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REAGENTLESS ELECTROCHEMICAL BIOSENSOR BASED ON THE MULTI-WALL CARBON NANOTUBES AND NANOGOLD PARTICLES COMPOSITE FILM

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1. ABSTRACT

A novel method was used to prepare the nano-composite by assembling nanogold (NG) particles on the multiwall carbon nanotubes (MWNTs) surface. The nano-composite could be immobilized on a glassy carbon (GC) electrode to get a novel modified electrode. The electrode can easily immobilize the horseradish peroxidase (HRP) molecules to construct a reagentless biosensor. The NG particles in the composite film have a good biological compatibility. And due to the existence of quinone groups on the MWNTs surface, the MWNTs can promote the electron transfer between enzymes and electrode surface. The biosensor shows a good stability and responds to H_2O_2 in the range from 2.0 μM to 3.5 mM with a detection limit of 1.0 μM.

2. INTRODUCTION

Carbon nanotubes are a new member of the carbon family with unique geometrical, mechanical, electronic, and chemical properties (1,2). Since the carbon nanotubes were discovered in 1991, most fundamental researches on nanotubes have been focused on their growth mechanism (3), their adsorption properties (4), refinement of production and purification methods (5-7), and direct measurements of various physical properties (8-12). Practical applications as tips in scanning probe microscopy (13-15), and novel electrodes have been reported (16). In particular, the research of carbon nanotube modified electrode attracted much attention during the past several years (33-37). This modified electrode can utilize the properties of carbon nanotubes and avoid the complicated technology of controlling the carbon nanotubes. The carbon nanotube modified electrodes have been confirmed that it can promote the electrochemical reaction of some biological small molecules, proteins and enzymes. Some mechanisms have been used to not only explain the electrochemical reaction processes on the carbon nanotube surface and but also to facilitate the development of biosensors.

In order to further exploit the application of the carbon nanotube in various fields, the modification of the carbon nanotube and the composites are highly attractive. Gu *et al* (45) enhanced the solubility of carbon nanotube in organic solvent by the derivation with the amino-alkyl chain. Adsorption of nano-sized particles from the colloidal suspensions onto substrates and their organization in two- or three-dimensional assemblies represents a current active research field in different fields (27). Fitzmaurice (28) and coworkers reported the carbon nanotube template self-assembly of nanogold particles and the subsequent thermal processing of the above assemblies to form continuous polycrystalline gold nanowires extending over many micrometers. Ajayan *et al* (46,47) selectively attached nanogold particles to the carbon nanotubes surface in different methods. It was proved that nanogold particles could provide an environment similar to the native environment for redox proteins. We have proved that the HRP molecules have high activity on the nanogold particles surface (29). It will be beneficial that two kinds of nanomaterials are assembled to the electrode surface to fabricate the composite film modified electrode.

Biosensors are highly selective due to the high substrate specificity of the enzyme and the interference free indication of the reaction product. They offer the possibility of real-time analysis that is important for the rapid measurement of body analytes. Since Clark and coworkers

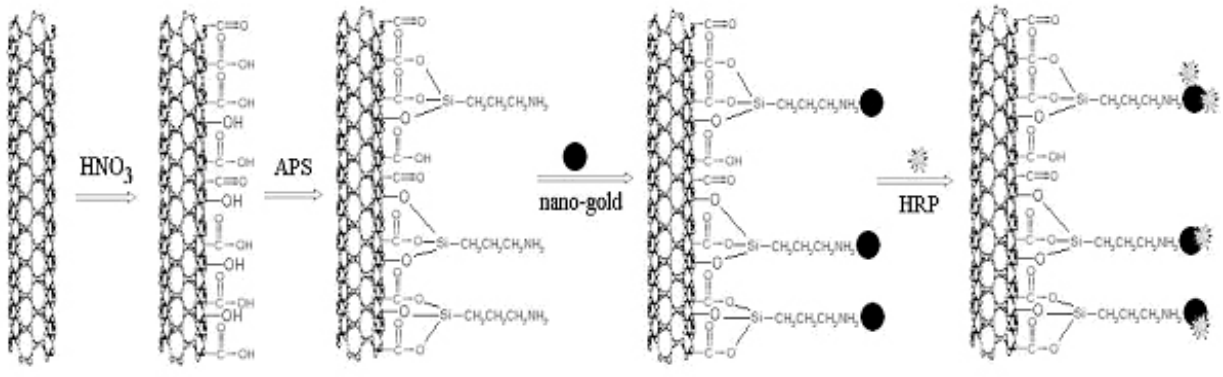


Figure 1. Preparation process of the biosensor.

