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The application of graphene for *in vitro* and *in vivo* electrochemical biosensing

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ABSTRACT

Advances in analysis are required for rapid and reliable clinical diagnosis. Graphene is a 2D material that has been extensively used in the development of devices for the medical proposes due to properties such as an elevated surface area and excellent electrical conductivity. On the other hand, architectures have been designed with the incorporation of different biological recognition elements such as antibodies/antigens and DNA probes for the proposition of immunosensors and genosensors. This field presents a great progress in the last few years, which have opened up a wide range of applications. Here, we highlight a rather comprehensive overview of the interesting properties of graphene for *in vitro*, *in vivo*, and point-of-care electrochemical biosensing. In the course of the paper, we first introduce graphene, electroanalytical methods (potentiometry, voltammetry, amperometry and electrochemical impedance spectroscopy) followed by an overview of the prospects and possible applications of this material in electrochemical biosensors. In this context, we discuss some relevant trends including the monitoring of multiple biomarkers for cancer diagnostic, implantable devices for *in vivo* sensing and, development of point-of-care devices to real-time diagnostics.

Contents

1. Introduction

Carbon nanomaterials have been a promise in electronic and optoelectronic mostly due to their high electron mobility, elevated surface area and excellent electrical and thermal conductivities

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(Janegitz et al., 2014a; Pingarrón and Villalonga, 2015; Saxena and Das 2016; Zhu et al., 2015). Carbon nanotubes, fullerenes and graphene are the most notorious materials for the biosensors field and, among these, graphene is the most remarkable one (Boujakhrout et al., 2015; Cincotto et al., 2015; Gao and Duan, 2015; Janegitz et al., 2014b; Kuila et al., 2011). First isolated by Andre Geim and Kostya Novoselov in 2004 at University of Manchester, graphene is a 2D single atomic carbon layer (Novoselov et al., 2004). Its atomic thickness combined with the sp^2 hybridization provides unique mechanical, electrical and thermal properties to this nanomaterial. Compared to carbon nanotubes, graphene surface area is 2-fold larger (2630 $cm^2 g^{-1}$) and over 500-fold larger than graphite's ($\sim$10 $cm^2 g^{-1}$) (Pumera, 2009). Arranged in a honeycomb pattern, graphene strength is exceptional and, as a 2D material, it shows a great flexibility since it can be deformed in an extra degree of freedom if compared to 3D materials. Such flexibility is favorable for wearable devices development (Matzeu et al., 2015; Wang et al., 2014). With these outstanding properties, not only wearable sensors and biosensors can be manufactured applying graphene but also energy storage devices (Bae et al., 2011; Gwon et al., 2011).

Graphene also exhibits zero-energy band gap with linear energy dispersion, which allows electrons to travel faster than in materials with other energy dispersions pattern or a non-zero energy gap (Wang et al., 2010). Besides, regarding its application in sensors and biosensors, high electron motility is by far the most outstanding property that, even at room temperature, exceeds 15,000 cm $V^{-1} s^{-1}$ (Su et al., 2015). Another interesting property of graphene for biosensors, especially in the case of graphene oxide (GO), is the capability for quenching fluorescence, which has been widely applied for fluorescent-label sensor (Gao et al., 2014; Liu et al., 2012d). In particular, fluorescence resonance energy transfer (FRET)-based sensors have employed GO to quench fluorescence in the fluorescent label, e.g., quantum dots (Dong et al., 2010), fluorescein (Chang et al., 2010) and up converting phosphors (Wang et al., 2011).

Biosensors are analytical devices known for their selectivity, simple preparation, relatively low cost and versatility. Furthermore, their easy miniaturization, mainly in the case of electrochemical devices, is an unquestionable attractive. Concisely, in a biosensor, the transducer receives and converts signal from the reaction of an analyte with a biological recognition element into an electrical measurable signal. Biological elements can be enzymes (Gamella et al., 2006; Sanz et al., 2005; Vicentini et al., 2013), antibodies/antigens (Figueiredo et al., 2015; Ojeda et al., 2012; Serafin et al., 2011), plants (Li et al., 2002; Liawruangrath et al., 2001; Oungpipat et al., 1995; Vieira and Fatibello-Filho, 1998) and animal-tissues (Mascini et al., 1982; Wu et al., 2005), or microorganisms (Holden et al., 1999; Tecon and van der Meer, 2008), genetically modified or wild type, capable of detecting or response to a specific molecule, a group of molecules or a surrounding condition. Employing graphene in the development of these devices has resulted in high-performance sensors for a great variety of analytes from clinical to environmental analysis as further described in this review.

In electrochemical biosensors, the biological material can be directly immobilized on an electrode by adsorption (Baby et al., 2010; Qin et al., 2012), covalent attachment (Song et al., 2011b), or encapsulation within a coating layer of a permeable conductive polymer or cross-linking reagent (Janegitz et al., 2015; Liu et al., 2012b; Unnikrishnan et al., 2013; Xu et al., 2010). These several strategies lead to different biosensor architectures from monolayer to multilayers (Li et al., 2013; Qin et al., 2012). In addition, numerous approaches for graphene functionalization have been accomplished to obtain more suitable properties for each application, becoming, for example, graphene not only easier to handle but also a more appropriate environment for biomolecules attachment (Kuila et al., 2011; Pumera, 2011).

In this review, we address the use of graphene in biosensing. We have combined well-established procedures to obtain and functionalize graphene-family nanomaterials with methods to immobilize biological recognition elements on these materials to electrochemical monitoring of biomolecules under *in vitro* and *in vivo* conditions. Moreover, we highlight the use of this nanomaterial in point of care diagnosis.

