Adv Biochem Eng Biotechnol (2019) 168: 215–226
DOI: 10.1007/10_2018_65

Published online: 13 July 2018

Ionic Liquids as Stabilization and Refolding Additives and Solvents for Proteins

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Contents

Abstract This chapter focuses on recent advances in the use of ionic liquids as additives and solvents in protein applications. The solvent properties of ionic liquids can be tuned by the appropriate selection of cation and anion. The effects of different kinds of ionic liquids on protein stability and refolding behavior have been investigated and reported. The ionic liquid properties affect the intermolecular interactions of proteins, inducing different formations and folding behavior. These effects also vary with the concentration of ionic liquids. Although many of the associated mechanisms are not completely clear, some of this behavior may be attributed to the kosmotropicity of the ions and their Hofmeister effects.

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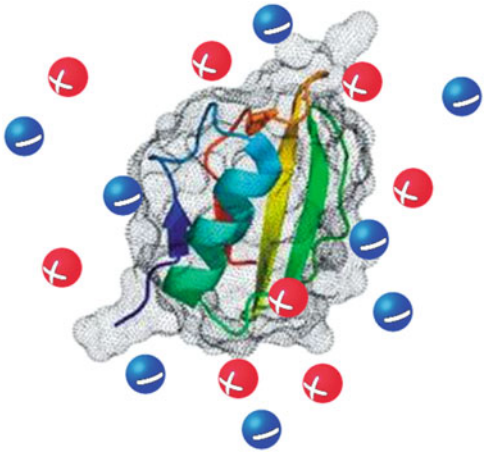
Graphical Abstract



Keywords Additive, Aggregation, Component ion, Protein refolding, Protein stability, Solvation

1 Introduction

Ionic liquids (ILs) have emerged as novel solvents possessing attractive features that are difficult to achieve in ordinary molecular solvents [1, 2]. Hence, ILs are extensively used in a wide range of fields, including chemistry, chemical engineering, biotechnology, and pharmaceuticals [3]. ILs have attracted particular interest as solvents and/or additives for biotechnology and biochemistry applications, namely biocatalysis [4], biotransformation [5], biopreservation [6], and bioseparation [7]. Recently, several reviews focusing on advances in the use of ILs in applications involving biomolecules have been published [8, 9].

Although ILs have been used as novel solvents for biomolecules, it is difficult to dissolve biomolecules in neat ILs and still retain their native structure and activity, regardless of the component ions of the IL. However, some enzymes that show activity as biocatalysts under anhydrous conditions, for example, lipase, show catalytic reactivity when dispersed in neat ILs. To preserve the higher-order structure of biomolecules dissolved in neat ILs, additional procedures are required, such as chemical modification of the protein surface [10] and the addition of specific molecules to the solvent [11]. However, surface modification involves tedious procedures and additional molecules in the IL complicate the system. Thus, the widespread application of neat ILs as protein solvents remains impractical until a significant breakthrough is achieved. For this reason, most studies to date have

used mixtures of ILs and conventional molecular solvents for biomolecular applications.

A recent interesting application of such IL/solvent mixtures is their use as additives for protein refolding [12]. By choosing component ions with suitable properties and optimizing the IL concentration, the solubility and the stability of proteins can be improved, apparently through reduced self-aggregation. This chapter discusses the effects of the component ions and the concentration of the IL in the solvent on the stability and structure of dissolved proteins. Specifically, it focuses on accomplishments in the field of protein stability and folding behavior in IL/water mixtures.

2 Effect of ILs on the Intermolecular Interactions of Proteins

Protein aggregation is one of the most important problems in industrial production and storage processes. Protein aggregation and structural stability also play important roles in protein misfolding diseases such as Alzheimer's and Parkinson's diseases. Moreover, self-assembled structures, including amyloid fibrils and nonamyloid fibrillar aggregates of globular proteins such as bovine serum albumin (BSA), human serum albumin (HSA), and ovalbumin, are of great importance in several scientific areas [13]. ILs have shown great potential in controlling protein structures and morphologies. By understanding the effects of the functional groups of ILs on protein structures, it may be possible to achieve deeper insight into IL-induced structural modification of proteins.

