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Direct electrochemistry of hemoglobin entrapped in dextran film on carbon ionic liquid electrode

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Abstract. Direct electrochemistry of hemoglobin (Hb) entrapped in the dextran (De) film on the surface of a room temperature ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate ($BMIMPF_6$) modified carbon paste electrode (CILE) has been investigated. UV-Vis and FT–IR spectroscopy showed that Hb retained its native structure in the De film. Scanning electron microscopy (SEM) indicated an uniform film was formed on the electrode surface. Cyclic voltammetric experiments indicated that the electron transfer efficiency between Hb and the electrode was greatly improved due to the presence of the De film and ionic liquid, which provided a biocompatible and higher conductive interface. A pair of well-defined and quasi-reversible redox peak was obtained with the anodic and cathodic peaks located at –0·195 V and –0·355 V in pH 7·0 phosphate buffer solution, respectively. The electrochemical parameters were calculated by investigating the relationship of the peak potential with the scan rate. The fabricated De/Hb/CILE showed good electrocatalytic ability to the reduction of H_2O_2 with the linear concentration range from $4{\cdot}0 \times 10^{-6}$ to $1{\cdot}5 \times 10^{-5}$ mol/L and the apparent Michaelis–Menten constant (K_M^{app}) for the electrocatalytic reaction was calculated as 0·17 μM.

Keywords. Hemoglobin; dextran; direct electrochemistry; ionic liquid; cyclic voltammetry.

1. Introduction

Protein electrochemistry has gained more interests in recent years for its potential applications in biosensors and bioreactors. The mechanism of direct electron transfer (DET) between proteins and the electrode surface can also provide useful information on the structure variation, ligand binding and electron transfer process in biological system.[1,2] Due to the deep burying of the electroactive prosthetic groups, the partly adsorptive denaturation of the proteins on the electrode surface or the unfavourable orientations at the electrodes, the heterogeneous electron transfer process of biomacromolecules is rather slower at the traditional electrode surface. Efforts were devoted to establish different kinds of promoters or the supporting films to facilitate the direct electron transfer between the electroactive center and the electrode surface. Different kinds of immobilized methods have been successfully applied to fabricate the protein modified films such as insoluble surfactants, hydrogel polymers, polyelectrolytes and nanoparticles,[3–8] which provided a suitable microenvironment for the proteins to keep their native structures and gave the protein molecules more freedom in orientation. Then the direct electron transfer between the protein and the electrode surface can be easily achieved.

Room temperature ionic liquids (RTILs) have many specific physicochemical properties such as high chemical and thermal stability, relatively high ionic conductivity, negligible vapor pressure and wide electrochemical windows.[9–11] RTILs have been widely used in the field of electrochemistry,[12–15] organic synthesis,[16,17] inorganic synthesis,[18,19] liquid–liquid extraction processes.[20] Because of the higher ionic conductivity and wider electrochemical windows, RTILs have been used in electrochemistry and electroanalysis.[21,22] Buzzeo[23] and Endres[24] reviewed the recent progresses of RTILs in electrochemistry. Opallo *et al* investigated the ion transfer processes occurring across RTILs/aqueous solution interface

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with different electrode supports for RTIL phase such as electroactive ceramic carbon electrode, carbon nanofibres electrode or carbon ionic liquid electrode (CILE).[25–27] Yu *et al* described the formation and electrochemical application of molecular films of water-miscible imidazolium-based IL on glassy carbon electrode (GCE).[28] Li *et al* used single-walled carbon nanotube (SWCNT) and 1-hexyl-3-methylimidazolium hexafluorophosphate ($HMIMPF_6$) to make a gel microelectrode for studies on the electrochemical oxidation of nitric oxide.[29] Sun *et al* fabricated a *N*-butylpyridinium hexafluorophosphate ($BPPF_6$) modified carbon paste electrode and investigated its electrocatalytic behaviours to ascorbic acid.[30] RTILs have been used in the field of protein electrochemistry. Different kinds of RTILs composite material modified electrodes have been devised. For example, Li *et al* used a chitosan/$BMIMPF_6$ for the immobilization of hemoglobin (Hb) and investigated its electrochemical behaviours.[31] Sun *et al* also fabricated different ionic liquid modified carbon paste electrodes and further used it as the basal electrode for the protein electrochemistry.[32–34]

