Anal Bioanal Chem (2007) 387:1553–1559
DOI 10.1007/s00216-006-1064-3

ORIGINAL PAPER

Immobilization of hemoglobin on SBA-15 applied to the electrocatalytic reduction of H_2O_2

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Received: 4 October 2006 / Revised: 27 November 2006 / Accepted: 30 November 2006 / Published online: 3 January 2007

Abstract The direct electron transfer between hemoglobin (Hb) and an electrode was realized by first immobilizing the protein onto SBA-15.The results of the immobilization showed that the adsorption was pH-dependent with a maximum adsorption near the isoelectric point of the protein, and SBA-15 with a larger pore diameter showed greater adsorption capacity for Hb. UV–vis spectroscopy and nitrogen adsorption analysis indicated that Hb was adsorbed within the channel of SBA-15 and no significant denaturation occurred to the protein. The Hb/SBA-15 composite obtained was used for the fabrication of a Hb biosensor to detect hydrogen peroxide. A pair of well-defined redox peaks at −0.337 and −0.370 V on the Hb/SBA-15 composite modified glassy carbon electrode was observed, and the electrode reactions showed a surface-controlled process with a single proton transfer at a scan rate range from 20 to 1,000 mV/s. The sensor showed a fast amperometric response, a low detection limit (2.3×10^{-9} M) and good stability for the detection of H_2O_2. The electrochemical results indicated that the immobilized Hb still retained its biological activity.

Keywords Hemoglobin · Immobilization · SBA-15 · Direct electron transfer · Biosensor

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Introduction

Hemoglobin (Hb) is a soft globular heme protein which has four electroactive iron hemes, a molar mass of approximately 64,500 g/mol and a protein dimension of 5.3 nm × 5.4 nm × 6.5 nm [1]. The isoelectric point (pI) of Hb is about 6.8–7.0 [2]. Hb has important biochemical functions, such as in electron transport, dioxygen transport, storage and dioxygen-related chemical transformations [3]. Since heme proteins are important in living systems, it is necessary to know their biological activity. Hb can be used as a model molecule for the study of the direct electron transfer between heme proteins and an electrode because of its commercial availability and relatively well known structure; however, it is difficult to do this owing to its high molar mass and large protein size. Up to now, great efforts have been carried out to realize the direct electron transfer of Hb [4–12].

The appearance of mesoporous materials such as M41S (Mobil composite of matter) [13] has created new possibilities for immobilizing proteins and other macromolecules. Mesoporous materials usually possess high specific surface areas and pore volumes, and well-ordered pore structures. Many papers have been published on the utilization of these materials [14–17]. However, the pore diameter of M41S is relatively small (in the range 2–10 nm), which limits the immobilization of macromolecules with large dimensions [14, 17]. Compared with M41S, SBA-15 (Santa Barbara) has larger pore diameters [18, 19]. The great increase in the pore size (up to 30 nm) achieved by the different synthesis process makes SBA-15 quite suitable for immobilizing macromolecules [20–24].

In cyclic voltammetry and amperometry experiments, the protein and the carrier were usually mixed together and then immobilized on the electrode [8, 25]. Nevertheless, it

may be hard to realize the whole activity of the proteins by such a method. However, by immobilization, proteins can retain their activities better than their free state [14, 15]. This is because immobilization on solid supports permits highly selective catalysis to be performed for enzymes using materials that are chemically and mechanically robust and readily separated from reaction mixtures [14, 15, 26]. Besides, the stability of enzymes may also be enhanced as a result of separation of enzyme molecules adsorbed on the surface of supports.

The electrochemical properties of heme proteins on mesoporous silica were investigated by Dai et al. [27–29]. HMS with different pore sizes was used for the direct electron transfer between proteins and an electrode. In the present work, the influence of both the pH values of the Hb solution and the pore diameters of SBA-15 on adsorption of Hb was studied. The resulting Hb/SBA-15 composite was used to fabricate a Hb sensor to detect hydrogen peroxide. Direct electron transfer between Hb and a glassy carbon electrode (GCE) was realized. The immobilized Hb exhibited a pair of well-defined peaks at −0.337 and −0.370 V. This sensor displayed fast amperometric response, a low detection limit and good stability for the detection of H_2O_2. All the results suggested that the immobilization provided a desirable way to retain its bioactivity.

