NEW MODIFIED ELECTRODES WITH HRP IMMOBILIZED IN POLYMERIC FILMS FOR PARACETAMOL ANALYSIS

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Abstract

In order to establish the configurations of biosensors that are able to detect paracetamol in various matrices, it was studied the behaviour of some modified electrodes with an electroconductive polymeric film. Polypyrrole films deposition on glassy carbon and screen-printed electrodes was developped by several methods. The electroanalytical characterization of the deposited films was performed by chronoamperometry in the presence of an enzyme immobilized into the polymeric films. Chronoamperometric study performed with glassy carbon and planar electrodes based on graphite, modified with horseradish peroxidase embedded in polypyrrole films, proved the existence of interactions between the immobilized enzyme and the electroactive species of paracetamol (N-acetyl-p-benzo-quinone imine-NAPQI), enzymatically generated in the presence of hydrogen peroxide.

Rezumat

În vederea elaborării unor biosenzori capabili să detecteze paracetamolul din diferite matrici a fost studiat comportamentul electrozilor modificaţi cu filme electroconductoare de polipirol. Depunerea filmelor de polipirol pe suprafaţa electrozilor de carbon vitros şi electrozilor planari imprimaţi a fost realizată prin mai multe metode. Caracterizarea electroanalitică a filmelor depuse a fost realizată prin cronoamperometrie, în prezenţa enzimei imobilizate la nivelul filmelor polimerice. Studiile de cronoamperometrie efectuate pe ambele tipuri de electrozi modificaţi cu peroxidaza din hrean, incorporată în filmul de polipirol, au demonstrat existenţa intercţiunilor între enzima imobilizată şi speciile electroactive ale paracetamolului (N-acetil-p-benzo-chinon imina-NAPQI), generate enzimatic în prezenţa peroxidului de hidrogen.

Keywords: Screen-printed electrodes, Horseradish peroxidase, Paracetamol.

Introduction

Electrochemical methods play an important role in the pharmaceutical, biomedical and environmental analysis, because they are sensitive, selective, and inexpensive and because, most times, excipients or matrices do not interfere in the analysis and the sample can be prepared by simply dissolving the substance in an appropriate solvent.

Design, fabrication and applications of amperometric sensors and biosensors have gained considerable importance in recent years [1-6]. Small, inexpensive sensors with high selectivity towards specific chemical compounds, molecules and ions are highly desirable for a great variety of application including environmental monitoring, food and pharmaceutical industries and medicine. For particular application, like monitoring micro-size environmental samples or *in situ* determination in the human body, the size of the sensor is critical and requires very small dimensions.

Biosensors based on the conducting polymers are well suited to the requirements of modern biological analysis, multiparametric assays, high information density and miniaturization [7].

The most important step out of the selection of the enzyme and the electrode material (the transducer) is the immobilization strategy. The immobilization of the biocompounds needs to ensure the stability of the enzyme on the electrode surface and to maintain, in most cases, the hydration of the microenvironment which ensures the enzyme's biocatalytic activity and prolongs the life of the biosensors. Several methods were used to immobilize the enzyme at the electrode surface, like adsorption, cross linking, covalent binding, biological membranes, magnetic microparticles, entrapment in sol-gel, anchoring by specific affinity interactions [8-16].

The enzyme immobilization into an electrochemical polymer or polymerizable matrices was also successfully used in the development of the amperometric biosensors [17-21].

Horseradish peroxidase (HRP) has been a useful tool in biomedical and pharmaceutical analysis. The wide use of HRP is due to its commercial availability in a relatively pure form at reasonably low prices, its stability, and its high turnover on a variety of substrates. HRP has been applied to the detection and quantification of neurotransmitters, immunoassays for tumor markers, antigen detection, DNA analysis, and the detection of carnitine, cholesterol, glucose, lactate, clozapine, thiols, hydrogen peroxide, phenols [22-36].

Recently, screen printed electrodes (SPEs) have demonstrated promising potential, especially in the development of rapid analysis and biosensor fabrication. [37].

