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Electrochemistry and electrocatalysis of myoglobin intercalated in Mg_2Al–Cl layered double hydroxide and ionic liquid composite material

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ABSTRACT

A novel biocompatible nanocomposite prepared by Mg_2Al–Cl layered double hydroxide (LDH) and ionic liquid (IL) 1-carboxyl-methyl-3-methylimidazolium tetrafluoroborate was used as the matrix for the immobilization of myoglobin (Mb). The IL-LDH-Mb composite was characterized by UV–vis adsorption spectroscopy and the results indicated that Mb retained its native structure in the IL-LDH nanocomposite. The IL-LDH-Mb bionanocomposite was modified on the surface of carbon ionic liquid electrode to get an Mb modified electrode. Electrochemical experiments showed that direct electrochemistry of Mb in the composite was realized with a pair of well-defined redox peaks appeared, which could be attributed to the specific microenvironment provided by layer structured LDH and high ionic conductive IL present for Mb molecule. The modified electrode exhibited good direct electrocatalytic ability to the reduction of trichloroacetic acid and hydrogen peroxide with good stability and reproducibility. Different kinds of real samples were detected by the modified electrode with satisfactory results. So the IL-LDH nanocomposite provided a novel and efficient platform for the immobilization of enzymes, which had potential applications in the fabrication of third-generation biosensors.

1. Introduction

Studies on direct electron transfer between native redox proteins and underlying electrode have aroused greatly interests due to the potential applications in the fields of bioreactor, biofuel cell and biosensors [1,2]. The investigation can provide the model of the electron transfer mechanism in biological systems and establish the foundation for the biodevices. However, the electroactive centers of redox proteins are often embedded within the structure of biomacromolecules, and the direct electrochemistry between the electroactive center and the substrate electrode is difficult to occur. Also the direct adsorption of redox proteins onto the surface of a bare metal electrode often results in the change of the conformations or the denaturation of structure with consequent loss of bioactivity and electrochemical activity. Therefore various kinds of film modified electrodes have been devised to provide a specific microenvironment for the proteins immobilization, which can keep their structure and remain their activity. Also the direct electron transfer rate between the protein and the underlying electrode can be enhanced [3]. The supporting materials used in the protein film modified electrode include biopolymers, nanomaterials, hydrogel, ionic liquids and so on, and direct electrochemistry of protein can be easily realized due to the specific properties of the materials. Among the materials used inorganic materials have exhibited some properties such as the regular structure, chemical inertia, biocompatibility, good mechanical and thermal stability, and low cost. Layered double hydroxides (LDHs), which are also named as hydrotalcite like materials or anionic clays, have been used as the supporting materials in the protein electrochemistry [4]. LDHs can be described with formula of $[M^{II}{}_{1-x}M^{III}{}_{x}(OH)_2]^{x+}(A^{n-})_{x/n}\cdot mH_2O$, where M^{II} is the divalent metal ions and M^{III} is the trivalent ones. The excess positive charge of x^+ can be compensated by interlayer counter anions, A^{n-}. LDHs can immobilize negatively charged biomolecules due to the high order layered structure with the positively charge. Also LDHs exhibit a high versatility of physical and chemical properties such as multivalent chemical compositions, adjustable textural properties, large surface areas with preparation variables [5,6]. Some electrochemical biosensors with LDHs as support matrices have been successfully developed with remarkable catalytic performances [7]. The adsorption of heme proteins or enzymes on the LDHs can result in a stable biohybrid and direct electron transfer rate between the electroactive heme sites of the

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proteins and the underlying electrode can be accelerated [8]. Shan et al. immobilized polyphenol oxidase on a Zn–Al LDH and applied the detection of different phenol derivatives [9]. Li et al. investigated the direct electrochemistry of hemoglobin (Hb) on a sodium dodecylsulfonate functionalized Zn–Al LDH modified glassy carbon electrode [10]. Chen et al. studied the direct electrochemical behavior of horseradish peroxidase (HRP) on Ni–Al LDH nanosheets modified glassy carbon electrode [11]. Due to the specific interactions occurred between redox proteins and different LDH materials, electrochemical sensors based on LDHs have been successfully used for the investigation on the direct electrochemistry of proteins. However, LDHs also exhibit some disadvantages such as poor charge transport activity and lower electroactivity, which limit the applications in the field of electrochemistry.

