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Direct electrochemistry of horseradish peroxidase immobilized in a chitosan–[C_4mim][BF_4] film: Determination of electrode kinetic parameters

Jenny S. Long [a], Debbie S. Silvester [a], Gregory G. Wildgoose [a], Annette-E. Surkus [b], Gerd-Uwe Flechsig [b], Richard G. Compton [a,*]

[a] Department of Chemistry, Physical and Theoretical Chemistry Laboratory, Oxford University, South Parks Road, Oxford OX1 3QZ, United Kingdom
[b] Universität Rostock, Institut für Chemie, Abt. für Analytische, Technische und Umweltchemie, Dr.-Lorenz-Weg 1, 18051 Rostock, Germany

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ABSTRACT

The direct electrochemistry of a HRP–chi–[C_4mim][BF_4] film (where HRP = horseradish peroxidase, chi = chitosan, and [C_4mim][BF_4] = the room temperature ionic liquid (RTIL) 1-butyl-3-methylimidazolium tetrafluoroborate) has been studied by cyclic voltammetry on a glassy carbon electrode. The mechanism for the electrochemical reaction of HRP is suggested to be EC for the reduction, and CE for the following re-oxidation, as the oxidative peak potential remained approximately unchanged across the scan rate range. The half wave potential of HRP reduction was found to be pH dependent, suggesting that a concomitant proton and electron transfer is occurring. Using theoretical simulations of the experimentally obtained peak positions, the standard electron transfer rate constant, k_0, was found to be 98 (±16) s^{-1} at 295 K in pH 7 phosphate buffer solution, which is very close to the value reported in the absence of ionic liquid. This suggests that the ionic liquid used here in the HRP–chi–[C_4mim][BF_4]/GC electrode does not enhance the rate of electron transfer. k_0 was found to increase systematically with increasing temperature and followed a linear Arrhenius relation, giving an activation energy of 14.20 kJ mol^{-1}. The electrode kinetics and activation energies obtained are identical to those reported for HRP films in aqueous media. This leads us to question if the use of RTIL films provide any unique benefits for enzyme/protein voltammetry. Rather the films may likely contain aqueous zones in which the enzymes are located and undergo electron transfer.

1. Introduction

The study of the direct electrochemistry of metalloenzymes is important not only for the understanding of electron kinetics in biological systems, but also for the development of third generation biosensors [1,2]. Third generation biosensors are based on direct electron transfer [2] and, unlike first and second generation biosensors, have the advantage of functioning without a mediator, allowing a simplified reaction system and superior selectivity [2]. Observing the direct electron transfer to metal centers of enzymes is often difficult as the redox centers may be buried deep within the enzyme structure. One commonly used approach to overcome this problem is to immobilize the enzyme onto an electrode surface using a variety of films or binders which promote electron transfer [3]. Horseradish peroxidase (HRP) is one of the most commonly studied enzymes in the fabrication of electrochemical biosensors. HRP has been immobilized on many electrode substrates using a myriad of different films including hydrogels [4,5], DNA networks [6], Nafion films [7,8] and carbon nanotube composites [9,10]. Recently chitosan films have also been used to successfully immobilize enzymes and promote electron transfer [11].

Chitosan (Chi), β-1,4-poly-D-glucosamine (see Fig. 1), is a linear polysaccharide made by the deacetylation of chitin and is made up of linked glucosamine units together with a percentage of *N*-acetyl-glucosamine units [12]. It demonstrates an excellent ability to form films, is naturally occurring, non-toxic and biocompatible with many enzymes, providing a favourable microenvironment for immobilization of HRP [11]. In addition to the development of new polymer films to immobilize enzymes, many researchers are investigating the use of room temperature ionic liquids in composites.