In this paper, a Hb modified electrode was fabricated by using dextran (De) film and CILE. Hb is a commonly used heme protein, which contains two α and two β subunits, each of them has an electroactive iron heme as prosthetic group. It can store and transport oxygen in muscle cells. Hb is often used as model for the investigation of the electron transfer reaction of heme proteins. De is a biocompatible materials and Hb can keep its native molecular structure in the De film. Experimental results indicated that the direct electron transfer of Hb was achieved and the fabricated De/Hb/CILE biosensor showed good electrocatalytic activity to the reduction of H_2O_2.

2. Experimental

2.1 *Reagents*

Bovine hemoglobin (Hb, MW. 64500) was purchased from Tianjin Chuanye Biochemical Limited Company. Room temperature ionic liquid 1-butyl-3-methyl-imidazolium hexafluorophosphate ($BMIMPF_6$) was obtained from Hangzhou Kemer Chemical Limited Company. Dextran (De, average M_W 20000) was obtained from China Guoyao Chemical Reagents Limited Company. 0·1 mol/L phosphate buffer solutions (PBS) with various pH values were prepared by mixing stock standard solutions of K_2HPO_4 with KH_2PO_4 and adjusted to the pH with 0·1 mol/L H_3PO_4 or NaOH. All the other chemicals were of analytical reagent grade and doubly distilled water was used in all the experiments.

2.2 *Apparatus*

The electrochemical measurements were performed on a CHI 750B electrochemical workstation (Shanghai CH Instrumentation, China) with a conventional three-electrode system composing a Hb film modified CILE as working electrode, a platinum wire as auxiliary electrode and a saturated calomel electrode (SCE) as reference electrode. UV-Vis absorption spectra were recorded on a Cary 50 probe spectrophotometer (Varian Company, Australia) with the wavelength range from 300 to 650 nm. FT–IR spectra were recorded by a Tensor 27 FT–IR spectrophotometer (Bruker Company, Germany). Scanning electron microscopy (SEM) was performed on a JSM-6700F scanning electron microscope (Japan Electron Company, Japan). The pH values were measured with a pHs-25 acidity meter (Shanghai Hongyi Instrument Factory, China).

2.3 *Electrode preparation*

The $BMIMPF_6$ modified carbon paste electrode (CPE) was prepared by mixing $BMIMPF_6$ with graphite powder at a ratio of 25/75 (w/w) in an agate mortar. The homogeneous paste was packed into a cavity of glass tube with the diameter of 4.0 mm. The electrical contact was obtained with a copper wire connected to the paste in the end of tube. The surface of CILE was polished by smoothing it on a weighing paper. The traditional CPE was prepared by hand-mixing of graphite powder with liquid paraffin at a ratio of 70/30 (w/w) in an agate mortar with the procedure similar to that of the CILE.

The Hb modified CILE was prepared with the following procedure. A 10·0 μL, of 20.0 mg/mL Hb was cast on the surface of CILE and left it to dry at room temperature. Then a 10·0 μL of 6·0 mg/mL De solution (in pH 7·0 PBS) was applied on the electrode surface, which could form a stable film and fix the Hb tightly on the surface of electrode. A 10 mL beaker was covered over the electrode so that water could evaporate slowly in air and an uniform film electrode could be formed. Other modified electrodes such as De/CILE or De/Hb/CPE were prepared by same procedure.

2.4 *Procedures*

Electrochemical experiments were performed at room temperature by using a CHI 750B electrochemical workstation with cyclic voltammetry. The test solutions were deoxygenated by bubbling pure nitrogen through them for at least 15 min. The experiments were carried out with the electrochemical cell kept in an atmosphere of pure nitrogen.

