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Direct Electrochemistry of Hemoglobin in a Nafion and CuS Microsphere Modified Carbon Ionic Liquid Electrode and its Electrocatalytic Behavior

W. Sun*, R.-F. Gao, R.-J. Zhao, H.-T. Zhu and K. Jiao

Key Laboratory of Eco-Chemical Engineering of Ministry of Education, College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042,P.R.China

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Direct electrochemistry of hemoglobin (Hb) was realized on a Nafion and CuS microsphere composite film modified carbon ionic liquid electrode (CILE) with *N*-butylpyridinium hexafluorophosphate ($BPPF_6$) as binder. Scanning electron microscopy (SEM), UV-Vis absorption spectroscopy and cyclic voltammetry were used to characterize the fabricated Nafion/CuS/Hb/CILE. Experimental results showed that a pair of well-defined quasi-reversible redox peaks appeared with the formal potential as -0.386 V (*vs*. SCE) in pH 7.0 Britton-Robinson (B-R) buffer solution, which was attributed to the Hb heme Fe(III)/Fe(II) redox couples. The electrochemical parameters of Hb in the composite film were carefully investigated with the charge transfer coefficient (α), the electron transfer number (n) and the electron transfer rate constant (k_s) as 0.505, 1.196 and 0.610 s^{-1}, respectively. The composite film provided a favorable microenvironment for retaining the native structure of Hb. The presence of CuS microspheres showed great improvement on the electron transfer rate of Hb with the CILE, which maybe due to the contribution of specific characteristics of CuS microspheres and the inherent advantages of ionic liquid on the modified electrode. The fabricated Hb modified electrode showed good electrocatalytic ability in the reduction of H_2O_2. The proposed bioelectrode can be used as a new third generation H_2O_2 biosensor.

Keywords: *N*-Butylpyridinium hexafluorophosphate, CuS microsphere, Carbon ionic liquid electrode, Hemoglobin, Hydrogen peroxide

INTRODUCTION

Room temperature ionic liquids (RTILs), which are ionic compounds composed of organic cations and various anions, represent a new class of green solvents for electrochemical applications. As non-aqueous polar solvent, RTILs have many unique properties such as high chemical and thermal stability, relatively high ionic conductivity, negligible vapor pressure and wide electrochemical windows. These properties make them suitable as electrolytes and solvents in the field of electrochemistry [1,2]. As for electroanalytical chemistry, RTILs can be used as not only the supporting electrolyte but also the modified materials [3]. Maleki *et al*. applied *N*-octylpyridinum hexafluorophosphate as a binder for the construction of a high-performance carbon composite electrode [4]. RTILs modified electrodes have also been used for the detection of electroactive substances, such as ascorbic acid (AA) and dopamine (DA) with the advantage of high sensitivity and good anti-fouling ability [5-7]. Some biopolymers or nanomaterials, such as chitosan, Nafion or carbon nanotubes (CNTs) have been mixed with ILs to form biocompatible matrixes for investigations on the direct

*Corresponding author. E-mail: sunwei@qust.edu.cn

electrochemistry of redox proteins [8-11].

With the development of material chemistry, microspherical materials have aroused great interest due to their unique structural, optical and surface properties [12]. Microspheres have many potential applications in the fields of chemistry, biotechnology and material science. And they have been used in artificial cells, shape-selective adsorbents, lightweight fillers, and as catalysts or waste removers [13,14]. Various kinds of inorganic or polymer materials with hollow spherical structure have been synthesized using different templates such as silica sols [15], liquid drops [16], polymer micelles [17] and emulsion [18]. Of these, copper sulfide microsphere can be fabricated through different methods [19-22] and used as novel gas sensoring materials [23].

Direct electrochemistry of redox proteins on modified electrodes has been widely studied for its potential applications in biosensor and bioreactor. Due to the difficulty of direct electron transfer between protein and the surface of bare electrode, film modified electrodes have been devised as effective tools for the investigation of protein electrochemistry. Various substances such as insoluble surfactants [24], hydrogel [25], composite film [26] and nanoparticles [27] have been explored as the immobilization matrix. Proteins can retain their native structure in the film and the direct electron transfer rate of protein with electrode can often be greatly enhanced.

