

MOLECULAR INTERACTION MECHANISMS OF BULKY IONS ON AQUEOUS NEUTRAL MACROMOLECULES

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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Abstract

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M.S. in Chemistry

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The ion specific effects on the solubility of macromolecules have been demonstrated for the first time more than a century ago yet the molecular level detailed picture of ion – macromolecule interactions has not been fully drawn. When considering the effects of salts on macromolecules, it was generally considered that the overall effect is dominated by the anions, in which the counter cations acting mainly for the charge balance in the medium. Recently, the chloride salts of tetra alkyl ammonium (NR_4^+) ions refuted such assumptions via demonstrating that weakly hydrated cations can interact with macromolecules as effectively as weakly hydrated anions (I^- , NO_3^- , SCN^-). In this thesis, we studied how tetra alkyl ammonium cations (NR_4^+) behave when in conjunction with surface-active anions (I^- , NO_3^-) and whether the ion effects on macromolecules are additive. When we examined the phase transition temperature changes of tetra alkyl ammonium iodide (NMe_4I , NEt_4I , NPr_4I , NBut_4I) salts on PNIPAM, a thermo-responsive polymer that enables to construct a thermodynamic model. A surprising salting-out behavior was observed, particularly for tetra butyl ammonium iodide (NBut_4I) even though a strong salting-in behavior is expected due to the presence of two surface-active ions. To elucidate the mechanism

of this non-additive behavior, we investigated the solubilized and collapsed states of the macromolecules using ^1H -NMR and ATR-FTIR experiments, respectively. Our experimental results indicated that when the cation and counter-anion are bound simultaneously, the charge of the macromolecular surface remains close to neutral, and thus, a salting-out behavior can occur. On the other hand, when the hydrophobicity of the cation decreases, this cooperative binding is partially disrupted, and the weakly hydrated anion becomes dominant, and clear salting-in effect is observed. In the second part, tetraphenyl-borate (TPB^-) and tetraphenyl-phosphonium (TPP^+) ions are also investigated, which are bulky molecular ions with charge asymmetry. Hydration spectroscopy (MCR-Raman) demonstrated the influence of charge asymmetry on the interaction of pi electrons of the phenyl groups and hydration water via the dangling O-H vibrational band shifts. In light of this, the effects of these ions on three different model neutral macromolecules (PNIPAM, PDEA and ELP) were investigated. The anion with the strong water interaction (TPB^-) exhibited a salting-out behavior, while the cation with the weakest water interaction (TPP^+) exhibited a salting-in action. NMR and ATR-FIR experiments demonstrated that the tetraphenyl phosphonium ion (TPP^+), in particular, showed an apparent non-site-specific interaction with the macromolecule. Consequently, the different hydration fingerprints of hydrophobic ions directly correlated with the well-known strongly hydrated, and weakly hydrated ion concepts, and clearly rationalize their effect on neutral macromolecule.

Keywords: Ion Specific Effect, Hofmeister Series, Non-Additivity, Bulky Ions, Super Chaotropic Ions, PNIPAM, Bio-interfaces, Hydration Spectroscopy, LCST

Özet

BÜYÜK İYONLARIN SULU ÇÖZELTİLERDEKİ NÖTR MAKROMOLEKÜLLER İLE OLAN ETKİLEŞİM MEKANİZMLARININ İNCELENMESİ

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İyonların makromoleküllerin çözünürlüğü üzerindeki spesifik etkileri ilk kez bir asırdan daha uzun bir süre önce gösterilmiş olmasına rağmen iyon-makromolekül etkileşimlerinin moleküler düzeydeki detaylı resmi henüz tam olarak çizilmemiştir. Tuzların makromoleküller üzerindeki etkileri düşünüldüğünde, genel etkinin genellikle anyonlar tarafından domine edildiği ve karşıt katyonların esas olarak ortamdaki yük dengesi için hareket ettiği düşünülüyordu. Son zamanlarda, tetra alkil amonyum (NR_4^+) iyonlarının klorür tuzları, zayıf hidratlanmış katyonların, zayıf hidratlanmış anyonlar (I^- , NO_3^- , SCN^-) kadar etkili bir şekilde makromoleküllerle etkileşime girebileceğini göstererek bu varsayımları çürütmüştür. Bu tezde, tetra alkil amonyum katyonlarının (NR_4^+) yüzey aktif anyonlar (I^- , NO_3^-) ile birlikte nasıl davrandığını ve iyonların makromoleküller üzerindeki etkilerinin katkısız olup olmadığını inceledik. Termodinamik bir model oluşturmaya olanak tanıyan termotepkisel bir polimer olan PNIPAM üzerindeki tetra alkil amonyum iyodür (NMe_4I , NEt_4I , NPr_4I , NBut_4I) tuzlarının faz geçiş sıcaklığı değişimlerini incelediğimizde, iki

yüzey aktif iyonun varlığı nedeniyle güçlü bir makromolekül çözünürlük artışı beklenmesine rağmen, özellikle tetra butil amonyum iyodür (NBut₄I) için şaşırtıcı bir çözünürlük azaltma davranışı gözlemlendi. Bu eklemeli olmayan davranışın mekanizmasını açıklamak için, sırasıyla ¹H-NMR ve ATR-FTIR deneylerini kullanarak makromoleküllerin çözünmüş ve çökmüş hallerini inceledik. Deneysel sonuçlarımız, katyon ve karşı anyon aynı anda bağlandığında, makromoleküler yüzeyin yükünün nötre yakın kaldığını ve böylece makromolekülün çözünürlüğünün azalabileceğini gösterdi. Diğer taraftan, katyonun hidrofobisitesi azaldığında, bu kooperatif bağlanma kısmen bozulur ve zayıf hidratlanmış anyon baskın hale gelir ve molekülün çözünürlüğünün arttığı gözlenir. İkinci bölümde, yük asimetrisine sahip hacimli moleküler iyonlar olan tetrafenil borat (TPB⁻) ve tetrafenil fosfonyum (TPP⁺) iyonları da incelenmiştir. Hidrasyon spektroskopisi (MCR-Raman), sarkan O-H titreşim bandı kaymaları yoluyla fenil gruplarının pi elektronlarının ve hidrasyon suyunun etkileşimi üzerinde yük asimetrisinin etkisini göstermiştir. Bunun ışığında, bu iyonların üç farklı model nötr makromolekül (PNIPAM, PDEA ve ELP) üzerindeki etkileri incelenmiştir. Güçlü su etkileşimine sahip anyon (TPB⁻) tuzlanma davranışı sergilerken, en zayıf su etkileşimine sahip katyon (TPP⁺) tuzlanma eylemi sergilemiştir. NMR ve ATR-FIR deneyleri, özellikle tetrafenil fosfonyum iyonunun (TPP⁺) makromolekülle belirgin bir şekilde bölgeye özgü olmayan bir etkileşim gösterdiğini ortaya koymuştur. Sonuç olarak, hidrofobik iyonların farklı hidrasyon parmak izleri, bilinen güçlü hidratlı ve zayıf hidratlı iyon kavramlarıyla doğrudan ilişkili olup, nötr makromolekül üzerindeki etkilerini açıkça ortaya koymaktadır.

Anahtar Kelimeler: İyona özel etkiler, Hofmeister serileri, Toplamsal olmayan etkiler, Büyük hacimli iyonlar, PNIPAM, Biyo-arayüzler, Hidrasyon spektroskopisi, LCST

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List of Abbreviations

A: Alanine

ALS: Alternating Least Square

ATR-FTIR: Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy

ELPs: Elastin Like Polypeptides

G: Glycine

LCST: Lower Critical Solution Temperature

MCR: Multivariate Curve Resolution

NMR: Nuclear Magnetic Resonance Spectroscopy

P: Proline

PDEA: *poly*- (N-N- diethyl acrylamide)

PNIPAM: *poly*- (N-isopropyl acrylamide)

RDF: Radial Distribution Function

SCS: Solute Correlated Spectrum

SFG: Sum Frequency Generation

TAAI: Tetra alkyl ammonium Iodide

TBAI: Tetra butyl ammonium Iodide

TEAI: Tetra ethyl ammonium Iodide

TMAI: Tetra methyl ammonium Iodide

TPAI: Tetra propyl ammonium Iodide

TPA: Tetra phenyl arsonium

TPB: Tetra phenyl borate

TPP: Tetra phenyl phosphonium

V: Valine



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Chapter 1 – Introduction

Macromolecules are affected by many factors in their solution environment. One of the important effects that should be discussed in particular is ion effects on macromolecules generally referred as “ion-specific effects”. Ion-specific effects play an important role in processes such as protein folding, liposome stabilization, protein crystallization, purification, and self-assembly. Although ion-specific effects are not a new concept, unfortunately the molecular-level detailed clear picture has not been established, yet. For this reason, ion-specific effects are still a highly-investigated subject and are an important research topic not only in macromolecule modulation but also in many fields such as electrochemistry, polymer chemistry, and soft robotics.^{1–13} In this chapter, it is aimed to present studies on how ions behave at interfaces in solutions, starting from the examples where ion-specific effects were first examined.

1.1 History of Ion Specific Effect: Hofmeister Series

Franz Hofmeister conducted the first studies on ion-specific effects in the late 19th century. In his experiments on egg white protein (albumin), he observed that different salts had different effects on protein structures and presented an ion series that is still valid by systematically examining different anion and cation salts. The direct effects of salts on egg proteins were first demonstrated in two separate papers titled: "About regularities in the protein precipitating effects of salts and the relationship of these effects with the physiological behaviors of salts" and "About the water withdrawing

effect of salts".^{14,15} Figure 1 shows that the ion series can be examined through two different behaviors. These two behaviors are called "salting-in" and "salting-out". The ions on the right side of the series are salting-in ions and basically increase the solubility of macromolecules in solutions. The most basic principle at this point is that these ions easily interact with the macromolecule inducing a charge even at a neutral surface. In this way, the interaction with the solvent can be increased, and solubility can be enhanced. The left side of the ion series is salting-out ions. The feature of these ions is to reduce macromolecule solubility and stabilize the complex structures. Such property has already been used in many areas. For instance, it is possible to roughly separate some proteins from protein solutions using different salts at different concentrations by salting out principle.^{16,17} The basic mechanism here is that ions are quite far from the macromolecule's surface and can easily displace solvent molecules from the macromolecule's surfaces. In addition to this, there are many exceptional salting-out mechanisms for ions and osmolytes.

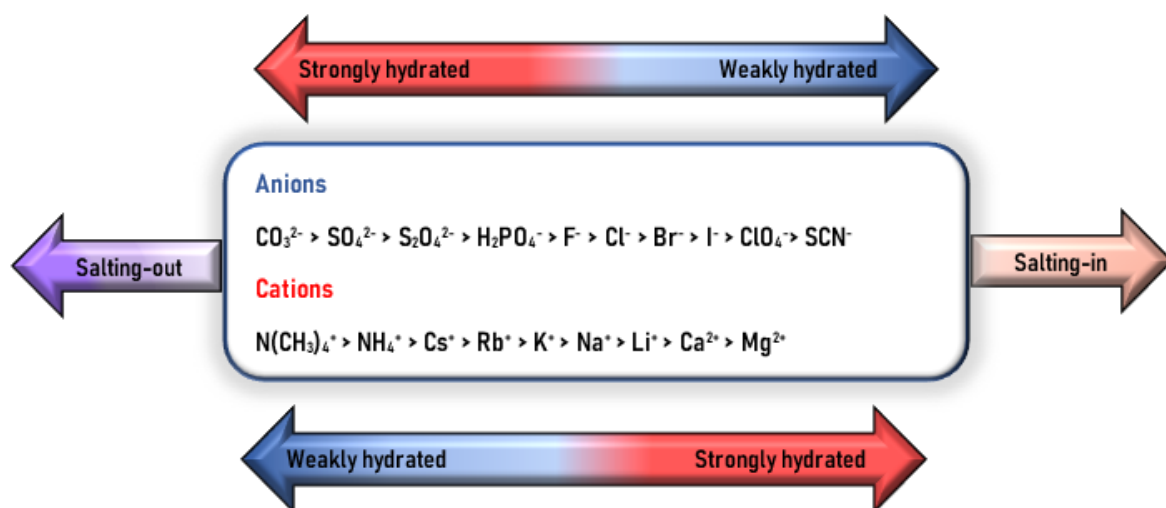


Figure 1: Hofmeister ion series for different cations and anions. The right side of series corresponds to salting-out, however the left side is salting-in. Also, hydration properties of ions are key to commenting on being salting-in or out.¹⁸

Another important feature of the ion series is that the hydration of the ions is related to these two behaviors. The decrease or increase in the solubility of macromolecules depends on the interactions with the solvent, which depends on how hydrated the ions are. For example, anions such as iodide (I^-) and thiocyanate (SCN^-) are weakly hydrated anions and can easily interact with macromolecules. On the other hand, salting out anions in the Hofmeister series are strongly hydrated ions. This situation is opposite for the cations, and it is possible to mention that cations are not considered as important as the anions for interactions since the salting-in cations are strongly hydrated due to having high charge densities. For salting-out ions, that are strongly hydrated anions and weakly hydrated cations. For cations, it is observed that the charge densities are low and the interaction doesn't pay the dehydration penalty when bound to the macromolecule interface. For the anions, being strongly hydrated dramatically reduces interaction probability of the ions with neutral macromolecules.

This effect is generally called "excluded volume effect" and is one of the well-known salting-out mechanisms.^{19,20} Especially Hofmeister's first paper titled "About the water withdrawing effect of salts" attempted to elucidate the mechanisms underlying protein stabilization. Additionally, the terms "chaotropic" and "cosmotropic" are also used widely in the literature, based on the interaction of ions with bulk water molecules and their effect on macromolecular solubility. Salting-out ions are called cosmotropic meaning order-bringing to the hydration shells, and the salting-in ions are called "chaotropic," meaning chaos-creating in the hydration and thus their intermolecular interactions are more favorable.^{14,15,18,21}

The important role of ion hydration in the Hofmeister effect also gives rise to the idea of using water as a probe. In this regard, techniques such as terahertz spectroscopy have repeatedly demonstrated that water is, in fact, the primary agent, and that significant information can be obtained by asking questions about it.²² Havenith and colleagues have demonstrated that terahertz light can provide important information about the collective hydrogen bond dynamics of water, based on the picosecond time scale of water's hydrogen bond dynamics. Static and time-resolved terahertz measurements for many anions and cations in the Hofmeister series, along with changes in terahertz absorption coefficients depending on the type and concentration of salts, have shown where the collective behavior of the hydration waters of some ions lies. As shown in Figure 2, the hydration waters of ions can be observed in spectra between 3 and 300 cm^{-1} .²²⁻²⁴ Similarly, techniques such as 2D-IR or MCR-Raman can provide information about the dynamics of hydration waters. 2D-IR is a nonlinear spectroscopy technique, and dynamic information can be obtained by modulating the time difference between successive pulses of the laser.²⁵⁻³⁰ MCR-

Raman, on the other hand, allows us to obtain information about interfacial waters by iteratively extracting bulk water spectra using the Alternating Least Squares method.^{31–38} Another new contemporary technique is the third-order nonlinear optical spectroscopy, also known as Hyper Raman spectroscopy or Correlated Vibrational Spectroscopy, which studies self-interacting, solute-like water molecules based on the polarization of incident and signal light. PPP polarization provides spectral information about solute-interacting (cross-correlated) water molecules, while other polarization combinations, such as SSS and SPP, give information on self-interacting, bulk-like water molecules.^{39,40} While each method may have its drawbacks, they are unique and state-of-the-art methods employed to paint a general picture of bulk water and solute hydrations.

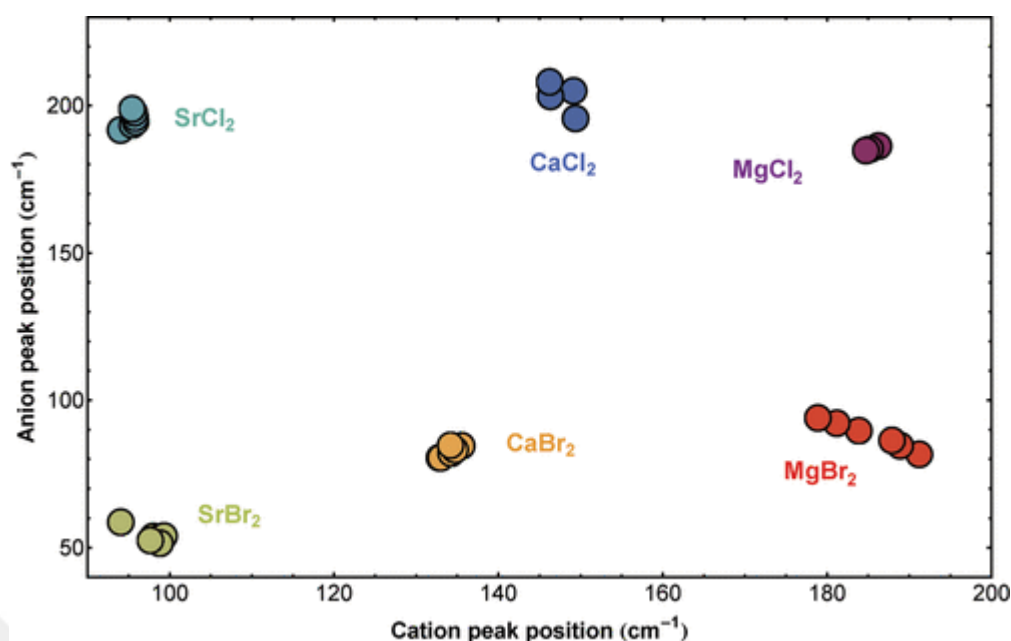


Figure 2: Terahertz absorption frequencies in the region of 3-200 cm^{-1} (0.1-10 THz) for hydration of salt. With dilution experiments, cation and anion hydration water terahertz absorption frequencies can be deduced. (The figure is adapted from ref 22)

1.2 Universality of Hofmeister Series and Ion specific Effect on Different Macromolecules

Proteins are challenging to understand due to their large number and diversity of amino acids, the complexity of intramolecular interactions, and possibly containing post-translational modifications. As a consequence, it is often more promising to go back to the basics and use model macromolecules such as simpler polypeptides or polyamides that can be considered the backbone of proteins. In the literature, Robinson and Jenks used N-acetyltetraglycine ethyl ester as a small model molecule consisting of four glycine residues connected by ester functional groups. Glycine, the smallest

amino acid, is abundant in the cell and thus makes it easier to investigate such model molecules compared to larger proteins with bulky side chains or charged groups. In addition, glycine has the advantage of being structurally simplest (its $-R$ group is only a hydrogen atom). At the same time, the protonation state of the structure can be pH-dependent in polypeptides.

In a similar study, Nandi and Robinson⁴¹ examined the interaction of different ions—particularly with peptide bonds—using oligopeptides of varying chain lengths instead of tetraglycine and also incorporated other hydrophobic amino acids such as valine and alanine. Regardless of the end groups, both studies showed that weakly hydrated anions (I^- , Br^- , SCN^-) and strongly hydrated cations (Li^+ , Mg^{2+}) were much more likely to bind, consistent with Hofmeister's early observations.⁴¹ Figure 3a shows the structure of ester-capped tetraglycine, 3b displays mongolicin and diglycine, and 3c shows ester-capped polypeptides containing other amino acids.^{42,43}

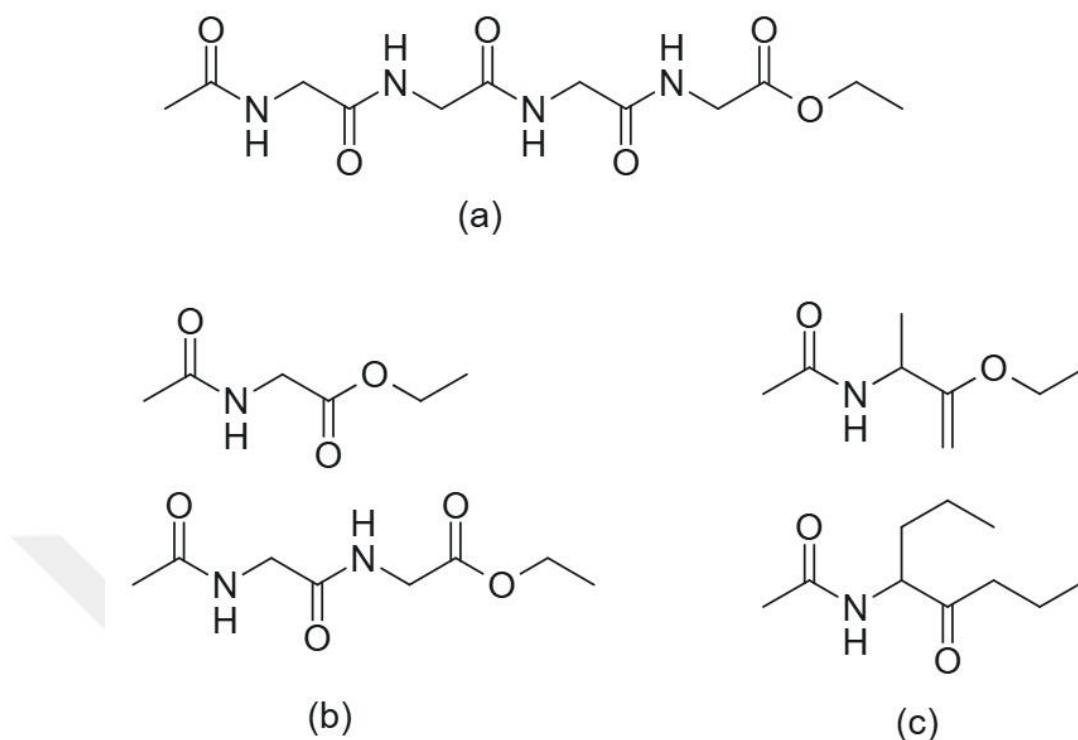


Figure 3: A) Structure of methyl ester capped poly glycine (tetraglycine) B) Structure of ester capped monoglycine and diglycine C) Structure of methyl ester capped amino acids. Methyl groups come from alanine, and propyl are coming from functional group of alanine and norvaline amino acids respectively.

Controlling polymers in different phases has been one of the most extensively studied topics, and many polymers have been developed to respond differently to external stimuli. Responses to external factors such as pH, temperature, and light have led to the use of polymers in areas such as controlled drug delivery systems and soft robotics. One of the important factors in selecting model molecules is their chemical similarity to complex biomolecules such as proteins, and the ability to derive thermodynamic insights is also crucial. At this point, it is necessary to test the universality of ion effects and explain the underlying mechanisms accordingly.

Many macromolecules have been used as models in the literature. PNIPAM (poly(*N* - isopropylacrylamide)) and PDEA (poly(*N*, *N* - diethylacrylamide)) are particularly important as amide-based macromolecules (structures shown in **Figure 4**). These two polymers exhibit thermo-responsive behavior and are among the most widely employed model macromolecules. Their structures differ only in the side chains, yet they can mimic the hydrophobic character of proteins when the side groups are hydrophobic, such as isopropyl or diethyl. In addition to these polymers, more biocompatible macromolecules have also been studied. Examples include polyoxazolines, sugar-based polymers such as hydroxypropyl cellulose and methylcellulose, polyethylene glycol, and one of the most realistic models, elastin-like polypeptides. Many studies have been carried out on these types of polymers, and more detailed mechanistic insights have been obtained by analyzing their thermo-responsive properties through thermodynamic measurements.⁴⁴⁻⁵¹

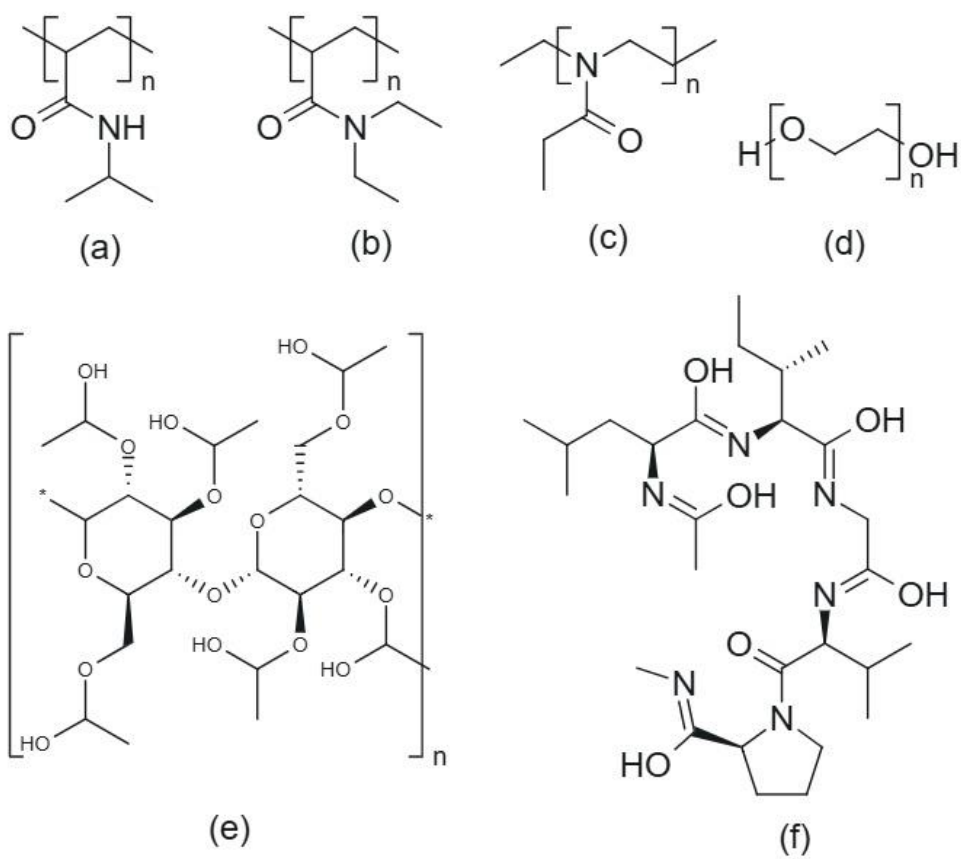


Figure 4: Structure of various thermos-responsive polymers. **A)** Structure of PNIPAM (Poly-N-Isopropyl acrylamide) **B)** Structure of PDEA (Poly-N-Diethyl acrylamide) **C)** Structure of poly (2 -ethyl-2 oxazoline), **D)** Structure of Polyethylene oxide **E)** Structure of Hydroxypropyl Cellulose, **F)** Elastin like polypeptide pentameric unit.

