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Impact of Imidazolium based Ionic Liquids on the Structure and Stability of Lysozyme

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Abstract

Various types of water-miscible aprotic ionic liquids with different cations (1-ethyl-3-methylimidazolium, 1-butyl-3-methylimidazolium, 1-octyl-3-methylimidazolium) and anions (ethylsulfate and chloride) were used as co-solvents to investigate the stability of Lysozyme. Different techniques such as fluorescence, thermal absorption and circular dichroism spectroscopy have been used for the study. Fluorescence results reveal that the addition of ionic liquids (1-ethyl-3-methylimidazolium ethyl sulfate and 1-ethyl-3-methylimidazolium) increases the hydrophobicity around the tryptophan environment in Lysozyme. circular dichroism analysis and temperature dependant studies were done to investigate the stability of the protein. From the circular dichroism analysis, it was observed that the ionic liquids keep the native structure of protein intact. Thermal denaturation studies depicted that the melting temperature of the protein increased in the presence of ionic liquids (1-ethyl-3-methylimidazolium ethyl sulfate and 1-ethyl-3-methylimidazolium), which indicates the stabilization of the protein.

1. INTRODUCTION

Biological processes such as protein kinetics, stability, and activity are largely influenced by the physical and chemical properties of solvent/co-solvent, environmental condition, additives, etc.[1-4] Generally, the biological processes are governed by the three-dimensional structure of the protein at its folded or native state.[2,3] Chemically, the three-dimensional native states of proteins are stable due to the existence of several weak interactions such as hydrogen bonds, ionic and hydrophobic interactions with solvents as well as other entities present in a medium. The signal of these weak interactions is dependent on the local/global chemical environment. Keeping these in mind, it is very much interesting to study the stability (local and global) of protein in different co-solvent conditions.[5,6] In this regard, ionic liquids (ILs) can act as a potential solvent as they have already shown promising applications in various pharmaceutical, biomedical and biotechnological research fields because of their unique properties such as negligible vapour pressure, good thermal stability, tunable viscosity, water miscibility, favourable solvating properties, and non-ionizing nature.[7-11] Due to the tunable properties as discussed above, ILs are used as designer solvents.

Various studies carried out by many research groups have shown that most of the ILs acted as stabilizers for biomolecules.[12-17] Lau, R M et al. observed that the enzyme, CALB retains its native structure in the $[Et_3NMe][MeSO_4]$ by maintaining its acitivity.[18] Water miscible ammonium and imdazlium based ILs was found to improve the activity and stability of CALB.[17] In another work, it was investigated that lysozyme effictivley refolds in ammonium based ILs.[19] Byrne et al. reported that ethylammonium nitrate

increases the thermal stability of lysozyme and provides long-term stabilization against aggregation.[15] It was observed that [chol]Cl markedly increases the thermal stability of the two enzymes, hen egg white lysozyme and α-lactalbumin.[20] Further, a work by Wei and Danielson explains the structural stability of cyt c in methyl ammonium and ethyla ammonium formate.[21] It was reported that ammonium, phosphonium and imidazolium ILs are able to stabilize the native structure and activity of a-chymotrypsin (CT).[12] Recently, Kumar, A et al. explored that imidazolium-based ILs act as stabilizers for the insulin structure.[22]

There were few reports demonstrating that some proteins are highly denaturated by the ILs. Baker and Heller observed that human serum albumin and equine heart cytochrome c were destabilized by 1-butyl-3-methylimidazolium chloride.[13] Furthermore, Constantinescu et al. pointed out that the native structure of protein is also perturbed by some ILs.[16] Recent work by Mandal et al. reveals the destabilizing effect of 1-butyl-3-methylimidazolium octylsulfate on lysozyme.[23] Thus, it is very desirable to know the effect (stabilizing or destabilizing) of ILs on the protein's structure and stability and a molecular level understanding is needed to gain a deeper insight into the nature of interactions between ILs and protein. To gain a mechanistic insight, we explore the effect of imidazolium based ILs as a function of various concentrations on the structure and stability of lysozyme with the help of UV-Vis, Fluoroscence and Circular-dichroism spectrophotometric methods. For the comparison among different types of ILs, we have used four different types varying the cations and anions. (Table 1) The chemical structures of ILs are given in Figure 1a.

