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A Biosensor Based on Immobilization of Horseradish Peroxidase in Chitosan Matrix Cross-linked with Glyoxal for Amperometric Determination of Hydrogen Peroxide

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Abstract An amperometric biosensor for hydrogen peroxide (H_2O_2) was developed via an easy and effective enzyme immobilization method with the "sandwich" configuration: ferrocene-chitosan: HRP: chitosan-glyoxal using a glassy carbon electrode as the basic electrode. In order to prevent the loss of immobilized HRP under optimized conditions, the biosensor surface was cross-linked with glyoxal. Ferrocene was selected and immobilized on the glassy carbon electrode surface as a mediator. The fabrication procedure was systematically optimized to improve the biosensor performance. The biosensor had a fast response of less than 10 s to H_2O_2, with a linear range of 3.5×10^{-5} to 1.1×10^{-3} M, and a detection limit of 8.0×10^{-6} M based on S/N = 3.

Keywords: horseradish peroxidase, biosensor, hydrogen peroxide, chitosan

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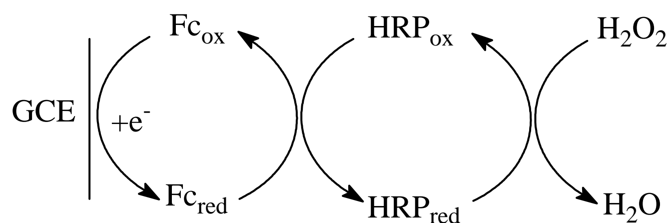
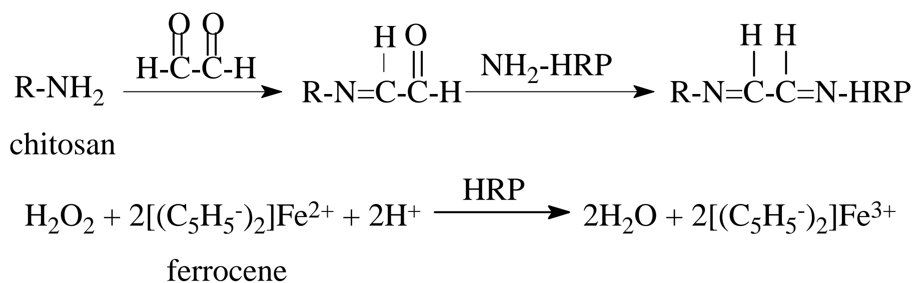
1. Introduction

The determination of hydrogen peroxide is of practical importance in chemical, biological and clinical as well as many other fields. Extensive techniques have been developed for this purpose, amongst which peroxidase modified electrodes are frequently used in recent years, particularly horseradish peroxidase (HRP). In order to improve the stability and activity of the enzyme immobilized on electrode surface, a variety of methods have been tried such as adsorption, entrapment

in a porous matrix, covalent binding and electrochemical copolymerization. Immobilization of enzyme may lead to changes in enzyme structure and hence causes the kinetics, stability and specificity differing from that of the enzyme in homogeneous solution [1]. Therefore, great interest is devoted to find immobilization matrix, which can retain its specific biological function. In recent years, there has been a significant increase of interest in using natural polymers as an immobilization matrix for enzymes, living organisms, cell carriers. Different types of polysaccharides such as agar [2], alginate [3], carrageenan [4], chitin [5] and chitosan [6] have been used as immobilization matrices. Chitosan, a copolymer of glucosamine and N-acetyglucosamine units linked by 1-4 glucosidic bonds, can be obtained by N-deacetylation of chitin, which is the second most abundant natural polymer [7]. Chitosan was selected as the matrix for immobilization of the enzyme because of an unusual combination of its properties, which includes an excellent membrane-forming ability, high permeability toward water, good adhesion, biocompatibility, non-toxicity and high mechanical strength. In addition, chitosan has the susceptibility to chemical modifications due to the abundant in amino groups, which not only facilitate the immobilization of enzymes in chitosan by covalent binding [8] but also adsorb metal ions [9-13] and various organic halogen substances [14,15], then prevents the enzyme from damage. Moreover, chitosan can form a thermally and chemically inert film that is insoluble in water. So chitosan has been widely used in the preparation of sensors in recent years [1,16-22].