2. Electroanalytical methods

Modern analytical chemistry has as basic premises the development of simple and low cost instrumentations for the monitoring of analytes in different samples, miniaturization of the analytical devices and, thus, the reduction of the consume of chemical reagents and the waste generation. In recent review papers, the concept of Green Analytical Chemistry is well defined, and the previous premises and a number of other pertinent recommendations are presented in order to become the analytical routines more environmentally friend and safety (Armenta et al., 2008; Gałuszka et al., 2013; Tobiszewski et al., 2010). In this scenario, electroanalytical techniques appear as important analytical tools. These methods have a number of interesting features, as lower cost of instrumentation and maintenance, potentiality for miniaturization, operational simplicity and small reagent and solvent consumption (Shao et al., 2010; Yadav et al., 2013). The main electroanalytical techniques explored for sensing and biosensing purposes are the potentiometry, cyclic voltammetry (CV), differential pulse voltammetry (DPV), square-wave voltammetry (SWV), amperometry and, electrochemical impedance spectroscopy (EIS).

Potentiometry is a classic electrochemical method employed in a wide range of electroanalytical applications. The potentiometric measurements are performed using an experimental setup constituted by a reference electrode and an indicator electrode, both connected to a potentiometer. In the potentiometric method, the substances are analysed when the electrochemical equilibrium is reached at an appropriate indicator electrode at zero current (Bagotsky, 2005). The potential difference established between the electrodes is dependent of the activity of the target analyte in according with the Nernst equation (Bagotsky, 2005). Portable and/or disposable potentiometric (bio)sensors are reported in literature to provide different determinations at a versatile and simple manner. Zuliani et al., (2014) reported the preparation of a portable potentiometric strip sensor based on a planar screen printed substrate for direct pH measurement in saliva samples. pH monitoring in saliva is an important indicator of oral healthy, Gastroesophageal Reflux Disease (GERD), among others. In another example, Jaworska et al., (2013) studied the performance of a disposable potentiometric sensors designed by using graphene or multi-walled carbon nanotubes. The proposed sensor showed enhanced analytical performance toward Na^+ ions detection (lower limit of detection and better selectivity) comparatively to previously reported devices. In a recently published work, Tarasov et al., (2016) proposed a disposable potentiometric biosensor for direct serological diagnosis. In this study, the viral pathogen Bovine Herpes Virus-1 (BHV-1) was determined.

CV is widely used for investigation of the redox behaviour of electroactive species as well as for the characterization of novel electrodic surfaces. This technique is supported in the following experimental base: the potential is ramped linearly to forward and backward with the time. The potential scan direction can be positive (anodic scan) or negative (cathodic scan). In analytical terms, CV technique found little application because the measurements

are affected by the capacitive current, and the faradaic current of interest is undetectable at low concentration levels of the target analyte. To solve this drawback, pulsed voltammetric techniques were developed. The most applied pulsed voltammetric techniques for analytical purposes are DPV and SWV. Potential applications in these techniques are performed in order to minimize the capacitive current in the measurements (Brett and Brett, 1993; Mirceski et al., 2013, 2007). Amperometry is based on the application of a fixed potential by a defined interval time under hydrodynamic conditions, being the analytical signal the anodic or cathodic current resulting from an interfacial redox reaction proportional to the analyte concentration. Pulsed voltammetric techniques and amperometry are frequently the most selected electroanalytical techniques for biosensing. Moreover, these techniques have been chosen successfully as electrochemical transduction for those procedures dedicated to the use of disposable (bio)sensors and portable electrochemical instruments. A portable potentiostat/galvanostat equipped with on-line data transmission and a global positioning system (GPS) was developed and applied for *in situ* determination of metals in water samples by Santos et al. (2015b). Square-wave anodic stripping voltammetry (SWASV) was explored to perform the simultaneous determination of Pb^{2+} and Cd^{2+}. A portable amperometric potentiostat was designed by Huang et al. (2007) to provide the specific bilirubin biosensing. Song et al. (2011a) proposed a disposable amperometric biosensor for catechol monitoring. The biosensor architecture was based on screen-printed electrodes (SPE) modified with a conjugate of graphene oxide and tyrosinase assembled gold nanoparticles. Simultaneous biosensing of tumor markers using disposable voltammetric biosensors was a challenge investigated by Lai et al. (2011). Carcinoembryonic antigen (CEA) and α-fetoprotein (AFP) were quantified as model target analytes using a multiplexed electrochemical immunoassay designed from the combination of glucose oxidase-functionalized silica nanosphere tags and a disposable immunosensor array. Other examples of electroanalytical applications for the voltammetric and amperometric techniques using portable devices and disposable sensors can be found in the literature. Recent advances in the use of these techniques for graphene-based biosensors dedicated to *in vivo*, *in vitro* and point-of-care diagnosis are discussed in the next sections.

EIS technique is explored to obtain high precise and quality information about electrochemical features of interfaces. Some traditional research fields, which EIS is widely used, are the development of batteries, fuel cells, and corrosion. Basically, EIS is based on the application of an AC voltage on the system and measure the current response from this perturbation. From the results, the interfacial region can be modeled as an equivalent circuit constructed from circuit elements such as resistors and capacitors. The quantification of these parameters is rigorous and sensitive to interfacial changes. Considering this aspect, EIS has been investigated for analytical purposes. Very low variations of analyte concentration or the occurrence of chemical/biological events with the molecules at the electrode interface can modify the electrical properties of the interface. This approach is a novel and promising trend in the development of highly sensitivity biosensors (Lisdat and Schäfer, 2008; Randviir and Banks, 2013; Xu and Davis, 2014). Loaiza et al. (2011) proposed a disposable impedimetric sensor for determination of concanavalin A (Con A). The determination was based on the impedimetric monitoring of the binding event between Con A and thiolated carbohydrate derivatives immobilized on a screen-printed carbon electrode (SPCE) modified with gold nanoparticles. The binding event caused an increase in the charge-transfer resistance (R_{ct}) of the ferri/ferrocyanide redox probe and, this R_{ct} change was used as analytical signal. Loo et al. (2012) developed a label-free and highly sensitive impedimetric aptasensor for thrombin. The disposable biosensor was outlined using SPEs modified with GO and a specific thrombin DNA aptamer. Again, the detection was based on the R_{ct} changes for a selected redox probe after the binding event between thrombin and the aptamer.

