Interaction of proteins with ionic liquid, alcohol and DMSO and in situ generation of gold nano-clusters in a cell

Somen Nandi¹ · Sridip Parui¹ · Ritaban Halder¹ · Biman Jana¹ ·
Kankan Bhattacharyya^{1,2}

Received: 24 September 2017 / Accepted: 23 October 2017 / Published online: 16 November 2017
© International Union for Pure and Applied Biophysics (IUPAB) and Springer-Verlag GmbH Germany 2017

Abstract In this review, we give a brief overview on how the interaction of proteins with ionic liquids, alcohols and dimethyl sulfoxide (DMSO) influences the stability, conformational dynamics and function of proteins/enzymes. We present experimental results obtained from fluorescence correlation spectroscopy on the effect of ionic liquid or alcohol or DMSO on the size (more precisely, the diffusion constant) and conformational dynamics of lysozyme, cytochrome c and human serum albumin in aqueous solution. The interaction of ionic liquid with biomolecules (e.g. protein, DNA etc.) has emerged as a current frontier. We demonstrate that ionic liquids are excellent stabilizers of protein and DNA and, in some cases, cause refolding of a protein already denatured by chemical denaturing agents. We show that in ethanol–water binary mixture, proteins undergo non-monotonic changes in size and dynamics with increasing ethanol content. We also discuss the effect of water–DMSO mixture on the stability of proteins. We demonstrate how large-scale molecular dynamics simulations have revealed the molecular origin of this observed phenomenon and provide a microscopic picture of the immediate environment of the biomolecules. Finally, we describe how favorable interactions of ionic liquids may be

utilized for in situ generation of fluorescent gold nano-clusters for imaging a live cell.

Keywords Binary mixture · Conformational dynamics · Folding/unfolding · Ionic liquid · MD simulation

Introduction

A room temperature ionic liquid (RTIL) consists of a bulky organic ion (cation) and a small inorganic counter ion (anion). Interactions of RTILs among each other and with other small and large biomolecules are currently attracting great interest because of the widespread applications of RTILs in green chemistry and electrochemistry, as well as for their ability to impart stability and long-term storage of proteins, enzymes and nucleic acids (e.g. DNA) (Baker et al. 2004; Benedetto and Ballone 2016; Chandran et al. 2012; Egorova et al. 2017; Fujita et al. 2007; Saha et al. 2016; Watanabe et al. 2017). The interaction of an ionic liquid with a biomolecule or a drug involves both electrostatic (through the ionic part of RTILs) and hydrophobic actions (with long-chain organic moieties of RTILs). Ionic liquids have also been demonstrated to enhance efficacy of traditional drugs and thereby has opened up new avenue in drug research (Egorova et al. 2017).

The interaction of RTIL with biomolecules has been studied quite extensively by X-ray and neutron scattering techniques. Neutron scattering experiments provide a microscopic picture of the interactions of ionic liquids with phospholipids, peptides/proteins and nucleic acids (e.g. DNA) (reviewed recently by Benedetto and Ballone 2016). These studies and the accompanying molecular dynamics (MD) simulations have examined the relative contribution of columbic interaction and other weak interactions such as dispersion, hydrogen bonds, steric effects etc. Microscopic details, such as the

This article is part of a special issue on ‘Ionic Liquids and Biomolecules’ edited by Antonio Benedetto and Hans-Joachim Galla

✉ Biman Jana
pcbj@iacs.res.in

✉ Kankan Bhattacharyya
kankan.bhattacharyya@gmail.com

¹ Department of Physical Chemistry, Indian Association for the Cultivation of Science, Jadavpur, Kolkata 700 032, India

² Department of Chemistry, Indian Institute of Science Education and Research Bhopal, Bhopal, Madhya Pradesh 462 066, India

location of the ionic liquid cations in the vicinity of the polar head group of lipids (Benedetto and Ballone 2016) or minor grooves of DNA (Chandran et al. 2012) or near a particular residue of a protein, have been shown to depend on the structure of the biomolecule and the ionic liquid itself.

The structure and self-assembly of neat RTIL itself is quite interesting. Early computer simulations predicted a nanoscopic structural organization with clear demarcation of the organic and ionic parts (Bhargava et al. 2007; Canongia Lopes and Padua 2006; Wang and Voth 2005). This organization was subsequently confirmed by small angle x-ray scattering (Russina et al. 2017; Triolo et al. 2007). One major consequence of the nanoscopic organization is the wide variation of local viscosity in neat ionic liquid. This phenomenon has been explored in recent fluorescence correlation spectroscopy (FCS) studies which monitor diffusion of the probe where the diffusion coefficient (D_i) is a function of local viscosity (η). The FCS data give the D_i from which the hydrodynamic radius (r_H) is calculated using the Stokes–Einstein equation,

$$D_i = K_B T / 6\pi\eta r_H \quad (1)$$

The micro-heterogeneity in neat ionic liquid gives rise to multiple values of local viscosity (Patra and Samanta 2012; Sasmal et al. 2011a). It is observed that while the average viscosity of the ionic liquids (1-pentyl-3-methyl imidazolium bromide {[PMIM][Br]} and fluoroborate {[PMIM][BF₄]} is about 100-fold greater than that of water, in different regions of the neat RTIL the viscosity varies by a factor of about 10. Further, the diffusion coefficient depends on the charge of the solute. An anionic dye (Coumarin 343) diffuses nearly two-fold slower than a neutral dye (Coumarin 480) (Sasmal et al. 2011a), which highlights the predominance of Coulombic interaction between solute and solvent in an ionic liquid (Sasmal et al. 2011a).

Many aspects of the interactions of an ionic liquid with a biomolecule have been reviewed recently (Benedetto and Ballone 2016; Egorova et al. 2017; Watanabe et al. 2017). However, there has yet to be a survey of current investigations on the interaction of ionic liquid with biomolecules using FCS. FCS studies can provide information on the size and time-scale of the conformational dynamics of a protein. The experimental techniques described above have elucidated several interesting features of the conformational stability and dynamics of proteins in different aqueous binary mixtures (Amin et al. 2016; Chattoraj et al. 2014; Ghosh et al. 2015a). However, there is still a need for a better understanding of the molecular details of the systems, including, for example (1) solvent quality and physical properties of the binary mixture at different compositions (Banerjee et al. 2012; Ghosh et al. 2013, Roy et al. 2012); (2) location of the water and non-aqueous solvent molecules [ethanol/ionic liquid/dimethyl sulfoxide (DMSO)] around a protein and their

distance from a specific residue (Bhattacharjya and Balaram 1997; Deshpande et al. 2005); (3) structural changes induced by non-aqueous solvent (folding/unfolding); (4) the effect on an ionic liquid on the biological function of the protein. Atomistic MD simulation studies provide the answer to these critical questions by providing the time-dependent trajectories of the protein molecules along with surrounding solvent molecules, as well as detailed descriptions of the inter-molecular interactions in atomic resolution. In this overview, we place special emphasizes on the study of such interactions using FCS as an experimental technique. We begin with a three-component system, i.e. a protein (lysozyme) in a mixture of water and an ionic liquid (Ghosh et al. 2015a). In this case the FCS data are supported by large-scale MD simulations. We then proceed to more complicated systems with more than three components, i.e. protein, water, ionic liquid and a denaturant [guanidine hydrochloride (GdnHCl)/urea]. Computer simulations of such systems are too complicated at the present time and have yet to be explored. We show that the effect of an ionic liquid on a protein in a non-native state (unfolded or molten globule) is quite interesting. We then discuss the behavior of a protein in other binary mixtures, such as water–ethanol and water–DMSO mixtures.