We employed the protein, Lysozyme because of its broad and well-characterized applications.[5,6] Lysozyme, N-acetyl-muramic-hydrolase, is a bacteriolytic enzyme commonly found as a single peptide chain consisting of 129 amino acids (~14 kD). In Lysozyme polypeptide sequence, the nature of amino acid side chain and its arrangement play a significant role in generating hydrophobicities and polarities in the molecular structure. Although, Lysozyme is found as a monomer in nature (Hen egg white is an easy and rich source for Lysozyme), it is more active in dimeric or polymeric form. Dimeric form exhibits therapeutic, antiviral and anti-inflammatory properties.[24,25] Therefore, because of their wide applications we used Lysozyme to study its stability as a function of different types of above mentioned ILs with varying concentrations. Very limited number of studies in the literature on the effect of ILs on the stability of Lysozyme make this study very intresting not only from academic view but also from the industrial aspect. The effect of employed ILs in this study on the thermal stability of Lysozyme as a function of concentration was not studied earlier as per our knowledge.

2. MATERIALS AND METHODS

2.1 Materials

Chicken egg white Lysozyme (Product No- MB098) was purchased from Himedia. The protein was used as such received from Himedia without any further purification. $EmimESO_4$ (Product No-51682), EmimCl (Product No-30764) and BmimCl (Product No-94128) were purchased from Sigma-Aldrich and used as it is. Whereas, OmimCl was synthesized as per the procedure given in Figure 1b.[26] 18 MΩ.cm Milli-Q water

(Millipore, Bedford, MA) was used as the solvent to prepare 0.01 M phosphate buffer (pH -7.4), which was used to prepare samples for all the experiments.

2.2 Synthesis Of 1-Octyl-3-Methylimidazolium Chloride

To a 100 ml round bottom flask equipped with reflux condenser and magnetic stirring bar connected with a nitrogen cylinder, 10 ml (125 mmol) of 1-methyl imidazole and 12.6 ml toluene was added to an ice bath. After 5 min of stirring 23.4 ml (138 mmol), 1-chloro octane was added. The solution was heated to reflux at 125 °C for 72 h, yielding a two-phase mixture of [OmimCl] and toluene. Then the toluene was decanted and the product was recrystallized with acetone three times. The final product was then dried in a schlenk line at 80 °C for 7 h before use.

^{1}H NMR ($CdCl_3$, 400 MHz), δ(ppm) : 0.69 (t), 1.00 (m), 1.70 (m), 3.98 (s), 4.17 (t), 4.68 (s), 5.27 (s), 7.38 (d) and 8.44 (d)

2.3 Methods

2.3.1 UV-Visible Spectroscopy

The ultraviolet spectra and thermal profiling of Lysozyme were studied by using UV-Vis spectrophotometer (Agilent, Cary 100) equipped with a peltier system for maintaining the temperature of the sample. For measurements, 10 μM protein solution, containing ILs of varying concentrations, 0, 0.2, 0.6 and 1 M, were employed. The ILs absorption was subtracted from the respective protein sample containing IL to remove the effect of ILs. For thermal denaturation studies, absorbance at 280 nm was observed at different

temperatures. Thermal profiling of Lysozyme in the absence of IL was taken as control. Temperature range for the study was maintained at 30 to 90 ^{o}C.

2.3.2 Steady-State Fluorescence Spectroscopy

The fluorescence measurements were done using Horiba Jvon Spectrometer (Fluoromax-4P). In order to perform the signal-based fluorescence measurements, we took the advantage of fluorescent properties of Trp residue as an intrinsic fluorophore. Trp was excited at 295 nm to avoid the interference from Tyr (tyrosine) and Phe (Phenylalanine) and the fluorescence was collected as a function of wavelength (or steady-state) in the range of 325 to 450 nm. The effect of ILs on the Trp-emission of Lysozyme was analyzed in terms of the fluorescence signal. For measurements, 10 μM protein solution containing ILs of varying concentrations (0 - 1 M) were used. The width of the excitation and emission slit was set to 5 and 5 nm, respectively. Fluorescence signals are corrected by subtracting blank solutions containing only ILs. This kind of correction was validated in earlier reports.[27,28]

2.3.3 Circular Dichroism Spectroscopy

Tertiary structures of the protein was studied by a circular dichroism (CD) spectrophotometer (JASCO-1500) at room temperature under constant nitrogen flush. The scan speed (100 nm/min) was fixed with a response time of 1 s and 1 nm bandwidth. Each spectrum was collected by averaging three spectra. The tertiary structure of Lysozyme was monitored by using near-UV (250–300 nm) (0.1 cm path length cuvette) spectra. The protein's concentration was maintained at 100μM and IL concentration was

kept at 0.2, 0.6 & 1.0 M. Each sample spectrum was obtained by subtracting the appropriate background (without protein) from the experimental protein spectrum.

