Direct Electrochemistry and Electrocatalysis of Myoglobin with Ionic Liquid through Multilayers Film on Carbon Ionic Liquid Electrode

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Multilayers of myoglobin (Mb) with ionic liquid 1-ethyl-3-methylimidazolium tetrafluoroborate ([EMIM]BF₄) was assembled on carbon ionic liquid electrode (CILE) based on the electrostatic attraction between the negatively charged Mb and the positively charged imidazolium ion of IL. The CILE was fabricated with 1-ethyl-3-methylimidazolium ethylsulfate ([EMIM]EtOSO₃) as the modifier, which exhibited imidazolium ion on the electrode surface. Then Mb molecules were assembled on the surface of CILE step-by-step to get a {IL/Mb}_n multilayer film modified electrode. UV-Vis adsorption and FT-IR spectra indicated that Mb remained its native structure in the IL matrix. In deaerated phosphate buffer solution (pH 7.0) a pair of well-defined quasi-reversible redox peaks appeared with the apparent formal potential ($E^{0'}$) as -0.212 V (*vs.* SCE), which was the characteristic of Mb heme Fe(III)/Fe(II) redox couples. The results indicated that the direct electron transfer of Mb was realized on the modified electrode. The {IL/Mb}_n/CILE displayed excellent electrocatalytic ability to the trichloroacetic acid reduction in the concentration range from 2.0 to 22.0 mmol/L with the detection limit of 0.6 mmol/L (3 σ). The proposed method provides a new platform to fabricate the third generation biosensor based on the self-assembly of redox protein with ILs.

Keywords: Myoglobin; Direct electrochemistry; Carbon ionic liquid electrode; 1-Ethyl-3-methylimidazolium tetrafluoroborate.

INTRODUCTION

Investigations on the direct electrochemistry of proteins or enzymes can provide a good model for the mechanistic studies on electron transfer in biological systems, which can help us understand the energy transform and material metaboly in life processes. However, it is difficult for the redox proteins in solutions or on the surface of conventional electrode to exchange electrons directly with the naked solid electrodes because of the embedment of the redox center of proteins in polypeptide chain structures. In these circumstances, an important object for biochemists is to achieve efficient electrical contact between the protein and the electrode surface. Various methods including sol-gel,¹ electropolymerization,² covalent-binding,³ layer-by-layer assembly⁴ and direct embedded in biocompatible membrane⁵ were used to immobilize the protein on the electrode surface and then enhance the electron transfer rate of redox proteins.

Ionic liquids (ILs) are molten salts that composed of organic cations and either organic or inorganic anions. They have exhibited many specific characteristics such as high chemical and thermal stability, negligible vapor pressure and high conductivity, which make them environmental friendly solvents used in various electrochemical processes. In recent years the applications of ILs in the fields of electrochemistry and electroanalysis have received much attention.^{6,7} ILs can be used as a novel electrode material for catalysis or sensing, or as the supporting electrolyte for the electrochemical reaction.⁸ Maleki et al.⁹ reported the simultaneous determination of dopamine, ascorbic acid and uric acid using carbon ionic liquid electrode (CILE). Sun et al.¹⁰ also applied CILE to the simultaneous

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detection of adenine and guanine. Recently the direct electrochemistry of redox proteins has also been achieved with different types of ILs. Due to the biocompatibility of the ILs, the activity of protein shows similar or even higher effect in ILs than in the conventional organic solvents. For example Wang et al.¹¹ investigated the direct electrochemistry and electrocatalysis of heme proteins with IL as electrolyte. Gao et al.¹² reported direct electrochemistry of myoglobin (Mb) on DNA modified CILE. Sun et al.¹³ electrodeposited Co nanoparticles on CILE and further used it as the platform to construct a Mb electrochemical biosensor. Chen et al.¹⁴ investigated the direct electrochemistry of horseradish peroxidase (HRP) in the Nafion-IL composite film. Zhang et al.¹⁵ used IL-hyaluronic acid biocomposite material modified electrode to realize the direct electrochemistry of Mb. Safavi et al.¹⁶ investigated the direct electrochemistry and electrocatalytic of hemoglobin (Hb) on CILE with octylpyridinium chloride ([OcPy][Cl]). Sun et al.¹⁷ fixed Mb in Nafion-BMIMPF₆ composite film on surface of CILE and studied the direct electrochemical behaviors.