As we will see in the next sections, all the briefly revised electroanalytical techniques have been applied in the development of graphene-based biosensors to conduct determinations under *in vitro* and *in vivo* environment, and develop point-of-care electrochemical devices.

3. Biosensors based on graphene applied to *in vitro* analysis

As commented in the previous section, graphene offers unique properties as a 2D nanocarbon platform for immobilizing biological recognition species. Recent relevant reports dedicated to development of biosensors based on graphene with application to *in vitro* analysis are discussed in details in this section. From this revision, it will be possible to observe the main bioanalytical problems that have been accessed by using graphene-based biosensors, the experimental details and challenges encountered related to the *in vitro* analysis. Performing a research in the Web of Science database, it was noted that the first works were published only from the beginning of this decade, with a growing number of publications over the years. This aspect revealed that the

Table 1
Electrochemical biosensors based on graphene applied to *in vitro* and *in vivo* analysis and point-of-care devices.

Analyte	Electrode	Linear range (μmol L^{-1})	Limit of detection (μmol L^{-1})	Reference
Glucose	GOD-GO/ME	10–1000	–	Hasan et al., 2015
NADH	Gr-DNAtetrahedron-AuNPs/Au	1.0×10^{-9}–1.0×10^{-4}	1.0×10^{-9}	Li et al., 2015b
Hydrogen peroxide	HRP-AuNPs-PDDA-GO-Chit/GCE	0.0198–1.04	0.00795	Yu et al., 2015
MicroRNA-21	InP-Gr/PGE	–	3.1×10^{-6}	Kilic et al., 2015
MicroRNA-21	MB-LNA-DenAu-Gr/GCE	1.0×10^{-7}–7.0×10^{-5}	6.0×10^{-8}	Yin et al., 2012
Nitric oxide	RGD-peptide-Gr	–	0.025	Guo et al., 2012
Glucose	(IL-RGO/S-RGO)n-GOD-Nafion/GCE	10–500	3.33	Gu et al., 2012
Glucose	Gr/ZnO@Pd/GOD/Nafion/GCE	20–500	2.39	Gu et al., 2014
L-lactate	Gr/ZnO@Pd/LOD/Nafion/GCE	20–500	2.52	Gu et al., 2014
Glucose	Gr-PANI-AuNPs-GOD/SPCE	200.0–11,200	100	Kong et al., 2014
S3-TH/D1-NPG	AuNPs-Gr/SPWPE	8.0×10^{-8}–5.0×10^{-4}	2.0×10^{-10}	Lu et al., 2012

GOD: glucose oxidase; GO: graphene-oxide; ME: microelectrode; AuNPs: gold nanoparticles; GCE: glassy carbon electrode; Gr: graphene; Au: gold electrode; HRP: horseradish peroxidase; PDDA: poly (diallyldimethylammonium chloride); Chit: chitosan; InP: inosine-substituted probe; MB: integrated hairpin molecule beacon; LNA: sulfydryl functionalized locked nucleic acid; DenAu: dendritic gold nanostructure; PGE: pencil graphite electrode; S-RGO: sulfonic acid (SO_3^-) functionalized graphene; IL-RGO, amineterminated ionic liquid functionalized graphene; LOD: L-lactate oxidase; PANI: polyaniline; SPCE: screen-printed carbon electrode; S3: ssDNA; TH: thionine; D1: dsDNA, NPG: nanoporous gold; SPWPE: screen-printed working paper electrode.

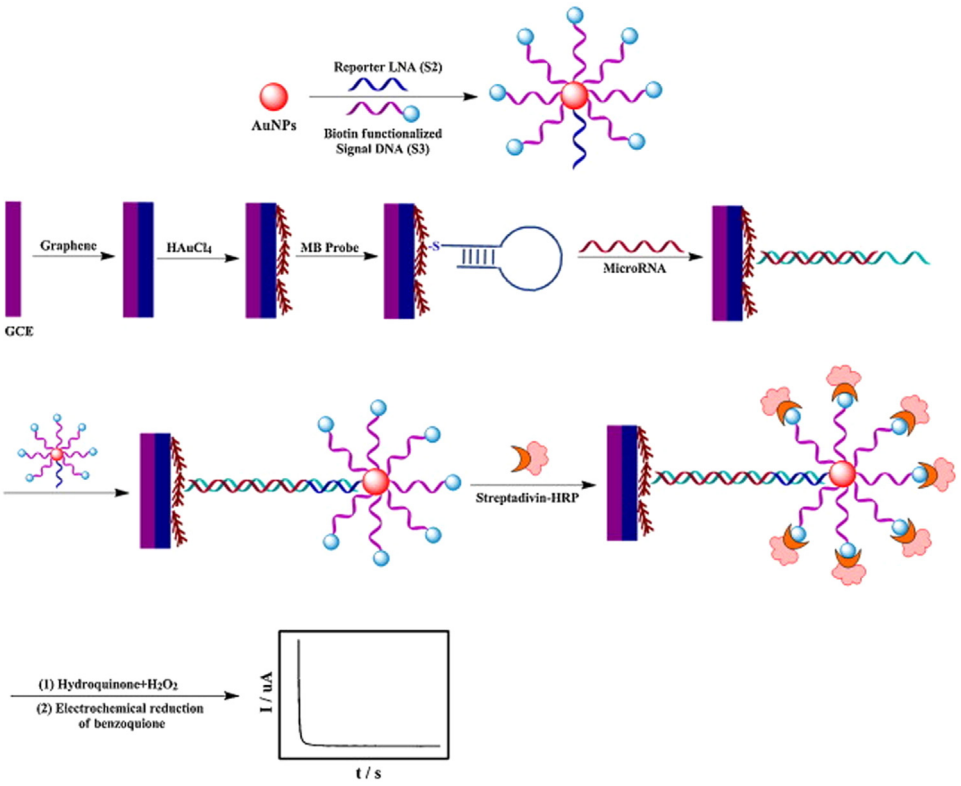

Fig. 1. Chronoamperometry determination of microRNA hybridization through three steps of amplification. Reprinted with permission from (Yin et al., 2012), Copyright Elsevier.

