Enzymes–Graphene Platforms for Electrochemical Biosensors Design With Biomedical Applications

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Contents

Abstract

Due to the growing need for sensitive, reliable, reusable, fast, and cheap devices for the detection of analytes which have an important role in diagnosis of different diseases, in metabolic disorders, in monitoring treatment of serious diseases such as cancers, the sensing field has attracted huge interest from the scientists. The majority of the traditional methods that are currently in use are invasive, expensive, and laborious.

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Moreover, highly specialized operators and sophisticated instrumentations are usually required. Taking these into account, the introduction of electrochemical sensors and biosensors avoid a lot of the disadvantages associated with most of the used analytical techniques. The biggest contribution to this development was the use of different nanomaterials as transducers of the analytical signal. The properties, such as high mechanical strength, good electrical conductivity, and ability to serve as efficient signal transducers, make carbon-based nanomaterials, including graphene, ideal materials for biosensor applications. Furthermore, graphene presents high surface area that can be easily modified in different ways to be adapted for the immobilization of various biocompounds for the construction of biosensors. Recent advances regarding the use of graphene and graphene materials for the immobilization of several enzymes for biosensor development and their applications for the detection of chemical and biological species are presented with focus on different enzymes immobilization techniques. In the end, the future trends for the development of graphene-based biosensors in biomedical field are also discussed.

ABBREVIATIONS

ADH	alcohol dehydrogenase
AuNPs	gold nanoparticles
β-CD	β-cyclodextrin
CHER	cholesterol esterase
ChOx	cholesterol oxidase
CLEA	cross-linking enzyme aggregates
CLEC	cross-linking enzyme crystals
CNTs	carbon nanotubes
DA	dopamine
DET	direct electron transfer
EDC	1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide
FAD	flavin adenine dinucleotide
FDH	D-fructose dehydrogenase
GO	graphene oxide
GCEs	glassy carbon electrodes
GOx	Glucose oxidase
HRP	horseradish peroxide
MET	mediated electron transfer
MNP	magnetic nanoparticles
NHS	*N*-hydroxysuccinimide
PB	Prussian blue
PAMAM	ethylenediamine core polyamidoamine G-4 dendrimers
PtNPs	platinum nanoparticles
rGO	reduced graphene oxide
SPE	screen-printed electrode
Tyr	tyrosinase
XOx	xanthine oxidase

1. INTRODUCTION

There is an enormous interest in the use of carbon-based nanomaterials, especially carbon nanotubes (CNTs) and graphene for the development of biosensors, including the enzymatic ones. This is mainly due to the ability of these nanomaterials to improve the analytical characteristics of macroscale biosensors, as well as to facilitate the development of some completely new nanoscale biosensors. Carbon nanomaterials are among the most commonly used nanomaterials for the development of biosensors since they allow reaching many of the important key issues such as the design of the biosensing interface so that the analyte selectively interacts with it; the achievement of efficient transduction of the biorecognition step; improved sensitivity and selectivity of the biosensor; faster response time in very sensitive systems; good compatibility with biological matrices for testing in complex biological samples and for in vivo applications; fabrication of viable biosensors that can operate inside cells; multiplexing biosensors for testing multiple analytes with only one device.

Electrochemical biosensors are analytical devices capable of converting the biochemical reaction into analytical and measurable signal. They belong to the large class of biosensors which associate to a transducer a biological element (Hulanicki, Glab, & Ingman, 1991). Therefore, biosensors can be categorized according to the biological recognition element (immuno, enzymatic, DNA, and whole-cell biosensors) or the signal transduction method (optical, colorimetric, mass-based, electrochemical, and thermal biosensors) (Fig. 1).

Electrochemical biosensors are attractive analytical devices for biomedical analysis featuring several advantages: detection of the substrate is made without prior separation; the sensitivity could arrive at ppm or ppb levels, higher selectivity (even specificity), high benefit/cost ratio, simple use, and the rapid data collection. Enzyme-based biosensors are the most used biosensing platforms surpassing the immunosensors (using antibodies as recognition element), the genosensors (using nucleic acids), the aptasensors (using aptamers), and the cell-based sensors.

This is due to the fact that enzymes have high affinity toward corresponding substrates, being able to catalyze several biochemical reactions, being relatively easily regenerated several times. The recognition system of a biosensor depends on the enzyme–substrate relation (Kurbanoglu, Ozkan, & Merkoçi, 2017). Enzymes can be immobilized onto the surface of a transducer

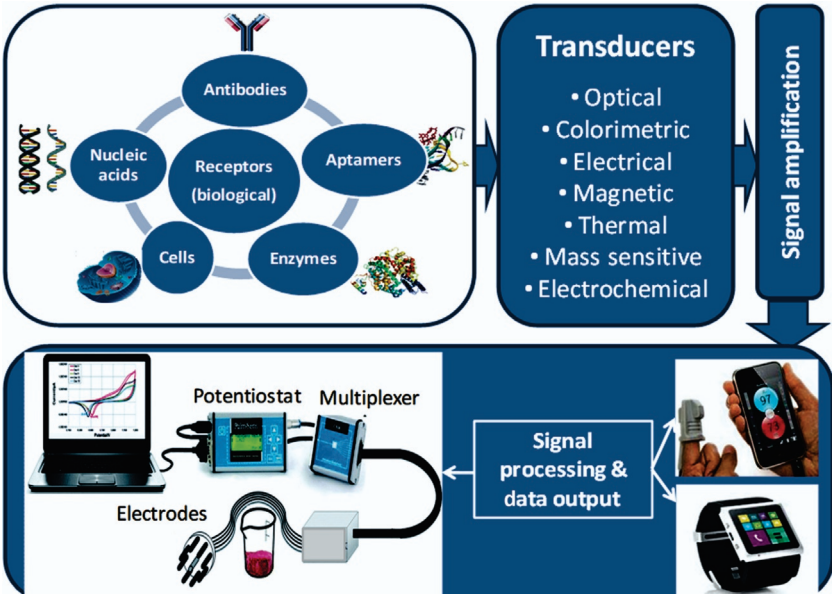


Fig. 1 The schematic representation of a biosensor design.

via several immobilization techniques, each technique having its own limitation regarding the best orientation of the enzymes toward substrate.

The reduced stability, the limited lifetime due to the rapid loss of enzyme activity, the interferences from complex sample matrices, and biofouling in case of in vivo measurements are some of the main disadvantages of the electrochemical biosensors (Castillo et al., 2004; Chaubey & Malhotra, 2002). Nanomaterials are excellent building blocks, generally used in electrochemical biosensors fabrication in order to modify transducers or to enhance their electrochemical signals, also eliminating most of the above-mentioned disadvantages.

The interest of graphene as a nanomaterial employed for biosensors design is due to the success of previous use of CNTs. CNTs are usually referred to as rolled-up graphene sheets, and both allotropes have a meshwork of sp^2-hybridized carbon atoms. Because these nanomaterials have the same composition, it was initially thought that the properties are similar. But practice has proved that this is not always true, and the differences in structure and properties open new ways for further developments in biosensors.

Any biosensor based on graphene should ideally be designed to exploit the unique properties of this nanomaterial. The ideal structure of graphene is

completely two-dimensional. It comprises a single layer of sp^2-hybridized carbon atoms joined by covalent bonds to form a flat hexagonal lattice (Castro Neto, Guinea, Peres, Novoselov, & Geim, 2009). Actually, there are different structures unified under the name of graphene. This is the consequence of the difficulty in isolating single layers of graphene in a controlled manner, meaning that what is termed graphene, actually comprises stacks of graphene layers, each containing different numbers of atomic sheets.

Therefore, it is mandatory to accurately assess the number of graphene layers, this being usually done by Raman spectroscopy (Gupta, Chen, Joshi, Tadigadapa, & Eklund, 2006), atomic force microscopy (Novoselov et al., 2004), scanning electron microscopy (SEM) (Grodecki, Jozwik, Baranowski, Teklinska, & Strupinski, 2016), contrast spectroscopy (Ni et al., 2007), and low-energy electron microscopy (Hibino et al., 2008). The above-mentioned methods can be used in order to determine if there are single-layer graphene, bilayer, few-layer graphene, or multilayer graphene. Even if it is demonstrated that the nanomaterial that is intended to be used for the development of biosensors is single-layer graphene, there are other problems that occur and must be taken into consideration. For example, the single-layer graphene is never atomically flat because of its flexibility. Usually the graphene sheets tend to fold, curl, or corrugate (Novoselov, Jiang, et al., 2005).

The mechanism of action of CNTs in electrochemical biosensors is not yet fully elucidated. Unfortunately, in the case of graphene and graphene compounds, the situation is even more unclear. This is mainly due to the fact that the studies dealing with the electrochemical properties of graphene were performed with different forms of graphene (Yang et al., 2010). Despite of this, there is still evidence that graphene and its derivatives can exhibit better electrochemical performance compared with other transducers such as glassy carbon, graphite, or CNTs. It was observed that the excellent electrochemistry of graphene may be attributed to its edges, while the functional groups and defects probably are also important in the case of graphene compounds. The electronic behavior of graphene is the most studied property. This originates in the delocalized π bonds above and below the basal plane, which arise from the sp^2 hybridization responsible for the layered structure of graphene (Castro Neto et al., 2009). The delocalized electrons, and the quality of the graphene, create high electrical conductivity and mobility. The mobility was measured at room temperature and a value of $15{,}000\,cm^2\,V^{-1}\,s^{-1}$ was obtained in graphene with maximum three layers (Novoselov, Geim, et al., 2005), while in the case of single layer

graphene, a mobility of 230,000 $cm^2\ V^{-1}\ s^{-1}$ has been measured at temperature around absolute zero (Bolotin et al., 2008).