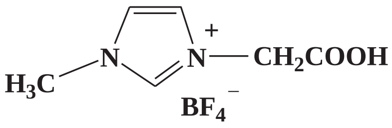
Ionic liquids (ILs) are ionic compounds composed of organic cations and various anions [12], which represent a new class of green solvents in the field of chemistry. As a non-aqueous polar solvent, ILs have exhibited many unique properties such as high chemical and thermal stability, relatively high ionic conductivity, negligible vapor pressure and wide electrochemical windows [13]. Due to these specific characteristics, ILs can be used as the electrolytes, the modifier on chemically modified electrode and the binder in carbon paste electrode (CPE). By incorporating ILs into the conventional CPE, carbon ionic liquid electrode (CILE) exhibits an excellent electrochemical behavior with its applications in the electrochemical sensors. CILE has been elucidated with the advantages such as high conductivity, good stability and antifouling ability with a layer of IL film presented on its surface [14]. CILE can be used as a basal electrode for protein immobilization and the direct electrochemistry of protein has been realized on different kinds of CILE. Safavi et al. studied the electrochemistry of Hb on CILE with octylpyridinium chloride [15]. Our group also applied different CILEs for the investigation on the direct electrochemistry of proteins [16–18].

In this paper a Mg_2Al–Cl based LDH was synthesized and used as the host matrix for protein immobilization with myoglobin (Mb) as the model redox protein. In order to increase the whole conductivity of the LDH-Mb biocomposite, a carboxyl functionalized IL was used as the enhancer in the composite, which had high ionic conductivity and good biocompatibility. Also a CILE was used as the working electrode due to its better performances. Then the IL-LDH-Mb composite modified CILE was fabricated with electrochemical behaviors carefully investigated. Experimental results indicated that direct electron transfer of Mb was realized in the composite film modified electrode. The electrocatalytic behaviors of Mb modified electrode to the reduction of trichloroacetic acid and hydrogen peroxide were also achieved, which indicated that an unmediated third-generation electrochemical biosensor was successfully fabricated.

2. Experimental

2.1. Apparatus and reagents

Equine heart myoglobin (Mb, Sigma), ionic liquid 1-hexylpyridinium hexafluorophosphate ($HPPF_6$, Lanzhou Greenchem ILS, LICP, CAS, 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 without further purification. 1-Carboxyl-methyl-3-methylimidazolium tetrafluoroborate ($CMMIMBF_4$) and $Mg_2Al(OH)_6Cl_3{\cdot}nH_2O$ (Mg_2Al–Cl) LDH were synthesized according to the references. 0.1 mol/L phosphate buffer solutions (PBS) with various pH values were used as the supporting electrolyte. All the other chemicals used were of analytical reagent grade and all aqueous solutions were prepared with doubly distilled water.



Scheme 1. The molecular structure of $CMMIMBF_4$.

All the electrochemical measurements were executed on a CHI 750B electrochemical workstation (Shanghai CH Instrument, China). A conventional three-electrode electrochemical cell was used with an Mb modified electrode as working electrode, a platinum wire as auxiliary electrode and a saturated calomel electrode (SCE) as reference electrode. All the potentials were reported with respect to this reference. UV–vis absorption spectroscopy was recorded on Cary 50 probe spectrophotometer (Varian Company, Australia). Scanning electron microscopy (SEM) was recorded on a JSM-6700F scanning electron microscope (Japan Electron Company, Japan) and transmission electron microscopy (TEM) was obtained on a JEOL JEM-2010HT transmission electron microscope (Japan Electron Company, Japan).

