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Application of Fe_3O_4 mesoporous sphere modified carbon ionic liquid electrode as electrochemical hemoglobin biosensor

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ABSTRACT

A new electrochemical hemoglobin (Hb) biosensor was constructed based on a Fe_3O_4 mesoporous spheres modified carbon ionic liquid electrode (CILE), which showed excellent electrocatalytic ability towards the reduction of trichloroacetic acid. CILE was prepared by using N-hexylpyridinium hexafluorophosphate ($HPPF_6$) as the modifier and the binder in the carbon paste. Ultraviolet-visible and Fourier transform infrared spectroscopic results indicated that Hb molecules retained the native structure in the chitosan and Fe_3O_4 mesoporous spheres composite film. Electrochemical results indicated that a pair of well-defined redox peaks appeared in 0.1 mol/L phosphate buffer solution (PBS) with the formed potential ($E^{0'}$) as −0.287 V (vs. SCE), indicating that the direct electron transfer of Hb in the composite film was realized. Electrochemical behaviors of Hb were carefully investigated with the electrochemical parameters such as electron transfer coefficient (α), electron transfer number (n), and heterogeneous electron transfer rate constant (k_s) calculated. Due to the specific characteristics of Fe_3O_4 mesoporous spheres present on the electrode surface, the electron transfer rate of Hb was greatly promoted. The Hb modified electrode exhibited excellent electrocatalytic properties with wider linear range and lower detection limit.

1. Introduction

Direct electrochemistry of proteins can be used for the investigation on the electron transfer mechanism in the biological system [1,2]. To achieve the direct electron transfer of redox proteins, protein film voltammetry has been widely used, which can provide a favorable microenvironment for keeping the molecular structure and biocatalytic ability of the proteins [3,4]. Different approaches such as direct casting, nanoparticle, layer-by-layer, polymers, etc., have been used for the fabrication of protein film modified electrodes [5–7]. Proteins can retain their native structures and remain the biocatalytic ability in the films, and then the direct electron transfer of proteins can be realized with a pair of well-defined redox peaks appeared on the cyclic voltammogram. The fabricated protein modified electrode show potential applications in biomedical devices, enzymatic bioreactors and third generation biosensors [8].

As a new kind of green solvent, ionic liquids (ILs) have attracted intensive interest because of their unique physicochemical properties such as higher ionic conductivity, wider electrochemical windows, lower toxicity, higher chemical stability and negligible vapor pressure [9]. In last decade ILs have been widely used in the fields of electrochemistry and electroanalysis [10]. One of its applications is to construct new kinds of modified carbon paste electrode. By mixing carbon powder, IL and/or paraffin together, carbon ionic liquid electrode (CILE) can be fabricated with many advantages including wider electrochemical windows, better anti-fouling ability and higher detection sensitivity [11,12]. Zhang et al. studied the electrochemical behavior of rutin on CILE [13]. Sun et al. [14–16] applied different CILEs for the detection of electroactive substances such as adenosine, guanosine and hydroquinone. Also CILE can be used as the substrate electrode for the immobilization of redox proteins. Sun et al. [17,18] investigated the direct electrochemistry of redox proteins on the carbon nanotube modified CILE. Fe_3O_4 nanoparticle is a typical superparamagnetic nanomaterial with the properties such as good biocompatibility, strong superparamagnetism, low toxicity and easy preparation process, which had been used in the fabrication of electrochemical biosensor. Reetz et al. [19] reported mechanically stable lipases/Fe_3O_4 nanoparticle/sol–gel biocatalysts by simultaneous entrapment of lipase and nanostructured Fe_3O_4 in hydrophobic sol–gel materials. Cao et al. [20] reported an electrochemical biosensor based on the heme proteins immobilized on Fe_3O_4 nanoparticles. On the other hand, the biopolymer chitosan (CTS) offers excellent characteristics such as biocompatibility, good film forming ability, nontoxicity,

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physiological inertness and high mechanical strength. Thus, it has been extensively used for the immobilization of enzymes and the construction of biosensors [21,22].

