Direct Electrochemistry of Hemoglobin Immobilized in the Sodium Alginate and SiO$_2$ Nanoparticles Bionanocomposite Film on a Carbon Ionic Liquid Electrode

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In this paper a carbon ionic liquid electrode (CILE) was fabricated by using a room temperature ionic liquid of 1-butyl-3-methylimidazolium hexafluorophosphate (BMIMPF$_6$) as binder. By using the CILE as basal electrode, the hemoglobin (Hb) molecule was immobilized on the surface of CILE with a sodium alginate (SA) hydrogel and SiO$_2$ nanoparticles organic-inorganic composite material. The direct electrochemical behaviors of Hb in the bionanocomposite film were further studied in a pH 7.0 Britton-Robinson (B-R) buffer solution. A pair of well-defined quasi-reversible cyclic voltammetric peaks of Hb was obtained on SA/nano-SiO$_2$/Hb/CILE with the formal potential (E^0) at -0.355 V (*vs.* SCE), which was the characteristic of heme Fe(III)/Fe(II) redox couples. The formal potential of Hb Fe(III)/Fe(II) couple shifted negatively with increasing pH of solution with a slope of -45.2 mV/pH, which indicated that a one electron transfer accompanied with one proton transportation. The immobilized Hb showed good electrocatalytic manner to the reduction of trichloroacetic acid (TCA).

INTRODUCTION

Studies on the direct electron transfer (DET) between electrode and redox proteins have aroused great interests for decades [1-3]. The research results can be used for the investigation of biosensors and biochemical synthesis. Because the protein will lost its bioactivity or even denature on the bare working electrode, numerous methods had been devised about protein electrochemistry based on different kinds of modified electrodes. Many immobilization methods for proteins had been proposed such as covalent attachments [4], sol-gels [5,6], polymers [7,8] and layer-by-layer [9-11]. When proteins are immobilized on the electrode surface, they

can be separated from the reaction medium with improved stability, higher sensitivity and faster response. In recent years nanomaterials are beginning to be used in the protein films for their unique advantages [12-14]. The activity of enzyme can be retained in the nanomaterial composite film and the electron transfer efficiency can be greatly enhanced between the active center of protein and the electrode due to the specific properties of nanoparticles. Although nanoscience had been developed greatly, the majority of the nanomaterials used in protein electrochemistry were focused on carbon nanotube (CNT) [15-18] and colloidal gold nanoparticles [19-21].

Recently, room temperature ionic liquids (RTILs) have been used in protein electrochemistry. RTILs are a new kind of "green" solution with specific properties such as wide potential windows, high thermal stability, negligible vapor

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pressure and good ionic conductivity [22-24]. RTILs are compounds composed of ions that exist at the liquid state around room temperature or related temperature range. Because of their higher ionic conductivity and wider electrochemical windows, RTILs have been used in electrochemistry and electroanalysis. Several groups have combined RTILs with some matrices to form novel composite materials for the electrode modification [25-28]. Dong *et al*. [29] mixed RTILs with different carbon nanomaterials to form RTILs-carbon composite materials for use as modifiers in proteins electrochemistry. Li *et al*. [30] fabricated a novel chitosan-BMIMBF$_4$ composite material to immobilize enzymes. Sun *et al*. [31] also immobilized hemoglobin (Hb) in the Nafion and CaCO$_3$ nanopartile film and investigated its bioelectrochemistry on an ionic liquid modified carbon paste electrode. Maleki *et al*. [32] applied N-octylpyridinum hexafluorophosphate (OPFP) as a binder for the construction of a high-performance carbon composite electrode. Sun [33] also combined N-butylpyridinium hexafluorophasphate (BPPF$_6$) with carbon powder to make a BPPF$_6$ modified carbon paste electrode. As a new kind of modified electrode, the electrochemical behaviors of some electroactive substances such as uric acid [34], dopamine [35], nitric oxide [36], and ascrobic acid [37] on the RTILs modified electrode were also reported.

