface, CLEAs can be covalent bonded to support by own cross-linked agent, allowing to ally vantagens

from CLEAs to inherent properties of the support, strategy which can produce unique catalysts. Among many supports, magnetic nanoparticles (MNP), such as magnetite (Fe_3O_4) has gained attention due to its high surface area, loading capacity, low toxicity and mainly, magnetic properties, which allow selective recovering from the reaction medium by applying an external magnetic [18], [19], [20].

MNPs can be functionalized through several approaches, including chitosan-based surface, silica-based surface, graphene-based surface, amino-based surface, which have been summarized in review papers [21], [22]. In one recent study MCLEAs of cellulases on amino-functionalized MNPs showed a better activity at a wider temperature range and pH values than that of the free cellulase and achieved an activity recovery of 74% on carboxymethylcellulose after six reaction cycles [23]. However, hydrolysis yield on bamboo biomass by MCLEAs was only 21% compared to 44% achieved with free cellulases at a biomass concentration of 0.075%. In the other study, cellulase MCLEAs on aminosilane-coated MNPs retained about 45% of its maximum activity at pH above 4.89 while free cellulase lost its activity sharply [24]. In addition, MCLEAs retained about 65% of its maximum activity at 80 °C and had improved thermal stability at 65 °C. Lastly, MCLEAs retained 30% of its initial activity through 6 cycles of reuse.

Despite the interest in cellulase immobilization, the studies on MCLEAs of cellulases are still very limited. In addition, the mechanisms of the improved stability and enzyme activity of cellulase MCLEAs have not been fully understood. Furthermore, previous studies on cellulase immobilized were mainly focused on the hydrolysis of biomass to fermentable sugars for producing biofuels (e.g., ethanol), which will not be economically feasible in the near future due to the relatively high enzyme and immobilization cost and low value of biofuels. In this study, cellulase MCLEAs were further studied. MCLEAs were firstly prepared on chitosan-modified MNPs with the use of different precipitation agents. MNPs and MCLEAs were characterized, including dynamic light scattering (DLS) and zeta potential analyses to understand colloidal properties of MCLEAs, which were correlated with the activity of MCLEAs for the first time. Furthermore, the MCLEAs was used to hydrolyze cellulose from sugarcane bagasse and straw and assessed to cello-oligosaccharides (COS) production, an important class of biologically active carbohydrates with prebiotic properties [25], [26], [27]. In addition to monitor the hydrolysis yield, the degree of polymerization (DP) of the COS obtained were also determined. Since higher molecular weight COS are currently produced in small amounts and with a 500 times more expensive cost than those with lower DP [28], [29], the results indicated that the MCLEAs may be used to produce COS with higher DP for higher value application compared to the use of free cellulases.

2. EXPERIMENTAL SECTION

2.1. Materials

Iron(II) chloride tetrahydrate ($\geq 99\%$), iron(III) chloride hexahydrate (97%), chitosan with low molecular weight and 70% deacetylation degree, glutaraldehyde (25%), cellulase from *Trichoderma longibrachiatum*, PEG 600, citric acid ($\geq 99.5\%$) and Bradford reagent were purchased from Sigma-Aldrich (USA). Ammonium sulfate ($\geq 99.5\%$), ethanol (96%), carboxymethylcellulose sodium salt (CMC), 3,5-dinitrosalicylic acid (98%) (DNS) and sodium phosphate dibasic ($>99\%$) were acquired from Neon (Brazil) and ammonium hydroxide (28%) was obtained from Merck (USA). All the starting materials and solvents were used without further purification. Sugarcane bagasse and straw were obtained from the São Martinho Mill (Américo Brasileiro, São Paulo, Brazil).

2.2. Characterization

Magnetic nanoparticles and MCLEAs were characterized by powder X-ray diffraction (PXRD), Fourier transform infrared (FT-IR) spectroscopy, thermogravimetric analyses (TGA) and dynamic light scattering (DLS). PXRD patterns were obtained using a Rigaku model RINT 2000 diffractometer, in the 2θ range 5–70°, with Cu K α radiation ($\lambda = 1.5418 \text{ \AA}$), a scanning speed of 1 s/step, and a step size interval of 0.02°. For magnetite, PXRD diffractogram was simulated using Mercury 3.0 software and Cu K α radiation ($\lambda = 1.5418 \text{ \AA}$) and CIF file AMCSD-2400, crystal system cubic and space group Fd3m. FTIR spectra were acquired in the range 4000–400 cm^{-1} , with resolution of 4 cm^{-1} and 32 scanning, using KBr pellets and a PerkinElmer FT-IR Frontier spectrophotometer. TGA measurements were performed using a PerkinElmer TGA 4000 thermogravimetric analyzer, with heating from room temperature to 800 °C at a rate of 10 °C min^{-1} , under a N_2 atmosphere (20 mL min^{-1}). DLS measures were carried out in a Malvern Instruments Zetasizer Nano Series System in quartz cuvettes and performed in triplicates at 25 °C.

In order to better understanding the effect of pH and temperature in the activity of MCLEAs and free cellulase, DLS measurements were carried out at the same net conditions studied in the enzymatic activity assay. At the same Malvern Instruments

Zetasizer Nano Series System, zeta-potential (ζ -potential) measures were obtained to evaluate the influence of pH at colloidal stability of MCLEAs.

Molecular weights (Mn and Mw), polydispersity index (PDI) and degree of polymerization (DP) of COS obtained after hydrolysis of cellulose from sugarcane bagasse and straw mixture were characterized by a Malvern Instrument GPC analysis system, using three conjoined chromatography columns (SHODEX – Ohpak SB-806M HQ) at 45 °C, where aqueous NaNO₃ 0.1 mol L⁻¹ with flow rate of 1 mL min⁻¹ was used as eluent and polystyrene (Malvern instrument) as the calibration standard. The retention volume was determined by a refraction index detector. DP values were calculated as the ratio between molecular weight (Mn) and the molecular mass of glucose (180.16 g mol⁻¹).

2.3. One-step synthesis of chitosan-coated magnetic nanoparticles (NP-CH)

NP-CH was synthesized in an one-step processing by conventional co-precipitation method using iron(II) and iron(III) in alkaline medium [30]. In a three-neck round-bottom flask equipped with a reflux condenser and argon atmosphere were added with 100 mL of Fe²⁺-Fe³⁺ (1:2) solution and 125 mg of chitosan. The mixture was kept under a stirring speed of 400 rpm at 50 °C until complete dispersion of chitosan. The precipitation of NP-CH was carried out with a dropwise addition of 30 mL of ammonium hydroxide, followed by keeping the stirring at 400 rpm at 50 °C for 30 min. NP-CH were recovered by using a permanent magnetic, washed several times and stored in ultrapure water until characterization and MCLEAs synthesis.

2.4. Synthesis of cellulases MCLEAs

MCLEAs were obtained using glutaraldehyde as a cross-linker and three different precipitation agents: ammonium sulfate (MCLEA-AS), PEG 600 (MCLEA-PEG) and ethanol (MCLEA-ET). For MCLEAs synthesis, 100 mg of NP-CH and 120 µL of glutaraldehyde were dispersed in 10 mL of phosphate-buffered saline (pH 7) and kept under stirring at 120 rpm and room temperature. After 1 h, 10 mL of 5 mg mL⁻¹ cellulase solution in acetate buffer were added and the mixture was stirred at 120 rpm and room temperature for 1 h. After that, 10 mL of precipitation agent were added to the above mixture, and the new mixture was stirred at 120 rpm and 4 °C for 12 h to form cellulase aggregates. After enzyme immobilization, MCLEAs were recovered by magnetic adsorption, washed 3 times with distilled water and stored in acetate buffer until characterization, enzyme activity assay and COS production.

2.5 Determination of enzyme loading efficiency

Enzyme loading efficiency was determined by two methods. The first method was based on protein loss in the solution before and after immobilization using Bradford assay [31]. Enzyme loading efficiency was calculated based on the following Equation:

$$\text{Enzyme loading efficiency (\%)} = \frac{C_0V_0 - C_sV_s}{C_0V_0} \quad \text{Eq. 1}$$

where C_0 is the initial protein concentration (mg mL⁻¹), V_0 the initial volume of protein (mL), C_s the protein concentration in the total supernatant (mg mL⁻¹) and V_s is the total volume of supernatant (mL).

The second method was based on TGA of MCLEAs and the calculation method was presented in Eq. 2. For this purpose, enzyme loading efficiency (%) was obtained from the ratio between final protein mass calculated by mass loss attributed to cellulase in the TGA curves of the MCLEAs and the initial protein mass used in the immobilization processes.

$$\text{Enzyme loading efficiency (\%)} = \frac{\text{Final protein mass}}{\text{Initial protein mass}} \times 100 \quad \text{Eq. 2}$$

2.6. Enzyme activity assay (optimal pH and temperature condition)

The activities of MCLEAs and free cellulase were determined by measuring of reducing sugar production after reaction of each sample with CMC, based on DNS method and using glucose as a standard [32]. The reaction mixture contained 0.9 mL of 0.44% CMC solution in citrate-phosphate buffer (pH 5) and 0.1 mL of enzyme solution. The CMC hydrolysis reaction was carried out for 1

h at 30, 37, 45, 50, 55, 63 and 70 °C, after which 1.5 mL of DNS reagent was added to stop the reaction, followed by heating the mixture in boiling water for 5 min to allow color formation. After cooling the reaction solutions to room temperature, the optical density (OD) of the solution was measured at 540 nm using a UV-Vis spectrophotometer. Reducing sugar concentration was calculated based on OD values with the use of glucose as the standard. In the respective optimal temperature condition for each sample, was determined the optimal pH (3, 4, 5, 6 and 7) for enzymatic activity as described above. The amount of enzyme needed to catalyze the release of 1 μ mol of reducing sugar equivalent per min was defined as one unit (1 U) of enzymatic activity.

For free cellulase and all MCLEAs, the enzymatic activity was expressed as the relative activity, i.e., the ratio between the activity on each set of values of pH and temperature and the activity in the optimal condition. Lastly, for each MCLEA was calculated the activity recovery value, the ratio between the MCLEA and the free cellulase activity.

2.7. MCLEA reusability assay

Reusability assay was carried out in the optimal conditions of pH and temperature using 0.44% CMC solution in citrate-phosphate buffer as substrate. In an Erlenmeyer flask was added 9 mL of CMC solution and 1 mL of respective MCLEA. After 1 h of reaction under stirring of 120 rpm, MCLEAs were magnetically recovered, washed with citrate-phosphate buffer and submitted to new reaction, whereas the obtained hydrolysates were evaluated to UV-Vis measure to determine the reducing sugar production in each cycle as well total production after all cycles.

2.8. Cellulose obtained from sugarcane bagasse and straw mixture

Cellulose was obtained from sugarcane bagasse and straw mixture (1:1 w/w) after thermic and alkali pre-treatment. Delignification was carried out in autoclave using a 1000 mL Erlenmeyer flask with 2.5% (w/v) NaOH solution and 1:20 solid-liquid fraction, at temperature of 110 °C. After 20 min of delignification process, the solid fraction obtained was recovery and washed several times until pH 7 and lignin removal. After this, the cellulosic fraction was dried at room temperature and characterized regarding cellulose, lignin, hemicellulose and ash content. Dried cellulose showed 59.68 ± 1.42 % of cellulose, 11.06 ± 1.59 % of lignin, 26.68 ± 0.44 % of hemicellulose and 1.48 ± 0.62 % of ash.

