NANOSTRUCTURED VS. CARBONACEOUS BIOSENSORS
Comparative Studies for Detection of Phenolic Compounds

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Abstract: The biosensing properties of tyrosinase biosensors were investigated for two different immobilization matrixes: carbon paste and Langmuir-Blodgett thin film. In both cases the electron mediator was the lutetium (III) bisphthalocyaninate. The electrochemical responses of biosensors towards phenol and catechol were analyzed and compared. The tyrosinase maintains its bioactivity well within the immobilization matrices. A clearly defined reduction current proportional to the phenolic compounds concentration was observed in cyclic voltammetry, which attributed to the reduction of enzymatically produced quinone at the electrode surface. It was demonstrated that the biosensor based on Langmuir-Blodgett thin film shows the best performances in terms of kinetics and detection limit for the phenolic compounds analyzed.

1 INTRODUCTION

A considerable number of phenolic compounds, extensively distributed throughout the environment, are important pollutants in medical, food and environmental matrixes. They are used in numerous industrial processes such as fabrication of paper, polymers, drugs, dyes, and pesticides (Hill, 2004). They are ones of the most important contaminants in soil and surface water (Manahan, 1991). Almost all of them are easily absorbed and have been shown to have negative effects on animal health (Bukowska and Kowalska, 2004). Taking into consideration their high toxicity and persistence in the environment, the determination of phenolic compounds becomes an important theme. For quantification of phenolics, several methods were developed such as colorimetry, gas chromatography, liquid chromatography, and capillary electrophoresis, fluorescence, and electrochemical methods (Moldoveanu and Kiser, 2007; Ma et al, 2005; Kovács et al, 2011). However, these analysis methods are relatively time-consuming, difficult to perform requiring complex samples pre-treatment, and may not be suitable for in situ monitoring. These inconveniences diminish its practical applications. Electrochemical sensors and biosensors can be a possible alternative to these techniques. Electrochemical analytic technique based on biosensors is an attractive method due to simplicity, low expense, high sensitivity and possibility of miniaturization. Enzymes are complex proteins that produce a specific chemical reaction in other substances without themselves being modified carrying out as biocatalysts by lowering the activation energy (Palmer, 1991). For the detection of phenolic compounds, biosensors based on tyrosinase have been developed (Carralero et al, 2006; Cosnier et al, 2001; Tsai and Chiu, 2007). Tyrosinase catalyzes the transformation of monophenols to diphenols and also the reaction of o-diphenols to o-quinones (Kazandjian and Klibanov, 1985). Several methods have been used for the immobilization of tyrosinase onto various substrates including carbon paste immobilization (Kumar Vashist et al, 2011; Granero et al, 2010), sol–gel immobilization (Zejli et al, 2008), physical adsorption (Shiddiky and Torriero, 2011), Langmuir–Blodgett thin films (Cabaj, 2010; Apetrei, 2011; Pavinatto, 2011), electrochemical entrapment of enzyme within conducting polymer or composite matrix (Ameer and Adeloju, 2009). Langmuir–

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Blodgett (LB) technique offers a possibility of developing an ultra-thin film with well-organized structure on molecular scale (Roberts, 1990). On the other hand, this technique is considered as a suitable immobilization method for biosensor because this can produce well-ordered thin films and can control the quantity of biocomponents by the number of deposited layers.

The immobilization of the enzyme tyrosinase into carbon paste electrodes has resulted in a number of biosensor configurations that have been shown to be relatively sensitive, specific, and durable in the detection and measurement of phenols (Granero et al, 2010). Various aspects concerning their construction and operation have been studied and optimized including the use of different binders (Rogers et al, 2001) and the use of redox mediators (Yin, 2010).

In this paper, carbon paste biosensors and LB biosensors based on tyrosinase and lutetium (III) bisphthalocyaninate (as electron mediator) have been prepared and their capability to detect phenolic compounds has been compared. For this purpose, phenol and catechol have been analyzed in aqueous solutions. The response dependences and amperometric characteristics including sensitivity, kinetics, linear range and limits of detection of the prepared enzyme electrode in the detection of phenolic compounds have been investigated.

