Feature Article

An Amperometric Biosensor Based on the Coimmobilization of Horseradish Peroxidase and Methylene Blue on a Carbon Nanotubes Modified Electrode

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Abstract
A novel hydrogen peroxide biosensor has been constructed based on the characteristics of the carbon nanotube. The multiwall carbon nanotube (MWNT) was used as a coimmobilization matrix to incorporate horseradish peroxidase (HRP) and electron transfer mediator methylene blue (MB) onto a glassy carbon electrode surface. Cyclic voltammetry and amperometric measurements were employed to demonstrate the feasibility of methylene blue as an electron carrier between the immobilized peroxidase and the surface of glassy carbon electrode. The amperometric response of this resulting biosensor to H_2O_2 shows a linear relation in the range from 4 μM to 2 mM. The detection limit was 1 μM when the signal to noise ratio is 3. The presence of dopamine and ascorbic acid hardly affects the sensitive determination of H_2O_2. This biosensor also possesses very good stability and reproducibility.

Keywords: Biosensor, Horseradish peroxidase, Methylene Blue, Carbon nanotubes

1. Introduction

The determination of hydrogen peroxide is of great relevance, ascribable to both the fact that it is the product of the reactions catalyzed by a large number of oxidase enzymes and that it is essential in food, pharmaceutical, and environmental analysis [1–4]. Techniques for detecting H_2O_2 include spectrometry [5], chemiluminescence [6], titrimetry [7], and electrochemical methods [8–12]. Among these procedures, the electrochemical analysis offers improved sensitivity, extended dynamic range and rapid response time. The direct electrochemical detection of H_2O_2 often requires relatively high overpotentials. However, hydrogen peroxide can be detected enzymatically at low applied potentials by employing peroxidase as bioelectrocatalysts for its electrochemical reduction. Among peroxidase, HRP has been the most widely studied in the development of enzyme-based amperometric biosensors. Although immobilization of enzymes on electrode surfaces is critical for stability, reproducibility, sensitivity and lifetime and so on, several valuable methods have been developed such as adsorption, covalent linking, incorporation of conducting polymer and bulk modified composite methods.

Amperometry coupling peroxidase with mediators is one of the most sensitive procedures. Such mediated hydrogen peroxide biosensors could provide a detection limit as low as about 10^{-7} M by employing an electron transfer mediator such as tetrathiafulvalene [13], $[Ru(NH_3)_5py]^{2+}$ [14], ferrocene derivatives [15], or organic dyes such as meldola blue [16], phenazine methosulfate [17] and methylene blue [18]. In order to construct a biosensor for multiple uses, immobilizing a soluble or insoluble mediator on the electrode surface is preferable. Such an electrode could provide a relatively stable response toward a target enzyme or other inert potential redox species at the redox potential of the mediator and requires no addition of mediator to the solution. Water-soluble dyes attached to graphite electrode surface [17, 18] or some polymers [19, 20] have been studied.

Carbon nanotubes are new and interesting members of the carbon family offering unique mechanical and electronic properties combined with chemical stability. Since the discovery of carbon nanotubes in 1991 [21], a series of experimental and theoretical researches have been directed toward their production, purification, mechanical and electronic properties, and electrical conductivity [22]. Hill et al. demonstrated the application of the carbon nanotube as electrode material, and they found that proteins and enzymes could be effectively immobilized on and within carbon nanotubes [23–26]. Li et al. cast the carbon nanotube on a glassy carbon (GC) electrode and investigated the electrochemical and electrocatalytic behaviors [27]. Ajayan et al. reported the fast electron transfer kinetics on multiwalled carbon nanotube microbundle electrode [28]. Crooks et al. reported the fabrication and characterization of electrodes constructed from single carbon nanotubes [29]. So far, the carbon nanotubes for coimmobilizing enzymes and mediators and their applications to the preparation of biosensors, to our best knowledge, have not been reported.

In this article, the multiwall carbon nanotube was firstly modified onto the GC electrode surface, then the HRP molecules and electron transfer mediator MB were coim-

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mobilized onto MWNT surface to prepare the H_2O_2 sensor. The surface coverage of the sensor is determined by the spectrophotometric method, cyclic voltammetry and amperometric measurements are employed to demonstrate its biological affinity to H_2O_2, sensitivity and stability, as well as the feasibility of methylene blue as an electron mediator between peroxidase and glassy carbon electrode. The aim of the present study is to develop a novel hydrogen peroxide biosensor by the characteristics of the carbon nanotube.