2.9. Hydrolysis of cellulose from sugarcane bagasse and straw

Cellulose hydrolysis assay was carried out in water-bath with stirring of 120 rpm, at same optimal conditions determined in enzymatic activity assay for free cellulase enzyme and each MCLEAs. In a 125 mL Erlenmeyer flask, 25 mL of acetate buffer, 2.5 mL of enzymatic solution and 1.25 g of cellulose from sugarcane bagasse and straw mixture were added and the mixture was kept under heating and stirring by 24 h. After reaction, the supernatant was recovered by filtration and submitted to UV-Vis analysis based on DNS method to reducing sugar quantification and GPC analysis for qualitative characterization of COS produced. Hydrolysis efficiency was defined as ration between reducing sugar produced by units of enzymatic activity employed in the hydrolysis reaction.

3. RESULTS AND DISCUSSION

3.1. Synthesis and characterization of NP-CH and MCLEAs

The one-step synthesis by coprecipitation method led to a satisfactory obtention of chitosan-coated magnetic nanoparticles. As shown in Fig. 1, matching curves were obtained between the PXRD patterns of NP-CH and simulated profile of magnetite (Fe_3O_4), indicating formation of this crystalline phase. In respect to cellulase enzymes immobilization, independent of precipitation agent used, no changes in crystalline structure of NP-CH was observed, which is an important aspect to maintaining of magnetic properties of nanoparticles and, consequently, of reusability capacity of biocatalyst.

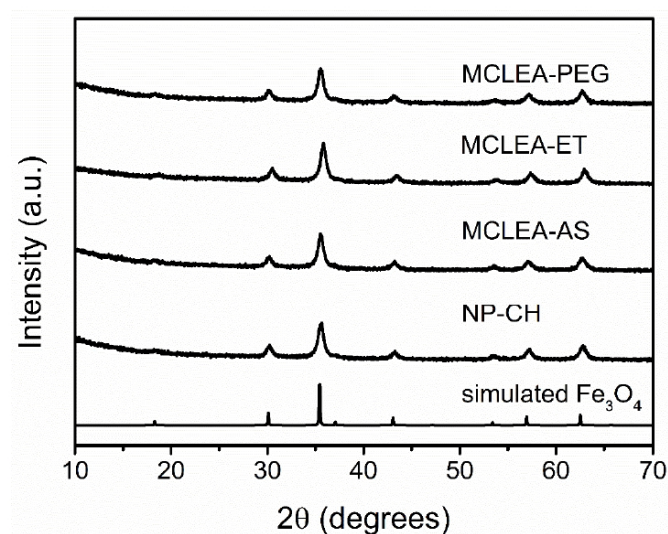


Fig. 1. PRXD patterns simulated of magnetite (Fe_3O_4), and as-synthesized NP-CH and of all MCLEAs obtained with different precipitation agents.

The peaks broadening viewed in the diffractograms of Fig. 1 can be used as an evidence of the nanometric scale of NP-CH and MCLEAs and [33], indeed, as shown in Tab. 1, measures of hydrodynamic diameter (Dh) performed by DLS help to confirm this evidence. NP-CH showed a Dh of almost 65 nm and after enzyme immobilization was observed an increase in the Dh of nanoparticles attributed to aggregation and cross-linker of cellulase enzyme with the magnetic nanoparticle surface. Besides that, this increase in the Dh of NP-CH is an indicative of enzyme immobilization. The highest Dh (121.93 nm) was observed using ethanol as precipitation agent. Both NP-CH and all MCLEAs showed a small polydispersity index (PDI), always lower than 0.20, indicating a satisfactory homogeneity of particle-size distribution.

Tab. 1. Hydrodynamic diameter (Dh, mean $\pm$ standard deviation) and polydispersity index (PDI, mean $\pm$ standard deviation) for NP-CH and MCLEAs obtained with different precipitation agents.

Sample	Dh (nm) $\pm$ sd	PDI $\pm$ sd
NP-CH	65.23 $\pm$ 2.23	0.20 $\pm$ 0.04
MCLEA-AS	118.45 $\pm$ 1.00	0.16 $\pm$ 0.01
MCLEA-ET	121.93 $\pm$ 0.71	0.10 $\pm$ 0.01
MCLEA-PEG	103.35 $\pm$ 0.23	0.11 $\pm$ 0.01

The surface modification of magnetic nanoparticles and cellulase immobilization can also be observed by the FTIR spectra showed in Fig. 2. In accordance with that reported in the literature, the FTIR spectrum of NP-CH (Fig 2a), the presence of chitosan was confirmed with the bands assigned to stretching (ν) of the O–H and N–H bonds at 3334 cm^{-1} ; C–H at 2896 cm^{-1} ; C–N at 1382 cm^{-1} and C–O–C at 1079 ; as well to bending mode (δ) of N–H bond at 1654 cm^{-1} [30]. The band centered at 580 cm^{-1} in the FTIR spectrum of NP-CH was attributed to characteristic $\nu\text{Fe-O}$ of the magnetite structure [34]. Regarding the cellulase immobilization, the presence of the enzyme in all MCLEAs synthesized was confirmed by characteristic bands of protein structure of cellulase (Fig 2b) at 1609 cm^{-1} assigned to stretching of the C=O bond and at 1384 attributed to bending mode of N–H bond. The band centered at 1245 cm^{-1} can be assigned to interaction of the carbonyl group of cellulases enzymes with a hydroxyl group of NP-CH nanoparticles [35].

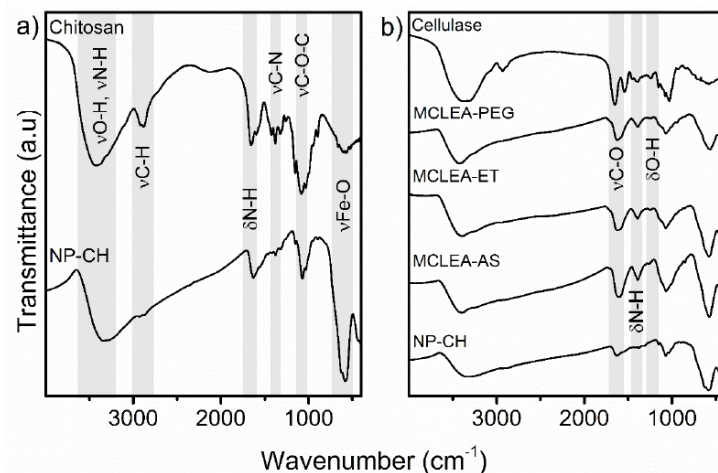


Fig. 2. (a) FTIR spectra of chitosan and NP-CH. (b) FTIR spectra of free cellulase enzyme, NP-CH and all MCLEAs obtained with different precipitation agents.

Quantitative analysis of immobilization is showed by TGA and Bradford protein assay. The Fig. 3 shows the TGA curves for NP-CH, chitosan, free cellulase enzymes and all MCLEAs synthesized. The TGA curve of NP-CH (black solid line) showed a mass loss of ~8% from room temperature until ~400 °C, attributed to the thermal decomposition of chitosan molecules (black dashed line). When compared to NP-CH TGA curve, an increase in the mass loss from room temperature until ~400 °C of 13.77% for MCLEA-AS, 14.66 % for MCLEA-PEG and 15.75% for MCLEA-ET were observed and assigned to thermal decomposition of cellulase enzymes cross-linked to NP-CH surface.

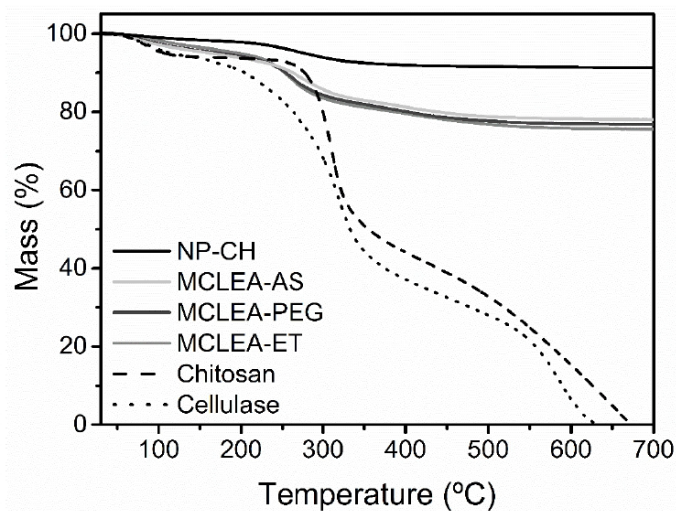


Fig. 3. TGA curves for NP-CH, free cellulase enzyme, chitosan and all MCLEAs synthesized with different precipitation agents.

These mass losses were used to determine the enzyme loading efficiency, which is shown in Tab. 2. In all cases, independent of precipitation agent used in the MCLEAs syntheses, an enzyme loading efficiency higher than 40% was obtained,

especially when ethanol was used as precipitation agent (47.25% of loading efficiency). Bradford protein assay also was carried out in order to assess the enzyme loading efficiency. In all cases, an immobilization efficiency upper to 50% and higher than those calculated by TGA was observed, mainly when PEG was used as precipitation (59.68% of efficiency). In general, a difference near at 10% was observed between the enzyme loading efficiency obtained by TGA and by Bradford protein assay. In the Bradford method, the protein loading onto the support surface is calculated indirectly measuring the initial and final protein concentration in supernatant immobilization media, using bovine serum albumin (BSA) as standard. Aspects as significant differences in the molecular weights, amino acid sequence and exposed structure to aqueous solutions between BSA and many enzymes have been found as some limitations of Bradford method [36], [37]. Meanwhile, TGA measures the amount of protein onto support surface directly. Despite that TGA have shown a smaller loading efficiency than the Bradford method, in summary the results illustrated in this work are satisfactory when compared to many reported works [35], [38], [39]. In addition, highest cellulase loading efficiency have been observed to chitosan-coated magnetic NP, such as the 66% observed by Sánchez-Ramírez and co-workers [35], when compared to the almost 15% for aminosilane-coated magnetic nanoparticles [38], both obtained at similar conditions of initial enzyme concentration used in this work and by Bradford protein assay. In order to enhance the stability parameters, chitosan, a biodegradable polymer, have been extensively used as a coating agent in enzyme immobilization on magnetic nanoparticles surface, mainly because can provide a positively charged surface, while acts as an adhesive enhancer for simple immobilization processes [21].

Tab. 2. Cellulase loading efficiency obtained by TGA and Bradford protein assay.

Sample	Enzyme loading efficiency (%)	
	TGA	Bradford protein assay
MCLEA-AS	41.31	51.74
MCLEA-ET	47.25	57.08
MCLEA-PEG	43.98	59.68

Indeed, the enzyme loading efficiency is an important aspect when considering the synthesis of satisfactory MCLEAs, but not the only one involved. In many cases, the immobilization can be attached to enzyme denaturation and, consequently, to significant losses in the enzymatic activity [40]. Thus, aspects as enzymatic activity and their stability in function of pH and temperature, recovery activity percentage and reuse capacity also are fundamental when has been thought in practice applications.

3.2. Enzymatic activity assay

Aiming determination of optimal operational conditions of cellulose hydrolysis were observed the effect of pH and temperature in the MCLEAs activity. Fig. 4 shows the effect of temperature in the relative enzymatic activity of the free cellulase enzyme and MCLEAs. Changes in the temperature of CMC hydrolysis reaction provided significant changes in the relative activity of all samples. As already established in the literature, free cellulase enzymes shows a higher activity at 50 °C [41] [42]. An increase in the optimal temperate to 55 °C was observed when ethanol and ammonium sulfate were used as precipitation and to 63 °C with PEG.

