Ana Paula Miranda Vieira

Síntese e avaliação do efeito antibiofilme de um novo nanosistema composto por nanopartículas magnéticas de óxido de ferro, quitosana e clorexidina

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Síntese e avaliação do efeito antibiofilme de um novo nanosistema composto por nanopartículas magnéticas de óxido de ferro, quitosana e clorexidina

Dissertação apresentada à Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, como parte dos requisitos para a obtenção do título de Mestre em Ciência Odontológica – Área Saúde Bucal da Criança.

Orientador: Prof. Dr. Douglas Roberto Monteiro
Coorientador: Prof. Tit. Alberto Carlos Botazzo Delbem
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Resumo

VIEIRA, A.P.M. **Síntese e avaliação do efeito antibiofilme de um novo nanosistema composto por nanopartículas magnéticas de óxido de ferro, quitosana e clorexidina**. 2018 55f. Dissertação (Mestrado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2018.

Objetivo: Este estudo sintetizou um novo nanosistema carreador de clorexidina (CLX) baseado em nanopartículas magnéticas de óxido de ferro (NMOFs) e quitosana (QS), e avaliou seu efeito antimicrobiano sobre biofilmes de *Candida albicans* e *Streptococcus mutans*: **Metodologia:** O nanosistema NMOFs-QS-CLX foi preparado pela interação de CLX sobre NMOFs revestidas com QS, e caracterizado por difração de raios X, espectroscopia de absorção na região do infravermelho médio com transformada de Fourier, microscopia eletrônica de transmissão e dispersão dinâmica de luz. A concentração inibitória mínima (CIM) do nanosistema NMOFs-QS-CLX capaz de inibir as cepas no estado planctônico foi determinada de acordo com o método de microdiluição em caldo. Na sequência, biofilmes simples e mistos de *C. albicans* e *S. mutans* foram formados durante 24 horas em poços de placas de 96 poços na presença do nanosistema contendo CLX a 39 (NMOFs-QS-CLX39) ou 78 µg/mL (NMOFs-QS-CLX78). Ainda, biofilmes pré-formados (24 horas) foram tratados durante 24 horas com o nanosistema nas mesmas concentrações. O efeito antibiofilme foi avaliado através da contagem do número de células cultiváveis, quantificação da biomassa total e avaliação da atividade metabólica. Controles apropriados foram incluídos em todas as análises. Os dados foram analisados pelo teste de Kruskal-Wallis e por ANOVA a um critério, seguidos dos testes Student-Newman-Keuls e Holm-Sidak, respectivamente ($\alpha = 0,05$). **Resultados:** Os resultados de caracterização confirmaram a formação do nanosistema NMOFs-QS-CLX, sem alteração das propriedades cristalinas das NMOFs. Além disso, as bandas de absorção características de cada composto foram identificadas no espectro do infravermelho, e o diâmetro médio do nanosistema foi menor que 40 nm. Os resultados de CIM mostraram que o nanosistema foi ligeiramente mais efetivo do que a CLX na inibição do crescimento dos microrganismos testados. Biofilmes

formados na presença do nanosistema NMOFs-QS-CLX39 atingiram patamares quantitativos similares àqueles observados para CLX a 78 µg/mL. Ainda, para os biofilmes simples e mistos, o nanosistema NMOFs-QS-CLX78 mostrou efeitos redutores superiores ou similares àqueles encontrados para CLX a 78 µg/mL e NMOFs-QS-CLX39. **Conclusão:** O nanosistema NMOFs-QS-CLX foi efetivo tanto na inibição da formação de biofilmes como sobre biofilmes pré-formados de *C. albicans* e *S. mutans* em culturas simples ou mistas. O desenvolvimento desse nanosistema instaura inúmeras possibilidades para exploração de terapias baseadas em nanopartículas magnéticas como carreadoras de drogas usadas na área médico-odontológica.

Palavras-chave: Clorexidina, nanopartículas, sistemas de entrega de drogas, biofilmes, *Candida albicans*, *Streptococcus mutans*.

Abstract

VIEIRA, A.P.M. **Synthesis and evaluation of the antibiofilm effect of a new nanosystem composed by iron oxide magnetic nanoparticles, chitosan and chlorhexidine**. 2018 55f. Dissertação (Mestrado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2018.

Aim: This study synthesized a new chlorhexidine(CHX)-carrier nanosystem based on iron oxide magnetic nanoparticles (IONPs) and chitosan (CS), as well as evaluated its antimicrobial effect on biofilms of *Candida albicans* and *Streptococcus mutans*. **Method:** The IONPs-CS-CHX nanosystem was prepared by CHX interaction to CS-coated IONPs and characterized by X-ray powder diffraction, Fourier transform infrared spectroscopy, transmission electron microscopy and dynamic light scattering. Minimum inhibitory concentration (MIC) of the IONPs-CS-CHX nanosystem was determined according to the broth microdilution assay. After, mono- and dual-species biofilms of *C. albicans* and *S. mutans* were formed for 24 hours into wells of 96-well plates in the presence of the nanosystem containing CHX at 39 (IONPs-CS-CHX39) or 78 μg/mL (IONPs-CS-CHX78). Moreover, pre-formed biofilms (24 h) were treated for 24 h with the nanosystem at the same concentrations. The antibiofilm effect was determined by quantification of cultivable cells, total biomass and metabolic activity. Appropriate controls were included in all analyzes. Data were analyzed by Kruskal-Wallis' test and one-way ANOVA, followed by Student-Newman-Keuls and Holm-Sidak tests ($\alpha = 0.05$). **Results:** Characterization results confirmed the nanosystem formation without altering the crystalline properties of the IONPs. In addition, the characteristic absorption bands of each compound were identified in the infrared spectrum, and the mean diameter of the nanosystem was lower than 40 nm. MIC results showed that the nanosystem was slightly more effective than CLX in inhibiting the growth of the microorganisms tested. Biofilms formed in the presence of IONPs-CS-CHX39 attained similar quantitative levels to those observed for CHX at 78 μg/mL. Further, for mono- and dual-species biofilms, IONPs-CS-CHX78 showed similar or superior antibiofilm effects when compared with IONPs-CS-CHX39 and free CHX at 78 μg/mL. **Conclusion:** The IONPs-CS-CHX nanosystem was able to

reduce biofilm formation and pre-formed biofilms of *C. albicans* and *S. mutans* in single or mixed cultures. The development of this nanosystem establishes several possibilities for exploration of magnetic nanoparticle-based therapy as drug carrier used in health area.

Keywords: Chlorhexidine, nanoparticles, drug delivery systems, biofilms, *Candida albicans*, *Streptococcus mutans.*

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Sumário

.

Abstract

This study synthesized and characterized a chlorhexidine (CHX)-carrier nanosystem based on iron oxide magnetic nanoparticles (IONPs) and chitosan (CS), as well as evaluated its antimicrobial effect on mono- and dual-species biofilms of *Candida albicans* and *Streptococcus mutans*. Physico-chemical results confirmed the formation of a nanosystem with size smaller than 40 nm. Antimicrobial susceptibility testing for planktonic cells showed that the strains were slightly more susceptible to the nanosystem than to CHX alone. In general, biofilm quantification assays (cultivable cells, total biomass and metabolic activity) revealed that CHX-containing nanosystem at 78 μg ml^{-1} displayed similar or superior antibiofilm effects when compared to its counterpart at 39 μg ml^{-1} and free CHX at 78 μg ml^{-1}. These findings highlight the potential of CS-coated IONPs as CHX carrier for fighting biofilm-associated oral diseases.

