Electron Transfer Process of Poly(ethylene oxide)-Modified Cytochrome c in Imidazolium Type Ionic Liquid

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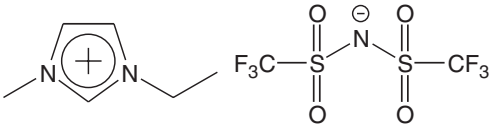
Modification of cytochrome c (cyt.c) by poly(ethylene oxide) (PEO) chains enabled the protein soluble in 1-ethyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide ionic liquid without denaturation. However, as the solution was full of ions, suitable supporting electrolyte (KCl) was essential for the electron transfer reaction of cyt.c in the ionic liquid to attain adequate ion size for the active center.

Construction of biosensor, biochip, and bioreactor frequently requires the fixing of corresponding biomaterials on the electrode without denaturation. These biomaterials play their functions only in an aqueous medium. However, the aqueous medium cannot satisfy the feasible requirement as a matrix. Practically available temperature range of the aqueous system is not wide enough, and it is scarcely possible to realize a long-term stability of proteins in an aqueous medium. It is essentially important to find non-aqueous matrix in which these biomolecules can play their roles stably without denaturation. We have already reported that salt-containing polyethers are excellent non-aqueous media as a reaction field for biomaterials.[1] Polyethers, especially poly(ethylene oxide) (PEO) is unique linear and polar polymer having repeating unit similar to water molecule. The redox reactions of various molecules were carried out in liquid and solid polyethers without even a drop of water.[2,3]

On the other hand, ionic liquids (ILs) have extremely high ion content, high ionic conductivity, low viscosity, non-volatility, and flame resistance. Accordingly, these have been investigated as novel and safe ion conductive matrices[4] as well as reaction solvents.[5] Furthermore, some of these have a wide potential window to allow various electrochemical reactions.[6] ILs are also expected as stable solvents in the biological field. The enzymatic reaction of lipase in ILs has been reported.[7] Kimizuka et al reported that the ether-containing IL solubilized glucose oxidase (containing carbohydrates) because of their IL has relatively good affinity with carbohydrates.[8] In the case of heme proteins, ILs are expected to expand the condition for practical use and to develop novel functions. As one of heme proteins, cyt.c has already been introduced into ILs.[9] Cyt.c was preliminarily dissolved in organic solvent and then mixed with ILs. This procedure is effective for only rigid and soluble proteins in organic solvents. This cannot be applied for most of proteins that are soft and water-soluble.

We have been studying the redox reaction of various PEO modified proteins dissolved in various organic solvents as well as in PEO.[3,10] Furthermore, it has been revealed that PEO-modified proteins, dissolved in solvent PEO, show their functions over a wide temperature range[11] and a long term stability[12] that are impossible in an aqueous medium. Since the PEO chains have high affinity with ions, PEOs are expected to be soluble in ILs. They are indeed soluble in a variety of ILs. This strongly pushed us to examine the solubilization of PEO-modified heme-proteins in ILs without denaturation. In the present study, cyt.c, modified with PEO chains (PEO-cyt.c), was dissolved in 1-ethyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide(**1**), one of typical ionic liquids. The electron transfer reactions of thus dissolved PEO-cyt.c were analyzed by highly sensitive spectroscopic method.



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Horse heart cyt.c (Type VI), purchased from Sigma Chemical Co., was used without further purification. PEO-cyt.c was synthesized according to our previous paper.[3] PEO monomethyl ethers with average molecular weight of 550, 1000, 2000, and 5000 were purchased from NOF Co. Ltd. The number-average modification degree of PEO chains was determined by titration of unreacted amino groups of cyt.c with 2,4,6-trinitrobenzenesulfonic acid.[13] For the present study, cyt.c was modified with 14.3 chains of PEO_{550} (PEO_{550}-cyt.c(14.3)), 15.4 chains of PEO_{1000} (PEO_{1000}-cyt.c(15.4)), 6.1 or 13.5 chains of PEO_{2000} (PEO_{2000}-cyt.c(6.1 or 13.5)), and 9.7 chains of PEO_{5000} (PEO_{5000}-cyt.c(9.7)), respectively to compare the effect of both PEO chain length and degree of modification on the electrochemical response. Lithium bis(trifluoromethanesulfonyl)imide (LiTFSI), a gift from Sumitomo 3M, was used as received. The salt, **1** was synthesized according to previous method[4] The structure of the obtained **1** was confirmed with ^{1}H-NMR spectroscopy. A Karl Fisher moisture titrator (MKS-210; Kyoto Electronics Co.) was used to determine the water content of **1** as 0.37 wt% in average. The visible absorption spectrum and the electrochemical redox reaction of PEO-cyt.c dissolved in **1** (0.1 mM) was studied with optical waveguide (OWG) spectrophotometer SIS-50, System Instruments Inc. The detailed description was mentioned elsewhere.[14] For the OWG analysis,[14] an optical glass waveguide LaSK n1 ($20 \times 65 \times 0.4$ mm, refractive index; 1.75) was purchased from Sumita Optical Glass Co. The electrochemical cell system constructed on the optical glass waveguide[15] with carbon working electrode (3×40 mm), together with Pt and Ag wires (0.5 mm) as counter and reference electrode, respectively.

