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REVIEW

Hydrogen peroxide biosensor based on the immobilization of horseradish peroxidase on γ-Al_2O_3 nanoparticles/chitosan film-modified electrode

Xiaojuan Liu · Liqiang Luo · Yaping Ding · Yanhong Xu · Fei Li

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Abstract An amperometric biosensor based on horseradish peroxidase (HRP) and γ-Al_2O_3/chitosan composite film at a glassy carbon electrode has been developed. Hydrogen peroxide (H_2O_2) was detected with the aid of ferrocene monocarboxylic acid mediator to transfer electrons between the electrode and HRP. The morphology and composition of the modified electrode were characterized by scanning electron microscopy and electrochemical impedance spectroscopy. The electrochemical characteristics of the biosensor were studied by cyclic voltammetry and amperometry. The effects of HRP concentration, the applied potential, and the pH values of the buffer solution on the response of the sensor were investigated for optimum analytical performance. The proposed biosensor showed high sensitivity (0.249 A M^{-1} cm^{-2}) and a fast response (<5 s) to H_2O_2 with the detection limit of 0.07 μM. The linear response range of the enzyme electrode to H_2O_2 concentration was from 0.5 to 700 μM with a correlation coefficient of 0.9998. The apparent Michaelis-Menten constant of the biosensor was calculated to be 0.818 mM, exhibiting a high enzymatic activity and affinity for H_2O_2.

Keywords Horseradish peroxidase · γ-Al_2O_3 · Hydrogen peroxide · Biosensor · Glassy carbon electrode

X. Liu · L. Luo · Y. Ding (✉) · Y. Xu
College of Sciences, Shanghai University,
99 Shangda Road,
Shanghai 200444, People's Republic of China
e-mail: wdingyp@sina.com

F. Li
The State Key Laboratory of High Performance Ceramics and Superfine Microstructure, Shanghai Institute of Ceramics,
Chinese Academy of Sciences,
1295 Dingxi Road,
Shanghai 200050, People's Republic of China

Introduction

The sensitive and accurate detection of hydrogen peroxide (H_2O_2) is of great interest to researchers because of its importance in the pharmaceutical, clinical, and industrial settings [1–4]. Many techniques have been employed for the determination of H_2O_2, such as titrimetry, spectrometry, chemiluminescence, and electrochemistry [5–7]. Among these methods, amperometric enzyme-based biosensors have received considerable attention due to its convenience, high sensitivity, and selectivity [8, 9].

Horseradish peroxidase (HRP) is a heme enzyme with a molar weight of ~44 kDa, which catalyzes the oxidation of a wide variety of substrates by H_2O_2 or related compounds [10–12]. Because of deep embedding of electrochemical prosthetic groups in protein structure, proteins exhibit a rather slow rate of heterogeneous electron transfer at conventional electrode. Therefore, the immobilization of enzyme is essential to the performance of biosensors [13]. In order to achieve direct electron transfer between HRP and electrode, surfactant, polymer, sol-gel, inorganic materials, etc. as matrices for immobilization of HRP have been investigated [10]. Among the matrices, inorganic materials are more attractive because of their regular structure, good mechanical, chemical, and thermal stabilities [14].

Chitosan (CHT) is a polysaccharide consisting of the functional groups −OH and $-NH_2$, and possessing many properties such as good film-forming ability, chemical inertness, high mechanical strength, high hydrophilicity, and biocompatibility [15–18]. Therefore, CHT is an attractive structural material and has been studied widely as a support for immobilization of enzymes and the construction of amperometric biosensors [19–22].