Glutaraldehyde was usually used as a protein cross-linking agent in the fabrication of biosensor [6], while little use of glyoxal can be found in this area. The present work was attempt to develop a simple and robust amperometric hydrogen peroxide biosensor based on HRP immobilized by chitosan film cross-linked with glyoxal. We selected the “sandwich” configuration: ferrocene-chitosan: HRP: chitosan-glyoxal. The chitosan film cross-linked with glyoxal permitted high enzyme loadings due to the cross-linking reaction between the amino group of chitosan and aldehyde group of glyoxal. This cross-linking reaction simultaneously improves the stability of the chitosan film. There was no obvious redox reaction between the HRP and the H_2O_2 if no electron mediators existed in the redox system. Among the various mediators, ferrocene is a satisfactory candidate for the amperometric hydrogen peroxide sensor by measuring the reduction current. The one-electron oxidation of ferrocene to the stable ferricenium cation is a simple electron transfer reaction, in that there are no complications arising from adsorption or associated chemical reactions [23]. Therefore, ferrocene is utilized as the mediator for the determination of hydrogen peroxide in many studies [24-30], although the ferricinium as the water-soluble oxidized form of ferrocene is easily leached out from the electrode surface and leads to the instability of the enzyme electrode. In this study, ferrocene and HRP were immobilized on the glassy carbon electrode surface in sequence. We expected that the good membrane-forming ability of chitosan could improve the stability of ferrocene on the electrode. As a mediator, ferrocene accelerated the redox reaction. The mechanism of the electrons transfer is as the following:





2. Experimental

2.1 Apparatus and reagents

HRP (EC 1.11.1.7, RZ > 3.0, 250 u/mg) was purchased from ACROS; chitosan (MW 5.8×10^5, 90.7% deacetylation) was made from chitin (ACROS). The other chemicals were of analytical grade and used without further purification. The supporting electrolyte was 0.02 M phosphate buffer (PBS) of pH 7.0. All aqueous solutions were prepared in doubly distilled water.

Electrochemical measurements were performed with a three-electrode system comprising a platinum wire as auxiliary electrode, a saturated calomel electrode as reference electrode, against which all potentials were quoted, and the modified glassy carbon electrode (3.0 mm diameter) as working electrode. The electrodes were connected to a 273A Potentiostat/Gawanostat (EG&G, USA). All electrochemical experiments were carried out in a conventional electrochemical cell holding 10.0 mL PBS at room temperature. In addition, the ferrocene-modified electrode exhibited almost the same response in air-saturated and nitrogen-bubbled hydrogen peroxide solution, which suggested that the ferrocene-mediated biosensor was insensitive to the presence of dissolved oxygen. Therefore, all the electrochemical processes were in aerated solutions. A magnetic stirrer and a bar provided convective transport during the amperometric experiments.

2.2. Procedures

2.2.1. Preparation of the enzyme electrode

Chitosan solution (1.0%) was prepared by dissolving chitosan powder in 0.8% acetic acid solution; the viscous solution was stirred at 250 rpm for 3 h at room temperature. Glassy carbon electrodes were polished on a leather kit before each experiment with alumina slurry, rinsed thoroughly with doubly distilled water, then washed successively with acetone and doubly distilled water in ultrasonic bath and dried in air.

The biosensor was constructed with the following procedure: 1) 10.0 µL of ferrocene ethanol solution (40 mM) was pipetted onto the surface of the electrode and dried in atmosphere. 2) 5.0 µL of chitosan solution was dropped onto the surface of the electrode, and allowed to dry at room temperature. 3) 10.0 µL of HRP solution (1.0 mg of HRP dissolved in 0.5 mL of 0.02 phosphate buffer, pH 7.0, containing 7 µL of glycerol) was dropped onto the surface of the GCE, and distributed gently over the entire surface to ensure the complete coating of the GCE by the HRP solution (the presence of a small amount of glycerol in the enzyme solution acts as an emollient). Then, the electrode was left to dry at 4° C. 4) 5.0 µL of chitosan solution was dropped to the above dried layer to stabilize and prevent

HRP from dissolving in solution. 5) 5.0 μL 0.025% glyoxal was dropped and allowed to dry at room temperature. Finally, the biosensor was immersed in a pH 7.0 phosphate buffer and kept at 4 °C overnight to remove the excess HRP from the electrode surface. The biosensor was stored in pH 7.0 phosphate buffer at 4 °C when it was not used.

