



Biomimetic biosensor based on lipidic layers containing tyrosinase and lutetium bisphthalocyanine for the detection of antioxidants

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ABSTRACT

This paper describes the preparation of a biomimetic Langmuir–Blodgett film of tyrosinase incorporated in a lipidic layer and the use of lutetium bisphthalocyanine as an electron mediator for the voltammetric detection of phenol derivatives, which include one monophenol (vanillic acid), two diphenols (catechol and caffeic acid) and two triphenols (gallic acid and pyrogallol). The first redox process of the voltammetric responses is associated with the reduction of the enzymatically formed *o*-quinone and is favoured by the lutetium bisphthalocyanine because significant signal amplification is observed, while the second is associated with the electrochemical oxidation of the antioxidant and occurs at lower potentials in the presence of an electron mediator. The biosensor shows low detection limit (1.98×10^{-6} – 27.49×10^{-6} M), good reproducibility, and high affinity to antioxidants (K_M in the range of 62.31–144.87 μ M).

The excellent functionality of the enzyme obtained using a biomimetic immobilisation method, the selectivity afforded by enzyme catalysis, the signal enhancement caused by the lutetium bisphthalocyanine mediator and the increased selectivity of the curves due to the occurrence of two redox processes make these sensors exceptionally suitable for the detection of phenolic compounds.

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1. Introduction

Recently, a lot of attention has been paid to antioxidants which are a broad group of active biological substances common in foods (Lucarini and Pedulli, 2010). Polyphenols as food antioxidants are of great interest due to their health benefits as they decrease the risks of cancer and coronary cardiopathy in humans (Crozier et al., 2009; Mello et al., 2010). Furthermore, polyphenols influence the quality and the organoleptic characteristics of foods and affect their antioxidant capacity (del Alamo and Nevaes, 2006; Rodríguez-Méndez et al., 2008a).

Phenolic compounds have been analysed using various methods that include chromatographic, electrophoretic and optic techniques (Kartsova and Alekseeva, 2008). Among various available techniques, electrochemical techniques have often been considered superior, due to their higher sensitivity and inherent portability. In particular, amperometric biosensors have proven to be suitable for phenols determination because of their good selec-

tivity, sensitivity and potential for miniaturization (di Fusco et al., 2010; Gutés et al., 2005). A variety of foods and beverages such as beer (El Kaoutit et al., 2007), tea (Ghindilis et al., 1992), vegetable extracts (Franzoi et al., 2009), olive oil (Busch et al., 2006) and wine (Sanz et al., 2005) have been analysed using amperometric biosensors.

Amperometric biosensors based on the enzyme tyrosinase have been widely used for phenol determination due to their high sensitivity (Lind and Siegbahn, 1999; Imabayashi et al., 2001). The detection and quantification of the reaction within the biosensor can be based on various principles, such as detection of oxygen consumption (Campanella et al., 1992), direct reduction of liberated *o*-quinone (Li et al., 1998) or reduction of the *o*-quinone using a redox mediator such as hexacyanoferrate (Bonakdar et al., 1989), conducting polymers (Wang et al., 2009) or cobalt phthalocyanine (Tanimoto de Alburquerque and Franco Ferreira, 2007).

Metallophthalocyanines (MPcs) are interesting choices as biosensing mediators because of their varied electrochemical activity and catalytic properties (Zagal et al., 2009; Nyokong, 2008). Compared with the parent MPcs, lanthanide bisphthalocyanines (LnPc₂), in which two Pc rings are coordinated with a lanthanide, can be a good alternative due to their rich electro-

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chemical behaviour associated with a range of accessible oxidation states (de Saja and Rodríguez-Méndez, 2005). Although thin films of LuPc₂ have been investigated by our group as the sensing material for voltammetric electrodes in a variety of applications (Rodríguez-Méndez et al., 2008b; Rodríguez-Méndez et al., 2009), their capabilities as electron mediators have not yet been analysed.

The appropriate immobilisation of the enzyme on a solid matrix is a key factor in retaining the enzyme's specific biological function (Goto et al., 2010; Schmidt et al., 2008). Tyrosinase biosensors have been immobilised using a range of techniques that include immobilisation onto carbonaceous electrodes via the cross-linking step by glutaraldehyde (Sanz et al., 2005; Zhao et al., 2009) or immobilisation in polymeric matrixes (Yildiz et al., 2006). The layer-by-layer (LbL) and the Langmuir–Blodgett (LB) techniques are of special interest for enzyme immobilisation because they allow the preparation of biomimetic systems where the enzyme is adsorbed in a lipidic layer via COOH group interaction (Cajab et al., 2009; Caseli et al., 2009). Electrodes incorporating lipids can enhance the enzymatic activity by preserving the conformation of the enzyme (Goto et al., 2010; Schmidt et al., 2008). It has been shown that the activity of horseradish peroxidase (HRP) adsorbed in LB films of phospholipids was ca. 23% higher than when in solution (Schmidt et al., 2008).

