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Novel enzyme biosensor for hydrogen peroxide via supramolecular associations

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ABSTRACT

A polythiolated-β-cyclodextrin polymer was synthesized and used as a coating material for gold electrodes. The functionalized electrodes were employed for immobilizing adamantane-modified horseradish peroxidase via supramolecular associations. The enzyme-containing electrode was used as an amperometric biosensor device with 1 mM hydroquinone as electrochemical mediator. The biosensor exhibited a fast amperometric response (10 s), a good linear response toward H_2O_2 concentrations between 28 μM and 5.5 mM, and a low detection limit of 7 μM. The biosensor showed a sensitivity of 109 μA/M cm^2 and retained 98% of its initial electrocatalytic activity after 40 days of storage at 4 °C in 50 mM sodium phosphate buffer pH 7.0. The host–guest supramolecular nature of the immobilization method was confirmed by cyclic voltammetry.

1. Introduction

Amperometric enzyme biosensors are considered a promising technology for the rapid, cheap and accurate quantification of important chemical compounds (Terry et al., 2005; Vianello et al., 2006; Xu et al., 2002). These devices have many advantages including simplicity, real time detection, high specificity and compact size. For these reasons, the development of novel strategies for constructing these electroanalytical devices continues to receive considerable attention.

During the fabrication of biosensors, the strategy employed for immobilizing the active biocatalyst constitutes one of the key aspects to be considered because enzymes can be easily inactivated or released from the electrodes if they are not properly immobilized. Within the past few years, several strategies have been employed for immobilizing enzymes on electrode surfaces, including covalent attachment, physical adsorption, and film deposition (Chaniotakis, 2004; Chen et al., 2003; Takashima and Kaneto, 2004). Recently, a supramolecular-based approach was described for immobilizing enzymes chemically functionalized with cyclodextrin derivatives on 1-adamantane-capped metal electrodes (Villalonga et al., 2007a). In addition, enzymes modified with adamantane units have been successfully immobilized on cyclodextrin-capped electrodes via the formation of host–guest inclusion complexes (Fragoso et al., 2002; Villalonga et al., 2007b,c,d). These enzyme electrodes with supramolecular architecture were further used for constructing robust enzyme biosensors showing high sensitivity toward xanthine and L-phenylalanine.

On the other hand, the sensitive and accurate quantification of H_2O_2 is of great importance because this inorganic compound is an essential mediator in biology, medicine and chemistry, as well as an important contaminant for several industrial products and wastes (Schumb et al., 1995). In addition, H_2O_2 is a by-product of highly selective oxidases commonly employed in biosensor design (Vojinović et al., 2006; Alonso Lomillo et al., 2005). That is why novel methods for immobilizing peroxidases (in special horseradish peroxidase (HRP, EC 1.11.1.7, H_2O_2 oxidoreductase)) on electrodes surfaces have been extensively developed, in order to construct robust H_2O_2 biosensors. In this regards, numerous biosensors has been constructed by the immobilization of the enzyme on electrodes coated with organic and inorganic materials, including: DNA films (Song et al., 2006), nanoAu/thionine/poly(p-aminobenzene sulfonic acid) composites (Gao et al., 2007), electrodeposited ZrO_2 films (Tong et al., 2007), sodium alginate (Ding et al., 2008; Camacho et al., 2007a), nanosheet-based ZnO microsphere with porous nanostructures (Lu et al., 2008), and Au-modified TiO_2 nanotubes (Kafi et al., 2008).

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Supramolecular-based strategies have been also employed for preparing H_2O_2 biosensors by using cyclodextrins as molecular receptor (Camacho et al., 2007b, 2008). As part of these studies, the present paper describes the use as of a polythiolated-β-cyclodextrin polymer (pCDSH) as capping material for gold electrodes for the non-covalent immobilization of HRP through the formation of host–guest supramolecular associations. This electrode with supramolecular design was further used as a biosensor device for H_2O_2 quantification.

The novel approach for biosensor construction presented here is based on the improved functional characteristics conferred to enzymes in the presence of cyclodextrin derivatives (Villalonga et al., 2005a,b, 2007e; Fernández et al., 2004). In fact, it has been demonstrated that the formation of multivalence supramolecular associations between cyclodextrin derivatives and enzymes yield an increased structural stabilization to these biocatalysts. This effect is specially remarkable when cyclodextrin polymers are employed as molecular receptor, which supports the present use of pCDSH as coating material for the Au electrode. On the other hand, the highly hydrophilic microenvironment provided by the oligosaccharide derivatives contributes to a favourable catalytic performance of enzymes.