Singh and Kang [14] reported the self-assembly of BSA into microparticles, microrods, and long helical fibers mediated by three different imidazolium-based ILs with long alkyl chains, which are called "IL surfactants (ILSs)." Functionalization of the alkyl chain of the ILs with amide and ester moieties, which are capable of hydrogen bonding, had a remarkable effect on the size and shape of the resulting self-assembled BSA structures compared to those obtained using the non-functionalized IL. The formation of self-assembled structures at different IL concentrations was monitored by dynamic light scattering (DLS), zeta potential, fluorescence, and circular dichroism measurements. Figure 1 shows the variation of count rate monitored by DLS of aqueous solutions of BSA as a function of the concentration of various ILs. The ILs interacted with BSA as monomers at low IL concentrations; in contrast, at higher IL concentrations, the monomers underwent hierarchical self-assembly to form morphologically distinct aggregated complexes. The differences in the nature of the prevailing interactions between BSA and ILs in the different IL concentration regimes were demonstrated by the variations in size, surface charge, and degree of unfolding or refolding of BSA. The IL functionalized with a flexible, hydrogen-bonding-prone ester moiety induced refolding of BSA, although the refolded structure was different from the native structure. The ester-type IL also

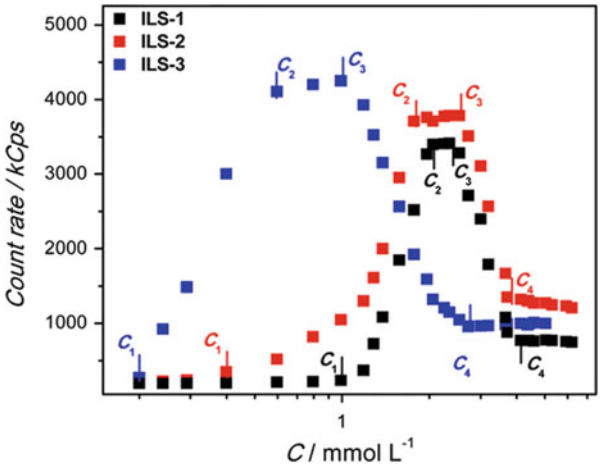


Fig. 1 Count rate observed by dynamic light scattering in aqueous solutions of BSA as a function of concentration of ILSs (Singh and Kang [14])

showed a distinct set of interactions with BSA compared to the other systems, affording long, right-hand-twisted amyloid fibers of BSA in a certain concentration range (Fig. 2).

Similarly, Mangialardo et al. [15] reported the effect of IL cations on the structure of hen egg white lysozyme (HEWL). HEWL, suspended in the ammonium-based ILs 2-methyl ethyl ammonium nitrate, ethyl ammonium nitrate, propyl ammonium nitrate, and butyl ammonium nitrate, was subsequently washed and then analyzed using Raman spectroscopy. The refolding enhancement properties of the ILs were found to depend on the cation structure. Cations with long alkyl chains influenced the proportion of ordered β-aggregates in the fibril conformation and prevented the conversion of β-sheets into α-helices. In contrast, ethyl ammonium nitrate, which has a short alkyl chain, induced both fibril melting and refolding of the secondary structure.

3 ILs as Refolding Additives

Hofmeister reported the effects of coexisting salts on the solubility of proteins in 1888. In the so-called "Hofmeister series," ions are ranked on the basis of their salting-in (protein-solubilizing) and salting-out (protein-desolubilizing) effects. Ions that tend to solubilize and denature proteins are classified as chaotropes. Conversely, ions that induce the structuring of water and hydrogen bonding, accelerate the aggregation of proteins, and stabilize the protein structure are categorized as kosmotropes [16]. Ammonium sulfate, which is one of the most widely used salting-out agents, is employed in protein folding methods as a stabilizer and is categorized as a kosmotrope. Similarly, sugars, polyols, betains, and hydrophilic polymers have also been used to stabilize proteins [17]. The effect of IL addition on the stability and folding behavior of dissolved proteins has also been investigated [18], and in this section the use of ILs as protein refolding additives is summarized.