In this work, a biosensor based on LB films of tyrosinase for the detection of phenol derivatives has been prepared. The enzyme has been incorporated in a monolayer of arachidic acid so as to mimic biological systems. A lanthanide bisphthalocyanine (the lutetium bisphthalocyanine), which is a new type of electron mediator, has been incorporated in the nanostructured films and its capability as an electron mediator has been tested. Cyclic voltammetry has been applied to study the detection of five phenolic species including one monophenol (vanillic acid), two diphenols (catechol and caffeic acid) and two triphenols (gallic acid and pyrogallol). The electrochemical behaviour of the biosensor (kinetics, detection limit and selectivity) has been analysed and the role of the lutetium bisphthalocyanine as an electron mediator has been discussed.

2. Materials and methods

All chemicals and solvents (Aldrich Chemical Ltd.) were of reagent grade and used as supplied. Mushroom tyrosinase (from mushroom EC 232-653-4), with a noted activity of 5370 U/mg of solid (Catalog No. T3824-250KU), was purchased from Sigma Chemical Co. (USA). A 67 µg mL⁻¹ solution of tyrosinase in buffer phosphate 0.01 M (pH 7) was used for the enzyme immobilisation. The lutetium (III) bisphthalocyaninate (LuPc₂) was synthesized and purified in the neutral radical state following a previously published procedure (Linaje et al., 2000).

Solutions of phenolic compounds including one monophenol (vanillic acid), two diphenols (catechol and caffeic acid) and two triphenols (gallic acid and pyrogallol) were prepared in phosphate buffer 0.01 M (pH 7). However, in the case of the caffeic acid, a 12% ethanol aqueous solution was used instead of water in order to improve the solubility.

LB films were prepared in a KSV 5000 System 3 Langmuir–Blodgett trough equipped with a Wilhelmy plate to measure the surface pressure. Films containing tyrosinase and arachidic acid (Tyr/AA) (Fig. 1a) were prepared by spreading a chloroform solution (1 × 10⁻⁵ M) of arachidic acid (AA) onto a water subphase (NaCl 0.1 M, phosphate buffer 0.01 M of pH 7 in ultrapure water – Millipore MilliQ; 20 °C). After the evaporation of the solvent, 10 µL of a 67 µg µL⁻¹ solution of tyrosinase in 0.01 M phosphate buffer (pH 7) was injected drop by drop underneath the air/water interface. The increase of the surface pressure indicated the adsorption of the enzyme to the floating molecules of AA. The

increase in the surface pressure reached a plateau after 80 min. Molecules were compressed using a symmetrical compression system. At a surface pressure of 40 mN m⁻¹, 20 monolayers were deposited onto the ITO glass surface. The substrate speed used was 2 mm min⁻¹. LB films were built by Y-type deposition with a transfer ratio close to 1.

To prepare films of lutetium bisphthalocyanine and arachidic acid (LuPc₂/AA) (Fig. 1b), a mixture (1:1) of arachidic acid (AA) and lutetium bisphthalocyanine (LuPc₂) was dissolved in chloroform (1 × 10⁻⁵ M) and spread onto the subphase. The molecules were compressed at a speed of 10 mm min⁻¹ to obtain a floating ordered monolayer (Rodríguez-Méndez et al., 1992). At a surface pressure of 20 mN m⁻¹, 20 monolayers of Langmuir–Blodgett films were deposited onto ITO glass.

In order to prepare films containing tyrosinase, arachidic acid and lutetium bisphthalocyanine (Tyr/AA/LuPc₂) (Fig. 1c), a 19-monolayer film of LuPc₂/AA was prepared using the method described in the previous paragraph. Then, the barriers were opened and the water surface cleaned. Then a mixture of LuPc₂/AA was spread onto the water subphase and 10 µL of the solution of tyrosinase were injected drop by drop underneath the air/water interface and adsorbed to a floating monolayer. Then, 20 monolayers were transferred to the ITO glass by Y-type deposition with a transfer ratio close to 1.