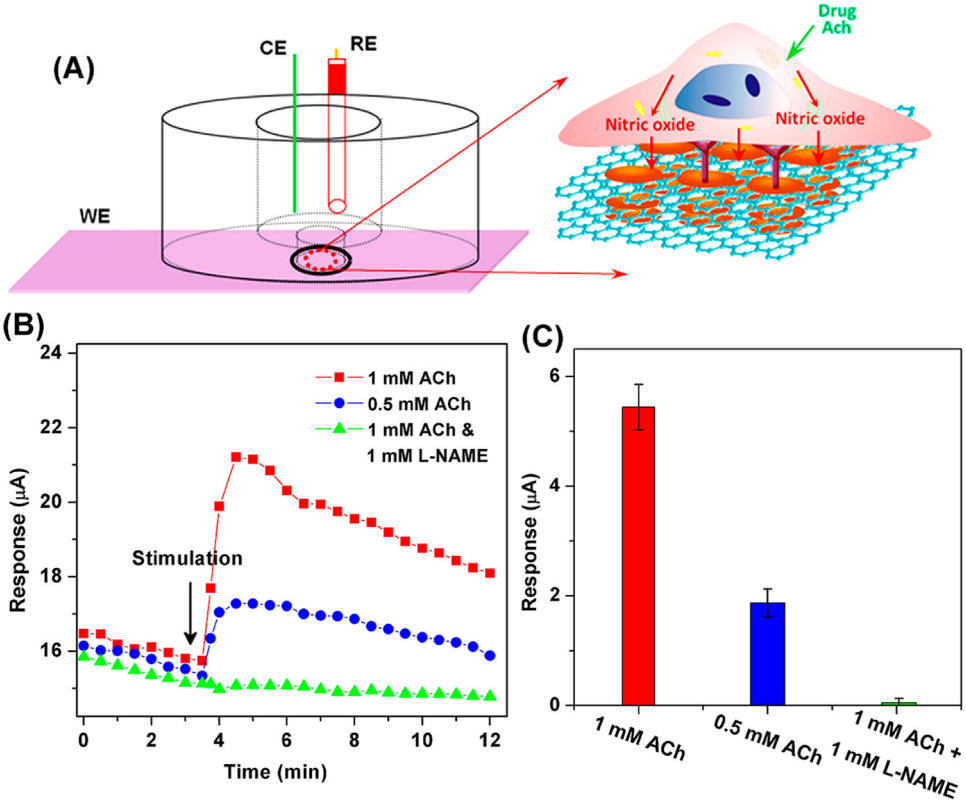

Fig. 2. **(a)** Scheme showing the setup for live-cell assay. **(b)** Real time monitoring of nitric oxide molecule released from the attached cells on RGD-peptide covalently bonded graphene biofilm in cell culture medium. The drug was added at the time indicated by the arrow. **(c)** Current responses of the cultured cells on RGD-peptide covalently bonded graphene biofilm toward different drugs. Acetylcholine (Ach) is a model drug to stimulate cell nitric oxide release and NG-nitro-L-arginine methyl ester (L-NAME) is a specific nitric oxide inhibitor. Reprinted with permission from (Guo et al., 2012), Copyright American Chemical Society.

application of graphene to development of electrochemical biosensors for *in vitro* analysis is a recent research field, and it is in full development. Table 1 comprises a list of reported works related to graphene-based biosensors applied to *in vitro* and *in vivo* analysis and, point-of-care devices. Different architectures have been designed with the incorporation of different biological recognition elements, as antibodies/antigens, enzymes and DNA probes for the proposition of immunosensors, enzymatic biosensors and genosensors.

An electrochemical biosensor based on graphene for determination of microRNA-21 (miRNA-21) was proposed by Yin et al. (2012). The abnormal expression of miRNA-21 is associated with the development of different solid tumors (at least 11 types) and, therefore, miRNA-21 can be used as an important biomarker for clinical diagnosis of cancer. A schematic representation of the proposed assay is displayed in Fig. 1. Firstly, a glassy carbon electrode (GCE) was modified with graphene nanosheets and dendritic gold nanostructure (DenAu), followed by the immobilization of sulfhydryl functionalized locked nucleic acid (LNA) integrated hairpin molecule beacon (MB) probe. This probe was employed to capture miRNA-21 from standard solutions and cell environment. After hybridization of miRNA-21, the proposed electrode was subjected to hybridization with multifunctional encoded DNA–AuNPs–LNA bio bar codes in which gold nanoparticles (AuNPs) were used to increase the number of oligonucleotide strands and, accordingly, the sensitivity. The biosensor reproducibility was accessed from the comparison of the response for six different electrodes and a relative standard deviation (RSD) of 10.05% was reported, this value being acceptable for this type of determination. The biosensor was successfully applied for monitoring of miRNA-21 expression from human hepatocarcinoma cell line (BEL-7402) and normal human hepatic cell line (L02).

The determination of microRNA-21 using graphene-based biosensor was also investigated more recently by Kilic et al. (2015) In this study, a disposable pencil graphite electrode modified with graphene (GME) was developed for the immobilization of inosine-substituted probe (InP), and this probe has undergone at hybridization with the target. The hybridization event was monitored using EIS and DPV measurements. The limit of detection obtained for microRNA-21 was equal to 2.09 μg mL^{-1}, and the applicability of the proposed biosensor was demonstrated from the analysis of microRNA-21 in cell lysates of breast cancer cell line (MCF-7) and hepatoma cell line (HUH-7). These two works reported important results in regarding the microRNA-21 electrochemical biosensing. However, as commented, the abnormal expression of miRNA-21 is associated with various types of cancer. Therefore, the design of graphene-based biosensors with great versatility in terms of applicability to different types of tissue is an analytical challenge to be explored in the future.