3. RESULTS AND DISCUSSION

3.1. Absorption Measurements

Absorption spectra of Lysozyme in the presence of different ILs are presented in Figure 3 (a-d). The spectra classified into two regions; a) 200-250nm (refers to the backbone polypeptide chain and corresponds to π–π* transition) and b) 260-290nm (refers to aromatic amino acid residues, i.e., Trp, Tyr, and Phe and corresponds to n–π* transition).[29] From Figure 3, it was observed that the absorbance at 280nm was decreased in the presence of ILs. In case of EmimESO4, the decrease is more profound with a blue shift in absorption maxima as a function of concentration. The observed changes may be as a result of the changes in the local environment around aromatic amino acid residues.[30] But in case of other ILs, there is a marginal decrease in absorbance was noticed with the increase in the hydrophobic moiety (i.e., from ethyl to butyl to octyl) in the imidazolium ring of the ILs.

From the absorption results, it is concluded that the hydrophobic groups have more impact on the local environment of aromatic residues which cause those perturbations. When considering the peak at lower wavelength range, it can be noticed that, the signal decreases in the presence of ILs, and a red shift was observed in all the ILs as a function of concentration. It is known that when amide moieties are exposed to aqueous medium they undergo low energy π–π*. In presence of ILs, the water in contact with peptide

backbone was replaced by ILs. It means the ILs change the polarity of the solvent medium, which lowers the π–π* transition energy causing a bathochromic shift. These observations may be attributed to disturbances in the microenvironment of the peptide backbone structure.[31] This kind of results was earlier reported in protein-IL interaction studies.[31,32] From the above observations, it is proved that the protein forms a complex with the ILs.[33]

3.2. Steady-State Fluorescence Study

Fluorescence spectroscopy has been widely applied to study interactions between protein and ligand and simultaneously, also to explore the quenching mechanism, binding constants, and binding sites. We used the intrinsic fluorescence of Lysozyme to explore the interaction between ILs and Lysozyme. From the crystal structure and amino acid sequence, it is evident that Lysozyme has several solvent exposed and unexposed tryptophan(s).[34] As shown in Figure 2, Lysozyme contains six Trp residues at positions 28, 62, 63, 108, 111, and 123. The Trp residues at positions 28, 108, 111, and 123 present in the α-domain, and the residues at positions 62, 63 and 108 are in the substrate binding sites. Trp 62 & 63 are situated close to the hinge region between the α and β domains and they are found to be highly susceptible to chemical modifications as they are more exposed to the solvent medium whereas Trp 108 is in hydrophobic core, away from the aqueous medium.[35,36] According to the detailed studies reported earlier, Trp 62 and 108 are found to have more impact on the fluorescence emission of Lysozyme, whereas other four residues have small contributions.[37,38] Therefore, the exposed Trp 62 and 63 residues are supposed to be the active site of binding and the fluorescence emission study

by exciting at 295 nm could provide the information of local environment around these Trp residues.

The emission spectra of Lysozyme in ILs were shown in Figure 4 (a-d). In the presence of ILs, $EmimESO_4$ & EmimCl, a sharp blue shift of the emission maxima was observed (345 nm to 336 nm). This is assigned to the change in local environment around fluorophore residues upon interaction with the IL indicating the movement of fluorophores toward a more hydrophobic environment, which is possible during stabilization of Lysozyme. The stabilization effect is evidenced by thermal denaturation studies discussed later. In case of BmimCl & OmimCl, a small blue shift of emission maxima observed (345 nm to 342 nm) which may be possible either by stabilization of Lysozyme where the fluorophores move towards hydrophobic core or by the interaction between hydrophobic groups of IL and the fluorophores during unfolding process. In these ILs, the unfolding process occurs considering the results obtained from thermal denaturation studies and circular dichroism studies discussed later.

Fluorescence quenching mechanisms: Quenching of Lysozyme fluorescence by ILs depend differently on the type of IL as evident from the Figure 4. It indicates different mechanism of interaction with Lysozyme. Quenching of the fluorescence can be static (ground state complex formation between fluorophore and quencher) or dynamic (excited state collision of fluorophore and quencher). These mechanisms can be distinguished by the Stern−Volmer plot, which give the idea about quenching efficiency of ILs after

binding with Lysozyme in the vicinity of fluorophore and protein backbone. The quenching process can be described by the Stern–Volmer equation (Eq.1) [39,32,40-42]

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q \tau_0 [Q] \qquad (1)$$

where F_0 and F are the fluorescence signal of BSA in the absence and presence of quencher IL, [Q] is the quencher concentration, and $K_{SV} = Kq\tau_0$ is the Stern- Volmer quenching constant (Kq is the bimolecular quenching constant, and τ_0 is the lifetime of fluorescence in the absence of quencher). K_{sv} values were obtained from the slope of the Stern-Volmer plot and Kq values were calculated by considering $\tau_0 = 10^{-8}$. Kq values were found to be less than the largest dynamic bimolecular quenching constant in aqueous medium, which is 2×10^{10} L $mol^{-1}s^{-1}$, indicating that the quenching of Lysozyme fluorescence by ILs is dynamic. It reveals that the quenching of fluorescence is mainly by the interaction of IL with Lysozyme and the unfolding of Lysozyme in the present case doest not contribute much to the decreased fluorescence signal.

