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# Reagentless biosensor based on layer-by-layer assembly of functional multiwall carbon nanotubes and enzyme-mediator biocomposite*

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**Abstract:** A simple and controllable layer-by-layer (LBL) assembly method was proposed for the construction of reagentless biosensors based on electrostatic interaction between functional multiwall carbon nanotubes (MWNTs) and enzyme-mediator biocomposites. The carboxylated MWNTs were wrapped with polycations poly(allylamine hydrochloride) (PAH) and the resulting PAH-MWNTs were well dispersed and positively charged. As a water-soluble dye methylene blue (MB) could mix well with horseradish peroxidase (HRP) to form a biocompatible and negatively-charged HRP-MB biocomposite. A $(PAH\text{-}MWNTs/HRP\text{-}MB)_n$ bionanomultilayer was then prepared by electrostatic LBL assembly of PAH-MWNTs and HRP-MB on a polyelectrolyte precursor film-modified Au electrode. Due to the excellent biocompatibility of HRP-MB biocomposite and the uniform LBL assembly, the immobilized HRP could retain its natural bioactivity and MB could efficiently shuttle electrons between HRP and the electrode. The incorporation of MWNTs in the bionanomultilayer enhanced the surface coverage concentration of the electroactive enzyme and increased the catalytic current response of the electrode. The proposed biosensor displayed a fast response (2 s) to hydrogen peroxide with a low detection limit of $2.0\times10^{-7}$ mol/L ($S/N$=3). This work provided a versatile platform in the further development of reagentless biosensors.

**Key words:** Reagentless biosensor, Layer-by-layer assembly, Horseradish peroxidase-methylene blue (HRP-MB) biocomposite, Functional multiwall carbon nanotubes, Hydrogen peroxide

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## 1 Introduction

Enzyme-based biosensors play an important role in various industries, such as food, manufacturing, clinic, and environment (Xu *et al*., 2002; Terry *et al*., 2005; Ozoemena and Nyokong, 2006; Deng *et al*., 2008). Recently, mediators have been employed in enzyme-based biosensors in order to shuttle electrons between the redox enzyme and the electrode surface (Li *et al*., 2003; Ricci *et al*., 2003). Solution-phase mediators may cause electrode contamination and operation inconvenience. In order to overcome the above-mentioned drawbacks and improve the performances of the biosensors, the immobilization of the mediator on a solid support provides a new way to construct reagentless biosensors.

Until recently, various methods including cross-linking, physical entrapment, and covalent bonding for the co-immobilization of enzyme and mediator on the electrode surface, have been reported. For example, Xu *et al*. (1998) fabricated a reagentless hydrogen peroxide biosensor, based on the co-immobilization of horseradish peroxidase (HRP) and the redox dye thionine by their cross-linking with glutaraldehyde on the surface of a glassy carbon electrode. Qiu *et al*. (2008) constructed an amperometric sensor for glucose by entrapping glucose oxidase (GOD) in a chitosan composite doped with

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ferrocene-modified multiwall carbon nanotubes which acted as an electron transfer mediator. Ma *et al*. (2009) used 2,6-pyridinedicarboxylic acid (PDC) polymer as the matrix to covalently immobilize thionine as a mediator, and then successfully adsorbed hemoglobin on the nano-Au which was electrodeposited onto a thionine-modified electrode surface. Among the above methods, time-consuming and uncontrollable fabrication processes were involved and the film thickness was difficult to control. Therefore, a new method was required for the preparation of biosensors with controllability and high order.

The layer-by-layer (LBL) assembly offering an easy and inexpensive process for multilayer formation has attracted much attention in recent years (Fendler, 1996; Hammond, 2004; Shi *et al*., 2007; Ou *et al*., 2008). Various charged materials, such as nanotubes (Mamedov *et al*., 2002), organic dyes (Cooper *et al*., 1995), and proteins (Liu and Lin, 2006), could be used as components to prepare multilayer films with high order at nanoscale via LBL assembly. Furthermore, the LBL method is conducted in aqueous solutions under mild conditions, which retains the activities of biomolecules (Ariga *et al*., 2007; Yao and Hu, 2010).

