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Diffusion and Structure in Complex Fluids: I. Axial Diffusion in Membranes II. Proteins in Ionic Liquids

Malvika Bihari
University of Massachusetts Amherst, mbihari@polysci.umass.edu

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DIFFUSION AND STRUCTURE IN COMPLEX FLUIDS:
I. AXIAL DIFFUSION IN MEMBRANES
II. PROTEINS IN IONIC LIQUIDS

A Dissertation Presented

by

MALVIKA BIHARI

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2010

Polymer Science and Engineering
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II. PROTEINS IN IONIC LIQUIDS

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Approved as to style and content by:

____________________________________
David A. Hoagland, Co-Chair

____________________________________
Thomas P. Russell, Co-Chair

____________________________________
Surita Bhatia, Member

____________________________________
David A. Hoagland, Department Head
Polymer Science and Engineering
DEDICATION

My Parents

Dr. (Mrs) Minakshi Shrivastava and Dr. M.B. Shrivastava
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I would like to thank my advisors Prof. David A. Hoagland & Prof. Thomas P. Russell for their constant interest, encouragement and invaluable cooperation throughout my research work. I am grateful to them for their guidance and help that they bestowed on me right from the inception to the successful completion of this endeavor.

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ABSTRACT

DIFFUSION AND STRUCTURE IN COMPLEX FLUIDS:
I. AXIAL DIFFUSION IN MEMBRANES
II. PROTEINS IN IONIC LIQUIDS

SEPTEMBER 2010

MALVIKA BIHARI, B.TECH., GOVERNMENT CENTRAL TEXTILE INSTITUTE KANPUR

M.TECH., INDIAN INSTITUTE OF TECHNOLOGY DELHI

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor David A. Hoagland and Professor Thomas P. Russell

Geometrically hindered motions of a single large solute (particle or polymer) can be imaged in real time via optical microscopy. The dynamics of fluorescent colloidal particles near surfaces and in porous membranes were monitored using confocal microscopy. A method of analysis to estimate diffusivity of particles in the axial direction by observing their intensity fluctuations was developed. The intensity fluctuations correspond to the Brownian motion of the particles in the axial direction. The method was successful in capturing the hindered diffusion of particles close to surfaces and in pores. This study provides a novel route to monitor the dynamics of particles, including biomacromolecules, near surfaces, through porous substrates and biological tissues.

Ionic liquid (IL) as a medium for room temperature preservation of biomacromolecules has been proposed and, to investigate the possibility,
physicochemical and enzymatic properties of proteins in the neat hydrophilic IL, ethylmethyl imidazolium ethyl sulfate [EMIM][EtSO4] were studied. Spectroscopic techniques were employed to probe the secondary and tertiary structure of proteins whereas light scattering and viscometry were used to estimate the hydrodynamic size. The secondary structure of the protein was retained in the ionic liquid but the tertiary structure was found to change. Alterations in protein conformation/activity were investigated after transfer of the dissolved protein from the IL to buffer. Further, suitability of ionic liquid gels as protein encapsulation and preservation media was assessed.
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1.1 Axial Diffusion in Membranes

Microscopy methods to study polymer and colloid dynamics typically image solute motions in the focal plane, ignoring or preventing solute excursions normal to this plane. Confined particle diffusion has previously been assessed by microscopic methods, such as single particle tracking,\(^1\) evanescent light scattering,\(^2\),\(^3\) reflection interference contrast microscopy,\(^4\) total internal reflection fluorescence microscopy,\(^5\)-\(^9\) and combined optical tweezers/digital video microscopy.\(^10\) Similarly, macroscopic approaches based on measurements of solute flux have been applied to membranes having cylindrical pores.\(^11\)-\(^13\)

For a sphere of radius \(a\) suspended in a bulk fluid of viscosity \(\eta\) and temperature \(T\), the free solution diffusion coefficient, symbolized \(D_0\), is well represented by the Stokes-Einstein equation, \(D_0 = kT/6\pi\eta a\).\(^{14\, 15}\) Inside a pore or near a wall, \(D\) is less than \(D_0\), manifesting hydrodynamic interactions with fixed, no-slip surfaces.\(^{16\, 17}\) Motion perpendicular to a surface is more impeded than motion parallel, so diffusion is anisotropic for particle-surface separations comparable to \(a\).\(^{18}\) Corrections for the influence of fixed surface(s) on sphere hydrodynamic drag were first developed long ago, and these sorts of corrections underlie well-established theories for hindered diffusion of spherical solutes in various sorts of porous materials.\(^7\),\(^16\)

For steady translation of a sphere near a planar surface, perpendicular and parallel hydrodynamic correction factors \(\lambda_\perp\) and \(\lambda_//\) connect \(D_0\) to \(D_\perp\) and \(D_//\),
perpendicular and parallel diffusion coefficients. For \( z/a \) less than \( \sim 5 \), these correction factors are well approximated as a power series in \( a/z \), where \( z \) is the distance from surface to sphere center,

\[
\frac{D_\perp}{D_0} = \lambda_\perp^{-1} = 1 - \frac{9}{8} \left( \frac{a}{z} \right) + \frac{1}{2} \left( \frac{a}{z} \right)^3 + O \left( \frac{a}{z} \right)^4
\]

(1)

and

\[
\frac{D_\parallel}{D_0} = \lambda_\parallel^{-1} = 1 - \frac{9}{16} \left( \frac{a}{z} \right) + \frac{1}{8} \left( \frac{a}{z} \right)^3 + O \left( \frac{a}{z} \right)^4
\]

(2)

Accuracy worsens as proximity of the sphere to surface increases, where hindrance is enhanced and is more easily measured. However, for the range of \( z/a \) experimentally examined, \( \sim 3 < z/a < \sim 30 \), the above approximations are accurate to within \( \pm 1\%-2\% \). Across this span, \( D_\perp \) and \( D_\parallel \) are predicted to deviate from \( D_0 \) by 4 to 36\% and 2 to 20\%, respectively. Previous experiments are in accord with these predictions.\(^{10}\)

Numerous theories for hindered diffusion in geometrically complex environments have been formulated, with much attention directed at porous materials such as ultrafiltration membranes that can be idealized as thin films penetrated by cylindrical pores. Here, hydrodynamic interactions of solutes with fixed surfaces, the pore walls, reduce solute mobility compared to the bulk solution. The axial diffusion coefficient \( D_z \) can be approximated by a power series in \( a/R \) where \( R \) is the pore radius. One classical result is the Renkin equation, derived from the friction expression for steady motion of a sphere along the centerline of a cylindrical pore under the approximation that \( a/R \) is small,
\[
\frac{D_z}{D_0} = K_d = 1 - 2.104 \left( \frac{a}{R} \right) + 2.104 \left( \frac{a}{R} \right)^3 - 0.95 \left( \frac{a}{R} \right)^5 + ... \tag{3}
\]

The approximation holds for \( a/R < -0.4 \). More accurate numerical results, valid for all radial positions, are available for larger \( a/R \).\textsuperscript{19}

Hindered diffusion \textit{inside} a membrane pore has rarely been experimentally assessed. In the membrane field, a more common measurement is the membrane permeability, the product of \( K_d \) with the equilibrium partition coefficient \( \Phi \), given for a cylindrical pore as \( [1-(a/R)]^2 \). The membrane permeability can be deduced from a macroscopic determination of solute diffusion flux, while \( K_d \) requires a microscopic determination of solute motion. The latter requires solute position be resolved at better than the pore length, usually tens of microns or less.

The aim of this study is to examine:

1) The hindered diffusion of Polystyrene latex beads in the axial direction using confocal microscopy.
2) Monitor the hindrance effect in track etched polycarbonate membranes of 2 micron pore size and determine the diffusivity.

The confocal microscope has a unique ability, to detect light coming from the focal region and reject light coming from outside this region. This feature has been utilized to visualize the motion of the beads in the focal region. The intensity fluctuations or “flickering” result from Brownian motion of the beads in the axial direction. These intensity fluctuations were analyzed to extract the diffusivity of beads in the vertical \( (z) \) direction. As a small solute moves axially across the region bisected by the focal plane of the confocal fluorescence microscope, its imaged intensity tracks with the
microscope’s point spread function (PSF), which decays steeply on either side of the focal plane. The intensity, thereby, reveals the axial position, affording an opportunity to study dynamics in the third dimension, if images are collected in a time sequence at a high enough rate. While dependent on optics, the PSF has an axial spread of as little as \(~1 \, \mu\text{m}\). The aim here is to examine the dynamics of small confined particles and polymers via analysis of flickering. This approach has significant advantages over two-dimensional imaging for some common confinement geometries, especially those associated with the approach and passage of solutes through thin film membranes. Many theories for membrane diffusion have been proposed, but rarely has the diffusion mechanism been monitored \textit{in situ}, as described here. The approach can readily be extended to study of solute motions in biological tissues or adhered films, where controlled drug delivery is an important potential application.

In this first implementation, solutes are limited to spherical, narrow size-distributed, fluorescent latex particles of colloidal dimension, and their concentration is extremely dilute, conditions that highlight individual particles of common dynamical and optical properties. Two methods of analysis have been used. The methods have elements of single particle tracking, whereby \(D\) can be obtained by Einstein’s one-dimensional diffusion equation, \(< \Delta z^2 > = 2 D \Delta t\), where \(< \Delta z^2 >\) is the mean-square axial displacement incurred over a time interval \(\Delta t\). However, there are some limitations to the applicability of this formula when viewing the particle dynamics in the axial direction. The width of the PSF defines the focal volume within which the particle motion can be monitored. A sampling bias not admitted in the Einstein equation is generated due to the lack of visibility beyond the boundary represented by the PSF. Only
those particles that are on one side of this boundary can be seen. Once they exit the focal volume and re-enter, they are treated as new particles; particle identity would be retained under the Einstein equation. In order to account for the experimental bias due to the loss of this identity, equations for mean square axial displacement in the presence of an adsorbing boundary have been derived, where in one case, called the time lag method, the MSD depends on the starting position of the particle and averaging over all distances (δ) from the adsorbing boundary, leads to the expression $< (z - \delta)^2 > = 2ID\Delta t$ where I = 0.8934. Although, Einstein’s one dimensional diffusion equation is valid for the shortest time interval, the adsorbing boundary formula applies at larger $\Delta t$. In the second case, called the distribution method, the mean-square axial displacement for particle trajectories originating at the adsorbing plane has been derived and found to scale as $4D\Delta t$. This leads to the normalized distribution of $z^2/t$ to be

$$f(z^2/t) = \frac{1}{4D} e^{-z^2/4Dt}.$$  

Theories for sphere diffusion near a planar wall or in a cylindrical pore are essentially exact, so any discrepancy between theory and experiment can be attributed to nonidealities or inaccuracies in the experiment, reflecting perhaps long range interactions of solute with wall or polydispersity of solute size. Confinement geometries in which sphere diffusion is theoretically well understood were chosen for this study to allow a critical assessment of the proposed experimental method for measuring diffusion coefficient.
1.2 Protein preservation in Ionic Liquid - Polymer Gels

Proteins are complex, fragile macromolecules that tend to become unstable when not in their native environment. Protein preservation methods should enable protein storage for extended periods of time without loss of original structure and activity. Proteins are susceptible to inactivation and degradation by several methods during storage, including chemical (oxidation, deamidation, crosslinking and hydrolysis), physical (adsorption/binding to storage vessel, aggregation, unfavourable pH and temperature) and biological (aqueous solutions of proteins stored at 4°C are prone to microbial contamination) pathways.

Protein-based pharmaceuticals and therapeutics are known to cure various diseases, but a major challenge is maintaining proteins in their native state during processing and storage to point of use. A few stabilized liquid protein products, such as zinc insulin solution and human serum albumin, have a two-year shelf life, but most protein solution products do not have such a long shelf life even at refrigerator temperatures. Storage requirements are distinct for each protein, with each method having advantages and drawbacks. Some of the common methods of protein storage are refrigerating solutions at 4°C, freezing formulations that are then stored at -20°C or lower temperatures (-20 to -80°C) and lyophilization. Liquid formulations may be incapable of withstanding stresses associated with agitation and freeze-thawing whereas frozen formulations are degraded by formation of cracks within ice during freezing. Often excipients like glycerol, PEG and sugars are added to protein formulations to prevent damage caused by sub zero temperatures and formation of ice during freezing. Sugars like sucrose and trehalose act as cryoprotectants and their effectiveness is
ascribed to their ability to form glassy/amorphous solids, inhibit the formation of ice crystals, and tendency to be excluded from the immediate vicinity of the biological molecule. Stability of frozen formulations therefore requires strict maintenance of temperature because slight changes can cause thawing or crystallization of excipients. This makes shipping and storage inconvenient.

Lyophilization or freeze drying is the most commonly used method of protein storage. Freeze drying can be defined as the drying of a substance by freezing it and removing the associated solvent by sublimation. There are mainly three stages involved in this process. The first stage is freezing; the material is rapidly cooled below its eutectic point (lowest temperature at which the solid or liquid phases of the material can coexist) or glass transition temperature to ensure that sublimation and not melting occurs at later stages. The next stage is primary drying where 95% of water in the material is sublimated by placing it under vacuum and applying heat to accelerate sublimation. The amount of heat to be supplied is governed by the latent heat of sublimation. The last stage is secondary drying. The aim here is to get rid of unfrozen moisture or bound moisture; secondary drying is governed by a materials’ adsorption isotherm.

Lyophilization also has some disadvantages. Klibanov et al discovered that the presence of moisture in small amounts irreversibly denatured lyophilized bovine serum albumin and reversibly denatured several other lyophilized proteins. The lyophilization process generates a variety of stresses to denature proteins. These include (1) low temperature stress, (2) freezing stresses, associated with formation of ice
crystals, increased ionic strength, changed pH, and phase separation, and (3) drying stress (removing of the protein hydration shell).\textsuperscript{25}

Lyophilization of pharmaceutical proteins requires great attention to product stability, formulation design, and ease of administration. In many cases, protein stability can be enhanced by development of a validated lyophilization procedure. However, as mentioned above, lyophilization has drawbacks and not all proteins can be successfully lyophilized.\textsuperscript{25, 26}

So, the potential drawbacks associated with frozen formulations combined with the expense of maintaining the frozen state during shipping, has stimulated an interest in developing alternate techniques for stabilization and storage of proteins. The aim of this study is to examine the structure and stability of proteins in neat hydrophilic ionic liquid (IL) and explore the possibility of using IL-based gels to preserve proteins. To achieve the overall objective of encapsulating proteins in IL-polymer gels, the following objectives were individually explored:

1) Study the behavior of proteins in neat IL

2) Fabrication of biocompatible polymer gels using IL

Chapter 3 describes IL-Protein systems and Chapter 5 provides a detailed account of IL-polymer biocompatible gels.

