Available online at www.sciencedirect.com
SCIENCE DIRECT
Biosensors and Bioelectronics 21 (2006) 1529–1535
BIOSENSORS & BIOELECTRONICS
www.elsevier.com/locate/bios

Amperometric biosensor for hydrogen peroxide based on ferrocene-bovine serum albumin and multiwall carbon nanotube modified ormosil composite

Vijay Shyam Tripathi, Vivek Babu Kandimalla, Huangxian Ju *

Key Laboratory of Analytical Chemistry for Life Science (Education Ministry of China), Department of Chemistry, Nanjing University, Nanjing 210093, China

Received 25 April 2005; received in revised form 1 July 2005; accepted 6 July 2005
Available online 11 August 2005

Abstract

A novel amperometric biosensor for hydrogen peroxide (H_2O_2) was developed by entrapping horseradish peroxidase (HRP) in a new ormosil composite doped with ferrocene monocarboxylic acid–bovine serum albumin conjugate and multiwall carbon nanotubes (MWNTs). The ormosil was prepared using 3-(aminopropyl)triethoxysilane and 2-(3,4 epoxycyclohexyl)-ethyltrimethoxy silane as monomers. The encapsulated conjugate showed excellent electrochemistry and acted as an electron transfer mediator. The presence of MWNTs improved the conductivity of the composite film. This matrix showed a biocompatible microenvironment for retaining the native activity of the entrapped HRP and a very low mass transport barrier to the substrate, which provided a fast amperometric response to H_2O_2. The proposed H_2O_2 biosensor exhibited a linear range of 0.02–4.0 mM with a detection limit of 5.0 μM (S/N = 3) and a K_M^{app} value of 2.0 mM. It could be used for flow injection analysis of hydrogen peroxide with a liner range from 0.02 to 4.5 mM, sensitivity of 0.042 μA/mM and analytical time of 20 s per sample. This biosensor possessed good analytical performance and storage stability.

Keywords: Ormosil; Ferrocene monocarboxylic acid–bovine serum albumin conjugate; Multiwall carbon nanotubes; Horseradish peroxidase; Hydrogen peroxide; Biosensors

1. Introduction

The rapid, accurate, reliable and reagentless analysis of hydrogen peroxide is of great importance in chemical, biological, clinical and many other fields. Most of the analytical techniques for hydrogen peroxide determination, such as chromatographic, colorimetric, photometric and electrochemical methods, are generally time-consuming and difficult for automation detection. Electrochemical sensors, such as amperometric biosensors, have been appeared as the most convenient tools for hydrogen peroxide determination (Mulchandani and Pan, 1999; Liu and Ju, 2002; Yu and Ju, 2002; Delvaux et al., 2004). The stability and electrochemical characteristics of biosensor are governed by the enzyme immobilization on the transducer surface. The analytical performance of the biosensors depends on both the immobilization process and the matrix used for immobilization of the enzyme. Sol–gel technology has been widely used for the fixing or efficient encapsulation of biocatalysts on transducer surface (Gill, 2001; Kandimalla et al., in press). Several HRP amperometric biosensors have been proposed for the determination of hydrogen peroxide using sol–gel technology (Wang et al., 1998, 2000).

Sol–gel matrices possesses chemical inertness, physical rigidity, negligible swelling in aqueous solution, high photochemical and thermal stability and optical transparency. To improve the porosity and biocompatibility of sol–gel matrices organically modified sol–gels (ormosils) have been developed (Pandey et al., 2001a), which could efficiently

* Corresponding author. Tel.: +86 25 83593593; fax: +86 25 83593593.
E-mail address: hxju@nju.edu.cn (H. Ju).

0956-5663/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2005.07.006

retain the biocatalytic activity of the entrapped enzymes, such as glucose oxidase and horseradish peroxidase (HRP) (Pandey et al., 2001a, 2003). However, the conductive properties of ormosils are poor for the biosensing applications. Thus, the conductive materials like carbon nanotubes (Gong et al., 2004) and graphite (Pandey et al., 2001b, 2003) have been doped in these matrices to enhance their conductive properties. A hydrogen peroxide sensor has also been prepared by doping palladium in ormosil (Pandey et al., 2001a).

