Bioprocess Biosyst Eng (2009) 32:537–544
DOI 10.1007/s00449-008-0275-8

ORIGINAL PAPER

Amperometric hydrogen peroxide biosensor based on covalently immobilizing thionine as a mediator

Liping Ma · Ruo Yuan · Yaqin Chai · Shihong Chen · Shujuan Ling

Received: 21 July 2008 / Accepted: 20 October 2008 / Published online: 7 November 2008

Abstract A novel amperometric hydrogen peroxide biosensor based on the immobilization of hemoglobin on the 2,6-pyridinedicarboxylic acid (PDC) polymer, thionine and nano-Au was successfully fabricated. In this strategy, PDC polymer acted as the matrices to covalently immobilize the thionine, and then hemoglobin was successfully adsorbed on the nano-Au which was electro-deposited on to thionine modified electrode surface. The preparation process of modified electrode was characterized with electrochemical impedance spectroscopy and atomic force microscope. The analytical performance of proposed biosensor toward H_2O_2 was investigated by cyclic voltammetry and chronoamperometry. The resulted biosensor exhibited fast amperometric response (within 6 s) to H_2O_2, and linear range was from 9.1 μM to 5.0 mM with the detection limit of 2.6 μM (S/N = 3). The apparent Michaelis–Menten constant (K_M^{app}) was evaluated to be 3.2 mM. Furthermore, the resulted biosensor showed good stability and reproducibility.

Keywords 2,6-Pyridinedicarboxylic acid · Thionine · Nano-Au · Hemoglobin · Biosensor

Introduction

The importance of determination of H_2O_2 lies in the fact that H_2O_2 plays an important role in clinical, chemical, biological, environmental and many other fields [1, 2]. Techniques for detecting H_2O_2 include spectrofluorimetry [3], chemiluminescence [4], electrochemical methods [5–7] and so on. Among these procedures, amperometric enzyme-based biosensors have received considerable attention, because they offer improved sensitivity, extended dynamic range and rapid response time [8]. However, it is usually difficult to achieve direct electron transfer between the enzyme and the electrode surfaces due to the deeply embedded redoxactive center [9], unfavorable orientation and denaturation of enzyme. Therefore, suitable materials and methods for enzyme immobilization on the electrode surface are very important for obtaining highly sensitive and selective biosensor [10]. Recently, some amperometric biosensors based on various nano-materials such as Au nanoparticles [11], multiwall carbon nanotubes [12] and CuO nanoparticles [13] have been reported for the determination of H_2O_2 in our laboratory. Among various nano-materials, nano-Au are attracting great interest in the preparation of the biosensors, because it not only can act as the immobilizing carrier to provide a relatively higher enzyme loading on the surface of the electrode but also can serve as the signal particle to magnify the detection signal and to greatly enhance the accuracy and lower the detection limit in the target materials detection. Up to now, many methods have been reported for the fabrication of nano-Au, such as chemical reduction, sonochemical reduction, radiolysis, photolysis, thermolysis, etc. [14]. However, the results are not satisfactory due to the time-consuming of preparation and the poor stability of as-prepared colloids [15]. Compared with the above methods, electrochemical reduction of $HAuCl_4$ solution is a short time-consuming, rather simple and clean method to form nanoparticles on an electrode, which can be convenient for immobilization of enzyme.

L. Ma · R. Yuan (✉) · Y. Chai · S. Chen · S. Ling
Key Laboratory on Luminescence and Real-Time Analysis, Ministry of Education, College of Chemistry and Chemical Engineering, Southwest University, 400715 Chongqing, China
e-mail: yuanruo@swu.edu.cn

L. Ma
e-mail: ping319@163.com

At the same time, the sizes of the nanoparticles can be easily controlled by different conditions of electrochemical deposition [16]. Recently, amperometry coupling enzyme with mediators is one of the most sensitive procedures [8]. A series of organic dyes as mediators fixed on the electrode surface mainly depend on electrostatic adsorption [17, 18] or electrochemical polymerization [19–21]. However, they all have some weakness such as poor stability and small peak current. In order to overcome these drawbacks and improve the performance of the biosensor, thionin (Thi) was selected as a perfect electronic mediator to construct the biosensor in this work. This is because Thi has a good electrochemical reversibility, stability and a fast charge transfer characteristic. More importantly, compared with the chemical structure of the other electronic mediators such as toluidine blue, azure A and methylene blue, the Thi's has more amino groups which is more beneficial for carbodiimide reaction to take place and adsorbing nano-Au. In view of these merits, a simple and novel amperometric hydrogen peroxide biosensor has been constructed based on the 2,6-pyridinedicarboxylic acid (PDC) polymer, Thi and nano-Au in this report. Here, the PDC, acted as a support of the redox mediator to provide enough carboxyl, was initially electropolymerized on to glassy carbon electrode (GCE) surface, and then we concentrated on covalent modification that involves the coupling of the amino groups of the Thi to the carboxyl groups of the PDC, using carbodiimide reaction that selectively forms amide linkages only with carboxyl groups [22]. Subsequently, nano-Au was electro-deposited on to Thi-modified electrode surface, which could not only attach enzyme tightly, but also prevent the leakage of Thi from the modified electrode surface. Finally, hemoglobin (Hb) as a model simulation enzyme was adsorbed on to the nano-Au layer to obtain biosensor for the detection of H_2O_2. The proposed biosensor shows good repeatability, long-term stability and a low cost.

