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Direct electrochemistry and electrocatalysis of hemoglobin in gelatine film modified glassy carbon electrode

Hui Yao [a,b], Nan Li [a], Jin-Zhong Xu [a], Jun-Jie Zhu [a,*]

[a] *Key Lab. of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, PR China*
[b] *College of Applied Chemistry, Shenyang Institute of Chemical Technology, Shenyang 110142, PR China*

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Abstract

Direct electrochemical and electrocatalytic behavior of hemoglobin (Hb) immobilized on glass carbon electrode (GCE) containing gelatine (Gel) films was investigated. The characteristics of Hb/Gel film modified GC electrode were performed by using SEM microscopy, UV–vis spectroscopy and electrochemical methods. The immobilized Hb showed a couple of quasi-reversible redox peak with a formal potential of −0.38 V (versus SCE) in 0.1 M pH 7.0 PBS. The formal potential changed linearly from pH 4.03 to 8.41 with a slope value of −52.0 mV pH^{-1}, which suggested that a proton transfer was accompanied with each electron transfer (ET) in the electrochemical reaction. The Hb/gelatine/GCE displayed a rapid amperometric response to the reduction of H_2O_2 and nitrite.

Keywords: Hemoglobin; Gelatine; Coimmobilize; Direct electron transfer; Electrocatalysis; Electrochemistry

1. Introduction

The direct electron transfer between biomacromolecules (i.e., enzymes and proteins) and electrode surface has received considerable attention in recent years [1,2]. The mechanism of direct electron transfer from protein to electrode surface and their electrocatalytic reactions can serve as a desirable models to understand the electron transfer behavior in biological systems, which may elucidate the relationship between their structures and biological functions [3]. In addition, redox-active proteins are potentially useful in the fabrication of electrochemical biosensors and biomedical devices [4]. However, the electron transfer rate of redox proteins is rather slow at conventional electrodes. Usually, the protein electroactive center embeds deeply in the protein structure. Unfavorable orientation of protein molecules on electrode surface may block the electron transfer between electrode and protein electroactive centers. Moreover, the adsorption of protein molecules onto bare electrode surface may lead to their denaturation, which also decreases direct electron transfer rate [5,6]. Therefore, one of the main challenges is to select host matrix to provide a suitable microenvironment for the proteins and enhance the direct electron transfer between the proteins and underlying electrodes. Until now, plenty of materials have been used to promote electron transfer between redox proteins and electrodes, such as insoluble surfactants [7], polymers [8], clay [9], egg-phosphatidylcholine [10,11], zirconium dioxide nanoparticles [12], and so on [13–15]. However, to our knowledge, there has been no report by using gelatine to perform the direct electron transfer in a biosensor system. In this paper, direct electrochemical and electrocatalytic behavior of hemoglobin (Hb) immobilized on glass carbon electrode (GCE) by gelatine films was investigated.

Hemoglobin (Hb) is a kind of polypeptide enzymes consisting of four subunits and has a molecular weight of approximately 64,500 g mol^{-1}. A heme (iron porphyrin) group in each subunit acts as the active center [16]. The polypeptide chains fold into several segments that serve to stabilize the conformation of iron heme through hydrophobic interaction and hydrogen bonding [17]. Hb is an ideal biomolecule for the study of electron transfer reactions of heme proteins because of its important role in oxygen transport and storage, commercial availability, a known and documented structure, enzyme-like redox catalytic activity, and numerous studies of its electron transfer characters have been performed, both homogeneous and heterogeneous [18].

* Corresponding author. Tel.: +86 25 83594976; fax: +86 25 83317761.
E-mail address: jjzhu@nju.edu.cn (J.-J. Zhu).

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Gelatine (Gel) is a linear polypeptide that consists of different amounts of 18 amino acids, where glycine, praline and hydroxyproline are the most abundant [19]. Gelatine was selected here because of its excellent membrane-forming ability, good adhesion, biocompatibility, and nontoxicity [20,21], which are important for preparing the biosensor and functional devices. To our knowledge, there are no reports by using gelatine to construct Hb modified electrode. To gain further information about Hb/Gel film, some analytical techniques such as electrochemical method, scanning electron micrographs (SEM), and UV–vis absorption were employed. Electrochemical results show conclusively that Hb has fine reversible redox process on gelatine-modified GC electrodes and can keep its catalytic activity to H_2O_2 and nitrite.

