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The Sonogel–Carbon materials as basis for development of enzyme biosensors for phenols and polyphenols monitoring: A detailed comparative study of three immobilization matrixes

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Abstract

Three amperometric biosensors based on immobilization of tyrosinase on a new Sonogel–Carbon electrode for detection of phenols and polyphenols are described. The electrode was prepared using high energy ultrasounds (HEU) directly applied to the precursors. The first biosensor was obtained by simple adsorption of the enzyme on the Sonogel–Carbon electrode. The second and the third ones, presenting sandwich configurations, were initially prepared by adsorption of the enzyme and then modification by mean of polymeric membrane such as polyethylene glycol for the second one, and the ion-exchanger Nafion in the case of the third biosensor. The optimal enzyme loading and polymer concentration, in the second layer, were found to be 285 U and 0.5%, respectively. All biosensors showed optimal activity at the following conditions: pH 7, −200 mV, and 0.02 mol l^{-1} phosphate buffer.

The response of the biosensors toward five simple phenols derivatives and two polyphenols were investigated. It was found that the three developed tyrosinase Sonogel–Carbon based biosensors are in satisfactory competitiveness for phenolic compounds determination with other tyrosinase based biosensors reported in the literature. The detection limit, sensitivity, and the apparent Michaelis–Menten constant K_m^{app} for the Nafion modified biosensor were, respectively, 0.064, 0.096, and 0.03 μmol, 82.5, 63.4, and 194 nA μmol^{-1} l^{-1}, and 67.1, 54.6, and 12.1 μmol l^{-1} for catechol, phenol, and 4-chloro-3-methylphenol. Hill coefficient values (around 1 for all cases), demonstrated that the immobilization method does not affect the nature of the enzyme and confirms the biocompatibility of the Sonogel–Carbon with the bioprobe. An exploratory application to real samples such as beers, river waters and tannery wastewaters showed the ability of the developed Nafion/tyrosinase/Sonogel–Carbon biosensor to retain its stable and reproducible response.

Keywords: Biosensors; Sonogel–Carbon; Tyrosinase; Phenols; Polyphenols; Amperometry

1. Introduction

The choice of a suitable electrode is a key stage in amperometric biosensor development process, since it is in intimate contact with the immobilized enzyme. A good electrical conductivity and sensitivity towards products are also important factors, and the chemical and physical inertias against the contacting solution are the basic conditions.

Due to the compatibility between the inorganic support and the immobilized biochemical species, the employment of sol–gel technology to produce ceramics based biosensitive materials has received increasing interest in recent years (Tsionsky et al., 1994; Rabinovich and Lev, 2001; Wang, 1999; Collinson and Howells, 2000; Jin and Bernnam, 2002; He and Toh, 2006). Special advantages, including the relative porosity, chemical inertness, simplicity of preparation, negligible swelling in solutions, low temperature encapsulation, and high sensitivity, have been recognized for sol–gel electrodes based sensors and biosensors. The entrapped species, such as chemical and biological molecules, magnificently preserve their chemical properties

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or bioactivities. However, it has been demonstrated that the greater interest in sol–gel sensing stems from the fact that many chemical and biochemical reactions that occur in solution can be accomplished in the pores of sol–gel host (Collinson and Howells, 2000; Jin and Bernnam, 2002). Furthermore, judicious selection of an approach to accomplish porous surfaces can increase the stability and sensitivity of the resultant sol–gel electrode. Various methods including variation of precursors, sol–gel synthesis conditions, silane:solvent ratio, and so on, have been proposed. All these strategies include the use of relative high concentrations of alcohol, and the later evaporation of this component involves an inevitable shrinkage of sol–gel matrix with the time thus affecting its porosity (Lev, 1992). Our group proposed a novel sol–gel procedure based on the use of sonocatalysis to obtain solid carbon composite electrodes, called by us Sonogel–Carbon electrodes (Hidalgo-Hidalgo-de-Cisneros et al., 2001; Cordero-Rando et al., 2002). High energy ultrasounds (HEU) are applied directly to the precursors, ultrasonic cavitation is achieved, the materials shrinkage is avoided, and the pore size can be controlled. In addition, thanks to this strategy hydrolysis with acidic water is promoted in the absence of any supplementary solvent in only few seconds operation time. The so-called Sonogel–Carbons are of high density, exhibiting fine texture and homogeneous structure; the presence of spectroscopic grade graphite renders them conductive. This matrix offers an alternative route for developing new composite electrodes with a large variety of structures and shapes.

These electrodes show very favourable electroanalytical properties for their use as amperometric sensors and, furthermore, they can easily permit the incorporation of numerous receptor molecules at the Sonogel–Carbon materials, which can notably improve the sensitivity and selectivity compared to classical electrodes (Ballarin et al., 2002, 2003; Cordero-Rando et al., 2005).

In the present study, several sensitive biosensors based on Sonogel–Carbon electrode and the enzyme tyrosinase, were developed. The objective designed for those biosensors was to seek new electrochemical biosensors for phenols and polyphenols determination. The influence of additive-protective polymers, such as polyethylene glycol (PEG) and perfluorinated-Nafion ion-exchanger on the surface of the biosensor were explored. The experimental parameters affecting the response of all resulting biosensors are discussed.

Sensing performances and kinetic characterisations of the developed biosensors were investigated toward seven phenolic compounds. The obtained data were compared to similar systems in the literature.

