Appl Biochem Biotechnol (2009) 152:418–427
DOI 10.1007/s12010-008-8238-8

Immobilization of Hemoglobin on the Gold Colloid Modified Pretreated Glassy Carbon Electrode for Preparing a Novel Hydrogen Peroxide Biosensor

Yang Liu · Qi-Yu Jiang · Shuang-Yun Lu · Ye Zhang · Hai-Ying Gu

Received: 18 January 2008 / Accepted: 31 March 2008 /
Published online: 30 August 2008

Abstract A novel hydrogen peroxide (H_2O_2) biosensor was developed by immobilizing hemoglobin on the gold colloid modified electrochemical pretreated glassy carbon electrode (PGCE) via the bridging of an ethylenediamine monolayer. This biosensor was characterized by UV-vis reflection spectroscopy (UV-vis), electrochemical impendence spectroscopy (EIS) and cyclic voltammetry (CV). The immobilized Hb exhibited excellent electrocatalytic activity for hydrogen peroxide. The Michaelis–Menten constant (K_m) was 3.6 mM. The currents were proportional to the H_2O_2 concentration from 2.6×10^{-7} to 7.0×10^{-3} M, and the detection limit was as low as 1.0×10^{-7} M (S/N=3).

Keywords Biosensor · Glassy carbon electrode · Gold colloid · Hemoglobin · Hydrogen peroxide

Introduction

In pharmaceutical, environmental, food, and other fields, determination of hydrogen peroxide is of great practical importance. Many techniques have been employed for detecting hydrogen peroxide, such as titrimetry, photometry, chemiluminescence, and electrochemistry. Electrochemical biosensors based on the direct electrochemistry of heme proteins have received considerable interest because of its high sensitivity and selectivity.

Hemoglobin (Hb), which is an ideal molecule for studying the mechanism of electron transfer reactions of heme proteins, comprises four polypeptide subunits (two α and two β subunits) [1]. It is well known that the catalytic activity of Hb is attributed to its iron heme group acting as the electroactive center. Study on the direct electrochemistry of Hb is of

Y. Liu · Q.-Y. Jiang · S.-Y. Lu · Y. Zhang · H.-Y. Gu (✉)
Institute of Analytical Chemistry for Life Science, School of Public Health,
Nantong University, Nantong 226007, China
e-mail: hygu@ntu.edu.cn, guhy99@21cn.com

significance in biology, which would provide a model for researching the mechanism of electron transfer in real biological systems and establish a foundation for fabricating the third-generation biosensors without mediators or promoters [2].

Because of embedding the electroactive centers deeply, adsorptive denaturation and unfavorable orientations of Hb onto electrode surfaces [3–5], many efforts have been made to enhance the electron transfer of Hb by using mediators or promoters. Great efforts have been made to increase the electron transfer kinetics, and the most commonly used methods include specific surface modifications of the electrodes with polymer films [6, 7], surfactants [8] and biomembranes [9, 10]. However, the insufficient reactivity and stability of some modified electrodes seem to impede their applications.

Gold colloid refers to a uniformly dispersed system consisting of 1–100 nm gold nanoparticles in the solution. The formation of gold nanoparticles can be observed by a change in color since small gold nanoparticles are red. The colors of gold colloid can be changed with the sizes of nanoparticles. It processed in unique properties of nanoparticles such as volume, surface, quantum size, and macro quantum tunneling effect, optical property, catalytic property, chemical reactivity, and so on [11]. Gold colloid exhibits better catalytic activity of H_2O_2 than metallic gold due to their high surface area-to-volume ratio and their interface-dominated properties [12–14]. It has been widely investigated that biomolecules were immobilized on gold nanoparticle surface in recent years [15–17]. Gold nanoparticles can enhance the electrocatalytic response of H_2O_2 because it is similar to that of a conducting wire or electron-conducting tunnel and provide more binding sites for the coupling of molecules, including proteins, enzymes, and antibodies [18–21]. Indeed, it has been reported that direct adsorption of Hb onto gold nanoparticles would not be denatured and retain their biological activity [22–24]. In our previous work, the adsorption characteristic of gold colloid, protein biosensors based on Hb/gold colloid/Au electrode and cellular biosensors based on hepatocytes or blood red cells on the surface of gold nanoparticles have been reported [25–27].

