Hydrogen Peroxide Biosensor with a Supramolecular Layer-by-Layer Design

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A new sensor design is reported for the construction of an amperometric enzyme biosensor toward H_2O_2. It was based in the supramolecular immobilization of alternating layers of horseradish peroxidase (either modified with 1-adamantane or β-cyclodextrin-branched carboxymethylcellulose residues) on Au electrodes coated with polythiolated β-cyclodextrin polymer. The analytical response of the electrodes, using 1 mM hydroquinone as an electrochemical mediator, increases when the number of enzyme layers increases. The biosensor having three enzyme layers showed a sensitivity of 720 μA/M cm^2 and a detection limit of 2 μM and retained 96% of its initial activity after 30 days of storage. The host−guest supramolecular nature of the immobilization method was confirmed by cyclic voltammetry.

During the past few years, several covalent and physical procedures have been employed to immobilize enzymes on electrode surfaces in order to construct reliable, stable biosensor devices.[1−3] Recently, we have described a novel supramolecular-based approach for immobilizing adamantane-modified enzymes and other proteins on metal electrodes capped with cyclodextrin (CDs) derivatives.[4,5] This strategy, based on the ability of CDs to form highly stable inclusion complexes with 1-adamantane derivatives,[6] can be potentially exploited to increase the number of enzyme molecules loaded on the electrode surface by the formation of multiple layers of the biocatalysts via host−guest interactions. In the present work, we describe an innovative method for the preparation of an enzyme biosensor for H_2O_2 through the layer-by-layer supramolecular immobilization of chemically modified horseradish peroxidase (HRP, EC 1.11.1.7, H_2O_2 oxidoreductase) forms on Au electrodes capped with a polythiolated βCD polymer (pCD). H_2O_2 was selected as a target compound for this study because of its essential role as a mediator in biology, medicine, and chemistry as well as its negative impact as an environmental contaminant.[7] In addition, H_2O_2 is a byproduct of highly selective oxidases commonly employed in biosensor design.[8,9]

The approach used here to prepare the functionalized Au electrode is based on its previous activation with pCD and the further immobilization of the first enzyme layer by host−guest associations with HRP modified with 1-adamantanyl residues (HRP-ADA). The second layer was then prepared by coimmobilization of the enzyme, previously glycosidated with carboxymethylcellulose branched with mono-6-butylenediamino-6-deoxy-β-cyclodextrin residues (HRP-CMC-CD), and further layers were formed by the sequential coimmobilization of HRP-ADA and HRP-CMC-CD forms (Figure 1). The enzyme electrode prepared via supramolecular associations of HRP layers was finally used to construct an amperometric biosensor for H_2O_2.

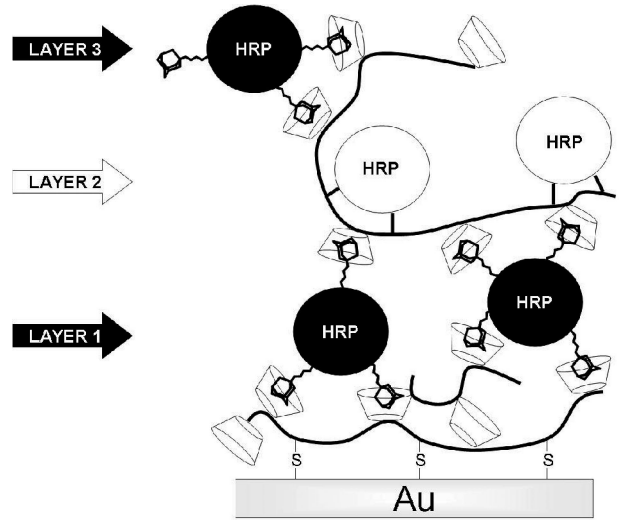

Figure 1. Layer-by-layer supramolecular immobilization of HRP-ADA and HRP-CMC-CD on a pCD-coated Au electrode.

