Impact of Ammonium based Ionic liquids on the conformation of Bovine serum albumin (BSA)

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Certificate

This is to certify that the dissertation entitled **"Impact of Ammonium based Ionic liquids on the conformation of Bovine serum albumin (BSA)"** submitted to the Department of Chemistry, National Institute of Technology, Rourkela-769008 in partial fulfilment of the requirement for the award of M.Sc. degree in Chemistry is a record of original work done by **Mr. Sangram Keshari Bagh** under my supervision and guidance.

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Sangram Keshari Bagh

Declaration

I, **Mr**. **Sangram Keshari Bagh**, hereby declare that all my research works are original and no part of this report had been submitted for any other degree or diploma. All the given information and works done are true to my sense and knowledge.

Place: Rourkela **Sangram Keshari Bagh**

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Abstract

In this study, impact of different ammonium based ionic liquids on the structure of Bovine serum albumin (BSA) was investigated by different spectroscopic methods like, UV-vis, fluorescence and CD spectroscopy. Fluorescence results reveal that the ILs has no significant impact on the environment of tryptophan residues. From the Far-UV CD results, it was observed that these ILs increase the α-helicity of the protein, BSA. Near-UV CD study confirms the fluorescence results whereas it shows the alteration of tertiary structure of BSA especially around disulphide bonds.

CONTENTS

CHAPTER-1

INTRODUCTION

1.1 Overview of protein

Proteins are large biological molecules, which consist one or more chains of amino acids. Primarily proteins differ from each other in their amino acid sequence, which can be known by the gene sequencing. Mostly, proteins occur in cells or tissues as a three-dimensional folded structure, which determines its function. Naturally only 20 amino acids have been found and the primary structures of different proteins can be obtained by the permutation and combination of these amino acids providing them a unique function. Proteins are biopolymers formed by the condensation of amino acids. In order to function properly most of the proteins found in nature adopt a specific conformation called the folded state or native state. The maintenance of a protein's structure in a thermodynamically stable form in its native state is of prime importance in biological processes. The stability of a protein depends on temperature, pressure, its hydration capacity and most importantly on its solvent's properties. Generally, a globular protein is stabilized by a balance between the intramolecular interactions of the protein's functional groups as well as with the co-solvent particles. Co-solvents play a variety of important roles in biochemical interactions between the functional groups of biomolecules. Since proteins are present in a solution that contains both water and co-solvent molecules, therefore one must explicitly consider the protein–co-solvent interactions to reveal the impact of co-solvents on the native structure of protein.

1.2 *Native and denatured state* -

The native state of a globular protein is defined as the small ensemble of compact conformations, reached under folding conditions in vivo or in vitro, in which the protein is stable and performs the required function & this state is characterized by a low amount of entropy as well as energy, because all protein atoms are kept by mutual interaction in a well-defined geometry. Intramolecular enthalpy is also relatively low because many attractive interactions are satisfied. Solvent molecules surrounding the folded protein in its native state take instead advantage of a large amount of entropy, since they are not involved in interactions with the hydrophobic protein interior.

By perturbing the folding conditions like increasing temperature, changing the solvent pH or adding a chemical denaturant, the protein can unfold & reach a much more complex and heterogeneous ensemble of conformations, which is called the denatured or unfolded state. It is difficult to characterize the structural properties of the denatured state, since it strongly depends on the unfolding conditions. Experimental techniques like infrared and circular dichroism

spectroscopy provide lots of information on secondary structure of protein. At the point, when a high percentage of secondary structure is lost, which is the situation under complete unfolding conditions, the unfolded state assumes a random coil structure. The denatured state is characterized by high conformational entropy, because native interactions are lost and residues are free to assume a large set of arrangements. Intramolecular enthalpy may be higher because of loss of native interactions. The solvent molecules get in contact with hydrophobic parts of the protein chain, which were buried in the native state. This fact causes water molecules to assume more ordered conformations in order to minimize the contact with nonpolar groups, which reduces the entropy of the solvent relatively to the folded state. Therefore, one can understand the entropy increase as a driving force for folding. When the folding conditions is restore in vitro, the spontaneous refolding of a denatured protein is usually initiated so that molecule is able to find the native state spontaneously.

1.3 *Solvent stabilization of protein Structure* -

The stability of the native proteins is affected strongly by a variety of substances that act at high concentration (usually $\geq 1M$). We refer these substances as co-solvents, which may be solid or liquid. Some of these substances are known to stabilize protein structure, where others to destabilize it, whereas still others can act either as stabilizers or destabilizers, which depends on their concentration and on the solution pH. The cosolvent-protein interaction is a surface phenomenon where more water is structured around the protein molecule, a phenomenon known as preferential hydration, which is one of the major reasons to the stabilization of the protein [2].

