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Application of the electrochemical concepts and techniques to amperometric biosensor devices

É. Lojou · P. Bianco

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Abstract This review addresses the most salient features concerning amperometric biosensor devices. In a first section, the electrode processes together with the basic principles of amperometric methods currently used in biosensor electrochemistry are described. In the second section, several strategies that combine the control of the electrode surface structure with stable immobilization are examined. The third section presents examples of amperometric biosensors developed for medical or environmental applications.

Keywords Amperometric biosensors · Modified electrodes · Immobilized enzymes

1. Introduction

A sensor is a small analytical detector used for direct measurement of an analyte in a sample matrix. Such a device must be capable of responding continuously and reversibly without perturbing the sample under investigation. Chemical (or biochemical) sensors consist of a transduction element covered with a chemical (or biological) recognition layer. This layer interacts with the target analyte, and the changes resulting from this interaction are translated by the transduction element to quantifiable, either electrical, optical, mass, or thermal signals.

Electrochemical sensors represent an important subclass of chemical sensors in which an electrode is used as the transduction element (Fig. 1). The aim is to generate an electrical signal which relates to the concentration of an analyte. In electrochemical biosensors [1] the analytical capability of electrochemical techniques is combined with the specificity of biological recognition processes. In this configuration, a bio-specific agent is immobilized at the surface of an electrode, which converts the biological recognition process into a quantitative amperometric or potentiometric response. The selectivity of a biosensor is enhanced by immobilizing a sensitive and selective biological element (typically an enzyme) within close proximity of the sensor [2]. Such devices have found a vast range of important applications in the fields of clinical, industrial, environmental, and agricultural analyses. The field of biosensors is interdisciplinary, and advances occur from progress in several disciplines, e.g. the development of microelectronics and microcomputers, research in biotechnology. New transducer technology (such as fiber optics, integrated microelectronic biosensors, piezocrystals, and surface acoustic wave methods), various methods of measurement (such as flow injection analysis), and the use of a large range of biological and biochemical systems (e.g., antibodies, bacteria, cells, organelles, liposomes, and tissues) have greatly expanding the areas of research linked to biosensors, as reviewed in several articles and books [3–7].

Miniaturization is critical for any in vivo, health care application, and useful in physiological studies, and in multisensor probes and arrays [8]. Microelectronics are linked to miniaturization because of the small circuitry and electronics produced by photolithographic semi-conductor technology. The goal of coupling biosensors and bioelectronics is to produce a biochip, a device which consists of the integration of a biosensor system with a microchip to form a sensing bio-electronic system, where multiple sensors and electrochemical processing electronics are on the same chip. The limitations can be in biosensor construction. It is necessary to

É. Lojou · P. Bianco (✉)
Unité de Bioénergétique et Ingénierie des Protéines, Institut de Biologie Structurale et Microbiologie—CNRS, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, France
e-mail: bianco@ibsm.cnrs-mrs.fr

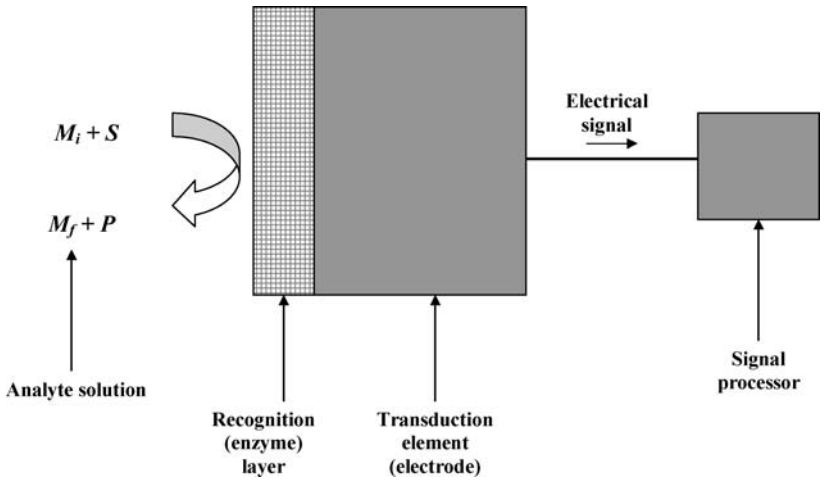

Fig. 1 The basic design of an electrochemical biosensor. The enzyme is immobilized on the electrode surface in the recognition layer. It catalyzes the reaction $S + M_i \rightarrow P + M_f$, where S is the substrate (i.e. the analyte) and M_i the initial form of the electroactive mediator which is "viewed" by the electrode. They are transformed via the electrochemical reaction into the product, P, and the resulting form of the initial M_i species, M_f

master the molecular structure at the surface of the electrode to meet specific applications. To overcome these problems, all-chemical methods of construction using molecular technology are required [9].

Two main categories of electrochemical biosensors may be distinguished, depending upon the nature of the biological process: (i) biocatalytic devices utilizing enzymes, cells, bacteria, or tissues as immobilized components, and (ii) affinity sensors based on antibodies/antigens, membrane receptors, or nucleic acids. The biological materials provide selectivity for the biosensor, but limitations can be observed because of poor transducer's performances. It is easy to guess that a high level of sophistication is necessary to produce easy-to-use, compact, and inexpensive devices.

Three broad classifications of electrochemical methods have to be considered in the field of biosensors [10]:

(1) Potentiometric methods include zero-current potentiometry and methods in which current of controlled amplitude is applied to the working electrode.
(2) Amperometric methods consider all techniques in which current is measured, including constant-potential amperometry and amperometric measurements made in response to a variety of applied potential waveforms in voltammetric methods.
(3) Impedance spectrometry is based on impedance, conductance, or capacitance measurements.

It is beyond the scope of this review to describe the innumerable combinations of biological and sensor elements possible, so only enzyme-modified electrodes [11] working as *amperometric* biosensors will be discussed. The favored configuration for biosensors utilizes amperometry by measuring directly electric current after "translation" of the (bio)chemical signal into an electronic one [12].

This mini-review sets out to describe the most important features concerning the construction of biosensor devices and to illustrate their use with examples taken from applied bioelectrochemistry. In the first section, however, we will briefly give an overview of electrode processes together with the basic principles of amperometric methods currently used in biosensor electrochemistry.

2. Detection of the electric signal

The basis of all controlled-potential techniques is the measurement of the current response related to the concentration of the target analyte to the potential applied to the working electrode. This objective is accomplished by monitoring the transfer of electrons during the redox process of the analyte (Fig. 2):

$$\mathrm{Ox} + \mathrm{ne}^- \Leftrightarrow \mathrm{Red} \tag{1}$$

where Ox and Red are the oxidized and reduced forms of the electrochemically active species. For systems governed by the laws of the thermodynamics, the potential of the electrode can be used to control the concentration of the oxidized form, C_O, and reduced form, C_R, of the electroactive species at the electrode surface according to the Nernst equation

$$E = E^\circ + (2.3RT/nF) \log C_O/C_R \tag{2}$$

where E° is the standard potential, R the universal gas constant, T the Kelvin temperature, n the number of electrons transferred in the reaction, and F the Faraday constant. The current resulting from a change in oxidation state of the electroactive species is termed the faradaic current; it is a direct measure of the rate of the reaction.

