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Endeavour to simplify the frustrated concept of protein-ammonium family ionic liquid interactions

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Abstract

The stupendous attention grabbed by ionic liquids (ILs) among various physical and chemical sciences has been attributed to their unique and designer nature. In the past few years, the role of ILs in protein folding/unfolding has been rapidly growing. In sight of the increasing importance of ILs, it is enviable to systematize the ion effects on protein properties such as structure stability, activity and enantioselectivity. Various studies available in the literature show ILs as a potential solvent media for many enzymatic reactions as well as in various protein folding/unfolding studies. Various reviews by many researchers focus on the synthesis, application and general properties of the ILs however, the review focussing the effect of various ILs on the activity, structure and stability of protein is still missing. Also, according to best of our knowledge there is no single review available throughout the literature which focuses the effect of same family of IL on different proteins. Therefore, the paramount need is to have a complete knowledge of the biomolecules particularly amino acids (AAs) and proteins in a particular IL family. The cynosure of the present perspective is to nose around the performance of a list of proteins and protein model compounds in presence of ammonium-based ILs. This perspective presents a survey of all the key developments from the available reports and also our past and present experience related to proteins and ammonium-based ILs. Additionally, we have tried to put the available information in chronological order in most of the cases. The use of ammonium family ILs as co- solvent for various proteins model compounds and proteins have been outlined. This perspective can act as barometer for reckoning the various advancements made in this field and also galvanize further cogitation of various untouched aspects of this research area.

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Introduction

The regular local folds of polypeptide chains make up the proteins. The amino acid (AA) sequence which directs the formation of unique three-dimensional structure defines the most captivating problem of protein folding. Three dimensional structure of protein particularly arises because of sequence of amino acids (AAs) present in the polypeptide chains which fold to create compact domains.[1] This three-dimensional network is maintained by several interactions such as ionic effects, hydrogen bonding and hydrophobic interactions. These interactions may either be disturbed by changes of different types in the environment of protein eventually leading to denaturation or inactivation via their unfolding.[2] In particular; even modest heating can lead to disruption of these stabilizing interactions. Therefore, the changes in the environment of protein can lead to changes in its three-dimensional structure. There is a wide range of work on proteins however; it is still almost an unanswered question as to which conformation the protein will migrate on experiencing a change either in pH, temperature or changing solvent conditions. The changes in the environmental condition might affect the choice between folding, misfolding and unfolding. The native conformation of protein which is the self assembly of protein has been the most important argument in biophysical chemistry.[1-3]

A remarkable way to perturb the conformational states of protein is the manipulation of solvent environment. Co-solvents can alter properties of proteins and can induce structural effects through biomolecular interactions of its functional groups with co-solvent molecules.[4-8] In this regard, ionic liquids (ILs) represent great interesting compounds having wide variety of applications in scientific and engineering fields particularly, in biophysical chemistry and bioengineering. Their use includes as additives, co-solvents or as reaction media for various chemical reactions. ILs are basically organic salts which are liquid below 100 ^{0}C. ILs are made up of combination of organic cation (e.g. ammonium, imidazolium, phosphonium, cholinium, pyridinium and etc.) with a variety of anions (e.g. Cl^-, Br^-, I^-, SO_4^- PF_6^-, $AlCl_4^-$, $CF_3SO_3^-$, CH_3COO^-, NO_3^-, BF_4^-, CF_3COO^- and etc.).[9] The cation is bulky with small anion, this combination of cation and anion give rise to big difference in their size which does not allow packing of lattice generally happening in many inorganic salts.[10]

Since the present perspective gives a detailed account of the effect of ammonium-based ILs on protein stability, therefore, it becomes almost mandatory to gives some detailed account of the properties of protic ionic liquids (PILs). Their formation takes place by the

transfer of a proton from a bronsted acid to a bronsted base. The basic contrasting feature which separates PIL from other ILs is the presence of proton-donor as well as acceptor sites. Ethyl ammonium nitrate (EAN) is the first discovered PIL in 1914 by Walden.[11] The distinguishing feature which separates EAN from other ILs is that just like water it can form three-dimensional hydrogen bonded structure.[12] All PILs are having a proton which is available for hydrogen bonding.[13] Among available PILs, EAN has grabbed most of the attention due to resemblance in properties and behaviour to that of water.[12, 14] EAN serves as compatible solvent for many proteins. The potential application of EAN for proteins includes improving solubility of proteins, as precipitating agent, also as an additive.[13,15] There is a variety of new ILs which have been synthesized and used as solvent in many chemical processes.[16] These ILs have been regarded as an alternative of organic solvents which is mainly due to broad liquid range, high thermal stability, non-flammability, solvation ability towards a wide range of solute.[17] The main application of ILs in the field of biophysical chemistry includes its usage in increasing the stability and enantioselectivity of enzymes, as refolding and crystallization additive and also as inhibitor of aggregation of various proteins.[18,19] In general, it is shown by literature that IL's polarity, hydrophobicity, miscibility with water are the key factors influencing the activity and stability of various proteins as well as enzymes.[20,21] There are numerous studies available in the literature which have revealed that bio-catalytic activity and thermal stability of proteins can be improved in the ILs.[22-27]

Research in the field of protein-IL interaction has remained a prospective avenue of investigation for years. Particularly investigations related to the stability of proteins in the presence of ILs are still in its early stages. More details describing the role of ILs in protein folding/unfolding studies has emerged from a range of both experimental[18-29] and theoretical[30-36] studies. The role of ILs in reversible folding-unfolding patterns of globular protein has been recognized by Sankaranarayanan et al.[28, 29] suggesting that beyond a certain concentration of ILs, there is a complete transition from helical to beta sheet conformation that ultimately can lead to a pre-fibrillar state. Zhang et al.[30] using molecular dynamic (MD) simulation studies explained the temperature dependence of methionine ligand dissociation and rebinding dynamics of cytochrome c (cyt c) in an aqueous solution. Recently, the studies related to protein ILs interaction are garnering mushrooming interest within scientific community.[37-39]

A number of comprehensive reviews on ILs with proteins have been published related to enzyme catalysis, Hofmeister series effect.[23, 40-44,] These studies suggest that modification

of the cation and anion structure of ILs have vast impact on the structure, stability and activity of enzymes however, the need is to assess the behaviour of different enzymes and proteins when they are exposed to same family of ILs. Is there any generalized trend observed? Is the impact of these ILs are totally depending on the protein used or there is any summarized output which can be put forward that can help to continue the research further in this field? All these questions need to be answered. Therefore, in order to search for these answers one has to dig deep inside the field of one family of IL and their interactions with various proteins so that we could get one simple thumb rule in predicting the behaviour of these ILs on proteins in future studies as well. In spite of the current thrust in research in this field, systematic characterization of interaction between protein and IL is far from being completely understood, hence, scrupulous systemization of the topic from varied view point is the need of the time. Simply elucidating the interaction between IL and protein would not be enough, it is really important to put emphasis on effect of at least one family of ILs on conformation, stabilization, activity as well as dynamics of the various proteins/enzymes involved.

The need of the time is to systematically characterize the effect of different ILs on the stability of different proteins. If careful analysis of these enzymatic reactions is carried out, it is revealed that the physiochemical properties of IL can play a pivotal role in altering the structure, stability as well as the activity of enzymes. Studies related to protein-IL interactions show that same protein can show different stability as well as activity behaviour in the presence of different ILs. A lot of confusion arises on the role of particular IL in stabilizing/destabilizing particular protein. Still there is no particular trend which can guide us in depicting the effect of ILs on protein stability. It is really essential to analyze the behaviour of different proteins in the presence of same family of IL. Therefore, the behaviour of particular protein in the presence of specific IL family can be gathered that would be helping in choosing and designing an IL which can serve as best solvent media for enzymatic reaction. An exquisite three-dimensional arrangement of protein's AA side chains defines the structural and functional properties of proteins, which are in turn influenced by the surrounding environment; therefore each specific family of IL may have distinct impact on the activity, enantioselectivity and protein stability. In this respect, recently ammonium-based ILs are emerging as best biocompatible solvents in many biomedical and various other scientific applications.

The aim here is to accomplish high degree of systemization of the information available in the open literature related to behaviour of different proteins in the presence of ammonium-based ILs. Major correlation between structure and activity of different proteins in the presence of ILs have been highlighted, the majority of the extent of our study have been focussed on the ammonium family ILs due to their biocompatibility as well as numerous applications.

The perspective recognizes the progress in understanding the phenomenon of protein stabilization in the presence of ammonium-based ILs. The fast growing applications of ammonium-based ILs through a range of applications varying from industry to biotechnology have been highlighted in the earlier portion of the perspective. Then, we have considered protein model compounds such as AAs as well as cyclic peptides (CDs) stability in ammonium-based ILs. In the latter section of the perspective, the effect of these ILs on individual proteins has been emphasized. Also some portions of the perspective have been devoted to study of same protein in presence of both ammonium as well as imidazolium-based ILs, depicting the contrast in behaviour of same protein under two family of ILs for the sake of comparison. Eventually, the perspective assesses the biocompatible behaviour of ammonium-based ILs in various studies related to proteins, ranging from suitable co-solvent media to protein crystallizing agent.

Important application of ammonium-based ILs in different scientific fields

The potential of IL as green solvent has opened burgeoning new areas of its application ranging from electrochemistry to biotechnology. As mentioned before, there are many advantages of ILs, despite the aforesaid advantages; recently many researchers are concentrated on the potential risk of these ILs regarding toxicity and biocompatibility. Among the various choices of ILs, ammonium-based ILs are seen to be most encouraging and safer alternative which can be used for the growth of pharmaceutical and various medical related applications[45-54]. The various applications of ammonium-based ILs in various fields are increasing day by day which is schematically presented in Scheme 1. The potential application of the ammonium family ILs in the field of proteins dates back to 1984 in which EAN was used to check the activity and stability of alkaline phosphatase.[45] Further, in 2001, the transesterification reaction of α-chymotrypsin (CT) was carried out by Lozano et al.[46] Recently, Heimer et al.[47]showed the potential application of methylammonium formate (MAF) and ethylammonium formate (EAF) for the oxidation of peptides. EAN has also been

used in the field of protein crystallization by Byrne and Angell[48] where the solubility of hen egg white lysozyme (HEWL) in aqueous EAN solution was exploited for the production of crystals of the protein. Of late, in 2014, Chen et al.[49] have shown the use of hydroxyl ammonium ILs for protein extraction thereby opening ways of potential use of ammonium-based ILs in extraction of other bio-analytes. Aside from the use of ammonium-based ILs in the field of protein stabilization studies, Zhao et al.[50] in 2002 highlighted the prospective role of ammonium-based ILs in industrial catalysis processes. Further in 2004, the use of ammonium-based ILs for electrochemical capacitors was highlighted by Sato et al.[51] The critical review by Galiński and co-workers in 2006, showcased the exploitation of ammonium-based ILs as electrolytes.[52] Roughly at the same time Pernak et al.[53] synthesized hydrophobic ammonium-based ILs which are air and moisture stable having potential application for wood preservation. Role of these ILs as lubricants or additives were focussed by Qu et al.[54] The composite material resulting from the combination of zeolites and ammonium-based ILs having latent relevance as hydrophilic conducting fillers for proton exchange membrane fuel cell.[55] Recent days they are receiving a very good response from the industrial point because of their high surface activity, less aqueous toxicity as well as resistance to oxidation and reduction processes.[51] There are various other applications of ammonium-based ILs apart from above mentioned, which includes development of biocompatible materials which can be used in pharmaceutical industry,[56] in chemical engineering as extracting agent[57] ammonium salts used as herbicides known as herbicidal ILs[58], energy storage devices[59], as extractants[60], fuel cell application[61], desulfurization of fuel[62] polymeric ILs in electrochemistry as solid electrolyte,[63] in polymer field as polymeric ILs[64]. Basically emphasizing the role of ammonium family ILs in biomedical applications, various efforts in the development of biocompatible and biodegradable materials can lead to enhanced electrical and pH-sensitive drug delivery systems.[56] The growing utilization of these ILs are rapidly increasing year by year and their growing importance is schematically represented in Scheme 2.