In this paper, a novel H_2O_2 sensor (HRP/γ-Al_2O_3/CHT/GCE) was constructed with gamma alumina (γ-Al_2O_3)

nanoparticles used as a matrix for HRP immobilization. Nano-Al_2O_3, has gained much research interest for a long time. In recent years, the research on the application of nanosized γ-Al_2O_3 in electrochemistry and electroanalysis has been rather intense which is mostly due to γ-Al_2O_3 particles' special properties such as porosity, large specific surface, good adsorption, high reaction activation, and cheapness [23]. Additionally, the biosensor based on γ-Al_2O_3 has the following advantages: firstly, γ-Al_2O_3 can react with $-NH_2$, −COOH groups of the enzyme by the hydrogen bonds on its surface, and the stability of the biosensor can be improved. Secondly, the porous matrix not only provides a large surface area for relative higher enzyme loading, but also decreases the resistance of material transport [24]. Our experiments show that the immobilization of enzymes on γ-Al_2O_3 is well suited for the preparation of biosensors. This γ-Al_2O_3 modified electrode exhibits a high degree of adsorption and reaction activation for the reduction of H_2O_2.

Experimental

Materials

HRP was purchased from Dingguo Biological Technology Co. Ltd. (Beijing), ferrocene monocarboxylic acid (FMCA 97%) used as an electron mediator was obtained from Sigma-Aldrich (USA), 30% H_2O_2 was bought from Shanghai Taopu Chemical Plant and the stock solution of H_2O_2 was diluted from this. γ-Al_2O_3 was purchased from Shanghai Gona Powder Technology Co. Ltd., CHT, Na_2HPO_4 and NaH_2PO_4 were bought from Sinopharm Chemical Reagent Co. Ltd. Phosphate buffer solution (PBS) was prepared by mixing stock solution of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 and adjusting the pH with 0.1 M H_3PO_4 or 0.1 M NaOH. All other reagents were of analytical grade and used as received without further purification. All aqueous solutions were prepared with doubly deionized water.

Measurements and apparatus

Cyclic voltammetry and amperometry were performed with a conventional three-electrode system using a CHI 660C electrochemical workstation (Shanghai, China). A saturated calomel electrode, a Pt foil electrode, and a glassy carbon electrode (GCE, Ø 3 mm) were used as the reference electrode, the counter electrode, and working electrode, respectively. For steady state amperometric experiments, the working potential was set at 0 mV and the solution was stirred gently with a magnetic stirrer. The electrochemical impedance spectroscopy (EIS) was recorded on Solartron 1255B Frequency Response analyzer/SI 1287 electrochemical interface (Scribner Associates, Inc.) with the frequency range of 10^{-1} to 10^5 Hz. Scanning electron microphotographs (SEM) measurements were carried out on a scanning electron microscope (JEOL JSM-6700F) at 15 kV. Nitrogen adsorption-desorption isotherms were obtained at −196 °C on a specific surface area and porosity analyzer (mMK-TriStar 3000). All the other measurements were carried out at a room temperature.

Preparation of enzyme electrode

A 0.5% (*w/v*) CHT solution was prepared by dissolving appropriate amount of CHT flakes in 0.1 M acetic acid and stirring for 1 h at room temperature until complete dissolution. A 2.0 mg γ-Al_2O_3 was dispersed in 1 mL of 0.5% CHT solution and the mixture was sonicated for 2 h. Finally, a high-dispersed colloidal solution was formed. The suspension of HRP/γ-Al_2O_3/CHT was prepared by mixing a stock solution of 5 mg mL^{-1} HRP with the colloidal solution of γ-Al_2O_3/CHT (2 mg mL^{-1}) in a 1:1 volume ratio. The obtained suspension was stored in a refrigerator when it was not in use.

Before each experiment, GCE was polished with 0.05 μm α-alumina powder. The polished electrode was successively sonicated in 1:1 aqueous HNO_3, ethanol, and doubly deionized water; and finally, allowed to dry at room temperature. The pre-prepared suspension of HRP/γ-Al_2O_3/CHT was agitated for 1 min before use and then 6 μL of the mixture was dropped on the surface of GCE. For comparison with HRP/γ-Al_2O_3/CHT/GCE, HRP/CHT/GCE was fabricated with the similar procedure.

