Colloids and Surfaces B: Biointerfaces 87 (2011) 61–66

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Effect of the structure of imidazolium cations in $[BF_4]^-$-type ionic liquids on direct electrochemistry and electrocatalysis of horseradish peroxidase in Nafion films

Lu Lu [a], Xirong Huang [a,b,*], Yinbo Qu [b]

[a] Key Laboratory of Colloid & Interface Chemistry of the Education Ministry of China, Shandong University, Jinan 250100, PR China
[b] State Key Laboratory of Microbial Technology of China, Shandong University, Jinan 250100, PR China

ARTICLE INFO

Article history:
Received 23 February 2011
Received in revised form 12 April 2011
Accepted 30 April 2011
Available online 7 May 2011

Keywords:
Room-temperature ionic liquids
Horseradish peroxidase
Direct electrochemistry
Bioelectrocatalysis
Structural effect
Nafion films

ABSTRACT

The direct electrochemistry and bioelectrocatalysis of horseradish peroxidase (HRP) in Nafion films at glassy carbon electrode (GCE) was investigated in three $[BF_4]^-$-type room-temperature ionic liquids (ILs) to understand the structural effect of imidazolium cations. The three ILs are 1-ethyl-3-methylimidazolium tetrafluoroborate ([Emim][BF_4]), 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF_4]) and 1-hexyl-3-methylimidazolium tetrafluoroborate ([Hmim][BF_4]). A small amount of water in the three ILs is indispensable for maintaining the electrochemical activity of HRP in Nafion films, and the optimum water contents decrease with the increase of alkyl chain length on imidazole ring. Analysis shows that the optimum water contents are primarily determined by the hydrophilicity of ILs used. In contrast to aqueous medium, ILs media facilitate the direct electron transfer of HRP, and the electrochemical parameters obtained in different ILs are obviously related to the nature of ILs. The direct electron transfer between HRP and GCE is a surface-confined quasi-reversible single electron transfer process. The apparent heterogeneous electron transfer rate constant decreases gradually with the increase of alkyl chain length on imidazole ring, but the changing extent is relatively small. The electrocatalytic reduction current of H_2O_2 at the present electrode decreases obviously with the increase of alkyl chain length, and the mass transfer of H_2O_2 via diffusion in ILs should be responsible for the change. In addition, the modified electrode has good stability and reproducibility; the ability to tolerate high levels of F^- has been greatly enhanced due to the use of Nafion film. When an appropriate mediator is included in the sensing layer, a sensitive nonaqueous biosensor could be fabricated.

1. Introduction

Room-temperature ionic liquids (ILs), which consist of organic cations and organic/inorganic anions, are molten salts at ambient temperature or temperatures lower than 100 °C. As a novel, nonaqueous and polar solvent, ILs have been widely used in electrochemical science due to their unique physical and chemical properties including nonflammability, high conductivity, wide electrochemical windows, good chemical and thermal stability, negligible vapor pressure, good dissolubility towards inorganic and organic substances, etc.

In the field of bioelectrochemistry and bioelectrocatalysis, ILs have been used primarily as modifying materials of bioelectrodes to improve the performance of the biosensors. In this respect, many reports have been released [1,2]. The sensing layer is usually prepared by immobilizing heme-containing proteins or redox enzymes in IL-based composite materials. The IL-based composite materials include IL/carbon nanotube [3–9], IL/metallic nanoparticles [10–14], and IL/graphene [15,16], etc. They have been demonstrated to be effective for the development of highly sensitive, selective and reproducible biosensors. Most of the results indicate that ILs have positive effects on the performance of bioelectrodes, however, there are some reports showing that ILs have little effects [17] or negative effects [18,19] on the performance of bioelectrodes. The analysis has proved that the effects of ILs in bioelectrochemistry and bioelectrocatalysis are correlated with not only the nature but also the usage of ILs. It follows that the effects of ILs and their mechanism are necessary to be further clarified.

