0273-2289/00/89/0127/$13.00

Biosensors Based on Immobilization of Biomolecules by Electrogenerated Polymer Films

New Perspectives

Serge Cosnier

Laboratoire d'Electrochimie Organique et de Photochimie Rédox, UMR CNRS 5630, Université Joseph Fourier Grenoble 1, BP 53, 38041 Grenoble, Cedex 9, France, E-mail Serge.Cosnier@ujf-grenoble.fr

Abstract

The concept and potentialities of electrochemical procedures of biomolecule immobilization are described. The entrapment of biomolecules within electropolymerized films consists of the application of an appropriate potential to an electrode soaked in an aqueous solution containing monomer and biomolecules. This method of biosensor construction is compared with a two-step procedure based on the adsorption of an aqueous amphiphilic pyrrole monomer-biomolecule mixture on an electrode followed by the electropolymerization of the adsorbed monomers. Another approach is based on the electrogeneration of polymer films functionalized by specific groups allowing subsequently the attachment of biomolecules. The immobilization of biomolecules on these films by covalent binding or noncovalent interactions is described.

Index Entries: Avidin; biosensor; biotin; enzyme; functionalized polymer; polypyrrole.

Introduction

The ingenious concept of combining the recognition properties of macromolecular biological molecules to the sensitivity of electrochemical devices has led to the emergence of biosensors as valuable analytical tools for the monitoring of target analytes in different technological areas. For three decades, biosensors have been the subject of increasing research effort and now constitute a major component of mainstream analytical chemistry *(1,2)*.

*Author to whom all correspondence and reprint requests should be addressed.

In recent years, there has been a growing interest in the development of microfabricated electrochemical biosensors. However, the stable and reproducible immobilization of macromolecular biomolecules such as enzyme, oligonucleotide, or antibody on conductive microsurfaces with complete retention of their recognition properties remains a crucial problem for the commercial development of miniaturized biosensors. Effectively, most of the conventional procedures of biomolecule immobilization, such as crosslinking, covalent binding, and entrapment in gels or membranes, suffer from low reproducibility and poor spatially controlled deposition.

Apart from these conventional methods, the immobilization of biomolecules in or on electropolymerized films is gaining importance *(3–5)*. One major advantage of electrochemical deposition procedures is the possibility of precisely electrogenerating a polymer coating over conductive microsurfaces of a complex geometry.

The electrochemical method involves the entrapment of biomolecules in organic polymers during their electrogeneration on an electrode surface. The polymer formation is carried out by controlled potential electrolysis of an aqueous solution containing monomers and biomolecules. Another electrochemical method involves, initially, the electropolymerization of functionalized conducting polymers. Then the attachment of biomolecules to the polymer surfaces can be obtained by chemical grafting or by affinity of the biomolecules at the functional group *(6–8)*. In comparison to the physical entrapment of biomolecules within polymer films such as polypyrrole, polythiophene, polyacetylene, or polyaniline, this approach allows better access of substrate to the immobilized biomolecules and facilitates macromolecular interactions.

In this article, I review the principles and advantages of these electrochemical methods of biomolecular immobilization for the construction of biosensors.

Electrochemical Entrapment of Biomolecules in Organic Polymers

Electrochemical entrapment of biomolecules in organic polymers involves the application of an appropriate potential to the working electrode soaked in aqueous solution containing both biomolecule and electropolymerizable monomer. Biomolecules present in the immediate vicinity of the electrode surface are thus incorporated into the growing polymer. In addition, this entrapment occurs without chemical reaction, which could affect the activity of the entrapped biomolecules. The advantage of the electrochemical polymerization is that films can be prepared easily in a rapid one-step procedure. Furthermore, this method enables exact control of the thickness of the polymer layer based on the measurement of the electrical charge passed during the electrochemical polymerization. Most of the electrochemically deposited polymers used for the immobilization of

