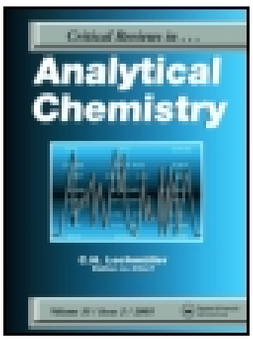

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Electrochemical Enzyme Biosensors Revisited: Old Solutions for New Problems

Tiago Monteiro[a] and Maria Gabriela Almeida [b]

[a]UCIBIO-REQUIMTE, Faculdade de Ciências e Tecnologia, Universidade NOVA de Lisboa, Caparica, Portugal; [b]CiiEM, Instituto Universitário Egas Moniz, Monte de Caparica, Caparica, Portugal

ABSTRACT

Worldwide legislation is driving the development of novel and highly efficient analytical tools for assessing the composition of every material that interacts with Consumers or Nature. The biosensor technology is one of the most active R&D domains of Analytical Sciences focused on the challenge of taking analytical chemistry to the field. Electrochemical biosensors based on redox enzymes, in particular, are highly appealing due to their usual quick response, high selectivity and sensitivity, low cost and portable dimensions.

This review paper aims to provide an overview of the most important advances made in the field since the proposal of the first biosensor, the well-known hand-held glucose meter. The first section addresses the current needs and challenges for novel analytical tools, followed by a brief description of the different components and configurations of biosensing devices, and the fundamentals of enzyme kinetics and amperometry. The following sections emphasize on enzyme-based amperometric biosensors and the different stages of their development.

KEYWORDS

Amperometry; analysis and diagnostics; biosensors; electron transfer principles; redox enzymes

Introduction

Worldwide demands for better analytical tools

The last decades have witnessed a remarkable increase in the social and political awareness for the need of monitoring and controlling many agricultural practices and industrial processes. The surveillance of these anthropogenic activities aims to minimize their impact on the environment and human health. For this reason, the most important government agencies including the European Union council,[1,2] the Food and Drug Administration (FDA)[3] and the Environmental Protection Agency (EPA)[4] in the United States, the World Health Organization (WHO)[5] and the Food and Agriculture Organization (FAO) of the United Nations, as well as some National authorities, have promulgated rules and directives to restrict the level of many chemical and biological species in environmental samples (waters, soils and air), foodstuff and industrial products. Ranging from simple inorganic ions and molecules to complex organic compounds and microorganisms, the list of substances to be monitored is expanding very quickly due to the permanent recognition of new hazardous species. This challenges the Analytical Sciences to develop innovative and improved methodologies that enable the straightforward inspection of complex matrices and, at the same time, the delivery of fast and accurate results.[6–13]

Looking into another direction, clinical diagnosis and research in biomedical sciences are also demanding optimal sensors tailored for the specific needs of real-time analysis, either *in vitro* or *in vivo*. Quite often, not only the identification and quantification of physiological species is required, but data on their distribution in space and time is also entailed. The real-time *in vivo* measurement of neurotransmitters in the extracellular space, for instance, is essential for the quantitative understanding of health and disease.[14,15] On the other hand, the fast point-of-care testing (POCT) of time-critical parameters like blood metabolites and cardiac markers accelerates decision-making in the emergency room and intensive care units.[16,17] Moreover, many POCT can be self-administered, thus reducing the frequency of visits to hospitals, clinics or physician offices and making patients much more responsible for managing their own condition(s).[7] Theranostics, the combination of diagnosis with therapy for individuals, is a recent trend in personalized medicine that aims not only to improve the efficacy of medications through a better administration but also to tailor treatments to individual patients based on test results.[18]

The request for novel and better analytical tools does not come only from the *environmental monitoring, industrial processing* and *health care* fields. In fact, the toxicology and forensic sciences have also to deal with the pressure of a short time of analysis, low detection limits and accuracy of identification, as illustrated by the current needs for both bulk and trace detection of toxins and illicit substances in order to address safety issues like the adulteration or poisoning of food and beverages and the illegal trafficking of drugs across borders.[19,20] The fight against doping in sports, as issued by the World Anti-Doping Agency, also demands the employment of quick, very sensitive and reliable analytical assays.[21] Furthermore, the contemporary challenges and needs for homeland defense and security are driving the R&D of optimized surveillance systems for providing either a warning or the early detection of

CONTACT Maria Gabriela Almeida mg.almeida@fct.unl.pt CiiEM, Instituto Universitário Egas Moniz, Monte de Caparica, 2829-511, Caparica, Portugal.
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chemical (e.g., neurotoxins, explosives) and biological (highly pathogenic bacteria, spores or virus) warfare agents that can be potentially used in terrorist activities.[8,20,22–25]

In conclusion, there is no doubt that the continuous research into the effective detection and quantification of a wide range of analytes is crucial to face all sorts of risks for the public health and the environment. If possible, the diagnosis solutions should be offered in a miniaturized format; thus, allowing the shift from central laboratories into non-laboratory settings. Still, taking analytical chemistry to the field is a major challenge. One of the most appealing strategies relies on the integration of biological and technological elements in a compact fashion, the so-called *biosensor*. Besides enhancing the selectivity and sensitivity of detection, biosensing instruments turn complex laboratory analysis into cheap, simple and quick tasks.

The history of biosensors begins in the early 1960s with a major breakthrough in analytical chemistry, namely the construction of a glucose biosensor based on the coupling between an enzyme (glucose oxidase, GO*x*) and an electrode transducer, as proposed in the pioneering work of Clark and Lyons (this and other milestones were reviewed elsewhere[26–28]). About 10 years later, this innovative strategy made possible the launch of the first commercial glucose analyzer (Yellow Springs Instrument Company) followed, in the late 1980s, by a number of hand-held devices for glucose self-monitoring, which had an enormous impact in clinical diagnosis. Thanks to these small and simply to use instruments, the blood glucose levels in patients with *diabetes mellitus* can be monitored by themselves, at home, with very little time and effort.[26,28–30] Thereafter, the concept of enzyme electrodes attracted much attention and was further expanded to other biological materials and transducing platforms. The rapid technological evolution that has taken place in the field, led to a plethora of biosensing apparatus that can vary in both the biorecognition and/or transducing strategies as well as in the type of application. Still, most of the biosensors commercially available are targeted for health care and biomedical applications like glucose, lactate, creatinine, urea and cholesterol testing, where one can find the best market opportunities and the potential revenues are significant.[16,31,32] Numerous scientific articles, review manuscripts, book chapters and full books devoted to the investigation, development and application of Biosensors are available in the literature (only a few could be referred herein). This not only reflects the intensive activity in this research area for almost six decades, but also expresses the versatility of biosensing devices either in terms of principles, formats or purposes.

Analytical tools: Needs and challenges

Ideally, a good quality analytical method should meet a long list of requirements. First, *selectivity* (ability to distinguish the analyte from interferences, expressed as a percentage of response variation) and *sensitivity* (slope of the calibration curve within the linear concentration range) should be optimized. The *detection limit* (lowest concentration that can be detected with an acceptable signal-to-noise ratio, usually 3xS/N) should be low enough and the *dynamic range* (range of concentrations displaying a linear response) should cover as many orders of magnitude as possible, in order to avoid sample dilution or preconcentration. The quantification must also be *accurate* and *precise* (low relative standard deviation, RSD) and needs to be validated in real samples through standard techniques.[33,34]

In addition, a good analytical performance often requires a short turnaround time and a high speed of analysis, which is especially critical in emergency situations.[17,33–36] This means that the protocol should be *simple* to execute, even by untrained personnel.[35,37] Sample processing should be fast and the *throughput* (number of individual samples per unit of time) should be high.[34] Preferably, the method should provide simultaneous information on several analytes through a multisensing approach.[15,38] Evidently, automation of techniques would be advantageous for the achievement of these last goals.

To enable on-site measurements, analytical devices should be *portable*, i.e., they need to be small but robust. Also, they should deliver a stable response and have a long shelf life (expressed in reference to the initial sensitivity).[35–37] For implantable applications, the materials have to be necessarily compatible with the human body (implants should not affect the normal functioning of the host medium and vice-versa).[15,36,39] Very importantly, employed materials must not be harmful to the environment.[34]

In order to be competitive, the new analytical method should be cheaper and more efficient than conventional protocols, unless it is so innovative that it is interesting by itself.[40,41]

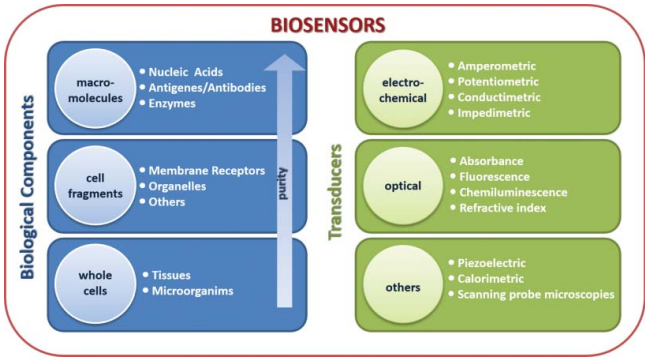
Last but not the least, the analytical target should have a potential market niche, while the testing system should be suitable for mass production. These two features are essential to make the sensor commercially viable.[37,40,41]

Biosensors

Definition

To meet the aforementioned requirements, the Analytical Sciences found in Nature a very good help. Indeed, molecular recognition phenomena in living systems are usually very selective, sensitive and quick; normally, enzymes, antibodies, nucleic acids and biological receptors play the central role in such processes. Taking this into consideration, one of the most important strategies for the development of improved analytical tools relies in the combination of a biological element that is able to recognize the analyte in complex samples, with a physicochemical transducer that translates the biorecognition event into a discrete or continuous electronic signal proportional to the analyte concentration. Ultimately, the outcome is a self-contained integrated apparatus that provides a quantitative readout, although semi-quantitative (*above* or *below* a specific threshold) or qualitative (*yes* or *no*) results could be sufficient for certain applications.[36,41]

According to the IUPAC recommendations, analytical instruments which do not incorporate a biological sensing element cannot be considered biosensors, even if applied to the analysis of biological samples. Furthermore, a biosensor should be a reagent-free device, i.e., the addition of specific reagents is not supposed to happen – this does not include the enzymes



Scheme 1. Examples of biological elements and transducers commonly used in the construction of biosensing devices. Biological components can range from highly pure macromolecules to single cells (bacteria and fungi) or even complex tissues (from plant or animal sources).

co-substrates naturally present in sample matrices. Additionally, no pre-separation steps or sample processing should make part of the analytical assay.[36]

Scheme 1 enumerates the biological and transducer elements usually employed to construct biosensors. Electrochemical and optical transducers are the most common.[42,43]

Some combinations of biological components and transducing systems work particularly well. The electrochemical enzyme-based biosensors are of special interest since they usually meet the most important criteria for a good analytical performance. Indeed, the coupling between the selectivity and sensitivity of biological catalysts with the portability and low cost of electrochemical instrumentation, enables the mass production of sensing systems that are not affected by the turbidity of samples and are well suited for the real-time and on-site analysis of complex samples.