Room temperature ionic liquids (RTILs) are salts composed entirely of ions that exist in the liquid state at and around 298 K. They are made up of a bulky asymmetric organic cation and a weakly coordinating organic or inorganic anion. As ionic liquids are entirely composed of ions, they have a very high intrinsic conductivity making them favourable solvents in electrochemistry [13,14], with no need for additional supporting electrolyte. Their wide electrochemical window, near-zero volatility and high thermal stability also make them durable and robust solvents whilst their significantly desirable properties have been exploited in many fields including gas sensing [15], organic synthesis [16] and catalysis [17]. Recently RTILs have become popular in the field of biosensing and biocatalysis [18]. RTILs have been shown to be favourable media for biocatalysis, with many enzymes showing greater stability, activity and higher selectivity in RTILs [19]. The thermal stability of HRP

* Corresponding author. Tel.: +44 1865 275 413; fax: +44 1865 275 410.
E-mail address: richard.compton@chem.ox.ac.uk (R.G. Compton).

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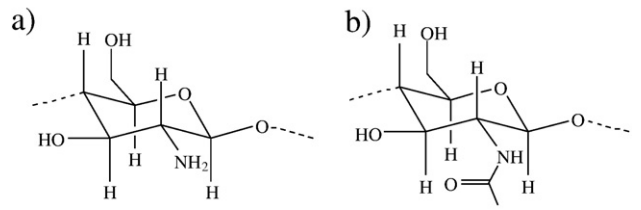

Fig. 1. Components of chitosan: a) Glucosamine structural unit and b) *N*-acetylglucosamine structural unit.

is reported to be increased in aqueous mixtures of 1-butyl-3-methylimidazolium tetrafluoroborate [C_4mim][BF_4] relative to aqueous buffer solutions [20], whilst Lu et al. [21] have developed a new composite material based on [C_4mim][BF_4] and chitosan for the immobilization of HRP. They demonstrated that the enzyme retained its native structure and activity within the film. [C_4mim][BF_4] and chitosan form a homogeneous solution which, when dried in air, forms a stable film on the electrode surface. Lu et al. [21] proposed that the ionic liquid, due to its inherent conductivity, facilitates electron transfer from the HRP heme group (Fe(III) at its resting state) to the electrode surface, enhancing the voltammetric signal.

In this work, the electrochemical behaviour of a glassy carbon electrode modified with a horseradish peroxidase–chitosan–[C_4mim][BF_4] film (HRP–chi–[C_4mim][BF_4]/GC electrode) is studied at different electrolyte pHs (pH 6–8) and over a range of temperatures (295–323 K). The cyclic voltammetric response over a range of different scan rates at each pH and temperature is recorded and analysed to extract the kinetic parameters of the HRP–chi–[C_4mim][BF_4]/GC electrode. The electrode kinetics and activation energies obtained are identical to those reported for HRP films in aqueous media. This in turn leads us to question whether the use of RTIL films provides any unique benefits for enzyme or protein voltammetry.

2. Experimental

2.1. Chemical reagents

Horseradish peroxidase (EC 1.11.1.7, Type VI, 255 U g^{-1}) and chitosan (from crab shells, minimum 85% deacetylated) were purchased from Sigma. 1-Butyl-3-methylimidazolium tetrafluoroborate ([C_4mim][BF_4], high purity, halide content <100 ppm) was kindly donated by Merck KGaA and was used as received. Sodium phosphate monobasic dihydrate (puriss), sodium phosphate dibasic (anhydrous, 99%) and acetate buffer solution were all purchased from Aldrich and used without further purification. All other reagents were of analytical reagent grade and used without further purification. 0.05 M phosphate buffer solutions at pH 6–8 were prepared from appropriate solutions of sodium phosphate monobasic and sodium phosphate diabasic. The pH of the buffer was adjusted using hydrochloric acid or sodium hydroxide solutions as necessary. All aqueous solutions were made using ultra-high quality grade water from a Millipore (Vivendi, UK) UHQ grade water system with a resistivity of not less than 18.2 MΩ cm at 298 K.

