HOFMEISTER CHEMISTRY:
WEAK CATION BINDING TO PROTEIN BACKBONES

&

BIOMOLECULAR SIZE INFLUENCE ON SPECIFIC ION EFFECTS

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by
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ABSTRACT

Hofmeister Chemistry: Weak Cation Binding to Protein Backbones & Biomolecular Size Influence on Specific Ion Effects (August 2014)

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The behavior of solutes in aqueous solutions was first shown over a century ago to be ion specific. Such ion specificity has been characterized and named as the lyotropic or Hofmeister series. These recurring ion trends have been elucidated for a wide range of biologically important processes including aggregation of polymers/proteins, protein folding, and enzymatic activities. In this dissertation, we investigated two major issues yet to be solved in ion specific effects on biomacromolecular systems. First, cation associations to the protein backbone was investigated with three different model systems. A simple amide molecule, butyramide, was utilized as a monomer unit for protein backbone. FTIR spectroscopy is employed to monitor the amide I band, and coupled with vibrational sum frequency spectroscopy (VSFS), which shows aligned water hydration at the air/butyramide/water interface. Contact paired cation binding is monitored via a salt concentration dependent new carbonyl stretch peak (1645 cm\(^{-1}\)), which is ~25 cm\(^{-1}\) blue shifted from the original resonance (1620 cm\(^{-1}\)). Moreover, enhancement in the degree of aligned water molecules upon cation absorption in the hydration of the butyramide monolayer at the air/water interface demonstrated a complementary set of evidence. Only strongly hydrated cations (Mg\(^{2+}\), Ca\(^{2+}\), Li\(^{+}\)) demonstrated these spectral changes. Weakly
hydrated cations (Na\(^+\), K\(^+\)), on the other hand, did not show any evidence for contact paired binding to simple amides.

The molecular mechanism of cation-protein backbone interactions was further investigated on neutral biomacromolecules. The hydrophobic collapse of poly (N-isopropyl acrylamide) (PNIPAM) and elastin like polypeptides (ELPs) was exploited to glean the effect of 11 alkali and alkaline earth metal chloride salt. The lower critical solution temperature (LCST) data was modeled to a simple equation where weakly hydrated cations once again show no clue for cation binding to neutral biomolecules. These cations mainly salt macromolecules out with surface tension effects. Very weak cation binding, however, was shown for strongly hydrated cations. Moreover, an additional bulk salting-in effect was shown for strongly hydrated cations, where ion hydration thermodynamics demonstrated a direct correlation with the specific salting in effects.

The second major topic focused in this thesis was the molecular size influence on specific ion effects. It was investigated by using various poly(N,N diethyl acrylamide) (PDEA) from oligomers to large polymers, along with some simple amide molecules; N-methyl acetamide (NMA), and N,N Diethyl acetamide. Hydrophobic collapse of PDEA was probed as a function of three representative Hofmeister anions (SO\(_4^{2-}\), Cl\(^-\), SCN\(^-\)). Molecular size changes showed no apparent influence with kosmotropic SO\(_4^{2-}\), and Cl\(^-\) anions. Nevertheless, chaotropic, SCN\(^-\), anion binding was dramatically influenced from molecular size. Similar results were achieved from the Raman spectrum of the model systems where the shifts in the C-H stretch peaks were monitored. Overall, weakly hydrated anion exclusion (K_D > 3M), and a strong anion binding (K_D ~ 130mM) were observed for simple amides and large polymers, respectively. The molecular size
dependence was also elucidated for the hydration of biomolecules. A gradual increase in the ice-like (3200 cm$^{-1}$) water peak, which is attributed to weakly hydrated sites at the biomolecular surface, was monitored as the macromolecular size increases. This hydration behavior must play a dominant role for the underlying molecular mechanism for the macromolecular size effect.
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CHAPTER I

INTRODUCTION

Hofmeister Series

Ions influence the behavior of solutes in aqueous solutions. This phenomena was discovered by Franz Hofmeister more than 12 decades ago.\textsuperscript{1,2} The first trend in ion specific effects was shown and named after him, called the Hofmeister series. In the original work, the precipitation of egg white protein was monitored with a series of sodium salts.\textsuperscript{2} Later, a wide range of physical and biological processes have been shown to follow the recurring anionic trend; including aggregation of polymers/proteins, protein folding, and enzymatic activities, as well as colloidal assembly, micelle stabilization, and many others.\textsuperscript{3–13} The typical ionic trend can be seen in figure 1.1. Anions to the left of chloride are strongly hydrated and lead to greater salting-out behavior. These ions are traditionally called kosmotropes. Whereas, ions to the right of the series are weakly hydrated and named as chaotropes along with a salting in property for proteins and polymers. Chloride is often found to be the dividing point between salting-in and salting-out behavior in this series.

In contrast to the anions, the Hofmeister effect for cations is usually weaker.\textsuperscript{6,14,15} Ions on the left salt proteins/macromolecules out of solution, while ions on the right lead to salting-in behavior (Figure 1.1). Curiously, the most effective cations for salting proteins into solution are the ones that are most strongly hydrated, while cations that are weakly hydrated lead to salting-out behavior. This is opposite of the anion series. Moreover, despite being less pronounced than the anionic series, specific cation effects
have direct importance for protein folding, protein-protein interactions, cell signaling, protein aggregation, enzyme catalysis, and even biotechnology.\textsuperscript{16-20}

Even though the specific ion effects have been known over a century, there are many anomalies that remain to be deconvoluted to achieve a unified mechanism of ion specificity at a wide range of physical and biological systems. For instance, the Hofmeister series has been shown to reverse when charged residues are involved at the substrate surface.\textsuperscript{21-24} Namely, strongly hydrated anions (i.e. SO$_4^{2-}$) salted in proteins and weakly hydrated anions (SCN$^-$) salted out. This reversal mechanisms has not been fully elucidated. Further, even though the influence of ions for neutral systems has been known over a century, the molecular level mechanisms for anion and cation binding to protein backbones were only elucidated recently.\textsuperscript{14,25} In this pioneering work, NMR spectroscopy, Molecular Dynamics (MD) simulation along with hydrophobic collapse behavior of polypeptides were utilized. Weakly hydrated anions were shown to bind a hybrid alpha proton binding site at the methylene groups of the protein backbone. The neighbor electron withdrawing groups, carbonyl and N-H groups, leave backbone methylene hydrogens partially positively charged. Moreover, the weakly hydrated nature of these sites made anion binding energetically more favorable by reducing the dehydration penalty upon ion binding.\textsuperscript{25} The molecular level mechanism of cation binding is one of the focus of this dissertation, and discussed in Chapter 2&3. Bulk ion solvation contributions and the influence of macromolecular size on specific ion effects are also elucidated and discussed in details in Chapter 3&4.
Figure 1.1. The direct Hofmeister Series for anions (top line), and cations (bottom line).
Model Systems

The mechanism of ion specific effects on biological systems have extensively been studied, yet the overall underlying chemical mechanism is still partially unclear due to two main reasons. First, the degree of structural and compositional complexity of naturally occurring biological systems is quite substantial. For instance, diverse amino acids variance, especially the ones with formal charges at physiological pH, strongly dominates the observables as such other major (or minor) components of specific ion effects may not be displayed, and understood clearly. Second, naturally occurring biological systems could be affected by secondary ion effects, such as structural changes of proteins due to ionic strength deviations. Nevertheless, model molecules have been widely used to investigate ion specific effects on various isolated chemical groups. These molecules vary from simple amide molecules to large biomacromolecules like Poly(N-isopropyl acrylamide) (PNIPAM), Poly (N,N diethyl acrylamide) (PDEA) and Elastin–Like polypeptides (ELPs).

Small model molecules

Simple amide molecules, such as N-Methyl acetamide (NMA), N,N-Dimethyl acetamide (DMA), and butyramide, along with single amino acids (i.e. glycine, and valine) (Figure 1.2 a-e) have been employed as model systems to mimic the amide groups on protein backbone. On one hand, their usage is beneficial due to their chemical simplicity and limited structural variations in numerous experimental conditions. On the other hand, lacking of the peptide bond rises many questions about the validity for mimicking protein backbone. Thus, any conclusion must only be drawn with the results
from small molecules after complementary results with macromolecule counterparts agree with it. (Figure 1.2 f-h)

**Poly (N-isopropyl acrylamide) (PNIPAM) and Poly(N,N diethyl acrylamide) (PDEA)**

PNIPAM and PDEA are synthetic polymers composed of N-isopropyl acrylamide, and N, N diethyl acrylamide monomer units, respectively. These polymers have the amide groups on the side chains as oppose to polypeptides and proteins. This modification yields identical amide groups for the entire polymer, as well as more exposed amide sites for any possible interactions with ions, osmolytes and other additives. Moreover, hydrophobic isopropyl and diethyl groups on the amide nitrogen enable these polymers to further imitate proteins also at the hydrophobic parts. These polymers are thermo-responsive, and undergo a phase transition via hydrophobic collapse/aggregation at certain temperature. \(^{33}\) This phase transition is called as lower critical solution temperature (LCST). The polymer is soluble and forms clear solution below transition temperature and turn to a milky white solution above the LCST value. (Figure 1.3 a, b) This process mimics an inverse cold denaturation of proteins. Typical cold denaturation temperatures of proteins are below the freezing point of water, whereas, aqueous solutions of these thermo-responsive polymers yield LCSTs around ambient temperatures, which makes them a great model system to be investigated under a wider variety of conditions.
Figure 1.2. The structure of model molecules that have been utilized to mimic protein backbone. a) N-methyl acetamide (NMA), b) Dimethyl acetamide (DMA), c) butyramide, d) glycine, e) valine, f) Poly(N-isopropyl acrylamide) (PNIPAM), g) Poly(N,N dimethyl acrylamide)(PDEA) and h) A representative pentameric ELP amino acid sequence (VPGXG).
Figure 1.3. a) A schematic drawing of thermo-responsive polymers and polypeptides in soluble and collapsed/aggregated states, b) the pictures of soluble and precipitated 10 mg/ml PNIPAM solutions at ambient and 35°C (above LCST value) temperatures, respectively.
**Elastin-Like Polypeptides (ELPs)**

Polypeptides are the most realistic protein model among biomacromolecules, where elastin-like polypeptides (ELPs) were one well-known example. ELPs are formed by Val-Pro-Gly-X-Gly pentapeptide repeat units \(^{34,35}\), where X serves as a guest residue, which can be every amino acid other than proline. ELPs have –SKGPG and –WP sequences on the N, and C termini, respectively. Tryptophan residue on the C terminus enables one to measure exact concentration of ELPs in solution with an extinction coefficient of 5690 M\(^{-1}\)cm\(^{-1}\) at 280nm. These polypeptides were synthesized by recursive directional ligation (RDL) technique. The details of the technique can be found in the literature.\(^{35}\) The simplicity of the technique allows one to create a large library of ELPs with a wide size and sequence variations.

ELPs undergo phase transition, similar to thermo-responsive polymers, from an extended state at temperatures below to collapsed and aggregated form at temperatures above LCST. (Fig 1.3 a&b) However, this collapse involves a subtle secondary and tertiary structural changes mainly β-turn/β-spiral as oppose to PNIPAM, and PDEA polymer models.\(^{36-38}\) Moreover, the LCST values of these polypeptides are tunable, and can be altered by genetically engineering the amino acid composition along with the chain length of the polypeptide. These polypeptides are named in the form of \(X_a Y_b Z_c - n\), where X, Y, and Z stands for the amino acid identity of the guest residues. The values a, b, and c denote the ratios of each guest amino acid. The total number of pentapeptide repeat units is indicated by the value n. For example, ELP VG-120 is 600 amino acid polypeptide where it contains 60 valine and 60 glycine at the guest residues.
ELPs are also very advantageous for their laboratory handling, and easy expression and purification steps. It can simply be expressed in *E. coli* strain BLR/DE3 by inserting desired DNA gene for the corresponding ELP sequence.\textsuperscript{34} These DNA genes also carry ampicillin resistant gene for reducing the risk of any contaminations in the incubation step. Expressed ELPs are separated from the nucleic acids by removing the aggregates upon treating with poly(ethyleneimine), just after the cells are lysed by sonication, and organelles and insoluble cell components are removed. Thermo-responsive property of ELPs are used for purification from other proteins and polypeptides. A series of heating and cooling cycles above and below LCST values of desired ELPs are performed, respectively, that yields high purity polypeptides, when coupled with selective collection of ELPs by centrifugation.

**Methods**

**Light Scattering Measurements**

The thermo-responsive behavior of these polymers and polypeptides have been employed as a macroscopic observable in numerous studies.\textsuperscript{25,28,29,39,40} These LCST values can be tuned by small changes in aqueous solutions including pH, as well as, variations in identity and concentrations of ions, osmolytes and other additives. Moreover, the LCST values can easily be measured by light scattering measurements. In our lab two instruments have been utilized for these measurements; linear temperature gradient device, and melting point apparatus.

**Linear temperature gradient device:** Our group designed an instrument, over a decade ago, based on a dark field microscopy in order to measure LCST values of thermo-
responsive polymers and polypeptides quickly and accurately by measuring the light scattering from samples.\textsuperscript{28,41–43} Two parallel brass tubes circulating hot and cold fluids is placed with 5 mm gap from each other. This generates a linear temperature gradient in between, where it is in accordance with Fourier heat diffusion theory.\textsuperscript{44} Aqueous solutions of biomacromolecules are filled to rectangular glass capillary tubes (100 μm x 1 mm x 2 cm) and placed parallel to the thermal gradient. (Fig 1.4a) The LCST value is measured with a dark field microscope image. The collapsed form scatters light significantly, yet the soluble polymers/proteins do not. The scattering generates a dark section in the image. LCST temperature is determined by measuring the lengthwise position of this phase transition. A sample image and a line scan of scattering data can be seen in figure 1.4b.

