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Electrocatalytic reduction of oxygen by bilirubin oxidase in hydrophobic ionic liquids containing a small quantity of water

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ABSTRACT

We have examined a direct electron transfer reaction of bilirubin oxidase (BOD) at a gold nanoparticle (AuNP) electrode in the mixtures of ionic liquid (IL) and water. No catalytic current induced by BOD was observed when neat ILs were used as an electrolyte solution. However, BOD-induced catalytic currents were observed when small quantities of water were added to hydrophobic ILs. The BOD-modified AuNP electrode provided a current density as high as 218 $\mu A\,cm^{-2}$ in 1-(2-hydroxyethyl)-3-methylimidazolium bis(trifluoromethane-sulfonyl)imide ([C2OHmim][Tf_2N]) containing 7.4 wt% H_2O. To investigate the effect of the added water to ILs on the enzymatic activity we compared the physicochemical properties of the IL/H_2O mixtures. There was a certain relation between the water activity (a_W) of the IL/H_2O mixtures and the current density of BOD. The BOD was confirmed to catalyze the four-electron reduction of O_2 to H_2O in the [C2OHmim][Tf_2N]/H_2O mixture by the data with cyclic voltammetry measurements using a rotating ring-disk electrode.

1. Introduction

There is a consensus that ionic liquids (ILs) have superior quality as solvents and totally different properties from those of molecular liquids [1,2]. In many cases, ILs usually exhibit negligible vapor pressure and high thermal, chemical, and electrochemical stability along with widely tunable properties, such as polarity, hydrophobicity, and solvent miscibility. Therefore, ILs attract attention as a superior sustainable solvent with respect to such advanced points as safety, separation, refinement, and reuse. Furthermore, application of ILs as reaction media for biomolecules has also been explored [3–5]. Use of enzymes in ILs was particularly anticipated because enzymes are the ultimate green catalysts. We anticipate improvements in the reactivity of enzymes in these ILs to be facilitated by the dissolution of previously insoluble substrates and the reduction of the amount of solvent for the separation or extraction of products. Improvements in the product selectivity could also be expected when ILs were adopted for enzymatic reactions.

However, it is very difficult to treat proteins keeping their activity in ILs. Most proteins exhibit very low solubility in general ILs, and their enzymatic activities are often lost even when they are successfully dissolved in the ILs. Homogeneous dissolution of proteins in ILs is not always be essential for the study of ILs as protein solvents, as was demonstrated in previous reports in which enzymes dispersed in ILs showed excellent activity compared with those in aqueous buffer solutions [6,7]. However, with the goal of optimizing reaction efficiency and application, many groups have attempted to obtain homogeneous protein-dissolved IL systems. Certain neat ILs have been reported to dissolve proteins [8,9]. There are such a few methods to introduce enzymes into ILs without denaturation as supramolecular complexation of enzymes using crown ethers [10,11], chemical modification of enzymes using polyethylene oxide [12–15], and immobilization of enzymes by reversed micelles [16,17]. We have studied ILs/H_2O mixtures as novel solvents for proteins [18–22]. Choline dihydrogenphosphate ([ch][dhp]) containing 20 wt% of water (about 3 water molecules per ion pair) (Hy[ch][dhp]) has been reported to dissolve cytochrome *c* (cyt *c*) while maintaining higher ordered structure and active center at high cyt *c* concentrations, and the cyt *c* dissolved in Hy[ch][dhp] maintained its structure even at high temperature that would be impossible in buffers [18–20]. We can also confirm the activity of enzymes under appropriate conditions in Hy[ch][dhp] [21,22]. Thus, Hy[ch][dhp] has a potential to be a solvent for proteins as substitute for buffer solutions. However, [ch][dhp] is very unusual among hydrophilic ILs because the mixtures of most other hydrophilic ILs and water cause the denaturation of proteins [23,24]. This is generally comprehensible that hydrophilic molecules compete with proteins to fight over hydration. Hydrophobic organic solvents are known to be better for keeping activity of proteins compared with hydrophilic ones.

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Therefore, it is also interesting to examine the use of enzymes in hydrophobic ILs. It is noted here that small amount of water is indispensable for most proteins. Enzymatic activity has been reported to improve in hydrophobic ILs that contain small amounts of water [25]. It is suggested that the quantity of free (not strongly bound) water in the system becomes the index of the enzymatic activity, similar to organic solvent [25]. In this study, we prepared hydrophobic IL containing different amount of water to examine the effects of water content in hydrophobic ILs on the activity of enzymes. We chose bilirubin oxidase (BOD) as a model enzyme because BOD is known to exchange electrons directly with electrode without any mediator [26,27]. Since BOD catalyzes the four-electron reduction of O_2 to H_2O under mild conditions (ambient temperature, near-neutral pH, and ambient pressure), BOD is expected to function as the cathodal catalyst of enzymatic biofuel cells [28,29]. We have also reported the direct electron-transfer (DET)-type reaction catalyzed by BOD using a gold nanoparticle (AuNP)-modified gold electrode [30]. In this system, we can easily assess the activity of BOD in various solvents by measuring the current density of the BOD-modified electrode. It is not necessary to consider the effects of the diffusion of the enzymes.

