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Immobilization of hemoglobin on zirconium dioxide nanoparticles for preparation of a novel hydrogen peroxide biosensor

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Abstract

Direct electrochemistry and thermal stability of hemoglobin (Hb) immobilized on a nanometer-sized zirconium dioxide (ZrO_2) modified pyrolytic graphite (PG) electrode were studied. The immobilized Hb displayed a couple of stable and well-defined redox peaks with an electron transfer rate constant of $(7.90 \pm 0.93)\,s^{-1}$ and a formal potential of -0.361 V (-0.12 V versus NHE) in 0.1 M pH 7.0 PBS. Both nanometer-sized ZrO_2 and dimethyl sulfoxide (DMSO) could accelerate the electron transfer between Hb and the electrode. Spectroscopy analysis of the Hb/ZrO_2/DMSO film showed that the immobilized Hb could retain its natural structure. This modified electrode showed a high thermal stability up to 74 °C and an electrocatalytic activity to the reduction of hydrogen peroxide (H_2O_2) without the aid of an electron mediator. The electrocatalytic response showed a linear dependence on the H_2O_2 concentration ranging from 1.5 to 30.2 μM with a detection limit of 0.14 μM at 3σ. The apparent Michaelis–Menten constant K_M^{app} for H_2O_2 sensor was estimated to be (0.31 ± 0.02) mM, showing a high affinity.
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Keywords: Biosensors; Electrocatalysis; Hydrogen peroxide; Hemoglobin; Direct electron transfer; Zirconium dioxide; Thermal stability

1. Introduction

Hemoglobin (Hb) is a molecule with four electroactive iron hemes and a molar mass of approximately 64,500 g mol^{-1}. Its electrochemistry has been described in a number of publications, such as early on dropping mercury electrodes (Scheller et al., 1974; Scheller, 1977; Kuznetsov et al., 1977) and later on membrane modified PG electrodes (Ye and Baldwin, 1988; Han et al., 2002) and indium oxide electrodes (Kawahara and Ohno, 1998). These works indicated that the bare solid electrodes resulted in an irreversible denaturation of Hb and showed a usually slow electron transfer. Great efforts have been made to facilitate electron transfer for Hb by using different mediators, promoters or special electrode materials (Lu and Dong, 1990; Rusling et al., 1993, 1998; Nassar et al., 1995; Ciureanu et al., 1998; Chen et al., 1999; Ma et al., 2000; Sun et al., 2000; Fan et al., 2001). These works provided a significant understanding of electron transfer mechanism between Hb and electrodes. Among these, the surface film technique was a main method. This method incorporated Hb in a biomembrane-like microenvironment, such as those in polymer containing methylene blue (Ye and Baldwin, 1988), lipid-bilayer (Rusling, 1998), liquid-crystal film of didodecyldimethylammonium bromide (Ciureanu et al., 1998), amphiphilic polymer polyacrylamide (Sun et al., 2000) and egg-phosphatidylcholine (Han et al., 2002) films, which improved the electrochemical properties of the immobilized Hb and eliminated the need for an electron transfer mediator.

Owing to the close similarity of hemoglobin with peroxidases, some researchers have observed the pseudo peroxidase activity of the Hb immobilized in SP sephadex (Fan et al., 2001), egg-phosphatidylcholine (Han et al., 2002) and kieselgubr (Wang et al., 2002) films and on colloidal gold nanoparticles (Gu et al., 2001). This property results in an electrocatalytic activity of the immobilized Hb to the reduction of hydrogen peroxide (H_2O_2) and has been used for preparation of H_2O_2 sensors.

Recently, there has been an increasing interest in the immobilization of proteins on nanoparticles (Zhao et al., 1992;

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Xiao et al., 2000; Gu et al., 2001; Shipway et al., 2000). The nanoparticles can retain the bioactivity of proteins to a large extent and accelerate the electron transfer between proteins and electrodes. However, these works were almost performed at colloidal gold nanoparticles. In this work, zirconium dioxide (ZrO_2) nanoparticles with 35 nm-diameter are cast on a pyrolytic graphite electrode by dispersing them in dimethyl sulfoxide (DMSO) to immobilize Hb, as an example of proteins to study the applications of ZrO_2 nanoparticles in protein immobilization and biosensing. The direct electrochemical behavior of protein at the ZrO_2 nanoparticle modified pyrolytic graphite (PG) electrode is studied for the first time. The ZrO_2 nanoparticles and adsorbed DMSO result in an increase of the cathodic peak current of Hb for 5.1 times and a good thermal stability of the immobilized Hb. The peak current increase of the immobilized Hb with increasing temperature from 9 to 74 °C shows an Arrhenius-type temperature dependence. On the ZrO_2 nanoparticles Hb retains its bioactivity and displays a high affinity to H_2O_2, producing a novel H_2O_2 sensor for a quick measurement of H_2O_2 down to 0.14 μM.

