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Research review paper

Immobilization strategies to develop enzymatic biosensors

Audrey Sassolas [1], Loïc J. Blum, Béatrice D. Leca-Bouvier *

Université de Lyon, Université Lyon1, CNRS, UMR5246, Institut de Chimie et Biochimie Moléculaires et Supramoléculaires, Laboratoire de Génie Enzymatique et Biomoléculaire, F-69622, Villeurbanne cedex, France

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ABSTRACT

Immobilization of enzymes on the transducer surface is a necessary and critical step in the design of biosensors. An overview of the different immobilization techniques reported in the literature is given, dealing with classical adsorption, covalent bonds, entrapment, cross-linking or affinity as well as combination of them and focusing on new original methods as well as the recent introduction of promising nanomaterials such as conducting polymer nanowires, carbon nanotubes or nanoparticles. As indicated in this review, various immobilization methods have been used to develop optical, electrochemical or gravimetric enzymatic biosensors. The choice of the immobilization method is shown to represent an important parameter that affects biosensor performances, mainly in terms of sensitivity, selectivity and stability, by influencing enzyme orientation, loading, mobility, stability, structure and biological activity.

Contents

* Corresponding author. Tel.: +33 472 448214; fax: +33 472 447970.
E-mail address: leca@univ-lyon1.fr (B.D. Leca-Bouvier).
[1] Present address: Laboratoire IMAGES EA 4218, Université de Perpignan Via Domitia, 52 avenue Paul Alduy bât. S, 66860 Perpignan Cedex, France.

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1. Introduction

Environmental monitoring, food industry or clinical analyses require the development of selective tools for biomolecule detection. Biosensors can be regarded as complementary tools to classical analytical methods (e.g. high performance liquid chromatography) due to their unique feature, their inherent simplicity, relative low cost, rapid response and proneness to miniaturization, thereby allowing continuous monitoring.

A biosensor is a device composed of two intimately associated elements (Fig. 1):

- a bioreceptor, that is an immobilized sensitive biological element (e.g. enzyme, DNA probe, antibody) recognizing the analyte (e.g. enzyme substrate, complementary DNA, antigen). Although antibodies and oligonucleotides are widely employed, enzymes are by far the most commonly used biosensing elements in biosensors.
- a transducer, that is used to convert the (bio)chemical signal resulting from the interaction of the analyte with the bioreceptor into an electronic one. The intensity of generated signal is directly or inversely proportional to the analyte concentration. Electrochemical transducers (Thévenot et al., 1999) are often used to develop biosensors (Sarma et al., 2009; Shah and Wilkins, 2003). These systems offer some advantages such as low cost, simple design or small dimensions. Biosensors can also be based on gravimetric, calorimetric or optical detection.

The first biosensor was described by Clark and Lyons in 1962 (Clark and Lyons, 1962) who immobilized glucose oxidase (GOD) on the surface of an amperometric oxygen electrode via a semipermeable dialysis membrane in order to directly quantify glucose concentration in a sample. Since then, numerous enzyme sensors have been developed for determining various substances such as glucose (D'Orazio, 2003; Wang, 2001, 2008; Wilson and Gifford, 2005), cholesterol (Arya et al., 2008) or lactic acid (Mo and Smart, 2004) in biological fluids (blood, serum, urine) (Malhotra and Chaubey, 2003), for toxicity analysis in environmental monitoring (Andreescu and Marty, 2006; Rogers, 2006), for food and quality control (Mello and Kubota, 2002) and in the biomedical and drug sensing arena.

Immobilization of enzymes is an important feature in designing the biorecognition part of enzyme based biosensors. Many books and comprehensive reviews have been written on enzyme immobilization thus reporting thousands of protocols (Cao, 2005a, 2005b; Guisan, 2006; Hermanson et al., 1992; Hermanson, 2008; Mulchandani and Rogers, 1998; Mateo et al., 2007; Minteer, 2011). Various immobilization strategies can be envisioned: adsorption, covalence, entrapment, cross-linking or affinity (Andreescu and Marty, 2006; Arya et al., 2008; Choi, 2004a) (Fig. 2). Table 1 presents the main advantages and drawbacks of each immobilization method. In some cases, enzyme immobilization protocols are also based on the combination of several immobilization methods. For example, an enzyme can be pre-immobilized on beads by adsorption, affinity or covalence before further entrapment in a porous polymer.

Enzyme immobilization appears as a key factor to develop efficient biosensors with appropriate performances such as good operational and storage stability, high sensitivity, high selectivity, short response time and high reproducibility. Immobilized biomolecules have to maintain their structure, their function, to retain their biological activity after immobilization, to remain tightly bound to the surface and not to be desorbed during the use of the biosensor. Moreover, an ideal biosensor has to be stable for long-term application. The type of immobilization method affects activity and stability of enzymatic biosensors. Factors such as accuracy of measurements, the sensor-to-sensor reproducibility and operational lifetimes are drastically influenced by enzyme stability. Since the analytical performances of a biosensor are strongly affected by the immobilization process, intensive efforts have been done to develop successful immobilization strategies in order to assure greater sensitivity and stability of biosensors.

Each immobilization method presents advantages and drawbacks. The choice of the most appropriate and judicious technique also depends on the enzyme nature, the transducer and the associated detection mode. The best method of enzyme immobilization varies if the biosensor application requires maximum sensitivity or rather focuses on stability. Reproducibility, cost and difficulty of the immobilization process also need to be considered. Sensitivity decreases if immobilization causes enzyme denaturation or conformational changes or if the enzyme has been modified, especially on its active site. A better sensitivity is obtained with oriented immobilization of enzymes on the transducer surface or by selecting the nature of the spacer arm between the enzyme and the support under covalent binding. Properly oriented enzymes correctly expose their active site to the solution phase. Rather long and flexible spacers such as polyethylene glycol can avoid steric hindrances and limited mobility of the immobilized enzymes. Numerous immobilization techniques involve random distribution or poor orientation of enzyme molecules inducing a partial or a total loss of activity due to enzyme denaturation or blocking of the active site from substrate accessibility. Techniques based on affinity interactions between enzymes and (strept)avidin molecules, lectins or sugars allow to immobilize enzymes in an ordered and site-specific manner in order to develop efficient biosensors (Campas et al., 2004). In the same way, self-assembled monolayer-based immobilization reduces the number of random orientations, generates uniform, reproducible and stable structures with high coverage (Campas et al., 2004).

The use of nanomaterials (e.g. conducting polymer nanowires, carbon nanotubes, nanoparticles) for the design of biosensing devices constitutes an exciting and recent approach to improve the performance of detection platforms. The extremely promising prospects of nanomaterials are due to their unique properties. Carbon nanotubes

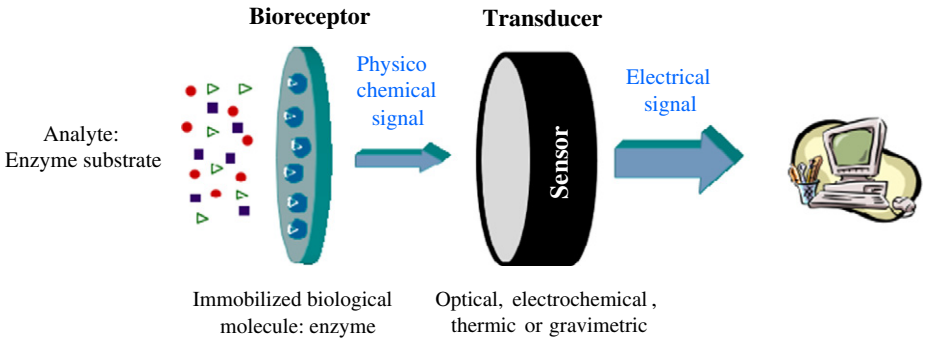

Fig. 1. Scheme of a biosensor.

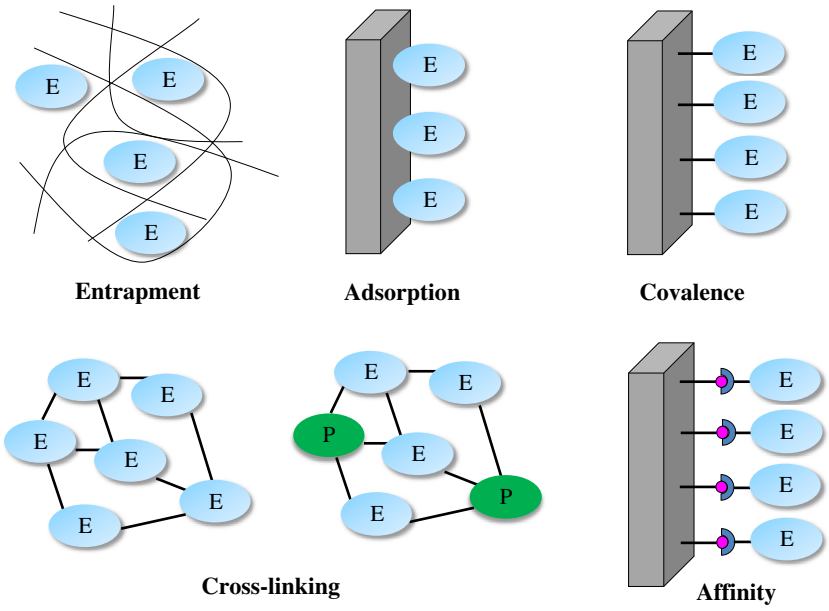

Fig. 2. Schematic representation of the main different methods of enzyme immobilization. E: enzyme, P: inert protein.

have remarkable electrical, mechanical and structural properties (Wang, 2005b). They can enhance the electrochemical reactivity of biomolecules and can promote the electron-transfer reactions of proteins. Nanoparticles of different compositions and sizes have also been used in last years as versatile and sensitive tracers for electronic, optical or microgravimetric transduction of different biomolecular recognition events (Wang, 2005a). Excellent conductivity of metal nanoparticles allows them to enhance the electron transfer between redox center in proteins and electrode surface (Luo et al., 2006; Pingarron et al., 2008). Biomolecules have also been immobilized with nanostructure materials by adsorption, covalence or entrapment to develop sensitive biosensors. Nanoparticles are interesting immobilization surfaces presenting a large surface area (Luo et al., 2006). Moreover, direct adsorption of enzymes onto bulk metal surfaces frequently results in denaturation of the protein and loss of bioactivity which can be avoided if enzymes are first adsorbed onto metal nanoparticles before being electrodeposited on the electrode surface (Liu et al., 2005). SiO_2 nanoparticles are also excellent matrices for enzyme immobilization due to their good biocompatibility and easy preparation (Luo et al., 2006).

In this work, different immobilization methods used to develop optical, electrochemical or gravimetric enzymatic biosensors, based or not on nanomaterials, are reviewed.

2. Entrapment

Enzymes can be immobilized in three-dimensional matrices such as an electropolymerized film, an amphiphilic network composed of polydimethylsiloxane (PDMS), a photopolymer, a silica gel, a polysaccharide or a carbon paste. Table 2 presents performances of some biosensors based on enzyme-entrapping matrices.

This immobilization is easy to perform. Enzyme, mediators and additives can be simultaneously deposited in the same sensing layer. There is no modification of the biological element so that the activity of the enzyme is preserved during the immobilization process. Biosensors based on physically entrapped enzymes are often characterized by increased operational and storage stability. However, limitations such as leaching of biocomponent and possible diffusion barriers can restrict the performances of the systems.

2.1. Electropolymerization

Electrochemical polymerization (or electropolymerization) is a simple and attractive approach for the controlled immobilization of enzymes on electrode surfaces. This one-step method consists in applying an appropriate potential or current to the transducer soaked in an aqueous solution containing both enzyme and monomer molecules.

Table 1
Advantages and drawbacks of the five basic immobilization methods.

	Binding nature	Advantages	Drawbacks
Adsorption	Weak bonds	○ Simple and easy ○ Limited loss of enzyme activity	○ Desorption ○ Non-specific adsorption
Covalent coupling	Chemical binding between functional groups of the enzyme and those on the support	○ No diffusion barrier ○ Stable ○ Short response time ○ High enzyme activity loss	○ Matrix not regenerable ○ Coupling with toxic product
Entrapment	Incorporation of the enzyme within a gel or a polymer	○ No chemical reaction between the monomer and the enzyme that could affect the activity ○ Several types of enzymes can be immobilized within the same polymer	○ Diffusion barrier ○ Enzyme leakage ○ High concentrations of monomer and enzyme needed for electropolymerization
Cross-linking	Bond between enzyme/cross-linker (e.g. glutaraldehyde)/inert molecule (e.g. BSA)	○ Simple	○ High enzyme activity loss
Affinity	Affinity bonds between a functional group (e.g. avidin) on a support and affinity tag (e.g. biotin) on a protein sequence	○ Controlled and oriented immobilization	○ Need of the presence of specific groups on enzyme (e.g. His, biotin)

Table 2
Biosensors based on enzymes entrapped within a polymer.