Experimental

Reagents and apparatus

Bovine (horse heart) Hb, Thi, chloroauric acid ($HAuCl_4$), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxy sulfo succinimide (NHS) and PDC were all purchased from Sigma Chemical Co. (St Louis, MO, USA). Hydrogen peroxide (H_2O_2, 30%, w/v solution) was purchased from Chemical Reagent Co. (Chongqing, China). The concentration of the more diluted hydrogen peroxide solutions prepared from 30% hydrogen peroxide was determined by titration with potassium permanganate. All other chemicals employed were of analytical grade and were used as received. All solutions were prepared using double distilled water and all experiments were done at ambient temperature (25 ± 2 °C).

All electrochemical measurements were carried out in a conventional three-electrode cell controlled by a CHI 660A electrochemical workstation (CHI Instruments, Chenhua Co., Shanghai, China). The modified Au disk electrode (diameter 4.0 mm) was used as the working electrode. A platinum electrode was used as an auxiliary electrode and a saturated calomel electrode (SCE) served as the reference electrode. All potential values given refer to SCE. Electrochemical impedance spectroscopy (EIS) measurements were carried out with a Model IM6e (ZAHNER Elektrick Co., Germany) in the presence of a 1.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)6]$ (1:1) mixture as a redox probe in 0.1 M PBS (pH 6.0) at a bias potential of 0.17 V. The alternative voltage was 5 mV and the frequency range was 50 MHz to 10 kHz. Atomic force microscope (AFM) experiments were conducted with a SPA400/SPI3800N (Seiko Instruments Inc., Japan) using the dynamic force mode at room temperature. A 100-μm scanner and a Si_3N_4 tip with a nominal spring constant of 40 N/m and a typical frequency of 300 kHz were applied in all image collections. All materials have been modified onto the cleaned gold sheet. After modified, the gold sheet surface was gently rinsed with doubly distilled water and dried in air, and then immediately subjected to AFM imaging.

Fabrication of the hydrogen peroxide biosensor

The GCE was polished to a mirror by using 0.3 and 0.05 μm alumina slurry, and ultrasonically cleaned in ethanol and double distilled water respectively. After being cleaned, the GCE was dipped in a 0.01 M PDC solution containing 0.1 M KCl and was conditioned by cyclic sweeping 20 cycles in the potential range from 0.0 to 2.0 V at 60 mV/s [23], and then immersed in a solution of EDC (100 mg/ml) and NHS (100 mg/ml) for 30 min. After that, the activated electrode was immersed immediately in Thi solution for 2 h. Subsequently, the obtained electrode was performed in the $HAuCl_4$ solution (1%) by applying a constant potential of −0.2 V for 30 s to form a nano-Au layer [16]. Finally, the resulting electrode was immersed in Hb solution (5 mg/ml, 0.1 M pH 6.0 PBS) for 8 h to obtain the hydrogen peroxide biosensor (Hb/nano-Au/Thi/PDC/GCE), which was stored in a refrigerator (4 °C) when not in use. The schematic illustration of the stepwise preparation of the hydrogen peroxide biosensor is shown in Fig. 1. For comparison, the Thi/PDC/GCE and nano-Au/Thi/PDC/GCE were prepared similarly.

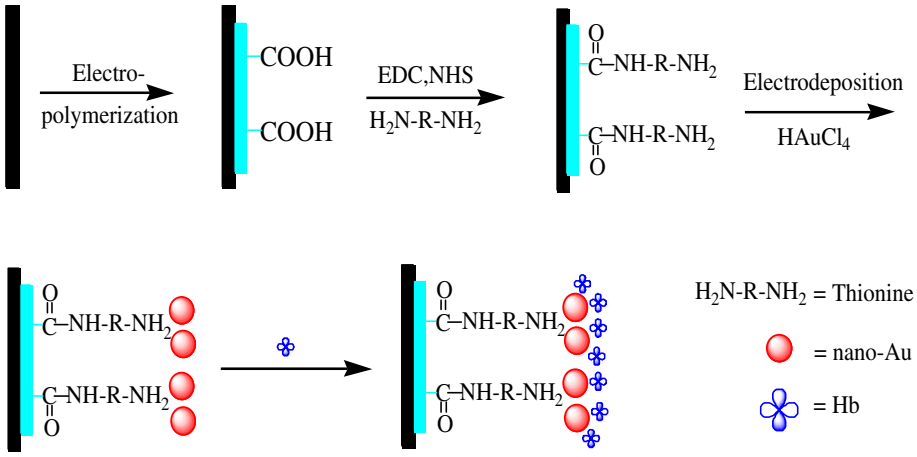

Fig. 1 Illustration of the preparation process of modified electrode

Results and discussion

Electrochemical impedance spectroscopy and atomic force microscopy characterization of self-assembly process

