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Permselective and enzyme-entrapping behaviours of an electropolymerized, non-conducting, poly(*o*-aminophenol) thin film-modified electrode: A critical study

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ABSTRACT

Non-conducting polymeric films synthesised by the electrooxidation of *o*-aminophenol on a platinum electrode in acetate or phosphate buffer displayed an interesting permselective behaviour, which proved valuable in minimising the electrochemical interferences from ascorbate, acetaminophen, cysteine and urate sample molecules in amperometric detection mode. The electrosynthesis of poly(*o*-aminophenol) (p(oAP)) film showed also useful as permselective membrane for enzyme immobilization as demonstrated by the production of an interference-free glucose oxidase biosensor. In this respect, the glucose response time, $t_{0.95}$, evaluated in batch addition experiments, was lower than 5 s while the calibration curve was linear up to 10 mM of glucose with a sensitivity of 69.7 nA/mM. Both the permselective behaviour and the enzyme-entrapping property of the film were critically compared with the relevant studies until now reported. With respect to the sophisticated but complex approaches described elsewhere, this study shows that simply a proper optimization of p(oAP) electrosynthesis and its permselective behaviour is the key to improve significantly the selectivity of the resulting analytical devices.

1. Introduction

Permselective film-based chemically modified electrodes (Bard and Faulkner, 2001), produced by electropolymerization of suited organic molecules, have proved useful for the assembly of novel analytical devices. Electropolymerization, in fact, has several advantages like simplicity of the experimental setup, ease of control and capability to address the modification solely on the device sensor while permselectivity leads to a significant improvement of sensitivity, selectivity and stability of the electrochemical device.

An interesting application of permselective films arises from their ability to entrap biomolecules within the polymer during the electropolymerization step, allowing the production of novel enzyme electrodes. This approach shows the advantages of electropolymerization while permitting an enhanced selectivity of the sensor. Indeed, poly(*o*-phenylenediamine) (Malitesta et al., 1990; Centonze et al., 1992a; Dempsey et al., 1993; Palmisano et al., 1994, 1995; Wang et al., 1996), polyphenol (Bartlett and Caruana, 1992; Bartlett et al., 1992), poly(2-naphthol) (Ciriello et al., 2000; Guerrieri et al., 2006) and overoxidized polypyrrole (Centonze et al., 1992b; Palmisano et al., 1993, 1995; Guerrieri et al., 1998, 2006) have been successfully used for the fabrication of a series of biosensors capable even of real sample analysis (Centonze et al., 1992a,b; Palmisano et al., 1993, 1994; Guerrieri et al., 1998).

The production of these films usually involves the electropolymerization of hetero-atom substituted aromatic compounds like phenol, phenylenediamines and pyrrole. In this respect, we have studied in the past (Ciriello, 1995, 1999; Guerrieri et al., 2006) some related molecules possessing different functional groups and/or having a dissimilar number of carbon atoms. The use of heterobifunctional phenyl derivatives like aminophenols was appealing to us since both functional groups can either be involved in the electropolymerization step either affect the charge of the resulting polymer. Moreover, the possibility to switch with pH the electrochemistry of the isomer *o*-aminophenol (oAP) and its polymer from the electroactive, conducting to electroinactive, passivating form (Kunimara et al., 1988; Barbero et al., 1989), suggested us to study the oAP polymer and to apply the insulating form p(oAP) as a novel permselective, enzyme-entrapping membrane (Ciriello, 1995, 1999).

Several studies reported the use of p(oAP) polymer either as permselective membrane (Miland et al., 1996; Lobo-Castanon et al., 1997) either for enzyme-entrapping, mainly in glucose biosensors

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(Zhang et al., 1996; Pan et al., 2004a,b, 2005a,b; Ye et al., 2005; Li and Lin, 2007). Although confirming the above approach, these studies reported also unclear and sometimes contradictory results in terms of transduction, interferent rejection and analytical performances of the sensors. For example, to lower the oxidation overpotential of hydrogen peroxide produced by the enzyme, conventional disk electrodes like platinum, gold or carbon paste (Miland et al., 1996; Lobo-Castanon et al., 1997) were replaced by platinized (Zhang et al., 1996), carbon nanotubes (Pan et al., 2005b; Ye et al., 2005) and Pt-polypyrrole nanocomposite glassy carbon (Li and Lin, 2007) electrodes but their application in biosensing does not show, in our opinion, the expected increase in selectivity. Similarly, prussian blue and metallophthalocyanines modified electrodes are known to catalyze hydrogen peroxide oxidation: unfortunately, when coupled with p(oAP) polymers (Pan et al., 2004a; Ye et al., 2005), the expected lowering of the hydrogen peroxide detection potential was not apparently allowed and hence the resulting permselectivity was not improved as expected. Even a p(oAP)/copper-modified gold electrode was proposed as glucose biosensor (Pan et al., 2005a) as copper is known to oxidize glucose: of course, in this case the proposed device cannot be still considered a biosensor since a direct, not enzymatic mediated, transduction approach was used.