As supporting electrolytes, ILs have been widely used in fundamental electrochemical science, however, there are few reports on the application of ILs in the field of bioelectrochemistry and bioelectrocatalysis. Xiong et al. immobilized some heme proteins in *N*,*N*-dimethylformamide (DMF)/chitosan composite material, and studied their electrochemical and biological activities in 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF_4]) [20,21]. Ding et al. designed a modified electrode by adsorbing

* Corresponding author at: No. 27 Shanda Nanlu, Shandong University, College of Chemistry & Chemical Engineering, Jinan 250100, PR China. Tel.: +86 531 88365433; fax: +86 531 88365433.
E-mail address: xrhuang@sdu.edu.cn (X. Huang).

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doi:10.1016/j.colsurfb.2011.04.037

myoglobin on the surface of a graphite electrode, and explored the direct electron transfer of myoglobin and the electrocatalytic reduction of O_2 in 1-(2-hydroxyethyl)-3-methyl imidazolium tetrafluoroborate ([Hemim][BF_4]) [22]. Lately, Wang et al. devised several enzyme electrodes by immobilizing some heme proteins in agarose hydrogel films, and investigated their activity, stability and the electrocatalysis of H_2O_2 in hydrophobic 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF_6]) and hydrophilic [Bmim][BF_4], respectively [23,24]. These case studies indicate that the effectiveness of the direct electrochemistry of an enzyme is correlated with the hydrophobicity or hydrophilicity of ILs. However, no attempts have been made to correlate the effect of ILs with their composition especially their cations' structure.

As a kind of heme-containing proteins, horseradish peroxidase (HRP) has been widely used in bioelectrochemistry and bioelectrocatalysis. HRP has currently been demonstrated to be electrochemically active as well as biologically active in [Bmim][PF_6] when immobilized in agarose hydrogel film [23]. In the present paper, HRP is immobilized in a Nafion film, the direct electrochemistry and bioelectrocatalysis of the immobilized HRP on a glassy carbon electrode (GCE) in three different [BF_4]$^-$-type ILs are investigated by means of cyclic voltammetry etc., and the effect of the alkyl chain length of imidazolium cations on the direct electrochemistry and electrocatalysis of HRP is discussed. To highlight the effect of cations as well as prevent any negative effect of F^- dissociated from [BF_4]$^-$, a perfluorinated sulfonate ionomer, Nafion, instead of neutral agarose is used to immobilize HRP on GCE, and no mediator (electron-transfer-accelerating agents) is added in the film. The present study is helpful to further clarify the effects of ILs and their mechanism in bioelectrochemistry and bioelectrocatalysis, it also provides a scientific support for developing biosensors for detecting water-insoluble materials in nonaqueous medium.

2. Experimental

2.1. Reagents

Horseradish peroxidase (HRP, EC 1.11.1.7, 250 U mg^{-1}) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China); HRP stock solution (10 mg mL^{-1}) was prepared by dissolving HRP in 50 mmol L^{-1} pH 7.0 phosphate buffer solution (PBS); ILs [Emim][BF_4] (1-ethyl-3-methylimidazolium tetrafluoroborate, 99%), [Bmim][BF_4] (1-butyl-3-methylimidazolium tetrafluoroborate, 99%) and [Hmim][BF_4] (1-hexyl-3-methylimidazolium tetrafluoroborate, 99%) were provided by Shanghai Chengjie Chemicals Co. Ltd. (Shanghai, China) and used without further purification; agarose was purchased from Spanish Biowest (distributed in Hong Kong, China); perfluorinated ion exchange resin (Nafion, 5 wt.%) was obtained from Sigma Co. (St. Louis, USA); H_2O_2 (A. R., 30 wt.%) was purchased from Tianjin Bodi Chemical Industry Stock Co. Ltd. (Tianjin, China). All other reagents were of analytical grade and used as received. Triply distilled water was used throughout the experiments.