The prepared LB films were immersed in a 2.5% (v/v) glutaraldehyde solution (in phosphate buffer 0.01 M of pH 7) for 20 min at room temperature. As established in previous papers, a Schiff base is formed between the C=O groups of the glutaraldehyde and the amines of the enzyme (Wang et al., 2009; Cajab et al., 2009; Migneault et al., 2004). As demonstrated by FTIR spectroscopy, the lutetium bisphthalocyanine does not react with the glutaraldehyde nor with the enzyme. The biosensor was washed using phosphate buffer and dried.

The electrochemistry was carried out in an EG&G PARC 263A potentiostat/galvanostat using a conventional three-electrode cell. The LB films were used as working electrodes. The reference electrode was Ag|AgCl/KCl 3 M and the counter electrode was a platinum plate. Cyclic voltammograms were registered from -0.5 to +0.5 V at a sweep rate of 0.05 V s⁻¹ (except when indicated otherwise).

3. Results and discussion

3.1. Sensing properties of Langmuir–Blodgett films (AA/LuPc₂, Tyr/AA, and Tyr/AA/LuPc₂) towards caffeic acid

In a first set of experiments, the sensing properties of AA/LuPc₂, Tyr/AA, and Tyr/AA/LuPc₂ nanostructured LB films were tested by exposing the electrodes towards a phosphate buffer (0.01 M) and a solution of caffeic acid (10⁻⁴ M in buffer phosphate). The comparison of the responses of the three electrodes can provide information about the enzymatic activity and of the electron mediator effect of the LuPc₂. The formation and characterisation of the Langmuir and Langmuir–Blodgett (LB) films will be discussed elsewhere. In summary, the arachidic acid molecules and the LuPc₂ molecules are organised with an edge on configuration, and the enzymes are adsorbed into the AA/LuPc₂ layers by electrostatic interactions.

As expected, the voltammograms obtained when using the Tyr/AA electrode immersed in a buffer phosphate did not show relevant peaks. The response of the LB films covered with LuPc₂/AA immersed in buffer phosphate (scan range from -0.5 V to +0.5 V) showed the expected redox pair at E_{1/2} = -0.35 V associated with the one electron reduction (Ln(III)Pc₂/Ln(II)Pc₂⁻) of the phthalocyanine ring (de Saja and Rodríguez-Méndez, 2005; Yilmaz et al., 2003)

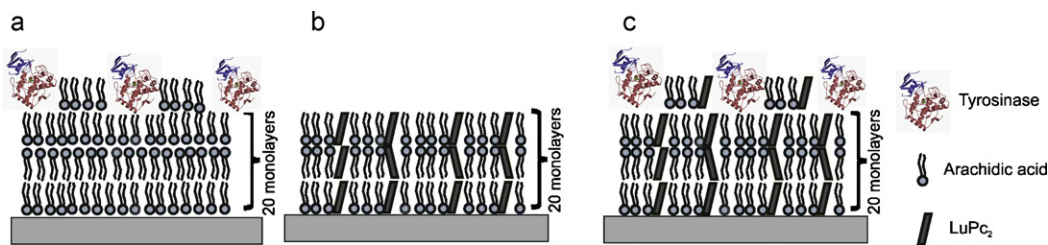


Fig. 1. Scheme of the sensors with structure (a) Tyr/AA; (b) LuPc₂/AA and (c) Tyr/AA/LuPc₂.

The response of both films towards caffeic acid (10^{-4} M) is illustrated in Fig. 2a and b respectively. Voltammograms obtained using an LB of Tyr/AA as the working electrode were characterised by a small reversible peak at $E_{1/2} = -0.21$ V. This peak is associated with the two electron enzymatic oxidation of caffeic acid to the corresponding *o*-quinone that is reduced back to caffeic acid at a low applied potential. This result is in good agreement with results published using tyrosinase-based sensors where the two electron cathodic peak corresponding to the reduction *o*-quinone appears at ca. -0.15 V vs. Ag/AgCl (Arecchia et al., 2010; Sanz et al., 2005). It is worthwhile noting that the voltammograms do not show the peak at ca. 0.5 V associated with the electrochemical (and not enzymatic) oxidation of the caffeic acid which is observed when unmodified glassy carbon electrodes are used (Kilmartin et al., 2001; Giacomelli et al., 2002).

The response of the LB film LuPc₂/AA showed the peak at -0.25 V associated with the reduction of the phthalocyanine ring (Fig. 2b). In addition, a weak peak associated with the electrochemical oxidation of the caffeic acid (Kilmartin et al., 2001; Giacomelli et al., 2002) appears at 0.38 V. Obviously, due to the absence of the enzyme, the reduction of the *o*-phenol acid associated with the enzymatic activity could not be observed.