2.2. Synthesis of Mg_2Al–Cl LDH

Mg_2Al–Cl LDH was prepared by the coprecipitation method at controlled pH based on Refs. [19,20] with the procedure described as follows. A mixed solution of $MgCl_2{\cdot}H_2O$ and $AlCl_3{\cdot}H_2O$, with a Mg^{2+}/Al^{3+} molar ratio (r) of 2 and a total concentration of 0.5 mol/L, was added slowly in a reactor containing decarbonated water, and the pH of solution was maintained at 9.5 during the coprecipitation by the simultaneous addition of a 4.0 mol/L $NH_3{\cdot}H_2O$ solution. Then the suspension was aged at room temperature with stirring for 24 h. The final product was centrifuged and washed several times with decarbonated water, and finally dried in air at room temperature for physical characterization or kept as a fresh aqueous gel (20 wt%). All experiments were carried out under N_2 atmosphere to avoid or at least minimize the contamination by CO_2.

2.3. Synthesis of $CMMIMBF_4$

The carboxyl functionalized IL was synthesized based on the reported procedure [21]. To a stirred ethanol solution of 1-methylimidazole (0.10 mol), a bromoacetic acid (0.10 mol) in 10 mL acetone was gradually added under −5 to 0 °C. The resulting solution was stirred for another 2 h under the same temperature, then allowed to stir for an additional 8 h under ambient temperature. The solvent was removed by rotary evaporation under reduced pressure to give the deep brown-reddish viscous liquid 1-carboxyl-methyl-3-methylimidazolium bromide (CMMIMBr). Then a solution of the resulted CMMIMBr in 50 mL of anhydrous MeOH was stirred and potassium fluoborate (0.20 mol) was added. The mixture was stirred for further 48 h under room temperature. Potassium salt was filtrated off and the filtrate was concentrated under reduced pressure. The residual potassium salt was removed by filtrating again. The viscous liquid was dried under vacuum to afford $CMMIMBF_4$ (21.43 g, 94%), which appeared as pale yellow viscous liquid with its structure shown in Scheme 1.

2.4. Fabrication of IL-LDH-Mb modified electrode

$HPPF_6$ based CILE was fabricated by hand-mixing 0.8 g of $HPPF_6$ and 1.6 g of graphite powder in a mortar and ground carefully. A portion of resulting homogeneous paste was packed firmly into a glass tube cavity ($\Phi = 4$ mm) and the electrical contact was

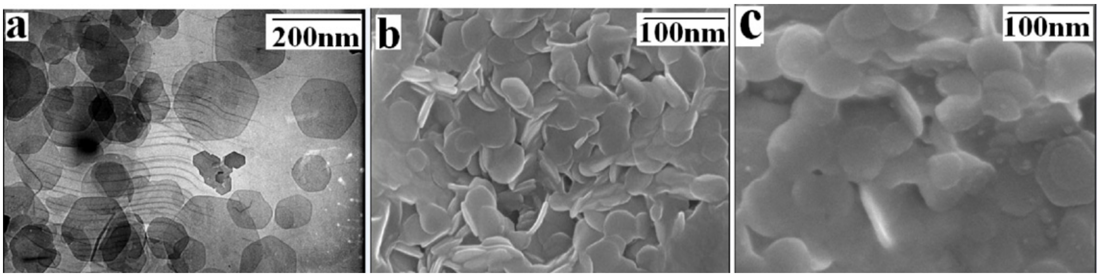


Fig. 1. TEM of (a) LDH, SEM of (b) LDH/CILE and (c) IL-LDH-Mb/CILE.

established through a copper wire to the end of the paste in the inner hole of the tube. The surface of CILE was polished on a weighing paper just before use.

The modified electrode was prepared by the following method. Firstly the mixture solution contained 15.0 mg/mL Mb, 0.5 mg/mL Mg_2Al–Cl LDH and 10% $CMMIMBF_4$ was prepared and mixed homogenously. Then 10.0 μL of the IL-LDH-Mb suspension solution was dropped onto the CILE surface directly and allowed to dry at room temperature, which gave the modified electrode denoted as IL-LDH-Mb/CILE. For comparison other electrodes such as LDH-Mb/CILE, Mb/CILE and IL-Mb/CILE were prepared by the similar procedure.