Electrochemical impedance spectroscopy is a powerful tool for studying the interface properties of surface-modified electrodes and has been successfully used to characterize the formation of multiplayer films on different substrates. The typical impedance spectrum includes a linear part at lower frequency range corresponding to the diffusion limited process and a semicircle part at higher frequencies representing the electron transfer limited process. The semicircle diameter of EIS equals the electron transfer resistance, R_{et}. This resistance controls the electron transfer kinetics of the redox probe at the electrode interface [12, 24]. In this paper, the stepwise assembly of the hydrogen peroxide biosensor was characterized by EIS, and the results are presented in Fig. 2. It can be seen that the bare GCE exhibited a very low R_{et}, which implied low transfer resistance (curve a). After PDC was electropolymerized on the GCE surface (curve b), the diameter of the semicircle increased dramatically, which indicated that the electropolymerized film has been formed on the electrode surface, and it blocked the electron transfer of the electrochemical probe. This may be ascribed to the weak conductivity of the film. When Thi and nano-Au was assembled on the electrode, curve c was almost a straight line, this maybe attribute to the excellent electrochemical activity of Thi, as well as the good conductivity of nano-Au. After Hb was attached on the surface of nano-Au monolayer, the semicircle increased remarkably (curve d), and it might be caused by the obstruction of the macromolecular structure of Hb to the electron transfer. From the above phenomenon, we could obviously confirm the success of the assembly of the electrode.

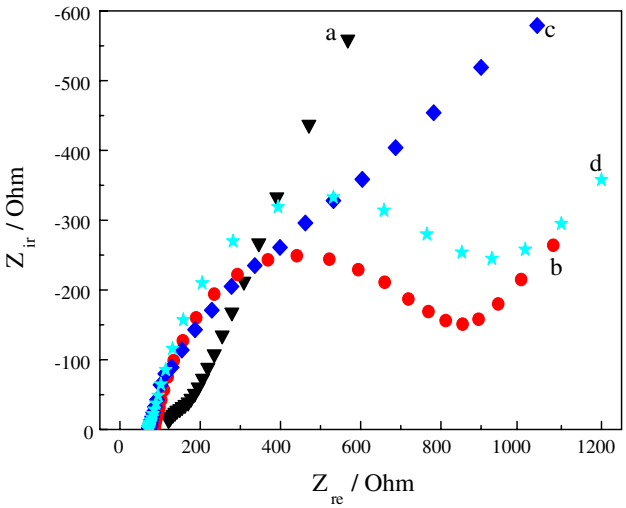

Fig. 2 Electrochemical impedance spectroscopy (*EIS*) of different electrodes in 5 mM $[Fe(CN)_6]^{4-/3-}$ + 0.1 M KCl solution: **a** bare GCE, **b** PDC/GCE, **c** nano-Au/Thi/PDC/GCE, **d** Hb/nano-Au/Thi/PDC/GCE

In order to further confirm the assembly of nano-Au and Hb on the electrode, the AFM of nano-Au/Thi/PDC film and Hb/nano-Au/Thi/PDC film were performed. The results are shown in Fig. 3. From images at different magnification (Fig. 3a, c), we can find that the surface of the nano-Au/Thi/PDC film shows rough structure with numerous globular nanoparticles well distributed in the AFM images, and the size of the nanoparticles is relative homogeneous with a mean size of around 177 nm. Their root mean square (RMS) roughness and thickness are 82.38 and 511.04 nm respectively. However, the Hb/nano-Au/Thi/PDC film presents an evident change in morphology, a layer of compact and smooth floccule can be observed on the nano-Au surface, and the RMS and thickness increased to 84.63 and 515.45 nm respectively (Fig. 3b, d), which indicated that an amount of Hb have been successfully adsorbed on the nano-Au/Thi/PDC/GCE surface.

