

## **A H<sub>2</sub>O<sub>2</sub> Biosensor Based on Immobilization of Horseradish Peroxidase in a Gelatine Network Matrix**

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**Abstract:** A simple and promising H<sub>2</sub>O<sub>2</sub> biosensor has been developed by successful entrapment of horseradish peroxidase (HRP) in a gelatine matrix which was cross-linked with formaldehyde. The large microscopic surface area and porous morphology of the gelatine matrix lead to high enzyme loading and the enzyme entrapped in this matrix can retain its bioactivity. This biosensor exhibited a fast amperometric response to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). The linear range for H<sub>2</sub>O<sub>2</sub> determination was from 2.5×10<sup>-5</sup> to 2.5×10<sup>-3</sup> M, with a detection limit of 2.0×10<sup>-6</sup> M based on  $S / N = 3$ . This biosensor possessed very good reproducibility.

**Keywords:** H<sub>2</sub>O<sub>2</sub> biosensor, horseradish peroxidase, gelatine matrix.

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### **Introduction**

During the past few decades, numerous investigations have been carried out on electrochemical biosensors, due to the possibility of combining speed, selectivity and sensitivity in a low cost chemical analysis method [1,2,3]. Our target is to construct the simplest possible biosensor which can be used for measurement purposes, consisting of only a thin layer of protein such as an enzyme or antibody. Many materials, such as chitosan [4,5], agar [6], alginate [7], carrageenan [8], Nafion [9], TCAP [10], DDAB [11], Eastman AQ polymer [12], etc. have been used as immobilization matrices, but only a few reports have been cited in the literature concerning the use of gelatine as a matrix.

Gelatine consists of different amounts of 18 amino acids, where glycine, proline and

hydroxyproline are the most abundant. It is obtained from cattle skins or bones through an alkaline treatment process [13]. Gelatine was selected here because of its unusual combination of properties, which includes excellent membrane-forming ability, good adhesion, biocompatibility, and nontoxicity. The optimized conditions of the enzyme electrode were studied, and the analytical performance of the biosensor was evaluated.

Different factors can impede direct electron transfer between electrodes and proteins, including the deep burial of the electroactive cofactors in the protein structure, adsorption denaturation of the protein at the electrode surface, and unfavorable orientation of the protein structure. In order to resolve the problem, a series of organic dyes have been used as electrode surface modifiers, such as methylene blue, which is a cationic dye whose electrochemical properties are very well known in the solution phase, and the dye has also been used as a redox indicator since its formal potential,  $E^{\circ}$ , is between 0.08 and  $-0.25$  V (vs. SCE) in the solution with pH between 2 and 8. This redox potential is close to that of most biomolecular redox potentials and a modified carbon electrode based on using this dye as an electron mediator system may be of great interest [14]. In this work, methylene blue was used as mediator.

## 2. Experimental

### 2.1. Reagents

Horseshoe peroxidase (HRP, BE 1841) was obtained from Sigma Company (USA). Methylene blue (MB, not purified before use) and  $H_2O_2$  (30% w/v solution) was purchased from Shanghai Chemical Reagent Company respectively. The concentration of the more diluted hydrogen peroxide solutions prepared was determined by titration with cerium (IV) to a ferroin endpoint. All other reagents were of analytical grade. All the solutions were prepared with doubly distilled water.

### 2.2. Apparatus and measurements

All electrochemical experiments were carried out in a three-electrode cell controlled by CHI 660 Electrochemical workstation (CH Instruments, USA). An electrode with gelatine and HRP was used as the working electrode. Reference and counter electrodes were a saturated calomel reference electrode (SCE) and platinum wire, respectively.

### 2.3. Preparation of biosensor

Gelatine solution (1%) was prepared by dissolving gelatine flakes in hot doubly distilled water. Gold working electrodes (0.5 mm diameter) were polished successively with 1.0, 0.3, and 0.05  $\mu$ m alumina powder on chamois leather, and rinsed thoroughly with doubly distilled water between each polishing step. Next, the polished electrode was sonicated in acetone and doubly distilled water and the allowed to dry at room temperature. HRP (0.2 mg) was dissolved in gelatine solution (1%, 0.5 mL) and then formaldehyde solution (10  $\mu$ L) was added into the enzyme solution. The mixture was hand-mixed completely. The gold electrode was coated with a 10  $\mu$ L drop of the resulting mixture, and then was left for at least 24 h at 4°C. HRP was thus entrapped in the gelatine crosslinked membrane. Then the

