

# Application of Cobalt Oxide Nanoflower for Direct Electrochemistry and Electrocatalysis of Hemoglobin with Ionic Liquid as Enhancer

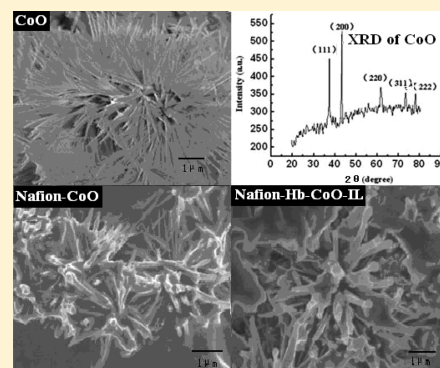
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**S** Supporting Information

**ABSTRACT:** A composite bionanomaterial was prepared by combining Nafion, flowerlike cobalt oxide (CoO) nanoparticles, hemoglobin (Hb), and ionic liquid (IL) 1-ethyl-3-methylimidazolium tetrafluoroborate. Then it was further applied on the surface of a carbon ionic liquid working electrode fabricated with 1-ethyl-3-methylimidazolium ethylsulfate as the modifier. Ultraviolet–visible and Fourier transform infrared spectroscopic results indicated that Hb molecules in the composite film retained the native structure. Cyclic voltammetric results showed that a pair of well-defined redox peaks appeared in 0.1 mol L<sup>-1</sup> pH 4.0 phosphate buffer solution, indicating that the direct electron transfer of Hb with the underlying electrode was realized. The results could be attributed to the synergistic effect of CoO nanoflower and IL in the composite film, which provided a specific microenvironment to keep the native structure of Hb and promoted the electron transfer rate of Hb. The electrochemical parameters of Hb on the modified electrode were carefully calculated. The composite material modified electrode showed excellent electrocatalytic ability toward the reduction of different substrates such as trichloroacetic acid and H<sub>2</sub>O<sub>2</sub>, which exhibited advantages such as high sensitivity, good stability, and wide dynamic range. Therefore, it has potential application in third-generation electrochemical biosensors.



## 1. INTRODUCTION

Direct electrochemistry of redox proteins can establish a desirable model for fundamental studies on the redox mechanism of proteins in biological systems,<sup>1</sup> which may elucidate the relationship between their structures and biological functions. Meanwhile, studies on direct electron exchange between proteins and underlying electrodes can also provide a platform for fabricating biosensors, enzymatic bioreactors, and biomedical devices.<sup>2</sup> Different kinds of protein film modified electrodes have been devised to achieve the direct electron transfer of redox proteins with working electrodes, and the film can provide a favorable microenvironment for keeping the molecular structures and biocatalytic abilities of the proteins.<sup>3,4</sup> Many methods including direct casting, layer by layer, sol–gel, etc. have been used for the fabrication of protein film electrodes.<sup>5,6</sup> In general proteins can retain their native structures and retain biocatalytic ability in the films under the selected conditions. The immobilized proteins on the modified electrode often exhibit a promoted electron transfer rate with well-defined redox peaks from the heme active center appearing.

Ionic liquids (ILs) are salts composed of ions, which exist in the liquid state at ambient temperature. They have many unique physicochemical properties including high ionic conductivity, wide electrochemical windows, and excellent solubility with many substances.<sup>7</sup> ILs have exhibited potential applications in

the field of electrochemistry and electroanalysis.<sup>8,9</sup> Wei and Ivaska<sup>10</sup> reviewed the applications of ILs in electrochemical sensors. ILs can be used as the electrolyte or as the binder/modifier in chemically modified electrodes. Wang et al. investigated the direct electrochemistry of heme proteins in agarose hydrogel films with IL as the electrolyte.<sup>11</sup> Ding et al. studied direct electrochemistry of myoglobin with 1-(2-hydroxyethyl)-3-methylimidazolium tetrafluoroborate as supporting electrolyte.<sup>12</sup> With the use of IL as binder and modifier, a new kind of modified electrode, named the “carbon ionic liquid electrode” (CILE), was successfully fabricated as a basal working electrode in electrochemical detection. For example, Sun et al. investigated the direct electrochemical behaviors of hemoglobin (Hb) in a sodium alginate film modified CILE.<sup>13</sup> Safavi et al. fabricated a glucose sensor based on a nanoscale nickel hydroxide modified CILE.<sup>14</sup> Zheng et al. also applied a CILE to the electrochemical detection of calcium dobesilate.<sup>15</sup>

In recent years, nanostructured materials have attracted great attention due to their specific properties and potential applications. Nanomaterials have exhibited many different physical and chemical properties from the bulk materials due to the small

