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Talanta 68 (2006) 721–727
Talanta
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Composite film of carbon nanotubes and chitosan for preparation of amperometric hydrogen peroxide biosensor

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Received 21 January 2005; received in revised form 10 May 2005; accepted 20 May 2005
Available online 15 July 2005

Abstract

A new amperometric biosensor for hydrogen peroxide was developed based on cross-linking horseradish peroxidase (HRP) by glutaraldehyde with multiwall carbon nanotubes/chitosan (MWNTs/chitosan) composite film coated on a glassy carbon electrode. MWNTs were firstly dissolved in a chitosan solution. Then the morphology of MWNTs/chitosan composite film was characterized by field-emission scanning electron microscopy. The results showed that MWNTs were well soluble in chitosan and robust films could be formed on the surface. HRP was cross-linked by glutaraldehyde with MWNTs/chitosan film to prepare a hydrogen peroxide biosensor. The enzyme electrode exhibited excellent electrocatalytic activity and rapid response for H_2O_2 in the absence of a mediator. The linear range of detection towards H_2O_2 (applied potential: −0.2 V) was from 1.67×10^{-5} to 7.40×10^{-4} M with correction coefficient of 0.998. The biosensor had good repeatability and stability for the determination of H_2O_2. There were no interferences from ascorbic acid, glucose, citrate acid and lactic acid.

Keywords: MWNTs; Chitosan; HRP; Electrocatalysis; H_2O_2

1. Introduction

Since carbon nanotubes were discovered in 1991 [1], they have been studied and used in many fields because of their unique properties. Carbon nanotubes include two different types: single-wall carbon nanotubes (SWNTs) and multiwall carbon nanotubes (MWNTs). About these two types of nanotubes, many papers have been reported [1–5].

The solubility and chemical modification of carbon nanotubes attract considerable interests [6–9]. By surface modification, new properties of carbon nanotubes can be observed and the resulted new materials will be applied widely. The solubility of carbon nanotubes is very important. The resolved methods are divided into two categories: direct solubility of carbon nanotubes in solvents and the attachment of functional groups to carbon nanotubes. The first category is very convenient and simple. Raw carbon nanotubes are insoluble in water and many other solvents. But carbon nanotubes after purification can form black suspensions in ethanol, DMF, CTAB [10–11], Nafion [12], 3-aminopropyltriethoxysilane [13], etc. The excellent solubility in these solvents facilitates the application of carbon nanotubes in many fields. Wang et al. [12] reported that MWNTs dissolved in Nafion could be applied to construct amperometric sensor for hydrogen peroxide. It has been reported that SWNTs pretreated by H_2SO_4/H_2O_2 solution were soluble in buffer solutions with various values of pH [14]. Thionine can also improve the solubility of SWNTs by strong interaction between them [7]. The primary amine of thionine attached onto the surface of SWNTs can be used to further modify SWNTs. In the second category, functional groups are attached to carbon nanotubes by the carboxylic acid formed in acidic pretreatment. These functional groups can improve the solubility of carbon nanotubes in common organic solvents.

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0039-9140/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2005.05.030

With good electronic properties and electric conductivity, carbon nanotubes have been applied in electrochemical research [15–23]. The direct electron transfer of enzymes such as cytochrome c [23], glucose oxidase [10], catalase [24], horseradish peroxidase, myoglobin [25] and hemoglobin [11] in the presence of carbon nanotubes can be observed. Carbon nanotubes also show electrocatalytic activities towards H_2O_2, NADH, ascorbic acid, dopamine, catechol, homocysteine [12,26–28]. The good catalytic activities towards these molecules open their applications in amperometric sensors.

Chitosan is a biological cationic macromolecule with primary amines. It has been widely applied because of its good biocompatibility and film-forming ability. Because of abundant amines which posed high affinity for nanoparticles, gold nanoparticle film formed on the electrode has been used to immobile enzymes towards determination of hydrogen peroxide and glucose [29].

