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Influence of the immobilization procedures on the electroanalytical performances of *Trametes versicolor* laccase based bioelectrode

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ABSTRACT

In this work three different immobilization methods (physico-chemical, electrostatic and covalent) of fungal laccase from *Trametes versicolor* (TvL) on multi-walled carbon nanotubes electrodes (MWCNTs) have been characterized and compared. To this aim, in particular, the following immobilization agents were used: polyazetidine prepolymer (PAP), Nafion solution and succinimide-carbodiimide (EDC-NHS). The comparison of these procedures has been realized by evaluating the enzymatic activity of the resulting bioelectrodes and their catalytic efficiency for oxygen reduction in the presence of the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS) as non-phenolic redox mediator and catechol as phenolic one. Another aspect taken into account was the diffusion evaluation of several mediators as ABTS, dopamine, catechol and caffeic acid across the immobilizing layer at the pH range where the enzyme shows the maximum activity. The experimental results put in evidence the better performances of TvL-PAP-MWCNTs biosensor in terms of bioelectrochemical and diffusion properties, allowing us to asses that PAP is the best immobilizing agent thanks to its good permeability to mediators and the ability to keep the enzyme bioelectrochemical properties.

1. Introduction

Laccases (EC 1.10.3.2) are cuproproteins belonging to the group of blue oxidase enzymes [1,2]. They are widely distributed in fungi [3], higher plants [4], and in some bacteria [5]. Laccase is a polyphenol oxidase catalyzing the oxidation of several inorganic substances as phenol, associated to the reduction of oxygen to water. Laccases have four neighboring copper atoms distributed among different binding sites and classified into three types: copper types 1, 2 and 3. Copper type 1 is involved in electron capture and transfer, copper type 2 activates molecular oxygen, while copper type 3 is responsible for oxygen uptake. Mediator oxidation using laccase is one-electron reaction, which generates a free radical [6].

Laccases are extensively studied oxidoreductase enzymes [7,8] because of their potential application to many industrial processes including decolourization of dyes [9], pulp delignification [10], oxidation of organic pollutants [11] and the development of biosensors [12,13] or biofuel cells [14,15].

In the case of laccase-based biosensor the enzymatic immobilization on the electrode surface is an important aspect to enhance the overall operational performance [16]. An optimal immobilization procedure should ensure activity and stability of the protein and, at the same time, provide a good accessibility of substrate and mediator molecules to the active site of the enzyme [17,18].

This paper reports a bioelectrochemical characterization of several immobilization procedures, employed for the development of an optimized *Trametes versicolor* (TvL) based biosensor on multi-walled carbon nanotubes screen printed electrode (MWCNTs-SPE). The choice of MWCNTs is due to their high surface area matrix, conductivity, flexibility and reactivity properties improving the enzyme biosensor performances [19].

In particular the influence of three different immobilizing approaches, by physico-chemical entrapment via polyazetidine prepolymer (PAP), by an electrostatic attraction within a Nafion layer and by covalent linkage using succinimide and carbodiimide have been reported [20–29].

In order to check the efficiency of these methods, we have compared the kinetic and analytical parameters of the resulting TvL based biosensors with respect to a non-phenolic (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) diammonium salt, ABTS) and phenolic (catechol) compound.

A comparative investigation on the pH dependence of catalytic current and formal potential was evaluated for four mediators (ABTS, dopamine, catechol and caffeic acid).

A characterization of the modified surface of the electrode after the deposition of PAP and Nafion was performed by means of Atomic Force Microscopy (AFM) experiments (see Supplementary Information).

Furthermore, the diffusion coefficients of the investigated mediators using the same electrode surface before and after PAP or Nafion

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deposition were determinate in the pH range 4.0–5.5, where TvL showed the maximum activity, to evaluate the layer permeability.

Finally the electrochemical characterization of the TvL-PAP-MWCNTS-SPE biosensor was studied by means of cyclic voltammetry (CV) experiments with respect to all the redox mediators proposed.