Recently, carbon nanotubes (CNTs) have been conducted in LBL assembly due to their specific characteristics including high surface-to-volume ratio, high chemical stability, and good electric conductivity (Kim *et al*., 2008; Sheng and Zheng, 2009). In order to subject CNTs to the electrostatic LBL assembly, charged sites have to be attached to the carbon nanotube structures. Wrapping CNTs with polyelectrolytes is an effective method. For example, multiwall carbon nanotubes (MWNTs) could interact with poly(allylamine hydrochloride) (PAH) and the resulting PAH-MWNTs are well dispersed and positively charged.

In this work, a type of reagentless biosensor, based on electrostatic LBL assembly of functional MWNTs and enzyme-mediator biocomposites, was proposed. HRP was chosen as a model enzyme and methylene blue (MB) was selected as a model mediator. By the use of a simple premixing procedure, a well-biocompatible and negatively-charged HRP-MB biocomposite was obtained. A (PAH-MWNTs/HRP-MB)$_n$ multilayer film was then prepared based on electrostatic LBL assembly of positively-charged PAH-MWNTs and negatively-charged HRP-MB on a gold electrode. Due to the excellent biocompatibility of HRP-MB biocomposite and the uniform LBL assembly, the immobilized HRP could retain its natural bioactivity and MB could efficiently shuttle electrons between HRP and the electrode. The incorporation of MWNTs in the bionanomultilayer enhanced the surface coverage concentration of the electroactive enzyme and increased the catalytic current response of the electrode. The prepared biosensor exhibited an electrocatalytic activity to the reduction of hydrogen peroxide with a fast response, a low detection limit, and an effective anti-interference capability.

## 2 Materials and methods

### 2.1 Reagents

HRP (EC 1.11.1.7, 250 U/mg) was purchased from Shanghai Lizhu Dongfeng Biotechnology Co., Ltd., China. MB was purchased from Shanghai Reagent Company. MWNTs were purchased from Nanoport Co., Ltd. (Shenzhen, China). 3-Mercapto-1-propanesulfonic acid sodium salt (MPS) was obtained from Aldrich Chemical Company, Inc., USA. Poly(sodium-*p*-styrene-sulfonate) (PSS; $M_w$ 70000) and PAH ($M_w$ 15000) were both purchased from Sigma-Aldrich Co., Ltd., UK. Dilute $H_2O_2$ solutions were all freshly prepared from a 30% $H_2O_2$ solution. All other chemicals were of analytical grade and used without further purification. Water used in this work was doubly distilled.

### 2.2 Preparations of functional MWNTs and HRP-MB biocomposite

The carboxylated MWNTs were obtained according to the procedures reported previously (Liu L.J. *et al*., 2008). The carboxylated MWNTs (1 mg/ml) were then dispersed in 0.04 mol/L barbital buffer (pH 9.0) containing 1 mg/ml PAH with 5 min ultrasonication. The resulting PAH-wrapped MWNTs (PAH-MWNTs) were well dispersed.

The HRP-MB biocomposite was obtained by a simple premixing procedure. Briefly, 1 mg/ml HRP and 0.33 mg/ml MB were mixed in 0.04 mol/L barbital buffer (pH 8.0) to form a homogeneous solution. The obtained HRP-MB biocomposite was well biocompatible.