3. Results and discussion

3.1 *UV-Vis absorption spectra*

The positions of the Soret absorption band of heme may provide information about possible denaturation of heme protein, and particularly on conformational change in the heme group region. Therefore, UV-Vis spectroscopy is a useful tool for conformational study of heme region.[35] If Hb is denatured, the Soret band will change or disappear. Figure 1 showed the UV-Vis absorption spectra of De and Hb mixture in different pH buffer solution. For natural Hb in water, the Soret band appeared at 405·7 nm (curve d). In pH 10·0 and 7·0 buffer solution, the Soret band appeared at 407·0 nm and 405·9 nm (curves b and c), respectively, which was very close to that of the native Hb. Since the difference of Soret band was less, the results suggested that Hb in De film was not distinctly denatured and its secondary structure was kept as the native state of Hb. However, when the pH value was changed to 2·0, the Soret band moved to 365·1 nm with the peak shape being smaller and broader (curve a), which indicated the denaturation of Hb in the more acidic solution. The results indicated that De might be a fine film material and Hb molecule in the De film had similar secondary structure to its native state in aqueous solution.

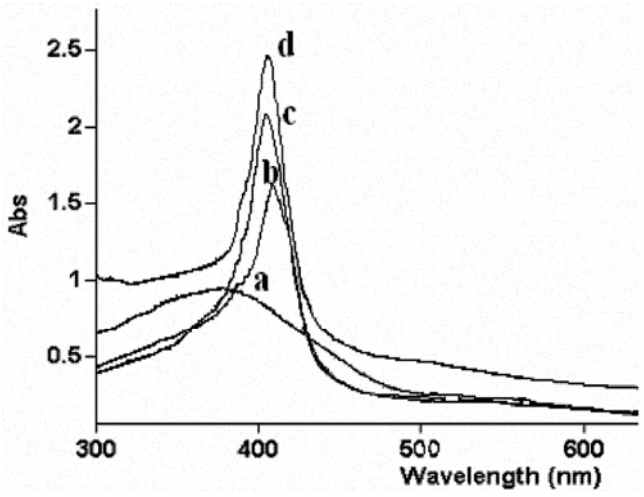

Figure 1. UV-Vis absorption spectra of De/Hb in pH; (a) 2·0; (b) 10·0; (c) 7·0 PBS; (d) Hb in water solution, respectively.

3.2 *FT–IR spectra*

FT–IR spectroscopy is also used to check the conformational integrity of the heme proteins. The characteristics of amide I and amide II bands of protein infrared bands provide the detailed information on the secondary structure of polypeptide chain. The amide I band (1700–1600 cm^{-1}) is caused by C=O stretching vibration of peptide linkages in the backbone of protein and amide II band (1620–1500 cm^{-1}) is assigned to a combination of N–H bending and C–N stretching vibration.[36] If Hb is denatured, the intensities and shape of amide I and II band will diminish or even disappear. Figure 2 showed the FT–IR spectra of Hb in the De film. The amide I and amide II bands of Hb in De film appeared at 1654·09 cm^{-1} and 1539·18 cm^{-1} (figure 2b), which had the same position to the native state of Hb spec-

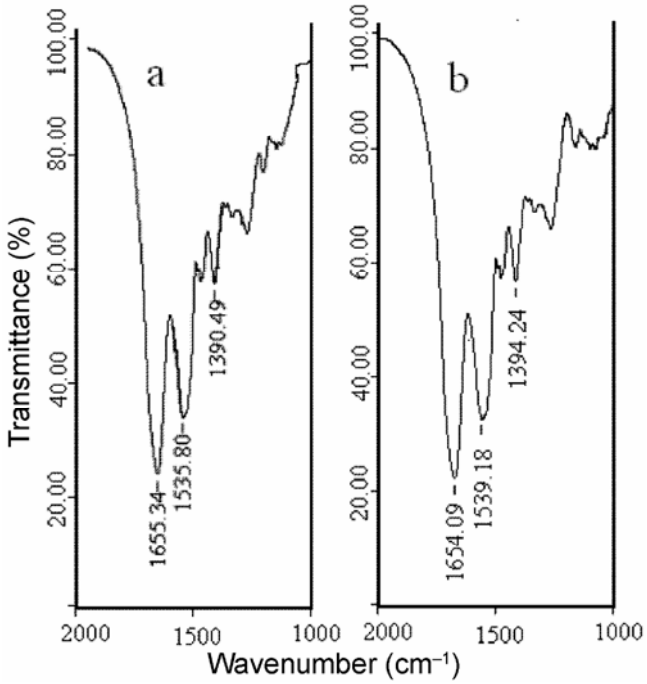

Figure 2. FT–IR spectra of the films of (a) Hb; (b) De/Hb.

