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Electropolymerizable Amphiphiles: Convenient Tool for Electrical Wiring of Enzymes and Miniaturization of Biosensors

Serge Cosnier, Laurence Allien, Liliane Coche-Guérente, Christophe Innocent, Pierre Labbé and Pascal Mailley

Laboratoire d'Electrochimie Organique et de Photochimie Rédox, URA CNRS 1210, Université Joseph Fourier, Grenoble 1, BP 53, 38041 Grenoble Cedex 9, France

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A novel biosensor fabrication based on the electropolymerization of amphiphilic pyrrole monomer-enzyme mixtures previously adsorbed on an electrode is described. This simple and rapid procedure provides fast-response and sensitive amperometric sensors for galactosides, phenols, glucose and several neurotransmitters. The possible applications of this method for multienzymatic sensor and microbiosensor elaboration as well as for electrical connection of immobilized enzymes are illustrated.

1. Introduction

Owing to some advantages over other methods, including high selectivity and simple use, biosensors are used in many applications both in synthetic and analytical chemistry. Recently, we reported a novel approach for the fabrication of amperometric biosensors without membranes which provides inexpensive, mechanically robust, sensitive and fast-response bioelectrodes.[1] This biosensor fabrication is based on the electropolymerization of pyrrole amphiphilic monomer-enzyme mixtures previously adsorbed on an electrode surface (Fig. 1). The entrapment of enzymes in polymer films such as polypyrrole by electrochemical polymerization of a solution containing monomers and enzymes suffers from severe limitations. Indeed, not only a large amount of monomers but also of enzymes

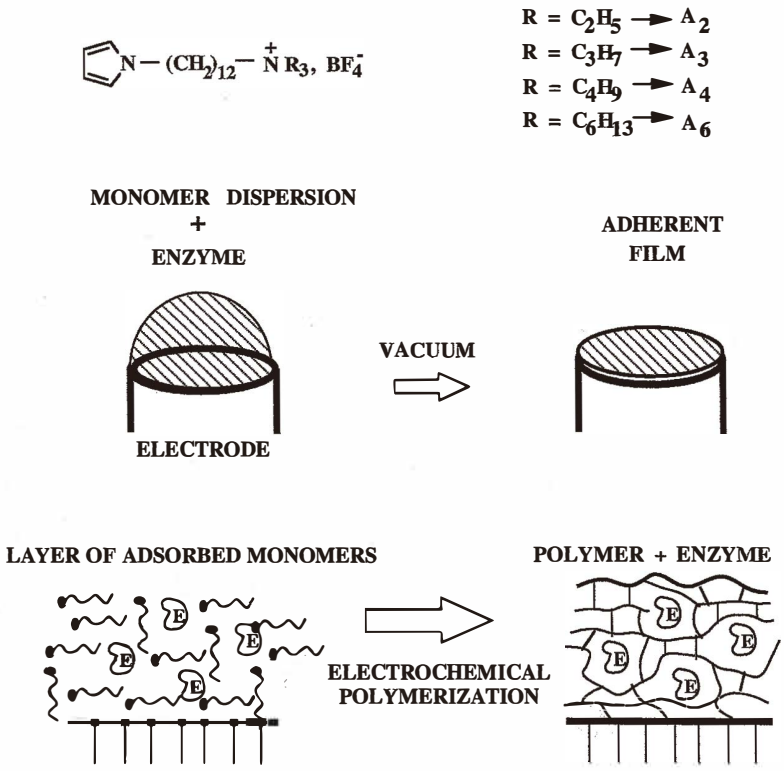

Fig. 1. Structure of the amphiphilic pyrrole monomers and principle of the biosensor fabrication.

must be used. Moreover, the quantity and actual activity of the enzyme embedded in the polymer cannot be easily determined without enzyme denaturation. The significant advantages of our method over other conventional electrochemical enzyme entrapments are mainly the controllability of the biopolymer composition and a better solute permeation through the polymeric matrices.[2]

Here we report the characteristics of this kind of biosensor exemplified with a polyphenol oxidase (tyrosinase), as well as the potential applications of this method of enzyme immobilization.