However, for the electron transfer and sensitive detection of hydrogen peroxide based on sol–gel technology, mediator is highly necessary for HRP based biosensors, which is generally added in the detection solution (Wang et al., 1998, 2000; Yu and Ju, 2002). The encapsulation of mediator, such as ferrocene derivatives in a siloxane homopolymer, for amperometric detection of peroxides has been reported (Armada et al., 2004). However, leakage has been a main problem for the entrapment of low molecular weight mediator in sol–gel matrices. This limitation can be dissolved through direct cross-linking of ferrocene derivatives with long poly(oxyethylene) chain (Okawa et al., 1999) or conjugation with *m*-phenylenediamine polymer (Mulchandani and Pan, 1999). Another way is to bind covalently ferrocene derivatives to flexible dendritic or polymeric back bone and then the resulting materials are deposited on to the surface or incorporated into the carbon paste electrodes (Losada et al., 1997; Koide and Yokoyama, 1999). This paper demonstrated a novel approach for both encapsulation of the mediator in ormosil and improvement of the conductivity using both ferrocene monocarboxylic acid–bovine serum albumin (FMC–BSA) conjugate and multiwall carbon nanotubes (MWNTs). BSA is a well-known inert protein with a 30–35 reactive primary amino groups and can provide thermostabilization to several enzymes through hydrophobic interactions (Chang and Mohaney, 1995). Compared with other high molecular weight active proteins, BSA is cheap and can conjugate a number of redox molecules (mediator) by that good electron mediation and high signal can be generated. This work used BSA to entrap ferrocene mediator, thus a reagentless biosensor for hydrogen peroxide could easily be developed by entrapping horseradish peroxidase in the new ormosil composite doped with FMC–BSA conjugate and MWNTs. The latter has appeared as highly conductive and catalytic nanomaterials in electrochemical applications due to their unique mechanical, chemical and electrical properties (Gavalas et al., 2001; Gong et al., 2004; Jiang et al., 2004). The presence of MWNTs in the composite facilitated the electron transfer among immobilized enzyme, mediator and electrode. The conjugation of mediator ferrocene to inert protein BSA was comparatively convenient and could inhibit its leakage away from electrode surface. The proposed biosensor exhibited good analytical performance towards the quantification of hydrogen peroxide.

2. Experimental

2.1. Chemicals

Horseradish peroxidase (HRP EC 1.11.17, Sigma), ferrocenemonocarboxylic acid (FMC, Sigma), poly ethyleneglycol (PEG, Sigma), *N*-hydroxysuccinimide (NHS, Sigma), bovine serum albumin (BSA, Sigma), 3-(aminopropyl)triethoxysilane (APTES, Fluka), *N*-(3-dimethylaminopropyl)*N*-ethylcarbodiimidhydrochlorid (EDC, Merck-Schuchardt, Hohenbrunnbei München) and 2-(3,4 epoxycyclohexyl)-ethyltrimethoxy silane (Epoxy, Fluka) were purchased from standard sources and used as received. Multiwall carbon nanotubes (length 1–2 μm, external diameter 10–20 nm and surface area 40–300 $m^2\,g^{-1}$) were procured from Shenzhen Nanotech Port Co. (China). 30% H_2O_2 purchased form Shanghai Jinlu Chemical engineering Ltd. Co., China). 0.1 M pH 6.8 phosphate buffer (PBS) was used as electrolyte solution.

2.2. Electrode modification

FMC–BSA conjugate was prepared using a carbodiimide coupling reaction (Padeste et al., 2000) in a molar ratio of 1:30 (BSA:FMC). The glassy carbon electrode (GCE, 3-mm diameter) was polished with emery paper (No. 2000), 0.3- and 0.05-μm alumina slurry on a woollen cloth, respectively, then sonicated under a water bath for 10 min followed by rinsing thoroughly with doubly distilled water. MWNTs were sonicated in water bath for 20 min and ethanol bath for another 30 min and dried at 80 °C for 30 min. Twenty-five milligrams per millilitre of MWNTs suspension was prepared.

The FMC–BSA/MWNTs/ormosil composite was prepared by mixing 125.0 μl double distilled water with 25.0 μl 4% PEG, 17.0 μl sol–gel precursors APTES, 9.0 μl Epoxy, 4.0 μl 25 mg/ml MWNTs suspension, 10.0 μL FMC–BSA conjugate (2.64 mg protein/ml) and 2.0 μl 0.1 M HCl sequentially. 3.0 μl of the resulting homogeneous solution was cast on well-polished glassy carbon electrode surface, which was kept at room temperature (28 °C) for 4 h and 4 °C for 20 h for the formation of FMC–BSA/MWNTs/ormosil composite film. The formed ormosil film was washed with PBS and stored at 4 °C till use. For the preparation of HRP incorporated composite film, 10.0 μl 3700 IU/ml HRP in pH 6.8 PBS was added to the mixture before casting.

