J Sol-Gel Sci Technol (2008) 45:157–163
DOI 10.1007/s10971-007-1671-2

ORIGINAL PAPER

Structural characteristics and cyclic voltammetric behaviour of Sonogel-Carbon tyrosinase biosensors. A detailed comparative study of three immobilization matrixes

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Received: 13 July 2007 / Accepted: 10 December 2007 / Published online: 25 December 2007

Abstract Structural characteristics an cyclic voltammetry of three amperommetric biosensors based on immobilization of tyrosinase on a Sonogel-Carbon electrode for detection of phenols are described. Cyclic voltammetry was applied to study the electrochemical behaviour of the electrode and the electrochemical reaction on the electrode surface. Scanning electron microscopy, X-ray energy dispersive spectroscopy and atomic force microscopy were used for the structure characterization of the electrode surface, enzyme film and polymers coatings. The influence of additive-protective polymers, such as polyethylene glycol and perfluorinated-Nafion ion-exchanger on the surface of the biosensor were explored.

Keywords Biosensor · Sonogel-Carbon · Tyrosinase · Catechol · Cyclic voltammetry · Scanning electron microscopy · Atomic force microscopy · X-ray energy dispersive spectroscopy

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1 Introduction

The sol–gel process is a chemical technique that enables to prepare a wide variety of oxide compounds at far lower temperatures than conventional methods. In this field, we proposed a novel sol–gel procedure [1, 2] based on the use of sonocatalysis to yield solid carbon composite electrodes. By means of sonocatalysis, high-energy ultrasounds are applied directly to the precursors, and ultrasonic cavitation is achieved so that hydrolysis with acidic water is promoted in the absence of any additional solvent [3, 4]. Thanks to the phenomenon of ultrasonic cavitation, sol–gel reactions occur in a unique environment, leading to gels with special characteristics. These so-called sonogels are mainly of high density, with a fine texture and homogeneous structure. The mix of sonogel with spectroscopic grade graphite leads to a new type of sol–gel electrode called by us the Sonogel-Carbon electrode. The electrode preparation procedure can be found in [1, 4].

The Sonogel-Carbon electrodes show the general good properties of other CCE's (Ceramic Carbon Electrodes). Besides, in comparison with other carbon electrodes, they exhibit especially favourable electrochemical properties, such as broad operational range of voltage and very low values of observed capacity (C_{obs}). Moreover, the electrodes show direct response for a large group of organic compounds and several metallic ions [2]. These reasons, besides their stability, make them very appropriate to be used as electrochemical sensors.

The sol–gel technology is very used to produce semiconductors composites [5] and optical devices [6, 7]. Currently, in the field of electrochemistry, it is applied to the construction of amperometric sensors and biosensors for the determination of phenolic compounds [8–10]. The choice of a suitable electrode is a key stage in the

development of amperometric biosensors due to the contact and interaction with the immobilized enzyme. A good electrical conductivity and a sufficient sensitivity are also important factors, as well as the chemical and physical inertias against the solution. 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. Nevertheless the applications of sonogels are not very extended yet [11–15].

In the present study, the behaviour of biosensors based on Sonogel-Carbon electrode and the enzyme tyrosinase are studied. Cyclic voltammetry was applied to study the electrochemical characteristics of the electrode and the electrochemical reaction on the electrode surface. Scanning electron microscopy, X-ray energy dispersive spectroscopy and atomic force microscopy were used for the structure characterisation of the electrode surface, enzyme film and polymers coatings. The influence of additive-protective polymers, such as polyethylene glycol and perfluorinated-Nafion ion-exchanger on the surface of the biosensor were explored. We carried out a previous electroanalytical study of these biosensors [14], and some amperometric and kinetic results then attained are now compared with the studies described in this paper.

2 Experimental

2.1 Reagents and materials

Methyltrimethoxysilane (MTMOS) was from Merck and HCl was from Panreac. Graphite powder (spectroscopic grade RBW) was from SGL Carbon. Mushroom tyrosinase (E.C. 1.14.18.1, 3,000 U mg^{-1}) was purchased from Sigma. KH_2PO_4 and K_2HPO_4 for phosphate buffer were from Fluka; boric acid, ortho-phosphoric acid, acetic acid and sodium hydroxide for Britton-Robinson buffer solution were from Merck. Nafion-perfluorinated ion-exchange resin (Cat. No. 27, 470-4) 5% (weight/volume) in a mixture of lower aliphatic alcohols and water was obtained from Aldrich; working solutions were prepared by diluting it in adequate volume of methanol (80%, weight/volume, 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 without further purification. Nanopure water was obtained by passing twice-distilled water through a Milli-Q system (18 MΩ cm). Cathecol was of analytical grade, and purchased from Merck.

