Permselective Behavior of an Electrosynthesized, Nonconducting Thin Film of Poly(2-naphthol) and Its Application to Enzyme Immobilization

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Abstract
The electrooxidation of 2-naphthol in phosphate buffer at pH 7 leads to the formation of a nonconducting polymer of poly(2-naphthol) on a platinum electrode. Such a resulting thin film displays an interesting permselective behavior, which proved useful in minimizing the interference of ascorbate, acetaminophen, cysteine, and urate sample molecules. Electrochemical detection in flowing streams was used to investigate the relevance of permselectivity for sensor development. Nonconducting poly(2-naphthol) film demonstrated useful also as a novel permselective membrane for glucose oxidase immobilization. The glucose response time, $t_{0.95}$, evaluated in batch addition experiments, was lower than 4 s. The calibration plot was linear up to 15 mM of glucose with a sensitivity of 2.2 nA/mM.

Keywords: 2-Naphthol, Electropolymerization, Modified electrodes, Permselective electrodes, Enzyme immobilization

1. Introduction

The realization and use of chemically modified electrodes based on permselective thin films produced on electrodes surface by electropolymerization of suited organic molecules has gained a growing interest from a technological and analytical point of view. Electropolymerization, indeed, possesses a considerable number of advantages compared to classical routes of electrodic modification, including simplicity, easiness in the control of the electrochemical process and ability to locate the modification exclusively onto the electrode surface. As a result, size exclusion, ion exchange, and hydrophobic interactions, just to mention the main mechanisms usually involved in the pemselective behavior of electrochemically prepared polymers, lead to an improved enhancement of sensitivity, selectivity, and stability of relevant electrode surfaces. To cite some examples of this approach, polymer films electroproduced by 1,2-diaminobenzene [1] and some phenolic and amino-aromatic compounds [2–3] proved useful for the realization of novel pH and ion sensors. Moreover, controlled growth of polyaniline, polyphenol and polypyrrole films allowed the development of selective flow injection measurements as well as major improvements in complex chromatogram acquisition [4]. Interestingly, the use of nonconducting polymers has been also exploited for the realization of novel amperometric enzyme electrodes. With respect to previous strategies used in biosensor construction, this approach offers several advantages, such as two-dimensional control of enzyme immobilization and thickness, miniaturization, multilayer and/or multienzyme structures facilities, while permitting a significant permselectivity. In particular, the use of nonconducting polymers such as poly-*o*-phenylenediamine [5–10], polyphenol [11–12] and overoxidized polypyrrole [9, 13–15] resulted in an improved biosensor selectivity so to allow their employment for real sample analysis [6, 8, 13–15].

The majority of the approaches exploiting permselective films has been mainly focused on the electropolymerization of a number of hetero-atom substituted aromatic compounds such as phenol, phenylenediamines, pyrrole and their derivatives. With the aim to develop novel passivating films to be used as permselective, ultrathin membranes for selective analysis as well as for enzyme immobilization, we are currently investigating similar organic compounds which share in their backbone different functional groups and/or possess a higher number of carbon atoms. In this respect, the use of fused polycyclic hydrocarbon derivatives like substituted naphthalenes seems promising because of their greater hydrophobic interactions eventually involving a tighter structure in the polymer with less degree of solvatation and impaired diffusion of solvatated molecules through the film. While the formation of conducting polymers has been reported [16–19] in organic solvents such as acetonitrile, the electrooxidation of naphthol isomers at several electrodes usually leads to a passivating film from both alkaline [20] and mild acidic [21] aqueous/methanolic mixtures. Moreover, reduction of interferences response at a platinum electrode using electropolymerized films of diaminonaphthalene and aminonaphthol derivatives has been recently reported [22]. Unfortunately, the electropolymerization of relevant monomers was carried out at extreme pH values in these cases, so precluding eventually any effective enzyme codeposition during their growth, if desired.

In the present article, the electrosynthesis of a nonconducting, poly(2-naphthol) thin film at a platinum electrode in neutral buffer solution and the permselective behavior of the relevant modified electrode are presented. While an effective permselective behavior was found of interest in minimizing interference effects by ascorbate, acetaminophen, cysteine and urate, this work demonstrates also that nonconducting films of poly(2-naphthol) can be used as a novel permselective membrane for glucose oxidase immobilization.