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sizes. The ability to tailor the size and the structure of nanomaterials offers wide prospects for designing novel sensors and enhancing the performance of the bioanalytical assay.<sup>16</sup> The applications of nanomaterials in bioanalytical chemistry can result in developing new nanodevices for biological, medical, and electronic applications. Nanostructured materials of metal oxide semiconductors have attracted considerable interest in bioanalytical applications such as bioelectronics and biosensors due to their properties such as high surface area, nontoxicity, biocompatibility, ease of fabrication, chemical and photochemical stability, and good electrochemical catalytic activity. In protein electrochemistry nanostructured materials with porous structures can greatly enhance the active surface area available for protein immobilization and facilitate direct electron transfer between the redox proteins and the electrodes. Metal oxide nanoparticles such as manganous oxide,<sup>17</sup> zirconium oxide,<sup>18</sup> titanium oxide,<sup>19</sup> iron oxide,<sup>20</sup> and zinc oxide<sup>21</sup> have been successfully used for the immobilization of enzymes with applications in biosensors. Due to the structural stability and small size of inorganic nanoparticles, a favorable microenvironment can be established for redox proteins and enzymes to transfer electrons with underlying electrodes. In addition the small pores in metal oxide nanoparticles could act as substrate-transport channels to decrease the mass transfer resistance for efficient biocatalytic processes. Recently, several works have been reported with cobalt oxide (CoO) nanomaterial for biotechnological applications. CoO is a versatile semiconductive inorganic material, which is known to be highly reactive and has attracted much attention due to its applications in many areas. In the field of biosensors, CoO nanomaterial with a large surface area can serve as the matrix for the immobilization of biomolecules. The nanostructure of CoO greatly enhances the active surface available for the binding of biomolecules, and no work has been reported using flowerlike CoO nanoparticles for protein electrochemistry. In the present paper, Hb was mixed with Nafion, CoO nanoflower, and 1-ethyl-3-methylimidazolium tetrafluoroborate to get a new composite material, which was further modified on the surface of the CILE. Nafion is a commonly used film material in electrochemical sensors with advantages such as excellent film-forming ability, high water permeability, and biocompatibility, and can form a stable membrane on the electrode surface and immobilize the enzyme on the electrode surface. Hb retained its native structure in the composite material due to the biocompatibility of IL and CoO nanoflower. Then the direct electrochemistry and bioelectrocatalytic activity of the immobilized Hb were investigated by electrochemical techniques. The electrocatalytic ability of this biosensor for the reduction of trichloroacetic acid and hydrogen peroxide were further evaluated.

## 2. EXPERIMENTAL SECTION

**2.1. Apparatus and Reagents.** Ionic liquids 1-ethyl-3-methylimidazolium ethylsulfate ([EMIM]EtOSO<sub>3</sub>) and 1-ethyl-3-methylimidazolium tetrafluoroborate ([EMIM]BF<sub>4</sub>) were purchased from Hangzhou Kemer Chemical Ltd Co. Bovine hemoglobin (Hb, MW 64 500, Sigma), Nafion (5% ethanol solution, Sigma), graphite powder (average particle size 30  $\mu\text{m}$ , Shanghai Colloid Chemical Co., China), trichloroacetic acid (TCA, Tianjin Kemiou Chemical Ltd Co., China), and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, Yantai Sahe Chemical Ltd Co., China) were used as received. As the supporting electrolyte, 0.1 mol L<sup>-1</sup> phosphate buffer solutions (PBS) with various pH values were used. Flowerlike

CoO nanoparticles were prepared according to ref 22. All other chemicals used were of analytical reagent grade, and doubly distilled water was used in the experiments.

A CHI 440A electrochemical workstation (Shanghai CH Instrument, China) was used for the electrochemical measurements. A conventional three-electrode system was used with a composite film modified electrode as working electrode, a platinum wire as auxiliary electrode, and a saturated calomel electrode (SCE) as reference electrode. Ultraviolet–visible (UV–vis) absorption spectra and Fourier transform infrared (FT-IR) spectra were recorded on a Shimadzu UV-1700 spectrophotometer and a Varian 600 FT-IR spectrophotometer, respectively. Scanning electron microscopy (SEM) was performed on a JSM-6700F scanning electron microscope (Japan Electron Company, Japan). X-ray powder diffraction (XRD) pattern was operated on a Japan Rigaku D/Maxr-A X-ray diffractometer equipped with graphite monochromatized high-intensity Cu K $\alpha$  radiation ( $\lambda = 1.54178 \text{ \AA}$ ).

**2.2. Synthesis of the CoO Nanoflower.** The CoO nanoflower was synthesized with the following procedure. In the first step, nanoflower of cobalt hydroxide carbonate was successfully obtained via a hydrothermal process at a low temperature. In detail, 0.582 g (2.0 mmol) of Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O and 0.6 g (10.0 mmol) of CO(NH<sub>2</sub>)<sub>2</sub> were dissolved respectively into 50 mL of water under stirring. After 10 min of slight stirring, the obtained homogeneous solution was further transferred into a Teflon-lined stainless steel autoclave. Then, the autoclave was sealed and maintained at 365 K for 8 h in an electric oven. After the equipment cooled to room temperature naturally, the substrate with purple precursors grown on it was taken out and rinsed with distilled water several times in order to remove the residue. In the second step, the as-synthesized precursors were annealed under a constant flow of argon (50 sccm) at 700 K (with a rate of 10 K/min) for 4 h, allowing for the complete pyrolysis of cobalt hydroxide carbonate to pure flowerlike CoO nanoparticles.

**2.3. Preparation of Modified Electrode.** The CILE was fabricated by hand mixing 0.20 mL of [EMIM]EtOSO<sub>3</sub>, 0.80 mL of liquid paraffin, and 3.2 g of graphite powder in a mortar and grinding carefully. A portion of the resulting homogeneous paste was packed firmly into a glass tube cavity ( $\Phi = 4 \text{ mm}$ ). Electrical contact was established through a copper wire to the end of the paste in the inner hole of the tube, and the surface of the CILE was polished by smoothing on a weighing paper.

