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Direct electron transfer and enzymatic activity of hemoglobin in a hexagonal mesoporous silica matrix

Zhihui Dai, Songqin Liu, Huangxian Ju*, Hongyuan Chen

Department of Chemistry, Institute of Analytical Science, State Key Laboratory of Coordination Chemistry, Nanjing University, Nanjing 210093, PR China

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Abstract

The direct electrochemistry of hemoglobin (Hb) immobilized on a hexagonal mesoporous silica (HMS)-modified glassy carbon electrode was described. The interaction between Hb and the HMS was investigated using UV-Vis spectroscopy, FT-IR, and electrochemical methods. The direct electron transfer of the immobilized Hb exhibited two couples of redox peaks with the formal potentials of −0.037 and −0.232 V in 0.1 M (pH 7.0) PBS, respectively, which corresponded to its two immobilized states. The electrode reactions showed a surface-controlled process with a single proton transfer at the scan rate range from 20 to 200 mV/s. The immobilized Hb retained its biological activity well and displayed an excellent response to the reduction of both hydrogen peroxide (H_2O_2) and nitrate (NO_2^-). Its apparent Michaelis–Menten constants for H_2O_2 and NO_2^- were 12.3 and 49.3 μM, respectively, showing a good affinity. Based on the immobilization of Hb on the HMS and its direct electrochemistry, two novel biosensors for H_2O_2 and NO_2^- were presented. Under optimal conditions, the sensors could be used for the determination of H_2O_2 ranging from 0.4 to 6.0 μM and NO_2^- ranging from 0.2 to 3.8 μM. The detection limits were 1.86×10^{-9} M and 6.11×10^{-7} M at 3σ, respectively. HMS provided a good matrix for protein immobilization and biosensor preparation.

Keywords: Biosensors; Hemoglobin; Hexagonal mesoporous silica; Glassy carbon electrode; Direct electron transfer; Hydrogen peroxide; Nitrate

1. Introduction

Hemoglobin (Hb), an important respiratory protein in red cells, has a molecular weight of approximately 64,500 and consists of four polypeptide chains, each with one electroactive iron heme group (Weissbluth, 1974). It is an ideal model molecule for the study of electron transfer reactions of heme proteins and also for biosensing and electrocatalysis (Ye and Baldwin, 1988). Facilitation of the electron transfer between Hb and electrode is very challenging since Hb has an extended three-dimensional structure and the inaccessibility of the electroactive center. Great efforts have been made to enhance its electron transfer by using mediators, promoters or some special electrode materials (Ciureanu et al., 1998; Chen et al., 1994, 1999; Fan et al., 2001; Han et al., 2002; Lu and Dong, 1990; Nassar and Rusling, 1996; Rusling, 1998; Sun et al., 2000; Yang et al., 1999; Ye and Baldwin, 1988). Among these works, incorporating Hb in an organic membrane was a main method. The direct electron transfer has been achieved by incorporating Hb in polyacylamide hydrogel (Sun et al., 2000), didodecyldimethylammonium bromide (Ciureanu et al., 1998), SP Sephadex (Fan et al., 2001), DNA (Nassar and Rusling, 1996), Nation (Huang et al., 1996), biomembrane-like dimyristoyl phosphatidylcholine (Yang et al., 1999), poly(ester sulfonic acid) (Yang et al., 1999) and egg-phosphatidylcholine (Han et al., 2002).

In comparison with the organic membrane, inorganic materials are intrinsically more stable matrices because of their layered oxide structure. Recently, a series of inorganic porous materials, such as clay (Lei et al., 1999), montmorillonite (Fan et al., 2000), NaY molecular sieves (Liu et al., 1997; Liu et al., 1999), sol–gel (Yu and Ju, 2002) have been proven to be promising as the immobilization matrices due to their regular structure, good mechanical, thermal and chemical stability. As an enzyme immobilization matrix, molecular sieves can incorporate with enzymes through physical or chemical actions without employing the conventional cross-linkers. However, the porous diameter of molecular sieves is usually too small and it always contains

* Corresponding author. Tel.: +86-25-3593593; fax: +86-25-3593593.
E-mail address: hxju@nju.edu.cn (H. Ju).

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alumina. For example, NaY is a microporous molecular sieves with a silica–alumina molar ratio of 1.5–3 (Rolison, 1990). Both vacancy of its pores and the surface can adsorb water easily due to the presence of alumina, which disadvantages the immobilization of enzymes. In order to improve the analytical performance of biosensors based on the immobilization of enzymes on the molecular sieves, we here use hexagonal mesoporous silica (HMS) for the first time to immobilize the protein for the preparation of biosensor. Hexagonal mesoporous silica, firstly synthesized by Tanev (Tanev et al., 1994), has a defined crystalline structure with the pores of nanoscale dimension, which can be tailored through the choice of surfactant, auxiliary chemicals and reaction conditions (Kresge et al., 1992). Many investigators have explored the suitability of this material for potential applications due to its large specific surface area, high porosity, hydrophobic and controllable pore size (Corma, 1997; Davis, 2002; Kageyama et al., 1999; Mal et al., 2003; Stein et al., 2000; Ying et al., 1999). To our knowledge, however, the immobilization of protein or enzyme on such molecular sieves-modified surface has not been reported in connection with amperometric biosensors.