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2. Experimental

2.1. Chemicals

2-Naphthol (2-NAP), ascorbic acid (AA), uric acid (UA), acetaminophen (AC, 4-acetamidophenol or paracetamol) and L-cysteine (CYS) were obtained from Aldrich (Aldrich-Chemie, Steinheim, Germany). Potassium ferrocyanide and hydrogen peroxide stock solutions were obtained from Carlo Erba (Milano, Italy). Hexaammineruthenium(III) chloride was purchased from Alfa Products. Glucose oxidase (GOD, type VII from *Aspergillus niger*, 162000 units/g) and β-D-glucose (G) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All the other chemicals were of analytical grade. 2-NAP was purified by sublimation at 90 °C under vacuum. Stock G 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. 2-NAP solutions were thoroughly sonicated at room temperature. Other solutions were prepared just before their use.

2.2. Apparatus and Electrodes

Batch electrochemical experiments were carried out by an AMEL (Milan, Italy) Model 466 polarographic analyzer. The electrochemical cell was a conventional three electrode system with a platinum counter electrode and a Ag/AgCl (KCl saturated) reference electrode. The platinum 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, Princeton, NJ, USA) RDE Model 616 equipped with a platinum disk electrode (4 mm diameter) embedded into a PTFE body. Unless otherwise stated, rotation rate was 500 rpm. Signals were recorded at a Linseis (Linseis, GmbH - Germany) LY 18100 $X-Y$ recorder.

A Hewlett-Packard (Norwalk, CT, USA) series 1050 pump module was used for the flow-injection (FI) experiments. A reversed - phase chromatographic column (5 µm packing, 250 × 4.6 mm) fitted between the pump and the injection valve assured enough back pressure for proper operation of the pump pulse damper, even at low flow rate. Two Rheodyne (Cotati, CA, USA) Model 7125 injection valves were used; the upstream valve equipped with a large sample loop (i.e., 1 mL) was used for the "in-situ" preparation of modified electrodes (vide infra), while the downstream valve, equipped 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 a Ag/AgCl, 3 M NaCl reference electrode was used. A single thin layer flowcell gasket (Bioanalytical Systems, Inc., West Lafayette, IN, USA) 0.005 thickness was used. A PTFE tubing (0.5 mm ID) was used to connect the sample injection valve to the electrochemical cell. FI signals were recorded on a Servogor (BBC Goerz Metrawatt) Model 120 stripchart recorder.

2.3. Preparation of Modified Electrodes

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

Unless otherwise stated, poly-2-naphthol (p(2-NAP)) films were electrochemically grown on platinum electrodes by cyclic voltammetry (potential range −0.1 to +0.8 V (vs. Ag/AgCl, KCl satd.) at 50 mV/s) using a 3.7 mM 2-NAP solution in a phosphate buffer ($I = 0.1$ M, pH 7). "In-situ" electrode modification was performed by injecting a 1 mL plug of a 2-NAP solution (vide ante) in the FI system at a constant deposition potential of +0.8 V (vs. Ag/AgCl, KCl satd.) and at a flow rate of 0.1 mL/min. When not in use, the flow cell assembled with the modified electrode was left in the FI system running at a 0.1 mL/min flow rate.

Electrochemical enzyme immobilization on platinum electrodes was typically performed by cyclic voltammetry (potential range 0 to +0.9 V (vs. Ag/AgCl, KCl satd.) at 50 mV/s) or at a constant potential of +0.8 V (vs. Ag/AgCl, KCl satd.) (deposition time 20 minutes) by using a 3.7 mM 2-NAP solution containing 500 U/mL of GOD in a phosphate ($I = 0.1$ M, pH 7) or acetate ($I = 0.1$ M, pH 5.2) buffer. To allow enzyme adsorption on the electrode surface, the platinum electrode was preliminary held at open circuit in the deposition solution for 15 min before commencing the electrochemical deposition step. After the deposition, the enzyme electrode was washed in a phosphate buffer solution by rotating at 1000 rpm for 15 min to remove any weakly adsorbed enzyme.