1.2.1 Proteins in Ionic Liquids

Proteins have been studied previously in nonaqueous media, and in fact, nonaqueous enzymology has been a major area of biotechnological research and
development. Enzymes show a striking behavior in organic solvents and have been found to be stable and catalyze reactions that are impossible in water. There is a notion that enzymes are less active in organic solvents than in water, which is not always true. Klibanov has elucidated ways of enhancing enzyme activity in organic solvents.\textsuperscript{27} ILs are salts with melting points below 100°C and in their molten states are almost entirely composed of ions. Room temperature ionic liquids (RTILs) exist as liquids at or close to room temperature. Attributes of ILs that have attracted interest are near-zero vapor pressure, high thermal stability as compared to organic solvents and polarity, hydrophobicity and solvent miscibility that can easily be tuned. ILs contribute no volatile organic compounds (VOCs) to the atmosphere because of their negligible vapor pressure and are therefore sometimes termed “green” solvents. Since an immense number of ILs can be envisaged by switching of cations and anions, they are also viewed as “designer” solvents.\textsuperscript{28-30}

The interest in ILs for biocatalysis and biopreservation stems from the desire to replace volatile organic solvents by nonvolatile ILs.\textsuperscript{29} Due to variable miscibility of ionic liquids and water, a wide variety of studies of proteins in both aqueous and anhydrous ionic liquids have been conducted. ILs have been explored as media for liquid-liquid extraction of biomolecules, protein preservation and biocatalysis.\textsuperscript{31} The earliest work on the use of ionic liquids and proteins involved an aqueous mixture of ethyl ammonium nitrate (EAN), the oldest known IL, which was found to increase the stability and activity of alkaline phosphatase for concentrations of EAN in aqueous solutions up to 10\% (v/v).\textsuperscript{32}
The effect of various ions on the stability of proteins in aqueous solutions can be described by the Hofmeister series. The Hofmeister series is a set of cations and anions arranged according to their effect on protein stability. Kosmotropicity and chaotropicity are loosely correlated with the series and basically describe the interaction of ions with water and their effect on the structure of water. The Hofmeister effects have been used to explain the stability of enzymes in aqueous mixtures of ILs. It is, however, worth mentioning that there is ambiguity in defining the kosmotropicity and chaotropicity of ions in ILs. According to the Hofmeister series, kosmotropic anions and chaotropic cations stabilize the proteins in aqueous solutions but this does not seem valid for predicting the compatibility of enzymes and aqueous ILs. For instance, the chaotropic chloride and tetrafluoroborate anions have been found to enhance the catalytic activity and thermostability of some enzymes whereas partial unfolding and denaturation of proteins in ionic liquids with kosmotropic acetate anions have also been reported in literature.

Enzymes such as chloroperoxidase, horse radish peroxidase, subtilism, papain and β-galactosidase have been studied in aqueous solutions of ILs. There is a disagreement in the literature as to whether Hofmeister series can be used to rationalize enzyme-IL compatibility. It appears that an IL that stabilizes one enzyme destabilizes another. Another hypothesis is that proteins lose stability in the presence of ions or solvents that interact more strongly with the unfolded enzyme than with the native one.

Hydrophobic or nearly anhydrous ILs have also been used for biocatalysis of lipases and proteases. The efficiency of lipase-catalysed reactions in ILs varies
depending on the type of lipase and the IL. For instance, the enzyme Candida Antarctica Lipase B (CaLB) was active in ILs of 1-alkyl-3-methylimidazolium and 1-alkyl-pyridinium families containing anions such as tetrafluoroborate, hexafluorophosphate, and bis(trifluoromethanesulfonyl)imide but deactivated in ILs containing nitrate, lactate and ethylsulfate anions, which were classified as strongly coordinating anions. Addition of water caused reactivation of enzyme with substantial recovery of activity. Other enzymes that have been studied in nearly anhydrous ILs are α-chymotrypsin, esterases, hydrolases and heme proteins and have been shown to be either active or inactive in them. A theoretical basis for predicting the compatibility of enzymes and anhydrous ILs has not yet been developed, although a number of causative factors have been discussed, such as the cation H-bond donating capability, logarithm of partition coefficient (log P), formation of hydrogen-bonded nanostructures, and solvent viscosity. None of the explanations are generally applicable.

Other studies of proteins in ILs involve covalent modification of proteins by attaching PEO chains to induce solubility and maintain activity in ILs.

It is believed that enzyme-compatible ILs generally do not interact strongly with the enzyme or cause it to dissolve. It has been reported in literature that solubility and activity are mutually exclusive events. A suspended protein is able to maintain its activity than a dissolved one (which often denatures or becomes inactive). The reason for this is that strongly coordinating ions of IL that dissolve the protein rupture the bonds required for protein stability and cause irreversible unfolding of the protein. An exception to this is an IL (choline dihydrogen phosphate + 20% water) that not
only dissolves the protein cytochrome c but allows the protein to retain activity for over a year.\textsuperscript{22, 40} But lysozyme in the same IL was denatured.\textsuperscript{41}

There is no theoretical basis for predicting the compatibility of aqueous or anhydrous ILs and enzymes, and the experimental data are not consistent with any of the arguments. But it is believed that ILs are tolerated to higher concentrations than water miscible organic solvents, and also, the activities of proteins have been found to be higher than those observed in conventional organic solvent.\textsuperscript{32} The current understanding of proteins in ILs has not completely evolved. Hence, a detailed knowledge of the relationship between protein solubility and stability in ILs is needed.

\subsection*{1.2.2 Ionic Liquid –polymer biocompatible gels}

Ionic liquid - polymer composites, so called gel polymer electrolytes or ion gels, gained attention for applications in electrochemical devices such as battery, capacitors and electrochromic cells. Angell and coworkers\textsuperscript{30} had described a new class of polymer electrolytes for secondary lithium batteries, termed “polymer” in “salt”, which were rubbery solid electrolytes possessing high ionic conductivities. The dimensionally stable polymer electrolytes showed significant advantages over liquid electrolytes from both the mechanical property and fabrication standpoints. Watanabe et al\textsuperscript{30} reported polymer electrolytes containing chloroaluminate-based ILs for battery applications but their moisture sensitivity, resulting in the formation of hydrochloric acid (which is highly corrosive), proved to be a major deterrent in further electrochemical device-based applications. Thus, began the exploration of new free-standing polymer electrolytes
produced by incorporating chemically stable nonchloroaluminate ILs into appropriate polymer matrix.\textsuperscript{30,42,43}

Watanabe el al\textsuperscript{44} reported \textit{in situ} free radical polymerization of vinyl monomers like methyl methacrylate (MMA), acrylonitrile (AN), vinyl acetate (VA), styrene and 2-hydroxyethyl methacrylate (HEMA) in 1-ethyl-3- methyl imidazolium tetrafluoro borate and 1-butyl pyridinium tetrafluoro borate. HEMA was found to form transparent flexible films for monomer concentrations up to 60\%, and above that, phase separation occurred resulting in translucent films. Compatibility of HEMA in the latter IL was good but the IL itself had low ionic conductivity.\textsuperscript{44} Other groups also prepared transparent MMA\textsuperscript{45} and HEMA\textsuperscript{43} gels in hexafluoro phosphate and fluorohydrogenate based-ILs respectively. These transparent, mechanically strong and highly conductive polymer gels were distinctly different from conventional polymer gels in terms of non-volatility and high thermal stability. Since ILs had the ability to be tailored for proton conduction, and lithium ion conduction, the use of ion gels as new polymer electrolytes had the potential to be used in related fields, like fuel cells, lithium batteries and solar cells.\textsuperscript{43}

Little literature on the use of polymer-IL gels as media for biopreservation exists. One of the probable reasons for this is the lack of information on the toxicity and biocompatibility for many ionic liquids. Firestone et al have reported protein encapsulation in a photocured gel using a mixture of a polymerizable ionic liquid monomer (1-decyl-3-methylimidazolium acrylate), PEG co-monomer, water and a photoinitiator.\textsuperscript{46} The aim of this work is to demonstrate the feasibility of protein preservation by encapsulation in polymer-IL gels. Eventually, with the development of
biocompatible ionic liquids, biopreservation in IL-polymer gels could be considered a novel means of protein preservation at room temperature.

The criteria for development of a suitable material for protein encapsulation are efficient stabilization of protein-based vaccines and therapeutics, potential to enable room temperature storage of these, reduction in cost of the new formulation over lyophilized formulation, easy transportation (no refrigeration required) and usage, and, allows protein storage in high concentrations which is not possible with aqueous sugar based protein stabilization techniques. ILs seem to be promising candidates that could meet most of the above mentioned criteria, though it is unclear whether liquid-based formulations can be rendered sufficiently stable to withstand the stresses associated with shipping and storage. However, if IL based gels are used for storage of proteins, the stability enhancing properties of ILs coupled with a polymer matrix in the form of a gel could make handling and transportation of such products easier. Additional advantages would be the possibility of using these systems directly as vehicles for drug delivery by using polymers and ILs that are biocompatible. Measuring small aliquots of liquids is tedious but, if the gels could directly be cut, weighed and used, handling would become much easier.

1.3 Dissertation Overview

Geometrically hindered motions of a single large solute (particle or polymer) can be imaged in real time via optical microscopy. When particle motions are perpendicular to a planar substrate, confocal microscopy offers a convenient method to probe such dynamics. Confocal microscope has a unique ability to detect the light coming from the
focal region and reject the light coming from outside this plane by means of a pinhole. In Chapter 2, we report a detailed study of the dynamics of fluorescent colloidal particles near a solid wall and within pores of a planar membrane using confocal microscopy. A method of analysis to estimate diffusivity of particles in the axial direction by observing their intensity fluctuations was developed. Intensity fluctuations arise from particle motions in-and out-of the focal volume. Thus, the intensity fluctuations which correspond to the Brownian motion of the particles in the axial direction were analyzed to extract the diffusivity of the particles. Theories for hydrodynamically hindered diffusion are well developed, and experimental data were compared to theoretical predictions. The method was successful in capturing the trend of hindered diffusion close to surfaces and in membrane pores. This study provides a novel route to monitor the dynamics of particles including biomacromolecules near surfaces, through porous substrates and biological tissues.

Ionic liquids, with and without added water, are currently being explored as media for room temperature preservation of biomacromolecules such as proteins so that the stringent requirements for storage which ensure stability can be relaxed. An ability to preserve biomacromolecules at room temperature would be a significant advantage towards widespread development and application of protein based therapeutics. Chapter 3 describes the solubility and structure of proteins α-chymotrypsin and cytochrome-c in neat hydrophilic ionic liquid, ethylmethyl imidazolium ethyl sulfate [EMIM][EtSO4]. Molecular dissolution of the two proteins in the IL was achieved by moderate heating (60°C). Dynamic light scattering and dilute solution viscometry were used to measure the dissolved protein size which was found to be slightly larger in the IL than in buffer,
suggesting different solvation or protein unfolding. Spectroscopic methods such as UV-Vis, fluorescence, FTIR and CD provided detailed insights into protein structure in both environments. The spectroscopic analysis revealed largely unchanged secondary structure from buffer for cytochrome c but changed tertiary structure. IL-dissolved cytochrome c had heightened peroxidase activity, supporting the same conclusions.

Chapter 4 reports transfer of IL-dissolved cytochrome c to buffer by dialysis and ensuing alterations to protein conformation. Effect of salt concentration on back extraction of cyt c from IL to buffer is discussed. Further, physicochemical and enzymatic properties of the recovered protein were determined by optical and vibrational spectroscopies and peroxidase activity assay. Spectroscopic methods (UV-Vis, fluorescence, FTIR, CD) show largely unchanged secondary structure but changed tertiary structure for the recovered cytochrome c.

In Chapter 5, suitability of biocompatible polymer-ionic liquid gels as protein encapsulation and preservation media was assessed. Optically transparent and mechanically robust poly(2-hydroxyethyl methacrylate)-IL gels were prepared by free radical polymerization at varying monomer-IL ratios. Additionally, the mechanical properties of IL-HEMA gels of various compositions were evaluated. The gels were patterned at micron and nanometer size scales by imprint lithography and imaged via scanning electron microscopy. Further, a few IL based systems including physical gels of PEO-IL were imaged by transmission electron microscopy to illustrate the ability to image “wet” gels due to the negligible vapor pressure of ILs.
1.4 References


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CHAPTER 2
AXIAL DIFFUSION IN MEMBRANES

2.1 Introduction

Microscopy methods to study polymer and colloid dynamics typically image solute motions in the focal plane, ignoring or preventing solute excursions normal to this plane. Confined particle diffusion has previously been assessed by microscopic methods, such as single particle tracking,\(^1\) evanescent light scattering,\(^2, 3\) reflection interference contrast microscopy,\(^4\) total internal reflection fluorescence microscopy,\(^5-9\) and combined optical tweezers/digital video microscopy.\(^10\) Similarly, macroscopic approaches based on measurements of solute flux have been applied to membranes having cylindrical pores.\(^11-13\)

For a sphere of radius \(a\) suspended in a bulk fluid of viscosity \(\eta\) and temperature \(T\), the free solution diffusion coefficient, symbolized \(D_0\), is well represented by the Stokes-Einstein equation, \(D_0 = kT/6\pi\eta a\).\(^14, 15\) Inside a pore or near a wall, \(D\) is less than \(D_0\), manifesting hydrodynamic interactions with fixed, no-slip surfaces.\(^16, 17\) Motion perpendicular to a surface is more impeded than motion parallel, so diffusion is anisotropic for particle-surface separations comparable to \(a\).\(^18\) Corrections for the influence of fixed surface(s) on sphere hydrodynamic drag were first developed long ago, and these sorts of corrections underlie well-established theories for hindered diffusion of spherical solutes in various sorts of porous materials.\(^7, 16\)

For steady translation of a sphere near a planar surface, perpendicular and parallel hydrodynamic correction factors \(\lambda_\perp\) and \(\lambda_\parallel\) connect \(D_0\) to \(D_\perp\) and \(D_\parallel\).
perpendicular and parallel diffusion coefficients. For $z/a$ less than ~5, these correction factors are well approximated as a power series in $a/z$, where $z$ is the distance from surface to sphere center,

$$\frac{D_\perp}{D_0} = \lambda_\perp^{-1} = 1 - \frac{9}{8} \left( \frac{a}{z} \right) + \frac{1}{2} \left( \frac{a}{z} \right)^3 + O\left( \frac{a}{z} \right)^4$$ (1)

and

$$\frac{D_\parallel}{D_0} = \lambda_\parallel^{-1} = 1 - \frac{9}{16} \left( \frac{a}{z} \right) + \frac{1}{8} \left( \frac{a}{z} \right)^3 + O\left( \frac{a}{z} \right)^4$$ (2)

Accuracy worsens as proximity of the sphere to surface increases, where hindrance is enhanced and is more easily measured. However, for the range of $z/a$ experimentally examined, $\sim3<z/a<\sim30$, the above approximations are accurate to within $\pm1-2\%$. Across this span, $D_\perp$ and $D_\parallel$ are predicted to deviate from $D_0$ by 4 to 36% and 2 to 20%, respectively. Previous experiments are in accord with these predictions.\(^{10}\)

Numerous theories for hindered diffusion in geometrically complex environments have been formulated, with much attention directed at porous materials such as ultrafiltration membranes that can be idealized as thin films penetrated by cylindrical pores. Here, hydrodynamic interactions of solutes with fixed surfaces, the pore walls, reduce solute mobility compared to the bulk solution. The axial diffusion coefficient $D_z$ can be approximated by a power series in $a/R$ where $R$ is the pore radius. One classical result is the Renkin equation, derived from the friction expression for steady motion of a sphere along the centerline of a cylindrical pore under the approximation that $a/R$ is small,
\[
\frac{D_z}{D_0} = K_d = 1 - 2.104 \left( \frac{a}{R} \right) + 2.104 \left( \frac{a}{R} \right)^3 - 0.95 \left( \frac{a}{R} \right)^5 + \ldots
\] (3)

The approximation holds for \( a/R < \sim 0.4 \). More accurate numerical results, valid for all radial positions, are available for larger \( a/R \).\(^{19}\)

Hindered diffusion inside a membrane pore has rarely been experimentally assessed. In the membrane field, a more common measurement is the membrane permeability, the product of \( K_d \) with the equilibrium partition coefficient \( \Phi \), given for a cylindrical pore as \([1-(a/R)]^2\). The membrane permeability can be deduced from a macroscopic determination of solute diffusion flux, while \( K_d \) requires a microscopic determination of solute motion. The latter requires solute position be resolved at better than the pore length, usually tens of microns or less.