Stock solutions of the phenolic compound (0.01 mol L^{-1}) were prepared daily by dissolving the appropriate amount in 10^{-2} mol L^{-1} phosphate buffer at pH 7. Glass capillary tubes, i.d. 1.15 mm, were used as the bodies for the composite electrodes.

2.2 Instrumentation

Cyclic voltammetry (CV) measurements were performed with an Ecochemie Autolab PGSTAT20 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 from Misonix Inc., equipped with a 13 mm titanium tip was used; the microprocessor controller allows precise control of output power, according to sample volume and power setting. The ultrasonic processor was enclosed inside a sound-proof chamber during operation.

All electrochemical experiments were carried out in a cell containing 10 mL of 0.01 mol L^{-1} phosphate buffer at pH 7 and 26 °C; the three-electrodes system consisted of a tyrosinase-modified Sonogel-Carbon, an Ag/AgCl (3 M KCl) and a platinum wire as working, reference and auxiliary electrodes, respectively.

Cyclic voltammograms were recorded in the range from 0.7 to −0.4 V at different scan rates, from 25 to 500 mV s^{-1}. Measurements were carried out under N_2 atmosphere when it was required.

Scanning electron microscopy (SEM) studies were carried out on a QUANTA 200 (FEI Company) operating at 20 keV and equipped with a Microanalyzer (EDAX) to perform X-ray energy dispersive spectroscopy (EDS).

Surface topological studies were performed using an atomic force microscope (AFM, Veeco Nanoscope IIIa) in tapping mode. Phosphorus (n) doped silicon cantilevers, with spring constants in the range 20–80 N m^{-1}, were used. Calibration of the microscope was achieved by imaging calibration gratings supplied by the manufacturer. AFM images were examined for artifacts, and reproducibility was checked in the usual way, i.e. by changing the AFM cantilever and either by moving (during the experiment) the sample in the x or y directions or by varying the scanning angle and frequency.

2.3 Biosensors preparation procedure

To prepare the Sonogel-Carbon, 500 μL of MTMOS and 100 μL of 0.2 M HCl were mixed and then insonated during 5 s with the high-power ultrasonic processor; in this way the mixture is subjected to the phenomenon of ultrasonic cavitation, by which the sol–gel process begins, avoiding the use of alcoholic solvent and reducing drastically the time needed to get an unique phase. To do the

insonation in a right way, the tip of the probe must be just contacting the surface of the mixture (only about 1 mm of length must be introduced). After insonation, 1 g of graphite powder was added and homogeneously dispersed in the sonosol obtained. The whole procedure for the fabrication of unmodified Sonogel-Carbon electrodes has been described previously [1, 4, 13]. Before modification, the electrodes were polished with No. 1200 emery paper 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 0.1 mol L^{-1} phosphate buffer of pH 7.4 giving an enzyme concentration of 57,000 U mL^{-1}. In the first step 5 μL of this solution (285 U) were placed onto the surface of an unmodified Sonogel-Carbon electrode and let it 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 dropped on the enzyme film. So three kinds of biosensors were used: Sonogel-Carbon biosensors coated with Nafion solution at a percentage of 0.5%, Sonogel-Carbon biosensors coated with PEG solution at a percentage of 0.5% (according to the configuration previously optimised), and uncoated Sonogel-Carbon biosensors. The resulting biosensors were stored during at least 8 h to dry in the refrigerator at 4 °C. Moreover, it is worth to mention that all biosensors were washed carefully with deionised water before and after the manipulation and stored immersed in phosphate buffer solution of pH 7 at 4 °C.