Keywords: Chlorhexidine, magnetic nanoparticles, drug delivery systems, biofilms, *Candida albicans*, *Streptococcus mutans*.

Introduction

The oral cavity exhibits a complex microbiota in which microorganisms of several species may be organized in well-structured communities, adhered to different surfaces and enfolded by a matrix of self-synthesized exopolymeric material, constituting the so-called oral biofilms (Remis et al. 2010). Biofilms constitute one of the etiological factors of various diseases, such as dental caries and oral candidoses (Beloin et al. 2014). *Streptococcus mutans* is considered a cariogenic bacterial species responsible for dental caries due to its ability to tolerate and proliferate at low pH, producing lactic acid through carbohydrate metabolism, which creates a favorable environment to dental demineralization (Krzyściak et al. 2014). On the other hand, *Candida albicans* is the main species of the genus *Candida* associated with oral candidoses (Kulak et al. 1997). Usually, *Candida* species are commensal in healthy individuals, but may become pathogenic in immunosuppressed and elderly patients, as well as in complete denture wearers (Pittet et al. 1994; Kulak et al. 1997; Zollner-Schwetz et al. 2008). Furthermore, synergistic interactions between *S. mutans* and *C. albicans* may occur in oral biofilms, favoring dental caries development and higher *Candida* adhesion to acrylic surfaces (Ribeiro et al. 2012; Metwalli et al. 2013; Falsetta et al. 2014).

Chlorhexidine (CHX) is a drug widely employed against pathogenic oral biofilms, being found in several oral health care products (Scheibler et al. 2017). Although it has been regarded as a gold standard antimicrobial (Setu et al. 2011), it has a limited effect on mature biofilms (Shen et al. 2011; Bonez et al. 2013; do Vale et al. 2017) and may be toxic to different cell types (Tu et al. 2015; Abbaszadegan et al. 2016). In addition, Bhardwaj et al. (2016) revealed that CHX induced expression

of vancomycin resistant genes in *Enterococcus faecium*, which reinforces the possibility of cross-resistance between CHX and antibiotics (Saleem et al. 2016; Wand et al. 2017). All these aspects justify the search for alternative strategies to improve CHX effectiveness on oral biofilms, reducing its concentration of use.

Within this context, nanotechnology has been employed as an excellent alternative to improve the effect of antimicrobial agents by using drug delivery nanosystems. Such systems may protect the drug from a rapid degradation or release, thus increasing its bioavailability and reducing the dose required for therapeutic success (Khan et al. 2015). Among the compounds used as drug carriers, iron oxide magnetic nanoparticles (IONPs) draws attention due to their higher surface energy, good reactivity and biocompatibility (Wilczewska et al. 2012; Chatterjee et al. 2014; Li et al. 2016). Notably, IONPs surface may be coated with different compounds for improving their biocompatibility and aiding in the stabilization and functionalization of the generated nanosystems (Liakos et al. 2014; Hou et al. 2015). Chitosan (CS) is a biocompatible and biodegradable adhesive polymer (Kas 1997) widely used in the functionalization of IONPs (Assa et al. 2016; Mukherjee and De 2016; Shi et al. 2016; Nehra et al. 2017). In addition to its antimicrobial and hemostatic properties, CS is also a permeabilizer with good performance in drug delivery to the oral mucosa (Senel et al. 2000; Badwan et al. 2015).

Recent studies have demonstrated great antimicrobial potential of nanosystems based on IONPs (El Zowalaty et al. 2015; Gao et al. 2016; Tung le et al. 2016; Nehra et al. 2017). El Zowalaty et al. (2015) reported a higher antimicrobial activity of the IONPs-CS-Streptomycin nanosystem on Gram-negative than on Gram-positive bacteria, also highlighting its action against *Mycobacterium tuberculosis.* In turn, topical applications of catalytic IONPs plus hydrogen peroxide (H_2O_2) ceased

the progression of carious lesions in rodents, whereas treatment with H_2O_2 alone did not show considerable effects (Gao et al. 2016). For CHX, however, no study evaluated its antibiofilm effect when associated to CS-coated IONPs.

In view of the above, the aim of the present study was to synthesize a new CHX-carrier nanosystem based on IONPs and CS, as well as to evaluate its antimicrobial effect on *C. albicans* and *S. mutans* biofilms. The null hypothesis was that the antimicrobial effect of the IONPs-CS-CHX nanosystem would not differ from that found for CHX alone.

Materials and methods

Preparation of the IONPs-CS-CHX nanosystem

The colloidal suspension of IONPs (Fe_3O_4 - magnetite) was kindly provided by *nChemi* company, São Carlos, São Paulo, Brazil. A homogeneous CS (Sigma-Aldrich, St. Louis, MO, USA) solution was prepared according to the protocol described by Mengistu Lemma et al. (2016), with modifications. Briefly, CS was solubilized in 2% acetic acid (Sigma-Aldrich) under constant magnetic stirring, during 24 h at room temperature. For IONPs coating with CS, 10 ml of colloidal IONPs at 1400 µg ml^{-1} were added to 10 ml of CS solution at the same concentration, in a simple mixing process. Next, CHX (500 µg; Sigma-Aldrich) was directly solubilized in 700 µg ml^{-1} IONPs-CS and maintained under constant magnetic stirring (1 h) for obtaining the IONPs-CS-CHX nanosystem.

Characterization assays

X-ray powder diffraction (XRD) was used to characterize the crystalline structure of the particles sample (Souza Neto et al. 2015). A Shimadzu XRD-6000 diffractometer (Shimadzu, Tokyo, Japan) was employed in this analysis with CuKα radiation (λ = 1,54056 Å), at 30 kV and 30 mA. Measurements were performed in the range of 10° ≤ 2θ ≤ 80° with continuous scanning at 2° min^{-1}. The structural identification of the samples was performed by comparing the obtained diffractograms with standardized tables available in "Powder Diffraction Standards - Powder Diffraction File (PDF)". Fourier transform infrared spectroscopy (FTIR) was used to identify the functional groups present in the samples of IONPS, IONPs-CS and IONPS-CS-CHX. All spectra were recorded in the range of 4000-400 cm^{-1}, using the Spectrum 400 spectrometer (Perkin Elmer, Waltham, USA) (Souza Neto et al. 2015). In order to obtain information regarding morphology and average size, transmission electron microscopy (TEM) and dynamic light scattering (DLS) were used. Then, the samples were deposited on a carbon coated 200 mesh copper grid, dried at room temperature and analyzed in a transmission electron microscope (Tecnai G2 F20 HRTEM; FEI Company, Hillsboro, USA) at 200 kV. Further, DLS was performed at a fixed angle of 173° on a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, United Kingdom) equipped with 50 mW 533 nm laser and a digital auto correlator.

Antimicrobial effect of the IONPs-CS-CHX nanosystem

Artificial saliva

Artificial saliva (AS; pH 6.8) was used as culture medium for biofilm formation, according to the formulation recommended by Lamfon et al. (2003), with minor alterations. Instead of glucose, AS was supplemented with 1% sucrose. All reagents were purchased from Sigma-Aldrich.

