ROLE OF IONIC LIQUIDS ON STABILITY OF BOVINE SERUM ALBUMIN

A Dissertation

Submitted for the partial fulfillment

FOR THE DEGREE OF

MASTER OF SCIENCE IN CHEMISTRY

Under The Academic Autonomy

NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA

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CERTIFICATE

This is to certify that the dissertation entitled ***"Role of Ionic Liquids on the stability of Bovine Serum Albumin"*** being submitted by Ms.Eeti Chatterjee to the Department of Chemistry, National Institute of Technology, Rourkela, Odisha, for the award of the degree in Master of Science is a record of bonafide research carried out by her under my supervision and guidance. To the best of my knowledge, the matter embodied in the dissertation has not been submitted to any other University/ Institute for the award of any Degree or Diploma.

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ACKNOWLEDGEMENT

With deep regards and profound respect, I avail the opportunity to express my deep sense of gratitude and indebtedness to **Dr. Harekrushna Sahoo**, Department of Chemistry, National Institute of Technology, Rourkela, for introducing the present project topic and for his inspiring guidance, constructive criticism and valuable suggestion throughout the project work. I most gratefully acknowledge his constant encouragement and help in different ways to complete this project successfully.

I acknowledge my sincere regards to **Dr. B. G. Mishra (HOD, Dept. of Chemistry)**and all the faculty members, Department of Chemistry, NIT Rourkela for their enthusiasm in promoting the research programme in Chemistry and for their kindness and dedication to students.

I specially record my deep appreciation and thanks to Dr**. Suman Jha (Asst. Professor, Dept. of Life Sciences)** for his suggestion regarding this project. I would also like to thank to **Dr. Usharani Subuddhi (Asst. Professor, Dept. of Chemistry)**and her Ph.D. scholar for giving me the necessary permission to use their laboratory facilities whenever I needed. I am also thankful to **Dr. G. Hota (Associate Professor, Dept. of Chemistry) and Dr. N. Panda (Associate Professor, Dept. of Chemistry)** for giving me the permission to use their laboratory.

It's my pleasure to thank **Dr. Priyabrat Dash (Asst. Professor, Dept. of Chemistry)** and his Ph.D. students (Basanti Ekka and Lipika Rout) and also my classmates Shubhasmin Rana, Nilendri Rout, Chinmayee Priyadarshini and Aurobinda Mohanty for their co-operation and continuous encouragement throughout the entire period of the project work and specially thanks for making a friendly environment in the laboratory.

Last but not the least, I also take the privilege to express my deep sense of gratitude to my Family, for selflessly extending their ceaseless help and moral support at all times.

Eeti Chatterjee

DECLARATION

I, Eeti Chatterjee hereby declare that this project report entitled **"Role of Ionic Liquids on stability of Bovine Serum Albumin"** is originally carried out by me under the supervision of Dr. Harekrushna Sahoo, Dept. of Chemistry, National Institute of Technology, Rourkela and the present work or any part of this work has not been presented in any other University or Institute for the award of other degree regarding to my belief.

Eeti Chatterjee

ABSTRACT

Bovine Serum Albumin (BSA) in water and PBS gets affected by the introduction of different ionic liquids. The impact of the model ionic liquids, i.e., 1-ethyl-3-methylimidazolium sulfate, 1-ethyl-3-methylimidazolium chloride, and 1-butyl-3-methylimidazolium chloride, on the tryptophan environment (Trp-environment) was determined by using fluorescence technique. The effect of different aprotic ionic liquids with the variations in cations and anions is very much significant. The presence of ionic liquids alters the Trp environment present in BSA, i.e., presence of hydrophobic and longer alkyl chain moieties disrupt the hydrophobic environment of tryptophan in the native BSA structure. Similarly, anions like sulfate and chloride have significant impact. Surprisingly, chloride exerts less disruption towards the Trp-environment compared to sulfate, which violates the Hofmeister series (as per Hofmeister series, sulfate should have more impact on the stability compared to chloride).