1.4 *Bovine Serum Albumin (BSA)-A model protein*:-

To study the effects of cosolvents on protein, Bovine Serum Albumin (BSA) is taken as the model protein. BSA can be derived from cows and it is used as a standard protein to track the protein concentrations. “Fraction V” is another name of BSA, which refers to the fifth fraction of the original Edwin cohn purification methodology. This particular method is first used with human albumin for medical use and later it was used for the production of BSA. The primary structure of BSA is composed of 583 amino acid residues [23], the sequence has 17 disulphide bonds resulting in nine loops formed by the bridges, which contains multiple cysteine and 8 pairs of disulphide bonds similar to HAS [24].

According to the amino acid sequence, the structure of BSA shows that it is composed of three homologous domains [25]. The circular dichroism measurement shows that the secondary structure of BSA contains α-helix, β-sheet, turn and random coil with 48.7 %, 0%, 10.9% and 30.7% contribution respectively [26]. It has been suggested that the α-helices are uniformly placed in the subdomains and in the connections between the domains. Most of the residues in the long loops (except at the end) and the regions linking the domains possibly form α-helices, whereas the intra-domain hinge regions are mainly non-helical structure. The three long helixes in the subdomain are considered as principle elements of the structure. These run parallel with each other, and a trough is formed owing to the middle helix being slightly lower in position. The helices are mainly linked together by disulphide bridges.

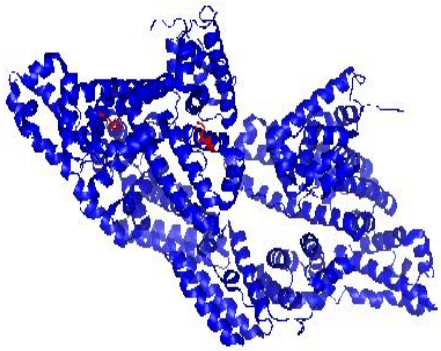


Figure 1: Crystal structure of BSA (PDB ID: 4F5S) representing highlighted tryptophan (red in color).

The N-terminal have 18 residual single peptide is cut off from the precursor protein on secretion; hence the initial protein product contains 589 amino acid residues [27]. For the efficient expression and purification of a mature BSA protein (containing 585 amino acids), additional 4 amino acid is cleaved [28]. There are several applications of BSA; following are a few of them.

1. It has numerous biochemical application including ELISA (Enzyme Linked Immunosorbent Assay), immunoblots and immunohistochemistry. Also it is used as a nutrient in cell and microbial structure.
2. It can be used for the stabilizing of some enzymes during digestion of DNA and to prevent adhesion of the enzyme to the reaction tubes, pipet tips and to the other vessels.

It is commonly used as a standard protein marker to determine the unknown quantity of other proteins by comparing with the known quantity of BSA. Because of its stability, it can also be used to increase the signal in assay. The other properties of BSA that made it a widely used protein are, it is less effective towards many biochemical reactions, cheaper in cost and large quantities of it can be readily purified from bovine blood.

1.5 *Ionic liquids-The Cosolvent*:-

Ionic liquids (ILs) have been described as molten salts that are entirely ionic in nature, compose of both cationic and anionic species and having a melting point below 100 ° C. If they are liquid at room temperature then they are termed as room temperature ionic liquids (RTILs). The choice of cations & anions has a great influence on their properties. Usually, ionic liquids consist of a large organic cations and an organic or inorganic anions. The structural modification of ionic liquids can be made either to the anions, cations, or to the substituents on the cations or anions, so that an almost limitless number of ionic liquids are possible. Hence, by changing the cation or anion of ionic liquids, their physical properties can be modified according to the requirements of a process. These properties include melting point, solubility, density, viscosity, hydrophobicity etc. Ionic liquids have a special place in the current scientific literature due to their unique properties, they are different from the conventional organic solvents. These have very low vapour pressure, wide liquid range, low flammability, high thermal stability, etc.

1.6 *Ion-specific effects on protein stability in aqueous environments: the Hofmeister series*

1.6.1 Thermal stability of proteins

May be of bigger importance than the utilization of ILs as slick solvents for proteins is the likelihood to control the solvent properties of aqueous solutions. While variables, for example, the solvent polarity, H-bond qualities or hydrophobicity of ILs have impact on protein stability, they don't appear to give all inclusive instruments. The ion-specificity of the watched impacts coordinates consideration regarding Hofmeister effects.

$$K^+ > Na^+ > [Me_4N]^+ \parallel Li^+ > [chol]^+ > [Et_4N]^+$$
$$\approx [C_2mim]^+ \approx [gua]^+ > [C_4mpyr]^+ > [C_4mim]^+$$
$$\approx [Pr_4N]^+ > [C_6mim]^+ \approx [Bu_4N]^+$$

$$[SO_4]^{2-} > [dhp]^- > [ac]^- > F^- > Cl^- \parallel [EtOSO_3]^- > [BF_4]^-$$
$$\approx Br^- > [TfO]^- > I^- > [SCN]^- \approx [dca]^- \gg [Tf_2N]^-$$