### 2.3 Preparation of polyelectrolyte precursor film

Prior to modification, the bare Au electrode was thoroughly polished with emery paper and alumina slurry in order of 1.0, 0.5, 0.03 μm, followed by ultrasonication in water. Next, the electrode was immersed in a freshly prepared piranha solution (30% $H_2O_2$ and 98% $H_2SO_4$; 1/3, v/v) for 40 min. After this, the electrode was rinsed with doubly distilled water and electrochemically pretreated with cyclic potential scanning between 1.5 and –0.2 V in 0.2 mol/L $H_2SO_4$ solution to obtain a clean gold electrode. Afterwards, the pretreated electrode was immersed in 0.02 mol/L MPS aqueous solution for 12 h to create a negative monolayer. The resulting electrode was then sequentially immersed in 1 mg/ml polycation PAH and polyanion PSS solutions for 20 min. PAH and PSS solutions were both prepared with pH 12.0 glycine-NaOH buffer including 0.4 mol/L NaCl. Thus, a PAH/PSS polyelectrolyte precursor film was successfully assembled on the gold electrode surface.

### 2.4 Preparation of (PAH-MWNTs/HRP-MB)$_n$ bionanomultilayer

The Au electrode modified with polyelectrolyte precursor film was then sequentially dipped into the PAH-MWNTs and HRP-MB solutions for 40 min. Thus, the (PAH-MWNTs/HRP-MB)$_n$ bionanomultilayer was prepared on the Au electrode via LBL assembly. The assembly process is shown in Fig. 1.

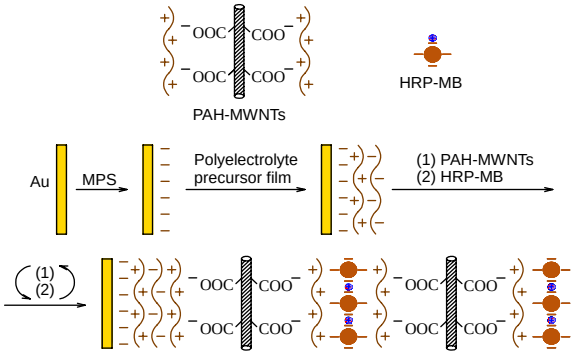


**Fig. 1 Schematic illustration of LBL assembly of PAH-MWNTs and HRP-MB on an Au electrode modified with a polyelectrolyte precursor film**

The Si-wafer used for scanning electron microscope (SEM) characterization and the poly(ethylene terephthalate) (PET) film used for ultraviolet-visible (UV-Vis) characterization were pretreated in 1.5 mol/L NaOH for 20 min to create a negatively-charged surface. The procedure used to assemble the (PAH-MWNTs/HRP-MB)$_n$ bionanomultilayer on Si-wafer or PET film was the same as that used for the Au electrode.

### 2.5 Worthington method

The Worthington method involved a reaction system of $H_2O_2$ and 4-amino antipyrine, which was catalyzed by HRP (Jiang *et al*., 2004). The experiment included three steps. Firstly, PET films were cut into the same size (1 cm×1 cm). Secondly, six bilayers of PAH/HRP-MB or PAH-MWNTs/HRP-MB based on LBL assembly of PAH or PAH-MWNTs and HRP-MB were assembled on the PET film. Thirdly, the modified PET film was immersed in a reaction system of $H_2O_2$ and 4-amino antipyrine. The system was catalyzed by HRP. After 5 min, the film was taken out and a spectrophotometer was used to measure the absorption of the red product of the reaction system at about 500 nm.

### 2.6 Characterization and electrochemical measurements

UV-Vis spectra were recorded using a Shimadzu UV-2550 spectrophotometer (Shimadzu International Trading Co., Ltd., Shanghai, China). The morphology of the bionanomultilayer was characterized with the use of a Sirion-100 instrument (FEI, USA) at an operation voltage of 5.0 kV. The zeta potential was measured using a Malvern Zetasizer 3000HSA (Malvern Instruments Ltd., Malvern, UK) to determine the effect of surface-charge in the PAH-MWNTs and HRP-MB solutions. Electrochemical measurements were carried out by a CHI 650 electrochemical analyzer (CH Instrument Company, Shanghai, China). A conventional three-electrode cell was used. A bare or modified gold electrode was used as a working electrode, and a platinum disk was used as an auxiliary electrode, with a saturated calomel electrode (SCE) used as a reference.