2. Materials and methods

2.1. Reagents and apparatus

Horseradish peroxidase (HRP, Type VI, 2×10^5 U/mg vs. 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) was purchased from Sigma (USA). Hydroquinone, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and H_2O_2 were purchased from Merck (Germany). β-Cyclodextrin (βCD) was obtained from Amaizo (USA) and used as received. All other chemicals were of analytical grade.

Cyclic voltammetry and amperometric experiments were performed using an AMEL 7050 potentiostat/galvanostat and the data were acquired using Junior Assist software (Amel Electrochemistry, Milan, Italy). A conventional three-electrode system was employed in all electrochemical studies. The working electrode was a gold disk (surface area 3.04 mm^2) modified with pCDSH and the immobilized enzyme. An Ag/AgCl/KCl (3 M) and a Pt-wire were used as reference and counter electrodes, respectively. Bioelectrode measurements were carried out at 25 °C in 0.1 M sodium phosphate buffer pH 7.0 (working volume 20 ml). The solution was exhaustively deaereated before each electrochemical experiment and 1 mM hydroquinone was employed as electrochemical mediator. The solutions were stirred at 300 rpm with a magnetic bar during amperometric studies. For analytical determination 25 mM H_2O_2 solutions (in 50 mM sodium phosphate buffer pH 7.0) were freshly prepared.

The mass variation during the chemisorption of pCDSH of the gold surface and the further immobilization of adamantane-modified HRP (HRP-ADA) was followed by a Seiko EG&G QCA922 quartz balance, oscillating at a nominal frequency of 9.5 MHz.

Spectrophotometric determinations were performed using an Ultrospec 2100 UV/VIS spectrophotometer and the data were acquired using the Swiff II software (Pharmacia Biotech, Uppsala, Sweden).

2.2. Preparation of adamantane-modified horseradish peroxidase (HRP-ADA)

A reaction mixture containing 10 mg of HRP, 10 mg of 1-adamantanecarboxylic acid and 10 mg of EDAC, dissolved in 5 ml of 100 mM sodium phosphate buffer pH 6.0, was stirred for 1 h at room temperature (28 °C) and then for 16 h at 4 °C. The solution was further dialyzed against 50 mM sodium phosphate buffer pH 7.0. The degree of modification of amino groups in the enzyme was determined by measuring the amount of free amino groups with o-phthalaldehyde using glycine as standard (Bruneel and Schacht, 1993). HRP concentration was estimated from the absorbance at 403 nm using the molar absorption coefficient, $\varepsilon_{403\,nm} = 10{,}000\ M^{-1}\ cm^{-1}$ (Salomi and Mitra, 2007).

2.3. Preparation of the polythiolated-β-cyclodextrin polymer (pCDSH)

For synthesizing the polythiolated-βCD polymer 5 g of βCD were dissolved in 50 ml of 10% (w/v) NaOH and treated with 10 ml of epichlorohydrine. The solution was stirred for 8 h and then another 5 ml of epichlorohydrine were added and stirring was continued overnight at room temperature. The solution was concentrated to about 15 ml and poured over cold ethanol (4 °C) in order to precipitate the polymer. The precipitate was crushed several times with ethanol in a mortar until a fine precipitate was obtained. The polymerized βCD was then washed again with ethanol and acetone and dried under high vacuum overnight.

In order to thiolate the polymer 2 g of the polysaccharide were dissolved in 20% (w/v) NaOH (50 ml) and tosyl chloride (2 g) in CH_3CN (5 ml) were added. The system was stirred for 24 h, neutralized with 2 M HCl, and kept at room temperature until a fine powder was formed. The solid was filtered, repeatedly washed with 5 mM HCl and dried at 60 °C under high vacuum overnight. The tosylated polymer was dissolved in dried DMF (50 ml) and thiourea (6 g) was added. The reaction mixture was stirred at 80 °C for 20 h and then the solution was treated with 20% (w/v) KOH under continuous stirring. The solution was further acidified with saturated $KHSO_4$ solution and then concentrated in vacuum to about 15 ml. The mixture was poured over cold acetone in order to precipitate the polythiolated polymer. The solid was then filtered and finally dried with P_2O_5 under vacuum.

