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BIOELECTRODE BASED ON TYROSINASE ENTRAPMENT IN ELECTROPOLYMERIZED MATRIX FOR AMPEROMETRIC DETECTION OF PHENOLIC COMPOUNDS

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ABSTRACT. Based on simplicity and low enzyme denaturation, physical methods for enzyme immobilization were extensively used for biosensor construction. In this context, two "*in situ*" obtained polymer matrix, polyaniline and poly-amphiphilic pyrrole, were compared for tyrosinase immobilization on Pt electrodes, in order to construct bioelectrodes for phenol amperometric detection. Mixtures of tyrosinase and the corresponding monomer were electropolymerized, in the presence of a supporting electrolyte (0.1 M $LiClO_4$), at 0.75 and 0.45 V *vs.* SCE for amphiphilic pyrrole derivative and for aniline, respectively. Steady state amperometric measurements, performed at -180 mV *vs.* SCE (aqueous buffer, KCl 0.1 M, phosphate tampon pH=7) and -50 mV *vs.* SCE (0.1 M $C_6H_5CH_2N(CH_2)_3Cl$ in $CHCl_3$), were used to estimate the bioelectrodes electroanalytical parameters. It was established that the polypyrrole matrix has a higher efficiency for enzyme retention resulting in higher bioelectrode sensitivity both in aqueous or chloroform media.

INTRODUCTION

The development of new amperometric biosensors continues to be a rapidly growing research field. When a redox enzyme is used as an active component in such systems, two basic aspects must be considered [1]: (i) the method of assembly of the enzyme electrode; (ii) the electrical contact of the bioelectrocatalyst within this assembly.

One method that offers a high rate of electron transfer between enzyme and electrode is the enzyme entrapment whitin conducting polymers. The most studied conducting polymers have been polyacetylene, polythiophene [2], polypyrrole and its derivative [3], and polyaniline [4].

They are easily to prepare by electrochemically oxidizing of the substrate on the electrode surface. The solvent used, and more particularly the counter anion present in solution, have a major effect on the polymer properties, especially on its conductivity and selectivity.

Phenols and phenol derivatives, due to their high toxicity and environmental persistence, represent a class of compounds of primary interest for water quality monitoring [5]. Recently, it was shown that a widely group of phenols could be detected aqueous media employing amperometric biosensors incorporating tyrosinase (polyphenol oxidase, PPO) [6-18].

The use of PPO for phenol detection in non-aqueous media has been studied intensively due to some advantages of this system [6, 12, 13]. Thus, PPO remains active when, entrapped within a thin aqueous film, is deposited on an electrode surface. Moreover, PPO is not soluble into organic solvents. Hence, there is not necessary a covalent immobilization as in aqueous media, the physical retaining of enzyme on the electrode being successful. On the other hand, the substrate concentration range is more extended because in nonaqueos media the polymerization of o-quinone, the product of the phenol oxidation, is less important than in aqueous solutions. In the same time, the prevention of electrode fouling by such polymerization products increases the biosensor lifetime [3, 18]. Nevertheless, eventually the hydration layer of the enzyme is slowly removed and the enzyme becomes dried and inactivates. Consequently, to avoid the enzyme denaturation the bioelectrode should be stored before using into humid atmosphere.

Summarizing, three kinds of effects should be considered when the influence of the solvent nature on the bioelectrode behavior is examined [19, 20]:

- the general effects due to the solvent hydrophobicity
- the solvent effect on the catalytic activity of the enzyme
- the solvent effect on the solution mass transport properties

This paper presents a comparison of the main electroanalytical parameters for phenol amperometric detection, performed with two different biosensors incorporating tyrosinase. PPO was physically entrapped in two different polymer matrix, obtained by ”*in situ*” electropolymerization of aniline (Figure 1) and an amphiphilic polypyrrole derivative [18] (Figure 2). The influence of a highly hydrophobic nonaqueous solvent (chloroform) on the PPO activity, when the enzyme was entrapped whitin amphiphilic polypyrrole matrix was also investigated.