## 3 Results and discussion

### 3.1 Characteristics of HRP-MB

The HRP-MB biocomposite was prepared by premixing HRP and MB in solution. The UV-Vis

spectra of MB, HRP, and HRP-MB solutions from 300 to 900 nm were shown in Fig. 2. The absorption peak of MB solution was observed at 665 nm (Fig. 2a). The HRP solution showed a typical absorption peak around 403 nm (Fig. 2b). The HRP-MB solution showed two absorption bands located at 665 and 404 nm (Fig. 2c), which were similar to those of the native solutions of MB and HRP. According to above results, the HRP-MB composite was well biocompatible, and HRP mixed in the current system could retain its natural bioactivity.

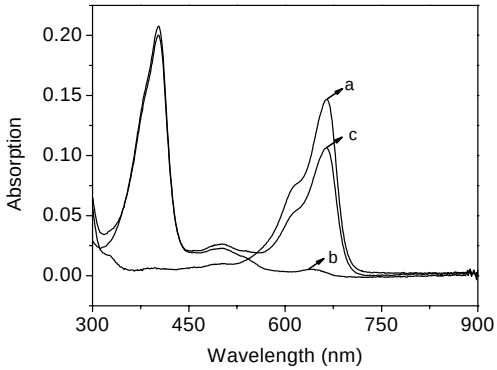


**Fig. 2 UV-Vis absorption spectra of MB (a), HRP (b), and HRP-MB (c) solutions**

### 3.2 Preparation of (PAH-MWNTs/HRP-MB)$_n$ bionanomultilayer

In the pH 8.0 solution, the HRP molecules, with an isoelectric point at 7.2, were introduced to be negatively charged. MB was a cationic dye. Negatively-charged HRP could interact with positively-charged MB to form an HRP-MB composite (HRP/MB, mole ratio 3/1). The carboxylated negatively-charged MWNTs could interact with polycations PAH (PAH/MWNTs, mole ratio 1/1). The zeta potential measurements were performed to determine the effect of surface-charge in PAH-MWNTs and HRP-MB solutions. The zeta potential values of PAH-MWNTs at pH 9.0 and HRP-MB at pH 8.0 were +34.9 and –3.2 mV, respectively. The result clearly identified that the PAH-MWNTs and HRP-MB were positively and negatively charged, respectively.

A schematic representation of Au/precursor film/(PAH-MWNTs/HRP-MB)$_n$ fabrication is showed in Fig. 1 and involved three steps. Firstly, MPS was assembled on the bare gold electrode to create a negatively-charged surface through covalent bonding between the sulfonic groups and the gold electrode. Secondly, polycations PAH and polyanions PSS were sequentially assembled on the MPS-modified electrode through electrostatic adsorption. The fabricated polyelectrolyte precursor film with outermost negative charge was used as a base for the next assembly. Thirdly, a (PAH-MWNTs/HRP-MB)$_n$ bionanomultilayer film was prepared by electrostatic LBL assembly of positively-charged PAH-MWNTs and negatively-charged HRP-MB. The influence of the bilayer number of PAH-MWNTs/HRP-MB on the response of the biosensor was investigated. The sensitivity of the modified electrode increased with the bilayer number growing from one to six. Therefore, six bilayers were chosen.

### 3.3 Characterization of (PAH-MWNTs/HRP-MB)$_n$ bionanomutilayer

Fig. 3 showed the UV-Vis spectra of six bilayers of PAH/HRP-MB (Fig. 3a) and PAH-MWNTs/HRP-MB (Fig. 3b) on PET films by Worthington method. The absorption peak at about 500 nm represented the activity of HRP immobilized on the PET film. As can be seen, the absorption peak of six bilayers of PAH-MWNTs/HRP-MB was obviously higher than that of PAH/HRP-MB, which indicated that the surface coverage concentration of the electroactive enzyme was enhanced by the usage of MWNTs. This result identified that MWNTs with a highly-accessible surface area could be used as predominant materials for enzyme immobilization.