firstly reported that enzymes can be immobilized at the electrochemical detectors to form enzyme electrodes and they may be utilized to sense their specific analytes, the electrochemical biosensors are developing quickly (17-20) and are considered as the foremost research field in the 21st Century. Some biosensors based on carbon nanotube have been developed and have some very good properties, especially Wang group's results (48-52).

The determination of hydrogen peroxide is of great relevance, which can be ascribable to both facts 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 (21-24). Among peroxidase, HRP has been 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. Because the activity center of the enzyme is hidden in the inner of the enzyme molecules, the direct electron transfer between the electrode and the enzyme molecules is difficult. Mediators are artificial electron transferring agents that can readily participate in the redox reaction with the biological component and thus facilitate the rapid electron transfer. For example, ferrocene and quinone derivatives etc as mediators are reported (25,26,38)**.** Biosensor with mediators is one of the most sensitive methods. It is sometimes necessary that the mediators should be co-immobilized on the electrode surface with the enzyme molecules. However, the stability of the mediators on the electrode is usually associated with the stability of the prepared biosensor. The mediator that is derived from the matrix is more stable than the mediator that is immobilized on the matrix.

To the best of our knowledge, such a composite film for the fabrication of the biosensors, which is based on the combination between the biological compatibility of nanogold particle and an electron mediator produced on the surface of carbon nanotubes, has not been reported. In this paper, a new method has been developed to prepare the nanogold and carbon nanotube composite, and a new electrochemical reaction process associated with the couple of hydroquinone and quinone on the carbon nanotube surface was proposed, finally a novel biosensor has been fabricated by the nanocomposite. In the course of constructing this biosensor, the oxygen-containing functional groups are firstly formed on the carbon nanotubes surface by acidic oxidation; in the following step, the amino groups on them are derived through the reaction between the (3-Aminopropyl) trimethoxysilane (APS) and the hydroxyl, then the nanogold particle is adsorbed onto the modified nanotubes surface to construct a composite film; finally the HRP molecules are assembled on the composite film. The preparation process of the biosensor was shown in Figure 1. The quinone formed by acidic oxidation on the carbon nanotubes surface acts as an electron mediator shuttling the electrons between the enzyme and electrode. Experimental results show the biosensor has a good electrochemical response for the H_2O_2 with a low detection.

3. MATERIALS AND METHODS

3.1. Reagents and apparatus

Horseradish peroxidase (HRP) was obtained from Sigma. H_2O_2 (30% w/v solution) was 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) (43), treated with HCl and oxidized with HNO_3 in turn during the purification process. Nanogold particles were prepared according to the reference (29). All other chemicals were of analytical grade. All the solutions were prepared with doubly distilled water.

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.

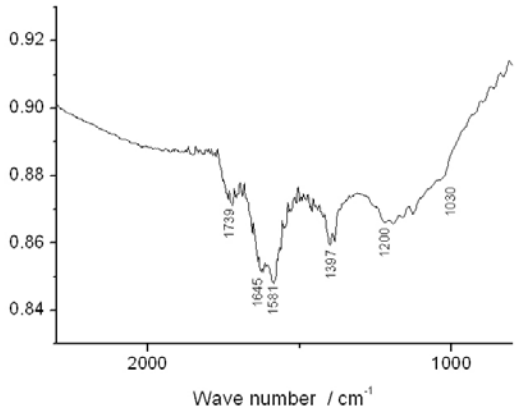


Figure 2. FT-IR spectrum of the MWNT oxidized with HNO_3.

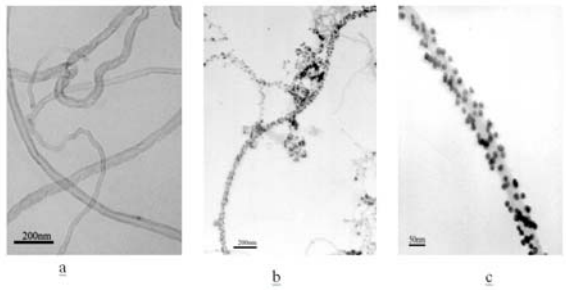


Figure 3. The transmission electron microscopy photos of (a) the MWNTs; (b) and (c) the MWNTs adsorbed nanogold particles.