2. Experimental

2.1. Reagents

Hemoglobin (Hb, bovine blood) was obtained from Sigma and used as received without further purification. Gelatine was purchased from the Shanghai Reagent Co. (China). The 0.1 mol L^{-1} phosphate buffers of various pH were prepared by mixing the stock solutions of Na_2HPO_4 and NaH_2PO_4 and adjusted by 0.1 mM NaOH and 0.1 mM H_3PO_4 solutions. Gelatine solution (1%) was prepared by dissolving gelatine flake in warm doubly distilled water. All solutions were made up with redistilled water. H_2O_2 (30% w/v solution) was purchased from Shanghai Chemical Reagent Company. The concentration of the more diluted hydrogen peroxide solutions prepared was determined by titration with cerium(IV) to a ferroin endpoint. All other chemicals were of analytical grade and used without further purification.

2.2. Apparatus and measurements

All electrochemical experiments were carried out in a three-electrode cell controlled by CHI 660 Electrochemical workstation (CH Instruments, USA). The working electrode was an Hb/gelatine-modified GC electrode. Reference and counter electrodes were a SCE and platinum wire, respectively.

Electrochemical impedance spectroscopy (EIS) experiments were performed in 0.1 M KNO_3 solution containing 5.0 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ (1:1), using an alternating current voltage of 5.0 mV. The impedance measurements were performed at an open circuit potential of 180 mV within the frequency range of 10^{-2} to 10^5 Hz.

Prior to the electrochemical measurements, buffer solutions were purged with high-purity nitrogen for at least 15 min, and then a nitrogen environment was kept over the solution in the cell. All experiments were performed at room temperature.

UV–vis absorption spectra were obtained with UV-3100 spectrophotometer (SHIMADZU) at room temperature.

The images for scanning electron micrographs (SEM) were obtained with a JEOL-4000EX electron microscope.

2.3. Electrode modification

Glassy carbon electrodes (GCE, 3 mm diameter) were polished successively with 1.0, 0.3, and 0.05 μm alumina powder on silk, and rinsed thoroughly with doubly distilled water between each polishing step. Next, the polished electrode was sonicated in 1:1 nitric acid, acetone and doubly distilled water and allowed to dry at room temperature.

0.1 mg Hb was dissolved in 0.1 ml of gelatine solution (1%), and then 3 μl of formaldehyde solution was added into the enzyme solution. The mixture was hand-mixed completely. The GCE was coated with a drop of 10 μl the resulting mixture, and then was left for at least 24 h at 4 °C. Thus, Hb/gelatine electrodes were obtained. The enzyme biosensors were stored at 4 °C in a refrigerator when not in use.

3. Results and discussion

3.1. Characteristics of Hb entrapped in gelatine films

3.1.1. Scan electron microscopy

The SEM images (Fig. 1) exhibit the morphology of (a) Gel, (b) Hb and (c) cross-linked Gel/Hb layers. Obviously, the SEM image of Hb cross-linked gelatine layer (Fig. 1c) was different from that of gelatine film (Fig. 1a) and Hb layer (Fig. 1b). Some small flowerlike accumulations with different shapes were observed in Hb cross-linked gelatine film, which was formed by aggregates of Hb molecules with gelatine during compressing process. The image gave the evidence of Hb immobilized in gelatine film.