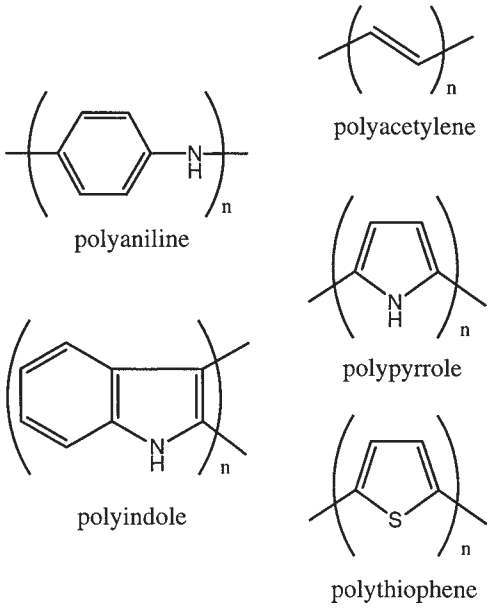

Fig. 1. Conducting polymer films used for the entrapment of biological macromolecules.

biomolecules are conducting polymers since their formation (contrary to a nonconducting polymer) is not restricted to the elaboration of very thin films. Among these conducting polymers (e.g., polyacetylene, polythiophene, and polyaniline), polypyrrole and its derivatives play the leading role owing to their versatile applicability (Fig. 1).

Among the different biomolecules, the entrapment within polypyrrole matrices has been mainly focused on enzyme molecules, particularly oxidases. Nevertheless, few examples have been devoted to the immobilization of coenzymes, antibodies, cells, and biological tissue, illustrating the wide potentialities of this method for retention of biomolecules *(9–11)*. The most widely held opinion is that the immobilization of biomolecules in electrogenerated polymers is partly owing to electrostatic interactions between negatively or positively charged proteins and polymer films, the proteins being incorporated within the polymer as counterions. However, most often the electropolymerization process requires very high concentrations of a supporting electrolyte or buffer. As a result, the incorporation of protein molecules in the growing polymer film is more likely owing to the presence of protein molecules in the immediate vicinity of the electrode surface and does not result from specific electrostatic interactions.

A slightly different approach for the integration of biomolecules into polymer films is based on an amphiphilic pyrrole ammonium **1** (Fig. 2). Contrary to the preceding electropolymerizable monomers, the polymerization of this surfactant can be achieved in aqueous solutions without a supporting electrolyte. This promotes the incorporation of negatively charged biomolecules during the polymer formation. The capacity of the

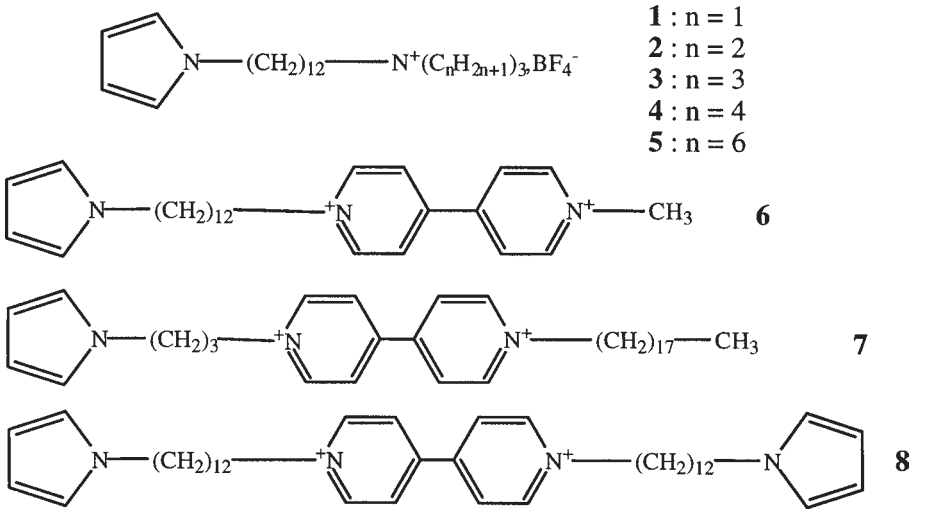

Fig. 2. Structure of the amphiphilic pyrrole derivatives **(1–8)**.