For assessing the benefits and limitations of these rankings it is worthwhile to note that the single-ion separation underlying the ion series is only meaningful at low salt concentrations, strictly speaking requiring extrapolation of the measured data towards infinite dilution of the ILs. By contrast, applications usually concern high concentrations of ILs, where mutual interference and co-operative effects of cations and anions may render ion rankings qualitative and may result in an interchange in the positions of the ions. The basic assumption is that the ions have different capacity to enhance or to break the H-bonded bulk structure of water, which will affect protein hydration. Ions of high surface charge density are believed to be ‘‘structure makers’’, which enhance the H-bonded network. Large ions of low charge should act as ‘‘structure breakers’’, which destroy this network. In the biochemical literature the two types of ions are denoted as ‘‘kosmotropes’’ and ‘‘chaotropes, respectively. An optimum protein stabilization requires the combination of a chaotropic cation with a kosmotropic anion. For discussing the molecular basis of the observed salt effects it is optimize to first summarize some experimental results:

- In contrast to the dominance of anion over cation effects in the case of inorganic salts 35 cation variation in ILs results in similarly large effects as anion variation. This increased variability concerns only the destabilizing site of the Hofmeister series. Results for homologous cations show that the destabilizing tendency is closely related to the hydrophobicity of the organic cations.
- Most molecular anions of ILs do not form homologous series and their effects on proteins do not easily fit into a simple ordering scheme, except for the tentative conclusion that an increasing hydrophobicity of the anion increases the destabilizing tendency.

1.7 *Stability of protein in presence of ILs*

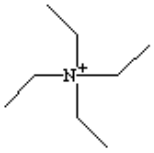
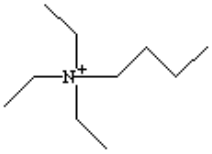
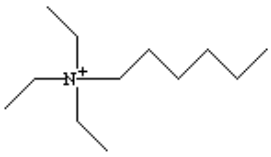
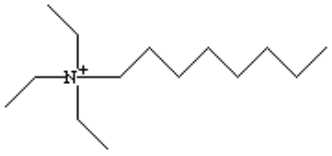
The action and dependability of native protein show peripheral changes in the vicinity of solvent environment. Ionic liquids speak to a somewhat differing class of co-solvents and that are blends of different particles, which are liquids at or near to room temperature. Then again, there are fast physiological hassles inside the cell hence the protein needs to keep up its native folded structure for proper working. Be that as it may, these physiological anxieties are overcome in the vicinity

of biocompatible ILs. ILs in light of the ammonium salts speak to the promising biocompatible solvent media for adjustment of native structure of biomolecules. In any case, ammonium ILs gives an exceptionally extraordinary study in a mixture of modern applications.

Ionic liquids have been produced as effective choices for natural solvents in conventional substance forms, and all the more as of late they have been investigated as trades for fluid media in catalyst based methodologies. Ionic liquids, organic salts with a melting temperature below 100°C, are a class of materials that have considerable potential to provide advances in the liquid formulation of protein pharmaceuticals. Organic salts with melting temperatures moderately above room temperature may easily be used as an ionic liquid solvent/aqueous co-solvent at room temperature by slightly hydrating the salt. The exciting feature of ILs is that because they consist of chemically distinct ions, the hydrogen-bonding character and the water miscibility can be essentially "tuned" to the application, depending on the nature of the cation and anion in the mixture [1,2]. Recent work has on the stability of BSA in different ionic liquids. Therefore, we planned to investigate the effect of ionic liquids on the structure of protein, BSA. The following ionic liquids (table-1) were employed in this work [17].

1.8 ***Proposed Ionic Liquid in the experiment***

Table-1: The Nomenclature, Acronyms, and Chemical Structure of the ILs Used in This Study

Entry	Ionic Liquid	Acronym	Cation	Anion
1	N,N,N,N-Tetraethylammonium Bromide	$[N_{2222}]Br$		Br^-
2	N,N,N-Triethylbutylammonium Bromide	$[N_{2224}]Br$		Br^-
3	N,N,N-Triethylhexylammonium Bromide	$[N_{2226}]Br$		Br^-
4	N,N,N-Triethyloctylammonium Bromide	$[N_{2228}]Br$		Br^-

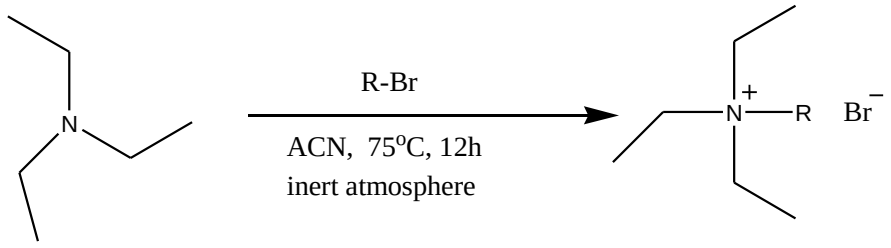
CHAPTER-2

MATERIALS AND METHODS

2.1 Materials - Bovine Serum Albumin (BSA) was purchased from Sigma Aldrich and used without further purification. All BSA solutions were prepared in the 0.01 M phosphate buffer solution (pH 7.4). Deionized water was used in preparation of aqueous solutions. All other common reagents were off analytical grade from commercial sources. ILs were synthesized as per the reported work (scheme-1)

Scheme-1

Ammonium-based bromide Ionic Liquids used in our study, abbreviated as $[N_{2222}]Br$, $[N_{2224}]Br$, $[N_{2226}]Br$, and $[N_{2228}]Br$ were synthesized by the following procedures.