Here, we fabricated Hb modified electrode with the nanometer-sized gold colloid self-assembled on the electrochemically pretreated glassy carbon electrode (PGCE) surface via the bridging of ethylenediamine monolayer, and then used UV-vis reflection spectroscopy (UV-vis), electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) to characterize the modified electrode. The Hb immobilized on nanometer-sized gold colloid/ethylenediamine/PGCE maintains its biological activity and exhibits excellent electrocatalytic response for the reduction of H_2O_2. This new method has more advantages than that proposed in our previous work in terms of analytical performances [25].

Materials and Methods

Materials

Hb (~90%, bovine blood) was purchased from Sigma Co. and used without further purification. Ethylenediamine and $LiClO_4$ were purchased from Sigma Co. Gold Chloride ($AuCl_3$ $HCl4H_2O$, Au % > 48%, the Shanghai No. 1 Reagent Factory) and all other chemicals were of analytical grade. Hydrogen peroxide [30% (*w*/*w*)] was from Shanghai Chemical Plant, and its dilute solution was prepared daily. Phosphate buffer solutions (PBS, 0.2 M) with different pH values were prepared by mixing the standard stock solutions of Na_2HPO_4 and NaH_2PO_4 by adjusting pH with 1.0 M H_3PO_4. Other chemicals were of analytical grade. All solutions were prepared with twice-distilled water.

Apparatus and Methods

The electrochemical experiments were performed CHI 660 Electrochemistry Working Station (CH Instruments Co., USA). The electrochemical cell consisted of a three-electrode system where the modified glassy carbon electrode (Φ=3 mm) was used as a working electrode, a platinum wire as a counter electrode and a saturated calomel electrode (SCE) as a reference electrode. All measurements were carried out at the temperature of 25±0.2 °C in a Faraday cage. Amperometric experiments were carried out in a stirred system by applying a potential of −200 mV to the working potential. Current-time data were recorded after a steady-state current had been achieved. All experimental solutions were deaerated by bubbling highly pure nitrogen for 30 min, and nitrogen atmosphere was kept over the solution during measurements. The UV-vis spectra of the samples were recorded using a UV-2450 spectrophotometer (Shimadzu, Japan). EIS experiments were performed in 0.10 M KNO_3 solution containing 5.0 mM $Fe(CN)_6^{-3}/Fe(CN)_6^{-4}$ (1:1). The impedance measurements were performed at an open circuit potential of 0.17 V within the frequency range of 10^{-2}–10^5 Hz.

Electrode Modification

All glasswares used in the following procedures were cleaned in a bath of freshly prepared 3:1 HNO_3–HCl, rinsed thoroughly in twice-distilled water and dried in air. Gold chloride and Na_3Citrate solutions need to be filtered through a 0.22 μm microporous membrane filter prior to use. Preparations were stored in brown glass bottles at 4°C. Gold colloids were prepared according to the literature [16] by adding Na_3Citrate solution to a boiling $AuCl_3$ HCl aqueous solution. The molar ratios of $AuCl_3$ HCl/Na_3Citrate were 1.0. The diameter of the colloidal gold particles was 31±2.5 nm measured by transmission electron microscopy (TEM).

The glassy carbon electrodes were polished firstly with fine emery paper and then with 0.3 μm alumina slurry on microcloth pads followed by rinsing with water and ethanol and briefly cleaning in an ultrasonic bath. PGCE was achieved by anodic oxidation at +1.8 V followed by potential cycling in the potential range over −0.8 to +0.1 V according to our previous report [27]. The cleaned PGCE was electrochemically modified in ethanol containing 0.10 M ethylenediamine and 0.10 M $LiClO_4$ with cyclic sweep from 0.0 to 1.4 V (20 mVs^{-1}) for five cycles. The resulting ethylenediamine monolayer modified PGCE was rinsed thoroughly with twice-distilled water and soaked in water for 10 h to remove the physically adsorbed ethylenediamine. Then, it was dipped into the gold colloid for 10 h and the hemoglobin solution (20 mg ml^{-1} in pH 8.0 PBS) for 20 h, respectively. Finally, the resulting Hb/gold colloid/ethylenediamine/PGCE was washed with water and stored in pH 7.2 PBS at 4°C for use. To characterize Hb by UV-vis reflection spectrum, the ITO electrode was modified with the same procedures as those for the Hb/nanometer-sized gold colloid/ethylenediamine /PGCE.