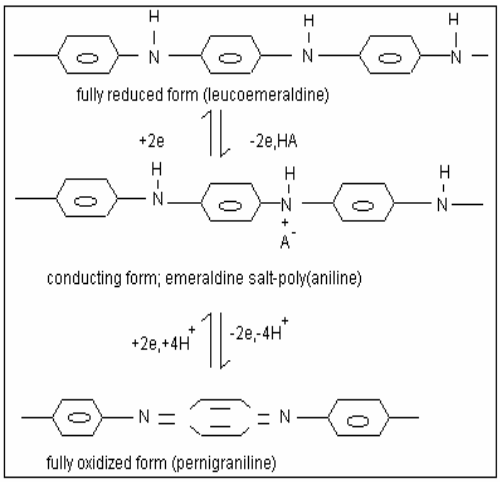

Figure 1. Oxidation of aniline to polyaniline showing switching modes [2].

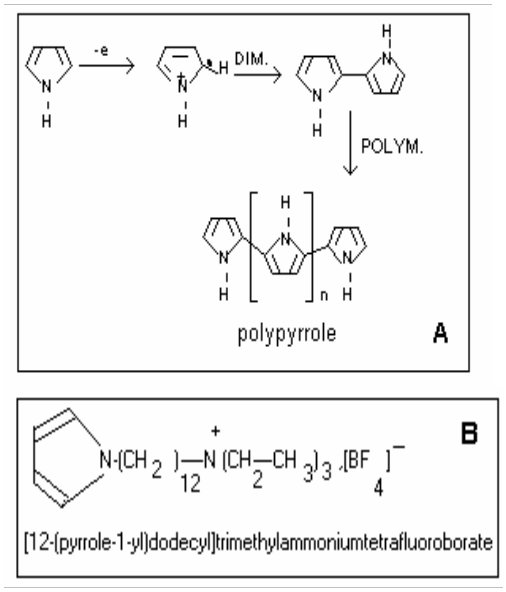

Figure 2. Oxidative polymerization of pyrrole (A) [2] and structure of the amphiphilic pyrrole (B) [18].

EXPERIMENTAL

Reagents

Tyrosinase (EC 1.14.18.1, from mushroom, 4200 Sigma units/mg) was purchased from Sigma. Phenol, chloroform, aniline, KH_2PO_4, K_2HPO_4 and $LiClO_4$ were obtained from Merck and used as received. The amphiphilic pyrrole derivative, [12-(pyrrole-1-yl) dodecyl] trimethylammonium tetrafluoroborate], was generously supplied as a gift by Dr. Serge Cosnier, LEOPR, Grenoble, France.

0.1M $LiClO_4$ and 0.1M phosphate buffer (pH 7, obtained by mixing of the corresponding volumes of 0.1M KH_2PO_4 and 0.1M K_2HPO_4) were used as supporting electrolytes for aqueous solutions for electropolymerisation and for amperometric measurements, respectively. 0.1M $C_6H_5CH_2N(CH_2)_3Cl$, supplied from Aldrich, was employed as supporting electrolyte for voltammetric and amperometric measurements in chloroform solutions.

Electrochemical measurements

All measurements were performed using a computer-assisted potentiostat (Autolab-PGSTAT-10, Eco Chemie, Utrecht, The Netherlands), connected to a conventional electrochemical cell equipped with three

electrodes. The bioelectrode was the working electrode. In both aqueous and non-aqueous experiments, a saturated calomel electrode (SCE) was used as reference electrode and a Pt-foil as counter electrode.

Steady state amperometric measurements were done as follows: the bioelectrode was immersed in 10 ml of testing solution (aqueous or non-aqueous) at room temperature, and poised at the desired value of the applied potential. When the recorded signal attained a stable value, a known volume of standard solution of substrate (phenol) was added under a vigorous stirring. Subsequently, the signal variation corresponding to the reduction of enzymatically produced o-quinone was recorded for 1-2 min. Thus, the calibration curve was constructed by means of successive additions of small volumes of standard aqueous solution of substrate. Before using the bioelectrode was kept at 5^{o} C in a humid atmosphere. The procedure presented above was repeated unchanged in all tests carried out using the amperometric bioelectrode in both aqueuos and chloroform solutions.

