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ScienceDirect
Bioelectrochemistry 69 (2006) 41 – 48
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

Immobilization of tyrosinase on poly(indole-5-carboxylic acid) evidenced by electrochemical and spectroscopic methods

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Received 1 September 2005; received in revised form 24 November 2005; accepted 28 November 2005
Available online 19 January 2006

Abstract

A conducting, polymeric film of poly(indole-5 carboxylic acid) has been prepared by electrochemical polymerization for covalent immobilization of an enzyme belonging to the family of phenoloxidases-tyrosinase. The polymer was characterized by cyclic voltammetry, UV–VIS and Raman spectroscopy in a buffer solution. As the polymer contains pendant carboxylic groups one-step carbodiimide method was used to immobilize tyrosinase on the polymer matrix. Immobilization of tyrosinase was confirmed by surface enhanced resonance Raman scattering spectra (SERRS) and by cyclic voltammetry as well. Tyrosinase was shown to retain its biological activity when being immobilized on the polymer surface. As proved by the electrochemical and spectroelectrochemical (UV–VIS) experiments, tyrosinase covalently bonded to the polymer matrix effectively catalyzes oxidation of catechol. The reduction current of *o*-quinones was measured as a function of catechol concentration. The linear dependence was found to be 15 μM of catechol with sensitivity of 250 mA/M cm^2.

Keywords: Tyrosinase; Immobilization; Poly(indole-5 carboxylic acid); Raman spectra

1. Introduction

In recent years there has been a growing interest in the development of microbiosensors based on conducting polymers (CP) and enzymes [1–8]. Electrically conducting polymers have significant flexibility in the available chemical structure which can be easy modified as required. They have also the ability to transfer efficiently electric charge produced during the biochemical reaction to electronic circuit. Another advantage in application of CP is that they can be directly chemically or electrochemically synthesized on electrodes of any size and geometry. Their unique properties like electrochemically controlled reversible doping–undoping, accompanied by significant changes in conductivity, redox and spectroscopic properties allows to use them as a suitable matrix in different biosensor constructions. The crucial problem in construction of such devices is to find the way for stable, reproducible immobilization of enzymes, preserving their biological activity. The simplest way of enzyme immobilization on the CP surface is the physical adsorption, where the binding forces involved hydrogen bonds, coulombic interaction, etc. [2,7,9,10].

The other way, widely used, is entrapment of enzymes in the CP matrix during electrochemical deposition of the polymeric films [2,11–16]. However such entrapment generates the steric hindrance and reduces the catalytic activity since accessibility of reagents to biomolecules is limited. One of the ways to elude this problem is to apply the electropolymerized, functionalized polymers where the attachment of enzymes can be carried out directly onto polymer surface by chemical binding.

The quantification of phenolic compounds in environmental, industrial, and food samples is of great interest. The electrooxidation of phenols received attention because of the possibility of amperometric and voltammetric detection. Unfortunately, the oxidation of phenols at solid electrodes produces the phenoxy radicals which couple to form a passivating polymeric film at the electrode surface [17–19]. Many strategies have been developed to overcome this problem, one of them was to apply the enzyme sensors. Tyrosinase (polyphenol oxidase, PPO) is a very well known enzyme which catalyzes the oxidation of phenols and

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doi:10.1016/j.bioelechem.2005.11.001

diphenols to orto-quinones in the presence of dioxygen [4,8]. The simplified reactions are as follows:

$$\text{Phenol} + \text{PPO}(\text{O}_2\} \rightarrow \text{catechol}$$

$$\text{Catechol} + \text{PPO}\{\text{O}_2) \rightarrow o - \text{quinone} + \text{H}_2\text{O}$$

o-Quinones can be electrochemically reduced to *o*-diphenols without any electron transfer mediator. It suggested the construction of amperometric sensors based on the reduction of *o*-quinones. However quinones may spontaneously react with each other to form oligomers or may be attacked by nucleophiles [20,21]. In effect, the PPO modified electrode may be blocked by some products or by insulating polymer film. Thus various PPO-biosensors containing different matrix and mediators were constructed. They involve carbon paste, glassy carbon electrode modified with gold nanoparticles, Nafion membranes, sol–gel systems, clays, self-assembled monolayers [22–30] and conducting polymers [14–16,31–34]. However, to our knowledge, there are only a few papers reporting covalent attachment of PPO on functionalized conducting polymer [35,36].