Finally, inspired by the idea of the ionic–liquid drug conjugate, we describe how the conjugate of ionic liquid with gold salt may be used to generate fluorescent gold nano-clusters (Au-NCs) in a live cell for cell imaging.

Interaction of a protein with an ionic liquid

As the protein surface has a fair amount of both hydrophobic and hydrophilic residues, ionic liquid having both characteristics interacts favorably with proteins. In general, because of this favorable interaction with a protein, an ionic liquid displaces (“strips”) water molecules from the immediate vicinity of the protein, and the residual water molecules at the protein surface tend to form small clusters (Micaelo and Soares 2008). Computer simulations of the enzyme serine protease cutinase in two ionic liquids, namely 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF₆]) and BMIM-nitrate ([BMIM][NO₃]), indicate that the structure of the protein crucially depends on the water content (Micaelo and Soares 2008). This has implications for the thermostability of the protein at high temperatures and at low hydration.

Results from neutron scattering and other experimental studies suggest that the enzyme xylanase retains its structure and activity in low concentrations of ionic liquids, such as 1-ethyl-3-methyl-imidazolium acetate ([EMIM][OAc]) or EMIM-ethyl sulfate ([EMIM][EtSO₄]) (Jaeger and Pfaendtner 2013). MD simulations suggest that at a high concentration of ionic liquid, the enzyme loses its activity not because of unfolding but due to the kinetic trapping of the

organic cation counter part of the ionic liquids in the binding pocket, resulting in a dampening of the dynamic motion at the active site (Jaeger and Pfaendtner 2013).

The effect of an ionic liquid on the stability of a protein is diverse and depends on the nature of both the protein and the ionic liquid. For example, while cytochrome c (Cyt C) is stabilized in ethylammonium nitrate (Jaganathan et al. 2015), the same ammonium-based ionic liquids are shown to cause unfolding of heme proteins (Jha et al. 2014). However, *Candida antarctica* lipase B becomes aggregated in [EMIM][EtSO₄], [EMIM][NO₃] and 1-ethyl-3-methylimidazolium-dicyanamide {[EMIM][N(CN)₂]} and loses its activity (Sate et al. 2007).

Several research groups have applied FCS technique in order to understand the effect of ionic liquid on the structure and dynamics of a protein (Pabbathi et al. 2013; Sasmal et al. 2011b; Sen Mojumdar et al. 2012). We have already noted in this review that FCS is efficient in determining the size, or more precisely the r_H of a protein as well as the time scale of conformational dynamics [i.e. relaxation time (τ_R)] of a protein. This can be understood as follows. In FCS study, a fluorescent probe is covalently attached to a specific residue of a protein. The fluorescence intensity of the probe varies with time and may be monitored by confocal microscopy. The fluctuation in fluorescence intensity occurs due to two factors. First, the protein may diffuse in and out of the focal volume, and the time constant of this process gives the D_t and hence the size of the protein (by applying Eq. 1). Second, during conformational dynamics of the protein, the distance of the fluorescent probe from a residue containing mainly free amino groups (e.g. tryptophan, histidine etc.) varies; for example, when the amino group comes in close proximity of the fluorescent probe (i.e. ~ 1 Å) electron transfer from the amine to the fluorescent probe causes quenching of fluorescence (Pabbathi et al. 2013, Sasmal et al. 2011b).

To elucidate how the use of FCS and MD simulation can coherently explain the interaction of ionic liquid with protein in molecular detail, we describe here a specific example using the protein (enzyme) lysozyme (Ghosh et al. 2015a). Lysozyme is a 129-residue enzyme that catalyzes the hydrolysis of the glycoside bond at cell membranes (Phillips 1967). This protein contains four helices, namely, the A (5–15), B (25–36), C (88–101), and D (109–115) helices (Fig. 1). The active site of lysozyme is the deep cleft between Glu-35 and Asp-52. For the FCS experiment, lysozyme was covalently labeled with a fluorescent probe, Alexa Fluor 488 at Lys-33 (helix B) and Lys-97 (helix C) (Chattoraj et al. 2014). Thus, the locations of the probe are very close to the active site as well as in the neighborhood of the six tryptophan residues of lysozyme (Trp-28, -62, -63, -108, -111 and -123) (Fig. 1).

The FCS data for lysozyme in an aqueous solution of an ionic liquid is fitted to a model with a single diffusion component and one conformational relaxation time (Ghosh et al.

Fig. 1 Structure of lysozyme. Reprinted from Chattoraj et al. (2014), with the permission of AIP Publishing, Melville

2015a). It is observed that the r_H of the native protein decreases from ~ 18 Å in 0 M ionic liquid to ~ 11 Å in 1.5 M RTIL (Fig. 2). The conformational dynamics of the protein is found to be accelerated upon addition of the ionic liquid and the corresponding τ_R also decreases from ~ 65 μ s in 0 M RTIL to ~ 5 μ s in 1.5 M RTIL (Fig. 2). Both of these results are quite surprising because (1) binding of the ionic liquid is likely to increase the size of the protein; (2) the presence of highly viscous ionic liquids around the protein is expected to dampen the conformational dynamics of the protein.

The observed experimental results may be reconciled in terms of a microscopic picture obtained from MD simulation (Ghosh et al. 2015a). The radial distribution function (RDF) of water, [PMIM]⁺ (RTIL cation) and Br[−] (RTIL anion) as a function of distance from the protein surface is described in Fig. 3. It is readily seen that there is a decrease in water population in first solvation shell of the protein while the density of [PMIM]⁺ is dramatically increased, thereby demonstrating that preferential solvation of protein by [PMIM]⁺ causes depletion of water from the protein surface. Inspection at molecular level reveals that lysozyme has negatively charged amino acids (7 Asp and 2 Glu) which drag [PMIM]⁺ towards themselves. Preferential solvation of [PMIM]⁺ thus operates via the favorable interaction with these negatively charged amino acids (Fig. 3e). Near the protein surface, the mole fraction of heavy atoms of [PMIM]⁺ at 1.5 M concentration of RTIL is ~ 1.3 -fold higher than that in the bulk of the binary mixture (Fig. 3b). As the distance increases, the ratio gradually decreases and eventually reaches the bulk-like value at ~ 30 Å, which illustrates that the protein is fenced by a [PMIM]⁺-rich shell having a thickness of ~ 30 Å (Fig. 3c) (Ghosh et al. 2015a).