2. Experimental

2.1. Reagents

Horseradish peroxidase (HRP) was obtained from Sigma. Methylene blue (MB, not further purified before use) was purchased from Chroma. Ortho-aminophenol(OAP) and H_2O_2 (30% w/v solution) were purchased from Shanghai Chemical Reagent Company. The concentration of the more diluted hydrogen peroxide solutions prepared was determined by titration with cerium(IV) to a ferroin endpoint. The MWNTs were produced by chemical vapor deposition (CVD) [31], and treated with HCl. All other chemicals were of analytical grade. All the solutions were prepared with doubly distilled water.

2.2. Apparatus

CHI660 Electrochemistry workstation (CHI Co. USA) was used for electrochemical measurements. A three-electrode system incorporating this H_2O_2 biosensor as the working electrode, a saturated calomel reference electrode (SCE) and a platinum wire counter electrode were used for the measurements. A magnetic stirrer and a stirring bar provided the convective transport for the amperometric experiments.

UV spectra were obtained in the range of 320 – 600 nm on a type BRAIC 1200 UV instrument (Beijing, China) with quartz cuvettes (path length 1 cm) at room temperature. The images for transmission electron micrographs (TEM) were obtained with a JEOL-JEM 200CX electron microscope.

2.3. Fabrication of the H_2O_2 biosensor

The glassy carbon electrode with a diameter 4 mm was polished with 1.0, 0.3, and 0.05 μm aluminium oxide, rinsed thoroughly with deionized water, sonicated in deionized water and ethanol, and dried in air before use. 10 mg of MWNT was dispersed with the aid of ultrasonic agitation in tetrahydrofuran (THF) to give a black solution. The MWNT film was prepared by dropping a solution of MWNT in THF on the GC electrode surface and then evaporating the solvent under an infrared heat lamp. 10 μL 1 g/L HRP prepared in pH 7.0 PBS was spread on electrode surface. The matrix was allowed to dry slowly in air at room temperature. 0.1 mmol/L MB solution was added on the electrode surface and dried at room temperature. During this process the dye was incorporated into the carbon nanotube matrix. Then the electrode was rinsed with doubly distilled water and immersed into the blank PBS solution to remove the non-firmly adsorbed dye molecules and HRP molecules until the steady electrochemical response.

3. Results and Discussion

3.1. Physical Characterization

Hill et al. [25] directly got the image of which the protein and enzyme had been immobilized in carbon nanotubes by the high resolution transmission electron microscopy (HRTEM). We used the TEM to study the interaction between the HRP and carbon nanotube. The TEM images confirmed that the HRP molecules have been immobilized in carbon nanotubes. Figure 1a shows the carbon nanotubes without HRP molecules, Figure 1b the carbon nanotubes with adsorbed HRP molecules. Comparing the two images, it can be directly observed that some HRP molecules are adsorbed along the carbon nanotubes. The result shows that the carbon nanotubes can be investigated as a support for peroxidase.

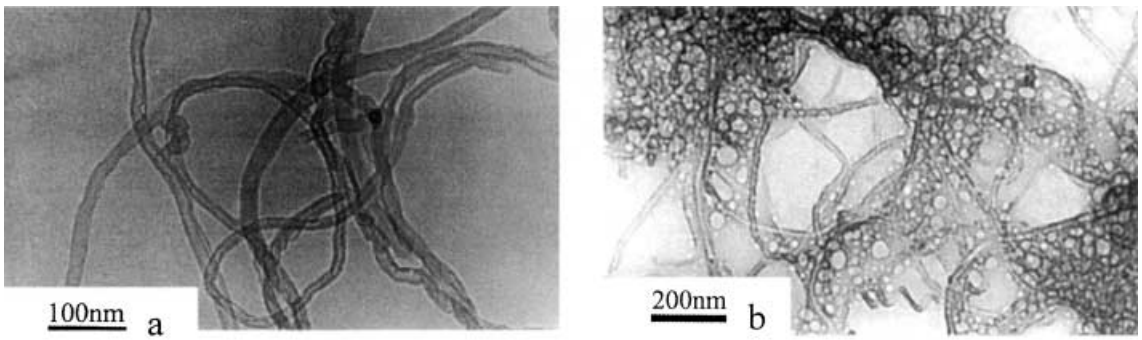

Fig. 1. Transmission electron microscopy photos of a) the MWNTs; b) the MWNTs adsorbed HRP molecules.