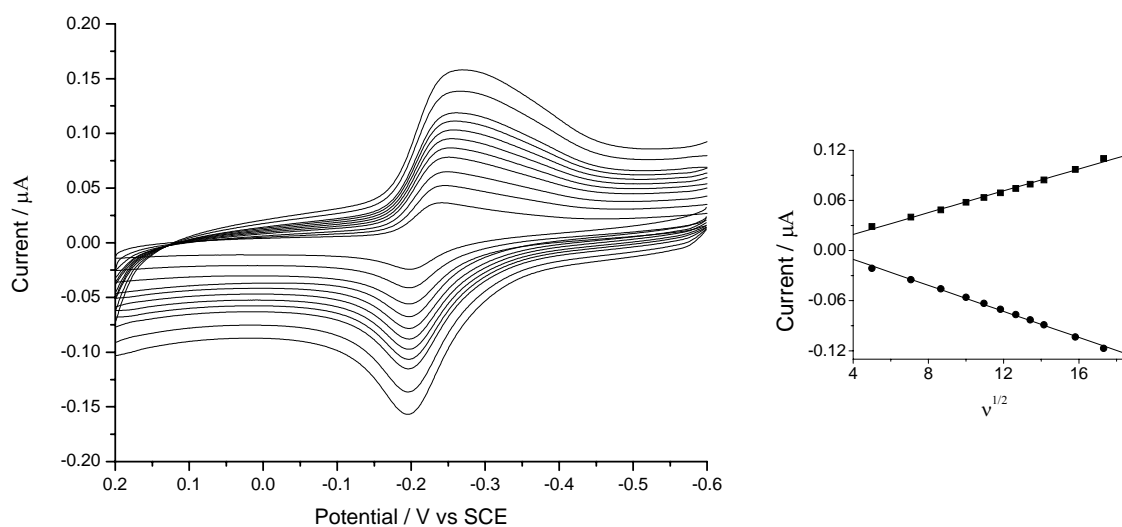
biosensor was immersed into 0.1 mmol/L MB solution. During this process the dye was incorporated into the gelatine matrix. The electrode was then rinsed with doubly distilled water and immersed into the blank PBS solution to remove the non-firmly adsorbed dye molecules until a steady electrochemical response was achieved. The enzyme biosensor was stored at 4°C in a refrigerator when not in use.

### 3. Results and Discussion

#### 3.1. Electrochemical studies

##### 3.1.1. Electrochemical Characteristics of the Coimmobilization of HRP in Gelatine Membrane Modified Au Electrode

Typical cyclic voltammograms of MB/HRP in 0.1 M phosphate buffer solution (pH 7.0) at different scan rates are shown in Figure 1. It is clear that the potentials of the anodic peak and the cathodic peak hardly change with the scan rate,  $\nu$ , i.e., the peak potential is independent of the scan rate in the range between 25 and 300  $\text{mV s}^{-1}$ . Both the anodic peak current and the cathodic peak current are proportional to  $\nu^{1/2}$  at the above scan rate range, suggesting that the electrode reaction is a diffusion-controlled process [15].

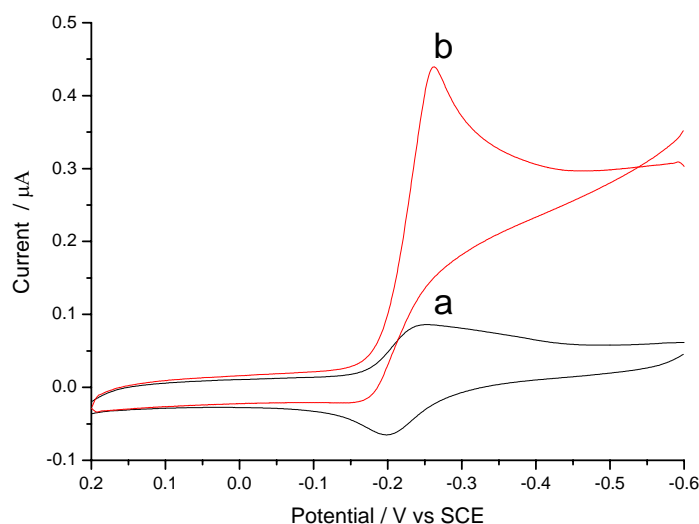


**Figure 1.** Typical cyclic voltammograms of MB-HRP/gelatine and the plot of the wave current at different scan rates (from inner to outer): 25、50、75、100、120、140、160、180、200、250、300  $\text{mV/s}$ .

