We report here a novel bioelectrode based on self-assembled multilayers of polyphenol oxidase intercalated with cationic polyallylamine built up on a thiol-modified gold surface. We use an immobilization strategy previously described by Hodak J. et al. (Langmuir 1997, 13, 2708–2716) Quartz crystal microbalance with electro-acoustic impedance experiments were carried out to follow quantitatively the multilayer film formation. The response of the self-assembly polyphenol oxidase–polyallylamine electrodes toward different metabolically related catecholamines was studied, to evaluate enzyme kinetics. For the analyzed compounds, only dopamine and its metabolite Dopac gave catalytic currents at applied potential close to 0 V. These responses were proportional to the number of polyphenol oxidase-immobilized layers and were also controlled by the enzymatic reaction. The combination of microgravimetric and electrochemical techniques allowed us to determine the kinetic enzymatic constants, showing that the decomposition rate for the enzyme–substrate complex is slower than the enzymatic reoxidation step.

Enzymatic biosensors are very useful tools in satisfying analytical requirements, particularly those of specificity, for biochemistry, pharmacology, industry, and environmental sciences.1,2

Enzyme immobilization on the sensing electrode surface is one of the most important points to be considered in biosensor design. The selected procedure to immobilize the enzyme has to be able to stabilize the macromolecule, to allow diffusion of substrates and products as well as to ensure an efficient electron transfer.3 Although many immobilization methods have been employed, very few can control the amount and spatial distribution of the biocatalyst.4 In relation to this, the uptake of enzymes in multilayer assemblies is very promising, not only from the analytical point of view but also from the view of basic enzymology. The self-assembling procedure, first introduced by Decher et al. is based on the electrostatic interactions between ionic macromolecules of different charge.5–10 In this approach, the electrode surface is first derivatized in order to have a stable surface charge excess. Then the multilayer film is built up by the layer-by-layer intercalation of positive and negative polyelectrolytes.11–17 This procedure has also been employed in association with a variety of systems including redox polyelectrolytes,13,14,17 clay15 and recognizing molecules such as DNA,8,9 and virus10 as well as other proteins with selective functions.11–13 Several authors have assembled mono- and bienzyme films under aerobic conditions12,16 or have used an artificial redox mediator as cosubstrate.13,17 In the case of enzyme electrodes, the enzyme is adsorbed at the appropriate pH with respect to the isoelectric point, so that it carries an excess of positive or negative charge on its surface.11–13,17

Electrochemical Behavior of Polyphenol Oxidase Immobilized in Self-Assembled Structures Layer by Layer with Cationic Polyallylamine

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Many advantages of this methodology derive from the possibility to control the amount of enzyme deposited in each immobilization step, which has been proved to be constant and reproducible. Interferences can be minimized by the proper selection of the charge of the topmost layer exposed to the electrolyte and also by the coimmobilization of complementary enzymes. To improve the selectivity of the biosensor, steric and electrostatic factors, as well as the hydrophobicity of the matrix environment, have to be controlled. This can be managed by the proper selection of the polyelectrolyte.

Polyphenol oxidase (PPO) has been proved to be very useful in the analytical determination of phenols and catechols such as neurotransmitter substances and related metabolites. These very similar molecules are also involved in redox reactions with ascorbic acid, ubiquitous in biological samples. These characteristics pose interference problems when a particular substrate has to be analyzed, as in the case of dopamine (Do), whose control is necessary in patients with Parkinson’s disease.

We report here a novel bioelectrode based on self-assembled multilayers of PPO intercalated with cationic polyallylamine (PAA) built up on a thiol-modified gold surface, using an immobilization strategy previously described in the literature. We have used a quartz crystal microbalance with electroacoustic impedance (QCM) to follow quantitatively the multilayer film formation as well as amperometric measurements of the catalytic response, to evaluate enzyme kinetics. The analytical performance of the bioelectrode toward several catecholamines, including Do, was also studied.

