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A third-generation hydrogen peroxide biosensor based on Horseradish Peroxidase (HRP) enzyme immobilized in a Nafion–Sonogel–Carbon composite

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ABSTRACT

A third-generation biosensor based on HRP and a Sonogel–Carbon electrode has been fabricated with the aim of monitoring hydrogen peroxide in aqueous media via a direct electron transfer process. The redox activity of native HRP, typical of thin-layer electrochemistry, was observed. The charge coefficient transfer, α, and the heterogeneous electron transfer rate constant, k_s, were calculated to be 0.51 ± 0.04 and $1.29 \pm 0.04\ s^{-1}$, respectively. Topographic study by atomic force microscopy (AFM) shows that the enzyme may have been introduced inside the ionic cluster of the Nafion. The immobilized HRP exhibited excellent electrocatalytical response to the reduction of H_2O_2 and preserved its native state after the immobilization stage. Several important experimental variables were optimized. The resulting biosensor showed a linear response to H_2O_2 over a concentration range from 4 to 100 μM, with a sensitivity of 12.8 nA/μM cm^{-2} and a detection limit of 1.6 μM, calculated as (3 S.D./sensitivity). The apparent Michaelis–Menten constant K_m^{app} was calculated to be 0.295 ± 0.020 mM. The biosensor showed high sensitivity as well as good stability and reproducibility. The performance of the biosensor was evaluated with respect to four possible interferences.

1. Introduction

The study of direct electron transfer (DET) between metalloproteins and metal surfaces is not only one of the most interesting subjects in the field of biosensors but also can make a major contribution to improving the competitiveness and performance of a significant group of biosensors categorized as "third-generation" [1–5]. Horseradish peroxidase (HRP) is possibly the typical model for these studies, as it relies on heme activity, predominant in nature [4,5]. HRP utilizes hydrogen peroxide to oxidize a wide variety of organic and inorganic one-electron donor compounds, such as aromatic phenols, phenolic acids, indoles, amines, and sulfonates [6]. The bioelectrocatalytic cycle involves two essential steps: the first is the oxidation of native HRP by H_2O_2 to the intermediate compound I involving a rapid two-electron exchange process. The second step consists of two successive one-electron reductions, generating the native enzyme from the compound I with the intermediate formation of the compound II, in which each electron is supplied by one molecule of the substrate [5,6]. In the absence of a substrate donor, and for the specific case of a peroxidase appropriately immobilized on an electrode, the two electrons may be delivered by the electrode, regenerating the native enzyme without any mediator. Thus, by means of the particular phenomenon of direct electron transfer, as defined in the case of HRP enzyme, peroxide presence can be detected in this way with an extremely high selectivity, and the electrode can be thus considered as a substrate. Carbonous materials have demonstrated their capacity for acting as electron donors in the construction of third-generation HRP-based biosensors [7–14]. Our group has worked on the development of a new ceramic composite electrode based on the combination of sono-catalysis methodology and the use of carbon grains to produce a new kind of sol–gel material [15–17]. The use of this electrode as a support for oxido-reductase enzymes has been shown to be satisfactorily competitive with other biosensors [18]. In this paper, we continue the series of reports on the performance of our electrode in respect of one of the most fascinating questions in the biosensor field, the DET mechanism. HRP has been used as a model protein to build the unmediated third-generation peroxide biosensor. Having constructed it simply by doping our electrode with the enzyme and Nafion surfactant, we report here its optimization regarding pH and potential, its physico-chemical characterization, and the study of possible interferences.

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2. Experimental

2.1. Reagents

Methyltrimethoxysilane (MTMOS) was obtained from Merck (Darmstadt, Germany) and HCl was from Panreac (Barcelona, Spain). Graphite powder (spectroscopic grade RBW) was from SGL Carbon (Ringsdorff, Germany). Horseradish peroxidase (E.C. 1.11.1.7, 269 U mg^{-1}) was purchased from Sigma (Steinheim, Germany). KH_2PO_4/K_2HPO_4 and acetic acid/sodium acetate for phosphate or acetate buffer were from Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany) respectively. Nafion-perfluorinated ion-exchange resin (Cat. No. 27, 47-4) 5% (w/v) in a mixture of lower aliphatic alcohols and water, and glutaricdialdehyde, 25 wt% solution in water were obtained from Aldrich (Steinheim, Germany). Peroxide 30% H_2O_2 Perhydrol was from Merck (Darmstadt, Germany). Nanopure water was obtained by passing twice-distilled water through a Milli-Q system (18 MΩ cm, Millipore, Bedford, MA). All interfering compounds tested in this work (dopamine (Dopa), − (−) epinephrine (Epi), uric acid (UA), and ascorbic acid (AA)) were of analytical grade, used as received, and purchased from Merck or Sigma. Stock solutions of these compounds (0.1 mol L^{-1}) were prepared daily by dissolving the appropriate amount either in 0.05 mol L^{-1} buffer solution, ethanol or acetic acid, depending on the solubility of the compound.