Strains and growth conditions

Two standard reference strains from American Type Culture Collection (ATCC) were employed in the current study: *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175. Glycerol stock cultures of *C. albicans* and *S. mutans* stored at -80˚C were streaked onto Sabouraud Dextrose Agar (SDA; Difco, Le Pont de Claix, France) and Brain Hearth Infusion Agar (BHI; Difco) plates, respectively. Aerobic cultivation was used for *C. albicans*, while *S. mutans* was cultivated in 5% CO_2, both at 37°C. After 24 h, colonies of each strain were separately inoculated in Sabouraud Dextrose Broth (SDB; Difco) and BHI broth (Difco), respectively for *C. albicans* and *S. mutans*, and incubated overnight under the same conditions described above. Afterwards, the cells of both species were harvested by centrifugation (8000 rpm, 5 min), washed twice in phosphate buffered saline (PBS; pH 7, 0.1 mol l^{-1}) and resuspended in AS at 1 x 10^7 and 1 x 10^8 cells ml^{-1}, respectively for *C. albicans* and *S. mutans*, based on the protocol described by Arias et al. (2016).

Determination of the minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the IONPs-CS-CHX nanosystem against the tested strains was determined by the broth microdilution assay, as detailed earlier (Arias et al. 2016). MIC values were defined as the lowest concentration of the compound without visible microbial growth after 48 h. IONPs, CS and CHX were also evaluated as controls. For this purpose, all compounds were diluted in specific culture media to achieve the same concentrations obtained during the dilution of the IONPs-CS-CHX nanosystem. All experiments were performed on three different days, each in triplicate.

Biofilm assays

The effects of the IONPs-CS-CHX nanosystem on mono- and dual-species biofilm formation by *C. albicans* and *S. mutans*, as well as on pre-formed biofilms of these species were assessed. For mono-species biofilm formation, 200 µl of inoculum (1 x 10^7 cells ml^{-1} in AS for *C. albicans*; 1 x 10^8 cells ml^{-1} in AS for *S. mutans*) were pipetted in the wells of 96-well plates (Costar, Tewksbury, USA), while 100 µl of each microbial suspension (2 x 10^7 cells ml^{-1} for *C. albicans* + 2 x 10^8 cells ml^{-1} for *S. mutans*) were inoculated together for dual-species biofilm formation (Arias et al. 2016). All microtiter plates were incubated during 2 h at 37°C in 5% CO_2. Next, AS was removed and the wells, washed once with PBS. The nanosystem was diluted in AS to attain final CHX concentrations of 39 (IONPs-CS-CHX39) and 78 µg ml^{-1} (IONPs-CS-CHX78), corresponding to 50- and 100-fold the lowest nanosystem MIC, respectively. These compounds were then pipetted in the wells containing adhered cells, and the plates were incubated for 24 h (37°C, 5% CO_2) in order to allow biofilm formation.

Regarding the effect on pre-formed biofilms, 200 µl of inoculum in single or mixed cultures were inoculated into wells, as detailed above, and the plates were incubated for 24 h. Posteriorly, 24-h biofilms were treated with the IONPs-CS-CHX39 and IONPs-CS-CHX78 nanosystems during 24 h. For both biofilm assays, 110 µg ml^{-1} IONPs, 110 µg ml^{-1} CS and 78 µg ml^{-1} CHX were used as controls. Untreated biofilm was considered the negative control (NC).

Quantification of cultivable cells, total biomass and metabolic activity

After the treatment periods, the resulting biofilms were washed with 200 µl of PBS to remove unbound cells, resuspended in PBS and then scraped from the wells. Biofilm

suspensions were homogenized by vortexing (90 s), serially diluted in PBS and plated on specific culture media for mono- and dual-species cultures, as detailed elsewhere (Arias et al. 2016; do Vale et al. 2017). The colony-forming units (CFUs) were enumerated after 24-48 h of incubation, and a logarithmic transformation was applied on the results. For quantification of total biomass and metabolic activity, the well-established methods of crystal violet (CV) staining and XTT reduction were employed, respectively, as previously described (Fernandes et al. 2016). Biofilm quantification results were standardized according to the area of the wells (log_{10} CFU cm^{-2} and absorbance cm^{-2}).

Structural analysis of biofilms

The effects of the IONPs-CS-CHX nanosystem on biofilm formation and on pre-formed biofilms were also examined by scanning electron microscopy (SEM). Shortly, dual-species biofilms of *C. albicans* and *S. mutans* were grown in 24-well plates (Costar) containing 1 ml of standardized microbial suspension, and treated with different concentrations of the nanosystem, as detailed above. Afterwards, biofilms were processed according to a previously reported protocol (Fernandes et al. 2016), and qualitatively analyzed in an electron microscope (FEG-VP Supra 35; Carl Zeiss, Jena, Thüringen, Germany).

Statistical analysis

All biofilm experiments were performed in triplicate, on three different occasions. SigmaPlot software (version 12.0; Systat Software Inc., San Jose, USA) was used in the statistical analysis of the results obtained. For the effect on biofilm formation, the results of *C. albicans* CFU in dual-species biofilm, as well as those of metabolic

activity of mono-species biofilm of *S. mutans* did not show a normal distribution (Shapiro-Wilk's test) and were analyzed by Kruskal-Wallis' test followed by Student-Newman-Keuls' test ($\alpha = 0.05$). For all other conditions, data showed normal distribution (Shapiro-Wilk's test), and significant differences among the treatments were assessed by one-way analysis of variance (ANOVA) followed by Holm-Sidak's *post-hoc* test. P-values lower than 0.05 were reported as statistically significant.

Results

Characterization assays

The XRD patterns for IONPs, IONPs-CS, and IONPs-CS-CHX nanosystem are represented in Figure 1a. All compounds showed typical peaks of face-centered cubic system (PDF file n° 86-1362) with crystallographic planes represented by the (1 1 1), (2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1), (4 4 0), (6 2 0) and (5 3 3) indices, characteristic of magnetite (Fe_3O_4) with a spinel structure. The diffractogram observed for IONPs was similar to those found for IONPs-CS and IONPs-CS-CHX, indicating that the amount of CS and CHX used in the nanosystem synthesis did not modify the crystalline structural properties of the nanoparticles.

Figure 1 also shows the FTIR spectra for IONPs, IONPs-CS and IONPs-CS-CHX. For IONPs (Fig. 1b), absorption peaks at approximately 3440 cm^{-1} and 1635 cm^{-1} migth be attributed to H-O-H stretching and deformation vibrations, respectively, while the signal observed at 580 cm^{-1} corresponds to the Fe-O stretching vibration, which is characteristic of magnetite. Representative peaks of IONPs and CS were observed in Figure 1c, indicating that the nanoparticles were effectively coated by the polymer. The band at 3440 cm^{-1} may also be associated with N-H and O-H stretching

of CS (Fig. 1c). In the bands related to C-H stretching present in the CS pyranoside ring, peaks around 2865 cm^{-1} and 1410 cm^{-1} might represent symmetrical and asymmetrical vibrations (Fig. 1c). In addition, peaks close to 1650 cm^{-1} and 1055 cm^{-1} might be attributed to C=O (amide group of CS) and -C-O-C- (in glycosidic linkage) stretching vibrations, respectively (Fig. 1c). It was possible to note that the main absorption bands of IONPs and CS remained in the nanosystem FTIR spectrum after CHX incorporation (Fig. 1d). Further, the occurrence of signals around 3300 cm^{-1}, 1550 cm^{-1}, 1080 cm^{-1} and 835 cm^{-1} might be assigned for N-H imines, N-H amine, and both Cl- and CH- bonded to aromatic functional group, respectively, which confirm the presence of CHX in the nanosystem (Fig. 1d).

