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Investigation of biosensor signal bioamplification: Comparison of direct electrochemistry phenomena of individual Laccase, and dual Laccase-Tyrosinase copper enzymes, at a Sonogel-Carbon electrode

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Abstract

Direct electrochemistry of *Trametes versicolor* Laccase (LAC) was found at a Sonogel-Carbon electrode. The bioamplification, performed by dual immobilization of this enzyme and *Mushroom* Tyrosinase (TYR), of the bio-electrocatalytic reduction of O_2 was investigated. The calculated α transfer coefficients were 0.64 and 0.67, and the heterogeneous electron-transfer rate constants were 6.19 and 8.52 s^{-1}, respectively, for the individual LAC and dual LAC-TYR-based Nafion/Sonogel-Carbon bio-electrodes. The responses of the dual enzymes electrode to polyphenols were stronger than those of the individual LAC or TYR biosensors. Hypotheses are offered about the mechanism of bioamplification. The surfaces of the biosensors were also characterized by AFM.

Keywords: Dual enzymes based biosensor; Direct electrochemistry; Signal bioamplification; Sonogel-Carbon electrode

1. Introduction

To improve the sensitivity of biosensors based on polyphenoloxidase enzymes, several research groups have described different signal amplification approaches. Among these the addition of reducing, pre-concentrating molecules and/or the integration of pairs of enzymes on a single transducer are particularly notable. This last strategy is very interesting because it permits the sensitivity to be enhanced and the range of substrates detected to be increased [1–6]. Nevertheless, the mechanism of signal bioamplification underlying this strategy is not fully elucidated in the bibliography. One of the aims of this work is to contribute to the better understanding of this phenomenon.

Further, in addition to the study of the redox activity of proteins, investigation of the direct electron transfer (DET) between an electrode and a metalloenzyme is considered to be a highly attractive research area in the biosensors field. This is because of its potential application in the research and development of highly sensitive biosensors, effective biofuel cells, and selective routes of biosynthesis. Therefore, since the first reports on the DET between cytochrome *c* and bipyridyl-modified gold [7] or tin doped indium oxide [8] electrodes were communicated in 1977, a number of papers have been published giving detailed information on electrochemical reaction mechanisms of redox proteins and enzyme film at various types of electrode [9–13]. The electrode material may play a vital role in the production of a bio-electrochemical device able to access the desired information.

The carbon-based electrodes have been used widely as materials for studying metalloenzyme bio-electrocatalysis [13–20]. This preference possibly began from the fact that carbon can act as a primary electron donor to native electroactive site of

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enzymes [11]. Ceramic-carbon composite electrodes, made by mixing organic–inorganic sol–gel material and carbon powder, represent a new kind of carbon electrode. These composites are rigid, porous, and easily modifiable by chemical and biological recognition components [21,22].

Our group has made a significant contribution to this subject; we have devised a new method for fabricating these composites by applying high energy ultrasounds. The materials we have developed have been termed Sonogel-Carbon [23]. This matrix offers an alternative route for developing new composite sensors and biosensors with a wide variety of structures and shapes. It presents favourable electroanalytical properties when used for amperometric sensors and biosensors, as well as an excellent sensitivity compared to classical electrodes [24–26]. In this context, we report here the use of Sonogel-Carbon as a transducer for the investigation of the very interesting phenomenon of DET. Cyclic voltammograms of two biosensors based on enzymes with copper centres and Sonogel-Carbon are discussed in detail. The first biosensor is based on modification by Laccase, while the second is based on a dual Laccase and Tyrosinase enzymatic modification. Our research into signal bioamplification by mixing these enzymes in the same biosensor has demonstrated that this strategy cannot only affect the selectivity of the resulting bioprobe, but can also improve its sensitivity compared to that achieved with biosensors made from individual enzymes. This phenomenon has been demonstrated not only in the case when the analyte is typically a substrate of two enzymes, but also when the substrate is especially reactive with only one of them [27]. In the present paper, we try to give an explanation using the information obtained from the direct electrochemistry of the two biosensors; the anticipation of an explanatory mechanism is also provided.

2. Experimental

2.1. Reagents

Methyltrimethoxysilane (MTMOS) was obtained from Merck (Darmstad, Germany) and HCl was obtained from Panreac (Barcelona, Spain). Graphite powder (spectroscopic grade RBW) was obtained from SGL Carbon (Ringsdorff, Germany). *Mushroom* Tyrosinase (E.C. 1.14.18.1, 3000 U mg^{-1}) and *Trametes versicolor* Laccase (E. C. 1. 10. 3. 2, 23.3 U mg^{-1}) were obtained from Fluka (Steinheim, Germany). KH_2PO_4/K_2HPO_4 and acetic acid/sodium acetate for phosphate or acetate buffer were acquired from Fluka (Buchs, Switzerland) and Merck (Darmstad, Germany), respectively. Nafion-perfluorinated ion-exchange resin (Cat. No. 27, 470-4) 5% (w/v) in a mixture of lower aliphatic alcohols and water, and Glutaricdialdehyde, 25 wt% solution in water, were obtained from Aldrich (Steinheim, Germany). Pure water was obtained by passing twice-distilled water through a Milli-Q system (18 MΩ cm, Millipore, Bedford, MA). Phenolic compounds were of analytical grade and obtained from Merck, Sigma or Panreac.