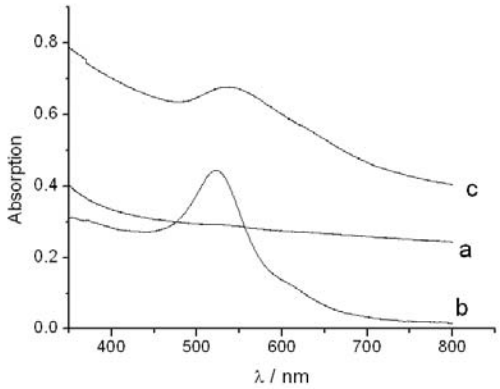


Figure 4. The UV-vis absorption spectra of MWNTs (a), nanogold particles (b) and NG/MWNTs (c) in aqueous solution.

UV spectra were obtained in the range of 200nm~800nm on a type BRAIC 1200 UV instrument (Beijing, China) with quartz cuvette (path length 1 cm) at room temperature. The images for transmission electron micrographs (TEM) were obtained by using a JEOL-JEM 200CX electron microscope.

3.2. Preparation of the nano-gold /MWNT composite film modified electrode

The GC electrode with a diameter 4 mm was polished in turn with 1.0, 0.3, and 0.05 μm aluminum oxide, rinsed thoroughly with deionized water, sonicated in deionized water and ethanol, and dried in air. 10 mg of MWNT was dispersed with the aid of ultrasonic agitation in tetrahydrofuran (THF) to give a black solution. Firstly, the MWNT film was prepared by dropping the MWNT solution to the GC electrode surface and then evaporating the solvent with an infrared heat lamp. The MWNT film modified electrode (denoted as MWNT/GC) was placed into the 1% APS solution for 30 min and then rinsed with water (denoted as APS/MWNT/GC). Finally, the electrode was placed into the gold colloid solution for 24 hours. The nano-gold MWNT composite film modified GC electrode (denoted as NG/MWNT/GC) was successfully prepared.

3.3. Fabrication of the H_2O_2 biosensor

The NG/MWNT/GC was placed into the PBS (pH 7.0) solution containing 1 g/L HRP for 12 hours to assemble the enzyme to the electrode surface (denoted as HRP/NG/MWNT/GC). In order to remove the non-firmly adsorbed HRP molecules, the electrode was rinsed with doubly distilled water and immersed into the blank PBS solution until the electrochemical response did not change.

4. RESULTS AND DISCUSSION

4.1. FT-IR and TEM characterization

FTIR is one of the powerful techniques available to study the surface properties (30). In our experiments, the FTIR was used to confirm the existence of quinone groups on the MWNTs surface. Figure 2 shows the FTIR curve of the MWNTs dealt with acidic oxidation. The spectral bands at 1739, 1200 and 1040 cm^{-1} are indicative of the production of ester groups and the band at 1645 cm^{-1} is assigned to the C=O stretching mode of quinone groups, while the band at 1581 cm^{-1} is assigned to C=C stretching mode located near the newly formed oxygenated group (31,32).

The TEM images of the MWNTs and the nanogold particles modified MWNTs are shown in Figure 3. It can be seen that the diameter of MWNTs is about 50 nm, and the length is about several μm (Figure 3(a)) and nanogold particles (*ca*. 14 nm) can be regularly assembled on the modified MWNTs surface (Figure 3 (b), (c)). The coverage of the nanogold particles is about 60% on the MWNTs surface. When the MWNTs were only oxidized by the HNO_3, the nanogold particles with negative charge hardly adsorb on the MWNTs surface. After the MWNTs surface was treated with APS, the amino group can be formed on the MWNTs surface. The nanogold particles can be strongly combined with the MWNTs surface by the electrostatic interaction. The composite film was suitable for preparing the biosensor. Although the MWNTs could also adsorb the nanogold particles when the MWNTs did not oxidize, there were not quinone groups on the MWNTs surface and the MWNTs were not suitable for developing a biosensor.

