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Direct electron-transfer of native hemoglobin in blood: Kinetics and catalysis

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Abstract

A novel approach that uses nature biological tissues, fish blood, for the study of the direct electron-transfer of hemoglobin and its catalytic activity for H_2O_2 and NO_2^- is observed. The direct electron-transfer of hemoglobin in red blood cells in fish blood on glassy carbon electrode was observed for the first time. By simply casting fish blood on GC electrode surface and being air-dried, a pair of well-defined redox peaks for HbFe (III)/HbFe (II) appeared at about −0.36 V (vs SCE) at the fish blood film modified GCE in a pH 7.0 phosphate buffer solution. Ultraviolet visible (UV/VIS) characterization and the enhancement of the redox response of Hb by adding pure Hb in fish blood suggested that Hb preserved the native second structures in the fish blood film. Optical micrographs showed that the RBCs retained its integrity in blood. Hb in blood/GCE maintained its activity and could be used to electrocatalyze the reduction H_2O_2 and NO_2^-.

Keywords: Direct electrochemistry; Hemeglobin (Hb); Blood; Electrocatalytic activity; Red blood cells (RBCs)

1. Introduction

Hemoglobin (Hb), a heme protein that contains two α and two β subunits, each of which has one heme as a prosthetic group, is the main substance in red blood cells (RBCs). Due to its important role in oxygen and carbon dioxide transport and storage, Hb is one of the most widely studied proteins. Although Hb does not function biologically or physiologically as an electron carrier, it was usually used as model systems to study electron-transfer properties of proteins. Direct electron-transfer of Hb at a variety of modified electrodes has been investigated [1–5]. The electrode modifiers can be inorganic materials, organic compounds [6,7] or even pure biological materials. Compared with organic compounds, inorganic materials are intrinsically more stable catalysts because of their layered oxide structure. Recently, a series of inorganic porous materials such as clay [8], montmorillonite [9], porous alumina [10] and sol–gel matrix [11] have been proven to be promising as the immobilization matrices. But these materials cannot provide real biological environments for proteins. Lu recently developed a novel approach that immobilized Hb on electrodes by pure biological materials, yeasts cells, to study the direct electrochemistry of Hb [12]. The utilization of real biological materials for the direct electron-transfer of proteins and biosensors is a new current [1]. Blood is the main biological tissue that contains Hb for vertebrates. In this work, we found for the first time that direct electron-transfer of native Hb in blood can be easily obtained when casting fish blood on a glassy carbon (GC) electrode and being air-dried. This is interesting as it is difficult to achieve the direct electron-transfer of commercial redox proteins that contain impurities or denaturalized proteins. Thus, the complicated blood film might provide the suitable native biological environments for the direct electron-transfer of Hb. Furthermore, Hb existed in blood films on electrode surface was proved to retain their native structures and natural catalytic capacity towards the reduction of hydrogen peroxide and nitrite. The present work not only foresaw the possibility of studying electron-transfer processes of proteins in vivo but also opened a new way for fabricating biosensing system based on biological tissues.

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2. Materials and methods

2.1. Reagents and apparatus

Hemoglobin was purchased from the Sigma Chemical Co. Grass carp and *Carassius auratus* were purchased from a market near Wuhan University (China). Human blood samples were from the hospital of Wuhan University (China). Phosphate buffer solutions (PBS) (0.1 mol L^{-1}) with various pH values were prepared by mixing stock standard solution of Na_2HPO_4 and KH_2PO_4 and adjust the pH with 0.1 M H_3PO_4 or NaOH. The concentration of Hb was 8 mg/ml in PBS. $NaNO_2$ and H_2O_2 were freshly prepared before use. All solutions were prepared from double-distilled water.

Electrochemical measurements were performed with a three-electrode system comprising a platinum wire as auxiliary electrode, a saturated calomel electrode (SCE) as reference, against which all potentials were quoted and the blood/GC electrode as working electrode. All electrochemical experiments were carried out with a computer-controlled electrochemical workstation (CHI 830). All solutions were held under a nitrogen atmosphere during the electrochemical experiments.

UV/VIS spectroscopy was recorded on a TU-1901 UV/VIS spectrophotometer (Purkinje General Instrument Co. Ltd., Beijing, China). The blood or blood-Hb film for UV/VIS was prepared by depositing the blood sample or the mixture of blood and Hb onto glassy slide and was dried in air overnight. A film only containing Hb on a glass slide was used as a control. The reference was an uncoated glassy glide.