**Melting Point Apparatus, MPA 100:** A simpler and conventional system is also utilized for light scattering measurements, where it is originally designed for measuring melting points of organics and minerals, called OptiMelt\textsuperscript{TM}, an automated melting point apparatus (MPA 100, Stanford Research Systems) (Figure 1.5a). This system is designed for a wider temperature window (0°C – 400°C) with high accuracy (~0.05°C). Aqueous solutions of desired samples are loaded to one side sealed capillary tubes, then placed into the 3 capillary sample holders. Measurements could be done at various heating rates from 0.1°C/min to 10°C/min. However, a heating rate of 1°C/min is used in a standard measurements. The built-in camera continuously monitor and record the light scattering of each sample separately. The software provides data in light scattering vs temperature format, which can be seen in figure 1.5b. OptiMelt\textsuperscript{TM} has few advantages over the linear temperature gradient device, where it gives the absolute temperature value of the phase transition and also have a wider temperature window.
Figure 1.4. a) Schematic drawing of the temperature gradient device, and b) the light scattering scan of 10 mg/ml PNIPAM in aqueous solution with the temperature gradient device. The LCST value is assigned to the intersection of the plateau at lower temperatures and the linear part of the light scattering transition. The inset shows the corresponding dark field microscopy image. Figure a, b adapted from ref 42.
Figure 1.5. a) A picture of automated melting point apparatus, Optimelt™, and b) a representative Optimelt™ light scattering data as a function of temperature for 6.4 mg/ml ELP V5-120 in 0.4M NaCl solution. The red dots are the data point and the dotted blue lines were employed to assign the LCST value. The intersection of the two blue lines was assigned to LCST value. Figure 1.5b adapted from the supporting information of ref 29.
Vibrational Spectroscopy (infrared and Raman)

Infrared spectroscopy measures the vibrational fingerprints of molecular vibration via absorption of incident beam. This process is only active for vibrational modes that results a dipole moment change of a chemical bond. In our lab a Nicolet 470 Fourier transform infrared (FTIR) spectrometer which was equipped with a single bounce ZnSe crystal (Pike Technologies, Madison, WI), and a MCT detector (Thermo Electron Corp., Madison, WI), which was cooled by liquid nitrogen. Another widely used vibrational spectroscopy technique, is called Raman spectroscopy. It is based on inelastic scattering of a monochromatic light. This process is only active when there is a change in the polarizibility of a vibrational state. (The details of our Raman system can be seen in the MCR-Raman section page-17.)

Vibrational spectroscopy, by using these two complementary techniques, provides unique chemical information of molecular vibrations of chemical bonds. It has been employed to gather wealth of chemical information including characterization of molecules, conformational changes of protein structures, and probing binding interactions.\textsuperscript{40,45–47} Vibrational spectroscopy was utilized in this study to monitor the molecular level picture of ion-model molecules association in aqueous solution. Specifically, we probed the changes in the model molecules upon ion binding.

Interfacial Spectroscopy

Ion-protein interactions could also be monitored with an indirect yet very effective measurements by monitoring changes in the vibrational signatures of water at the biomolecules and/or ion hydration shells. These measurements could potentially provide
unique information, however, it requires an interfacial specific spectroscopy. A well-known surface specific technique is vibrational sum frequency spectroscopy (VSFS). Moreover, a new method, called MCR-Raman spectroscopy, based on Raman spectroscopy and analytical multivariate curve resolution techniques was also developed to gather solvent correlated water spectrum at the hydration shells of the desired solute.

**Vibrational Sum Frequency Spectroscopy (VSFS):** The so called VSFS, is a second-order nonlinear spectroscopy. In this technique, spatially and temporally overlapped infrared ($\omega_{\text{IR}}$) and visible ($\omega_{\text{vis}}$) input beams generates an output beam at the sum frequency ($\omega_{\text{SFG}}$) (eqn. 1.1).$^{48-50}$

$$\omega_{\text{SFG}} = \omega_{\text{IR}} + \omega_{\text{vis}}$$

(eqn. 1.1)

The intensity of sum frequency beam ($I_{\text{SFG}}$), is not only directly proportional to the intensities of input infrared ($I_{\text{IR}}$) and visible ($I_{\text{vis}}$) beams, but also proportional to the square of the effective second-order nonlinear susceptibility ($X_{\text{eff}}^{(2)}$).

$$I_{\text{SFG}} \propto \left| X_{\text{eff}}^{(2)} \right|^2 \cdot I_{\text{IR}} \cdot I_{\text{vis}}$$

(eqn. 1.2)

The second order non-linear susceptibility tensor can be further expressed with the following equation; (eqn. 1.3)$^{48-50}$

$$X_{\text{eff}}^{(2)} = X_{\text{NR}}^{(2)} + X_{\text{R}}^{(2)} = X_{\text{NR}}^{(2)} + \sum_q \frac{A_q}{\omega_{\text{IR}} - \omega_q + i\Gamma_q}$$

(eqn. 1.3)

where $X_{\text{NR}}^{(2)}$, $X_{\text{R}}^{(2)}$ are frequency independent nonresonant and frequency dependent resonant terms, respectively. Frequency dependent term is proportional to oscillator strength ($A_q$), also inversely proportional to vibrational frequencies ($\omega_{\text{IR}}, \omega_q$) and the peak width of the vibrational transition ($\Gamma_q$).
VSFS includes a combination of infrared and Raman transitions, see Figure 1.6. Since selection rules of each transition applies, sum frequency has a unique vibrational selection rules. It only generates signal from non-centrosymmetric molecules, and media. Namely, VSFS is silent to bulk and only active at the anisotropic surfaces, including air/water, quartz/water, and air/desired molecule/water interfaces.\textsuperscript{15,48,51–53} VSFS also probes the relative conformation information.

In our SFG system, the 1064 nm fundamental laser beam with a 17 picosecond pulse width, was generated with a Nd:YAG laser (PY61C, Continuum Inc., Santa Clara, CA), where the laser beam was produced by cavity dumping technique. The energy and pulse frequency of generated beam were 50mJ/pulse and 20Hz, respectively. Then, generated 1064 nm laser beam was sent to optical parametric/amplifier (OPG/OPA) system (Laser Vision, Bellevue, WA). The input 1064 beam was divided with a beam splitter. One part forms 532 nm input light by doubled frequency for angle-tuned down conversion stage, which involves a dual potassium titanyl phosphate (KTP) nonlinear crystals, and produces the 1.35 to 1.85 μm idler beams. Delayed fundamental 1064nm laser was then combined with the idler beams at angle tuned potassium titanyl arsenate (KTA) crystals. The software controlled positions of KTP/KTA stages produced the tunable infrared beam, where the intensity of the beam is 600 μJ/pulse at 3200 cm\(^{-1}\). Then, this tunable infrared beam spatially and temporarily overlapped with fixed 532 nm visible beam. The resultant sum frequency beam was collected by a photomultiplier tube (Hamamatsu, Japan). The collected data was processed by a boxcar integrator to improve the signal to noise ratio. In VSFS, the polarization combination choice was very critical, and sps (s-sum frequency, s-visible, and p-infrared) polarization, was used in all the VSFS spectra in this dissertation.
Figure 1.6. a) A linear combination of infrared and visible input beams generate the sum frequency beam. The scheme indicates the IR absorption and a Raman process by a visible absorption, and SFG emission. b) The schematic diagram showing the principle of vibrational sum frequency spectroscopy (VSFS) on a Gibbs monolayer at air/butyramide/water interface with ssp polarization combination. The input beams are temporarily and spatially overlapping at the surface and creating sum frequency signal.
**Multivariate Curve Resolution (MCR) Raman Spectroscopy**

The Raman spectroscopy was performed using a home built micro-Raman system. Briefly, 514.5 nm Krypton/Argon (Spectra-Physics Inc, Model 2018-RM) laser source was used as an illumination beam with 100mW at the sample stage. Elastic (Rayleigh) and inelastic (Raman) scatterings were collected with a 20x objective (Nikon Japan, Plan APO 20x/0.75 DIC M, w.d. 1.0 cm) Then, the Rayleigh contribution was filtered with a 514 nm notch filter (Semrock Inc., 514.5 StopLine™ Notch filter, E-grade). The signal was collected with a liquid nitrogen cooled CCD detector (Princeton Instruments Inc., PyLoN detector with 1340x100pixels), after the light passed through spectrograph (Princeton Instruments Inc., IsoPlane SCT 320) which was equipped with 600 g/mm grating.

Measured Raman spectra were processed with Multivariate Curve Resolution (MCR), which is a wide class of algorithms used to separate individual input components from a mixture spectra. Among others self-modelling MCR (SMCR) was used in this study, yet other tested MCR methods *i.e.* Alternating Least Squares (ALS) MCR yielded identical output spectra. The details of the SMCR algorithm can be found elsewhere.\(^{54-56}\) Briefly, the hydration (solvent correlated) spectrum of a solute could be achieved by subtracting the pure bulk water component from a measured Raman spectrum of aqueous solute solution. It was demonstrated that clean and reproducible solvent correlated spectra could only be achieved with providing a very high signal to noise (S/N > 5000/1) input Raman spectra.

MCR-Raman technique lacks of rigorous spectroscopic efforts to measure interfacial specific spectroscopic information, yet it started to be considered as an alternative to second order non-linear optical measurements. The hydration information of
the air/substrate/water could at least be complemented with hydration information in bulk aqueous solutions. In this dissertation, MCR-Raman was employed to measure hydration shell spectra of different sized biomacromolecules.
CHAPTER II

SPECTROSCOPIC EVIDENCE FOR WEAK CATION BINDING TO AMIDES IN AQUEOUS SOLUTIONS

Introduction

The biologically relevant cation-protein backbone interactions has been widely investigated in many studies in the literature over the last five decades. Yet, reported results showed no agreement, where mostly tight cation binding was concluded.$^{30,57-63}$ In this chapter, we also focused on the same problem, and investigated the salt interactions with butyramide molecule (Figure 1.2c) as a simple mimic of biologically relevant cation interactions with protein backbones. Two orthogonal spectroscopic techniques including attenuated total reflection Fourier transform infrared (ATR-FTIR) and vibrational sum frequency spectroscopy (VSFS) are utilized to run experiments in aqueous metal chloride solutions. In the former, which provided information about contact pair formation, the response of the amide I band to the nature and concentration of salt was monitored in bulk aqueous solutions. In the latter set of complementary experiments, the effect of cation identity and concentration was investigated at the air/butyramide/water interface. In these studies, metal ion-amide binding led to the ordering of the adjacent water layer. Such experiments were sensitive to the interfacial partitioning of cations in either a contact pair with the amide or as a solvent separated pair.

The binding site for cations to protein backbone should involve the carbonyl oxygen of the amide.$^{64}$ However, it has been a particular challenge to obtain quantitative binding constant information for metal cations with amide moieties. There are a few
structural studies in the literature focusing on amide-cation interactions in non-aqueous media\textsuperscript{65,66} and in the solid phase.\textsuperscript{58} Five decades ago, Bello and coworkers reported qualitative association of Li\textsuperscript{+} and Ca\textsuperscript{2+} with the simple amide-containing compounds, N-methyl acetamide (NMA) and N,N dimethyl acetamide (Figure1.2 a&b), at very high concentrations of the organic molecule in aqueous solutions by viscosity and calorimetric methods.\textsuperscript{57–59} In the solid state, these same authors found adducts between cations and amide-containing molecules by X-Ray crystallography. Later, Robinson and coworkers attempted to study cation and anion association with end-capped mono, di, tri and tetra glycine molecules by solubility measurements.\textsuperscript{60,61} A few NMR studies show amide-Li\textsuperscript{+} interactions via the chemical shift of the carbonyl carbon.\textsuperscript{62,63} These studies were performed either in non-aqueous solutions or with molar concentrations of the organic molecules, making quantitative binding information unobtainable. A few recent molecular dynamics simulations have concluded that the interactions of cations with amide moieties are actually quite strong.\textsuperscript{30,32,67} Some studies even claimed the association of Na\textsuperscript{+} and K\textsuperscript{+} with the amide containing side chains of poly(N-isopropyl acrylamide) (PNIPAM) dominated over the interactions of even the most weakly hydrated anions. In sharp contrast, other MD simulations found that the interactions of anions are indeed dominant.\textsuperscript{25,31,68} In order to experimentally elucidate cation-amide interactions we have directly tested the association between butyramide, a model amide-containing molecule, and the chloride salts of Na\textsuperscript{+}, K\textsuperscript{+}, Li\textsuperscript{+}, Ca\textsuperscript{2+}, and Mg\textsuperscript{2+}.

Materials and Methods

Materials. All inorganic salts (\( \geq 99.9\% \)) (from Aldrich, MO and Fisher, NJ) and butyramide (\( \geq 99\% \)) were used as received. Aqueous solutions were prepared from purified
water with a minimum resistivity of 18.1 MΩ.cm. (NANOpure Ultrapure Water System, Barnstead, Dubuque, IA). D$_2$O samples were prepared with heavy water which was obtained from Cambridge Isotope Laboratories (99.98%, Andover, MA). The d-butyramide samples were prepared by dissolving butyramide in D$_2$O and vacuum drying the samples, which led to the exchange of NH protons by deuterium from the heavy water.$^{69}$ This deuterium exchange process was performed a minimum of three times. ATR FTIR samples were prepared by addition of the desired amount of anhydrous salts to a 100 mM $d$-butyramide solution in D$_2$O. The amide I bands can have contributions from the C-N stretching mode and the NH$_2$ bending mode as well as the C=O stretch.$^{70}$ By performing the experiments in D$_2$O with fully exchanged ND$_2$ groups, the overwhelming contribution to the amide I band is just from the C=O stretch. Also anhydrous, high purity salts ($\geq 99.99\%$) and heavy water were needed to attenuate the interference of the broad water bending peak background in the amide I region. It should be noted that VSFS samples were prepared in H$_2$O by the addition of desired amount of salt to 300 mM butyramide in 10 mM Tris-HCl buffer at pH 7.0. In this case, the goal was to monitor the interfacial water OH signal in the presence of a Gibbs monolayer of butyramide. (see Figure 2.7b)

**VSFS Measurements.** Our VSFS system can be found in the methods section in chapter 1 (p-14).$^{71,72}$ VSFS experiments were performed at room temperature using a 35 mL sample of a 300 mM butyramide solution with the desired salt type and concentration. This was poured into a Langmuir trough (Model 601M, Nima, U.K.). Some butyramide molecules partitioned to the interface to form a Gibbs monolayer under these conditions. A fixed frequency visible beam at 532 nm and a tunable infrared beam were spatially and temporally aligned to the air/butyramide/water interface. All VSFS experiments were
performed with the ssp polarization combination (s-sum frequency, s-visible, and p-infrared).