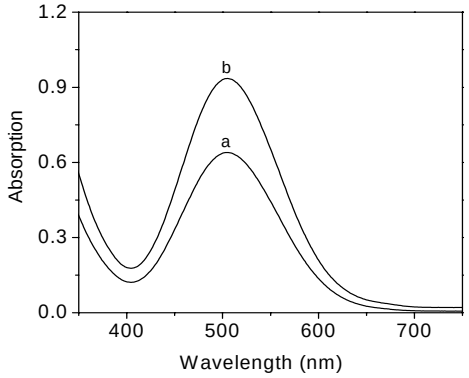


**Fig. 3 UV-Vis absorption spectra of six bilayers of PAH/HRP-MB (a) and PAH-MWNTs/HRP-MB (b) on PET films**

The LBL assembly of (PAH-MWNTs/HRP-MB)$_n$ bionanomultilayer was also characterized by SEM.

Fig. 4 showed the SEM images of different bilayers of PAH-MWNTs/HRP-MB immobilized on the Si-wafer surface. When the first bilayer of PAH-MWNTs/HRP-MB was immobilized, MWNTs were sparsely dispersed on the Si-wafer surface (Fig. 4a). After the bilayer number increased to three, an obvious increase in MWNTs coverage was observed (Fig. 4b). Finally, when the bilayer number increased to six, the Si-wafer surface was fully covered with MWNTs (Fig. 4c). From the high magnification image (Fig. 4d), high-density carbon nanotubes were clearly visible. A uniform $(PAH\text{-}MWNTs/HRP\text{-}MB)_n$ multilayer film was formed on the substrate surface.

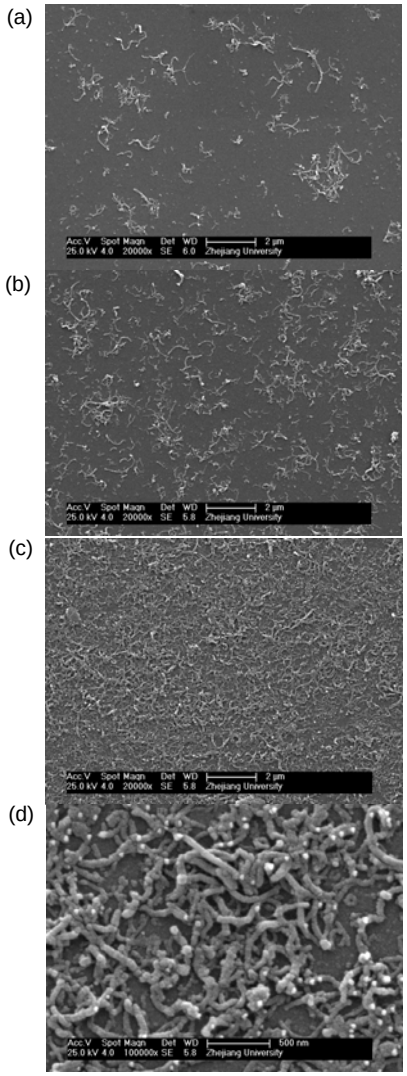


**Fig. 4 SEM images of one bilayer (a), three bilayers (b), and six bilayers (c) of PAH-MWNTs/HRP-MB on the Si-wafer surface, and six bilayers of PAH-MWNTs/HRP-MB at high-magnification (d)**

