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Nanofibrous membrane based tyrosinase-biosensor for the detection of phenolic compounds

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ABSTRACT

A tyrosinase-modified electrode is described to be used as amperometric biosensor for the detection of phenolic compounds in food. The enzyme has been immobilized by drop-coating on a glassy carbon electrode covered by a polyamidic nanofibrous membrane prepared by electrospinning. With respect to others, the selectivity of the designed tyrosinase-biosensor resulted modified by the presence of the nanostructured coating which seems to affect the permeability of phenols as a function of the pH of the solution and of their dissociation constants. The biosensor exhibits a response time of 16 s, a detection limit of 0.05 μM, and a linearity up to 100 μM (slope: $-304\,nA\,\mu M^{-1}$; intercept: −191 nA, $r^2 = 0.996$, $n = 19$). Among others, it can be successfully used for monitoring in real time the release kinetics of phenols encapsulated in polymeric microcapsules.

1. Introduction

Phenolic compounds constitute a very important and widespread class of substances. They are widely used as additives in the chemical industry and as germicides and pesticides in agronomic practices [1,2]. Some of them are considered pollutants when released in the environment due to their toxicity [3,4]. However, phenols naturally occur in vegetables, fruits, and teas preventing degenerative diseases through anti-oxidative action and/or the modulation of several protein functions [5,6].

The determination of phenols is of paramount importance in both environmental analysis and in food quality characterization. For these purposes, many methods are available, such as gas chromatography [1] and spectrophotometry [7]. A large number of amperometric biosensors for the determination of phenolic compounds have been developed [8–10]. Most of them are based on the immobilization of tyrosinase on different electrode supports [11–14].

Nanotechnology offers new materials for the design of sensitive nanostructured transducer surfaces and, thus, new methods for the preparation of electrochemical biosensors [15]. Besides nanoparticles [16], nanowires [17] and nanotubes [18], membranes comprised of randomly oriented fibers ranging from microns to nanometers in diameter (nanofibers) can be a promising support for the build-up of biosensing devices [19]. Nanofibrous membranes are nanostructures with large specific surface area and high total porosity. These are important features to achieve high enzymes loadings and efficient transport of the analytes through the membrane toward the electrode surface.

In this work, a nanofibrous membrane based on tyrosinase-biosensor for the detection of phenolic compounds has been developed. The effect of the nanofibrous membranes on the tyrosinase-biosensor selectivity has been explained. Performance characteristics of the designed biosensor have been evaluated by means of chronoamperometry in standard solutions and in real samples.

2. Experimental

2.1. Reagents

Nylon-6, glucose, fructose, galactose, ascorbic acid, cysteine, caffeic acid, potassium chloride, monosodium phosphate and the organic solvents as methanol, acetic acid and, formic acid (98%), bovine serum albumin and glutaraldehyde were all purchased from Sigma–Aldrich. Deionized water was obtained from a Milli-Q system (Millipore).

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2.2. Electrospinning

Membrane of nylon-6 were prepared by electrospinning. Briefly, 8 g of nylon-6 pellets were dissolved in 26.6 mL of formic acid (88%). A syringe was filled with this solution and flowed at a rate of 0.03 mL h^{-1} thanks to a KDS100 syringe pump (KD-Scientific). The needle of the syringe was linked to the Spellman SL150 high voltage power supply (24 kV). A 80 mm diameter copper wire ring was positioned at 80 mm in front of the needle and grounded. The electrospinning was stopped at 20 min.

2.3. Sensor preparation

The membranes were mounted on a glassy carbon electrode (BAS MF-2012). First, the electrode was mechanically polished on alumina slurry of 0.3 μm on soft cloth pads. Then the electrode was thoroughly washed with distilled water, sonicated in water for 5 min, washed again with distilled water and, finally, left to dry. The top of the electrode was coated with a portion of the nanofibrous membrane. The membrane was kept tight on the electrode surface thanks to a plastic o-ring.

2.4. Enzyme immobilization

The enzyme tyrosinase (Tyr) was immobilized on the nylon nanofibers by a drop coating procedure. 5 μL of a suspension containing 1.41 g L^{-1} of tyrosinase (7571700 U L^{-1}), 6 g L^{-1} of BSA and 0.5% (w/v) of GA have been deposited on the electrode surface. After a drying for 15 min, the biosensor has been stored in PB 0.1 M pH 6.5 (4 °C).