2.3. Electrochemical studies

Electrochemical experiments were performed on a BAS-100B electrochemical analyzer (Bioanalytical Systems Inc.) with a three-electrode system comprising a platinum wire as auxiliary electrode, a saturated calomel electrode (SCE) as reference and HRP/FMC–BSA/MWNTs/ormosil composite modified GCE as working electrode. The cyclic voltammet-

ric (CV) and amperometric experiments were performed in a thermostated electrochemical cell at 28 °C. The electrolyte solutions were purged with highly pure N_2 for at least 10 min to remove O_2 and kept under N_2 atmosphere during the measurements. Amperometric experiments were carried out in a stirred system by applying a potential step of +220 mV to the working electrode and adding successively freshly prepared aliquots of H_2O_2 standard solution to the solution. Current–time data were recorded after steady state current achieved. The difference of the response (i_{ss}) upon addition of H_2O_2 and the background current (i_0) was reported as current i.

2.4. FIA experiments

The FIA experiments were carried out with a thin-layer flow cell equipped with above three electrodes that were connected to BAS 100B at a poised potential of +220 mV. A BT00-100M peristaltic pump (Baoding, China) was used to deliver flow stream at a flow rate of 1.0 mL min^{-1}. 0.1 M pH 6.8 PBS was used as carrier buffer.

3. Results and discussion

3.1. Morphologies of ormosil composite film

Scanning electron micrographs (SEMs) were used to evaluate the physical appearance and surface characteristics of the ormosil composite (Fig. 1). The ormosil film was very smooth and crack free (Fig. 1a). After MWNTs were doped in the ormosil film, the SEM showed homogeneous distribution of the MWNTs in the film (Fig. 1b). The further entrapment of FMC–BSA in the/MWNTs/ormosil composite resulted in more bright particles of FMC–BSA conjugate molecules, which were distributed regularly and showed an islandlike structure (Fig. 1c). These features would help in retaining of dopants for long time and improving the electrochemical behavior of the film. The presented gelatin protocol was very simple and fast and it did not require sonication for the homogenization of the precursors.

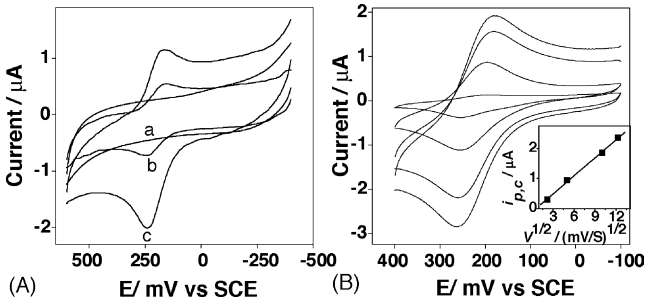

Fig. 2. Cyclic voltammograms of (A) ormosil (a), FMC-BSA/ormosil (b) and FMC–BSA/MWNTs/ormosil composite film modified electrodes (c) in 0.1 M pH 6.8 PBS at 100 mV/s and (B) FMC–BSA/MWNTs/ormosil composite film modified electrode in 0.1 M pH 6.8 PBS at 5, 25, 100 and 150 mV/s (from internal to external). *Inset*: Plot of anodic peak current vs. $v^{1/2}$.

3.2. Electrochemical behavior of FMC–BSA/MWNTs/ormosil composite

The cyclic voltammogram of the FMC-BSA/ormosil modified electrode showed a couple of obvious redox peaks +165 and +235 mV at 100 mV/s (Fig. 2A). Compared curve b with curve a in Fig. 2A, this couple of redox peaks could be attributed to the reduction and oxidation of the immobilized ferrocene group. In the composite film of MWNTs/ormosil, the reduction and oxidation peak currents of the conjugate increased from 0.45 to 0.86 μA and 0.39 to 1.33 μA (curve c in Fig. 2A). Although larger background current occurred in presence of MWNTs the FMC–BSA/MWNTs/ormosil composite film showed a better CV shape, which was due to the close interaction of MWNTs with the mediator. The doped MWNTs improved greatly the conductivity of the ormosil film and the electron shuttle between the mediator and electrode. Furthermore, with a continous cyclic scan the voltammetric responses of both modified electrodes were stable, while the cyclic voltammograms of both the FMC/ormosil and the FMC/MWNTs/ormosil composite film modified electrodes showed decreasing peak currents (not shown), indicating the leakage of FMC from electrode surface. Thus, the encapsulation of FMC in a conjugated form was significant.