There are several papers in literature dealing with electrochemical acetaminophen detection. In particular, there was reported the spectrophotometric and electroanalytical study of acetaminophen and ascorbic acid in effervescent oral solid formulations, using a carbon paste electrode, magnetized nanoporous silica based microparticles were used for HRP immobilization and applied for amperometric peroxidase-based biosensor development, biosensor based on HRP zirconium alkoxide porous gel deposed on glassy carbon support was reported for acetaminophen amperometric determination and screen printed electrodes modified with zirconium alkoxide or/and carbon nanotubes were realized [21,38-41]. The HRP biocomponent of the biosensor is immobilized onto the SPE surface by entrapment into a porous zirconium alkoxide gel-polyethyleneimine film and the resulting device allowed the amperometric detection of acetaminophen in the presence of hydrogen peroxide.

In the present work, the HRP biocomponent was immobilized either on the glassy carbon electrode or on the transducer surface (the working electrode of the SPE) by entrapment into the electrochemicaly generated polypyrrole film, by several methods. The resulting devices were used to monitor the amperometric signal produced by the electrochemical reduction of the enzymatically-generated, electroactive oxidized species of acetaminophen (N- acetylbenzoquinoneimine - NAPQI) in the presence of hydrogen peroxide.

$$\text{Paracetamol} + H_2O_2 + 2H^+ + \text{HRP} \rightarrow \text{NAPQI} + 2H_2O$$

$$\text{NAPQI} + 2e^- + 2H^+ \rightarrow \text{Paracetamol [40].}$$

Such an approach allows avoiding interferences from oxidizable analytes.

Materials and Methods

An AUTOLAB PGSTAT 30 potentiostat/galvanostat (Ecochemie, The Netherlands) equipped with specific soft GPES was employed for electrode preparation and other voltammetric and amperometric determinations. All measurements were performed at room temperature, under continous stirring (20-23 ˚C).

Solid electrodes made from glassy carbon (d = 3 mm) purchased from Radiometer Analytical (Hach Lange) were used as working electrodes in electrochemical determination. Experiments were conducted in 3-electrode geometry, a platinum wire auxiliary electrode, and Ag/AgCl (3 M KCl) reference electrode.

Screen-printed electrodes type DS 110 purchased from Dropsens® (Spain) were also used for electrochemical measurements. In this case the

electrochemical cell is composed by a graphite working electrode (d = 4 mm), a graphite auxiliary electrode and a silver pseudoreference electrode, with silver electric contacts and ceramic substrate. These electrodes are ideal for working with microvolumes, for decentralized assays or to develop specific sensors.

Deionised water was used for preparation of buffers and all other aqueous solutions. All chemicals were of analytical grade, and used as received, without further purification.

Horseradish peroxidase was purchased from Sigma Aldrich.

Polypyrrole and horseradish peroxidase (HRP) modified electrode preparation

Glassy carbon were polished using alumina on a polishing pad, rinsed with deionised water, ethanol and once again with deionised water. Electrodes were sonicated for 10 minutes in deionised water to remove any alumina particles and finally dried in N_2 flux. The polished electrode was placed in a 0.5 M NaOH solution and the electrochemical activation of the electrode was performed by potentiostatic method at 0.9 V for 100 s. This electrode was used for the preparation of modified electrode.

The polypyrrole-HRP modified electrodes were obtained by three different methods. To avoid the risk of enzyme folding and blocking of the electrode surface by the biocomponent, a thin polypyrrole film was first deposited on screen printed electrode surface by immersing the working electrode into a 0.05 M pyrrole solution in 0.1 M $LiClO_4$ and sweeping the potential for one complete cycle, either between 0 and + 0.8 V or 0 and +1.4 V *versus* SCE (saturated calomel electrode) for the glassy carbon electrodes and either between 0 and + 1.0 V or 0 and +1.6 V *versus* Ag pseudo-reference with a scan rate of 0.1 V s^{-1} for the SPEs.

Method 1. The polypyrrole-HRP film was then grown onto the electrode already covered by the thin polypyrrole layer by immersing this electrode into a 0.48 g L^{-1} HRP and 0.05 M pyrrole solution in 0.1 M $LiClO_4$ and cycling three times the potential between 0 and + 0.8 V *versus* SCE for the glassy carbon electrodes and between 0 and + 1.0 V *versus* Ag/AgCl for the screen-printed electrodes with a scan rate of 0.1 V s^{-1}.

Method 2. The polypyrrole-HRP film was then grown onto the electrode already covered by polypyrrole layer by immersing this electrode into a 0.53 g L^{-1} HRP and 0.05 M pyrrole solution in 0.1 M $LiClO_4$ and cycling two times the potential between 0 and + 0.8 V vs. Ag/AgCl for the glassy carbon electrodes and between 0 and + 1.0 V vs. Ag/AgCl for the screen printed electrodes, with a scan rate of 0.1 V s^{-1}.