2.2.2 Measuring procedure

Cyclic voltammetric experiments were performed in an unstirred solution at a scan rate of 100 mV s^{-1}. In amperometric experiments, −100 mV was selected as the applied potential, moreover, a steady-state background current had to be obtained before H_2O_2 standard solution was added into the stirred buffer solution.

3. Results and discussion

3.1. Optimization of experimental variables

Ferrocene as a mediator, the effect of its concentration on the biosensor response was investigated by varying the concentration of mediator (10.0-50.0 mM). As shown in Fig. 1, the biosensor response to H_2O_2 increased sharply with the increase of ferrocene concentration from 10 to 40 mM. Typically, when the mediator concentration was low, the biosensor response was limited by the enzyme-mediator kinetics. A further increase of the concentration of mediator resulted in a slight decrease in current response as the response signal was limited by the enzyme-substrate kinetics. Thus, the concentration of mediator was fixed at 40.0 mM for all the subsequent experiments.

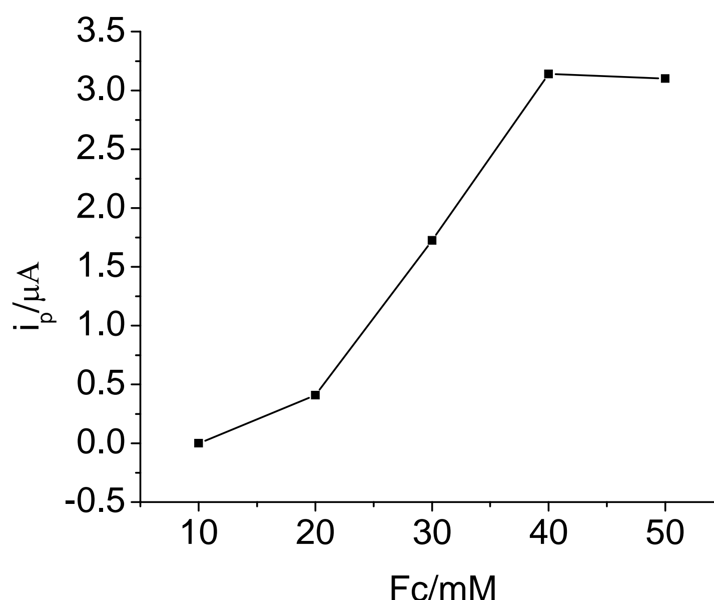


Figure 1. Influence of ferrocene concentration on the biosensor response studied by amperometric measurement for 9.68×10^{-5} M H_2O_2 in 10.0 mL 0.02 M phosphate buffer solution (pH 7.0).

The effect of applied potential on the biosensor response was tested and the results are shown in Fig. 2. The biosensor response to H_2O_2 increased with the change of applied potential from –200 mV to

100 mV. The highest sensitivity was obtained at –100 mV. A further increase of the anodic potential resulted in decrease in current response and then –100 mV was selected as the applied potential in amperometric detection.