1.2.1 Ion-specific Effect on Thermo-responsive Polymers

The most commonly used polymers among thermos-responsive polymers are PDEA and PNIPAM. The most important feature of these polymers is that they exhibit phase change above a certain temperature in the solution phase. It is possible to observe this phase change macroscopically. Above the temperature called LCST (Lower Critical Solution Temperature) or aggregation temperature, these polymer solutions show a milky appearance. PNIPAM appears to be a clear solution under room

temperature, but when the temperature is around 35 °C, light is scattered as a result of the aggregation of the polymers, and it gives a milky appearance. Additionally, one of the important aspects of this phase change is that the process is reversible. When the solution is cooled again after aggregation, the solution turns to clear again.⁴ Herein, it is possible to compare this phase change to a protein that denatures as the temperature decreases. The difference is that this process occurs as the temperature decreases and is reversible. For this reason, thermo-responsive polymers such as PNIPAM serve as a model that can switch between two phases. Of course, the mentioned LCST temperature is very important to obtain thermodynamic information because this temperature can be controlled and changed using the concentration and the type of the salt. When the LCSTs of PNIPAM are measured as a function of salt concentration, it is possible to see two different behaviors depending on the salt type.⁴ LCST (Lower Critical Solution Temperature) measurements for these two salts on PNIPAM solution in water were conducted with light scattering experiments where the light scattering intensity is measured with increasing temperature. The offset point of the vertical and inclined line is called LCST, and it was measured in different NaI and NaCl solutions in the range of 0 and 1.5 Molar. A detailed explanation of the experiment can be found in the Materials and Methods section. Figure 5 shows the phase change profiles of PNIPAM solutions for NaCl and NaI as a function of concentration.^{18,21} As seen for NaCl, a linear monotonic decrease is observed. This is a characteristic situation for salting-out behavior. On the other hand, for NaI, there is a very different behavior with respect to NaCl. Especially in concentration ranges up to 0.2 molar, a non-linear increase and then a decrease is observed. The non-linear change or the increase of LCST followed by a decrease as a function of salt concentration as in the case of NaI

characterizes as the salting-in behavior.^{18,21,50} As seen here, Na^+ is the common cation and only the counter-anion has changed. When LCST curves are compared, it is clear that I^- ion interacts with PNIPAM in contrast to chloride, and the literature has already shown that it interacts with weakly hydrated ions such as iodide, especially to acidic hydrogens such as alpha carbon on macromolecules.¹⁸

To understand the change in the LCST as a function of concentration and salt type and to interpret the obtained results thermodynamically, an empirical model is introduced. In this model, it is possible to define how LCST changes with concentration utilizing two main terms. The first term represents a linear change and here “ c ” refers to the degree of linear LCST decrease. The c value is tied with the degree of salting-out. The second term is the non-linear term called Langmuir type isotherm. This term is added to model the saturable binding interaction of the ions in salting-in behavior. The important parameter here is the dissociation constant, K_d expresses how strongly the ion is bound to the macromolecule. As an analogy, one can exemplify with IC_{50} used for drugs to inhibit some enzymes in biochemistry. The K_d value can also be expressed as the concentration required to saturate half of the available interaction sites, where the unit of K_d is molarity. Thus, the small value of K_d means that the binding is strong which is a salt-specific parameter.^{4,21,50,52}

$$T_{\text{LCST}} = T_0 + c[M] + \frac{B_{\text{max}}[M]}{K_D + [M]} \quad \text{Equation 1}$$

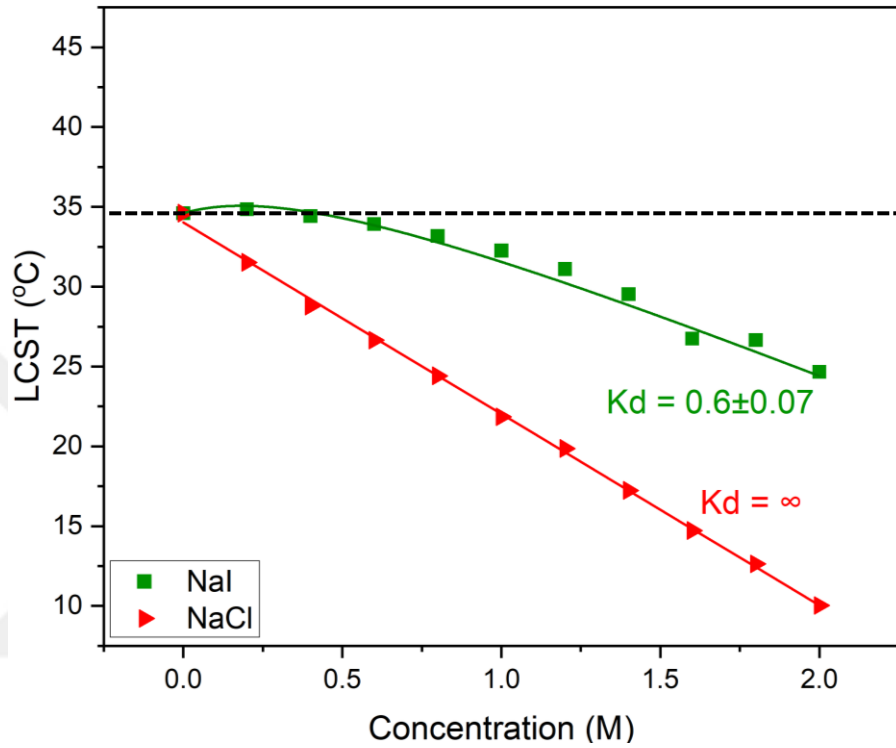


Figure 5: LCST (Lower Critical Solution temperature) measurements with respect to Salt Concentration in range of 0 to 1.5 M. Polymer solutions are 5 mg/ml in water. The green curve is for NaI and red curve for NaCl. Measurements were conducted via Optimelt (Stanford Inc.) in temperature change rate is 1 °C/min. (The figure adapted from ref 18)

The K_d values are inversely proportional to binding strength and in order to calculate this coefficient, when the LCST of macromolecule as a function of salt concentration is plotted as in Figure 5. For example, NaCl shows only a linear monotonic decrease between 0 and 2 molar.^{18,21} There is no non-linearity in any

concentration range. In this case, the non-linear term approaches to a value of 0, and the K_d value approaches to infinity. Of course, there is no chemically infinite molarity, but for NaCl, binding interaction cannot be shown even if the solubility limit is reached. For NaI, on the other hand, a non-linear LCST curve is observed especially between 0 - 1 M, which means that some ions from the salt (in this case, iodide) interact with certain functional groups of PNIPAM. Indeed, it has been shown numerously that iodide ions interact with the alpha carbon hydrogens on the backbone of PNIPAM.^{4,21} NMR experiments performed at similar concentration range show that the chemical shifts of alpha carbon hydrogens, that are adjacent to electron withdrawing functional groups, have a similar curve as in the LCST data.^{18,21}

1.2.2 Other Model Macromolecules: Sugar-Based Polymers, Polyethylene Oxides and Elastin Like Polypeptides.

The Hofmeister series is universal and thus the specific ion effects have been demonstrated on various macromolecules. Herein, the most important factor that plays a role in the solubility of a macromolecule is the ions being present in the solvent, and their distinct effects on macromolecules. No matter how much the structure of the macromolecule alters, the location of the ions in the Hofmeister series remain the dominant factor on the observed effects. In the previous section, we discussed the LCST, or phase transition temperature measurements made specifically for PNIPAM and PDEA and how this temperature changes as a function of salt nature and concentration with an empirical model. Now it is necessary to discuss thermo-responsive polymers and polyamides other than PNIPAM and PDEA.

One of the most important applications of macromolecular modulation is undoubtedly controlled drug release and biosensors.^{53–57} In this direction, the biocompatibility of the polymer plays an important role. Sugar-based polymers are biocompatible polymers and can be used in cancer treatment methods such as phototherapy.⁵⁸ Hydroxypropyl cellulose is a polymer that exhibits a reversible phase transition with an aggregation temperature like PNIPAM or PDEA. Unlike polymers such as PNIPAM and PDEA, the phase transition temperature is around 45 °C and has been tried to be used for the controlled study of photosensitizers by combining with many drugs. Methyl cellulose is also a thermo-responsive polymer like HPC, and its LCST value is very high, and is about 75 °C degrees. Hohenschutz and colleagues extensively studied the interactions of ions with sugar-based polymers using SAXS, H-NMR, and COSY especially on MC cellulose gels.^{46,48} In addition to simple ions, the binding mechanisms related to Kegging structures for sugar-based dissolved polymers have also been studied on HPC (Hydroxy propyl cellulose). Kegging structure and super-chaotropic ions will be discussed in detail in the following sections.⁵⁹

Polyethylene oxide and poly ethylene glycol (PEG) are other biocompatible polymers that show thermo-responsivity according to its different sizes. The aggregation temperature for PEG-300000 is around 90 °C. Cremer and coworkers studied the interactions of PEG polymers of various sizes with weakly hydrated ions such as SCN⁻ and studied the change of the structure of water molecules at the polymer interface with hydration shell spectroscopy as a function of polymer chain length.⁵¹ Deyerle and Zhang studied the change of PEO micellization and critical micellization concentration with Hofmeister Series ions. They showed by turbidimetry experiments

that the change of CMT (Critical micellization temperature) increases especially with weakly hydrated ions, which is coherent with other amide-based polymers LCST behavior in solution phase such as PDEA and PNIPAM.⁶⁰

Among thermoresponsive polymers, one of the most important one is undoubtedly Elastin-like Polypeptides. These polypeptides are used and designed for various applications, especially in tissue engineering. They exhibit reversible phase separation behavior similar to the previously mentioned PNIPAM, PDEA, MC, HPC, and PEG.^{45,61,62} Their general structure can actually be expressed as a repetition of five amino acid units. These five amino acid units are called pentameric units and are repeated in long polypeptides in the form of V-P-G-X-G. Here, V, P and G, refers to valine, proline, and glycine, respectively. Amino acid X is a guest residue and can be different amino acids containing charged, hydrophilic, or hydrophobic groups that can be altered. Naturally, the aggregation temperature varies depending on this amino acid X. Regarding hydrophobicity, using more hydrophobic amino acids at the guest residue reduces the aggregation temperature of ELPs, such as 28 °C for 100% Valine, while decreasing hydrophobicity can increase it to over 40 °C. Additionally, the number of repeats of this pentameric unit, or the length of the polypeptide, are also important for the shape and temperature of phase transition. ELPs can be viewed as a model bridge between polyamides polymers like PNIPAM and PDEA and real proteins. ELPs are also capable of mimicking intrinsically disordered proteins, exhibiting some beta turn and beta structures. Regarding initial studies on ion-specific effects with these ELPs, Zhang and Cho showed that strongly hydrated anions (SO_4^{2-} , Cl^-) reduce the aggregation temperature in a linear, and monotonic manner.⁴⁵ This is characteristic salting-out behavior, also seen for other model molecules.^{18,45,49} For

weakly hydrated anions, the binding interaction was demonstrated through NMR, VSFG, ATR-FTIR, and turbidimetry experiments, and the similar trends were observed for the polypeptides as previously reported polymers such as PDEA and PNIPAM.¹⁸

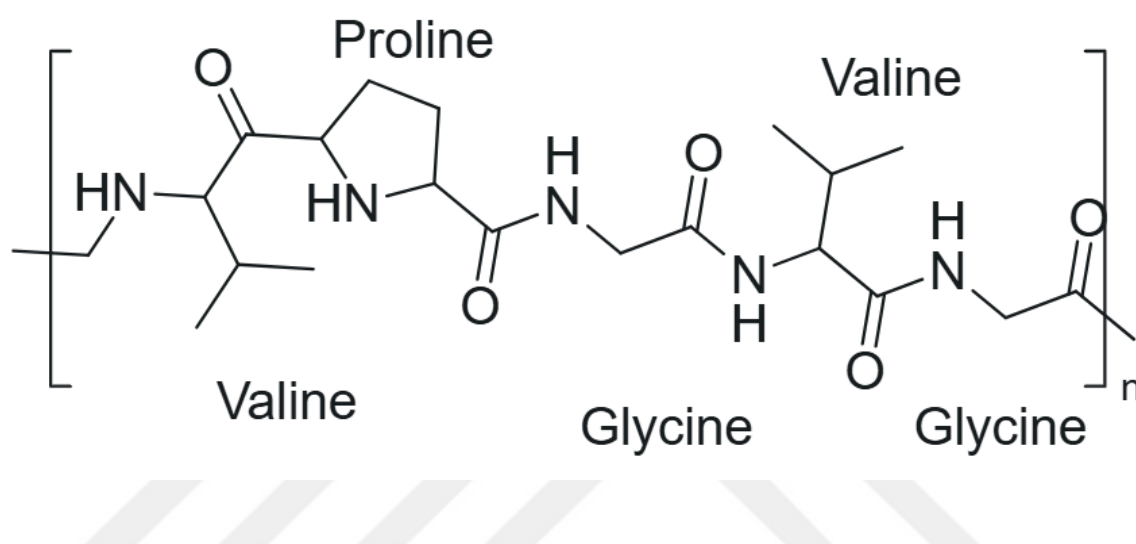


Figure 6: Molecular structure of Elastin-like polypeptide used by Cho and Rembert. Pentameric unit is used as Valine-Proline-Glycine-X-Glycine, (X is any amino acid except for proline) especially in ion-macromolecule interactions studies Cho and Rembert used valine as X residue.⁶³

1.2.3 Anion Dominant Ion Specific Effect, Interaction Sites of Anions

Ion-specific effects have been shown to be universal across many polymers and are widely demonstrated of ion interactions in model macromolecules.^{18,21,44–46,59} Many studies have primarily focused on the weakly hydrated (i.e. SCN^- , I^- , NO_3^-) and strongly hydrated (SO_4^{2-} , F^- , Cl^-) anions, emphasizing that hydration properties are closely linked to the salting-in and salting-out behaviors. Understanding these effects at a site-specific level is crucial. In particular, it is necessary to determine where

interactions occur in different polymers by focusing on anion-specific effects and how these interactions depend on polymer length, charge, and functional groups, as reported in the literature.^{18,21,44–46,59}

Although Hofmeister initially proposed a series for cations, subsequent spectroscopic and computational studies have demonstrated that salt effects are generally dominated by anions. Consequently, many investigations have used the anion as a variable to generalize and illustrate salt effects. Weakly hydrated anions such as SCN^- , NO_3^- , and I^- , discussed in earlier chapters, can interact with macromolecules and induce charges even on neutral polymers. These ions are referred to as salting-in or chaotropic ions.^{64–67}

Thermo-responsive model polymers, particularly PNIPAM, have been extensively employed to study these phenomena. Measurements of the LCST (lower critical solution temperature) or aggregation temperature in the presence of different salts allow researchers to determine whether the polymer exhibits salting-in or salting-out behavior, depending on salt type and concentration. Figure 5 shows LCST profiles for NaCl and NaI: NaCl demonstrates a linear monotonic decrease (salting-out), whereas NaI exhibits a non-linear change (salting-in), particularly at low concentrations. For NaCl, both ions remain excluded from the polymer-water interface, causing the counter cation (Na^+) to act primarily as a spectator. In contrast, NaI shows a general binding trend involving the iodide ion, which is known to significantly enhance macromolecule solubility.

The structure of PNIPAM (see Figure 4a) consists of a backbone with two types of carbon atoms (alpha and beta) attached to the amide group, whose hydrogens are

also designated as alpha and beta. The amide nitrogen is bonded to an isopropyl hydrophobic group, giving rise to four main potential sites of interaction. $^1\text{H-NMR}$, highly sensitive to the chemical environment, can be employed to probe the ion-macromolecule interactions. Salt concentration dependent changes in hydrogen chemical shifts reveal the specific interaction sites for the ions. An empirical model based on the two main fundamental terms of equation 1 can be applied to describe these effects. Changes in the individual chemical shifts can then be analyzed as a function of ion concentration using Equation 2.^{4,18,21,68}

$$\Delta\delta = -c [M] + \frac{\Delta\delta_{\max}[M]}{K_d + [M]} \quad \text{Equation 2}$$

The Concentration-dependent $^1\text{H-NMR}$ chemical shift experiments show that weakly hydrated anions, such as I^- , SCN^- , and NO_3^- , interact mainly with the alpha carbon hydrogens in the PNIPAM backbone. The similarity between the interaction nature obtained from NMR experiments and the K_d derived from the LCST profile in Figure 5 indicates that the counter cation (Na^+) has negligible effect in the case of NaI. From a chemical intuition perspective, one of the most acidic, partially positively charged and weakly hydrated hydrogens in PNIPAM is the alpha carbon hydrogen. Since anions are negatively charged, they preferentially interact with relatively positively polarized hydrogens. It should be noted, however, that this generalization applies only to neutral macromolecules; the presence of other charged groups can lead to dominant charge-charge (Coulombic) interactions.

In addition to experimental observations, computational studies support the interaction of weakly hydrated ions with relatively acidic hydrogens. Okur and

colleagues employed MD simulations to calculate the distribution functions of ions around the PNIPAM backbone.¹⁸ An important advantage of simulations is the ability to separate the distributions of cations and anions. The distribution functions for Na₂SO₄ and NaSCN salts are shown independently. As expected, proximal distributions indicate that SO₄²⁻ and Na⁺ ions do not accumulate significantly around PNIPAM. In contrast, weakly hydrated ions such as SCN⁻ show significant accumulation approximately 0.75 nm from the polymer backbone, which is towards the carbon atoms of the backbone. In addition to proximal distributions, preferential binding constants (K_d) can also be calculated.¹⁸ The study by Okur and colleagues demonstrates that NaSCN exhibits considerably stronger binding to PNIPAM than Na₂SO₄. This observation is consistent with LCST measurements: for NaSCN, the K_d value exceeds the solubility limit, whereas for Na₂SO₄, K_d is approximately 0.22 M, corresponding to the linear monotonic decrease in LCST.

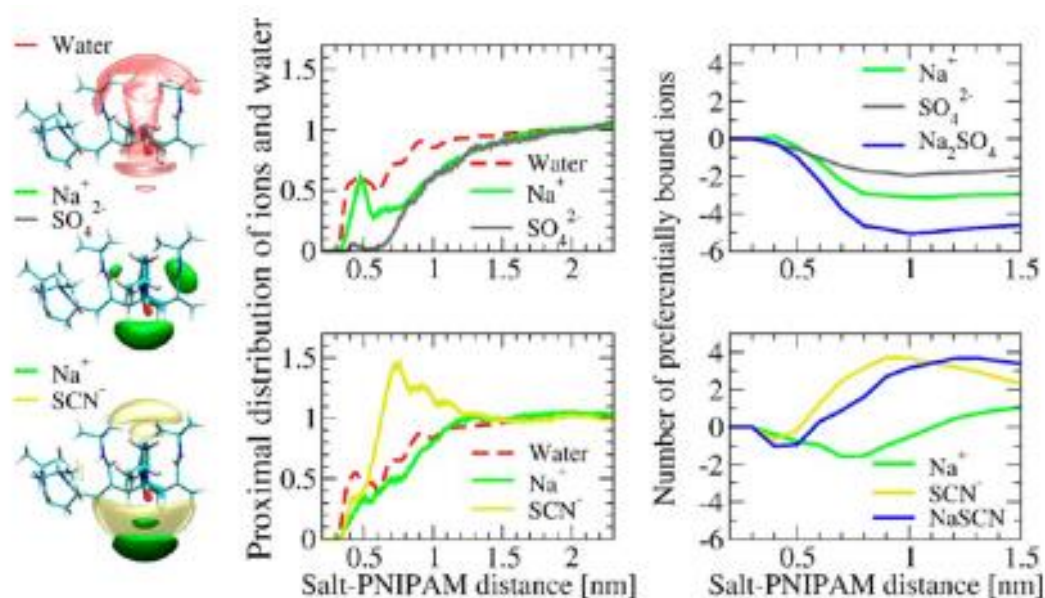


Figure 7: Proximal distribution of ion around PNIPAM chain for Na_2SO_4 and NaSCN . Proximal distribution shows that Na_2SO_4 is depleted from the polymer surface because the distribution function is not above 1 for any distance from the polymer backbone. On the other hand, NaSCN proximal distribution shows dramatic accumulation at 0.75 nm from the backbone, which corresponds to the polymer backbone itself. Preferential binding numbers show that the accumulation of NaSCN is thanks to the binding of SCN^- , not Na^+ because in both salts, preferential binding numbers are negative for Na^+ ion. (Figure is adapted from ref 18).

Other studies have been done on ELP (Elastin-like Polypeptides). They are very important model macromolecules for the transition to the real group structure of ELPs. Structures consist of pentameric repeating units containing V-P-G-X-G (Valine-Proline-Glycine-X-Glycine). Cho et al.⁴⁵ and Rembert et al.⁶³ looked at the conditioning of weakly hydrated anions with ELPs. They used 120 pentameric repeats with ELP and valine as X amino acid. It was seen that chemical shifts of different hydrogens in the ELP structure were examined as a function of salt concentration with ^1H -NMR experiments. They showed that weakly hydrated anions such as SCN^- and I^-

were affected by hydrogen in the structure in Figure 7. As can be seen, these anions do not interact with the isopropyl valine, but with the hydrogens attached to the alpha carbons of proline and glycine and with the amide NH group. When it is looked at the acidity of hydrogens chemically and intuitively, the anionic interaction is with the alpha carbon hydrogens, as in PNIPAM.