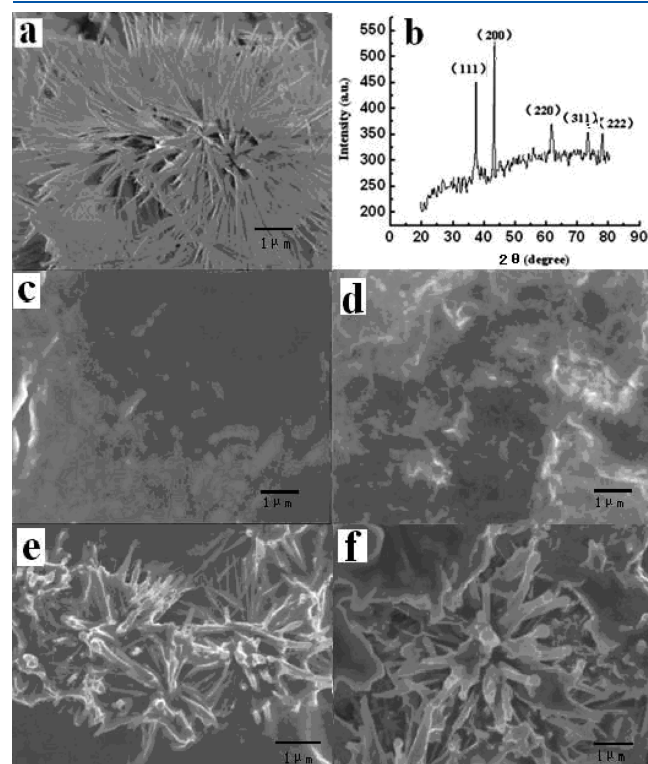
The modifier was prepared by mixing 500  $\mu\text{L}$  of 10.0 mg mL<sup>-1</sup> Hb, 100  $\mu\text{L}$  of 1.0 mg mL<sup>-1</sup> CoO nanoflower, 200  $\mu\text{L}$  of 0.5% Nafion, and 60  $\mu\text{L}$  of [EMIM]BF<sub>4</sub> together, then diluting to 1.0 mL with 0.1 mol L<sup>-1</sup> PBS (pH 7.0), and sonicating homogeneously. Then 8.0  $\mu\text{L}$  of the prepared Nafion–Hb–CoO–IL mixture was cast on the surface of the CILE, and it was left to dry at room temperature to form a stable film. The resulting electrode was named “Nafion–Hb–CoO–IL/CILE” and was stored at 4 °C when not in use. Other modified electrodes including Nafion–Hb–CoO/CILE, Nafion–Hb–IL/CILE, Nafion–Hb/CILE, etc. were prepared by similar procedures and used in the experiment for comparison.

**2.4. Procedures.** Electrochemical measurements were carried out in a 10 mL electrochemical cell containing 0.1 mol L<sup>-1</sup> PBS, which was purged with highly purified nitrogen for 30 min prior to a series of experiments and maintained in a nitrogen atmosphere during the experiments. UV–vis spectroscopic experiments were performed with a mixture solution of certain

concentrations of Hb, Nafion, CoO nanoflower, and [EMIM]BF<sub>4</sub>. The Nafion–Hb–CoO–IL film assembled on a glass slide was used for FT-IR measurements.

### 3. RESULTS AND DISCUSSION

**3.1. SEM Images of Modified Electrode.** Figure 1a shows typical SEM images of the prepared CoO nanoparticle. It can be seen that the samples are flowerlike structures composed of CoO rods. Each of the rods has one end outside and another end bound to other rods. The flowerlike CoO nanoparticle has a large surface area, which is beneficial for enzyme immobilization.

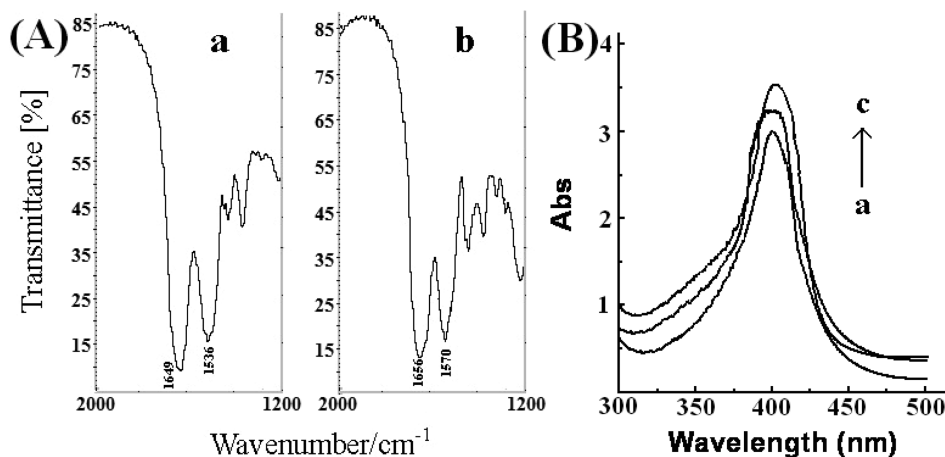


**Figure 1.** (a) SEM image and (b) XRD result of CoO nanoflower and SEM images of (c) CILE, (d) Nafion–Hb–IL/CILE, (e) Nafion–CoO/CILE, and (f) Nafion–Hb–CoO–IL/CILE.

