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Amperometric sensor for hydrogen peroxide based on electric wire composed of horseradish peroxidase and toluidine blue-multiwalled carbon nanotubes nanocomposite

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Abstract

A kind of nanocomposites with good dispersion in water was prepared through noncovalent adsorption of toluidine blue (Tb) on multiwalled carbon nanotubes (MWCNT) for electric communication between horseradish peroxidase (HRP) and electrode. The nanocomposites could be conveniently cast on electrode surface. With the aid of chitosan, HRP was then immobilized on the nanostructure to form a reagentless amperometric sensor for hydrogen peroxide. UV–vis spectroscopy and electrochemical impedance spectroscopy were used to characterize the adsorption of Tb on MWCNT. The presence of both Tb as mediator of electron transfer and MWCNT as conductor enhanced greatly the enzymatic response to the reduction of hydrogen peroxide. The novel biosensor exhibited fast response towards hydrogen peroxide with a detection limit of 1.7×10^{-6} M and the linear range extended up to 4×10^{-4} M without the interference of ascorbic acid and uric acid. The Michaelis–Menten constant (K'_m) of the immobilized HRP was evaluated to be 0.16 mM.

Keywords: Amperometric sensor; Carbon nanotubes; Toluidine blue; Nanocomposites modified electrode; Hydrogen peroxide

1. Introduction

In recent years carbon nanotubes (CNTs) have been extensively used for electrochemical study and preparation of electrochemical sensors due to their electrical conductivity, unique structural and catalytic properties. However, a major barrier for the preparation of CNTs-based biosensors is the insolubility of CNTs in most solvents. Although the challenge of solubilizing CNTs has been addressed by several methods, including temporarily dispersing in some organic solvent [1,2], oxidation treatment [3] and polymer wrapping or grafting [4–7], these methods possibly impair the chemical properties of CNTs or decrease their conductivity. Therefore, the new strategy for improving the solubility of CNTs and keeping their pristine mechanical and electronic properties has attracted considerable attention. The CNTs exhibit a special sidewall curvature and possess a π-conjugative structure with a highly hydrophobic surface, which allow them to interact with some aromatic compounds [8] such as anthracene derivatives [9], methylene blue (MB) [10], and thionine [11] through π–π electronic and hydrophobic interactions. By use of these interactions, this work prepared a kind of nanocomposites through noncovalent adsorption of toluidine blue (Tb) on multiwalled carbon nanotubes (MWCNT). The nanocomposites showed good dispersion in water, and could thus be conveniently cast on an electrode surface for preparation of biosensors.

The integration of redox mediators and CNTs has been used for development of electrochemical sensors [12–18]. Gorski proposed two β-nicotinamide adenine dinucleotide (NADH) amperometric sensors by covalently attaching Azure dye [12] and Tb [13] as redox mediators to polysaccharide chains of chitosan, which were then mixed with CNTs and cast on electrode to provide remarkable synergistic augmentation of sensor responses. Other amperometric sensors for NADH have been prepared with thionine adsorbed CNTs [14], which also show the synergistic augmentation and sensitive response to nitrite [15], and poly(Tb)/MWCNT composite formed on a glassy carbon electrode through electropolymerization [16]. One amperomet-

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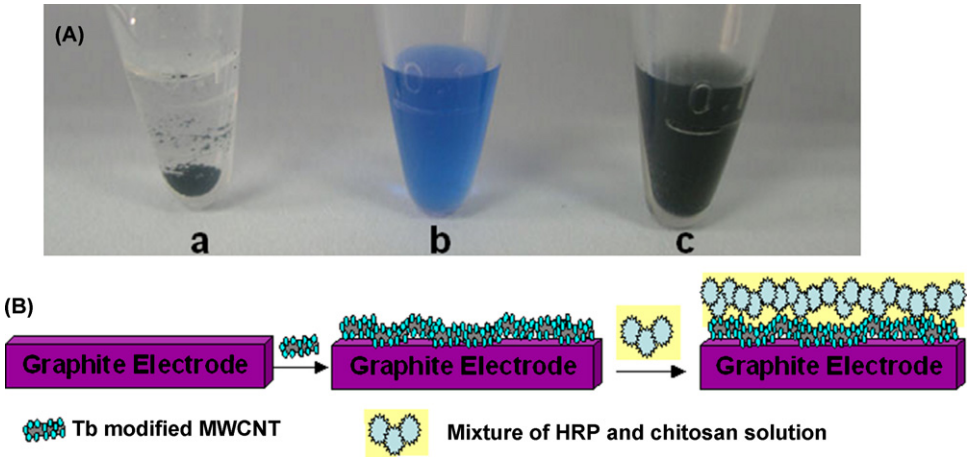