2.5. Procedure

The electrochemical measurements were carried out in a 10 mL electrochemical cell containing 0.1 mol/L pH 7.0 PBS. Prior to the measurements, PBS was deaerated with highly purified nitrogen for 30 min, and then a nitrogen atmosphere was maintained over the cell during the experiments. Cyclic voltammetric experiments were performed between −0.7 and 0.2 V with the scan rate of 100 mV/s. All the electrodes were stored at 4 °C in a refrigerator when not in use.

3. Results and discussion

3.1. Characteristics of the composite material

The synthesized Mg_2Al–Cl LDH was characterized by TEM with the result shown in Fig. 1a, which indicated that the LDH was nanoscaled flake with a diameter of 20–40 nm and the thickness of several nanometers. The uniform-sized and monodispersed nanoflakes can be suspended stable in the solution for more than 6 months. Scanning electron microscopy (SEM) was used to characterize and compare the morphologies of different modified electrodes. On the surface of LDH/CILE the LDH was randomly stacked to form a three-dimensional interface (Fig. 1b). On the IL-LDH-Mb/CILE (Fig. 1c) LDH was covered and connected with a continuous membrane with the increase of the dimension, which could be attributed to the presence of high viscous IL that blend the LDH and Mb together. The differences of the SEM images indicated that the composite material was formed on the CILE surface.

3.2. UV–vis spectroscopic results

UV–vis absorbance spectrum can provide the conformational change of heme proteins. The possible denaturation of heme proteins can be speculated from the shape and position of the Soret absorption band [22]. As shown in Fig. 2, Mb had a Soret band at 410.0 nm in PBS (curve a). After mixing Mb with different materials such as LDH, IL or IL-LDH together, the absorption peak also appeared at 410.0 nm without any changes (curves b–d). Obviously, the same absorption position in different matrix was attributed to the Soret band of Mb and the results indicated that Mb molecules remained their native structure in the LDH and IL nanocomposite materials. LDH is an inorganic material with good biocompatibility, which can act as a protective matrix to the increase of the bioactivity and lengthening the lifetime of biomolecules [23]. At the same time IL has been proven to be a green solvent that can retain the enzyme activity and stability than the traditional organic or aqueous solution [24]. So the native structure of Mb was kept after mixing with the IL-LDH composite material.

3.3. Electrochemical impedance spectra of the modified electrodes

Electrochemical impedance spectroscopy (EIS) can provide detailed information on the change of the surface property of the modified electrodes. The impedance spectra include a semicircle portion and a linear portion. In general the semicircle diameter at higher frequencies corresponds to the electron-transfer resistance (Ret) and the linear part at lower frequencies corresponds to the diffusion process. Fig. 3 showed the electrochemical impedance spectra of different modified electrodes with the frequency swept from 10^5 to 0.1 Hz. The Ret value of Mb/CILE was estimated to be 197.7 Ω (curve a), which was due to the presence of Mb molecules on the electrode surface that blocked the approach of the redox probe $[Fe(CN)_6]^{3-/4-}$. On the LDH-Mb/CILE (curve b), the Ret value was decreased to 128. 9 Ω, indicated that the multilayers of LDH on the electrode surface could adsorb more $[Fe(CN)_6]^{3-/4-}$. While on the IL-LDH-Mb/CILE the Ret value was further decreased with almost a straight line appeared (curve c). The result indicated that the addition of high conductive IL in the composite material decreased the resistance of the electrode interface and enhanced the conductivity of the composite film. The EIS changes of the modification process also indicated that LDH and IL were successfully immobilized on the electrode surface.