Figure 1b shows the X-ray powder diffraction (XRD) pattern of the final product. All the diffraction peaks can be indexed to the standard raw salt cubic CoO with the lattice constant  $a = 4.261 \text{ \AA}$ , which is consistent with the value in the standard card (JCPDS Card No. 48-1719), and no other peaks of impurities are observed. Typical SEM images of the different composite material modified electrodes were further recorded. On the CILE a continuous and smooth layer of graphite appeared, which was attributed to the high viscosity of the IL used that bound the graphite together (Figure 1c). On the Nafion–Hb–IL/CILE (Figure 1d) a rough surface appeared, indicating the presence of the composite on the electrode surface. On the Nafion–CoO/CILE the CoO nanoflower could be easily observed with a layer of Nafion covering the nanoflower (Figure 1e). On the Nafion–Hb–CoO–IL/CILE (Figure 1f), CoO nanoflower was embedded uniformly in the composite membrane, indicating the full contact of the materials used.

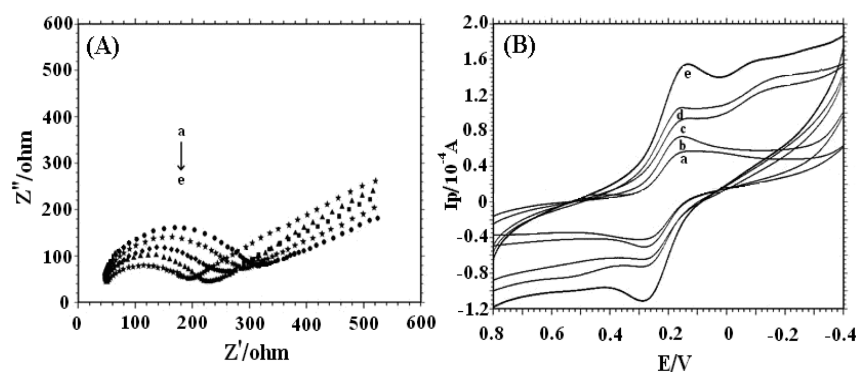
**3.2. Spectroscopic Results.** FT-IR spectroscopy is a sensitive method to probe into the secondary structure of proteins. The shape and position of the amide I and II infrared absorbance bands of proteins provide detailed information on the secondary structure of the polypeptide chain. The amide I band at  $1700\text{--}1600 \text{ cm}^{-1}$  is attributed to the C=O stretching vibration of the peptide linkage in the backbone of protein. The amide II band at  $1600\text{--}1500 \text{ cm}^{-1}$  is caused by the combination of N–H in-plane bending and C–N stretching vibration of the peptide groups.<sup>23</sup> If a protein molecule is denatured, the intensity and shape of the amide I and II bands will diminish or even disappear.<sup>24</sup> As shown in Figure 2A, the FT-IR peaks of two amide bands of pure Hb were located at  $1649$  and  $1536 \text{ cm}^{-1}$ , respectively (Figure 2A(a)). In the mixture the positions of amide I and II bands appeared at  $1656$  and  $1570 \text{ cm}^{-1}$  (Figure 2A(b)). The similarities of the two spectra suggested that Hb retained the essential features of its native structure in the bionanocomposite materials.

In UV–vis absorption spectra the Soret absorption band from the four iron heme groups of heme proteins may provide information on the conformational integrity of the proteins and the possible denaturation or conformational change about the heme region.<sup>3</sup> As shown in Figure 2B, the Soret band of Hb appeared at  $400.0 \text{ nm}$  in water (curve a) and at  $401.0 \text{ nm}$  in  $0.1 \text{ mol L}^{-1}$  pH 4.0 PBS (curve b). The mixture of Nafion–Hb–CoO–IL also gave the absorption peak at  $401.0 \text{ nm}$  (curve c).



**Figure 2.** (A) FT-IR spectra of (a) Hb and (b) Nafion–Hb–CoO–IL film. (B) UV–vis absorption spectra of (a) Hb in water solution, (b) Hb in pH 4.0 PBS, and (c) Nafion–Hb–CoO–IL in pH 4.0 PBS.





**Figure 3.** (A) EIS for (a) Nafion-Hb/CILE, (b) CILE, (c) Nafion-Hb-IL/CILE, (d) Nafion-Hb-CoO/CILE, and (e) Nafion-Hb-CoO-IL/CILE in the presence of  $5.0 \text{ mmol L}^{-1} [\text{Fe}(\text{CN})_6]^{3-/4-}$  and  $0.1 \text{ mol L}^{-1} \text{ KCl}$  with frequencies swept from  $10^4$  to  $0.1 \text{ Hz}$ . (B) Cyclic voltammograms of (a) Nafion-Hb/CILE, (b) CILE, (c) Nafion-Hb-IL/CILE, (d) Nafion-Hb-CoO/CILE, and (e) Nafion-Hb-CoO-IL/CILE in a mixture solution of  $10.0 \text{ mmol L}^{-1} [\text{Fe}(\text{CN})_6]^{3-/4-}$  and  $0.1 \text{ mol L}^{-1} \text{ KCl}$  solution with scan rate of  $100 \text{ mV s}^{-1}$ .

Obviously, the same position of the Hb Soret band suggested that Hb in the composite matrix retained its native structure. Nafion is a sulfonated tetrafluoroethylene based fluoropolymer-copolymer, which has been widely used in electrochemical biosensors due to its good film-forming ability, biocompatibility, good water permeability, and high mechanical adhesion. ILs are green solvents with the properties of high stability and good biocompatibility. Proteins and enzymes can retain their stabilities and activities better in ILs than in conventional organic solvent and aqueous solutions.<sup>25</sup> Also, CoO nanoparticle processes excellent biocompatibility. Therefore, Hb can retain its native structure in the Nafion-CoO-IL biocompatible materials.