Fig. 1. (A) Photos of (a) pristine MWCNT, (b) 0.5 mM Tb solution and (c) Tb–MWCNT solution containing 2 mg mL^{-1} MWCNT, and (B) fabrication process of the sensor.

ric sensor for hydrogen peroxide based on the electrocatalytic reduction by thionine immobilized covalently on MWCNTs has been reported [17]. Chen and co-workers [18] prepared a sensor for hydrogen peroxide by using tetrahydrofuran to disperse MWCNTs and then incorporating horseradish peroxidase (HRP) and electron transfer mediator MB in the matrix. Obviously the presence of tetrahydrofuran would decrease the activity of the immobilized enzyme, and the hydrophobic surface of CNTs was also a disadvantage to the enzymatic reaction. This work conveniently used the Tb adsorbed noncovalently on MWCNT as a mediator for electric communication between HRP and its substrate to prepare a hydrogen peroxide biosensor by using chitosan to immobilize HRP on the film of Tb–MWCNT nanocomposites. The chitosan provided good biocompatibility for maintaining the activity of the immobilized HRP. The constructed sensor showed good stability and reproducibility. The synergistic effects of Tb as a mediator for electron transfer and MWCNT as an excellent conductor on the enzymatic reaction between HRP and hydrogen peroxide produced a sensitive reagentless biosensor for hydrogen peroxide.

2. Experiments

2.1. Chemicals and instruments

Horseradish peroxidase (HRP) (240 U mg^{-1}) was purchased from Huamei Bioengineering Company (China). Stock solution of 2 mg mL^{-1} HRP was prepared by directly dissolving HRP in 0.2 M phosphate buffer solution (PBS) (pH 7.0) and stored at 4 °C. Toluidine blue and chitosan (85%) were purchased from Sigma. One weight precent chitosan solution was prepared by dissolving chitosan flakes in 0.1 M HCl and adjusting the pH to 5.0 with NaOH solution. Multiwalled carbon nanotubes (average diameter of about 20 nm) were purchased from Nanoport Co. Ltd. (Shenzhen, China) and used as received without any pretreatment. Other reagents were commercially available and all of analytical reagent grade. Aqueous solutions were prepared with doubly distilled water, 0.2 M PBS (pH 7.0) was always employed as supporting electrolyte and deaerated by bubbling nitrogen prior to the experiments.

Amperometric, cyclic voltammetric and electrochemical impedance spectroscopic experiments were performed on a CHI 660B Electrochemical Workstation (CH Instruments Inc., USA). All experiments were carried out using a conventional three-electrode system with a modified graphite electrode as working, a platinum foil as auxiliary, and a saturated calomel electrode as reference electrodes. Ultraviolet–vis absorption (UV–vis) spectra were recorded with a Lambda 35 UV–vis spectrometer (Perkin-Elmer instruments, USA).

2.2. Preparation of soluble nanocomposites

Two milligrams of pristine MWCNT was added into 1 mL 0.5 mM Tb solution. After the mixture was sonicated for 1 h at room temperature, the resulting suspension was filtered with a millipore porous filter (0.45 μm, Millipore). The sediment was then washed with distilled water to remove nonadsorbed Tb. The obtained Tb–MWCNT nanocomposites could be dispersed in 1 mL doubly distilled water to obtain a stable nanocomposites suspension, as shown in Fig. 1A.

2.3. Preparation of HRP/Tb–MWCNT-modified electrode

After the home-made graphite electrode (inner-diameter: 3.0 mm; outer-diameter: 4.6 mm) was abraded with fine silicon carbide paper, it was successively polished with 0.3 and 0.05 μm alumina slurry, and sonicated in water and absolute ethanol. Three microlitters of Tb–MWCNT suspension was spread onto the electrode surface and allowed to dry in air. After washed with water to remove any loosely adsorbed species, the Tb–MWCNT-modified electrode was further modified by dropping 3 μL mixture of 2 mg mL^{-1} HRP solution and 1 wt% chitosan solution. The resulting HRP/Tb–MWCNT-modified electrode was washed with doubly distilled water and stored at 4 °C in air. The fabrication process of the modified electrode was shown in Fig. 1B. For comparison, the HRP/Tb or HRP/MWCNT-

modified graphite electrode was prepared by coating TB or MWCNT and then the mixture of HRP and chitosan on electrode surface.