3.2. Measurements of Peroxidase Activity of HRP Immobilized onto a MWNT Layer

In order to determine the amount of active HRP immobilized on the MWNT modified GC electrode, a variant of the spectrophotometric method described in the Section 2. was employed. For this purpose, an HRP-MWNT modified electrode was immersed in a spectrophotometric cuvette containing OAP (0.5 mM) and H_2O_2 (0.1 mM) for 15 min, then the electrode was removed from the solution, and the absorption curve resulted from the generation of the oxidized OAP was recorded. The intensity of absorption spectra of solutions containing OAP and H_2O_2 under same conditions increased with increasing (through the addition of a stock HRP solution) amounts of HRP was recorded, and the results are shown in Figure 2. In all cases there was an absorption band at 436 nm due to formation of the oxidized form of OAP whose concentration could be correlated to the amount of active enzyme occurred. Using the absorbance resulting from the HRP immobilized on the electrode (0.554) the amount of immobilized active enzyme was determined to be 4.0×10^{-13} mol. This value is close to 1/8 of the surface coverage of active enzyme of 3.2×10^{-12} mol/cm^2. The surface coverage is not high. We thought it is depended on the properties of the MWNT.

3.3. Electrochemical Characterization of the Coimmobilization of Horseradish Peroxidase and Methylene Blue on a Carbon Nanotubes Modified GC Electrode (MB-HRP/NME)

Typical cyclic voltammograms of MB-HRP/NME in 0.1 M phosphate buffer solution (pH 6.5) at different rates are shown in Figure 3. The $E_{1/2}$ shifted negatively for 25 mV in

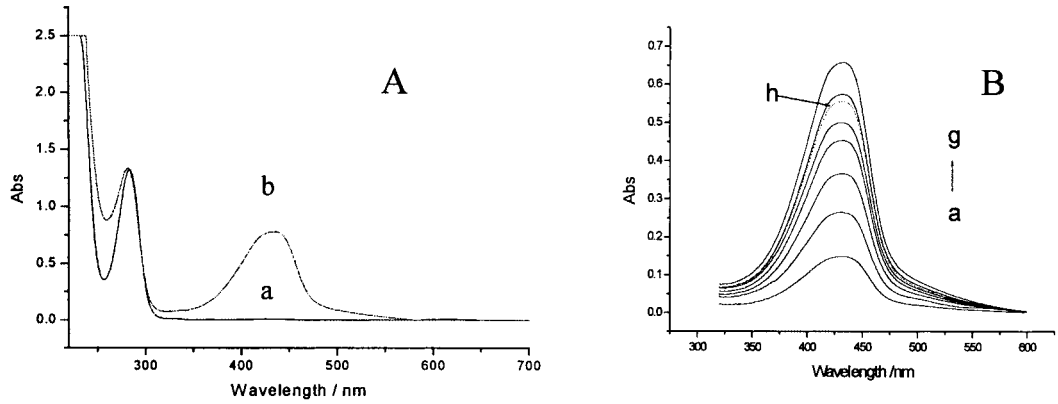

Fig. 2. A) Absorption spectra of solutions containing OAP (0.5 mM) and H_2O_2 (0.1 mM) in 5.0 mM pH 7.0 PBS in the absence of HRP (a) and in the presence of HRP (b). B) Absorption spectra at HRP concentrations of a) 1, b) 2, c) 3, d) 4, e) 5, f) 6, g) 7 ng/mL in the same solution. Spectra were recorded 15 min after the addition of the HRP. Trace (h) corresponds to the spectrum of a MB-HRP/NME after it had been immersed for 15 min in the same solution.