2.4. Electrochemical Measurements

Unless otherwise stated, all the electrochemical measurements were performed using a detection potential of +0.650 V in a phosphate buffer ($I = 0.1$ M). Solutions and carrier stream were air-saturated and the temperature was ambient. In batch mode, G response was obtained by measuring the current difference after the injection of the desired amount of G in a stirred buffer. Unless otherwise stated, the flow rate in FI experiments was 0.2 mL/min.

3. Results and Discussion

3.1. Voltammetric Behavior of 2-Naphthol

A representative cyclic voltammogram of 2-naphthol (2-NAP) on a platinum electrode in phosphate buffer solution at pH 7 is shown in Figure 1. Two anodic peaks, A1 and A2, were observed during the first anodic scan while no cathodic process was notable during the reversal scan, thus suggesting that both the electrochemical processes corresponding to A1 and A2 peaks were irreversible. Apparently, after the first scan cycle (see scan 2 in Figure 1), the electrochemistry of 2-NAP was completely suppressed, even after the abatement of diffusion layer at the electrode surface; moreover, a significant background current decrease was observed. Although no presence of film was evident on the electrode surface, the electrodic modification was so effective and firm to require chemical etching (i.e., oxidation with nitric acid) or mechanical polishing of the electrode surface to restore the usual electrochemistry of bare platinum. These vol-

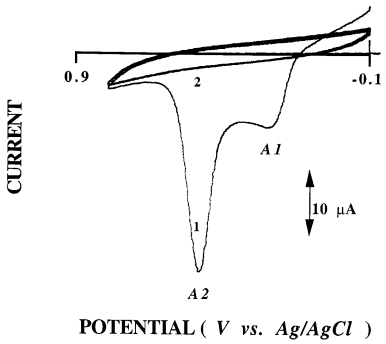

Fig. 1. Cyclic voltammograms of a bare platinum disk electrode (3 mm diameter) in a 6 mM 2-NAP/phosphate buffer solution ($I = 0.1$ M, pH 7). Scan rate: 50 mV/s. Other conditions as described in Section 2. Numbers refer to the scan cycle.

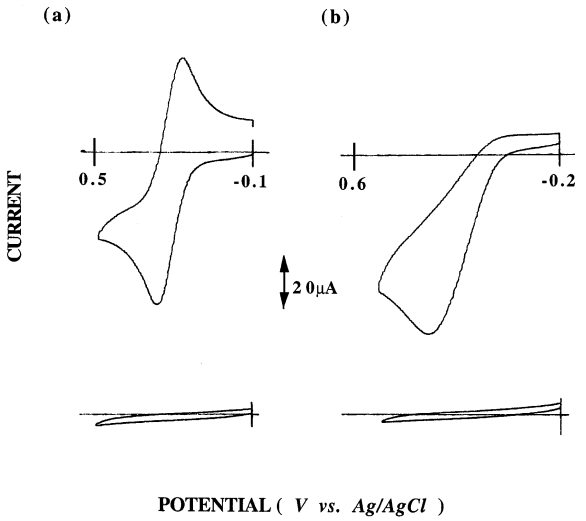

Fig. 2. Permselective behavior of a p(2-NAP) modified platinum electrode towards a) ferrocyanide and b) ascorbic acid electrochemistry. Cyclic voltammograms relevant to a) ferrocyanide (5 mM) and b) ascorbic acid (5 mM) in a phosphate buffer solution ($I = 0.1$ M, pH 7) at a bare platinum electrode (upper curves) and at a p(2-NAP) modified platinum electrode (lower curves). Electrode diameter: 3 mm; scan rate: 50 mV/s. Electrode modification as described in Section 2.

tammetric behaviors imply that the electrochemical oxidation of 2-NAP at platinum electrode in neutral buffer solution leads to the irreversible formation of a passivating film of poly(2-NAP) on the electrode surface which does not allow any further 2-NAP electron transfer. A more detailed study of 2-NAP electrochemistry as well as an XPS characterization of the relevant polymeric film will be reported elsewhere.

3.2. Permselective Behavior of Poly(2-naphthol) Film

The permselective behavior of platinum electrodes modified by a passivating film of poly(2-NAP) was investigated by either cyclic voltammetry or constant potential amperometric detection in a flow injection (FI) system.