Results and Discussion

Construction of Nanometer-sized Gold Colloid/ethylenediamine/PGCE and Immobilization of Hb

The resulting PGCE contained oxygen and oxygen-containing functional groups such as [27]. Many studies have revealed that the electrode surface structure become very porous

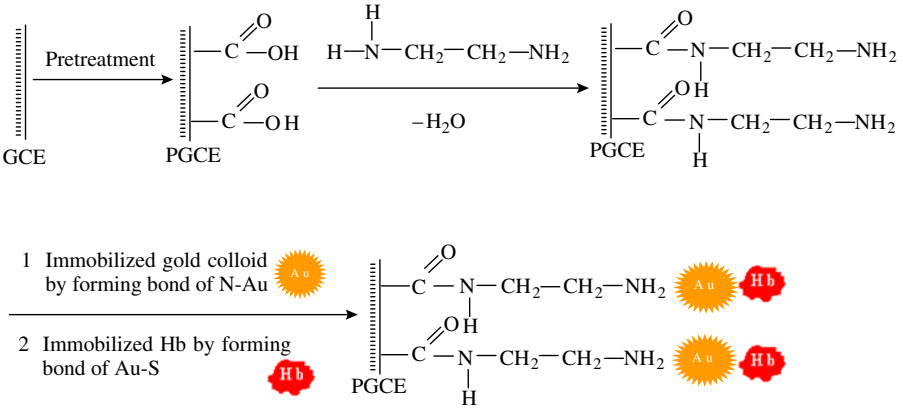

Fig. 1 The modification procedure of Hb/nanometered-sized gold colloid/ethylenediamine/PGCE

with an enhanced electrode surface area [28]. Such functional group will increase the density of active sites at the electrode surface and improve the electrode surface of the reaction. Electrochemical modification of ethylenediamine at PGCE was carried out in ethanol containing 0.10 M ethylenediamine and 0.10 M $LiClO_4$ by cyclic voltammetry through dehydration [27]. And then, gold colloid was modified by the bond of Au–N between the gold colloid and ethylenediamine. At last, Hb was assembled by the interaction of Au from colloidal gold and −SH from Hb, as illustrated in the following scheme (Fig. 1). With regard to the interaction of Au and −SH of protein, many reports have been certified [25–28].

Electrochemical Impedance Spectroscopy

EIS was applied to monitor the whole process in preparing modified electrodes, which could provide useful information between steps and often be used for probing the changes of surface-modified electrodes.

Figure 2 exhibits the EIS curves of each fabricated procedure, shown as Nyquist plot. The semicircle part at higher frequency is corresponding to the electron transfer limited step and the diameter of the semicircle is equivalent to the electron transfer resistance, which controls the electron transfer kinetics of the redox probe at the electrode interface; the linear part at lower frequencies corresponds to the diffusion process. During the fabricated procedure, regular differences of EIS were observed. Curve a in Fig. 2 shows EIS of the bare glass carbon electrode (GCE). When ethylenediamine was assembled on the PGCE surface (curve b), the semicircle increased, suggesting that ethylenediamine film acted as an insulating layer that made interfacial charge transfer inaccessible. Then, gold colloids were immobilized on the ethylenediamine/PGCE through Au–N, and the semicircle (curve c) was obviously reduced compared to ethylenediamine/PGCE (curve b). The reason may be that nanometer-sized gold colloid modified on the PGCE by ethylenediamine played an important role of a conducting wire or an electron-conducting tunnel, which makes it easier for the electron transfer to take place [29]. The adsorption of Hb (curve d) on the modified electrode made the semicircle increased greatly again. The impendence change of modification process indicated that Hb, a big biological macromolecule, had attached to the electrode surface.