Graphene also presents an ambipolar electric field effect in which negative gate voltages induce large concentrations of holes, while positive voltages induce large numbers of electrons (Castro Neto et al., 2009). There are other electronic properties and all of these are related to the number of layers in the graphene sheet. For example, a single-layer graphene sheet is a zero-gap semiconductor, while three-layer graphene sheet is a semimetal. The extent of overlap between the conduction and valence bands increases with the number of layers and similar behavior with that of graphite is observed at about 15 layers of graphene (Grüneis et al., 2008).

Raman and infrared spectroscopy can provide useful information about the optical properties of graphene derivatives. Raman spectroscopy is also used in order to identify the number of layers in graphene sheets that is related with the modifications observed in spectra (Ferrari et al., 2006). Thus, there are two highly characteristic Raman bands in graphene: the G band at approximately 1580 cm^{-1} and the D band at about 2700 cm^{-1} (Gupta et al., 2006). Another D band appears at about 1350 cm^{-1}, this being due to the edges, defects, and impurities of the graphene sheets (Ferrari et al., 2006).

Electrochemical enzymatic biosensors based on graphene nanocomposites have been designed for the determination of a wide range of molecules from various fields.

This chapter will focus only on the use of graphene in enzymatic electrochemical biosensors development for the detection of some compounds of biomedical interest. The latest advances in electrochemical biosensors that use graphene will be further discussed and their performances will be critically compared.

2. ELECTROCHEMICAL BIOSENSORS: GENERAL CONSIDERATION

2.1 Definition of Biosensors

Sensors are small or miniaturized devices designed for the monitoring of the physical, chemical, or biochemical properties of specific analytes in order to provide qualitative and quantitative information. Chemical sensors are related to the presence and concentration of analyte by responding selectively to the modification of the properties of the analyte's features such as electrical, optical, thermal, or gravimetric features. Biosensors can be

defined as devices with a thin layer serving as support to immobilized biomolecule that contains biorecognition sites. The biomolecules may be enzymes, proteins, receptors, antibodies, oligo- or polynucleotides, microorganisms, cells, or even tissues that display specific interactions with target analyte.

According to IUPAC, an electrochemical biosensor is "a self-contained integrated device that is capable of providing specific quantitative or semiquantitative analytical information by using a biological recognition element (biochemical receptor) in direct spatial contact with a transduction element." Therefore, an electrochemical biosensor contains three parts:

- the biological recognition element
- the transduction element (converting the signal in a quantified result)
- the signal processor (displaying the signal).

However, it must be taken into account that there is a difference between a biosensor and a bioanalytical system. The last one requires additional processing steps, such as reagent addition. According to this definition, there is no size constraint on a biosensor, which can be macroscopic (commercial glucose biosensors), can be composed of a macroscopic device containing a micro or nanoscale-sensing element (field-effect transistor type devices), or the entire device can be on the nanoscale (Yang et al., 2010).

The discovery of biosensors is related to the early 1960s, when Clark and Lyons first reported on amperometric glucose detection using the enzyme glucose oxidase (GOx) (Clark & Lyons, 1962). These sensors were applied for monitoring biological processes, for the detection of biomolecules. The majority of biosensors developed for glucose detection to date are electrochemical ones, these being preferable due to their ease of use, low-cost, small size and relatively fast response, good detection limit, simplicity, rapidity, reliability, less sample requirement, high sensitivity, and less bulky instrumentation. Electrochemical biosensors are potentiometric, amperometric, or conductimetric and are based on the monitoring of the signal generated from electrochemical reactions of the analytes or in response to the presence of different chemical species related to the analytes, thus producing/consuming ions/electrons, leading to the generation of an electrochemically measurable signal (current, potential, charge accumulation, and impedance) that is analyzed by the electrochemical detector. In the case of enzyme-based electrochemical biosensors, the analyte is recognized by enzymes that are immobilized on the working electrode surface, and their catalytic activity causes electron transfer, produces electric current, or contributes to voltage production.

2.2 Nanomaterials: Modifiers for Transducers in Biosensors

Important steps in the development of the biosensors were made with the discovery of nanomaterials and of various nanofabrication tools, such as electron beam lithography, focused ion beam, and nanoimprint lithography (Liu & Guo, 2012). Various nanomaterials coupled with enzymes as biorecognition element led to electrochemical biosensors with high surface area, excellent conductivity, biocompatibility, and (bio)catalytic activity resulting in ultrasensitive and selective biosensing. These nanomaterials include CNTs (Lahiff, Lynam, Gilmartin, O'Kennedy, & Diamond, 2010; Liu et al., 2010; Wang, 2005), nanoparticles (Wilson, 2008), nanowires (Arlett, Myers, & Roukes, 2011; Song et al., 2010), and graphene (He, Wu, Yin, & Zhang, 2012; Huang et al., 2011; Ratinac, Yang, Gooding, Thordarson, & Braet, 2011). A wide range of substances with biomedical importance have been detected by employing graphene-based biosensors (Bollella et al., 2017; Lawal, 2018; Song et al., 2016).

The use of nanosized materials in electrochemical biosensors improved the performance factors such as sensitivity, response time, and limit of detection up to great extent. An ideal nanomaterial must sum up some fundamental properties like ease in preparation, availability, abundance of functional groups, and chemical stability for the use as a suitable matrix in biosensor fabrication. The presence of nanomaterial on the electrode surface facilitates fast electron transfer kinetics, enhances the electroactive surface area, and increases the biomolecule loading. Furthermore, the large surface area increases the binding affinity of immobilized biomolecule via the multivalent immobilization techniques.

Graphene is in the forefront of nanomaterials used for the elaboration of biosensors, and this fact is due to their unique electronic, physical, and chemical properties. The spectacular development of nanomaterial-based electrochemical biosensors is also correlated with their significant advantages compared to conventional optical, biochemical, and biophysical methods. These advantages are high sensitivity, high resolution for localized detection, easy method for integration, and real-time and nondestructive detection ability.

2.3 Enzymatic Biosensors

Enzymes were the first molecular recognition elements used in biosensors technology. Enzymes are macromolecular biological catalysts composed of aminoacids generating globular proteins with 3D structure. Their specific

structure (which may also include cofactors) is responsible for the high catalytic activity and specificity of the enzyme for one type of substrate molecule.

An enzymatic biosensor is an analytical device that combines enzyme with transducer and converts signal proportional to target analyte/substrate concentration with high catalytic activity, selectivity, and specificity. Appropriate immobilization of enzyme on the surface of nanomaterials not only enhances the concentration of the biorecognition molecule at the appropriate location but also protects the biomolecules from being inactivated.

The most challenging issue remains the establishment of an efficient and lasting electrical communication between enzymes and the electrode concerning the electrons transfer. Two main strategies were employed for the electric wiring of the active site of the enzyme to the electrode:

– direct electron transfer (DET) (the active site is in direct electronic communication with the electrode surface);
– mediated electron transfer (MET) (using redox mediators).

The most important parameters for DET are the immobilization strategy, the orientation of the enzyme, and the localization of the active site in the protein. Meanwhile, the key parameters for MET envisage: the immobilization strategy for the enzyme and the redox mediator, the redox potentials and structure of the redox mediator, and the electron transport via the redox mediator film.

2.3.1 Strategies for the Immobilization of Enzymes for Biosensors Development

The immobilization of enzymes represents the process through which this bioelement is fixed or localized in a certain position (that is well established) on a special support (electrode in the case of electrochemical biosensors). It is very important to mention that the immobilization process must not affect the catalytic activity of the enzyme, allowing the sensitive, repetitive, and continuous detection of the target analyte. A good biosensor requires optimum amount of enzyme loading together with the preservation of its optimal activity. In order to achieve this, it is important to avoid the use of harsh chemical treatment or exposure to harsh conditions such as high temperature. Immobilization of enzymes on the surface of graphene and graphene compounds provides platforms suitable for the development of biosensors taking advantage of their electronic and optical properties for signal transduction. The best strategy for a successful fabrication of an enzyme biosensor is to elaborate a configuration for the electrode active surface that allows the direct transfer of the electrons from the redox center of the

enzyme to the transducer. In this respect, several immobilization strategies are used, such as physical adsorption or covalent immobilization of large biomolecules onto the surface of previously immobilized graphene or graphene derivatives (Gorecka & Jastrzebska, 2011).

The ideal enzyme immobilization strategy must meet several basic criteria, such as:

- employing mild conditions;
- allowing the immobilization of large quantities of enzyme;
- providing substrate with a large surface area vs a small total volume for enzyme;
- removing barriers to mass transport both for the substrate and the product;
- providing a chemically and mechanically robust system.

The immobilization strategy is usually chosen depending on the desired application, and the large number of possible applications was a good reason for the continuous development and improvement of the methods used for immobilization. Another very important issue is represented by the selection of the most appropriate support for the immobilization of a particular biocompound. Selection criteria differ among one another, depending on the biocatalyst of interest, but there are still few basic principles that must be considered for the right choice of the support material such as:

- high chemical, physical, and biological stability during processing and reaction;
- high mechanical strength;
- low or no effect both for the immobilized biocompound and the target analyte that should be detected;
- the presence of adequate functional groups for binding the biocompound;
- adequate physical characteristics such as porosity, swelling, or particle characteristics;
- high loading capacity;
- biodegradability;
- low costs for large-scale production (Gorecka & Jastrzebska, 2011).

There are two categories of immobilization procedures, namely, reversible and irreversible immobilization, respectively. The selection of the immobilization technique and support depends on different factors. The parameters that influence the above selection in case of biosensors are the nature of enzyme, the type of transducer, physicochemical properties of the analyte, and the operating conditions.

Reversible immobilization methods include adsorption, metal chelation, and disulfide bounds formation.