In this paper, earlier unpublished results (Ciriello, 1995, 1999) concerning the electrosynthesis of non-conducting poly(oAP) thin films at a simple disk platinum electrode and its use as novel permselective membranes for glucose oxidase immobilization will be described and critically compared with similar studies until now reported.

2. Experimental

2.1. Chemicals

o-Aminophenol (oAP) was purchased by Fluka (Fluke Chemie, Switzerland). Ascorbic acid, uric acid, acetaminophen and L-cysteine were obtained from Aldrich (Aldrich-Chemie, Germany) while potassium ferrocyanide, potassium ferricyanide and hydrogen peroxide stock solutions from Carlo Erba (Italy). Glucose oxidase (GOD, type VII from *Aspergillus niger*, 162,000 units/g) and β-D-glucose were obtained from Sigma Chemical Co. (USA). All the other chemicals were of analytical grade. oAP was purified by ethylacetate recrystallization. Stock glucose solutions were prepared in bidistilled water or buffer, allowed to mutarotate overnight at room temperature before their use and stored in the dark at 4 °C. The monomer and interferent solutions were prepared just before their use.

2.2. Apparatus and electrodes

Batch electrochemical experiments were done with an AMEL (Italy) mod. 466 polarographic analyser. The electrochemical cell was a three electrode system with a platinum counter electrode and an Ag/AgCl/KCl (sat'd) reference electrode. The working electrode consisted of a polycrystalline platinum disk (1 or 3 mm diameter) embedded in a PTFE body. Rotating disk electrode (RDE) experiments were performed by an EG&G (EG&G Princeton Applied Research, USA) RDE mod. 616 equipped with a platinum disk electrode (4 mm diameter) embedded into a PTFE body. Unless otherwise stated, rotation rate was 1000 rpm.

Electrochemical quartz crystal microbalance (EQCM) experiments were performed with a mod. QCA917 SEIKO EG&G system coupled with a mod. 263A EG&G PAR potentiostat/galvanostat, both controlled by a desktop computer running a Model 270/250 Research Electrochemistry software version 4.23 (EG&G). AT-cut 9 MHz platinum-plated (geometrical area of the electrode surface of 0.2 cm^2) quartz crystals (EG&G) were used, mounted on a Well-Type all-Teflon electrochemical cell (SEIKO EG&G) with a platinum wire counter electrode and an Ag/AgCl/KCl (sat'd) reference electrode. A single quartz crystal was used for each experiment and used as received. Electrochemical determination of the electrode area gave a value of 0.202 ± 0.002 cm^2 ($n = 5$) by using potassium ferricyanide electrochemistry in 1 mM potassium nitrate (a diffusion coefficient of 7.19×10^{-6} cm^2 s^{-1} was used for ferricyanide as in Bucur et al., 1978). Silver electrodeposition experiments (Gabrielli et al., 1991) showed frequency changes linear up to 20 μg of mass increase, with a sensitivity of $10.7 \pm 0.3 \times 10^8$ Hz g^{-1} ($n = 3$) as calculated by Sauerbrey's equation.

A Hewlett-Packard (Norwalk, CT, USA) series 1050 pump module was used for the flow-injection (FI) experiments. A chromatographic column (5 μm packing, 250 mm × 4.6 mm) fitted between the pump and the injection valves assured enough back pressure for proper operation of the pump pulse damper. Two Rheodyne (Cotati, CA, USA) model 7125 injection valves were used: the upstream valve equipped with a 1 ml sample loop was used for the "*in situ*" preparation of modified electrodes, while the downstream valve, with a 20 μl loop, was used for the sample injection. An EG&G model 400 electrochemical detector including a thin-layer electrochemical cell with a platinum working electrode (3 mm diameter) and an Ag/AgCl/NaCl (3 M) reference electrode was used. A single thin-layer flowcell gasket (Bioanalytical Systems, Inc., USA) 0.005" thickness was used.

2.3. Preparation of modified electrodes

Before each modification, the platinum working electrode was cleaned by few drops of a hot nitric acid solution followed by extensive washing with bidistilled water, alumina (0.05 μm particles) polishing procedure, extensive washing and sonication in bidistilled water. Then, the electrode was immersed in a 0.5 M sulphuric acid solution and its potential cycled typically between −0.255 and +1.225 V vs. Ag/AgCl/KCl (sat'd) at 200 mV s^{-1} until a steady-state cyclic voltammogram was obtained. The cleaning scans ended with the electrode in the reduced state. The electrochemical pretreatment was followed by copious rinsing with bidistilled water.

