Electrochemistry of Hemoglobin in the Chitosan and TiO_2 Nanoparticles Composite Film Modified Carbon Ionic Liquid Electrode and Its Electrocatalysis

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Direct electron transfer of hemoglobin (Hb) in the chitosan (CTS) and TiO_2 nanoparticles (nano-TiO_2) composite films was achieved by using a room temperature ionic liquid of 1-butyl-3-methylimidazolium hexafluorophosphate ($BMIMPF_6$) modified carbon paste electrode (CILE) as the basal electrode. UV-Vis and FT-IR spectroscopy indicated that Hb in the film retained the native structure. Electrochemical investigation indicated that a pair of well-defined quasi-reversible redox peaks of Hb heme Fe(III)/Fe(II) was obtained with the formal potential located at -0.340 V (*vs*. SCE) in pH 7.0 phosphate buffer solution (PBS). The electrochemical parameters such as the electron transfer coefficient (α), the electron transfer number (n) and the standard electron transfer rate constant (k_s) were got as 0.422, 0.93 and 0.117 s^{-1}, respectively. The fabricated CTS/nano-TiO_2/Hb/CILE showed good electrocatalytic ability to the reduction of trichloroacetic acid (TCA) and hydrogen peroxide (H_2O_2), which exhibited a potential application in fabricating a new kind of third generation biosensor.

Key Words: Hemoglobin, TiO_2 nanoparticles, Ionic liquid, Carbon paste electrode, Electrochemistry

Introduction

Direct electron transfer of redox proteins has been shown great importances in understanding the mechanistic study on the electron exchange in biological system and the fabrication some new kinds of biosensors or bioreactors. Due to the difficulty of direct electron transfer of protein on bare electrode, protein film modified electrodes fabricated with different methods such as layer-by-layer assembly, absorption *etc.* have been developed. Because the modified film can provide a favorable microenviroment for proteins to retain their activity and enhance the direct electron transfer rate between the protein with basal electrodes, protein film electrochemistry had been greatly developed in recent years.[1]

As a green solvent, room temperature ionic liquids (RTILs) had aroused great attentions in the field of chemistry due to their excellent physicochemical properties, such as high ionic conductivity, wide electrochemical windows, good solubility, negligible vapor pressure and chemical stability.[2,3] Sun *et al.* investigated the direct electrochemistry of hemoglobin (Hb) in the sodium alginate (SA) film on a 1-butyl-3-methylimidazolium hexafluorophosphate ($BMIMPF_6$) modified carbon paste electrode.[4] Maleki *et al.* applied *N*-octylpyridinum hexafluorophosphate (OPFP) as a binder for the construction of a high-performance carbon composite electrode and carefully investigated the electrochemical behaviors of carbon ionic liquid electrode (CILE) toward different electro-active compounds.[5] Wang *et al.* investigated the electrochemistry and electrocatalysis of heme proteins entrapped in agarose hydrogel/ionic liquid films modified glassy carbon electrode (GCE).[6] Zein *et al.* had reported the electropolymerization of benzene in 1-hexyl-3-methyl- imidazolium tris(pentafluoroethyl) trifluorophasphate modified electrode.[7] Yu *et al.* described the direct electron transfer of horseradish peroxidase (HRP) in a water-miscible imidazolium-based ionic liquid film modified glassy carbon electrode (GCE).[8]

In this paper a new Hb composite film modified CILE was fabricated by using chitosan (CTS) and TiO_2 nanoparticles. CTS has been widely reported as an immobilization matrix for the preparation of biosensor and bioreactor,[9-12] which exhibit excellent film-forming ability as a biocompatible, biodegradable and nontoxic natural biopolymer. TiO_2 nanoparticles have been also revealed to afford good biocompatibility, large surface area, good dispersing properties and fast electron transfer ability, which had been applied to immobilize protein or enzyme for either mechanistic study or the fabrication of electrochemical biosensors. Zhang *et al.* has investigated direct electrochemistry and electrocatalysis of HRP in simple dropcasting TiO_2 nanoparticles films.[13] Li *et al.* used nanocrystalline TiO_2 films on electrodes to entrap heme proteins such as cytochrome C (Cty c), Hb, myoglobin (Mb) and the direct electrochemistry of these proteins were observed.[14] Zhou *et al.* reported that the Hb molecules entrapped in the TiO_2 nanoparticles could exhibit direct electrochemistry and show good electrocatalytic activity toward hydrogen peroxide.[15] In this work the Hb modified electrode (CTS/nano-TiO_2/Hb/CILE) was constructed by using CTS and nano-TiO_2 as the film forming materials. Experimental results indicated that the direct electron transfer of Hb with the electrode was achieved on the modified electrode. The Hb in the film retained its native structure and exhibited good electrocatalytic activity to the reduction of trichloroacetic acid (TCA) and hydrogen peroxide (H_2O_2).