Stock solutions of the phenolic compounds (0.1 mol L^{-1}) were prepared daily by dissolving the appropriate amount in ethanol. Glass capillary tubes, i.d. 1.15 mm, were used as the bodies for the composite electrodes.

2.2. Apparatus

Electrochemical measurements were performed with an Autolab PGSTAT20 (Ecochemie, Utrecht, The Netherlands) potentiostat/galvanostat interfaced with a personal computer, using the AutoLab GPES software for waveform generation and data acquisition and elaboration.

A 600-W model, 20 kHz ultrasonic processor (Misonix Inc., Farmingdale, NY) equipped with a 13 mm titanium tip was used.

Surface topological studies were performed using an atomic force microscope (AFM) Veeco Nanoscope IIIa, in tapping mode. Phosphorus (*n*) doped silicon cantilevers, with spring constants in the range 20–80 N m^{-1}, were used. Calibration of the microscope was achieved by imaging calibration gratings supplied by the manufacturer. AFM images were examined for artifacts, and reproducibility was checked in the usual way, i.e. by changing the AFM cantilever and by either moving (during the experiment) the sample in the *X*- or *Y*-directions or by varying the scanning angle and frequency.

2.3. Methods

2.3.1. Electrochemical transducer preparation

The unmodified Sonogel-Carbon electrode was prepared as described previously [23,24]. Before modification, the electrodes were polished with emery paper No. 1200 to remove extra composite material, gently wiped with weighing paper, and electrochemically pre-treated by dipping them in 0.05 mol L^{-1} sulphuric acid. Finally, they were polarized in the three-electrodes cell by voltage cycling from −0.5 to 1.5 V (five cycles). The electrodes with similar current background were selected, carefully washed with MiliQ water and let to dry at ambient temperature after their biological modification.

2.3.2. Biosensors fabrication

The dual LAC-TYR Sonogel-Carbon-based biosensor was fabricated as follows: adequate quantities of the enzymes LAC and TYR were dissolved in 30 μL of 0.2 mol L^{-1} pH 6 phosphate buffer solutions. At this enzymatic solution, 1.25 μL of glutaricdialdehyde were added, set to polymerize in ultrasonic bath for 3 min, and modified by adding 3.5 μL of Nafion 5%. From the resulting solution, adequate quantities were deposited on the top of the Sonogel-Carbon electrodes with a μ-syringe and allowed to dry under ambient conditions. Finally, the resulting biosensors had 23–100 Units/Electrode of LAC and TYR, respectively, ≈ 0.9% of glutaricdialdehyde and 0.5% of Nafion. The same method was used to prepare individual LAC and TYR-based biosensors and the resulting bio-probes have 23 U and 100 Units/Electrode of LAC and TYR, respectively, and the same quantities of Nafion and glutaricdialdehyde as the dual bi-enzymatic biosensor. Before their use, all biosensors were dipped in stirred buffer solution for 15 min, to eliminate the

excess of non-absorbed enzymes, rinsed with the same buffered solution and stored immersed in the buffered solution at 4 °C when not in use.

2.4. Measurements

Cyclic voltammetry were carried out in an electrochemical cell containing 25 mL of an aerated 0.05 mol L^{-1} acetate buffer solution at pH 5; the three-electrodes system consisted of a enzyme-modified Sonogel-Carbon electrode as working electrode, and a Ag/AgCl (3 M KCl) and a platinum wire as reference and auxiliary electrodes, respectively. Potential range and scan rate are shown in the respective figures.

Tapping mode AFM measurements were performed over different regions of all samples to check for sample surface homogeneity. All AFM images selected to be shown here are representative of the sample's surface topology. For comparison, the scanned area is always 500 × 500 nm^2.