2. Materials and methods

2.1. Apparatus and reagents

Methyltrimethoxysilane (MTMOS) was from Merck (Darmstad, Germany) and HCl was from Panreac (Barcelona, Spain). Graphite powder (spectroscopic grade RBW) was from SGL Carbon (Ringsdorff, Germany). Mushroom tyrosinase (E.C. 1.14.18.1, 3000 U mg^{-1}) was purchased from Sigma (St. Louis, MO, USA). KH_2PO_4 and K_2HPO_4 for phosphate buffer were from Fluka (Buchs, Switzerland). Nafion-perfluorinated ion-exchange resin (Cat. No. 27, 470-4) 5% (w/v) in a mixture of lower aliphatic alcohols and water was obtained from Aldrich (Steinheim, Germany); working solutions were prepared by diluting with methanol (80%, w/v, Merck). Polyethylene glycol (PEG, MW 550) was from Aldrich; working solutions were prepared by diluting with water. All reagents were of analytical grade or higher, and used as received. Nanopure water was obtained by passing twice-distilled water through a Milli-Q system (18 MΩ cm^{-1}, Millipore, Bedford, MA). All phenolic compounds tested in this work (cathecol, phenol, hydroquinone, 4-chlorophenol, 4-chloro-3-methylphenol, catechin, and gallic acid) were of analytical grade, and purchased from Merck, Sigma, or Panreac.

Stock solutions of the phenolic compounds (0.01 mol l^{-1}) were prepared daily by dissolving the appropriate amount either in 0.02 mol l^{-1} phosphate buffer at pH 7 or in ethanol, depending on the phenolic compounds solubility. More dilute standards were prepared by suitable dilution with 0.02 mol l^{-1} phosphate buffer solution at pH 7, which was also used as the supporting electrolyte.

Glass capillary tubes, i.d. 1.15 mm, were used as the bodies for the composite electrodes.

Chronoamperometric measurements were performed with an Autolab PGSTAT20 Ecochemie, Utrecht, The Netherlands) potentiostat/galvanostat interfaced with a personal computer, using the AutoLab software GPES for waveform generation and data acquisition and elaboration.

A 600-W model, 20 kHz ultrasonic processor (Misonix Inc., Farmingdale, NY) equipped with a 13 mm titanium tip was used. The ultrasonic processor was enclosed inside a soundproof chamber during operation.

All electrochemical experiments were carried out in a cell containing 25 ml of an aerated 0.02 mol l^{-1} phosphate buffer solution at pH 7 and 22 ± 2 °C; the three-electrodes system consisted of a tyrosinase-modified Sonogel–Carbon electrode as working electrode, and a Ag/AgCl (3 mol l^{-1} KCl) and a platinum wire as reference and auxiliary electrodes, respectively. A magnetic stirrer and stirring bar were used to provide continuous convective transport during the amperometric measurement.

2.2. Procedure

2.2.1. Biosensor fabrication

The unmodified Sonogel–Carbon electrode was prepared as described previously (Hidalgo-Hidalgo-de-Cisneros et al., 2001; Cordero-Rando et al., 2002). Before modification, the electrodes were polished with emery paper No. 1200 to remove extra composite material, wiped gently with weighing paper, thoroughly washed with deionised water, and allowed to dry at room temperature.

Tyrosinase powder was dissolved in 0.2 mol l^{-1} phosphate buffer of pH 7.4 giving an enzyme concentration of 57,000 U ml^{-1}. In the first step, 1.5, 3, or 5 μl of this solution (corresponding to 85, 171, or 285 U, respectively) were placed onto the surface of an unmodified Sonogel–Carbon electrode and

allowed to adsorb and dry at room temperature. In the second step, when biosensors with polymeric coatings were prepared, 1.5 μl of Nafion or PEG solution were spread on the enzyme film. Three kinds of electrodes were used: Sonogel–Carbon biosensors coated with Nafion solutions at different percentages (0.1, 0.25, 0.5, 0.75, and 1%), Sonogel–Carbon biosensors coated with PEG solutions at different percentages (0.5, 1, 2, 5, 10, and 20%), and uncoated Sonogel–Carbon biosensors. The resulting biosensors were stored for a minimum of 8 h to dry in a refrigerator at 4 °C. Moreover, it is worth to mention that all biosensors were washed carefully with buffer solution before and after each manipulation, and were stored by immersing in a phosphate buffer solution of pH 7 at 4 °C when not in use.

3. Result and discussion

3.1. Optimisation of the biosensor fabrication procedure

3.1.1. Influence of the carbon powder

The response of a tyrosinase-modified electrode is usually limited by the electrochemical back reduction of the quinone leading to the diphenolic compound. The choice of the carbon powder affects significantly the sensitivity of ceramic–carbon based biosensors, because the limiting electrochemical reaction of the enzymatic products takes place on the grain of the carbon materials (Rabinovich and Lev, 2001). The carbon selected in this paper was the RW-B carbon graphite powder, which showed by cyclic voltammetry an observed lower capacity "C_{obs}" than the other studied graphites (Cubillana-Aguilera et al., 2006).

3.1.2. Study of enzyme loading and polymer coating percentage

The study of the effect of enzyme loading was carried out using 85, 171, and 285 U of enzyme, a fixed amount of polymer coating, and three kinds of biosensors: Sonogel–Carbon coated with 0.5% Nafion (Nafion/Tyr/Sonogel–Carbon), Sonogel–Carbon coated with 10% PEG (PEG/Tyr/Sonogel–Carbon), and uncoated Sonogel–Carbon (Tyr/Sonogel–Carbon). All the electrodes were polarized at −200 mV in presence of 10 μmol l^{-1} of catechol in 0.02 mol l^{-1} phosphate buffer. The best response was obtained for the Nafion coated biosensor and the worse one for PEG coated biosensor. For this latest, no response was obtained for an enzyme loading of 85 U, but an increasing signal arises for 171 and 285 U. For both the Nafion coated and the uncoated biosensors, a linear relationship between enzyme loading and electrochemical response was obtained, with a regression coefficient of 0.997 and 0.998, respectively. Therefore, loading by 285 U of enzyme was adopted for the preparation of all biosensors in this work.