In this paper, a room temperature ionic liquid *N*-butylpyridinium hexafluorophosphate ($BPPF_6$) was used as a binder to make a carbon ionic liquid electrode (CILE). Hemoglobin (Hb) was immobilized on the surface of CILE by the simple casting method. Then, CuS microspheres and Nafion were coated on the surface of Hb/CILE step by step to get a modified electrode denoted as Nafion/CuS/Hb/CILE. CuS microspheres act as enhancers to accelerate the electron transfer rate of the redox proteins. Nafion is a proton-conductive and biocompatible perfluorosulfonate linear polymer that exhibits excellent film-forming ability, which has been used for the immobilization of enzymes [28,29]. The presence of Nafion on the electrode surface can form a stable film to enhance the stability of protein immobilized on the modified electrode and prevent the leakage of Hb from electrode surface into the solution. The immobilized Hb retained its native structure in the composite film and experimental results indicated that the electrochemical response of Hb was greatly enhanced with the appearance of a pair of well-defined quasi-reversible redox peaks. The modified electrode exhibited high electrocatalytic ability in the reduction of H_2O_2.

EXPERIMENTAL

Apparatus

A CHI 750B electrochemical workstation (Shanghai CH Instrument, China) was used for all the electrochemical experiments with a traditional three-electrode system consisting of a Nafion/CuS/Hb/CILE (Φ = 4 mm) as the working electrode, a platinum wire as an auxiliary electrode and a saturated calomel electrode (SCE) as a reference one. Scanning electron microscopy (SEM) and UV-Vis absorption spectra were obtained on JSM-6700F scanning electron microscope (Japan Electron Company) and Cary 50 probe UV-Vis spectrophotometer (Varian Company, Australia), respectively.

Reagents

Bovine hemoglobin (Hb, MW. 64500, Tianjin Chuanye Biochemical Limited Company) was dissolved in Britton-Robinson buffer solution (pH 8.0). *N*-Butylpyridinium hexafluorophosphate ($BPPF_6$, Hangzhou Chemer Chemical Limited Company), and graphite powder (Shanghai Colloid Chemical Plant, average diameter of 30 μm) were used as received. 0.5% Nafion ethanol solution (Sigma) was prepared and stored at 4 °C. CuS microspheres (average diameter of 300 nm) were prepared by a reported soft template method [30] and dispersed in ethanol. 0.2 M Britton-Robinson (B-R) buffer solutions with various pH values were used as the supporting electrolytes and deoxygenated with nitrogen for 30 min before the experiments. All the other chemicals were of analytical reagent grade and double-distilled water was used throughout. The experiments were performed at a temperature of 25 ± 2 °C.

Procedure

CILE was prepared according to a previous report [31]. 3.0 g of graphite powder and 1.0 g of $BPPF_6$ were mixed thoroughly in a mortar and heated at 80 °C for about 1 h to form a homogeneous carbon paste. A portion of the carbon

paste was filled into one end of a glass tube (Φ = 4 mm) and a copper wire was inserted through the opposite end to establish an electrical contact. After polishing the surface of CILE carefully on a piece of weighing paper, the following procedure was followed to fabricate an Hb modified electrode. First, a 5 µl of 15.0 mg ml^{-1} Hb solution was evenly pipetted onto the surface of the CILE and spread gently over the entire surface. The electrode was left in the air to dry under ambient conditions for about 6 h to get Hb/CILE. Second, a 5 µl of the 3.0 mg ml^{-1} CuS microspheres suspension solution was pipetted on the Hb modified CILE and dried at room temperature. Finally, a 5 µl of the 0.5% Nafion solution was spread onto the electrode and dried to get a modified electrode denoted as Nafion/CuS/Hb/CILE. Other modified electrodes such as Nafion/CILE, Nafion/CuS/CILE and Nafion/Hb/CILE were prepared using a similar procedure.