Experimental

Materials

Bovine heart Hb was purchased from Sigma and was used without further purification. Copolymer poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol) (Pluronic P123, molecular weight 5,800, $EO_{20}PO_{70}EO_{20}$) was obtained from Aldrich, and the silica source tetraethyl orthosilicate (TEOS) was from Shanghai Chemical Reagent Co.

Sample preparations

The synthesis of SBA-15 with various pore diameters was performed as described in the literature [18, 19]. In a typical process, 4.0 g of P123 was dissolved in 30 g of water and 120 g of 2 M HCl solution with stirring at 35 °C. Then, 8.5 g of TEOS was added to the aforementioned solution with stirring at 35 °C for another 24 h. The mixture was then transferred to an autoclave and heated to 100 or 150 °C and aged for 24 h. The solid product was recovered by filtration, rinsed with deionized water and dried at room temperature. Calcination was carried out in an air atmosphere at 550 °C for 6 h with a heating rate of 1 °C/min to remove the template. The final product was recorded as SBA-15-X (X is 100 or 150, the temperature used in the synthesis in degrees Celsius).

Instrumentation

Powder X-ray diffraction patterns were obtained using an ARL X'TRA X-ray diffractometer using Cu Kα radiation. Nitrogen adsorption and desorption isotherms were measured at −196 °C with a Micromeritics ASAP 2020 analyzer. The specific surface area was determined using the standard Brunauer–Emmett–Teller method, while the pore size distribution was calculated by the Barrett–Joyner–Halenda (BJH) method. UV–vis absorption spectra were recorded using a Shimadzu UV-2401PC spectrophotometer.

Adsorption of Hb

A series of standard Hb solutions with the concentration ranging from 0.2 to 4.0 μM were prepared by dissolving different amounts of Hb in phosphate buffer solution (PBS) with different pH values (pH 5.2, 7.2 and 9.2 respectively). In each adsorption experiment, 50 mg of SBA-15-X was put into 10 mL of Hb solution. After stirring for 1 h at 25 °C, the mixture was separated by centrifugation. The amount of Hb adsorbed onto SBA-15 was determined by UV absorption at 406 nm of the supernatant liquid before and after Hb adsorption.

Preparation of silica sol and fabrication of the biosensor

Silica sol was prepared according to the method given in literature [30] by mixing 600 μL ethanol, 50 μL TEOS, 10 μL of 5 mM NaOH and 60 μL H_2O in a small test tube at room temperature. After sonicating, this sol was formed and stored at 4 °C.

The GCE was first polished with 1.0-, 0.3- and 0.05-μm alumina powder successively, followed by rinsing thoroughly with doubly distilled water. The polished electrode was then sonicated in acetone and doubly distilled water and finally allowed to dry at room temperature. The Hb/SBA-15 composite immobilized under the optimal conditions was selected to check the bioactivity of protein. The resulting composite was resuspended in 0.5 mL water and 10 μL of this suspension was deposited onto the electrode surface. The electrode was then left to dry at room temperature. Then 5 μL silica sol was added to encapsulate the composite. The electrode was then left to dry and stored for at least 24 h at 4 °C. The biosensor was stored under the same conditions when not in use.

Cyclic voltammetry and amperometry experiments were performed using a CHI660B workstation (Shanghai Chenhua, Shanghai). All experiments were carried out using a conventional three-electrode system, where the

GCE modified with Hb/SBA-15 as the working electrode, a platinum wire as the auxiliary electrode and a saturated calomel electrode as the reference electrode. All solutions were deoxygenated by highly pure nitrogen before and during the measurements.

Results and discussion

Instrumentation

All X-ray diffraction patterns and nitrogen adsorption isotherms of the as-prepared materials after calcination at 550 °C were in accord with the reported data [18, 19]. Textural properties of all the samples before and after adsorption based on the nitrogen adsorption are collected in Table 1. The data given in Table 1 show that the Hb molecules were adsorbed within the channels of SBA-15. The Hb molecules would also have been adsorbed onto the outer surface area of SBA-15 during the adsorption process. For the mesoporous materials, the inner surface accounts for most of the total surface area. There would be few Hb molecules on the outer surface and they would fall off during centrifugation and would not interfere with the electrochemical properties of Hb.