The presence of a polymer layer on the surface of a working electrode reduces in general the diffusion coefficients of both the substrates and the reaction products through the membrane, which consequently should increases the response time of the biosensor and decreases its absolute signal. In this paper, the effect of different percentages of Nafion and PEG coatings were studied.

For Nafion coated biosensors (Fig. 1A), the best response was obtained for a 0.5% content of polymer. Nafion is a polymer that includes in its structure a hydrophobic organic fluorocarbon region and a hydrophilic ion-cluster region (Liu et al., 1997); up to 0.5% of Nafion, the presence of the two regions provides a medium completely compatible with the enzyme, which favours its stability and mobility; concurrently, the action as exchanger of the polymer facilitates the penetration and pre-concentration of the analyte (Nistor et al., 1999) and consequently an increase in the response is obtained. However, for higher percentages of Nafion, it must be taken into account several unfavourable effects: the additional increase in the hydrophobic character and in the alcoholic content forwarding the Nafion solution leads to the denaturising of the enzyme. Furthermore, additional increase in the film thickness leads to a decrease of the diffusion coefficient, and consequently to lower sensitivities.

A different effect can be observed for PEG coated biosensor (Fig. 1B). PEG is a high hydrophilic polymer that has

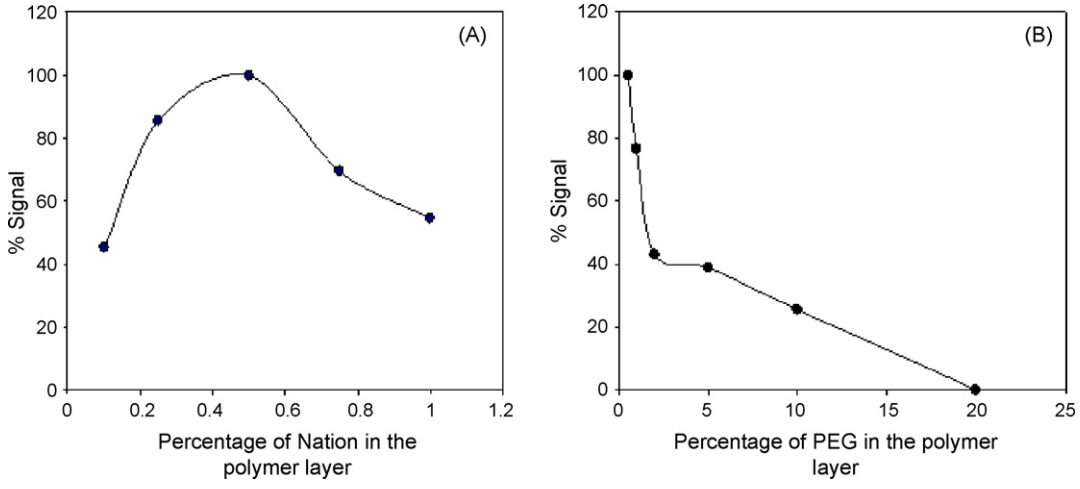

Fig. 1. Effect of the percentage of Nafion (A) and PEG (B) on the steady-state response of the Tyr/Sonogel–Carbon biosensors. Tyrosinase loading 285 U/electrode, catechol concentration 10 μmol l^{-1}, PB 0.02 mol l^{-1} (pH 7.0), and applied potential −200 mV vs. Ag/AgCl. $I_{0.5\%\ coating}$ are 461 ± 18 and 107 ± 7 nA, in 'A' and 'B', respectively.

demonstrated a protective effect against the denaturation of phenoloxidase enzymes (Nakamoto and Machida, 1992; Wagner and Nicell, 2001). The presence of a moderate thickness PEG layer favours the stability of the enzyme and facilitates the penetration of the analyte through micro-channels in the polymer (Cordero-Rando et al., 2005). However, for a thicker layer, a considerable decrease in the diffusion coefficients occurs pushing to find a concentration of compromise. A percentage of 0.5% of PEG in the second layer was selected for this type of biosensor.

3.2. Optimisation of experimental parameters

The strong dependence of the response of the enzymatic biosensors obtained by the adsorption method on the pH, ionic strength of the solvents, applied potential as well as the p*I* of the proteins, requires a detailed study of these parameters in order to achieve optimal response and to characterize the way of enzyme entrapment.

The effect of the operational potential on the response for all biosensors was studied from −400 to 200 mV in a 0.02 mol l^{-1} buffer solution containing 10 μmol l^{-1} of catechol. As expected, for the two membrane modified biosensors the reductive detection of the liberated quinone starts at 100 mV, increases sharply up to 0 mV, reaches a plateau in the interval between −100 and −200 mV and decreases by 70 and 80%, at the potential value of −400 mV, for both the Nafion and the PEG modified biosensors. On the other hand, for the unmodified biosensor the plateau was very short, the optimal potential was obtained for −200 mV, and no response was observed at extreme polarisations. It has been shown that a gradual decrease of the response at negative potentials is very common for similar biosensors to those studied in this paper (Sanz et al., 2005; Rajesh et al., 2004; Campuzano et al., 2003; Kim and Lee, 2003; Freie et al., 2002). This can be attributed to an undesired phenomenon leading to a possible polymerization of the *o*-quinones to form non-conductive polymers occurring at extreme potentials. This reduces the active surface of the electrode and thus decreases the electrode response. However, the two polymeric coated electrodes exhibit a greater resistance to this phenomenon than the uncoated electrode, which confirms the protective effect of the second layer. An optimal potential of −200 mV was selected for all biosensors in all subsequent experiments.