RESULTS AND DISCUSSION

SEM Images

Figure 1 shows the scanning electron microscopic (SEM) images of CuS microspheres, CILE, Hb/CILE and Nafion/CuS/Hb/CILE, respectively. CuS microspheres were in good dispersion with the average diameter of 300 nm (Fig. 1a). On the surface of the CILE (Fig. 1b), a more uniform surface was formed and no separated carbon layer could be observed,

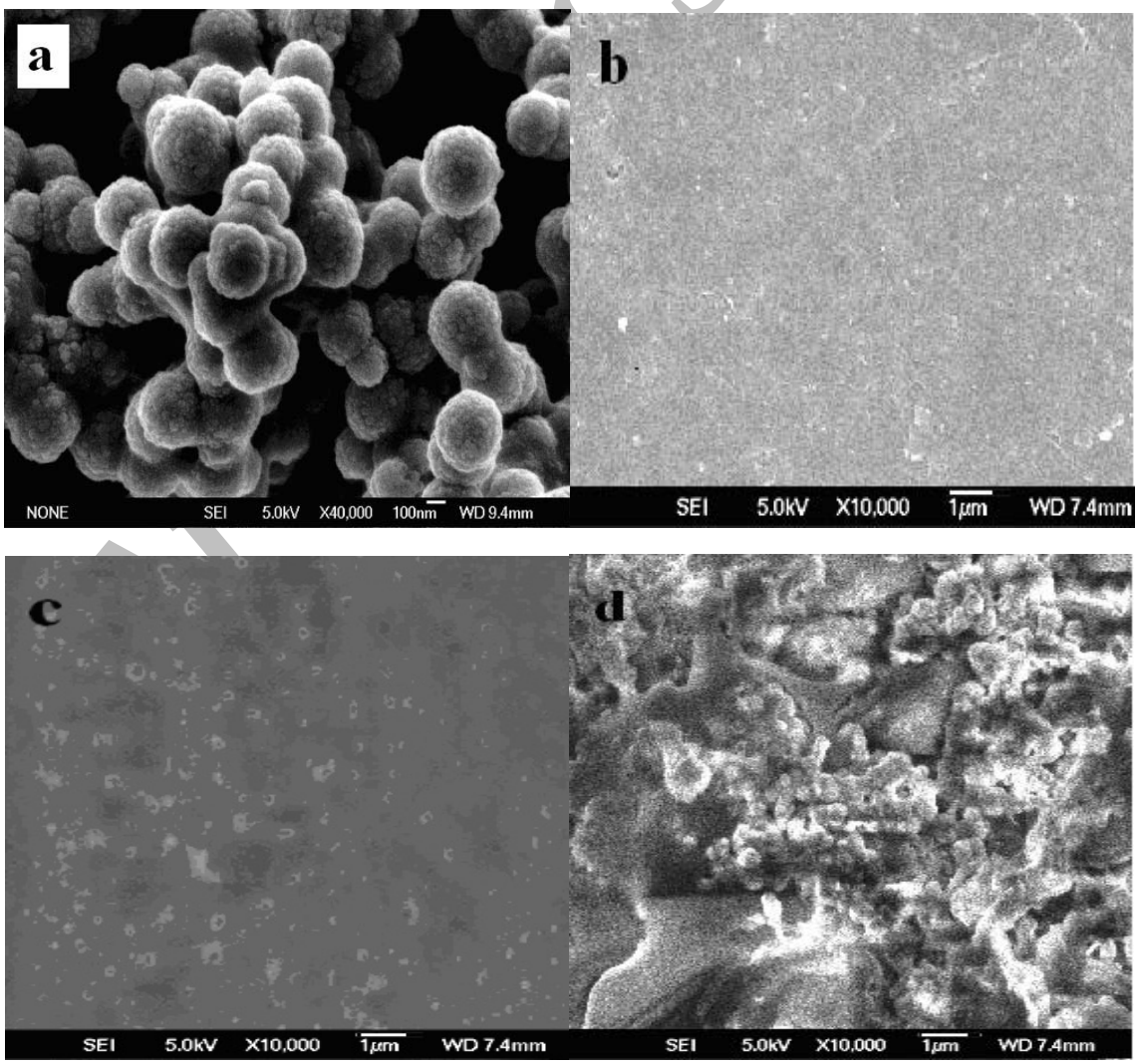

Fig. 1. SEM images of CuS microspheres (a), CILE (b), Hb/CILE (c) and Nafion/CuS/Hb/CILE (d).

which was caused by mass of RTILs embedded in carbon layers and bridged each layer of carbon flakes. On the Hb/CILE (Fig. 1c), the aggregation of the immobilized Hb molecules was distributed regularly showing a network-like structure. As for Nafion/CuS/Hb/CILE (Fig. 1d), some spherical particles were surrounded by unshaped membrane, which could have been due to the fact that Hb, CuS microspheres and Nafion interlocked together. So, the Hb molecules and CuS microspheres were tightly fixed onto the surface of CILE with the help of Nafion film.