3. Results and discussion

3.1. Characteristics of Tb–MWCNT

Tb can be easily attached onto MWCNT through the strong π–π stocking force between two conjugated frames. Fig. 1A compares the dispersion of pristine MWCNT, Tb, and Tb–MWCNT nanocomposites. It is difficult to disperse the pristine MWCNT in water because of its hydrophobic surface (photo a, Fig. 1A). Tb is a kind of purple dye and can be easily dissolved in water (photo b, Fig. 1A). Its adsorption on MWCNT greatly improved the dispersion of MWCNT in water due to the hydrophilicity of the adsorbed Tb molecules. The resulting Tb–MWCNT nanocomposites can be easily dispersed in water (photo c, Fig. 1A). The suspension of the nanocomposites is stable for at least 2 weeks, which highly facilitates the application of MWCNT in development of biosensors.

The UV–vis spectrum of Tb showed two characteristic absorption peaks of Tb around 288 and 633 nm and one absorption peak ascribed to the absorption of Tb dimer at 598 nm [19] (curve a, Fig. 2). The suspension of oxidized MWCNT in water did not cause any special absorbance except a broad absorption peak at 250 nm (curve c, Fig. 2) [10], which was the typical UV absorption of MWCNT. The two characteristic absorption peaks of Tb could be observed in Tb–MWCNT nanocomposite suspension (curve b, Fig. 2), indicating the presence of Tb molecules on MWCNT. However, the absorption wavelength of one characteristic peak shifted from 288 to 275 nm, and the absorption peak at 598 nm disappeared (curve b, Fig. 2). In comparison with

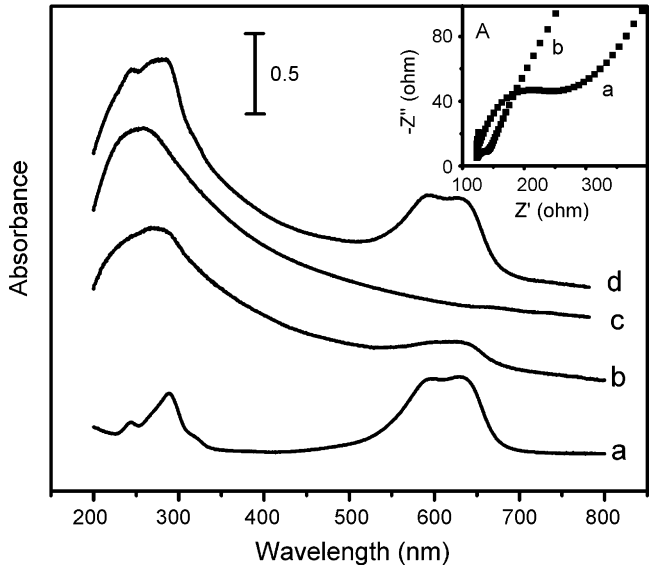

Fig. 2. UV spectra of (a) Tb, (b) Tb–MWCNT nanocomposites, (c) oxidized MWCNT, and (d) the arithmetical addition of the UV spectra of Tb and oxidized MWCNT. Inset: Nyquist diagrams of (a) Tb-modified and (b) Tb–MWCNT nanocomposites-modified electrodes in 1.0 M KCl containing 10 mM $[Fe(CN)_6]^{3-/4-}$.

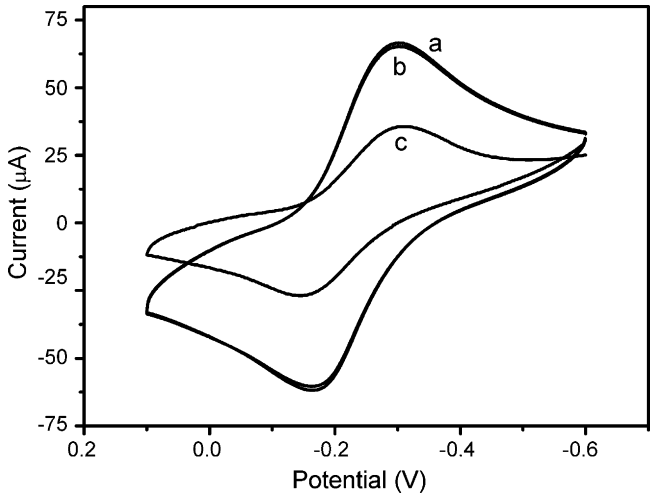

Fig. 3. Cyclic voltammograms of Tb–MWCNT nanocomposites-modified electrode at the first (a) and the 20th (b) scanning circles, and HRP/Tb–MWCNT-modified electrode (c) in 0.2 M PBS (pH 7.0) saturated with nitrogen at 100 mV s^{-1}.