Different concentrations of phosphate buffer around pH 7 value were tested in order to establish the possible influence of the ionic strength on the electrochemical response for all Sonogel–Carbon biosensors. The experiments were carried out in an electrochemical cell containing different concentrations of phosphate buffer in the range of 0.001–0.2 mol l^{-1} in presence of 10 μmol l^{-1} catechol concentration. For the three enzyme biosensors, a relative flat electrochemical response is observed up to 0.02 mol l^{-1} with relative standard deviations of 1.7, 6.5, and 11.1% for Nafion, PEG, and unmodified biosensors, respectively. The same order of variation was observed in the extreme value of 0.001 mol l^{-1} PB, but with response decreases of 44.6, 61.4, and 76.1%, respectively, compared to their optimum values. For the sake of use an ionic strength that does not cause any important change in the microenvironment of the enzyme, a buffer concentration of 0.02 mol l^{-1} was used in following experiments.

It should be pointed out that the enzyme activity and biosensor stability can be strongly affected by the pH of the medium. In order to understand this behaviour, we have studied the influence of this parameter in the pH range of 4.5–9.5, in 0.02 mol l^{-1} buffer solution containing 10 μmol l^{-1} catechol. Contrarily to the Nafion and PEG modified biosensors, for which a small variation in the response were found for pH values between 5.5 and 7.5 (11.3 and 18.2%, respectively), the unmodified biosensor shows clearly an optimal response at pH 7. A wide optimal pH range of 5–8 has been reported for free tyrosinase (Daigle and Leech, 1997; Horowitz et al., 1970), so it seems that the matrix and the coating used for the immobilization of the enzyme in our experiments did not affect its behaviour. However, a decrease of the electrochemical response was observed for all biosensors for pH values lower than 5.5 or higher than 7.5. For the extreme value of pH 4.5, the activity kept 63% using the Nafion modification, 50% with PEG, and finally no unregenerate response in the case of uncoated biosensor. The inactivation of the uncoated biosensor at a pH below the isoelectric point of the enzyme tyrosinase (4.9) can be assigned to desorption of the entrapped enzyme into the silicate sonogel electrode surface. It seems that the presence of the layer of the coating prevents in great extension the leak of the enzyme. The pH 7 for the medium was selected as optimal for all subsequent tests, considering the highest sensitivity and stability obtained at this pH.

3.3. Reproducibility and stability

The reproducibility of the biosensors were evaluated by mean of 10 repetitive measurements with each electrode in 0.02 mol l^{-1} PB solution, pH 7, containing 5 μmol l^{-1} of catechol. Relative standard deviations of 3.9, 4.8, and 13.1% were obtained for Nafion, PEG, and unmodified tyrosinase biosensors, respectively. The operational storage stability of biosensors were tested by amperometric measurements every day, when it was possible, using a 10 μmol l^{-1} concentration of catechol. According to the description in the literature about the evolution of the electrochemical response of biosensors made by simple physical adsorption of the enzyme on electrode surface, all the biosensors decrease their current response with respect to the first days, and reach a steady value after several days. For the Nafion-modified biosensor, a long lifetime (38 days) was observed, and a reproducible value, with a R.S.D. of 4.3% from 6th to 16th day, was obtained. In the case of the PEG modification the relative stable response (R.S.D. 9.01%) was from 5 to 10 days with a lifetime of 22 days. However, a short lifetime (10 days) and a non-significant plateau response in the evolution of the simply adsorbed enzyme biosensor was observed.

The relative stability of the three biosensors can be attributed to two factors. The first of them is the biocompatibility of the Sonogel–Carbon electrode with the enzyme tyrosinase, probably because of the inorganic–organic structure of the Sonogel. The second is the inertness of this material; the shrinkage phenomenon was avoided thanks to the exclusion of the use of organic solvent in the sonogeling process, and the porosity and

active surface were fixed. However, additional considerations must be taking into account to explain the differences showed between the stabilities of the three enzymatic electrodes. On one hand, it must be necessary to consider the differences in the immobilization methods of the enzyme on the surface of the electrode, so the presence of the polymers can act as entrapment matrix of the enzyme tyrosinase on the Sonogel–Carbon electrode surface. On the second hand, the quinone products of the enzymatic reaction inactivate the enzyme due to the interaction between the enzyme's active sites and phenoxy radicals, or passivate the electrode surface via the formation of non-conductive etheric polymer films (Cosnier et al., 2001; Kalibanov et al., 1983; Wood and Ingraham, 1965), with the consequent alteration in the operational stability of the biosensor. PEG and Nafion have widely proved their protective role in this aspect, probably because they offer preferable sites for the adsorption of the phenoxy radicals than the enzyme structure.

3.4. Sensing performances of the three tyrosinase based biosensors

Fig. 2A shows an example of the amperometric response when the three biosensors are polarized at a potential of −200 mV, and after successive additions of catechol to an aerated, stirred pH 7, 0.02 mol l^{-1} PB solution. The increase in the reduction current was detected and the response time (with 95% steady state) was achieved at about 3.5, 11, and 20.5 s for Nafion, PEG, and unmodified biosensors, respectively. These values were generally faster or similar to those reported in literature (Kim and Lee, 2003; Liu et al., 2000, 2005a,b; Yu et al., 2003; Wang et al., 2002). This faster response can be probably attributed to the well-demonstrated sensitivity of the surface of the Sonogel–Carbon electrode (Cordero-Rando et al., 2002) and to the rapid accessibility of the substrate from the bulk solution to the simply adsorbed enzyme.