Results and discussion

Characterization

The surface morphologies of γ-Al_2O_3 and HRP/γ-Al_2O_3/CHT composite films modified GCE were examined by SEM (Fig. 1). Figure 1a shows the surface structure contains a high degree of porosity which largely enhances the active surface of γ-Al_2O_3 and the coatings on the glassy carbon surface formed from the (γ-Al_2O_3)-C_2H_5OH suspension are compact, which is consistent with the isotherms of γ-Al_2O_3. Figure 1b indicates that the enzyme of HRP (white part) is entrapped into the γ-Al_2O_3/CHT composite films, which can be proved by the increase of the electron transfer resistance (R_{et}) of HRP/γ-Al_2O_3/CHT.

Figure 2 shows the nitrogen adsorption-desorption isotherms and pore size distribution of γ-Al_2O_3. The isotherms are type IV with hysteresis loops according to the IUPAC classification, which are characteristics of the

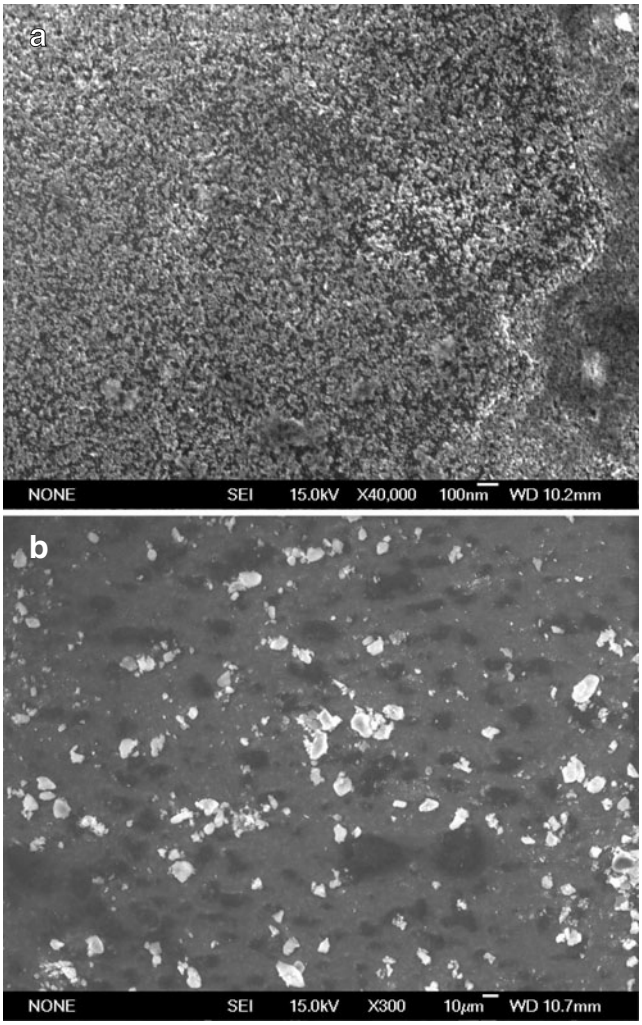

Fig. 1 SEM of γ-Al_2O_3 (**a**), and HRP/γ-Al_2O_3/CHT (**b**)

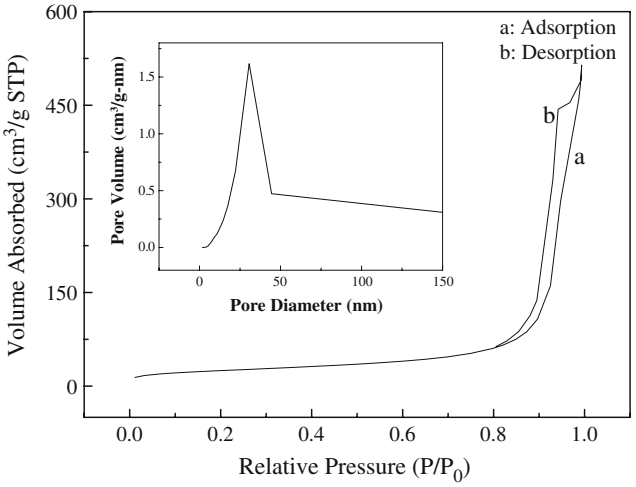

Fig. 2 N_2 adsorption and desorption isotherms for γ-Al_2O_3, and corresponding pore size distribution curve (*inset*) obtained from adsorption branches of the isotherms using Barrett-Joyner-Halenda method