In our report we describe novel results of immobilization of tyrosinase on the surface of electrochemically polymerized poly(indole-5 carboxylic acid) (PIn5COOH). Indole-5 carboxylic acid (In5COOH) was for the first time electropolymerized by Waltman et al. [37]. Subsequently Bartlett et al. [38] investigated an influence of pH on the redox behaviour of PIn5COOH in aqueous solutions. The molecular structure of PIn5COOH in acidic aqueous solution was determined by Talbi et al. [39]. The polymer was shown to contain pendant carboxylic groups. Because the carboxylic groups allow chemical binding of enzymes we used one-step carbodiimide method to immobilize tyrosinase onto the PIn5COOH surface [40]. The immobilization of tyrosinase on the polymer film was proved by in situ surface enhanced resonance Raman spectra (SERRS). By using electrochemical and spectroelectrochemical experiments an evidence of activity of thus immobilized tyrosinase with respect to catechol was given.

2. Experimental

Electropolymerization of indole-5-carboxylic acid was carried out on a Pt disc (geometric surface area 0.03 cm^2), Pt gauze or ITO (indium tin oxide coated glass) electrode from acetonitrile solution containing 10^{-2} M of monomer and 0.1 M $LiClO_4$. Before electropolymerization the Pt electrodes were polished to a mirror finish with alumina powder (disc) or were annealed in flame (gauze). ITO electrodes were cleaned in sonic bath.

For polymer film formation cyclic voltammetry was used and the potential range was set from −0.2 V to 1.1 V and back to −0.2 V at a sweep rate of 10 mV/s. After polymerization the electrode was carefully washed with a mixture of acetonitrile and water and finally with pure water. On such prepared surface, a drop of PPO solution was added and a crystal of binding agent *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDAC) was also added. After about 10 min the electrode was washed with water and used for measurements. PPO solution was prepared by dissolution of 10^{-4} g in 0.5 ml of water. To enhance the Raman spectra a thin layer of Ag was deposited on the polymer surface by dipping the formerly reduced polymer into 0.1 M $AgNO_3$ solution.

All electrochemical measurements were carried out by using AUTOLAB equipped with the potentiostat PGSTAT (Ecochemie, Netherlands). The measurements were made in a three-electrode cell with a platinum gauze counter electrode and an aqueous 1 M silver chloride electrode as the reference. Optical spectra were recorded using a double beam UV–VIS spectrophotometer (Lambda 12, Perkin-Elmer). Raman spectra were recorded with Jobin-Ivon Spex T64000 spectrometer equipped with a 1024×256 pixel nitrogen-cooled CCD detector and an Olympus BX40 microscope with a 50× long-distance objective. The 488 and 647.1 nm lines of the Kr^+–Ar^+ laser (Laser-Tech, the LJ-800 model) were used to excite the Raman spectra. To avoid intensive heating of the sample the laser power at the sample was not higher than 10 mW.

Indole-5-carboxylic acid, CH_3CN, $LiClO_4$ were purchased from Aldrich and used as received.

Mushroom tyrosinase (EC 1.14.18.1) exhibiting activity of ca. 4800 units/mg of solids and EDAC were supplied by Sigma. Phosphate buffer was prepared using KH_2PO_4 and NaOH (POCh). Catechol was of analytical grade (from POCh). All solutions were prepared with milliQ water (Millipores).

3. Results and discussion

3.1. Electrochemical and Raman evidences of PPO immobilization

Fig. 1 presents cyclic voltammograms (CV) obtained during electropolymerization of In5COOH acid in acetonitrile solution. The first CV curve exhibits only one anodic peak at 0.95 V, which is associated with a monomer oxidation. During successive cycles this peak shifts to less positive potentials and the oxidation current of a monomer increases.

The polymer exhibits two characteristic redox processes in acetonitrile solution. The first is taking place at 0.1–0.2 V (see inset in Fig. 1). It is believed that this process can be ascribed to the creation of radical cations and the formation of a very stable polycationic intermediate form [41]. The second process takes place at 0.9 V, so it coincides with the oxidation of monomer. This process, accompanied by deprotonation of the intermediate state, leads to a fully oxidized polymer form.

Our further experiments showed that the thickness of the polymeric films was crucial for PIn5COOH application in aqueous solutions. If the films were too thick the polymer layer striped off the Pt electrode surface. Usually, the polymeric films were produced by applying four deposition cycles. To control film thickness we monitored the charge passed during polymerization reaction. The charge consumed by film doping was subtracted from the value of the polymerization charge. For that purpose a CV curve was recorded in a monomer free acetonitrile solution after each polymerization cycle (see inset in Fig. 1). For experiments with tyrosinase and catechol we