An inverse microscope (Axiovert 200 M, Zeiss) under 40×, 0.6 NA was used to observe the red blood cells (RBCs) and a high resolving power digital camera to image (3900×3090 pixel, AxioCam, Zeiss). The blood was deposited onto the glass slide and dried for the optical micrographs.

2.2. Preparation of the electrode modification

A GC electrode was first polished with an alumina (particle size of about 0.05 mm)/water slurry on silk, then washed with nitric acid (1:1), ethanol and double-distilled water in an ultrasonic bath for about 5 min in turn. 5 μl of the blood samples or the mixture of blood and 8 mg/ml Hb (with the ratio 1:1) was dropped to the freshly cleared GC electrode surface and dried in air for about 8 h. The electrode was thus able to be ready for use.

3. Results and discussion

3.1. Electrochemical properties of Hb in blood/GC electrode

Fig. 1 shows the cyclic voltammograms of the bare GC electrode, the blood/GC electrode and the Hb–blood/GC electrode in 0.1 mol L^{-1} PBS (pH 7.0), whose blood was from grass carp dead from the absence of oxygen. A pair of well-defined and nearly symmetrical redox peaks is observed at blood/GC (curve b) and Hb-blood/GC electrode (curve c) while no redox peaks appeared at the bare GC electrode (curve a). There was also no redox peaks for remained blood serum (BS) by separated Hb from blood/GC electrode. The formal potential (E^{σ}), defined as the average the cathodic peak potential (E_{pc}) and the anodic peak potential (E_{pa}), is nearly the same for both blood/GC and Hb-blood/GC except for a lower peak current of blood/GC, indicating that the redox peaks for blood/GC was attributed to Hb in blood. The E_{pa} and E_{pc} for blood/GC electrode are −0.338 V and −0.382 V, respectively, with E^{σ} of −0.36 V and the separation of peak potentials (ΔE_p) of 44 mV. Moreover, the ratio of the cathodic current to the anodic current is close to one. These electrochemical parameters suggest that Hb in blood undergoes a quasi-reversible redox process at blood/GC electrode.

In order to verify which segment of blood is responsible for the direct electron-transfer of Hb in blood, blood serum (BS) and blood corpuscle (BC) were separately used to modify GC electrode for examining their electrochemical signals. No redox peaks were observed at BS/GC and Hb-BS/GC electrodes, suggesting that BS cannot facilitate the direct electron-transfer between Hb and the electrode surface. However, a pair of well-defined redox peaks is observed at the BC/GC electrode. The E^{σ} for BC/GC electrode is −0.36 V, which is close to that at blood/GC and indicates the similar microenvironments for Hb at the two electrodes. RBCs provide a suitable microenvironment for Hb to retain its native conformation in blood, and Hb can obtain suitable orientation in the effect of potential on the electrode, thus the direct electrochemistry of Hb in blood can be obtained (Scheme 1). This is reasonable as the direct electrochemistry of redox proteins have been widely reported in surfactants films with properties similar to the cell membrane of blood cells.

The surface coverage (Γ) was estimated from integration of the reduction peak of the CVs according to $\Gamma = Q/nFA$, where Q is the charge involved in the reaction, n the number of electron transferred, F Faraday constant, and A the electrode area, with a value of 1.09×10^{-11} mol/cm^2 for the blood/GC electrode. The theoretical monolayer coverage for Hb is 4.6×10^{-12} mol/cm^2, which were estimated taking into account the crystallographic dimensions of 6.4 nm×5.5 nm×5.0 nm for it, assuming one molecule with the long axis parallel to the electrode surface, only those Hb molecules in the inner layers that closed to the