Behaviour of model protein compounds in the presence of ammonium-based ILs

As it is well known that proteins are made up of peptide bonds which are covalent chemical bonds formed between AAs. As suggested by Tietze et al.[43] in their review that smaller parts of proteins such as AAs and peptides need to be separated from proteins when studies related to synthetic chemistry, biochemistry and structural biology are concerned, keeping in view they possess different functions and interaction modes. However, before

coming to effect of ammonium-based ILs on biomolecules such as protein, it sometimes become essential to see the effects of the same family ILs on these smaller parts of proteins taking into account the wide application of CDs in drug production and their use in group additivity schemes to evaluate the protein folding studies. Attri and Venkatesu [65] quantified the bimolecular interaction between functional groups of the protein and ILs by reporting the apparent transfer free energies ($\Delta G'_{tr}$) of a homologues series of CDs from water to aqueous solutions of ILs using solubility measurements. The impact of ammonium-based ILs such as diethylammonium acetate (DEAA), triethylammonium acetate (TEAA), diethylammonium dihydogen phosphate (DEAP), triethylammonium dihydogen phosphate (TEAP), diethylammonium sulphate (DEAS) and triethylammonium sulfate (TEAS) were studied on the series of CDs such as cyclo(Gly-Gly), cyclo(Ala-Gly), cyclo(Ala-Ala), cyclo(Leu-Ala), and cyclo(Val-Val). The $\Delta G'_{tr}$ values for CDs from water to ILs were positive indicating stabilization of native structures of these CDs. The ILs followed the order TEAS>DEAS>TEAA>DEAA>TEAP>DEAP in their stabilizing tendency towards the CDs which shows sulphate anion to be strong stabilizers, acetate as moderate and phosphate as weak stabilizers.[65] In terms of role of cation, TEA^+ behaved as strong stabilizers while DEA^+ substituted behaved as weak stabilizers for CDs structure. The estimation of transfer free energy ($\Delta g'_{tr}$) of peptide backbone unit was carried out using the experimental results. Their findings conclude that the peptide bond, the peptide backbone unit, the alanyl residue and the valyl residue play a major role in protein folding/unfolding whereas the side chains play a less significant role in stability of protein.[65] Preferential exclusion of the IL from the surface of CDs was documented as the reason for stabilization, thereby unfavourable interactions between ILs and CDs were the main reason accounted for the model compound stability.

Keeping in view the fact that properties of protein are basically dependent on the nature and arrangement of AAs. In this regard, Vasantha et al.[66,67] studied the interactions of AAs such as alanine (Ala), valine (Val), leucine (Leu), histidine (His), tryptophan (Trp), and tyrosine (Tyr) with ammonium-based ILs such as DEAA, DEAS, TEAA, TEAS, TEAP and trimethylammonium acetate (TMAA) through $\Delta G'_{tr}$ using solubility measurements. The results suggested unfavourable interaction between ILs and AA which stabilizes the structure of AA and the ILs followed the sequence: TEAS> DEAS >TEAP >TEAA >DEAA>TMAA. This order was explained on the basis of variation of alkyl chain at the substituted ammonium cation as well as on the basis of solvation of IL. HSO_4^- competes effectively for the water molecules which are associated with the surface of AA because of strong its strong

interaction with the water molecules which leads to strong solvation of the ILs resulting in exclusion from the AA surface. This explains the more unfavourable interaction in case of TEAS as compared to TEAA. HSO_4^- acted as best stabilizer for the AA, $H_2PO_4^-$ and CH_3COO^- as moderate and weak stabilizer thus, obeying the Hofmeister order as HSO_4^- > $H_2PO_4^-$ > CH_3OO^-.

In order to deepen our understanding of the interaction between IL and AAs, it is necessary to assess the particular interaction of specific IL with AAs that might be helpful in detour the trend of the effect of ILs on the stability of AAs nature. Analysis of the solubility behaviour of Ala, Val, His, Leu, Trp and Tyr in DEAA, DEAS, TEAA, TEAS, TEAP, and TMAA reveals that the solubility of these AAs decrease in a monotonic fashion with the increasing concentration of ILs indicative of dominant salting-out effect. This solubility behaviour of AAs cannot be explained based on individual effect of cation and anion of IL since there exists a balance between competitive interaction of the cation, anion and water with the AA. This complex interaction plays the pivotal role in determining the preferential interaction and solvation of the IL with AA[66]. In this context it was suggested that a common basis of salting-in and salting-out phenomenon is the competition between water-AA side chain and IL-AA side chain and water-IL interactions[66]. The water affinity of AA decreases with increasing hydrophobicity as the side chain increases. Further, it is suggested that electrostatic forces are dominant at each IL concentration. In addition, the $\Delta G'_{tr}$ values of AA increase with increasing concentration of IL. Basically, due to difference in cation and anion species in different ILs, different ILs are solvated to different extent. Finally, it can be concluded that strongly solvated ILs promote salting-out effect and stabilize AA[66].

The systematic and quantative estimation of $\Delta G'_{tr}$ for glycine peptides (GPs) such as glycine (Gly), diglycine (Gly_2), triglycine (Gly_3) and tetraglycine (Gly_4) was carried out in the presence of DEAA, DEAS, TEAA, TEAS, TEAP, and TMAA.[68-70] Positive values of $\Delta G'_{tr}$ for GPs from water to ILs were observed which indicate that the interaction between ILs and GPs are unfavourable resulting in stabilization of the structure of model protein compound. Further m-values were obtained to get the mechanistic events of role of IL in enhancing the stability of the model compounds. DEAS > TEAA > TEAS > DEAA > TMAA > TEAP is the order followed by the ILs for Gly. Overall the results suggest that ammonium cation with acetate anion have comparable stabilities on stabilizing GPs. The IL containing TEA^+ cation such as TEAA acted as strong stabilizer while DEAA was found to be weak stabilizer for all the investigated GPs. Furthermore, it can be concluded from their results that

anion variation has significantly more influence on stability of GP as compared to cation variation.

With the objective of getting a glimpse of behaviour of these model compounds particularly AAs in other ILs, we have analyzed some of the available literature for the purpose of viewing the fact that whether the other IL is also following the same trend as that of ammonium family IL or there is some difference in the behaviour of these ILs. Recently Zafarani-Moattar et al.[71,72] have shown imidazolium-based ILs acting as destabilizer for the AA serine (Ser). They observed the ILs 1-(2-carboxyethyl)-3-methylimidazolium chloride [HOOCEMIM][Cl] and 1-carboxyethyl-3-methylimidazolium chloride [HOOCMMIM][Cl] were imposing favourable interaction with the AA as indicated by negative $\Delta G'_{tr}$values leading to destabilization of the AA. However, one of the recent studies by Vasantha et al.[73] suggests completely opposite behaviour of the same AA in ammonium-based ILs such as DEAA, TEAA, TEAP, DEAS, TMAA and TEAS. They show stabilization of Ser in ammonium family ILs. Unfavourable interaction between ILs and AA was accounted for the stabilization of Ser. The preferential attraction of water molecules by the surface of the AA ultimately leading to exclusion of ILs leads to stabilization of AA. Therefore, the ILs which are preferentially excluded are generally highly hydrated resulting in stabilization of the native state of the protein without any perturbation to the structure of the biomolecules. The results obtained so far on the stability of these protein model compounds in ammonium-based ILs portray them as stabilizing agents.

From the above studies on exploring the thermodynamic contribution of ammonium ILs on AAs, CDs and GPs suggest that there is no symmetry in ILs for the stability of these model compounds. However, some of the observations can be generalized from these studies and put forward for carrying out protein folding/unfolding studies further. Foremostly, the ammonium-based ILs through unfavourable interactions for all the model compounds behaved as stabilizers. Another point to be considered should be the contribution of anions of ILs in stabilization of these model compounds. In CDs the Hofmeister series was followed in sulphate only however, in case of AAs the anions followed the Hofmeister series completely. For Gly and Gly_4, ILs containing sulphate as the anion proved to be more efficient as stabilizing agent when compared to ILs containing phosphate however, for Gly_2 and Gly_3 phosphate anion containing ILs dominated over sulphate containing ILs. Obviously, the above results suggest that anion played the overruling role in stabilization of these model compounds. Keeping in view the role of cations of ILs it can be concluded by averaging the

results of many model compounds, TEA^+ behaved as strong stabilizer as compared to lower one, DEA^+. The possible explanation of this behaviour was based on viscosity effect. Since ILs having more viscosity slows down the conformational changes of the biomolecules leading to more compact structure. Finally, from the above discussions ammonium-based ILs can be considered as stabilizer for the protein model compounds when compared with imidazolium family ILs.

Behaviour of different proteins in presence of ammonium-based ILs

The role of ammonium-based ILs on the stability and activity of lysozyme

Lysozyme consists of 129 AAs residues.[74] Lysozyme forms the part of innate immune system. It protects us from danger of bacterial infection. It functions by attacking the protective cell wall of bacteria. Many attention-grabbing studies related to ammonium-based IL interaction with lysozyme have been carried out focussing the role of these ILs. A study by summers and flowers[18] on HEWL showed that EAN stabilized lysozyme against irreversible thermal denaturation. EAN was found to protect the enzyme since, on thermal denaturation the disulphide bonds remain intact with exposed hydrophobic core which results in intermolecular association of the hydrophobic core consequentially leading to aggregation. Nevertheless, the interaction between ethyl group of EAN and hydrophobic portion of the protein protect it from intermolecular association. The electrostatic interactions of its secondary structure were stabilized by the charged group of the IL. Denatured-reduced HEWL was found to regain 75% of its activity in EAN. Renaturation of denatured HEWL with disulfide bonds intact can produce properly folded protein however; renaturation of reduced HEWL is unproductive due to competition between aggregations and refolding. Nevertheless, chemically denatured-reduced HEWL in the presence of EAN led to high yield of active protein.[18] One major point to be noted that the structure of HEWL was denatured in the high concentration of EAN while in the presence of lower concentration of EAN high yield of active protein was obtained. Butylammonium nitrate (BAN) also played the role of renaturation of HEWL, however the yield of active protein was low as compared to EAN.[18]

Byrne et al.[19] reported the thermal refolding as well as extended period stabilization of lysozyme with concentration > 200 mg/mL. To achieve the refolded fraction of the protein EAN was used. About 97 % protection against aggregation in a single unfold-refold cycle was obtained. Triethylammonium methane sulfonate [TEA][MS] was able to refold 97% of thermally denatured HEWL.[19] Again in another study by Byrne and Angell showed the use of

EAN to dissolve the fibril of lysozyme.[75] For the sake of comparison two other ammonium-based ILs such as [TEA][MS] and triethylammonium triflate [TEA][Tf] were also used. EAN was found to be effective dissolving agent since dissolution was complete and immediate.[75]

Four different ammonium-based ILs based on cation chain modifications were used to check the stability and activity of HEWL using circular dichroism (CD) spectroscopy by Mann and co-workers.[76] The refolding ability of propylammonium formate (PAF) after heating the protein to 90 ^{0}C was more as compared to EAF at higher concentration, since lysozyme refolded at 62.5 wt% of PAF while it was between 25 wt% to 50 wt% for EAF. This was explained on the basis of refolding mechanism of both the IL. On unfolding of lysozyme there is favourable interaction between hydrophobic core of the protein and hydrophobic side chain of EA^+. This adsorption of the cation leads to procurement of net positive charge which prevents aggregation of the protein. Further, on cooling, the desorption of EA^+ is required for refolding of the protein. However, for refolding to occur the refolding energy must be ample to overcome the cation adsorption energy. At low concentration of EAF this condition is possible and refolding occurs at 25 wt% of EAF. Conversely, at higher concentration the energy of refolding is insufficient to overcome the cation adsorption resulting in not refolding of the protein due to the presence of ILs which are adsorbed. PAF was most effective in promoting refolding at concentrations of ~62 wt% however, its higher concentrations led to denaturation of the protein. Due to the increased hydrophobicity of the cation in PAF as compared EAF self-aggregation of PAF in water is greater which leads to lowering of the energetic cost of desorption of cation of PAF as compared to EAF which results in refolding of the protein at higher PAF concentrations.

