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Nafion–ZrO$_2$ nanoparticle–ionic liquid nanobiocomposite for the direct electrochemistry of myoglobin

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Abstract A nanobiocomposite was perpared by mixing zirconia nanoparticle with ionic liquid 1-ethyl-3-methylimi-dazolium tetrafluoroborate and Nafion together, which was further used to immobilize myoglobin (Mb) on the surface of carbon ionic liquid electrode. UV–Vis and FT-IR spectroscopic results confirmed that Mb remained its native structure in the nanobiocomposite material. Cyclic voltammetric results showed that a pair of well-defined and quasi-reversible redox peaks appeared with the formal potential as −0.219 V (vs. saturated calomel electrode) in pH 7.0 phosphate buffer solution, indicating that the direct electrochemistry of Mb was realized on the modified elecctrode. Mb exhibited a surface-controlled process and the electrochemical parameters were calculated. The Mb electrode showed good electrocatalytic ability to the reduction of trichloroacetic acid within the concentration range from 1.0 to 16.0 mmol/L and the Michaelis–Menten constant was calculated as 7.378 mmol/L. The proposed bioelectrode exhibited good stability and repeatability with the potential application in third-generation biosensor.

Keywords Zirconia nanoparticle · Ionic liquid ·
Myoglobin · Carbon ionic liquid electrode · Direct
electrochemistry

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Introduction

Direct electrochemistry of proteins or enzymes on the electrode can provide basic information on the fabrication of biosensors, bioreactors, and biomedical devices. The research results can also establish the model for mechanism investigation on the electron transfer between enzymes in biological system [1]. However, the direct electron transfer of protein with traditional electrodes is difficult to be realized due to the slow electron transfer rate on the electrode surface. The reason can be ascribed to the deep burying of the electroactive prosthetic groups or the unfavorable orientations of the proteins on the electrode interface. Great efforts have been made with film-modified electrodes, which can provide a favorable microenvironment for remaining the protein structure and promoting the electron transfer rate [2]. Successful applications include the films of proteins with surfactants, biopolymers, polyelectrolyte, nanomaterials, and so on, which have been reported to facilitate the fast and reversible electron transfer between the underlying electrode and proteins or enzymes [3–6].

Recently, ionic liquids (ILs) have also been used in the protein film electrochemistry. As a new kind of green solvent, ILs have exhibited some specific physicochemical properties, including negligible vapor pressure, wide potential windows, good ionic conductivity, and catalytic ability [7, 8]. Buzzeo [9] and Sun [10] reviewed the progresses of ILs in the field of electrochemistry and analytical chemistry. ILs can be used as not only the electrolyte but also the modifier in the protein electrochemistry. Wang et al. reported the direct electrochemistry and electrocatalysis of heme proteins entrapped in agarose hydrogel films on glassy carbon electrode (GCE) with IL 1-butyl-3-methylimidazo-lium hexafluorophosphate as the electrolyte [11]. Rozniecka et al. [12] studied the ion-transfer processes across ILs/ aqueous solution interface with different working electrodes

such as carbon ceramic electrode and carbon paste electrode (CPE) as the ILs phase-supporting matrix. Long et al. investigated the direct electrochemistry of horseradish peroxidase immobilized in a chitosan/1-butyl-3-methylimidazolium tetrafluoroborate composite film [13]. ILs can also be incorporated as a new kind of binder for CPE, and the fabricated carbon ionic liquid electrode (CILE) shows many advantages than the traditional carbon electrode [14, 15], so CILE had been used as a new working electrode for electroanalysis. Safavi et al. constructed a pyridinium-based CILE for the direct immobilization of hemoglobin (Hb) [16]. Sun et al. entrapped redox proteins on the CILE surface with different film-forming materials [17–19].

With the development of nanoscience, the application of nanomaterials in the fields of electrochemical biosensors has aroused great interests due to specific characteristics of nanostructure materials, such as the large surface area, high chemical stability, and good biocompatibility. Different kinds of nanoparticles have been used in the protein electrochemistry with the structure and biocatalysis of proteins remained [20]. ZrO_2 nanoparticles have attracted increasing attention in electrochemical biosensors due to their good biocompatibility, low toxicity, and high active surface areas. For example, Liu et al. studied the direct electrochemistry and thermal stability of Hb immobilized on the ZrO_2 nanoparticles' modified pyrolytic graphite electrode [21]. Yang et al. reported the direct electron transfer reactivity of glucose oxidase on ZrO_2 nanoparticles' modified electrode [22]. Qiao et al. investigated the direct electron transfer and electrocatalysis of myoglobin (Mb) in poly(ethylene glycol) and ZrO_2 nanoparticles' layer-by-layer films [23].