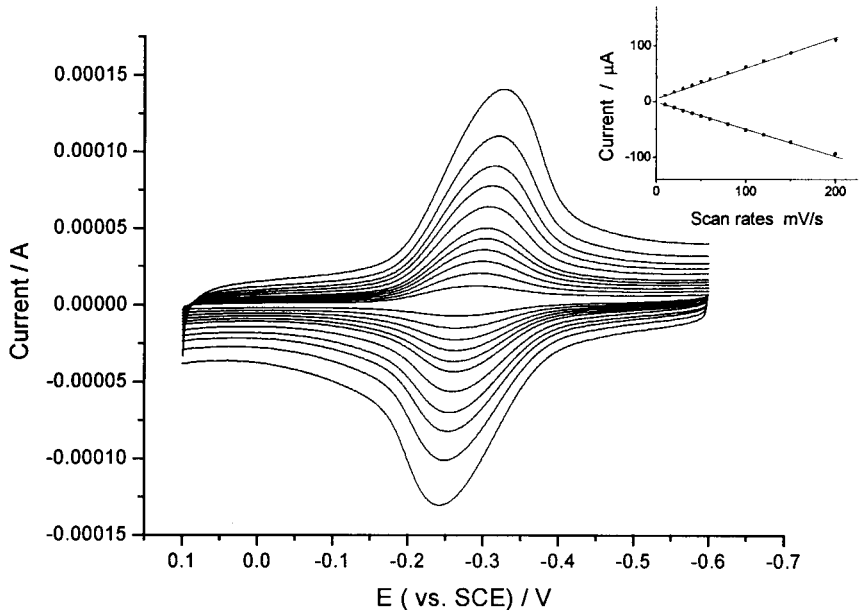

Fig. 3. Typical cyclic voltammograms(a) of MB-HRP/NME and the calibration plot of the wave current at different scan rates (from inner to outer): 10, 20, 30, 40, 50, 60, 80, 100, 120, 150, 200 mV/s.

comparison with that of the redox process of free MB in the buffer solution, and the peak currents of the MB-HRP/NME are proportional to the scan rates up to 200 mV/s, indicating the surface redox reaction of the MB-HRP/NME. Furthermore, no essential decrease of the peak current was found, after the potential swept cyclically from −0.6 to 0.2 V at a scan rate of 10 mV/s for 100 cycles. It is indicated that the MB molecules are immobilized on the MWNT surface.

3.4. Electrochemical Response to Hydrogen Peroxide

The cyclic voltammagrams of resulting electrode in the absence and presence of H_2O_2 are shown in Figure 4. A significant increase of the cathodic peak currents was generated in 1 mM H_2O_2 solution. A little peak-potential shift toward negative direction with the increase of H_2O_2 concentration was observed. The reaction mechanism of the sensor was summarized as follows [30]:

The HRP reduces hydrogen peroxide to water.

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

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

$$HRP\text{-}I + (MB)_{red} \rightarrow HRP\text{-}II + (MB)_{ox}$$

$$HRP\text{-}II + (MB)_{red} \rightarrow HRP + (MB)_{ox}$$

$$(MB)_{ox} + 2e \rightarrow (MB)_{red}$$

The MB can then be recycled at the electrode as the mediator leading to an increase of its reduction current.

3.5 Optimization of Hydrogen Peroxide Monitoring

In order to determine the optimal working potential for the H_2O_2 sensing, a plot of chronoamperometric current vs. working potential was made as shown in Figure 5. It was observed from Figure 5 that the steady-state current increased with an increase of cathodic potential, and when the potential reached −0.3 V, the electroreduction of H_2O_2 hardly changed.

The pH effect on the response of the sensor is shown in Figure 6. It can be observed that the current response of the sensor was suitable in the pH range 4–7; however, it dropped quickly from pH 7 to 9. So buffer solution of pH 5.0 was used

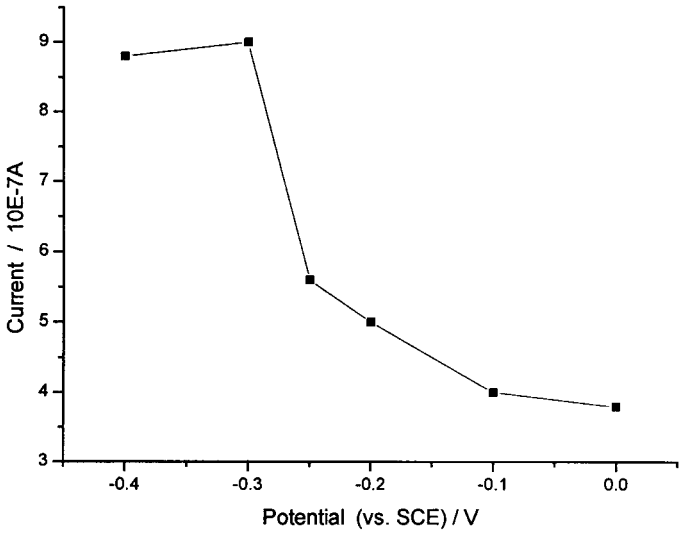

Fig. 5. Effect of the working potential on the response of the sensor. Experimental conditions: 0.10 mM H_2O_2 in pH 7.0 PBS.