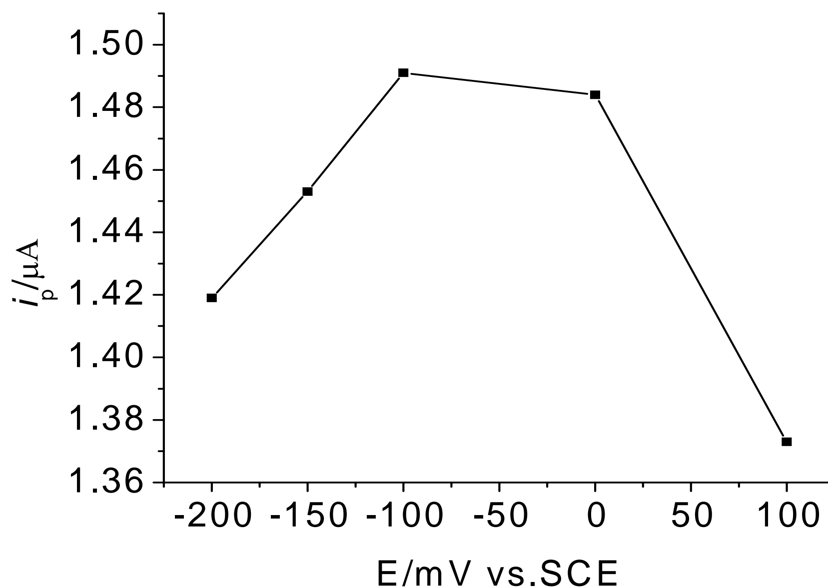


Figure 2. Effect of applied potential on the biosensor response studied by amperometric measurement for 9.68×10^{-5} M H_2O_2 in 10.0 mL 0.02 M pH 7.0 PBS.

The effect of the pH of supporting electrolyte on the biosensor response was tested in the range of 6.0 to 8.5, as shown in Fig. 3.

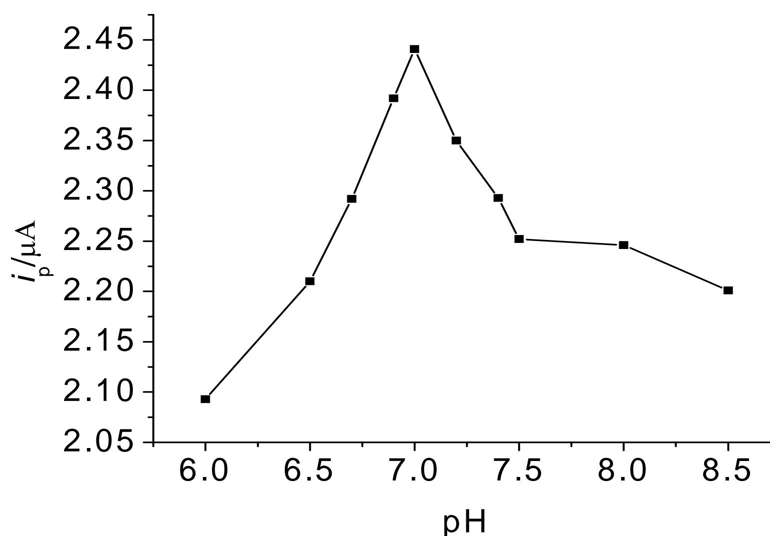


Figure 3. Effect of pH of the buffer solution on the biosensor response was tested with various pH values containing 4.84×10^{-5} M H_2O_2.

From the graph, pH 7.0 was easily selected for the remainder of the experiments. Moreover, pH 7.0 is close to the optimum pH observed for soluble peroxidase [31] and avoids the possible dissolution of the chitosan film under the acidic conditions.

The effect of the concentration of chitosan used for the preparation of the biosensor was investigated by varying the concentration with 0.25%, 0.5% and 1.0%, which is shown in Fig. 4.

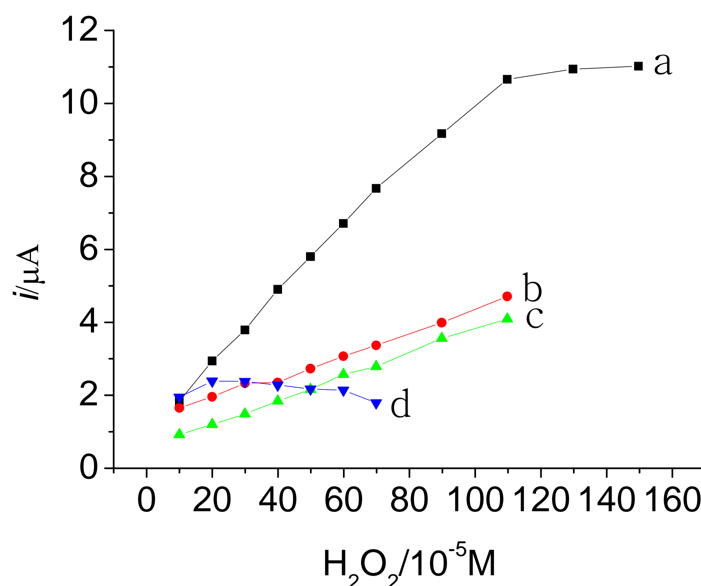


Figure 4. Effect of the chitosan concentration and glyoxal on the biosensor response studied by calibration plots for H_2O_2 in 10.0 mL 0.02 M pH 7.0 PBS. The chitosan film was prepared with a solution: (a) 1% chitosan with 0.025% glyoxal; (b) 0.5% chitosan with 0.025% glyoxal; (c) 0.25% chitosan with 0.025% glyoxal and (d) 1% chitosan without glyoxal.