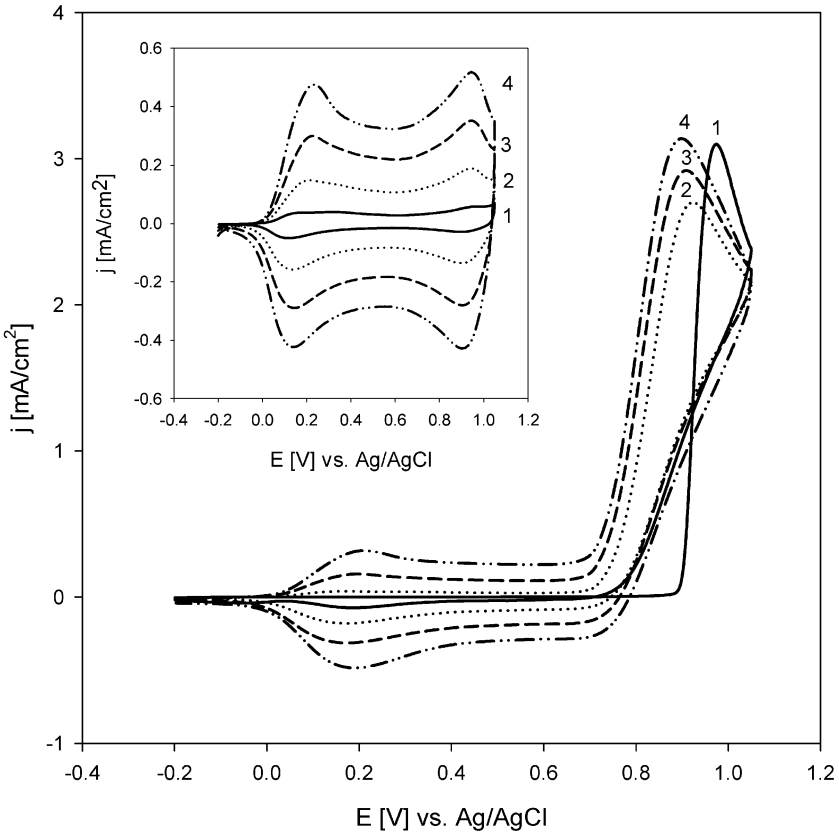

Fig. 1. CV curves recorded during electropolymerization of In5COOH on Pt electrode in acetonitrile solution containing 0.1 M $LiClO_4$ and 0.01 M of monomer, scan rate 10 mV/s^{-1}, 1, 2, 3, 4—subsequent cycles. The inset shows CV curves recorded on the Pt/PIn5COOH electrode in a monomer free solution after (1), (2), (3), and (4) cycle of electrodeposition, scan rate 10 mV/s.

applied the polymer films obtained with 0.55–0.57 C/cm^2 charge passed. To estimate the thickness of the polymer films the following rough assumptions were made:

- the number of electrons involved in the polymerization of a monomer is 2 (as for pyrrole) [42], density of the polymer is similar to that of polypyrrole and amounts to 1.5 g/cm^3 [42],
- the total charge measured in the subsequent cycles was exclusively consumed by the monomer and polymer oxidation, i.e. there were no side reactions. The outcome thickness value estimated with these assumptions was about 2 μm.

It has been shown by Bartlett et al. that the polymer is stable and electroactive in aqueous solution in the broad pH range up to pH 5 [38]. We investigated more carefully the electrochemical properties of the polymer in buffered solutions at pH 6.5 and we found that the polymer is still electroactive and conducting. However, to obtain the reproducible CV curves at higher pH the polymer should be stabilized by continuous cycling (about 20–30 cycles). Fig. 2a presents the CV curves recorded in buffered solution (pH=6.5) at different sweep rates. As may be seen only a single redox process is observed. It can be ascribed to the transition from undoped, neutral form of the polymer to the partially oxidized form of the polymer containing radical cations. The plot of square root of a sweep rate $v^{1/2}$ was linear (not shown here). The latter suggests that the charge transfer in the polymeric film is limited by diffusion of the counter ions. Immobilization of tyrosinase on the polymer surface resulted in a decrease of anodic and cathodic currents. It is clearly seen in Fig. 2b, where the CV curves recorded on Pt/PIn5COOH and Pt/PIn5COOH/PPO electrodes in buffered solutions are compared. The CV curves were recorded for different sweep rates and the results obtained at 2 mV/s were used to determine the average charge consumed in the oxidation and reduction of the polymeric films. These charge values were evaluated by the redox peak integration and for a given sample (Fig. 2b) were found to be 1.4×10^{-3} C for Pt/PIn5COOH electrode and 8.2×10^{-4} C for Pt/Pin5COOH/PPO electrode. By using a simple equation: $\Gamma = Q/nFA$, where Γ is the surface concentration of electroactive sites (mol/cm^2), Q is the average charge spent on the oxidation and reduction of the polymeric film, n is a number of electrons involved in the redox process of the polymer (in this case 2), F is the Faraday constant, A is an area of the polymer surface (usually a geometric area of electrode) we can estimate the changes in Γ after PPO immobilization and in consequence the percentage of the surface electroactive sites involved in the PPO binding. We found that depending on the sample about 40–50% of the surface electroactive sites were involved in immobilization of PPO.