In this paper, a novel nanobiocomposite material was fabricated by mixing ZrO_2 nanoparticle with IL 1-ethyl-3-methyl-imidazolium tetrafluoroborate ([EMIM]BF_4) and Nafion together, which was used to immobilize Mb on the surface of a CILE. By combining the advantages of the substances used, such as the good biocompatibility of ZrO_2 nanoparticle, the high ionic conductivity of IL, and the good film-forming of Nafion, the biocomposite material can provide a favorable microenvironment for protein. Experimental results indicated that direct electron transfer process was realized with a pair of well-defined cyclic voltammetric peaks appeared, so the direct electrochemistry and electrocatalysis of Mb on the modified electrode was carefully investigated in the present work.

Experimental

Apparatus

CHI 1210A electrochemical workstation (Shanghai CH Instrument, China) was used for all the electrochemical measurements. A conventional three-electrode system was used with a Mb-modified electrode as working electrode, a platinum wire as auxiliary electrode, and a saturated calomel electrode (SCE) as reference electrode. UV–Vis absorption and FT-IR spectra were recorded on Cary 50 probe spectrophotometer (Varian Company, Australia) and Tensor 27 FT-IR spectrophotometer (Bruker Company, Germany), respectively.

Chemicals

IL 1-ethyl-3-methylimidazolium ethylsulfate ([EMIM]EtOSO$_3$) and [EMIM]BF_4 were purchased from Hangzhou Chemer Chemical Ltd. Co.. Bovine myoglobin (MW. 17,800, Tianjin Chuanye Biochemical Ltd. Co.), Nafion (5 %, Sigma), graphite powder (average particle size 30 μm, Shanghai Colloid Chemical Co.), and trichloroacetic acid (TCA, Tianjin Kemiou Chemical Limited Company) were used as received. ZrO_2 nanoparticles were synthesized according to the reported paper [24], which was spherical with the average diameter of 30 nm. A series of 0.1 mol/L phosphate buffer solutions (PBS) with different pH value were used as the supporting electrolyte for electrochemical measurements. All the other chemicals used were of analytical reagent grade and doubly distilled water was used in all experiments.

Preparation of modified electrode

CILE was fabricated by mixing 0.20 mL of [EMIM]EtOSO$_3$, 0.80 mL of liquid paraffin, and 3.2 g of graphite powder together in a mortar and ground carefully to get a homogeneous carbon paste. A portion of the modified carbon paste was packed firmly into a glass tube cavity (Φ=4 mm) with a copper wire inserted through the opposite end of the paste as the electrical contact. The surface of CILE was polished by smoothing on a piece of weighing paper just before use.

The nanobiocomposite material was prepared by mixing 15.0 mg of Mb, 75 μL of [EMIM]BF_4, 150 μL of 0.5 % Nafion solution, and 50 μL of 0.5 g/L ZrO_2 nanoparticle suspension solution together and diluting it to 1.0 mL with 0.1 mol/L PBS (pH 7.0). Then, 7.0 μL of the prepared Nafion–Mb–ZrO_2–IL mixture solution was cast on the surface of the newly prepared CILE and dried at room temperature to form a stable film. The fabricated electrode was denoted as Nafion–ZrO_2–IL–Mb/CILE and other modified electrodes such as Nafion–Mb/CILE, Nafion–ZrO_2–Mb/CILE, etc., were also fabricated with the similar procedures for comparison. All the modified electrodes were stored at 4 °C in a refrigerator when not in use.

Electrochemical measurements

Electrochemical measurements were carried out 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 under nitrogen atmosphere during the experiments. UV–Vis spectroscopic experiments were performed with a mixture solution containing certain concentration of Mb, Nafion, ZrO_2 nanoparticle, and [EMIM]BF_4 with doubly distilled water. The Nafion–ZrO_2–IL–Mb film assembled on a glass slide was used for FT-IR measurements.

Results and discussion

Spectroscopic results

UV–Visible absorption can give structural information about the environmental surrounding of the heme group of Mb. If the protein structure was changed or the protein was denatured, the Soret band would shift or disappear [25]. As shown in Fig. 1a, Mb had its Soret band at 408.0 nm in the aqueous solution (curve a). Both the Soret band of Mb in IL and Nafion solution appeared at 407.5 nm (curves b and c). After mixing Mb with Nafion–ZrO_2–IL solution, the Soret band appeared at 407.3 nm (curve d), which shifted only 0.7 nm compared with that of native Mb. The results indicated that Mb in the Nafion–ZrO_2–IL composite kept its secondary structure similar to that of the native Mb, which was attributed to the biocompatibility of Nafion, ZrO_2 nanoparticles, and IL used in the experiment.