The aim of this study is to examine:

1) The hindered diffusion of Polystyrene latex beads in the axial direction using confocal microscopy.

2) Monitor the hindrance effect in track etched polycarbonate membranes of 2 micron pore size and determine the diffusivity.

The confocal microscope has a unique ability, to detect light coming from the focal region and reject light coming from outside this region. This feature has been utilized to visualize the motion of the beads in the focal region. The intensity fluctuations or “flickering” result from Brownian motion of the beads in the axial direction. These intensity fluctuations were analyzed to extract the diffusivity of beads in the vertical \((z)\) direction. As a small solute moves axially across the region bisected by the focal plane of the confocal fluorescence microscope, its imaged intensity tracks with the
microscope’s point spread function (PSF), which decays steeply on either side of the focal plane. The intensity, thereby, reveals the axial position, affording an opportunity to study dynamics in the third dimension, if images are collected in a time sequence at a high enough rate. While dependent on optics, the PSF has an axial spread of as little as \( \sim 1 \, \mu\text{m} \). The aim here is to examine the dynamics of small confined particles and polymers via analysis of flickering. This approach has significant advantages over two-dimensional imaging for some common confinement geometries, especially those associated with the approach and passage of solutes through thin film membranes. Many theories for membrane diffusion have been proposed, but rarely has the diffusion mechanism been monitored \textit{in situ}, as described here. The approach can readily be extended to study of solute motions in biological tissues or adhered films, where controlled drug delivery is an important potential application.

In this first implementation, solutes are limited to spherical, narrow size-distributed, fluorescent latex particles of colloidal dimension, and their concentration is extremely dilute, conditions that highlight individual particles of common dynamical and optical properties. Two methods of analysis have been used. The methods have elements of single particle tracking, whereby \( D \) can be obtained by Einstein’s one-dimensional diffusion equation, \( \langle \Delta z^2 \rangle = 2D\Delta t \), where \( \langle \Delta z^2 \rangle \) is the mean-square axial displacement incurred over a time interval \( \Delta t \). However, there are some limitations to the applicability of this formula when viewing the particle dynamics in the axial direction. The width of the PSF defines the focal volume within which the particle motion can be monitored. A sampling bias not admitted in the Einstein equation is generated due to the lack of visibility beyond the boundary represented by the PSF. Only
those particles that are on one side of this boundary can be seen. Once they exit the focal volume and re-enter, they are treated as new particles; particle identity would be retained under the Einstein equation. In order to account for the experimental bias due to the loss of this identity, equations for mean square axial displacement in the presence of an adsorbing boundary have been derived, where in one case, called the time lag method, the MSD depends on the starting position of the particle and averaging over all distances (δ) from the adsorbing boundary, leads to the expression \( <(z-\delta)^2> = 2ID\Delta t \) where I = 0.8934. Although, Einstein’s one dimensional diffusion equation is valid for the shortest time interval, the adsorbing boundary formula applies at larger \( \Delta t \). In the second case, called the distribution method, the mean-square axial displacement for particle trajectories originating at the adsorbing plane has been derived and found to scale as \( 4D\Delta t \). This leads to the normalized distribution of \( z^2/t \) to be

\[
f(z^2/t) = \frac{1}{4D}e^{-z^2/4Dt}.
\]

Theories for sphere diffusion near a planar wall or in a cylindrical pore are essentially exact, so any discrepancy between theory and experiment can be attributed to nonidealities or inaccuracies in the experiment, reflecting perhaps long range interactions of solute with wall or polydispersity of solute size. Confinement geometries in which sphere diffusion is theoretically well understood were chosen for this study to allow a critical assessment of the proposed experimental method for measuring diffusion coefficient.
2.2 Theory

According to the statistical theory of diffusion, the normalized probability density $P(z,t)$ for a solute particle undergoing one-dimensional Brownian motion follows the diffusion equation

$$\frac{\partial P(z,t)}{\partial t} = D \frac{\partial^2 P(z,t)}{\partial z^2}$$

(4)

with boundary/initial conditions conveying how solute enters/exits the domain. To extract $D$ from one-dimensional single particle trajectories observed in the focal volume, each imaged particle can be thought to enter this volume at time $t=0$, when the particle is first detected. All such particles will eventually exit the domain, returning later to $z=0$ or reaching the far side at $z=l$. Viewed in this manner, Equation 4 is solved with effective adsorbing boundary conditions at $z=0$ and $z=l$. For the initial period when $l$ is large compared to $(Dt)^{1/2}$, and ignoring particles occupying the focal volume at the start of the observation period, particle motion can be interpreted as transient diffusion into a semi-infinite half-space, $0<z<\infty$. A particle fluctuating about $z=0$ is, thus, counted as a new particle at each of its entries, resetting $t$ to zero several times.

If particles appear and adsorb at the same boundary, equation 4 has just the trivial solution $P(z,t)=0$. To avoid this outcome, particles are envisaged to appear first at $z=\delta$, where $\delta$ is a small positive displacement. Then, as described in Crank\textsuperscript{20}, $P(z,t)$ can be written as the sum of probability densities for a positive instantaneous unit source at $z=\delta$ and a negative instantaneous unit source at $z=-\delta$. Particle distributions from the two sources sum to zero at $z=0$, creating the needed adsorbing boundary conditions. Since probability densities from neither source reflect the $z=0$ boundary
condition, in keeping with the solution to Equation 4, the two densities evolve into mirroring (but opposite) Gaussian distributions, with a sum of,

\[ P(z,t) = \frac{1}{N} \left[ e^{-(z-\delta)^2/4Dt} - e^{-(z+\delta)^2/4Dt} \right] \]  

(5)

where N is an unspecified normalization constant. For \( \delta \ll (Dt)^{1/2} \), the condition for particle trajectories emanating close to the boundary, expansion of the exponentials and evaluation of N leads to the explicit form

\[ P(z,t) = \frac{z}{2Dt} e^{-z^2/4Dt} \]  

(6)

valid for \( 0 < z < \infty \). This probability density describes surface-initiated random walks terminated when the walker later touches the initiating (adsorbing) surface; note that \( \delta \) does not appear in \( P(z,t) \). The second moment about \( z=0 \) of this probability density is obtained through integration by parts,

\[ <z^2> = 4Dt \]  

(7)

This moment is larger, by a factor of two, than the corresponding moment associated with one-dimensional diffusion from an instantaneous unit source in free space. The difference arises in the preferential termination, by adsorption at \( z=0 \), of the lower \( z \) particle trajectories. As typical in transient diffusion, a composite variable \( z^2/t \) collapses the separate \( z \)- and \( t \)- dependences of \( P(z,t) \). From Equation 6, the normalized distribution \( f(z^2/t) \) for the composite variable obeys,

\[ f(z^2/t) = \frac{1}{4D} e^{-z^2/4Dt} \]  

(8)

Equations 7 and 8 underlie the “distribution” method for determining \( D \). As their two functional forms indicate, the accuracy in \( D \) hinges on reducing the relative uncertainty
in the average of the ratio \( z/(Dt)^{1/2} \). At the shortest observation time, \( t=1.625 \) s, the error in \( z \) (±0.05 \( \mu \)m) is not much smaller than \((Dt)^{1/2} \) (≈0.1 \( \mu \)m), so the ratio is obtained only to low accuracy. Fortunately, with the error in \( z \) nearly constant, accuracy improves with increasing \( t \). Eventually, when \((Dt)^{1/2}\) approaches 1, a transient diffusion analysis becomes inappropriate.

A related means to determine \( D \) is the “time lag” method, which tracks mean-squared displacement as a function of lag time \( \tau \). Each particle position serves as an initial “point source” for a succession of diffusion experiments of increasing \( \tau \), sampled at increments of the shortest observation time. By letting \( \tau \) replace \( t \), Equation 5 describes the particle distribution evolved from an instantaneous point source at \( z=\delta \).

The corresponding mean-squared particle displacement as a function of \( \tau \) is computed

\[
<(z-\delta)^2> = \frac{1}{N} \int_{0}^{\infty} (z-\delta)^2 \left[ e^{-\frac{(z-\delta)^2}{4Dt}} - e^{-\frac{(z+\delta)^2}{4Dt}} \right] dz
\]

(9)

Differently than in free space, the mean-squared displacement depends on the starting position, and so, to average across a set of particle trajectories extending into the half-space, \(<(z-\delta)^2>\) must be weighted with the probability density for \( \delta \), which is identical to the distribution of \( z \) given in Equation 6. The final expression can be expressed in the form

\[
<(z-\delta)^2> = 2IDt
\]

(10)

where

\[
I = \frac{\infty}{\int_{0}^{\infty}} \frac{x^2 e^{-x^2}}{0} \left[ \frac{\int_{0}^{\infty} (x-y)^2 e^{-(x-y)^2} \left( 1-e^{-xy} \right) dx}{\int_{0}^{\infty} e^{-(x-y)^2} \left( 1-e^{-xy} \right) dx} \right] dy
\]

(11)
with x and y serving as dummy variables. Termination of particle trajectories at z=0 lowers I from unity. Numerical evaluation yields I=0.8934.

The preceding time lag analysis supposes that a returning particle always adsorbs at z=0. Actually, as monitored by experiment, z is sampled only at discrete values of \( \tau \); between sampled time points, a particle occasionally will drift back-and-forth through z=0 without impact from the artificial adsorbing boundary condition, i.e., the observed particle trajectory is not terminated, as assumed in theory. A proper general accounting for this effect is difficult. However, since no trajectories are eliminated by boundary adsorption over the interval of the shortest (first) time lag (1.625 s), the distribution of z tracks with the expectation for free one-dimensional diffusion. In this limit, the density distribution is Gaussian and \( \langle (z-\delta)^2 \rangle = 2Dt \). As \( \tau \) grows from the first time lag, there is a transition from this higher value of \( \langle (z-\delta)^2 \rangle \) to the one calculated with a rigorously adsorbing boundary. The transition, occurring over approximately the first 10 time lags, is evident in experimental data. Unfortunately, since \( (Dt)^{1/2} \) is just an order-of-magnitude smaller than \( I \), experimental data in the limit for an adsorbing boundary, are convoluted onto finite size effects, which begin to surface when \( \tau \sim (0.1)l^2/D \approx 50 \) s, i.e., at \( \sim 20^{th} \) time lag.

2.3 Experimental

2.3.1 Materials

Polystyrene latex beads (Fluoresbrite® YG microspheres) of 0.5 microns diameter with yellow green fluorescence (451/486) were purchased from Polysciences.
The beads were dispersed in a solution of viscosity 120 cP prepared by mixing glycerol and TBE buffer solution (0.5x Tris-Borate-EDTA with 45 mM Tris-borate and 1 mM EDTA) in the ratio of 85:15 by weight. The viscosity of the glycerol-buffer solution was measured by Ubbelohode viscometer at a temperature of 20°C. The dilution of the beads (volume fraction ~$10^{-4}$) was sufficient to neglect any particle-particle interactions. Cyclopore track - etched polycarbonate membranes (25 mm diameter) of 2 micron pore size were procured from Whatman. PDMS (SYLGARD 184) was purchased from Dow Corning.

2.3.2 Microscopy

Images were collected at 8-bit resolution (512x512 pixels) on a Leica TCS SP2 inverted confocal laser scanning microscope equipped with HCX PL APO 40x/1.25-0.75 oil immersion objective ($\alpha$/0.17/E). With excitation at 458 nm, using a 458/514 DD excitation beam splitter, the detection wavelength was set to 480-520 nm. The scanning speed was 400 Hz and the pinhole was set to 0.5 airy units. The acquisition time for one frame under these settings was 1.635 seconds. The experiments were performed at 20±0.8°C. As described later, Image J [a public domain, Java-based image processing program developed at the National Institutes of Health (NIH)] was used to extract intensity values from stored files.

2.3.3 Sample Preparation

A 10:1 (by weight) resin:crosslinker PDMS mixture was spin coated onto a glass coverslip as a thin layer that was cured at room temperature for 48 hours. For
studies of diffusion in cylindrical pores, a track-etched membrane was placed onto the PDMS layer and on the membrane was pipetted a few µl of diluted bead suspension. The PDMS layer prevents slippage of the membrane. A concave glass slide with 0.5 mm depression was used to seal the suspension against the surface using an adhesive tape. This sandwich arrangement precluded solvent evaporation and associated flow. For studies of diffusion near a planar surface, the bead suspension was directly pipetted onto the PDMS layer.

2.3.4 Visualization

Movies of Brownian motion near the PDMS surface were acquired in the microscope’s x-y-t mode for ~30 min (1000 frames). In this mode, images in a single horizontal plane are collected as a function of time, the interval per image (1.635 s) much less than the characteristic time, ~5-10 min, for bead diffusion across the PSF (~1 µm). The focus knob of the microscope allowed the observation depth (distance from coverslip to focal plane) to be varied and precisely measured with respect to reference beads that were fixed on the PDMS.

2.4 Data Analysis

2.4.1 Optical Calibration

The PSF, quantifying the intensity distribution of a point object about the focal plane, depends on the optical characteristics of the sample, coverslip, objective lens, wavelength and the microscope. Here, the term PSF is informally attached to the axial intensity distribution of a single imaged bead of 0.5 micron diameter, not the
hypothesized point object. The PSF thereby serves as a “calibration curve” for measuring axial displacement of a bead relative to the focal plane. The maximum of the PSF superimposes with the focal plane, and the PSF drops on either side, reaching a null background outside the focal volume. Beads of the current investigation were small enough that the PSF displays a single peak.

To determine the PSF for beads above a planar surface, a sparse field of beads was deposited onto the PDMS coated coverslips by evaporation, glycerol-buffer solution was pipetted as a droplet over the beads, and the droplet was sealed with a second concave glass slide as described in the Experimental section. Employing a galvanometer-driven stage, bead images were gathered from 3 µm below to 3 µm above the focal plane at 40 nm axial or z increments. The images were processed to obtain the numerical values of intensities. The maximum gray value of the pixel within the selection was taken as the intensity value for a bead in that particular frame. Since the images were acquired at 8 bit resolution, the intensities range from 0 to 256.

To determine the PSF for beads trapped in membrane pores, calibration curves were derived analogously from stationary beads attached to pore walls. Attachment in this case was achieved only after the study of bead diffusion, the excess fluid wicked from the membrane by paper towel, the coverslip with membrane heated to 50ºC for 10 min, and the membrane rewetted and resealed before calibration.

The PSF is sensitive to spherical aberrations caused by refractive index mismatch between the objective lens immersion media and the sample. These aberrations decrease the peak intensity of the PSF, increase the peak full-width-at-half-maximum (FWHM), and create asymmetry about the focal plane. Refractive indices of
the current set-up, \( \eta_{\text{immersion}} = 1.515 \), \( \eta_{\text{PDMS}} = 1.451 \), \( \eta_{\text{solvent}} = 1.451 \), and \( \eta_{\text{membrane}} = 1.586 \), were chosen to reduce this mismatch to the extent possible. Aberrations are less pronounced at low numerical aperture (NA) but severely distort the PSF with increasing observation depth.\(^{25-28}\) The objective lens used has a correction collar that tunes an iris in the pupil plane of the lens, altering NA. Before constructing the PSF, the correction collar was adjusted to ensure that the PSF was symmetric above and below the focal plane. This was achieved by tracking the reflection from the PDMS-coverslip interface\(^{29}\) and at the collar’s optimal setting, this reflection has maximum intensity and sharpness and minimum axial width.