Experimental

Reagents. Bovine hemoglobin (Hb, MW. 64500, Tianjin Chuanye Biochemical Limited Company), room temperature ionic liquid of 1-butyl-3-methylimidazolium hexafluorophosphate ($BMIMPF_6$, Hangzhou Kemer Chemical Limited

Company), chitosan (CTS, minimum 95% deacetylated, Dalian Xindie Chemical Reagents Limited Company), TiO_2 nanoparticles (nano-TiO_2, particle size 60 nm, Shandong Jinker Chemical Limited Company), graphite powder (average particle size 30 μm, Shanghai Colloid Chemical Plant) and trichloroacetic acid (TCA, Tianjin Kemiou Chemical Limited Company) were used as received. 0.1 mol L^{-1} 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^{-1} H_3PO_4 or NaOH. All the other chemicals were of analytical reagent grade and doubly distilled water was used in all the experiments.

Apparatus. The electrochemical measurements such as cyclic voltammetry and electrochemical impedance spectroscopy (EIS) were performed on a CHI 750B electrochemical workstation (Shanghai CH Instrumention, China) with a conventional three-electrode system composing of a Hb film modified carbon ionic liquid electrode (CILE) as working electrode, a platinum wire as auxiliary electrode and a saturated calomel electrode (SCE) as reference electrode. UV-Vis absorption spectra and FT-IR spectra were recorded on a Cary 50 probe spectrophotometer (Varian Company, Australia) and 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).

Electrode preparation. The $BMIMPF_6$ modified carbon paste electrode (CILE) was prepared according to the reference.[4] $BMIMPF_6$ was mixed with graphite powder at a ratio of 25/75 (w/w) in an agate mortar. The homogeneous paste was packed into a cavity of glasstube with the diameter of 4.0 mm. The electrical contact was got 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 Hb modified CILE was prepared with the following procedure: A 10.0 μL of 20.0 mg mL^{-1} Hb was casted on the surface of CILE and left it to dry at room temperature. Then a 6.0 μL of 4.0 mg mL^{-1} nano-TiO_2 solution was applied on the electrode surface and left to dry at room temperature. Finally, a 6.0 μL of 4.0 mg mL^{-1} CTS (1.0 % HAC) solution was applied on the electrode surface and dried. During these steps 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. The fabricated electrode was denoted as CTS/nano-TiO_2/Hb/CILE and stored at 4 °C when not in use. For comparison other modified electrodes such as CTS/nano-TiO_2/CILE, CTS/Hb/CILE etc. were prepared with the similar procedures.

Procedures. Electrochemical measurements were performed at room temperature of 20 °C by using a CHI 750B electrochemical workstation with cyclic voltammetry. The working buffer solutions were deoxygenated by bubbling high pure nitrogen thoroughly for at least 15 min just before the experiments and the nitrogen atmosphere environment was kept in the electrochemical cell during the procedure. The three-electrode system was immersed in a 10 mL cell containing 0.1 mol/L pH 7.0 PBS and scanned in the potential range from +0.2 to –0.9 V (*vs*. SCE) at the scan rate of 100 mV/s. Electrochemical impedance spectroscopy (EIS) was preformed with applied perturbation amplitude as 10 mV by using 10.0 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ as the redox probe with the frequencies swept from 10^5 to 10^{-2} Hz.

Results and Discussion

UV-Vis absorption spectra. The position of the Soret absorption band of heme may provide information about possible denaturation of heme protein, and particularly on the conformational change in the heme group region. Therefore, UV-Vis spectroscopy is a useful tool for conformational study of heme region.[16] If Hb is denatured, the Soret band will change or even disappear. The UV-Vis absorption spectra of Hb composite film in different pH buffer solution were shown in Figure 1. As for natural Hb, the Soret band appeared at 409.0 nm (curve a). At pH 7.0 and 10.0, the Soret bands of Hb composite film appeared at 407.0 nm and 408.0 nm (curve b and c), respectively, which were very close to that of the native Hb, suggesting that the Hb in CTS/nano-TiO_2 films suffered almost no denaturation. However, when the pH value was changed to pH 2.0, the Soret band moved to 366.0 nm with the peak shape turned to smaller and broader (curve d), which indicated the denaturation of Hb to a certain extent in the extreme acidic solution. The results also indicated that Hb can keep its native state in the CTS/nano-TiO_2 films in a wide range of pH range.