Influence of pH values and pore size on the adsorption

The influence of pH values on the adsorption of Hb is shown in Fig. 1 by using SBA-15-150 as the adsorbent. It can be seen that the amount of Hb adsorbed onto SBA-15 first increased from pH 5.2 to 7.2, and then decreased from pH 7.2 to 9.2. The maximum amount of Hb absorbed (3.9 μmol/g) appears at pH 7.2, near its pI. The result was similar to that in [24]. The protein is positively charged at a pH below the pI and negatively charged at a pH above the pI. The pI of the silica surface of mesoporous materials is around 3.60 [16]; hence, the adsorbent surface is negatively charged at all the pH values studied here. From pH 5.2 to 7.2, the main driving force for the adsorption of Hb included the Coulombic repulsion between Hb molecules and electrostatic attraction existed in Hb and the silanol groups of the adsorbent. With the increase of pH, the positive charge of Hb was reduced and thus the repulsion between Hb molecules decreased. Although the electrostatic attraction between Hb and the silica surface should be reduced correspondingly, the protein molecules occupied less space than at lower pH, which implied SBA-15 could hold more protein molecules.

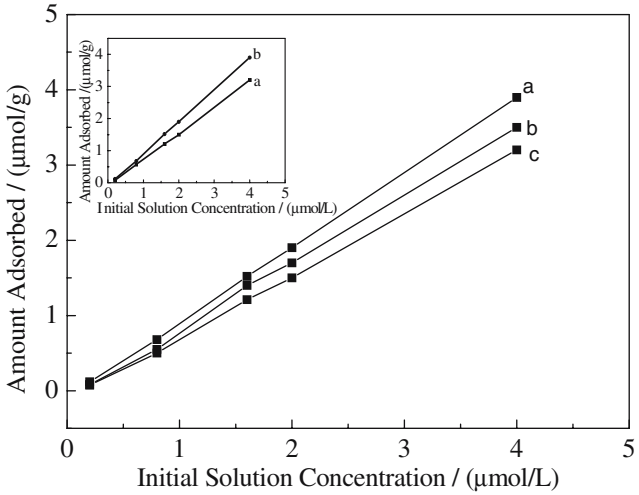

Fig. 1 Adsorption isotherms of hemoglobin (*Hb*) on SBA-15-150 at different pH: **a** pH 7.2; **b** pH 5.2; **c** pH 9.2. *Inset*: Effect of pore diameter on adsorption (pH 7.2): **a** SBA-15-100; **b** SBA-15-150

When the pH was close to the pI of Hb, the net charge of Hb was almost zero; hence, the Coulombic repulsive force between the protein molecules was minimal and the space that a Hb molecule occupied was also the least, resulting in the maximum adsorption capacity of the material for Hb.

The surface of Hb was negatively charged when the pH of the solution was above the pI. The main acting force for the adsorption of Hb included the Coulombic repulsion between the negatively charged Hb molecules and the electrostatic repulsion between Hb molecules and negatively charged silanol groups of the adsorbent, leading to the decrease of adsorption of Hb on SBA-15.

The effect of pore diameter was also investigated. The inset in Fig. 1 shows the adsorption isotherms of SBA-15 with different pore diameters at pH 7.2. The result reveals that SBA-15 with a larger pore diameter tends to immobilize more protein. This may be due to the existence of micropores in the material [31–33]. SBA-15 synthesized at lower

Table 1 Specific surface area and specific pore volume of SBA-15 before and after hemoglobin adsorption at pH 7.2

Sample	Micropore area (m^2/g)	Before adsorption			After adsorption (C_{Hb}=4.0 μM)		
		Pore diameter[a] (nm)	A_{BET}[a] (m^2/g)	V_p (cm^3/g)	Pore diameter[a] (nm)	A_{BET}[a] (m^2/g)	V_p (cm^3/g)
SBA-15-100	112	6.3	776	1.17	5.7	457	0.56
SBA-15-150	45	10.6	423	1.13	6.5	302	0.54