3. Results and discussion

3.1. Morphologies

As shown in Fig. 1, tapping mode AFM was used to evaluate the structure of the silica-based Sonogel material (a), the composite Sonogel-Carbon electrode (b), and the modification with Nafion alone (c) or with the mixtures of Nafion and individual

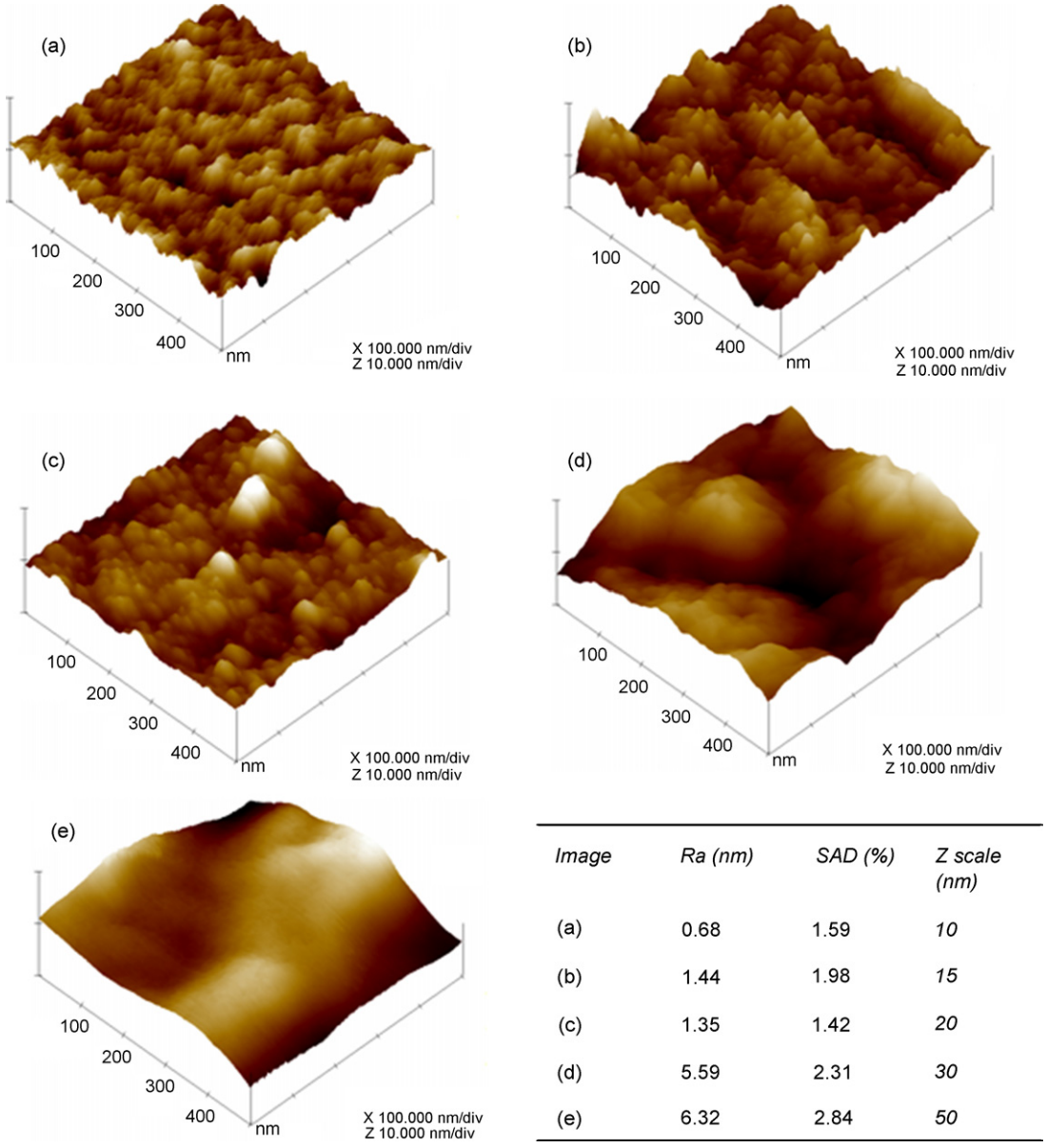

Image	Ra (nm)	SAD (%)	Z scale (nm)
(a)	0.68	1.59	10
(b)	1.44	1.98	15
(c)	1.35	1.42	20
(d)	5.59	2.31	30
(e)	6.32	2.84	50

Fig. 1. FM images of (a) Sonogel; (b) Sonogel-Carbon Composite; (c) Sonogel-Carbon coated with 0.5% Nafion; (d) individual LAC-Nafion/Sonogel-Carbon; and (e) dual LAC-TYR-Nafion/Sonogel-Carbon biosensors. Note that the value of one *Z*-axis division increases from (a) to (e) and is always lower than in *X* and *Y*-axes (100 nm).