2.2. Preparation of electrodes

A glassy carbon electrode (GCE, 3 mm in diameter) was first polished with alumina slurry on chamois leather. After that, it was cleaned sequentially in an ultrasonic cleaner with 1:1 (v/v) nitric acid, acetone and triply distilled water for 5 min, respectively. Finally, the GCE was dried with nitrogen gas.

The Nafion/GCE was prepared by uniformly coating 10 μL Nafion on the surface of the treated GCE, followed by drying under a beaker in a ventilative environment.

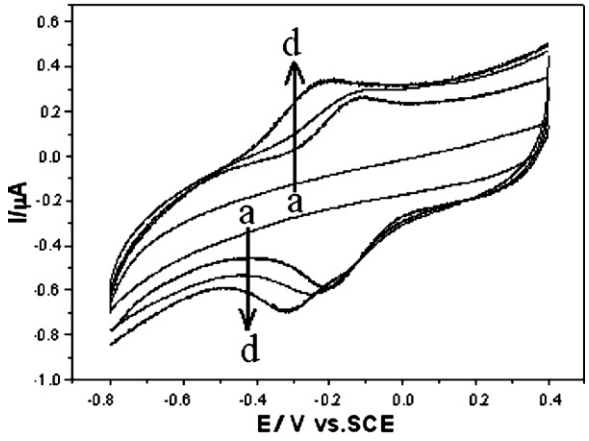

Fig. 1. Cyclic voltammograms of Nafion/GCE (a) in [Bmim][BF_4] containing 6.0% (v/v) H_2O and Nafion/HRP/GCE (b–d) in [Bmim][BF_4] (b), [Hmim][BF_4] (c) and [Emim][BF_4] (d) containing 6.0% (v/v) H_2O. The scan rate was 60 mV s^{-1}.

To prepare the Nafion/HRP/GCE, 8 μL HRP stock solution was first coated on the surface of the treated GCE. After drying, 10 μL Nafion was then coated on the resulting GCE, followed by drying under a beaker in a ventilative environment.

The method for fabricating Agarose/HRP/GCE was reported elsewhere [23].

When not in use, the aforementioned electrodes were stored at 4 °C.

2.3. Electrochemical measurements

Cyclic voltammetry was performed on a CHI 630 Electrochemical Analyzer (Shanghai Chenhua Co., China). The three-electrode system was composed of a working electrode (Nafion modified GCE (Nafion/GCE), HRP and Nafion modified GCE (Nafion/HRP/GCE) or HRP and agarose modified GCE (Agarose/HRP/GCE)), a platinum wire counter electrode and a saturated calomel electrode (SCE) reference electrode. Prior to measurements, electrolytes were deaerated with high purity nitrogen for 20 min. All the measurements were carried out at 28 ± 1 °C.

2.4. Spectroscopic characterization of the structure of HRP

HRP films on glass slides instead of GCE were prepared using the same procedure as for fabricating HRP electrodes. The glass slides with HRP films were separately immersed in the three ILs with or without H_2O. After 20 min, UV–vis absorption spectra (380–440 nm) were recorded on a UV–vis spectrophotometer (UV-2500, Shimadzu Co., Japan).

3. Results and discussion

3.1. Electrochemical behaviors of Nafion/HRP/GCE in ILs

Fig. 1 shows the cyclic voltammograms of Nafion/HRP/GCE in the three ILs containing 6.0% H_2O. Without HRP, i.e., at Nafion/GCE, no redox peaks appear; in the presence of HRP, i.e., at Nafion/HRP/GCE, however, there are two obvious redox peaks. These results indicate that the direct electron transfer between HRP and GCE happens in the three media. In contrast to aqueous medium, the redox signal of the HRP electrode was larger in the ILs, indicating that ILs facilitate the direct electron transfer of HRP. The reversibility of the electron transfer process, however, is different, depending upon the IL used and the scan rate. At lower scan rates, the process in the three media is quasi-reversible based on the peak potential separation and the

Table 1
Electrochemical parameters from CVs of Nafion/HRP/GCE in different ionic liquids containing 6.0% (v/v) H_2O (ν = 60 mV s^{-1})*.