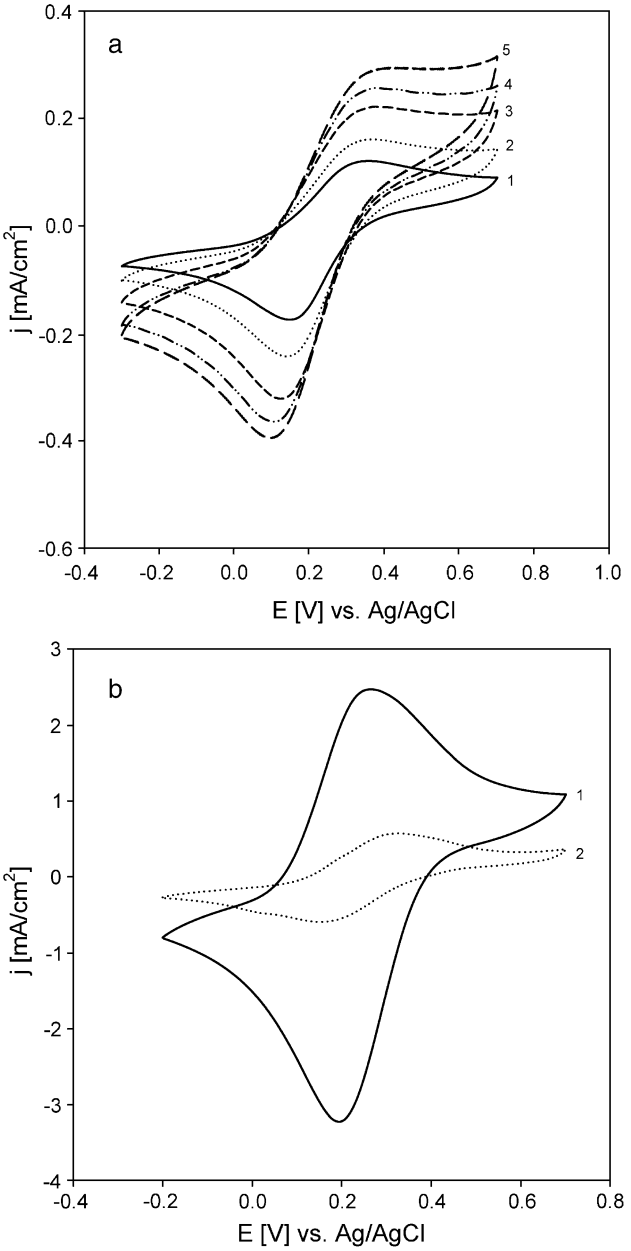

Fig. 2. CV curves recorded on: (a) Pt/ PIn5COOH electrode in buffer solution (pH=6.5); scan rates: 10 (1), 20 (2), 40 (3), 60 (4), 80 (5) mV/s^{-1}; (b) Pt/ PIn5COOH (1) and Pt/PIn5COOH/PPO (2) electrodes in buffer solution; scan rate 40 mV/s.

To find evidence for tyrosinase immobilization on the PIn5COOH film the IR spectra have been recorded with the external reflection technique (IRRAS). Unfortunately, it appeared that the bands that may be diagnostic of tyrosinase (amide I and II bands at 1660 and 1550 cm^{-1}) strongly overlap with the C=O and phenyl ring stretching bands of PInCOOH (at 1670, 1620 and 1570 cm^{-1}). Thus, to solve this problem we decided to perform in situ spectroelectrochemical Raman experiments.

In-situ resonance Raman spectra of PIn5COOH film deposited on a Pt electrode were already reported by Talbi et al. [39]. These measurements were however performed at strongly acidic solutions (2.5 M $HClO_4$), for selected potentials, related to the voltammetry curves. By exciting the Raman spectra with violet (457.9 nm) and red (676.4 nm) lines at least three different species were identified for various oxidation states. With regard to optimal enzymatic activity of tyrosinase in almost neutral medium (pH 6.5), we recorded the Raman spectra at a phosphate buffer solution at pH=6.5. As follows from spectroelectrochemical UV–VIS experiments (compare Fig. 8a) we may expect pre-resonance Raman spectra at both 488 and 647.1 nm excitation lines applied in our experiments.