The response towards caffeic acid of the LB films containing Tyr/AA/LuPc₂ was tested in the range from -0.5 V to $+0.5$ V at a scan rate of 0.050 V s⁻¹ (Fig. 2c). The cyclic voltammogram consisted of three definite redox processes: one anodic wave at -0.22 V associated with the reduction of the phthalocyanine ring (that confirms the presence of the electron mediator); a second peak (cathodic) at -0.02 V related to the reduction of the *o*-quinone to caffeic acid that occurs after the enzymatic process (that will be referred to as peak I), and a third peak (anodic) at 0.38 V associated with the electrochemical oxidation of the caffeic acid (this peak will be referred to as peak II).

In the previous literature studies, it has been established that the oxidation peak (peak II) appears at 0.45 V (at pH 3) and the formal potential increases by 0.066 V per pH unit (Kilmartin et al., 2001). Taking into account these previous data, it can be concluded that the presence of LuPc₂ facilitates the oxidation of caffeic acid, which occurs at lower potentials than in carbon electrodes (0.38 V vs. 0.45 V), thus demonstrating an electrocatalytic effect of the phthalocyanine.

In addition, the electron mediator increases drastically the intensity of the signals of both the enzymatic (peak I) and the electrochemical process (peak II) with respect to the signals obtained in absence of the electron mediator. The coupling of the oxidation of caffeic acid facilitated by the LuPc₂ and the reduction of the *o*-quinone forms a reaction cycle, which results in an amplification of the signal (Fig. 3).

Kinetic studies were carried out by registering the cyclic voltammograms of the nanostructured biosensor at different scan rates (from 0.010 to 0.210 V s⁻¹). In the enzymatic process (peak I), the peak currents were proportional to sweep rates (linear equation of the plot was $y = -0.066x - 0.6322$; $R^2 = 0.9973$), pointing to a charge transfer limited process (due to the electrochemical activity of the enzyme deposited in the surface of the electrode). From the slope of this line and using the Laviron equation:

$$I_p = \frac{n^2 F^2 v A \Gamma}{4RT}$$

where Γ is the surface coverage of the redox species (caffeic acid adsorbed to the electrode) (mol cm^{-2}), A is the electrode area (cm^2), v is the potential sweep rate, n is the number of electrons (two for the oxidation–reduction of caffeic acid) and n , I_p , F , R and T have their usual meanings (Bard and Faulkner, 2001), the total surface coverage calculated was 1.77×10^{-8} mol cm⁻².

The intensity of peak II (electrochemical oxidation) increased linearly with the square root of the scan rate (linear equation of the plot was $y = 0.8536x + 0.6372$; $R^2 = 0.9973$) indicating the dominance of the diffusion controlled processes according to the Randles–Sevcik equation.

$$I_p = 2.687 \times 10^5 n^{3/2} v^{1/2} D^{1/2} A C$$

where I_p is the peak current, A is the electrode surface area, D is the diffusion coefficient, and C is the bulk concentration. From the slope of the I_p vs. $v^{1/2}$ plot, the calculated diffusion coefficient D was 7.87×10^{-5} cm² s⁻¹. This value is in accordance with those found in the literature for carbon electrodes (Bard and Faulkner, 2001; Kallel Trabelsi et al., 2004).

The effect of the concentration of caffeic acid in the response of the sensor was studied by immersing the electrode in solutions with concentrations ranging from 10 to 490 μM . As observed in Fig. 4, the intensity of both the enzymatic and the electrochemical

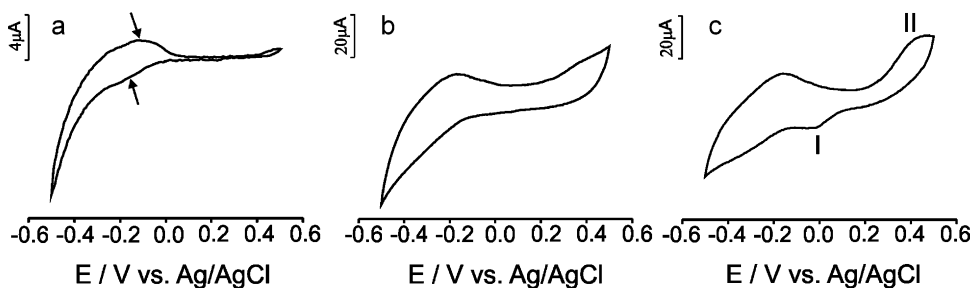


Fig. 2. Voltammetric response of an ITO covered with an LB film of (a) AA/Tyr; (b) AA: LuPc₂ and (c) Tyr/AA/LuPc₂ immersed in caffeic acid 10^{-4} M (phosphate buffer 0.01 M at pH of 7).