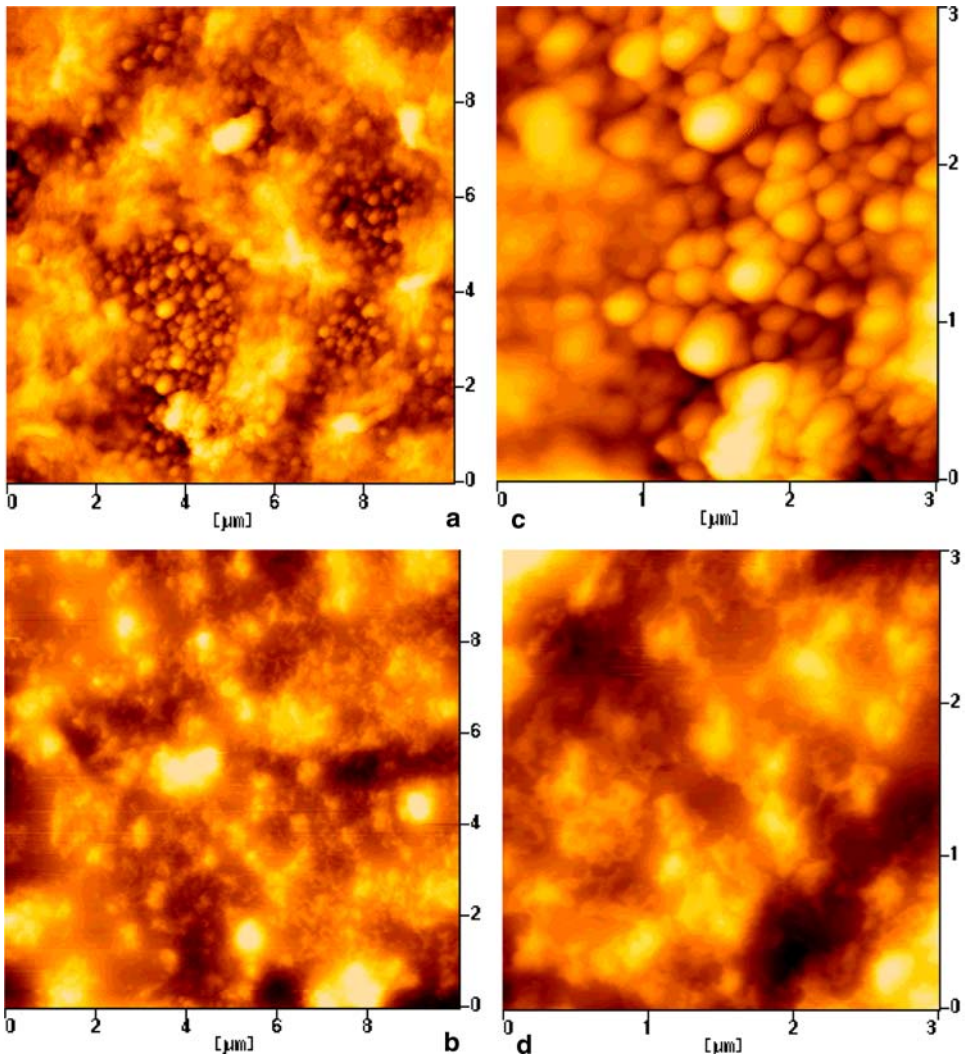

Fig. 3 Atomic force micrographs of the modified electrodes **a** nano-Au/Thi/PDC, **b** Hb/nano-Au/Thi/PDC. **c**, **d** Corresponding to images at different magnification

Electrochemical characteristics of hydrogen peroxide biosensor

Figure 4 displays the cyclic voltammetries (CVs) of the hydrogen peroxide biosensor in 0.1 M PBS (pH 6.0) at scan rates ranging from 20 to 450 mV/s. With the increasing scan rate, the anodic peak potential shifted to a positive direction and the cathodic peak potential shifted in a negative direction; the redox peak currents are proportional to the square root of scan rate (inset of Fig. 4), indicating a diffusion limited redox process.

The effect of pH on formal potential of biosensor at various pH values in the presence 0.35 mM H_2O_2 was studied. The results are shown in Fig. 5. It is found that both the cathodic and the anodic potential shifted in a negative direction with an increase of pH (inset of Fig. 5). The formal potentials $E^{0'}$, which is estimated as the midpoint of cathodic and anodic peak potentials, varied linearly with pH from 4.5 to 8.0, and the correlation equation is $E^{0'} = -0.051$ pH + 0.077. The slope of −0.051 V/pH is very close to the

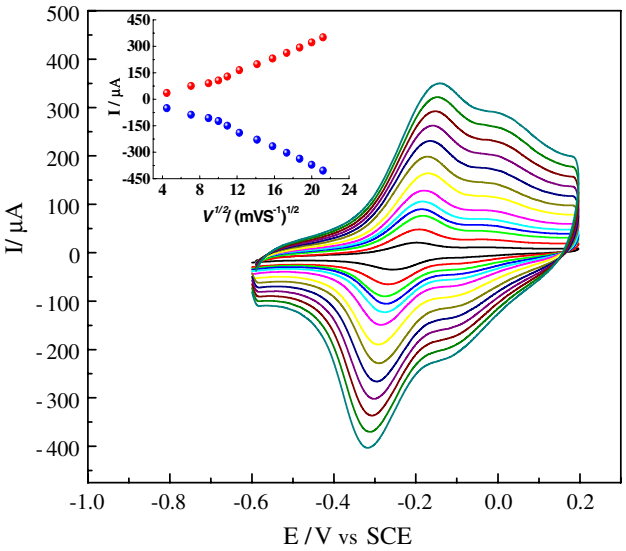

Fig. 4 Cyclic voltammograms of the biosensor at different scan rates (from inner to outer): 20, 50, 80, 100, 120, 150, 200, 250, 300, 350, 400 and 450 mV/s in 5 ml 0.1 M PBS (pH 6.0). Inset: plots of peak currents versus $v^{1/2}$