In this work, Hb is incorporated into HMS to modify a glassy carbon electrode. The immobilized Hb shows an enhanced electron transfer for its heme Fe(III) to Fe(II) redox couple. The very simple process constructs two high-performance biosensors. The results obtained from FT-IR, UV-Vis spectroscopy and CV show an interaction between the Hb and HMS. The modified molecular sieves provides a desirable microenvironment to retain the bioactivity of Hb. The prepared sensor has been used for the determinations of H_2O_2 and NO_2^-.

2. Experimental

2.1. Reagents

Hb (bovine blood) was purchased from Sigma and used as received. Hydrogen peroxide (30% (w/v) solution) was purchased from Shanghai Biochemical Regent Co. and $NaNO_2$ from Nanjing Chemical Reagent Factory (China). The concentrations of more diluted hydrogen peroxide solutions were determined by titration with cerium(IV) to a ferroin end point (Hurdis et al., 1954). Polyvinyl alcohol (PVA, average degree of polymerization: 1800 ± 100) was purchased from Shanghai Laize Factory of Fine Chemicals. Octodecylamine (ODA) was obtained from Shanghai Chem. Co. and tetraethylorthosilicate (TEOS) from Beijing Chem. Co. All other chemicals were of analytical grade and used without further purification. Phosphate buffer solutions (PBS, 0.1 M) with various pH values were prepared by mixing stock standard solutions of K_2HPO_4 and KH_2PO_4 and adjusting the pH with H_3PO_4 or NaOH. All the solutions were prepared with doubly distilled water.

Table 1
Pore characterization of HMS

A_{BET} (m^2/g)	V_{total} (cm^3/g)	a_0 (nm)	D (nm)	L (nm)
718	0.86	6.54	4.04	2.50

A_{BET}: total specific surface area; V_{total}: total pore volume; a_0: lattice parameter; D: pore parameter; and L: wall thickness.

2.2. Preparation of HMS and its colloidal solution

HMS was prepared following a recipe similar to that reported by Tanev (Tanev et al., 1994). In brief, ODA was added to the mixture of water and ethanol (molar ratio, 36:6.5) and stirred until a homogeneous solution occurred at 50 °C. TEOS was added slowly to this solution under vigorously stirring followed by the further stirring for about 15 min and keeping it at 50 °C for 24 h. The final molar ratio was 1.0TEOS:0.27ODA:6.5EtOH:36H_2O. After the system was cooled to room temperature, a white solid was obtained. After the white solid was dried in air at room temperature, the organic molecules occluded in the mesopores were removed by direct calcination at 823 K for 6 h to produce HMS. The lattice parameter, total specific area, wall thickness, pore volume and diameter of the HMS, obtained using a Micromeritics ASAP 2000 instrument by the adsorption and desorption isotherms of N_2 and calculated according to the method of Barrett et al. (Barrett et al., 1951) from the adsorption branch, were listed in Table 1.

Thirty milligrams of HMS was dispersed into 10 ml water to obtain a suspension of 3 mg/ml HMS. The obtained suspension of 100 μl was then mixed with 5 μl 3% PVA solution of ethanol/water (1:1 (v/v)) to produce a HMS colloid that was used for the following work.

2.3. Electrode modification

The glassy carbon electrodes (GCE, 3 mm in diameter) were polished to a mirror-like finish with 1.0, 0.3, and 0.05 μm alumina slurry (Beuhler) followed by rinsing thoroughly with doubly distilled water. The electrodes were successively sonicated in 1:1 nitric acid, acetone and doubly distilled water, and then allowed to dry at room temperature. Two microliters 0.1 mM Hb (in 0.1 M pH 7.0 PBS) and 2 μl HMS colloidal solution were dropped on the pretreated GCE surface, respectively, and allowed to dry under ambient conditions for 3 h. The modified electrode was rinsed with doubly distilled water for twice or thrice and then immersed into 0.1 M pH 7.0 PBS until a stable electrochemical response of Hb was observed. When not in use, the obtained Hb/HMS electrode was stored in 0.1 M pH 7.0 PBS at 4 °C.

2.4. Apparatus and measurements

Cyclic voltammetric and amperometric measurements were performed on 270 electrochemistry system (EG&G,

USA). All electrochemical experiments were carried out in a cell containing 5.0 ml 0.1 M PBS at room temperature (20 ± 2 °C) and using a platinum wire as auxiliary, a saturated calomel electrode as reference and the Hb/HMS electrode as working electrode. All solutions were deoxygenated by bubbling highly pure nitrogen for at least 10 min and maintained under nitrogen atmosphere during the measurements. The amperometric experiments were carried out by applying a potential of −400 mV for H_2O_2 and −800 mV for $NaNO_2$ on a stirred cell at (20 ± 2 °C). The sensor responses were measured as the difference between total and residual currents. Fourier transform infrared (FT-IR) spectra were obtained on a NEXUS 670 (Nicolet) FT-IR instrument at room temperature. UV-Vis absorbance spectroscopy was performed using a UV-2201 spectrophotometer (Shimadzu, Kyoto, Japan).