**3.3. Electrochemical Characteristics of Modified Electrodes.** Electrochemical impedance spectroscopy (EIS) was performed in a mixture solution of  $5.0 \text{ mmol L}^{-1} [\text{Fe}(\text{CN})_6]^{3-/4-}$  and  $0.1 \text{ mol L}^{-1} \text{ KCl}$  with the frequency range swept from  $10^4$  to  $0.1 \text{ Hz}$ . Nyquist plots of different modified electrodes are shown in Figure 3A, and the semicircle diameter was equal to the electron transfer resistance ( $R_{\text{et}}$ ). On the CILE the  $R_{\text{et}}$  value was obtained as  $99.6 \Omega$  (curve b). After incorporation of Hb on the CILE, the  $R_{\text{et}}$  value of Nafion-Hb/CILE was increased to  $121.3 \Omega$  (curve a), indicating that the presence of nonconductive Hb hindered the electron transfer of the redox probe. On the Nafion-Hb-IL/CILE and Nafion-Hb-CoO/CILE the  $R_{\text{et}}$  values were decreased to  $80.7$  and  $58.9 \Omega$  (curves c and d), indicating that the presence of high conductive IL or CoO nanoflower in the film could increase the interface conductivity. On the Nafion-Hb-CoO-IL/CILE the smallest  $R_{\text{et}}$  value ( $40.3 \Omega$ ) appeared (curve e), which indicated that the synergistic effect of IL and CoO nanoflower in the composite increased the conductivity of the modified film. Therefore, different modification procedures of the electrode resulted in different electron transfer resistances and Hb was successfully immobilized on the electrode surface.

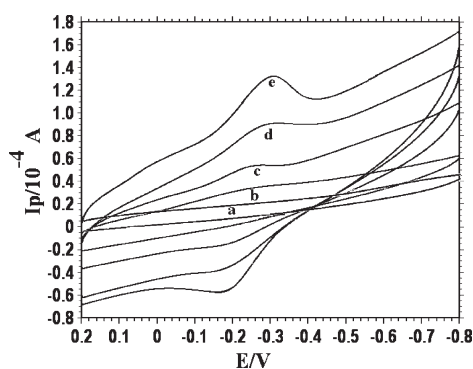
Potassium ferricyanide is a commonly used probe to evaluate the performance of modified electrodes with the typical cyclic voltammograms shown in Figure 3B and the electrochemical data summarized in Table 1. On the bare CILE (curve b) a pair of well-defined redox peaks appeared, indicating the good reversibility of the CILE. On the Nafion-Hb/CILE the redox peak currents decreased apparently (curve a), indicating that the presence of Hb on the electrode surface hindered the electron transfer. On the Nafion-Hb-IL/CILE the redox peak currents were bigger (curve c) than that of Nafion-Hb/CILE, which may be due to the presence of high ionic conductive IL in the composite

**Table 1. Electrochemical Data of Different Modified Electrodes in  $10.0 \text{ mmol L}^{-1}$  Ferricyanide Solution with Scan Rate as  $100 \text{ mV s}^{-1}$**

electrode	$I_{\text{pa}}$ ( $\mu\text{A}$ )	$E_{\text{pa}}$ (V)	$I_{\text{pc}}$ ( $\mu\text{A}$ )	$E_{\text{pc}}$ (V)	$I_{\text{pa}}/I_{\text{pc}}$	$\Delta E_{\text{p}}$ (V)
CILE	41.72	0.283	49.62	0.151	0.84	0.132
Nafion-Hb/CILE	35.95	0.298	38.54	0.116	0.93	0.182
Nafion-Hb-IL/CILE	46.69	0.284	52.55	0.152	0.89	0.132
Nafion-Hb-CoO/CILE	50.36	0.283	56.32	0.150	0.89	0.133
Nafion-Hb-CoO-IL/CILE	68.46	0.289	84.99	0.143	0.81	0.146

film. On the Nafion-Hb-CoO/CILE, the redox peak currents also increased (curve d), which was attributed to the presence of CoO nanoflower in the composite film. CoO nanoparticle has properties such as huge surface area, efficient catalytic activity, and good electron transfer ability which can facilitate the electron transfer rate. On the Nafion-Hb-CoO-IL/CILE the redox peak currents increased greatly (curve e) and were bigger than those of Nafion-Hb-IL/CILE and Nafion-Hb-CoO/CILE, indicating that the synergistic effects of CoO nanoflower and IL in the composite film promoted the electron transfer rate of the redox probe. Also the cyclic voltammograms of the modified electrodes in the  $0.1 \text{ mol L}^{-1} \text{ KCl}$  solution were recorded with the results shown in Figure S1 in the Supporting Information. It can be seen that no electrochemical responses appeared in the selected potential range.

The faradic current ( $I_{\text{p}}$ ) of ferricyanide showed a good linear relationship with the square root of the scan rate ( $\nu^{1/2}$ ) in the range from  $10.0$  to  $500.0 \text{ mV s}^{-1}$ , so the electrode reaction was controlled by a semi-infinite linear diffusional process. According to the Randles-Sevcik equation,  $i_{\text{p,c}} = (2.69 \times 10^5) n^{3/2} A D^{1/2} C^* \nu^{1/2}$ , where  $i_{\text{p,c}}$  is the reduction peak current (A),  $n$  is the electron transfer number,  $A$  is the electroactive surface area ( $\text{cm}^2$ ),  $D$  is the diffusion coefficient of  $\text{K}_3[\text{Fe}(\text{CN})_6]$  in the solution ( $\text{cm}^2 \text{ s}^{-1}$ ),  $C^*$  is the concentration of  $\text{K}_3[\text{Fe}(\text{CN})_6]$  ( $\text{mol cm}^{-3}$ ), and  $\nu$  is the scan rate ( $\text{V s}^{-1}$ ), the electroactive surface area of different modified electrodes can be estimated. On the basis of this method, the average electroactive area of CILE, Nafion-Hb/CILE, Nafion-Hb-IL/CILE, Nafion-Hb-CoO/CILE, and Nafion-Hb-CoO-IL/CILE were calculated as  $0.198$ ,  $0.131$ ,  $0.215$ ,  $0.222$ , and  $0.427 \text{ cm}^2$ , respectively. The results indicated that the presence