the arithmetic addition of the UV spectra of toluidine blue and the oxidized MWCNT (curve d, Fig. 2), the blue shift resulted from the interactions between Tb and MWCNT and the steric barrier for the free conformation of Tb on MWCNT [20], and the disappearance of Tb dimer [11] was due to the formation of Tb–MWCNT nanocomposites.

The Tb–MWCNT nanocomposites-modified electrode was stable (Fig. 3) and showed low electron transfer impedance, R_{et}, due to the presence of MWCNT (inset, Fig. 2). The cyclic voltammogram of the modified electrode showed a couple of redox peaks at −0.300 and −0.165 V (curve a, Fig. 3), which were ascribed to the reduction and oxidation of the adsorbed TB. After continuously scanning for 20 cycles in PBS the cyclic voltammogram remained the same behavior (curve b, Fig. 3). The R_{et} value of $[Fe(CN)_6]^{3-/4-}$ at Tb–MWCNT-modified electrode was about 24 Ω, while it was 204 Ω at Tb-modified electrode. The MWCNT in the nanocomposites membrane was excellent conductor for electron transfer between the enzymatic system and electrode.

3.2. Electrocatalytic reduction of H_2O_2 on HRP/Tb–MWCNT-modified electrode

Chitosan displays excellent biocompatibility, nontoxicity, and high mechanical strength, and has thus been used as immobilization matrix for protein and biomolecule [21]. After the mixture of chitosan and HRP was coated on Tb–MWCNT-modified electrode, the cyclic voltammetric peak currents of Tb–MWCNT decreased and the separation of peak to peak hardly changed (curve c, Fig. 3). The decrease in the peak currents resulted from the block of the chitosan–HRP membrane to the diffusion of proton needed in the redox process of Tb. The same peak-to-peak separation indicated the presence of HRP–chitosan membrane did not affect the electron transfer rate.

At a bare graphite electrode the reduction of H_2O_2 showed great overpotential (Fig. 4A). In the potential window studied

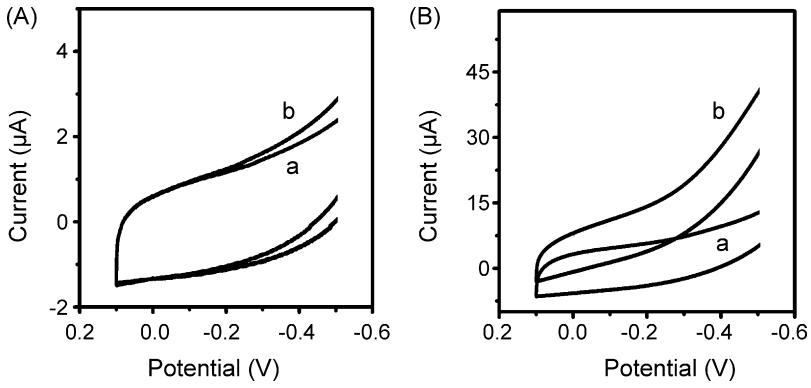

Fig. 4. Cyclic voltammograms of (A) bare and (B) HRP/MWCNT-modified electrodes in (a) 0.2 M PBS (pH 7.0) saturated with nitrogen and (b) 10 mM H_2O_2 at 100 mV s^{-1}.

the response of H_2O_2 was very low. Upon addition of 10 mM H_2O_2 an increase of 0.24 μA was detected at −0.4 V, while the value was about 17.3 μA at HRP/MWCNT-modified electrode (Fig. 4B).

The HRP/Tb-modified electrode exhibited only a pair of redox peak at of Tb (curve a, Fig. 5A). Upon addition of 10 mM H_2O_2, the reduction peak current increased greatly (from 18.6 to 42.9 μA) with slightly negative shift of peak potential and the oxidation peak current decreased (curve b, Fig. 5A), indicating an obvious electrocatalytic process of Tb to the enzymatic reduction of H_2O_2 by the immobilized HRP. The Tb acted as an electron transfer mediator. At HRP/Tb–MWCNT-modified electrode the electrocatalytic process was further enhanced (Fig. 5B). The increase in reduction peak current was about 64.4 μA (from 27.1 to 91.5 μA), which was about 2.65 times the electrocatalytic response at HRP/Tb-modified electrode. The electrocatalytic mechanism could be followed that reported previously [22].