Procedure - Triethylalkylammonium bromides were prepared by the alkylation of triethylamine (100 mmol) with corresponding alkyl halide i.e. ethyl bromide, N-butyl bromide, N-hexyl bromide, N-octyl bromide (100 mmol) in solvent acetonitrile (20 ml). The reaction mixture was stirred vigorously at a temperature of $75^{O}C$ for 12 h in an oil-bath, which was fitted with a reflux condenser. The solid product was washed thoroughly with hexane, filtered and dried under vacuum at $75^{O}C$ for 6 h to obtain the pure ILs.

2.2 Methods

2.2.1 Absorption-spectra - Absorption spectra for BSA in different concentration of ionic liquid solution were measured at room temperature by using Cary-100 UV–Visible spectrophotometer. Absorbance values were recorded in the range 200–320 nm. Absorption measurements were carried out by keeping the concentration of BSA at 2×10^{-6} M while varying the ionic liquid concentration from 2×10^{-3} M to 2×10^{-6} M. The ILs absorption was subtracted from the respective protein sample containing IL to remove the effect of ILs.

2.2.2 Fluorescence spectra - The fluorescence measurements were done using Horiba Jvon Spectrometer (Fluoromax-4P). Excitation wavelength was fixed at 295 nm to avoid the interference from Tyr (tyrosine) and Phe (Phenylalanine) and the fluorescence was collected as a function of wavelength in the range of 305 to 450 nm. Excitation and emission slit width were fixed at 5nm.

2.2.3 Circular dichroism spectra - Secondary and tertiary structures of the proteins were studied by a circular dichroism (CD) spectrophotometer (JASCO-1500) at room temperature under constant nitrogen flush. The scan speed (200nm/min) was fixed with a response time of 1 sec and 1 nm bandwidth. Each spectrum was collected by averaging three spectra. The protein's concentration was maintained at 2 μM & 100μM for far-UV & near-UV studies respectively. The ionic liquids concentration were varied from 2×10^{-3} M to 2×10^{-6} M. Each sample spectrum was obtained by subtracting the appropriate background (without protein) from the experimental protein spectrum.

CHAPTER-3

RESULT AND DISCUSSION

3.1 Absorption spectra - Absorption spectra of BSA in aqueous solution exhibits two absorption peaks at 200–250 nm and 260–300 nm, respectively. The first peak in the lower wavelength region (200–220 nm) reflects the backbone framework of the protein and it corresponds to the π-π* transition. The second peak in the higher wavelength region (260–300 nm) relates to the aromatic amino acids such as tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) and the result of the n-π* transition.