Trichloroacetic acid (TCA) is an important organohalide pollutant present in the environment. It is widely used in biochemistry for the precipitation of macromolecules such as proteins, DNA and RNA. Also TCA can be found in the drinking water due to the chlorine disinfection and is the major metabolite of the trichloroethylene and tetrachloroethylene.¹⁸ So it is necessary to establish sensitive methods for the TCA detection.

In this paper a carbon ionic liquid electrode (CILE) was fabricated by using 1-ethyl-3-methylimidazolium ethylsulfate ([EMIM]EtOSO₃) as the binder. Then the Mb and ionic liquid 1-ethyl-3-methylimidazolium tetrafluoroborate ([EMIM]BF₄) was alternatively adsorbed on the electrode surface to get a $\{IL/Mb\}_n/CILE$. Experimental results indicated that the direct electron transfer of Mb was realized in the LBL film with a pair of well-defined redox peaks appeared. The $\{IL/Mb\}_n/CILE$ showed good electrocatalytic activity to the reduction of trichloroacetic acid (TCA) with high sensitivity.

EXPERIMENTAL

Chemical

Ionic liquids 1-ethyl-3-methylimidazolium ethylsulfate ([EMIM]EtOSO₃) and 1-ethyl-3-methylimidazolium tetrafluoroborate ([EMIM]BF₄) were purchased from Hangzhou Kemer Chemical Limited Company. Horseheart myoglobin (Mb, MW.17800, Sigma), graphite powder (average particle size \leq 30 µm, Shanghai Colloid Chemical Plant) and trichloroacetic acid (TCA, Tianjin Kermel Chemical Reagent Co. Ltd.) were used as received. 0.1 mmol/L phosphate buffer solutions (PBS) were used as supporting electrolyte, which was deaerated for 30 min with high-purity nitrogen and kept under the nitrogen atmosphere during the experiments. Other chemicals were of analytical reagent grade and the solutions were prepared with doubly distilled water.

Apparatus

Electrochemical experiments were performed on a CHI 750B electrochemical workstation (Shanghai CH Instrument, China) with a conventional three-electrode system, where a Mb modified electrode was used as the working electrode, a platinum wire as auxiliary electrode and a saturated calomel electrode (SCE) as reference electrode. UV-Vis spectroscopy was recorded on a Cary 50 probe spectrophotometer (Varian Company, Australia) and FT-IR spectra were performed with a Tensor 27 FT-IR spectrophotometer (Bruker Company, Germany).

Fabrication of carbon ionic liquid electrode

The following procedure was used to prepare the CILE. Typically 200 μ L of [EMIM]EtOSO₃, 800 μ L of liquid paraffin and 3.2 g of graphite powder were put into an agate mortar and hand-mixed carefully. Then a portion of carbon paste was packed firmly into a glass tube with the diameter of 4 mm. A copper wire was inserted into the end of the paste in the inner hole of the tube to make the electrical contact and the CILE surface was polished on a weighing paper to get a smooth interface.

Construction of $\{IL/Mb\}_n$ multilayer film

The newly prepared CILE was used as the basal electrode for the multilayer film assembly. Firstly, the CILE was immersed in 10.0 mg/mL Mb solution (in pH 8.0 PBS) for 40 min to adsorb a layer of Mb on the surface of CILE. The obtained electrode was rinsed with water and dried in air. Then Mb/CILE was alternately immersed in [EMIM]BF₄ and Mb solution (10.0 mg/mL in pH 8.0 PBS) for 40 min, with intermediate water washing and air drying. This procedure was repeated for the desired number of bilayers (n) to get the $\{IL/Mb\}_n$ multilayer films on the surface of CILE.

RESULTS AND DISCUSSION

UV-Vis spectroscopy

UV-Vis spectroscopy is a conventional tool for identifying the secondary structure of redox protein in different matrix and it is used to investigate the influence of IL on the structure of Mb. Due to the hydrophilic properties of IL [EMIM]BF₄, Mb solution (in pH 8.0 PBS) can easily mixed with [EMIM]BF₄. And the Uv-Vis spectra were recorded by a transmission model. The position of the Soret band of the Mb molecules can provide the information about the conformational change in the heme protein, which is sensitive to the changes of microenvironments around the heme site.¹⁹ Fig. 1 showed the UV-Vis adsorption spectra of Mb and its mixture with IL. The Soret absorption band of native Mb molecules appeared at 409.1 nm in water (curve a). After mixed with [EMIM]BF₄, the Soret absorption band appeared at 410.3 nm (curve b), which was about 1.2 nm shift to the red direction. The results suggested that Mb remained its native structure in the IL matrix, which indicated that the biocompatible IL had the ability to provide a favorable microenvironment to stabilize enzyme.²⁰