2. Experimental

2.1. Materials

Hb was obtained from Sigma and used without further purification. Three milligram per milliliter Hb solution was stored at a temperature of 4 °C as stock solution. ZrO_2 (35 ± 3 nm) is a gift from Xinxing Chemicals Group of Jiangsu (Yixing, Jiangsu, China). Other reagents were of analytical reagent grade. 0.1 M phosphate buffer solutions with different pH values were prepared by mixing the stock standard solutions of K_2HPO_4 and KH_2PO_4 and adjusting the pH with 0.1 M H_3PO_4 or NaOH. All solutions were made up with twice-distilled water.

2.2. Electrode preparation

The substrate PG electrodes with a geometric area of 3.78 mm^2 were polished before each experiment with 1.0, 0.3 and 0.05 μm α-alumina slurry, respectively, rinsed thoroughly with doubly distilled water between each polishing step, then sonicated in 1:1 (HNO_3:H_2O (v/v)) nitric acid solution, acetone and doubly distilled water successively and allowed to dry at room temperature.

For preparation of a Hb entrapped ZrO_2 nanoparticle modified electrode (Hb/ZrO_2/DMSO/PG), a ZrO_2 suspension was firstly obtained by dispersing 2.0 mg ZrO_2 nanoparticle powder in 1.0 ml DMSO. Aqueous mixture of 10 μl of Hb solution and 10 μl of ZrO_2 suspension was spread onto the surface of the substrate PG electrode. Alternatively, only 10 μl Hb solution, ZrO_2/DMSO suspension, Hb/ZrO_2/water suspension containing 1.5 mg Hb and 1.0 mg ZrO_2 in 1.0 ml water or Hb/DMSO solution obtained by dissolving 1.5 mg Hb in 1.0 ml DMSO was cast onto the PG electrode to form Hb/PG, ZrO_2/DMSO/PG, Hb/ZrO_2/PG or Hb/DMSO/PG modified electrode, respectively. A small bottle was fit tightly over the electrode for 2 h to ensure the slow evaporation of water and the formation of more uniform film. The film was then dried and aged overnight in a sealed flask kept at a constant temperature of 18 °C. Prior to electrochemical experiments, the electrode was rinsed thoroughly with doubly distilled water and kept in PBS at 4 °C.

2.3. Electrochemical measurements

Electrochemical measurements were performed with a conventional three-electrode system with the modified PG electrode as working electrode, a platinum wire as auxiliary electrode, and a saturated calomel electrode (SCE) as reference against which all potentials were measured. The electrodes were connected to a BAS-100B electrochemical analyzer (Bioanalytical System, USA). Cyclic voltammetric measurements were done in a unstirred electrochemical cell thermostated at 18 ± 0.2 °C. The thermal stability of the Hb/ZrO_2/DMSO/PG was examined by increasing the cell temperature and recording cyclic voltammograms after keeping the corresponding temperature for 20 min. All experimental solutions were deoxygenated by bubbling highly pure nitrogen for 15 min and maintained under nitrogen atmosphere during measurements. AC impedance experiments were carried out with the PGSTAT30/FRA2 system (Autolab, The Netherlands) in 0.1 mM $Fe(CN)_6^{3-/4-}$ solution.

UV-Vis absorbance spectroscopy was performed using a UV-2201 spectrophotometer (Shimadzu; Kyoto Japan). Fourier transform infrared (FT-IR) spectra were recorded on a Vector 22 FT-IR spectrometer (Bruker). Hb solution, Hb/DMSO solution, Hb/ZrO_2/water or Hb/ZrO_2/DMSO mixture was cast on a Teflon chip, respectively. After the membrane on the chip was dried in air, it was stripped off and tabletted with KBr powder for FT-IR measurement. Twenty scans were collected and averaged for each spectrum.