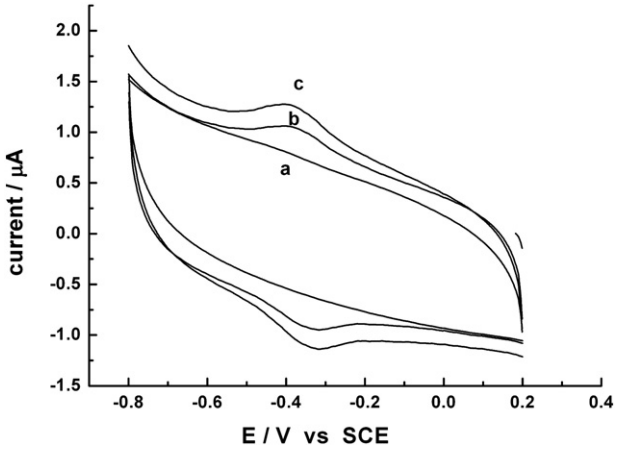
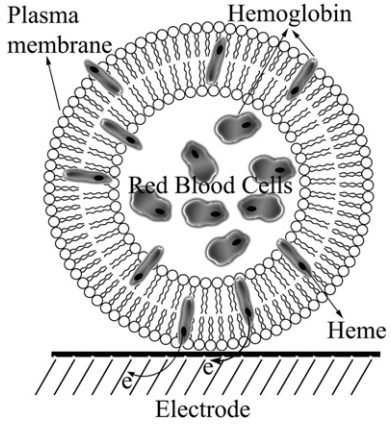


Fig. 1. Cyclic voltammograms of (a) bare GC; (b) blood/GC; (c) Hb-blood/GC in 0.1 mol L^{-1} pH 7.0 PBS at a scan rate of 200 mV/s.



Scheme 1. The process of the electron-transfer of Hb on blood/GC electrode.

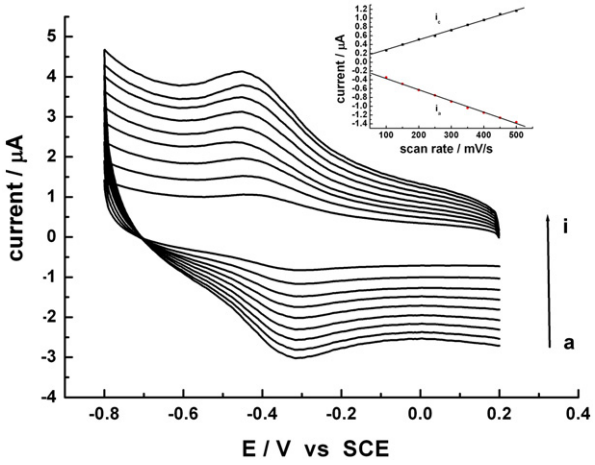


Fig. 2. Cyclic voltammograms of the blood/GC in 0.1 mol L^{-1} pH 7.0 PBS at 100, 150, 200, 250, 300, 350, 400, 450, 500 mV/s. (Inset) Plot of cathodic peak current and anodic peak current vs scan rate.

electrode can exchange electrons with the electrode. The electron-transfer rate (k_s) of Hb in blood/GC electrode was evaluated based on the equation derived by Laviron [13] for diffusionless CV. The value of k_s, 4.27±0.13 s^{-1}, suggested reasonably fast electron-transfer.

Comparing with other works in this field, we can find that E^{σ} and k_s depend on the materials used to immobilize the proteins, suggesting that the electron-transfer between proteins and the electrode is affected by the local environment of protein. The k_s of Hb in our work is similar to that in regenerated silk fibroin film [2], didodecyldimethylammonium bromide (DDAB) film [5] and sol–gel film [11], and smaller than SP sephadex [14] and some other materials (Table 1). Speculating upon these differences, possible explanations included that different electrodes and methods were used. According to the theoretics of electron-transfer, we know that the electron-transfer rate can be facilitated with favorable orientation of proteins, which could be adjusted by electrostatic force when the electrode surface is modified with films possessing positive or negative charge. Armstrong et al. have pointed that the edge face of the pyrolytic graphite (PG), subjected to standard polishing procedures in air, contains a variety of hydrophilic C–O functional groups, which expected to interact favorably with the protein [16]. It is also well known that the edge plane pyrolytic graphite electrode is considerably more conducting, which facilitates the electron-transfer between the protein and the electrodes. Thus, a higher value of k_s was obtained in some work. The E^{σ} of Hb in blood/GC electrode is more negative than those in the DDAB film [5] and in regenerated silk fibroin film [2], more positive than that in the lecithin film [17]. This confirms a specific influence of film environment on E^{σ} of heme proteins. Film components may change formal potential by the interaction with the protein or by their influence on the electrode double-layer.