Unless otherwise stated, poly(*o*-aminophenol) (p(oAP)) films were electrochemically grown on platinum electrodes by cyclic voltammetry (potential range −0.1 V/+0.9 V vs. Ag/AgCl/KCl (sat'd) at 50 mV s^{-1}) using a 5 mM oAP solution in phosphate buffer (*I* 0.1 M, pH 7). "*In situ*" electrode modification was performed by injecting a 1 ml plug of an oAP solution in the FI system at a constant deposition potential of +0.8 V vs. Ag/AgCl/KCl (sat'd) and at a flow rate of 0.1 ml min^{-1}. The carrier stream was phosphate buffer (*I* 0.1 M, pH 7). When not in use, the flow cell assembled with the modified electrode was left in the FI system run at 0.1 ml min^{-1} flow rate.

Electrochemical enzyme immobilization on platinum electrodes was typically performed by cyclic voltammetry (potential range 0 V/+0.9 V vs. Ag/AgCl/KCl (sat'd) at 50 mV s^{-1}) or at a constant potential of +0.8 V vs. Ag/AgCl/KCl (sat'd) (deposition time 20 min) by using a 5 mM oAP solution containing 500 U ml^{-1} of GOD in phosphate (*I* 0.1 M, pH 7) or acetate (*I* 0.1 M, pH 5.2) buffer. To allow any enzyme adsorption, the electrode was preliminary held at open circuit in the deposition solution for 15 min before commencing the electrochemical deposition step. After deposition, the enzyme electrode was washed in phosphate buffer by rotating at 1000 rpm for 15 min to remove any weakly adsorbed enzyme and stored at 4 °C in the dark in phosphate (*I* 0.1 M, pH 7) buffer when not in use.

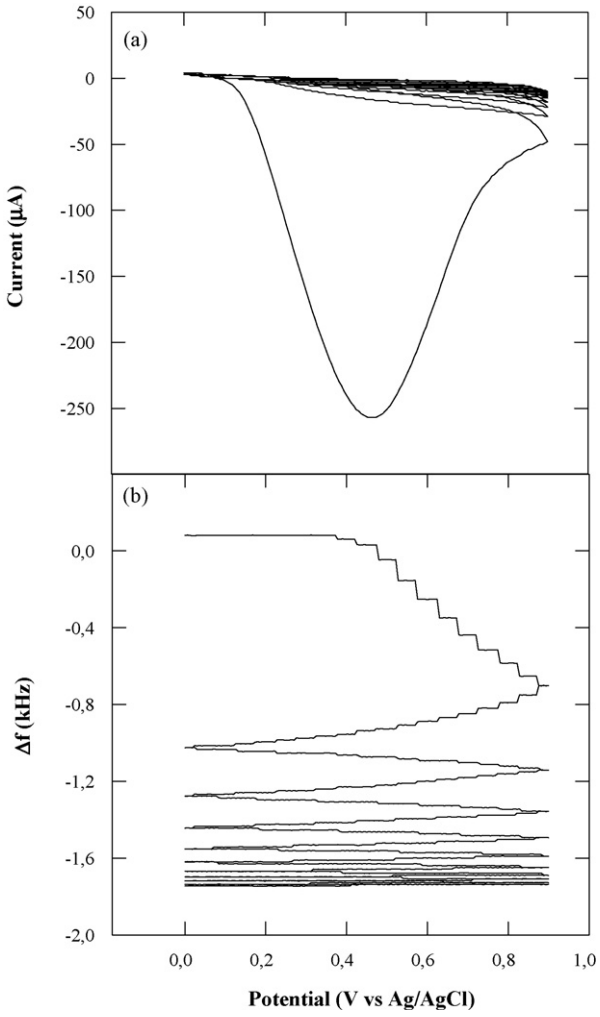

Fig. 1. Voltammetric (a) and microgravimetric (b) profiles observed during the p(oAP) electrosynthesis at a Pt quartz crystal electrode immersed in a 5 mM oAP/phosphate buffer solution (*I* 0.1 M, pH 7). Scan rate: 50 mV s^{-1}; other conditions as described in Section 2.

2.4. Electrochemical measurements

Unless otherwise stated, all the electrochemical measurements were performed using a detection potential of +0.650 V vs. Ag/AgCl/KCl (sat'd) in phosphate buffer (*I* 0.1 M) and the flow rate in FI experiments was 0.2 ml min^{-1}. Solutions and carrier stream were air-saturated and the temperature was ambient (22 °C).