2.5. Electrochemical measurements

Electrochemical experiments were performed using a three-electrode system with a silver/silver chloride double junction reference electrode (model 90-02, BAS), a 1 cm^2 platinum counter electrode and a glassy carbon electrode (model MF-2012, BAS) as working electrode. The potentiostat used was an CHI 1010 potentiostat (CH Instrument, Austin Texas). The sensitivity of the corresponding biosensor was monitored amperometrically at the working potential of −0.2 V vs. Ag/AgCl electrode. All measurements were carried out in 0.1 M phosphate buffer adjusted to pH 6.5. Temperature control at 25.0 ± 0.1 °C was achieved using a circulating bath.

2.6. Tyrosinase activity assay

Tyrosinase activity was measured by a spectrophotometric assay at room temperature using respectively pyrocatechol, caffeic acid and epicatechin, as substrates. After the addition of 10 μL of enzyme stock solution (1261.95 U mL^{-1}) to a 3 mL cuvette containing an excess of substrate (5 mM) in phosphate buffer 0.1 M (pH 6.5), the solution was immediately mixed. The increasing of absorbance at 420 nm as a function of time (indicating the formation of the o-quinone specie) was monitored with a Cary 100Bio UV–vis spectrophotometer (Varian) to determine the initial rate of enzyme activity. The initial rate was calculated from the linear part of the progressive curve (over the first 2 min).

3. Results and discussion

3.1. Functioning of tyrosinase-biosensor

In tyrosinase-biosensor, the tyrosinase immobilized on the nylon nanofibrous membrane catalyzes the oxidation of phenols to o-quinones in the presence of molecular oxygen. The produced o-quinone is reduced again into phenol at the surface of the GC electrode at reductive working potentials.

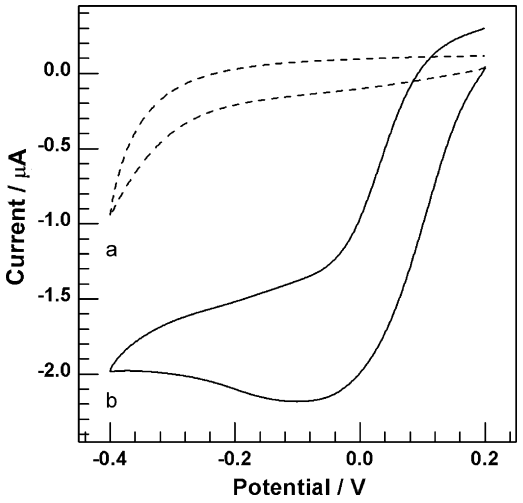

Fig. 1. Cyclic voltammetric response of the tyrosinase-biosensor in a phosphate buffer solution (0.1 M, pH 6.5) in absence (a) and in presence (b) of pyrocatechol 100 μM. Scan rate, 5 mV s^{-1}.

Fig. 1 shows the cyclic voltammogram of the tyrosinase-biosensor in a phosphate buffer solution (0.1 M, pH 6.5) in the absence (a) and in the presence (b) of pyrocatechol (100 μM) at the scan rate of 5 mV s^{-1}. Upon addition of the substrate, a cathodic peak corresponding to the reduction o-quinone appears at around −0.1 V vs. Ag/AgCl (Fig. 1b).

3.2. Selectivity of the biosensor

Preliminary experiments have been conducted to investigate on the influence of the nylon nanofibrous membrane on the tyrosinase-biosensor selectivity. It is well known that mushroom tyrosinase is able to use mono-, di- and tri-hydroxyphenols as substrates with different affinity [20]. Consequently, the response of biosensors based on tyrosinase as the biological component generally lacks in selectivity. Therefore, it has been investigated if the overall selectivity of the biosensor may be improved by using nylon nanofibrous membrane as enzyme supporting element and as electrode coating in the tyrosinase-biosensor set up. This hypothesis is supported by a previous work where nylon nanofibrous membranes acted as selective barrier to the electrochemical polyphenol oxidation, as a function of the pH of the solution and of the polyphenols dissociation constants [21].

To show the influence of the nanofibrous coating on biosensor response, solutions containing different phenols have been analyzed by chronoamperometry (−0.2 V vs. SCE) at the glassy carbon electrode coated with the nylon nanofibrous membrane, where the enzyme was covalently bound. The proposed tyrosinase-biosensor responded to some ortho-di-hydroxyphenols (pyrocatechol, caffeic acid and epicatechin) with a sensitivity which decreases following this order: pyrocatechol > caffeic acid > epicatechin. Regarding pyrocatechol, this result confirmed what reported in literature [22]. But, relatively to the other two substances, other tyrosinase biosensors [13] showed a higher sensitivity for epicatechin than caffeic acid, contrarily to our results. This suggested again that nylon nanofibrous membrane may influence tyrosinase-biosensor selectivity.