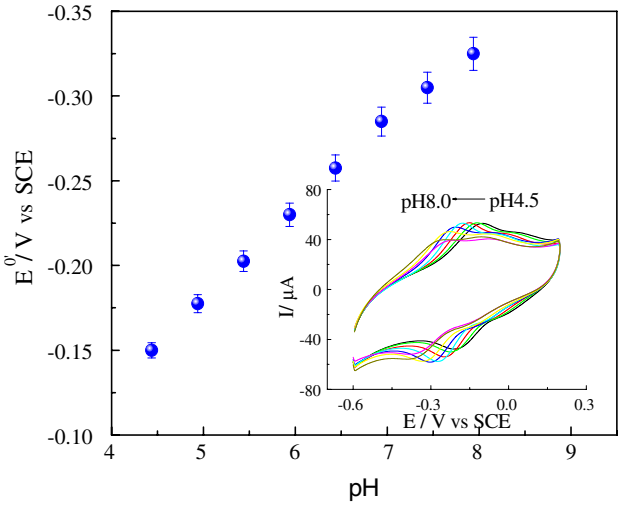

Fig. 5 Effect of pH on formal potential of biosensor at various pH values from 4.5 to 8.0 in the presence 0.35 mM H_2O_2. Inset: CVs of biosensor in 0.1 M PBS at a scan rate of 50 mV/s

−0.059 V/pH, which indicated that proton participated in the electron transfer process. Moreover, the number of proton equals to that of electron [25].

The electrocatalytic behavior of the biosensor toward H_2O_2 was investigated by CV in 0.1 M PBS of pH 6.0. No obvious electrocatalytic reductions of H_2O_2 were observed at the electrodes modified with Thi/PDC films and nano-Au/Thi/PDC films respectively after 0.7 mM H_2O_2 was added to the electrochemical cell (Fig. 6a, b). However, the bioelectrocatalytic reaction of H_2O_2 occurred at the biosensor. With the addition of H_2O_2, the reduction currents increased and the oxidation currents decreased clearly (Fig. 6c), indicating that the Thi was an efficient mediator to shuttle electron transfer between the bioactive center of Hb and the GCE surface. Hb catalyzed H_2O_2 reduction according to the following schemes [26].

$$H_2O_2 + Hb_{(Red)} \rightarrow Hb_{(Ox)} + H_2O \quad (1)$$

$$Hb_{(Ox)} + Thi_{(Red)} \rightarrow Hb_{(Red)} + Thi_{(Ox)} \quad (2)$$

$$Thi_{(Ox)} + 2H^+ + 2e^- \rightarrow Thi_{(Red)} \quad (3)$$

where $Hb_{(Red)}$ and $Hb_{(Ox)}$ represent the reduced form and the oxidized form of Hb; $Thi_{(Red)}$ and $Thi_{(Ox)}$ represent the reduced form and the oxidized form of Thi, respectively.

Optimization of experimental conditions

For biosensor it is a very important to work under right circumstance of pH and potential. The effect of pH on the biosensor was investigated in the pH range 4.5–8.0 in the presence of 0.35 mM H_2O_2. Inset of Fig. 5 unveiled that the peak current increased clearly from pH 4.5 to pH 6.0,

