Hydrogen Peroxide Biosensor Based on Myoglobin Immobilized on Silver Nanoparticles Decorated Oxidized Multi-Walled Carbon Nanotubes

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A novel H_2O_2 biosensor has been fabricated based on the direct electrochemistry and electrocatalysis of myoglobin(Mb) immobilized on silver nanoparticles decorated oxidized multi-walled carbon nanotubes(o-MWCNT). Numerous carboxylic acid groups on o-MWCNT enhanced their biocompatibility to provide a favorable microenvironment for Mb to retain its bioactivity. The inherent conductivity of Ag nanoparticles could realize fast electron transfer between Mb and the electrode. The key analytical parameters relative to the biosensor performance such as pH and applied potential were optimized. Under optimum conditions, the amperometric determination of H_2O_2 was performed with a linear range of 2 µM to120 µM and a detection limit of 0.3 µM.

1. INTRODUCTION

H_2O_2 is one of the products of reactions catalyzed by many oxidase enzymes in many natural biological and environmental processes [1]. Also, H_2O_2 is released into the environment in either large or small quantities as it is widely used in many industrial processes as an oxidizing, bleaching and sterilizing agent. However, too high levels of H_2O_2 can give rise to functional and morphologic disturbances due to that it is extremely toxic to cells [2, 3]. Therefore, the determination of H_2O_2 is of great importance in environmental sciences and biochemistry as well as in food, pharmaceutical and clinical analysis. Among many techniques developed for this purpose, a third-generation amperometric biosensor, based on the direct electron transfer between an electrode and immobilized redox proteins,

is especially promising because of its practical advantages including operation simplicity, low expense, and suitability for real-time detection [4].

Myoglobin(Mb) is considered to be an ideal model protein for the study of the electron transfer of heme molecules because of its commercial availability, moderate cost as well as well-known and documented structure. However, it has been demonstrated that on conventional solid electrodes, the fast electron transfer between Mb and the electrode is not possible because the redox center of proteins is embedded in polypeptide chain structures and the proteins are easily absorbed on the electrode surface [5]. Therefore, great efforts such as using mediators, surfactant [6], gold nanoparticles[7], nanoparticles composite[8], nanotubes[9] and layered titanate nanosheets [10] have been devoted to explore new immobilization methods and supporting materials that accelerate the electron transfer and maintain the enzymatic activity. The good biocompatibility and conductivity of supporting materials is essential for obtaining good enzymatic stability and fast electron transfer.

Multi-walled carbon nanotubes(MWCNT) have enormous potentials as components of nanoscale electronic devices and biosensors, as a result of their ability to promote electron transfer reactions and high thermal capacity [11, 12].
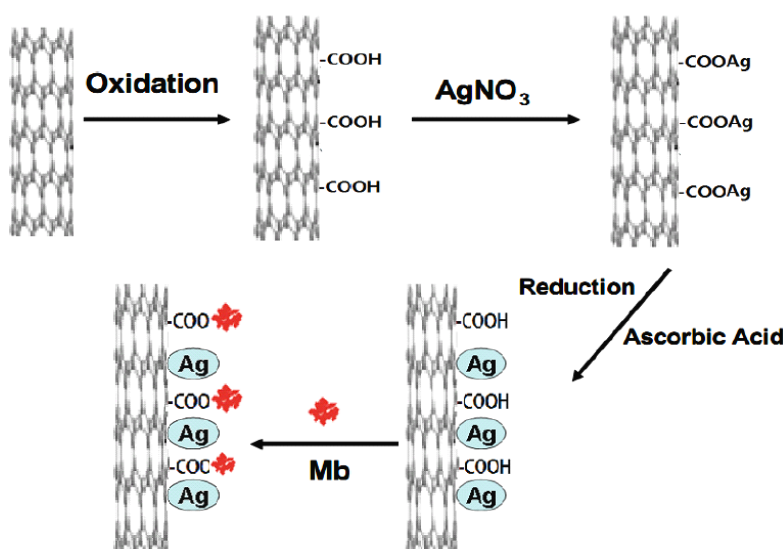

Figure 1. Schematic representation for immobilizing of Mb on the Ag/MWCNT composites.