In spite of their passivating surface covering, voltammetric experiments carried out at p(2-NAP) modified platinum electrodes (Pt/p(2-NAP)) in a pH 7 phosphate buffer supporting electrolyte showed anodic and cathodic potential limits very close to those usually observed at bare platinum electrodes. This means that both hydrogen and oxygen evolution reactions can easily occur at such a modified electrode indicating that small molecules, such as water (as well as hydrogen peroxide, vide infra), can efficiently permeate through the film.

A different behavior was observed for relatively larger molecules. In this respect, Figure 2 compares the voltammetric behavior of a) ferrocyanide and b) ascorbic acid (AA) at both bare (top curves) and Pt/p(2-NAP) (bottom curves) electrodes. As can be seen, the ferro/ferricyanide reversible redox couple as well as the irreversible oxidation of AA were both completely suppressed at the modified electrode. This is apparently consistent with the presence of a continuous, insulating film on the platinum electrode surface, free from bulky pin-holes or defects allowing electroactive molecules to reach the electrode surface. Similar permselective behaviors have been reported for other insulating polymers such as poly-*o*-phenylenediamine [5–10], overoxidized polypyrrole [9, 13–15], polyphenol and its derivatives [4, 11–12].

To get further insights about the permselective behavior of p(2-NAP) film, the electrochemistry of a positively charged probe was also investigated. Thus, hexaammineruthenium(III) was chosen because of its similar size to ferrocyanide and its fast electron transfer kinetic at platinum electrodes. Cyclic voltammetry studies (not shown) of hexaammineruthenium(III) at a p(2-NAP) modified platinum electrode showed also for this molecular probe a complete suppression of its reversible electrochemistry. These voltammetric experiments suggested to us that ion exchange cannot be limiting in the mechanism of exclusion controlling the permselective behavior of p(2-NAP) films, at least for the electrochemical probes here investigated. It is worth to note that an opposite behavior, i.e., a striking electrostatic control on analyte permeation, has been reported in the case of overoxidized polypyrrole thin films grown on graphite and platinum electrodes [23].

3.3. In Situ Electrodic Modification and Antiinterferent Properties

Since platinum electrodes modified with p(2-NAP) films displayed an interesting and promising permselective behavior towards both anionic and cationic species, further studies were carried out to verify the analytical usefulness of such an electropolymerized film. In this respect, electrochemical detection in FI system was used to investigate the relevance of its permselective behavior for the development of novel sensors. Indeed, the possibility of an exclusion of significant interferences in real sample analysis could greatly enhance the selectivity of FI measurements.

As already outlined, electropolymerization possesses the significant advantage in that electrodic modification can be achieved in a single-step by an all-chemical procedure possessing a high spatial control. Accordingly, such features were herewith investigated to prepare a p(2-NAP) platinum modified electrode "in situ", i.e., in a closed system consisting of a FI system provided of a conventional platinum based thin-layer amperometric cell. Figure 3 shows the relevant potentiostatic current–time profile (solid line) recorded during the injection of a 1 mL plug of a 5 mM 2-NAP solution in the carrier stream at 0.1 mL/min. As can be seen, 2-NAP exhibits a sharp current drop, quite different from that observed for hydrogen peroxide (dashed line in the figure) under the same hydrodynamic conditions. Such a different

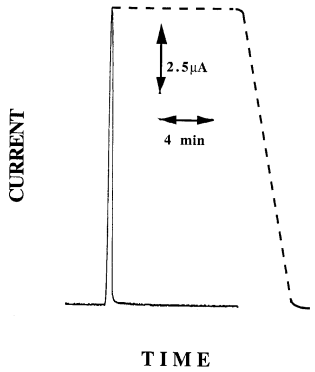

Fig. 3. In-flow current–time profiles for the oxidation of 5 mM 2-NAP (solid line) and 1 mM H_2O_2 (dashed line) solutions obtained during the injection of a 1 mL plug solution in a phosphate buffer ($I = 0.1$ M, pH 7) carrier stream. Applied potentials: +0.80 V and +0.65 V (vs. Ag/AgCl) for 2-NAP and H_2O_2, respectively. Other conditions as described in Section 2.

behavior suggests that the current–time profile relevant to 2-NAP electrooxidation cannot be due to the trailing edge of the injected plug but, as already observed in the case of quiescent solutions (vide ante), to a strong passivation of electrode surface in spite of a continuous renewal of the diffusion layer. These experimental findings show that, as in the case of poly-*o*-phenylenediamine [6], in situ electrodic modification by 2-NAP electrooxidation within a FI system is as effective as the classical batch electrochemical techniques.