Ionic liquids	I_{pa}[a] (μA)	I_{pc}[b] (μA)	E_{pa}[c] (mV)	E_{pc}[d] (mV)	ΔE_p[e] (mV)	$E^{0\prime}$[f] (mV)
[Emim][BF_4]	0.217	0.285	−254	−318	64.0	−286
[Bmim][BF_4]	0.192	0.234	−120	−192	72.0	−156
[Hmim][BF_4]	0.132	0.171	−130	−205	75.0	−168

* Each datum was an average of three replicate determinations.
[a] Anodic peak current.
[b] Cathodic peak current.
[c] Anodic peak potential.
[d] Cathodic peak potential.
[e] Peak potential separation ($\Delta E_p = E_{pa} - E_{pc}$).
[f] Formal potential ($E^{0\prime} = (E_{pa} + E_{pc})/2$).

peak current ratio. At the scan rate of 60 mV s^{-1}, [Hmim][BF_4] gives rise to the worst reversibility. Table 1 shows the corresponding anodic and cathodic peak currents (I_{pa}, I_{pc}), peak potentials (E_{pa}, E_{pc}), peak potential separation (ΔE_p) and formal potential ($E^{0\prime}$). It follows that I_p, ΔE_p and $E^{0\prime}$ are correlated with the nature of ILs used.

3.2. Effect of the water content in ILs on the electroactivity of HRP

Fig. 2 shows the water content dependence of the cathodic peak currents of HRP in different ILs. It can be seen that the dependence curve is very similar in the three ILs. For [Emim][BF_4], [Bmim][BF_4] and [Hmim][BF_4], the optimum water content ranges are 7.0–6.0%, 6.5–6.0% and 5.0–3.0%, respectively. These results indicate that the presence of a small amount of water in $[BF_4]^-$-type ILs as supporting electrolytes is vital to the electrochemical activity of HRP in Nafion film. The curves can be explained as follows. Without addition of water, cations passed into the film via ion exchange interact with the water on the surface of the proteins due to the hydrophilicity of ILs, leading to the loss of the structural water of HRP that is needed to maintain the catalytic activity of HRP (similar phenomenon was observed in some polar organic solvents). As a result, the electroactivity of HRP decreases. In the presence of small amounts of water, however, the aforementioned negative effect lessens [24]. With the gradual increase of the water content, HRP is satisfied with the structural water; moreover, it exhibits some structural flexibility due to moderate swelling of the film (they favor for the electron transfer), resulting in an increase in the current. Further addition of water, however, may not only increase the resistance of the film, but also turn the supporting electrolyte into a H_2O/IL binary mixture with conductivity and viscosity being quite different from the pure ILs (Table 2 presents the related physiochemical parameters of the pure ILs used) [25,26]. The decrease of the reduction peak current at higher water content should be a synergistic effect of these factors. The difference of optimum water content range in the three ILs can be explained as follows. The hydrophilicity of the cations deceases with the increase of alkyl chain length on imidazole ring [26]. At the same water content, the least hydrophilic [Hmim][BF_4] contains the most free water. As a result, the least water in [Hmim][BF_4] is needed to maintain the optimum activity of HRP. The fact that the optimum water content range in [Emim][BF_4] is close to that in [Bmim][BF_4] is due to their close hydrophilicity.

Table 2
The ionic conductivity and viscosity of the present three ionic liquids.

Ionic liquids	κ (mS cm^{-1}) [27]	η (mPa s) [28]
[Emim][BF_4]	15.7 (17.9)[a]	36.9 (26.0)[b]
[Bmim][BF_4]	4.38 (5.33)[a]	99.2 (61.3)[b]
[Hmim][BF_4]	1.79 (2.40)[a]	191 (110)[b]

[a] Data obtained at 298 K, data in parentheses at 303 K;
[b] Data obtained at 298 K, data in parentheses at 308 K.