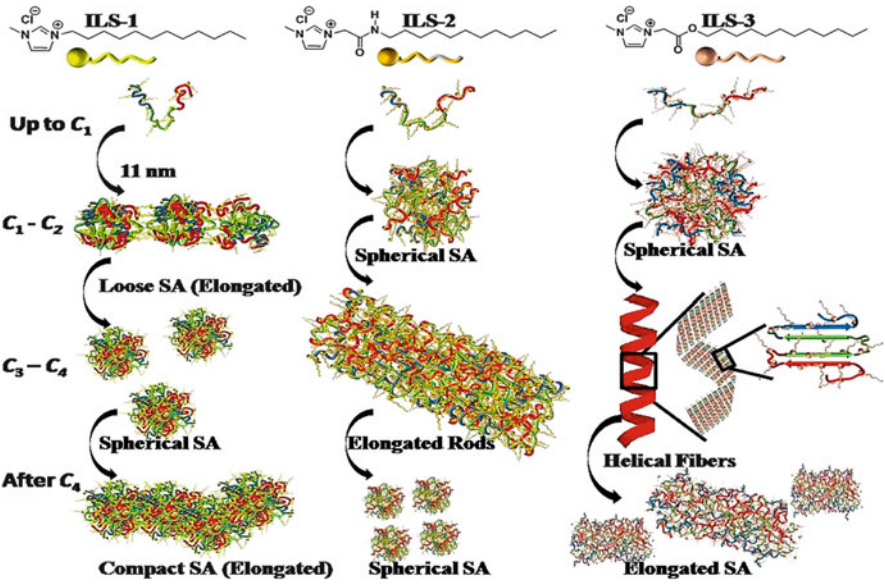


Fig. 2 Schematic view showing various self-assembled structures of BSA formed by mediation of different ILSs in different concentration regimes (Singh and Kang [14]). SA refers to self-assembly

3.1 Cosolute Systems with Urea-Induced Solubilization and Refolding

To solubilize protein aggregates, a relatively low concentration (0.5–2.0 M) of a chaotropic additive, such as urea or guanidine hydrochloride, is generally used to dissolve the aggregates, and then the additives are gradually removed during the protein refolding procedure. However, this method results in a limited refolding rate and low efficiency caused by reaggregation of the protein. Some ILs have been reported to be effective for protein refolding when used in conjunction with chaotropic additives. Attri et al. [19] reported that the addition of 100 μL of the protic IL triethyl ammonium phosphate induced structural refolding of the enzymes α-chymotrypsin and succinylated Con A from the urea-induced chemically denatured states (3 or 4 M urea in 2 mg/mL enzyme solution). Bae et al. [20] investigated the effect of additives, including metal cofactors, organic cosolvents, and imidazolium-based ILs, on the refolding of horseradish peroxidase (HRP) that had undergone urea-induced chemical denaturing. Refolding of the denatured sample was initiated by tenfold dilution of the sample with a refolding buffer containing the additives. Among the tested ILs, the highest HRP activity was observed with 1-ethyl-3-methylimidazolium chloride (1.0 vol%). The effect of the ILs on HRP refolding was attributed to Hofmeister effects. The HRP refolding yield increased significantly, up to 75%, with the addition of ILs, whereas yields of 45% and 10%

were obtained in refolding buffers containing hemin and without hemin, respectively. The effect of temperature on the refolding of HRP was also studied, and the highest and most stable refolding yield was obtained at 4°C.

Bisht et al. [21] reported the refolding behavior of urea-denatured lysozyme in the presence of hydrophobic ILs. Ammonium-based ILs with the trifluromethylsulfonyl imide anion (1 vol%) were added to a lysozyme solution pretreated with urea. Structural refolding was observed for some hydrophobic ammonium-based ILs using several optical methods, although it was unclear how the refolding and the activity of the lysozyme in ILs were related.

Molecular dynamics (MD) studies have been used to provide insight into the detailed mechanisms by which urea and ILs affect protein refolding dynamics, many of which remain unclear. For example, an MD simulation conducted by Ghosh et al. [22] showed that triethylammonium acetate efficiently counteracts the urea-induced denaturation of a small S-peptide analogue, even when the preservative IL is present in a very low molar ratio with respect to the denaturant urea. Further studies might help to improve the understanding of the refolding mechanism related to various ILs.

3.2 Aggregation Inhibition

Small molecular additives are frequently employed to inhibit aggregation during the refolding procedure [23]. Summers and Flowers [24] were the first to explore the ILs tetraethyl and tetrabutyl ammonium nitrate as additives for protein refolding. These ILs effectively suppressed the aggregation of HEWL and led to a significant increase in refolding yields during oxidative refolding at concentrations up to 5% (0.5 M). However, neat tetraalkyl ammonium nitrates were found to denature the proteins.