V_p total pore volume
[a] Calculated from the adsorption branch

temperature has more micropores and a larger micropore area (Table 1). The decrease in surface area for SBA-15-100 and SBA-15-150 after adsorption was 319 and 121 m^2/g and the maximum amount of Hb immobilized was 3.2 and 3.9 μmol/g for the two materials. From these data, the specific surface capacity for SBA-15-100 and SBA-15-150 was calculated to be 0.01 and 0.032 μmol/m^2, respectively. So it can be concluded that the higher percentage of micropores in the surface in SBA-15-100 than in SBA-15-150 led to the lower capacity of SBA-15-100, since the micropores could not immobilize Hb as well because of their small pore diameters. As shown in Table 1, the average adsorption pore diameter of two samples calculated by using the BJH method was 10.6 and 6.3 nm, respectively. However, the pore size distribution of SBA-15-100 showed that the maximum primary mesopore (main channel) diameter was centered at 7.6 nm, which more than 6.3 nm (not shown). The reason for the difference between the two pore diameters was that 6.3 nm was the average pore size of the mesoporous and microporous pores, and the pore diameter of the microporous pores was much smaller that of the mesoporous pores. This explained why SBA-15-100 could also immobilize Hb. The direct electrochemistry of heme proteins on another hexagonal mesoporous silica HMS was investigated by Dai et al. [27–29], and the effect of pore size on adsorption of protein was the same as we found here. Though HMS and MCM-41 (one type of M41S) possess structures similar to that of SBA-15, their pore sizes were much smaller, so it was impossible to immobilize Hb in their channels. Immobilization of proteins on the outer surface of mesoporous materials was not stable, so MCM-41 was not suitable for the direct electron transfer between Hb and an electrode.

UV–vis absorption spectroscopic analysis

UV–vis spectroscopy is a useful tool for monitoring the possible change of the Soret absorption band in the heme group region [34]. The band shift may provide information on the possible denaturation of heme protein, particularly conformational change. Figure 2 shows the UV–vis spectra of SBA-15, Hb/SBA-15 and Hb in 0.1 M pH 7.0 PBS, respectively. It can be seen that both free Hb and immobilized Hb showed a maximum absorbance at 406 nm. The absorption peak was attributed to the Soret band of Hb, suggesting no significant denaturation occurred to the protein after it had been immobilized on SBA-15 [35].

Direct electron transfer of the Hb/SBA-15 composite modified electrode

The cyclic voltammograms (CVs) of different electrodes are given in Fig. 3. No peak appeared at the SBA-15-

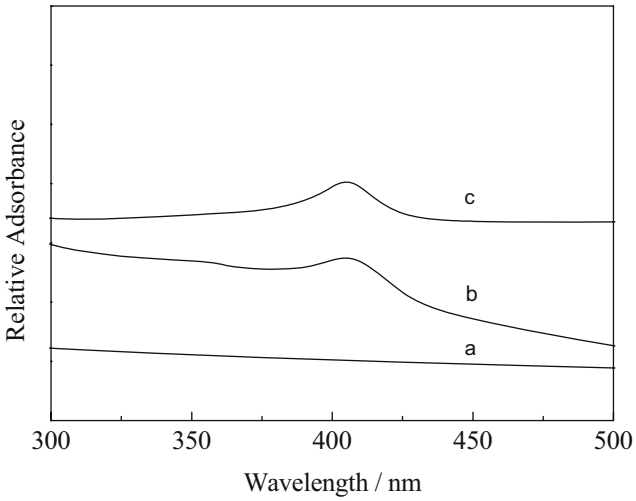

Fig. 2 UV-vis spectra of **a** SBA-15 and **b** Hb/SBA-15 on a quartz slide; **c** free Hb in pH 7.0 phosphate buffer solution (*PBS*)

modified electrode, which indicated that SBA-15 was not electroactive. When the electrode was modified with Hb (the electrode was prepared by directly dropping a 4 μM Hb solution onto the surface of the electrode), only an irreversible and small reduction peak was observed. A couple of well-defined redox peaks were observed at the Hb/SBA-15 composite modified electrode at −0.370 and −0.337 V, which contribute to the Fe(III)/Fe(II) center of the immobilized Hb, suggesting that the immobilization of Hb on SBA-15 may have a more favorable orientation and facilitate the direct electron transfer between Hb and the electrode.