3 Results and discussion

3.1 Cyclic voltammetric studies

The current that flows through an amperometric sensor introduced in a solution containing an inert background electrolyte (that provides the necessary electrical conductivity), and an electroactive species have two components: the non-faradaic current and the faradaic current. A non-faradaic process involves the accumulation of charges at the electrode/solution interface; the structure formed in this process is called the electrical double layer: The double layer works as a capacitor. The double layer capacitance depends on the solution composition and also depends on potential applied to the electrode, and it is a measure of the ability of the electrical double layer to store electrical charge as a capacitor. Non-faradaic processes occurring at electrodes cause a flow of non-faradaic currents (also called charging currents or capacitive currents), and account for the charging rate of the electrical double-layer at an electrode–solution interface. This current does not involve any chemical reactions (charge transfer), it only causes accumulation (or removal) of electrical charges on the electrode and in the electrolyte solution near the electrode. Non-faradaic currents are usually non-specific and they are rarely used to provide analytical signals; however, these currents contribute to the background noise and limit the detectability of controlled-potential techniques. Faradaic currents correspond to the electroreduction or electrooxidation of electroactive substances and, accordingly, are associated with electron transfer across the interface; species present in bulk solution may be reduced or oxidized only if they are brought to the electrode surface via a mass transport process (diffusion, migration and convection). In particular, the term diffusion describes faradaic current whose magnitude is controlled by the rate at which an electroactive species diffuses toward an electrode–solution interface. If the rate of the overall process is controlled by the mass transport the process is called reversible [16].

Cyclic voltammograms were recorded for different scan rates from 25 to 500 mV s^{-1}; for scan rates below 25 mV s^{-1} an excessive noise was obtained for the background signal. The measurements were carried with the three types of biosensors. Figure 1 shows the cyclic

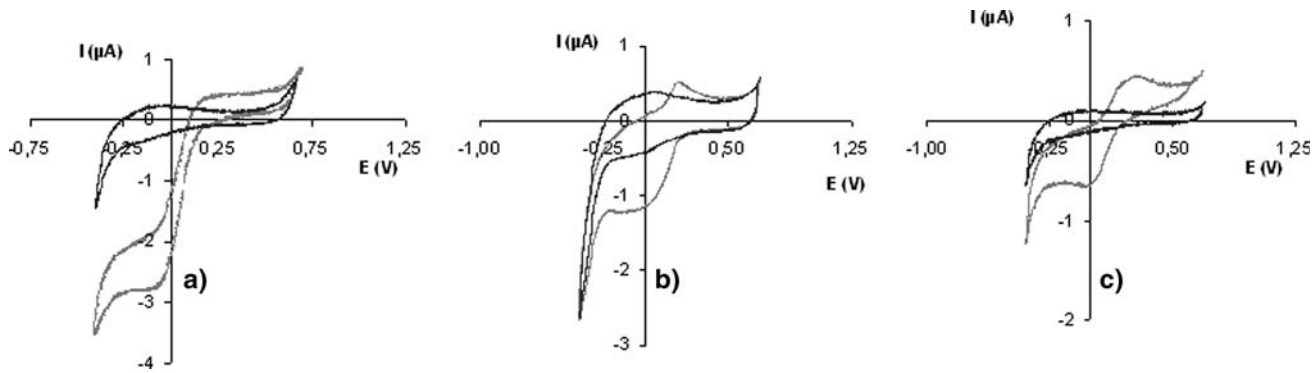

Fig. 1 Cyclic voltammograms for: (**a**) Nafion-coated, (**b**) PEG-coated, and (**c**) uncoated tyrosinase biosensors at a scan rate of 50 mV s^{-1} for the background electrolyte (—, black line) and for a 0.01 mol L^{-1} cathecol concentration (—, grey line)

voltammograms at a scan rate of 50 mV s^{-1} for the background electrolyte and for a 0.01 M cathecol concentration. No electrochemical signal is obtained when the electrolyte support is recorded. However, it is clear the influence of the scan rate; when this parameter increases the residual current increase too, and this reveals a high contribution of charging (non-faradaic) currents to the background current under the conditions used.

To evaluate the behaviour of the biosensors the corresponding values of experimental observed capacity, parameter defined as $C_{obs} = i/v$, where i is the average anodic and cathodic current density and v is the potential scan rate, were obtained. In these calculations, those parts of the voltammograms where faradaic intensities prevailed upon capacitive intensities were not considered [17]; likewise double layer capacity was calculated by making cyclic voltammetry scans at different scan rates, and plotting average values of anodic and cathodic density currents for each scan rate versus them. The result is a line with a slope which is the value of the mentioned parameter for the biosensor electrode under the established conditions [18].