The oxidized MWCNT(o-MWCNT) would have numerous -COOH groups on their surfaces which could interact with $-NH_2$ groups of Mb. Thus, Mb could be immobilized on o-MWCNT surfaces with good biocompatibility. Silver nanoparticles, which are easy to synthesize, have attracted considerable attentions due to their unique characteristics, such as small granule diameter, large specific surface area and high conductivity [13, 14]. In order to take full advantage of the two kinds of nanomaterials, it is desirable to create Ag/MWCNT composites, so that the unique properties of each material can be integrated. Therefore, based on their integrated properties, Ag/MWCNT composites can be used as supporting materials to immobilize Mb for its direct electrochemistry.

In the present work, silver nanoparticles were deposited on the o-MWCNT surfaces to synthesize Ag/MWCNT composites by a simple chemical plating method. The o-MWCNT surfaces have numerous carboxylic acid groups which could play important role to adsorb Ag^+ ions and could obtain a specific nucleation of silver nanoparticles [15]. The as-prepared Ag/MWCNT composites was successfully applied to immobilize Mb on the glassy carbon electrode surface. Schematic illustration of the fabrication process was given in Fig. 1. Thus, based on the direct electrochemistry of Mb, a stable and sensitive biosensor for hydrogen peroxide was demonstrated.

2. EXPERIMENTAL

2.1. Chemicals and instrumentation

Mb (from equine skeletal muscle) was purchased from Sigma and used without further purification. Multi-walled carbon nanotubes (MWCNT) were obtained from Shenzhen Nanotech Port Co., Ltd. (Shenzhen, China) with a typical diameter of 10–20 nm. The purity was more than 95%. Other chemicals were purchased from Sinopharm Chemical Reagent Co. Ltd. (China) and used without further purification. The 0.1M phosphate buffer solutions (PBS) at various pH values were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4, and then adjusting the pH with 0.1 M H_3PO_4 or NaOH.

All electrochemical measurements were carried out on a CHI660A electrochemical workstation (ChenHua Instrument Company of Shanghai, China). A conventional three electrode system was used with a saturated calomel electrode (SCE, all potentials are given against this reference) as the reference electrode, a platinum wire as the counter electrode, and a modified glassy carbon electrode (GCE, 3mm in diameter) as the working electrode. Transmission electron microscopy (TEM) images were obtained by using JEM-200CX (JEOL Ltd., Japan). The chemical composition of the Ag/MWCNT composites was analyzed by EX-250 energy dispersive spectrometer (EDS) (HORIBA, Japan).

2.2. Preparation of Ag/MWCNT composites

O-MWCNT were prepared as follows: 10 mg MWCNT were treated by stirring the MWCNT in a flask that containing 12 ml mixture of sulfuric acid/nitric acid (3:1 by volume) and refluxing at 60℃ in US wave cleaner for 3 h. After filtration and repeated washing with deionized water, the o-MWCNT was dried at 80℃ under vacuum for 8 h and stored in desiccator for further use. The procedure of in situ chemical reduction coprecipitation to synthesize Ag/MWCNT was as follows: 5 mL of 3 mg/mL o-MWCNT suspension was mixed with 5 mL of 6 mM $AgNO_3$ solution with stirring for 10 min, and 1 mL of 0.1 M ascorbic acid were added to the stirring mixed solution, which results in the reduction of Ag^+ ions to Ag precipitation. Then, the Ag decorated MWCNT composites were separated from the solution by centrifugation, and washed by multiple steps with deionized water.