Figure 4 showed the spectra of aqueous dispersions of nanogold particles, the MWNTs and NG/MWNTs composite. The absorption spectrum of nanogold particles revealed a plasmon absorption band maximum (λ_{max}) at

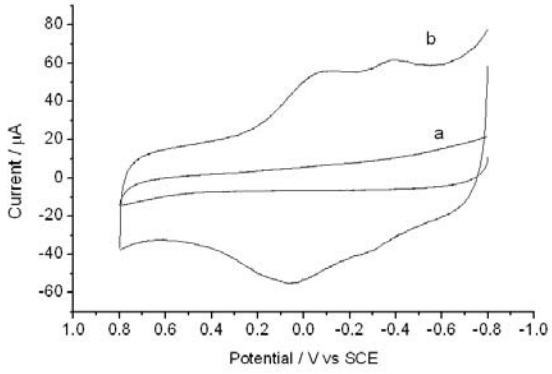


Figure 5 Typical cyclic voltammograms of the bare GC electrode (a) and the MWNT/GC (b) at pH 6.0 PBS scan rate 0.05 V/s.

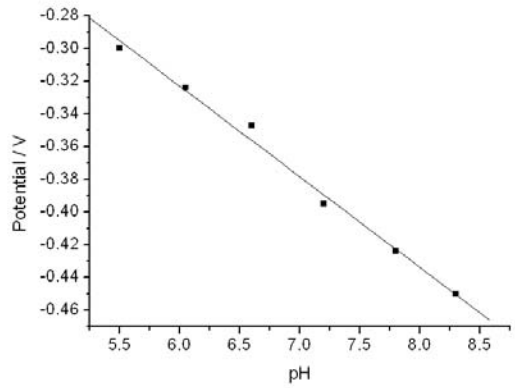


Figure 6. The calibration plot between the $E_{1/2}$ and the different pH.

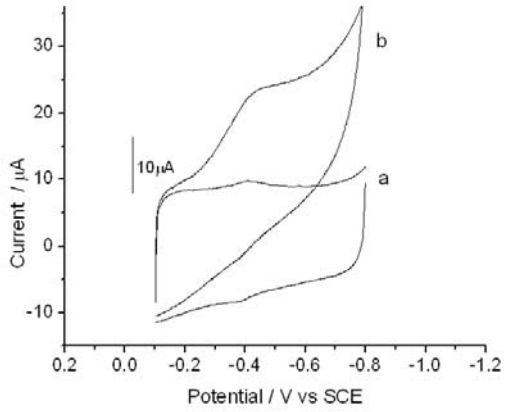


Figure 7. The cyclic voltammograms of the H_2O_2 sensor in the absence of H_2O_2 (a), and in the presence of 1.0 mM H_2O_2 (b) at a scan rate of 10 mV/s in pH 7.0 PBS

520 nm. There was not absorption peak in the MWNTs spectra. The MWNTs coated with nanogold particles caused a red-shift in λ_{max} and an expansion in band. The red-shift in λ_{max} was due to the coupling of the surface plasmon in neighboring MWNTs. That indicated that the nanogold particles behaved as discrete but coupled particles and not as a thin, continuous metal shell (42). The result showed that the character of the nanogold particle was still reserved.

4.2. Electrochemical behavior of the nanogold/MWNTs composite film

The nanogold/MWNTs film modified GC electrode was placed into pH 7.0 PB solution, two pairs of redox peaks were observed in Figure 5. The first pair of peaks with formal potential –0.075 V corresponds to the redox of the carboxylic acid group on the carbon nanotubes surface (33,34). According to the result of FT-IR experiment, there are the quinone groups on the surface of the MWNTs, and none of other elements was introduced on the MWNTs surface except the carbon, oxygen, hydrogen in preparing and purifying process. This pair of peaks with formal potential –0.399 V should be attributed to the redox of the quinone group. The lower formal potential may result from the big ring with conjugated system in the MWNTs structure. The result is in agreement with that report in the literatures (38-41).