3. Results and discussion

3.1. Electrochemistry of o-aminophenol

A representative cyclic voltammogram of *o*-aminophenol (oAP) on a bare platinum electrode in phosphate buffer at pH 7 is shown in Fig. 1.a. A single peak with a significant diffusional tailing was observed during the first anodic scan while any cathodic process was notable during the reversal scan. After the first scan cycle, the electrochemistry of oAP was still evident, but reduced, and completely suppressed after about ten scans, even after the stirring of the electrolyte to remove any diffusion layer at the electrode/solution interphase. Although any film was visibly evident on the electrode surface, the electrodic modification was so effective and firm to require chemical etching (*e.g.* oxidation with nitric acid) and/or mechanical polishing of the electrode surface to restore the usual electrochemistry of bare platinum. Comparable electrochemical behaviours have been observed at the higher pH values (*e.g.* in borate buffer) as well as in mild acidic media (*e.g.* in acetate buffer). All this implies that the electrochemical oxidation of oAP at platinum electrode in the pH range 4–9 leads to the irreversible formation of a passivating film of poly(*o*-aminophenol) on the electrode surface (here p(oAP)) which does not allow, after full electrode coverage, any further oAP electron transfer.

Similar behaviours were observed from the relevant microgravimetric profiles, *i.e.* the frequency–potential curves, in EQCM mode under the same experimental conditions (see Fig. 1.b). The frequency decreased significantly with the first scan, due to film formation onto the conducting spot of the quartz crystal, and more slowly during the successive scans. Notably, in the first scan cycle, polymer deposition started at potential values proximal to the corresponding anodic peak and continued even during the cathodic scan. Moreover, about half of the overall mass change was observed during the successive scan cycles, even if the oAP electrochemistry was significantly depressed (see Fig. 1.a). These findings indicate that much of the electrochemical charge is spent almost for the initial electrochemical formation of monomer (and/or oligomers) soluble radicals, while polymerization carries on until complete passivation of the electrode surface, which stops radical generation. Note however that the growth of p(oAP) film and the relevant electrode passivation is not so fast and/or exhaustive as in the case of other passivating monomers like 2-naphthol (Ciriello et al., 2000). By the relevant frequency change and using a polymer density value in the range of 1.3–1.5 g cm^{-3} (Skotheim, 1986), a thickness of about 57–65 nm can be estimated for a nearly uniform film onto the electrode surface.

3.2. Permselective behaviour of poly(o-aminophenol) film

In spite of their passivating surface covering, voltammetric experiments carried out at p(oAP) modified platinum electrodes (Pt/p(oAP)) in a pH 7 phosphate buffer displayed anodic and cathodic potential limits very close to those observed at bare platinum electrodes; both hydrogen and oxygen evolution reactions can so easily occur at such modified electrodes meaning that small molecules like water (and hydrogen peroxide, *vide infra*) can efficiently permeate through the film. Larger molecules behaved differently: for example, the electrochemistry of ferrocyanide and ascorbate was completely suppressed at the modified electrode. This suggests the presence of a continuous, insulating film on the electrode surface, free from bulky pin-holes or defects allowing larger electroactive molecules to reach the electrode.

As already outlined, electropolymerization can be achieved in a single-step by an all-chemical procedure possessing a high spatial control: accordingly, this approach was used to prepare a p(oAP) platinum modified electrode "*in situ*", *i.e.* in a flow-injection (FI) system using a conventional platinum disk based thin-layer amperometric cell as detector. Fig. 2.a shows the relevant potentiostatic current–time profile (solid line) at a bare platinum electrode, for the injection of a 1 ml plug of a 5 mM oAP solution in the carrier stream (phosphate buffer, *I* 0.1 M, pH 7) at 0.1 ml min^{-1}. A sharp current drop was observed, quite different from the steady-state current observed for hydrogen peroxide (dashed line in figure) under the same hydrodynamic conditions. The different behaviour suggests that the current–time profile relevant to oAP electrooxidation cannot be due to the trailing edge of the injected plug but to the passivation of electrode surface notwithstanding the continuous renewal of the diffusion layer. Additional 1 ml plug injections of the same oAP solution (see Fig. 2.b and c) showed anyway that the electrode surface can be further passivated. These findings support that