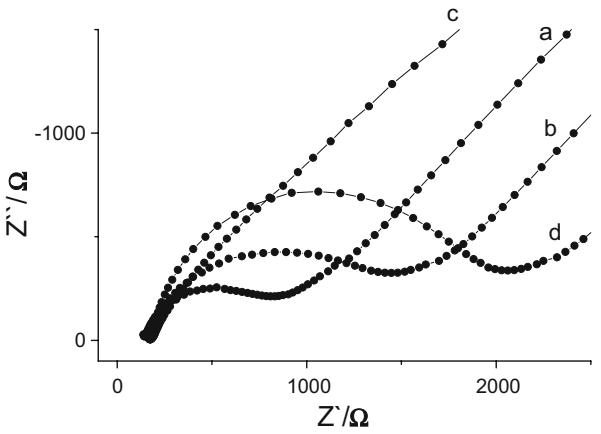

Fig. 2 The EIS of: (*a*) bare GCE; (*b*) ethylenediamine/PGCE; (*c*) nanometer-sized gold colloid/ethylenediamine /PGCE and (*d*) Hb/nanometer-sized gold colloid/ethylenediamine/PGCE

UV-vis Reflection Spectroscopic Analysis

Some biological macromolecules such as Hb, myoglobin (Mb), and horseradish peroxidase (HRP) and cytochrome C have a sensitive Soret absorption at about 410 nm. The shape and positions of the Soret absorption bands could provide information about possible denaturation of heme proteins [30]. And we know that absorption bands of gold nanoparticles solution were at about 520 nm [31]. So UV-vis reflection spectra was employed to investigate the electrode modification processes. Electrochemical pretreated ITO electrode served a reference. The ethylenediamine/pretreated ITO electrode (curve a) showed no peak in the Fig. 3 (curve a). And we can see that absorption bands of gold colloid/ethylenediamine/pretreated ITO (527 nm; curve b) were very similar to those of gold nanoparticles solution (curve c; 526 nm), showing the presence of the gold nanoparticles in the surface of ethylenediamine/pretreated ITO. When Hb was modified in the gold colloid/ethylenediamine/pretreated ITO (curve d), its peak (408 nm) was very close to that in solution (curve e; 406 nm): a difference of only 2 nm was observed. This showed that Hb maintained biological activity on the functional interface of nanometer-

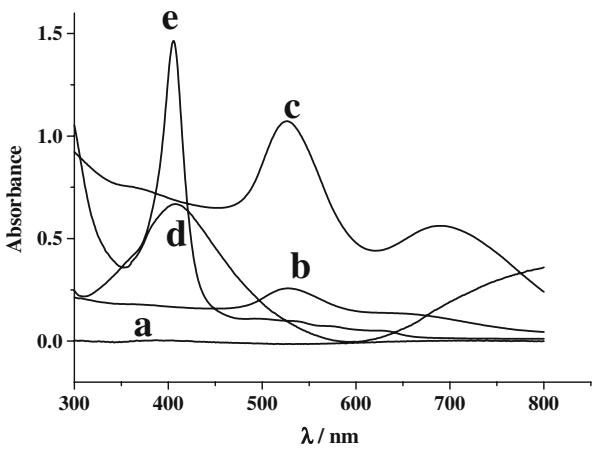

Fig. 3 UV-vis reflection absorption of (*a*) ethylenediamine/pretreated ITO, (*b*) nanometer-sized gold colloid /ethylenediamine/pretreated ITO, (*c*) gold nanoparticles solution, (*d*) Hb/nanometer-sized gold colloid/ethylenediamine/pretreated ITO, (*e*) 0.50 mg ml^{-1} Hb in pH 8.0 PBS

sized gold colloid. We also found that after the immobilization of Hb, the peak of gold colloid was disappeared (curve e). The reason may be that gold colloid was covered by Hb.

Direct Electrochemistry of Hb Immobilized on the Nanometer-sized Gold Colloid/Ethylenediamine/PGCE

In Fig. 4, no peak (curve a) was observed at the bare GCE in pH 7.0 PBS. When Hb was modified on the gold colloid/ethylenediamine/PGCE (curve b), a pair of peaks at PGCE disappeared and Hb gave a couple of quasi-reversible redox peaks at about −0.20 and −0.10 V at 50 mV s^{-1}, which is the characteristic of heme Fe(III)/Fe(II) redox couple of the protein [25].