The CVs of the Hb/SBA-15 modified electrode display a well-defined peak shape at different scan rates (Fig. 4). Obviously, both the anodic and cathodic peak currents of the immobilized Hb increased linearly with the increase of

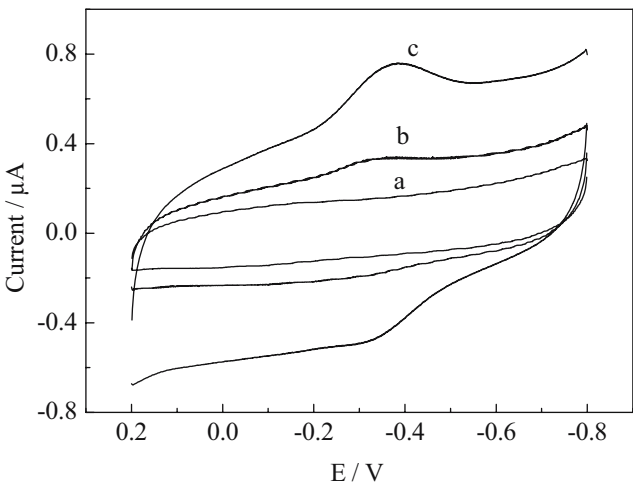

Fig. 3 Cyclic voltammograms of **a** SBA-15/glassy carbon electrode (*GCE*), **b** Hb/GCE and **c** Hb/SBA-15/GCE in pH 7.0 PBS at 100 mV/s

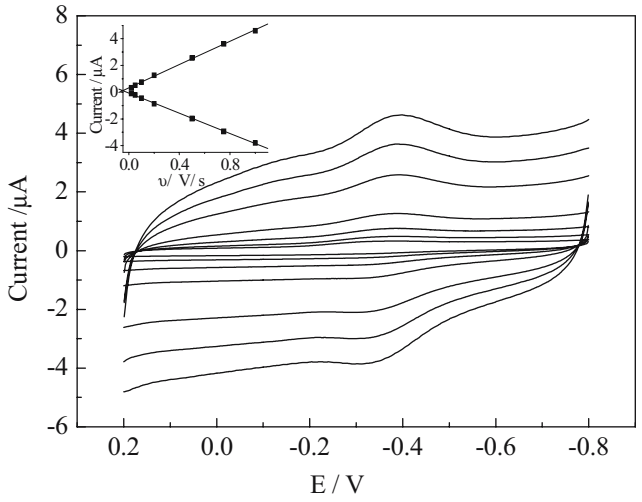

Fig. 4 Effect of scan rate on the cycle voltammograms of Hb/SBA-15/GCE in pH 7.0 PBS; the scan rate was 20, 50, 100, 200, 500, 750 and 1,000 mV/s (from *inner* to *outer*). *Inset*: Peak current vs. scan rate

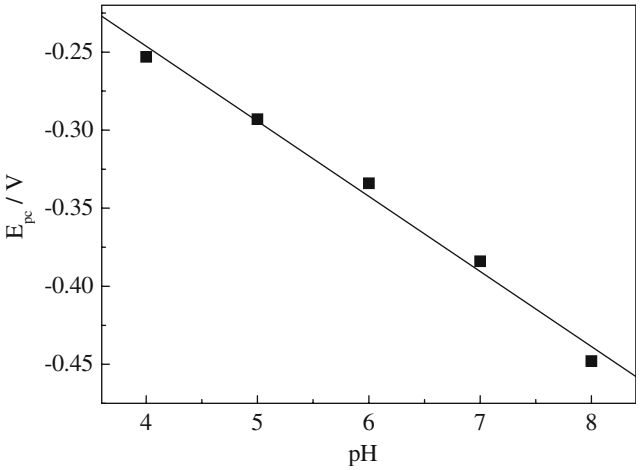

Fig. 5 Effect of pH on the formal potential of Hb/SBA-15/GCE in 0.1 M PBS

the scan rate (Fig. 4, inset), indicating that the electrode process is surface-controlled.