FT-IR spectra. FT-IR spectroscopy is also used to check the conformational integrity of the heme proteins. The shape and position of amide I and amide II infrared bands provide the detailed information on the secondary structure of polypeptide chains. 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.[17] If Hb is denatured, the intensities and shapes of amide I and II band will diminish or even disappear. Figure 2

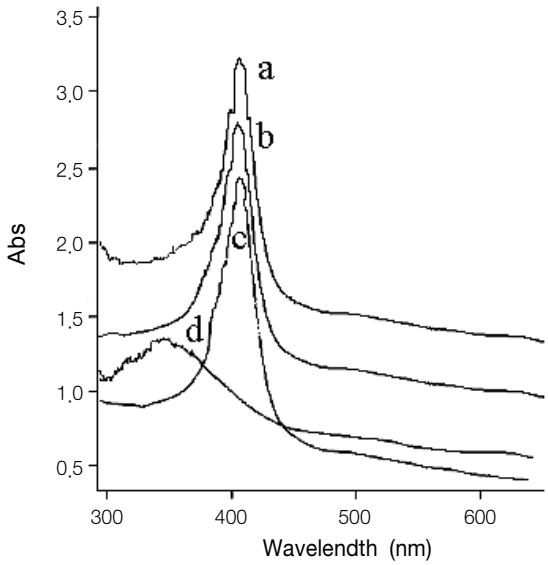

Figure 1. UV-Vis absorption spectra of Hb in water solution (a), CTS/nano-TiO_2/Hb in pH 7.0 (b), 10.0 (c) and 2.0 PBS (d), respectively.

showed the FT-IR spectra of Hb in the CTS/nano-TiO_2 films. The amide I and amide II bands of Hb in CTS/nano-TiO_2 films appeared at 1653.55 cm^{-1} and 1538.66 cm^{-1} (Figure 2b), which had almost the same position with the native state of Hb spectrum at 1655.34 cm^{-1} and 1535.80 cm^{-1} (Figure 2a). These results also showed that Hb retained the essential features of its original structure in the CTS/nano-TiO_2 films.

SEM images of modified electrodes. Scanning electron microscopy (SEM) was used to characterize and compare the morphologies of the different modified electrodes with the results shown in Figure 3. On the surface of the CILE (Figure 3A), a more uniform and smooth surface was formed, which was attributed to the good solubility and dispersing ability of $BMIMPF_6$ toward graphite powder. The high viscosity of $BMIMPF_6$ could also act to bridge the isolated carbon flake. At the Hb/CILE (Figure 3B), a networkline structure was observed with the aggregation of the immobilized Hb molecules regularly distributed. Figure 3C was the image of a nano-TiO_2/Hb/CILE, which showed a rough surface structure with many nanosized particles or spheriform granules, indicating the presence of TiO_2 nanoparticles. The spheriform granules on the surface of CILE was possibly attributed to the partly aggregations of nanoparticles. With the further modification of CTS on nano-TiO_2/Hb/CILE a stable film could be found (Figure 3D). The differences of the SEM images indicated that Hb existed in different microenvironments with the stepwise modification on electrode surface.

Direct electrochemistry of Hb. Figure 4A showed the cyclic voltammograms of diferent modified electrodes in pH 7.0 PBS at the scan rate of 100 mV s^{-1}. On the CILE (curve a) and CTS/nano-TiO_2/CILE (curve b) no redox peak responses appeared, which indicated no electroactive substances existed on the surface of electrode. The bigger background current of CTS/nano-TiO_2/CILE than that of bare CILE was attributed to the prescence of CTS/nano-TiO_2 films on the electrode surface, which increased the thickness of the electrode interface. On the Hb/CILE, there was no redox peak responses appeared, which was due to the unstable of the Hb on the surface of CILE and Hb maybe dissolved into the PBS gradually (curve c). On the CTS/Hb/CILE a small pair of redox peaks was observed (curve d), which indicated that Hb in the CTS film could take part in the electron transfer with the CILE. CILE was a new kind of modified electrode with many specific advantages such as high conductivity, provision of excellent electron transfer ability and good anti-fouling properties. A thin layer of IL cationic ions were present on the surface of CILE,[5,18-20] which was also considered to be able to play an inherent catalytic ability toward the electrochemical reaction of Hb. At the same time CTS was a biocompatible biomacromolecules and often used as a immobilization material to fix the biomolecules on the surface of biosensors. While on the CTS/nano-TiO_2/Hb/CILE a pair of well-defined quasi-reversible redox peaks was observed (curve e) with the increase of electrochemical responses, which was attributed to the presence of TiO_2 nanoparticles incorporated in the film. TiO_2 has been reported to be commonly used in the fabrication of dye-sensitized solar cells and electrochromic devices with good conductivity and biocompatibility. Due to the co-contribution of CTS, TiO_2 nanoparticles and IL present on the surface of CILE, the direct electrochemistry of Hb, which was derived from the heme Fe(III)/Fe(II) redox couples, was achieved in the pH 7.0 PBS.

