

FABRICATION OF A HYDROGEN PEROXIDE BIOSENSOR BASED ON A SELF-ASSEMBLE COMPOSITE OXIDE FILM

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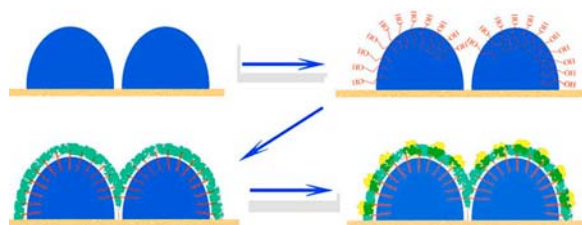
1. ABSTRACT

A titania film with horseradish peroxidase (HRP) was immobilized on indium tin oxide (ITO) electrodes to act as a biosensor of hydrogen peroxide via the self-assembling and sol-gel route. The film was characterized by the atomic functional microscopy, electrochemical and UV-vis spectroscopy methods. The resulting biosensor exhibited fast amperometric response and good stability to hydrogen peroxide (H_2O_2). The linear range for H_2O_2 determination was from 2×10^{-6} to 1.45×10^{-3} M, with a detection limit of 4×10^{-6} M based on $S/N=3$. The apparent Michaelis-Menten constant of the biosensor to H_2O_2 was estimated to be 1.5 mM, showing the HRP on the films had the higher biologic activities.

2. INTRODUCTION

The method of enzyme immobilization is very important for the construction of a good biosensor. The sol-gel process attracted much attention owing to its advantages such as physical rigidity, chemical inertness, high photochemical and thermal stability and negligible swelling in both aqueous and organic solution (1). The surface sol-gel process is an excellent chemical method for the fabrication of metal oxide nanocomposite films (2-4). The TiO_2 as one of the sol-gel matrices has been used to immobilize the enzyme to prepare the biosensors (5-7). TiO_2 can be widely used as a substance to support the biomolecules because of its high biocompatibility, and the TiO_2 nanostructure have been proved that it can adsorb

Fabrication of a hydrogen peroxide biosensor



Scheme 1. The schematic representation of possible growth mechanism of the modified process.

the enzyme and enhance the active surface area (8-10). TiO_2 matrices could be obtained through the hydrolysis of titanium alkoxide. The problem of this method is that the process of evaporation of sol-gel needs a long time to prevent the film from cracking. On the other hand, in order to obtain a suitable titanium alkoxide solution, complexing molecules or acids was added to act as stabilizing agents to hinder the condensation reactions in traditional ways, which leads to the rapid formation of a dense inorganic network yielding poorly structured materials (11-12). Meanwhile the existence of those complexes and acids could destroy the activity of biomolecules.

ITO electrode was the conductive glass covered with the nanostructured SnO_2 film. Some biosensors have been prepared by modifying the ITO electrode surface. The prepared process often firstly derived the -OH groups on the SnO_2 surface by the chemical methods. The -OH groups on the surface of materials can form the Ti-O bond in the hydrolysis process of sol-gel (7). So the TiO_2 sol-gel film can be combined with the SnO_2 on the ITO electrode surface by the Ti-O bond. This bond effect will make the film to strongly immobilize on the electrode. The composite structure of nano-sized TiO_2 - SnO_2 combined the properties of SnO_2 and TiO_2 together to form the enzyme biosensor.

In this work, A novel route that combined the self-assembling and sol-gel technique was used to fabricate a biosensor. Firstly, the electrode surface was modified with hydroxyl, and then immersed into the solution of $\text{Ti}(\text{O}^n\text{Bu})_4$. Then the $\text{Ti}(\text{O}^n\text{Bu})_4$ hydrolyzed into TiO_2 in the HRP enzyme solution. The combination of forming titania film and the enzyme fixing could save a lot of time and the whole process became controllable. This process made the enzyme immobilize on the surface of electrode well and the fabricated biosensors had fast response and good stability.