In order to verify the usefulness of p(2-NAP) electrodic modification on the selectivity of FI measurements, some of the main potential interferent species occurring in real samples were tested with respect to hydrogen peroxide response. Figure 4a and b compare the FI amperometric responses of hydrogen peroxide, uric acid (UA), acetaminophen (AC), ascorbic acid (AA), and cysteine (CYS) at a bare and at a p(2-NAP) modified platinum electrode, respectively, for concentration values near the upper limits of their respective physiological concentration ranges as far as interferents. As can be seen, the presence of a passivating film of p(2-NAP) gives rise to a significant and, in some cases, dramatic change of the FI peak intensities. Interferences due to AA, UA, and AC were completely suppressed whereas CYS showed some moderate permeation. In particular, the electrodic modification reduced the UA response by a factor of 2300 whereas CYS permeation was reduced one hundred-fold. Furthermore, such a permselective behavior did not change significantly during all the time (i.e., some weeks) of continuous or discontinuous use of these electrodes; this comportment corroborates the stability already outlined in the voltammetric experiments (vide ante). The ability of p(2-NAP) films to reject both UA and AC seems promising for real sample analysis applications; conventional antiinterferent membranes such as those based on cellulose acetate, at the contrary, show poor discrimination towards AC, which at the present is the most serious interferent in hydrogen peroxide–detecting biosensors.

In spite of the passivating layer, hydrogen peroxide, on the contrary, permeates significantly through the film producing a valuable signal (thirty-fold signal reduction only). Interestingly, the FI time profiles (compare Fig. 4a and b) were practically identical, irrespective of the electrode employed. This suggests that the electropolymerized p(2-NAP) film, being very thin, assures a fast response which is comparable with that obtained at the unmodified platinum electrode. All these features were quite appealing since it anticipates the possibility to use p(2-NAP) films as a permselective membrane in amperometric biosensor.

3.4. Enzyme Immobilization

One of the most interesting features of electrosynthesized polymer films is their ability to entrap, during their growth onto the electrode surface, biological molecules like enzymes. More importantly, the use of nonconducting, permselective thin films allows the realization of amperometric biosensors with fast

