2017

Improved Protein Characterizations using Ionic Liquids: PAGE and MALDI-MS

Punprabhashi Vidanapathirana
Louisiana State University and Agricultural and Mechanical College, punprabhashi@gmail.com

IMPROVED PROTEIN CHARACTERIZATIONS USING IONIC LIQUIDS: PAGE AND MALDI-MS

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Chemistry

by
Punprabhashi Vidanapathirana
B.S., University of Colombo, Sri Lanka, 2010
August 2017

DEDICATION

I dedicate this work to my beloved parents, the reason for whom I am today and to my wonderful husband, who supported me to achieve this goal with his eternal love and care

ACKNOWLEDGMENTS

I appreciate all the people in graduate school who helped me to achieve my goals, especially:

Prof. Isiah M. Warner, my Ph.D. advisor for his proper guidance, support, encouragement, and care throughout the Ph.D. program.

Prof. Doug Gilman, Prof. Megan Macnaughtan, and Prof. Chandra Theegala for serving as my doctoral Committee and valuable support towards my dissertation.
Dr. Farhana Hasan for introducing me to protein separation studies and guidance throughout my projects.

Prof. Anne Grove for her assistance with protein separation technique development and valuable suggestions.

Prof. Kermit Murray for helpful comments and suggestions on protein detection studies using MALDI mass spectrometry.

Dr. Jack Losso and Dr. Samuel Washington for their contributions with IEF technique development.

Dr. Noureen Siraj, Dr. Susmita Das, Dr. Girija Sahasrabudhe, Dr. Pratap Chhotaray, and Dr. Rocio Perez for their valuable discussions on projects.

Anuja Pande for her contribution on cationic gel electrophoresis technique development.

Kaitlyn Mussio, Michael Brands, and Abdul-Rahman Ezzir for their contributions on experiments.

Past and present Warner Research Group for their helpful comments and suggestions.

Funding source for projects, National Science Foundation

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

α-CHCA	Alpha cyanohydroxycinnamic acid
α-Chy	Alpha chymotrypsinogen
α-Lac	Alpha lactalbumin
β-cas	Beta casein
BSA	Bovine Serum albumin
Cat	Catalase
CD	Circular dichroism
CMC	Critical micelle concentration
C_4PBr	4-Butene-4-methylpyridinium bromide
C_8PBr	8-Octene-4-methylpyridinium bromide
C_{11}PBr	11-Unedcene-4-methylpyridinium bromide
CTAB	Cetyltrimethylammonium bromide
Cyt c	Cytochrome c
IEF	Isoelectric focusing
ILs	Ionic liquids
ILS	Ionic liquid surfactants
IPG	Immobiline pH gradient
PAGE	Polyacrylamide gel electrophoresis
MALDI	Matrix assisted laser desorption ionization
Myo	Myoglobin
Ova	Ovalbumin

Rib b	Ribonuclease b
SDS	Sodium dodecyl sulfate
TOF	Time-of-flight
Tr	Transferrin
TCA	Tricholoroacetic acid

ABSTRACT

Protein separation by polyacrylamide gel electrophoresis (PAGE) and identification by matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS) are primary tools of protein analysis. In these techniques, surfactants are used in protein sample preparation in order to enhance the protein solubility. Conventional surfactants have shown limitations in protein analysis due to the structural complexity of proteomes, resulting in low resolution. The research goal of this dissertation is to address some of these limitations by applying novel cationic ionic liquid surfactants (ILS), *N*-alkyl-4-methyl pyridinium bromide (C_nPBr where n=4, 8, 11). The ILS would be suitable candidates to be used in PAGE protein separations as a result of positive cooperative binding to proteins at low concentrations of ILS and protein denaturing ability at room temperature. These compounds were used as buffer additives in ILS-PAGE protein separation and matrix additives in MALDI-MS protein identification. Anionic ILS-PAGE was used to separate a mixture of acidic proteins by applying ILS in sample and running buffers. Protein separation was improved for transferrin and ovalbumin, which were resolved as multiple bands of isoforms. In cationic ILS-PAGE, ILS were applied in polyacrylamide gels in addition to sample and running buffers. Separation of both acidic and basic proteins as sharp bands with high resolution is a major advancement of this technique. Cationic ILS-PAGE was used to resolve ribonuclease b glycoforms as multiple protein bands. In contrast, the same protein was migrated as a single band in Sodium dodecyl sulfate (SDS)-PAGE. Moreover, alpha antitrypsin glycoforms were resolved as multiple spots by two dimensional (2D) Isoelectric focusing (IEF)/ILS-PAGE. Furthermore, C_4PBr and C_8PBr ILS were applied as matrix additives with MALDI matrix, α-cyanohydroxycinnamic acid (CHCA), to perform protein sample analysis as well as rat brain tissue profiling. ILS showed high protein signal intensity at low concentrations

(0.02% (w/v)) in protein samples compared to SDS, cetyltrimethyl ammonium bromide (CTAB), and no surfactants present (blank). A large number of new protein peaks were acquired from tissue sample as compared to the absence of ILS in the matrix. These results show the applicability of ILS in improved protein identification by MALDI imaging mass spectrometry.

CHAPTER 1: INTRODUCTION

1.1. Background

Proteins are essential macromolecules with diverse biological functions, such as enzyme catalysis, regulation of biological activities as hormones, molecular transportation, act as structural proteins, and involve in immunity reactions as antibodies in living organisms.[1] Protein structure and function are significantly affected by various disease stages[2] and changes in cellular environments.[3] Hence, studies on proteins are essential to diagnose progression of diseases and to discover new medicines.

Proteomics is the analysis of the whole protein content of a cell, tissue or an organism.[4] The proteome undergoes structural and functional changes based on different biological conditions and pathological stages.[5,6,7] Compared to the complexity of a genome, which codes a particular proteome of a living organism, a proteome is more complex due to the additional post translational modifications and alternate splicing during protein synthesis.[4] Hence, proteome analysis becomes extremely challenging even for a single cell. In order to analyze structural and functional properties, a proteome should undergo a sequential analytical process known as proteomic workflow. A typical proteomic work flow includes protein extraction from cells or tissues, separation, purification, and identification.[8] Solvent extraction[9] and solvent precipitation[10] are typical methods of protein extractions, while gel electrophoresis,[11] IEF,[12] and liquid chromatography[13] are some of the major tools used in protein separations and purifications. Then protein identification is performed by use of mass spectrometry.[14]

Proteins with different structural and functional properties demand variable agents and techniques to separate and identify them. For example, SDS-PAGE, the most common protein

separation technique, shows unexpected migration patterns for certain hydrophobic proteins due to charge heterogeneity.[15,11,16] Such proteins require separation techniques, which are more compatible with protein charges and capable of denaturing complex protein structures. Similarly, mass spectrometry identification of proteins, particularly in tissue analysis, often requires new agents to improve protein signal intensity and resolution. As a result, a growing interest is being placed on the development of novel techniques and agents that have capacity to enhance the efficacy of protein separation and identification.

1.2. Protein Structure

Protein structures can be classified into several classes, primarily based on amino acid sequences. Protein conformation, which results due to variable amino acid interactions, determines the protein structure.[17]

1.2.1. Protein Primary Structure

Two termini of a polypeptide chain are identified as C-terminus and N-terminus, in which the C-terminus is a carboxylic group and N-terminus is an amino group. The α-amino group of one amino acid can react with the α-carboxyl group of the adjacent amino acid by a condensation reaction. These bonds, known as peptide bonds, can link multiple amino acids to form linear polypeptide chains, known as protein primary structures (Figure 1.1). Every protein has a unique amino acid sequence in the primary structure.

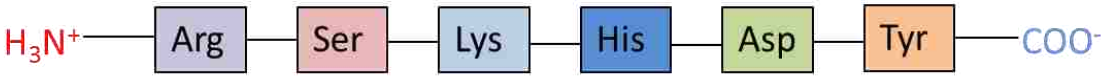


Figure 1.1. Primary structure of a protein with the N-terminus on left and the C-terminus on right

1.2.2. Protein Secondary Structure

Spatial interactions between nearby amino acid residues in a primary structure leads to a secondary structure. The two major types of protein secondary structure are α-helix and β- sheets are shown in Figure 1.2.

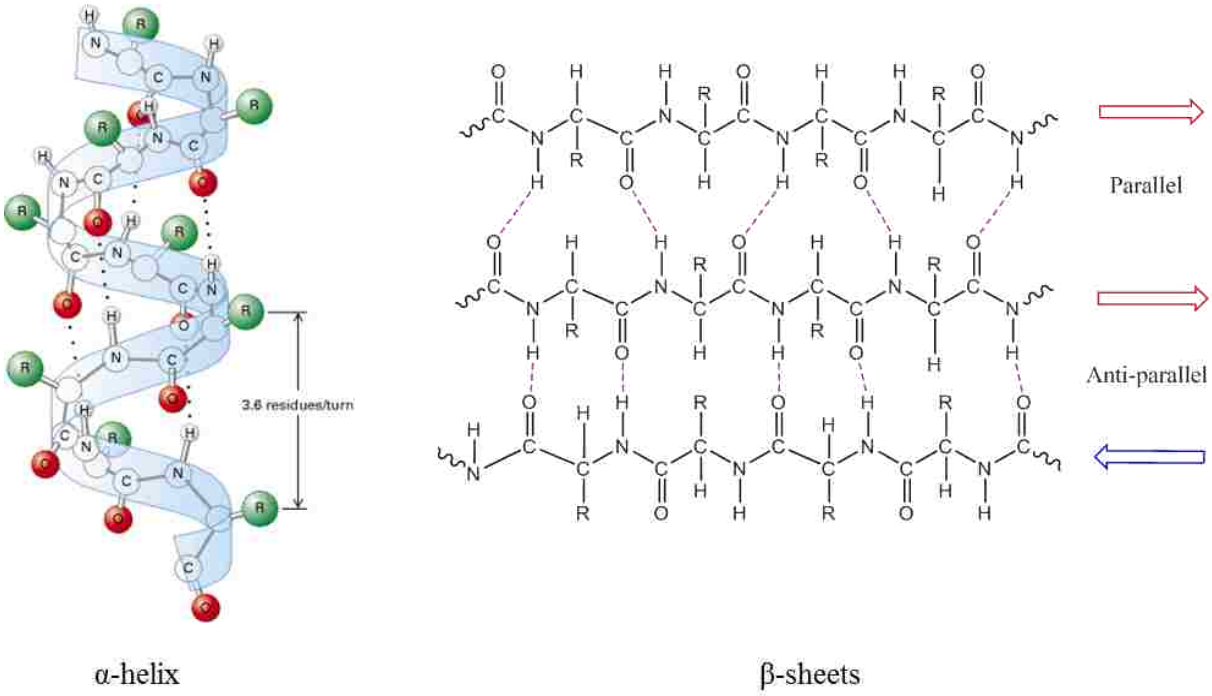


Figure 1.2. Schematic diagram of an alpha helix and a beta sheet. Dotted lines in α-helix and dashed lines in β-sheets represent hydrogen bonds, which stabilize the structures. Source for the alpha helix- (http://www.nslc.wustl.edu/courses/bio2960/labs/02Protein_structure/ps2011.htm)

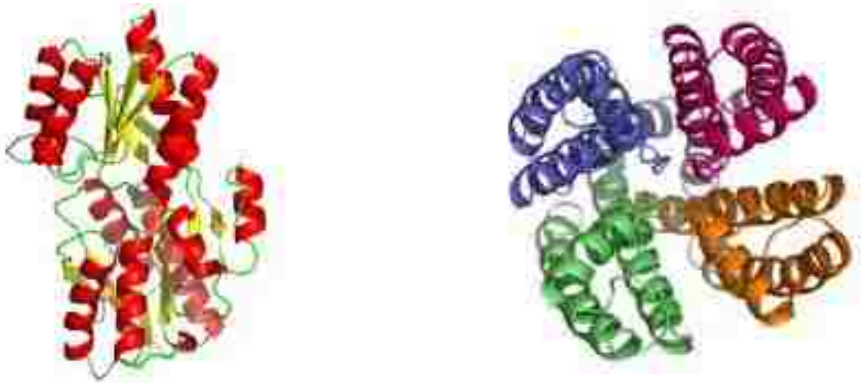
An α-helix is a spiral conformation that is tightly coiled in clockwise direction. The back-bone of this conformation consists of the polypeptide chain contributing 3.6 amino acid residues into one coiled turn. The stability of the conformation maintains by two factors. First and most importantly, oxygen atoms of carbonyl groups hydrogen bond to hydrogen atoms of amino groups, so each peptide makes two hydrogen bonds. The atoms involved in hydrogen bonds linearly arranged through the entire length of the polypeptide chain to stabilize the conformation.

The other factor is the spatial separation of amino acid side chains (R groups) by projecting them outward from the backbone. (See α-helix structure in Figure 1.2)

Beta-sheet conformation of protein secondary structure occurs when polypeptide strands closely lie on top of each other. The conformation stabilizes by lateral hydrogen bonding interactions between carbonyl oxygen and amino hydrogen of polypeptide chains, which are closer together. Beta-sheet conformation can be parallel, i.e. C-terminus and N-terminus of adjacent polypeptide chains are at the same direction, or antiparallel.

1.2.3. Protein Tertiary Structure

The folded protein secondary structure leads to a globular and more compact conformation, which is known as a tertiary structure (Figure 1.3-A). Folding (of globular proteins) is driven by exclusion of water and interactions between R groups, which can stabilize the fold.



(A) (B)

Figure 1.3. Tertiary structure of Arabinose binding protein (A) (Source- http://chemistry.umeche.maine.edu/CHY431/Proteins10.html) and quaternary structure of a potassium ion channel protein from *Streptomyces lividans* (B) (Source- http://chemistry.umeche.maine.edu/MAT500/Proteins12.html)

In addition, tertiary structures stabilize by strong, covalent disulfide bonds between sulfur atoms in cysteine amino acid side chains, charge–charge interactions between oppositely charged species in the amino acid side chains, hydrogen bonds, and Van der Waals interactions.

The quaternary structures are the combination of several polypeptide units together, which are known as multimers, i.e. dimer, trimer, tetramer, etc. (Figure 1.3-B). The conformational stability of the quaternary structure maintains by the same interactions, which occur in the tertiary structure.

1.3. Protein Stability

In general, most proteins are in a two-state model, folded (native) and unfolded (denatured) states.[18] Native folded states can be reversibly transformed into an unfolded states therefore, in cells and tissues these two states are in an equilibrium.[19,20]

$$N \leftrightarrow \mathrm{D} \tag{1.1}$$

Here, N is the concentration of native protein and D is the concentration of denatured protein in the equilibrium. The stability of the native state is marginally higher (-5 to -10 kCal/mol) than the unfolded state.[19] Transitions from lower energy state, i.e. folded state to the higher energy state (unfolded state) occur in the presence of a protein-denaturing agent. In such an environment, hydrogen bonds, disulfide bonds, electrostatic interactions, and Van der Waal interactions, which maintain the tertiary structure, is destroyed.

Protein denaturation achieves by using various conditions: temperature and pH variations, addition of chemical agents, and the application of a mechanical stress. Herein, I will be focused on the first three factors since those are being extensively used. High temperature conditions (~60 ºC- 100 ºC) are often caused denaturation by weakening the spatial interactions and

irreversibly changing the native structure. Exposure of proteins to a low pH (< ~4) environment can also be led to protein unfolding.[21] Urea and guanidine chloride are two most commonly used chaotropic agents. At high concentrations, these substances weaken the hydrogen bond network between solvent molecules. This leads to reduction of hydrophobic effect of proteins. As a result, native state becomes unstable.[22] All these denaturing conditions of proteins are applied in protein separation, a major step in proteomic workflow.

1.4. Protein Separation

Protein separation is performed by various analytical techniques, such as reversed phase chromatography and hydrophobic interaction chromatography based on protein polarity;[23] size exclusion chromatography,[24] gel electrophoresis,[25] and gel filtration technique[26] based on protein size and shape; capillary electrophoresis,[27] IEF,[28] and ion exchange chromatography[29] based on protein charge; etc. Polyacrylamide gel electrophoresis (PAGE) is by far the most commonly used technique for separation of protein mixtures.[30,31,32]

1.4.1. Polyacrylamide Gel Electrophoresis (PAGE)

PAGE is a widely used technique for protein separation and purification based on molecular weight and/or charge. The technique involves sieving of proteins with different charges and masses through a polyacrylamide gel matrix in an applied electric field. PAGE can be used to separate proteins in their native (native PAGE)[33,34,35] or denatured form (denaturing PAGE).[36,37,38] The latter is achieved with the addition of surfactants in sample, gel, and running buffer preparations. PAGE can be classified into two based on nature of the surfactants used; anionic or cationic.

Anionic PAGE uses anionic surfactants with basic buffers, while cationic surfactants at low pH buffers are used in cationic PAGE. Protein separation by use of PAGE with anionic surfactant, SDS, which is known as SDS-PAGE is the most common anionic PAGE technique used to estimate the molecular weight of proteins.[39,30, 40] SDS is used in protein sample, polyacrylamide gel, and running buffer preparations in SDS-PAGE.[30] Typically, for most proteins, monomeric SDS in the sample buffer binds to proteins in a ratio of 1.4 g of SDS/1 g of protein.[41,42] Binding of SDS to any protein is predominantly hydrophobic and causes the tertiary structure to be unfolded.[42] Reducing agents in sample buffer aid with disulfide bond cleavages. At SDS concentrations greater than the critical micelle concentration (CMC), hydrophobic domains of proteins interact with the inner core of SDS micelles causing the proteins to be unfolded. Proteins are further unfolded as a result of hydrophobic and electrostatic interactions between SDS and proteins, yielding linear chains of negatively charged SDS-protein complexes. In an electric field, these charged complexes sieve through gel pores from cathode to anode. The electrophoretic mobility is a unique function of protein molecular weight as most of the SDS-protein complexes under investigation have a constant negative charge.[43] In other words, the plot of logarithm of molecular weight vs. protein migration distance in SDS-PAGE protein separation often yields a linear relationship.

Even though SDS has been extensively used to denature and enhance protein solubility in extractions and separations, several deficiencies are associated with it in protein separation followed by identification. For example, defects in SDS-PAGE are known even though it reveals accurate molecular weight information for most proteins. For some proteins, particularly in membrane proteomics, anomalous protein migrations are observed where protein migrations do not correlate to the molecular weight, often known as ‘gel shifts’.[41,44]

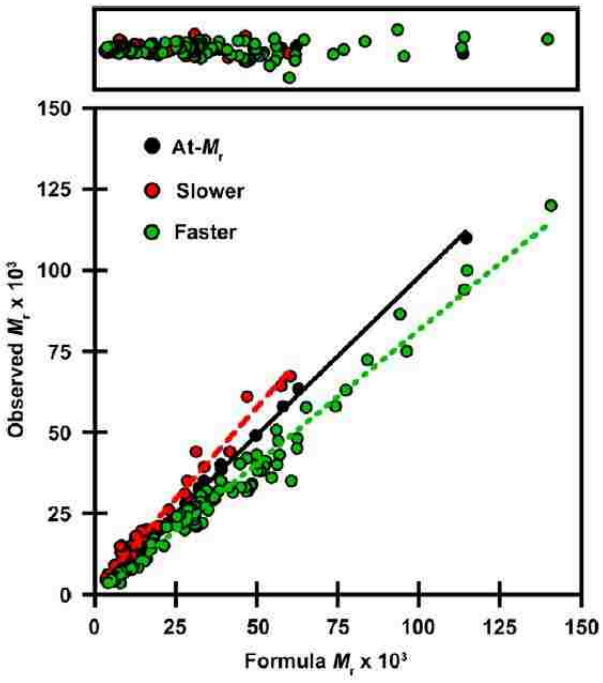


Figure 1.4. A plot of estimated molecular weight from SDS-PAGE (observed M_r) vs. molecular weight derived from the amino acid sequence (formula M_r) for transmembrane proteins running at formula M_r (n=28, black dots), slower than formula M_r (n=48, red dots), and faster than formula M_r (n=92, green dots).[45]

Figure 1.4 shows such an example, a systematic study performed by Rath et al. to investigate the relationship between the estimated protein molecular weight from SDS-PAGE and the molecular weight derived from amino acid sequence of the protein, for a series of trans membrane proteins. These anomalous protein migrations have been occurred due to numerous biological factors: alternative splicing, posttranslational modifications, and endoproteolytic processes occur in membrane proteins.[45] All these factors may change the molecular weight derived from the amino acid sequence hence, migrate at a different speed. In addition, various levels of SDS binding to hydrophobic proteins also could affect the migration speed. Even though the maximum binding ratio of SDS to protein is 1.4 g of SDS/1 g of protein, in another study, Rath et al. reported that the ratio varies from 3.4-10 g of SDS/1 g of membrane proteins.[41] This suggests that membrane proteins denature into variable extents depending on the SDS

loading level to such proteins. Consequently, SDS-PAGE separation of these types of proteins does not solely based on molecular weights but, variable protein shapes, extent of unfolding, and protein charges also affect differential migration patterns of SDS-PAGE.[11,16,15] For this reason, alternative surfactants and techniques are in need, which are more suitable in separating membrane proteins.

Cationic PAGE has been used in membrane protein separations by use of 2D-PAGE [46,44,47,48] and 1D-PAGE.[49,50] In contrast to anionic PAGE, cationic surfactants, such as cetyltrimethylammonium bromide (CTAB),[44,49] 16-benzyldimethyl-n-hexadecylammonium chloride (16-BAC),[46,48] tetradecyltrimethylammonium bromide (TDAB),[51] and cetylpyridinium chloride[52] along with acidic sample and running buffers are employed in cationic PAGE. Interaction of proteins with these surfactants can form positively charged surfactant-protein complexes under acidic pH leading to a migration from anode to cathode.[44] Similar to SDS-PAGE, molecular weight-based separations are obtained using cationic PAGE technique as well.[51,44]

A significant portion of research related to membrane protein separations reported using cationic PAGE. Amory et al. used TDAB-based cationic PAGE to separate a phosphorylated catalytic intermediate of purified ATPase of the *Schizosaccharomycespombe* yeast with a high resolution. It has been revealed that after separation of proteins by CTAB-PAGE the enzymatic activity was retained owing to the low CMC of the surfactant, which reduced the denaturation effect.[50] In a recent study, CTAB-PAGE was able to detect post translational modifications of nuclear proteasomes, which were not visible in SDS-PAGE.[49] These modifications were known to possess negative charge heterogeneity, which are more compatible with cationic CTAB-

PAGE. In recent years, ionic liquid-based surfactants have shown much interest in protein separation chemistry due to unique chemical properties associated with them.[53,54,55]

1.4.2. Ionic Liquids

Ionic liquids (ILs) are organic salts with melting point below 100 ºC. ILs that are liquids at room temperature are known as room temperature ILs. In contrast, those that are solids at room temperature are known as frozen ILs. The presence of a bulky cation and/or anion in ILs makes a loosely packed lattice structure. This leads to a low melting point compared to conventional salts, which have high melting points. Figure 1.5 shows some of the common cations and anions, which are used in ILs synthesis. The cation of ILs is generally bases on different types of organic compounds, such as dialkylimidazolium-, phosphonium-, *N*-alkylpyridinium-, alkylammonium-, etc. The anion may be organic or inorganic, for some examples, hexafluorophosphate, tetrafluoroborate, bis(perfluoromethanesulfonyl)imide, bis(trifluoromethanesulfonyl)imide, chloride, and bromide.

ILs have achieved considerable attention due to various physicochemical properties, e.g., high conductivity,[56,57] low volatility,[58,59] high thermal stability,[60,61] non-flammability,[62,63] etc. An attractive property of ILs is the ability to tune those properties for a desired task by changing counter ions. In most cases, this performs simply by an ion exchange reaction. For this reason, numerous types of ILs are being exploited in different applications, for instance, light emitting diodes (LED),[64] stable solvents for organic synthesis,[65] sensors,[66] and surfactants in separations.[67]

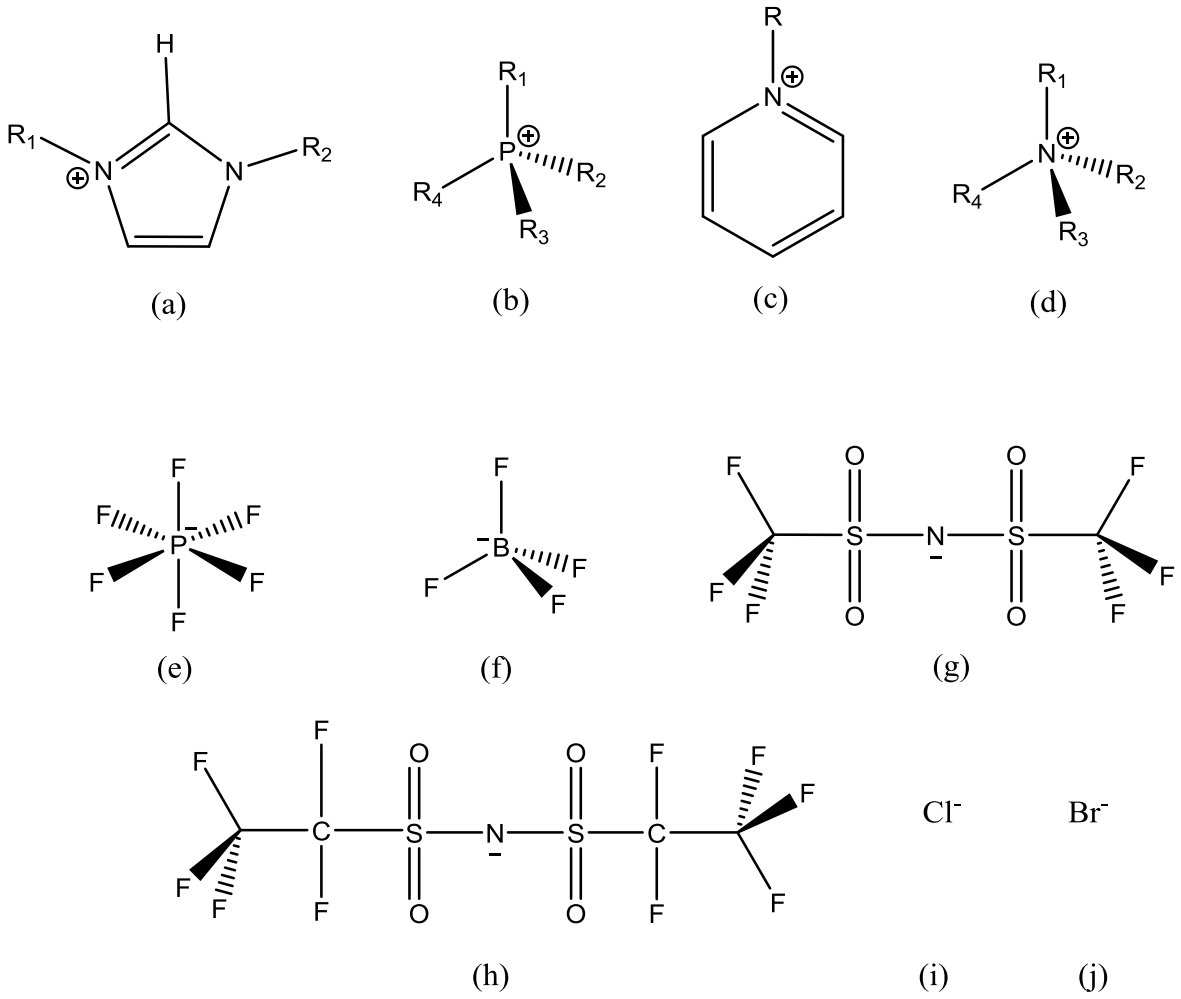


Figure 1.5. Common cations; dialkylimidazolium (a), phosphonium (b), *N*-alkylpyridinium (c), and alkylammonium (d) and anions; hexafluorophosphate (e), tetrafluoroborate (f), bis(trifluoromethanesulfonyl)imide (g), bis(pentafluoroethanesulfonyl)imide (h), chloride (i), and bromide (j)

1.4.3. Ionic Liquid-based Surfactants in Protein Separation

ILs have been used as surfactants for protein separation using various analytical techniques. Zhang et al. reported the application of 3-methylimidazolium-based ILs for IL-assisted SDS-PAGE separation of human serum proteins.[68] In this study, ILs were applied in polyacrylamide gel matrix, while SDS was employed in protein sample and running buffers to perform protein separation under standard SDS-PAGE conditions. Serum proteins with low and moderate

molecular weights were separated with a high resolution using this technique. In another study, isopropylammonium formate ILs as buffer modifiers, have been used in mobile phase of reverse phased-high performance liquid chromatography (HPLC).[69] This technique has successfully been used to separate subunits of a variety of proteins including glutamate dehydrogenase and lactate dehydrogenase. Furthermore, 1-alkyl-3-methylimidazolium-based ILs have been used as coating agents in fused silica capillaries and buffer additives in running buffer of capillary electrophoresis (CE) for rapid and efficient separation of basic proteins.[70] Inclusion of ILs in capillary coating has resulted to yield an improved sharp protein peak on electropherograms of CE.

1.5. Protein Identification

Protein identification by mass spectrometry is a critical step in proteomic workflow. Two soft ionization methods in mass spectrometry, electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI), are widely used in protein and peptide analysis. The latter technique has more advantages in direct analysis of proteins.[71]

1.5.1. Protein Identification by MALDI-MS

MALDI-MS is a highly sensitive technique, which used to identify biomolecules with large molecular weights.[72] Tremendous efforts and strategies have been made by use of MALDI-MS to identify protein biomarkers in cells and tissue extracts,[73] urine,[74] blood serum and plasma,[75] cerebrospinal fluid,[76] fingerprints,[77] etc. In addition, it has been used to analyze proteins in microorganisms[78] and food.[79] Furthermore, MALDI imaging mass spectrometry (IMS) technique has been used for spatial analysis of protein distribution with unbiased visualization on tissue samples.[80]

The performance of MALDI-MS protein detection is affected by low abundance and hydrophobicity of proteins.[71] The latter affects the protein solubility, which is a key factor for the efficient co-crystallization of proteins and matrix in sample. Researchers have employed surfactants as matrix additives to solubilize hydrophobic proteins in samples to eliminate the bias of mass detection in favor of hydrophilic proteins over hydrophobic proteins.[81,82]

The presence of surfactants, in particular, SDS, in MALDI-MS sample preparation has shown counter effects, such as reduction of signal-to-noise ratio[83] and mass resolution[84] in protein identification. Signal suppression due to this anionic surfactant has been attributed to the partial precipitation of protein-surfactant ion pairs below the CMC and formation of surfactant coatings on protein-matrix crystals above the CMC.[84] As a result, it is not possible to recover the total protein signal at any concentration of SDS. Consequently, mass spectrometry compatible surfactants as matrix additives have been exploited in MALDI-MS that have capacity to solubilize hydrophobic proteins and improve mass spectral quality.[85,86,87] For an instance, anionic surfactant, sodium 3-((1-(furan-2-yl)undecyloxy) carbonylamino) propane-1-sulfonate has been used to increase protein solubility of an in-gel protein digestion sample prior to mass spectral identification of peptides.[85] The quality of mass spectra was not negatively affected as a result of removal of the surfactant including other contaminants by centrifugation and solid phase extraction. In another study, sodium laurate has been used as an efficient protein extracting agent, similar to SDS, in membrane proteomics.[87] However, in the presence of the surfactant, mass spectra were significantly suppressed, which recovered to some extent after removing the surfactant by phase transfer method. None of these surfactants showed an improvement in the presence of the surfactant or applications in protein separation, in addition to identification.

1.6. Instrumentation Techniques

1.6.1. Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gels are prepared by polymerization of acrylamide monomer along with N,N'-methylenebisacrylamide cross linker in the presence of a suitable catalyst for anionic or cationic PAGE (Figure 1.6).

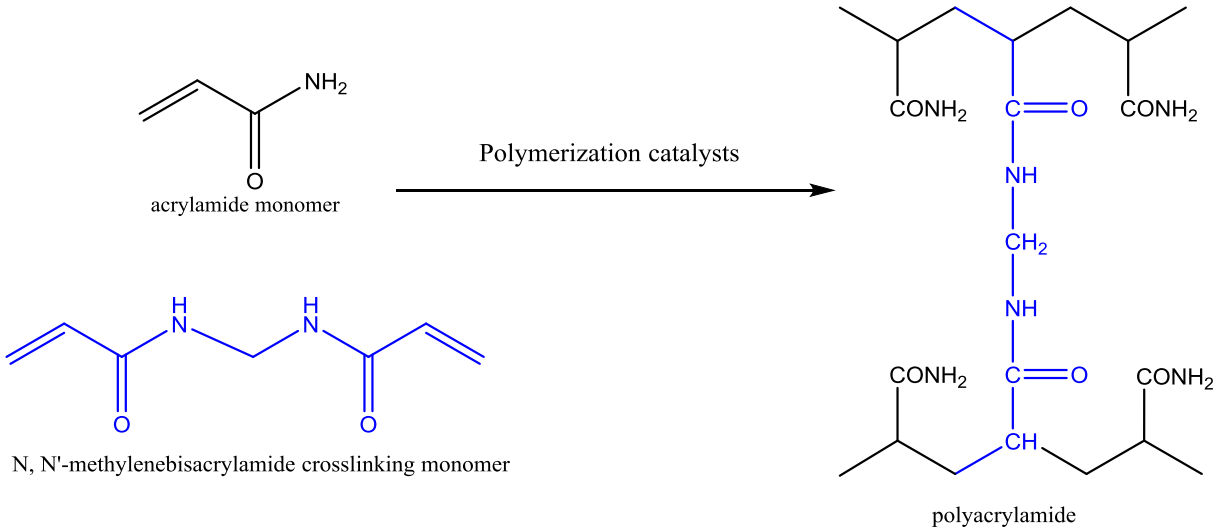


Figure 1.6. Reaction for polyacrylamide gel polymerization

The pore sizes of the gel matrix are affected by acrylamide composition, total percentage of both monomers (T), and percentage of bisacrylamide cross linker with respect to the total monomer concentration (C). Primarily, higher proportions of cross linker leads to small pores, therefore, suitable to separate proteins with relatively low molecular weight and vice versa.[88] Thus, careful selection of %T and %C are critical for an optimal protein separation.

Two PAGE techniques can be identified based on distribution of pH, pore sizes, and voltage in gel medium; continuous and discontinuous. In continuous PAGE, a homogenous buffer system with uniform pH and a single layer of gel media are used.[89] In contrast, multiple gel

layers, i.e., stacking and resolving, which have different pore sizes and gradient pH buffer systems are used in discontinuous PAGE. In this system, protein samples are concentrated into a sharp zone inside large pores of the stacking gel layer, reducing sample diffusion prior to the separation. As a result, highly resolved protein bands are often yielded.

Bio-Rad Mini PROTEAN Tetra Cell, a vertical gel electrophoresis system, was used in all the PAGE protein separations that are discussed in this dissertation. In anionic PAGE experiments, Bio-Rad precast gels were used at 200 V for 40 minutes. In cationic PAGE experiments, polyacrylamide gels containing ILS were prepared in the lab using Bio-Rad glass plates, i.e., one short plate and one spacer plate. Cationic PAGE experiments were performed at 200 V for 10 minutes followed by 300 V for 50 minutes.

1.6.2. Isoelectric Focusing (IEF)

Isoelectric focusing (IEF) is an electrophoretic technique, which is used for protein separations based on the isoelectric point (pI) of proteins. For this purpose, an immobilized pH gradient (IPG) in a polyacrylamide gel matrix uses as a gel strip with a range of pH (3-12).[90] Iso-electric focusing was performed using an Ettan IPGphor (GE healthcare) unit. Protein separation is performed by first, immersing the IPG strip in an aqueous protein sample for rehydration. As explained in section 1.2.1., proteins can have either a net negative or positive charge depending on the pH of the medium. In an applied electric field, proteins will migrate towards the electrode, which is oppositely charged to proteins (Figure 1.7-A).

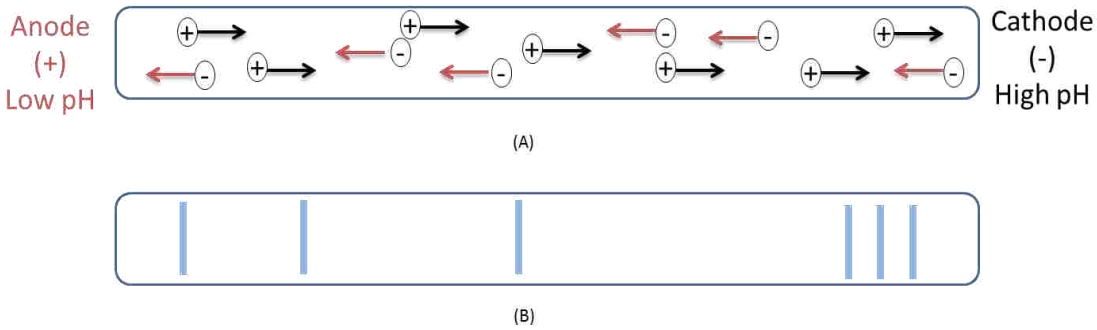


Figure 1.7. Schematic diagram, which illustrates the protein separation mechanism in IEF; proteins with net charges are migrated in an electric field towards anode or cathode (A). After the separation, proteins appear as sharp bands along the gel strip (B)

During this migration through pH gradient, proteins can be protonated or deprotonated. Eventually, proteins reach to a point where the net charge is zero, in other words, pI of the protein. This will stop the migration and condense proteins to a particular pH region, which is same as the pI of each protein in the mixture (Figure 1.7-B).

1.6.3. Fluorescence Spectroscopy

Fluorescence spectroscopy is an analytical technique with high sensitivity and can be used to analyze molecular environments at micro molar concentrations. In this technique, a fluorophore is irradiated at a specific wavelength to excite it from ground energy state to higher vibrational levels of excited states (S1 and S2). Energetic relaxation of fluorophore from the first excited state to the ground state through emission of radiation is known as fluorescence. This energy transition occurs in the same spin state, i.e., from S1 to S0. After the energy absorption, the fluorophore may undergo several other radiative and non-radiative processes. A Jablonski diagram, which illustrates the processes, is shown in Figure 1.8.

One of the non-radiative processes is relaxation of a molecule from S_2 to the lowest vibrational energy level of S_1, known as internal conversion, is a rapid process occurs in 10^{-12} s.