LAC (d) or dual LAC-TYR (e). The table in Fig. 1 shows the roughness (R_a) values for each of the images. This parameter is calculated by the AFM analysis software as the arithmetic average of the absolute values of the surface height deviations measured from the mean plane within the scanned area:

$$R_a = \frac{1}{n}\sum_{j=1}^{n}|z_j|$$

Additionally, the table shows the percentage of *surface area difference* (*S.A.D.*) between the three-dimensional area of the image and its projected two-dimensional area, expressed as:

$$\text{S.A.D.} = \left(\frac{\Sigma(\text{surface area})_i}{\Sigma(\text{projected area})_i} - 1\right) \times 100$$

As seen in Fig. 1, films of the silica Sonogel (a) were dense, with pore sizes around 10 nm. Since the pores were of smaller size than the carbon particles, this result implies that, during the formation of the Sonogel-Carbon composite, the graphite particles do not get inside the silica pore; instead, the gel is formed around the graphite particles, and the conductivity of the final formed composite is promoted by a percolation mechanism between these particles. When Nafion was deposited (c) on the surface of the Sonogel-Carbon electrode (b), a slight decrease in the roughness and in surface area difference of the composite can be observed, but the same porous structure is conserved and a new granular aspect is generated for the composite. Biomodification of the composite Sonogel-Carbon by a mixture of enzyme and Nafion produces a significant increase in the roughness, as well as a considerable gain in the surface area difference from 1.42% to 2.3–2.84%, while preserving the granular aspect due to Nafion, as shown in Fig. 1 (c–e). In addition, the AFM phase detection technique, applied to these three samples, does not show any significant differences over the entire surface, i.e., it must be homogeneous in composition. These results lead us to think that the enzyme may possibly be introduced inside the ionic cluster region of the Nafion.

3.2. Electrochemistry of dual LAC-TYR and individual LAC-based enzyme electrodes

Fig. 2 shows the current–potential curves firstly for bare and Nafion-coated Sonogel-Carbon, and lastly for LAC, TYR and dual LAC-TYR-based Sonogel-Carbon biosensors. As can be seen, the voltammogram of the TYR-based biosensor is similar to that of the Nafion-coated electrode; furthermore, when the scan rate is varied between 25 and 500 mV s^{-1}, this latter biosensor does not show any Faradaic current. For these reasons, we focussed all the studies on the individual LAC and dual LAC-TYR-based biosensors. The curves of the non biomodified Sonogel-Carbon electrodes are almost flat, indicative of purely capacitive behaviour. The curves (voltammograms) of the LAC and LAC-TYR-based biosensors are different and manifest redox waves with a proportional increase in the capacitive current due to the enzymatic modification of the electrode. Reversible double-layer capacitance can be obtained from the relationship $C_{DL} = j/\upsilon$, where j is the current density in the

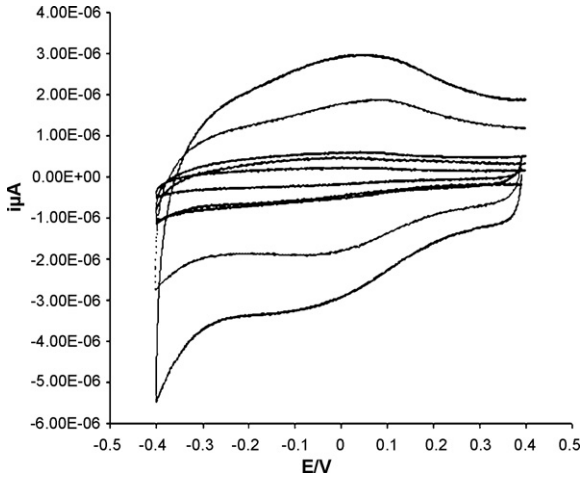

Fig. 2. Cyclic voltammograms of five electrodes (from inner to outer curves): Sonogel-Carbon, Nafion/Sonogel-Carbon, TYR-Nafion/Sonogel-Carbon, LAC-Nafion/Sonogel-Carbon, and LAC-TYR-Nafion/Sonogel-Carbon, respectively. Medium: aerated acetate pH 5 buffer solution. Scan rate: 200 mV s^{-1}.

plateau current regions and υ is the scan rate [28]. We have varied the scan rate from 50 to 500 mV s^{-1} for these four electrodes, and no current corresponding to Faradaic process has been found in the case of non bio-modified electrodes, contrary to the case of the biosensors. Furthermore, to calculate C_{DL} in the case of the biosensors, the Faradaic current was eliminated using a linear correction from the peak's beginning to its end. From the slope of the linear curve representing a limiting current density (for the same absolute cathodic and anodic values) versus scan rate, capacitance values were obtained for all cases as summarised in Table 1. An increase of capacitance with the degree of modification can be seen. So, the unmodified electrode shows the lowest capacitance and the electrodes modified with Nafion and the two enzymes show the highest capacitance value (i.e. 10 times higher than that of the unmodified electrode). These results are in accordance with the morphology study, and can be attributed to the limitation of charge movement through the modification layers, and to the changes in the active surface exposed to the electrolyte, as seen in the AFM studies.