2.2. Instrumental

Electrochemical experiments were performed using a three-electrode system comprising of a film-modified glassy carbon electrode (BAS technicol, USA) as the working electrode, a platinum coil as the counter electrode and a saturated calomel electrode (SCE, Radiometer, Copenhagen, Denmark) as the reference electrode. Prior to use, the glassy carbon electrode (GCE, 3 mm diameter) was successively polished using a 1.0 μm to 0.10 μm diamond spray (Kemet, UK). After each polishing step the electrode was sonicated and rinsed with water and ethanol respectively. The effective area of the GCE was estimated to be *ca.* 0.0475 cm^2 using a Randles–Sevčik analysis of the variation of the peak current with the square root of scan rate in 1.0 mM $[Fe(CN)_6]^{3-}$/$[Fe(CN)]_6]^{4-}$. Phosphate buffer solution (0.05 M pH 7) was used as the electrolyte unless stated otherwise, and all buffer solutions were purged with nitrogen for at least 30 min prior to the recording of voltammograms, and a nitrogen atmosphere was maintained during the course of the experiment. For experiments where the electrolyte was studied at increased temperatures, the cell was placed in an oil bath, and a thermostated, heated stirrer plate was used to maintain a constant temperature.

2.3. Preparation of HRP–chi–[C_4mim][BF_4] modified film electrode

Aqueous chitosan solution (pH 5, 6 mg mL^{-1}) was prepared following a literature procedure [22]. The chitosan flakes were dissolved in hot (353–363 K) 0.05 M hydrochloric acid and left to cool to room temperature. After cooling, the pH of the solution was carefully adjusted to pH 5 using sodium hydroxide solution. The chitosan solution was then filtered through a cellulose film of pore size 0.465 μm and stored at (269 K) when not in use. 6 mg mL^{-1} HRP solution was prepared by dissolving the lypholized powder in pH 7 phosphate buffer solution.

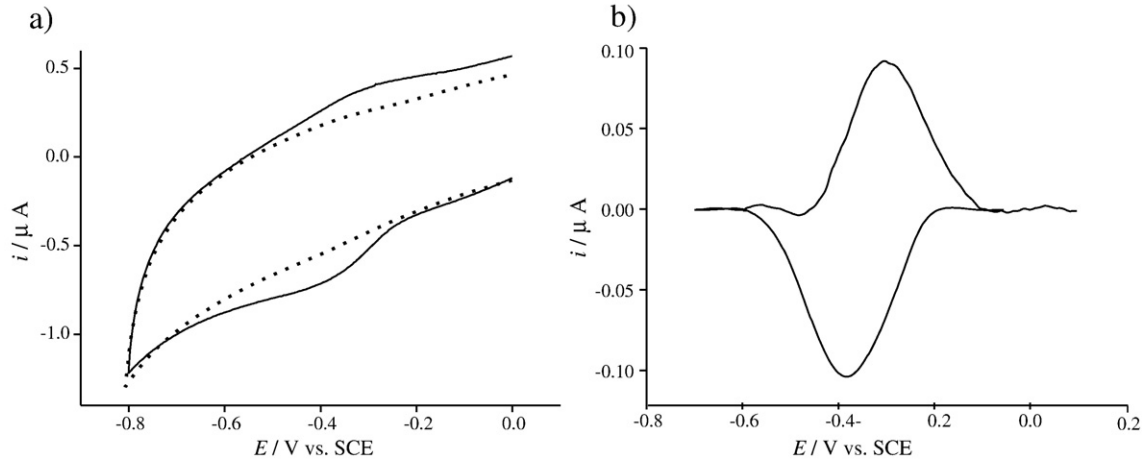

Fig. 2. a) Cyclic voltammetry (CV) of HRP–chi–[C_4mim][BF_4]/GC electrode (solid line) and HRP–chi/GC electrode (dotted line) in 0.05M pH 7 phosphate buffer solution, scan rate 0.2 V s^{-1} at 295 K. b) Baseline correct voltammogram for HRP–chi–[C_4mim][BF_4]/GC electrode from the CV presented in a).

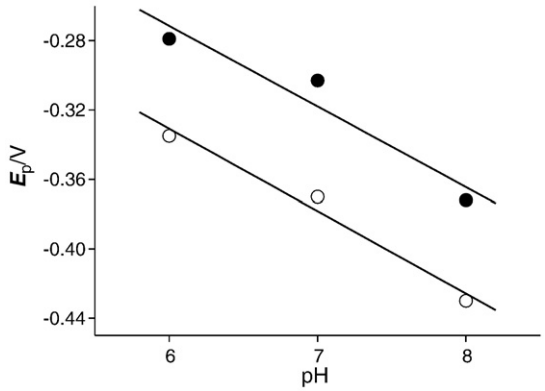

Fig. 3. Peak potentials, $E_{p,red}$ (○) and $E_{p,ox}$ (●), as a function of pH for HRP–chi–[C_4mim][BF_4]/GC electrode.