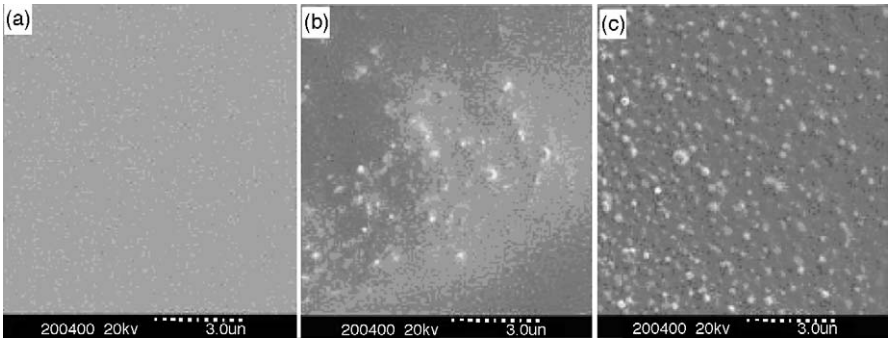

Fig. 1. Scanning electron micrographs of (a) ormosil film, (b) MWNTs/ormosil composite film and (c) FMC–BSA/MWNTs/ormosil composite film.

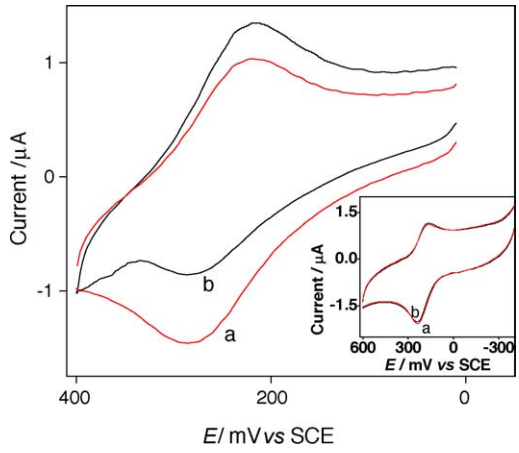

Fig. 3. Cyclic voltammograms of HRP/FMC–BSA/MWNTs/ormosil composite film modified electrode (a) in absence and (b) in presence of 6.0 mM H_2O_2 in 0.1 M pH 6.8 PBS at 100 mV/s. *Inset*: Cyclic voltammograms of FMC–BSA/MWNTs/ormosil composite film modified electrode in absence (a) and presence (b) of 6.0 mM H_2O_2 in 0.1 M pH 6.8 PBS at 100 mV/s.

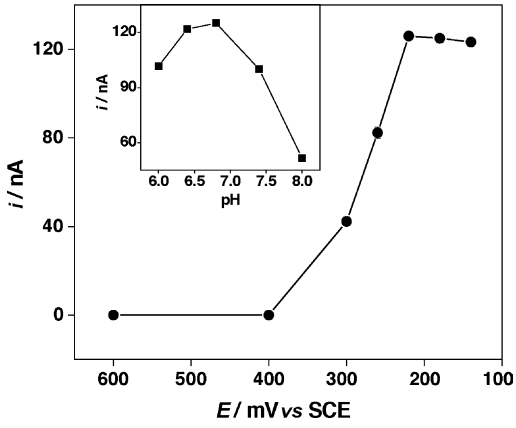

Fig. 4. Influence of applied potential on amperometric response of biosensor in 0.1 M pH 6.8 PBS containing 3.0 mM H_2O_2. *Inset*: Effect of buffer pH on amperometric response at an applied potential of +220 mV.

With an increasing scan rate the CV peak currents of the FMC-BSA/ormosil modified electrode increased and the peak potentials almost kept at the constant values in the scan rate range of 5–150 mV/s (Fig. 2B). The plot of the reduction peak current versus the square root of scan rate showed a linear relation (inset in Fig. 2B), which might be attributed to a slow electron hoping across the matrix of the composite membrane.