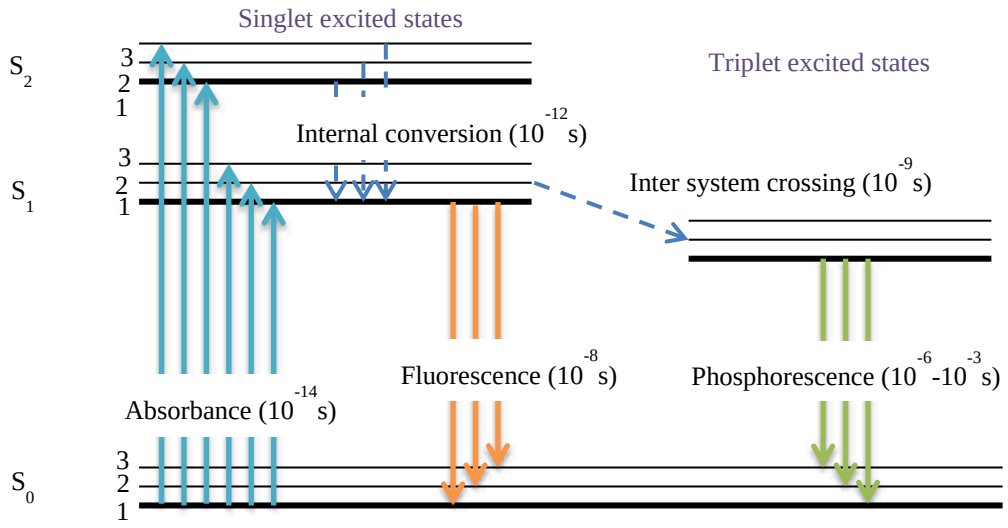


Figure 1.8. Jablonski diagram, which shows possible radiative (solid lines) and non-radiative (dashed lines) transitions

Fluorescence emission occurs within 10^{-8} s after the completion of internal conversion. For this reason, fluorescence emission happens from a thermally equilibrated, lowest vibrational level of S_1 state. Another non-radiative transition is intersystem crossing, where a spin conversion occurs from singlet state (S_2) to the first triplet state (T_1). The emission from T_1 to S_0 is a radiative transition, known as phosphorescence and always occurs at longer wavelengths as compared to the fluorescence.

The analytical instrument used to measure fluorescence was a SPEX Fluorolog-3 spectrofluorimeter (model FL3-22TAU3). Figure 1.9 shows the basic units of the instrument; light source, excitation and emission monochromators, sample holder, and detector (photomultiplier tube). A xenon arc lamp (450 W) was used as the light source in the fluorescence studies discussed in this dissertation. Excitation and emission monochromators are gratings, which are used to filter a specific wavelength. As the beam of light is passed through excitation monochromator, it is filtered through an adjustable slit before entering into the sample chamber. The emission monochromator is placed at a 90º angle to the light source in order to

collect the emission light and minimize scattered light. The emission light is then passed through emission monochromators, where a fixed wavelength range is selected by the user. It is then filtered through an adjustable slit and forward to the photomultiplier tube. Finally, fluorescence spectrum is generated after amplifying the signal by the detector.

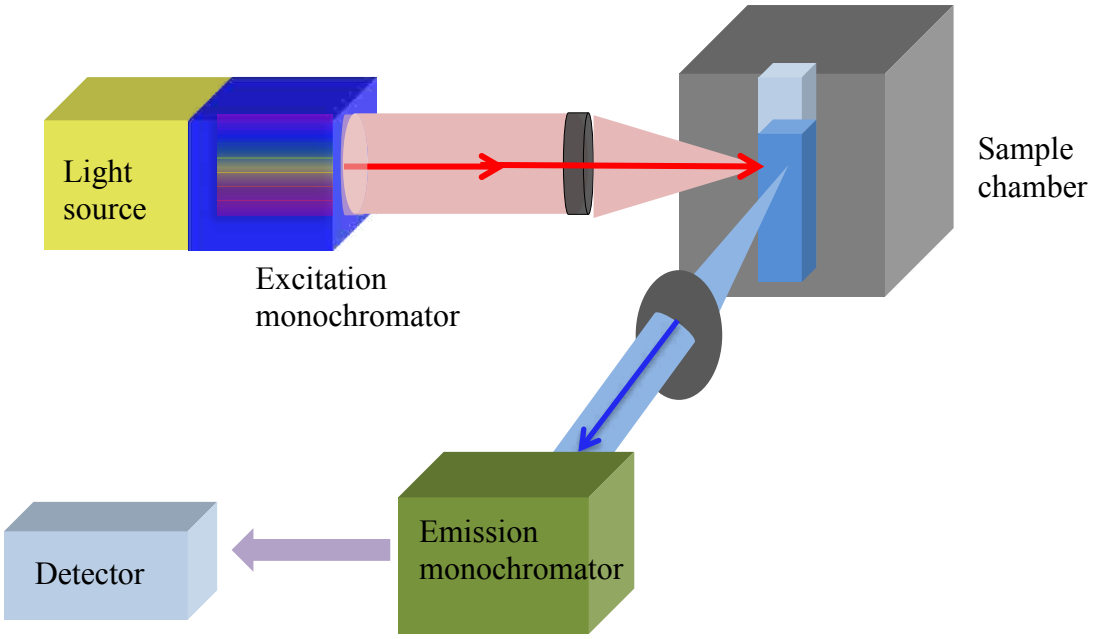


Figure 1.9. Major components of fluorescence spectrophotometer

The molecular environments of proteins can be studied using intrinsic protein fluorescence; three fluorescent amino acids, phenylalanine, tyrosine, and tryptophan located in proteins are acted as fluorophores.[91,92,93,94] Tryptophan is the major fluorophore, which is widely used as a reporter molecule.[95] Excitation wavelength of Tryptophan lies between 295-305 nm and emission occurs near 350 nm. It is recommended to excite tryptophan at 295 nm to avoid interference from tyrosine spectral emission.[96] Spectral shifts in tryptophan emission are due to changes in local environments of the protein. Hence, it reveals useful information on protein structural changes, denaturation, and protein- ligand interactions. Tryptophan emission is highly affected by different types of quenchers (amino and carboxyl groups, peptide bonds, disulfides,

and amides) and polarity of surroundings as well.[97] When the protein is in a completely non-polar environment, it shows a characteristic blue-shifted emission (hypsochromic effect). As tryptophan residue is exposed to a polar solvent or formed hydrogen bonds with water, the emission is red-shifted (bathochromic effect). These variable emission characteristics are useful to understand different stages of protein unfolding.

1.6.4. Circular Dichroism (CD)

CD is a valuable analytical technique used to examine the protein structure in a solution. The principle of this technique is based on absorption of two components, clockwise (R) and anti-clockwise (L) rotations of circularly polarized light by a chiral chromophore (Figure 1.10). If R and L components are absorbed in equal extents or not absorbed at all, the total absorbance will generate a polarized radiation on the same plane as the incident light. If two components are different, the radiation will be elliptically polarized. The circular polarized absorbance is measured by use of a spectropolarimeter and reported the ellipticity in degree. Hence, the CD spectrum is a plot of ellipticity (θ) versus wavelength (λ).

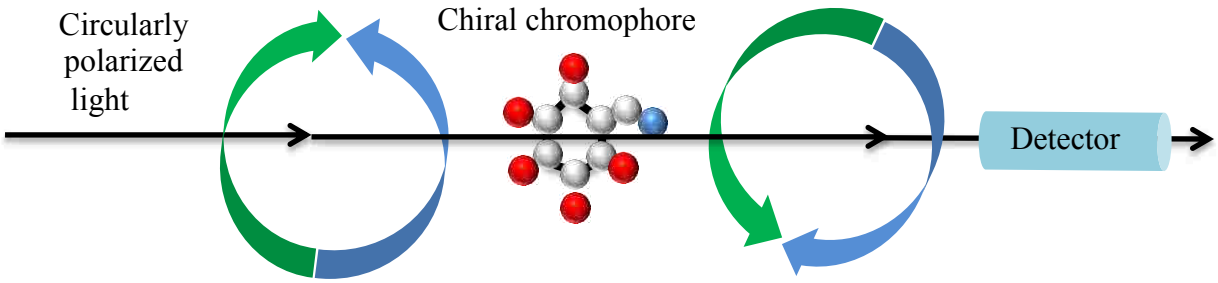


Figure 1.10. The principle of Circular dichroism

CD exhibits characteristic spectra at different finger print regions for protein secondary and tertiary structures. Protein secondary structures; α-helix, anti-parallel β sheets, and β turns show different spectra at far-UV region (170-250 nm). These absorption characteristics of the

secondary structure are due to the peptide bonds with transitions from $n \rightarrow \pi^*$ (around 220 nm) and $\pi \rightarrow \pi^*$ (around 190 nm). Tertiary structures of proteins can be identified due to absorption by aromatic amino acids, phenyl alanine, tryptophan, and tyrosine leading to the absorbance in the near-UV region (250-320 nm). These amino acids absorb at characteristic wavelengths; phenyl alanine in between 255-270 nm, tryptophan around 290 nm, and tyrosine in between 275-282 nm.

Moreover, CD spectra can reveal structural changes due to ligand binding as well. The concentration, in which such changes may occur also can be detected using a range of concentrations of ligands. In addition, protein denaturation in the presence of a denaturing agent or with increased temperature can be monitored due to loss of CD signal. All of these characteristics are widely applied in protein biochemistry, protein synthesis, and analysis. An AVIV spectrophotometer (model 62DS) was used to collect CD data in this dissertation.

1.6.5. Matrix Assisted Laser Desorption Ionization (MALDI)-Mass Spectrometry (MS)

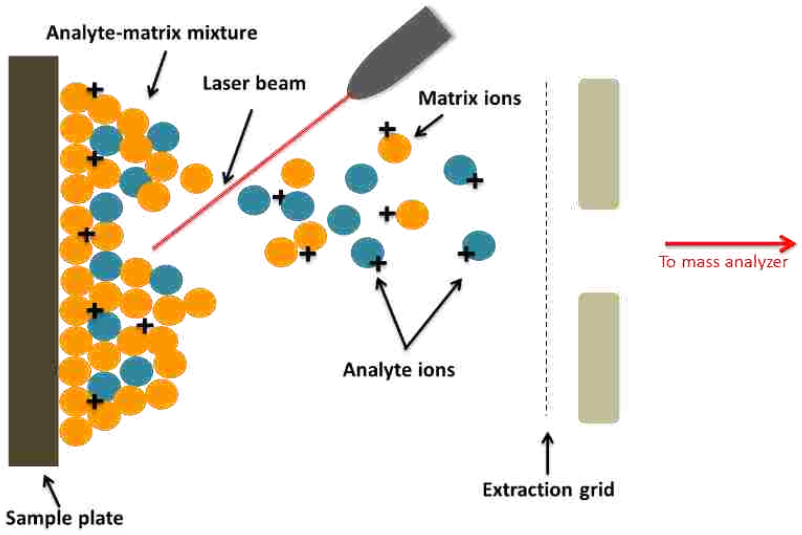


Figure 1.11. Schematic diagram, which shows the ionization process in MALDI-MS

MALDI-MS is a soft ionization mass spectrometry technique, which uses to analyze masses of biopolymers including peptides and proteins. In this technique, suitable chromophores, such as α-cyanohydroxycinnamic acid (CHCA), sinapinic acid, 2,5-dihydroxybenzoic acid (DHB), and succinic acid are used as matrix components during sample preparation. For protein characterization studies discussed in this dissertation, a MALDI time-of-flight (TOF) mass spectrometer (Bruker, ultrafleXtreme) with Nd:YAG laser (355 nm, 3 ns pulse width) was used. During the analysis, a protein sample irradiates with the laser beam. Then, the matrix mixture desorbs into the gas phase. As a result, proton transfer can occur from matrix to analyte or vice versa, which leads to protonation or deprotonation of analytes and matrix molecules (Figure 1.11). These charged ionic species then migrate through an electric field at different speeds depending on mass to charge ratio (m/z) and finally, leading to detection by a mass detector.

1.7. Scope of Dissertation

In this dissertation, the application of novel, cationic ionic liquid surfactants, *N*-alkyl-4-methyl pyridinium bromide (C_nPBr where n=4, 8, 11), in protein separations by PAGE and identification by MALDI-MS will be discussed. Chapter 2 will be focused on synthesis and the application of *N*-alkyl-4-methylpyridinium bromide-based ILS in separation of acidic proteins including ovalbumin and transferrin isoforms by anionic PAGE. In this study, intrinsic fluorescence spectroscopy and circular dichroism studies will be used to explain protein-ILS interactions and denaturing effects of ILS. In chapter 3, the application of the same ILS in cationic PAGE for separation of both acidic and basic proteins will be discussed. Furthermore, the application of the technique to resolve ribonuclease b glycoforms will be reported in detail. As another application of the cationic PAGE technique, 2D-PAGE (IEF/ILS-PAGE) separation of complex proteins will be demonstrated in chapter 4. Separation of alpha antitrypsin

glycoprotein and cytochrome c protein subunits using this technique will be discussed and compared with conventional 2D-PAGE methods in the same chapter. Chapter 5 highlights the application of the same ILS as matrix additives in MALDI-MS for detection of proteins. The signal-to-noise-ratio improvement in the presence of ILS will be discussed compared to 0% surfactants and conventional surfactants. In addition, application of the ILS in MALDI-MS profiling mode experiments to improve protein analysis of a rat brain tissue sample will be investigated.

1.8. References

1. Petsko, G. A.; Ringe, D., *Protein structure and function*. New Science Press: 2004.

2. Suresh, S.; Spatz, J.; Mills, J.; Micoulet, A.; Dao, M.; Lim, C.; Beil, M.; Seufferlein, T., Reprint of: connections between single-cell biomechanics and human disease states: gastrointestinal cancer and malaria. *Acta biomaterialia* **2015,** *23*, S3-S15.

3. Harada, R.; Tochio, N.; Kigawa, T.; Sugita, Y.; Feig, M., Reduced native state stability in crowded cellular environment due to protein-protein interactions. *Journal of the American Chemical Society* **2013,** *135* (9), 3696.

4. Kim, M.-S.; Pinto, S. M.; Getnet, D.; Nirujogi, R. S.; Manda, S. S.; Chaerkady, R.; Madugundu, A. K.; Kelkar, D. S.; Isserlin, R.; Jain, S., A draft map of the human proteome. *Nature* **2014,** *509* (7502), 575-581.

5. Budak, H.; Akpinar, B. A.; Unver, T.; Turktas, M., Proteome changes in wild and modern wheat leaves upon drought stress by two-dimensional electrophoresis and nanoLC-ESI–MS/MS. *Plant Molecular Biology* **2013,** *83* (1-2), 89-103.

6. Emery, S. J.; Pascovi, D.; Lacey, E.; Haynes, P. A., The generation gap: Proteome changes and strain variation during encystation in Giardia duodenalis. *Molecular and biochemical parasitology* **2015,** *201* (1), 47-56.

7. Romero, R.; Erez, O.; Maymon, E.; Chaemsaithong, P.; Xu, Z.; Pacora, P.; Chaiworapongsa, T.; Done, B.; Hassan, S. S.; Tarca, A. L., The maternal plasma proteome changes as a function of gestational age in normal pregnancy: a longitudinal study. *American Journal of Obstetrics and Gynecology* **2017**.

8. Wiśniewski, J. R.; Duś, K.; Mann, M., Proteomic workflow for analysis of archival formalin-fixed and paraffin-embedded clinical samples to a depth of 10 000 proteins. *PROTEOMICS-Clinical Applications* **2013,** *7* (3-4), 225-233.

9. Wilson, A. M.; Bailey, P. J.; Tasker, P. A.; Turkington, J. R.; Grant, R. A.; Love, J. B., Solvent extraction: the coordination chemistry behind extractive metallurgy. *Chemical Society Reviews* **2014,** *43* (1), 123-134.

10. Crowell, A. M.; Wall, M. J.; Doucette, A. A., Maximizing recovery of water-soluble proteins through acetone precipitation. *Analytica chimica acta* **2013,** *796*, 48-54.

11. Kawashima, A.; Kigoshi, T.; Katoh, Y.; Ishikawa, Y.; Shawki, H. H.; Inoue, N.; Tamba, M.; Matsuda, M.; Okamura, N., CABCOCO1, a novel coiled-coil protein With calcium-binding activity, is localized in the sperm flagellum. *Molecular reproduction and development* **2016,** *83* (10), 912-926.

12. Anderson, J. C.; Peck, S. C., Detection of protein phosphorylation and charge isoforms using vertical one-dimensional isoelectric focusing gels. *Plant MAP Kinases: Methods and Protocols* **2014**, 39-46.

13. Prasad, B.; Evers, R.; Gupta, A.; Hop, C. E.; Salphati, L.; Shukla, S.; Ambudkar, S. V.; Unadkat, J. D., Interindividual variability in hepatic organic anion-transporting polypeptides and P-glycoprotein (ABCB1) protein expression: quantification by liquid chromatography tandem mass spectroscopy and influence of genotype, age, and sex. *Drug Metabolism and Disposition* **2014,** *42* (1), 78-88.

14. Sakamoto, A.; Matsumaru, T.; Yamamura, N.; Suzuki, S.; Uchida, Y.; Tachikawa, M.; Terasaki, T., Drug Transporter Protein Quantification of Immortalized Human Lung Cell Lines Derived from Tracheobronchial Epithelial Cells (Calu-3 and BEAS2-B), Bronchiolar–Alveolar Cells (NCI-H292 and NCI-H441), and Alveolar Type II-like Cells (A549) by Liquid Chromatography–Tandem Mass Spectrometry. *Journal of pharmaceutical sciences* **2015,** *104* (9), 3029-3038.

15. Chaijarasphong, T.; Nichols, R. J.; Kortright, K. E.; Nixon, C. F.; Teng, P. K.; Oltrogge, L. M.; Savage, D. F., Programmed ribosomal frameshifting mediates expression of the α-carboxysome. *Journal of molecular biology* **2016,** *428* (1), 153-164.

16. Gazzola, D.; Pasini, G.; Tolin, S.; Curioni, A.; Vincenzi, S., CHARACTERIZATION OF CHITINASE ISOFORMS FROM GRAPE JUICE. *Italian Journal of Food Science* **2017,** *29* (1).

17. Anfinsen, C. B., Studies on the principles that govern the folding of protein chains. 1972.

18. Arnold, U.; Ulbrich-Hofmann, R., Kinetic and thermodynamic thermal stabilities of ribonuclease A and ribonuclease B. *Biochemistry* **1997,** *36* (8), 2166-2172.

19. Creighton, T. E., Protein folding. *Biochemical journal* **1990,** *270* (1), 1.

20. Dill, K. A.; Shortle, D., Denatured states of proteins. *Annual review of biochemistry* **1991,** *60* (1), 795-825.

21. Rabbani, G.; Ahmad, E.; Zaidi, N.; Fatima, S.; Khan, R. H., pH-Induced molten globule state of Rhizopus niveus lipase is more resistant against thermal and chemical denaturation than its native state. *Cell biochemistry and biophysics* **2012,** *62* (3), 487-499.

22. Salvi, G.; De Los Rios, P.; Vendruscolo, M., Effective interactions between chaotropic agents and proteins. *Proteins: Structure, Function, and Bioinformatics* **2005,** *61* (3), 492-499.

23. Xiu, L.; Valeja, S. G.; Alpert, A. J.; Jin, S.; Ge, Y., Effective protein separation by coupling hydrophobic interaction and reverse phase chromatography for top-down proteomics. *Analytical chemistry* **2014,** *86* (15), 7899-7906.

24. Fekete, S.; Beck, A.; Veuthey, J.-L.; Guillarme, D., Theory and practice of size exclusion chromatography for the analysis of protein aggregates. *Journal of pharmaceutical and biomedical analysis* **2014,** *101*, 161-173.

25. Dunn, M. J., *Gel electrophoresis of proteins*. Elsevier: 2014.

26. Nuñez, J. K.; Kranzusch, P. J.; Noeske, J.; Wright, A. V.; Davies, C. W.; Doudna, J. A., Cas1–Cas2 complex formation mediates spacer acquisition during CRISPR–Cas adaptive immunity. *Nature structural & molecular biology* **2014,** *21* (6), 528-534.

27. Han, X.; Wang, Y.; Aslanian, A.; Fonslow, B.; Graczyk, B.; Davis, T. N.; Yates III, J. R., In-line separation by capillary electrophoresis prior to analysis by top-down mass spectrometry enables sensitive characterization of protein complexes. *Journal of proteome research* **2014,** *13* (12), 6078-6086.

28. Eulitz, M. In *Purification of Myeloma Proteins by Iso-electric Focusing*, Protides of the Biological Fluids: Proceedings of the Seventeenth Colloquium, Bruges, 1969, Elsevier: 2014; p 481.

29. Fekete, S.; Beck, A.; Fekete, J.; Guillarme, D., Method development for the separation of monoclonal antibody charge variants in cation exchange chromatography, Part I: Salt gradient approach. *Journal of pharmaceutical and biomedical analysis* **2015,** *102*, 33-44.

30. Brunelle, J. L.; Green, R., One-dimensional SDS-polyacrylamide gel electrophoresis (1D SDS-PAGE). *Methods in enzymology* **2014,** *541*, 151.

31. Nigg, P. E.; Pavlovic, J., Characterization of Multi-subunit Protein Complexes of Human MxA Using Non-denaturing Polyacrylamide Gel-electrophoresis. *JoVE (Journal of Visualized Experiments)* **2016,** (116), e54683-e54683.

32. Kabir, M. R.; Afrose, R.; Shahidullah, A.; Hossain, M. A.; Paul, S. K.; Rahman, M.; Kobayashi, N., Usage of Silver-Stained Polyacrylamide Gels Electrophoresis (PAGE) for Detection of Rotavirus Infection by Direct Identification of Viral Nucleic Acid. *Bangladesh Journal of Medical Science* **2016,** *15* (2), 154.

33. Weber, M.; Weber, F., Monitoring activation of the antiviral pattern recognition receptors RIG-I and PKR by limited protease digestion and native PAGE. *Journal of visualized experiments: JoVE* **2014,** (89).

34. Umetsu, K.; Yuasa, I.; Hashiyada, M.; Adachi, N.; Watanabe, G.; Haneda, T.; Yamazaki, K., The art of traditional native PAGE: The APLP 48-ID assay for human identification. *Legal Medicine* **2016,** *19*, 28-31.

35. Moyano, M. F.; Mariño-Repizo, L.; Tamashiro, H.; Villegas, L.; Acosta, M.; Gil, R. A., ICPMS analysis of proteins separated by Native-PAGE: Evaluation of metaloprotein profiles in human synovial fluid with acute and chronic arthritis. *Journal of Trace Elements in Medicine and Biology* **2016,** *36*, 44-51.

36. Sharma, A. K.; Plant, J. J.; Rangel, A. E.; Meek, K. N.; Anamisis, A. J.; Hollien, J.; Heemstra, J. M., Fluorescent RNA labeling using self-alkylating ribozymes. *ACS chemical biology* **2014,** *9* (8), 1680-1684.

37. Conway, J. W.; McLaughlin, C. K.; Castor, K. J.; Sleiman, H., DNA nanostructure serum stability: greater than the sum of its parts. *Chemical Communications* **2013,** *49* (12), 1172-1174.

38. Selvadurai, K.; Wang, P.; Seimetz, J.; Huang, R. H., Archaeal Elp3 catalyzes tRNA wobble uridine modification at C5 via a radical mechanism. *Nature chemical biology* **2014,** *10* (10), 810-812.

39. Swain, S.; Panigrahi, K.; Baisakh, B., Estimation of Seed Storage Protein and Molecular Weight Determination in Micro Mutant Lines of Green Gram (Vigna radiate (L.) Wilczek) by SDS-PAGE. *Trends in Biosciences* **2014,** *7* (19), 2912-2917.

40. Kinoshita, E.; Kinoshita-Kikuta, E.; Karata, K.; Kawano, T.; Nishiyama, A.; Yamato, M.; Koike, T., Specific glutamic acid residues in targeted proteins induce exaggerated retardations in Phos-tag SDS-PAGE migration. *Electrophoresis* **2017**.

41. Rath, A.; Glibowicka, M.; Nadeau, V. G.; Chen, G.; Deber, C. M., Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *Proceedings of the National Academy of Sciences* **2009,** *106* (6), 1760-1765.

42. Bhuyan, A. K., On the mechanism of SDS-induced protein denaturation. *Biopolymers* **2010,** *93* (2), 186-199.

43. Reynolds, J. A.; Tanford, C., The gross conformation of protein-sodium dodecyl sulfate complexes. *Journal of Biological Chemistry* **1970,** *245* (19), 5161-5165.

44. Buxbaum, E., Cationic electrophoresis and electrotransfer of membrane glycoproteins. *Analytical biochemistry* **2003,** *314* (1), 70-76.

45. Rath, A.; Deber, C. M., Correction factors for membrane protein molecular weight readouts on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. *Analytical biochemistry* **2013,** *434* (1), 67-72.

46. Hartinger, J.; Stenius, K.; Högemann, D.; Jahn, R., 16-BAC/SDS–PAGE: a two-dimensional gel electrophoresis system suitable for the separation of integral membrane proteins. *Analytical biochemistry* **1996,** *240* (1), 126-133.

47. Godl, K.; Wissing, J.; Kurtenbach, A.; Habenberger, P.; Blencke, S.; Gutbrod, H.; Salassidis, K.; Stein-Gerlach, M.; Missio, A.; Cotten, M., An efficient proteomics method to identify the cellular targets of protein kinase inhibitors. *Proceedings of the National Academy of Sciences* **2003,** *100* (26), 15434-15439.

48. Nothwang, H. G.; Schindler, J., Two-dimensional separation of membrane proteins by 16-BAC-SDS-PAGE. *Membrane Proteomics: Methods and Protocols* **2009**, 269-277.

49. Pitcher, D. S.; de Mattos-Shipley, K.; Wang, Z.; Tzortzis, K.; Goudevenou, K.; Flynn, H.; Bohn, G.; Rahemtulla, A.; Roberts, I.; Snijders, A. P., Nuclear proteasomes carry a constitutive posttranslational modification which derails SDS-PAGE (but not CTAB-PAGE). *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics* **2014,** *1844* (12), 2222-2228.

50. Akins, R. E.; Levin, P. M.; Tuan, R. S., Cetyltrimethylammonium bromide discontinuous gel electrophoresis: Mr-based separation of proteins with retention of enzymatic activity. *Analytical biochemistry* **1992,** *202* (1), 172-178.

51. Amory, A.; Foury, F.; Goffeau, A., The purified plasma membrane ATPase of the yeast Schizosaccharomyces pombe forms a phosphorylated intermediate. *Journal of Biological Chemistry* **1980,** *255* (19), 9353-9357.

52. Mócz, G.; Bálint, M., Use of cationic detergents for polyacrylamide gel electrophoresis in multiphasic buffer systems. *Analytical biochemistry* **1984,** *143* (2), 283-292.

53. Yang, X.; Zhang, S.; Yu, W.; Liu, Z.; Lei, L.; Li, N.; Zhang, H.; Yu, Y., Ionic liquid-anionic surfactant based aqueous two-phase extraction for determination of antibiotics in honey by high-performance liquid chromatography. *Talanta* **2014,** *124*, 1-6.

54. Rajabi, M.; Ghanbari, H.; Barfi, B.; Asghari, A.; Haji-Esfandiari, S., Ionic liquid-based ultrasound-assisted surfactant-emulsified microextraction for simultaneous determination of three important flavoring compounds in plant extracts and urine samples. *Food Research International* **2014,** *62*, 761-770.

55. El-Hady, D. A.; Albishri, H. M., Alkyl imidazolium ionic liquid based sweeping-micellar electrokinetic chromatography for simultaneous determination of seven tea catechins in human plasma. *Journal of Chromatography B* **2014,** *969*, 224-229.

56. Monti, D.; Jónsson, E.; Palacín, M. R.; Johansson, P., Ionic liquid based electrolytes for sodium-ion batteries: Na+ solvation and ionic conductivity. *Journal of Power Sources* **2014,** *245*, 630-636.

57. Ye, Y.; Sharick, S.; Davis, E. M.; Winey, K. I.; Elabd, Y. A., High hydroxide conductivity in polymerized ionic liquid block copolymers. *ACS Macro Letters* **2013,** *2* (7), 575-580.

58. Zhou, D.; Zhou, R.; Chen, C.; Yee, W.-A.; Kong, J.; Ding, G.; Lu, X., Non-volatile polymer electrolyte based on poly (propylene carbonate), ionic liquid, and lithium perchlorate for electrochromic devices. *The Journal of Physical Chemistry B* **2013,** *117* (25), 7783-7789.

59. Huang, K.; Xia, S.; Zhang, X.-M.; Chen, Y.-L.; Wu, Y.-T.; Hu, X.-B., Comparative study of the solubilities of SO2 in five low volatile organic solvents (sulfolane, ethylene glycol, propylene carbonate, N-methylimidazole, and N-methylpyrrolidone). *Journal of Chemical & Engineering Data* **2014,** *59* (4), 1202-1212.

60. Liu, J.; Wang, F.; Zhang, L.; Fang, X.; Zhang, Z., Thermodynamic properties and thermal stability of ionic liquid-based nanofluids containing graphene as advanced heat transfer fluids for medium-to-high-temperature applications. *Renewable Energy* **2014,** *63*, 519-523.

61. Maton, C.; De Vos, N.; Stevens, C. V., Ionic liquid thermal stabilities: decomposition mechanisms and analysis tools. *Chemical Society Reviews* **2013,** *42* (13), 5963-5977.

62. Lin, X.; Kavian, R.; Lu, Y.; Hu, Q.; Shao-Horn, Y.; Grinstaff, M., Thermally-responsive, nonflammable phosphonium ionic liquid electrolytes for lithium metal batteries: operating at 100 degrees celsius. *Chemical Science* **2015,** *6* (11), 6601-6606.

63. Salem, N.; Abu-Lebdeh, Y., Non-flammable electrolyte mixtures of ringed ammonium-based ionic liquids and ethylene carbonate for high voltage li-ion batteries. *Journal of The Electrochemical Society* **2014,** *161* (10), A1593-A1601.

64. Parker, S. T.; Slinker, J. D.; Lowry, M. S.; Cox, M. P.; Bernhard, S.; Malliaras, G. G., Improved turn-on times of iridium electroluminescent devices by use of ionic liquids. *Chemistry of materials* **2005,** *17* (12), 3187-3190.

65. Feldmann, C.; Ruck, M., Ionic Liquids–Designer Solvents for the Synthesis of New Compounds and Functional Materials. *Zeitschrift für anorganische und allgemeine Chemie* **2017,** *643* (1), 2-2.

66. Jamali, T.; Karimi-Maleh, H.; Khalilzadeh, M. A., A novel nanosensor based on Pt: Co nanoalloy ionic liquid carbon paste electrode for voltammetric determination of vitamin B 9 in food samples. *LWT-Food Science and Technology* **2014,** *57* (2), 679-685.

67. Liu, Y.; Shamsi, S. A., Combined use of chiral ionic liquid surfactants and neutral cyclodextrins: Evaluation of ionic liquid head groups for enantioseparation of neutral compounds in capillary electrophoresis. *Journal of Chromatography A* **2014,** *1360*, 296-304.

68. Zhang, T.; Gai, Q.; Qu, F.; Zhang, Y., Ionic liquid-assisted SDS-PAGE to improve human serum protein separation. *Electrophoresis* **2011,** *32* (20), 2904-2910.

69. Zhou, L.; Danielson, N. D., The ionic liquid isopropylammonium formate as a mobile phase modifier to improve protein stability during reversed phase liquid chromatography. *Journal of Chromatography B* **2013,** *940*, 112-120.

70. Corradini, D.; Nicoletti, I.; Bonn, G. K., Co-electroosmotic capillary electrophoresis of basic proteins with 1-alkyl-3-methylimidazolium tetrafluoroborate ionic liquids as non-covalent coating agents of the fused-silica capillary and additives of the electrolyte solution. *Electrophoresis* **2009,** *30* (11), 1869-1876.

71. Wang, S.; Xiao, Z.; Xiao, C.; Wang, H.; Wang, B.; Li, Y.; Chen, X.; Guo, X., (E)-Propyl α-Cyano-4-Hydroxyl Cinnamylate: A High Sensitive and Salt Tolerant Matrix for Intact Protein Profiling by MALDI Mass Spectrometry. *Journal of The American Society for Mass Spectrometry* **2016,** *27* (4), 709-718.

72. Hillenkamp, F.; Jaskolla, T. W.; Karas, M., The MALDI process and method. *MALDI MS: A Practical Guide to Instrumentation, Methods, and Applications, Second Edition* **2014**, 1-40.

73. Meding, S.; Nitsche, U.; Balluff, B.; Elsner, M.; Rauser, S.; Schöne, C. d.; Nipp, M.; Maak, M.; Feith, M.; Ebert, M. P., Tumor classification of six common cancer types based on proteomic profiling by MALDI imaging. *Journal of proteome research* **2012,** *11* (3), 1996-2003.

74. Van Swelm, R. P.; Laarakkers, C. M.; Kooijmans-Otero, M.; de Jong, E. M.; Masereeuw, R.; Russel, F. G., Biomarkers for methotrexate-induced liver injury: urinary protein profiling of psoriasis patients. *Toxicology letters* **2013,** *221* (3), 219-224.

75. Sandanayake, N. S.; Camuzeaux, S.; Sinclair, J.; Blyuss, O.; Andreola, F.; Chapman, M. H.; Webster, G. J.; Smith, R. C.; Timms, J. F.; Pereira, S. P., Identification of potential serum peptide biomarkers of biliary tract cancer using MALDI MS profiling. *BMC clinical pathology* **2014,** *14* (1), 7.

76. Greco, V.; Pieragostino, D.; Piras, C.; Aebersold, R.; Wiltfang, J.; Caltagirone, C.; Bernardini, S.; Urbani, A., Direct analytical sample quality assessment for biomarker investigation: qualifying cerebrospinal fluid samples. *Proteomics* **2014,** *14* (17-18), 1954-1962.

77. Ferguson, L. S.; Wulfert, F.; Wolstenholme, R.; Fonville, J. M.; Clench, M. R.; Carolan, V. A.; Francese, S., Direct detection of peptides and small proteins in fingermarks and determination of sex by MALDI mass spectrometry profiling. *Analyst* **2012,** *137* (20), 4686-4692.

78. Nomura, F., Proteome-based bacterial identification using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS): A revolutionary shift in clinical diagnostic microbiology. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics* **2015,** *1854* (6), 528-537.

79. Sassi, M.; Arena, S.; Scaloni, A., MALDI-TOF-MS platform for integrated proteomic and peptidomic profiling of milk samples allows rapid detection of food adulterations. *Journal of agricultural and food chemistry* **2015,** *63* (27), 6157-6171.

80. Gessel, M. M.; Norris, J. L.; Caprioli, R. M., MALDI imaging mass spectrometry: spatial molecular analysis to enable a new age of discovery. *Journal of proteomics* **2014,** *107*, 71-82.

81. Cohen, S. L.; Chait, B. T., Influence of matrix solution conditions on the MALDI-MS analysis of peptides and proteins. *Analytical chemistry* **1996,** *68* (1), 31-37.

82. Patel, E.; Clench, M. R.; West, A.; Marshall, P. S.; Marshall, N.; Francese, S., Alternative surfactants for improved efficiency of in situ tryptic proteolysis of fingermarks. *Journal of The American Society for Mass Spectrometry* **2015,** *26* (6), 862-872.

83. Botelho, D.; Wall, M. J.; Vieira, D. B.; Fitzsimmons, S.; Liu, F.; Doucette, A., Top-down and bottom-up proteomics of SDS-containing solutions following mass-based separation. *Journal of proteome research* **2010,** *9* (6), 2863-2870.

84. Amado, F. M.; Santana-Marques, M. G.; Ferrer-Correia, A.; Tomer, K. B., Analysis of peptide and protein samples containing surfactants by MALDI-MS. *Analytical Chemistry* **1997,** *69* (6), 1102-1106.

85. Saveliev, S. V.; Woodroofe, C. C.; Sabat, G.; Adams, C. M.; Klaubert, D.; Wood, K.; Urh, M., Mass spectrometry compatible surfactant for optimized in-gel protein digestion. *Analytical chemistry* **2013,** *85* (2), 907-914.

86. Chang, Y.-H.; Gregorich, Z. R.; Chen, A. J.; Hwang, L.; Guner, H.; Yu, D.; Zhang, J.; Ge, Y., New mass-spectrometry-compatible degradable surfactant for tissue proteomics. *Journal of proteome research* **2015,** *14* (3), 1587-1599.

87. Lin, Y.; Huo, L.; Liu, Z.; Li, J.; Liu, Y.; He, Q.; Wang, X.; Liang, S., Sodium laurate, a novel protease-and mass spectrometry-compatible detergent for mass spectrometry-based membrane proteomics. *PloS one* **2013,** *8* (3), e59779.

88. Shi, Q.; Jackowski, G., One-dimensional polyacrylamide gel electrophoresis. *Gel electrophoresis of proteins: A practical approach, 3rd ed. Oxford University Press, Oxford* **1998**, 1-52.

89. Maurer, H. R., *Disc electrophoresis and related techniques of polyacrylamide gel electrophoresis*. Walter de Gruyter: 1978.

90. Garfin, D. E., Gel electrophoresis of proteins. *Essential cell biology* **2003,** *1* (7), 197-268.

91. Das, S.; Sylvain, M. R.; Fernand, V. E.; Losso, J. N.; El-Zahab, B.; Warner, I. M., Positive cooperative mechanistic binding of proteins at low concentrations: A comparison of poly (sodium N-undecanoyl sulfate) and sodium dodecyl sulfate. *Journal of colloid and interface science* **2011,** *363* (2), 585-594.

92. Ghisaidoobe, A. B.; Chung, S. J., Intrinsic tryptophan fluorescence in the detection and analysis of proteins: a focus on Förster resonance energy transfer techniques. *International journal of molecular sciences* **2014,** *15* (12), 22518-22538.

93. Chan, F. T.; Schierle, G. S. K.; Kumita, J. R.; Bertoncini, C. W.; Dobson, C. M.; Kaminski, C. F., Protein amyloids develop an intrinsic fluorescence signature during aggregation. *Analyst* **2013,** *138* (7), 2156-2162.

94. Chan, F. T.; Pinotsi, D.; Kaminski Schierle, G.; Kaminski, C. F., Structure-specific intrinsic fluorescence of protein amyloids used to study their kinetics of aggregation. *Bio-nanoimaging: protein misfolding and aggregation* **2014**.

95. Chen, Y.; Barkley, M. D., Toward understanding tryptophan fluorescence in proteins. *Biochemistry* **1998,** *37* (28), 9976-9982.

96. De, S.; Girigoswami, A.; Das, S., Fluorescence probing of albumin–surfactant interaction. *Journal of colloid and interface science* **2005,** *285* (2), 562-573.