3. EXPERIMENTAL DETAILS

3.1. Reagents and Apparatus

Horseradish peroxidase (HRP, BE 1841) was obtained from Sigma (USA). $\text{Ti}(\text{O}^n\text{Bu})_4$, Ortho-aminophenol (OAP) and H_2O_2 (30% w/v solution) were purchased from Shanghai Chemical Reagent Company(Shanghai, China). ITO electrodes were obtained from Asahi glass in Japan. Phosphate buffer solution (PBS) was prepared for detection. All the other reagents used in the experiments were of analytical grade, and were used without further purify. All the solutions were prepared using doubly distilled water.

CHI660 Electrochemistry workstation (CHI Co. USA) was used for electrochemical measurements. All measurements were carried out by using a three-electrode system consisting of an ITO electrode as the working electrode, a saturated calomel electrode as the reference electrode (SCE), and a platinum wire as the counter electrode. A magnetic stirrer and a stirring bar provided the convective transport for the amperometric experiments. All experiments were carried out in a thermostated at $30^\circ\text{C} \pm 0.5^\circ\text{C}$. All experimental solutions were deoxygenated before measurement. UV spectra were obtained in the range of 300nm-600nm on a type BRAIC 1200 UV instrument (Beijing, China) with quartz cuvette (path length 1 cm) at room temperature. The AFM images were recorded on a SPI 3800N atomic force microscope.

3.2. Fabrication of the H_2O_2 biosensor

The ITO electrode was immersed into the acetone, alcohol, and doubly distilled water in turn, washing with ultrasonic for 15 min. After the electrodes were cleaned, they were put into 5 mol/L NaOH solution for 5-6 hours to make the electrode surface with the hydroxy film, and then dried by flushing with nitrogen gas. A titania film with HRP was deposited on the electrode by immersing the solid substrate into 100 mM $\text{Ti}(\text{O}^n\text{Bu})_4$ in 1:1 (v/v) toluene/ethanol for 3 min, followed by rinsing in ethanol for 1 min, and then the electrodes were dipped into the 2 mg/mL HRP solution for about 90 minutes.

4. RESULT AND DISCUSSION

4.1. AFM observations of the biosensor

The surface morphology was study with AFM technique in preparing process of the biosensor. It is very an important factor to the biosensor performance.

The possible growth mechanism could be illustrated as follows and a schematic representation of the procedure is shown in Scheme 1. The AFM observations (Figure 1) revealed the true situation of the electrode surface in the procedures. Figure 1a shows an AFM image of the ITO electrode surface. The surface shows the characteristic grain-subgrain microstructure, with an average grain size of 50-100nm, which may be the nanostructured SnO_2 on ITO electrode. After the electrode was immersed into 5 mol/L sodium hydroxide solution, the -OH groups were attached on the ITO surface. This result is shown in figure 1b. Then the ITO electrode was immersed in the $\text{Ti}(\text{O}^n\text{Bu})_4$ solution, and put it into ethanol for 1 min, at last the modified electrode was immersed into 2 mg/ml horseradish peroxidase solution for about 90 min. There were two processes happened in the enzyme solution. The first step is to make $\text{Ti}(\text{O}^n\text{Bu})_4$ hydrolyze into TiO_2 . The hydroxyl covering on the electrode surface could provide an ideal matrix to form the Ti-O bond, thus the film could attach on ITO electrode tightly and regularly. On the other hand, because the TiO_2 sol-gel formed Ti-O-Ti bond to become a reticular structure in the process of hydrolyzation, when the electrode was in the horseradish peroxidase solution, the biomolecules could entrap in the network of sol-gel or adsorb on the electrode surface. This