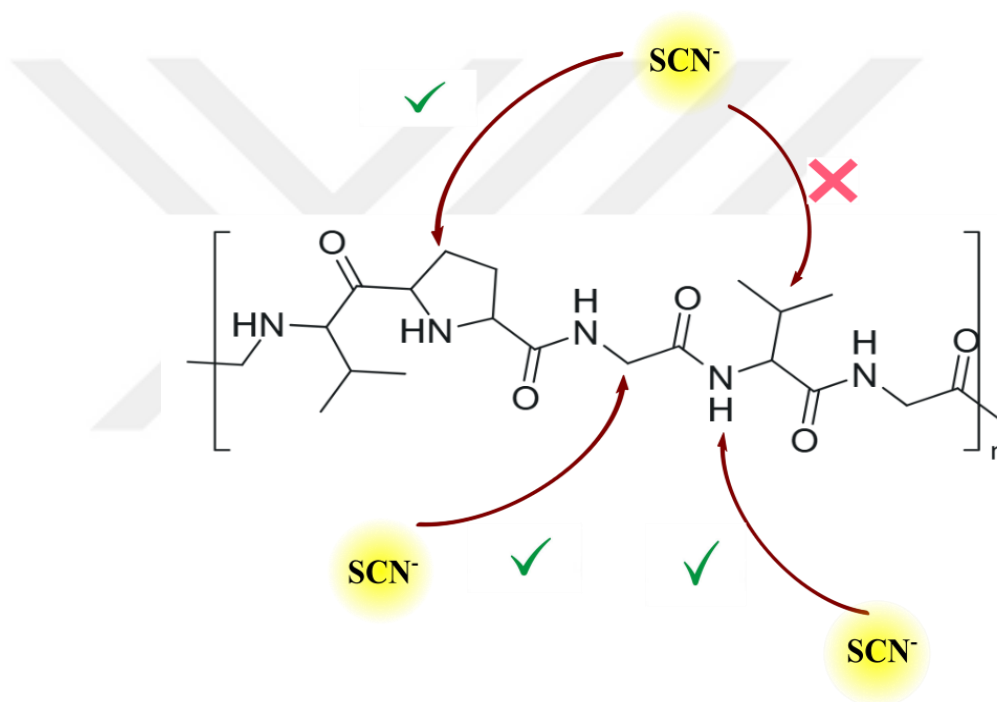


Figure 8: Specific interactions with weakly hydrated ions such as SCN^- for VPGVG-120 ELP (Elastin like polypeptide). It has been shown by NMR that it interacts with relatively acidic hydrogens in the structure. (Figure is inspired from ref 35)

In addition to ELP and polyamides, the effects of weakly hydrated anions on interactions can also be shown on PEO (polyethylene oxide). Okur and his colleagues examined the interactions of different parts of PEO polymer chains of different lengths with weakly hydrated ions such as SCN^- . First, no interaction of SCN^- ion with PEO

monomers, namely ethylene glycol, was observed. Binding of SCN^- ion to a pentameric polyethylene oxide was observed.⁵¹ In addition, another aim of Okur's study was to examine site-specific interactions as in PEO. Since NMR is a technique sensitive to chemical environment, it is possible to separate the hydrogens attached to the carbon in the middle or at the end of the polymer. In this direction, when the interactions of SCN^- ion are examined, it was observed by NMR measurements that the ions interact mostly with the hydrogens in the middle of the polymer for PEO-5. When the water structure around the polymer is examined with MCR-Raman, the intensity of the peak at 3450 cm^{-1} (disordered water) increased and the peak intensity of the peak at 3250 cm^{-1} (ordered water) decreased with increasing polymer length. The most disordered hydrating water molecules here are the water found in the middle of the polymer, while the more ordered hydration water structure was found at the ends of the polymer (termini) chain. When the ^1H -NMR and MCR-Raman results were combined, it was concluded that the disordered hydration water molecules in the middle of the molecules facilitates the ion-macromolecule interaction, while the more-ordered waters around the end groups of the macromolecule makes ion binding unfavorable with higher dehydration penalty of the ions to the polymer. This study demonstrated that even the monomer chemistry is the same in the polymer, differences in hydration within the polymer (end and middle groups) can lead to variations in ion-binding affinities.⁵¹

1.3 Cationic Interactions, Ion Pairing Scenarios and Interaction Mediated Salting-out Behavior

The ion specific effect on different macromolecules such as poly-oxazoline, hydroxypropyl cellulose, amide-based polymer (PNIPAM and PDEA), elastin-like polypeptides (ELPs) and PEG (polyethylene glycol) has been discussed in the previous section. Up to this section, it has generally been shown that the anion specific effect is a more dominant factor than the cation specific effect. Although Hofmeister has shown the cationic series, various studies have shown that the anionic interaction is more effective and dominant than the cationic interaction.^{21,50,51,69} The effect of cationic interactions on different molecules will be discussed. As introduced in the Hofmeister series section, strongly hydrated cations such as Ca^{2+} and Mg^{2+} or Li^{+} are salting-in cations.⁷⁰ On the other hand, weakly hydrated cations such as hydrophobic ions or simple monovalent ions such as Na^{+} or K^{+} are salting-out ions. Different studies used simple cations such as Na^{+} , K^{+} to investigate only the effect of anionic interactions on different molecules.⁷¹ On the other side, divalent cations which carry a higher charge than simple monovalent cations are examined to better understand the cationic binding.

72–75

1.3.1 Weak Interaction of Salting-in Cations

One of the most investigated cations in the Hofmeister context is the Ca^{2+} ion. The first study on its interaction with neutral small molecules was carried out by Okur and coworkers, who investigated the effects of Ca^{2+} together with Na^+ ions on the amide oxygen of deuterated butyramide. Firstly, the solubility limit for NaCl (about 5 M) was examined in relation to the vibrational chemical shift of the carbonyl functional group of amides. In the IR spectroscopy, the amide oxygen peak of deuterated butyramide appeared at $\sim 1620\text{ cm}^{-1}$, and its position did not change significantly upto NaCl concentrations.

In contrast, when the solubility limit of CaCl_2 was reached, the amide oxygen band of deuterated butyramide has an additional band that is blue shifted $\sim 20\text{ cm}^{-1}$. This indicates that simple monovalent ions such as Na^+ do not have an apparent strong interaction with the amide oxygen, whereas divalent cations like Ca^{2+} do interact with relatively negatively charged moieties. When the interaction is compared between cations and weakly hydrated anions, it can be concluded that even strongly hydrated cations such as Ca^{2+} interact much weaker with respect to weakly hydrated anions such as SCN^- or I^- .⁶⁹

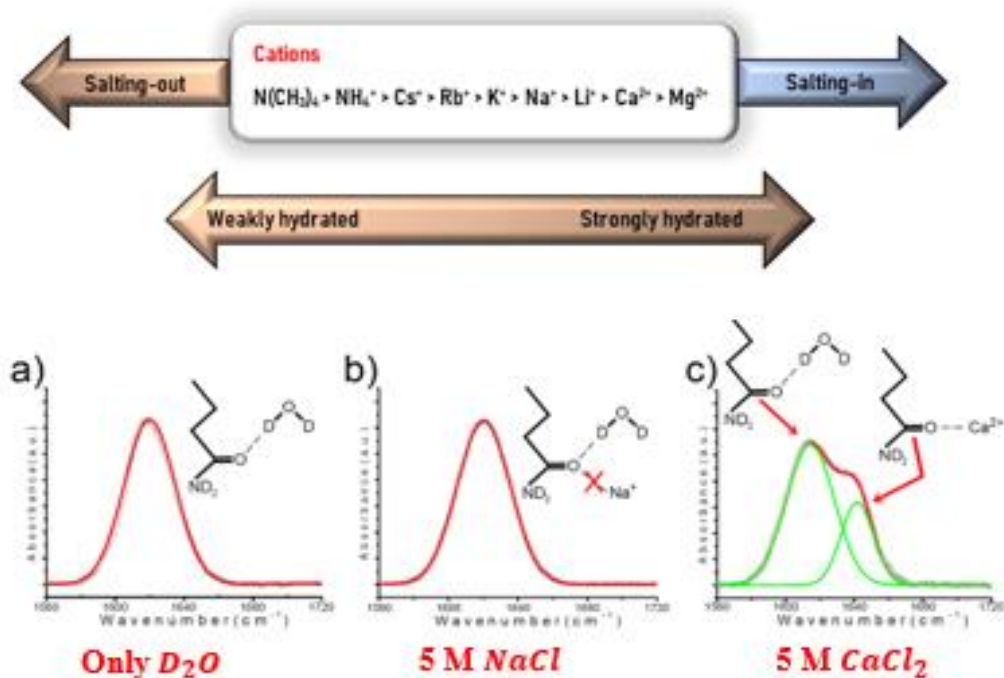


Figure 9: Interaction of Ca^{+2} cation with amide oxygen of deuterated butyryl amide. Ca^{2+} is in the right side of Hofmeister series which means that it is considered as a salting-in ion. A) Shows position of amide stretching at 1630 cm^{-1} in D_2O . B) Show the same region in presence of 5 M of NaCl, solubility of NaCl is about 5.5 M in D_2O . C) It shows a new amide oxygen band in presence of 5 M $CaCl_2$. According to intensity ratio only %30 percent of amide oxygens are interacting with Ca^{2+} ions. (Figure is adapted from ref 68)

1.3.2 Ion Pairing Scenarios

A previous study investigating the interaction of calcium ions was performed on deuterated butyramide, a small molecule model compared to macromolecules.⁶⁹ Bruce and coworkers were the first to study LCST measurements of PNIPAM (poly(N-isopropylacrylamide)) by salting out the counter anion, chloride.

As shown in Figure 6, the LCST profiles for simple ions, excluding Li^+ , Ca^{2+} , and Mg^{2+} , exhibited a direct monotonic linear decrease, whereas for these specific ions,

the decrease was non-linear. Since the empirical model described in Equation 1 was insufficient to capture this new phase transition trends, a non-linear polynomial terms d and f (Equation 3, Figure 10) were incorporated in addition to the linear salting-out term to account for the partial salting-in effect. Molecular dynamics simulations were conducted to determine site specific ion interactions on PNIPAM. The radial distribution function revealed that cations were relatively concentrated at approximately 0.75 nm and 1.25 nm away from the polymer backbone. Consequently, this study demonstrated that salting-in cations weakly interact with the amide oxygen of PNIPAM. These findings clearly show that strongly hydrated cations only interact weakly with the carbonyl groups of macromolecules, while chloride anions cooperate with these cations to form ion pairs at the surface of macromolecules at elevated salt concentrations.⁷⁶

$$T_c = T_0 + ac_s + dc_s + fc_s \quad \text{Equation 3}$$

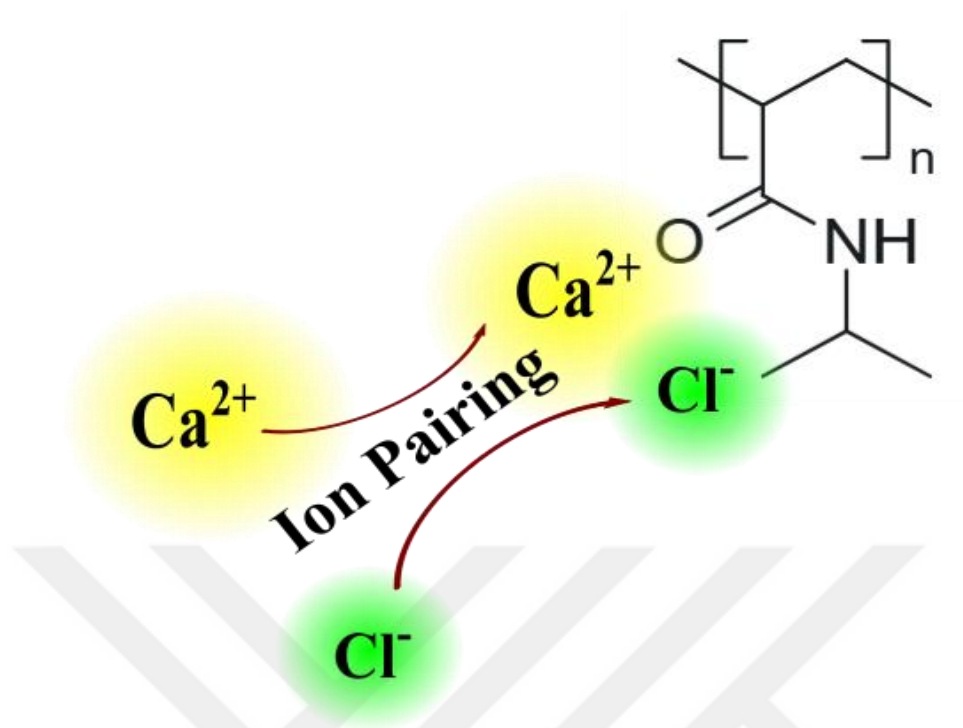


Figure 10: Illustration of Ca^{2+} interaction of amide oxygen of PNIPAM backbone, as it is shown in in all atom MD, chloride also accumulated about 1.25 nm means pendant isopropyl group of PNIPAM, so overall interaction mechanism is explained with solvent separated ion pairing scenario. (Figure is inspired from ref 77)

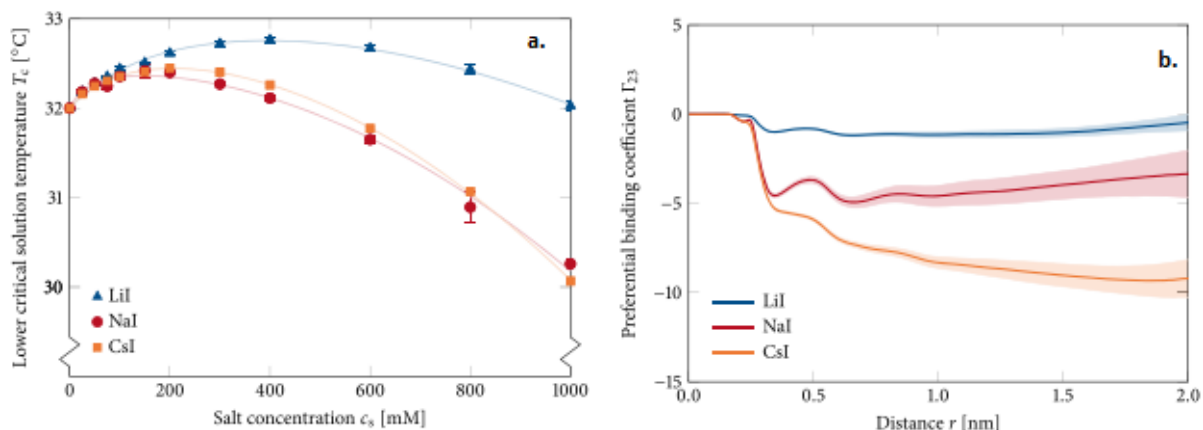


Figure 11: A) In MD simulation chemical species are labeled and in this case 1 is water, 2 polymer and 3 is ions. Preferential binding constants for Iodide salts B) and LCST measurements If Γ_{23} is more than 0, it means there is ion partition, otherwise there is depletion. (The figure is adapted from ref 76)

Iodide salts of different cations were investigated and LCST and preferential binding coefficients are calculated by MD simulation for a system where the counter anion is also surface active, that is I^- rather than Cl^- .^{76,77} In this case, even if anion is a salting-in ion, there is a depletion of the ion from the amide surface. For strongly hydrated cations such as Li^+ , depletion of ion is less than others. According to LCST measurements of these salts, at low concentrations, there is salting-in behavior, but it turns into salting-out as the salt concentration increases. At high concentration, higher salting-out behavior is observed in CsI and again ion pairing scenario is considered, yet this time not at the surface of macromolecules. In the mechanism, Cs^+ ions ion-pairs with I^- ions and overall behavior is depletion both ions from the amide surface. In addition to these, the radial distribution function (RDF) from the polymer surface for cation, anion and (ion-pairs) are determined, and it is seen that RDF intensity is decreasing dramatically, if only anion and salt are compared. As a result, partitioned

I^- ions are paired with Cs^+ ions in the solution and since cation is depleted, anion is also depleted from the macromolecular surface.⁷⁷ To summarize the ion specific mechanism here, although the anion has the energetics to bind to the molecular surface, cations like Cs^+ indirectly pull these anions into the solution and trigger a dominant salting-out behavior. Note that this mechanism is one of the rare examples where cation and anion work cooperatively, and it can be called an ion-pairing in solution mechanism.

No matter how much salt is dissolved in aqueous solution, they do not have to stay apart at the polymer-solvent interface. One important example of this new effect was shown by Bruce in the mixture of sodium sulfate and sodium iodide salts in the presence of PNIPAM.⁷⁸ For a solution of PNIPAM where the concentration of Na_2SO_4 was kept constant at 1 M and NaI concentration gradually increased, LCST measurements showed a decrease in LCST up to 0.2 M of NaI. Although, iodide is a salting-in ion and it can interact with macromolecules, a non-additive behavior of salt mixtures was observed. The vibrational sum frequency generation experiment on PNIPAM monolayers implies that 3200 cm^{-1} (ordered tetrahedrally structured water) peak is increasing, but 3400 cm^{-1} (disordered water molecules) decreased with the addition of NaI. All atom molecular dynamic simulation studies on 20-mer PNIPAM also showed that some Na^+ cations are ion pairing with SO_4^{2-} ions and thus the water molecules around I^- are getting denser, meaning that I^- is more strongly hydrated and it is more difficult for it to interact with PNIPAM. As such, bulk ion pairing scenarios such as CsI and Na_2SO_4 show non-additive ion-specific effects.^{77–79}

Another important macromolecule surface ion pairing scenario was observed in guanidinium thiocyanate salt.^{80–83} Guanidinium is a well-known denaturant. When

the LCSTs of guanidinium salts are examined, the LCST decreases in the counter anion salts, including SCN^- , Cl^- , NO_3^- , and SO_4^{2-} . The surprising effect in this study was the guanidinium salt with SCN^- . World-best denaturant salts-out at low to medium (0.1 – 1.5 M) salt concentrations. ATR-FTIR experiments were performed to examine the collapsed form of the macromolecule to observe the role of the salt ions in the precipitation of ELP. In the case of guanidinium sulfate, there were no ions in the collapsed ELPs for any of salts, which meant that the polymer did not collapse with the salts. In the case of guanidinium thiocyanate, ATR-FTIR experiments showed that the ELP collapsed with both guanidinium and thiocyanate ions. When the same observation was supported with coarse-grained simulations, it was calculated that there were too many ion pairs on the surface of the macromolecule in the guanidinium thiocyanate case.⁸⁴ In addition, the excessive reduction of ion pairs was reflected as a significant increase in the radius of gyration and solubilizing of the polymer as the salt concentration increases. As a result, two important conclusions can be drawn. First, ions can interact as ion pairs, and if there is an interaction salting-in behavior is definitely not necessary to be observed. Secondly, when ion pairs are considered as cations and anions, they do not add any net charge on the surface and therefore do not increase the interaction with the solvent; can lead to salting-out behavior as illustrated in Figure 12.

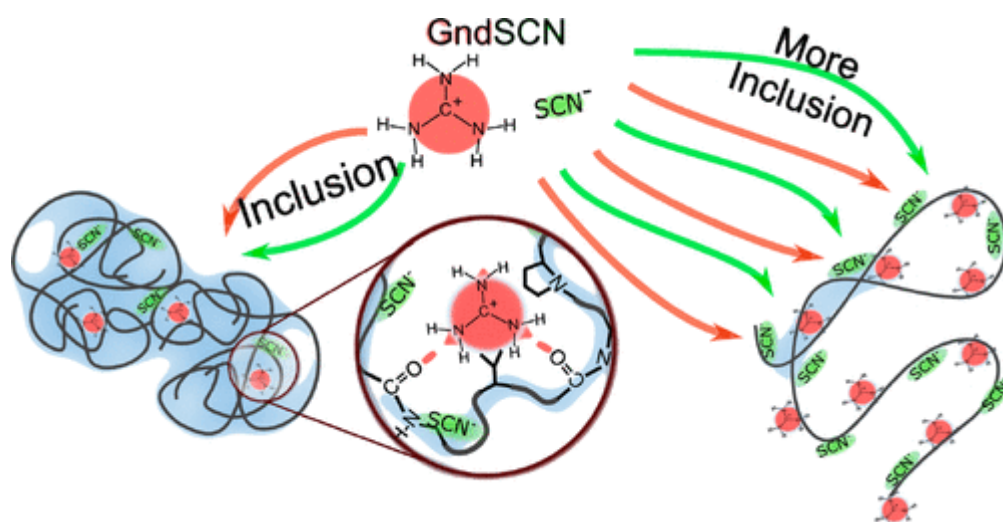


Figure 12: Mechanism for Salting-out behavior of GndSCN salt on ELPs. Both the cation and anion undergo inclusion in collapsed state of ELP, since overall net charge will be close to zero and solvent macromolecule interaction will not be enhanced, overall behavior is salting-out instead of salting-in. Therefore, interaction of ion can induce salting-out as well. (The figure is adapted from ref 84).

1.4 New Ions to Hofmeister Series: Bulky and Nanoscopic Ions

The ions discussed in the Hofmeister series so far were mainly simple, mostly monoatomic salt ions. The binding mechanisms of weakly hydrated anions and the weak interactions of cations have been demonstrated. In this section, the focus is on the extremes of the newly studied ions, namely the extremely weakly hydrated cations and anions to complement the shortcomings of the extremes of the Hofmeister series in the synthesis of new inorganic complex salts and the modulation of macromolecules. Hydrophobic ions are ions with many hydrophobic groups around the center, which compensate for their interactions with water only by their excess

surface area. Even if the individual interactions with water are weak in number, they can disrupt the tetrahedral structure of water molecules. In this way, the high entropic penalty is paid for their solvation as much as a highly hydrated ion. Since Hofmeister's early work, many ions and molecules have been studied to generalize and drawing a picture of the interactions of ions or osmolytes. With the explanation of the deep meanings of terms such as hydrophobicity^{85,86}, the development of solution thermodynamics and mechanics and experimental methods^{22,23,25,87}, the interest in the extreme parts of the Hofmeister series is also increasing. Contrary to expectations, complex structured ions (such as tetra alkyl ammonium, boron clusters⁸⁸⁻⁹¹, Keggin structured -polyoxometallates,^{46-48,92-96} tetra phenyl borate (TPB) and tetra phenyl phosphonium (TPP))^{97,98} have been observed to interact with very interesting mechanisms and have become promising molecules for various applications in this direction.⁹⁹ These bulk ions do not have to be really hydrophobic feature, in literature bulky and strong water structure breaking ions were shown spectroscopically. Fayer and his colleagues has shown that tetra fluoroborate anion has significant hydrogen bond capability thanks to the presence of four fluorine atoms. The FTIR experiments, showed the water -OH stretching apparently split, indicating different environments for water molecules. Such -OH region splitting in IR measurements also used as a probe for hydrogen bond exchange dynamics between ion-water and water-water. Result of time correlated 2D-IR experiments shows that tetrafluoroborate-water dynamics (9.4 ps) is much slower compared to water-water hydrogen bond exchange (2.2 ps).^{100,101} Tetrafluoroborate-water interaction also reveals new questions on bulky ions even if there is no distinct -OH region splitting. Hydrogen bond dynamics could be getting slower when there is strong interaction with water and also with high

hydrophobic area. In the light of these facts, in this thesis, more extreme parts of Hofmeister series is investigated and the main purpose is to understand how bulky ions interact with macromolecules mechanistically. At this point correlation with hydrogen bond dynamics of water and solvation phenomena will be the main concepts to be addressed.

1.4.1 Tetra Alkyl Ammonium Cations and Their Interactions

It was observed that cations generally do not interact as strongly as the anions, even for the divalent cations. Many studies indicate that cations often act as spectators, although they can participate in interaction mechanisms under exceptional circumstances, such as ion pairing. A striking feature of the mostly utilized cationic Hofmeister series is the absence of very weakly hydrated cations. These ions are typically hydrophobic with very low charge densities. As highlighted in the Hofmeister series, ion hydration plays a crucial role in salting-in or salting-out behaviors. As such, it is reasonable to expect that very large, weakly hydrated cations interact with macromolecules and can induce salting-in behavior.

One original study on this topic focused on the influence of tetraalkylammonium chloride salts on PNIPAM. These cations consist of four hydrophobic groups surrounding a nitrogen center, and their hydrophobicity can be tuned by the length and branching of the alkyl chains. Increasing the alkyl chain length is expected to enhance the hydrophobicity of the cation, and consequently, its effect on macromolecular solubility.