Systematic trends in the effects of imidazolium-based ILs on protein refolding were investigated by Lange et al. [25]. The renaturation of two model proteins, HEWL and an anti-oxazolone single-chain antibody fragment, were investigated in the presence of a series of *N*′-alkyl- and *N*′-(hydroxyalkyl)-*N*-methylimidazolium chlorides with alkyl chain lengths of two to six carbon atoms. All the investigated ILs acted as refolding enhancers, and in some instances performed even better than L-arginine hydrochloride, which is one of the most widely used additives for protein renaturation. The most favorable effect on refolding was observed at a concentration of about 1 M, although the optimal concentration varied slightly depending on the protein and IL used. The ability of the ILs to suppress protein aggregation and increase stability was determined to be the key feature responsible for renaturation. Hydrophobic imidazolium cations with longer alkyl chains increased destabilization, whereas cations with hydroxyl-terminated alkyl chains improved protein stability. Similar results in terms of alkyl chain length, IL concentration, and prevention of aggregation during protein refolding were also reported for *N*-alkylpyridinium and *N*-alkyl-*N*-methylpyrrolidinium ILs by Yamamoto et al. [26] (Fig. 3).

Variation of the anion was found to have a profound effect on the renaturation of the recombinant plasminogen activator (rPA) [12], with anions other than chloride

leading to a reduction in refolding yields. 1-Ethyl-3-methylimidazolium ([C_2mim]) salts with different anions were screened as additives for in vitro refolding of rPA. The renaturation yields of rPA in the presence of 2-(2-methoxyethoxy)ethyl sulfate ($MDEGSO_4^-$), ethyl sulfate ($EtSO_4^-$), and hexyl sulfate ($HexSO_4^-$) were qualitatively correlated with the hydrophobicity of the three salts. The influence of the anion on the efficacy of the IL as a refolding enhancer for rPA decreased in the following order: Cl^- > $MDEGSO_4^-$ > acetate > tosylate > diethyl phosphate > $HexSO_4^-$. In vitro refolding of rPA was promoted more effectively by [C_2mim][Cl] (refolding yield of 10% at 1.2 M) than by L-arginine hydrochloride (refolding yield of 14% at 3 M), which has been possibly the most widely used additive for in vitro refolding over the last two decades.

These reports reveal that IL additives can act as direct enhancers of protein refolding. Moreover, by preferentially binding to and being slightly or moderately chaotropic for proteins, IL additives prevent protein aggregation.

3.3 Use of Vesicle Systems

Bharmoria et al. [27] reported a vesicle-forming biamphiphilic IL that induced significant folding alterations in protein structures. The binding behavior of 3-methyl-1-octylimidazolium dodecylsulfate ([C_8mim][$C_{12}OSO_3$]) with BSA in an aqueous medium at pH 7.0 was investigated at different concentrations. Both the cation and anion of this IL are amphiphilic in nature, and the IL acts as a catanionic surfactant. At low concentrations, [C_8mim][$C_{12}OSO_3$] induced a small amount of unfolding of BSA because of the formation of monomer complexes via exothermic

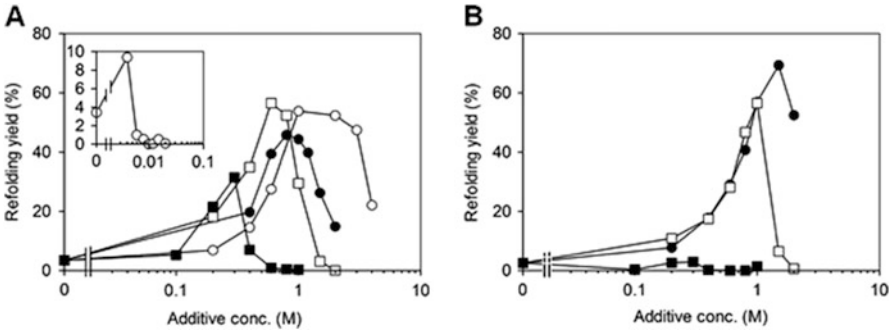


Fig. 3 Refolding yields (recovered activities) of denatured and reduced lysozyme obtained by dilution with refolding buffers containing carious *N*-alkylpyridinium chlorides and *N*-alkyl-*N*-methylpyrrolidinium chlorides as a function of concentrations. (**a**) *N*-Alkylpyridinium chlorides: *N*-ethyl- (open circles), *N*-butyl- (closed circles), *N*-hexyl- (open squares), *N*-octyl- (closed squares), and *N*-dodecyl- (inset, open circles) pyridinium chlorides. (**b**) *N*-Alkyl-*N*-methylpyrrolidinium chlorides: *N*-butyl- (closed circles), *N*-hexyl- (open squares), *N*-hexyl- (open squares) *N*-methylpyrrolidinium chlorides (Yamamoto et al. [26])