In this paper the direct electrochemistry of hemoglobin (Hb) was realized on the CTS and Fe_3O_4 mesoporous spheres composite material modified CILE. An ionic liquid N-hexylpyridinium hexafluorophosphate ($HPPF_6$) was used as the binder for the preparation of CILE, which had been used for the electrochemical detection [14]. Maleki et al. [23] carefully investigated the electrochemical performances of CILE, and the experimental results indicated that CILE combined many good features of different types of carbon electrodes such as glassy carbon electrode (GCE) and edge plane pyrolytic graphite. Also CILE processed the advantages of carbon paste electrode (CPE) such as low cost, simplicity of preparation, surface modification and renewal. The favorable electrochemical response, higher conductivity and inherent electrocatalytic ability observed on CILE toward biomolecules make it a good candidate for construction of biosensors. By combination of the advantages of different materials used, direct electrochemistry of Hb was realized on the composite film modified electrode. The electrocatalytic ability of the fabricated electrode to the reduction of trichloroacetic acid was further investigated carefully.

2. Experimental

2.1. Apparatus and reagents

Ionic liquid N-hexylpyridinium hexafluorophosphate ($HPPF_6$, >99%, Lanzhou Greenchem. ILS. LICP. CAS., China), bovine hemoglobin (Hb, MW 64500, Tianjin Chuanye Biochemical Limited Company, China), chitosan (CTS, Dalian Xindie Limited Company, China), graphite powder (average particle size 30 μm, Shanghai Colloid Chemical Company, China) and trichloroacetic acid (TCA, Tianjin Kemiou Chemical Limited Company, China) were used as received. 0.1 mol/L pH 7.0 phosphate buffer solutions (PBS) were used as the supporting electrolyte. All the other chemicals used were of analytical reagent grade and doubly distilled water was used in the experiments.

All the electrochemical experiments were executed on a CHI 750B electrochemical analyzer (Shanghai CH Instrument, China). A standard three-electrode system was used with an Hb 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 was recorded on Cary 50 probe spectrophotometer (Varian Company, Australia) and Fourier transform infrared (FT-IR) spectra was performed on Tensor 27 FT-IR spectrophotometer (Bruker Company, Germany), respectively. A JSM-6700F scanning electron microscope (SEM, Japan Electron Company, Japan) was used to record the scanning electron microscopy.

2.2. Preparation of Fe_3O_4 mesoporous spheres

Fe_3O_4 mesoporous spheres were synthesized based on the reported procedure [24]. In brief, 1.2 g of $FeCl_3{\cdot}6H_2O$ were dissolved in 75 mL of glycol and the mixed solution was stirring for 30 min. Then 3.6 g of NaAc was added to the solution followed with stirring for 1 h. The resulting solution was transferred to a 100 mL Teflon-lined stainless-steel autoclave and heated at 200 °C for 16 h, and then cooled naturally to the room temperature. The product was collected by filtration, washed with distilled water and absolute ethyl alcohol. After the product was dried at 60 °C for 6 h, Fe_3O_4 mesoporous spheres were obtained.

2.3. Preparation of the modified electrode

CILE was prepared by a reported procedure [14], 1.6 g of $HPPF_6$ and 3.2 g of graphite powder were mixed carefully in a mortar, heated at 60 °C for 10 min and ground to form a homogeneous carbon paste. Then the prepared ionic liquid modified carbon paste was tightly packed into a cavity of glass tube (4.0 mm diameter) and the electrical contact was established via a copper wire. The surface of CILE was polished to a smooth surface for the further use.

The modified electrode was prepared with the following procedure. Typically a solution containing 20.0 mg/mL Hb and 0.33 mg/mL Fe_3O_4 mesoporous spheres was prepared and mixed homogeneously. Then 10.0 μL of the prepared Hb–Fe_3O_4 mixture was cast on the surface of CILE and left it to dry at room temperature to get an electrode denoted as Hb–Fe_3O_4/CILE. Finally, 5.0 μL of 1.0 mg/mL CTS solution (in 1% HAc solution) was spread onto the surface of the Hb–Fe_3O_4/CILE to get the final modified electrode (CTS/Hb–Fe_3O_4/CILE). For comparison, different modified electrodes such as CTS/CILE, CTS/Fe_3O_4/CILE and CTS/Hb/CILE, etc. were fabricated with similar drop-casting procedure.