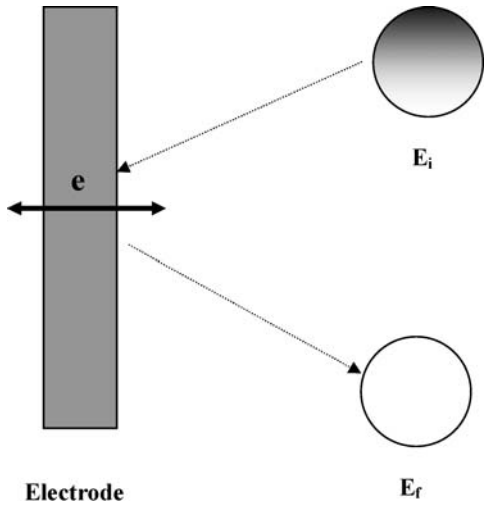

Fig. 2 The electroactive species diffuses toward the electrode surface where it exchanges electron(s), then the electrochemically transformed species diffuses in turn from the electrode surface to the bulk solution

Several potential excitations are available, but we restrict here to chronoamperometry and very popular cyclic voltammetry.

2.1. Chronoamperometry [13]

In chronoamperometry, the potential of the working electrode is stepped from a value at which no faradaic reaction occurs to a potential at which the surface concentration of the electroactive species becomes nil. Mass transport under these conditions is supposed to be solely diffusion-controlled. The current-time curve reflects the change in the concentration gradient in the vicinity of the surface. The current at a planar electrode decays with the time as given by the Cottrell equation:

$$i(t) = nFACD^{1/2}/\pi^{1/2}t^{1/2} \quad (3)$$

where A is the electrode area, C the bulk concentration of the electroactive species, and D its diffusion coefficient.

Chronoamperometry is currently used for *in vivo* analysis by pulsing the potential of the electrode repetitively at fixed time intervals.

2.2. Cyclic voltammetry [13]

Cyclic voltammetry (CV) is the most versatile electroanalytical technique for the qualitative study of the stability and homogeneous reactions of species which may be produced in an electrode reaction. Moreover, CV may be used to obtain the quantitative kinetic data once the mechanism of the reaction is understood. Briefly, CV consists of cycling the potential of an electrode dipped in an unstirred solution from an initial value to a predetermined limit where the direction of the scan is reversed, and measuring the resulting current. The excitation signal for CV is linear potential scan with a triangular wave form. The current is the response signal to the potential excitation signal. The voltammogram is a display of current (vertical axis) versus potential (horizontal axis) as shown in Fig. 3. Because the potential varies linearly with time, the horizontal axis can also be thought as a time axis. Important parameters of a CV curve are the magnitude of the (anodic and cathodic) peak currents, and of the respective peak potentials. For a redox couple in which both species rapidly exchange electrons with the working electrode (i.e. for a reversible electrochemical couple), the formal reduction potential is centered between the anodic and cathodic peak potentials.

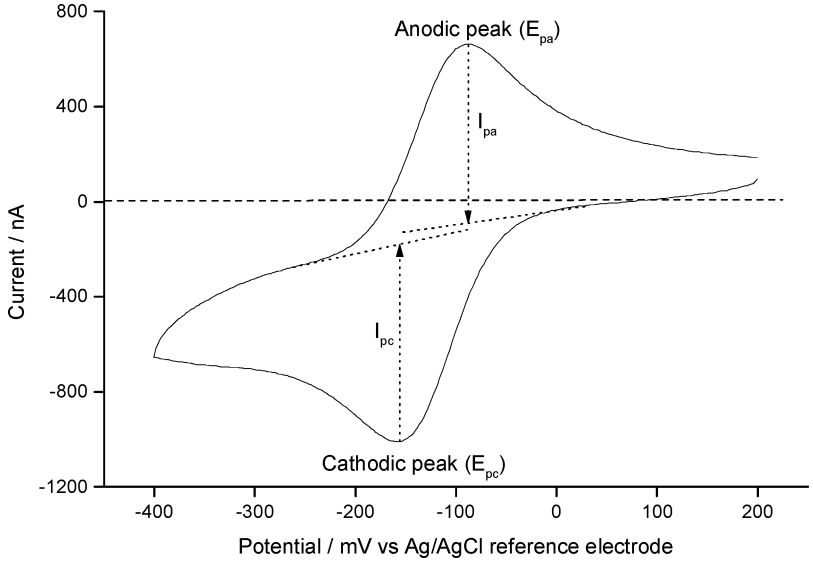

Fig. 3 A typical cyclic voltammogram obtained in the case of a reversible (fast) electrochemical system. Cyclic voltammogram at a pyrolytic graphite electrode of 150 μM $Ru(NH_3)_6^{3+}$ in 10 mM Tris chloride buffer solution, pH 7.6. Scan rate, 20 mV s^{-1} I_{pa} and I_{pc} represent the magnitudes of the anodic and cathodic peak currents, E_{pa} and E_{pc}, the anodic and cathodic peak potentials, respectively

One of the most important applications of cyclic voltammetry deals with qualitative diagnosis of homogeneous chemical reactions that precede or follow the heterogeneous electron-transfer processes occurring at the electrode-solution interface. They directly affect the available surface concentration of the electroactive species. One of the simplest electrode reactions is the electrochemical/chemical mechanism (EC) (also called "following chemical reaction") in which the electrogenerated species reacts with a solution component Z at a rate characterized by the rate constant k. The catalytic regeneration mechanism is a variation of this type of mechanism, in which the initial electroactive species is regenerated by the homogeneous reaction, as follows:

$$R \Leftrightarrow \mathrm{O} + e^- \tag{4}$$

$$O + Z \overset{k}{\rightarrow} R + Z' \tag{5}$$

This situation is frequently encountered in the reaction of an enzyme (E), a substrate (S), and an electroactive mediator (M). The mediator shuttles electrons between the electrode and the enzyme. Changes in the shape of the cyclic voltammograms (as illustrated in Fig. 4 for the reaction between an enzyme, hydrogenase, a substrate, hydrogen, and a mediator, cytochrome c_3 [14]), resulting from the chemical competition for the electrochemical reactant or product, allow the reaction pathways to be elucidated [15, 16]. A well-known example of clinical importance is the reaction of glucose oxydase ($= E$), glucose ($= S$), and a fast redox system, benzoquinone ($= M$).