Classification

Biosensors may be classified according to the nature of the biorecognition element, the mode of signal transduction or a combination of both. Biosensors may be further grouped according to the type of target analyte. In parallel, they can also be classified as *catalytic* or *affinity* systems, depending on the conferring principle of biological selectivity.[36]

Catalytic biosensors

In this class of biosensors, besides the already mentioned purified enzymes, any cell organelle, whole cell or tissue slice displaying appropriate catalytic activity can be employed as biorecognition element[1]. In fact, these biological materials are rich sources of enzymes that may be employed directly, with minimal preparation. Due to the stabilization of biocatalysts in their natural environment, they display improved lifetimes and are less affected by experimental conditions (inhibitors, temperature, pH, ionic strength, solvents, etc.). Moreover, given that no purification steps are involved, they are much cheaper than isolated proteins. On the other hand, response times are usually slower, the detection is less selective and the catalytic activity is lower.[33,44] Microorganisms, in particular, can be more complicated to use, especially if they have to be kept alive during measurements (nutrients must be supplied, and wastes removed) as for the whole-cell sensors used in pollution control. To surpass these issues, genetically modified microorganisms have been applied to the development of microbial biosensors.[44–47]

All catalytic biosensors rely on the measurement of the steady-state concentration of a detectable species (typically, a low molecular weight compound[48]), either lost or formed over a biocatalytic reaction. The very first biosensors, for example, were based on oxidase enzymes like GO*x*, which not only depend on an electroactive species such as oxygen, but also produce another electroactive compound, hydrogen peroxide. Therefore, both the co-substrate and product can be monitored through an amperometric method.[29,44,49]

Besides the enzymes substrates and co-substrates, indirect monitoring of *inhibitors* or *activators* is also possible,[36,50] a strategy often followed to detect several contaminants of waters and soils. Enzyme inhibition can be either reversible or irreversible; in the latter case, the biosensors regeneration can be compromised, precluding the continuity of its use.[51]

Affinity biosensors

This second class of biosensors comprises all detection systems based on the formation of a stable complex between the biological component and the analyte species, like the binding of antibodies, nucleic acids and biological receptors with their affinity ligands. In such non-catalytic biosensors, equilibrium is generally reached and no further net consumption of analyte occurs.[36,44,52] For this reason, affinity biosensors normally offer a narrow linear operating range of concentrations.[36]

On top of the high selectivity for their targets, oligonucleotide hybridization and immunochemical reactions often possess remarkably high affinity constants. Consequently, sensors based on these molecular recognition principles usually display extremely low detection limits. On the other hand, the complex formation may be *irreversible*, so they may not be reusable or able to monitor the analyte concentration continuously.[52] Hence, affinity biosensors are better suited for single-use configurations. Despite other options, optical and mass-sensitive analytical techniques are usually engaged in signal transduction.

For a long time, biological recognition elements were assumed to be isolated from biological materials only. Presently, *aptamers*, artificial short oligonucleotides sequences (ssDNA or RNA) synthesized *in vitro* that bind tightly to a specific target (amino acids, drugs, proteins, among other molecules), are also being employed in affinity biosensors.[53–55]

Configurations

As previously mentioned, some biosensing devices are not fitted to be used repeatedly, neither following an intermittent fashion nor a continually (or nearly continually) mode. On the contrary, they are designed to be used one single time and discarded immediately after. In practical terms, biosensors can adopt one of the following configurations:

[1]Some antibodies have demonstrated to possess catalytic activity.

i) Single use

Single use biosensors are unable to monitor the analyte concentration over time. As so, all disposable and non-regenerative devices fall in this category.[36] The single use approach clearly dominates the biosensor market.[56] The typical outcome is a small, user friendly instrument with digital readout, coupled to a throw-away sensing system. An enzyme modified thin film electrode associated to a portable potentiostat, like the home blood glucose biosensor, illustrates the concept very well.

Because single use biosensors are manufactured through a mass production technology and are not prone to calibration, reproducibility within batches is extremely important.[44]

ii) Intermittent use

These biosensors can be rapidly and reproducibly regenerated after each assay. Therefore, they can be reused many times, with the maximum number of utilizations depending on the biosensing system. Instruments based on intermittent use are typically benchtop units that can be easily calibrated and eventually automated.[56]

iii) Continuous use

Continuous use biosensors are similar to the previous ones in regard to reusability, since they can be easily regenerated between measurements. The main difference is the fact that the sensor is dipped into the sample (*in situ* systems), continually displaying readings that reflect the analyte concentration as a function of time.[56] They can also be integrated with on-line process monitoring schemes (entailing continuous or discontinuous sampling of a process stream)[57] or implanted in cells or human tissues.[39,58]

Enzyme amperometric biosensors

Redox enzymes and transduction strategies

According to the Protein Data Bank (https://www.rcsb.org/stats/enzyme), about 15% of the enzymes known today are oxidoreductases. They catalyze electron transfer (ET) reactions that are fundamental for a wide range of biological functions, such as gene regulation, cell signaling, energy conversion (photosynthesis and respiration), drug detoxification and cellular defense. In general, redox enzymes make use of non-polypeptide groups, so called co-factors, to carry out catalysis. These groups can either participate directly in the enzyme active site or be involved in internal charge transfer processes, thereby donating (or accepting) electrons to the catalytic center. Redox cofactors may be *organic* such as quinones, flavines and nicotinamide adenine dinucleotide (phosphate) [NAD(P)/NAD(P)H)] or *inorganic* like metal clusters composed of copper, iron or molybdenum. They can be covalently (prosthetic groups) or non-covalently bound to the protein backbone; alternatively, coenzymes can freely diffuse and bind to the catalytic site during turnover. The redox potentials of enzyme cofactors are confined to a relatively narrow potential window of −1 to +1 V vs normal hydrogen electrode (NHE).[52,59–61]

The substrates of many redox enzymes are extremely important from the analytical point-of-view. This is the case of a large number of sugars (e.g., glucose or lactate) that are oxidized by the oxidases[31,62] and/or dehydrogenases superfamilies[63] and many phenol and xenobiotic compounds that are monooxygenated by enzymes from the tyrosinases[64,65] and cytochrome P450 families,[66] respectively. Also, some substrate reducing enzymes exhibit catalytic activity in the presence of relevant analytes like nitrite.[67] In all situations, the substrate recognition event is associated to an ET process, which makes electrochemical methods excellent platforms for signal transduction.[52]

Depending on the property to be measured (current, potential or impedance), the electrochemical methods applied in the construction of biosensors can be organized in three main categories: amperometric, potentiometric and impedimetric, respectively.[36,49,68] This article will focus only on those techniques that measure current because they are the most appropriate to translate redox reactions into electronic signals and are also suited for mass production.[68] However, the direct electronic communication between protein cofactors and electrode surfaces is normally difficult to achieve. The distance between both partners is too long to enable direct electron transfer (DET), unless the redox groups are correctly oriented toward the electrode interface and not deeply buried inside the polypeptide chain.[69,70] In order to enable the amperometric transduction of biocatalytic reactions, a number of alternative approaches have been developed based on the electrochemical monitoring of electroactive co-substrates and products or, alternatively, on the enzyme coupling to redox mediators.

In another perspective, the enzyme–enzyme coupling has been exploited to amplify the sensitivity of electrochemical biosensors as well as to expand the applicability of biosensing technology to other analytes. The experimental strategy is typically based on the recycling of substrate molecules between at least one pair of enzymes, in a sequential (cascade) or cyclic fashion. The biosensor architecture may involve as well additional enzymes for the removal of interfering species through competition reactions.[44,60]

Fundamental aspects of enzyme kinetics

This section aims to briefly remind the basic concepts of enzyme kinetics (a detailed description can be found in any Biochemistry text book). The simplest mechanism for an enzyme (E) catalyzed reaction, involving only one single reactant (substrate, S), starts with the fast and reversible formation of the enzyme–substrate complex (ES), which breaks down slowly and irreversibly into the free enzyme and the reaction product (P), as follows[40,44,59,71]:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P \quad (1)$$

In the above scheme, each elementary reaction is characterized by a rate constant: k_1 (s^{-1}), and k_{-1} (s^{-1}) correspond to the forward and reverse rate constants, respectively, for the substrate binding step, while k_2 (s^{-1}) is the rate constant for the catalytic reaction. It is assumed that $k_{-1} >> k_2$, so that the

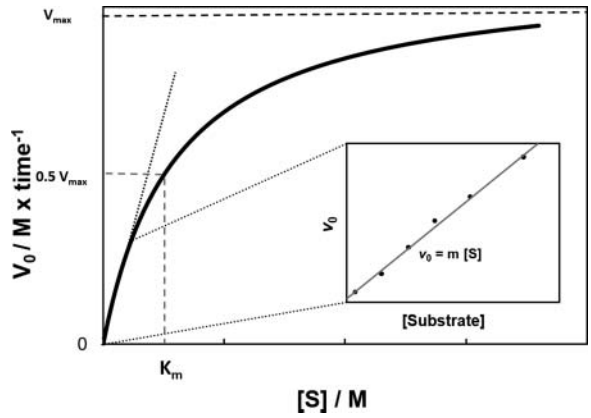


Figure 1. The Michaelis–Menten curve for an enzyme-catalyzed reaction: the initial reaction rate v_0 as a function of $[S]_0$, at a constant $[E]$, is described by a rectangular hyperbola. At very high $[S]_0$, the enzyme is saturated and v_0 reaches the maximum value ($v_{0,max}$). At low $[S]_0$, the reaction rate is directly proportional to $[S]_0$. This region of the plot is analytically useful for substrate quantification, the slope (m) of the calibration curve (straight line) providing the sensitivity of detection. When v_0 is one-half of $v_{0,max}$, the substrate concentration corresponds to the Michaelis–Menten constant, K_m.

formation of *ES* can be treated as a rapid equilibrium process and the second step is rate-limiting. The reaction rate v (M s^{-1}) can thus be defined as the product of the smaller rate constant k_2, commonly denoted as the catalytic constant[2] (k_{cat}), and the concentration of the *ES* complex:

$$v = k_{cat} \times [ES] \tag{2}$$

It is more practical, though, to describe the enzyme kinetics in terms of the easily measurable initial substrate concentration ($[S]_0$) and the initial reaction rate (v_0), and as exemplified in Figure 1. The mathematical relationship between these two quantities follows a Michaelis–Menten profile (rectangular hyperbola), given by the equation:

$$v_0 = \frac{v_{0,max} \times [S]_0}{K_m + [S]_0} \tag{3}$$

where $v_{0,max}$ is the maximum initial reaction rate and K_m (M) is the Michaelis–Menten constant, defined as

$$K_m = \frac{k_{-1} + k_2}{k_1} \tag{4}$$

Note that if $k_{-1} >> k_2$ as previously assumed, K_m can be expressed as the ratio k_{-1}/k_1, which is equivalent to the dissociation constant (K_d) for the *ES* complex. Hence, the K_m value provides an indication of the binding strength between an enzyme and a given substrate or, in other words, the enzyme affinity for the substrate[3] (a low K_m signifies that the enzyme binds the substrate strongly; conversely, a high K_m indicates a lower affinity for the substrate).

[2]The catalytic constant, k_{cat}, is also known as the *turnover number* because it represents the number of substrate molecules converted to product per second and per enzyme molecule; the higher the k_{cat}, the more rapid the reaction.

[3]However, care should be taken when using K_m as an estimate for the K_d of the *ES* complex because a high K_m could also be the result of a very large k_{cat} [see Eq. (4)].