3.1.2. UV–vis absorption spectra

UV–vis spectroscopy is also a useful conformational probe for heme proteins. The shape and position of the Soret absorption band of heme may provide information about possible denaturation of heme proteins, especially conformational change in the heme group region [22,23]. Fig. 2 shows the UV–vis spectra of the Gel, Hb and Gel/Hb cross-linked composite in water

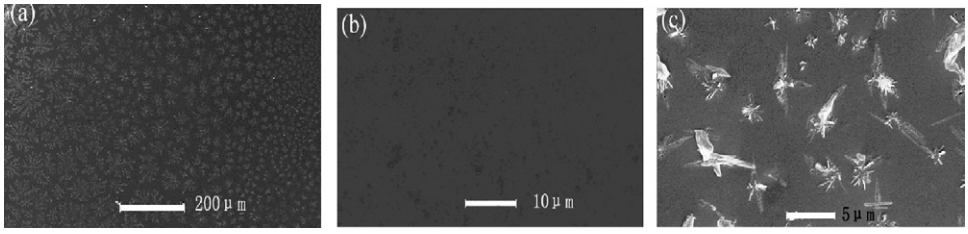

Fig. 1. SEM images of (a) gelatine, (b) Hb and (c) cross-linked Hb + gelatine films.

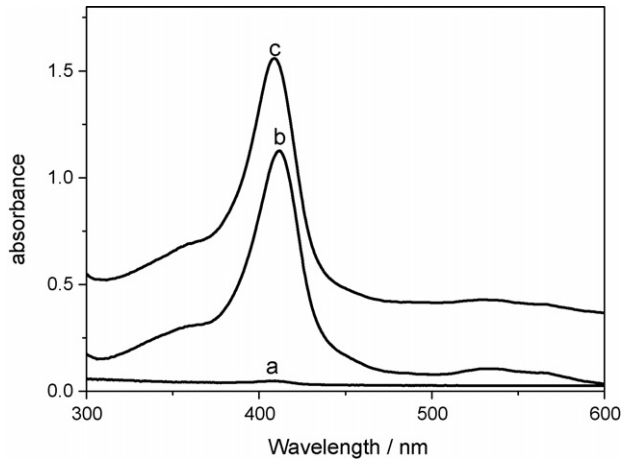

Fig. 2. UV–vis spectra curves: (a) gelatine, (b) Hb, (c) cross-linked Hb + gelatine.

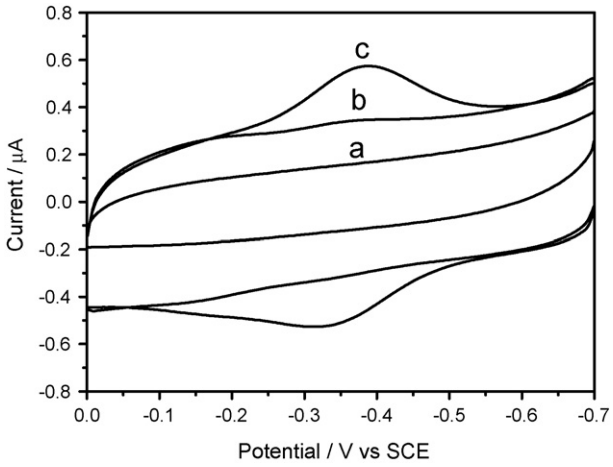

Fig. 3. Cyclic voltammograms of bare GC electrode (a), Gel/GC electrode (b), and Hb-Gel/GC electrode (c) in 0.1 M pH 7.0 PBS.

from 300 to 600 nm. For Gel (curve a in Fig. 2), no prominent peaks were observed among this range. The native Hb gave a heme band at 410 nm (curve b in Fig. 2). For Hb cross-linked with Gel, the absorption band was blue shifted by only 2 nm at 408 nm (curve c in Fig. 2). The slight shift may be due to the interaction between gelatine and proteins. Such interaction did not destroy the proteins' structure and change the fundamental microenvironment. A shift of only several nanometers suggests that the environment of protein is slightly changed and no significant denaturation occurred [24].