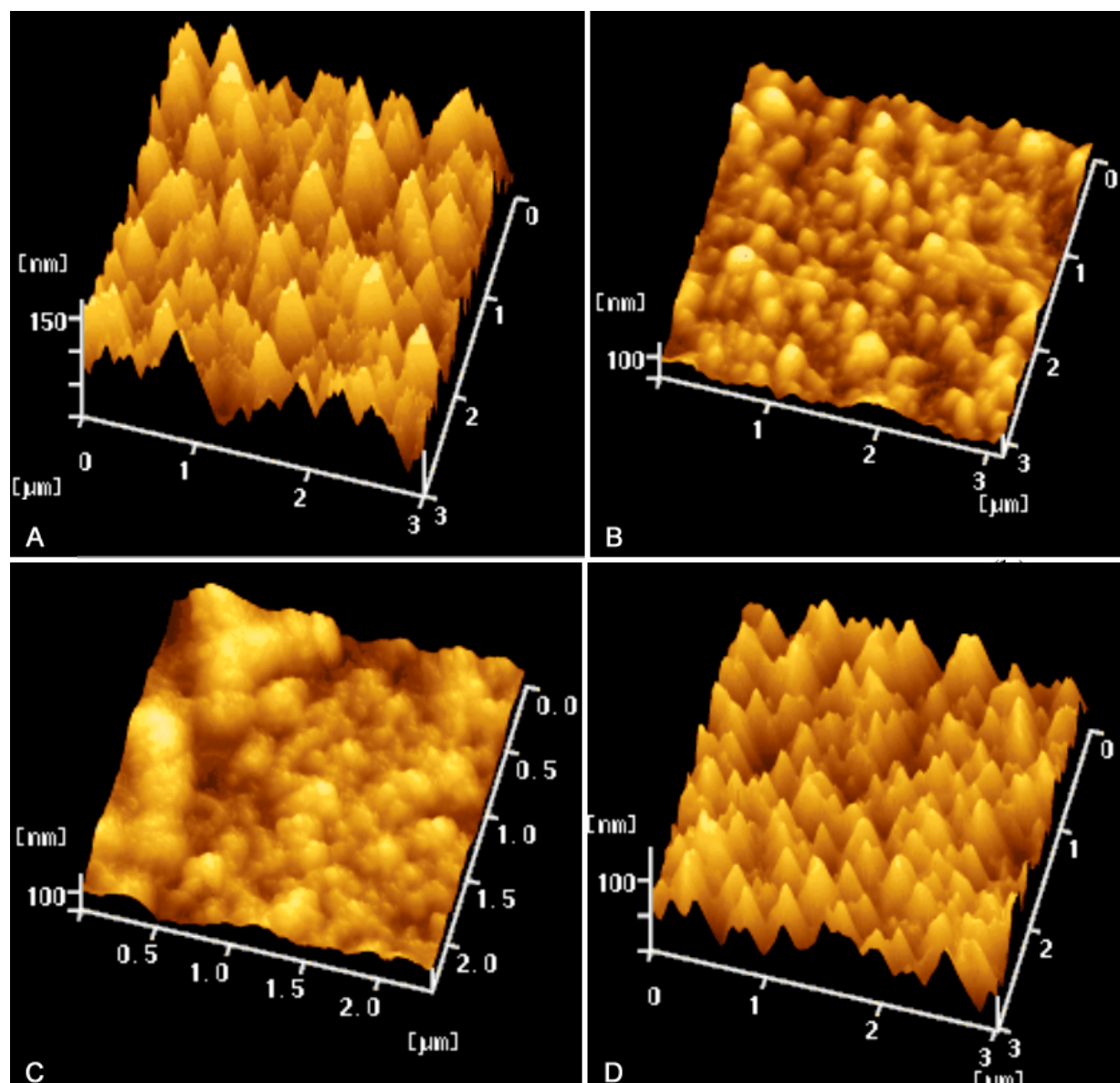


Figure 1. AFM images of electrode surface. (a) bare ITO electrode surface , (b) the electrode surface after immersing the ITO electrode in 5.0 mol/L NaOH for 5 hours, (c) the electrode surface of TiO₂ with HRP, (d) the electrode surface of TiO₂ film, putting the hydroxylated electrode into 100 mM Ti(OnBu)₄ (1:1 ethanol and toluene) for 3 minutes, then put it into ethanol and pure water for 1 minute respectively.