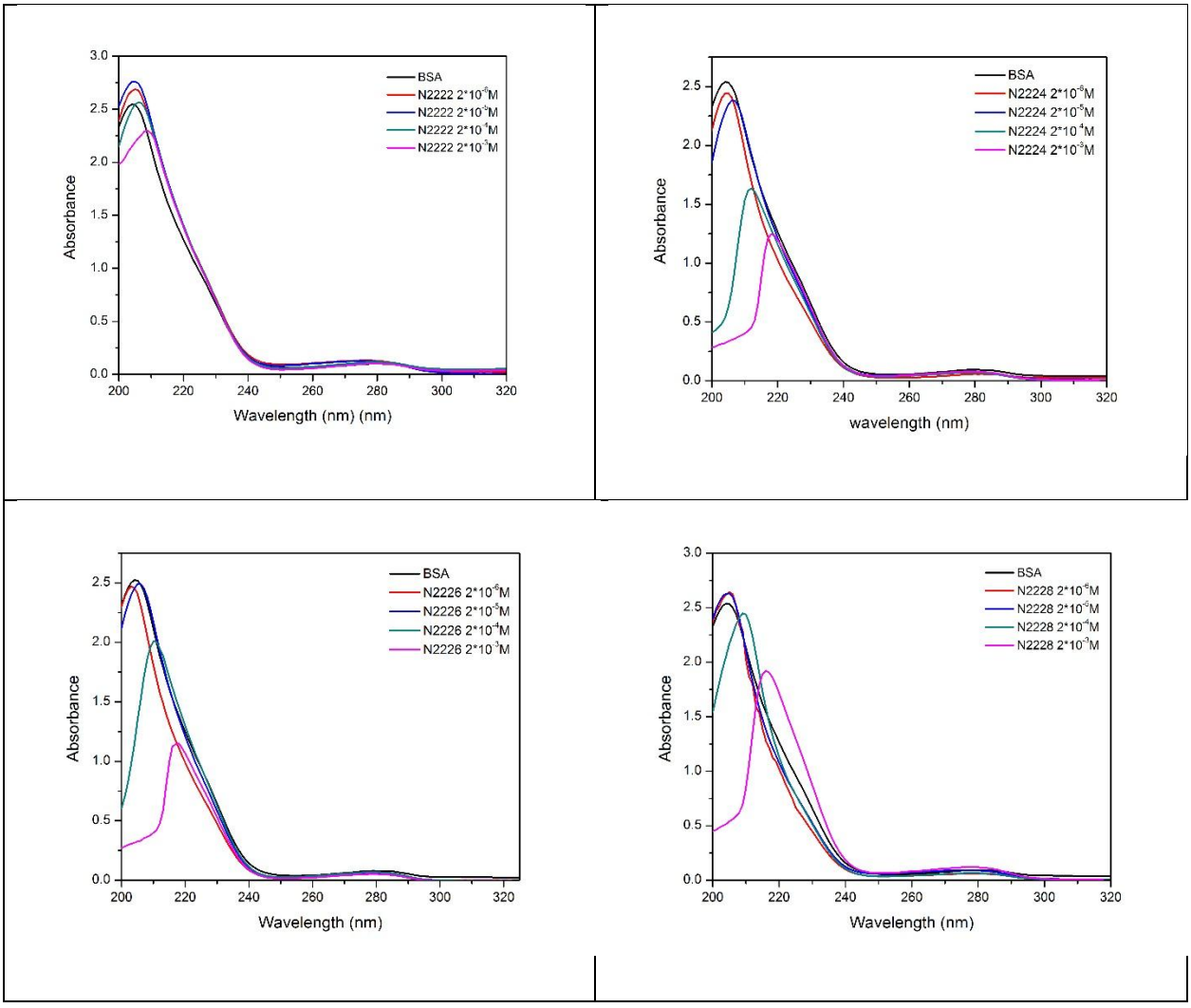


Figure 2: Absorption spectra of BSA in various ILs and as a function of their concentrations.

Absorption spectra of BSA with different concentrations of ILs were shown in Figure 2. The results indicated that the absorption spectra of BSA underwent changes when ILs was added. The maximum absorption of BSA decreased regularly with a red shift with increasing concentration of the ILs, lead to obvious hypochromic effect at 223 nm. It is probable that the increased polarity of the solvent induced the shielding of the peptide group from the aqueous environments and lowered the energy of the π-π* electron clouds assisting the low-energy π-π* transition. This lower energy π-π* transition appears as the bathochromic shift in the peak of BSA in the lower wavelength region [6]. These changes arise from the disturbance of the micro-environment around the polypeptide caused by the interaction of respective ILs with BSA. The peak of BSA corresponding to the aromatic amino acid residues (Trp, Tyr, and Phe) remains unchanged irrespective of the type of IL. This peak appears to have a maximum at 278 nm in each of the studied buffer condition. The small change in the intensity of this peak of BSA was observed from the samples containing IL with various concentrations. The observed minor changes may be the results of the change in micro-environment around aromatic amino acid residues due to the conformational change in the protein structure [10].

3.2 Fluorescence spectra - Since the fluorescence intensity of fluorophore molecule is highly sensitive to the molecular environment, therefore, fluorescence technique serves as an efficient tool in the investigation of molecular environment in the vicinity of chromophore molecule. In case of proteins, the aromatic amino acid residues, such as tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) are responsible fluorophore molecule. However, it was observed that, when enzyme is excited at or above 295 nm, the contributions from Tyr or Phe residues became negligible. Based on these structural features of tryptophan residues in BSA, it was assumed that the effect of solvent on the emission spectra of the BSA attributed to the change in environmental polarity of the tryptophan residue [3, 4]. This change in environmental polarity of tryptophan residues may be caused either by solvent polarity or the structural change in BSA.