Another ammonium-based IL 2-methoxy ethylammonium formate (MEOAF) which differed from EAF in alkyl chain length without substantial change in hydrophobicity as compared to EAF was proved to be more efficient in re-naturing the protein at 50 wt%. Reduced strength of hydrophobic interaction among $MOEA^+$ and hydrophobic core of lysozyme resulted in refolding occurring at higher IL concentration. Enhanced thermal stability of lysozyme in ethanolammonium formate (EtAF) was because it interacted in a different way with lysozyme when compared to other IL which did not contain alcohol. It was suggested that the electrostatic interaction between EA^+ and lysozyme and EtA^+ and lysozyme were almost same however, the presence of an alcohol group in EtA^+ creates an extra as hydrogen bond donor/acceptor leading reduced hydrophobic interaction with

lysozyme. In case of MEOAF there is presence of ether oxygen which can act only as hydrogen bond acceptor.[76]

All the above studies relates the bimolecular interactions between ammonium family IL and lysozyme on the other hand, experiments carried out by Judge et al.[77]exhibit the use of IL as precipitating agent and additive for the crystallization of lysozyme. tetraethylammonium bromide [TEA][Br] induced lysozyme crystallization in the concentration range (9-27% w/v). The precise changes in the solution condition such as pH, ionic strength was considered the main reason behind the effect the ammonium-based IL on lysozyme crystal morphology and crystal size which ultimately led to change in protein solubility or crystal growth. In a report by Mangialardo et al.[78] on the investigation of fibrillar lysozyme treated with four ammonium-based ILs having varying alkyl chain, the refolding efficiency of the ILs were evaluated. The fibrillar conformation is significantly reduced by the action of EAN which was the shortest chain IL. The long chain IL, propylammonium nitrate (PAN) and BAN did not help in driving fibrillar lysozyme in the direction of native conformation. They seem to operate by partly dissolving the fibril leading the protein in a partly β un-aggregated conformation. However, 2- methoxy ethyl ammonium nitrate (MEOAN) containing an ether group failed to act as a refolding additive. Basically these long chains ILs (PAN, BAN and MEOAN) lead to such a conformation of protein which contains β around 70% which is neither close to fibrillar structure nor to the native structure of the protein. Thus, their result clearly signifies the dependence of refolding capability of PIL on the cation structure.[78] It was unveiled that the mechanism of interaction of IL with the protein depended on the alkyl chain length of the cation of the IL.

From the above studies related to the role of ammonium-based ILs on lysozyme suggest the role of ammonium family ILs as refolding additive, fribrilizing agent, precipitating agent, additives for protein crystallization, prevention of aggregation, renaturing agent as well as stabilizers against thermal unfolding. Also, based on the above experimental results EAN can be termed as refolding additive from the thermally as well as chemically denatured lysozyme. Further, talking particularly about the cations having various hydrogen bond donor sites result in more effective coordination to the protein thereby stabilizing the biomolecule structure in more efficient manner. Also irrespective of the ILs, very high concentration results in protein destabilization.

The influence of ammonium-based ILs on the activity and stability of α-chymotrypsin (CT)

CT is a proteolytic enzyme comprising of 245 AAs with the catalytic triad made up of His 57, Asp 102 and Ser 195 which is the reactive group.[79, 80] It consists of total five disulphide bonds. It consists of total eight Trp residues. It is regarded as one of the most valuable enzymes for understanding the protein folding and unfolding. In 2001, Lozano et al.[46] reported the use of methyl trioctylammonium bis(trifluoromethyl) sulfonyl amide [MTOA][Tf_2N] as a reaction media to synthesize N-acetyl-L-tyrosine propyl ester by transesterification with CT, which showed the suitability of ammonium-based IL for the reaction media.[46] The synthetic activity decreased with decrease in polarity of the ILs. The main outcome of the result is the dependence of synthetic activity of CT on the polarity of IL irrespective of the different family of ILs. Hydrophobicity of the ILs played an important role when the stability of the enzyme in the assayed media is concerned. Since, increase in hydrophobicity of the system results in reduction of the interaction between protein and ions by maintaining the essential water layer surrounding the protein thus resulting in enhanced stability of the enzyme. Later in 2003, Lozano et al.[81] found that the polarity of IL can play a major role when the stability of the enzyme is analyzed in the assayed media. The half life time of CT in IL kept an inverse relation with the polarity of the solvent. The half life time of CT in [MTOA][Tf_2N] which is having lowest polarity among all the used ILs was 13.5 times higher than that of in 1-propanol thus hinting to the fact that CT was responsive to deactivation by the increase in poarity. The evaluation the activity of the CT dissolved in a range of ammonium-based ILs such as diethanolammonium chloride (DEACl), diethanolammonium methanesulfonate (DEAMS), N,N-dimethylethanolammonium acetate (DMEAAc), N,N-dimethylethanolammonium glycolate (DMEAGly), N-butyldiethanolammoniumtrifluoromethanesulfonate [BDEA][TFMS], bis(2-methoxyethyl)ammonium sulfamate [DMOEA][SN] and 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF_6]) using N-acetyl-L-phenyl-alanine ethyl ester (APEE) as the substrate was carried out by Falcioni et al.[82] On contrary to the expectation, CT was found to be inactive in all studied ILs. However, surprisingly it maintained some of the activity in [Bmim][PF_6].The misfolding/unfolding of CT in DEACl was assumed to be the reason of loss in activity. In another study by Attri and Venkatesu[83] suggested that longer hydrophobic ammonium cations carrying longer alkyl chain are weak stabilizer while small alkyl chains are strong stabilizers for CT structure. The reason for the observed behaviour was

unfavourable interaction of the ILs with the disulphide bonds of CT while the stabilizing ILs tend to be excluded from the enzyme surface.

The aforementioned studies carried out by different groups using CT suggest that the physical properties of ILs such as polarity, hydrophobocity play major role in their stabilizing behaviour towards CT. Another major point comes out here is that the maintenance of the native structure of CT is important for preservation of the activity of the enzyme. Except hydroxyl-based ammonium ILs, all other ammonium-based ILs proved to either as a suitable solvent for carrying out various reactions including transesterification or as a stabilizing co-solvent.

The role of ammonium-based ILs on the stability of Heme proteins

It is well known that haemoglobin (Hb), myoglobin (Mb) and cyt c all come under the category of heme proteins. All of them play important role in numerous biological processes particularly involving transport of oxygen and electron transfer in life processes. In 2011, Wei and Danielson[84] studied the structural stability of cyt c in alkylammonium formate (AAF) ILs using spectroscopic methods. It was put forward by the authors that even in high concentration of 50-70% AAF/water or AAF/phosphate buffer, the native structure of cyt c was maintained however, the activity of cyt c in 80% AAF/water was decreased as compared to cyt c activity in buffer. Also, they suggested that the thermal denaturation of the enzyme followed different pattern as compared to its denaturation by increasing concentration of AAF.[84] Another stabilization study of cyt c in ammonium-based ILs was carried out by Jaganathan et al.[85] using MD simulation. The hydrogen bonding between functional groups of protein and water as well as between EAN and water plays important role in the stability of cyt c in EAN. Assembly of EAN around the hydrated protein is supposed to impart increased stability to the protein. The electrostatic interaction and hydrogen bonding between cation and anion in EAN leads to generation of ionic domains.[86] This solvophobic force[87] compels the alkyl group to cluster in apolar domains. The above interaction mechanism suggested by Jaganathan et al.[85] is consistent with sponge like structure of EAN with alternating layers of non-polar and charged regions as suggested by X-ray reflectivity studies by Niga et al.[86] Therefore, the organized assembly of EAN around the hydrated protein is providing stability to the protein.

The ion specific effect of ammonium-based ILs on Mb by Attri et al.[88] showed that phosphate-based IL to be strong stabilizer, sulphate to be weak in stabilizing the heme protein

while acetate was found to destabilize the protein which was mainly because of weak hydration and increase in hydrophobicity of anion having stabilizing effect on the native state of the protein.[88] Also viscosity of the IL can play dominant role in their tendency towards stabilization of the protein.[89]The conformational changes taking place in biomolecules slow down in high viscosity IL such as TEAP (64.31 mPa.s) as compared to trimethylammonium dihydrogen phosphate (TMAP) (7.62 mPa.s) which allows the protein to maintain a more compact structure. Also increase in the alkyl chain of the cation of water-soluble ILs can lead to less water absorption.[90] There is less local arrangement of hydrogen bonds of water molecules around the higher alkyl chain length of the cation. This leads to strong exclusion of less hydrated TEAP from the water molecules at the surface of protein in comparison to TMAP.

So far literature considered anion effect on protein stability [91] recently, we for the first time showed the effect of cation chain length on the structural stability of well known heme proteins Hb and Mb.[92] We observed unfolding of heme protein induced by cations. A series of ammonium family ILs having hydroxide anion with variation in alkyl chain length of the cation such as tetrabutylammonium hydroxide (TBAH), tetrapropylammonium hydroxide (TPAH), tetraethylammonium hydroxide (TEAH) and tetramethylammonium hydroxide (TMAH) were used. Unexpectedly, it was found out that in the presence of these ILs the thermal stability of both the heme proteins decreased leading to unfolding of protein at lower temperature as compared to buffer. The enhancement of the peptide sequence solubility by the ILs and preferential interaction of ILs with protein functional groups were the main reason behind observed structural distorations of the heme proteins. The trend of the distortion effect followed TMAH > TEAH > TPAH > TBAH, thus, short alkyl chain containg cation to be more denturing as compared to more hydrophobic long chain cation. This trend indicates that shorter alkyl chain interacts strongly with the hydrophobic part of the protein. The same reason as stated by Attri et al.[88] based on viscosity of ILs was the cause of the observed behaviour since TBAH has the highest viscosity (6.69 mPa s) while TMAH is having the lowest viscosity (2.77 mPa s). Also due to large size of the higher alkyl chain cation they are little away from the surface of the protein resulting in less contact of the cation and the protein surface.