97. Watson, M. D.; Peran, I.; Zou, J.; Bilsel, O.; Raleigh, D. P., Selenomethionine Quenching of Tryptophan Fluorescence Provides a Simple Probe of Protein Structure. *Biochemistry* **2017,** *56* (8), 1085-1094.

CHAPTER 2: IONIC LIQUIDS AS BUFFER ADDITIVES IN IONIC LIQUID-POLYACRYLAMIDE GEL ELECTROPHORESIS SEPARATION OF MIXTURES OF LOW AND HIGH MOLECULAR WEIGHT PROTEINS[*]

2.1. Introduction

Large scale studies of proteins have received widespread public attention because of the significance of proteomics for medical diagnosis and treatment. The progress of proteomics is strongly dependent on the development of protein separation techniques and MS technology.[1] Many classical approaches become inefficient because of the complexity of protein structures and their sequence and folding motifs.[2] A number of methods have been used for protein separations, which includes liquid chromatography,[3-5] size exclusion chromatography,[6] capillary electrophoresis[7-9] and gel electrophoresis.[10-13] The ordinary gel-based electrophoresis techniques, SDS-PAGE,[14-17] 2-DE,[15,16,18] Blue-native,[19,20] and Native-PAGE [21,22] are important and the most commonly used methods for protein separation.

SDS-PAGE has become and remains the standard method for identification, purification, and structural analyses of proteins. SDS, as a solubilizing agent for proteins, was first introduced by Laemmli in 1970.[23,24,25] The amphiphilic properties of SDS are very important to the separation because at concentrations greater than the critical micelle concentration (CMC), surfactant monomers spontaneously self-assemble into micellar structures. Such micelles denature, solubilize and bind to proteins.[26] Negatively charged SDS-protein complexes are formed having the same mass to charge ratio; therefore, charge is eliminated from the migration mechanism and separation is solely based on relative molecular masses.[27] Although SDS has been used in PAGE

[*]I would like to thank Dr. Farhana Hasan for her guidance and support to work on this chapter. It is reproduced by permission of The Royal Society of Chemistry
http://pubs.rsc.org/en/content/articlelanding/2015/ra/c5ra11559k/unauth#!divAbstract

for decades, there are some limitations of PAGE due to joule heating and long measurement times. In addition, some proteins migrate anomalously in SDS-PAGE.[28,29]

In addition, the presence of long chained alkyl sulfates in unpurified SDS may lead to multiple bands as single protein complexes with one or more SDS monomer.[29] Even by use of pure SDS, carbohydrate-bearing, highly basic and highly acidic proteins migrate anomalously in gel electrophoresis.[30-33] Another deficiency in SDS-PAGE is that SDS may crystallize at low temperatures,[34,35] producing artifacts in gels, which then affect the resolution of proteins.[36] In Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), SDS forms sodium adducts with proteins and thus reduces the accuracy of protein identification.[37] To overcome these limitations, various other detergents have been used to replace SDS. For example, in 1984 two cationic detergents, cetyltrimethylammonium bromide (CTAB) and cetylpyridinium chloride were used to replace SDS.[38] However, these cationic detergents required a ten-fold increase in concentration as compared to SDS. In another study by Ross and coworkers,[34] an acid labile surfactant (ALS) reportedly exhibited similar denaturing and electrophoretic properties as SDS. The authors revealed that ALS decomposes at low pH and the negative charge on the ALS-protein complex may not be as high as with SDS. Limitations of SDS-PAGE require development of new surfactants that can discriminate protein types and molecular weights. Therefore, a simple modification of SDS-PAGE for complex protein sample separation may provide great potential for widely used applications in protein analysis.

Ionic liquids are a group of organic salts consisting of ions (cations and anions), having appreciable liquid ranges and melting points below 100 $^{\circ}$C. These salts have low melting points because of the larger size of one or both ions and thus low symmetry.[39] There are two broad categories of ILs: room temperature ILs (RTILs) that melt below 25 °C and frozen ILs (FILs),

which are typically solids at room temperature (>25 °C) but, melt below 100 °C. ILs have attracted extensive attention in recent years and have aroused considerable interest in biological catalysis[40] and protein separations[41,42] due to their unique properties i.e. low volatility, negligible vapor pressure, nonflammability, high thermal stability, and wide electrochemical window. The chemical and physical properties of these salts can be obtained by choosing suitable cationic and/or anionic constituent. Thus, ILs are regarded as ‘‘designer solvents’’ because of the tunability, which increases their potential applications. ILs which are considered as cationic surfactants (ILS), because of the amphiphilicity of their cation,[43] play an important role in separation, such that they have been used either in the stationary phase of HPLC,[44] reverse phase liquid chromatography (RPLC)[45] and gas chromatography (GC)[46,47] or as a buffer modifier in capillary electrophoresis (CE).[48-50] ILS based monolithic column for CE have been reported recently.[51] In mass spectrometry, ILS have been used as MALDI matrices and ion pairing reagents in ESI.[52]

Herein, we report the first ILS-PAGE technique, in which hydrophobic ILS were used as buffer additives in both sample and running buffers for separation of five acidic proteins: Cat, Tf, BSA, Ova, and α-Lact. The ILS used in this study were chosen based on their significant physiochemical properties, i.e. solubility in aqueous solution, good conductivity, and good hydrophobicity. For this purpose, novel, *N*-alkyl-4-methylpyridinium bromide ionic liquids with different alkyl chain lengths i.e. 1-butene-4-methylpyridinium bromides (C_4PBr), 1-octene-4-methylpyridinium bromide (C_8PBr) and 1-undecene-4-methylpyridinium bromide (C_{11}PBr) have been synthesized. These ILS are categorized as RTILS because they are viscous at room temperature and follow the normal temperature requirements for IL salts. In this regard, the influences of chain lengths and concentration of these ILS in sample and running buffers on

protein separation have been investigated. As compared to standard Laemmli protocol for SDS-PAGE, no heating of sample buffer was required in ILS-PAGE. Therefore, we report CD studies which prove that these ILS denature the protein without heating. Fluorescence studies were performed in order to understand ionic liquid-protein interactions. In studies of surfactant-protein interactions, BSA was used as a model protein due to its well-established primary structure, water solubility, and versatile binding capacity.[53]

2.2. Experimental Section

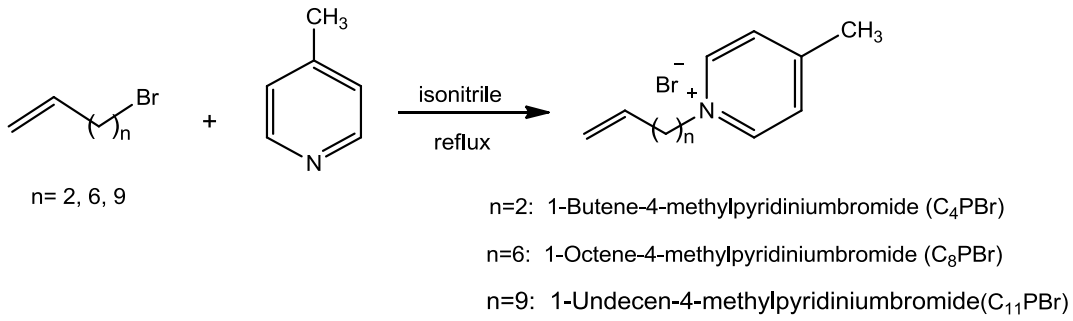
2.2.1. Materials

All proteins: Cat (bovine liver, 250 kDa, p*I* 5.4), Tf (human, 80 kDa, p*I* 5.5), BSA (bovine, 66 kDa, p*I* 4.7), Ova (egg, 45 kDa, p*I* 4.9), and α-Lact (bovine milk, 14 kDa, p*I* 4.2) were purchased from Sigma Aldrich (St. Louis, MO, USA) in pure form (≥ 95%). Proteins were reconstituted into 3 mg/ml aliquots with 10 mM phosphate buffer at pH 7.4 and stored at -20 ºC. Standard precast 4-20% Tris-HCl gradient polyacrylamide mini gels were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Tris-Glycine buffer was used for 1D-gel electrophoresis and all other studies. Reagents used to prepare the running buffer, sample buffer, and the destaining solution were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Ultrapure water (18.2 MΩ) was obtained using an Elga PURELAB Ultra purifier (Lowell, MA, USA). All reagents were used as received without further purification.

2.2.2. Synthesis and Characterization of Ionic Liquid Surfactants

Pyridinium based ILS with different alkyl chain lengths i.e. C_4PBr, C_8PBr, and C_{11}PBr were synthesized and characterized by use of nuclear magnetic resonance (^{1}H-, ^{13}C-NMR) and

electrospray ionization (ESI)-mass spectrometry (MS). The instruments used for ESI-MS and NMR were Agilent 6210 TOF LC/MS and Bruker AV 400 respectively.



Scheme 2.1. Reaction for the 4-methylpyridinium-based ionic liquid synthesis

1-Butene-4-methylpyridiniumbromide (C_4PBr)

To acetonitrile, 4-bromo-1-butene (10 g, 0.074 M) and 4-methylpyridine (1.02 eq., 7 g, 0.075 M) were added and the mixture was refluxed at 80 ºC for 24h. After 24h, acetonitrile was evaporated under a vacuum, which then resulted yellow viscous liquid. TLC in 90% $CHCl_3$: MeOH showed that the product was pure (16 g, 94% yield).

^{1}H-NMR ($CDCl_3$, 400 MHz): δ 9.33 (d, J= 6.52, 2H), 8.36 (d, J= 6.62 Hz, 2H), 5.82 (m, 1H), 4.97 (m, 1H), 4.84 (m, 2H), 2.71 (m, 2H), 2.26 (s, 3H); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 158.4, 144.1, 140.5, 128.3, 119.8, 59.4, 35.5, 22.2; (MS, ESI) m/z 148 (M^+)

Other ILS, 1-Octene-4-methylpyridinium bromide (C_8PBr) and 1-Undecene-4-methylpyridinium bromide (C_{11}PBr) were synthesized following the same procedure mentioned above and characterized by ^{1}H-NMR, ^{13}C-NMR, and ESI/MS.

1-Octene-4-methylpyridiniumbromide (C_8PBr)

^{1}H-NMR ($CDCl_3$, 400 MHz) (C_8PBr) : δ 9.43 (d, J =6.68Hz, 2H), 7.98 (d, J=6.28Hz,2H), 5.79 (m, 1H), 5.75 (m,1H), 5.72 (m,1H), 4.90 (m, 2H), 2.86 (s, 3H), 2.39 (m, 2H), 2.0 (m,2H),

1.36 (m,6H); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 158.5, 144.1, 138.4, 128.7, 114.2, 60.7, 33.2, 31.5, 28.2, 25.6, 22.06; (MS, ESI) m/z 204 (M^+)

1-Undecene-4-methylpyridiniumbromide (C_{11}PBr)

^{1}H-NMR ($CDCl_3$, 400 MHz) (C_{11}PBr) : δ9.30 (d, J =6.6Hz, 2H), 7.89 (d, J=6.28Hz,2H), 5.77 (m, 1H), 5.73 (m,1H), 5.71 (m, 1H), 4.80 (m, 1H), 2.63 (s, 3H), 2.0 (m,2H), 1.96 (m,2H), 1.29 (m, 10H); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 158.7, 144.2, 139.09, 128.8, 114.1, 61.1, 31.8, 29.2, 29, 28.9, 28.8, 26, 22.2; (MS, ESI) m/z 246 (M^+)

2.2.3. Critical Micelle Concentration (CMC)

The CMCs of the ILS, C_4PBr, C_8PBr, and C_{11}PBr, were determined by use of surface tension measurements at the room temperature with KSV Sigma 703 digital tensiometer. These CMCs were determined in both deionized water and 25 mM Tris/192 mM glycine buffer (pH 8.4).

2.2.4. Instrumentation

Fluorescence studies were performed using a SPEX Fluorolog-3 spectrofluorimeter (model FL3-22TAU3; Jobin Yvon, Edison, NJ) equipped with a 450 W xenon lamp and R928P photomultiplier tube (PMT) emission detector. A quartz cuvette with an optical pathlength of 0.4 cm was used and bandwidths of both the excitation and emission monochromators were set at 3 nm. Excitation of Tryptophan (Trp) was performed at 295 nm, while emission spectra were measured in the range of 300-500 nm. Fluorescence spectra reported here were obtained from proteins at concentration of 1 mg/ml in 25 mM Tris/ 192 mM Glycine (pH 8.4). CD data were obtained using an AVIV Model 62DS (AVIV Associates, Lakewood, N.J.) spectrophotometer at 25 °C, fitted with a 1 mm path length quartz cell. The CD spectra of native protein samples in 25 mM Tris/192 mM Glycine (pH 8.4) were acquired at concentrations that produced optimal CD

signals. All CD scans were conducted in triplicate using the far UV (200-240 nm) and near UV (240-320 nm) regions of the spectrum. Average CD spectra were recorded after correcting for background intensity of the buffer. The CD response is reported as ellipticity and displayed in units of millidegree (mdeg).

A Mini-PROTEAN 3 Electrophoresis Module from Bio-Rad Laboratories (Hercules, CA, USA) was used for PAGE separations. A constant voltage of 200 V was applied for each separation. Gels were placed in plastic containers and set on a shaker (Midwest Scientific, St. Louis, MO, USA) during staining and destaining. Typical staining and destaining times for SDS-PAGE were used. Protein bands were analyzed for each gel using a Kodak Gel Logic 200 Image Analyzer (Rochester, NY, USA).

2.2.5. Binding Parameters

Binding parameters of C_4PBr, C_8PBr, and C_{11}PBr, interacting with BSA were determined by use of the fluorometric titration method. Each protein (1 mg/mL) was allowed to equilibrate for 30 min with concentrations of C_4PBr, C_{11}PBr (0-35 mM), and C_8PBr (0-95 mM) in 25 mM Tris/ 192 mM Glycine (pH 8.4) at 25 ºC. In biological systems where a ligand binds to a receptor (macromolecule), Scatchard plot (v/c vs v)[54,55] is used to determine the binding properties from different regions of the isotherm: binding constant for each region and number of ligand binding sites.

The various parameter characteristics of such analyses were determined as mentioned below:

Fraction of surfactant bound, $\alpha = (I-I_0) / (I_m-I_0)$

The concentration of the bound surfactant, $S_b = \alpha$ [Total surfactant]

The concentration of free ILS = 1 - [bound ILS]

The parameter, $v = \alpha$ [Total surfactant] / [Total protein]

The concentration of free surfactant, c = [Total surfactant] (1-α)

Where I_0 is the fluorescence intensity of the protein in the absence of ILS (C_4PBr, C_8PBr or C_{11}PBr), I is the fluorescence intensity when the protein and ILS are in an equilibrium, and I_m is the fluorescence intensity when the protein is saturated with ILS. Each linear portion of a Scatchard plot was given a linear fit and the equilibrium binding constant (*K*) and number of binding sites (*n*) for a particular concentration region were obtained from the slope and the intercept respectively.

2.2.6. Electrophoretic Separation

Standard 10× running buffer (RB) stock solution contained 25 mM Tris and 192 mM Glycine (pH 8.4). The RB solution was prepared by pipetting an appropriate amount of ILS (or SDS) into a volumetric flask, dissolving it with 50 mL of running buffer stock solution, and diluting up to a final volume of 500 mL with ultrapure water (18.2 MΩ). Concentrations of 0.0125%, 0.025%, 0.05%, 0.1%, and 0.5% (w/v) ILS were used in the running buffer for optimization and validation of separations. The sample buffer (SB) was prepared in 2 mL Eppendorf tubes by combining appropriate amounts of ultrapure water, 50 mM Tris-HCl (pH 6.8), glycerol, bromophenol blue, and 0.025%, 0.05%, 0.25%, 0.5%, 1% (w/v) of ILS (or SDS). Proteins from stock solution (3 mg/mL) were added into the SB at protein:SB ratio of 1:20. The reducing agent, β-mercaptoethanol, was added at 5% (v/v) into the SB. The running buffer was loaded into the inner and outer chambers of the Mini-PROTEAN 3 module. For ILS, no heating of samples was needed. However, for SDS, each sample was heated at 95 °C for 5 minutes on a dry bath incubator purchased from Fisher Scientific (Pittsburgh, PA, USA). Fifteen microliters (15 µL) of sample were loaded into each well of the 4-20% Tris-HCl gradient mini gels. Migration time was 40 minutes for all separations. After each separation,

gels were rinsed with ultrapure water (18.2 MΩ), stained with approximately 20 mL of Coomassie Blue Stain, and placed on a shaker. Gels were destained using ultrapure water (18.2 MΩ) until a clear background was visible.

2.3. Results and Discussion

2.3.1. Critical Micelle Concentration

Surface tension measurements were performed to evaluate the surface activity of three pyridinium ILS in water and 25 mM Tris/192 mM glycine buffer (pH 8.4). Figure 2.1 depicts the surface tension (γ) verses concentration (C) plots for C_nPBr (n= 4, 8, 11) in water and in buffer at 25 ^oC. The surface tension of ILS both in water and buffer progressively decreases with an increase in concentration. After that, it reaches a plateau region indicating that micelles are formed, and the concentration of the break point corresponds to the CMC. The values of the CMC decreases in order of C_4PBr > C_8PBr > $C_{11}PBr$, in accordance with increased hydrophobicity owing to an extension of the hydrocarbon chain. Increasing the hydrophobic chain length decreases the water solubility of ILS, and causes close packing of the ILS within micelles, which facilitates the formation of micelles at a lower concentration.[56] The CMC also depends on the ionic strength or salt effect. Hydrophobic interactions can be enhanced by an increase in ionic strength.[57] The reduction in the CMC in the presence of electrolytes for ionic detergent is likely due to a reduction in the electronic environment surrounding the ionic head groups. Addition of electrolytes decreases the repulsion between similarly charged ionic head groups within a micelle and therefore, the detergent monomers can pack more tightly, and thus the CMC is reduced.[58] The overall CMC in buffer is lower as compared to the CMC in water (Table 2.1).

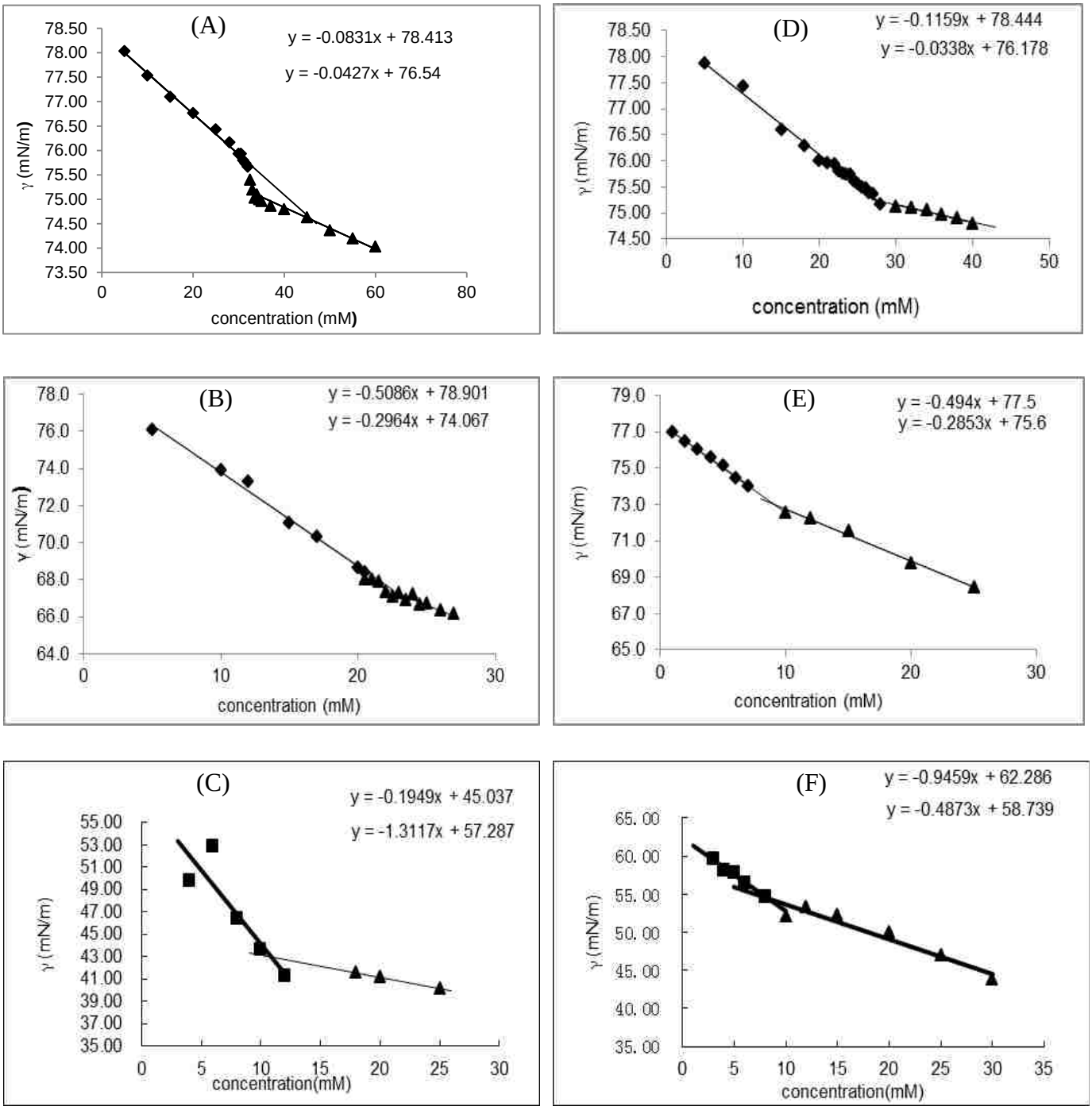


Figure 2.1. Tensiometric analysis of CMC of C_4PBr (A and D), C_8PBr (B and E), C_{11}PBr (C and F) in water (A-C) and 25 mM Tris/glycine buffer (D-F) respectively

Table 2.1. CMC of ionic liquids; 1-butene-4-methylpyridiniumbromide (C_4PBr), 1-octene- 4-methylpyridiniumbromide (C_8PBr), and 1-undecene-4-methylpyridiniumbromide (C_{11}PBr).

Ionic liquid Surfactants	**CMC in water (mM)**	**CMC in buffer (mM)**
C_4PBr	46.36	27.60
C_8PBr	21.50	9.10
C_{11}PBr	11.24	7.73

2.3.2. Separation of Proteins by ILS-PAGE

Separation of a mixture of five proteins, i.e. Cat, Tf, BSA, Ova, and α-Lact (p*I* 4-6), has been evaluated by using ionic liquids in PAGE. ILS with C_4, C_8, C_{11} alkyl chains (C_4PBr), (C_8PBr) and (C_{11}PBr) were used as buffer additives in both sample and running buffers. SDS was completely replaced by use of ILS in PAGE. The electrophoretic migration patterns of proteins in mixtures as well as purified form were studied. The electropherogram displayed in Figure 2.2 was obtained by use of 0.025% (w/v) C_4PBr in sample buffer and 0.025% (w/v) C_4PBr in running buffer.

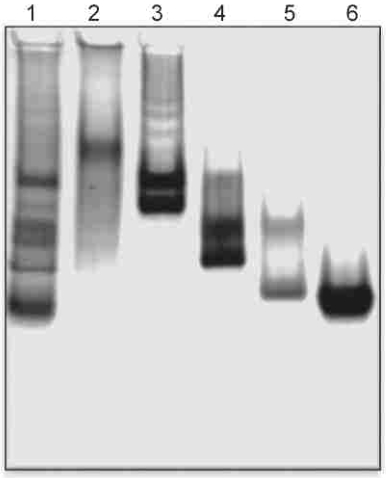


Figure 2.2. ILS-PAGE with 0.025% (w/v) C_4PBr in protein mixture (1); Cat, 250 kDa (2); Tf, 80 kDa (3); BSA, 66 kDa (4); Ova, 45 kDa (5); and α-Lact, 14 kDa (6).

Cat (250 kDa) in lane 2 appeared as a single band, while Tf (80 kDa, lane 3) separated as two highly stained bands which could be its β_1 and β_2 isoforms. [59] BSA (66 kDa, lane 4) appeared as multiple bands, which may be due to the formation of oligomers.[60] Ova (45 kDa, lane 5) separated as two bands of its S and I isoforms [61] and lastly a single band of α-Lact (14 kDa, lane 6). A similar pattern for individual protein migrations was observed in bands analyzed in mixture (lane 1)**.**

2.3.3. Comparison of ILS-PAGE and SDS-PAGE

The resolution and migration distances of individual proteins, separated by optimum concentration of 0.025% (w/v) C_4PBr in running buffer, 0.025% (w/v) sample buffer and ordinary SDS-PAGE were compared (Figure 2.3).

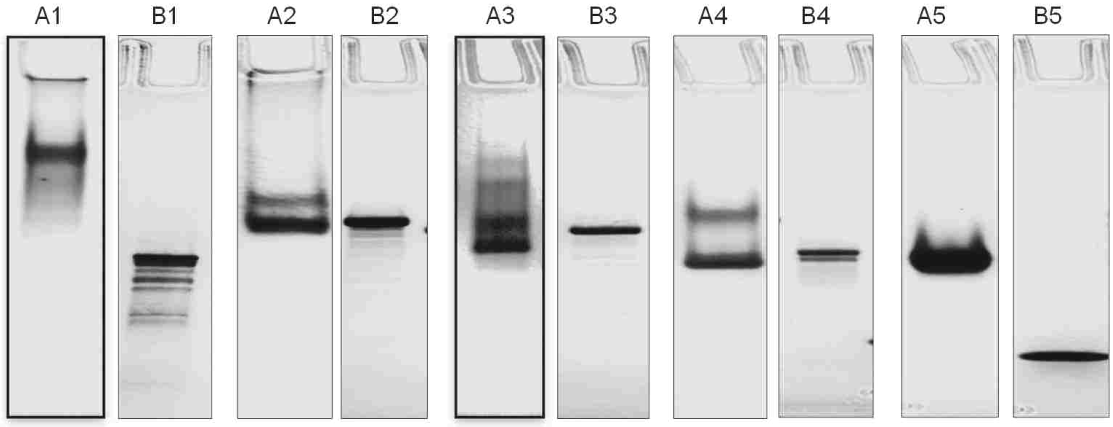


Figure 2.3. Separation of Cat (1), Tf (2), BSA (3), Ova (4), and α-Lact (5) in C_4PBr-PAGE (lanes A) and SDS-PAGE (lanes B)

In standard SDS-PAGE, all samples were heated at 95 ^oC for 5 minutes, while no heating was involved in ILS-PAGE. A difference was observed in the separation pattern of Cat, Tf, BSA, and Ova. Cat is a homotetrameric enzyme, composed of four subunits. [62] There is one major band of each subunit (approximately 62.5 kDa) appeared [63] in SDS-PAGE, having the same R_f value

as BSA, while in C_4PBr-PAGE, Cat (A1) separated as a single band in the range of 250 kDa. Tf and Ova exist as isoforms; β_1, β_2 [59] and S, I [61] respectively, due to small variations in their carbohydrate structures. Separation of isoforms is difficult because of the smaller difference in their R_f values and involved multiple steps of separation (chromatographic and electrophoretic etc.). The unique property of ILS-PAGE is that the isoforms of Tf and Ova could easily be separated as shown in A2 and A4 respectively, while SDS-PAGE failed to separate any of these isoforms. Tf appeared as single band and if closely observed, Ova appeared as two bands (B4), which were very close and difficult to separate by SDS-PAGE. BSA showed multiple bands (A3) in ILS-PAGE, which could be due to the formation of oligomers. [60] In SDS-PAGE, BSA was separated as a single band (B3). Examination of the results showed that the mobility distance of a lower molecular weight protein (α-Lact) in ILS-PAGE was decreased as compared to SDS-PAGE. The mobility distance could be increased by varying the conditions of PAGE, which are concentration of surfactant, running time of the gel, and voltage.

ILS-PAGE performance of protein samples at room temperature indicates that ILS itself solubilizes the protein in its hydrophobic environment, thus, disrupting its tertiary structure and denaturing the protein, which was later confirmed by CD studies. In ILS-PAGE, sample buffer concentration was 0.025% (w/v) ILS, which was 100-fold less than the standard SDS-PAGE concentration (2% (w/v)). Running buffer concentration in ILS-PAGE was 0.025% (w/v) ILS, while in SDS-PAGE, the running buffer concentration of SDS was 0.1% (w/v), which wass 5-fold more than ILS-PAGE. Higher separation resolution of Tf and Ova was achieved in case of ILS-PAGE, eventhough the migration distance of α–Lact decreased. In ILS-PAGE, separation was achieved by using lesser amount of surfactant as compared to SDS-PAGE. No heating of

sample buffer will have a direct impact on reduction in time and energy usage when multiple samples are being prepared simultaneously.

2.3.4. Factors Affecting the Separation of Protein Mixture in ILS-PAGE

Different factors affecting the separation of mixtures of five acidic proteins, i.e. (p*I* 4- 6), Cat, Tf, BSA, Ova, and α-Lact, have been studied, which are as follows:

2.3.4.1. Different Concentration in Sample Buffer and Alkyl Chain Length of ILS Cation

We examined the effects of different alkyl chain lengths of C_nPBr (n= 4, 8, 11) as a buffer additive on the separation of a mixture of five proteins (Cat, Tf, BSA, Ova, and α-Lact). The concentration of ILS in the sample buffer was varied, while it was held constant (0.025% (w/v)) for the running buffer. In Fig. 2.4A, 0.025% (w/v) of C_4PBr, C_8PBr and C_{11}PBr were used as sample buffer additives respectively, which produced separation of five proteins. α-Lact (14 kDa) exhibited shorter migration distance in case of C4 and C8 as compared to C11.This indicated that low molecular weight protein migration was retarded by C4 and C11 ILS. At lower concentrations, 0.025% and 0.05% (w/v), of both C_4PBr and C_8PBr gave clear separation (Fig. 2.4A, B). Geng et al. have reported that ILS with long alkyl chain form complex with protein by hydrophobic and electrostatic interactions.[64] Stronger hydrophobic interactions could damage the ILS-protein complex, so at higher concentrations, 0.25%-1% (w/v), of C_8PBr and C_{11}PBr in running buffer damage the ILS-protein complex, which deteriorate the separation of protein mixture (Fig. 2.4E). The results indicate that, at higher concentrations of longer alkyl chain ILS, as buffer additive in ILS-PAGE result poor separation of proteins. Overall, shorter alkyl chain (C4, C8) ILS at lower concentration as buffer additive were suitable for protein mixture separation.

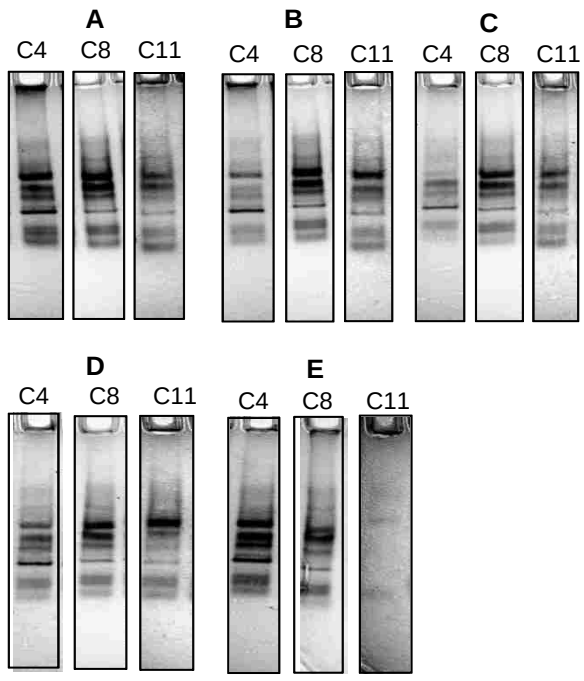


Figure 2.4**.** ILS-PAGE of protein mixture (Cat, 250 kDa; Tf, 80 kDa; BSA, 66 kDa; Ova, 45 kDa; α-Lact, 14 kDa) with C_4-C_{11}PBr concentration (w/v) of (A) 0.025% (w/v) (B) 0.05% (C) 0.25% (D) 0.5% (E) 1% in sample buffer, while running buffer concentration is 0.025% (w/v) of C_4PBr–C_{11}PBr.

2.3.4.2. Different Concentrations of ILS in Running Buffer

C_4PBr ILS at a low concentration in sample buffer, 0.025% (w/v) was chosen to optimize the concentration of ILS in running buffer as it gave optimum separation in the previous section. In this regard, the effect of different concentrations of C_4PBr ILS (0.1-0.0125% w/v) in running buffer on the separation of protein mixture (Cat, Tf, BSA, Ova, and α-Lact) and their migration patterns were investigated. The resolution of proteins was poor and the larger molecular weight protein exhibited shorter migration distance at higher (0.1-0.05% (w/v)) and very low (0.012% (w/v)) concentrations of C_4PBr in the running buffer. The higher concentrations of ILS made the Ova (45 kDa) band virtually disappear, while α-Lact (14 kDa) appeared as a dark condense band (Fig. 2.5A, B). These results showed that using a lower concentration, 0.025% (w/v) of C_4PBr,

better performance, resolution, and mobility distance of proteins were improved (Fig. 2.5C), and this was the optimized concentration of ILS in running buffer. As compared to ordinary SDS-PAGE, 0.025% (w/v) of C_4PBr in running buffer enhanced the mobility distance of all proteins except α-Lact, which could be modified by changing the parameters of gel electrophoresis, and by running the gel at lower voltage for longer time periods. The change in electrophoretic mobility of proteins in ILS-PAGE could be due to the positive charge and hydrophobic alkyl

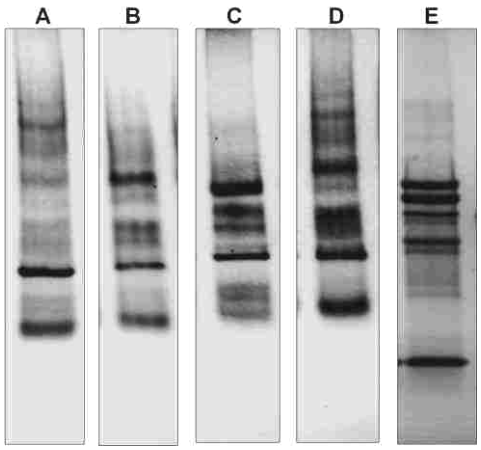


Figure 2.5. Effects of different concentrations (w/v) of C_4PBr in running buffer (A) 0.1%, (B) 0.05%, (C) 0.025%, and (D) 0.0125% for separation of protein mixture. Sample buffer concentrations were kept constant at 0.025% (w/v) in A, B, C, and D comparing 0.025% (w/v) (C) with ordinary SDS (E)

Chain in the ILS cation, which produce electrostatic and hydrophobic interactions with proteins. This may play an important role in changing their electrophoretic mobility.

2.3.5. Separation Mechanism of ILS-PAGE

In SDS-PAGE, protein separation is entirely dependent upon the molecular mass of proteins.[27,65,66] In contrast, ILS-PAGE separation is based on charges on proteins and their molecular masses. Based on this study, we have proposed the following mechanism of protein

separation in ILS-PAGE. ILS in solution first interact or form complex with protein and induce conformational changes in protein structure, which results in protein unfolding, exposing the acidic amino acids on the surface of protein, and thus proteins acquire negative charges due to the alkaline pH of the electrode buffer. Geng, et al. have reported that ILS with long alkyl chain form complexes with proteins by electrostatic and hydrophobic interactions.[64] The charge strengths of proteins depend upon the difference in buffer pH and p*I* of the protein. Thus, the migration of proteins in ILS-PAGE could be the function of their net charge and molecular size.[67] Electrophoretic mobility is directly related to net charge and inversely related to mass of protein.

Other factors that could be involved in protein separation are hydrophobic and electrostatic interactions, as well as hydrogen bonding. Better resolution of Tf and Ova was achieved in ILS-PAGE, due to the stronger hydrophobic interactions between pyridine possessing longer alkyl-chain ionic liquids and proteins,[57,68] even though the concentrations of ILS used in sample and running buffer were far below the CMC of this ILS. Hydrophobic interaction increases from C_4PBr > C_8PBr > C_{11}PBr. Electrostatic interactions also play a role, possibly between the negatively charged sites on proteins and ILS-cation. ILS can also bind to proteins by H-bonding interactions, which are between their cationic head groups and amino acid residue at the surface of protein. Fluorescence and CD studies, which are discussed later, also support this supposition for this phenomenon. In conclusions, ILS could be promising alternative surfactants to perform electrophoresis separations in the future. However, these surfactants are not yet ready to replace SDS without optimization of other separation parameters (e.g. buffer composition, voltage and running time of gel).

2.3.6. Fluorescence Studies

For macromolecules, fluorescence measurements can provide information regarding binding mechanism of a ligand to a protein.[69] The fluorescence of intrinsic Trp residue was used to determine the binding of BSA to three ligands, i.e. C_4PBr, C_8PBr, and C_{11}PBr.The binding properties of SDS to BSA has been reported earlier.[70,71] BSA has two Trp residues in the native state, one buried in the hydrophobic pocket and the other located towards the outer surface (solvent accessible). To examine whether C_nPBr binds to BSA, fluorescence measurements were performed in 25 mM Tris/192 mM Glycine buffer (pH 8.4) at 25 ºC. In this study, a titration method was adopted in which BSA concentration was held constant (10 µM), while varying the concentration of C_nPBr (n=4, 8, 11). The excitation wavelength for Trp is 295 nm, while the maximum emission wavelength (i.e. λ_{max}) is 352 nm in the absence of C_nPBr (n=4, 8, 11). The Trp emission of BSA was gradually quenched with increasing concentration of ILS. The initial blue shift was accompanied by a red shift of the maximum emission peak until the point of saturation was reached, after which there was no additional quenching or shifting of emission maxima even by further increasing the concentration of ILS (Fig. 2.6).

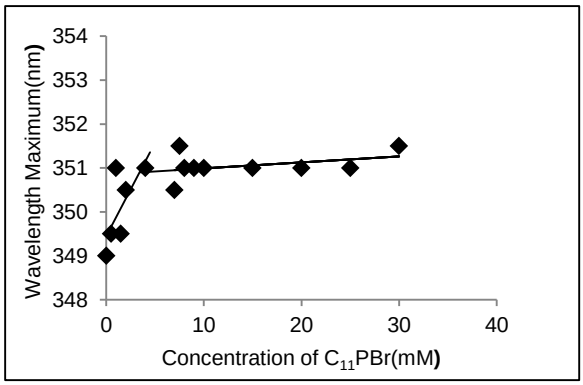


Figure 2.6. Fluorescence wavelength maxima shift of Trp in the presence of increasing concentration of C_{11}PBr in association with BSA (10 uM), determined by steady state fluorescence (λ_{ex} =295 nm, 25 ^{o}C).