The morphology and size of the nanoparticles were observed by TEM and DLS. As can be seen in Figure 2, IONPs had a predominantly spherical shape (Fig. 2a) with an average diameter of 21 ± 7 nm (Fig. 2b). IONPs were completely covered by CS, maintaining their spherical shape (Fig. 2c), and the average diameter of the IONPs-CS compound was estimated at 29.9 ± 10.7 nm (Fig. 2d). It was also possible to observe that CHX was homogeneously distributed in the organic layer of CS on IONPs (Fig. 2e), and the diameter of the IONPs-CS-CHX nanosystem was 33.6 ± 10.7 nm (Fig. 2f).

Antimicrobial effect of the IONPs-CS-CHX nanosystem

MIC

For both species analyzed, MIC values of IONPs-CS-CHX were slightly lower than those observed for CHX applied alone (Table 1). Regardless of the evaluated compound, *S. mutans* was more susceptible than *C. albicans*. Moreover, IONPs and

CS were not able to inhibit *C. albicans* and *S. mutans* growth at the highest concentration tested (140 µg ml^{-1}).

Effect on biofilm formation

Mono- and dual-species biofilms of *C. albicans* and *S. mutans* developed in the presence of CHX, IONPs-CS-CHX39 and IONPs-CS-CHX78 displayed significantly lower number of CFUs when compared to the NC (Table 2). In addition, nanosystems showed statistically similar effects on CFUs in comparison to CHX, except for mono-species biofilm of *C. albicans*, in which the reduction promoted by IONPs-CS-CHX78 (4.27-log_{10}) was statistically higher than that observed for CHX (1.77-log_{10}) and IONPs-CS-CHX39 (1.80-log_{10}), as compared to the NC. For all strains, IONPs and CS did not significantly reduce the number of CFUs compared to the NC, except for the treatment of *S. mutans* in mono-species culture with IONPs, which promoted a significant decrease of 1.93-log_{10} ($p = 0.012$).

For total biofilm biomass, the IONPs-CS-CHX78 nanosystem promoted the highest reductions when compared to the NC, with values of 78.8 ($p < 0.001$), 63.4 ($p < 0.001$) and 68.5% ($p = 0.049$), respectively for *C. albicans*, *S. mutans* and mixed biofilms (Table 2). However, no significant differences were observed among CHX, IONPs-CS-CHX39 and IONPs-CS-CHX78. Biofilms formed in the presence of IONPs showed statistically similar biomass to that verified for the NC, whereas CS had a significant reducing effect only for mono-species biofilms.

Comparing to the NC, *C. albicans* and dual-species biofilms grown in the presence of CHX, IONPs-CS-CHX39 and IONPs-CS-CHX78 showed pronounced reductions in the metabolic activity, ranging from 93.3 to 100% ($p < 0.001$), despite the differences among the treatments were not significant (Table 2). However, the

metabolism was not significantly reduced when those biofilms were formed in the presence of IONPs and CS. For *S. mutans*, no treatment significantly reduced biofilm metabolism in comparison to the NC.

Dual-species biofilm developed in the presence of IONPs (Fig. 3b) displayed an arrangement similar to that noted for the NC (Fig. 3a), being characterized by multilayers of hyphae covering the surfaces, few yeasts and agglomerates of cocci attached to the fungal cells. Comparing to the NC, biofilms formed in the presence of CS (Fig. 3c), CHX (Fig. 3d) and IONPs-CS-CHX39 (Fig. 3e) revealed less dense structures, with lower and higher number of hyphae and yeasts, respectively. The greatest disruption in biofilm formation was seen for IONPs-CS-CHX78, which showed some yeasts and sparse cocci, both partially covering the surface (Fig. 3f).

Effect on pre-formed biofilms

Regarding the CFU quantification for *C. albicans* in mono-species biofilm, only the treatments with CHX and nanosystems promoted significant reductions when compared to the NC, and IONPs-CS-CHX78 was significantly more effective than CHX and IONPs-CS-CHX39, achieving a 3.04-$\log_{10}$ decrease (Table 3). For this fungus in dual-species biofilm, only the treatments with IONPs-CS-CHX39 and IONPs-CS-CHX78 had significant effects on the number of CFUs, reaching reductions of 1.57-$\log_{10}$ and 3.25-$\log_{10}$, respectively, as compared to the NC. It was also possible to note a significant difference between these treatments ($p = 0.004$). For *S. mutans* in mono- and dual-species biofilms, CHX, IONPs-CS-CHX39 and IONPs-CS-CHX78 were effective in reducing the CFU number, attaining decreases ranging from 3.06- to 5.85-$\log_{10}$, compared to the NC.

For mono-species biofilm of *C. albicans*, treatments with CS, CHX, IONPs-CS-CHX39 and IONPs-CS-CHX78 promoted significant reductions in the total biomass of 19.6 ($p = 0.033$), 28.5 ($p = 0.003$), 29.1 ($p = 0.003$) and 44.3% ($p < 0.001$), respectively, as compared to the NC (Table 3). Contrarily, no treatment significantly decreased *S. mutans* biofilm biomass, whereas for dual-species biofilm only the IONPs-CS-CHX78 nanosystem was able to promote a significant reduction in comparison to the NC (44%; $p = 0.008$).

Treatment with the IONPs-CS-CHX78 nanosystem was more effective in reducing *C. albicans* biofilm metabolism (94.9%; $p < 0.001$) than CHX (53%; $p = 0.001$), when compared to the NC (Table 3). Comparing to the NC, treatments with CS, CHX, IONPs-CS-CHX39 and IONPs-CS-CHX78 exhibited significant decreases in *S. mutans* biofilm metabolism, ranging from 92.6 to 98.7%, but without statistically significant differences among these treatments. For dual-species biofilms, metabolic activity was only significantly reduced after treatment with CHX (89.7%; $p < 0.001$) and IONPs-CS-CHX78 (94.4%; $p < 0.001$), as compared to the NC, and these treatments did not differ from each other.

The NC (Fig. 3g), IONPs (Fig. 3h) and CS (Fig. 3i) groups demonstrated a compact arrangement of interconnected cellular elements (yeasts, hyphae and cocci) totally covering the surfaces. After treatment with CHX (Fig. 3j) and IONPs-CS-CHX39 (Fig. 3k), slightly less dense structures were observed when compared to the NC, and the greatest disruption was seen for IONPs-CS-CHX78 (Fig. 3l). Energy dispersive spectroscopy (EDS) mapping for biofilm treated with IONPs-CS-CHX78 (Fig. 3m-3q) showed the presence of C, O and Fe atoms. In addition, it was possible to note Fe atoms (which are characteristic of IONPs) homogenously distributed in the sample (Fig. 3n).