Hb is a kind of biological macromolecule. The direct electron transfer of Hb at usual bare glass carbon electrode is impossible. Gold nanoparticles were used as an efficient electron-conducting tunnel. Experimental results showed nanometer-sized gold colloid have large specific surface area, good biocompatibility, and suitability for many surface immobilization mechanism [25]. It is possible that the Hb attached to the surface of nanometer-sized gold colloid has more spatial freedom in its orientation. So the nanometer-sized colloidal gold facilitated direct electron transfer between the heme site of the immobilized Hb and the electrode surface [31, 32].

Figure 5 shows the cyclic voltammograms of the Hb/gold colloid/ethylenediamine/PGCE in PBS (pH 7.0) at different scan rates. With increasing scan rate from 20 to 150 mV s^{-1}, the redox peak currents increased. A linearity of the peak currents with scan rates indicated that the redox process was confined to involvement of adsorption in the total electrode process [33], which also showed that Hb modified on the electrode was stable. This tells us that all the electroactive ferric hemoglobin [Hb Fe(III)] in the film is reduced to ferrous hemoglobin [Hb Fe(II)] on the positive potential to negative potentials and that the Hb Fe(II) produced is oxidized to Hb Fe(III) on the reverse [34]. According to Laviron's equation [35]:

$$i_p = \frac{nFQv}{4RT}$$

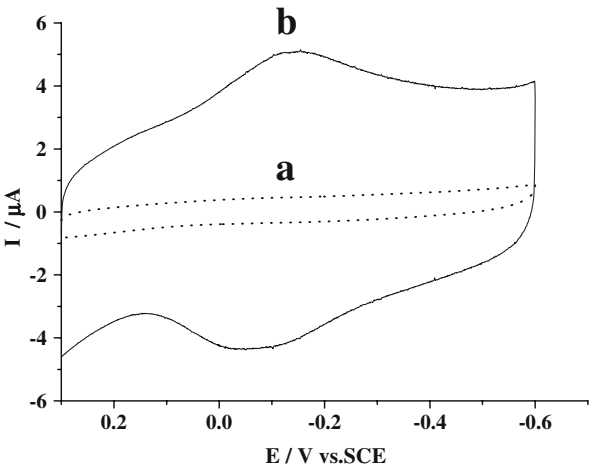

Fig. 4 Cyclic voltammograms of the different electrode in pH 7.0 PBS at a scan rate of 50 mV s^{-1}: (*a*) bare GCE, (*b*) Hb/nanometer-sized gold colloid/ethylenediamine/PGCE

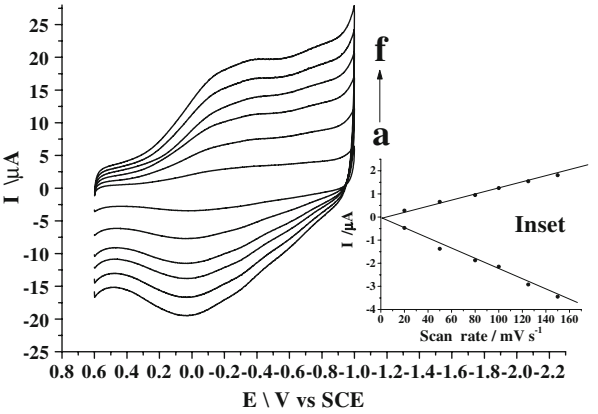

Fig. 5 Cyclic voltammograms of Hb/nanometer-sized gold colloid/ethylenediamine/PGCE in pH 7.0 PBS at scan rates of (*a*) 20, (*b*) 50, (*c*) 80, (*d*) 100, (*e*) 125, (*f*) 150 mV s^{-1}. *Inset*, plot of peak current vs. scan rates

From the slope of the $i_p \propto v$, n was calculated to be 1.3. Therefore, the redox of Hb on a gold colloid/ethylenediamine/PGCE is a single ET reaction.

Amperometric Response to H_2O_2

Previous reports showed that proteins, such as Hb, Mb, HRP, and cytochrome C which contained heme groups, could display electrochemical response to H_2O_2 [25, 26, 36, 37]. When H_2O_2 was added to the PBS (pH 7.0), we can see an increase in reduction peak at about −0.20 V, which indicated that the immobilized Hb kept excellent electrocatalytic activity (Fig. 6).