2 EXPERIMENTAL

2.1 Chemicals and Solutions

Carbon paste was made with graphite powder (High purity Ultracarbon®, Ultra F purity) mixed with high purity mineral oil (Nujol, Fluka). The sources of materials and reagents used were as follows: arachidic acid, phenol, catechol from Sigma; tyrosinase (EC 1.14.18.1, from mushroom) was purchased from Sigma. A $67\mu g \cdot \mu L^{-1}$ solution of tyrosinase in buffer phosphate 0.01 M (pH=7) was used for the enzyme immobilization.

The buffer was prepared from potassium monobasic and dibasic phosphate salts (pH 7) from Aldrich. All the aqueous solutions were prepared using 18 MΩ·cm MilliQ water (Millipore).

The lutetium (III) bisphthalocyaninate ($LuPc_2$ was synthesized and purified in their neutral radical state following previously published procedures.

2.2 Biosensor Construction

2.2.1 Carbon Paste based Biosensor

Carbon paste electrodes were prepared as previously reported by mixing graphite powder and the bisphthalocyanine (15%, w/w). Nujol was used as the binder of the composite mixture. Carbon pastes were packed into the body of a 1mL plastic syringe and compressed. A metallic copper wire was used as a contact.

The enzyme, tyrosinase (Tyr), was immobilized on the above carbon paste electrodes by a casting technique followed by cross-linking. 5µL of 0.01 M phosphate buffer (pH 7.0) containing $67\mu g \cdot \mu L^{-1}$ of enzyme, was added onto carbon paste electrode surface. After drying, the biosensor was exposed to a 2.5% (v/v) glutaraldehyde solution (in phosphate buffer 0.01M of pH 7) for 20 minutes at room temperature. The enzyme-immobilized electrode was dried at 10ºC and rinsed with phosphate buffer solution thrice to remove any unbound enzyme from the biosensor surface and was further dried at 10ºC and stored at 4ºC.

2.2.2 Langmuir-Blodgett based Biosensor

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, $LuPc_2$ and arachidic acid (Tyr/$LuPc_2$-AA) were prepared by spreading a chloroform solution (10^{-5} M) of arachidic acid and $LuPc_2$ onto a water subphase (NaCl 0.1M, phosphate buffer 0.01M of pH 7 in ultrapure water – Millipore MilliQ; 20ºC). After the evaporation of the solvent, 10µL of a $67\mu g \cdot \mu L^{-1}$ solution of tyrosinase in 0.01M phosphate buffer (pH 7) was injected drop by drop underneath the air/water interface.

Molecules were compressed using a symmetrical two barrier compression system. At a surface pressure of $30mN \cdot m^{-1}$, 20 monolayers were deposited onto the ITO (indium tin oxide) surface. The substrate speed used was $2mm \cdot min^{-1}$. LB films were prepared by Y-type deposition with a transfer ratio close to 1. The biosensor was washed using phosphate buffer, dried at 10ºC and stored at 4ºC.

2.3 Apparatus

Electrochemical experiments and analytical testing were carried out in a 100 mL electrochemical cell using a platinum electrode as the counter electrode and a biosensor as the working electrode. The

potentials were measured and referred to a Ag/AgCl/KCl 3M electrode. Electrochemical measurements were carried out with an EG&G PARC Model 263 potentiostat/galvanostat (Princeton Applied Research Corp.).

The electrochemical experiments were carried out in 0.01 M phosphate buffer solution (PBS) of pH=7 as supporting electrolyte.

3 RESULTS AND DISCUSSIONS

The suitable immobilization of the enzyme in solid substrates is crucial for the development of the biosensors. The structure of the matrix used for immobilize Tyr should contribute to the preservation of enzyme functionality.

3.1 Cyclic Voltammetry Studies

The response towards phenolic compounds of the biosensors was registered in the range from -0.5 V to +0.5V at a scan rate of 0.050 $V \cdot s^{-1}$ (Figure 1).

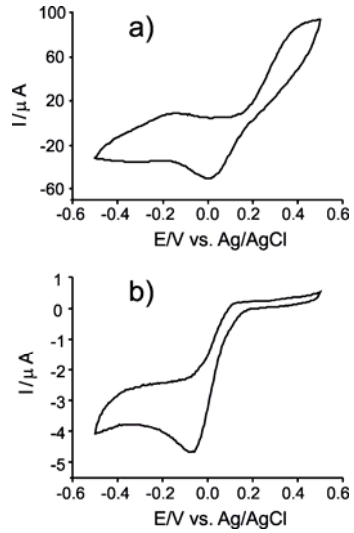

Figure 1: Cyclic voltammograms of biosensors immersed in $4 \cdot 10^{-4}$ M catechol (in PBS, pH=7); a) Tyr/$LuPc_2$-AA, b) Tyr/$LuPc_2$-CP.