3. Results and discussion

3.1. Spectroscopic analysis of the Hb/ZrO_2/DMSO film

Fig. 1 shows the UV-Vis spectra of different solutions. The solutions of Hb and Hb/DMSO and the suspensions of Hb/ZrO_2/water and Hb/ZrO_2/DMSO showed a largest absorbance at 405.4 nm, while no absorption for both pure DMSO and ZrO_2 suspension was observed. Obviously, this absorption peak was attributed to the Soret band of Hb (George and Hanania, 1953). Previous studies have demonstrated that the absorption band would diminish upon the full protein denaturation (George and Hanania, 1953; Nassar et al., 1995). Thus, the Hb mixed in the suspensions

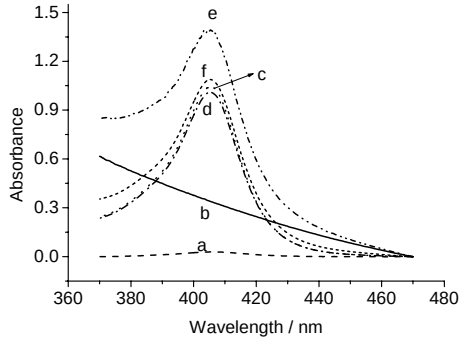

Fig. 1. UV-Vis spectra of DMSO (a); ZrO_2/DMSO (b); Hb (c); Hb/DMSO (d); Hb/ZrO_2 (e) and Hb/ZrO_2/DMSO (f) solutions.

of ZrO_2/DMSO and ZrO_2/water and pure DMSO could retain its native structure.

As well known, the shapes of the infrared absorption bands of amide I and amide II in Hb molecule can provide detailed information on the secondary structure of the polypeptide chain (Kauppinen et al., 1981). The absorption band of 1700–1600 cm^{-1} for amide I is caused by C=O stretching vibration of peptide linkages in the protein's backbone. The absorption band around 1620–1500 cm^{-1} for amide II results from a combination of N–H bending and C–N stretching. The complete denaturation of Hb will eliminate the distinctive absorption bands of amide I and amide II (Song et al., 1992; Nassar et al., 1995). Fig. 2 shows the FT-IR spectra of Hb, Hb/ZrO_2, Hb/DMSO and Hb/ZrO_2/DMSO films. The absorption bands for amide I and amide II in the Hb/ZrO_2/DMSO film were located at 1653.13 and 1540.96 cm^{-1}, respectively, which were nearly the same as those obtained for the protein itself (1653.38 and 1540.86 cm^{-1}), while these absorption bands in the Hb/ZrO_2 or Hb/DMSO film deviated slightly from these value, indicating an interaction between Hb and ZrO_2 or DMSO when only Hb and ZrO_2 or Hb and DMSO existed in the film. Both

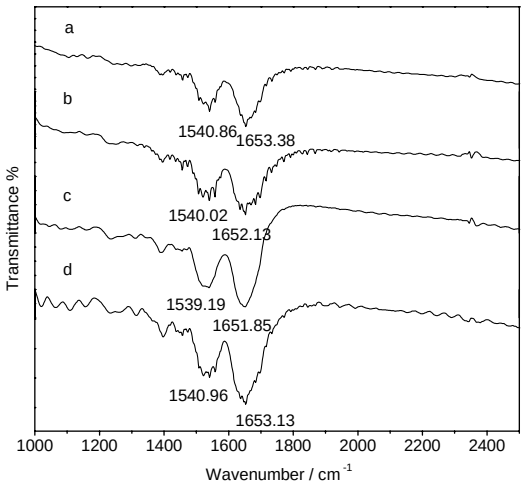

Fig. 2. FTIR spectra of Hb (a); Hb/DMSO (b); Hb/ZrO_2 (c) and Hb/ZrO_2/DMSO (d) films.

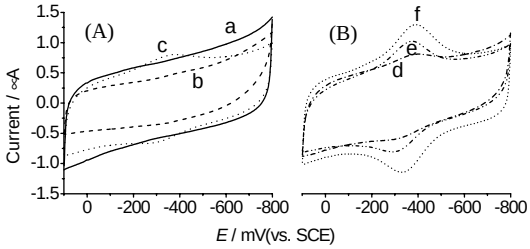

Fig. 3. Cyclic voltammograms of PG (a, solid line), ZrO_2/DMSO/PG (b, dash line), Hb/PG (c, dot line), Hb/ZrO_2/PG (d, short dash dot line), Hb/DMSO/PG (e, short dash dot dot line) and Hb/ZrO_2/DMSO/PG (f, short dash line) in 0.1 M pH 7.0 PBS at 50 mV s^{-1}.