pCD (MW = 1.3×10^4, −SH content = 87 mol/mol polymer) was synthesized by the polymerization of βCD with epichlorohydrin as previously described[10] and further functionalization with thiol groups.[11] HRP-ADA and HRP-CMC-CD were respectively prepared by modification of the native enzyme (2×10^5 U/mg vs 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic

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(1) Chaniotakis, N. A. *Anal. Bioanal. Chem.* **2004**, *378*, 89.
(2) Chen, X.; Jia, J.; Dong, S. *Electroanalysis* **2003**, *15*, 608.
(3) Takashima, W.; Kaneto, K. *Sens. Actuators, B* **2004**, *102*, 271.
(4) Villalonga, R.; Cao, R.; Hernández, J.; Camacho, C. *Chem. Commun.* **2007**, 942.
(5) Fragoso, A.; Caballero, J.; Almirall, E.; Villalonga, R.; Cao, R. *Langmuir* **2002**, *18*, 5051.
(6) Szejtli, J. *Chem. Rev.* **1998**, *98*, 1743.
(7) Schumb, W. C.; Satterfield, C. N.; Wentworth, R. L. *Hydrogen Peroxide*; Reinhold: New York, 1995.
(8) Vojinoviċ, V.; Esteves, F. M. F.; Cabral, J. M. S.; Fonseca, L. P. *Anal. Chim. Acta* **2006**, *565*, 240.
(9) Alonso Lomillo, M. A.; Ruiz, J. G.; Muñoz Pascual, F. J. *Anal. Chim. Acta* **2005**, *547*, 209.
(10) Villalonga, M. L.; Reyes, G.; Fragoso, A.; Cao, R.; Villalonga, R. *Biotechnol. Appl. Biochem.* **2005**, *41*, 217.

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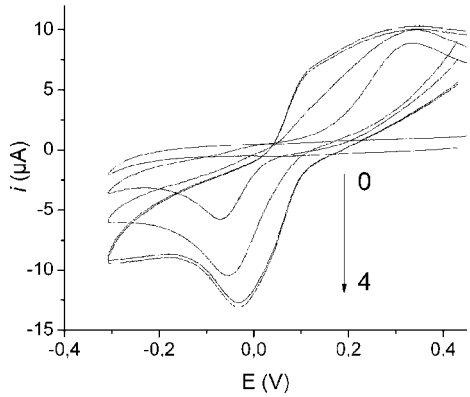

Figure 2. Cyclic voltammograms of the enzyme electrodes at 50 mV/s in 0.1 M sodium phosphate buffer at pH 7.0 containing 1.0 mM hydroquinone in the presence 600 μM H_2O_2. Numbers on the arrow represent the number of HRP layers on the electrode surface.

acid) with 1-adamantane carboxylic acid and βCD-branched carboxymethylcellulose,[12] as described elsewhere for xanthine oxidase.[4] The HRP-ADA form retained 87% of its initial activity after modification of 78% of the enzyme amino groups with 1-adamantane residues. However, HRP retained 73% of its catalytic activity after the attachment of 1.6 mol of βCD-branched carboxymethylcellulose polymer to the protein surface, as determined by the colorimetric quantification of carbohydrates in the conjugated enzyme by the phenol−sulfuric acid method.

To prepare the layer-by-layer enzyme electrode, a clean Au disk electrode ($A = 3.04$ mm^2) was dipped in an aqueous solution of pCD (10 mg/mL) for 4 h and then exhaustively washed with distilled water. The first enzyme layer was then immobilized by dipping the modified electrode in a 2 mg/mL HRP-ADA solution in 20 mM sodium phosphate buffer (pH 7.0, 24 h, 4 °C). The electrode was then washed with cool buffer solution and kept at 4 °C until use. The second layer was similarly prepared by using HRP-CMC-CD solution, and additional layers were coimmobilized by the sequential incubation of the electrode in HRP-ADA or HRP-CMC-CD solution.