The above mentioned studies clearly demonstrate that ammonium-based ILs act as stabilizing agents for the heme proteins in most of the cases. Again, EAN plays the role of biocompatible solvent with its peculiar characteristics of sponge like structure with

alternating non-polar and charged regions. Also the alkyl chain of the cation of IL is imposing greater impact as far as the stability of heme proteins particularly Mb and Hb are concerned. The higher alkyl chains having more viscosity are comparatively more stabilizing as compared to shorter alkyl chain cations having less viscosity.

The influence of ammonium-based ILs on the activity and stability of Lipases

Lipases constitute family of enzyme which hydrolyze triglycerides. Candida antartica lipase B (CALB) consists of 317 AA residues having molecular weight of 33.27 kDa.[93] The enzyme is having Ser-His-Asp catalytic triad in its active site. In the study by Lozano et al.[94]described how with the increase in hydrophobicity of the functional side chain of the cation both synthetic activity and selectivity decreased since an increase in the hydrophobicity of the functional side chain of the cation of IL leads to increase in free water molecules which acts as nucleophile acceptors so produce a loss in selectivity. The worst activity was found to be in most hydrophilic IL (3-Hydroxypropyl)-trimethylammonium bis(trifluoromethylsulfonyl)imide ([C_3OHtma][NTf_2]) which could be due to disruption of the hydration shell around the protein caused by strong hydrogen-bond interaction of the functional side chains of the cation with the essential water molecules.[94]

Lau et al.[95] studied the dissolution of CALB in EAN and the reaction rate of transesterification of ethyl butanoate was 10 times slower thus leading to the conclusion that the ILs that interact suitably sturdily with the proteins affecting its dissolution bring about structural changes that results in loss of activity. However, contrary to above expectation modest transesterification activity was maintained in triethylmethylammonium methyl sulphate ([Et_3MeN][$MeSO_4$]) even though it dissolved CALB. Loss of activity in the EAN is caused by changes in the enzyme secondary structure as confirmed by FT-IR spectroscopy, however, in the latter case the native structure of the enzyme was preserved in agreement with the preservation of catalytic activity. IL must imitate water with respect to hydrogen bonding when dissolution of protein is concerned at the same time the interaction among IL and protein should be moderate otherwise stronger interactions can result in severance of the hydrogen bonds responsible for the maintenance of the secondary structure of the protein resulting in enzyme unfolding. Thus, in order to maintain the activity of IL-dissolved enzymes there should be a balance of hydrogen bond accepting and donating properties. Some of the ions which do not easily penetrate the matrix of the protein will lead to dissociation of many inter molecular hydrogen bonding to create few new ones could lead to maintenance of activity. [95]

In 2005, Diego et al.[96] reported the stabilization of CALB using water-immiscible ILs such as butyltrimethylammonium bis(trifluoromethylsulfonyl) imide ([btma][NTf_2]) and 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide [Emim][NTF_2]. The synthetic activity of CALB being highest in imidazolium-based IL followed by ammonium-based IL thus, proving the suitability of both the ILs for butyl butyrate synthesis. The addition of aqueous solution of enzyme to the IL should not be considered as dissolved rather it is included because of the formation of strong ionic matrix in water immiscible ILs, thus providing a suitable micro environment for the catalytic action to take place and also resulting in enhanced enzyme stability. Specific interaction between the enzyme and the solvent or interaction between the solvent and essential water molecules around the enzyme can account for the above explanation. Maintenance of the secondary structure of the enzyme is the main reason of the preservation of the catalytic activity of the enzyme in these ILs indicating protection of the native conformation of the enzyme by these against deactivation. As compared to hexane, the synthetic activity as well stability of CALB were improved in water-immiscible ILs such as [btma] [NTf_2] at 2% v/v water content and 50 ^{0}C. The low solubility of water in the ILs can be the reason leading to preservation of critical water molecules in the enzyme's microenvironment.[46, 97-99] Therefore, it can be suggested that water immiscible ILs form strong ionic matrix in which CALB molecules can be retained in an adequate microenvironment which results in a supramolecular net, thus keeping the protein in active conformation. The stabilization of the enzyme was supposed to be related to evolution of α-helix to β-sheet resulting in a more compact conformation which is able to exhibit activity.

Free and immobilized lipases were used for the synthesis of butyl propionate in reaction media which consisted of nine different ILs such as [Bmim][PF_6], 1-methyl-3-octylimidazolium hexafluorophosphate [Omim][PF_6], 1-butyl-3-methylimidazolium tetrafluoroborate [Bmim][BF_4], 1-hexyl-3-methylimidazolium tetrafluoroborate ([Hmim][BF_4]), 1-methyl-3-octylimidazolium tetrafluoroborate [Omim][BF_4], 1-butyl-2,3-dimethylimidazolium hexafluorophosphate [Bdmim][PF_6], 1-butyl-2,3-dimethylimidazolium tetrafluoroborate [Bdmim][BF_4], 1-ethyl-3-methylimidazolium ethylsulfate [Emim][ES] and cocosalkyl-pentaethoxi-methylammonium methylsulfate ([CPMA][MS]). High level of synthetic activity was obtained for Novozym 435 which was immobilized CALB in ammonium-based IL, [CPMA][MS].[100] No particular reason behind such behaviour was mentioned. It was explained based on the structure of IL which suited the reaction specificity

due to the philicity among the alkyl chain of the substrate and cation of the IL. The different synthetic activity of different lipases in the same IL suggests different structural conformation of different lipases was responsible for the discrepancy in results. The synthetic activity of free CALB in [CPMA][MS] increased proportionally with temperature while free free T. lanuginosus (TLL) only showed a slight increase in activity in the same IL. As already explained, the stabilization of enzyme in water immiscible tetraalkly ammonium cation based IL as a result of preservation of the active conformation of the enzyme in the IL net which safeguards the hydrated conformation of the enzyme however, in the present case [CPMA][MS] which is water miscible IL, different types of interactions between enzyme and IL such as hydrogen bonding, van der Waals interaction can result in supramolecular cage which is able to maintain the native conformation of the enzyme,[101] on the other hand its miscibility with water results in increased enzyme deactivation at higher temperature.

The different structural conformation of the enzyme under assay was the basic reason behind the obtained activities of free and immobilized lipases. The formation of supramolecular net as a consequence of enzyme-IL interaction was another highlighting point. Also the view cannot be generalized that ILs which interact sufficiently strongly with the protein will always result in loss of activity, therefore, changes in the structure of enzyme on dissolution will be the deciding factor in this case. Hydrophobicity of the functional side chains can play a major role in enzyme activity as well as selectivity towards a particular reaction. The above studies clearly show the hurdles involved in predicting a simple thumb rule mechanism to predict the behaviour of CALB in ammonium-based ILs. Generally, in all the cases the ammonium-based ILs proved to be stabilizers however, the mechanism varied depending on the miscibility of IL with water which in turn affected the hydrogen bonding between enzyme and ILs.

The effects of ammonium-based ILs on the activity and stability some other proteins

Succinyl concanavalin A (S Con A) is basically known to exist as dimer in nature[102]. It is obtained by the treatment of Con A with succinic anhydride[102]. Structural changes of S Con A in the presence of DEAP, DEAS, TEAP and TEAS were investigated using fluorescence and CD spectroscopy.[102] Combination of kosmotropic anion and chaotropic cation stabilized the native structure of S Con A as revealed from the thermodynamic profile. From the transition temperature (T_m) analysis, it is revealed that TEAP and DEAP as strong stabilizers, TEAS as moderate and DEAS to be weak stabilizer. The CD spectra of S Con A in these ILs

were having more pronounced β-structure. The more hydrophobic alkyl chain decreases the protein-anion interaction; therefore they acted as strong stabilizers while small alkyl chain cation being more hydrophilic leads to increased anion-protein interaction. Also using spectroscopic and NMR technique, DEAP and TEAP were found to be refolding additives for the S Con A, however rest of the ILs failed to do so. The mechanism suggested was on thermal denaturation of S Con A, there is favourable interaction between its exposed hydrophobic core and alkyl side chains of DEA^+ and TEA^+ leading to adsorption of DEA^+ and TEA^+ to hydrophobic regions of S Con A. This created electrostatic repulsion that prevented S Con A aggregation. In another work by Attri and Venkatesu[103] on S Con A using ammonium family ILs having different kosmotropicity with chaotropic trialkylammonium cation such as TEAP, TMAA, TMAP and trimethylammonium hydrogen sulfate (TMAS). It was suggested that again ammonium-based ILs was proved to be compatible solvent for the S Con A. The unfavourable interaction between ILs and surface of protein helped in formation of hydration layer around the protein, in the intervening time ILs led to enhancement of water structure and formation of hydrated layer with water molecules. All these resulted in inability of the surface of protein to interact with the hydration layer around the IL consequently non-binding interaction between IL and surface of protein eventually leading to preferential removal of IL from the surface of protein. In this case again IL consisting of long chain substituted cation with phosphate anion such as TEAP was strong stabilizer as compared to short chain cation consisting IL such as TMAP. Once again the IL having high viscosity stabilized the enzyme more as compared to IL having less viscosity. Two things happen when alkyl chain of the cation is increased. Firstly, the increase in alkyl chain length results in less water absorption, secondly the arrangement of hydrogen bonds of water molecules are less locally ordered in case of higher alkyl chain length. Therefore, TEAP is less hydrated when compared to TMAP thus more strongly excluded from the water molecules present at the surface of protein.

Serum albumins play an important role in the transport and deposition of a variety of endogenous and exogenous substances in blood.[104] The primary structure consists of single chain of about 580 AAs[105] residues while the secondary structure consists of 67% of α- helix of six turns and 17 disulphide bridges. There is almost 70% similarity in sequence of BSA (bovine serum albumin) with HSA (human serum albumin). EAN [>97%] and 2-hydroxyethylammonium formate (HEF) were used as solubilizers, adsorption solvents and supporting electrolytes for electrochemical analysis of human serum albumin (HSA) and

bovine serum albumin (BSA). It was observed that the ILs competed for the electrode surface with the analyzed proteins. Seeing from the point of view of co-adsorption processes, there was no interference of EAN while only small decrease in Tyr and Trp peak was observed in presence of [HEF].[106] However, a stronger co-adsorption effect was observed in case of imidazolium family ILs.

Laccase is a member of the family of multicopper oxidases. It is a glycoprotein which catalyses the oxidation of orthopehols, paraphenols, polyamines. It consists of a typical three-domain fold with T1 mononuclear copper centre in domain 3 and also a trinuclear copper cluster which is positioned between domain 1 and 3. [107] The very first report regarding activity and stability of laccase in ammonium-based IL by Feder-Kubis and Bryjak [108] reveal that ammonium-based water immiscible ILs increased the stability of the enzyme even more than buffer. The IL composed of same anion (NTf_2) with ammonium cation such as heptyl[(1*R*,2*S*,5*R*)-(–)-menthoxymethyl]dimethylammonium and decyl[(1*R*,2*S*,5*R*)-(–)-menthoxymethyl]dimethylammonium differing in the alkyl chain length. Laccase was found to be exceptionally stabilized in the presence of heptyl [(1*R*,2*S*,5*R*)-(–)-menthoxymethyl]dimethylammonium cation containing IL. Interestingly, ammonium family ILs bearing shorter alkyl chain was better than their longer counter parts. The ILs based on heptyl substituent which is shorter alkyl chain IL as compared to decyl supported very good enzymatic activity as well as stability. Therefore, it was revealed that the influence of ILs on the conformational stability is governed by the cation structure of IL. Another work on laccase[109] revealed that as compared to 1-butyl-3-methylimidazolium trifluoromethanesulfonate [Bmim][TfO] and 1-butyl-1-methylpyrrolidinium trifluoromethanesulfonate ([Bmpyr][TfO]), tetramethylammonium trifluoromethanesulfonate ([TMA][TfO]) stabilized laccase by its interaction with water surrounding laccase which is leading to shrinking of the surface area exposed to solvent since its direct interaction with enzyme is difficult to occur[91, 102, 110] which supports the fact that enzymes are stabilized by chaotropic cation as revealed in their study by Zhao.[111] Further, it was also revealed that at pH which is lower than the isoelectric point (pI) of the enzyme, the anion dominates to the laccase and IL interaction however, at pH which is higher than the pI of the enzyme, the interaction is subject to influenced more by cation, thereby depending on the nature of the ion whether chaotropic or kosmotropic, the enzyme will be stabilized or destabilized at that pH.