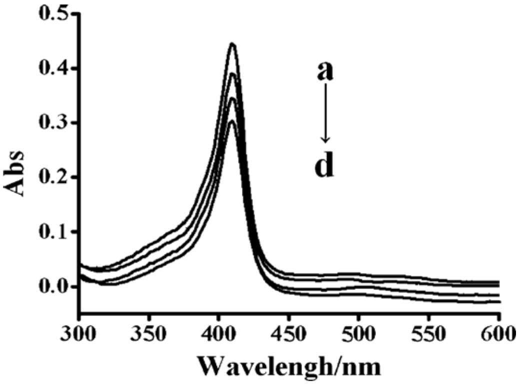


Fig. 2. UV–vis absorption spectra of Mb (a), LDH-Mb (b), IL-Mb (c) and IL-LDH-Mb (d) with pH 7.0 PBS.

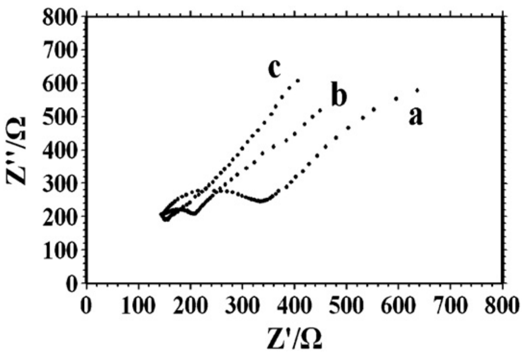


Fig. 3. Nyquist plot of EIS for (a) Mb/CILE, (b) LDH-Mb/CILE, (c) IL-LDH-Mb/CILE in the presence of a solution of 5.0 mmol/L $[Fe(CN)_6]^{3-/4-}$ and 0.1 mol/L KCl with the frequencies ranging from 10^5 to 0.1 Hz.

3.4. Direct electrochemistry of the modified electrodes

Electrochemical properties of Mb modified electrodes were studied by cyclic voltammetry with the results shown in Fig. 4. In a 0.1 mol/L pH 7.0 PBS only a small oxidation peak appeared on the Mb/CILE (curve a), indicating an irreversible electrode process. While on the LDH-Mb/CILE a pair of unsymmetrical redox peaks appeared with small redox peak current (curve b). LDHs have exhibited some intrinsic properties such as a wide range of possible chemical compositions, adjustable textural properties and preparation variable, which make LDHs have effective host structures for the immobilization of biomolecules [25]. On the IL-Mb/CILE (curve c) the redox peak currents increased, which was attributed to the presence of high conductive IL on the electrode surface that could improve the electron transfer rate of Mb with the substrate electrode. On the IL-LDH-Mb/CILE a pair of stable and well-defined redox peaks appeared (curve d) with the electrochemical response bigger than that of IL-Mb/CILE and LDH-Mb/CILE. The results indicated that the interaction of LDH with IL could result in a stable nanocomposite with high stability and good conductivity, which provided a specific three-dimensional microenvironment for the immobilization of Mb and accelerated the electron transfer rate. Based on the cyclic voltammetric results, the apparent formal peak potential ($E^{0\prime}$) was calculated as −0.204 V with the peak-to-peak separation (ΔE_p) as 0.096 V (curve d), indicating a quasi-reversible electrochemical process.

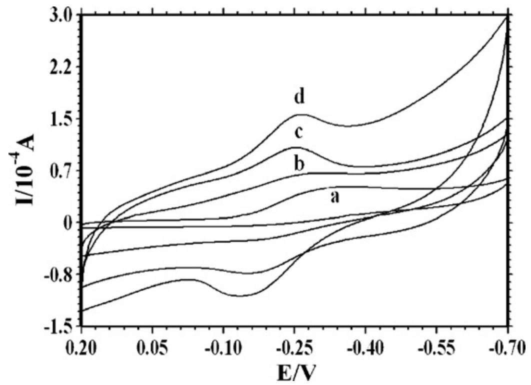


Fig. 4. Cyclic voltammograms of Mb/CILE (a), LDH-Mb/CILE (b), IL-Mb/CILE (c) and IL-LDH-Mb/CILE (d) in 0.1 mol/L PBS (pH 7.0) at the scan rate of 0.1 V/s.