Discussion

The growing microbial resistance to classical antibiotics has stimulated the development of studies focusing on the creation of new compounds to combat infectious diseases (Beloin et al. 2014). In this sense, the current study synthesized a CHX-carrier nanosystem and evaluated its antibiofilm effect on two important oral pathogens, aiming to potentiate the antimicrobial effect of one of the most commonly used drugs in Dentistry (Bonez et al. 2013). The results allowed to partially reject the null hypothesis, since *C. albicans* and *S. mutans* in planktonic state were slightly more susceptible to the nanosystem than to CHX alone. In addition, the IONPs-CS-CHX78 nanosystem showed superior antibiofilm effect when compared to CHX for some of the parameters analyzed.

As for nanosystem synthesis, IONPs were superficially modified by CS and, posteriorly, the IONPs-CS compound was charged with CHX. This method used in the synthesis and functionalization was effective, as confirmed by all nanosystem characterization assays (Figs. 1 and 2). IONPs coating with CS was performed in acid medium, which might favor the electrostatic interactions and/or hydrogen bonds between protonated groups of amines present in the CS and negatively charged groups on IONPs surface (Assa et al. 2016). Also, CHX may have established covalent bonds or chemical interactions with CS, given that CHX ability to bind to CS was reported in a study where CHX-loaded CS microspheres were able to promote prolonged release of the drug into oral cavity (Giunchedi et al. 2002).

MIC results showed that the IONPs-CS-CHX nanosystem was slightly more effective at inhibiting microorganisms' growth than free CHX, whereas the controls (IONPs and CS) were not able to impede *C. albicans* and *S. mutans* growth (Table

1). These findings reveal that there was no synergistic interaction among the compounds present in the nanosystem against the microorganisms tested, but a tendency of improvement of the CHX action when attached to CS-coated IONPs. Previous studies have also reported an improved antimicrobial effect of some drugs when carried by IONPs (Hussein-Al-Ali et al. 2014; Wang et al. 2017), including CHX (Tokajuk et al. 2017). CHX is a biguanide derivative with broad spectrum of action against bacteria and fungi. Its well-known mechanisms involve disturbances in permeability or attacks on membranes with consequent protein denaturation, respectively when at low or high concentrations (Karpinski and Szkaradkiewicz 2015; Tokajuk et al. 2017). However, the functionalization of IONPs with CHX showed to be able to modulate these mechanisms of action, being the induction of oxidative stress, as well as the oxidation of cellular structures, additional mechanisms proposed when CHX was associated with IONPs (Tokajuk et al. 2017).

As the maturation stage of a biofilm is determinant for its antimicrobial susceptibility, the present study evaluated the effect of the IONPs-CS-CHX nanosystem on biofilm formation and on pre-formed biofilms of *C. albicans* and *S. mutans.* In general, quantification results for mono- and dual-species biofilms revealed that IONPs-CS-CHX78 had similar or superior effects in comparison to CHX alone (Tables 2 and 3). Furthermore, biofilms formed in the presence of IONPs-CS-CHX39 attained similar quantitative levels to CHX at 78 μg ml^{-1} (Table 2). These findings are extremely relevant and indicate that CHX binding to a magnetic nanocarrier did not reduce its effectiveness against biofilms, and emphasize the possibility of using nanosystems containing lower concentrations of CHX, which might even decrease possible side effects related to this drug. Analyzing together all biofilm quantification data, it is possible to infer that the reductions in total biomass

promoted by CHX-containing compounds are related to decreases in CFU number and in production of extracellular matrix. This last phenomenon might be a reflection of the reductions in metabolic activity observed for mono- and dual-species biofilms. Additionally, quantitative results are in line with SEM observations, where it was possible to note that IONPs-CS-CHX78 promoted the most notable structural changes in biofilms (Fig. 3).

For all biofilms evaluated, IONPs alone were not able to significantly decrease the CFU number, total biomass and metabolic activity, when compared to NC (Tables 2 and 3). These results are conflicting with others described in the literature. Borcherding et al. (2014), for instance, found that IONPs induced biofilm formation by *Pseudomonas aeruginosa*. On the other hand, Chifiriuc et al. (2013) showed an inhibitory effect of IONPs on the development of mature fungal biofilms. The strain type as well as the IONPs concentration are factors that may interfere in the antimicrobial effect (Dinali et al. 2017), which could explain the divergences noted in the above-mentioned studies.

An inverse trend was observed for free CS, which exhibited significant inhibitory effect on biomass and metabolism for some mono-species biofilms, despite having no effect on the number of cells (Tables 2 and 3). Taken together, these findings suggest a reducing effect of CS on biofilm extracellular matrix. A previous study demonstrated that this polysaccharide inhibited *C. albicans* and *S. mutans* growth at concentrations of 1.5 and 0.5 mg ml^{-1} (Costa et al. 2014), respectively, which are higher than those used in the nanosystem synthesis of the current study (110 µg ml^{-1}). Thus, CS may have contributed to the antibiofilm action of the nanosystem by its effect on the extracellular matrix, altering the CHX release profile or facilitating drug penetration into deeper layers of the biofilm. EDS mapping for

dual-species biofilm treated with IONPs-CS-CHX78 (Fig. 3) reinforces this last hypothesis, considering that the presence of dispersed and homogeneously distributed Fe atoms in the sample may be an indicative of the nanosystem capacity to penetrate in the biofilm layers.

From a clinical point of view, the results of the present study suggest that the IONPs-CS-CHX nanosystem may be used in order to fight biofilm-associated oral diseases as preventive or therapeutic agent. Furthermore, this nanotechnology-based therapy could act as an alternative to mouth-rinse formulations containing CHX and ethanol in their composition, considering possible harmful effects of this alcohol (Tokajuk et al. 2017). However, as this is the first report on antibiofilm effect of the IONPs-CS-CHX nanosystem, future studies should assess its interaction with other pathogens, bearing in mind the polymicrobial nature of the oral cavity and possible implications of its *in vivo* use. Another important aspect to be explored is the magnetic property of IONPs, which may allow the delivery to a target location, as well as the CHX release profile from the nanosystem and its cytotoxic effects. All these relevant topics will contribute to define treatment protocols closer to clinical reality.

In conclusion, the IONPs-CS-CHX nanosystem was able to reduce biofilm formation and pre-formed biofilms of *C. albicans* and *S. mutans* in single or mixed cultures. Moreover, IONPs-CS-CHX78 showed similar or superior antibiofilm effects when compared with IONPs-CS-CHX39 and free CHX. The development of this nanosystem establishes several possibilities for exploration of magnetic nanoparticle-based therapies as drug carrier used in Dentistry.

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Declaration of interest statement

No conflict of interest declared.

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Table and figure captions

Table 1. Minimum inhibitory concentration (MIC) values for iron oxide magnetic nanoparticles (IONPs), chitosan (CS), chlorhexidine (CHX), and IONPs-CS-CHX nanosystem against the tested strains.

Table 2. Mean (SD) values for the quantifications assays of mono- and dual-species biofilms of *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 grown in the presence of 110 μg ml^{-1} iron oxide magnetic nanoparticles (IONPs), 110 μg ml^{-1} chitosan (CS), 78 μg ml^{-1} chlorhexidine (CHX), and nanosystem containing CHX at 39 (IONPs-CS-CHX39) and 78 μg ml^{-1} (IONPs-CS-CHX78).