In the present studies, the VSFS spectrum for each sample was taken a minimum of three times. The spectra were collected from 2700 cm\(^{-1}\) to 3800 cm\(^{-1}\), which covers the CH stretch, OH stretch, and NH stretch regions. The oscillator strength (O.S.) of the water peaks was calculated by fitting the spectra to equation 1.3 by using MATLAB software (version 7.12.0.635). The relative phase of each peak was confirmed by employing the Maximum Entropy Method (MEM) to calculate the imaginary part of \(X_{\text{eff}}\). where a detailed description of this method can be found in ref 72.

**ATR-FTIR Measurements.** Infrared spectra were collected with a Nicolet 470 FTIR spectrometer which was equipped with a Pike Miracle ATR attachment that contained a single-bounce ZnSe crystal (Pike Technologies, Madison WI) and an MCT detector (Thermo Electron Corp., Madison, WI), which was cooled by liquid nitrogen. All spectra were collected at 2 cm\(^{-1}\) resolution with 256 scans over a window from 1000 cm\(^{-1}\) to 4000 cm\(^{-1}\). One level of zero-filling as well as the Black-Harris apodization function were employed.

Each sample was measured a minimum of three times. Moreover, an otherwise identical salt solution without butyramide was used as a background and measured just before each sample measurement was made. This background was subtracted from each sample spectrum. Spectral fitting was performed using Origin (version 7.0, Microsoft, Northampron, MA). The number of Gaussian peaks required to fit a given spectrum was determined using a second derivative test. The least error sum method was employed to
check the quality of the spectral fitting and all spectral fits shown in this chapter have the lowest least error sum.

**Results**

**ATR-FTIR Measurements of d-butyramide.** Figure 2.1 shows the amide I band from d-butyramide in (a) pure D₂O, (b) a D₂O solution containing 5 M NaCl, and (c) a D₂O solution containing 5 M CaCl₂. The spectrum with 5 M NaCl and without salt gave rise to a single peak at 1620 cm⁻¹ that showed no variation in intensity with increasing concentration of the sodium salt. This peak could easily be fit by a single Gaussian curve. By contrast, the amide I band split into two distinct resonances with 5 M CaCl₂. These could be fit by two separate Gaussian curves at 1615 cm⁻¹ and 1645 cm⁻¹ with the lower frequency peak showing a slight red-shift from the pure D₂O case. In fact, this peak continuously red-shifted and decreased in intensity as more CaCl₂ was added to solution (Figure 2.2a). The higher frequency peak did not appear to show a frequency shift, but continuously rose in intensity as CaCl₂ was added.
Figure 2.1. FTIR spectra of the amide I band for $d$-butyramide in a) D$_2$O, b) 5M NaCl, and c) 5M CaCl$_2$ in D$_2$O. The schematic diagrams associated with each spectrum show the type of cation and water interactions with the amide in each case. The gray circles represent the FTIR spectral data, and the red lines are the overall fits to the data. For c), green curves are also provided showing the two individual Gaussian fits to the overall spectrum.
Amide bands consist of a combination of the carbonyl stretch, the NH bend, and CN stretch. However, the amide I band from small molecules, like butyramide, arises almost exclusively from the carbonyl stretch in D$_2$O and appears at 1620 cm$^{-1}$. By contrast, the same amide I band appears at 1715 cm$^{-1}$ in air or vacuum. The red-shift in water compared with air can be explained by the hydrogen bonding of water molecules to the organic molecule. The amide I band will also red-shift relative to air when bonded to the NH of urea, the NH of another amide, or through contact pairing with a metal ion. However, these latter red shifts are almost always smaller than the one for water, which involves significant charge transfer to the σ* orbital of water’s OH bond. As a consequence, the amide I band appears to blue-shift in bulk aqueous solution upon interaction with most species other than water. For metal ions, this phenomenon is not limited to amides. For example, the CN stretch of thiocyanate and the carbonyl stretch of acetone both show a blue-shift in aqueous solution when a divalent metal ion displaces water to form a contact pair. As such, the higher frequency peak in Figure 2.1c can be assigned to a contact pair between Ca$^{2+}$ and the amide, while the lower frequency peak should represent water bonded species. Indeed, these assignments are consistent with the increasing intensity of the higher frequency peak and the attenuation of the lower frequency peak as salt is added to solution. The slight red-shift of the lower frequency peak with salt concentration may be caused by the increasing interactions of Ca$^{2+}$ with oxygen atoms from water molecules in the amide’s hydration shell. It should be noted, however, that the dielectric constant of water changes from 78.5 to 48.4 as the CaCl$_2$ concentration is increased from 0 M to 4M, which may also influence the peak position of the water-bonded amide.
Figure 2.2. FTIR spectra of the amide I band for d-butyramide at a) CaCl$_2$ concentrations from 0 to 5M, b) MgCl$_2$ from 0 to 4M, and c) LiCl from 0 to 9M.
Figure 2.3. FTIR spectra of the amide I band for d-butyramide at a) NaCl concentrations from 0 to 5M, and b) KCl from 0 to 3M.
Data analogous to that of CaCl$_2$ for the amide I spectra of $d$-butyramide is shown for MgCl$_2$ and LiCl at various concentrations (Figure 2.2b&c). Once again, a higher frequency peak rose in intensity while the 1620 cm$^{-1}$ fell and red-shifted for Mg$^{2+}$ and only fell for Li$^+$. The high frequency peak occurred at 1649 cm$^{-1}$ with 4 M MgCl$_2$ and 1652 cm$^{-1}$ with 9 M LiCl. The slightly higher frequency blue shift for the Li$^+$-amide interaction is consistent with more modest amide bond polarization. Additional data for the amide I band was collected in the presence of KCl as a function of concentration. As with NaCl, no evidence was found within experimental error for either a frequency shift or an intensity change of the amide I band peak at 1620 cm$^{-1}$ even with a saturation concentration of KCl (see Figure 2.3).

The salt concentration dependence of the FTIR peaks in Figure 2.2 can be employed to obtain quantitative data about the fraction of metal ions in direct contact with the amide oxygen. This is done by plotting the ratio of the area under the high frequency peak to the total area under the amide I band at various salt concentrations (Figure 2.4). As can be seen in Figure 2.4, the fraction of contact pairs between metal ions and amides rose linearly with increasing concentrations of CaCl$_2$, MgCl$_2$ and LiCl. The slopes were considerably steeper for the divalent cations compared with LiCl and the slope for Ca$^{2+}$ was slightly steeper (more favorable binding) compared with Mg$^{2+}$. Since, NaCl and KCl led to no observable peak splitting or shifts, their binding fractions should be zero under all conditions. Such data are in good agreement with a direct cationic Hofmeister series, although some series report Mg$^{2+}$ rather than Ca$^{2+}$ as the strongest salting-in agent: $^{79}$

\[ \text{Ca}^{2+} > \text{Mg}^{2+} > \text{Li}^+ > \text{Na}^+ \approx \text{K}^+ \]
Figure 2.4. The binding fraction of metal bound amide vs. total amide concentration for all employed salts.
The data in Figure 2.4 cannot be employed to directly abstract equilibrium dissociation constant information, since even Ca$^{2+}$ simply does not bind tightly enough to get beyond the linear portion of the binding curve by saturation concentration. Nevertheless, nearly 30\% of the carbonyl oxygen binding sites were occupied at 5 M CaCl$_2$. If one extrapolates from this value to the concentration at which 50\% of the sites would be occupied by Ca$^{2+}$, an approximation of $K_d$ value of 8.5 M would be obtained. By analogy the apparent $K_d$ values would be 9.5 M for Mg$^{2+}$ and 20.2 M for Li$^+$. All these values are beyond the saturation concentrations of the respective salts and therefore hypothetical. Moreover, they are sufficiently weak that they border on being merely statistical. In other words, they are not greatly different from what one would expect if the free energy difference between metal ion and water binding to the carbonyl oxygen were zero. On the other hand, contact pair formation for both Na$^+$ and K$^+$ are unfavorable and should have a positive free energy compared with the water solvation to both sites. As such, they are excluded from the amide carbonyl oxygen/water interface.

**VSFS measurements of butyramide.** FTIR of the amide I band primarily provides information on contact pair formation between metal ions and the carbonyl oxygen of the amide. Therefore, it is important to use a complementary spectroscopy to glean information on the overall partitioning of cations to the amide interface. VSFS can provide such information as long as one ion preferentially partitions to the amide/water interface.$^{72,81}$ This will be the case for metal ions in chloride solutions, as chloride interactions with the amides are negligible.$^{72}$ Yet, butyramide, as a simple amide molecule, must be characterized over some changes in the subphase such as pH, concentration, and monolayer coverage to ensure all the spectral changes are resulted from ion adsorption.
Characterization of air/butyramide/water interface. In a first set of control experiments, the VSFS spectra in the 2800 to 3800 cm\(^{-1}\) range were probed at pH 3, 7 and 10 (Figure 2.5). In all conditions, two dominant peaks appeared in the CH range at 2880 cm\(^{-1}\) and 2940 cm\(^{-1}\) correspond to the CH\(_3\) symmetric stretch and a Fermi resonance, respectively.\(^{81,82}\) On the other hand, the water region (3100 cm\(^{-1}\) - 3600 cm\(^{-1}\)) appears to look unusual compared with the literature spectra.\(^{83,84}\) In fact, four bands can be identified. The two broadest, around 3200 cm\(^{-1}\) and 3420 cm\(^{-1}\) correspond to the usual assignments of more ordered and less ordered interfacial water structure, respectively. However, two relatively sharp peaks are also found near 3180 cm\(^{-1}\) and 3390 cm\(^{-1}\). These resonances are caused by the symmetric and asymmetric NH\(_2\) stretch modes.\(^{85}\) The lower frequency NH\(_2\) resonance constructively interferes with the water bands, whereas the higher frequency peak interferes destructively. The latter leads to a dip in the spectrum. The pH changes between 3 and 10 did not result any spectral changes within experimental error. This is consistent with the idea that the NH\(_2\) moiety is not titratable in this pH range.\(^{86}\) Next, experiments were performed with a D\(_2\)O subphase, which verified that all the NH and OH groups could be exchanged for deuterium (Figure 2.6a). Only the CH stretch peaks remained unchanged under these conditions, as they must be. Butyramide molecules at the interface were further tested for monolayer quality under different bulk butyramide concentrations. Figure 2.6b showed the VSFS spectra for a series of butyramide concentrations in the subphase ranging from 200 mM to 900 mM in H\(_2\)O. Under these conditions, the oscillator strength (O.S.) of the CH\(_3\) symmetric stretch peak at 2880 cm\(^{-1}\) was enhanced by approximately 50% by increasing butyramide from 200 mM to 900 mM in the subphase.
Figure 2.5. The VSFS spectra of air/butyramide/water interface at different pH’s at the subphase. The tested pH’s could be seen in the legend. The pH of the subphases were adjusted by adding NaOH and HCl as needed.
Figure 2.6. a) VSFS spectra of Gibbs monolayer of butyramide at 300 mM concentration on H$_2$O and D$_2$O subphases. b) VSFS spectra of Gibbs monolayers of butyramide at varying concentration of the organic in H$_2$O. The inset spectra show a blow up of the 2800 – 3000 cm$^{-1}$ region.
This is consistent with a modestly higher number density and/or better monolayer ordering as the amount of the organic molecule was increased. Nevertheless, the water region remained unchanged in the same concentration region. Thus, any changes in the water region could be attributed to ion absorption to the amide monolayer when salt solutions are used in the subphase.

**Ion effects to the air/butyramide/water interface.** Figure 2.7 shows the VSFS spectra of the air/butyramide/water interface (schematic can be seen in Figure 1.6b p-16) with 4 M chloride salt solutions of Ca\(^{2+}\), Mg\(^{2+}\), Li\(^+\), Na\(^+\), 3.8 M K\(^+\) and pure H\(_2\)O in the subphase. The presence of different salts in the subphase strongly affected the water orientation. Indeed, the adsorption of cations should cause net water orientation with the OH group facing toward the butyramide monolayer in agreement with other interfacial ion adsorption studies.\(^{72}\) (see Figure 2.7b) Moreover, a greater degree of cation adsorption over its counter ion should correspond to a greater extent of water ordering.\(^{81}\) In fact, the presence of 4 M CaCl\(_2\) and MgCl\(_2\) in the subphase dramatically enhance the intensity of the OH stretch peaks, whereas 4 M LiCl leads to more moderate water signal enhancement. In sharp contrast, the presence of 4 M NaCl and 3.8 M KCl hardly perturb the water structure within experimental error. The order of the intensity enhancements for the 3200 cm\(^{-1}\) and 3420 cm\(^{-1}\) peaks closely track the cationic Hofmeister series observed for the FTIR data. One could argue that different salts in the subphase cause differences in Gibbs monolayer density and/or ordering, where these could interfere with the ion specific effects.
Figure 2.7. a) VSFS spectra for Gibbs monolayer of butyramide at the air/water interface. For each spectrum the subphase contained 4 M of the respective salt as indicated in the legend, with only exception of KCl, which was measured in a saturated salt solution (~3.8 M) b) The schematic diagram of the air/butyramide/water interface with (right) /without (left) absorbed cations. The red arrows indicate the electric field at the interface.
Figure 2.8. VSFS spectra of 300 mM butyramide Gibbs monolayer at the air/D$_2$O interface. Each subphase contained 4M of given salts as indicated in the legend except KCl, which was measured with a nearly saturated solution (3 M). The inset shows a blow up of the C-H stretching region.
In order to test the effect of salts to the density and/or orientation of butyramide Gibbs monolayer at the air/water interface, analogous experiment to figure 2.7 were measured in D₂O solvated subphase with the same salts and concentrations except KCl solution was at 3M due to solubility limit of the salt. (Figure 2.8). The VSFS spectra with the monovalent salts were identical in the CH stretch range; however, the oscillator strength (O.S.) of the 2880 cm⁻¹ feature increased ~28% in intensity with the divalent salts. This is consistent with slightly better ordering and/or slightly higher number density for the monolayer in the case of the divalent salts. In fact, divalent salts have twice the chloride concentration leading to a modest salting-out effect. Indeed, there is generally a modest increase in monolayer ordering as any salt is added. Nevertheless, the underlying water structure was essentially insensitive to these moderate deviations in monolayer structure as seen from Figure 2.6b. This means that any actual changes in water region (3000 – 3600 cm⁻¹) that occurred in Figure 2.7 must have been from ion absorption to air/butyramide/water interface.