mesoporous materials. The inset shown in Fig. 2 presents the pore size distribution curve centered around 30 nm by Barrett-Joyner-Halenda method.

EIS can provide useful information on the impedance changes of the electrode surface during the fabrication process. The Nyquist plot of the EIS includes a semicircular portion and a linear portion. The semicircular portion at higher frequencies corresponds to the electron transfer-limited process and its diameter is equal to R_{et}, which controls the electron transfer kinetics of the redox probe at the electrode interface. Meanwhile, the linear part at lower frequencies corresponds to the diffusion process [25]. Figure 3 displays the Nyquist plots of the EIS of bare (a) GCE, (b) CHT/GCE, (c) γ-Al_2O_3/CHT/GCE, (d) HRP/CHT/GCE, and (e) HRP/γ-Al_2O_3/CHT/GCE in 5.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) solution containing 0.1 M KCl. After pure CHT was dropped on the GCE, an increase of R_{et} (200 Ω) was observed (curve b). The reason is that the CHT film hinders the diffusion of ferricyanide toward the electrode surface. For γ-Al_2O_3/CHT/GCE, the value of R_{et} was found to be 100 Ω, implying that the incorporation of γ-Al_2O_3 facilitates electron transfer (curve c). Further immobilization of enzymes, the R_{et} of HRP/CHT/GCE (curve d) and HRP/γ-Al_2O_3/CHT/GCE (curve e) increase to 1200 Ω and 1100 Ω, respectively, and it might be caused by the hindrance of the macromolecular structure of HRP to the electron transfer. The above results clearly confirm that HRP is immobilized successfully onto the electrode.

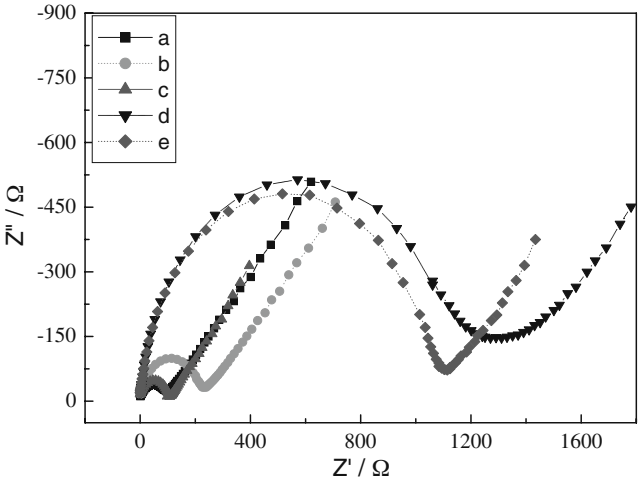

Fig. 3 EIS of bare GCE (**a**), CHT/GCE (**b**), γ-Al_2O_3/CHT/GCE (**c**), HRP/CHT/GCE (**d**), and HRP/γ-Al_2O_3/CHT/GCE (**e**) in 5.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) containing 0.1 M KCl