CONTENTS

List of Figures:

List of Schemes:

CHAPTER-1

INTRODUCTION

INTRODUCTION

1.1 CONCEPT 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.

Following are the four different stages of protein:

1. The sequence of different amino acids of the protein forms its primary structure. These primary structures are held together by peptide bonds, which are made during the translation. Alternatively, these are called as polypeptide chains.
2. The secondary structure of proteins refers to the repetition of local structures, which are stabilized by hydrogen bonds. Alpha helix and Beta strand structures come under the secondary structure of protein. Two helices or Beta strands are interconnected by a un structured region, called as loop.
3. In molecular biology, the tertiary structure of a protein refers to its three dimensional structure [1]. This kind of structure is formed by the packing of secondary structure elements into protein domains [2]. Proteins that are formed by the gathering of separate, folded polypeptide chains give rise to the quaternary structure of protein. The tertiary structure is stabilized by the formation of hydrophobic core, salt bridges, hydrogen bonds, disulphide bonds etc. The basic functions of proteins can be controlled by its tertiary structure.
4. Many proteins are actually the combination of more than one polypeptide chain, which are also known as protein subunits. Multiple subunits of proteins refer to a quaternary structure, which functions as a single protein complex.

1.2 Serum Albumin:

Serum Albumin, a very stable, highly soluble multi domain protein, is commercially available at high purity and low cost. It does not contain any prosthetic group or bulky appending carbohydrates. It is of elliptical shape with low intrinsic velocity. Albumin is a very stable protein although more than 50 human albumins exists which are slight variants of the 585 amino acid sequence. The structure of Human Serum Albumin (HSA) has been revealed by the high resolution X-ray image of the protein [3,4].Since, serum albumin molecule can change its structure and conformation with variation in environmental conditions and with binding of ligands, therefore, this particular protein can be described as a very flexible protein. Besides this, albumin has a resilient structure and regains its shape easily owing to the disulphide bridges, which provides strength in physiological conditions. [5]

Albumin is the most abundant extracellular protein. It contains total 60% of the total serum content in human. It is a single polypeptide with 585 amino acids having molecular weight of 66.2kD and is expressed in the liver. It differs from the extracellular proteins by its primary structure. Structurally, it has multiple cystein (Cys-34) and has two tryptophan. X-ray crystallography studies reveals that the secondary structure of serum albumin consists of approximately 67% of α-helix as well as there are 9 loops and 17 disulphide bridges giving a heart shaped 3D structure [3, 6]. The tertiary structure of serum albumin is composed of three domains I, II, and III, and each domain is constituted of two sub-domains A and B.

1.2.1 Function of Serum albumin:

Albumin is the most abundant protein present in blood plasma having typical concentration of 50 g/L and it has been one of the most extensively studied protein. Some of the most important and commonly studied proteins are Human Serum Albumin (HSA), Bovine Serum Albumin (BSA), Equin Serum Albumin (ESA) and Rat Serum Albumin (RSA).

Physiological Roles of albumin:

1. **Maintenance of the colloid osmotic pressure (COP):**

   Albumin is responsible for the 75-80% of the osmotic pressure. It constitute the main protein in the blood plasma. Therefore it can be considered as the COP gradient rather than the absolute plasma value. It determines the flow of fluid in and out of the capillaries [5].

2. **Binding and transport of drugs:**

   It is responsible for the transportation of drugs and reduces the serum concentration of the compound. Albumin has four different binding sites with varying specificity for defferent substances. It can be considered as the carrier of numerous exogenous and endogenous compounds in the blood [3, 6].

3. **Acid Base balance:**

   Albumin is a negatively charged protein present in high concentration in the plasma. It contributes heavily towards the "Anion gap". The concentration of anions and cations in plasma should be equal so that the remaining anions come predominantly from albumin, inorganic phosphate and hemoglobin. Thus, when the concentration of albumin is high in blood plasma, the anion gap should be narrow [5].