Practically, in the case of enzyme-based electrodes, an immobilized layer of enzyme is selected to catalyze a reaction, which generates (or consumes) a detectable species, based on the scheme given above in Fig. 1:

$$S + M_i P \overset{Enzyme}{\rightarrow} + M_f \tag{6}$$

where M_i and M_f represent the mediator under its initial (i.e. before the electron-exchange reaction) and final form (after the electron-exchange reaction), respectively. The success and performances of enzyme electrodes depend largely on the quality of the immobilization of the enzyme layer when working under different conditions (pH, temperature, ionic strength, stability of the biomolecules, working time, storage conditions, etc.). Enzyme electrodes can suffer from some drawbacks resulting from undesirable adsorption of inhibiting molecules, and interfering compounds.

Enzyme-modified electrodes must obey several mandatory conditions as selectivity for the analyte of interest, maintain of the integrity of the active site, rapidity of the response, and, if possible, low cost from commercial considerations. In the following section, we will examine the main schemes that can be used to construct enzyme-immobilized electrodes which meet the requirements above, with the objective to enhance surface coverage with active enzyme while maintaining a rapid time response

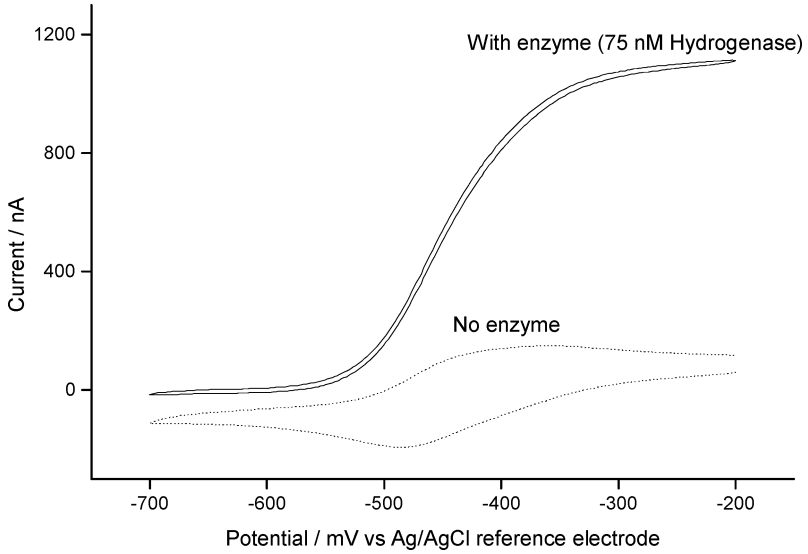

Fig. 4 An example of homogeneous enzymatic reaction: the catalyzed oxidation of hydrogen (the substrate) via hydrogenase (the enzyme) in the presence of cytochrome c_3 (the mediator). The catalytic scheme is [14]:
$\frac{1}{2}\mathrm{H_2} + \mathrm{Hase_{ox}} \rightarrow \mathrm{H^+} + \mathrm{Hase_{red}} + e^-$
$\mathrm{Hase_{red}} + \mathrm{Cyt\,c_{3ox}} \rightarrow \mathrm{Hase_{ox}} + \mathrm{Cyt\,c_{3\,red}}$
$\mathrm{Cyt\,c_{3red}} \Leftrightarrow \mathrm{Cyt\,c_{3ox}} + 4e^-$.
Cyclic voltammetry at a pyrolytic graphite electrode of 20 μM *Desulfovibrio fructosovorans* cytochrome c_3 in 10 mM Tris chloride buffer solution, pH 7.6, in the absence and in the presence of 75 nM hydrogenase. Scan rate, 5 mV s^{-1}

and overcoming the problems associated with electrode fouling.

3. Construction of enzyme-based electrodes

3.1. Principle

Two possible configurations available for an amperometric biosensor electrode system can be proposed [8]:

(1) The electrode is dipped into a buffer solution containing the enzyme and a mediator. On addition of substrate, the change in concentration of mediator may be monitored with respect to time.
(2) In a second configuration, the enzyme is attached to (or in close vicinity of) the sensing electrode itself. Satisfactory running relies on diffusion of substrate to the enzyme, and on diffusion of the enzymatically generated (reduced or oxidized) form of the mediator from the immobilized enzyme to the working electrode. It is clear that immobilization of enzymes onto the electrode surface is a crucial step. Success for obtaining satisfactory and stable responses depends upon the efficiency of immobilization.

In this mini-review, only systems involving enzymes attached directly (or very close) to the working electrode will be considered.

3.2. Immobilization of enzymes at electrode surfaces

The immobilization of enzymes that are confined to an electrode surface offers several attractive features. When the enzyme is immobilized, it can be readily separated from the reaction mixture and re-used. Furthermore, by controlling the microenvironment of the immobilized enzyme, high sensitivity, fast response, and prevention of electrode fouling and interferences from other species in solution can be achieved. Methods for immobilizing enzymes at electrode surfaces can be viewed as variants closely related to those classically used to prepare electrodes that are gathered under the generic term of "chemically modified electrodes" [17–20].

There are several strategies to immobilize an enzyme onto an electrode surface (Fig. 5). They are briefly reviewed under.

3.2.1. Membrane electrode (Fig. 5(A))

The simplest approach, initially described in several reports [21, 22] and more recently revisited [23], is to imprison the enzyme between the electrode surface and a dialysis membrane having a cutoff inferior to the size of the enzyme, while the substrate is able to freely diffuse through the dialysis membrane. This technique offers several advantages: only small amounts of enzyme are used, the integrity of the enzyme is preserved, several parameters (especially, pH and ionic strength) can be modified without renewing of the enzyme sample.

3.2.2. Adsorption (Fig. 5(B))

Physical adsorption of the enzyme on the electrode surface is also a very simple technique. Theoretically, this strategy could be used for any enzyme (since proteins are supposed to adsorb spontaneously and irreversibly on solid surfaces [24]). After the contact step, the molecules which have not been adsorbed are removed by washing. The electrode surface is randomly, and often completely, covered with the enzyme, thus possibly hindering electron transfers. The technique is not widely applicable because of possible enzyme leaching from the electrode surface. The procedure suffers also from susceptibility to changes in pH, ionic strength, and temperature. Nevertheless, successful catalytic studies have been carried out using enzymes adsorbed on an electrode [25, 26], thus opening promising avenues for further research.

3.2.3. Covalent attachment (Fig. 5(C))

This technique provides a more stable immobilized enzyme, and it is widely applicable. The covalent attachment generally involves three main steps [24], i.e. (1) activation of the support, (2) enzyme coupling, and (3) removal of loosely bound enzymes. The optimal experimental conditions for each step have to be determined. Attachment to the support can be accomplished via chemicals such as silanes or, in the case of carbon material, after treatment with, e.g., carbodiimide or glutaraldehyde [27]. A drawback for covalent coupling is the decrease in (or even loss of) activity which can affect the fixed enzyme.