2.4. Procedure

Electrochemical measurements were performed in a 10 mL electrochemical cell containing 0.1 mol/L PBS, which was purged with highly purified nitrogen for 30 min prior to experiments and maintained in a nitrogen atmosphere during the experiments. UV-Vis spectroscopic experiments were performed with a mixture solution of Hb, CTS, and Fe_3O_4 mesoporous spheres with pH 7.0 PBS. The CTS/Hb–Fe_3O_4 and Hb film assembled on a glass slide was used for FT-IR measurements.

3. Results and discussion

3.1. SEM images

SEM images were recorded to identify the surface morphologies. As shown in Fig. 1A, the synthesized Fe_3O_4 material exhibited as mesoporous ball with an average diameter of 400 nm. Also the holes existed on the rough surface of mesoporous spheres could be obviously observed. On CTS/Hb–Fe_3O_4/CILE (Fig. 1B) a uniform surface appeared with rough interface, which was due to the good film forming abilitity of CTS that could incorporate the materials used tightly on the electrode surface.

3.2. Spectroscopic results

In FT-IR spectroscopy the shape and position of amide I and II infrared absorbance bands of Hb can provide information on the secondary structure of the polypeptide chain. The amide I band (1700–1600 cm^{-1}) is attributed to the C=O stretching vibration of the peptide linkage in the backbone of protein. The amide II band (1600–1500 cm^{-1}) is caused by the combination of N—H inplane bending and C—N stretching vibration of the peptide groups [25]. The shape of amide I and amide II bands will diminish or even disappear if Hb molecule is denatured [26,27]. As shown in Fig. 2Aa, the two amide bands of pure Hb were located at 1649 and 1533 cm^{-1}. After mixing with CTS and Fe_3O_4 mesoporous spheres, the position of amide I and II bands appeared at 1647 and 1533 cm^{-1} (Fig. 2Ab). The similarities of the amide band positions suggested that Hb retained its native structure after immobilized in the CTS–Fe_3O_4 composite film.

In UV–vis absorption spectrum the Soret absorption band from the four iron heme groups of heme proteins can also provide

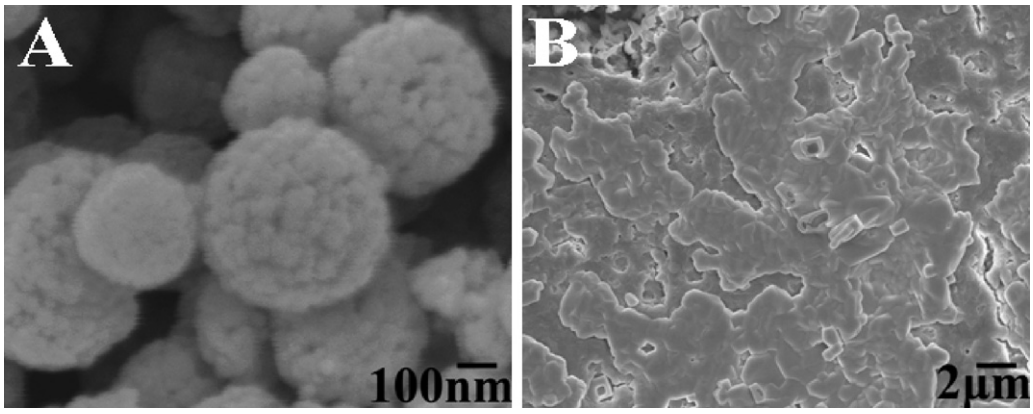

Fig. 1. SEM images of (A) Fe_3O_4 mesoporous spheres and (B) CTS/Hb–Fe_3O_4/CILE.

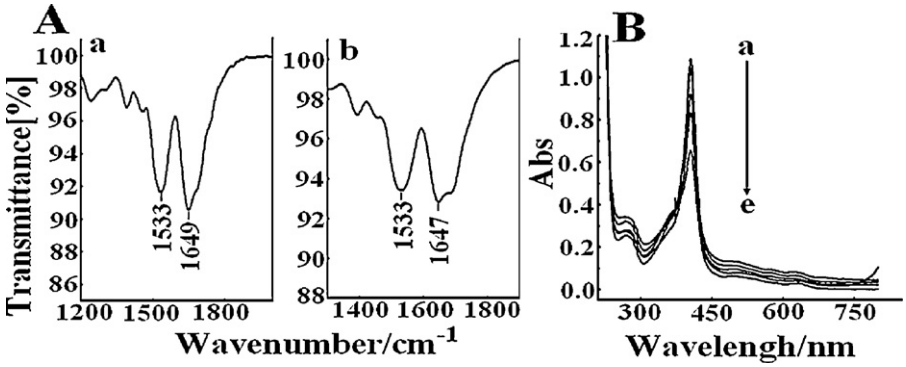

Fig. 2. (A) FT-IR spectra of (a) Hb film and (b) CTS–Hb–Fe_3O_4 film; (B) UV–vis absorption spectra of Hb in water (a), CTS–Hb (b), Hb (c), Hb–Fe_3O_4 (d) and CTS–Hb–Fe_3O_4 (e) in pH 7.0 PBS.