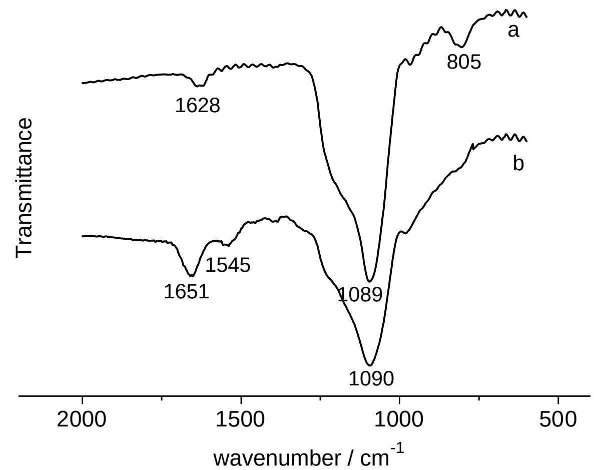

Fig. 2. FT-IR spectra of HMS (a) and Hb/HMS (b).

3. Results and discussions

3.1. Spectroscopic analysis of HMS and Hb/HMS

Fig. 1 shows the UV-Vis spectra of HMS, Hb and Hb/HMS, respectively. The Soret band, sensitive to variation of the microenvironment around the heme site, for the entrapped Hb is located at 408 nm, shifting only 2 nm toward the red in comparison with that of natural Hb in solution. This result suggests no significant denaturation occurred (George and Hanania, 1953; Nassar et al., 1995). The slight shift in the Soret band and the decrease in its absorbance indicate that the interaction of HMS with the Hb indeed existed due to the surface potential energy and adsorption properties of HMS particles (Lei and Deng, 1996; Lei et al., 1999). Such interaction did not destroy the structure and did not change the fundamental micro-environment of Hb.

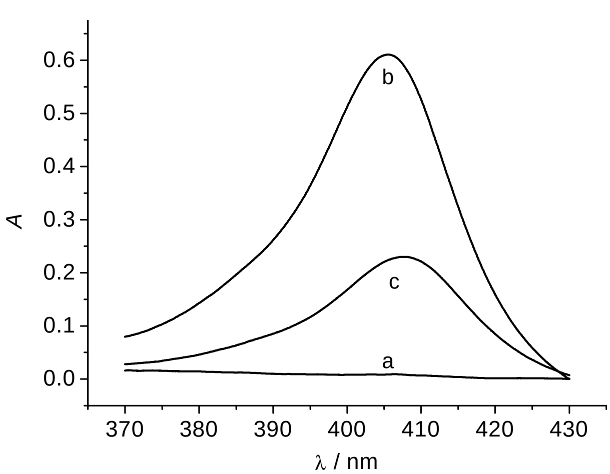

Fig. 1. UV-Vis spectra of 3.0 mg/ml HMS colloidal solution (a), 0.1 mM Hb in 0.1 M pH 7.0 PBS (b), and 0.1 M pH 7.0 PBS containing 3.0 mg/ml HMS colloid and 0.1 mM Hb (c).

The interaction between HMS and Hb could be demonstrated with the FT-IR spectra of HMS and Hb/HMS. It could be seen from Fig. 2 that the IR spectrum of HMS showed the adsorption bands at 1628, 1089, and 805 cm^{-1}. The peak at 1628 cm^{-1} was attributed to the vibration of adsorbed water and the others were from the frame symmetric and asymmetric flexible vibrations of Si groups (Anderson and Klinowski, 1986) in HMS. As is well known, the IR spectrum of Hb showed the adsorption bands at around 1650 and 1550 cm^{-1}, which were attribute to the shapes of the amides I and II infrared absorbance bands of Hb. They can provide the detailed information on the secondary structure of the polypeptide chain (Kauppinen et al., 1981). The amide I band (1700–1600 cm^{-1}) is caused by C=O stretching vibrations of peptide linkages in the protein backbone. The amide II band (1620–1500 cm^{-1}) results from a combination of N–H bending and C–N stretching. Upon adsorption of Hb on HMS, the band at 1628 cm^{-1} produced from adsorbed water was covered by the adsorption band of Hb at 1650 and 1550 cm^{-1}. The effect of Hb intercalation on pore size could be observed from the shift of frame vibration band (Liu et al., 1997). A slight shift of 1089 cm^{-1} absorbance band displayed most of Hb molecules did not enter the mesopores. A complete disappearance of the band at 805 cm^{-1} might result from the interaction between the Hb and some specific sites of HMS.