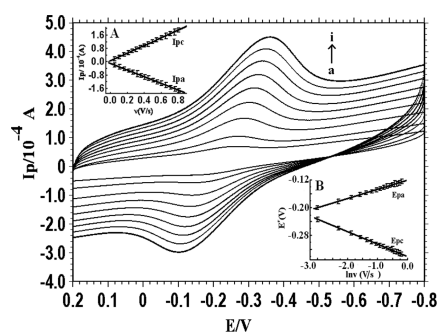


**Figure 4.** Cyclic voltammograms of (a) CILE, (b) Nafion-Hb/CILE, (c) Nafion-Hb-IL/CILE, (d) Nafion-Hb-CoO/CILE, and (e) Nafion-Hb-CoO-IL/CILE in pH 4.0 PBS with scan rate of  $100 \text{ mV s}^{-1}$ .

of IL and CoO nanoflower in the composite film greatly improved the effective surface area and accelerated the electron transfer between the redox couple in bulk solution and the electrodes.

**3.4. Optimal Conditions for the Modified Electrodes.** To obtain the stablest and highest cyclic voltammetric responses of the Hb modified electrode, the ratio of Hb, CoO, IL, and Nafion in the mixture solution were optimized by varying their concentrations in the control experiments. The results indicated that the amount of CoO nanoflower and IL can greatly influence the electrochemical responses of the modified electrode. The presence of CoO nanoflower can provide a specific interface with high surface area, but excessive addition of CoO nanoflower can result in the cracking of the composite film. The addition of IL in the composite film can enhance the whole conductivity of the film due to its high ionic conductivity, which facilitates the electron transfer rate of Hb with the electrode. However, excessive amounts of IL [EMIM]BF<sub>4</sub> would cause the instability of the composite film due to its hydrophilic property. Also Nafion played important roles in the stability of the modified electrode. When the composite without Nafion added was used, the modifier could be easily leaked out from the electrode surface into the buffer solution, which proved the function of Nafion with good film-forming ability. Nafion has been widely used in electrochemical biosensors as a selective and protective coating material for enzyme immobilization. Therefore, the mixture solution containing  $5.0 \text{ mg mL}^{-1}$  Hb,  $0.1 \text{ mg mL}^{-1}$  CoO nanoflower, 0.1% Nafion, and 6% [EMIM]BF<sub>4</sub> was selected as the optimal ratio for the fabrication of modified electrode.

**3.5. Direct Electrochemistry of the Modified Electrodes.** Figure 4 shows typical cyclic voltammograms of different modified electrodes in deaerated  $0.1 \text{ mol L}^{-1}$  PBS. On bare CILE (curve a) no electrochemical signal appeared, indicating that no redox substances were present on the electrode surface. On Nafion-Hb/CILE (curve b) a pair of small and unsymmetric redox peaks appeared, indicating a quasi-reversible electrochemical process. After the addition of IL in the composite film, the redox peak current increased on the Nafion-Hb-IL/CILE (curve c), which could be attributed to the presence of IL. IL has advantages such as high ionic conductivity and good biocompatibility, so the direct electron transfer rate of Hb was promoted. On the Nafion-Hb-CoO/CILE (curve d) the redox peak currents also increased gradually with values higher than that of Nafion-Hb/CILE (curve b) and the peak shape became more symmetrical. The results can be attributed to the presence

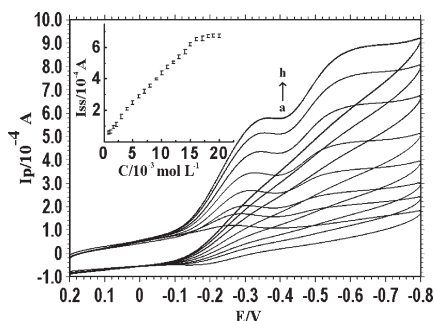


**Figure 5.** Cyclic voltammograms of Nafion-Hb-CoO-IL/CILE in pH 4.0 PBS with different scan rates (from a to i, 50, 100, 200, 300, 400, 500, 600, 700, and  $800 \text{ mV s}^{-1}$ , respectively). Inset A: Linear relationship of (a) cathodic and (b) anodic peak currents versus scan rate. Inset B: Linear relationship of peak potential (a)  $E_{pa}$  and (b)  $E_{pc}$  versus  $\ln v$ .