According to the phenomenon of hydrotropism, first described by Neuberg in 1916,¹⁰² a molecule that is not sufficiently hydrophobic to self-aggregate can increase the solubility of other hydrophobic molecules. Tetraalkylammonium cations are less hydrophobic than classical surfactants, yet numerous studies have shown that these quaternary ammonium salts modulate the solubility of many hydrophobic molecules. For example, Saylor first demonstrated that NMe₄Br increased the solubility of benzene and nitrobenzene.¹⁰³ Bockris further showed that by increasing the alkyl chain length, ammonium to tetrabutylammonium salts enhanced the solubility of aromatic acids, with the effect becoming more pronounced as the chain length increased.¹⁰⁴

This salting-in behavior was later observed for many heterocyclic compounds, including aniline and various aromatic acids.^{105–107} Studies on macromolecules revealed that quaternary ammonium salts altered the denaturation temperatures of lysozyme, as measured by differential scanning calorimetry (DSC), with ammonium and tetramethylammonium showing the smallest effect.¹⁰⁸ Similar observations were reported for ribonuclease, where unfolding temperatures decreased in the presence of hydrophobic quaternary ammonium salts.^{13,109–111}

Ertekin and Okur investigated the effects of tetra alkyl ammonium (methyl, ethyl, propyl and butyl) chloride salts on the PNIPAM thermo-responsive polymer and measured the phase transition temperature depending on the concentration. It was expected that the PNIPAM LCST profiles would have a linear monotonic decrease due to the lack of effect of these large ions, but as seen in Figure 13, it was observed that there was a non-linear LCST change, especially as the hydrophobicity increased. A non-interacting chloride anion was utilized with the focus of observing the cationic

interaction. ^1H -NMR measurements on the chemical shifts of the macromolecules were performed. When the chemical shift changes of the PNIPAM peaks were measured with increasing salt concentration, an interaction of the hydrophobic pendant isopropyl groups of PNIPAM was clearly observed. Normally, considering the polar group-charge interactions, it is expected that the hydrophobic cations would interact with the amide oxygen. Yet, the hydrophobic-hydrophobic interaction can also occur and dominates for the hydrophobic tetra alkyl ammonium cations.⁶⁸

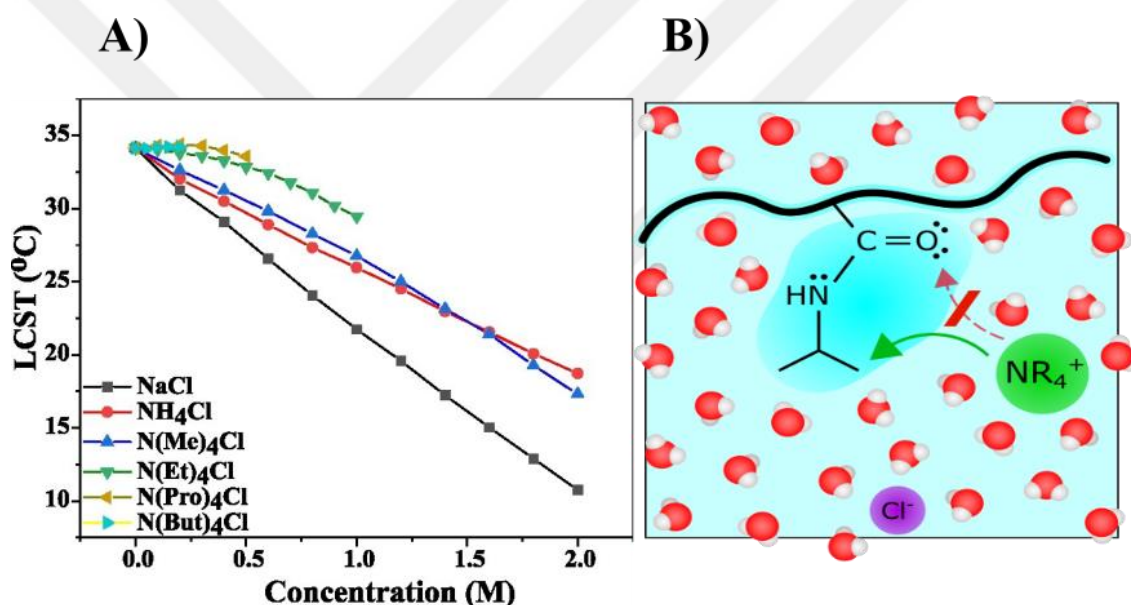


Figure 13: A) LCST measurements of 5mg/ml PNIPAM solutions in aqueous QA (Quaternary ammonium salts) with chloride counter anion. When Hydrophobicity of alkyl chain increases, non-linear change in LCST is increasing and it is a sign of interaction. B) Interaction site of QA cations with PNIPAM pendant isopropyl parts according to NMR chemical shift experiments. (The figure is adapted from ref 68)

1.4.2 Tetraphenyl Phosphonium and Tetraphenyl Borate Ions

In the 1960s, Grunwald put forward the TPA-TPB (tetraphenyl arsonium, and tetraphenyl borate) hypothesis on charge asymmetric bulky ions. As shown in Figure 14, these ions, which are surrounded by four phenyl rings around a positive (phosphonium or arsonium) or negative (borate) center, were used as reference anions and cations because the charge was surrounded by a large insulator.¹¹² Later, the differences in the hydration enthalpies of these two ions refuted the idea of the presence of only insulators around the charge.⁷⁵ These two ions have very important roles in the membrane biophysics. The most important of these is that tetraphenyl-borate can pass through the cell membrane 5 orders of magnitude more easily than the tetraphenyl-arsonium.¹¹³ The adsorption and penetration of such hydrophobic ions on membranes provides information about the charge's behavior at that membrane or the surface. Additionally, the hydrophobic ions can easily pass through mitochondrial membranes. Accordingly, they can be used as drug delivery agents in the treatment of mitochondrial diseases.¹¹³ Miyauchi and his colleagues have conducted many studies on the passive passage of these ions in different lipid bilayer models. Ions such as tetraphenyl-phosphonium (TPP^+) can pass through membranes passively without the use of any passage mediating protein or polypeptides, and this is mainly driven by concentration gradient of the salt ions.

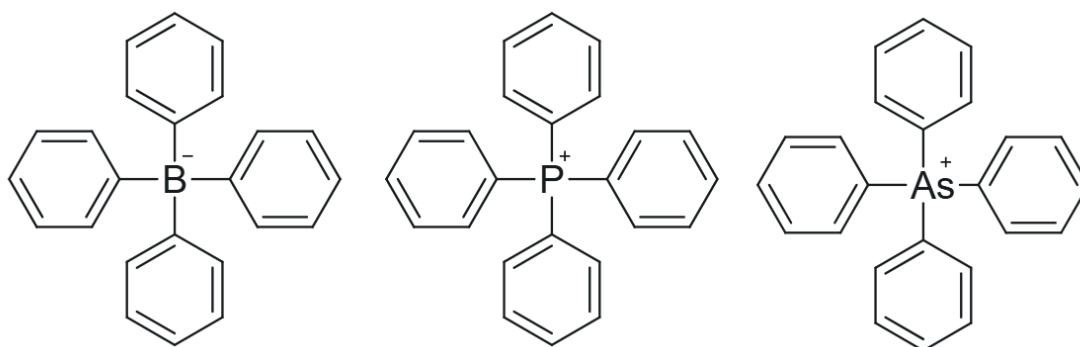


Figure 14: Structure of tetraphenyl ions, borate is negatively charged, phosphonium and arsonium are positively charged ions.

Another important factor here is that an electrochemical gradient is required for the transport of these ions, and this is followed by processed employing ATP in the cell.¹¹⁴ Indeed, when oligomycin ATPase inhibitors are added to HeLa cells, the entry of TPP⁺ decreases.¹¹⁵ Many areas of application have been considered for these ions, and many cellular factors, especially membrane potentials, have been investigated for processes such as drug transport. The fact that TPP⁺ and TPB⁻ ions have opposite charges but are close in charge density or size raises questions about ion-specific effects. It has been discussed numerously and shown with examples of difference between the anionic and cationic effects. These two ions can provide new insight and a new perspective on our understanding on the ion specific effects by only having charge asymmetry. Tetraphenyl borate and arsonium ions can be utilized as two reference ions to understand the effect of charge asymmetry at interfaces. One would first check the surface tension influence of these ions at the air-water interface. As shown in Figure 15, the changes in surface tension are similar as a function of concentration of these two salt ions. Yet, it is, in fact, questionable to employ air-water

interface as a universal model for biosurfaces. Looking at the structures of the ions, the charge at the center affects the electron density on the phenyl groups, and one might expect an altering interaction when these ions interact with water. The NMR data show that the peaks around 7-8 ppm, aromatic rings, are quite different. This is shown to be due to the difference in charge at the center, which can increase or decrease the electron distribution on the phenyl groups. VSFG (Vibrational Sum Frequency Generation) experiments were conducted to determine the extent to which these ions are distributed at the air-water interface and whether charge asymmetry plays a role in surface propensity. As a result, in experiments where salt concentrations were held constant for both ions (tetraphenyl borate (TPB^-) and tetraphenyl arsonium (TPA^+)), the peak intensities from the salts were almost identical when examined separately. When examined together with phase analyses, it can be concluded that the only difference is the orientational direction of the water molecules, as expected in the case of asymmetric charge.¹¹⁶

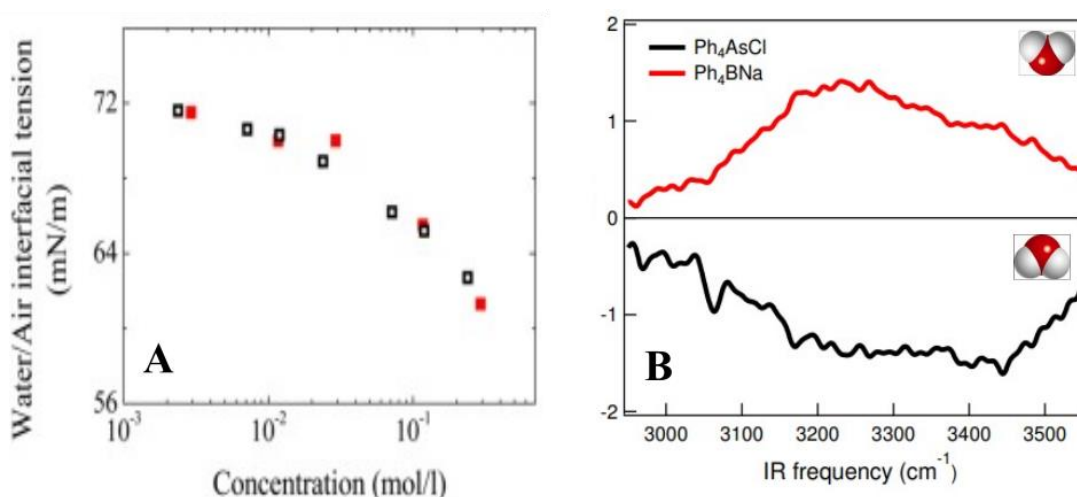


Figure 15: Air-Water Interface propensity investigation for TPA⁺ (Tetraphenyl arsonium) and TPB⁻ (Tetraphenyl borate) A) Air-Water interface surface tension changes with respect to ion concentration counter ions for these salts were Cl⁻ and Na⁺ ions that have interface propensity. Both ions show same decreasing trend in surface tension measurements. B) Sum Frequency Generation Scattering measurements to investigate how TPA⁺ and TPB⁻ are distributed. Peak intensities are the same, but signs are different. The imaginary part of second order susceptibility has imaginary and real part and imaginary part is correlated with orientation of dipoles. (The figure is adapted from ref 116)

Unlike the air-water interface, oil-water interfaces are a system that can better mimic biosurfaces. Here, the VSFG (Vibrational Sum Frequency Generation) experiments revealed significant differences between the ions in the peak intensity and position at the oil/water interface of oil droplets.^{117,118} The reason for the difference is that the relatively hydrophobic ion can migrate to the nonpolar phase but cannot do so at the air/water interface. In addition to VSFG experiments, the MCR-Raman method was applied to investigate the hydration properties of tetraphenyl arsonium (TPA⁺) and tetraphenyl borate (TPB⁻) ions. The hydration water layer

around these bulky ions differ from the bulk water can be separated, and it could be associated with interfacial or hydration water spectrum.^{31,119–123} Previously, it was demonstrated that the interaction between the phenyl group and water was investigated using simple aromatic molecules, such as benzene and phenol.^{121,122,124,125} The interaction between the phenyl group and water was demonstrated, showing that the water-OH group interacts with the phenyl delocalized electrons, similar to ion- π interactions. For the phenol molecule, the stretching of the dangling -OH group was determined to be $\sim 3610\text{ cm}^{-1}$. For different aromatic molecules with electron-donating and withdrawing groups, this value should be altered via red or blue shifting.¹²³ In light of the interaction of phenol or benzene with water. Same dangling -OH determination can be held for tetraphenyl-borate and tetraphenyl-arsonium. The electron density difference because of the central change should play a role in the water interaction. Thus, the MCR-Raman method was utilized to find dangling-OH stretching band. Tetraphenyl borate ions have a more electron-rich phenyl group, so interaction with water-OH is more stronger and thus red-shifted to 3578 cm^{-1} compared to the one for tetraphenyl arsonium, that is blue shifted to 3641 cm^{-1} .^{31,97,126}

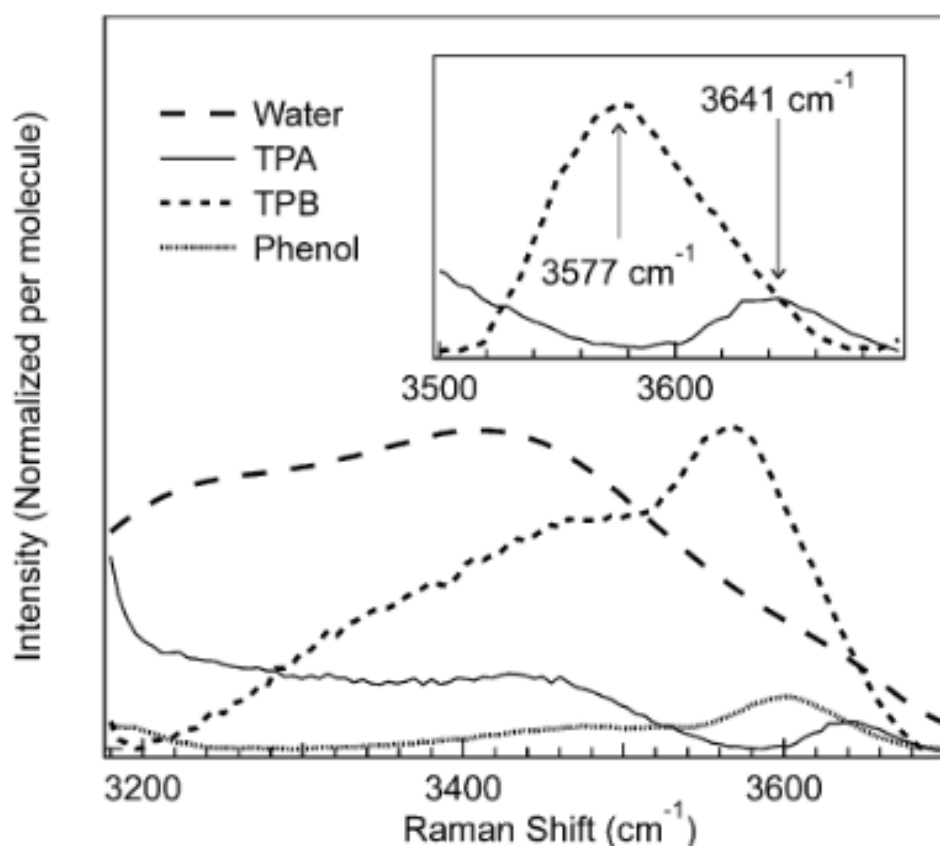


Figure 16: MCR (Multivariate Curve Resolution)-Raman Spectra of Water, TPA+ (Tetraphenyl arsonium), TPB-(Tetraphenyl borate) and Phenol in water -OH stretching region. Dangling -OH values were 3641 and 3577 cm^{-1} respectively. Phenol -OH stretching is between the ions. Negative charged center is better electron donating than phenol hydroxyl group. Therefore, interaction with water is more prominent in TPB- than phenol. Tetraphenyl arsonium has positively charged center, so electrons could from phenyl groups are attracted and interaction with water is diminished. (The figure is adapted from ref 97)

1.5 Motivation and Aim of The Study

The interactions of tetra alkyl ammonium cations demonstrate that anions do not always dominate the Hofmeister ion series. The interactions of these large cations with the hydrophobic portions of the polymer on the PNIPAM model molecule were demonstrated site-specifically by NMR experiments. This point led to the conclusion that the weakly hydrated ions on the left side of the Hofmeister ion series, particularly those related to the cation, i.e. hydrophobic cations, are actually salting-in. This suggests that only monovalent cations such as Na^+ and K^+ are salting-out, and that both extremes of the cation series have a salting-in action. The fact that large cations interact as strongly as weakly hydrated anions has raised many other questions. When discussing specific ion effects, we refer to both additive and non-additive behaviors. Although literature examples address the dominant behavior of anions, the interactions of greasy cations and weakly hydrated (salting-in) anions together are also of interest in this thesis. Therefore, this thesis aimed to further investigate tetraalkylammonium chloride salts by using counter anions such as Iodide and nitrate, which are expected to be spectator anions as chloride and have been repeatedly shown to interact with the surface of macromolecules. The primary objective was to mechanistically and site-specifically investigate how hydrophobic cations interact with salting-in anions and how they affect the behavior of macromolecules in solutions. In addition to tetra alkyl ammonium ions, the thesis also aimed to investigate the interaction mechanisms of tetraphenyl-borate (TPB^-) and tetraphenyl phosphonium (TPP^+) ions, which have been used as reference ions where significant asymmetry has been observed. In this context, in addition to polyamide-based polymers such as PDEA and PNIPAM, ELP (Elastin-

like Polypeptide) has been used as a better model for intrinsically dissociated polypeptides. Mechanistic studies, particularly LCST measurements, were conducted using ATR-FTIR, NMR, and hydration shell spectroscopy (MCR-Raman) techniques to generalize the fundamental mechanism of large hydrophobic ions. In particular, MCR-Raman provided a fundamental basis for the investigation of charge-asymmetry-rich TPP^+ and TPB^- ions and their interaction mechanisms. It was demonstrated spectroscopically for the first time that ion hydration is directly dictating the salting-in and salting-out behaviors of salts on neutral macromolecules.

Chapter 2- Materials and Methods

In the chapter information on the methods and materials utilized in experiments are provided. The basic properties of the chemicals used, the lyophilization procedure used for long-term storage and handling of polymer samples, LCST (Lower Critical Solution Temperature), ^1H -NMR, ATR-FTIR measurements, along with hydration shell spectroscopy (MCR-Raman measurements, a unique method for probing waters of hydration and obtaining information about solutions), will be described in detail.

2.1 Solution Preparation and General Information about Chemicals

All tetra alkyl ammonium salts, NMe_4I (Tetra methyl ammonium iodide), NEt_4I (Tetra ethyl ammonium iodide), NPr_4I (Tetra propyl ammonium iodide), NBu_4I (Tetra butyl ammonium iodide), NaTPB (Sodium tetra phenyl borate), TPPCl (Tetra phenyl phosphonium chloride) employed were in their pure form, exceeding 99% purity. DSS (-(Trimethylsilyl)-1-propanesulfonic acid sodium salt) having purity of 98%. was used as a reference salt in NMR measurements Ideally, it was prepared at a 2 mM concentration in D_2O and had a purity of 97% (Sigma Aldrich). The stock solutions of the salts were prepared with deionized water, with a resistance of around 18.2 $\text{M}\Omega$. All glass and metal materials used in preparing the stock solutions were soaked in 1% Deconex solution for 24 hours, then rinsed at least ten times with deionized water and dried carefully. Among the polymers used, poly (*N*-isopropyl acrylamide) (PNIPAM)

was obtained by Sigma Aldrich with a molecular weight of 85,000 Daltons and a PDI of 1.5. Poly (N-N diethyl acrylamide) (PDEA) was purchased from Polymer Source Inc. with an average molecular weight of 55,000 Daltons and a PDI of 1.16.

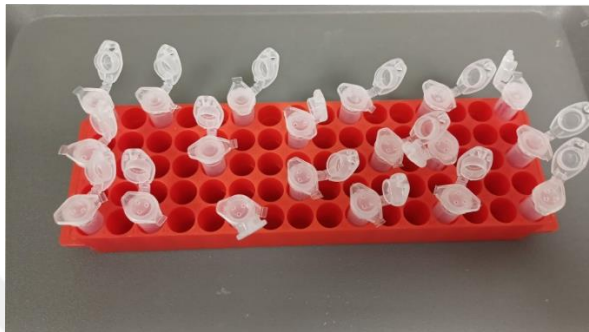
2.2 Lyophilization of Polymer Solutions

In experiments, a small amount of polymer is utilized in order to prepare them in an identical manner for every sample lyophilization procedure was utilized.

The procedure of lyophilization is as follows. The first step is to prepare a stock polymer solution in deionized water. Since PDEA and PNIPAM are soluble in water below the phase transition temperature (LCST, around 30 degrees Celsius), it was kept in the fridge at 4 °C for a day. This waiting time is to ensure polymer solubilization in the stock solution. Based on the experimental requirements, it was decanted in 2 ml Eppendorf tubes. In the LCST measurements 5 mg/ml polymer concentration with a total sample volume of 100 microliters is standard. For NMR samples at least 400 microliters of sample polymer solution were required for a standard sample volume to fill the NMR tubes. In the lyophilization process, after addition of stock polymer solution to the Eppendorf tubes, all samples were dipped into liquid nitrogen and allowed to freeze. When all samples are frozen in liquid nitrogen (in few minutes), they are transferred to a desiccator within a sample rack. The desiccator was vacuumed to a 5×10^{-4} Torr pressure, and all samples were left for at least 24 hours. Here, the main intention was to evaporate water with other volatile impurities. All Eppendorf tubes were covered with another lid with 3 small holes. After 24 hours, all samples are taken from the desiccator, and they are covered with their own lids. These samples

look like dry polymers, and they could be stored even at room temperature for a year. In this way polymer samples were dried, and samples were unified in terms of the amount of material from the stock solution.

A)



B)



Figure 17: Lyophilization process of polymer solutions. Samples are frozen-dried by liquid nitrogen before they are transferred to desiccator. **A)** Samples are capped with an extra lid with three small holes to ensure water evaporation. **B)** Desiccator system connected to vacuum; samples are waited for at least 24 hours.

2.3 Lower Critical Solution Temperature (LCST) Measurements

The three macromolecular models employed in this study, namely PNIPAM, PDEA, and Elastin-like- polypeptides (ELP) that exhibit thermo-responsive behavior. This behavior is the aggregation and phase separation of polymers from the soluble phase above a certain temperature, and this process is fully reversible. This phase transition temperature is known as the Lower Critical Solution Temperature. Light scattering measurements are performed to measure the LCST values, but before this, the desired solution must be added to previously prepared dried or lyophilized polymer samples. The desired salt solutions were usually added to the lyophilized dry polymer samples to maintain a final polymer concentration of 5 mg/ml. The amount of solutions added was usually around 100 microliters. After the aqueous solution was introduced, the Eppendorf tubes were thoroughly mixed using a vortex and refrigerated at 4°C for at least 24 hours to ensure the polymer is completely dissolved in the solution. The Optimelt MPA100 (Stanford Research Systems) is a device used for measuring the melting points of salts. The basic operating principle of this device is to measure dramatic changes in light scattering intensity by increasing the temperature at a specific rate. Pre-prepared samples are divided equally into three one-side-open capillary tubes and placed in the sample slots within the Optimelt (Stanford Inc). The Optimelt is connected to a computer, and experimental parameters can be customized with the MeltView 2.0.7 software. Images from the device's camera can also be observed in real time using the MeltView application. Temperature ranges are generally set wide, from 15 to 65 degrees Celsius. This temperature range can be

adjusted before each sample is measured, depending on the salt's effect and concentration. The temperature ramp rate is kept at 1 degree per minute.

During the measurement range, the camera collects information about light scattering intensity. When the LCST temperature was reached for the macromolecule, the light scattering intensity increases dramatically as the macromolecule aggregates. Since the sample is divided into three capillary tubes, there are three data points for each sample, and the average, and standard deviation of the data points are reported. The LCST point is set to onset of the intensity increase. Figure 18 shows the setup and a generic measurement performed on the software. LCST data are typically measured as a set for a single polymer and are measured as a function of salt or osmolyte concentration. The LCST values and their salt concentration dependent increase followed by a subsequent decrease, or the direct monotonic linear decrease, provide information about the fundamental influence of salt ions or osmolyte on the macromolecule. The thermodynamic model fitted for salting-in and salting-out behaviors is discussed in detail in the introduction section.

All data fitting is performed in Origin software. In the non-linear fit section of the fitting settings, the desired function and the parameters are defined on Origin. As in Equation 1, parameters such as K_d , B_{max} , are the variables where their values are known by data fitting. T_0 is defined when the LCST value of the polymer used for LCST is known without any salt, i.e. in deionized water. The function was iterated until the R^2 values were minimized, and the chemically informative apparent binding constant K_d and salt-out factor c are calculated and reported.

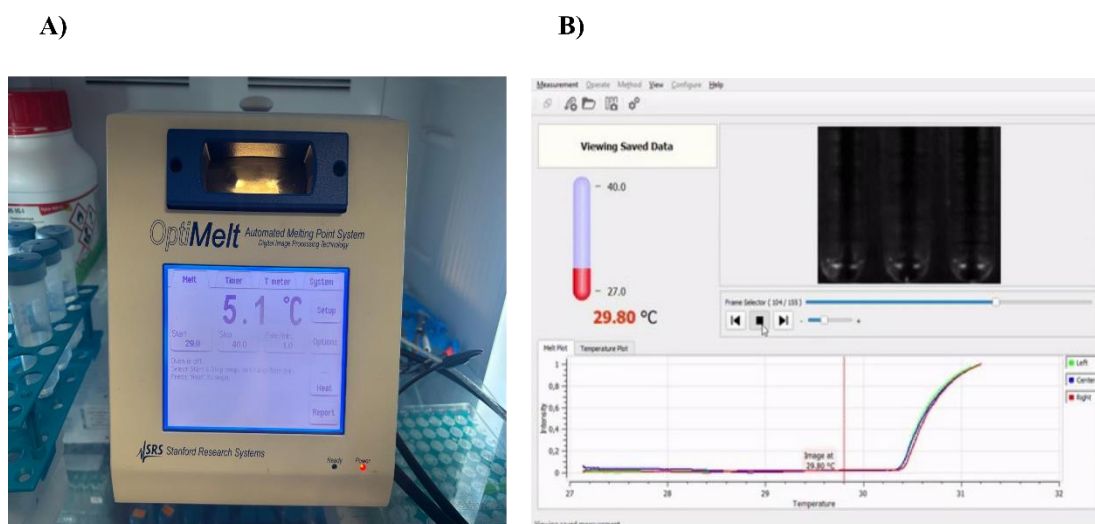


Figure 18: LCST (Lower Critical Solution Temperature) Measurements A) Optimelt (Stanford Inc.) system. Device is operated in fridge, samples are divided into three capillary tubes and inserted to corresponding cavities (It is enlightened with yellow light) that is imaged with camera. B) Meltview Screen for examining light scattering intensity change with respect to temperature. The Camara measures light scattering intensity simultaneously and it can be observed by software.