3.2. Direct electrochemistry of Hb/HMS-modified electrode

Fig. 3 shows the cyclic voltammograms of different electrodes in 0.1 M pH 7.0 PBS at 100 mV/s. No peak was observed at both GCE and the HMS-modified GCE, which showed HMS was electroinactive in the potential window. The Hb-modified GCE also showed the response of Hb, but there was only an irreversible reduction peak and the response was smaller than that of the Hb/HMS-modified

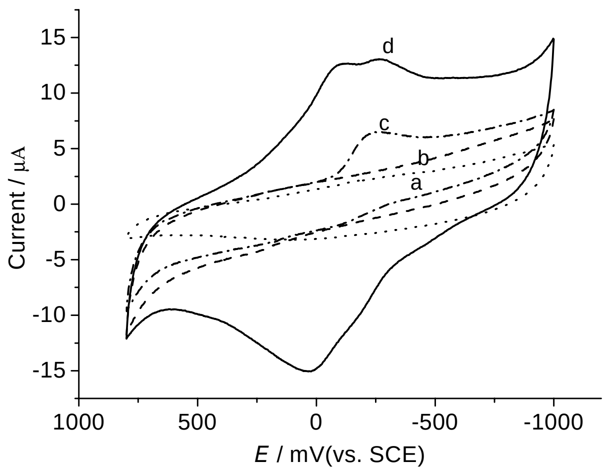

Fig. 3. Cyclic voltammograms of GCE (a), HMS-modified GCE (b), Hb-modified GCE (c) and Hb/HMS-modified GCE (d) in 0.1 M pH 7.0 PBS at 100 mV/s.

GCE. The Hb/HMS electrode exhibited two couples of stable redox peaks that were attributed to the redox of immobilized Hb. For the first couple, the anodic and cathodic peak potentials were at 22 and −96 mV, respectively, and for the other couple they were at −186 and −278 mV, respectively. The worse reversibility and smaller response of Hb-modified GCE than the Hb/HMS electrode indicated that the adsorption of Hb on HMS played an important role in facilitating the electron transfer between the electroactive center of Hb and GCE.

The two couples of redox peaks in Fig. 3d with the formal potentials of −0.037 and −0.232 V resulted from the adsorbed Hb and the intercalated Hb, respectively. The presence of many positive-charged acidic SiOH groups on the surface of HMS (Teraoka et al., 2001) made the oxidation of Hb more difficult thermodynamically. Thus, the adsorbed Hb showed a more positive formal potential. The limited space of the mesopores made the reduction of the electroactive center of the intercalated Hb more difficult, resulting in a more negative formal potential.

The effect of scan rate on the response of the immobilized Hb was shown in Fig. 4. With increasing scan rate, the redox peak currents of the Hb increased linearly, and the peak-to-peak separation also increased, indicating a surface-controlled process (Murray, 1984). From the integration of the reduction peaks of the Hb/HMS-modified GCE, the average surface coverage of Hb was calculated to be 1.27×10^{-9} mol/cm^2 that was much larger than that of monolayer coverage.

The small peak-to-peak separation indicated a fast electron transfer rate. The electron transfer rate constant k_s could be estimated with the formula $k_s = mnF\upsilon/RT$ when the peak-to-peak separation was less than 200 mV (Laviron, 1979), where m is a parameter related to the peak-to-peak separation. The peak-to-peak separation was 104, 118, 121 and 126 mV at 50, 100, 150, and 200 mV/s, respectively, producing an average k_s value of $0.92 \pm 0.18\,s^{-1}$ that was much larger than that of Hb immobilized on a Au colloid–cysteamine-modified gold electrode of $0.49\,s^{-1}$ (Gu et al., 2001). The fast electron transfer rate resulted from the strong interaction between Hb molecules and HMS. Thus, HMS can provide a microenvironment for Hb to undergo facile electron transfer reaction.

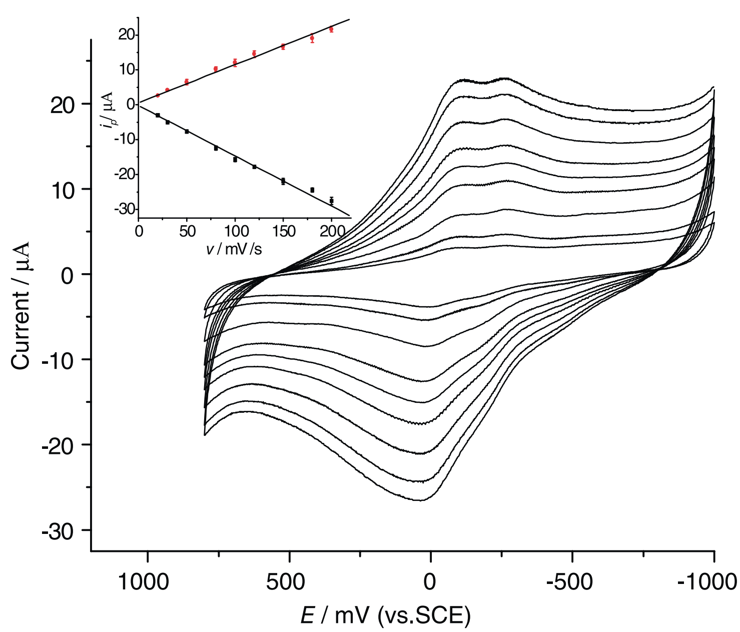

Fig. 4. Cyclic voltammograms of Hb/HMS-modified GCE in 0.1 M pH 7.0 PBS at 20, 30, 50, 80, 100, 120, 150, 180, and 200 mV/s. Inset: plot of peak current vs. scan rate.