3.3. Effect of the structures of the cations in ILs on heterogeneous electron transfer kinetics

The apparent heterogeneous electron transfer rate constant (k_s) is an important kinetics parameter of the electron transfer process. To obtain the parameter, we recorded the cyclic voltammograms of Nafion/HRP/GCE in different ILs at different scan rates. In [Bmim][BF_4] containing 6.0% H_2O (see Fig. 3), with the increase of the scan rate, both the anodic and the cathodic peak currents increase linearly and at the same time, the E_{pa} and the E_{pc} values shifted positively and negatively, respectively, but the $E^{0\prime}$ remained

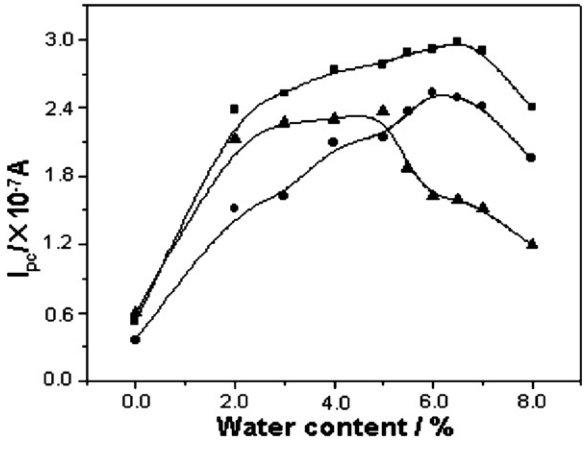

Fig. 2. Plots of cathodic peak current of Nafion/HRP/GCE versus water content (v/v) in [Emim][BF_4] (■), [Bmim][BF_4] (●) and [Hmim][BF_4] (▲), respectively. Scan rate was fixed at 60 mV s^{-1}. Each datum was an average of three replicate determinations.

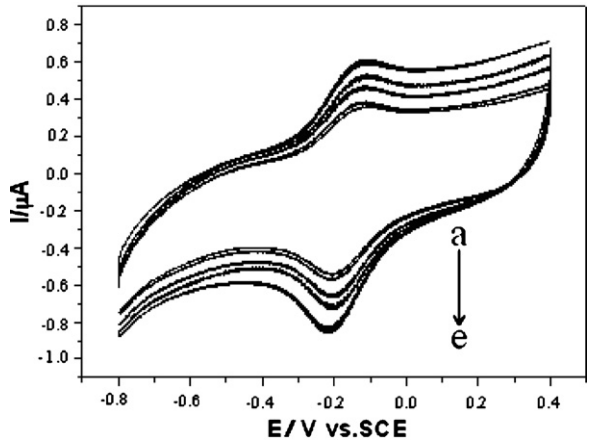

Fig. 3. Cyclic voltammograms of Nafion/HRP/GCE in [Bmim][BF_4] containing 6.0% (v/v) H_2O. The scan rates (from a to e) were 60, 70, 80, 90, 100 mV s^{-1}.