Figure 7 shows a typical current-time plot for the Hb/gold colloid/ethylenediamine/PGCE. A potential of −0.20 V (vs. SCE) was chosen as the detection potential for the amperometric determination of H_2O_2, where the risk for interfering reactions of oxygen was minimized, and then the background current would decrease to the lowest level as our experiment. As can be seen, the direct electrocatalytical reduction of H_2O_2 was effective, and the reduction current increased steeply within 2 s to reach a stable value.

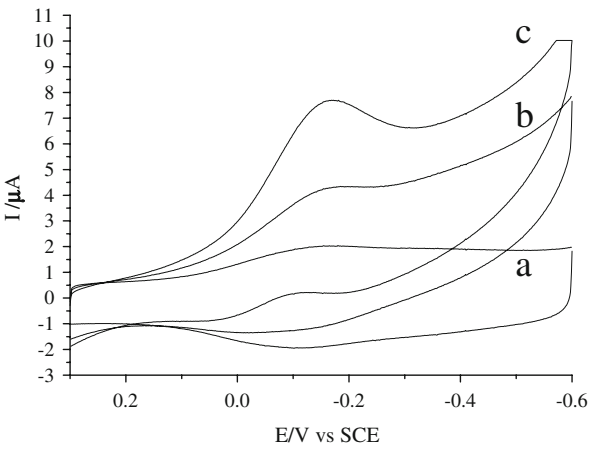

Fig. 6 Cyclic voltammograms of Hb/nanometer-sized gold colloid/ethylenediamine/PGCE in pH 7.0 PBS at a scan rate of 50 mV s^{-1} (*a*) in the absence of H_2O_2, (*b*) and (*c*) in the presence of 1×10^{-4} M and 3×10^{-4} M H_2O_2, respectively

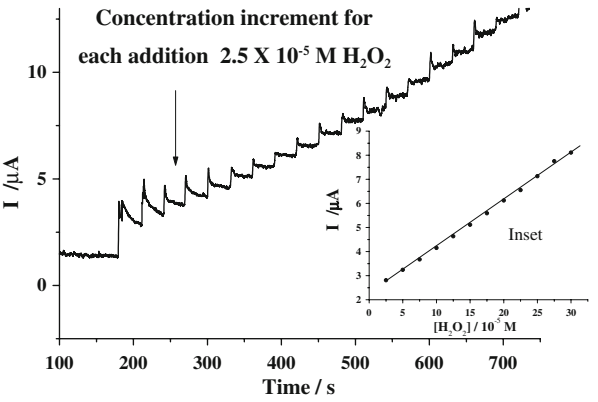

Fig. 7 Amperometric responses with successive additions of 2.5×10^{-5} M H_2O_2. *Inset*, the linear calibration curve of electrocatalysis current vs. H_2O_2 concentrations

Good linearity was found in the plot of peak current vs. H_2O_2 concentration. The response was linear in the range of 2.6×10^{-7} to 7.0×10^{-3} M, and the detection limit was as low as 1.0×10^{-7} M (S/N=3). The linear regression equation is I (μA)=2.2258+0.1958 $[H_2O_2]$ ($\times10^{-5}$ M), with a correlation coefficient γ=0.9993. The RSD was 5.4% for eight successive determinations at H_2O_2 concentration of 2.5×10^{-4} M.

From Table 1, the linear range of this biosensor was much wider than those of other biosensors and chemical sensors based on Hb/gold colloid–cysteame-modified Au electrode [25], Hb/zirconium nanoparticles modified GCE [38] and Au–Pt nanoparticles [39] or platinum nanoparticles [40] modified electrodes. In this proposed method, the limit of detection of 1.0×10^{-7} M was lower than those listed in Table 1. Response time was quicker, its cost was also lower, and procedure was very easy for operation. Therefore, the Hb/gold colloid/ethylenediamine/PGCE can be used as an amperometric biosensor for determining H_2O_2 in solution without any other mediator.