The film solution was made up from the stock solutions and contained 3 mg mL^{-1} HRP, 1.5 mg mL^{-1} chitosan, and 5% (v/v) [C_4mim][BF_4] in 0.05 M pH 5 acetate buffer solution. The HRP–chi–[C_4mim][BF_4] modified electrode was made by casting a 3 μL aliquot of this film solution onto the surface of the polished GC electrode. The electrode was then covered with a small beaker to allow the water to evaporate slowly in air to form a uniform film. The HRP–chi/GC electrode was prepared in the same way without the ionic liquid. The dry electrodes were stored at 269 K when not in use, and the electrodes were soaked in pH 7 phosphate buffer solution for 30 min before electrochemical measurements were recorded, to ensure that any physically adsorbed HRP or [C_4mim][BF_4] was removed.

3. Results and discussion

3.1. Voltammetric behaviour of HRP–chi–[C_4mim][BF_4]/GC electrode

Typical cyclic voltammetry of a HRP–chi–[C_4mim][BF_4]/GC electrode in pH 7 phosphate buffer solution is shown in Fig. 2a. The voltammetry gives rise to a pair peaks which are attributed to the direct electron transfer between the HRP (Fe^{3+}/Fe^{2+}) and the electrode. Fig. 2b shows the baseline corrected voltammetry, obtained by fitting the baseline of the cyclic voltammogram using a 6th order polynomial and then correcting the data to this baseline. From the baseline corrected voltammetry, the reductive peak potential, $E_{p,red}$ was found to be −0.37 V, with a corresponding oxidative peak, $E_{p,ox}$, at −0.31 V (0.2 V s^{-1}). The formal potential E_f^o (estimated as $(E_{p,red}+E_{p,ox})/2$) for HRP (Fe^{3+}/Fe^{2+}) was −0.34 V and the peak separation was 60 mV, which was in agreement with the literature [11,21,23]. The peak currents for the forward and reverse peaks are almost equal, indicating a quasi-reversible electrochemical process. The enzyme electrode (dotted line, Fig. 2a) without ionic liquid (HRP–chi/GC) did not exhibit any peaks [24].

Using Faraday's laws, calculating the charge from the area under the peaks, the surface coverage of HRP is estimated to be 2.01×10^{-11} mol cm^{-2} (assuming a one electron transfer, and electrode area of 0.0475 cm^2). Therefore, the total amount of HRP deposited on the electrode surface is *ca.* 2.05×10^{-10} mol (molecular weight of HRP is *ca.* 44,000) [2], giving the percentage of electroactive HRP on the electrode surface as *ca.* 0.47%, which is higher than that reported in the absence of ionic liquid (0.2%) [11]. This suggests that only the enzymes closest to the electrode and with the correct orientation are involved in electron transfer.

3.2. Influence of pH

The cyclic voltammetry of the HRP–chi–[C_4mim][BF_4] shows a strong dependence on the pH of the solvent (phosphate buffer solution), indicating that concomitant proton and electron transfer is occurring. Both the reductive and oxidative peak potentials for the HRP(Fe^{3+}/Fe^{2+}) redox couple shift to more negative potentials with increasing pH (Fig. 3).

The peak potentials were plotted against the pH and the gradients of the lines for the change in the potential of the reductive and oxidative peaks with pH are −47.5 mV pH^{-1} and −46.5 mV pH^{-1} respectively. These are close to the theoretical value for the concomitant transfer of n electrons coupled with n protons, here $n=1$ (−57.6 mV pH^{-1}) [25]. Yamazaki et al. [26] have proposed that a heme-linked ionizing group in the HRP is responsible for the pH dependence, and the pK_a of the reduced enzyme (Fe^{2+}) is *ca.* 7 (there are seven isozymes for HRP and the pK_a reported is for the two most abundant forms B and C) [27]. The overall electrochemical reaction can be expressed as:

$$\mathrm{HRP}(\mathrm{Fe}^{3+}) + e^- + \mathrm{H}^+ \overset{k_1}{\rightleftharpoons} \mathrm{HRP}(\mathrm{Fe}^{2+}). \quad (1)$$

3.3. Influence of the potential scan rate

Cyclic voltammetry was obtained for the HRP–chi–[C_4mim][BF_4]/GC electrode in phosphate buffer solution over scan rates of 0.1 to 6.0 V s^{-1}, and is shown in Fig. 4. As the scan rate is increased, the cathodic and anodic peak currents increase simultaneously, whilst the reductive peak moves to more negative potentials.