FT-IR spectroscopy

FT-IR spectroscopy is very sensitive to the conformational changes of the protein,²¹ and the conformational changes will affect the electron transfer reactivity. The amide I and amide II bands of proteins provide detailed information on the secondary structure of polypeptide chain. The amide I band (1700–1600 cm⁻¹) is caused by C=O stretching vibrations of peptide linkages in the protein's backbone and the amide II band (1620–1500 cm⁻¹) is attrib-



Fig. 1. UV-Vis absorption spectra of Mb (a) in aqueous solution, (b) in the IL matrix.

uted to the combination of N–H bending and C–N stretching. Fig. 2 showed the typical FT-IR spectra of Mb film and IL-Mb film, the amide I and amide II bands of Mb in the IL matrix (1639 and 1573 cm⁻¹) are very similar to those obtained in Mb film (1655 and 1539 cm⁻¹), suggested that Mb retained its native structure in the IL matrix.

Cyclic voltammograms of {IL/Mb}_n/CILE

According to the reference²² a layer of IL film was present on the surface of CILE. Due to the specific of IL structure, the positive charges existed on the surface of CILE with the presence of the organic cationic ions. While the isoelectric point (pI) of Mb molecule is at 6.8, so the Mb molecules have the net negative charges in pH 8.0 PBS, which can interact with the positive charged imidazolium ions on CILE surface by the electrostatic force. Then Mb and IL [EMIM]BF4 were assembled on the surface of CILE by a step-by-step procedure to make a {IL/Mb}_n/CILE. Firstly the Mb molecules were adsorbed on the CILE to get an electrode denoted as Mb/CILE, which was further immersed in [EMIM]BF4 to adsorb a layer of IM cationic ions on the surface of Mb/CILE. After several repetition in IL and Mb solution, a {IL/Mb}_n multilayer film was formed on the surface of CILE. The multilayer assembly process was characterized by electrochemical method and Fig. 3 showed the cyclic voltammetric results of {IL/Mb}_n/CILE with different assembly cycles (n). It can be seen that a pair of well-defined quasi-reversible redox peaks appeared with almost equal height of peak currents at different n value from 1 to 4, indicating the direct electron transfer of Mb was realized on the multilayer film modified electrode. So the $\{IL/Mb\}_n$ film was favorable to realize the electro-



Fig. 2. FT-IR spectra of (a) Mb and (b) IL-Mb film.

chemical reaction of Mb. IL is a biocompatible green solvent with high ionic conductivity, and it can provide a suitable microenvironment for Mb to retain its native structure and enhance the electron transfer rate with the underlying electrode. From Fig. 3 it can be seen that the redox peak current increased with the assembly cycles of IL/Mb adsorption bilayers up to 3 and then began to decrease slowly with the further increase of the assembly number. The results was similar to a recent report about the sol-gel processed ionic liquid-hydrophilic carbon nanoparticles layerby-layer film modified electrode,²³ which may be attributed to the neutralization of the imidazolium groups that resulted in the less adsorption of Mb molecules in the multilayer film. While no redox peaks appeared in the selected potential range when Mb was not present on the electrode surface. To get the biggest voltammetric response the assembly cycles were fixed at three in the following experiments. The surface concentration of electroactive Mb in the {IL/Mb}_n/CILE was further calculated to explain the corresponding electrochemical results. According to the Faraday's law (Q = nFA Γ^*), where F is Faraday constant and Γ^* is the surface concentration of electroactive Mb, Q can be calculated from the integration charge of the reduction peak, A is the area of CILE, and n stands for the number of electron transferred. Based on the cyclic voltammetric reduction peak area of different {IL/Mb} multilayer films, the value of Γ^* was calculated as 2.63×10^{-9} mol/cm² for the first layer. With the increase of the assembly cycle number from 2 to 4, the values of Γ^* were calculated to be 8.67 × 10^{-9} mol/cm², 1.45×10^{-8} mol/cm² and 1.20×10^{-8} mol/cm², respectively. The maximum value of $1.45 \times 10^{-8} \text{ mol/cm}^2$



Fig. 3. Cyclic voltammograms of the {IL/Mb}_n film electrode in pH 7.0 PBS with assembly number as 1,2,3,4 (a-d) at the scan rate of 100 mV/s.

appeared at the number of 3, which also indicated that 3 layers of multifilm gave the maximum electrochemical responses. All the Γ^* values calculated were larger than the theoretical monolayer value of $1.89 \times 10^{-11} \text{ mol/cm}^{2,11}$ which indicated that several layers of Mb in the multilayer films took part in the electron transfer with the underlying electrode.