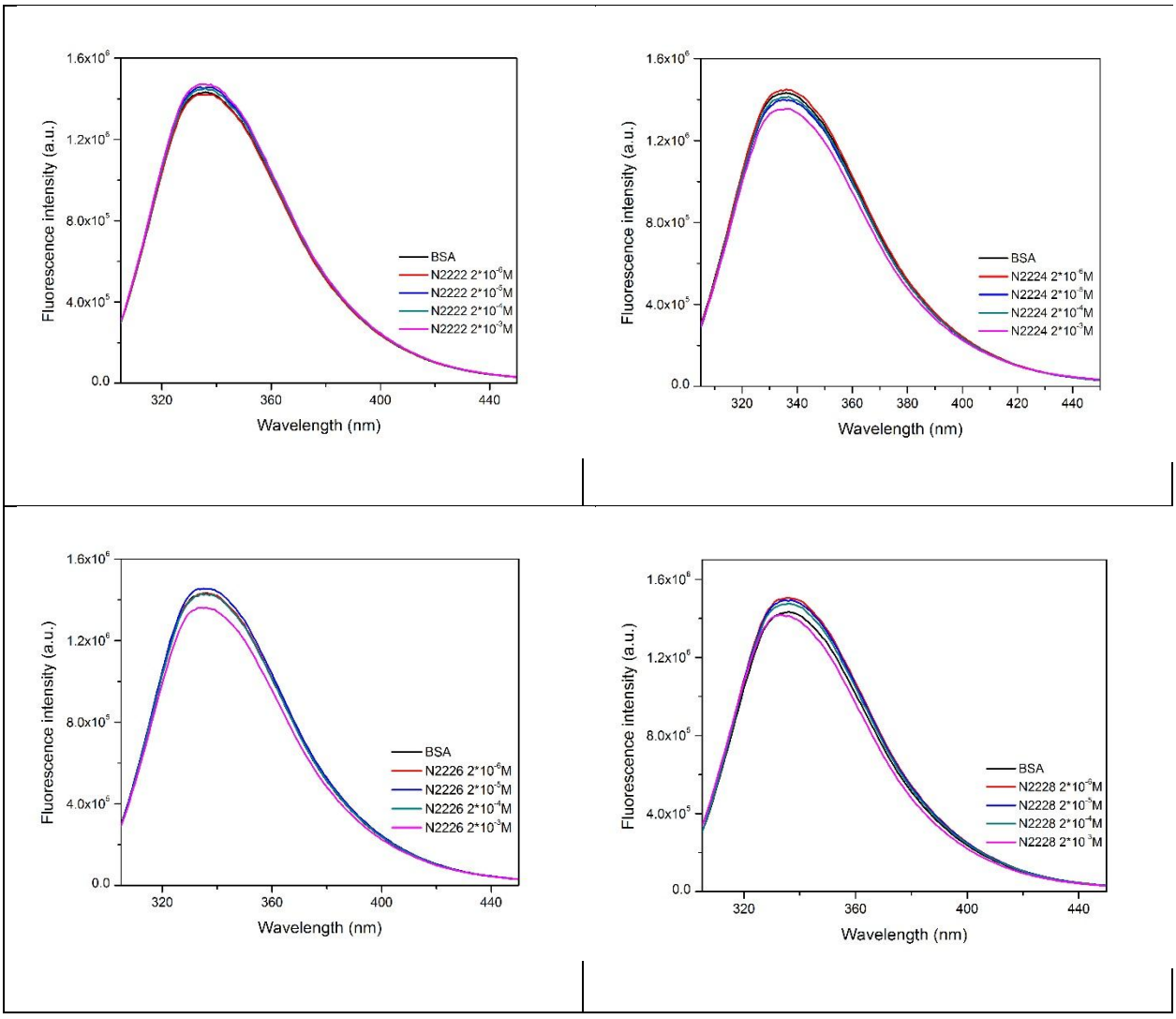


Figure 3: Fluorescence spectra of BSA in the absence and presence of various ILs and as a function of their concentrations. (Same notations as mentioned in UV spectra)

It is well known that the increase in hydrophilic environment around Trp residues may cause the shifting in the emission peak towards higher wavelength and the shifting in the emission peak toward lower wavelength corresponds to increasing in the hydrophobic microenvironment around Trp residues. Therefore, the Trp residue shielded from the aqueous environment undergoes blue shift with high quantum yield. It might be possible that this shielding effect may provide a more stable and compact native structure to the protein in the solvent medium [5]. From figure 3, it was observed the fluorescent intensity

decreases with increasing the concentration of the ionic liquids except in case of N2222 (???) where it was in reverse order. In all the cases, it was observed that there was no shift of emission maximum, which proves the environment of tryptophan was not changed in all the studied ILs [21, 22]. The quenching of emission intensity in presence of higher concentration of IL was attributed to the complex formation between BSA and IL.

3.3 Circular dichroism spectra

CD measures the absorption difference between left-handed and right-handed polarized light arising from structural asymmetry. The absence of regular structure produces zero CD single, while ordered structures produce a spectrum containing both positive and negative outputs [12, 13]. The near-UV CD spectral region (250-350 nm) is sensitive to certain aspects of a proteins tertiary structure. The signals in this region are associated with phenylalanine (254, 256, 262 and 267 nm), tyrosine, (276 and 283 nm) tryptophan (280-300 nm) and disulfide chromophores, which give rise to broad, but weak signals [14]. The signals in this region are sensitive to the chromophores local environment and consequently, the proteins tertiary structure. As such, near UV CD spectra provide a 'fingerprint' of protein morphology, but little quantitative structural information can be derived from this region.

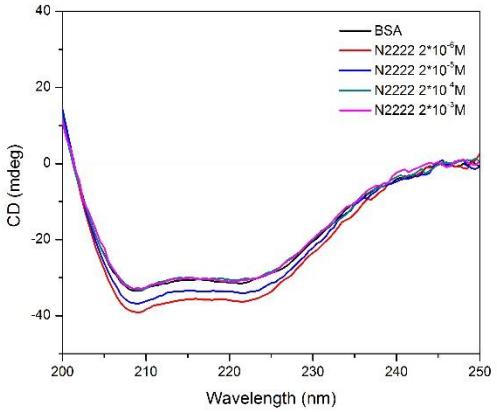
Secondary structural content can be calculated from the far-UV CD spectral region (190-250 nm). α- helix percentage of BSA were calculated as follows after expressing CD results in terms of mean residue ellipticity (MRE) in deg-cm^2 dmol^{-1} according to the following equation:

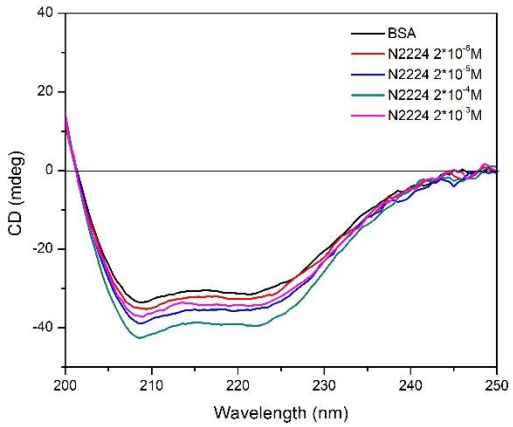
$$\mathrm{MRE} = \frac{\text{observed CD}(m\,\text{deg})}{C_P n l \times 10}$$

Where; Cp is the molar concentration of the protein, n is the number of amino acid residues (583 for BSA) and l the path-length (0.1 cm) [6]. The alpha-helical contents were determined from MRE values at 208 nm using the following equation:

$$\alpha - \text{helix}(\%) = \frac{-\text{MRE}_{208} - 4000}{33000 - 4000}$$

α- helix percentage of BSA (51%) in ILs				
ILs \ Conc.	2×10^{-6} M	2×10^{-5} M	2×10^{-4} M	2×10^{-3} M
N2222	62	60	50	50
N2224	54	61	69	59
N2226	60	57	50	57
N2228	59	63	53	





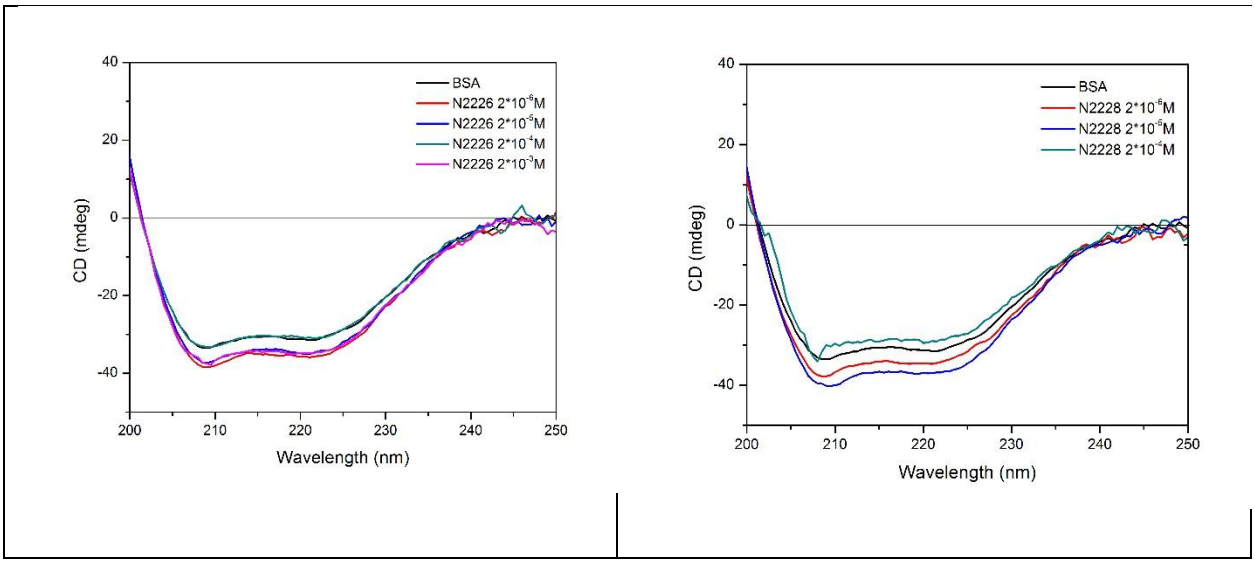
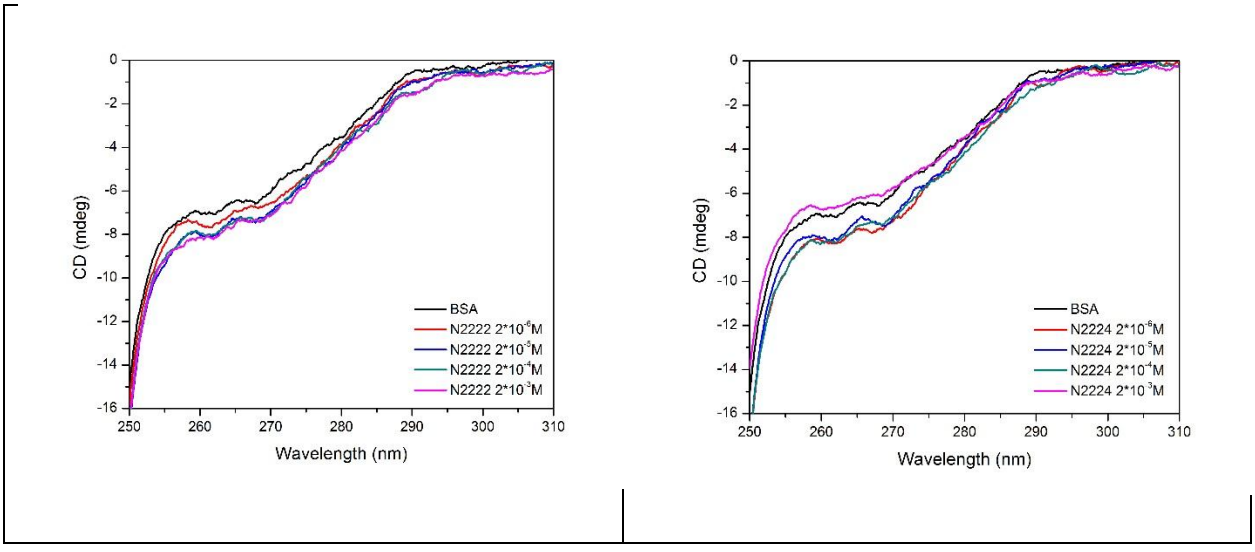