surfactant **1** to be used in microbiosensor fabrication has been investigated for the immobilization of glutamate oxidase and polyphenol oxidase (PPO) on platinum and carbon microelectrodes (diameter 8–30 μm) *(12,13)*. The resulting modified microelectrodes reveal an excellent sensitivity for glutamate (32 mA/[*M*·cm^2]) and dopamine (59 mA/[*M*·cm^2]), respectively. Note that the poly **1**-PPO microelectrode provides an attractive detection limit for dopamine, namely 50 n*M*, which is the lowest concentration value that can be detected using microbiosensors based on PPO. Moreover, preliminary in vivo experiments have demonstrated the mechanical stability of the poly(amphiphilic pyrrole) films toward the penetration of brain tissue *(12)*.

However, the immobilization of biomolecules by electrochemical entrapment in conducting or nonconducting polymer films requires high concentrations of monomer and biomolecules during the electropolymerization process. Consequently, the accurate amount of biomolecules immobilized within the polymeric network cannot be estimated by the simple difference between the biological concentrations before and after the electropolymerization step. This special feature constitutes a handicap for the kinetic characterization and the optimization of multienzyme electrodes.

An original strategy to overcome these drawbacks involved the immobilization of monomer and enzyme together, by adsorption, on the electrode surface before the electropolymerization step. Taking advantage of the adsorption and electropolymerization properties of amphiphilic monomers (Fig. 2), an original two-step procedure of biosensor construction has been developed *(5,14)*. This strategy of enzyme immobilization is based first on the solubilization of biomolecules in an aqueous dispersion of amphiphilic pyrrole. Then the aqueous mixture is spread and dried on

Table 1
Properties of Biomolecule Retention
of Poly(amphiphilic pyrrole) Host Matrices *(15–19)*

Enzyme	Molecular weight (kDa)	Entrapped enzyme[a] in polymeric film (%)	p*I*
Flavin reductase	28.5	90	4.9
Horseradish peroxidase	40	60	7.2
Galactose oxidase	68	87	12.0
Choline oxidase	72	85	4.5
Polyphenol oxidase	128	70	4.7
Glucose oxidase	160	24	4.3
Nitrate reductase	200	48–72	4.2
Xanthine oxidase	275	8	—

[a]Defined as the ratio between the amount of entrapped enzyme over that of deposited enzyme. The amount of entrapped enzyme was determined from the difference between the enzyme quantity deposited on the electrode surface and the enzyme amount lost during the electropolymerization step.

an electrode surface. The electropolymerization of the adsorbed monomers in an aqueous electrolyte provides the irreversible entrapment of biomolecules in the resulting polypyrrolic matrix.

The main advantage of this procedure of enzyme immobilization is the possibility of controlling and improving the composition of the biomolecule-polymer layer, particularly the loading of biomolecules. In contrast with the classic electrochemical method of entrapment of biomolecules, the polymer is formed from an adsorbed layer of monomer and biomolecules in an aqueous electrolyte free of biomolecules and monomer. Consequently, the loss of biomolecules in the aqueous electrolyte during the polymer formation can be determined by measuring the biological activity or the protein content of the electrolyte medium. Therefore, the true quantity of biomolecules entrapped in the polymer can be determined by the simple difference between the initially deposited amount and the amount lost in the electrolyte *(15–17)*.

The efficiency of enzyme entrapment by different amphiphilic pyrrole monomers was investigated with enzymes of molecular weight from 28.5 to 275 kDa *(15–19)*; Table 1 summarizes the results. It appears that these host polymers efficiently entrap enzymes of low molecular weight whatever their isoelectric point (p*I*). Contrary to the main classic monomers such as pyrrole, aniline, phenol, or diaminobenzene, all amphiphilic pyrroles are positively charged. Consequently, they can display electrostatic interactions with the negatively charged residues of the protein shell. This electrochemical procedure of biomolecular immobilization is widely applicable to biomolecules whatever their p*I*. However, their retention properties decrease drastically for enzyme molecules above 275 kDa.