result could make the biomolecules immobilize on the electrode surface stably. In figure 1c, we can observe that those white bright parts are biomolecules. It is obvious that the enzyme immobilized on the surface of the titania film well. In order to explain the existence of TiO₂ on electrode surface, the electrode with Ti(OⁿBu)₄ was put into aqueous solution to hydrolyze into TiO₂, it was found that the existence of nanosized TiO₂ on the electrode (Figure 1d).

4.2. UV-visual absorption spectra

In order to detect the activity of HRP immobilized on the electrode, the spectrophotometric method described in the Section 2 was employed. For this

purpose, The electrode was immersed in a spectrophotometric cuvette containing OAP (0.5 mM) and H₂O₂ (0.1 mM) for 20 min, then it was removed from the solution, and the absorption curve resulted from the generation of the oxidized OAP was recorded. Figure 2 showed the UV-visual absorption after the ITO electrode with TiO₂ film dipped into 2 mg/mL HRP solution for a period of time. It illuminates that when the electrode with TiO₂ film immersed into the enzyme solution for about 90 minutes, the absorption reached the maximum stabilization. Therefore, we immersed the electrodes into the enzyme solution for about 90 minutes. In all cases there was an absorption band at 425 nm due to the formation of the

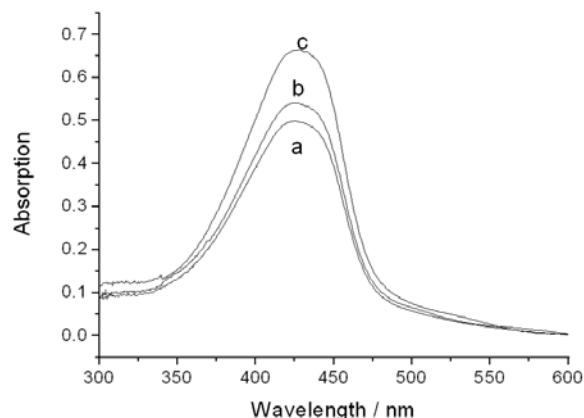


Figure 2. UV-Visual absorption spectra of solutions containing OAP (0.5 mM) and H_2O_2 (0.1 mM) in 5.0 mM pH 7.0 PBS in the presence of HRP. Different curves correspond to the ITO electrode modified with TiO_2 film dipped into HRP solution for different time: (a) 30 minutes, (b) 60 minutes, (c) 90 minutes.

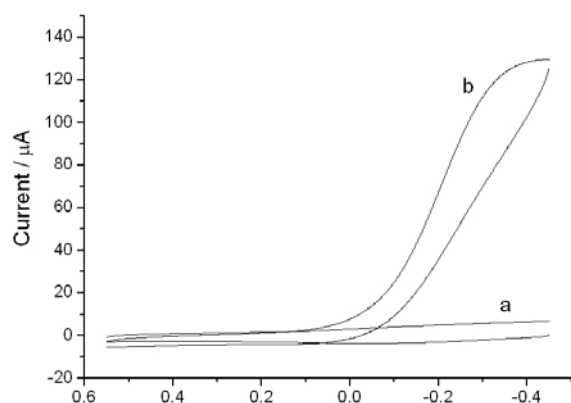


Figure 3. Cyclic voltammograms of HRP/ TiO_2 /ITO in 0.1 M pH = 7.0 PBS in the absence (a) and in the presence (b) of 0.45 mM H_2O_2 .