2. Materials and Methods

2.1 *Reagents*

Tyrosinase (EC 1.14.18.1., from mushrooom, 3870 U mg^{-1}), glucose oxidase (EC 1.1.3.4, from *Aspergillus niger,* 150 U mg^{-1}), ascorbate oxidase (EC 1.10.3.3, from *Cucurbita* species, 265 U mg^{-1}) and galactose oxidase (EC 1.1.3.9, from *Dactylium*

dendroides, 165 U mg^{-1}) were purchased from Sigma. Glutamate oxidase (EC 1.4.3.11, from *Streptomyces* species)[3] was kindly donated by Dr. Hitoshi Kusakabe of Yamasa Shoyu Ltd., Japan. The synthesis of amphiphilic pyrrole monomers has been previously described.[4] (Ferrocenylmethyl)dimethyl pyrrolyidodecylammonium tetrafluoroborate was obtained by refluxing in tetrahydrofuran (ferrocenylmethyl)dimethylamine with an excess of 1-iodo-12-pyrrolyidodecane. All chemicals were obtained commercially and were of the highest purity available.

2.2 *Measurements and apparatus*

All electrochemical studies were performed with a conventional three-electrode potentiostatic system. The electrochemical equipment has been described elsewhere.[5] The working electrode was a platinum or glassy carbon disk (5 mm diameter) polished with 1 μm diamond paste. The microelectrode was a platinum disk (50 μm diameter) fabricated by Professor M. Tsacopoulos and Mr. J.-L. Munoz (Experimental Ophthalmology Laboratory). Potentials are reported versus an SCE reference electrode. All measurements were carried out in the phosphate buffer solutions saturated with oxygen by stirring. Glucose, glutamate and galactosides were monitored at 0.5 V by electrochemical oxidation of the liberated hydrogen peroxide. The amperometric detection of phenols and catechols was performed at – 0.2 V by the electrochemical reduction of enzymatically generated *o*-quinones.

2.3 *Enzyme immobilization*

The coating of Pt and C disk electrodes was achieved by direct application on the electrode surface, by means of a syringe, a known amount of an aqueous solution containing amphiphilic pyrrole and enzyme. Water was evaporated under vacuum. Then the resulting modified electrode was transferred to an aqueous 0.1 M $LiClO_4$ solution, and polymerization of the adsorbed amphiphilic pyrroles was carried out by controlled potential electrolysis for 20 min at 0.75 V.

3. Results and Discussion

Our method of biosensor fabrication has been employed for the immobilization of a polyphenol oxidase (tyrosinase) as an enzyme model to illustrate the enzymatic and electrochemical characteristics of this kind of biosensor (Fig. 2). The characteristics (amount of immobilized enzyme, response time and sensitivity) of this biosensor and the effect of enzyme loading on the biosensor response as well as on its kinetic parameters have also been studied and it was found that the enzymatic reaction is the rate limiting step in this system owing to the good permeability of the polymeric films.[6] The sensitivity of the enzyme electrode varies over the range of 1–300 mA M^{-1}, depending on the substrate, illustrating the selectivity of the biosensor afforded by enzyme catalysis (Table 1). Furthermore, this biosensor provides very low limits of detection with very fast response times regardless of the tested substrate. In particular, with dopamine, whose determination is of biological importance, the detection limit is 5×10^{-8} M. This value is markedly lower than

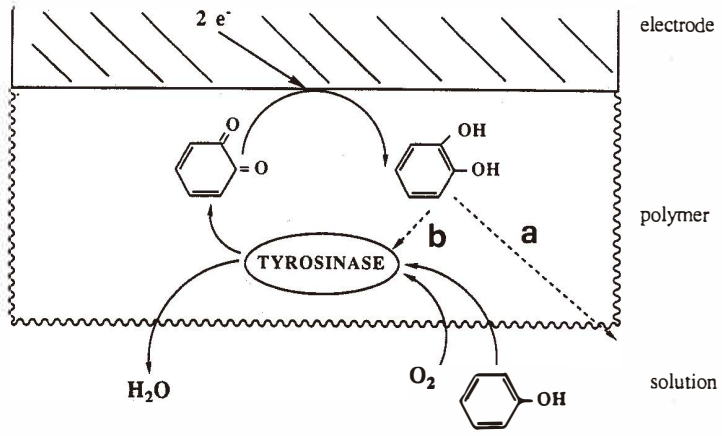

Fig. 2. Description of the electroenzymatic cycle for the detection of phenol as the substrate: (a) diffusion to the solution; (b) enzymatic reoxidation. Applied potential: – 0.2 V; air-saturated 0.1 M phosphate buffer (pH 6.5).

Table 1
Bioanalytical performance of the poly-A_2-tyrosinase electrode using various substrates.