Because higher concentration of acetic acid would not be favorable for enzyme immobilization, the concentration of acetic acid used for preparing chitosan solution was relatively low (0.8%). Thus, a thicker chitosan film was not tried, as it was difficult to prepare more concentrated chitosan solution with the acetic acid (0.8%). Furthermore, if the concentration of chitosan was higher and the immobilization film was thicker, it would lead to a higher diffusion barrier for the substrate and mediator. However, when the concentration of chitosan was low, the amount of immobilized enzyme was low, and the response of the biosensor was low and saturated rather early. It can be seen from Fig. 4, the biosensor prepared with 1.0% chitosan solution and 0.025% glyoxal (curve a) had a higher current response and wider linear response range than that prepared with 1.0% chitosan solution without glyoxal (curve d). It can be seen also that the response of the biosensor decreased with the decreasing of chitosan concentration in the presence of 0.025% glyoxal (curve b and c). The above results suggest that the improvement of response in the presence of glyoxal could be due to the cross-linking reactions between the amino groups of chitosan and the aldehyde groups of glyoxal, and amino groups of HRP and the aldehyde groups of glyoxal. More amino groups in 1.0% chitosan solution can be cross-linked with the aldehyde groups and thus more HRP can be immobilized on the biosensor. HRP and chitosan could not be cross-linked directly except via the glyoxal acting as a "bridge". Nevertheless, the activity of the enzyme and the stability of the biosensor would be damaged if the concentration of the glyoxal were too high. Therefore, we selected 0.025% glyoxal as the cross linker.

3.2. Voltammetric behavior of the enzyme electrode

The electrochemical behavior of the enzyme electrode was studied using cyclic voltammetry. Fig. 5 shows the cyclic voltammograms of the ferrocene-modified GCE (a) and ferrocene-modified enzyme GCE (b) in 0.02 M pH 7.0 phosphate buffer solution.

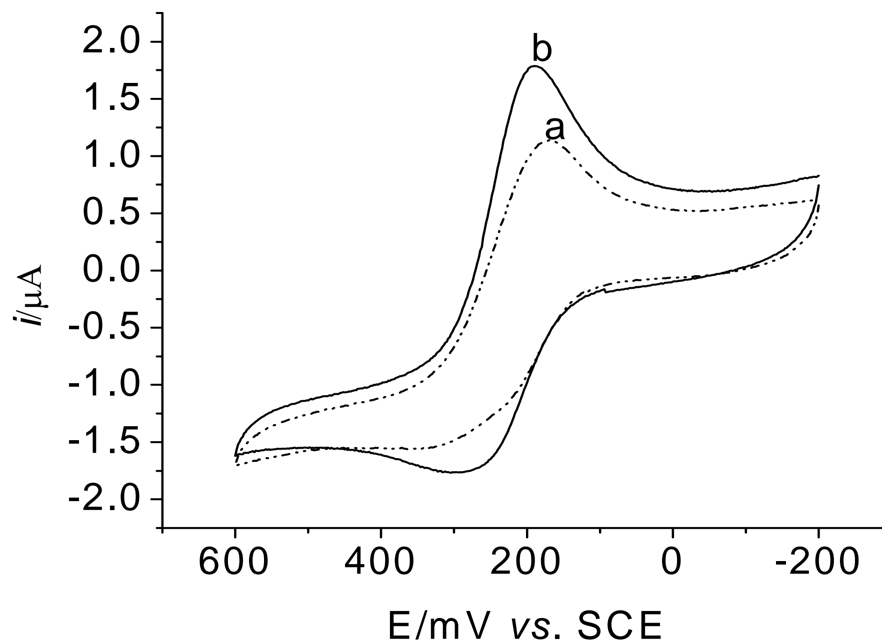


Figure 5. Cyclic voltammograms of CS/Fc electrode (a) and CS/HRP/Fc (b) obtained in 10.0 mL 0.02 M pH 7.0 PBS. Scan rate 100 mV s^{-1}.