The values of the C_{obs} for the three types of biosensors are shown in Table 1. As observed, for each biosensor the C_{obs} values are very similar in all scan rate range, although the values are different for each type of biosensor according to the sequence PEG-biosensor > Nafion-biosensor > Uncoated-biosensor (the higher values for the PEG-biosensor, the smaller ones for the uncoated-biosensor). As it is well known, when an additional film is placed on the electrode surface, the electrochemical characteristics of the active surface change, and the residual current increase due to the presence of the polymer. The same conclusions can be extracted from the values of the double layer capacity; in the case of the uncoated biosensor the value is in the range for many solid electrodes (100–200 μF cm^{-2}) [19], whereas it is higher for the other two biosensors (174.7, 347.2 and 552.9 μF cm^{-2} for uncoated, Nafion-coated and PEG-coated biosensors, respectively). It must be mentioned that the behaviour of the Nafion-coated is always better than the PEG-coated biosensor, which agrees with our previous results related to electrochemical applications of the two coated-biosensors.

Table 1 Observed capacity (μF cm^{-2}) for the three biosensors at different scan rates

Scan rate (V s^{-1})	Observed capacity (μF cm^{-2})		
	Uncoated-biosensor	Nafion-coated biosensor	PEG-coated biosensor
0.025	153.1	303.2	498.7
0.050	148.8	328.9	545.1
0.100	155.8	344.7	569.8
0.200	165.3	355.3	563.9
0.300	169.8	349.9	551.4
0.400	172.5	352.8	545.2
0.500	172.3	341.2	563.3

The C_{obs} values obtained for an unmodified Sonogel-Carbon electrode are always lower than for the biosensors, due to the composition of the active surface; for a scan rate of 100 mV s^{-1}, we have reported [4] a C_{obs} value of 28.0 μF cm^{-2} for an unmodified Sonogel-Carbon electrode, while values of 155.8, 344.6 and 569.8 μF cm^{-2} are now reported for uncoated, Nafion-coated and PEG-coated biosensors, respectively. It can be mentioned that although these C_{obs} values have been obtained for the same scan rate, the electrolyte support used in this work is not the same than previously. The value of the double layer capacity for an uncoated biosensor is similar than that reported previously. Compared with other sol–gel biosensors, but not tyrosinase biosensors [20], C_{obs} given in the literature for low scan rates and double layer capacity are over 700 μF cm^{-2}, much higher values than those reported in this paper for the Sonogel-Carbon biosensors. This behaviour agrees with our previous study of the Sonogel-Carbon electrodes.

Figure 1 shows the cyclic voltammograms for the three types of Sonogel-Carbon tyrosinase biosensors; in all cases the voltammograms show a background current without detectable signal. After addition of catechol (Fig. 1a), a reduction current with the shape of a catalytic wave is obtained, with a maximum current value at a potential of about −20 mV. The reduction current is due to the reduction of quinone species liberated from the enzymatic reaction catalyzed by the tyrosinase on the enzyme electrode. The appearance of reduction current indicates that tyrosinase has been successfully immobilized on the electrode surface, and retains its biological activity in the film.

In a catalytic system, the rate constant of the enzymatic reaction (k_c) and the potential scan rate (v) establish the shape of the cyclic voltammograms. If k_c/a is high (where $a = nFv/RT$) the reduced specie (cathecol in our case) is continually replenished at the electrode; then no peak is obtained and a limiting or plateau (steady state) current is observed, i.e. the electrochemical response (current in our case) do not change in time. This current is independent of the potential scan rate and a function of k_c, and no peak is obtained in the anodic scan. This is the result obtained for a Nafion-coated Sonogel-Carbon biosensor. When the rate constant of the enzymatic reaction is not so high, an important influence of the diffusion process is observed; the plateau current is reached at lower current values, and peak-shape signals appear in both the cathodic and anodic scans. The uncoated Sonogel-Carbon biosensors show this behaviour (Fig. 1c); for these electrodes, the cathodic peak current is proportional to $v^{1/2}$, as expected for a diffusion-controlled process. It is clear that the Nafion

film improves the enzymatic reaction, and provides an adequate interface to carry out the enzymatic process, without hindering the electrochemical reduction on the electrode surface. For the uncoated biosensors, the interaction between the enzyme deposited on the electrode and the catechol from the solution is more difficult, and thus less favoured the enzymatic reaction; the absence of a polymeric film worsens the retention on the enzyme film of the quinone species liberated from the enzymatic reaction, and consequently worsens too the later electrochemical reduction to catechol on the electrode surface. PEG-coated biosensors (Fig. 1b) show a behaviour in which contribution of both phenomena can be observed; a limited or plateau current is obtained, but with a less value than that found for Nafion-coated biosensor. Furthermore, a peak is obtained at a potential similar than that described for uncoated biosensor, although the peak height for the PEG-coated biosensor is higher. For both types of biosensors (PEG-coated and uncoated) a shift of the peak potentials towards more negative values is observed; this fact is in accordance with a diffusion controlled process. Thus, for a PEG-coated biosensor a contribution of both the catalytic reaction and the diffusion process must be assumed.