**EXPERIMENTAL SECTION**

**Reagents.** Solutions were prepared with Milli Q (Millipore) deionized water and chemicals used as received. Thiol solution, 2.0 × 10⁻³ M, was prepared with 3-mercapto-1-propanesulfonic acid (Aldrich) in 1.6 × 10⁻³ M sulfuric acid (M erck). Polyelectrolyte solutions contained 0.16% w/w polyallylamine hydrochloride (Aldrich) in deionized water and commercial PPO (EC 1.14.18.1) (Sigma) in the concentration range from 0.84 to 0.96 g L⁻¹ in 1.0 ± 10⁻² M TRIS buffer (J. K. Barker), pH 6.6 ± 0.3. Air-saturated 0.10 M phosphate (M erck) buffer solutions were the base electrolyte in amperometric measurements. Solutions of pyrocatechol (Mallinckrodt), Dopac, adrenaline, noradrenaline (NA), L-DOPA, L-α-methyl-DOPA, and isoproterenol (Sigma) of different concentrations were prepared in phosphate buffer.

**Electrochemical Measurements.** The working electrodes were gold sheets of 2.2 ± 0.1 cm² geometric area. The cleaning procedure included polishing with 600-grade emery paper followed by a careful sonication in deionized water, heating in sulfuric/nitric acid mixture for 2 h, immersion in “piranha” solution (1:3 H₂O₂/98% H₂SO₄) for 20 min, and rinsing with ultrapure water. Caution: “piranha” solution is very corrosive and must be handled with care at all times. The surfaces were then stabilized by cycling in 0.5 M sulfuric acid between 0.20 and 1.55 V until a reproducible cyclic voltammetry corresponding to a clean surface was obtained. This cleaning procedure was repeated before each experiment.

A platinum mesh was the counter electrode, and a saturated calomel was the reference. All potentials in the text are reported with respect to this reference.

**Preparation of PPO-PAA Assemblies.** We employed the method of Hodak et al. to build up layer-by-layer supramolecular structures by reverting the surface charge of the topmost layer as depicted in Figure 1. The first step was the derivatization of the gold surface by soaking the electrode in thiol solution for 30 min, followed by a careful rinsing in deionized water. Thiol adsorption brings a negatively charged surface due to exposed sulfonate groups (Figure 1A). The following steps consisted of alternated immersions in the cationic polyelectrolyte and anionic enzyme solutions for 5 and 10 min, respectively. As amine groups of PAA in deionized water are 25% protonated, the polycation is readily adsorbed on the thiol layer, generating a positive residual charge on the exposed surface (Figure 1B). The following immersion in buffered pH 6.6 PPO solution modifies the surface in two ways: the protonated amine fraction decreases to 15% and the anionic PPO (isoelectric point IP = 4.7) is adsorbed on the surface.
positively charged surface (Figure 1C). As PPO is itself a polyanion, the new exposed surface was negative, favoring the adsorption of a second PAA layer (Figure 1D). The second and third steps were repeated several times, depending on the desired number of bilayers.

For blank experiments, PPO—PAA multilayers were prepared with denatured enzyme. The last was obtained by heating the protein solution for 10 min in boiling water. The lack of enzymatic activity was checked spectrophotometrically.

**Electroacoustic Measurements.** AT-cut quartz crystals (10 MHz) of 10-mm diameter (International Crystal Manufacturing Co., Inc., Oklahoma City, OK) with rugose gold electrodes (1 μm) of 0.196-cm² active area were employed.

The complex voltage divider to measure the resonant frequency and both components of the quartz crystal modified Butterworth—Van Dyke (BVD) equivalent circuit has been described elsewhere. In brief, a 10-MHz sinusoidal voltage (5 mV peak to peak) generated by a voltage-controlled oscillator (VCO) connected to the D/A output of a Keithley data acquisition system 575 was applied. Both the input V_i and output V_o voltage moduli were amplified and rectified with an ideal diode circuit, and the resulting signals were measured with an A/D converter of the Keithley data acquisition system 575. An AT-386 computer generated the perturbation ac signal and calculated the ratio of the circuit transfer function modulus, i.e., |V_o/V_i| as a function of the VCO output signal frequency. The sample rate was 10⁴ s⁻¹; thus, a complete transfer function spectrum (50 kHz and 100 points) was taken in 10 ms. To correct for any shift of the VCO, the extreme frequencies were measured with a HP5334B frequency meter via an IEEE-488 interface. Calibration of the dc-rectified signals was achieved by applying the read level functions of the HP5334B to the amplified rf signals used for frequency measurement. The transfer function spectrum, modulus of V_o/V_i, as a function of frequency around 10 MHz, was obtained in real time for the quartz crystal in contact with the solutions.