FT-IR spectroscopy is further used to probe the secondary structure of proteins. The positions of amide I and II infrared absorbance bands of Mb provide detailed information on the secondary structure of the polypeptide chain. The amide I band at 1,700–1,600 cm^{-1} is attributed to the C=O stretching vibration of the peptide linkage in the backbone of protein. The amide II band at 1,600–1,500 cm^{-1} is caused by the combination of

N—H in plane bending and C—N stretching vibration of the peptide groups [26]. As shown in Fig. 1b, the FT-IR spectra of amide I and II bands of native Mb were located at 1,655 and 1,539 cm^{-1}, respectively (Fig. 1b (a)). After mixing Nafion, Mb, ZrO_2 nanoparticles, and IL together, the spectra of amide I and II bands appeared at 1,649 and 1,549 cm^{-1} (Fig. 1b (b)). The similarities of the two spectra further suggested that Mb retained the essential features of its native structure after immobilized in the Nafion–ZrO_2–IL composite film, which could be attributed to the biocompatible materials used.

Direct electrochemistry of Nafion–ZrO_2–IL–Mb/CILE

Figure 2 showed the typical cyclic voltammograms of different modified electrodes in pH 7.0 PBS at the scan rate of 100 mV/s. On the Nafion-Mb/CILE (curve a) an unsymmetric redox peaks appeared with the reduction peak current greater than the oxidation peak current, indicating an irreversible electrode process. With the addition of ZrO_2 nanoparticles or IL into the composite film, the electrochemical responses increased with more reversible behaviors (curves b and c). The results can be attributed to the presence of IL or ZrO_2 nanoparticles in the composite film, which enhanced the electron transfer rate of the Mb. IL is a green solvent with high ionic conductivity, which can improve the whole conductivity of the composite materials and enhance the electron transfer rate of Mb. ZrO_2 nanoparticle is classified as a wide band gap semiconductor with different applications in the fields such as fuel-cell technology, catalysis, and photochemical processes. ZrO_2 nanoparticles have exhibited the properties such as excellent chemical inertness and good biocompatibility, which has been widely used in the electrode modification. Wang et al. reported that the ZrO_2 sol-gel carbon composite electrode could decrease the overpotential of electrooxidation of
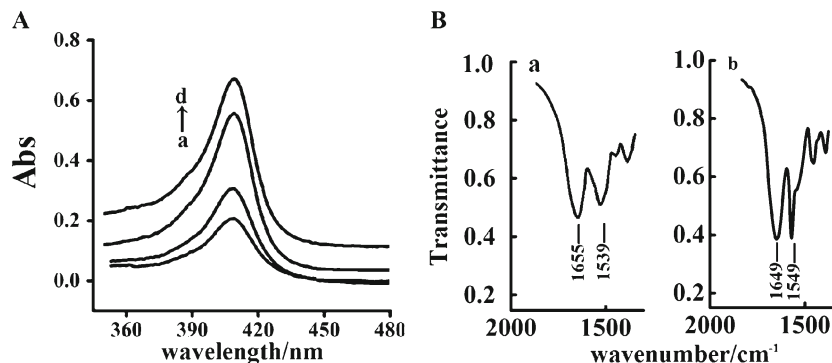
A

B

Fig. 1 **a** UV–Vis absorption spectra of (*a*) 0.026 mg/mL Mb in water and (*b*) 0.039 mg/mL Mb with IL, (*c*) 0.060 mg/mL Mb with Nafion, (*d*) 0.075 mg/mL Mb with Nafion, ZrO_2, IL mixture solution in 0.1 mol/L pH 7.0 PBS. The concentrations of IL, Nafion, ZrO_2 nanoparticles were 0.75 %, 0.075 %, and 2.5 μg/mL, respectively. **b** FT-IR spectra of (*a*) Mb and (*b*) Nafion–Mb–IL–ZrO_2 film
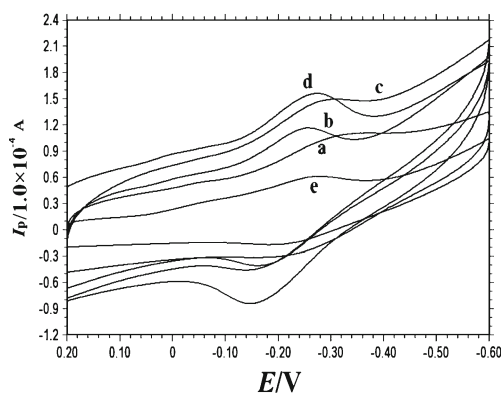
Fig. 2 Cyclic voltammograms of (a) Nafion–Mb/CILE, (b) Nafion–ZrO$_2$–Mb/CILE, (c) Nafion–IL–Mb/CILE, (d) Nafion–ZrO$_2$–IL–Mb/CILE and (e) Nafion–ZrO$_2$–IL–Mb/CPE in pH 7.0 PBS with scan rate of 100 mV/s