As observed in Figure 1, at low concentrations of the substrate ($[S]_0 << K_m$), v_0 is linearly related to $[S]_0$, as in a first-order reaction [Eq. (5)]. From the analytical standpoint, the range of concentrations that verify this condition defines the limits of the calibration curve.

$$v_0 = \frac{v_{0,max} \times [S]_0}{K_m} \tag{5}$$

Because the decomposition of the *ES* complex [Eq. (1), second step] is rate-limiting, at high substrate concentrations ($[S]_0 >> K_m$) the enzyme is steadily saturated by the substrate. Consequently, v_0 is no longer dependent on $[S]_0$ and the catalytic reaction obeys a zero-order kinetics: $v_0 = v_{0,max}$ (Figure 1, plateau-like region). At this stage, v_0 reaches its maximum value and is limited only by the experimental conditions (temperature, pH, ionic strength), the total enzyme concentration ($[E]_0$) and the catalytic constant (k_{cat}). According to this

$$v_{0,max} = k_{cat} \times [E]_0 \tag{6}$$

The Michaelis–Menten equation [Eq. (3)] also tell us that at $v_0 = v_{0,max}/2$, $K_m = [S]_0$; that is, the K_m value corresponds to the initial substrate concentration at which the reaction rate is half-maximum.

In the construction of enzyme electrodes, it is desirable to obtain the highest $v_{0,max}$ and lowest K_m possible values, thereby, providing a fast reaction rate combined with a high affinity (selectivity) for the substrate/analyte.[71]

It should be mentioned that the theoretical approach presented above is rather simplistic. Actually, the assumptions and simplifications underlying the kinetic model of Michaelis–Menten were not explained here. Moreover, most enzyme reactions involve more than one substrate; some are prone to allosteric effects and may lessen the catalytic activity in the presence of inhibitors.

In the context of cyclic voltammetry (CV) and steady-state techniques, the transduction of the catalytic activity of a redox enzyme into an electrical signal may be accomplished by DET or, alternatively, by means of a reversible redox couple (one of the two forms should serve as a co-substrate to the enzyme). In general, if both substrate and co-substrate exhibit a Michaelis–Menten behavior, the resulting catalytic currents (i_{cat}) can be fitted to the following equation:

$$i_{cat} = \frac{i_{max} \times [S]_0}{K_m + [S]_0} \tag{7}$$

where i_{max} represents the maximum catalytic current that is observed at enzyme saturating conditions (Figure 2, black curve).[72–75][4]

This relationship may be exploited for sensing the enzyme substrate. The slope of the calibration curve ($\Delta i_{cat}/\Delta[S]_0$), at first-order kinetics, represents the sensitivity of the biosensor; it can be expressed in $A \cdot M^{-1}$ or $A \cdot M^{-1} \cdot cm^{-2}$. It must be stressed that when the enzyme is immobilized and the kinetics is fast,

[4]Complete theoretical descriptions on bioelectrocatalytic models and their influencing factors were presented by several authors, such as Bartlett[84] and Limoges,[72,73] among others.

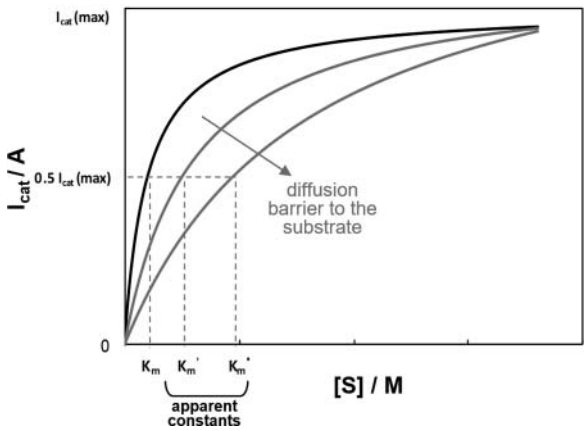


Figure 2. Catalytic current as a function of the concentration of the enzyme substrate. Typically, a Michaelis–Menten profile is observed, which is largely non-linear (black curve). Though, the immobilization membrane acts as a barrier to the substrate diffusion; thereby, reducing its local concentration. In such diffusion-control conditions (gray curves), the linearity is extended and the response is no longer controlled by the enzyme kinetics. Also, the apparent Michaelis constant (K_m^{app}) increases several times.

the overall reaction rate and, therefore, the sensitivity of detection may be partly influenced by mass transfer limitations (Figure 2, gray curves), which critically depend on the thickness and permeability of the surface sensing coat.[40]

Amperometric methods – theoretical aspects

Dynamic voltammetric techniques are very useful to monitor enzyme-catalyzed redox reactions; thereby, elucidating the properties of both biosensor constructs and immobilized enzymes. CV, in particular, is normally used to evaluate the reversibility of the heterogeneous ET reaction, as well as to assess the mass transport regime that governs the electrode response (diffusion or surface control) and to detect chemical reactions that could be coupled to the electrochemical process (one of the simplest schemes corresponds to the EC mechanism where the interfacial ET process is followed by a homogeneous chemical reaction).[75–77] Nonetheless, to obtain quantitative information on the analyte of interest, chronoamperometric measurements are preferentially made.[52,78]

No matter the technique, the transducer element is usually a solid *working electrode* made of a noble metal like platinum or gold, or a carbonaceous material such as glassy carbon, boron doped diamond, graphite paste, pyrolytic graphite, carbon nanotubes (CNTs), graphene or nanoparticles (NPs).[40,49,52] It can be used in a wide range of *size* (micro-to-macro), *thickness* (bulky, thick or thin film), and *shape* (planar, fibers, rods), either *alone* or in an *array* platform.[40,49] In chronoamperometric assays, the working electrode is poised at a constant potential[5] (E) with respect to a *reference electrode*[6] (usually a silver wire coated with a layer of silver chloride, Ag/AgCl) in order to drive an interfacial redox reaction (electrochemical oxidation or reduction of an electroactive species)[35,49,68]. The current (i) thus generated is proportional to the concentration of the analyte in the sample; to be more precise, it is a direct measurement of the electrochemical reaction rate, as described by the Faraday's law:

$$i = nFAv \tag{8}$$

where n is the number of electrons transferred in the electrochemical reaction, F is the Faraday constant, v is the oxidation or reduction rate (in mol s^{-1} cm^{-2}) and A is the electrode area (cm^2). The reaction rate depends on the heterogeneous ET rate and on the mass transport of redox reactants to the surface.[40] By increasing the overvoltage (deviation from the equilibrium potential), the rate of ET can be considerably accelerated; consequently, the concentration of the electroactive species at the electrode surface becomes zero.[44] Under these conditions, the overall process is controlled by diffusion, i.e., the current output (i) is limited by the maximum rate of mass transport. At a planar electrode and a non-stirred solution, the diffusion-controlled current decays with time (t) and depends on the bulk concentration of the electroactive species (C), according to the Cottrell equation:

$$i = nFAC\sqrt{\frac{D}{\pi t}} \tag{9}$$

where D is the diffusion coefficient. Despite the progressive broadening of the diffusion layer into the bulk solution, the current intensity never reaches zero due to the natural convection; even after long time intervals, low currents are still observed.[40] In many practical applications, solution convection is forced either through rotating the working electrode or flowing samples across the electrode, as in flow analysis methods.[40,49] Under such conditions, the mass transport to the electrode interface is controlled by the diffusion of redox species through a much thinner layer (δ) and therefore, the steady state is reached in a very short time. Still, the current depends on the analyte concentration, as described by the following equation:

$$i = \frac{nFADC}{\delta} \tag{10}$$

Because any redox active substance being electrochemically converted at lower potentials will contribute to the total current and interfere with the analytical quantification, the choice of the operating potential is crucial for the selectivity of the analysis. From this standpoint, the working potential should be kept as low as possible (absolute values); in other words, the applied potential should be close to zero volts (versus the standard hydrogen electrode).[44] Therefore, the driving force (electrode potential) should be both sufficient to generate a diffusion controlled current and small enough to minimize electrochemical interferences.

To sum up, amperometric analysis is relatively complex and can be considered as a succession of ET and mass transport events; the rate of each step depends on several parameters,

[5] It is the absence of a sweeping potential that distinguishes amperometry from voltammetry.[49]

[6] If the resulting currents are very low (from 10^{-9} to 10^{-6} A), the reference electrode can carry the charge from electrolysis without changing its formal potential and these two electrodes are sufficient to set up the electrochemical cell. If not, a third *auxiliary electrode* made of an inert conducting material, like Pt, is employed in a three-electrode arrangement. In this way, the charge from electrolysis passes through the counter electrode; thus, avoiding the polarization of the reference electrode.[36,40,49,68]

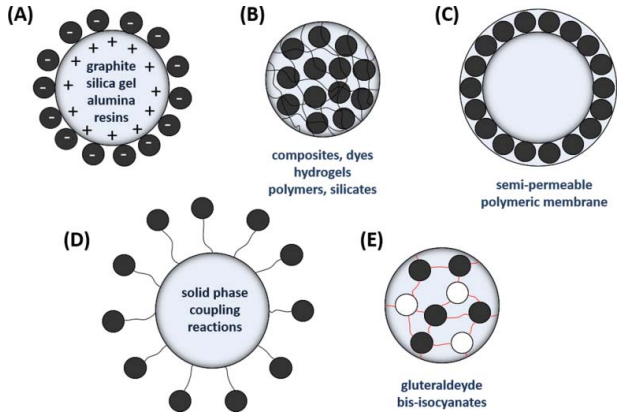
namely the nature of the electrode surface, the working potential and the operating conditions. Obviously, further coupling of chemical events like enzyme-catalyzed reactions increases the degree of complexity of the whole process. Besides charge transfer, diffusion, convection and experimental conditions (including the electrode features), the overall sensor current can also be dependent on some other factors, including enzyme kinetics and mass transport of the enzyme substrate. The latter are also influenced by the type and characteristics of the enzyme immobilization layer. The next section describes very briefly the most popular methods and their main pros and cons.

Enzyme immobilization on electrodes surfaces

Methods

Enzyme electrodes have been extensively studied over the years. As a consequence, a multitude of materials and physical or chemical techniques for the modification of electrodes with enzymes are well established and described in detail in the literature.[77–79] In the context of biosensors manufacturing, the main goals of enzyme immobilization are i) to enable the close contact between the protein and the transducer surface while preserving the catalytic competence and ii) to avoid the leakage of the biological component to the sample. This means that the microenvironment of the immobilized enzyme should be carefully designed in order to maintain the enzyme activity and, at the same time, to optimize the analytical performance of the biosensor.[40,49,60,78] Various strategies have been successfully used for enzyme immobilization on electrochemical transducers (the most important ones are summarized in Scheme 2). Although the immobilization procedure may somewhat disturb the conformation of the enzyme or partly block the active site, some methods can even improve enzyme stability.[49] In some situations, the immobilization method can also have a functional role during signal transduction.