The modified lumped-element BVD electrical equivalent circuit described elsewhere, which consists of a static capacitance, C_o, in parallel with a motional branch, was used to extract electrical parameters that can be related to mass and viscoelastic properties of the enzyme multilayer. The motional arm impedance is the sum of the impedance due to the unperturbed or bare quartz with L_o, C_o, and R_o; the liquid load with L_i and R_i; and the viscoelastic film load with L_i and R_i (Z = Z_o + Z_i + Z_l). The motional arm lumped elements, R and L, and the static capacitance, C_o, of the modified BVD equivalent circuit were obtained by nonlinear fit of the experimental transfer function spectrum data |V_o/V_i(ω)|, to the analytical expression of the BVD transfer function:

\[
\frac{V_o}{V_i} = \frac{\sqrt{\frac{(\omega L - \frac{1}{\omega C})^2 + R^2}{\sqrt{(\omega L - \frac{1}{\omega C})^2 + \frac{\omega L}{C_o} - \frac{C_o}{\omega C} - \frac{1}{\omega C} + R + \frac{RC_o}{C_o} + R^2}}}}{\sqrt{\frac{(\omega L - \frac{1}{\omega C})^2}{\omega L - \frac{1}{\omega C} + \frac{C_o}{\omega C} + \frac{1}{\omega C}}}}
\]

where C_o is the measuring capacitance in series with the BVD, \( \omega = 2 \pi f_o \) and \( f_o \) is the resonant frequency of the AT-quartz crystal.

**Quartz Crystal Impedance.** For a system of two nonpiezoelectric layers attached to the quartz resonator: a viscoelastic film (f) and a viscous liquid electrolyte overlayer (l), Martin et al. derived an expression for the total electrical equivalent impedance Z in terms of the surface mechanical impedance Z_m.

\[
Z = \frac{2\pi L_o}{\pi \sqrt{\mu_o \rho_o}} Z_m = R + jX_L
\]

where \( X_L = \omega L \), \( L_o = 7.5 \times 10^{-5} \), and \( \rho_o = 2650 \) kg m⁻³ is the density of the quartz.

The mechanical impedance of each nonpiezoelectric layer on the quartz can be described:

\[
Z_m = \sqrt{G \tanh(kd)}
\]

where G = G’ + jG” is the complex shear modulus of the nonpiezoelectric layer at 10 MHz, k = |\omega|/\sqrt{\mu_o \rho_o}², the wave propagation constant, and d is the thickness of the nonpiezoelectric layer of density \( \rho_o \).

If the layer is thick enough (d → ∞), the shear wave vanishes in the bulk of the layer, and no energy is transferred from this point. In this case, \( \tan(hk) \sim -1 \) and \( Z_m = Z_m^\infty = (\rho_o G)^{1/2} \), where the superscript * indicates the semi-infinite approximation.

For small changes in \( X_{LM} \), corresponding to a small deposited mass, the variation in resonant frequency can be approximated as linear on \( X_{LM} \), and the Sauerbrey equation holds:

\[
\Delta f_o = \frac{-2 \pi f_o^2}{\sqrt{\mu_o \rho_o}} \frac{\Delta m}{A}
\]

where A is the active area of the piezoelectric resonator and \( \Delta m \) is the variation in the electrode mass (Sauerbrey mass).

If two nonpiezoelectric layers are successively attached to the crystal, the following expression describes the surface impedance:

\[
Z_s = \frac{2\pi L_o}{\pi \sqrt{\mu_o \rho_o}} \left( \frac{Z_m^\infty \tanh(kd_f) + Z_l^\infty \tanh(kd_l)}{1 + \frac{Z_m^\infty}{Z_l^\infty} \tanh(kd_f) \tanh(kd_l)} \right)
\]

where the subscript f denotes the viscoelastic film underlayer and the subscript l the liquid overlayer, respectively.

If the mechanical interaction between both layers is negligible, the denominator tends to unity and additivity of the layer impedances holds; that is,

\[
Z_s = \frac{2\pi L_o}{\pi \sqrt{\mu_o \rho_o}} \left( Z_m^\infty \tanh(kd_f) + Z_l^\infty \tanh(kd_l) \right)
\]

References:

In the case of two layers with comparable mechanical impedances, the interaction between both layers cannot be neglected and the full eq 5 should be used. We can describe the contribution of the viscoelastic layer to the total quartz crystal impedance $Z_{\text{Q}}$ at 10 MHz in terms of film properties, \(^{24,25}\)

\[
Z_s = R_f + j\omega L_f = \frac{2\omega L_0}{\tau \sqrt{\mu_f \rho_f}} \left( \sqrt{\frac{\rho_f}{G_f}} \tanh \left( j\omega d_f \sqrt{\frac{\rho_f}{G_f}} \right) \right)
\]

where $d_f$ is the film thickness, $\rho_f$ its density, and $G_f$ the viscoelastic complex shear modulus ($G'_f + jG''_f$) with $G'_f$ the storage modulus and $G''_f$ the loss modulus of the viscoelastic film at 10 MHz.