Electrochemical behavior of the HRP/γ-Al_2O_3/CHT-modified electrode

Figure 4 displays cyclic voltammograms of HRP/γ-Al_2O_3/CHT/GCE and HRP/CHT/GCE in 0.1 M PBS (pH 6.0) containing 0.5 mM FMCA at scan rate of 0.1 V s^{-1}. A reduction peak is observed on HRP/CHT/GCE (curve a) and HRP/γ-Al_2O_3/CHT/GCE (curve d) without H_2O_2, which is attributed to the redox of FMCA. However, the HRP/γ-Al_2O_3/CHT/GCE shows relatively high amperometric response due to the facilitated electron transfer by γ-Al_2O_3 nanoparticles. It can be seen from Fig. 4 that a small response was observed for HRP/CHT/GCE in the presence of 100 μM (curve b) or 200 μM (curve c) H_2O_2, but the HRP/γ-Al_2O_3/CHT/GCE (curve e, f) shows a remarkably increased reduction current in the same amount of H_2O_2, which could be attributed to γ-Al_2O_3 nanoparticles not only facilitating the electron transfer between the HRP and GCE, but also providing a friendly microenvironment for the immobilized HRP. Therefore, HRP/γ-Al_2O_3/CHT was chosen as optimal film to fabricate the H_2O_2 sensor.

Figure 5 illustrates cyclic voltammograms of the same biosensor at different scan rates from 0.02 to 0.4 V s^{-1} in 0.1 M PBS (pH 6.0) containing 0.5 mM FMCA. With the increase of the scan rate, the peak currents of the modified electrode increase and the peak potentials almost keep at the constant values. The anodic and cathodic peak currents are found to be linearly proportional to the square root of scan rate, indicating the electrochemical reaction of HRP/γ-Al_2O_3/CHT modified electrode is a diffusion-controlled process [26].

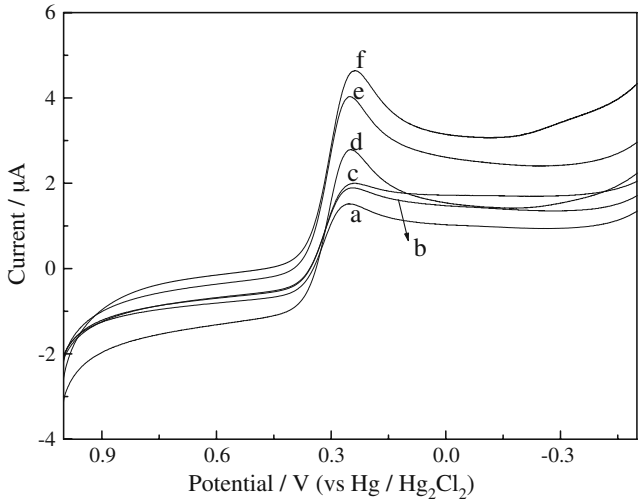

Fig. 4 Cyclic voltammograms of HRP/CHT/GCE with 0 μM (**a**), 100 μM (**b**), 200 μM (**c**), H_2O_2 and HRP/γ-Al_2O_3/CHT/GCE with 0 μM (**d**), 100 μM (**e**), and 200 μM (**f**) H_2O_2 in 0.1 M PBS (pH 6.0) containing 0.5 mM FMCA at a scan rate of 0.1 V s^{-1}

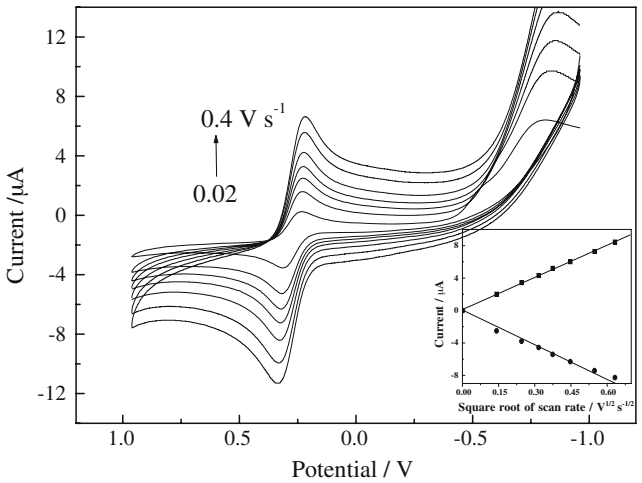

Fig. 5 Cyclic voltammograms of HRP/γ-Al_2O_3/CHT/GCE in 0.1 M PBS (pH 6.0) containing 0.5 mM FMCA at different rates. *Inset plot* the relationship of peak currents versus the square root of scan rates