2. Experimental

2.1. Chemicals and reagents

Fungal laccase from *Trametes versicolor* was supplied by Fluka (Buchs, Switzerland) (EC 1.10.3.2, activity: 30.6 U mg^{-1}) and stored at −18 °C. All the redox mediators used (ABTS, dopamine, catechol and caffeic acid) were from Sigma-Aldrich (Buchs, Switzerland). Solutions of mediators were prepared in 0.015 M Britton-Robinson (B-R) buffer at the pH range 4.0–8.0 immediately before use. The reactants employed for protein immobilization were as follows: polyazetidine prepolymer (PAP®), donated by Hercules Inc. (Wilmington DE, USA), Nafion® 117 solution (purum, ~5% solution in a mixture of lower aliphatic alcohols and water), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride, Commercial grade (EDC) and N-hydroxysuccinimide, 98% (NHS) were supplied by Sigma-Aldrich (Buchs, Switzerland). Other chemicals were all of analytical grade. High purity deionized water (Resistance: 18.2 M Ω × cm at 25 °C; TOC < 10 μg L^{-1}) obtained from Millipore (Molsheim, France) has been used throughout experiments.

2.2. Laccase immobilization procedures

For the laccase immobilization three different methods were used. The use of PAP matrix gave a physico-chemical immobilization, the Nafion solution allowed an electrostatic attraction and finally the covalent immobilization is realized by means of carbodiimide (EDC) and N-hydroxysuccinimide (NHS). In this way a comparison between different immobilization techniques was carried out. The methods used were performed as the following way:

Method 1—PAP: TvL-PAP-MWCNTs-SPE was prepared by depositing 3 μL of a solution of PAP containing 2.5 U μL^{-1} TvL to the MWCNTs electrode surface and let to dry for about 48 hours at room temperature.

Once formed, PAP embedding *Trametes versicolor* was equilibrated for 10 min in measuring buffer before starting the electrochemical measurement.

Method 2—Nafion: a Nafion 117 solution (5% w/v) was diluted with ethanol and NaOH solution to give a stock solution of 1%, pH 5.0.

TvL-Nafion-MWCNTs-SPE was prepared by spreading 3 μL of this solution containing 2.5 U μL^{-1} TvL to the surface of the MWCNTs working electrode, and then let to dry overnight at room temperature.

The resulting enzyme electrode was equilibrated for 10 min in measuring buffer before starting the electrochemical measurement.

Method 3—EDC/NHS: the carboxyl functions on the surface of MWCNTs-SPE were activated with 3 μL of a mixture containing 0.5 mM EDC and 0.1 mM NHS in water and 2.5 U μL^{-1} of laccase for about 20 min. After removing EDC–NHS mixture and rinsing the surface of the MWCNTs working electrode with 0.015 M B-R buffer solution pH 5.0, electrochemical measures were carried out.

2.3. Electrochemical experiments

Electrochemical measurements were performed in a 5 mL thermostated glass cell (model 6.1415.150, Metrohm, (Herisau, Switzerland)) with a conventional three-electrode configuration. An $Ag/AgCl/KCl_{sat}$ (198 mV vs. NHE) was used as reference electrode (cat. 6.0726.100, Metrohm, (Herisau, Switzerland)) and a glassy carbon rod as counter electrode (cat. 6.1248.040, Metrohm, (Herisau, Switzerland)). Different working electrodes were employed, in particular: for the electron transfer studies and for the investigation of the effect of pH on catalytic current and on formal redox potential of mediators, a MWCNTs-SPE from DropSens (Oviedo, Spain) with a surface diameter of 3 mm was used; on the other hand, for the determination of the diffusion coefficients, a Pt microdisk (PtM) diameter 20 μm embedded in a glass (Amel, Italy), in presence and in absence of PAP and Nafion, was employed. The PtM electrode radius was determined before each measurement.

All experiments were carried out with a μ-Autolab potentiostat from EcoChemie (Utrecht, The Netherlands) and at 25 °C in oxygen atmosphere.