electrostatic interactions with the charged amino acid residues on the surface of the protein. As the concentration was increased, protein refolding was observed, until the critical aggregation concentration of $[C_8mim][C_{12}OSO_3]$ was reached. This aggregation resulted from crosslinking of $[C_8mim][C_{12}OSO_3]$ via electrostatic interactions at one end and hydrophobic interactions at the other. Above the critical aggregation concentration, a small amount of BSA unfolding was again observed until the critical vesicular concentration was reached, which suggested that aggregation of $[C_8mim][C_{12}OSO_3]$ at the BSA surface occurred via cooperative electrostatic and hydrophobic interactions in this concentration regime. BSA remained stable against folding alterations in the vesicular and postvesicular regimes because of the strong cooperative hydrophobic and electrostatic interactions among $[C_8mim][C_{12}OSO_3]$ ions (Fig. 4). BSA in the postvesicular concentration regime of $[C_8mim][C_{12}OSO_3]$ showed for a month high stability against aggregation, which is the major cause of protein destabilization. Similar results have been reported for the stability and activity of the enzyme cellulase [28]. This work provided insights into the design of surface-active ILs as artificial chaperones for protein stabilization.

4 Hydrated ILs as Refolding Fields

Hydrated ILs are prepared by mixing ILs with a small amount of water, such that there is almost no free water. The threshold for the existence of free water was suggested to be seven or more water molecules per one ion pair, regardless of the ion structure [29]. Depending on the IL, the IL concentration in hydrated ILs can be greater than 4 M; hence, it is predicted that their effects on proteins are different from those of ILs used as additives in solution. By selecting an optimized ion structure and

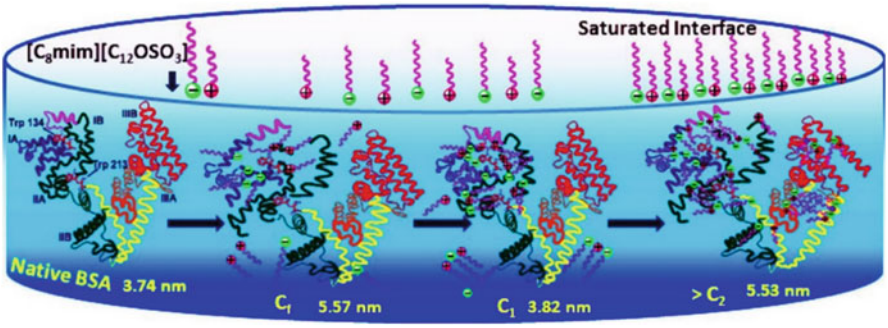


Fig. 4 Folding alterations in BSA at variable concentration of $[C_8mim][C_{12}OSO_3]$ (Bharmoria et al. [28]). C_f signifies the maximum unfolding concentration resulting from association of biamphiphilic IL monomers to BSA; C_1 corresponds to the critical aggregation concentration up to which refolding of BSA occurs as a result of cross-linking of IL ion on BSA, forming BSA − IL aggregate complexes; $>C_2$ shows the post-vesicular regime where BSA − IL aggregates and BSA adsorbed on vesicles exist

water content, hydrated ILs can achieve the dissolution of some proteins, at the same time maintaining their higher-order structure. One of the most effective hydrated ILs as a biomolecular solvent that dissolves biomolecules but maintains the higher-order structure was the pair composed of the cholinium cation and the dihydrogen phosphate anion ([ch][dhp]) [30]. Improvements of the long-term stability and thermal stability of proteins after dissolution were observed in hydrated [ch][dhp] [31]. The refolding of aggregated cellulase, which is a protein heterologously expressed in *Escherichia coli*, was recently reported to occur in hydrated [ch][dhp] without the use of denaturants or additives [32]. A common method for regenerating aggregated proteins that are abundantly expressed in *E. coli* (inclusion bodies) is the use of a high concentration of denaturant to dissolve aggregates after cell breakage by sonication. However, excess amounts of denaturant inhibit protein refolding and recovery, even after dialysis or dilution, and reaggregation of the dissolved proteins occurs during this step. Furthermore, problems, such as the generation of large amounts of wastewater and the long time required to remove the denaturant, make this procedure impractical. A more efficient and easily performed method is needed to regenerate aggregated proteins. Notably, ILs and hydrated ILs have potential as novel refolding solvents that are able to dissolve aggregated protein and/or induce refolding behavior directly. The solubility of aggregated cellulase was not high, but dissolved cellulase was observed to have a folded state similar to that of its native state in hydrated [ch][dhp]. The dissolution and restructuring patterns of aggregated cellulase in hydrated ILs differed depending on the component ions of the IL. When other imidazolium-type ILs (1-ethyl-3-methylimidazolium methylphosphate and 1-ethyl-3-methylimidazolium tetrafluoroborate) were used, unsatisfactory results were obtained, which demonstrated the difficulty of dissolving aggregated cellulase, even in the hydrated state.