3.3. Cyclic voltammetry behaviour in the absence of organic mediators

Laccase and Tyrosinase belong to the same family of oxidase enzymes with active copper centres. Laccase contains four copper atoms with different electron paramagnetic resonances: Type 1 or blue (T_1), Type 2 or normal (T_2), and Type

Table 1
Double-layer capacitances of Sonogel-Carbon and enzyme/Sonogel-Carbon electrodes

Electrodes	C_{DL} (mF cm^{-2})
Sonogel-Carbon	0.083 ± 0.06
Nafion/Sonogel-Carbon	0.236 ± 0.019
LAC-Nafion/Sonogel-Carbon	0.565 ± 0.102
LAC-TYR-Nafion/Sonogel-Carbon	0.959 ± 0.106

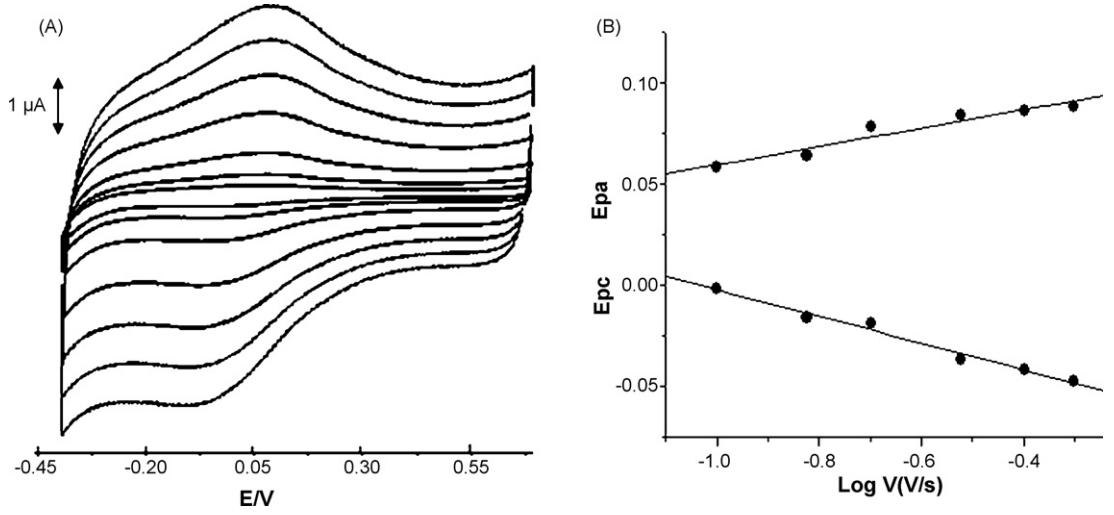

Fig. 3. (A) Effect of scan rate on the cyclic voltammograms (from inner to outer curves) of the LAC-Nafion/Sonogel-Carbon biosensor, from 100 to 500 mV s^{-1}. (B) Dependence of peak potentials versus scan rate in logarithmic scale. Conditions as in Fig. 1.

3 or coupled binuclear copper site (T_3), that catalyze the oxidation of hydrogen atom from the hydroxyl group of various aromatics, mainly the large number of phenolic compounds, polyamines, lignins and aryl diamines, as well as some inorganic ions, coupled to the reduction of molecular dioxygen to water [29]. It can be assumed that the donor substrates react initially near the T_1 and one electron is transferred to a T_2/T_3 cluster site where oxygen is reduced via four electrons exchange. Tyrosinase (monophenol monoxygenerase) contains two copper T_3 type centres, and therefore catalyzes two different oxygen-dependent reactions: the *o*-hydroxylation of monophenols to *o*-diphenols (cresolase activity), and the successive oxidation of *o*-diphenols to *o*-quinones (catecholase activity) [30]. For special kinds of immobilization and electrode, LAC (of different origin) and TYR (from *Mushroom*) have demonstrated electrochemical activities without any mediator [11]. This electron transfer can be described as a tunnelling process between the enzyme catalytic centre and the electrode.