Table 3. Mean (SD) values for the quantifications assays of mono- and dual-species pre-formed biofilms of *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 treated with 110 μg ml^{-1} iron oxide magnetic nanoparticles (IONPs), 110 μg ml^{-1} chitosan (CS), 78 μg ml^{-1} chlorhexidine (CHX), and nanosystems containing CHX at 39 (IONPs-CS-CHX39) and 78 μg ml^{-1} (IONPs-CS-CHX78).

Figure 1. X-ray diffraction patterns (a) and FTIR spectra (b, c and d) for IONPs, IONPs-CS, and IONPs-CS-CHX nanosystem. FTIR, Fourier transform infrared spectroscopy; IONPs, iron oxide magnetic nanoparticles; CS, chitosan; CHX, chlorhexidine.

Figure 2. TEM images (a, c and e) and histograms of particle size distribution obtained by DLS (b, d and f) for IONPs (a and b), IONPs-CS (c and d), and IONPs-

CS-CHX nanosystem (e and f). TEM, transmission electron microscopy; DLS, dynamic light scattering; IONPs, iron oxide magnetic nanoparticles; CS, chitosan; CHX, chlorhexidine.

Figure 3. SEM images of dual-species biofilms (*C. albicans and S. mutans*) formed in the presence of different compounds for 24 h (a-f), and pre-formed biofilms treated with the same compounds during 24 h (g-l). SEM, scanning electron microscopy; NC, negative control; IONPs, iron oxide magnetic nanoparticles; CS, chitosan; CHX, chlorhexidine. Magnification: 2500x. Bars, 10 µm. Energy dispersive spectroscopy mapping for biofilm treated with IONPs-CS-CHX78 (m-q) showing the presence of Fe, O and C atoms. Magnification: 1000x. Bars, 20 µm.

Table 1. Minimum inhibitory concentration (MIC) values for iron oxide magnetic nanoparticles (IONPs), chitosan (CS), chlorhexidine (CHX), and IONPs-CS-CHX nanosystem against the tested strains.

Species	**IONPs** MIC (μg ml^{-1})	**CS** MIC (μg ml^{-1})	**CHX** MIC (μg ml^{-1})	**IONPs-CS-CHX** MIC (μg ml^{-1})
S. mutans	> 140	> 140	0.78 – 1.56	0.78
C. albicans	> 140	> 140	6.25 – 12.5	3.125 – 6.25

Table 2. Mean (SD) values for the quantifications assays of mono- and dual-species biofilms of *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 grown in the presence of 110 μg ml^{-1} iron oxide magnetic nanoparticles (IONPs), 110 μg ml^{-1} chitosan (CS), 78 μg ml^{-1} chlorhexidine (CHX), and nanosystem containing CHX at 39 (IONPs-CS-CHX39) and 78 μg ml^{-1} (IONPs-CS-CHX78).

	NC	IONPs	CS	CHX	IONPs-CS-CHX39	IONPs-CS-CHX78
Number of cultivable cells - Log_{10} CFU cm^{-2} (SD)						
Mono-species biofilms						
C. albicans	6.09 (0.33)^{a}	5.97 (0.34)^{a}	5.99 (0.04)^{a}	4.32 (0.02)^{b}	4.29 (0.12)^{b}	1.82 (0.15)^{c}
S. mutans	5.17 (0.45)^{a}	3.24 (0.39)^{b}	5.02 (0.29)^{a}	0.33 (0.58)^{c}	1.14 (0.86)^{c}	1.17 (0.81)^{c}
Dual-species biofilms						
C. albicans	6.43 (0.07)^{a}	6.38 (0.13)^{a}	5.15 (0.15)^{ab}	3.74 (0.38)^{bc}	3.80 (0.41)^{bc}	0.52 (0.46)^{c}
S. mutans	4.59 (0.62)^{a}	3.88 (0.80)^{a}	4.56 (0.88)^{a}	0.56 (0.09)^{b}	1.17 (0.19)^{b}	1.16 (0.24)^{b}
Total biomass – Absorbance (570 nm) cm^{-2} (SD)						
Biofilms						
C. albicans	1.61 (0.09)^{a}	1.62 (0.08)^{a}	0.65 (0.16)^{b}	0.47 (0.06)^{bc}	0.44 (0.06)^{bc}	0.34 (0.12)^{c}
S. mutans	0.82 (0.12)^{a}	0.81 (0.04)^{a}	0.41 (0.07)^{b}	0.46 (0.02)^{b}	0.39 (0.05)^{b}	0.30 (0.12)^{b}
Dual-species	1.24 (0.37)^{a}	1.25 (0.30)^{a}	0.81 (0.45)^{ab}	0.57 (0.15)^{ab}	0.58 (0.19)^{ab}	0.39 (0.14)^{b}
Metabolic activity - Absorbance (490 nm) cm^{-2} (SD)						
Biofilms						
C. albicans	1.20 (0.15)^{ab}	1.33 (0.09)^{a}	0.98 (0.18)^{b}	0.04 (0.03)^{c}	0.08 (0.03)^{c}	0.01 (0.01)^{c}
S. mutans	0.18 (0.07)^{a}	0.14 (0.04)^{a}	0.02 (0.03)^{a}	0.01 (0.01)^{a}	0.01 (0.02)^{a}	0.01 (0.01)^{a}
Dual-species	1.08 (0.16)^{a}	1.28 (0.13)^{a}	0.93 (0.31)^{a}	0.04 (0.04)^{b}	0.03 (0.01)^{b}	0.00 (0.00)^{b}

Note: Within each quantification assay and type of biofilm, different lowercase letters denote significant differences among the treatments (1-way ANOVA or Kruskal-Wallis' test followed by Holm-Sidak's or Student-Newman-Keuls' tests, respectively; $p < 0.05$). NC: negative control (untreated biofilms). SD = standard deviation.

Table 3. Mean (SD) values for the quantifications assays of mono- and dual-species pre-formed biofilms of *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 treated with 110 μg ml^{-1} iron oxide magnetic nanoparticles (IONPs), 110 μg ml^{-1} chitosan (CS), 78 μg ml^{-1} chlorhexidine (CHX), and nanosystems containing CHX at 39 (IONPs-CS-CHX39) and 78 μg ml^{-1} (IONPs-CS-CHX78).