Substrate	Sensitivity (mA M^{-1})	Detection limit (M)
Catechol	300	2×10^{-9}
Phenol	70	5×10^{-9}
p-cresol	50	5×10^{-9}
Dopamine	11	5×10^{-8}
Noradrenaline	7	5×10^{-7}
L DOPA	2	8×10^{-7}
L-tyrosine	1	8×10^{-7}

those normally reported ($\geq 10^{-6}$ M) for the tyrosinase-based sensors previously described in the literature.[7,8]

One of the advantages of this procedure of biosensor fabrication is the possibility of controlling the enzyme location in the polymeric matrices. For example, the effect of an additional polymer coating on electrode performance has been evaluated in the case of bioelectrodes containing galactose oxidase (GAO). The poly-A_2-GAO electrode, prepared as described above presents relative rates of oxidation of several galactosides which are similar to those reported for free galactose oxidase (Table 2). A poly-A_2/poly-A_2-GAO electrode is realized by additional adsorption and electropolymerization of A_2 on a poly-

Table 2
Substrate specificity of free GAO and of GAO-based biosensors.

Galactoside (molecular weight)	Raffinose (504)	Galactose (180)	Lactose (342)	Maltose (342)
Enzyme system				
Free GAO	100	83–70	8–4	4–1
Poly-A_2-GAO Electrode "monolayer"	100	75	7	0.5
Poly-A_2/poly A_2-GAO Electrode "multilayer"	100	150	134	20

A_2-GAO film. The substrate specificity of such a "multilayer" biosensor differs markedly from the substrate specificity of the preceding "monolayer" biosensor (Table 2), showing a modulation of the biosensor selectivity due to the steric hindrances afforded by the outer polymeric layer.[9]

Moreover, the sequential adsorption and electropolymerization of different enzyme-monomer mixtures leads to the fabrication of multi-enzymatic materials with ordered enzyme location. Thus sensors containing two enzymes for glucose determination in the presence of interfering agents have been fabricated. Immobilization of ascorbate oxidase (AO) or tyrosinase in the inner layer and glucose oxidase in the outer layer prevented interference due to the direct electrochemical oxidation of ascorbate or acetaminophen on a platinum electrode at +500 mV. Owing to the enzymatic oxydation of ascorbate and acetaminophen by the immobilized AO and tyrosinase these electrochemical interference effects are drastically reduced.

$$\text{4-acetamidophenol} + O_2 \xrightarrow{\text{Tyrosinase}} \text{4-acetamido-1,2 benzoquinone} + H_2O$$

$$\text{ascorbate} + O_2 \xrightarrow{\text{AO}} \text{dehydroascorbate} + H_2O$$

Figure 3 shows how ascorbate interferes with the measurement of glucose. As demonstrated by the calibration curve obtained with the poly-A_2-GOD/poly-A_2-AO electrode, the interference effects can be drastically reduced by appropriate adjustment of the amount and location of ascorbate oxidase in the biocatalyst layer without decreasing the biosensor sensitivity to glucose.

One of the most noteworthy advantages of this electrochemical procedure of enzyme immobilization is the possibility of functionalizing microelectrodes. In particular, we have fabricated an implantable microbiosensor for L-glutamate with the aim of detecting *in vitro* and *in vivo* extracellular levels of this neurotransmitter. This sensor includes a platinum disk (diameter 50 μm) modified by oxidative electropolymerization of a previously adsorbed amphiphilic pyrrole-glutamate oxidase mixture. The functioning principle is based on the amperometric detection of enzymatically generated H_2O_2 in the presence of