The bioelectrode preparation

Amphiphilic polypyrrole matrix

The technique of enzyme entrapment into polypyrrole matrix consisted in the electro-polymerization of the amphiphilic pyrrole monomer, after the adsorption on electrode surface of a mixture of enzyme and monomer [7, 8]. Thus, 2.5 mg of monomer were ultrasonically dispersed in 1 mL of distillated water and 3 mg of PPO was added per mL of dispersion. A volume of 10μL from the above-described mixture was deposited on the Pt disk electrode (3mm diameter) and the water was removed by keeping the coated electrode during 2h hours under reduced pressure. Finally, the monomer-enzyme film was electropolymerized in a 0.1M $LiClO_4$ aqueous solution, by controlled potential electrolysis. Figure 3 presents the current-time dependence observed during the amphiphilic pyrrole electropolymerization.

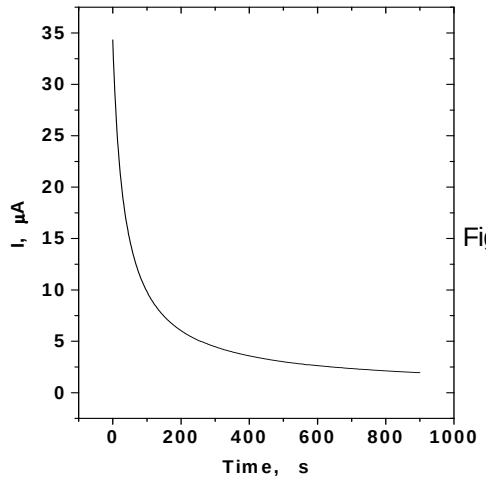

Figure 3. Current variation during potentiostatic electropolymerization of the amphiphilic pyrrole monomer. Experimental conditions: applied potential, 0.76 V *vs.* SCE; supporting electrolyte, 0.1M $LiClO_4$ aqueous solution.

Polyaniline matrix

3 mg of PPO was dispersed in 1 mL of fresh distilled aniline. The Pt disk electrode (φ = 3mm) was coated with 10 μL from the above-described mixture. Subsequently, the electrode was dried during 2h under reduced pressure and was covered with a polyethylene membrane. The aniline electropolymerization was performed at 0.45 V *vs.* SCE in a 0.1M $LiClO_4$ aqueous solution.

RESULTS AND DISCUSSION

The new strategy recently proposed to obtain reagentless phenol amperometric biosensors, based on PPO entrapment in a polypyrrole matrix obtained by "*in situ*" electropolymerization [7, 8], was first extended to polyaniline and finally used for phenol detection in a nonaqueous solvent ($CHCl_3$). Thus, two similar biolelectrodes using enzyme matrix based on polyaniline and a pyrrole derivative polymer were prepared and compared for phenol amperometric detection. Furthermore, the bioelectrode showing the best electroanalytic characteristics was checked for use in a nonaqueous solvent.

Comparison between polyaniline and amphiphilic polypyrrole matrix

Steady state amperometric measurements were performed with both investigated bioelectrodes at an applied potential of -180 mV *vs.* SCE. The corresponding calibration plots are shown in figure 4. The bioelectrode