	NC	IONPs	CS	CHX	IONPs-CS-CHX39	IONPs-CS-CHX78
Number of cultivable cells - Log_{10} CFU cm^{-2} (SD)						
Mono-species biofilms						
C. albicans	6.42 (0.11)^{a}	6.33 (0.09)^{a}	6.39 (0.12)^{a}	4.67 (0.19)^{b}	5.86 (0.19)^{c}	3.38 (0.03)^{d}
S. mutans	6.02 (0.24)^{a}	5.57 (0.25)^{a}	4.46 (0.41)^{b}	0.17 (0.15)^{c}	0.19 (0.33)^{c}	0.19 (0.33)^{c}
Dual-species biofilms						
C. albicans	6.49 (0.17)^{a}	6.40 (0.16)^{a}	5.78 (0.05)^{ab}	5.66 (0.35)^{ab}	4.92 (0.71)^{b}	3.24 (0.63)^{c}
S. mutans	6.11 (0.34)^{a}	6.01 (0.21)^{a}	5.95 (0.46)^{a}	1.04 (0.47)^{b}	3.05 (0.19)^{c}	1.01 (0.52)^{b}
Total biomass – Absorbance (570 nm) cm^{-2} (SD)						
Biofilms						
C. albicans	1.58 (0.04)^{a}	1.55 (0.13)^{ab}	1.27 (0.12)^{bc}	1.13 (0.14)^{cd}	1.12 (0.06)^{cd}	0.88 (0.12)^{d}
S. mutans	0.59 (0.11)^{a}	0.66 (0.18)^{a}	0.52 (0.17)^{a}	0.58 (0.11)^{a}	0.49 (0.09)^{a}	0.42 (0.12)^{a}
Dual-species	1.50 (0.41)^{a}	1.49 (0.43)^{a}	1.22 (0.32)^{ab}	1.08 (0.15)^{ab}	1.10 (0.23)^{ab}	0.84 (0.15)^{b}
Metabolic activity - Absorbance (490 nm) cm^{-2} (SD)						
Biofilms						
C. albicans	0.98 (0.04)^{a}	1.09 (0.09)^{a}	0.98 (0.18)^{a}	0.46 (0.08)^{b}	1.00 (0.17)^{a}	0.05 (0.04)^{c}
S. mutans	0.81 (0.36)^{a}	0.63 (0.46)^{a}	0.03 (0.02)^{b}	0.06 (0.05)^{b}	0.02 (0.01)^{b}	0.01 (0.01)^{b}
Dual-species	1.07 (0.06)^{a}	1.06 (0.26)^{a}	0.94 (0.27)^{a}	0.11 (0.03)^{b}	1.12 (0.10)^{a}	0.06 (0.02)^{b}

Note: Within each quantification assay and type of biofilm, different lowercase letters denote significant differences among the treatments (1-way ANOVA followed by Holm-Sidak's test, $p < 0.05$). NC: negative control (untreated biofilms). SD = standard deviation

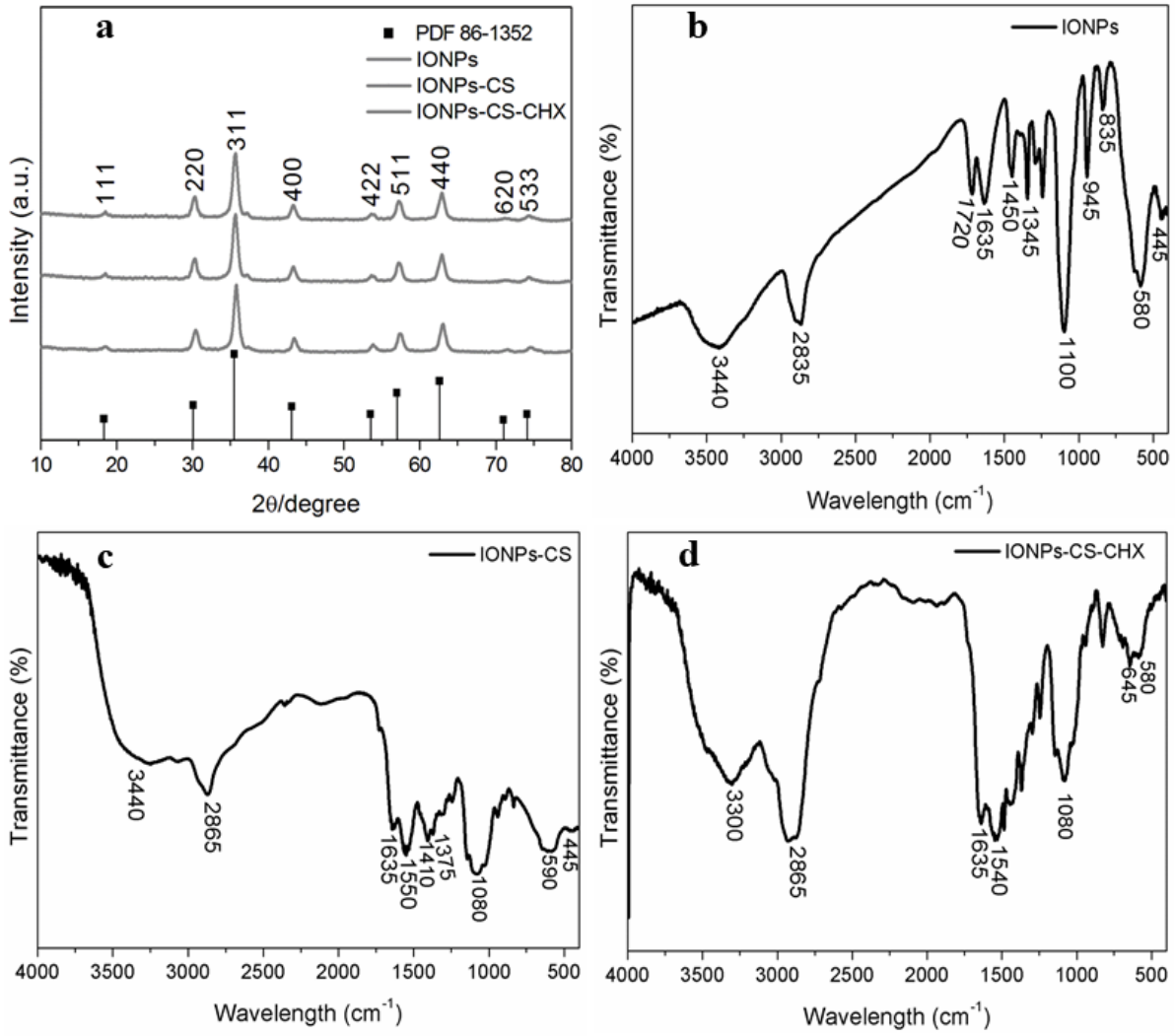

Figure 1. X-ray diffraction patterns (a) and FTIR spectra (b, c and d) for IONPs, IONPs-CS, and IONPs-CS-CHX nanosystem. FTIR, Fourier transform infrared spectroscopy; IONPs, iron oxide magnetic nanoparticles; CS, chitosan; CHX, chlorhexidine.