FT-IR and UV-Vis spectroscopic experiments suggested that the Hb in Hb/ZrO_2/DMSO film was not grossly denatured and retained its native secondary structure.

3.2. Direct electrochemistry of Hb/ZrO_2/DMSO modified electrode

Fig. 3 shows the cyclic voltammograms of different electrodes in 0.1 M pH 7.0 phosphate buffer solution. The Hb/ZrO_2/DMSO/PG gave a couple of stable and well-defined redox peaks at −345 and −378 mV at 50 mV s^{-1} (curve f in Fig. 3B), while no redox peak was observable at both substrate PG electrode (curve a in Fig. 3A) and ZrO_2/DMSO/PG (curve b in Fig. 3A), the ZrO_2/DMSO/PG displayed a low background current. Obviously, the response of the Hb/ZrO_2/DMSO/PG was attributed to the redox of the electroactive centers in the immobilized Hb. On the other hand, the Hb/PG (curve c in Fig. 3A) and the Hb/ZrO_2/PG (curve d in Fig. 3B) also displayed a couple of redox peaks of Hb. But these peak currents were much smaller than those at the Hb/ZrO_2/DMSO/PG. Thus, the presence of DMSO played an important role in accelerating the electron transfer between Hb and the electrode. This property can be seen from curve e in Fig. 3B for the Hb/DMSO/PG. The increasing peak currents due to the presence of DMSO were attributed to the decrease of the dielectric constant of the microenvironment around Hb molecules, which decreased the reorganization energy of biological electron transfer (Zhou, 1994). The response at the Hb/DMSO/PG, however, was 2.4 times smaller than that of the Hb/ZrO_2/DMSO/PG. Thus, ZrO_2 nanoparticles were more important for facilitating the electron exchange. ZrO_2 nanoparticles provided a three-dimensional stage and some of the restricted orientations also favored the direct electron transfer between the protein molecules and the conductor surface.

The formal potential ($E^{0\prime}$) of the heme $Fe^{III/II}$ couple in Hb/ZrO_2/DMSO/PG, estimated as the midpoint of anodic and cathodic peak potentials, was −361 mV (−120 mV versus NHE) at pH 7.0. This value was similar to those reported previously (Nassar and Rusling, 1996; Chen et al., 1999; Wang et al., 2002) and was more negative than that obtained

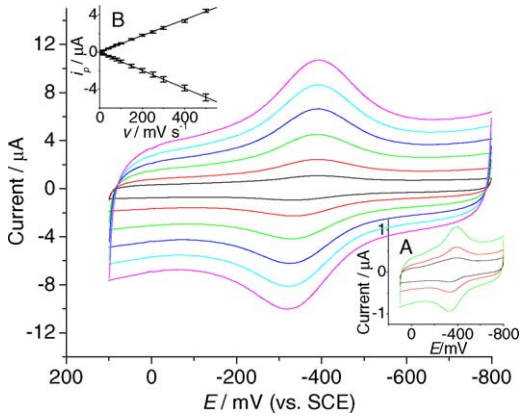

Fig. 4. Cyclic voltammograms of Hb/ZrO_2/DMSO/PG in 0.1 M pH 7.0 PBS at 40, 80, 150, 300, 400 and 500 mV s^{-1} (from lowest to highest peak currents). Insets: cyclic voltammograms of Hb/ZrO_2/DMSO/PG in 0.1 M pH 7.0 PBS at 10, 20, 40 mV s^{-1} (A) and plot of peak current vs. scan rate (B).