VSFS spectra of air/butyramide/water interface as a function of CaCl₂ and MgCl₂ concentrations are shown in Figure 2.9 a&b, respectively. The water structure peaks increase continuously as a function of CaCl₂, as well as MgCl₂ concentrations. LiCl solutions showed behavior similar to divalent salts, yet the water structure ordering is slightly more modest (Figure 2.10). On the other hand, no appreciable change in the water spectrum is observed by adding NaCl, or KCl to the subphase up to the saturation points, as can be seen in Figure 2.11.
Figure 2.9. VSFS spectra of the air/butyramide/water interface as a function of a) CaCl$_2$ and b) MgCl$_2$ concentrations.
Figure 2.10. VSFS spectra of butyramide Gibbs monolayer at the air/water interface as a function of LiCl.
Figure 2.11. VSFS spectra of the air/butyramide/water interface as a function of a) NaCl and b) KCl concentrations.
In order to obtain more quantitative information, the VSFS spectra need to be fit to equation 1.3 in order to obtain the oscillator strength for the water bands. Figure 2.12 provides such O.S. strength data for the 3200 cm\(^{-1}\) peak as a function of salt concentration for the five chloride salts. Linear increasing trends are observed for Ca\(^{2+}\), Mg\(^{2+}\), and Li\(^+\) cations with the same ordering in slopes as found with FTIR in Figure 2.4. Such close agreement is remarkable because the VSFS data report on interfacial water structure ordering, while the FTIR data correspond to the carbonyl stretch of the amide moiety in bulk solution. Also, the Na\(^+\) and K\(^+\) cations showed negligible changes in their 3200 cm\(^{-1}\) O.S. values. Again, this is in good agreement with the FTIR data. Such results further indicate that the free energy of partitioning Na\(^+\) and K\(^+\) to an amide-containing monolayer is unfavorable. It should be noted that essentially O.S. of the 3420 cm\(^{-1}\) peak follows the same trends as reported in Figure 2.12.
Figure 2.12. Oscillator Strength of 3200 cm$^{-1}$ peak as a function of different salt concentrations in the subphase.
Discussion & Conclusion

In proteins, the driving force for cation-polypeptide interactions should generally involve electrostatic interactions between negatively charged carboxylate side chains and cations.\textsuperscript{18,19,27,87} Such interactions can be far tighter than anion-peptide backbone interactions and even dominate the phase behavior of a protein under appropriate conditions.\textsuperscript{27} As shown above, however, the interactions of cations with amides are extremely weak with Ca\textsuperscript{2+} giving rising to the tightest apparent binding. By contrast, the measured $K_d$ values for weakly hydrated anions with their respective peptide binding sites are substantially tighter. For example, SCN$^-$ yields a $K_d$ value of ~200 mM in various spectroscopic and thermodynamic measurements.\textsuperscript{28,72} Thus, anion affinity for amide binding sites is nearly 2 orders of magnitude tighter compared with the most strongly interacting cations (e.g. Ca\textsuperscript{2+} vs. SCN$^-$). Furthermore, the nature of cation and anion binding for uncharged polypeptide backbones is significantly different. Indeed, strongly hydrated cations tend to bind whereas only the most weakly hydrated anions accumulate around protein backbones.\textsuperscript{15,25,31} This difference in Hofmeister series properties must first and foremost be understood in light of the very different binding sites for cations and anions. As noted above, the amide oxygen is the key binding site for cations. By contrast, the binding site for Hofmeister anions to peptide backbones involves a combination of the amide nitrogen and the adjacent hydrophobic methylene unit.\textsuperscript{25}

Both Na$^+$ and K$^+$ are known to bind with model amide molecules/polypeptides in the gas phase.\textsuperscript{88} The situation in aqueous solutions is far more complex because in the absence of binding, the amide oxygen should hydrogen bond to water and the cation will be surrounded by its hydration shell. These interactions need to be at least partially
disrupted in order for cation-amide contact pairing to take place. Herein, we have shown spectroscopic evidence for very weak binding behavior for Ca\(^{2+}\) and Mg\(^{2+}\), nearly statistical binding for Li\(^{+}\), and exclusion for Na\(^{+}\) and K\(^{+}\) from the amide oxygen. Until now, MD simulations have found evidence for varying degrees of binding of Na\(^{+}\) and K\(^{+}\) cations with the amide oxygen in aqueous solutions.\(^{25,30-32,67}\) Such differences amongst the simulations presumably reflect the challenges associated with accounting for the very small differences in free energy associated with the bound and unbound states. Nevertheless, the spectroscopic data are most consistent with those simulations that do not find tight associations between metal cations and the carbonyl oxygen’s of amides.

The findings with the butyramide molecule were tested with a more realistic model systems, PNIPAM and ELPs in the next chapter, in order to further test the validity of our results for applying to biomacromolecules.

Contributions: Halil I. Okur measured and analyzed all presented data.
CHAPTER III

THE EFFECT OF HOFMEISTER CATIONS ON THE SOLUBILITY OF
BIOMACROMOLECULES: PNIPAM AND ELASTIN-LIKE
POLYPEPTIDES

Introduction

Herein, we further investigated the cation-protein backbone association mechanism, with polymer/polypeptide model systems. The thermo-responsive property of poly(N-isopropyl acrylamide) and elastin-like polypeptide (ELP), were employed as an observable for the effect of cations on the macroscopic behavior of biomacromolecules. In this chapter, the lower critical solution temperatures (LCST) were measured for a series of alkali, and alkaline earth metal cations chloride salts. This LCST data were modeled with an empirical equation where it proposed a threefold salt effects on macromolecules; a salt dependent salting out effect due to surface tension changes of aqueous salt solutions, and a very weak direct (contact paired) cation binding to amide carbonyl oxygen, along with an additional salting-in effect where only effective at elevated salt concentrations. The latter two effects were further investigated to achieve a molecular level picture. Attenuated Total Reflection-FTIR (ATR-FTIR) spectroscopy was utilized as a complementary technique to elucidate contact pair cation binding. A new salt concentration dependent carbonyl stretching peak rose around 1650 cm$^{-1}$ only for strongly hydrated (Mg$^{2+}$, Ca$^{2+}$, Sr$^{2+}$, Ba$^{2+}$ and Li$^+$), and NH$_4^+$ cations as a strong indication for contact pair formation with PNIPAM. Weakly hydrated (Cs$^+$, Rb$^+$, K$^+$, Na$^+$) cations, on the other hand, showed no clue for apparent direct binding to the same binding site. Moreover, both direct binding and the
exponential term components of LCST data from our empirical model presented a linear correlations for the bulk $\Delta H$ and $\Delta S$ and $\Delta G$ of hydration of the cations. These correlation gave clues about the nature of ion-macromolecule interactions where indeed, bulk ion properties actually do influence the macroscopic macromolecule behaviors.

Despite the widespread role of cations in biological fluids including creating vital cell potentials, efficient signaling transduction events, and maintaining enzyme/protein structure and functions $^{3,19,89}$, there is no common understanding of cationic Hofmeister effect on neutral proteins and polypeptides. The main reasons for this is the minor contribution of cations to specific ion effects as compare to counter anions, as well as the influences of the identity of the protein/macromolecule system, the nature of counter anion, the pH of the solution, along with other experimental conditions interfere with the obtained results. Thus, the overall mechanism of how positively charged ions interact with neutral biomacromolecules and why the influence of cations follow a specific order in many neutral systems have been ambiguous. Recently, Gao and coworkers, have proposed a simple mechanism on specific Hofmeister ion effect.$^{90}$ In this model, cations have a dual influence on protein backbones; including a direct contact pair binding and a solvent involved interaction to amide oxygen. The latter effect was described by the polarization of water molecules in the hydration shells of cations, as a result it strengthen the H-bonding in aqueous solution.$^{90,91}$ Yet, there is no experimental studies directly supporting this mechanism. More simulation and experimental results are needed to sum up the cation-macromolecule interactions under a unified molecular mechanism explanation.

**LCST of PNIPAM and ELPs for anion-biomacromolecules interactions.** The power of thermodynamic phase transition temperature measurements were shown in numerous
studies. Specifically, the LCST of macromolecules, polypeptides and proteins were shown to lead a molecular level mechanism for Hofmeister anions biomacromolecule interactions both at the backbone and at charged side chains. Cremer group demonstrated the LCST of PNIPAM as a function of 11 sodium salts, where the data can be seen in Figure 3.1a. These set of data elucidated a trifold mechanism for specific anion effects on the solubility of polymers/proteins. First, a salting in effect was observed due to direct weakly hydrated anion (SCN⁻, ClO₄⁻, I, NO₃⁻, Br⁻) binding to amide moieties of the polymer. Second, a surface tension mediated salting out effect was proposed again for weakly hydrated anions. Indeed, this dual mechanism for weakly hydrated anions gave a reasonable explanation for their curved LCST behavior as a function of salt concentration. Third, a salting out effect in combination of an excluded volume effect along with surface tension for strongly hydrated anions (CO₃²⁻, SO₄²⁻, S₂O₃²⁻, H₂PO₄⁻, F⁻) was also proposed where the salting out capability of these ions was shown to be proportional to their corresponding hydration entropies. This work was further proven by Cho et.al. where the proposed findings with PNIPAM was shown to be applicable to the phase transition behavior of ELPs. The LCST data of ELPs as a function of the same 11 sodium salts can be seen in Figure 3.1b. In both systems the apparent binding constants (salting-in), surface tension, excluded volume effects (salting-out) were followed an anionic Hofmeister Series.

The same strategy was utilized in this chapter to monitor any differential cation interactions. Specifically, the putative direct ion binding and solvent involved ion interactions to the amide moieties, as well as any indirect ion effects on the amide, and hydrophobic groups. Moreover, other proposed mechanisms could be tested such as the role of dispersion forces in hofmeister effects.
Figure 3.1. The LCST behavior of thermo responsive macromolecules a) 10 mg/ml PNIPAM solution as a function of 11 Hofmeister anions where sodium was chosen to be the counter cation. b) 6.4 mg/ml ELPs solution as a function of the same 11 Hofmeister salts. The salt identity can be seen in the legends. Figure a, b were adapted from ref 28 and ref 29, respectively.
Experimental

Materials. High purity inorganic salts of NaCl, KCl, LiCl, CsCl, RbCl, NH₄Cl, NMe₄Cl, CaCl₂, BaCl₂, SrCl₂, and MgCl₂ were purchased from Sigma Aldrich (> 99 % purity). Low conductivity deionized water, obtained from a NANOpure Ultrapure (Barnstead, Dubuque, IA) water system with a minimum resistivity of 18 MΩ.cm was used to prepare all the salt solutions for LCST measurements. Freeze dried PNIPAM and ELP V₅-120 samples were dissolved in desired salt solutions with a 10 mg/ml final polymer/polypeptide concentration. The phase transition temperature measurements were taken with an automated melting point apparatus (Optimelt MPA 100, Stanford Research Systems), with ramping rate of 0.5°C/min in all the measurements. All LCST values reported in this study are highlyrepeatable and the shown data is an average of six measurements.

PNIPAM polymer was synthesized by free radical polymerization of its monomer, N-isopropyl acrylamide. A detailed synthesis method can be found elsewhere. This synthetic route yielded 4 different molecular weights. Among all, 1.78 × 10⁴ g/mol fraction was used for all the measurements in this study.

A detailed procedure for ELP preparation can be seen in the Methods section (p-8). Briefly, ELP V₅-120 polypeptide consists of 120 pentapeptide units of amino acid sequence –VPGVG- is expressed in BR/DE3 E.coli cells. pET plasmids of the amino acid sequence were constructed using recursive directional ligation (RDL) process. Resulting plasmids were transformed into cells and the ELP plasmid containing cells were incubated for 24 hours in high nutrient growth media (TB Dry ™). Cells were lysed using sonication (Sonicator® 3000 system; Mandel scientific company Inc.). ELP was purified from nucleic
acids, other cell lysates, and proteins using a polyethyleneimine treatment along with a series of ‘inverse transition cycling’ steps. The molecular weight and purity of obtained ELP was confirmed by typical SDS-PAGE and CuCl$_2$ staining method. Finally, ELPs were further purified via dialyzing against deionized water.

**ATR-FTIR Measurements.** Anhydrous inorganic salts NaCl, KCl, LiCl, CsCl, RbCl, NH$_4$Cl, NMe$_4$Cl, CaCl$_2$, BaCl$_2$, SrCl$_2$, and MgCl$_2$ were purchased from Sigma Aldrich and VWR (> 99 % purity). Salt stock solutions are prepared with heavy water which is obtained from Cambridge Isotope Laboratories (99.98%, Andover, MA). The lyophilized PNIPAM samples extra processed before adding the designated salt solution by simply dissolved in D$_2$O and relyophilized three times. This process exchanges amide hydrogen at N-H position to deuterium. Moreover, this exchange yields an amide I band at 1625 cm$^{-1}$ which is prominently carbonyl stretching (C=O). FTIR sample solutions were also kept at 10 mg/ml macromolecule concentration to be consistent with thermodynamic measurements.