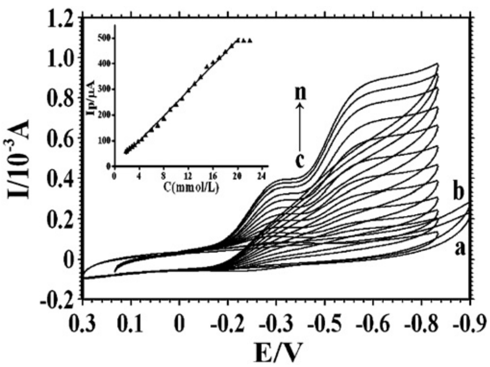


Fig. 5. Cyclic voltammograms of IL-LDH/CILE in the presence of (a) 0 mmol/L, (b) 4.0 mmol/L TCA in 0.1 mol/L pH 7.0 PBS and IL-LDH-Mb/CILE in the presence of 1.0 mmol/L, 1.6 mmol/L, 2.0 mmol/L, 3.8 mmol/L, 6.0 mmol/L, 8.0 mmol/L, 10.0 mmol/L, 12.0 mmol/L, 14.0 mmol/L, 16.0 mmol/L, 18.0 mmol/L, 20.0 mmol/L TCA (curves c–n) with the scan rate as 100 mV/s (Inset was the linear relationship of catalytic reduction peak currents and the TCA concentration).

Cyclic voltammograms of IL-LDH-Mb/CILE at different scan rates were further recorded in the scan rate range from 50 to 600 mV/s. With the increase of scan rate, the redox currents increase gradually. Two linear relationships between the redox peak currents and scan rate were calculated with the linear regression equations as I_{pa} (μA) = −103.58υ (V/s) − 22.56 (n = 18, γ = 0.995) and I_{pc} (μA) = 237.67υ (V/s) − 8.21 (n = 18, γ = 0.999). These results indicate that the redox process is a typical surface controlled process [26]. Then the surface concentration (Γ^*) of electroactive Mb in the nanocomposite film was estimated from the integration of the reduction peak in cyclic voltammograms. According to the following equation: $\Gamma^* = Q/nAF$, where Q is the charge involved in the reaction and A is the effective electrode area (obtained according to the Randles–Sevcik equation). The surface concentration of electroactive Mb (Γ^*) was calculated with the result as 2.28×10^{-9} mol/cm^2. While the total amounts of Mb cast on the electrode surface was 5.35×10^{-8} mol/cm^2, so the percentage of electroactive Mb on the electrode surface was calculated as 4.2%. This suggested that multi-layer of immobilized Mb in the IL-LDH film participated in the electron transport process.

The influence of buffer pH on the electrochemical response of Mb on the modified electrode was also investigated. The formal peak potentials ($E^{0\prime}$) of Mb were negatively shifted with the solution pH in the range from 4.0 to 7.0 and a linear regression equation was got as $E^{0\prime}$ (mV) = −50.9 pH − 58.6 (n = 7, γ = 0.997). The slope value of −50.9 mV/pH was close to the theoretical value of −57.6 mV/pH for a reversible one proton coupled one electron transfer process. The result can be attributed to the effect of the protonation states of transligands to the heme iron and amino acids around the heme or to the protonation of water molecules coordinated to the center that may exist at different pH values [27]. So the electrochemical reaction occurred on the Mb modified electrode was a quasi-reversible one-electron transfer coupled with a single-proton transportation process with the equation represented as Mb Fe(III) + H^+ + e → Mb Fe(II) [28].