In bulk water, the effective radius of the protein with one layer of water, obtained from MD simulation (~ 17.5 Å), is in good agreement with the obtained experimental value (~ 18 Å). This agreement implies that in pure water, protein diffuses with one layer of water. Based on results from both the FCS and MD simulation studies, the effective radius of the dry protein in pure water is ~ 12.5 Å. The reduction in size of the

Fig. 2 Variation of hydrodynamic radius (r_H) and time constant of conformational relaxation [relaxation time (τ_R)] of lysozyme as a function of room temperature ionic liquid (RTIL) concentration. Reprinted from Ghosh et al. (2015a), with the permission of AIP Publishing, Melville

protein by ~ 5 Å in the presence of ionic liquid may be attributed to the “stripping off” of the water layer and structural change of the protein. Preferential solvation of the RTIL cation leads to the formation of a nanoscopic cage with an overall radius of ~ 42 Å. This study demonstrated that a sphere with a radius of ~ 42 Å corresponds to a diffusion time (τ_D) of ~ 1134 μs. However, the experimental τ_D (obtained from FCS study) of lysozyme is 302 μs, which corresponds to a radius of ~ 11 Å. It would appear that the “dry and dehydrated” protein undergoes local motion within the nanoscopic cage of the RTIL cation.

The effective radius of dry protein in RTIL–water mixtures is somewhat less than that in pure water, suggesting a structural compaction of the protein upon addition of the RTIL. The distribution of distance between the two domains (α -domain: residues 1–37 and 88–129; β -domain: residues 41–84; Fig. 4a) around the hinge region of lysozyme in water reveals a bimodal distribution, with the two peaks corresponding to the closed form (~ 18 Å) and open form (~ 19 Å), respectively, of the protein. In 1.5 M RTIL, a single peak corresponding to the closed form reflects a structural collapse due to domain closure of lysozyme (Fig. 4b) (Ghosh et al. 2015a). Thus, the

Fig. 3 **a** Radial distribution function [$g(r)$] of water (W), the 1-pentyl-3-methyl imidazolium cation ([PMIM] $^+$), and the bromide anion (Br^-) from the protein surface (P). **b** The relative mole fraction of heavy atoms of [PMIM] $^+$ and water compared to those in bulk as a function of distance from the protein surface. **c** Schematic representation of RTIL-rich

solvation shell around the lysozyme protein. **d** Representative snapshot of RTIL cation (D1, blue), water (D2, red), and superposition of both around lysozyme (D3). **e** Representative snapshot showing interaction of the negatively charged Glu and Asp with the [PMIM] $^+$. Reprinted from Ghosh et al. (2015a), with the permission of AIP Publishing, Melville

Fig. 4 **a** Schematic representations of the α -domains (blue) and β -domains (red) of lysozyme. **b** Distribution of domain distances of lysozyme in bulk water and in aqueous RTIL solution. Open, closed refer to open and closed form, respectively, of the protein. Reprinted from Ghosh et al. (2015a), with the permission of AIP Publishing, Melville

decrease in the r_H is partly due to domain closure of the protein and partly due to dehydration.

We now discuss an interesting case where an ionic liquid by itself causes unfolding of a protein, human serum albumin (HSA), while it causes refolding of the protein if it is unfolded beforehand by a denaturing agent, such as GdnHCl (Chowdhury et al. 2012; Sasmal et al. 2011b). In this study, the single cysteine residue (cys-34) of HSA is site specifically covalently labeled by the fluorophore 7-dimethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM) (Fig. 5a). From the FCS data, it is evident that both GdnHCl and the ionic liquid separately cause unfolding of the HSA protein, resulting in an increase in size (i.e. r_H) from ~ 38 to 61 Å (Sasmal et al. 2011b). To the contrary, the HSA protein experiences compaction, with a r_H of ~ 41 Å, in a mixture of both GdnHCl and ionic liquid (Sasmal et al. 2011b).

In order to understand the immediate environment of the protein in the presence of ionic liquid, we carried out a solvation dynamics study by monitoring the emission of the probe, CPM, covalently attached to the protein. The average solvation time is ~ 650 ps in the native state of HSA, ~ 260 ps in 1.5 M ionic liquid and ~ 60 ps in the presence of 6 M GdnHCl (Chowdhury et al. 2012). This implies unfolding of the protein by ionic liquid or GdnHCl and subsequent exposure of the

probe (CPM) to the polar environment or to the bulk water. When 1.5 M RTIL is added to the protein already denatured by the addition of 6 M GdnHCl, there is a ~ 5 nm red shift in the emission maximum of CPM which reflects a more polar environment, and the time scale of solvation dynamics becomes approximately twofold faster (~ 30 ps). These changes indicate that the immediate environment of the solvation probe (CPM) is very different from that in the native state of the protein in the presence of both ionic liquid and GdnHCl, despite the more compact structure of the protein. This results in the local enrichment or accumulation of the ionic liquid and GdnHCl near the protein, thereby providing a confinement which induces the collapse (Chowdhury et al. 2012). Using computer simulations, Xia et al. (2012) have also shown that the protein tends to collapse in the mixture of denaturants (aqueous urea and GdnHCl). These authors also explained this phenomenon in terms of confinement caused by the excess denaturants.

A protein may form non-native folding intermediate states [i.e. partially unfolded states called molten globules (MG)] upon addition of various denaturants [e.g. sodium dodecyl sulfate (SDS), urea etc.] or by changing the pH of the solution. In the case of HSA protein, two non-native states (i.e. MG-I and MG-II) were obtained by just changing the pH (in acidic

Fig. 5 Structure of 7-dimethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM)-labeled human serum albumin (HSA) (a) and horse heart cytochrome c (b). Reprinted with permission from Sasmal et al. (2011b) and Sen Mojumdar et al. (2012), respectively. Copyright (2011) and (2012) held by the American Chemical Society, Washington D.C.

pH) (Chowdhury et al. 2013). HSA has a multi-domain structure, and it is possible to monitor the fluctuations in the inter-domain distance by Förster resonance energy transfer (FRET). In FRET studies, a protein (or DNA) is labeled by two fluorescent probes, one donor (D) and another acceptor (A). The efficiency of FRET (ϵ_{FRET}) is inversely proportional to the sixth-power of the donor–acceptor distance ($R_{\text{D-A}}$). In the previous section, we noted that the separate addition of GdnHCl and the ionic liquid caused unfolding of the protein, giving rise to states of the same overall size ($r_{\text{H}} \sim 61 \text{ \AA}$). The results of the FRET study suggest that actually there are considerable structural differences between these two states obtained upon the separate addition of GdnHCl and the ionic liquid. The same is also observed for the two MG states (Chowdhury et al. 2013). In the non-native states, the presence of multiple values of FRET efficiency implies the presence of different conformers.