Table 1
Electrochemical parameters of Hb in various films

Films	pH	E^{σ} (V) vs SCE	Average k_s (s^{-1})	Method/ electrode	References
Blood	7.0	−0.36	4.27	CV/GE	This work
Silk fibroin-Hb	7.0	−0.334	1.51	CV/GE	[2]
DDAB-Hb	5.5	0.084±0.006 (vs SHE)	2.3±0.4	CV/PG	[5]
PTFE-Hb	7.3	−0.281	1.9	CV/GE	[15]
(Silica sol–gel)-Hb	7.0	−0.113 (vs SHE)	1.58	CV/CPE	[11]
Hb-SP sephadex	5.5	−0.28	102.12	SWV/PG	[14]

The peak currents for blood/GC electrode are linearly proportional to scan rate from 0.1 to 0.5 V s^{-1} (Fig. 2). Furthermore, integration of reduction peaks at different scan rates gave nearly constant charge (Q) values. All these are characteristic of diffusionless, thin-layer electrochemical behavior, that is, nearly all electroactive proteins in the films are reduced on the forward cathodic scan, with full conversion of the reduced proteins back to their oxidized forms on the reversed anodic scan. The potential separation between anodic and cathodic peaks (ΔE_p) is larger than the theoretical value of 0 mV for a surface process, which is probably attributed the long distance from the heme of Hb to the electrode surface due to the deep-buried electroactive centers in polypeptides.

3.2. Influence of pH on the voltammetry

Nearly reversible voltammograms were obtained in the tested pH range (3.0–10.0), with stable and well-defined peaks (Fig. 3). The CV of blood/GC electrode showed a strong dependence on the solution pH. An increase of pH caused a negative shift in both cathodic and anodic peak potentials for Hb, indicating that protons are involved in the electrode reaction of Hb. In general, all changes in cyclic voltammetry peaks with pH are reversible in the pH range from 3 to 10. When the modified electrode was transferred from a pH 7.0 solution to a pH 3.0 solution, an asymmetric redox CV was observed, which can be restored into well-defined symmetric redox peaks when the modified electrode was transferred back to the buffer pH 7.0. This is likely due to a reversible pH-induced conformational change at low pHs. This can also be proved by UV/VIS experiments.

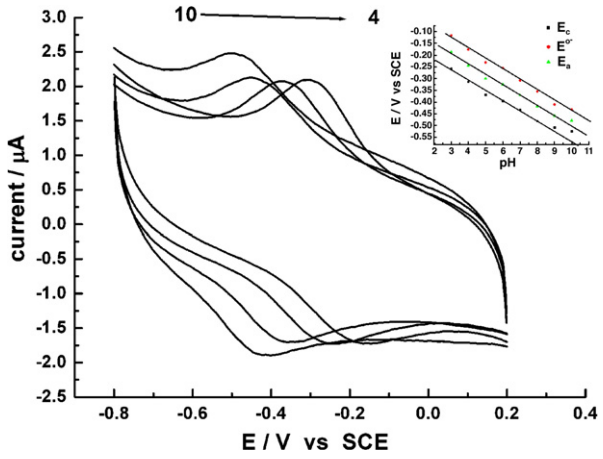


Fig. 3. Cyclic voltammograms of blood/GC in 0.1 mol L^{-1} PBS at different pHs. Scan rate: 300 mV/s. (Inset) Plots of E_a, E_c and E^{σ} vs pH for blood/GC at a scan rate of 300 mV/s.

The E^{σ} of Hb in the modified electrode has a linear relationship with pH from 3.0 to 10.0, with a slope −46.4 mV pH^{-1}. This value is close to the expected −58 mV pH^{-1} at 20 °C for a one electron and one proton reaction. Thus, the electron-transfer between Hb and the electrode for blood/GC electrode can be presented by

$$Hb_{heme}Fe(III) + H^+ + e^- \leftrightarrow Hb_{heme}Fe(II) \quad (1)$$

3.3. UV/VIS spectroscopy

The location of the Soret absorption band of prosthetic heme group provides information about possible denaturation of heme proteins. Fig. 4a shows the UV/VIS spectra of Hb film, blood film and blood-Hb film on glass slides immersed in pH 7.0 PBS. The Soret bands for them are 405.2, 404.4 and 404.6 nm, respectively. It suggests that there is no change in the environment of heme group in blood. Comparing with the UV/VIS spectrum of haematin hydrochloride that has a broad band at about 384.2 nm, the UV/VIS spectrum of blood shows an obvious peak band at 404.4 nm, suggesting that the heme group of Hb is not removed from the heme pocket and retains its native environment in blood. Fig. 5 shows the optical micrographs of RBCs in fish blood on glass slide. The results showed that RBCs retained their native integrity in blood when it was dried on the glass glide. The diameter of RBCs is about 5–6 μm.