Adsorption represents the simplest and the most commonly used method of reversible immobilization. It is often used for the immobilization of cells and enzymes on different substrates, including graphene-based ones and it is also found in various biotechnological processes. In this case, the immobilization is based on weak forces such as van der Waals forces, ionic, hydrophilic and hydrophobic interactions, hydrogen bonds, and even affinity binding (Bucur, Danet, & Marty, 2004; Mateo et al., 2006).

Disulfide binding is a reversible immobilization method applied especially in the case of enzymes. In fact, this may be considered a covalent bonding, as there are stable covalent bonds formed between activated support and free thiol group (for example, on cysteine) in the biological element. However, this method is classified as a reversible immobilization, while those bonds can be easily broken using suitable agent under mild conditions (Neves-Petersen, Snabe, Klitgaard, Duroux, & Petersen, 2006).

Metal chelation is widely used in chromatography, while it is very important to ensure an easy regeneration of the support without reducing the immobilization yield. The immobilization is based on the ability of the side chains of certain amino acids (histidine, tryptophan, tyrosine, cysteine, and phenylalanine) to substitute weakly bonded ligands in the metal ions that have been immobilized by chelating group covalently bound to a solid support (Afag & Iqbal, 2001).

Irreversible immobilization methods include covalent bonding, cross-linking, entrapment, and encapsulation.

Covalent bonding represents one of the most frequently used methods for enzyme immobilization. This is mainly due to the stability of the bonds formed between the enzyme and the support, which prevents enzyme detachment during the elaboration and detection protocol. There is a wide variety of covalent bounding strategies for enzyme immobilization, all being related on the type of the matrix and substrate. The first strategy involves the generation of functional groups at the surface of the electrode via a polymer matrix without its modifications, while the second one implies the modification of the support matrix in order to generate activated groups. These groups are usually electrophile ones that will react with strong nucleophile ones from the biocompound. There are three different bounding methods that are exploited: the first one involves binding via the side chains of lysine (ε-amino group), the second one involves the thiol group in cysteine, and the third one

involves carboxylic group of aspartic and glutamic acids. Furthermore, in this method only the functional groups which are not essential for the catalytic activity of the enzyme are allowed for bonding (Mateo et al., 2006).

Entrapment is an irreversible method where immobilized particles or cells are entrapped in a support matrix or inside fibers. Electropolymerization is an example of entrapment methodology in which the enzyme is mixed with a monomer, followed by electrochemical polymerization, where the enzyme becomes embedded into the polymer matrix. The incorporation of the enzyme into the matrix is often promoted through electrostatic interactions. An important condition for unaltered preservation of the enzyme activity is to avoid the use of harsh chemicals during the polymerization process (Gao, Wang, Diao, Luo, & Dai, 2010).

Encapsulation is another irreversible immobilization method, similar to entrapment. The major difference is that in this process the biocompound molecules are restricted by the membrane walls that have a form of a capsule. However, there are no restrictions for the movement within the capsule space. The membrane is semipermeable, allowing for free flow of substrates and nutrients (when cells are used as a biocatalyst), yet keeping the biocatalyst inside. The factor determining this phenomenon is the proper pore size of the membrane. This method is suitable for multienzyme systems used in sequential enzymatic reactions, as several different bioelements may be incorporated into the same capsule (Cao, 2005a).

Cross-linking represents an irreversible method of enzyme immobilization that does not require a support. It is a useful method to stabilize adsorbed biomaterials, and it generally involves the residues which are not involved in catalysis (e.g., amino group of lysine residues). There are two main strategies used for cross-linking of enzymes, both involving the use of glutaraldehyde which reacts with amino groups from protein surface:

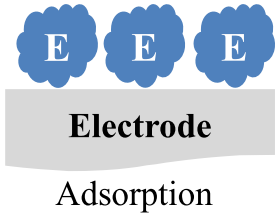
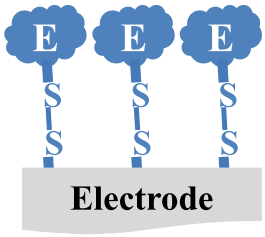
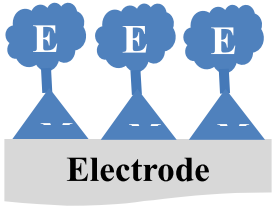
(a) Cross-linking enzyme aggregates (CLEA).

(b) Cross-linking enzyme crystals (CLEC).

An important factor is to use the proper cross-linking agent, glutaraldehyde being the most frequently employed, as it is inexpensive and readily commercial available, but it is unsuitable for immobilization of some enzymes (e.g., nitrilases). Other cross-linking agents are dextran, polysaccharide, bis-isocyanate, bis-diazobenzidine, diazonium salts, and functionally inert proteins, such as bovine serum albumin or ovalbumin (Cao, 2005b; Sheldon, 2007).

The advantages and drawbacks of the above-presented methods for enzyme immobilization are comparatively presented in Table 1.

Table 1 Advantages and Drawbacks of Different Immobilization Methods

Immobilization Method	Advantages	Drawbacks
 Adsorption	– easy in preparation; – low cost; – the possibility to reload the support.	– a very high rate of leakage due to the weak interactions between the support and the biocatalyst; – low reproducibility due to the impossibility to control the enzyme loading; – high susceptibility to changes in ambient conditions (pH, temperature, ionic strength).
 Disulfide bounding	– the possibility to influence the reactivity of thiol groups by alteration of pH; – the possibility to chemically activate the support before the immobilization process.	– the need to establish ideal conditions for fast and reproducible immobilization and the achievement of high coverage rates in biomolecules.
 Metal chelation bounding	– reversibility of immobilization can be attained by exposing the surface to a stronger chelator or by using excess of competitor ligand.	– high difficulty in obtaining reproducible immobilization of enzyme onto the electrodes; – long duration due to the large number of steps involved.

Continued

Table 1 Advantages and Drawbacks of Different Immobilization Methods—cont'd

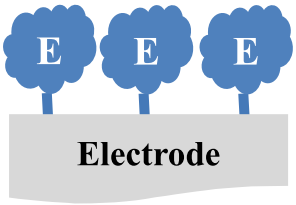
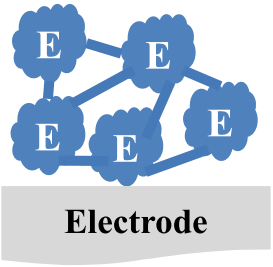
Immobilization Method	Advantages	Drawbacks
 Covalent bounding	– unlimited contact between the enzyme and the substrate; – increased stability of the enzyme.	– the biosensor usability is determined by the direction of the enzyme binding. The highest activity level is achieved only if the active center of the enzyme is not involved in binding with the support; – the activity of enzyme may be lost during the covalent bonding while this is usually carried out using chemicals and at different pH ranges. – is rarely applied for immobilization of cells due to the fact that agents used for covalent bonds formation are usually cytotoxic.
 Entrapment	– increased stability of the enzyme at the electrode surface; – great enhancement of sensor response due to the increase in the surface area, as well as the improvement in the electrical communication between the redox centers of the enzyme and the electrode; – simple one-step preparation; – exclusion of electroactive and surface-active interferences; – control of film thickness; – localization of biocatalysts onto tiny electrode surfaces.	– high costs of immobilization; – diffusion limitations; – deactivation during immobilization; – abrasion of support material during usage; – low loading capacity with biocompound as it has to be incorporated into the support matrix: • small pores—low loading while adsorption can be done only on external surface; • big pores—leaking and decreases the loading.

Table 1 Advantages and Drawbacks of Different Immobilization Methods—cont'd

Immobilization Method	Advantages	Drawbacks
 Encapsulation	– limited access to the microcapsule interior protects the bioelement from the harsh environmental conditions; prevents leakage; – increases the efficiency; no chemical modifications of the capsule material are required for the immobilization; – the activity of the bioelement remains intact.	– the necessity for a very strict pore size control is difficult in the case of small enzymes; – this technique cannot be applied for enzymes similar in size to their reaction product.
 Cross-linking	– low costs; high specificity and activity of the enzyme; – easier recycling; in CLEC method enzymes are much more stable and resistant to denaturation by heat or organic solvents and to proteolysis; – the addition of salts, organic solvents or nonionic polymers determine the aggregation of enzymes which maintain their activity, and increase their stability (CLEA).	– low mechanical stability (CLEA); low reproducibility (CLEA); and low activity retention (CLEA); – the necessity for very high purification of enzyme and crystallization before use is laborious and time consuming (CLEC); – the size of obtained aggregates of enzymes with diameter no greater than 10 μm, because substrate particle may lay in the same size range in a heterogeneous reaction system, and can cause problems for enzymes recovery in continuous processes.

3. GRAPHENE AND GRAPHENE COMPOUNDS USED FOR BIOSENSORS DESIGN

Graphene is the youngest carbon allotrope being discovered in 2004 by Geim and Novoselov who described it as "more than just a flat crystal"; since then, its properties were intensively studied leading to a wide range of nanocomposites (in combination with polymers and nanoparticles) applied in various areas. The use of electrochemical enzymatic biosensors based on graphene in the pharmaceutical and biomedical fields has significantly increased due to their sensitivity, specificity, cost-effective, simplicity, and possibility of miniaturization becoming promising tools for portable devices such as point-of-care (POC) devices (Bo, Zhou, & Guo, 2017).