We have used Bovine Serum Albumin (BSA) protein for this experiment. The intrinsic fluorescence of tryptophan present is used for the stability study of the protein.

1.2.2 Difference between HSA and BSA:

HSA and BSA are the most commonly studied serum albumin proteins. Almost 76% homology and a repeating pattern of disulphide occurs between HSA and BSA. The major difference between these two proteins is the number and position of tryptophan residues present. HSA has only one tryptophan, located at position 214 and it is equivalent to the tryptophan present in BSA at position 212 which is buried in a hydrophobic pocket at sub domain IIA. [8] BSA has two tryptophan located at position 212 and 134, the later one is more exposed to solvent than that of trp-212 and is found at sub domain IB [4, 5]. Because of the medical importance, low cost, ready availability, and unusual ligand binding properties, BSA is used as the model protein [3, 7]

We are studying the local environment of fluorescent amino acid like phenylalanine, tyrosine, tryptophan and cystiene present in the protein using fluorescence spectroscopy. Moreover, we are very much interested to study the local environment of tryptophan amino acid which is present in BSA protein.

1.2.3 BSA protein (Bovine Serum Albumin) and its structure:

BSA can be derived from cows and it is used as an standard for protein concentration. "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.

Structure of BSA:

The primary structure of BSA is composed of 583 amino acid residues, the sequence has 17 disulphide bonds resulting in nine loops formed by the bridges. It contains multiple cysteine and 8 pairs of disulphide bonds similar to HAS [3].

According to the amino acid sequence, the structure of BSA shows that it is composed of three homologous domains [9, 10]. 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 [11, 7].it has been suggested that the α-helices are uniformly placed in the subdomains and in the connections between thedomains. 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 helices 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 [6].

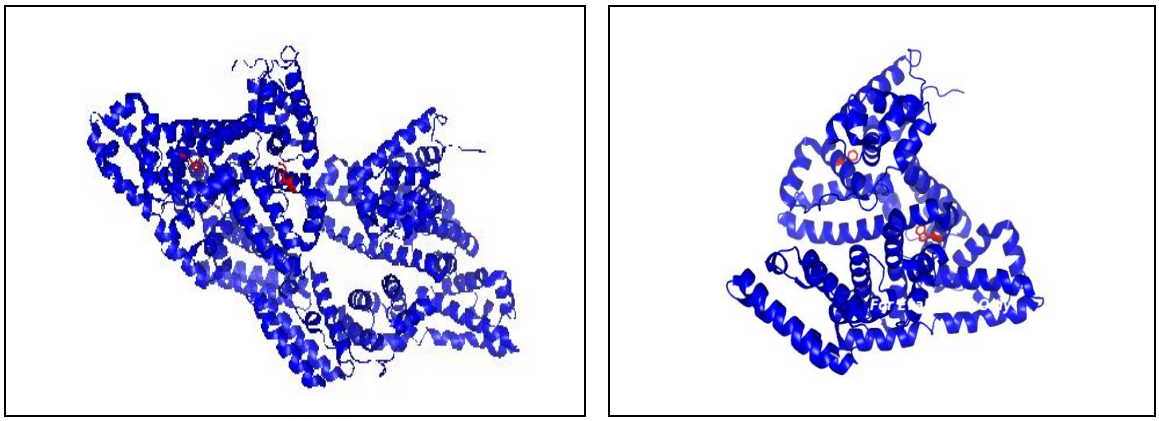


Figure 1: Crystal structure of BSA (PDB ID: 4F5S). Left panel represents the dimeric form where as right panel shows the monomeric form with the highlighted tryptophan (red in color).