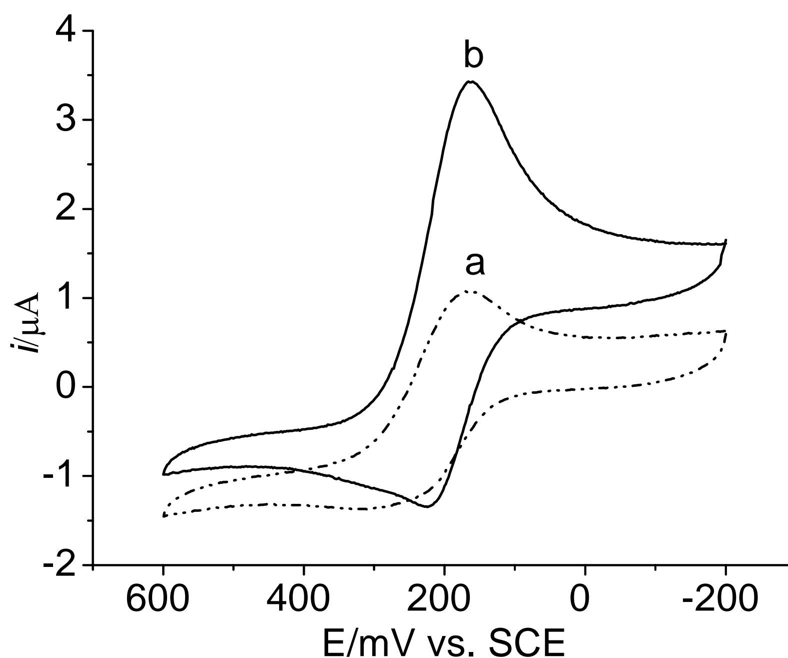


Figure 6. Cyclic voltammograms of CS/Fc electrode (a) and CS/HRP/Fc (b) in 10.0 mL 0.02 M pH 7.0 PBS containing 1.94×10^{-4} M H_2O_2. Scan rate 100 mV s^{-1}.

It can be seen that both the electrodes have only one pair of quasi-reversible redox peaks of ferrocene and the redox peak currents are increased slightly in the presence of HRP. Thus, the presence of HRP is in favor of the electron transfer between ferrocene and electrode surface. The cyclic voltammograms of the ferrocene-modified electrode and ferrocene-modified enzyme electrode in 0.02 M pH 7.0 phosphate buffer solution in the presence of H_2O_2 are shown in Fig. 6. The cathodic peak current of the ferrocene-modified enzyme electrode was increased significantly when 1.94×10^{-4} M H_2O_2 was added (b), which indicates that H_2O_2 oxidizes Fc_{red} to Fc_{ox} in the presence of HRP, and the Fc_{ox} is subsequently reduced at the electrode surface. No obvious increase of the cathodic peak current of the ferrocene-modified electrode was observed in the presence of 1.94×10^{-4} M H_2O_2 (a). These results suggest that HRP can be immobilized well in the chitosan film and remain its bioactivity.

3.3. Amperometric response of the HRP electrodes

Fig. 7 shows the typical current-time response of the HRP biosensor for successive additions of H_2O_2 under the optimized experimental conditions. It is clear that a rapid and sensitive response to H_2O_2 is achieved.