Graphene is the basic building block for graphitic materials of all other dimensionalities. Graphene is a single layer of sp^2-hybridized carbon atoms that can be folded into fullerenes, rolled into 1D CNTs (Dai, 2002) and can be stacked to obtain 3D graphite. The pristine graphene can be oxidized to graphene oxide (GO) containing oxygen functionalities. It was observed that high amount of oxygen functional groups on the surface of graphene lowers the electron transfer rate. The reduction of GO leads to reduced graphene oxide (rGO) that presents less oxygen functionalities, but allows the recovery of the electrical properties of pristine graphene that was lost after oxidation to GO. All these graphene derivatives can be functionalized, and the composite materials based on graphene are widely used for the immobilization of biomolecules or for the detection of various analytes. The electron transfer rate is high at the edge plane instead of basal plane of graphene. Each carbon atom on the nanocarbon surface is exposed to the environment, and any small changes in the environment can result in drastic changes in the electrical properties of the nanocarbon device, thus forming the basis for ultrasensitive biosensing (Schedin et al., 2007; Wang et al., 2011).

The overall electronic properties of the sensing devices based on graphene and biomolecules can be altered through one of the following mechanisms: surface charge-induced gating, charge transfer, or doping between carbon nanomaterials and biomolecules, a scattering potential across carbon nanomaterials, and Schottky barrier modification between carbon nanomaterials and metal electrodes (Liu & Guo, 2012). The pH, ionic strength, and the type of ions present in the analyzed environment also have a strong influence on the above-mentioned parameters (Heller et al., 2010). Another important feature of graphene is that its dimension is

comparable with that of the molecules to be detected and this important property allows the detection of single-molecule events.

Graphene is a molecular chemical entirely composed of carbon atoms which gives high compatibility to biocompounds and allows controlled and selective functionalization of their surface (Liu & Guo, 2012). Despite its unique properties, graphene can stack together damaging its electrochemical performances. In order to minimize the aggregation level of the graphene sheets, some strategies have been employed such as: noncovalent, covalent functionalization, intercalation of nanomaterials, and structure engineering leading to high exposure of active sites and high utilization of catalysts. Graphene metallic nanocomposites containing metal, metal oxides, or metal alloy nanostructures have improved the biosensor performances for different target analytes due to their synergistic effects (Khalil, Rahmati, Julkapli, & Yehye, 2018; Maduraiveeran, Sasidharan, & Ganesan, 2018).

4. ENZYMES USED FOR ELECTROCHEMICAL BIOSENSORS BASED ON GRAPHENE

4.1 Oxidases-Based Biosensors

4.1.1 GOx-Based Biosensors

GOx is a large, dimeric protein having the molecular weight of 160 kDa. It contains one flavin adenine dinucleotide (FAD) unit per monomer, seated deep inside the enzyme. These redox active prosthetic groups are not covalently bound and may be released from the protein during denaturation. The active site where glucose binds is just above the FAD-binding site, in a deep pocket (Fig. 2). The oxidation of glucose to gluconolactone is performed at the FAD level.

In recent years, graphene has been widely used as a high performance two-dimensional material in the development of biosensors based on GOx and the DET between enzyme and electrode in the absence of any mediator is very desirable. The DET property and enzyme activity of GOx on graphene surface without and with mediators were studied. Experimental results showed that the biosensor had no response to glucose in mediator-free solutions, even though the DET of GOx was observed, indicating that the GOx with DET property lacked enzymatically catalytic activity. Two types of GOx adsorbed on rGO/GCE surface (Fig. 2) are described, one with DET property (DET-GOx), other with enzyme catalytic activity (Act-GOx). The DET-GOx might suffer a massive structural rearrangement when

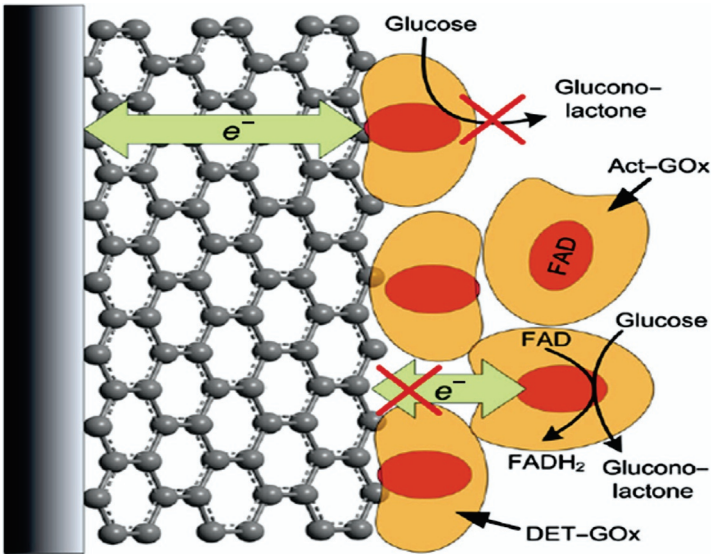


Fig. 2 Illustration of GOx adsorbed on the surface of electrochemically reduced GO-modified GCE (Liang et al., 2015).

adsorbed onto graphene surface, which shortens the distance of FAD to graphene and facilitates the DET with GOx and inactivates the enzyme at the same time (Liang et al., 2015).

4.1.2 Cholesterol Oxidase-Based Biosensors

The determination of cholesterol is of great significance in clinical diagnosis of coronary heart disease, atherosclerosis, myocardial infarction, and brain thrombosis. Among the various biosensors of cholesterol, cholesterol oxidase (ChOx)-based electrochemical biosensors are especially attractive because they offer simple, rapid, and inexpensive means of quantifying cholesterol. Amperometric cholesterol biosensors are based on the ChOx enzyme, which catalyzes the oxidation of cholesterol to 4-cholesten-3-one and generating H_2O_2 in the presence of oxygen. Thus, the quantification of cholesterol can be achieved via electrochemical detection of the enzymatically liberated H_2O_2.

A simple and ultrasensitive cholesterol biosensor based on GO and gold nanoparticles (AuNPs) comediated enzymatic silver deposition was designed by immobilizing ChOx, cholesterol esterase, and GO onto the surface of

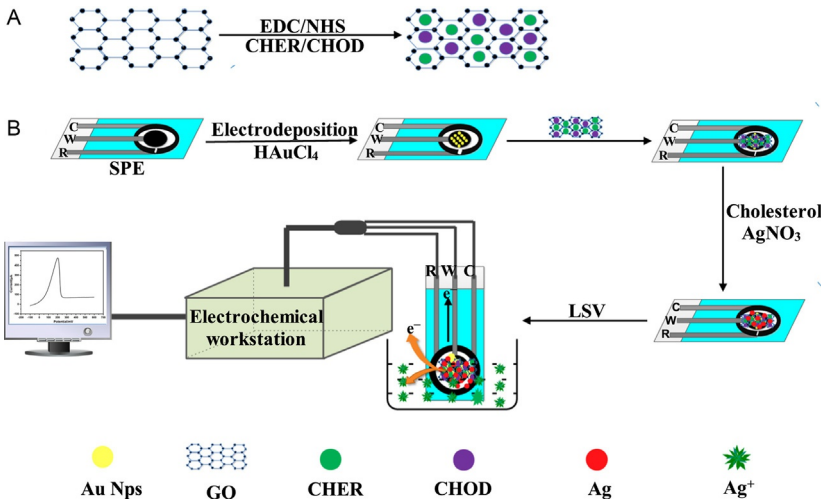


Fig. 3 Electrochemical detection of cholesterol based on GO and AuNPs comediated enzymatic Ag deposition (*LSV*, linear sweep voltammetry; *SPE*, screen-printed electrode) (Huang et al., 2018).

AuNPs modified screen-printed carbon electrode (Fig. 3). First, the carboxyl groups on GO were activated using a mixture of 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) at room temperature followed by the addition of cholesterol esterase (CHER) and cholesterol oxidase (CHOD) solutions and the mixture was incubated at 4°C for 3 h. First step of the experimental protocol used for the preparation of the cholesterol biosensor was the electrochemical generation of AuNPs onto the activated electrode. Then, the enzyme mixture solution was used in order to increase the specificity of the sensor (see the schematic representation of the experimental protocol in Fig. 3). The cholesterol was hydrolyzed to generate hydrogen peroxide which can reduce the silver ions found in the solution to metallic silver that was quantified by anodic stripping voltammetry measurement. The peak current of Ag increased with the increasing cholesterol concentration in a wide range and a limit of detection of 1 ng mL^{-1} was obtained (Huang et al., 2018).

4.1.3 Xanthine Oxidase-Based Biosensors

Xanthines are a group of alkaloids commonly used for their effects as mild stimulants and as bronchodilators, notably in the treatment of asthma

symptoms. Therefore, the detection of these compounds is important, and it has been performed also using electrochemical biosensors. Xanthine oxidases (XOxs) are a class of enzymes that generate reactive oxygen species. These enzymes catalyze the oxidation of hypoxanthine and can further catalyze the oxidation of xanthine to uric acid. These enzymes play an important role in the catabolism of purines in humans.