In accordance with the type of protein retention, the immobilization technique can be organized into three major groups: (i) physical adsorption; (ii) entrapment (behind or within a porous membrane, polymer, clay, past or ink); and (iii) chemical binding (cross-linking and covalent attachment to functionalized supports). Not surprisingly, the combination of two or more methods is often seen. The multiplicity of variants within each group is so vast that the description and discussion of each one was left out of this article (an overview of the different immobilization techniques has been reviewed by Sassolas et al.[80]). Briefly, one can say that covalent bonding is the most stable immobilization strategy. Although the presence of suitable functional groups on both proteins and supports is mandatory and the covalent linkage often damages the native conformation of the enzyme, thus, reducing the stability, the catalytic activity and the substrate affinity.[33,40,49,77] On the contrary, the physical adsorption, based on van der Waals attractive forces between enzymes and solid electrodes, is one of the oldest and simplest methods for enzyme immobilization. It is normally sufficient for short-term studies but makes the bioelectrode vulnerable to leaching and fouling. Moreover, proteins often denature if in direct contact with noble metals or carbon surfaces.[33,40,49,52] Another easy way to attach a protein molecule to the electrode surface is to entrap it using a porous



Scheme 2. The protocols for enzyme (•) immobilization onto electrode surfaces can be classified in five main groups, according to the chemical and physical principles. Proteins may be (A) simply adsorbed through electrostatic interactions, (B) encapsulated within a porous matrix or (C) entrapped behind a semi-permeable membrane or film. Alternatively, enzyme molecules can be (D) covalently attached to the support or (E) cross-linked via bifunctional agents.

membrane, as simple as a dialysis membrane.[40,49,81] The covering of the enzyme layer with an appropriate permselective outer film can also help to:[36,40]

- improve the stability and lifetime of the sensor, by reducing leakage of the reacting layer components into the sample solution;
- protect the sensing surface from fouling;
- increase the intrinsic selectivity by preventing interfering species from entering and interacting with the reaction layer, usually based on a charge-, polarity- or size-exclusion discriminating mechanism.
- Interestingly, these protective membranes can also work as diffusion barriers to the enzyme substrates.[36] This phenomenon has important implications on the analytical performance of biosensors.

With the aim of mimicking the natural environment of biocatalysts thereby preserving their biological activity, and/or improving the electronic communication between enzymes and transducers, highly advanced and less detrimental immobilization procedures have been developed over the last two decades. All methods require the premodification of the electrode surface with a mono- or multi-layered film made of suitable polyions, synthetic detergents or lipids, or, alternatively, with more sophisticated assemblies composed of nanostructured materials. Very often, the bioactive membranes are designed with molecular level control. But still, protein binding is based on the very same principles discussed above, i.e., electrostatic interactions, covalent attachment and encapsulation.

Effects on analytical parameters

The immobilization procedure can have a direct impact on the structure and properties of the biocatalyst. For instance, the enzyme interaction with the matrix can induce conformational changes that usually decrease the affinity to the substrate (increase of K_m) or cause partial or total inactivation (decrease of i_{max}). A non-uniform distribution of the protein also affects its access to both the substrate molecules and the transducing interface. This can be reflected on the apparent kinetic constant, normally an average of the values from the different enzyme populations. A diffusion effect also occurs if the matrix

interacts with substrates, products, protons or intermediates. Moreover, the pH and temperature can change significantly in the protein microenvironment. All these effects can surely have a great influence on the overall performance of the biosensor, including the stability, selectivity, sensitivity, linear range, detection limit and response time.[44,82]

The influence of the immobilization layers or outer coats on substrate diffusion must be emphasized. The interaction of the analyte with any sort of matrix slows down mass transport, which may become the rate-limiting step of the biosensor response. This means that the measured currents are lower and so are the sensitivities of detection. Additionally, the time of response increases (the thicker the membrane, the longer the time), and there are risks of inhibitory effects from the accumulation of species in a confined space. The lower limit of detection can be negatively affected, as well. On the other hand, mass transfer control has important advantages in biosensing applications, namely (i) the linear ranges are extended, and (ii) because it becomes independent of the enzyme kinetics, the response is less affected by variations on the experimental conditions (pH, temperature, ionic strength).[40,82] One easy way to check the predominance of mass transfer resistance over enzyme kinetics is to compare the K_m^{app} of the sensor with the K_m value measured in solution; under diffusion control, K_m^{app} should be several times higher than K_m (Figure 2, gray curves).[83]

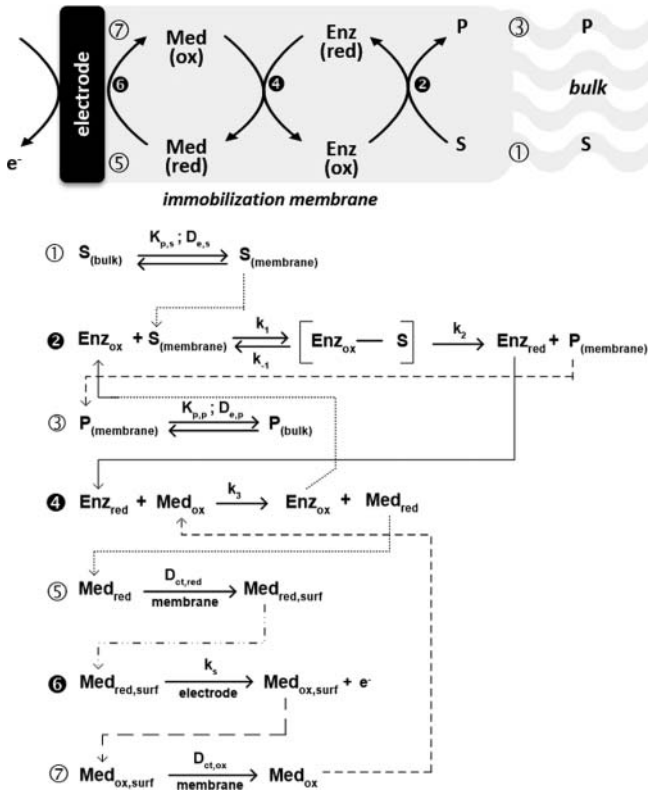
In summary, the imposition of a diffusion barrier to the substrate through a suitable outer membrane can be extremely useful for the modulation of many important analytical parameters.

The theoretical modeling of the biosensor operation provides a better understanding of the effects of immobilization on the enzyme kinetics and should help to find improved biosensor architectures; thus, optimizing the overall performance. However, given that the enzyme kinetics is coupled to the partition and diffusion of substrate and product, it is necessary to solve a highly intricate set of diffusion-reaction equations (the Michaelis–Menten kinetics for the enzyme-catalyzed reaction means that the resulting diffusion equations are also non-linear). Although heterogeneous ET should be fast, in certain conditions, the rate of the intermediate ET steps can also contribute for the amperometric current (Scheme 3). Furthermore, the development of a suitable model requires the knowledge of a large number of sensor parameters (thickness, partition and diffusion coefficients of each species for each membrane or layer, distribution of biocatalytic activity within the sensor layers, transducer operating properties, etc.), which normally are not easy to assess.[36,52,84]

The problem of deriving a complete mathematical model to describe the diffusion processes and enzyme catalyzed reactions within uniform films containing immobilized enzymes (and mediators) has attracted considerable attention in the literature.[59] Several models were proposed for limiting cases, but no consistent treatment for the many possible situations has been provided so far.

Electron-transfer modes in amperometric biosensors

A fundamental condition for the development of effective amperometric biosensors displaying high sensitivities and



Scheme 3. Schematic model depicting the sequence of events (charge transfer, mass transport and catalytic reaction) involved in the mediated response of an amperometric enzyme (e.g., oxidase) biosensor. The process is triggered by the presence of the analyte (enzyme substrate, *S*), which needs to cross the solution/electrode interface and diffuse through the enzyme containing membrane; this first step (1) depends on the partition coefficient K_S and diffusion coefficient of the substrate $D_{e,S}$. This is sequentially followed by two homogeneous electron transfer reactions, the enzyme catalysis (step 2) and the reduction of the redox mediator (step 4), which are characterized by the rate constants k_2 (k_{cat}) and k_3 (intermolecular ET), in that order. The reduced mediator diffuses to the electrode surface (step 5, which is controlled by the propagation charge constant, $D_{ct,red}$) and delivers the reducing equivalents through a heterogeneous ET reaction (step 6), ideally at a fast rate (k_s). Then, the resulting oxidized mediator moves through the membrane (step 7, $D_{ct,ox}$) till it finds a novel reduced enzyme molecule. Meanwhile, the enzyme product (*P*) diffuses to the bulk solution (step 3, dependent on the diffusion ($D_{e,P}$) and partition (K_P) coefficients). The rate-determining step, typically the enzyme catalysis or the mass transport of the substrate (a kinetic or diffusion controlled process, respectively), governs the overall rate and thus the magnitude of the amperometric signal. Note: the mass transport events are numbered in white, whereas the charge transfer processes (either homogeneous or heterogeneous) are numbered in black. Med_{ox} and Med_{red} represent the mediator in the oxidized and reduced forms, respectively, while *surf* means surface.

prompt responses is the establishment of a fast ET from the biological component to the electrode (or vice-versa). At first look, the easiest way to do it would be the establishment of a direct electrochemical communication between the enzyme cofactors and the electrode surface through electron tunneling mechanisms.[70,85,86] However, as discussed previously, the redox active sites are usually deeply buried inside the insulating polypeptide shell, precluding DET with electrodes. In addition, because the protein molecules are randomly orientated within the immobilization layer, not all of them are facing the electrode surface properly, further increasing ET distances. Indeed, the direct electrochemistry of proteins is only possible when ET pathways are sufficiently short, which is rarely observed at bare electrodes.[48,52,70] A second prerequisite, aiming at the production of reagent-free devices, is the tight and functional immobilization of redox enzymes on transducer surfaces. This means

that alternative electron tunneling pathways should be provided, enabling a fast ET and, at the same time, avoiding the diffusion of redox species to the bulk solution.[60]

To circumvent this problem, several experimental strategies were proposed over the years. According to the stage of integration, amperometric biosensors were earlier grouped into three generations. More recently, this classification tends to be based on the ET principle. In first-generation biosensors, the biocatalytic event is detected by measuring an electroactive co-substrate or product of the enzymatic reaction, while in second-generation biosensors the biorecognition reaction is monitored through synthetic or natural redox mediators. Finally, third-generation biosensors rely on a more advanced approach where oxidoreductases are able to establish DET with transducing interfaces.[87,88]

First-generation biosensors

The simplest approach for the construction of amperometric biosensors based on oxidoreductases, involves the monitoring of the current change produced by the oxidation (or reduction) of a freely-diffusing natural co-substrate, in the presence of the enzyme main substrate – the true analyte.[86] In a way, the natural electroactive co-substrate works as an electron shuttle between the enzyme and the transducer surface. This was the ET strategy employed in the early era of biosensors research, mostly using oxygen dependent enzymes, including *oxidases* (concomitant production of hydrogen peroxide, Eq. (11)) and later adapted to several *monooxygenases* (enzymatic production of water, Eq. (12))[44,65]:

$$\underset{\text{(analyte)}}{\text{Substrate}} + \underset{\text{(electroactive)}}{O_2} \xrightarrow{\text{oxidase}} \text{Product} + \underset{\text{(electroactive)}}{H_2O_2} \qquad (11)$$

Substrate: glucose, lactate, cholesterol, ethanol, etc.

$$\underset{\text{(analyte)}}{\text{Substrate}} + \underset{\text{(electroactive)}}{O_2} \xrightarrow{\text{oxygenase}} \text{Product} + H_2O \qquad (12)$$

Substrate: tyrosine, catechol, bilirubin, etc.