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

2.2. Apparatus

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

UV–vis spectra were recorded with a spectrophotometer (Jasco V-550, from Japan), using the Jasco 32 software.

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 sound-proof chamber during operation.

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 of 20–80 N m^{-1}, were used. The microscope was calibrated by imaging the calibration grids 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 by either moving (during the experiment) the sample in the *x* or *y* directions or by varying the scanning angle and frequency.

2.3. Methods

2.3.1. Electrochemical transducer preparation

The unmodified Sonogel–Carbon electrode was prepared as described previously [15,16]. Before modification, the electrodes were polished with emery paper no. 1200 to remove excess composite material, gently wiped with weighing paper and electrochemically pre-treated by dipping them in 0.05 mol L^{-1} sulphuric acid. The three-electrode cell was polarized by voltage cycling from −0.5 to 1.5 V for five cycles. Electrodes with analogous current background were selected, washed carefully with Milli-Q water and left to dry at ambient temperature after their biological modification.

2.3.2. Biosensors fabrication

The enzyme peroxidase was used as a biological sensing element. A quantity of 2.54 mg of the enzyme HRP was dissolved in 30 μL of 0.2 mol L^{-1} pH 7 phosphate buffer solution. A volume of 1.25 μL of glutaricdialdehyde was added to this enzymatic solution, set to polymerize in an ultrasonic bath for 3 min, and modified by adding 3.5 μL of 5% Nafion solution. From the resulting solution, appropriate quantities were deposited on top of the previously prepared Sonogel–Carbon electrodes with a μ-syringe and allowed to dry under ambient conditions. The resulting biosensor had 54 units of enzyme, approximately 0.9% of glutaricdialdehyde and 0.5% of Nafion. Before its use, the enzyme electrode was dipped in a stirred buffer solution for 15 min to eliminate the excess of non-absorbed enzyme, rinsed with the same buffered solution and stored immersed in buffer at 4 °C when not in use.

2.4. Measurements

2.4.1. Electrochemical

Electrochemical experiments were carried out in a cell containing 25 mL of an aerated 0.05 mol L^{-1} adequate buffer and pH, at 22 ± 2 °C. The three-electrode system consisted of an enzyme-modified Sonogel–Carbon electrode as working electrode, an Ag/AgCl (3 M KCl) and a platinum wire as reference and auxiliary electrodes, respectively. For amperometric measurements, a selected potential was applied to the working electrode and the background current was recorded until the steady state was reached. The respective quantities of H_2O_2 were added to the cell and the corresponding current–time curves were recorded. The biosensor response was measured as the difference between the total and the background currents. A magnetic stirrer and stirring bar were used to provide continuous convective transport.

2.4.2. Optical

A quantity of 5.1 mg of HRP was dissolved in 60 μL of phosphate buffer solution at pH 7. From this solution, 30 μL was diluted in 10 mL of the same buffered solution. This is taken as the sample for the spectrum of the native enzyme. With the remaining solution, the same crosslinkage procedure was applied, as described in Section 2.3.2, and its result was diluted in 10 mL of the buffer solution, and taken as a reference of the enzyme state in our immobilization matrix. The blank used in all cases was a simple buffer solution. It is important to note that this methodology was carried out assuming that the enzyme cannot be regenerated by simple dilution if denaturation takes place in the crosslinking step, and if that occurs the same phenomena can be noted in the case of the immobilized enzyme, so the biosensor is maintained immersed in the solution for its entire life-time.

2.5. Morphology characterization

Tapping mode AFM measurements were performed over different regions of all samples to check for sample surface homogeneity. All AFM images selected to be shown here are representative of the surface topology of samples. For comparison, the scanned area is always 500 nm × 500 nm.