Analyte	Immobilized enzyme	Detection mode	Detection limit (M)	Linearity range (M)	References
Electropolymerization					
Polypyrrole					
Glucose	GOD	Amperometry	ND	Up to 1×10^{-2}	Uang and Chou (2003)
Glucose	GOD	Amperometry	2.1×10^{-6}	2.5×10^{-6}–5×10^{-3}	Njagi and Andreescu (2007)
Phenol	Tyrosinase	Amperometry	3.10^{-8}	5×10^{-8}–7×10^{-5}	
Phenol	Tyrosinase	Amperometry	1×10^{-8}	Up to 1×10^{-5}	Cosnier et al., (1999a)
Glucose	GOD/HRP	Amperometry	3×10^{-5}	3×10^{-5}–2.43×10^{-3}	Zhu et al. (2007)
H_2O_2	HRP	Amperometry	ND	1×10^{-4}–2×10^{-3}	Li et al. (2007)
Glucose	GOD	Amperometry	2×10^{-4}	Up to 5×10^{-2}	Wang and Musameh (2005)
LEV	HRP	Amperometry	9.81×10^{-6}	ND	Alonso-Lomillo et al. (2009)
Nitrate	Nitrate reductase	Potentiometry	1.5×10^{-5}	1×10^{-4}–5×10^{-3}	Sohail and Adeloju (2008)
Polyaniline					
Glucose	GOD	Amperometry	8×10^{-6}	2.5×10^{-5}–3×10^{-3}	Eftekhari (2004)
Hypoxanthine	XOD	Amperometry	8×10^{-7}	1×10^{-6}–4×10^{-4}	Hu et al. (2000)
Glucose	GOD	Amperometry	1×10^{-6}	5×10^{-6}–5×10^{-3}	Shan et al. (2008)
Photopolymerization					
Glucose	GOD	ECL	1×10^{-5}	Up to 1×10^{-3}	Corgier et al. (2005)
Lactate	LOD	ECL	3×10^{-6}	Up to 1×10^{-4}	
Lactate	LOD	ECL	2×10^{-6}	2×10^{-6}–2×10^{-4}	Marquette et al. (2003)
D-lactate	D-lactate dehydrogenase	Amperometry	3×10^{-5}	5×10^{-5}–1×10^{-3}	Avramescu et al. (2002)
Acetaldehyde	Aldehyde dehydrogenase	Amperometry	1×10^{-6}	5×10^{-6}–5×10^{-4}	
Ethanol	ADH/NADH oxidase	Amperometry	ND	3×10^{-7}–1×10^{-4}	Leca and Marty (1997)
Chlorpyrifos ethyl oxon	AChE	Amperometry	1.24×10^{-9}	ND	Andreescu et al. (2002a)
Paraoxon	AChE	Amperometry	1.91×10^{-8}	ND	
Dichlorvos	AChE	Amperometry	7×10^{-11}	ND	Valdes-Ramirez et al. (2008a)
Dichlorvos	AChE	Amperometry	9.6×10^{-11}	2×10^{-10}–1×10^{-8}	Valdes-Ramirez et al. (2008b)
Omethoate	AChE	Amperometry	1×10^{-7}	Up to 3×10^{-6}	Bucur et al. (2005b)
Choline	ChOD	Amperometry	2.5×10^{-9}	Up to 1×10^{-4}	Leca et al. (1995a, 1995b)
Silica sol–gel					
Glucose	GOD	Amperometry	ND	2×10^{-3}–4×10^{-2}	Li and Tan (2000)
Glucose	GOD	Amperometry	ND	Up to 7×10^{-3}	Choi et al. (2005)
Glucose	GOD	ECL	8.2×10^{-6}	1×10^{-5}–1×10^{-2}	Zhu et al. (2002b)
Glucose	GOD	ECL	2.6×10^{-5}	5×10^{-5}–1×10^{-2}	Zhu et al. (2002a)
Glucose	GOD	Amperometry	3×10^{-7}	1×10^{-5}–6×10^{-3}	Noorbakhsh et al. (2008)
Glucose	GOD	Amperometry	ND	1×10^{-4}–5×10^{-3}	Couto et al. (2002)
Glucose	GOD	Amperometry	1.9×10^{-5}	6×10^{-5}–4.4×10^{-3}	Liu and Sun (2007)
ATP	GOD/HK	Amperometry	ND	5×10^{-7}–2×10^{-5}	
ACh	AChE/ChOD	Amperometry	6×10^{-7}	1×10^{-6}–1.5×10^{-3}	Yang et al. (2005)
Choline	ChOD	Amperometry	5×10^{-7}	Up to 1.6×10^{-3}	
Choline	ChOD	Amperometry	10^{-7}	5×10^{-6}–1×10^{-4}	Song et al. (2006)
H_2O_2	HRP	Amperometry	ND	2×10^{-5}–4×10^{-3}	Li and Tan (2000)
Phenol	Tyrosinase	Amperometry	ND	5×10^{-5}–6×10^{-4}	
Catechol	Tyrosinase	Amperometry	3.5×10^{-7}	1×10^{-6}–1×10^{-4}	Kim and Lee (2003)
Xanthine	XOD/SOD/HRP	Fluorescence	2×10^{-8}	Up to 3.5×10^{-6}	Salinas-Castillo et al. (2008)
Cholesterol	CholOD	Amperometry	1.4×10^{-6}	4×10^{-6}–1×10^{-4}	Shi et al. (2005)
Cholesterol	CholOD	Amperometry	1.2×10^{-7}	1×10^{-6}–8×10^{-5}	Li et al. (2003)
Carbaryl	AchE	Amperometry	1×10^{-8}	ND	Bucur et al. (2006)
Ethanol	ADH	Amperometry	ND	5×10^{-4}–2.5×10^{-3}	Jena and Raj (2006)
Lactate	LDH	Amperometry	ND	1×10^{-4}–6×10^{-4}	
Heavy metals	Urease	Fluorescence	1×10^{-5}	1×10^{-5}–2.3×10^{-4}	Tsai et al. (2003)
L-Phe	L-amino acid oxidase	Fluorescence	2×10^{-5}	2×10^{-5}–1×10^{-2}	Gavalas et al. (2004)
Polysaccharide-based gel					
Chitosan					
NH_4^+	Glutamate DH	Colorimetry	5×10^{-6}	5×10^{-6}–5×10^{-4}	Azmi et al. (2009)
Phenol	Tyrosinase	Amperometry	5×10^{-11}	1×10^{-10}–2.3×10^{-8}	Wang et al. (2002)
Choline	ChOD (+DEAE beads)	ECL	ND	4×10^{-7}–1.3×10^{-4}	Sassolas et al. (2009b)
Ethanol	ADH	Amperometry	5.2×10^{-7}	ND	Lee and Tsai (2009)
Lactate	LOD	Amperometry	5×10^{-6}	Up to 8×10^{-4}	Cui et al. (2007)
Lactate	LDH	Amperometry	7.6×10^{-7}	5×10^{-6}–1.2×10^{-4}	Tsai et al. (2007)
Cholesterol	CholOD	Amperometry	4.79×10^{-6}	Up to 3×10^{-4}	Tsai et al. (2008)
Agarose					
Choline	ChOD (+DEAE beads)	ECL	ND	8×10^{-7}–1.3×10^{-4}	Sassolas et al. (2009b)
Dopamine	Tyrosinase	Amperometry	9×10^{-7}	2×10^{-6}–1×10^{-5}	Tembe et al. (2006)
Catechol	Tyrosinase	Amperometry	6×10^{-6}	6×10^{-5}–8×10^{-4}	Tembe et al. (2007)
Carbon paste electrodes					
Gentisic acid	Polyphenol oxidase	Amperometry	5×10^{-5}	Up to 2×10^{-4}	Pedano and Rivas (2000)
Glucose	GOD/HRP	Amperometry	ND	1×10^{-2}–5×10^{-2}	Yabuki et al. (1992)
Glucose	GOD/HRP	Amperometry	ND	2.8×10^{-5}–2.8×10^{-4}	Rondeau et al. (1999)
Glucose	GOD	Amperometry	6×10^{-4}	Up to 3×10^{-2}	Rubianes and Rivas (2003)
Glucose	GDH/diaphorase	Amperometry	1×10^{-5}	Up to 8×10^{-4}	Antiochia and Gorton (2007)
Glucose	Pyranose oxidase	Amperometry	ND	2×10^{-4}–3×10^{-2}	Odaci et al. (2008)
Dopamine	HRP	SWV	9×10^{-6}	9.9×10^{-5}–1.6×10^{-3}	Fritzen-Garcia et al. (2009)

Monomer oxidation gives rise to a radical cation which can either react with a second radical cation or with a neutral monomer in order to obtain a dimer that is then oxidized. Finally, a polymer is formed at the electrode surface. Enzyme molecules that are present in the immediate vicinity of the electrode surface are physically incorporated within the growing polymer network.

Most of electropolymerized films used for biomolecule immobilization are conducting polymers such as polyaniline, polypyrrole or polythiophene (Fig. 3). Owing to their conductivity, the thickness of the polymer films can be easily controlled and is not restricted to thin films contrary to non-conducting polymers.

Polypyrrole (PPy), in particular, presents a relatively stable electrical conductivity and can be electrosynthesized under biocompatible conditions such as low oxidation potential and neutral pH.

The simple one-step immobilization method does not involve any chemical reaction between the monomer and the biomolecule that could affect its activity. Furthermore, this method enables the exact control of the thickness of the polymer layer and hence the modulation of the immobilized amount of biomolecules. It is also compatible with miniaturization. However, this method usually requires high concentrations of monomer (0.05–0.5 M) and enzyme (0.2–3.5 $mg.ml^{-1}$). In addition, the accurate amount of biomolecules entrapped within the polymer network cannot be estimated by a simple difference between the biological concentrations before and after the electropolymerization step (Cosnier, 2007). Conducting polymers can act as electron promoters thus representing an interesting property for the fabrication of electrochemical biosensors (Rahman et al., 2008).

Pionnering works (Bartlett and Whitaker, 1987; Foulds and Lowe, 1986; Umana and Waller, 1986) described the entrapment of GOD within the growing network of conducting polypyrrole. Since these first reports, numerous biosensors based on enzymes entrapped in electropolymers onto electrode surfaces have been described (Ahuja et al., 2007; Malhotra et al., 2006).

Galvanostatic immobilization of nitrate reductase with β-NADH within a conducting PPy film was successfully used to design a potentiometric biosensor for nitrate detection (Sohail and Adeloju, 2008). The presence of NADH in close vicinity of nitrate reductase enabled higher efficiency of the enzyme-catalyzed reaction and improved the sensitivity of the biosensor. Nitrate was detected in a linear concentration range between 1×10^{-4} M and 5×10^{-3} M (detection limit of 1.5×10^{-5} M). The reproducibility of the measurements was quite high (relative standard deviation of 1.9%, n = 6). Recently, a selective and sensitive HRP-based biosensor was developed to detect a novel antiepileptic, Levetiracetam (LEV) (Alonso-Lomillo et al., 2009). The response of the biosensor to LEV was based on chronoamperometric detection of reduced LEV formed upon HRP catalysis in the presence of oxidized LEV and H_2O_2. The enzyme was immobilized in a polypyrrole film during electropolymerization by cyclic voltammetry. This biosensor showed a detection limit of 9.8×10^{-6} M. The biosensor was applied for the determination of LEV in pharmaceutical drugs and spiked human plasma samples. The obtained results showed good agreement with the theoretical values.

In another example, cholesterol oxidase (CholOD) was entrapped in poly(3,4-ethylenedioxypyrrole) (PEDOP) in order to develop an amperometric biosensor for cholesterol detection (Türkarslan et al., 2009). The responses of the enzyme electrode were measured via monitoring the oxidation current of H_2O_2 at +0.7 V vs. Ag/AgCl in the absence of any mediator. The detection limit was 4×10^{-4} M and the response time was 150 s. This biosensor kept its maximum relative activity during 20 days.

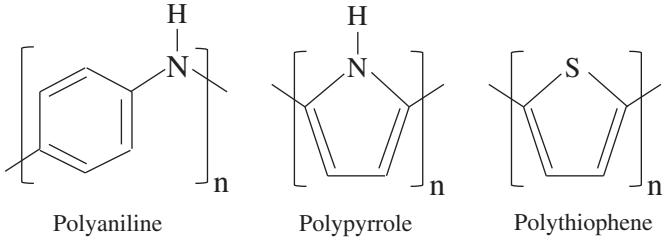

Fig. 3. Polyaniline, polypyrrole and polythiophene structures.

Polyaniline has attracted much attention due to various remarkable characteristics such as controllable conductivity, charge transfer capability and environmental stability. This polymer was described as an interesting material for fabrication of biosensors because it can act as an effective mediator for electron transfer in redox or enzymatic reactions. Polyaniline's transport properties, electrical conductivity or rate of energy migration, provide an enhanced sensitivity (Dhand et al., 2010; Wei and Ivaska, 2006). A biosensor for hypoxanthine detection was developed by entrapment of xanthine oxidase within a polyaniline film electropolymerized on a sodium montmorillonite-methyl viologen carbon paste modified electrode (Hu et al., 2000). The biosensor was based on the electrochemical detection of oxygen consumed by the enzymatic reaction catalyzed by xanthine oxidase. Incorporation of methyl viologen and sodium montmorillonite into the carbon paste allowed to obtain an excellent catalytic activity for oxygen reduction. Hypoxanthine was linearly detected in the range from 1×10^{-6} M to 4×10^{-4} M (detection limit of 8×10^{-7} M). This biosensor could be reused for at least 200 measurements over a period of 2 weeks and 60% of the initial enzyme activity was retained after 5 weeks. This enzymatic biosensor was very selective. Polyaniline film acted not only as an immobilization matrix but also as a permselective membrane blocking interfering species. An amperometric biosensor for detection of phenolic compounds and based on an in situ electropolymerized polyaniline–polyacrylonitrile composite film was also developed (Xue and Shen, 2002; Xue et al., 2002). Tyrosinase was immobilized during electropolymerization of aniline on a polyacrylonitrile-coated Pt working electrode. This biosensor allowed to detect catechol in a linear range comprised between 5×10^{-8} M and 7.5×10^{-5} M. After 100 measurements, the enzyme electrode retained about 97% of its initial activity. When the electrode was stored at 4 °C, the response to 20 μM catechol remained unchanged for 6 months.

Non-conducting polymers are emerging matrices for immobilization of biomolecules. By definition, a non-conducting polymer prepared by electropolymerization possesses a high resistivity (Yuqing et al., 2004). The growth of such polymers is self-limited and the film that is formed is much thinner than typical conducting polymer films. Accordingly, substrates and products diffuse rapidly to and from the enzyme. Non-conducting polymers are permselective so that they prevent interference from electroactive species in the sample. In addition to good selectivity, high sensitivity, fast response time and good reproducibility can be expected (Yuqing et al., 2004). Insulating electropolymerized films like polyphenol, poly(o-phenylenediamine), poly(o-aminophenol) and overoxidized polypyrrole have been used in biosensors (Yuqing et al., 2004).

An amperometric biosensor for glucose detection based on GOD entrapment in an electropolymerized poly(o-aminophenol) (POAP) film on copper-modified gold electrode was developed (Pan et al., 2005). Electrode surface was modified with copper that can electrochemically oxidize glucose and thus increase the sensitivity. Detection limit was 1×10^{-5} M glucose. This biosensor presented excellent reproducibility, storage stability (72% of electrochemical response retained after 30 days) and allowed to detect efficiently glucose contents in human blood samples. Poly(o-phenylenediamine) (POPD) was also used for the development of biosensors, allowing the diffusion of small redox active species like hydrogen peroxide to the electrode surface, whereas many other species, greater in size, are retarded at the polymer/solution interface (Mazeikiene and Malinauskas, 2002).

In some cases, electropolymers are associated with nanomaterials that act as intermediates between the redox center of the enzyme and

the electrode, so facilitating electron transfer. An amperometric glucose biosensor was developed by simultaneous immobilization of GOD and horseradish peroxidase (HRP) in a PPy film electropolymerized on a single-walled carbon nanotube (SWCNT)-coated electrode (Zhu et al., 2007). The linear response range extended from 3×10^{-5} M to 2.4×10^{-3} M glucose. An amperometric biosensor for phenol derivative was also developed by a one-step electropolymerization process in a medium containing carbon nanotubes, pyrrole and HRP (Korkut et al., 2008). Linear range, sensitivity and detection limit were determined for 18 different phenol derivatives (e.g. m-cresol, 2-chlorophenol). The lowest detection limit was found to be 2.7×10^{-8} M for p-benzoquinone and the highest detection limit was found to be 2.8×10^{-5} M for 2,4-dimethylphenol. This system retained 70% of its initial activity after 700 measurements for 1 month. An amperometric biosensor based on the co-immobilization of CNTs and GOD within an electropolymerized PPy film was also developed (Wang and Musameh, 2005). Voltammetric growth profiles indicate that CNTs were incorporated within the PPy film for maintaining its electrical neutrality in an analogous manner to the doping of PPy by small inorganic anions. Detection limit was 2×10^{-4} M of glucose.