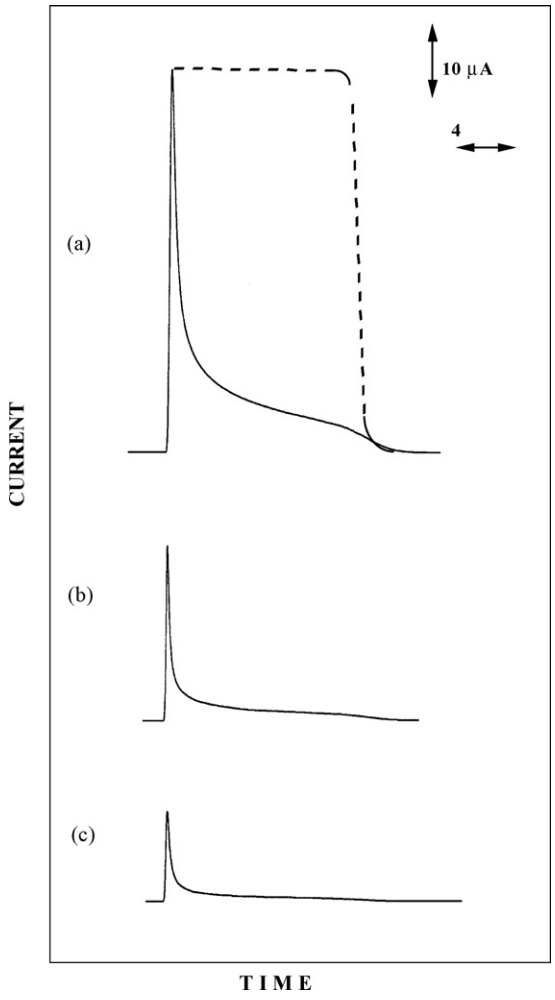

Fig. 2. In-flow current–time profiles (a) for the oxidation of 5 mM oAP (solid line) and 1 mM H_2O_2 (dashed line) solutions obtained during the injection of a 1 ml plug solution in a phosphate buffer (*I* 0.1 M, pH 7) carrier stream. Curve (b) and (c) refer to additional 1 ml plug injections of the same oAP solution. Applied potentials: +0.80 and +0.65 V vs. Ag/AgCl/KCl (sat'd) for oAP and H_2O_2, respectively; other conditions as described in Section 2.

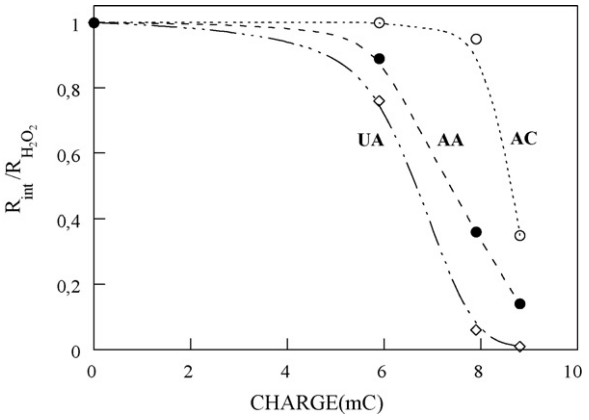

Fig. 3. $R_{int}/R_{H_2O_2}$ ratios (see text for their definitions) relevant to ascorbic acid (AA), uric acid (UA) and acetaminophen (AC) as function of the charge involved in the *in situ* p(oAP) electrosynthesis in the FI system. Experimental conditions as in Fig. 2; ratios for cysteine were omitted for sake of clarity.

in situ electrodic modification by monomer electrooxidation within a FI system is as effective as the classical batch electrochemical technique (Centonze et al., 1992a; Ciriello et al., 2000).

To test the permselective behaviour of the film and for a critical comparison with similar studies until now reported, the electrochemical responses of some of the main potential interferent species occurring in real samples (*e.g.* serum), namely uric acid (UA), acetaminophen (AC), ascorbic acid (AA) and cysteine (CYS), were compared with respect to hydrogen peroxide ones both on bare and modified platinum electrodes. The extent of electrode modification was monitored by the charge under the current–time profiles during the *in situ* electrode modification as due to the injection of variable amounts of a 5 mM oAP solution. The ratio $R_{int} = r_{i,m}/r_{i,u}$ (where $r_{i,m}$ and $r_{i,u}$ are the FI responses of interferent *i* at a modified and unmodified electrode, respectively) was then evaluated for each interferent. Since the hydrogen peroxide response was depressed by the film as well, the permselective behaviour of the film toward hydrogen peroxide detection was then evaluated by the ratio $R_{int}/R_{H_2O_2}$, where $R_{H_2O_2}$ is defined similarly to R_{int}. Fig. 3 shows the relevant ratio $R_{int}/R_{H_2O_2}$ for some interferents as a function of the charge involved during the *in situ* film formation (*i.e.* electrode modification degree). The interferent rejection improved significantly with the charge but was anyway significantly different for each test molecule. Indeed, at about 8 mC, the UA and AC interferences were practically suppressed while the AA rejection grade was as for hydrogen peroxide; however, an increase of about 1 mC definitively reduced the interferent effects even for AA. After that, the film became completely insulating so interference and hydrogen peroxide responses cannot be further measured. The test molecules bear the same charge sign at pH 7 and their sizes are somewhat similar, so ion-exchange and size-exclusion effects should be ruled out to justify this different permselective behaviour. The electrogravimetric experiments (see Fig. 1.b) showed a significant mass increases at the corresponding charges so a thickness effect might be considered.

Figs. 4.a and b compare the FI responses of hydrogen peroxide with UA, AC, AA and CYS at a bare and at a p(oAP) modified platinum electrode, respectively, for concentration values near the upper limits of their respective physiological concentration ranges as far as interferents. The FI time profiles were practically identical suggesting that the electropolymerized p(oAP) film, being very thin, assures a fast response. In contrast, the presence of a passivating film of p(oAP), as here optimized (*i.e.* electrode modification charge of about 9 mC) gave rise to a significant and, in some cases, dramatic change of the FI peak intensities. Interference due to UA was practically suppressed whereas AA, AC and CYS showed still a somewhat but very low permeation. Hydrogen peroxide, on the contrary, permeates significantly through the film producing a valuable signal (almost one-half signal reduction only, $R_{H_2O_2} = 0.43$). This permselective behaviour did not change significantly during the use of these electrodes (*i.e.* some weeks).