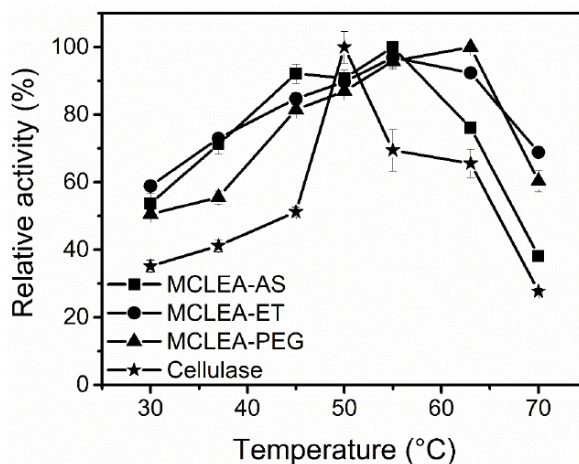


Fig. 4. Effect of temperature at pH 5 in the relative enzymatic activity of free cellulase enzyme and MCLEAs obtained with different precipitation agents.

In all evaluated sample was observed the same effect of the temperature in the activity of enzymes, i.e., increase in the relative activity from low temperatures until the respective optimal temperature with subsequent activity losses. For each kind of enzyme there is an optimal temperature which the largest number of molecular collisions as possible is observed, allowing thus, the maximum reaction rate without an enzymatic denaturation [43]. However, high temperatures lead to rupture of native enzyme structure and, consequently, to significant loss in the enzymatic activity, as well observed in Fig. 4. Besides that, free enzymes tend to be more sensitive to temperature than when are in immobilized form as MCLEAs. For free cellulase, the relative activity can vary as much as 50% from optimal temperature until 45 °C and 30% until 63 °C. Meanwhile, in MCLEAs cases, changes around optimal temperature provide variation in the relative activity as little as 3% for MCLEA-PEG and MCLEA-ET, and 7% for MCLEA-AS. Thus, MCLEAs demonstrated significantly improved in preserving enzymatic activity after changes in optimal temperature compared with free cellulase. Besides that, the aggregates obtained here shows a higher thermal stability in the enzyme activity than others previously reported works, e.g., when compared to variation of approximately 20% around optimal temperature observed to cellulase immobilized on silica-coated [44] or chitosan-coated [42] magnetic nanoparticles, synthesized by physical adsorption and covalent bond, respectively. The cross-linked method have produced biocatalysts by inexpensive and effective synthesis, while precipitation agent produces physical aggregates of enzyme molecules without perturbation of their tertiary structure, cross-linker agent glutaraldehyde held it covalent bonded to nanoparticle surface, leading to obtention of highly stable and reusable biocatalysts [45]. Faced it, the cross-linking reaction employed in this work show as an important strategy to enhance thermal stability of the immobilized enzyme when compared to other immobilization method.

Particle-size distribution (PSD) measures performed by DLS were used to reach a better understanding of the temperature effect in the activity of free cellulase enzymes and MCLEAs. As shown in Fig. 5a, increasing in the temperature affects the PSD profile of cellulase enzyme. In the optimal temperature of 50 °C and below it, the native structure of cellulase appears partially aggregated near at 7 nm and 54 nm. However, above the 50 °C, PSD profile is severely affected by temperature increasing which led to formation of large aggregates near at 100 and 500 nm, especially in high temperature as 63 and 70 °C. This change in the PSD profile of free cellulase enzyme suggests that denaturation mechanism of cellulase enzyme by temperature effect is tied to protein aggregation, which can be causing changes in the cellulase structure as steric block of the active site. For MCLEAs, independently of the type of precipitation agent used, variations in the optimal temperature did not induce changes in the PSD profile (Fig. 5a-c), suggesting an excellent colloidal stability of these materials, which can be used to justify the most significant stability of their enzymatic activity if compared to free cellulase. The aggregation of nanoparticles has been shown to significantly reduce the activity of nanomaterials, resulting in inferior performance [46] and, therefore, one of the essential advantages of the enzyme immobilization have been to enhance the enzymatic catalytic stability [47], as shown in this work. Furthermore, nanoparticles colloidal stability, or lack thereof, will determine the dispersity and diffusion of nanomaterials as MCLAs, important aspect when goal the employment of heterogenous catalysts.

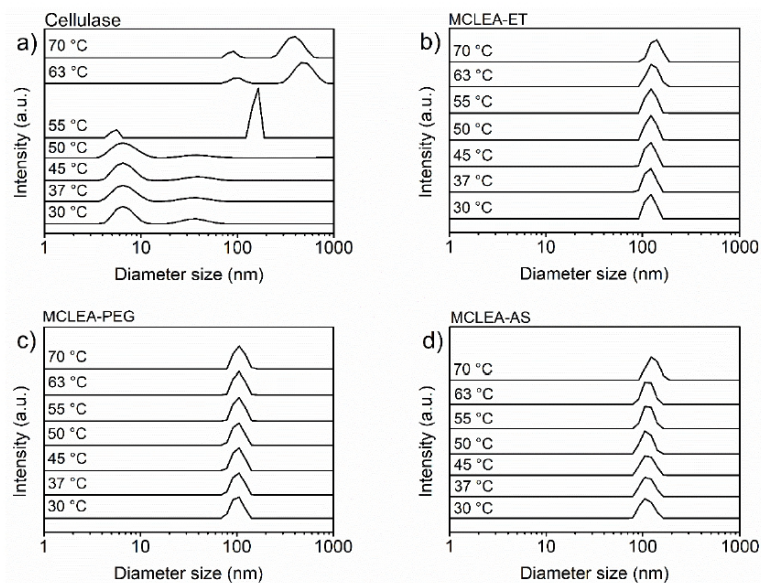


Fig. 5. Effect of temperature in the particle-size distribution of cellulase and MCLEAs obtained with different precipitation agents.

Also was observed the pH effect on the enzymatic activity of free cellulase and MCLEAs in their respective optimal temperature and these results are shown in Fig. 6. The free cellulase shows the highest relative activity at pH 5, while all MCLEAs at pH 4. The presence of charges in support surface makes the concentration of charged species been different in the enzyme microenvironment in relationship to bulk solution [45] and, due to this charge gradient, usually has been reported changes in the optimal pH of enzymes after immobilization process [39].

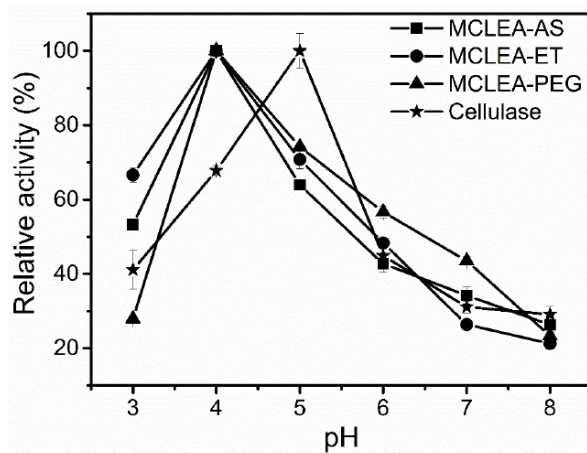


Fig. 6. Effect of pH at optimal temperature in the relative enzymatic activity of free cellulase enzyme and MCLEAs obtained with different precipitation agents.

However, the obtention of MCLEAs did not increase the stability of enzymatic activity due to changes in the pH of CMC hydrolysis reaction. However, colloidal behavior of these samples can be helpfully to understand it. The pH plays an essential role in the colloidal stability of free and immobilized enzyme since dissociation reactions affect the surface charge of molecules and particles, which determines the surface potential and aggregation state [48]. As shown in Fig. 7, for free cellulase enzyme, both above and below of the optimal pH 5, was observed severe changes in its PSD profile. In more acid medium there was a formation of aggregates with almost 800 nm and more alkaline medium nearly to 200 nm.

All MCLEAs showed in pH 4 its highest relative activity and, in general, the same effect of pH in PSD profile. For MCLEA-ET, increases in pH led to decreases in mean particle-size from 177 nm to almost 140 nm, while decreases in pH led to an aggregation with a mean particle-size nearly to 230 nm. In MCLEA-AS case, was observed decreases in mean particle-size from 270 nm at pH 4 until around 140 nm with increases in pH, and pH decreases led to increases in mean particle-size nearly to 320 nm. Lastly, for MCLEA-PEG increases in pH led to decreases in mean particle-size from 188 nm to almost 120 nm, while decreases in pH led to an aggregation with a mean particle-size nearly to 260 nm.

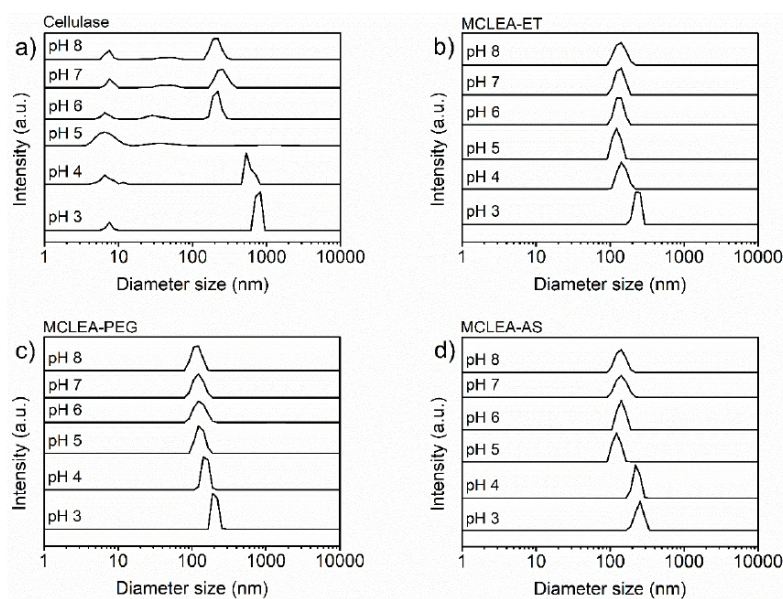


Fig. 7. Effect of pH in the particle-size distribution of cellulase and MCLEAs obtained with different precipitation agents.

It is well established at colloidal science that changes in pH produce changes in charge of particle surface and, consequently, its aggregation state. The Fig. 8 shows the ζ -potential curve in the function of pH for each MCLEA. At pH 3, especially the MCLEA-AS and MCLEA-PEG, show a smaller ζ -potential, and its proximity with isoelectric point justify the observed increase in mean particle-size for these samples. On the other hand, in higher pH, increases in the ζ -potential led to an increase in the colloidal stability of particles and, due to it, to a smaller increasing in the mean particle-size of MCLEAs was observed.

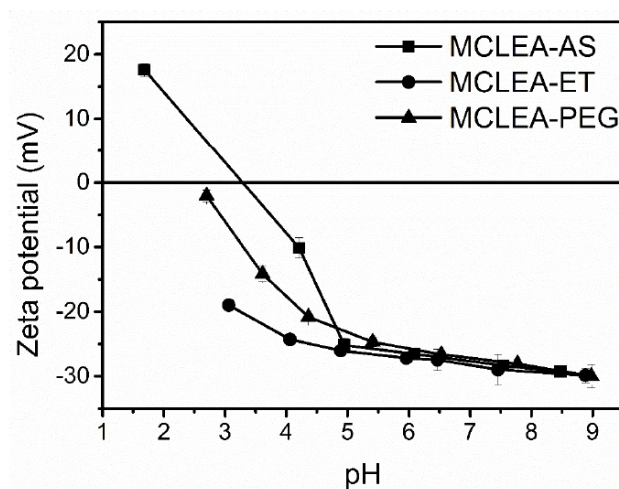


Fig. 8. ζ -potential curve in function of pH for the MCLEAs obtained with different precipitation agents.