Effect of solution pH on direct electron transfer of Hb

In most cases the electrochemical behavior of proteins is highly dependent on the pH of the solution. In this research, CVs of the Hb/SBA-15 composite modified electrode showed a strong dependence on the solution pH (Fig. 5). All the changes in the peak potential and current caused by pH (from 4 to 8) were reversible. For example, the CVs for the Hb/SBA-15 composite modified electrode at pH 7.0 were reproduced after the Hb/SBA-15 composite modified electrode had been immersed in pH 8.0 buffer and then returned to the pH 7.0 buffer. $E^{0\prime}$ of the heme Fe^{III}/Fe^{II} redox couple for the Hb/SBA-15 electrodes showed a linear relationship with pH in the range 4.0–8.0, with a slope of −48.1 mV/pH. This value was a little smaller than that of −57.8 mV/pH at 18 °C for a reversible one-proton-coupled single-electron transfer during electrochemical reduction [36, 37], and this may be due to the effect of the protonation states of ligands *trans* to the heme iron and amino acids around the heme or to the protonation of the water molecules coordinated to the center [37]. Thus, the electrochemical reduction reaction of Hb may be described as follows [38]:

$$\text{Hb heme Fe(III)} + H^+ + e^- = \text{Hb heme Fe(II)}$$

Electrocatalytic reduction of H_2O_2 by the Hb/SBA-15 composite modified electrode

On the basis of the excellent electrochemical behavior of the Hb/SBA-15 composite, it was immobilized on the surface of the GCE and used to construct a sensor. The CVs of the Hb/SBA-15 and SBA-15 modified electrodes in pH 7.0 PBS before and after the addition of H_2O_2 are shown in Fig. 6. Both the reduction and the oxidation current for the Hb/SBA-15 composite modified GCE increased with the addition of H_2O_2. However, no current was observed on the SBA-15 modified electrode, indicating that the Hb/SBA-15 composite can act as a catalyst for the reduction of H_2O_2. Under optimal conditions, the electrode modified with the Hb/SBA-15 composite displays an amperometric response to H_2O_2 with linear ranges from 1×10^{-8} to 1×10^{-7} M (Fig. 6, inset) and a detection limit of 2.3×10^{-9} M at a signal-to-noise ratio of 3. The linear regression equation was $y=0.091x+0.0272$. From the slope of 0.091 μM/μA, the sensitivity of the proposed Hb sensor

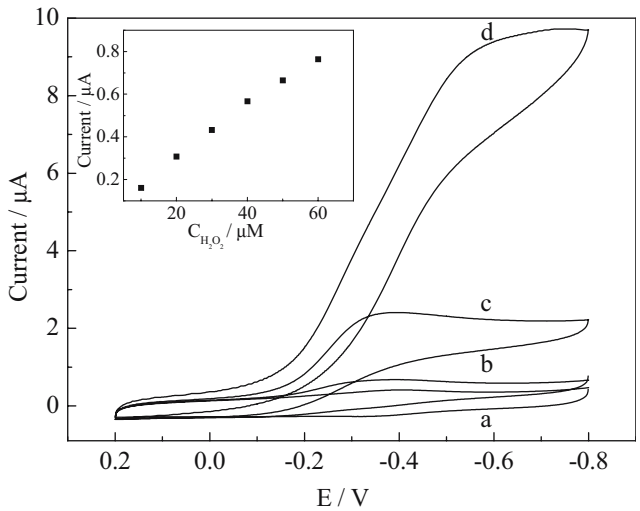

Fig. 6 Cyclic voltammograms of **a** SBA-15 with 5×10^{-4} M H_2O_2 and Hb/SBA-15/GCE in pH 7.0 PBS with different H_2O_2 concentration at 100 mV/s; **b** 5×10^{-5} M; **c** 5×10^{-4} M; **d** 1×10^{-3} M. *Inset*: The linear fitting program of the cathodic peak current of Hb for H_2O_2

was calculated to be 1.29 A/M/cm^2, which was larger than that of 0.74 [8], 0.243 [34] and 0.6 A/M/cm^2 [40]. Compared with the results obtained by others [41, 42], the present method of immobilization has improved sensitivity but a narrower concentration range.