The ability of p(oAP) films to reject both UA and AC seems hence promising for real sample analysis applications. Conventional anti-interferent membranes like cellulose acetate, at the contrary, show poor discrimination towards AC, which is the most serious interferent in hydrogen peroxide-detecting biosensors (Wilson and Thevenot, 1990). As far as we know, the present p(oAP) modified platinum electrode displays the top performances in terms of interferent rejection ability (compare with Miland et al., 1996; Zhang et al., 1996; Pan et al., 2004a,b, 2005a,b; Ye et al., 2005; Li and Lin, 2007). The rejection performances here observed were almost similar to those reported for platinized electrodes (Zhang et al., 1996) but significantly better than Prussian blue ones (Pan et al., 2004a,b). Indeed, only highly organised electrodic structures (Ye et al., 2005; Li and Lin, 2007) gave apparently better results for AA and UA but rejection for AC and CYS was not reported. We conclude here that

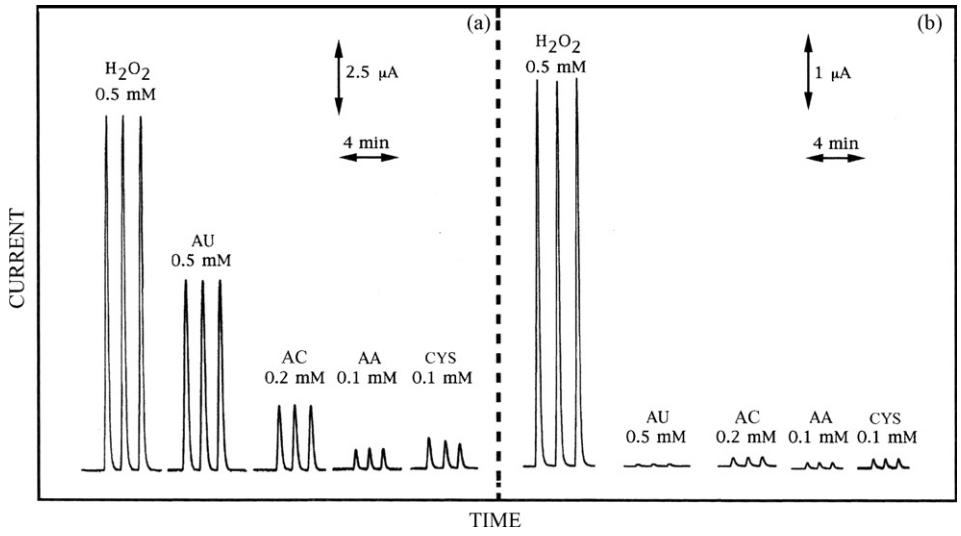

Fig. 4. Flow-injection responses for triplicate injections of 0.5 mM H_2O_2, 0.5 mM uric acid (UA), 0.2 mM acetaminophen (AC), 0.1 mM ascorbic acid (AA) and 0.1 mM cysteine (CYS) at a (a) bare and a (b) p(oAP) modified platinum electrode. Injection volume: 20 μl; charge for p(oAP) electrosynthesis: 9 mC; detection potential: +0.7 V; flow rate: 0.2 ml min^{-1}; other conditions as described in Section 2.

simply a proper tuning of the electrode modification is required for optimal selectivity: larger or unusual electrodic surfaces and/or catalytic modifiers seem then not the key to obtain successfully anti-interferent features.

3.3. Enzyme immobilization

Glucose oxidase (GOD) was immobilised by electrochemical polymerization of oAP onto platinum surface in the presence of GOD in the electrodeposition solution. Besides a plain mechanical entrapment of the enzyme during the film growth, GOD immobilization can mainly occur also by adsorption at the platinum surface (Szucs et al., 1989): indeed a 65–70% contribution only due to pure GOD adsorption was reported for a poly(*o*-phenylenediamine) modified platinum electrode (De Benedetto et al., 1994). Therefore, the electrode was allowed to stand at open circuit in the deposition solution before the electrochemical deposition step in order to control any enzyme adsorption. Cyclic voltammograms relevant to oAP oxidation at platinum electrode in a pH 5.2 acetate or pH 7 phosphate, *I* 0.1 M buffer solution containing GOD (see Fig. S1 in supporting information) were similar to those observed for oAP (see Fig. 1.a); anyway, the current decay with the scan cycles was slowed down by the presence of GOD, a feature not reported elsewhere (Zhang et al., 1996). As the electrochemical oxidation of oAP is mainly a surface process (*vide ante*), this suggests that GOD could compete with the products of oAP electrooxidation for the adsorption at the electrode surface, thus slowing down the p(oAP) film growth. The above difference confirms the significant adsorption of GOD at the electrode surface, therefore representing an effectiveness test of enzyme immobilization. GOD was also immobilised by potentiostatic deposition at +0.8 V vs. Ag/AgCl/KCl (sat'd). Even so, the current–time transients relevant to film deposition showed a slower time decay when GOD was present in solution. Both electrochemical techniques proved useful for immobilization, do not showing any difference in term of performances of the biosensor obtained.