In the very first approach, the catalytic reaction was followed by measuring the consumption of molecular oxygen using a Clark-type electrode, which promotes the reduction of dissolved oxygen into water [Eq. (13)] at a platinum cathode poised at −0.6 V vs Ag/AgCl (Scheme 4A)[33]:

$$2O_2 + 4H^+ + 2e^- \rightarrow 2H_2O \qquad (13)$$

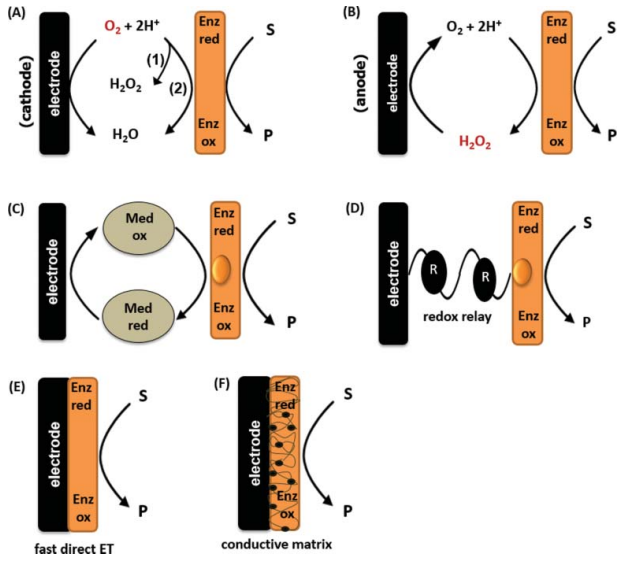
Basically, first-generation biosensors were oxygen sensors covered with an enzyme layer (usually an oxidase encapsulated within a porous membrane). Despite being highly innovative and working reasonably well, this biosensing system has raised several problems. First, the electrode response is dependent on the concentration of dissolved O_2, which may vary from sample to sample, leading to the misreading of oxygen consumption. This is especially critical in *in vivo* measurements, where fluctuations in the partial pressure of oxygen are expected. Second, due to the low solubility of O_2 in aqueous media, the concentration of this secondary substrate may be not enough to guarantee the full oxidation of the main substrate (analyte), often demanding sample dilution.[86] Last, the working potential is quite negative, so it generates high background currents and other electroactive species may interfere with the results.[29,33,44,89,90]

To overcome these drawbacks, the amperometric monitoring of the natural co-substrate oxygen was replaced by the anodic detection of the reaction product – hydrogen peroxide [Scheme 4B, Eq. (14)]. This strategy was well accepted in the scientific community and is still employed in modern days. Actually, this was the measuring principle used in the first glucose meter commercialized by *Yellow Springs*, which may be considered as the archetypal first-generation biosensor[91]:

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \qquad (14)$$

Still, the oxidation of hydrogen peroxide requires high operating voltages (*ca.* +0.7 V vs Ag/AgCl), which are sufficient to oxidize interfering species usually present in physiological samples, like ascorbate, urate and acetaminophen. And once again, the biosensor response depends on a minimum oxygen concentration.[28,29,33,90]

In order to improve the selectivity of detection, the biosensing layers can be covered with ion-selective polymers, such as the negatively charged Nafion (a perfluorosulfonated ionomer), that can block the free access of interfering species to the electrode surface.[86] Another alternative approach employs a dual electrode system where a blank electrode (without the



Scheme 4. Schematic representations of the working principles in enzyme-based amperometric biosensors. First, generation biosensors are based on the electroactivity of (A) natural enzyme substrates or (B) products (1: oxidase; 2: oxygenase). Second, generation biosensors exploit the electrochemical response of artificial redox mediators, either in the soluble form (C) or covalently bound to the immobilization matrix (D). In the third generation biosensors, the enzyme displays a non-mediated electrochemical response; it may exchange electrons directly with the working electrode (E) or via a conducting polymer (F). The oxidized and reduced states of the enzyme are depicted by Enz_{ox} and Enz_{red}, whereas Med_{ox} and Med_{red} represent the mediator in the oxidized and reduced forms, respectively.

biological recognition element) measures the background current. This self-referencing methodology has been particularly useful for *in vivo* measurements. The subtracted signal is fairly independent of oxygen fluctuations and interference from endogenous species or drugs.[14,15] However, these strategies are transversal and can be employed in any one of the three classes of amperometric biosensors.

Second-generation biosensors

Synthetic and natural mediators

The introduction of artificial *redox mediators* (molecules that can shuttle electrons between the enzyme redox centers and the electrode) in the ET pathway, in the place of molecular oxygen, provided major advances in biosensing technology (Scheme 4C and D). As prerequisites, redox mediators should not only display a fast and reversible electrochemistry, but as alternative electron acceptors of oxidases, they should also be able to compete with freely diffusing oxygen for the enzyme reoxidization. In other words, ET mediators must react with the reduced oxidase very quickly during the catalytic cycle, yet not many mediators are able to fully satisfy this condition, so the possibility of oxygen interference still exists. Additionally, an ideal synthetic mediator should meet the following requirements[29,52,76,89,92,93]:

- The overpotential for the regeneration of the oxidized mediator should be relatively low. In this way, the current response can be obtained at a lower potential than that of the direct oxidation of hydrogen peroxide, and so the risks of oxidation of interfering compounds are greatly reduced.
- The reduced form of the mediator should not readily react with oxygen.
- They must be sufficiently soluble, in both the oxidized and the reduced forms, to rapidly diffuse between the active site of the enzyme and the electrode surface.
- The reduction potential must be independent of pH.
- If possible, the chemicals should not be toxic.
- Compared to the formal reduction potential of the enzyme redox co-factors, the reduction potential of the mediator should be more positive for oxidative biocatalysis, or more negative for reductive biocatalysis.
- In general, the electroactive charge carriers employed in biosensing applications are low molecular-weight compounds like tetrathiafulvalene, tetracyanoquinodimethanide, quinone derivatives, organic dyes (Prussian blue, methylene blue, toluidine blue, viologens, phenazines) and metal complexes (e.g., ferrocene derivatives, ferricyanide, Ru- and Os-complexes), among others.[29,70,76,89] In particular, ferricinium ions have demonstrated to be efficient electron acceptors for glucose oxidase. The derivatization of this iron complex was very useful to tune the formal reduction potential over several hundred mV (from 0.1 to 0.4 V vs saturated calomel electrode)[94] and adapt it to that of the proteins redox groups. These and other examples are listed in Table 1.[76,95–98]

The employment of non-physiological electron carriers in biosensors offers several advantages over first-generation devices. First, the turnover rate is not limited by the O_2 partial pressure, thus facilitating the control of the sensor performance.[32] Second, as biosensors can be operated at moderately low electrode potentials, the analysis is less susceptible to interferences from electroactive compounds.[28,29] Some mediators can lower the working potential to a value close to 0 V vs Ag/AgCl.[15,28] In such cases, permselective membranes might be omitted.[93]

Very importantly, the use of electron acceptors other than oxygen allows the direct or indirect exploitation of other oxidases that do not depend on this co-substrate (e.g., dehydrogenases, peroxidases), as well as substrate reducing enzymes that were out of the first-generation biosensors. For example, secondary enzyme substrates that cannot be directly regenerated at bare electrodes at fairly low potentials, such as the freely diffusing cofactor NAD(P)H, which only undergoes irreversible electrochemical oxidation at high overvoltages (over 1 V vs NHE), may be indirectly monitored at a potential much closer to the thermodynamic value (*ca.* −0.32 V vs NHE),[7] by the electronic coupling to a proper artificial redox mediator (e.g., meldola blue and Prussian blue).[99–102] In this way, the very important and abundant class of NAD(P)H dependent oxidoreductases like those from the dehydrogenases and cytochromes P450 families, may be used as biological recognition elements in amperometric biosensors, as extensively reviewed by Gorton[63,98] and by Katz and Willner.[92] Consequently, the range of analytes potentially detected through this technology was greatly expanded. Worth mentioning are the reduced NADH-glucose dehydrogenase (GDH) and the pyrroloquinoline quinone-glucose dehydrogenase (PQQ-GDH), that despite having the same primary substrate of GO*x*, they are absolutely different from the latter. PQQ-GDH, in particular, has the advantage of not depending on the oxygen level. On the other hand, it is less selective and stable and very expensive.[29,32,57,90,103]

An alternative strategy to accomplish the electronic communication between enzymes and electrodes lies in the employment of the enzymes *physiological partners* as electronic mediators, thus, mimicking the charge transfer processes that take place in biological systems. Specifically, small redox proteins involved in the natural ET chains (e.g., cytochromes and blue copper proteins) and that are able to display a fast and reversible electrochemical response on solid electrodes are used to shuttle redox equivalents from (to) the biological catalyst to (from) the transducer surface. This means that the electronic mediation step is based on the selective interaction between two complementary proteins. On the contrary, synthetic electron carriers are also able to catalyze unspecific electronic reactions, which may lessen the selectivity of detection.[52,87,92] Furthermore, because most synthetic mediators are toxic agents, their replacement by harmless biological entities turns the method more friendly to the environment.[93] However, even though the successful application in bioelectroanalysis of a number of enzyme/mediator couples, such as the copper nitrite reductase (NiR)/pseudoazurine from *Alcaligenes faecalis*

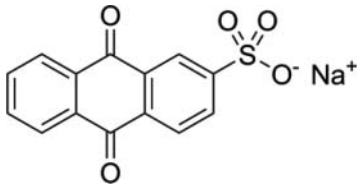
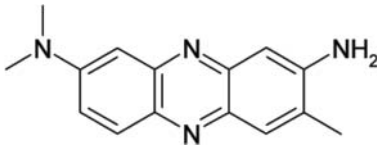
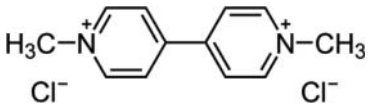
[7] Since the formal potential of the NAD(P)/NAD(P)H redox couple is relatively low, only biosensors based on the oxidation of NAD(P)H have been developed. Otherwise, one would have to employ working potentials below the formal potential, making the system prone to interfering reactions, including the direct electrochemical reduction of oxygen.[63]

Table 1. Selected examples of synthetic redox mediators commonly used in amperometric biosensors.

Mediator	$E^{0'}$/mV vs NHE	Reference
Tetrathiafulvalene	542	76
Ferricyanide	430	76
Ferrocene	410	76
Tetracyanoquinodimethane	370	76
Benzoquinone	280	76
Phenazine methosulfate	80	76
Ruthenium hexamine	47	95
Methylene blue	11	76

(*Continued on next page*)

Table 1. (*Continued*).