2.3. Preparation of Mb/Ag/MWCNT modified GCE

Prior to use, the glassy carbon electrode was carefully polished with a leather containing 0.05 μm Al_2O_3 slurry and then ordinal ultrasonically cleaned in ethanol and distilled water. 5μL of Ag/MWCNT suspension was dropped on the pretreated GCE surface and dried at room temperature to form Ag/MWCNT/GCE. Then the Ag/MWCNT/GCE was immersed into 0.1M PBS containing 4 mg mL^{-1} Mb for 12 h at 4℃ in refrigerator. The resulting Mb/Ag/MWCNT/GCE was then rinsed throughout with double-distilled water to wash away the loosely adsorbed Mb molecules. For comparison, MWCNT/GCE and Mb/MWCNT/GCE were also prepared with the same procedure. All Mb-modified electrodes were stored in 0.1M pH 7.0 PBS at 4℃ in refrigerator when not in use.

3. RESULTS AND DISCUSSION

3.1. Characterization of Ag/MWCNT composites

The microstructure of Ag/MWCNT composites were observed by TEM. Figure 2 shows the TEM image of the deposition of Ag on the o-MWCNT. As can be seen, Ag nanoparticles with a uniform size of about 4–8 nm were well dispersed onto the MWCNT surfaces, although a few aggregates were observed. The numerous carboxylic acid groups on o-MWCNT could play important role to adsorb Ag^+ ions. The Ag precipitation on the o-MWCNT surfaces according to the reaction:

$$-COO^-Ag^+ + 1/2C_6H_8O_6 \rightarrow -COO^- + Ag + 1/2C_6H_6O_6 + H^+.$$
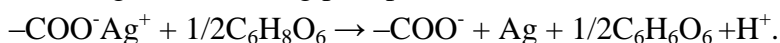
In this case, Ag nanoparticles were in situ generated from the $AgNO_3$ aqueous solution at room temperature and attached to the convex surfaces of o-MWCNT, forming the Ag/MWCNT composites.
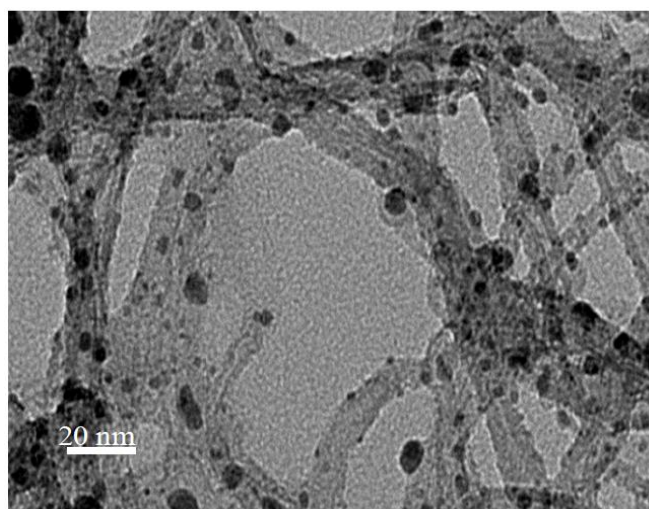

Figure 2. TEM image of Ag/MWCNT composites

The presence of the elemental silver, oxygen and carbon can be seen in the graph presented by the EDS analysis, which indicated the reduction of silver onto the o-MWCNT surfaces (Fig. 3). The

Ag content of 58.11% in weight and 4.40% in atom show that large amount of Ag has deposited on the o-MWCNT. This is attributed to the o-MWCNT give a uniform coverage of carboxylic groups on the MWCNT surface, which could absorb a lot of Ag+.
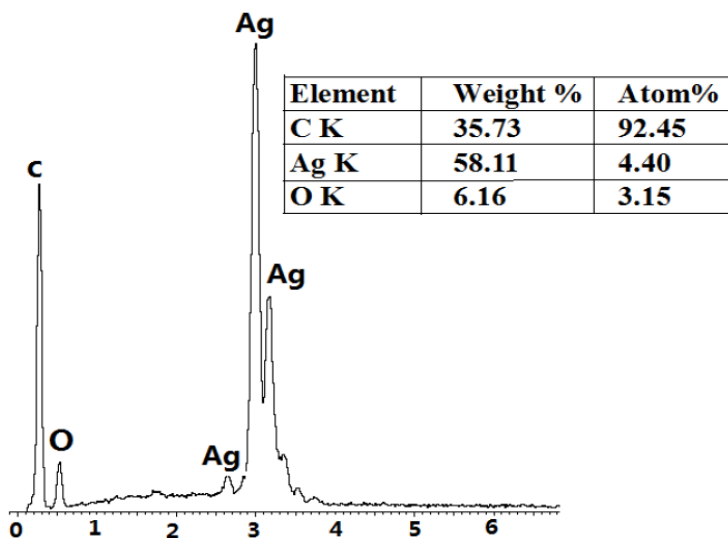