2.4. Diffusion coefficient determination

The Randles–Sevcik equation, usually used for the electrochemical determination of the diffusion coefficients of electroactive species, requires the knowledge of their concentrations. This is not a problem when the species are free in solution; conversely, when a redox compound is entrapped or immobilized to some extent, its concentration in the embedding layer is generally different to the bulk concentration; moreover, it is often difficult and sometimes impossible to specify [30]. In the present work we used the normalized form of the Cottrell equation [31], which allows the diffusion coefficients of the selected redox compounds to be calculated without having to know their concentrations in the PAP or Nafion layer, respectively:

$$i_d = \pi^{1/2} nFD^{1/2} cr^2 / t^{1/2} + 4nFDcr \quad (1)$$

Normalizing Eq. (1) with respect to the steady state current yields:

$$i_d / i_{d,ss} = (\pi^{1/2} / 4) r (Dt)^{-1/2} + 1 \quad (2)$$

where i_d is the diffusion current, $i_{d,ss}$ the steady state current, D the diffusion coefficient, t is the time elapsing after the application of an appropriate potential step and r is the radius of the microdisk electrode. A plot of $i_d/i_{d,ss}$ vs. $t^{-1/2}$ is a straight line with an intercept of one and slope S. If r is known, one can evaluate D:

$$D = \pi r^2 / 16S^2 \quad (3)$$

This procedure allows the direct determination of D. The radius can be evaluated by performing a similar experiment using a redox species of known diffusion coefficient.

In practice, the PtM electrode was used as working electrode; before every measurements the electrode radius was calculated from the steady state current using 4 mM $K_4Fe(CN)_6$ in 1 M KCl solution (under these conditions, the diffusion coefficient of $Fe(CN)_6^{4-}$ is reported to be $D = 6.32 \times 10^{-6}$ cm^2 s^{-1}), as reported in literature [32]. The electrode was first polished on alumina powder of different size (0.3 and 0.05 μm) and then ultrasonically cleaned in water for about 5 min. The PAP or Nafion-coated electrode was prepared by spreading 3 μL of a film solution containing an adequate amount of inert protein, bovine albumin serum (BSA), over the electrode surface and allowing it to dry overnight.

All the measurements were the average of at least 10 replicated measurements.

2.5. Measurement procedures

The amperometric measurements allowed us to study the influence of pH, in a pH range 4.0–8.0, on the catalytic current for each mediator (0.05 mM).

CV measures were carried out with a bare electrode (MWCNTs-SPE), in order to study the dependence of the mediator formal potential (E^0), determined as the mean value of the anodic and cathodic peak potentials, on pH. Mediator solutions (0.25 mM) were prepared in B-R buffer in the same range.

CV responses were recorded in 5 mL of mediator solutions of 0.25 mM, prepared in buffer solution pH 5.0. Potential was scanned from −0.2 V to 0.8 V vs. Ag/AgCl/KCl_{sat}, after holding the electrochemical system at the initial potential for 10 s; all voltammograms were recorded at scan rate of 5 mV s^{-1}. For each mediator a new PAP-MWCNTs-SPE was dipped into the cell. To assess the real catalytic property of the *Trametes versicolor* biosensor towards the studied mediators, CV experiments were performed immobilizing the enzyme on the electrode surface by PAP matrix as described before.

Typical voltammetric behavior of our biosensor in the presence of each mediator (0.25 mM) was carried out.

3. Results and discussion

3.1. Immobilization systems

Three different enzyme immobilization systems were tested: the physico-chemical adsorption (PAP), electrostatic attraction (Nafion) and covalent coupling (EDC/NHS).

To assess the effective catalytic properties of the three biosensors proposed toward a non-phenolic mediator (ABTS) and a phenolic one (catechol), CV experiments were performed (see below). Typical voltammetric behavior of the laccase immobilizing systems in the presence of the two mediators was obtained. From these experiments, kinetic parameters for ABTS and for catechol were determined by monitoring the variation of catalytic steady state current at increasing mediator concentration and compared for the three used strategies, respectively. Amperometric detection was carried out at an appropriate potential (E_{ABTS} = 0.0 V and $E_{catechol}$ = −0.2 V). Calibration curves of ABTS for the different systems are reported in Fig. 1. The higher sensibility, related to the slope value, was found to be 0.28 μA mg^{-1} L for the TvL-PAP-MWCNTs electrode as reported in Fig. 1 inset and in Table 1.