Because the oxidative polymerization of amphiphilic pyrrole leads to an electroactive skeleton, the deposition and electropolymerization of

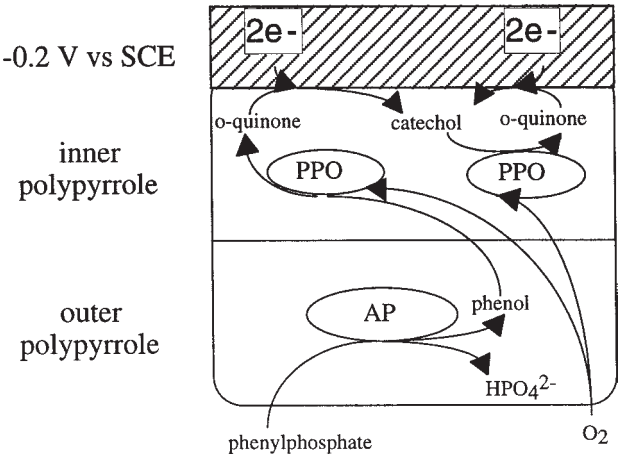

Fig. 3. Schematic of the functioning principle of an AP-PPO electrode.

additional amphiphilic monomer-biomolecule mixtures can be easily performed. This property allows the elaboration of multienzyme structures exhibiting a heterogeneous enzyme location. For instance, the sequential adsorption and electropolymerization of monomer **2** with PPO as inner layer and monomer **2** with alkaline phosphatase (AP) as outer layer provides a bienzyme electrode for the amplified detection of phenylphosphate *(20)*. The functioning principle of the resulting biosensor is based on the enzymatic hydrolysis of the AP substrate (phenylphosphate) followed by the enzymatic oxidation of phenol into *o*-quinone, which is then detected via its reduction at –0.2 V vs saturated calomel electrode (SCE) at the electrode surface (Fig. 3). Because the amperometric detection generates by reduction catechol, another PPO substrate, the latter can undergo successive cycles of enzymatic oxidation-electrochemical reduction. This induces an amplification of the biosensor response by a factor of 9.7.

The coimmobilization of organic or inorganic additives and protein in poly (amphiphilic pyrroles) films constitutes a convenient strategy to optimize either the biocompatibility of the host polymers or the amperometric transduction step. Thus, the anion-exchange properties of the poly (pyrrole-ammonium) films also can be used to incorporate negatively charged redox mediators able to establish an electrical communication between the electrode surface and immobilized enzymes. For instance, a bienzyme sensor (poly **2**-glucose oxidase [GOD]-peroxidase-$Fe[CN]_6^{4-}$ electrode) involving a wired peroxidase has been prepared for the interference-free determination of glucose *(21)*.

Another approach is to simultaneously entrap additives and biomolecules in poly (amphiphilic pyrroles) films. It has been previously observed that the electrochemical entrapment of enzymes in poly (amphiphilic pyrroles) films always induces a strong decrease in their specific activity, which could be owing to the hydrophobic character of these host

organic matrices. A possible way to preserve the activity of entrapped enzymes could be to modify the enzyme microenvironment by incorporating hydrophilic additives within the polypyrrole host matrices. For this purpose, hydrophilic laponite clay nanoparticles have been entrapped in various poly **2**-enzymes films. The coimmobilization of laponite particles with either cholesterol oxidase or cholesterol oxidase and cholesterol esterase has been accomplished, providing a marked improvement in the analytical performance of both biosensors for the amperometric detection of free and total cholesterol.

Attachment of Biomolecules to Electrogenerated Organic Polymers

The physical entrapment within electrogenerated polymers drastically reduces the accessibility to the immobilized biomolecules. The steric constraints generated by the surrounding polymer, in particular, may hinder the formation of specific antigen-antibody binding or the hybridization of complementary oligonucleotides. An attractive alternative to the entrapment of biomolecules involves the covalent binding of the biomolecules to polymer films bearing adequate functional groups. The main advantage of this sequential procedure—electropolymerization and covalent binding—lies in the possibility of using optimal conditions for each step. In particular, the initial formation of polymer films can be performed under electrolysis conditions, which are deleterious for biomolecules.