Quantitatively, different precipitation agents showed different effect in the cross-linking and enzyme aggregation, consequently, in the enzymatic activity and activity recovery of the MCLEAs. Tab. 3 shows the enzymatic activity value for the MCLEAs and free cellulase enzyme and recovery activity value for all MCLEAs. Activity recovery is a number that gives an idea of the success of immobilization process, whereof the activity of the immobilized is divided by total starting activity of the free enzyme [49].

For MCLEAs, the highest activity enzymatic was observed using ethanol as precipitation agent (193.42 U g^{-1} for MCLEA-ET), while was obtained an activity of 123.40 and 130.90 U g^{-1} when ammonium sulfate and PEG were used as precipitation agent, respectively. Besides having the higher enzymatic activity, ethanol also has proportioned the higher activity recovery (43.53%) when compared to 29.46 and 27.77 % of MCLEA-PEG and MCLEA-AS, respectively. Other than reducing the dielectric constant of medium leading to enzyme precipitation on support surface, organic solvents as ethanol interact with hydrophobic portion of enzymes, providing their aggregation due to electrostatic interactions between groups with opposite charge in the hydrophilic portion of enzyme, without prejudice the native protein structure and consequent denaturation [50]. When compared to free cellulase enzyme, decreases in the activity recovery values and activity losses after immobilization processes for MCLEAs can be attributed to factors as the presence of multipoint attachment to support which can result in changes enzyme conformation, block up of active site of cellulase and losses of enzyme flexibility [21], [22]. Although have been observed losses in activity after immobilization process, the obtained values to activity recovery shows comparatively close and, in some cases, higher to some works reported in literature, e.g., to 30% showed for cellulase immobilized on silica-coated nanoparticles [51]; to 37% observed in cellulase on chitosan-coated nanoparticles [35]; and to 40% seen for cellulase immobilized on polyvinyl alcohol/ F_2O_3 magnetic nanoparticles [52].

Tab. 3. Enzymatic activity and activity recovery for free cellulase enzyme and MCLEAs (mean $\pm$ sd) and their respective condition of pH and temperature.

Sample	Enzymatic activity (U g^{-1})	Activity recovery (%)	Optimal condition	
			pH	T ($^{\circ}\text{C}$)
Cellulase	444.24 ± 5.34	-	5	50
MCLEA-ET	193.42 ± 2.22	43.53 ± 6.78	4	55
MCLEA-AS	123.40 ± 1.70	27.77 ± 5.24	4	55
MCLEA-PEG	130.90 ± 2.35	29.46 ± 3.11	4	63

Frequently, the reduced enzymatic activity, low stability faced to changes in operational conditions and losses in efficiency enzymatic, characterized by low values in activity recovery, difficult the employment of immobilized enzymes in industrial process [53]. However, the synthesis of cross-linked enzyme aggregates and their immobilization on magnetic support, as the MCLEAs obtained in this work, can be usefully in to overcome these challenges, due principally to their satisfactory values of enzymatic activity,

activity recovery and colloidal stability. Furthermore, the magnetic properties of MCLEAs can improve their reusability capacity, favoring the employment in many reaction cycles, which can significantly increase yield in process and decrease industrial cost.

3.3. Reusability assay

Fig. 9 shows the relative activity of MCLEAs after successive cycles of CMC hydrolysis. Different precipitation agents have changed the reuse capacity of MCLEAs. Samples obtained with ammonium sulfate and ethanol, MCLEA-AS and MCLEA-ET, respectively, retained almost 90% of relative activity after 3 cycles, losing their hydrolytic capacity only in the seventh cycle. Better results were observed with PEG as precipitation agent. Like the other samples, MCLEA-PEG retained 90% of relative activity after 3 cycles, however, in the fifth cycle yet retained 80% of relative activity, losing its hydrolytic capacity only in the ninth cycle.

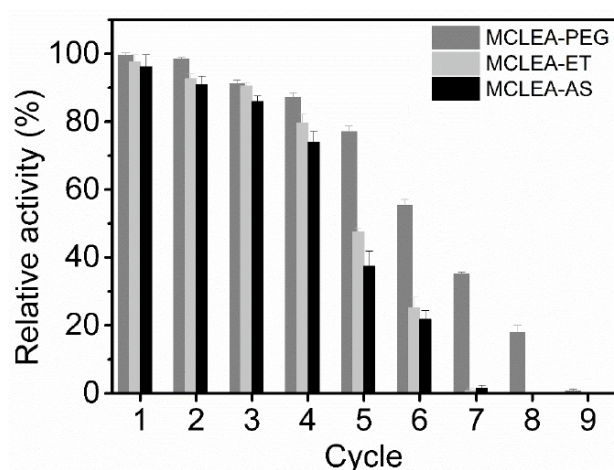


Fig. 9. Activity loss over after successive cycles of CMC hydrolysis using different MCLEAs.

The Tab. 4 shows some results reported in the literature about recovery capacity of cellulase immobilized on magnetic nanoparticle surface.

Tab. 4. Reported results about recovery capacity of cellulase immobilized on magnetic nanoparticles.

Sample	Substrate	Experimental condition	Cycles	Retained activity (%)	ref
Fe ₃ O ₄ -chitosan	CMC	pH 5 and 50 °C	5	75	[54]
Zn-Ferri-te-glutaraldehyde	CMC	pH 4 and 60 °C	6	40	[55]
Fe ₃ O ₄ -silica	CMC	pH 3.8 and 50 °C	6	30	[24]
Fe ₃ O ₄ -silica	CMC	pH 4 and 50 °C	7	77	[44]

The activity loss after successive hydrolysis reaction can be attributed to factors as enzyme inhibition by products or denaturation by long time heating [39]. However, these effects showed less expressive in the MCLEA-PEG. Nonionic polymers as PEG have been extensively used as precipitation agents for different enzymes and proteins, due its neutrality, wide range of molecular mass e water solubility, which make it highly versatilely for enzyme precipitation [56]. Even not showing the higher values for enzymatic activity and activity recovery, the best reusability capacity allows it the highest reducing sugar production after successive cycles, once it were produced 30.74, 14.53 and 9.53 mg mL⁻¹ of sugar by MCLEA-PEG, MCLEA-ET and MCLEA-AS, respectively.

From the viewpoint of industrial applications, the reusability capacity of MCLEAs is a variable of great importance and represent a big challenge and a high cost in biomass conversion processes due to the high amount of cellulase needed to cellulose hydrolysis [57]. Thus, there is a need to improve application of MCLEAs in different biomass conversion process, as shown in this work.

3.4. Cellulose hydrolysis and cello-oligosaccharides (COS) production

COS are carbohydrates biologically active with physiological properties that can benefit the health and growth of animals and humans due to their metabolic functions and ability to stimulate the growth of probiotic bacteria [25], [26], [27]. In this context, several methods have been employed to produce COS, either by polymerization from glucose or cellobiose monomers or by partial hydrolysis of the cellulose. Polymerization by both chemical [58] and enzymatic [59] approaches are challenging due to the technical and cost barriers (e.g., regioselectivity and stereoselectivity by catalytic synthesis and high enzyme cost). COS can also be produced by acid hydrolysis of cellulose, however, this method is nonselective and accompanied by co-product formation and low COS yields [60]. Lastly, enzymatic hydrolysis carried out by cellulases complex lead to mainly glucose production, as result of the synergism between endoglucanases, exoglucanases and β -glucosidases [3], [4].

Faced to importance of this class of compound and technical difficulties in its obtention is that arise the needed of alternative approaches for COS production. Thus, MCLEAs and free cellulases were used in the production of COS from hydrolysis of cellulose obtained from alkali pretreated sugarcane bagasse and straw. The data of hydrolysis efficiency are shown in Tab. 5. The highest hydrolysis efficiency (38.14 mg U^{-1}) was observed for free enzyme. Even MCLEAs not showing the best results, if one takes into account the numbers of possible cycles due to magnetic nanoparticles support it allows the highest yields of sugar production. As discussed for enzymatic activity, cellulose hydrolysis efficiency loss can be attributed to alteration of active site structure after cellulase immobilization on NP-CH. Moreover, considering that cellulose is a biopolymer with high molecular weight, diffusion process to the active site of the immobilized cellulase and steric impediments for enzyme-substrate interactions also need to be taken into account when evaluating its efficiency loss [61], [62].

Among MCLEAs prepared in this work, the highest hydrolysis efficiency were observed for MCLEA-PEG (9.98 mg U^{-1}) and MCLEA-ET (7.65 mg U^{-1}). Interestingly, MCLEA-AS e MCLEA-PEG presented similar enzymatic activity (Tab. 3), but MCLEA-PEG showed highest efficient compared to MCLEA-AS in cellulose hydrolysis, probably due to PEG influence in the diffusion capacity of MCLEA.

Tab. 5. Hydrolysis efficiency obtained by free cellulase and MCLEAs in cellulose hydrolysis.

Sample	Hydrolysis efficiency (mg U^{-1})
Cellulase	38.14
MCLEA-AS	1.36
MCLEA-ET	7.65
MCLEA-PEG	9.98

The hydrolyzed produced by both free and immobilized cellulase were investigated by GPC (Fig. 10), which allowed molecular mass determination and confirm cello-oligosaccharides production from cellulose hydrolysis.

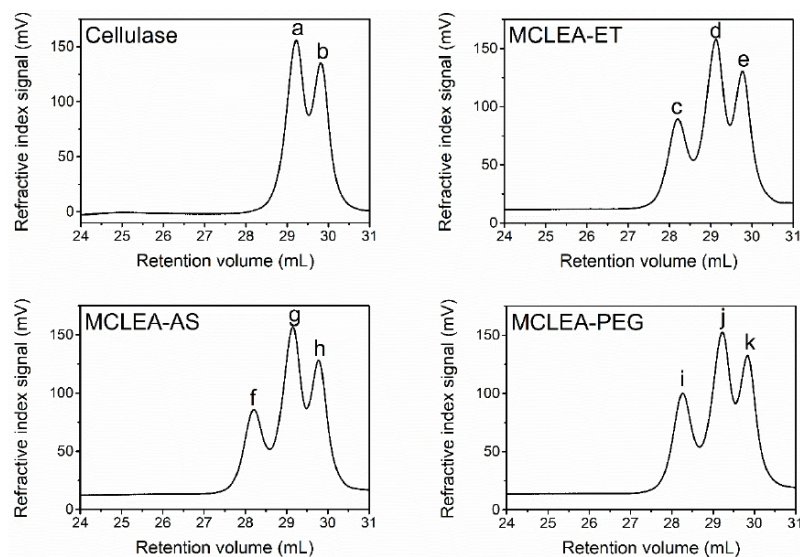


Fig. 10. Chromatogram of hydrolysates obtained from cellulose hydrolysis carried out by free cellulase enzymes and MCLEAs. The peaks a to k are analyzed in Tab. 5.

Although they have not been more efficient than free cellulase, MCLEAs led to a wide variety of products in hydrolysis of cellulose from sugarcane bagasse. COS with DP 3 and 6 were obtained in the hydrolysis of cellulose carried out by both free cellulase and all MCLEAs. However, MCLEAs also can produce COS with DP 12. Currently, higher molecular weight cello-oligosaccharides are produced in small amounts and with a 500 times more expensive cost than those with lower DP [28], [29]. Thus, a low cost-effective process for COS with DP 12 production as developed in our work will enable sustainable industrial applications.