Fig. 4b shows the effect of pH on the Soret band for Hb in blood. It can be seen that the blood film absorption spectra in the range of pH 4.0–10.0 exhibits a strong heme Soret band at 405 nm. However, when pH was decreased to below 3.0, the 405 nm Soret band absorption disappeared, while a new broad 380 nm band appeared, indicated that there is a great change in heme pocket. The change is reversible. When the blood film was transferred to pH 7.0 PBS from pH 3.0 solution, the Soret band at 405 nm reappeared. The reversibility of change in conformation should be attributed to a highly specific and tightly folding conformation of proteins [18]. At strong acid

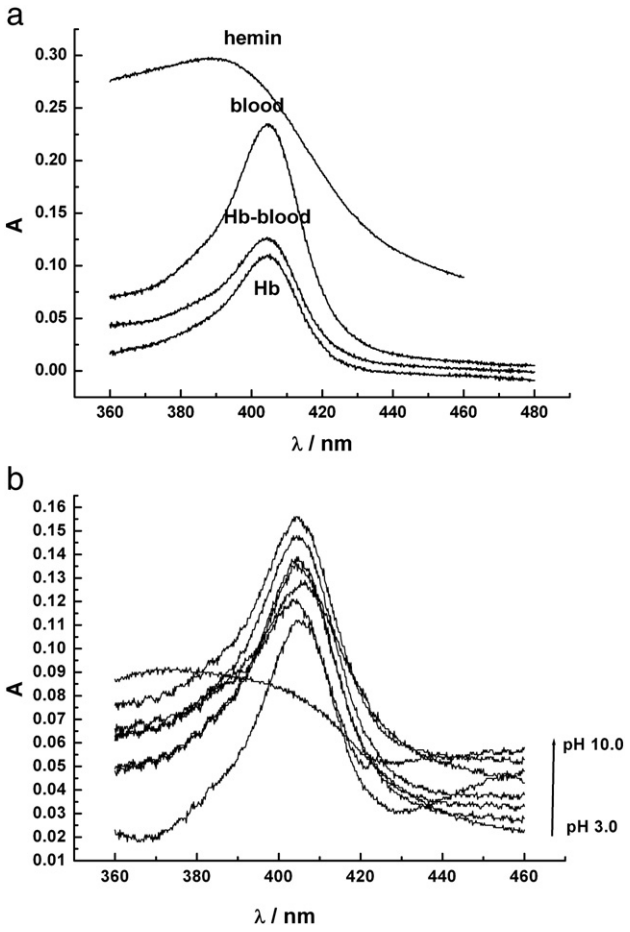


Fig. 4. (a) UV/VIS spectra of Hb, Hb-blood, blood and hemin films on glass slides. (b) pH dependence of UV/VIS spectra of blood film on a glass slide.

solutions, the heme group became unfolded and acted as a kind of "glue" that tends to hold the surrounding protein structure together. This state was a transient intermediate and was changed into its native structure easily when it was immersed in the buffers of suitable pH.

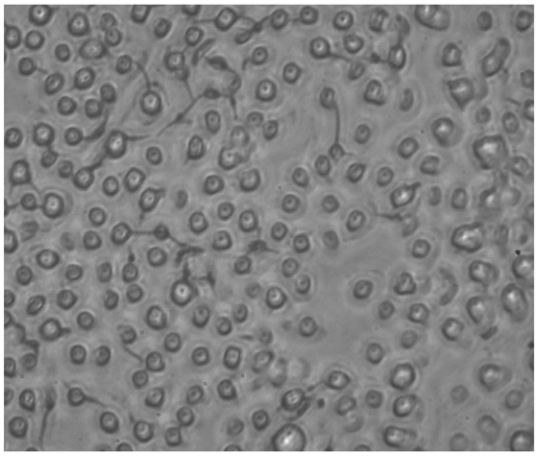


Fig. 5. The optical micrograph of red blood cells.