2.4 Investigation of the Collapsed State of Macromolecules via ATR-FTIR Measurements

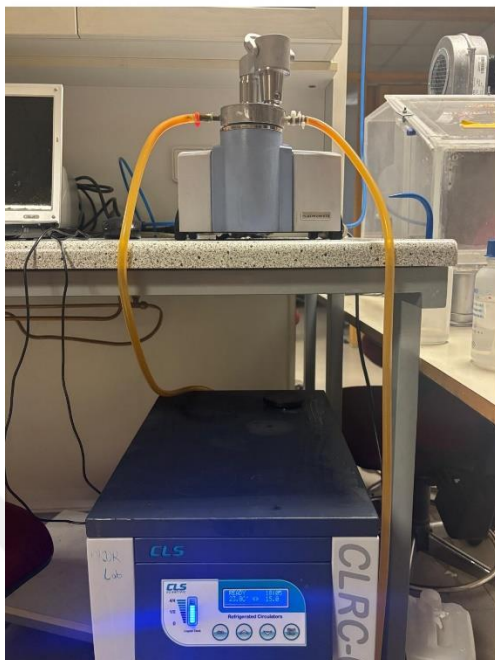
The primary purpose of the ATR-FIR experiments in this study is to investigate the collapsed state of a macromolecule above the phase transition temperature (LCST). They were used to independently explore the interaction site of salting-in ions with macromolecules. As such, if an ion interacts with the macromolecule, its vibrational bands should also be present in the spectrum of the collapsed state macromolecule. The most important factor in these experiments is to measure the macromolecule above its LCST. Therefore, to increase the temperature in the ATR crystal, a perforated iron plate connected to the circulation bath (CLRC-05C, CLS Scientific) is used for the

sample solutions. After starting the circulation bath (CLRS-05C), the temperature is generally adjusted to 10 degrees Celsius above the LCST, and connected to the ATR crystal. The metal plate has maintained at least one hour to allow the temperature to stabilize and to ensure that the ATR crystal temperature is above the LCST. We employed the Bruker Alpha ATR-FTIR setup with monolithic diamond as ATR crystal and tungsten carbide as the infrared light source was utilized for these measurements. The measurements are performed with 2 cm^{-1} resolution with at least 32 scans. Once the temperature is above the LCST of the polymer in the sample solution conditions, the background measurement was taken, and approximately 50 microliters of sample solutions were located on the ATR crystal for the measurements. The polymer concentrations in the samples were 20 mg/ml. When the sample takes on a milky appearance upon contact with the ATR crystal, the temperature is confirmed to be above the LCST, and the measurement is initiated.

Since macromolecular aggregation is a slow kinetic process, and solution evaporation can interfere, these measurements were taken at various time intervals, initial, after 1, 3, 5, and 7 minutes. The primary initiative here is to roughly see the time at which macromolecular aggregation occurs before the solvent is significantly evaporated, and all reported data here are from minute 3 samples.

In ATR-FTIR spectra, the penetration depth varies with wavenumber; therefore, ATR Corrected spectra were implemented. Moreover, as the refractive index change the penetration depth of the evanescent wave also alters for the collapsed polymer samples, its effect is negligible due to relatively low concentration of polymer utilized which is 20 mg/mL. In other words, the sample is mainly consisting of solvent water.

A)



B)

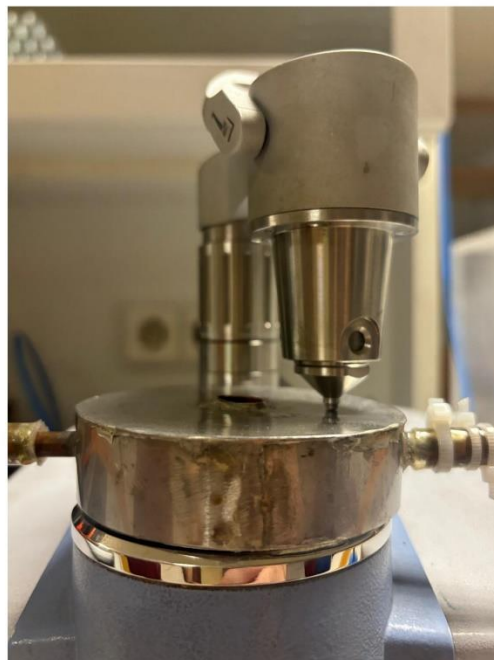


Figure 19: ATR-FTIR measurements for investigation of collapsed state of thermos-responsive macromolecules. A) Circulation bath (CLS) connected to metal plate for providing temperature above LCST of sample. B) Metal plate on ATR-FTIR crystal (Monoclinic Diamond). Throughout the hole in the middle, 50 microliter sample is introduced, and it is examined by time dependent 0,1,3,5 and 7 minutes.

2.5 Investigation of Soluble State of Macromolecules via NMR Measurements

^1H -NMR is used to determine the site-specific binding behavior of macromolecules below the LCST (in the solubilized state).^{4,21,51,52,63,127} A similar application is also found in supramolecular chemistry to understand host-guest chemistry.^{128–130} NMR measurements were conducted in 600 MHz (Agilent Inc.) with about 500 μl samples with 5 mg/ml polymer concentrations. Free Induction Decay (FID) time is 1.703 ms and pulse time is 3.9 microseconds. Wilmad 527-PP-7 NMR tubes with 5 mm OD and 0.38 mm wall thickness were utilized. Reference tube is coaxial reference tube with reference chemical DSS (Sodium trimethylsilylpropanesulfonate 97% purity in D_2O). The polymer samples were prepared from lyophilized form to a final 5 mg/ml concentration to a total of at least 500 microliters. The chemical shifts of the model macromolecules in the soluble state are known in literature.^{49,50} As indicated in the figure, the chemical shift peaks can be easily resolved at a polymer concentration of 5 mg/ml. Having a separate reference tube outside eliminates any possible interaction of the reference molecule used with the desired sample.



Figure 20: NMR samples with tubes and coaxial insert tubes. The main aim is to eliminate effect of reference molecule (DSS), so NMR reference tube is inserted separate tube with same paramagnetic properties.

$$\Delta\delta = -c [M] + \frac{\Delta\delta_{\max}[M]}{K_d + [M]} \quad (\text{Equation 3})$$

NMR analyses were performed using Origin Software, including the positioning of the reference peak position. As illustrated in **figure 21** the chemical shift peaks of the polymers used do not overlap with the DSS reference peaks. Polymer peaks are significantly broader than the reference peaks, and DSS peaks are identified by their splitting patterns and analyzed through chemical shifts. Depending on the salt concentrations used, the chemical shifts of the polymer peaks were analyzed based on their full-width half maximums, and the chemical shift changes were calculated by taking the peak positions when the polymer was in salt only as a reference. The resulting chemical shift changes are modelled with Equation 3 and that is similar to

Equation 1. The resulting concentration-dependent change in the chemical shift peaks were fit and K_d values were calculated for the regions where binding was observed.

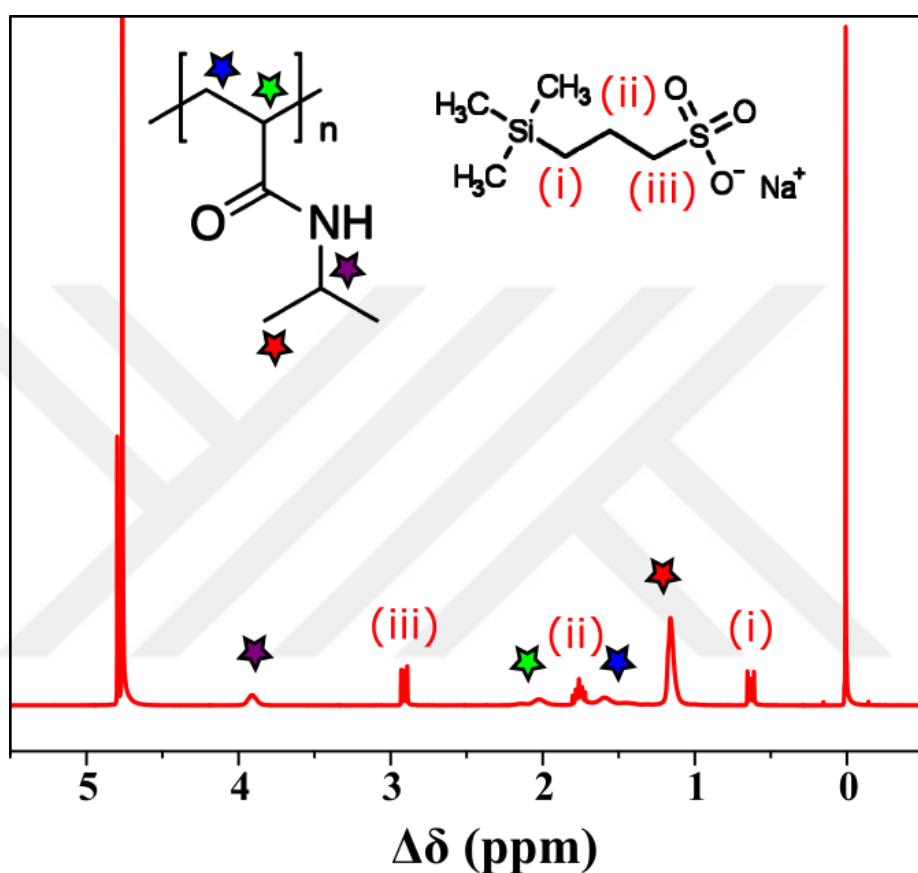


Figure 21: Peak position of one the model macromolecule PNIPAM (poly-*N* - isopropyl acrylamide) in D_2O . Reference molecule DSS peaks are also identified in the region of 0 - 3 ppm. No DSS peaks overlapped with PNIPAM or peaks and it another used model macromolecule i.e. PDEA (poly-*N*-*N*-diethyl acryl amide)

2.6 Hydration Spectroscopy Raman Measurements

Raman Spectroscopy is a vibrational spectroscopy based on light scattering. In addition to characterization of materials, Raman can be a unique method to explore solvation shells adjacent to solute molecules. When the solute and solvent spectra are utilized in conjunction with an alternating least squares algorithm of multivariate curve resolution, a clear vibrational spectrum of the hydration shell is achieved.^{31,119,120} By means of the Alternating Least Squares algorithm, bulk water spectrum can be deconvoluted from the sample spectra, and vibrational fingerprints of the hydration shell can be achieved. Experimentally, a Raman spectroscopy setup with low noise, utilizing continuous laser source with 532 nm (Green Light) at 50mW power. The Fundamental Laser source is a DPPS (Diode Pump Solid State) provided by Ventus with 50 mW power. To reduce the any possible damaging effect of laser light 90 % attenuation was implemented. The Raman optical setup is a Jobin Yuon Horiba with a Andor CCD (Charge Coupled Device) camera. CCD was employed 0.01 electron/pixel/second dark current response provided by thermoelectrically cooled with a Peltier system up to -69 °C. Laser light was directed with a 10 x Objective lens to expose light to the sample. Scattered light was collected with the same objective and directed to a 600 grooves/mm grating to a 1024×256-pixel CCD camera (Andor Inc.). All measurements were conducted with 5-minute integration time and samples were prepared in quartz cuvettes 12.5 × 12.5 × 45 mm dimensions.

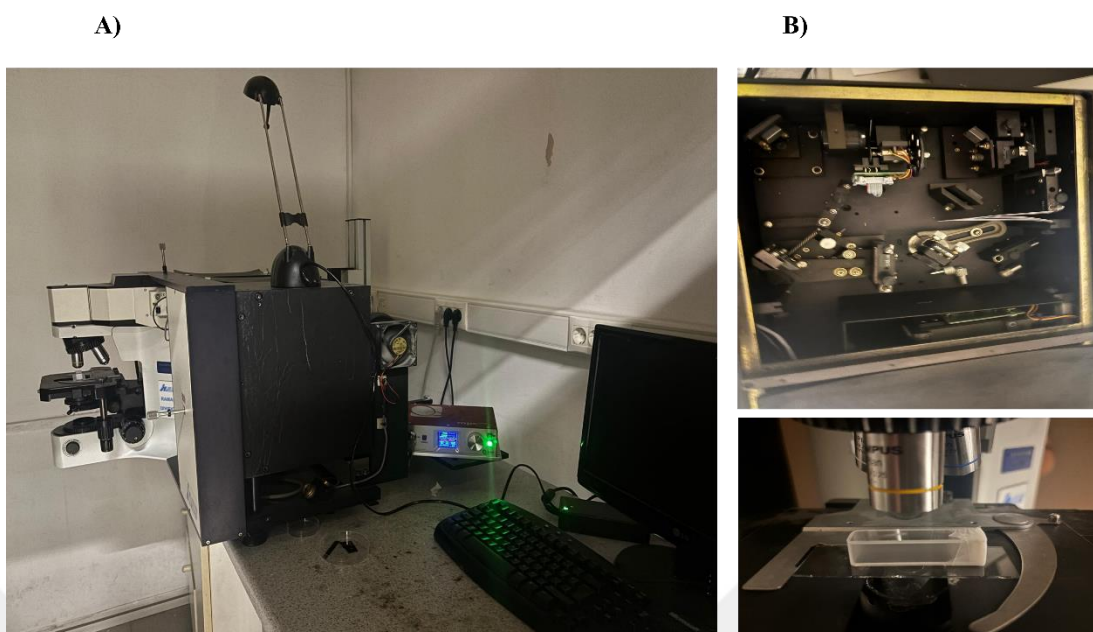


Figure 22: Raman Spectroscopy Measurements for hydration shell investigation. **A)** Raman setup with 532 nm green DPPS (Diode Pumps Solid State Ventus 50 mW power). **B)** Optical Setup (Yuon Horiba with CCD camera) Light is focused with 10x objective lens.

2.6.1 Multivariate Curve Resolution (MCR) Raman Process: Hydration Spectroscopy

Multivariate curve resolution algorithm is an analysis method utilized to calculate the relative contributions of various components in a data set. The SMCR-Raman method is used to distinguish between perturbed water and bulk water in a solution. The spectra of solution samples and pure water are measured using the Raman setup described above. Mathematically speaking, the resulting solution and water spectra can be expressed using the D matrix and this matrix is segmented into three components, i.e. bulk water, solute, and hydration water^{119,122,131,132}. The corresponding equation is:

$$D = S \times C + E \quad \text{Equation 3}$$

In Raman MCR method, D matrix is spectra of two solvents (i.e. Ethanol-Water Mixture), so overall D matrix has several rows and N points (number of points between 400 and 4000 cm^{-1}). In solvation case, there are three main components, and they are basically “Bulk Water”, “Solute” and “Hydration Water”. Contribution factors are represented in C matrices. Lastly, E is a residual error matrix and when it is minimized overall equation is solved in the optimized way.

The overall matrix equation can be extended in a simpler way as follows (Equation 4):

$$D(\text{Pure Water}) = S_{\text{Bulk}} C_{\text{Bulk}}(\text{Pure Water}) + S_{\text{Hydration}} C_{\text{Hydration}}(\text{Pure Water})$$

Here, “Solute” and “Hydration” terms are zero because spectrum is only for pure water. Therefore, overall equation for pure spectrum for solvent is (Equation 5):

$$D(\text{Pure Water}) = S_{\text{Bulk}} C_{\text{Bulk}}(\text{Pure Water}) \text{ and } C_{\text{Bulk}} = 1$$

Conceptually, the main idea is to decompose every spectrum in D matrix to three main components as multiplication of S and C matrices. Since pure water spectrum does not have solute has only bulk contribution. When this bulk contribution subtracted from D matrix only Solution related components remained. The main goal is to subtract bulk water contribution form overall solution.

$$D = \alpha S_{\text{Bulk}}(\text{Pure Water}) + S C_{\text{Hydration}}(\text{Solution}) + \text{Constant}$$

Alpha (α) factor is a number between 0-1 to express bulk water contribution and it is found by iterative algorithm called Alternation Least Square (ALS). Iteration control

and propagation were provided according to chemical and mathematical boundaries. These boundaries are: Any point in solute correlated spectrum cannot be negative and since corresponding solute correlated spectrum is hydration water overall area should be minimized. The “Constant” is for base shift correction for ensuring that pure water and solute correlated (SC) spectra are on the same baseline. Consequently, obtained solute correlated spectrum is acquired by subtraction of maximum amount of bulk water contribution by providing minimized and non-negative spectrum.

First, Raman spectra are plotted using Origin Software, focusing on the spectrum between 2600 and 3800 cm^{-1} . The pure water and solution spectra are normalized to the -OH stretching region. The algorithm is then run with the water sample and the X-axis, respectively, using the Igor software running the MCR algorithm. The algorithm iteratively calculates the spectrum numerically and obtains the solute correlated spectrum (hydration shell spectrum) by minimizing the total area and ensuring that all points are positive. The resulting spectrum is referred to as SC (Solute Correlated) in the literature and in this thesis.

Chapter 3 - Results and Discussion

Weakly hydrated hydrophobic cations (Greasy cations) show apparent binding behavior on neutral macromolecules. For the cationic Hofmeister series, it does not touch weakly hydrated cations, and it was shown that they can bind to macromolecules as strongly as weakly hydrated anions.⁶⁸ Ion-specific effects are generally considered additive. In some cases, different interaction behaviors can be found, and they are associated with a non-additive ion-specific effect. In the first part of this chapter, the greasy cations will be investigated with weakly hydrated (salting-in) anions such as nitrate (NO_3^-) and Iodide (I^-) as a counter anion. In the second part of the chapter, charge asymmetry of greasy I ons is investigated. The influence of tetraphenyl-borate and -phosphonium ions on macromolecules and their interaction mechanisms are presented with their spectroscopic hydration properties.

3.1 Surface Charging Dictates the Phase Behavior of Macromolecules: Non-Additivity of Tetra Alkyl Ammonium Cation with Weakly Hydrated Anions

The influence of tetra alkyl ammonium cation, and the counter anions are whether additive or not, the Iodide salts (a weakly hydrated, surface-active anion) were employed rather than the chloride ones. Indeed, comparison of the LCST curves of PNIPAM with NaCl or NaI reveal a non-linear change for NaI, especially at low concentrations (see figure 5). A good indication for direct anion – macromolecule interaction. Numerous studies on Iodide anion in neutral macromolecules such as PNIPAM and PDEA, have shown that it interacts with the hydrogens bonded to the

alpha carbon positions in the polymer via NMR experiments.^{45,51} More recently, our group studied with the tetra alkylammonium chloride salts and demonstrated that these cations interact with hydrophobic pendant isopropyl groups of PNIPAM.⁶⁸ As such, one would expect an additive ion behavior for alkyl ammonium iodide salts and thus a salting-in effect, as both ions (cation and anion) interact with macromolecule surfaces and tend to partition to the polymer-solvent interfaces in solution.

The first set of experiments conducted at this point was to determine the LCST curves of the model macromolecule PNIPAM as a function of tetra alkylammonium iodide (TAAI) salt concentration. The degree of greasiness of the cation can be controlled by the carbon number of the alkyl chains of the tetra alkyl ammonium cations. A fixed 5 mg/ml polymer solution with varying salts at different concentrations was prepared, and LCST values were measured up to points close to the solubility limits of the salts. Figure 23 shows the LCST curves of PNIPAM. It is first noted that the LCST curve of PNIPAM as a function of NaCl salt decreases up to 1 M concentration and exhibiting a linear, monotonic decrease, and this is often noted as characteristic salting-out action. In contrast, the interaction between macromolecule and NaI, exhibits a non-linear LCST curve. Such nonlinear curve can be modelled with a Langmuir type binding isotherm and represents characteristic salting-in action of salt. Considering salts with increasing hydrophobicity, tetraethyl ammonium iodide (TEAI) has a 1.25 M solubility. With this salt the LCST of PNIPAM increases continuously as the salt concentration increases, as expected for salt where both the cation and anion are surface active. The Tetra propylammonium iodide (TPAI) is a relatively poorly soluble salt (~ 0.5 M), and its LCST curve, like TEAI, shows a continuously increasing trend. Comparing tetraethyl (TEAI) and tetra propyl

ammonium iodide (TPAI) salts, it can be said that the salting-in effect is relatively reduced compared to tetraethyl iodide. The low solubility salts are plotted separately, shown in Figure 23b. These salts are tetramethyl ammonium iodide (TMAI) and tetra butyl ammonium iodide (TBAI). Their solubility limits are 0.15 and 0.05 molar, respectively. TMAI like tetraethyl- and tetra propyl-ammonium iodide salts, exhibits a continuously increasing LCST and a salting-in behavior. The most significant and interesting results in this set of experiments belong to tetra butyl-ammonium iodide (TBAI). one would expect a direct salting-in behavior like the tetraethyl- and tetra propyl-ammonium iodide salts, while the LCST results indicate that tetra butyl-ammonium salt exhibits a salting-out behavior. The LCST profile is linear and monotonically decreasing, similar to the effect of NaCl. The salting-out behavior of NBu₄I, contrary to expectations, can serve as an indication for non-additive behavior.

In the next step after having these phase transition temperature results, is to explore this phenomenon mechanistically and explain how it will apply to different weakly hydrated ions in the presence of wide range of polymers and macromolecules. The most important step at this point is to rationalize this salting-out behavior using spectroscopic methods, primarily ¹H-NMR and ATR-FTIR.

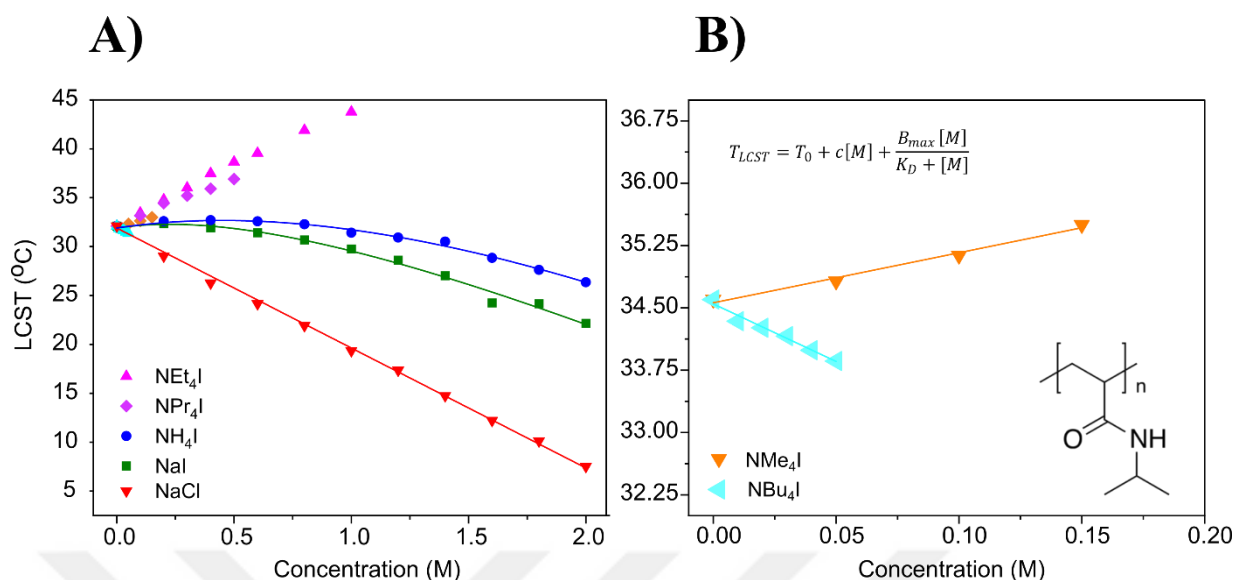


Figure 23: LCST of PNIPAM in presence of Tetra alkyl ammonium iodide solution. A) the graph shows the LCST for NaCl, NaI, NH₄I, NEt₄I and NPr₄I salts and B) the graph shows the NMe₄I and NBut₄I salts. For NMe₄⁺ and NEt₄⁺, NPr₄⁺ the LCST is directly increasing and shows a clear salting-in effect. NBut₄I shows reversed behavior, where a linear monotonic decrease that is associated with salting-out behavior is observed.

To generalize this behavior of tetra alkyl ammonium cations, LCST measurements were also performed with different counter anions. The model macromolecule was kept identical, i.e. PNIPAM, and the transition phase was measured as a function of salt concentrations. The LCST curves were investigated for tetraethyl and tetra butyl cations. As seen in Figure 24A, when examining the LCST curves of the tetraethyl ammonium (TEA⁺) cation for different counter anions (I⁻, F⁻, NO₃⁻, Cl⁻), an increase was observed for the weakly hydrated, surface-active anions, that are iodide and nitrate ions up to 0.6 molar. On the other hand, the salting-out behavior was dominantly observed for the surface inactive anions, i.e. fluoride and chloride anions. When we compare the position of anions in the Hofmeister series with

respect to their salting-in and salting-out actions, these results are expected.^{18,21} Based on these results one can conclude that for tetraethyl ammonium salts, the anion effect is still dominant. The tetraethyl-ammonium cation is not greasy enough to interact as much as tetra butyl-ammonium (TBA^+) salt with the polymer surface, so it is expected that the anion still governs the general phase transition behavior. On the other hand, in Figure 24B, when tetra butyl-ammonium salts were utilized with NO_3^- and I^- anions, a clear decrease was observed, highlighting that the tetra butyl ammonium cation (TBA^+) exhibits salting-out behavior regardless of the weakly hydrated anion present. The reappearance of the salting-out behavior with the nitrate counter anion supports the non-additive behavior of this cation with the surface-active anions.