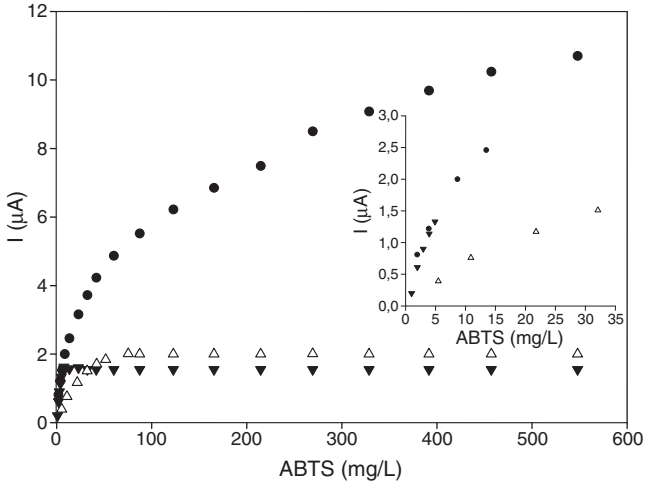


Fig. 1. Catalytic current responses of (▼) PAP, (●) EDC/NHS and (△) Nafion TvL-MWCNTs-modified electrodes obtained by chronoamperometry as function of ABTS concentration. Inset: Detail of linear range of the biosensors. Experimental conditions: 0.015 M B-R buffer, pH 5.0.

Table 1
Analytical characterization of TvL-MWCNTs biosensors for two mediators (ABTS and catechol), with different methods of immobilization, obtained by batch amperometry at fixed potential (E_{ABTS} = 0 V and $E_{catechol}$ = −0.2 V) in stirred solutions, in 0.015 M B-R buffer, pH 5.0.

Electrode	Mediator	Linear range (mg/L)	Slope (μA mg^{-1} L)	r^2	LOD (mg/L)
TvL-PAP-MWCNTs	ABTS	1.00–4.90	0.28 ± 0.02	0.998	0.30
	Catechol	0.07–2.28	0.53 ± 0.01	0.994	0.02
TvL-Nafion-MWCNT	ABTS	3.23–32.09	0.040 ± 0.004	0.998	0.97
	Catechol	0.15–7.20	0.15 ± 0.01	0.998	0.05
TvL-EDC/NHS-MWCNT	ABTS	0.47–13.44	0.14 ± 0.01	0.992	0.14
	Catechol	0.26–14.82	0.14 ± 0.01	0.999	0.08

The current-concentration dependence of the analyzed compounds was modelled by using a Michaelis–Menten nonlinear fitting thus allowing the calculation of the main kinetic parameters; results obtained are summarized in Table 2. By comparing the calculated K_M^{app} of these compounds, with the different laccase modified electrodes we observed that the smaller values were obtained using the TvL-PAP-MWCNTs electrode suggesting that this immobilization method maintains the active site accessibility of the enzyme better than the other ones. Moreover the higher value of I_{max}/K_M^{app} ratio with the TvL-PAP-MWCNTs electrode put in evidence a better catalytic efficiency [33,34]. Furthermore, the three TvL-based sensors were tested for their analytical properties using ABTS and catechol as mediators. The results obtained are reported in Table 1. Comparison of the data highlights, that TvL-PAP-MWCNTs sensor displays a higher slope, thus indicating a better sensitivity for the two compounds.

On the basis of data shown in Tables 1 and 2, we can assess that the TvL-PAP-MWCNTs is the best configuration to optimize the biosensor performances. Therefore, basing on our findings, it could be concluded that the best enzyme immobilizing strategy is the PAP entrapment (Scheme 1).

3.2. Influence of pH to catalytic current for mediators

In order to define the TvL-PAP-MWCNTs biosensor, different experiments were carried out.