### 3.4 Electrochemical characteristics of the developed electrode

Fig. 5 showed the cyclic voltammetries (CVs) of gold electrodes modified with six bilayers of PAH-MWNTs/HRP and PAH-MWNTs/HRP-MB in 0.2 mol/L PBS (pH 7.0) at a scan rate of 100 mV/s. No obvious redox peaks could be observed on the electrode modified with six bilayers of PAH-MWNTs/HRP (Fig. 5a) under this potential range as the lack of electron mediator between HRP and the electrode. In contrast, the electrode modified with six bilayers of PAH-MWNTs/HRP-MB displayed a couple of well-defined redox peaks at –0.301 and –0.235 V (Fig. 5b), which was similar to the character of MB directly added in the solution. After the addition of 3.0 mmol/L hydrogen peroxide, the reduction peak current increased while the oxidation peak current decreased (Fig. 5c). This result demonstrated that MB was an efficient mediator for transferring electrons between HRP and the electrode. HRP catalyzed hydrogen peroxide reduction according to the following schemes (Kafi *et al*., 2008):

$$H_2O_2+2H^++HRP_{Red}\rightarrow HRP_{Ox}+2H_2O,$$
$$HRP_{Ox}+MB_{Red}\rightarrow HRP_{Red}+MB_{Ox},$$
$$MB_{Ox}+2H^++2e\rightarrow MB_{Red}.$$

In the above, $HRP_{Ox}$ and $HRP_{Red}$ represented the oxidized and reduced forms of HRP, and $MB_{Ox}$ and $MB_{Red}$ represented the oxidized and reduced forms of MB, respectively.

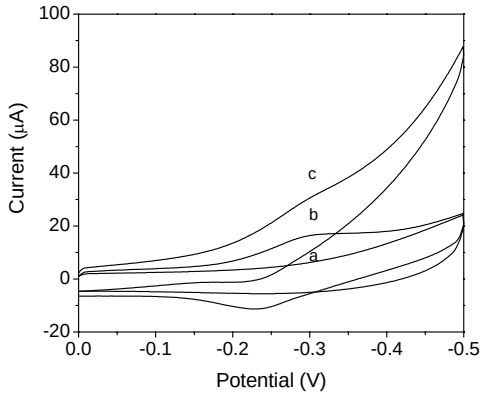


**Fig. 5 Cyclic voltammograms of Au electrodes modified with six bilayers of PAH-MWNTs/HRP (a) and PAH-MWNTs/HRP-MB in the absence (b) and presence (c) of 3.0 mmol/L hydrogen peroxide in 0.2 mol/L PBS (pH 7.0) at a scan rate of 100 mV/s**

### 3.5 Influences of potential and pH

The dependence of the biosensor response on the applied potential was studied (Fig. A1). With the increasing potential from –0.25 to –0.05 V, the sensitivity of the biosensor decreased. The background current decreased similarly to the trend of the sensitivity. In order to obtain higher sensitivity and lower background, and also avoid interference at high negative applied potentials, –0.2 V was chosen as working potential for this study.

The pH of the measurement solution affected the activity of immobilized HRP, resulting in the dependence of the biosensor response on pH (Fig. A2). Both of the sensitivity and the background current decreased from pH 6.0 to 8.0. The biosensor showed an optimum sensitivity of response and lower background to hydrogen peroxide at pH 7.0. Therefore, the suitable pH of the biosensor was set at pH 7.0.

### 3.6 Amperometric response of the developed reagentless biosensor

Amperometric measurements were carried out in a stirred 0.2 mol/L PBS (pH 7.0) at an applied potential of –0.2 V. Fig. 6a presented the current responses of Au electrodes modified with six bilayers of PAH/HRP-MB and PAH-MWNTs/HRP-MB on consecutive addition of 10 μmol/L hydrogen peroxide. The current response of Au electrode modified with six bilayers of PAH-MWNTs/HRP-MB was much higher than that of PAH/HRP-MB. The result indentified that the introduced MWNTs increased the catalytic current response of the electrode. A typical amperometric response of Au electrode modified with six bilayers of PAH-MWNTs/HRP-MB to successive addition of hydrogen peroxide was shown in Fig. 6b. The maximum steady-state current was within 2 s. The linear response range of the biosensor was from $3.0\times10^{-7}$ to $3.2\times10^{-5}$ mol/L and from $3.2\times10^{-5}$ to $2.8\times10^{-4}$ mol/L with the linear regression equations of $I$ (μA)=0.670+0.033$c$ (μmol/L) ($R$=0.9991, $n$=16) and $I$ (μA)=1.468+0.011$c$ (μmol/L) ($R$=0.9974, $n$=11), respectively. Based on a signal to noise ration of 3,