Element	Weight %	Atom%
C K	35.73	92.45
Ag K	58.11	4.40
O K	6.16	3.15

Figure 3. Energy dispersive spectrum (EDS) of the Ag/MWCNT composites.

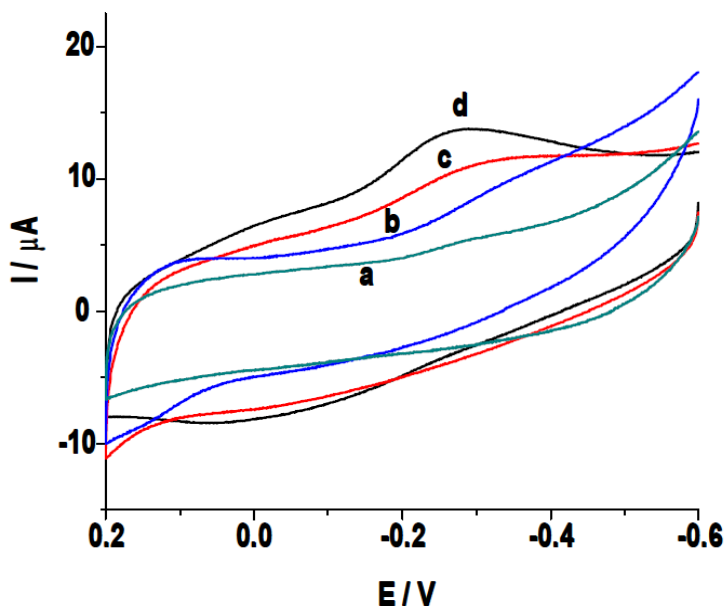*3.2 Direct electrochemistry of Mb in Ag/MWCNT/GCE*

Figure 4. CVs of (a) MWCNT/GCE, (b) Ag/MWCNT/GCE, (c) MB/MWCNT/GCE, and (d) Mb/Ag/MWCNT/GCE in 0.1M N$_2$-saturated PBS (pH 7.0) at a scan rate of 80 mVs^{-1}.

The direct electrochemistry of Mb immobilized on Ag/MWCNT modified GCE was investigated in 0.1M nitrogen-saturated PBS. Fig. 4 shows the typical cyclic voltammograms (CVs) of Mb at different modified electrodes in the potential range of 0.2～-0.6V. No redox peaks were observed on the MWCNT/GCE(curve a) and Ag/MWCNT/GCE(curve b) indicating that there were no

electroactive substances to react at this range. However, A pair of stable and well-defined quasi-reversible redox peaks (curve d) of Mb for the $Mb(Fe^{III})/Mb(Fe^{II})$ redox couple transformation were found at Mb/Ag/MWCNT/GCE with Epa $=-0.01$V and Epc $=-0.26$V. The results may be caused by the direct electron transfer between Mb and the underlying electrode. The different electrode modification materials could affect the formal potential of heme protein, which maybe attributed to the effect of different microenvironment on the direct electron transfer[16-18]. Although a pair of redox peaks of Mb could also be observed at Mb/MWCNT/GCE (curve c), the peak currents were much smaller than those observed at Mb/Ag/MWCNT/GCE, indicating that the presence of Ag nanoparticles could provide a more favorable microenvironment and greatly facilitate the electron transfer between Mb and the underlying electrode.