From the CVs of $[Fe(CN)_6]^{3-}$ at HRP/Tb and HRP/Tb–MWCNT-modified electrodes, the presence of MWCNT increased the apparent surface of electrode for 1.6 times. Thus, the change of 2.65 times in the electrocatalytic response was attributed to the enlarged electrode surface, the good conductivity of MWCNT and the synergy of MWCNT and Tb in the Tb–MWCNT nanocomposites. The nanocomposites acted as the electric wire between the enzymatic system and electrode.

3.3. Applied potential and optimal pH for amperometric detection of H_2O_2

The amperometric response of the HRP/Tb–MWCNT-modified electrode to 20 μM hydrogen peroxide was investigated over the potential range of −0.1 to −0.6 V. The response increased sharply from −0.1 to −0.3 V and reached its peak value at −0.4 V, similar to the trend of cyclic voltammetric response. Thus, −0.4 V was selected as the applied potential for detection of H_2O_2.

The activity of immobilized HRP was related to the pH of detection solution. At the applied potential of −0.4 V the amperometric response increased quickly from pH 4.5 to 6.5, and decreased from pH 7.0 to 9.5, which was in agreement with that for the HRP immobilized on electrode surface through gold nanoparticles and sol–gel [23]. Thus, the presence of Tb–MWCNT nanocomposite did not change the optimal pH value for the enzymatic reaction.

3.4. Performance of the amperometric sensor

Amperometric measurements were performed in a stirred 0.2 M PBS (pH 7.0) at an applied potential of −0.4 V. Fig. 6 shows the typical current–time responses at both HRP/Tb and HRP/Tb–MWCNT-modified electrodes for successive addition of H_2O_2. At HRP/Tb–MWCNT-modified electrode a sharp increase of current was observed after each addition of H_2O_2,

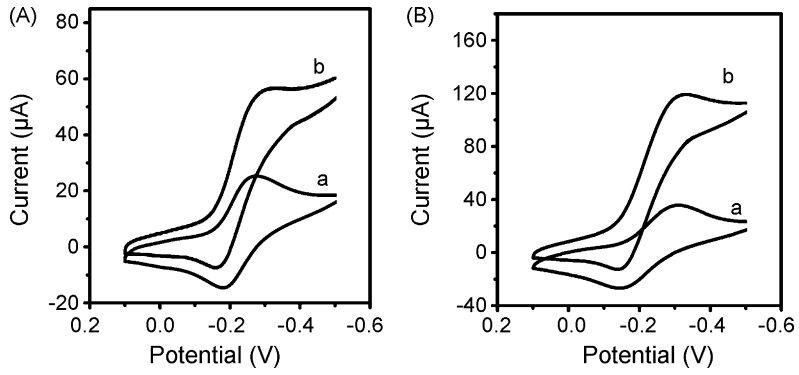

Fig. 5. Cyclic voltammograms of (A) HRP/Tb and (B) HRP/Tb–MWCNT-modified electrodes in (a) 0.2 M PBS (pH 7.0) saturated with nitrogen and (b) 10 mM H_2O_2 at 100 mV s^{-1}.

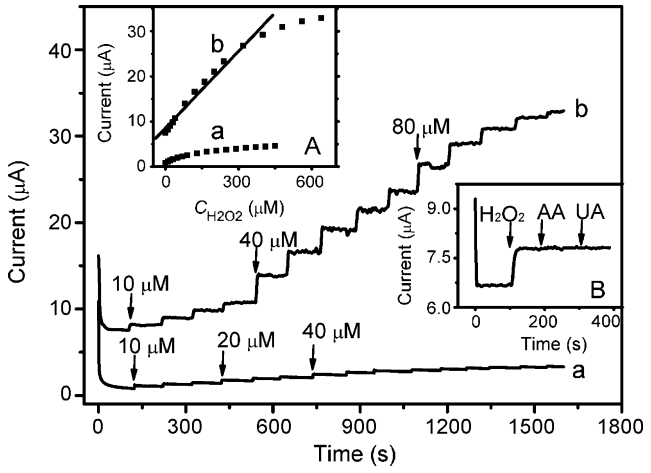

Fig. 6. Amperometric responses of (a) HRP/Tb and (b) HRP/Tb–MWCNT-modified electrodes at an applied potential of −0.4 V to successive addition of H_2O_2 in a stirred 0.2 M PBS (pH 7.0) saturated with nitrogen. Inset (A) calibration curves at (a) HRP/Tb and (b) HRP/Tb–MWCNT-modified electrodes, and (B) amperometric response of 10 μM H_2O_2, 10 μM AA, and 10 μM UA at an applied potential of −0.4 V in a stirred 0.2 M PBS (pH 7.0) saturated with nitrogen.