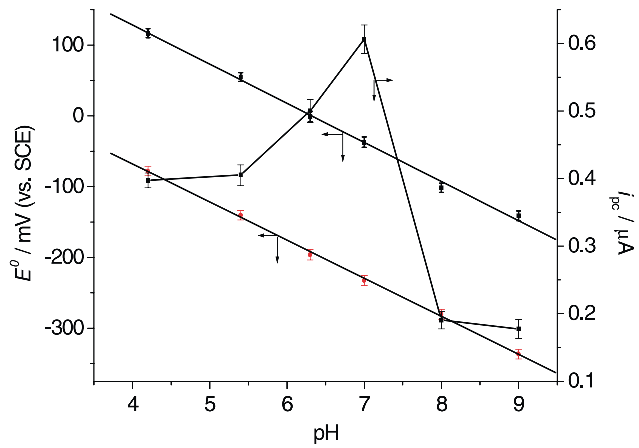

Fig. 5. Plots of formal peak potentials and cathodic peak current vs. pH.

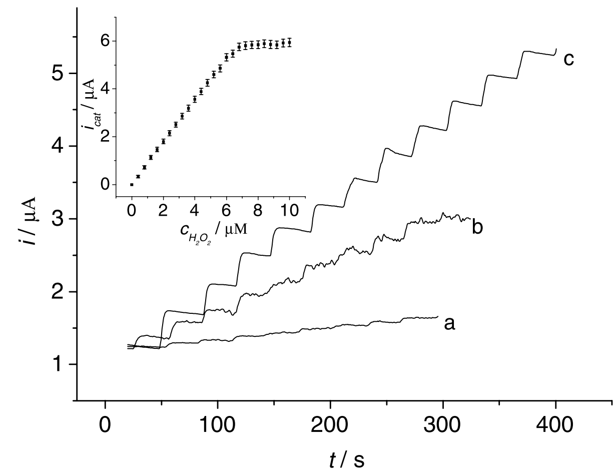

Fig. 6. Amperometric responses of HMS (a), Hb (b) Hb/HMS (c) modified GCEs at −400 mV upon successive additions of 5 μl 0.4 mM H_2O_2 to 5.0 ml 0.1 M pH 7.0 PBS. Inset: plot of catalytic current at Hb/HMS-modified GCE vs. H_2O_2 concentration.

3.3. Effect of solution pH on direct electron transfer of Hb

The direct electrochemistry of Hb immobilized on HMS showed a strong dependence on solution pH (Fig. 5). An increasing of solution pH caused a negative shift in both cathodic and anodic peak potentials, and the maximum peak currents of Hb occurred at pH 7.0. All changes in voltammetric peak potentials and currents with pH were reversible. For example, the cyclic voltammogram for the Hb/HMS-modified electrode at pH 9.0 was reproduced after immersion in pH 4.2 buffer and then returned to the pH 9.0 buffer. Plots of the formal potentials of the two couples of redox peaks versus pH (from 4.2 to 9.0) produced two lines with the slopes of –55.2 and –53.8 mV/pH, respectively, which was close to the expected value of –58.0 mV/pH for a single proton transfer coupled to reversible single electron transfer. The slope indicated one proton participating in a single electron transfer process (Bond, 1980) for neutralizing the excess charge that accumulated at the interface upon electrochemical reduction. The surface controlled electrode process indicated the diffusion of proton was very fast. Therefore, the electrode process could be expressed as follows (Trushina et al., 1997):

$$\mathrm{HbFe(III)} + \mathrm{H^+} + \mathrm{e^-} = \mathrm{HbFe(II)}$$

3.4. Electrocatalysis of Hb/HMS to reduction of H_2O_2

The amperometric responses of the HMS, Hb and Hb/HMS-modified GCEs upon successive additions of H_2O_2 to 0.1 M pH 7.0 PBS at an applied potential of –400 mV were shown in Fig. 6. The HMS-modified GCE showed a much smaller response to H_2O_2 than both Hb and the Hb/HMS-modified GCEs at the same H_2O_2 concentration. Thus, the Hb/HMS-modified GCE showed an electrocatalytic response to the reduction of H_2O_2. The low response resulted from the direct reduction of H_2O_2 at negative potential. The signal of the Hb-modified electrode was noisy, so it could not be used for detection of H_2O_2. Upon addition of an aliquot of H_2O_2 to the buffer solution, the reduction current increased steeply and then reached a stable value. The enzyme electrode achieves 95% of the steady-state current in less than 5 s. The results demonstrated clearly that the electrocatalytic response was very fast. Although the current steps for the catalyzed signal displayed a decreasing current over time, we did not observe the difference among the signals determined for several times at the same concentration after H_2O_2 was added for 30 s. The catalytic current was stable and reproducible after 30 s. The decrease was due to the uneven concentration of H_2O_2 on the electrode surface that resulted from the addition of new H_2O_2 solution.