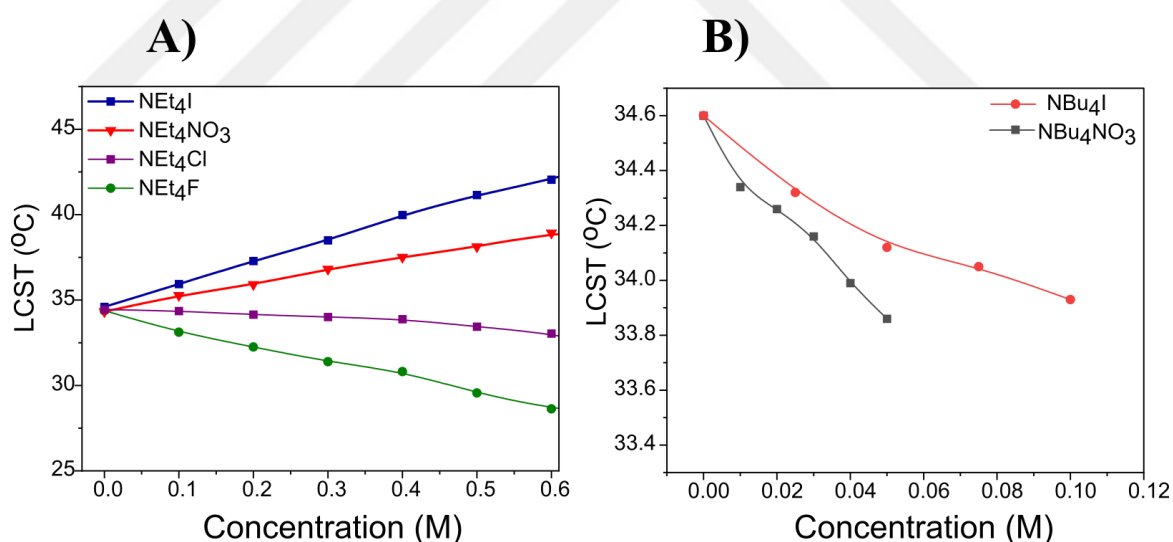


Figure 24: **A)** LCST measurements of PNIPAM in tetraethyl ammonium cation with different counter anion. **B)** LCST measurements of PNIPAM in tetra butyl ammonium cation with different counter anion. The polymer concentration is kept at 5 mg/ml.

3.1.1 Determination of Interaction Sites in Solubilized Macromolecules

To elucidate how tetra alkyl ammonium iodide salts bind to macromolecules, the change in the chemical shift of the polymer peaks were investigated as a function of the salt concentration. As in the LCST measurements, PNIPAM was employed as a model macromolecule. As described in the Materials and Methods section, NMR measurements were performed with an external reference tube with a DSS reference dissolved in D₂O. As shown in the figure 21, four main hydrogen peaks can be identified for PNIPAM: the alpha (2.1 ppm) and beta (1.6 ppm) carbon hydrogens that form the polymer's backbone. Peaks originating from the polymer's hydrophobic side chain can be classified as isopropyl methyl hydrogens (1.2 ppm) and -CH hydrogens (3.9 ppm). These hydrogens and the reference peaks are assigned in figure 21. The interaction side of large cations is shown to be isopropyl group of PNIPAM, and the interaction site of iodide ion is the alpha carbon.^{18,21,45,51} The interaction becomes particularly pronounced as hydrophobicity increases. Both the tetra butyl ammonium cation (TBA⁺) as well as the weakly hydrated anions interact with the macromolecules, but the binding effect can become difficult to detect as hydrophobicity of the cation decreases. However, the LCST measurements with tetraethyl- or tetra propyl-ammonium cations indicate that salting-in interaction occurs when these cations are used instead of TBA⁺. An inference can be made from the ratio of the binding strengths, or K_d values, at these two interaction sites. When the counter anion is iodide, the strongest salting-in effect is observed with TEAI. An unexpected salting-out is observed with TBAI salts, expected behavior was salting-in because both cation and anion are surface active ions, as observed in LCST measurements. These two salts

were selected as two distinct cases among tetra alkyl ammonium iodide (TBAI) salts, and their binding at these interaction sites were investigated. Please note that no significant apparent binding interaction was observed at other chemical sites, which means, the salt dependent change in the chemical shift of the corresponding peak is linear. The equation 2 models the NMR data as a linear and Langmuir binding isotherm, as shown below.

Figure 25 shows the chemical shift changes at the interaction sites of the PNIPAM polymer with the greasy cations and weakly hydrated anion in tetraethyl ammonium iodide (TEAI) and tetra butyl ammonium iodide(TBAI) solutions. Since hydrophobic interactions dominate the greasy cation, their interaction is associated with the isopropyl group hydrogens (shown in figure 25 with black curves), and the alpha carbon hydrogens for the iodide anion (shown in figure 25 with red curves) were demonstrated. A non-linear shift was only observed at the alpha carbon hydrogens in the tetraethyl ammonium iodide salt, see Figure 25d, while a linear monotonic decrease was observed for the tetraethyl ammonium cation binding site. When the interaction is directly linear, the minimum dissociation constant (K_d) is assumed to be at least twice the concentration range.

The ratio of K_d between anions and K_d cation ratio ($K_d(\text{anion})/K_d(\text{cation})$) is a useful scale to demonstrate which ion binding is dominant. For TEAI salt, the ratio has a maximum value of 0.17, which indicates the binding is anion dominated. Figure 25b shows the change in the chemical shift position for the tetra butyl ammonium iodide (TBAI) salt at the same binding sites. Note that the salt concentration scale utilized for TBAI is an order of magnitude more diluted due to salt solubility issues. Here, unlike the TEAI salt, both sites show a clear binding behavior. Interestingly, when the binding

constants for the alpha carbon (anion binding site) are compared for these two salts, the anion binding gets tighter with a significant decrease in the apparent K_d values from 0.20 M (TEAI) to 0.013 (TBAI) M, indicating that the binding of the anion is enhanced when the counter cation is different in the solution.

For tetra butyl ammonium iodide (TBAI), the $K_d(\text{Anion})/K_d(\text{Cation})$ ratio is approximately 1.6, indicating similar binding affinities of the anion and the cation relative to tetraethyl ammonium iodide (TEAI), where the cation binding is slightly tighter. Overall, the NMR experiments provide two important insights. First, as hydrophobicity of the cation increases, cation binding gets significantly tighter, while surface net charging decreases because cation and anion bind affinities reaching similar values. Secondly, anion binding increases in conjunction with cation binding, suggesting a cooperative mechanism between these two ions at the polymer-solvent interface.

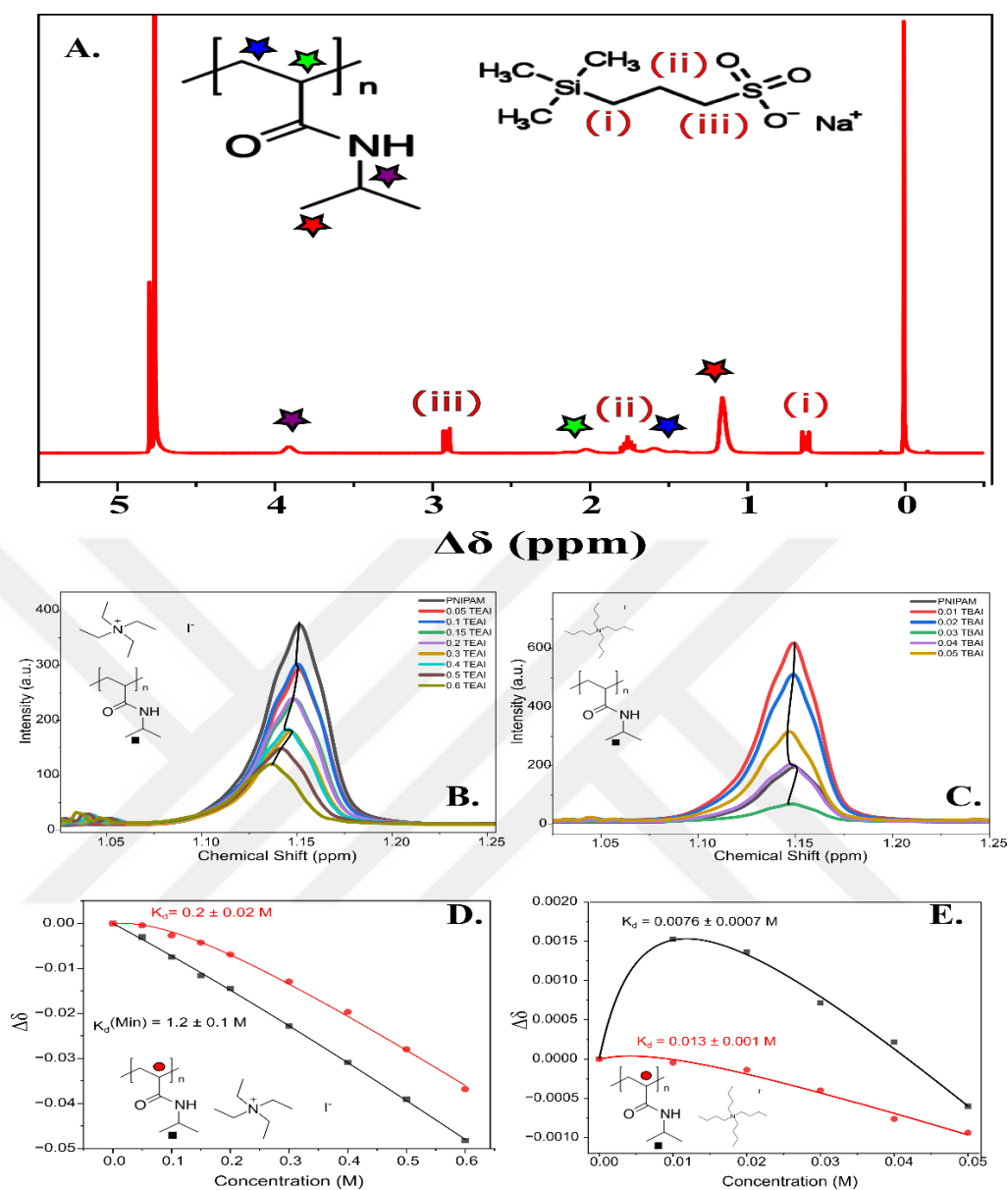


Figure 25: **A)** Solution ^1H -NMR measurements PNIPAM solution. **B)** Isopropyl peak chemical shift changes for TEAI. **C)** Isopropyl peak chemical shift changes for TBAI. **D)** Chemical shift changes with respect to salt concentration in the range of 0-0.6 M for TEAI. Alpha carbon hydrogens peak non-linear change due to binding iodide but there for cation interaction site (isopropyl hydrogens) only linear monotonic decrease has appeared. **E)** TBAI binding curves for alpha carbon and isopropyl hydrogens. Since cation is more hydrophobic, clear binding to isopropyl hydrogen are apparent.

After this step, it is useful to recall the LCST curves, as the non-additive behavior exhibited by tetra butyl ammonium iodide (TBAI) in the LCST curves may actually be explained by testing tetra propyl ammonium iodide (TPAI) salt. Compared to TEAI, the salting-in influence of the salt is less pronounced for the TPAI. The NMR experiments of TPAI will provide an integral piece of the puzzle for the molecular-level mechanism. Figure 26 shows the chemical shift changes of PNIPAM for the isopropyl and alpha carbon hydrogens of TPAI on the PNIPAM macromolecule ranging up to 0.3 M salt concentration. As expected, binding to the iodide was clearly observed. Furthermore, while the binding was not pronounced for the TEA^+ cation, it was pronounced for the tetra propyl cation (TPA^+), but not as pronounced as for tetra butyl ammonium when the K_d value compared. The $K_d(\text{anion})/K_d(\text{anion})$ ratio was found to be approximately 0.5. This ratio is intermediate between the two previous measurements when compared with TEAI and TBAI. This supports the previously mentioned mechanism of charge neutrality at the interface when the cation and anion are bound together. Since tetra propyl ammonium is between tetraethyl- and tetra butyl-ammonium in terms of hydrophobicity, its binding affinity concerning the anion binding is also between TEAI and TBAI

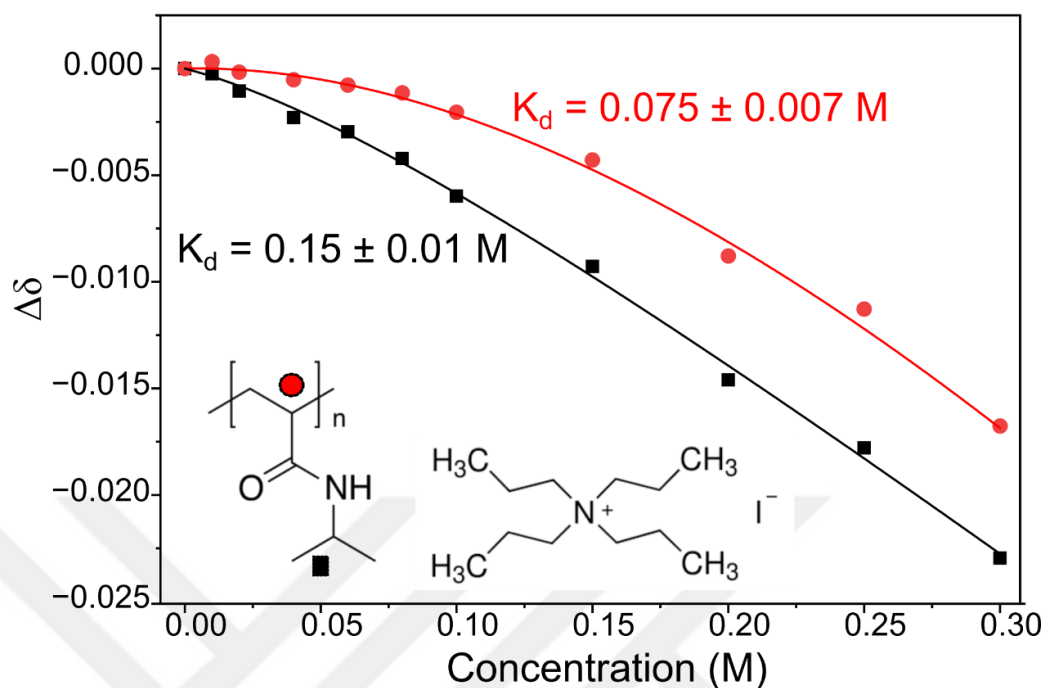


Figure 26: ^1H -NMR measurements for PNIPAM polymer in tetra propyl ammonium iodide solution in concentration range 0 - 0.3 M. TPAI non-linear chemical shift changes are associated with greasy cation interaction. Anion binding is also apparent for iodide interactions. K_d ratio is about 0.5 and it is between K_d ratio of tetraethyl ammonium iodide (TEAI) and tetra butyl ammonium iodide (TBAI).

Based on the NMR results, it is possible to conclude that the expected salting-in salt behavior of TBAI is actually related to the binding affinity of the cation and anion and their relative proportions. To further investigate these findings, in addition to the solubilized state of the macromolecule, another important step is to examine the salt's interactions in the collapsed state above the LCST.

3.1.2 Analysis of the collapsed state of Macromolecules via ATR-FTIR Spectroscopy

The NMR measurements analyze the interactions of ions while the macromolecule is solvated in water, whereas ATR-FTIR measurements can be used to understand the presence of ions in the collapsed state (aggregated) polymers above the phase transition temperature.^{18,68,84} As mentioned in the Materials and Methods section, to ensure the macromolecule is in the collapsed state, the ATR crystal containing the sample is heated with a metal plate, leading the macromolecule solution to phase transition.

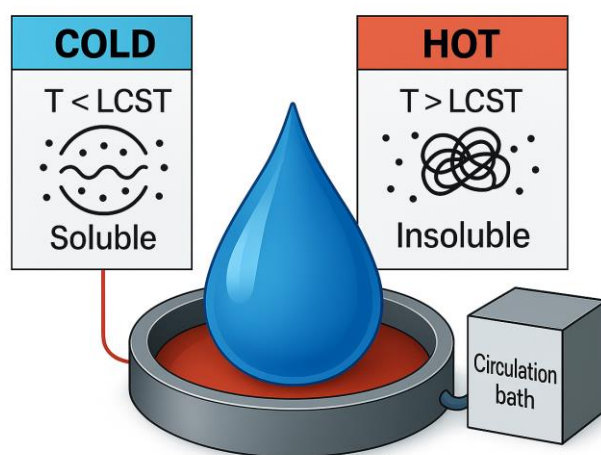


Figure 27: An illustration for ATR-FTIR measurements above LCST of model macromolecule. Above LCST ion can be excluded from or included to collapsed state. Especially, for salting-in ions inclusion of ions are also observed increase in ion peak intensity while polymer is collapsing. However, for excluded volume effect, it is not possible to see change in ion intensity even macromolecules are accumulated on ATR-FTIR crystal.

NMR measurements provide important information about the interactions between the isopropyl and alpha carbons in TEAI. It was observed that the interaction from the TEA⁺ cation was not apparent by NMR, but the iodide interaction was confirmed by the alpha carbon hydrogen chemical shift changes. On the other hand, in TBAI, the interaction with the PNIPAM isopropyl group and the alpha carbon hydrogens were observed. TEA⁺ and TBA⁺ exhibited two distinct behaviors. While the iodide counterion can be used in NMR experiments, this is not useful to utilize in the ATR-FTIR measurements. This is simply due to the presence of no vibrational bands of monoatomic ions. As a consequence, salts of another weakly hydrated anion, nitrate ion (NO₃⁻), can be examined with ATR-FTIR spectroscopy. Since the LCST plots for tetra butyl ammonium nitrate (TBAN) exhibit a non-additive behavior similar to tetra butyl ammonium iodide (TBAI), the nitrate ion is assumed to demonstrate the same unexpected behavior. As described in the Materials and Method section, measurements were performed immediately (0 minutes), and after 1, 3, 5, 7 minutes of sample exposure to heated ATR crystal surface. the measurement after 3 minutes was reported in the thesis. Yet, other time intervals still show the same results. The samples were measured at temperatures above the LCST temperature (experimental temperature was 55 Celsius) , it was ensured by the presence of milky solution on the crystal.

ATR-FTIR measurements were performed with tetraethylammonium nitrate (TEAN) with the model macromolecule PNIPAM, see Figure 28. Tetraethylammonium is seen in the region of 975 - 1025 cm⁻¹, and it is assigned to R-C-N stretching. In the same region, there is no other peak that is overlapped by the vibrational bands of solvent water or PNIPAM. The Peak intensities at 1005 cm⁻¹ show

that there is no significant intensity difference between aqueous salt solution (salt-water) and aqueous salt solution with PNIPAM (salt-water-PNIPAM), and this indicates there is no strong interaction between the cation and polymer. At the same time, there is no ion exclusion from the collapsed polymer. Figure 28b shows a broad nitrate peak between 1325 - 1410 cm^{-1} . A double C-H band at 1387 cm^{-1} , and 1370 cm^{-1} can be seen from PNIPAM. When water-salt and water-salt-polymer systems were compared concerning nitrate peak intensities, it was clearly observed that nitrate bands increased dramatically with the presence of the collapse state of PNIPAM. Such an accumulation of ions in the presence of collapsed PNIPAM is an excellent indication of salting-in behavior.

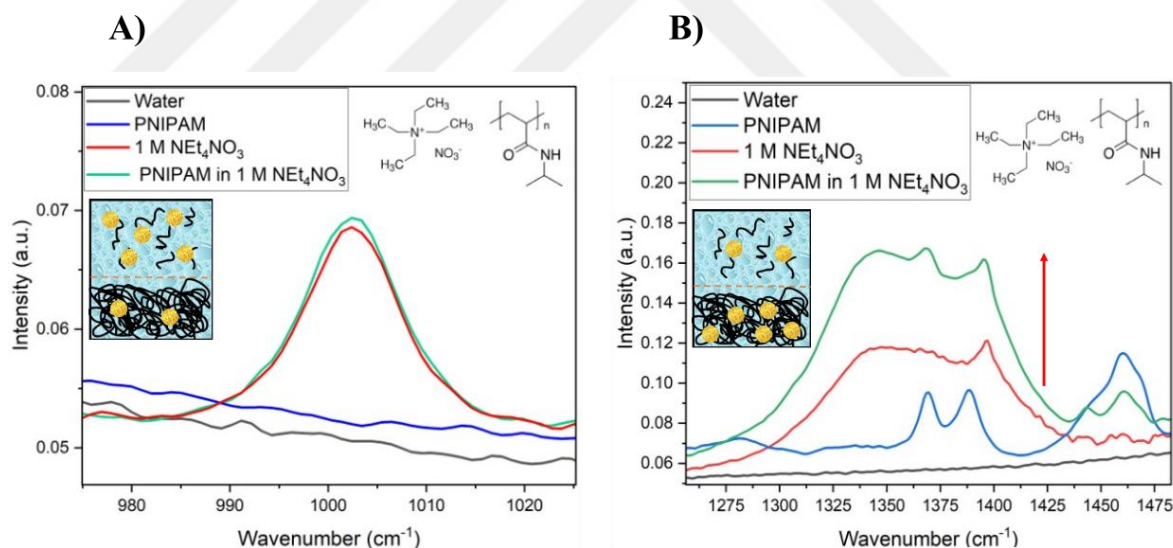


Figure 28: ATR-FTIR measurements above LCST of PNIPAM with 1 M NEt_4NO_3 . A) Peak at 1005 cm^{-1} corresponds R-C-N stretching from NEt_4^+ cation. No significant intensity changes with collapsing of PNIPAM. B) The broad band at 1300–1420 cm^{-1} corresponds to NO_3^- vibrational bands.

To illustrate the interaction mechanism of tetraethyl ammonium cation with weakly hydrated anions, we first observed an increase in the LCST curves of tetraethyl ammonium with weakly hydrated anions, i.e. nitrate and iodide anions. Using a weakly hydrated ion such as iodide or ATR-FTIR experiments (nitrate), the salting-in behavior can be reconstructed by considering the collapsed state. The NMR and ATR-FTIR experiments indicate that the interaction occurs mainly from the anions, and because the cation is not strongly bound, it cannot compensate for the surface charge generated during the ion binding.

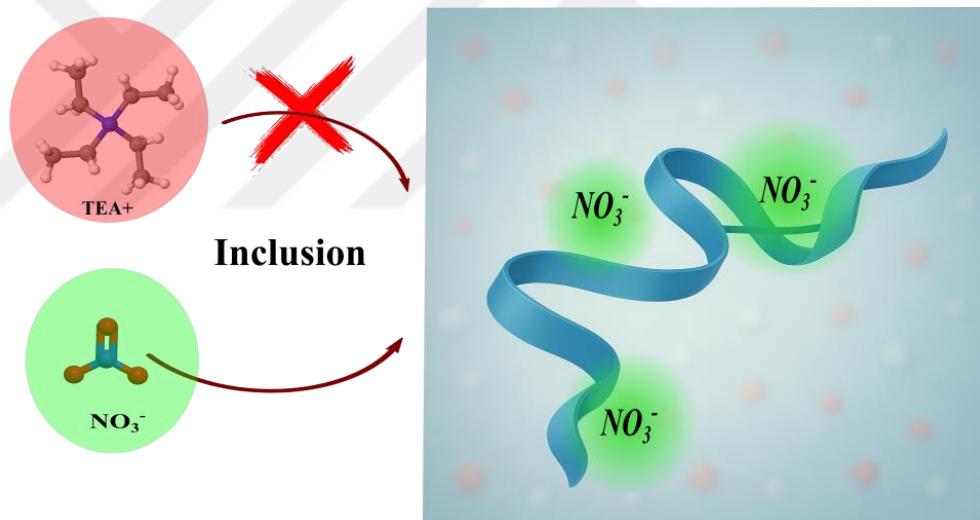


Figure 29: Salting-in mechanism of tetraethyl ammonium nitrate (NEt_4NO_3), the main interaction is dominated by anion and weak cation interaction cannot compensate charging by counter anion. Overall, net charge will be negative and this way, interaction with water will be increased which means solubility of macromolecule increase.

Another important case is the behavior of tetra butyl ammonium nitrate salt in its collapsed state. The same process was repeated for TBAN. The R-C-N stretch peak

frequency of the tetra butyl ammonium cation (TBA^+) is slightly red shifted to around 875 cm^{-1} , as shown in Figure 30a. The factor that complicates this experiment for tetra butyl ammonium is its relatively low solubility where the solubility limit is around 0.1 M. When the R-C-N stretch band overlaps with the liberation modes of water, it is relatively more difficult to observe and analyze the peak. In **figure 30a**, the 0.1 M tetra butyl ammonium chloride cation peak is included for comparison. While the peak is not resolved in the same region for 0.1 M tetra butyl ammonium nitrate (TBAN), the peak starts to appear clearly in the presence of the PNIPAM-water-salt system. In this case, if a peak is not visible in the water-salt mixture but is visible in the water-PNIPAM-salt mixture, this indicates the accumulation and favorable interaction of tetra butyl ammonium cation. In the same figure (b), a nitrate peak can be distinguished around 1325 cm^{-1} . As in the case of tetraethyl ammonium nitrate, the intensities of the nitrate peaks increase as the polymer collapses, which was expected from a weakly hydrated anion.