The red shift and quenching in the Trp emission maxima of BSA with increasing concentration of the ligand are attributed to changes in the conformation of the proteins.[72] Such a conformational change induced by binding of ligand to the protein leads to exposure of Trp residue to a relatively hydrophobic domain. Fluorescence quenching increases with increasing the alkyl chain length of C_nPBr (n=4, 8, 11), so hydrophobic interactions increase from C_4PBr to $C_{11}PBr$.[57] Hence, it is speculated that hydrophobic interactions play an important role in the interaction of C_nPBr with protein. In order to confirm this, NaCl was added to Tris-Glycine buffer (pH 8.4), and the concentration was increased from 0.1 to 1.0 M. At the higher NaCl concentration (1.0 M), fluorescence intensity of Trp dramatically decreases with increasing $C_{11}PBr$ concentration (from 1.5 to 4.0 mM) compared to the system containing 0.1 M NaCl (Fig. 2.7).

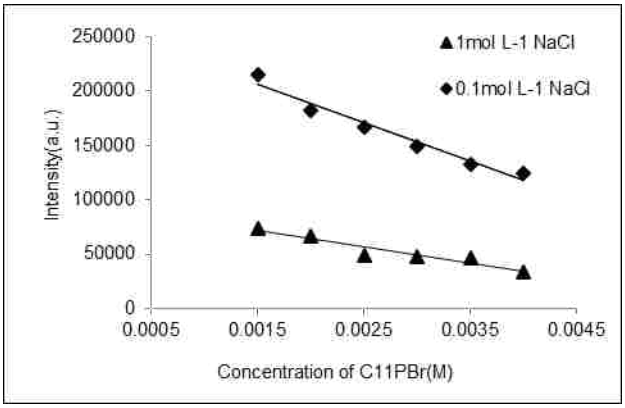


Figure 2.7. Effects of $C_{11}PBr$ on the fluorescence intensity of BSA at different NaCl concentration (0.1, 1 mol L^{-1})

The ionic strength of the system increases due addition of NaCl, which could enhance hydrophobic interactions between ionic liquid and protein. [57,73] This is attributed to a contention that hydrophobic interactions between ILS and protein plays an important role in protein separation by ILS-PAGE.

2.3.6.1.Binding Studies and Scatchard Analysis

Scatchard plots demonstrate the types of binding, particularly when multi-site ligand binding is suspected.[74] Generally, there are four characteristic binding regions: (a) specific binding to high energy sites on the protein, which are believed to be electrostatic, (b) noncooperative binding, (c) cooperative binding, where protein unfolding is believed to occur and a marked increase in binding, and (d) saturation, in which micelles co-exist with the saturated protein and no further binding occurs.[71]

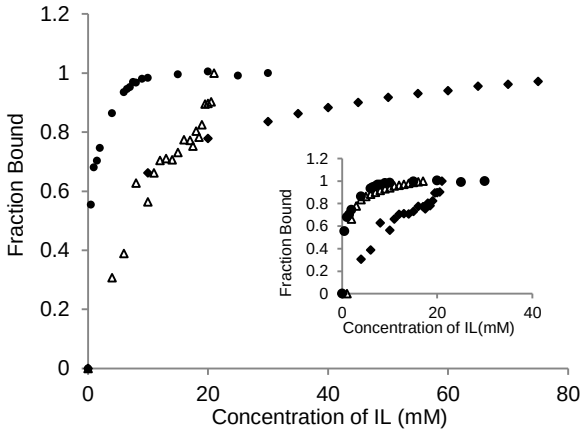


Figure 2.8. Fraction bound of C_{11}PBr (circles), C_8PBr (triangles) and C_4PBr (solid diamonds) to BSA (10 μM) with increasing concentration of C_{11}PBr, C_8PBr,(0-30 mM), C_4PBr (0-80 mM).

The binding isotherms for ILS to BSA suggest that the concentration for saturation binding was determined by the alkyl chain length of the ILS. Fig. 2.8 suggests that for the ILS with the longest alkyl chain length, saturation binding was attained at much lower concentration followed by the other two ILS in the order of decreasing chain length. This observation indicates that hydrophobicity of the ILS is a very important factor, which directs ILS-BSA complexation. Also, interactions between ILS and BSA is found to be primarily hydrophobic.

Scatchard plots for binding of $C_{11}PBr$, C_8PBr, and C_4PBr suggest that the binding mechanism of the three ionic liquids to BSA is significantly different from each other. For SDS, Scatchard analysis with BSA was reported earlier by Das, et al.[70] Scatchard plots characteristic at different concentration regions suggest that binding of these ILS to BSA follows separate mechanism in various concentration regions.

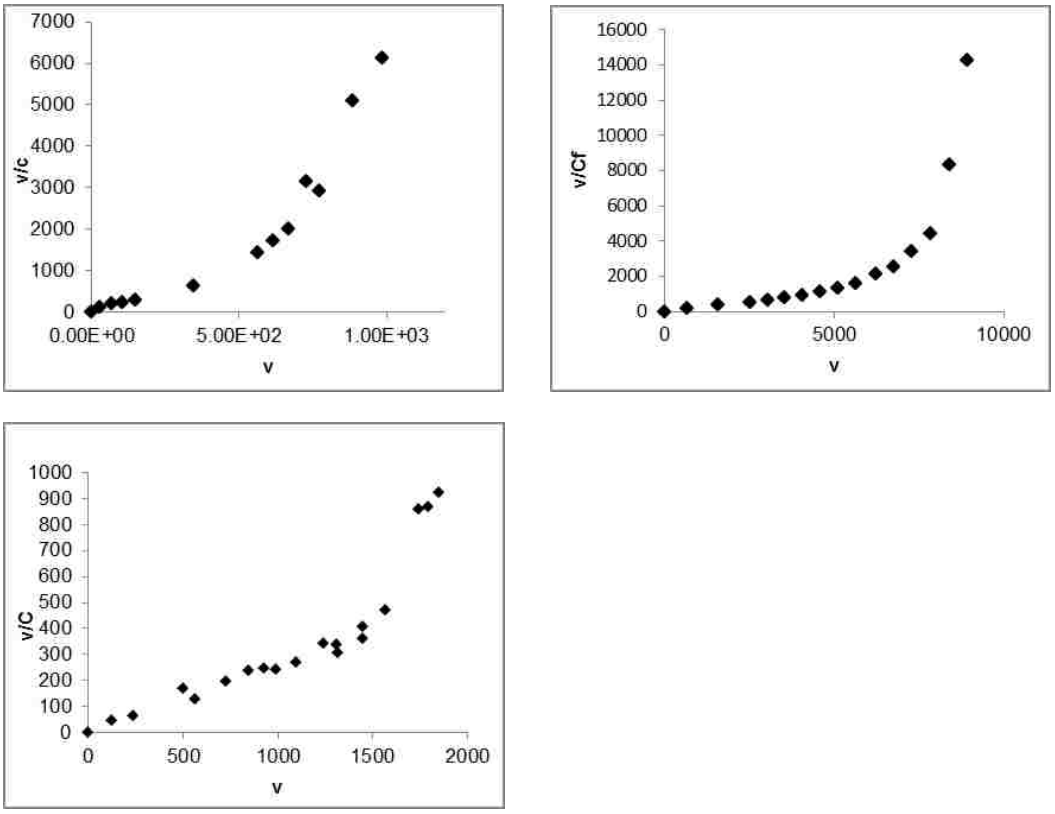


Figure 2.9. Scatchard plots of BSA with (A) $C_{11}PBr$, (B) C_8PBr, (C) C_4PBr

Scatchard analysis (Fig. 2.9., Table 2.2.) revealed that ILS produce a highly cooperative binding mechanism in low concentration regions. The binding mechanism of ILS to BSA is in direct opposition to what has been reported earlier for conventional anionic surfactant SDS.[70] The concave downward Scatchard plot revealed that $C_{11}PBr$ shows positive cooperative binding

with BSA in the low concentration region (0-1 mM) (Figure 2.9.), while SDS showed negative cooperative binding with BSA in a similar low concentration region.

Below 2 mM, C_{11}PBr showed positive cooperative binding, which revealed that binding of ILS to one site on BSA increases the C_{11}PBr binding affinity to subsequent sites of the protein.

Table 2.2. Types of cooperative binding for ILS in the low concentration region

ILS	Regions	BSA
C_{11}PBr	Region1 Region2 Region3 Region4	sp, + + - n.sp
C_8PBr	Region1 Region2 Region3 Region4	sp - n.sp
C_4PBr	Region1 Region2 Region3 Region4	sp + - n.sp

Sp=specific binding, + = positive cooperative binding, - = negative cooperative binding, n.sp= nonspecific binding.

Above 2 mM, C_{11}PBr exhibited negative cooperative binding which revealed that in this concentration region, BSA was saturated with ILS and thus binding at one site lowers the binding affinity at adjacent site. At higher concentrations (3.4-5.4 mM), SDS showed positive cooperative binding followed by a linear region characteristic of non-specific binding.[54] The highest binding of SDS with BSA occurs in this region (4.1-5.4 mM) because of micellization. At higher concentrations (7-15 mM), C_{11}PBr exhibited nonspecific binding which revealed that with increasing surfactant concentration, BSA saturated with ILS, no further binding occurs, and micelles co-exist with saturated protein.[71] Summarizing the results from intrinsic fluorescence data for all ILS studied for BSA, it is apparent that C_{11}PBr binding to protein is more cooperative than C_8PBr and C_4PBr at lower concentrations. We attribute this phenomenon to hydrophobicity

(hydrophobic domain) that exists in C_{11}PBr more than C_8PBr and C_4PBr. Therefore for BSA, binding occurs primarily through hydrophobic interactions.

2.3.7. Protein Denaturation Monitored by CD Studies

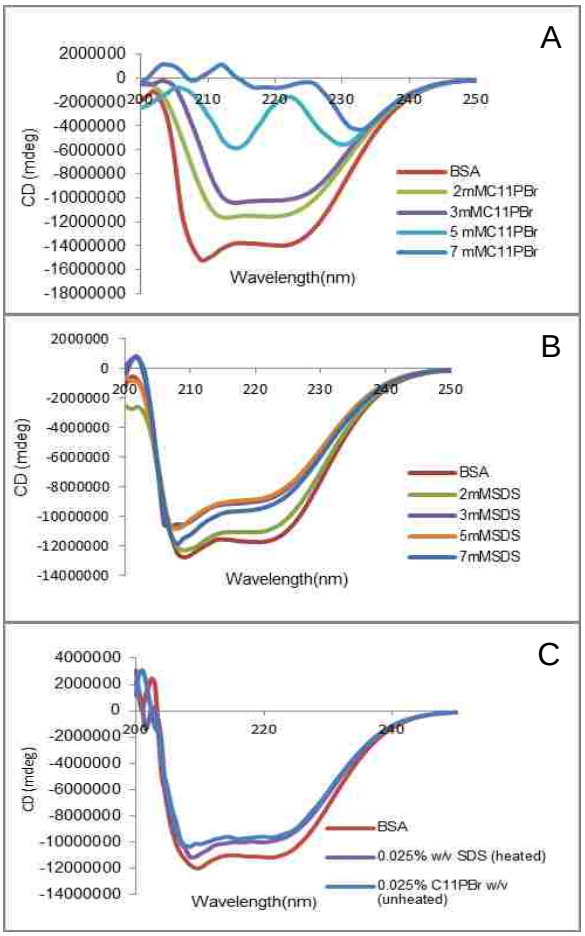


Figure 2.10. The CD spectra of BSA (10 μM) with increasing concentration of C_{11}PBr (A) SDS (B), in the presence of heated sample of 0.025% (w/v) (0.86 mM) SDS and unheated 0.025% (w/v) (0.86 mM) C_{11}PBr (C). The buffer was 25mM Tris/192 mM Glycine at pH 8.4 and 25^{o}C.

In order to investigate the influence of ILS (C_{11}PBr) on protein conformation as compared to the conventional surfactant SDS, CD spectra of BSA in the absence and presence of C_{11}PBr were obtained from 200-250 nm (Fig. 2.10). CD spectrum of BSA consists of two negative bands in the ultraviolet region at 208 (π-π* transition) and 222 nm (n-π* transition), which is a

characteristic of α-helical structure of a protein.[75] In this study, we found that interaction of C_{11}PBr with BSA caused a dramatic increase in band intensity at all wavelengths of far UV CD with a significant shift in peak position (Fig. 2.10A). This clearly indicates that there was a significant conformational change occurring and BSA begins to unfold.[76] The shift in CD signal for the 208 nm minimum at 2 mM suggests a decrease in α-helical content in the presence of C_{11}PBr. The change in secondary structure of BSA is quite significant as the ellipticity increases at both wavelengths (208, 222 nm) with increasing concentration of IL from 0-7 mM (Fig. 2.10A). In the case of SDS (Fig. 2.10B), there was a small increase in ellipticity from 0-5 mM concentration, which suggests that SDS did not appreciably change the conformation of BSA. The results clearly indicates major changes in the protein structure, namely a significant decrease in α-helical content in protein. This may be caused by interactions between the IL and BSA, which leads to a swelling of biomacromolecule and exposing of hydrophobic residues.[77] Thus, some of the original α-helices are broken to give a more open disordered structure.

One of the denaturation processes of proteins in standard SDS-PAGE is to heat the sample buffer at 95 $^{\circ}$C for 5 minutes. However, in ILS-PAGE, it was observed that IL itself denature the protein without heating, and this was confirmed by CD studies. The CD spectra in Fig. 2.10C showed that there is an increase in ellipticity along with a significant shift at 208 nm for unheated 0.86 mM (0.025% (w/v)) C_{11}PBr. The heated 0.86 mM (0.025% (w/v)) SDS showed a smaller increase in ellipticity with no shift at 208 nM. The above results suggest that C_{11}PBr denatured the protein at lower concentration without heating as compared to the conventional surfactant, which required heating of sample buffer.

2.4. Conclusions

In this chapter, an ILS-PAGE method is established for the separation of low and higher molecular weight, acidic proteins, using C_nPBr (n=4, 8, 11) as buffer additives in both sample and running buffers. The effects of ILS with four, eight, and eleven carbons have been evaluated. Better separation was achieved with shorter carbon chain (C4, C8) ILS at lower concentration. The longer carbon chain (C11) ILS at higher concentration gave poor separation. The stronger hydrophobic interactions between longer carbon chain ILS and protein could distruct the protein-ILS complex, which would deteriorate the separation of proteins. This method improves separation and resolution of transferrin and ovalbumin in comparison to ordinary SDS-PAGE. Overall resolution of proteins in ILS-PAGE is improved even though the migration distance of α-Lact decreases. However, this can be improved by optimization of other separation parameters (e.g., buffer compositions, voltage of instrument, and running time of gel). The experimental data obtained from intrinsic fluorescence studies showed that binding of ILS to protein is more cooperative at low concentration, as compared to the mostly negative cooperative binding with SDS. In addition, CD studies revealed that ILS denature the protein without heating. Further studies are underway in our laboratory to explore the applications of these ILS and their molecular micelles for separation of hydrophobic proteins.

2.5. References

1. Chen, C. H. *Analytica chimica acta* **2008**, *624*, 16.
2. Wang, H.; He, Y.; He, X.; Li, W.; Chen, L.; Zhang, Y. *Journal of separation science* **2009**, *32*, 1981.
3. D'Attoma, A.; Heinisch, S. *Journal of Chromatography A* **2013**, *1306*, 27.
4. Yang, G.; Zhang, X.; Lei, H.; Niu, W.; Bai, L. *Analytical Letters* **2013**, *46*, 1477.

5. Wagner, K.; Racaityte, K.; Unger, K.; Miliotis, T.; Edholm, L.; Bischoff, R.; Marko-Varga, G. *Journal of Chromatography A* **2000**, *893*, 293.

6. Chen, X.; Ge, Y. *PROTEOMICS* **2013**, *13*, 2563.

7. Wu, R.; Wang, Z.; Zhao, W.; Yeung, W. S.; Fung, Y. S. *Journal of chromatography. A* **2013**, *1304*, 220.

8. IKI, N.; YEUNG, E. *Journal of chromatography. A* **1996**, *731*, 273.

9. Albarghouthi, M. N.; Stein, T. M.; Barron, A. E. *Electrophoresis* **2003**, *24*, 1166.

10. Dodd, D. W.; Hudson, R. H. *Electrophoresis* **2007**, *28*, 3884.

11. Schägger, H.; Von Jagow, G. *Analytical biochemistry* **1987**, *166*, 368.

12. Wang, W.; Lu, J. J.; Gu, C.; Zhou, L.; Liu, S. *Analytical chemistry* **2013**, *85*, 6603.

13. Hsieh, J.-F.; Chen, S.-T. *Biomicrofluidics* **2007**, *1*, 014102.

14. Burre, J.; Beckhaus, T.; Schagger, H.; Corvey, C.; Hofmann, S.; Karas, M.; Zimmermann, H.; Volknandt, W. *Proteomics* **2006**, *6*, 6250.

15. Li, X.; Cao, J.; Jin, Q.; Xie, C.; He, Q.; Cao, R.; Xiong, J.; Chen, P.; Wang, X.; Liang, S. *Journal of cellular biochemistry* **2008**, *104*, 965.

16. Yang, X.; Clifton, J.; Huang, F.; Kovac, S.; Hixson, D. C.; Josic, D. *Electrophoresis* **2009**, *30*, 1185.

17. Zgoda, V. G.; Moshkovskii, S. A.; Ponomarenko, E. A.; Andreewski, T. V.; Kopylov, A. T.; Tikhonova, O. V.; Melnik, S. A.; Lisitsa, A. V.; Archakov, A. I. *Proteomics* **2009**, *9*, 4102.

18. Addis, M. F.; Tanca, A.; Pagnozzi, D.; Rocca, S.; Uzzau, S. *Proteomics* **2009**, *9*, 4329.

19. Wittig, I.; Braun, H.-P.; Schägger, H. *Nature Protocols* **2006**, *1*, 418.

20. Aivaliotis, M.; Karas, M.; Tsiotis, G. *Journal of proteome research* **2007**, *6*, 1048.

21. Arndt, C.; Koristka, S.; Bartsch, H.; Bachmann, M. In *Protein Electrophoresis*; Springer: 2012, p 49.

22. Rothe, G. M.; Purkhanbaba, H. *Electrophoresis* **1982**, *3*, 43.

23. Ghosh, S. *Colloids and Surfaces B: Biointerfaces* **2008**, *66*, 178.

24. Ghosh, S. *Journal of Colloid and Interface Science* **2001**, *244*, 128.

25. Laemmli, U. K. *Nature* **1970**, *227*, 680

26. Jungblut, P.; Thiede, B.; Zimny-Arndt, U.; Müller, E.; Scheler, C.; Wittmann-Liebold, B.; Otto, A. *Electrophoresis* **1996**, *17*, 839.

27. Shapiro, A. L.; Vinuela, E.; Maizel, J. V., Jr. *Biochemical and biophysical research communications* **1967**, *28*, 815.

28. Reynolds, J. A.; Tanford, C. *Proceedings of the National Academy of Sciences* **1970**, *66*, 1002.

29. Malhotra, M.; Sahal, D. *International journal of peptide and protein research* **1996**, *48*, 240.

30. Allen, R. C.; Saravis, C. A.; Maurer, H. R. *Gel electrophoresis and isoelectric focusing of proteins*; de Gruyter Berlin, 1984.

31. Margulies, M. M.; Tiffany, H. L. *Analytical biochemistry* **1984**, *136*, 309.

32. Andrews, A. T. *Electrophoresis: theory, techniques, and biochemical and clinical applications*; Clarenden Press, 1986.

33. Hames, B. D.; Rickwood, D. *Gel electrophoresis of proteins: a practical approach*; IRL Press, 1981.

34. Ross, A. R.; Lee, P. J.; Smith, D. L.; Langridge, J. I.; Whetton, A. D.; Gaskell, S. J. *Proteomics* **2002**, *2*, 928.

35. Furthmayr, H.; Timpl, R. *Analytical biochemistry* **1971**, *41*, 510.

36. Cannon-Carlson, S.; Tang, J. *Analytical biochemistry* **1997**, *246*, 146.

37. Zhang, N.; Li, L. *Analytical chemistry* **2002**, *74*, 1729.

38. Zardoya, R.; Diez, A.; Mason, P. J.; Luzzatto, L.; Garrido-Pertierra, A.; Bautista, J. M. *BioTechniques* **1994**, *16*, 270.

39. Krossing, I.; Slattery, J. M.; Daguenet, C.; Dyson, P. J.; Oleinikova, A.; Weingärtner, H. *Journal of the American Chemical Society* **2006**, *128*, 13427.

40. van Rantwijk, F.; Sheldon, R. A. *Chemical reviews* **2007**, *107*, 2757.

41. Shu, Y.; Chen, X.-W.; Wang, J.-H. *Talanta* **2010**, *81*, 637.

42. Ge, L.; Wang, X.-T.; Tan, S. N.; Tsai, H. H.; Yong, J. W.; Hua, L. *Talanta* **2010**, *81*, 1861.

43. Qiu, H.; Zhang, Q.; Chen, L.; Liu, X.; Jiang, S. *Journal of separation science* **2008**, *31*, 2791.

44. Chitta, K. R.; Van Meter, D. S.; Stalcup, A. M. *Analytical and bioanalytical chemistry* **2010**, *396*, 775.

45. Wang, Y.; Tian, M.; Bi, W.; Row, K. H. *International journal of molecular sciences* **2009**, *10*, 2591.

46. Zhang, M.; Liang, X.; Jiang, S.; Qiu, H. *TrAC Trends in Analytical Chemistry* **2014**, *53*, 60.

47. Buszewski, B.; Studzińska, S. *Chromatographia* **2008**, *68*, 1.

48. Corradini, D.; Nicoletti, I.; Bonn, G. K. *Electrophoresis* **2009**, *30*, 1869.

49. Wu, X.; Wei, W.; Su, Q.; Xu, L.; Chen, G. *Electrophoresis* **2008**, *29*, 2356.

50. Kašička, V. *Electrophoresis* **2010**, *31*, 122.

51. Tang, S.; Liu, S.; Guo, Y.; Liu, X.; Jiang, S. *Journal of Chromatography A* **2014**, *1357*, 147.

52. Joshi, M. D.; Anderson, J. L. *Rsc Advances* **2012**, *2*, 5470.

53. Hu, Y.-J.; Liu, Y.; Pi, Z.-B.; Qu, S.-S. *Bioorganic & medicinal chemistry* **2005**, *13*, 6609.

54. Notides, A. C.; Lerner, N.; Hamilton, D. E. *Proceedings of the National Academy of Sciences* **1981**, *78*, 4926.

55. Perlmutter-Hayman, B. *Accounts of Chemical Research* **1986**, *19*, 90.

56. Zana, R.; Benrraou, M.; Rueff, R. *Langmuir* **1991**, *7*, 1072.

57. Yan, H.; Wu, J.; Dai, G.; Zhong, A.; Chen, H.; Yang, J.; Han, D. *Journal of Luminescence* **2012**, *132*, 622.

58. Rosen, M. J.; Kunjappu, J. T. *Surfactants and interfacial phenomena*; John Wiley & Sons, 2012.

59. Görögh, T.; Rudolph, P.; Meyer, J. E.; Werner, J. A.; Lippert, B. M.; Maune, S. *Clinical chemistry* **2005**, *51*, 1704.

60. Babcock, J.; Brancaleon, L. *International journal of biological macromolecules* **2012**.

61. Covaciu, M.; Olaru, F.; Petrescu, I. *OVALBUMIN ISOFORMS ¬PURIFICATION AND DENATURATION / RENATURATION STUDIES*, 2004; Vol. 5.

62. Aydemir, T.; Kuru, K. *Turkish Journal of Chemistry* **2003**, *27*, 85.

63. Walicke, P.; Varon, S.; Manthrope, M. *The Journal of neuroscience* **1986**, *6*, 1114.

64. Geng, F.; Zheng, L.; Yu, L.; Li, G.; Tung, C. *Process biochemistry* **2010**, *45*, 306.

65. James, T. L.; Milos, N. C. *Analytical Biochemistry* **2007**, *367*, 134.

66. Lacher, N. A.; Wang, Q.; Roberts, R. K.; Holovics, H. J.; Aykent, S.; Schlittler, M. R.; Thompson, M. R.; Demarest, C. W. *Electrophoresis* **2010**, *31*, 448.

67. Ramos, Y.; Gutierrez, E.; Machado, Y.; Sánchez, A.; Castellanos-Serra, L.; González, L. J.; Fernández-de-Cossio, J.; Pérez-Riverol, Y.; Betancourt, L.; Gil, J. *Journal of proteome research* **2008**, *7*, 2427.

68. Jiang, T.-F.; Gu, Y.-L.; Liang, B.; Li, J.-B.; Shi, Y.-P.; Ou, Q.-Y. *Analytica chimica acta* **2003**, *479*, 249.

69. Ojha, B.; Das, G. *The Journal of Physical Chemistry B* **2010**, *114*, 3979.

70. Das, S.; Sylvain, M. R.; Fernand, V. E.; Losso, J. N.; El-Zahab, B.; Warner, I. M. *Journal of colloid and interface science* **2011**, *363*, 585.

71. Gelamo, E.; Silva, C.; Imasato, H.; Tabak, M. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology* **2002**, *1594*, 84.

72. Chakraborty, T.; Chakraborty, I.; Moulik, S. P.; Ghosh, S. *Langmuir* **2009**, *25*, 3062.

73. Melander, W.; Horváth, C. *Archives of biochemistry and biophysics* **1977**, *183*, 200.

74. Ercolani, G. *Journal of the American Chemical Society* **2003**, *125*, 16097.

75. Gull, N.; Chodankar, S.; Aswal, V.; Sen, P.; Khan, R. H. *Colloids and Surfaces B: Biointerfaces* **2009**, *69*, 122.

76. Peyre, V.; Lair, V.; André, V.; le Maire, G.; Kragh-Hansen, U.; le Maire, M.; Møller, J. V. *Langmuir* **2005**, *21*, 8865.

77. Nielsen, A. D.; Borch, K.; Westh, P. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology* **2000**, *1479*, 321.

CHAPTER 3: CATIONIC IONIC LIQUID SURFACTANT-POLYACRYLAMIDE GEL ELECTROPHORESIS FOR ENHANCED SEPARATION OF ACIDIC AND BASIC PROTEINS WITH SINGLE-STEP RIBONUCLEASE B GLYCOFORMS SEPARATION

3.1. Introduction

Polyacrylamide gel electrophoresis (PAGE) has long been a major tool for electrophoretic mobility-based protein separation.[1,2,3,4,5] In this technique, ionic surfactants are employed; these surfactants bind to proteins and usually cause protein unfolding. Anionic PAGE [6,7,8] and cationic PAGE [9,10,11] are two types of PAGE techniques that depend on the type of surfactant, i.e. anionic and cationic respectively, buffer pH, and the direction of electrophoretic mobility.

The most widely used PAGE technique — sodium dodecyl sulfate (SDS)-PAGE is anion-based; SDS is an anionic surfactant. SDS-PAGE has been extensively used for mass-based protein separations for the past several decades.[12,13,14,15] In this technique, SDS-treated protein samples are boiled and then loaded onto SDS-based polyacrylamide gels, while employing a needed voltage for electrophoresis. SDS has been shown to homogeneously bind to most proteins, thus conferring the same negative charge to all surfactant-protein complexes. Therefore, upon application of voltage, these protein complexes migrate from cathode to anode, and the distance of migration is dependent primarily on the mass of proteins. This is because the mass of the complexed SDS molecules is negligible compared to the protein mass.

The SDS-PAGE technique is the primary technique for protein separation. However, many examples of anomalous protein migration patterns have been reported, particularly for membrane protein separations.[16,17,18] These problems may arise due to differential surfactant binding to hydrophobic domains in more complex membrane proteins.[19,18, 20] The amount of SDS actually interacting with every amino acid residue within a given protein is greatly affected by the

presence of inter/intra-molecular bonds (characteristic of the protein tertiary structure). For example, intact disulfide bonds in lysozyme limit SDS binding to this protein.[21] In such a non-reduced state, this protein is more compact and migration in the electric field is anomalously fast. In contrast, a higher number of SDS molecules bind to reduced lysozyme and the completely denatured protein migrates slower due to less compactness and loss of the tertiary structure. Similar to non-reduced lysozyme proteins, complex membrane proteins with intact hydrophobic interactions tend to bind less amount of SDS. As a result, they migrate faster than the calibration standards.

It should be noted that glycoproteins are commonly present in membrane protein fractions and that SDS-PAGE is not an efficient separation technique for such proteins. Separation of glycoproteins by use of SDS-PAGE often yields broad and fuzzy bands due to extensive negative charges on their carbohydrate side chains.[22,20] This significantly reduces the sensitivity and resolution of the technique. Similarly, subunits of post-translationally modified protease complexes are not visualized using SDS-PAGE. However, such systems are observed to be well resolved in cetyltrimethyl ammonium bromide (CTAB)-PAGE, a cationic PAGE technique.[9] This difference in separation has been ascribed to incompatibility between highly negatively charged hydrophobic protein subunits in anionic PAGE. Therefore, use of cationic surfactants, which can neutralize excess negative charges on proteins, has been suggested for overcoming this deficiency.[22]

Cationic PAGE depends on cationic surfactants binding to proteins and forming positively charged surfactant-protein complexes often at low pH values. In contrast to SDS-PAGE, these complexes migrate from anode to cathode in an electric field. In addition to CTAB, [9-10, 22] numerous other cationic surfactants have been suggested for use in cationic PAGE, including

benzyldimethyl-n-hexadecylammonium chloride (16-BAC),[23,24] tetradecyltrimethylammonium bromide,[25] and cetylpyridinium chloride.[26]

Cationic surfactants have mostly been used to separate membrane proteins with post-translational modifications, more commonly, glycoproteins.[22,23,9] Glycoproteins often exist as variable glycosylated populations of the same protein, which are known as, glycoforms. Glycoform analysis is important as the attached glycans may control protein function and conformation. These different glycoforms may reflect the cell environment, physiological conditions, as well as disease states.[27,28] Thus far, none of the one dimensional (1D)-PAGE techniques have been able to separate glycoforms into species with distinct migration values. SDS- PAGE (1D) generally separates glycoproteins into broad bands due to protein heterogeneity.[29] As a result, further analyses of proteins present in those bands are often required. Typically, this process involves releasing glycans using in-gel deglycosylation followed by glycan profiling using mass spectrometry.[20,27,29] An isoelectric focusing (IEF)/SDS-PAGE (2D) technique has been used to reveal protein separations due to different pI and/or masses of glycoforms.[30] However, this technique often under-represents the amount of total proteins as a result of the low solubility power of the non-ionic and zwitterionic surfactants employed in IEF. Therefore, surfactants with strong solubility power as well as high resolving power in 1D-PAGE are desired in order to overcome those limitations, especially, for glycoform separations. In the study outlined in this manuscript, we demonstrate that certain ionic liquid surfactants (defined as surfactants that would be liquid in pure form) can effectively separate ribonuclease b glycoforms into multiple bands at room temperature.

Ionic liquid surfactants (ILS) are amphiphilic organic salts with a cation and an anion that have melting points below 100 °C in the pure state. This class of materials exhibits various

tunable physicochemical properties, which make them ideal candidates for analytical applications where conventional surfactants are less suitable or not applicable. High conductivity, non-volatility, non-flammability, tunable hydrophobicity, and high chemical stability are some of the properties of ILS, which make them suitable for PAGE applications. In a previous study, we have used *N*-alkyl-4-methylpyridinium bromide ILS, 1-butene-4-methylpyridinium bromide (C_4PBr), 1-octene-4-methylpyridinium bromide (C_8PBr), and 1-undecene-4-methylpyridinium bromide (C_{11}PBr) as buffer additives in the sample and running buffers for anionic PAGE separation of a mixture of acidic proteins.[31] Observation of high resolving power of the technique, protein binding characteristics, and protein denaturation ability by use of ILS suggested their further applications in protein separation.

Herein, we report the development of a cationic PAGE technique using the three ILS. The application described here is unique and truly impactful for protein separations. In this study, these ILS were employed in three components of the PAGE separation, i.e. the discontinuous polyacrylamide gels, sample and running buffers. The optimization of concentrations of the ILS in those three components is also described. A major advantage of this approach as compared to the previous study is use of ILS to separate both acidic and basic proteins with a wide range of molecular weights. Most importantly, a one-step separation of Rib b glycoforms is achieved using this cationic ILS-PAGE approach. To the best of our knowledge, this is the first report of successful separation of Rib b glycoforms into multiple discrete bands using a 1D-PAGE technique.

3.2. Materials and Methods

3.2.1. Materials

Four (4)-Picoline, 4-Bromo-1-butene, 8-Bromo-1-octene, 11-Bromo-1-undecene, bovine serum albumin (BSA), β-casein (β-Cas), myoglobin (Myo), ribonuclease b (Rib b), ovalbumin (Ova), α-chymotrypsinogen (α-Chy), cytochrome c (Cyt c), CTAB, tris, glycine, urea, potassium dihydrogen phosphate, potassium monohydrogen phosphate, ascorbic acid, $FeSO_4$, pyronine Y, and α-cyanohydroxycinnamic acid (CHCA) were purchased from Sigma Aldrich (Milwaukee, WI). SDS, acrylamide/bisacrylamide (37.5:1) (12.5%), methylene bis acrylamide (MBA), 2-mercaptoethanol, IEF reagents, immobilized pH gradient (IPG) gels, 2D electrophoresis precast gels, and Bio-safe Coomassie Blue were obtained from Bio-Rad Laboratories, Inc. (Hercules, Ca). Hydrogen peroxide (30% solution) and phosphoric acid were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ).

3.2.2. Ionic Liquid Surfactants Synthesis

C_nPBr (n=4,8,11) ILS were synthesized and characterized according to our previous procedure.[31] In brief, 4-Picoline (7g, 0.075 M) and n-Bromo-1-alkene, where n=4, 8, 11 (0.02 eq, 10 g, 0.074 M) were separately reacted by refluxing at 80 °C overnight. Pure products, yellow color viscous liquids, were obtained under the specified conditions and characterized by use of ESI-MS.

3.2.3. Instrumentation

Discontinuous polyacrylamide mini gels were freshly prepared by casting a stacking gel on top of a resolving gel. For this purpose, Bio-Rad short and spacer glass plates (with integrated

1.00 mm spacers) and 10 well combs were used. Polyacrylamide gel electrophoresis was performed by use of a Bio-Rad Mini-PROTEAN® Tetra vertical electrophoresis system (Hercules, CA, USA). Electrophoresis was started at 200 V, 25 mA and continued for 10 minutes until proteins were first entered the resolving gel. Then, proteins were separated at 300 V, 80 mA for 50 minutes or until the tracking dye reached the bottom of the gel. Gel images were captured using a Kodak Gel Logic 200 Image Analyzer (Rochester, NY).

MALDI-MS analyses were performed by use of a Bruker UltrafleXtreme MALDI-time of flight (TOF) mass spectrometer with an Nd:YAG laser (355 nm wavelength and 3 ns pulse duration). Mass spectra were acquired using positive ion linear mode at 25 kV acceleration potential and analyzed by use of Bruker FlexAnalysis 3.3 software.

3.2.4. Sample Buffer Preparation for ILS-PAGE

Urea (450 mg) was added to a mixture of glycerol (100 µL), ILS (0.02%-2% (w/v)), ultrapure water (400 µL), and pyronine Y tracking dye (5% (w/v), 20 µL), which then vortexed for 1 minute. The buffer was stored at room temperature.

3.2.5. Protein Sample Preparation for ILS-PAGE

Individual protein samples (1 mg/mL) and protein mixtures (1 mg/mL of each protein) were freshly prepared by dissolving appropriate amounts of proteins in ultrapure water. The protein sample (2.5 µL), 2-mercaptoethanol (2.5 µL) and sample buffer (47.5 µL) were mixed together to prepare a protein sample buffer for application in gel electrophoresis.

3.2.6. Running Buffer Preparation for ILS-PAGE

ILS (0.02%-0.1% (w/v)) was added to glycine (150 mM) and phosphoric acid (50 mM) to prepare 500 ml of running buffer (pH 3).

3.2.7. ILS Discontinuous Gel Preparation

The resolving gel (12.5% (w/v), 5 mL) was made first by preparing a solution of urea (3 M), phosphate buffer (1.25 mL, pH 2.1), ILs (0.02%-0.5% (w/v)), acrylamide/bisacrylamide (12.5% (w/v)), MBA (2% (w/v), 0.150 mL), ascorbic acid (4 mM), and $FeSO_4$ (8 µM). Then H_2O_2 (0.001% (w/v)) was added to initiate the polymerization. The solution was vortexed, quickly poured into the assembled glass plates, and allowed to polymerize for about 20 minutes. Next, the stacking gel solution (2.5 mL) containing urea (3 M), phosphate buffer (0.625 mL, pH 4.1), ILS (0.02%-0.5% (w/v)), acrylamide/bisacrylamide (4.00% (w/v)), MBA (2% (w/v), 0.293 mL), ascorbic acid (4 mM), and $FeSO_4$ (8 µM) was prepared. Then, H_2O_2 (0.002% (w/v)) was added, the solution vortexed, and poured onto the resolving gel. The comb was inserted quickly and the gel was allowed to polymerize overnight.

3.2.8. Cationic ILS-PAGE Experiment

The gel was assembled into the gel box, the running buffer was poured into the buffer tank and the gel box with assembled gel. Protein samples (15 µL) were loaded into the wells at room temperature. The system was closed and electrodes connected such that proteins migrated from anode to cathode. After completion of electrophoresis, the gel was fixed with gel fixation solution (methanol: acetic acid: water-4:1:5), maintained on a shaker for 1 hour, followed with changes in the fixation solution every 15 minutes. After that, the gel was stained with Bio-safe

Coomassie Blue for 1 hour on the shaker. Finally, gel images were captured after washing with deionized water for at least 1 hour.

3.2.9. Anionic SDS-PAGE

SDS-PAGE gel (15% (w/v)) was casted and assembled into the gel-running tray already containing 1x Laemmli buffer (running buffer). Each protein sample (15 µL) was loaded into each well after boiling sample buffer containing SDS at 95 °C. The samples were then allowed to separate from cathode to anode, using a voltage of 200 V for 50 minutes. After completion of electrophoresis, the gel was carefully rinsed with distilled water and stained using Bio-Safe Coomassie Blue for about 30 minutes, after which it was destained with a destaining solution (Glacial Acetic Acid and Methanol). Finally, gel images were captured after re-rinsing the gels in distilled water.