Zhang et al. [30] reported that carbon nanotubes dissolved in biocompatible chitosan could be applied for electrochemical measurements based on dehydrogenase enzymes. Luo et al. [31] reported that nanocomposite of chitosan and carbon nanotubes could be electrodeposited and used for biosensors. Compared with other solvents, chitosan can prevent biological molecules from denaturing. Moreover, enzymes can be attached to chitosan molecules by the primary amines of them. Glutaraldehyde is often used to link enzymes and chitosan and many biosensors have been developed by this method. A hydrogen peroxide biosensor has been reported [32]. But it was used to determine H_2O_2 in the presence of a mediator. Here, the work was firstly reported to develop a hydrogen peroxide biosensor based on HRP cross-linked by glutarahyde with MWNTs/chitosan composite film on a glassy carbon electrode (GCE). Moreover, hydrogen peroxide could be measured in the absence of a mediator. MWNTs were dissolved in a chitosan solution and then MWNTs/chitosan solution was dropped onto the surface of GCE and this resulted in composite film of MWNTs/chitosan formed on the electrode. Glutaraldehyde was used as the cross-linked reagent to attach HRP onto the composite film. Electrochemical property of MWNTs/chitosan and electrocatalytical activity of the enzyme electrode were characterized by cyclic voltammetric methods. The amperometric response for H_2O_2, repeatability and stability of the enzyme electrode were also studied.

2. Experimental

2.1. Chemical and reagents

Low molecular weight chitosan (Mw 4000) was obtained from Aldrich and used as received. HRP ($RZ \geq 3.0$, $250\,U\,mg^{-1}$) was from Sino-American biotechnology Co. (Beijing, China) and used without further purification. Glutaraldehyde was obtained from ACROS and diluted to 7% before use. MWNTs purchased from Shenzhen Nanotech. Port. Co. Ltd. (Shenzhen, China) were purified according to the reported literature. MWNTs were purified and shortened by fluxing in 3 M HNO_3 for 48 h. The purified MWNTs were characterized by field-emission scanning electron microscopy (FE-SEM) (Fig. 1A) and the average diameter is 20–60 nm. Hydrogen peroxide (30%) was from Beijing Chemical Reagent Co. (Beijing, China). Unless otherwise stated all chemicals and reagents used were of analytical grade. All solutions were prepared using double distilled water. The phosphate buffer solutions were used as supporting electrolytes by mixing solution Na_2HPO_4 and NaH_2PO_4.

2.2. Apparatus

Cyclic voltammetric and amperometric experiments were carried out with a CHI 630B (Shanghai, China). A three-electrode system was employed with a GCE as working electrode, an Ag/AgCl (saturated potassium chloride) as reference electrode, a platinum foil as counter electrode. All solutions were deoxygenated by pure nitrogen for at least 15 min, and a continuous flow of nitrogen was maintained in the experiments.

The FE-SEM images were obtained on PHILIPS XL-30 ESEM. The samples for FE-SEM were prepared by drop-casting one drop of the MWNTs/chitosan ($10\,mg\,ml^{-1}$) solution onto the indium-doped tin oxide (ITO) conductive glass and dried in air before experiments. The samples were coated with thin Au film to well characterize the film of MWNTs/chitosan.

2.3. Procedures

2.3.1. Preparation of the MWNTs/chitosan coated electrode

A 0.5 wt.% chitosan solution was prepared according to the reported method [30]. A 5 mg chitosan was dissolved in 1 ml 0.05 M HCl and pH of solution was adjusted to ~5.0 with concentrated NaOH. MWNTs were dissolved in 500 μl of 0.5 wt.% chitosan solution with the aid of ultrasonic agitation. This resulted in a homogeneous black solution. By this method, 5 and $10\,mg\,ml^{-1}$ black solutions were prepared. A $2.5\,mg\,ml^{-1}$ solution were prepared by diluting with 0.5 wt.% chitosan solution.

The GCE (diameter 3 mm) was first polished with alumina slurry (followed by 1.0, 0.3 and 0.05 μm) and ultrasonically cleaned with ethanol and double distilled water and dried in nitrogen. The MWNTs/chitosan coated electrode was prepared by dropping a 5 μl of MWNT solution on the electrode. Before used for experiments, the electrode was put in air at the room temperature for some time in order to evaporate solvent. The chitosan coated GCE was prepared by the same procedure except for dropping a 5 μl of 0.5 wt.% chitosan solution on the electrode surface.