The relation of the pH and formal potential was also studied. The experimental results indicated the formal potential gradually shifted in more negative direction with the pH increase as shown in Figure 6. The slope is 55.4 mV/pH, which corresponds to a two-electron and two-proton reaction mechanism of Q/H_2Q.

$$Q + 2e + 2H^+ \rightarrow H_2Q$$

When the nanogold particles and HRP were assembled on the surface of the MWNTs as a biosensor, the two pairs of peaks still were observed. The curve was shown in Figure 7(a). The biosensor has a good catalytic response to the H_2O_2. A significant increase of the cathodic peak currents was observed in the presence of 1 mM H_2O_2 as shown in Figure 7(b). We suggested the quinone be recycled in the electrode reaction as a mediator leading to an increase of its reduction current.

In order to determine the contribution of each electrode component, the electrodes with different functional surface were studied and the results are shown in Figure 8. It was obvious that all the functional surface of the MWNT/GC, the APS/MWNT/GC and the NG/MWNT/GC hardly responded to H_2O_2. Only the surface of NG/MWNT/GC immobilized by HRP molecules well responded to H_2O_2. Here HRP is a biological catalyst which promotes the reduction of H_2O_2 with the aid of hydroquinone on the surface of MWNTs.

In order to confirm the action of nanogold particles, it was studied that MWNTs directly adsorbed HRP molecules to prepare a biosensor. A comparison between the biosensor prepared by the MWNTs unmodified nanogold particles and by MWNTs composite film was shown in Figure 8. The current response of the biosensor of composite film was obviously larger than that of unmodified nanogold particles. The result showed that the enzymes have high activity on the nanogold particles surface.

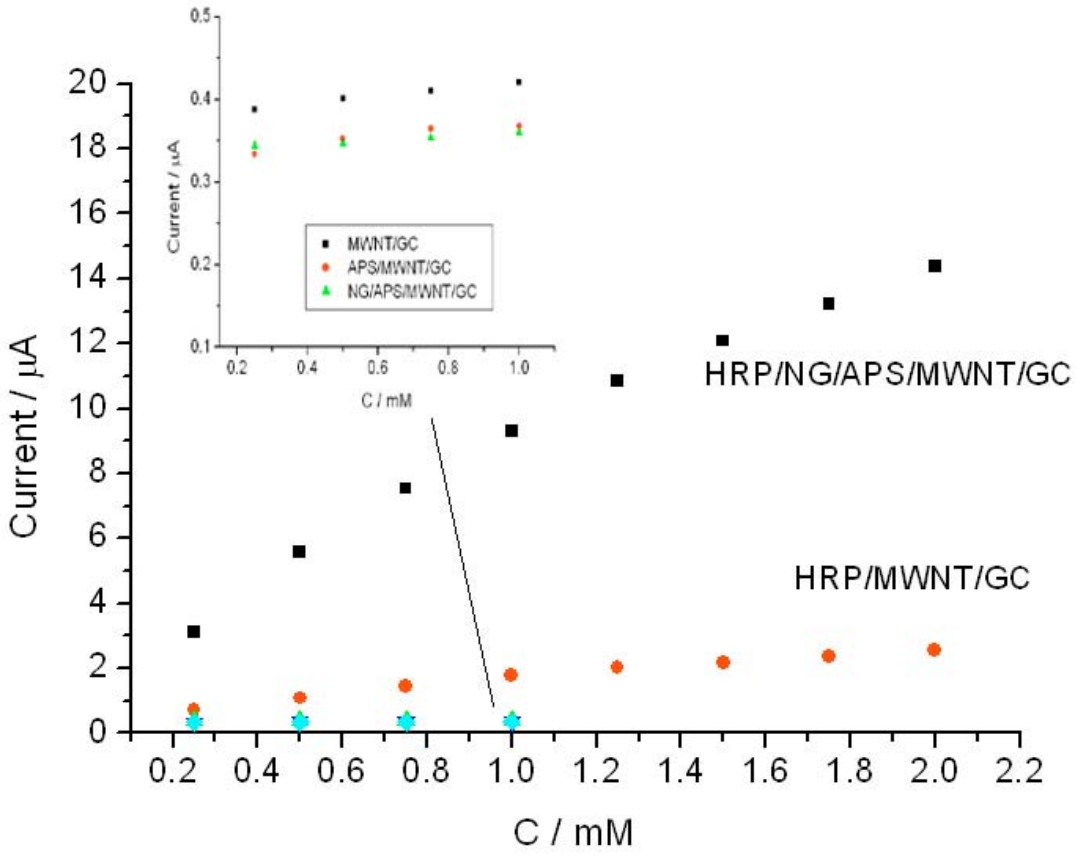
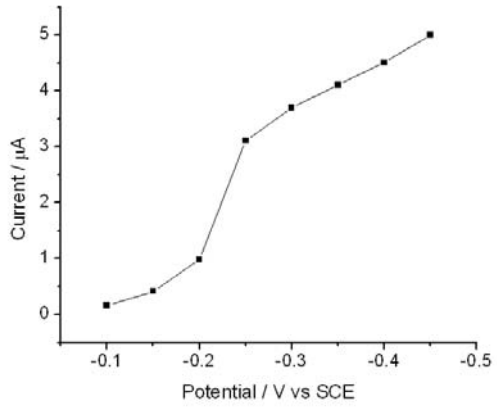