When small molecules bind independently to a set of equivalent sites on a biomolecule, the equilibrium between free and bound molecules is given by the following equation (Eq.2).[43]

$$log\frac{F_0 - F}{F} = logK_b + n\,log[Q] \qquad (2)$$

Where, n is the number of binding sites and K_b is the binding constant. The values of n and K_b were obtained from the slope and intercept of the plot of $log(F_0 - F)/F_0$ versus log[Q] respectively.

The values of n and K_b were shown in table 2. Gibbs energy for the binding of the IL to the protein can be calculated from the following equation (Eq.3).[44]

$$\Delta G_b = -\ 2.303RT\ \log\ K_b \qquad (3)$$

The "n" value is helpful to know the number of binding sites and to locate the binding site in Lysozyme. The calculated "n" value is closer to unity indicates that there was one independent class of binding sites on Lysozyme with IL. K_b values reveal that the reactivity of ILs with Lysozyme follows the trend OmimCl > $EmimESO_4$ > BmimCl > EmimCl which are in agreement with UV difference spectra where the strongest effect observed for OmimCl and $EmimESO_4$. The negative ΔG_b values indicate the binding interaction between the ILs and Lysozyme is a spontaneous process.

The low values of K_b suggest very weak interactions between ILs and Lysozyme. In general, ligand - protein complexes have the binding affinity in the range of 10^5-10^8 $Lmol^{-1}$.[45] However, lower binding constants (10^2-10^4 $Lmol^{-1}$) have been reported for several ligand-protein complexes.[44,39,41,42] Recently, imidazolium ILs have been reported to possess very weak interactions (binding constants in the order of 10^2) with BSA having ΔG_b values in the range of -10 to -15 kJ mol^{-1}.[32] The low free energy values are attributed to the use of higher concentration of ILs, by which the IL-IL interactions dominate the IL-lysozyme interactions in the formation of micelle.[46] Hence the driving force for IL binding with lysozyme gets reduced.

3.3 Thermal Denaturation Studies

Figure 5a shows the thermal denaturation curve of Lysozyme in the buffer solution, as monitored by absorption measurements. Thermal denaturation of Lysozyme in absence and presence of ILs was found to follow a single step, two state transition. These results are in accordance with previous reports where single step, two state transition unfolding was observed.[47-49]

Experimentally, the fraction of unfolded molecules (Melting temperature, T_m) can be measured by taking the first derivative of the sigmoidal curve of the absorbance vs temperature graph.[50,51]

The effect of different ILs ($EmimESO_4$, EmimCl, BmimCl, and OmimCl) on thermal stability of Lysozyme were illustrated in Figure 5b. (The complete analysis included in supplementary information).

Figure 5b shows that some of the ILs ($EmimSO_4$ & EmimCl) rapidly increase the T_m of the Lysozyme and some (BmimCl & OmimCl) follow the reverse order, which also depend on the concentration of the IL. These observations reveal that the native structure of Lysozyme keeps intact in the presence of some ILs. The stability of the proteins in the presence of ILs follows the trend $EmimESO_4$ > EmimCl > BmimCl > OmimCl and the stability is found to be higher at low concentrations of EmimCl. The increased thermal stability may be due to the interaction of ILs (cationic moiety) with the negatively charged residues on the surface of Lysozyme (via electrostatic, hydrophobic forces /

hydrogen bonding through amine protons). It is assumed that in this case these ILs do not interact with the functional groups of Lysozyme. The extra stabilization of $EmimESO_4$ can be predicted by the interaction of both cation, $Emim^+$ as well as anion, ESO_4^- with oppositely charged sites of Lysozyme via electrostatic and hydrophobic forces. In case of BmimCl & OmimCl, with increasing concentration, the disruption is found to be more and follows the trend of 0.2 M < 0.6 M < 1.0 M. It means when the size of the hydrophobic group in the cation moiety increases, the protein is more prone to disruption. The unfolding of protein in these ILs may be due to the interactions between ions of the ILs and the functional groups of protein (specifically hydrophobic interactions between the hydrophobic core of protein and hydrophobic groups of the ILs) and the repulsive intramolecular interactions between the protein functional groups which may disrupt the protein's native structure.[52,53]

Further, to have a better understanding on the structural changes in ILs we performed CD analysis.