Nicolet 470 FT-IR spectrometer with a Pike Miracle attachment which contained a single bounce diamond coated ZnSe crystal (Pike Technologies, Madison, WI) and liquid nitrogen cooled MCT detector (Thermo Electron Corp, Madison, WI) is utilized to employ ATR FT-IR measurements. Samples were measured three times and subtracted automatically from heavy water spectrum by OMNIC software (Thermofisher) Each spectrum was collected by averaging 256 scans from 1000 to 4000 cm$^{-1}$ range with a 2 cm$^{-1}$ resolution. The least error some method was employed in order to check quality of the spectral fittings.
**Results**

**LCST data of PNIPAM with various chloride salts.** The LCST values of PNIPAM biomacromolecule were measured in the presence of increasing concentration of various monovalent chloride salts (Figure 3.2a). Chloride salts of Li\(^+\), Na\(^+\), K\(^+\), Rb\(^+\), Cs\(^+\), NH\(_4^+\) and NMe\(_4^+\) were employed up to 2 molar concentrations for this purpose. The aqueous solution of PNIPAM undergoes phase transition at 31.14±0.05°C in deionized water. The phase transition temperatures yield gradual decreasing trends with a start of curving upward from linearity with increasing salt concentration for all monovalent salts with the exception of NMe\(_4^+\). Interestingly, this deviation is more dominant at higher salt concentrations, as well as being more pronounced for more strongly hydrated cations (Li\(^+\)), and NH\(_4^+\). Weakly hydrated monovalent cations, on the other hand, yield more linear like trends. Overall, the efficiency of cations to salt out PNIPAM from solution is as follows.

\[ \text{Cs}^+ \geq \text{Na}^+ \geq \text{K}^+ \geq \text{Rb}^+ > \text{NH}_4^+ > \text{Li}^+ > \text{NMe}_4^+ \]

The small LCST differences between weakly hydrated monovalent cations (Cs\(^+\), Rb\(^+\), K\(^+\) and Na\(^+\)), and the opposite curvature for NMe\(_4^+\) are not insignificant and will be discussed later. Figure 3.2b showed a second set of LCST data taken with multiple alkaline-earth metal ions. Divalent cations also caused a decreasing LCST trend for PNIPAM as a function of salt concentration. With the difference of more prominent curving upward trends for all divalent cations as compare to monovalent ones. Overall, divalent cations also precipitate out macromolecule from solution with the following order:

\[ \text{Ba}^{2+} > \text{Sr}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+} \]
Figure 3.2. The LCST values of 10mg/ml PNIPAM in the presence of metal chloride salts of a) monovalent, and b) divalent cations. The salt identity of each curve can be seen in the legends of the graphs. Each data point represents an average of six measurements and the associated experimental standard deviations were within the size of the points used to plot the data. (The lines are curve fit the data to eqn 3.2) All error bars were within the data points drawn.
Table 3.1. Tabulated literature values of polarizibility, delta surface tension increments of metal chloride salts per mole, and calculated c values of the LCST data of PNIPAM and ELP V5-120.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Polarizibility (cm³ mol⁻¹)</th>
<th>Δδ* (mN L/m mol)</th>
<th>csurf (PNIPAM) (T M⁻¹)</th>
<th>csurf (ELP V5-120) (T M⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>Li⁺</td>
<td>0.08</td>
<td>1.58 ± 0.05</td>
<td>-12.25</td>
<td>-13.70</td>
</tr>
<tr>
<td>Na⁺</td>
<td>0.65</td>
<td>1.66 ± 0.05</td>
<td>-12.88 ± 0.09**</td>
<td>-14.40 ± 0.11**</td>
</tr>
<tr>
<td>K⁺</td>
<td>2.71</td>
<td>1.53 ± 0.1</td>
<td>-12.19</td>
<td>-13.27</td>
</tr>
<tr>
<td>Rb⁺</td>
<td>4.1</td>
<td>1.56</td>
<td>-11.85</td>
<td>-13.53</td>
</tr>
<tr>
<td>Cs⁺</td>
<td>6.9</td>
<td>1.56</td>
<td>-12.05</td>
<td>-13.53</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>4.7</td>
<td>1.36 ± 0.1</td>
<td>-10.55</td>
<td>-11.8</td>
</tr>
<tr>
<td>NMe₄⁺</td>
<td>22.9</td>
<td>0.6</td>
<td>-8.0</td>
<td>-9.00</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>-0.7</td>
<td>3.09 ± 0.1</td>
<td>-23.9</td>
<td>-26.8</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1.56</td>
<td>3.21 ± 0.15</td>
<td>-24.9</td>
<td>-27.85</td>
</tr>
<tr>
<td>Sr²⁺</td>
<td>2.65</td>
<td>3.25 ± 0.1</td>
<td>-25.1</td>
<td>-28.2</td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>5.17</td>
<td>2.97 ± 0.2</td>
<td>-23.05</td>
<td>-25.76</td>
</tr>
<tr>
<td>SCN⁻</td>
<td>17</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl⁻</td>
<td>8.63</td>
<td>1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>13.79</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* surface tension data is taken from ref 93, 94 and averaged from the multiple values of chloride salts.

csurf refers to the surface tension component of each salt salting-out effect. These csurf values were calculated from NaCl data fit to eqn. 3.2., no appreciable binding to PNIPAM and ELP V5-120 biomacromolecules were assumed to be negligible. The details of the calculation could be seen in data fitting section.

(SCN⁻, SO₄²⁻ and Cl⁻ values is also added for comparison. Surface tension values are for sodium salts.)
Data fitting. The LCST data (Figure 3.1) of 11 hofmeister anions with these very same biopolymers had been modelled successfully with the following equation, which consists of a constant, a linear term and a Langmuir binding isotherm (eqn. 3.1).28,29

\[
T = T_0 + c[M] + \frac{B_{max}[M]}{K_d + [M]}
\]

(eqn. 3.1)

where \(T_0\) is the LCST of polymer/polypeptide in the absence of salt. \([M]\) is the molar concentration of salt and \(c\) is a constant having units of temperature/molarity. \(K_d\) is the apparent dissociation constant of the anion-polymer interaction. \(B_{max}\) is a constant having units of temperature, which is related to the increase in phase transition temperature of polymer due to direct anion binding. Nevertheless, the LCST data of biomacromolecules with hofmeister cations cannot be fitted to eqn. 3.1. An additional non-linear salting in term is needed where it is more pronounced at higher salt concentrations. Henceforth, the phase transition data for both PNIPAM as well as ELPs were perfectly fitted to the following equation:

\[
T = T_0 + c[M] + A[M]^2
\]

(eqn. 3.2)

where \(T_0\), and \(c\) are identical coefficients/constants as described for eqn. 3.1. The new term, \(A[M]^2\), was empirical. It is the best fit function to LCST data among \([M]^{1/2}\), \([M]^{3/2}\), \([M]^2\), \([M]^3\), higher orders of concentration powers, and exponentials. This nonlinear salting in behavior is not specific to LCST of thermo responsive biomacromolecules. Similar trends can also be seen in the solubility of aliphatic and aromatic hydrocarbons as a function of salt concentration.95,96 The LCST data of PNIPAM for cations could not fit to a Langmuir binding isotherm like anions (except NMe₄Cl data), and one can claim that this is an evidence for a very weak nature of direct (contact pair) cation binding. In other words, the
direct cation binding for all alkali and alkali-earth metal cations is only very subtle and at the linear part of a Langmuir isotherm, at least with the salt concentrations which were employed in this chapter.

\[
\sigma_{\text{salt}} = \Delta \delta_{\text{salt}} \cdot x \frac{c_{\text{NaCl}}}{\Delta \delta_{\text{NaCl}}}
\]  

(eqn. 3.3)

The \(\sigma_{\text{surf}}\) values were calculated using eqn. 3.3 simply from the ratio of the surface tension increments of individual salts per mole and using NaCl values as a reference. Since, it was previously shown for both PNIPAM and ELPs biomacromolecules that the \(c\) constant (linear portion) of eqn. 3.1 is directly proportional to the salt specific surface tension increments for sodium salts of weakly hydrated (chaotropic) anions including chloride.\(^{28,29}\) Calculated \(\sigma_{\text{surf}}\) constants for PNIPAM and ELP V5-120, \(\Delta\) surface tension values per mole of all employed salts, along with the polarizibility constants of the cations were tabulated in Table 1.

The underlying reason for an \(A[M]^2\) expression in eqn 3.2 was not clear. However, there are only very few possible mechanism which can yield a salting in effect. It can involve some direct or indirect (solvent shared, solvent separated) ion interactions to the polymers, along with a limiting correction to the surface tension effect. This interaction was negligible at low (< 0.25 M), and become significant at higher (>1 M) salt concentrations. Figure 3.3a & b show the salting in contributions of direct cation binding and the \(A[M]^2\) contribution to the LCST values, for all employed cations as a function of salt concentration, respectively. Linear direct binding curves in Figure 3a validated substantially weak direct cation binding. Among all cations, only NMe₄⁺ reached the nonlinear portion of a Langmuir isotherm to some extent and can be fitted with eqn. 3.1
with an apparent 2.5±0.6 M $K_d$ value. Indeed, NMe$_4^+$ cation is the only tested cation where its polarizibility is comparable to weakly hydrated anions (Table 3.1). This was shown to be a key property to interact with weakly hydrated sites on polymer/protein surfaces.\textsuperscript{21}

A[M]$^2$ term contribution to LCST values is quite significant and went as high as 12°C at 2M MgCl$_2$, whereas its effect is much smaller at the lower concentrations; such as 3°C at 1M and only 0.19°C at 0.25M MgCl$_2$. This new ion effect demonstrated an ion specific behavior and followed recurring cationic hofmeister series, as follows:

NMe$_4^+$ < Cs$^+$ < Rb$^+$ < K$^+$ < NH$_4^+$ < Na$^+$ < Li$^+$ < Ba$^{2+}$ < Sr$^{2+}$ < Ca$^{2+}$ < Mg$^{2+}$

Only ammonium cation places out of its original Hofmeister series. It is worth to mention, it was the only cation that potentially H-bond with the amide groups.
Figure 3.3. The contribution of individual effects to LCST of PNIPAM a) the residual LCST data for the linear $c[M]$ term after known surface tension contribution was subtracted. B) The residual salting in contribution of $A[M]^2$ term in eq 3.2. (The individual colored curves correspond to each cation and can be seen in the legend of each graph)
**ATR FT-IR measurements of PNIPAM.** The amide I band (carbonyl stretching peak) is shown to be susceptible to local hydration environments, as well as the hydrogen bond strength with solutes and/or cosolute. A blue shifted new C=O stretch resonance rose for amide-amide H-bonding,\textsuperscript{69} amide-urea,\textsuperscript{39} along with amide-contact pair cation binding interactions demonstrated and discussed in Chapter 2. Figure 3.4 a, b&c show the PNIPAM spectrum in 2M NaCl, 2M CsCl and as a function of LiCl concentration in D\textsubscript{2}O solvated medium, respectively. Amide I band of PNIPAM gave rise to a peak at 1622 cm\textsuperscript{-1} in D\textsubscript{2}O solvated solution. This resonance had some degree of asymmetry due to residual N-H groups remain after deuteration.\textsuperscript{70} Nevertheless the asymmetry was on the red side (lower wavenumbers) of the main C=O stretching peak, and the interference with the cation contact pair binding results, which is blue shifted in nature, was negligible. Figure 3.4 a, and b showed the peak position or the peak intensity of carbonyl stretching peak were not affected in 2M NaCl, and 2M CsCl solutions. In contrast, increasing LiCl salt concentration yielded two separate peaks at 1622 and 1650 cm\textsuperscript{-1}. The former peak is the heavy water solvated carbonyl stretching frequency and, the latter is blue shifted C=O band where the Li\textsuperscript{+} cation contact paired to the carbonyl oxygen.\textsuperscript{14} Here only LiCl salt could be tested up to 4 M salt solutions since the LCST values of PNIPAM at this salt concentrations were around 10°C, quite higher than all other employed salts. (the experiments were performed at least 5°C lower than LCST value). Moreover, the intensity of this new peak increased proportional to the salt concentration, where the contact paired bound portion of amide oxygen can reach up to 10% of the all available binding sites. Other strongly hydrated cations (Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, Sr\textsuperscript{2+}, Ba\textsuperscript{2+}) showed similar behavior and yielded a secondary blue shifted C=O stretching band.
Figure 3.4. The FTIR spectra of 10 mg/ml PNIPAM in a) 2M NaCl b) 2M CsCl, and c) various LiCl salt concentrations. The PNIPAM spectrum in D₂O is added to the each graph as a reference. All FT-IR measurements were performed at 2°C to achieve an experimental condition at least 5°C lower than the LCST temperature of PNIPAM in all tested salt concentration.
Figure 3.5. The FTIR spectra of 10 mg/ml PNIPAM in a) monovalent, b) divalent metal chloride salt solutions. The salt concentration and identity can be seen in the legends. The PNIPAM spectrum in D$_2$O is added to each graph as a reference. All FT-IR measurements were performed at 2°C to achieve an experimental condition at least 5°C lower than the LCST temperature of PNIPAM in all tested salt concentrations. Ba$^{2+}$ and Sr$^{2+}$ metal salts were performed in 1.8 M solutions.
In sharp contrast, there were no clues for a blue shifted shoulder peak for other weakly hydrated cations (K\(^+\), Rb\(^+\)) (Figure 3.5a,b). \(\text{NH}_4^+\) data, in Figure 3.5a, yielded some substantial differences where the red shifted region of the original carbonyl peak varied mostly due to an interfered N-H stretching peak of ammonium cation. It should be noted that, the hydrogen exchange of \(\text{NH}_4^+\) with the solvent molecules can also affect the quality of this spectrum. Overall, these FTIR results further elucidated the weak contact pair cation binding to amide oxygen in biomacromolecules for strongly hydrated cations as well as the findings in previous chapter were validated to be applicable for biomacromolecules.