The cyclic voltammogram of the Tyr/$LuPc_2$-AA biosensor in $4 \cdot 10^{-4}$ M catechol (pH 7.0 phosphate buffer solution) showed a redox pair at $E_{1/2}$=-0.24V associated with the one electron reduction of the phthalocyanine ring (de Saja and Rodriguez-Mendez, 2005). The peaks related with catechol appear at +0.01V (cathodic peak associated with the reduction of the enzymatically formed o-quinone to catechol) and at +0.40V (anodic peak associated to the electrochemical oxidation of the catechol), respectively.

The cyclic voltammogram of the Tyr/$LuPc_2$-CP biosensor in the same solution do not show the peaks related with phthalocyanine. As is show in the Figure 1b, only the peak corresponding to enzymatic reduction of the o-quinone to catechol appearing at -0.07V is observed.

The results are similar in the case of phenol analysis. The peak pair corresponding to $LuPc_2$ is clear only in the case of Tyr/$LuPc_2$-AA biosensor. Additionally, only the reduction peak of the enzymatically formed o-quinone at biosensor surface is observed. In the case of LB biosensor the peak appear at +0.01V and in the case of CP biosensor at -0.07V.

The presence of reduction peak indicates that the immobilization process retains the biological activity of tyrosinase in both solid substrates.

3.2 Kinetic of the Biosensors

Kinetic studies were performed by registering the cyclic voltammograms of the biosensors at different scan rates, from 0.02 to 0.20$V \cdot s^{-1}$ (Figure 2).

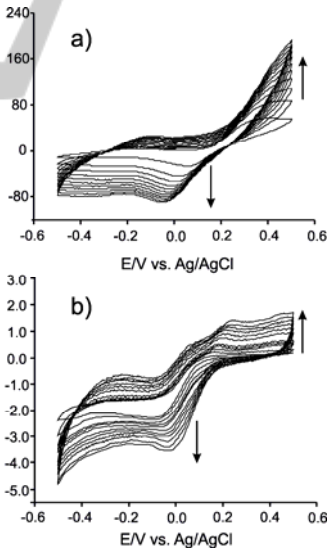

Figure 2: Cyclic voltammograms of biosensors a) Tyr/$LuPc_2$-AA, b) Tyr/$LuPc_2$-CP registered at different scan rates. Electrolyte solution was $4 \cdot 10^{-4}$ M catechol (in PBS 0.01M, pH=7).

In both cases, the cathodic peak currents were proportional to sweep rates pointing to a charge transfer limited process due to the catalytic activity of the enzyme deposited in the surface of the electrode. The principal parameters of linear regression equation of the plots I vs. V were

presented in the Table 1.

Table 1: Quantitative data obtained from kinetic studies for cathodic peak.

	Tyr/LuPc$_2$-AA		
	m	R^2	Γ / mol·cm^{-2}
Phenol	-0.1124	0.988	$2.20 \cdot 10^{-9}$
Catechol	-0.2812	0.986	$1.77 \cdot 10^{-8}$
	Tyr/LuPc$_2$-CP		
	m	R^2	Γ / mol·cm^{-2}
Phenol	-0.0036	0.981	$7.04 \cdot 10^{-10}$
Catechol	-0.0068	0.976	$4.28 \cdot 10^{-9}$

m- is the slope of the plots I_c vs. v

The trends observed when immersing the biosensors in phenolic compounds solutions were similar. In both cases, the same o-quinone is enzymatically formed, which is electrochemically reduced at biosensor surface. Tyr/LuPc$_2$-AA biosensor showed a fast electron transfer between the phenolic compounds and LB thin film. When the carbon matrix was used as support material, the electron transfer was difficult and the signals showed a smaller intensity. The differences are in the range of two orders of magnitude.

From the slope of this line and using the Laviron equation:

$$I_c = n^2 F^2 \nu A \Gamma / 4 R T \quad (1)$$

where Γ is the surface coverage of the redox species (o-quinone) (mol·cm^{-2}), A is the electrode area (cm^2), ν is the potential sweep rate and n, I_c, F, R and T have their usual meanings (Bard and Faulkner, 2001), the total surface coverage could be calculated.