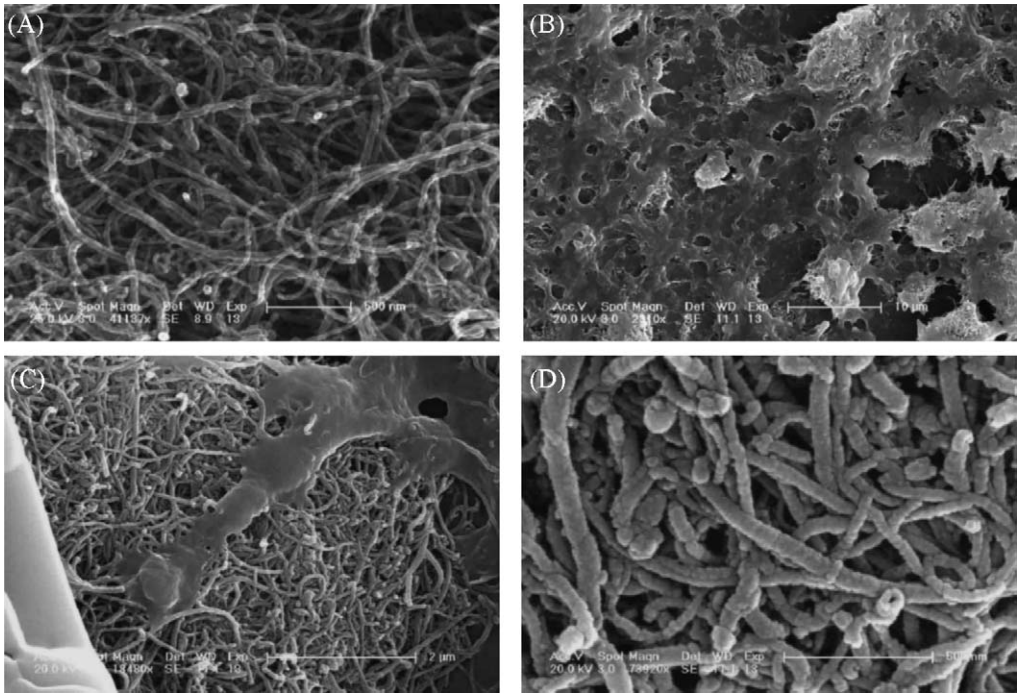

Fig. 1. FE-SEM images of MWNTs purified by 3 M HNO_3 and of MWNTs/chitosan composite films on the surface of ITO glass: (A) MWNTs purified by HNO_3, (B) MWNTs/chitosan composite. (B), (C), and (D) corresponding to images at different locations and magnification.

2.3.2. Preparation of the enzyme electrode

The MWNTs/chitosan coated electrode was dipped in a 7% glutaraldehyde solution for 30 min. After that, the electrode was rinsed with water to remove excess physical adsorbed glutaraldehyde and dried in nitrogen. Then it was immersed in a 20 mg ml^{-1} HRP solution (pH 6.9) for 30 min. After rinsed clearly, the enzyme electrode was stored in the buffer solution at 4 °C before use.

3. Results and discussion

3.1. FE-SEM images of MWNTs/chitosan composite

As a common biomacromolecule, chitosan is insoluble at alkaline and neutral, but it can well dissolve in acidic solution (pH < 6). In acidic medium, the amino functions of chitosan are protonated and this results in polycations appeared. As we all know, sidewalls and end of carbon nanotubes are functionalized with carboxylic acid groups after purification in 3 M HNO_3. So MWNTs are negative charged because of the presence of carboxylic acid groups. When MWNTs and chitosan were mixed by ultrasonication, chitosan molecules were adsorbed on the surface of MWNTs by electrostatic interaction which resisted MWNTs aggregating and resulted in forming a black solution. This indicated that MWNTs were well soluble in chitosan. Fig. 1B–D corresponded to FE-SEM images of 10 mg ml^{-1} MWNTs/chitosan solution dropped on an ITO glass. Robust and nonuniform thin film of MWNTs/chitosan could be produced on the surface of the ITO glass (Fig. 1B). Fig. 1C and D showed the images of different locations and magnifications. These images revealed that robust and nonuniform film of MWNTs could be formed on the ITO surface by dissolving them in a chitosan solution.