However the spectra appeared to be rather weak, with poor signal to noise ratio. Generally, independent on the excitation laser line they exhibited two bands at potentials corresponding to the reduced state (a) (at about 1340 cm^{-1} and a broad, featureless band centered about 1600 cm^{-1}) (Fig. 3). The oxidized state (b) is characterized by rather sharp band at 1633 cm^{-1} and several very weak features at 1145, 1205, and 1460 cm^{-1} (Fig. 3). The spectra were independent on the wavelength of the excitation line. To enhance the Raman spectrum of PIn5COOH, silver particles were deposited on the polymer film by dipping the formerly reduced polymer (deposited on the Pt electrode) into 10^{-1} M $AgNO_3$ solution. This procedure resulted in strong Raman spectra owing to the surface enhancement by the Ag particles that have been created on the polymer matrix. A series of thus recorded surface enhanced resonance Raman spectra of PIn5COOH film at pH 6.5, for several electrode potentials, is presented in Fig. 4. As may be seen a strong band at about 1350 cm^{-1} and a broad envelope of medium intensity, in which three components may be distinguished at 1575, 1600 and about 1625 cm^{-1}, are characteristic of the spectrum recorded at potential range, corresponding to reduced form of

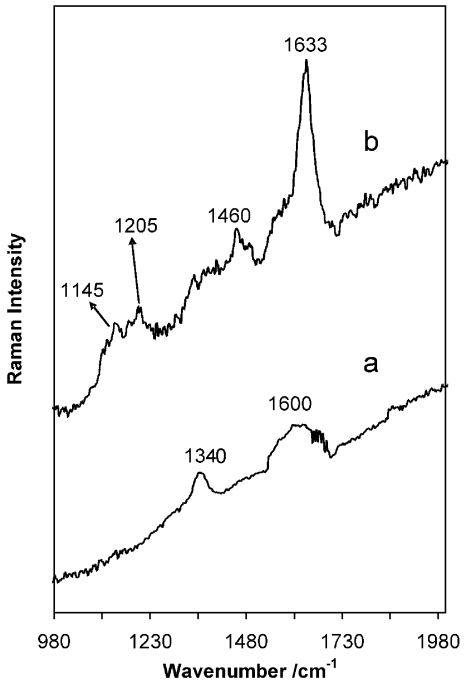

Fig. 3. Pre-resonance Raman spectra of PIn5COOH film recorded in buffer solution (pH=6.5) at two potentials corresponding to the reduced (a) and oxidized (b) states (λ_{exc}=488 nm).

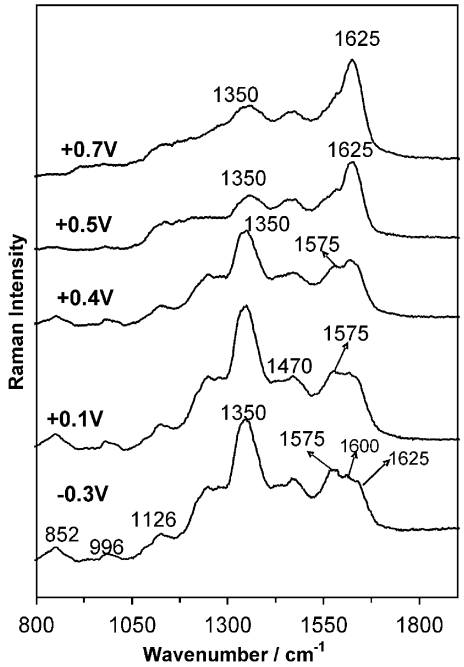

Fig. 4. SERRS spectra of PIn5COOH film covered with Ag particles, recorded in buffer solution (pH=6.5) at several potential values (λ_{exc}=488 nm).

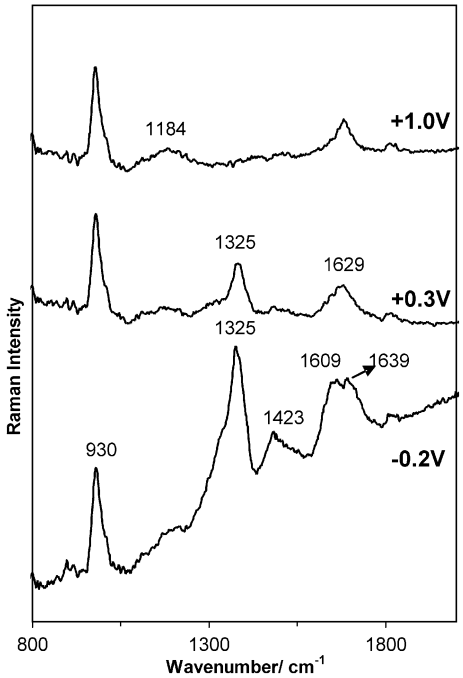

Fig. 5. SERRS spectra of PIn5COOH film recorded in 2 M $HClO_4$ at several potential values. The band at 930 cm^{-1} is due to ClO_4^- anion (λ_{exc}=488 nm).