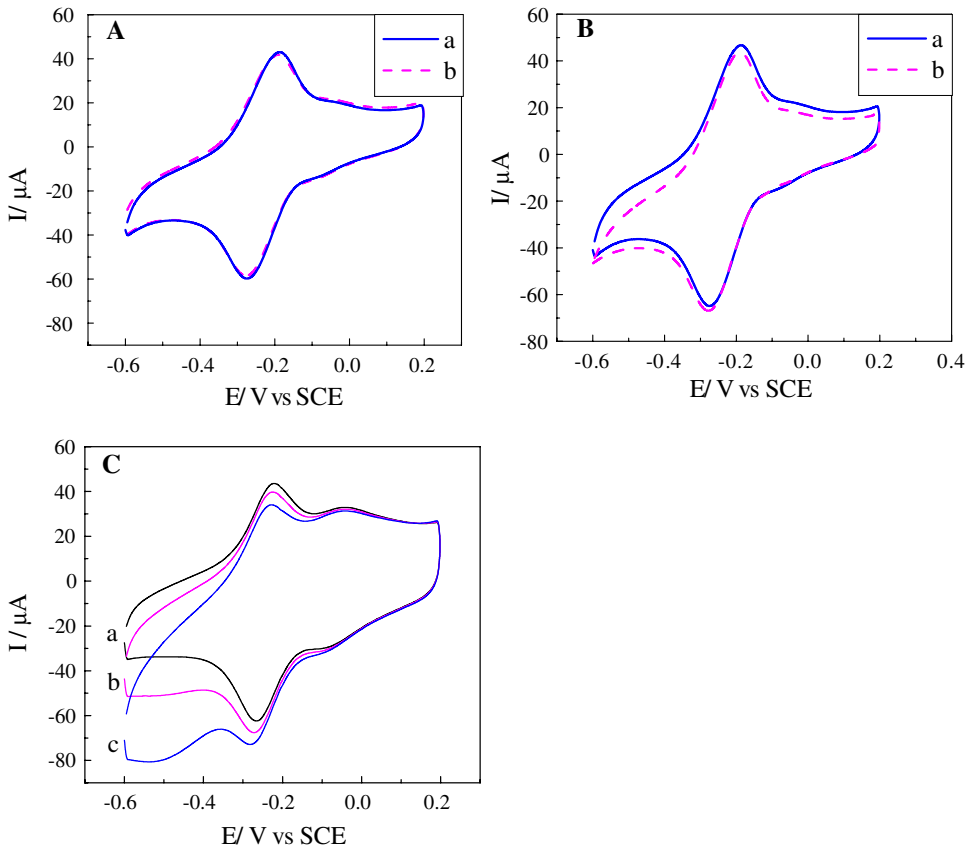

Fig 6 **a** Cyclic voltammograms of the Thi/PDC/GCE in 0.1 M PBS (pH = 6.0) in the absence (*a*) and presence of 0.7 mM H_2O_2 (*b*) at a scan rate of 50 mV/s. **b** Cyclic voltammograms of the nano-Au/Thi/PDC/GCE in 0.1 M PBS (pH = 6.0) in the absence (*a*) and presence of 0.7 mM H_2O_2 (*b*) at a scan rate of 50 mV/s. **c** Cyclic voltammograms of the biosensor in 0.1 M PBS (pH = 6.0) in the absence (*a*), presence of 0.35 mM H_2O_2 (*b*) and 0.7 mM H_2O_2 (*c*) at a scan rate of 50 mV/s

and reached the maximum at pH 6.0, then the further increase of buffer pH led to decrease of peak current. It can be viewed that this biosensor exhibited an optimum sensitivity of response to H_2O_2 at pH 6.0. Therefore, we selected pH 6.0 phosphate buffer for working media solution to determine H_2O_2.

The influence of applied potential on the biosensor was also investigated from 0 to −0.5 V in the presence of 0.35 mM H_2O_2 (the graph not shown). Although amperometric current increased gradually as the applied potential increased from 0 to −0.5 V, an applied potential of −0.3 V was chose to detect H_2O_2. The reason is that we not only could get enough current responses but also could minimize the risk for interfering reactions of oxygen and other electroactive substrates in the solution at −0.3 V.

Calibration curve and chronoamperometric response

Inset of the Fig. 7 illustrates typical current–time curves of the biosensor to reduction of H_2O_2 under the optimal condition. The reduction current increases obviously with increasing concentration of H_2O_2. Within 6 s, 95% of steady catalytic current was obtained after injecting H_2O_2 each time. There is an excellent linear relation of the current with concentration of H_2O_2 from 9.1 μM to 5.0 mM, as shown in the Fig. 7. The linear regression equation is i (μA) = −6.4 − 2.4 [H_2O_2] (mM) with a correlation coefficient of 0.998 (n = 31). The detection limit for H_2O_2 on this Hb/nano-Au/Thi/PDC/GCE is estimated to be 2.6 μM (S/N = 3), lower than that on the Hb/Au/ITO electrode [27] and on the Hb/Au–ZrP/chitosan/GCE [28].

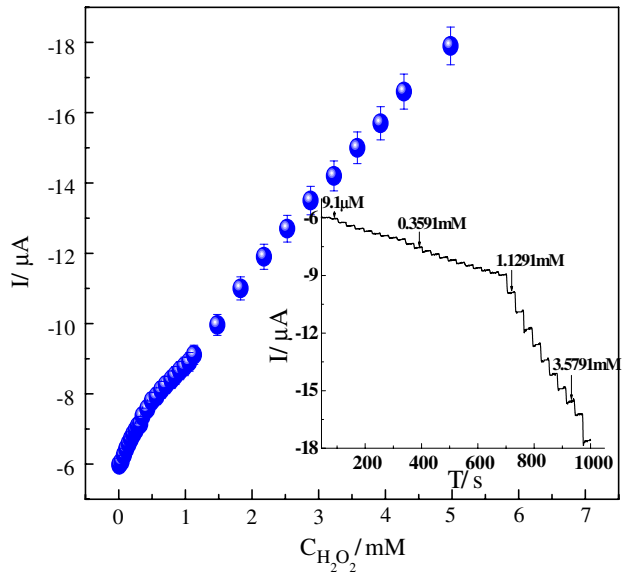

Fig. 7 The calibration curve of the biosensor; Inset: typical current–time response of the biosensor on successive injection of different concentration H_2O_2 into a stirred solution of 0.1 M PBS (pH 6.0) at an applied potential of −0.3 V in the time intervals of 30 s

The apparent Michaelis–Menten constant (K_M^{app}), which gives an indication of the enzyme–substrate kinetics, can be obtained from the Lineweaver–Burk equation [29]:

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_M^{app}}{I_{max}C}$$

where I_{ss} is the steady-state current after the addition of substrate, I_{max} is the maximum current under saturated substrate condition and C is the bulk concentrate of the substrate. The apparent Michaelis–Menten constant can be obtained by an analysis of the steady-state current versus the H_2O_2 concentration. Accordingly, the K_M^{app} value of the proposed biosensor was found to be 3.2 mM (Fig. 8), smaller than the reported value for HRP incorporated in Au nanoparticles and chitosan composite film (K_M^{app} = 4.51 mM) [30] and in poly(thionine) film (K_M^{app} = 28 mM) [25], indicating that Hb immobilized in nano-Au/Thi/PDC/GCE possessed a high biological affinity to H_2O_2.