Real-time monitoring of nitric oxide (NO) expressed by living cells is an important analytical challenge. NO level in the cell environment is involved in the parthenogenesis of Parkinson's disease and tumor angiogenesis. However, its real time quantification is difficult due to the fast reaction between NO and molecular oxygen taking place in the biological system. In this scenario, Guo et al. (2012) developed a biofilm based on RGD-peptide functionalized graphene, where the RGD-peptide provided desired biomimetic properties for superior human cell attachment and growth on the film surface to allow real time detection of NO. The electrochemical response of the proposed biofilm was investigated by cyclic voltammetry at human umbilical vein endothelial cell culture without and with 10 μmol L^{-1} NO. The applicability of the proposed electrochemical biosensor was tested from the real-time monitoration of NO released by human endothelial cells cultured and attached on the biofilm. Fig. 2(a) presents the setup assay. In Fig. 2(b) and (c) we observe the NO response in the presence of

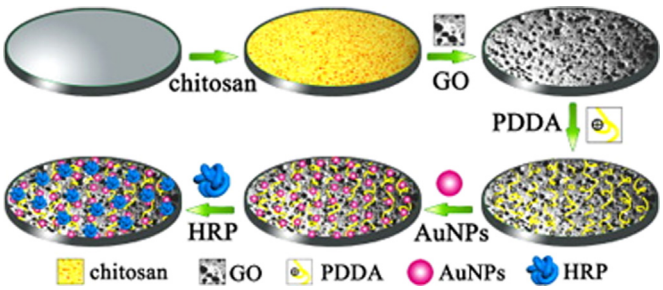

Fig. 3. Schematic process for the construction of the electrochemical biosensor. Reprinted with permission from (Yu et al., 2015) Copyright Elsevier.

Ach, L-NAME and a mixture of Ach and L-NAME, which was able to discriminate and quantify different NO concentrations in the cell culture. There are various nitric oxide biosensors in literature. However, the applicability of the designed methodology is frequently limited to analysis of biological fluid samples, and these approaches are different of the clinical diagnostic reality.

Xu et al. (2013) designed an electrochemical competitive immunoassay for the quantification of Bax protein in tumor cells using glassy carbon electrodes modified with a thionine–graphene composite (TH–GN). The determination of this pro-apoptotic protein in tumor cells is relevant for medical monitoring of chemotherapeutic treatment, once the Bax protein deficiency has been associated with resistant to the apoptotic effects of chemotherapeutic drugs. Under the optimized experimental conditions, the analytical curve was linear in the MCF-7 cell concentration of 2.5×10^3 to 1.6×10^5 cells mL^{-1}, with a limit of detection of 800 cells mL^{-1}. Moreover, the electrochemical biosensor was employed for determination of Bax protein expression on cell surfaces.

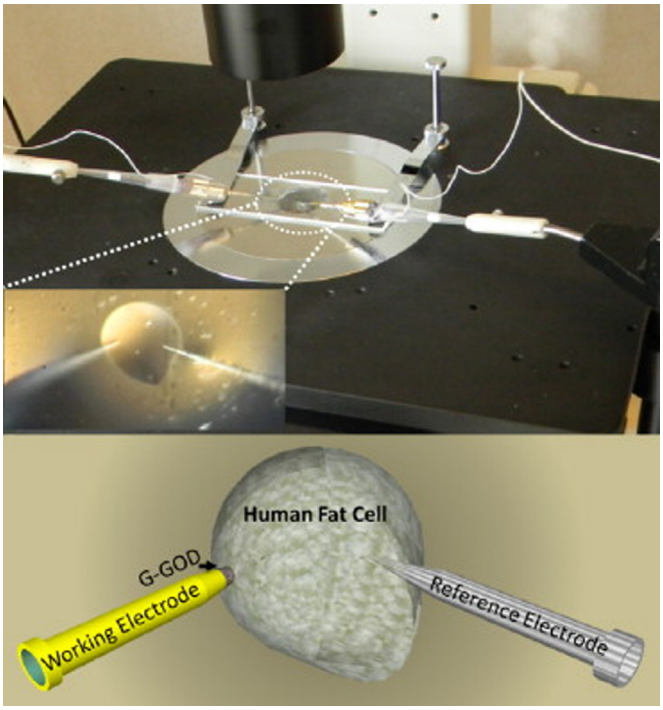

Fig. 4. Experimental setup used for selective measurements of the intracellular glucose concentration (top) with a schematic illustration (bottom). Microscopic images of a single human adipocyte are taken during measurements. As-measured values of intracellular glucose are comparable to other sophisticated techniques such as NMR. Reprinted with permission from (Hasan et al., 2015), Copyright Elsevier.

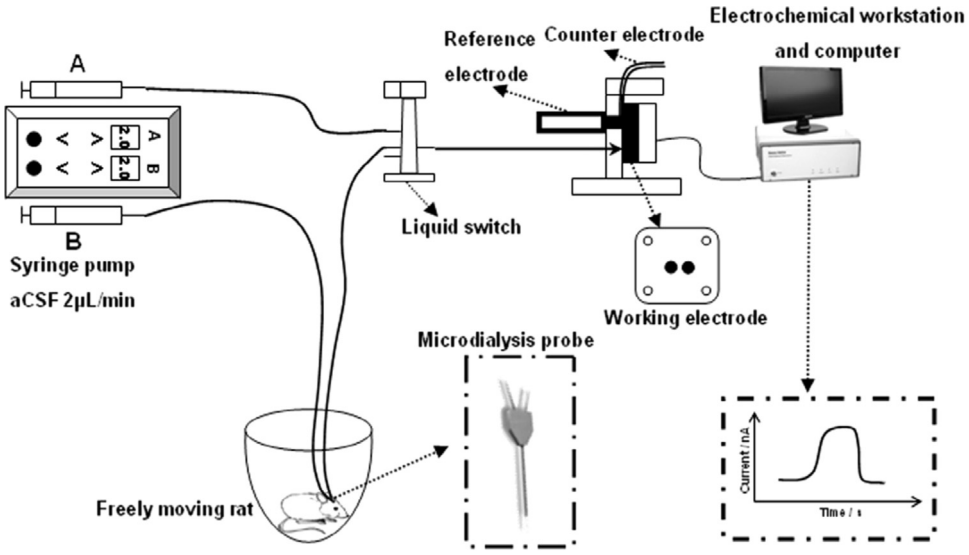

Fig. 5. Schematic diagram of the on-line electrochemical method with oxidase modified biosensors for simultaneous and continuous monitoring of glucose and lactate in the brain of freely moving rats. Reprinted with permission from (Gu et al., 2014), Copyright Elsevier.