The peaks analysis for chromatograms from Fig. 10 with their respective weight-average molecular weight (Mw), number-average molecular weight (Mn), PDI (Mw/Mn) and DP are shown in Tab. 5.

Tab. 5. Molar mass determination for each peak of Fig. 10. Software used to peak analysis: Omni SEC v. (5.1).

Sample	Peak	Mn (Da)	Mw (Da)	PDI (Mw/Mn)	DP
Cellulase	a	1,061	1,104	1.04	6
	b	584	609	1.04	3
	c	2,244	2,350	1.05	12
MCLEA-ET	d	1,104	1,136	1.03	6
	e	599	627	1.04	3
	f	2,203	2,312	1.05	12
MCLEA-AS	g	1,081	1,112	1.03	6
	h	597	623	1.04	3
	i	2,122	2,217	1.04	12
MCLEA-PEG	j	1,029	1,054	1.02	6
	k	568	592	1.04	3

Currently, industrial applications of COS have been found in food and pharmaceutical industries, however, biorefineries based on the hydrolysis of lignocellulosic biomass will certainly encounter in these compounds an important raw material for their processes [63], [64]. In this context, the diversity of COS produced by MCLEAs make this materials an important tool in the obtention of value-added chemical from lignocellulosic biomass.

4. CONCLUSIONS

Magnetic cross-linked enzymes aggregates were synthesized based on immobilization of cellulases in chitosan-coated magnetic nanoparticles using different precipitation agents (ethanol, ammonium sulfate and PEG 600). In quantitative terms, ethanol

and PEG 600 show more effective as precipitation agent than the ammonium sulfate. After cellulase immobilization, highest values of enzymatic activity (193.42 U g^{-1}) and activity recovery (43.53 %) were observed with the use of ethanol as precipitation agent. While the use of PEG led to MCLEAs with the highest reusability capacity, which retained 80% of initial relative activity after five cycles of reaction. As consequence, the use of PEG resulted in better performing MCLEA, since that allowed, in general, a sugar production two times higher than the observed for other precipitation agents in the CMC hydrolysis.

Dynamic light scattering (DLS) analyses showed that the colloidal stability can be applied to reach a better understanding of thermal stability of cellulases in their both free and MCLEA form. Free cellulase show a low thermal stability and alterations in its particle-size distribution faced to changes in temperature suggests that such low thermal stability can be associated to a denaturation mechanism based on molecular aggregation, which can lead to steric block of active site. Differently, its immobilized form, i.e., the MCLEAs, showed a higher thermal stability, which can be assigned to high colloidal stability obtained independently of precipitation agent used. In addition, DLS measurements reported here for the first time to assess the thermal stability of MCLEAs, show as an interesting approach to better understand the operational behavior of other enzymes and proteins as a whole, faced to several denaturation conditions.

Lastly, while free cellulase can produce only COS with low DP (3 and 6), a variety of COS with different DP (3, 6 and 12) were obtained by MCLEAs applied in hydrolysis of cellulose from sugarcane bagasse and straw. Thus, due to higher value-added of COS obtained by MCLEAs, in addition to their important prebiotic properties, the cellulases immobilization method employed in this work can enable a sustainable production of value-added chemical from lignocellulosic biomass.

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**Magnetic crosslinked porcine pancreatic lipase aggregates
for transesterification process**

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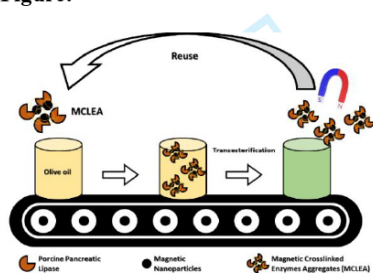
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GA Figure:



GA Text: Magnetic crosslinked enzyme aggregates
finding application on olive oil transesterification. It
may be magnetically separated and reused for 6
cycles.

Magnetic crosslinked porcine pancreatic lipase aggregates for transesterification process

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Abstract

Lipases have been used in industrial processes as biocatalysts for transesterification reactions. The synergism between enzymes and magnetic properties may be reached by using magnetic nanoparticles (MNPs) as support to immobilize them in aggregate structures, denominated by magnetic crosslinked enzyme aggregates (MCLEA). One of the advantages of such supports is the possibility of using magnetic separation for enzyme recovery, reducing costs and allowing reuse in continuous systems. Here, porcine pancreatic lipase (PPL) was immobilized onto functionalized magnetite support (Fe_3O_4 -APTS) with a protein binding efficiency of 78.94%. Physical and chemical properties of the NPs and immobilized lipase were characterized by X-ray diffraction (XRD), transmission electron microscopy (TEM), infrared spectroscopy (FTIR), dynamic light scattering (DLS), zeta potential, vibrating sample magnetometer measurements (VSM), and ^{57}Fe Mössbauer spectroscopy. The immobilized lipase additionally exhibited improved stability across wide pH and temperature ranges compared with free lipase. The immobilized derivate also attained good reusability, maintaining 61.37% of its initial activity after 6 reaction cycles. Through magnetic behavior and also because of its surface modification to crosslinking the enzyme, the MCLEA produced in this work has enhanced the biocatalytic activities of PPL.

Keywords: Immobilization, porcine pancreatic lipase, magnetic nanoparticles, hydrolytic activity, MCLEA

Introduction

Enzymes are applied efficiently in different sectors such as the food and pharmaceutical industries.¹ Currently, there is great interest in the use of enzymes in the production of biofuels as many chemical reactions can render them economically competitive.^{2–6} According to the support and production method selected, the activity and reusability of the enzyme are strongly influenced,^{7,8} conferring greater stability and resistance across different pH and temperature ranges, in addition to preserving enzyme activity over several catalytic cycles.⁹

For the biocatalysts to succeed, the support must be inert and compatible with the enzyme and must not interfere with its native protein structure or compromise its biological activity.¹⁰ The best support options are solid carriers that interact with the enzyme by adsorption, covalent bonding, encapsulating, confinement, or crosslinking.^{11,12} Previous studies have indicated that carbon nanotubes, silica mesopores, nanofibers, nanocomposites, nanorods, and MNPs are all feasible nanomaterials for support.¹³

Among the different supports, magnetite nanoparticles (Fe_3O_4) emerge as a promising material that plays an essential role in enzyme immobilization. Fe_3O_4 is colloidally stable, exhibits good liquid dispersion, and may be easily applied on an industrial scale.^{14–16} However, Fe_3O_4 nanoparticles are prone to oxidation in the environment necessary for reactions, so a protective shell of silica or other inert material is required to achieve chemical stability.^{17,18} Furthermore, Fe_3O_4 can be functionalized with a wide range of functional groups capable of immobilizing enzymes on its surface, without losing the magnetic properties that allow easy separation from the reaction medium through an external magnetic field.¹⁹

Lipases have been the most used biocatalysts in industrial processes and can be applied in several types of industries, being one of the most historically important groups of biocatalysts for biotechnological applications.^{20,21} They found applications in the synthesis of fine chemicals, such as agrochemicals, therapeutics, cosmetics, and flavors; new biopolymeric materials, and biodiesel production.¹⁹

Lipases (E.C.3.1.1.3) are classified as hydrolases, mainly hydrolyzing triglycerides (TAGs) to glycerol and fatty acids.²² These enzymes are classified based on their origin: plant, animal, and microbial lipases. Animal lipase is low-cost and obtained from the pancreas of cattle, sheep, or swine.²³ Lipases operate at the interface between lipids and water and therefore possess an α -helical amphipathic 'cap' region of the protein that covers the catalytic residues of the active site.²³ A closed cap protects the active site from the environment and makes interaction with the substrate impossible; an open cap allows the protein to perform its catalytic activity.²⁴

Crosslinked enzyme aggregates (CLEAs) are an excellent technology for the immobilization of enzymes that, when used in conjunction with surface-functionalized magnetic nanoparticles, can form magnetic crosslinked enzyme aggregates (MCLEAs).²⁵ In addition to the biocatalytic properties of CLEAs, such as increasing enzyme thermostability compared to free enzymes,²⁶ MCLEAs add the magnetic properties of Fe_3O_4 nanoparticles, becoming advantageous during magnetic separation. Other advantages of using MCLEAs include a higher proportion of enzyme-substrate; improved reusability; reduced chance of contamination; improvements in activity, process control, catalytic process, functional effectiveness, product stability, and level of continuous operation.²⁷

Some scientific literature has already described the immobilization of porcine pancreatic lipase (PPL) with a magnetic carrier and characterized hydrolytic activity in olive oil,^{28,29} however, the use of MNPs to immobilize a PPL enzyme was not discussed based on its magnetic properties. This work aimed to evaluate the immobilization of PPL onto MNPs and its application in biocatalysts reactions. The hydrolytic activity was evaluated for free and immobilized PPL to determine the catalytic viability of immobilized PPL in the transesterification of olive oils to produce biodiesel. The enzyme aggregates obtained in this work have demonstrated that the concept test of these magnetic supports and their crosslinking with the enzyme lead to cutting-edge materials that will certainly improve biodiesel synthesis.

Materials and methods

Chemicals

Iron(II) chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), iron(III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), diethanolamine (DEA), diethylene glycol (DEG), glutaraldehyde (25%), phosphate buffer (pH 7.4), porcine pancreatic lipase (PPL), polyethyleneglycol ($1 \times 10^6 \text{ g mol}^{-1}$), 3-aminopropyltriethoxysilane (APTS) and gum arabic powder were purchased from Sigma-Aldrich Co. (Burlington, USA). Sodium hydroxide (NaOH) and commercial ethanol were purchased from Synth (São Paulo, Brazil). Sodium chloride was purchased from J. T. Baker, S.A. (Phillipsburg, USA).

Synthesis of MNPs functionalized with amine group

The Fe_3O_4 MNPs were obtained by the precipitation in alkali media. Briefly, 2 mmol of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and 4 mmol of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were dissolved in 80 mL of DEG/DEA (1:1, v/v) and, separately, 16 mmol of NaOH was dissolved in 80 mL of DEG/DEA (1:1, v/v). The solutions were magnetically stirred in a round flask, under an argon atmosphere. The reaction took place at 200°C for 8 h. After cooling to ambient temperature, the black precipitate was centrifuged and washed with alcohol/water (1:1, v/v) three times. Then, the sample was dried in a dissector and denominated by Fe_3O_4 .³⁰

For functionalization, 200 μL of APTS was added to 20 mL of Fe_3O_4 suspension. The system was magnetically stirred for 24 h and then centrifuged and washed with alcohol/water (1:1, v/v) three times. The precipitate was denominated by Fe_3O_4 -APTS.

MCLEAs obtention

An aliquot of 5 mL of Fe_3O_4 -APTS (0.9 g mL^{-1}) was added to a Falcon tube containing 18 mL of phosphate buffer (0.01 mol L^{-1} , pH 7.4). Subsequently, 2 mL glutaraldehyde (25%) were added and the solution was vortexed for 1 h, at room temperature, to obtain the aggregate structures. The system was centrifuged and the precipitate was washed with alcohol, water, and phosphate

buffer solution (1:1:1) to remove any excess glutaraldehyde, at least 10 times until the supernatant turns colorless. Then, the magnetic aggregate was suspended in 20 mL of phosphate buffer, followed by the addition of 50 mg of PPL and 0.01 g of polyethylene glycol (1000 kDa). Vortexing was performed for 5 min before the solution was maintained for 16 h at 6 °C. The suspension was centrifuged and the precipitate was washed with phosphate buffer (pH 8.0, 1 mol L⁻¹) to remove any free PPL. The sample was denominated by Fe₃O₄-PPL.