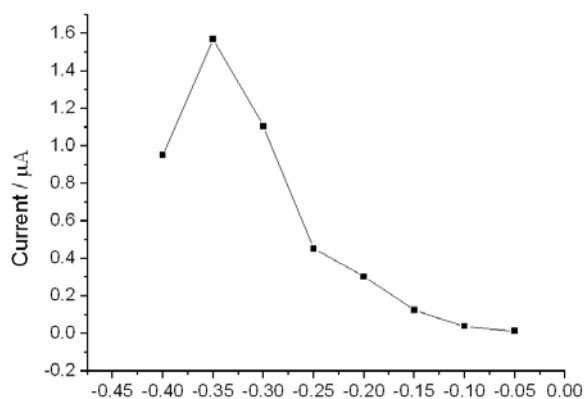


Figure 4. Effect of the working potential on the response of the sensor. Experimental conditions: 0.10 mM H_2O_2 in pH=7.0 PBS.

oxidization of OAP whose concentration could be correlated to the amount of active enzyme occurred.

4.3. Electrochemical research

Figure 3 showed cyclic voltammetric behavior of the enzyme electrode in the 0.1 mol/L pH=7.0 PBS. In the absence of H_2O_2 , there was no obvious current was observed (Figure 3a). When 0.45 mM H_2O_2 was added into the solution, a large catalytic current can be observed (Figure 4b). To verify whether the current was due to nonenzymatic reduction of H_2O_2 , an experiment in the absence of HRP was performed. Only a small catalytic current response could be observed at the modified electrode in the absence of HRP. However, the enzyme electrode could catalyze the hydrogen peroxide quite well. These results corresponded to the former report of Dong group (13). The appearance of the catalytic current may be due to the direct electron transfer from the HRP molecules to the bulk electrode surface, because the HRP molecules could immobilize in the sol-gel well.

4.4. Optimization of experimental variables

In order to determine the optimal working potential for H_2O_2 sensing, a plot of chronoamperometric current vs. working potential was made. It was observed that the steady-state current changed with an increase of applied potential from -0.05 V to -0.35V (figure 4). The maximum response of the biosensor to H_2O_2 was -0.35V. However, the response at -0.30 V was also good, and the noise was less than the potential -0.35V. Hence, the potential of -0.30V was selected as the optimized monitoring potential.

The effect of pH on the sensor was investigated in the pH range from 4.3 to 9.0 in the presence of 0.05 mM H_2O_2 . Figure 5 showed the effect of pH on the performance of the HRP sensor. When the pH was lower or higher, the sensors exhibited low response current. It can be observed that the current response of the electrode was suitable in the pH range 5~7.5; however, it dropped quickly from pH value 7.5 to 9.0. Therefore, we chose pH = 6.0 phosphate buffer solution in the experiments.

The effect of temperature on the biosensor was examined between 15 and 55 °C as shown in figure 6. It was observed that an increase of temperature enhanced the sensitivity of the electrode to hydrogen peroxide, which reached a maximum value at 30°C. However, the further increasing temperature led to a decrease of the response possibly. The experimental results showed the best optimum temperature for the sensor was 30°C. At this temperature it was more suitable for the existence of the enzyme and activities of the enzyme could be kept well.

4.5. The dynamical research

Figure 7 displayed a typical current-time response curve of HRP- TiO_2 sensor for successive additions of H_2O_2 under the optimal experimental conditions. A well-defined, stable and fast amperometric response could be observed at -0.3 V with successive injections of 0.005 mM H_2O_2 for ten times, 0.05 mM H_2O_2

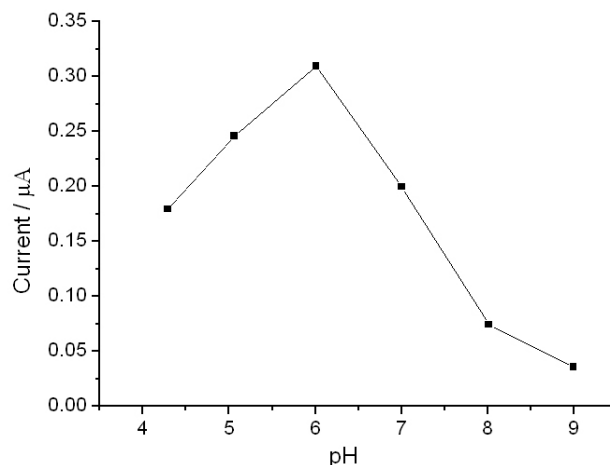


Figure 5. Effect of the pH on the response of the sensor to 0.05 mM H₂O₂ in PBS.