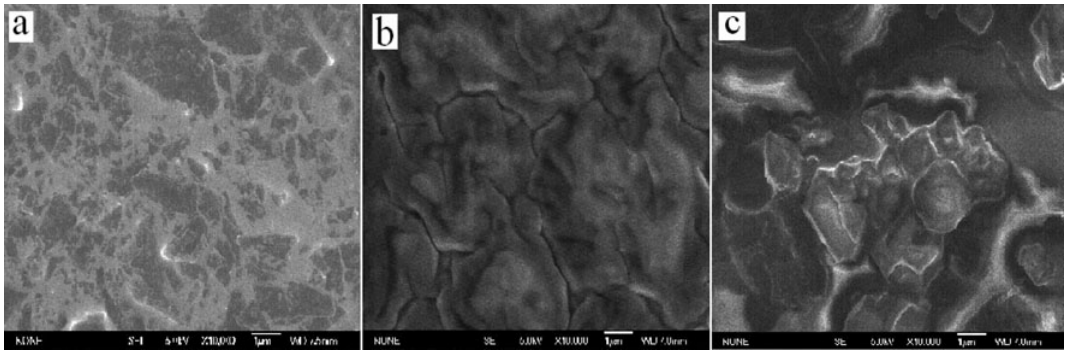

Figure 3. SEM images of (**a**) CILE; (**b**) Hb/CILE; (**c**) De/Hb/CILE.

trum at 1655·34 cm^{-1} and 1535·80 cm^{-1} (figure 2a). These results showed that Hb retained the essential features of its original structure in the De film.

3.3 *Scanning electron microscopy*

Scanning electron microscopy (SEM) was used to characterize the morphology of different kinds of modified electrodes and the results were shown in figure 3. The SEM image of CILE (figure 3a) showed a fairly uniform surface, which was due to the embedded of $BMIMPF_6$ between the carbon layers. As an organic ionic liquid with good solubility and high viscosity, the presence of RTIL is capable of better dispersing the graphite powder in the paste than the conventional mineral oils and bridge the carbon flakes. After Hb was cast on the surface of CILE, the electrode surface looked more loose and uneven with the protrusion assignable of Hb (figure 3b). While on the surface of the De/Hb/CILE (figure 3c), an uniform film was formed, which indicated that De had good film forming ability and could interlock with Hb together to form a stable film.

3.4 *Direct electrochemistry*

Figure 4 showed the cyclic voltammograms of different electrodes in pH 7.0 PBS at the scan rate of 100 mV/s. It can be seen that no redox peaks appeared on the CILE (curve a) and De/CILE (curve b). However, when the Hb was entrapped in the De film and modified on the surface of CILE, direct electron transfer between Hb and CILE was easily achieved and a pair of well-defined, quasi-reversible redox peaks were observed in the cyclic voltammogram (curve c), which was attributed to the heme Fe (III)/Fe (II) redox couple. For comparison, the direct electrochemistry of the De/Hb/CPE was also investigated with the cyclic voltammograms as shown in figure 5. Only a small reduction peak appeared without the oxidation peak (curve c), indicating the irreversible electrochemical process on the De/Hb/CPE. Due to the presence of ILs in the carbon paste, CILE had showed some advantages such as biocompatible interface and high electron transfer rate. So the CILE played an important role in realizing the direct electron transfer with Hb molecules on the surface. At the same time De film is an electro-inactive substance in the potential ranges and it has good biocompatibility with polymeric network. The De film on the electrode provided suitable microenvironments to maintain the natural conformation of Hb and kept the bioactivity of Hb effectively.