1.2.4 Properties of BSA:

The full length BSA protein has 583 amino acid residues.[12] The N-terminal having 18 residual single peptide is cut off from the precursor protein on secretion, hence the initial protein product contains 589 amino acid residues. For the efficient expression and purification of a mature BSA protein(containing 585 amino acids), additional 4 amino acid is cleaved. [13, 14]

1.2.5 Application of BSA:

1. It has numerous biochemical application including ELISA (Enzyme Linked Immunosorbent Assay), immunoblots and immunohistochemistry. It is also 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. [15]
3. 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. [12]

1. 3 ROLE OF IONIC LIQUIDS:

Because of the binding of two or more proteins, interaction occurs between proteins. DNA replication is carried out by protein – protein interaction, where large molecules are built from a large number of organized protein components. The interaction of proteins is involved in Bio-Chemistry, Quantum Chemistry, Molecular Dynamics, Chemical Biology and Signal Transduction etc. [16] This kind of interactions of proteins occurs at the core of the interatomic system of all living cells.

Importance:

1. A part of a protein complex can be form by the interaction of protein for a long time. A protein may be carrying another protein (eg. In case of the nuclear improteins from cytoplasm to nucleus and vice versa), or for the modification of protein it may interact with another protein (eg. A phosphate group will be added to a target protein by a protein kinase). This kind of modification of protein refers to the change in protein – protein interaction.
2. Some specific proteins having SH_2 domains can only bind to other proteins when they are phosphorylated on the amino acid tyrosin while bromo domains specifically recognize acetylated lysines.[17] Protein study gives new therapeutic approaches for various diseases.

1.3.1 ionic liquid

Ionic liquid is a salt which is present in liquid state and its melting point is below 100^{o}C. Ionic liquids are composed of ions and short lived ion pairs. The decomposition or vaporization of any salt without melting, results ionic liquid. Crystalline or glassy ionic solids can be prepared by the cooling of ionic liquids. These are the normal solvents mainly consists of bulky, non-symmetric organic cations such as imidazolium, pyrolidinium, pyridinium, ammonium or organic anions such as tetrafluoroborate, bromide ions etc.

Ionic liquids are poor conductors of electricity. Along with this, it has various unique properties such that, negligible vapour pressure, good thermal stability, tunable viscosity, miscibility with water and organic solvents. Ionic liquids are highly viscous, low toxic and non-ionizing in nature. Non-flammable and non-volatile property of ionic liquid makes it an good option for the

development. The selectivity and variety of chemical reactions can be alter by the ionic nature of the ionic liquids. The cationic and anionic properties of ionic liquids have some basic properties such as, polarity, hydrophobicity and other chemical and physical properties. Ionic liquids can be used as a designer solvents because of their tunable property and it increases the potential application. Due to the delocalization of the molecular charge of some typical ions present in ionic liquids show moderate polarization charge densities. [16, 17]

The commonly used cations in ionic liquids are hexafluorophosphate, ethylsulphate, 1-alkylpyridinium, 1,1-dialkylpyridinium, 1-ethyl-3-methyl imidazolium, 1,3-dialkyl imidazolium, tetra alkylammonium, tetra alkylphosphonium, 1-alkyl pyrrolinium, 1,3-dialkyl trizolium, hexaalkylgyaninium1-alkylthiazolium. Commonly used anions are chloride, bromide, iodide, nitrate, nitride, isothiocyanate, nitrite, [amino acetate]$^-$,[p-$MeC_6H_4SO_{3]}^-$, $[CF_3CO_2]^-$,$[OCN]^-$, $[N(CN)_2]^-$, $[Al(Et)Cl_3]^-$, $[Al_2Cl_7^{]-}$, $[Al_3Cl_{10}]^-$, $[Al_2(Et)_2Cl_5]^-$, $[Al(CH_2CF_3)_4]^-$, $[MeCO_2]^-$, $[SbF_6]^-$, $[P(C_2F_5)_3F_3]^-$, $[BF_4]^-$, $[(RO)_2PO_2]^-$, $[ROSO_3]$, $[B(oxalate)_2]^-$, $[B(C_6H_{44}\text{-}CF_3)]^-$, $[C(CN)_3]^-$, $[F(HF)_n]^-$, $[(CF_3SO_2)_2N]^-$, $[CF_3SO_3]^-$, $[ROSO_3]^-$, $[(RO)_2PO_2]^-$, $[PF_6]^-$. [18, 19, 20]