NADH [27]. Due to the ability to retain the native structure and bioactivity of the redox proteins, ZrO$_2$ nanoparticles had also been assembled with proteins to get the electrochemical biosensors. Liu et al. immobilized Hb on the ZrO$_2$ nanoparticles' modified pyrolytic graphite electrode for the preparation of a H$_2$O$_2$ biosensor [21]. Qiao et al. assembled poly(ethylene glycol) and ZrO$_2$ nanoparticles layer-by-layer to get a {PEG/ZrO$_2$}$_n$ film, which was further used to absorb Mb with direct electron transfer realized [23]. Zhao et al. realized the direct electrochemistry of heme proteins immobilized on the self-assembled ZrO$_2$ nanofilm-modified GCE [28]. The presence of ZrO$_2$ nanoparticles can provide a three-dimensional structure to absorb more Mb molecules on the electrode surface and facilitate the direct electron transfer between protein molecules and the electrode interface. While on the Nafion–ZrO$_2$–IL–Mb/CILE (curve d), a more symmetric redox peak appeared with the voltammetric responses better than that of Nafion–IL–Mb/CILE and Nafion–ZrO$_2$–Mb/CILE, respectively. The results indicated the synergistic effects of ZrO$_2$ nanoparticles and IL presented in the composite film, which could promote the electron transfer rate between

Mb and the electrode. For comparison, the Nafion–ZrO$_2$–IL–Mb composite materials were also cast on the surface of traditional CPE. Only a pair of small redox peaks was observed on the Nafion–ZrO$_2$–IL–Mb/CPE (curve e), indicating the superiority of CILE to CPE. Since CILE is prepared with IL as the modifier, which has the characteristics such as good ionic conductivity and wide electrochemical windows with a layer of IL present on the electrode surface [14], the best electrochemical response was achieved on the Nafion–ZrO$_2$–IL–Mb/CILE.

From the cyclic voltammogram of Nafion–ZrO$_2$–IL–Mb/CILE, the cathodic peak potential (E_{pc}) and the anodic peak potential (E_{pa}) appeared at −0.269 and −0.169 V, respectively. The formal potential ($E^{0'}$), which was calculated from the midpoint of E_{pa} and E_{pc}, was obtained as −0.219 V (vs. SCE). The result was the typical characteristic of electroactive heme Fe(III)/Fe(II) redox couple of Mb [29]. The peak-to-peak separation (ΔE_p) was obtained as 100 mV, and the ratio of redox peak current was nearly in unity. The results indicated that the direct electrochemistry of Mb was realized and the Nafion–ZrO$_2$–IL composite film could provide conductive pathway to facilitate the electron transfer between the immobilized protein and underlying electrode.

Influence of scan rate

The influence of scan rate on the electrochemical response of Nafion–ZrO$_2$–IL–Mb/CILE was investigated in the range from 100 to 500 mV/s. As shown in Fig. 3, the redox peak current increased gradually with the shift of the redox peak potential, indicating a quasi-reversible process. Both the redox peak currents increased linearly with scan rate and the linear regression equations calculated as $I_{pa}(\mu A)=94.92\nu(mV/s)+1.305$ ($\gamma=0.997$) and $I_{pc}(\mu A)=-82.32\nu(mV/s)-3.245$ ($\gamma=0.997$), respectively, indicating the electron transfer was a typical surface-controlled thin-layer