With increasing H_2O_2 concentration the response increased. Under optimal conditions, the linear response range of the sensor to H_2O_2 concentration was from 0.4 to 6.0 μM with a correlation coefficient of 0.9998 ($n = 16$) (inset in Fig. 6). From the slope of 0.88 μA μM^{-1}, the detection limit was estimated to be 1.86×10^{-9} M at a signal-to-noise of 3. The mean steady-state current of 4.0 μM H_2O_2 for six determinations was 3.6 μA with a RSD of 3.6%.

When the concentration of H_2O_2 was higher than 6 μM, a platform was observed, showing a characteristic of the Michaelis–Menten kinetic mechanism. The apparent Michaelis–Menten (K_M^{app}) could be obtained from the electrochemical version of the Linweaver–Burk equation (Kamin and Willson, 1980). The K_M^{app} value for the enzymatic activity of the Hb/HMS to H_2O_2 was determined to be 12.3 ± 0.84 μM that was much smaller than that of Hb in a SP Sephadex membrane of 1.9 mM (Fan et al., 2001). Thus, the Hb entrapped in the HMS matrix was of a higher affinity to H_2O_2 and a higher enzymatic activity to H_2O_2 reduction.

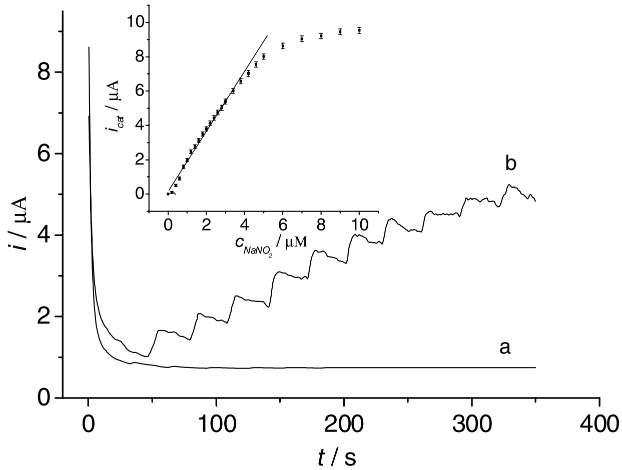

Fig. 7. Amperometric responses of HMS- (a) and Hb/HMS-modified (b) GCEs at −800 mV upon 10 successive additions of 5 μl 0.2 mM $NaNO_2$ to 5.0 ml 0.1 M pH 7.0 PBS. Inset: plot of catalytic current at Hb/HMS-modified GCE vs. $NaNO_2$ concentration.

3.5. Electrocatalysis of Hb/HMS to reduction of $NaNO_2$

Fig. 7 shows the amperometric responses of the HMS and Hb/HMS-modified GCEs to $NaNO_2$ in 0.1 M pH 7.0 PBS at an applied potential of –800 mV. No response is observable at the HMS-modified GCE. Upon addition of an aliquot of $NaNO_2$ to the buffer, the reduction current of NO_2^- increased steeply and then reached a stable value, indicating a fast reduction rate. The current was produced from the reduction of NO that came from the slow disproportionation reaction of nitrite (Barley et al., 1986; Younathan et al., 1992). This reaction and the uneven concentration of NO_2^- on the electrode surface when new NO_2^- solution was added the buffer resulted in the current decrease over time. After a reaction time of 30 s, the current was stable and reproducible. With increasing $NaNO_2$ concentration, the amperometric response increased. Inset in Fig. 7, shows the calibration curve of the Hb/HMS-modified GCE. The linear response range of the sensor to $NaNO_2$ concentration was from 0.2 to 3.8 μM with a correlation coefficient of 0.9957 ($n = 18$). From the slope of 1.79 μA μM^{-1}, a detection limit of 6.11×10^{-7} M was obtained at a signal-to-noise of 3. At 3.8 μM $NaNO_2$ concentration the RSD for six determinations of amperometric response was 5.1%. At $NaNO_2$ concentrations higher than 3.8 μM, the calibration curve showed a platform. The K_M^{app} value of the Hb/HMS to $NaNO_2$ was estimated to be 49.3 ± 1.32 μM, indicating a higher affinity of the Hb/HMS-modified electrode to NO_2^-.