1.3.2 APPLICATION OF IONIC LIQUID IN VARIOUS FIELD: -

1. **Pharmaceutical Industries :-** Three kind of generations of ionic liquids are used in pharmaceutical industries. The first generation refers to the physical and chemical properties such as, density, viscosity, solubility, chemical stability etc. The second generation is important for the tunable properties of ionic liquids and the third generation is the recent generation which involves pharmaceutical ingredients. Many pharmaceutical drugs are formed by using ionic liquids which provide many wonderful properties compared to usual counter parts. [21, 22]
2. **Antibacterial Activity:-** Ionic liquids are very much active against gram-positive and gram-negative bacteria fungi and algae. The ionic liquid 1-alkylquinolinium ionic liquid has a vigorous activity against microorganisms which grown in both planktonic and sessile mode of growth. Neutralization of Tetrabutylammonium hydroxide results in the formation of some room temperature ionic liquids like tetra butyl ammonium salts such that, formate acetate, propionate, benzoate, salicylate etc. [23, 24, 25]

Imidazolium, phosphonium and ammonium cations of ionic liquids act as anti cancer. The preliminary structure plays an important role towards the anti tumor and anti toxic properties and it also gives more information about the chain length of alkyl substitution. Because of the good toxicology and solubility properties of ionic liquids, these are an excellent option for biotechnological application. Ionic liquids are also known as greener solvents because they are non-volatile and can made from nontoxic components. Based on the above advantages, we used ionic liquids (as a function of the type of cations and anions). [26, 27, 28]

1.3.3 IMPORTANCE OF IMIDAZOLIUM CATION:-With a small change in imidazolium cations, the physical properties changes drastically. The density of ionic liquid increases with the increase in molecular weight of anion while it decreases with the increase in alkyl chain length.

1.3.4 IMPORTANCE OF ANION:-The thermal stability increases with the increase in the size of the anion. The heat capacity also increases with the increasing number of anions in the ionic liquids. In room temperature the anions of ionic liquid has greater effect on the hydrogen bonding in comparison to cation. [29]

1.3.5 IMPORTANCE OF EMIM SULFATE:-

Presence of chloride ion increases the viscosity of ionic liquid where as presence of water and other co-solvents reduce the viscosity. 1-Ethyl-3-methyl imidazolium ethylsulfate is less expensive, water miscible, air stable, low viscous and it can be easily prepared by using diethyl sulphate in halide free manner.

1.3.6 IMPORTANCE OF CHLORIDE ANION:-

Viscosity is a physical parameter that depends directly on the concentrations of the ionic liquids. When viscosity increases the cohesive force also increases through hydrogen bonding between the chloride ion and the protons of the imidazolium ring. But presence of chloride ion decreases the density of ionic liquid. In case of proton NMR spectra of ionic liquid contain chloride ion causes the downfield shift. [30]

We are studying the local environment of fluorescent amino acid like phenylalanine, tyrosine, tryptophan and cystiene present in the protein using fluorescence spectroscopy. Moreover, we

are very much interested to study the local environment of tryptophan amino acid which is present in BSA protein.

1.4 Aim of the present work

The basic goal of this research work is to point out the effects of different ionic liquids, which are common and widely used, on the protein stability and particularly, study of the Trp-environment holds the primary aspect. In this regards, the ionic liquids are varied on the basis of their anions (keeping fixed the cation) and cations (keeping fixed the anion). For this purpose, fluorescence spectroscopy technique is used as the basic and fundamental instrumentation.