3.2.4. Gel/Polymer entrapment (Fig. 5(D))

This technique uses mild conditions, and a wide variety of matrix hosts are available. Other advantages are that most enzymes can be easily incorporated into polymers, and a high concentration of active enzyme can be immobilized. The most currently employed matrices are hydrogels such as alginate, collagen, cellulose triacetate, polyacrylamide, gelatin, silicone rubber, and poly(vinyl alcohol). Drawbacks can occur from the size and structure of polymer, and possible deactivation by radicals formed during polymer formation. Leaching of the enzyme can also be observed [28].

More specifically, the entrapment in electropolymerized films is based on the electrochemical oxidation of a monomer species dissolved in a solution containing an enzyme to form a polymer at the electrode surface. Advantage is gained in the

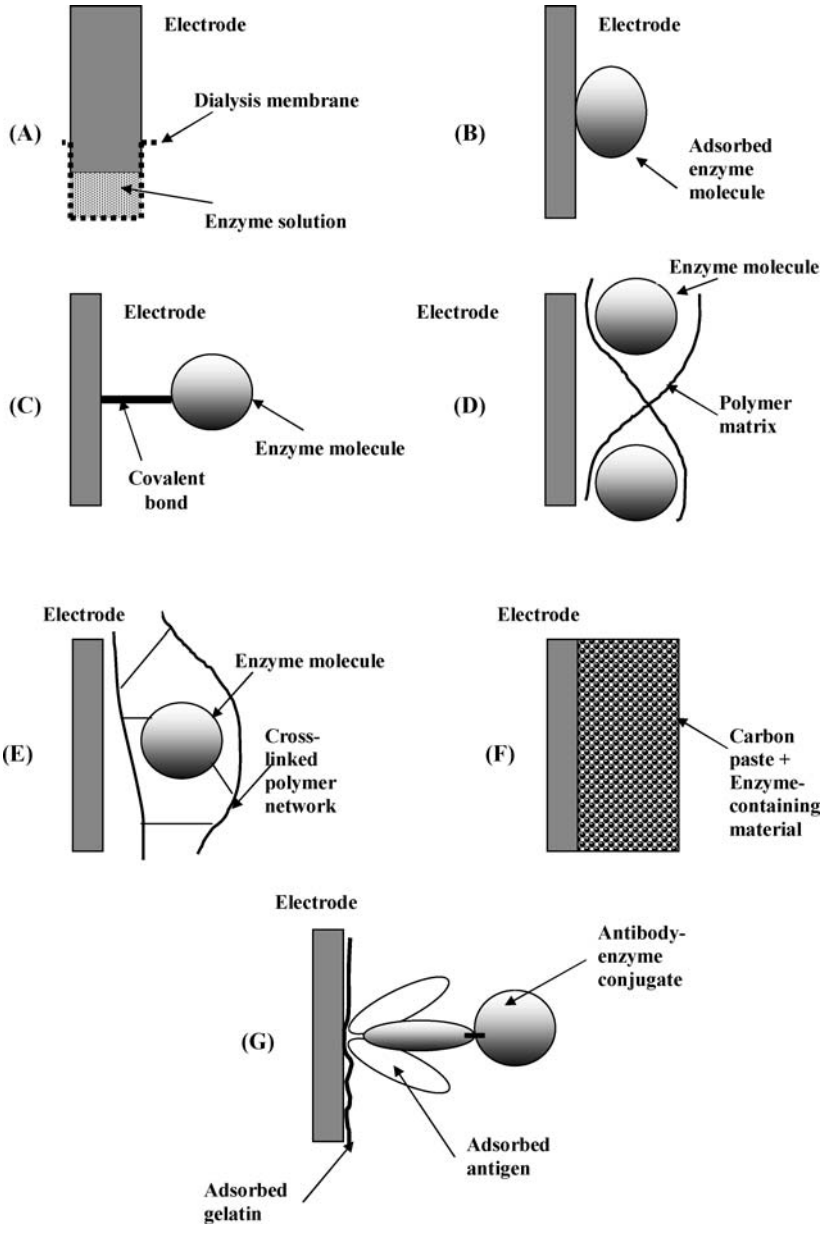

Fig. 5 Scheme of the main types of enzyme-modified electrodes. (A) Membrane electrode; (B) Modification of the electrode surface through adsorption; (C) Covalent bonding; (D) Gel/polymer entrapment; (E) Entrapment via cross-linking; (F) Entrapment of the enzyme-containing material in a carbon-paste electrode; (G) Antigen-Antibody assembly on an electrode

control of the polymer film thickness and hence the amount of enzyme confined in the close vicinity of the electrode. Drawbacks can result from instability of the enzymes when using a large potential range for performing polymerization [29].

3.2.5. Cross-linking (Fig. 5(E))

Adsorption and entrapment are often supplemented with cross-linking to prevent leaching of the enzyme. It is also possible to use cross-linking alone. Because of the presence of lateral functions, enzymes can be cross-linked by employing bifunctional agents [30, 31]. The enzymes can be linked either with each other or with another, "inert", protein. The difficulties with this method are that experimental conditions are not always easy to control.

Moreover, it is possible that the integrity of the enzyme is not totally conserved after the cross-linking step.

3.2.6. Tissue and bacteria electrodes (Fig. 5(F))

The limited stability of enzymes when under their isolated state, and the fact that some enzymes are expensive or not stable when extracted from tissues have promoted the direct use of cellular materials as components of enzyme electrodes. Carbon-paste matrix mixed with tissues can be used as fast-responding and sensitive sensors, when introduced in the cavity of an electrode [32, 33]. In another configuration, whole cells have been immobilized at carbon electrode

surfaces to control their hydrogenase activity in hydrogen uptake and evolution reactions [14, 34].

3.2.7. Immunological reactions (Fig. 5(G))

Most reported affinity biosensors are based on immunological reactions involving the recognition of an antigen by an antibody binding site to form an antibody/antigen complex [35]. The antibody is a protein which binds to foreign molecules, i.e. antigens. The remarkable selectivity of antibodies is based on the stereospecificity of the binding site for the antigen. In such enzyme "immunosensors", the antibody is attached to the transducer (i.e. the electrode) and in contact with the solution containing the antigen (i.e. the analyte). After the reaction is accomplished, the sensor is washed to eliminate components which have not reacted. The sensor is placed in a solution containing the substrate capable of selectively reacting with the enzyme, and the electrochemical measurement is performed [36]. In fact, these immunosensors are relatively short-lived because of poor sensitivity due to nonspecific binding. Best and most reproducible behaviors can be obtained when a convenient protein is adsorbed as a sacrificial antigen on the electrode surface (covered with gelatin to hinder nonspecific adsorption). In a second step, the adsorbed antigen is recognized by a labeled antibody to form a second layer endowed with catalytic activity [37].