The CVs of Mb/Ag/MWCNT/GCE in PBS (pH 7.0) with different scan rates from 10 to 100 mVs^{-1} were shown in Fig. 5. With the increase of scan rate, the anodic and cathodic peak potentials of the Mb showed a small shift and the redox peak currents increased linearly(Fig. 5 inset), indicating a surface-controlled electrode process[19].
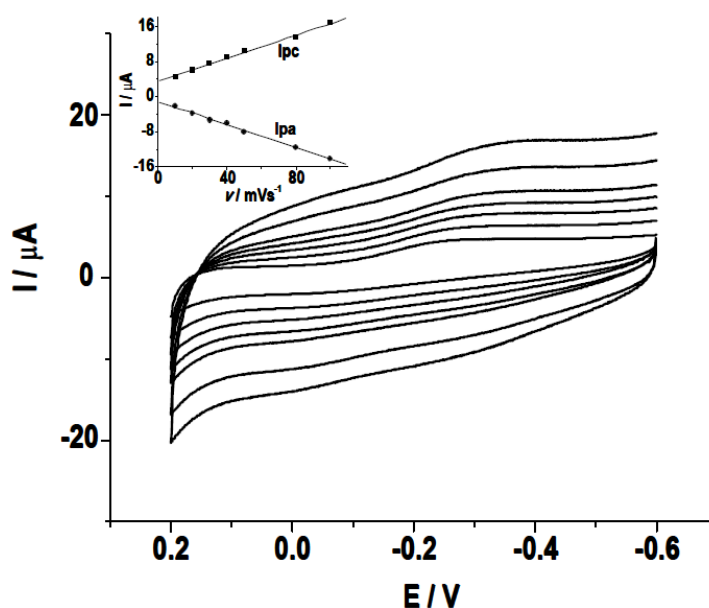

Figure 5. CVs of Mb/Ag/MWCNT/GCE in pH 7.0 PBS with the scan rates from inner to outer as 10, 20, 30, 40, 50, 80 and 100 mVs^{-1}, respectively. Inset: linear relationship of the peak currents vs. the scan rates.

3.3 Electrocatalytic reduction of H_2O_2

The electrocatalytic reduction activity of the Mb immobilized on Ag/MWCNT/GCE to H_2O_2 was investigated. Fig. 6 shows cyclic voltammograms of the modified electrode in the absence and presence of H_2O_2. With the addition of 0.3 mM H_2O_2 into 0.1M PBS (pH 7.0), it was observed that the reduction peak current increased, while the oxidation peak current decreased (curve a), showing a typical electrocatalytic reaction behavior of Mb toward H_2O_2. When $MbFe^{III}$ was reduced directly to

MbHFe^{II} at Mb/Ag/MWCNT/GCE, MbHFe^{II} could be oxidized by H$_2$O$_2$ to produce MbFe^{III} quickly. The chemical reaction increased the reduction current of MbFe^{III} to form the catalytic current. The electrocatalytic mechanism could be expressed as follows:
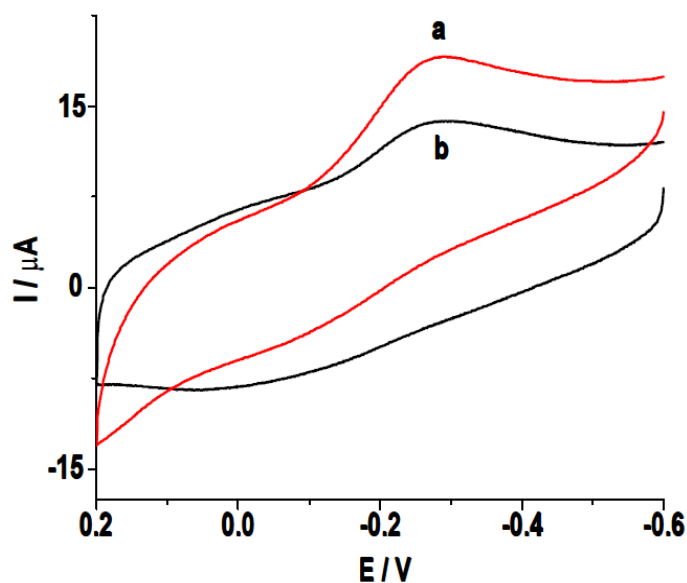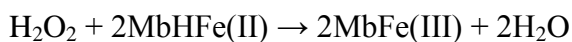
$$H_2O_2 + 2MbHFe(II) \rightarrow 2MbFe(III) + 2H_2O$$