3.2.10. MALDI-MS Analysis of Ribonuclease b Protein Bands

A separate cationic ILS-PAGE experiment was performed to analyze Rib b protein bands. The same amount (15 µL) of protein sample was loaded into all 10 wells of the gel and the separation was performed using C_4PBr ILS (0.02% (w/v) in sample buffer, 0.05% (w/v) in gel, and 0.05% (w/v) in running buffer). After completion of electrophoresis run, the gel was vertically divided into two parts, i.e., five protein lanes in each. One part of the gel was stained with coomassie blue, while the other part was used for MALDI-MS analysis without staining to avoid interferences from the stain. The two distinct bands of Rib b protein on the stained gel piece were used as a reference to determine the corresponding position of the two protein bands on the unstained gel. The two parts were aligned to form a complete gel, and then two narrow horizontal excisions on the unstained part of the gel were performed at positions matching the

protein bands on the stained part. The two horizontal gel parts were cut into small pieces and placed in two separate eppendorf tubes. A saturated CHCA solution in TFA (1% (v/v)) and acetonitrile (60% (v/v)) was added to each tube using a sufficient volume to cover all the gel parts. These tubes were then vortexed at a high speed overnight. Finally, the matrix solution which contained extracted proteins was retrieved and analyzed by use of MALDI-MS.

3.2.11. Two Dimensional IEF/SDS PAGE Analysis of Ribonuclease b Protein

The first dimension, IEF, was performed as described below. A lyophilized powder of Rib b protein (5 mg) was dissolved in 2 mL of rehydration buffer containing 8 M urea, 2% (w/v) CHAPS, 50 mM dithiothreitol (DTT), 0.2% (w/v) Bio-Lyte® 3/10 ampholytes, and a trace amount of bromophenol blue. The sample was diluted five times with rehydration buffer and 625 µL was applied into a focusing tray. An IPG gel strip (pH 3-10) was placed in the tray maintaining the gel side down. Care was taken not to introduce air bubbles. After that, mineral oil (1 mL) was overlaid to avoid evaporation of the rehydration buffer. After 12 hours of rehydration, focusing was performed in two steps. The first step was 100 V for 2 hours, followed by 200 V for 1 hour.

After completion of IEF, the gel strip was removed from the tray and excess oil was removed by vertically holding up the strip. Then, the strip was placed in a disposable equilibration tray maintaining gel side up. Equilibration buffer (2 mL) containing 6 M urea, 2% (w/v) SDS, 0.375 M tris-HCl (pH 8.8), 20% (w/v) glycerol, and 2% (w/v) DTT was added to the tray containing the gel strip and the tray was placed on an orbital shaker for gentle shake. After 10 minutes, the buffer was carefully drained. Fresh equilibration buffer (2 mL) with 6 M urea, 2% (w/v) SDS, 0.375 M tris-HCl (pH 8.8), 20% (w/v) glycerol, and 3.75% (w/v) iodoacetamide was added to

the tray, which was placed on the orbital shaker for another 10 minutes to complete the equilibration.

After the equilibration, the gel strip was removed and briefly dipped in a tris-glycine-SDS running buffer. The gel strip was then laid gel side up on the back plate above the IPG well of the 2D electrophoresis (2DE) precast gel. Then, the 2DE gel was vertically placed in a test tube rack, while the gel strip was still resting on the back plate. A low melting point agarose sealing solution was pipetted into the IPG well in order to overlay on top of the 2DE gel. After that, the gel strip was pushed down into the well with forceps. The 2DE gel containing the IPG strip was mounted in the gel box of the electrophoresis system to perform anionic PAGE at 200 V for 40 minutes. After completion of electrophoresis, the gel was removed and washed with deionized water for 15 minutes with three changes every 5 minutes. Then the gel was stained with Bio-Safe Coomassie Blue for 1 hour and then de-stained with deionized water for at least 1 hour before capturing the gel image.

3.3. Results and Discussion

3.3.1. Optimization of Ionic Liquid Surfactant Concentration in Cationic PAGE

The three ILS used in this study have a common cationic head group (4-methyl pyridinium) but, three different alkyl chain lengths (C4, C8, and C11) as shown in Figure 3.1(A). ILS were applied in polyacrylamide gels, protein sample buffers, and electrophoresis running buffer in ILS-PAGE experiments. As the first step, concentrations of ILS in polyacrylamide gels were optimized. Then, the concentrations in sample and running buffers, and the effects of the ILS alkyl chain length were evaluated by comparing three different ILS-PAGE techniques.

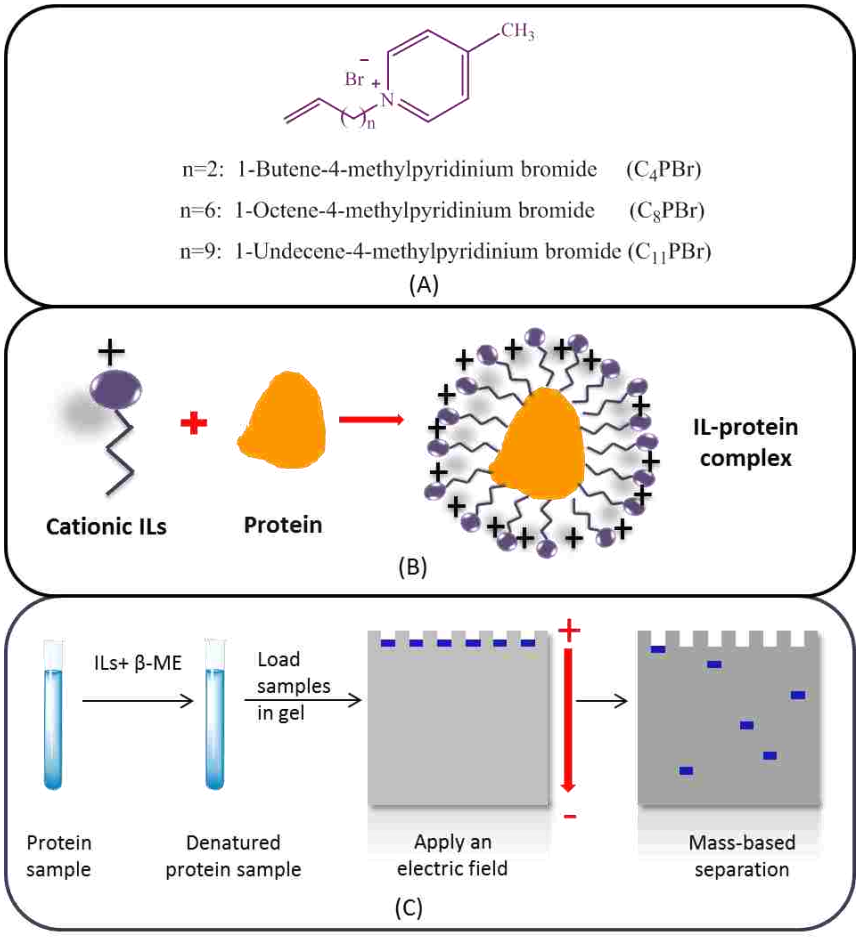


Figure 3.1. Cationic PAGE with 4-methylpyridinium-based ILS. (A) The generic structure of the ILS with 4-methyl pyridinium cation and variable alkyl chain lengths. (B) Formation of cationic ILS-protein complex upon interaction between ILS and protein. (C) Stepwise experiments in cationic ILS-PAGE

3.3.1. Optimization of ILS Concentration in Polyacrylamide Gels

Five different proteins, i.e., BSA, β-Cas, Myo, Rib b, and Cyt c with known molecular weights were used in order to demonstrate the effects of different concentrations of ILS in polyacrylamide gels for protein separation. Four different concentrations of each ILS were chosen (0.02%, 0.05%, 0.1%, and 0.5% (w/v)) in polyacrylamide gel preparation, while ILS concentration in sample buffer (0.02% (w/v)) and running buffer (0.05% (w/v)) were maintained constant. Of the three ILS studied, the best results were obtained when C_4PBr ILS was

employed, so the results obtained with this ILS were reported in this chapter. The data of the other two ILS tested were presented on the Appendix A (Figure S1). Figure 3.2 displays the electropherograms of protein migration patterns at different concentrations of ILS in polyacrylamide gels. An increase on the protein migration was observed when ILS concentrations varied from 0.02% (w/v) through 0.1% (w/v) although at 0.5% (w/v) the mobility was reduced. Sharp protein bands were obtained at low concentrations, 0.02% and 0.05% (w/v) of C_4PBr ILS. At all the concentrations, Rib b protein was resolved in two bands, this behavior is discussed in detail in section 3.3.2. High concentrations of ILS, 0.1% and 0.5% (w/v), affected band width and intensity as well as resulted in broad and distorted bands. In addition, at the highest concentration of ILS (0.5% (w/v)), protein migration was reduced.

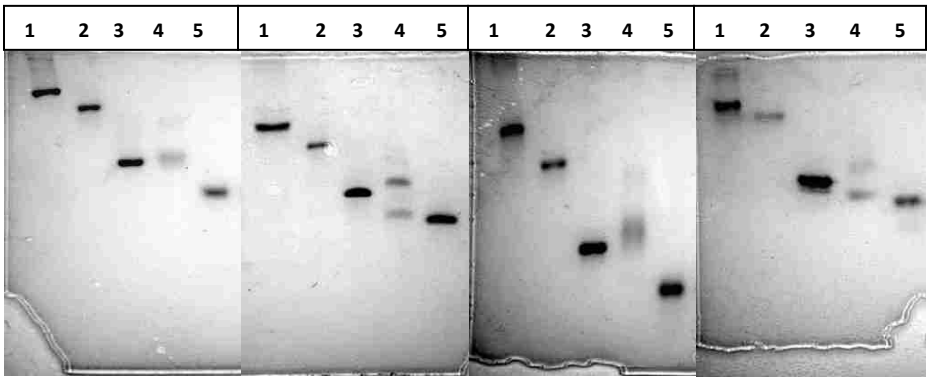


Figure 3.2. Polyacrylamide gel images obtained from C_4PBr-PAGE showing migrations of five proteins, (1) BSA (pI-4.8, 66 kDa), (2) β-cas (pI-5.13, 23.6 kDa), (3) Myo (pI-6.8, 16.9 kDa), (4) Rib b (pI-9.45, 14.7 kDa), and (5) Cyt c (pI-10, 12.3 kDa) at different concentrations, (A) 0.02%, (B) 0.05%, (C) 0.1%, and (D) 0.5%, (w/v) of C_4PBr ILS in gels. C_4PBr concentrations in sample buffer (0.02% (w/v)) and running buffer (0.05% (w/v)) were the same for all four gels.

A brief overview of the protein separation mechanism is described below in order to explain this behavior. ILS-protein complexes can be formed by binding proteins to ILS present in the sample buffer. These complexes may have overall positive charges as a result of the cationic

charges of the ILS (see Figure 3.1 (B)). Therefore, these cationic complexes will migrate from anode to cathode during cationic PAGE experiments (Figure 3.1(C)). Due to the presence of the 4-methylpyridinium cations of ILS in the gel, these ILS-protein complexes will be electrostatically stabilized while they are migrating. As a result, highly resolved sharp protein bands were obtained at 0.05% (w/v) of ILS in the gel as compared to the bands obtained at the other concentrations analyzed. As well as at 0.05% (w/v) of ILS, the electrophoretic mobility of proteins was increased in comparison with the 0.02% (w/v) concentration as a result of higher conductivity, without reducing the quality of separation (Figure 3.2. (A) and (B)). Although the electrophoretic mobility was further increased when the ILS concentration was 0.1% (w/v), the resolution of Rib b was reduced (lane 4 in Fig 3.2 (C)). At 0.5% (w/v), band diffusion occurred, perhaps due to excessive heating produced in gels with very high conductivity. At the same time, repulsive forces between cationic ILS-protein complexes and ILS present in gels (0.1% and 0.5% (w/v) ILS) may also hamper the resolution of protein bands. Considering all these effects, band diffusion and protein band resolution, 0.05% (w/v) ILS concentration was identified as the optimum concentration for use in our gels.

3.3.1.1. Optimization of Ionic Liquid Surfactants Concentration in Running Buffer and Sample Buffer using a Mixture of Proteins

The effects of ILS concentrations in running buffer and sample buffer were investigated using a mixture of five proteins; BSA (pI-4.8, 66 kDa), Ova (pI-4.6, 44.3 kDa), α-Chy (pI-8.8, 25.7 kDa), Myo (pI-16.9, 14.7 kDa), and Cyt c (pI-10, 12.3 kDa). (A different set of proteins with discrete molecular weights were used to minimize overlapping of protein bands.)

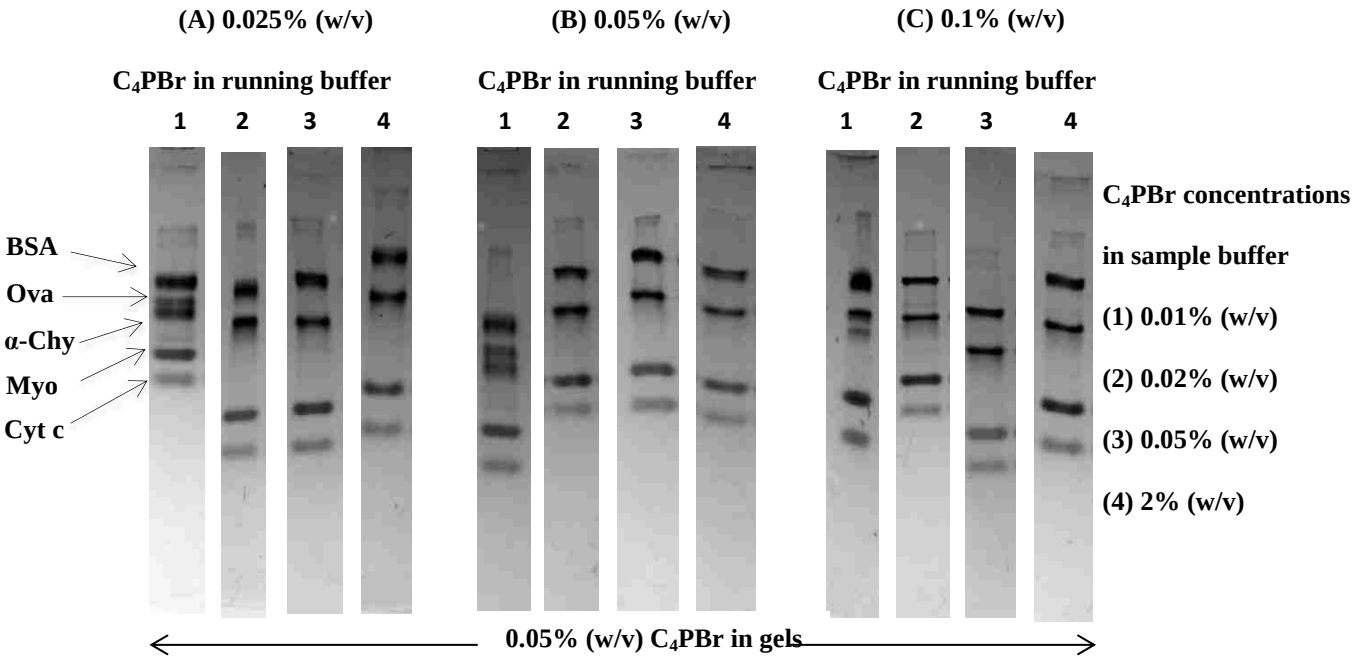


Figure 3.3**.** C_4PBr-PAGE separation of a mixture of five proteins; BSA (pI-4.8, 66 kDa), Ova (pI-4.6, 44.3 kDa), α-Chy (pI-8.8, 25.7 kDa), Myo (pI-16.9, 14.7 kDa), and Cyt c (pI-10, 12.3 kDa) from top to bottom in A-1, B-1, and C-1, using different C_4PBr running buffers; (A) 0.025% (w/v) (B) 0.05% (w/v), and (C) 0.1% (w/v) and sample buffers; (1) 0.01% (2) 0.02% (3) 0.05% (4) 2% (w/v) with 0.05% (w/v) C_4PBr gels. Note that Ova band disappears when high ILS concentrations were included in sample buffers using all three running buffers.

This protein mixture was separated by use of C_4PBr- PAGE and employing four different C_4PBr concentrations in the sample buffer, i.e., 0.01%, 0.02%, 0.05%, and 2% (w/v). The C_4PBr concentrations in the running buffer were varied from 0.025% to 0.1% (w/v). The optimum C_4PBr concentration in the gel, i.e., 0.05% (w/v), was used for all separations in this experiment. All five proteins showed single bands when the ILS concentration was 0.01% (w/v) in the sample buffer, regardless of the ILS concentration in the running buffer (Figure 3.3-A1, B1, and C1). Ova protein was not resolved when higher ILS concentrations were used in the sample buffer. Protein band resolution (compare Ova and α-Chy protein bands in A-1, B-1, and C-1 gel images in Fig. 3.3) and migration distances increased with increasing ILS concentration in the running buffer. This was due to higher ionic conductivity generated with higher ILS

concentrations, which aided separation. We concluded that the lowest concentration of ILS in the sample buffer (0.01% (w/v)) and the highest concentration in the running buffer (0.1% (w/v)) are the optimum conditions for separation of protein mixtures using ILS-PAGE.

3.3.1.2. Effects of Alkyl Chain Length in Ionic Liquid Surfactants

ILS with three different alkyl chain lengths, i.e., 4, 8, and 11 carbon atoms in C_4PBr, C_8PBr, and C_{11}PBr, respectively, were used for protein separation. ILS concentration in gels, sample buffer, and running buffer were separately optimized for each employed ILS-PAGE techniques. The optimized ILS concentrations are shown in the Table 3.1.

Table 3.1. Optimized ILS concentrations in C_4PBr, C_8PBr, and C_{11}PBr-PAGE

ILS-PAGE	Concentration of ILS in gel (w/v) %	Concentration of ILS in Sample Buffer (w/v) %	Concentration of ILS in Running Buffer (w/v) %
C_4PBr-PAGE	0.05	0.01	0.10
C_8PBr-PAGE	0.05	0.02	0.05
C_{11}PBr-PAGE	0.05	0.02	0.05

The previous mixture of five proteins, which were used for optimization studies of running and sample buffer concentrations, was employed for this experiment. Figure 3.4 displays the electropherograms of three ILS-PAGE separations of protein mixture. As can be seen, C_4PBr-PAGE and C_8PBr-PAGE produced five well resolved protein bands, but reduced resolution in C_{11}PBr-PAGE. Similarly, in our previous study, where these ILS were used as buffer additives in anionic ILS-PAGE protein separations, C_{11}PBr ILS resulted in poor band resolution as compared to C_4PBr and C_8PBr.[31]

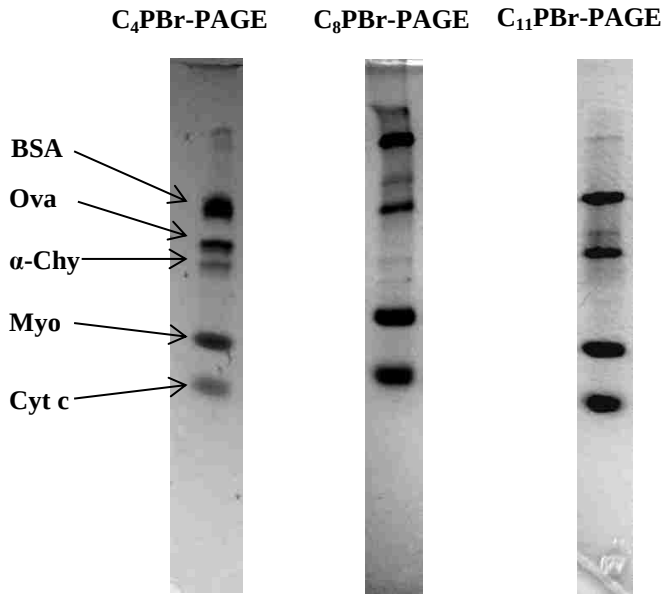


Figure 3.4. Separation of mixture of five proteins; BSA (pI-4.8, 66 kDa), Ova (pI-4.6, 44.3 kDa), α-Chy (pI-8.8, 25.7 kDa), Myo (pI-16.9, 14.7 kDa), and Cyt c (pI-10, 12.3 kDa) using cationic PAGE, C_4PBr-PAGE using C_4PBr ILS (0.05% (w/v) in gel, 0.01% (w/v) in sample buffer, and 0.1% (w/v) in running buffer), C_8PBr-PAGE using C_8PBr ILS (0.05% (w/v) in gel, 0.02% (w/v) in sample buffer, and 0.1% (w/v) in running buffer), and C_{11}PBr-PAGE using C_{11}PBr ILS (0.05% (w/v) in gel, 0.02% (w/v) in sample buffer, and 0.1% (w/v) in running buffer) from left to right respectively.

Thus, as the alkyl chain becomes longer, hydrophobic interactions between hydrophobic domains of the proteins and the ILS become stronger. These strong interactions may deteriorate protein separation by reducing resolution.[32] Evidently, shorter alkyl chains, i.e., C4 and C8, are much better suited for cationic ILS-PAGE.

3.3.2. Separation of Ribonuclease b Glycoforms in ILS-PAGE and Comparison with SDS-PAGE

In section 3.3.1., we observed that BSA, β-cas, Myo, and Cyt c proteins migrated as sharp, intense single bands at optimum ILS concentration in gel (reproduced in Figure 3.5(A)). In contrast, Rib b protein (lane 4) migrated as two sharp bands. Rib b is a glycoprotein, in which a

range of oligomannose structures (Man_5 $GlcNAc_2$ to Man_9 $GlcNAc_2$) are attached to a single N-glycosylation site to generate five possible glycoforms.[33,34,35,] These masses of glycoforms have been distinguished by MALDI-MS and reported.[33]

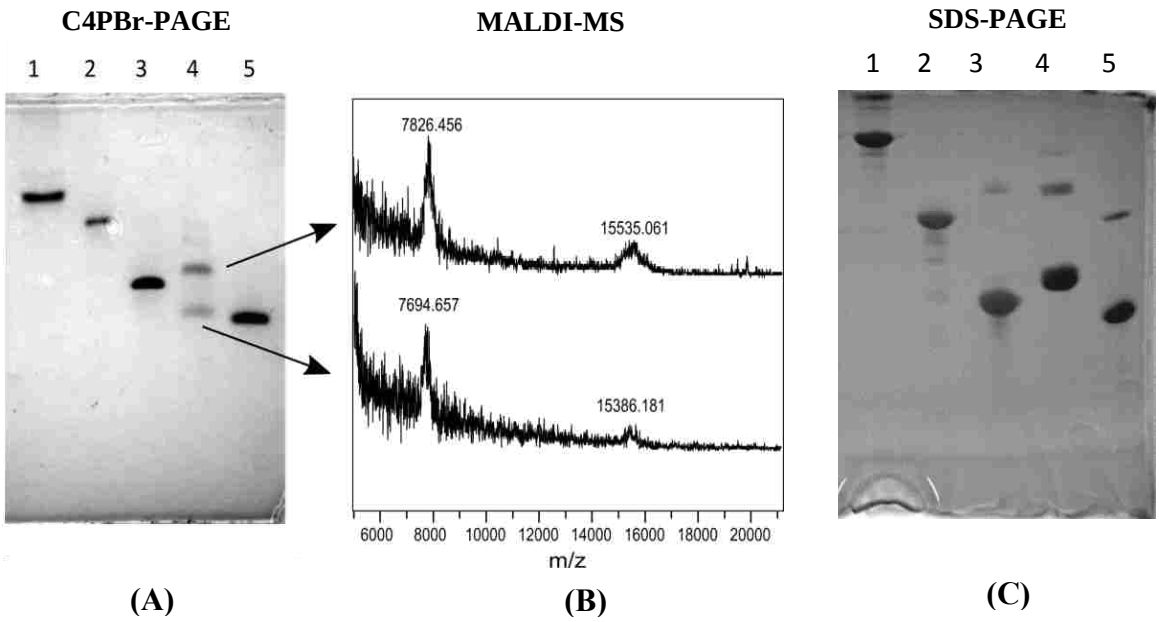


Figure 3.5**.** Electropherogram of (A) cationic C_4PBr -PAGE with C_4PBr concentrations of 0.05% (w/v) in gel, 0.02% (w/v) in sample buffer, and 0.05% (w/v) in running buffer (B) MALDI mass spectra of 2 protein bands extracted from Rib b (lane 4) (C) anionic SDS-PAGE. The same set of five proteins; (1) BSA (pI-4.8, 66.5 kDa), (2) β-cas (pI-5.1, 23.6 kDa), (3) Myo (pI-6.8, 16.9 kDa), (4) Rib b (pI-9.45, 14.7 kDa), and (5) Cyt c (pI-10.0, 12.3 kDa) were used in both SDS-PAGE and ILS-PAGE.

In this study, the two bands observed in lane 4 in Figure 3.5(A) were further analyzed by extracting the proteins from the gels and determining the mass of proteins in each band by use of MALDI-MS. The mass of protein corresponding to the top band was revealed as 15,535.061 (m/z) and the bottom band contained protein with a mass of 15,386.181 (m/z) (Figure 3.5(B)). Both masses matched two of the Rib b glycoform masses previously reported. As gleaned from reported data, the top band correspond to a glycoform of Rib b with Man_9 $GlcNAc_2$, with an

average mass reported as 15,548 (m/z) and the bottom band represent the protein modified with Man_8 $GlcNAc_2$ (average mass of 15,386 (m/z))[33]. The technique proposed in this manuscript, ILS-based cationic PAGE, allows separation of two glycoforms of Rib b protein. In contrast, these glycoforms migrated as a single predominant band in SDS-PAGE (anionic PAGE) as shown in figure 3.5(C). The mass of the additional band observed at the top of the lane did not appear in the mass range of Rib b glycoforms (Figure S4) illustrating the failure of conventional 1D-PAGE to separate these glycoforms.

3.3.3. IEF/SDS-PAGE reveals negative charge heterogeneity on Ribonuclease b Protein

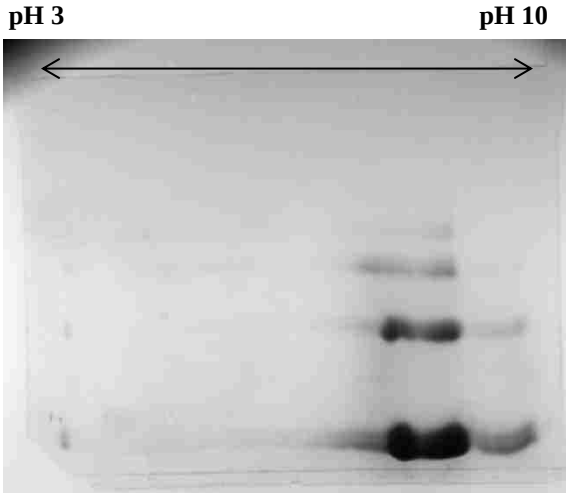


Figure 3.6. 2D map (IEF/SDS-PAGE) of Rib b protein. IEF was carried out in pH 3-10 range. Striated protein pattern reflect charge heterogeneity of Rib b protein.

In order to understand the reason, the separation of Rib b glycoforms in ILS-PAGE, but not in standard SDS-PAGE, we examined the properties of these glycoproteins. For this purpose, Rib b glycoprotein was subjected to isoelectric focusing (IEF)/SDS-PAGE. A 2-D map showed numerous spindle-shaped, 'striated', and slanted focusing patterns as shown in Figure 3.6. These characteristics reflect the charge heterogeneity of the protein.[36] Since the patterns are slanting towards the positive end, it is reasoned that the charges are anionic. These anions may not be

compatible with SDS-PAGE[9], but are compatible with ILS-PAGE separations. As a result, two of the Rib b glycoforms were readily visualized in ILS-PAGE.

3.3.4. A Proposed Mechanism for Cationic ILS-PAGE

A better linear plot of R_f vs. log molecular weight was obtained for five proteins (Figure 3.7) under the optimum conditions of C_4PBr ILS in gels, sample buffer, and running buffer as compared to that of SDS-PAGE (Figure S5).

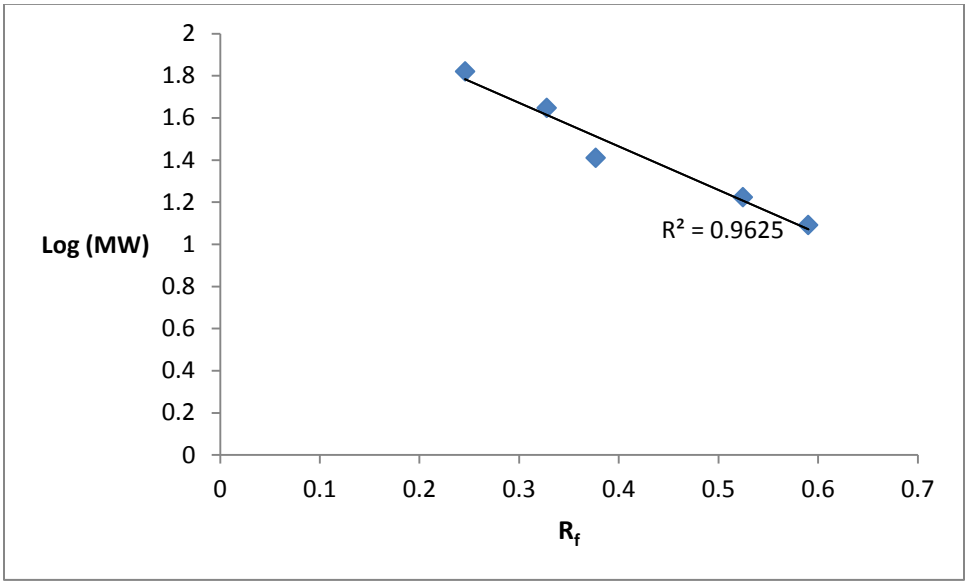


Figure 3.7 The plot of R_f vs. log molecular weight of proteins; BSA (pI-4.8, 66 kDa), Ova (pI-4.6, 44.3 kDa), α-Chy (pI-8.8, 25.7 kDa), Myo (pI-16.9, 16.7 kDa), and Cyt c (pI-10, 12.3 kDa) separated by C_4PBr-PAGE at optimum C_4PBr IL concentrations; 0.05% (w/v) in gel, 0.02% (w/v) in sample buffer, and 0.1% (w/v) in running buffer

The linearity of the plot suggests that cationic ILS-PAGE protein separations are primarily mass-based. The mechanism of mass-based protein separations is a result of the binding of ILS to proteins via electrostatic interactions, hydrogen bonding, and hydrophobic interactions.[31] In our previous study, we have shown that, when proteins are dissolved in ILS sample buffers, at

very low concentrations, ILS can undergo cooperative binding to proteins, i.e., binding of ILS to a certain site of protein enhances binding of ILS to multiple sites of proteins.[31] In general, protein unfolding is believed to occur with this type of binding mechanism.[37] As a result, intrinsic amino acids can be exposed to the solvent. The protein sample buffer has a neutral pH; the exposed amino acids have native charges. ILS can further interact with the native charges of the amino acids and bind to the surfaces of unfolded regions of proteins. Thus, positively charged ILS-protein complexes are formed, which migrate from anode to cathode through polyacrylamide gels where ILS are present. Those ILS can further stabilize the positive charges of the complexes. ILS present in the running buffer assist in separation by generating conductivity, which is required for electrophoretic mobility of proteins during the electrophoresis experiment.

3.4. Conclusions

In summary, a cationic ILS-PAGE technique has been developed for the first time using ILS in gel, sample buffer, and running buffer. Both acidic and basic proteins were separated using this novel technique. Optimum separations were obtained by use of C_4PBr ILS at 0.05% (w/v) in gel, 0.1% (w/v) in the running buffer, and 0.01% (w/v) in the sample buffer. For the first time, effective separation of two Rib b glycoforms was observed using 1D-PAGE. This finding becomes more significant when analyzing different glycans and deglycosylated units in Rib b glycoforms, which serves as a model protein in glycoproteomics. For this purpose, glycoforms can be directly fractionated without pretreatment using cationic ILS-PAGE. Use of IEF/SDS-PAGE allowed confirmation of the negative charge heterogeneity of Rib b. The cationic charges of the ILS were more compatible with the negative charge distribution of the glycoprotein. This suggests that this ILS-PAGE technique has the potential to overcome current deficiencies in other types of glycoprotein separations as well.

3.5. References

1. Raymond, S., Acrylamide gel electrophoresis. *Annals of the New York Academy of Sciences* **1964,** *121* (2), 350-365.

2. Chrambach, A.; Rodbard, D., Polyacrylamide gel electrophoresis. *Science* **1971,** *172* (3982), 440-451.

3. Schägger, H.; Von Jagow, G., Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical biochemistry* **1987,** *166* (2), 368-379.

4. Kang, D.; Gho, Y. S.; Suh, M.; Kang, C., Highly sensitive and fast protein detection with coomassie brilliant blue in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Bulletin-Korean chemical society* **2002,** *23* (11), 1511-1512.

5. Lesse, A. J.; Campagnari, A. A.; Bittner, W. E.; Apicella, M. A., Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Journal of immunological methods* **1990,** *126* (1), 109-117.

6. Nowakowski, A. B.; Wobig, W. J.; Petering, D. H., Native SDS-PAGE: high resolution electrophoretic separation of proteins with retention of native properties including bound metal ions. *Metallomics* **2014,** *6* (5), 1068-1078.

7. Alba, F. J.; Bartolomé, S.; Bermúdez, A.; Daban, J.-R., Fluorescent Labeling of Proteins and Its Application to SDS-PAGE and Western Blotting. *Detection of Blotted Proteins: Methods and Protocols* **2015**, 41-50.

8. Kinoshita, E.; Kinoshita-Kikuta, E.; Kubota, Y.; Takekawa, M.; Koike, T., A Phos-tag SDS-PAGE method that effectively uses phosphoproteomic data for profiling the phosphorylation dynamics of MEK1. *Proteomics* **2016**.

9. Pitcher, D. S.; de Mattos-Shipley, K.; Wang, Z.; Tzortzis, K.; Goudevenou, K.; Flynn, H.; Bohn, G.; Rahemtulla, A.; Roberts, I.; Snijders, A. P., Nuclear proteasomes carry a constitutive posttranslational modification which derails SDS-PAGE (but not CTAB-PAGE). *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics* **2014,** *1844* (12), 2222-2228.

10. Karadimitris, A.; Kleijnen, M. F., Bortezomib Amplifies Effect on Intracellular Proteasomes by Changing Proteasome Structure☆. **2015**.

11. Simpson, R. J., CTAB-PAGE. *Cold Spring Harbor Protocols* **2010,** *2010* (4), pdb. prot5412.

12. Carraro, U.; Catani, C., A sensitive SDS-PAGE method separating myosin heavy chain isoforms of rat skeletal muscles reveals the heterogeneous nature of the embryonic myosin. *Biochemical and biophysical research communications* **1983,** *116* (3), 793-802.

13. Karpe, F.; Hamsten, A., Determination of apolipoproteins B-48 and B-100 in triglyceride-rich lipoproteins by analytical SDS-PAGE. *Journal of lipid research* **1994,** *35* (7), 1311-1317.

14. Berber, I.; Cokmus, C.; Atalan, E., Characterization of Staphylococcus species by SDS–PAGE of whole-cell and extracellular proteins. *Microbiology* **2003,** *72* (1), 42-47.

15. Kinoshita, E.; Kinoshita-Kikuta, E.; Koike, T., Phos-tag SDS-PAGE systems for phosphorylation profiling of proteins with a wide range of molecular masses under neutral pH conditions. *Proteomics* **2012,** *12* (2), 192-202.

16. Rath, A.; Deber, C. M., Correction factors for membrane protein molecular weight readouts on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. *Analytical biochemistry* **2013,** *434* (1), 67-72.

17. Rath, A.; Cunningham, F.; Deber, C. M., Acrylamide concentration determines the direction and magnitude of helical membrane protein gel shifts. *Proceedings of the National Academy of Sciences* **2013,** *110* (39), 15668-15673.

18. Shi, Y.; Mowery, R. A.; Ashley, J.; Hentz, M.; Ramirez, A. J.; Bilgicer, B.; Slunt-Brown, H.; Borchelt, D. R.; Shaw, B. F., Abnormal SDS-PAGE migration of cytosolic proteins can identify domains and mechanisms that control surfactant binding. *Protein Science* **2012,** *21* (8), 1197-1209.

19. Rath, A.; Glibowicka, M.; Nadeau, V. G.; Chen, G.; Deber, C. M.; Engelman, D. M., Detergent Binding Explains Anomalous SDS-Page Migration of Membrane Proteins. *Proceedings of the National Academy of Sciences of the United States of America* **2009,** *106* (6), 1760-1765.

20. Li, L.; Wang, C.; Qiang, S.; Zhao, J.; Song, S.; Jin, W.; Wang, B.; Zhang, Y.; Huang, L.; Wang, Z., Mass Spectrometric Analysis of N-Glycoforms of Soybean Allergenic Glycoproteins Separated by SDS-PAGE. *Journal of Agricultural and Food Chemistry* **2016**.

21. Dunker, A. K.; Kenyon, A. J., Mobility of sodium dodecyl sulphate-protein complexes. *Biochemical Journal* **1976,** *153* (2), 191-197.

22. Buxbaum, E., Cationic electrophoresis and electrotransfer of membrane glycoproteins. *Analytical biochemistry* **2003,** *314* (1), 70-76.

23. Hartinger, J.; Stenius, K.; Högemann, D.; Jahn, R., 16-BAC/SDS–PAGE: a two-dimensional gel electrophoresis system suitable for the separation of integral membrane proteins. *Analytical biochemistry* **1996,** *240* (1), 126-133.

24. Macfarlane, D. E., Use of benzyldimethyl-n-hexadecylammonium chloride ("16-BAC"), a cationic detergent, in an acidic polyacrylamide gel electrophoresis system to detect base labile protein methylation in intact cells. *Analytical biochemistry* **1983,** *132* (2), 231-235.

25. Amory, A.; Foury, F.; Goffeau, A., The purified plasma membrane ATPase of the yeast Schizosaccharomyces pombe forms a phosphorylated intermediate. *Journal of Biological Chemistry* **1980,** *255* (19), 9353-9357.

26. Mócz, G.; Bálint, M., Use of cationic detergents for polyacrylamide gel electrophoresis in multiphasic buffer systems. *Analytical biochemistry* **1984,** *143* (2), 283-292.