Influence of HRP amount on sensor fabrication

The amount of the enzyme in composite is a vital factor affecting the analytical sensitivity of the biosensor. By use of composite solutions containing different HRP concentration and a fixed γ-Al_2O_3 concentration (2 mg mL^{-1}) for dropping on the surface of the GCE, the change of amperometric current with HRP amount under constant H_2O_2 concentration (0.08 mM) is shown in Fig. 6. The current response increases as increasing HRP concentration and achieves a maximum value at 5.0 mg mL^{-1}. With the increasing of HRP concentration from 5.0 to 10.0 mg mL^{-1}, the sensitivity reduces gradually. So, an optimum loading of 5.0 mg mL^{-1} HRP was used for subsequent experiments.

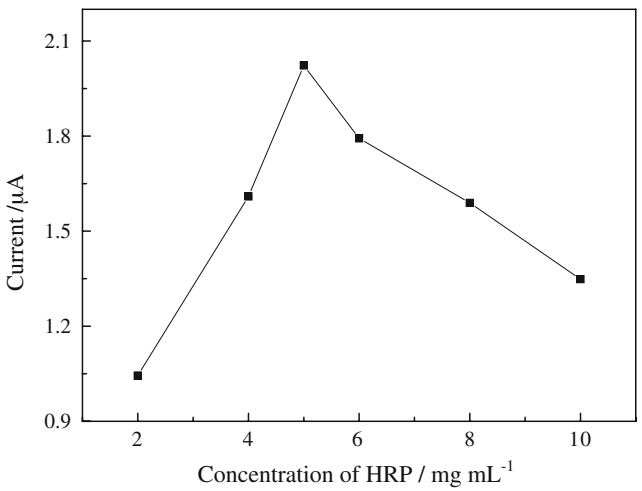

Fig. 6 Effect of HRP concentration on the biosensor response studied by amperometry under constant H_2O_2 concentration (0.08 mM) in 0.1 M PBS (pH 6.0) containing 0.5 M FMCA. *Applied potential* 0 V

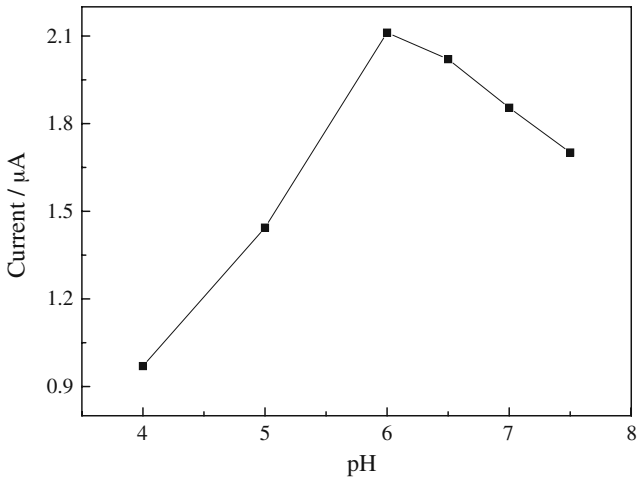

Fig. 7 Effect of pH on the biosensor response studied by amperometry under constant H_2O_2 concentration (0.08 mM) in 0.1 M PBS (pH 6.0) containing 0.5 M FMCA. *Applied potential* 0 V

Optimization of measurement variables

The effect of pH on the modified electrode response was investigated under constant H_2O_2 concentration (0.08 mM) in 0.1 M PBS (pH 6.0) containing 0.5 M FMCA and the results are displayed in Fig. 7. The biosensor response increases with increasing pH value from 4.0 to 6.0, and achieves a maximum value at 6.0, then decreases from 6.0 to 7.5. So pH 6.0 PBS was chosen as the supporting electrolyte for the further experiments.