The respiratory protein horse heart Cyt C (Fig. 5b) forms two important non-native folding intermediate states (MG states: MG-I and MG-II) upon addition of a surfactant (SDS and urea) (Sen Mojumdar et al. 2012). The effect of an ionic liquid, the RTIL [PMIM][Br] on the size of the protein in the native state, the MG state and completely unfolded states is drastically different (Fig. 6; see Sen Mojumdar et al. 2012). In the native and MG-I states, the size of the protein increases (implying unfolding) with increasing concentration of the ionic liquid. However, addition of the ionic liquid causes the protein in the unfolded state and in the MG-II state to decrease in size. These effects imply that in the first case (i.e. native and MG-I states), the ionic liquid acts as a denaturant, whereas in the second case (i.e. MG-II and denatured states) the same

ionic liquid serves as a protein stabilizer. The results demonstrate the complex interplay of the interaction of protein residues with each other and also with added ionic liquid /or denaturants (Sen Mojumdar et al. 2012). Computer simulation of such multi-component systems is quite complex and may be a subject of future studies.

Effect of alcohol and DMSO on proteins

The stability, function and conformational dynamics of proteins are highly dependent on their interaction with the surrounding solvent molecules, temperature, pressure etc. Water is a natural solvent for proteins and particularly important for their functional stability inside living organisms. The conformational stability and dynamics of different proteins that play important roles in living organisms have been investigated in water under different external conditions (varying temperature, pressure etc.). The introduction of an aqueous binary mixture (mixture of another liquid with water) into such studies provides another dimension to this area of research. Even without any change to the external conditions, the composition of such a binary mixture can be varied to produce solvents of different qualities in which proteins can show widely different behaviors. As well, changing the partner non-aqueous liquid can change both the bulk solvent properties and direct interaction with the protein molecules and therefore provide an array of different conditions to probe the stability and function of proteins. The binary mixtures of water with non-aqueous liquids, such as ionic liquid, alcohol or DMSO are increasingly being used for various reasons. Since amphiphilic molecules (such as methanol, ethanol, DMSO, RTIL etc.) possess both hydrophobic and hydrophilic moieties, such molecules may produce different effects in water. The hydrophilic groups tend to interact favorably with water by forming hydrogen bonds while, in contrast, hydrophobic groups avoid water and tend to self-aggregate by breaking the water structure, a phenomenon often referred to as “hydrophobic hydration.”

The composition-dependent effect of the ethanol–water mixture (“liquor”) on protein is a subject of great interest (Fukasawa and Sato 2011; Giugliarelli et al. 2013; Juárez et al. 2012; Lousa et al. 2012; Martin et al. 2008; Mattos and Ringe 2001; Nemzer et al. 2013; Ortore et al. 2011; Tanaka et al. 2001). Further, Klibanov and co-workers extensively used enzymes in a binary mixture of water with another solvent (Klibanov 2001; Rariy and Klibanov 1997). Nuclear magnetic resonance and circular dichroism (CD) spectroscopy have been applied to elucidate the effect of ethanol on the structure of bovine serum albumin (Avdulov et al. 1996) and of DMSO on hen egg-white lysozyme (Bhattacharjya and Balaram 1997). The effect of ethanol on lysozyme has also been studied by the dynamic light scattering method

Fig. 6 Variation in the r_{H} of cytochrome c (Cyt C) in the native, molten globule (MG-I, MG-II) and denatured states. Reprinted with permission from Sen Mojumdar et al. (2012). GdnHCl Guanidinium hydrochloride, a denaturing agent. Copyright (2012) held by the American Chemical Society, Washington, D.C.

(Calandrini et al. 2000). CD spectra suggest that the structure of lysozyme changes from the native α helix to the β sheet with the formation of amyloid protofilaments at high concentration of ethanol (Goda et al. 2000; Sasahara and Nitta 2006).

Bagchi and coworkers hinted about a structural transition in binary mixtures of water (with alcohol and DMSO) and explained several anomalous observations of the bulk binary mixture along with conformational dynamics of proteins (Banerjee et al. 2012; Ghosh et al. 2013). These researchers analyzed partial unfolding of the chicken villin head piece (HP-36) in different binary mixtures in terms of the structural change in the bulk binary mixture and specific interactions with the protein. They further showed that the barrier between the folded to unfolded state as well as the stability of these two states in pure water is modulated as a function of the composition of DMSO or ethanol (Banerjee et al. 2012; Ghosh et al. 2013; Roy et al. 2012).

We will now discuss a few selected examples on this topic. According to the crystallography study performed by Deshpande et al. (2005), there are two ethanol binding sites near the active site of lysozyme, with one found near Asn-59 in the sugar binding cleft and the other found near Glu-7 and Cys-6 in helix A. Upon the addition of ethanol to an aqueous solution of this protein, the alcohol molecules are sequestered around the proximity of these hydrophobic residues, as indicated by the blue-shifted emission spectra of tryptophan, and the ethanol molecules gradually displace the weakly bound water molecules (as many as 16 bound water molecules) in the vicinity of this protein (Deshpande et al. 2005). More recently, using a combined simulation and experimental studies, Bagchi and co-workers also detected non-monotonous (oscillatory) transitions in the structure of myoglobin in an ethanol–water mixture, which led them to talk about oscillations and hint at percolation transition from their bulk solution (Ghosh et al. 2015b).

Upon the addition of ethanol to an aqueous solution of lysozyme, the changes of r_H of lysozyme exhibit a non-monotonic and oscillatory dependence on alcohol content (Chattoraj et al. 2014). First, the size (r_H) decreases from 19 Å in the native state to 13 Å at 3% ethanol (v/v). The size increases to 21 Å at 9% ethanol, 26 Å at 32.5% ethanol, and 29 Å at 68.5% ethanol (all v/v) (Fig. 7). However, there are two minima—at 17 Å at 19.5% ethanol and 19 Å at 49% ethanol (both v/v). Thus, size of the lysozyme molecule fluctuates by as much as 16 Å between a compact, collapsed structure with $r_H = 13$ Å and a partially unfolded structure of $r_H = 29$ Å. Such a large change in r_H is quite significant for a protein with four di-sulfide bonds (S–S).

A similar oscillation in the conformational dynamics and in the rate for the fast folding/unfolding dynamics is also observed. The τ_R of lysozyme fluctuates between 15 and 240 μ s in an ethanol–water mixture (Chattoraj et al. 2014).

This non-monotonic oscillating behavior in the structure and dynamics of lysozyme is caused by the percolation of ethanol molecules around the tryptophan residues, which leads to competition of the mutual interaction among ethanol, water and lysozyme.