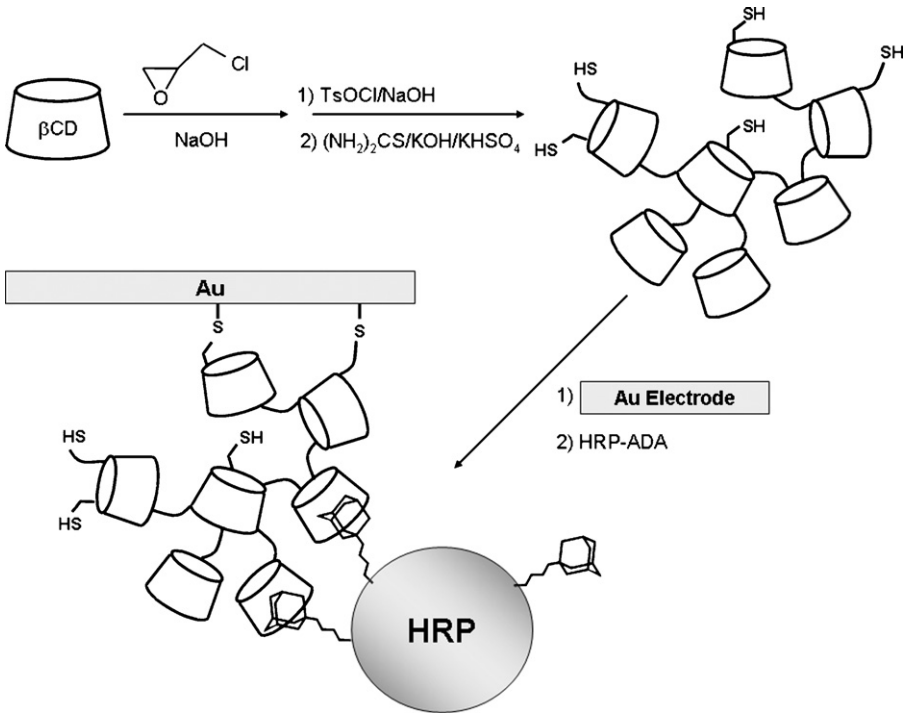
The polymer was characterized by conventional NMR techniques and analyzed by size exclusion chromatography on Fractogel EMD BioSEC (S) (1.6 cm × 80 cm) equilibrated with 100 mM NaCl in 50 mM sodium acetate buffer pH 5.0 and using dextrans as molecular weight standards. The degree of modification was estimated by quantification of the free thiol groups according to the Ellman method using L-cysteine as standard (Ellman, 1959).

2.4. Supramolecular immobilization of HRP-ADA on pCDSH-coated Au electrodes

Firstly, the disk gold-electrode was exhaustively cleaned by dipping in concentrated HNO_3 for 1 h, followed by rinsing in absolute EtOH and double distilled water. The capped-electrode was prepared by immersing a disk gold electrode into a 2-mg/ml aqueous solution of pCDSH followed by rinsing with EtOH and doubly distilled water. This modified electrode was further dipped into 2 ml of 50 mM sodium phosphate buffer pH 7.0 containing 5 mg HRP-ADA. The mixture was shaken at 4 °C for 12 h, then exhaustively washed with cool (4 °C) 50 mM sodium phosphate buffer pH 7.0 and finally kept at 4 °C in the same buffer solution until used.

3. Results and discussion

The synthesis of the polysaccharide used as coating material for the gold-electrode was carried out through a three-step reac-



Scheme 1. Preparation of the HRP-modified electrode.

tion scheme. The first step involved the cross-linking of βCD with epichlorohydrin in alkaline medium, to give a randomly polymerized material with an average molecular mass of 1.3×10^4, as determined by gel filtration chromatography. In the second step, the above obtained polymer was treated with tosyl chloride in alkaline medium with the aim of activating to the remaining hydroxyl groups of the polymer. Finally, the tosylated polysaccharide was reacted with thiourea, following by treatment with KOH and $KHSO_4$, in order to randomly introduce thiol groups into the macromolecular structure. Colorimetric titration of the polymer with the Ellman reagent revealed an average of 87 mol –SH groups per mol of polymer.

The polythiolated polymer was further used as a coating material for the gold surface through the chemisorption of several thiol groups of the polymer to the metal surface. The amount of polymer attached to the gold surface was determined as 0.65 μg/cm^2 (about 0.05 nmol/cm^2) according to quartz microbalance experiments.