Figure 8. Steady-state amperometry measurement of current vs. peroxide concentration in stirred pH 7.0 PBS in the biosensor assembled with nanogold particles (a) and that unassembled nanogold particles (b). (A) MWNT/GC; (B) APS/MWNT/GC; (C) NG/APS/MWNT; (D) HRP/NG/APS/MWNT/GC.



4.3. Optimization of the experimental conditions

In order to obtain the optimal response of H_2O_2, the effect of working potential on the steady-state current of H_2O_2 was examined (shown in Figure 9). It was observed that the steady-state current increased with the negative shift of cathodic potential, and when the potential reached –0.25 V, the change of the steady-state current became smaller.

The pH effect on the response of the sensor is shown in Figure 10. The current response increased with pH in the range of pH 4.0 ~ 7.0 and then decreased with the pH further increase. Thus buffer solution of pH 7.0 was selected to obtain a stable, reproducible and maximal amperometric response in the subsequent work.

4.4. Steady-state amperometric response to hydrogen peroxide

Figure 11 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 biosensor to H_2O_2. The time required to reach 95% of the maximum steady-state current is less than 5 s. Figure 12 displays the calibration plot of the biosensor. The biosensor responds to H_2O_2 in the range from 2 μM to 3.5 mM. The detection limit is down to 1.0 μM when the signal to noise ratio is 3.The relative standard deviation is 4.5% for 8 repetitive measurements to 0.5 mM H_2O_2 solution. The reproducibility of four electrodes made by the same process shows a variation coefficient of 5.1% for the current measured at 0.1 mM H_2O_2.

After measurement, the biosensor was rinsed with doubly distilled water and stored at 4 □. The stability of this biosensor was shown in Figure 13. It was found that the biosensor could keep its original response for at least three weeks. Because the quinone is on the MWNTs surface and never falls out from them, it is of great importance to keep the stability of the biosensor.

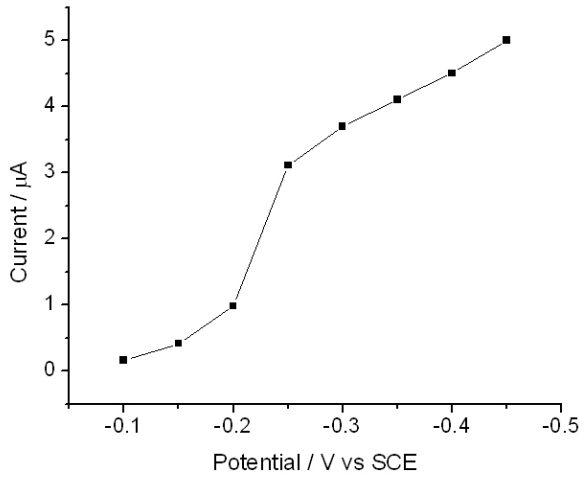


Figure 9. Effect of the working potential on the response of the sensor. Experimental conditions: 0.25 mM H_2O_2 in pH 7.0 PBS.