The parameters for Michaelis–Menten kinetics were determined from the steady-state currents and the electrochemical version of the Lineweaver–Burk equation [21]:

$$\frac{1}{I} = \frac{1}{I_{max}} + \frac{K_M^{app}}{I_{max}\,[S]}$$

where I is the steady-state current after the addition of analyte, [S] is the concentration of analyte, I_{max} is the maximum rate of the enzymatic reaction (or maximum current measured for a saturated analyte), and K_M^{app} is the apparent Michaelis–Menten constant. With respect to previous results, I_{max} values vary according to the sequence Nafion-coated > PEG-coated > uncoated biosensor, the same that has been found for the contribution of catalytic reaction in cyclic voltammetry studies in presence of cathecol. This fact corroborate that the environment offered by Nafion to the immobilized tyrosinase is more convenient for enzyme free mobility than that offered by PEG, avoiding the problems associated to the surface saturation by the reaction products observed in the case of the non-protective biosensor [14].

3.2 SEM and EDS studies

The structural characterization of the Sonogel-Carbon biosensors was performed; for each sample, the SEM and EDS studies were performed on the same equipment and at the same time, using the instrumental parameters mentioned in Sect. 2. The samples were distributed as follows: Nafion-coated, PEG-coated and uncoated Sonogel-Carbon tyrosinase bionsensors. The two modes in which these samples were distributed were: not used polished electrode and used polished electrode.

Figure 2 shows the SEM micrographs. For the Nafion-coated biosensor a characteristic film of the polymer is observed; the presence of PEG also generates a distinctive film, but in this case it can be observed fissures in the coating. No characteristic film is shown for the uncoated biosensor.

The EDS spectra for the Nafion-coated biosensors show the presence of F, due to the chemical composition of Nafion coating; when these biosensors are used a less amount of F is observed, since a certain amount of the coating is removed from the electrode surface by dissolution into the electrolyte support. No F is found for PEG-coated and uncoated biosensors. Figure 3 shows the EDS spectra for the Nafion-coated (before and after use) and PEG-coated (before use) tyrosinase polished biosensors.

A small amount of Si is found for the biosensors before use; neither the tyrosinase coating nor the Nafion or PEG coatings have Si in their composition, so the presence of Si must be attributed to the silane of the Sonogel material. When these electrodes are used the EDS spectra reach the surface of the Sonogel more easily; so, the amount of Si increases for the used biosensors.

The presence of P and K can be explained in the same terms. P and K are components of the phosphate buffer, and appear for the three biosensors before use; after use, the dissolution of the buffer occurs, and it led to the lost of the components of the phosphate buffer, and P and K do not appear in the EDS spectra of the biosensors after use.

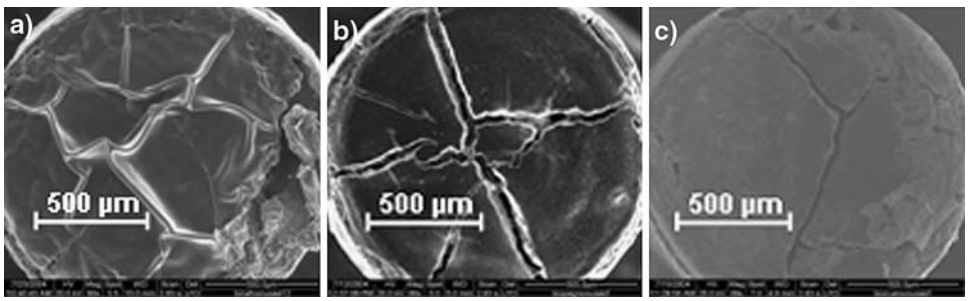

Fig. 2 SEM micrographs for (**a**) Nafion-coated, (**b**) PEG-coated, and (**c**) uncoated tyrosinase polished biosensors