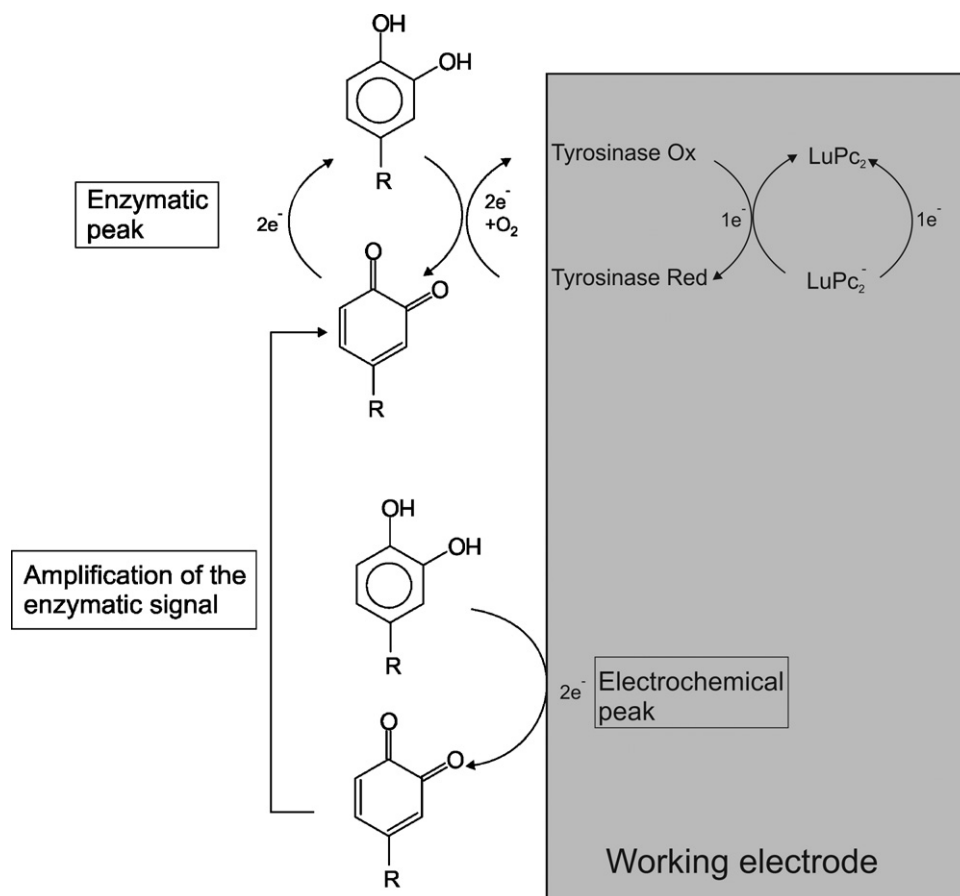


Fig. 3. Scheme of the reactions that take place at the working electrode.

peak increased with caffeic acid concentration. A linear response was observed in the 10–400 μM range (inset of Fig. 4), with sensitivities of 0.093 $\mu\text{A}/\mu\text{M}$ for the enzymatic process (peak I) and 0.162 $\mu\text{A}/\mu\text{M}$ for the electrochemical oxidation (peak II). The corresponding detection limits were calculated according to the $3s_b/m$ criterion, where m is the slope of the calibration graph, and s_b was estimated as the standard deviation ($n = 7$) of the voltammetric signals from different solutions of the substrate at the concentration level corresponding to the lowest concentration of the calibration plot. The detection limits calculated were 1.98 μM for the enzymatic process (peak I) and 2.09 μM for the electrochemical process (peak II).