When additivity holds and eq 6 can be used, the motional impedance for the modified BVD equivalent circuit is given by the sum of the unperturbed resonator $Z_0 = R_0 + j\omega L_0$, the liquid load $Z_l = R_l + j\omega L_l$, and the viscoelastic film load $Z_f = R_f + j\omega L_f$ which contribute to the mechanical impedance of the composite resonator, $Z = Z_0 + Z_l + Z_f$.

**RESULTS AND DISCUSSION**

PAA and PPO Adsorption in Assembled Structures. As was discussed elsewhere,\(^ {13}\) the use of the QCM to monitor the study of the electrostatic adsorption of PAA onto the thiolated gold surface involves a strong viscoelastic effect due to the rheological properties of the polycation solution. Because of this, the decrease in the crystal resonance frequency observed after PAA adsorption cannot be assigned directly to a mass increase. This fact could be evidenced by impedance measurements.

Figure 2 shows the time course of quartz crystal electroacoustic impedance parameters, $\Delta X_{\text{lf}}$ and $\Delta R_f$, during the adsorption of cationic PAA onto the thiolated gold surface (Figure 2A) and the first layer of adsorbed PPO. Additions of 0.5-mL aliquots of PAA 0.48% (w/w) to 1.0 mL of deionized water. Quartz crystal (10 MHz, AT-cut), 0.196-cm\(^2\) active area. Controlled convective conditions.

Figure 2. Changes in electroacoustic impedance parameters, $\Delta X_{\text{lf}}$ (○) and $\Delta R_f$ (+), during cationic PAA adsorption on (A) gold electrode modified with sodium 3-mercaptop-1-propanesulfonate and (B) the first layer of adsorbed PPO. Additions of 0.5-mL aliquots of PAA 0.48% (w/w) to 1.0 mL of deionized water. Quartz crystal (10 MHz, AT-cut), 0.196-cm\(^2\) active area. Controlled convective conditions.

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Figure 3 shows the time course of quartz crystal electroacoustic impedance parameters, $\Delta X_{\text{lf}}$ (○) and $\Delta R_f$ (+), during anionic PPO adsorption on the sixth layer of adsorbed PAA. Addition of 0.25-mL aliquot of PPO 2.52 mg mL\(^{-1}\) to 0.50 mL of 10 mM TRIS buffer pH 6.3. Quartz crystal (10 MHz, AT-cut), 0.196-cm\(^2\) geometric area. Sensibility 46 ng $\Omega^{-1}$ cm\(^{-2}\). Convective conditions.

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Figure 2 shows the time course of quartz crystal electroacoustic impedance parameters, the reactive impedance $\Delta X_{\text{lf}}$, and the motional damping resistance $\Delta R_f$ for the adsorption of cationic PAA, the first one onto the thiolated gold surface (Figure 2A) and the second one onto a previously adsorbed anionic PPO layer (Figure 2B). For both PAA layers, variations in the two impedance parameters were observed at the beginning of the adsorption step in a way that precludes direct correlation of $\Delta X_{\text{lf}}$ and mass.\(^ {13}\) Successive layers of adsorbed PAA showed similar impedance variations. As the parameters remained constant for adsorption times longer than 5 min, this period was chosen as the PAA adsorption time in each step of the assembly procedure.

Figure 3 shows typical changes in $\Delta X_{\text{lf}}$ and $\Delta R_f$ transients during the electrostatic adsorption of PPO onto the top layer of...
the Au–thiol–cationic PAA electrode after injection of enzyme solution. After 15 min PPO was added to the buffer solution, $\Delta X_L$ reached a steady-state value and it can be observed that $\Delta R_f$ change in parallel, with $\Delta R_f = 0.1 \Delta X_L$. The same behavior was observed for consecutive PPO layers, independently of their number. The total change in $\Delta R_f$ for 10 successive bilayers was lower than 4% of total $\Delta X_L$. This behavior was found in all the experiments. As a result, we could assign the variations of $\Delta X_L$ to mass changes related to the PPO uptake. The Sauerbrey equation is therefore applicable as follows:\textsuperscript{13}

$$\Delta m = \frac{A\sqrt{\mu_0\rho_0}}{8\pi r_f^2\rho_0} \Delta X_{L_f} \quad (8)$$

As previously stated, the steady state was achieved in 15 min, so this time was selected for each enzyme adsorption step.

