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Bioelectronic tongue based on lipidic nanostructured layers containing phenol oxidases and lutetium bisphthalocyanine for the analysis of grapes

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ABSTRACT

In this work, a multisensor system formed by nanostructured voltammetric biosensors based on phenol oxidases (tyrosinase and laccase) has been developed. The enzymes have been incorporated into a biomimetic environment provided by a Langmuir–Blodgett (LB) film of arachidic acid (AA). Lutetium bisphthalocyanine ($LuPc_2$) has also been introduced in the films to act as electron mediator. The incorporation of the enzymes to the floating layers to form Tyr/AA/$LuPc_2$ and Lac/AA/$LuPc_2$ films has been confirmed by the expansion in the surface pressure isotherms and by the AFM images. The voltammetric response towards six phenolic compounds demonstrates the enhanced performance of the biosensors that resulted from a preserved activity of the tyrosinase and laccase combined with the electron transfer activity of $LuPc_2$. Biosensors show improved detection limits in the range of 10^{-7}–10^{-8} mol L^{-1}. An array formed by three sensors AA/$LuPc_2$, Tyr/AA/$LuPc_2$ and Lac/AA/$LuPc_2$ has been employed to discriminate phenolic antioxidants of interest in the food industry. The Principal Component Analysis scores plot has demonstrated that the multisensor system is able to discriminate phenols according to the number of phenolic groups attached to the structure. The system has also been able to discriminate grapes of different varieties according to their phenolic content. This good performance is due to the combination of four factors: the high functionality of the enzyme obtained using a biomimetic immobilization, the signal enhancement caused by the $LuPc_2$ mediator, the improvement in the selectivity induced by the enzymes and the complementary activity of the enzymatic sensors demonstrated in the loading plots.

1. Introduction

The determination of phenols, the main antioxidants in foods, has been widely investigated using traditional techniques including spectroscopy, chromatography and electrochemical methods (Mello et al., 2010; Bartosz, 2013).

A promising approach in food analysis consists in the use of electronic tongues which are multisensor systems based on a number of low-selective sensors and use advanced mathematical procedures for processing the electrochemical signals, based on pattern recognition and/or multivariate data analysis (Vlasov et al., 2005; Tahara and Toko, 2013). Electronic tongues provide global information about the sample instead of information about specific compounds.

Electrochemical sensors are the most widely used sensing units in electronic tongues. They include potentiometric (Ciosek and Wroblewski, 2011), amperometric (Scampicchio et al., 2008), voltammetric (Winquist et al., 2011; Escobar et al., 2013; Rodriguez-Mendez et al., 2008) or impedimetric sensors (Cabral et al., 2009).

Arrays of voltammetric electrodes chemically modified with electroactive materials (i.e. phthalocyanines) have demonstrated to be particularly interesting for the analysis of phenolic compounds (Gay et al., 2012; Ceto et al., 2012a; Parra et al., 2006). When using such electrodes, voltammograms show redox peaks produced by the electrode material and by the solution. In addition, the interactions between the electrode and the solution (i.e. electrocatalytic activity of the sensing material) produce shifts in the peak positions and changes in their intensity. In this way, each electrode produces a distinct response towards different solutions. The intrinsic complexity, richness and cross-selectivity

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of the signals generated by an array of voltammetric electrodes are an advantage because each curve contains large amount of information about the sample (Rodriguez-Mendez et al., 2008; Winquist et al., 2011).

Phthalocyanines (MPc) and their sandwich type lanthanide derivatives ($LnPc_2$) are among the most suitable materials for electrochemical sensors due to their well-known electrocatalytic properties (Zagal et al., 2010; Bouvet et al., 2013; Rodriguez-Mendez et al., 2008). They have demonstrated to behave as excellent modifiers for the detection of a variety of analytes including polyphenolic compounds (Gay et al., 2012; Ceto et al., 2012a; Matemadombo et al., 2012). Nanostructured electrochemical sensors based on phthalocyanines can be prepared using the Langmuir–Blodgett (LB) technique (Arrieta et al., 2003; Volpati et al., 2008).

On the other hand, electrochemical biosensors are an interesting alternative for the analysis of phenols due their high sensitivity and selectivity. They contain phenol oxidase enzymes such as tyrosinase or laccase combined with appropriate electron mediators such as metallic nanoparticles, graphene and conducting polymers among others (Karim and Fakhruddin, 2012). It has been demonstrated that MPcs and $LnPc_2$ can also be used as electron mediators in tyrosinase biosensors (Yin et al., 2010; Fernandes et al., 2011; Apetrei et al., 2011). The LB technique is of special interest in the field of biosensors because using this method enzymes can be immobilized in a nanostructured lipidic layer with a structure similar to that of the biological membranes. This biomimetic environment can help to preserve the functionality of the enzyme (Soloducho et al., 2009; Fernandes et al., 2011; Apetrei et al., 2011). In addition, using the LB technique, the enzyme and the electron mediator can be co-inmobilized in a single sensitive layer, facilitating the electron transfer between the enzyme and the electrode.