The apparent Michaelis-Menten constant $\left(K_m^{app}\right)$ was calculated to evaluate the catalytic activity of immobilized protein by the Lineweaver–Burk equation [43]:

$$1/I_{ss}=1/I_{max}+K_m^{app}/I_{max}C,$$

where I_{ss} is the steady current after the addition of substrate (which can be obtained from amperometric experiments), C is the bulk concentration of the substrate and I_{max} is the maximum current measured under the saturated substrate condition. The apparent Michaelis–Menten constant K_m^{app}, which gives an indication of the enzyme–substrate kinetics, was determined to be 1.85 μM for the Hb/SBA-15 composite modified electrode. The value was much smaller than that previously reported [4, 27, 40, 44, 45]. This could be ascribed to the fact that Hb and SBA-15 were not simply put together but were combined by a first immobilization method. The Hb molecules were more tightly adsorbed onto the SiOH groups of SBA-15 in this way than those reported [27–29]; hence, the stability of the protein was greatly improved. This provided a relative favorable orientation and was beneficial for the realization of the whole activity of the Hb. All this suggested that there was a large catalytic activity of Hb immobilized on SBA-15 toward H_2O_2. Direct electrochemistry of Hb was observed at a carbon nanotube interface by Zhao et al. [45]. The Michaelis–Menten constant was calculated to be 675 μM in their work, which is much larger than the value obtained in our research and meant the affinity to H_2O_2 was better in our work. Besides, the method used here was relatively simpler and the sensitivity we achieved was higher than that of Zhao et al. [45].

Additional experiments were carried out to test the reproducibility and stability. No obvious change was found after the Hb/SBA-15 modified electrode was immersed in PBS and stored in a refrigerator at 4 °C for 20 h and when the biosensor was stored in a dry state at 4 °C for 20 days. The relative standard deviation of the peak current in six successive determinations at a H_2O_2 concentration of 0.03 μM was 3.62% for the Hb/SBA-15 modified GCE.

Conclusions

Direct electron transfer between Hb and an electrode was realized by a first immobilization method. It was found that the immobilization of Hb on SBA-15 was significantly affected by the pH of Hb solution and the pore diameter of SBA-15. The maximum loading of Hb was achieved near its pI. As for the pore diameter, the result showed that SBA-15 with a larger pore diameter tended to load more protein. By immobilization, the direct electron transfer between Hb and the electrode was strengthened. The sensor constructed using the Hb/SBA-15 composite obtained showed a high response sensitivity and a lower detection limit for the detection of H_2O_2. So such a method of immobilization has provided a convenient way to fabricate new protein biosensors.

Acknowledgements This work was supported by the National Natural Science Foundation of China (grant nos. 20635020, 20575026 and 90606016), the National Basic Research Program of China (grant no. 2003CB615804), Jiangsu National Natural Science Foundation (BK2006114) and the Modern Analysis Center of Nanjing University.