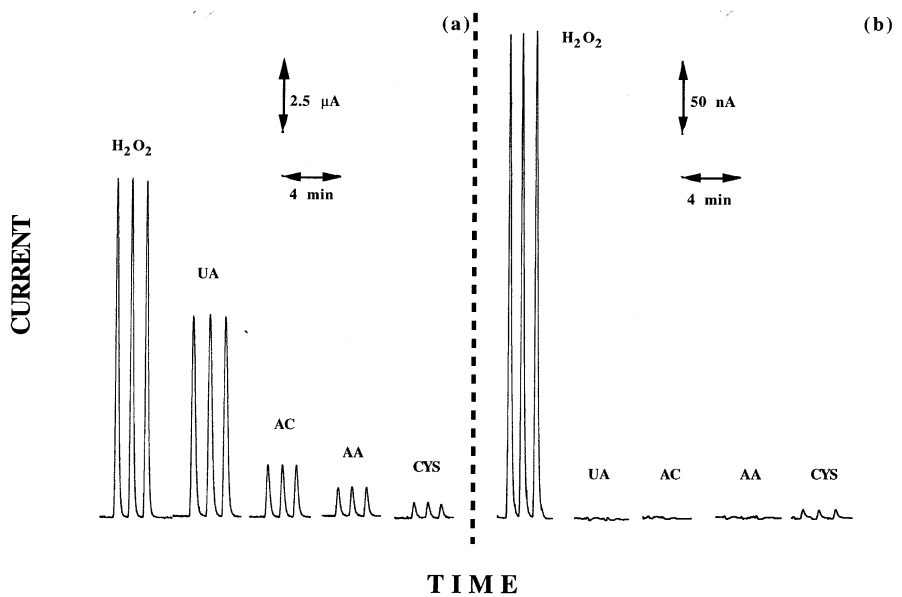

Fig. 4. Flow-injection responses for triplicate injections of 0.5 mM H_2O_2, 0.5 mM uric acid (UA), 0.2 mM acetaminophen (AC), 0.1 mM ascorbic acid (AA) and 0.1 mM cysteine (CYS) at a) a bare and b) a p(2-NAP) modified platinum electrode. Injection volume: 20 μL. Other conditions as described in Section 2.

response and improved antiinterferent and antifouling properties. Here, the immobilization of the enzyme glucose oxidase (GOD) was investigated by electrochemical polymerization of 2-NAP 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 by adsorption at the platinum surface [24, 25]. Therefore, the platinum electrode was allowed to stand at open circuit in the deposition solution before the electrochemical deposition step in order to control any enzyme adsorption.

Figure 5 shows a typical cyclic voltammogram relevant to 2-NAP oxidation at platinum electrode in a pH 7 phosphate buffer solution containing GOD. With respect to the voltammetric behavior of 2-NAP already shown in Figure 1, two meaningful differences were observed. Although the current densities of peak A2 were similar in both cases, peak A1 was much less intense in the presence of GOD in solution. The different voltammetric behavior with the subsequent scans was another important feature between voltammograms shown in Figure 1 and 5. As can be seen, the current decay with the scan cycles was less pronounced in the presence of GOD. As the electrochemical oxidation of 2-NAP is mainly a surface process (vide ante), these experimental findings suggest that GOD may compete with the products of 2-NAP electrooxidation for the adsorption at the electrode surface, thus slowing down the p(2-NAP) film growth and consequently the electrode surface passivation. Similar results have been observed also for the electropolymerization of pyrrole in the presence of GOD [13]. This difference in the electrochemical behavior also implies a significant adsorption of GOD at the electrode surface, therefore representing an indicative parameter of the goodness of enzyme immobilization. GOD immobilization was also investigated by potentiostatic deposition at +0.8 V (vs. Ag/AgCl, KCl satd.). Again, in agreement with the above findings, the current–time transients (not shown) relevant to film deposition showed a slower time decay when GOD was present in solution. Both electrochemical techniques proved useful for GOD immobilization and did not show any significant difference in term of biosensor performances. Nevertheless, the potentiostatic technique was preferred for biosensor preparation because of its simplicity and easiness in the control of the electrochemical process.

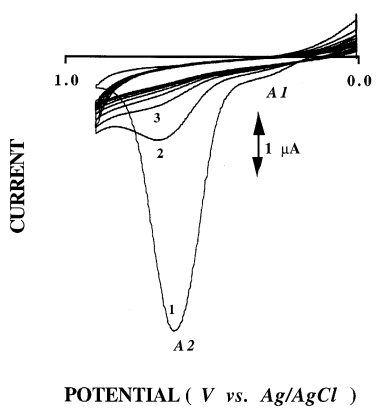

Fig. 5. Cyclic voltammograms of a bare platinum disk electrode (1 mm diameter) in a 3.7 mM 2-NAP/phosphate buffer solution ($I = 0.1$ M, pH 7) containing glucose oxidase (500 U/mL). Scan rate: 50 mV/s; open circuit enzyme adsorption time: 15 min. Other conditions as described in Section 2. Numbers refer to the scan cycle.

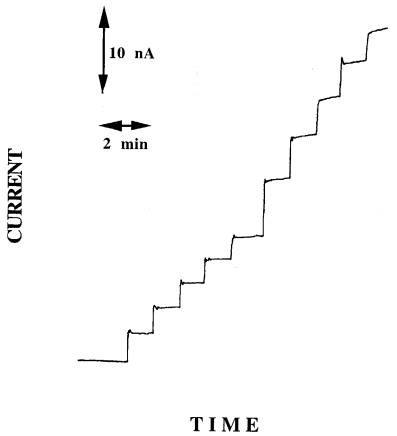

Fig. 6. Current–time responses of a typical rotating disk Pt/GOD/p(2-NAP) electrode for successive addition of glucose standard solutions to an air-saturated, phosphate buffer supporting electrolyte ($I = 0.1$ M, pH 7). Glucose additions in the figure refer to total glucose concentrations of 1.0, 2.0, 3.0, 4.0, 5.0, 7.9, 10.3, 12.7, 15.0, and 17.4 mM in supporting electrolyte; rotation rate: 500 rpm. Other conditions as described in Section 2.