Mediator		$E^{0'}$/mV vs NHE	Reference
Sodium anthraquinone-2-sulfonate		−225	96
Neutral red		−325	98
Methyl viologen		−440	98

and the cytochrome cd_1-NiR/cytochrome-c_{552} from *Marinobacter hydrocarbonoclasticus*, this very interesting ET route has been scarcely exploited up to now. In fact, there are not many options available, given that the physiological partner of the enzyme should be previously identified and isolated. Alternatively, horse heart cytochrome *c*, a versatile electroactive protein, has proven to work as an effective electron carrier in a number of electrochemical biosensors based on non-related oxidoreductases isolated from very different organisms, namely the cytochrome *c* peroxidase from *Saccharomyces cerevisae*,[104] the cellobiose dehydrogenase from *Phanerochaete crysosporium*,[105] the laccase from *Trametes versicolor*,[106] the bilirubin oxidase from *Myrothecium verrucaria*,[107,108] the human[109] and the chicken liver sulfite oxidases[110] and the cow milk xanthine oxidase,[111] among others. Earlier it was shown that monolayers of microperoxidase-11 or de *novo*-synthesized proteins containing a heme group assembled on solid electrodes were able to establish the electronic coupling with cytochrome-dependent enzymes, like the nitrate reductase from *Escherichia coli*.[92] It should be stressed that, no matter the proteins pair, the electrode design is a very important issue, since it should guarantee both catalytic turnover and vectorial ET from (or to) the redox enzyme.

Configurations based on diffusional mediators

In the simplest configuration, redox mediators are just added to the sample solution and work as freely-diffusing ET shuttles.[70,92] However, this is far from being a reagent-free device, as stipulated in the biosensor definition. Alternatively, the soluble electron carrier may be entrapped within or next to the enzyme containing layer, normally a polymeric matrix. This undemanding approach paved the way for the successful application of amperometric biosensors in single-use devices (*MediSense*, now owned by Abbott, was the first company to launch a second-generation product for glucose monitoring, the *Exatech*; the electronic mediation was provided by a ferrocene derivative).[26,29]

Still, both continuous and intermittent applications are not feasible due to the inevitable leaching of the mediator into the bulk solution that causes a signal decrease over time. To prevent the redox mediator leakage, several different strategies for integrating redox mediators into biosensing coats have been proposed. One of them is the adsorption of less soluble synthetic mediators on the electrode surface followed by the immobilization of the enzyme in a second layer. Although the mediating species is less prone to leak, the problem of signal instability is not completely solved yet. Another possibility is the mixing of the redox mediator into a carbon paste (graphite powder/binder) together with (or underneath) the biological recognition element, but once more the progressive leakage of the charge carrier molecules cannot be ruled out. Further incorporation of additives in the carbon paste may improve both stability and response time of the sensor.[52,70,92,112]

Electro-enzymes

A more integrative approach is the covalent attachment of electron mediators to the enzyme itself, at a proper binding site (side chains of amino acids at the protein periphery, inner redox co-factors or active site), creating the so-called *electro-enzymes*. Such protein modification with redox-relays made possible the *non-diffusional* electron shuttling between the redox active groups deeply buried in the protein shell and the transducer interface. However, when incorporated within a conducting polymer like polypyrrole, the ability of free-diffusing electro-enzymes to display fast heterogeneous ET disappears. This problem was partially solved by linking the redox moiety to a flexible spacer chain; in this way, the modified enzyme and the mediator were relatively free to exchange electrons, which then move to the transducer surface through the polymer wire. Still, the ET process was not efficient, most likely

due to the low density of redox relays. Besides, the chemical modification of the polypeptide chain was often detrimental to the native conformation of the enzyme and consequently, to the catalytic activity and stability.[92,113]

A major progress in the field was achieved by Willner and co-workers through the surface-reconstitution of apoenzymes. Specifically, after removal of the FAD cofactor from the native GO*x*, the apoenzyme was reconstituted on a gold surface functionalized with a monolayer of semi-synthetic FAD groups, covalently linked to PQQ redox-relay units, i.e., an Au-PQQ-FAD assembly. In this way, the redox mediator (PQQ) is covalently attached to both the enzyme cofactor and to the electrode support, with optimal distances and orientations. Such surface engineering of GO*x* enabled the optimal arrangement of all ET entities, and thus an excellent electrical communication between the redox protein and the transducing surface. This enzyme electrode construct exhibited unprecedented high turnover rates, which made the catalytic activity insensitive to oxygen.[114]

A similar strategy was employed to build Au-PQQ-NAD^+ electron relays, which enabled the mediated electronic communication of NAD-dependent enzymes, like lactate dehydrogenase (LDH), with the transducing surface. The cross-linking of the affinity complex formed between LDH and the PQQ-NAD^+-monolayer functionalized electrode allowed the assembly of a fully integrated biosensor.[114] The concept of surface reconstitution of apoenzymes with other relay linkers was elaborated further to improve the electrical communication with transducers (this topic was conveniently reviewed by Fruk and colleagues in reference[115]).

Electroactive polymers

Instead of the direct functionalization of proteins, the covalent binding of redox active groups to the backbone of the immobilization matrix is a more appealing route to improve ET pathways between the redox enzymes and electrode surfaces. Indeed, the use of flexible polymers onto which mediating functionalities were chemically attached was a major advance in the development of reagent-free devices. Enzymes are incorporated within the polymeric network and surrounded by a large number of redox relays. Regardless of the spatial orientation, the electrical wiring of the enzyme is assured through an electron-hopping mechanism, i.e., a cascade of short distance charge transfer reactions between adjacent redox relays. Frequently, the rate-limiting step is the intermolecular ET between the protein cofactor and the closest mediator group.[52,60]

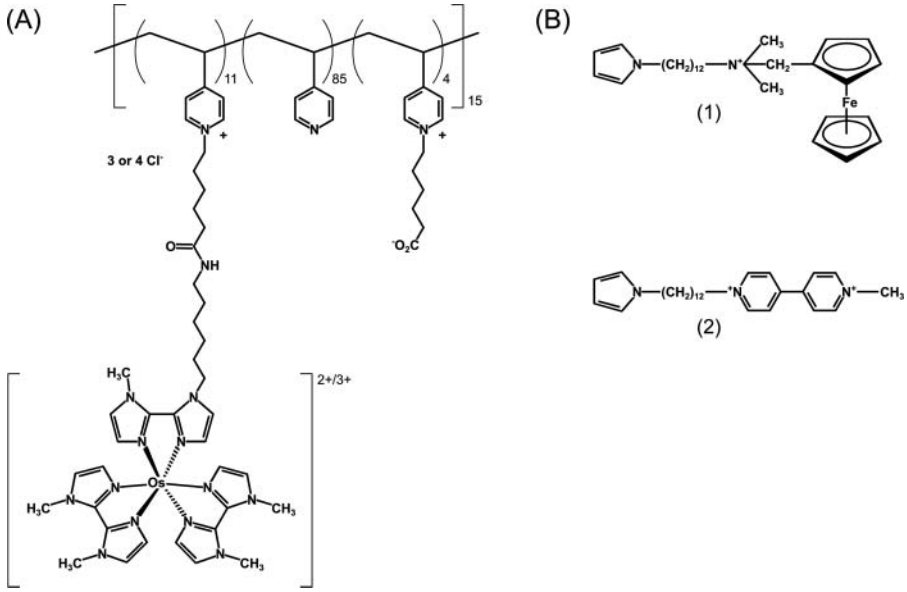
The employment of suitable electroactive polymers has another important advantage: it connects electrically multiple enzyme layers, producing current densities much higher than those produced by an enzyme monolayer.[29] In practical terms, two main approaches (hydrogels vs conductive polymers) can be followed.

At this point, it is important to note that, on the one hand, biosensors based on electrically wired enzymes show features of mediated ET, just like second-generation biosensors, but on the other hand, of direct and integrated electronic communication, as entailed in third-generation biosensors.[70] Because of this, there is some confusion in the literature concerning the classification of these biosensors. As pointed out before, the trend today is to categorize the first, second and third generations in agreement with the signal transfer mode, i.e., mediated ET through freely diffusing physiological substrates, mediated ET via additional charge carriers (regardless of the integration level) and non-mediated ET, respectively. Following this criterion, we have decided to consider electroactive polymers based electrodes as second-generation biosensors. There are reported cases of direct electrical contact of enzymes to electrodes through non-functionalized conducting polymers (the conducting network resembles molecular wires around the entrapped enzymes).[75] Since there is no requirement for a redox mediator, these enzyme electrodes should be included in the group of third-generation biosensors.

Redox active hydrogels. Perhaps the most popular strategy to achieve the effective electrical wiring of redox enzymes is the entrapment of the protein molecules within the generally called *redox hydrogels*, first described by Heller et al. in 1990 with the GO*x* enzyme model.[116,117] These insoluble but water-swollen polymer networks are formed of a adequately cross-linked poly (vinylpyridine), poly(vinylimidazole), poly(acrylic acid), or poly(allylamine) to which redox mediators like Os-complexes are covalently bound through mobile polymer segments (see example in Scheme 5A). Redox hydrogels conduct electrons by a self-exchange mechanism, which is based on rapid collisions between reduced and oxidized redox centers.[29,118] Upon optimal ligand replacing, the redox potentials of the wiring hydrogels could be downshifted to very low oxidizing potentials (−69 mV vs SCE), greatly improving the selectivity against electrooxidizable interferents, such as urate and ascorbate ions.[119]

Biosensing layers are usually prepared by drop-drying on top of the electrode a solution containing the enzyme, the mediator-modified polymer and a crosslinker. Remarkably, owing to the hydrophilic properties of the redox polymer, i) both the substrate/product of GO*x* (glucose/gluconolactone, respectively) as well as other water-soluble ions are able to diffuse across the resulting electron conducting phase,[29] ii) the microenvironment is enzyme-friendly, and iii) the matrix provides the biocompatibility needed for *in vivo* measurements. Actually, since redox hydrogels are able to meet the most important demands for implementing implantable biosensors (stability, no leachable constituents and biological compatibility), they have been used in the construction of amperometric electrodes capable of being inserted in living tissues. A commercial product based on this technology is the glucose meter *FreeStyle Navigator*, developed by *TheraSense* (later acquired by Abbott). This needle type sensor measures subcutaneous glucose levels about every minute, and transmits results to a pager-like device that saves the information and provides high and low threshold level warnings.[15,26,29,32]

The response of glucose biosensors based on wired enzymes was further improved by Mano and his coworkers. Not only the question of hydrogel optimization was addressed,[120] but also protein engineering tools were used in order to produce more effective forms of both GO*x*[121,122] and PQQ–GDH.[123,124]



Scheme 5. Structural formula of (A) a poly(4-vinylpyridine) redox hydrogel bearing flexible 13 atom long spacers that tether the osmium redox units to the polymer backbone and enable effective electron-transferring collisions[29]; (B) electropolymerized conducting polymers composed of amphiphilic pyrrole derivatives functionalized by an electron shuttle system, namely a ferrocene (a) or viologen (b) group.[126]