2.2. Entrapment in an amphiphilic network

An original optical biosensor based on an amphiphilic polymer network was developed to detect hydroperoxides. This network was composed of an hydrophilic phase of poly(2-hydroxyethyl acrylate) (PHEA) and an hydrophobic phase of PDMS (Bruns and Tiller, 2005; Hanko et al., 2006). HRP and diammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) were co-immobilized in this matrix. The principle was based on the swelling properties of the polymers. Depending on solvent polarity, each phase independently swelled. ABTS and enzyme could diffuse from hydrophilic solution into the swollen hydrophilic phase of the network. On drying, the phase shrank, entrapping the indicator reagent (ABTS) and the enzyme in a hydrophilic and enzyme-friendly environment.

This matrix has been used to optically detect butylhydroperoxides (BuOOH) (Hanko et al., 2006). Immobilized HRP catalyzes the oxidation of ABTS by BuOOH:

$$BuOOH + 2ABTS + 2H^{+} \xrightarrow{HRP} BuOH + H_2O + 2ABTS^{+\cdot}$$

A rapid and linear increase in absorbance was observed, indicating the formation of $ABTS^{\cdot+}$ radical cations. The indicator reagent is often spatially separated from the immobilized enzymes (e.g. it is dissolved in the measurement solution or immobilized in a separate layer of the sensor) but, in this case, the chromophoric indicator was co-immobilized with the enzyme in the optically clear and transparent matrix. In a dry atmosphere at 4 °C, the biosensor was stable for at least 2 weeks.

2.3. Photopolymerization

The poly(vinyl alcohol)-bearing styrylpyridinium groups (PVA-SbQ), a soluble pre-polymer bearing photo-crosslinkable groups, has largely been used to entrap enzymes. It was synthesized for the first time by Ichimura and Watanabe (1982a, 1982b, 1984). To form an insoluble matrix, polymerization is initiated by light exposition (Fig. 4).

Table 2 presents performances of some biosensors based on enzyme-entrapping PVA-SbQ photopolymer.

An amperometric ethanol biosensor based on the co-immobilization of alcohol dehydrogenase (ADH), NADH oxidase and NAD-dextran in a PVA-SbQ matrix was developed (Leca and Marty, 1997). Dynamic range extended from 3×10^{-7} M to 1×10^{-4} M ethanol and response time was lower than 2 min. More than 80 reproducible measurements could be performed without requiring cofactor addition, pointing out a good operational stability. A PVA-SbQ-based biosensor was also

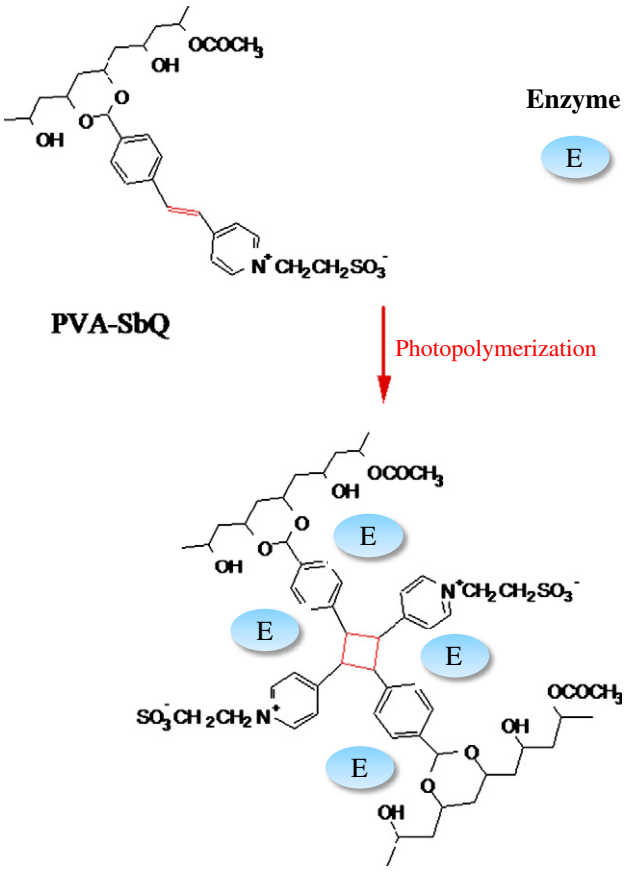

Fig. 4. PVA-SbQ entrapment of enzymes.

developed for the detection of pesticides in water miscible organic solvents (Andreescu et al., 2002a). In this work, p-aminophenol was used as the acetylcholinesterase (AChE) substrate instead of acetylthiocholine. AChE was immobilized on a screen-printed electrode (SPE) surface by entrapment in a PVA-SbQ polymer. Detection limits as low as 1.9×10^{-8} M paraoxon and 1.24×10^{-9} M chlorpyrifos ethyl oxon were obtained when experiments were carried out in a phosphate buffer containing 5% acetonitrile.

In some cases, enzyme was successfully pre-immobilized by ionic interactions with charged beads before entrapment in the photopolymer in order to limit enzyme leaching (Leca and Blum, 2000; Leca et al., 2001; Marquette et al., 2003; Sassolas et al., 2009a).

PVA-SbQ is not the only photopolymer that can be used to develop enzymatic biosensors. Recently, azide-unit pendant water-soluble photopolymer (PVA-AWP) was used to entrap protein phosphatase (Campas et al., 2007) or AChE (Galezowska et al., 2008; Valdes-Ramirez et al., 2008b). An amperometric acetylcholinesterase biosensor has been developed for quantification of pesticides in a phosphate buffer containing 5% acetonitrile using three different acetylcholinesterases (Valdes-Ramirez et al., 2008b). Enzymes were immobilized on cobalt(II) phthalocyanine-modified electrodes by entrapment in PVA-AWP. The biosensor based on genetically engineered AChE (B394) showed a detection limit of 9.6×10^{-11} M of dichlorvos. The developed biosensor was used for the determination of pesticides in real apple samples.

Acrylated polyurethane has also been used as a photopolymeric membrane entrapping glucose oxidase to design an amperometric biosensor for glucose detection (Puig-Lleixa et al., 2001). A pre-polymer solution containing the acrylated urethane oligomer, a cross-linker and a photoinitiator was used. The authors claim that the polymer can be prepared with a fast and simple process and that it presents a higher mechanical stability than some hydrogels that are used for enzyme immobilization, with a long-term stability of ca. 3 months. Poly(2-hydroxyethyl methacrylate)

(polyHEMA) photopolymeric films containing glucose oxidase and ferrocene have also been reported to design an amperometric biosensor for glucose (Bean et al., 2005). Leaching of both mediator and enzyme was prevented. In this case, the monomer 2-hydroxyethyl methacrylate (HEMA) was mixed with an initiator before ferrocene and GOD addition and further exposition to UV radiation.

2.4. Sol–gel process

Sol–gel process is based on the ability to form solid metal or semi-metal oxides via the aqueous process of hydrolytically labile precursors (e.g. ester of silicic, polysilicic acid, alkoxide, halide of aluminum) (Gill and Ballesteros, 2000).

Sol–gel process involves hydrolysis of alkoxide precursors under acidic (or alkaline) conditions followed by condensation of the hydroxylated units, which leads to the formation of a porous gel (Fig. 5). First, a low-molecular weight metal alkoxide precursor molecule such as tetramethoxysilane (TMOS) or tetraethoxysilane (TEOS) is hydrolyzed in the presence of water at acid (or alkaline) pH, resulting in the formation of (Si–OH) groups. In the second step, the condensation reaction between silanol moieties at alkaline (or acidic) pH results in the formation of siloxane (Si–O–Si) polymers, creating a matrix in which an enzyme can be successfully entrapped (Campas and Marty, 2006; Gupta and Chaudhury, 2007; Jeronimo et al., 2007; Kandimalla et al., 2006).

Silica gels are highly porous, showing physical rigidity, chemical and biological inertness and thermal stability. However, these matrices suffer from considerable shrinkage during the drying process, which leads to fracture of the material and pore collapse. Recent reports suggest that the use of additives might help to overcome these problems by decreasing internal stress and shrinkage of the materials. For example, the use of trehalose or glycerol as a drying control chemical additive is considered as an interesting possibility in the sol–gel process (Desimone et al., 2008). In the same way, some polymers such as the natural polymer chitosan, poly(ethyleneglycol) or Nafion can also be used to prevent cracking (Choi et al., 2005).

Sol–gel process is used classically to immobilize enzymes in order to develop biosensors (Gill and Ballesteros, 2000; Kandimalla et al., 2006).

The sol–gel technology is increasingly used for the development of optical biosensors (Jeronimo et al., 2007). For example, an electrochemiluminescent (ECL) biosensor for glucose detection was developed with a GOD-immobilizing silica matrix formed on a glassy carbon electrode surface (Zhu et al., 2002a). Glucose was detected in a concentration range comprised between 5×10^{-5} M and 1×10^{-2} M (detection limit of 2.6×10^{-5} M). After 1 month, the biosensor stored at 4 °C retained about 70% of its original sensitivity. A fluorescent biosensor for xanthine detection based on the immobilization of xanthine oxidase (XOD), superoxide dismutase (SOD) and peroxidase, in a silica matrix coupled to the Amplex Red probe was also described (Salinas-Castillo et al., 2008). Xanthine determination was based on a sequence of reactions (Fig. 6). First, xanthine was oxidized to uric acid and superoxide radical. Catalytic dismutation of the radical resulted in the formation of H_2O_2, which reacted with non-fluorescent Amplex Red to produce highly fluorescent resofurin. This system allowed to detect xanthine up to 3.5×10^{-6} M (detection limit of 2×10^{-8} M). This biosensor remained stable for 2 weeks under appropriate storage conditions.

Numerous electrochemical sol–gel-based biosensors were also described. A system based on GOD-immobilizing silica gel formed onto a glassy carbon electrode allowed to detect glucose from 6×10^{-5} M to 4.4×10^{-3} M (Liu and Sun, 2007). A biosensor for ATP detection was developed by co-immobilization of GOD and hexokinase (HK) in a silica gel. ATP detection was based on competitive enzymatic reactions for glucose (Fig. 7). In the presence of ATP, the reaction (3) consumed glucose, resulting in the decrease of glucose concentration for reaction (1) and thus of the electrochemical response due to oxidation of hydrogen peroxide at the electrode surface reaction (2). This biosensor allowed to detect ATP between 5×10^{-7} M and 2×10^{-5} M. The electrode-to-electrode reproducibility was satisfactory (relative standard deviation of 5.6%). The stability of the biosensor was estimated over a period of 3 weeks. The response to ATP decreased by about 35% during 4 days and then remained remarkably stable.

Silica gels have also been used in association with nanostructures such as CNTs or gold NPs. An electrochemical biosensor for cholesterol detection was reported. First, platinum-decorated CNTs (CNT-Pt) were formed on the surface of a waxed graphite electrode (Shi et al., 2005). Then, CholOD was immobilized within a silica gel on the CNT-Pt modified surface. CNT-Pt electrodes showed higher catalytic activities than CNT electrodes for the reduction of hydrogen peroxide. This system showed a linear range between 4×10^{-6} M and 1×10^{-4} M (detection limit of 1.4×10^{-6} M). A glucose biosensor was also developed by entrapping GOD in a silica matrix formed at the surface of modified

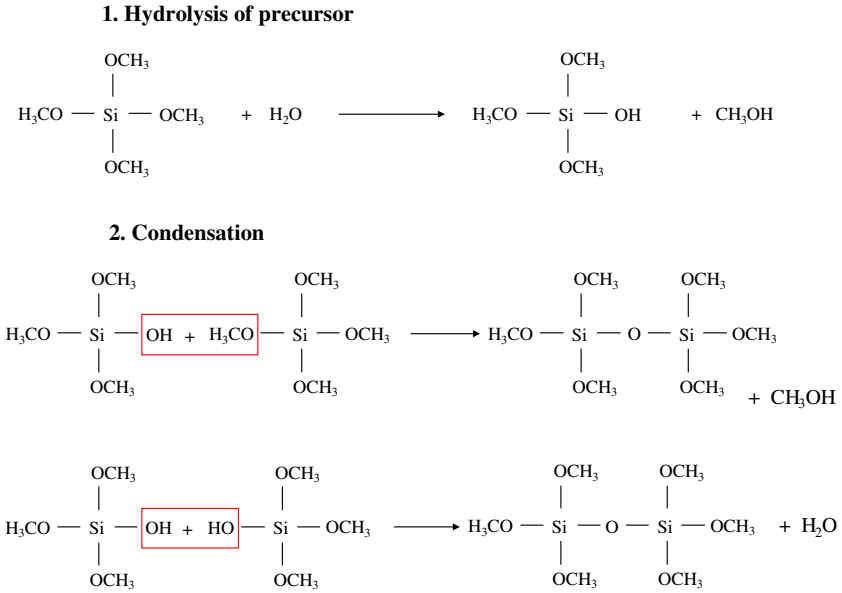

Fig. 5. Sol–gel process composed by two steps: hydrolysis of TMOS (1) and condensation of silanol groups (2).

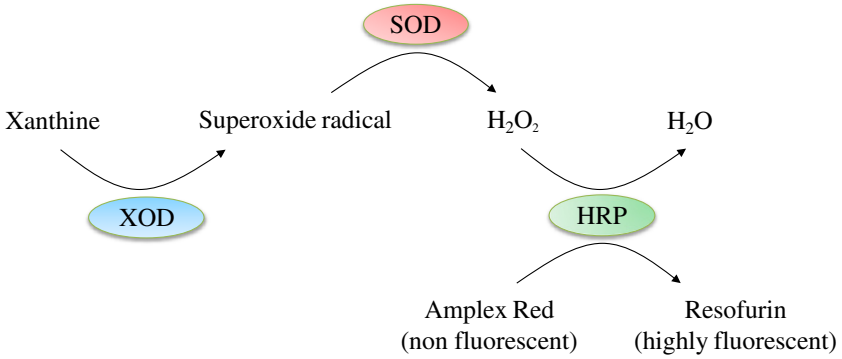

Fig. 6. Scheme of resofurin formation catalyzed by the coupled XOD-SOD-HRP enzymatic system (Salinas-Castillo et al., 2008).

multi-walled CNT/Celestine blue modified glassy carbon electrode (Noorbakhsh et al., 2008). This biosensor allowed to detect glucose between 1×10^{-5} M and 6×10^{-3} M (detection limit of 3×10^{-7} M). Glucose determination was performed in human serum samples and no interference from electroactive species was observed.

Electrochemical biosensors based on integrated assembly of lactate or ethanol dehydrogenase and gold nanoparticles (AuNPs) in a silica gel were also developed (Jena and Raj, 2006). (3-Mercaptopropyl)trimethoxysilane was used for designing a three-dimensional silica gel on a gold electrode. This precursor is known to form a high-thiol 3D structure which has a strong affinity for gold. Thus, enzyme-encapsulated gel could be chemisorbed onto the electrode surface (Fig. 8). Because of the thiol groups distributed throughout the network, nanosized AuNPs could be conveniently self-assembled on the thiol groups present both inside and on the network surface. Then, hydroxylamine-mediated growth of AuNPs was achieved in order to improve the sensitivity. Entrapped enzyme catalyzed the oxidation of lactate or ethanol in the presence of NAD^+ and AuNPs catalyzed the oxidation of enzymatically generated NADH. This biosensor could detect lactate and ethanol concentrations as low as 1×10^{-4} and 2×10^{-5} M, respectively.