UV-Vis Absorption Spectra

UV-Vis absorption spectroscopy is a useful tool to check the conformational integrity of heme proteins. Figure 2 shows UV-Vis absorption spectra of Hb in the composite film with different pH values of buffer solution. The Soret band of Hb in water solution appeared at 405 nm (curve a), which was an intrinsic heme-group property. The absorption spectra of the mixture of Hb with Nafion and CuS at different pHs of external buffer solutions were also tested. In the range of pH values between 5.0 and 9.0, the Soret band appeared at almost the same position as for the native Hb solution (curve c→f), indicating that Hb essentially retained its native conformation in CuS and Nafion mixture solutions with the pH range from 5.0 to 9.0. When the pH value of buffer solution shifted to a more acidic or more basic direction, the Soret band became smaller and broader. For example, at the pH value of 2.0 and 10.0, the Soret bands were located at 370 nm and 410 nm (curve b and g), respectively, with the absorbance curve deformed, suggesting that Hb in the film might undergo the denaturation to a certain extent.

Direct Electrochemistry of Hemoglobin

Figure 3 shows the cyclic voltammograms of different modified electrodes in pH 7.0 B-R buffer solution at scan rate of 100 mV s^{-1}. No voltammetric responses were observed on CILE, Nafion/CILE and Nafion/CuS/CILE, indicating that no electroactive substance existed on the electrode surface. Regarding Nafion/Hb/CILE, a pair of small redox peaks appeared (curve e), indicating that direct electron transfer rate was slow between the Hb and CILE. Accordingly, CILE had the advantages such as high chemical and thermal stability, relatively high ionic conductivity, wide electrochemical

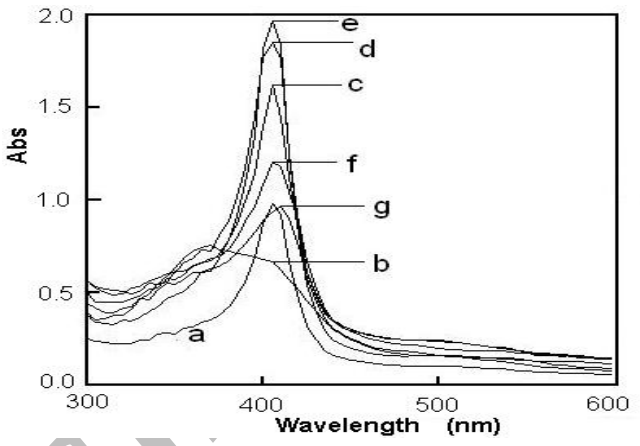

Fig. 2. UV-Vis absorption spectra of Hb in water (a) and Nafion/CuS/Hb in pH (b) 2.0, (c) 5.0, (d) 6.0, (e) 7.0, (f) 9.0, (g) 10.0 B-R buffer solution.

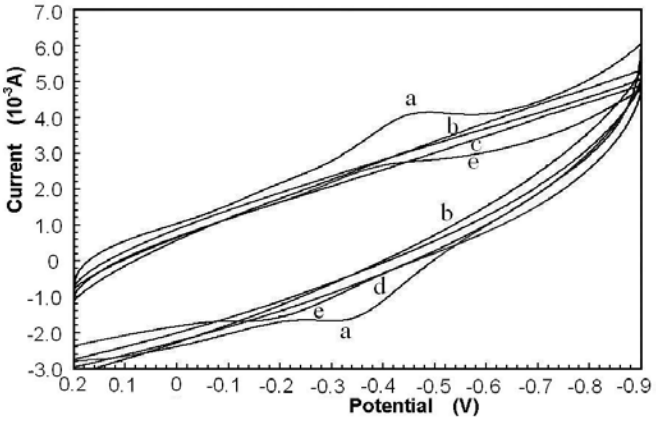

Fig. 3. Cyclic voltammograms of (a) Nafion/CuS/Hb/CILE, (b) CILE, (c) Nafion/CuS/CILE, (d) Nafion/CILE and (e) Nafion/Hb/CILE in pH 7.0 B-R buffer solution at scan rate of 100 mV s^{-1}.

windows and good biocompatibility [32]. A layer of IL was also present on the surface of CILE, which played important roles in accelerating the direct electron transfer rate of Hb. After the CuS microspheres were added into the film, a pair of well-defined quasi-reversible cyclic voltammetric peaks appeared (curve a), indicating that the presence of CuS microspheres in the film could greatly enhance the electron transfer rate and improve the redox response. CuS

microspheres have the characteristics such as small crystal size, large surface area, tunable porosity and good conductivity [33]. The addition of CuS microspheres into the Hb film helped to assemble the Hb molecules and form a biocompatible porous hybrid film. Under the synergetic effect of CuS microspheres and IL in the composite film interface, the direct electron transfer of Hb was realized successfully.