the information on the conformational integrity and the possible denaturation or the conformational change about the heme region [3]. As shown in Fig. 2B, the Soret band of Hb appeared at 405.0 nm in water (curve a) and pH 7.0 PBS (curve c). After mixing Hb with CTS, Fe_3O_4 and CTS–Fe_3O_4, respectively, the absorption value also appeared at 405.0 nm without changes (curves b, d and e), which suggested that Hb in the composite film retained its native structure. The results also proved the biocompatibility of Fe_3O_4 mesoporous sphere and CTS with protein. Fe_3O_4 nanoparticle is a typical nanomaterial with the properties such as good biocompatibility, strong superparamagnetism and so on [20]. CTS is an abundant natural biopolymer originated from the exoskeleton of crustaceans, which is biocompatible, biodegradable without toxicity [28]. So the CTS–Fe_3O_4 composite film was a suitable immobilization matrix for redox proteins.

3.3. Electrochemical characteristics of the modified electrodes

Potassium ferricyanide is commonly used as a probe to evaluate the performance of different modified electrodes with the typical cyclic voltammograms shown in Fig. 3. On CILE a pair of well-defined redox peaks appeared (curve b) with the peak-to-peak separation (ΔE_p) as 0.079 V (vs. SCE), indicating the good reversibility on CILE. On CTS/CILE the redox peak currents increased apparently (curve a). CTS is a positively charged biopolymer and the negatively charged $[Fe(CN)_6]^{3-/4-}$ could be easily adsorbed on the electrode surface, so the voltammetric response increased correspondingly. On CTS–Hb/CILE the redox peak current decreased greatly (curve d), indicating the presence of Hb molecules in the composite film hindered the electron transfer rate. While on CTS/Hb–Fe_3O_4/CILE the redox peak currents increased (curve c), which may be due to the presence of semiconductor Fe_3O_4 mesoporous sphere with high surface area in the composite film accelerated the electron transfer. The differences of cyclic voltammograms indicated different immobilization steps on the electrode surface and the successful fixation of Hb on the electrode surface.

3.4. Direct electrochemistry of the modified electrodes

Direct electrochemistry of the immobilized Hb was further carefully investigated by cyclic voltammetry. Fig. 4 showed the typical cyclic voltammograms of different modified electrodes in a deaerated 0.1 mol/L PBS. On bare CILE (curve a) and CTS/CILE (curve b) no electrochemical responses were observed at the selected potential range, which indicated that no electroactive substances present on the electrode surface. The increase of the background current could

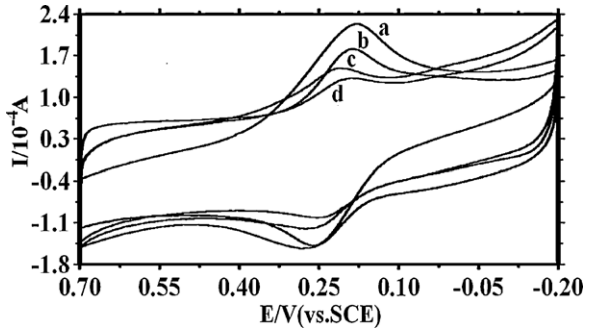

Fig. 3. Cyclic voltammograms of (a) CTS/CILE, (b) CILE, (c) CTS/Hb–Fe_3O_4/CILE and (d) CTS/Hb/CILE in a mixture solution of 1.0 mmol/L $K_3[Fe(CN)_6]$ and 0.5 mol/L KCl with the scan rate as 100 mV/s.