2.5. Entrapment in a polysaccharide-based gel

Enzymes can also be entrapped in a polysaccharide-based gel (e.g. alginate, chitosan or agarose). Contrary to synthetic polymers such as polyacrylamide, these matrices are biocompatible, non toxic, provide natural microenvironment to the enzyme and also give sufficient accessibility to electrons to shuttle between the enzyme and the electrode.

Alginate is derived from brown seaweed extracts (*Phaeophyceae*) and is composed of 1,4-linked β-D-mannuronic acid and α-L-guluronic acid (Fig. 9). Sodium alginate is a monovalent salt that is widely used because of its good gelification ability in the presence of divalent cations (e.g. Ca^{2+}, Cu^{2+}). Enzyme entrapment in an alginate gel is easy to perform (Cosnier et al., 2006; Haider and Husain, 2007; Munjal and Sawhney, 2002; Palmieri et al., 1994; Prakash et al., 2008). However, this matrix presents some drawbacks: gel is very porous and its stability is poor so that it can cause enzyme leaching. A biosensor for glucose detection was developed by immobilizing GOD in an alginate gel (Cosnier et al., 2006). First, sodium alginate and enzyme were mixed and 25 µl of this solution were deposited on a platinum electrode which was then soaked in 0.1 M $CaCl_2$ solution (30 min) in order to initiate gelification. Immobilization of GOD into alginate was a very mild procedure providing a gentle polysaccharide environment but leading to a very low sensitivity which could be most likely attributed to enzyme release. Values of response time of various biosensors based on different immobilization matrices were shown to be in good agreement with their permeability values. The most permeable matrix exhibited the faster response but gave the lower sensitivity, thus pointing out that the amount of active immobilized enzyme must also be taken into account.

Chitosan is a derivative of chitin, a natural polyaminosaccharide found in the exoskeleton of crustaceans or insects and in fungal cell wall (Peter, 1995). Chitosan is obtained by N-deacetylation of chitin and it is characterized by the degree of deacetylation. Thus, chitosan is a copolymer of N-acetyl-D-glucosamine and D-glucosamine (Fig. 10). Chitosan is insoluble in water, but the presence of amino groups induces its solubility in acidic solutions below pH about 6.5, the degree of deacetylation necessary for that being 80–85% (Krajewska, 2004). An amperometric biosensor was developed to detect phenols by immobilizing tyrosinase in a chitosan film on a carbon electrode (Wang et al., 2002). The responses to phenol were linear in the range from 1×10^{-10} M to 2.3×10^{-8} M (detection limit was 5×10^{-11} M). The positively charged matrix displayed a good anti-interference ability to substances such as cysteine and glucose. This biosensor retained 75% of the initial enzymatic activity for at least 79 days. Recently, an optical biosensor based on glutamate dehydrogenase immobilized in a chitosan film was developed for the determination of ammonium in water samples (Azmi et al., 2009). The enzyme requires the cofactor NADH and ammonium for the enzymatic conversion of α-ketoglutarate to L-glutamate. During the reaction, NADH was oxidized in NAD^+ thereby making possible the indirect monitoring of ammonium by measuring the consumption of NADH at a wavelength of 340 nm. A linear

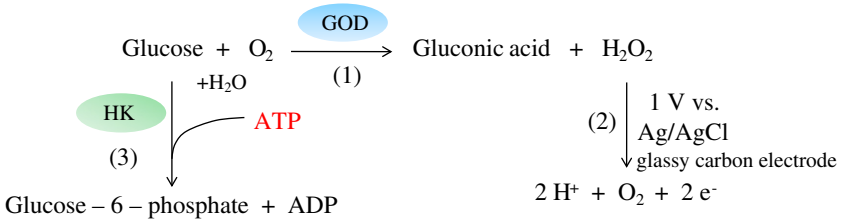

Fig. 7. Competitive enzymatic reactions.
Adapted from (Liu and Sun, 2007.

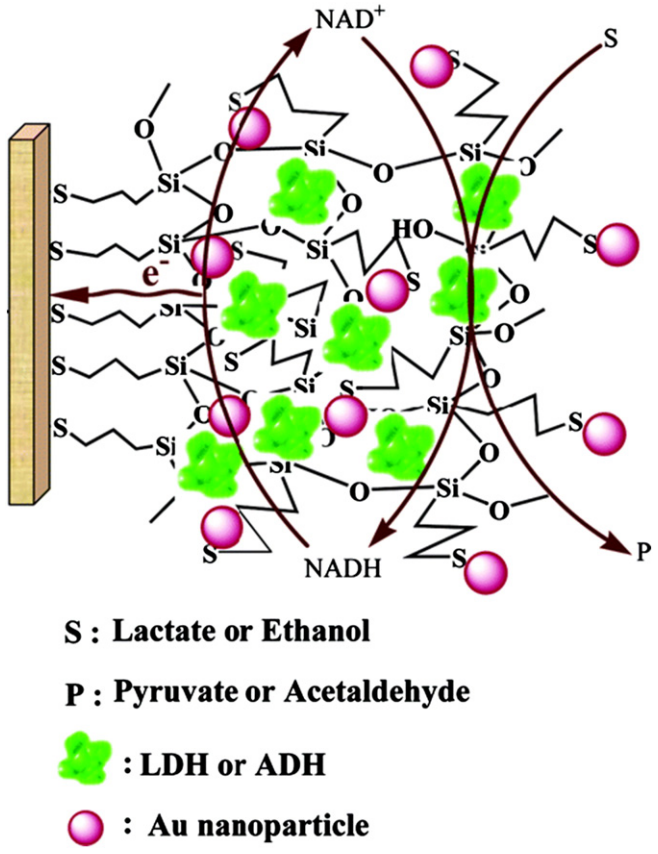

Fig. 8. Schematic representation of the bioelectrocatalytic sensing of lactate and ethanol using an integrated assembly (Jena and Raj, 2006).

response was obtained in the ammonium concentration range from 5×10^{-6} M to 5×10^{-4} M. The biosensor was stable for at least 1 month when stored dry at 4 °C.

CNT-chitosan nanocomposites were also developed to detect biomolecules such as lactate (Cui et al., 2007; Tsai et al., 2007), ethanol (Lee and Tsai, 2009) or cholesterol (Tsai et al., 2008). CNTs interweaved with chitosan on the electrode and directly improved the conductivity of the composite. MWCNT worked as numerous nanoelectrodes throughout the composite providing much better electron transfer paths. The inclusion of MWCNT in chitosan was reported to raise the current responses, to decrease the electrooxidation potential of NADH and to prevent electrode surface fouling. Recently, an amperometric biosensor was developed for ethanol detection by co-immobilizing MWCNTs and alcohol dehydrogenase (ADH) within a chitosan matrix on a glassy carbon electrode (Lee and Tsai, 2009). Detection limit was

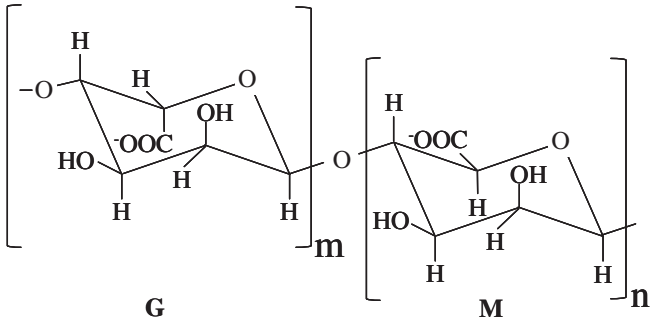

Fig. 9. Chemical structure of alginate, composed of guluronic acid (G) and mannuronic acid (M) units.

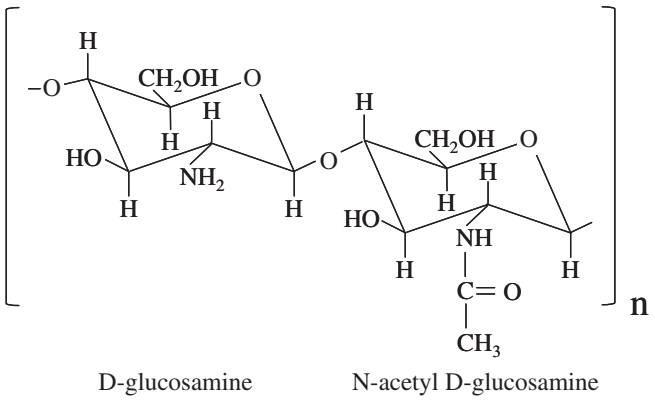

Fig. 10. Chemical structure of chitosan.

5.2×10^{-7} M. This biosensor was used for the analysis of ethanol in beer, red wine and spirit.

Agarose gels (Fig. 11) have also been used for enzyme immobilization. Agarose powder is dissolved in a buffer by heating (Shumyantseva et al., 2004). This mixture gellifies when temperature decreases. An enzyme incorporated in the mixture during gelling can be entrapped. An electrochemical biosensor based on tyrosinase immobilized in a composite biopolymeric film of agarose and guar gum was developed (Tembe et al., 2006; Tembe et al., 2007). This composite enzyme-entrapping matrix was formed on a glassy carbon electrode. For example, dopamine was detected by the direct reduction of the biocatalytically liberated quinine species at −0.18 V vs. Ag/AgCl. This biosensor could be reused up to 15 assays and had a shelf life of more than 2 months.

In some cases, to avoid enzyme leakage from the porous immobilization matrix, the biological element (choline oxidase) was pre-immobilized by ionic interactions on positively-charged beads before entrapment in a polysaccharide-based gel (Sassolas et al., 2009b). Detection of low choline concentrations (8×10^{-7} M–1.3×10^{-4} M) was reported.

2.6. Entrapment in a carbon paste

Carbon paste, a mixture of carbon (graphite) powder and a binder (pasting liquid) is a popular electrode material used for the preparation of various electrodes, sensors and detectors (Svancara et al., 2009). Carbon paste used as an electrode material is a convenient matrix for the incorporation of biological components. It allows an intimate contact between incorporated enzymes, mediator and sensing sites permitting a fast electron transfer. It is versatile, stable and the surface is easily renewed with good reproducibility. Carbon paste electrodes modified with enzyme are prepared by first mixing enzyme solution and graphite powder. Then, the resulting enzymatic powder is mixed with mineral oil (e.g. paraffin). The final paste is filled into a plastic cylindrical cartridge (Boujtita et al., 1996; Rondeau et al., 1999; Serban et al., 2004).

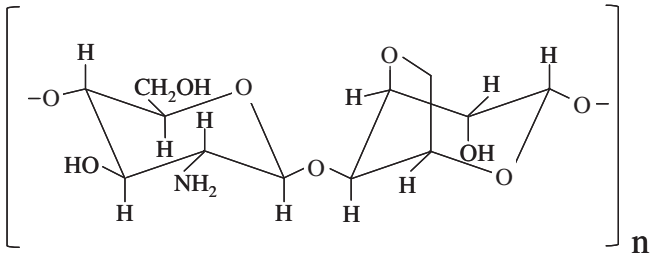

Fig. 11. Chemical structure of agarose.

An amperometric ethanol biosensor was designed using alcohol dehydrogenase in a NAD^+ modified carbon paste electrode (Boujtita et al., 1996). The type and amount of pasting liquid were shown to present a great effect on sensitivities and linear ranges of the biosensors.

An electrochemical glucose biosensor was developed by incorporating GOD, HRP and ferrocene into a carbon paste matrix (Rondeau et al., 1999). The proximity of these three components enhanced the electron transfer within the electrode. Simple polishing on a paper sheet easily renewed the electrode surface. Moreover, the applied working electrode was low (−0.05 V vs. Ag/AgCl) decreasing the interference from electroactive compounds and thus increasing the selectivity of the biosensor. This system allowed to detect glucose between 2.8×10^{-5} and 2.8×10^{-4} M. The paste presented a good shelf life after 4 months (no decrease in sensitivity). This immobilization method was also adapted to develop a rapid and a sensitive automated method for glucose monitoring in drinks (Serban et al., 2004).

Rivas' group described carbon nanotube paste electrodes obtained by dispersion of MWCNTs within mineral oil (Rubianes and Rivas, 2003). A highly selective and sensitive glucose biosensor was developed by incorporation of GOD within the carbon paste without using any metal, redox mediator or anti-interference membrane. The resulting carbon nanotube carbon paste electrode combined the attractive advantages of composite materials such as the feasibility to incorporate different substances, low background currents, easy surface regeneration, and the ability to promote electron-transfer reactions. Glucose was linearly detected up to 3×10^{-2} M with a detection limit of 6×10^{-4} M. An amperometric biosensor for continuous glucose monitoring based on MWCNTs/graphite/GOD packed needle-type electrode was also described (Jia et al., 2008). A mixture of MWCNTs, graphite powder and enzyme was compactly pressed into the cavity at the end of a glass capillary with electrical contact to its inner end with a copper wire. Then, the end of capillary was soaked in paraffin oil and dried at 50 °C. After optimization, the biosensor displayed good sensitivity and stability (14 days), and a detected range up to 2×10^{-2} M of glucose. A binderless CNT/GOD composite packed in a needle device was also prepared for amperometric detection of glucose (Wang and Musameh, 2003). This biosensor showed 80% remaining activity after 24 h thermal stress at 90 °C. Moreover, it was shown that CNTs can act as mediators, since the biosensor could operate at very low potential, eliminating interferences from other electroactive species.

A biosensor for dopamine detection was developed using a carbon paste electrode containing peroxidase-immobilizing pegylated polyurethane nanoparticles (Fritzen-Garcia et al., 2009). In the presence of H_2O_2, enzyme catalyzed the oxidation of dopamine to o-quinone that was electrochemically reduced back to dopamine on the surface of the electrode. Generated currents were directly proportional to dopamine concentrations. Enzyme was adsorbed by electrostatic interactions on nanoparticles thus conferring a favorable environment for peroxidase, improving its stability. Dopamine was detected from 9.9×10^{-5} M to 1.6×10^{-3} M. A good storage stability was obtained over a 200 day period. This modified carbon paste electrode was validated by determination of dopamine in pharmaceutical products.

2.7. Clay-modified electrodes

The use of inorganic host matrices such as silica or clays is an alternative to organic polymers that are widely used for immobilizing enzymes in biosensors. Cationic and anionic clays are two-dimensional layered inorganic solids with open structures favorable to interactions with enzymes and intercalation of redox mediators. They are suitable host matrices for enzyme immobilization in biosensors thus forming biohybrids (Mousty, 2004 and references therein; Mousty, 2010 and references therein). Cross-linking with glutaraldehyde is generally required to prevent release of enzymes into solution. Many enzymes such as GOD, polyphenol oxidase, HRP or urease have been immobilized in clay inorganic matrices for glucose, phenol derivatives, H_2O_2 or urea detection, respectively. Layered double hydroxides also called anionic clays are synthetic inorganic materials with a well-defined lamellar structure, composed of positively charged hydroxide layers enabling intercalation of anions in the nanosized interlayer space. These low cost biocompatible compounds presenting anion-exchange and intercalation capacity become increasingly used to incorporate enzymes. The presence of clays provides a favorable environment to enzyme activity and leads to improved analytical characteristics of the biosensors. For instance, layered double hydroxides — urease nanohybrids were used in a biosensor for urea detection (Vial et al., 2006). The sensitivity varied with the urease loading and with the permeability of the biomembrane, in correlation with the biocompatibility of the matrix and steric hindrance limiting diffusion of the substrate to the enzyme active site. Good performances were obtained in terms of sensitivity, linear ranges and long-term stability.