3. Results and discussion

3.1. Characterization of biosensor surface morphology

As shown in Fig. 1, tapping mode AFM was used to evaluate the structure of the silica-based Sonogel material (a), the composite Sonogel–Carbon electrode (b), and the modification with Nafion alone (c) or with the mixture of Nafion and HRP enzyme

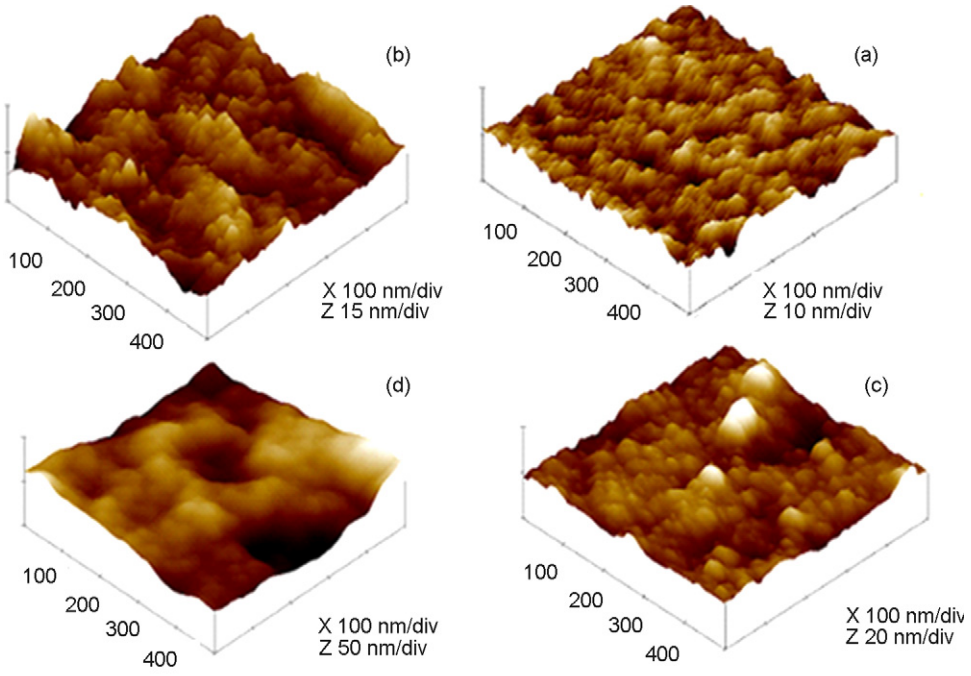

Image	a	b	c	d
Ra nm	0.68	1.44	1.35	6.22

Fig. 1. AFM images of (a) Sonogel material, (b) Sonogel–Carbon composite, (c) Sonogel–Carbon coated by 0.5% Nafion, and (d) HRP–Nafion–Sonogel–Carbon biosensor. The table shows the corresponding roughness values.

(d). The table in Fig. 1 shows the roughness (Ra) values for each of the images. 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. As seen in Fig. 1(a), the film of the silica Sonogel was dense, with pore sizes around 10 nm. Hence, the pore sizes were smaller than the size of carbon particles; this result implies that, during the formation of the Sonogel–Carbon composite, the graphite particles do not get inside the silica pores; instead, the gel probably forms around the graphite particles, and the conductivity of the final composite formed is promoted by a mechanism of percolation between these particles. When Nafion is deposited on the surface of the Sonogel–Carbon electrode, a slight decrease in the roughness can be observed, the same porous structure is conserved and a new granular aspect for the composite is generated. Bio-modification of the composite Sonogel–Carbon by a mixture of enzyme and Nafion results in a significant increase in the roughness, as well as a considerable gain in the surface area from 1.42% to 2.3–2.84%, while preserving the granular aspect due to Nafion, as shown in Fig. 1(c)–(d). In addition, the AFM phase detection technique, applied to this sample, does not show any significant difference over the entire surface, therefore it must be homogeneous in composition. These results lead us to think that the enzyme may be introduced inside the ionic cluster region of the Nafion (for more information, see the schematic presentation in [19]).