In general, the mass transfer resistance increases with the presence of the polymer film and consequently the response time of the biosensor increases too. However, the experimental results show that an opposite effect is obtained in our case: the response times for Nafion and PEG modified biosensors are six and two times faster than the response time for the non-coated biosensor. This is probably due, as we have previously mentioned, to the exchanger-like behaviour of Nafion and to the hydrophilic nature of PEG, respectively. This overcomes the decrease in the diffusion coefficients caused by the presence of the coating film.

Tyrosinase adsorbed on the surface of the Sonogel–Carbon electrode is capable to catalyze the oxidation of phenol, catechol and their derivatives such as chlorophenols and polyphenols. In order to study the selectivity and the analytical performances of the three Sonogel–Carbon biosensors, the response of five monophenols such as cathecol, phenol, 4-chlorophenol (4-ClPh), 4-chloro-3-methylphenol (4-Cl-3MPh), hydroquinone and two polyphenols such as (+)-catechin, and gallic acid, were investigated in the optimised conditions. Calibration curves for catechol are shown in Fig. 2B. The limit of detection (LOD), limit of quantification (LOQ) and the sensitivity of the system were all statistically calculated from the linear range of the calibrations curves, as follows (Miller and Miller, 1993):

$$\text{LOD (or LOQ)} = \frac{k \times S_B}{b}$$

where S_B is the standard deviation of the blank measurement, b the sensitivity of the method (determined as the slope of the calibration curve), and k is a statistical constant (values of 3 and 10 in the case of LOD and LOQ, respectively, are largely accepted). Table 1 summarizes performance characteristics of the developed biosensors for different phenolic compounds. It can be seen that Nafion modification has a notable and favourable effect on the sensitivity of the bioprobe for all the phenolics substrates tested except for the polyphenol catechin, for which the

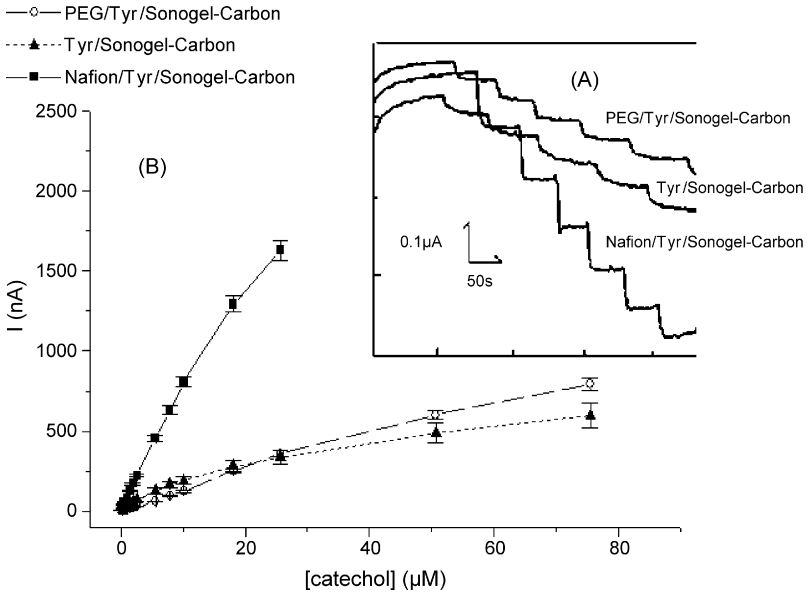

Fig. 2. (A) Steady-state current–time response curves for increasing catechol concentration (2 μmol l^{-1} steps). Stirred aerated 0.02 mol l^{-1} PB solution at pH 7 and an applied potential of −200 mV, (B) calibration plots vs. catechol for the three types of biosensors, deduced from the corresponding amperograms.

Table 1
Analytical performances and kinetic factors of proposed biosensors, for phenolic compounds

Compounds	Electrode	LOD (μmol l^{-1})	LOQ (μmol l^{-1})	Sensitivity (nA μmol^{-1} l^{-1})	h	K_m^{app} (μmol l^{-1})	I_{max} (nA)	I_{max}/K_m^{app} (nA μmol^{-1} l^{-1})
Catechol	Nafion/Tyr/Sonogel	0.064	0.210	82.5 ± 0.7	1.03	67.1	5905	89.0
	PEG/Tyr/Sonogel	0.930	2.39	12.3 ± 0.6	1.10	97.1	1381	15.8
	Tyr/Sonogel	0.193	0.640	26.9 ± 0.6	1.04	19.9	588.1	29.6
Phenol	Nafion/Tyr/Sonogel	0.096	0.200	63.4 ± 1.0	1.15	54.6	3793	69.4
	PEG/Tyr/Sonogel	0.696	2.32	22.9 ± 1.5	1.00	57.8	2895	50.1
	Tyr/Sonogel	0.734	2.38	17.7 ± 1.0	1.26	9.8	173.3	17.7
4-Chlorophenol	Nafion/Tyr/Sonogel	0.294	0.980	74.8 ± 2.3	0.99	24.5	2520	106
	PEG/Tyr/Sonogel	0.476	1.59	31.7 ± 1.4	1.09	27.9	1135	40.7
	Tyr/Sonogel	1.70	3.58	24.6 ± 0.6	0.92	10.5	294.8	28.0
4-Chloro-3-methylphenol	Nafion/Tyr/Sonogel	0.030	0.090	194 ± 5.1	0.93	12.1	2269	188
	PEG/Tyr/Sonogel	0.368	1.23	40.2 ± 0.9	0.97	22.9	1276	55.8
	Tyr/Sonogel	0.802	2.67	24.7 ± 1.4	0.97	10.0	427.7	42.8
Hydroquinone	Nafion/Tyr/Sonogel	3.50	11.6	2.60 ± 0.1	0.97	4160	1306	3.13
	PEG/Tyr/Sonogel	8.83	29.4	$35.9 \times 10^{-2} \pm 5.3 \times 10^{-2}$	0.93	476	303.7	0.390
	Tyr/Sonogel	8.64	28.8	$66.2 \times 10^{-2} \pm 12.0 \times 10^{-2}$	1.21	133	124.3	0.930
Gallic acid	Nafion/Tyr/Sonogel PEG/Tyr/Sonogel Tyr/Sonogel	85.0	287	$1.60 \times 10^{-2} \pm 0.2 \times 10^{-2}$	1.02	720	51.30	7.00×10^{-2}
Catechin	Nafion/Tyr/Sonogel	1.25	4.15	5.36 ± 0.3	1.11	163.9	953.3	5.81
	PEG/Tyr/Sonogel	2.21	7.36	4.72 ± 0.5	1.08	166.7	823.9	4.94
	Tyr/Sonogel	0.604	2.01	8.02 ± 0.5	1.03	13.9	116.2	12.6