In addition to aberrations, the mismatch of immersion medium and sample refractive index also causes a shift in the actual position of the imaged optical slice from the actual mechanical distance moved by the stage along the optic axis depending on the NA and the degree of mismatch. Calculations of the difference between apparent and actual focal plane position are extensively reported.\(^{30}\) For small NA, the shift in focus \( \Delta f \) can be approximated by the following equation: 

\[
\Delta f = \left( \frac{\eta_{\text{immersion oil}}}{\eta_{\text{solvent}}} \right) \Delta s
\]

where \( \Delta s \) is the depth of observation.\(^{31}\) Applying this formula, the actual surface-focal plane displacement was 0.95x the displacement registered on the stage micrometer.

The intensity vs. axial position data of the beads were processed in the following manner: the baselines were set to zero by subtracting the background intensity value from the intensity data. Then the first moments were taken to center the curves about the focal plane by reploting the data as \( I_i \) vs. \( (z_i - \sum I_i z_i / \sum I_i) \) where \( z_i \) denotes the axial position and \( (z_i - \sum I_i z_i / \sum I_i) \) denotes the distance from the focal plane. The calibration curve was subsequently prepared by averaging these curves from multiple beads. A
Gaussian curve described the data in the region of interest i.e. where the intensity varies sharply with distance from the focal plane. The calibration curve from multiple beads obtained above was thus fitted by a Gaussian of the form \( I = I_0 + Ae^{-\frac{(z-z_c)^2}{2w^2}} \) using two parameters \( w \) and \( A \) where \( w \) and \( A \) are the width and amplitude of the Gaussian respectively, \( z_c \) is \( z \) coordinate corresponding to the focal plane (set to zero) and \( I_0 \) corresponds to the baseline which was also set to zero. The intensity is given by \( I \) expressed as a function of the distance from the focal plane \( z \). The full width at half maximum, FWHM \( (w_f) \) of the Gaussian can be determined from \( w \) using the relation \( 2w = \frac{w_f}{\sqrt{\ln 4}} \). A typical PSF calibration curve, with fitted Gaussian, is shown in Fig. 2.1, and the inset provides associated \( A \) and FWHM values; the latter characterizes the length scale over which the \( z \) position can be accurately monitored for a bead, in this case, \(~1.2 \mu m\). This calibration curve was used to analyze the real-time experiments to retrieve the distances moved by the randomly moving beads based upon the fluctuations in their intensities. The horizontal line indicates a cutoff below which intensity data were discarded because of low precision and poor fit by the Gaussian. A bead outside the Gaussian region was not assigned a position. A separate calibration curve was prepared for each experiment, the cutoff intensities varying from 15 to 30, as determined by the goodness of the Gaussian fit.
Figure 2.1. A typical calibration curve (filled squares) fitted with a Gaussian (line) of the form $I = I_0 + Ae^{-(z-z_0)^2/(2w^2)}$ using two parameters $w$ and $A$. FWHM is calculated from $w$ using the relation $2w = \frac{W_i}{\sqrt{\ln 4}}$. Each calibration curve (filled squares with error bars here) is obtained by averaging several beads. All intensity values below 16 are discarded for further analysis due to deviations between the measured and fitted curve.

Since the experiments were done at different depths of observation (1, 3, 5 and 10 microns) from the substrate and the PSF was determined at the surface of the coverglass, it was necessary to ensure that the FWHM of the PSF remained constant over the entire range of depths observed. Because the glycerol-buffer mixture and PDMS\textsuperscript{32} have identical refractive index, observing a dynamic bead in suspension in the glycerol-buffer solution at a focal plane 10 \textmu m from PDMS is optically equivalent to observing a stationary bead deposited on a PDMS layer 10 \textmu m thicker. The procedure used was as follows: different thicknesses of PDMS were spin coated on coverglasses by varying the spin coating speed. The minimum thickness (25 microns) corresponds to the one used in experiments. The rest of the sample preparation was same as mentioned earlier for constructing calibration curves. The thickness of the
PDMS layer was measured by a profilometer (Dektak3). The correction collar was adjusted only in the presence of the thinnest PDMS layer, 25 µm. Without disturbing the settings of the correction collar or the microscope, calibration curves were made using coverglasses with higher thicknesses of PDMS. Fig. 2.2 shows calibration curves at different PDMS layer thickness, and Table 2.1 summarizes corresponding values of A and FWHM. Spherical aberrations have no discernable impact for bead-surface separation up to 11 µm, but at larger separation, they become prominent, precluding measurements of bead z-displacements in the far field region. This limitation is unfortunate, as Equations 1 and 2 suggest that, for a 0.5 µm diameter sphere, a separation ~50 µm from a planar surface is needed for the full transition to $D_0$. Hence, the calibration curve taken at the surface of the coverglass can be used to estimate the position of beads at different observation depths up to 10 microns used in present experiments.
Figure 2.2. Calibration curves obtained at different depths into the sample. As the vertical distance from the substrate increases, there is a decrease in maximum intensity and an increase in the width of the calibration curve. The curves with open circle and triangle overlap showing that the calibration curves remain valid up to an observation depth of 11 microns from the substrate. As the distance increases to 18 microns, a loss in maximum intensity and an increase in width of the calibration curve can be observed.

Table 2.1. Summary of the parameters $A$ and FWHM at varying distances from the substrate

<table>
<thead>
<tr>
<th>Thickness of PDMS (µm)</th>
<th>Observation depth (µm)</th>
<th>Highest Peak Intensity (A)</th>
<th>FWHM (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0</td>
<td>142 ±0.6</td>
<td>1.25 ±0.006</td>
</tr>
<tr>
<td>36</td>
<td>11</td>
<td>141 ±0.7</td>
<td>1.25 ±0.007</td>
</tr>
<tr>
<td>43</td>
<td>18</td>
<td>119 ±0.7</td>
<td>1.37 ±0.009</td>
</tr>
<tr>
<td>63</td>
<td>38</td>
<td>91 ±0.4</td>
<td>18.03 ±0.089</td>
</tr>
</tbody>
</table>

2.4.2 Diffusion Measurements

To ensure accuracy, each diffusion experiment was performed at the same lateral position as a calibration curve, with the depth of observation kept less than or equal to 10 µm to make the FWHM of the calibration curve constant. For study of diffusion near a planar surface, after constructing the calibration curve, the mixed
solvent was gently wicked away and replaced by the bead suspension in glycerol-buffer solution before the diffusion experiment proceeded with other conditions unchanged. Identical experimental conditions and settings of the microscope were used for the determination of the calibration curve as well as performing real time diffusion experiments. Diffusion in membrane pores was measured only at one depth of observation, 3 μm, falling about halfway along the pore length, and as noted, the calibration curve was obtained after the diffusion study.

Each movie displays multiple bead trajectories, often with several beads visualized simultaneously. The z-position of the bead image of highest intensity, extracted from all the bead trajectories in a movie, is assigned to the focal plane. The intensity of this bead image typically falls 1-5% less than A, so intensities across the entire movie are rescaled to make this intensity equal to A. Each trajectory typically varies from 30 to 200 frames and 75-150 such trajectories were analyzed for each observation depth.

2.4.3 Time Lag Method

The normalized intensity vs. time data are referenced to the calibration curve and absolute values of z are extracted as a function of time for each bead. Due to the calibration curve’s symmetry about z = 0, this scheme cannot distinguish positive from negative z, but fortunately, a bead travels only a small portion of the FWHM over the time interval between frames. The corresponding displacement error, incurred as a bead moves through z = 0, is thus small and infrequent. MATLAB (The Mathworks, Inc.) is used to obtain bead displacement over all accessible time lags, and the mean-
squared bead displacement (MSD) vs. time is determined by averaging. This MSD vs. time data was used to obtain particle diffusivity.

2.4.4 Distribution Method

In this approach, only those trajectories that start at the adsorbing boundary (when the particles begin to be seen) are considered. In other words, the particles already present in the focal volume are ignored. The initial position of the particle was set to zero and other positions in the trajectory were re-adjusted to give the distance from adsorbing boundary layer. Note that here \( z = 0 \) is different from that of time lag method where \( z = 0 \) corresponds to the focal plane. Here \( z = 0 \) corresponds to the adsorbing boundary. The distribution of \( z^2/t \) was then fit to equation 8 to obtain the diffusion coefficient.

2.5 Results and Discussion

2.5.1 Bead Size

To measure \( D \) in the manner proposed, all beads must be identical in both geometry and optical properties. To confirm the former, beads dispersed in the glycerol-buffer suspension were initially examined by dynamic light scattering using an ALV scattering instrument (ALV/SP-125 goniometer, ALV-5000 correlator, Thorn EMI photomultiplier) operated with a 514.5 nm argon laser (Coherent Innova 70). First-, second-, and third-order cumulant analyses of the correlation function offer significantly different values for the first cumulant, a feature indicating size polydispersity roughly over the span \( 240 \text{ nm} < a < 360 \text{ nm} \). Contin analysis of correlation function produces a
single peak, the maximum at 340 nm but the breadth suggesting polydispersity. Beads dried onto a coverslip were thus imaged by scanning electron microscopy (JEOL 6320F SEM) to generate images such as the one shown in Fig. 2.3a, and a size histogram, derived from many such images, displayed in Fig. 2.3b. The radius given for the beads by their supplier is too small, their number average radius by SEM is equal to 320±20 nm. The bulk diffusivity according to the Stokes-Einstein equation is calculated taking the average radius of beads as 320±21 nm, \( \eta = 119±3 \) cP and \( T = 293±0.8 \) K. This value corresponds to \( D_0 = 5.6±0.4 \times 10^{-11} \) cm\(^2\)/s. (Fig. 2.3a contains a few beads aggregated by drying; only unaggregated beads were considered in size analysis.)

![Figure 2.3a. Scanning electron micrograph of dried latex beads of size 0.5 microns. It is visually clear that the beads are not perfectly monodisperse.](image-url)
Figure 2.3b. Histogram showing the polydispersity in size of beads. The number average size is 639 nm with a standard deviation of 42 nm.

2.5.2 Diffusion Near a Planar Surface

2.5.2.1 Time Lag Analysis

Fig. 2.4a shows the intensity vs. time trace for a typical bead, and Fig. 2.4b shows the accompanying plot of z vs. time, where z is the distance from the focal plane. Note that this bead entered and left the focal volume from one side, never crossing the focal plane during the period of observation. If the displacement $\Delta z$ over time interval $\tau$ is a random variable, as expected for Brownian motion, $\Delta z$ follows a Gaussian distribution. Fig. 2.4c shows the distribution of $\Delta z$ (i.e. displacement between adjacent frames) for the shortest interval sampled, $\tau=1.635$ s, and the experimental measurements closely follow the Gaussian form. Only one case is shown in Fig. 2.4c that corresponds to a distance of 10 µm from the substrate. Table 2.2 provides the values of diffusivity.
obtained using the shortest time lag at various observation depths with the diffusivity being lowest for beads observed at 1 µm from the substrate and highest at 10 µm from the substrate, as would be expected. The diffusivity at a distance of 10 µm from the substrate is close to the bulk diffusivity obtained by Stokes-Einstein equation. The qualitative trend that diffusion is hindered closer to a substrate is evident. Table 2.3 compares the ratios of diffusivities \( \frac{D_z}{D_0} \) where \( D_0 = 5.6 \pm 0.4 \times 10^{-11} \text{ cm}^2/\text{s} \) obtained experimentally with those calculated theoretically using equation 1. The results are in reasonable agreement given the high error associated with each diffusivity value.

![Graph](image)

Figure 2.4a. The graph shows variations in intensity with time for a bead moving about the focal plane. Inset shows intensity fluctuations for longer time frame. It can be seen that when there is no bead in the focal volume, the intensity falls to the background value.
Figure 2.4b. The graph shows the corresponding values of distance from the focal plane based on the calibration curve for the bead shown in fig. 2.4a.

Figure 2.4c. Displacements for shortest time interval (1.635 seconds) follow a Gaussian form.

Table 2.2. The values of diffusion coefficients measured at vertical distances 1, 3, 5 and 10 microns from the substrate using the two methods of analysis are shown.  

<table>
<thead>
<tr>
<th>Distance from substrate (µm)</th>
<th>Time Lag Method</th>
<th>Distribution Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5.69±0.87</td>
<td>5.03±0.50</td>
</tr>
<tr>
<td>5</td>
<td>5.21±0.70</td>
<td>6.03±0.51</td>
</tr>
<tr>
<td>3</td>
<td>5.20±0.92</td>
<td>3.81±0.52</td>
</tr>
<tr>
<td>1</td>
<td>4.08±0.99</td>
<td>4.29±0.44</td>
</tr>
</tbody>
</table>
Table 2.3. The values of diffusion coefficients measured at vertical distances 1, 3, 5 and 10 microns from the substrate using the time lag method are shown. Experimental and theoretical values of the ratios of diffusivities are also shown.

<table>
<thead>
<tr>
<th></th>
<th>$D_z$ ($10^{-11} \text{cm}^2/\text{sec}$)</th>
<th>$D_z/D_0$ ($z = 3,5,10$)</th>
<th>$D_z/D_0$ ($\text{Theory}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_1$</td>
<td>4.08±0.99</td>
<td>0.72±0.18</td>
<td>0.65±0.023</td>
</tr>
<tr>
<td>$D_3$</td>
<td>5.20±0.92</td>
<td>0.92±0.18</td>
<td>0.88±0.007</td>
</tr>
<tr>
<td>$D_5$</td>
<td>5.21±0.70</td>
<td>0.92±0.14</td>
<td>0.92±0.004</td>
</tr>
<tr>
<td>$D_{10}$</td>
<td>5.69±0.87</td>
<td>1.00±0.17</td>
<td>0.96±0.002</td>
</tr>
</tbody>
</table>

Fig. 2.5 shows MSD vs. time for beads separated from the PDMS surface by 1, 3, 5 and 10 µm. The curves show a significant deviation from linearity which is expected for free one-dimensional diffusion. This arises because, as mentioned earlier, the entire trajectory of a particle cannot be monitored and the view is limited to the motion of a diffusing particle within the focal volume defined by the Gaussian PSF. As explained in the theory, this sampling bias can be effectively treated and analyzed by assuming the presence of an adsorbing boundary where the particles enter the focal volume, taking into account the starting position of the particles. As given by equation 10, the mean-squared displacement in this case is not $2Dt$ as is the case for free one dimensional diffusion equation but lower by a factor of $I$ (=0.8934). This qualitatively explains the curvature observed is the MSD vs time data shown in Fig. 2.5. This adsorbing boundary approximation is not exact and, therefore, the resulting value of $I$ is somewhat higher than that observed experimentally. However, for the first time lag (1.625 s), no trajectories are eliminated by boundary adsorption and hence the distribution of $\Delta z$ is Gaussian and equal to $2Dt$. 
Figure 2.5. The graph shows MSD vs. time for beads at vertical distances of 1, 3, 5 and 10 microns from the substrate. The trend that diffusion is hindered as beads approach the coverglass is clearly visible. Error bars have been omitted for clarity. A distinct curvature with increasing time can be seen in this plot that arises due to the experimental limitation of not being able to monitor the dynamics of particle diffusion beyond the focal volume defined by the width of the PSF.

2.5.2.2 Distribution Analysis

Fig.2.6a shows the corresponding values of subsequent displacements for the particle shown in Fig. 2.4b from the adsorbing boundary by setting the particle’s initial position to zero. Here, \( z = 0 \) corresponds to the adsorbing boundary. In Fig.2.6b the distribution of \( z^2/t \) (for the case of 10 µm from the substrate) up to \( \sim 17 \) seconds is fitted with equation 8 as derived in theory. The values of diffusivity obtained are listed in table 2.2. As seen from equation 7 and 8, the MSD of particles close to the adsorption boundary is \( 4Dt \) as opposed to \( 2Dt \) which is the case for free one dimensional diffusion.

The diffusivity values are close to the bulk diffusivity obtained using the Stokes-Einstein equation. However, unlike the time lag method, the values of diffusivity at various distances from the substrate do not follow the qualitative trend of hindered
diffusion. One of the probable reasons is the error in the values of diffusivity that is greater than the difference between the diffusivities at various observation depths.