Figure 6. CVs of the Mb/Ag/MWCNT/GCE with 0.3 mM H$_2$O$_2$ (a) and without H$_2$O$_2$ (b) in 0.1M N$_2$-saturated PBS (pH 7.0) at a scan rate of 80mVs^{-1}.

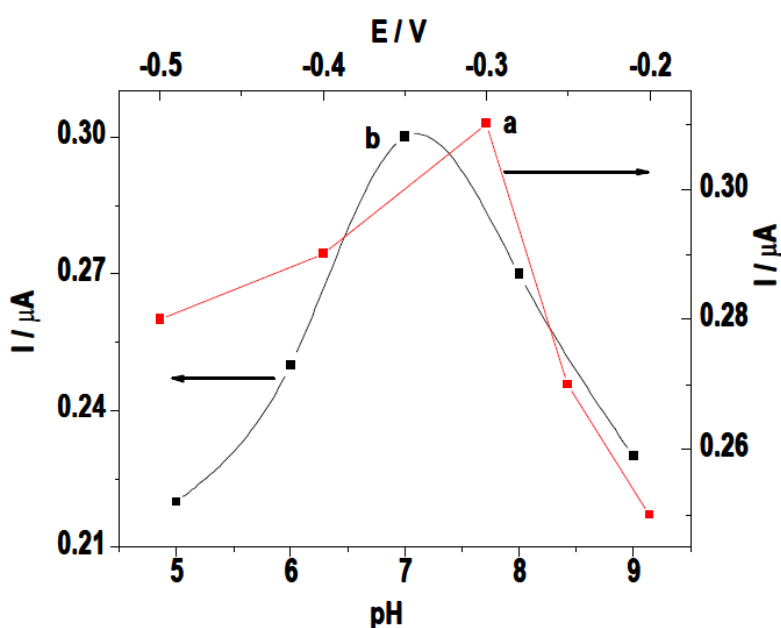*3.4 Optimization of experimental conditions*

Figure 7. Effect of the applied potential (a) and pH (b) on the amperometric response of Mb/Ag/MWCNT/GCE to 30 μM H$_2$O$_2$.

The amperometry is the most used method to evaluate the electrocatalytic activity of electrochemical sensors and enzyme-based biosensors. To optimize the suitable applied potential, different potentials have been performed. As shown in Fig. 7a, the current increased rapidly with the applied potential shifting from −0.2 to −0.3V. At a potential more negative than −0.3 V, a slight decrease in the current was observed. The current approached a maximum value at −0.3 V, so the potential of −0.3 V was chosen for further measurements.

The pH value of electrolyte will affect the three-dimension extending structure of protein, and will also affect the electrochemical response[20]. The dependence of the biosensor response on pH of the measurement solution was investigated. As shown in Fig. 7b, The amperometric response current increased from pH 5.0 and reached a maximum at pH 7.0. Then, the current response was found to be decreased from pH 7.0 to 9.0. The neutral pH is close to the physiological environment of Mb and can retain its activity effectively. Thus pH 7.0 was chosen for the following measurements.