Method 3. The polypyrrole-HRP film was then grown onto the electrode already covered by polypyrrole layer by immersing this electrode into a 0.30 g L^{-1} HRP and 5×10^{-5} M pyrrole solution in 0.05 M $LiClO_4$ and cycling three times the potential between 0 and + 0.8 V vs. SCE for the glassy carbon electrodes and between 0 and + 1.0 V vs. Ag/AgCl for the screen printed electrodes, with a scan rate of 0.05 V s^{-1}.

These biosensors were rinsed with phosphate buffer solution under constant stirring and then kept in phosphate buffer at pH=7 overnight, at 5°C. Enzyme electrodes were conditioned for 5 minutes in phosphate buffer solution at pH=7, prior using.

Results and Discussion

Polypyrrole-HRP modified glassy carbon electrodes

Figure 1 shows the superposed cyclic voltammograms of the 10^{-2} M paracetamol solution in phosphate buffer (pH 7.4) with the unmodified glassy carbon electrodes and the polypyrrole-HRP modified electrodes. These voltammograms were done with the aim to settle the value of the reduction potential of NAPQI which is necessary for the chronoamperometric determinations.

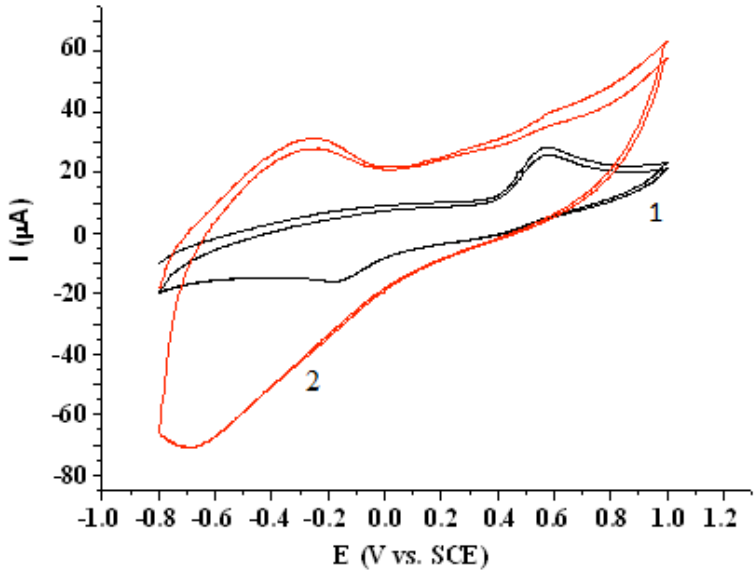

Figure 1

Cyclic voltammograms of the 0.01 M paracetamol solution in phosphate buffer (pH 7.4) with glassy carbon electrode (1) unmodified and (2) modified with polypyrrole and HRP (scan rate 0.1 V s^{-1})

From these voltammograms it can be seen that the value of the potential for the reduction of NAPQI (the paracetamol oxidation product) at the unmodified electrode is – 0.175 V vs. SCE. The reduction of NAPQI at the polypyrrole-HRP modified electrodes was not clearly observed because of the highly capacitive current of the polypyrrole film.

Figure 2 (curve 1) shows the current – time recording for successive addition of different volumes of 0.01 M paracetamol solution with the glassy carbon electrode modified with polypyrrole and HRP (method 1). The obtained current response related to NAPQI reduction was well-defined, stable and fast, with the response time of about 20 s. The inset figure shows the calibration curve of the electrode, with a linear detection range of 9.36 – 83.669 μM (R^2 = 0.9923) and a detection limit of 8.4 μM. The correlation between paracetamol concentration and the value of the current is expressed by the following equation:

$$-I\ (\mu A) = 4.5345 \times [\text{Paracetamol}](mM) + 1.2 \times 10^{-3}$$

Curves 2 and 3 of Fig. 2 clearly show the absence of interfering reduction current from hydrogen peroxide itself. They clearly indicate the effective role of hydrogen peroxide in the catalyzed oxidation of paracetamol by HRP.