In this study, cyclic voltammetry was applied to illustrate the electron transfer between these multicopper enzymes and the surface of the Sonogel-Carbon electrode. Two biosensors have been compared in this respect, individual LAC/Sonogel-Carbon and a dual LAC-TYR/Sonogel-Carbon. As can be seen in the voltammograms of Fig. 2, redox processes were not observed for the uncoated or the Nafion-coated Sonogel-Carbon electrodes. In contrast, two redox peaks can be seen for the LAC/Sonogel-Carbon and LAC-TYR/Sonogel-Carbon bio-electrodes: the reduction/oxidation potential peaks are around −14 and 86 mV (vs. Ag/AgCl), with $\Delta E_p = 100$ mV and $E_m = 36$ mV, for the LAC-based biosensor, while the peaks are −131 and 3 mV, with $\Delta E_p = 116$ mV and $E_m = -64$ mV, for the dual LAC-TYR-based biosensor. The separation of peak values, much larger than 60 mV/n, prove the quasi reversible behaviour in the two electrocatalytic biosensors [28]. On the other hand, it can also be observed that the reduction currents start at 300 and 330 mV for LAC, and LAC-TYR-based biosensors, respectively; these val-

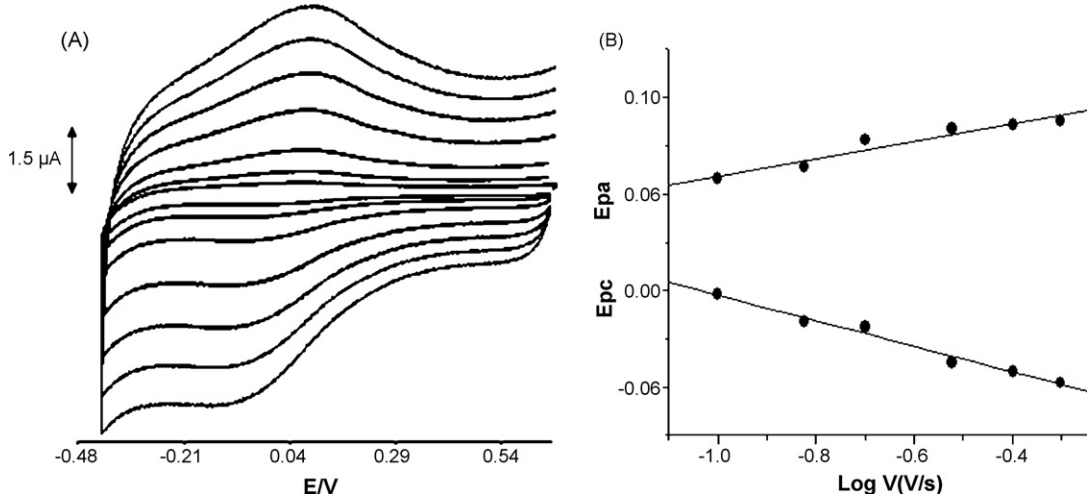

Fig. 4. (A) Effect of scan rate on the cyclic voltammograms (from inner to outer curves) of the dual LAC-TYR-Nafion/Sonogel-Carbon biosensor, from 50 to 500 mV s^{-1}. (B) Dependence of peak potentials versus scan rate in logarithmic scale. Condition as in Fig. 1.

ues are more negative than others reported for similar Laccase: around 550 mV at pH 3.5 for *Trametes Ochrasera*, and *Trametes Hirsuta* Laccases entrapped at a HOPG electrode surface [31], and around 450 mV at pH 5.5 for *Polyperous versicolor* Laccase adsorbed on the surface of a pyrolytic graphite electrode [16]. Moreover, the quasi-reversible couple that appears for our two biosensors is similar to that reported by Lee et al. [16], when 2,9-dimethyphenanthroline was added to the supporting electrolyte, and also similar to that shown for Laccase, from *Trametes Hirsuta*, immobilized on a bare gold electrode in aerated citrate–phosphate buffer at pH 4 [32].

Figs. 3(A) and 4(A), show, respectively, the cyclic voltammograms of the two biosensors at different scan rates, from 0.05 to 0.5 V s^{-1}. The anodic and cathodic peaks show linear increase in current intensity with scan rate, thus indicating a surface control electrode process (data not shown). The average covered surface can be calculated from the Faraday's law as follows: $Q = nFA\Gamma_m$; where Q is the integrated peak value, A is the surface electrode (0.0103 cm^2), and n is the number of transferred electrons assumed equal to 4, so this is the number of electrons necessary to reduce the molecular oxygen near the T_2/T_3 cluster site of active enzymes. From the cathodic peaks recorded at a scan rate of 0.5 V s^{-1}, values of 1.7×10^{-10} and 1.9×10^{-10} mol cm^{-2} for LAC and dual LAC-TYR-based Sonogel-Carbon biosensors, respectively, were obtained. These values are much higher than that obtained by a saturated pyrolytic graphite electrode surface with *Polyperous versicolor* Laccase [16], which indicates the performance of our immobilization matrix and electrode, taking into account the similar mass for the two Laccase enzymes (about 70 kDa).