From the cyclic voltammetric results the anodic (Epa) and cathodic (Epc) peak potentials were obtained as –0·195 V and –0·355 V (vs SCE), respectively. The apparent formal potential ($E^{0\prime}$), which was calculated from the equation as $E^{0\prime} = (E_{pa} + E_{pc})/2$, was got as –0·257 V and was close to the previous report.[37] The peak-to-peak potential separation (ΔE_p) at 100 mV/s was 0·16 V and the ratio of redox peak current (I_{pa}/I_{pc}) was approximately to be 1·0. These features also indicated that the direct electron transfer of Hb on the surface of CILE in the De film was achieved.

With the increase of the scan rate the redox peak currents increased gradually. In the scan rate range from 50 to 250 mV/s the peak currents increased linearly with the scan rate (figure 6), which were characteristics of surface-confined thin-layer electrochemical behaviours. So the electrochemically active Hb Fe (III) in the film on the CILE surface was reduced to Hb Fe (II) on the forward scan and on the reverse scan the Hb Fe (II) produced was converted to Hb Fe (III).

By integration of the cathodic peak currents of cyclic voltammogram, the average surface concentration (Γ*) of electroactive species could be calculated according to the following equation:

$$Q = nFA\Gamma^*, \tag{1}$$

where Q is charge passing through the electrode with full reduction of electroactive Hb in the film, n is the number of electron transferred, F is the Faraday's constant, A is the area of electrode. From the experimental results the average surface concentration of electroactive Hb (Γ*) on the electrode surface was estimated to be $2{\cdot}64 \times 10^{-9}$ mol/cm^2. Because Hb was cast onto the surface of CILE and the total amount of Hb in the film was calculated as $2{\cdot}47 \times 10^{-8}$ mol/cm^2. So the electroactive Hb on the electrode surface accounted for 10·7% of the total amount of Hb in the film, which was larger than the reported values of 1·3% (ref. 38) and 2·0%.[31] The results indicated that only the Hb molecules close to the electrode surface could exchange the electron with the CILE.

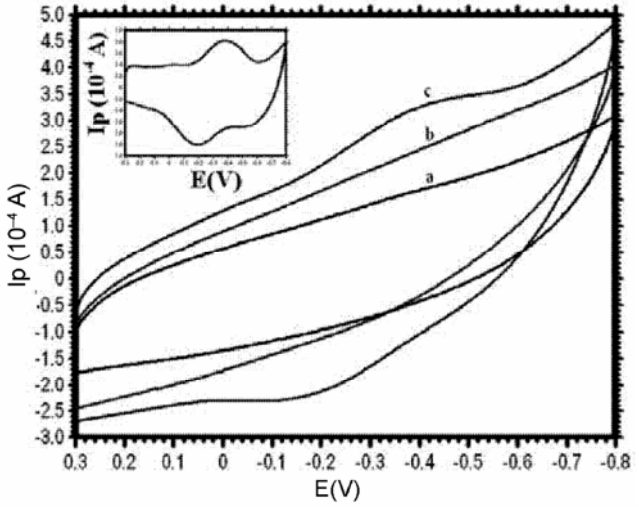

Figure 4. Cyclic voltammograms of (a), bare CILE; (b), De/CILE; (c), De/Hb/CILE at the scan rate of 100 mV/s in pH 7·0 PBS (inset: the background-subtracted cyclic voltammogram of De/Hb/CILE using data presented in curve b).