Kumar and Venkatesu[112] showed that some of the ammonium-based ILs such as TMAS, TEAS, TMAP, TEAP and TMAA can facilitate the prevention of Insulin (*In*) from

its aggregation, since the monomeric form of *In* is the active form. Not only the ammonium-based ILs were helpful in preventing it from self-aggregation nevertheless, it also proved to be useful in controlling the thermal instability of the protein.[112] An exceptional example of enzyme catalysis in low-water system was focussed by Falcioni et al.[82] Subtilisin was found to be active in DEACl which was surprising since PILs containing chloride anion was considered to be detrimental for the activity of enzymes.[82] The probable reason for this typical behaviour was hypothesized to be coordination of the two cation hydroxyls to chloride which overcomes the denaturing capability of chloride anion.

Therefore, from above results it is revealed that stabilization of proteins in ILs depends on the type of interaction exhibited by proteins and ILs. Generally, stabilizing ILs interact unfavourably with the proteins therefore, they are excluded from the protein surface indicating adoption of a completely folded structure by the protein with least exposed surface area to water molecules. Alternatively, ILs augment the water structure, forming a hydration layer with water molecules, also there is formation of hydration layers by the surrounding network of proteins with water molecules. This leads to inability of polypeptide chain of protein to interact with the hydration layer around the IL. There is non-binding between IL and the protein surface, and eventually IL is preferentially expelled by the surface of the biomolecule, which causes the IL to stabilize the native structure of protein[8,91,92].

On the other hand, some of the results show that ILs destabilize the native structure of the protein. In this case, IL interacts preferentially with protein functional groups that lead the protein to be preferentially bonded with the ions of ILs. Obviously, there are favourable interactions between the ions of the ILs and surroundings of protein. There are also direct protein-IL interactions which lead to major conformational changes in the protein structure thereby promoting protein unfolding[8,91,92]. For the sake of clarity and distinction, we have explicitly shown these mechanisms in Scheme 3.

It is of particular interest to emphasize on the role of ILs and their specific feature as solvents particularly for proteins. Additionally, the enzyme's activity, specificity and stability are often related to the solvent properties of ILs such as polarity, viscosity, hydrophobicity, ion kosmotropicity as well as hydrogen-bond basicity and nuclophilicity of anions. Generally, the polarity of ILs is found to be closer to lower alcohols based on solvatochromic studies.[113,114] The viscosities of ILs are more than that of conventional organic solvents.[16] In this context, it was suggested that high viscosity of ILs slows down the

conformational changes taking place in the protein which ultimately results in maintenance of native structure of the enzyme as well as its activities.[40,102,103] Further, hydrophobicity of ILs which is expressed in terms of log P scale is another important feature of solvent property affecting the enzyme performance in ILs. The kosmotropicity of ions based on well known Hofmeister series is another important aspect affecting the solvent property of ILs[41,89].

It is noteworthy to shed some light on the effects of ions on solvation on general basis. ILs are made up of ions with the fact that in aqueous solution hydrophillic ILs dissociate into individual ions.[89] Therefore, combination of effects of ions on the solvent structure and the specific interaction between the ions and the biomolecule needs special attention. Taking the general case, the ions can influence both the surface of the protein as well as the structure of water. Viewing from the mechanistic point of view, the process involved in modulation of an aqueous solution in solvating a polar surface. This is based on the division of interfacial region around a solute into three layers assuming each layer to be one water molecule thick.[115] Solvation layer being defined as the first water layer around the protein surface while the second and third layer designated as transition layer and bulk surface.[115] The insertion of ions into the third interfacial water layer modifies the interaction of second interfacial water layer in helping the first water layer to solvate the protein surface. The insertion of strongly hydrated anion into the third water layer results in inability of the second water layer in helping first hydration layer to solvate the protein. This ultimately leads the protein to minimize its solvent exposed surface area by becoming more compact.[116-120] However, when a weakly hydrated anion gets inserted into the third water layer, the second water layer is now free to help the first water layer to solvate the surface of protein. Now the protein attempts to unfold, thus facilitating the maximization of its solvent exposed area.[121]

Also, solvation is ordered by interfacial tension between solvent and solute. Talking in terms of the interfacial tension between solvent and solute, it has been found that dissolved solutes play a role in altering the surface tension at water and protein interface. Dissolved solutes in an indirect manner increase the surface tension at the interface.[122] This increase in surface tension occurs because of creation of a complex geometry near the interface which makes it difficult for water molecules to increase their interaction in the interfacial region therefore, preference of water molecules an interior location away from interface thus creating a force which attempts to minimize the amount of interface. Therefore, if any substance increases the surface tension of water will be preferentially excluded from the interface. This increase in surface tension is the main reason of strongly hydrated solute near

the non-polar portion of the surface of the protein and induces driving force for the burial of non-polar surface.[121]

Effect of alkyl chain length of cation of ammonium-based IL on the stability of proteins

After going through all the literature regarding the effect of ammonium family ILs on the stability of proteins, one crucial aspect which should be emphasized is the effect of alkyl chain length of the cation of IL. Generally, the anions are supposed to have a more dominant impact as compared to cations for the stability of proteins; however, the literature survey shows that the effect of alkyl chain length of the cation on the protein stability is almost as important as that of anions. The study related to protein model compounds by Atria and Venkatesu[65] shows that higher alkyl chain length cation such as TEA^+ to be strong in stabilizing the CDs as compared to DEA^+. Again the same group found the same result for lysine peptides.[68-70] On contrary to the above result, Mangialardo et al.[78] found out that short chain IL, EAN was efficient in taking the fibrillar lysozyme towards its native conformation while higher members with increased chain length such as PAN and BAN failed to do so. Attri and Venkatesu [83] found out that ammonium cation with longer hydrophobic group were weak stabilizer to CT while small alkyl chain cation containing IL was strong stabilizer. Conversely, Jha et al.[92] bring into being that the thermal stability of the heme proteins Mb and Hb decreased with decrease in alkyl chain of the cation. Thus, the short alkyl chain cation containing IL TMAH was more denaturing than more hydrophobic long chain cation TBAH. For the protein S Con A, more hydrophobic alkyl chain cation acted as strong stabilizer while smaller counterparts acted as weak stabilizer.[102] Interestingly, for laccase the story was somewhat different. The IL consisting of shorter heptyl substituent in the cationic part was better than the IL consisting of larger decyl substituent in the cationic part as far as enzymatic activity and stability are concerned.[108] From the above studies, it is concluded that the interaction between the cation of the IL with the protein is dependent on the alkyl chain of the cation as well as on the nature of protein. That is why, we did not get one pattern of stabilization/destabilization of the protein in ILs. However, if one goes by the majority of study it can be interpreted that in most of the cases the cation containing more hydrophobic alkyl chain are more efficient in stabilizing the protein as compared to smaller alkyl chain cation having less hydrophobicity.

Novel character of ammonium-based ILs towards various stresses

As it is known, proteins are multifarious systems exhibiting huge number of conformations. However, there are various stresses in the environment such as heat, cold, high salinity etc. which are fatal for the conformational stability of proteins which need to be handled. So far, literature shows that ammonium-based ILs can prove to be helpful in overcoming these stresses. There is literature which shows that even in the presence of the denaturing agents the protein conformation can be protected in the presence of ammonium family ILs. Also, as we know that the problem of protein aggregation is one among the various causes of major economic loss in the field of biotechnology and pharmaceutical industries that also can be overcome in the presence of these ammonium-based ILs. The detrimental effects of protein aggregation is not only limited to manufacturing process also protein mis-folding can result in various diseases such as Alzheimer, Parkinson and other diseases. Therefore, it is really important to put emphasis on the novel behaviour of the ammonium family ILs so that these analyses can prove beneficial in carrying out further research in the area of protein stabilization to achieve various goals. The story of the novel behaviour of ammonium-based ILs starts with the EAN. As already been discussed, EAN serves as a solvent for different proteins for renaturation purpose as well as in crystallization studies. Also quite evident from these studies, EAN serves as good solvent for the proteins due to hydrophobic, ionic nature and its ability to form hydrogen bonds. Aggregation of proteins is one of the major challenges faced during their renaturation process, EAN has proved very effectual in preventing this aggregation.[18] As mentioned before, the renaturation of lysozyme against thermal denaturation using EAN by Summers and Flowers in 2000.[18] Further, in 2007, Byrne et al.[19] showed the protecting role of EAN against aggregation. In 2012, Mangialardo et al.[78] showed the refolding behaviour of EAN by driving the fibrillar conformation of lysozyme towards native conformation. The fibrillar conformation of lysozyme which mainly contains β-aggregates was considerably decreased in the presence of EAN.

After EAN the tradition was further carried out by other ammonium family ILs. Attri et al.[123] showed attenuation of the deleterious action of well known chaotrope, urea, on the CT by TEAA using CD, fluorescence and NMR techniques. It was found that TEAA and urea mixture substantially increased the T_m values which showed the counterbalance of the urea induced denaturation of CT. The core point is that the damaging effect on CT in 5 M urea was counteracted by only 1 M TEAA. The reason behind this behaviour was devised to be the ability of acetate ion to readily form H-bonds leading to strengthening of water-water and water-urea interactions and decrease in urea-CT hydrogen bonds. The increased

kosmotropicity of the acetate anion due to high viscosity *B*-coefficient was the foremost reason for the observed behaviour.[123]

In another study by Attri et al.[124] it was proved that TEAP acted as refolding additive from the urea-induced chemical denatured state for CT and S Con A. As compared to water which refolded up to 3 M urea denatured S Con A, TEAP refolded up to 5 M urea-induced denatured S Con A. All the local polar interactions and disruption of non-local interactions from the denatured state of S Con A may be renatured by TEAP. In case of CT, water failed to refold chemically-denatured CT structure, however, TEAP refolded up to 3 M urea-induced structure of CT significantly recovering both secondary and tertiary perturbed structure. In order to differentiate between the above mentioned studies, both the phenomenon counteraction and refolding abilities of IL are presented in Scheme 4. Further, TEAA renatured the CT structure from thermal denatured at 80 ^{0}C therefore, suggesting TEAA to act as refolding additive for thermally unfolded structure of CT.[91]There is favourable interaction between the exposed hydrophobic core of CT with the hydrophobic alkyl side chain of TEA^{+} resulting in adsorption of TEA^{+} to the hydrophobic region. On heating this adsorption is exposed which leads to electrostatic repulsion which prevents the aggregation of the enzyme. The coordination of cation and anion to the charged residues leads to stabilization of the secondary structure of the enzyme. In one of the studies by Attri and Venkatesu[103] in 2012, TEAP was shown to be acting as an efficient refolding agent for thermally denatured S Con A. The protein S Con A was renatured from its thermally denatured state in the presence of TEAP. The suggested mechanism was same as in case of TEAA acting as refolding additive for CT. [91]