Enzymatic immobilization yield

The immobilization yield was evaluated through protein dosage carried out by Bradford assay, according to previous work.³¹ Equation 1 was employed for immobilization yield determination:

$$\text{Immobilization yield (\%)} = \left(1 - \frac{P_f}{P_i}\right) \times 100 \quad (1)$$

where P_f corresponds to the protein concentration of free PPL and P_i is the protein concentration of the immobilized Fe₃O₄-PPL sample.

Hydrolytic activity of lipase immobilized with MNPs

The hydrolytic activity was determined by the olive oil emulsion method proposed by Bassan *et al.*³⁰ 2.5 mL of olive oil and water emulsion (1:1 v/v) and 2 mL of phosphate buffer (0.1 mol L⁻¹, pH 8.0) were mixed. The reaction media was kept under stirring at 150 rpm in a thermostatic bath. 2 mL of the immobilized enzyme solution (1 mg mL⁻¹) was added, keeping the system under stirring at 37 °C for 5 min. After the incubation period, 10 mL of a mixture of ethanol and acetone (1:1) and 10 mL of a previously standardized KOH solution (0.05 mol L⁻¹) were added. Excess KOH was titrated with HCl (0.05 mol L⁻¹). The enzymatic activity was calculated according to Equation 2:

$$A = \frac{(V_b - V_a) \cdot M \cdot 10^{-6}}{t \cdot m} \quad (2)$$

where A is the hydrolytic activity (U g^{-1}), M is the molarity of the HCl solution (mol L^{-1}), t is the incubation time (min), V_b is the blank volume (L), V_a is the sample volume (L) and m is the mass of the biocatalyst (g).

Determination of the optimum pH and temperature

To study the influence of pH on free and immobilized lipase, the analyses were carried out at a constant 37°C , varying the buffer's pH from 3 to 10. After establishing the optimum pH value of the enzyme, the pH was kept constant and the temperatures were evaluated between 30 and 60°C to determine the optimum temperature for biocatalyst activity.³⁰ Na_2HPO_4 (0.1M) and Citric acid (0.1M) were employed to prepare a buffered solution comprising between pH 3.0 to 8.0. Buffers made from Na_2CO_3 (0.1M) and Na_2HPO_4 (0.1M) were used for pH range of 9 and 10.

Thermal stability

To compare thermal stability, free and immobilized enzymes were incubated in 1 mL of phosphate buffer (0.1 mol L^{-1}) at pH 7.0 and pH 8.0, respectively, at 60°C for 4h, 8h, 12h, and 24h. Storage stability and reaction temperature of free and immobilized preparations were studied during storage at 4°C and at reaction time by evaluating their activity for 4h, 8h, 12h, and 24h.²⁵ The reusability of the enzyme was evaluated in the transesterification reaction of olive oil. After reaction at 60°C , the immobilized derivative was separated by a magnet and washed three times with *n*-hexane and then three times with water. After this treatment, the immobilized enzyme was resuspended in a new aliquot of substrates for the next catalytic cycle.

Instrumentation

The Fourier transform infrared (FTIR) spectra were recorded by a PerkinElmer Frontier model spectrometer (Waltham, USA), equipped with Attenuance Total Reflectance (zinc selenide crystal) apparatus. Spectra were recorded by 128 scans, with 4 cm^{-1} resolution. Transmission

electron microscopy (TEM) analysis was carried out with a Philips CM120 microscope (Amsterdam, Netherlands). The software ImageJ (version 1.45s) was used for image treatment. The X-ray diffraction (XRD) patterns of samples were obtained in a Siemens D5005 diffractometer (Berlim, Germany) using $K\alpha$ radiation, with a wavelength (λ) of 0.15418 nm. Measurements were recorded at the 2θ scan interval from 10° to 80° , with a scan speed of 0.02° per second. The thermogravimetric analysis (TG) of the samples was performed on Netzsch STA 409/CD equipment (Ulm, Germany) under dry airflow of 100 mL min^{-1} . Zeta potential was determined by the Zetasizer Nano ZS, Malvern (Malvern, UK). The samples were dispersed in an aqueous solution of 0.001 mol L^{-1} NaCl and titrated in the pH range of 3 to 10. The hydrodynamic diameter was determined by the Zetasizer Nano ZS, Malvern (Malvern, UK), by dynamic light scattering (DLS) technique, which samples were dispersed in deionized water. The magnetization measurements were performed in a vibrating sample magnetometer (VSM) from LakeShore, model 7400 (Westerville, USA), at 300K. The Mössbauer spectra were recorded at 300 K in a spectrometer from SEECO (Westerville, USA) operating in the transmission mode, using a ^{57}Co gamma-ray radiation source with 12 mCi activity. The spectra were fit using Normos90 software; the isomer shifts are reported relative to $\alpha\text{-Fe}$ at 300 K.

Results and Discussion

Characterization of lipase immobilization onto magnetic nanoparticle support.

The X-ray diffractograms of synthesized (Fe_3O_4), functionalized ($\text{Fe}_3\text{O}_4\text{-APTS}$), and immobilized ($\text{Fe}_3\text{O}_4\text{-PPL}$) samples are shown in Figure 1. The diffraction peaks correspond to the inverse-spinel structure of magnetite, consistent with its standard structure (JCPDS card No. 39-1346), and no secondary phase was observed. The estimative of crystallite diameter was calculated from the Scherrer equation, finding 9.75 nm for Fe_3O_4 , 9.94 nm for $\text{Fe}_3\text{O}_4\text{-APTS}$, and 10.17 nm for $\text{Fe}_3\text{O}_4\text{-PPL}$ samples. The samples showed crystallite sizes close to the bare NPs. **It is known that the APTS functionalization molecules are covalently bonded to the nanoparticles' surfaces, thus, the**

chemical modification of the NPs surface may lead to thin layer of a different iron oxide. According to the diffractograms, for all samples, and within the XRD sensitivity we conclude that there are no second phases.

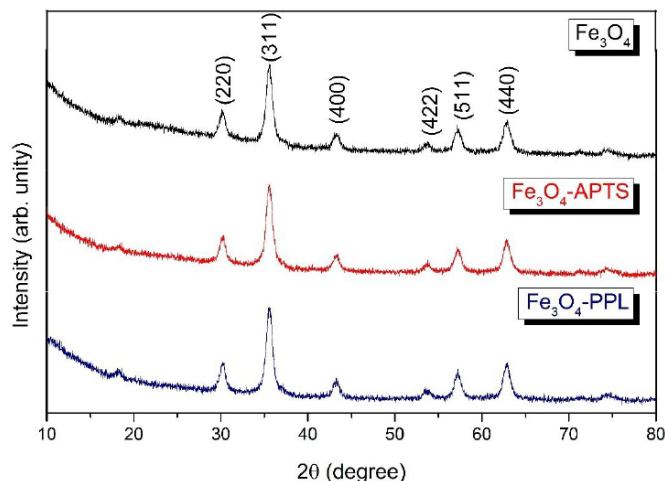


Figure 1. XRD pattern of Fe_3O_4 , Fe_3O_4 -APTS, and Fe_3O_4 -PPL.

The TEM image of Fe_3O_4 -APTS particles is shown in Figure 2A. This micrograph displays sphere-like NPs which are homogeneous in shape and size. Figure 2B shows the histogram of nanoparticle sizes. The sizes of 135 NPs were evaluated and the data of frequency versus size was fit to a lognormal distribution. The average nanoparticle size was found to be approximately 5.1 nm. The results obtained from XRD and TEM analyses are in accordance with the data reported in the scientific literature.^{32,33} Based on the results obtained from XRD regarding the crystallite size, which refers to the average size of individual crystallites in a solid material and pertains to the smallest crystal or polycrystalline solid comprising the crystalline structure of the material, it is observed that this size is slightly larger than the average particle diameter found in TEM. A particle is composed of an aggregation of multiple crystals, and when it is small enough, it can refer to a single crystal. Thus, the average particle size is never smaller than the crystallite size. The

underestimated average value in TEM is primarily attributed to the limited number of counts and the presence of aggregates, which hinders the accurate determination of the real size of nanoparticles. However, considering the obtained standard deviation, it is evident that the values are in agreement.

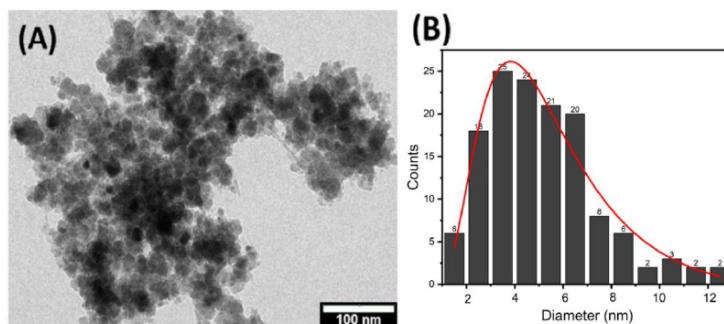


Figure 2. A) TEM images of Fe_3O_4 -APTS sample. B) Histogram distribution of Fe_3O_4 -APTS.

Since the magnetic core structure was preserved after the functionalization steps, the surface modification was evaluated by FTIR, as shown in Figure 3. For the Fe_3O_4 sample, the bands observed at $\sim 3200\text{ cm}^{-1}$ and $\sim 1600\text{ cm}^{-1}$ were attributed to the presence of water (O-H stretching, H-O-H deformation, respectively). The intense bands observed at 597 and 445 cm^{-1} were attributed to the characteristic Fe-O bond of iron oxide.³⁴ After APTS functionalization, a significant increase in the intensity of the bands at ~ 3395 and 1625 cm^{-1} was observed and a broadening and displacement can be attributed to an increase in the contribution of O-H groups derived from silanol and to the N-H folding of primary amines present in the APTS molecule. The bands at 2922 cm^{-1} and 2853 cm^{-1} were attributed to symmetric and asymmetric stretching of C-H, as well as the weak bands in the regions 1450 cm^{-1} and 1384 cm^{-1} were attributed to C-H folding. A weak and broad band observed in the region 1065 cm^{-1} was attributed to the ν Si-O stretching, corroborating the formation of a Si-O coating on the surface of MNPs³⁵. After functionalization with the PPL enzyme, a broader and more intense band at $\sim 3450\text{ cm}^{-1}$ was observed because of the contributions of O-H and N-H bonds

due to the presence of the enzyme, as well as a significant increase in the bands of C-H. In addition, the main bands associated with proteins were observed on free PPL and Fe_3O_4 -PPL samples, such as amine I ($\sim 1641 \text{ cm}^{-1}$), amide II ($\sim 1560 \text{ cm}^{-1}$), and amide III ($\sim 1400\text{--}1200 \text{ cm}^{-1}$).^{36–38} No additional band was observed on the Fe_3O_4 -PPL sample, once the crosslinking reaction between aldehydes groups from glutaraldehyde and amine groups from PPL and Fe_3O_4 -APTS sample took place on the same absorption region from proteins. On the other hand, the primary amine (N-H stretching at 3550 cm^{-1}) is only present on the free PPL sample, confirming the consumption of amines during the crosslinking step.

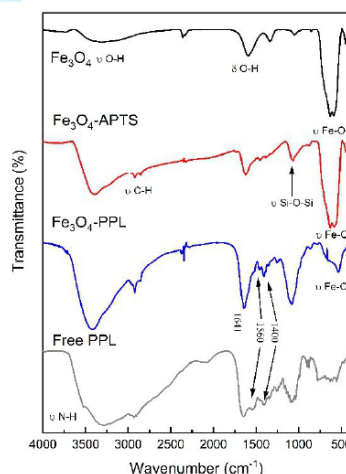


Figure 3. FTIR spectra of Fe_3O_4 , Fe_3O_4 -APTS, Fe_3O_4 -PPL, and free PPL samples.