3.3. Electrochemical response of HRP/FMC–BSA/MWNTs/ormosil composite to H_2O_2

Upon addition of 6.0 mM H_2O_2 to electrolyte an obvious electrocatalytic response was observed. The reduction peak current of the HRP/FMC–BSA/MWNTs/ormosil composite film increased and the oxidation peak current decreased (Fig. 3), while the FMC–BSA/MWNTs/ormosil composite film modified electrode did not show any change (inset in Fig. 4). The formal potential of ferrocene derivative FMC-BSA was obtained to be about +200 mV (versus SCE) from its redox peak potentials, which was more positive than that of −220 mV (versus SCE) for native HRP in solution (Harbury, 1957). These phenomena illustrated that the HRP was entrapped efficiently and catalyzed the reduction of H_2O_2 in presence of ferrocene. This process might be explained by the biocatalytic oxidation of ferrocence moieties in the composite film by HRP in the presence of hydrogen peroxide and re-reduction of ferrocenium groups at the applied potential (Armada et al., 2004, 2003). The porous structure of the composite film made the substrate easy to approach the immobilized enzyme and facilitated the appropriated interaction of the enzymatic redox centers with the ferrocence moieties. Thus, the composite film modified electrode could be used as a biosensor for detection of hydrogen peroxide.

3.4. Optimization of measurement variables

Firstly, the effect of applied potential on the amperometric response of the biosensor was evaluated in the range of +600 to +100 mV. The reduction of H_2O_2 was already observed at an applied potential of around +400 mV. Upon decreasing the applied potential from +400 to +220 mV, the steady-state current increased due to the increased driving force for the fast reduction of the mediator at the lower potentials (Fig. 4). The reduced form of the mediator reduced further the oxidized form of HRP to form reduced HRP, which catalyzed the reduction of H_2O_2. The response approached a plateau value at +220 mV, which was selected as the working potential for amperometric determinations of H_2O_2. The working potential was much more positive than those of −150 mV (Yu and Ju, 2002), −100 mV (Wang et al., 2000), –300 mV (Xiao et al., 2000) and +100 mV (Mulchandani and Pan, 1999), indicating a lower overpotential for H_2O_2 reduction at the proposed biosensor.

With increasing of buffer pH the amperometric response of the biosensor increased till pH 6.8. The further increase of buffer pH led to decrease of the response, indicating that the catalytic response were controlled by the enzymatic activity in this region. Decrease in the response at high pH was possibly due to the decrease of enzymatic activity. The optimal pH value was closer to other reports (Okawa et al., 1999; Delvaux et al., 2004). Enzymatic activity usually varies with its vicinity (microenvironment), hence the optimal pH value of HRP is varied from 5.5 to 7.4 in different reports (Okawa et al., 1999; Mulchandani and Pan, 1999; Yu and Ju, 2002; Delvaux et al., 2004).

The concentration of enzyme in composite was also an important factor affecting the amperometric response of the biosensor. With increasing of enzyme level on electrode surface up to 0.55 IU per electrode the catalytic current increased (Fig. 5a), the response then reduced slowly due to the increased protein concentration in ormosil composite, which restricted the approach of the substrate to the immo-

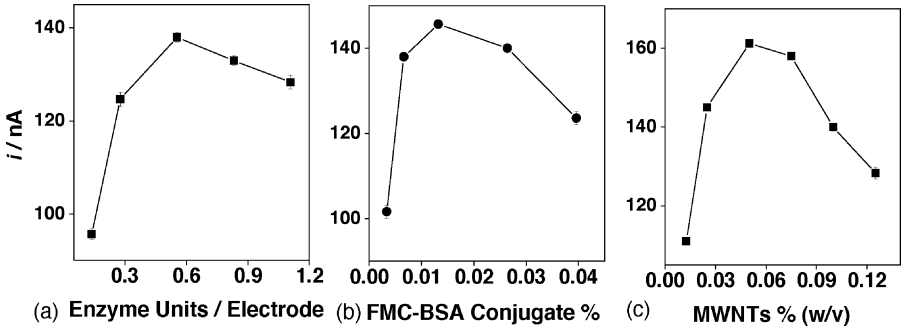

Fig. 5. Dependence of amperometric response of biosensor in 0.1 M pH 6.8 PBS containing 3.0 mM H_2O_2 at +220 mV on amounts of (a) enzyme loading, (b) FMC–BSA conjugate and (c) MWNTs in FMC–BSA/MWNTs/ormosil composite film.