Similar calibration curves were obtained for polypyrrole-HRP electrodes prepared by methods 2 and 3. Table I summarizes the resulted data. For all three electrodes, the deviation from the linearity at higher concentration of the substrate may occur from the saturation of the immobilized enzyme, and/or inhibition of the immobilized enzyme.

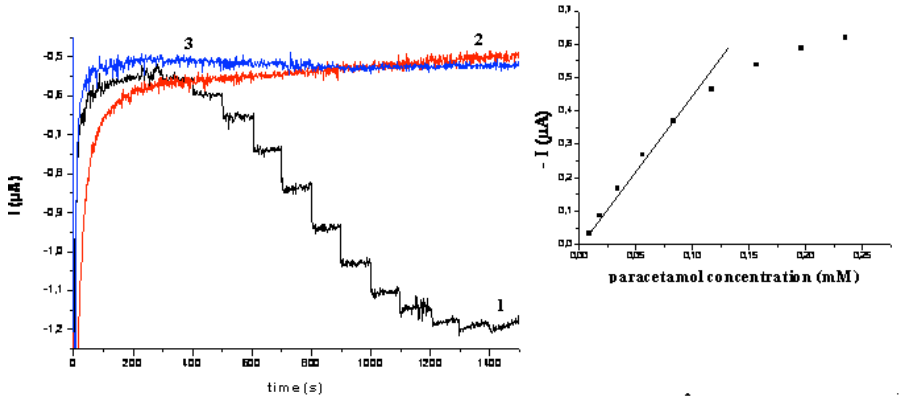

Figure 2

Current – time recording for 5 μL; 10 μL; 15 μL; 25 μL; 35 μL; 45 μL; 55 μL; 65 μL of (1): 0.01 M paracetamol solution in 0.2 mM H_2O_2 [40] in phosphate buffer pH=7.4; (2) 0.01 M paracetamol solution in phosphate buffer pH=7.4 and (3) 0.2 mM H_2O_2 in phosphate buffer pH=7.4 at polypyrrole-HRP modified glassy electrode (method 1). Inset: Calibration curve of the electrode. The applied working potential was – 0.175 V vs. SCE

In order to confirm the mechanism of the biocatalytic peroxidation of paracetamol, current-time curves were performed in the same conditions for the unmodified glassy carbon electrodes and for the electrodes modified only with the polypyrrole film.

Figure 3 (curve 2) shows the current – time recording for successive addition of different volumes of 0.01 M paracetamol solution with the glassy carbon electrode modified with polypyrrole and HRP (method 1) in comparison with the unmodified glassy carbon electrode (curve 1) and with the glassy carbon electrode modified only with polypyrrole (method 1, without the enzyme, curve 3).

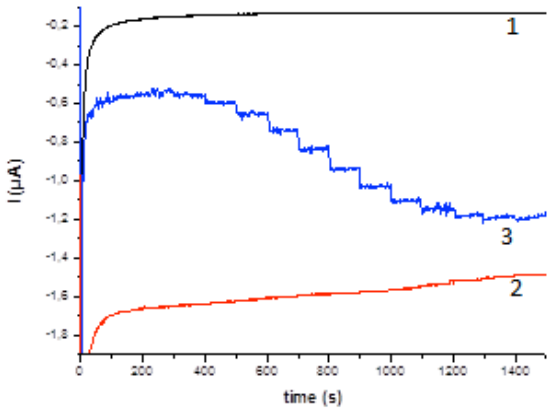

Figure 3

Current – time recording for 5 μL; 10 μL; 15 μL; 25 μL; 35 μL; 45 μL; 55 μL; 65 μL of: 0.01 M paracetamol solution in 0.2 mM H_2O_2 in phosphate buffer pH=7.4 for the (1) unmodified glassy carbon electrode, (2) polypyrrole modified electrode (without the enzyme) and (3) polypyrrole-HRP modified electrode (method 1). The applied working potential was – 0.175 V vs. SCE

The mechanism of the enzymatic process is therefore confirmed: the enzyme oxydates paracetamol resulting its electroactive specie NAPQI, which is firther reduced on the electrodes surface at the determined potential -0.175 V vs SCE. The turnover of the HRP is assured by the H_2O_2 0.2 M (Figure 4).