Direct electrochemistry of {IL/Mb}₃ /CILE

From the curve c of Fig. 3, it can be seen that a pair of well-defined and stable symmetric redox peaks appeared on the {IL/Mb}₃/CILE with the anodic (Epa) and cathodic (Epc) peak potential located at -0.283 and -0.141 V (vs. SCE). The apparent formal potential $(E^{0'})$, which is calculated form the midpoint of Epa and Epc, was got as -0.212 V. The result was the typical characteristic of Mb heme Fe(III)/Fe(II) redox couples,²⁴ which indicated that the direct electron transfer of Mb with electrode was achieved in the layer-by-layer film with [EMIM]BF4. It has been reported that the refolding of protein is improved with the activity remained in the IL that containing tetrafluoroborate (BF_4) and hexafluorophosphate (PF_6) anions.²⁵ Typical electrochemical responses of the {IL/Mb}3/CILE at different scan rates were further investigated with the cyclic voltammograms shown in Fig. 4. In the scan rate range from 30 to 300 mV/s a pair of well-defined redox peaks appeared on the {IL/Mb}₃/CILE. The oxidation and reduction peak currents increased linearly with the scan rate, and two linear regression equations were plotted as $Ipc(\mu A) =$ $28.918 + 1.023 \upsilon (mV/s) (\gamma = 0.995)$ and $Ipa(\mu A) = -44.288$ -0.722υ (mV/s) ($\gamma = 0.992$), indicating the electrode pro-



Fig. 4. (A) Cyclic voltammograms of {IL/Mb}₃/CILE with different scan rates (from a to k as 30, 50, 80, 100, 120, 150, 180, 200, 220, 250, 300 mV/s) in pH 7.0 PBS; (B) Plot of the cathodic and anodic peak current with scan rate (υ); (C) The relationship of the cathodic and anodic peak potential against lnυ.

cess was a typical diffusionless surface-confined electrochemical process. With the increase of scan rate the oxidation peak moved to more positive direction and the reduction peak moved to the negative direction. The peak-topeak separation (ΔEp) was increased gradually, indicating a quasi-reversible electrode process. So the Laviron's model^{26,27} was used to calculate the electrochemical parameters such as the charge transfer coefficient (α) and the electron transfer rate constant (k_s) . By exploring the relationship of the redox peak potential with the value of lnu, two straight lines were got with the linear regression equations as $Epc(V) = -0.0274 - 0.063 lnv (mV/s) (\gamma = 0.994)$ and Epa(V) = -0.300 - 0.0352lnu (mV/s) ($\gamma = 0.994$), then the values were calculated with α as 0.36 and k_s as 0.45 s⁻¹, respectively. The k_s value was in accordance with the previous reported values, 28,29 indicating a fast electrochemical kinetic process.

Electrocatalytic to TCA

It has been reported that redox proteins with heme groups can catalyze different kinds of substrates. So the fabricated {IL/Mb}₃/CILE was used to test the electrocatalytic activity to the reduction of TCA. Fig. 5 showed the cyclic voltammograms obtained at above modified electrode in the presence of different concentrations TCA added in the oxygen free PBS. On the CILE without Mb modification, the direct reduction of TCA was found to be more than -1.1 V (curves a and b). While on the {IL/Mb}₃/CILE the reduction peak appeared at -0.31 V with the disappearances of the oxidation peak, which was the typical characteristic



Fig. 5. (A) Cyclic voltammograms of CILE in the presence of (a) 0, (b) 1.0 mmol/L TCA in 0.1 mol/L pH 7.0 PBS and cyclic voltammograms of {IL/Mb}₃/CILE in pH 7.0 PBS containing different concentrations of TCA (from c to h: 0, 4.0, 6.0, 8.0, 10.0, 12.0 mmol/L TCA) with the scan rate of 100 mV/s. (B) The relationship of the catalytic reduction peak current with the TCA concentration.