From the cyclic voltammograms the anodic and cathodic peak potentials were located at -0.479 V and -0.198 V (*vs.* SCE) (Figure 4B curve f). The formal potential ($E^{0'}$), which can be calculated from the equation of $E^{0'} = (Epa + Epc)/2$, was got as -0.340 V, which was close to the previous reports.[21] The peak to peak potential separation (ΔEp) at 100 mV s^{-1} was 0.281 V and the ratio of redox peak current (Ipa/Ipc) was approximately to be 1.0. While on the CTS/Hb/CILE, the ΔEp value was 0.318 V with small redox peak currents (Figure 4B

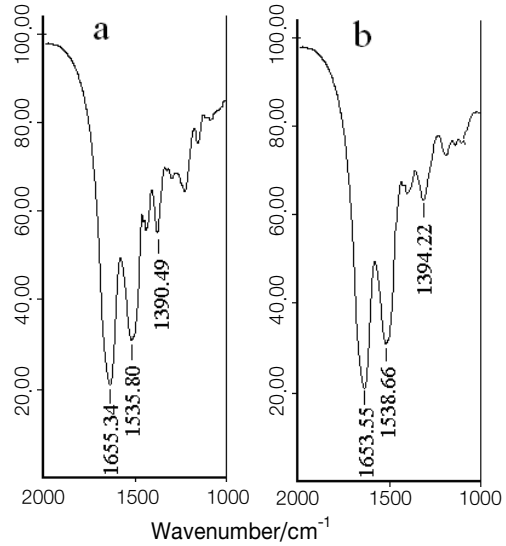

Figure 2. FT-IR spectra of Hb (a) and CTS/nano-TiO_2/Hb film (b).

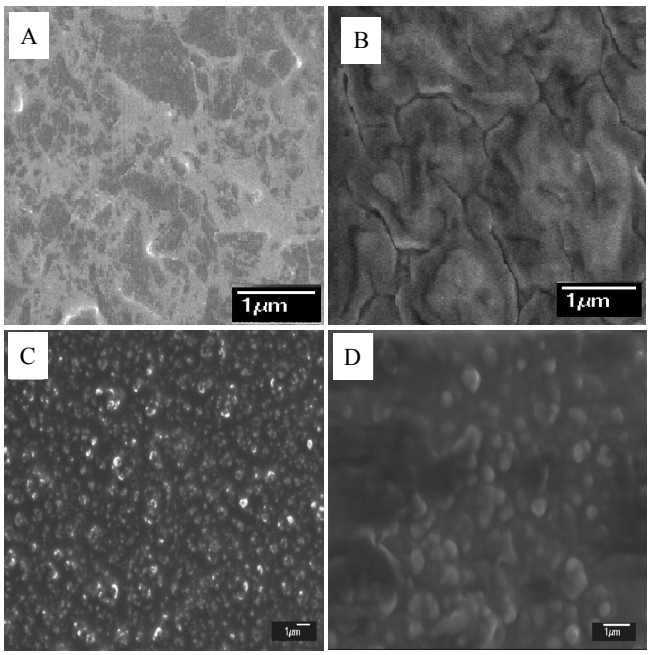

Figure 3. SEM top views of CILE (A), Hb/CILE (B), nano-TiO_2/Hb/CILE (C) and CTS/nano-TiO_2/Hb/CILE (D).

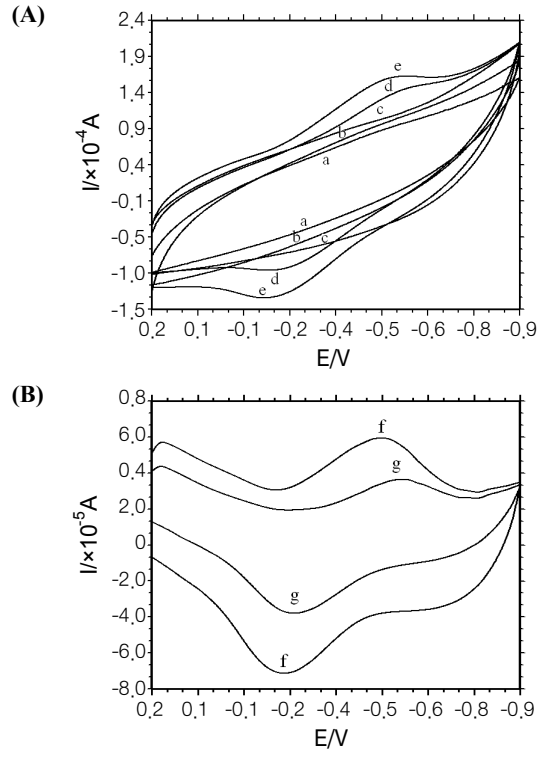

Figure 4. (A) Cyclic voltammograms of bare CILE (a), CTS/nano-TiO_2/CILE (b), Hb/CILE (c), CTS/Hb/CILE (d) and CTS/nano-TiO_2/Hb/CILE (e) at the scan rate of 100 mV s^{-1} in pH 7.0 PBS. (B) The background-subtracted cyclic voltammograms of CTS/nano-TiO_2/Hb/CILE (f) and CTS/Hb/CILE (g) using data in curve b.