CHAPTER-2

MATERIALS AND METHODS

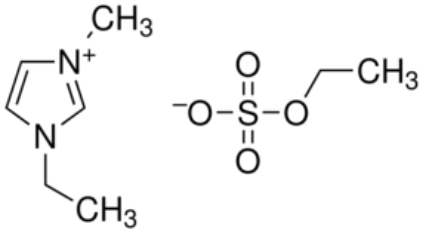
2.1 MATERIALS:

2.1.1 Protein used:

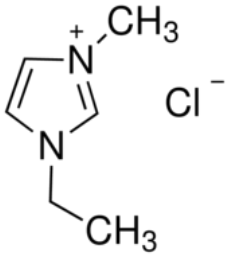
Bovine Serum Albumin (Product No- A2153) was purchased from Sigma Aldrich. The native fluorescence of tryptophan present is used for the stability study of protein. It was used without further purification.

2.1.2 Ionic Liquid used:

1-Ethyl-3-methyl imidazolium ethyl sulphate ($C_8H_{16}N_2O_4S$) [MSDS No: 51682] and 1-Ethyl-3-Methyl imidazolium chloride ($C_6H_{11}ClN_2$) [MSDS No: 30764] was purchased from Sigma Aldrich.



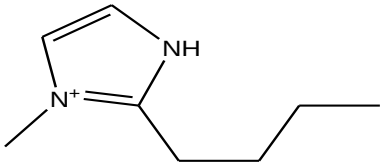
(1-Ethyl-3-methyl imidazolium ethyl sulphate)



(1-Ethyl-3-Methyl imidazolium chloride)

Apart from these two commercially available ionic liquids,we have used one more ionic liquid synthesized in Dr. Priyabrat Dash's laboratory.

I. 1-Butyl-3- methyl imidazolium chloride (BMImCl)



1-butyl 3-methyl imidazolium chloride

2.1.3 Solvents used:

Deionised water was used for all experiments. Ionic Liquids were used as the additives in solvents.

2.1.4 Instrumentation:

The steady-state fluorescence measurements were recorded using *Horiba Jobin Yvon Spectrofluorimeter (Fluoromax-4P).*

2.1.5 Preparation of BSA solution:

The BSA stock solution was prepared in deionised water freshly every time. The required protein concentration (~100 µM) was prepared on measuring the amount of protein and crosschecking the concentrations using its molar absorption coefficients. The protein solution was allowed for the homogeneous mixing before starting the measurements.

2.1.6 Preparation of Ionic liquid solution:

The stock solution of the three different Ionic liquids was prepared by weighing required amount followed by the addition of solvent. Then the dilution was done to obtain the required concentration for the targeted experiments.

2.2 METHODS:

2.2.1 Measurement of Absorption Spectrum:

The main elements of UV-VIS spectrophotometer are a light source, a monochromator and a detector. The monochromator works as a diffraction grating to dispense the beam of light into various wavelengths. The detectors role is to record the intensity of the light which has been transmitted.

Before the samples are run, a reference must first be taken. This calibrates the spectra to screen out an spectral interference. In this case milipore water was used as solvent as reference.

2.2.2Measurement of Steady-State Fluorescence Spectrum:

The spectrofluorimeter is an instrument which takes the advantage of intrinsic or extrinsic fluorescent properties of the compounds in order to provide information regarding their concentration and chemical environment in a sample. A certain excitation wavelength is selected, and the emission is observed at a wavelength scan to record the intensity versus wavelength, also called as steady-state emission spectra. The slit widths are also fixed for a particular experiment at which the best output signal is obtained. In the present experiment, the excitation wavelengths selected was 295 nm (to avoid the excitation of tyrosin, Phenyl alanine & Cysteine of protein) and the emission spectra were recorded in the range of 325 nm-450 nm, with slit widths of 3/5 nm.