The elaboration of functionalized polymer films has been investigated either by direct electropolymerization of functionalized monomers or by chemical or electrochemical postfunctionalization of deposited conducting polymer films. For example, the chemical nitration of a polypyrrole film followed by the electrochemical reduction of the *in situ*–generated nitro groups provides a polymer film functionalized by amino groups *(6)*. A more investigated approach involves the electropolymerization of pyrroles, azulenes, thiophenes, dithienylpyrroles, and dithienylbenzene derivatives functionalized by amino or carboxylic groups. The immobilization of biomolecules onto these polymers was later performed by chemical grafting *(22,23)*. More recently, the electrochemical polymerization of thiophene and pyrrole derivatives functionalized by easy leaving groups (*N*-hydroxysuccinimide and *N*-hydroxyphtalimide esters, respectively) has led to attractive precursor polymers *(7,24)*. The latter have been applied to the immobilization of GOD and oligonucleotide and used as an amperometric biosensor for glucose and complementary nucleotide, respectively.

A more promising strategy of attachment of biomolecules that preserves their biological activity is the immobilization onto polymers by affinity instead of covalent binding. The attachment of biomolecules to sensor surfaces can be achieved via the simple formation of an avidin-biotin bridge with biotinylated biomolecules or avidin-conjugated biomolecules. The protein avidin will bind four biotins, a vitamin, by a