3. Adsorption

Enzyme adsorption onto solid supports represents the easiest method of physical immobilization (Choi, 2004a). Enzyme is dissolved in solution and the solid support is placed in contact with the enzyme solution for a fixed period of time. The unadsorbed enzyme is then removed by washing with buffer. The adsorption mechanisms are based on weak bonds such as Van der Waal's forces and electrostatic and/or hydrophobic interactions. This technique does not involve any functionalization of the support and is generally non-destructive for enzyme activity. Table 3 presents performances of some biosensors based on adsorbed immobilized enzymes.

Although this immobilization method causes little or no enzyme inactivation, this technique presents drawbacks: enzymes are loosely bound to the support and desorption of the enzyme resulting from changes in temperature, pH and ionic strength, appears to be the main problem. Thus, biosensors based on adsorbed enzyme suffer from poor operational and storage stability. Another drawback is the non-specific adsorption of other proteins or substances.

3.1. Physical adsorption

Physical adsorption consists of simple deposition of an enzyme onto a surface and its attachment through weak bonds. This immobilization strategy has widely been used to develop enzymatic biosensors.

An amperometric biosensor based on AChE immobilization was described by simple adsorption of the enzyme on SPEs (Bonnet et al., 2003). To increase the operational stability of this system, the electrodes were washed with a series of suitable washing buffers which removed the superficial and the excess of adsorbed enzyme. In this way, the electrodes were stable for eight continuous assays and conserved the same activity for 50 days under vacuum storage. LOD and lactate dehydrogenase (LDH) were co-immobilized on polyaniline films by physical adsorption (Chaubey et al., 2000). LOD catalyzed the oxidation of L-lactate into pyruvate, which is the substrate of the reaction catalyzed by LDH. Thus, in the presence of NADH, L-lactate was regenerated from pyruvate. Regeneration of L-lactate by substrate recycling provided an amplification of the sensor response allowing to measure lactate at low concentrations. H_2O_2 produced as a result of the enzymatic reaction was electrochemically measured. The detection limit was 5×10^{-5} M of L-lactate. This biosensor was stable for more than 3 weeks at 4 °C.

An amperometric glucose biosensor was developed using nano scale PPy tubes that provide a higher exposed surface area compared to that of flat surface electrodes (Ekanayake et al., 2007). GOD was physically adsorbed on a PPy nanotube modified electrode. Glucose was linearly detected between 5×10^{-4} M and 1.3×10^{-2} M. A response time of about 3 s was observed. Due to the enhanced

Table 3
Biosensors based on adsorbed enzyme.

Analyte	Immobilized enzyme	Immobilization technique	Detection method	Detection limit (M)	Linearity range (M)	References
H_2O_2	HRP	Physical adsorption	Amperometry	2×10^{-6}	8×10^{-6}–3×10^{-3}	Wang et al. (2009a)
Lactate	LOD/LDH	Physical adsorption	Amperometry	5×10^{-5}	ND	Chaubey et al. (2000)
Glucose	GOD	Physical adsorption	Amperometry	ND	5×10^{-4}–1.3×10^{-2}	Ekanayake et al. (2007)
Glucose	GOD	Physical adsorption	Amperometry	4×10^{-4}	5×10^{-4}–1.75×10^{-2}	Chu et al. (2007)
Glucose	GOD	Electrostatic interactions	Amperometry	8.3×10^{-5}	5×10^{-5}–5.5×10^{-3}	Liu et al. (2007)
Choline	ChOD	Layer-by-layer	Amperometry	5×10^{-7}	5×10^{-7}–1×10^{-4}	Shi et al. (2006)
Glucose	GOD	Layer-by-layer	Amperometry	ND	2.5×10^{-3}–1×10^{-2}	Miscoria et al. (2006b)
Glucose	GOD	Layer-by-layer	Amperometry	ND	Up to 1×10^{-2}	Miscoria et al. (2006a)
Glucose	GOD	Layer-by-layer	Amperometry	5.8×10^{-5}	Up to 5×10^{-3}	Zhao and Ju (2006)
Glucose	GOD	Layer-by-layer	Amperometry	2.5×10^{-6}	5×10^{-6}–6.5×10^{-4}	Xu et al. (2007)
Glucose	GOD	Layer-by-layer	Amperometry	5×10^{-7}	1×10^{-5}–4.5×10^{-3}	Xu et al. (2008)
Glucose	GOD	Layer-by-layer	Amperometry	1×10^{-5}	1×10^{-4}–1.1×10^{-2}	Zhao and Ju (2006)
Glucose	GOD	Layer-by-layer	Amperometry	7×10^{-6}	1.5×10^{-5}–6×10^{-3}	Liu and Lin (2006)
Choline	ChOD	Electrochemical doping	Amperometry	ND	1×10^{-7}–1×10^{-4}	Zhang et al. (2007)
H_2O_2	HRP	Electrochemical doping	Amperometry	2.5×10^{-4}	2.5×10^{-4}–5×10^{-3}	Mathebe et al. (2004)
Urea	Urease	Lipidic microenvironment	Potentiometry	5×10^{-3}	1×10^{-2}–6.8×10^{-2}	Singhal et al. (2002a)
Lactose	Lactase/GalOD	Lipidic microenvironment	Amperometry	ND	5.6×10^{-2}–3.3×10^{-1}	Sharma et al. (2004b)

adsorption property, the authors thought that it should be possible to work with reduced amounts of enzyme, so that the production cost of the biosensor could be reduced. Recently, a disposable H_2O_2 biosensor based on HRP adsorbed on AuNPs electrodeposited onto ITO electrode surface (Wang et al., 2009a) was developed. AuNPs were very efficient to retain the enzyme activity and to promote electron transfer. Amperometric responses to H_2O_2 showed a linear relationship in the concentration range extending from 8×10^{-6} M to 3×10^{-3} M (detection limit of 2×10^{-6} M). This biosensor presented an excellent reproducibility, a high selectivity and long-term stability (about 83% of the initial response remained after 12 weeks).

3.2. Electrostatic interactions

Enzymes can be electrostatically immobilized onto charged surfaces. If the isoelectric point of the enzyme is lower than the pH value of the solution, enzyme is negatively charged and thus can be bound to a positively charged support. Techniques of layer-by-layer deposition and electrochemical doping method based on electrostatic adsorption have been performed to develop enzymatic biosensors.

3.2.1. Layer-by-layer deposition

Electrostatic immobilization of enzymes by the technique of layer-by-layer deposition was first described by Decher in 1991 (Decher and Hong, 1991). This method is based on alternate layers of polyelectrolyte and enzyme with opposite charges (Zhao et al., 2006). Polycations that have predominantly been used in layer-by-layer films include poly(allylamine), poly(L-lysine), poly(ethyleneimine), poly(dimethyldiallylammonium chloride) (PDDA) poly(allylamine hydrochloride) and chitosan (or chitosan derivatives). The most commonly used polyanions are poly(styrenesulfonate) (PSS), poly(vinylsulfonate), poly(anilinepropanesulfonic acid), poly(acrylic acid) and poly(methacrylic acid) (Zhao et al., 2006). Initially, an electrode has to be modified with a charged layer. This negatively (or positively) charged surface is then immersed in a polycation (or polyanion) solution to form the first positively (or negatively)-charged layer. Negatively-charged enzymes are immobilized on the polycationic electrode through electrostatic forces. These deposition processes are carried out repeatedly to obtain the desired number of layers. Layer-by-layer approach is generic and provides a strategy to rationally design the properties of immobilized films (Campas and O'Sullivan, 2003). For example, an amperometric biosensor was developed using ChOD and 6-O-ethoxytrimethylammoniochitosan chloride (EACC) multilayer films deposited on a Prussian blue (PB)-modified platinum electrode (Shi et al., 2006). This chitosan derivative has an excellent film-forming ability, high permeability toward water and small molecules, good adhesion, non-toxicity, high mechanical strength and good biocompatibility. The PB/(EACC/ChOD)$_{10}$ film-coated biosensor presented a linear range between 5×10^{-7} M and 1×10^{-4} M. This biosensor retained around 85% of its initial current response to choline after 2 month. An amperometric glucose biosensor was also developed by the alternate deposition of a quaternized chitosan derivative and GOD on a Nafion-modified electrode (Miscoria et al., 2006b). The sensitivity of the biosensor increased up to the 10th bilayer. After the 11th bilayer, the response decreased drastically, probably due to the destabilization of the multistructure. This system was very selective since the presence of uric acid and ascorbic acid did not interfere with the electrochemical response.

An amperometric glucose biosensor based on layer-by-layer adsorption of GOD and dendrimer-encapsulated Pt nanoparticles on multiwalled CNTs was described (Xu et al., 2007). First, CNTs were functionalized in order to present negatively charged carboxylic groups. Alternating PDDA and PSS monolayers were first adsorbed onto CNTs surface, successively. Then, positively dendrimer-encapsulated Pt nanoparticles (Pt-DENs) and negatively GOD molecules were alternatively adsorbed onto the PSS/PDDA/CNts layer until the desired number of layers was obtained. The layer-by-layer technique provided a favorable microenvironment to keep the bioactivity of enzyme and to prevent enzyme leakage. Glucose was linearly detected between 5×10^{-6} M and 6.5×10^{-4} M (detection limit of 2.5×10^{-6} M). The same group also developed an amperometric glucose biosensor based on self-assembling GOD and dendrimer-encapsulated Pt nanoparticles on nanofibrous polyaniline (Xu et al., 2008). In this case, glucose was linearly detected from 1×10^{-5} M to 4.5×10^{-3} M (detection limit of 5×10^{-7} M). After storage at 4 °C for 20 days, this biosensor retained 85% of response. Recently, a novel strategy based on the deposition of enzyme layers on cyclodextrin-modified electrodes through complementary supramolecular interactions between adamantane-appended enzymes and cyclodextrin-capped gold nanoparticles was also reported (Fragoso et al., 2009).

3.2.2. Electrochemical doping

Enzymes can also be immobilized by electrochemical doping. By controlling pH, the negatively or positively charged enzymes can be doped into the conductive polymer film during its oxidation or reduction process, respectively. During oxidation, the polymer becomes positively charged. At a given pH value of the solution, negatively-charged enzyme molecules can be incorporated into the conductive polymer during the oxidation process to form an enzyme electrode (Zhang et al., 2007). This procedure has been used to develop

biosensors for cholesterol (Wang and Mu, 1999), choline (Langer et al., 2004; Zhang et al., 2007), glucose (Mu and Xue, 1996; Mu et al., 1991), H_2O_2 (Mathebe et al., 2004) or galactose (Mu, 1994) detection. Characteristics of glucose biosensors prepared by simple physical adsorption or electrochemical doping on/in a polyaniline film have been compared (Mu and Xue, 1996). X-ray photoelectron spectroscopy (XPS) experiments revealed that the binding force between enzyme and polyaniline film is stronger with electrochemical doping than with adsorption. GOD simply adsorbed on the polymer desorbed more easily than that electrochemically doped in the film. More recently, an amperometric biosensor for H_2O_2 detection has also been developed using polyaniline nanoparticles synthesized with dodecylbenzylsulfonic acid (Morrin et al., 2005). Nanoparticles were electrodeposited on the surface of a glassy carbon electrode. Then, HRP was electrostatically adsorbed to the nanoparticle-modified surface by electrochemical doping. This biosensor format exhibited higher signal-to-signal background ratios and shorter response times than previous polyaniline biosensor configurations.

3.2.3. Pre-immobilization on ion-exchanger beads

Enzymes can be electrostatically immobilized on diethylaminoethyl (DEAE) anion exchanger beads. Many oxidases have been pre-adsorbed on positively-charged DEAE-beads before being entrapped in a biological (e.g. polysaccharide) (Sassolas et al., 2009b) or a chemical polymer (e.g. photopolymer or silica gel) (Leca and Blum, 2000; Leca et al., 2001; Marquette et al., 2003; Sassolas et al., 2009a, 2009b; Tsafack et al., 2000b). The pre-immobilization on beads allowed to avoid enzyme leakage from the porous immobilization matrix. For example, an electrochemiluminescent choline biosensor was developed (Leca and Blum, 2000). Because of its low isoelectric point value (pI = 4.5), ChOD could be efficiently immobilized on DEAE beads by ionic interactions without risk of release from the support when in use at pH 7 or higher. Then, ChOD-DEAE beads were entrapped in a PVA-SbQ matrix formed on a SPE. This biosensor allowed choline detection from 2×10^{-9} M to 1×10^{-4} M (Leca and Blum, 2000). In the same way, an ECL choline biosensor based on polyluminol was developed by entrapping ChOD-DEAE beads within silica, agarose, chitosan or alginate gels (Sassolas et al., 2009b).

3.3. Retention in a lipidic microenvironment

Langmuir–Blodgett (LB) technology allows to build up lamellar lipid stacking by transferring a monomolecular film formed at an air/water interface onto a solid support, with an accurate control of the thickness and of the molecular organization (Girard-Egrot et al., 2005). Based on the self-assembly properties of amphiphilic biomolecules at the air/water interface, this technique offers the possibility to prepare ultrathin layers suitable for enzyme immobilization.

Enzymes can easily be adsorbed onto pre-formed LB films. However, the release of protein molecules due to the weakness of their association with the lipidic surface remains a major drawback.

In order to develop a biosensor for choline detection, ChOD was inserted in a hydrophilic or hydrophobic environment of behenic acid LB films and the biosensing layer was directly coated on a Pt electrode (Girard-Egrot et al., 1998). In this work, the enzyme was sandwiched between the heads or between the tails of the lipidic layers. The detection limit was 2.7×10^{-4} M in the hydrophobic enzyme environment and 2.5×10^{-5} M in the hydrophilic one.

LB films of poly-3-hexylthiophene (P3DT)/stearic acid (SA) (Malhotra et al., 2005) have been used for immobilization of galactose oxidase (GalOD) (Sharma et al., 2004a), lactase (Sharma et al., 2004b) or glucose oxidase (Singhal et al., 2002b). Enzyme mixed in a solution of P3DT/SA in chloroform was spread onto air–water interface of LB trough. These P3DT/SA/enzyme monolayers were then transferred onto indium-tin oxide coated glass plates. In the same way, urease was immobilized in mixed monolayers of poly(N-vinyl carbazole) and stearic acid formed at an air–water interface (Singhal et al., 2002a). The enzyme catalyzed the decomposition of urea into ammonium ions that were detected by potentiometry using an ammonium analyzer. Urea was detected between 1×10^{-2} M and 6.8×10^{-2} M. This electrode could be reused about 10 times and the shelf-life of this biosensor was 5 weeks.