According to the cyclic votammogram of Nafion/CuS/Hb/CILE, the oxidation and reduction peak potentials were obtained as -0.326 V and -0.445 V (*vs*. SCE), respectively. The apparent formal potential ($E^{0'}$) was calculated as -0.386 V (*vs*. SCE), which was located at the characteristic potential of the heme Fe(III)/Fe(II) redox couples of heme proteins [34]. The results suggest that the Hb molecules entrapped in the composite film retained the electrochemical activity.

Figure 4A shows the cyclic voltammograms of Nafion/CuS/Hb/CILE with different scan rates in pH 7.0 B-R buffer solution. A pair of roughly symmetric redox peaks appeared with almost equal heights of the redox peak currents in the scan rate from 0.07 to 0.50 V s^{-1}. The results indicate that all the electroactive Hb Fe(III) in the film were reduced to Hb Fe(II) on the forward scan and then reoxidized to Hb Fe(III) on the reverse scan. A good linear relationship was found (Fig. 4B) between the redox peak current and scan rate with the results as Ipc (μA) = 31.29 + 241.03υ (V s^{-1}) (R = 0.992) and Ipa (μA) = -11.87-323.99υ (V s^{-1}) (R = 0.994), which were the characteristic of surface-controlled thin-layer electrochemical behavior. The surface concentration (Γ*) of electroactive Hb in the film was calculated according to the following equation [35]: Q = nFAΓ*, (Q is the charge passing through the electrode with full reduction of electroactive Hb in the film, A is the area of the CILE, n is the electron transfer number for each Hb molecule, F is Faraday's constant and Γ* is the surface concentration of the electroactive substance). The value of Γ* was calculated as 4.52×10^{-9} mol cm^{-2}, while the concentration of Hb cast on the CILE surface was 9.70×10^{-9} mol cm^{-2}. So, 46.6% of the total amount of Hb in the film took part in the electron transfer. The theoretical monolayer coverage of Hb on the electrode surface was about 2.0×10^{-11} mol cm^{-2}, so it can be concluded that several layers of Hb immobilized on the electrode surface took part in the electrochemical reaction. The results indicate that the three-dimensional porous structure of the CuS microspheres