bilized enzyme and reduced the conductivity of the composite or both. Similarly, the level of FMC–BSA conjugate also influenced greatly the signal (Fig. 5b). The maximum response occurred at the conjugate amount of 0.013% (w/v) in the composite. When the amount increased from this value to 0.04% (w/v) the amperometric decreased by 15%. The response decrease might be due to the restriction of the electron transfer and the transnational degree of movement of encapsulated ferrocene and the decrease of enzyme-substrate kinetics. Although MWNTs could improve the conductive property of the composite film, which increased the amperometric response of the resulting biosensor, a quick decrease of the response was observed when the MWNTs amount in the ormosil film was more than 0.05% (w/v) (Fig. 5c). The similar appearance was also reported for determination of dopamine and ascorbic acid (Jiang et al., 2004). Hence, the amounts of 0.55 IU per electrode, 0.013% (w/v) conjugate and 0.05% (w/v) MWNTs were used for biosensor preparation.

3.5. Analytical performance of biosensor

Under optimized conditions the typical current–time response showed a good analytical performance (Fig. 6).

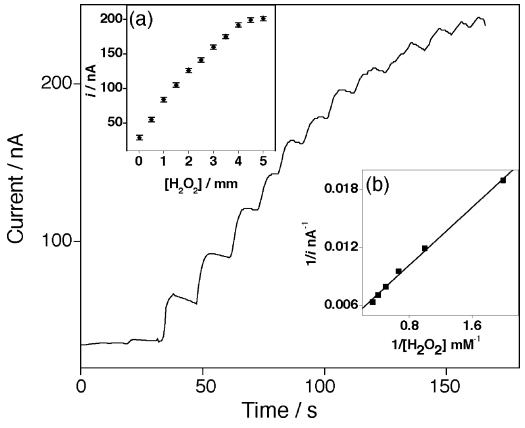

Fig. 6. Typical amperometric response of biosensor at +220 mV to successive addition of 0.5 mM H_2O_2 to 0.1 M pH 6.8 PBS. *Inset*: (a) Calibration curve and (b) Lineweaver–Burk plot.

Upon addition of H_2O_2 the amperometric response increased and reached a maximum value within 5 s, indicating a fast response. The faster response than those of HRP in silica sol–gel (Li et al., 1996) and sol–gel/hydrogel composite film (Wang et al., 2000) was mainly due to the biocompatible microenvironment, good porosity and well conductive properties of the MWNTs doped ormosil composite. The sudden current decrease was observed at low H_2O_2 concentration due to the fast catalysis of HRP, which led to a uneven concentration on electrode surface. With the increasing H_2O_2 concentration the amperometric response increased linearly in the range from 0.02 to 4.0 mM ($R = 0.998$) (inset in Fig. 6). The detection limit was 5.0 μM at a signal to noise ratio of 3. At higher H_2O_2 concentration the plot showed a Michaelis–Mentien type response. The apparent Michaelis–Mentien constant K_M^{app} was calculated to be 2.0 mM from the Lineweaver–Burk plot (Kamin and Wilson, 1980) (inset in Fig. 6). This K_M^{app} value was smaller than those of 4.8 mM (Li et al., 1996) and 4.6 mM (Wang et al., 2000) for H_2O_2 biosensors based on sol–gel, 7.6 mM based on sol–gel gold nanotubes (Delvaux et al., 2004), and close to those of 2.3 mM based on gold colloid/cysteamine modified gold electrode (Xiao et al., 2000) and 2.5 mM based on siloxane homopolymer (Armada et al., 2004). Low K_M^{app} value indicated that the enzyme immobilized in the ormosil composite retained its activity with a low diffusion barrier.