3.2.8. Other methods

Other less conventional techniques based on non-manual approaches for the deposition of enzyme-containing polymer films have been described [38], such as screen-printing techniques combined with photoinduced cross-linking, spin-coating followed with photolithographic structuring and lift-off and piezo-actuated microdispensing.

Layer-by-layer organized film assembly by alternate adsorption of charged macromolecules provides a way for constructing a molecular architecture in the direction perpendicular to an electrode. In the assembly procedure one can use different proteins and polyions. Efficient devices can be built up provided that alternating negatively and positively charged species are assembled [39]. Successful electrode-driven catalysis of the oxidation of styrene has been studied using films containing myoglobin or cytochrome P450 [40].

4. Mediated electron-transfers between enzymes and electrodes

The electroactive centers of most redox enzymes are generally buried deep inside the protein structure. Considering the electrode and the redox center of an enzyme as a donor-acceptor pair, it appears that the thick polypeptidic layer surrounding the active center(s) provides an effective kinetic barrier to electron transfer. The insulation of the active center usually can hinder any direct electron transfer with bulk electrodes. Very few non-mediated direct biocatalytic reactions have been observed (except, for example, the direct electroreduction of O_2 and H_2O_2 by laccase and horseradish peroxidase, respectively). Challenges facing the development of efficient amperometric biosensors can be met by developing methods based on "mediated" electron-transfer processes [41]. The direct electron communication between redox enzymes and electrodes can be established by summoning artificial charge-carriers (i.e. electron-transfer mediators) as intermediates. These compounds have a wide range of structures and properties, including a wide range of redox potentials. In shuttled charge-transfer processes, the mediator is repetitively cycled between its oxidized and reduced states. Catalytic current densities as high as 50 μA/cm^{-2} can be obtained routinely [42]. A mandatory condition is that the mediator provides rapid reaction with the enzyme as with the electrode. Several approaches have been designed to enhance the electron transfers between redox enzymes and the electrodes. These methodologies range from the use of soluble enzymes with diffusing electron mediators to more sophisticated multi-component templates. The development of these efficient methodologies has resulted in the construction of numerous amperometric biosensors including bioreactors and biofuel cells.

Several situations can be encountered in mediated electron-transfer between redox enzymes and electrodes [43]. We will limit here to the cases of electron-transfer involving diffusing mediators or mediator-functionalized electrodes (Fig. 6).

4.1. Electron-transfer reactions controlled by diffusing mediators

The enzyme and the mediator are dissolved in the solution. The mediator acting as electron relay is able to enter the enzyme body up to the active center where electrons are exchanged, and then to re-diffuse from the protein toward the external solution and the electrode. Ferrocene derivatives, organic dyes, hexaferricyanide, ruthenium complexes have been largely employed as mediators in the case of soluble redox enzymes lacking direct contact with the transducers.

4.2. Dissolved enzymes at mediator-modified electrodes

This situation is interesting for the study of the interactions between enzymes and mediators. The electrochemical kinetics of electrodes modified with various mediators (especially viologens) have been studied upon their interaction with

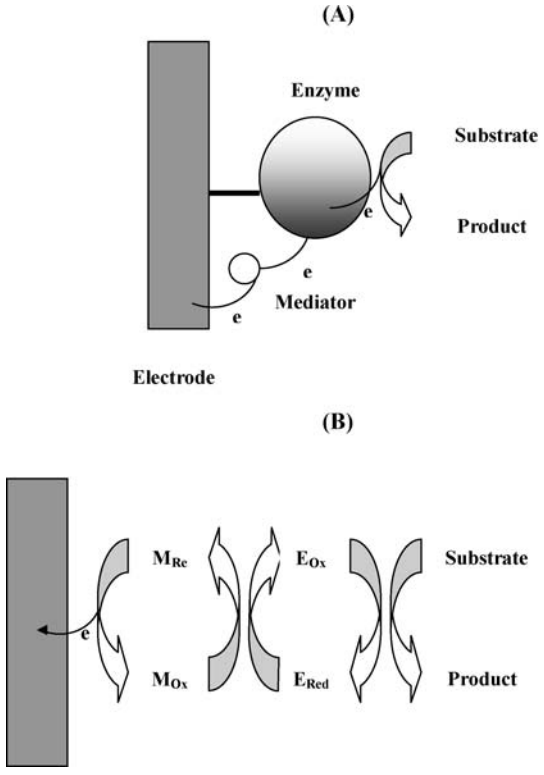

Fig. 6 (A) Scheme of an electron-transfer reaction provided by a mediator diffusing in a solution containing the substrate to be detected. (B) Sequence of events that occurs in the mediated system

freely diffusing enzymes. Such studies have revealed affinity association of the enzyme molecules with the mediator-modified electrode surfaces. As a consequence, such constructions have allowed the development of integrated biocatalytic systems of mediators and enzymes resulting from the lateral cross-linking of the affinity complex generated onto the electrode surface.

4.3. Dissolved mediators at enzyme-modified electrodes

In this situation, the enzyme is immobilized as described in paragraph 3 and the mediator is able to freely diffuse inside the solution up to the modified electrode surface. Detectable amperometric responses depend upon the amount and efficiency of active enzyme fixed on the electrode surface. Improvements in the amperometric responses can be attained by depositing a controllable number of the enzyme layers in multilayer assemblies [44].

4.4. "Wired" enzyme electrodes

It is possible to establish an electrical connection of active centers of enzyme to electrodes through an electrical "wiring", especially in the case of enzymes which are not able to exchange electrons directly with the electrochemical transducer [45]. Well-known examples of "wiring mediators" used for the detection of glucose by glucose oxidase are osmium bipyridine complexes which establish facile exchange of electrons between the bound metal centers and the active sites of the enzyme. One of the coordination sites of the osmium center is occupied by the N-atom of an imidazole or pyridine moiety of the poly(vinylimidazole) or poly(vinylpyridine) unit. These polymeric units react with a diepoxide to form a cross-linked, three-dimensional redox polymer which remains immobilized on the electrode surface. In this case, electron transfer between the active site and the electrode surface is achieved by "electron-hopping" between the osmium centers attached to polyvinyl units. For this reason, the active sites can be considered to be "wired" to the electrode surface.