For example, a novel layer-by-layer biosensor architecture was prepared with poly(dopamine)-modified magnetic nanoparticles (MNP) coated with fourth-generation ethylenediamine core polyamidoamine G-4 dendrimers (PAMAM) through covalent bounding, then decorated with platinum nanoparticles (PtNPs). This nanohybrid was layered on glassy carbon electrodes (GCEs) coated with a GO-carboxymethylcellulose hybrid nanomaterial through electrostatic interactions. The nanostructured surface was then employed as scaffold for the covalent immobilization of the enzyme XOx through a glutaraldehyde-mediated cross-linking. The enzyme electrode allowed the amperometric detection of xanthine in the 50 n*M*–12 μ*M* range, with a high sensitivity of 140 mA M^{-1} cm^{-2} and low detection limit of 13 n*M*. The biosensor exhibited high reproducibility and repeatability, and was successfully tested for the quantification of xanthine in fish. Fig. 4

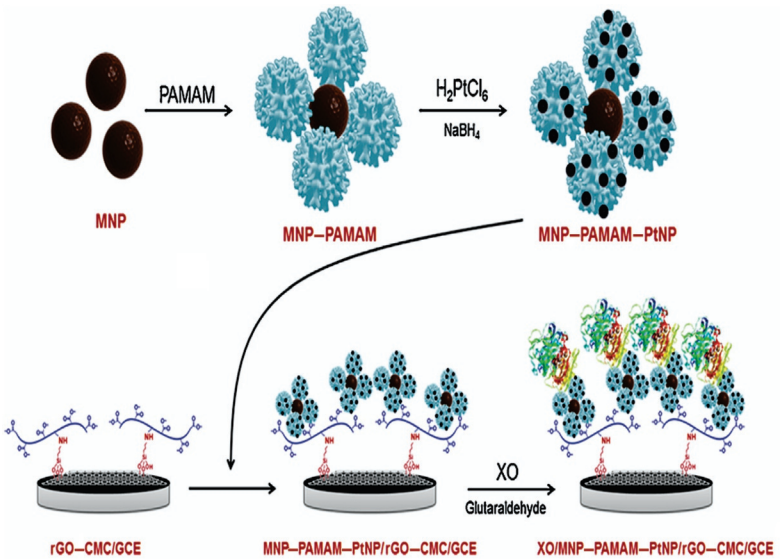


Fig. 4 Preparation of MNP–PAMAM–PtNP and XOx/MNP–PAMAM–PtNP/rGO–CMC/GCE enzyme electrode (Borisova et al., 2016).

shows the steps involved in the preparation of the hybrid nanomaterial and the assembly of the biosensor (Borisova et al., 2016).

Other oxidases used for the development of biosensors with biomedical applications include tyrosinase (Tyr), bilirubin oxidase, lysine oxidase, laccase, and β-galactosidase.

Peroxidases (enzymes that catalyze the substrate oxidization by using H_2O_2 as the oxidizing agent) were also often employed for biosensors development, the most used being horseradish peroxide (HRP) (more details and examples about the uses of HRP in biosensors are presented in Table 3).

4.2 Dehydrogenases-Based Biosensors

Some dehydrogenases including alcohol dehydrogenase (ADH), D-fructose dehydrogenase (FDH), and glutamate dehydrogenase were also employed to develop various electrochemical biosensors. Ethanol biosensors based on ADH employed various platforms based on graphene and Au nanorods and bimetallic nanoparticles. The enzyme was immobilized by physical adsorption and cross-linking with glutaraldehyde (Li, Lu, & Deng, 2013; Tig, 2017).

Electrochemical biosensors based on other types of enzyme such as alkaline phosphatase and urease were also reported.

4.3 Examples of Enzyme-Based Biosensors With Graphene

4.3.1 Biosensors for Pharmaceuticals

The intensive use of drugs in the treatment of various health problems and illness, especially in large cities with many hospitals, can lead to their spreading in the environment and possible contamination especially if released into environment in their complete form, without metabolization. Many of these drugs have a high toxicity and some even mutagenic and carcinogenic potential, posing and causing environmental damage, even at low concentrations (Lutterbeck, Baginska, Machado, & Kümmerer, 2015).

The monitoring of the environmental levels of drugs is therefore essential to minimize the risks associated with these drugs. In addition to environmental and occupational monitoring, the monitoring of plasma concentrations of some drugs is of great importance. This is because ideal treatment in different illness depends on the personalized therapeutic schemes, which are adapted according to the treatment response of each patient. The clinical monitoring of the plasmatic concentration of antineoplastic drugs is also important for the optimization of therapy and management of side effects

because at doses that are too high, side effects and toxicity can occur, while at doses that are too low, the desired effects can be reduced or even lost (Florea et al., 2015).

Considering the large number and variety of different electrochemical biosensors based on enzymes and graphene derivatives already described in the literature, it is clear that this subject has been gaining importance. It is a promising scientific area which could bring great advantages to the human health and to the environment.

Some examples related to this subject can be mentioned. The highly sensitive and selective artesunate (an antimalarial drug) detection in pharmaceutical formulation and biological fluids was achieved by using indium tin oxide electrode electropolymerized with GO-polyaniline nanocomposite film on which horseradish peroxidase was immobilized by using glutaraldehyde as cross-linker. The analytical performances of this biosensor included a linear range of 0.05–0.40 ng mL^{-1}, a limit of detection of 0.012 ng mL^{-1}, and a sensitivity of 0.15 mA ng mL^{-1} (Radhapyari, Kotoky, Das, & Khan, 2013).

The same cross-linkage procedure was used for laccase immobilization on a hybrid nanomaterial based on rGO doped with Sb_2O_5 thin film. The electrochemical biosensor was tested for estriol (an endocrine disrupting compound) determination with high selectivity, fast analytical response, high sensitivity (275 mA M^{-1}), low limit of detection (11 n*M*), and a wide linear range (25 n*M*–1.03 m*M*) (Cincotto et al., 2015).

4.3.2 Biosensors for Physiological Compounds and Metabolites

4.3.2.1 Detection of Glucose

Glucose plays an important role in the diagnosis and management of diabetes mellitus; therefore, its accurate determination was the purpose of a wide range of biosensors. Glucose enzymatic biosensors contain the enzyme GOx which catalyzes the oxidation of glucose to gluconolactone by consuming oxygen and liberating H_2O_2, the process being illustrated by the following equations:

$$\text{Glucose} + \text{GOD/FAD} \rightarrow \text{Gluconolactone} + \text{GOD/FADH}_2 \quad (1)$$

$$\text{GOD/FADH}_2 + \text{O}_2 \rightarrow \text{GOD/FAD} + \text{H}_2\text{O}_2 \quad (2)$$

$$\text{FAD} + 2\text{H}^+ + 2\text{e}^- \leftrightarrow \text{FADH}_2 \quad (3)$$

GOx is an oxidoreductase enzyme which has the active center (FAD) deeply embedded in the protein shell, being the reason why the DET

between FAD and the electrode surface is difficult. The first generation of glucose biosensors measured the oxygen consumption during the catalytic process, while the second generation quantified the H_2O_2 output. The glucose oxidation by GOx in the absence of oxygen is characteristic for the real third-generation glucose biosensors, the reduced form of the enzyme being oxidized at the electrode surface.

Various types of electrodes (GCE, screen-printed electrode (SPE), Au, Sn, Pt electrodes, porous gold electrode) modified with graphene (GO, rGO, nitrogen-doped graphene, graphene nanodots), polymers (polyethyleneimine, polyvinyl alcohol, polyaniline, poly(3,4-ethylenedioxythiophene), polyamidoamine dendrimer), and metallic nanoparticles (AuNPs, Fe_3O_4NPs, Co_3O_4NPs, ZnO, PtNPs) have been tested for the elaboration of sensors for the determination of glucose with outstanding analytical performances: low limit of detection, high sensitivity, wide linear range, good stability, and improved selectivity toward possible interfering substances (uric acid, ascorbic acid, dopamine (DA), NADH, acetaminophen, cholesterol, glycine, D-galactose, urea, L-phenylalanine, L-tyrosine). The characterization of the hybrid nanocomposites concerning its composition, morphology, enzyme modification, and electrochemical properties was achieved by various techniques such as: UV–vis spectrophotometry, Raman and FTIR spectroscopy, ATR, XRD, EDS, SEM, TEM, CV, EIS, amperommetry, linear sweep voltammetry, and differential pulse voltammetry (Feng, Cheng, Pan, & Zheng, 2015).

The noncovalent immobilization of the enzyme was performed by using polymer entrapment in chitosan, nafion, electrostatic interactions (with Fe_3O_4, rGO), π–π interaction (between the pyrene-modified enzymes and graphene), and physical adsorption. The covalent strategies for enzyme immobilization included the amide bond formation by using glutaraldehyde as cross-linking reagent and succinimidyl ester moiety.

The electrocatalytic oxidation of glucose, lactate, and glutamate was achieved on Au electrode modified with polystyrene beads (500 nm diameter) covered by a layer of Prussian blue (PB); after the template removal, the thiol graphene was deposited in the cavities. The enzymes GOx, glutamate oxidase, and lactate oxidase were immobilized by binding to (3-glycidyloxypropyl) trimethoxysilane. The overall protocol used for sensor elaboration and the SEM images obtained during biosensor elaboration are presented in Fig. 5 (Li et al., 2016).

Table 2 contains some examples of biosensors for glucose based on graphene derivatives.

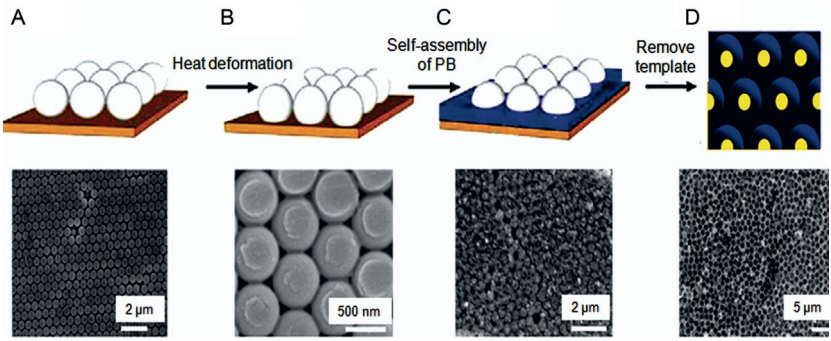


Fig. 5 The Prussian blue film formation on the Au electrode surface by using polystyrene beads (Li, Peng, Chu, Jiang, & Jin, 2016).