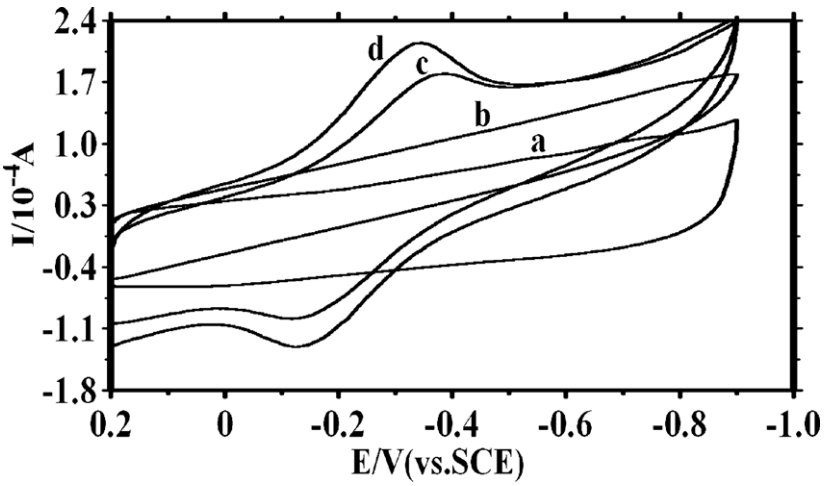

Fig. 4. Cyclic voltammograms of (a) CILE, (b) CTS/CILE, (c) CTS/Hb/CILE and (d) CTS/Hb–Fe_3O_4/CILE in pH 7.0 PBS with the scan rate as 100 mV/s.

be ascribed to the presence of IL in the carbon paste. By using IL as the binder in CPE, a layer of IL film was present on the surface of graphite power, which could act as the double layer with increase of the capacitance, so the background current was increased greatly. On CTS/Hb/CILE a pair of unsymmetric redox peaks appeared (curve c), which indicated that the direct electron transfer of Hb with CILE had taken place. CILE had been elucidated with the advantages such as higher conductivity and better reversibility. So the direct electron transfer of Hb can be realized on the CILE. While on CTS/Hb–Fe_3O_4/CILE (curve d), a pair of well-defined redox peaks appeared with increased electrochemical response, indicating that the presence of Fe_3O_4 mesoporous sphere on the electrode surface played important roles in facilitating the electron transfer of Hb. Fe_3O_4 mesoporous sphere have exhibited good biocompatibility, better conductivity and bigger surface area with many small holes on the surface, which could adsorb more Hb molecules with the electroactive center of Hb molecules more closer to the sphere and a three-dimensional structure could be formed. So the direct electron transfer of Hb was easily realized on the modified electrode. The formal potential ($E^{0\prime}$) was calculated as −0.287 V, which was the characteristic of the heme Fe(III)/Fe(II) redox couples of the protein [29].

The composition of the modifier on the electrode was optimized to get the stablest and highest electrochemical responses of Hb. By varying the ratio of Hb, Fe_3O_4 mesoporous spheres and CTS, direct electrochemistry of Hb modified electrode was investigated and compared. Experimental results showed that the amount of Fe_3O_4 mesoporous spheres had great influences on the redox responses of the modified electrode. The presence of Fe_3O_4 mesoporous spheres provided a specific interface with high surface area, but excessive amounts of Fe_3O_4 mesoporous spheres on the electrode surface would increase the thickness of the film. Due to the good film forming ability of CTS, the composite without CTS film was not stable and the modifier could be easily leaked out to the buffer solution. The experimental results indicated that the composite film that containing 20.0 mg/mL Hb, 0.33 mg/mL Fe_3O_4 mesoporous spheres and 1.0 mg/mL CTS was the optimal ratio for the fabrication of modified electrode.

3.5. Electrochemical behaviors of the CTS/Hb–Fe_3O_4/CILE

The influence of scan rate on the electrochemical responses of CTS/Hb–Fe_3O_4/CILE was further investigated in the range from 50.0 to 500.0 mV/s with the result shown in Fig. 5A. With the increase of scan rate a pair of symmetric redox peaks appeared with the almost equal height of redox peak currents, indicating a quasi-reversible electrode process. 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 the redox peak currents increased linearly with scan rate (Fig. 5B), demonstrating that the electrode process of Hb was a surface-controlled thin-layer electrochemical reaction. With the increase of scan rate the value of ΔE_p was also increased gradually, so the electrochemical parameters of the Hb modified electrode reaction can be calculated according to the Laviron's equation [30].