the polymer (−0.3 V to 0.1 V vs. Ag/AgCl). Starting from 0.2 V some changes in the spectrum are observed. Namely, the relative intensity of the 1350 cm^{-1} band drops down, while that of 1625 cm^{-1} feature considerably increases. These diagnostic bands have the following assignment: the band about 1350 cm^{-1} is ascribed to the stretching vibration localized on the pyrrole ring while the bands at the vicinity of 1600 cm^{-1} are due to stretching vibrations of the phenyl ring [43,44]. Possible assignment of the 1575 cm^{-1} band is the asymmetric stretching vibration of the COO^- group. This band was not observed in the Raman spectra of the polymer without deposited Ag. It may be understood, if one takes into account that in the resonance or pre-resonance Raman spectra of PIn5COOH only the bands due to vibrations of the aromatic rings are resonantly enhanced. Lack of the 1575 cm^{-1} band in the surface enhanced Raman spectra of PIn5COOH in acidic medium (2 M $HClO_4$), where the carboxylic groups are protonated, confirms its assignment to the stretching vibration of the COO^- group (see Fig. 5). The 1575 cm^{-1} band survives as a shoulder on a low frequency side of the 1625 cm^{-1} band at positive potentials (Fig. 4) that corresponds to the oxidized form of the polymer at pH 6.5.

Fig. 6 presents a series of the SERRS spectra of the PIn5COOH film on which tyrosinase has been immobilized. As may be seen there are some differences between these spectra and the respective spectra of the polymer film without the enzyme, when compared at the same electrode potentials. The frequency of the band assigned to the pyrrole ring stretching vibrations (1335 cm^{-1}) differs by about 15 cm^{-1} and the band at 1575 cm^{-1} is not observed in the spectra of PIn5COOH with attached tyrosinase, thus giving evidence of a covalent binding of the enzyme with participation of the carboxylic groups. Moreover, a new band at 1385 cm^{-1} appears at potentials corresponding to the oxidation of the polymer matrix (>0.3 V). Its intensity increases at the expense of the 1335 cm^{-1} band, which totally disappears at 0.7 V. All the potential-induced changes of the spectrum are totally reversible. The 1385 cm^{-1} band was not observed for un-

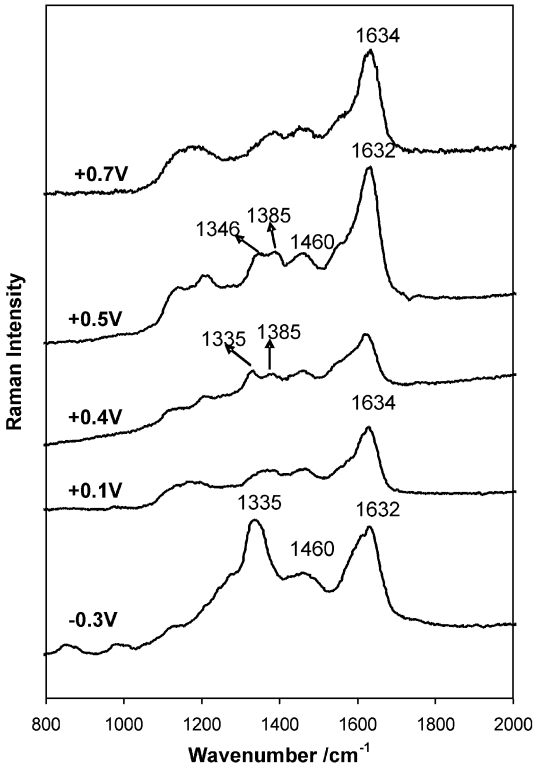

Fig. 6. SERRS spectra of PIn5COOH film with covalently immobilized tyrosinase recorded in buffer solution (pH=6.5). The Ag particles were deposited on the polymer film (λ_{exc}=488 nm).

modified PIn5COOH film at pH 6.5. However, we found this band in the spectrum of the oxidized form of PIn5COOH in 2 M $HClO_4$ at 0.8 V (λ_{exc}=647.1 nm—spectra not shown). The 1380 cm^{-1} band was also reported by Talbi et al. [39] for PIn5COOH film in acidic medium and it was ascribed to the C=N stretching vibration of the oxidized polymer. Appearance of the C=N bond is however connected with deprotonation of the pyrrole ring. Such a process is observed in acidic medium during the second redox process, which starts at potentials more positive than 0.7 V. Nevertheless, this band was observed by Talbi et al. [39] already at 0.2 V as a weak feature and considerably gained in intensity at the expense of 1332 cm^{-1} at more positive potentials (>0.7 V). Similar phenomenon may be observed in the SERRS spectra of PIn5COOH with immobilized tyrosinase at nearly neutral medium (see Fig. 6), thus suggesting the proton exchange at the pyrrole ring during the redox process. Perhaps this proton exchange is facilitated by the presence of the NH_3^+ groups of amino acid residues of the polymer-bound tyrosinase and this may be the reason for not observing this process in unmodified PIn5COOH matrix.

3.2. Electrochemical and UV–VIS evidences of bioactivity of Pt/PIn5COOH/PPO electrode

To prove that the Pt/PIn5COOH/PPO electrode is bioactive the electrochemical and spectroscopic measurements were carried out. Fig. 7 illustrates a typical current–time dependence for PIn5COOH/PPO electrode upon the successive addition of catechol to air-saturated, buffered solution (pH 6.5) under stirring.