4. Cross-linking

Immobilization of enzymes by cross-linking with glutaraldehyde or other bifunctional agents such as glyoxal or hexamethylenediamine is another well-known approach to develop biosensors. The enzyme can be either cross-linked with each other or in the presence of a functionally inert protein such as bovine serum albumin. This method is attractive due to its simplicity and the strong chemical binding achieved between biomolecules. The main drawback is the possibility of activity losses due to the distortion of the active enzyme conformation and the chemical alterations of the active site during cross-linking. Table 4 presents performances of some biosensors based on enzymes immobilized by cross-linking.

Several conductometric biosensors based on the immobilization of enzymes in a gel obtained by co-reticulation with glutaraldehyde in the presence of BSA have been reported for the detection of different molecules such as heavy metals (Berezhetskyy et al., 2008), nitrite (Zhang et al., 2009) or pollutants (Gogol et al., 2000). For example, a biosensor was developed for heavy metal ion determination by cross-linking alkaline phosphatase with BSA in saturated glutaraldehyde vapor on the electrode surface (Berezhetskyy et al., 2008). Storage stability in buffer solution at 4 °C was more than 1 month.

A gravimetric glucose biosensor was also reported for selective detection of blood glucose levels. GOD was immobilized onto cantilever surface by cross-linking with glutaraldehyde and BSA (Pei et al., 2004). The enzyme-functionalized microcantilever underwent bending due to a change in surface stress induced by the reaction between glucose and immobilized GOD. No external physical parameters, such as potential or current, were applied during the measurements, so the selectivity only depended on the enzyme reaction. This biosensor was very selective and species such as catechol, ascorbic acid and 4-acetaminophen had no effect on the response of the cantilever biosensor.

A disposable alcohol biosensor was also developed using the cross-linking method to immobilize ADH and NAD^+ on a Nafion-Meldola blue modified SPE (Luo et al., 2008). A mediator was used in order to decrease the potential required for NADH oxidation. This biosensor presented a good specificity, reproducibility, storage stability, accuracy and provided a fast response. It was successfully used for the measurements of serum alcohol.

Recently, an amperometric glucose biosensor was developed by immobilizing GOD onto ZnO nanotube-modified electrode by cross-linking (Kong et al., 2009). Glucose was linearly detected from 5×10^{-5} M to 1.2×10^{-2} M (detection limit was 1×10^{-6} M). This biosensor presented a good anti-interference ability and long-term stability since the system retained around 7% of its initial activity after 60 days.

A needle-type biosensor was also developed for monitoring blood glucose in fish (Yonemori et al., 2009). Cross-linking between GOD and BSA was induced by addition of glutaraldehyde. Continuous glucose monitoring was performed by implanting the sensor into the eye of the fish. The eyeball scleral interstitial fluid glucose concentrations could be measured continuously between 3.9×10^{-3} M and 2.3×10^{-2} M.

Enzyme immobilization by cross-linking and by entrapment in a carbon paste can also be combined to develop enzymatic biosensors (Pereira et al., 2007; Santos et al., 2006; Santos et al., 2007). For this purpose, a mixture of MWCNTs modified with methylene blue, enzyme, BSA and glutaraldehyde was added to a graphite powder. The homogeneous paste obtained with mineral oil was placed into the cavity of a

Table 4
Biosensors based on cross-linked enzymes.

Analyte	Immobilized enzyme	Detection method	Detection limit (M)	Linearity range (M)	References
Glucose	GOD	Amperometry	ND	70–420 $mg.dl^{-1}$	Yonemori et al. (2009)
Glucose	GOD	Amperometry	1×10^{-6}	5×10^{-5}–1.2×10^{-2}	Kong et al. (2009)
Glucose	GOD	Amperometry	ND	5×10^{-4}–2.5×10^{-2}	Su et al. (2004)
Glucose	GOD	Amperometry	2×10^{-5}	Up to 10^{-3}	Barsan and Brett (2009)
Choline	ChOD/HRP	Amperometry	3×10^{-6}	5×10^{-6}–6×10^{-4}	Yang et al. (2004)
Monosodium glutamate	L-glutamate OD and L-glutamate DH	Amperometry	0.02 $mg.l^{-1}$	0.02–3 $mg.l^{-1}$	Basu et al. (2006)
Cholesterol	CholOD and CholE	Amperometry	ND	2–50 $mg.dl^{-1}$	Basu et al. (2007)
Hypoxanthine	XOD	ECL	1×10^{-7}	6×10^{-7}–2×10^{-4}	Lin et al. (2008)
Acetylcholine	AchE/ChOD	Amperometry	1×10^{-7}	Up to 1×10^{-4}	Guerrieri et al. (2006)
Urea	Urease	Conductimetry	1×10^{-5}	1×10^{-5}–1.3×10^{-3}	Mikkelsen and Rechnitz (1989)
Catechol	Tyrosinase	Amperometry	ND	5×10^{-6}–1×10^{-3}	De Albuquerque and Ferreira (2007)
Fluoride	Tyrosinase	Amperometry	ND	1×10^{-6}–2×10^{-5}	Asav et al. (2009)
Cd^{2+}	PAL	Conductimetry	0.5 ppm	0.5–50 ppm	Berezhetskyy et al. (2008)
Ethanol	ADH	Amperometry	1.1×10^{-5}	Up to 5×10^{-3}	Luo et al. (2008)

glass tube. Based on this principle, amperometric biosensors for phenolic compound (Santos et al., 2007), ethanol (Santos et al., 2006) or lactate (Pereira et al., 2007) detection were reported. Amperometric responses obtained with ADH- and LDH-based biosensors showed excellent sensitivity, operational stability (about 95% of the activity was maintained after 300 measurements) and wide linear range (5×10^{-5} M to 1×10^{-2} M and 1×10^{-4} M and 1×10^{-2} M for ethanol and lactate, respectively). When NAD^+ was incorporated into the carbon paste, the sensitivity was higher than the one obtained using NAD^+ free in solution.

5. Covalent immobilization

Covalent coupling of enzymes to polymeric supports is a popular chemical immobilization method used to develop enzymatic biosensors. For this purpose, biocatalysts are bound to the surface through functional groups that they contain and that are not essential for their catalytic activity. The binding of the enzymes to the solid support is generally carried out by initial activation of the surface using multifunctional reagents (e.g. glutaraldehyde or carbodiimide), followed by enzyme coupling to the activated support, then the removal of excess and unbound biomolecules. The carrier support can either be an inorganic material (e.g. controlled pore glass), a natural (e.g. cellulose) or synthetic polymer (e.g. nylon). Membranes (e.g. Immunodyne, Ultrabind) that are already pre-activated have also been used. Covalent immobilization can be performed directly onto the transducer surface or onto a thin membrane fixed onto the transducer.

Numerous protocols for activating solid surfaces have been described (Cao, 2005a; Choi, 2004b; Hermanson et al., 1992) (Table 5). The most used procedures to develop enzymatic biosensors (using glutaraldehyde or carbodiimide) are briefly described below. An increased stability of the enzyme is obtained but high amounts of bioreagent are required and reproducibility is generally poor. Table 6 presents performances of some biosensors based on covalently immobilized enzymes.

5.1. Activation of carboxylic groups

Carbodiimides allow the binding between the carboxyl groups of a support and the amino function of an enzyme (Fig. 12A). N-hydroxysuccinimide (NHS) can be associated to carbodiimide in order to improve immobilization efficiency (Fig. 12B). This procedure is widely used to develop enzymatic biosensors.

An amperometric cholesterol biosensor was developed by cholesterol oxidase immobilization on a gold working electrode using a self-assembly approach (Shen and Liu, 2007). A thiol, 3-mercaptopropionic acid (MPA), was self-assembled onto the working electrode. Then, EDC was allowed to bind the carboxylic groups of the MPA with the amino groups of the enzyme. Cholesterol was detected from 6.5×10^{-4} M to 7.8×10^{-3} M. This biosensor suffered from interferences due to biological species and the storage stability was not satisfactory (after 4 days, the response decreased to 20% of its original value).

Rahman et al. widely used this immobilization strategy to develop enzymatic biosensors for the detection of glutamate (Rahman et al., 2005), glucose (Rahman et al., 2005) or lactate (Rahman et al., 2009). Recently, these authors developed an amperometric biosensor for lactate detection based on poly-5,2′-5′, 2″-terthiophene-3′-carboxylic acid (pTTCA) and multiwall CNT composite on a gold electrode (Rahman et al., 2009). In this work, LDH and NAD^+ were co-immobilized on the free carboxylic groups of pTTCA/multiwall CNT composite layer using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and

Table 5
Covalent immobilization methods of enzymes.

Enzyme group	Surface group	Activation methods	References
$-NH_2$	$-NH_2$	Glutaraldehyde	Delvaux and Demoustier-Champagne (2003) and Ferreira et al. (2003)
	−OH	Epichlorohydrin	Hermanson et al. (1992) and Tumturk et al. (1999, 2000)
		Silane epoxide	Petri et al. (2004)
		Cyanuric chloride	Tumturk et al. (1999)
		Cyanogen bromide	Hermanson et al. (1992) and Wilchek and Miron (2003)
		Tosyl chloride	Hermanson et al. (1992) and Moreno et al. (1997)
		Thiol silane + succinimide ester	Bhatia et al. (1991)
		Chlorotriazine	Moreno et al. (1997)
	−COOH	Carbodiimide	Delvaux and Demoustier-Champagne (2003) and Hermanson et al. (1992)
		Acyl azoture	Coulet et al. (1974)
		Carbonyldiimidazole	Hermanson et al. (1992)
−COOH	$-NH_2$	Carbodiimide	Wu et al. (1994)
−SH	−SH	2,2′-dipyridyldisulfur	Hermanson et al. (1992)

Table 6
Biosensors based on covalently immobilized enzymes.

Analyte	Immobilized enzyme	Detection method	Detection limit (M)	Linearity range (M)	References
Carboxylated surface					
Choline	ChOD/HRP	Amperometry	1×10^{-7}	1×10^{-6}–8×10^{-5}	Rahman et al. (2004)
Glutamate	Glutamate oxidase	Amperometry	1×10^{-7}	2×10^{-7}–1×10^{-4}	Rahman et al. (2005)
K^+	Pyruvate oxidase	Amperometry	3×10^{-7}	1×10^{-6}–1×10^{-4}	Rahman et al. (2006)
Lactate	LDH	Amperometry	1×10^{-6}	5×10^{-6}–9×10^{-5}	Rahman et al. (2009)
H_2O_2	HRP	Amperometry	5×10^{-8}	Up to 8×10^{-6}	Yu et al. (2003)
Aminated surface					
Carbodiimide coupling					
Urea	Urease	Amperometry	ND	1.6×10^{-4}–5×10^{-3}	Rajesh et al. (2005)
Phenol	Tyrosinase	Amperometry	9×10^{-7}	1.8×10^{-6}–1.7×10^{-4}	Rajesh and Kaneto (2005)
Catechol	Tyrosinase	Amperometry	7×10^{-7}	1.3×10^{-6}–1.1×10^{-4}	
p-Cresol	Tyrosinase	Amperometry	1.1×10^{-6}	2.1×10^{-6}–1.68×10^{-4}	
Cholesterol	CholOD	Absorbance	6.5×10^{-4}	1.3×10^{-3}–1.3×10^{-2}	Arya et al. (2007)
Cholesterol	CholOD	Amperometry	6.5×10^{-4}	6.5×10^{-4}–1×10^{-2}	Dhand et al. (2007)
Dopamine	Tyrosinase	Amperometry	ND	5×10^{-6}–1.2×10^{-4}	Zhou et al. (2007)
Glucose	GOD	Amperometry	2.6×10^{-5}	1×10^{-4}–2.7×10^{-2}	Yang et al. (2003)
Glutaraldehyde					
H_2O_2	HRP	Amperometry	5×10^{-7}	5×10^{-6}–6.5×10^{-5}	Gao et al. (2004)
L-Phe	Phe DH	Amperometry	2.5×10^{-5}	Up to 9.1×10^{-3}	Villalonga et al. (2008)
Glucose	GOD	Amperometry	9×10^{-6}	Up to 8×10^{-3}	Sun et al. (2006)
Glucose	GOD	Amperometry	9×10^{-6}	1.8×10^{-5}–1.1×10^{-3}	Nan et al. (2009)
Cholesterol	CholE/CholOD	Amperometry	ND	1.3×10^{-3}–1.3×10^{-2}	Singh et al. (2006)
Phenol	Tyrosinase	Amperometry (VC)	2×10^{-7}	1×10^{-6}–2×10^{-4}	Zhou and Zhi (2006)
Catechol	Tyrosinase	Amperometry	ND	2×10^{-7}–8×10^{-5}	Wang et al. (2009b)

NHS. This biosensor allowed to detect lactate between 5×10^{-6} M and 9×10^{-5} M (detection limit 1×10^{-6} M) and it could be used for 1 month without any significant loss in sensitivity. This system was also successfully used for the analysis of L-lactate in commercial milk and human serum samples. The same conducting polymer was used to covalently immobilize glutamate oxidase (Rahman et al., 2005). A needle-type amperometric biosensor for glutamate detection was designed. This system exhibited a wide linear range between 2×10^{-7} M and 1×10^{-4} M. The biosensor was implanted into the rat brain for in vitro monitoring of the extracellular glutamate released by cocaine stimulation.

Enzymes can also be covalently immobilized on functionalized nanotubes present on an electrode surface. An amperometric biosensor for H_2O_2 detection was described by immobilizing HRP onto the ends of SWCNTs by using EDC to promote amide linkages between carboxyl-terminated nanotubes and lysine residues of the enzyme (Yu et al., 2003). Detection limit was 5×10^{-8} M of H_2O_2. In the same way, a biosensor for glucose detection based on covalent

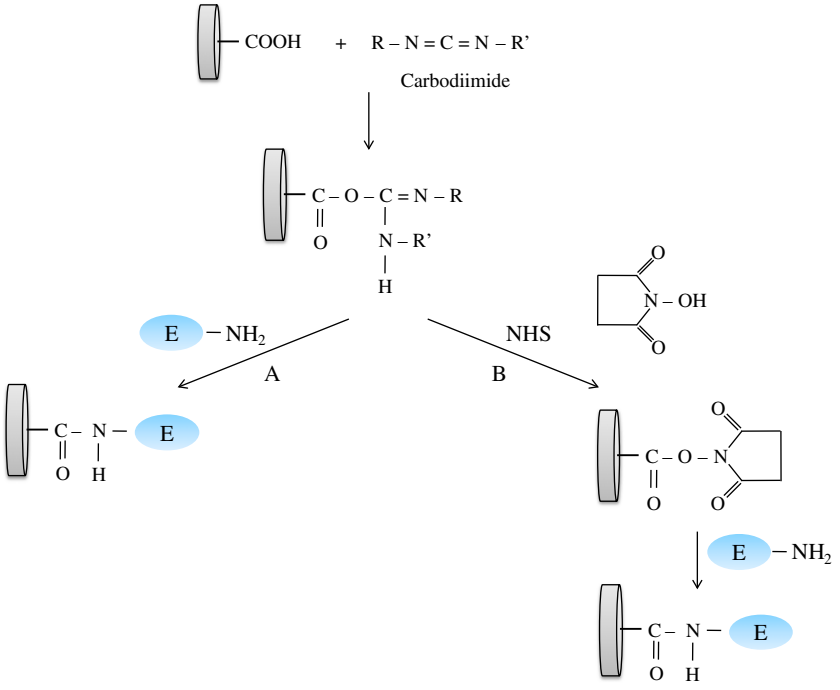

Fig. 12. Enzyme immobilization on carboxylated surface by carbodiimide coupling (A) without or (B) with NHS.

immobilization of GOD on SWCNTs was developed (Xue et al., 2003). The biosensor exhibited good operational and storage stability, with 90% of activity retained after 4 months.