3.2. Electrochemical properties of MWNTs/chitosan coated electrode

The electrochemical and electrocatalytic activity of SWNTs have been studied and the redox of carboxylic acid groups was observed in cyclic voltammograms [20]. Fig. 2 showed the cyclic voltammograms of bare and MWNTs/chitosan coated GCE in phosphate buffer solution (pH 6.9) at a scan rate of 100 mV s^{-1}. A couple of peaks, the cathodic and anodic peak potentials at about −100 and −25 mV were observed (curve b). This attributed to the reduction and oxidation of carboxylic acid groups at the surface of MWNTs. These groups were reduced to $-CH_2OH$ coupled with four electrons and the background current was very large compared with that of bare GCE (curve a) because of the increased surface charge [20]. This showed that chitosan did not affect electrochemical activity of MWNTs. The peak current did not obviously change after 10 cyclic scans. This indicated the MWNTs/chitosan film on the electrode surface was very stable.

The electrochemical activity of MWNTs/chitosan composite coated GCE with different content of MWNTs in phosphate buffer solution was also studied. With amount of MWNTs increasing, peak current increased. Electrochemical activity of MWNTs/chitosan modified GCE could be

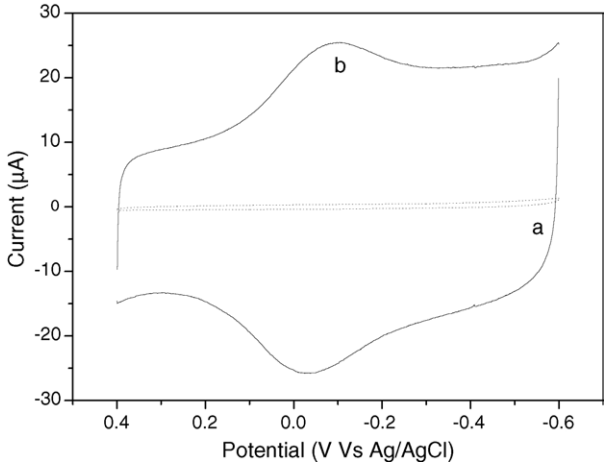

Fig. 2. Cyclic voltammograms in phosphate buffer solution (0.1 M, pH 6.9) at a scan rate of 100 mV s^{-1}: (a) bare GCE and (b) 5 ml 2.5 mg ml^{-1} MWNTs/chitosan coated GCE.

adjusted by changing amount of MWNTs in chitosan. Carbon nanotube showed electrocatalytic activities for many molecules such as ascorbic acid, NADH, H_2O_2, etc. So the MWNTs/chitosan modified GCE could be used to determine many substrates and the catalytic behaviors should be adjusted. Moreover, the primary amines of chitosan could be chemically modified with other biomolecules. This could be applied to develop different type of enzyme biosensors.

3.3. *Electrocatalysis for H_2O_2 of the enzyme modified GCE*

Many methods were reported for immobilizing HRP to develop biosensors. Gold nanoparticle, biomembrane and surfactants were often used. Tang et al. [33] reported that the direct redox reaction of HRP was observed when HRP was immobilized in a lipid film. The third generation hydrogen peroxide biosensor based on self-assemble gold nanoparticles was reported [34]. The reaction of glutaraldehyde with a primary amino group was also applied to covalently bind enzyme and compounds [35]. Here, the bifunctional reagent glutaraldehyde was used to cross-link MWNTs/chitosan composite and HRP.

Electrocatalyical behavior of the enzyme electrode was characterized by cyclic voltammetric experiments. Fig. 3 showed cyclic voltammograms of the enzyme modified GCE in pH 6.9 phosphate buffer solution at a scan rate of 20 mV s^{-1}. There were obviously redox peaks of carboxylic acid before cross-linking (curve a). But peak current slightly decreased for enzyme electrode (curve b) and this possibly attributed to the resistance of electron transfer.