The PPO surface concentration $\Gamma_{\text{PPO}}$ calculated from the $\Delta X_L$ shift (eq 8) for each enzyme layer was estimated assuming a molar mass of 120,000 g mol$^{-1}$ and 100% enzymatic purity degree.

Of Electrochemical Response of Catechols. It is well known that catechol derivatives electrooxidize on different carbon and metal electrodes to the corresponding quinone in a quasi-reversible two-electron process according to\textsuperscript{20–22,29}

$$\text{HO-} \quad \text{O} \quad \text{C} \quad \text{R} \quad + 2H^+ + 2e^- \quad (9)$$

The same products can be obtained enzymatically, provided the catechols are substrates of PPO.

To check the reactivity of self-assembled PPO electrodes toward catechol derivatives, a set of cyclic voltammograms for the different substrates was performed. Figure 5 shows I–E

Figure 4 shows the time dependence of $\Gamma_{\text{PPO}}$ for successive PPO layers. The mean surface concentration value, averaged over 36 bilayers was $1.2 \times 10^{-11}$ mol cm$^{-2}$ per layer. To our knowledge, no data related to the molecular area of PPO are available, so no estimation of the number of moles involved in each layer could be accomplished. However, crystallographic dimensions of glucose oxidase (GOX) have allowed the estimation of a value of $4.7 \times 10^{-12}$ mol cm$^{-2}$ for a close-packed monolayer of the enzyme.\textsuperscript{13} In the case that the PPO molecular size was not very different from that of GOX, it could be concluded that the enzyme molecules were aggregated in an amount equivalent to several monolayers. However, the quartz crystal microbalance overweightes proteins when comparing the wet and dry QCM-determined masses due to the inclusion of water and ions together with the protein.\textsuperscript{28}

When the stationary enzyme mass ($m_{\text{PPO}}$) from experiments such as those of Figure 4 were plotted against the number of bilayers ($n_b$), a linear relationship was obtained for $n_b$; see inset in Figure 4. This is clear evidence that every PAA layer behaved in a similar way toward electrostatic adsorption of PPO. As previously reported, for glucose oxidase, some dispersion was observed for the first layers.\textsuperscript{13}


profiles at 0.020 V s\(^{-1}\) for electrodes modified either by six or seven enzyme–polycation bilayers. Voltammograms were recorded in buffer solution pH 6.6 with and without (4–5) \(\times 10^{-4}\) M substrate. For NA and L-\(\alpha\)-methyl-DOPA, irreversible responses were obtained, as can be seen in Figure 5A. A similar behavior was observed for adrenaline, isoproterenol, and L-DOPA (not shown). Potentials more negative than \(-0.2\) V were necessary to reduce the corresponding quinones. On the other hand, Do, pyrocatechol, and Dopac presented less irreversible responses (Figure 5B), although the kinetics were more sluggish than those reported in the literature for unmodified gold surfaces.\(^{29}\) As the potential limit in the positive scan was far beyond the catechol oxidation potential (0.20 V), the reduction waves observed involved quinone derivatives enzymatically formed as well as electrochemically generated eq 9 backward.

Suitable potential values for the amperometric detection of quinones enzymatically formed were \(-0.10, -0.15,\) and \(-0.20\) V for Do, pyrocatechol, and Dopac, respectively. These potential values were selected from I–E profiles such as those of Figure 5B. The lack of electrochemical response at these potentials for NA, L-\(\alpha\)-methyl-DOPA, adrenaline, isoproterenol, and L-DOPA is a real advantage for the analytical determination of Do in the presence of such substrates, which otherwise would interfere.