In this paper, a new type of bionanocompatible composite material, fabricated by using sodium alginate (SA) and SiO$_2$ nanoparticles, was used to entrap hemoglobin (Hb) at the surface of an ionic liquid modified carbon paste electrode. SA is a biocompatible, biodegradable and nontoxic natural biopolymer. It is a linear hydrophilic polysaccharide composed of β-D-mannuronic (M) and α-L-guluronic (G) acids. SiO$_2$ nanoparticle is an inorganic nonmetallic material, which can give the protein molecules more freedom in orientation and provide an environment similar to the native environment for electron transfer of redox protein [38,39]. The composite materials of SA and nano-SiO$_2$ are biocompatible and nontoxic, which was further used to immobilize Hb molecule on the surface of CILE and the direct electrochemistry of Hb was carefully investigated. The results indicated that the addition of nano-SiO$_2$ in the composite film could greatly enhance the direct electron transfer of Hb. The SA/nano-SiO$_2$/Hb/CILE showed good electrocatalytic behaviors to the reduction of

trichloroacetic acid (TCA).

EXPERIMENTAL

Chemicals

The ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate (BMIMPF$_6$, Hangzhou Kemer Chemical Limited Company), bovine hemoglobin (Hb, MW. 64500, Tianjin Chuanye Biochemical Limited Company), sodium alginate (SA, Tianjin Yuanhang Chemical Limited Company), graphite powder (average particle size 30 μm, Shanghai Colloid Chemical Plant), trichloroacetic acid (TCA, Tianjin Kemiou Chemicals Company) were used as received. SiO$_2$ nanoparticle (average particle size 20 nm) was kindly provided by our Department of Material Chemistry, which was prepared by the methods in the reference [40]. Other chemicals used were of analytical reagents grade and a series of 0.2 M Britton-Robinson (B-R) buffer solution was used as the supporting electrolyte.

Instruments

Cyclic voltammetric experiments were carried out on a CHI model 832 electrochemical workstation (Shanghai CH Instrumentation, China) with the traditional three-electrode system, which was composed of a Hb film modified carbon ionic liquid electrode as working electrode, a saturated calomel electrode (SCE) as reference electrode and a platinum wire as auxiliary electrode. All the test solutions were thoroughly deoxygenated with pure nitrogen for about 30 min prior to experiments and nitrogen atmosphere was kept during the experiments. Scanning electron microscopies (SEM) of the surface of modified electrode were obtained by a model JSM-6700F scanning electron microscope (Japan Electron Company). Cary 50 probe UV-Vis spectrophotometer (Varian Company, Australia) was used for recording absorption spectra.

Preparation of Modified Electrode

The traditional carbon paste electrode (CPE), composed of graphite powder and paraffin, was prepared according to the literature [41]. The BMIMPF$_6$ modified CPE (CILE) was prepared by mixing BMIMPF$_6$ with graphite powder at a ratio of 25/75 (w/w) in a mortar. The homogeneous paste was

packed into a cavity of glasstube with the diameter of 4.2 mm. The electrical contact was established with a copper wire connected to the paste in the tube. The electrode surface was smoothed on a weighing paper.

The Hb modified CILE was prepared with the following procedure. A 15.0 mg ml^{-1} Hb solution and 8.0 mg ml^{-1} nano-SiO$_2$ suspension solution (v/v = 1:1) was mixed together and 5.0 µl of the mixture was cast on the surface of CILE and dried for 6 h at room temperature. Then 5.0 µl of 2.0 mg ml^{-1} SA hydrogel was applied on the electrode surface and then the electrode was placed to dry for at least 6 h. A 10 ml beaker was covered over the surface of electrode so that a more uniform film could be formed at the electrode surface. SA/CILE and SA/Hb/CILE were also prepared in the similar procedure. All the modified electrodes were stored at 4 ºC in a refrigerator when not in use.

RESULTS AND DISCUSSION

Images of Modified Electrode

Figure 1 shows the scanning electron microscopic images of four different modified electrodes. At the traditional CPE (Fig. 1a), the carbon flakes appeared isolated irregularly and no conducting media is available between carbon layers. On the surface of CILE, a more uniform surface could be observed with no separated carbon layers (Fig. 1b), due to the good miscibility of BMIMPF$_6$ with the graphite powder. As a liquid solvent with high viscosity, the presence of BMIMPF$_6$ could disperse the graphite powder homogenously and bridge the isolated carbon flake. After Hb molecule was placed on the surface of CILE, the electrode surface looked more loose and uneven with the protrusion assignable of Hb (Fig. 1c). After SA and nano-SiO$_2$ were added ito the above film, the electrode surface looked bumpy with some donga (Fig. 1d), which was attributed to the presence of nanoparticles in the film. The differences of the images indicated that Hb existed in different microenvironments on the surface of CILE.