Coming back to EAN, not long ago study carried out by Jaganathan et al.[85] for denaturation of cyt c using urea in the presence of EAN showed that EAN can act as renaturing agent for the protein by the formation of tight organized assembly around the protein in which the beta strand state exists as local minimum energy state.[85] Of late, in 2013, Attri and Choi[125] showed that TEAP strongly attenuates the detrimental action of atmospheric pressure plasma jet (APPJ) on the enzyme CT. This ammonium-based IL TEAP is able to maintain the structural integrity as well as activity of CT even after the exposure of APPJ. The strong interaction of TEAP with the protecting water layer around CT results in protection of CT structure by not allowing the penetration of reactive oxygen species (ROS) radicals and other charged species to interact with the CT. Recently, Awanish and Venkatesu, [112] for the first time showed ammonium-based ILs as novel solvent for *In* for offsetting self-

aggregation of *In* in the presence of TMAS, TEAS, TMAP, TEAP and TMAA. Therefore, native structure of *In* was found to be stabilized in the presence ammonium family ILs by unfavourable interactions with the surface of protein.[112]

Byrne et al.[126] studied the stabilization of Tabacco mosaic virus (TMV) in ammonium-based ILs. Ethylammonium mesylate (EaMs) stabilized the tertiary structure of TMV with 10 ^{0}C improvement in the thermal stability of TMV. Propylammonium mesylate (PropMs) also followed the similar trend. Ammonium-based ILs proved to be successful in stabilization of virus too, with enhanced shelf-life, thus proving their novelty. The successful use of ammonium-based ILs is not only limited to protein stabilization but also they stabilize the virus. Kennedy et al.[127] used EAF, ethylammonium propionate (EAP), ethylammonium methanesulfonate (EAMs), ethylammonium pivalate (EAPv), ethylammonium trifluoroacetate (EATfA), ethylammonium acetate (EAA), EAN, ethanolammonium nitrate (EOAN), triethanolammonium nitrate (TEOAN) and diethanolammonium nitrate (DEAON) as additive for crystallization experiments for HEWL, trypsin and glucose isomerise in which they found that in most of the cases the ammonium-based ILs were effectual in increasing the size as well as quality of the crystals of the protein.[127]

Assessment of contrasting behaviour of ammonium-ILs and imidazolium-based ILs

The cornerstone of the present perspective has been protein stabilization in ammonium-based ILs however, keeping in view the wide applications and uses of imidazolium family ILs in various fields it is really important to throw some light on the protein stabilization in imidazolium-based ILs too, from the evaluation point of view. This comparison between the two families of ILs will give us future guidance in our choice of ILs in protein stability study and also give the reason behind choosing ammonium family ILs as our subject of this perspective. As reported in the preceding text, the stability of AAs in ammonium-based ILs indicated unfavourable interaction of ammonium family ILs with AAs leading to increase in their stability.[66-70] In recent studies by Zafarani-Moattar et al.[71, 72] on the interaction of Ser with [HOOCMMIM][Cl] or [HOOCEMIM][Cl] suggested favourable interaction between AA and ILs consequentially destabilization of Ser in both the imidazolium-based ILs. However, in one of our recent studies, the same AA was stabilized in ammonium-based ILs such as DEAA, TEAA, TEAP, DEAS, TMAA and TEAS through unfavourable interaction between ILs and AA ultimately resulting in stabilization of the AA.[73] When free CALB was assayed in water-miscible IL [CPMA][MS], best synthetic activity was obtained as compared

to other imidazolium-based ILs used.[100] Also there was an enhancement of synthetic activity of free CALB in [CPMA][MS] with the increase in temperature.[100]

The stability and activity of CT in the presence of both ammonium and imidazolium-based ILs such as TEAA, TEAP, 1-benzyl-3-methylimidazolium chloride [Bzmim][Cl] and 1-benzyl-3-methylimidazolium tetrafluoroborate [Bzmim][BF_4]) respectively, were explored by Attri et al.[91] The stability of the native CT varied from IL to IL, following the order TEAA > TEAP > [Bzmim][Cl] > [Bzmim][BF_4], thus, confirming that imidazolium family ILs were weak stabilizer for CT while ammonium-based IL were strong stabilizers and therefore more biocompatible ILs for CT stability. In terms of activity measurements, the larger alkyl chain ILs such as [Bzmim][Cl] and [Bzmim][BF_4] were bound to functional active site of the enzyme resulting in lower enzyme activity while lower alkyl chain of TEAA and TEAP were non-functional folded with enzyme resulting in higher enzyme activity. The contrasting nature of ammonium and imidazolium-based ILs is also consistent with Yu et al.[109] where among [Bmim][TfO],[Bmpyr][TfO] and [TMA][TfO], only [TMA][TfO] which is ammonium-based IL stabilized laccase while the rest imidazolium-based ILs destabilized it. Therefore, [TMA][TfO] not only acted as good stabilizing agent, also greatly helped in maintaining the catalytic efficiency of laccase. The studies related to protein model compounds by Vasantha et al.[68] showed that all the ammonium-based ILs such as TEAS, DEAS, TEAA, DEAA, TEAP, DEAP, TMAA, proved to be stabilizers for the model compounds, however, the same model compounds such as Gly, Gly_2, Gly_3 , Gly_4 and cyclo (Gly-Gly) when studied in the presence of imidazolium-family ILs having $[Bmim]^+$ cation with different anions such as chloride (Cl^-), bromide (Br^-), hydrogen sulphate (HSO_4^-), acetate (CH_3COO^-), thiocynate (SCN^-) showed different behaviour with the variation in anion of the IL.[128] The values obtained for $\Delta G_{tr}^{\cdot}$ were negative for cyclo(Gly-Gly) in 1-butyl-3-methyl imidazolium bromide ([Bmim][Br]) which indicated that Br^- based IL to behave as a strong destabilizer for the structure of cyclo(Gly-Gly).

Very recent study by Rodrigues et al.[129] showed the activity of TlL (Thermomyces lanuginosus lipase) in the presence of different families of ILs, which shows that IL containing ammonium cation results in higher activity as compared to imidazolium-based ILs thus adding value to our results that ammonium-based ILs showing higher stabilization as well as activity as compared to imidazolium family ILs. Further, evidence in support of ammonium-based ILs was reported by Das et al.[130] in 2007 where they tested the activity of horseradish peroxidase (HRP) in different imidazolium family ILs based on variation of

anion and they also tested the activity of HRP in specifically designed ammonium-based IL. For the first time, they reported the biocatalysis of HRP in the presence of IL based on the variation of cationic component. As compared to best performing imidazolium based IL 1-butyl-3-methylimidazolium trifluoromethanesulfonate [Bmim][CF_3SO_3], HRP was found to exhibit almost 6-fold higher activity in tetrakis(2-hydroxyethyl) ammonium triflouromethanesulfonate which was obviously ammonium-based IL.[130] As earlier discussed the work by Attri and Choi[125]clearly shown the stabilization of CT in TEAP, however the imidazolium-based ILs 1-butyl-3-methylimidazolium chloride [Bmim][Cl] and 1-methylimidazolium chloride ([Mim][Cl]) completely failed to offset the ruinous effect of APPJ on the CT structure.

Bose et al.[131] in 2010, studied the activity and stability of cellulase in the presence of both ammonium-based ILs as well as imidazolium-based ILs such as tris(2-hydroxyethyl)methylammonium methylsulfate (HEMA), [MimCl] and 1-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide ([Bmim][NTf_2]), ([Bmim][Cl]) respectively. They showed that HEMA provided stability to the enzyme and also increased the thermal resistance close to 100 ^{0}C. Therefore, HEMA proved to be novel as well as green medium for carrying hydrolysis of cellulose. Hence, this study again proved the superiority of ammonium family ILs over imidazolium-based ILs in providing stability to the enzymes. In continuation with their work on the activity and stability of cellulase, Bose and co-workers [132] in 2012, showed that again HEMA bestowed significant stability to the cellulase when compared to imidazolium-based ILs. Since in buffer, the T_m was ~55 ^{0}C which increased to ~75 ^{0}C in HEMA. Further, from the activity measurement studies it was concluded that both imidazolium and ammonium cations with similar anions showed opposite effect on the activity of cellulase, ammonium family ILs being stabilizer while imidazolium-based IL being destabilizer.[132] Our conclusion of ammonium-based ILs to be more biocompatible is consistent with the results obtained by Lau et al.[95] who showed that CALB dissolved in [Et_3MeN][$MeSO_4$] maintained its activity with preservation of native conformation while the imidazolium-based IL showed low activity. Ammonium-based hydrophobic IL butyltrimethylammonium bis(trifluoromethylsulfonyl)imide ([N_{1114}][NTf_2]) proved to be efficient stabilizing agent for cellulase activity with residual activity around 40% after 5 days. However, the imidazolium-based ILs [Bmim][Cl] behaved as deactivating agent for the enzyme. Also, [N_{1114}][NTf_2] acted as suitable agent for cellulase activity against the negative impact of [Bmim][Cl] which could be due to preservation of essential water around the

protein.[133] Further, Rantwijk et al.[134] showed dissolved CALB retained its activity in [Et_3MeN][$MeSO_4$] however, the other imidazolium-based ILs dissolved CALB with the loss in its activity.