No such oscillation in either size (r_H) or τ_R of lysozyme is observed in the case of the binary mixture of water and DMSO (Ghosh et al. 2014). Rather, the fast folding/unfolding dynamics exhibit a chevron-like plot (Fig. 7). First, the size of the protein (r_H) increases rapidly from ~ 18 Å at 0 mol% to ~ 33 Å at 5 mol% of DMSO content, reflecting the unfolding of the protein. The addition of more DMSO leads to a decrease in size to 18 Å at 30 mol% of DMSO (Fig. 7). In this binary solvent mixture, the blue-shifted emission maxima of tryptophan demonstrates that the DMSO molecules also preferentially accumulate near the tryptophan residues of the protein, like ethanol, to form a cluster through hydrophobic interaction. Bagchi and co-workers obtained exactly the same results for lysozyme in a DMSO–water binary solvent system (Roy et al. 2012). At a lower concentration of DMSO ($\sim 5\%$), the conformational flexibility of the protein was suppressed considerably. Further increases in the DMSO concentration (~ 15 – 20%) result in the formation of a collapsed state (Roy et al. 2012). The experimentally observed anomalies of the DMSO–water binary mixture can be assigned to a percolation-like phase transition at low DMSO concentrations (Banerjee et al. 2010; Roy et al. 2011). The unusual behavior of a linear hydrocarbon chain in the DMSO–water binary mixture at low DMSO concentrations has also been observed (Ghosh et al. 2011). The solvation dynamics study in the DMSO–water binary mixture reveals that there is a consistent slowdown of solvation dynamics at a certain mole percentage of DMSO (Roy and Bagchi 2013).

The addition of alcohol to the aqueous solution of Cyt c causes a non-monotonic variation in the size of the protein (Amin et al. 2016). This observation was further supported by the MD simulation study. The free energy landscape of Cyt C as a function of radius of gyration (R_g) at different ethanol content has been obtained from MD simulation (Amin et al. 2016). In water (i.e. 0% ethanol), the compact folded state of this protein is the most stable state, with a R_g of ~ 13 Å (Fig. 8a). At $X_{EtOH} = 0.07$ (19.6% v/v), the minimum shifts to ~ 14 Å, implying slight unfolding. The structure corresponding to this minimum suggests an increase in the distance between helix 2 and helix 3 compared to that in the native state (Fig. 8b). Upon further addition of ethanol, i.e. at $X_{EtOH} = 0.2$ (44.75% v/v), two minima corresponding to two different structures of Cyt C are observed. The first minimum indicates that the protein is slightly unfolded compared to the native state, whereas the second minimum, which is more stable, indicates significant unfolding (Fig. 8c). Free energy analysis at higher ethanol concentrations (e.g.

Fig. 7 Variations in the r_H and τ_R of lysozyme in an ethanol (EtOH)–water (left panel) and a dimethyl sulfoxide (DMSO)–water binary mixture (right panel). Reprinted from Chattoraj et al. (2014), with the permission of AIP Publishing, Melville, and from Ghosh et al. (2014), with of The Royal Society of Chemistry, London

$x_{\text{EtOH}} = 0.3$ (58% v/v) reveals a compact state with $R_g = 13.2$ Å (Fig. 8d) (Amin et al. 2016).

The contact map analysis between different amino acid residues of Cyt C shows that ethanol molecules disrupt several hydrophobic contacts that are present in the native state. However, at a high mole fraction of ethanol, these hydrophobic contacts begin to reappear. The breaking and re-formation

of these crucial contacts at different mole fractions of ethanol dictates the structural transition of the protein.

Among the four helices of Cyt C, the contact between the helix 2–helix 3 interface is found to be affected the most. At $x_{\text{EtOH}} = 0.0$, these two helix are at a distance of ~ 14 Å. This distance increases with the addition of ethanol up to $x_{\text{EtOH}} = 0.2$ (44.75% v/v). The snapshots for the stable

Fig. 8 Free energy profiles of Cyt C as a function of radius of gyration (R_g) in bulk water (a) and in an EtOH–water binary mixture at ethanol concentrations of $x_{\text{EtOH}} = 0.07$ (19.6% v/v) (b) $x_{\text{EtOH}} = 0.2$ (44.75% v/v) (c) and $x_{\text{EtOH}} = 0.3$ (58% v/v) (d). Reprinted from Amin et al. (2016), with the permission of AIP Publishing, Melville

conformations at different mole fractions of ethanol with the protein molecule surrounded by the solvent molecules depict that helix 2–helix 3 distances vary significantly depending on the different mole fraction of ethanol. There are not many contacts present between helix 1 and helix 4 even in the native state. The other interfaces (helix 1–helix 2 and helix 3–helix 4) do not change drastically upon the addition of ethanol.

The organization of the solvent around different stable structures of the protein at different concentrations of ethanol have been explored by calculating the RDF [$g(r)$] of water and ethanol molecules around a hydrophobic contact pair (residue 64–95). A gradual increase of the first peak of $g(r)$ is noteworthy as it indicates that the greater number of ethanol molecules accumulate in the close proximity of that contact pair with increasing R_g (Fig. 9a, b), suggesting a preferential solvation of the protein by ethanol molecules.

To the contrary, the $g(r)$ of the water molecules around the contact pair does not show any remarkable changes (Amin et al. 2016), and fewer water molecules are present around the contact pair at $X_{\text{EtOH}} = 0.2$ (44.75% v/v) than at $X_{\text{EtOH}} = 0.3$ (58% v/v) (Fig. 9c, d). Therefore, the compaction of the Cyt C structure at the 30% mole fraction ($X_{\text{EtOH}} = 0.3$) is probably due to structural transition in the bulk water–ethanol mixture, which results in a non-monotonic local composition near the hydrophobic contact pair.

Thus, as the size of the protein increases, more and more ethanol molecules accumulate around the hydrophobic pair of the protein, whereas the density of water does not change

significantly; this indicates that ethanol molecules interact preferentially with the protein as its size increases. The anomaly at $X_{\text{EtOH}} = 0.3$ can be explained by considering the stable structure of cytochrome c at $X_{\text{EtOH}} = 0.2$ and performing the MD simulation at $X_{\text{EtOH}} = 0.3$. The same hydrophobic contact pair was chosen which was present in native state but absent at $X_{\text{EtOH}} = 0.2$ and the number of ethanol and water molecules around these pairs at $X_{\text{EtOH}} = 0.2$ and 0.3 was calculated (Fig. 9c, d). At $X_{\text{EtOH}} = 0.2$, more ethanol molecules are present around that contact pair than water molecules, whereas at $X_{\text{EtOH}} = 0.3$, more water molecules are present around the contact pair than ethanol molecules. At higher ethanol concentrations, ethanol molecules are engaged in the formation of an ethanol–ethanol network, with the result that fewer ethanol molecules are freely available to interact with the protein. So, at higher concentrations of ethanol, a compact state is more stable. Further investigation of the composition-dependent anomaly of protein in the presence of aqueous binary mixtures needs to be performed.