Redox active conducting polymers. In the late 1980s, the research groups of Schuhmann and Bartlett, among others, demonstrated that the electrogeneration of conducting polymers such as polypyrrole (PPy), polyaniline (PAN) and polythiophene, in the presence of enzyme molecules, is a very convenient method for the entrapment of these biocatalysts on electrode surfaces and their subsequent use in sensor applications. The formation of the polymer is carried out by controlled potential electrolysis of an aqueous electrolyte containing both monomers and biomolecules.[60,125] Several advantages can be pointed out to the electrochemical polymerization over the classical methods for electrode modification: i) polymeric films can be easily and reproducibly prepared in a non-manual, one-step procedure; ii) the film thickness can be precisely controlled by just monitoring the amount of charge consumed during the electropolymerization process; iii) polymer coatings are electrochemically deposited on the electroactive area of the working electrodes solely, even if surfaces are small and with complex geometries; iv) the sensor selectivity may be improved due to size-exclusion mechanisms; v) the bioelectrode is fairly protected against fouling; vi) the construction of well-organized multi-layered assemblies is straightforward. Moreover, conducting polymers bearing redox active units at flexible side chains can be used to transport electrons between the enzymes ET sites and the electrodes, through an electron-hopping mechanism.[75,126,127] The polymer networks can be chemically modified after electrogeneration of the conducting film in a heterogeneous reaction; instead, mediator-attached monomers may be synthesized in advance and later electropolymerized in the presence of unmodified monomers, to avoid steric hindrance of the side chains.[70,75,126] Due to the wide variety of redox species that may be covalently linked to the pyrrole group, PPy is by far, the most used conducting polymer in molecular wiring. For instance, ferrocene units and osmium complexes were chemically incorporated within polypyrrole films, which were used for the entrapment of GO*x*. In an early stage, the ET rates between the protein redox centers and the transducing surface were generally low, owing to the low density of mediator groups. In addition, due to the hydrophobic nature of the polypyrrole skeleton, the glucose diffusion within the film was rather slow, leading to a low sensitivity within a broad linear range. In the case of the ferrocene-PPy films, the lifetime was also poor due to the instability of the redox groups. To improve the biosensors performance, the concentration of redox groups as well as the polymers hydrophilicity were increased.[60,70,75] Alternatively, a new generation of amphiphilic pyrroles functionalized with redox mediators, such as ferrocene derivatives or viologen groups, was synthesized by Cosnier and collaborators, a few years later (Scheme 5B). The amphiphilic monomers were dispersed in aqueous media and co-adsorbed with the enzyme molecules on the electrode surface. The electropolymerization step was performed in an electrolyte solution with neither monomers nor proteins; therefore, one could save reagents and better control the protein loading. This strategy has enabled the efficient electrical connection to electrode surfaces of not only the model catalyst GO*x* (via a ferrocene-PPy) but, very importantly, some other enzymes operating in the reductive pathway, namely nitrate[128] and nitrite[67] reductases (both through a viologen-PPy polymer).

Due to the strong linkage of the redox mediator to the immobilization matrix, biosensors composed of redox active polymers may be potentially used in intermittent (repetitive) or continuous analysis.

In general, redox active conducting polymers do not supplant the potentialities of redox hydrogels for the effective electrical wiring of most oxidases. Also, there are risks of protein denaturation following the electropolymerization step due to the intrinsic hydrophobic character of the host organic matrix.[129] Nevertheless, the ability to promote the non-manual and spatially controlled deposition of polymer layers, no matter the electrode size or shape, aside the control of the film thickness and enzyme loading, are great assets that drive the

use of electrogenerated polymers for the modification of enzyme electrodes (the topic was reviewed by Cosnier and Holzinger[128]). Interestingly, attempts have been made to design sensors which combine the advantages of redox hydrogels with those of conducting polymers.

Third-generation biosensors

Benefits and challenges

In spite of some proposals for second-generation biosensors turned out to be very effective and highly stable, there is a strong interest in developing third-generation biosensing devices (Scheme 4E and F), which are based on mediator free ET processes.[69,87]. Besides all the advantages of second-generation biosensors, these enzyme electrodes can offer some new benefits. One of the main advances is the absence of extra charge carriers, which greatly improves the selectivity of detection, especially when compared to synthetic mediators, since these non-specific catalysts are able to react not only with the enzyme of interest but also with many other redox species that may exist in the sample. Just depending on the enzyme selectivity and operating potential, biosensors based on DET are thus virtually free of interfering reactions. Moreover, the manufacture of these analytical instruments is much simpler and requires fewer reagents, thereby reducing production costs. Also, they avoid the use of toxic compounds, as the majority of artificial redox mediators is.[52,69,87,130] In the case of oxidase enzymes, the problems related with the low partial pressure of oxygen (first-generation biosensors) and with the slow intermolecular ET rates between the mediator and the protein redox co-factors (second-generation) are eliminated.

Unfortunately, as discussed in the beginning of this section, DET between proteins and signal transducers is often difficult to attain because the redox sites are shielded by the polypeptide chain, imposing a kinetic barrier that prevents fast interfacial ET. Even if direct electrochemical responses are observable, many heterogeneous ET reactions are rate-limited by conformational reorientations at the electrode–electrolyte interface.[131] In extreme cases, drastic conformational changes caused by the direct contact with metal or carbon surfaces denature proteins and make them lose the catalytic competence.[52,61,132] To overcome these drawbacks, improved electrode interfaces are required.

The reversible direct electrochemical response of a redox protein on solid electrodes was reported for the first time in 1977, for the small monoheme cytochrome *c* (horse heart), in two independent works: one using a tin doped indium oxide electrode[133] and the other, a gold surface modified with an ET promoter.[61,134] Afterwards, the DET and electrocatalysis of two large enzymes, the blue copper oxidase, laccase, and the heme-containing horseradish peroxidase (HRP), were observed at bare electrodes.[8][135]

Four decades have passed since then, much more examples were reported, showing catalytic activity, like alcohol dehydrogenase,[136] cytochrome *c* NiR (*cc*NiR)[137] and GO*x*.[138] Regarding the latter, one should mention that there is some debate on whether DET is truly achieved[139,140] and that some works were excluded from this section, because the claim of being third-generation biosensors was not accurate. In fact, the determination of glucose is often based on monitoring the electroreduction of O_2 at low potentials and its consumption by GO*x*, and conceptually, this approach falls into the category of first-generation biosensors.

Despite the progress made, the number of examples reporting the direct electronic coupling between enzymes and electrodes is still small (less than 1% of all oxidoreductases known today[130,141]). However, owing to the latest advancements in both surface and protein engineering techniques, much more papers on the subject are expected in the near future. Actually, many of the successful works published in recent years have greatly benefited from the advent of nanotechnology and nanomaterials, which brought significant advances to both fundamental and analytical applications.[142–145] In addition, the engineering of proteins is being increasingly used in the development of functional biosensors with improved signal transduction, stability or selectivity.[146,147]

Advanced materials for electrode interfaces

Supramolecular layered structures. The development of appropriate electrode interfaces and protein immobilization methods to facilitate heterogeneous ET has been accomplished with the help of many different techniques. Among these, one can point out the modification of electrode surfaces with supramolecular-ordered enzyme assemblies, organized in multi-layered architectures. For instance, the layer-by-layer (LbL) construction of enzyme-polyion films by means of electrostatic adsorption provides excellent control over thickness and enables the augmentation of enzyme loading.[148–150] Another example is the integration of membrane associated proteins in lipid bilayer films (either adsorbed or embedded) that mimic biological membranes. Both strategies combine experimental simplicity with the ability to fine-tuning the film characteristics (e.g., roughness, thickness and porosity) while preserving the molecular integrity of biomolecules. However, from the biosensing standpoint, they fail in terms of response stability. The link to the electrode interface is based on weak intermolecular interactions (electrostatic forces, dipole–dipole, hydrophobic or hydrogen bonds), so the protein may leak from the films.[75]

A variation of these techniques that overcomes stability issues relies on the specific and strong coupling between the protein *avidin* and the vitamin *biotin* (association constant $K_a = 10^{15}$ M^{-1}). In practical terms, it consists of making avidin–biotin–avidin bridges between previously biotinylated (or avidin-conjugated) enzyme molecules. Because each avidin molecule can bind four biotin units, the build-up of enzyme multi-layers is straightforward.[149,151]

The modification of metallic surfaces, like gold (Au), silver (Ag) and platinum (Pt), with highly ordered self-assembled monolayers (SAMs) of functionalized alkylthiols has also been giving an extremely valuable contribution to the direct electrochemistry of proteins. This is mainly due to the optimization of DET routes by forcing the protein to adopt a correct

[8]The surface chemistry of the electrode is of critical importance for the achievement of an effective electronic contact. The most successful electrode materials are noble metals modified with promoters and carbon materials like pyrolytic graphite, and metal oxides that have natural surface functionalities.[125]

orientation toward the transducer interface and providing the most favorable distance to the electrode. The interface properties can be easily tailored just by playing with the nature and amount of the functional end groups, which also allows the covalent attachment of proteins. Besides, the combination of metal NPs with SAMs has proved to be very useful for the enhancement of interfacial ET.

Definitely, the most remarkable developments for the third-generation biosensors were obtained through the construction of nanostructured architectures on electrodes surfaces. In point of fact, the employment of nanoscale materials, such as NP, nanotubes, nanowires and nanorods, has seen a remarkable growth during the last decade.[145,152–154] These nano-objects are frequently capable of promoting DET exchange with electrode surfaces. In practical terms, this means that the overvoltage required to drive the electrochemical reaction may decrease, whereas the resulting current density usually increases.

Carbon nanotubes and graphenes. The past years have brought fundamental innovations that added significantly to the utility of carbon materials in the (bio)electrochemistry field. This is the case of boron-doped diamond, fullerenes, and two of the most studied nanostructured materials – CNTs and graphenes.

CNTs are rolled-up one-atom-thick sheets composed of sp^2-bonded carbon atoms arranged in a honeycomb lattice. The nanodimensions, aspect ratio, surface chemistry (type and content of oxygenated groups) and unique electronic and mechanical properties make them excellent materials for (bio)chemical sensing.[142,154–157] The exposure of CNTs to chemical and/or physical treatments that oxidize the carbon surface shortens the CNTs, opens the end caps of the tubes and introduces structural defects on the side walls. Consequently, as the edge plane of pyrolytic graphite, the nanotubes surface becomes rich in reactive oxide functional groups (e.g., carbonyls, phenols, lactones, ethers and carboxylates) that have been demonstrated to be very helpful for the reactivity and electroactivity of these materials,[158–161] and thus for the improvement of all generations of amperometric enzyme electrodes. To illustrate this point, one should call the attention for perhaps one of the main achievements in the field reported by Wang and co-workers about 15 years ago, who was able to detect the oxidation of freely diffusing NADH[162] and H_2O_2[163] at much lower voltages, using CNTs coated electrodes. The highly sensitive, low-potential amperometric sensing of NADH and H_2O_2 has had a great impact in the development of amperometric biosensors based on dehydrogenases and oxidases, respectively.[142,152,157] Although these systems are not mediator-free biosensors, the direct electrical wiring of various oxidoreductases was observed too, shortly after the publication of Wang's work. Of particular note is the very elegant way to construct effective third-generation biosensors suggested by the groups of Gooding and Rusling, who independently proposed the attachment of heme containing enzymes (microperoxidase-11,[164] myoglobin and HRP[165]) to the end of vertically aligned single-walled CNTs. Thereafter, the concept of CNTs-forest arrays was combined to the method of surface reconstitution of apoenzymes to attain the direct molecular wiring of GO*x*[166]; the difference here is that the semi-synthetic FAD is not linked to any redox relay. However, a much simpler and widely used strategy is the stepwise deposition of non-oriented CNT multi-layers, creating porous networks capable of entrapping high protein loads.[156,167,168] One of the major obstacles to overcome in this approach is the poor solubility in aqueous media of the highly hydrophobic graphene cylinders, which hinders the obtaining of homogeneous dispersions and the subsequent production of thin films. To solve this problem, there are two main options: i) CNTs are suspended either in a pure organic solvent or a water solution containing a solubilization agent (e.g., detergents, polymers); ii) the end and/or sidewall of the graphene tubes are functionalized, making them much more hydrophilic.[156,160]