Figure 2.6a. The graph shows the distance from the adsorbing plane (z=0) for the particle trajectory shown in fig.2.4b. The distances from the focal plane have been rescaled by setting the particle’s initial position to zero. The dotted lines indicate the displacements incurred by the particle in subsequent time from the adsorbing boundary.

Figure 2.6b. The graph shows the normalized distribution of $z^2/t$ for the observation depth of 10 µm from the substrate. The solid line is the fit obtained by using the equation $f(z^2/t) = \frac{1}{4D}e^{-z^2/4Dt}$. 

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2.5.3 Diffusion in Membrane Pores

The analysis of experiments done in PC membranes was done similarly to the case when no membrane was present. Fig. 2.7 shows an overlay of the transmission image of the membrane and the fluorescent image of the PS beads obtained by confocal microscopy. This was done to confirm that the beads were indeed inside the pores of the membrane. Table 2.4 shows the diffusivity of beads in the pores of the membrane compared with the diffusivity of the beads in the absence of membrane obtained by both methods of analysis. As expected - due to the additional drag from the pore walls - the value of diffusivity obtained for the beads in the pores was lower than the value obtained at a distance of 3 microns without the membrane.

Figure 2.7. Overlay of the transmission image of the track-etched polycarbonate membrane and the fluorescent Polystyrene beads of 0.5 micron size (indicated by green arrows).
Table 2.4. Diffusivities in membrane and in the absence of membrane at an observation depth of 3 microns from the substrate

<table>
<thead>
<tr>
<th>Diffusion Coefficient (x $10^{-11}$ cm$^2$/sec)</th>
<th>Time Lag Method</th>
<th>Distribution Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC membrane</td>
<td>4.03±1.20</td>
<td>3.55±1.02</td>
</tr>
<tr>
<td>Without membrane</td>
<td>5.20±0.92</td>
<td>3.81±0.51</td>
</tr>
</tbody>
</table>

The Renkin equation gives the hindered diffusion coefficient of a spherical particle in cylindrical pores based on the diameter of the particles and the pores as mentioned in the introduction. SEM of the PC membrane of 2 micron pore size is shown in Fig. 2.8a and b.

![SEM micrograph of PC membrane](image1)

**Figure 2.8a and b.** SEM micrographs of the top-view and side-view of track-etched PC membrane of 2 micron pore size. The pores are obviously not monodisperse.

The pores are not monodisperse as can be seen visually in the SEM (Fig. 2.8a and b). The individual pores are elliptical, as opposed to circular, in cross-section and cannot be characterized by a single diameter. Even so, as expected, Fig. 2.9 shows a decreased diffusivity of the beads in the membrane pores as compared with diffusivity without the membrane. However, a simple theoretical model of spherical beads in
straight and cylindrical pores is not applicable due to the elliptical shape and tortuosity of the pores.

![Graph showing comparison of experiments in the membrane (filled squares) and without the membrane (filled circles) with the linear fits (solid line). The diffusivity in the membrane is indeed less than the corresponding case without the membrane.]

**Figure 2.9.** Comparison of experiments in the membrane (filled squares) and without the membrane (filled circles) with the linear fits (solid line). The diffusivity in the membrane is indeed less than the corresponding case without the membrane.

### 2.5.4 Experimental Errors

The discrepancy between the $D_\perp/D_0$ (designated as $D_z/D_0$) predicted by the theory for hindered diffusion and the value estimated from experimentally determined $D_z$ at various vertical distances above the substrate can be attributed to several factors. One of the probable sources of error could be the dispersity in the size of the beads which would result in a distribution of diffusivities estimated.

Another source of error could be from the instrument electronic noise that can cause a fluctuation in intensity of a bead from frame to frame. Since the intensity values were used to determine the distances moved by the beads undergoing brownian motion, a variation in intensity would result in a variation in the measured diffusivity. In order to
quantify this variation of intensity between frames, an experiment similar to the real
time experiments was performed on stationary beads. Images were recorded in the x-y-t
mode. Fig. 2.10 and Table 2.5 show a ~15% fluctuation in intensity of one stationary
bead over 200 frames.

Figure 2.10. The graph shows the intensity of a stationary bead in 200 frames. The
fluctuation in intensity about the mean value of 203.6 is about 15.7%.

Table 2.5. Statistical analysis of fluctuations in intensity

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum value</td>
<td>190</td>
</tr>
<tr>
<td>Maximum value</td>
<td>222</td>
</tr>
<tr>
<td>Range</td>
<td>32</td>
</tr>
<tr>
<td>Mean</td>
<td>203.585</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>7.36301</td>
</tr>
<tr>
<td>Standard error</td>
<td>0.5206</td>
</tr>
<tr>
<td>Median</td>
<td>203</td>
</tr>
<tr>
<td>First Quartile</td>
<td>197</td>
</tr>
<tr>
<td>Third Quartile</td>
<td>209</td>
</tr>
</tbody>
</table>

A third source of error could be the variation in fluorescence intensity from bead
to bead. The assumption in the analysis that all the beads have equal brightness may not
necessarily be true. A bead with intensity less than the highest value (for a movie) was
considered at some distance from the focal plane estimated from the calibration curve. However, there is a possibility that this bead is less fluorescent and is actually on the focal plane. This can introduce an error in the calculation of MSD and subsequently bulk diffusivity. It is not practically feasible to determine the brightness of individual beads while performing the real time experiments.

2.6 Conclusions

The diffusivity of colloidal particles in the axial direction using three different methods of analysis was measured. Diffusion coefficients of the particles in PC membrane pores were also measured. The time lag method was found to be the most accurate as the value of diffusivity obtained for the case of 10 µm was close to that predicted by Stokes-Einstein equation and the values of diffusivities at other distances from substrate agreed with the qualitative trend of hindered diffusion. Given the associated experimental errors, this trend is noteworthy.

Although the values of diffusivities estimated from distribution method are also close to that predicted by the Stokes-Einstein equation, this method fails to capture the qualitative trend of hindered diffusion as distance from the substrate decreases. This is reasonable to expect since the distribution method looks at fewer trajectories than the time lag method, as only the trajectories in which particles started at the adsorbing plane were considered in this analysis. In addition, unlike the time lag method, the distribution method does not employ internal averaging of trajectory data and thus results in poorer statistics. Also as explained in the theory, different methods analyze the data in different time regimes which allow for some difference between the resulting diffusivity values.
2.7 References


CHAPTER 3

PROTEIN STRUCTURE AND STABILITY IN NEAT IONIC LIQUID

3.1 Introduction

New techniques are sought for the stabilization and storage of proteins and protein-based pharmaceuticals, as current approaches suffer from protein degradation (for lyophilized formulations) or high cost of temperature control (for frozen formulations).\(^1\),\(^2\) As outlined in several recent reports,\(^3\)-\(^6\) ionic liquids (ILs) pose a new option, not just as media for protein preservation but also for biocatalysis. To evaluate prospects, more must be known about “rules” for IL-protein compatibility, protein conformation in ILs, and transfer of IL-dissolved proteins into aqueous environments. Here, the first two issues are addressed for a representative IL-protein pair that has properties conducive to in situ study of protein structure and activity.

ILs are salts with melting points below 100°C, and room temperature ionic liquids (RTILs) are a subset of ILs with melting points near or below room temperature. Attributes of ILs that attract most interest are near-zero vapor pressure, high thermal stability, broad miscibility, and polarity and hydrophobicity that can easily be tuned. Because of negligible vapor pressure, ILs sometimes are viewed as “green” solvents, and because an immense number of ILs can be envisaged by switching of cation and anion, they are also viewed as “designer” solvents.

Predicting the compatibility (i.e., molecular level mixing) of proteins and ILs is not yet possible. For neat ILs, i.e. those lacking measurable water or organic solvent, factors such as hydrogen-bonding, polarity (hydrophobicity, dielectric constant), liquid
nanostructure, and solvent viscosity could all be important.\textsuperscript{6} Even with compatibility, Hofmeister effects could impact stability and activity, as already found for some IL-buffer mixtures.\textsuperscript{6} Strong coordination of an IL ion to protein can be an important factor favoring dissolution, but the same factor tends to disrupt the intramolecular interactions responsible for native structure. The combination of slight solubility and measurable enzyme activity for Candida Antarctica lipase B in the IL triethyl methyl ammonium methylsulfate demonstrates that appropriate interactions are possible.\textsuperscript{7} Perhaps more promisingly, mixtures of the IL choline dihydrogen phosphate with water not only dissolve cytochrome c (cyt c), but kept at room temperature, sustain notable enzyme activity for over a year.\textsuperscript{8} The same IL denatures lysozyme.\textsuperscript{9}

We have studied structure and stability of the proteins α-Chymotrypsin and cytochrome c (cyt c) in the neat hydrophilic IL, ethylmethyl imidazolium ethylsulfate [EMIM] [EtSO\textsubscript{4}], a nontoxic and hydrophilic RTIL available commercially in large volumes. Protein sizes were characterized via dynamic light scattering (DLS) and viscometry, while protein structures were characterized by spectroscopic methods such as UV-Vis, Fluorescence, FTIR, and CD. Additionally, protein activity was measured in IL and buffer. Common ILs display broad UV absorbances that mask protein spectral features between 280 and 350 nm, precluding assessment of structure through typical spectroscopic methods. As described here, the difficulty is avoided with cyt c, fortuitously discovered to dissolve in [EMIM][EtSO\textsubscript{4}]. Thermal transitions of this hygroscopic liquid are absent above -60ºC, and at room temperature, viscosity is moderate, ~90 cp. Key to structural study is cyt c’s heme group, which outside the range of UV interference by IL, absorbs light in a manner sensitive to the nearby protein
conformation. Therefore, cyt c was chosen as a model to assess protein structure and stability in a neat hydrophilic IL.

Cyt c is a small globular protein (104 residues, M=12,384) with an isoelectric point of 10.3. Buried in a crevice lined with hydrophobic amino acids, the heme group in horse heart cyt c is covalently attached through Cys\textsuperscript{14} and Cys\textsuperscript{17}. Cyt c belongs to the class of iron-porphyrin containing proteins such as hemoglobin, myoglobin and catalase. Covalent linkage to the heme group on two sides provides significant stabilization of the overall protein structure. Axial coordination of the low spin heme iron with His\textsuperscript{18} and Met\textsuperscript{80} strongly stabilizes the native tertiary structure.\textsuperscript{10-12} The iron readily converts between its ferrous and ferric states, making cyt c an efficient biological electron transporter.\textsuperscript{13}

3.2 Experimental

3.2.1 Materials

Cyt c derived from horse heart and α-Chymotrypsin were purchased from Sigma-Aldrich and used without further purification while [EMIM] [EtSO\textsubscript{4}] was donated by Evonik-Goldsmit, Inc. Depending on protein concentration c, heating under vacuum at 60°C achieved apparent dissolution in IL, as reflected in visual homogeneity, after a period of 1 to 5 days. This preparation step also removes any residual water in IL to below the detection limit of optical refractometry, ~0.3 wt.% (The refractive indices of IL and water are 1.479 and 1.333, respectively.) These IL-protein mixtures were
stored under nitrogen between experiments. Aqueous protein solutions were prepared in 0.01M phosphate buffer at pH~7.2.

3.2.2 Characterization

3.2.2.1 Dynamic Light Scattering

Dynamic light scattering was performed on dissolved α-Chymotrypsin in both IL and buffer using an ALV scattering instrument (ALV/SP-125 goniometer, ALV-5000 correlator, Thorn EMI photomultiplier) operated with a 514.5 nm argon laser (Coherent Innova 70). The protein concentration was 10 mg/ml, sufficiently dilute to make the inferred protein size independent of concentration. Cumulants analysis of the correlation curve provided the mean decay rate as a function of q, the magnitude of the scattering vector. For diffusive decay, necessary for extraction of solute size, $\Gamma \alpha D q^2$, where D is the diffusion coefficient. From D, the hydrodynamic radius is calculated using the expression $R_H = kT/6\pi\eta D$ where $\eta$ is the solvent viscosity.

3.2.2.2 Viscometry

A semi-micro Ubbelohde viscometer (Cannon Instruments) was chosen for measuring the intrinsic viscosity $[\eta]$ of cyt c in both IL and buffer. The average Stokes radius $R_s$ was extracted from the experimentally determined $[\eta]$ via the standard relationship $R_s^3 = 3M[\eta]/10\pi N_A$, where $N_A$ is Avogadros number and $M$ is molecular weight. Solvent viscosities of IL and buffer were determined to be 93 cp and 0.96 cp, respectively, and for determining $[\eta]$, c varied from 2 to 10 mg/ml in IL and 40 to 80 mg/ml in buffer; both ranges are dilute, as verified by linear plots of specific viscosity.
versus c. Viscometry was performed in a nitrogen-purged glove bag to avoid moisture uptake by the hygoscopic IL.

3.2.2.3 UV-Vis Spectroscopy

A UV-Vis spectrophotometer (Perkin Elmer Lambda 25) recorded the absorption spectrum of cyt c solutions in standard 1-cm path-length cuvettes. The extinction coefficient measured at 409 nm, $1.11 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$, compares favorably to that reported in literature for the same buffer.  

3.2.2.4 Fluorescence Spectroscopy

For c equal to 0.05 mg/ml in buffer and 0.05 to 10 mg/ml in IL, fluorescence emission spectra were recorded (Perkin Elmer LS50B) at a fixed excitation wavelength of 280 nm.

3.2.2.5 FTIR Spectroscopy

FTIR spectra were obtained on a Fourier transform infrared spectrometer (Bruker Tensor 27) equipped with a LN-MCT detector and MIRacle, a single reflection horizontal ATR diamond crystal plate. A total of 100 scans at 2 cm$^{-1}$ resolution were averaged to get the reported, background subtracted spectra. Protein concentration was set to 2 mg/ml in 0.01M buffer at pH 7.2 and 18 mg/ml in IL.

3.2.2.6 Circular Dichroism

Soret region circular dichroism (CD) experiments were executed on a Jasco 720 spectrometer using 1-cm path length quartz cuvette. After 5 min equilibration of the
solution filled quartz cuvettes at 25 °C, spectra were recorded as three scan averages from 410 to 450 nm (Soret UV CD) with 16 seconds response time and 10 nm/min scan rate. The final spectra were obtained after subtracting the signal from a solvent filled cuvette. The protein concentration was 0.2 mg/ml in both buffer and IL.

3.2.2.7 Peroxidase activity assay

The peroxidase assay was initiated by adding hydrogen peroxide (H$_2$O$_2$) to a solution mixture of cyt c and guaiacol. The latter was then catalytically oxidized to its tetramer (tetraguaiacol) or similar compound $^{15}$ which is orange, allowing the reaction to be followed by monitoring absorbance at 470 nm. Assays were performed in 1.5 ml quartz cuvettes at guaiacol and H$_2$O$_2$ concentrations of 20 mM and 50 mM, respectively. Protein concentration was varied from 0.02-0.2 mg/ml. The rate of steady state reaction was taken as the maximum of the first derivative of the product formation curve.

3.3 Results and Discussion

3.3.1 Size of proteins

α-Chymotrypsin formed a clear and transparent solution upon dissolution in IL and fig. 3.1a and b show plots of $\Gamma$ vs. $q^2$ for buffer and IL. The hydrodynamic size ($R_h$) was then calculated using the Stokes-Einstein equation $R_h = kT/6\pi \eta D$ which was found to be $6.6\pm1$ nm in IL and $4.2\pm0.4$ nm in buffer. For both cases, further CONTIN analysis of the correlation curves indicates a single, narrow well resolved peak in the size distributions. The high absorbance of the IL in the UV region prevented further structural analysis of this protein.
Figure 3.1a. The diffusivity of α-Chymotrypsin dissolved in buffer was estimated from the slope of $\Gamma$ vs. $q^2$.