The values obtained were presented in Table 1. The highest surface coverage values were obtained in the case of Tyr/LuPc2-AA biosensor. This result suggests that in the LB thin film exist a greater number of active sites comparing with carbonaceous matrix. Therefore, the enzyme preserve better the biocatalytic activity when is immobilized in a biomimetic environment.

The intensity of peaks related to the electrochemical oxidation of phenolic compounds increases linearly with the square root of the sweep rate (Table 2) indicating a diffusion controlled processes according to the Randles-Sevcik equation.

$$I_a = 2.687 \cdot 105\ n^{3/2} \nu^{1/2} D^{1/2} A C \quad (2)$$

where I_a is the peak current, A is the electrode surface area, D is the diffusion coefficient, and C is the concentration. From the I_a, in function of $\nu^{1/2}$ plot, the diffusion coefficient D could be calculated.

Table 2: Quantitative data obtained from kinetic studies for anodic peak.

	Tyr/LuPc$_2$-AA		
	m	R^2	D / cm^2·s^{-1}
Phenol	0.2235	0.967	$6.32 \cdot 10^{-6}$
Catechol	0.6276	0.978	$7.87 \cdot 10^{-5}$
	Tyr/LuPc$_2$-CP		
	m	R^2	D / cm^2·s^{-1}
Phenol	0.0024	0.959	$5.40 \cdot 10^{-7}$
Catechol	0.0063	0.961	$5.34 \cdot 10^{-6}$

m- is the slope of the plots I_a vs. $v^{1/2}$

From the above results, could be concluded that the Tyr/LuPc$_2$-AA presents the fastest diffusion coefficients pointing that the electrochemical processes a more rapid in the case of nanostructured thin film.

3.3 Amperometric Response of the Biosensors

Figure 3 illustrates the amperometric response for the Tyr/LuPc$_2$-CP biosensor at -0.07V V (a) and for the Tyr/LuPc$_2$-AA biosensor at +0.01V V (b) after the addition of successive aliquots of phenol to the 0.01 M PBS (pH 7.0) under constant stirring. Definite reduction currents proportional to the concentration of phenol were observed, which results from the electrochemical reduction of o-quinone species enzymatically formed.

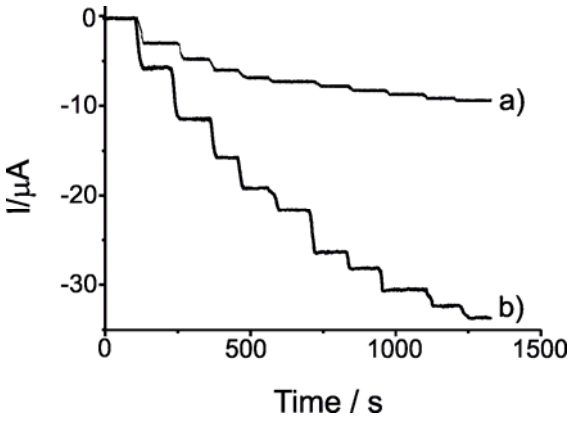

Figure 3: Amperometric response of a) Tyr/LuPc$_2$-CP and b) Tyr/LuPc$_2$-AA biosensors to phenol in 0.01 M PBS solution (pH=7).

The Tyr/LuPc$_2$-AA biosensor achieves 95% of steady-state current in less than 4 s. The response rate is much faster than that of 7 s obtained in the case of Tyr/LuPc$_2$-CP biosensor. The faster response could be attributed to a more rapid electron transfer between the enzymatically-produced quinone and

the biomimetic LB thin film comparing with carbon paste biosensor.

3.4 Effect of Phenolic Compounds Concentration

Figure 4 showed the relationship between the response current of the biosensors and the phenol concentration in PBS (pH 7.0) at +0.01V for Tyr/LuPc$_2$-AA biosensor and -0.07V for Tyr/LuPc$_2$-CP biosensor (calibration curves).