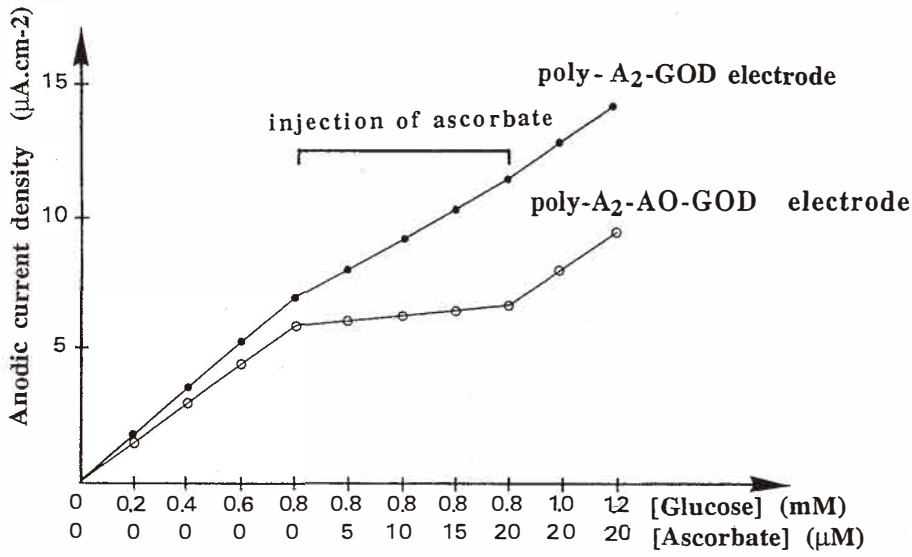

Fig. 3. Estimation of the bias introduced by ascorbate on the measurement of glucose by biosensors containing 0.2 mg of glucose oxidase. Applied potential: 0.5 V; air-saturated 0.1 M phosphate buffer (pH 7).

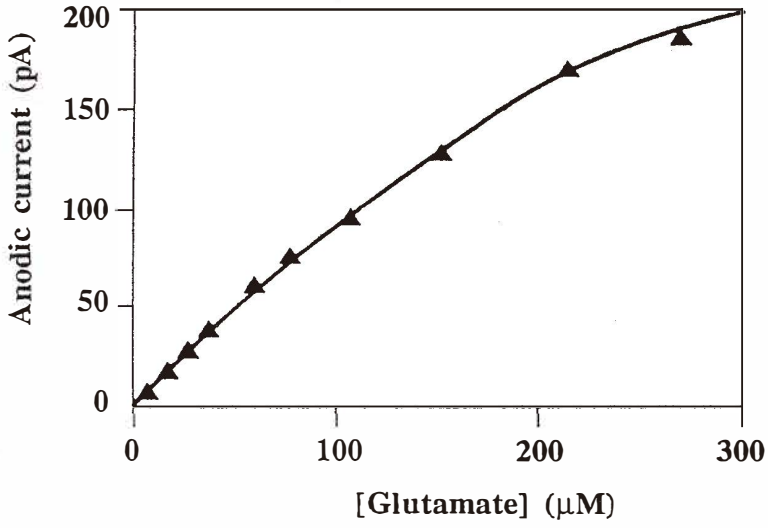

Fig. 4. Calibration curve for glutamate obtained with the poly-A_2-glutamate oxidase electrode. Same experimental conditions as for Fig. 3.

glutamate. Figure 4 shows the calibration curve obtained for glutamate in aqueous solution. This biosensor has been successfully applied to the detection of glutamate in isolated retinal photoreceptor cells of the honeybee drone, as well as in isolated tissue of rat hippocampus.

This strategy for biosensor fabrication has been applied to biosensors based on electrically wired enzymes. For this purpose, a new generation of amphiphilic pyrroles substituted by redox groups able to establish electron transfer with biological molecules has been synthesized. In particular, an amphiphilic pyrrole functionalized by a ferrocene group (Fc), which presents the same properties of dispersion, adsorption and electropolymerization in water as the initial amphiphilic pyrroles, has been applied to the entrapment of glucose oxidase. We have investigated, by cyclic voltammetry, the electrocatalytic behavior of the poly-Fc-GOD electrode. In the presence of glucose, the anodic peak increases and the cathodic peak decreases, indicating a catalytic behavior of the ferrocene groups towards the oxidation of glucose (Fig. 5). There appears to be an efficient coupling of the enzymatic reaction to the electrode surface through electron transport supported by ferrocene groups. Thus, the amperometric detection of glucose can be accomplished in anaerobic conditions (Fig. 6).

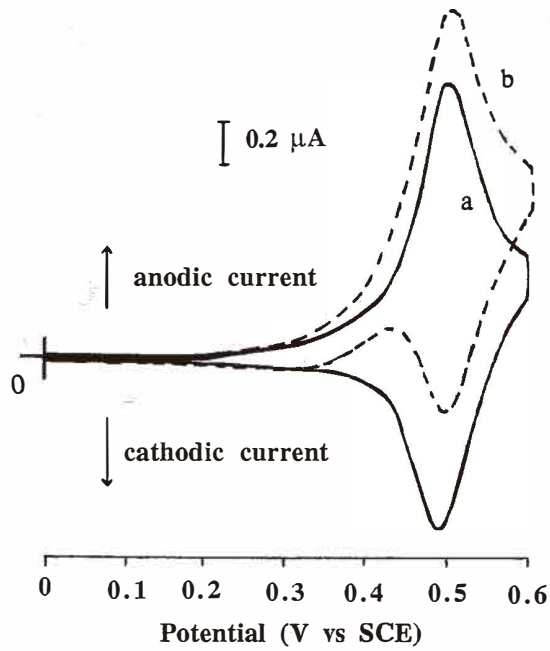

Fig. 5. Cyclic voltammograms for a poly-Fc-GOD electrode without glucose (dashed line) and with 20 mM glucose in argon atmosphere; scan rate 1 mV s^{-1}.