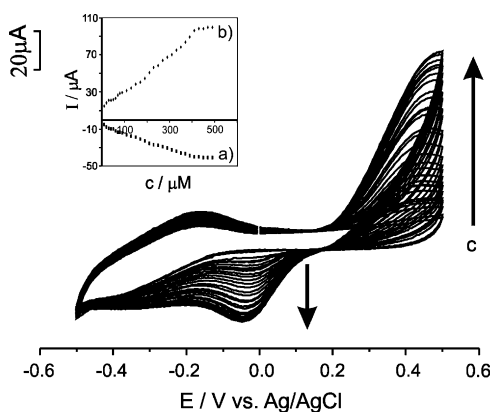


Fig. 4. Cyclic voltammograms of a Tyr/AA/LuPc₂ electrode immersed in caffeic acid solutions (10–490 μM). Inset: Plot of the intensity of (a) peak I (enzymatic process) and (b) peak II (electrochemical process) vs. the caffeic acid concentration.

The detection limits are in the range of those published for biosensors based on tyrosinase containing CoPc as electron mediator (Tanimoto de Albuquerque and Franco Ferreira, 2007; Ozsoz et al., 1996), being also in the range of polyphenol concentrations commonly found in foods (di Fusco et al., 2010).

From the above results, the Hill coefficient (h) can be calculated by representing the $\log[I_{\text{lim}}/(I_{\text{max}} - I_{\text{lim}})]$ vs. $\log[S_{\text{ox}}]$ (the logarithm of the oxidised substrate concentration). A Hill coefficient of 1.01 was calculated for the enzymatic process ($R^2 = 0.952$). The finding that the h parameter, calculated from the corresponding Hill's plot, was close to unity demonstrated that the kinetics of the enzymatic reaction fitted into a Michaelis–Menten type kinetics (Kurganov et al., 2001).

Using the Lineweaver–Burk equation and representing $1/I_{\text{lim}}$ vs. $1/[S_{\text{ox}}]$ it is possible to calculate the apparent Michaelis–Menten constant (from the slope) and the I_{max} (from the intercept). A K_M^{app} of 62.31 μM and an I_{max} of 37.87 μA were obtained. This value is smaller than that obtained for the free enzyme (2.30 mM as reported by Tanimoto de Albuquerque and Franco Ferreira, 2007). Such higher activity may be associated with the favourable environment for tyrosinase afforded by the lipidic layer, which may induce an enhanced catalytic rate because the enzyme structure is preserved and the active sites are adequately exposed, as it has been shown to occur for other enzymes (Schmidt et al., 2008). In addition, according to Cosnier and Innocent (1993), the caffeic acid generated by the electrochemical reduction of the enzymatically produced *o*-quinone (peak I) may enter into another enzymatic oxidation cycle providing a local increase in substrate concentration, and, consequently, an amplification of the electrode response.

We also stress that K_M^{app} is lower than the values usually reported for tyrosinase biosensors (in the range of mM) using

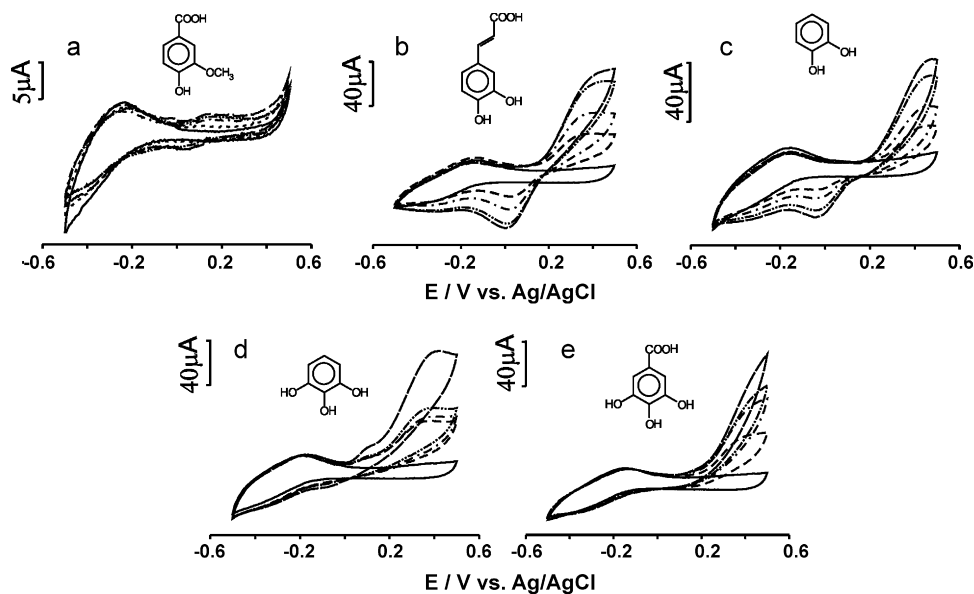


Fig. 5. Cyclic voltammograms of a Tyr/AA/LuPc₂ biosensor immersed in phosphate solutions of (a) vanillic acid; (b), caffeic acid; (c) catechol; (d) pyrogallol and (e) gallic acid at different concentrations (0 M, 1×10^{-4} M, 2×10^{-4} M, 3×10^{-4} M and 4×10^{-4} M).

other immobilisation methods. This proves the benefits of the biomimetic environment provided by the LB technique (Tanimoto de Albuquerque and Franco Ferreira, 2007; Schmidt et al., 2008; di Fusco et al., 2010).