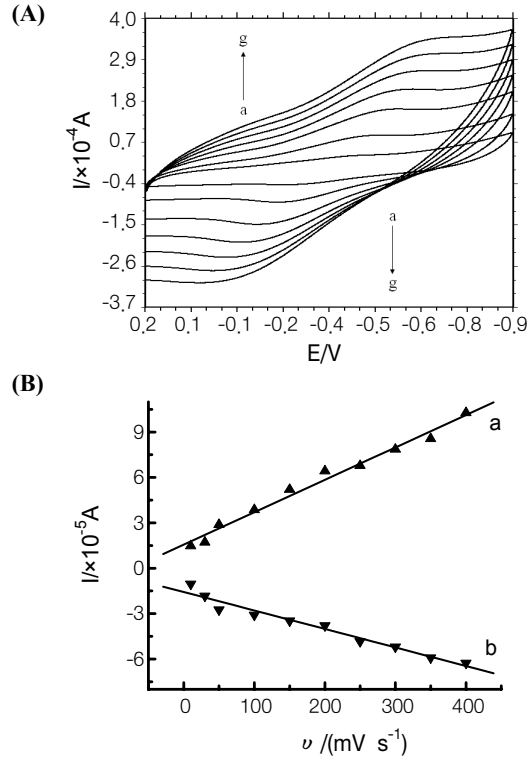

Figure 5. (A) Cyclic voltammograms of the CTS/nano-TiO_2/Hb/CILE in pH 7.0 PBS at the scan rate of 10, 50, 100, 150, 200, 250, 300 mV s^{-1} (from a to g). (B) The linear relationships of the cathodic (a) and anodic (b) peak current (Ip) *versus* the scan rate (υ) in pH 7.0 PBS.

curve g). All the results indicated that direct electron transfer of Hb in the CTS/nano-TiO_2 films was achieved on the surface of CILE and the presence of nano-TiO_2 facilitated the rate of electron transportation between the Hb molecules and the basal electrode.

Figure 5A showed the cyclic voltammograms of CTS/nano-TiO_2/Hb/CILE at different scan rates in pH 7.0 PBS. With the increase of the scan rate a pair of redox peaks appeared with the redox peak currents increased gradually, which indicated that the electrochemical 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). The peak currents were found to increase linearly with the scan rate in the range from 10 to 400 mV s^{-1} with the linear regression equations as Ipc(μA) = 0.021υ (V/s) + 0.168 (n = 10, γ = 0.992) and Ipa(μA) = -0.012υ(V/s) - 0.157 (n =10, γ = 0.981). And the results were shown in Figure 5B, which were characteristics of surface-confined thin-layer electrochemical behaviors.

According to the following equation:[22] $Q = nFA\Gamma^*$, the average surface concentration (Γ^*) of electroactive species could be calculated, where Q is the 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 geometric area of electrode. From the experimental results the average surface concentration (Γ^*) of electroactive Hb on the electrode surface was estimated to be 4.513×10^{-9} mol cm^{-2}. While the total amount of Hb casted in the film was calculated as 2.47×10^{-8} mol cm^{-2}. So the electroactive Hb on the electrode surface accounted for 15.07% of the total amount of Hb in the film, which was higher than the reported value of 2.0%.[23]

The relationship of the peak potentials with scan rate was further constructed, which could be used for the calculation of the electrochemical parameters of the electrochemical reaction. According to the Laviron's equations:[24,25]

$$Epc = E^{0'} - \frac{2.3RT}{\alpha nF}\log\upsilon \quad (1)$$

$$Epa = E^{0'} + \frac{2.3RT}{(1-\alpha)nF}\log\upsilon \quad (2)$$

$$\log k_s = \alpha\log(1-\alpha) + (1-\alpha)\log\alpha - \log\frac{RT}{nF\nu} - \frac{nF\Delta Ep\alpha(1-\alpha)}{2.3RT} \quad (3)$$

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

A linear relationship between the Ep with the logυ was established and two straight lines were shown in Figure 6. The linear regression equations were calculated as Epa(V) = 0.048logυ - 0.062 (n = 8, γ = 0.991) and Epc(V) = -0.066logυ-0.69 (n = 8, γ = 0.994). According to the equations (1) and (2) the electron transfer coefficient (α) and the electron transfer number (n) could be calculated and the results were got as 0.422 and 0.93, respectively. Based on the equation (3) the standard electron transfer rate constant (k_s) was further

calculated from the relationship of ΔEp with logυ and the result was got as 0.117 s^{-1}, which was bigger than the reported value.[26]

Effect of pH. 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 reaction of the heme proteins. The effect of pH on the direct electrochemistry of Hb was also examined. In the pH range from 4.0 to 8.5, a stable and well-defined cyclic voltammogram could be obtained and the formal potential ($E^{0'}$) of Hb showed a negative shift with the increase of buffer solution pH. A good linear relationship of $E^{0'}$ and pH value was got with the linear regression equation as $E^{0'}$(V) = -0.038 pH - 0.072 (n = 9, γ = 0.993). The slope was got as -38.0 mV pH^{-1}, which was reasonably smaller than the theoretical value of -56.0 mV pH^{-1} at 20 °C for a one proton-coupled single electron transfer process, which may due to the different microenvirment present for Hb molecules.