27. Küster, B.; Wheeler, S. F.; Hunter, A. P.; Dwek, R. A.; Harvey, D. J., Sequencing of N-linked oligosaccharides directly from protein gels: in-gel deglycosylation followed by matrix-assisted laser desorption/ionization mass spectrometry and normal-phase high-performance liquid chromatography. *Analytical biochemistry* **1997,** *250* (1), 82-101.

28. Zamfir, A. D.; Flangea, C.; Serb, A.; Zagrean, A.-M.; Rizzi, A. M.; Sisu, E., Separation and identification of glycoforms by capillary electrophoresis with electrospray ionization mass spectrometric detection. *Mass Spectrometry of Glycoproteins: Methods and Protocols* **2013**, 145-169.

29. Geyer, H.; Geyer, R., Strategies for analysis of glycoprotein glycosylation. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics* **2006,** *1764* (12), 1853-1869.

30. Lee, Y.; Kockx, M.; Raftery, M. J.; Jessup, W.; Griffith, R.; Kritharides, L., Glycosylation and Sialylation of Macrophage-derived Human Apolipoprotein E Analyzed by SDS-PAGE and Mass Spectrometry EVIDENCE FOR A NOVEL SITE OF GLYCOSYLATION ON SER290. *Molecular & Cellular Proteomics* **2010,** *9* (9), 1968-1981.

31. Hasan, F.; Vidanapathirana, P.; Das, S.; Fernand, V. E.; Siraj, N.; Losso, J. N.; Warner, I. M., Ionic liquids as buffer additives in ionic liquid-polyacrylamide gel electrophoresis separation of mixtures of low and high molecular weight proteins. *RSC Advances* **2015,** *5* (85), 69229-69237.

32. Zhang, T.; Gai, Q.; Qu, F.; Zhang, Y., Ionic liquid-assisted SDS-PAGE to improve human serum protein separation. *Electrophoresis* **2011,** *32* (20), 2904-2910.

33. Tarelli, E.; Byers, H. L.; Wilson, M.; Roberts, G.; Homer, K. A.; Beighton, D., Detecting Mannosidase Activities Using Ribonuclease B and Matrix-Assisted Laser Desorption/Ionization–Time of Flight Mass Spectrometry. *Analytical biochemistry* **2000,** *282* (2), 165-172.

34. Rudd, P. M.; Scragg, I. G.; Coghill, E.; Dwek, R. A., Separation and analysis of the glycoform populations of ribonuclease B using capillary electrophoresis. *Glycoconjugate journal* **1992,** *9* (2), 86-91.

35. Nwosu, C. C.; Huang, J.; Aldredge, D. L.; Strum, J. S.; Hua, S.; Seipert, R. R.; Lebrilla, C. B., In-gel nonspecific proteolysis for elucidating glycoproteins: a method for targeted protein-specific glycosylation analysis in complex protein mixtures. *Analytical chemistry* **2012,** *85* (2), 956-963.

36. Gianazza, E., Isoelectric focusing as a tool for the investigation of post-translational processing and chemical modifications of proteins. *Journal of Chromatography A* **1995,** *705* (1), 67-87.

37. Das, S.; Sylvain, M. R.; Fernand, V. E.; Losso, J. N.; El-Zahab, B.; Warner, I. M., Positive cooperative mechanistic binding of proteins at low concentrations: A comparison of poly (sodium N-undecanoyl sulfate) and sodium dodecyl sulfate. *Journal of colloid and interface science* **2011,** *363* (2), 585-594.

CHAPTER 4: TWO DIMENSIONAL (2D) IEF/ILS-PAGE AS A TOOL FOR COMPLEX PROTEIN SEPARATIONS

4.1. Introduction

Novel analytical techniques as tools to enhance the efficiency of proteomic work flow are in growing need with expansion of protein expression levels in living organisms. The three major steps in proteomic work flow are protein extraction, separation, and identification. Two dimensional (2D) polyacrylamide gel electrophoresis (PAGE) is a widely used separation technique for complex protein analysis.[1,2,3,4,5] The most common 2D-PAGE technique is isoelectric focusing (IEF) followed by SDS-PAGE (IEF/SDS-PAGE).

In the first dimension, IEF, an immobiline pH gradient gel strip is immersed in a protein sample allowing the proteins to be well rehydrated in sample buffer and absorbed into the gel. Upon application of a voltage, the proteins are migrated in the electric field based on charges. Protein migration ceases when they reach the pH, which is the same as the protein isoelectric point (pI). At this step, the proteins are separated based on charge. After that, in the second dimension, the gel strip with separated proteins is subjected to PAGE. Proteins are further separated based on molecular weights during this process. The IEF/SDS-PAGE technique has numerous applications. First, complex protein mixtures can be separated into polypeptide subunits such as isoforms,[5-6] which have variable pI values. Then, this technique can be used as a tool to make comparisons of protein expression patterns, which are derived from different cellular environments.[7] Finally, cellular responses for an external source such as drugs can be evaluated by monitoring at the protein behavioral patterns of 2D protein profiles.[8,9]

Most of the complex proteins are underrepresented in 2D protein profiles although IEF/SDS-PAGE serves as the state-of-the-art technique for complex protein separations.[10,11,12,13] This has

been identified as a result of poor solubility and complicated separation behavior.[14] Clemant et al. described a comparison study of human lymph and plasma protein profiling by use of 2D-PAGE and 1D-SDS-PAGE.[15] The former technique had poor resolving power resulting limited quantity of proteins. Some other techniques have been proposed as alternatives to IEF/SDS-PAGE for complex protein separations. Some examples are, double SDS-PAGE technique where 6M urea-SDS-PAGE is used in the first dimension and SDS-PAGE without urea is used in the second dimension,[16] blue-native-PAGE followed by SDS-PAGE,[17] and cationic PAGE followed by SDS-PAGE have been widely used in membrane proteomics.[18,19,20] The latter technique involves the use of a cationic surfactant, either benzyldimethyl-n-hexadecylammonium chloride (16 BAC)[20,21,22] or cetyltrimethylammonium bromide (CTAB),[23,24,25] in cationic PAGE along with acidic buffers.

This chapter describes the use of IEF in the first dimension and cationic ionic liquid surfactants (ILS)-PAGE in the second dimension to improve the resolution of complex protein separation. The application of 4-methylpyridinium bromide-based ILS (C_nPBr where n=4, 8, and 11) in cationic PAGE protein separations was described in chapter 3. The ILS concentration in gel, sample, and running buffers were optimized along with alkyl chain length in order to achieve the maximum resolution in protein separation by cationic ILS-PAGE. The results showed a high resolving power with C_4PBr concentrations of 0.05% (w/v) in gel, 0.01% (w/v) in sample buffer, and 0.1% (w/v) in running buffer. Moreover, this technique could successfully apply for one-step separation of ribonuclease b glycoforms. In contrast, SDS-PAGE of the same protein showed a single intense band with a low migration distance relative to the other proteins, showing an anomalous migration behavior. Further studies of ribonuclease b protein with IEF/SDS-PAGE showed negative charge heterogeneity, which induces better compatibility of

protein with cationic ILS compared to anionic SDS. These studies suggested the application of cationic ILS-PAGE in glycoprotein separations.

In this study, we describe the application of ILS-PAGE in 2D separation (IEF/ILS-PAGE) of proteins. First, the optimization of ILS in polyacrylamide gels for 2D separations will be discussed. Next, IEF/ILS-PAGE technique for separation of alpha-antitrypsin glycoprotein and cytochrome c protein will be discussed. Moreover, 2D maps of this technique will be compared with the results obtained with the conventional 2D-PAGE technique, IEF/SDS-PAGE.

4.2. Materials and Methods

4.2.1. Materials

Alpha antitrypsin, cytochrome c, 4-Picoline, 4-Bromo-1-butene, and buffer ingredients were purchased from Sigma Aldrich Inc. (Milwaukee, WI). *E. Coli* protein sample, rehydration/sample buffer, nanopure water, equilibration buffers containing SDS, iodoacetamide, overlay agarose solution, immobiline pH gradient (IPG) strips, Mini-protean TGX gels for SDS-PAGE, acrylamide/bisacrylamide (12.5%), methylene bisacrylamide (MBA), Bio-Safe Coomassie blue and Glycerol (30%) were purchased from Bio-Rad Inc. (Hercules, CA).

4.2.2. Instrumentations

An Ettan IPGphor unit (GE Healthcare, Piscataway, NJ) was used for the IEF experiments. Second dimension gel electrophoresis experiments were performed using a Bio-Rad Mini-PROTEAN® Tetra vertical units. Gel images were captured by using a Bio-Rad ChemiDoc™ XRS+ system with Image Lab™ Software.

4.2.3. Ionic Liquid Surfactant Synthesis

ILS was synthesized according to the previous report.[26] Briefly, 4-picoline (7 g, 0.075 M) and n-Bromo-1-butene (n=4,8,11) (10 g, 0.074 M) were mixed together and refluxed at 80 ºC overnight. The final product, which was viscous and yellow color was collected and characterized by ESI-MS.

4.2.4. Protein Sample Preparation, Rehydration, and Isoelectric Focusing (IEF)

A lyophilized powder of protein (0.5 mg) was dissolved in a rehydration sample buffer (1.25 mL). The sample (650 µL) was loaded into a rehydration tray and an IPG strip (pH 3-10) was laid in the sample, maintaining the gel side down. Mineral oil (650 µL) was overlaid on top of the protein sample to avoid sample evaporation during rehydration. The tray was closed and allowed the sample to rehydrate for about 12 hours. Finally, the IEF was performed at 200 V for 1 hour followed by 100 V for 2 hours.

4.2.5. Sample Equilibration

The gel strips were removed from the rehydration tray and transferred the gel side up into a dry and clean equilibration tray after the IEF step has been completed. An equilibration buffer I with urea (6 M, 20 mL), SDS (2%) for IEF/SDS-PAGE or ILS (0.02%) for IEF/ILS-PAGE, Tris-HCl (0.375 M, pH 8.8), Glycerol (20%), and DTT (2%) was added onto the gel strip. The tray was closed and put on an orbital shaker for 10 minutes. After that, the equilibration buffer was carefully drained from the tray and added the equilibration buffer II containing everything in equilibration buffer I except DTT that was replaced by iodoacetamide (3.75% (w/v). The tray was returned to the orbital shaker for another 10 minutes.

4.2.6. Second Dimension-PAGE

At the end of the equilibration, the gel strip was removed from the tray and briefly dipped in an electrophoresis running buffer. Then it was placed on top of a Mini-protean TGX gel with the aid of forceps. Mini-protean TGX gels that are specified for IPG gels of 7 cm were used for SDS-PAGE. ILS resolving gels were prepared according to the method described in chapter 3 (section 3.2.7.). Agarose sealing solution was poured inside the gel plates to seal the IPG and polyacrylamide gels. Care was taken to remove any air bubbles trapped underneath the gel strip. The gel was assembled in the gel box of electrophoresis apparatus. Electrophoresis running buffer was added into the gel box and to the buffer tank. Gel electrophoresis was performed at 200 V for 40 minutes for SDS-PAGE or 300 V for 40 minutes for ILS-PAGE.

4.2.7. Gel Staining and Imaging

SDS-PAGE gels were rinsed with deionized water for 3 times at every 5 minutes after the electrophoresis. ILS-PAGE gels were fixed with 40% methanol and 10% acetic acid for an hour with 4 changes at every 15 minutes. After these steps, the gels were immersed in Bio-safe coomassie blue for 1 hour followed by deionized water for another hour while gentle shaking. Finally, gel images were captured and protein spots were scanned by imagej software (National Institute of Health, Maryland, USA).

4.3. Results and Discussion

4.3.1. IEF/ILS-PAGE of *E. Coli* Cell Lysate

In Chapter 3, the optimization of ILS-PAGE for protein separation was discussed in detail (section 3.3.1). C_4PBr ILS at 0.05% (w/v) concentration in gels and 0.1% (w/v) concentration in

running buffer showed the optimum resolution in C_4PBr-PAGE protein separation. The efficacy of ILS-PAGE as a second dimension in 2D-PAGE was tested using an *E. Coli* cell lysate. Three ILS-PAGE techniques, C_4PBr-PAGE, C_8PBr-PAGE, and C_{11}PBr-PAGE, were applied at two ILS concentrations, i.e. 0.02% (w/v) and 0.05% (w/v) in second dimension polyacrylamide gels. Those two concentrations were chosen as they showed the maximum resolving power during 1D-PAGE. In addition, ILS were applied in equilibration and running buffer preparation at a fixed concentration (0.02% (w/v) and 0.1% (w/v) concentrations of each ILS respectively).

Figure 4.1 shows 2D maps of *E. Coli* cell lysate at different ILS conditions. The number of spots was increased when concentrations of ILS in second dimension gels were increased from 0.02% (w/v) to 0.05% (w/v) in both, IEF/C_4PBr-PAGE and IEF/C_8PBr- PAGE (Figure 4.1(B) and 4.1(D) respectively). A similar effect was observed in chapter 3 (section 3.3.1.) where 0.05% (w/v) concentrations of C_4PBr and C_8PBr were applied in gels of 1D-PAGE. At those conditions, proteins were separated as sharp bands with improved resolution as compared to 0.02% (w/v) of ILS in gels. This high resolution was claimed to achieve as a result of more stabilized ILS-protein complexes in the presence of a moderate ILS concentration in gels. An increased number of spots in 2D-PAGE gels in this study further supports this claim. Figure 1(E) and 1(F) show 2D maps of IEF/C_{11}PBr-PAGE at two different concentrations of C_{11}PBr, i.e. 0.02% (w/v) and 0.05% (w/v), in second dimension gels. Number of protein spots was reduced in both images compared to 1(B) and 1(D). In latter two images, 0.05% (w/v) concentration of C_4PBr and C_8PBr ILS were applied in second dimension gels of IEF/C_4PBr-PAGE and IEF/C_8PBr-PAGE respectively. Thus, C_{11}PBr ILS has a low impact on protein resolution as compared to C_4PBr and C_8PBr. Similar effects of ILS chain length was discussed in chapter 3 (section 3.3.2.).

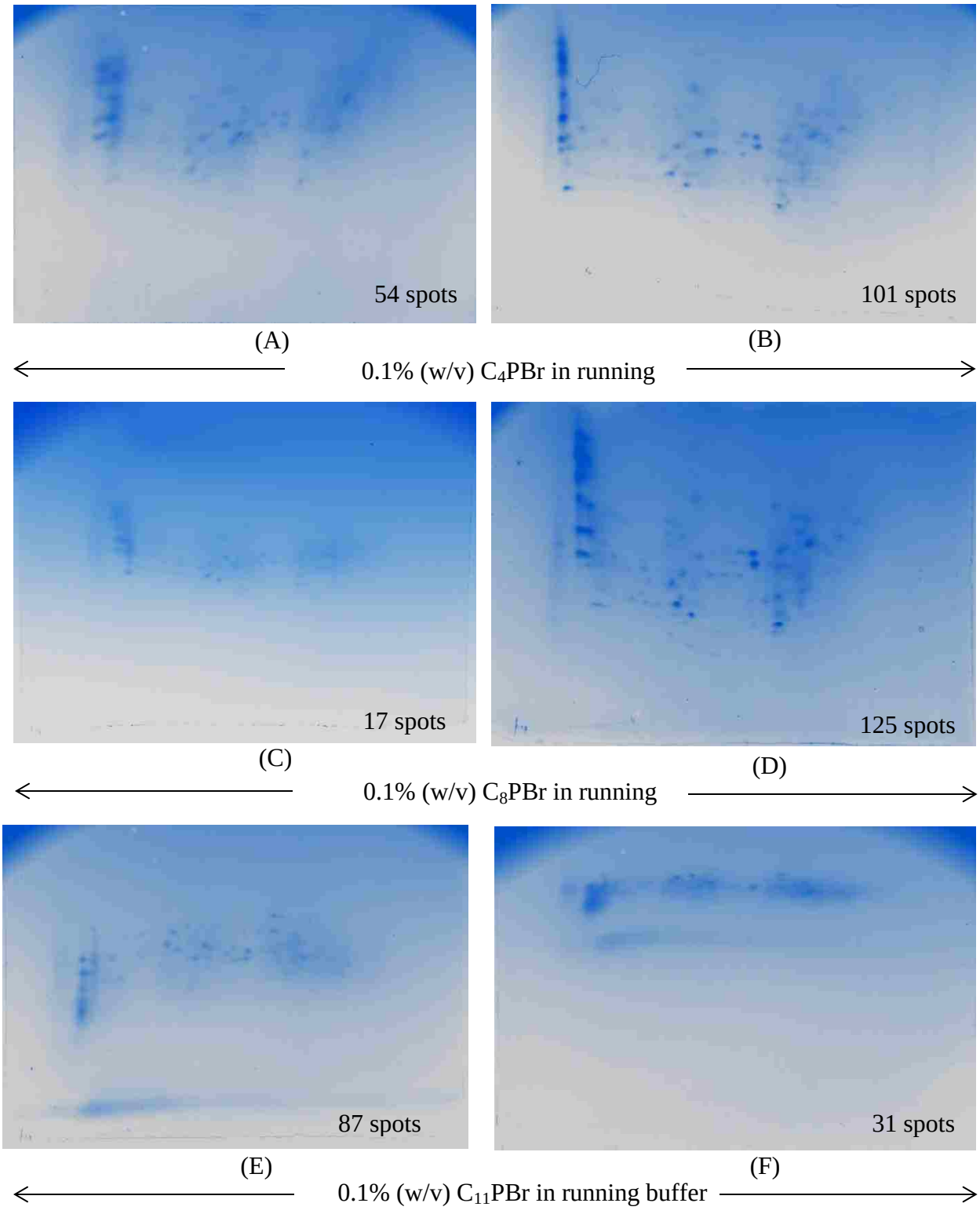
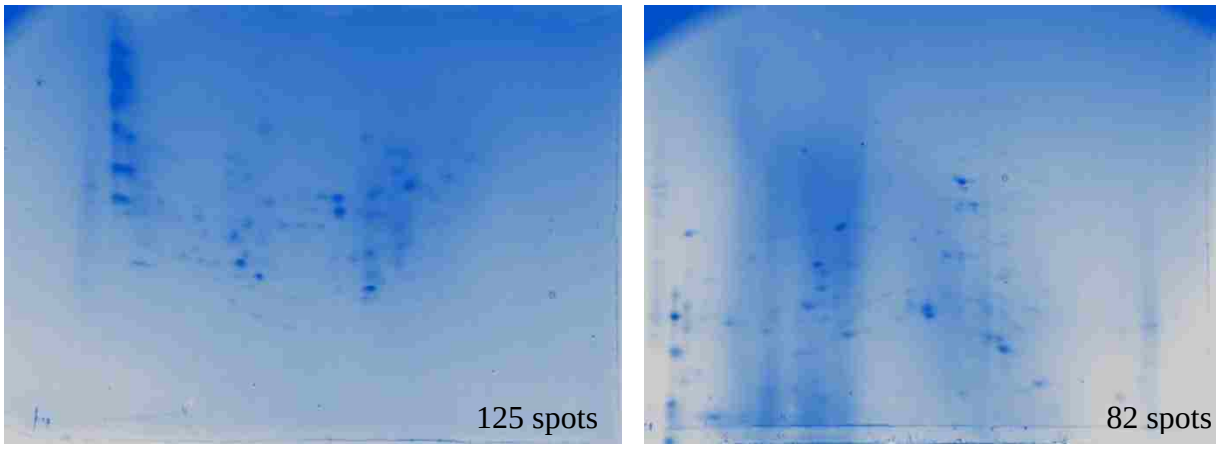


Figure 4.1. 2D profiles of *E. Coli* cell lysates at different ILS concentrations in gels (A) 0.02% (w/v) C_4PBr (B) 0.05% (w/v) C_4PBr (C) 0.02% (w/v) C_8PBr (D) 0.05% (w/v) C_8PBr (E) 0.02% (w/v) C_{11}PBr (F) 0.05% (w/v) C_{11}PBr. Number of spots obtained from imagej software are labeled on each gel image.

C_{11}PBr-PAGE showed poor band resolution in all the concentration tested, as a result of strong interactive forces between hydrophobic domains of the proteins and the ILS.

Next, the results obtained with IEF/C_8PBr-PAGE were compared with conventional method, IEF/SDS-PAGE (Figure 4.2.) The 2D map of latter showed only 82 spots as compared to IEF/ILS-PAGE 2D map, which showed 125 spots. In addition, vertical streaking was appeared on IEF/SDS-PAGE gel, which may have occurred due to poor solubility power or interference from salts[27] even though standard SDS conditions were applied. In contrast, IEF/C_8PBr-PAGE showed a clean 2D profile without vertical or horizontal streaking.



(A) (B)

Figure 4.2. 2D profiles of *E. Coli* obtained from (A) IEF/C_8PBr-PAGE and (B) IEF/SDS-PAGE

4.3.2. Separation of Alpha-antityrpsin Protein Subunits by IEF/C_4PBr-PAGE

In chapter 3, ILS-PAGE technique showed an enhanced resolution in Ribonuclease b glycoforms separation. Negative charge heterogeneity on this glycoprotein caused the cationic ILS-PAGE to be more compatible with the protein. In order to further investigate the effect of ILS-PAGE on separation of other glycoforms, human alpha-antitrypsin (A1PI) protein (52 kDa, pI-4.2-4.9) was chosen for two dimensional IEF/C_4PBr-PAGE. A1PI glycoprotein has five

different glycoforms with pI values ranging from 4.2-4.9 as a result of different –di and –tri antennary glycans, and a truncated N-terminus. Generally, three of the five glycoforms can be identified in an IEF experiments.[28]

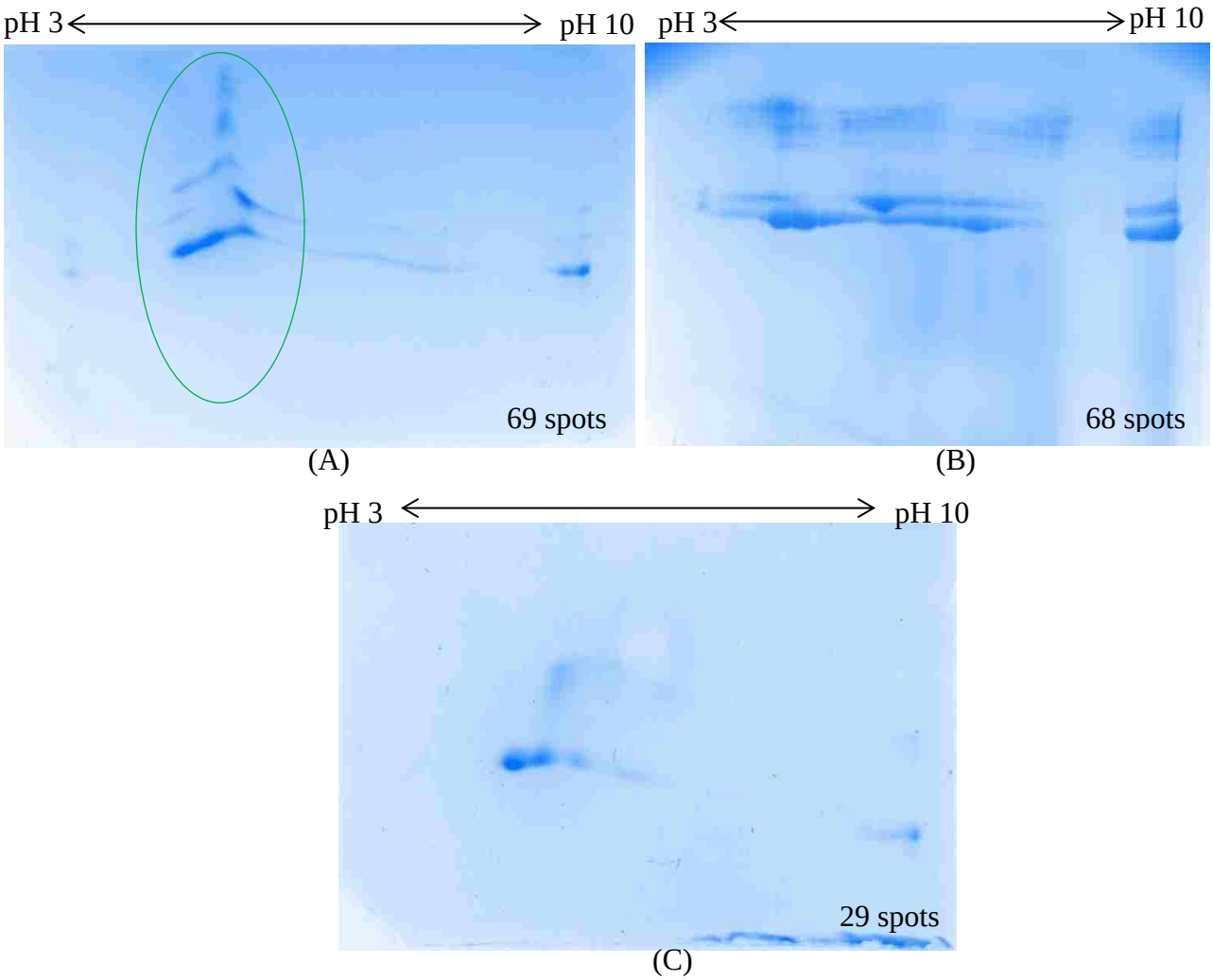


Figure 4.3. Separation of alpha-antitrypsin protein subunits by (A) IEF/C_4PBr-PAGE, (B) IEF/SDS-PAGE, and (C) IEF/CTAB-PAGE

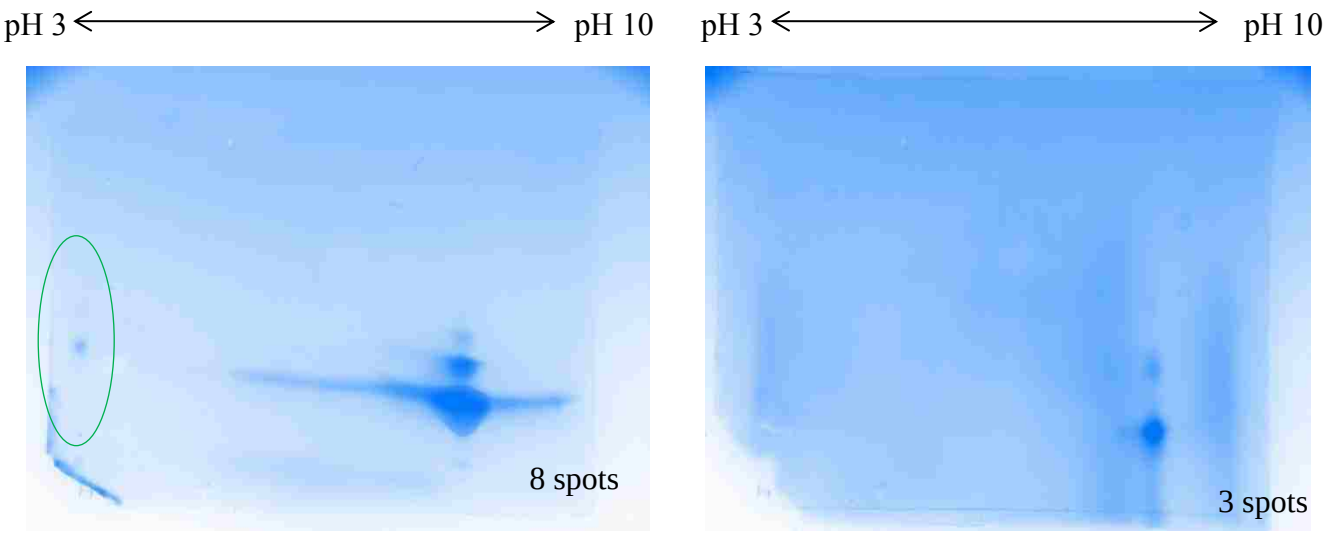
Figure 4.3 (A) shows the A1PI 2D profile of IEF/C_4PBr-PAGE. Multiple spots in the pI region of 4-5 (circled with green) were observed on this 2D map. As the spots are appearing on the same pI region of A1PI glycoforms, those should be subunits derived from the glycoforms. Even though, only three glycoforms are appeared in IEF (1st dimension) gels,[28] the second

dimension C_4PBr-PAGE could resolve other subunits related to these glycoforms as well. IEF migration of different glycoforms of A1PI protein is known to occur due to the presence of sialic acid residues, which have pK_a of 2.6.[29] In second dimension ILS-PAGE, sialic acid residues are deprotonated during the electrophoresis at pH 3.0. Ionic interactions between negatively charged sialic acid residues and cationic C_4PBr may have aided in resolving multiple subunits of the glycoforms. The IEF/C_4PBr-PAGE results for A1PI protein were compared with IEF/SDS-PAGE and IEF/CTAB-PAGE. The standard separation conditions were applied for those two conventional techniques. The 2D map of IEF/SDS-PAGE is shown in Figure 4.2 (B). Only two major spots were observed in the corresponding pI region (4.2-4.9) of A1PI glycoforms owing to the low resolution associated with the second dimension SDS-PAGE. It shows the incompatibility between anionic SDS surfactant and anionic sialic acid residues during electrophoresis running buffer conditions (pH 8.3). 2D map of IEF/CTAB-PAGE (Figure 4.2 (C)) showed one major band in the pI region of glycoforms, showing failure to resolve any subunits of the glycoprotein.

4.3.3. Separation of Cytochrome c Subunits by IEF/C_4PBr-PAGE

Cytochrome c protein (13 kDa, pI-10.5) was subjected to IEF followed by C_4PBr-PAGE. Figure 4.4 (A) shows the 2D map of IEF/C_4PBr-PAGE with 8 protein spots. One protein spot was observed at pH 3-4 region (circled in Figure 4.4 (A)). Cytochrome c protein consists of 24 basic and 15 acidic amino acid residues.[30] The latter includes two propionic acid residues with pI of 4.87, which are on the heme group of the protein. Hence, the acidic protein spot of IEF/C_4PBr-PAGE may represent the heme group with two propionic acids. Figure 4.3 (B) shows a 2D map (IEF/SDS-PAGE) of the same protein with three spots. No spots were appeared in pH

3-4 region on that 2D map. Overall, these results show the high resolving power of IEF/C_4PBr-PAGE for cytochrome c protein compared to conventional IEF/SDS-PAGE.



(A) (B)

Figure 4.4. Separation of Cytochrome c subunits by (A) C_4PBr-PAGE (B) SDS-PAGE

4.4. Conclusions

In summary, ILS-PAGE technique was applied in 2D IEF/ILS-PAGE for some of the complex protein separation. C_4PBr-PAGE and C_8PBr-PAGE could achieve better recovery of *E. Coli* protein separations among three ILS-PAGE techniques when they were applied in 2D PAGE. This suggests that ILS with short alkyl chains impose better interactions with proteins as compared to long chains.

Increased number of A1PI protein sub units, which have derived from A1PI glycoforms appeared in IEF/C_4PBr-PAGE as compared to conventional IEF/SDS-PAGE. This proves the high resolving power of ILS-PAGE on glycoprotein separations. Negative charge heterogeneity of these glycoforms in the presence of sialic acids may have caused the differential separation ability of IEF/SDS-PAGE and IEF/ILS-PAGE. However, a comparable resolution could not

achieve when ILS-PAGE was replaced by CTAB-PAGE, which is also a cationic PAGE technique. This shows some unique cationic surfactant properties associated with ILS caused them to make better interactions with proteins yielding a high resolution in separation. In addition, an acidic subunit of Cytochrome c protein could be resolved by using IEF/C_4PBr-PAGE proving better resolving power of IEF/ILS-PAGE. Future works on this study include application of ILS in the first dimension, IEF, as buffer additives to improve protein solubility and increase protein recovery in second dimension ILS-PAGE. This will help to resolve proteins with different charges that are not readily resolved by conventional 2D-PAGE techniques.

4.5. References

1. Sanchez, J. C.; Chiappe, D.; Converset, V.; Hoogland, C.; Binz, P. A.; Paesano, S.; Appel, R. D.; Wang, S.; Sennitt, M.; Nolan, A., The mouse SWISS-2D PAGE database: a tool for proteomics study of diabetes and obesity. *Proteomics* **2001,** *1* (1), 136-163.

2. Issaq, H. J.; Veenstra, T. D., Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE): advances and perspectives. *Biotechniques* **2008,** *44* (5), 697.

3. Wilson, N. L.; Schulz, B. L.; Karlsson, N. G.; Packer, N. H., Sequential analysis of N-and O-linked glycosylation of 2D-PAGE separated glycoproteins. *Journal of proteome research* **2002,** *1* (6), 521-529.

4. Caruso, G.; Cavaliere, C.; Foglia, P.; Gubbiotti, R.; Samperi, R.; Laganà, A., Analysis of drought responsive proteins in wheat (Triticum durum) by 2D-PAGE and MALDI-TOF mass spectrometry. *Plant Science* **2009,** *177* (6), 570-576.

5. Müller, D.; Schindler, P.; Coulot, M.; Voshol, H.; Van Oostrum, J., Mass spectrometric characterization of stathmin isoforms separated by 2D PAGE. *Journal of mass spectrometry* **1999,** *34* (4), 336-345.

6. Raikos, V.; Hansen, R.; Campbell, L.; Euston, S. R., Separation and identification of hen egg protein isoforms using SDS–PAGE and 2D gel electrophoresis with MALDI-TOF mass spectrometry. *Food chemistry* **2006,** *99* (4), 702-710.

7. Chen, G.; Gharib, T. G.; Huang, C.-C.; Taylor, J. M.; Misek, D. E.; Kardia, S. L.; Giordano, T. J.; Iannettoni, M. D.; Orringer, M. B.; Hanash, S. M., Discordant protein and mRNA expression in lung adenocarcinomas. *Molecular & cellular proteomics* **2002,** *1* (4), 304-313.

8. Celis, J. E.; Gromov, P., 2D protein electrophoresis: can it be perfected? *Current Opinion in Biotechnology* **1999,** *10* (1), 16-21.

9. Lilley, K. S.; Razzaq, A.; Dupree, P., Two-dimensional gel electrophoresis: recent advances in sample preparation, detection and quantitation. *Current opinion in chemical biology* **2002,** *6* (1), 46-50.

10. Galeva, N.; Altermann, M., Comparison of one-dimensional and two-dimensional gel electrophoresis as a separation tool for proteomic analysis of rat liver microsomes: cytochromes P450 and other membrane proteins. *Proteomics* **2002,** *2* (6), 713.

11. Chevallet, M.; Santoni, V.; Poinas, A.; Rouquié, D.; Fuchs, A.; Kieffer, S.; Lunardi, J.; Garin, J.; Rabilloud, T., New zwitterionic detergents improve the analysis of membrane proteins by two-dimensional electrophoresis. *Electrophoresis* **1998,** *19* (11), 1901-1909.

12. Molloy, M. P., Two-dimensional electrophoresis of membrane proteins using immobilized pH gradients. *Analytical biochemistry* **2000,** *280* (1), 1-10.

13. Rabilloud, T., Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis. *Electrophoresis* **1998,** *19* (5), 758-760.

14. Helling, S.; Schmitt, E.; Joppich, C.; Schulenborg, T.; Müllner, S.; Felske-Müller, S.; Wiebringhaus, T.; Becker, G.; Linsenmann, G.; Sitek, B., 2-D differential membrane proteome analysis of scarce protein samples. *Proteomics* **2006,** *6* (16), 4506-4513.

15. Clement, C. C.; Aphkhazava, D.; Nieves, E.; Callaway, M.; Olszewski, W.; Rotzschke, O.; Santambrogio, L., Protein expression profiles of human lymph and plasma mapped by 2D-DIGE and 1D SDS–PAGE coupled with nanoLC–ESI–MS/MS bottom-up proteomics. *Journal of proteomics* **2013,** *78*, 172-187.

16. Laßek, M.; Weingarten, J.; Acker-Palmer, A.; M Bajjalieh, S.; Muller, U.; Volknandt, W., Amyloid precursor protein knockout diminishes synaptic vesicle proteins at the presynaptic active zone in mouse brain. *Current Alzheimer Research* **2014,** *11* (10), 971-980.

17. Hashemi, A.; Gharechahi, J.; Nematzadeh, G.; Shekari, F.; Hosseini, S. A.; Salekdeh, G. H., Two-dimensional blue native/SDS-PAGE analysis of whole cell lysate protein complexes of rice in response to salt stress. *Journal of Plant Physiology* **2016,** *200*, 90-101.

18. Macfarlane, D. E., Two dimensional benzyldimethyl-n-hexadecylammonium chloride→ sodium dodecyl sulfate preparative polyacrylamide gel electrophoresis: a high capacity high resolution technique for the purification of proteins from complex mixtures. *Analytical biochemistry* **1989,** *176* (2), 457-463.

19. Navarre, C.; Degand, H.; Bennett, K. L.; Crawford, J. S.; Mørtz, E.; Boutry, M., Subproteomics: identification of plasma membrane proteins from the yeast Saccharomyces cerevisiae. *Proteomics* **2002,** *2* (12), 1706-1714.

20. Hartinger, J.; Stenius, K.; Högemann, D.; Jahn, R., 16-BAC/SDS–PAGE: a two-dimensional gel electrophoresis system suitable for the separation of integral membrane proteins. *Analytical biochemistry* **1996,** *240* (1), 126-133.

21. Braun, R. J.; Kinkl, N.; Zischka, H.; Ueffing, M., 16-BAC/SDS-PAGE analysis of membrane proteins of yeast mitochondria purified by free flow electrophoresis. *Membrane Proteomics: Methods and Protocols* **2009**, 83-107.

22. Nothwang, H. G.; Schindler, J., Two-dimensional separation of membrane proteins by 16-BAC-SDS-PAGE. *Membrane Proteomics: Methods and Protocols* **2009**, 269-277.

23. Longuespée, R.; Tastet, C.; Desmons, A.; Kerdraon, O.; Day, R.; Fournier, I.; Salzet, M., HFIP Extraction Followed by 2D CTAB/SDS-PAGE Separation: A New Methodology for Protein Identification from Tissue Sections after MALDI Mass Spectrometry Profiling for Personalized Medicine Research. *Omics: a journal of integrative biology* **2014,** *18* (6), 374-384.

24. Yamaguchi, Y.; Miyagi, Y.; Baba, H., Two-dimensional electrophoresis with cationic detergents, a powerful tool for the proteomic analysis of myelin proteins. Part 1: Technical aspects of electrophoresis. *Journal of neuroscience research* **2008,** *86* (4), 755-765.

25. Braun, R. J.; Kinkl, N.; Beer, M.; Ueffing, M., Two-dimensional electrophoresis of membrane proteins. *Analytical and bioanalytical chemistry* **2007,** *389* (4), 1033-1045.