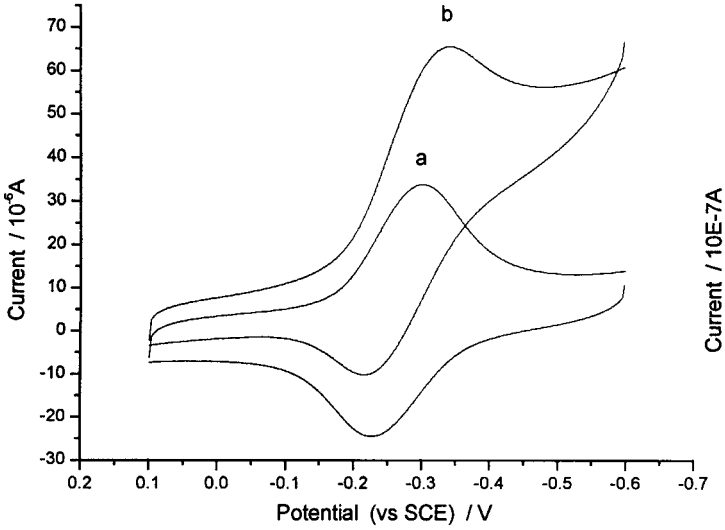

Fig. 4. Cyclic voltammograms of the H_2O_2 sensor in the absence of H_2O_2 (a), and in the presence of 1.0 mM H_2O_2 (b) at a scan rate of 50 mV/s in pH 7.0 PBS.

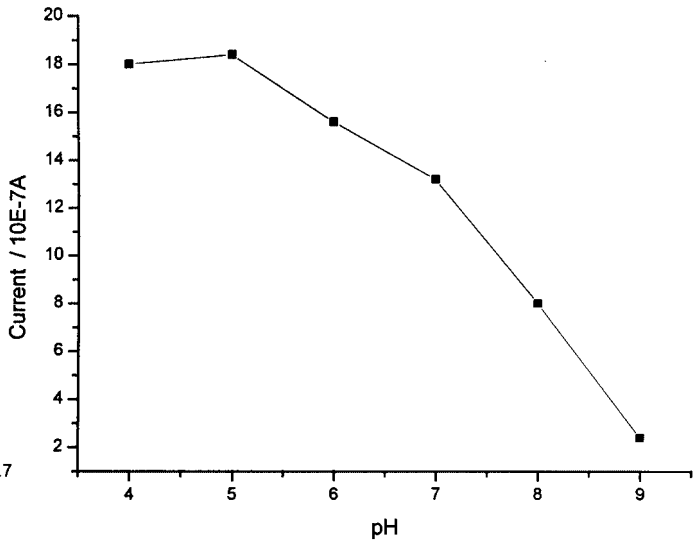

Fig. 6. Effect of the pH on the response of the sensor to 0.1 mM H_2O_2 in PBS.

for the following work to obtain stable, reproducible and maximal amperometric response.

The effect of temperature on the sensor was examined between 20 and 55 °C. The experiments showed that the response current is hardly affected by temperature in the range of 25 – 45 °C. According to the Langmuir adsorption, the adsorptive quantity decreases with the temperature increasing. But the rate of enzyme reaction increases with the temperature increasing. It is a probability that the increase of response currents resulted from the increase of rate of enzymatic reaction just compensates the decrease of response currents resulted from the decrease of adsorptive quantity of enzyme when temperature rises from 25 to 45 °C. The further increasing temperature gave rise to a decrease of the response because of the denaturation of the enzyme.