Electrocatalytic activity of the enzyme electrode for H_2O_2 was also studied in pH 6.9 phosphate buffer solution. Fig. 4 corresponded to the cyclic voltammograms of the enzyme electrode in buffer solution at a scan rate of 20 mV s^{-1}. When 30 μl of 50 mM H_2O_2 was added into 3 ml phosphate buffer solution, the reductive currents increased (curve b)

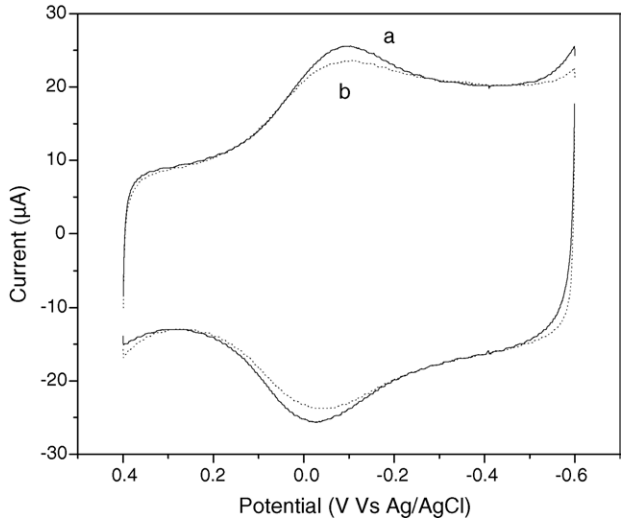

Fig. 3. Cyclic voltammograms in phosphate buffer solution (0.1 M, pH 6.9) at a scan rate of 20 mV s^{-1}: (a) MWNTs/chitosan composite film coated GCE and (b) enzyme modified GCE.

compared with that obtained from buffer solution without H_2O_2 (curve a). With amount of H_2O_2 increasing, the reductive peak currents increased and peak potential negatively shifted (curves b and c). When the content of H_2O_2 was more than 1.46 mM (curve d), reductive current did not obviously increase and this was character of electrocatalysis. This indicated the enzyme electrode showed excellent catalytic activity for H_2O_2. The electrocatalytic reduction mechanism of HRP towards H_2O_2 could be explained by the following cycles [36,37]:

$$\text{HRP} + H_2O_2 \rightarrow \text{Compound I} + H_2O$$

$$\text{Compound I} + e \rightarrow \text{Compound II}$$

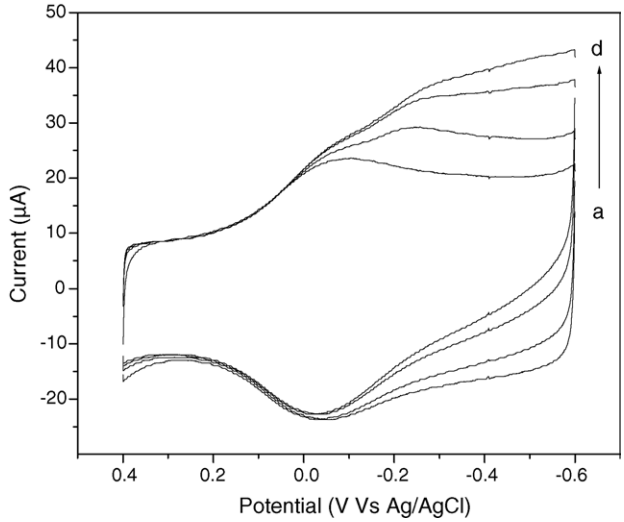

Fig. 4. Cyclic voltammograms of enzyme electrode in phosphate buffer solution (0.1 M, pH 6.9) with different amount of H_2O_2 at a scan rate of 20 mV s^{-1}: (a) 0, (b) 0.50 mM, (c) 1.46 mM, and (d) 2.38 mM.

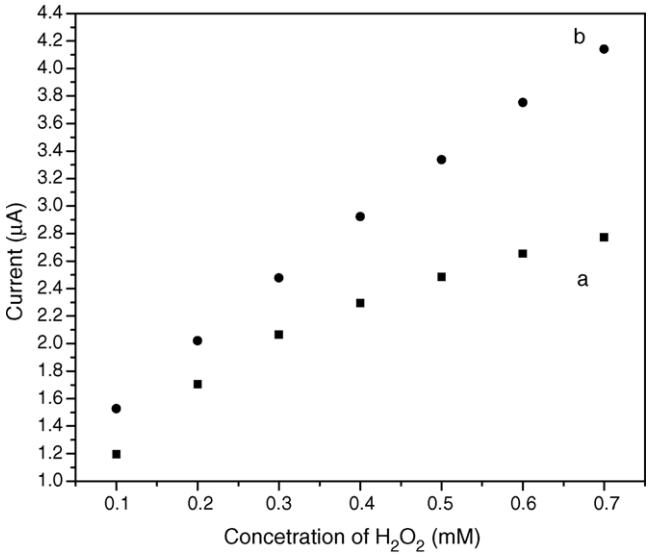

Fig. 5. Calibration curve for the concentration of H_2O_2 in the range of 0.1–0.7 mM in phosphate buffer solution (0.1 M, pH 6.9): applied potential, −0.2 V; (a) MWNTs coated GCE and (b) enzyme electrode.