sensitivity was 50% lower than that obtained for the unmodified electrode. The sensitivity for this modified biosensor follows the trend: 4-Cl-3MPh > catechol > 4-ClPh > phenol > (+)-catechin > hydroquinone > gallic acid, being different from those of unmodified and PEG modified biosensors. It can be concluded that the sensitivity sequence for a variety of phenolic compounds does not only depend on the varied activity of the enzyme tyrosinase but also on the nature of the protecting or immobilizing matrix. Furthermore, the trends and the affinity for the chlorinated phenols observed for this perfluorinated polymer modification is in accordance with that obtained by Nistor et al. (1999), in the case of Nafion/PPO-SGEs and different for that obtained with Nafion/PPO-CPE biosensor reported in the same paper, and proves that the selectivity sequence can be attributed to the nature of the surface electrode as well as the factors above mentioned.

With the aim of comparing the performances of the biosensors developed in this paper with those in the literature relayed on other strategy designs, we show in Table 2 some recently published biosensor results. It can be seen that the sensitivities of 193.5, 82.53, 74.8, and 63.36 nA μmol^{-1} l^{-1} for Nafion/Tyr/Sonogel–Carbon, 40.2, 12.18, 31.7, and 22.9 nA μmol^{-1} l^{-1} for PEG/Tyr/Sonogel–Carbon, and 24.7, 26.85, 24.5, and 17.73 nA μmol^{-1} l^{-1} for the unmodified Tyr/Sonogel–Carbon towards 4Cl-3-MPh, catechol, 4ClPh, and phenol, respectively, were better or similar to the values obtained with the majority of biosensors cited in Table 2 for: tyrosinase immobilized chemically on the carbon fiber (Freie et al., 2002), cross linked on SAM–Au electrode (Campuzano et al., 2003), entrapped with polypyrrole derivative (Rajesh et al., 2004, 2005; Rajesh and Kaneto, 2005), covalently immobilized by $MgFe_2O_4$–SiO_2 cross-shell to CPE (Liu et al., 2005a,b) or cross linked within a poly(thionine) mediator film on the GCE (Dempsey et al., 2004). However, these findings are worse than those obtained by the mediated Al_2O_3 sol–gel and $Fe(CN)_6^{4-}$ chitosan tyrosinase biosensor (Liu et al., 2000; Wang et al., 2002). It should be pointed out that the simple strategy for biosensors design we presented here could improve the LOD and the sensitivity by simple increasing the amount of enzyme loading.

3.5. Kinetic factors of the biosensors

In general, the dependence of the steady-state response of a biosensor on the substrate concentration is often described using the empirical Hill equation (Kurganov et al., 2001),

$$I = \frac{([C]/[C]_{0.5})^h}{1 + ([C]/[C]_{0.5})^h} I_{max}$$

where I_{max} is the maximum rate of the enzymatic reaction, $[C]_{0.5}$ the concentration of half saturation, and h is the Hill coefficient. In our case, the Hill coefficient is around 1 (see Table 1), thus the above expression turns into a classical Michaelis–Menten equation, and $[C]_{0.5}$ corresponds to the apparent Michaelis–Menten constant K_m^{app}. Moreover, as can be seen from Fig. 3, the Eadie–Hofstee representation for the different phenolic compounds tested shows two regions: the first one non-linear observed at low substrate concentrations, suggesting a diffusion control, and the second one, linear observed at high substrate concentration. This suggests an enzymatic control (Rubianes and Rivas, 2000). Consequently, the calculations of K_m^{app} and

Table 2
Analytical characteristic for some reported and suggested tyrosinase based biosensors