References

1. Peng S, Gao Q, Wang Q, Shi J (2004) Chem Mater 16:2675–2684
2. Kondo A, Fukuda H (1998) J Colloid Interface Sci 198:34–41
3. Gu HY, Yu AM, Chen HY (2001) J Electroanal Chem 516:119–126
4. Ma X, Sun Z, Zheng X, Li G (2006) J Anal Chem 6:669–672
5. Yang W, Bai Y, Li Y, Sun C (2005) Anal Bioanal Chem 382:44–50
6. Nadzhafova OY, Zaitsev VN, Drozdova MV, Vaze A, Rusling JF (2004) Electrochem Commun 6:205–209
7. Jia N, Wang L, Liu L, Zhou Q, Jiang Z (2005) Electrochem Commun 7:349–354
8. Liu S, Dai Z, Chen HY, Ju H (2004) Biosens Bioelectron 19:963–969
9. Feng J-J, Zhao G, Xu J-J, Chen H-Y (2005) Anal Biochem 342:280–286
10. Liu H-H, Wan Y-Q, Zou G-L (2006) Anal Bioanal Chem 385:1470–1476
11. Liu XJ, Ma X, Li GX (2005) Sens Actuators B 106:284–288
12. Tao WY, Pan DW, Liu YJ, Nie LH, Yao SZ (2005) Anal Biochem 338:332–340
13. Kresge CT, Leonowicz ME, Roth WJ, Vartulli JC, Beck JS (1992) Nature 359:710–712
14. Díaz JF, Balkus KJ (1996) J Mol Catal B 2:115–126
15. Takahashi H, Li B, Sasaki T, Miyazaki C, Kajino T, Inagaki S (2001) Micro Meso Mater 44–45:755–762
16. Deere J, Magner E, Wall JG, Hodnett BK (2002) J Phys Chem B 106:7340–7347
17. Jenny MK, Antje D, Geoffrey WS, Andrea JO (2001) Micro Meso Mater 44–45:769–774
18. Zhao D, Feng J, Huo Q, Melosh N, Fredrickson GH, Chmelka BF, Stucky GD (1998) Science 279:548–552
19. Zhao D, Huo Q, Feng J, Bradley FC, Galen DS (1998) J Am Chem Soc 120:6024–6036
20. Humphrey HPY, Wright PA, Botting NP (2001) Micro Meso Mater 44–45:763–768
21. Lei J, Fan J, Yu C, Zhang L, Jiang S, Tu B, Zhao D (2004) Micro Meso Mater 73:121–128
22. Hudson S, Magner E, Cooney J, Hodnett BK (2005) J Phys Chem B 109:19496–19506
23. Chong ASM, Zhao XS (2004) Appl Surf Sci 237:398–404
24. Vinu A, Murugesan V, Hartmann M (2004) J Phys Chem B 108:7323–330

25. Zhou LH, Xian YZ, Zhou YY, Hu J, Liu HL (2005) Acta Chim Sin 63:2117–2120
26. Tischer W, Kasche V (1999) Trends Biotechnol 17:326–335
27. Dai Z, Liu S, Ju H, Chen H (2004) Biosens Bioelectron 19:861–867
28. Dai Z, Xu X, Ju H (2004) Anal Biochem 332:23–31
29. Dai Z, Ju H, Chen H (2005) Electroanalysis 17:862–868
30. Wang QL, Lu GX, Yang BJ (2004) Biosens Bioelectron 19:1269–1275
31. Ryoo R, Ko CH, Kruk M, Antochshuk V, Jaroniec M (2000) J Phys Chem B 104:11465–11471
32. Hoang VT, Huang Q, Eić M, Do TO, Kaliaguine S (2005) Langmuir 21:2051–2057
33. Kruk M, Jaroniec M, Ko CH, Ryoo R (2000) Chem Mater 12:1961–1968
34. Theorell H, Ehrenberg A (1951) Acta Chem Scand 5:823–848
35. Nassa A-EF, Willis WS, Rusling JF (1995) Anal Chem 67:2386–2392
36. Nassar AE, Zhang Z, Hu N, Rusling JF, Kumosinski TF (1997) J Phys Chem B 101:2224–2231
37. Huang H, He P, Hu N, Zeng Y (2003) Bioelectrochemistry 61:29–38
38. Trushina E, Oda R, Landers J, Mcmurray C (1997) Electrophoresis 18:1890–1898
39. Ricci F, Amine A, Palleschi G, Moscone D (2003) Biosens Bioelectron 18:165–174
40. Karyakin AA, Karyakina EE, Gorton L (2000) Anal Chem 72:1720–1723
41. Feng J-J, Xu J-J, Chen H-Y (2006) Electrochem Commun 8:77–82
42. Yu J, Ju H (2003) Anal Chim Acta 486:209–216
43. Kamin RA, Wilson GS (1980) Anal Chem 52:1198–1205
44. Fan C, Wang H, Sun S, Zhu D, Wagner G, Li G (2001) Anal Chem 73:2850–2854
45. Zhao YD, Bi YH, Zhang WD, Luo QM (2005) Talanta 65:489–494