**LCST data of V\(_5\)-120 ELP with various chloride salts.** The LCST values of ELP V\(_5\)-120 were also measured with increasing concentration of the same monovalent and divalent metal chloride salts which were tested with PNIPAM (Figure 3.6 a&b). The aqueous solution of this hydrophobic ELP undergoes phase transition around 28.5°C. Again salts had dramatic effects on the temperature of this transition. Gradual decreasing trends in the phase transition temperature were observed with increasing concentration for all employed salts. Interestingly, ELP solubility was found to demonstrate a very similar behavior with PNIPAM, where it strongly depends on the chemical identity of positively charged ions. The efficiency of cations to salt out ELP from solution is as follows;

\[
\text{Na}^+ > \text{K}^+ > \text{Rb}^+ > \text{Cs}^+ > \text{NH}_4^+ > \text{Li}^+ > \text{NMe}_4^+ \\
\text{Ba}^{2+} > \text{Sr}^{2+} > \text{Mg}^{2+} > \text{Ca}^{2+}
\]

The LCST data were fitted to eq. 3.2, with the exception of \(\text{N(CH}_3)_4^+\), where it was fitted to eq 3.1 due to data trend gave a better fit. The fitting parameters, including A and c
coefficients as well as individual components of the gradual decreasing trends (surface tension and bulk components) were tabulated in Table 2. The strongly hydrated cations (Mg$^{2+}$, Ca$^{2+}$, Sr$^{2+}$, Ba$^{2+}$, Li$^+$) and NH$_4^+$ again showed a weak contact pair binding, as well as a larger A constant (A>1). Weakly hydrated cations (K$^+$, Na$^+$, Rb$^+$, Cs$^+$, NMe$_4^+$) have fairly linear decreasing trends with mostly quite small A values (A<1) (see Table 2) These weakly hydrated cations do not display contact pair binding interaction as well, other than NMe$_4^+$. Interestingly, only the fairly polarizable weakly hydrated cations show weak direct cation binding behavior compared to weakly hydrated anions. This interaction should involve some other mechanisms besides having carbonyl oxygen as a direct binding site.

The corresponding data of alkaline-earth metal cations (Figure 5b) is limited up to 1M concentration. Higher divalent cation concentrations (> 1M) demonstrated a strong amino-acid sequence dependent results on LCST of the polypeptide. This remarkable effect is under investigation in our lab, with various sequence and size variance in ELP’s (V$_5$-120, V$_5$A$_2$G$_3$-60, V$_5$A$_2$G$_3$-120, V$_5$A$_2$G$_3$-330 and VG-128). Interestingly, some preliminary data suggests the secondary structure changes of the polypeptide seemed to be the underlying reason for this behavior at higher divalent cation concentrations. (For the sake of the completeness of this dissertation, these data were not included in this chapter.)
Figure 3.6. The LCST values of 10mg/ml ELP V₅-120 in the presence of metal chloride salts of a) monovalent, and b) divalent cations. The salt identity can be seen in the legends. The associated experimental standard deviations are within the size of the points used to plot the data. (The lines were curve fit the data to eqn. 3.2 all employed salts, but N(CH₃)₄⁺ where eqn. 3.1 was used.) All error bars are within the data points drawn.
Table 3.2. The fitting parameters of $B_{\text{max}}$, $K_d$, $c$, $c$ residual ($c^i$), and $A$ values of each salt from the LCST data fit to eqn.3.2 and eqn.3.1.

<table>
<thead>
<tr>
<th>Salt</th>
<th>PNIPAM</th>
<th>ELP V5-120</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$c$ (T M$^{-1}$) Experimental</td>
<td>$c^i$ (T M$^{-1}$)</td>
</tr>
<tr>
<td>LiCl</td>
<td>-8.50</td>
<td>3.75</td>
</tr>
<tr>
<td>NaCl</td>
<td>-12.88</td>
<td>0</td>
</tr>
<tr>
<td>KCl</td>
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</tr>
<tr>
<td>RbCl</td>
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</tr>
<tr>
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<td>NH$_4$Cl</td>
<td>-8.42</td>
<td>2.13</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>-12.56</td>
<td>11.34</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>-15.04</td>
<td>9.86</td>
</tr>
<tr>
<td>SrCl$_2$</td>
<td>-15.06</td>
<td>10.04</td>
</tr>
<tr>
<td>NMe$_4$Cl*</td>
<td>-8.18</td>
<td>7.14±0.9</td>
</tr>
</tbody>
</table>

*NMe$_4$Cl data fit to eqn. 3.1 due to its distinct different trend as compare to other salt data.

c$^i$ is calculated simply by subtracting the surface tension effects from the experimental c value. The surface tension component were calculated by taking NaCl as a reference and further described in Data fitting section.
One can claim separation of a linear fit (c[M]) into two components may be over interpretation of these data. Nevertheless, the salting out (c values) of PNIPAM with Hofmeister anions were shown to be affected by two separate mechanisms.\textsuperscript{28,97} On one hand, chaotrope anions only salt out with a surface tension mediated mechanism. On the other hand, kosmotrope anions salt-out with a dual mechanism. The surface tension effect could again be seen, along with a secondary mechanism where it was directly correlated to the bulk salt solution thermodynamic parameters. Interestingly, the LCST behavior of PNIPAM clearly demonstrated this dual salting-out effect at elevated salt concentrations. Two step phase transitions, a direct experimental observable, were measured where the lower temperature one was correlated with the hydration enthalpy of anions, and the higher one agreed with the surface tension changes due to salt addition. Analogues to these Hofmeister anion study, here in this chapter, the linear part of the LCST data were separated into a surface tension and bulk aqueous solution components. $c^I$ represented the residual components after the surface tension component was subtracted from experimental $c$ coefficients. Interestingly, the residual values were positive (salt-in) for strongly hydrated cations, whereas they were shown to be negative for kosmotrope anions. Moreover, the residual $c$ values perfectly correlated with the bulk hydration thermodynamics. Figure 3.7 a, b &c showed this correlation with the delta S, delta H and delta G of hydration with the strongly hydrated cations, respectively. Since the surface tension effect was previously subtracted for Na\textsuperscript{+}, K\textsuperscript{+}, Rb\textsuperscript{+}, Cs\textsuperscript{+} cations, they had zero $c^I$ contribution, and showed no correlation with hydration thermodynamics (black data points).
Figure 3.7. The residual c values of LCST data of PNIPAM for all employed salts were graphed with the hydration thermodynamics of cations; a) $-\Delta S$ of hydration, b) $-\Delta H$ of hydration, and c) $-\Delta G$ of hydration. (The red lines were linear fits to red data points.)
Figure 3.8. The residual c values of ELP V5-120 LCST data for all employed salts were graphed with the hydration thermodynamics of cations; a) -ΔS of hydration, b) -ΔH of hydration, and c) -ΔG of hydration. (black points had small residual c value after the surface tension was subtracted.) (Green dashed lines are only serves as an eye guide to show the linear correlation except Mg\(^{2+}\) values.)
The same method was applied to the LCST data of ELP V₅-120. Figure 3.8 a,b &c showed the analogous data of hydration thermodynamics correlation for the residual c values of ELP V₅-120. A linear correlation of -ΔS of hydration, -H of hydration, and -G of hydration with the c¹ values can be seen. Again, only strongly hydrated cations data showed proportionality, but only Mg²⁺ data was out of the linear trend. Indeed, recent studies in our lab indicated that Mg²⁺ affected the LCST of ELPs different than other divalent cations at elevated salt concentrations, it was claimed to be affecting the secondary structure of the polypeptide. Figure 3.7 and Figure 3.8 are the first set of experimental data showing a bulk hydration contribution to macromolecule solubility.

**Discussion**

The cations employed in this study represented four subgroups, including strongly hydrated (Mg²⁺, Ca²⁺, Sr²⁺, Ba²⁺, Li⁺), with NH₄⁺, a H-bonding capable cation, and weakly hydrated (Na⁺, K⁺, Rb⁺, Cs⁺), along with hydrophobic and polarizable NMe₄⁺. Salting-in and salting-out behavior of cations on biomacromolecules, and polypeptides could be categorized under three main ion specific effects; a salting-out contribution due to the surface tension increments of aqueous solutions, and a salting in influence via direct contact paired cation binding to the amide oxygen, along with a ion specific bulk aqueous solution effects. These three mechanisms were also shown to be the dominant for the anionic Hofmeister series.²⁸,²⁹,⁹⁷ All tested cations salt-out with the surface tension effect. Nevertheless, their salting-in mechanisms deviated from one another. Strongly hydrated cations interacted with macromolecules through direct binding to amide oxygen (Figure 3.4, 3.5) as well as a significant bulk cation hydration thermodynamics (Figure 3.7, and Figure 3.8). NH₄⁺ performs a similar behavior compare to strongly hydrated cations, yet
the direct binding to amide carbonyl must be minimal, if there is any (Figure 3.5a). Weakly hydrated cations, on the other hand, had no apparent salting-in contribution, whereas, N(CH₃)₄⁺ demonstrated a totally different behavior. It yielded an LCST curve of PNIPAM similar to weakly hydrated anions. Indeed, its polarizibility constant (Table 3.1) was at least a factor of 3 larger from other cations and on the same order for weakly hydrated anions. One can claim that N(CH₃)₄⁺ interaction with polymers/polypeptides should be similar to weakly hydrated anions, and it should partition to the partially negative charges on the backbone and/or side chains. Yet, this hypothesis has not been experimentally tested.

Overall, all employed salts are salting out as a function of salt concentration at least up to 2 M. Moreover, the LCST data of PNIPAM and ELP V₅-120 are in very good agreement with each other. It can be proposed that amides being on the backbone or on the side chain doesn’t significantly alter the cation interaction with the neutral macromolecules. From this results one can claim the proposed cation interaction mechanisms should be universal, and must apply to any neutral polymer and/or protein system. The only significant difference between two model systems was the influence of Mg²⁺ ion, where a possible ion mediated secondary structure change of the polypeptide might occur. Moreover, the specific cation interactions with this polymers/polypeptides are in complete agreement with the detailed spectroscopic investigation of butyramide (Chapter 2). In all tested model molecules, a weak binding between the amide oxygen and the strongly hydrated cations were observed. The direct cation interaction to polymers are somewhat weaker than the simple amide molecules. This should be due to the presence of more sterically hindered amide moieties on the macromolecules. The strongly hydrated
cations should be paying an extra penalty for an unfavorable ion hydration shedding in the vicinity of hydrophobic groups to reach proxy of amide sites.

The ion-solute interaction in aqueous solutions has been investigated in the literature. However, no complete mechanism explaining the entire cationic Hofmeister series has been proposed yet. In an early work, it was proposed that the electrostatic effects of added salts, and the related changes in the dielectric constant of water dominate the ion effects in aqueous solutions. However, this explanation lacks of any ion specificity. Another viewpoint proposed by Long and McDevit, so called internal pressure theory, the ion effects were seen as volume changes in water upon salt addition. Moreover, this mechanism gave good correlation with the solubility of numerous hydrocarbons. Nevertheless, it gave significantly higher salting-out constants as compared to experimentally measured values. Later, scaled particle theory was proposed. Two counteracting ion effects were introduced including, a salting-out mechanism with the required free energy to create a solvation cavity for the solute in solution, and an attractive van der Walls interaction between the hydrophobic solute and the cavity of cosolvent. The former effect is in parallel to the surface tension effect since in general, salts makes the cavity formation harder as function of salt concentration. The drawback of this theory is it ignores any direct ion binding. There are numerous attempts to give a complete mechanism for ion macromolecule interactions, yet most lack experimental data, or a good representative model molecule. The proposed mechanism, in this chapter, by employed LCST data of PNIPAM and ELPs seems to cover all major possible ion interaction with neutral macromolecules. In charged systems, of course, the electrostatic interactions dominate and show orders of magnitude tighter ion binding interactions.
The A coefficient in eqn. 3.2 also correlated with the bulk ion hydration thermodynamics, where other attempts with various (hydration numbers, polarizibility, ionic volume) bulk solution parameters showed no correlation. The underlying mechanism of A coefficient was not clear. Yet correlations with bulk solution thermodynamics show a strong indication that it is related to bulk solution properties. However, it could simply be a second term of a Taylor expansion. Namely, it could potentially be just a correction to the bulk ion hydration or surface tension.

Conclusions

In summary, the specific cation effect of various chloride salts were investigated with two hydrophobic model biomacromolecules for shedding light onto the underlying molecular mechanism of cation interactions. The hydrophobic collapse process of thermo-responsive macromolecules even though dominated by a salting out behavior, specific cation effects could be observed. Specific very weak contact pair cation binding was shown for strongly hydrated cations. Same cations also showed a bulk solution effect that salts in dramatically. Weakly hydrated alkali cations do not interact with the polymers presumably through surface tension mechanisms. NH$_4^+$ and NMe$_4^+$ further demonstrated a hydrogen bonding, and a highly polarizable cations, respectively. They both salted out lesser than their alkali and alkali-earth counterparts.

Contributions: ELP Plasmids were donated by Chilkoti Group, Duke University. ELP expression was done by Jaibir Kherb and Halil I. Okur. The LCST data were originally taken by Jaibir Kherb, and Yanjie Zhang. The LCST data were retaken, analyzed and modelled by Halil I. Okur.
CHAPTER IV

BIOMOLECULAR SIZE INFLUENCE ON SPECIFIC ION EFFECTS

Introduction

Specific ion effects have been investigated over the last century. In the original Hofmeister studies the anionic effects were shown to be prominent as compare to their cationic counterparts for neutral polymer/polypeptide systems. This behavior, of course, can be altered when the electrostatic interactions between ions and charge residues are involved, and even reversed Hofmeister series have been demonstrated to achieved. Cremer group showed the anion binding to model molecules were in the expected order (~100s mM) in numerous studies involving various model polymers/polypeptides. In the last two chapters, the direct cation binding to neutral model molecules is shown to be only very limited. Thus, the binding affinities of cations are orders of magnitude weaker than the binding affinities of anions. However, other literature studies using various polymers, polypeptides, and proteins as well as small molecules as model systems showed a wide range of affinities for both anion and cation interactions with these molecules. Interestingly, no overall agreement can be seen for the partitioning and the binding affinities of the employed ions. This wide range of ion specific results have never been fully understood, and generally attributed to some problems in the experiments or simulations. Nevertheless, this effect could be due to a hidden physical phenomenon that has never been taken into account in the salt specific effects.