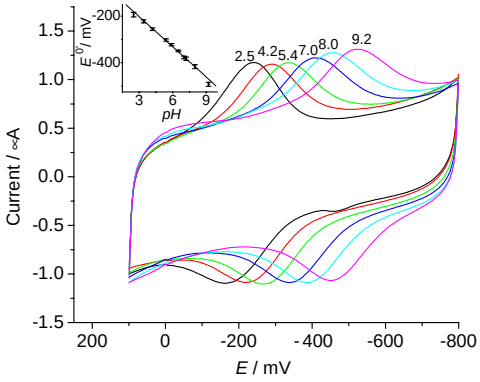

Fig. 5. Cyclic voltammograms of Hb/ZrO_2/DMSO/PG in PBS with various pH values at 50 mV s^{-1}. Inset: effect of pH on formal potential of Hb/ZrO_2/DMSO/PG in 0.1 M PBS.

in an aqueous solution (Lu and Dong, 1990). The negative shift of the peak potentials for the Hb modified electrode verses the Hb/ZrO_2 modified electrode was attributed to the presence of the ZrO_2 nanoparticles which can stabilize the oxidized form of Hb.

The cyclic voltammogram of the Hb/ZrO_2/DMSO/PG showed a nearly equal height of reduction and oxidation peaks at the same scan rate (Fig. 4). With an increasing scan rate ranging from 10 to 500 mV s^{-1} the anodic and cathodic peak potentials of the Hb showed a small shift and the redox peak currents increased linearly (inset B in Fig. 4), indicating a surface-controlled electrode process.

The peak-to-peak separations of the cyclic voltammograms of the Hb/ZrO_2/DMSO/PG at 100, 150, 200, 250, 300, 400 and 500 mV s^{-1} were 33, 36, 38, 40, 42, 49 and 56 mV, respectively. Supposing the charge transfer coefficient was between 0.3 and 0.7, the electron transfer rate constant k_s was estimated according to the model of Laviron (Laviron, 1979) with the formula $k_s = mnFv/RT$ to be $(7.90 \pm 0.93)\,s^{-1}$, where m is a parameter related to the peak-to-peak separation. This value was larger than that of $(2.3 \pm 0.4)\,s^{-1}$ for Hb immobilized in the didodecyldimethylammonium bromide film (Lu et al., 1997), suggesting a reasonably fast electron transfer between the immobilized Hb and the electrode due to the presence of ZrO_2 nanoparticles.

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

The effect of solution pH on the response of the Hb/ZrO_2/DMSO/PG was shown in Fig. 5. An increase of solution pH from pH 2.5 to 9.2 led to a negative shift of both reduction and oxidation peak potentials. In general, all changes in the peak potentials and currents with solution pH were reversible in the pH range from 2.5 to 9.2, that was, the same cyclic voltammograms could be obtained if the electrode was transferred from a solution with a different pH value to its original solution. Plot of the formal potential versus pH (from 2.5 to 9.2) (inset in Fig. 5) showed a line with a slope of −43.5 mV pH^{-1} ($R = 0.9956$), which was close to the expected value of −57.8 mV/pH at 291.15 K, indicating that one proton participated in the electron transfer process (Sun et al., 2000; Chen et al., 1999).

3.4. Impedance characterization of Hb/ZrO_2/DMSO/PG

The effect of Hb modified films on the impedance of PG electrode was investigated using AC impedance spectroscopy. An equivalent circuit was utilized to model the impedance data, thus enabling the extraction of electrical parameters from the impedance spectra. The data analysis results were listed in Table 1. It was clearly observed that the polarization impedance increased largely upon spreading of Hb to PG surface. ZrO_2 nanoparticles could decrease the polarization impedance, while DMSO increased slightly the polarization impedance due to its hydrophilic interface. The interaction of ZrO_2 and DMSO at Hb/ZrO_2/DMSO/PG,

Table 1
Data analysis of AC impedance spectra of different electrodes in 0.1 mM $Fe(CN)_6^{3-/4-}$

Electrodes[a]	*a*	*b*	*c*	*d*	*e*	*f*	*g*
Polarization impedance (Ω)	2000 ± 34[b]	2928 ± 53	1040 ± 14	5281 ± 68	3895 ± 55	6938 ± 61	2641 ± 29
Capacitance (μF cm^{-2})	11.4 ± 0.6	7.6 ± 0.3	0.5 ± 0.1	11.3 ± 0.4	6.8 ± 0.4	3.6 ± 0.3	8.5 ± 0.7

[a] (a) PG; (b) ZrO_2/PG; (c) ZrO_2/DMSO/PG; (d) Hb/PG; (e) Hb/ZrO_2/PG; (f) Hb/DMSO/PG and (g) Hb/ZrO_2/DMSO/PG.
[b] R.S.D. for three determinations.