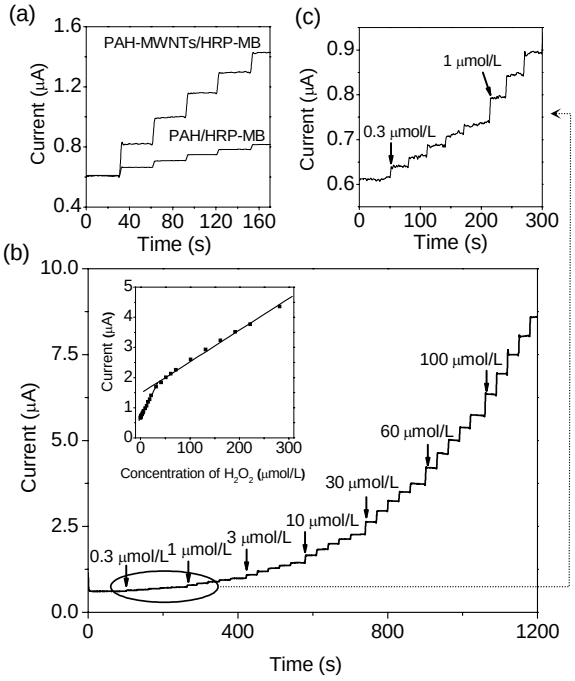


**Fig. 6 Current responses of Au electrodes**

(a) Current responses of Au electrodes modified with six bilayers of PAH/HRP-MB and PAH-MWNTs/HRP-MB on successive addition of 10 μmol/L hydrogen peroxide; (b) A typical amperometric response of Au electrode modified with six bilayers of PAH-MWNTs/HRP-MB to successive addition of hydrogen peroxide at an applied potential of −0.2 V in 0.2 mol/L PBS (pH 7.0) and calibration curve (inset); (c) Amplified response curve of (b) in 0–300 s

**Table 1 Comparison of the developed biosensor for $H_2O_2$ detection with other biosensors**

| Electrode | Response time (s) | Linear range (μmol/L) | Detection limit (μmol/L) | Study |
|---|---|---|---|---|
| Au/Precursor/(PAH-MWNTs/HRP-MB)$_6$ | 2 | 0.3–280.0 | 0.2 | This work |
| MWNTs/MG/HRP/GCE | <10 | 0.5–20.0 | 0.5 | Upadhyay *et al*., 2009 |
| HRP-MB/MWNTs/GC | <30 | 4–2000 | 1.0 | Xu *et al*., 2003 |
| MB-Bir/HRP/QY/GCE | 8 | 1.5–8100.0 | 1.3 | Yang *et al*., 2008 |
| HRP/Tb-MWNTs/GE | 8 | −400 | 1.7 | Liu Y. *et al*., 2008 |
| Au/CS-MWNTs-NB-HRP | 2 | 1–240 | 0.12 | Xi *et al*., 2009 |
| PVA/Hb/MWNTs/CILE | − | 1.2–30.0 | 0.5 | Sun *et al*., 2009 |

PAH: poly(allylamine hydrochloride); HRP: horseradish peroxidase; GCE: glassy carbon electrode; MB: methylene blue; MG: methylene green; MWNTs: multiwall carbon nanotubes; GC: glassy carbon; MB-Bir: MB-intercalated birnessite; QY: polyquaternium-10; Tb: toluidine blue; GE: graphite electrode; CS: chitosan; NB: nile blue; PVA: polyvinyl alcohol; CILE: carbon ionic liquid electrode

the detection limit was $2.0\times10^{-7}$ mol/L. The comparison of the prepared electrode with other reagentless electrodes fabricated with MWNTs or organic dye is listed in Table 1. It can be seen that the prepared electrode exhibited a faster response and a lower detection limit than those of other reported literature.