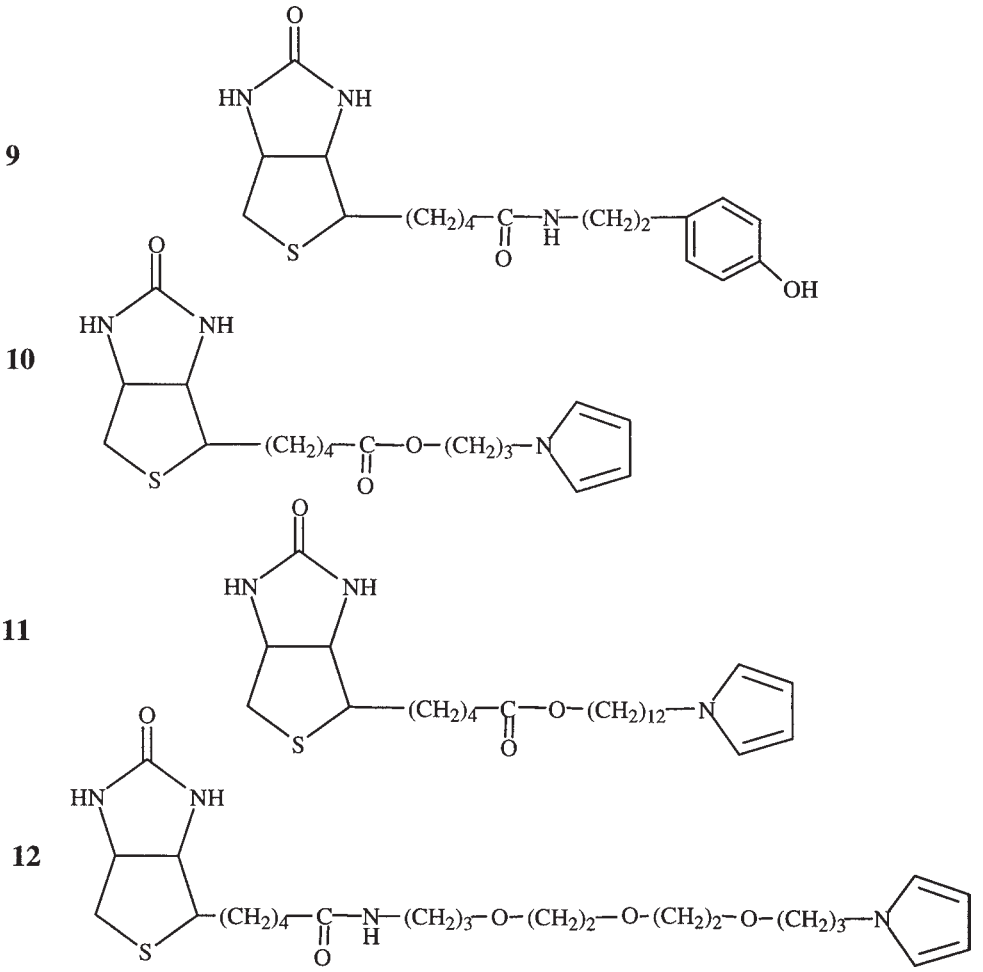

Fig. 4. Structures of biotin derivatives substituted by an electropolymerizable phenol or pyrrole group.

noncovalent interaction that is quasi-irreversible. Because of the high affinity of avidin for biotin (association constant $K_a = 10^{15}\ M^{-1}$), this coupling system has been extensively used for the immobilization of biomolecules *(25,26)*. In this context, an innovative electrochemical method of immobilization of biomolecules consists of the electrogeneration of biotinylated polymer films. Recently, the formation of biotinylated surfaces was attempted by electropolymerization of a biotin derivative (**9**) functionalized by a phenol group (Fig. 4) *(27)*.

Unfortunately, electropolymerization provides nonconductive polymeric films that passivate the electrode and prevent further film growth. Nevertheless, the efficient coupling of an avidin-conjugated β-galactosidase to the biotinylated film has shown that this kind of polymer film allows biotin sites to develop specific interactions with avidin. An alterna-

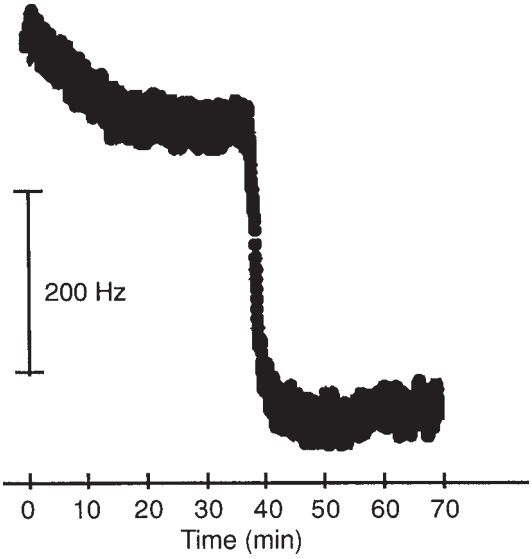

Fig. 5. Microbalance response of a 27-MHz gold-coated quartz crystal modified by a biotinylated polypyrrole film (poly **11**) to an avidin solution (0.5 mg/mL).

tive consists of the electrogeneration of conductive biotinylated polypyrrole films. The first example of a biosensor based on poly (pyrrole-biotin) films has been prepared by oxidative polymerization of the biotin derivative **10** (Fig. 4) *(8,28)*. The efficient fixation of avidin onto a biotinylated polypyrrole film was investigated by quartz microbalance experiments (Fig. 5). The 27-MHz quartz crystal microbalance (QCM) was modified by the electrogeneration of a poly **11** film onto one side of the Au electrode (surface area = 0.2 cm^2) of the QCM. In the presence of an aqueous solution of avidin (0.5 mg/mL), a rapid variation in frequency was clearly recorded for the modified QCM. This corresponded to an increase in mass of 540 ng/cm^2. Taking into account that the theoretical maximum coverage of an avidin monolayer was estimated as 5.48×10^{-12} mol/cm^2 *(29)*, the corresponding increase in mass is 373 ng/cm^2. Therefore, the poly **11** film can bind efficiently 1.4 avidin monolayer.