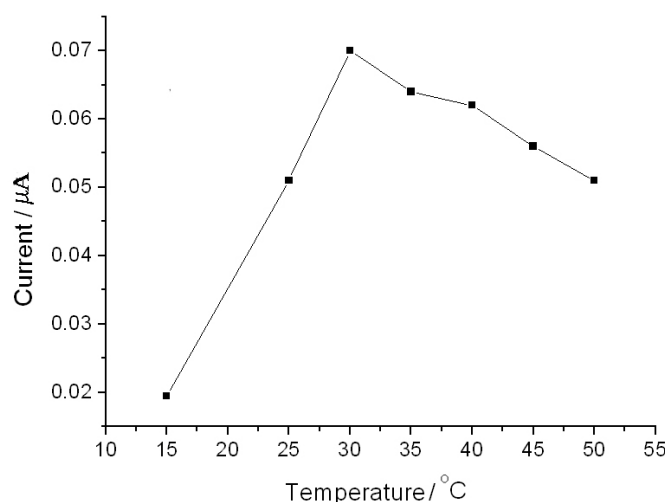


Figure 6. The effect of temperature on the response of the sensor to 0.05 mM H₂O₂ in pH = 7.0 PBS.

for ten times, 0.1 mM H₂O₂ for ten times and 1 mM H₂O₂ for six times into phosphate buffer solution and the time required to reach the maximum response was less than 5 s. The insert is the linear range spans the concentration of HRP from 0.002 to 1.45 mmol/L. The detection limit was 10⁻⁶ M when the signal to noise ratio is 3. It was clear that the rapid and sensitive response to H₂O₂ was achieved. Such a fast response can be attributed to the fast diffusion of the substrate molecule in the thin film. The AFM image of the enzyme electrode was shown in Figure 1c, indicating that the TiO₂ film can sustain the enzyme well. Different from previous reports, the enzymes were entrapped in the TiO₂ film when the Ti(OⁿBu)₄ was hydrolyzing. Therefore the enzyme was able to promote the electrode transfer, which could improve the sensitivity of this biosensor. Figure 8 showed a typical calibration graph of the steady-state current versus H₂O₂ concentration. The apparent Michaelis-Menten constant (K_M^{app}) is generally used to evaluate the biological activity of immobilized enzyme and it could be calculated according to the Michaelis-Menten equation (14):

$$K_M^{\text{app}} = \frac{i_{\text{max}} - i}{i} \times C$$

where i is the steady-state catalytic current, i_{max} is the maximum current measured under saturated substrate conditions, C refers to the HRP concentration and K_M^{app} stands for the apparent Michaelis-Menten constant of the system as a whole. In this work, the K_M^{app} was evaluated as 1.5 mmol/L. It suggested the HRP enzyme on the TiO₂ films had the higher biologic activities. Additionally it also revealed that the immobilized HRP on the films retained its native activity well.

5. CONCLUSION

In this paper, several merits of utilizing of the method should be highlighted. First, the as-prepared TiO₂ and HRP were immobilized on electrode, and quite different from those methods that TiO₂ adsorb HRP in structure. Therefore, the thin film with enzyme could