Figure 6 shows typical current responses at an enzyme modified rotating disk electrode Pt/GOD/p(2-NAP) relevant to successive additions of G to an air-saturated, phosphate buffer solution (pH 7). As in the case of hydrogen peroxide, the G response was quite fast probably because the polymer onto the electrode surface was very thin and the enzyme kinetic fast. The response time, $t_{0.95}$, evaluated in batch addition experiments, was less than 4 s in the worst cases and was likely mainly influenced

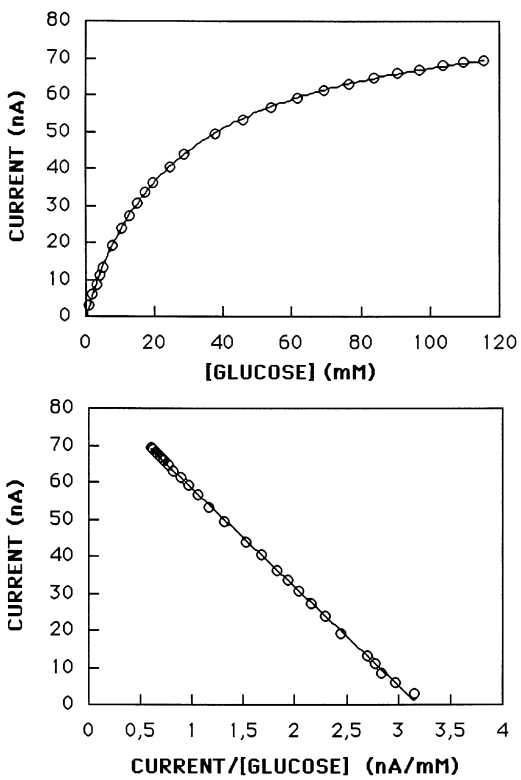

Fig. 7. Glucose calibration plot (upper graph) and relevant Eadie-Hofstee plot (lower graph) obtained at a typical rotating disk Pt/GOD/p(2-NAP) electrode. Experimental conditions as in Figure 6.

by the mixing time in these experiments. Figure 7 shows a typical G calibration plot obtained at a Pt/GOD/p(2-NAP) rotating disk electrode. The plot showed the well-known behavior expected for an enzyme-catalyzed reaction with linear and saturated response at low and high substrate concentration, respectively. Calibration plots were linear up to 15 mM with a typical sensitivity of 2.2 nA/mM. The relevant Eadie-Hofstee plots (see Figure 7) were linear suggesting that in the present case the enzymatic reaction is rate controlling [26–28]. Linear fitting of data gave a maximum current under saturating substrate conditions, I_{max}, of 85 nA and an apparent Michaelis-Menten constant, K'_m, of 27 mM at pH 7. Since reported values for the Michaelis-Menten constant of GOD lie around 20 mM [29], this suggests that GOD immobilization by p(2-NAP) electrosynthesis did not influence significantly the enzyme catalysis.

The stability of such enzyme electrodes was preliminary evaluated by monitoring their responses over a period of some weeks. After an initial fall in sensitivity, the response remained almost stable upon storage in buffer. In particular, the within-day ($n = 3$) and the between-days ($n = 7$) coefficients of variation for G response at 5 mM level were 6 % and 24 %, respectively.

4. Conclusions

The electrooxidation of 2-NAP at a platinum electrode in neutral buffer solution leads to the formation of a nonconducting, permselective thin film of p(2-NAP) which proved useful for electroactive interferent rejection of AA, AC, CYS, and UA. Electrochemical deposition of p(2-NAP) in the presence of GOD in solution permits the realization of a novel amperometric enzyme electrode which seems promising for G analysis in real samples. Work is in progress in this last direction.

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