3.5. Electrocatalytic properties of IL-LDH-Mb/CILE

As indicated by previously reports [29,30], Mb film modified electrodes often showed good electrocatalytic ability toward the reduction of organohalides such as ethylene dibromide and TCA with a large decrease of activation energy. So the electrocatalytic reduction of TCA at the IL-LDH-Mb/CILE was carefully investigated by cyclic voltammetry. As shown in Fig. 5, an increase in the reduction peak current at −0.334 V could be observed and accompanied by a decrease in the oxidation peak current on the addition of different amounts of TCA into the solution. In addition, another new reduction peak appeared at −0.558 V with the further increase of the TCA concentration (curves c–n), which was the typical characteristic of the electrocatalytic reaction for the TCA reduction. While

Table 1
Detection results of H_2O_2 in disinfector sample ($n = 3$).

Sample	Detected (mM)	Added (mM)	Found (mM)	Recovery (%)	RSD (%)
1	0.436	0.20	0.204	100.67	1.28
2	0.439	0.40	0.394	99.25	1.37
3	0.442	0.60	0.612	101.2	2.69

no redox peak was observed at the IL-LDH/CILE under the same conditions without Mb in the composite film (curves a and b). The results indicated that Mb on the electrode surface exhibited good catalytic behaviors. Based on the reference [31], the second reduction peak could be tentatively assigned to the highly reduced form of Mb [Mb Fe(I)], which was an active reductant that could dechlorinate di- and monochloroacetic acid after the dechlorination of TCA by Mb Fe(II). The overall electrocatalytic reduction mechanism for TCA can be proposed with the following equations:

$$\text{Mb Fe(III)} + e \rightarrow \text{Mb Fe(II)} \tag{1}$$

$$2\text{Mb Fe(II)} + Cl_3CCOOH + H^+ \rightarrow 2\text{Mb Fe(III)} + Cl_2CHCOOH + Cl^- \tag{2}$$

$$\text{Mb Fe(II)} + e \rightarrow \text{Mb Fe(I)} \tag{3}$$

$$2\text{Mb Fe(I)} + Cl_2CHCOOH + H^+ \rightarrow 2\text{Mb Fe(II)} + ClCH_2COOH + Cl^- \tag{4}$$

$$2\text{Mb Fe(I)} + ClCH_2COOH + H^+ \rightarrow 2\text{Mb Fe(II)} + CH_3COOH + Cl^- \tag{5}$$

The catalytic reductive peak current increased linearly with the TCA concentration in the range from 1.0 to 20.0 mmol/L with the linear regression equation as I_{ss} (μA) = 24.51C (mmol/L) + 1.84 with a correlation coefficient of 0.999 ($n = 23$). And the detection limit was calculated as 0.353 mmol/L at a signal-to-noise ratio of 3. At higher concentrations of TCA in the solution, a platform appeared in the catalytic peak current, which was in accordance with the Michaelis–Menten model. The apparent Michaelis–Menten constant ($K_M{}^{app}$), which could be obtained from the electrochemical version of the Lineweaver–Burk equation, was calculated as 15.78 mmol/L. The value was smaller than that of Mb entrapped in agarose hydrogel films in the ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate (177 mmol/L) [32], and Mb in a Nafion-ionic liquid composite film modified CILE (90.8 mmol/L) [33]. The results suggested that Mb immobilized on IL-LDH/CILE had good affinity to TCA. So the specific microenvironment provided by $CMMIMBF_4$ and LDH composite might contribute to the improvement of the affinity and good performances of the Mb biosensor.

The IL-LDH-Mb/CILE also showed good electrocatalytic ability to the reduction of H_2O_2. With the increase of the H_2O_2 concentration in the buffer solution, the reduction peak current increased with the decrease and disappear of the oxidation peak gradually, which demonstrated the typical electrocatalytic reduction process of H_2O_2. Meanwhile, the reduction peak current increased gradually and exhibited a linear relationship with H_2O_2 concentration in the range from 1.4 to 68.0 μmol/L with the linear regression equation as I_{ss} (μA) = 0.259C (μmol/L) + 20.00 ($\gamma = 0.998$) and the detection limit as 0.45 μmol/L (3σ). The value of $K_M{}^{app}$ could be calculated as 6.5 μmol/L by the electrochemical version of the Lineweaver–Burk equation, which was smaller than some previous reports [34–37].