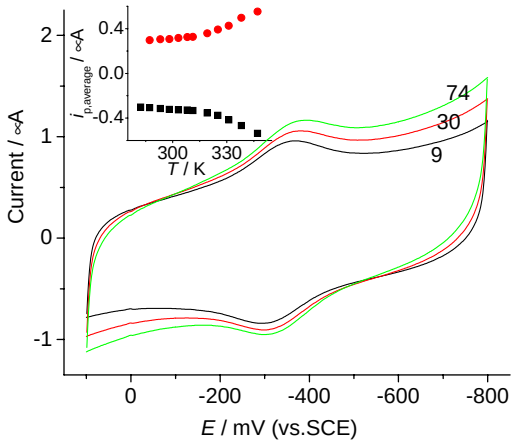

Fig. 6. Cyclic voltammograms of Hb/ZrO_2/DMSO/PG at 9, 30 and 74 °C in 0.1 M pH 7.0 PBS at 50 mV s^{-1}. Inset: plot of cathodic peak current (●) and anionic peak current (■) vs. temperature.

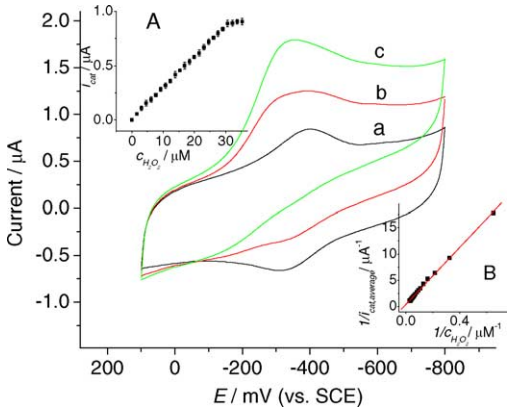

Fig. 7. Cyclic voltammograms of Hb/ZrO_2/DMSO/PG in 0.1 M pH 7.0 PBS containing 0, 15 and 35 μM H_2O_2 (from bottom to top) at 50 mV s^{-1}. Inset: plot of catalytic current vs. H_2O_2 concentration (A) and the Lineweaver–Burk plot (B).

however, could decrease greatly the polarization impedance resulted from the spreading of Hb. The spreading of both ZrO_2 suspension and DMSO to PG surface resulted in a decrease of capacitance. Thus, the redox reaction of Hb could easier take place on the Hb/ZrO_2/DMSO/PG than any other electrodes.

3.5. Thermal stability of Hb/ZrO_2/DMSO/PG

The immobilization of proteins and enzymes onto transducer surfaces can lead to a change of their behavior compared to that observed in homogeneous solution (Weetall, 1974; Bowers, 1986). Thermal stability is a measure of the ability of the biosensor to withstand elevations in temperature (Wang et al., 1997). Fig. 6 shows the cyclic voltammograms of the Hb/ZrO_2/DMSO/PG in pH 7.0 PBS at various temperatures. Both the anodic and cathodic peak currents of the Hb/ZrO_2/DMSO/PG increased with increasing temperature from 9 to 74 °C, displaying an expected Arrhenius-type temperature dependence (inset in Fig. 6). Meanwhile no peak was observed at the Hb/PG up to 52 °C (results not shown), indicating the denaturation of Hb. The increase in the thermal stability of the Hb/ZrO_2/DMSO/PG could be attributed to the presence of ZrO_2 nanoparticles. The immobilized Hb on hydrophobic ZrO_2 nanoparticles could greatly enhance the thermal stability due to the unusual conformational rigidity in this nonpolar binding environment (Wang et al., 1997).

3.6. Electrocatalysis of Hb/ZrO_2/DMSO/PG to reduction of H_2O_2

Cyclic voltammograms of the Hb/ZrO_2/DMSO/PG before and after injection of aliquots of H_2O_2 solution in pH 7.0 PBS were shown in Fig. 7. Upon addition of 32 μM H_2O_2 to the electrochemical cell the reduction peak current increased and the anodic peak current decreased dramatically, indicating a typical electrocatalytic reduction process of H_2O_2. Furthermore, the reduction peak current increased with the increasing H_2O_2 concentration. This phenomenon was not observed at a bare PG or a ZrO_2/DMSO/PG electrode. Thus, the catalytic reduction of H_2O_2 was due to the presence of Hb.