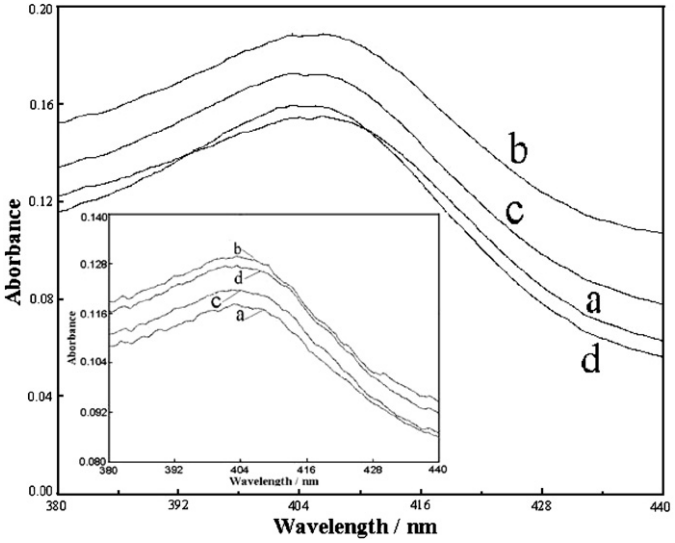

Fig. 4. UV–vis spectra of (a) HRP film, (b) [Emim][BF_4]/HRP film, (c) [Bmim][BF_4]/HRP film, and (d) [Hmim][BF_4]/HRP film. Inset: UV–vis spectra of (a) HRP film recorded after 20 min immersion in (b) [Emim][BF_4], (c) [Bmim][BF_4], and (d) [Hmim][BF_4] containing 6.0% (v/v) water.

almost unchanged. Similar changes in other two ILs were also observed. These results indicate that the direct electron transfer between HRP and GCE is a surface-confined thin-layer electrochemical process. According to Lavion's methods, we obtained a k_s value of 0.70 s^{-1}, 0.55 s^{-1} and 0.43 s^{-1} (each datum was an average of three replicate determinations) for water-containing (6.0%) [Emim][BF_4], [Bmim][BF_4] and [Hmim][BF_4], respectively. It can be seen that the k_s values of HRP on GCE are not large; moreover, they decrease with the increase of the carbon chain length on imidazolium cation in $[BF_4]^-$-type ILs. The orientation of HRP on the surface of GCE is random, so the change of k_s with the alkyl chain length can be explained as follows. The cations of ILs can pass into the film via ion exchange with Nafion. The entered cations may interact with HRP, altering the microenvironment of the heme or ferriporphyrin. The nitrogen on dialkyl imidazolium cation lacks a lone pair electron, so the cations have no possibility to affect the microenvironment via their coordination with Fe(III)/Fe(II) [19]. The cations may change the peripheral non-heme structure of HRP via electrostatic interaction, but evidences from UV–vis spectra shows that their influence seems to be same. It is seen from Fig. 4 that in the presence of a pure IL, the Soret band of HRP in the three cases blue-shifted ca. 4 nm. After immersion of the HRP film in the three ILs containing 6.0% H_2O for 20 min, the Soret band of HRP still occurs at the same position (see the inset of Fig. 4, the difference of peak height is due to the difference of the amount of HRP in the irradiated area), indicating that these H_2O-containing ILs had little effect on the microenvironment of the heme. It follows that the difference of k_s is mainly determined by the electron transfer resistance of the enzyme film. The increase of the resistance caused by the increase of both alkyl chain length on imidazolium cations passing into the film and free water in the film, slows down the electron transfer process.

3.4. Electrocatalytic reduction of H_2O_2 in different ILs

Fig. 5A–C show the cyclic voltammograms of the enzyme electrode in different water-containing ILs (6.0%) with or without H_2O_2. With the increase of the concentration of H_2O_2, the reduction peak current increases, while the oxidation peak current decreases. All these are characteristics of typical electrocatalytic reduction; i.e., this modified electrode can electrocatalytically reduce H_2O_2 in all