5. Examples of amperometric biosensors

5.1. Ferrocene-modified horseradish peroxidase for the determination of hydrogen peroxide

Horseradish peroxidase (HRP) is a heme-containing glycoprotein. Because of its availability in high purity and low cost, it is a privileged enzyme for sensing purposes. It catalyzes oxidation of a substrate by hydrogen peroxide:

$$\text{Substrate} + H_2O_2 \rightarrow \text{Product} + H_2O \quad (7)$$

The catalytic currents generated in the presence of peroxidase but in the absence of a mediator are very low at conventional electrodes. The strategy of covalently binding the mediator to the enzyme, in addition to retaining the mediator at the electrode surface has been adopted [46], thus offering a closer integration of the enzyme to mediator and mediator to electrode where the electron-transfer reactions occur.

The working electrode was a printed carbon electrode [46]. It was modified with adsorbed ferrocene-modified HRP. Figure 7 shows a cyclic voltammogram of the ferrocene-modified HRP electrode in the absence of hydrogen peroxide (dashed line). When a drop of a solution of hydrogen peroxide was placed on the surface of the electrode and the cyclic voltammogram again performed, a substantial cathodic current enhancement was observed and the anodic peak disappeared (solid line). This behavior results from the catalytic oxidation of the ferrocene groups by hydrogen peroxide and their reduction by the working electrode. The stability of the modified ferrocene-HRP electrode was assessed from measurements within a 16 days range: no variation in current values was detected within 5 days. The electrode has been shown to be suitable [46] for the determination of hydrogen peroxide in the range 1–50 μmol l^{-1}. The same

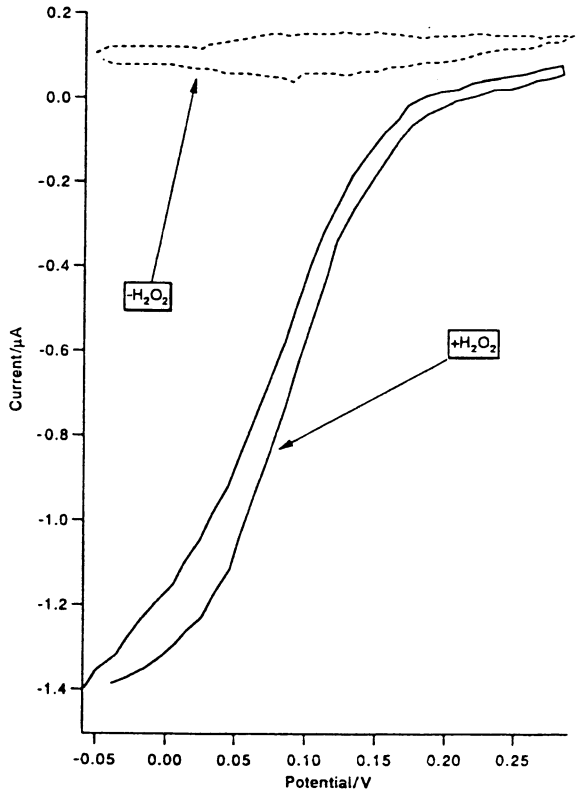

Fig. 7 Cyclic voltammograms at a printed electrode modified with adsorbed ferrocene-modified HRP, in the absence (dashed line) and presence (solid line) of 0.5 mmol l^{-1} hydrogen peroxide. Potential sweep, 50 mV s^{-1}. [W.-C. Tsai and A.E.G. Cass, *Analyst*, **120**, 2249 (1995)]—Reproduced by permission of The Royal Society of Chemistry

electrode can also be used for the determination of linoleic hydroperoxide, thus suggesting that such a biosensor is suitable for assessing the early onset of oxidative deterioration in foods (rancidity).

5.2. Amperometric biosensors for the detection of glucose

Due to the pivotal role of glucose in various physiological processes, much efforts have been devoted to the development of methods for detecting glucose in food and biological matrices, especially human blood. Among these methods, enzyme-based sensors are a privileged tool. The enzyme most widely used for glucose detection is glucose oxidase. The active site of this enzyme is a flavin adenine dinucleotide which exists in one of two forms (oxidized, FAD, or reduced, $FADH_2$). FAD oxidizes glucose to gluconic acid, and the $FADH_2$ generated can be oxidized to FAD by oxygen (hydrogen peroxide being a by-product of this reaction). Methodologies based on glucose oxidase catalytic reaction have been extensively plowed, and we have selected two different approaches to illustrate the versatility of amperometric biosensors.

5.2.1. Dissolved glucose oxidase at mediator-functionalized electrodes.

The enzyme is present in the analyte solution in contact with surface-immobilized redox mediator, in this case fullerene-C_{60} derivative acting as relay [47] (see paragraph 4.2). When a fullerene-C_{60}-functionalized gold electrode is dipped in a solution containing glucose oxidase, cyclic voltammetry reveals an efficient electrocatalyic current upon addition of glucose (Fig. 8). The catalytic current depends upon the glucose concentration (see inset in Fig. 8), and varies linearly in the range 0–60 mM.

5.2.2. Electrocatalytic activity of a glucose oxidase monolayer based on antigen-antibody assembly onto an electrode.

This approach uses the concept of molecular recognition to assembly successive monolayers of glucose oxidase on a glassy carbon electrode surface [44] (see paragraph 3.2.7). A polyclonal IGG purified from rabbit serum is adsorbed as a sacrificial antigen on the glassy carbon surface (Fig. 9). In a second step, the adsorbed IgG is recognized by anti-rabbit IgG covalently bound to glucose oxidase to form a second layer endowed with catalytic activity. Cyclic voltammetry reveals the occurrence of the catalytic oxidation of glucose after the addition of the substrate, as shown in Fig. 10 (solid line). The enzymatic activity and the rate constants of the catalytic reaction can be derived from the experimental curves. Once these rate constants are calculated, the measurement of the catalytic currents gives access to the amount of immobilized enzyme catalytically active [48, 49]. It has been established that self-assembly by antigen-antibody interactions does not decrease the enzyme activity.

This approach opens new avenues in the field of amperometric biosensors, when modifying the nature of the enzyme and constructing more sophisticated multi-enzymatic systems.

5.3. Cell- and tissue-modified electrodes

Cellular materials can be used directly as a source for enzymatic activity (see paragraph 3.2.6). This approach is very suitable to preserve the stability of delicate biological species and is more close to physiological processes. Two examples of such a methodology are given here.