Ionic liquid in drug research and live cell imaging

An ionic liquid can significantly enhance the efficacy of many drugs (Egorova et al. 2017). The main advantage of an ionic liquid is that it can be used to solubilize and emulsify many poorly soluble drugs and thus increase their bio-availability (Egorova et al. 2017). Another strategy is to convert drugs and

Fig. 9 **a, b** Radial distribution function (RDF) [$g(r)$] of the EtOH molecule around the hydrophobic contact pair (64–95) of Cyt C for different R_g values at different concentrations of ethanol. Inset shows corresponding RDF of water. **c, d** The distance-dependent coordination number of ethanol and water molecules around the hydrophobic contact pair (64–95). Reprinted from Amin et al. (2016), with permission of AIP Publishing, Melville

Fig. 10 **a** Radial distributions function of ionic liquid ions in different regions of DNA (phosphate backbone, major groove and minor groove). **b** Representative spatial distribution map of ionic liquid cations showing their association with the DNA backbone, major groove and minor

bio-active substance into ionic liquid salt form through suitable derivatives (Egorova et al. 2017). The imidazolium cation of an ionic liquid readily binds with negatively charged DNA (Fig. 10) and RNA. This technique is utilized to stabilize DNA (Chandran et al. 2012; Ding et al. 2010), perform fluorescence sensing of RNA inside biological cells (Shirinfar et al. 2013), transfect short interfering RNA (siRNA) and perform gene silencing inside a cell (Dobbs et al. 2009).

While metal nano-particles, which contain thousands of metal atoms, are non-fluorescent, small clusters of noble metal (e.g. gold, silver, copper etc.) containing 10–30 metal atoms are highly fluorescent (Dutta Choudhury et al. 2012; Stamplecoskie and Kamat 2014; Yau et al. 2013; Zheng et al. 2003). Fluorescent NCs are very important in research and can be used for cell imaging (Chattoraj and Bhattacharyya 2014). If the gold NCs are prepared outside the cell and used for cell imaging, then most of the time the NCs do not penetrate the nucleus of the cell (Chattoraj and Bhattacharyya 2014). In order to ensure the presence of gold NCs inside the nucleus, a conjugate of imidazolium cation and AuCl₄[−] anion is injected in the cell culture medium. Since the nucleus of a cell contains DNA, the imidazolium cation readily delivers the AuCl₄[−] anion to the inside of the nucleus, and highly fluorescent gold NCs are generated in situ inside the nucleus (Chattoraj et al. 2016). Significant differences between cancer and non-cancer cells are observed using this protocol, which also leads to visualization of the nucleolus inside the nucleus (Chattoraj et al. 2016).

Conclusion

In this review, through combined application of experimental (FCS) and computational (MD simulation) studies we have attempted to describe the complexity of the interaction of proteins with ionic liquids and binary mixtures containing co-

solvents like ethanol, DMSO etc. Ionic liquids differentially affect the structure and dynamics of proteins in their native, denatured and MG states, respectively. In general, ionic liquid displaces the hydration layer of the protein. Dehydrated lysozyme undergoes fast diffusion, with a smaller size and faster conformational dynamics inside a 30 Å-thick shell of ionic liquid. We have reported studies on detailed structural changes. Ionic liquids are also shown to bind with the nucleic acids (DNA and RNA) and have been used to generate gold NCs in situ inside a living cell (in particular, inside the nucleus) for live cell imaging.

Acknowledgements Biman Jana gratefully acknowledges the supercomputing facility CRAY at the Indian Association for the Cultivation of Science.

Funding Thanks are due to the Department of Science and Technology J. C. Bose Fellowship for a generous research grant to Kankan Bhattacharyya and to the Council of Scientific and Industrial Research for awarding fellowships to Somen nandi, Sridip Parui and Ritaban Halder.

Compliance with Ethical Standards

Conflicts of interest Somen Nandi, Sridip Parui, Ritaban Halder Biman jana and Kankan Bhattacharyya declare that they have no conflicts of interest regarding this work

References

- Amin MA, Halder R, Ghosh C et al (2016) Effect of alcohol on the structure of cytochrome C: FCS and molecular dynamics simulations. *J Chem Phys* 145:235102-1–235102-8. <https://doi.org/10.1063/1.4972015>
- Avdulov NA, Chochina SV, Daragan VA et al (1996) Direct binding of ethanol to bovine serum albumin: a fluorescent and ¹³C NMR Multiplet relaxation study. *Biochemistry* 35:340–347. <https://doi.org/10.1021/bi9513416>