The calibration curve of the Hb/ZrO_2/DMSO/PG to H_2O_2 concentration obtained with cyclic voltammetry was shown in inset A in Fig. 7. The linear response range of the sensor to H_2O_2 concentration was from 1.5 to 30.2 μM. The linear regression equation was $y = 0.028x + 0.015$ μA, with a correlation coefficient of 0.999. From the slope of 0.028 μA/μM the detection limit was estimated to be 0.14 μM at 3σ. The sensitivity of the proposed Hb sensor was 0.74 A M^{-1} cm^{-2}, which was larger than that of 0.6 A M^{-1} cm^{-2} (Karyakin et al., 2000) and 0.243 A M^{-1} cm^{-2} (Ricci et al., 2003), and slightly lower than that of 1 or 2 A M^{-1} cm^{-2} at Prussian blue modified platinum or gold screen-printed electrode (De Mattos et al., 2003). The high sensitivity of Hb modified electrode was due to the electrochemical characterization of intrinsic peroxidase activity of Hb.

The redox peaks for the direct electron transfer of the immobilized Hb and the electrocatalysis to the reduction of hydrogen peroxide occurred in the potentials more negative than −300 mV. 0.1 mM ascorbic acid and dopamine with the redox potentials more positive than 0 V (versus Ag/AgCl) at PG electrode (Han et al., 2002) did not interfere the determination of 30 μM hydrogen peroxide.

When the concentration of H_2O_2 was higher than 30 μM, a response platform was observed, showing a characteristic of the Michaelis–Menten kinetic mechanism. The apparent Michaelis–Menten constant (K_M^{app}), a reflection of both the enzymatic affinity and the ratio of microscopic kinetic constants, could be obtained from the electrochemical version of the linweaver–Burk equation (Kamin and Willson, 1980). The K_M^{app} value for the Hb/ZrO_2/DMSO/PG was determined to be (0.31 ± 0.02) mM. This value was smaller than that of immobilized horseradish peroxidase (Ferri et al., 1998; Xiao et al., 2000). It was also smaller than those of

0.975 mM for Hb incorporated kieselgubr film (Wang et al., 2002) and 1.9 mM for Hb incorporated SP sephadex membrane (Fan et al., 2001). Thus, the presence of ZrO_2 nanoparticles resulted in a higher affinity to H_2O_2, as shown on a Au colloid-cysteamine-modified gold electrode (Gu et al., 2001).

3.7. Stability of the Hb/ZrO_2/DMSO/PG

When the Hb/ZrO_2/DMSO/PG was not in use, it was store in PBS or in the refrigerator at 4 °C. It retained 91% of its initial current response after a two months storage. The relative standard deviation was 5.4% for six successive determinations at a H_2O_2 concentration of 20 μM. The relative standard deviation for determinations of H_2O_2 concentration at 20 μM was 6.6% for seven successive renewals. The fabrication of six electrodes, made independently with the same bare electrode, showed an acceptable reproducibility with a relative standard deviation of 5.2% for the current determined at 20 μM H_2O_2.

4. Conclusions

Hemoglobin can be effectively immobilized on zirconium dioxide nanoparticle modified pyrolytic graphite electrode to produce a fast direct electron transfer. On the ZrO_2 nanoparticles Hb retains its bioactivity and native structure. The immobilized hemoglobin displays a high affinity to hydrogen peroxide, which allows producing a novel H_2O_2 sensor for a quick measurement of H_2O_2 down to 0.14 μM. This work offers a way to build the new mediator-free sensors by immobilizing proteins or enzymes on ZrO_2 nanoparticles for determination of different substrates, such as glucose using glucose oxidase. This may also be a good alternative method for further study on the direct electrochemistry of redox proteins and their sensing application.

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References

Bowers, L., 1986. Applications of immobilized biocatalysts in chemical analysis. Anal. Chem. 58, 513A–530A.

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.

De Mattos, I.L., Gorton, L., Ruzgas, T., 2003. Sensor and biosensor based on prussian blue modified gold and platinum screen printed electrodes. Biosens. Bioelectron. 18, 193–200.

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.

Ferri, T., Poscia, A., Santucci, R., 1998. Direct electrochemistry of membrane-entrapped horseradish peroxidase. Part I. A voltammetric and spectroscopic study. Part II. Amperometric detection of hydrogen peroxide. Bioelectrochem. Bioenerg. 44, 177–181.