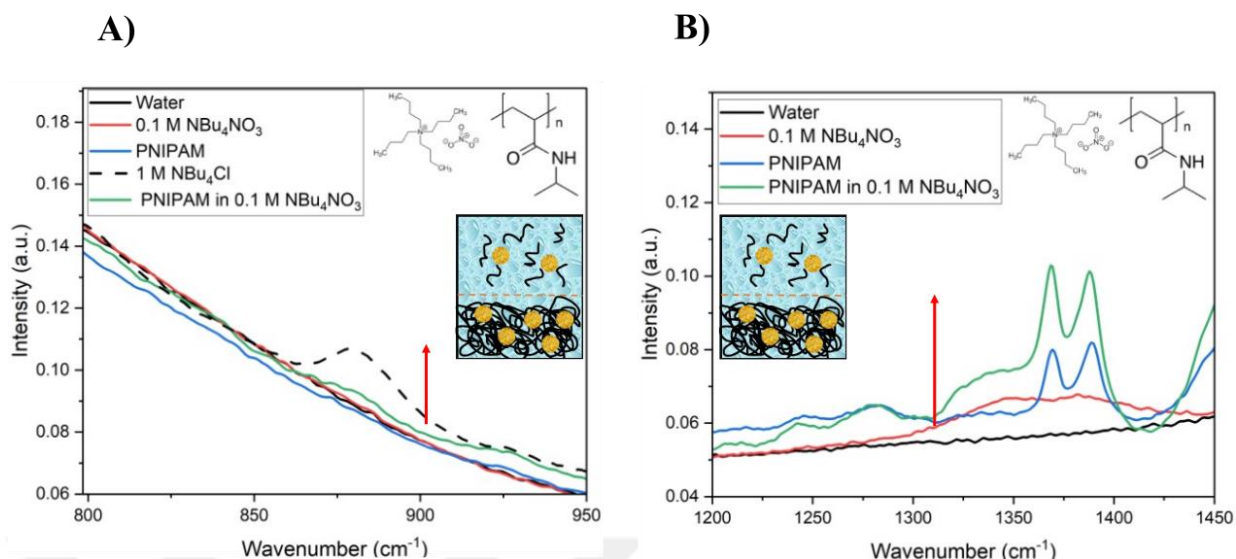


Figure 30: ATR-FTIR measurements above LCST of PNIPAM with 0.1 M NBu₄NO₃. A) The Peak at 875 cm⁻¹ corresponds R-C-N stretching from NBu₄⁺ cation.-C-N stretching intensity increased with collapsing of PNIPAM. B) Peak at 1325 cm⁻¹ corresponds to NO₃⁻ rocking mode. Since there is significant interaction between macromolecules, nitrate also accumulated with polymer.

The mechanism of salting-out behavior for tetra butyl ammonium iodide and nitrate salts can be interpreted using NMR and ATR-FTIR experimental results. K_d ratio of 1.65 with the NMR measurements indicating that the cation and anion have similar binding affinities. As can be seen in ATR-FTIR experiments, the co-presence of the cation and anion leads to diminishing net macromolecular surface charging via ion binding. This leads to coexistence of opposite charges on the polymer surface and that leads to the solubility decrease of the PNIPAM molecule.

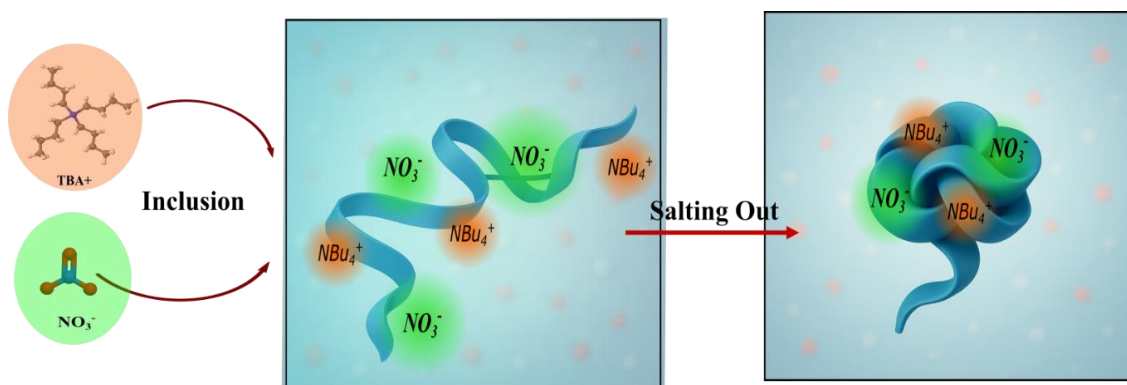


Figure 31: Salting-out mechanism of tetra butyl ammonium nitrate (NBu_4NO_3), co-binding of cation and anion cannot create charge duality. Overall, net charge will be close to neutral and this way, interaction with water will be decreased which means solubility of macromolecules decreases.

The salting-out mechanism of TBAI and TBAN salts, where anion and cation bind together, is reminiscent of a similar ion-macromolecule interaction mechanism as previously described. As it is mentioned in the introduction, Figure 12, guanidinium (Gnd^+) can bind to Elastin-like Polypeptides (ELP) due to its hydrogen bonding capacity, but the crucial point here is that macromolecular behavior can be modulated as the guanidinium counter anion is modified. For example, guanidinium sulfate exhibits pure salting-out behavior, interestingly the same cation exhibits the same salting-out behavior when the counter anion is a salting-in anion, i.e. thiocyanate (SCN^-). While the salting-out mechanism of Gnd_2SO_4 can be explained with pure excluded volume effect, the GndSCN salts out by both ions' accumulation to polymer surface. The binding of the cation and the readily binding anion stabilizes the total charge by binding together.

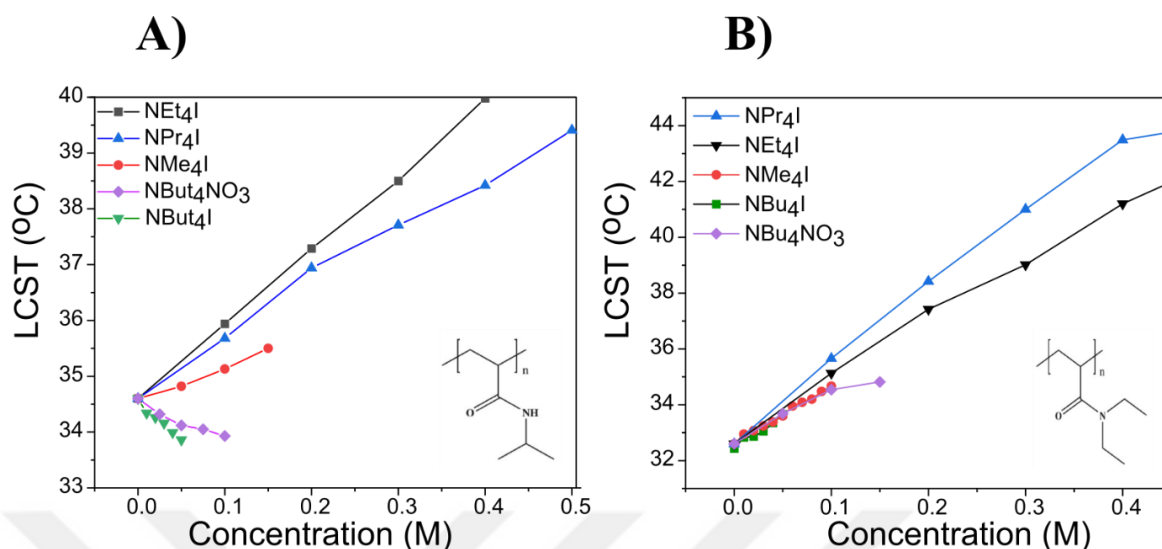


Figure 32: A) LCST measurements of PNIPAM in presence of tetra alkyl ammonium salt with iodide and nitrate counter anions. B) LCST measurements of PDEA in presence of same salts. Side chain chemistry of polymer was changed to di ethyl group from isopropyl. Salting-out behavior of tetra butyl ammonium iodide (TBAI) and nitrate (TBAN) is turned to salting-in with losing interaction site of tetra butyl.

On the basis of the findings from the spectroscopic measurements, it was put forward that the co-binding of anion and cation can lead to macromolecular stabilization. Greasy cations such as tetrabutylammonium were mentioned that are interacting with relatively hydrophobic groups of macromolecules. In the context of PNIPAM model macromolecule, the interaction site for greasy cation was the isopropyl group as shown in solution NMR measurements. For elucidating of tetra butyl ammonium iodide interaction site was the same, and binding is only observed on this specific site. Another set of experiments were designed to verify the universality of the findings, by changing the side chain chemistry of the PNIPAM polymer. To test potential interaction site another thermo-responsive macromolecule called PDEA (poly-*N,N*-diethyl acrylamide), only difference between these polymers are that the

hydrophobic group is changed to two ethyl groups instead of an isopropyl group (chemical structures are given in Figure 4a and 4b). The LCST measurements are performed in the same tetra alkyl ammonium salts, and it was observed that the unexpected salting-out behavior of tetra butyl ammonium iodide and nitrate is lost (see figure 32). In the light of these LCST measurements, it could be concluded that when the cation interaction side is changed and the binding affinity is altered, and thus the salting-out behavior is eliminated, by having an anion-dominant interaction that dictates a salting in behavior of aqueous macromolecules.

In conclusion, the main aim was to examine the interactions of recently observed greasy cations with weakly hydrated anions such as nitrate (NO_3^-) and Iodide(I^-), in addition to their interactions with weakly hydrated anions such as nitrate and Iodide. While the fact that both the anion and the cation are surface-active ions suggests that we would always observe salting-in behavior due to direct interaction. Salting-out behavior has been observed, particularly as the hydrophobicity of the cation increases (such as tetra butyl ammonium Iodide). H^1 -NMR experiments probed site-specific interactions on PNIPAM, providing insights into the mechanism in the solvated phase. Consequently, increasing hydrophobicity suggests that the anion and cation bind together and induce charge duality. Indeed, ATR-FTIR experiments in the collapsed phase also revealed cases where the cation and anion bind together. The key conclusion to draw here is that strong interactions between ions and macromolecules do not always imply salting-in behavior. Likewise in GndSCN salt on aqueous macromolecules, particularly when the cation and anion are surface active, excessive binding of one can render the surface charged, while simultaneous binding of similar strengths can also lead to salting-out behavior.⁸⁴ While such non-additive behaviors

are rare and the mechanisms are difficult to understand, a mechanism has been proposed by examining ion binding in soluble and collapsed states via a multi-instrumental approach.

3.2 Further Step on Bulky Ions: Ion Hydration Reveals the Behavior of Aqueous Macromolecules

It was demonstrated that greasy cations and nanoscopic ions have a tendency and affinity to be partitioned at the neutral polymer-solvent interfaces.^{18,21,46,59,68,92,95,99,133,134} In light of these studies, another set of commonly utilized bulky model ions is the tetra phenyl phosphonium (TPP) and tetra phenyl borate (TPB) ions (molecular structures shown in Figure 15), which possesses charge asymmetry due to the formal charge of its center atoms. These ions have been used as charge carriers in cell membranes and similar bilayer systems.^{113–116} These ions assumed as the charge is hidden within a hydrophobic phenyl layer.^{75,97,116} These ions were used as reference ions to determine the properties of other ions due to their dispersed charge and assumed similar hydration properties. Until the development of interface-specific spectroscopic methods, studying these ions at solvent interfaces was challenging. Various studies have shown that tetra phenyl borate (TPB) and tetra phenyl arsonium (TPA) ions do not have distinct air-water interface behavior, yet at oil-water interfaces represent different surface affinities.^{97,116} Dor Ben Amotz demonstrated the hydration difference between TPB and TPA ions using the MCR-Raman method. The hydration water spectra around TPB and TPA ions exhibit two distinct hydration water structure, and the dangling -OH stretch (O-H group pointing a non-hydrogen bonding groups) for TPB and TPA was determined to be at 3577 cm^{-1} and 3641 cm^{-1} for the hydration water, respectively.⁹⁷

The specific ion effects have been tied with the hydration properties of the surface and the ions^{18,97,116,117,135}. Spectroscopically, the hydration structure of macromolecules has been widely studied at the air/polymer/water interface. However, it is not quite possible to observe distinct differences in the hydration structure for the Hofmeister ions using the same methods. This is simply due to missing spectroscopic methods to measure hydration in bulk solution. Even the number of water molecules is not the same, and as is known from thermodynamic and MD simulation studies, the spectral fingerprints of hydration waters are not truly variable. Tetraphenyl borate (TPB) and tetraphenyl phosphonium (TPP) ions are special model ions that show distinct spectral differences the hydration structures, and in fact, their hydration properties can be attributed to effects on macromolecules. Therefore, in this thesis, we asked a question about whether the hydration differences of ions can be correlated with the solution phase behavior of macromolecules. In this regard, three model macromolecules (PNIPAM (*poly*-(N-isopropyl acryl amide), PDEA (*poly*-(N-N-diethyl acryl amide) and ELP (Elastin Like Polypeptide)) were investigated in the presence of tetraphenyl borate and phosphonium ions via using phase transition temperature MCR-Raman Spectroscopy, (LCST) ATR-FTIR and Solution Phase ¹H-NMR spectroscopy measurements.

3.2.1 MCR-Raman Spectroscopy to Reveal Hydration Water Spectra

Figure 33a shows the input Raman spectra of the sample solution and bulk water as it is measured. Figure 33b shows the hydration shell spectrum after MCR algorithm implementation. As it is seen, the -OH stretching region shows a broad band between 3000-3800 cm⁻¹ due to the hydrogen bond network, and its fast dynamic that

corresponds in picosecond time scales. Additionally, about 2800 to 3000 cm^{-1} alkyl group (-C-H) symmetric and asymmetric stretching bands are apparent clearly coming from the solute molecule, i.e. tertiary butyl alcohol. The frequency of the -OH stretch band region has been assigned to different average number of hydrogen bonds for the liquid water. While 3200 cm^{-1} corresponds to tetrahedrally coordinated water molecules (also referred as ice-like), the 3400 cm^{-1} region is associated with relatively disordered water molecules (water-like) indicating less than four hydrogen bonds. When MCR algorithm was implemented to the sample spectra, instead of broad -OH stretching region, more distinct hydration bands appear. Including the dangling O-H band at around 3660 cm^{-1} . In the solute correlated spectrum, this peak becomes more pronounced.

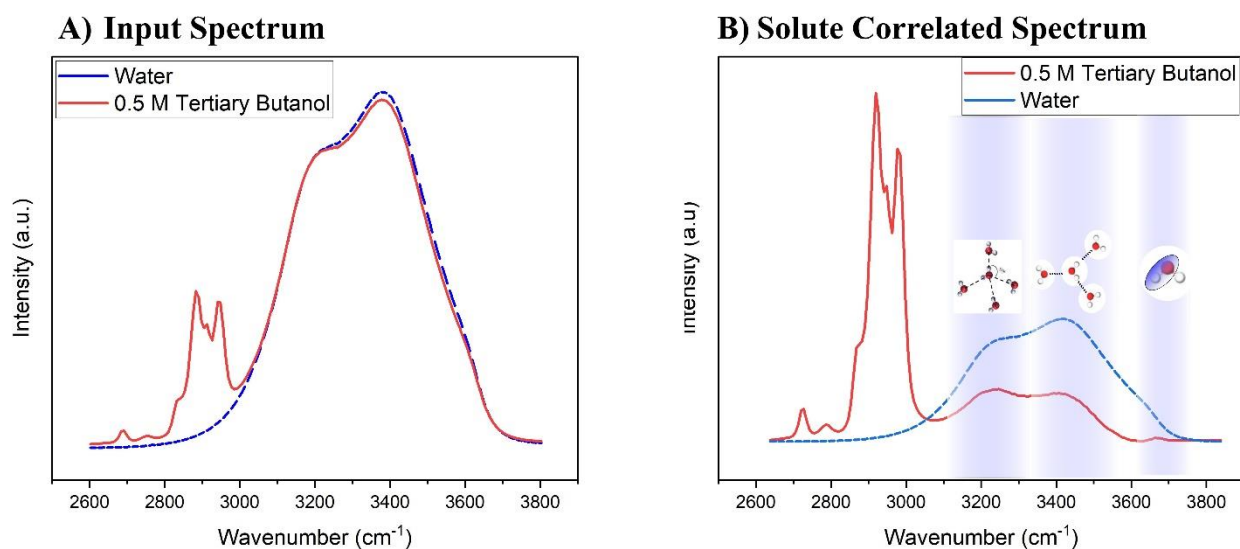


Figure 33: Implementation of MCR-Raman process to 0.5 M Tertiary Butanol. **A)** Raman Spectrum of 0.5 M Tertiary Butanol, 2800-3000 cm^{-1} corresponds to -CH stretching frequencies and 3000-3800 cm^{-1} corresponds to -OH stretching bands. **B)** MCR-Raman implemented 0.5 M Tertiary Butanol spectrum (Solute Correlated), -OH stretching region can be divided into tetrahedrally structured (around 3200 cm^{-1}), relatively disordered (3400 cm^{-1}) and dangling -OH (3600 cm^{-1}) frequency regions.

The same methodology was applied to the tetraphenyl borate (Na salt) and tetraphenyl phosphonium (Cl salts) salts. Previously, it was shown that dangling -OH stretchings are clearly in different frequencies and 64 cm^{-1} difference was reported.⁹⁷ In this study, same MCR-Raman measurements were conducted on tetraphenyl borate and phosphonium ions and as it is seen figure 34. In the hydration shell spectroscopy there is only a small ice-like water band, along with some disordered water band. The major difference was at the dangling O-H groups. For tetraphenyl borate and tetraphenyl phosphonium ions same dangling -OH have quite distinct positions. The

band is observed at 3578 cm^{-1} for tetraphenyl borate, while for tetraphenyl phosphonium, it is observed at 3641 cm^{-1} . A significant difference between these salt ions are observed that is 63 cm^{-1} . Note that, the shift in the dangling O-H band is an indication of weakening or strengthening of the interfacial O-H band of water. For instance, the dangling O-H band pointing directly air at the air/water interface, no interaction with any neighboring molecule, the position is at 3700 cm^{-1} .^{136–141} The interaction associated with the dangling O-H presented here differs from the interaction of hydrophobic hydration with other hydrophobic molecules, such as for the tetraalkyl ammonium cations or for tertbutyl alcohol, the dangling O-H frequency appears at 3660 cm^{-1} . The difference can be attributed to the water- π interaction: phenyl groups have delocalized electron clouds, and as in ion- π interactions, the relatively positively charged hydrogens of water interact with the delocalized π electrons. A small shift in the dangling O-H frequency was previously demonstrated for simple aromatic systems by changing the electron withdrawing and electron-donating side groups of benzene molecule.^{31,97,131}

In figure 34, in addition to the TPB^- and TPP^+ ions, it is also necessary to add the hydration spectrum of phenol molecule as a reference for dangling O-H position. The dangling O-H band appears at 3610 cm^{-1} . In the tetraphenyl borate ion, the center is negatively charged and interacts with water by increasing the electron density on the phenyl groups and thus a more red-shifted band at 3578 cm^{-1} is present. For the tetraphenyl phosphonium ion, the positively charged center reduces the electron density on the phenyl groups, and reducing the water- π interaction, and thus the dangling O-H band is observed at 3641 cm^{-1} .⁹⁷ The data presented here is similar to the MCR-Raman data previously shown for terphenyl borate and arsenium salts.⁹⁷ As

such, the central charge influence on the phenol π electron density and resulting O-H band is demonstrated in a clear fashion. Appendix A and B demonstrate the spectral fitting efforts in O-H stretch region to show the position and intensity of dangling O-H band of the MCR-Raman data, as well as the delta spectrum of the actual measured data to clarify the dangling O-H band do not appear as an algorithmic artifact for the both salt hydration. The next question is that if this hydration differences influence any solution phase phenomenon, such as their effects on solution phase behavior of neutral macromolecules.

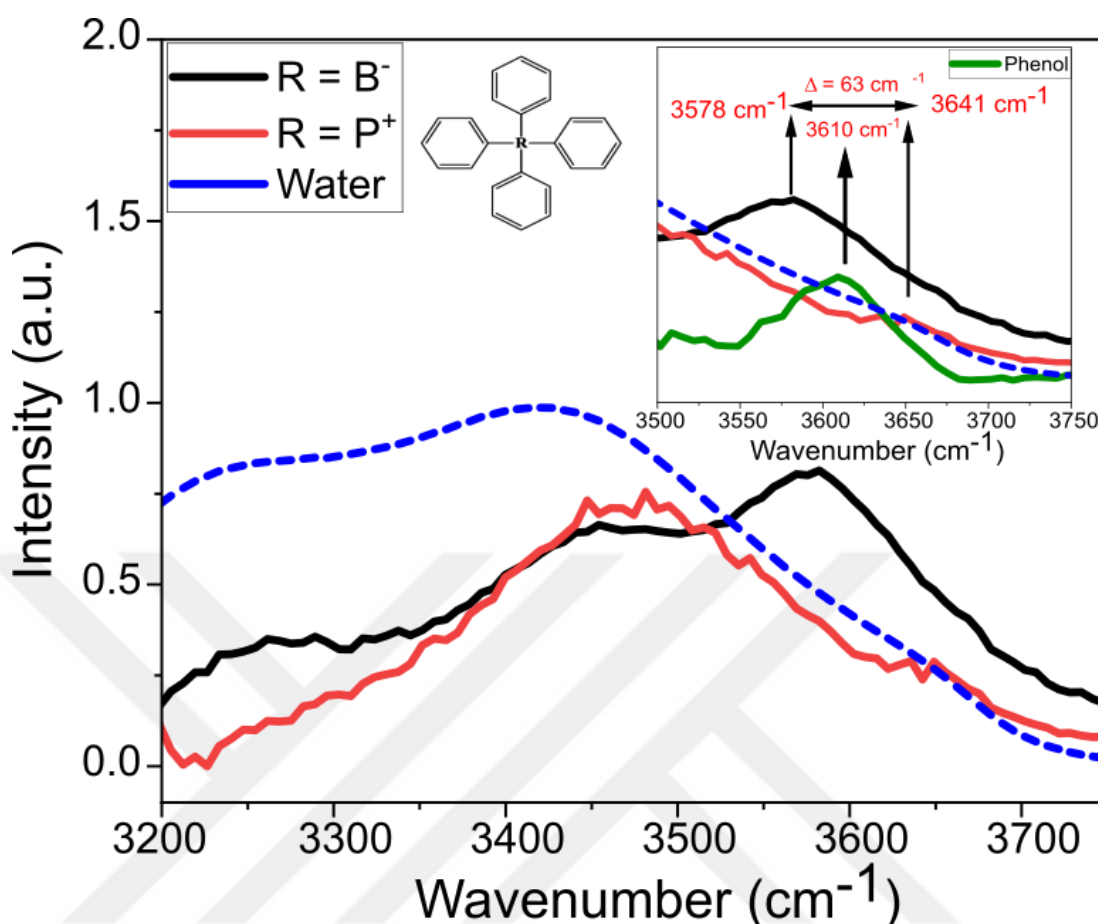


Figure 34: Hydration Shell Spectra for 0.1 M Sodium Tetraphenyl Borate (Black), 0.1 M Tetraphenyl Phosphonium Chloride (Red) and 0.3 M Phenol (Green) solutions in the spectral range of 3200 cm^{-1} - 3750 cm^{-1} . The dangling O-H stretching are at 3578 cm^{-1} for tetraphenyl borate and 3641 cm^{-1} for Tetraphenyl phosphonium. As a reference phenol dangling -OH stretching is also shown in 3610 cm^{-1} . The inset spectra show the $3500 - 3750\text{ cm}^{-1}$ region.

3.2.2 LCST Measurements on Neutral Model Macromolecules: PNIPAM, PDEA, and ELP

Obtaining spectroscopic hydration fingerprints of ions and observing significant differences is quite unexpected for many ions.^{31,97,116,120,131} It can be argued

that the interaction of water with delocalized electrons in tetraphenyl borate and tetraphenyl phosphonium ions is particularly effective in observing hydration differences. The elucidation of the salting-in and salting-out behavior of PNIPAM, PDEA, and ELP (**E**lastin-**L**ike **P**olypeptide) through their phase transition temperature (LCST) experiments will be a first step in understanding how the charge asymmetry affects different thermo-responsive macromolecules. As it is mentioned in introduction section that ELPs (**E**lastin **L**ike **P**olypeptides) have pentameric amino acid unit having VPGXG (Valine-Proline-Glycine-**X**-Glycine) and **X** can be any amino acid except for proline. The ELPs (Elastin-like polypeptides) employed in the experiments is V₅A₂G₃ with 120 pentameric repeating units. In figure 35, LCST curves for Sodium tetraphenyl borate and tetraphenyl phosphonium chloride salts on ELP, PDEA and PNIPAM are shown. In figure 35a, for all polymers, direct increase in LCST for all three polymers demonstrated that tetraphenyl phosphonium salts show salting-in behavior independently from the nature of neutral macromolecule. On the other hand, in figure 35b, tetraphenyl borate salt shows an initial dramatic increase and then steep decrease meaning these ions are surface active and interacts with macromolecules and they clearly salt-out above 0.04 M salt concentration.