4.3.2.2 Detection of Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a by-product which is hydrolyzed by oxidases in many biological processes playing an important role as a signaling molecule. Its determination by using electrochemical biosensors based on heme proteins such as hemoglobin (Hb), HRP, cytochrome *c* (Cyt *c*), catalase (CAT), myoglobin (Mb), and microperoxidase-11 (MP-11) is simple, selective, and sensitive. There are two types of heme protein-based H_2O_2 biosensors: mediated biosensors (second generation needs a mediator for the electronic transfer between the redox proteins and the underlying electrodes) and mediated-free biosensors (third generation with DET) (Zhang & Chen, 2017).

Among the heme proteins, HRP is the most used enzyme in H_2O_2 biosensors. HRP catalyzes the activation of peroxides and subsequent redox reaction as an electron acceptor (Eqs. 4–6). This redox enzyme contains 308 amino acid residues, a heme as prosthetic group (iron protoporphyrin type IX) and two calcium ions, being highly stable for chemical modifications (Nandini et al., 2013).

$$\text{HRP } (Fe^{3+}) + H_2O_2 \rightarrow \text{Compound I } (Fe^{4+}=O) + H_2O \quad (4)$$

$$\text{Compound I } (Fe^{4+}=O) + e^- + H^+ \rightarrow \text{Compound II} \quad (5)$$

$$\text{Compound II} + e^- + H^+ \rightarrow \text{HRP } (Fe^{3+}) + H_2O \quad (6)$$

Graphene-based nanomaterials have been widely employed for H_2O_2 detection because these nanocomposites are a microenvironment which maintains the bioactivity of the redox proteins and also facilitate the electron transfer.

Table 2 Examples of Enzyme-Based Biosensors for Glucose and Related Compounds

Electrode Configuration	Analyte	Linear Range	LOD	Sensitivity	References
rGO-AuNPs/CS-GOx/GCE	Glucose	0.1–1.3 m*M*	76 μ*M*	34 mA M^{-1} cm^{-2}	Bai and Shiu (2014)
rGO/AuNPs/GOx/GCE		0.2–2; 2–20 m*M*	17 μ*M*	56.93; 13.48 μA mM^{-1} cm^{-2}	Wang and Zhang (2013)
rGO-Fe_3O_4/MSPE		0.05–1 m*M*	0.1 μ*M*	5.9 μA mM^{-1}	Pakapongpan and Pooarporn (2017)
rGO-GOx/GCE		0.1–27 m*M*		1.85 mA mM^{-1} cm^{-2}	Unnikrishnan, Palanisamy, and Chen (2013)
AuNPs-PEI-GNS		1–100 μ*M*	0.32 μ*M*	93 μA mM^{-1} cm^{-2}	Rafighi, Tavahodi, and Haghighi (2016)
GOx/PVA-Fe_3O_4/Sn		0.005–30 m*M*	8 μ*M*	9.36 mA mM^{-1}	Sanaeifar et al. (2017)
GF/Co_3O_4-NPs-GOx/GCE		0.5–16.5 m*M*	0.05 m*M*	13.52 μA mM^{-1} cm^{-2}	Karuppiah, Palanisamy, Chen, Veeramani, and Periakaruppan (2014)
CS-GOx/GRA-PANI/Pt		10.0 μ*M*– 1.48 m*M*	2.769 μ*M*	22.1 μA mM^{-1} cm^{-2}	Feng et al. (2015)
GOx/rGO-MWNTs/AuNPs/GCE		10 μ*M*–2 m*M* 2 m*M*– 5.2 m*M*	4.1 μ*M* 0.95 m*M*	0.695 μA mM^{-1} cm^{-2} 0.2380 μA mM^{-1} cm^{-2}	Devasenathipathy et al. (2015)
GOx/rGO/borosilicate glass capillary		10–1000 μ*M*	—	—	Hasan et al. (2015)
GOx/GRA-PEDOT:PSS/SPCE		20–900 μ*M*	0.3 μ*M*	7.23 μA mM^{-1}	Wisitsoraat et al. (2013)
PRGO-AuNPs/GOx/GCE		0.14–4.0 μ*M*	0.06 μ*M*	15.04 mA mM^{-1}	Sabury, Kazemi, and Sharif (2015)
CS-GOX/PTBO/PB/rGO/GCE		20 μ*M*– 1.09 m*M*	8.4 μ*M*	59 mA M^{-1} cm^{-2}	Bai, Chen, and Shiu (2013)

Continued

Table 2 Examples of Enzyme-Based Biosensors for Glucose and Related Compounds—cont'd

Electrode Configuration	Analyte	Linear Range	LOD	Sensitivity	References
GOx/CS/AuNPs/PPy/rGO/GCE		0.2–1.2 m*M*	—	123.8 μA mM^{-1} cm^{-2}	Xue et al. (2014)
rGO/Fe_3O_4/GOx/GCE		0.5–10 m*M*	—	2.645 μA mM^{-1}	Wang et al. (2018)
GOx/Nafion/PANI-TNTs/GCE		10–2500 μ*M*	0.5 μ*M*	11.4 μA mM^{-1} 177.16 μA mM^{-1} cm^{-2}	Zhu, Liu, Wang, Huo, and Yan (2015)
GOx/G-PSE/SPE		0.1–1 m*M*	28.3 μ*M*	32.15 μA mM^{-1}	Chia, Tan, Khiew, Chin, and Siong (2015)
GOx/rGO-CNT-ZnO/GCE		10 μ*M*–6.5 m*M*	4.5 μ*M*	5.36 μA mM^{-1} cm^{-2}	Hwa and Subramani (2014)
GOx/PtNPs/PAMAM-GPTMS-rGO/GCE		10 μ*M*–8.1 m*M*	0.8 μ*M*	24.6 mA *M* cm^{-2}	Araque et al. (2014)
GOx (XOx)/NG/GCE	Glucose	Up to 1.2 m*M*	17 μ*M*	12.8 μA mM^{-1} cm^{-2}	Barsan, Prathish, Sun, and Brett (2014)
	Hypoxanthine		180 μ*M*	135.6 μA mM^{-1} cm^{-2}	
GOx (CAT)/graphene nanodots encaged porous gold electrode	Glucose	0.05–100 m*M*	30 μ*M*	—	Wang et al. (2015)
	H_2O_2	0.005–4 m*M*	1 μ*M*		
GOx (GMOx) (LOx)/tG/PB/Au	Glucose	0.01–0.8 m*M*	—	162 μA mM^{-1} cm^{-2}	Li et al. (2016)
	Glutamate	0.01–0.09 m*M*		93 μA mM^{-1} cm^{-2}	
	Lactate	0.01–0.2 m*M*		70 μA mM^{-1} cm^{-2}	

AuNPs, gold nanoparticles; *CAT*, catalase; *CS*, chitosan; *GCE*, glassy carbon electrode; *GF*, graphene flakes; *GMOx*, glutamate oxidase; *GOx*, glucose oxidase; *GPTMS*, (3-glycidyloxypropyl)trimethoxysilane; *GRA*, graphene; *Lox*, lactate oxidase; *MSPE*, magnetic screen-printed electrode; *MWCNTs*, multiwalled carbon nanotubes; *NG*, nitrogen-doped graphene; *PAMAM*, polyamidoamine; *PANI*, polyaniline; *PB*, Prussian blue; *PEDOT*, poly(3,4-ethylenedioxythiophene); *PEI*, polyethyleneimine; *PRGO*, partially reduced graphene oxide; *PSE*, 1-pyrenebutyric acid *N*-hydroxysuccinimide ester; *PSS*, polystyrene sulfonic acid; *PTBO*, poly(toluidine blue O); *PVA*, polyvinyl alcohol; *rGO*, reduced graphene oxide; *SPCE*, screen-printed carbon electrode; *tG*, thiol graphene; *TNTs*, TiO_2 nanotubes; *Xox*, xanthine oxidase.

Therefore, graphene (as GO, graphene functionalized with COO^-, graphene foam) was used in combination with metallic nanoparticles (such as PdNPs, AuNPs, CeO_2, MoS_2) or polymers (polydopamine, poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate), polypyrrole) for electrochemical sensing of H_2O_2. These nanocomposites were characterized by SEM/EDS, CV, DPV, and EIS.

The HRP enzyme was immobilized by using galvanostatic electrodeposition, covalent grafting to polydopamine, NHS/EDC system, or physical adsorption, encapsulation in biomimetic graphene capsules, nafion, and chitosan.

Some examples that illustrate the use of graphene for the elaboration of biosensors for H_2O_2 detection are presented in Table 3.

4.3.2.3 Detection of Other Analytes With Biomedical Relevance

4.3.2.3.1 Phenols The oxidation of phenolic compounds such as catechol, phenol, cresol, DA, and epinephrine is catalyzed by Tyr or polyphenol oxidase (PPO), a multifunctional oxidoreductase with two copper atoms in the active site, according to Eqs. (7) and (8):

$$\text{Catechol} + \text{tyrosinase } (O_2) \rightarrow o\text{-quinone} + H_2O \quad (7)$$

$$o\text{-Quinone} + 2H + 2e^- \rightarrow \text{catechol (at electrode)} \quad (8)$$

Catechol can also be oxidized by laccase, a multicopper oxidase, as follows:

$$\text{Catechol} + \text{laccase (oxy)} \rightarrow \text{1,2-benzoquinone} + \text{laccase (deoxy)} + 2H^+ + 2e^- \quad (9)$$

$$\text{Laccase (deoxy)} + O_2 + 4H \rightarrow \text{laccase (oxy)} + 2H_2O \quad (10)$$

Various graphene-based nanocomposites have been used for catechol and DA determination such as graphene nanoplatelets decorated with TiO_2 nanotubes, conducting polymer poly(3,4-ethylenedioxythiophene) doped with GO and Fe_2O_3, palladium–copper alloy nanocages supported on rGO, rGO functionalized with β-cyclodextrin (β-CD), and rGO functionalized with pyrrole-β-cyclodextrin. The enzymes were immobilized by using polymers (Nafion, polyethyleneimine, amphiphilic pyrrole), glutaraldehyde or physical adsorption (see Table 4).