In order to prepare a modified-enzyme suitable to form host–guest inclusion complexes with the polythiolated-βCD polymer HRP was chemically modified with 1-adamantanecarboxylic acid through a water-soluble carbodiimide catalyzed condensation reaction. As a result, 78% of the amino groups on the surface of HRP were modified with adamantane units. Most importantly, HRP retained 87% of its original activity after its conjugation. HRP-ADA was immobilized on the pCDSH-modified Au surface by dipping the electrode for 12 h at 4 °C in a 2 mg/ml HRP-ADA solution at pH 7.0. This immobilization process was confirmed by quartz microbalance measurements determining that an average of 114 ng/cm^2 (about 3 pmol/cm^2) was adsorbed on the pCDSH-coated Au surface. The overall approach employed for preparing the enzyme electrode is shown in Scheme 1.

The enzyme electrode prepared through the supramolecular strategy was further used as a biosensor device for H_2O_2 determinations. The cyclic voltammetric behaviour of the enzyme electrode in an unstirred and de-aerated solution of 1 mM hydroquinone in 0.1 M sodium phosphate buffer pH 7.0, was investigated at a 50-mV/s scan rate (Fig. 1). It has been previously demonstrated that hydroquinone can successfully act as electrochemical mediator for HRP through a diffusion controlled process, and 1 mM was experimentally determined as optimal concentration for this mediator (data not shown). Upon the addition of H_2O_2 for a 1.2-mM concentration, an enhancement in the cathodic current is clearly noted. Additional increases in the concentration of H_2O_2 up to 2.3 and 3.3 mM resulted in concomitant increases in the cathodic peak current. These results suggest that H_2O_2 oxidizes hydroquinone in the presence of HRP and that the benzoquinone produced is subsequently reduced at the surface of the electrode according to the

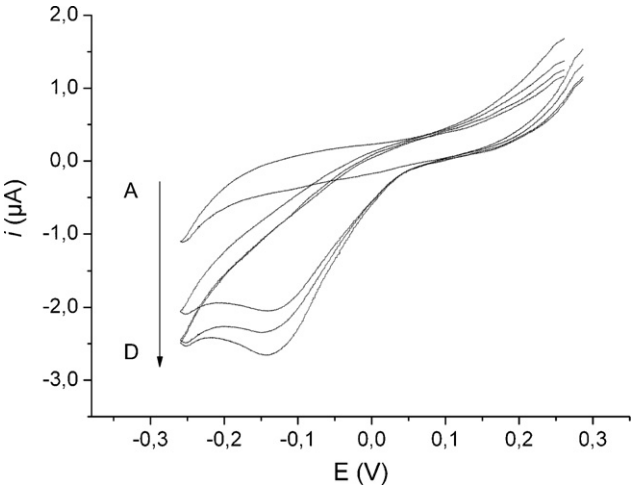


Fig. 1. Cyclic voltammograms of the H_2O_2 sensor at the scan rate of 50 mV/s in the 0.1 M sodium phosphate buffer pH 7.0 containing 1.0 mM hydroquinone in the absence (A) and the presence of 1.2 mM (B), 2.3 mM (C) and 3.3 mM (D) H_2O_2.

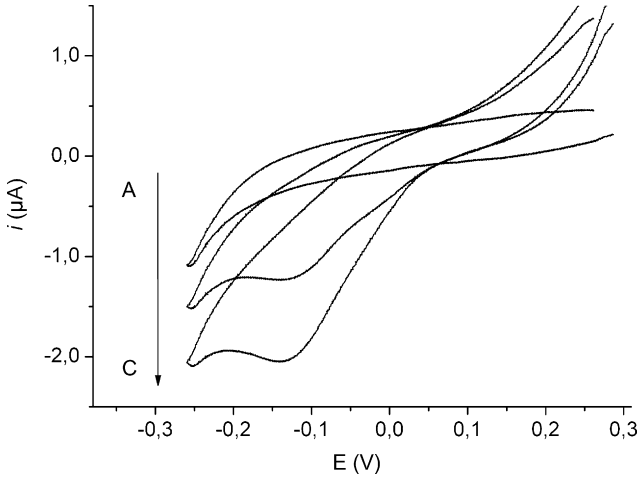


Fig. 2. Cyclic voltammograms of the H_2O_2 sensor at the scan rate of 50 mV/s in the 0.1 M sodium phosphate buffer pH 7.0 containing 1.0 mM hydroquinone in the absence (A) and the presence 1.2 mM H_2O_2 before (C) and after (B) 1 day incubation in saturated 1-adamantane carboxylic acid solution.