3.6. Steady-State Amperometric Response to Hydrogen Peroxide

Figure 7 shows the dynamic response of the sensor at a working potential of – 0.3 V with successive injections of H_2O_2. The trace clearly demonstrates the fast response and high sensitivity of the sensor to H_2O_2. The time required to reach 95% of the maximum steady-state current is less than 30 s. Figure 8 displays the calibration plot of the sensor. The sensor responses to H_2O_2 in the linear range from 4 μM to 2 mM. The detection limit was down to 1 μM when the signal to noise ratio is 3. In addition, the relative standard deviation is 4.5% for 8 repetitive measurements of 0.5 mM H_2O_2 solution.

After measurement, the sensor was rinsed with doubly distilled water and stored at 4 °C, and it was found that the current response decreased about 10% in 5 days, and 20% in about two weeks.

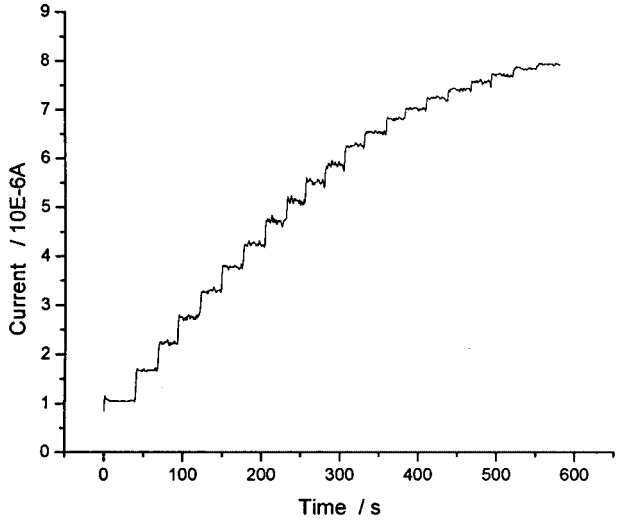

Fig. 7. Dynamic response of the H_2O_2 sensor to successive addition of 0.02 mM H_2O_2 steps in the solution at the applied potential of – 0.3 V.

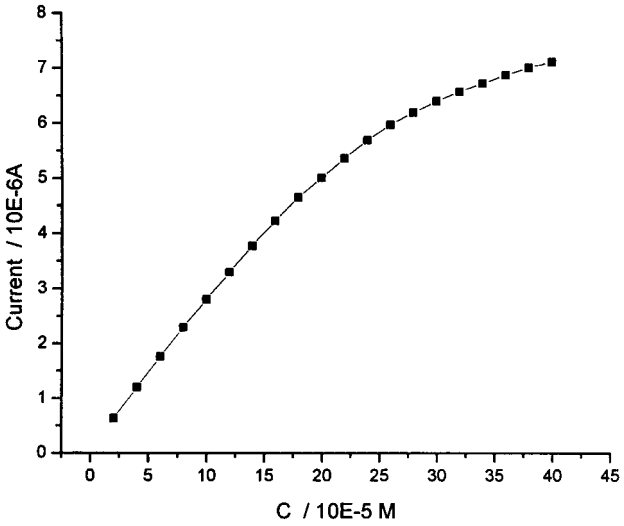

Fig. 8. Calibration plot between current and H_2O_2 concentration.

3.7. Determination of the Apparent Michaelis-Menten Constant

The apparent Michaelis-Menten constant (K_M^{app}), which gives an indication of the enzyme-substrate kinetics, can be obtained from the electrochemical version of the Lineweaver-Burk equation [32]:

$$1/I_{SS} = 1/I_{max} + K_M^{app}/I_{max}\, c$$

where I_{SS} is the steady-state current after the addition of substrate, c is the bulk concentration of the substrate and I_{max} is the maximum current measured under saturated substrate condition. The K_M^{app} was determined by analysis of the slope and intercept for the plot of the reciprocals of the steady-state current versus H_2O_2 concentration. The K_M^{app} value for the sensor was found to be 0.12 mM. This value was lower than that reported [33], giving clear evidence for the higher sensitivity of the sensor.

4. Conclusions

The experimental results prove that the nanotubes can be used as the carrier to coimmobilize the HRP and MB on the surface of GC electrode to develop a novel hydrogen peroxide biosensor. The biosensor possesses very good bioactivity, stability and reproducibility. The results also imply that more novel biosensors could be developed by means of the characteristics of carbon nanotube, even a single carbon nanotube biosensor is possible. The attractive properties of carbon nanotube enzyme electrodes may find various practical applications.

5. Acknowledgements

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