and the response reached 95% of the steady state value within 8 s. The HRP/Tb showed relatively low amperometric response. The linear calibration range of H_2O_2 at HRP/Tb–MWCNT-modified electrode extended up to 0.4 mM ($R = 0.990$, $n = 11$) (curve b, inset A in Fig. 6), a little higher than 0.34 mM at thionine–MWCNT-modified electrode [17] and much higher than 73.0 μM based on the direct electrochemical behavior of HRP entrapped in a zirconia enhanced grafted collagen trihelix scaffold [24] and 19 μM based on the immobilization of HRP on electropolymerized polyaniline films doped with CNTs [25]. The sensitivity of HRP/Tb–MWCNT-modified electrode was about 56.8 μA mM^{-1}, better than those of 49.0 μA mM^{-1} based on the direct electrochemistry of HRP immobilized on SWCNT-modified electrode [26] and 2.45 μA mM^{-1} at HRP and MB–SiO_2 nanocomposite coimmobilized gelatine matrix-modified electrode [27]. In comparison, the linear calibration range of the HRP/Tb-modified electrode was only up to 0.07 mM with the sensitivity of about 21.1 μA mM^{-1} (curve a, inset A in Fig. 6). Furthermore, the detection limit of HRP/Tb–MWCNT-modified electrode was estimated to be 1.7×10^{-6} M at a signal-to-noise ratio of 3, lower than those of 4.0×10^{-6} M reported in Ref. [27], 1.03×10^{-5} M at HRP cross-linked MWCNT/chitosan film [28], and 3.4×10^{-6} M based on the direct electrochemistry of hemoglobin [29].

The calibration curve of the sensor for H_2O_2 exhibited a typical characteristic of Michaelis–Menten kinetics at high concentrations. The apparent Michaelis–Menten constant (K'_m) was evaluated to be 0.16 mM derived from the Lineweaver–Burk equation. The value was similar to that of 0.12 mM at MB/HRP/CNT-modified electrode [18] and much smaller than the reported 23.85 mM based on the direct electrochemistry of HRP in sol–gel derived ceramic–CNT nanocomposite film [30], and 0.9 mM for HRP coimmobilized in MB–SiO_2 nanocomposite mixed gelatine matrix [27]. This result indicated that the immobilized HRP had higher biological affinity to H_2O_2.

The effects of common interfering species on the biosensor response were examined. After the injection of 10 μM H_2O_2, the amperometric response at the applied potential of −0.4 V increased greatly. The injection of the same concentration of ascorbic acid (AA) or uric acid (UA) did not cause interference to the amperometric response of H_2O_2 (inset B in Fig. 6).

To prove the precision and practicability of the proposed method, the reproducibility and storage stability of the biosensor were also examined. The relative standard deviation (R.S.D.) of the biosensor response to 10 mM H_2O_2 was 2.8% for eight successive measurements. When stored at 4 °C in air and measured everyday, it retained about 80% of its original sensitivity after 3 weeks. Both the reproducibility and the stability were better than those of 4.5% R.S.D. for eight repetitive measurements and retaining 80% of original sensitivity after 2 weeks at the electrode modified with MWCNT as a coimmobilization matrix to incorporate HRP and electron transfer mediator MB [18].

4. Conclusions

A simple method is proposed to prepare a kind of nanocomposites of MWCNT and electron transfer mediator with good dispersion in water. The nanocomposites can be conveniently used for preparation of amperometric biosensor by immobilizing enzyme on the surface of nanocomposites membrane with chitosan. The presence of MWCNT as a good conductor in the HRP/Tb–MWCNT system decreases the electron transfer impedance. The chitosan can maintain the enzymatic activity of the immobilized HRP. The synergy of MWCNT and Tb in the Tb–MWCNT nanocomposites enhances the electrocatalytic action of Tb to the enzymatic reduction of H_2O_2 by HRP and the electric communication between HRP and electrode, resulting in a sensitive amperometric sensor for H_2O_2. The proposed sensor shows good performance.

Acknowledgements

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