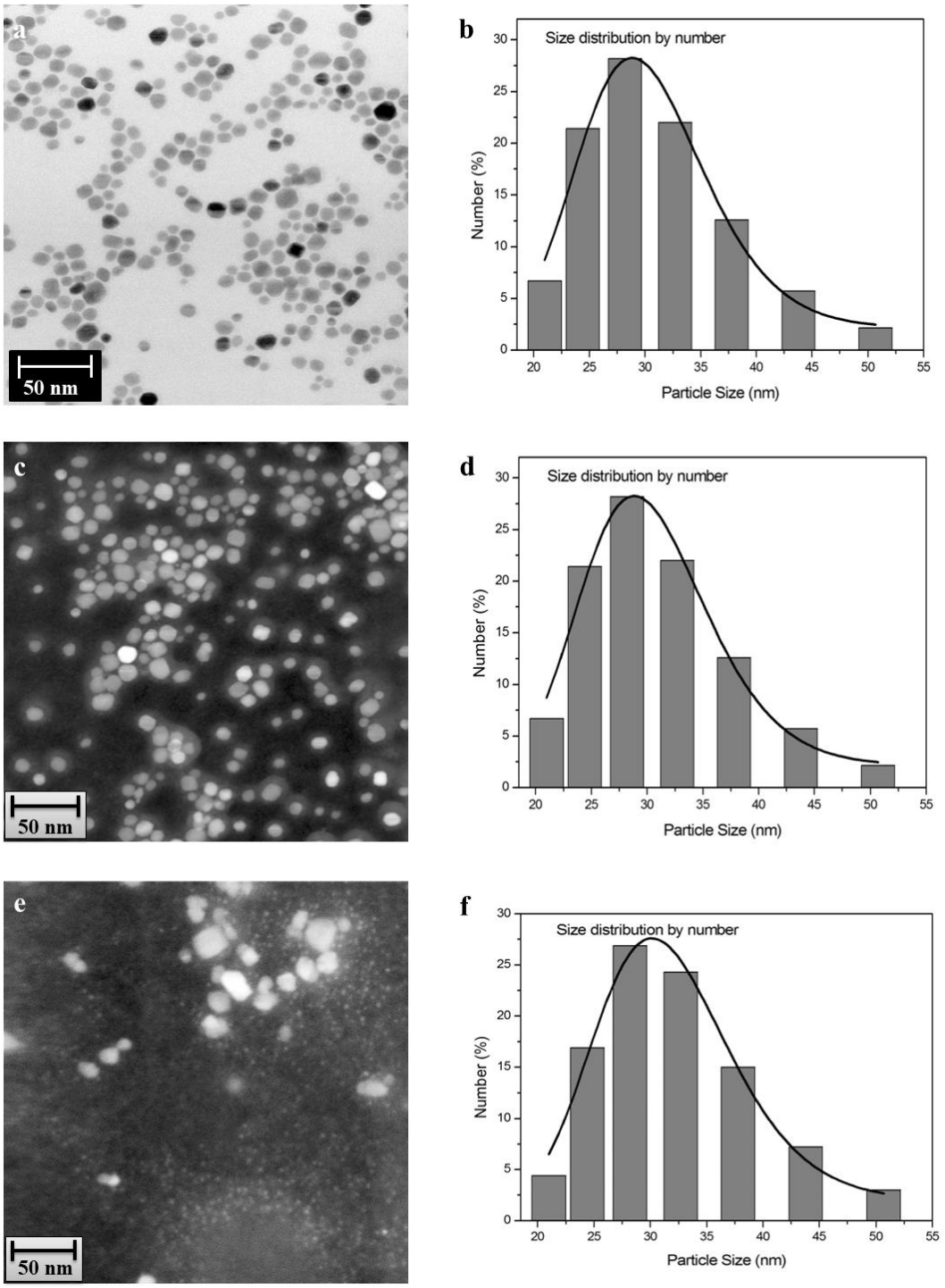

Figure 2. TEM images (a, c and e) and histograms of particle size distribution obtained by DLS (b, d and f) for IONPs (a and b), IONPs-CS (c and d), and IONPs-CS-CHX nanosystem (e and f). TEM, transmission electron microscopy; DLS, dynamic light scattering; IONPs, iron oxide magnetic nanoparticles; CS, chitosan; CHX, chlorhexidine.

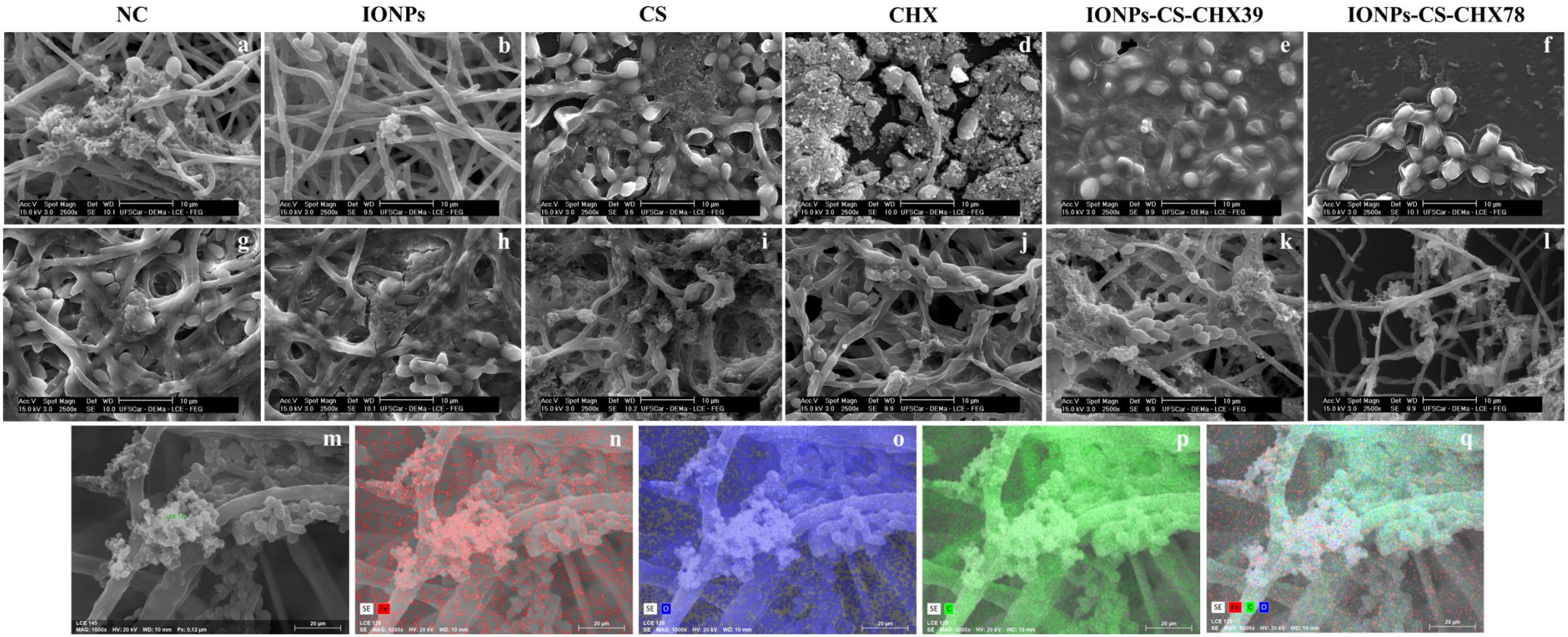

Figure 3. SEM images of dual-species biofilms (*C. albicans and S. mutans*) formed in the presence of different compounds for 24 h (a-f), and pre-formed biofilms treated with the same compounds during 24 h (g-l). SEM, scanning electron microscopy; NC, negative control; IONPs, iron oxide magnetic nanoparticles; CS, chitosan; CHX, chlorhexidine. Magnification: 2500x. Bars, 10 µm. Energy dispersive spectroscopy mapping for biofilm treated with IONPs-CS-CHX78 (m-q) showing the presence of Fe, O and C atoms. Magnification: 1000x. Bars, 20 µm.

Anexo A

Instruções aos autores: Biofouling

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