##### 3.1.2. Electrochemical Response to Hydrogen Peroxide

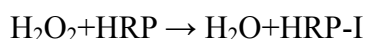
Figure 2 shows the cyclic voltammetric behavior of the enzyme electrode. In blank phosphate buffer,

the enzyme electrode only gives the electrochemical behavior of methylene blue. There is a pair of quasi-reversible anodic and cathodic waves. When 2.5 mM H<sub>2</sub>O<sub>2</sub> was added into the solution, cathodic peak current increased significantly. With an increase of reduction peak current, the oxidation peak current decreased. It was observed that a small peak-potential shifted toward negative direction with the increase of H<sub>2</sub>O<sub>2</sub> concentration. The reaction mechanism of the sensor was summarized as follows: [2]

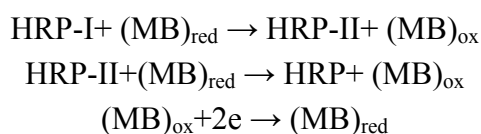


**Figure 2.** Cyclic voltammograms of the H<sub>2</sub>O<sub>2</sub> sensor in the absence of H<sub>2</sub>O<sub>2</sub> (a), and in the presence of 2.5 mM H<sub>2</sub>O<sub>2</sub> (b) at a scan rate of 100 mV/s in pH 7.0 PBS.

The HRP reduces hydrogen peroxide to water.



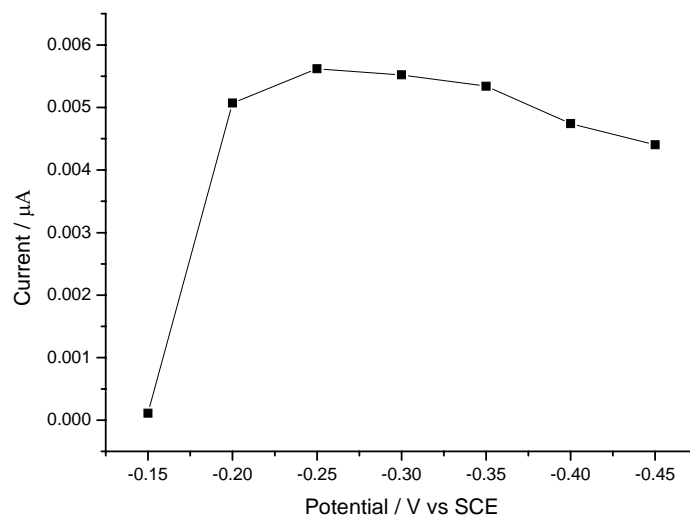
HRP can be regenerated by using a mediator through two separate one-electron steps,



The MB can then be recycled at the electrode as the mediator leading to an increase of its reduction current, which indicated that methylene blue incorporated in this matrix could effectively shuttle electrons between the Au electrode and the bioactive center of HRP in the membrane.

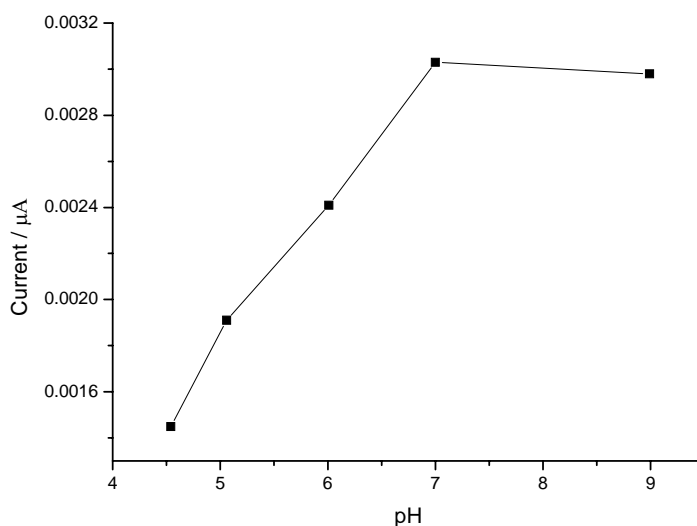
### 3.1.3. Optimization of Hydrogen Peroxide Monitoring

In order to determine the optimal working potential for the H<sub>2</sub>O<sub>2</sub> sensing, a plot of chronoamperometric current vs. working potential was made as shown in Figure 3. One may observe in Figure 3 that the steady-state current changed with an increase of applied potential from −0.15 to −0.5 V, and when the potential reached −0.25 V, the electroreduction of H<sub>2</sub>O<sub>2</sub> reached a plateau. Hence, the potential of −0.25V was selected as optimized monitoring potential.