Owing to the remarkable high affinity of avidin for biotin coupled to proteins or nucleic acids *(30)*, the attachment of biotinylated enzymes on biotinylated polypyrrole films has been investigated. For this purpose, a biotinylated GOD was chosen as the enzyme model. The successive immersion of the biotinylated polypyrrole electrode into an avidin solution and then into a biotinylated GOD solution led to the formation of an enzyme monolayer via the avidin-biotin bridge. Following this easy and fast procedure, several enzyme layers can be fixed on the polymer surface. Because the biotinylated GOD catalyzes the oxidation of glucose with the production of H_2O_2, glucose can be determined through the electrochemical oxidation of H_2O_2 at the surface of the platinum electrode. Figure 6 shows the resulting calibration curve for glucose obtained with biosensors based on one,

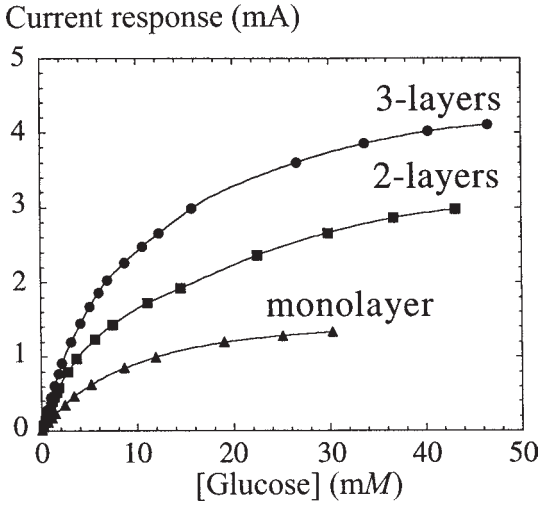

Fig. 6. Calibration curves for glucose obtained with biotinylated polypyrrole platinum electrodes modified by a monolayer, two layers, and three layers of biotinylated GOD. Applied potential: 0.6 V (vs SCE); air saturated: 0.1 *M* phosphate buffer (pH 7.0) kept under stirring.

two, and three enzyme layers. It appears clearly that the biosensor sensitivity for glucose increases approximatively with the number of enzyme layer, namely, 0.79, 1.65, and 2.16 mA/(M·cm^2) for monolayer, two layers, and three layers, respectively. Moreover, the response time of the biosensor (approx 10 s) remains identical for the three configurations. This demonstrates the successive elaboration of reproducible enzyme layers *(8)*.

The storage stability of the biosensor based on an enzyme monolayer stored dry at 4°C was evaluated by checking periodically its amperometric sensitivity to glucose. It appears that the biosensor retained 85 and 50% of its initial glucose sensitivity after 3 and 10 d, respectively. The synthesis of a biotin linked by a long hydrophilic arm to an electropolymerizable pyrrole group (**12**) has been recently reported (Fig. 4) *(31)*. The improvement in monomer solubility in water allows the electropolymerization of this biotin derivative in aqueous solutions. In addition, the hydrophilic arm may facilitate accessibility to the polymerized biotin groups. The efficient immobilization of streptavidin-conjugated R phycoerythrin on biotinylated films obtained by copolymerization of pyrrole and **12** was demonstrated by fluorescence measurements. Note that the intensity of the fluorescence can be related to the amount of biotin derivative involved in the copolymerization process.

Conclusion

Among the numerous procedures of biosensor construction, the entrapment of biomolecules within electropolymerized films constitutes a simple and efficient method that does not require additional chemicals.

Moreover, the electropolymerization of preadsorbed amphiphilic pyrrole-protein coatings offers specific advantages such as control of the real amount of entrapped biomolecules. Furthermore, the stability and permeability of electrogenerated polymer films in organic solvents are attractive properties for the elaboration of organic-phase biosensors.

In addition, the use of functionalized polymers bearing easy leaving groups or affinity sites for the chemical grafting or the noncovalent binding of biomolecules has opened interesting perspectives in the field of biosensor construction.

Acknowledgment

I am grateful to Dr. Alain Deronzier for continuous support of my work in the field of amperometric biosensors.