**Catalytic Activity of Immobilized PPO.** Figure 6 shows voltammetric waves for Do and pyrocatechol obtained when negative potential sweeps from 0.100 V were applied on seven-bilayer electrodes, after blank current subtraction. A similar behavior was observed for Dopac. In all cases, potentials higher than 0.1 V were avoided in order to prevent electrochemical oxidation of substrates. Considering the applied potential, the catalytic currents observed can be related to the electrochemical reduction of the enzymatically formed quinone derivatives, according to eq 9 backward. The very well-defined catalytic waves made evident the active state of the enzyme.

Figure 7 shows the chronamperometric response for successive additions of standard Do solution aliquots to an assembly of 12 bilayers prepared with denaturalized and native PPO, Figure 7a and b, respectively. In the former case, no reduction current was observed, while in the latter, well-defined stationary currents were evident. Differences between both amperometric curves demonstrate that the reduction currents in Figure 7b are undoubtedly due to the electroreduction of the enzymatically formed quinone derivative, eq 9 backward, as was discussed above. Steady-state currents \(I_{ss}\) were reached at times shorter than 120

---

Figure 7. Chronoamperometric currents at \(-0.1\) V on a self-assembled PPO–PAA electrode (12 bilayers). (a) Denaturalized PPO: addition of 400-, 500-, and 1000-\(\mu\)L aliquots of 1.4 \(\times 10^{-3}\) M Do. (b) Active PPO (484 units mg\(^{-1}\)): addition of 500- and 300-\(\mu\)L aliquots of 1.6 \(\times 10^{-3}\) M Do. Phosphate buffer solution pH 6.6. Initial volume 18 mL. Convective conditions. Air-saturated solutions.

Figure 8. Amperometric response of self-assembled PPO–PAA electrodes prepared with 4 (\(\bullet\)); 8 (\(\circ\)); 12 (\(\square\)) bilayers, 484 units mg\(^{-1}\) PPO toward Do at \(-0.1\) V. (A) \(I_{ss}\) vs \(c_{Do}\) curves, (B) Normalized Eadie–Hofstee plots. Phosphate buffer solution pH 6.6. Mechanical convective conditions. Air saturated solutions.
s after substrate addition. Similar responses for pyrocatechol and Dopac were obtained, reaching the stationary currents at electrolysis times shorter than 2.5 min.

Increase of the surface enzyme content, $\Gamma_{PPO}$, can easily be accomplished by increasing the number of bilayers. Accordingly, possible incidence of enzymatic matrix thickness on the electrochemical response and on the relative affinity of the electrode toward PPO substrates was checked using three different electrodes, each one prepared with 4, 8, and 12 bilayers. Plots of $I_{SS}$ versus Do concentration ($c_{D0}^{-}\mp$) performed with each electrode are shown in Figure 8. For a given substrate concentration, a linear relationship between the number of bilayers and $I_{SS}$ was obtained. The corresponding Eadie–Hofstee plots were linear with a regression coefficient higher than 0.9931 in the whole concentration range. This behavior allowed us to discard any diffusional resistance, either internal or external, as well as to evaluate the apparent Michaelis–Menten constant $K_M'$. Values of $3.7 \times 10^{-4}$, $4.0 \times 10^{-4}$, and $4.9 \times 10^{-4}$ M were calculated for an increasing number of bilayers. Differences between the three values were not significant. Figure 8B shows a normalized Eadie–Hofstee plot. It is clearly seen that experimental points fit the negative unitary slope expected for pure Michaelian control of the global electrode reaction rate very well.

Pyrocatechol and Dopac gave typical michaelian responses, as is shown in Figure 9. Linear Eadie–Hofstee plots were also found from which $K_M'$ was estimated. Results are summarized in Table 1. Although $K_M'$ values were quite similar, it is clear that $K_M'$ (pyrocatechol) $< K_M'$ (Dopac) $< K_M'$ (Do). A similar tendency in $K_M'$ values for these substrates was observed when PPO was included in carbon paste electrodes, $^{21}$ this being a clear indication that the observed results reflect intrinsic enzymatic properties rather than immobilization environmental effects.