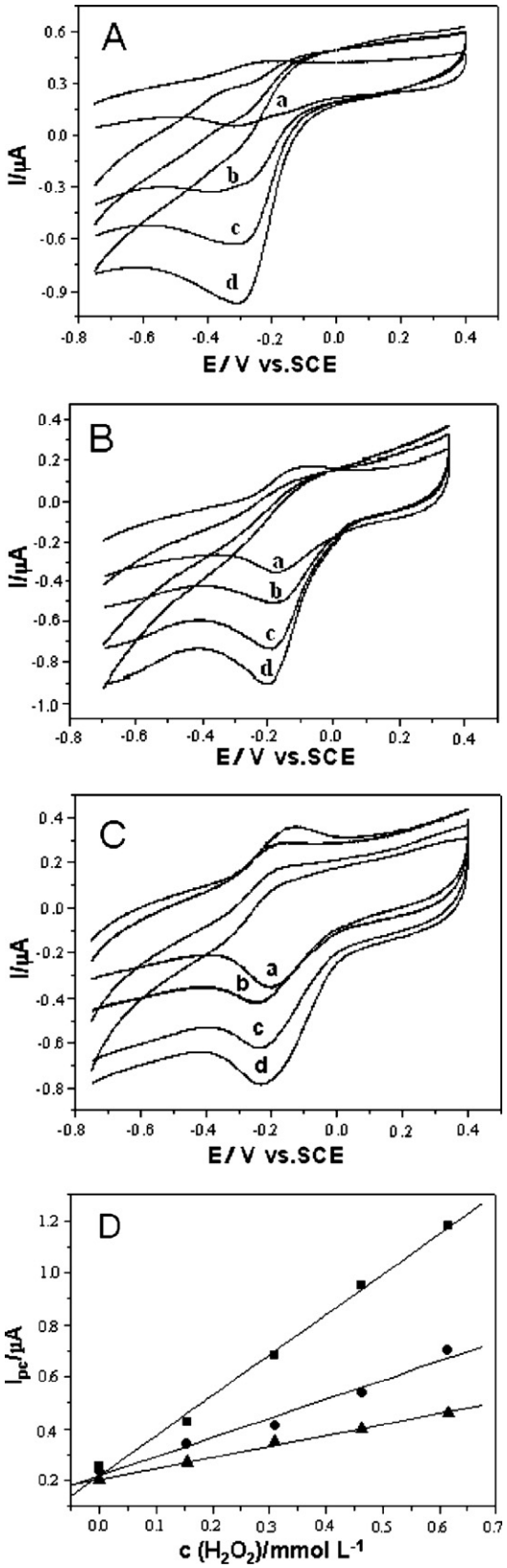

Fig. 5. Cyclic voltammograms of Nafion/HRP/GCE in [Emim][BF_4] (A), [Bmim][BF_4] (B) and [Hmim][BF_4] (C) containing 0 mmol L^{-1} (a), 0.156 mmol L^{-1} (b), 0.311 mmol L^{-1} (c), and 0.466 mmol L^{-1} (d) H_2O_2. The total water content was fixed at 6.0%. (D) The H_2O_2 concentration dependence of the catalytic reduction current at Nafion/HRP/GCE in H_2O-containing [Emim][BF_4] (■), [Bmim][BF_4] (●) and [Hmim][BF_4] (▲). Each datum in Fig. 5D was an average of three replicate determinations.

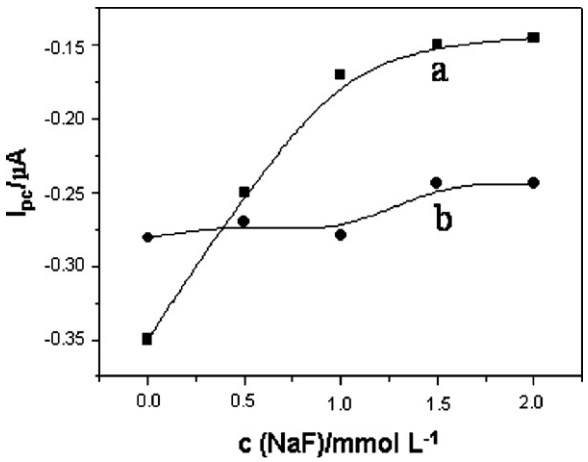

Fig. 6. Plot of the cathodic peak currents of Agarose/HRP/GCE (■) and Nafion/HRP/GCE (●) versus the concentrations of NaF in [Bmim][BF_4] containing 6.0% (v/v) H_2O. Scan rate: 60 mV s^{-1}. Each datum was an average of three replicate determinations.

the three ILs. At the same concentration of H_2O_2, the electrocatalytic reduction peak current decreases with the increase of alkyl chain length on imidazole ring (see Fig. 5D). As the same enzyme electrode was used for testing and the CVs of the electrode are quasi-reversible (though k_s is not large), the kinetics of the enzymatic catalysis is the limiting factor of the present catalytic current. For an immobilized enzyme, the mass transfer of substrates is usually the limiting factor of the biocatalysis, especially in highly viscous media [7,29]. In the present system, stirring the electrolytes did increase the catalytic current. So the remarked change of the catalytic current in different electrolytes can be explained as follows. With the increase of the alkyl chain length on imidazole ring, the viscosity of ILs increases greatly (see Table 2), and therefore the mass transfer of H_2O_2 via diffusion in ILs deceases largely, resulting in a great drop in the signal.