Fig. 3 a Cyclic voltammograms of the Nafion–ZrO$_2$–IL–Mb/CILE in pH 7.0 PBS with the scan rates (from 1 to 9) as 100, 150, 200, 250, 300, 350, 400, 450, and 500 mV/s, respectively; b Linear relationship of redox peak potential (E_{pa}) and (E_{pc}) versus lnν; c Linear relationship of cathodic and anodic peak current (I_p) versus scan rate (ν)
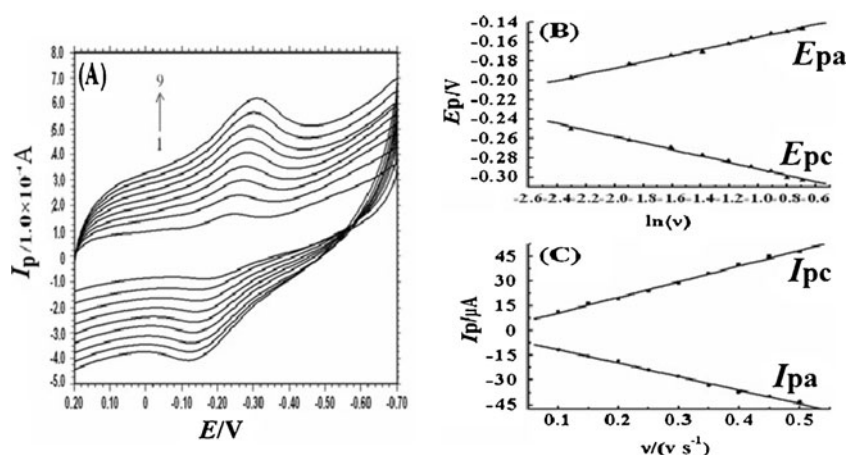
electrochemical reaction. With the increase of scan rate, the peak-to-peak separation (ΔE_p) also increased gradually. Based on the following Laviron's equations [30]:

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

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

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

Electrochemical parameters of the electrode reaction can be further calculated by exploring the relationship of the peak potential with the value of lnυ. Two straight lines were obtained with the equations as E_{pa}(V)=0.0319 lnυ(mV/s) − 0.123(γ=0.998) and E_{pc}(V)=−0.0335 lnυ(mV/s) − 0.325 (γ=0.996). Then the values of electron transfer coefficient (α) and the apparent heterogeneous electron transfer rate constant (k_s) were calculated as 0.499 and 0.813 s^{-1}, respectively. The k_s value was greater than the reported value of Nafion–IL composite film-modified CILE (0.532 s^{-1}) [31], indicating the presence of ZrO$_2$ nanoparticles played an important role for Mb to undergo a fast electron transfer. By integration the reduction peak of cyclic voltammetric curve and based on the Faraday's Law of Γ^*=Q/nAF (where Q is the charge involved in the reaction, n is the number of the electron transferred, F is the Faraday constant, Γ^* is the surface concentration of the electroactive substance), the surface concentration (Γ^*) of electroactive Mb on Nafion–ZrO$_2$–IL/CILE was calculated as 3.487× 10^{-11} mol/cm^2. The result was 2.2 times larger than that of the theoretical monolayer of Mb (1.58×10^{-11} mol/cm^2) [32], indicating the several layers of protein close to the electrode surface involved in the reaction. While the total amount of Mb cast on the electrode surface was 4.697× 10^{-10} mol/cm^2, 7.41 % of the Mb on the electrode surface took part in the electrochemical reaction, which was larger than a reported value [33]. The results demonstrated that the composite film provided a suitable three-dimensional microenvironment for Mb to remain its activity.

Electrocatalytic activity

Due to the peroxidase bioactivity of Mb, the Nafion–ZrO$_2$– IL–Mb/CILE showed good electrocatalytic ability to the reduction of TCA. Figure 4 showed the cyclic voltammograms of the Mb-modified electrode in 0.1 mol/L PBS with the addition of different amounts of TCA. It can be seen that
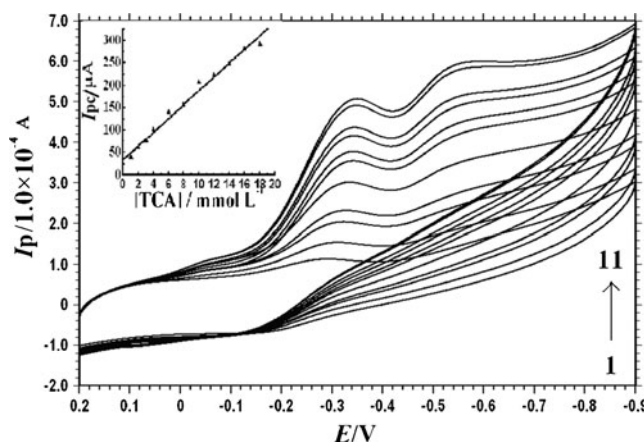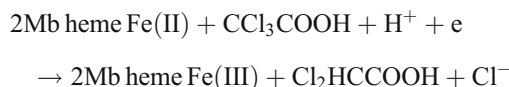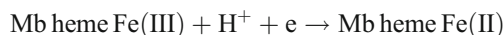