Direct Electrochemistry of Hb

Figure 2 shows the cyclic voltammograms of different Hb modified electrode in pH 7.0 B-R buffer solution. On SA/CILE (curve a) and SA/nano-SiO$_2$/CILE (curve b) no electrochemical responses can be found, indicating that no electroactive substances existed on the electrode surface. While on SA/nano-SiO$_2$/Hb/CILE a pair of well-defined redox peaks appeared (curve d), the result indicated that the direct electron transfer of Hb was achieved in the bionanocomposite film on the surface of CILE. On the surface of SA/nano-SiO$_2$/Hb/CPE the redox peaks were not very distinct with small peak currents (curve c). So the appearances of the
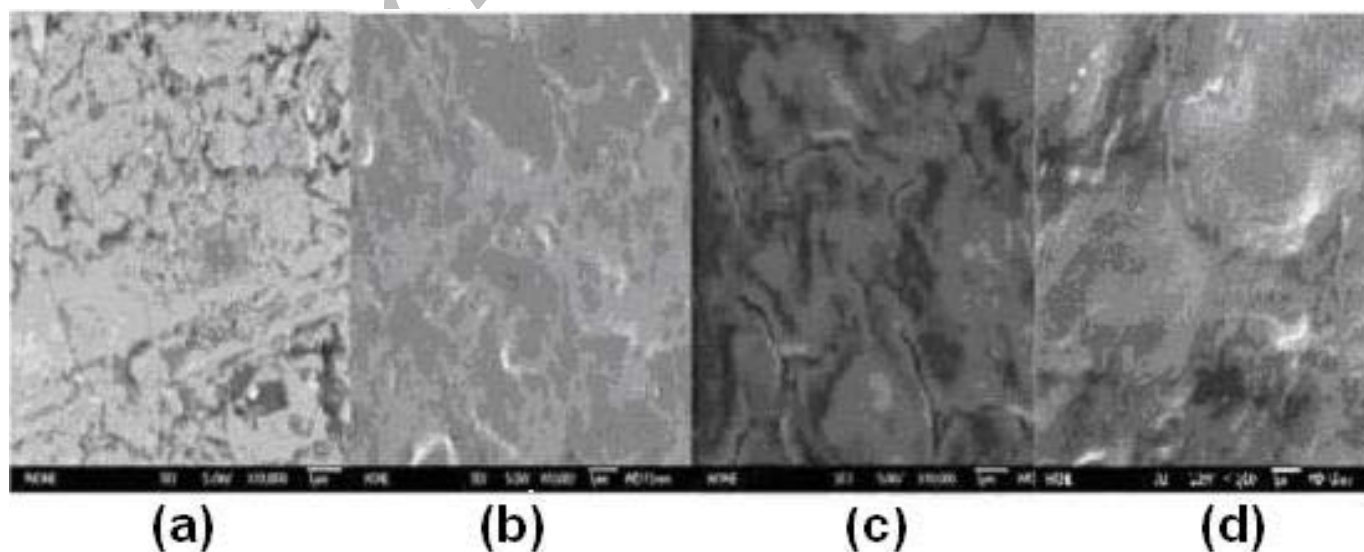

Fig. 1. SEM images of CPE (a); CILE (b); Hb/CILE (c); SA/nano-SiO$_2$/Hb/CILE (d).
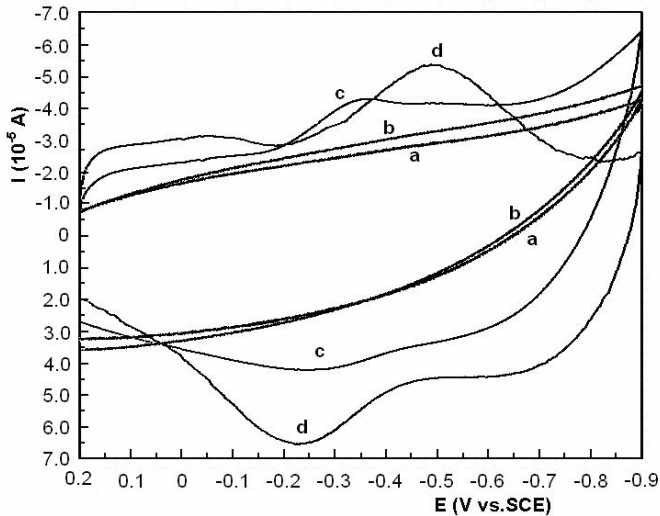
Fig. 2. Cyclic voltammograms of SA/CILE (a), SA/SiO₂/CILE (b), SA/nano-SiO₂/Hb/CPE (c) and SA/nano-SiO₂/Hb/ CILE (d) in pH 7.0 B-R buffer solution with the scan rate as 100 mV s⁻¹.
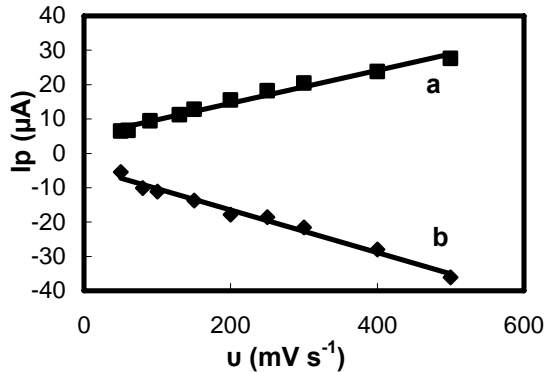