3.6. Stability and reproducibility of the H_2O_2 and $NaNO_2$ sensors

The Hb/HMS-modified GCE could retain the direct electrochemistry of the immobilized Hb at constant current values upon the continuous cyclic voltammetric sweep over the potential range from –1.0 V to +0.8 V at 100 mV/s. A storage period of a week in 0.1 M pH 7.0 PBS at 4 °C did not change the currents for the direct electron transfer and the responses to H_2O_2 and $NaNO_2$. After a month, the sensor retained 96% of its initial response to H_2O_2 and 93% of its initial response to $NaNO_2$. Thus, HMS was very efficient for retaining the bioactivity of immobilized Hb and preventing it from leaking out of the sensor. The fabrication reproducibility of six electrodes, made independently, showed an acceptable reproducibility with the relative standard deviations of 4.6 and 5.7% for the current determinations of 4.0 μM H_2O_2 and 2.0 μM $NaNO_2$, respectively.

4. Conclusions

Hemoglobin can be effectively immobilized in a hexagonal mesoporous silica matrix. The Hb/HMS-modified electrode shows a fast direct electron transfer of Hb. The uniform porous structure of HMS provides a microenvironment around the protein to retain the enzymatic bioactivity. The immobilized Hb displays a high affinity and high response sensitivity to both hydrogen peroxide and nitrite. The sensor shows a good reproducibility and stability. Hexagonal mesoporous silica provides an efficient strategy and a new promising platform for the study of electron transfer of proteins and the development of biosensors.

Acknowledgements

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References

Anderson, M.W., Klinowski, J., 1986. Zeolites treated with silicon tetrachloride vapor. Part 1. Preparation and characterization. J. Chem. Soc., Faraday Trans. 1 82, 1449–1469.

Barley, M.H., Takcuchi, K.J., Meyer, T.J., 1986. Electrocatalytic reduction of nitrite to ammonia based on a water-soluble iron porphyrin. J. Am. Chem. Soc. 108, 5876–5885.

Barrett, E.P., Joyner, L.G., Halenda, P.P., 1951. The determination of pore volume and area distributions in porous substance. I. Computations from nitrogen isotherms. J. Am. Chem. Soc. 73, 373–380.

Bond A.M., 1980. Modern Polarographic Methods in Analytical Chemistry. Dekker (Marcel), New York, p. 27.

Chen, H., Ju, H., Xun, Y., 1994. Methylene blue/perfluorosulfonated ionomer modified microcylinder carbon fiber electrode and its application for the determination of hemoglobin. Anal. Chem. 66, 4538–4542.

Chen, X., Hu, N., Zeng, Y., Rusling, J.F., Yang, J., 1999. Ordered electrochemically active films of hemoglobin, didodecyldimethylammonium ions, and clay. Langmuir 15, 7022–7030.

Ciureanu, M., Goldstein, S., Mateescu, M.A., 1998. Direct electron transfer for hemoglobin in surfactant films cast on carbon electrodes. J. Electrochem. Soc. 145, 533–541.

Corma, A., 1997. From microporous to mesoporous molecular sieve materials and their use in catalysis. Chem. Rev. 97, 2373–2419.

Davis, M., 2002. Ordered porous materials for emerging application. Nature 417, 813–821.

Fan, C., Wang, H., Sun, S., Zhu, D., Wagner, G., Li, G., 2001. Electron transfer reactivity and enzymatic activity of hemoglobin in a SP sephadex membrane. Anal. Chem. 73, 2850–2854.

Fan, C., Zhuang, Y., Li, G., Zhu, J., Zhu, D., 2000. Direct electrochemistry and enhanced activity for hemoglobin in a sodium montmorillonit film. Electroanalysis 12, 1156–1157.

George, P., Hanania, G., 1953. Spectrophotometric study of ionizations in methemoglobin. Biochem. J. 55, 236–243.

Gu, H.Y., Yu, A.M., Chen, H.Y., 2001. Direct electron transfer and characterization of hemoglobin immobilized on a Au colloid–cysteamine-modified gold electrode. J. Electroanal. Chem. 516, 119–126.

Han, X., Huang, W., Jia, J., Dong, S., Wang, E., 2002. Direct electrochemistry of hemoglobin in egg-phosphatidylcholine films and its catalysis to H_2O_2. Biosens. Bioelectron. 17, 741–746.

Huang, Q., Lu, Z., Rusling, J.F., 1996. Composite films of surfactants, nation, and proteins with electrochemical and enzyme activity. Langmuir 12, 5472–5480.

Kageyama, K., Tamazawa, J., Aida, T., 1999. Extrusion polymerization catalyzed synthesis of crystalline linear polyethylene nanofibers within a mesoporous silica. Science 285, 2113–2115.

Kamin, R.A., Willson, G.S., 1980. Rotation ring-disk enzyme electrode for biocatalysis kinetic studies and characterization of the immobilized enzyme layer. Anal. Chem. 52, 1198–1205.

Kauppinen, J.K., Moffatt, D.J., Mantsch, H.H., Cameron, D.G., 1981. Resolution of complex band contours by means of Fourier self-deconvolution. Appl. Spectrosc. 35, 271–276.