Fig. 4 Cyclic voltammograms of Nafion–ZrO$_2$–IL–Mb/CILE in 0.1 mol/L pH 7.0 PBS containing 0, 1, 2, 3, 4, 6, 8, 10, 12, 14, and 16 mmol/L TCA (from 1 to 11), respectively, with the scan rate as 100 mV/s. *Inset* is the linear relationship of catalytic reduction peak currents with the TCA concentration

a significant increase of the reduction peak current was observed at −0.35 V with the disappearance of the oxidation peak current. The more TCA added, the higher the reduction peak currents observed. This result illustrated that Mb in the composite film retained its biocatalytic activity and exhibited excellent electrocatalytic behavior towards the reduction of TCA, so the Mb-modified electrode could be used as a TCA electrochemical sensor. The catalytic reduction peak currents increased with the TCA concentration in the range from 1.0 to 16.0 mmol/L with the linear regression equation as I_{pc}(μA)=15.37C (mmol/L)+33.19 (n=10, γ= 0.994) and the detection limit as 0.33 mmol/L (3σ). The detection limit was smaller than that of the {IL/Mb}$_3$/CILE (0.6 mmol/L) [34] and {PDDA/Hb}$_8$-films on PG electrode (1.98 mmol/L) [35]. When the TCA concentration was more than 16.0 mmol/L, the peak current turned to level off, indicating a typical Michaelis–Menten kinetic process, so the apparent Michaelis–Menten constant (K_M^{app}) was further calculated by the electrochemical version of Lineweaver–Burk equation [36],

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

where I_{ss} is steady-state current after the addition of TCA, I_{max} is the maximum current measured under saturated TCA, and c is the TCA concentration, respectively. Based on this equation, the value of K_M^{app} was calculated as 7.378 mmol/L, which was lower than the reported value of 47.0 mmol/L [11] and that of Nafion–BMIMPF$_6$/Mb/CILE (90.8 mmol/L) [31]. It is well-known that the smaller K_M^{app} value represents a higher catalytic ability, so the Mb in the composite film-modified CILE exhibited excellent biocatalytic activity.

Based on the reference [37], the electrocatalytic reduction mechanism can be expressed with the following equations:

$$Mb\ heme\ Fe(III) + H^+ + e \rightarrow Mb\ heme\ Fe(II)$$

$$2Mb\ heme\ Fe(II) + CCl_3COOH + H^+ + e$$
$$\rightarrow 2Mb\ heme\ Fe(III) + Cl_2HCCOOH + Cl^-$$

Stability and repeatability of the Mb-modified electrode

The stability of Nafion–ZrO_2–IL–Mb/CILE was evaluated by examining the cyclic voltammetric peak currents after continuous scanning for 50 cycles and there was nearly no decrease of the voltammetric response, indicating that Nafion–ZrO_2–IL–Mb/CILE was stable in buffer solution. After the modified electrode was stored at 4 °C for 2 weeks, 95.0 % of the initial current response was retained. Six Nafion–ZrO_2–IL–Mb/ CILE were made by the same procedure independently, and the relative standard deviation for the determination of 5.0 mmol/L TCA was calculated as 3.6 %. All the results indicated the modified electrode had good repeatability and stability due to the unique biocompatible Nafion–ZrO_2–IL composite that could prevent the Mb molecules from denaturation.

Conclusions

In this paper, a nanocomposite material composed of ZrO_2 nanoparticle, IL, and Nafion was fabricated and used for the immobilization of Mb on the surface of a CILE. Due to the biocompatibility of the materials used, Mb molecules remained its native structure in the composite film. Direct electrochemistry of Mb on the modified electrode was achieved with a pair of well-defined redox peak appeared, which was due to the specific biocompatible and synergistic effects of the unique structure, excellent biocompatibility, and high ionic conductivity of the Nafion–ZrO_2–IL hybrid composite. The fabricated electrode showed good electrocatalytic ability to the reduction of TCA with wider linear range, lower detection limit, and good stability, which provided a new effective support matrix for the fabrication of third-generation electrochemical biosensor with the Nafion–ZrO_2–IL composite film.

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