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

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

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

Two straight lines were got between the E_p value with the logarithm of scan rate (Fig. 5C) with the equations as E_{pa}(V) = 0.057 log υ (V/s) − 0.326 (n = 15, γ = 0.998) and E_{pc}(V) = −0.142 log υ (V/s) − 0.071 (n = 15, γ = 0.996). Then the values of the electron transfer coefficient (α) and the heterogeneous electron transfer rate constant (k_s) were calculated as 0.29 and 0.478 s^{-1}, respectively. It is well-known that k_s reflects the local microenvironment of the protein immobilized on the electrode. The value obtained here is in the range of k_s for typical surface-controlled quasi-reversible electron transfer processes. This k_s is higher than that of Hb immobilized on a nanocrystaline titanium oxide modified electrode (0.137 s^{-1}) [31], sodium alginate and SiO_2 nanoparticle bionanocomposite film (0.077 s^{-1}) [32] and carbon nanotube (0.062 s^{-1}) [33]. The increase of the electron transfer rate of Hb was due to the specific characteristics of Fe_3O_4 mesoporous spheres with high surface and good conductivity.

By integration of the cyclic voltammetric curve, an almost unchanged charge value (Q) was got regardless of the change of scan rate. Based on the equation of $\Gamma^* = Q/nAF$, the surface concentration (Γ^*) of electroactive Hb was calculated as 9.75×10^{-9} mol/cm^2. While the electroactive surface concentration of Hb on CTS/Hb/CILE was calculated by the same method with the results as 1.88×10^{-9} mol/cm^2, which was much smaller than that of CTS/Hb–Fe_3O_4/CILE. The results indicated that the presence of Fe_3O_4 mesoporous sphere could provide a three-dimensional

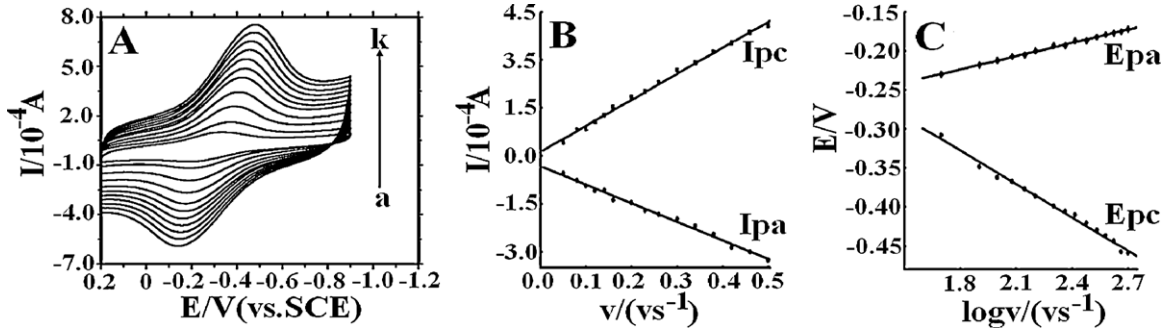

Fig. 5. (A) Influence of scan rate on the cyclic voltammograms of CTS/Hb–Fe_3O_4/CILE in pH 7.0 PBS; (B) the relationship of the cathodic and anodic peak current with scan rate (υ); (C) the relationship of the cathodic and anodic peak potential against log υ.

structure with more channels for the Hb molecules to exchange electrons. While the total amount of Hb casted on the electrode surface was 2.47×10^{-8} mol/cm^2, so 39.47% of the Hb molecules on CTS/Hb–Fe_3O_4/CILE took part in the electrochemical reaction, which was larger than some reported values [34,35]. The result also demonstrated that the multilayers of Hb in the composite film participated into the electron transfer process, which could be attributed to the specific sphere structure of Fe_3O_4 mesoporous with large surface area.