3.4. Catalytic activity

Fig. 6a shows the cyclic voltammertic curves for the blood/GC electrode by adding H_2O_2. The blood/GC electrode showed a pair of redox peaks attributed to hemeFe (III/II) in pH 7.0 PBS. When adding H_2O_2 into the buffer, the reduction peak at −0.40 V was increased, and this was accompanied by the disappearance of the oxidation peak. However, the direct reduction of H_2O_2 was not observed at bare GC or BS/GC electrode at the potential range from 0.2 to −0.8 V. These results are characteristics reduction of H_2O_2 by Hb in blood. The calibration curve shows that the reduction peak current increases linearly with the concentration of H_2O_2 in the range from 5×10^{-7} to 1.05×10^{-4} mol L^{-1} (Fig. 6b). When the concentration of H_2O_2 was higher than 3.5×10^{-4} mol L^{-1}, the calibration curve gradually tended to a plateau and then dropped down upon adding more H_2O_2, implying a progressive enzyme inactivation in the presence of higher concentration of H_2O_2, consistent with a Michaelis–Menten kinetics model. The

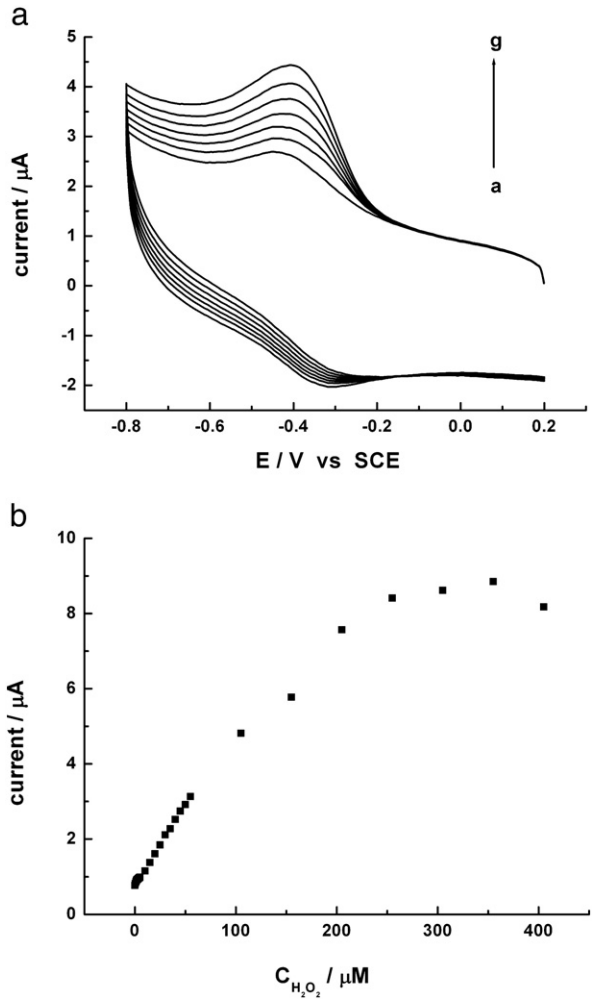


Fig. 6. (a) Cyclic voltammograms of blood/GC in 0.1 mol L^{-1} pH 7.0 PBS containing H_2O_2 (a) 0; (b) 0.5 μmol L^{-1}; (c) 1 μmol L^{-1}; (d) 1.5 μmol L^{-1}; (e) 2 μmol L^{-1}; (f) 2.5 μmol L^{-1}; (g) 3 μmol L^{-1}. (b) Plot of catalytic peak current vs the concentration of H_2O_2. At a scan rate of 300 mV/s.