3.4 CD Spectral Analysis

CD spectroscopy can be used to observe the secondary and tertiary structural changes occurred in the presence of ILs. The secondary structure of proteins can be calculated from the far-UV CD spectrum, but the working concentrations of ILs produce high values of HT voltage which leads to high noise in the spectrum. Therefore, we confined our study to near-UV CD spectral region (250–350 nm), which is sensitive to the chromophore's local environment and, consequently, the protein's tertiary structure.

The near-UV CD spectra of 100 µM Lysozyme in different ILs are presented in Figure 6 (a-d). The tertiary structure of Lysozyme is confirmed by a positive triplet-like signal in the range of 280–300 nm and which indicates the active conformation of Lysozyme.[54,19] The net positive CD bands are associated with the Trp and Tyr L_b transitions and net negative CD bands are associated with Trp L_a and disulfide transitions.[55] The positive triplet feature (signals at 283, 289 and 295 nm) of Lysozyme is considered to be obtained from the transitions of the Trp residues.[56] Therefore, any changes in CD spectra in the presence of ILs reveals the alteration of the environment of these aromatic amino acid residues indicating the unfolding of Lysozyme.

In the presence of $EmimESO_4$, the spectra were not changed much except the signal of the peak at 289 nm which decreases as a function of concentration. But, the width of the peak is lesser as compared to the native Lysozyme. This observation is attributed to the proteins compact structure in the IL. But in case of EmimCl, the signal of the triplet peak increases at lower concentration (0.2 M) and at higher concentration the triplet feature diminished, which means the protein is unfolded at higher concentration. Therefore, comparing these two ILs, we can conclude that the anion is playing a crucial role in stabilizing and destabilizing protein structure. It is also supported by the melting temperature of Lysozyme in the presence of these ILs.

When comparing the effects of the hydrophobic moiety present in ILs on protein structure, it was observed that, with increasing alkyl chain length in the imidazolium group of the ILs, the protein gets destabilized. In case of BmimCl at higher concentration

(0.6 M & 1.0 M), the signal of triplet signal was found to be higher, but those peaks are broader. This observation suggests, the presence of IL in the vicinity of Trp residues changes the local environment and consequently the tertiary structure altered.[19] Considering the effect of OmimCl, it was noticed that the triplet feature keeps intact at low concentration but with increasing concentration the triplet signal reduced and finally at 1.0 M the tertiary structure disappeared. These results are attributed to the increased hydrophobic interactions between protein hydrophobic core and the hydrophobic group of the ILs during the unfolding process. Recent simulation studies of protein conformation reveal that direct interaction between protein core - IL leads to destabilization of the protein.[57,58] During the process, ILs induce conformational changes in protein enabling ILs to directly bind to the protein surface, thereby unfolding of the biomolecule. Klahn et al. elucidate that the van der Waals interactions placed a dominant role in the interaction of the protein with the cation of the ILs, which leads to the disruption of the protein structure in the ILs.[57] These processes reduce the free energy evidenced by the decrease of T_m value and, therefore, destabilize the native structure of the protein.

4. CONCLUSION

In conclusion, among the ILs used in this study, $EmimESO_4$ acted as most promising and biocompatible IL for the native structure of Lysozyme at all studied concentrations followed by EmimCl at low concentration. From the results, it can be concluded that not only the IL type but also concentration of the ILs play a major role in stabilizing/destabilizing a protein. It was found that anion ESO_4 has a great influence on

increasing the thermal stability of Lysozyme compared to Cl ion and hydrophobicity of the cation influences the thermal stability profoundly in destabilizing protein. The present wotk explains the mechanism of lysozyme-IL interaciotns and it was observed that very weak interactions exist between lysozyme and ILs elucidated by spectrophotometric studies. We hope that the results obtained here will be useful in the selection and designing of ILs with varying combination of different cations and anions, which can provide green, biocompatible co-solvents for the stabilization of proteins in biochemical processes.

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Table 1. Briefing of employed Ionic liquids for protein stability investigation.