3.2. Absorbance measurements

Optical measurements were made to determine the effect of the immobilization step on the structure of the HRP. Heme absorption provides a very useful conformational test for the study of heme proteins. The spectrum of native HRP was compared with that of the enzyme crosslinked by glutaricdialdehyde, treated by ultrasound in a bath for 3 min, and modified with Nafion ion exchange. As shown in Fig. 2, the spectrum of the native enzyme is characterized by an asymmetric Soret band at 403 nm, and a shoulder at approximately 376 nm; at the higher wavelength range, two weak absorption bands (charge-transfer) at approximately 500 and 640 nm were observed. However, the spectrum of the enzyme after crosslinkage shows the same asymmetric band at 403 nm and the same two bands with unchanged λ_{max} at the higher wavelength range. Denaturation of the heme-enzyme was previously characterized by an increase of the intensity and the symmetry as well as the narrowing of the Soret band and by the blue-shifts of the two bands registered at the higher wavelength range [20–22]. In our case, the intensity of the Soret band increases by only 5.5%

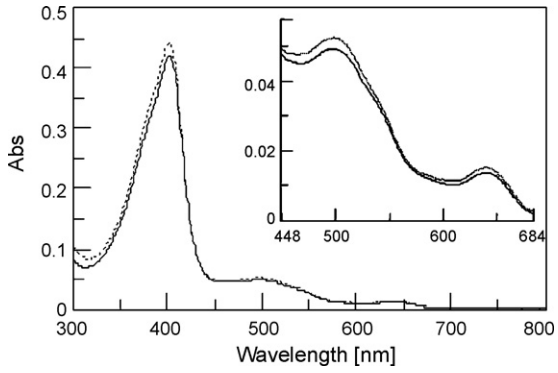

Fig. 2. UV–vis spectra of 0.25 mg mL^{-1} HRP in phosphate buffer solution (solid line) and the same quantity of the enzyme crosslinked and sonicated before its solubilization (discontinuous line).

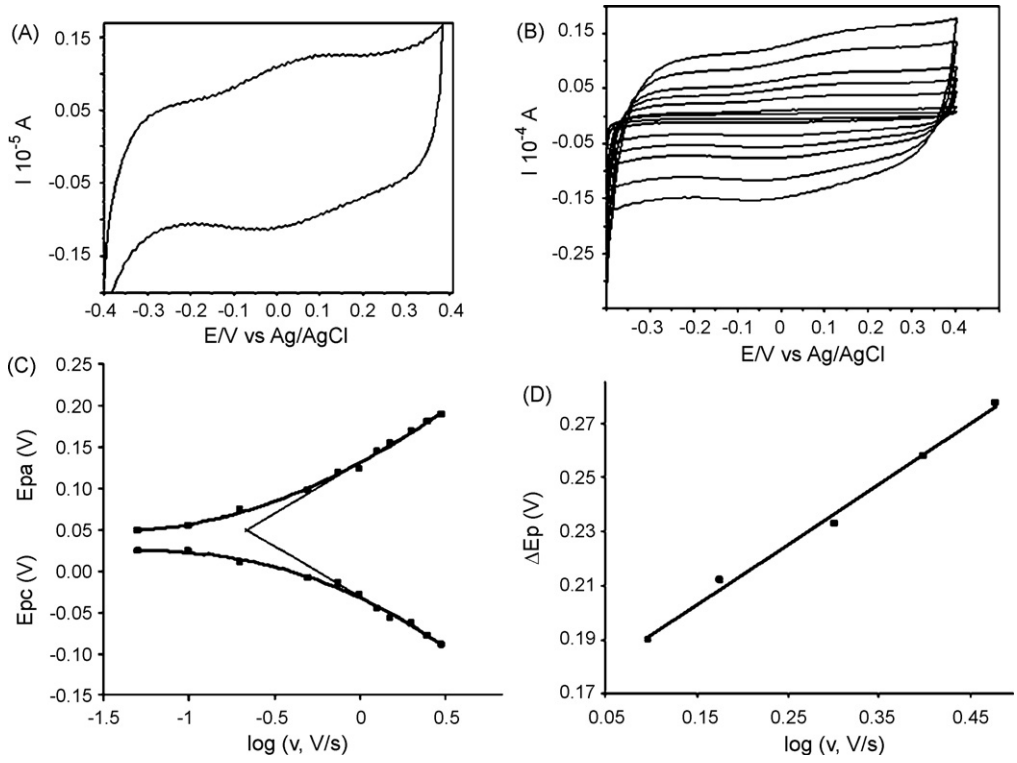

Fig. 3. (A) CV of native HRP–Nafion–Sonogel–Carbon biosensor at 0.2 V/s in PSB (0.05 mol L^{-1} in both KCl and pH 7 phosphate buffer); (B) effect of scan rate from inner to outer, at 0.1, 0.2, 0.5, 0.75, 1, 1.5, and 2 V/s; (C) plots of anodic and cathodic peaks potential vs. logarithm of the scan rate; (D) plot of ΔE_p vs. log(υ).

compared to that of the native HRP, and no shift was observed for all characteristic bands in both spectra. These results suggest that there exists a slight variation of structure in the vicinity of the heme group, and no significant denaturation occurs after its crosslinkage and sonification.