Figure 2 shows the cyclic voltammograms of the enzyme electrodes in the presence of 600 μM H_2O_2 and using 1.0 mM hydroquinone as electrochemical mediator.[13] A noticeable electrochemical transformation of H_2O_2 was observed after the immobilization of the first HRP layer, when compared with the electrodes coated only with the polythiolated βCD polymer. A subsequent increase in the number of enzyme layers yielded an increase in the cathodic current peaks, reaching a maximum

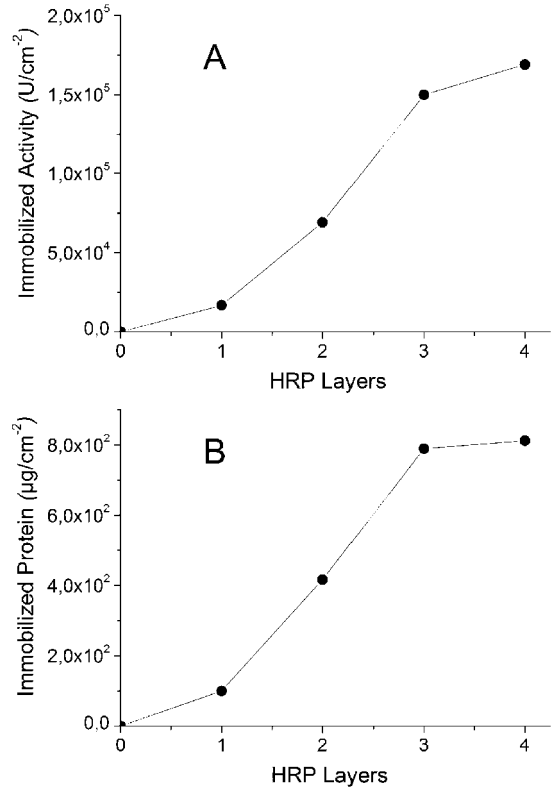

Figure 3. Effect of the number of HRP layer on the enzymatic activity (A) and protein (B) immobilized on the electrode surface.

value when the third HRP layer was immobilized. Further increases in the enzyme layers, by coimmobilization of the second layer of HRP-CMC-CD, did not cause a significant change in the bioelectrocatalytic transformation of H_2O_2. Similar behavior was observed by determining the immobilized HRP enzymatic activity (vs 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) as a function of the number of layers on the electrode surface (Figure 3A).

In addition, the enzyme electrodes were sealed in evacuated glass tubes containing 6 M HCl solution and incubated at 110 °C for 24 h. The hydrolyzed protein was further quantified by amino acid analysis, showing a similar pattern with regard to the number of enzyme layers (Figure 3B). These facts demonstrate that the increased bioelectrocatalytic activity shown by the multilayer electrodes was mediated by an increased number of immobilized HRP forms as a result of the subsequent formation of different enzyme layers on the metal electrode and not by conformational changes in the protein structure that could yield more active enzyme forms.

In the present biosensor, a polythiolated β-CD polymer was used to coat the gold electrode. This polymeric layer was alternated with β-CD-branched carboxymethylcellulose conjugated to HRP. The presence of β-CD favors the necessary permeability required for the diffusion of the substrate and mediator.[14] Nevertheless, the presence of both polymers increases the enzyme−electrode distance, a factor that can seriously affect the electron-transfer rate.[14] This characteristic should explain why when modifying the electrode with more than three enzyme layers no additional increase in the analytical response nor in sensitivity was observed (as can be seen below). On the contrary, within the first three layers a greater amount of enzyme is present without a significant loss in the electron-transfer rate. Additionally, a large number

(11) Polymerized βCD (2 g) was dissolved in 20% (w/v) NaOH (50 mL), and tosyl chloride (2 g) in CH_3CN (5 mL) was 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 to precipitate the polythiolated polymer. The solid was then filtered and finally dried with P_2O_5 under vacuum. The product was characterized by conventional NMR techniques.

(12) Valdivia, A.; Pérez, Y.; Ramírez, H. L.; Cao, R.; Villalonga, R, *Biotechnol. Lett.* **2006**, *28*, 1465.

(13) All electrochemical measurements were performed using an AMEL 7050 potentiostat/galvanostat, and the data was acquired using JuniorAssist software (Amel, Milan, Italy). A conventional three-electrode system was employed in all electrochemical studies. The working electrode was the Au disk covered with the enzyme forms. 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 at pH 7.0 (working volume 20 mL). The solution was exhaustively deaereated before each electrochemical experiment and stirred at 300 rpm during amperometric studies.