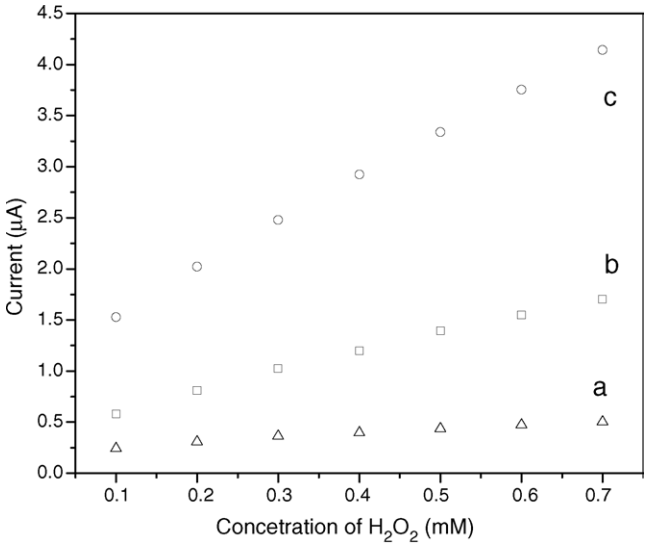

Fig. 6. Calibration curves of enzyme electrodes with different content of MWNT for concentration of H_2O_2 in the range of 0.1–0.7 mM in phosphate buffer solution (0.1 M, pH 6.9): applied potential, −0.2 V; (a) 2.5 mg ml^{-1}, (b) 5 mg ml^{-1}, and (c) 10 mg ml^{-1}.

$$\text{Compound II} + e \rightarrow \text{HRP}$$

HRP was firstly oxidized to a first intermediate (Compound I). Compound I obtained one electron and was reduced to the second intermediate (Compound II). At last, Compound II was reduced to the HRP on the electrode surface. So the reductive current increased in the presence of H_2O_2.

The effect of pH on the H_2O_2 biosensor response was also studied. The sensitivity of enzyme electrode was high between pH 6.0 and 7.0 which was consistent with that of the reported literature [32]. This indicated that the biosensor could be used for the determination of H_2O_2 in the range of pH 6.0–7.0.

In order to study the effect of MWNTs, electrocatalytic behavior towards H_2O_2 of the enzyme electrode without MWNTs was also studied. The amperometric response of enzyme modified GCE showed that the current did not increase when solutions of H_2O_2 were added into the buffer solution. So a mediator had to be used for this enzyme electrode [32]. This indicated that the presence of MWNTs was very important because of their good conductivity.

It was reported that MWNTs could also electrocatalyze H_2O_2 [12]. We also studied the catalytic behavior towards H_2O_2 of MWNTs/chitosan coated GCE. Fig. 5 corresponded to the calibration curve of the MWNTs/chitosan modified electrode and enzyme electrode in concentration of H_2O_2 between 0.1 and 0.7 mM. The amperometric response of MWNTs/chitosan modified electrode (curve a) was smaller than that from the enzyme electrode (curve b). Moreover, the sensitivity would decrease when the concentration of H_2O_2 was more than 0.3 mM. So the enzyme electrode showed higher electrocatalytic activity compared with the MWNTs/chitosan coated GCE. This suggested that HRP could also keep electrocatalytic activity and catalyze the reduction of H_2O_2. Good electrocatalyitc activity of the enzyme electrode for H_2O_2 was attributed to the presence of MWNTs and HRP. MWNTs showed good conductivity, electrocatalytic activity and biocompatibility which facilitated electron transfer between electrode and redox protein and these resulted in effective catalytic activity. The MWNTs/chitosan composite film could be used as conductive and sensing platform. HRP attached onto the surface still kept electrocatalytic activity and the modified electrode exhibited higher response and sensitivity for H_2O_2 reduction compared with that of MWNTs/chitosan modified GCE.