26. Hasan, F.; Vidanapathirana, P.; Das, S.; Fernand, V. E.; Siraj, N.; Losso, J. N.; Warner, I. M., Ionic liquids as buffer additives in ionic liquid-polyacrylamide gel electrophoresis separation of mixtures of low and high molecular weight proteins. *RSC Advances* **2015,** *5* (85), 69229-69237.

27. Lee, S.-H.; Ahn, J.-Y.; Min, J.; Kim, S. Y.; Wee, J.-H.; Sekhon, S. S.; Kim, Y.-H., The optimized conditions of two-dimensional polyacrylamide gel electrophoresis for proteomic analysis of Saccharomyces cerevisiae. *Toxicology and Environmental Health Sciences* **2014,** *6* (4), 256-260.

28. Chung, H.-S.; Kim, J.-S.; Lee, S. M.; Park, S. J., Additional N-glycosylation in the N-terminal region of recombinant human alpha-1 antitrypsin enhances the circulatory half-life in Sprague-Dawley rats. *Glycoconjugate journal* **2016,** *33* (2), 201-208.

29. Kolarich, D.; Weber, A.; Turecek, P. L.; Schwarz, H. P.; Altmann, F., Comprehensive glyco-proteomic analysis of human α1-antitrypsin and its charge isoforms. *Proteomics* **2006,** *6* (11), 3369-3380.

30. Guevremont, R.; Siu, K.; Le Blanc, J.; Berman, S. S., Are the electrospray mass spectra of proteins related to their aqueous solution chemistry? *Journal of the American Society for Mass Spectrometry* **1992,** *3* (3), 216-224.

CHAPTER 5: IONIC LIQUIDS AS SIGNAL ENHANCING MATRIX ADDITIVES FOR PROTEIN ANALYSIS USING MALDI-MS

5.1. Introduction

Novel analytical methodologies for use in proteomics have received considerable attention over the past few decades.[1,2,3,4] Protein extraction, separation/purification, and characterization are the main steps in a general proteomic analytical scheme. Protein characterization is an essential step for precise protein identification. In this regard, matrix assisted laser desorption ionization (MALDI)-mass spectrometry (MS) and electrospray ionization (ESI)- mass spectrometry (MS) are widely used techniques for characterization of proteins and peptides on the basis of molecular weight as well as structural analysis by tandem mass spectrometry as part of protein sequencing. Among these techniques, MALDI-MS is a robust technique with high tolerance for solvent background effects and high sensitivity for proteins.[5,6,7] However, the quality of the MALDI mass spectrum depends on sample preparation, matrix composition, and sample impurities.[8] The presence of trace surfactant impurities, which usually remain in the protein sample after protein extraction and separation can interfere with protein MALDI mass spectral acquisition.[7,9,10,11]

Surfactants are often used in protein extraction, separation, and analysis to increase solubility and stability of the sample.[12,13,14,15,16] Amphiphilic properties of surfactants lead to micelle formation in aqueous media at a concentration known as the critical micelle concentration (CMC). Surfactant micelles play a critical role in proteomics by aiding in unfolding and denaturing of proteins through the formation of protein-micelle complexes.[17] An anionic surfactant, SDS, is the most commonly employed surfactant in proteomics. Although SDS has been widely used in proteomics, mass spectral interferences are associated with protein analyses

using SDS in MALDI-MS.[18,19,20,21,22] Typical interferences include peak broadening associated with sodium protein adduct formation, low signal-to-noise ratio, and lower resolution.[21,22,] Therefore, it is important to remove SDS before MALDI-MS analysis in proteomic work flow. Common techniques used for removal of SDS include a C18 ZipTip cleanup method[10] and an ion exchange column chromatography technique.[10,20] Unfortunately, these removal processes require additional time and efforts that often cause a significant loss of proteins.[23] Ultimately, the presence of surfactants produces poor quality mass spectra and creates challenges for protein characterization.[10] In this regard, several studies have been performed to investigate the optimum concentration of SDS for use in MALDI analyses of proteins.[10,24,25,26,27] Zhang et al. reported that concentrations of SDS above 0.2% (w/v) are effective for MALDI analysis of peptides.[28] The MALDI-MS signal intensity is suppressed when SDS was used below the CMC due to the partial precipitation of proteins with SDS monomers.[27] However, interferences from SDS-matrix clusters were observed in MALDI mass spectra when high SDS concentrations were used.[28]

Alternative surfactants with useful properties have been proposed for proteomic work flow.[29,30,31] For example, Jiménez and coworkers have proposed novel anionic branched surfactants suitable for PAGE protein separation.[29] However, mass spectrometric compatibility of these surfactants has not been assessed in the study. A cationic surfactant, CTAB, has also been used for low molecular mass analysis, particularly for amino acids and peptides at a ratio of 1000:1 (matrix/CTAB) for suppression of matrix related background in MALDI-MS.[32] Furthermore, CTAB is a useful surfactant in cationic PAGE, particularly for 2D-PAGE protein separations.[33] A zwitterionic surfactant, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and a non-ionic surfactant, Triton X-100, have also been tested for protein sample preparation in MALDI-MS analysis.[34] It was determined that the presence of

Triton X-100 in protein samples caused a slight degradation of mass spectral quality, while the presence of CHAPS highly affected the spectra by producing a low signal-to-noise ratio.

Recently, ionic liquids-based surfactants have been proposed as novel surfactants for proteomics.[35] Ionic liquids have been previously used as MALDI matrices,[36,37] but not as surfactants in protein analysis by MALDI. In previous chapters, we studied 4-methyl pyridinium-based ionic liquid surfactants (ILS) as buffer additives for protein separation by anionic[35] and cationic PAGE. Herein, we report the role of these ILS as additives for protein characterization using MALDI-MS with a conventional matrix, α-cyanohydroxycinnamic acid (CHCA). Low vapor pressure, high thermal stability, and solubility of different proteins in ILS ensure the suitability of these ILS for applications in MALDI-MS for protein analyses. Three proteins of different molecular weights; i.e. Cytochrome c (Cyt c,~12 kDa), Bovine serum albumin (BSA, ~66 kDa), and Transferrin (Tr, ~78 kDa), were selected as analytes. Results obtained using ILS were compared to no surfactant added, SDS, and CTAB.

5.2. Experimental Section

5.2.1. Materials

The compounds 4-bromo-1-butene, 8-bromo-1-octene, 4-methylpyridinium, SDS, CTAB, BSA, Tr, Cyt c, and CHCA were purchased from Sigma Aldrich (Milwaukee, WI) and used as received. All solutions (surfactant, matrix, and protein) were prepared using triply deionized ultrapure distilled water (18.23 MΩ) from an ARIES high purity water system (ARIES filter works, West Berlin, NJ). Ethanol, methanol, and chloroform were purchased from Thermo Fisher Scientific (Fair Lawn, New Jersey). Rat brain tissue samples were provided from School of Veterinary Medicine, Division of Laboratory Animal Medicine at LSU.

5.2.2. Ionic Liquid Synthesis

Ionic liquids were synthesized and characterized using protocols established in our previous work.[35] Briefly, 4-methylpyridinium (7 g, 0.075 M) and 4-bromo-1-butene (for the synthesis of 1-butene-4-methylpyridinium IL (C_4PBr)) or 8-bromo-1-octene (for the synthesis of 1-ocene-4-methylpyridinium IL (C_8PBr)) (0.02 eq, 10 g, 0.074 M) were added separately into acetonitrile solvent and refluxed at 80 °C overnight. Acetonitrile was then evaporated by using a rotoary evaporator and the product was freeze dried. The products were yellow, highly viscous liquids and were characterized with ESI-MS.

5.2.3. Sample Preparation for Protein Analysis

Five concentrations of surfactant solutions, 0.02%, 0.05%, 0.1%, 0.5%, and 1% (w/v), were prepared by dissolving the appropriate quantities in ultrapure water. BSA, Tr, and Cyt c proteins were dissolved in ultrapure water to prepare protein stock solutions of 4.0×10^{-5} M. Protein samples were prepared by mixing 5 µL of protein stock solution with 5 µL of surfactant solution in a micro-centrifuge tube and vortexing at high speed for about 30 seconds. A saturated CHCA solution in 1% TFA and 60% acetonitrile/water was prepared as the matrix before performing MALDI-MS. This matrix solution (10 µL) was added into the protein sample (10 µL) and further vortexed for about 30 seconds. An aliquot of the analyte-and matrix solution (1 µL, 10 pmol) was pipetted onto the MALDI sample target and air dried at room temperature.

5.2.4. Tissue Sample Preparation

Horizontal sections of rat brain tissue samples were cut into pieces of 10 µm thickness. These samples were prepared with a Leica cryostat (CM 1850, Leica Microsystems, Germany). The

optimal cutting temperature (OCT) compound was used to fix the tissue on a cryostat. When fixing the tissue, care was taken to avoid contact of the exposed side of it with the OCT solution. They were thaw-mounted on microscope slides, which were coated with indium tin oxide (ITO) and stored at -80 °C. These horizontal sections were used for MALDI profiling mode experiments. Before use, the tissue section was vacuum dried (10 min) and washed for 30 s each with 70% ethanol and 95% ethanol followed by chloroform wash (15 s). Tissue section was vacuum dried again for 10 min before the application of matrix. Two ILS, C_4PBr and C_8PBr, were separately mixed with MALDI matrix (α-CHCA saturated with 1% TFA and 60% acetonitrile/water) at 0.02% (w/v) concentration. The matrix was applied by manual spotting (200 nL each) into 2×3 array of spots in each section and allowed to air dry. This process was repeated five times on each spot. One section was used as the control matrix for two ILS and the other section contained the matrix with C_4PBr and C_8PBr (each in a set of three spots).

5.2.5. Instrumentation

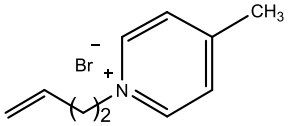
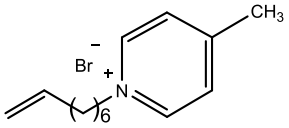
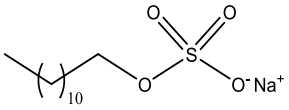
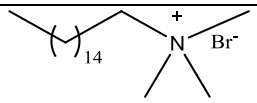
Surface tension measurements for CMC determinations were performed using a KSV Sigma 703 digital tensiometer at room temperature. Agilent 6210 electrospray TOF mass spectrometer at positive mode was used to acquire ESI mass spectra of ionic liquid surfactants. MALDI mass spectra were acquired using a MALDI-TOF mass spectrometer (UltrafleXtreme, Bruker) equipped with a Nd:YAG laser with 355 nm wavelength and 3 ns pulse duration. MALDI-TOF MS experiments were performed in positive ion linear mode with 25 kV acceleration potential. Complete sample analysis was performed and an average signal intensity was collected for each sample spot. Mass spectra were processed using FlexAnalysis 3.3 (Bruker). Light microscopic digital images were captured using a Leica DM RXA2 upright microscope equipped with Sensicam QE 12- bit, CCD camera and SlideBook 6.0 software.

5.3. Results and Discussion

5.3.1. Surfactant Properties of C_4PBr, C_8PBr, SDS, and CTAB

Surfactants have surface active properties due to a polar head group in combination with a hydrophobic tail. In the ILS studied here, 4-methylpyridinium is a cationic hydrophilic head group, while variable alkyl chains impart hydrophobic properties. Additionally, bromide anion was chosen as the counter ion in these ILS in order to attain water soluble characteristics for studies in aqueous protein solutions. Structures, molecular weights (MW) and CMC values for all surfactants (C_4PBr, C_8PBr, SDS, and CTAB) are reported in Table 5.1.

Table 5.1. Structures, MW, and CMC values of each surfactant used in this study

Surfactant	Structure	MW (g/mol)	CMC (mM)	CMC (w/v)%
C_4PBr		228	46.36	1.06
C_8PBr		284	21.50	0.61
SDS		288	8.10[38]	0.23[38]
CTAB		364	1.30[38]	0.05[38]

5.3.2. Protein Sample Analysis

In order to compare the role that surfactants play on the quality of MALDI mass spectra in terms of signal intensity, resolution, signal-to-noise ratio etc., three proteins of variable molecular weights (Cyt c, BSA, and Tr) and different surfactants (C_4PBr, C_8PBr, SDS, and CTAB) were used at the same concentration (0.02% (w/v)). Results obtained from these measurements are compared with MALDI mass spectra obtained in the absence of surfactant (Figure 5.1). Interestingly, with C_4PBr and C_8PBr ILS, Cyt c protein (m/z 12387) exhibited enhanced signal in the acquired MALDI mass spectra, as compared to the spectra obtained for the same protein in the absence of surfactant. In contrast, the presence of normal surfactants, i.e. SDS and CTAB, significantly reduced the signal intensity in comparison to the signal obtained for the spectrum of 0% surfactant (Figure 5.1(A)). BSA protein signals (m/z 66449) were also affected by two ILS in terms of enhanced signal intensity as compared to normal surfactants and the 0% surfactant (Figure 5.1(B)). The intensity of transferrin (m/z 79019) was significantly enhanced in the presence of C_8PBr ILS, thus giving a high signal-to-noise ratio as compared to C_4PBr, SDS, CTAB and 0% surfactant.

The above observations suggest that the presence of trace amounts of ILS do not suppress the protein signal; rather, they enhance signal intensity of MALDI mass spectra for improved characterization. The aggregate of all results suggest that ILS perform better than the conventional surfactants employed in this study. This is contrary to the general supposition that surfactants will often suppress the signal of MALDI-MS.[34] The concentration of ILS (0.02% (w/v)) used in this study was lower than the CMC of C_4PBr (1.06% (w/v)) and C_8PBr (0.61% (w/v)). This suggests that unlike SDS, micelle formation is not required for ILS to be effective in

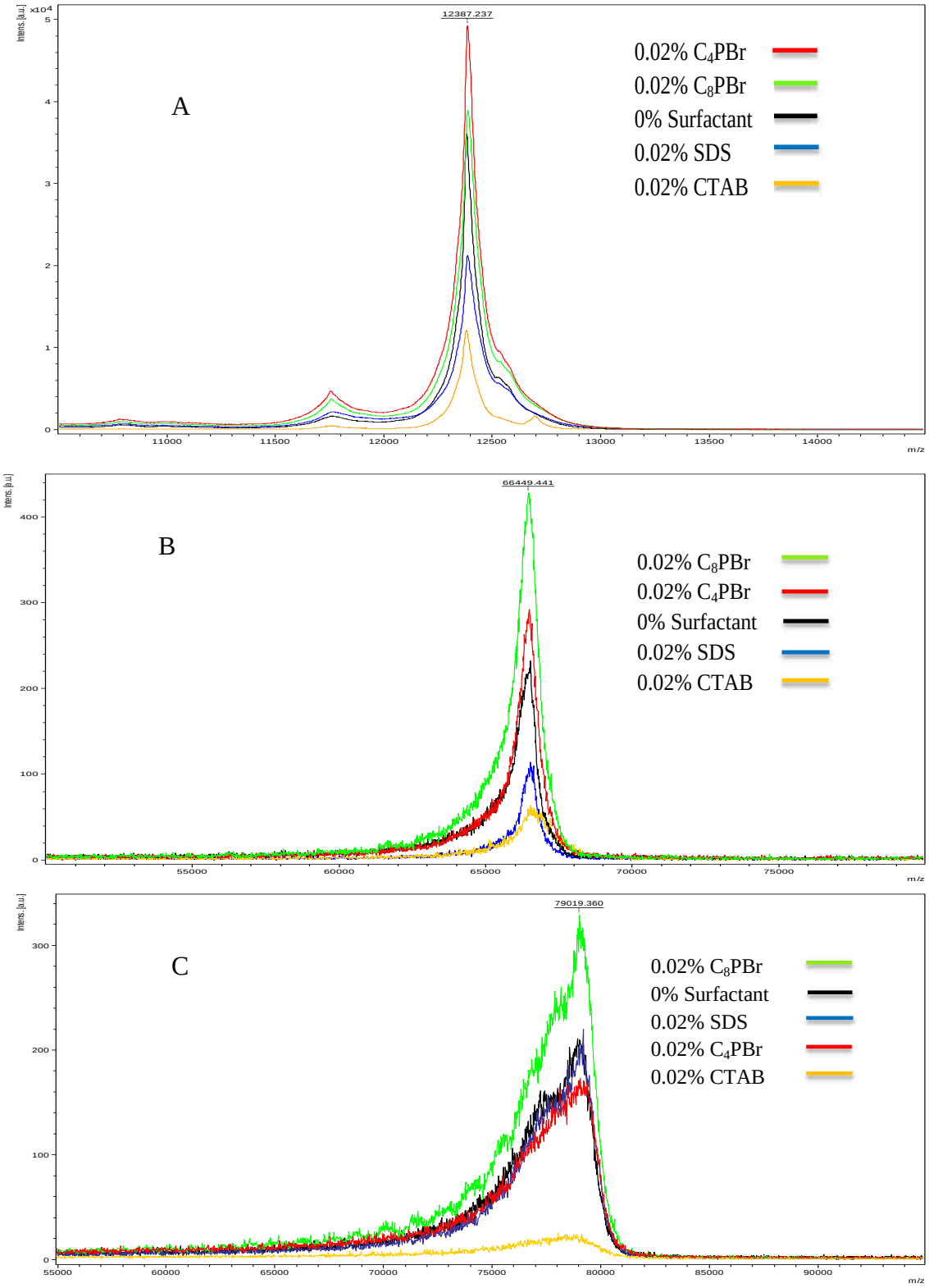


Figure 5.1. MALDI mass spectra of (A) Cyt c (B) BSA and (C) Tr (1.0×10^{-5} M, 10 pmol on the sample target) in the presence of different surfactants

MALDI-MS; rather, ionic interactions between ILS and proteins may play a larger role than the micelle. Since both ILS and CTAB are cationic surfactants, a comparison between the two may aid in an understanding of the role of cationic surfactants in MALDI-MS measurements. As observed in Figure 5.1, all proteins showed enhanced protein signals in the presence of 0.02% ILS as compared to the same concentration of CTAB. Since the anion is bromide in both surfactants, variation in MALDI signal is attributed to differences in cationic moiety and dissociated properties of ILS.

5.3.3. Concentration Effects

Table 5.2. MALDI-MS signal intensities of BSA, Cyt c, and Tr proteins in the presence of different concentrations of surfactants. (ND*-A signal was not detected at this concentration of surfactant.)

Conc.	**Cyt c Intensity (a.u.)**				**BSA Intensity (a.u.)**				**Tr Intensity (a.u.)**			
(w/v)	C4	C8	SDS	CTAB	C4	C8	SDS	CTAB	C4	C8	SDS	CTAB
0%	35067	35067	35067	35067	224	224	224	224	211	211	211	211
0.02%	49289	38694	21202	12093	292	428	114	61	173	329	204	24
0.05%	13500	5000	0	1000	220	331	53	11	212	381	51	8
0.1%	12000	7000	1500	510	163	130	124	5	111	472	64	7
0.5%	6000	1500	6000	ND*	95	27	90	ND*	272	286	5	ND*

Concentration effects of ILS for protein MALDI-MS signal intensities were also investigated. In this study, 0%, 0.02%, 0.05%, 0.1%, and 0.5% (w/v) concentrations of four different surfactants: C_4PBr, C_8PBr, SDS, and CTAB were used. The resultant signal intensities (in arbitrary units) are shown in Table 5.2. In the case of Cyt c and BSA proteins, highest MALDI-MS signal intensity was observed at 0.02% (w/v) of ILS concentration (both C_4PBr and C_8PBr). In contrast, MALDI-MS signals for Cyt c and BSA proteins were decreased at 0.02% (w/v) of SDS as compared to 0% (w/v) of surfactant and essentially suppressed at 0.05% (w/v) SDS. However, these signals recovered with increasing concentrations of SDS. A similar

observation has been reported for Cyt c and BSA protein MALDI-MS signal suppression at SDS subcritical concentrations (0.05%-0.1% (w/v)) by Amado et al.[27] This decrease in MALDI-MS signal at a subcritical concentration of SDS has been attributed to partial precipitation of proteins with SDS monomers in solution. Higher MALDI-MS signal for these proteins was achieved when an SDS concentration was used above its CMC value (0.23% (w/v)) due to the micelle formation. Hence, SDS micelle formation is claimed to be necessary for high protein signal intensity in MALDI-MS. However, other concentrations of SDS were not observed to achieve a significant increase of mass spectral signal intensity for any proteins in comparison to the 0% SDS solution. But, in our study, ILS caused a significant increase in MALDI-MS signal as compared to the absence of surfactant.

The highest signal intensity for Tr was acquired in the presence of 0.5% (w/v) C_4PBr and 0.1% (w/v) C_8PBr. Again for Tr protein, SDS additions exhibited similar behavior of diminished signal compared to 0% (w/v) as noted earlier for other proteins. The effect of cationic surfactant, CTAB, on MALDI intensity revealed that its addition caused a gradual decrease in signal as the concentration of surfactant increased from 0-0.1% (w/v). A protein signal was not observed at 0.5% (w/v) CTAB concentration.

After investigating the response of different surfactants, it was observed that ILS aid in MALDI-MS signal enhancement of proteins below the CMC value as compared to MALDI mass spectra free from surfactant. We have also examined ILS concentration effects on MALDI mass spectra above the CMC, and observed that protein signals tend to diminish at those concentrations (results not shown). However, normal surfactants can be used above the CMC value to obtain a better signal but, signals are not as good with 0% surfactant. In particular, SDS tends to diminish the signal at concentrations below the CMC, which suggests that the MALDI-

MS protein signal in the presence of SDS depends on the extent of micelle formation. In contrast, protein signal enhancement in the presence of ILS is attributed to ionic interactions.

5.3.4. Sample Homogeneity

Sample spot homogeneity was compared for all surfactants: C_4PBr, C_8PBr, SDS, and CTAB at low concentration (0.02% (w/v)). This evaluation focused on homogeneity of MALDI-MS sample spots. The study was performed by using a previously described procedure.[12] The sample spot was virtually divided into 9 regions and the laser was shot at the center of the each region to obtain a representative MALDI mass spectrum. The mass spectral intensities of the 9 regions in each sample spot were used for calculation of range, average, and relative standard deviation (RSD) (results are shown in Table 5.3).

Table 5.3. Range, average, and relative standard deviations (R.S.D.) of protein MALDI-MS intensities with different surfactants

Surfactant	**Intensity of Cyt C [M+H$^+$] (a.u.)**			**Intensity of BSA [M+H$^+$] (a.u.)**			**Intensity of Tr [M+H$^+$] (a.u.)**		
	Range	**Average**	**R.S.D. (%)**	**Range**	**Average**	**R.S.D. (%)**	**Range**	**Average**	**R.S.D. (%)**
Blank (0%)	13 699 – 19 757	17 243	12	63-613	298	48	0 - 11	3	143
0.02% C4PBr	15 360 – 37 930	24 391	30	51-292	131	58	16 - 99	36	68
0.02% C8PBr	5 424 – 1 4310	9 912	24	206-428	299	22	104- 330	146	49
0.02% SDS	0-1413	254	196	114-344	218	32	14 – 75	25	75
0.02% CTAB	4 422 – 14 798	8740	34	16-61	27	49	0 - 16	4	166

The maximum intensity for Cyt c was exhibited with C_4PBr, while the maximum intensities for BSA and Tr proteins were obtained with C_8PBr ILS, which are consistent with our previous

study. This suggests that the trends previously observed are reproducible. Computed RSD values were relatively low with two ILS as compared to normal surfactants, SDS and CTAB. RSD values can be used as measures of sample uniformity in MALDI-MS experiments.[39] Evaluation of RSD values indicates that uniformity of distribution within the sample spot is better in the presence of ILS. In other words, ILS provide greater homogeneity.

Further clarification of sample homogeneity of MALDI-MS sample spots for Cyt c is captured by use of an optical microscope. Figure 5.2 shows microscopic images of protein sample spots in the presence of different surfactants at the same concentration (0.02% (w/v)).

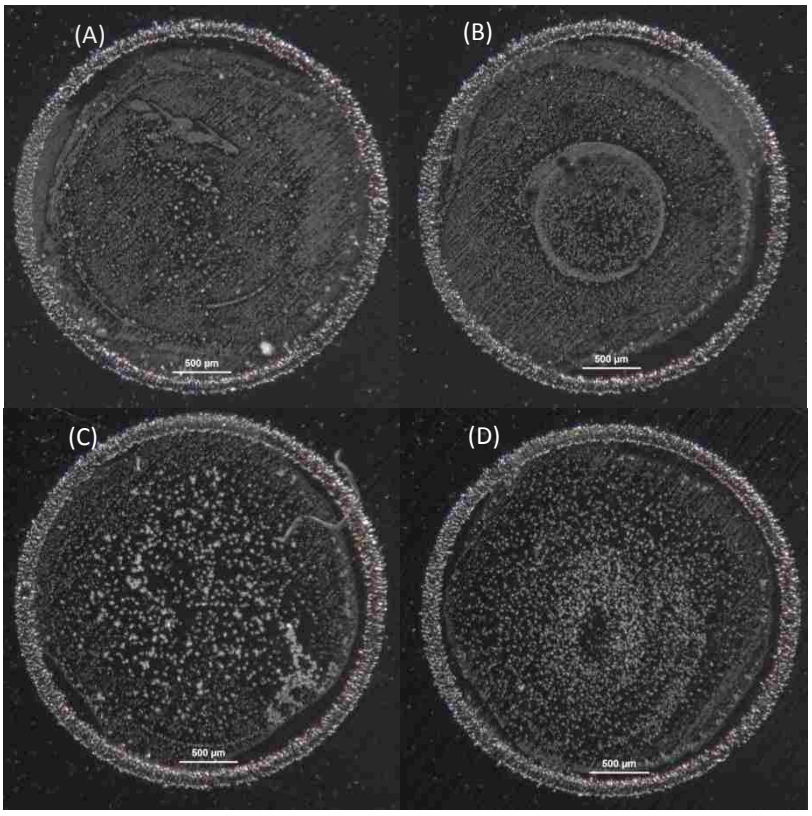


Figure 5.2. Optical microscopic images of Cyt C protein sample spots on the MALDI target plate. The protein sample has prepared by using different surfactants; (A) C_4PBr (B) C_8PBr (C) SDS (D) CTAB

An apparent difference in sample distribution is observed between sample spots containing ILS and conventional surfactants. C_4PBr and C_8PBr containing protein sample spots were noticeably transparent and continuous with a thin layer of small sized crystals (Figure 5.2 (A and B)). The thin layer covered a large area of sample spot and very little spaces were observed on the metal surface. In contrast, SDS and CTAB sample spots were visible with clusters of crystals, which were larger in size (Figure 5.2 (C and D)). In particular, for SDS sample spot, the surface coverage by crystals on the sample plate was very low.

The above observations provide a possible reason for the higher sensitivity observed for ILS. In order to act as signal enhancing agents in MALDI-MS, ILS either have to possess MALDI-matrix properties or act as protein-matrix crystallization modulators. Since no absorbance of light occurs for these ILS at the corresponding wavelength (355 nm) of the Nd:YAG laser (see appendix B for the absorbance spectra), this factor can be ruled out. In the presence of ILS, homogeneous sample distribution of the sample spot revealed an even distribution of matrix molecules throughout. This leads to an increase in the amount of matrix molecules, which are exposed to the surface and thus can absorb greater laser energy. Furthermore, high surface coverage on the sample spot by a very small thin crystal layer ensures the presence of a higher number of protein-matrix interaction sites and more efficient proton transfer from matrix to analyte. As a result, the number of protonated analyte molecules, which can be detected by use of the mass analyzer becomes greater. Finally, the signal intensity, which is a function of the amount of charged analytes, becomes higher in ILS protein samples, compared to SDS and CTAB protein samples.

5.3.5. Profile Mode Analysis of Rat Brain Tissue in the Presence of ILS

Finally, the effects of ILS for MALDI-MS profiling were evaluated using rat brain tissue samples. In this regard, two consecutive tissue sections were chosen to apply the MALDI matrix. MALDI profiling mass spectra acquired in this study are shown in Figure 5.3. The same location in two tissue sections was used for spectral peak comparison in the presence and absence of ILS. The presence of both C_4PBr and C_8PBr on the matrix increased the number of peaks in 29% and 111% respectively, compared to no ILS present in the matrix. It shows the efficiency of ILS in extracting biomolecules from tissue matrices.

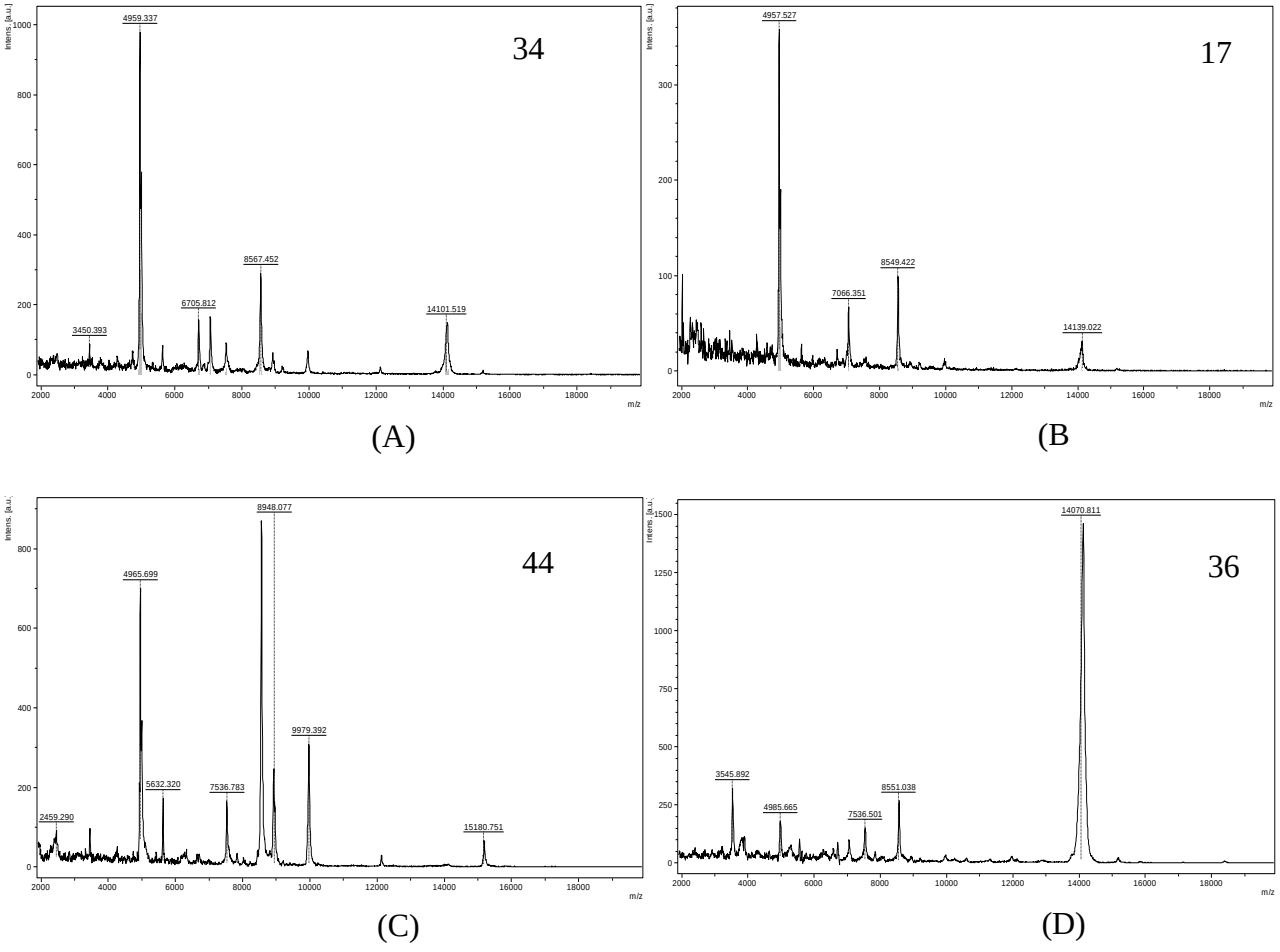


Figure 5.3. MALDI profiling mass spectra without ILS (upper) and with ILS (below) for C_4PBr (A and C) and C_8PBr (B and D)

The addition of C_4PBr resulted to obtain 39 new peaks out of 44 total peaks detected, whereas almost all the spectra (34 out of 36) acquired in the presence of C_8PBr were new peaks. The intensity of those new peaks shown by C_8PBr ILS was significantly greater than the peaks appeared from the control tissue sample. In terms of enhancing number of peaks, intensity of peaks, and acquiring new peaks, C_8PBr showed better performance than C_4PBr. This is due to strong hydrophobic interactions between tissue sample and C_8PBr surfactant, which enabled efficient extraction of hydrophobic proteins there by co-crystallization with the matrix. In contrast, C_4PBr with short alkyl chain makes weak hydrophobic interactions with tissue sample and extraction of less number of proteins.

5.4. Conclusions

The presence of trace amounts of conventional surfactants in protein samples results in poor MALDI-MS signal quality as compared to the absence of surfactants. However, surfactants based on ILS as matrix additives in protein sample analysis exhibited improved protein characterizations by use of MALDI-MS compared to the absence of surfactants. Concentration studies revealed that maximum MALDI-MS signal can be obtained at 0.02% (w/v) ILS, which is a lower concentration than the CMC. Hence, micelle formation is not necessary for MALDI-MS signal enhancement effect. In contrast, normal surfactants, i.e. SDS and CTAB, exhibited reduced MALDI-MS signal intensities at low concentrations (0.02% (w/v)) as compared to 0% surfactant. The concentration of SDS had to be increased above the CMC in order to obtain a considerable MALDI-MS signal for proteins, indicating that micelle formation is necessary to achieve better signal quality. However, signal could not be completely recovered even at higher concentrations of SDS compared to the absence of surfactant. Moreover, the presence of ILS represented better shot-to-shot reproducibility and acted as protein-matrix crystallization

modulators with better ionic interactions through formation of very homogeneous sample spots. As a result, high sensitivity in MALDI mass spectra could be achieved with ILS. It should be noted that this difference in behavior between ILS and normal surfactants suggest that ILS are more than just a collection of ions in solution. Rather they have distinct properties even when dissolved in aqueous solution.

Since the same ILS have been used as surfactants in polyacrylamide gel electrophoretic protein separations, high sensitivity in MALDI-MS analysis enhances applicability of these compounds in proteomics. In this case, the presence of low amounts of ILS in protein samples to be analyzed by MALDI-MS is not a concern, unlike other types of surfactants including SDS. Therefore, we conclude that, use of ILS-based surfactants will save time and cost in proteomics research, due to no need for removal of ILS from protein samples for purification.

Finally, MALDI profiling mode experiments showed better interactions of ILS with tissue samples to extract proteins. Furthermore, this suggests the applicability of ILS in MALDI imaging MS for analysis of novel proteins in tissue samples.

5.5. References

1. Vuckovic, D.; Dagley, L. F.; Purcell, A. W.; Emili, A., Membrane proteomics by high performance liquid chromatography–tandem mass spectrometry: analytical approaches and challenges. *Proteomics* **2013,** *13* (3-4), 404-423.

2. Alonso, A.; Marsal, S.; Julià, A., Analytical methods in untargeted metabolomics: state of the art in 2015. *Frontiers in bioengineering and biotechnology* **2015,** *3*, 23.

3. Walczak, J.; Bocian, S.; Trziszka, T.; Buszewski, B., Hyphenated Analytical Methods in Determination of Biologically Active Compounds in Hen's Eggs. *Critical Reviews in Analytical Chemistry* **2016,** *46* (3), 201-212.

4. Mateos, J.; Carneiro, I.; Corrales, F.; Elortza, F.; Paradela, A.; del Pino, M. S.; Iloro, I.; Marcilla, M.; Mora, M. I.; Valero, L., Multicentric study of the effect of pre-analytical variables

in the quality of plasma samples stored in biobanks using different complementary proteomic methods. *Journal of Proteomics* **2017,** *150*, 109-120.

5. Cramer, R.; Pirkl, A.; Hillenkamp, F.; Dreisewerd, K., Liquid AP-UV-MALDI Enables Stable Ion Yields of Multiply Charged Peptide and Protein Ions for Sensitive Analysis by Mass Spectrometry. *Angewandte Chemie International Edition* **2013,** *52* (8), 2364-2367.

6. Cramer, R.; Karas, M.; Jaskolla, T. W., Enhanced MALDI MS sensitivity by weak base additives and glycerol sample coating. *Analytical chemistry* **2013,** *86* (1), 744-751.

7. Wang, S.; Xiao, Z.; Xiao, C.; Wang, H.; Wang, B.; Li, Y.; Chen, X.; Guo, X., (E)-Propyl α-Cyano-4-Hydroxyl Cinnamylate: A High Sensitive and Salt Tolerant Matrix for Intact Protein Profiling by MALDI Mass Spectrometry. *Journal of The American Society for Mass Spectrometry* **2016,** *27* (4), 709-718.

8. Xu, Y.; Bruening, M. L.; Watson, J. T., Non-specific, on-probe cleanup methods for MALDI-MS samples. *Mass spectrometry reviews* **2003,** *22* (6), 429-440.

9. Pham, M. D.; Yu, S. S.-F.; Han, C.-C.; Chan, S. I., Improved mass spectrometric analysis of membrane proteins based on rapid and versatile sample preparation on nanodiamond particles. *Analytical chemistry* **2013,** *85* (14), 6748-6755.

10. Zhang, N.; Li, L., Ammonium dodecyl sulfate as an alternative to sodium dodecyl sulfate for protein sample preparation with improved performance in MALDI mass spectrometry. *Analytical chemistry* **2002,** *74* (7), 1729-1736.

11. Chang, Y.-H.; Gregorich, Z. R.; Chen, A. J.; Hwang, L.; Guner, H.; Yu, D.; Zhang, J.; Ge, Y., A New Mass Spectrometry-compatible Degradable Surfactant for Tissue Proteomics. *Journal of proteome research* **2015,** *14* (3), 1587.

12. Shibata, M.; Koeda, S.; Noji, T.; Kawakami, K.; Ido, Y.; Amano, Y.; Umezawa, N.; Higuchi, T.; Dewa, T.; Itoh, S., Design of New Extraction Surfactants for Membrane Proteins from Peptide Gemini Surfactants. *Bioconjugate Chemistry* **2016,** *27* (10), 2469-2479.

13. Reis, M. F. T.; Bonomo, R. C. F.; de Sousa, R. d. C. S.; Veloso, C. M.; Fontan, R. d. C. I.; Gandolfi, O. R. R., Optimization of protein extraction process from jackfruit seed flour by reverse micelle system. *Acta Scientiarum. Technology* **2016,** *38* (3), 283.