References

1. Turner, A. P. F., Karube, I., and Wilson, G. S. (1987), *Biosensors: Fundamentals and Applications*, Oxford University Press, New York.
2. Kauffmann, J. M. and Guilbault, G. G. (1992), *Bioanalytical Applications of Enzymes*, vol. 36, Suelt, C. H., ed., John Wiley & Sons, New York, pp. 63–113.
3. Bartlett, P. N. and Cooper, J. M. (1993), *J. Electroanal. Chem.* **363,** 1–12.
4. Schuhmann, W. (1995), *Mikrochim. Acta* **121,** 1–29.
5. Cosnier, S. (1997), *Electroanalysis* **9,** 894–902.
6. Schuhmann, W., Lammert, R., Uhe, B., and Schmidt, H.-L. (1990), *Sensors Actuators B* **1,** 537–541.
7. Korri-Youssoufi, H., Garnier, F., Srivasta, P., Godillot, P., and Yassar, A. (1997), *J. Am. Chem. Soc.* **119,** 7388, 7389.
8. Cosnier, S., Galland, B., Gondran, C., and Le Pellec, A. (1998), *Electroanalysis* **10,** 808–813.
9. Deshpande, M. V. and Hall, E. A. H. (1990), *Biosens. Bioelectron.* **5,** 431–448.
10. John, R., Spencer, M., Wallace, G. G., and Smyth, M. R. (1991), *Anal. Chim. Acta* **24,** 381–385.
11. Uchiyama, S., Hasebe, Y., and Tanaka, M. (1997), *Electroanalysis* **9,** 176–178.
12. Cosnier, S., Innocent, C., Allien, L., Poitry, S., and Tsacopoulos, M. (1997), *Anal. Chem.* **69,** 968–971.
13. Poitry, S., Poitry-Yamata, C., Innocent, C., Cosnier, S., and Tsacopoulos, M. (1997), *Electrochim. Acta* **42,** 3217–3223.
14. Cosnier, S. and Innocent, C. (1992), *J. Electroanal. Chem.* **328,** 361–366.
15. Cosnier, S. and Innocent, C. (1993), *Bioelectrochem. Bioenerg.* **31,** 147–160.
16. Coche-Guérente, L., Cosnier, S., Innocent, C., and Mailley, P. (1995), *Anal. Chim. Acta* **311,** 23–30.
17. Cosnier, S., Fontecave, M., Limosin, D., and Nivière, V. (1997), *Anal. Chem.* **69,** 3095–3099.
18. Cosnier, S., Galland, B., and Innocent, C. (1997), *J. Electroanal. Chem.* **433,** 113–119.
19. Leca, B., Morelis, R. M., and Coulet, P. R. (1994), *Talanta* **41,** 925–930.
20. Cosnier, S., Gondran, C., Watelet, J. C., De Giovani, W., Furriel, R. P. M., and Leone, F. A. (1998), *Anal. Chem.* **70,** 3952–3956.
21. Pospecu, I., Cosnier, S., and Labbé, P. (1997), *Electroanalysis* **9,** 998–1004.
22. Schuhmann, W., Huber, J., Mirlach, A., and Daub, J. (1993), *Adv. Mater* **5,** 124–126.
23. Röckel, H., Huber, J., Gleiter, R., and Schuhmann, W. (1994), *Adv. Mater* **6,** 567–571.
24. Hiller, M., Kranz, C., Huber, J., Bäuerle, P., and Schuhmann, W. (1996), *Adv. Mater* **8,** 219–222.

25. Pantano, P. and Kuhr, W. G. (1993), *Anal. Chem.* **65,** 623–630.
26. Hoshi, T., Anzai, J., and Osa, T. (1995), *Anal. Chem.* **67,** 770–774.
27. Yang, S. T., Witkowski, A., Hutchins, R. S., Scott, D. L., and Bachas, L. G. (1998), *Electroanalysis* **10,** 58–60.
28. Cosnier, S. and Le Pellec, A. (1999), *Electrochim. Acta* **44,** 1833–1836.
29. Frey, B. L., Jordan, C. E., Kornguth, S., and Corn, R. M. (1995), *Anal. Chem.* **67,** 4452–4457.
30. Wilchek, M. and Bayer, E. A. (1988), *Anal. Biochem.* **171,** 1–32.
31. Torres-Rodriguez, L. M., Roget, A., Billon, M., Livache, T., and Bidan, G. (1998), *J. Chem. Soc. Chem. Commun.* 1993, 1994.