Fig. 3. Plots of the cathodic (a) and anodic (b) peak current with the scan rate (υ).

redox peaks were attributed to the combination effect of nano-SiO₂ and ionic liquid modified electrode. The nano-SiO₂ could provide a three-dimensional stage and a more suitable microenvironment for protein. With the higher ratio of surface area of nano-SiO₂, the Hb on the nano-SiO₂ surface had more uniform orientation. While on the surface of CILE, an ionic liquid film also existed, which not only acted as a high ionic

conductive binder but also exhibited an inherent catalytic activity and ion exchange property. As indicated in a recent report [32], the ionic liquids modified carbon paste electrode showed excellent advantages, such as sensitivity, low-potential, simplicity, low-cost, and stable electrochemical sensing of biomolecules and other electroactive compounds. Thus, the direct electron transfer of Hb on the SA/nano-SiO₂/CILE was obtained with the co-contribution of the bionanocomposite SA/nano-SiO₂ film and the specific properties of ionic liquid present in the modified electrode.

The reduction and oxidation peak potentials were appeared at -0.48 and -0.23 V (*vs.* SCE), respectively. By the equation of $E^{0'} = (E_{pa} + E_{pc})/2$, the formal potential ($E^{0'}$) of Hb was estimated as -0.355 V (*vs.* SCE) and the peak-to-peak separation found to be 250 mV at a scan rate of 100 mV s⁻¹. The ratio of redox peak current I_{pa}/I_{pc} was 0.94 suggesting the occurrence of a quasi-reversible redox reaction at the surface of CILE.

The influence of the scan rate on the electrochemical response was carefully investigated. Both the reduction and oxidation peak currents increased linearly with the scan rate in the range from 40 to 500 mV s⁻¹ and the relationship of peak current with scan rate were shown in Fig. 3, which corresponded to a typical surface-controlled thin-layer voltammetric process.

With the increase of the scan rate, the redox peak potentials were also shifted gradually with an increase in the peak-to-peak separation. Thus, the electrochemical parameters could be calculated by the following Laviron's method [42,43]:

$$E_{pc} = E^{0'} - \frac{2.3RT}{\alpha nF} \log \frac{\alpha}{m} \tag{1}$$

$$E_{pa} = E^{0'} + \frac{2.3RT}{(1-\alpha)nF} \log \frac{1-\alpha}{m} \tag{2}$$

and

$$m = \frac{RT}{F} \cdot \frac{k_s}{n\upsilon} \tag{3}$$

$$\log k_s = \alpha \log(1-\alpha) + (1-\alpha) \log \alpha - \log(\frac{2.3RT}{nF\upsilon}) - \alpha(1-\alpha)\frac{nF\Delta E_p}{2.3RT} \tag{4}$$

where α is the electron transfer coefficient, n is the number of

electrons transferred in the rate determining reaction, k_s is the electron transfer rate constant, R is the gas constant, T is the absolute temperature, and ΔEp is the peak-to-peak separation.

Based on the above equation the electrochemical parameters could be further calculated. Figure 4 shows the linear relationship of the redox peak potentials with the ln υ with regression equations as Epc (V) = -0.0642 lnυ - 0.561 (n = 11, γ = 0.996) and Epa (V) = 0.0313 lnυ - 0.140 (n = 9, γ = 0.991). According to Eqs. (1) and (2), the value of αn was calculated as 0.400. Given 0.3 < α < 0.7, in general it can be concluded that n = 1 and α = 0.40; so, the redox reaction of Hb is a single electron transfer process. Based on Eq. (4), the electron transfer rate constant (k_s) was further calculated from the relationship of ΔEp with lnυ as 0.077 s^{-1}.