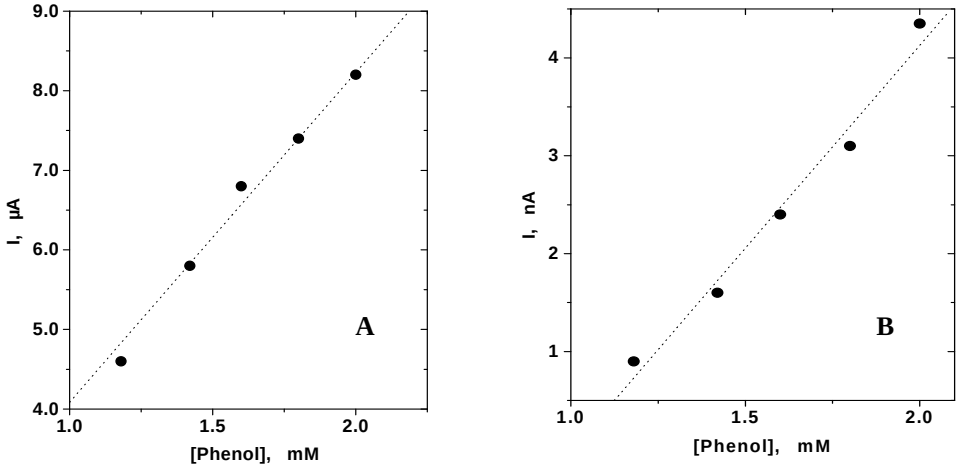

Figure 4. Amperometric response of the phenol biosensors: amphiphilic polypyrrole matrix ***(A)****;* polyaniline matrix ***(B)****.* Experimental conditions: working potential –180 mV *vs.* SCE; magnetically stirred buffer solutions (pH 7.0).

sensitivities, estimated as the slope of the linear range, were found to be strongly different: the bioelectrode based on polyaniline showed a sensitivity of (4,1 ± 0.3) μA M^{-1}, while that using amphiphilic polypyrrole had a higher sensitivity (4,4 ± 0.3) mA M^{-1}. The difference in the behavior of the two biosensors could be attributed to: (i) a higher efficiency for enzyme retention for the polypyrrole matrix; (ii) a high substrate permeability through the amphiphilic polypyrrole matrix; (iii) the matrix hydrophilicity, due to the amphiphilic structure of the pyrrole derivative [7, 8].

From figure 5 the response time for the bioelectrode using polypyrrole as polymer matrix for PPO entrapment can be estimated to be lower than 1 minute.

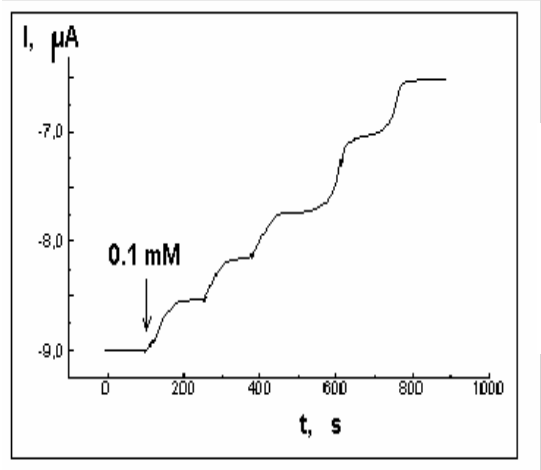

Figure 5. Amperometric response of the biosensor based on amphiphilic pyrrole matrix to successive additions of 0.1 mM phenol. Experimental conditions: see figure 4.

Summarizing, the bioelectrode using the polypyrrole matrix showed a high sensitivity, a low response time and a good operational stability. Thus, this variant was retained for all further investigations.

Phenol detection in chloroform

Cyclic voltammetry measurements performed in chloroform, in the absence and in the presence of phenol, proved that the bioelectrode based on polypyrrole matrix maintains its bioelectrocatalytic response for phenol (Figure 6). In the same time, it was noticed that even a lower applied potential than –180 mV *vs.* SCE can be used for amperometric measurements.