Catalytic activity. Usually, the immobilized Hb on the surface of electrode could offer a good electrocatalytic ability. The electrocatalytic activity of CTS/nano-TiO_2/Hb/CILE was examined with various substrates of biological or environmental significances such as trichloroacetic acid (TCA) and H_2O_2, respectively.

As shown in Figure 7, after the addition of TCA into the buffer solution, a significant increase of the reduction peak at -0.338 V (*vs*. SCE) was observed with the disappearance of the oxidation peak (curve c to j). While no appearance of reduction peak was observed on CTS/nano-TiO_2/CILE at the same solution (curve a and b). The result can be attributed to the presence of Hb in the composite film. The catalytic reduction peak currents increased with the TCA concentration in the range 1.0×10^{-4} to 6.0×10^{-3} mol L^{-1} with the linear regression equation as Ip(mA) =0.955C(mmol L^{-1}) + 1.364(n = 9, γ = 0.997) and the dection limit was calculated as 4.0×10^{-5} mol L^{-1} (3σ).

The apparent Michaelis-Menten constant (K_M^{app}), which

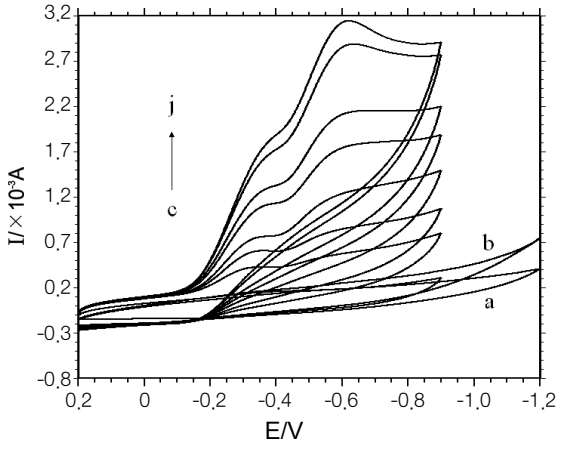

Figure 7. Cyclic voltammograms of CTS/nano-TiO_2/CILE in 0.1 mol L^{-1} pH 7.0 PBS containing (a) 0 and (b) 1.6×10^{-3} mol L^{-1} TCA solution, and CTS/nano-TiO_2/Hb/CILE in 0.1 mol L^{-1} pH 7.0 PBS containing 0, 2.0×10^{-4}, 4.0×10^{-4}, 1.0×10^{-3}, 1.2×10^{-3}, 3.2×10^{-3}, 3.8×10^{-3}, 4.0×10^{-3} mol L^{-1} TCA (curve c-j), respectively, with the scan rate as 100 mV s^{-1}.

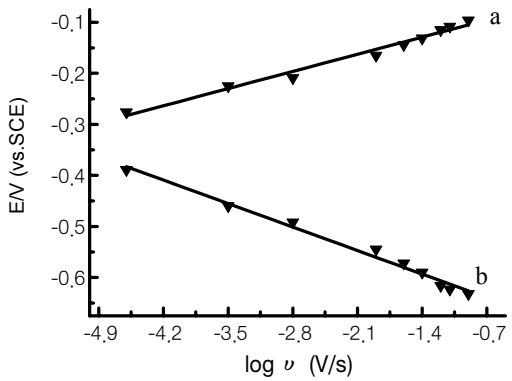

Figure 6. The relationships of the anodic (a) and cathodic (b) peak potential against logυ.

provides an indicative of the enzyme-substrate kinetics, was calculated from the electrochemical version of the Lineweaver-Burk equation.[27]

$$\frac{1}{I_{SS}} = \frac{1}{I_{max}} + \frac{K_M^{app}}{I_{max}C} \quad (4)$$

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.

The value of K_M^{app} was calculated by analysis of the slope and the intercept of the plot of the reciprocals of the reduction peak current versus TCA concentration. From the equation (4) the K_M^{app} value was got as 0.281 mM, which meaned a high enzymatic activity of immobilized enzyme.