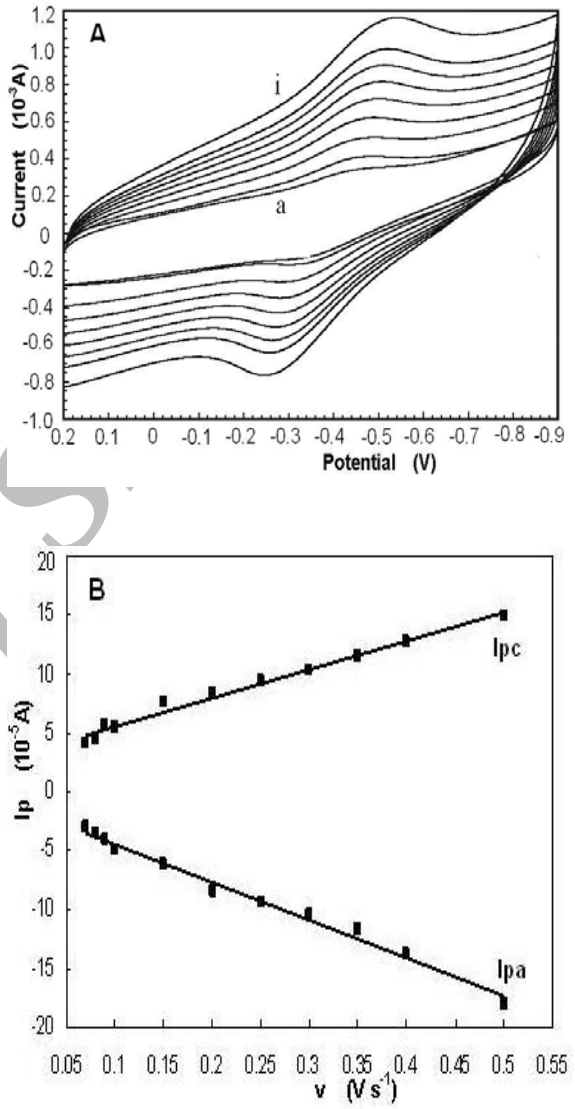

Fig. 4. (A) Cyclic voltammograms of Nafion/CuS/Hb/CILE with scan rate as 80, 100, 150, 200,250, 300, 350, 400, 500 mV s^{-1} (a → i) in pH 7.0 B-R buffer solution; (B) the plots of cathodic and anodic peak current with scan rate.

with a large surface area and high loading content of Hb contributed to the multi-layerity of protein participating in the electron transfer process.

The peak-to-peak separation (ΔEp) also increased gradually with the increase of scan rate, indicating that the electrode process turned to more irreversible. The

electrochemical parameters of Hb in the modified electrode were estimated using the following Laviron's equations [36]:

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

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

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

where α is the charge transfer coefficient, n is the number of electrons transferred and k_s is the electron transfer rate constant. The relationships of redox peak potentials with logarithm of scan rate (logυ) were established as E_{pc}(V) = -0.539-0.0932 logυ (R = 0.995) and E_{pc}(V) = -0.232 + 0.0977 logυ (R = 0.994). According to these equations, the value of α, n and k_s were calculated as 0.505, 1.196 and 0.610 s^{-1}, respectively. The k_s value was indicative of a quasi-reversible redox process of Hb.

The influence of buffer pH on the electrochemical responses of Nafion/CuS/Hb/CILE was recorded. Both the redox peaks of the Hb modified electrode shifted to the negative direction with the increase of the external buffer pH. By exploring the relationship of the apparent formal potentials ($E^{0'}$) with the pH value, a linear regression equation was established as $E^{0'}$(V) = -0.0452 pH -0.0738 (R = 0.998) in the pH range from 5.0 to 9.0. The result indicated that protons had taken part in the electrode reaction. The value of slope was -45.2 mV/pH, which was smaller than the theoretical value of -59.0 mV/pH at 25 °C for the reversible one-electron transfer coupled with single-proton transportation. This may be due to the influence of the protonation of residue ligands around the heme as well as that of water molecules coordinated with the central iron [37]. So the simplified equation for the electrochemical reaction of Hb can be expressed as [38]: Hb heme Fe(III) + H^+ + e^- ⇌ Hb heme Fe(II), where the charges on Hb molecules are omitted.

Electrocatalytic Reduction Towards H_2O_2

The Hb modified electrode showed good electrocatalytic activity to the reduction of H_2O_2 and the cyclic voltammograms of Nafion/CuS/Hb/CILE with different amounts of H_2O_2 in pH 7.0 B-R buffer are shown in Fig. 5A. After the addition of H_2O_2, a new and significant reduction peak appeared at -0.308 V, the oxidation peak decreased and eventually disappeared, which was characteristic of an electrochemical catalytic reaction of Hb immobilized in the composite film electrode. This could be attributed to the oxidation of Hb heme Fe(II) with H_2O_2 resulting in Hb heme Fe(III) quickly, and then the Hb heme Fe(III) is directly and electrochemically reduced to Hb heme Fe(II). The catalytic reduction peak current increased linearly with the H_2O_2 concentration in a range from 8.0 to 700.0 μM by cyclic voltammetry (Fig. 5B) with the regression equations as I_{ss} (μA) = 1.53 C (μM) + 3.37 (R = 0.997) and the detection limit as 4.0 μM (3σ).