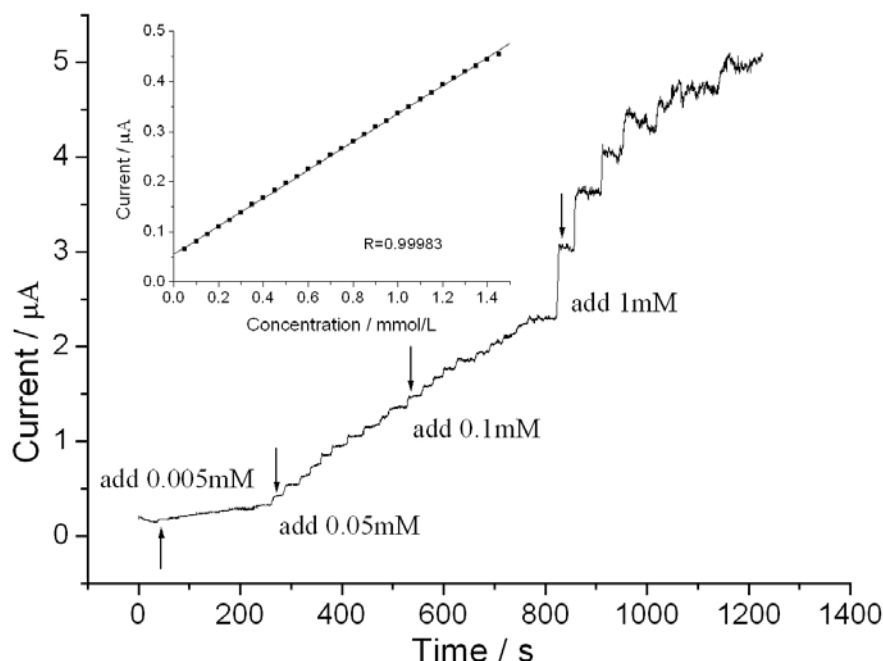


Figure 7. A typical current–time curve of HRP-TiO₂ electrode for successive addition of different concentration of H₂O₂ in 0.1 M deoxygenated phosphate buffer solution (pH=6.0) at the applied potential of –0.3V vs. SCE at 30 °C. The inset is calibration plots for the biosensor for HRP from 0.002 to 1.45 mmol/L.

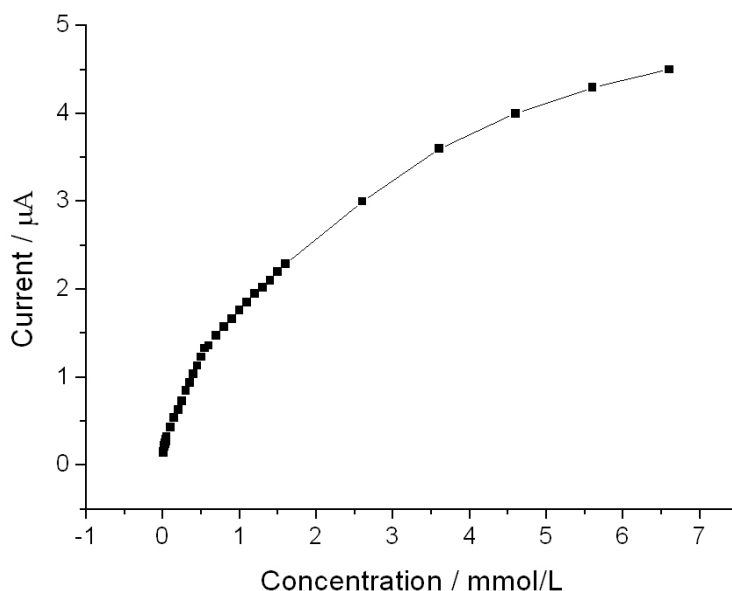


Figure 8. Calibration curve between current and H₂O₂ concentration. Experimental conditions as in Figure 7.

effectively facilitate the transfer of electrons. Second, the sensor can provide a two-dimensional interface to adsorb the enzyme, therefore, it could increase the enzyme loading. Third, the enzyme could be successfully entrapped into TiO₂ film. Thus it can avoid HRP leaching from the surface of electrode under the operation conditions. The resulting biosensor exhibits high sensitivity and good stability implying that this sensor can provide a suitable microenvironment for enzyme.

6. ACKNOWLEDGEMENTS

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