(14) Villalonga, R.; Cao, R.; Fragoso, A. *Chem. Rev.* **2007**, *107*, 3088.

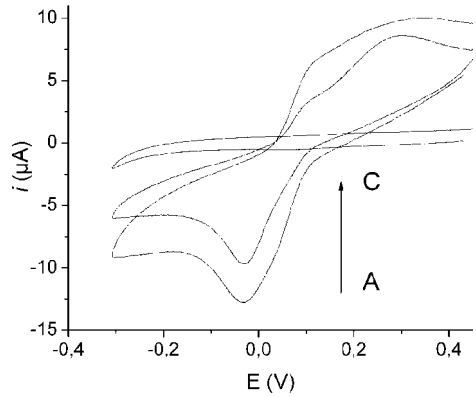

Figure 4. Cyclic voltammograms of the enzyme electrode having three layers of HRP at 50 mV/s in 0.1 M sodium phosphate buffer at pH 7.0 containing 1.0 mM hydroquinone in the absence (C) and the presence of 600 μM H_2O_2 before (A) and after (B) 24 h of incubation in a saturated 1-adamantane carboxylic acid solution.

of immobilized HRP layers should yield a low stable supramolecular architecture at the electrode surface.

To confirm the supramolecular nature of this multilayer assembly, the electrode containing three HRP layers was incubated at 4 °C in 0.1 M sodium phosphate buffer at pH 7.0 saturated with 1-adamantane carboxylic acid. It has been previously demonstrated that 1-adamantane derivatives can form the most stable inclusion complexes with CDs.[6] Consequently, the presence of saturated 1-adamantane carboxylic acid in the incubation media should disrupt possible host−guest interactions stabilizing the multilayer enzyme architecture at the electrode surface.

Figure 4 shows that the cathodic current significantly decreased after 24 h of incubation, suggesting that the enzyme derivatives were released from the electrode surface in the presence of 1-adamantane carboxylic acid. This fact was confirmed by detecting HRP protein molecules in the incubating solution, as quantified by amino acid analysis. According to these facts, it is clear that high concentrations of 1-adamantane carboxylic acid can disrupt the host−guest association between the enzyme layers and the pCD-coated Au electrode, supporting our supramolecular-based hypothesis. However, the remaining bioelectrocatalytic activity expresed after incubation of the electrode in concentrated 1-adamantane carboxylic acid solution can be justified by the multipoint nature of the immobilization strategy employed, yielding stable supramolecular structures than cannot be completely disrupted by the single 1-adamantane carboxylic acid molecules.

In a control experiment, the multilayer electrodes were incubated in 0.1 M acetic acid solution for 1 day at 4 °C. Under these conditions, neither HRP activity, carbohydrates, nor protein molecules (quantified by amino acid analysis) were detected in the incubating solution, confirming the supramolecular immobilization of the enzyme on the electrodes.

The functionalized electrodes prepared via layer-by-layer host−guest associations were used in the construction of amperometric biosensors for the quantification of H_2O_2. The enzyme electrodes showed maximum analytical responses at −50 mV and pH 7.0 when an optimized concentration of 1.0 mM hydroquinone was used as an electrochemical mediator. These optimum conditions were then employed for further experiments. Figure 5A shows the dynamic amperometric response of the electrodes, characterized by a fast bioelectrocatalytic response with 95% of the steady-state current being achieved in about 10 s for H_2O_2. Linear relations between the cathodic steady-state currents and H_2O_2 concentration in the range