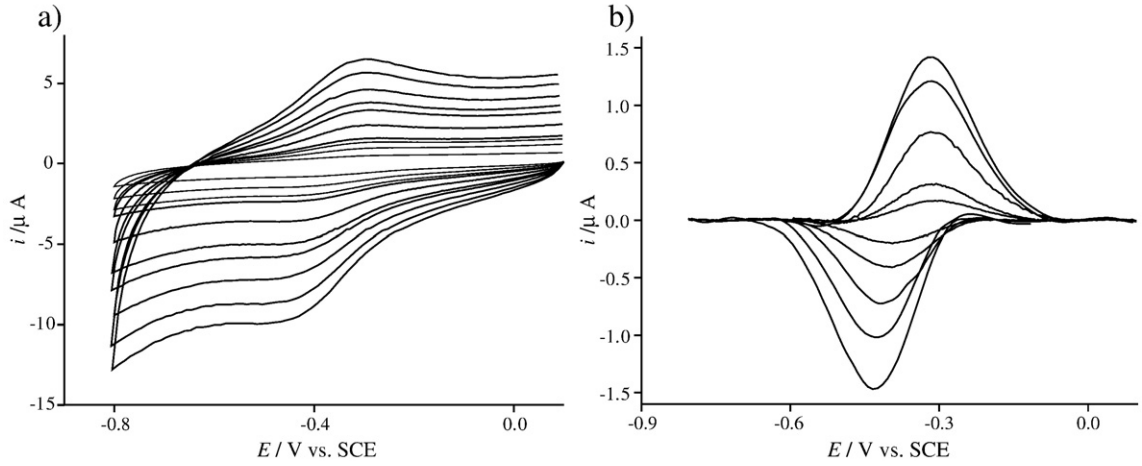

Fig. 4. a) Cyclic voltammetry of HRP–chi–[C_4mim][BF_4]/GC electrode in pH 7 phosphate buffer solution at scan rates of 0.2, 0.5, 0.8, 1.0, 1.5, 3.0, 4.0, 5.0 and 6 V s^{-1} at 295 K. b) Baseline corrected voltammetry for scan rates 0.5, 1.0, 2.5, 4.0 and 5.0 V s^{-1}.

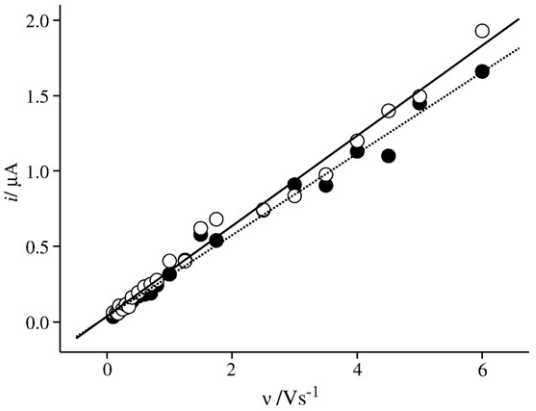

Fig. 5. Reductive (○) and oxidative (●) peak currents are presented as a function of scan rate for the HRP–chi–[C_4mim][BF_4]/GC electrode in pH 7 phosphate buffer solution. The linear fits for the reductive (solid line) and oxidative (dotted line) peak current trend are also presented.