Name of the Ionic liquid		Molecular Weight /gm	Availability*
Abbreviation	Chemical Name		
$EmimESO_4$	1-Ethyl-3-Methyl-Imidazolium EthylSulfate	236.29	Commercial
EmiImCl	1-Ethyl-3-Methyl-Imidazolium Chloride	146.62	Commercial
BmimCl	1-Butyl-3-Methyl-Imidazolium Chloride	174.67	Commercial
OmimCl	1-Octyl-3-Methyl-Imidazolium Chloride	230.78	Synthesized

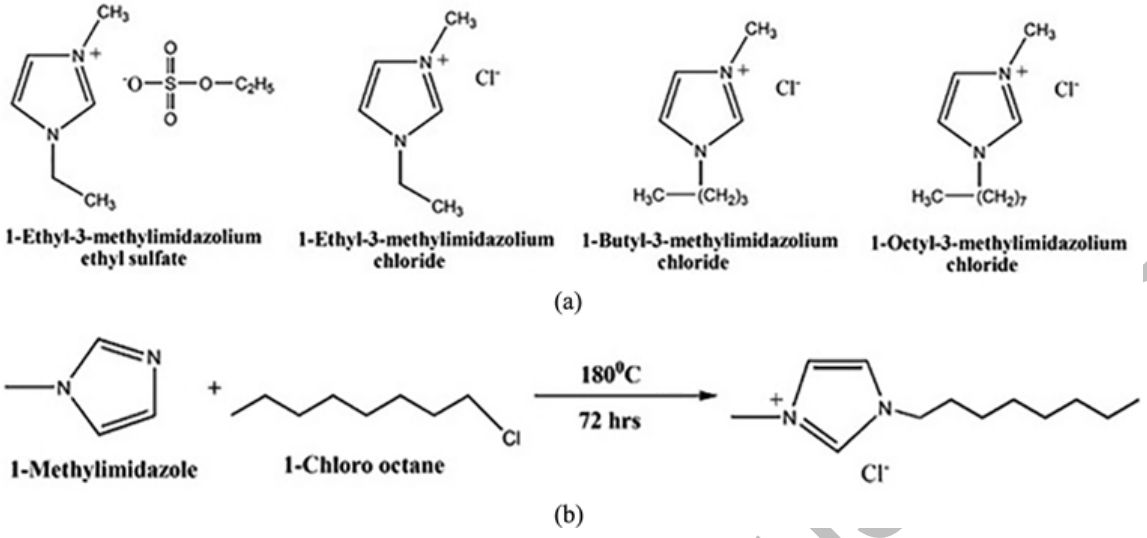
* Details are mentioned in "Materials and Methods" section

Table 2. Stern–Volmer quenching constant (K_{SV}), bimolecular quenching constant (kq), binding constant (K_b), binding sites (n) and Gibbs energy (ΔG_b) for the binding of Ionic iquids with Lysozyme at 298.15 K

IL	K_{SV}/Lmol^{-1}	K_q/Lmol^{-1}s^{-1}	K_b/Lmol^{-1}	n	ΔG_b/kJ mol^{-1}
$EmimESO_4$	1.71	$1.7*10^8$	1.61	0.79	-1.19
EmimCl	1.26	$1.2*10^8$	1.18	0.78	-0.42
BmimCl	1.44	$1.4*10^8$	1.37	0.80	-0.78
OmimCl	2.31	$2.3*10^8$	2.29	0.92	-2.06

* $EmimESO_4$: 1-Ethyl-3-Methyl-Imidazolium EthylSulfate, EmimCl: 1-Ethyl-3-Methyl-Imidazolium Chloride, BmimCl: 1-Butyl-3-Methyl-Imidazolium Chloride, OmimCl: 1-Octyl-3-Methyl-Imidazolium Chloride

Figure 1



(a)

(b)