**Figure 3.** Effect of working potential on the response of the biosensor. Experimental conditions: 0.05 mM  $\text{H}_2\text{O}_2$  in pH 7.0 PBS

The effect of pH on the enzyme electrode response was also investigated in the pH range 4.5~9.0 in the presence of 0.05 mM  $\text{H}_2\text{O}_2$  at working potential  $-0.25$  V (Figure 4). It could be observed that the current response of the electrode was suitable in the pH range 7.5-9, however, it dropped quickly from pH 7.0 to 4.5. Therefore, pH 7.0 phosphate buffer solution was selected in the experiments, this value is the optimum active spot for soluble peroxidase [16].

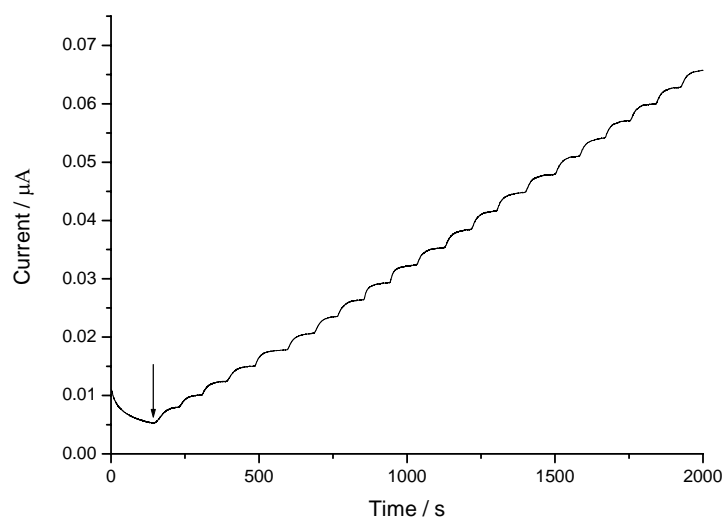


**Figure 4.** Effect of the pH on the response of the biosensor to 0.025 mM  $\text{H}_2\text{O}_2$  in PBS at the working potential of  $-0.25$  V.

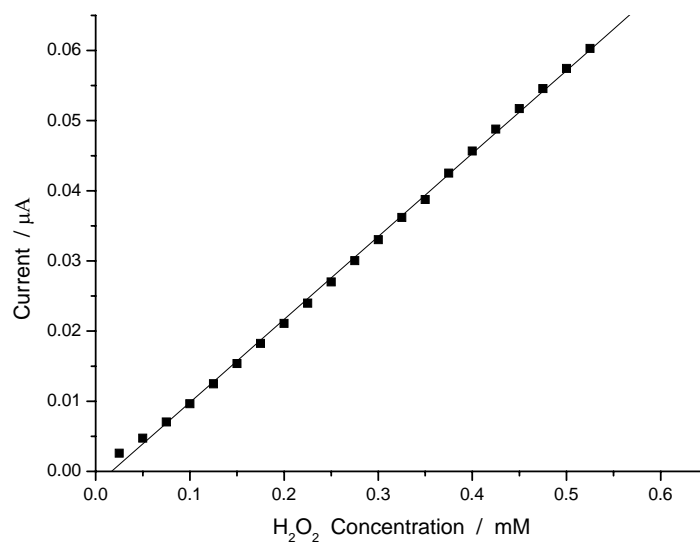
#### 3.1.4. Steady-State Amperometric Response to Hydrogen Peroxide

Figure 5 displays the dynamic response of the electrode under the optimal experimental conditions

with successive injections of  $\text{H}_2\text{O}_2$  to the phosphate buffer solution under stirring. The trace clearly demonstrates the fast response and high sensitivity of the electrode to  $\text{H}_2\text{O}_2$ . The response to  $\text{H}_2\text{O}_2$  is linear in the range from  $2.5 \times 10^{-5}$  to  $2.5 \times 10^{-3}$  M (Figure 6). The detection limit was  $2.0 \times 10^{-6}$  M when the signal to noise ratio is 3.



**Figure 5.** Dynamic response of the  $\text{H}_2\text{O}_2$  sensor to successive addition of 0.025 mM  $\text{H}_2\text{O}_2$  steps in the solution at the applied potential of  $-0.25$  V.



**Figure 6.** Calibration plot between current and  $\text{H}_2\text{O}_2$  concentration.

## Conclusions

In this paper, horseradish peroxidase was successfully entrapped in gelatine to make a H<sub>2</sub>O<sub>2</sub> sensor. The good biocompatibility of gelatine can make the HRP retain its native state in the films and show good electrocatalytic activity. Cyclic voltammetry and chronamperometric measurements have been used to illustrate the feasibility of MB in gelatine matrix as an electron transfer mediator between immobilized HRP and a gold electrode. This biosensor exhibited a fast amperometric response to H<sub>2</sub>O<sub>2</sub>. Work to exploit other enzyme electrodes using the new immobilization material is in progress.

## Acknowledgements

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