Both the cathodic and anodic peak currents increase linearly with scan rate (Fig. 5) which is indicative of a surface bound species [25]. The equations for the reductive peak current ($i_{p,red}$) and oxidative peak current ($i_{p,ox}$) from this plot are $i_{p,red}=0.299v+0.0374$ and $i_{p,ox}=0.270v+0.0336$ respectively (i_p: μA, v: V s^{-1}, with least-squares correlation coefficient, $R>0.994$). The surface coverage did not change significantly with scan rate, indicating that the HRP is immobilized on the surface of the electrode and not diffusing through the film. The data was baseline corrected and the peak potentials, $E_{p,red}$ and $E_{p,ox}$, were plotted against a logarithmic scan rate scale to construct a so-called 'trumpet plot' [1]. Interestingly, the $E_{p,red}$ is seen to move to more reducing potentials but $E_{p,ox}$ varies significantly less over this range of scan rates [28]. This suggests that the electrochemical reaction given in Eq. (1) can be written specifically, using Testa and Reinmuth notation, as:

$$\mathrm{HRP(Fe^{+3})} + e^- \overset{k_0}{\rightleftharpoons} \mathrm{HRP(Fe^{+2})} \quad \text{Electron transfer step } (E) \tag{2}$$

$$\mathrm{HRP(Fe^{+2})} + \mathrm{H^+} \overset{k_{max}}{\rightleftharpoons} \left[\mathrm{HRP(Fe^{2+})\text{–}H^+}\right] \quad \text{Chemical step } (C). \tag{3}$$

In effect, the reduction of the HRP is EC whilst the re-oxidation is CE. The oxidation of Fe^{2+} is hindered by a preceding chemical step.

Using a simulation program developed by the Armstrong group [29,30], in which a Butler–Volmer model is used and the electron transfer can be approximated by Eq. (4), we were able to fit the 'trumpet plots' and obtain rate constants for the electron transfer (k_0) [30]:

$$\frac{1}{k_1} = \frac{1}{k_{BV}} + \frac{1}{k_{max}} \tag{4}$$

where k_{BV} is the rate constant derived from the Butler–Volmer equation and k_{max} represents the rate of the reaction preceding the electron transfer. At low over-potential k_{BV} determines the rate whilst conversely at high over-potential k_{max} determines the maximum rate.

The simulation calculates cyclic voltammograms for different scan rates using a finite difference procedure [31]. The parameters input into the simulation were: k_0, k_{max}, peak separation at low scan rate, E_f^o at 1.0 V s^{-1}, the temperature (fixed) and $n_{apparent}$ (the number of electrons transferred) which was equal to 1. The simulation included corrections to account for the non-idealities seen in the cyclic voltammetry, including a small shift in E_f^o with increasing scan rate, a constant peak separation at low scan rate, and half height peak widths which are broader than theoretically expected. Whilst the curvature of the 'trumpet plots' (Fig. 6) are greatly affected by k_0, as detailed in the work of Jeuken et al. [30], k_{max} influences the peak positions only to a small extent over this scan rate range and the fittings of different k_{max} in the range 10^3–10^9 did not affect the overall fitting (although simulations with $k_{max}<1000$ fit poorly). Peak positions were, however, very sensitive to changes in k_0. The value obtained for k_0 at pH 7 at 295 K was 98 (±16) s^{-1}, which is very similar to the reported value with no ionic liquid [24] in the chitosan film, $k_0=104$ (±34) s^{-1} [11].

Further cyclic voltammetry was obtained by varying the electrolyte pH. Voltammetry was obtained at scan rates between 0.1 and 6.0 V s^{-1} and modeled to find k_0. There was no systematic variation of k_0 with pH. k_0 at pH 8 was slightly larger than at pH 6 and pH 7 (see Table 1). It is suggested that, as the pK_a for the heme-linked ionization is *ca.* 7 [26], this increase may be due to the fact that there is little proton transfer to the enzyme at pH 8.

Table 1
Measured k_0 values at different pH and temperature, obtained by fitting peak potentials as a function of scan rate, described in Section 3.3

Phosphate buffer pH	Temperature of electrolyte/K	k_0/s^{-1}	E_f^o/V
6	295	118±25	−0.33
7	295	98±16	−0.36
8	295	178±22	−0.41
7	303	107.3±18	−0.37
7	313	128±12	−0.38
7	323	161±19	−0.38

Formal potential used in the simulation is also given.