Figure 3.1b. The diffusivity of α-Chymotrypsin dissolved in IL was estimated from the slope of $\Gamma$ vs. $q^2$.

Dispersing cyt c in either IL or buffer endows a pronounced red color to the liquid due to the heme iron. In both cases, mixtures are homogeneous and fully transparent, strong evidences for protein dissolution. A more rigorous test for dissolution is the demonstration that the dispersed objects have a molecular size commensurate with their molecular weight which is normally accomplished by dynamic
light scattering. However, in the present case, due to protein coloration, attempts to perform dynamic light scattering under available instrument configurations failed, leading to pursuit of protein size determination through dilute solution capillary viscometry, which offers a slightly more difficult but more accurate size measurement. The extra difficulty arises from [EMIM][EtSO₄] hygroscopicity, which necessitated measurements in nitrogen atmosphere, and high viscosity, which made membrane filtration, serial dilutions, and flow measurements time-consuming. One full day was required for each [\eta] determination. In IL, [\eta] was 17±1 ml/g, and in buffer, [\eta] was 2.5±0.6 ml/g, corresponding to Stokes radii of 3.2±0.4 nm and 1.7±0.1 nm, respectively. Because of their magnitudes, both sizes confirm the molecular level mixing of the protein and solvent, and the current measurement in buffer is consistent with literature measurements in similar buffers. When denaturants (urea or guanidine HCl) are added to aqueous buffer, [\eta] for cyt c increases,¹⁶,¹⁷ much as observed in IL, and the increase can be attributed to an expansion of three-dimensional protein structure, i.e., a globule-to-coil transition driven by the loss of both the secondary and tertiary protein structure. By analogy, one might infer that cyt c in IL is also denatured, but other explanations for enlarged size cannot be discounted, for example, a thicker solvation layer. Unless strong association with added denaturant stabilizes molecular dispersion, protein denaturation in aqueous environments at finite c inevitably exposes hydrophobic residues that cause uncontrolled, disordered aggregation. In IL, the observed increase of cyt c size cannot be explained in terms of aggregation since the Stokes radius remains comparable to that in buffer and does not grow in time. All evidence points to molecularly disrupted, yet solution-stable cyt c, a motif possibly allowing protein
refolding upon transfer from IL to buffer. Whether the molecules possess a defined molecular structure different than the one adopted in buffer or adopt statistical, random coils cannot be ascertained through viscometry.

3.3.2 Spectroscopic Characterization

Due to its heme prosthetic group, cyt c displays characteristic absorption bands starting from the edge of the near UV and extending across the visible region. In comparison to other factors affecting UV-VIS absorption, the difference in refractive index between buffer and IL has negligible impact.\textsuperscript{18, 19} The UV-Vis spectra of neat IL, native cyt c dissolved in buffer, and cyt c dissolved in IL are shown in Figs. 3.2a, b and c.

![Graph](image)

**Figure 3.2a.** UV-Vis spectrum of neat IL, showing high absorbance upto ~350 nm.
Figure 3.2b. Soret and Q bands of cyt c in both 0.01 M, pH 7 phosphate buffer and IL.

Figure 3.2c. Magnified Q band with maximum at ~530 nm, characteristic of native cyt c. The inset shows a charge transfer band at ~695 nm for native cyt c, and this band absent in IL.

Absorption spectrum of native cyt c shows an intense maximum at 409 nm, the Soret band that reflects the spin state of iron, which is governed by axial ligands. With His$^{18}$ and Met$^{80}$ as the axial ligands, Iron in cyt c exists in a low spin state,$^{20}$ as indicated by a soret band with a maximum intensity at 409 nm. Cyt c in its high spin
states displays a blue shift of the Soret band to 394 nm. Less intense bands at around 530 nm, termed the Q bands, reflect $n\rightarrow\pi^*$ transitions of the porphyrin ring. Oxidation state can be determined by the shape of the Q bands manifested as two peaks (around 520 and 550 nm) in the reduced state but a single peak (around 530 nm) in the oxidized state. Another band in the visible spectrum, called the charge transfer band, is observed at a wavelength of 695 nm. This band reflecting coordination of Met$^{80}$ to heme iron, is lost when the S-Fe bond between Met$^{80}$ and iron is disrupted. Fig. 3.2a establishes that UV spectroscopy of cyt c in IL is impossible due to IL interference at wavelengths below ~360 nm. As seen from figs. 3.2b and c, the spectrum of cyt c in buffer has the three prominent bands, expected for the oxidized protein at 409, 530 and 695 nm. Cyt c in IL shows the same Soret band at 408 nm. However, the Q-band region in IL possesses not just a broad peak at ~530 nm but also a “shoulder” at ~550 nm, the latter indicating molecules in their reduced state. Expanding the spectral region beyond 650 nm, the inset to Fig 3.2c reveals complete loss of the charge transfer band in IL, a feature indicative of perturbation or loss of heme’s axial sulfur-coordinated ligand. With the Soret band essentially as for native protein, any new IL ligand does not alter iron spin state. Based on similarity in ligand structures, we speculate that the imidazolium cation complexes with oxidized iron, displacing Met$^{80}$, a scenario also proposed by investigators exploring the impact of exogenous nitrogeneous ligands such as imidazole on cyt c in buffer. It has been reported that the nitrogenous ligands displace the Met$^{80}$ as the sixth axial ligand of heme iron further characterized by the disappearance of the charge transfer band at 695 nm. No other differences in the UV-Vis spectra of native cyt c and cyt c-imidazole complex have been observed.
Figs. 3.3 a and b show the fluorescence emission spectra of the cyt c in buffer
and IL. Fluorescence spectroscopy interrogates the interactions of aromatic residues,
Trp\textsuperscript{59} in this case. Native cyt c has a weak fluorescence emission spectrum with no clear
maxima, a consequence of quenching by Forster energy transfer between closely spaced
heme group and Trp\textsuperscript{59}.\textsuperscript{12, 27} Fig. 3.3 a compares the emission spectra in buffer to that in
IL at a high concentration, 10 mg/ml, and a dramatic difference is noted. The maximum
around 300 nm in buffer corresponds to the first Stokes band of water.\textsuperscript{28} Fig. 3.3 b
reveals systematic changes in protein/IL spectrum across a lower range of cyt c
concentration, 0.05 mg/ml to 3 mg/ml. The IL’s inherent fluorescence decreases as the
protein concentration increases, and finally at sufficiently high cyt c concentration, this
fluorescence is efficiently quenched. As the protein concentration increases, it appears
that the IL’s emission is absorbed by the protein (cyt c has a high absorbance at ~409
nm reflected by the soret band in absorbance spectrum), as reported when the IL butyl
methyl imidazolium chloride [BMIM][Cl] was added to a buffer solution of cyt c at pH
7.4.\textsuperscript{29} Thus, no definitive conclusions about cyt c structure in IL can be drawn from
fluorescence measurements.
Figure 3.3a. For excitation at 280 nm, fluorescence emission spectrum of cyt c in 0.01 M, pH 7 phosphate buffer and IL.

Figure 3.3b. Emission from neat IL and IL with cyt c at c from 0.05 to 3mg/ml.

Far and near UV CD of cyt c in IL proved uninformative due to the high absorbance of IL in this wavelength region (200-350 nm), but there was no IL interference in the Soret region, which provides insight into the integrity of the heme unit. CD spectra in the Soret region (350-450 nm) for cyt c in buffer and IL are shown in Fig. 3.4. The spectrum in buffer exhibits a strong negative band at around 416 nm and a strong positive band at around 407 nm. The negative band results from interaction of the
Phe$^{82}$ side chain, positioned on the Met$^{80}$ side of the heme plane, with the heme group, and so this band reveals the integrity of Met$^{80}$ as axial ligand. Absence of this band for cyt c in IL indicates that the distance and/or orientation of the Phe$^{82}$ side chain with respect to the heme iron is perturbed.$^{11, 30}$ The absence likely reflects disruption of the Fe-S bond between the axial ligand Met$^{80}$ and Iron revealed through disappearance from UV-Vis spectrum of the charge transfer band. The band is also lost when buffer-dissolved cyt c is treated with denaturants such as guanidine HCl and urea.$^{31}$

![Figure 3.4. Soret region CD spectrum of cyt c in 0.01 M, pH 7 phosphate buffer and IL.](image)

Fig. 3.5 shows the FTIR spectra of cyt c in buffer and in IL as well as the IR spectrum of the IL itself. In buffer, peaks at 1653 and 1548 cm$^{-1}$ correspond to the Amide I and Amide II regions. In IL, corresponding peaks are at 1651 and 1542 cm$^{-1}$. Similarities in the two peak positions demonstrate that, for the most part, the protein’s native secondary structure remains intact in IL. Unlike the preceding UV/Vis and CD
experiments, FTIR assesses the secondary structure across the protein, not just in the vicinity of heme.

**Figure 3.5.** FTIR spectra in Amide I and II regions of neat IL, cyt c in 0.01 M, pH 7 phosphate buffer, and cyt c in IL.

### 3.3.3 Peroxidase activity

Taken together, the preceding measurements indicate that dissolution of cyt c in IL causes changes in protein structure, particularly a perturbation or loss of methionine as the sixth axial ligand of the heme unit. Cyt c acquires peroxidase activity when the coordinate bond between heme iron and met$^{80}$ sulfur breaks, a property demonstrated when cyt c complexes to substances such as cardiolipin and anionic lipids.$^{15, 32}$ We therefore measured cyt c peroxidase activity in IL and buffer. This activity relies on the accessibility of heme to substrates. In native cyt c, all six coordination Fe$^{3+}$ bonds of the heme unit are occupied (four with the nitrogen atoms of the tetrapyrrole ring and two with the nitrogen of His$^{18}$ and the sulfur of Met$^{80}$). A genuine heme peroxidase, such as horse radish peroxidase, has a free coordination position. Thus, increased peroxidase
activity in cyt c is associated with partial protein unfolding, and opening of the heme crevice, leading to an increased access to heme iron resulting from loss of its methionine ligand. In native cyt c, the heme group is deeply “buried” in the protein, resulting in a low rate of reaction with peroxide.\textsuperscript{15, 33, 34} The two impacts of IL on cyt c, molecular expansion and ligand disturbance, should both enhance peroxidase activity.

Fig. 3.6 compares peroxidase activities of cyt c dissolved in buffer and IL, and Table 3.1 shows the extracted slope of the steady state phase. The protein in IL shows 3 times higher peroxidase activity than in buffer. As a control, just prior to assay, sufficient IL was added to buffer-dissolved cyt c to create a mixture with the same composition and, here, peroxidase activity was lowest, an experiment and result demonstrating that increased activity in neat IL is generated by IL-induced conformational changes, not simply exposure to IL. From the low activity in the control, one might conclude that molecules in a mixture retain their native structure and hydration spheres, which in context of trends for neat IL, presages a complex, competitive interplay between solvation/coordination by water and IL. Protein behavior in IL-water mixtures will not be discussed here, but previous studies have also seen enhanced enzyme activity in such mixtures. It should also be noted that IL has a higher viscosity than buffer and yet shows higher peroxidase activity compared to buffer. One would assume the reaction to be diffusion limited; however, higher reaction rate observed for cyt c in IL proves that the changed structure of cyt c in IL causes it to have a higher peroxidase activity.
Figure 3.6. Cyt c peroxidase activity assay: comparison of activities in 0.01 M, pH 7 phosphate buffer and IL. In the control, IL was added to buffer prior to assay.

Table 3.1. Comparison of cyt c peroxidase activities in buffer, IL, and control (IL added after protein dissolution in buffer)

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\Delta A/\Delta t$ (Cyt c conc = 0.2mg/ml)</th>
<th>$\Delta A/\Delta t$ (Cyt c conc = 0.02mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyt c-Buffer</td>
<td>5.9 x10^{-4}</td>
<td>4.6 x10^{-4}</td>
</tr>
<tr>
<td>Cyt c-IL</td>
<td>18.4</td>
<td>15.5</td>
</tr>
<tr>
<td>Cyt c-Buffer +IL (control)</td>
<td>2.4</td>
<td>2.7</td>
</tr>
</tbody>
</table>

3.4 Conclusions

Because of its unique spectral features, cyt c has been used as a model protein for the study of protein structure in ILs. Via a combination of measurements we conclude that secondary structure of cyt c remains largely unchanged in the test IL but the tertiary structure changes significantly. Trends for cyt c in IL resemble those for acid denatured cyt c in buffer. In both cases, the Fe-S bond of the methionine ligand of the heme group is disrupted.
Dissolution of the protein in IL is apparently achieved by breakage of the Fe-S bond and complexation of the freed heme coordinate site with presumably, the IL cation or anion. Both proteins studied in this IL acquire a larger size than in buffer, supporting the view that strong interaction of the IL with the enzymes, that aids dissolution, also disrupts the structural integrity of the enzymes.

Our results support belief that dissolution of enzyme in neat IL requires a molecular interaction of sufficient strength to threaten the enzyme’s structural integrity. While dissolution in IL is accompanied by denaturation, reflected in an increase in overall molecular size, the denatured proteins in IL were never observed to aggregate or precipitate, a feature distinct from denaturation in buffer.
3.5 References


CHAPTER 4

PROTEIN TRANSFER FROM IONIC LIQUID TO BUFFER AND STRUCTURAL ANALYSIS

4.1 Introduction

The structure of cyt c changes in the IL as discussed in the preceding chapter. The focus here is to transfer the protein from the IL to the buffer and determine, by structure and activity measurements, the degree to which protein unfolding/denaturation is reversible. Initial studies on back extraction of dissolved protein from IL into 0.01 M, pH ~7 phosphate buffer resulted in protein precipitation. In order to achieve protein back extraction into the aqueous phase from the IL phase, various methods of protein precipitation, like salting in/out, addition of organic solvents, neutral polymers, polyelectrolytes and liquid-liquid based bioseparation methods, have been reviewed.