The most important parameter for use as a criterion in investigating the bioamplification procedure is the electron-transfer rate constant k_{ET}, which quantifies the direct electron-transfer efficiency in the two cases. Laviron's model [33] for a diffusionless electrochemical system has been used to determine this parameter, which makes it possible to deduce the transfer coefficient and the rate constant of an electrochemical reaction from the experimental study of the variation of the potential peak as a function of the scan rate. Figs. 3(B) and 4(B), show the linear dependence of the anodic and cathodic potential peaks on scan rate in logarithmic scale. According to the Laviron's criterion, when $n\Delta E_p > 200$ mV, the transfer coefficient α can be determined from the slope of the representation $E_p = f(\log(\upsilon))$ which equals $-2.3RT/\alpha nF$ and $2.3RT/(1-\alpha)nF$ for the cathodic and anodic peaks, respectively, and k_{ET} can be obtained from the following equation:

$$\log(k_{ET}) = \alpha\log(1-\alpha) + (1-\alpha)\log(\alpha) - \log\left(\frac{RT}{nF\upsilon}\right) - \alpha(1-\alpha)\frac{nF\Delta E_p}{2.3RT}$$

From these, we calculated $\alpha = 0.64$ and k_{ET} 6.19 s^{-1} for the Laccase biosensor, and 0.67 and 8.52 s^{-1} for the dual enzymes biosensor. As expected, the bioamplification does not affect the transfer coefficient and possibly reflects that the active sites present in the dual enzymes layer and in the individual enzyme layer do not differ qualitatively between the two cases. In addition, the k_{ET} values reported here for both cases are close to those obtained with *Coriolus versicolor* Laccase monolayer covering a pyrocarbon surface electrode [15], and lower than that obtained with Laccase, from the same origin, immobilized on highly dispersed colloidal graphite or carbon black [19].

3.4. Effect of organic mediator

Fig. 5(A), shows the voltammograms of the two biosensors when a 0.5 mM concentration of gallic acid was added in the electrochemical cell. As expected, the shape of the voltammograms changes completely from the unmediated voltammograms: the change can be summarised as the shift of the oxido-reduction peaks to positive potentials, and the appearance of the catalytic wave at a potential beyond −50 mV. The current collected in the catalytic wave, the origin of which is the reduction of the enzymatic product, is larger in the case of the dual enzymes modification than that obtained for the individual Laccase modification, and indicative of the signal bioamplification. Moreover, it is important to note that gallic acid is not an ideal substrate for Tyrosinase. Thus, in our investigation in this context, we found that the sensitivity of the LAC-based biosensor to gallic acid is 100 times stronger than that shown by the TYR-based biosensor and the response of the dual electrode is stronger than the sum of the responses of individual LAC and TYR-based biosensors [27].

Although this amplification has already been reported, the mechanism has not yet been adequately elucidated. Our results, especially the E_m potential, the average of covered surface and the calculated k_s for the two biosensors, may demonstrate that this amplification can also be manifested in catalytic bioelectroreduction of oxygen as well as in the DET manifested in all cases.

In the catalytic cycle of the two enzymes, oxygen is reduced to water without the intermediate formation of hydrogen peroxide [32]. Furthermore, it has been demonstrated that the presence of H_2O_2 increases the oxy-Tyrosinase content for Tyrosinase and the peroxide-level intermediate for Laccase [29]. Based on these findings, a speculative explanation of this signal amplification has been reported, in the generation of H_2O_2 between the two enzymes [2,6]. To check this assumption, we added peroxide to the solution in the case of TYR and LAC-based individual enzyme electrodes. In the presence of gallic acid no significant changes in the current was observed in the voltammogram for the individual TYR-based biosensor, because of the low sensitivity of this enzyme to gallate, although in the presence of catechol, as prototype substrate of this enzyme, the current registered at potential of −200 mV was amplified by approximately 30% compared with that without peroxide, and the irreversible peak was not changed, as seen in Fig. 5(B). On the other hand, the voltammograms for the individual LAC biosensor in the presence of gallic acid and peroxide displayed significant changes, as shown in Fig. 5(C). These changes are an amplification of the current of the peaks, the displacement of their potentials, and the variation in the waveform with an increase in the catalytic current up to the potential of about −200 mV.