The above discussion provides us a clear idea of distinction between the behaviour of different proteins in the ammonium and imidazolium family ILs. Still some perplexity is there regarding the behaviour of different families of ILs towards same protein. Therefore, in contemplation of clearing the doubt and confusions, we have taken some of the most widely studied proteins in these families of ILs. Cyt c was found to show different behaviour in the presence of different ILs. It did not retain its Fe(III)/Fe(II) redox activity in 1-butyl-3-methylimidazolium salts of bis(trifluoromethylsulfonyl)imide [bmim][tf_2N] and hexafluorophosphate [Bmim][PF_6].[135] Also, in 2009, it was found by Baker and Heller that cyt c was denatured in imidazolium-based ILs which was concentration dependent manner.[136] On the other hand, Baker et al.[137] distinctly demonstrated that EAN is the most biocompatible IL for cyt c as compared to imidazolium-based ILs. Further, Bihari et al.[138] showed that cyt c structure was denatured by ethylmethylimidazolium ethylsulfate [EMIM][$EtSO_4$]. The tertiary structure of the cyt c was affected on dissolution in [EMIM][$EtSO_4$]. Complex formation between heme coordination site and imidazolium cation was supposed to occur on dissolution.[138] As already explained in the preceding section, in 2011, Wei and Danielson[84] showed MAF and EAF to improve the structural stability of cyt c. As per our experience, the protein CT was stabilized in ammonium family ILs such as DEAA, DEAS, DEAP, TMAA, TMAS and TMAP[83,91] however, CT was destabilized in the presence of Hofmeister series of anions such as SCN^-, HSO_4^-, I^- with $[Bmim]^+$ cation.[139] Mb was also stabilized in ammonium-based ILs[88] although it was destabilized in the presence of SCN^-, I^-, Br^-, Cl^-, SO_4^{2-}, CH_3COO^- with $[Bmim]^+$ cation.[140] Kumar and Venkatesu[112]demonstrated that ammonium-based ILs were found to put a stop to its self-aggregation into an inactive form while *In* was denatured in the presence of imidazolium family ILs having SCN^-, I^-, $HSO4^-$, CH_3COO^- anions with $[Bmim]^+$ cation.[141]

Thus, it can be concluded that protein-IL interactions vary from protein to protein and IL to IL. It is unreasonable to conclude from a particular result that if a particular IL is destabilizing/stabilizing a particular protein it will destabilize/stabilize another protein also. Therefore, it is quite essential to observe the interactions between particular proteins with a set of ILs. In spite of various discrepancies in the results, finally based on our own experience and through different studies it would be reasonable to conclude that ammonium-based ILs

are more biocompatible for most of the biomolecules while imidazolium-family ILs being comparatively inferior than ammonium-family ILs as far as study related to protein folding/unfolding is concerned. For giving a clear idea of comparison between ammonium- and imidazolium-based ILs, we have diagrammatically displayed in Scheme 5. Our assumption is quite consistent with the results published Rodrigues et al.[129] Further, it is suggested that toxicity of IL is dependent more on cation as compared to anion with toxicity of cation following the trend ammonium< pyridinium<imidazolium<triazolium< tetrazolium for the organisms Vibrio fischeri and Daphnia magna however, at the same time for Escherichia coli K-12, water-miscible alkylammonium salts are usually toxic while short alkyl chains containing imidazolium ILs and methylpyrrolidium ILs are relatively non-toxic.[142,143] Also, addition of EAN to β-lactoglobulin resulted in the formation of α-helical structure with the disruption of tertiary structure. However, the formation of α-helical structure in EAN was without aggregation unlike 1-butyl-3-methylimidazolium nitrate ([Bmim][NO_3]).[144] Therefore, further research concerning ammonium-based ILs and proteins need to be carried out to clear all the confusions and development of better protein protecting solvents. The basic understanding of the stabilization/destabilization behaviour of proteins is necessary to protect the biological functioning of the proteins. Moreover, it can be anticipated that the stability of proteins can be controlled up to much extent by modifying the solvent property.

Conclusions

The results obtained up to now clearly give the potential role of ammonium-based ILs in the stabilization of proteins. The proteins have shown enhanced activity, stability as well as selectivity in the presence of ammonium family of ILs when compared to not only conventional organic solvent but also most common imidazolium family ILs. Among the various ammonium family ILs, EAN continues to rule the field of protein stabilization. The effect of these ammonium-based ILs on protein stability is highly governed by the type of anion, the alkyl chain length of the cation, viscosity, hydrophobicity, polarity of the IL. In general, anion played superseding effect in the stabilization of model compounds. Irrespective of the IL used, high concentration of it can be detrimental for the stability as well as activity of proteins. Water-immiscible ILs behaved as strong stabilizer for most of the proteins. Also in most of the cases, cation containing long alkyl chain length proved to be

more efficient in stabilizing the protein when compared to smaller one. However, it is important to realize that the ion interaction with protein depends on both the structure of the protein as well as on the IL used. Thus, future focus should be set on the encouragement of protein-IL research to achieve the goals related to protein stabilization. The above studies suggest ammonium-based ILs to be acting as stabilizers in most of the cases yet it cannot be considered the thumb rule. Although, many efforts have been put in this perspective to explain not only fragmentarily but totally the mechanisms of various interactions however, there are several phenomena which are yet to be fully understood. This is our hope that this perspective will be helpful in disclosing and shedding light on the various causes of the stabilization/ destabilization of proteins in the presence of ammonium-based ILs and further helpful in choosing the particular set of ILs for particular protein.

List of abbreviations

Name	**Abbreviation**
Amino acid	AA
Amino acids	AAs
Ionic liquids	ILs
Protic ionic liquids	PILs
Alanine	Ala
Valine	Val
Leucine	Leu
Histidine	His
Tryptophan	Trp
Tyrosine	Tyr
Serine	Ser
Glycine	Gly
Diglycine	Gly_2
Triglycine	Gly_3
Tetraglycine	Gly_4
Cyclic dipeptides	CDs
α-chymotrypsin	CT
Cytochrome c	cyt c
Haemoglobin	Hb

Myoglobin	Mb
Insulin	*In*
Candida antartica lipase B	CALB
Succinyl concanavalin A	S Con A
Human serum albumin	HAS
Bovine serum albumin	BSA
Horseradish peroxidase	HRP
Hen egg white lysozyme	HEWL
Apparent transfer free energy of model compounds	$\Delta G'_{tr}$
Apparent Transfer free energy of peptide backbone unit	$\Delta g'_{tr}$
N-acetyl-L-phenyl-alanine ethyl ester	APEE
Reactive oxygen species	ROS
Circular Dichroism	CD
Molecular Dynamic	MD
Isoelectric point	pI
Atmospheric pressure plasma jet	APPJ
Transition temperature	T_m

Name of Ionic Liquid	**Abbreviation**
Ethyl ammonium nitrate	EAN
Methylammonium formate	MAF
Diethylammonium acetate	DEAA
Triethylammonium acetate	TEAA
Propylammonium formate	PAF
Ethylammonium formate	EAF
Butylammonium nitrate	BAN
Propyl ammonium nitrate	PAN
Alkyl ammonium formate	AAF
Ethylammonium mesylate	EaMs
Ethylammonium propionate	EAP
Ethylammonium methanesulfonate	EAMs
Ethylammonium pivalate	EAPv

Ethylammonium trifluoroacetate	EATfA
Ethylammonium acetate	EAA
Ethanolammonium nitrate	EOAN
Propyl lammonium mesylate	PropMs
Diethylammonium dihydogen phosphate	DEAP
Diethylammonium hydrogen sulfate	DEAS
Triethylammonium sulfate	TEAS
Triethylammonium dihydrogen phosphate	TEAP
Trimethylammonium acetate	TMAA
Trimethylammonium dihydrogenphosphate	TMAP
Trimethylammonium dihydogen sulphate	TMAS
Triethylammonium methane sulfonate	TEAMS
Triethylammonium triflate	[TEA][Tf]
Tetramethylammonium trifluoromethanesulfonate	[TMA][TfO]
Tetraethylammonium bromide	[TEA][Br]
Tetramethylammonium hydroxide	TMAH
Tetraethylammonium hydroxide	TEAH
Tetrapropylammonium hydroxide	TPAH
Tetrabutylammonium hydroxide	TBAH
Ethanolammonium formate	EtAF
Diethanolammonium nitrate	DEAON
Diethanolammonium chloride	DEACl
Diethanolammonium methanesulfonate	DEAMS
N,N-dimethylethanolammonium acetate	DMEA Ac
N,N-dimethylethanolammonium glycolate	DMEA Gly
Triethanolammonium nitrate	TEOAN
Triethyl methyl ammonium methyl sulfate	$[Et_3MeN][MeSO_4]$
2-hydroxyethylammonium formate	HEF
2-methoxyethylammonium formate	MEOAF
2-methoxyethylammonium nitrate	MEAON
(3-Hydroxypropyl) -trimethylammonium Bis(trifluoromethylsulfonyl)imide,	$[C_3OHtma][NTf_2]$
Butyltrimethylammonium bis(trifluoromethylsulfonyl) imide	[btma] $[NTf_2]$

Cocosalkyl-pentaethoxi-methylammonium methylsulfate	[CPMA][MS]
N-butyldiethanolammonium trifluoromethanesulfonate	[BDEA] [TFMS]
Bis(2-methoxyethyl)ammonium sulfamate	[DMOEA] [SN]
Methyl Trioctylammonium Bis(Trifluoromethyl) Sulfonyl Amide	[MTOA][Tf_2N]
Butyltrimethyl-ammonium bis(trifluoromethylsulfonyl)imide	[N_{1114}][NTf_2]
1-methylimidazolium chloride	[Mim][Cl]
1-Ethyl-3-methylimidazolium ethylsulfate	[Emim][ES]
1-Ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide	[Emim][NTF_2]
Ethylmethylimidazolium ethylsulfate	[EMIM][$EtSO_4$]
1-ethyl-3-methylimidazolium ethylsulfate	[Emim][ES]
1-butyl-3-methylimidazolium chloride	[Bmim][Cl]
1-butyl-3-methylimidazolium bromide	[Bmim][Br]
1-butyl-3-methylimidazolium hexafluorophosphate	[Bmim][PF_6]
1-butyl-3-methylimidazolium nitrate	[Bmim][NO_3]
1-butyl-3-methylimidazolium tetrafluoroborate	[Bmim][BF_4]
1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide	[bmim][tf_2N]
1-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide	[Bmim][NTf_2]
1-butyl-3-methylimidazolium trifluoromethanesulfonate	[Bmim][TfO]
1-butyl-3-methylimidazolium trifluoromethanesulfonate	[Bmim][CF_3SO_3]
1-butyl-2,3-dimethylimidazolium tetrafluoroborate	[Bdmim][BF_4],
1-hexyl-3-methylimidazolium tetrafluoroborate	[Hmim][BF_4]
1-octyl-3-methylimidazolium hexafluorophosphates	[Omim][PF_6]
1-methyl-3-octylimidazolium tetrafluoroborate	[Omim][BF_4]
1-benzyl-3-methylimidazolium tetrafluoroborate	[Bzmim][BF_4]
1-benzyl-3-methylimidazolium chloride	[Bzmim][Cl]
1-(2-carboxyethyl)-3-methylimidazolium chloride	[HOOCEMIM][Cl]
1-carboxyethyl-3-methylimidazolium chloride	[HOOCMMIM][Cl]
1-butyl-1-methylpyrrolidinium trifluoromethanesulfonate	[Bmpyr][TfO]

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Figure Captions

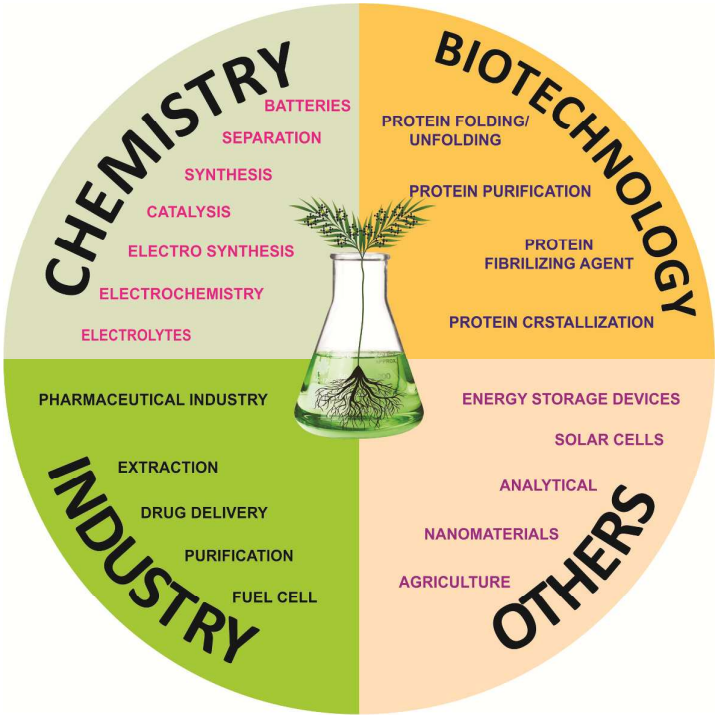
Scheme 1 Applications of ammonium-based ILs in various fields of scientific community.