Biosensor/mediation; immobilization; modification/electrode	Analytes	Sensitivity or slope	Detection limit (μmol l^{-1})	K_m (μmol l^{-1})	$tr_{95\%}$ (s)	Reference
Tyr/Alginate/GCE	Catechol	0.08	1		20	Abu-Rabeah et al. (2005)
Tyr/pyrrole-alginate/GCE	Catechol	0.35 nA μmol^{-1} l^{-1} cm^{-2}	0.4		20	
Tyr/MPA/AuE	Phenol, catechol, 4-Cl-3-MPhl, 4-ClPh	13.9, 34.2, 41, 73.2 nA μmol^{-1} l^{-1}	0.088, 0.11, 0.094, 0.15	146.4, 334.5, 60.4, 23.6		Campuzano et al. (2003)
Tyr/poly(amphiphilic pyrrole)/GCE	Phenol	680 nA μmol^{-1} l^{-1} cm^{-2}	0.010			Cosnier et al. (1999)
Tyr/amphiphilic substituted polypyrrol/SPCE	Catechol, catechin	409, 0.38 nA μmol^{-1} l^{-1}				Cummings et al. (2001)
Tyr/redox poly(thionine)/GCE	Phenol	44.7 nA μmol^{-1} l^{-1}	1	111		Dempsey et al. (2004)
Tyr/Cross Linked/CFμE	Phenol	13.5 nA μmol^{-1} l^{-1}	0.159			Freie et al. (2002)
Tyr/silicate sol–gel; Nafion/GCE	Phenol, catechol, 4-ClPh	46, 200, 120 nA μmol^{-1} l^{-1}	1, 0.35, 0.67	65×10^3, 75×10^3, 40×10^3	15 catechol	Kim and Lee (2003)
Tyr/$MgFe_2O_4$–SiO_2/CPE	Phenol	54.2 nA μmol^{-1} l^{-1}	0.6		20	Liu et al. (2005a)
Tyr/Al_2O_3, sol–gel/GCE	Phenol	127 nA μmol^{-1} l^{-1}	2×10^{-4}		4	Liu et al. (2000)
Tyr/ZnO sol–gel/GCE	Phenol, catechol	168, 166 nA μmol^{-1} l^{-1}	0.05, 0.08		15 catechol	Liu et al. (2005b)
Tyr/Ppy-CPE	Phenol, catechol	3.13×10^3, 4.75×10^3 nA μmol^{-1} l^{-1} cm^{-2}		11 catechol	20 catechol	Mailley et al. (2003)
Tyr/PTS-doped × polypyrrole/GCP	Phenol, catechol, 4-ClPh	17.1, 70.2, 24.3 nA μmol^{-1} l^{-1}	0.8, 1.5, 2.4		75, 40, 75	Rajesh et al. (2004)
Tyr/Fe^{2+} polypyrrole/ITO	Phenol	330 nA μmol^{-1} l^{-1} cm^{-2}	0.7		80s	Rajesh et al. (2005)
Tyr/poly-*N*-(3-aminipropyl) pyrrole/Stainless steel plat	Phenol, catechol	57.6, 71.4 nA μmol^{-1} l^{-1} cm^{-2}	0.9, 0.7		30, 35	Rajesh and Kaneto (2005)
Tyr/n-Au/GCE	Phenol, catechol, gallic acid	82, 107, 0.23 nA μmol^{-1} l^{-1}	0.21, 0.15, 7	140, 120, 440		Sanz et al. (2005)
Tyr/titania sol–gel/GCE	Phenol	103 nA μmol^{-1} l^{-1}	0.1	290	5	Yu et al. (2003)
Tyr/$Fe(CN)_6^{4-}$, chitosan/GCE	Phenol	150×10^3 nA μmol^{-1} l^{-1}	5×10^{-5}		2	Wang et al. (2002)
Tyr/TiO_2 sol–gel/GCE	Phenol	15.78×10^3 nA μmol^{-1} l^{-1} nA μmol^{-1} l^{-1} cm^{-2}	0.01		10	Zhang et al. (2003)
Tyr/Sonogel–Carbon, PEG/Tyr/Sonogel–Carbon, and Nafion/Tyr/Sonogel–Carbon	Catechol	26.9, 12.3, and 82.5 nA μmol^{-1} l^{-1} or 2.6×10^3, 1.2×10^3, and 8×10^3 nA μmol^{-1} l^{-1} cm^{-2}	0.193, 0.930, 0.064	19.88, 97.08, 67.11	20.5, 11, 3.5	This work

GCE: glassy carbon electrode; MPA-SAM: 3-mercaptopropionic acid (MPA) self-assembled monolayer (SAM); AuE: gold electrode; SPCE: screen printed carbon electrode; CFμE: carbon fiber micro electrode; PTS: *para*-toluene sulfonate; n-Au: gold nanoparticles; CPE: carbon paste electrode.

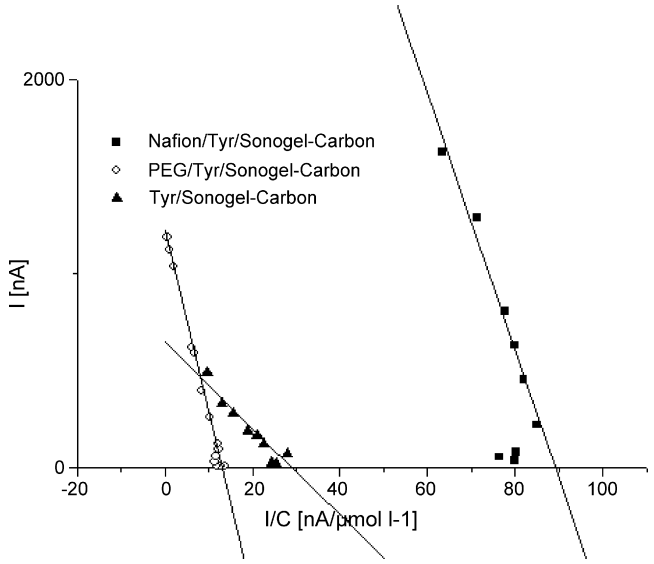

Fig. 3. Eadie–Hofstee plot obtained from catechol calibration curves using the three biosensors at optimum conditions.