To verify further this hypothesis, two solutions of pyrocatechol and caffeic acid (1 μM) respectively, have been analyzed at the glassy carbon electrode before and after its coating with the nylon nanofibrous membrane, when the enzyme was free in solution. At

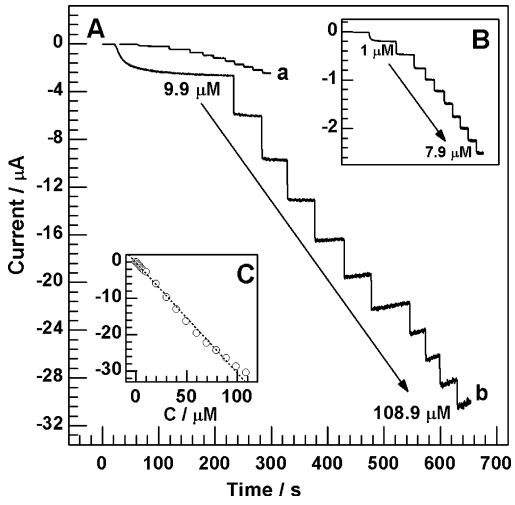

Fig. 2. (A) Amperometric response of the tyrosinase-biosensor at −0.2 V to successive additions of standard pyrocatechol, where line a corresponds to successive addition of 10 μL of standard pyrocatechol (1 mM), whether line b corresponds to successive addition of 10 μL of standard pyrocatechol (10 mM). (B) Zoom of line a. (C) Calibration curve of pyrocatechol.

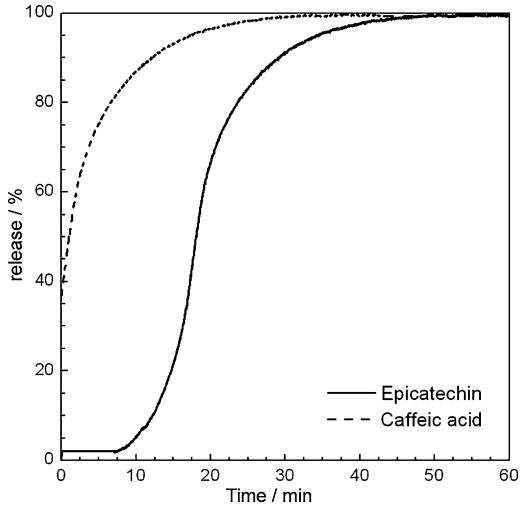

Fig. 3. Release profiles of epicatechin and caffeic acid in aqueous solution from spray-dried microcapsules.

the uncoated GC electrode the signal ratio of pyrocatechol respect to caffeic acid was 5:1. This ratio was obtained also by spectrophotometric measurements indicating that the result depends strictly on the specificity of tyrosinase. At the coated GC electrode the signal ratio of pyrocatechol respect to caffeic acid was instead 26:1. This change in the response of the tyrosinase-biosensor indicated the influence of nylon nanostructured membrane on its selectivity.

Next, our investigation was focused on the evaluation of the effect of tyrosinase immobilization on the response of the biosensor. Thus, two solutions of pyrocatechol and caffeic acid, respectively, have been analyzed at the GC electrode coated with the nylon nanofibrous membrane, where the enzyme was covalently immobilized. The resulting signal ratio of pyrocatechol respect to caffeic acid was then compared with the one obtained at the coated GC electrode when the enzyme was free in solution. A similar ratio (26:1) was achieved, revealing that (1) the selectivity of the biosensor is affected by the nanofibrous membrane at the same way either when the enzyme is free in solution or when the enzyme is immobilized through the nanostructured support; (2) the functioning of the catalytic site of the enzyme is not compromised by its immobilization on a nanofibrous environment.