We also find that the calibration curve gradually tended to a plateau and then dropped down when adding H_2O_2. This shows that Hb has a similar structure of peroxidase. The apparent Michaelis–Menten constant (K_m), which gives an indication of the enzyme-substrate kinetics, can be obtained from the line equation [29]:

$$\frac{1}{I_{SS}} = \frac{K_m}{I_{\max}} \cdot \frac{1}{C} + \frac{1}{I_{\max}}$$

Here, I_{ss} is the steady-state current after the addition of substrate, C is the bulk concentration of the substrate, and I_{max} is the maximum current measured under saturated

Table 1 Comparison of the proposed method with other biosensors and chemical sensors for determining H_2O_2.

Methods	Limit of detection (M)	Linear range (M)	Response time (s)
The proposed method	1.0×10^{-7}	2.6×10^{-7}–7.0×10^{-3}	2
Reported in [25]	1.2×10^{-7}	3.6×10^{-7}–8.6×10^{-4}	2
Reported in [38]	1.5×10^{-7}	8.0×10^{-4}–1.3×10^{-1}	5
Reported in [39]	1.6×10^{-5}	1.6×10^{-5}–2.0×10^{-3}	Null
Reported in [40]	1.0×10^{-6}	5.0×10^{-6}–2.0×10^{-2}	<10

substrate conditions. The K_m value of this H_2O_2 biosensor was found to 3.6 mM. The lower value of K_m indicated the Hb immobilized showed a high biological affinity to H_2O_2.

The Stability and Repeatability of the Hb/Gold Colloid/ethylenediamine/PGCE

It was studied by repeatedly recording the response current to H_2O_2. To investigate the reproducibility of the Hb/gold colloid/ethylenediamine PGCE, the response current to H_2O_2 at five electrodes prepared in the same way was recorded. The relative standard deviation was 2.9%. The modified electrodes were stored in pH 7.2 PBS under refrigeration at 4 °C. The modified electrode retained more than 85% of its initial sensitivity to H_2O_2 after 2 weeks. The good long-term stability may be attributed to the strengthened biocompatibility and stability of the nanoparticles [28].

Conclusion

The third-generation amperometric H_2O_2 biosensor was constructed by self-assembling ethylenediamine, gold colloid and Hb sequentially on the PGCE. The Hb/nanometer-sized gold colloid/ethylenediamine/PGCE was characterized by both spectroscopic and electrochemical techniques. The nanometer-sized colloidal gold was a promising alternative material for the modified electrode, in which direct electron transfer was possible between Hb and the modified electrode. The proposed biosensor showed a wider linear range and lower detection limit for determining H_2O_2 as compared to other biosensors and chemical sensors.

Acknowledgments This work was financially supported by the National Natural Science Foundation of China (Grant number: 20675042), the Social Development Item of Jiangsu Province (Grant number: BS2005030) and the Jiangsu Province Education Commission Natural Science Foundation (Grant numbers: JH03-041, 03KJB150107).

References

1. Lei, C., Wollenberger, U., Bistolas, N., Guiseppi-Elis, A., & Scheller, F. W. (2002). *Analytical Bioanalytical. Chemistry*, *372*, 235–239.
2. Scheller, F., Bistolas, N., & Liu, S. Q. (2005). *Advances in Colloid and Interface Science*, *116*, 111–120.
3. Li, Z, & Hu, N. (2003). *Journal of Electroanalytical Chemistry*, *558*, 155–165.
4. Li, Y., Liu, H., & Pang, D. (2004). *Journal of Electroanalytical Chemistry*, *574*, 23–31.
5. Han, X. J., Cheng, W. L., Zhang, Z. L., Dong, S. J., & Wang, E. K. (2002). *BBA-Bioenergetics*, *1556*, 273–277.
6. Lu, Q., Zhou, T., & Hu, S. (2007). *Biosensors & Bioelectronics*, *22*, 899–904.
7. Tao, W., Liu, Y., Pan, D., Nie, L., & Yao, S. (2004). *Bioelectrochemistry*, *65*, 51–58.
8. Chen, L., & Lu, G. (2007). *Sensors and Actuators B*, *121*, 423–429.
9. Han, X. J., Huang, W. M., Jia, J. B., Dong, S. J., & Wang, E. K. (2002). *Biosensors & Bioelectronics*, *17*, 741–746.
10. Yang, J., & Hu, N. F. (1999). *Biosensors & Bioelectronics*, *48*, 117–127.
11. Chen, W., Wu, W., Chen, H., & Shen, Z. (2003). *Science in China*, *46*, 330–338.
12. Welch, C. M., & Compton, R. G. (2006). *Analytical Bioanalytical Chemistry*, *384*, 601–619.
13. Burton, S. G. (1994). *Catalysis Today*, *22*, 459–487.
14. Yu, A., Liang, Z., Cho, J., & Caruso, F. (2003). *Nano Letters*, *3*, 1203–1207.
15. Yang, G., Ruo, Y., & Chai, Y. Q. (2008). *Colloids and Surfaces B*, *61*, 93–100.
16. Liu, S., Peng, L., Yang, X., Wu, Y., & He, L. (2008). *Analytical Chemistry*, *375*, 209–216.
17. Zhang, H., & Hu, N. (2007). *Biosensors & Bioelectronics*, *23*, 393–399.