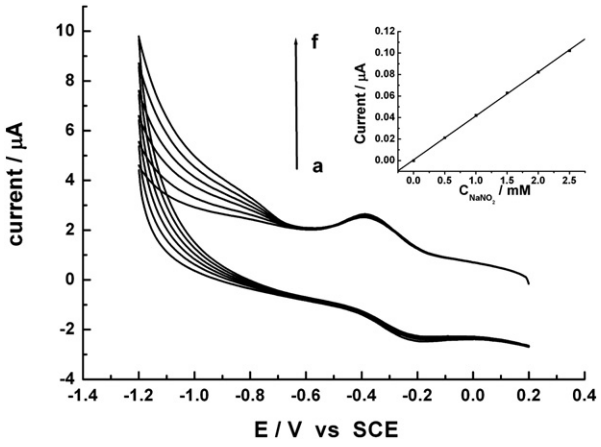


Fig. 7. Cyclic voltammograms of blood/GC in 0.1 mol L^{-1} pH 5.0 PBS containing NO_2^- (a) 0; (b) 0.5 mmol L^{-1}; (c) 1 mmol L^{-1}; (d) 1.5 mmol L^{-1}; (e) 2 mmol L^{-1}; (f) 2.5 mmol L^{-1}. (Inset) Plot of catalytic peak vs the concentration of $NaNO_2$. At a scan rate of 300 mV/s.

apparent Michaelis–Menten constant (K_m^{app}), which can provide an indication of the enzyme–substrate kinetics, can be obtained from the Lineweaver–Burk equation:

$$1/I_{ss} = 1/I_{max} + K_m^{app}/I_{max}C \quad (2)$$

Where, 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 substrate conditions. K_m^{app} can be obtained by the analysis of the slope and the intercept of the plot of reciprocals of the steady-state current versus H_2O_2 concentration. The K_m^{app} value for the elecrocatalytic activity of blood/GC electrode to H_2O_2 was determined to be 80.5 μmol L^{-1}. It was smaller than that of 896 μmol L^{-1} for a Hb/sol–gel film modified carbon paste electrode [9] and 427 μmol L^{-1} for a Hb/Triton X-100/PG electrode [19], which implies that the present modified electrode shows a higher affinity for H_2O_2.

Electrocatalytic reduction of nitrite was also studied by blood/GC electrode. Fig. 7 shows the cyclic voltammetric curves of blood/GC in pH 5.0 PBS upon addition of $NaNO_2$. The reduction peak can be observed at about −0.87 V. When the pH of the buffer was higher than 6.0, no electrocatalytic reduction peak of nitrite was observed by blood/GC electrode. It has been demonstrated that the reduction peak at −0.87 V arose from NO. NO is one of the products of the disproportionation reaction of nitrite in its acidic solution. The mechanisms can be expressed as followings:

$$2NO_2^- + 2H^+ \rightarrow 2NO + NO_3^- + H_2O \quad (3)$$

$$HbFe(II) + NO \rightarrow HbFe(II)-NO \quad (4)$$

$$HbFe(II)-NO + 2e^- + 2H^+ \rightarrow HbFe(II) + H_2O + N_2O \quad (5)$$

The reduction peak current is linearly dependent on the concentration of NO_2^- in the range from 5×10^{-4} to 3×10^{-2} mol L^{-1}.

3.5. Reproducibility and stability of the modified electrode

The stability and reproducibility of the modified electrode was also studied. The direct electrochemistry of blood/GC electrode can retain satisfying current values upon continues cyclic sweep over the potential range from −0.8 to 0.2 V at 200 mV/s. When the modified electrode was stored in air for about 2 weeks, it can retain >97% of its initial current response for its direct electron-transfer behavior. The fabrication of 6 blood/GC electrodes, made independently, showed an acceptable reproducibility with a R.S.D. of 3.7% for the current determined at a H_2O_2 concentration of 12 μM and 4.6% at a $NaNO_2$ concentration of 3 mmol L^{-1}.

4. Conclusions

Hb in pure blood films showed its direct electrochemical behavior when blood was immobilized on GC electrode. The formal potential (E^o) of Hb in blood is −0.36 V which attributed to the Fe (II)/Fe (III) couple of Hb in pH 7.0 PBS. The investigation of UV–VIS spectrum has shown that Hb in blood maintained its native conformation due to the native environment for Hb in blood. The conformation change is reversible in the pH range from 3.0 to 10.0. Optical micrographs indicated that the RBCs in blood can retain its native integrity. Hb in blood can catalyze the reduction of H_2O_2 and nitrite. The blood/GC electrode can be used to determine the concentration of H_2O_2 and NO_2^-.

Acknowledgements

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