5.2. Activation of amino groups

Carbodiimides also allow the binding between the amino groups of a support and the carboxyl function of enzyme (Fig. 13A).

Based on this immobilization principle, a biosensor for glucose detection was developed using a porous organic–inorganic hybrid sol–gel carbon composite (Yang et al., 2003). The presence of free amine groups on the sol–gel silicate backbone was used for the covalent immobilization of GOD using EDC and NHS. The biosensor showed a linear range from 1×10^{-4} M to 2.7×10^{-2} M (detection limit of 2.6×10^{-5} M) and a good stability (about 85% of its original stability remaining after 1 month).

Cholesterol oxidase was also immobilized onto a two-dimensional self-assembled monolayer of N-(2-amino-ethyl)-3-aminopropyl-trimethoxysilane deposited on ITO-coated glass plates using EDC and NHS (Arya et al., 2007). Cholesterol was linearly detected between 1.3×10^{-3} M and 1.3×10^{-2} M (detection limit of 6.5×10^{-4} M). The stability was found to be ca. 10 weeks upon storage at 4 °C and the electrode could be reused approximately 10 times.

Urease was immobilized on a poly(N-3-aminopropyl pyrrole-co-pyrrole) film having free amino groups via carbodiimide coupling reaction (Rajesh et al., 2005). Amperometric detection of urea was possible in the range extending from 1.6×10^{-4} M to 5×10^{-3} M. The principle was based on the use of pH-sensitive electrochemically active dissolved hematein molecule. This biosensor retained 80% of its initial enzyme activity for 2 months when stored at 4–6 °C. The same immobilization strategy was used to immobilize tyrosinase on poly(3-aminopropyl pyrrole) (Rajesh and Kaneto, 2005) in order to develop an amperometric biosensor for phenolic compound detection. For example, phenol was detected in the concentration range comprised between 1.8×10^{-6} M and 1.7×10^{-4} M. This electrode retained 80% of its activity after 3 months.

Enzyme immobilization can also be achieved using glutaraldehyde as the activating agent. A first Schiff-base reaction occurs between an aldehyde group of glutaraldehyde and an amine function of the support. Then, the second aldehyde group of glutaraldehyde reacts with an amine function of the enzyme (Fig. 13B).

An amperometric H_2O_2 biosensor was designed by the modification of screen-printed carbon electrodes with amino groups (Gao et al., 2004). It has been demonstrated that the electrochemical oxidation of thionine in neutral phosphate led to the modification of the SPEs with amino-groups which allowed covalent immobilization of HRP. H_2O_2 was detected in a linear range comprised between 5×10^{-6} M and 6.5×10^{-5} M with a detection limit of 5×10^{-7} M. When the enzyme electrode was stored at 4 °C, the biosensor remained stable over a 30 day period.

Phenylalanine dehydrogenase was covalently immobilized by glutaraldehyde on amino-activated cellulose membrane which was associated to a glassy carbon electrode in order to develop an amperometric biosensor for L-phenylalanine detection (Villalonga et al., 2008). The enzyme electrode showed a linear range extending up to 9.1×10^{-3} M with a detection limit of 2.5×10^{-5} M. The electrode was highly stable, retaining full initial analytic response after 16 days of storage at 4 °C in phosphate buffer.

Amino groups of the support can also be transformed into diazonium salts before chemical enzyme coupling. Immobilization of horseradish peroxidase (HRP) onto a diazonium functionalized screen-printed gold electrode has recently been successfully developed using a novel protocol (Radi et al., 2009). After electrochemical grafting of a p-nitrophenyl layer on the electrode surface, electrochemical reduction of the nitro groups was performed so that an aminated surface was obtained. Further chemical reaction with nitrous acid transformed the amine to diazonium derivative thus enabling chemical coupling of the enzyme with the diazonium group to form a covalent diazo bond. The designed biosensor allowed to detect H_2O_2 by amperometry. Detection limit was 2×10^{-6} M. The reproducibility of the biosensor response was investigated by analysis of the same concentration of H_2O_2 using 5 electrodes and a relative standard deviation of 5.5% was calculated.

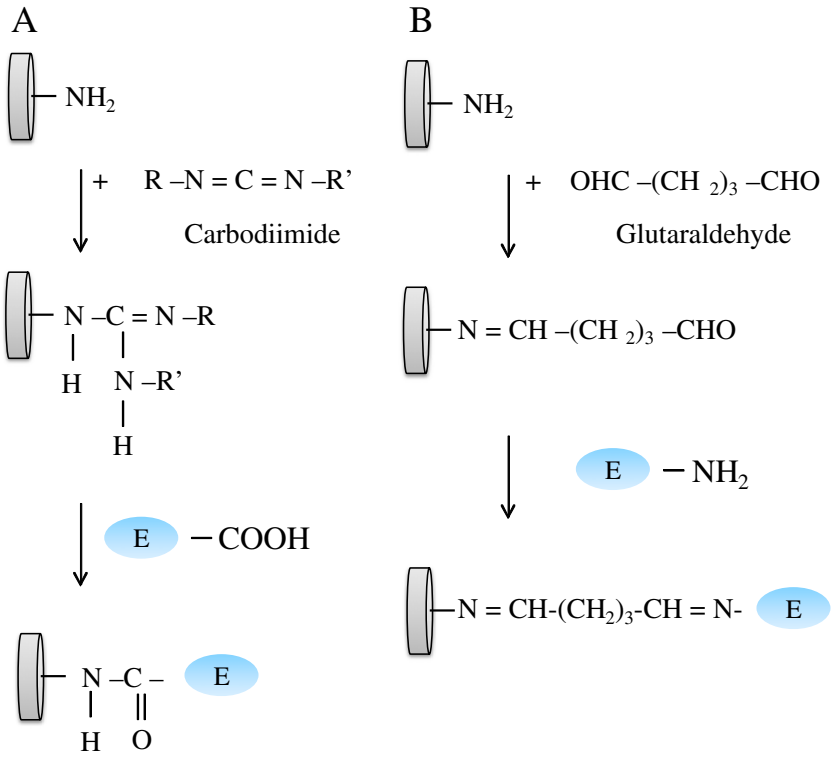

Fig. 13. Enzyme immobilization on an aminated surface by (A) carbodiimide or (B) glutaraldehyde coupling.

The operational stability was also tested and the immobilized enzyme retained its full activity for 15 measurements. Storage stability was acceptable since the immobilized HRP retained its full activity for 2 weeks and 75% of its initial activity after 3 weeks of storage at 4 °C.

Enzymes can also be immobilized onto pre-formed self-assembled monolayers (SAM) via covalent binding. First, alkanethiols with appropriate functionalities such as carboxylic acid or amine groups are chemisorbed onto the gold surface. Activation of the free terminal groups present on the pre-formed SAM has to be achieved using coupling agents such as glutaraldehyde or carbodiimide (Cabrita et al., 2005). SAM immobilization generates uniform structures with high coverage and provides reproducibility and stability to the protein assembly. SAM-based immobilization process was commonly used to immobilize a wide range of enzymes such as HRP (Dong and Li, 1997; Mendes et al., 2008) or cytochrome c oxidase (Dong and Li, 1997). Kubota et al. studied the effects of different self-assembled monolayers on HRP immobilization process to develop a biosensor for H_2O_2 detection (Mendes et al., 2008). Best results were obtained when HRP was bound to a SAM formed by cysteamine using glutaraldehyde. This biosensor showed a good linear response range between 3×10^{-6} M and 1×10^{-4} M (detection limit of 6×10^{-7} M).

5.3. Chemisorption

Thiol-containing enzymes can be directly immobilized on gold surface because of the strong affinity between thiols and gold substrates. The main advantages of this technique are the simplicity and the strong and stable biomolecule attachment. The enzyme can be modified to contain thiol moieties which bind to gold. For this purpose, McRipley and Linsenmeier used Traut's reagent to replace the primary amine groups of GOD with thiol groups (McRipley and Linsenmeier, 1996). The modified enzyme was then covalently bound to a gold electrode. However, this protocol required a long incubation time (250 h at 4 °C) for the preparation of the enzyme electrode. Site-directed mutagenesis was also used to introduce Cys into the enzyme sequence (Gwenin et al., 2007). The binding of the enzyme to the thiol-reactive surface was achieved in an ordered orientation (Campas et al., 2004; Hernandez and Fernandez-Lafuente, 2011). Based on this principle, an amperometric biosensor was developed for the detection of explosives containing nitroaromatic compounds. Nitroreductase was immobilized on a gold electrode surface without loss of enzyme activity through a sequence of six cysteine amino acids which were genetically incorporated into the native enzyme. The reversibility of this covalent immobilization of enzymes via their thiol groups can be underlined. Indeed, disulfide bonds can be broken by reaction with a suitable agent such as dithiothreitol (DTT) under mild conditions, thus allowing to easily regenerate the enzyme support for a new active enzyme coupling to be performed.

6. Affinity

Efforts have been achieved in order to develop biosensors based on oriented and site-specific immobilization of enzymes. A strategy is to create (bio)affinity bonds between an activated support (e.g. with lectin, avidin, metal chelates) and a specific group (a tag) of the protein sequence (e.g. carbohydrate residue, biotin, histidine). This method allows to control the biomolecule orientation in order to avoid enzyme deactivation and/or active site blocking. Several affinity methods have been described to immobilize enzymes through (strept)avidin-biotin, lectin-carbohydrate and metal cation–chelator interactions. An enzyme can contain affinity tags in its sequence (e.g. a sugar moiety) but, in some cases, the affinity tag (e.g. biotin) needs to be attached to the protein sequence by genetic engineering methods such as site-directed mutagenesis, protein fusion technology and post-transcriptional modification (Andreescu and Marty, 2006). Several affinity methods have been described to immobilize enzymes.

Table 7 presents biosensors based on enzymes immobilized by affinity.

6.1. Biotin-(strept)avidin

A strategy to immobilize enzymes is to use the strong affinity existing between biotin and (strept)avidin (dissociation constant of 10^{-15} M). Biotinylation of proteins can be achieved through a covalent coupling of biotin to the protein by the use of biotin-ester reagents that preferentially modify lysine residues (Nilsson et al., 1997). Enzymes can also be genetically biotinylated using a biotin acceptor peptide sequences fused to the C-terminus of enzyme (Zhang and Cass, 2000).

Biotinylated enzymes have been used to develop chemiluminescent biosensors for choline and acetylcholine (ACh) detection (Yao et al., 2002). Streptavidin was first entrapped in a polyacrylamide gel by using a very small quantity of glutaraldehyde. Then, this membrane was incubated in the presence of biotinylated ChOD and AChE. An outer membrane was also formed in order to prevent enzyme leaching. This system allowed to detect choline between 4×10^{-8} M and 1×10^{-4} M (detection limit of 2.5×10^{-9} M) and ACh between 6×10^{-8} M and 1×10^{-4} M (detection limit of 1×10^{-8} M). The immobilized enzymes were stable and retained 65% activity after 1 month. In the same way, an impedimetric biosensor for H_2O_2 detection was developed (Esseghaier et al., 2008). HRP conjugated with streptavidin was immobilized on a mixed self-assembled monolayer formed by 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(biotinyl) and

Table 7
Biosensors based on enzymes immobilized by affinity.

Analyte	Immobilized enzyme	Immobilization strategy	Detection method	Detection limit (M)	Linearity range (M)	References
Avidin/biotin						
Urea	Urease	Biotinylated urease	ChemFEC	ND	1×10^{-4}–1×10^{-1}	Barhoumi et al. (2008)
H_2O_2	HRP	Streptavidin-HRP	Impedance	1×10^{-5}	1×10^{-5}–1×10^{-1}	Esseghaier et al. (2008)
ACh	AChE/ChOD	Biotinylated-AChE/ChOD	CL	1×10^{-8}	6×10^{-8}–1×10^{-4}	Yao et al. (2002)
Catechol	Tyrosinase	Biotinylated PPy	Amperometry	ND	1×10^{-6}–4×10^{-5}	Mousty et al. (2001)
Catechol	Tyrosinase	Biotinylated PPy	Amperometry	2×10^{-7}	Up to 3×10^{-5}	Cosnier et al. (1999b)
Glucose	GOD	Biotinylated PPy	Amperometry	5×10^{-5}	Up to 2.4×10^{-3}	Cosnier et al. (2001)
Chelation						
Acetylthiocholine	AChE	NTA derivative	Amperometry	ND	1×10^{-6}–3×10^{-4}	Andreescu et al. (2001)
Paraoxon	AChE	NTA	Amperometry	2×10^{-9}	ND	Bucur et al. (2004a)
Sugar/lectine						
Chlorpyrifos	AChE	Con A	Amperometry	1×10^{-8}	ND	Bucur et al. (2004b)
Acetylthiocholine	AChE	Con A	Amperometry	ND	1×10^{-5}–1.1×10^{-4}	
Acetylthiocholine	AChE	Con A	Amperometry	ND	1×10^{-5}–1×10^{-4}	Bucur et al. (2005a)
Chlorpyrifos methyl-oxon	AChE	Con A	Amperometry	5×10^{-8}	ND	Bucur et al. (2004a)

16-mercaptohexadecanoic acid previously grafted on a gold electrode. This biosensor was able to detect 1×10^{-5} M of H_2O_2 and it was successfully tested in raw milk samples for H_2O_2 detection at a concentration of ca. 5×10^{-5} M. An electropolymerizable pyrrole-modified biotin (Cosnier and Lepellec, 1999) has also been used to develop several glucose and catechol biosensors (Cosnier et al., 1999b; Cosnier et al., 2001; Mousty et al., 2001). This strategy involved the electropolymerization of a biotin derivative. Then, the attachment of biomolecules to the electrode surface could be achieved by the functionalization of the resulting conducting polypyrrole by (strept)avidin and subsequent coupling using biotinylated molecules. This strategy was also used to develop a clinical biosensor for urea detection (Barhoumi et al., 2008). Biotinylated urease was immobilized through an avidin layer deposit on biotinylated polypyrrole coated chemical field effect capacitance (ChemFEC) devices. This system presented a linear urea concentration range from 1×10^{-4} M to 1×10^{-1} M.