I_{max} were accomplished from this later region and the results were summarized in Table 1 too. For the immobilized enzymes, different factors can affect the observed kinetic parameters, such as inter- and intra-diffusion of substrates and products of reaction, substrate steric and conformational effects, immobilization matrix which may causes the enzyme disfiguration, electrode active surface which influences on the conductivity of the electrode area, the amount of the enzyme at the surface, and the amplification of the biosensor response by the recycling process at the surface electrode. In general, comparing K_m^{app} obtained with each one of the three biosensors, it can be seen that this value is low for the Nafion/Tyr/Sonogel–Carbon and lower for Tyr/Sonogel–Carbon, compared with what is observed in the case of the PEG modified tyrosinase biosensor. On the contrary, regarding I_{max} it can be observed that this parameter was higher for the Nafion modification and very lower for the unmodified biosensor. These trends depend on several factors: thus, the maximum velocity I_{max}, which, is equal to $k_{+2}[E_T]$ (k_{+2} is the dissociation constant of the enzyme–substrate complex and $[E_T]$ is the total enzyme concentration), varies widely for a given enzyme concentration as well as with immobilization matrix and with enzyme denaturation or activation. The study of stability proves that a biosensor manufactured via adsorption end-up usually in the loss of a great quantity of enzyme initially adsorbed. Moreover, as we observed in the response time study, the environment offered by Nafion to the immobilized tyrosinase is more convenient for enzyme free mobility than that offered by PEG. These remarks, and surface saturation by the reaction product supposed in the case of the non-protective biosensor, would explain the obtained I_{max} trends comparing the three biosensors for all phenolic compounds. On the other hand, admitting the amplification phenomenon due to the electrochemical regeneration of the enzyme substrate observed in the case of enzyme-based biosensors, which reduces drastically the K_m^{app} parameter compared with that of the free enzyme (Cosnier et al., 1998; Cosnier and Innocent, 1993; Wang and Dong, 2000) and recalling the configuration of our three biosensors, it is clear that this phenomenon of substrate recycling is not accentuated in the three cases. It is logical since, in the first one, the biosensor is manufactured by a simple adsorption of the enzyme and the substrate recycling process occurs at the solution/electrode interface. In the two other cases, when the biosensors were made by sandwich configuration, such phenomenon occurs into the material and the internal mobility factor of substrate and these products must be taken into account. These considerations could explain the trends of K_m^{app} described above.

Furthermore, when comparing K_m^{app} and I_{max} obtained with the individual biosensors for the tested phenolic compounds, it can be seen, as assumed theoretically, that a lower Michaelis–Menten constant is always observed for those compounds with maximum velocity. Table 1 includes also the catalytic efficiency, I_{max}/K_m, which confirms the substrate sensitivity trend. Furthermore, for the same enzyme concentration, this ratio can reflect the specificity for different substrates. It can be observed, comparing the catecholase and phenolase activities, that the hydrophilic modification (PEG) encourages the phenolase activity; contrary to that, the hydrophobic Nafion modification do not alter this two basic activities of the enzyme tyrosinase. The presence of the modifier polymer promotes the specificity of the biosensor towards the chlorinated monophenols and has an inverse effect towards the polyphenol catechine, probably for the reason that the three rings of the (+)-catechin molecule cannot easily penetrate through the first layer to reach the enzyme.

3.6. Application to real samples

For studying the real feasibility of the tyrosinase-based Nafion modified Sonogel–Carbon electrode, the polyphenol and phenol content of four beers and four environmental water samples were estimated by standard addition analysis. In the first case, (+)-catechin was used as standard and two lagers and two black commercial beers were analyzed. In the second case, two Martil river water (located at the north of Morocco) sampled 200 m after and 50 m ahead of an industrial wastew-

Table 3
Application of Nafion/Tyr/Sonogel–Carbon biosensor to real samples

Samples	Total polyphenols catechin (eq. mg l^{-1})	s (eq. mg^{-1})	R.S.D. (%)	No. of exp. points
Beer application				
Lager 1	95.3	16.6	17.4	32
Lager 2	209.6	2.1	1.0	8
Black 1	252.3	9.6	3.8	12
Black 2	294.1	9.6	3.3	8
Samples	Total phenols catechol (eq. µg l^{-1})	s (eq. mg^{-1})	R.S.D. (%)	No. of exp. points
Environmental application				
River 1	146.0	4.9	3.4	8
River 2	525.9	9.6	1.8	8
Tannery 1	544.1	27.5	5.1	8
Tannery 2	124.1	5.7	4.6	12

ater release zone (including a chemical plant). The sampling includes also two tannery's wastewater. All the collected samples were analyzed using catechol as standard. The results, in standards equivalent, are showed in Table 3. As can be seen, the Nafion/Tyr/Sonogel–Carbon biosensor retains it stable and reproducible response in real samples and provides a good protection for the immobilized enzyme against possible inhibitors.

4. Conclusion

The present study has proved the feasibility of developing a Sonogel–Carbon material based biosensor for monitoring phenolic and polyphenolic compounds. The experimental variables studies, the Hill coefficient evaluation, and the stability manifested by the developed biosensors, prove the biocompatibility of the immobilization matrix proposed. The careful choice of the concentration of the modifier polymer can amplify the good analytical performance manifested by these biosensors. Detailed studies of the sensitivity and selectivity demonstrate that the biosensors can respond easily to different phenolic compounds. Preliminary results, for direct application of the Nafion/Tyr/Sonogel–Carbon to real samples, demonstrated a good stability and repeatability of these systems toward beer and wastewater containing phenols and polyphenols.

The satisfactory obtained LOD, sensitivity, and stability, encouraged us to continue our investigations with these systems to search for new applications. At the moment, we are trying to develop a similar biosensor for a total or relative polyphenols index determination, which could be of great help in the brewing industry, as a new analytical tool for beer quality indication since the presence of flavonols is in fact responsible for cloudiness in these alcoholic drinks.

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