Figure 8 shows the dependence of the current response of the modified electrode on the applied potential in the range from −0.1 V to 0.1 V under constant H_2O_2 concentration (0.08 mM) in 0.1 M PBS (pH 6.0) containing 0.5 M FMCA. With applied potential decreasing from 0.1 V to 0 V, the steady state current increases due to the increased driving force for the fast reduction of H_2O_2 at the lower potentials and approaches a maximum value at 0 V, then the response current decreases with applied potential decreasing from 0 V to −0.1 V. Therefore, 0 V was selected as the applied potential for amperometric measurement in subsequent experiments.

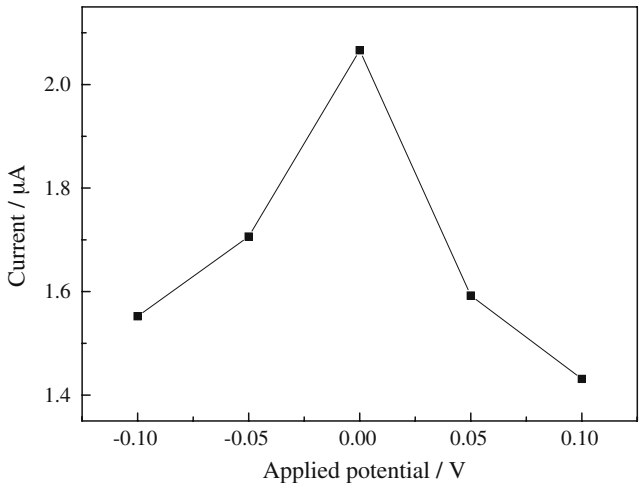

Fig. 8 Effect of applied potential on the biosensor response studied by amperometry under constant H_2O_2 concentration (0.08 mM) in 0.1 M PBS (pH 6.0) containing 0.5 M FMCA

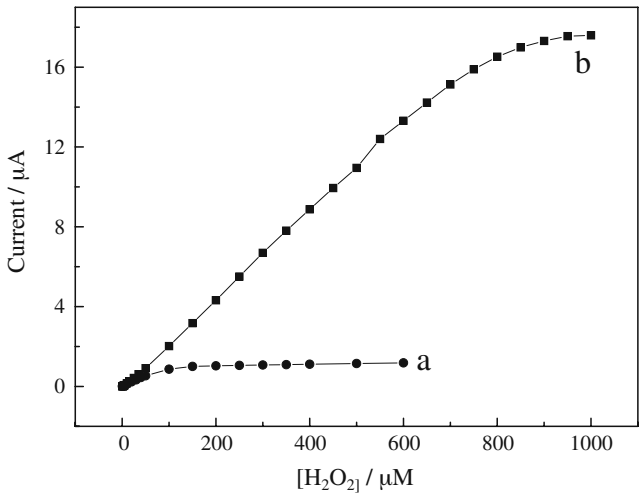

Fig. 9 Calibration curves between the catalytic current and the concentration of H_2O_2 obtained at **a** HRP/CHT/GCE and **b** HRP/γ-Al_2O_3/CHT/GCE in the 0.1 M PBS (pH 6.0) containing 0.5 M FMCA. *Applied potential* 0 V

Electrocatalytic properties of HRP/γ-Al_2O_3/CHT/GCE and enzymatic kinetic parameter

With increasing H_2O_2 concentration, the amperometric response of the enzyme electrode increased. The calibration curves under optimal conditions are shown in Fig. 9. The linear response range of HRP/γ-Al_2O_3/CHT/GCE (Fig. 9b) to H_2O_2 concentration is from 0.5 μM to 700 μM with a correlation coefficient of 0.9998 and a detection limit of 0.07 μM (S/*N*=3). The sensitivity of the biosensor is calculated to be 0.249 A M^{-1} cm^{-2}, which is higher than that (8.55 mA M^{-1} cm^{-2}, 8.44 mA M^{-1} cm^{-2}) obtained by other immobilized HRP electrode reported previously [27,