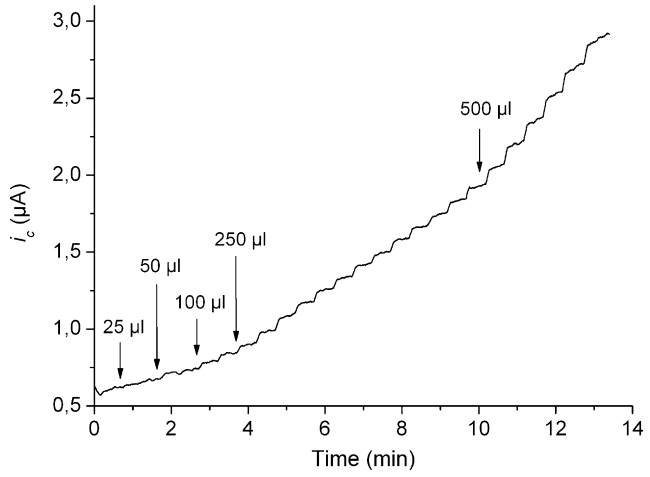
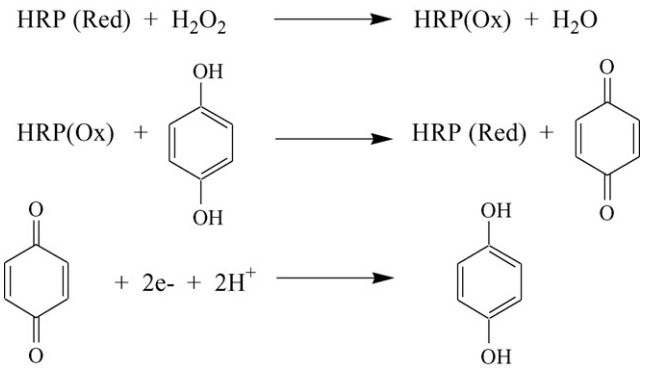


Fig. 3. Dynamic amperometric response of the biosensor to successive additions of 28 mM H_2O_2 solution. Applied potential: −300 mV; stirring rate: 300 rpm; temperature: 25 °C; initial working volume: 20 ml; supporting electrolyte: 0.1 M sodium phosphate buffer, pH 7.0 containing 1.0 mM hydroquinone.

following mechanism:



A cyclic voltammetry experiment was also carried out in order to demonstrate the supramolecular nature of the immobilization approach employed in this study. In this sense, the enzyme electrode was incubated at 4 °C in 0.1 M sodium phosphate buffer pH 7.0 saturated with 1-adamantane carboxylic acid. It is well known that CDs form highly stable inclusion complexes with adamantane derivatives (Cromwell et al., 1985). For this reason, it was expected that the presence of saturated 1-adamantane carboxylic acid in the incubation media should disrupt any possible host–guest interactions between the immobilized enzyme and the pCDSH-modified electrode.

The results of this experiment are illustrated in Fig. 2. As can be observed, the cathodic current significantly decreased after 1 day of incubation in 1-adamantane carboxylic acid solution. According to this fact, it can be suggested that the enzyme was released from the electrode surface in the presence of 1-adamantane carboxylic acid by its competitive supramolecular interaction with the βCD cavities of the pCDSH polymer attached to the metal surface. The fact that the equilibrium has not spontaneously shifted towards the total substitution of HRP-ADA by the free 1-adamantane carboxylic acid speaks about the multivalence nature of the supramolecular interaction between the modified-enzyme and the pCDSH polymer.

It should be noted that highly hydrophobic compounds as well as surfactant substances at high concentration can disrupt the host–guest interactions between βCD and 1-adamantane residues. For this reason, the stability of the enzyme biosensor here reported can be significantly affected if it is used in the presence of such kind of substances, limiting its practical applications.

The effect of the applied potential on the enzyme electrode response in the presence of 1.2 mM H_2O_2 was studied. The electrode response increased steadily with variations in the applied potential from 0 to −250 mV (vs. Ag/AgCl). A further increase of the anodic potential resulted in very small change in current response and for that reason −250 mV was selected as the working potential for the remainder of the experiments. It was also confirmed that the signal-to-noise ratio for the electrochemical response of the electrode toward H_2O_2 was lower at this value of applied potential.