Figure 4: Circular dichroism spectra of BSA showing the secondary structures in the presence and absence of various ILs as a function of their concentrations.

The near-UV CD spectra of 100 µM BSA in ILs were presented in figure 4. BSA tertiary structure is characterized by the presence of two minima at 261 and 268 nm which is also referred to the environment around disulphide bonds and phenylalanine residues (phenylalanine shows weaker bands). The environment around aromatic chromophores can be analyzed by the study of the region 270-300 nm.



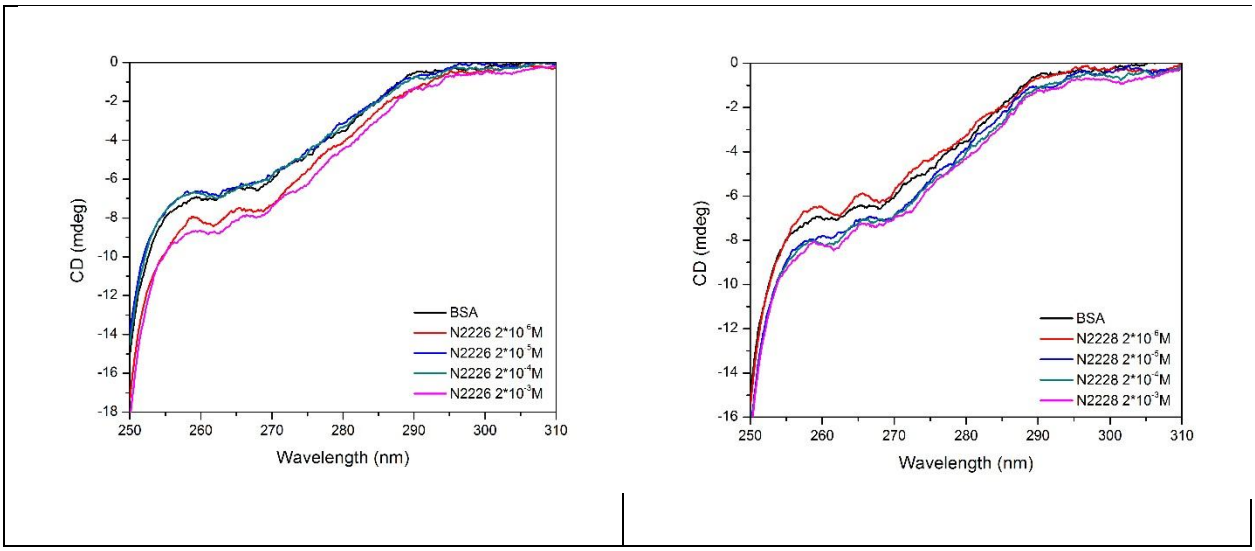


Figure 5: Near-UV CD spectra of BSA in various ILs (increase the Y-axis scaling a little more… may be from -18 to 1)

The near-UV CD spectra of BSA in ILs were shown in figure 5. From the results, it was observed that the ellipticity at 261 & 268 nm decreased in presence of all the ionic liquids at all concentrations. It means the tertiary structure of BSA was altered especially by the loss of asymmetry around disulphide bonds [7, 8]. It was also important to note that there was no significant change in ellipticity in the region 280-300 nm which confirms no change in environment around tryptophan residues which is in agreement with fluorescence data.

Conclusion

Among the studied ammonium based ionic liquids, it was observed that N2222 acts as a good stabilizer for the structure of BSA. That does not mean that the other ionic liquids are de-stabilizers. It was found that all the ionic liquids increase the α-helicity of BSA at low concentrations and they all kept the tryptophan environment as it is in native structure of BSA. Therefore, further studies like thermal stability, refolding in presence of these ionic liquids could make these ionic liquids a good solvent medium for protein structure and stability.

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