18. Lyon, L. A., Musick, M. D., & Natan, M. J. (1998). *Analytical Chemistry*, *70*, 5177–5183.
19. Wang, M. J., Wang, L. Y., Wang, G., Ji, X. H., Bai, Y. B., Li, T. J., et al. (2004). *Biosensors & Bioelectronics*, *19*, 575–582.
20. Lei, C. X., Yang, Y., Wang, H. G., Shen, L., & Yu, R. Q. (2004). *Analytica Chimica Acta*, *513*, 379–384.
21. Huang, S., Wei, H., & Lee, Y. (2007). *Composites Science and Technology*, *67*, 811–816.
22. Kamau, G. N., Guto, M. P., Munge, B., Panchagnula, V., & Rusling, J. F. (2003). *Electrochemical Catalysts*, *19*, 6976–6981.
23. Dai, Z. H., Liu, S. Q., Ju, H. X., & Chen, H. Y. (2004). *Biosensors & Bioelectronics*, *19*, 861–867.
24. Cai, C., & Chen, J. (2004). *Analytical Biochemistry*, *325*, 285–292.
25. Gu, H. Y., Yu, A. M., & Chen, H. Y. (2001). *Journal of Electroanalytical Chemistry*, *516*, 119–126.
26. Gu, H. Y., Sa, R., Yuan, S., Chen, H., & Yu, A. (2003). *Chemistry Letters*, *32*, 934–935.
27. Gu, H. Y., Lu, S., & Jiang, Q. (2006). *Analytical Letters*, *39*, 2849–2859.
28. Feng, J. J., & Chen, H. Y. (2005). *Journal of Electroanalytical Chemistry*, *585*, 44–54.
29. Zhao, J., Henkens, R. W., Stonehuerner, J., O'Daly, J. P., & Crumbliss, A. L. (1992). *Journal of Electroanalytical Chemistry*, *327*, 109–119.
30. George, P., & Hanania, G. (2007). *Biomaterials*, *28*, 2147–2154.
31. Yang, W., Cai, Y., & Sun, C. (2006). *Sensors and Actuators B*, *115*, 44–45.
32. Li, X. L., & Yuan, R. J. (2006). *Biotechnology*, *123*, 356–366.
33. Murray, R. W., & Bard, A. J. (1984). *Electroanalytical chemistry.* New York: Marcel Dekker.
34. Jiang, X., & Dong, S. J. (2006). *Electrochemistry Communications*, *8*, 1137–1141.
35. Laviron, E. (1979). *Journal of Electroanalytical Chemistry*, *101*, 19–28.
36. Lu, Z., Huang, Q., & Rusling, J. (1997). *Journal of Electroanalytical Chemistry*, *423*, 59–66.
37. Chen, S. M., & Teng, C. C. (2005). *Journal of Electroanalytical Chemistry*, *575*, 147–160.
38. Zong, S., Yong, C., Zhou, Y., & Ju, H. (2007). *Analytica Chimica Acta*, *582*, 361–366.
39. Chikae, M., Idegami, K., Kerman, K., Nagatani, N., Ishikawa, M., Takamura, Y., et al. (2006). *Electrochemistry Communications*, *8*, 1375–1380.
40. Yang, M. H., Qu, F. L., Lu, Y. S., Shen, G. L., & Yu, R. Q. (2008). *Talanta*, *74*, 831–835.