Ferri, T., Poscia, A., Santucci, R., 1998. Direct electrochemistry of membrane-entrapped horseradish peroxidase. Part I. A voltammetric and spectroscopic study. Part II. Amperometric detection of hydrogen peroxide. Bioelectrochem. Bioenerg. 45, 221–226.

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.

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. Fourier self-deconvolution: a method for resolving intrinsically overlapped bands. Appl. Spectrosc. 35, 271–276.

Karyakin, A.A., Karyakina, E.E., Gorton, L., 2000. Amperometric biosensor for glutamate using prussian blue-based "artificial peroxidase" as a transducer for hydrogen peroxide. Anal. Chem. 72, 1720–1723.

Kawahara, N.Y., Ohno, H., 1998. Thermal stability and electron transfer reaction of PEO-modified hemoglobin cast on an ITO electrode in polymer electrolytes. Electrochim. Acta 43, 1493–1497.

Kuznetsov, B.A., Shumakovich, G.P., Mestechkina, N.M., 1977. The reduction mechanism of cytochrome c and methemoglobin on mercury electrode. Bioelectrochem. Bioenerg. 4, 512–521.

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

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

Lu, Z., Huang, Q., Rusling, J.F., 1997. Films of hemoglobin and didodecyldimethylammonium bromide with enhanced electron transfer rates. J. Electroanal. Chem. 423, 59–66.

Ma, H., Hu, N., Rusling, J.F., 2000. Electroactive myoglobin films grown layer-by-layer with poly(styrenesulfonate) on pyrolytic graphite electrodes. Langmuir 16, 4969–4975.

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.

Ricci, F., Amine, A., Palleschi, G., Moscone, D., 2003. Prussian blue based screen printed biosensors with improved characteristics of long-term lifetime and pH stability. Biosens. Bioelectron. 18, 165–174.

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

Rusling, J.F., Nassar, A.-E.F., 1993. Enhanced electron transfer for myoglobin in surfactant films on electrodes. J. Am. Chem. Soc. 115, 11891–11897.

Scheller, F., Jaenchen, M., Etzold, G., Will, H., 1974. A conformational study of poly-L-lysine, metmyoglobin, cytochrome c, methemoglobin and glycogen phosphorylase b adsorbed at mercury electrode. Bioelectrochem. Bioenerg. 1, 478–486.

Scheller, F., 1977. Functional properties of adsorbed hemoproteins. Bioelectrochem. Bioenerg. 4, 490–499.

Shipway, A.N., Lahav, M., Willner, I., 2000. Nanostructured gold colloid electrodes. Adv. Meterol. 12, 993–998.

Song, Y.P., Petty, M.C., Yarwood, J., Feast, W.J., Tsibouklis, J., Mukherjee, S., 1992. Fourier transform infrared studies of molecular ordering and interactions in langmuir-blodgett films containing nitrostilbene and stearic acid. Langmuir 8, 257–261.

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

Wang, H., Guan, R., Fan, C., Zhu, D., Li, G., 2002. A hydrogen peroxide biosensor based on the bioelectrocatalysis of hemoglobin incorporated in a kieselgubr film. Sens. Actuators B 84, 214–218.

Wang, J., Liu, J., Cepra, G., 1997. Thermal stabilization of enzymes immobilized with in carbon paste electrodes. Anal. Chem. 69, 3124–3127.

Weetall, H.H., 1974. Immobilized enzymes: analytical applications. Anal. Chem. 46, 602A–612A.

Xiao, Y., Ju, H.X., Chen, H.Y., 2000. Direct electrochemistry of HRP immobilized an Au colloid-cysteamine monolayer modified gold electrode. Anal. Biochem. 278, 22–28.

Ye, J., Baldwin, R.P., 1988. Catalytic reduction of myoglobin and hemoglobin at chemically modified electrodes containing methylene blue. Anal. Chem. 60 (20), 2263–2268.

Zhao, J., Henkens, R.W., Stonehuerner, J., O'daly, J.P., Crumbliss, A.L., 1992. Direct electron transfer at horseradish peroxidase-colloidal gold modified electrodes. J. Electroanal. Chem. 327, 109–119.

Zhou, H.X., 1994. Effects of mutations and complex formation on the reduction potentials of cytochrome c and cytochrome c peroxidase. J. Am. Chem. Soc. 116, 10362–10375.