The effect of buffer pH on the cyclic voltammetric responses of the modified electrodes was further investigated. In the pH range from 4.0 to 9.0, a stable and well-defined cyclic voltammetric curves were obtained. With the increase of buffer pH the redox peak potential shifted to the negative direction, implying that protons were involved in the electrode process. A good linear regression relationship was got between the formal potential ($E^{0'}$) and pH with the equation as $E^{0'}$(V) = −47.7 pH + 3.15 (n = 7, γ = 0.997). The slope value of −47.7 mV/pH was smaller than the theoretical value of −56.0 mV/pH at 20 °C for a single-proton coupled reversible one-electron transfer process. The reason might be originated from the influence of the protonation states of transligands to the heme iron and amino acids around the heme, or the protonation of the water molecule coordinated to the central iron. However, it also could indicate that a single protonation accompanies with one electron transfer of Hb Fe(III) to electrode. So the electrochemical reaction equation could be expressed as: Hb heme Fe (III) + H^+ + e^- → Hb heme Fe (II).

3.6. Electrocatalytic activity

It is well-known that redox protein which contains a prosthetic group have good electrocatalytic activity towards different substrates including TCA, $NaNO_2$, H_2O_2, etc., which are important detection targets in the fields such as biochemistry and environmental analysis. TCA is a metabolic byproduct of CCl_4 under reduced oxygen tension. Also it is an analogue of acetic acid with three hydrogen atoms of methyl group replaced by chlorine atoms. TCA has been widely used in the field of biochemistry for the precipitation of biomacromolecules such as proteins, DNA and RNA. It can also be present in the drinking water as the result of Chlorine disinfection with the potential to kill normal cells. So it is necessary to establish sensitive method for TCA detection and the electrocatalytic reduction of TCA on CTS/Hb–Fe_3O_4/CILE were examined with the results shown in Fig. 6. It can be seen that the cathodic peak current increased dramatically at −0.362 V with the disappearance of the anodic peak after the addition of different amounts of TCA into PBS (curve a–l), which was the typical electrocatalytic behaviors of Hb immobilized on the electrode. The results indicated that the activation energy of TCA reduction was decreased greatly due to the presence of Hb in the modified electrode. In addition, another new reduction peak appeared at −0.63 V with the further increase of the TCA concentration. Based on the reference [36], the second reduction peak could be tentatively assigned to the highly reduced form of Hb Fe(I), which was an active reductant that could dechlorinate di- and monochloroacetic acid after the dechlorination of TCA by Hb Fe(II). The overall electrocatalytic TCA reduction mechanism can be expressed with the following equations:

$$\mathrm{HbFe(III)} + \mathrm{e} \rightarrow \mathrm{HbFe(II)} \quad (1)$$

$$2\mathrm{HbFe(II)} + \mathrm{Cl_3CCOOH} + \mathrm{H^+} \rightarrow 2\mathrm{HbFe(III)} + \mathrm{Cl_2CHCOOH} + \mathrm{Cl^-} \quad (2)$$

$$\mathrm{HbFe(II)} + \mathrm{e} \rightarrow \mathrm{HbFe(I)} \quad (3)$$

$$2\mathrm{HbFe(I)} + \mathrm{Cl_2CHCOOH} + \mathrm{H^+} \rightarrow 2\mathrm{HbFe(II)} + \mathrm{ClCH_2COOH} + \mathrm{Cl^-} \quad (4)$$

$$2\mathrm{HbFe(I)} + \mathrm{ClCH_2COOH} + \mathrm{H^+} \rightarrow 2\mathrm{HbFe(II)} + \mathrm{CH_3COOH} + \mathrm{Cl^-} \quad (5)$$

The catalytic reductive peak current increased linearly with the TCA concentration in the range from 2.4 to 20.0 mmol/L with the linear regression equation as I_p(mA) = 0.089c (mmol/L) + 0.015 (n = 21, γ = 0.998) and the detection limit as 0.033 mmol/L (3σ). The detection limit was smaller than that of Nafion-CdS-Hb modified CILE (10.0 mmol/L) [37] and carbon nanochips (CNCs))/Hb/Nafion

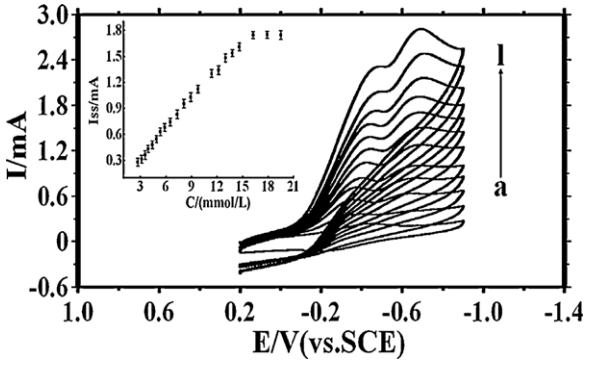

Fig. 6. Cyclic voltammograms of CTS/Hb–Fe_3O_4/CILE in pH 7.0 PBS containing 2.4, 3.0, 4.2, 4.8, 6.0, 7.2, 8.0, 10.0, 11.0, 12.0, 15.0, 16.0 mmo/L TCA (curve a–l), respectively, with the scan rate as 100 mV/s (inset was the linear relationship of catalytic reduction peak currents and the TCA concentration.