3.3. Cyclic voltammetry of native HRP

The cyclic voltammogram of the HRP-Nafion modified Sonogel–Carbon electrode showed a couple of anodic/cathodic redox peaks in 74 and 10 mV at scan rate of 200 mV/s (Fig. 3A). The anodic and cathodic peaks are of similar magnitude, with i_{pa}/i_{pc} ratio around unity and potential separation ΔE_p around 64 mV, indicating that the redox process is almost reversible. These peaks result from the redox process of native HRP, and could be attributed to the Fe^{2+}/Fe^{3+} couple in HRP. The formal potential E'^0 (0.24 ± 0.01 V/NHE) is close to that estimated for HRP embedded within a Nafion cysteine matrix [19,23], and that for other heme enzymes adsorbed on Nafion film [24]. It is about 0.2 V smaller than the mediated HRP immobilized in gold colloids/cysteine/Nafion [25], and it is more positive than that reported for HRP in solution determined by potentiometry (about −0.08 V/NHE) [26]. This result can be explained by the acidic microenvironment produced due to the highest negatively charged groups located within the micellar structure of Nafion especially when the enzyme is embedded in it, i.e., the same formal potential value was shown for HRP immobilized on a negatively charged matrix [27,28].

Fig. 3B illustrates the cyclic voltammetry of the same biosensor at different scan rates from 0.1 to 3 V/s. It was found that the peak currents increase as scan rate gets higher. In the range of scan rates from 0.1 to 1.25 V/s, the anodic and cathodic peaks show a linear increase in current intensity with scan rate (data not shown here) indicating a surface-controlled electrode process. The average of the covered surface can be calculated from Faraday's law, as follows: $Q = nFA\Gamma_m$; where Q is the integrated peak value, A is the surface electrode (0.0103 cm^2), and n is number of the transferred electrons (assumed to be equal to 1). From the area of the anodic peaks, the total amount of charge passing through the electrode has been calculated as 0.215 ± 0.034 μC and a value of $\Gamma_m = 2.15 \times 10^{-10}$ mol cm^{-2} of HRP immobilized on the Sonogel–Carbon surface, has also been derived. This value, slightly smaller than that reported for HRP adsorbed on carbon nanotube electrodes [7] and higher than that reported for HRP immobilized in a mixture of Nafion and active carbon powder [8], suggests that the Nafion–Sonogel–Carbon matrix acts as a sufficient promoter to achieve maximum enzyme activity. In order to obtain the kinetic parameters, Laviron's model for a surface-controlled electrochemical system was applied. It was found that, up to a scan rate of 1 V/s, ΔE_p was near 200 mV (Fig. 3C); thus the transfer coefficient α can be calculated from the slopes of the anodic and cathodic process, and the result is 0.51 ± 0.04. As illustrated in Fig. 3D, the variation of ΔE_p vs. log(υ) is linear, and from this fact, the heterogeneous electron transfer rate constant can be calculated as 1.29 ± 0.04 s^{-1}, which is of the same order of magnitude as that reported in the literature [7,8,11,19].

The bioelectrochemical catalytic reduction of peroxide by HRP immobilized on Nafion–Sonogel–Carbon was tested firstly by cyclic voltammetry, as shown in Fig. 4. When H_2O_2 0.5 mM was present in pH 7 buffer solution, its reduction was performed on the enzyme electrode, and an enhancement in the reduction current was observed. Non-diffusional current was observed and the wave has a typical form of electrocatalytic current. Similar pure bioelectrocatalytic current was registered for several heme enzyme-based biosensors in presence of peroxide [29–33] and this current has been attributed to the reduction of heme–oxygen complex as oxidized forms of the native enzyme because the applied potential is much more positive than the redox potential for ferric/ferrous transition. The more widely reported mechanism of the electrocat-