The arrows indicate the subsequent addition of 5 μM of catechol. The amperometric response of PIn5COOH/PPO electrode was examined at 0 V and −0.2 V. The latter potential is often used for reduction of *o*-quinones. However in our case the changes in reduction current after catechol addition at −0.2V were not satisfactory. At this potential the polymeric matrix is reduced, thus having low conductivity (see Fig. 2). An inset in Fig. 7 shows the amperometric current response at 0 V as a function of catechol concentration. The linear dependence is found to be approximately 15 μM of catechol with the slope in the range equal to 250 mA/M cm^2. A decrease in sensitivity of electrochemical detection observed at higher catechol concentration may be caused by a limited amount of PPO on the polymer surface and/or by chemical reactivity of *o*-quinones (oligomerization). In order to check the PIn5COOH/PPO activity in solution containing higher catechol concentration spectroelectrochemical measurements were carried out. The UV–VIS spectra of ITO/PIn5COOH electrode recorded at various potentials in buffered solutions are presented in Fig. 8a.

It can be seen that the fully neutral, undoped state of the polymer (−0.2 V) is characterized by one electronic π–π* transition. This transition is manifested by increasing absorption towards the UV part of the spectra. Polarisation of the

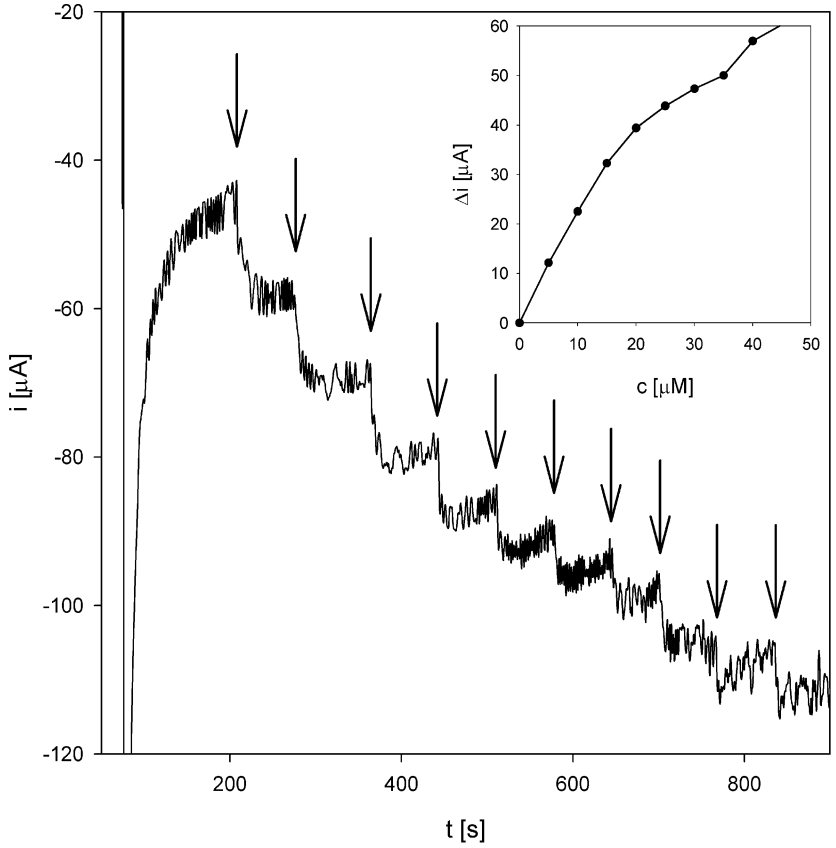

Fig. 7. Dependence of current on time and on successive addition of catechol for Pt/PIn5COOH/PPO electrode at 0 V. The inset shows the amperometric current response as a function of catechol concentration.