Data on lifetime indicate that LB films can be cycled up to 50 times. The decrease in signal intensity expressed as percentage of decay was lower than 4% over 50 continuous scan cycles. The reproducibility of the biosensor was explored at a caffeic acid concentration of 2.0×10^{-4} M. The mean steady-state current is 25.22 μA with a relative standard deviation (S.D.) of 2.6% for seven determinations. Regarding the reproducibility of the fabrication method, six sensors, independently constructed, showed a relative S.D. of 2.9% (calculated from the steady-state current obtained measuring a 2.0×10^{-4} M a caffeic acid solution).

3.2. Sensing properties of the Tyr/AA/LuPc₂ biosensor towards different antioxidants: kinetics and selectivity

Similar studies were carried out with different phenolic compounds, also which included one monophenol (vanillic acid), two diphenols (caffeic acid and catechol) and two triphenols (pyrogallol and gallic acid). The cyclic voltammograms for the five antioxidants at different concentrations are shown in Fig. 5. The Tyr/AA/LuPc₂ sensor produces a response towards all the antioxidants tested. However, important differences in the responses from one antioxidant to another could be observed. Peak positions and the detection limits are listed in Table 1.

In case of the response of the vanillic acid (a monophenol), the reduction of the quinone can be clearly observed at +0.035 V that increased its intensity with the concentration of the vanillic acid. It is worth noting that the anodic peak associated with the electrochemical oxidation cannot be observed in the studied range. According to the literature, the electrochemical oxidation of vanillic acid occurs at potentials higher than for other phenols (typically 0.7–0.8 V at pH 3) and this can explain the absence of this peak in the studied range (Lind and Siegbahn, 1999; Tanimoto de Albuquerque and Franco Ferreira, 2007).

Cyclic voltammograms registered in buffered solution of diphenols (caffeic acid and catechol) showed one intense cathodic wave at ca. 0.0 V (associated with the enzymatic process) and a very intense anodic wave associated with the electrochemical oxidation

of the substrate at 0.38 V (for catechol) and 0.44 V (for caffeic acid). These low potentials are due to the presence of easily oxidisable *ortho*-diphenol groups.

The response of biosensor in the presence of triphenols shows one intense peak at ca. 0.35 V related to the electrochemical oxidation of the compounds. The enzymatic reduction of the quinone appears as a very small peak at -0.05 V. This result could be related to the high reactivity of the produced *o*-quinones that may condense with other triphenols through a Michael type addition, yielding purpurogallin (in the case of pyrogallol) and purpurogallin-β-carboxylic acid (in the case of gallic acid) (Brown, 1967). The absence of enzymatic activity can also be related to the specificity of the enzyme and the size of the active site that prevents the fitting of a large molecule in the active site.

Note also that the electrochemical peak (peak II) appears at lower potentials than the values obtained in classical carbon electrodes as a consequence of the electrocatalytic activity of the lutetium bisphthalocyanine (Kilmartin et al., 2001). The catalysis was also more effective than using cobalt phthalocyanine, where values of E_p around of 0.65 V were found for the oxidation of catechol indicating that CoPc did not show any electrocatalytic activity (Tanimoto de Albuquerque and Franco Ferreira, 2007).

In summary, the biosensor formed by Tyr/AA/LuPc₂, produced two electrochemical signals with different positions and intensities. The biomimetic environment, combined with the electron mediator action and the catalytic activity of the LuPc₂, increased the intensity of the signals and shifted the electrochemical peak up to detectable values. This bi-modal sensor increases the selectivity towards phenolic compounds giving rise to a variety of responses. In all cases, the enzymatic peak (peak I) showed a linear dependence with the scan rate (fitting the Laviron equation) indicating that the kinetics of the process was dominated by the electron transfer, whereas the electrochemical process (peak II) fitted the Randles–Sevcik equation as a result of a kinetics controlled by diffusion. Table 1 lists the surface coverages (Γ) and diffusion coefficients (D) for all phenolic compounds studied. As observed in the table, the diffusion coefficients increase in the order catechol < pyrogallol < vanillic acid < gallic acid < caffeic acid and is the consequence of the increasing molar mass and size of the analytes analysed.