### 3.7 Reproducibility and stability of the reagentless biosensor

The reproducibility and storage stability of the biosensor were studied. Among the reproducibility experiment, each measurement was performed in a separate PBS solution with consecutive addition of 10 μmol/L $H_2O_2$ solution. The relative standard deviation (RSD) of the biosensor with eight successive measurements was 2.3%. The fabrication reproducibility was examined through measurements of six electrodes prepared in the same procedure independently and the RSD was 3.3%. When not in use, the modified electrode was stored in dry conditions at 4 °C in a refrigerator. After a storage period of one month, the biosensor retained approximately 86% of its original sensitivity. The 14% decay of the signal within the storage period involved the deviation. The result showed a long lifetime of the biosensor.

### 3.8 Selectivity and practicability of the prepared biosensor

The selectivity of the prepared $H_2O_2$ biosensor was evaluated through studying the influences of possible interfering substances including ascorbic acid, uric acid, and glucose. The addition of 0.2 mmol/L interfering substance into an electrochemical cell containing 0.02 mmol/L $H_2O_2$ did not cause any observable interference, which was due to the low working potential of –0.2 V used in the measurement of the biosensor.

The prepared biosensor could be applied to the $H_2O_2$ determination in biological and medical samples. The recovery of $H_2O_2$ in real sample was determined, in the range of 96.8%–102.7%.

## 4 Conclusions

A type of reagentless biosensor based on functional MWNTs and enzyme-mediator biocomposite was formed by LBL assembly. Due to the excellent biocompatibility of HRP-MB biocomposite and the uniform LBL assembly, the immobilized HRP could retain its natural bioactivity and MB could efficiently shuttle electrons between HRP and the electrode surface. The incorporation of MWNTs in the bionanomultilayer enhanced the surface coverage concentration of the electroactive enzyme and increased the catalytic current response of the electrode. The resulting biosensor provided favorable analytical characteristics for $H_2O_2$ including a faster response, a lower detection limit, and an effective anti-interference capability. This approach provided a versatile platform for the further development of reagentless biosensors.

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## Appendix

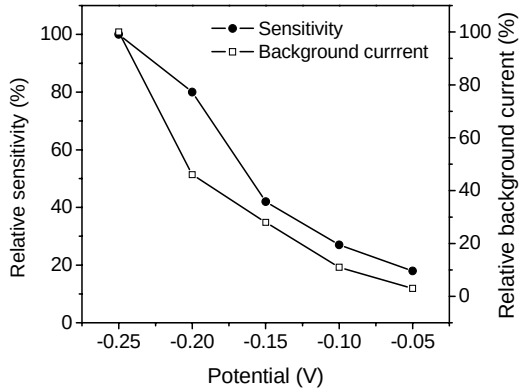


**Fig. A1 Dependence of the biosensor response on the applied potential for hydrogen peroxide in 0.2 mol/L PBS (pH 7.0)**

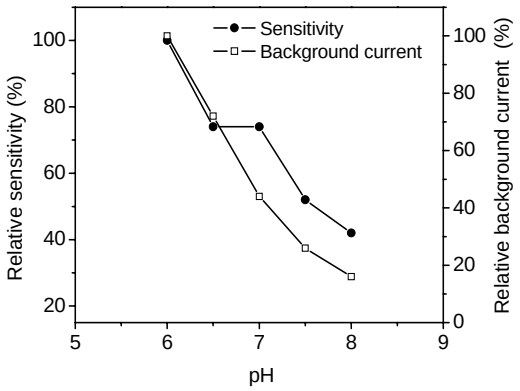


**Fig. A2 Dependence of the biosensor response on the pH of PBS (0.2 mol/L) for hydrogen peroxide at an applied potential of −0.2 V**