3.5. Amperometric response of the developed H_2O_2 biosensor

The amperometric response of Mb/Ag/MWCNT/GCE to H_2O_2 at an applied potential of −0.3V is illustrated in Fig. 8. Upon addition of H_2O_2, the biosensor responded rapidly to the substrates and could achieve 95% of the steady-state current within 5 s, indicating a fast amperometric response to H_2O_2 reduction. The amperometric response showed a linear relation with H_2O_2 concentration from 2 μM to120 μM with a correlation coefficient of 0.9991 (inset in Fig. 8). The linear regression equation was I(μA) = 0.086 + 0.007 [H_2O_2] (μM). The detection limit was estimated to be 0.3μM (S/N= 3), which was lower than that of some silver nanomaterials modified GC electrode [21, 22] and some HRP-based biosensors [23, 24].
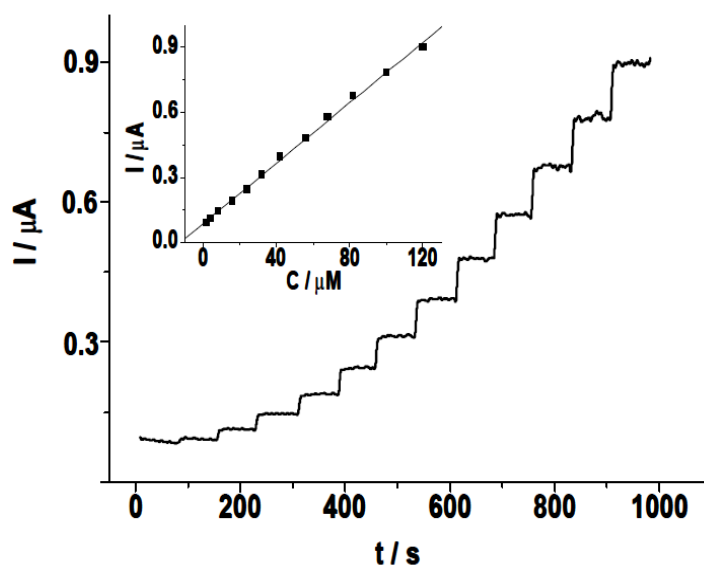

Figure 8. Typical amperometric response of the Mb/Ag/MWCNT/GCE to the addition of a certain amount of H_2O_2. Inset: calibration curve of the amperometric response current vs. the concentration of H_2O_2.

3.6 Precision, reproducibility, and stability of Mb/Ag/MWCNT/GCE

The precision and reproducibility of Mb/Ag/MWCNT/GCE was investigated. The precision was evaluated by successive determinations of 20 μM H_2O_2 for five times using the same modified electrode. The relative standard deviation (R.S.D.) was calculated to be 4.7%. The fabrication reproducibility was estimated by the measurements of 20 μM H_2O_2 with four different modified electrodes made at the same procedure. The R.S.D. of the measurement was 5.9%, showing a good reproducibility and acceptable precision.

The long-term stability of the electrode was also investigated. When Mb/Ag/MWCNT/GCE was not in use, it was stored in PBS at 4 ∘C and measured intermittently, the current response to 20 μM H_2O_2 decreased less than 10% over ten days. The good long term stability can be attributed to the excellent biocompatibility of the o-MWCNT, which can provide a favorable microenvironment for Mb to retain its bioactivity. Both biocompatibility of o-MWCNT and inherent conductivity of Ag nanoparticles enable the composite material to become an excellent biosensing platform for realizing direct electrochemistry and electrocatalysis of Mb.

3.7 Interference study and analysis of real samples

The electrochemical reduction of H_2O_2 is often interfered by some electroactive species. In order to assess the anti-interference capability of the present sensor, the electrochemical responses of some common interfering substances were studied. As for the proposed biosensor, amperometric steady-state curve has also been carried out at −0.3 V for successively adding equal amount of DA, UA and AA to electrochemical cell containing 20 μM H_2O_2, the current response of H_2O_2 was almost no changes. The results indicate that the biosensor has good selectivity.

As the proposed biosensor has good selectivity, reproducibility and stability, the real sample of our laboratory waste water containing H_2O_2 was detected using the proposed sensor. The real concentration of H_2O_2 was firstly detected by $KMnO_4$ method and the sample containing desirable concentration of H_2O_2 was then prepared by diluting suitable amount of waste water with 0.1M PBS (pH 7.0). The mean concentration of H_2O_2 in waste water was detected to be 35.6 μM, which was in good agreement with 37.2 μM detected by $KMnO_4$ method. Thus, the proposed method could be used as H_2O_2 sensor.