3.5. Stability, reproducibility and anti-interference of F^- of Nafion/HRP/GCE

In this section, we aimed at the potential application of the enzyme electrode. Based on the results in the preceding sections, it was found that among the three ionic liquids, [Bmim][BF_4] is the best one for analytical application due to its less negative reduction potential of H_2O_2 and better reversibility of the electron transfer process. Therefore, only in [Bmim][BF_4] were the stability, reproducibility and anti-interference of F^- of the enzyme electrode investigated.

Nafion/HRP/GCE was relatively stable in H_2O-containing [Bmim][BF_4]; after ten CV scan cycles, a small change in the cathodic peak current was observed with the relative standard deviation (RSD) being 1.0%. During storage, the cathodic peak current was measured every three days and the RSD of five sequential determinations was 4.2%; three weeks later, the electrode retained about 84.8% of the initial cathodic peak current. The reproducibility was also good; an RSD value of 5.4% in the cathodic peak currents was obtained for four modified electrodes prepared in the same way.

HRP is a heme-containing protein. Its active center (ferriporphyrin) is easily influenced by F^-. It is known that $[BF_4]^-$-type ILs can dissociate F^- under certain conditions [30], which will affect the bioactivity of HRP [31]. It has been reported that Nafion modified electrodes can assemble cations and reject anions in electrolytes [32]. So Nafion film not only facilitates the present study but also reduces the potential interference of F^-. As shown in Fig. 6, the cathodic peak currents of HRP in Nafion film scarcely decreases with the increase of NaF concentrations over a range of 0–2.0 mmol L^{-1} NaF. By contrast, the cathodic peak currents of HRP in agarose film decreases greatly with the increase of NaF concentration and a 50% reduction in the current is obtained at 1.0 mmol L^{-1} NaF. It follows that the Nafion film does resist F^- and favors the research of HRP thin-layer electrochemistry and detection in ILs containing F^-.

4. Conclusions

The prepared Nafion/HRP/GCE has good electroactivity and bioactivity in H_2O-containing $[BF_4]^-$-type ILs. The direct electron transfer between HRP and GCE is a surface-confined single electron transfer process. The presence of a small amount of water in the present three ILs is crucial for the expression of the electroactivity of HRP in Nafion film. The optimum water content range in each IL is different and decreases with the increase of alkyl chain length on imidazole ring, which is due to the difference of the hydrophilicity of ILs. In contrast to aqueous medium, ILs media facilitate the direct electron transfer of HRP, and the electrochemical parameters obtained in different ILs are obviously related to the nature of ILs. The apparent heterogeneous electron transfer rate constant decreases gradually with the increase of alkyl chain length on imidazole ring, but the changing extent is relatively small, which is primarily determined by the internal resistance of the modified film. The electrocatalytic reduction current of H_2O_2 at the present electrode decreases obviously with the increase of alkyl chain length, and the mass transfer of H_2O_2 via diffusion in ILs should be responsible for the change. In addition, the modified electrode has good stability and reproducibility; the ability to tolerate high levels of F^- has been greatly enhanced due to the use of Nafion film.

Acknowledgements

The authors gratefully acknowledge the financial support from State Key Laboratory of Microbial Technology of China, the Provincial Natural Science Foundation of Shandong (Y2008B13), the National Natural Science Foundation of China (20973103) and the National Basic Research Program of China (2011CB707400).

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