The enzyme loading, a well-known effect on biosensor kinetics never reported before for p(oAP)-based biosensors, was surely controlled by the enzyme concentration in the galvanic bath solution, whatever pure entrapment in the polymer matrix and/or adsorption onto the electrode surface are mainly involved in the immobilization process. Several Pt/GOD/p(oAP) modified electrodes were assembled, in triplicate, by using GOD solutions at several concentrations; their sensitivities were than tested in a phosphate buffer solution (*I* 0.1 M, pH 7) in batch mode and the relevant kinetic data (*i.e.* the apparent Michaelis-Menten constant K'_M and the maximum current response I_{MAX}) extracted. The sensitivity behaviour (see Fig. 5) strongly resembles an adsorption isotherm (even for I_{MAX} measurements); in contrast, the relevant K'_M values remained almost constant over the investigated GOD range, ruling out the diffusion-control kinetic expected at the high enzyme loadings (Mell and Maloy, 1975; Bartlett and Whitaker, 1987a,b). Therefore, the behaviours depicted in Fig. 5 reveal, unfortunately without distinguish them, the interplay between pure adsorption and entrapment of the enzyme, both controlling the response of the present biosensor. Note that the polymer thickness (*vide ante*) is almost ten times the size of a GOD molecule, so that a diffusion effect inside the polymer membrane would be indeed expected; due to the low enzyme amount adsorbed/entrapped in the present case, on the other hand, the mean free kinetic path of the enzyme catalysed reaction may be longer than the membrane thickness, extending well out in solution, so that an external to

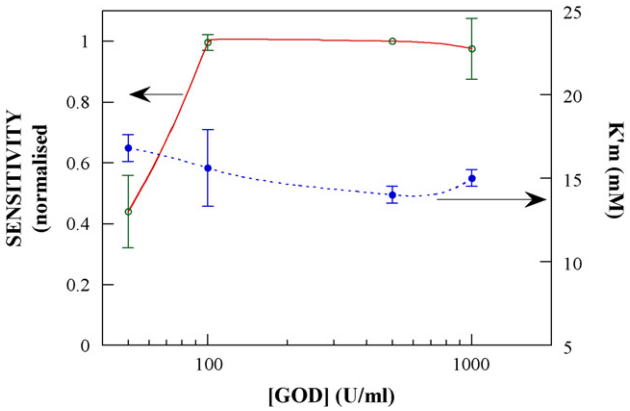

Fig. 5. Sensitivity (left scale) and apparent Michaelis-Menten constant K'_M (right scale) for Pt/GOD/p(oAP) biosensors ($n = 3$) assembled at several GOD concentrations (see text for further experimental details). Rotation rate: 1000 rpm; other conditions as described in Section 2.

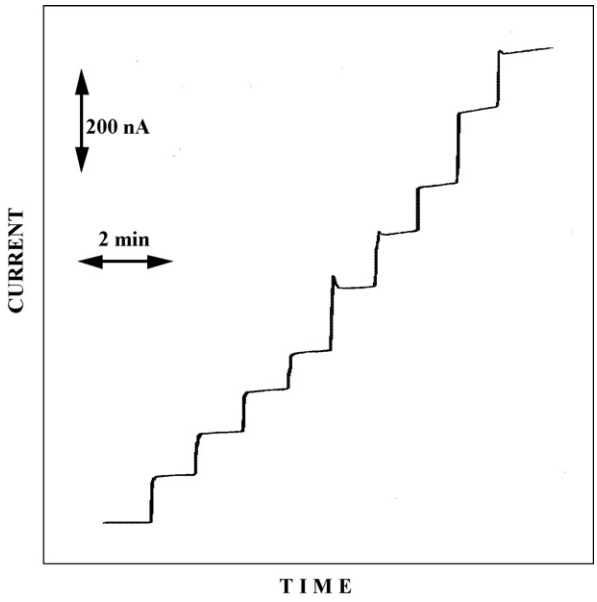

Fig. 6. Current–time responses of a typical rotating disk Pt/GOD/p(oAP) electrode for successive addition of glucose standard solutions to an air-saturated, phosphate buffer supporting electrolyte (*I* 0.1 M, pH 7). Glucose additions in figure refer to total glucose concentrations of 1.25, 2.49, 3.72, 4.95, 7.39, 9.80, 12.19, 16.91, 21.53 and 26.07 mM in supporting electrolyte. Rotation rate: 500 rpm; other conditions as described in Section 2.

membrane mass transport effect might be anticipated. This kind of kinetic control by enzyme catalysis was confirmed by studying the hydrodynamic behaviour of a rotating enzyme electrode (see the Hydrodinamic Behaviour section and Fig. S2 in the supporting information). Since responses increased lowering the rotation rate, this latter resulted a powerful tool for maximising glucose response.