The evaluation of $\Gamma_{PPO}$ through QCM experiments allowed us to estimate the enzyme turnover, $k_{cat}$. Under enzymatic control of the reaction rate,

$$ I_{SS} = nF\Delta V_E $$

where $v_E$ is the rate of the enzymatic reaction and $f$ is the “collection coefficient”, the fraction of enzyme product that is electrochemically reduced, which has to be related to the mass transport coefficients for enzyme product inside and outside the catalytic matrix. $^{30}$ Assuming that the catalytic mechanism can be described reasonably well by a “ping pong” reaction scheme, it follows that

$$ \text{Do} + \text{PPO(Cu(I)} \rightarrow \text{Cu(I))} \rightarrow \frac{k_1}{k_{-1}} \text{Kcat} $$

$$ \text{Do} - \text{PPO(Cu(I)} \rightarrow \text{Cu(I))} \rightarrow \text{DO} + \text{PPO(Cu(I)} \rightarrow \text{Cu(I))} + 2H^+ $$

and accordingly,

$$ v_E = \frac{k_{cat} \Gamma_{PPO}}{1 + \frac{K_M'}{c_{D0}^-} + \frac{K_M'}{c_{D0}^-}} = \frac{v_{max}}{1 + \frac{K_M'}{c_{D0}^-} + \frac{K_M'}{c_{D0}^-}} $$

where $v_{max}$ stands for the maximum rate and $c_{D0}^-$ is the Do bulk concentration, assuming that no concentration gradient developed outside the enzyme matrix. The rest of the factors have their usual meaning. Under oxygen saturation conditions ($K_M'(O_2)/c_{D0}^- \ll 1$), and the expression for enzyme rate, for Do, is

$$ v_E = \frac{k_{cat} \Gamma_{PPO}}{1 + K_M' / c_{D0}^-} $$

and the transduced current under steady state can be considered to be

$$ I_{SS} = nF\Delta V_E \frac{k_{cat} \Gamma_{PPO} c_{D0}^-}{K_M' + c_{D0}^-} $$

Since $I_{SS}$ is proportional to $\Gamma_{PPO}$ for different $c_{D0}^-$ (Figure 8), $f$ does not depend on the number of bilayers or on substrate concentrations, and accordingly, it can be treated as a constant. Thus, in case all PPO was active, from eq 15, $f_{cat} = 2.4\, \text{s}^{-1}$ was evaluated. As $f \leq 1$, and probably some fraction of the enzyme was immobilized, if $f_{cat} = 2.4\, \text{s}^{-1}$ was estimated.

### Table 1. Apparent Michaelis–Menten Constants for Self-Assembled PPO/PAA Systems

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_M' \times 10^4$/M</th>
<th>Linear regression coefficient $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrocatechol</td>
<td>$0.8 \pm 0.1$</td>
<td>0.9733</td>
</tr>
<tr>
<td>Dopac</td>
<td>$2.7 \pm 0.3$</td>
<td>0.9832</td>
</tr>
<tr>
<td>Dopamine</td>
<td>$4.2 \pm 0.9$</td>
<td>0.9861</td>
</tr>
</tbody>
</table>

$^a$ Smallest value obtained in each group of measurements.

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was actually inactive, it can be concluded that PPO is able to transform at least 2.4 molecules/s. In relation to the reaction rate of PPO reoxidation, $K_M'(O_2) = \frac{k_{cat}}{k_2}$. As $K_M'(O_2) = 1.4 \times 10^{-4} \text{ M}^{[31]}$ $k_2$ has the minimum value of $1.7 \times 10^{4} \text{ M}^{-1} \text{ s}^{-1}$, indicating that PPO regeneration rate (eq 12), proportional to $k_{O_2}^{2+}$, is at least 17 times greater than the enzyme–substrate complex dissociation rate. This value fully justifies eq 14.

**CONCLUSIONS**

MCQ studies allowed us to conclude that immobilization of PPO in self-assembled layers using cationic PAA is an excellent procedure in order to control the amount of the biocatalyst, this being proportional to the number of bilayers. The electrochemical response also confirms the linear correlation between catalytic activity and $I_{PPO}$.

In all cases analyzed, no diffusional limitations of the reaction rate were observed, irrespective of the number of assembled bilayers. The electrodes functioned under enzymatic control, with well-characterized Eadie–Hofstee plots. Apparent Michaelis–Menten constant values were similar to those observed when PPO was included in carbon paste electrodes.

The combination of microgravimetric and electrochemical techniques allowed us to determine the enzyme turnover number under the immobilized conditions, showing that the decomposition rate for the enzyme–substrate complex is slower than the enzymatic reoxidation step.

Given the observed differences in the values of the quinone reduction currents at potentials close to 0 V for different substrates, the self-assembled multilayers of PPO–PAA electrodes would be a good choice for the quantification of Do and its metabolite Dopac in the presence of other related compounds.

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