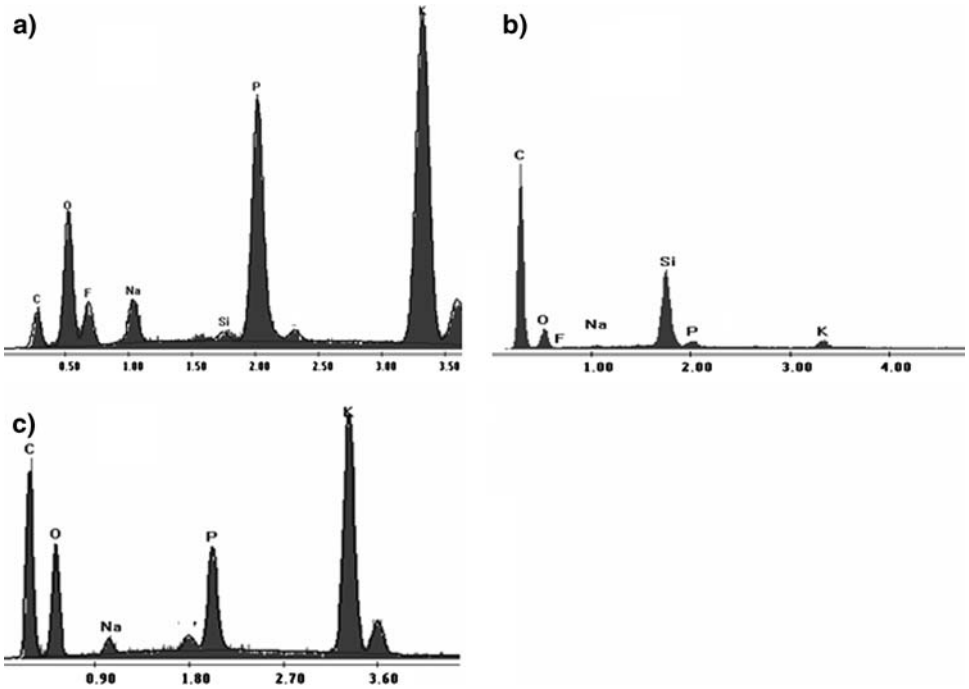

Fig. 3 EDS spectra for Nafion-coated (**a**, **c**), and PEG-coated (**b**) tyrosinase polished biosensors before (**a**, **b**) and after (**c**) use

Although a certain removal of the coatings can be assumed, the activity of the enzyme remains, and the presence of the polymer coating increases the biosensor stability. For the Nafion modified biosensor a lifetime of 38 days was observed, and a reproducible value, with a relative standard deviation, RSD, of 4.3% from 6th to 16th day, was obtained; for PEG coated biosensor the relative stable response (RSD 9.0%) was from 5th to 10th day with a lifetime of 22 days. However, for the uncoated biosensor a short lifetime (10 days) and a non-significant plateau response was observed [13]. It must be taken into account the biocompatibility of the Sonogel-Carbon electrode with the enzyme tyrosinase, probably because of the inorganic–organic structure of the Sonogel. With respect to the higher stability of the coated biosensors, the presence of the polymers favours the entrapment of the enzyme on the Sonogel-Carbon electrode surface, and the enzyme activity allows the use of the coated biosensor during a long lifetime, due to the dissolution of the buffer and/or the partial dissolution of the coating do not imply the lost of enzyme, and it remains on the electrode surface thanks to the biocompatibility with the Sonogel-Carbon material.

3.3 AFM studies

Tapping mode AFM measurements were performed over different regions of the samples to check for sample surface homogeneity. All AFM images selected to be shown here are representative of the samples surface topology. For comparison, the scanned area is always 2×2 μm^2.