When the H_2O_2 concentration was higher than 720.0 μM, the calibration curve tended to a plateau, which was a typical Michaelis-Menten kinetics behavior. The apparent Michaelis-Menten constant (K^M_{app}) can be calculated from the electrochemical version of the Lineweaver-Burk equation [39]:

$$\frac{1}{I_{SS}} = \frac{1}{I_{\max}} + \frac{K^M_{app}}{I_{\max}C}$$

where I_{ss} is the steady-state current after the addition of the substrate, C is the bulk concentration of the substrate, and I_{max} is the maximum current measured under saturated substrate conditions. K^M_{app} can be obtained by the analysis of the slope and the intercept of the plot of the reciprocals of the steady-state current *vs*. H_2O_2 concentration. In this system, the value of Nafion/CuS/Hb/CILE towards the electrocatalytic reduction of H_2O_2 was found to be 98.4 mM.

Reproducibility and Stability of the Nafion/CuS/Hb/CILE

The fabricated Hb modified electrode was used for 11 parallel determinations of 20.0 μM H_2O_2 solution and the relative standard deviation (RSD) was 3.8%. The stability of the Hb modified electrode was evaluated by examining the cyclic voltammetric response of Hb after continuously

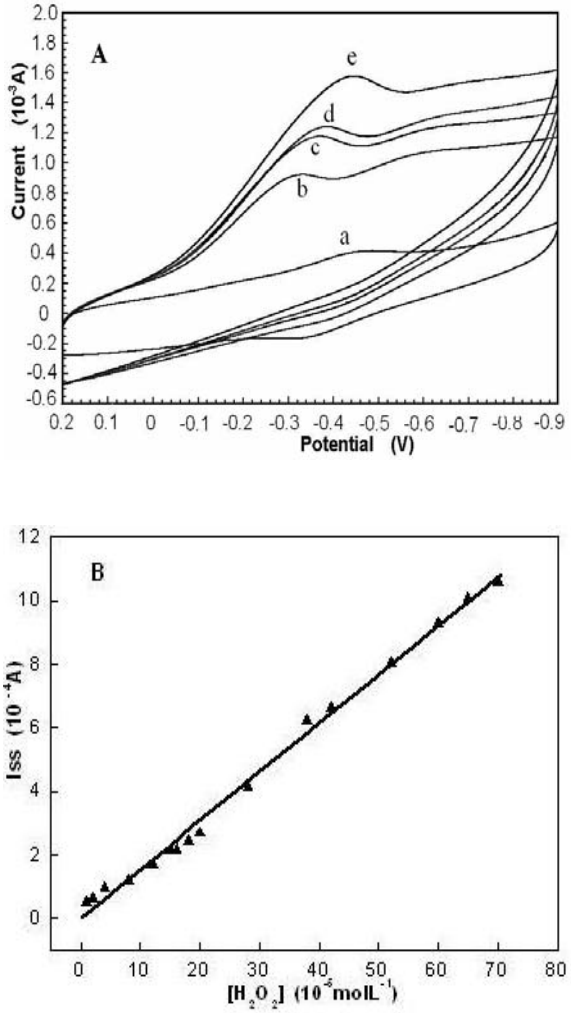

Fig. 5. (A) Cyclic voltammograms obtained at a Nafion/CuS/Hb/CILE in pH 7.0 B-R buffer solution with different amounts of H_2O_2 (from a → e 0, 280, 380, 420, 520 μM); (B) the calibration curve of catalytic reduction current *vs*. concentration of H_2O_2.

scanning for 50 cycles. There was nearly no decrease of the voltammetric peak currents. After the electrode was stored for two weeks at 4 °C refrigerator, 97.5% of the initial current response remained. After one-month storage the Hb modified electrode retained 93.4% of the initial current response. The results indicated that Nafion and CuS microspheres composite film was a suitable matrix for Hb immobilization and the bioactivity of Hb was retained.

CONCLUSIONS

By using an *N*-butylpyridinium hexafluorophosphate ($BPPF_6$) modified carbon paste electrode as the basal electrode, Hb was successfully immobilized on the surface of CILE with CuS microspheres and Nafion film. The resulting Hb modified electrode gave a pair of well-defined quasi-reversible redox peaks with the formal potential of -0.386 V (*vs*. SCE). The enhanced electrochemical behavior of Hb contributed to the synergetic function of CuS microspheres and ionic liquid $BPPF_6$, which were present in the composite film. The immobilized Hb molecules retained their native biocatalytical ability and the Nafion/CuS/Hb/CILE showed good electrocatalytic performance to the reduction of H_2O_2 with wider concentration range. In addition, Nafion/CuS/Hb/CILE showed good stability and reproducibility, which affords a potential application in third-generation mediator-free biosensor.

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