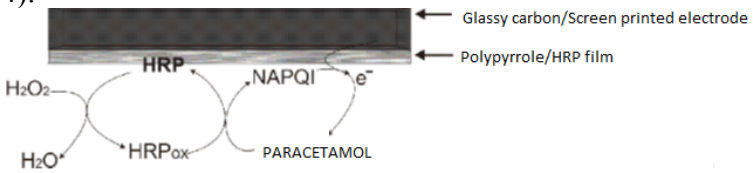

Figure 4

The mechanism of biocatalytic peroxidation and amperometric detection of paracetamol with the polypyrrrole HRP biosensor [40]

Table I

Limits of detection (LOD) and equations of the linear variations of the currents I measured by chronoamperometry at -0.175 V with paracetamol concentration at different glassy carbon/polyrrole-HRP electrodes

Method of preparation of the polyrrole-HRP electrode	Paracetamol dynamic concentration range (µM)	*I*-[paracetamol] equation	LOD (µM)
Method 1	9.3 – 83.7	$I/\mu A = 4.5345 \times [paracetamol]/mM + 1.2 \times 10^{-3}$	8.4
Method 2	9.3 – 83.7	$I/\mu A = 1.7063 \times [paracetamol]/mM + 2.5 \times 10^{-3}$	6.5
Method 3	9.3 – 83.7	$I/\mu A = 0.5148 \times [paracetamol]/mM - 0.6 \times 10^{-3}$	7.2

Polypyrrole-HRP modified screen printed electrodes

Figure 5 shows the superposed cyclic voltammograms for polypyrrole-HRP modified SPEs in phosphate buffer pH 7.4 with and without 5×10^{-4} M paracetamol. These voltammograms allow determining the value of the reduction potential which is necessary for chronoamperometric determinations. It can be seen that for these electrodes the operating potential for the reduction of NAPQI (the paracetamol oxidation product under the enzyme activity) is – 0.4 V vs. Ag/AgCl.

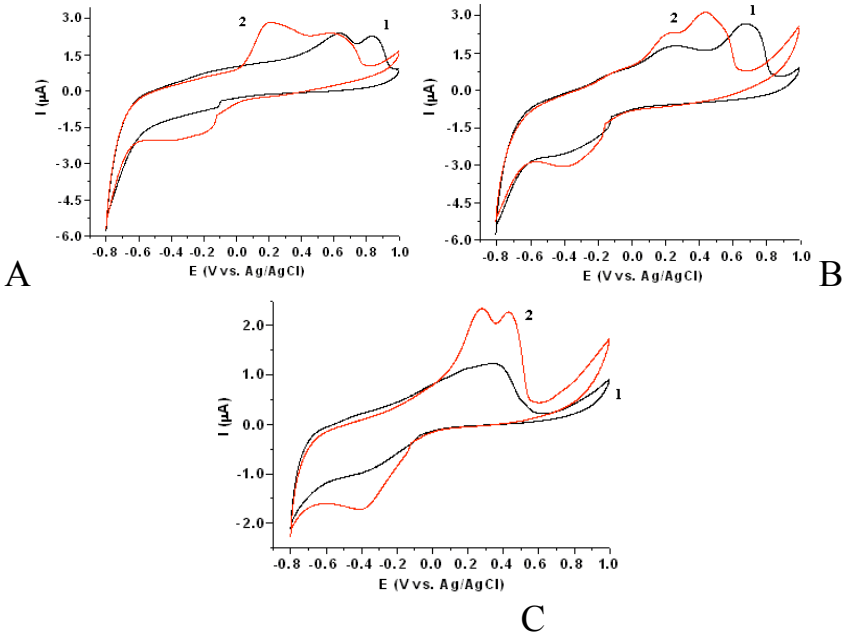

Figure 5

Cyclic voltammograms for graphite based screen printed electrodes modified with polypyrrole and HRP in phosphate buffer of pH=7.4 without (1) and with (2) $5 \cdot 10^{-4}$ M paracetamol solution; (A) method 1; (B) method 2; (C) method 3 (scan rate 0.1 V s^{-1})