3.6. Flow injection analysis

In order to evaluate the automatization possibility of the detection device and the stability of the biosensor in continuous operation, flow injection analysis was carried out at a poised potential of +220 mV. pH 6.8 PBS was used as a carrier buffer, the flow rate was optimized to be 1.0 ml/min based on the peak height and response time. As shown in Fig. 7, the amperometric response increased linearly. The linear range was from 0.02 to 4.5 mM ($R = 0.996$) with a sensitivity of 0.042 μA/mM. To check the reproducibility of response 2.0 mM H_2O_2 was injected for eight times subsequently, the response was stable with an RSD of 1.9% (108 ± 2 nA), indicating that the response was reproducible. Each analysis could be completed within 20 s (Fig. 7) due

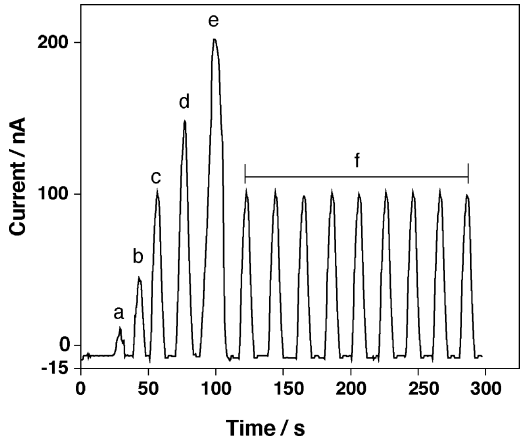

Fig. 7. Flow injection analysis at flow rate of 1.0 ml/min 0.1 M pH 6.8 PBS at an applied potential of +220 mV with successive injection of 0.02, 0.5, 2.0, 3.0 and 4.5 mM H_2O_2 (from peaks a to e) and eight successive additions of 2.0 mM H_2O_2 (f).

to the porous and conductive properties of the ormosil film.

3.7. Storage stability and fabrication reproducibility of the H_2O_2 biosensor

The storage stability was determined by storing the peroxide biosensor at 4 °C under dry condition. After a storage period of 3 months the biosensor kept 95% of its initial response, indicating good stability due to the high biocompatibility of the ormosil composite. Six biosensors made independently showed the relative standard deviations of 2.1% (47.6 ± 1.0 nA) and 1.3% (153 ± 2.0 nA) for current detection of 0.5 and 3.0 mM H_2O_2 at +220 mV, displaying an acceptable reproducibility.

3.8. Real sample analysis

In order to demonstrate the applicability of the proposed biosensor for real sample analysis 0.5 and 2.0 mM hydrogen peroxide solution were spiked in to milk samples, respectively. The recovery of the biosensor was 102% (0.51 ± 0.05 mM, $n = 3$) and 106% (2.13 ± 0.29 mM, $n = 3$), respectively. The milk sample without spiking H_2O_2 did not show any detectable signal.

4. Conclusions

This work demonstrates that the proposed FMC–BSA/MWNTs/ormosil composite is highly useful for the bioelectrochemical/biosensing applications. This composite possesses excellent conductive, entrapment and biocompatible properties and the doped ferrocene group can act as electron transfer mediator for preparation of reagentless biosensors. The proposed reagentless H_2O_2 biosensor exhibits wide linear detection range, acceptable reproducibility and operational and storage stability. The entrapped enzyme is highly stable and shows good affinity towards the substrates due to the less diffusional barrier.

Acknowledgments

This work is supported by the Distinguished Young Scholar Fund to H.X. Ju (20325518) and the NSFC (20275017). V.S.T. and V.B.K. are highly thankful to the post-doctoral fellowship from Nanjing University.

References

Armada, M.P.G., Losada, J., Cuadrado, I., Alonso, B., González, B., Casado, C.M., Zhang, J.B., 2004. Preparation of biosensors based in a siloxane homopolymer with interacting ferrocenes for the amperometric detection of peroxides. Sens. Actuators B 101, 143–149.

Armada, M.P.G., Losada, J., Cuadrado, I., Alonso, B., González, B., Ramírez-Oliva, E., Casado, C.M., 2003. Electrode modified with a siloxane copolymer containing interacting ferrocences for determination of hydrogen peroxid and glucose. Sens. Actuators B 88, 190–197.

Chang, B.S., Mohaney, R.R., 1995. Enzye thermostabilization by bovine serum albumin and other proteins: evidence for hydrophobic interactions. Biotechnol. Appl. Biochem. 22, 203–214.

Delvaux, M., Walcarius, A., Demoustier-Champagne, S., 2004. Electrocatylitic H_2O_2 amperometric detection using gold nanotube electrode ensembles. Anal. Chim. Acta 525, 221–230.

Gavalas, V.G., Andrews, R., Bhattacharyya, D., Bachas, L.G., 2001. Carbonnanotube sol–gel composite materials. Nano Lett. 1, 719–721.

Gill, I., 2001. Biodpoed nanocomposite polymers: sol–gel bioencapulates. Chem. Mater. 13, 3404–3421.