3.2. Electrochemical studies

3.2.1. Characterizations of the Hb/gelatine-modified GC electrode with CV and EIS measurements

The electrochemical behavior of the Hb immobilized in cross-linked gelatine film modified electrodes in 0.1 M PBS (pH 7.0) at 100 mV/s was studied by cyclic voltammetry. Fig. 3 shows the cyclic voltammograms obtained with bare GC electrode, gelatine-modified GC electrode and Hb entrapped in gelatine-modified GC electrode, respectively. No peaks were observed at both bare GCE and gelatine-modified GCE (curves a and b in Fig. 3), which indicated that gelatine was not electroactive in the potential window. However, when GC electrode was covered with a layer of Hb/Gel film, the cyclic voltammogram displayed a pair of stable, well-defined, and quasi-reversible redox peaks (curve c in Fig. 3). The formal potential $E^{\circ\prime}$ (taken as the average of reduction and oxidation peak potentials in the cyclic voltammogram) is about −0.38 V versus SCE, which is located at the characteristic potential of the heme Fe^{III}/Fe^{II} couples of the proteins [25,26]. The results present strong evidence that direct electron transfer between Hb and GC can be achieved in gelatine film. The peak-to-peak separation value (ΔE_P) is about 60 mV, indicating a fast electron transfer process. Thus the heme groups in Hb molecules still remained their structure and electrochemical activity.

In 0.1 M pH 7.0 PBS, typical cyclic voltammograms of Gel/Hb at different scan rates are shown in Fig. 4. It is clear that the potentials of the anodic peak and the cathodic peak hardly change with the scan rate, υ, i.e., and the peak potential is independent of the scan rate in the range between 25 and 600 mV s^{-1}. Both the anodic peak current and the cathodic peak current are proportional to υ at the above scan rate range, suggesting that electrode reactions are typical of the surface-controlled process.

Electrochemical impedance spectroscopy (EIS) was carried out to probe the changes of surface-modified electrodes. Fig. 5

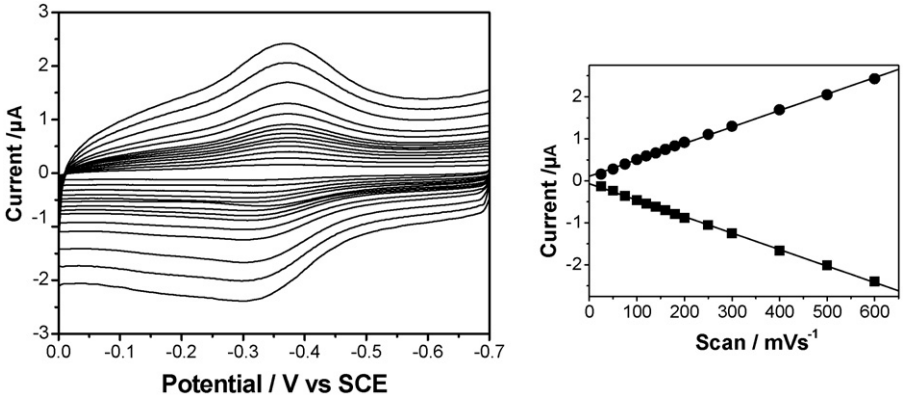

Fig. 4. Typical cyclic voltammograms of Hb-Gel/GCE at different scan rates (from inner to outer): 25, 50, 75, 100, 120, 140, 160, 180, 200, 250, 300, 400, 500, and 600 mV s^{-1} in the 0.1 M phosphate buffer (pH 7.0). Side figure shows the peak current dependence on the scan rate, υ.

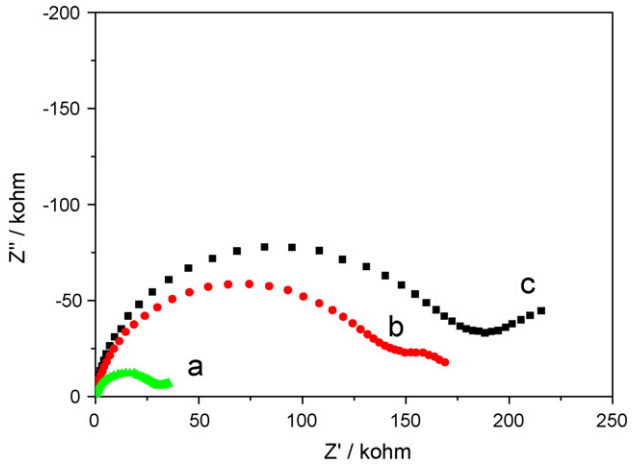

Fig. 5. EIS curves on (a) bare GC electrode, (b) gelatine-modified GC electrode, and (c) Hb/gelatine-modified GC electrode.