Basic Concepts of fluorimeter:

1) Excitation source is provided by a Xenon lamp.

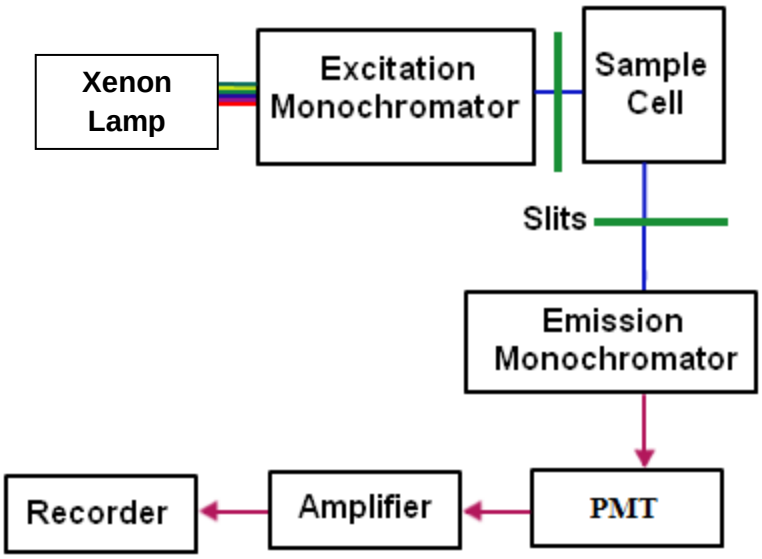
2) Light passes through an excitation filter before entering sample compartment. Prior to it, light passes through the excitation monochromator.

3) Light is absorbed by the fluorescent sample in the sample chamber.

4) After excitation of the fluorescent substance, the excited molecule returns to ground energy state and light with a longer wavelength (fluorescence) is emitted.

5) Fluorescent light passes through an emission filter. The excitation beam path is right angle to the emission beam in the set up.

6) The amount of light passing through the emission is measured with a photomultiplier Tube (PMT). Flourophores (in our case, it is tryptophan) preferentially absorb photons whose electric vectors are aligned parallel with transition moment .



Scheme 1: Schematic diagram for the Steady-State Fluorimeter setup.

2.3 Parameters studied:

2.3.1Solvent:

Ionic liquids are used as additives in Buffer and Water. Different concentrations of the Ionic liquid were taken to measure the fluorescence spectra. In this experiment basic buffer has been used which has pH at around 7.4. In order to avoid the pH parameter during result interpretations, we measured the pH of individual solutions in the presence and absence of ionic liquids.

Buffer						Water		
Ionic liquid						Ionic liquid		
	$EMImSO_4$	EMImCl	BMImCl			$EMImSO_4$	EMImCl	BMImCl
Conc.(M)	1.0	1.0	1.0	0.1	0.5	1.0	1.0	1.0
pH	7.5	7.5	7.3	6.9	7	7.3	6.3	8.2

CHAPTER-3

RESULT & DISCUSSION

3.1 Role of Anions:

***1-Ethyl-3-methylimidazolium Ethyl Sulfate* (*EMIMSO$_4$*):** Steady-state fluorescence measurements with $EMIMSO_4$ as an additive in water and PBS buffer (pH 7.4) indicate an alternation in both compactness of the Trp environment with respect to the native protein and thus, it allows the Trp to interact more efficiently with the solvent molecules compared to the native state.