of the CoO nanoflower in the composite film, which is biocompatible with a high surface area and can work as a mediator to shuttle electrons between Hb and the electrode. Therefore, the electron transfer rate of Hb can be accelerated. On the Nafion-Hb-CoO-IL/CILE (curve e) a more symmetrical redox peak appeared with a voltammetric response bigger than those of the Nafion-Hb-IL/CILE and Nafion-Hb-CoO/CILE, respectively. The composite made with these substances represents a synergistic function due to the combination of the high surface area of CoO nanoflower, the biocompatibility of Nafion, the high ionic conductivity of IL, and the advantages of CILE, which provided a favorable three-dimensional microenvironment for the immobilized Hb molecules and accelerated the electron transfer rate with the underlying electrode. From curve e the values of  $E_{pc}$  and  $E_{pa}$  were obtained as  $-0.278$  and  $-0.198 \text{ V}$ . The apparent formal potential ( $E^{0'}$ ), which is calculated by the midpoint of the redox peak potentials, was estimated to be  $-0.238 \text{ V}$  (vs SCE). The result was in accordance with the typical characteristics of Hb heme Fe(III)/Fe(II) redox couples.<sup>26</sup> The peak-to-peak separation ( $\Delta E_p$ ) was obtained as  $0.080 \text{ V}$  and the ratio of redox peak current was nearly unity, which indicated a quasi-reversible electrode process.

Electrochemical responses of Hb modified electrode were recorded in different pH buffer solutions to get the best results. In the pH range from 2.5 to 9.0 stable and well-defined cyclic voltammetric curves could be obtained and the biggest redox peak currents appeared at pH 4.0. Therefore, the pH 4.0 PBS was selected as the supporting electrolyte for the electrochemical investigation on the direct electrochemistry of Hb.

**3.6. Electrochemical Behaviors of the Modified Electrode.** The influence of scan rate on the electrochemical responses of Nafion-Hb-CoO-IL/CILE was further investigated with the results shown in Figure 5. With the increase of scan rate a pair of symmetric redox peaks appeared with a height almost equal to that of the redox peak currents and with an increase of the  $\Delta E_p$  value. The results indicated that all the electroactive Hb Fe(III) in the composite film was reduced to Hb Fe(II) on the forward scan and then reoxidized to Hb Fe(III) on the reverse scan. Both redox peak currents increased linearly with scan rate in the range from 50 to  $800 \text{ mV s}^{-1}$ , indicating that the electrode process was a surface-controlled thin-layer electrochemical reaction. The linear regression equations were calculated as  $I_{pa} (\mu\text{A}) = 2.309v (\text{V s}^{-1}) + 0.081$  ( $n = 16$ ,  $\gamma = 0.998$ ) and  $I_{pc} (\mu\text{A}) = -1.986v (\text{V s}^{-1}) - 0.095$  ( $n = 16$ ,  $\gamma = 0.998$ ) (inset A of

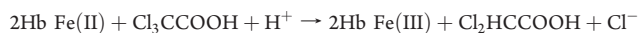


**Figure 6.** Cyclic voltammograms of Nafion-Hb-CoO-IL/CILE in 0.1 mol L<sup>-1</sup> pH 4.0 PBS containing different concentrations of TCA (from a to h, 0, 1.0, 1.6, 2.0, 4.0, 6.0, 8.0, and 12.0 mmol L<sup>-1</sup> respectively) with scan rate of 100 mV s<sup>-1</sup>. Inset: Relationship of catalytic reduction peak currents and TCA concentration.

Figure 5). With the increase of scan rate the value of  $\Delta E_p$  also increased gradually, indicating a quasi-reversible process. Therefore, the electrochemical parameters of the electrode reaction can be calculated according to Laviron's equation.<sup>27</sup> Two straight lines were obtained with the equations as  $E_{pa}$  (V) = 0.263 ln  $v$  - 0.122 ( $n = 16$ ,  $\gamma = 0.996$ ) and  $E_{pc}$  (V) = -0.0358 ln  $v$  - 0.340 ( $n = 16$ ,  $\gamma = 0.997$ ). Then the values of the electron transfer coefficient ( $\alpha$ ) and the apparent heterogeneous electron transfer rate constant ( $k_s$ ) were calculated as 0.423 and 1.571 s<sup>-1</sup>, respectively. It is well-known that the  $k_s$  value reflects the local microenvironment of the protein immobilized on the electrode. The value obtained here is in the range of  $k_s$  values for typical surface-controlled quasi-reversible electron transfer processes. Also, this value for  $k_s$  is higher than that for Hb immobilized on a Au colloid-cysteamine-modified gold electrode (0.49 s<sup>-1</sup>),<sup>28</sup> nanocrystalline titanium oxide film (0.137 s<sup>-1</sup>),<sup>29</sup> and carbon nanotubes (0.062 s<sup>-1</sup>).<sup>30</sup> The results showed that the electron transfer of Hb was facile due to the specific microenvironment provided by Nafion-CoO-IL nanocomposite film.

Based on the equation of  $\Gamma^* = Q/nAF$ , the surface concentration ( $\Gamma^*$ ) of electroactive Hb was calculated by integration of the cyclic voltammetric curve with the result of  $2.47 \times 10^{-9}$  mol cm<sup>-2</sup>. While the total amount of Hb cast on the electrode surface was  $9.88 \times 10^{-9}$  mol cm<sup>-2</sup>, 25.0% of the Hb molecules on the electrode surface took part in the electrochemical reaction, which was larger than some reported values.<sup>31,32</sup> The result also demonstrated that the composite film provided a specific three-dimensional structure for multilayer Hb to exchange electrons with the basal electrode.