Reproducibility, stability and selectivity of the hydrogen peroxide biosensor

The reproducibility of the current response of the biosensor was examined at a H_2O_2 concentration of 0.35 mM and the relative standard deviation was 3.8% (n = 10), displaying an acceptable reproducibility. Also, the proposed biosensor showed excellent long-term stability. When not in use, the biosensor was stored dry at 4 °C, during the first 2 days the signal had about 4.8% decreasing and in the next 2 weeks the current response decreased about 19.4% of its initial response, and 28.9% for 1 month. Selectivity is an important factor in practical use of the biosensor. Six interfering substances were used to examine whether they

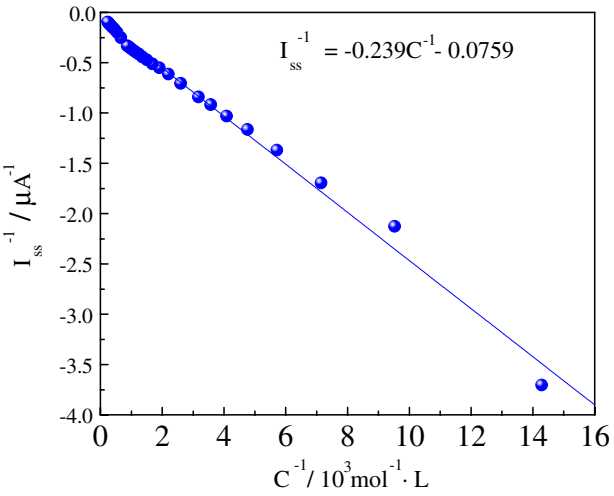

Fig. 8 Determination of Michaelis–Menten constant

Table 1 Interference experiment with the hydrogen peroxide biosensor

Possible interferences	Current ratio[a]
Glucose	1.002
Ethanol	0.998
L-Glutamic acid	0.983
L-Tyrosine	0.975
L-Cysteine	0.981
Ascorbic acid	1.052

[a] Ratio is the current from a mixture of 0.35 mM interfering substances and 0.35 mM H_2O_2 versus the current from 0.35 mM H_2O_2 alone. Assay solution: 0.1 M PBS (pH 6.0)

interfere the determination of H_2O_2 in our experiments. From the value of the current ratio in Table 1, we found that the degree of interference from interfering substances was no more than 1.7%. Thus, there was no evident interference in the measure of the biosensor, which should be owed to the low working potential of -0.3 V used in the determination of H_2O_2.

Conclusions

In conclusion, a simple and novel hydrogen peroxide biosensor has been successfully fabricated. It has following advantages. Firstly, compared with the electron mediator immobilized by electrostatic adsorption or electrochemical polymerization, the covalently immobilized electron mediator (Thi) exhibited bigger and steadier redox current, which enhanced sensitivity and lowered detection limit for H_2O_2 determination. Secondly, nano-Au, which was formed by electro-deposition on the electrode surface not only provided good biocompatibility for maintaining the activity of the immobilized Hb, but also avoided the leakage of the Thi from the modified electrode. Furthermore, the proposed biosensor exhibited long-term stability, excellent repeatability and low cost.

Acknowledgments Financial support of this work was provided by the National Natural Science Foundation of China (20675064), the Natural Science Foundation of Chongqing City (CSTC-2004BB4149, 2005BB4100), China and High Technology Project Foundation of Southwest University (XSGX 02), China.