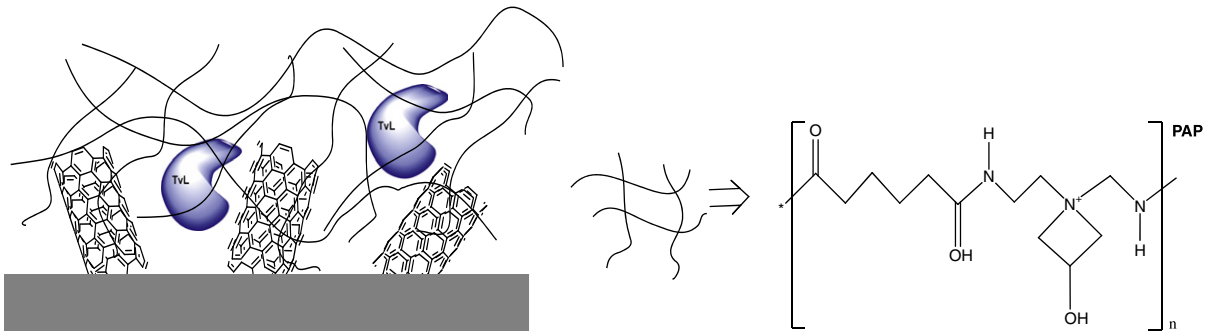
To investigate the optimum pH for enzyme activity, a study on the influence of pH was aimed to catalytic currents of the studied mediators (ABTS, dopamine, catechol and caffeic acid). As depicted in Fig. 2 only one trend is observed in pH range 4.0–8.0. All the mediators exhibited a bell-type pH dependence centered around pH interval 4.5–5.0. At pH extreme range 7.0–8.0, the activity of *Trametes versicolor* toward all compounds seemed to decay rapidly being negligible.

3.3. Influence of pH to formal redox potential for mediators

Fig. 3 shows formal redox potential of the studied mediators as a function of pH, in the range 4.0–8.0. The E^0 of ABTS remains constant in the pH range investigated, whereas the potentials of the other mediators (catechol, dopamine and caffeic acid) shift to more negative values as the pH increases.

Table 2
Calculated kinetic parameters of TvL-MWCNTs integrated systems with different immobilization procedures for two mediators: ABTS and catechol. Experimental conditions: 0.015 M B-R buffer, pH 5.0, E_{ABTS} = 0 V and $E_{catechol}$ = −0.2 V.

Electrode	Mediator	I_{max} (μA)	K_M^{app} (mM)	I_{max}/K_M^{app} (μA/mM)
TvL-PAP-MWCNTs	ABTS	3.0	0.013	231
	Catechol	5.5	0.076	72
TvL-Nafion-MWCNTs	ABTS	2.9	0.056	52
	Catechol	2.4	0.078	31
TvL-EDC/NHS-MWCNTs	ABTS	11.3	0.142	79
	Catechol	5.1	0.131	39



Scheme 1. TvL-PAP-MWCNTs biosensor and PAP structure.

This variation is linear and fits to the following equations. Catechol: $E^0 = -59.0\ pH + 641.4$; Dopamine: $E^0 = -67.9\ pH + 651.9$; Caffeic acid: $E^0 = -64.1\ pH + 671.6$. The values of the negative slopes are near the Nerstian value of 59 mV/pH units, indicating that their redox processes involve the same number of protons as electrons.

3.4. Determination of diffusion coefficients

In addition, for a thorough study, the determination of diffusion coefficients across PAP and Nafion layers was performed in the pH range 4.0–5.5, where the TvL activity has the maximum values (Fig. 2). We used the approach based on chronoamperometry and the Cottrell equation to determine the influence of the PAP or Nafion layer on the diffusion of the mediators above cited. The experimental results (D_{sol} without matrix, D_{PAP} with PAP matrix and D_{Nafion} with Nafion one, in $cm^2\ s^{-1}$) are reported in Table 3. By examining experimental data, D values in the presence of PAP and Nafion films are smaller with respect to those in their absence. The decrease of the diffusion coefficient values, when the mediators are forced to cross a matrix, with respect to their free diffusion from the solution to the electrode, is correlated to their strength as electrolytes, their charge status and their size.

Besides, comparing diffusion coefficients of these two immobilizing supports we can observe the following: (a) for non-phenolic redox mediator (ABTS) they are comparable as a result of size exclusion properties of both films; (b) for phenolic compounds the diffusion coefficients obtained with Nafion film present one order of magnitude larger than the ones obtained with PAP layer. It can be attributed to the presence of negatively charged sulfonate groups in the Nafion structure that promote the permeability of the compounds by electrostatic interactions; while the same mediators are much more hindered by PAP film.

Another aspect to take in consideration is the overall viscosity of the whole system. The presence of both matrices leads to a decreasing of D values, more evident for PAP membrane with respect to Nafion. Despite these results, compared to analogous works employing different matrices [35,36], the PAP layer shows better diffusion properties on the electrode surface for each electroactive species.