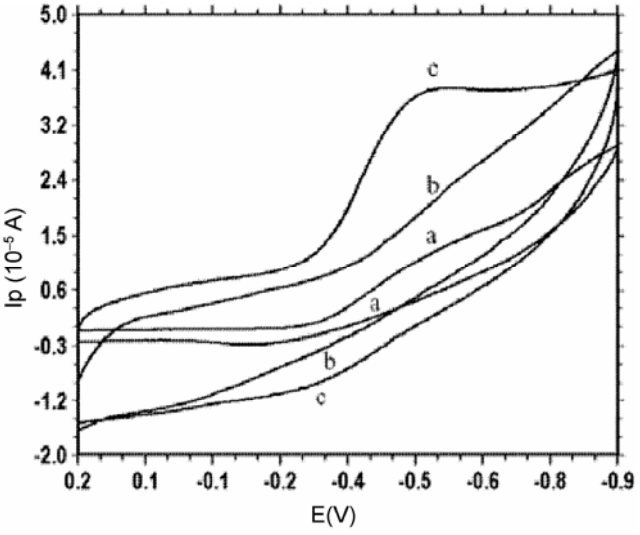

Figure 5. Cyclic voltammograms of (a), bare CPE; (b), De/CPE; (c), De/Hb/CPE at the scan rate of 100 mV/s in pH 7·0 PBS.

The relationship of the peak potentials with scan rate was further constructed, which could be used for the calculation of the electrochemical parameters. According to the Laviron's equations:[39]

$$Epc = E^{0\prime} - \frac{2{\cdot}3RT}{\alpha nF}\log v \tag{2}$$

$$Epc = E^{0\prime} - \frac{2{\cdot}3RT}{(1-\alpha)nF}\log v \tag{3}$$

$$\log k_s = \alpha\log(1-\alpha) + (1-\alpha)\log\alpha - \log\frac{RT}{nFv} - \frac{(1-\alpha)\alpha Fn\Delta Ep}{2{\cdot}3RT}, \tag{4}$$

where α is the electron transfer coefficient, n is the electron transfer number, k_s is the apparent heterogeneous electron transfer rate constant, v is the scan rate, $E^{0\prime}$ is the apparent formal potential and F is the Faraday's constant.

A linear relationship between the Ep with the $\log v$ was established and two straight lines were obtained with the results as shown in figure 7. The linear regression equations were calculated as $Epa(V) = 0{\cdot}047 \log v\,(\text{mV/s}) - 0{\cdot}085$ ($n = 7$, $\gamma = 0{\cdot}993$) and $Epc(V) = -0{\cdot}076 \log v\,(\text{mV/s}) - 0{\cdot}54$ ($n = 7$, $\gamma = 0{\cdot}992$). According to the (2) and (3) the electron transfer coefficient (α) and the electron transfer number (n) could be calculated and the result was 0·463 and 0·89, respectively. Based on the (4) the value of k_s was further calculated from the relationship of ΔEp with $\log v$ and the result was 0·428 s^{-1}.

The effect of buffer pH on the direct electrochemistry of Hb was also examined. It is well-known that most of the heme proteins exhibit a pH-dependent conformational equilibrium and the pH value of the

buffer solution influences the electrochemical reactions of the heme proteins. In the pH range from 5·5 to 9·0 stable and well-defined cyclic voltammograms could be obtained. The apparent formal potential ($E^{0\prime}$) of Hb showed a negatively shift with the increase of buffer solution. A good linear relationship of $E^{0\prime}$ and pH value was obtained with the linear regression equation as $E^{0\prime}$ (V) = –0·0447 pH +0·0076 (n = 8, γ = 0·992). The slope value was –44·7 mV/pH, which was reasonably close to the theoretical value of –56·0 mV/pH at 20°C for a one proton-coupled single electron transfer process. So the electrode process can be represented as Hb Fe(III) + H^+ + e $\rightleftharpoons$ Hb Fe(II).[40]

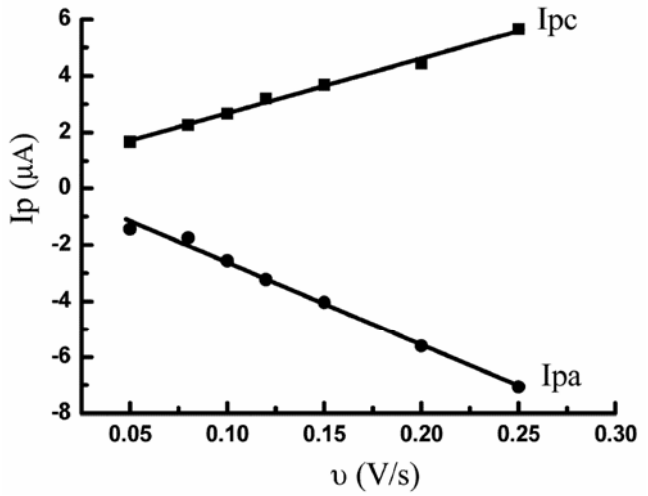

Figure 6. Linear relationship of redox peak current (Ip) versus scan rate (v) in pH 7·0 PBS.