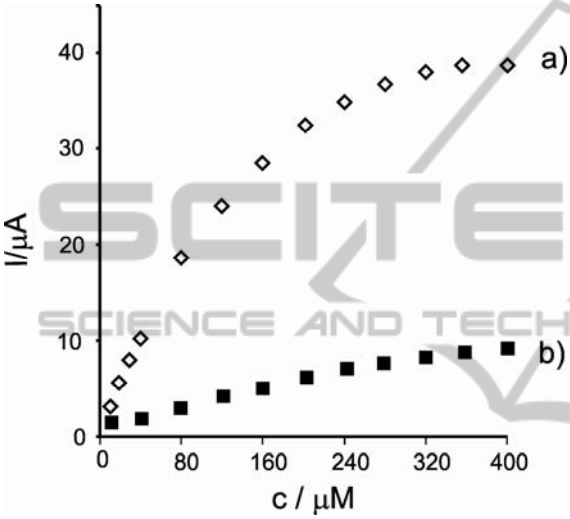

Figure 4: The calibration curve between the reduction current and the concentration of catechol in PBS (pH 7.0) of a) Tyr/LuPc$_2$-AA and b) Tyr/LuPc$_2$-CP biosensors to phenol in 0.01 M PBS solution (pH=7).

The response current of Tyr/LuPc$_2$-AA biosensor is linear with phenol concentration in the range from 10 to 120μM, indicating that the enzyme catalytic reaction of Tyr is the first-order reaction. Then, with further increasing catechol concentration, the current increases slowly, and the enzyme reaction shows a transition from first to zero-order. The sensitivity of the biosensors is 0.053μA·μM^{-1}. 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 amperometric 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 5.4 μM. The values obtained are lower than that obtained in the case of Tyr/LuPc$_2$-CP biosensor (the sensitivity is 0.0075μA·μM^{-1} and the detection limit is 8.57 μM). Therefore the Tyr/LuPc$_2$-AA biosensor has better quality performances comparing with Tyr/LuPc$_2$-CP biosensor.

From the calibration data, the Hill coefficient (h) can be calculated by representing the log[I/(I_{max}-I)] vs. log [S] (the logarithm of substrate concentration). A Hill coefficient of 1.09 was calculated for the reduction process of o-quinone formed from the enzymatic reaction on the electrode surface (R^2=0.952) for Tyr/LuPc$_2$-AA biosensor. In the case of Tyr/LuPc$_2$-CP biosensor a Hill coefficient of 0.94 was obtained. The value obtained for 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. The value slightly higher than 1 obtained for Tyr/LuPc$_2$-AA biosensor (h=1.09) demonstrates a positive cooperative effect between the occupied active sites. A negative cooperative effect between the occupied active sites takes place in the case of Tyr/LuPc$_2$-CP biosensor (h=0.94).

The apparent Michaelis–Menten constant (K_M) is calculated for the immobilized Tyr by using the linearization of Lineweaver-Burk expressed by eq. (3) (Shu and Wilson, 1976).

$$1/I = 1/I_{max} + K_M / (I_{max} \cdot [S]) \quad (3)$$

where I is the cathodic current, I_{max} is the steady-state current, K_M is the apparent Michaelis-Menten constant and [S] is the concentration of substrate.

The maximum current response and apparent Michaelis–Menten constant were calculated from the intercept and slope. The values obtained for both biosensors immersed in phenolic compounds solutions were presented in Table 3.

Table 3: Response characteristics of the biosensors to phenolic compounds.

	Tyr/LuPc$_2$-AA		
	LD / μM	I_{max} / μA	K_M / μM
Phenol	5.40	39.11	81.52
Catechol	1.80	45.65	24.56
	Tyr/LuPc$_2$-CP		
	LD / μM	I_{max} / μA	K_M / μM
Phenol	8.57	9.31	241.93
Catechol	8.19	11.61	92.42

In agreement with the inherent characteristic of Michaelis–Menten constant, the small the value of K_M, the stronger will be the affinity between Tyr and substrate. A highest I_{max} indicate a higher sensitivity of the biosensor (Kiralp and Toppare, 2006).

The values obtained indicate that the Tyr/$LuPc_2$-AA biosensor have highest quality performances.

4 CONCLUSIONS

It is demonstrated that the biomimetic LB thin film biosensor have the advantages of maintaining enzyme bioactivity, making the enzyme catalytic sites close and easily accessible to the substrate molecules comparing with tyrosinase-based carbon paste biosensor.

The kinetic studies demonstrate that Tyr/$LuPc_2$-AA biosensor have a fast electron transfer between the phenolic compounds and LB thin film. In the case of Tyr/$LuPc_2$-CP biosensor, the electron transfer was difficult and the signals showed a smaller intensity.

These advantages lead to significant improvement of the affinity, response sensitivity and detection limit of Tyr/$LuPc_2$-AA to phenol and catechol in pH 7.0 phosphate buffer.

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