4.1.1 Methods for protein precipitation

Hydrophobic, charged and polar groups are all important in determining the behavior of protein molecules in various solvents. Within the ionic strength range of zero to about 0.5 M, the increase in solubility of a protein (at a given pH and temperature) with increasing salt concentration is known as “salting in.” The salting-in range can be exploited for protein precipitation in two distinct ways. First, decreasing the salt concentration by dilution or dialysis may cause aggregation of proteins resulting in precipitation. Second, isoelectric precipitation can be used by exploiting the variation of solubility with pH without changing the ionic strength. It is observed that proteins
have a minimum solubility and precipitate near their isoelectric point. At the isoelectric point, pI, the net charge is zero, which minimizes electrostatic repulsion between protein molecules causing hydrophobic forces to attract the molecules to each other. This is called isoelectric precipitation.¹

One of the most widely used techniques in enzyme purification is the salting out of proteins using high concentrations of salt. Salting out largely depends on the hydrophobicity of the protein, whereas salting in depends on surface charge distribution and polar interactions with the solvent. The pH may change the solubility; a neutralization of charge may make the surface less polar. Solubility in salts is usually highest around neutral pH, where proteins have most charged groups. On the other hand, aggregation may occur more easily close to protein’s isoelectric point. A typical protein molecule in solution has hydrophobic patches on its surface (side chains of Phe, Tyr, Trp, Leu, Ile, Met, Val) which tend to aggregate in the presence of high salt concentrations. As the concentration of salt increases, the salt ions get hydrated, freely available water molecules become scarce. Therefore, there is a greater tendency to pull off the ordered “frozen” water molecules from the hydrophobic side chains, exposing the bare nonpolar areas that tend to aggregate. The nature of the salt is also extremely important. Those salts that bind and interact directly with the proteins have a destabilizing effect and are called chaotropic. The optimum salts are those that promote hydration of polar regions (and dehydration of hydrophobic regions) on the protein without direct interaction themselves. The salting out ability of anions follows the Hofmeister series, which for some common anions is $\text{SCN}^- < \text{ClO}_4^- < \text{NO}_3^- < \text{Br}^- < \text{Cl}^- < \text{acetate}^- < \text{SO}_4^{2-} < \text{PO}_4^{3-}$. As far as cations are concerned, monovalent ions should be used
with \( \text{NH}_4^+ > \text{K}^+ > \text{Na}^+ \). Ammonium sulfate is the most commonly used salt for salting out of proteins.\(^1\)

Addition of a miscible organic solvent, like ethanol or acetone, has been known to cause protein precipitation by lowering the dielectric constant of the aqueous solution. The decrease in dielectric constant results in an increased electrostatic attraction between the protein molecules leading to precipitation. An alternate explanation proposed for this mechanism is the decreased solvating power of water for charged hydrophilic protein molecules resulting in precipitation. Protein precipitation using organic solvents can be carried out at low temperatures to prevent denaturation of proteins.\(^2\)\(^3\) At low temperatures, the lack of conformational flexibility means that organic solvent molecules are unlikely to penetrate the internal structure of the protein and cause destabilization. But at higher temperatures, small organic molecules enter the cracks in the surface that occur spontaneously due to natural flexing of the structure, and attach themselves to hydrophobic internal residues causing denaturation. The salt concentration should also be kept low. If the salt concentration is high, then electrostatic aggregation is impaired, higher levels of organic solvents are needed and denaturation is more likely. On the other hand, at very low salt concentration, a very fine precipitate may be formed that can be difficult to sediment.\(^1\)

A variety of neutral polymers like poly(ethylene glycol), poly(vinyl pyrrolidone) and dextran can also precipitate proteins. PEG and other neutral polymers act as macromolecular crowding agents. The mechanism of precipitation is believed to be based on excluded volume effects. The polymer sterically excludes the protein from the solvent causing preferential hydration. This causes the proteins to concentrate and
crowd until they are no longer soluble and hence precipitate. The observations that the effectiveness of PEG as a precipitant increases with increasing size of the polymer and larger proteins require lower concentration of polymer supports the above explanation.

Polyelectrolytes, such as poly(acrylic acid), carboxy methyl cellulose and heparin, have been used for protein precipitation. Protein precipitation by this method is related to the net charge on the protein and the size and charge density of the polyelectrolyte. The binding of the oppositely charged protein molecules and polyelectrolyte leads to neutralization, formation of a multimolecular complex eventually leading to precipitation.

Liquid-liquid two-phase systems can also be used for protein precipitation. Aqueous, liquid-liquid, two-phase systems can be obtained by mixing two incompatible polymers or by mixtures of polymer and salt solution of high ionic strength. Some examples of polymer pairs commonly used are dextran-PEG, PEG-PVA or PEG-phosphate salt system. Partition of protein between the two phases depends on the surface properties of the protein, essentially, the exposed groups on the surface that come in contact with the phase components. The interaction between the protein and the components of each phase is complex and involves H-bonds, charge interactions, Van der Waals forces, hydrophobic interactions and steric effects. The partition depends on the molecular weight and chemical properties of the phase forming polymers and size and chemical properties of the partitioned proteins.

Another liquid-liquid extraction method is reverse micellar protein extraction. Liquid-liquid extraction by reversed micelles involves forward extraction of a
targeted solute from an aqueous feed phase into an organic phase followed by recovery of extracted solute into a fresh aqueous solution by back extraction. Various factors govern the partitioning of proteins between the micellar organic phase and the aqueous solution such as pH, ionic strength and type of salt of the aqueous phase. Parameters related to the organic phase like the concentration and type of surfactant, presence of co-surfactant, and type of solvent also affect the forward extraction.

Back extraction of proteins from the reverse micelles is achieved by electrostatic repulsion between the surfactant and the protein by adjusting the pH or through size exclusion by increasing the salt concentration in the aqueous stripping phase. Alternate methods are adding a second solvent to destabilize the reverse micelles and release the protein and addition of alcohols or counterionic surfactants to break the reverse micelles facilitating protein recovery.\textsuperscript{7-10}

A few studies related to transfer of proteins from IL phase to aqueous phase have been reported, the main focus of such studies being liquid-liquid extraction of proteins into ILs that are immiscible with water. The concentration of proteins is less than 1 mg/ml in these studies. In one case, a macrocyclic ligand (dicyclohexano-18-crown-6 ether) enabled transfer of cyt c into IL phase (hydroxyl group modified alkyl methylimidazolium bis(trifluoromethanesulfonylelimide) from water via supramolecular complexation and recovery was achieved by addition of potassium chloride that formed an inclusion complex with DC18C6.\textsuperscript{11} Wang et al studied extraction of BSA from aqueous phase to [BMIM][Cl] phase by addition of potassium monohydrogen phosphate. Salting out effect dominated protein extraction.\textsuperscript{12} Yu et al performed liquid-
liquid extraction of lysozyme using dye-modified IL [BMIM][PF₆]. Back extraction of lysozyme was achieved under high ionic strength (1M KCl under pH 9 -11).

The method used for back extraction of cyt c from IL to buffer in this study focused on the effect of type of salt (potassium/sodium chloride, ammonium sulfate), salt concentration and pH of the aqueous phase for back extraction. The extraction efficiency was determined by measuring the concentration of protein in the buffer after extraction by UV-Vis spectroscopy. The structure of recovered protein was measured by FTIR, UV-Vis, Fluorescence and CD (near, far and soret region) spectroscopy. Activity measurements were performed using cytochrome c peroxidase activity assay.

4.2 Experimental

4.2.1 Materials

Cyt c derived from horse heart and potassium chloride were purchased from Sigma-Aldrich and used without further purification while [EMIM] [EtSO₄] was donated by Evoniks, Inc. Regenerated cellulose dialysis tubes with MWCO ~3000 g/mol were procured from Spectrum. All aqueous solutions were prepared in 0.01M phosphate buffer at pH ~7.2. The protein dissolved in IL (5mg/ml) was dialysed against 0.01 M phosphate buffer with no or varying concentrations of KCl (0, 0.1, 0.3, and 1 M KCl). The dialysis was carried out for a period of 24 hours with a change of dialysate buffer after 12 hours.
4.2.2 Characterization

4.2.2.1 UV-Vis Spectroscopy

The absorption spectrum of cyt c was recorded with a UV-Vis spectrophotometer (Perkin Elmer Lambda 25) employing a 1-cm path length cuvette. Protein concentration was determined spectrophotometrically via the measured pH ~7 extinction coefficient of $1.06 \times 10^5 \text{M}^{-1}\cdot\text{cm}^{-1}$ at 410 nm.$^{14}$

4.2.2.2 Fluorescence Spectroscopy

Fluorescence measurements were performed with a xenon discharge lamp for excitation on a spectrometer (PerkinElmer LS50B). Emission spectra were recorded at a fixed excitation wavelength of 280 nm.

4.2.2.3 FTIR Spectroscopy

FTIR spectra were obtained on a Fourier transform infrared spectrometer (Bruker Tensor 27) equipped with a LN-MCT detector and MIRacle, a single reflection horizontal ATR diamond crystal plate. A total of 100 scans at 2 cm$^{-1}$ resolution were averaged to get the reported, background subtracted spectra. Protein concentration was set to 1 mg/ml.

4.2.2.4 Circular dichroism

Far, near and soret region circular dichroism (CD) experiments were carried out on Jasco 720 spectrometer using 0.1 (Far UV CD) and 1-cm path (Near and Soret region CD) length quartz cuvettes. After 5 min equilibration of the solution filled quartz
cuvettes at 25 °C, spectra were recorded as three scan averages from 200 to 250 nm (Far UV CD), 240 to 320 nm (Near UV CD) and 410 to 450 nm (Soret UV CD) with 16 seconds response time and 10 nm/min scan rate. The final spectra were obtained after subtracting the signal from a solvent filled cuvette. The protein concentration was 0.2 mg/ml in all the cases.

4.2.2.5 Peroxidase activity assay

The peroxidase assay was initiated by adding hydrogen peroxide (H$_2$O$_2$) to a solution mixture of cyt c and guaiacol. The latter was then catalytically oxidized to its tetramer (tetraguaiacol) or similar compound which is orange, allowing the reaction to be followed by monitoring absorbance at 470 nm. Assays were performed in 1.5 ml quartz cuvettes at guaiacol and H$_2$O$_2$ concentrations of 20 mM and 50 mM, respectively. Protein concentration was 0.2 mg/ml. The rate of steady state reaction was taken as the maximum of the first derivative of the product formation curve.

4.3 Results and Discussion

4.3.1 Transfer of protein from IL to buffer

Protein transfer from IL to buffer by dialysis resulted in protein precipitation initially. In the early stages of dialysis, as the buffer entered the dialysis tube, the salt content became very high in the aqueous-IL solution in the dialysis tube because IL is also essentially a molten salt and this resulted in protein precipitation. As dialysis proceeded and equilibrium was established between the contents of the dialysis tube and the buffer outside after exchange of IL through the membrane, the protein subsequently
dissolved in the buffer. However, some protein was lost due to aggregation. The schematic in Fig. 4.1 represents the early and late stages of dialysis in the dialysis tube.

![Schematic showing initial and final stages of dialysis of cyt c from IL into buffer.](image)

**Figure 4.1.** Schematic showing initial and final stages of dialysis of cyt c from IL into buffer.

KCl is considered to be a salting in salt according to the Hofmeister series. The percentage of protein transferred from IL to buffer as a function of molarity of KCl is plotted in Fig. 4.2. At no or very low salt concentration, as well as very high salt concentrations (1 M), the recovery of cyt c into buffer is low. It reaches a maximum value at some intermediate salt concentration (0.3 M in this case) which is consistent with the well-known bell shaped solubility curve for proteins in aqueous salt solutions.

![Graph showing extraction efficiency vs molarity of KCl](image)

**Figure 4.2.** Transfer of cyt c from IL to buffer with varying salt concentration.
4.3.2 Spectroscopic Characterization

The structure of protein transferred into buffer was compared to that of native protein in buffer solution (0.01 M Phosphate buffer with 0.3 M KCl). The UV-Vis spectrum of recovered and native protein is shown in Fig. 4.3. The Soret band for recovered cyt c shifts to 408 nm compared to native protein where a soret maximum of 409 nm is observed. The protein largely exists in the oxidized state similar to native protein indicated by the presence of a single band at 530 nm (Q band). The third band i.e. the charge transfer band, characteristic of native cyt c in buffer, is also observed in the recovered protein. However, it should be noted that this band around ~695 nm is observed only at high concentrations of recovered protein. At concentrations below 0.1 mg/ml, this band is not visible. This indicates that the sixth axial ligand, methionine, might be absent in the recovered protein.16

![Figure 4.3. Soret band and the Q band of native and recovered cyt c in 0.01 M phosphate buffer, 0.3 M KCl, pH ~ 7. The inset shows the CT band at 695 nm for both the cases.](image)
Fluorescence emission spectrum of recovered cyt c and native cyt c in buffer is shown in Fig. 4.4. Native cyt c has a weak fluorescence emission spectrum with no clear maxima, a consequence of quenching of fluorescence of the aromatic amino acid residue tryptophan (Trp$^{59}$) by Forster energy transfer between closely spaced heme group and Trp$^{59}$. Similarity of both the spectra suggests that the position of tryptophan relative to heme iron is intact.

![Fluorescence emission spectrum](image)

**Figure 4.4.** Fluorescence emission spectrum of native and recovered cyt c in 0.01 M phosphate buffer, 0.3 M KCl, pH ~ 7 using an excitation wavelength of 280 nm.

Fig. 4.5 shows the IR spectra of recovered and native cyt c in buffer. The presence of peaks at 1653 and 1542 cm$^{-1}$ corresponding to the Amide I and Amide II regions for both the cases suggests that the native secondary structure of cyt c remains intact upon recovery.
Figure 4.5. FTIR spectrum of native and recovered cyt c in 0.01 M phosphate buffer, 0.3M KCl, pH ~ 7 in the Amide I and II regions.

The far, near and Soret region CD spectra of recovered and native cyt c in buffer are shown in Figs. 4.6 a, b and c respectively. Far UV CD spectrum is sensitive to the secondary structure of proteins. The $\alpha$-helix, $\beta$-sheet or random coils each have a unique CD spectrum. $\alpha$ helices usually exhibit two negative peaks at 208, 222 nm, $\beta$ sheets show one negative peak at 218 nm and random coils have a negative peak at 196 nm. The far UV CD spectrum of cyt c exhibits two negative peaks at 208 and 222 nm characteristic of $\alpha$ helix.\textsuperscript{17, 18} The line shape of the far UV CD spectrum of recovered and native cyt c in buffer is similar indicating that the recovered protein remains largely $\alpha$ helical. However, differences in the intensity of the negative peak at 208 nm can be observed that suggest changes in the $\alpha$-helical content of the protein. The near UV CD spectrum provides information about the tertiary structural packing of the aromatic amino acid side chains. In the native protein, negative bands around 280 and 288 nm are assigned to the tertiary structural packing of Trp\textsuperscript{59} residue and the broad positive band around 250-270 nm is due to transitions in heme.\textsuperscript{17, 19, 20} The differences between the spectra of recovered and native cyt c in buffer suggest changes in the tertiary structure of
the recovered protein. From the Soret region CD spectrum, it can be seen that the negative peak at 416 nm present in native cyt c in buffer (manifesting interaction of the Phe\(^{82}\) side chain, positioned on the Met\(^{80}\) side of the heme plane, with the heme group, and revealing the integrity of Met\(^{80}\) as axial ligand) is not fully regained in the recovered protein. It might be inferred that the Met\(^{80}\)-iron ligation is disrupted in the recovered protein.\(^{21,22}\)

Figure 4.6a. Far UV CD spectrum of native and recovered cyt c in 0.01 M phosphate buffer, 0.3M KCl, pH ~ 7.
Figure 4.6b. Near UV CD spectrum of native and recovered cyt c in 0.01 M phosphate buffer, 0.3M KCl, pH ~ 7.

Figure 4.6c. Soret region CD spectrum of native and recovered cyt c in 0.01 M phosphate buffer, 0.3M KCl, pH ~ 7.

4.3.3 Peroxidase Activity

Cyt c acquires peroxidase activity when the coordinate bond between heme iron and Met80 sulfur breaks\textsuperscript{15, 23, 24}, as demonstrated previously, when cyt c dissolves in the IL. Fig. 4.7 compares peroxidase activities of native and recovered cyt c in buffer, and
Table 4.1 shows the extracted slopes of the steady state phase. It is found that recovered cyt c exhibits nine times higher peroxidase activity than native protein. This confirms the findings from preceding discussion, particularly a perturbation or loss of Met\textsuperscript{80} as axial ligand to the heme group.