Native cyt.c or thus prepared PEO-cyt.c was mixed with **1** and stirred slowly to reach the final concentration of 0.1 mM. Native cyt.c was insoluble in **1**, but cyt.cs modified with PEO_{550}(14.3), PEO_{1000}(15.4) or PEO_{2000}(6.1) were partly soluble. On the other hand, cyt.cs modified with PEO_{2000}(13.5) or PEO_{5000}(9.7) were well dissolved in **1**. Figure 1a shows the OWG absorption spectra of the IL containing native cyt.c (dotted line) and PEO_{2000}-cyt.c(13.5) (solid line). Since native cyt.c was insoluble in **1**, no absorption was observed. Against this, Soret band was clearly observed at 408 nm in the case of PEO_{2000}-cyt.c(13.5) in **1**. At least 10 chains of PEO with averaged molecu-

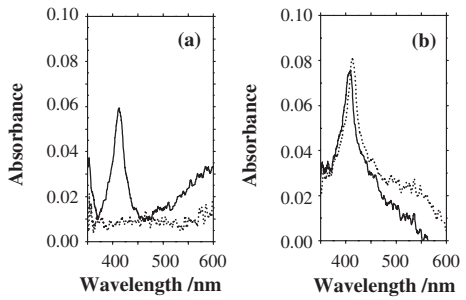

Figure 1. (a) OWG spectra of native cyt.c (dotted), and PEO_{2000}-cyt.c (solid) in **1**, (b) OWG spectra of oxidized (solid) and reduced (dotted) PEO_{2000}-cyt.c in KCl-saturated **1**.

lar weight of over 2000 should be required for the dissolution of cyt.c in **1**. This absorption spectrum clearly shows that dissolved PEO-cyt.c contains heme with ferric ion (Fe^{3+}) and that the vicinity of the heme is unchanged.

The redox activity of dissolved PEO_{2000}-cyt.c(13.5) in **1** was analyzed by OWG spectroscopy, which is useful technique for sensitive and convenient analysis of redox reaction of molecules in a small amount of solution or adsorbed on opaque electrodes.[15] Only μL range analysis is available which is suitable for expensive and highly valuable samples such as ILs. The electrochemical cell system was constructed on the waveguide.[15] PEO_{2000}-cyt.c(13.5) was dissolved in **1** (0.1 mM), and 150 μL of this solution was directly introduced into the cell system. OWG spectra were analyzed with applying the potential (−800 to +800 mV vs Ag, sweep rate: 5 mV/s). However, the spectral change based on the redox reaction of PEO-cyt.c was scarcely observed. The absorbance change at certain wavelengths as the function of the given potential was useful to obtain the electrochemical characteristics such as redox potential. At 414 nm, the λ_{max} of the reduced cyt.c, no absorbance change was found along with potential sweep (Figure 2a). This was explained as lack of small counter ions for heme in spite of extremely high density of ions in ILs. To carry out the redox reaction, small and suitable sized ion species were concluded to be essential for the electron transfer reaction in ILs. Since KCl is known to be a good electrolyte for the electrochemical reaction of heme-proteins in PEOs,[10–12] we added KCl as the supporting electrolyte in **1**. The solubility of KCl in **1** was low, i.e., less than 0.1 M. PEO_{2000}-cyt.c (13.5) (0.1 mM) was dissolved in this KCl-saturated **1** to carry out a similar analysis as mentioned above. The spectrum was the same as that without KCl (Figure 1b, solid line), indicating the oxidized form. When negative potential (−0.8 V vs Ag) was applied to this, the Soret band showed a red shift from 408 nm to 414 nm based on the reduction (Figure 1b, dotted line). After that, the λ_{max} returned

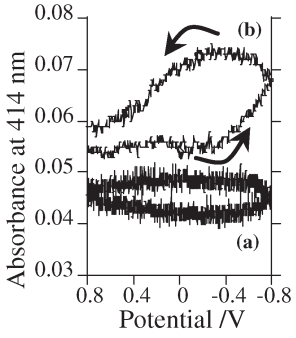

Figure 2. Effect of the given potential on the absorbance change at 414 nm of PEO_{2000}-cyt.c dissolved in (a) **1** only, and (b) KCl-saturated **1**.

to 408 nm by applying positive potential (+0.5 V vs Ag). Figure 2 shows the effect of KCl on the absorbance change at 414 nm with potential sweep. Since PEO-cyt.c was reduced at the negative potential, the absorbance increased even in the initial stage of positive scanning side. Hysteresis in the Figure 2b should be due to slow diffusion of PEO-cyt.c in ILs. The shape of potential absorbance intensity relationship reflects the diffusion coefficient, electron transfer rate, and so on. From the absorbance change as the function of the applied potential in Figure 2b, the oxidation–reduction potential of PEO_{2000}-cyt.c (13.5) dissolved in **1**/KCl mixed electrolyte solution was estimated to be about −0.3 V vs Ag. This redox reaction has been detected similarly after keeping the solution in a dry desiccator for over one month. This extreme stability is comprehensible as lack of water molecules similar to the case in PEO oligomers. No cyclic voltammogram of PEO-cyt.c in IL was detected because of slow electron-transfer reaction and low diffusion coefficient. Since the absorbance is proportional to the quantity of electricity that is the integrated product of current, spectroscopy is quite convenient and sensitive to follow the redox reaction when it was followed by absorbance change. This OWG spectroscopy is confirmed to be sensitive method to analyze the redox behavior of this kind of system.

Our results here show that the modification of PEO chains on the proteins is effective to dissolve the proteins into ILs. For cyt.c, at least 10 PEO chains with average molecular weight of over 2000 was required to solubilize it into ILs keeping the redox activity. It was also confirmed that a suitable electrolyte was essential to realize the smooth electron transfer reaction of heme-proteins in ILs. It is noteworthy that the organic bulky ions are inevitable for IL formation but they are definitely not suitable as electrolytes for proteins having active center in the folded polypeptide chain.

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