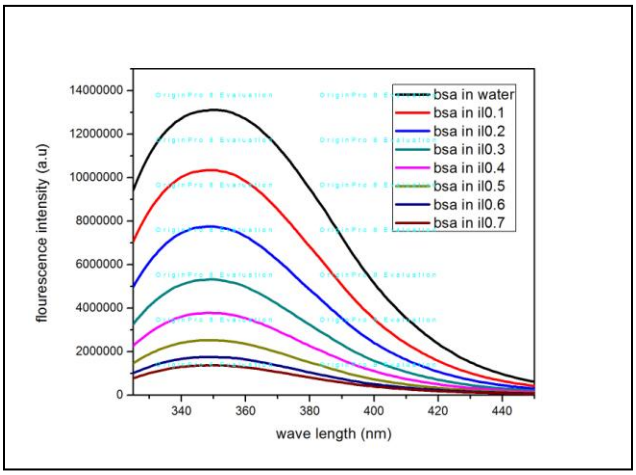
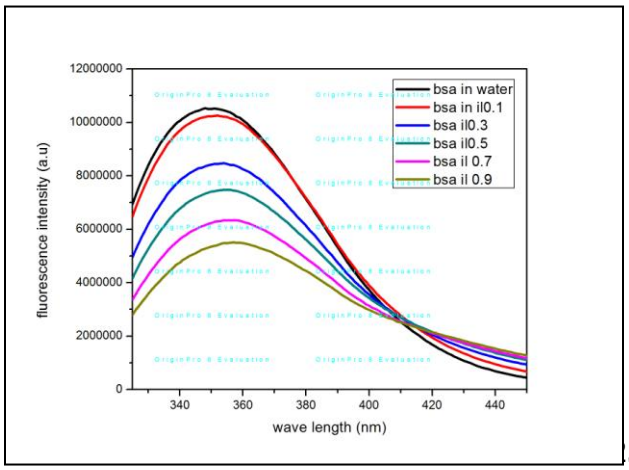
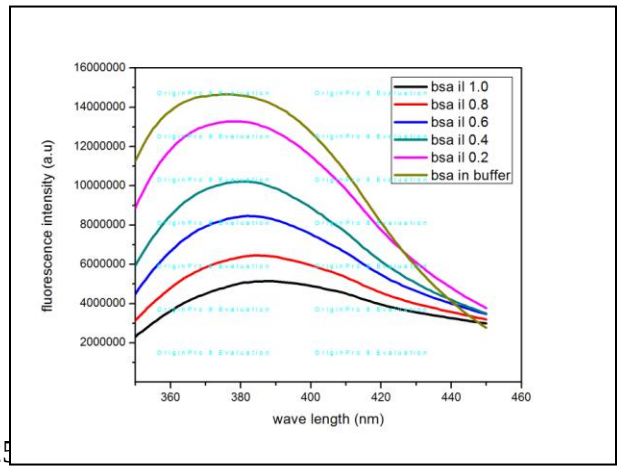


Figure 2: BSA emission spectra as a function of $EMIMSO_4$ concentration in water (1A).

***Ethylmethylimidazolium Chloride* (*EMImCl*):** Use of EMIMCl revealed the impact of anion on the protein stability compared to that of $EMIMSO_4$. Presence of EMIMCl as an additive in water and PBS buffer (pH 7.4) disrupts the compactness and increases the solvent exposure of the Trp environment with respect to the native protein. When EMIMCl is used as the additive in water or PBS medium, Trp environment in BSA gets less compact compared to the native Trp structure as a function of concentration of the ionic liquid as a result of which Trp pocket opens up and gets more exposed to the solvent.





F**igure 3:** BSA emission spectra as a function of EMIMCl concentration in water and PBS.

Trp shows a gradual decrease in fluorescence due to the quenching by the solvent molecules. The decrease in the fluorescence intensity could be as a result of the chaotropic nature of the chloride ion.

4. CONCLUSION:

It is observed that the destabilization of the protein or the disruption of the hydrophobic core depends on the alkyl groups attached to the imidazolium cation, i. e., with increasing in chain length, the destabilization/unfolding of BSA and disruption of the Trp-hydrophobic environment increases. From various experiments, it is concluded that the destabilizing characteristics of BSA increases with increase in the alkyl chain length or the chaotropic character of the individual cations and anions.

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