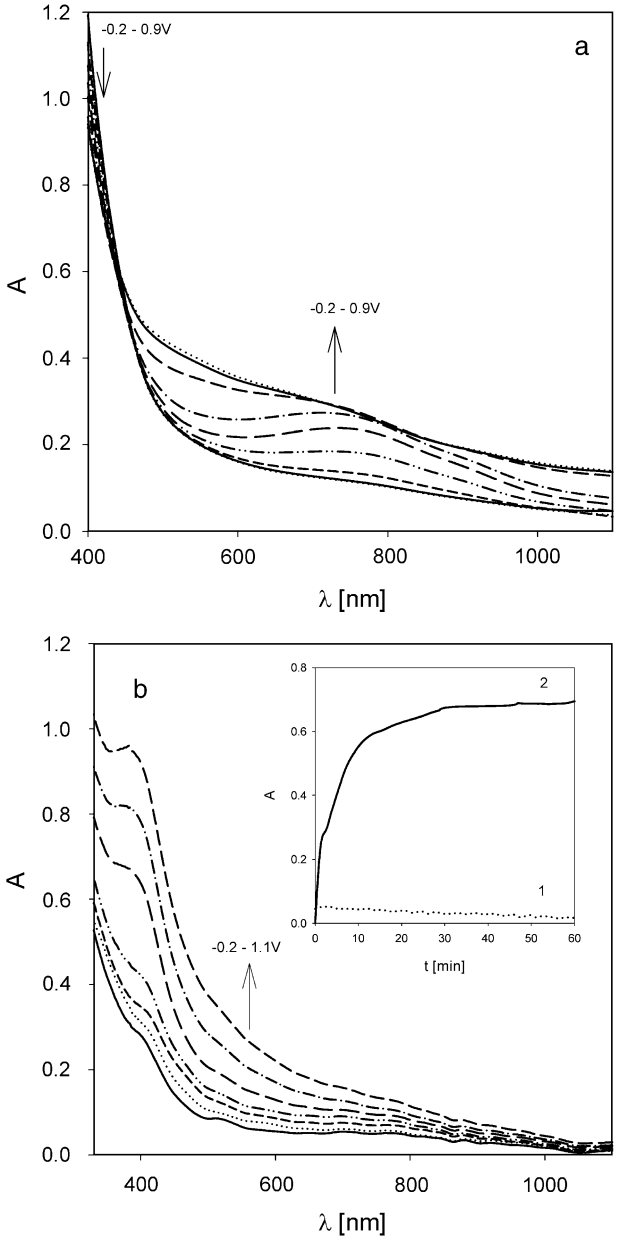

Fig. 8. UV–VIS absorption spectra of: (a) ITO/PIn5COOH electrode recorded in buffer solution (pH=6.5) at various potentials: −0.2, 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, 0.9 V; (b) ITO/PIn5COOH electrode recorded in buffer solution (pH=6.5) containing 5×10^{-3} M of catechol at various potentials: −0.2, 0.0, 0.3, 0.5, 0.7, 0.9, 1.1 V. The inset shows the changes of absorption in time for ITO/PIn5COOH (1) and ITO/PIn5COOH /PPO (2) electrodes at an open circuit potential and at constant wavelength of −400 nm.

electrode in the potential range from −0.2 V to 0.6 V, which corresponds to the oxidation of the polymer, results in a steady growth of the 770–780 nm band. This band grows at the expense of the band about 400 nm. At this wavelength absorbance decreases with increasing electrode potential.

In further experiments ITO/PIn5COOH/PPO electrode was used and the absorbance spectra were recorded as a function of time at constant wavelength of 400 nm and at an open circuit potential at which catechol is not oxidized electrochemically. This wavelength value was chosen because it corresponds to the maximum absorption of oxidation products of catechol (Fig. 8b). The results are shown as an inset in Fig. 8b for ITO/PIn5COOH and ITO/PIn5COOH/PPO electrodes. As may be seen for ITO/PIn5COOH/PPO electrode, absorbance at 400 nm increases with time, reaching a plateau after about 30 min. All these results indicate that PPO immobilized chemically on the polymer surface retains its biochemical activity. The Pt/PIn5COOH/PPO electrodes were also examined for the enzymatic stability at room temperature. The electrode showed enzyme inactivation after 10 h.

4. Conclusions

The present study has proven the suitability of functionalized conducting polymers with pedant groups to be used as a polymer matrix for one-step enzyme immobilization. It was confirmed by the surface enhanced resonance Raman scattering spectra that tyrosinase can be successfully bonded by chemical coupling to the free carboxylic group of electrochemically formed PIn5COOH. Two methods were used to prove that PIn5COOH/PPO electrode is bioactive. The reduction current of *o*-quinones was detected as a function of catechol concentration. The linear dependence was found to be 15 μM of catechol with sensitivity of 250 mA/M cm^2. Additionally, the products of catechol oxidation were recorded by detecting absorbance spectra vs. time at constant wavelength. All results point out that immobilized tyrosinase retains their bioactivity. In comparison with other CP based biosensor where PPO was entrapped in polymer matrix like polypyrrole, amphiphilic polypyrrole, polytiophene (PEDT), polyaniline–polyacrylonitrile [14–16], we obtained lower values of sensitivity. Our results are comparable with these obtained for tyrosinase-based poly(dicarbazole) electrodes formed by one-step method [35]. However Pt/PIn5COOH/PPO electrodes are not so stable like recently described poly *N*-(3-aminopropyl) pyrrole/PPO electrode also formed by one-step method [36].

Acknowledgements

This work was supported by the Ministry of Scientific Research and Information Technology in 2004–2007, Project No. PBZ 18-KBN-098/T09/2003. The authors are very grateful to DSc Robert Koncki for his help and advices concerning the tyrosinase immobilization.

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