Table 1
Peak positions of the redox processes observed when electrodes are immersed in different phenolic compounds. Detection limits calculated from the linear part of the curves. Surface coverages (Γ) and diffusion coefficients for the phenolic compounds studied.

Antioxidant	Type	Cathodic (enz. peak)		Anodic (enz. peak)		Anodic (electroc. peak)		Cathodic (enz. peak)		Number of electrons ^a	Γ (mol cm ⁻²)	D (cm ² s ⁻¹)
		E (V)	LD (μ M)	E (V)	LD (μ M)	E (V)	LD (μ M)	I_{\max} (μ A)	K_M (μ M)			
Vanillic acid	Monophenol	0.035	4.37	0.15	127.9	–	–	17.66	144.87	2	3.51×10^{-8}	3.37×10^{-5}
Caffeic acid	Diphenol	-0.025	1.98	–	–	0.44	2.09	37.87	62.31	2	7.48×10^{-8}	2.36×10^{-6}
Catechol	Diphenol	0.025	2.11	–	–	0.38	1.71	23.58	63.72	2	1.77×10^{-8}	7.87×10^{-5}
Galic acid	Triphenol	-0.050	16.8	–	–	0.35	21.46	13.25	124.54	2	5.24×10^{-8}	2.56×10^{-5}
Pyrogallol	Triphenol	-0.070	27.49	0.10	111.12	0.35	1.67	15.72	109.93	2	3.31×10^{-8}	5.67×10^{-5}

^a Number of electrons taken from Kilmarin et al.

The kinetic parameters and the analytical characteristics listed in Table 1 demonstrated that for the five phenolic compounds analysed, the kinetics of the enzymatic reaction (peak 1) fitted into a Michaelis–Menten type kinetics, as demonstrated by the h parameter close to 1 obtained from the Hill's plot.

Studies of the dependence of substrate reduction showed that K_M^{app} varied with the nature of the substrate. In general, lower K_M^{app} values were obtained for the phenolic compounds exhibiting a higher sensitivity. The sensitivity of the nanostructured biosensor decreased in the order diphenols > triphenols > monophenols. The greater value of I_{\max} and the lowest K_M^{app} were found for caffeic acid. These results are in accordance with results reported in the literature for other tyrosinase biosensor designs (Sanz et al., 2005; Bonakdar et al., 1989), thus revealing a good affinity of the enzyme immobilised on nanostructured hybrid film for these substrates.

The detection limits were in the range of 1.98×10^{-6} – 27.49×10^{-6} M for the enzymatic process. The lowest detection limits were found for caffeic acid and the highest for pyrogallol. In the case of electrochemical oxidation, the detection limits were in the range of 1.67×10^{-6} – 21.46×10^{-6} M. The lowest detection limits were found for pyrogallol and the highest for gallic acid. The coexistence of two redox processes increases information provided by the sensor, improving their selectivity.

4. Conclusions

A biomimetic sensor based on Langmuir–Blodgett films of tyrosinase incorporated in a lipidic layer and using lutetium bisphthalocyanine as electron mediator was electrochemically characterised as transducer for a voltammetric biosensor. The Tyr/AA/LuPc₂ biosensor showed a cathodic peak related to the reduction of the *o*-quinone to the phenolic compound that occurs after the enzymatic process, and one anodic peak associated with the electrochemical oxidation of the corresponding phenol. For the enzymatic process, the detection limits ranged from 1.98×10^{-6} M to 27.49×10^{-6} M, while for electrochemical process the limits varied from 1.67×10^{-6} M to 21.46×10^{-6} M. The lutetium bisphthalocyanine was proven to play an important role as electron mediator increasing the intensity of the signals, in addition to showing an electrocatalytic effect by shifting the peak position of the electrochemical peak to lower values. The coexistence of two electrochemical processes increased the selectivity towards phenolic compounds giving rise to distinct responses towards the five compounds analysed with competitive detection limits. When combined with the biomimetic environment, the electron mediator action and the catalytic activity of lutetium bisphthalocyanine increased the intensity of the signals and shifted the electrochemical peak up to detectable values. The observations presented here show that biomimetic sensors for phenol based on Tyr/AA/ LuPc₂ can be easily used in thin-layer cells and these sensors can conveniently serve for the determination of phenols.

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