4.3.2.3.2 Cholesterol Cholesterol is a lipid steroid metabolite with serious implication in cardiovascular diseases; therefore, its determination is very important for clinical diagnosis. Various electrochemical biosensors

Table 3 Examples of Enzyme-Based Biosensors for Hydrogen Peroxide

Electrode Configuration	Dynamic Range	LOD	Sensitivity	References
(HRP-Pd)/f-graphene/Gr	25 μ*M*–3.5 m*M*	0.05 μ*M*	92.82 μA mM^{-1} cm^{-2}	Nandini et al. (2013)
HRP/PDA/MB-CNTs/3D-G	0.2 μ*M*–1.1 m*M*	58 n*M*	227.8 μA mM^{-1} cm^{-2}	Liu, Wang, Wang, and Wang (2015)
PEDOT:PSS-rGO-AuNPs-HRP/SPGE	5–400 μ*M*	0.08 μ*M*	677 μA mM^{-1} cm^{-2}	Mercante et al. (2017)
rGO/Ppy nanotube/GCE	—	0.1 μ*M*	—	Cai et al. (2017)
GS-PSS/GRCAPS/ITO	0.01–12 m*M*	3.3 μ*M*	—	Fan et al. (2015)
Nafion/HRP/CeO_2-rGO/GCE	0.1–500 μ*M*	0.021 μ*M*	4.65 μA mM^{-1}	Radhakrishnan and Kim (2015)
HRP-MoS_2–GRA/GCE	0.2–1103 μ*M*	0.049 μ*M*	679.7 μA mM^{-1} cm^{-2}	Song, Ni, and Kokot (2014)
CS/HRP/Fc-rGO/GCE	0.35–150 μ*M*	0.10 μ*M*	—	Deng, Zhou, and Li (2013)

3D-G, 3D-graphene foam freestanding electrode; *AuNPs,* gold nanoparticles; *CS,* chitosan; *f-graphene*, graphene functionalized with COO^-; *Fc*, ferrocene; *GCE*, glassy carbon electrode; *Gr*, graphite; *GRA*, graphene; *GRCAPS*, graphene capsule; *GS-PSS*, graphene-poly(sodium 4-styrenesulfonate); *HRP*, horseradish peroxidase; *ITO*, indium tin oxide; *MB*, methylene blue; *PEDOT*, poly(3,4-ethylenedioxythiophene); *Ppy*, polypyrrole; *PSS*, polystyrene sulfonic acid; *rGO*, reduced graphene oxide; *SPGE*, screen-printed graphite electrode.

Table 4 Examples of Enzyme-Based Biosensors for Other Analytes

Electrode Configuration	Analyte	Dynamic Range	LOD	Sensitivity	References
Tyr/[Demim]Br/Nafion/GNPs–TNTs/GCE	Catechol	0.30–110 μ*M*; 110.5–390 μ*M*	0.055 μ*M*	—	Liu, Yan, Zhu, Zhang, and Liu (2015)
PEDOT-rGO-Fe_2O_3-PPO/GCE		0.04–62 μ*M*	7 n*M*	—	Sethuraman, Muthuraja, Anandha Raj, and Manisankar (2016)
Lac/rGO–PdCu NCs/GCE		0.005–1.155 m*M* 1.655–5.155 m*M*	1.5 μ*M* 2.0 μ*M*	12.65 μA mM^{-1} 5.51 μA mM^{-1}	Mei et al. (2015)
rGO/β-CD/Tyr/PEI/GCE	Dopamine	3.9–328.8 μ*M*	3.9 μ*M*	12.1 nA μM^{-1}	Fritea, Tertiş, Cosnier, Cristea, and Săndulescu (2015)
poly-[NEt^{4+}-Py]/Tyr/poly-[β-CD-Py/rGO]/GCE	Catechol	1 n*M*–25 μ*M*	1 n*M*	0.71 A M^{-1} cm^{-2}	Fritea, Le Goff, et al. (2015)
	Dopamine	27 n*M*–38.6 μ*M*	27 n*M*	0.012 A M^{-1} cm^{-2}	
GO&CHER&ChOx/AuNPs/SPE	Cholesterol	0.01–5000 μg mL^{-1}	0.001 μg mL^{-1}	0.084 μA cm^{-2} μg mL^{-1}	Huang et al. (2018)
ChOx/AuNs/GO-SH/Gr		0.05–11.45 m*M*	0.2 n*M*	273 mA mM^{-1} cm^{-2}	Nandini et al. (2013)
G/Ti(G)-3DNS/CS/ChOx/GCE		0.05–8.0 m*M*	6 μ*M*	3.82 μA cm^{-2} mM^{-1}	Komathi, Muthuchamy, Lee, and Gopalan (2016)
ChOx/AuNPs/TGPHs/GCE		0.05–590 μ*M*	0.017 μ*M*	—	Cao, Zhang, Chai, and Yuan (2013)

Continued

Table 4 Examples of Enzyme-Based Biosensors for Other Analytes—cont'd

Electrode Configuration	Analyte	Dynamic Range	LOD	Sensitivity	References
ADH–AuNRs-GN/GCE	Ethanol	5–377 μ*M*	1.5 μ*M*	102 μA mM^{-1} cm^{-2}	Li et al. (2013)
Naf/ADH/ERGO/P(L-cys)/Au-AgNPs/GCE		0.017–1.845 m*M*	5 μ*M*	0.644 μA mM^{-1}	Tig (2017)
ADH/ERGO–PTH/GCE		0.05–1.0 m*M*	0.3 μ*M*	2.8 μA mM^{-1} cm^{-2}	Li et al. (2013)
Sandwich-type sensor based on AuNPs/MgO nanoflowers and GO-AuNPs/GCE	miRNA	0.0001–100 p*M*	0.05 f*M*	—	Shuai, Huang, Zhang, Cao, and Jia (2017)
HRP-AuNPs–Ab2/CEA/MBs–Ab1/G	CEA	5–60 ng mL^{-1}	5 ng mL^{-1}	—	Jin et al. (2014)
FDH/TRGO	Fructose	0.7–8.8 m*M*	0.7 m*M*	14.5 μA mM^{-1} cm^{-2}	Sakinyte, Barkauskas, Gaidukevic, and Razumiene (2015)
β-Gal-GOx/P(1,5-DAN)/G/Pt	Lactose	0–60 μg mL^{-1}	1.3 μg mL^{-1}	1.33 μA μg^{-1} mL^{-1}	Nguyen et al. (2016)
BOx/GONP/NiNPs/ITO	Bilirubin	0.01–600 μ*M*	0.15 n*M*	—	Rawal, Chauhan, Tomar, and Gupta (2017)
BOx/GONP@Ppy/FTO		0.01–500 μ*M*	0.1 n*M*	—	Chauhan, Rawal, Hooda, and Jain (2016)

ZnPh/GO/Urs/GCE	Urea	0.4–22 μ*M*	0.034 μ*M*	44.53 μA mM^{-1}	Selvarajan, Suganthi, and Rajarajan (2018)
Urs-GLDH/GOS/ITO		3.3–19.9 m*M*	2.1 m*M*	2.6 μA mM^{-1} cm^{-2}	Abraham et al. (2014)
LOx/c-MWCNTs–SnO_2–G–CS/GCE	Lysine	0.99 μ*M*–0.16 m*M*	0.15 μ*M*	55.2 μA mM^{-1} cm^{-2}	Kacar, Erden, and Kilic (2017)
Lac/rGO-RhNP/GCE	17β-Estradiol	0.9–11 p*M*	0.54 p*M*	25.7 μA M^{-1} cm^{-1}	Povedano et al. (2017)

β-CD, β-cyclodextrin; *β*-Gal, galactosidase; *3DNS*, 3D nanostacks; *Ab*, antibody; *ADH*, alcohol dehydrogenase; *AgNPs*, silver nanoparticles; *AuNPs*, gold nanoparticles; *AuNRs*, gold nanorods; *AuNs*, gold nanostructures; *BOx*, bilirubin oxidase; *CEA*, carcinoembryonic antigen; *CHER*, cholesterol esterase; *ChOx*, cholesterol oxidase; *CS*, chitosan; *[Demim]Br*, brominated 1-decyl-3-methyl imid-azole; *ERGO*, electrochemically reduced graphene oxide; *FDH*, D-fructose dehydrogenase; *FTO*, fluorine-doped tin oxide; *G*, graphene; *GCE*, glassy carbon electrode; *GLDH*, glutamate dehydrogenase; *GN*, graphene nanosheets; *GNPs*, graphene nanoplatelets; *GO*, graphene oxide; *GONP*, graphene oxide nanoparticles; *GOS*, mesoporous silica particle embedded graphene oxide; *Gr*, graphite; *GR*, graphene; *HRP*, horseradish peroxidase; *ITO*, indium tin oxide; *Lac*, laccase; *Lox*, lysine oxidase; *MB*, methylene blue; *Naf*, Nafion; *NiNPs*, nickel nanoparticles; *PdCuNCs*, palladium–copper alloyed nanocages; *PEDOT*, poly(3,4-ethylenedioxythiophene); *PEI*, polyethylenimine; *poly-[NEt4+-Py]*, poly[12-(pyrrol-1-yl) dodecyl] triethylammonium tetrafluoroborate; *PPO*, polyphenol oxidase; *Ppy*, poly-pyrrole; *Py*, pyrrole; *P(L-Cys)*, poly(L-cysteine); *PTH*, polythionine; *P(1,5-DAN)*, 1,5-polydiaminonaphthalene film; *rGO*, reduced graphene oxide; *RhNPs*, rhodium nanoparticles; *TGPHs*, TiO_2-graphene-Pt-Pd hybrid materials; *TNTs*, TiO_2 nanotubes; *TRGO*, thermally reduced graphene oxide; *Tyr*, tyrosinase; *Urs*, urease; *ZnPh*, zinc phthalocyanine.

were developed based on nanocomposites containing GO, GO-SH, graphene sheets interconnected graphene decorated with AuNPs, TiO_2, and Pt–Pd hybrid material (see Table 4). The enzymes cholesterol oxidase and cholesterol esterase were immobilized by using NHS/EDC system for covalent bonding to graphene and chitosan as binder, respectively (see Table 4).