Figure 2

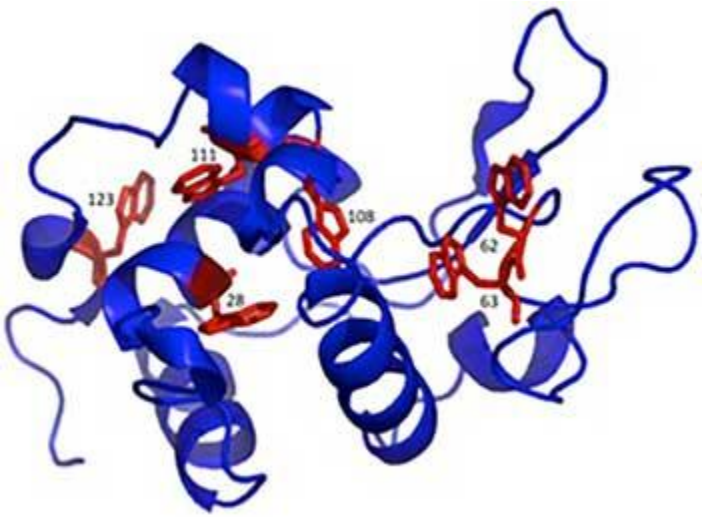


Figure 3

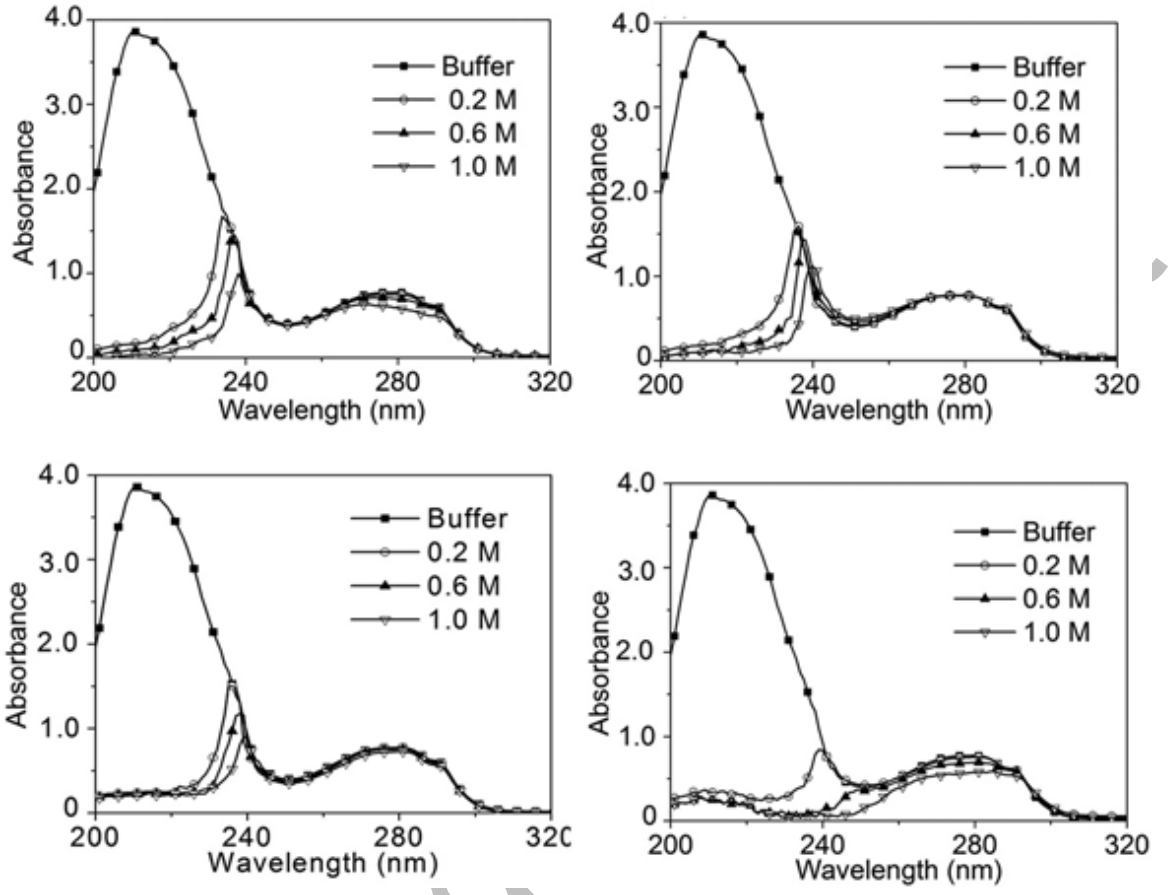