modified GCE (0.06 mmol/L) [38], indicating the higher sensitivity of the modified electrode. When the TCA concentration was more than 20.0 mmol/L, the current response turned to level off, which indicated a saturation of Hb catalytic reaction. So the apparent Michalies–Menten constant (K_M^{app}), which is an indicator of the enzyme–substrate reaction kinetics, is calculated to evaluate the biological activity of the immobilized Hb. By using the Lineweaver–Burk equation:

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

where I_{ss} is the steady-state current after the addition of substrate, c is the bulk concentration of substrate and I_{max} is the maximum current under saturated substrate solution. K_M^{app} can be obtained by the analysis of slope and intercept of the plot of the reciprocals of the steady-state current versus TCA concentration. The K_M^{app} of CTS/Hb–Fe_3O_4/CILE was estimated to be 14.9 mmol/L, which was smaller than that of Hb immobilized in agarose hydrogel films in 1-butyl-3-methylimidazolium hexafluorophosphate on GCE (47.0 mmol/L) [34], Nafion/CdS nanorods film modified CILE (98.5 mmol/L) [37] and CTS-MWCNTs-Hb film modified CILE (18.7 mmol/L) [18].

3.7. Anti-interfering activity

The potential interferences on CTS/Hb–Fe_3O_4/CILE to the determination of 4.0 mmol/L TCA were investigated. Under the selected conditions, the electrocatalytic reduction peak current of TCA was individually measured in the presence of different amount of interferents with the changes of the peak current recorded. Experimental results indicated that 100-fold concentration of Zn^{2+}, Ni^{2+}, Cu^{2+}, Mg^{2+}, Ca^{2+}, Cl^-, $SO_4{}^{2-}$, $N0_3{}^-$ did not interfere with the catalytic reduction peak with the peak current changes below ±5%. So CTS/Hb–Fe_3O_4/CILE exhibited good selectivity to the TCA detection.

3.8. Reproducibility and stability of the modified electrode

The stability of CTS/Hb–Fe_3O_4/CILE was investigated by examining the redox peak currents after 50 successively scanning and the voltammetric responses were not changed, indicating that CTS/Hb–Fe_3O_4/CILE was stable in buffer solution. The long-term storage stability of the Hb modified electrode was investigated by keeping the electrode at 4 °C when not use. 93.2% of the initial current response was retained after 2 weeks storage. After 30 days, the redox current response decreased about 9%. The results indicating that CTS/Fe_3O_4 nanocomposite material was very efficient for preventing the leakage of the proteins and retaining electrocatalytic activity of Hb. Five Hb modified electrodes were prepared by the same procedure independently and the relative standard deviation (RSD) for the determination of 7.0 mmol/L TCA was calculated as 2.6%, which indicated the modified electrode had good repeatability. The reproducibility of the Hb electrode was checked by successively detecting 7.0 mmol/L TCA for 6 times and the RSD value was got as 3.1%, demonstrating a good reproducibility of the prepared electrode.

4. Conclusions

In this paper a Fe_3O_4 mesoporous sphere was used as the modifier with a $HPPF_6$ based CILE as the substrate electrode, which provided a specific microenvironment for the further immobilization of Hb. Direct electrochemistry of Hb was realized in the CTS–Fe_3O_4 nanocomposite film modified electrode with the electrochemical parameters calculated. Due to the specific characteristics of Fe_3O_4 mesoporous sphere, the secondary structure and catalytic activity of Hb was remained. The CTS/Hb–Fe_3O_4/CILE showed good electrocatalytic ability to the reduction of TCA with wider dynamic range, small K_M^{app} value, low detection limit and long term stability. So the modified electrode has the potential application in the third-generation electrochemical biosensor.

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