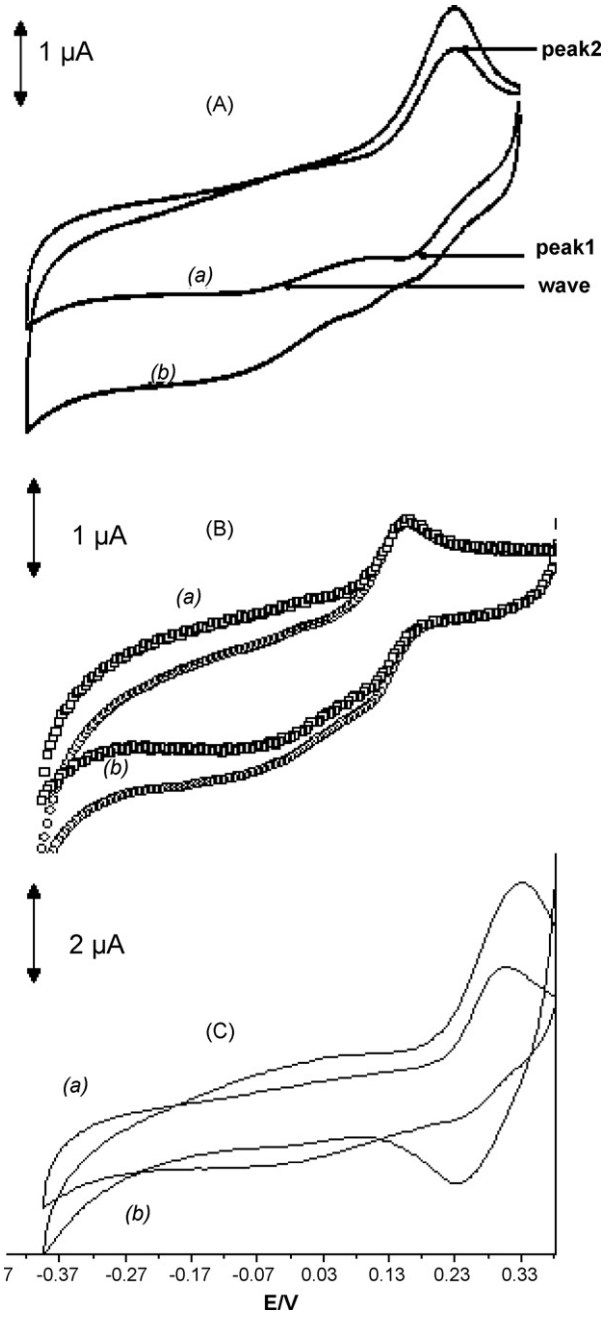

Fig. 5. Cyclic voltammograms of: [A] LAC-Nafion/Sonogel-Carbon (a), dual LAC-TYR-Nafion/Sonogel-Carbon (b) in absence of peroxide, [B] TYR-Nafion/Sonogel-Carbon in absence (a) and presence (b) of peroxide, and [C] LAC-Nafion/Sonogel-Carbon in absence (a) and presence (b) of peroxide. Condition: aerated acetate pH 5 buffer solution, 0.01 mM of peroxide for [B] and [C], 0.5 mM gallic acid for [A] and [C], and 0.5 mM of catechol for [B], scan rate 100 mV s^{-1}.

These results confirm a relative favourable effect on the catalytic cycle of the two enzymes, depending on the nature of the substrate, although this is not sufficient to give a clear response to the question: At what stage of the catalytic cycles of the enzymes does the peroxide have this effect? Nor do the results illustrate unequivocally the mechanism of the signal amplification in the case of dual enzymes electrode.

However, considering all the collective results accumulated in this research, we can postulate three hypotheses. The first hypothesis is that the generation of peroxide, which is also observed in the catalyzed TYR system, increases the intermediate peroxide-level in the LAC cycle, and this promotes a reduction of oxygen by this enzyme; the amplification of the parameter in the DET and the increase in the current of the peaks observed in Fig. 5(C) serve as the basis for this hypothesis. The second hypothesis is that the effectiveness of the TYR biocatalysis is promoted by a radical phenol, as well as by the peroxide generated by LAC; the relatively favourable effect of H_2O_2 on the catalytic wave in Fig. 5(B) is proof of that. Finally, simple explanations could be based on the data obtained in the characterization study of the surface and/or the use of the crosslinkage procedure to immobilize the enzymes in both biosensors. The increase in the protein quantity in the dual biosensor compared to that used for the individual one conserving the same percentage of glutaraldehyde can protect the enzymes from the undesirable desnaturation caused by inter-crosslinkage phenomena and also increase the sensitivity of the resulting biosensor. The roughness, together with the considerable gain in the surface area, demonstrated for the dual enzyme-based sensor (see table in Fig. 1) might be advantageous for mass transfer or for redox cycle-based amplification. In an attempt to confirm this third possible explanation, amperometric measurements were performed with three biosensors (TYR, LAC, and dual enzyme-based biosensors) in the presence of Ferulic acid. No response was obtained for the TYR-based biosensor, as a consequence of the ortho-occupation in this substrate; nevertheless, bioamplification phenomenon was observed in the case of the dual enzymes based biosensor, with an increase in the sensitivity and a decrease in the apparent Michaelis–Menten constant, when compared with the response of the individual Laccase biosensor.

Although the definitive confirmation of one of these mechanisms is not easy, the use of a multi-transducer could help to elucidate it, especially by proving the two first explanations, but this strategy is considerably limited by the distance between the active centres of the two different enzyme-based biosensors [6].

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