The surface functionalization was also evaluated by zeta potential. The zeta potential of the samples was measured as a function of pH, as shown in Figure 4. The isoelectric point (IEP) for the Fe_3O_4 sample is 2.9. Due to surface modification with APTS, the IEP for Fe_3O_4 -APTS shifted to 6.4, as expected for the increase in the density of amino groups on the surface of the nanoparticles.³⁹ Free PPL has an IEP of 5.4, a value close to the immobilized sample (IEP = 5.0). The changes in zeta potential features are caused by the functional groups identified by FTIR measurements for each nanoparticle functionalization step, corroborating both findings.

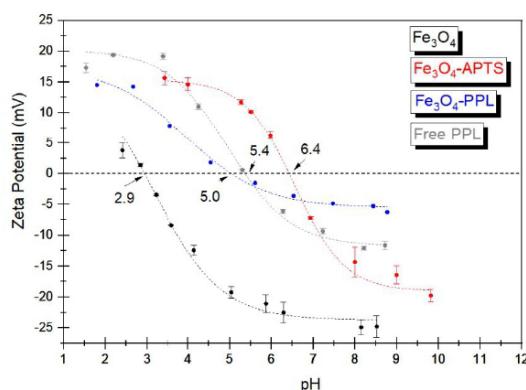


Figure 4. Zeta potential of Fe₃O₄, Fe₃O₄-APTS, Fe₃O₄-PPL, and free PPL.

Dynamic light scattering measurements were carried out to study the size distribution of samples in solution. Table 1 summarizes the average hydrodynamic diameter and polydispersity index (PDI), meanwhile, Figure 5 shows the distribution profile of samples. In general, all samples show a single distribution. The Fe₃O₄ sample shows an average hydrodynamic diameter of 234.1 nm. After being functionalized with APTS, the sample increased in hydrodynamic size to 554.1 nm. This increase in hydrodynamic diameter is related to surface charge. According to the zeta potential curves in Figure 4, the value of zeta potential decreases in modulus from -25 mV (Fe₃O₄) to -7 mV (Fe₃O₄-APTS), and the absence of electrostatic repulsion may result in an aggregation process. The hydrodynamic diameter observed for free lipase was 7.6 nm, which is expected for small proteins. Due to crosslinking, which occurs when many supported enzymes combine throughout the reaction with glutaraldehyde, the immobilization step also displayed an increase in hydrodynamic diameter to 1088 nm. These results confirm the surface modification of the magnetic carrier with lipase.

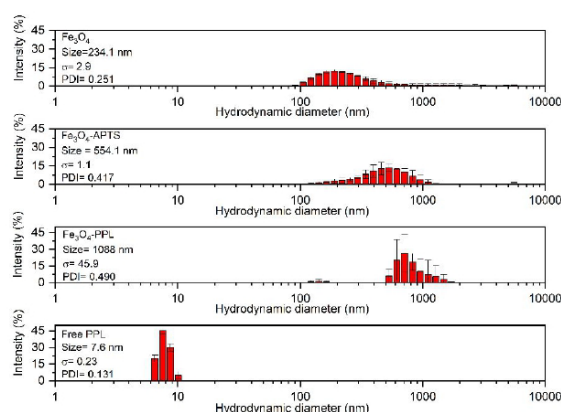


Figure 5. Hydrodynamic diameter distribution of Fe_3O_4 , Fe_3O_4 -APTS, Fe_3O_4 -PPL, and free PPL.

Table 1. Average hydrodynamic diameter (D_h) and polydispersity index (PDI) for Fe_3O_4 , Fe_3O_4 -APTS, Fe_3O_4 -PPL, and free PPL.

Sample	D_h (nm)	PDI
Fe_3O_4	234.1±2.9	0.251
Fe_3O_4 -APTS	554.1±1.1	0.417
Fe_3O_4 -PPL	1088±45	0.490
Free PPL	7.6±0.2	0.131

TG was employed to determine the amount of enzymes that effectively bonded to the magnetic carrier. This determination was accomplished by comparing the mass loss between the samples of Fe_3O_4 -APTS, Fe_3O_4 -PPL, and free PPL (Figure 6). The mass losses below 200 °C can be attributed to the removal of water. For Fe_3O_4 -APTS, mass loss was only 5.39% from 25 °C to 1000 °C, indicating good thermal resistance. The thermal behavior of Fe_3O_4 -PPL exhibited a mass loss of 69.81% from 600 to 800 °C. For free PPL, 96.14% of organic composition was lost up to a temperature of 650 °C. **This finding suggests that the immobilization may raise the temperature at which the enzyme decomposes to levels above those observed for free PPL.** The remaining mass of the Fe_3O_4 -PPL was 0.64 mg, representing 30.19% of the initial mass amount. By crossing the

tangent lines, the TG curve can be verified; at a temperature of 794 °C, they indicate a mass loss of 77.14%, therefore 22.86% is attributed to lipase on the magnetic support surface.

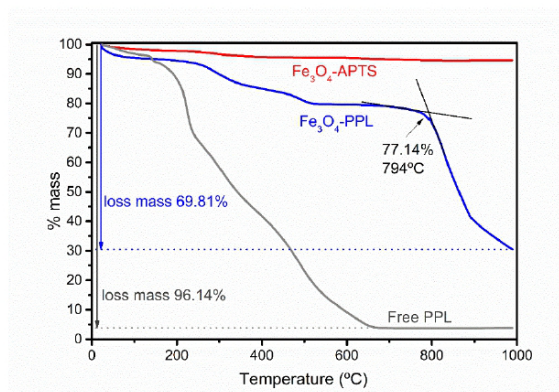


Figure 6. TG curves for Fe₃O₄-APTS, Fe₃O₄-PPL, and free PPL.

The hysteresis curves (Figure 7), recorded at 300 K, show remanent magnetization (M_r) and coercivity field (H_c) values of 1.31 emu g⁻¹ and 49 Oe for Fe₃O₄-APTS, and 0.13 emu g⁻¹ and 45 Oe for sample Fe₃O₄-PPL. In both samples, the small values of M_r and H_c indicate that a fraction of NPs is in the superparamagnetic regime and the rest is in the thermally blocked regime. The magnetization values measured at field strength of 1.5 T were 21.6 and 7.4 emu g⁻¹ for Fe₃O₄-APTS and Fe₃O₄-PPL, respectively. To obtain the magnetization, the measured magnetic moment is normalized by the sample mass; thus, the magnetization of sample Fe₃O₄-PPL is smaller because of the mass of the PPL. Superparamagnetic NPs in the absence of a magnetic field behave like a non-magnetic system; however, in the presence of a field, they are strongly attracted by magnets. In this sense, the Fe₃O₄-PPL NPs may be removed from the aqueous dispersion by using a magnet.

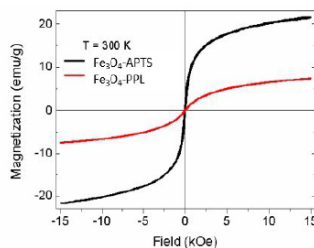


Figure 7. Hysteresis curves recorded at 300 K for samples Fe_3O_4 -APTS and Fe_3O_4 -PPL.

Figure 8A shows the spectrum of sample Fe_3O_4 -APTS, it was fit to two components: a doublet due to superparamagnetic NPs and a distribution of sextets ascribed to thermally blocked NPs (Table 2). The distribution of hyperfine magnetic field (Hhf) is displayed in Fig 8B and has its highest peak at 42 T, however, the magnetic field extends up to 50 T. The relative absorption area (RAA) of blocked particles was 65%. The distribution of Hhf shows fields noticeably smaller than expected for either bulk magnetite (46-49T) or maghemite (50 T).³⁴ This is because the whole system has not reached yet a blocked regime; in such a scenario, the Hhf would be larger, and it will be close to 49-50 T. The component doublet is related to superparamagnetic NPs, these are NPs with smaller size than the blocked ones. In both cases, the isomer shifts (IS) are in agreement with Fe^{3+} .³⁴

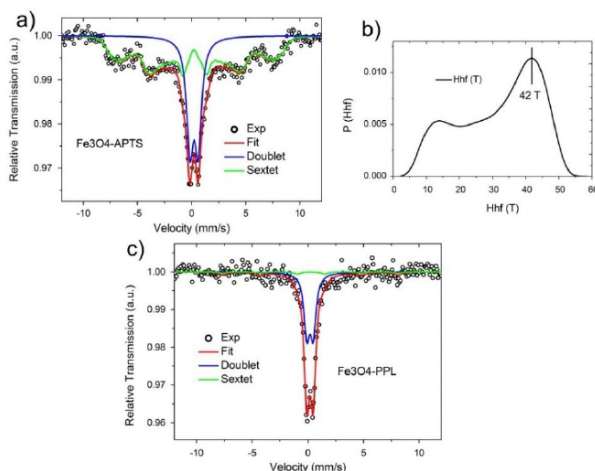


Figure 8. Mössbauer spectra recorded at 300 K for samples: a) Fe_3O_4 -APTS and c) Fe_3O_4 -PPL. b) is the histogram of the hyperfine magnetic field used to fit the spectrum of sample Fe_3O_4 -APTS.

Figure 8C shows the spectrum of sample Fe_3O_4 -PPL. In order to fit the spectrum we considered a sextet and a doublet. The sextet has Hhf and RAA of 48.8 T and 9 %, respectively, meanwhile the doublet has RAA of 91 %. This sample has a larger number of superparamagnetic NPs compared with sample Fe_3O_4 -APTS.

Superparamagnetism is a property occurring in small, single-domain magnetic NPs without magnetic interaction, it is characterized by a blocking temperature (T_b), above which the NPs are superparamagnetic and below T_b the NPs are blocked. Factors like relaxation time of moments between the easy axis of magnetization (τ), magnetocrystalline anisotropy (K), and volume of NPs (V) affect the blocking temperature (T_b). In an ensemble of no interacting single-domain NPs, the magnetic moments in each NP experience a relaxation time (τ) given by the Néel equation (3):

$$\tau = \tau_0 e^{\frac{KV}{K_B T}} \quad (3)$$

where K_B is the Boltzmann constant, T is temperature, τ_0 is a constant of the order of 10^{-9} - 10^{-11} s.

The relaxation is considered slow when τ is of the same order as the measuring time (τ_m), the value of τ_m depends on the experimental technique. For instance, τ_m is of 10 or 10^{-8} s for the techniques

magnetometry or Mössbauer spectroscopy, respectively. In case of $\tau = \tau_m$, then $T = T_b$ (blocking temperature). In case of existing a magnetic interaction between NPs the Néel equation is modified by adding a temperature term (T^*) ascribed to interactions between NPs (see equation 4) as discussed by Allia et al. in his model interacting superparamagnetic regime:⁴⁰

$$\tau_m = \tau_0 e^{\frac{KV}{k_B(T+T^*)}} \quad (4)$$

The theory indicates that the actual magnetic moments on magnetic dipolar interacting NPs are systematically larger than those obtained considering the size of the NPs. The expectations of the model has been confirmed in granular Cu-Co alloys interacting superparamagnets⁴⁰ and Fe-oxide NPs,⁴¹ among others.