The chloroform being a hydrophilic compound it can remove water from the enzymatic environment [20, 21], and, consequently, only saturated with water the bioelectrode would keep its response due to the preservation of the hydration layer around the enzyme. In order to check the effect of

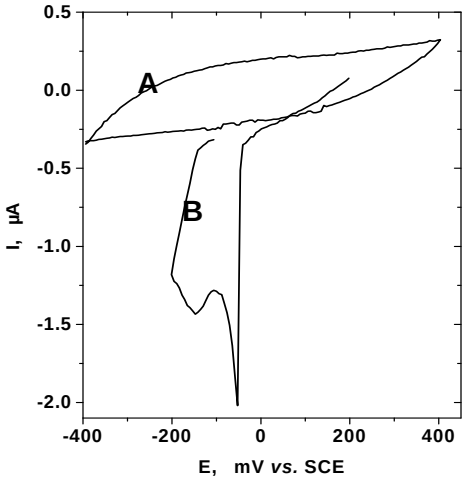

Figure 6. Cyclic voltammograms recorded in chloroform at tyrosinase based bioelectrode: in the absence of phenol ***(A)*** and in the presence of 0.1 mM phenol ***(B)***. Experimental conditions: working bioelectrode, tyrosinase entrapped in amphiphilic polypyrrole electropolymerized on Pt (3mm diameter); scan rate, 50 mV/s; starting potential, -400 mV ***(A)*** and +200 mV ***(B)***, *vs.* SCE.

chloroform on the operational stability of the polypyrrole based bioelectrode its amperometric response for 0.1 mM phenol was recorded after two hours of continuous immersion in chloroform. The remove of the enzyme hydration layer by chloroform was demonstrated by the absence of any response, if the experiment was done without any previous hydration. Contrarily, if the biosensor was kept one day in a humid atmosphere the signal to 0.1 mM phenol was recovered almost completly (~ 95%). The steady-state current was achieved typically after 5 min.

The calibration curve obtained by successive additions of standard phenol solution in chloroform (Figure 7) showed a linear response up to 2 mM with a lower sensitivity than that observed in water solutions (Table 1). The reason of the sensitivity decrease could be due to the gradual remove of the enzyme hydration layer and, as it was previously observed [22], to the difference between the solvent polarity, strongly influencing the reactivity of the enzyme active center.

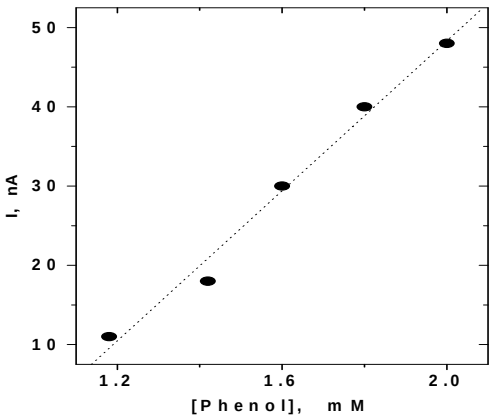

Figure 7. Calibration curve of the bioelectrode based on amphiphilic pyrrole matrix in chloroform containing 0.1M $C_6H_5CH_2N(CH_2)_3Cl$. Experimental conditions: applied potential, -50 mV *vs.* SCE; magnetically stirred solutions.

Table 1.

Sensitivity to phenol of the polypyrrole based biosensors recorded in two different media, at the same applied potential (-50 mV *vs.* SCE).

Solvent	**Sensitivity (nA/M)**	**Linear range (mM)**	**Regression coeff. / No. of exp. points**
H_2O	276	up to 2	0.9944 / 5
$CHCl_3$	47.3	up to 2	0.9934 / 5

CONCLUSIONS

This study demonstrates the advantages to use conducting polymers for bioelectrode construction, especially the amphiphilic polypyrrole, as polymer matrix for tyrosinase entrapment. It was observed a rapid stabilization of the background current, a high sensitivity to phenol and a good operational stability (higher than tree months) when tyrosinase was entrapped in amphiphilic polypirrole in order to construct amperometric biosensors.

On the other hand, a strong adherence of the enzyme matrix to the Pt electrode surface was observed in both water and chloroform solvent. Contrarily, the bioelectrode based on polyaniline matrix showed a low reproducibility and a poor operational stability.

Finally, this work demonstrated for the first time to our knowledge the suitability of organic phase tyrosinase bioelectrode using amphiphilic polypyrrole matrix for monitoring phenols in chloroform.

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