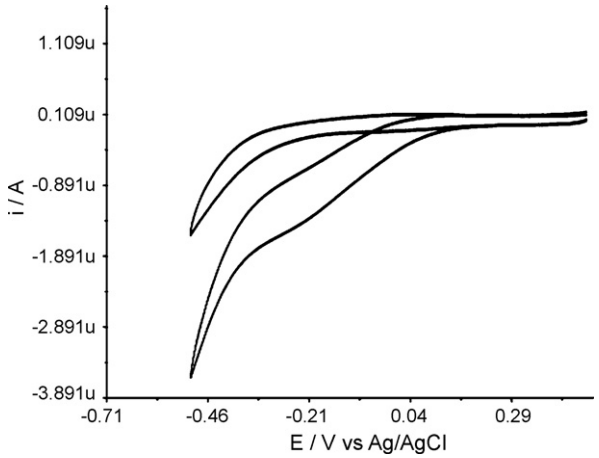

Fig. 4. CV of the HRP–Nafion–Sonogel–Carbon biosensor in the absence and presence of 0.5 mM H_2O_2, scan rate 50 mV s^{-1}; other parameters are the same as in Fig. 3.

alytic reduction of peroxide by HRP has been expressed as follows [34,35]:

$$HRP + H_2O_2 \rightarrow \text{compoundI}[(Fe^{IV}{=}O)P^{\bullet+}] + H_2O$$

$$\text{compoundI}[(Fe^{IV}{=}O)P^{\bullet+}] + 2e^- + 2H^+ \rightarrow HRP + H_2O$$

In which the second reaction occurs through the intermediate formation of compound II.

However this simple catalytic cycle did not give a logical explanation to the fact that the current is exponentially increased by the applied potential and ignores the possible further reduction of HRP[Fe^{III}] to HRP[Fe^{II}] when the cathodic scan passes the formal potential of this reaction. Other mechanism based on the fact that peroxide can acts as oxidant and reductant, especially for high concentration of H_2O_2 [36,37], introduces the step of one electron reduction of HRP[Fe^{III}] to HRP[Fe^{II}] at electrode and involves the molecular oxygen in the catalytic reduction of peroxide as follows [38–40]:

$$HRP[Fe^{III}] + H_2O_2 \leftrightarrow \text{CompoundI}[(Fe^{IV}{=}O)P^{\bullet+}] + H_2O$$

$$\text{compoundI}[(Fe^{IV}{=}O)P^{\bullet+}] + H_2O_2 \leftrightarrow HRP[Fe^{III}] + O_2$$

$$HRP[Fe^{III}] + e^- \leftrightarrow HRP[Fe^{II}]$$

$$HRP[Fe^{II}] + O_2 \leftrightarrow HRP[Fe^{II}{-}O_2]\,(\text{fast})$$

$$HRP[Fe^{II}{-}O_2] + 2e^- + 2H^+ \leftrightarrow HRP[Fe^{II}] + H_2O_2$$

Our results, especially the absence of any diffusional current, could be explained according this mechanism by the recycling of H_2O_2 at the intimate electrode surface. A demonstration of the dependence of this mechanism on oxygen presence is not possible in our case because, as it will be reported later, no difference has been founded in the biosensor response with and without oxygen. This result is in accordance with that reported by Wang and Lu [41] in his study on the effect of Nafion in the response of GOX-based biosensor acting under oxygen-deficit conditions.

3.4. Effect of potential and solution pH on peroxide determination

The effect of potential on the response of the HRP–Nafion–Sonogel–Carbon biosensor to H_2O_2 is shown in Fig. 5A. Steady-state current, related to the reduction of compound I and proportional to H_2O_2 concentration, was previously recorded at 0.00 mV. The slope of the calibration curve increases considerably when the applied potential decreases from 0.00 to −250 mV, which can be attributed to the increasing activation energy for the fast reduction of compound I at low potential [31]. The sensitivity approaches a maximum value at −250 mV. Thus, the value of −250 mV was selected as the working potential. This value is less than that previously reported [19,42,43]. The risk derived from the choice of working potential for this type of biosensor is the interference from oxygen. However, the calibration curve of H_2O_2 in air-saturated buffer has the same slope as that obtained in nitrogen-saturated buffer in accordance with a previously cited report [41]. Moreover, the chosen potential can minimize possible interferences.

The effect of the pH of the medium on the biosensor sensitivity to H_2O_2 in buffer solution is shown in Fig. 5B. The current value and sensitivity reach their maximum values at a pH value close to 7. The same result was observed for soluble HRP [26], indicating that the Nafion–gluta–Sonogel–Carbon matrix did not alter the optimum pH for oxido-reduction activity of the immobilized HRP.