of electrocatalytic reaction. So the presence of Mb molecules can great enhance the electrocatalytic of TCA with the reduction overpotential decreased for more than 0.8 V, which indicated the large decrease of the activation energy for the reduction reaction. The results indicated that the Mb Fe(II) produced in the electrode reaction was chemically oxidized by TCA and returned back to Mb Fe(III) with a catalytic cycle. When the concentration of TCA is higher than 4.0 mmol/L, a new reduction peak appeared at -0.54 V, which was attributed to the formation of highly reduced form of Mb that could dechlorinate di- and monochloroactetic acid after the dechlorination of TCA with the Mb Fe(II).³⁰ According to the reference,³¹ a highly reduced form of Mb could be produced in a didodecyldimethylammonium bromide (DDAB) film modified electrodes, which was proved by the spectroelectrochemical results. This highly reduced Mb Fe(I) acted as an active reductant during the dechlorination of trichloroethylenes. So the second reduction peak on the cyclic voltammograms with high concentration of TCA can be tentatively assign to the highly reduced form of Mb Fe(I), which can dechlorinate di- and monochloroacetic acid after the dechlorination of TCA by Mb Fe(II). Then the overall electrocatalytic reaction by the Mb molecules in the modified film may be deduced with the following equations.

$Mb Fe(III) + e \rightarrow Mb Fe(II)$	(1)
2Mb Fe(II) + Cl ₃ CCOOH + H ⁺ →	
$2Mb Fe(III) + Cl_2CHCOOH + Cl^-$	(2)
Mb $Fe(II) + e \rightarrow Mb Fe(I)$	(3)
2Mb Fe(I) + Cl ₂ CHCOOH + H ⁺ →	
$2Mb Fe(II) + ClCH_2COOH + Cl^{-1}$	(4)
2Mb Fe(I) + ClCH ₂ COOH + H ⁺ →	
$2Mb Fe(II) + CH_3COOH + Cl^{-1}$	(5)

The total reaction included the electrochemical reduction of Mb Fe(III) to Mb Fe(II) on the electrode, the reduction of TCA with Mb Fe(II) in the film, the further reduction of Mb Fe(II) to Mb Fe(I) in the modified film, the reduction of di- and monochloroactetic acid with formed Mb Fe(I), and the reoxidation of Mb Fe(I) back to Mb Fe(II). All the above results indicated that the Mb molecules in the LBL multilayer films retained its bioelectrocatalytic activity and exhibited excellent electrocatalytic ability to the reduction of TCA.

The reduction peak current increased with the TCA

concentration in the range from 2.0 to 22.0 mmol L⁻¹ and a linear regression equation was obtained as $Iss(\mu A) = 47.01C \text{ (mmol/L)} + 3.87 (\gamma = 0.997)$ with the detection limit as 0.6 mmol/L (3 σ), which was smaller than some previous results,^{32,33} indicating the proposed Mb bioelectrode exhibited higher sensitivity to TCA detection. The apparent Michaelis-Menten constant (K_M^{app}) can be calculated from the electrochemical version of the Lineweaver-Burk equation:³⁴

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_M^{app}}{I_{max}c}$$

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 the saturated substrate condition. Based on the above equation the K_M^{app} value was calculated as 21.36 mmol/L, which was smaller than some reported values.^{17,35}

Stability and reproducibility of the {IL/Mb}₃/CILE

The stability and reproducibility of the {IL/Mb}₃/ CILE was examined by cyclic voltammetry. Direct electrochemistry of the Mb modified electrode retained constant current values upon continuous 50 cyclic sweeps over the potential range from 0.2 to -0.7 V at 100 mV/s. The longterm stability of the electrode was also investigated. When the electrode was not in use, it was stored dry at 4 °C and measured intermittently. After 4 weeks storage period, the electrode still retained 92% of its initial current response. The good storage stability can be attributed to the excellent biocompatibility of IL, which could provide a favorable microenvironment to stabilize the Mb molecules in the multilayer film. Moreover, six bioelectrodes were fabricated based on the same procedure and the relative standard deviation (RSD) for the detection of 8.0 mmol/L TCA was 4.1%. These results indicated that the modified electrode exhibited good stability and reproducibility.

CONCLUSIONS

In this paper, [EMIM]BF₄ and Mb molecules were assembled step-by-step on the surface of CILE based on the electrostatic attraction. UV-Vis and FT-IR spectroscopic results indicated that Mb remained its native structure in the IL matrix. A pair of well-defined redox peaks appeared on the multilayer film modified electrode, indicating the direct electron transfer of Mb with the underlying electrode was realized. The result can be attributed to the specific characteristics of IL such as high ionic conductivity and good biocompatibility that provide a suitable microenvironment for Mb. The electrochemical behavior of Mb in the multilayer film was carefully investigated with the electrochemical parameters calculated. The Mb bioelectrode showed excellent electrocatalytic ability to the reduction of TCA. So the Mb and IL LBL multilayer film modified electrode provided an efficient platform for the preparation of a new third generation electrochemical biosensor.

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