The amount of MWNTs was very also important for the response of biosensors. So the effect of amount of MWNTs was studied. The relationship between response current and the amount of MWNTs was presented in Fig. 6. With the amount of MWNT increasing, response current and sensitivity of the enzyme electrode increased (curve a–c). So, 5 μl of 10 mg ml^{-1} MWNT solution was chose to obtain better sensitivity and response for H_2O_2 in our experiments.

3.4. Amperometric response of the enzyme electrode for H_2O_2

The current response of HRP modified GCE was investigated in the stirring buffer solution, and −200 mV was selected as the applied potential according to the results of cyclic voltammograms (Fig. 7). When solution of H_2O_2 was added into the buffer solution, the reductive currents increased rapidly and reached stability. This indicated it was a fast electrocatalytic process because of the biocompatibility of chitosan and good conductivity of MWNTs. Fig. 7 corresponded to the calibration curve of biosensor.

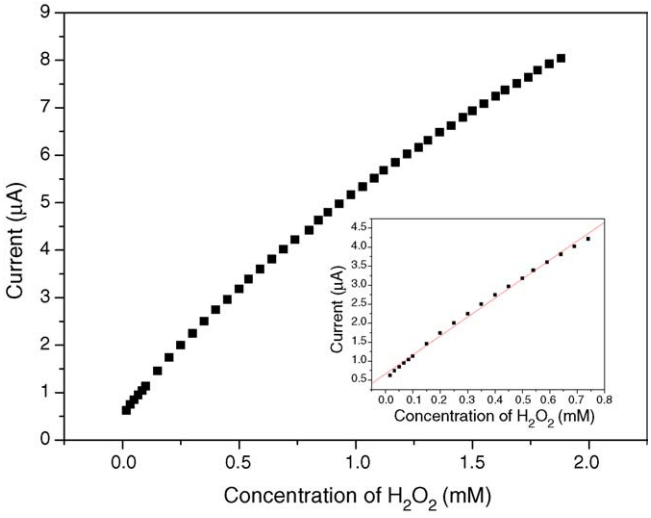

Fig. 7. Calibration curve of the enzyme electrode: applied potential, −0.2 V; supporting electrolyte, 0.1 M phosphate buffer solution (pH 6.9). Insert: the linear relationship between amperometric response with concentration of H_2O_2.

The currents had a good linearly relationship with the concentration of H_2O_2 in the range of 1.67×10^{-5} to 7.40×10^{-4} M, the regression equation was $Y = 0.659 + 4.995X$ with correction coefficient of 0.998 ($n = 19$). The sensitivity of this biosensor was 4.995 μA/mM and the detection limit was 1.03×10^{-5} M estimated at a signal-to-noise of 3. The repeatability of the sensor was also studied and relative standard deviation (R.S.D) was 3.3% ($n = 8$) for 0.1 mM H_2O_2. Ascorbic acid is often an interference for hydrogen peroxide biosensors [32,38]. But these were no interferences of ascorbic acid, glucose, citrate acid, lactic acid for the biosensor. Compared with other biosensors based on chitosan, the interferences of ascorbic acid were absence for the enzyme electrode. Moreover, the modified electrode still exhibited good electrocatalytic activity without a mediator. However, the catalytic efficiency for H_2O_2 of the enzyme electrode was not very high. So some works must be done to improve the catalytic activity and sensitivity. The enzyme electrode was stored in phosphate buffer solution (0.1 M, pH 6.9) and after 20 days, the response to 0.1 mM H_2O_2 of the biosensor only decreased 10%. This could be attributed to the biocompatibility and stability of composite film of MWNTs and chitosan.

4. Conclusion

A new amperometric biosensor for hydrogen peroxide was prepared based on composite film of MWNTs/chitosan. MWNTs/chitosan composite could be formed on the GCE and glutaraldehyde was used to cross-link HRP and MWNTs/chitosan composite. The resulted enzyme electrode could be applied for amperometric determination of H_2O_2 in the absence of a mediator. The amperometric experiments showed excellent electrocatalytical activity of the biosensor for H_2O_2. With good repeatability and stability, different enzyme biosensors can be prepared by this method.

Acknowledgements

This work was supported by the National Nature Science Foundation of China with the Grant (No. 20475052) and the National Key Basic Research Development project research on Human Major Disease Proteomics (No. 2001CB5102).

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