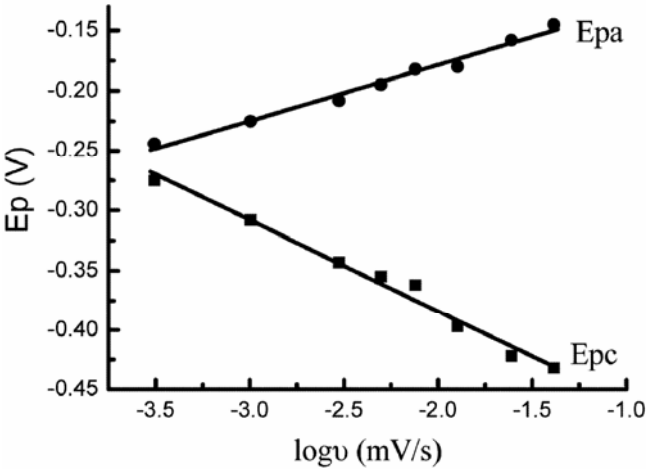

Figure 7. Linear relationship of the anodic (Epa) and cathodic (Epc) peak potential against log v.

3.5 *Electrocatalysis of De/Hb/CILE to H_2O_2*

Electrocatalytic reduction of H_2O_2 on De/Hb/CILE was investigated by cyclic voltammetry. With the addition of H_2O_2 into a pH 7·0 PBS in the electrochemical cell, an obvious increase of the cathodic peak current was observed with the decrease of the anodic peak current, which was the characteristic of an electrochemically catalytic reaction. The catalytic peak potential was located at –270 mV and the cathodic peak current increased with the increase of H_2O_2 concentration. While no electrochemical signals could be observed on the bare CILE and De/CILE in the same potential range. A linear relationship between the catalytic peak current and H_2O_2 concentration was obtained in the range of $4{\cdot}0 \times 10^{-6}$ to $1{\cdot}5 \times 10^{-5}$ mol/L with the linear regression equation as Iss(μA) = 0·013C (μmol/L) + 0·355 (n = 6, γ = 0·993) and the detection limit was 1.0×10^{-7} mol/L (3σ).

The apparent Michaelis–Menten constant (K_M^{app}), which provided to be an indication of the enzyme-substrate kinetics, was calculated from the electrochemical version of the Lineweaver–Burk equation.[41]

$$\frac{1}{I_{SS}} = \frac{1}{I_{\max}} + \frac{K_M^{app}}{I_{\max} C}, \tag{5}$$

where I_{ss} is the steady 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 condition.

By analysis of the steady-state current and the H_2O_2 concentration, the apparent Michaelis–Menten constant could be calculated as 0·17 μmol/L, which was lower than that of some previous reports.[42,43] Thus, the Hb immobilized in De film had good affinity to H_2O_2 and showed high electrocatalytic efficiency.

The De/Hb/CILE showed good stability. The Hb modified electrode was stored in a refrigerator at 4°C for 15 days with the peak current decreased for 3% and 30 days with the peak current decreased for 8%, The results showed good storage stability for the De/Hb/CILE.