Scheme 2 Depiction of growing importance of ammonium family ILs in protein related research fields in chronological order.

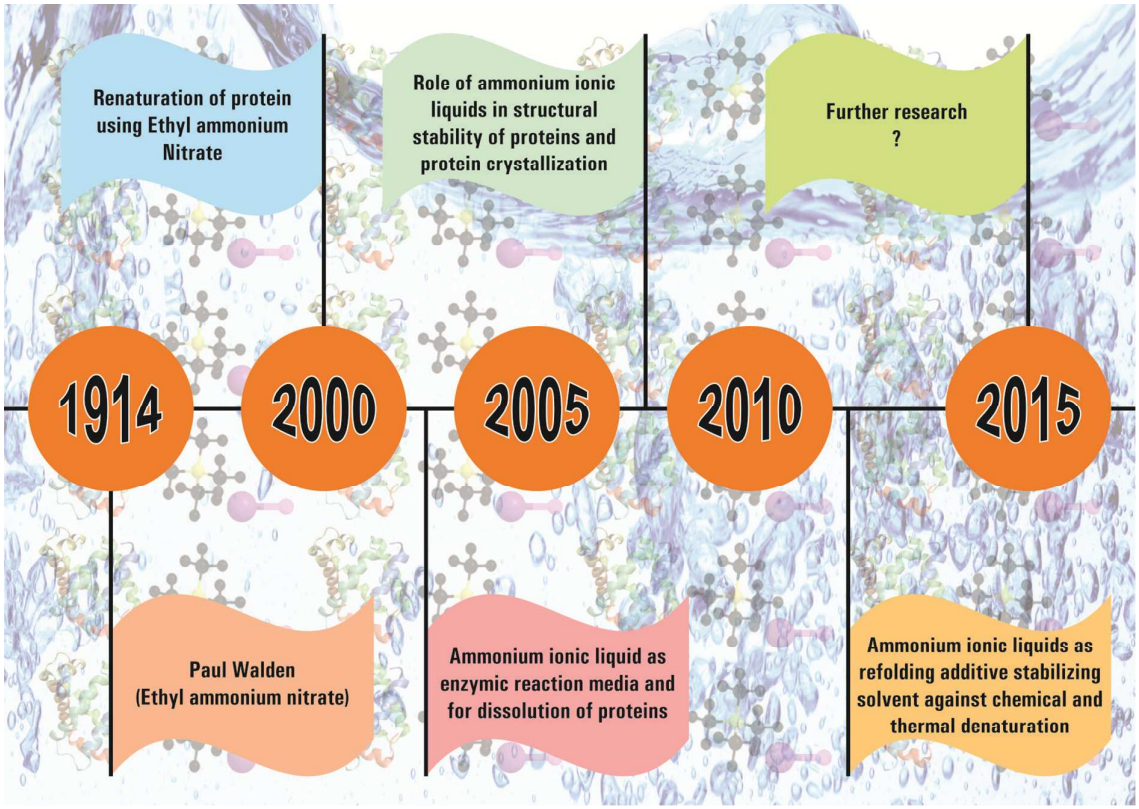
Scheme 3 Mechanism depicting different mode of stabilization and destabilization of proteins in the presence of ILs.

Scheme 4 The difference between the counteraction and refolding abilities of ILs against the denatured proteins: (A) The counteracting ability of ILs on urea-induced protein. (B) Refolding capability of ILs against chemically (urea) denatured proteins.

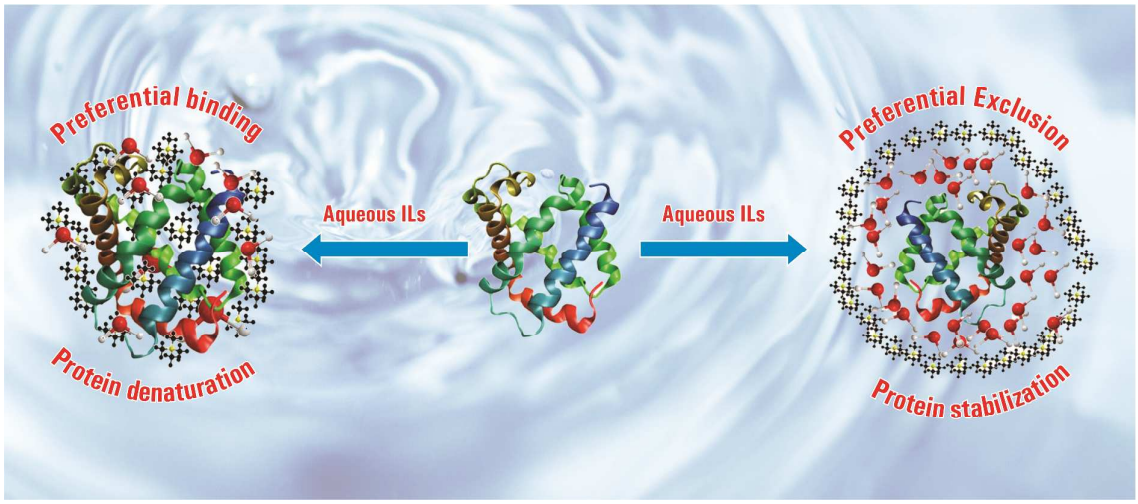
Scheme 5 The biocompatible behaviour of ammonium-based ILs as compared to imidazolium-based ILs for proteins.



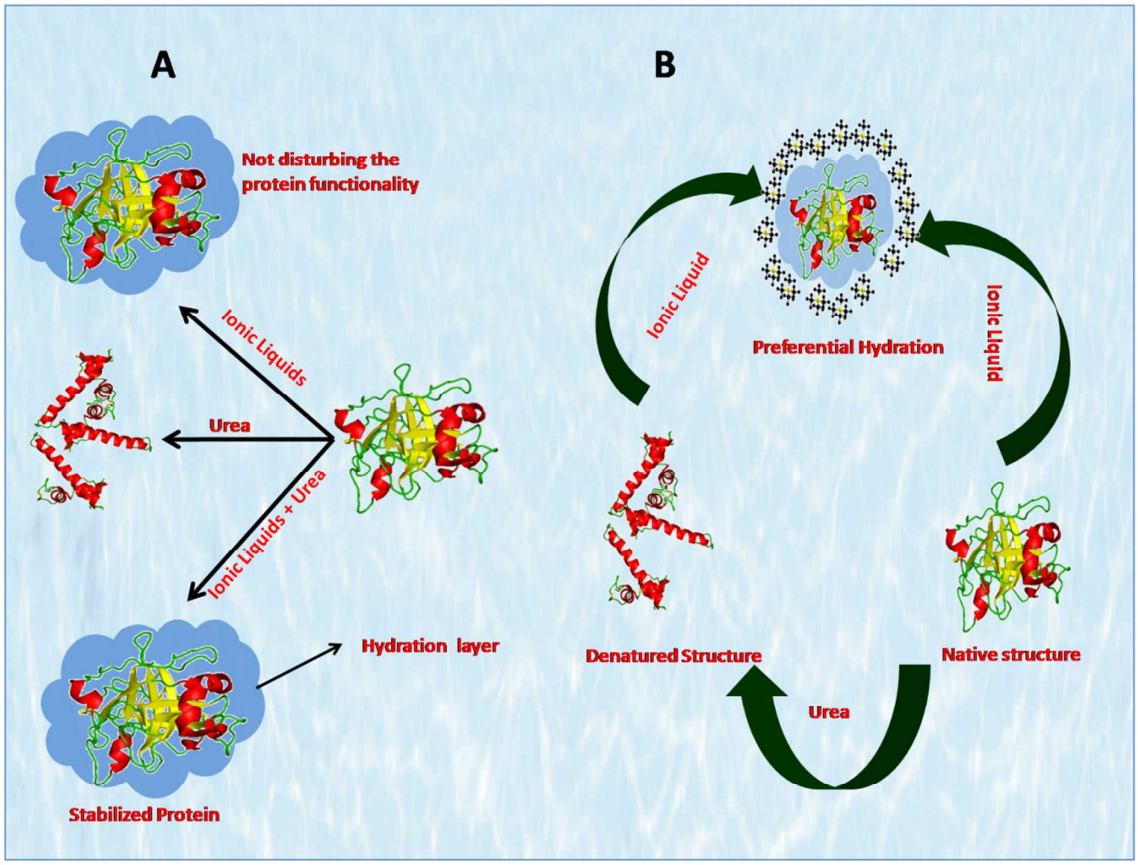
Scheme 1



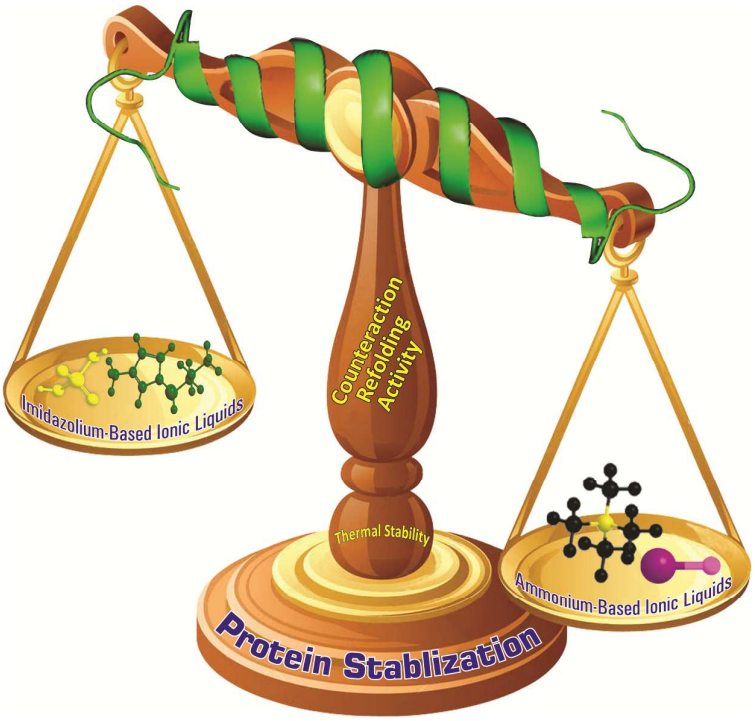
Scheme 2



Scheme 3



Scheme 4



Scheme 5

Indrani Jha has received her post graduate degree in physical chemistry from Department of Chemistry, University of Delhi, Delhi, India, in 2011. She has been awarded Junior Research Fellowship (JRF) from Council of Scientific and Industrial Research (CSIR), New Delhi, India in 2011. She is currently pursuing her Ph.D. at University of Delhi, Delhi, India. Her research interests include the study of the effects of ionic liquids on protein folding/unfolding. She is a recipient of the Indian Chemical Society Award for the presentation of poster for two consecutive years (2012 and 2013). Also, she has received Award for the best poster at 8th National Conference on Thermodynamics of Chemical, Biological and Environmental Systems-2013 by The Indian Thermodynamic Society in the Year 2013. In the same year, she was also awarded 3rd Prize for Poster presentation in International Conference on Recent Advances in Chemical Sciences. Further in year 2014, she has received Young Scientist Award (Professor Santi Ranjan Palit Memorial Award) in the year 2014 from the Indian Chemical Society of India.

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