14. Ward, K.; Cheng, S. I.; Stuckey, D. C., Protein separation using non-ionic and cationic surfactant precipitation. *Journal of Chemical Technology and Biotechnology* **2016,** *91* (10), 2563-2567.

15. Yamada, H.; Matsumura, C.; Yamada, K.; Teshima, K.; Hiroshima, T.; Kinoshita, M.; Suzuki, S.; Kakehi, K., Combination of SDS-PAGE and intact mass analysis for rapid determination of heterogeneities in monoclonal antibody therapeutics. *Electrophoresis* **2017**.

16. Banstola, B.; Grodner, E. T.; Cao, F.; Donnarumma, F.; Murray, K. K., Systematic assessment of surfactants for matrix-assisted laser desorption/ionization mass spectrometry imaging. *Analytica Chimica Acta* **2017,** *963*, 76-82.

17. Chandrababu, K. B.; Dutta, K.; Lokappa, S. B.; Ndao, M.; Evans, J. S.; Moradian-Oldak, J., Structural adaptation of tooth enamel protein amelogenin in the presence of SDS micelles. *Biopolymers* **2014,** *101* (5), 525-535.

18. Saveliev, S. V.; Woodroofe, C. C.; Sabat, G.; Adams, C. M.; Klaubert, D.; Wood, K.; Urh, M., Mass spectrometry compatible surfactant for optimized in-gel protein digestion. *Analytical chemistry* **2013,** *85* (2), 907-914.

19. Antharavally, B. S.; Mallia, K. A.; Rosenblatt, M. M.; Salunkhe, A. M.; Rogers, J. C.; Haney, P.; Haghdoost, N., Efficient removal of detergents from proteins and peptides in a spin column format. *Analytical biochemistry* **2011,** *416* (1), 39-44.

20. Botelho, D.; Wall, M. J.; Vieira, D. B.; Fitzsimmons, S.; Liu, F.; Doucette, A., Top-down and bottom-up proteomics of SDS-containing solutions following mass-based separation. *Journal of proteome research* **2010,** *9* (6), 2863-2870.

21. Caprioli, R. M.; Porter, N. A.; Norris, J. L., Cleavable surfactants and methods of use thereof. Google Patents: 2014.

22. Antharavally, B. S.; Mallia, A. K.; Salunkhe, A., Detergent removal from protein samples prior to mass spectrometry analysis. Google Patents: 2013.

23. Sánchez-Hernández, L.; Montealegre, C.; Kiessig, S.; Moritz, B.; Neusüß, C., In-capillary approach to eliminate SDS interferences in antibody analysis by capillary electrophoresis coupled to mass spectrometry. *Electrophoresis* **2017**.

24. Canas, B.; Pineiro, C.; Calvo, E.; López-Ferrer, D.; Gallardo, J. M., Trends in sample preparation for classical and second generation proteomics. *Journal of Chromatography A* **2007,** *1153* (1), 235-258.

25. Puchades, M.; Westman, A.; Blennow, K.; Davidsson, P., Removal of sodium dodecyl sulfate from protein samples prior to matrix-assisted laser desorption/ionization mass spectrometry. *Rapid communications in mass spectrometry* **1999,** (13), 344-9.

26. Funk, J.; Li, X.; Franz, T., Threshold values for detergents in protein and peptide samples for mass spectrometry. *Rapid communications in mass spectrometry* **2005,** *19* (20), 2986-2988.

27. Amado, F. M.; Santana-Marques, M. G.; Ferrer-Correia, A.; Tomer, K. B., Analysis of peptide and protein samples containing surfactants by MALDI-MS. *Analytical Chemistry* **1997,** *69* (6), 1102-1106.

28. Zhang, N.; Li, L., Effects of common surfactants on protein digestion and matrix-assisted laser desorption/ionization mass spectrometric analysis of the digested peptides using two-layer sample preparation. *Rapid Communications in Mass Spectrometry* **2004,** *18* (8), 889-896.

29. Ospinal-Jiménez, M. n.; Pozzo, D. C., Anionic Branched Surfactants as Alternative Denaturing Agents for Protein Separations. *Langmuir* **2014,** *30* (5), 1351-1360.

30. Gillespie, D. L.; Aguirre, M. T.; Ravichandran, S.; Leishman, L. L.; Berrondo, C.; Gamboa, J. T.; Wang, L.; King, R.; Wang, X.; Tan, M., RNA interference targeting hypoxia-inducible factor 1α via a novel multifunctional surfactant attenuates glioma growth in an intracranial mouse model. *Journal of neurosurgery* **2015,** *122* (2), 331-341.

31. Wang, S.; Xiong, N.; Dong, X.-Y.; Sun, Y., A novel nickel-chelated surfactant for affinity-based aqueous two-phase micellar extraction of histidine-rich protein. *Journal of Chromatography A* **2013,** *1320*, 118-124.

32. Guo, Z.; Zhang, Q.; Zou, H.; Guo, B.; Ni, J., A method for the analysis of low-mass molecules by MALDI-TOF mass spectrometry. *Analytical chemistry* **2002,** *74* (7), 1637-1641.

33. Mayer, U.; Ungerer, N.; Klimmeck, D.; Warnken, U.; Schnölzer, M.; Frings, S.; Möhrlen, F., Proteomic analysis of a membrane preparation from rat olfactory sensory cilia. *Chemical senses* **2008,** *33* (2), 145-162.

34. Rosinke, B.; Strupat, K.; Hillenkamp, F.; Rosenbusch, J.; Dencher, N.; Krüger, U.; Galla, H. J., Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) of membrane proteins and non-covalent complexes. *Journal of Mass Spectrometry* **1995,** *30* (10), 1462-1468.

35. Hasan, F.; Vidanapathirana, P.; Das, S.; Fernand, V. E.; Siraj, N.; Losso, J. N.; Warner, I. M., Ionic liquids as buffer additives in ionic liquid-polyacrylamide gel electrophoresis separation of mixtures of low and high molecular weight proteins. *RSC Advances* **2015,** *5* (85), 69229-69237.

36. Tholey, A.; Heinzle, E., Ionic (liquid) matrices for matrix-assisted laser desorption/ionization mass spectrometry—applications and perspectives. *Analytical and bioanalytical chemistry* **2006,** *386* (1), 24-37.

37. Abdelhamid, H. N.; Gopal, J.; Wu, H.-F., Synthesis and application of ionic liquid matrices (ILMs) for effective pathogenic bacteria analysis in matrix assisted laser desorption/ionization (MALDI-MS). *Analytica chimica acta* **2013,** *767*, 104-111.

38. Love, L. C.; Habarta, J. G.; Dorsey, J. G., The micelle-analytical chemistry interface. *Analytical Chemistry* **1984,** *56* (11), 1132A-1148A.

39. Armstrong, D. W.; Zhang, L.-K.; He, L.; Gross, M. L., Ionic liquids as matrixes for matrix-assisted laser desorption/ionization mass spectrometry. *Analytical chemistry* **2001,** *73* (15), 3679-3686.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

6.1. Conclusions

Application of ionic liquid surfactants (ILS) based on *N*-alkyl-4-methylpyridinium bromide were evaluated in anionic polyacrylamide gel electrophoresis (PAGE), cationic PAGE, two dimensional (2D) isoelectric focusing (IEF)/ILS-PAGE, and matrix assisted laser desorption ionization (MALDI)-mass spectrometric (MS) analysis of proteins. Ionic liquids have promising characteristics such as high conductivity, high thermal stability, tunable hydrophobicity, and non-flammability, which made them suitable in analytical applications. Additionally, ILS are ideal candidates to be chosen as buffer additives in PAGE and MALDI-MS as they are amphiphilic compounds with the above mentioned properties giving them distinct advantages.

In chapter 2, synthesis and characterization of *N*-alkyl-4-methylpyridinium bromide-based (C_nPBr where n=4, 8, and 11) ILS were described. These surfactants were applied in sample and running buffers to separate a mixture of acidic, low and high molecular weight proteins using anionic PAGE. C_4PBr-PAGE showed better protein separations at low concentrations of ILS in sample buffer as compared to other two ILS-PAGE. ILS with long alkyl chains reduced the quality of separation due to strong surfactant-protein interactions. In addition, this technique could resolve transferrin and ovalbumin isoforms as multiple bands, whereas anionic SDS-PAGE showed single protein bands for the same proteins. Fluorescence binding studies of the ILS revealed highly cooperative binding of proteins at very low concentrations. In contrast, SDS exhibited negative cooperative binding to proteins. Furthermore, circular dichroism (CD) studies showed strong denaturation properties of the ILS at room temperature. These fluorescence and CD studies brought insights of ILS to be further used in protein separation studies.

In order to improve the separation efficacy for both acidic and basic proteins, the ILS-PAGE technique was modified as cationic PAGE. In this regard, the ILS were used in polyacrylamide discontinuous gel preparation in addition to sample and running buffers at acidic pH media. The proteins migrated from anode to cathode under these conditions. Similar to anionic PAGE, ILS with the shortest alkyl chain length, C_4PBr, showed optimum protein separations at very low concentrations of the surfactant. In contrast, ILS with long alkyl chain, C_{11}PBr, showed poor resolution in separation. Proteins were separated with a high resolution at C_4PBr concentrations of 0.05% (w/v) in gel, 0.01% (w/v) in sample buffer, and 0.1% (w/v) in running buffer. Under the optimum conditions, ribonuclease b glycoprotein was separated into its glycoforms, which appeared as multiple bands. The same protein migrated as a single band in anionic SDS-PAGE, which showed the incompatibility with the glycoprotein. Hence, significant improvement of the cationic ILS-PAGE technique is the high resolution associated with separation of glycoprotein subunits due to charge compatibility.

This was further proved by applying cationic ILS-PAGE in 2D-PAGE separation of complex proteins as described in chapter 4. An increased number of protein subunits, which derived from alpha antitrypsin glycoforms appeared in 2D profile of IEF/ILS-PAGE as compared to the conventional IEF/SDS-PAGE technique. As most glycoproteins possess negative charge heterogeneity due to the presence of sialic acid residues attached to glycans, anionic SDS-PAGE could not resolve those subunits. In these cases, cationic ILS-PAGE can be successfully applied to resolve glycoprotein subunits.

In chapter 5, the application of two ILS, C_4PBr and C_8PBr, as a signal enhancing matrix additives for the detection of proteins by use of MALDI-MS was discussed. The presence of low concentrations (0.02% (w/v)) of ILS in protein samples resulted in increased signal intensity as

compared to 0% surfactants. Conventional surfactants such as SDS and CTAB reduced the protein signal intensity unless they were applied at higher concentrations above the CMC. Additionally, ILS showed better shot-to-shot reproducibility in MALDI-MS acquisitions and more homogeneous crystals were observed as compared to SDS and CTAB. These properties may enhance efficient proton transfer from matrix to protein, which resulted to increase the signal intensity. All these results suggest the compatibility of ILS in MALDI-MS detection of proteins.

6.2. Future Directions

All the fascinating properties of the ILS gave insights to apply them in further studies on PAGE protein separation and MALDI-MS identification. For an instance, anionic SDS-PAGE technique will be modified by applying the ILS in polyacrylamide gels, while using SDS in protein sample and running buffers at SDS-PAGE standard conditions. This ILS-SDS-PAGE technique is expected to improve the separation of low and moderate molecular weight proteins as ILS in gels can stabilize excessive negative charges on SDS-protein complexes during electrophoresis. This would help to enhance the electrophoretic mobility, hence, protein separation resolution.

The application of cationic ILS PAGE for membrane proteins, in particular, glycoproteins separation is another task to be done in future. For this purpose, a series of glycoproteins: Immunoglobulin, Mucin, α1-Acid, and Invertase will be tested using 1D-cationic ILS-PAGE technique. Analysis of glycoforms of these proteins will help to reveal biological conditions of the tissue source. At the optimized conditions of cationic ILS-PAGE, glycoforms are expected to be resolved in a single step similar to Rib b protein.

In addition, novel 2D PAGE methods based on cationic ILS-PAGE will be investigated to separate complex proteins, which have charge heterogeneities. For an example, such proteins will be separated using cationic ILS-PAGE in the first dimension followed by SDS-PAGE in the second dimension. The advantage of using the former in the first dimension is the ability of ILS to solubilize, denature, and resolve a higher number of proteins including those with negative charge heterogeneity. During the second dimension, SDS-PAGE will resolve the proteins into sub categories to obtain a detailed 2D profile. In another study, as a follow up to chapter 4, ILS are expected to be applied in IEF sample buffer in the first dimension to enhance solubility of hydrophobic proteins and to increase the recovery. Then, ILS-PAGE will be performed in the second dimension, in a similar way as described in the same chapter.

Finally, the ILS will be applied in MALDI-imaging MS (IMS) to analyze hydrophobic proteins in tissue samples. MALDI-MS profiling mode results suggested the possibility of interactions between tissue sample and ILS. C_8PBr ILS, in particular, showed the ability to extract new proteins as a result of its hydrophobicity. The application of this ILS in tissue samples analysis using MALDI-IMS would enhance the protein detection sensitivity.

APPENDIX A: SUPPORTING INFORMATION FOR CHAPTER 3

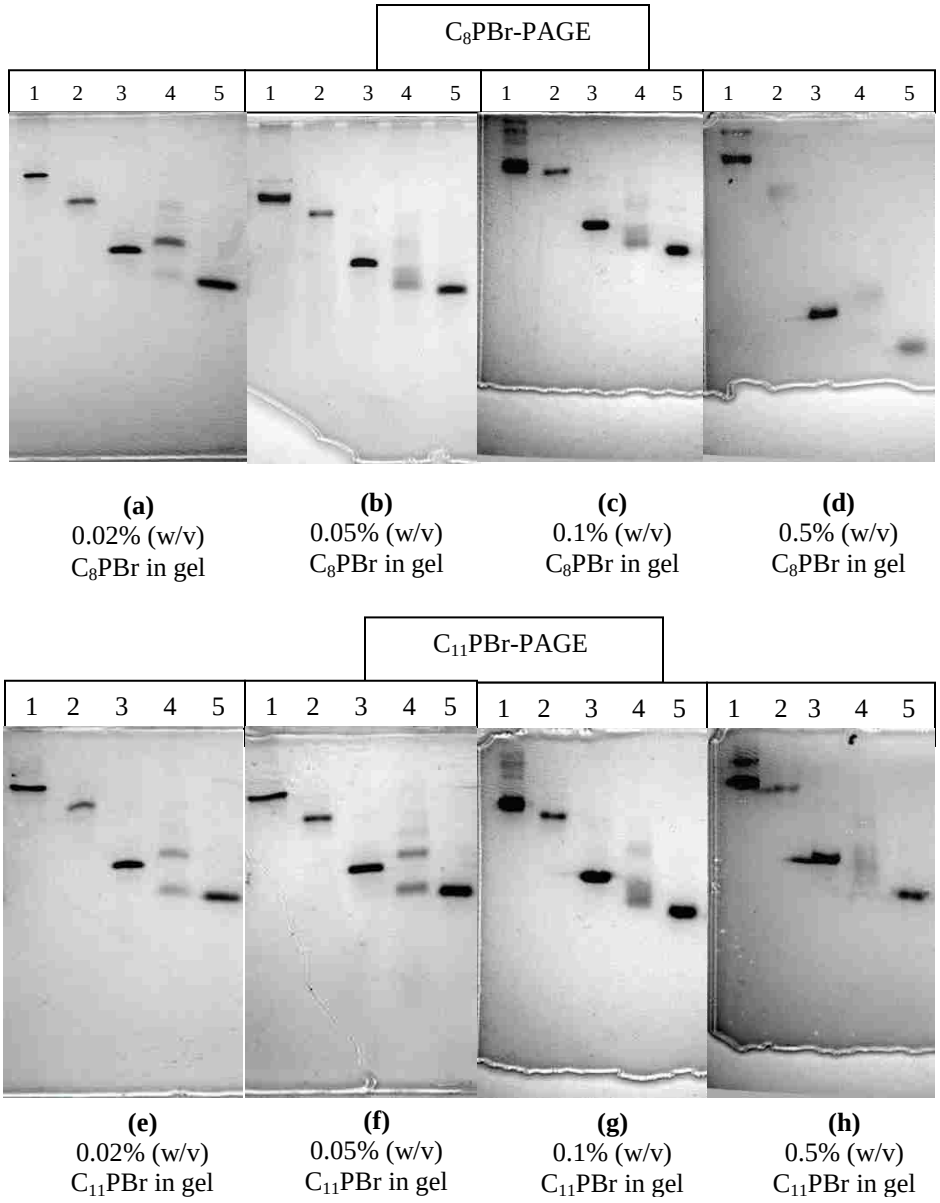


Figure S1. Optimization of IL concentration in polyacrylamide gels for C_8PBr-PAGE and C_{11}PBr-PAGE. Five proteins; (1) BSA (pI-4.8, 66 kDa), (2) β-cas (pI-5.13, 23.6 kDa), (3) Myo (pI-6.8, 16.9 kDa), (4) Rib b (pI-9.45, 14.7 kDa), and (5) Cyt c (pI-10, 12.3 kDa) were used for this study. Different concentrations; 0.02%, 0.05%, 0.1%, and 0.5%, (w/v) of C_8PBr and C_{11}PBr ILs were included in gels of C_8PBr-PAGE and C_{11}PBr-PAGE respectively. Sample buffer concentration (0.02% IL (w/v)) and running buffer concentration (0.05% IL (w/v)) were the same for all eight gels. In C_8PBr-PAGE, 0.05% (w/v) IL concentration, gel (b), was selected as the optimum concentration in gels due to sharp bands obtained in lane 1,2,3, and 5. Even though Rib b protein in lane 4 of the same gel appeared as a diffused band, better migration was observed as compared to 0.02% (w/v) in gel (a), which also showed comparable sharp bands. In C_{11}PBr-PAGE, 0.05% (w/v) IL concentration, gel (f), was selected again as the optimum concentration in gels due to higher resolution and sharp bands obtained as compared to the other concentrations.

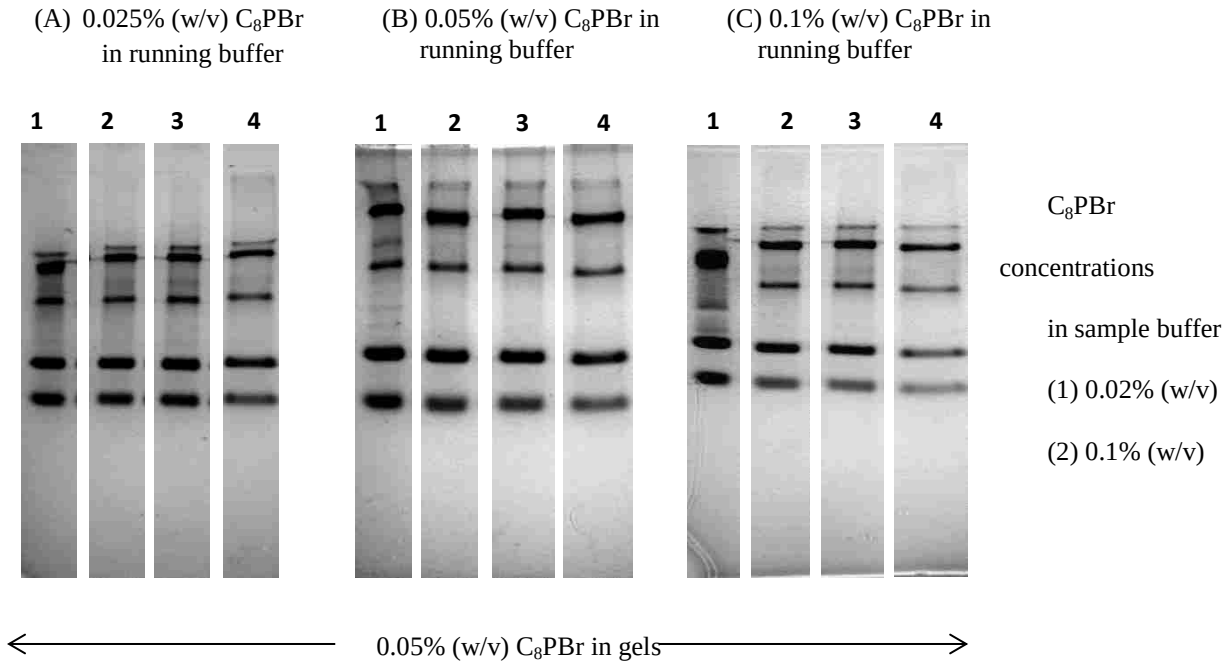


Figure S2. Optimization of C_8PBr IL in running and sample buffers of C_8PBr-PAGE separation of a mixture of five proteins; BSA (pI-4.8, 66 kDa), Ova (pI-4.6, 44.3 kDa), α-Chy (pI-8.8, 25.7 kDa), Myo (pI-16.9, 14.7 kDa), and Cyt c (pI-10, 12.3 kDa). Three different C_8PBr concentrations in running buffers; (A) 0.025% (w/v) (B) 0.05% (w/v), and (C) 0.1% (w/v) were used. The IL concentration in sample buffers were varied as; (1) 0.02%, (2) 0.1%, (3) 0.5%, and (4) 2% (w/v).. Same concentration of C_8PBr (0.05% (w/v)) was included in all the gels. Five resolved, sharp protein bands were observed in B-1 (at 0.05% (w/v) IL in running buffer, 0.02% (w/v) IL in sample buffer, and 0.05% (w/v) IL in gel). So those conditions were used as the optimum concentrations for C_8PBr-PAGE.

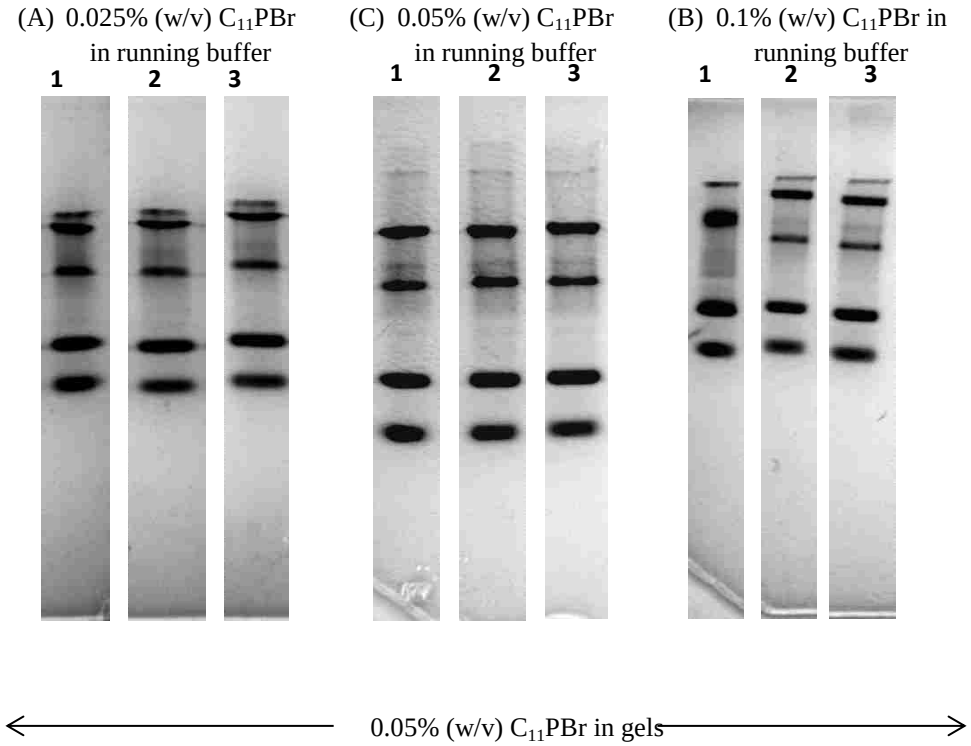


Figure S3. Optimization of C_{11}PBr IL concentration in running and sample buffers of C_{11}PBr -PAGE separation of a mixture of five proteins; BSA (pI-4.8, 66 kDa), Ova (pI-4.6, 44.3 kDa), α-Chy (pI-8.8, 25.7 kDa), Myo (pI-16.9, 14.7 kDa), and Cyt c (pI-10, 12.3 kDa). Three different C_{11}PBr concentrations in running buffers; (A) 0.025% (w/v) (B) 0.05% (w/v), and (C) 0.1% (w/v) were used. The IL concentration in sample buffers were varied as; (1) 0.02%, (2) 0.05%, and (3) 0.1% (w/v). Same concentration of C_{11}PBr (0.05% (w/v)) was included in all the gels. The best resolution was achieved at 0.05% (w/v) concentration of IL in running buffer and 0.02% (w/v) IL in sample buffer. So those conditions were used as the optimum concentrations for C_{11}PBr -PAGE.

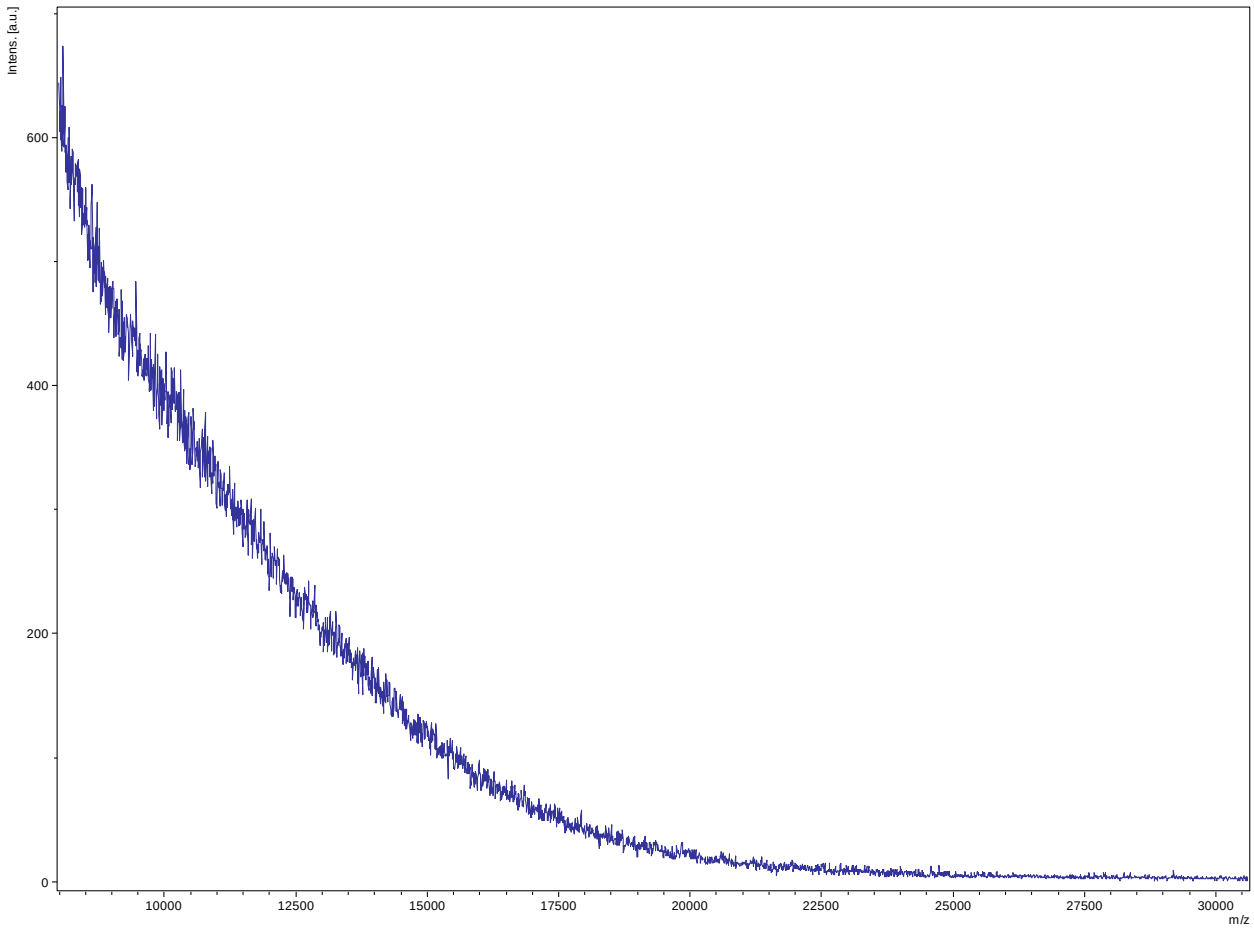


Figure S4. MALDI mass spectra obtained for the protein band observed on the top of rib b lane in SDS-PAGE. No signal was observed in the mass range of 14 kDa-16 kDa, which shows the absence of rib b glycoforms in that protein band

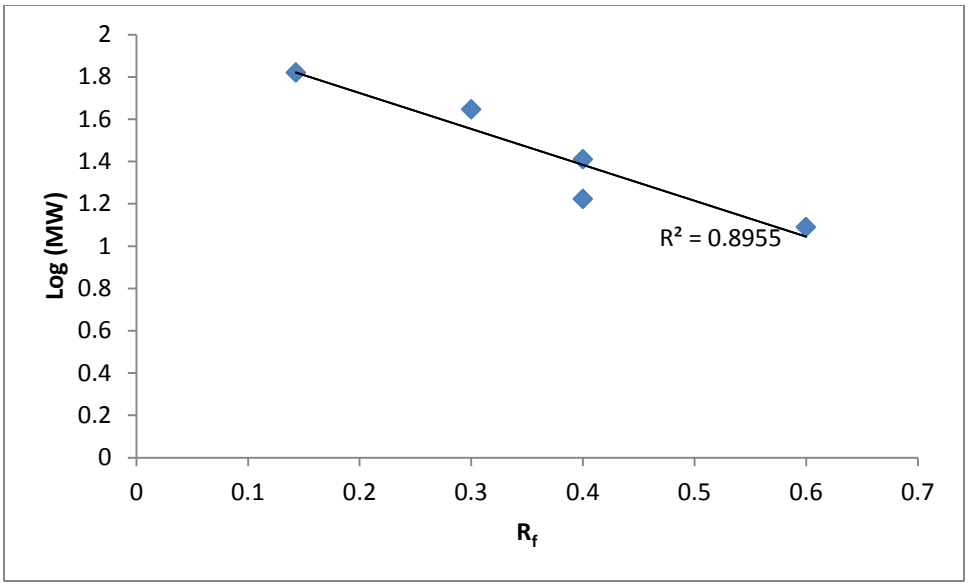
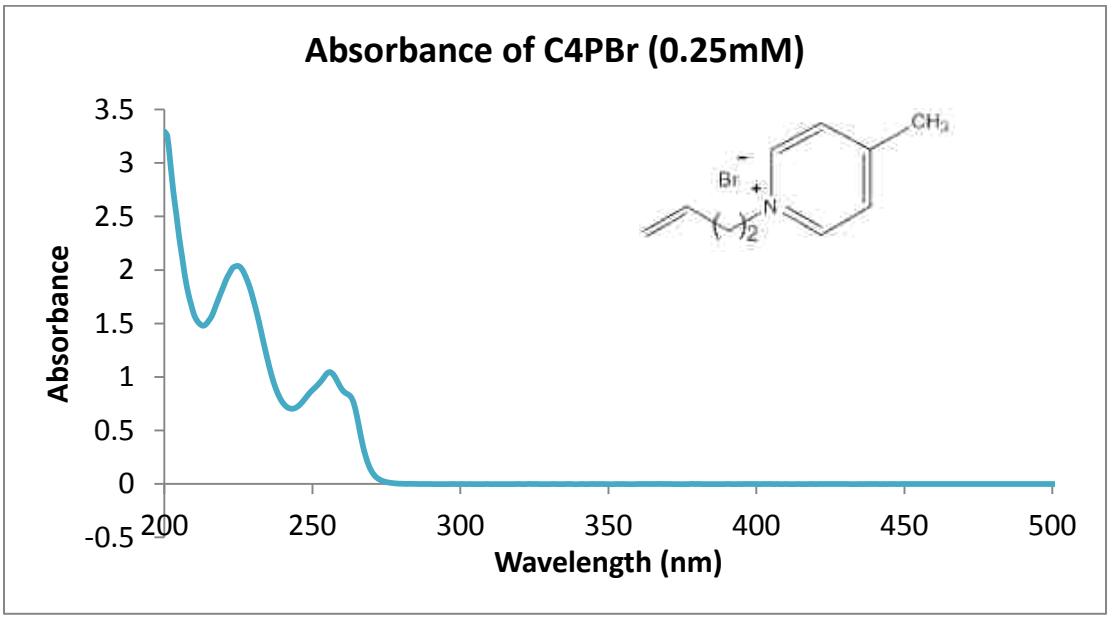
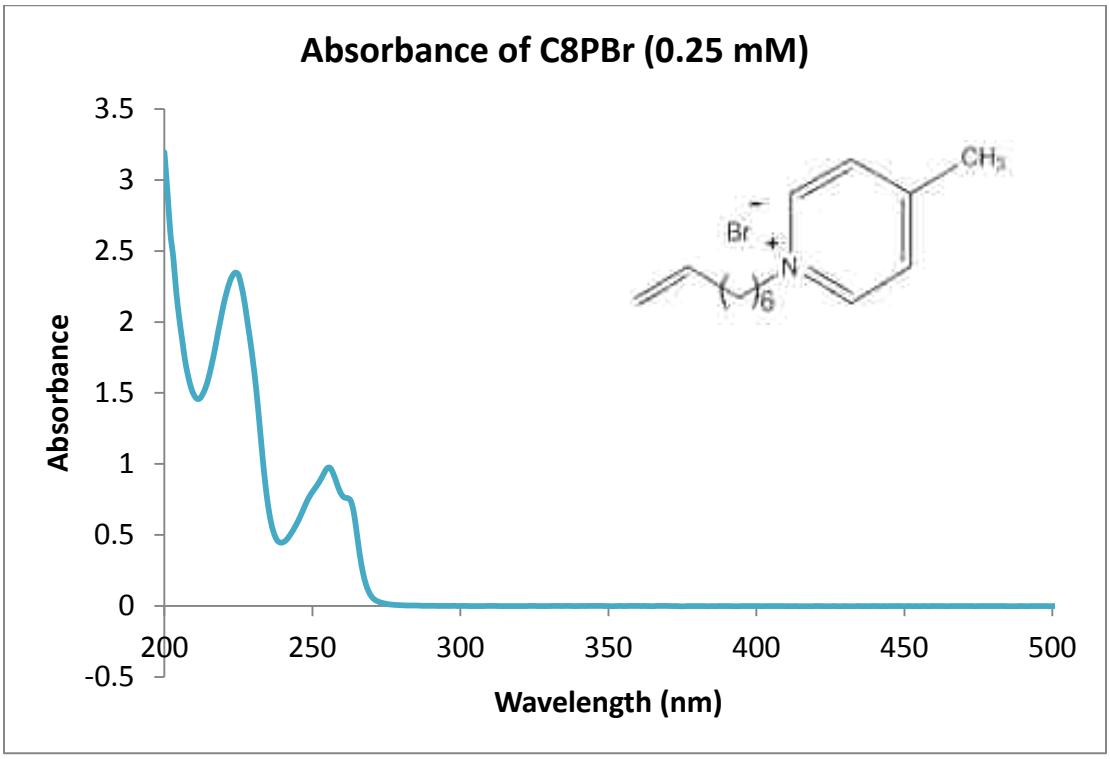


Figure S5. The plot of R_f vs. log molecular weight of proteins; BSA (pI-4.8, 66 kDa), Ova (pI-4.6, 44.3 kDa), α-Chy (pI-8.8, 25.7 kDa), Myo (pI-16.9, 16.7 kDa), and Cyt c (pI-10, 12.3 kDa) separated by SDS-PAGE at Laemmli conditions

APPENDIX B: SUPPORTING INFORMATION FOR CHAPTER 5





APPENDIX C: LETTER OF PERMISSION

RE: Reuse of an article as a theses chapter

Gill Cockhead <CockheadG@rsc.org>

Wed 4/12/2017 1:08 AM

To: Punprabhashi Vidanapathirana <pvidan1@lsu.edu>;

Dear Punprabhashi

The Royal Society of Chemistry (RSC) hereby grants permission for the use of your paper(s) specified below in the printed and microfilm version of your thesis. You may also make available the PDF version of your paper(s) that the RSC sent to the corresponding author(s) of your paper(s) upon publication of the paper(s) in the following ways: in your thesis via any website that your university may have for the deposition of theses, via your university's Intranet or via your own personal website. We are however unable to grant you permission to include the PDF version of the paper(s) on its own in your institutional repository. The Royal Society of Chemistry is a signatory to the STM Guidelines on Permissions (available on request).

Please note that if the material specified below or any part of it appears with credit or acknowledgement to a third party then you must also secure permission from that third party before reproducing that material.

Please ensure that the thesis states the following:

Reproduced by permission of The Royal Society of Chemistry

and include a link to the paper on the Royal Society of Chemistry's website.

Please ensure that your co-authors are aware that you are including the paper in your thesis.

Regards

Gill Cockhead
Publishing Contracts & Copyright Executive

Gill Cockhead
Publishing Contracts & Copyright Executive
Royal Society of Chemistry,
Thomas Graham House,
Science Park, Milton Road,
Cambridge, CB4 0WF, UK
Tel +44 (0) 1223 432134

Follow the Royal Society of Chemistry:
www.rsc.org/follow

Winner of The Queen's Award for Enterprise, International Trade 2013

-----Original Message-----
From: pvidan1@lsu.edu [mailto:pvidan1@lsu.edu]
Sent: 11 April 2017 17:30
To: Gill Cockhead <CockheadG@rsc.org>
Subject: Reuse of an article as a theses chapter

Name: Punprabhashi Vidanapathirana
Message: I want to include a paper which I am the second author (RSC Adv., 2015, 5, 69229), as a chapter in my theses. Can you please let me

know the proper way to get journal permission for that?

VITA

Punprabhashi Vidanapathirana was born in Galle, Sri Lanka in 1985. She is the elder child in the family having one brother. She attended Morawaka primary school for her primary education and received a full scholarship at grade 6 to attend the top ranked girl's school in Sri Lanka, Visakha Vidyalaya. She completed high school in there and started collage at the University of Colombo where she received her Bachelor of Science degree in Computational Chemistry in 2010.

She moved to United States in 2011 and joined the Department of Chemistry at Louisiana State University as a Ph.D. candidate in Analytical Chemistry. She performed her graduate research studies under the supervision of Prof. Isiah M. Warner. In addition, she worked as a graduate teaching assistant in the Department of Chemistry. Punprabhashi anticipates graduating with her Ph.D. from Louisiana State University in August 2017.