The catalytic reactivity of CTS/nano-TiO_2/Hb/CILE toward H_2O_2 was also investigated by cyclic voltammetry. With the addition of H_2O_2 into the test solution, a new reduction peak appeared at -0.445 V and increased with the increase of the H_2O_2 concentration, accompanied with the decrease and gradually disappearance of Hb oxidation peak. The results indicated that the oxidation of Fe(II) to Fe(III) by H_2O_2 was occurred. The linear relationship was also obtained between the reduction peak current and the concentration of H_2O_2 in the range from 1.0×10^{-5} to 1.4×10^{-4} mol L^{-1} with the linear regression equation as Ip(μA)=0.219 C(μmol L^{-1}) + 2.731 (n = 8, γ = 0.998). When the concentration of H_2O_2 exceeded 4.0×10^{-4} mol L^{-1}, the reduction peak current tended to level off, indicating a saturation of enzyme catalytic activity towards substrate.

Since Hb has the similar electroactive center as the peroxidase, so it exhibits certain peroxidase activity. The electrode reaction mechanism of bioelectrochemical reduction of H_2O_2 might be schematically expressed by following equations:[28]

$$\text{HbFe(III)} + H_2O_2 \rightarrow \text{Compound I} + H_2O \quad (5)$$

$$\text{Compound I} + H_2O_2 \rightarrow \text{HbFe(III)} + O_2 + H_2O \quad (6)$$

$$\text{HbFe(III)} + e^- \rightarrow \text{HbFe(II) (at electrode)} \quad (7)$$

$$\text{HbFe(II)} + O_2 \rightarrow \text{HbFe(II)} - O_2 \text{ (fast)} \quad (8)$$

$$\text{HbFe(II)} - O_2 + 2H^+ + 2e^- \rightarrow \text{HbFe(II)} + H_2O_2 \text{ (at electrode)} \quad (9)$$

By analysis of the steady-state current and the H_2O_2 concentration, the apparent Michaelis-Menten constant was calculated as 4.192 μM, which was lower than that of some previous reports.[29,30] Thus the Hb immobilized in CTS/nano-TiO_2 films afforded a good affinity to H_2O_2 and exhibited a high electrocatalytic efficiency.

Electrochemical impedance spectroscopy. Electrochemical impedance spectroscopy (EIS) is an effective tool for studying the interface properties of suface-modified electrodes. The value of electron transfer resistance (Ret), which is the semicircle diameter at high frequencies in the Nyquist plot of impedance spectroscopy, reflects the interfacial electron transfer ability. Therefore, Ret can be used to describe the interface properties of the electrode. Its value varies with the stepwise modification of electrode surface. By using 10.0 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ solution as the redox probe and with the frequencies range from 0.01 to 10^5 Hz, the typical results of AC impedance spectra of the different modified electrodes such as bare CILE (curve a), nano-TiO_2/CILE (curve b), CTS/CILE (curve c), Hb/CILE (curve d), CTS/Hb/CILE (curve e) and CTS/nano-TiO_2/Hb/CILE (curve f) were shown in Figure 8, respectively. It can be seen that the Ret value of the bare CILE exhibited as 110 Ω, which implied the characteristic of a diffusional process (curve a). After the bare CILE was coated with nano-TiO_2, the semicircle increased slightly (curve b) and the Ret value was 165 Ω. Nano-TiO_2 is a kind of semiconductive material, so the presence of nano-TiO_2 film on the CILE surface can hinder the interfacial electron transfer of ferricyanide to the electrode. The semicircle was further increased at CTS/CILE (curve c), indicating that CTS was film-forming material and interfere with the diffusion of ferricyanide toward the electrode surface. After the bare CILE was coated with Hb, the semicircle increased again (curve d) and the Ret value was 322 Ω, which was bigger than that of CTS/CILE, indicating the presence of Hb further hindered the electron transfer. After CTS was coated on the Hb/CILE, the semicircle obviously increased (curve e) with the Ret value as 383 Ω, indicating that Hb had been successfully immobilized. Subsequently, with the CTS and nano-TiO_2 coated on the Hb/CILE the semicircle increased again (curve f) and the Ret value was 520 Ω, this maybe ascribe to the increase of the thickness of the modified film.

The cyclic voltammetric experiment with ferricyanide was also used to monitor the electron transfer of different modified electrodes. As shown in Figure 9, a pair of well-defined redox peaks was observed at the bare CILE (curve a). When the nano-TiO_2 was coated on the surface of the CILE (curve b), the peak current was smaller than that of the bare CILE, which was due to presence of semiconductor nano-TiO_2 on the electrode surface. While at the CTS/CILE (curve c), the peak current was smaller than that of the nano-TiO_2/CILE, which due to the presence of CTS hindered the electron transfer rate. In comparison with the CTS/CILE, the peak current of Hb/CILE (curve d) was further decreased, indicating the presence of Hb film on the CILE. On the CTS/Hb/CILE a further decrease of peak current appeared (curve e). With the incorporation of nano-TiO_2 in the film, the peak current was the smallest, indicating the electron transfer was difficult due to the present of modified materials on the electrode surface (curve f). The results were consistent with the data obtained from EIS experiments. On the basis of the EIS and cyclic voltammetric results, we can conclude that Hb was successfully immobilized on the CTS/TiO_2 composite films.