5.3.1. Control of the hydrogenase activity at cell-coated carbon electrodes.

Hydrogenases are enzymes that catalyze the reversible reaction $H_2 \Leftrightarrow 2H^+ + 2e^-$, in the presence of a suitable electron donor/acceptor [50]. Some hydrogenases are involved

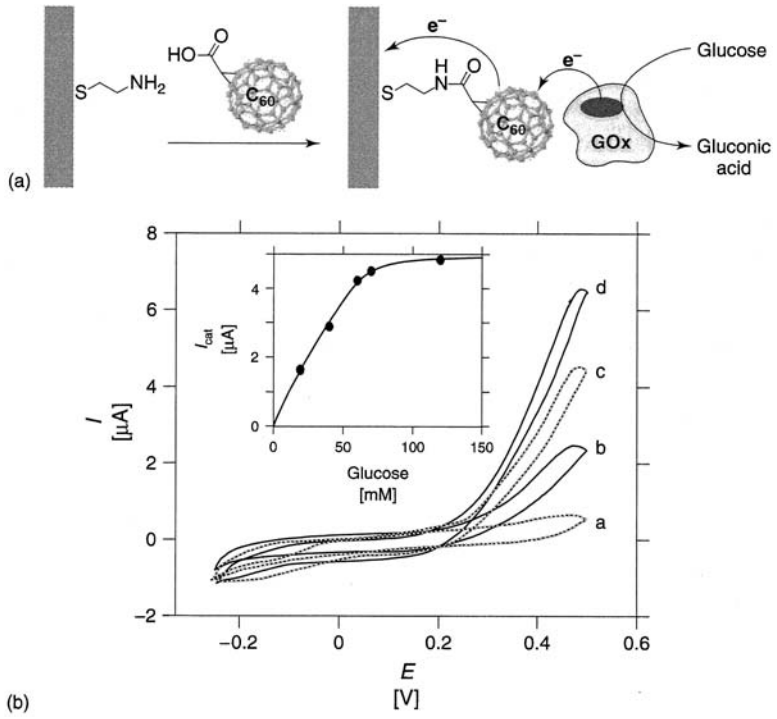

Fig. 8 (a) Assembly of a fullerene-C_{60} monolayer on a modified gold electrode for the catalyzed oxidation of glucose through glucose oxidase. (b) Cyclic voltammograms at the fullerene-C_{60} modified electrode in the presence of 2 mg ml^{-1} glucose oxidase and glucose at increasing concentrations: (a) 0, (b) 20, (c) 40, (d) 100 mM. Scan rate, 5 mV s^{-1}. Inset: calibration curve for the concentration dependence of the catalytic current. [E. Katz, A.N. Shipway, and I. Willner, in *Bioelectrochemistry*, edited by G.S. Wilson (Wiley-VCH, Weinheim, 2002), p. 567]—Reproduction by permission of WILEY-VCH Verlag

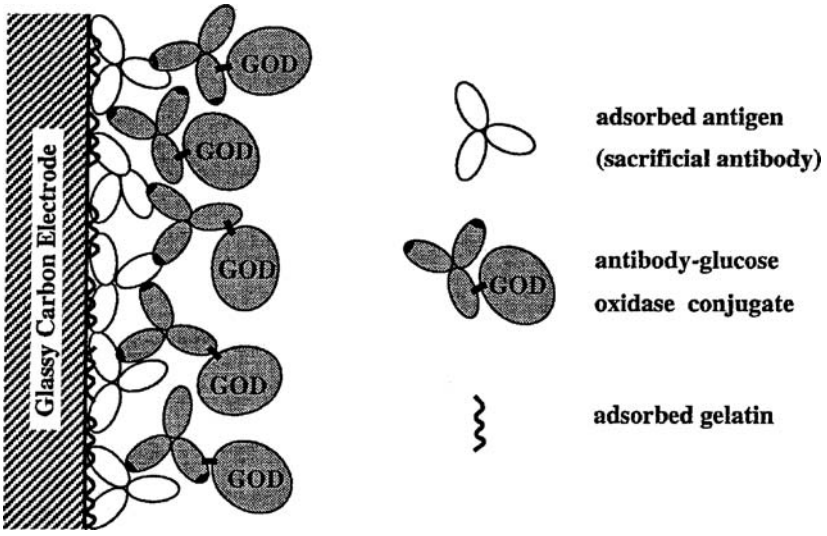

Fig. 9 Scheme of the modified monolayer glucose oxidase glassy carbon electrode obtained after adsorption of a sacrificial antibody and reaction with an enzyme antibody conjugate. Gelatin hinders nonspecific adsorption of the conjugate. [C. Demaille, J. Moiroux, J.M. Savéant, and C. Bourdillon, in *Protein Architecture*, edited by Y. Lvov and H. Möhwald (Marcel Dekker, Inc., New York, 2000), p. 317]—Reproduction by permission of Marcel Dekker

in hydrogen uptake and evolution reactions occurring e.g. in sulfate-reducing bacteria (see paragraph 2.2 and Fig. 4 for the catalytic reaction monitored by *Desulfovibrio fructosovorans* hydrogenase in the presence of its physiological partner, cytochrome c_3, under hydrogen as substrate [14]). In order to understand the hydrogen cycling mechanism for further applications to fuel cells, exhaustive studies have been performed using whole bacteria cells [34]. A glassy carbon electrode was modified by a coating of whole cells. The anodic and cathodic activities were studied in the presence of methyl viologen used as a mediator. Typical sigmoidal-shaped waves (Fig. 11) were observed either in hydrogen uptake or in hydrogen evolution conditions. It has been suggested that sufficient protection of the enzyme in the cell body is ensured, so that no denaturation can occur. This approach opens a promising way for the development of whole cell-based biosensors.

5.3.2. Mushroom tissue-modified electrodes: a dopamine-biosensor

A very interesting development in the field of electrochemical biosensors is the use of catalysts such as animal- or plant tissues and cells (see paragraph 3.2.6). The employ-

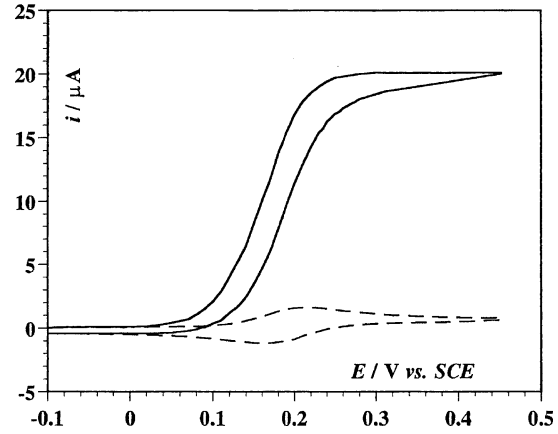

Fig. 10 Cyclic voltammograms at a glassy carbon electrode modified with a monolayer of glucose oxidase, in the presence of 0.1 mM ferrocene methanol in pH 8 phosphate buffer: no glucose (dashed line), plus 0.5 M glucose (solid line). [C. Demaille, J. Moiroux, J.M. Savéant, and C. Bourdillon, in *Protein Architecture*, edited by Y. Lvov and H. Möhwald (Marcel Dekker, Inc., New York, 2000), p. 323]—Reproduction by permission of Marcel Dekker