6.2. Metal ion–chelator

The strong affinity link between a metal cation and a chelator such as nitrilotriacetic acid (NTA), imidodiacetic acid (IDA) or tag poly(histidine) can also be used to develop enzymatic biosensors. This method is based on the principle of Immobilized Metal Affinity Chromatography (IMAC), currently used for protein purification and separation. Immobilization of the metal ion on a chromatographic resin by chelation allows the separation of histidine-tagged proteins from untagged proteins. The bound molecule can be eluted from the resin by reducing the pH and increasing the ionic strength of the buffer or using EDTA or imidazole. Based on this principle, enzymes with His residues in their structure can be easily attached to a support containing a metal chelate. However, few His residues are present on enzyme surface and are accessible for binding to a chelate-modified surface. To solve this problem, genetic engineering methods allow the production of tagged enzyme by attaching His at a specific position of the protein. A biosensor for lactate detection based on this immobilization method has been described (Halliwell et al., 2002). First, polyaniline-polyacrylate films were formed on an electrode. This copolymer could be loaded with Ni^{2+} ions, which acted as coordination sites for histidine residues present on his-tagged lactate dehydrogenase. NTA was also commonly used as a chelator for selective enzyme attachment. In this case, four of six coordinations of Ni^{2+} ions were occupied by the four ligands of the NTA chelate, while the other two positions were occupied by water or buffer molecules which could be selectively replaced by the His tag that were incorporated in the enzyme sequence (Andreescu et al., 2001; Campas et al., 2004). This strategy also allowed to immobilize His-tagged AChE on functionalized graphite used for SPE fabrication (Andreescu et al., 2003). The graphite was modified in order to incorporate a NTA group that could interact with nickel ions. Then, immobilization of the enzyme was possible via a histidine tail. The linear range for acetylthiocholine detection extended from 1×10^{-6} M to 6×10^{-5} M. This immobilization allowed to develop an electrochemical biosensor for glucose detection using an histidine-tagged GOD (Haddour et al., 2005). In this study, the pyrrole monomer functionalized with an NTA group was electropolymerized on a platinum electrode (Fig. 14). Then, copper ions interacted with NTA groups. In the presence of the enzyme, a complex between GOD-His, Cu^{2+} ions and NTA was formed. Biosensors based on His tag affinity method can be easily regenerated since His or the Ni^{2+} ions can be removed from the electrode surface using imidazole or EDTA as competitive agents, respectively.

The oriented and site-specific immobilization of a highly sensitive genetically engineered acetylcholinesterase (B394) with a hexa(histidine) tail by affinity interactions on metal chelate-functionalized magnetic microbeads carrying Ni-IDA complexes was also reported (Istamboulie et al., 2007). A very sensitive detection of chlorpyriphos-oxon and chlorfenvinphos was underlined, corresponding to as low as 1.3×10^{-11} M (IC10). The ability to easily control the charging/discharging of the electrode surface by application of a magnetic field provided reusability of the same electrode for several analyses by simply removing the magnet and recharging the surface with fresh enzyme functionalized beads.

HRP modified with histidine was also immobilized on IDA-Sepharose beads entrapped in a PVA-SbQ photopolymer to develop a hydrogen peroxide sensor (Tsafack et al., 2000a). Detection limit was 6×10^{-8} M. Pre-immobilization on IDA-Sepharose beads enhanced the sensing layer stability and enabled the immobilization of a larger amount of enzyme.

6.3. Lectin-carbohydrate

Affinity immobilization was also described between a sugar moiety naturally present in some enzymes such as AChE and concanavalin A (Con A) deposited onto a surface. Con A is a lectin which has multiple sites with high affinity for carbohydrates (Andreescu and Marty, 2006). First, the lectin is immobilized on a support and then glycosidic enzyme is bound to specific lectins. The enzyme and Con A have to operate under the same experimental conditions (e.g. temperature, pH buffer) and measurement solutions does not contain carbohydrates that could have a higher affinity for the lectin than the enzyme carbohydrate chain (Campas et al., 2004). Contrary to strategies based on avidin/biotin and metal ions/chelator, this method is based on a tag that is naturally present in the enzyme. Thus, no enzyme modification is necessary. The affinity of lectins for carbohydrate chains provides reversible immobilization, good steric substrate accessibility and protection of the enzyme against proteolytic digestion. Strong affinity links between Con A and the mannose residues of AChE allowed to develop amperometric biosensors for acetylthiocholine and insecticide detection (Bucur et al., 2004a, 2004b, 2005a). Detection limit for chlorpyrifos was 1×10^{-8} M. Different configurations of biosensors have been tested. For example, a sugar was covalently linked to the amino groups of the working electrode surface and a bioaffinity bridge was created between this saccharide and AChE via Con A (Bucur et al., 2005a). This immobilization process led to a sandwich structure:

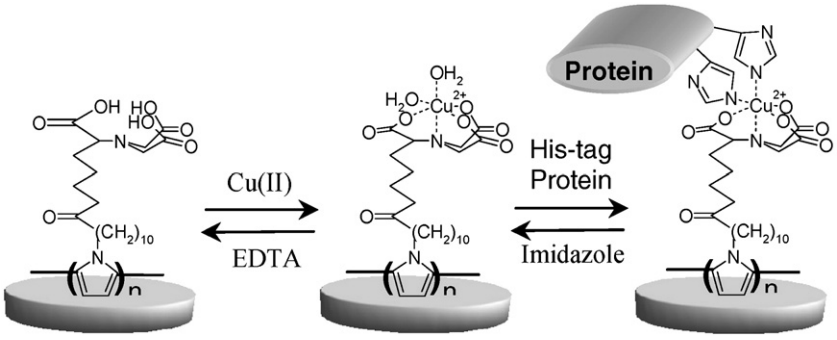

Fig. 14. Schematic representation of the reversible immobilization of histidine-tagged biomolecules to an electrogenerated poly(pyrrole)-NTA film (Haddour et al., 2005).

electrode-carbohydrate-Con A-AChE. In this case, acetylthiocholine was detected from 1×10^{-5} M to 1×10^{-4} M. Detection limit for chlorpyrifos was 1×10^{-8} M. An operational stability of more than 10 measurements was registered, while the active surface of the electrode was successfully reloaded for three consecutive times without any important change of the analytical performances.

The maximum quantity of glycoenzymes that can be immobilized using concanavalin A is limited to a monolayer. The layer-by-layer self-assembly technique allows to obtain organized molecular assemblies with alternate layers of concanavalin A and glycoenzyme. Enzymes, such as HRP (Liu et al., 2008; Yang et al., 2006), GOD (Anzai et al., 2000) or LOD (Anzai et al., 2000), have been incorporated into multilayers without any chemical modification. The response of the electrode modified by the Con A/enzyme multilayers was related to the number of assembled bilayers of Con A/enzyme (Yang et al., 2006). The increase of the number of bilayers induced an increase of the enzyme quantity immobilized on the electrode surface and thus exhibited a better sensitivity of the biosensor to the enzymatic substrate. On the other hand, thicker layers of assembled film could obstruct the diffusion of the substrate. Based on this principle, an inhibition biosensor for the detection of sulfide was described (Liu et al., 2008). The determination of this contaminant was achieved in a linear range of 1×10^{-7}–3.9×10^{-5} M with a detection limit of 5×10^{-8} M. This biosensor exhibited high sensitivity and selectivity and could successfully be applied for the detection of waste water sample, collected from a final outfall of a local power plant, a sugar refinery and a tannery industry. The biosensor retained about 82% of its original response after 60 days.

7. Conclusion

Since Clark and Lyons described the first glucose biosensor in 1962 (Clark and Lyons, 1962), tremendous progress have been achieved in the field of enzymatic biosensors. The choice of an appropriate immobilization technique is essential for fabrication of useful biosensors (Leca-Bouvier and Blum, 2010). Physical adsorption is the simplest immobilization method since it consists in contacting the enzyme solution with the surface for a defined period of time and subsequently washing off any non-adsorbed biomolecules. However, this technique suffers from biomolecule desorption from the support due to weak binding and reversible nature of the binding. Enzymes can also be immobilized by entrapment in three-dimensional matrices such as electropolymers, photopolymers or silica gels. This strategy is simple and no chemical reaction between a monomer and the enzyme can affect the activity. However, enzymes can be released from the porous matrix. Cross-linking is also a very popular immobilization technique of enzymes but this method involves the use of a multifunctional reagent such as glutaraldehyde which is toxic and causes loss of enzyme activity. Covalent binding of enzymes to a surface is another conventional immobilization method which is carried out by initial activation of support using a coupling agent, followed by enzyme binding to the activated surface. Glutaraldehyde and carbodiimide coupling are the most exploited techniques for covalent attachment of enzymes on various matrices. SAM immobilization has the advantage to allow the covalent binding and the orientation of the enzyme on the surface. Enzymes can also be immobilized via affinity bonds between a functional group of the support (e.g. avidin, lectin, metal chelates) and a specific group (e.g. biotin, carbohydrate, histidine) naturally present or genetically engineered at a specific location in the enzyme sequence which does not affect the activity or the folding of the protein. Affinity interactions allow to create highly ordered structures on biosensing supports. Several immobilization strategies can also be combined to develop enzymatic biosensors. For example, enzyme can be pre-immobilized on beads by adsorption, covalence or affinity before being entrapped in a porous polymer (Sassolas et al., 2009b; Tsafack et al., 2000a) or in a carbon paste (Fritzen-Garcia et al., 2009).

Simplicity, cost, processing time and reproducibility of the immobilization process have to be taken into consideration. The choice of the most appropriate and judicious immobilization technique allowing to design a more or less sensitive or stable biosensor also depends on the enzyme nature, the transducer and the associated detection mode. Whatever the immobilization method, enzymes have to retain their biological activity after immobilization and not to be desorbed during the operational use of the biosensor. Sensitivity (detection limit) and selectivity of biosensors are directly related to the accessibility and activity of the immobilized enzymes. Covalent linking allows a better stability to be obtained. Improved accessibility can be achieved via a linker that avoids unwanted steric hindrance. Enzymes that are randomly oriented can lose their substrate binding ability. More controlled enzyme immobilization can be performed by chemical modification of enzymes with tags such as biotin, histidine or thiol groups prior to immobilization. Optimized immobilization includes proper and uniform orientation of the enzyme and minimum enzyme modification. Higher activity is obtained with a repeatable, uniform and oriented immobilization compared to adsorption or random immobilization resulting in loss of activity.

The influence of the immobilization method of an enzyme on the resulting biosensor performances can be compared. For instance, a higher lifetime has been noticed for AChE entrapped in a PVA-SbQ matrix compared to immobilization by metal–chelate affinity (Andreescu et al., 2002b). This could be explained by the protective effect of the polymer matrix on the AChE activity. Entrapment of GOD by the sol–gel technique was also reported to give rise to a higher stability of the biosensor than cross-linking with glutaraldehyde (Barsan et al., 2007). On another hand, entrapment of AChE in PVA-SbQ gave rise to a higher detection limit for pesticide detection than immobilization by metal–chelate affinity, related to the presence of a diffusion barrier (Andreescu et al., 2002b). In the same way, entrapment of GOD by the sol–gel technique allowed a lower sensitivity to glucose to be obtained compared to glutaraldehyde cross-linking (Barsan et al., 2007).

A biosensor results from the intimate association between the sensing layer represented by the immobilized enzyme and the transducer related to a given detection method. The performances of a biosensor are obviously directly influenced, not only by the enzyme and the immobilization method, but also by the transducer and the detection mode. Considering glucose detection for instance, using GOD as the immobilized enzyme and amperometry as the detection method, analytical ranges are shown to be different according to the immobilization process involving entrapment (Table 2), adsorption (Table 3), cross-linking (Table 4), covalence (Table 6) or affinity binding (Table 7), thus pointing out the influence of the immobilization method on the biosensor performances. On the other hand, the detection method is also important. The same enzyme (GOD) immobilized with the same immobilization method (entrapment in a silica gel) gives rise to different linear ranges for glucose detection according to the detection method, such as amperometry or ECL (Table 2).

More attention should be paid to the protein structure property before selecting the way of immobilization (Ren and Yu, 2011). On the basis of the analysis of the protein surface amino acid residues, it is possible to predict to favor a given type of immobilization matrix to successfully immobilize the enzyme on it, thus taking the orientation adjustment of protein on the immobilization matrix into consideration. The strategy to select the immobilization matrix based on the analysis of protein surface structure has already proven to be successful, for instance for an oriented adsorptive immobilization of esterase BioH on a hydrophilic-modified support (Ren and Yu, 2011).

From a practical point of view, interferences by sample constituents are an important problem influencing the performances (Jia et al., 2010). Although enzymes are generally very specific and selective,

electrochemical detection can be hardly influenced by electroactive species (e.g. uric acid or ascorbic acid) present in biological samples. Various methods have been reported to improve the selectivity of enzyme-based biosensors. The choice of immobilization method can minimize the effect of electroactive species. For example, electropolymerized films are not only used as immobilization matrices but also as barriers to eliminate interferences. For example, o-phenylenediamine was used for GOD immobilization on Pt electrodes by in situ one-step electrochemical polymerization (Malitesta et al., 1990). This permselective membrane also allowed to block interfering species, improving the selectivity of the biosensor.

Nanomaterials including nanoparticles, nanowires and nanotubes have recently attracted considerable attention for the design of electrochemical (Agui et al., 2008; Ahammad et al., 2009; Hu and Hu, 2009; Liu et al., 2005; Luo et al., 2006; Rivas et al., 2007; Wang, 2005b) and optical (for instance, based on electrochemiluminescence) (Bertoncello and Forster, 2009; Hazelton et al., 2008; Qi et al., 2009) enzyme biosensors due to their unique physical and chemical properties. Nanomaterials prepared from metals, carbon or polymeric species allow to develop new biosensors that exhibit high sensitivity and stability. Their high conductivity properties have been reported to enhance the electron transfer between the enzyme redox center and the electrode surface. These compounds are also known to present an advantageous high surface area for loading enzymes. Various designs of nanomaterial-based enzymatic biosensors have been reported. Enzymes can be immobilized on functionalized nanomaterials by adsorption (Wang et al., 2009a) or covalence (Xue et al., 2003). In other cases, enzymes and nanomaterials can be co-entrapped in a polymer (Wang and Musameh, 2005) or in carbon paste electrodes (Jia et al., 2008).

Magnetic particles have also been used as bioimmobilization platforms, magnetic carriers of biomolecules. Biofunctionalized magnetic nanoparticles can be obtained by traditional enzyme immobilization methods such as covalent binding, adsorption, specific affinity interactions or entrapment in porous surface layers (Stanciu et al., 2009 and references therein). Advantages in terms of high enzyme loading, easy manipulation and control of the bioassembly to a specific location on the transducer surface, possibilities of regeneration and reuse can be underlined. The control of charging/discharging of the transducer surface by application of a magnetic field provides reusability of the same electrode for several analyses.

In spite of the numerous immobilization methods existing, it is interesting to find innovative immobilization techniques that can be exploited to develop new enzymatic biosensors.

It can be noted that the immobilization methods described in this review are generic and can be adapted not only to enzymes but also to other biomolecules such as nucleic acids (Sassolas et al., 2008) or antibodies (Jung et al., 2008).

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