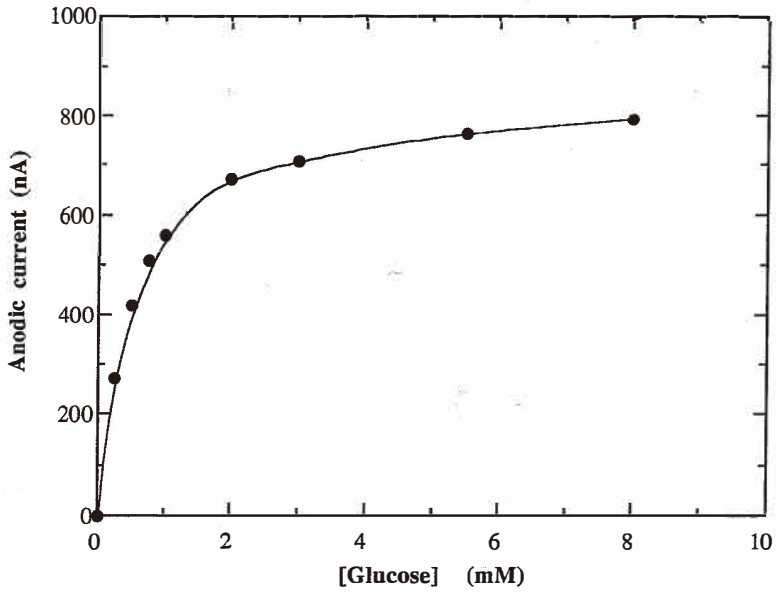

Fig. 6. Calibration curve for glucose obtained with the poly-Fc-GOD electrode. Applied potential: 0.5 V; argon-saturated 0.1 M phosphate buffer (pH 7).

4. Conclusion

We have demonstrated that our procedure of enzyme immobilization is a convenient and powerful strategy for biosensor fabrication. Moreover, this method allows the functionalization of microelectrodes and the formation of biostructure complexes.

We also showed that enzyme entrapment and its effective electrical connection to the electrode surface can be simultaneously accomplished via functionalized amphiphilic pyrroles.

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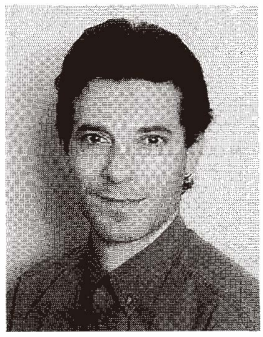
References

1 S. Cosnier and C. Innocent: J. Electroanal. Chem. **328** (1992) 361.

2 L. Coche-Guérente, S. Cosnier, C. Innocent, P. Mailley, J.-C. Moutet, R.M. Morélis, B. Leca and P. R. Coulet: Electroanalysis **5** (1993) 647.

3 H. Kusakabe, Y. Midorikawa, T. Fujishima, A. Kuninaka and H. Yoshino: Agric. Biol. Chem. **47** (1983) 1323.
4 L. Coche-Guérente, A. Deronzier, B. Galland, J.-C. Moutet, P. Labbé, G. Reverdy, Y. Chevalier and J. Amhrar: Langmuir **10** (1994) 602.
5 S. Cosnier, A. Deronzier and J.-C. Moutet: J. Mol. Cat. **45** (1988) 143.
6 S. Cosnier and C. Innocent: Bioelectrochem. Bioenerg. **31** (1993) 147.
7 J. Wang and M. S. Lin: Anal. Chem. **60** (1988) 1545.
8 A. Navaratne, M. S. Lin and G.A. Rechnitz: Anal. Chim. Acta **237** (1990) 107.
9 S. Cosnier and C. Innocent: Anal. Lett. **27** (1994) 1429.



Serge Cosnier is Chargé de Recherche, CNRS, at the University Joseph Fourier of Grenoble, France, where he is heading with Professor P. Labbé the bioelectrochemistry team. He received his D. Sc. in 1988 from the University of Grenoble. He was an Alexander von Humbold Postdoctoral Fellow at the Technische Universität Munich (1989–1990). Since 1991 he has been working in the field of modified electrodes as biomimetic devices. He is also working on the development of biosensors via the immobilization of enzymes by organic polymers and inorganic materials.