A large set of literature examples can be given to the wide range of anion binding affinities, including the followings. Blake et.al. showed weakly hydrated I⁻ anion were
repelled from the hydrophobic hydration spheres of small molecules, including trimethylamine, and trimethylamine N-Oxide.\textsuperscript{104} On the other hand, Cremer group and others showed weakly hydrated anions bind tight to large macromolecules where the most weakly hydrated anions have as tight as 50 mM $K_d$ value.\textsuperscript{25,72} Paterová \textit{et.al.} tested C, and N capped neutral triglycine as a model molecule for anion binding and showed a weaker SCN$^{-}\text{ binding with a } K_d$ value larger than 1M.\textsuperscript{105} Curiously, these literature results seem to give a correlation between the size of the macromolecule and the binding affinity of anions, where anion binding is tighter for macromolecules and weaker, or even repulsive from the surface of small molecules. This hypothesis needs to be investigated systematically, where different sized polymers, oligomers and monomers of the same chemical specie are utilized.\textsuperscript{106}

Herein, we have elucidated the effect of macromolecular size on ion-biomacromolecule binding on $N,N$ diethyl acrylamide based monomers, oligomers and the polymers. Two techniques were utilized for this purpose, including the lower critical solution temperature (LCST) measurements for monitoring macroscopic behavior of the various sized polymers, as well as Raman spectroscopy of the C-H stretching bands to gain molecular level picture of the ion-biomolecule interactions. These experiments could inform the current picture of ion specific effects in mixed environments, where large macromolecules coexist with oligomers and small molecules, like all biological fluids.

**Experimental**

**Materials.** NaCl, Na$_2$SO$_4$ (>99.9 %) and NaSCN (>98%, 99.99%) salts (from Sigma Aldrich, MO and Fluka) were used as received. Aqueous solution samples were prepared
with heavy water (99.98%, Cambridge Isotope Laboratories, Andover, MA) in order to eliminate the interference between the O-H and C-H stretching peaks. Different sized PDEA samples were vacuum dried and dissolved in desired salt solutions where the final unit cell concentration of the biomolecule kept to be 250mM for both LCST and Raman measurements. PDEA, and tested salts are not susceptible to pH changes, thus no buffers were added to sample solutions. The phase transition temperatures were measured with an automated melting point apparatus (Optimelt MPA 100, Stanford Research Systems), with a ramping rate of 1°C/min in all measurements. The reported LCST data in this chapter is an average of minimum 3 measurements, if not 6, and it is highly repeatable. The resulted LCST curves as a function of salt concentration were modelled to the following equation. This is the same equation with the eqn. 3.1 in Chapter 3. Briefly, T₀ is the LCST of polymer in pure D₂O, [M] is the molar concentration of tested salt, and c is a coefficient with unit of temp(°C)/molarity. Bₘₐₓ is another coefficient with units in temperature (°C), and Kₐ is the dissociation constant of the anion-biomolecule interaction.

\[ T = T_0 + c \frac{[M]}{K_d + [M]} \]  

(eqn 4.1)

**PDEA synthesis.** Detailed synthesis section can be seen in ref 106. Briefly, a mixture of N,N diethyl acrylamide, CuCl₂, and 2-propanol was deoxygenated. Me₆TREN was added, and the mixture was stirred for 20 min for the formation of the CuCl₂/Me₆TREN complex. The initiator, deoxygenated by bubbling with argon for 30 min and then added to begin the polymerization at ambient temperature. The reaction was monitored with gel permeation chromatography (GPC) of small portion of aliquots every 2 hours to synthesize the desired molecular weight. The reaction was terminated by bubbling air for 3 min. The reaction mixture was filtered first with a short neutral alumina, followed by a passed through a short
column of neutral alumina and Sephadex LH-20 column for purification. The latter applied 5 times to achieve ultra-pure and narrow polydispersity of the substrate.

**Raman Measurements.** A detailed description of our Raman system can be seen in the methods section. Briefly, 514.5 nm Krypton/Argon (Spectra-Physics Inc., Model 2018-RM) laser source was used as an illumination beam with 100mM power at the sample stage. The objective was located perpendicular to the incident laser beam, and the signal was collected with a 20x objective (Nikon Japan, PLAN APO 20x/0.75 DIC M, w.d. 1.0 cm) Signal was collected with a liquid nitrogen cooled CCD detector (Princeton Instrument Inc., PyLoN) after passing through 514nm notch filter (Semrock Inc., 514.5 StopLine™) and spectrograph (Princeton Instrument Inc., IsoPlane SCT 320) with 1800 g/mm grating.

The Raman spectra were further processed with Savitzky-Golay function and 2800-3100 cm⁻¹ (CH stretching peaks) region fit to gaussian functions for the corresponding C-H stretching peaks. All spectral fits shown here have the lowest least error sum.

**Results**

**The solubility of different sized PDEA as a function of salt concentration.** Figure 4.1a plots the LCST data of various sized PDEA molecules at 250 mM monomer concentration as a function of NaCl concentration. First, the LCST values in D₂O changed as a function of macromolecular size of PDEA. The phase transition occurs at 36.32°C, 35.15°C, 31.74°C, and 31.73°C for 18 mer, 29 mer, 43 mer and 81 mer of PDEA polymers, respectively. The monomer unit concentration of the polymers were kept the same in all measurements, yet there is a 4.5°C difference in temperature between the 18 mer and 81 mer polymers. Also the 9 mer does not show any phase transition behavior at all.
Figure 4.1. The LCST data for various sized PDEA polymers at fixed 250mM monomer unit concentration as a function of a) NaCl, b) Na₂SO₄ concentration in D₂O. Figure 4.1b is kept at the same concentration scale with figure 4.1a for clarity. (The size of the polymer is as in the legend) (The symbols are the measured data points, and curves are the fit to eqn. 4.1) All error bars are within the data points drawn.
Figure 4.2. a) The LCST curves of various sized PDEA polymers at fixed 250mM monomer unit concentration as a function of NaSCN concentration. b) The curves represents the Langmuir binding isotherms’ after the linear fraction (c[M]) is subtracted. K_d values were 1.18 M, 0.94 M, 0.68 M, and 0.6 M for 18mer, 29mer, 43mer, and 81mer, respectively. (The size of the polymer is as in the legend) (The symbols are the measured data points, and curves are the fit to eqn. 4.1) All error bars are within the data points drawn.
The size of the polymer affects the LCST values without any salt added. One could claim a macromolecular size effect at least for the intra/inter molecular interactions was demonstrated via PDEA polymers.

As can be seen in Figure 4.1a, the LCST values gradually decrease as the NaCl concentration in solution increased. It is worth noting that all the curves had very similar slopes as a function of chloride salts. Thus, one can claim that NaCl salt affect all different sized macromolecules very similarly. In an analogous experiment the transition temperature of the same four PDEA polymers and oligomers were probed as function of Na$_2$SO$_4$ concentration. As can be seen in Figure 4.1b, again all the curves are linearly changed with all different PDEA polymers and oligomers, and again with very similar negative slopes. Nevertheless, the salting-out effect of SO$_4^{2-}$ salts were quite dominant as compare to chloride salt. This result is on the expected trend, where sulfate is a well-known characteristic stabilizer and chloride is generally defined as a dividing line between chaotropes and kosmotropes.

Figure 4.2a plots the LCST curves of PDEAs with increasing NaSCN concentrations. By contrast to other employed salts, the LCST curves were non-linear, along with a deviation in the curve trends as a function of molecular size. These LCST data fit to eqn. 4.1, where apparent dissociation constant of SCN$^-$ ion can be calculated. It yields a factor of two difference between the smaller oligomer and the largest tested polymers of PDEA. The apparent K$_d$’s were in the range of 1200 mM – 600 mM, where smaller oligomers yielded a weaker anion binding. Indeed, the macromolecular size effect on ion-biomolecule interactions can be seen significantly with the weakly hydrated anion, whereas, strongly hydrated anions only salt-out independent to molecular size. It is worth mentioning that
only weakly hydrated anions were shown to directly bind to the polymer surfaces, strongly hydrated anions only effect via either surface tension and/or bulk hydration properties, at least for the neutral biomacromolecular systems.\textsuperscript{28,29,95} In order to clearly demonstrate the differences in the dissociation constants of the SCN\textsuperscript{-} ion binding, Figure 4.2b plotted the residual curves for each PDEA polymer/oligomer after the linear part (c[M]) in eqn.4.1 was subtracted. Tighter anion binding can clearly be seen for the larger PDEA macromolecules.

A linear combination of a Langmuir binding isotherm and a linear term has been shown to be a good model to explore specific anion effects, at least for the tested ion concentrations.\textsuperscript{28,29} All LCST data shown above fit to eqn. 4.1 and the fitting parameters were tabulated in Table 4.1 for side by side comparisons. The only apparent macromolecular size effect can be seen for SCN\textsuperscript{-} direct binding, all the other tested salts were insensitive to macromolecular effects.

Phase transition measurements of PDEA polymers demonstrated a very distinct trend where anion binding gets weaker as the size of the macromolecule gets smaller. Unfortunately, smaller oligomers and simple amide molecules do not have this phase transition behavior. Thus, Raman spectroscopy was utilized as a complementary technique to further investigate macromolecular size effect. The C-H stretch bands of all different molecular weight polymers including larger polymers and small molecules were monitored as a function of the same sodium salts.
Table 4.1. The fitting parameters of LCST data to eqn. 4.1 for all tested PDEA polymers.

<table>
<thead>
<tr>
<th>Macromolecule Size</th>
<th>NaSCN</th>
<th>NaCl</th>
<th>Na\textsubscript{2}SO\textsubscript{4}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ka [mM]</td>
<td>B\textsubscript{max} (°C)</td>
<td>C (°C/[M])</td>
</tr>
<tr>
<td>18mer</td>
<td>1180±130</td>
<td>35.7±4.1</td>
<td>-7.65±0.86</td>
</tr>
<tr>
<td>29mer</td>
<td>940±80</td>
<td>28.1±2.3</td>
<td>-6.65±0.54</td>
</tr>
<tr>
<td>43mer</td>
<td>680±100</td>
<td>22.4±2.7</td>
<td>-7.85±0.76</td>
</tr>
<tr>
<td>81mer</td>
<td>600±30</td>
<td>19.9±0.3</td>
<td>-7.35±0.42</td>
</tr>
</tbody>
</table>

NaCl and Na\textsubscript{2}SO\textsubscript{4} fit to a linear line, and have no contribution from direct ion binding which yields B\textsubscript{max} value to be zero, and Ka becomes undefined.
The macromolecular size effect probed with Raman spectroscopy. Figure 4.3a shows Raman spectrum of PDEA polymer with 81 repeating monomer units in the 2850 cm\(^{-1}\)-3100 cm\(^{-1}\) region. Mainly five distinct PDEA peaks can be seen. The backbone CH asymmetric stretching and CH\(_2\) asymmetric stretching rose at 2916 cm\(^{-1}\) and 2905 cm\(^{-1}\), respectively. These two resonances have only moderate peak intensities. Side group yields three peaks at 2882.5, 2940.8 and 2980 cm\(^{-1}\) for CH\(_3\) symmetric stretching, CH\(_2\) asymmetric stretching, along with CH\(_3\) asymmetric stretching bands, respectively. Side chain peaks are more distinct and yields higher intensity as compare to backbone peaks. These assignments are in complete agreements with the literature values.\(^46\) Yet, the peak at 3021 cm\(^{-1}\) where it rose as a shoulder band to asymmetric CH\(_3\) stretch resonance must be due to some Fermi resonances, yet this peak has not well characterized in the literature. Among all C-H peaks, asymmetric CH\(_2\) was the most susceptible to presence of salt solutions. Thus, shifts in this peak was utilized as a probe to monitor ion-biomolecule interactions. Similar approaches have been successfully utilized for NMR, FT-IR, and Raman spectroscopies.\(^{14,25,104}\) Figure 4.3b shows the change in the peak position for the CH\(_2\) asymmetric stretch band as a function of NaSCN concentration for all employed PDEA molecules, where a clear macromolecular size effect can be seen. In order to quantitatively investigate the Raman data points, equation 4.1 was adapted in the following form;

\[
\Delta \text{Peak position shift (cm}^{-1}) = c[M] + \frac{B_{\text{max}}[M]}{K_d+[M]} \quad \text{(eqn. 4.2)}
\]

where c, B\(_{\text{max}}\) and K\(_d\) are identical terms with eqn. 4.1 where only peak position changes were used instead of temperature changes.
Figure 4.3. a) Raman spectrum of PDEA (81mer) in D$_2$O. b) The change in the CH$_2$ asymmetric stretch peak position as a function of NaSCN concentration for different sized PDEA polymers. (The dots are data points and curves are the fits to eqn. 4.2) The spectra are offseted by 0.5 cm$^{-1}$ for clarity. Each data point is average of two scans.
Figure 4.4. The Δ Raman peak shift for different sized PDEA molecules as a function of NaSCN concentration. The residual peak shift are calculated simply subtracting the linear contribution (c[M]) from eqn.4.2. The apparent $K_d$ values are also added for each tested molecular size.
All tested PDEA polymers, oligomers were fit to eqn. 4.2, where apparent dissociation constants ($K_d$) values showed a direct macromolecular size correlation. The residual Raman peak shifts, after the linear portion was subtracted, as a function of NaSCN can be seen in Figure 4.4. The $K_d$ values were around 930 mM for 9mer and 130 mM for 81mer of PDEA polymer. The apparent $K_d$ values were 580 mM and 250 mM for 29mer and 43mer, respectively. Other tested ions (Cl$^-$ and SO$_4^{2-}$) only cause subtle to no shifts for this resonance. The overall ion binding interaction was tighter in spectroscopic measurements compare to the findings of macroscopic hydrophobic polymer collapse. The reason for this phenomenon was explained in a recent study.$^{25}$ A weaker ion effect is generally seen for a macroscopic thermodynamic measurement where, the result is simply an integration of the ion effect over the entire polymer surface. On the other hand, spectroscopic measurements can only monitor the most significant peak shifts, presumably the tightest binding site. Thus, spectroscopy yields tighter overall ion binding.