References

1. Tripathi VS, Kandimalla VB, Ju HX (2006) Amperometric biosensor for hydrogen peroxide based on ferrocene-bovine serum albumin and multiwall carbon nanotube modified ormosil composite. Biosens Bioelectron 21:1529–1535
2. Shi CG, Xu JJ, Chen HY (2007) Electrogenerated chemiluminescence and electrochemical bi-functional sensors for H_2O_2 based on CdS nanocrystals/hemoglobin multilayers. J Electroanal Chem 610:186–192
3. Pa'zdzioch-Czochra M, Wide'nska A (2002) Spectrofluorimetric determination of hydrogen peroxide scavenging activity. Anal Chim Acta 452:177–184
4. Hanaoka S, Lin JM, Yamada M (2001) Chemiluminescent flow sensor for H_2O_2 based on the decomposition of H_2O_2 catalyzed by cobalt(II)-ethanolamine complex immobilized on resin. Anal Chim Acta 426:57–64
5. Qiu JD, Peng HZ, Liang RP et al (2007) Synthesis, characterization, and immobilization of Prussian blue-modified Au nanoparticles: application to electrocatalytic reduction of H_2O_2. Langmuir 23:2133–2137
6. Zhou H, Gan X, Wang J et al (2005) Hemoglobin-based hydrogen peroxide biosensor tuned by the photovoltaic effect of nano titanium dioxide. Anal Chem 77:6102–6104
7. Lin TY, Wu CH, Brennan JD (2007) Entrapment of horseradish peroxidase in sugar-modified silica monoliths: toward the development of a biocatalytic sensor. Biosens Bioelectron 22:1861–1867
8. Liu BH, Liu ZJ, Chen DD et al (2000) An amperometric biosensor based on the coimmobilization of horseradish peroxidase and methylene blue on a β-type zeolite modified electrode. Fresenius J Anal Chem 367:539–544
9. Wang JW, Gu M, Di JW et al (2007) A carbon nanotube/silica sol–gel architecture for immobilization of horseradish peroxidase for electrochemical biosensor. Bioprocess Biosyst Eng 30:289–296
10. Zhang Q, Zhang L, Li JH (2007) DNA-hemoglobin-multiwalls carbon nanotube hybrid material with sandwich structure: preparation, characterization, and application in bioelectrochemistry. J Phys Chem C 111:8655–8660
11. Cao SR, Yuan R, Chai YQ et al (2007) A mediator-free amperometric hydrogen peroxide biosensor based on HRP immobilized on a nano-Au/poly 2, 6-pyridinediamine-coated electrode. Bioprocess Biosyst Eng 30:71–78
12. Chen SH, Yuan R, Chai YQ et al (2007) Amperometric third-generation hydrogen peroxide biosensor based on the immobilization of hemoglobin on multiwall carbon nanotubes and gold colloidal nanoparticles. Biosens Bioelectron 22:1268–1274
13. Miao XM, Yuan R, Chai YQ et al (2008) Direct electrocatalytic reduction of hydrogen peroxide based on Nafion and copper oxide nanoparticles modified Pt electrode. J Electroanal Chem 612:157–163
14. Roucoux A, Schulz J, Patin H (2002) Reduced transition metal colloids: a novel family of reusable catalysts. Chem Rev 102:3757–3778
15. Chen HJ, Wang YL, Wang YZ et al (2006) One-step preparation and characterization of PDDA-protected gold nanoparticles. Polymer 47:763–766
16. He XL, Yuan R, Chai YQ et al (2007) A new antibody immobilization strategy based on electro-deposition of gold nanoparticles and Prussian blue for label-free amperometric immunosensor. Biotechnol Lett 29:149–155
17. Gua TT, Hasebe Y (2004) Peroxidase and methylene blue-incorporated double stranded DNA–polyamine complex membrane for electrochemical sensing of hydrogen peroxide. Analytica Chimica Acta 525:191–198
18. Lawrence NS, Wang J (2006) Chemical adsorption of phenothiazine dyes onto carbon nanotubes: toward the low potential detection of NADH. Electrochem Commun 8:71–76
19. Qu FL, Yang MH, Jiang JH et al (2007) Novel poly (neutral red) nanowires as a sensitive electrochemical biosensing platform for hydrogen peroxide determination. Electrochem Commun 9:2596–2600
20. Du P, Liu SN, Wu P et al (2007) Single-walled carbon nanotubes functionalized with poly(nile blue A) and their application to

dehydrogenase-based biosensors. Electrochim Acta 53:1811–1823
21. Gao Q, Cui XQ, Yang F et al (2003) Preparation of poly(thionine) modified screen-printed carbon electrode and its application to determine NADH in flow injection analysis system. Biosens Bioelectron 19:277–282
22. Shobha Jeykumari DR, Ramaprabhu S, Sriman Narayanan S (2007) A thionine functionalized multiwalled carbon nanotube modified electrode for the determination of hydrogen peroxide. Carbon 45:1340–1353
23. Yang J, Yang T, Feng YY et al (2007) A DNA electrochemical sensor based on nanogold-modified poly-2, 6-pyridinedicarboxylic acid film and detection of PAT gene fragment. Anal Biochem 365:24–30
24. Liu YJ, Yin F, Long YM et al (2003) Study of the immobilization of alcohol dehydrogenase on Au-colloid modified gold electrode by piezoelectric quartz crystal sensor, cyclic voltammetry, and electrochemical impedance techniques. J Colloid Interface Sci 258:75–81
25. Xiao Y, Ju HX, Chen HY (1999) A reagentless hydrogen peroxide sensor based on incorporation of horseradish peroxidase in poly(thionine) film on a monolayer modified electrode. Anal Chim Acta 391:299–306
26. Gao FX, Yuan R, Chai YQ et al (2007) Amperometric hydrogen peroxide biosensor based on the immobilization of HRP on nano-Au/Thi/poly (p-aminobenzene sulfonic acid)-modified glassy carbon electrode. J Biochem Biophys Methods 70:407–413
27. Zhang JD, Oyama M (2004) A hydrogen peroxide sensor based on the peroxidase activity of hemoglobin immobilized on gold nanoparticles-modified ITO electrode. Electrochim Acta 50:85–90
28. Feng JJ, Xu JJ, Chen HY (2005) Synergistic effect of zirconium phosphate and Au nanoparticles on direct electron transfer of hemoglobin on glassy carbon electrode. J Electroanal Chem 585:44–50
29. Kamin AR, Wilson GS (1980) Rotating ring-disk enzyme electrode for biocatalysis kinetic studies and characterization of the immobilized enzyme layer. Anal Chem 52:1198–1205
30. Tangkuaram T, Ponchio C, Kangkasomboon T et al (2007) Design and development of a highly stable hydrogen peroxide biosensor on screen printed carbon electrode based on horseradish peroxidase bound with gold nanoparticles in the matrix of chitosan. Biosens Bioelectron 22:2071–2078