The influence of pH on the enzyme electrode response upon additions of H_2O_2 was also determined. The electrode response exhibited an almost perfect bell shape curve, with a maximum value for the cathodic transformation at pH 7. Thus, this value of pH was selected as optimum for further experiments. Similar value of optimum pH has been reported for HRP-based electrodes (Camacho et al., 2007a,b).

Under these optimized experimental conditions the amperometric response of the enzyme electrode to successive additions of H_2O_2 was further evaluated. Fig. 3 shows the typical current–time dynamic response of the HRP-modified electrode towards H_2O_2. The electrode showed a rapid and sensitive bioelectrocatalytic response, reaching about 95% of the steady-state current in about 10 s after each addition of H_2O_2. However, it should be noted that this amperometric response was slightly higher than the previously reported for electrodes based on HRP immobilized on electropolymerized polyaniline films doped with carbon nanotubes (Luo et al., 2006) and a composite material of chitosan and room temperature ionic liquid 1-butyl-3-methyl-imidazolium tetrafluoroborate (Lu et al., 2006). This fact could be explained by lower difussional rate of the electroactive substances to the metal surface, due to the steric hindrance of the polymeric structures covering the electrode.

Fig. 4 shows that the analytical response of the biosensor was linear in the range of 28 μM to 5.5 mM (i_c(μA) = 0.38 × c(H_2O_2/mM) + 0.02), with a correlation coefficient of 0.998 (n = 6) and a sensitivity of 125 μA/M cm^2. The detection limit of the biosensor was found to be 7 μM based on a signal-to-noise ratio of 3. This value was significantly better than the detection limit previously reported for other H_2O_2 biosensors, based on HRP immobilized on composite film of carbon nanotubes and chitosan (Qian and Yang, 2006) and electropolymerized

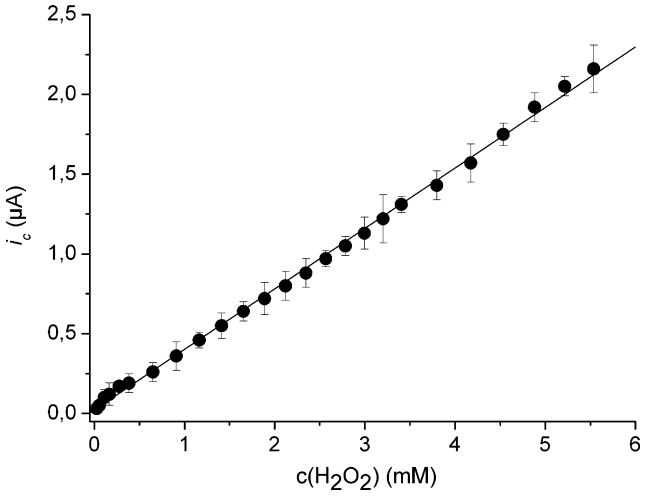


Fig. 4. Calibration curve of the biosensor in the range of 70 μM to 8.8 mM H_2O_2 concentration. Applied potential: −300 mV; stirring rate: 300 rpm; temperature: 25 °C; initial working volume: 20 ml; supporting electrolyte: 0.1 M sodium phosphate buffer, pH 7.0 containing 1.0 mM hydroquinone.

polyaniline films doped with carbon nanotubes (Luo et al., 2006). On the other hand, the detection limit of this electrode is similar to the previously constructed by using perthiolated cyclodextrin as capped material for Au electrodes (Camacho et al., 2007b).

The enzyme electrode showed $K_{M(app)}$ and I_{MAX} values of 58 mM and 23 μA, respectively. These constants were calculated by considering that the enzymatic transformation of H_2O_2 follows a Michaelis–Menten kinetics model and using the Lineweaver–Burk plots according to the following equation:

$$\frac{1}{I_S} = \frac{K_M}{I_{MAX}} \times \frac{1}{S} + \frac{1}{I_{MAX}}$$

where I_S is the steady-state current observed during the amperometric experiments, K_M the Michaelis–Menten constant, S the concentration of H_2O_2, and I_{MAX} the maximum steady-state current that can be achieved from the system. It should be notified that the $K_{M(app)}$ and I_{MAX} values determined for this electrode were better than the previously reported for HRP immobilized on Au electrodes coated with perthiolated cyclodextrin (Camacho et al., 2007b). This fact suggests that the pCDSH polymer provides a more favourable microenvironment for HRP bioelectrocatalytic activity.