Basing on these findings, the use of the TvL-PAP-MWCNTs biosensor seems to be preferable with respect to the TvL-Nafion-MWCNTs one because despite the latter shows better diffusion to film interface for all redox compounds, the first biosensor has demonstrated its better performance in terms of denaturing action toward the laccase enzyme and its sensitivity to redox mediators, as demonstrated by kinetic and analytical parameters.

3.5. Voltammetric detection of laccase-catalysed oxidation of mediators

Mediators taken into account were characterized in their main electrochemical parameters for the chosen immobilizing system. Slow-scan voltammograms recorded in 0.25 mM mediator solutions with 0.015 M B-R buffer, pH 5.0 employing PAP-MWCNTs-SPE, in presence and in absence of immobilized laccase, are shown in Fig. 4. All the compounds generate well-shaped almost reversible or quasi-reversible signals with peak potential separation values (ΔE_p) between 230 and 100 mV and formal potentials between (E^0) between 270 and 501 mV (vs. $Ag/AgCl/KCl_{sat}$). Chemical stability of the different redox species generated in the corresponding electrode

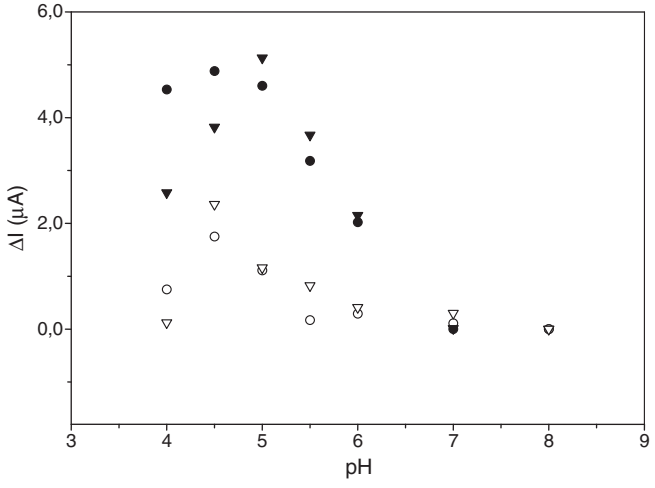


Fig. 2. Dependence of the catalytic current on pH for TvL-PAP-MWCNTs-modified electrode. Absolute values of catalytic current were plotted. The redox mediators employed were: (▼) ABTS, (▽) dopamine, (○) catechol, (●) caffeic acid. Experimental conditions: 0.015 M B-R buffer, pH 4.0–8.0.

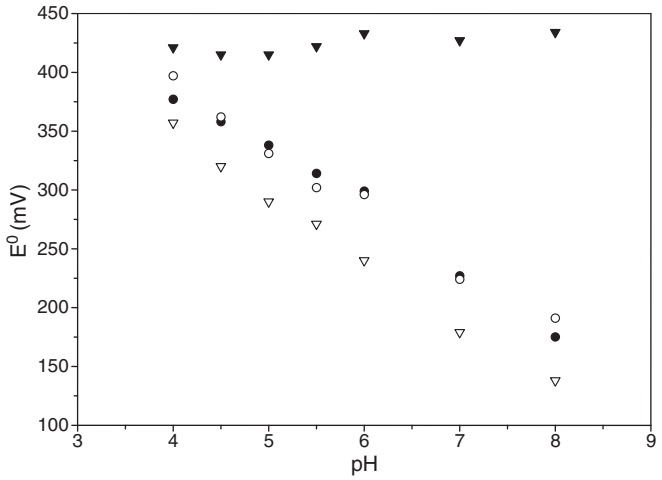


Fig. 3. Formal redox potential of (▼) ABTS, (▽) dopamine, (○) catechol, (●) caffeic acid as a function of pH. Experimental conditions: 0.015 M B-R buffer, pH 4.0–8.0.

Table 3
Diffusion coefficients of mediators without membrane and with PAP and Nafion ones in 0.015 M B-R buffer, pH 4.0–5.5.