![Absorbance at 470 nm vs Time (sec)](image)

**Figure 4.7. Cyt c peroxidase activity assay: comparison of activity of native and recovered cyt c in 0.01 M phosphate buffer, 0.3 M KCl, pH ~ 7.**

**Table 4.1. Comparison of Peroxidase Activity of native and recovered cyt c in buffer**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native cyt c</td>
<td>$5.6 \times 10^{-4}$</td>
</tr>
<tr>
<td>Recovered cyt c</td>
<td>50</td>
</tr>
</tbody>
</table>

**4.4 Conclusions**

Cyt c dissolved in neat IL was transferred to buffer containing varying amounts of salting-in salt, KCl, by dialysis. The recovered cyt c largely retained its native secondary structure while the tertiary structure was not regained after transfer to buffer. The protein acquires peroxidase activity probably by loss of Met\textsuperscript{80} ligand. Strongly coordinating ILs that dissolve proteins also tend to disrupt the native conformation of...
proteins and are not suitable for biopreservation. While changes in structure of cyt c were observed upon transfer to buffer from IL, complete denaturation or microbial contamination evident (visually and by UV VIS spectroscopy-data not shown) in cyt c stored in buffer at room temperature in just a short period of 1 month was not observed. Protein stored in IL for over 4 months, when transferred to buffer gave similar results as shown here, irrespective of the length of time it was stored in IL at room temperature.
4.5 References


CHAPTER 5
IL-POLYMER BIOCOMPATIBLE GELS

5.1 Introduction

Ionic liquid - polymer composites, so called gel polymer electrolytes or ion gels, gained attention for applications in electrochemical devices such as battery, capacitors and electrochromic cells. Angell and coworkers\textsuperscript{1} had described a new class of polymer electrolytes for secondary lithium batteries, termed “polymer” in “salt”, which were rubbery solid electrolytes possessing high ionic conductivities. The dimensionally stable polymer electrolytes showed significant advantages over liquid electrolytes from both the mechanical property and fabrication standpoints. Watanabe et al\textsuperscript{1} reported polymer electrolytes containing chloroaluminate-based ILs for battery applications but their moisture sensitivity, resulting in the formation of hydrochloric acid (which is highly corrosive), proved to be a major deterrent in further electrochemical device-based applications. Thus, began the exploration of new free-standing polymer electrolytes produced by incorporating chemically stable nonchloroaluminate ILs into appropriate polymer matrix.\textsuperscript{1-3}

Watanabe et al\textsuperscript{4} reported \textit{in situ} free radical polymerization of vinyl monomers like methyl methacrylate (MMA), acrylonitrile (AN), vinyl acetate (VA), styrene and 2-hydroxyethyl methacrylate (HEMA) in 1-ethyl-3- methyl imidazolium tetrafluoro borate and 1-butyl pyridinium tetrafluoro borate. HEMA was found to form transparent flexible films for monomer concentrations up to 60\%, and above that, phase separation occurred resulting in translucent films. Compatibility of HEMA in the latter IL was
good but the IL itself had low ionic conductivity.\textsuperscript{4} Other groups also prepared transparent MMA\textsuperscript{5} and HEMA\textsuperscript{3} gels in hexafluoro phosphate and fluorohydrogenate based-ILs respectively. These transparent, mechanically strong and highly conductive polymer gels were distinctly different from conventional polymer gels in terms of non-volatility and high thermal stability. Since ILs had the ability to be tailored for proton conduction, and lithium ion conduction, the use of ion gels as new polymer electrolytes had the potential to be used in related fields, like fuel cells, lithium batteries and solar cells.\textsuperscript{3}

Little literature on the use of polymer-IL gels as media for biopreservation exists. One of the probable reasons for this is the lack of information on the toxicity and biocompatibility for many ionic liquids. Firestone et al have reported protein encapsulation in a photocured gel using a mixture of a polymerizable ionic liquid monomer (1-decyl-3-methylimidazolium acrylate), PEG co-monomer, water and a photoinitiator.\textsuperscript{6} The aim of this work is to demonstrate the feasibility of protein preservation by encapsulation in polymer-IL gels. Eventually, with the development of biocompatible ionic liquids, biopreservation in IL-polymer gels could be considered a novel means of protein preservation at room temperature.

The criteria for development of a suitable material for protein encapsulation are\textsuperscript{7} efficient stabilization of protein-based vaccines and therapeutics, potential to enable room temperature storage of these, reduction in cost of the new formulation over lyophilized formulation, easy transportation (no refrigeration required) and usage, and, allows protein storage in high concentrations which is not possible with aqueous sugar based protein stabilization techniques.
ILs seem to be promising candidates that could meet most of the above mentioned criteria, though it is unclear whether liquid-based formulations can be rendered sufficiently stable to withstand the stresses associated with shipping and storage. However, if IL based gels are used for storage of proteins, the stability enhancing properties of ILs coupled with a polymer matrix in the form of a gel could make handling and transportation of such products easier. Additional advantages would be the possibility of using these systems directly as vehicles for drug delivery by using polymers and ILs that are biocompatible. Measuring small aliquots of liquids is tedious but, if the gels could directly be cut, weighed and used, handling would become much easier.

In this work, ionic liquid chemical gels using a biocompatible polymer have been prepared with the aim of protein preservation, since prior work has shown the potential of ILs as protein preservation media. The protein cytochrome c was encapsulated in a 40:60 (HEMA: IL) composition gel and the structure characterized by spectroscopic methods.

Additionally, the mechanical properties of IL-HEMA gels of various compositions were evaluated and the gels were patterned on the micron and nanometer size scale by imprinting. The patterned gels were imaged by SEM and SFM. Further, a few IL based systems including physical gels of PEO-IL were imaged by TEM to illustrate the ability to image “wet” gels.
5.2 Experimental

5.2.1 Materials

The monomer, initiator and crosslinker (HEMA, AIBN and EGDMA) were purchased from Sigma Aldrich. HEMA was vacuum distilled to remove inhibitors. Chemically crosslinked gels of poly (2-hydroxy ethyl methacrylate) of different monomer-ionic liquid compositions (varying from 10%–90%) were prepared in EMIM ethylsulphate by free radical polymerization. The concentration of initiator and crosslinker were 0.2% and 4% based on monomer weight respectively. The polymerization was carried out in nitrogen atmosphere at a temperature of 60°C.

5.2.2 Characterization

5.2.2.1 Spectroscopic Characterization

5.2.2.1.1 UV-VIS

The absorption spectrum of cyt c encapsulated in IL-HEMA gels was recorded with a UV-Vis spectrophotometer (Perkin Elmer Lambda 25).

5.2.2.1.2 Circular Dichroism

Soret-region circular dichroism (CD) experiments were performed on Jasco 720 spectrometer. After 5 min equilibration of the cytochrome c encapsulated IL-HEMA gel at 25 °C, spectra were recorded as three scan averages from 410 to 450 nm (Soret UV
CD) with 16 seconds response time and 10 nm/min scan rate. The final spectra were obtained after subtracting the signal from IL-HEMA gel.

5.2.2.2 Tensile Testing

The stress-strain curves of IL-HEMA gels of various compositions were measured on an Instron 4411 tensile testing machine equipped with a 100 N load cell at a crosshead speed of 1 mm/min. Dogbone shaped samples complying with ASTM D1708 standard, were cut using a standard die and the thickness was measured using micrometer calipers.

5.2.2.3 Transmission Electron Microscopy (TEM)

Bright-field TEM was performed on a (JEOL-1200EX) TEM operating at an accelerating voltage of 56 kV. Samples of nanoparticles in IL and physical gels of IL-PEO were pipetted onto carbon coated copper grids that were placed in vacuum oven for ~2 days to remove traces of water before observation under TEM.

5.3 Patterning

The gels with over 30% monomer concentration were topographically patterned on the micrometer and nanometer length scale using imprint lithography. First, a template was generated by pouring poly(dimethyl siloxane) (PDMS) resin and SYLGARD 184 crosslinker (10:1 wt. ratio) into a master mold (patterned silicon wafer made by photolithography for micron-sized patterns and poly(carbonate) mold prepared from HD-DVD disk\textsuperscript{9} for nanometer-sized patterns) and cured for 1 h at 60°C. The flexible PDMS film was then peeled from the mold and placed face down on a solution
containing the gel components which, upon heating to 60°C, were polymerized and crosslinked. After reaction for 8-10 h, PDMS was gently removed, exposing the gel pattern. Before imaging, the gels were typically stored at 60°C under nitrogen for several days. The patterned gels were observed by scanning electron microscope (SEM) (JEOL 6320F) and scanning force microscope (SFM) (Nanoscope III, Digital Instruments Co.). Samples were not coated with gold due to inherently conductive nature of ILs.

5.4 Results and Discussion

5.4.1 Polymerization

Clear, colorless, mechanically robust gels were obtained at monomer compositions greater than 10 wt.%; at lower HEMA concentrations, crosslinked products were too weak and sticky to handle. Poly(HEMA) and [EMIM][EtSO₄] form compatible binary systems even at monomer concentrations as high as 90%. Fig. 5.1 shows the clear and transparent gels obtained at various monomer-IL compositions.
Figure 5.1. Poly (HEMA) gels with different monomer compositions. [Gel composition (monomer: ionic liquid) from left to the right: 40:60; 20:80; 10:90]

The mechanical properties of these gels were examined at monomer concentrations varying from 15 wt.% to 75 wt.%; across this range, the tensile modulus rises from 0.3 MPa to 100 MPa, as shown in Fig. 5.2. Neat poly (HEMA) is a brittle solid. Therefore, as the concentration of monomer increases, an increase in modulus is observed. 15% (HEMA concentration) gels have the lowest modulus as would be expected.
5.4.2 Protein encapsulation in HEMA-IL gel

Cytochrome c was dissolved in IL and gels of HEMA-IL of 40:60 composition were prepared by addition of initiator and crosslinker. UV-Vis spectrum of cyt c encapsulated in HEMA-IL gel is shown in Fig. 5.3. The Soret band for encapsulated protein is observed at 408 nm. The protein largely exists in the oxidized state similar to native protein indicated by the presence of a single band at 530 nm (Q band). The charge transfer band at ~ 695 nm is absent in this case indicating the loss of methionine as sixth axial ligand.¹⁰ This spectrum is similar to the one obtained for cyt c in IL as discussed in Chapter 3.
Figure 5.3. UV-Vis spectrum of cyt c encapsulated in HEMA-IL gel.

CD spectrum of cyt c encapsulated in HEMA-IL gel is shown in Fig. 5.4. Here too, lack of a negative band at 416 nm indicates that distance and orientation of the phenyl alanine side chain on the methionine side of the heme plane with respect to the heme iron is perturbed.\textsuperscript{11, 12} The similarity of the uv-vis and CD spectrum of encapsulated cyt c to cyt c in IL indicate that the polymer PHEMA does not cause additional changes in the structure of the protein than those observed in neat IL.
5.4.3 Patterning of IL-HEMA gels

Gel surfaces were topographically patterned on the micrometer and nanometer length scales by imprint lithography. The schematic is shown in Fig. 5.5.

![Schematic of imprint lithography](image)

Figure 5.5. Imprint lithography for poly(HEMA)-[EMIM][EtSO₄] gels.

Fig. 5.6 shows SEM images of a 30-40 wt.% monomer gel patterned with a PDMS mold imposing raised line features of 700 nm (left) and 200 nm (right) nominal width; below 20 wt.% monomer, with moduli less than 1MPa, pattern replication was
partial or absent, presumably because gels were too soft or too adhesive to PDMS. However, excellent replication is seen for all more concentrated gels.

Figure 5.6. SEM image of 40 wt.% surface-patterned poly(HEMA)-[EMIM][EtSO₄] gel. Nominal line widths are 700 nm (left) and 200 nm (right). Scale bar is 1µm.

To assure the absence of imaging artifacts, gel patterns with features of ~200 nm were also examined by tapping mode SFM, the resulting height (left) and phase (right) images shown in Fig. 5.7.

Figure 5.7. SFM height (left) and phase (right) images of 40 wt.% poly(HEMA)-[EMIM][EtSO₄] gel surface-patterned with (nominally) 200 nm width lines raised by 60 nm from 200 nm width trenches (10 µmx10 µm areas).

Table 5.1 compares mold and gel feature sizes measured by SFM, and a 20% shrinkage in width is found for the nominal 200 nm raised lines, but there seems to be
no accompanying width change for the intervening trenches; the line heights are somewhat less than in the template. These size differences from the original templates likely reflect the relaxation of polymerization stresses by shape change upon removal of the gel from its rigid support or leaching out of IL with time. Reasonably accurate replication at such small size scales for a soft gel is remarkable. It should be noted that the samples for SEM were not coated with gold. No charging of the sample was observed exhibiting the conductive nature of gels.

Table 5.1. Comparison of Feature Sizes between Mold and Patterned Gel

<table>
<thead>
<tr>
<th></th>
<th>Line Width (nm)</th>
<th>Trench Width (nm)</th>
<th>Line Height (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mold</td>
<td>232±20</td>
<td>173±20</td>
<td>65±10</td>
</tr>
<tr>
<td>Gel</td>
<td>187±14</td>
<td>186±17</td>
<td>40±5</td>
</tr>
</tbody>
</table>

5.4.4 TEM of IL based systems

Gels and other solvated polymer microstructures, in general, cannot be directly imaged by electron microscopy due to the volatility of the solvents used to form the gel. Typically, these “soft” systems are dried or cryogenically cooled prior to imaging, which can alter the morphology. By using ionic liquids (ILs) as the solvating medium, solvent volatility is not an issue, providing an alternate approach in gaining insight into these systems. In addition, ILs are usually highly conductive, eliminating the need to use a metal coating to prevent charging. While several IL-based “gel-polymer electrolytes” have been studied by electron microscopy to assess film structure\textsuperscript{13,14}, broader prospects of the IL approach for imaging of “wet” polymer gels has not been explored. More
broadly, *in situ* electron microscopy affords a real-space image for the determinations of the nano- and meso-scale microstructure of model gels.

Iron oxide nanoparticles and ferritin were dispersed in [EMIM] [EtSO₄]. The diameter of the former was ~15 nm and that of the latter was ~12 nm. Fig. 5.8 and 5.9 show the TEM images of Iron oxide and ferritin in IL. The white spots indicate beam damage. IL is susceptible to beam damage.

![Figure 5.8. Iron oxide nanoparticles dispersed in IL. Particles seem to aggregate indicating that IL is not a good solvent for these particles.](image)

![Figure 5.9. Ferritin dispersed in IL.](image)
Poly(ethylene oxide) (PEO) forms opaque physical gels in [EMIM] [EtSO₄] at room temperature.⁵¹ 15 wt % PEO was dissolved in IL at ~70°C, cast on carbon coated copper grid and placed in vacuum oven for 2 days to remove all traces of moisture before imaging by TEM. Figs. 5.10 and 5.11 show the PEO crystallites from different regions of the sample. Beam damage of IL in Fig. 5.10 indicates the presence of IL in the sample.

Figure 5.10. PEO-IL physical gels.

Figure 5.11. Spherulites of PEO from another region of the sample.

However, it is difficult to show the presence of IL in other samples. But similarity in texture of a bulk gel of PEO-IL prepared on a Si wafer (by drop casting the 15 wt.% PEO-IL solution) and imaged by optical microscopy shown in Fig. 5.12 with
the TEM images might indicate that IL is present in the sample imaged by TEM. It might also allude to the fractal nature of these gels.

![Image of bulk PEO-IL gel on silicon wafer](image)

**Figure 5.12. Optical microscopy image of bulk PEO-IL gel on silicon wafer.**

### 5.5 Conclusions

Non-drying, biocompatible and mechanically robust gels of PHEMA and IL were prepared. The gels were used as a model system for encapsulation of protein cyt c. The structure of the protein was found to be the same as that in the neat IL proving that the polymer has the potential to be used as a protein preservation medium by a suitable choice of proteins and ILs. The PHEMA-IL gels were patterned in the micron and nanometer size range and imaged by SEM proving the versatility of such systems to be used as gel polymer electrolytes with interdigitated electrodes or as substrates for cell adhesion and proliferation, as it is known that nanoscale features on a substrate enhance cell adhesion and growth. The negligible vapor pressure of ILs allows imaging “wet” gels by TEM, a scenario not possible with conventional hydrogels.
5.6 References