Thus, the magnetic dipolar interparticle interaction becomes stronger when the NPs are too close, in this condition, a given NP feels the magnetic field from the neighbor NPs and their magnetic moments become blocked. This energy decreases when the NPs are moved far apart each other. Therefore, the functionalization of magnetite NPs with PPL will increase the distance between two neighbor NPs by twice the size of the PPL molecule and, thus, more particles will turn superparamagnetic.

Table 2. Hyperfine parameters from fittings of spectra recorded at 300 K for samples Fe₃O₄-APTS and Fe₃O₄-PPL

	Fe ₃ O ₄ -APTS		Fe ₃ O ₄ -PPL	
	Doublet	Sextet	Doublet	Sextet
IS (mm/s)	0.343	0.321	0.301	0.350
QS (mm/s)	0.753	0.084	0.570	0.051
H _{hf} (T)	--	44.2	--	48.8
RAA (%)	35	65	91	5

Catalytic activity of MCLEAs in olive oil transesterification.

MNPs are excellent materials for use as carriers to immobilize enzymes since they can be stored for a long time and have high stability at neutral pH.⁴² Protein concentration was used to compute the immobilization yield. After 16 hours of immobilization, Fe₃O₄-PPL has a protein concentration of 17.98 µg mL⁻¹ compared to 22.78 µg mL⁻¹ in free PPL, yielding an immobilization rate of 78.84%.

The effect of pH on the activity of the free and immobilized enzymes was evaluated. The highest activity of immobilized and free lipase was established as 100% and other pH conditions were listed as relative activities (Figure 9A). The results show that optimal activity for free PPL occurs at pH 7 while its immobilized form occurs at pH 8, indicating that Fe₃O₄-PPL influences the optimal pH. In addition, the immobilized derivative showed less variation in activity over a wide range of pH 6 to 9, while the free enzyme showed a great loss of activity when reaching pH 8 and 9. A possible explanation for the increase in pH stability is that the immobilization helps maintain the proper enzyme conformation under harsh pH conditions.⁴³ Additionally, it has been previously demonstrated that the open conformation of lipases is more active when the number of α -helices is reduced.⁴⁴ Therefore, immobilization might shield the enzyme-active regions from pH changes.

The activities of free and immobilized lipase were also investigated concerning the temperature (figure 9B). The Fe₃O₄-PPL sample did not show great activity variations between 30 °C and 50 °C. At 50 °C, the immobilized derivative had an increase in its activity, reaching its maximum at an optimum temperature of 60 °C. The results obtained from Fe₃O₄-PPL had better resistance to high temperatures than other supports used for PPL immobilization described in the literature, such as chitosan-SBA-15 nanocomposites and Fe₃O₄-chitosan.^{45,46}

Thermal stabilities of the free and lipase immobilized were studied by measuring the activities of the lipase after incubation in phosphate buffer (Figure 9C). The results show that the soluble enzyme and the immobilized preparation retain almost 40% of the activity after 24 h of incubation at 60 °C. Despite both curves had shown similar behavior, immobilized sample show advantages from the free one according to its reusability, as will be discussed in the next

paragraphs. The storage temperature of 4 °C was also evaluated during incubation of 24 h, and Fe_3O_4 -PPL presented 62% of relative hydrolytic activity compared to free PPL with 59% (Figure 9D). According to the results, the Fe_3O_4 -PPL loses activity more gradually after 12 hours, whereas the activity decline in the free sample is more pronounced. Despite the short storage stability in phosphate buffer, the immobilized sample shows better strength than free PPL.

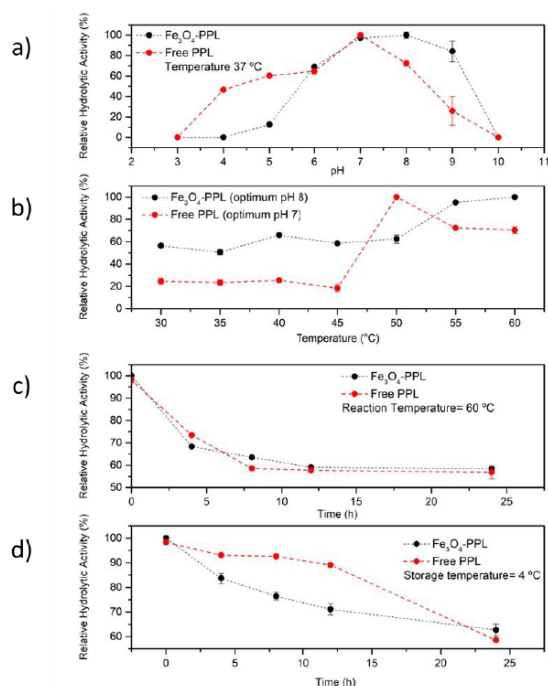


Figure 9. A) Relative hydrolytic activity (%) of Fe_3O_4 -PPL and free PPL as a function of pH. B) Relative hydrolytic activity (%) of Fe_3O_4 -PPL and free PPL as a function of temperature. C) Thermal stability at 60 °C for 24 h. D) Storage stability at 4 °C for 24 h.

The reusability of the biocatalyst has been also investigated to offset the cost of enzymes when compared to homogeneous and chemical catalysts. After each reaction, the immobilized lipase was recovered with a magnet and the next batch was carried out with a new substrate. Fe_3O_4 -PPL showed a good ability to be used repeatedly (Figure 10). The initial activity before any recovery was set as 100% and, after 6 cycles, 61.37% of the initial activity of the derivative was

maintained. Although the cycles lead to a reduction in activity, immobilization has the ability to lessen any modification to the enzyme's structure that could cause denaturation. This finding is good for reducing operational costs in real industrial processes. Similar outcome was attained in an earlier study using PPL immobilization on a magnetic graphene oxide platform, in which recyclability kept good activity (around 90%) for the first 6 cycles.⁴⁷ The literature reports similar behavior for immobilizations involving PPL and magnetic nanoparticles,⁴⁸ metal-organic frameworks (MOFs),⁴⁹ and zeolite supports,⁴³ where activity has kept above 50% after 6 cycles.

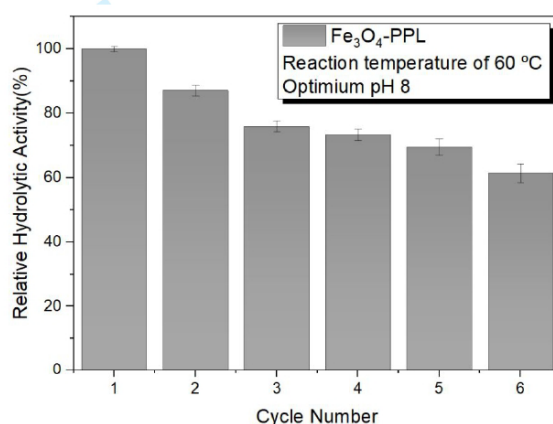


Figure 10. Relative hydrolytic activity (%) as a function of number of cycles using Fe_3O_4 -PPL.

Conclusions

The obtaining and characterization of MCLEAs were successful. The functionalization and immobilization did not alter the crystal structure of the magnetic core. Besides, FTIR and zeta potential measurements confirmed functionalization with APTS and PPL immobilization. The enzyme immobilization also reveals a higher thermal resistance of MCLEA than compared with free lipase. The Mössbauer spectra of sample Fe_3O_4 -PPL showed mainly a doublet, indicating that this sample is in the superparamagnetic regime. Regarding enzymatic activity, immobilized PPL presented higher activity at 60 °C than free PPL. The free PPL had an optimum pH of 7.0, while

the Fe₃O₄-PPL immobilized derivative gave an optimum pH of 8.0. The immobilized lipase exhibited remarkably stability across a wide range of pH and temperature compared to the free one, along with good reusability after 6 cycles. The immobilization method has shown to be an excellent candidate for transesterification processes and the Fe₃O₄-PPL biocatalyst will be trialed in the production of biodiesel in future works.

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Author Contributions

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V. Considerações Finais e Hipótese de Estudo

Esta tese de livre docência resume as principais atividades de pesquisa desenvolvidas pelo proponente durante sua carreira como professor e pesquisador. A maioria das pesquisas relacionam-se com materiais magnéticos. Devido a propriedade de interação dos momentos magnéticos destes materiais com um campo magnético externo, diferentes aplicações podem surgir, sejam pelo uso como suporte magnético ou mesmo como transdutor de sinal externo quando na presença de um campo magnético externo alternado. O aquecimento das nanopartículas magnéticas quando na presença de campo magnético alternado, possibilita a uso destas nanopartículas como transdutor de sinal externo em estímulo interno para aplicações em biomedicina. Além disso, esta propriedade de aquecimento pode também servir para modular outras aplicações biotecnológicas como, por exemplo, suporte para enzimas, biocatalizadores amplamente utilizados na indústria. As enzimas possuem condições ótimas para melhor rendimento nas reações por elas catalisadas. Assim, processos biotecnológicos devem acontecer em condições de temperatura e pH que permitam o máximo rendimento nas reações. Na maioria das aplicações, as enzimas acabam não sendo recuperadas se forem aplicadas em sua forma livre, assim, o foco de muitas pesquisas consiste no desenvolvimento de suportes que possibilitem o reuso das enzimas em novos ciclos produtivos sem perda em sua eficiência e atividade recuperada. A atual pergunta de pesquisa que o proponente tem buscado responder trata-se de avaliar a influência de um campo magnético externo alternado nas propriedades biocatalíticas de enzimas quando as mesmas estão em suportes com propriedades magnéticas: nanopartículas magnéticas ou grafeno decorado com nanopartículas magnéticas.

Na cinética química, a teoria das colisões estabelece os 3 fatores que determinam o sucesso de uma reação baseada na velocidade das colisões suficientemente energéticas. Tais fatores consideram: a) rapidez com a qual ocorrem as colisões, dados pela densidade de colisão (Z_{A-B}), ou seja, o número colisões A-B por unidade de tempo e por unidade de volume; b) a probabilidade de uma colisão ser bem sucedida dependendo da energia cinética das moléculas e energia de ativação (E_a) e c) o requerimento estérico de orientação adequada. Em meio líquido, devido a processos difusionais que surgem pela presença das moléculas do meio reacional, as moléculas reagentes permanecem próximas durante mais tempo, conhecido como efeito “gaiola”. Desta forma, a velocidade de reações em solução será influenciada pelos fatores a) frequência dos encontros, b) duração do encontro e c) energia e orientação.

No caso de reações catalisadas por enzimas, a pergunta que norteia a pesquisa central do proponente é qual a influência de um campo magnético alternado nos processos difusionais,

duração e frequência dos encontros de reações em solução quando o biocatalizador estiver em um suporte magnético. Estudos preliminares realizados pelo grupo indicam que quanto menor a frequência do campo magnético alternado maior será o rendimento da reação. Este fato sugere que frequências de campo magnético muito alto podem alterar processos difusionais diminuindo o tempo de contato entre os reagentes, levando a consequente diminuição na atividade enzimática. Outra questão que norteia a pesquisa é entender como o aquecimento provocado pelas NP magnéticas na presença de um campo alternado (magneto hipertermia) pode alterar o rendimento dos biocatalizadores. Algumas reações enzimáticas acontecem em meio viscoso, a hipótese é que quando aquecidas, as NP magnéticas irão alterar localmente a viscosidade da mistura reacional, alterando o funcionamento das enzimas. Além da frequência, a intensidade do campo magnético deve também ser considerada. Assim, espera-se que estudos em andamento no grupo coordenado pelo proponente possam contribuir para a elucidação e modelagem de modelos que considerem a influência de parâmetros do campo magnético alternado nas propriedades catalíticas observadas.

VI. Referências

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