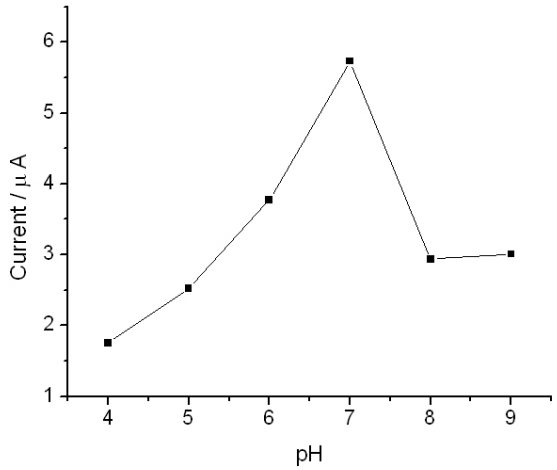


Figure 10. Effect of the pH on the response of the sensor to 0.25 mM H_2O_2 in PBS.

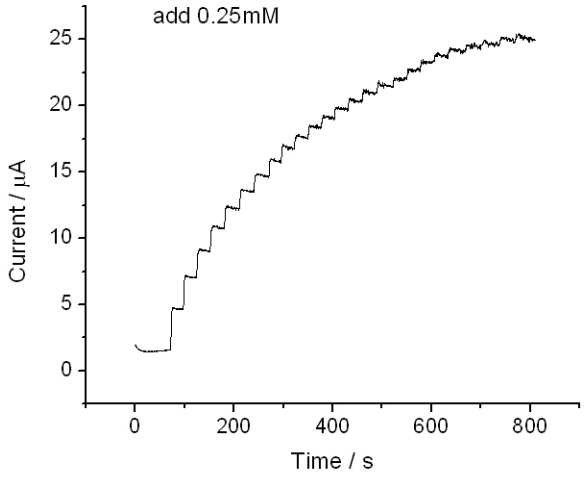


Figure 11. Dynamic response of the H_2O_2 sensor to successive addition of 0.25 mM H_2O_2 steps in the solution at the applied potential of -0.3 V.

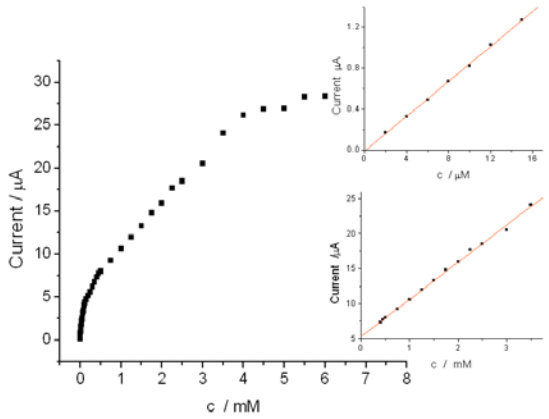


Figure 12. The calibration plot between the current and the different concentration H_2O_2

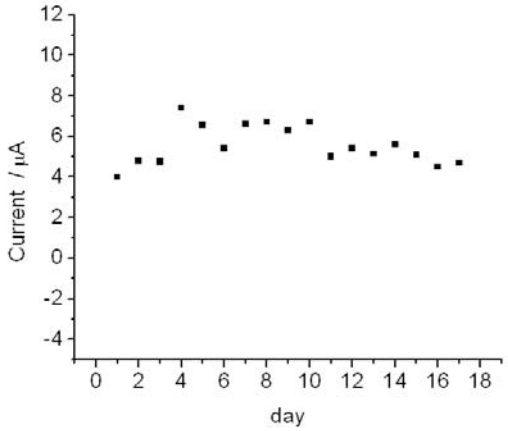


Figure 13. Effect of storage time on the response ratio of the biosensor to H_2O_2.

4.5. 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 (44):

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

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 0.2 mM.

5. CONCLUSION

A new composite film in combination of the characters of nanogold particle and MWNT can be achieved by means of the multistep assembled method. A novel biosensor has been constructed with excellent performance based on the composite. The results provide a possibility of constructing the new functional biomaterials. Thus, it is worthwhile for fabricating functional biosensors to further develop the multi-functional composite materials and various assembly techniques.

6. ACKNOWLEDGEMENTS

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Abbreviations: MWNTs: multiwall carbon nanotubes, HRP: horseradish peroxidase, GC: glassy carbon, NG: nano-gold, APS: (3-Aminopropyl)trimethoxysilane, K_M^{app}: Michaelis-Menten constant.

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