shows the curves of bare GC, Gel/GC, and Hb/Gel/GC, presented as Nyquist plot (Z'' versus Z'). Generally, the semicircle portion, observed at high frequencies, corresponds to the charge transfer limiting process. The charge transfer resistance R_t can be directly measured as the semicircle diameter [27]. As we can see that when gelatine was droped onto the GC surface (curve b), the semicircle dramatically increased, suggesting that gelatine film acted as an insulating layer and barriers that made interfacial charge transfer inaccessible. Next, Hb was immobilized on gelatine film (curve c), the semicircle also increased, indicating that Hb had been successfully immobilized on gelatine film.

3.2.2. *Effects of solution pH on direct electron transfer of Hb/Gel modified GCE*

The direct electrochemistry of Hb immobilized on GCE by incorporated with gelatine film shows a dependence on solution pH (Fig. 6). An increase of solution pH from pH 4.03 to 8.41 led to a negative shift of both reduction and oxidation peak potentials. The slope value of formal potential was −52 mV/pH (side in Fig. 6), which is close to the reported value of −55.7 mV/pH [28] and −52 mV/pH [29], respectively. The results indicate that a single protonation accompanies the electron transfer of Hb Fe(III) to electrodes and the catalytic process of electron transfer is nearly reversible. And the results can also indicate that gelatine film can provide a suitable microenvironment for Hb.

3.2.3. *Catalytic reactivity*

Electrocatalytic behavior of Hb/Gel film was examined and characterized by CV with various substrates such as hydrogen peroxide and nitrite.

Fig. 7 shows the electrocatalytic property of Hb/gelatine films toward hydrogen peroxides. In blank phosphate buffer, the protein electrode only gives the electrochemical behavior of Hb (Fig. 7a), a pair of quasi-reversible anodic and cathodic peaks. When 0.6 mM H_2O_2 was added into the solution, significant increase in the reduction peak at about −0.38 V was observed, and the oxidation peak current decreased (Fig. 7b) simultaneously. Thus, a potential of −0.38 V (versus SCE) is chosen as the detection potential for the amperometric determination of H_2O_2, where the risk for interfering reactions of oxygen and other electroactive substrates in the solution is minimized and then the background current would decrease to the lowest level. It was reasonable to think that the catalytic peak came from the interaction between Hb and H_2O_2. The electrocatalytic process can be expressed as follows [30,31]:

$$H_2O_2 + 2HbHFe(II) \rightarrow 2HbFe(III) + 2H_2O$$

These results are the characteristics of electrochemical catalytic reduction of H_2O_2 by Hb immobilized onto cross-link gelatine film modified electrode. The side curve gave the amperometric responses of the Hb/Gel modified electrode with successive additions of H_2O_2 to pH 7.0 PBS at −0.38 V. There was a linear relationship between catalytic reduction peak current and the concentration of H_2O_2 in the range from 50 μM to 1.2 mM. The detection limit of 3.4×10^{-6} M is estimated at a signal-to-noise ration of 3, which is similar to others' reports [12,13]. The results show that Hb entrapped into gelatine film can act as a very effective peroxidase-like biocatalyst to catalytic the reduction of H_2O_2.