3.3. Performance characteristics of Tyr-biosensor

The performance of the biosensor was investigated by means of chronoamperometry technique. The current response given by the electrochemical reduction of the o-quinone resulting from the enzyme reaction was detected at −0.2 V vs. SCE. A typical amperometric response of the Tyrosinase-biosensor to successive standard additions of pyrocatechol is shown in Fig. 2(lines a and b). The 90% of the steady-state response time is reached within 16 s, suggesting that the substrate molecule can diffuse through the pores of the nanofibrous membrane. The reduction currents appeared proportional to the phenol concentration. The resulting calibration plot for pyrocatechol (shown in Fig. 2(C)) is linear over the 1–100 μM range (slope: −304 nA μM^{-1}; intercept: −191 nA, $r^2 = 0.996$, $n = 19$). The estimated detection limit (calculated using the linear regression technique from Miller and Miller [23]) is 0.05 μM.

3.4. Stability, reproducibility and lifetime

The stability of the biosensor was investigated by monitoring the changes in the slopes of the linear plot of independent standard addition experiments at the concentration range from 1 to 10 μM and from 10 to 100 μM of pyrocatechol (PB 0.1 M, pH 6.5). The stability of the biosensor was estimated from the relative standard deviation (rsd%) of the average slopes ($n = 10$). The stability at the concentrations range from 1 to 10 μM was 6.4% ($n = 10$). The stability at the higher concentration range (10–100 μM) was worse, with a rsd% of 9.7% ($n = 10$). Such decrease of the biosensor stability is probably due to a progressive accumulation of pyrocatechol during its electro-oxidation at the electrode surface till the formation of a polymeric film (passivation) at higher concentrations [24].

A lot-by-lot reproducibility of the nylon nanofibers biosensors was evaluated by the response variation of 10 tyrosinase-biosensors, prepared in different days. The amperometric steady-state current ($i_{ss} - i_0$), generated with a stirred solution of pyrocatechol 1 μM showed a relative standard deviation of 13.81% ($n = 10$).

One of the reasons to immobilize an enzyme is to improve its lifetime, allowing its use over a long period of time. Thus, the biosensor lifetime was estimated by measuring its response to 1 μM pyrocatechol solution everyday for 1 month. The lifetime results longer than 2 weeks. This range of time can be considered acceptable, if we take into account the cheapness and easiness of the preparation of this biosensor.

3.5. Effect of interfering substances

To identify possible interferents which may affect the signal with a different mechanism than the analyte, the effect on the biosensor response of typical chemical compounds present in food products has been examined. Amperometric analysis of buffered solutions of pyrocatechol (10 μM), to which sugars and vitamin C (10 μM) were added, has been carried out. Addition of α-glucose, α-fructose, α-galactose, lactose, sorbitol did not cause observable changes in the biosensor response. Also, ascorbic acid was not detectable at the applied working potential. However, addition of ascorbic acid in a solution containing pyrocatechol causes a decrease of the catechol reduction current because of the homogeneous reduction of the o-quinone to phenol [25].

3.6. Biosensing polyphenols in real samples: monitoring of release kinetics

The functioning of tyrosinase-biosensor has been tested in real samples. In particular, the release of phenols (epicatechin or caffeic acid) from spray-dried microcapsules made of a mixture of maltodextrin/shellac have been monitored by the tyrosinase-biosensor. The current generated by the release of epicatechin or caffeic acid in phosphate buffer (0.1 M pH 6.5) at −0.2 V has been registered for 60 min. The release profiles of epicatechin and caffeic acid are presented in Fig. 3. The total amount of epicatechin and caffeic acid was released respectively after 40 and 30 min of stirring in an aqueous solution. These results have been confirmed by colorimetric analysis (Folin-Ciocolteau method) [26]. In addition, this experiment showed that chronoamperometry may be an useful and easy-handling tool for monitoring in real time the release kinetics of compounds from complex matrices.

4. Conclusion

In conclusion, we demonstrate the suitability of nylon-6 nanofibrous membranes based tyrosinase electrodes for the biosensing of phenolic compounds. The results confirmed that nylon nanostructured membrane have an influence on the electrochemical detection of phenolic compounds improving tyrosinase-biosensor selectivity. Furthermore, the functioning of the catalytic transduction remains unaffected after the covalent immobilization of the enzyme through the nanofibrous membrane.

The proposed biosensor provided high sensitivity, long lifetime, and excellent reproducibility. However, the operational stability of the biosensor resulted dependent on analyte concentration. Biosensor performance in terms of reproducibility appeared excellent only in the presence of low concentrations of phenols. Testing the functioning of tyrosinase-biosensor in real samples revealed that chronoamperometry may be a useful and easy-handling tool for monitoring in real time the release kinetics of compounds from complex matrices.

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