Mediator		ABTS	Catechol	Dopamine	Caffeic acid
pH 4.0	D_{sol}	4.0×10^{-6}	2.3×10^{-5}	1.9×10^{-5}	1.6×10^{-5}
	D_{PAP}	4.1×10^{-8}	4.6×10^{-7}	5.7×10^{-8}	2.7×10^{-7}
	D_{Nafion}	4.9×10^{-8}	6.3×10^{-6}	4.8×10^{-7}	7.5×10^{-8}
pH 4.5	D_{sol}	2.4×10^{-6}	3.0×10^{-5}	2.5×10^{-5}	0.4×10^{-5}
	D_{PAP}	2.2×10^{-7}	1.7×10^{-7}	2.4×10^{-7}	5.6×10^{-7}
	D_{Nafion}	5.5×10^{-8}	4.4×10^{-6}	4.6×10^{-7}	8.2×10^{-7}
pH 5.0	D_{sol}	4.5×10^{-6}	5.5×10^{-5}	4.4×10^{-5}	1.5×10^{-5}
	D_{PAP}	3.9×10^{-8}	4.0×10^{-7}	6.4×10^{-8}	3.1×10^{-8}
	D_{Nafion}	8.3×10^{-8}	2.9×10^{-6}	6.8×10^{-7}	1.2×10^{-6}
pH 5.5	D_{sol}	4.4×10^{-6}	2.0×10^{-5}	5.1×10^{-5}	1.5×10^{-5}
	D_{PAP}	11.7×10^{-8}	5.9×10^{-7}	2.6×10^{-8}	1.6×10^{-8}
	D_{Nafion}	12.4×10^{-8}	2.5×10^{-6}	2.9×10^{-7}	8.1×10^{-7}

processes is confirmed by the anodic to cathodic peak ratio (i_{pa}/i_{pc}), which approaches to 1 in all cases.

Enzyme-mediated reactions were carried out in mediator solution in oxygen atmosphere. A significant change in the shape of each redox mediator was observed. All of them are characterized by the disappearance of the anodic process due to the catalytic oxidation of the mediator, and a significant enlargement of the cathodic process related to the reduction of the oxidized form of the mediator generated by the enzymatic reaction. The shape of these voltammograms is typical of catalytic redox processes taking place at the electrode solution interface, which has been widely described in the literature [37–39]. Steady-state values of the cathodic current were always observed. The stability of the different redox species of a laccase mediator is very important when laccase-mediated systems are applied to different industrial processes since it is known that the actual oxidant of the target molecule is the oxidized form of the mediator, but not the enzyme itself.

4. Conclusions

In this paper the influence of three enzyme immobilization techniques on the performances of Laccase based MWCNTs electrodes has been evaluated. The kinetic and analytical properties of the resulting biosensors were tested in the presence of two redox mediators: ABTS and catechol. Results indicate that the TvL-PAP-MWCNTs biosensor shows better performances if compared with the other ones.

In order to shed deeper light on the behavior of the selected system, different experiments were carried out. Firstly, the dependence of the catalytic current and of the formal redox potential as a function of pH was investigated with respect to some redox mediators: ABTS, catechol, dopamine and caffeic acid. Then the diffusion coefficients across PAP and Nafion layers for the proposed mediators were studied, in the pH range 4.0–5.5, where the maximum TvL activity was found. In this pH range the polyazetidine matrix has excellent diffusion properties on the electrode surface for each electroactive species.