The influence of pH on the Pt/GOD/p(oAP) electrodes was studied over the pH range of 4–9 (see the Influence of pH section and Fig. S3 in the supporting information). Just about the native enzyme, maximal activity was observed at about pH 6 while K'_M at this pH was about 24 mM.

3.4. Analytical performances of biosensor

Fig. 6 shows typical current responses at an enzyme modified rotating disk electrode Pt/GOD/p(oAP) relevant to successive additions of glucose to an air-saturated, phosphate buffer solution (*I* 0.1 M, pH 6). As for hydrogen peroxide, the glucose response was quite fast since the thin thickness of the polymer onto the electrode surface. The response time, $t_{0.95}$, evaluated in batch addition experiments, was less than 5 s and likely influenced by the mixing time in these experiments. Glucose calibration curves obtained at a Pt/GOD/p(oAP) rotating disk electrode behaved as expected for an enzyme-catalysed reaction and were linear up to about 10 mM with a typical sensitivity of 0.6 mA M^{-1} cm^{-2}. The relevant Eadie-Hofstee plots were linear and independent of rotation rate suggesting that the enzymatic reaction is rate controlling without any "in-membrane" diffusional contributions. Linear fitting of data gave a I_{MAX} value of 12 μA cm^{-2} and a K'_M, of 23.4 mM at pH 6. The *in situ* modification ability of this approach (*vide ante*) was used successfully to modify the platinum working electrode of a typical thin-layer electrochemical cell coupled with a FI analysis system. Since of sensitivity and low response time, fast and sensitive FI peaks were observed for glucose injections so as to appear promising for glucose analysis of real samples.

Undoubtedly, the present biosensor, based on a simple Pt disk electrode, displayed a low sensitivity (compare with Pan et al., 2004a,b, 2005a,b; Ye et al., 2005; Li and Lin, 2007) showing the usefulness of using larger electrode surface and/or highly organised electrodic structures to increase the enzyme loading and hence sensitivity. Nevertheless, glucose levels of clinical relevance (*e.g.* blood glucose levels) span well above the micromolar detection limits allowed in these last cases, so that optimal sensitivity is expected to be obtained in real sample analysis applications even with the present (low sensitivity) approach. The biosensor sensitivity can be alternatively further increased in the present case by lowering the rotation rate (or flow rate) with the notable decrease of the faradic interference effects, as known diffusion-controlled. Indeed, a wide linear range is an important requirement for glucose sensing in blood. Pt-polypyrrole nanocomposite glassy carbon electrodes (Li and Lin, 2007) and the present Pt/GOD/p(oAP) sensor are the only ones reaching the highest upper limits, while in the remaining cases (Zhang et al., 1996; Pan et al., 2004a,b, 2005a,b; Ye et al., 2005) dropping in the low millimolar range, below the normal glucose levels. Obviously their application to direct and/or continuous glucose monitoring in blood (like electrodes for fingertip blood sample, artificial pancreas devices) appears impracticable since the required sample dilution is disallowed in these techniques.

Stability studies were performed by monitoring the response of several Pt/GOD/p(oAP) electrodes for some weeks. After a steady fall in sensitivity during the first ten days, lowering the sensitivity up to about 20% of its initial value, the response remained almost stable for at least further 2 weeks upon storage in buffer, even if no particular effort was made to avoid bacterial growth in the storage media. In these conditions, the within-day ($n = 3$) and the between-days ($n = 7$) coefficients of variation for glucose response at 5 mM level were 6% and 24%, respectively.

4. Conclusions

The electrooxidation of oAP at a platinum electrode in neutral buffer solutions leads to the formation of a non-conducting, permselective thin film of p(oAP) which proved successfully for both electroactive interferent rejection and GOD immobilization. Both the permselective and kinetic behaviours of the sensor were investigated, giving light to interesting features never studied and reported before.

From a comparison with similar sensors reported elsewhere, the use of large and/or highly organised electrodic surfaces appears an essential requirement to significantly increase the sensitivity but at the expense of a low linear range. The present biosensor does not display these limitations so that appears promising for glucose level monitoring in blood samples.

We conclude here that simply a proper tuning of the electrode modification is required for optimal selectivity: larger or unusual electrodic surfaces, even combined with catalytic modifiers, seem then not the key to obtain successfully analytical performances in the present case.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.08.004.

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