4. CONCLUSIONS

Mb was immobilized on the Ag/MWCNT/GCE and the direct electron transfer of Mb was realized with the aid of the Ag/MWCNT composite. Because of the good biocompatibility and conductivity of o-MWCNT and Ag nanoparticles, the resulting Mb/Ag/MWCNT/GCE exhibited good electrocatalytic performances to H_2O_2, indicating that the Ag/MWCNT composite can not only retain the enzyme activity of Mb effectively, but also largely promote the direct electron transfer. The

Mb/Ag/MWCNT/GCE showed good precision, reproducibility and long-term stability which exhibited promising application as a new third-generation H_2O_2 sensor.

References

1. Y.F. Yang and S.L. Mu, *Biosens. Bioelectron.* 21 (2005) 74.
2. M.C.Y. Chang, A. Pralle, E.Y. Isacoff and C.J. Chang, A Selective, *J. Am. Chem. Soc.* 126 (2004)15392.
3. E.W. Miller, A.E. Albers, A. Pralle, E.Y. Isacoff and C.J. Chang, *J. Am. Chem. Soc.* 127(2005) 16652.
4. E.E. Ferapontova, *Electroanalysis* 16(2004)1101.
5. A. Heller, *Acc. Chem. Res.* 23 (1990) 128.
6. F. Wang and S.H. Hu, *Colloid Surf. B: Biointerfaces* 63(2008)262.
7. W.W. Yang, Y.C. Li, Y. Bai and C.Q. Sun, *Sens. Actuators B* 115(2006)42.
8. J.J. Feng, G. Zhao, J.J. Xu and H.Y. Chen, *Anal. Biochem.* 342(2005)280.
9. X.J. Zhao, Z.B. Mai, X.H. Kang and X.Y. Zou, *Biosens. Bioelectron.* 23(2008)1032
10. L. Zhang, Q. Zhang and J.H. Li, *Adv. Funct. Mater.* 17(2007)1958.
11. M.J. Moghaddam, S. Taylor, M. Gao, S. Huang, L. Dai and M.J. McCall, *Nano Lett.* 4(2004) 89.
12. G. Liu and Y. Lin, *Electrochem. Commun.* 8(2006)251.
13. M. Ghalkhani, S. Shahrokhian and F. Ghorbani-Bidkorbeh, *Talanta* 80(2009)31.
14. D. Tang, R. Yuan and Y.Q. Chai, *J. Phys. Chem. B* 110(2006)11640.
15. T.W. Ebbesen, H. Hiura, M.E. Bisher, M.M.J. Treacy, J.L. Shreeve-Keyer and R.C. Haushalter, *Adv. Mater.* 8(1996)155.
16. L.S. Duan, Q. Xu, F. Xie and S.F. Wang, *Int. J. Electrochem. Sci.* 3(2008)118.
17. W.W.Yang, Y.C. Li, Y. Bai and C.Q. Sun, *Sensors Actuators B: Chem.* 115(2006) 42
18. S.Z. Zong, Y. Cao and H.X. Ju, *Electroanalysis* 19(2007)841.
19. Z.H. Dai, S.Q. Liu, H.X. Ju and H.Y. Chen, *Biosens. Bioelectron.* 19(2004) 861.
20. H.H. Liu, Z.Q. Tian, Z.X. Lu, Z.L. Zhang, M. Zhang and D.W. Pang, *Biosens. Bioelectron.* 20(2004)294.
21. K. Cui, Y.H. Song, Y. Yao, Z.Z. Huang and L.Wang, *Electrochem. Commun.,* 10 (2008)663.
22. Z.L. Liu, B. Zhao, Y. Shi, C.L. Guo, H.B. Yang and Z. Li, *Talanta*, 81 (2010) 1650.
23. H.S.Yin, S.Y. Ai, W.J. Shi and L.S. Zhu, *Sens. Actuators B-Chem.*, 137(2009)747.
24. C. Xiang, Y. Zou, L.X. Sun and F. Xu, *Sens. Actuators B-Chem.*, 136(2009)158.