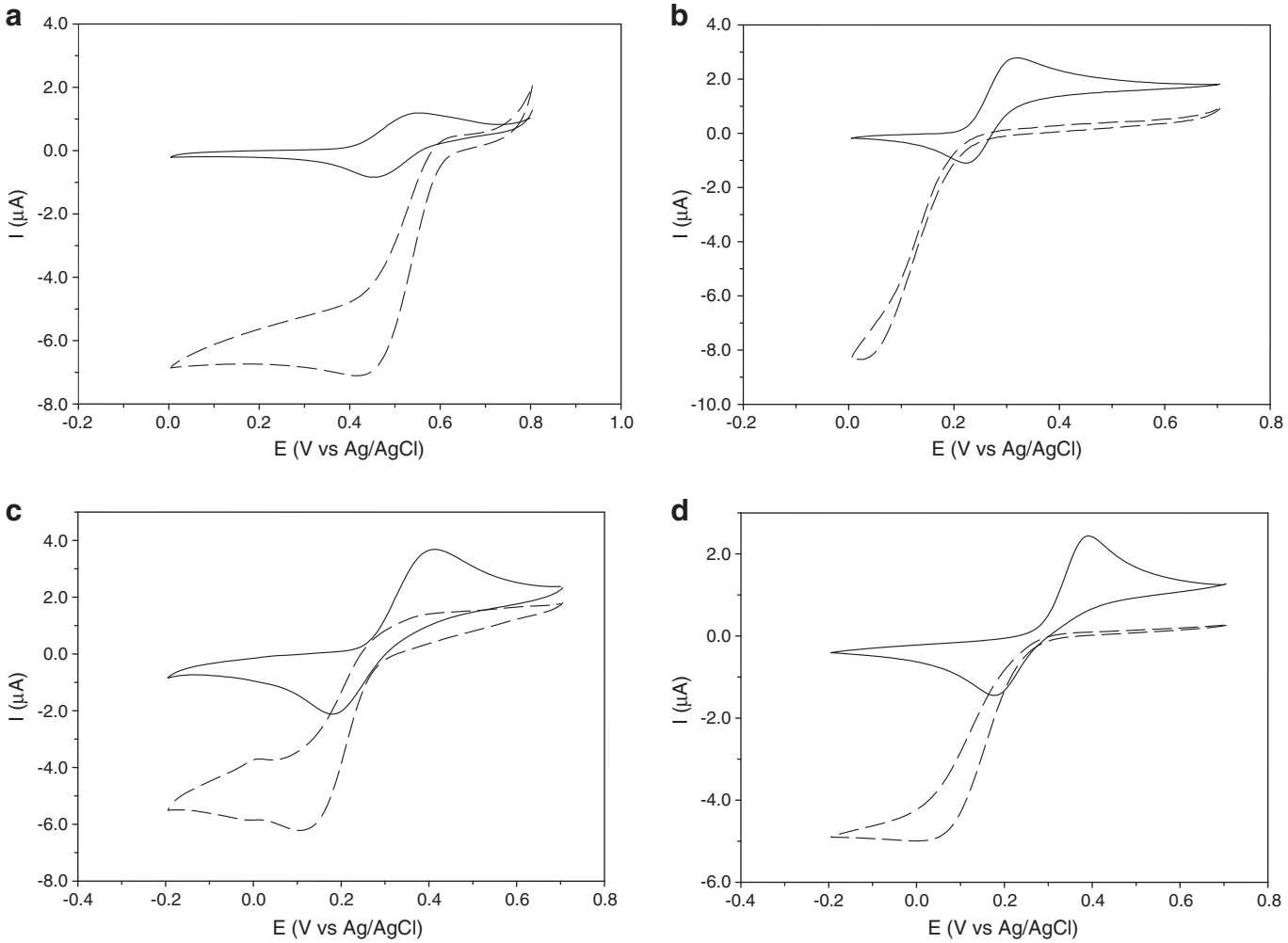


Fig. 4. Cyclic voltammograms of 0.25 mM redox mediator, in absence (solid line) and in presence (dashed line) of TvL immobilized with PAP onto the electrode surface, recorded in 0.015 M B-R buffer, pH 5.0 at a scan rate of 5 mV s^{-1}. The redox mediators employed were: (a) ABTS, (b) dopamine, (c) catechol, (d) caffeic acid.

Finally, mediators taken into account were characterized by cyclic voltammetry for this particular system.

The results indicate the suitability of PAP as an enzyme immobilizing agent for the development of laccase based biosensors taking into account its good permeability toward the redox mediators and the maintenance of bioelectrochemical properties ensuring a good stability and reproducibility of the enzymatic membrane onto the electrode surface.

From the experimental results obtained in the electrochemical characterization of Laccase based MWCNTs electrodes developed with the three immobilization procedures, it is evident that the physico-chemical immobilization performed by means of PAP, represents a more favorable environment for the enzymatic protein due to its capacity to have an adequate mobility allowing an optimal orientation of the redox center for its interaction with analytes and electrode surface. Moreover PAP ensures a good catalytic retention of the enzymatic protein as well as an optimal contact time with analytes in order to improve the catalytic efficiency of the resulting biosensors as confirmed by the I_{max}/K_M^{app} ratio.

From this work we can confirm the great potentiality of PAP as laccase immobilizing agent not only for the development of biosensor for analytical applications, but also as cathode in biofuel cells.

Supplementary materials related to this article can be found online at doi:10.1016/j.microc.2011.08.001.

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