Tapping mode AFM was used to evaluate the structure of the Nafion-coated, PEG-coated and uncoated tyrosinase biosensors; Figure 4 shows the AFM images for the three biosensors. Table 2 shows the roughness (R_a) values for each image. This parameter is calculated by the AFM analysis software as the arithmetic average of the absolute values of the surface height deviations measured from the mean plane within the scanned area:

$$R_a = \frac{1}{n}\sum_{j=1}^{n} |z_j|$$

The table also shows the percentage of Surface Area Difference (S.A.D.) between the three-dimensional area of the image and its projected two-dimensional area, expressed as:

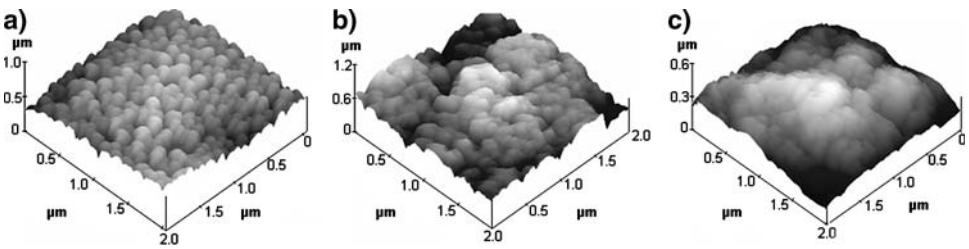

Fig. 4 AFM images for: (**a**) Nafion-coated, (**b**) PEG-coated, and (**c**) uncoated tyrosinase biosensors. For (**a**) x and y axes 0.5 μm/div; z axis 0.5 μm/div; for (**b**), x and y axes 0.5 μm/div; z axis 0.6 μm/div; for (**c**), x and y axes 0.5 μm/div; z axis 0.3 μm/div

$$S.A.D. = \left[\frac{\sum(\text{surfacearea})_i}{\sum(\text{projected area})_i} - 1\right] \times 100$$

In Table 2 R_a and S.A.D. data for Sonogel and Sonogel-Carbon materials are included. According to these values, it can be observed an important increase in the roughness and a considerable gain in the surface area difference when the Sonogel-Carbon material is bio-modificated; the incorporation of both a tyrosinase film and a polymer-tyrosinase film led to an increase of the studied parameters. It must be mentioned that the Sonogel and Sonogel-Carbon materials are polished before use, whereas the biosensors are not polished in order to avoid the loss of biomaterial.

The incorporation of the polymer coating causes an increase of about 30 and 50% in R_a values (for PEG and Nafion, respectively) with respect to that obtained for an uncoated tyrosinase biosensor. Although Nafion coated biosensor exhibits the highest values for R_a and S.A.D., the AFM images (Fig. 4) show great differences between this biosensor and the other ones. When Nafion is deposited on the surface of the Sonogel-Carbon electrode, a porous structure is observed and a distinctive granular aspect is generated; this film shows smoothness of its structure, and particle sizes of about 0.1 μm. PEG coated biosensor shows a different porosity, with particle sizes between 0.1 and 0.4 μm. No granular aspect is observed for uncoated biosensor.

4 Conclusions

The Sonogel-Carbon material shows adequate characteristics for the fabrication of tyrosinase biosensors. The cyclic voltammograms for the Sonogel-Carbon tyrosinase biosensors in presence of catechol show a reduction current with the shape of catalytic wave, and it indicates that tyrosinase has been successfully immobilized on the electrode surface, and retains its biological activity in the film. C_{obs} values follow the sequence PEG-biosensor > Nafion-biosensor > Uncoated-biosensor (the higher values for the PEG-biosensor, the smaller ones for the uncoated-biosensor), and this fact can be related to the modification of the biosensor surface.

In the SEM micrographs it can be observed a distinctive film of the polymers, Nafion or PEG. The EDS spectra show the presence of the components of the Nafion coating (F) and buffer solution (P, K), as well as this one of the silane (Si). The biocompatibility with the Sonogel-Carbon material and the presence of the polymers favours the entrapment of the enzyme on the Sonogel-Carbon electrode surface. The AFM studies reveal a porous structure with a distinctive granular aspect for Nafion and PEG coated biosensors, with smaller particle size for the first one.

Table 2 Roughness and Surface Area Difference values for the three biosensors, and the sonogel and Sonogel-Carbon materials

	Roughness (R_a, nm)	Surface Area Difference (S.A.D., %)
Nafion-coated biosensor	38.3	119.1
PEG-coated biosensor	25.2	103.1
Uncoated biosensor	19.7	94.9
Sonogel material	0.7	1.6
Sonogel-Carbon material	1.4	2.0

Acknowledgements The authors are grateful to FEDER (Fondo Europeo de Desarrollo Económico y Regional) and the Ministerio de Educación y Ciencia of Spain (Project CTQ2004-03708/BQU) and to the Junta de Andalucía for financial support, and Agencia Española de Cooperación Internacional (AECI) through a grant to Mohammed ElKaoutit.

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