3.6 *Electrochemical characteristics of modified electrodes*

Electrochemical impedance spectroscopy (EIS) is a valuable method to give information on the imped-

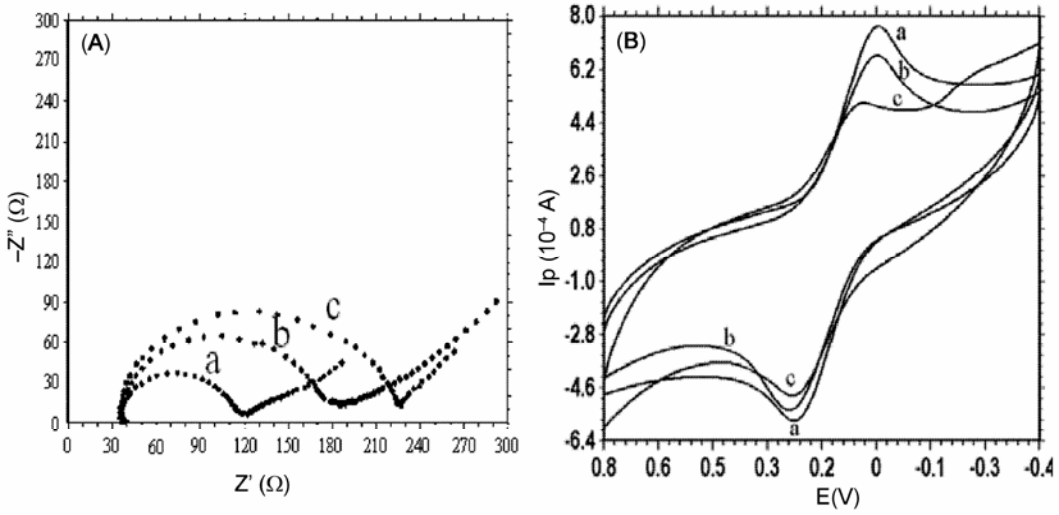

Figure 8. (**A**) Electrochemical impedance spectroscopy for curve (a), bare CILE; curve (b), De/CILE; (c), De/Hb/CILE in the presence of 10·0 mmol/L $[Fe(CN)_6]^{3-/4-}$ and 0·1 mol/L KCl with the frequency range from 10^5 to 0·1 Hz. (**B**) Cyclic voltammograms of curve (a), bare CILE; curve (b), De/CILE; curve (c), De/Hb/CILE in 0·1 mol/L KCl solution containing 1·0 mmol/L $[Fe(CN)_6]^{3-}$ with the scan rate of 100 mV/s.

ance changes of modified electrode and the semicircle diameter of EIS equals to the electron transfer resistance (Ret). By using $1{\cdot}0 \times 10^{-4}$ mol/L $Fe(CN)_6^{3-/4-}$ solution as the redox probe with the frequencies sweep from 10^5 to 0·1 Hz, the EIS of different electrodes were shown in figure 8(A). The Ret of bare CILE was estimated to be 72·0 Ω (curve a), which was due to the presence of highly conductive RTILs in the mixture with graphite powder. The Ret value of De/CILE was got as 108·2 Ω (curve b), indicating the presence of De film could hinder the electron transfer of $Fe(CN)_6^{3-/4-}$. However, the Ret value of De/Hb/CILE was further increased to 134·5 Ω (curve c), which was larger than that of bare CILE and De/CILE. This might be due to the presence of Hb in the film, which further hindered the transfer of electrons. Cyclic voltammograms of different electrodes were further recorded in $Fe(CN)_6^{3-}$ solution with the results shown in figure 8(B). On CILE a pair of well-defined quasi-reversible redox peaks were observed (curve a). While on the De/CILE (curve b) the redox peak current decreased, which was due to the presence of De on the electrode surface. On the De/Hb/CILE (curve c) the redox peak current was further decreased, indicating the presence of Hb molecules in the De film hindered the electron transfer rate. The results were in consistent with that of EIS, which indicated the successful fabrication of the De and Hb modified electrode.

4. Conclusions

In this paper, an ionic liquid $BMIMPF_6$ modified carbon paste electrode (CILE) was constructed and Hb was immobilized on the surface of CILE with the help of De film by layer-to-layer casting method. The presence of De film provided a biocompatible microenvironment for the Hb. UV-Vis and FT–IR spectra indicated that Hb retained its secondary structure in the film. The CILE provided a biocompatible interface with high conductivity and excellent electron transfer efficiency. The De/Hb film modified CILE exhibited good electrochemical catalytic reduction of H_2O_2, which could be further used for the construction of H_2O_2 biosensor.

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