Stability and reproducibility of the modified electrode. Hb can be embedded in the film of CTS and nano-TiO_2 to form a stable CTS/nano-TiO_2/Hb modified electrode. The modified electrode was evaluated by examining the cyclic voltammetric peak currents, no obvious changes of cyclic voltammetric response of the electrode can be observed after the CTS/nano-TiO_2/Hb/CILE was stored at 4 °C in a refrigerator for 2 weeks, which suggested that the bioelectrode had an excellent stability. The reproducibility of the CTS/nano-TiO_2/Hb/CILE was estimated by the electrochemical responses for the 11 parallel determinations of 6.0×10^{-5} mol L^{-1} H_2O_2 and the relative standard deviation (RSD) was got as 4.7%. The

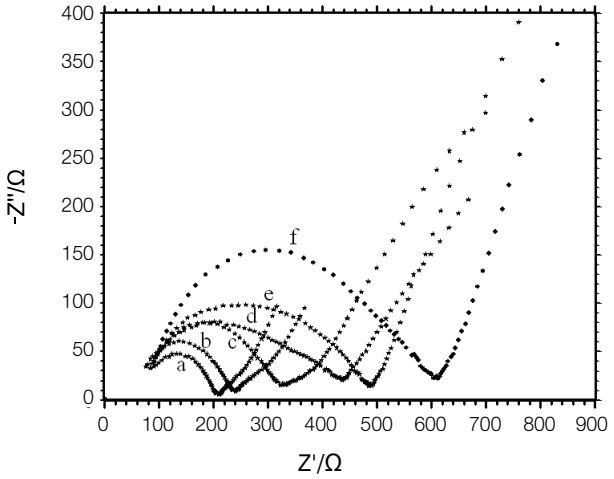

Figure 8. Electrochemical impedance spectroscopy for bare CILE (a), nano-TiO_2/CILE (b), CTS/CILE (c), Hb/CILE (d), CTS/Hb/CILE (e) and CTS/nano-TiO_2/Hb/CILE (f) in the presence of 10.0 mmol/L $[Fe(CN)_6]^{3-/4-}$ and 0.1 mol L^{-1} KCl with the frequencies swept from 10^5 to 10^{-2} Hz.

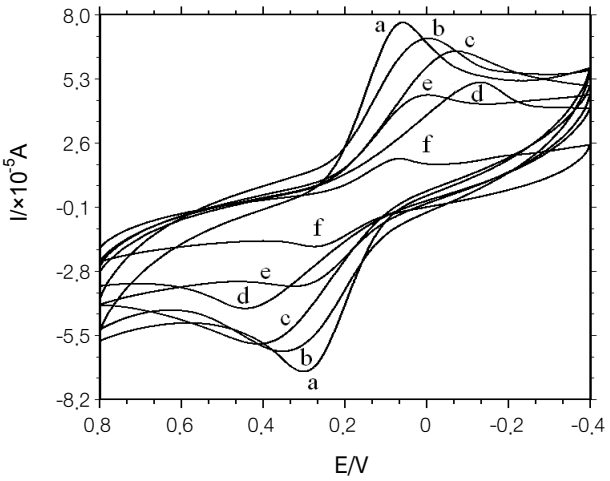

Figure 9. Cyclic voltammograms of bare CILE (a), nano-TiO_2/CILE (b), CTS/CILE (c), Hb/CILE (d), CTS/Hb/CILE (e) and CTS/nano-TiO_2/Hb/CILE (f) in 0.1 mol L^{-1} KCl solution containing 10.0 mmol L^{-1} $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1). Scan rate: 100 mV s^{-1}.

repeatability of the modified electrodes was also studied. Six Hb modified electrodes were made by the same procedure independently and the RSD of 5.1% was calculated for the same solution, which indicated the modified electrode had good repeatability.

Conclusions

In summary, a room temperature ionic liquid $BMIMPF_6$ modified carbon paste electrode (CILE) was constructed and used as the basal electrode for the investigation of direct electrochemistry of Hb. By immobilizing the Hb molecules in the composite film of CTS and TiO_2 nanoparticles, the direct electron transfer of Hb involving the heme Fe(III)/Fe(II) redox couples was easily realized. The CTS/nano-TiO_2/ Hb/ CILE showed good stability and the protein in the film retained their native structures. The electrochemical behaviours of Hb in the film were carefully investigated with the electrochemial parameters calculated. Meanwhile, the CTS/ nano-TiO_2/Hb film CILE exhibited good electrochemical catalytic reduction towards TCA and H_2O_2. Therefore, the CTS/nano-TiO_2 composite films could be useful for the fabrication of a third generation biosensor.

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