Figure 6 shows the current – time recording of the graphite screen-printed electrode modified with polypyrrole and HRP by method 1 (see details in the experimental part) to successive different volumes of 0.01 M paracetamol solution addition. Substrate concentration was changed by stepwise addition of the concentrate solution to a stirred buffer. The obtained current response was well defined, stable and fast, with the response time of about 25 s. The inset of Fig. 4 shows the calibration curve of the electrode, with a linear detection range of 3.1 – 83.7 μM (R^2 = 0.9976) and a detection limit of 26.71 μM. The correlation between paracetamol concentration and the value of the current is expressed by the following equation:

$$- I\ (\mu A) = 13.56 \times [\text{Paracetamol}]\ (mM) + 0.0509.$$

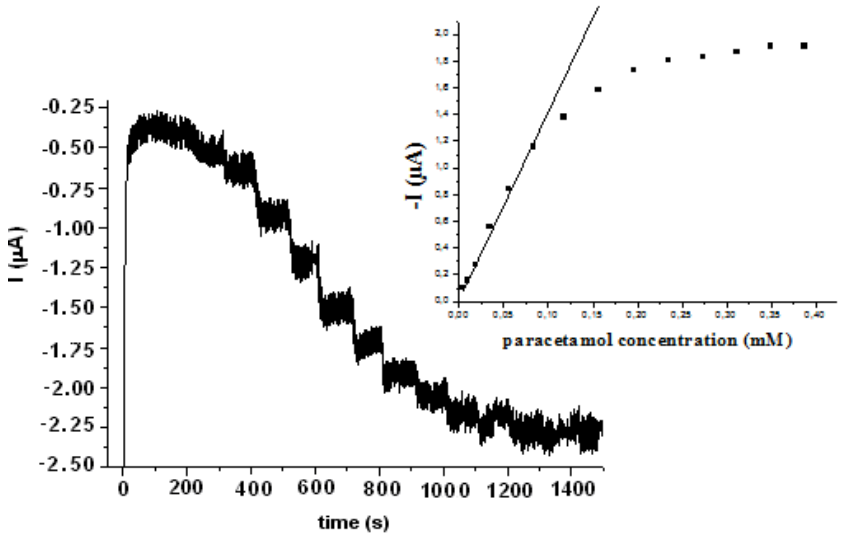

Figure 6

Current – time recording for 5 μL; 10 μL; 15 μL; 25 μL; 35 μL; 45 μL; 55 μL; 65 μL 0.01 M paracetamol solution in 0.2 mM H_2O_2 in phosphate buffer pH=7.4 at graphite SPE modified with polypyrrole and HRP (method 1). Inset: Calibration curve of the electrode. The applied working potential was – 0.35 V vs. Ag/AgCl

Similar calibration curves were obtained by polypyrrole-HRP electrodes prepared by methods 2 and 3. Table II summarises the obtained data. For all three electrodes, the deviation from the linearity at higher concentration of the substrate may occur from the saturation of the immobilized enzyme, and/or inhibition of the immobilized enzyme.

Table II

Limits of detection (LOD) and equations of the linear variations of the currents I measured by chronoamperometry at -0.35 V with paracetamol concentration at different SPE/polyrrole-HRP electrodes

Method of preparation of the polyrrole-HRP electrode	Paracetamol dynamic concentration range (µM)	*I*-[paracetamol] equation	LOD (µM)
Method 1	3.1 – 83.7	I/µA = 13.56 × [paracetamol]/mM + 0.0509	26.71
Method 2	3.1 – 55.9	I/µA = 24.823 × [paracetamol]/mM + 0.0002	2.76
Method 3	3.1 – 55.9	I/µA = 13.233 × [paracetamol]/mM + 0.0634	1.52

Conclusions

The main objective was to study some polymeric films modified electrodes behaviour with the aim of biosensors elaboration, which has to be able to detect and quantify pharmaceuticals in different matrix. Polypyrrole films deposition on planar glassy carbon and screen printed electrodes was made by several methods. The electroanalytical characterization of the deposited films was performed by using the chronoamperometry method in the presence of the horseradish peroxidase, an enzyme which was immobilized into the polymeric films. Using the planar electrodes based on graphite modified with peroxidase embedded in polypyrrole film, the existence of interactions between the immobilized enzyme and the electroactive species of paracetamol enzymatically generated in the presence of the hydrogen peroxide was proved. The method used for these determinations was the chronoamperometry. In the case of the glassy carbon electrodes modified with polypyrrole films and HRP the dynamic linear detection range was the same for the three methods. For both electrode configurations (glassy carbon or SPE), the highest sensitivity was obtained for electrodes prepared by method 1 and 2, respectively, that provide thicker polypyrrole films. This allows confirming that in spite of the thickness of the native polypyrrole film, its overoxidation is efficient to ensure the easy accessibility of the incorporated enzyme.

Acknowledgments

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