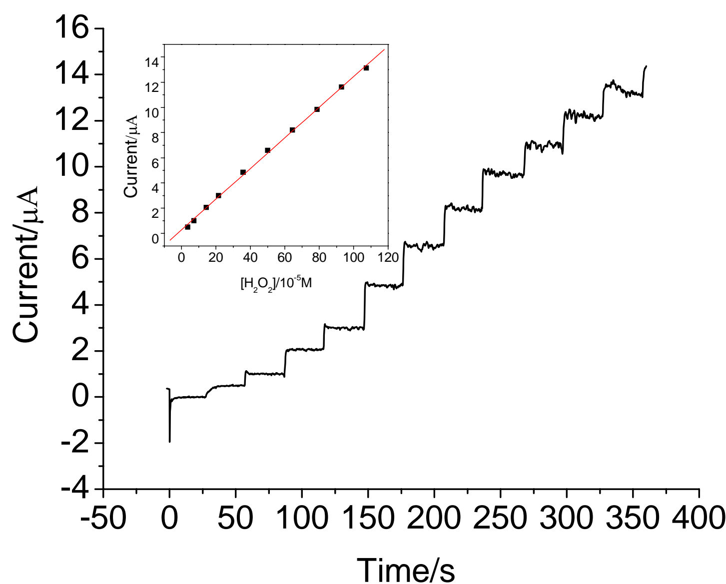


Figure 7. A typical current-time response curve for successive additions of 10, 10, 20, 20, 40, 40, 40, 40, 40, 40 and 50 μL 35.8 mM H_2O_2 for the enzyme electrode in 10.0 mL 0.02 M pH 7.0 PBS. Applied potential: -100 mV. The insert plot shows a calibration curve of current with H_2O_2 concentration.

The insert plot shows that the response of the biosensor to H_2O_2 has a linear relationship in the range of 3.5×10^{-5} to 1.1×10^{-3} M with the regression equation: $i_p/\mu A = 1.22\times10^4\, c + 0.29$ (c in M, r = 0.9998). The detection limit of the biosensor was found to be 8.0×10^{-6} M based on S/N = 3 and the sensitivity of the biosensor was 172.4 μA mM^{-1} cm^{-2} which was higher than that reported before [32]. The apparent Michaelis-Menten constant (K_m^{app}) is calculated to be 2.0 mM according to the Lineweaver-Burk equation, which was smaller than those reported for HRP immobilized by sol-gel/hydrogel film [33]. The high sensitivity and the small K_m^{app} mean that the immobilized HRP possessed a higher enzymatic activity. When the concentration of H_2O_2 was 3.1×10^{-4} M, the relative

standard deviation of six successive determinations was 4.1% (n = 6) with a mean current of 4.2 μA.

3.4. Selectivity against interference and storage stability

Eight possible interfering substances were used to evaluate the selectivity of the HRP electrode. The current obtained for each interfering substance at a concentration of 1.0 mM in the presence of 4.26×10^{-5} M H_2O_2 was used as an indicator for the enzyme electrode selectivity in comparison with the H_2O_2 alone. The results of the interference study are listed in Table 1. Glucose, sucrose, citric acid, oxalic acid, cysteine, nitrate and fluride did not cause any observable interference except ascorbic acid. Ascorbic acid can reduce the $(C_5H_5^-)Fe^{3+}$ produced in the peroxidase catalyzed reaction and thus, interferes in the determination of H_2O_2.

The stability of the HRP sensor was investigated by amperometric measurements in the presence of H_2O_2. No decrease of current was observed after the electrode was tested 20 times continuously. Another, the sensor's stability was examined with intermittent measuring the current response to H_2O_2 standard solution every 5 days in the period of 1 month. The catalytic current response could retain about 75%. The decrease of the current response may be due to the leach of ferrocene from the electrode surface to solution.

Table 1. Possible interferences tested with the enzyme biosensor.

Possible interference	Current ratio[a]
Ascorbic acid	0.21
Cysteine	0.86
Sucrose	0.97
Citric acid	0.98
Nitrate	0.99
Glucose	1.00
Fluride	1.00
Oxalic acid	1.00

[a]Ratio of currents for mixtures containing 1.0 mM interfering substance and 4.26×10^{-5} M H_2O_2 to that for 4.26×10^{-5} M H_2O_2 alone.

4. Conclusions

In this article, we introduced a new type of amperometric H_2O_2 biosensor based on the immobilization of HRP and ferrocene in chitosan matrices with a "sandwich" configuration. The abundant amino groups present in chitosan provide a biocompatible environment for HRP immobilization. The developed HRP sensor exhibits high sensitivity and a fast response to H_2O_2. Moreover, it also shows a very good reproducibility and stability.

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