Biosensing of hydrogen peroxide (H_2O_2) in living cells is a well-researched topic in literature. Yu et al. (2015) explored the layer-by-layer self-assembly technique for fabricating a H_2O_2 biosensor based on HRP enzyme immobilized on a AuNPs decorated GO interface. The scheme of the biosensor preparation is showed in Fig. 3. Thus, firstly GCE was modified with a chitosan (CS) film through an electrodeposition method, generating a positively charge electrode surface. Next, the obtained CS-GCE surface was dipped into GO, poly (diallyldimethylammonium chloride) (PDDA), AuNPs and HRP solutions, for preparation of the biosensor architecture. The cellular flux of H_2O_2 was determined in two kinds of living cells (normal cells of neutrophils and K562 cancer cells) under exposition to ascorbic acid, which stimulated the generation of H_2O_2 due to its cytotoxic activity. This type of biosensor is important because cellular flux of H_2O_2 is higher in the case of carcinogen cells.

Glucose measurement at intracellular environment was an analytical challenge investigated by Hasan et al. (2015). In this work, a potentiometric biosensor was developed from the coating of a fine borosilicate glass capillary with graphene and subsequent immobilization of glucose oxidase (GOD) enzyme. The basic scheme for construction of a two-electrode electrochemical potentiometric cell that can be represented by following scheme: Ag/AgCl | Cl^- || buffer | graphene, was adopted. The variation in the electrochemical cell voltage (EMF) is the key to measure the changes in electrolyte composition. Regarding the practical application of the proposed analytical approach, the intracellular glucose concentration was determined in single human adipocytes. For this purpose, the experimental setup shown in Fig. 4. As can be seen, a glucose selective working microelectrode was prepared on a micromanipulator, and moved into a position at the same level as the cell. The working and the reference microelectrodes were pushed through the cell membrane and into the cell. So, the intracellular glucose concentration in a human adipocyte obtained using the glucose potentiometric biosensor was very similar to those recorded using a more sophisticated analytical technique (Nuclear Magnetic Resonance Spectroscopy NMR). Thus, it is interesting note that this potentiometric glucose biosensor has real potentiality for future commercial applications, due to simplicity and wide range of glucose concentration response.

A novel DNA biosensor based on graphene and origami technology for determination of dihydronicotinamide adenine dinucleotide (NADH) was explored by Li et al. (2015b). NADH is considered an important biomarker because it is related with a number of lethal diseases and infections. The biosensor consisted of a gold disk electrode modified with AuNPs, DNA tetrahedron and graphene. The modified gold electrode provided an enhanced oxidation signal for NADH detection at a potential of +0.28 V *vs.* Ag/AgCl by using DPV. In addition, the selectivity of the biosensor was proved through the NADH determination in simulative cellular environment. The proposed biosensor presented a wide linear concentration range and low limit of detection, representing an important advance for the NADH biosensing. However, the developed methodology was not tested toward NADH quantification in real samples.

4. Biosensors based on graphene applied to *in vivo* analysis

As highlighted before, the development of biosensors using nanomaterials as graphene is an area of intense research in the biomedical field, especially in clinical diagnosis and therapy (Feng and Liu, 2011; Lin et al., 2015). Graphene effect *in vivo* has been shown to be related to its physical-chemical properties that depend on how it was synthesized or modified (Pinto et al., 2013). Besides, graphene has high surface area that allows a good biocompatibility with biomolecules such as enzymes, cells, proteins, DNA and antibodies (Fang and Wang, 2013; Gan and Zhao, 2015; Li et al., 2015a; Liu et al., 2012c; Wang et al., 2012).

The use of *in vivo* electrochemical devices is an important step to understand the physiology and pathological processes in living organisms, especially in humans (Zhang et al., 2014). *In vivo* methods, in general, are featured by a set of advantages and disadvantages. They are appropriate to evaluate the performance of the analytical method when applied in complex medium, which different sources of interference are found. On the other hand, *in vivo* studies involve the use of model animals and, thus, the extrapolation for humans is performed. The assays can be laborious, invasive and costly. In addition, the analytical frequency is low and there is a lack of certified standard methods (Carbonell-Capella et al., 2014). On the other hand, nowadays, electrochemistry has shown immense potential for *in vivo* applications. Numerous advantages have been obtained such as direct and rapid measurement, instrumental simplicity, accuracy, stability, high sensitivity and selectivity, minimally invasive implantable devices and others (Jackowska and Krysinski, 2013; Wang, 2008; Zhang