3.6. Stability and reproducibility of IL-LDH-Mb/CILE

The stability of IL-LDH-Mb/CILE was evaluated by examining the cyclic voltammetric peak currents after continuous scanning for 40 cycles and there was nearly no decrease of the voltammetric response, indicating that IL-LDH-Mb/CILE was stable in buffer solution. The long-term stability of the IL-LDH-Mb/CILE was examined by storing the modified electrode at 4 °C in a refrigerator and measured intermittently. The current response to 5.0 mmol/L TCA decreased 8.5% over 3-weeks, indicating the good stability of the modified electrode. The results can be attributed to the biocompatibility of IL-LDH composite film, which could prevent the leakage of enzyme and retain its electrocatalytic activity. Seven Mb modified electrodes were made by the same procedure independently and the relative standard deviation (RSD) for the determination of 5.0 mmol/L TCA was calculated as 4.5%, which indicated the modified electrode had good repeatability.

The precision of the proposed electroanalytical method was further investigated based on a within-day and between-day basis. The within-day precision is based on the relative standard deviation (RSD) of the five replicated analysis of two different concentration of analyte in the same day. While the between-day precision is determined by the same concentration of analyte detected for the five consecutive days. Based on this procedure the within-day precision for the detection of 5.0 mmol/L and 10.0 mmol/L TCA were performed by the modified electrode with the RSD values as 2.56% and 2.82%. Also the between-day precision for the detection of 5.0 mmol/L and 10.0 mmol/L TCA was calculated as 2.98% and 3.46%. The results indicated the modified electrode exhibited better precision for the electrochemical detection of TCA.

3.7. Real samples analysis

In order to verify the application of the modified electrode, the H_2O_2 concentration in the real sample of disinfector was detected. The disinfector was produced by Shandong Lircon Disinfection Technology Limited Company. Under the selected conditions with the experimental procedure and by using the standard addition method, the determination results were shown in Table 1. It can be seen that the results were satisfactory with the recovery in the range of 99.25–101.20% and the RSD value in the range of 1.37–2.69%. The results indicated the modified electrode could be used for the H_2O_2 sample analysis.

The IL-LDH-Mb/CILE was further applied to the detection of H_2O_2 residues in the real milk samples with the standard addition method. H_2O_2 is often used as the preservative agent or the stabilizer in commercially milk products. But the residual H_2O_2 in the milk must be eliminated before the consumption due to its harmful to the skin and the human health. Aseptically packaged milk was purchased from a local supermarket in Qingdao, China, which was diluted by 0.1 mol/L pH 7.0 PBS and detected with the general procedure. The result were summarized in Table 2 and it could be seen that no H_2O_2 residues were detected in the samples with the recovery in the range of 96.6–102.6%, including that the

Table 2
Detection results of H_2O_2 in the milk sample ($n = 3$).

Sample	Detected (μM)	Added (μM)	Found (μM)	Recovery (%)	RSD (%)
Aseptically packaged milk	0	20.00	19.32	96.60	3.61
		30.00	30.47	101.57	4.23
		40.00	41.04	102.60	4.68

modified electrode could be used for the determination of H_2O_2 concentration in the milk samples with satisfactory results.

4. Conclusion

In this work an Mg_2Al–Cl LDH was synthesized and further mixed with a carboxyl functionalized IL to get a new composite material, which was used for the immobilization of Mb molecules on the electrode surface. By using CILE as the substrate electrode, a new electrochemical Mb biosensor was fabricated and the direct electrochemistry of Mb was investigated on the modified electrode. Due to the specific characteristics of LDH and IL, Mb remained its native structure and biocatalytic activity. The fabricated electrochemical Mb sensor showed good electrocatalytic ability to the reduction of TCA in the concentration range from 1.0 to 20.0 mmol/L and the reduction of H_2O_2 in the concentration range from 1.4 to 68.0 μmol/L. So the combination of LDH with IL and Mb provided a new platform for the construction of third-generation electrochemical biosensor with better performance.

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