- Baker SN, McCleskey TM, Pandey S, Baker GA (2004) Fluorescence studies of protein thermostability in ionic liquids. *Chem Commun* 0: 940–941. <https://doi.org/10.1039/b401304m>
- Banerjee S, Roy S, Bagchi B (2010) Enhanced pair Hydrophobicity in the water–dimethylsulfoxide (DMSO) binary mixture at low DMSO concentrations. *J Phys Chem B* 114:12875–12882. <https://doi.org/10.1021/jp1045645>
- Banerjee S, Ghosh R, Bagchi B (2012) Structural transformations, composition anomalies and a dramatic collapse of linear polymer chains in dilute ethanol–water mixtures. *J Phys Chem B* 116:3713–3722. <https://doi.org/10.1021/jp2085439>
- Benedetto A, Ballone P (2016) Room temperature ionic liquids meet biomolecules: a microscopic view of structure and dynamics. *ACS Sustain Chem Eng* 4:392–412. <https://doi.org/10.1021/acssuschemeng.5b01385>
- Bhargava BL, Devane R, Klein ML, Balasubramanian S (2007) Nanoscale organization in room temperature ionic liquids: a coarse grained molecular dynamics simulation study. *Soft Matter* 3:1395–1400. <https://doi.org/10.1039/B710801J>
- Bhattacharjya S, Balam P (1997) Effects of organic solvents on protein structures: observation of a structured helical core in hen egg-white lysozyme in aqueous dimethylsulfoxide. *Proteins Struct Funct Genet* 29:492–507. [https://doi.org/10.1002/\(SICI\)1097-0134\(199712\)29:4<492::AID-PROT9>3.0.CO;2-A](https://doi.org/10.1002/(SICI)1097-0134(199712)29:4<492::AID-PROT9>3.0.CO;2-A)
- Calandrini V, Onori G, Santucci A (2000) Effect of 1-alkanols on the native conformation of lysozyme. *Phys Chem Chem Phys* 2:4143–4146. <https://doi.org/10.1039/b003746j>
- Canongia Lopes JN, Padua AA (2006) Nano-structural organization in ionic liquids. *J Phys Chem B* 110:3330–3335. <https://doi.org/10.1021/jp056006y>
- Chandran A, Ghoshdastidar D, Senapati S (2012) Groove binding mechanism of ionic liquids: a key factor in long-term stability of DNA in hydrated ionic liquids? *J Am Chem Soc* 134:20330–20339. <https://doi.org/10.1021/ja304519d>
- Chattoraj S, Bhattacharyya K (2014) Fluorescent gold nano-cluster inside a live breast cell: etching and higher uptake in cancer cell. *J Phys Chem C* 118:22339–22346. <https://doi.org/10.1021/jp506745p>
- Chattoraj S, Mandal AK, Bhattacharyya K (2014) Effect of ethanol–water mixture on the structure and dynamics of lysozyme: a fluorescence correlation spectroscopy study. *J Chem Phys* 140:115105–1–115105–8. <https://doi.org/10.1063/1.4868642>
- Chattoraj S, Amin MA, Mohapatra S, Ghosh S, Bhattacharyya K (2016) Cancer cell imaging by in situ generated gold Nano-clusters. *ChemPhysChem* 17:61–68. <https://doi.org/10.1002/cphc.201500731>
- Chowdhury R, Mojumdar SS, Chattoraj S, Bhattacharyya K (2012) Effect of ionic liquid on the native and denatured state of a protein covalently attached to a probe: solvation dynamics study. *J Chem Phys* 137:0551041–0551048. <https://doi.org/10.1063/1.4739922>
- Chowdhury R, Chattoraj S, Mojumdar SS, Bhattacharyya K (2013) FRET between a donor and an acceptor covalently bound to human serum albumin in native and non-native states. *Phys Chem Chem Phys* 15:16286–16293. <https://doi.org/10.1039/c3cp52296b>
- Deshpande A, Nimsadkar S, Mande SC (2005) Effect of alcohols on protein hydration: crystallographic analysis of hen egg-white lysozyme in the presence of alcohols. *Acta Cryst D* 61:1005–1008. <https://doi.org/10.1107/S0907444905009364>
- Ding Y, Zhang L, Xie J, Guo R (2010) Binding characteristics and molecular mechanism of interaction between ionic liquid and DNA. *J Phys Chem B* 114:2033–2043. <https://doi.org/10.1021/jp9104757>
- Dobbs W, Heinrich B, Bourgogne C et al (2009) Mesomorphic imidazolium salts: new vectors for efficient siRNA Transfection. *J Am Chem Soc* 131:13338–13346. <https://doi.org/10.1021/ja903028f>
- Dutta Choudhury S, Ray K, Lakowicz JR (2012) Silver nanostructures for fluorescence correlation spectroscopy: reduced volumes and increased signal intensities. *J Phys Chem Lett* 3:2915–2919. <https://doi.org/10.1021/jz301229m>
- Egorova KS, Gordeev EG, Ananikov VP (2017) Biological activity of ionic liquids and their application in pharmaceuticals and medicine. *Chem Rev* 117:7132–7189. <https://doi.org/10.1021/acs.chemrev.6b00562>
- Fujita K, MacFarlane DR, Forsyth M et al (2007) Solubility and stability of cytochrome c in hydrated ionic liquids: effect of Oxo acid residues and Kosmotropicity. *Biomacromolecules* 8:2080–2086. <https://doi.org/10.1021/BM070041O>
- Fukasawa T, Sato T (2011) Versatile application of indirect Fourier transformation to structure factor analysis: from X-ray diffraction of molecular liquids to small angle scattering of protein solutions. *Phys Chem Chem Phys* 13:3187–3196. <https://doi.org/10.1039/c0cp01679a>
- Ghosh R, Banerjee S, Chakrabarty S, Bagchi B (2011) Anomalous behavior of linear hydrocarbon chains in water–DMSO binary mixture at low DMSO concentration. *J Phys Chem B* 115:7612–7620. <https://doi.org/10.1021/jp110549h>
- Ghosh R, Roy S, Bagchi B (2013) Solvent sensitivity of protein unfolding: dynamical study of chicken villin headpiece subdomain in water–ethanol binary mixture. *J Phys Chem B* 117:15625–15638. <https://doi.org/10.1021/jp406255z>
- Ghosh S, Chattoraj S, Chowdhury R, Bhattacharyya K (2014) Structure and dynamics of lysozyme in DMSO–water binary mixture: fluorescence correlation spectroscopy. *RSC Adv* 4:14378–14384. <https://doi.org/10.1039/c4ra00719k>
- Ghosh S, Parui S, Jana B, Bhattacharyya K (2015a) Ionic liquid induced dehydration and domain closure in lysozyme: FCS and MD simulation. *J Chem Phys* 143:125103–1–125103–8. <https://doi.org/10.1063/1.4931974>
- Ghosh R, Samajdar RN, Bhattacharyya AJ, Bagchi B (2015b) Composition dependent multiple structural transformations of myoglobin in aqueous ethanol solution: a combined experimental and theoretical study. *J Chem Phys* 143:015103–1–015103–11. <https://doi.org/10.1063/1.4923003>
- Giugliarelli A, Sassi P, Paolantoni M et al (2013) Vibrational circular dichroism spectra of lysozyme solutions: solvent effects on thermal denaturation processes. *J Phys Chem B* 117:2645–2652. <https://doi.org/10.1021/jp311268x>
- Goda S, Takano K, Yamagata Y et al (2000) Amyloid protofilament formation of hen egg lysozyme in highly concentrated ethanol solution. *Protein Sci* 9:369–375. <https://doi.org/10.1110/ps.9.2.369>
- Jaeger VW, Pfaendner J (2013) Structure, dynamics, and activity of xylanase solvated in binary mixtures of ionic liquid and water. *ACS Chem Biol* 8:1179–1186. <https://doi.org/10.1021/cb3006837>
- Jaganathan M, Ramakrishnan C, Velmurugan D, Dhathathreyan A (2015) Understanding ethylammonium nitrate stabilized cytochrome c—molecular dynamics and experimental approach. *J Mol Struct* 1081:334–341. <https://doi.org/10.1016/j.molstruc.2014.10.049>
- Jha I, Attri P, Venkatesu P (2014) Unexpected effects of the alteration of structure and stability of myoglobin and hemoglobin in ammonium-based ionic liquids. *Phys Chem Chem Phys* 16:5514–5526. <https://doi.org/10.1039/C3CP54398F>
- Juárez J, Alatorre-Meda M, Cambón A et al (2012) Hydration effects on the fibrillation process of a globular protein: the case of human serum albumin. *Soft Matter* 8:3608–3619. <https://doi.org/10.1039/c2sm06762e>
- Klibanov AM (2001) Improving enzymes by using them in organic solvents. *Nature* 409:241–246. <https://doi.org/10.1038/35051719>
- Lousa D, Baptista AM, Soares CM (2012) Analyzing the molecular basis of enzyme stability in ethanol/water mixtures using molecular dynamics simulations. *J Chem Inf Model* 52:465–473. <https://doi.org/10.1021/ci200455z>
- Martin SR, Esposito V, De Los Rios P et al (2008) Cold denaturation of yeast frataxin offers the clue to understand the effect of alcohols on