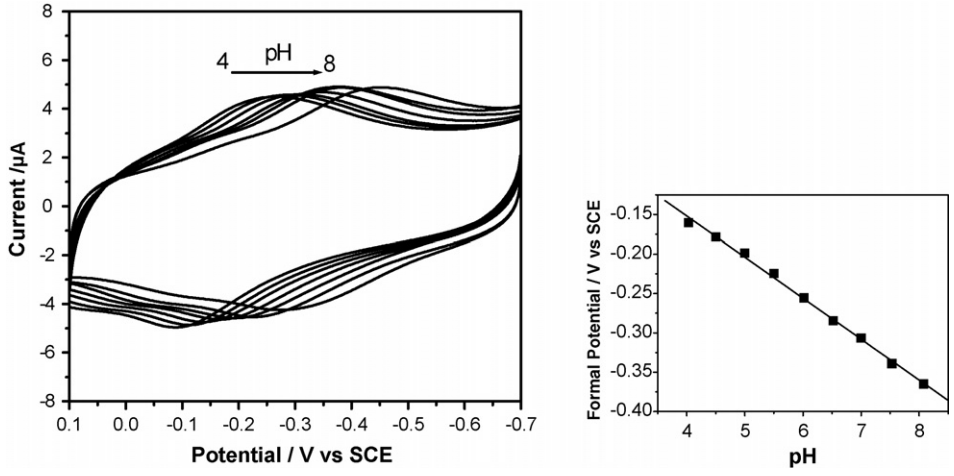

Fig. 6. Cyclic voltammograms of Hb-Gel/GCE in 0.1 M PBS with various pH values at scan rate of 100 mV s^{-1}. Side figure shows effect of pH on formal potential.

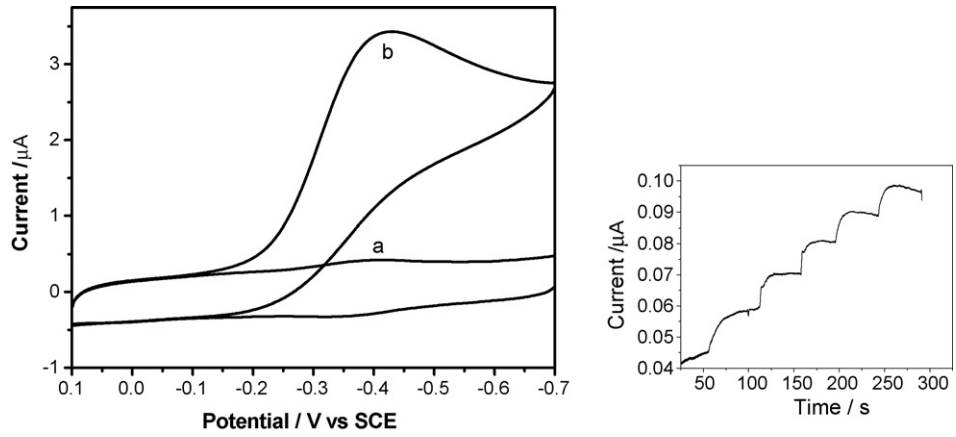

Fig. 7. Cyclic voltammograms of Hb-Gel/GCE in the absence of H_2O_2 (a) and in the presence of H_2O_2 0.6 mM (b) in 0.1 M pH 7.0 PBS at scan rate of 100 mV/s. Side curve is current–time plot with Hb/gelatine/GC upon the addition of 0.05 mM H_2O_2 in the 0.1 M phosphate buffer solution at pH 7.0 at −0.38 V.

Further experiments also found that the Hb immobilized in gelatine film electrode has the function as an electrochemical nitrite reductase. When $NaNO_2$ was added into pH 7.0 0.1 M PBS, a new catalytic reduction peak at about −0.82 V was observed (data not shown), and the peak current increases with further addition of $NaNO_2$, while no obvious peak appeared at gelatine-modified electrode without Hb immobilization. It indicates a promoting action of immobilized Hb on the gelatine film electrode for the electrochemical reduction of nitrite.

The modified electrode showed an acceptable repeatability and reproducibility. Five independent determinations at a H_2O_2 concentration of 50 μM show a relative standard deviation (R.S.D.) of 5.6%. The modified electrode had a long-term life for about 60-day period, which may be attributed to the strengthened biocompatibility and stability of gelatine.

4. Conclusions

We have successfully developed a novel and simple biosensor. In this paper, we first used gelatine-modified GCE as working electrodes. The cross-linked polypeptide substrate, gelatine, can form networks and provide a suitable biomembrane-like microenvironment for Hb, which greatly facilitated the electron exchange between Hb and electrodes. Hemoglobin can be effectively immobilized on GC electrode surface by incorporated with gelatine. The films are easily prepared and stable for a month or more, as long as the protein is stable. Electrode modified with this Hb/Gel film proposed high electrochemical activity and shows a fast direct electron transfer of Hb. Moreover, this modified electrode shows an electrochemical activity to the reduction of H_2O_2, NO_2^-, and so on without the aid of any electron mediator.

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