The number of water molecules per [ch][dhp] pair clearly influenced the renaturation procedure. The spectral intensity decreased when the number of water molecules per ion pair in hydrated [ch][dhp] was increased. This behavior indicated that the dissolution ability of hydrated [ch][dhp] for aggregated cellulase decreased with increasing water content, even though the viscosity of hydrated [ch][dhp] decreased with increasing water content. Furthermore, the position of the fluorescence spectral maxima, which indicates the folding state of the protein, was affected by the water content (Fig. 5). Fluorescence spectra with a spectral maximum around 340 nm were observed for both native cellulase and cellulase in hydrated [ch][dhp] with four, seven, or ten water molecules per ion pair, which suggests that the aggregated cellulase dissolved in hydrated [ch][dhp] had a folding state similar to that of its native structure in buffer. In contrast, the fluorescence maximum showed a blue shift to 300 nm when cellulase was dissolved in [ch][dhp] with around 2,800 water molecules per ion pair, that is, a 20 mM solution. This spectral shift indicated the formation of an aggregated state similar to that observed in buffer and in water. In [ch][dhp] with 50 water molecules per ion pair, the spectrum had two peaks at around 300 and 340 nm, suggesting that cellulase was present in two different states: the native folded state and the aggregated state. Renaturation and correct refolding of the aggregated cellulase dissolved in hydrated [ch][dhp] was observed. Furthermore,

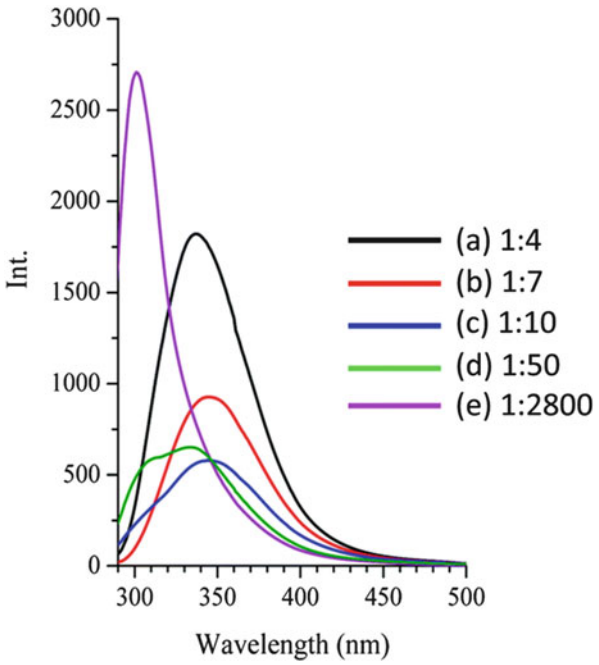


Fig. 5 Fluorescence spectra of supernatants mixed with white precipitate in [ch][dhp] containing (a) 4, (b) 7, (c) 10, (d) 50, and 2,800 water molecules per ion pair (Fujita et al. [32])

cellobiose was produced by the refolded cellulase after desalination of [ch][dhp]. Understanding and controlling the water state in hydrated ILs may facilitate the development of improved and convenient methods for the renaturation of aggregated proteins.

5 Concluding Remarks

ILs have diverse solvation properties that can be tuned by the proper choice of cation and anion. For applications in biotechnology and bioscience, many kinds of ILs have been investigated at different concentrations. ILs have been reported to have notable effects on protein activity, solubility, stability, separation, crystallization, morphology, refolding, and unfolding. Some of this behavior can be attributed to the kosmotropicity of the ions and their Hofmeister effects [33]. Other effects, however, are difficult to explain because of the complexity and variety of IL moieties, as well as their distinct behavior depending on the concentration of water molecules in the solution. The potential of neat or hydrated ILs for bioapplications has not yet been fulfilled. Studies on the mechanisms of biocompatible hydrated ILs and the interactions among biomolecules, ions, and water may reveal useful information for understanding intercellular phenomena.

Acknowledgments This research was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (KAKENHI, No. 17H01225). K.F. is grateful for the fund from the Asahi Glass Foundation. Reproduced from Fujita et al. 2016 with permission from The Royal Society of Chemistry.

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