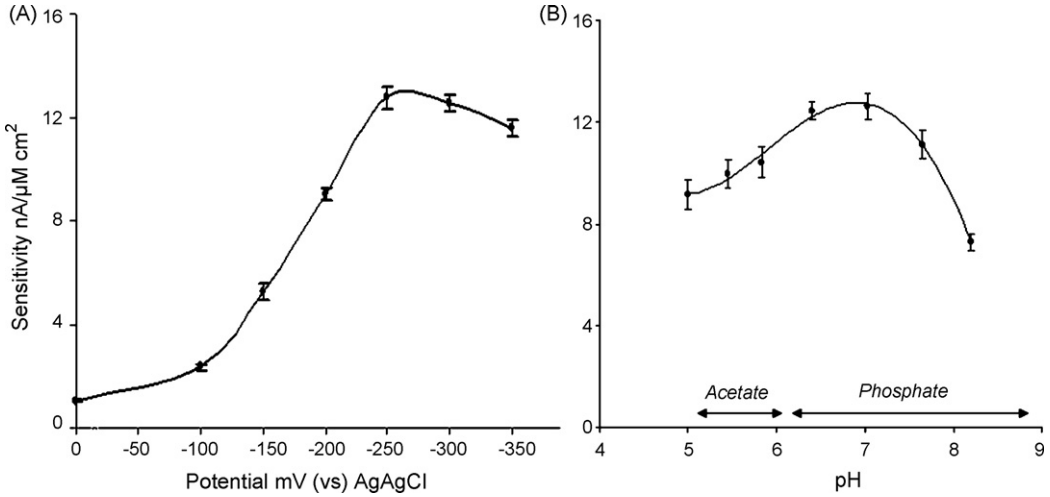

Fig. 5. Effect of potential (A) and pH (B) on the response of Nafion–HRP–Sonogel–Carbon biosensor. The sensitivities are deduced from steady-state current recorded at different potentials (A) and at −250 mV (B), in 0.05 mol L^{-1} PSB.

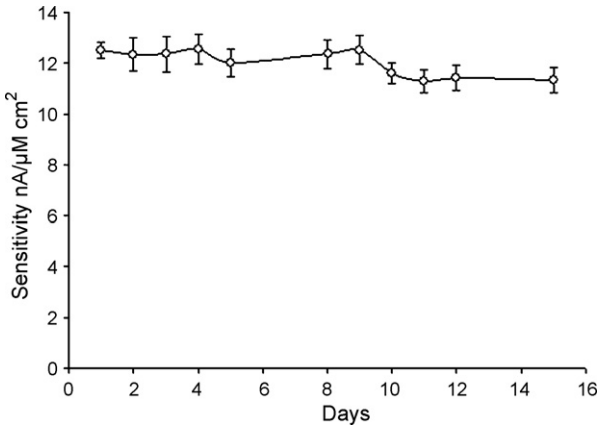

Fig. 6. Life-time profile of Nafion–HRP–Sonogel–Carbon peroxide biosensor.

3.5. Stability and reproducibility of biosensor

The stability of the biosensor response constitutes a critical factor in the fabrication of biosensors. Many different criteria were considered in this respect. The repeatability of HRP-based biosensors was calculated by nine repetitive calibration curves of H_2O_2 recorded on the same day and using the same electrode in the optimum working conditions previously established. A relative standard deviation of 5.3% was obtained. The useful working life of the biosensors was checked by performing repetitive measures every day under the same working conditions, using the same biosensor and constructing two calibrations curves of H_2O_2; when the biosensor was not in use it was stored immersed in buffered solution at 4 °C. The profile of biosensor life-time evolution is summarized in Fig. 6. The biosensor retains 91% of its initial sensitivity after 15 days of operation, and maintains its reproducible response with a 95% confidence interval for the first week. Finally, the reproducibility of the method of enzyme immobilization was also evaluated by comparing the first-day response of five HRP-based Sonogel–Carbon biosensors. A R.S.D. of 10.5% was obtained for the slope of the calibration curves constructed in the same conditions.