et al., 2014). In the last two decades, some devices have been proposed for use *in vivo* including electrochemical sensors (Caffrey et al., 2015; Grant et al., 2001; Gyetvai et al., 2009; Harreither et al., 2013; Hurst and Clark, 2003; Jacobs et al., 2011; Liu et al., 2012a, 2015; Ragones et al., 2015; Shao et al., 2013; Swamy and Venton, 2007; Wang et al., 2001; Zhang et al., 2007); and biosensors (Abel and von Woedtke, 2002; Chai et al., 2013; Deng et al., 2008; Edagawa et al., 2014; Lin et al., 2013; Lowry and Fillenz, 2001; Lu et al., 2013; Pohanka et al., 2009; Ren et al., 2013; Ricci et al., 2007; Santos et al., 2015a; Tian et al., 2005; Yu et al., 2011a, 2011b; Zhang et al., 2004; Zhu et al., 2009). In this context, recently, graphene has also been used in electrochemical sensors (Arvand and Ghodsi, 2013; Manibalan et al., 2015; Zhu et al., 2011) and biosensors (Gu et al., 2014, 2012) for *in vivo* applications. The purpose of this section is to present a brief revision of the last 15 years about electrochemical biosensors based on graphene for *in vivo* applications. Since graphene was first prepared, few works about electrochemical biosensors modified with graphene for application *in vivo* environment have been reported (Chen et al., 2010). Interesting study for application *in vivo* was reported in the literature by Gu et al. (2012). The authors developed an amperometric biosensor for sensitive *in vivo* detection of glucose integrated with on-line microdialysis system using multilayer films arranged by layer-by-layer (LBL) self-assembling of amine-terminated ionic liquid (IL-NH_2), and sulfonic acid functionalized graphene coated with a thin film of Nafion® on a GCE. In a second study, carried out by Gu et al. (2014), an dual-enzyme electrochemistry biosensor for continuous and simultaneous monitoring of glucose and L-lactate using a graphene hybrid was developed. These studies demonstrated the construction of an effective on-line analytical system through integration of microdialysis in the electrochemical detection of glucose and L-lactate in the brain of rats (Fig. 5). We can emphasize that microdialysis is a minimally invasive sampling technique for extracellular fluid analysis in a selected brain area of animals. It can be applied to monitor many endogenous molecules as well as hormones, glucose, drugs, and others.

As expected, the increase of studies *in vitro* systems will lead increment of *in vivo* applications. An example of a study published recently by Zhang et al. (2015) developed multifunctional glucose biosensors from Fe_3O_4 nanoparticles modified chitosan/graphene nanocomposites together with oxidase enzyme using graphite electrode.

5. Point-of-care electrochemical biosensors based on graphene

Point-of-care (POC) diagnostics devices require low cost, fast response, stability, reliability, portability, miniaturized form, disposability and easy-to-be used. They have received considerable attention mainly in hospitals because of the great need of illness fast diagnostics, allowing thus the choice of the best treatment for the patients (Loncaric et al., 2012; Wang, 2006). In this sense, POCs facilitate the realization of laboratory tests, bringing the possibility of analyses much closer to the bedside of the patient (Adiguzel and Kulah, 2012; Luppa et al., 2011). POC systems can process clinical samples using different devices in a variety of settings, for medical clinical, laboratories, hospital and even to the remotest places. Basically, POC systems can be used for various purposes beyond clinic diagnostic as for detection of explosives, environmental studies and food safety analysis (Kumar et al., 2015).

The development of biosensors for point-of-care diagnostics using biological material and graphene can bring many advantages in terms of stability, robustness, shelf lifetime, sensitivity and selectivity considering the advantageous characteristics of these modifiers. We are highlighting here, the studies of graphene-based biosensors for POC diagnosis of the last years. Kong et al. (2014) detected glucose in human blood using a disposable screen-printed carbon electrode modified with graphene/polyaniline/Au nanoparticles/glucose oxidase biocomposite. In this study, the applicability and validity of the method to whole blood samples were evaluated with satisfactory results. Lu et al. (2012) developed a SPE simple, low-cost, disposal device based on DNA immobilized onto gold nanoparticles/graphene for detection of thionine. Under optimal conditions, the genosensor showed high sensitivity, good precision, and excellent performance in human serum assay. The device developed can be easily applied for point-of-care testing with success in the public health and environmental monitoring. Ge et al. (2015) reported a low-cost, simple, portable and sensitive electrochemical biosensor based on a hybrid material of 3D gold nanoparticles/graphene, ionic liquid and Concanavalin A (Con A) in screen printed paper electrodes for the detection of K-562 cell in point-of-care testing. Con A enzyme immobilized onto the electrode surface was applied to capture K-562 cells, and ionic liquid served to enlarge electrochemical window, by improving biocompatibility and conductivity provided by gold nanoparticles and graphene. Biosensors arrays were also proposed based on different modifications. One example is the glucose biosensor based on immobilization of glucose oxidase on platinum nanoparticles/graphene/chitosan nanocomposite film reported by Wu et al. (2009). In another interesting work, a paper sensor modified with poly (3,4-ethylenedioxythiophene)-poly (styrenesulfonate) (PEDOT:PSS) and reduced graphene oxide (RGO) composite for cancer detection was described (Kumar et al., 2015). A sensitive immunosensor for cancer biomarker detection was also reported based on dual signal amplification strategy of graphene sheets and multienzyme functionalized carbon nanospheres (Du et al., 2010). An electrochemical aptasensor was accomplished based on graphene-3,4,9,10-perylenetetracarboxylic dianhydride as platform and functionalized hollow PtCo nanochains for determination of thrombin (Peng et al., 2012). Teixeira et al. (2014) proposed a biosensor using oriented antibodies and graphene screen-printed electrodes for detection of human chorionic gonadotropin at picogram levels. A clear example of a graphene-based biosensor that can be applied as POC diagnostics is the device reported by Kailashiya et al. (2015) consisted of a sensitive electrochemical biosensor based on antibody and GO on a GCE for detection of platelet-derived microparticles (PMPs). The biosensor was highly specific for PMPs using the technique of electrochemical impedance spectroscopy especially when evaluated blood samples obtained from patients diagnosed with acute myocardial infarction and healthy patients. In addition, POC could be implanted in a minimally-invasive way and most importantly, the wireless device senses heart and brain electrical activity (Ghafar-Zadeh, 2015) (Fig. 6).

In Fig. 7 is shown a POC device for monitoring in real-time of bacteria on tooth using impedance measurement techniques (Ghafar-Zadeh, 2015; Mannoor et al., 2012). The designed device is innovative in the scenario of point-of-care diagnostics, providing high analytical sensitivity and selectivity toward bacteria detection, easy biotransferability, and wireless and battery-free operability. Further works will be undertaken in order to enhance the applicability, including the miniaturization of the device and *in vivo* tests (Mannoor et al., 2012). To sum up, recent published works of graphene-based biosensors with POC configuration devices present advantages such as simplicity, portability, easy-to-use and low cost. Furthermore, POCs exhibit good sensitivity, selectivity, stability, and reproducibility in the electrochemical measurements. These devices offer characteristics that make them promising for a wide range of applications, especially clinical diagnostics.