2. Experimental

2.1. Materials

Bilirubin oxidase (BOD; EC 1.3.3.5) from *Myrothecium verrucaria* (Amino Enzyme Inc.) was used as received. 1-Butyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide ([C4mim][Tf_2N]) (Kanto Chemical Co.) was purchased and used without further purification. Both 1-ethyl-3-methylimidazolium methylphosphonate ([C2mim][(MeO)(H)PO_2]) and 1-(2-hydroxylethyl)-3-methylimidazolium bis(trifluoromethanesulfonyl)imide ([C2OHmim] [Tf_2N]) were synthesized by the method reported previously [31,32]. Structure of the ILs (Fig. 1) was confirmed using ^{1}H NMR (400 MHz, JEOL Ltd.). All of the prepared ILs were dried *in vacuo* before use. The IL/H_2O mixtures were checked to contain the desired amount (in weight %) of water by Karl Fischer titration method (MKC-510N, Kyoto Electronics Co.). The water activity (a_W) of the IL/H_2O mixtures was measured using water activity measurement apparatus (Pawkit, Decagon Devices, Inc.). The measurements were conducted by sealing the sensor into the open ends of 15 mL plastic vials that were maintained at 25 °C until a constant reading was achieved.

2.2. Kamlet–Taft parameter measurement

The Kamlet–Taft parameters (π^*: dipolarity, α: hydrogen-bond acidity, and β: hydrogen-bond basicity) are often used to specify the hydrogen-bonding abilities of ILs [33]. Three different dyes, *N*,*N*-diethyl-4-nitroaniline, 4-nitroaniline, and Reichardt's dye, were used. For the solvatochromic measurements, the solutions of the dyes in the IL were placed in a quartz cell with a light-path length of 0.1 mm, and their visible spectra were recorded. The Kamlet–Taft parameters (π^*, α, and β) were calculated according to the following equations:

$$\nu(\text{dye}) = \frac{1}{\lambda_{\max(\text{dye})} \times 10^{-4}} \quad (1)$$

$$E_T(30) = \frac{28\,592}{\lambda_{\max(\text{Reichardt's dye})}} - 8.6898 \quad (2)$$

$$\pi^* = 0.314(27.52 - \nu_{N,N\text{-diethyl-4-nitroaniline}}) \quad (3)$$

$$\alpha = 0.0649E_T(30) - 2.03 - 0.72\pi^* \quad (4)$$

$$\beta = \frac{1.035\nu_{N,N-\text{diethyl-4-nitroaniline}} + 2.64 - \nu_{4-\text{nitroaniline}}}{2.80} \quad (5)$$

2.3. Preparation of BOD-modified AuNP electrodes

For the electrochemical studies of BOD, a gold nanoparticle (AuNP)-modified gold electrode was prepared first, in which the electron-transfer current is known to be enhanced by the greatly increased surface area of the electrode. The AuNPs were synthesized as described in the literature [30]. The particle diameter of the AuNPs was estimated to be approximately 15 nm based on the UV–vis spectrum of the solution. A polycrystalline gold electrode (diameter: 1.6 mm, BAS, Inc.) and a rotating ring-disk electrode (diameter: 4 mm, Pt ring electrode, 5 mm inside diameter and 7 mm outside diameter, BAS, Inc.) were polished with water on aluminum oxide lapping film sheets and wiped out with clean cloth, and the concentrated AuNP dispersion was dropped gently onto the surface of the Au electrode with subsequent drying under air. The AuNP coating procedure was repeated three times to allow the AuNPs to accumulate. The AuNP-modified gold electrode and the rotating ring-disk electrode were then immersed in a solution of BOD in a 100 mM phosphate buffer (pH 7.0) for 2 h to immobilize the enzymes on the electrodes. The rotating ring-disk electrode was used after wiping out the Pt-ring region with a clean cloth. When the BOD-modified AuNP electrode was used as a working electrode in an O_2-saturated phosphate buffer solution, well-defined cathodic wave was observed. The current density retained about 94% of its initial value after 24 h in the buffer solution [30]. This indicates that BOD is effectively immobilized and maintains its activity on the electrode at enough time.

2.4. Electrochemical measurements

The electrochemical experiments were performed using an ALS Electrochemical Analyzer (ALS 702B). All of the experiments were conducted with a three-electrode cell in which Pt wire and Ag wire electrodes were used as a counter electrode and a reference electrode, respectively. All of the potentials cited in this paper refer to the Ag wire electrode, with a potential of +350 mV *vs.* the normal hydrogen electrode (NHE). All of the measurements were conducted at room temperature (25 °C).

The enzyme-modified and enzyme-unmodified rotating ring-disk electrodes were attached to the shaft of an electrode rotator (RRDE-2, BAS, Inc.) for the rotating ring-disk electrode measurements. Before the measurements with the rotating ring-disk electrodes, O_2 was purged by bubbling highly purified O_2, and the measurements were performed in O_2-saturated buffers and IL/H_2O mixed solutions.

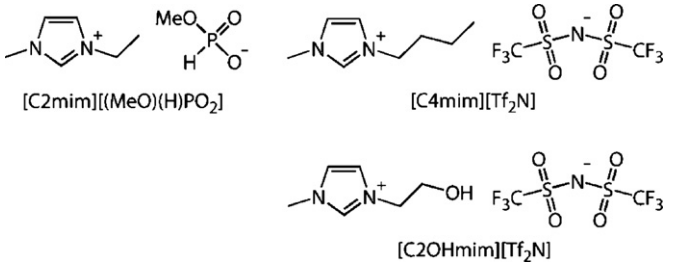

Fig. 1. The chemical structures of the ionic liquids used in this study.