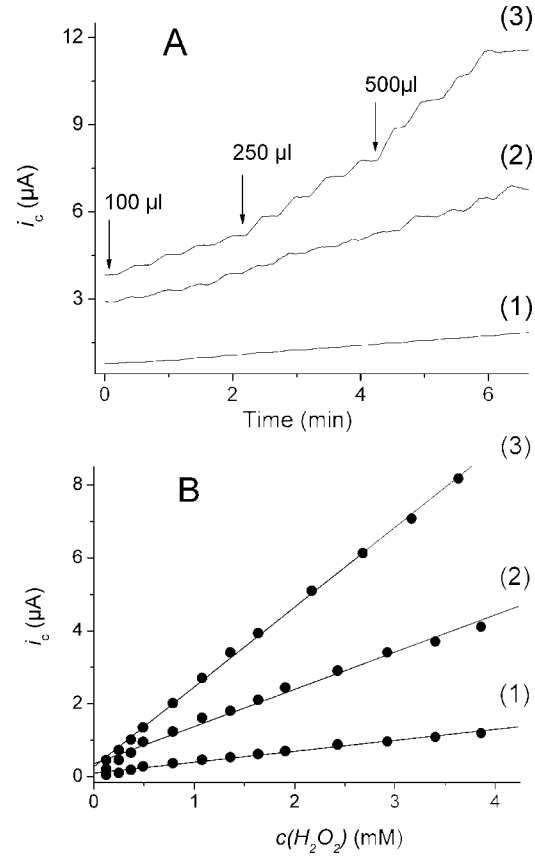

Figure 5. (A) Dynamic amperometric response of the electrodes to successive additions of aliquots of 25 mM H_2O_2 solution. (B) Calibration curves for the electrodes. Applied potential, −50 mV; stirring rate, 300 rpm; temperature, 25 °C; initial working volume, 20 mL; supporting electrolyte, 0.1 M sodium phosphate buffer at pH 7.0 containing 1.0 mM hydroquinone. Numbers in parentheses represent the number of HRP layers on the electrode surface.

of 100 μM−3.9 mM were also observed for the electrodes (Figure 5B), with correlation coefficients of about 0.997 (n = 6).

Interestingly, the slope of the calibration curves, that is, the sensitivity of the biosensors, increased when the number of HRP layers increased. Electrodes containing one to three enzyme layers showed sensitivity values of 98, 340, and 720 μA/M cm^2, respectively. Similar improvement was observed for the detection limit of the biosensors, which showed values of 15 (layer 1), 6 (layer 2), and 2 μM (layer 3) for a signal-to-noise ratio of 3.

The value of K_M for the enzyme electrodes was not significantly affected by the number of HRP layers on the surface, showing an average value of 7.5 mM. On the contrary, I_{MAX} increased when the number of enzyme layers increased from 1 to 3, showing I_{MAX} values of 2.1, 13.7, and 21.2 μA, respectively.

According to these results, the electrode having three HRP layers was employed for further studies. The reproducibility of this electrode was determined with respect to H_2O_2 concentrations of 125 and 600 μM, and the relative standard deviations were 3.7 and 8.4%, respectively (n = 10). This biosensor was also very stable, retaining 96% of its initial electrocatalytic response after 30 days of storage at 4 °C in 50 mM sodium phosphate buffer at pH 7.0. This high stability suggests that the supramolecular associations prevented the loss of the enzyme forms from the electrode surface or that the presence of βCDs in the microenvironment of the HRP derivatives improved their functional stability as previously reported for other biocatalysts.[14]

The selectivity of the biosensor was determined in the presence of potential interfering substances, such as D-glucose, ethanol,

lactic acid, L-cysteine, ascorbic acid, L-tyrosine, acetic acid, and citric acid. The cathodic current response obtained for each possible interferant at 2.5 mM concentration in the presence of 600 μM H_2O_2 was used as an indicator of biosensor selectivity, in comparison with the reading alone. The electrocatalytic response of the enzyme biosensor was significantly affected only in the presence of ascorbic acid and L-cysteine, with decreases in the cathodic current to about 78 and 91%, respectively.

In conclusion, this is the first report dealing with the preparation of an enzyme electrode with a layer-by-layer architecture based on supramolecular associations. Experiments are in progress to explore the application of this approach in the construction of multienzymatic biosensors.

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Supporting Information Available: Synthesis and characterization methods employed for the CD and HRP derivatives prepared. This material is available free of charge via the Internet at http://pubs.acs.org.

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