The influence of buffer pH on the electrochemistry of Hb was investigated. With the increase of solution pH from 4.0 to 8.5, a negative shift in both the reduction and the oxidation peak potentials was observed. A linear regression equation of the formal potential ($E^{0\prime}$) against pH was constructed as $E^{0\prime}$ (V) = -0.0452 pH - 0.0347 (n = 9, γ = 0.998). The slope value foun to be -45.2 mV/pH, which was lower than that of the expected value of -59.0 mV/pH (25 ºC) for an ideal reversible one proton transfer couple to one electron transfer process. This should be due to different microenvironments of the Hb in the film and the slightly changes of the conformation of the peptide backbone with the variation of the external solution [44]. But the conclusion can also be drawn that one proton and one electron were attended in the electron transfer process and the electrode equation could be expressed as:

$$\text{Hb Fe(III)} + \text{e} \xleftrightarrow{\text{H}^+} \text{Hb Fe(II)}$$
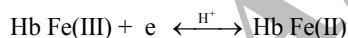
where the charges on Hb species have been omitted.

The surface concentration of electroactive protein (Γ*) could be estimated by integration of cyclic voltammetric reduction peaks using the equation of Q = nFAΓ*, where Q is the charge passing through the electrode with full reduction of electroactive Hb in the composite film, A is the geometric area of the CILE, n and Γ have their usual meanings. According to this method, the average Γ* value was calculated as 1.18 × 10^{-10} mol cm^{-2}. The value obtained was about 6 times higher than that of the theoretical monolayer about 1.89 × 10^{-11} mol cm^{-2} [25]. The result indicated that several layers of Hb
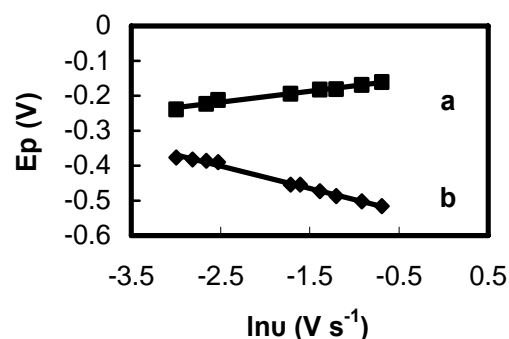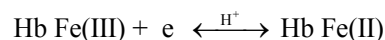

Fig. 4. The relationship of the anodic (a) and cathodic (b) peak potential with the lnυ.
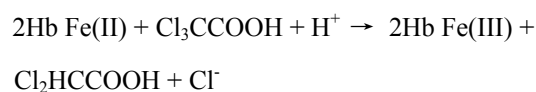
entrapped in the bionanocomposite film may participated in the electron-transfer process. The relative amount of electroactive Hb on the electrode surface was 2.81% of the total amount of Hb deposited on the electrode surface, which was higher than that in reference [30].

Electrocatalytic Behavior to Reduction of Trichloroacetic Acid

The SA/nano-SiO$_2$/Hb/CILE showed good electrocatalytic ability to the reduction of trichloroacetic acid (TCA) and the cyclic voltammograms are shown in Fig. 5. When TCA was added into a pH 7.0 B-R buffer, an increase in the reduction peak current at about -0.41 V was observed with the disappearance of the oxidation peak (curves b-e), which indicated that Fe(II) was reacted with the organohalides. While on bare CILE, the reduction peak appeared at -1.0 V, so the presence of Hb composite film could lower the reduction overpotential of TCA for about 0.6 V. The results indicated that a large decrease in activation energy for the reaction. According to reference [45], the reaction process was proposed as follows:

$$\text{Hb Fe(III)} + \text{e} \xleftrightarrow{\text{H}^+} \text{Hb Fe(II)}$$

at electrode

$$2\text{Hb Fe(II)} + \text{Cl}_3\text{CCOOH} + \text{H}^+ \rightarrow 2\text{Hb Fe(III)} +$$
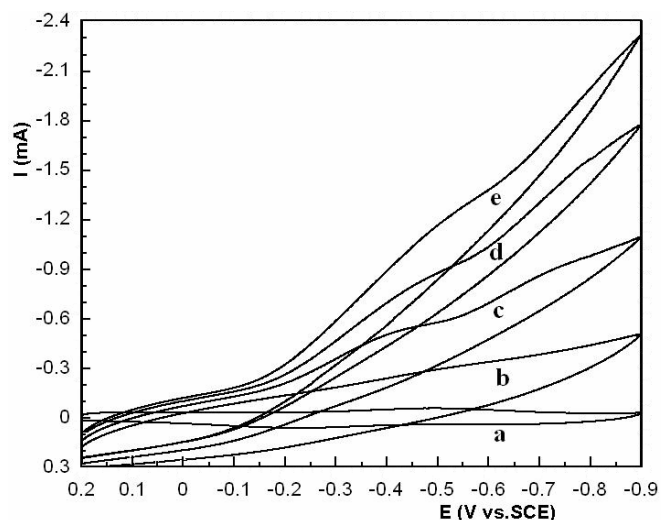$$\text{Cl}_2\text{HCCOOH} + \text{Cl}^-$$

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Fig. 5. Cyclic voltammograms of SA/nano-SiO₂/Hb/CILE in a pH 7.0 B-R buffer solution containing 0, 37.5, 62.5, 112.5 and 137.5 μM TCA (curve a-e) with the scan rate as 100 mV s⁻¹.
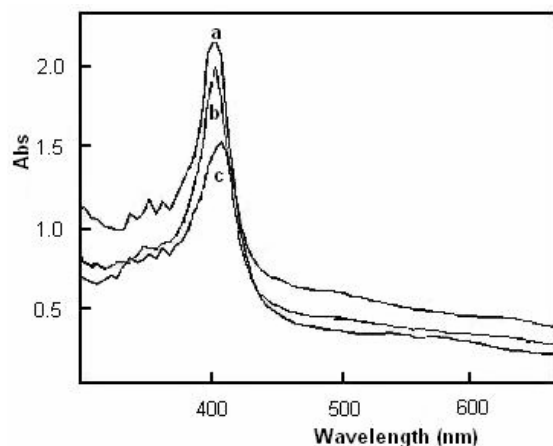

Fig. 6. UV-Vis absorption spectra of Hb in water solution (a), SA/nano-SiO₂/Hb in pH 5.0 (b) and pH 10.0 (c) B-R buffer.

The reduction peak current was proportional to the concentration of the TCA in the range of 6.25 ~ 137.5 μM with a linear regression equation of Ip (μA) = 5.64 C (μM) + 114.83 (n = 9, γ = 0.993) and a detection limit of 5.0 μM (3σ). The apparent Michaelis-Menten constant (K_M^{app}) could be

calculated from the electrochemical version of the Lineweaver-Burk equation [46]:

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

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 saturated substrate condition. By analysis the slope and the intercept of the plot of the reciprocals of the reduction peak current versus TCA concentration, the value of K_M^{app} was determined as 0.28 mM, which was an indicative of the enzyme-substrate kinetics. The smaller value of K_M^{app} means the higher enzymatic activity of immobilized enzyme. The calculated K_M^{app} value is smaller than that of Pang's reports [25], indicating that Hb immobilized on the SA/nano-SiO₂ film had good affinity to TCA.

UV-Vis Spectroscopy

UV-Vis Soret absorption band of Hb provides the information on the conformational integrity of the heme group region of heme protein. If Hb is denatured, the spectroscopy of Soret band will change or disappear. Figure 6 shows the UV-Vis absorption spectra of Hb and its mixture with SA and nano-SiO₂ in different buffer pH. As for Hb in water, the Soret band appeared at 404.6 nm (curve a). The position of the Soret band would change along with the pH of the external solution. When pH was located at 10.0 (curve c), the Soret band began to decrease and moved to 410 nm, which indicated a small denaturation of Hb. At pH 5.0 buffer, the Soret band appeared at 405.1 nm (curve b), which was close to the 404.6 nm of native Hb. The results indicated that Hb in the SA/nano-SiO₂ film maintained its native secondary structure in the pH range from 5.0 to 10.0.

CONCLUSIONS

In this paper the direct electrochemistry of Hb in the SA and nano-SiO₂ organic-inorganic composite film was achieved on an ionic liquid BMIMPF₆ modified carbon paste electrode. Hb could retain its bioactivity in the SA/nano-SiO₂ bionanocomposite materials, and the presence of nano-SiO₂ in

the film could greatly enhance the direct electron transfer efficiency of Hb on the CILE. The fabricated SA/nano-SiO$_2$/Hb/CILE showed good electrocatalytic ability to the reduction of TCA, which has potential application in biosensors and bioelectrochemistry.

ACKNOWLEDGEMENTS

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