The spectroscopic investigation was extended with the simple amide molecules, where $N$-Methylacetamide (NMA) and Diethylacetamide (DEA) molecules were tested as a function of NaSCN and NaCl salt concentrations. Figure 4.5 a&b shows the analogous C-H resonance shift data for the most prominent peak shift for DEA and NMA molecules, where CH$_2$ asymmetric stretch peak for DEA, along with CH$_3$ asymmetric stretch peak for NMA were monitored. Curiously the peak shift trends were perfectly linear as a function of salt concentration for employed salts, lack of any non-linear contributions. These data points fit perfectly only the linear portion of eqn. 4.2, with $K_d$ values larger than 3M.
Figure 2.5. The Raman peak position shift as a function of salt concentration a) asymmetric CH$_2$ stretching band of N,N Diethyl acetamide and b) asymmetric CH$_3$ band of N-methyl acetamide. Each data point is average of two scans.
This linear curves are a strong indication for apparent ion exclusion from the surface of simple amide molecules. A similar ion expulsion mechanism has drawn for another weakly hydrated anion (I⁻) from the hydrophobic hydration of tetramethylamine oxide molecule.104 Again, other C-H bands show the same trends with NaSCN and NaCl concentration increments, but with a smaller overall peak shift. (Data not shown) The simple amide molecules represents one end of this macromolecular size scale and showed no apparent binding even demonstrated ion exclusion. Interestingly, only the weakly hydrated anion binding gradually gets tighter as the size of the macromolecule increase.

**MCR-Raman solvent correlation spectra of PDEA polymers.** The spectroscopic Raman data, along with the thermodynamic LCST measurements demonstrate a clear macromolecular size dependence for weakly hydrated anion-biopolymer binding. Nevertheless, these two sets of data provide no clue for the underlying molecular level picture. One can argue that hydration of the solute molecule plays a key role based upon Dr. Chandler work where a dramatic alteration in the hydration thermodynamics was shown as a function of molecular size.107 Thus we further investigated the employed model molecules with MCR-Raman spectroscopy. This technique have been utilized to monitor solvent correlated spectrum of the solute molecules in bulk aqueous solutions.56,104,108,109 The details of this technique can be seen in the Methods section of chapter 1 (p-17).

Figure 4.6a shows the spectra of the solute with its correlated water hydration, so called “solvent correlated (SC) spectrum”, of different sized PDEA polymers. The spectra of all tested molecules were identical in 2800 cm⁻¹-3100 cm⁻¹ region. Namely, the C-H peaks remained the same as the molecular size changes from 9 mer to 81 mer. However, the water region (3100 cm⁻¹-3700 cm⁻¹) yielded some dramatic changes. Traditionally, this
region has been classified as two distinct populations, including an ice-like and water-like water molecules in both bulk and surface studies.\textsuperscript{72,83,110} The ice-like waters have more H-bonds and rose as a resonance around 3200 cm\textsuperscript{-1} in the spectrum, whereas, water-like band appear around 3400 cm\textsuperscript{-1} and have lesser H-bonds per water molecule. The solute hydration spectrum of PDEA gradually varied as the size of the molecule increases. The smallest PDEA oligomer, 9mer, showed prominently water-like hydration, where ice-like water contribution was only subtle. This water hydration behavior progressively changes and 81 mer shows an equal intensity for both water peaks.

Hydration of solute molecules was further elucidated for small amide molecules. Figure 4.6b displayed the SC spectra of NMA and DMA molecules. Low frequency region (3000 cm\textsuperscript{-1}) showed the tail from the C-H stretch peaks. Interestingly, the solvent correlated spectrum of DMA molecule is identical with the 9mer PDEA, where 3400 cm\textsuperscript{-1} is higher in intensity and the 3200 cm\textsuperscript{-1} peak is only subtle. NMA spectrum, on the other hand, seems a little different, where a small 3350 cm\textsuperscript{-1} contribution can be seen. It must be noted that N-H stretching band of the solute also give rise to the spectrum at this frequency. Overall, small oligomers and small amide molecules show more water-like hydration spectrum, whereas, a molecular size dependent 3200 cm\textsuperscript{-1} grow as the polymers grow in size. This hydration differences must play a fundamental role for the ion partitioning to biomolecule surfaces. (see Figure 4.7 for schematic representation) Indeed, weakly hydrated anions (SCN\textsuperscript{-}) displayed a tighter binding with more ice-like hydrated molecular sizes. SC spectrum presumably only yields information about the first hydration layer of water. More ice-like water hydration must leave more weakly hydrated sites on the biomolecular surface due to more H-bonding to the bulk water. Indeed, this weakly hydrated surface sites are
Figure 4.6. a) The solvent correlated MCR-Raman spectra of different sized PDEA polymers and oligomers at ambient conditions. (The data is normalized to per monomer unit.) b) The water region of the solvent correlated spectra of the simple amide molecules; N-methyl acetamide (NMA) and N,N dimethyl acetamide (DMA). The 9 mer and 81 mer of PDEA data is added for direct comparison.
Figure 4.7. The schematic representation of weakly hydrated anion, SCN$^-$, binding to monomer, oligomer, and polymer. The dark blue line around each molecule indicates the tight hydration. The red dashed lines show weakly hydrated sides around these molecules (corresponds to 3200 cm$^{-1}$ peak in Figure 4.6). The extent of weakly hydrated sites seem to directly proportional to tight weakly hydrated anion binding.
more susceptible for weakly hydrated ion binding, as such yields tighter SCN$^-$ binding with larger macromolecules. SC spectra of these model molecules indicated the polymer hydration plays a key role in macromolecular size effect on ion binding.

**Discussion and Conclusions**

The thermodynamic phase transition and spectroscopic Raman measurements along with solvent correlated MCR-Raman demonstrated a very clear macromolecular size effect for ion binding. The LCST measurements (Figure 4.1, and Figure 4.2) show the overall polymer behavior as a function of sodium salts of strongly and weakly hydrated anions. SO$_4^{2-}$ and Cl$^-$ anions only salted out the PDEA polymer. NaSCN, however, yielded a non-linear phase transition behavior as the salt concentration increases. Moreover, the data modeled to eqn. 4.1, where K$_d$ values were achieved. Tighter binding of weakly hydrated anion was shown with larger polymers and weaker for the smaller ones. LCST measurements cannot show the entire size spectrum due to the limitations of phase transition that only occur above a certain molecular size. Thus, LCST measurements only applied for PDEA polymers bigger than 9 mer. Spectroscopic Raman measurements had no low end molecular size limitations, and C-H stretch peak shifts demonstrated a very similar molecular size effect as a function of NaSCN salt concentration. (Figure 4.3 and Figure 4.4) The peak position shift data were modelled with an equation analogues to eqn. 4.1 and again yield K$_d$ values in agreement with the findings from macroscopic measurements, but the spectroscopic data elucidated a tighter binding as compare due to monitoring only a single binding site as compare to overall polymer surface.$^{25}$ Figure 4.8 plots the dissociation constants of weakly hydrated anions as a function of macromolecular size where number of monomer units is used as a size unit.
Figure 4.8. The overall dissociation constants achieved by LCST (red), and Raman measurements (blue) in this study are plotted against the molecular size of the model molecules, along with some literature data.
As can be seen in the figure, the size effect is more dramatic from monomers to oligomers. It varied from ion excluded \( (K_d > 3 \text{M}) \) from the surface of simple amide molecules to weak anion binding \( (K_d = 0.93 \text{M}) \) to weakly hydrated sites on the oligomers (9 mer PDEA) surfaces. The effect is more modest in the larger sizes the ion binding only varies a factor of 3 from 29mer to 4000mer. These results are qualitatively in agreement with Chandler’s explanation for cavity formation in aqueous solutions. In those simulations a dramatic hydration energy increase for hollow cavity formation as a function of cavity size is shown.\(^{107}\) Nevertheless, our results show a more modest changes as the size of the solute molecules increase. It should also be noted that a hollow cavity may not fully represent a polymer/protein surface.

The SC spectra as a function of molecular weight provided a strong spectroscopic evidence for water hydration variations as a function of solute size, where a gradual increase in the more ordered (ice-like) water molecules in the hydration of solute molecules as a function of molecular size. The more ice-like water hydration should yield more weakly hydrated polymer surface sites due to a strongly interacting hydration water molecules with the bulk water. Indeed, weakly hydrated sites are shown to be more susceptible for weakly hydrated anion binding. The tightest SCN\(^{-}\) binding and the more intense 3200 cm\(^{-1}\) water peak occurs for larger PDEAs, 81 mer, whereas weakest binding and subtle ice-like water peak was observed for smaller PDEA, 9 mer, and simple amide molecules. This set of hydration data gives a molecular level insight to macromolecular size effect.

Molecular size effect shown for PDEA polymers/oligomers should be universal, and these results should apply to other amide involved polymers (PNIPAM) and
polypeptides i.e. ELPs, along with naturally occurring proteins. However, testing other systems might be challenging due to the difficulty of achieving controlled molecular sized models with high size precision. Presence of charged residues may shift, or alter this size effect. In order to achieve a full understanding of the molecular size effect, it must also be investigated on a series of charged systems.

These results have an implication in biophysics. Different sized biomolecules coexist in all biological fluids including cytoplasm, and extracellular fluids. The role of ions may not be the same for small building blocks of life such as amino acids, glucose, and many others along with functional naturally occurring macromolecules. This complex puzzle has not been fully solved. Nevertheless, our findings here show that ion behaviors are specific to macromolecular size. These ion specific results at least for weakly hydrated anions have direct application since weakly hydrated anions i.e. iodide could change the functionality of naturally occurring proteins, but have no such influence on small molecules. Such effects are completely missing for strongly hydrated anions. Moreover, there is no evidence for the macromolecular size effect for positively charged ions, at least for the systems tested in Chapter 2, and Chapter 3.

Contributions: PDEA polymers and oligomers were synthesized by Chen Chen from Allcock group. LCST, Raman, and MCR-Raman samples were measured, analyzed and modeled by Halil I. Okur.
CHAPTER V

CONCLUSION & FUTURE DIRECTIONS

Ion specific effects on biological interfaces control a wide range of physiologically important processes; including hydrophobic aggregation/collapse, protein folding, enzyme kinetics, and enzymatic activity, along with phase transition of lipids. Biological fluids show a direct manifestations of these effects by being very sensitive and selective to both the identity and the concentration of ions. One well-known example is K⁺ concentration is actively maintained two orders of magnitude higher over Na⁺ in the cytoplasm via Na⁺- K⁺ ion pumps. Interestingly, the Hofmeister series has been shown to recur in many of the processes named above, as well as some non-physiological processes. Typically, a direct Hofmeister series is observed, but when strong electrostatic interactions are involved a reversed order can also be seen.²¹,¹⁰⁵ The anionic Hofmeister series is more prominent and shown to recur in over a thousand studies. Cationic effects, however, are mostly shown for ion pairing interactions with formally charged residues. Moreover, cation specificity has not been elucidated experimentally on neutral systems (i.e. protein backbones) due to the smaller contribution to specific ion effects. In this dissertation cation interactions with the neutral protein backbones were elucidated using three different model molecules, including a simple amide molecule, butyramide, polymers with pendant amide groups (PNIPAM), and elastin-like polypeptides (ELPs). First, a weak cation binding to simple amide molecules is demonstrated with vibrational spectroscopy coupled with surface specific nonlinear VSFS. Only strongly hydrated cations (Ca²⁺, Mg²⁺, Li⁺) displayed a weak affinity towards amide carbonyl. Cation binding was monitored via the appearance of a new amide carbonyl resonance, along with the alignment of the hydration water at the
air/butyramide/water interface as a function of salt concentration. Weakly hydrated cations (Na\(^+\) and K\(^+\)) show no evidence for cation-amide interactions. Interestingly, these findings with simple amide molecules completely agreed with the phase transition behavior of the biomacromolecules as a function of alkaline and alkali earth metal cations. Again no direct cation binding was observed for weakly hydrated cations, and a weak binding with strongly hydrated ones. Furthermore, LCST measurements displayed an additional bulk ion hydration thermodynamics mediated salting-in effect. These experimental results solidify the small effect of cations as compared to the anionic Hofmeister effect on neutral polymer/protein systems.

Hofmeister ion effects have been investigated with a wide range of model systems where the molecular sizes vary from a single monomer unit to large macromolecules. Even though it is fundamentally important, the macromolecular size effect has not been fully elucidated. For instance, naturally occurring biological systems exist with a mixture of monomers, oligomers and biomacromolecules. A series of various sized PDEA polymers along with few single amide molecules were examined against three representative Hofmeister anions via thermodynamic phase transition as well as spectroscopic Raman measurements. Strongly hydrated SO\(_4^{2-}\) and Cl\(^-\) anions yielded no difference for various sizes and only salt-out for all molecular weights. Weakly hydrated SCN\(^-\), however, demonstrated two orders of magnitude difference for anion binding to the biomolecules. Namely, SCN\(^-\) was shown to be repelled from small amide molecules (Kd >3-5M). The anion binding gradually got tighter as a function of molecular weight, and yielded a 130 mM dissociation constant for the 81mer. This dramatic anion binding deviation for the weakly hydrated anions were based on the hydration changes of the biomolecules, and
formed more weakly hydrated sites as a function of macromolecular size. These sites were shown to be the binding sites for weakly hydrated anions. Salting-in, salting-out behavior of cations, on the other hand, appeared not to be affected from the macromolecular size variations. Simple amide molecules and large biomacromolecules yielded the same weak cation binding results for only strongly hydrated cations.

Even though it has been investigated more than one hundred years, there are still many new areas to explore that display ion-specific effects. For example, as a follow up study, the macromolecular size effect should easily be elucidated for charged polymers, proteins and nucleic acids. Electrostatic interactions should also be effected by the size related hydration changes. Over the last decade the underlying mechanism of protein-protein aggregation and liquid phase transition of proteins were displayed. This fundamental understanding of ion effects on biomacromolecules can be used to solve pharmaceutical industry problems, such as storing and transporting solutions of Immunoglobulin G (IgG) protein without losing its functionality. There are many more physical and biological processes that follow the Hofmeister series and are yet to be explored. This list is quite long and has practical examples from tofu and cheese manufacturing to understanding the mechanism of lightning.
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Paterova J., Rembert K.B., Heyda Y, Kurra Y., Okur H.I., Liu W.R,


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