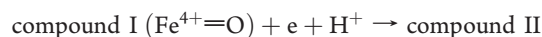
**3.7. Electrocatalytic Activity.** The Nafion-Hb-CoO-IL/CILE showed good electrocatalytic ability for the reduction of TCA and H<sub>2</sub>O<sub>2</sub>. Figure 6 shows cyclic voltammograms of the Hb modified electrode in 0.1 mol L<sup>-1</sup> PBS containing different amounts of TCA. With the increase of TCA concentration a significant increase of the reduction peak current was observed with the disappearance of the oxidation peak, demonstrating a typical electrocatalytic reduction reaction. According to ref 33, the reaction process was proposed as follows:



The catalytic reduction peak currents increased with the TCA concentration in the range from 0.8 to 16.0 mmol L<sup>-1</sup> with the

linear regression equation as  $I_p$  ( $\mu\text{A}$ ) = 0.385C (mmol L<sup>-1</sup>) + 0.399 ( $n = 18$ ,  $\gamma = 0.999$ ) and the detection limit as 0.27 mmol L<sup>-1</sup> (3 $\sigma$ ). When the TCA concentration was more than 16.0 mmol L<sup>-1</sup>, the peak current turned to level off, indicating a typical Michaelis-Menten kinetic process. Therefore, the apparent Michaelis-Menten constant ( $K_M^{\text{app}}$ ) can be further calculated from the electrochemical version of the Lineweaver-Burk equation.<sup>34</sup> On the basis of the voltammetric results the value of  $K_M^{\text{app}}$  was calculated as 7.04 mmol L<sup>-1</sup>, which was lower than the reported value of 47.0 mmol L<sup>-1</sup> for Hb-agarose film modified electrode.<sup>31</sup> Therefore, the immobilized Hb on the Nafion-CoO-IL modified electrode exhibited a high affinity for TCA.

The Nafion-Hb-CoO-IL/CILE also showed good electrocatalytic ability for the reduction of H<sub>2</sub>O<sub>2</sub>. With the addition of H<sub>2</sub>O<sub>2</sub> into pH 4.0 PBS, the reduction peak current was greatly enhanced corresponding with the decrease of the oxidation peak current. The more the H<sub>2</sub>O<sub>2</sub> added, the larger the reduction peak current got, which indicated that the Hb immobilized in the composite film could act as an effective catalyst to the reduction of H<sub>2</sub>O<sub>2</sub>. The mechanism of catalytic reduction of the immobilized Hb to H<sub>2</sub>O<sub>2</sub> is exemplified by the following schemes:<sup>35</sup>



The catalytic peak current changed linearly with H<sub>2</sub>O<sub>2</sub> concentration in the range from 8.0 to 140.0  $\mu\text{mol L}^{-1}$  with the linear regression equation as  $I_p$  ( $\mu\text{A}$ ) = 1.84C ( $\mu\text{mol L}^{-1}$ ) + 2.56 ( $n = 15$ ,  $\gamma = 0.998$ ) and the detection limit as 2.67  $\mu\text{mol L}^{-1}$  (3 $\sigma$ ), which was lower than some previous reports.<sup>36,37</sup> When the concentration of H<sub>2</sub>O<sub>2</sub> was more than 140.0  $\mu\text{mol L}^{-1}$ , a plateau of the peak current appeared, indicating a typical Michaelis-Menten kinetic process. Therefore, the apparent Michaelis-Menten constant ( $K_M^{\text{app}}$ ) was further calculated from the electrochemical version of the Lineweaver-Burk equation with the result of 0.016 mmol L<sup>-1</sup>.

**3.8. Stability of the Modified Electrode.** The stability of the Nafion-Hb-CoO-IL/CILE was investigated with two methods. First, the modified electrode was evaluated by examining the cyclic voltammetric peak currents after continuous scanning for 50 cycles. Nearly no decrease of the voltammetric response appeared, indicating that the Nafion-Hb-CoO-IL/CILE was stable in buffer solution. The storage stability of the Hb modified electrode was investigated by keeping the electrode at 4 °C when not in use. After 2 weeks storage, 97.1% of the initial current response was retained. After 1 month of testing, the peak current response decreased about 9.8%. The relatively good stability of the Hb electrode can be attributed to the biocompatibility of the Nafion-CoO-IL composite material, which prevented the leakage of the proteins and retained the electrocatalytic activity of the protein. Six Hb modified electrodes were prepared by the same procedure independently, and the relative standard deviation (RSD) for the determination of 6.0 mmol L<sup>-1</sup> TCA was calculated as 3.5%, which indicated that the modified electrode had good repeatability.

## 4. CONCLUSIONS

In this paper a composite material composed of Nafion, CoO nanoflower, and IL was fabricated and further applied on the surface of the CILE to get a new kind of electrochemical Hb



biosensor. UV–vis and FT-IR spectroscopic results indicated that Hb in the composite film retained its native structure. Cyclic voltammetric results showed that a pair of well-defined redox peaks appeared, indicating that the direct electron transfer of Hb in the composite film with the underlying electrode was realized. The results were related to the synergistic manner of the substances used, including the high surface area of CoO nanoflower, the high ionic conductivity of ILs, the biocompatibility and good film-forming ability of Nafion, and the excellent electrochemical properties of the CILE. The electrochemical behaviors of Hb in the modified electrode were carefully investigated with the electrochemical parameters calculated. The Nafion–Hb–CoO–IL/CILE showed good electrocatalytic ability for the reduction of TCA and H<sub>2</sub>O<sub>2</sub>. Therefore, the composite material can provide a favorable microenvironment for the immobilization of redox proteins and the modified electrode has potential applications in biosensors and biocatalysis.

## ■ ASSOCIATED CONTENT

**S Supporting Information.** Cyclic voltammograms of different modified electrodes in 0.1 mol L<sup>−1</sup> KCl. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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