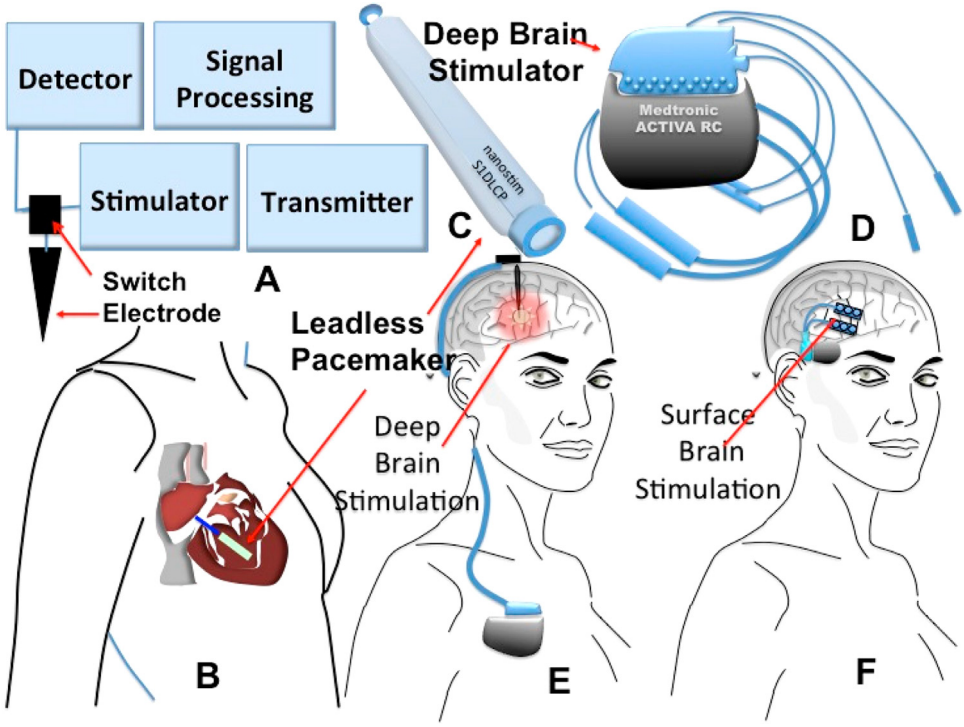

Fig. 6. State of the art of implantable devices for brain POC applications: (A) simplified diagram of implantable system; (B) the schematic of new wireless pacemaker placed in the heart; (C) photo of this leadless pacemaker commercialized by Nanosim Inc. (D) Brain stimulator commercialized by Medtronics for epilepsy point-of-care purposes implanted: (E) under the skull for surface brain stimulation or (F) stimulator placed above the chest for deep brain stimulation. Reprinted with permission from (Ghafar-Zadeh, 2015), Copyright MDPI - Open Access Publishing.

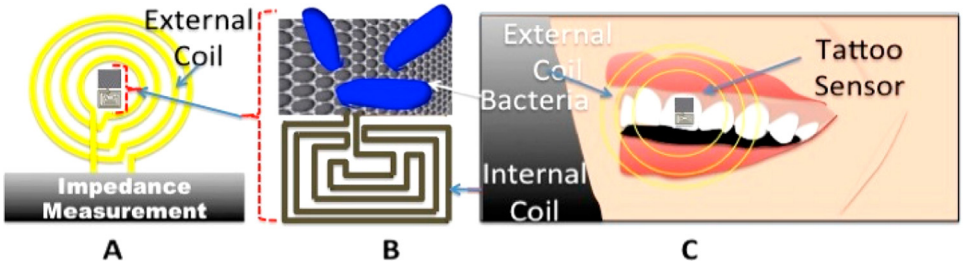

Fig. 7. Remote Bio-sensing technique: Simplified diagram of (**A**) sensing technique measuring the impedance change due to the presence of bacteria; (**B**) sensing electrodes on soft substrate; (**C**) attached electrodes on tooth. Reprinted with permission from (Ghafar-Zadeh, 2015), Copyright MDPI - Open Access Publishing.

6. Conclusion

Graphene, the youngest carbon nanomaterial, is a great 2D nanomaterial that has been extensively applied in several areas. In addition, to be a relative new material, the improvement of synthesis processes, the incorporation of other nanomaterials, and the interaction with biomolecules are challenges. Electrochemical devices based on graphene present interesting properties derived from the structure and high surface area, these improving the analytical characteristics, and allowing the preparation of disposable electrochemical biosensors designed for various targets, in order to early detection of diseases in a less invasive way.

7. Future perspectives

Biosensors for *in vivo* and *in vitro* diagnosis have been developed in the recent years, which can lead low concentrations and fast responses, allowing a choice for medical treatment. These are still recent research topics, and more effort should be spend by the researchers to address different challenges. Firstly, the synthesis of graphene-based platforms for different biological recognition species using low cost materials and reproducible routes is required. For example, the use of paper and plastic as low cost materials, is expected, these allowing the construction of interesting and cheaper alternatives for manufacturing point-of-care disposable biosensors. In addition, there is a trend in biosensing for the development of versatile and disposable devices to perform accurate and sensitive simultaneous determination of multiples target analytes (e.g., detection of more than one biomarker). In this sense, studies dedicated to investigate the mechanisms and parameters affecting the detectability of graphene-based biosensors toward different biological targets should be proposed in the near future. Moreover, the performance of the graphene-based biosensor devices must be increasingly tested at complex samples and *in vivo* conditions. The miniaturization and portability of the electrochemical devices are necessary research lines for the future commercial success of the developed technologies.

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