Table 1 Ratio of currents for mixtures containing 0.1 mM interfering substance and 0.1 mM H_2O_2 to that for 0.1 mM H_2O_2 alone

Interfering reagent	Current ratio
L-Tyrosine	1.0027
Leucine	1
Glutamate	1
Alanine	0.9995
Glucose	0.9972
Uric acid	0.9821
Dopamine	0.8245

28] and the sensor reaches 95% of the steady state current within 5 s. For HRP/CHT/GCE (Fig. 9a), it can be seen that the catalytic reduction current of H_2O_2 is linearly proportional to the H_2O_2 concentration in the range of 0.5–50 μM with a correlation coefficient of 0.9991 and a low sensitivity of 0.1137 A M^{-1} cm^{-2}. This fact demonstrates clearly that the HRP/γ-Al_2O_3/CHT composites as an immobilization matrix for HRP are superior to HRP/CHT composites and the presence of γ-Al_2O_3 nanoparticles remarkably improves the catalytic activity of the biosensor to the determination of H_2O_2.

The apparent Michaelis-Menten constant (K_M') can give an indication of the enzyme-substrate kinetics. It could be calculated from the electrochemical version of the Lineweaver-Burk equation: $1/i_{ss} = 1/i_{max} + K_M'/(ci_{max})$, where i_{ss} is the steady state current after the addition of substrate, i_{max} is the maximum current under saturated substrate conditions, and c in μM is the concentration of substrate (H_2O_2), the K_M' is the Michaelis-Menten constant [29]. In our work, K_M' is calculated to be 0.818 mM, which is smaller than those of HRP in DNA-silver nanohybrids of 1.30 mM [8], HRP in methylene green incorporated in Nafion film of 2.10 mM [30], HRP in a tetrathiafulvalene-tetracyanoquinodimethane/multi-walled carbon nanotubes film of 4.04 mM [29] and HRP doped with ZrO_2 of 8.01 mM [31]. It indicates that HRP immobilized in HRP/γ-Al_2O_3/CHT film retains its bioactivity and has a high biological affinity to H_2O_2.

Reproducibility and stability of the biosensor

The reproducibility of the response of the enzyme electrode was investigated at 0.08 mM H_2O_2. The relative standard deviation (RSD) determined with the same enzyme electrode is found to be about 1.47% (n=17). For the reproducibility of four electrodes, prepared independently, the RSD is 3.49% at 0.08 mM H_2O_2. The stability of the biosensor under storage was investigated by measuring the biosensor response in 0.08 mM of H_2O_2. It retains 90.0% of its initial current response to H_2O_2 after a month.

Interference

Selectivity is an important factor in the performance of an enzyme sensor. Seven possible interfering substances were used to evaluate the selectivity of the enzyme electrode. The interference experiments were performed in PBS at optimal conditions by comparing the response current of 0.1 mM H_2O_2 plus 0.1 mM each interfering substance with the 0.1 mM H_2O_2 alone. The results of the interference study are summarized in Table 1. Only dopamine interferes significantly in the determination of H_2O_2 and the decrease of the response may be attributed to consumption of H_2O_2 involving oxidation of dopamine.

Conclusion

In this paper, we have introduced a simple and versatile approach for the fabrication of a H_2O_2 biosensor based on γ-Al_2O_3. This technique is reliable and simple. The experimental results clearly exhibit that the immobilized HRP possesses excellent catalytic ability and well-retained activity. The developed biosensor shows a rapid response, a wide linear range, and a high sensitivity under optimum conditions. This work shows it is hopeful that nanostructured γ-Al_2O_3 could be used to immobilize other enzymes to construct a range of biosensors.

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