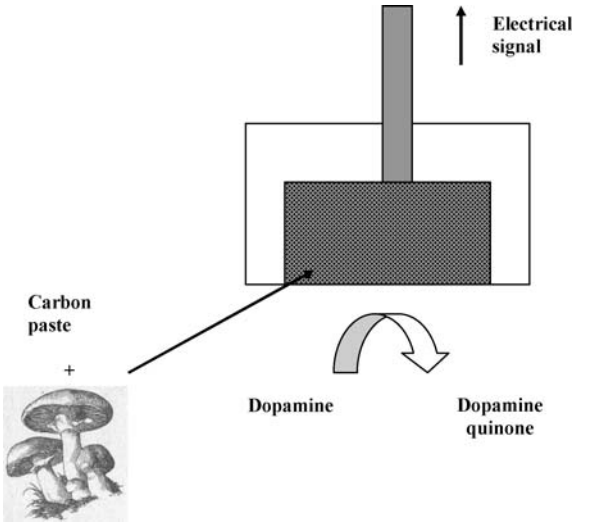

Fig. 12 Scheme of a mushroom tissue/carbon paste electrode for the detection of dopamine [33]

ment of tissues slices as catalysts is attractive because of their high stability, high level of activity, and low cost. An approach based on a mixed-plant tissue/carbon paste electrode has been described [32, 33] (Fig. 12). The detection of dopamine at a mixed-mushroom (or other vegetal products such as pear, peach or potato) tissue/carbon paste electrode has been performed. The biocatalytic activity of this electrode arises from the fact that these natural tissues contain polyphenol oxidase, which catalyzes the conversion of dopamine to dopamine quinone. Flow injection measurements have given a detection limit of about 1×10^{-6} M (i.e. 20 ng) dopamine. Selective detection of dopamine in the presence of ascorbic acid has also been demonstrated. The results suggest that this type of "mixed" electrode could be widely applicable to other types of biosensors.

6. Marketing of amperometric biosensors

Biosensor research is ongoing and further applications are continually being developed. Biosensor research and marketing during the 1990s focused primarily on medical and healthcare applications, mainly for glucose detection. Because of the increased range of applications for biosensors, their global market is now worth an estimated $ 4 billion per year. Some examples of commercially available biosensors are given in Table 1.

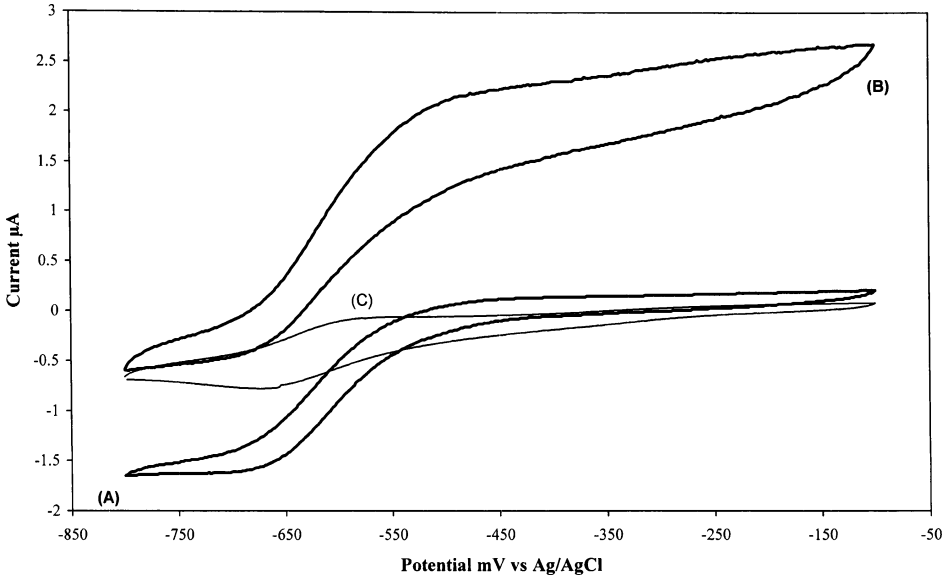

Fig. 11 Cyclic voltammograms at a *Desulfovibrio fructosovorans* cell-modified glassy carbon electrode in the presence of 100 μM methyl viologen, (A) in nitrogen-saturated 0.1 M acetate buffer, pH 5.6, (B) in hydrogen-saturated 0.1 M Tris chloride buffer, pH 8.5. Curve (C) is obtained at the bare glassy carbon electrode. Scan rate, 20 mV s^{-1}. [É. Lojou and P. Bianco, *Electroanalysis*, **16**, 1093 (2004)]—Reproduced by permission of WILEY-VCH, Verlag

Table 1

Target compound	Biosensor type
Glucose	Enzyme-based
Benzene	Antibody-based
Fermentation monitoring	Antibody-based
Herbicides/pesticides	Antibody-based
Microbial contamination	Enzyme-based
Metals	Micro-organism or enzyme based
Ammonia	Enzyme-based

Biosensors can be used in a wide range of applications within many different industries and health problems. Applications fall in three main categories, as follow.

6.1. Environmental monitoring

As an example, bioluminescence-based biosensors are now widely used throughout Europe and have been adopted as a screening procedure for testing water quality in France, Germany, Spain and Sweden. On-line biosensors have been used successfully for detecting specific microorganisms in drinking water, food and pharmaceutical products.

6.2. Monitoring of manufacturing processes

"Real time" information is often required about the formation of product to indicate how well the process is performing. Biosensors offer an efficient means in applications including fermentation monitoring, process water monitoring, detection of trace compounds in treated/untreated waste.

6.3. Safety monitoring

There is an ongoing legislative requirement in industry for the rapid and sensitive monitoring of substances that are hazardous for human health (an example: kits for the detection of benzene, that are commercially available). There are also applications for on-line biosensors to detect water contamination and to monitor air and surface in areas such as pharmaceuticals and cosmetics manufacture.

Economically speaking, the costs of biosensors vary depending on their applications. Although instrumentation usually required can be costly, biosensors offer a competitive method of analysis and eventually low cost per test. The use of biosensors can result in significant time savings and reduced waste production.

7. Conclusion and perspective

The present account has addressed insights into amperometric biosensors by describing the most current methods of construction. Some examples are given on the applications of these biosensors to practical problems related to health, food, or energy supply. Of course, other methodologies may be envisaged to expand the area of biosensors to other analytical tasks. For example, the development of new biosensors can be seen as essential to solving the inherent difficulties of a variety of challenging in situ and in vivo analyses. Other types of biosensors exist (such as potentiometric devices), but in all cases, an important challenge is miniaturization of biosensors: the interest is double, (i) by limiting the amount of immobilized enzyme, and (ii) by yielding tools for direct use and possible implantation in living tissues. Recent developments have shown that laser patterning can be used to make biosensors with enhanced sensitivity [8]. Increase of the biological material in these patterned structures can be accomplished by using dendrimer-like chemistries. In consequence, very promising ways toward "ideal" amperometric biosensors have been opened.

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