Figure 4

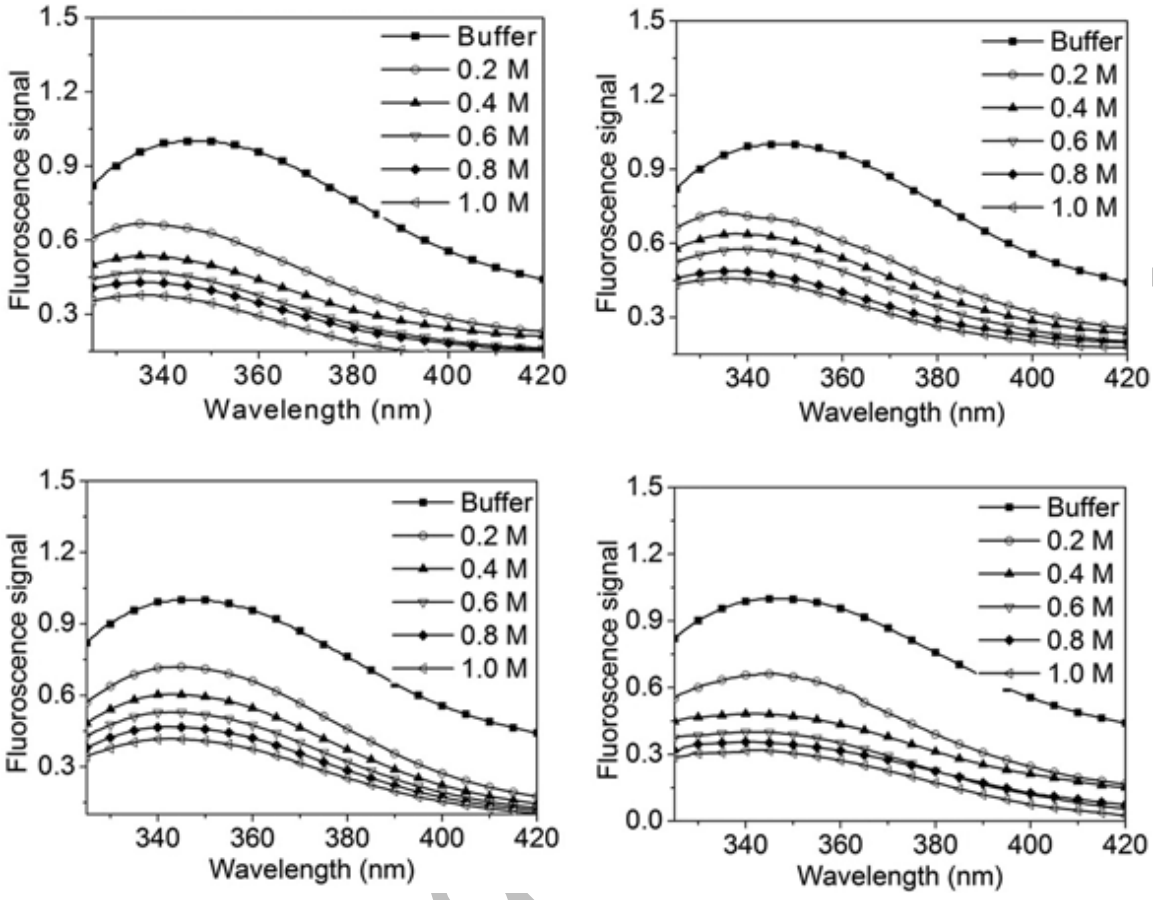


Figure 5

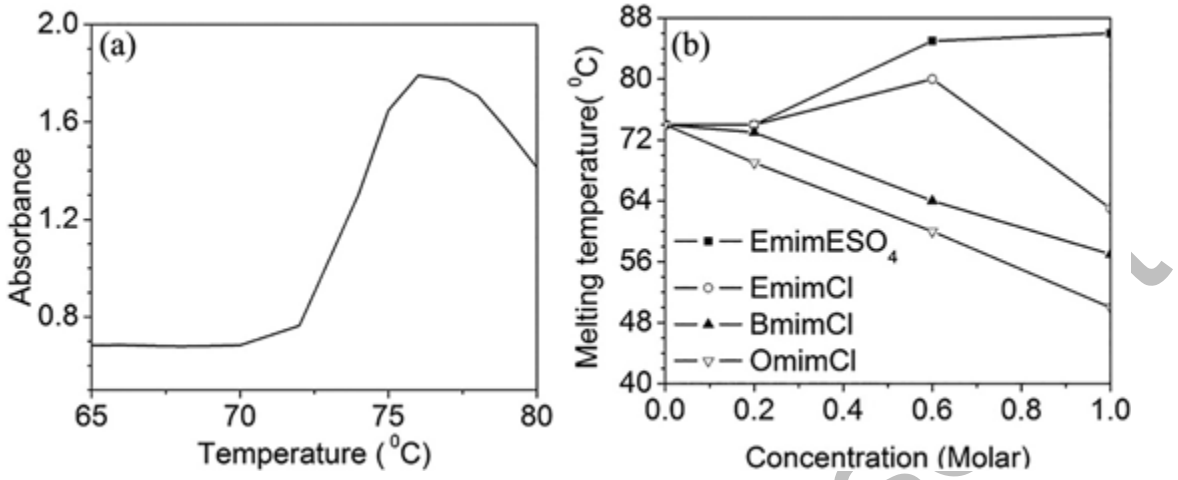


Figure 6