4.3.2.3.3 Alcohol Ethanol biosensors employed various platforms such as graphene–Au nanorods hybrid nanosheets, bimetallic nanoparticles onto which poly(L-cysteine) and electrochemically rGO were deposited. Yeast ADH used in biosensors is a Zn-containing metalloenzyme catalyzing the interconversion between alcohols and aldehydes or ketones, oxidation reactions which are coupled with the reduction of NAD^+ coenzyme.

4.3.2.3.4 Other Analytes Other substances with biochemical importance and useful for clinical diagnosis, such as miRNA, carcinoembryonic antigen (CEA), fructose, lactose, bilirubin, urea, lysine, and estradiol, have been determined using various graphene-based biosensors. The employed nanocomposites consisted in MgO nanoflower and GO–AuNPs, magnetic beads and enzyme-labeled antibody-AuNP bioconjugate, thermally rGO, graphene/1,5-polydiaminonaphthalene film, GO nanoparticles decorated with NiNPs/polypyrrole, mesoporous silica particle embedded GO, carboxylated multiwalled CNTs–tin oxide nanoparticles–graphene–chitosan, RhNPs deposited on rGO. The enzymes (alkaline phosphatase, HRP, FDH, β-galactosidase, GOx, bilirubin oxidase, urease, glutamate dehydrogenase, lysine oxidase, laccase) have been immobilized by specific reaction between avidin and biotin, physical adsorption, glutaraldehyde, electrostatic interactions, and amide covalent bind (NHS/EDC).

For the detection of DA a first approach developed by our team consisted in the Tyr immobilization in polyethylenimine (PEI) film. The biosensor was elaborated by layer-by-layer (LBL) deposition of: rGO (1 mg/mL, chemical reduction of GO with ascorbic acid), β-CD (1 mg/mL), Tyr (1 mg/mL), and PEI (1 mg/mL, hydroalcoholic solution 1:1). The optimization process included the variation of rGO and Tyr layers, the most sensitive configuration for DA determination was based on one layer of 5 μL rGO and 50 μg Tyr. Furthermore, the Tyr immobilization onto the graphene layer can be reinforced by the inclusion complexes between hydrophobic residues from Tyr shell with β-CD. This biosensor exhibited

good analytical performances such as: linear range from 3.9 to 328.8 μ*M*, limit of detection of 3.9 μ*M*, sensitivity of 12.1 nA/μ*M*, recovery rates of 96.9% and 100.7% for pharmaceutical formulation, 97.25% and 100.19% for urine samples, 98.18% and 101.92% for serum samples, respectively. Interferences studies have been also performed with propylene glycol, ethanol, metabisulfite, glucose, uric acid, and ascorbic acid without any significant modification of the DA signal (Fritea, Tertiş, et al., 2015).

The second strategy was based on Tyr entrapment in an amphiphilic pyrrole derivative [12-(pyrrol-1-yl) dodecyl] triethylammonium tetrafluoroborate] which was electropolymerized at the electrode surface. rGO was noncovalent functionalized with β-CD-pyrrole (β-cyclodextrin-11-pyrrolyl-1-undecyl carboxylic acid amide) leading to an electropolymerizable graphene framework in which the pyrrole derivative bearing a CD moiety acted as an aqueous dispersant (due to the β-CD presence) and also as an electropolymerizable unit (due to the pyrrole presence) generating electrodeposited graphene sheets.

Two possibilities have been tested for the graphene-based biosensor elaboration. The two-step deposition procedure was performed by electrodeposition of poly-(β-CD-pyrrole/rGO) layer followed by the electropolymerization of an adsorbed mixture of amphiphilic pyrrole and Tyr. The one-step technique consisted in the direct electropolymerization of the mixture containing β-CD-pyrrole/rGO, amphiphilic pyrrole, and Tyr. Both configurations (bilayer and mixed layer) generated a maximum current density and sensitivity two times more increased and a lower LOD than in the absence of the electrodeposited graphene layer when the biosensors were tested for catechol and DA amperometric determination. In the case of catechol sensing, the analytical performances recorded on the bilayer configuration were maximum intensity of 171.4 μA cm^{-2}, sensitivity of 0.71 A M^{-1} cm^{-2}, LOD 2.8 n*M*, and linear range 2.8 n*M*–25 μ*M*; meanwhile for the mixed layer were 162.1 μA cm^{-2}, 0.71 A M^{-1} cm^{-2}, 1 n*M* and 1 n*M*–25 μ*M*, respectively. The analytical parameters for DA determination included a maximum intensity of 17.4 μA cm^{-2}, sensitivity of 0.012 A M^{-1} cm^{-2}, LOD 27 n*M*, and linear range 27 n*M*–38.6 μ*M* recorded on the bilayer configuration; meanwhile the parameters obtained on the mixed layer configuration were 13.1 μA cm^{-2}, 0.011 A M^{-1} cm^{-2}, LOD 27 n*M*, linear range 27 n*M*–37 μ*M*, respectively (Fritea, Le Goff, et al., 2015).

In conclusion, the combination of the rGO with CD, Tyr, and PEI enhanced the electroanalytical performances of the biosensor allowing a selective and sensitive detection of DA.

In addition, a new nanostructured graphene framework reinforced by a polypyrrole film was designed and electropolymerized on GCEs leading to the elaboration of rGO-based biosensors applied for catechol and DA sensing.

5. FUTURE TRENDS

The electrochemical biosensors present some advantages compared with other analytical methods including a sensitive, selective, simple, fast, and inexpensive determination of biomolecules. Furthermore, they can be integrated into portable and miniaturized devices such as POC with applications in diagnosis and clinical areas. The great need to bring the laboratory tests much closer to the patient bedside was solved by developing POC diagnostics devices based on electrochemical sensing which offer all the advantages mentioned before, adding also the miniaturized form, portability, easy-to-be used, and disposability.

The main disadvantages of conventional detection methods orientated the researchers to find innovative strategies that will satisfy the demands of POC devices. A set of criteria that an ideal POC device needs to satisfy is currently used by researchers, so-called ASSURED criteria, where the acronym stands for A-affordable, S-sensitive, S-selective, U-user-friendly, R-rapid and robust, *E*-equipment-free, and D-deliverable to end users (Ciui, Jambrec, Sandulescu, & Cristea, 2017; Yu, Maslova, & Hsing, 2017). Electrochemical biosensors are good candidates for future POC devices due to their features and advantages. However, there are more criteria from this list that need to be satisfied. Although several examples of electrochemical biosensors were given through this chapter, their application in the detection of some pharmaceutical compounds and other hazardous compounds in real samples are at the beginning. Those compounds could inhibit the enzymes; therefore, the integration of nanomaterials and other improvements regarding the sensitivity and selectivity of the resulting biosensors must be considered.

Compared to other instrumental methods of chemical analysis, electrochemical biosensors are relatively easy to miniaturize. The main trends in modern electroanalysis include the development of biosensors based on progress in chemical and biochemical methods of molecular recognition, the development of measuring devices of a large integration scale, including sensor arrays, the use of new materials, including nanosized and nanostructured materials, as well as the introduction of flow systems. In order to become ready for the market, a great demand in developing lab-on-a-chip and paper platforms for less assays steps and with interest for *in field* applications are needed, as well as biocompatible devices (for in vitro analysis).

The employment of new materials for easy to use and multidetection devices are also requirements for successfully, environmentally, and human friendly biosensors for biomedical analysis.

6. CONCLUSIONS

The outstanding electrochemical properties of graphene and graphene-based materials determined their intense use as platforms for the construction of a wide range of electrochemical biosensors with improved analytical behavior. Graphene and its composites have shown great potentials in biosensing even that there are issues to be solved in the future: the reproducibility and the stability of the nanocomposites with graphene that must be improved and the possible (and not yet entirely proved) toxicity of the graphene that limits its application to in vivo analysis.

Enzymes, with their high catalytic activity and specificity, have been intensively included in biosensors development using various immobilization methods such as adsorption, disulfide binding, metal chelation, covalent bonding, entrapment, encapsulation, and cross-linking.

Several important physical, chemical, electrical, and optical properties of graphene were summarized, which make them one of the best materials used for the transduction of signals associated with the recognition of analytes. Moreover, there is a huge category of analytes that can be considered for detection using graphene-based platforms such as biomarkers for different diseases, metabolites, and drugs. Besides, graphene can serve as scaffolds for immobilization of biomolecules on their surface. The electrocatalytic activity of graphene-modified transducers and electrodes toward hydrogen peroxide and NADH permits effective low-potential amperometric detection of various important analytes for a wide range of applications with practical relevance. Furthermore, graphene-based biosensors have huge potential for in vivo detection with less cytotoxicity, high sensitivity, and long-term stability for reliable POC diagnostics under physiological conditions. These applications are not restricted to the biomedical field but also have various applications in the food quality assurance, water purification, agriculture, industry, and environment monitoring.

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