Some attempts have been carried out to develop arrays of biosensors containing phenol oxidases for the detection of phenols (the so-called bioelectronics tongues) (Ceto et al., 2012b; Ghasemi-Varnamkhasti et al., 2012). It has been demonstrated that arrays of biosensors combine the advantages of classical arrays of electrochemical sensors that provide global information about the sample, with the specificity of the enzyme-substrate reaction typical of biosensors. However, in these previous works only non-nanostructured sensors have been used.

The purpose of this work is to develop a bioelectronic tongue based on an array of nanostructured biosensors combining the sensing properties of two different phenol oxidases-tyrosinase and laccase-, and to evaluate its capability of discrimination towards phenols of interest in the food industry. The enzymes have been incorporated into LB films of arachidic acid using lutetium bisphthalocyanine as the electron mediator. Cyclic voltammetry has been applied to detect six phenolic compounds including one monophenol, three orto-diphenols and two triphenols. The complementarity achieved by the different sensors and the electron mediator capability of the bisphthalocyanine will be discussed.

2. Materials and methods

2.1. Chemicals

All chemical and solvents were of reagent grade. Deionized water (resistivity of 18.2 $M\Omega\,cm^{-1}$) was used to prepare subphases and solutions.

Laccase, from Trametes versicolor (EC number: 1.10.3.2, activity of 20.7 $U\,mg^{-1}$) and Tyrosinase (from mushroom EC 232-653-4), noted activity of 3610 $U\,mg^{-1}$ were purchased from Sigma Chemical. 70 $\mu g\,mL^{-1}$ solutions of tyrosinase and laccase were prepared in buffer phosphate 0.01 mol L^{-1} (pH=7.0) (PBS).

The lutetium (III) bisphthalocyaninate ($LuPc_2$) was synthesized following a previously published procedure (Linaje et al., 2000).

2.2. Langmuir and Langmuir–Blodgett films

Isotherms and LB films were prepared in a KSV 5000 Langmuir–Blodgett trough (KSV Instruments, Finland) equipped with a Wilhelmy plate to measure the surface pressure.

According to a previously published method, LB films were prepared using a PBS–NaCl subphase (NaCl 0.1 M, phosphate buffer 0.01 M of pH=7.0 in ultrapure water) (Apetrei et al., 2011).

Mixed films containing arachidic acid (AA) and lutetium bisphthalocyanine ($LuPc_2$) were prepared by spreading 250 μl of a mixture 10:1 ($AA/LuPc_2$) dissolved in chloroform (1×10^{-5} mol L^{-1}) onto the PBS–NaCl subphase. The surface-area isotherms were measured by compressing the floating molecules at a speed of 10 mm min^{-1}.

At a surface pressure of 40 mN m^{-1}, 20 monolayers were deposited onto previously cleaned ITO glass surface, by Y-type deposition with a transfer ratio close to 1.

LB films containing enzyme, arachidic acid and lutetium bisphthalocyanine ($Enz/AA/LuPc_2$), were prepared in two steps. First, 10 monolayers of $AA/LuPc_2$ were deposited using the method described in the previous paragraphs. Then, 10 monolayers of $Enz/AA/LuPc_2$ were deposited onto the $AA/LuPc_2$ layers as follows: 250 μl of the $AA/LuPc_2$ solution were spread onto the PBS–NaCl subphase. When the solvent was evaporated, 100 μl of a 70 $\mu g\,ml^{-1}$ solution of the corresponding enzyme in 0.01 mol L^{-1} PBS were injected drop by drop underneath the air/liquid interface. Barriers were compressed at a speed of 10 mm min^{-1}. At a surface pressure of 40 mN m^{-1}, 10 monolayers of $Enz/AA/LuPc_2$ were deposited onto ITO glass with a substrate speed of 3 mm min^{-1}. Films were built by Y type deposition with a transfer ratio close to 1.

After preparation, LB films of $Enz/AA/LuPc_2$ were treated with glutharaldehyde to form covalent bonds between the enzymes and the amphiphilic molecules (Pavinatto et al., 2011).

Langmuir films were analyzed with Brewster Angle Microscopy (BAM) using a KSV MicroBAM.

AFM images were registered in LB films deposited onto ITO using a MultiMode Scanning Probe Microscope Model MMAFM-2 from Digital Instruments.

2.3. Electrochemical measurements

The electrochemical measurements were carried out in an EG&G PARSTAT 2273 potentiostat/galvanostat using a conventional three-electrode cell. The LB films were used as the working electrode. The reference electrode was Ag/AgCl/KCl 3 mol L^{-1} and the counter electrode was a platinum plate. Cyclic voltammograms were registered at a sweep rate of 0.1 V s^{-1}.

2.4. Phenols and grapes

10^{-3} mol L^{-1} stock solutions of phenolic compounds including one monophenol (vanillic acid), two orto-diphenols (catechol and caffeic acid), one para-diphenol (hydroquinone) and two triphenols (gallic acid and pyrogallol) were prepared by solving the corresponding compound in PBS. Solutions with lower concentration were prepared by dilution.

Grapes of five different varieties (*Tempranillo*, *Garnacha*, *Cabernet*, *Prieto Picudo and Mencía*) were harvested in 2012 in the Castilla y León region (Spain) by the Agrotechnological Institute of the regional Government (ITACYL), and by a cellar of the region