- protein stability. *J Am Chem Soc* 130:9963–9970. doi: <https://doi.org/10.1021/ja803280e>
- Mattos C, Ringe D (2001) Proteins in organic solvents. *Curr Opin Struct Biol* 11:761–764. [https://doi.org/10.1016/S0959-440X\(01\)00278-0](https://doi.org/10.1016/S0959-440X(01)00278-0)
- Micaelo NM, Soares CM (2008) Protein structure and dynamics in ionic liquids. Insights from molecular dynamics simulation studies. *J Phys Chem B* 112:2566–2572. <https://doi.org/10.1021/jp0766050>
- Nemzer LR, Flanders BN, Schmit JD et al (2013) Ethanol shock and lysozyme aggregation. *Soft Matter* 9:2187–2196. <https://doi.org/10.1039/c2sm27124a>
- Ortore MG, Mariani P, Carsughi F et al (2011) Preferential solvation of lysozyme in water/ethanol mixtures. *J Chem Phys* 135:245103-1–245103-9. <https://doi.org/10.1063/1.3670419>
- Pabbathi A, Patra S, Samanta A (2013) Structural transformation of bovine serum albumin induced by dimethyl sulfoxide and probed by fluorescence correlation spectroscopy technique and additional methods. *Chemphyschem* 14:2441–2449. <https://doi.org/10.1002/cphc.201300313>
- Patra S, Samanta A (2012) Microheterogeneity of some Imidazolium ionic liquids as revealed by fluorescence correlation spectroscopy and lifetime studies. *J Phys Chem B* 116:12275–12283. <https://doi.org/10.1021/jp3061202>
- Phillips DC (1967) The hen egg-white lysozyme molecule. *Proc Natl Acad Sci USA* 57:484–495
- Rariy RV, Klibanov AM (1997) Correct protein folding in glycerol. *Proc Natl Acad Sci USA* 94:13520–13523
- Roy S, Bagchi B (2013) Chemical unfolding of chicken villin headpiece in aqueous Dimethyl Sulfoxide solution: Cosolvent concentration dependence, pathway, and microscopic mechanism. *J Phys Chem B* 117:4488–4502. <https://doi.org/10.1021/jp308589b>
- Roy S, Banerjee S, Biyani N, Jana B, Bagchi B (2011) Theoretical and computational analysis of static and dynamic anomalies in water–DMSO binary mixture at low DMSO concentrations. *J Phys Chem B* 115:685–692. <https://doi.org/10.1021/jp109622h>
- Roy S, Jana B, Bagchi B (2012) Dimethyl sulfoxide induced structural transformations and non-monotonic concentration dependence of conformational fluctuation around active site of lysozyme. *J Chem Phys* 136:115103-1–11510310. <https://doi.org/10.1063/1.3694268>
- Russina O, Celso FL, Plechkova NV, Triolo A (2017) Emerging evidences of mesoscopic-scale complexity in neat ionic liquids and their mixtures. *J Phys Chem Lett* 8:1197–1204. <https://doi.org/10.1021/acs.jpclett.6b02811>
- Saha D, Kulkarni M, Mukherjee A (2016) Water modulates the ultraslow dynamics of hydrated ionic liquids near CG rich DNA: consequences for DNA stability. *Phys Chem Chem Phys* 18:32107–32115. <https://doi.org/10.1039/C6CP05959G>
- Sasahara K, Nitta K (2006) Effect of ethanol on folding of hen egg-white lysozyme under acidic condition. *Proteins Struct Funct Bioinforma* 63:127–135. <https://doi.org/10.1002/prot.20782>
- Sasmal DK, Mandal AK, Mondal T, Bhattacharyya K (2011a) Diffusion of organic dyes in ionic liquid and giant micron sized ionic liquid mixed micelle: fluorescence correlation spectroscopy. *J Phys Chem B* 115:7781–7787. <https://doi.org/10.1021/jp202090x>
- Sasmal DK, Mondal T, Sen Mojumdar S et al (2011b) An FCS study of unfolding and refolding of CPM-labeled human serum albumin: role of ionic liquid. *J Phys Chem B* 115:13075–13083. <https://doi.org/10.1021/jp207829y>
- Sate D, Janssen MHA, Stephens G, Sheldon RA, Seddon KR, Lu JR (2007) Enzyme aggregation in ionic liquids studied by dynamic light scattering and small angle neutron scattering. *Green Chem* 9: 859–867. <https://doi.org/10.1039/B700437K>
- Sen Mojumdar S, Chowdhury R, Chatteraj S, Bhattacharyya K (2012) Role of ionic liquid on the conformational dynamics in the native, molten globule, and unfolded states of Cytochrome C: a fluorescence correlation spectroscopy study. *J Phys Chem B* 116:12189–12198. <https://doi.org/10.1021/jp307297s>
- Shirinfar B, Ahmed N, Park YS et al (2013) Selective fluorescent detection of RNA in living cells by using Imidazolium-based cyclophane. *J Am Chem Soc* 135:90–93. <https://doi.org/10.1021/ja3112274>
- Stamplecoskie KG, Kamat PV (2014) Size-dependent excited state behavior of glutathione-capped gold clusters and their light-harvesting capacity. *J Am Chem Soc* 136:11093–11099. <https://doi.org/10.1021/ja505361n>
- Tanaka S, Oda Y, Ataka M et al (2001) Denaturation and aggregation of hen egg lysozyme in aqueous ethanol solution studied by dynamic light scattering. *Biopolymers* 59:370–379. [https://doi.org/10.1002/1097-0282\(20011015\)59:5<370::AID-BIP1034>3.0.CO;2-Z](https://doi.org/10.1002/1097-0282(20011015)59:5<370::AID-BIP1034>3.0.CO;2-Z)
- Triolo A, Russina O, Bleif HJ, Di Cola E (2007) Nanoscale segregation in room temperature ionic liquids. *J Phys Chem B* 111:4641–4644. doi: <https://doi.org/10.1021/jp067705t>
- Wang Y, Voth GA (2005) Unique spatial heterogeneity in ionic liquids. *J Am Chem Soc* 127:12192–12193. <https://doi.org/10.1021/ja053796g>
- Watanabe M, Thomas ML, Zhang S et al (2017) Application of ionic liquids to energy storage and conversion materials and devices. *Chem Rev* 117:7190–7239. <https://doi.org/10.1021/acs.chemrev.6b00504>
- Xia Z, Das P, Shakhovich EI, Zhou R (2012) Collapse of unfolded proteins in a mixture of denaturants. *J Am Chem Soc* 134:18266–18274. <https://doi.org/10.1021/ja3031505>
- Yau SH, Varnavski O, Goodson T III (2013) An ultrafast look at au Nanoclusters. *Acc Chem Res* 46:1506–1516. <https://doi.org/10.1021/ar300280w>
- Zheng J, Petty JT, Dickson RM (2003) High quantum yield blue emission from water-soluble Au₈ Nanodots. *J Am Chem Soc* 125:7780–7781. <https://doi.org/10.1021/ja035473v>