CNTs can also be used as additives in composite materials (e.g., carbon paste,[112,156] carbon conductive inks for screen printed electrodes[169] or combined with electrogenerated conducting polymers[152,167]). In all these cases, enzyme molecules may connect to functionalized nanotubes not only at their open ends, but also throughout the side walls (via simple hydrophobic interactions), or at the functionalized structural defects (through electrostatic attraction forces, direct or indirect covalent binding and affinity interactions).[167,168,170]

Regardless the methodology used, CNTs can plug into the enzymes and thus shorten the ET distances to their redox centres, which results in an efficient electrical wiring.[153,156] This is very useful in the construction of third-generation biosensors, provided that biocatalytic activity is maintained.[171]

The properties and potential applications of *graphene* have become hot topics of research in the past 10 years.[172–174] The chemical structure of CNTs and graphene is identical, but the latter appears as a flat two-dimensional sheet with a single-atom thickness.[174,175] Similarly, this carbon material is biocompatible, inexpensive, versatile and tunable, with good mechanical and electrical properties.[175–177] In the last years, graphene has been replacing CNTs in sensing applications, due to its superior performance; also, it is more feasible from a commercial point-of-view, since it is produced from a low-cost material (graphite) without any hazardous byproducts.[178] As CNTs, graphene is highly hydrophobic, which makes it difficult to disperse in an aqueous medium. Consequently, the sheets tend to restack, reforming graphite. Here again, these handicaps can be circumvented by functionalizing the carbon material through a sequential oxidation and reduction process; the resulting product – the reduced form of graphene oxide (rGO) – is simultaneously soluble and conductive.[175] Therefore, it is an excellent matrix for the development of electrochemical biosensors. Numerous works have been published on this matter and were reviewed elsewhere.[174,179,180] Very briefly, in what concerns third-generation biosensors, rGO have been mainly used to promote DET of GO*x*, the favorite enzyme model; several approaches have been pursued in order to immobilize the biorecognition element either directly on graphene oxide, without resorting to cross-linking agents,[176] or using more complex graphene-based nanocomposites with cross-linked enzyme.[178] In addition to polymers, graphene can be combined with other materials (e.g., CNTs, metals and metal oxides), producing composites with new functions.[177] For example, combining graphene oxide and MWCNT can produce a synergetic effect that results in a fast DET between GOx

and the electrode.[181] Also, the combination of magnetic NPs and rGO can result in a fast DET, due to the present graphene material, while preventing the immobilized biomolecules from leaching away from the electrode's surface, owing to the presence of an external magnetic field.[177]

Despite the unique properties of these 2D carbon sheets and the broad applications found in the biosensing field, a critical analysis of these and other works shows that the introduction of graphene materials in the R&D of third-generation biosensors did not cause the breakthrough that was initially expected, a decade ago. In our understanding, the gain in using graphene over the vastly explored CNTs was not that remarkable.

Metal nanoparticles. The application of metal (or semiconductor) NPs, especially gold NPs (AuNPs), in the field of analytical electrochemistry has also attracted much attention, due to their remarkable physicochemical characteristics, namely high surface-to-volume ratio, high surface energy and good biocompatibility. These are valuable features for the direct electrical wiring of redox enzymes. Together with the freedom in orientation, the extremely low dimensions of NPs (comparable to those of biological molecules) makes the distances between the redox centers of proteins and metal particles exceptionally low, thereby, facilitating electron tunneling. Also, surface modification of metal NPs is both simple and versatile. One of the most frequent modifications is the chemisorption of alkanethiols monolayers (SAMs), which may not only help the interaction and immobilization of enzyme molecules, but also allows the covalent attachment of NP ensembles to bulk electrodes. Because the ratios of surface area to volume of NPs are much higher than their macrosized counterparts, electrochemical interfaces modified with NP provide larger electrochemically active areas and better signal-to-noise ratios. Consequently, NP-modified interfaces behave as nanoelectrode arrays which, in principle, can lower the detection limit of the electroactive species.[144,182–185]

In bioelectrochemical applications, NPs act as relay units of enzyme integrating supramolecular structures that tailor the electrochemical-sensing interface. A great variety of examples, each one adopting a different architecture, can be found in the literature, as comprehensively listed by Pingarron et al. in a review paper concerning the use of AuNPs in electrochemical biosensors. The enzyme systems can vary from the classical models GO*x*, HRP and dehydrogenases to the less explored biocatalysts, tyrosinase and xanthine oxidase. Regarding the modification strategies, they range from the electrostatic or covalent attachment of redox proteins on AuNPs coated with a SAM further tethered to a gold-electrode surface, through the cross-linking of enzyme molecules on glassy carbon electrodes modified with electrodeposited AuNPs, to more complex nanostructured assemblies obtained by the co-incorporation of enzymes and AuNPs into sol-gel networks[114,182,183] or polymer-enzyme-NP films obtained by one-pot protocols,[186] just to cite a few.

The surface reconstitution of the apo-GO*x* or apo-GDH on FAD-functionalized AuNPs associated with gold electrodes was also accomplished. The aligned, reconstituted enzymes on the electrode surfaces revealed effective electrical connection, delivering exceptionally fast DET rates. As for the electrode-CNTs-FAD-GO*x* assemblies previously mentioned, AuNPs have acted as nanoelectrodes that mediate charge transport from the FAD center to the electrode.[187]

It is worth to mention that besides Au, other metals have been successfully applied in the development of nanostructured third-generation biosensors. For example, cobalt oxide (Co_3O_4) and Ag NPs have been applied in the development of Hb based biosensors, respectively.[188,189] Tungsten oxide (WO_3) NPs were used to improve the ET of immobilized *cc*NiR,[190] and titanium oxide (TiO_2) nanosheets and Pt NPs have been used to improve the catalytic response of GO*x* bioelectrodes toward glucose, respectively.[191,192] Iron oxide (Fe_3O_4) magnetic NPs coupled with hemi/ad-micelles have been used to increase the adsorption capability and favor the orientation of Hb on carbon screen printed electrodes, promoting a fast and effective immobilization of the enzyme and also facilitating the DET process.[193] Additionally, the conjugation of metallic NPs with other nanomaterials like CNTs has also attracted a considerable interest, with the resulting composites often displaying synergetic properties,[183] as mention previously.

Conclusions and future perspectives

A look into the present needs for safety and protection of human beings and Nature shows a significant shift in the way analytical problems are solved, with the main ambitions now being prevention and diagnosis, using cheap, multiplex and decentralized technologies. Consequently, R&D of enzyme-based electrochemical biosensors will most likely continue to be a very active domain of Analytical Sciences, targeting key applications in environmental protection, process industries, homeland defense and biomedical markets. Innovative proposals to either replace the existing methodologies or detect the emerging analytes are thus expected.

Since the pioneer work of Clark and Lyons reporting the first enzyme electrode, numerous efforts have been done to improve the electronic connection between the transducing interfaces and the ET sites of enzymes, as well as the stabilization of signals and the elimination of interferences. However, despite the significant progress in the field, only a few sensors have reached markets other than the glucose analysis. Even though, none of them has come closer to the great commercial success of glucose meters, which assist about 8% of the world population[194] (the volume of sales of lactate meters, the second most sold biosensor device, is at least 100-fold smaller).[20,41] Besides, there has been an over attention on glucose sensing. Actually, GO*x* is often chosen for the evaluation of new sensor designs, due to its outstanding properties and high availability, albeit the technology could potentially serve other niche markets.[41,52,152]

Truth be told, the implementation of biosensor devices is still facing several obstacles, many of which are related with the inherent properties of the biological catalyst, such as the poor stability over time and the weak performance in harsh conditions. The design of integrated systems and the lack of compatibility with automated fabrication technologies are other concerns for a successful business venture.[20,42] Therefore, in order to make possible the widespread adoption of biosensor

technology, the R&D efforts will have to continue. A special attention should be given to several key areas, listed as follows:

- Frequently, there is a lack of knowledge on the catalytic properties and kinetic mechanisms of numerous enzymes. Hence, focused research on fundamental understanding of both *enzyme kinetics* and intra- and inter-molecular *ET processes* could lead to a qualitatively better control and prediction of the bioelectrocatalytic behavior.
- Enzyme availability can also constitute an economic obstacle to the cost-effective mass production of biosensors. In certain cases, the use of modern molecular biology tools might solve the problem by *protein overexpression*.
- *Protein engineering* could also be very useful to tailor the enzyme properties (turnover, selectivity and stability), to facilitate the charge transfer processes (optimizing protein orientation and ET distances) and the protein immobilization (integrating specific attachment tails).[147,195] The design of *de novo* enzymes is also foreseen[146,147].
- The search for *novel enzyme systems* is extremely important. The identification and isolation of new biocatalysts from other sources (e.g., cell membranes, extremophiles) that can selectively recognize interesting substrates would make possible the expansion of biosensor technology to novel target analytes.

Nonetheless, the advances in *nanotechnology* and *surface sciences* over the last decade have been paving the way for the construction of original and efficient bioelectronic systems, including novel configurations of catalytic biosensors. Actually, multi-faceted approaches have been proposed on a regular basis to improve the transducer interface. Very often, these proposals enabled the direct electronic coupling of redox enzymes and the delivery of highly sensitive, selective and stable devices. In addition, the characterization of the resultant nanostructured surfaces has benefitted from the constant progress in high-resolution surface imaging techniques like the electron and scanning probe microscopies [transmission electron microscopy (TEM), scanning electron microscopy (SEM), scanning tunneling microscopy (STM) and atomic force microscopy (AFM)], which can give detailed information about coverage and topographical heterogeneity. AFM and STM, in particular, have brought about an unprecedented capacity to visualize the electrode interface *in situ*, sometimes even with molecular resolution. Furthermore, the coupling of electrochemical cells to both techniques enables the dynamic microscopic characterization of surface modification and interfacial electrochemical ET.[35,149,195–198] A wide variety of other well-established techniques are also being used to probe and map surface monolayers and thin films. Of particular note are the spectroscopic methods like ellipsometry, electrochemical impedance spectroscopy (EIS), X-ray photoelectron spectroscopy (XPS), surface plasmon resonance (SPR), Fourier transform-infrared (FT-IR) spectroscopy, infrared reflection absorption spectroscopy (IRRAS) and Raman spectroscopy.[35,198–201] Both vibrational and the fluorescence spectroscopies can be enhanced by metal interfaces [e.g., surface enhanced infrared absorption (SEIRA) and surface enhanced resonance Raman (SERRS)] and performed *in situ* with simultaneous potential control.[195,202] Besides offering the chance to characterize the interface between electrodes and solutions, the combined spectroelectrochemical approach represents a powerful tool for interrogating the structure and reaction dynamics of immobilized proteins that exchange electrons directly with the electrode surface.[203,204]

To conclude, the future of R&D on biosensing devices should be settled on stronger collaborative approaches, bringing together expertise in biochemistry, electrochemistry, nanotechnology, materials science, electronics and biotechnology. Such interdisciplinary projects will certainly boost the indexes of biosensor applications.

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ORCID

Maria Gabriela Almeida http://orcid.org/0000-0002-4508-7379

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