3.6. Chronoamperometry response, calibration curve, and kinetic analysis

Fig. 7A shows an example of the chronoamperometric response of the biosensor to different concentrations of hydrogen peroxide. Additions of H_2O_2 to the solution resulted in an apparent increase in the reduction current. The enzyme electrode reached 95% of steady-state current within 35 s, which indicates a fast diffusion of the hydrogen peroxide from the bulk solution to the enzyme active sites and an easy mobility of the enzyme trapped in the Nafion matrix. Fig. 7B also illustrates calibration curve of peroxide obtained from the steady-state current amperomograms. The sensitivity of the biosensor to H_2O_2 is 12.8 nA/μM cm^{-2}, and the linear range spans an H_2O_2 concentration from 4 μM to 100 μM with a correlation coefficient of 0.9997 ($n = 7$). The sensor has a detection limit of 1.6×10^{-6} M calculated as (3 S.D./sensitivity), where S.D. is the blank deviation and 3 is a commonly accepted statistical parameter [44]. The use of the Eadie–Hofstee transformation from the Michaelis–Menten equation is quite efficient in the kinetic analysis of immobilized enzymes [18]. For amperometric biosensors the reaction rates are substituted with steady-state currents, and the algebraic Eadie–Hofstee transformation of Michaelis–Menten equation can be expressed as follows: $I = I_{max} K_m^{app}(I/C)$. Here I is the steady-state current, C the concentration of substrate, K_m^{app} the apparent Michaelis–Menten constant, and I_{max} is the intercept on the current axis. K_m^{app} was calculated to be 0.295 ± 0.020 mM. The K_m^{app} parameter is independent of the enzyme concentration and it is well known that the lower K_m^{app} values are reported for highly sensitive amperometric biosensors, as a consequence of the substrate recycling phenomenon [45,46]. Therefore it was appropriate to perform a comparative study of this parameter with that reported in literature. We found that the K_m^{app} values obtained with the proposed biosensor are lower than that reported for HRP immobilized in a pure sol–gel matrix [47], or for sol–gel functionalized by Nafion-methylene green [42], as well as for the case of HRP immobilized in different matrices [9,12,48]; these findings indicate that the present electrode and immobilization matrix exhibit a good affinity for direct electron transfer between the compound I and the carbon composite.

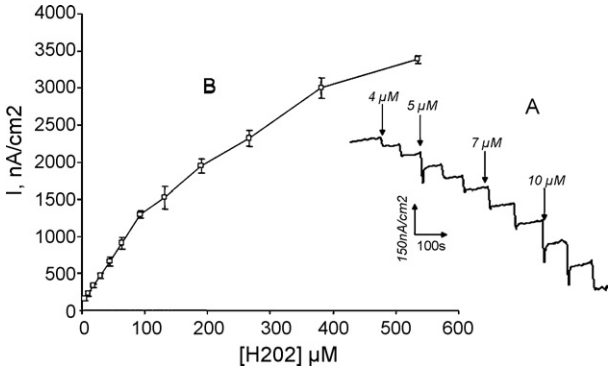

Fig. 7. Typical current–time response and calibration curves of H_2O_2 recorded with the Nafion–HRP–Sonogel–Carbon biosensor at optimum conditions (pH 7 PSB and −250 mV).

3.7. Effect of interferences

Studies of amperometric-based HRP biosensors concern not only the sensing of hydrogen peroxide and small organic peroxides but also the combination with other hydrogen peroxide producing enzymes, e.g. glucose oxidase. The experimental work for measuring the effect of possible interfering substances on the current of a HRP-modified electrode was carried out by comparison of sensitivities before and after the construction of calibration curves in the presence of interferents. Four such substances were used to evaluate the selectivity of the electrode: ascorbic acid, uric acid, dopamine, and epinephrine were tested at the 0.4 mM level. This concentration is equal to or higher than the concentrations that are expected to be found in human blood and urine [49]. Table 1 lists the variation of the sensitivity in the presence of these interfering substrates. Dopamine and epinephrine, as a neurotransmitter phenolic derivative model, show a favourable interfering effect due to the mediated ET mechanism. These compounds may compete with carbon grains in the electron donation process and also increase the sensitivity. Uric and ascorbic acids have the inverse effect, probably due to the adsorption of uric acid on the electrode surface, as was

Table 1
Effect of possible interferents on the response of the biosensor

Interferents	% response
Blank	100
UA	92.54
AA	89.12
Dopa	111.41
Epi	120.14

reported for hydrophobic substances on carbon paste electrodes [50] and for direct reaction between ascorbic acid and H_2O_2 [51].

4. Conclusions

In this paper, we report the successful immobilization of HRP on our Sonogel–Carbon electrode by the combination of crosslinked glutaricdialdehyde and the addition of protective Nafion. AFM studies show that the HRP enzyme is probably introduced within the ion cluster region of the Nafion. We have studied the direct electron transfer behaviour of the immobilized HRP, and developed a biosensor with excellent performance for the determination of H_2O_2. As an extension of this work, we are now investigating the combination of HRP with other enzymes also generating H_2O_2 as a product of their catalytic cycle.

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