Kresge, C., Leonowicz, M., Roth, W., Vartuli, J., Beck, J., 1992. Ordered mesoporous molecular sieves synthesized by a liquid-crystal template mechanism. Nature 359, 710–712.

Laviron, E., 1979. General expression of the linear potential sweep voltammogram in the case of diffusionless electrochemical systems. J. Electroanal. Chem. 101, 19–28.

Lei, C., Deng, J., 1996. Hydrogen peroxide sensor based on coimmobilized methylene green and horseradish peroxidase in the same montmorillonite-modified bovine serum albumin–glutaraldehyde matrix on a glassy carbon electrode surface. Anal. Chem. 68, 3344–3349.

Lei, C., Lisdat, F., Wollenberger, U., Scheller, F.W., 1999. Cytochrome c/clay-modified electrode. Electroanalysis 11, 274–276.

Liu, B., Hu, R., Deng, J., 1997. Characterization of immobilization of an enzyme in a modified Y zeolite matrix and its application to an amperometric glucose biosensor. Anal. Chem. 69, 2343–2346.

Liu, B., Yan, F., Kong, J., Deng, J., 1999. A reagentless amperometric biosensor based on the coimmobilization of horseradish peroxidase and methylene green in a modified zeolite matrix. Anal. Chim. Acta 386, 31–39.

Lu, Z., Dong, S., 1990. Rapid redox reaction of hemoglobin at methylene green modified platinum electrode. Electrochim. Acta 35, 1139–1143.

Mal, N., Fujiwara, M., Tanaka, Y., 2003. Photocontrolled reversible release of guest molecules from coumarin-modified mesoporous silica. Nature 421, 350–353.

Murray, R.W., 1984. In: Bard, A.J. (Ed.), Electroanalytical Chemistry, vol. 13. Dekker (Marcel), New York, pp. 191–368.

Nassar, A.-E.F., Rusling, J.F., 1996. Electron transfer between electrodes and heme proteins in protein–DNA films. J. Am. Chem. Soc. 118, 3043–3044.

Nassar, A-E.F., Willis, W.S., Rusling, J.F., 1995. Electron transfer from electrodes to myoglobin: facilitated in surfactant films and blocked by adsorbed biomacromolecules. Anal. Chem. 67, 2386–2392.

Rolison, P., 1990. Zoelite-modified electrodes and electrode-modified zeolites. Chem. Rev. 90, 867–878.

Rusling, J.F., 1998. Enzyme bioelectrochemistry in cast biomembrane like films. Acc. Chem. Res. 31, 363–369.

Stein, A., Melde, B., Schroden, R., 2000. Hybrid inorganic–organic mesoporous silicates-nanoscopic reactors coming of age. Adv. Mater. 12, 1403–1419.

Sun, H., Hu, N., Ma, H., 2000. Direct electrochemistry of hemoglobin in polyacrylamide hydrogel films on pyrolytic graphite electrode. Electroanalysis 12, 1064–1070.

Teraoka, Y., Ishida, S., Yamasaki, A., Tomonaga, N., Yasutake, A., Izumi, J., Moriguchi, I., Kagawa, S., 2001. Synthesis and characterization of tin oxide-modified mesoporous silica by the repeated post-grafting of tin chloride. Micro. Meso. Mater. 48, 151–158.

Tanev, P.T., Chibwe, M., Pinnavaia, T.J., 1994. Titanium-containing mesoporous molecular sieves for catalytic oxidation of aromatic compounds. Nature 368, 321–323.

Trushina, E., Oda, R., Landers, J., Mcmurray, C., 1997. Determination of nitrite and nitrite reduction by capillary ion electrophoresis. Electrophoresis 18, 1890–1898.

Weissbluth, M., 1974. Molecular Biology: Biochemistry and Biophysics, vol. 15. Springer, New York, p. 68.

Yang, J., Hu, N., 1999. Direct electron transfer for hemoglobin in biomembrane-like dimyristoyl phosphatidylcholine films on pyrolytic graphite electrodes. Bioelectrochem. Bioenerg. 48, 117–127.

Yang, J., Hu, N., Rusling, J.F., 1999. Enhanced electron transfer of hemoglobin in play (ester sulfonic acid) films on pyrolytic graphite electrodes. J. Electroanal. Chem. 463, 53–62.

Ye, J., Baldwin, R.P., 1988. Catalytic reduction of myoglobin and hemoglobin at chemically modified electrodes containing methylene blue. Anal. Chem. 60, 2263–2268.

Ying, J., Mehnert, C., Wong, M., 1999. Synthesis and applications of supramolecular-templated mesoporous materials. Angew. Chem. Int. Ed. Engl. 38, 56–77.

Younathan, J.N., Wood, K.S., Meyer, T.J., 1992. Electrocatalytic reduction of nitrite and nitrosyl by iron protoporphyrin dimethyl ester immobilized in an electropolymerized film. Inorg. Chem. 31, 3280–3285.

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