The reproducibility of the analytical response of the HRP-modified electrode was examined at H_2O_2 concentrations of 0.31 and 2.3 mM, and the mean current responses were 0.17 and 0.9 μA, with relative standard deviation of 3.3% and 7.5%, respectively ($n = 10$). On the other hand, the mean current response and the R.S.D. of four equivalently prepared electrodes towards 1.2 mM H_2O_2 were 0.46 μA and 7.6%, respectively. Comparing with previous reports, the present biosensor has better reproducibility than those prepared by covalent immobilization of HRP on sodium alginate (Camacho et al., 2007a).

The selectivity of the sensor was evaluated in the presence of several potential interfering substances (Table 1). The current obtained at 2.5 mM concentration of each possible interfering species in the presence of 1.2 mM H_2O_2 was used as an indicator of the biosensor selectivity. The enzyme biosensor showed unaffected electrochemical signals in the presence of ethanol, glucose, lactic acid and citric acid. However, ascorbic acid and L-cysteine caused interference in the quantification of H_2O_2 with a decrease in the cathodic current in about 67% and 81%, respectively. It is well known that ascorbic acid can reduce the oxidation form of mediators, destroying the circulation of catalytic reaction and interfering

Table 1
Possible interferences tested with the enzyme biosensor.

Possible interferences	Current ratio (±R.S.D.)[a]
Ethanol	1.08 ± 0.06
Citric acid	1.07 ± 0.03
D-Glucose	1.00 ± 0.03
Ascorbic acid	0.67 ± 0.09
Lactic acid	1.07 ± 0.06
L-Cysteine	0.81 ± 0.09

[a] Ratio of cathodic currents for mixtures containing 2.5 mM interfering substances and 1.2 mM H_2O_2, compared to that for 1.2 mM H_2O_2.

in the quantification of H_2O_2 (Miao and Tan, 2001; Wang et al., 2003). On the other hand, HRP is inhibited by thiol-containing compounds, such as L-cysteine (Sariri and Jafarian, 2006). Considering these facts, it should be expected low specificity for the biosensor in the presence of highly antioxidant compounds or thiol-containing substances. Similar results have been previously described for other HRP-based H_2O_2 biosensors (Miao and Tan, 2001; Wang et al., 2003).

The HRP-based biosensor was tested for real sample analysis by using milk samples from local supplies. No detectable analytical signals were detectable in the raw samples. However, the biosensor showed excellent electrocatalytic response after addition of H_2O_2 up to 310 μM and 2.3 mM. The average recovery of the biosensor for these measurements was 106% (330 ± 10 μM, $n = 6$) and 96% (2.20 ± 0.12 mM, $n = 6$), respectively. On the other hand, the R.S.D. for 10 repetitive measurements of 2.3 mM H_2O_2 was 8.2%.

The long-term stability of the electrode was investigated by measuring the electroanalytical response to 1.2 mM H_2O_2 after different storage intervals at 4 °C in 50 mM sodium phosphate buffer pH 7.0. The enzyme-modified electrode exhibited a very good long-term stability retaining about 98% of its initial activity after 40 days of storage. This remarkable stability could be justified by the occurrence of a multivalence supramolecular association between HRP-ADA and the pCDSH-capped Au-electrode as mentioned above. Such multivalence supramolecular interaction is stable enough to prevent the dissociation of the enzyme from the electrode surface for long incubation periods. From a practical point of view, the high storage stability showed by the biosensor constitute a very interesting result, allows to evaluate this kind of supramolecularly functionalized enzyme electrodes as reliable and stable analytical devices for biomedical, chemical and environmental applications.

4. Conclusions

A novel immobilization strategy based on the formation of host–guest supramolecular associations was reported for the construction of a biosensor device for H_2O_2. A gold-electrode was coated with a polythiolated-βCD polymer and further employed as support for the non-covalent immobilization of HRP-ADA via host–guest interactions. The method combines the advantages of supramolecular associations and the immobilizations of enzymes in thin film polymers. That is the reason for which the obtained enzyme electrode was highly robust and showed good sentitivity and fast electroanalytical response toward H_2O_2. Based on these results, it is suggested that this combined supramolecular-based strategy is a useful tool for preparing stable amperometric enzyme biosensors.

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