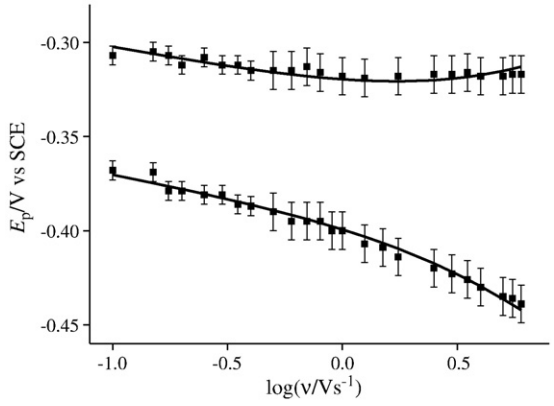

Fig. 6. Experimental (■) and simulated peak positions (line) are presented as a function of scan rate for HRP–chi–[C_4mim][BF_4]/GC electrode in pH 7 phosphate buffer solution at 295 K.

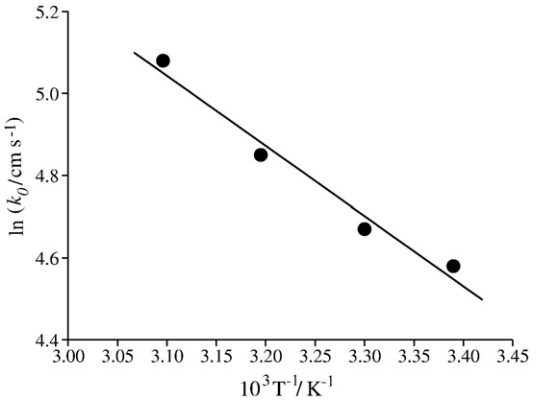

Fig. 7. Natural log of the standard rate constant is presented against the inverse of temperature for HRP–chi–[C_4mim][BF_4]/GC electrode in pH 7 phosphate buffer solution. Rate constants were obtained from the theoretical fit of the experimental peak positions.

3.4. Influence of temperature

The temperature dependence of the kinetics was also investigated by varying the temperature of the electrolyte. Cyclic voltammetry at a range of scan rates from 0.1 to 6.0 V s^{-1} was obtained at four temperatures (295, 303, 313 and 323 K) for the HRP–chi–[C_4mim][BF_4]/GC electrode by submerging the cell in an oil bath and maintaining a constant temperature with a heated magnetic stirrer plate. The k_0 values were obtained from the data by fitting the 'trumpet plots' (described in Section 3.3), and are displayed in Table 1. As expected, the k_0 value increased systematically with temperature and the activation energy, E_a, for the electron transfer was calculated using the Arrhenius equation:

$$k_0 = A \exp\left(\frac{-E_a}{RT}\right). \quad (5)$$

Fig. 7 shows a plot of ln k_0 vs $1/T$ for the HRP–chi–[C_4mim][BF_4]/GC electrode. A straight line was observed (least-squares correlation coefficient = 0.987), with a gradient of −1708 K, corresponding to an activation energy of 14.20 kJ mol^{-1}, which is comparable with the activation energy calculated by the increase in catalytic activity for the reduction of hydrogen peroxide, H_2O_2, with temperature (14.84 kJ mol^{-1}) [4], both diffusion controlled processes; the former being the diffusion of the proton and the latter the diffusion of H_2O_2 to the enzyme.

4. Conclusions

The direct electrochemistry of HRP has been studied by cyclic voltammetry on a HRP–chi–[C_4mim][BF_4] film-modified GC electrode. The reduction is believed to proceed *via* an EC mechanism containing a proton transfer step, as deduced by strong dependence of the cyclic voltammetry on the pH of the solvent. The reduction and oxidation peaks (corresponding to the Fe^{3+}/Fe^{2+} redox centre) were baseline corrected to a 6th order polynomial, and theoretically modelled using a 'trumpet plot' [1]. The standard electron transfer rate constant, k_0 was found to be 98 (±16) s^{-1}, close to that in the absence of ionic liquid (104 ± 16 s^{-1}) [11], suggesting that the use of RTILs in the enzyme film may in fact provide no unique benefits in enzyme/protein voltammetry (with the exception of enhanced voltammetric signals). In addition, an Arrhenius plot of k_0 with T gives an activation energy for HRP reduction that is almost identical to that reported for HRP films in aqueous media.

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