Gong, K., Zhang, M., Yan, Y., Su, L., Mao, L., Xiong, S., Chen, Y., 2004. Sol–gel derived ceramic-carbon nanotube nanocompsote electrodes: tunable electrode dimension and potential electrochemical applications. Anal. Chem. 76, 6500–6505.

Harbury, H.A., 1957. Oxidation and reduction potentials of horseradish peroxidase. J. Biol. Chem. 225, 1009–1024.

Jiang, L., Lu, C., Jian, L., Peng, Z., Lu, G., 2004. A chitosan multiwall carbon nanotube modified electrode for simultaneous detection of dopamine and ascorbic acid. Anal. Sci. 20, 1055–1059.

Kamin, R.A., Wilson, G.S., 1980. Rotating ring disk enzyme electrode for biocatalysis kinetic studies and characterization of the immobilized enzyme layer. Anal. Chem. 50, 1198–1205.

Kandimalla, V.B., Tripathi, V.S., Ju. H.X. Immobilization of biomolecules in sol–gels: biological and analytical applications. Crit. Rev. Anal. Chem., in press.

Koide, S., Yokoyama, K., 1999. Electrochemical characterization of an enzyme electrode based on a ferrocene-containing redox polymers. J. Electroanal. Chem. 468, 193–201.

Li, J., Tan, S.N., Ge, H., 1996. Silica sol–gel immobilized amperometric biosensor for hydrogen peroxide. Anal. Chim. Acta 335, 137–145.

Liu, S.Q., Ju, H.X., 2002. Renewable reagent less hydrogen peroxide sensor based on direct electrontransfer of HRP immobilized on colloidal gold-modified electrode. Anal. Biochem. 307, 110–116.

Losada, J., Cuadrado, I., Moan, M., Casado, C.M., Alonso, B., Barranco, M., 1997. Ferrocenyl silicon based dendrimers as mediators in amperometric biosensors. Anal. Chim. Acta 338, 191–198.

Mulchandani, A., Pan, S., 1999. Ferrocene-conjugated *m*-phenylenediamine conducting polymer-incorporated peroxidase biosensors. Anal. Biochem. 267, 141–147.

Okawa, Y., Nagano, M., Hirota, S., Kobayashi, H., Ohno, T., Watanabe, M., 1999. Tethered mediator biosensor. Mediated electron transfer between redox enzyme and electrode via ferrocene anchored to the electrode surface with long poly (oxyethylene chain). Biosens. Bioelectron. 14, 229–235.

Padeste, C., Grubelnik, A., Tiefenauer, L., 2000. Ferrocene-avidin conjugates for bioelectrochemical applpications. Biosens. Bioelectron. 15, 431–438.

Pandey, P.C., Upadhyay, S., Tiwari, I., Sharma, S., 2001a. A novel ferrocene-encapsulated palladium-linked ormosil-based electrocatalytic biosensor. The role of the reactive functional group. Electroanalyis 13, 1519–1527.

Pandey, P.C., Upadhyay, S., Tiwari, I., Tripathi, V.S., 2001b. An ormosil based peroxide biosensor a comparative study on direct electron transport from horseradish peroxidase. Sens. Actuators B 72, 224–232.

Pandey, P.C., Upadhyay, S., Shukla, N.K., Sharma, S., 2003. Studies on electrochemical performance of glucose biosensor based on ferrocene encapsulated ormosil and glucose oxidase modified graphite past electrode. Biosens. Bioelectron. 18, 1257–1268.

Wang, B., Li, B., Deng, Q., Dong, S., 1998. Amperometric glucose biosensor based on sol–gel organic-inorganic hybrid material. Anal. Chem. 70, 3170–3174.

Wang, B., Zhang, J., Cheng, G., Dong, S., 2000. Amperometric enzyme electrode for the determination of hydrogen peroxide based on sol–gel/hydrogel composite. Anal. Chim. Acta 407, 111–118.

Xiao, Y., Ju, H.X., Chen, H.Y., 2000. Direct electrochemistry of HRP immobilized on a coolid/cystamine modified gold electrode. Anal. Biochem. 278, 22–28.

Yu, J.H., Ju, H.X., 2002. Preparation of porous titania sol–gel matrix for immobilization of horseradish peroxidase by vapor deposition method. Anal. Chem. 74, 3579–3583.