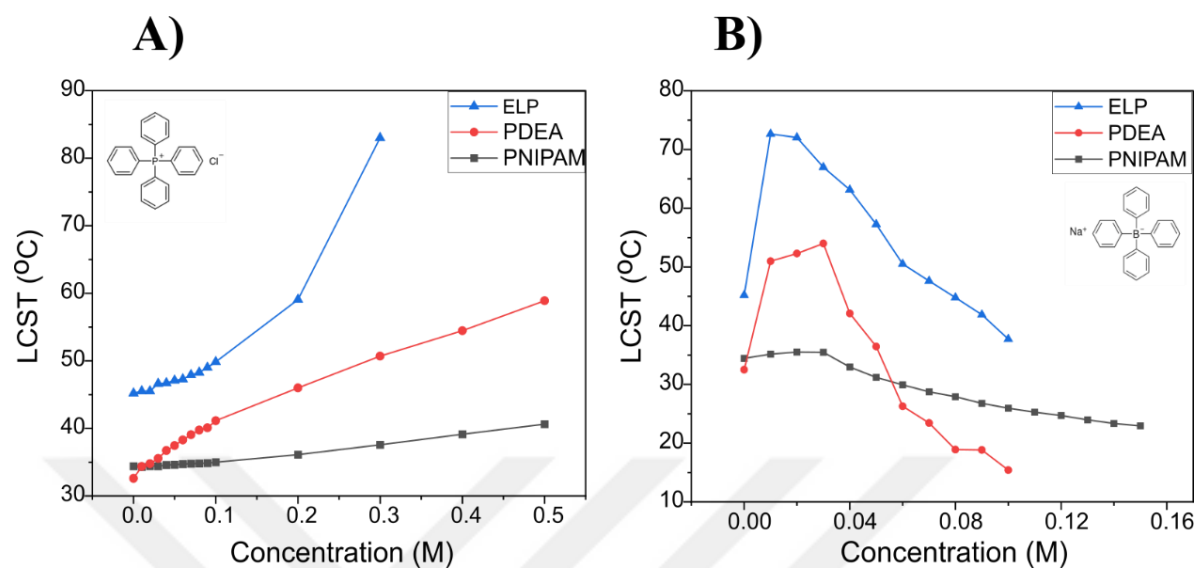


Figure 35: LCST measurements of ELP, PDEA and PNIPAM in presence of sodium tetraphenyl borate and tetraphenyl phosphonium chloride. **A)** In the range of 0-0.5 M concentration of TPP chloride LCST measurements. Solubility limit for salt is 0.5 M and for ELP in 0.4 M and above concentrations could not be measured due to high LCST points (over boiling point of water). For all three sets of experiments, there is direct dramatic increase in LCST which is characteristic for salting-in behavior. **B)** LCST curves for Sodium tetraphenyl borate salt in 0-0.15 M range. Above 0.04 M all polymers show decrease in the LCST, meaning a clear salting-out behavior.

The LCST curves show two different behaviors for different distinct bulky ions, independent of the polymer chemical structure. Tetraphenyl borate, which interacts more strongly with water, exhibited a salting-out behavior. While tetraphenyl phosphonium, which interacts weaker with water because of higher electron densities on phenyl group, exhibited only salting-in behavior. This behavior has been claimed for many decades for the Hofmeister series but without any strong spectroscopic evidence.^{18,21} Namely, the strongly hydrated ions predominantly cause a salting-out

action for macromolecules, and the weakly hydrated ions give rise a salting-in action via a surface adsorption process. The dangling O-H band position here serves as a probe to determine the degree of ion - water interaction. Between TPB^- and TPP^+ . As it is demonstrated in figure 34, the tetraphenyl borate is more strongly interacting with hydration water molecules evident by the more red shifted dangling O-H band, that also show a strong salt-out at elevated ($> 0.04 \text{ M}$) salt concentrations. The picture alters to a clear salting-in effect with tetraphenyl phosphonium ion, that can be classified as weakly hydrated with the least red-shifted dangling O-H band in its hydration.

This is clear spectroscopic evidence that can prove the long claimed strongly- and weakly-hydrated ions and their proposed influence on the solubility of macromolecules. Here, one can argue if the charge nature has a dominant influence on the effect. It is well-known in the literature that large polarizable ions can interact with the macromolecular surfaces for anions and also for cations.^{18,21,69}

3.2.3 Interaction Sites of Charged Asymmetric Ions: Solution NMR Measurements

This difference in hydration properties and the observed behavioral differences in PNIPAM, PDEA, and ELP raise the question of which site interacts with these model polymers. In this context, we used the PNIPAM model molecule to investigate the interactions of tetraphenyl borate and phosphonium salts in solutions. The hydration spectroscopy measurements indicate that the TPB^- ion interacts more strongly with water, while the TPP^+ ion interacts less strongly with water molecules. Therefore, based on the LCST profiles, it is conceivable that the bond strengths and sites may differ.

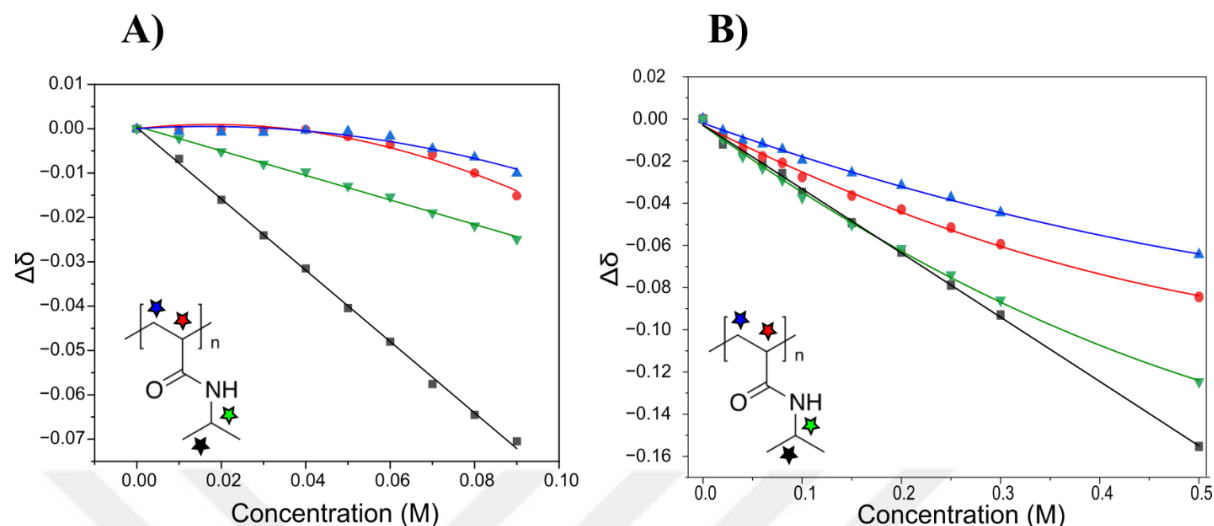


Figure 36: H-NMR measurements of PNIPAM in tetraphenyl borate and phosphonium salt solutions. Possible interaction site in structure is given colorful stars. **A)** NMR measurements in tetraphenyl borate (Na^+ counter cation) in concentration range of 0-0.09 M (In D_2O , solubility decreases from 0.15 M) For alpha and beta carbon hydrogen, non-linear change in chemical shift is observed. On the other hand, -CH and Isopropyl hydrogens show only linear monotonic decrease. **B)** NMR measurements in tetraphenyl phosphonium (Cl^- counter anion), all sites of PNIPAM show monotonic decrease, trend in change in chemical shifts are not perfectly linear but it does not have mean that there is binding in those sites.

Figure 36a shows the chemical shift of all sites of PNIPAM with increasing tetraphenyl borate ion (Na^+ counter cation) concentration. The four different interaction sites, alpha, beta, -CH, and isopropyl hydrogens, are marked with the same colored stars with symbols. Examining the change in chemical shift, the interaction sites of the tetraphenyl borate ion are seen as the alpha and beta carbon hydrogen. It is evident from the nonlinear chemical shift profiles. On the other hand, the isopropyl and -CH (tertiary carbon) hydrogens, which are part of the hydrophobic group, show

a linear monotonic decrease. From these data, bulky anion interacts with the same binding site of the common Hofmeister anions (SCN^- , I^-). Since the ion center is still negatively charged, the observation that the interaction occurs with relatively acidic hydrogens is similar to that observed previously in anion interactions. Although the phase transition behavior is salting-out, at low salt concentrations the solubility of PNIPAM increases ($< 0.04 \text{ M}$). As such the NMR and LCST data are in correlation. Figure 36b shows the concentration-dependent chemical shift changes for the same sites in PNIPAM for tetraphenyl phosphonium. Here, a direct decrease is observed for all four hydrogen species. The monotonic, decrease indicates no strong interaction^{18,68}, although LCST data clearly indicating a salting-in effect. Considering the hydration properties of these two ions tetraphenyl phosphonium is more-weakly hydrated and thus should be more surface active. Moreover, the NMR data does not support the macroscopic phase transition temperature data.

Although ^1H -NMR measurements may not reveal the interaction for tetraphenyl phosphonium, another strategy is to examine the collapsed state of the macromolecule above the LCST. The interaction of such bulky ions may not be site-specific and can be hidden from the NMR measurements. Therefore, ATR-FTIR measurements are an important step in determining whether the salt is present in the collapsed state of the macromolecules.

3.2.4 Collapsed State Analysis for Explaining Interactions of Tetraphenyl Ions: ATR-FTIR Measurements

To determine whether ion binding is measurable from the precipitated state of polymers, the temperature was adjusted above the LCST using a metal plate, as previously studied for tetra alkyl ammonium iodide salts. For PNIPAM, PDEA, and

ELP, the circulating bath salt concentration was adjusted to 10°C above the LCST (Figure 27), while the tetraphenyl borate and tetraphenyl phosphonium ion concentrations were kept constant at 0.1 molar. To ensure precipitation of the polymer, the polymer concentration was adjusted to 20 mg/ml, and again the analysis was performed on the resulting spectra using a 3 minutes reference time.

An increase in salt peak intensity with the collapsed phase of the macromolecules indicates that the salt ions partition to the macromolecule rich phase due to the binding of the corresponding ion. In this case, the Ph-C-B (1030 cm^{-1}) and Ph-C-P (1110 cm^{-1}) stretch bands and phenyl out of plane C-C-H bending ($700\text{--}750\text{ cm}^{-1}$) of tetraphenyl -borate and -phosphonium ions as the polymer peaks increase. Figure 37 shows six different spectra for TPP salt in different polymers, and two columns corresponds to two different spectral regions associated with salt and every row is corresponding PNIPAM, PDEA and ELP polymers respectively. Figures 37a&b show the Ph-C-P stretching (1110 cm^{-1}) and phenyl out of plane bending ($700\text{--}750\text{ cm}^{-1}$) in solutions using the PNIPAM as a model. As can be seen, the salt peaks are clearly visible for both salts and an increase in the salt peaks with the presence of the collapsed state of PNIPAM in two different regions meaning is visible. Such an increase indicates that there is an interaction of TPP^+ cation to PNIPAM. Figures 37c&d show the salt peaks for the same spectral regions when the model macromolecule is PDEA. Similar to the PNIPAM polymer, the salt peak intensities increase in the both regions. Finally, as seen in Figures 37e&f, the interaction of TPP^+ salt in the collapsed state is also visible for ELP macromolecules, with moderate intensity increase in the salt related vibrational peaks. Thus, it is possible to explore the ion interaction by the changes in peak intensities that is quite dramatic intensity

increase in the ATR-FTIR experiments for tetraphenyl phosphonium salts for the three model macromolecules.

The interaction of the tetraphenyl phosphonium ion, which was not apparent in the solution ^1H -NMR experiments, was clearly observed in the collapsed state, supporting the notion that the interaction should be not a site-specific interaction. The same treatment was done and illustrated in Figure 38 on also TPB^+ salt for the same spectral regions and it was seen that there are also increase in their peaks even peak intensities that are relatively weak due to the nature of salt. Already, binding of TPB^+ was shown with LCST and NMR measurements and now supported with the ATR-FTIR experiments.

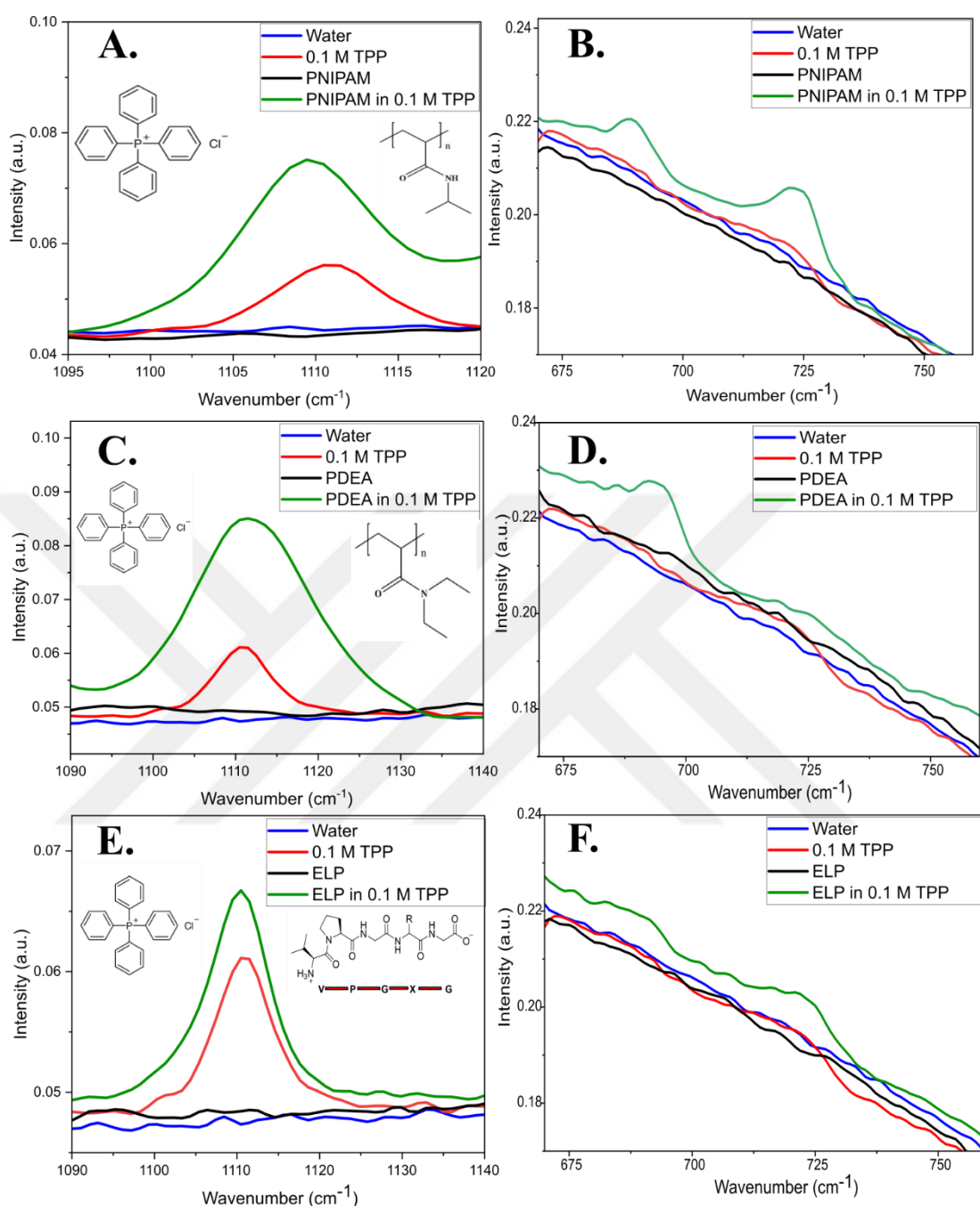


Figure 37: ATR-FTIR measurements above LCST for TPP^+ salt for PNIPAM (A,B), PDEA (C,D) and ELP (E,F) two spectral regions which are Ph-C-P stretching (1110 cm^{-1}) and Phenyl out of plane bending ($700\text{--}750 \text{ cm}^{-1}$) (Slope is coming from water peaks) were investigated inducing collapsed state. In addition to increase in peaks for both regions with TPP^+ , broadening in 1110 cm^{-1} corresponds to bound and non-bound salts and reveals as broader peak.

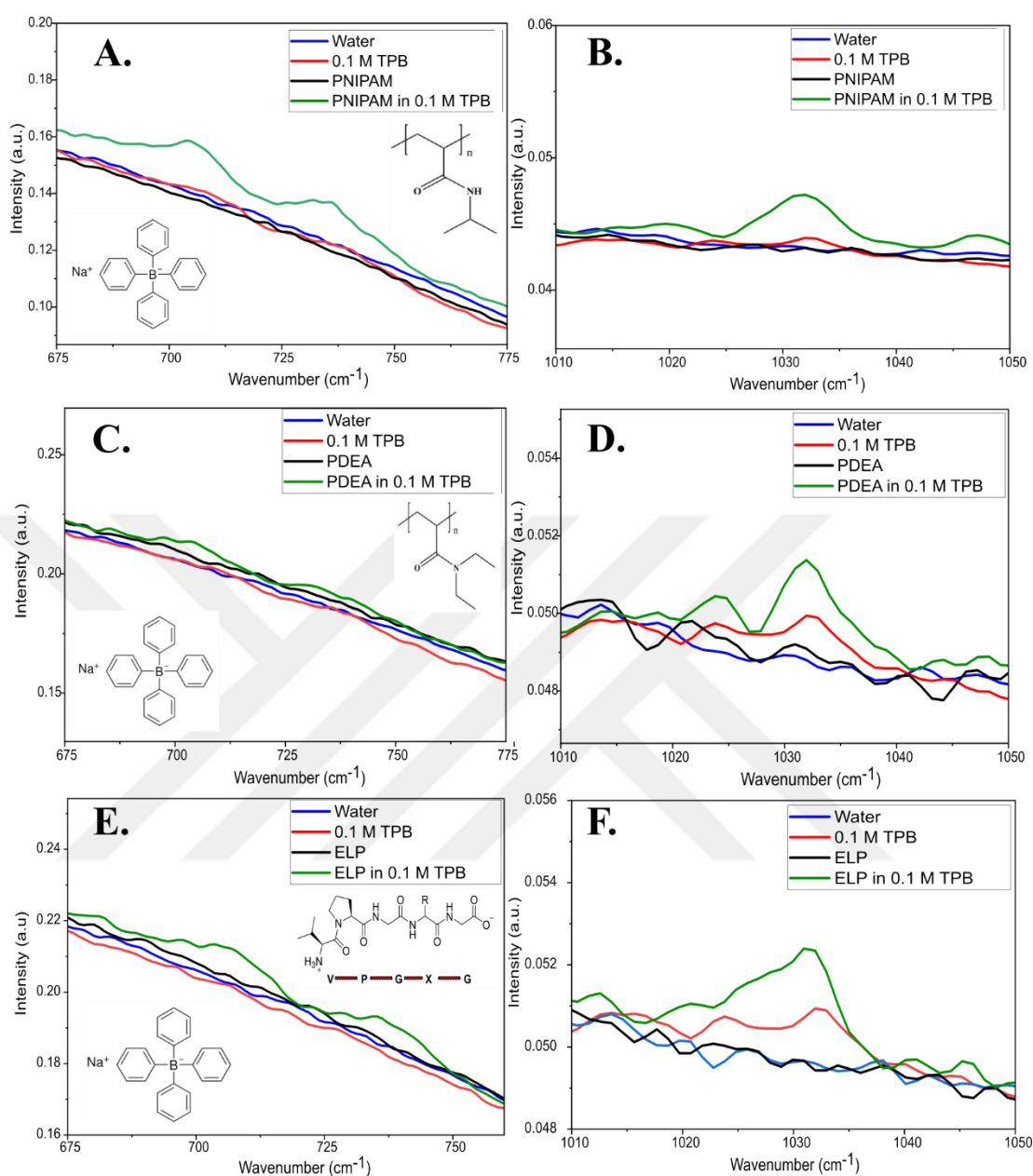


Figure 38: ATR-FTIR measurements above LCST for TPB⁻ salt for PNIPAM (A,B), PDEA (C,D) and ELP (E,F) two spectral regions which are Ph-C-P stretching (1030 cm^{-1}) and Phenyl out of plane bending (700-750 cm^{-1}) (Slope is coming from water peaks) were investigated inducing collapsed state.

In summary, a significant 63 cm^{-1} shift on the dangling O-H band of the hydration spectroscopy of tetraphenyl borate and phosphonium ions, whose charge asymmetric centers are expected to interact with water, was observed in the π -water interaction. Such dramatic difference in the hydration is utilized as spectroscopic evidence to show weakly and strongly hydrated ions and their salting-in and salting-out influence on neutral macromolecules. The LCST curves were demonstrated for the thermo-responsive model polymers; PNIPAM, PDEA, and ELP. Indeed, a clear salting-out was observed at high concentrations for tetraphenyl borate, while only salting-in was observed for tetraphenyl phosphonium salts. The charge asymmetry cannot be the underlying difference, due to both cationic and anionic bulky ions being surface active. The solution-phase NMR experiments and ATR-FTIR showed that both TPB⁻ and TPP⁺ ions interact with the polymer surface. Regarding the scientific significance of this study, a direct correlation between the hydration fingerprints and their influence on the solution phase behavior of aqueous macromolecules was shown with molecular level mechanisms. In addition to spectroscopic finding, as a next step the dangling OH frequency shift can be supported with Ab-initio Molecular Dynamics (MD) simulations.

Chapter 4 - Conclusion

Many questions remain unanswered about the differential effects of ions on macromolecules, beginning with Franz Hofmeister in the late 19th century.^{18,21,93,127} Even with the advancement of spectroscopic methods, determining accurate analyses using chemical heuristics remain crucial. Recently, the cationic interaction that has been long underestimated and often neglected, were shown to as strong as of binding weakly hydrated anions when the cations are highly hydrophobic. In this thesis, inspired by the intriguing interaction of weakly hydrated cations such as tetra alkyl ammonium cations, a primary goal was to explore how these cations could be manipulated by weakly hydrated anions, which are surface-active molecules. The selection of tetra alkyl ammonium iodide salts in this direction, and the dominance of the salting-out process in a system containing two surface-active ions, made more sense. As expected, hydrophobicity would reduce the salting-out behavior, and even an unforeseen salting-out process was observed for tetra butyl ammonium iodide (TBAI). To further elucidate the mechanisms of intermolecular interactions, we examined the dissolved and collapsed states of the model macromolecule using spectroscopic methods. Because ¹H-NMR experiments allow us to provide site-specific information on the model macromolecule through interaction, we were able to analyze the cation and its specific processes in terms of the binding constant (K_d) ratios. For tetraethylammonium iodide (TEAI), the $K_d(\text{anion})/K_d(\text{cation})$ ratio is very low (0.17), while for tetra propyl ammonium iodide (TPAI), a ratio of 0.5 and approximately 1.7 is sufficient as hydrophobicity increases. Two key factors can be considered for the patterning of the macromolecule. If the hydrophobicity of the initial layer is controlled, binding can be controlled, and thus the surface can be neutralized

by cooperative binding with the anion during the solution phase. In the case of tetraethyl ammonium iodide (TEAI), the salting-out process, where anion inertia is dominant, is still observed. In addition to NMR experiments, simultaneous binding of both the anion and cation was observed for the tetra butyl ammonium nitrate salt during ATR-FTIR experiments above the LCST (using the opposing anion nitrate). In both NMR and ATR-FTIR experiments, cooperative interactions of the ions can still be observed, and salt-out behavior can be observed. While direct interactions of ordinary ions are typically salted-in, this non-additive behavior is an exception in tetra alkyl ammonium iodide salts. Consequently, surface charge determines macromolecular stabilization in the aqueous phase. In addition to the tetra alkyl ammonium cation, the charged-asymmetric tetraphenyl borate (TPB^-) and tetraphenyl phosphonium (TPP^+) ions, which have been studied with interest for years by increasing hydrophobicity, were also investigated. While many studies typically fail to demonstrate significant differences in air-water circuits, MCR-Raman measurements were conducted with a different -OH frequency, indicating that these ions have very different interfacial surfaces due to their π - H_2O interactions.^{31,35,97,116,131} Phosphonium, with a positive center, interacts less with phosphonium, while borate, with a negative center, interacts more with water. Based on this, an application of this method was conducted to study the effects of the salts of these two ions on three different model macromolecules (PNIPAM, PDEA, and ELP) using LCST measurements. TPB^- salt showed a significant decrease in LCST profiles, initially rising, and then salting out. TPP^+ , which interacts less in other countries, exhibited a salting-out effect. The most important aspect of this experiment is that the specifically observed hydration differences are clearly observed in the LCST plots, independent of the model macromolecule. While

many previous studies focused on hydration and macromolecular continuation, the study on these two ions provides the clearest example. To establish molecular order, NMR and ATR-FTIR experiments were conducted with the tetraphenylphosphonium model macromolecule. While NMR experiments indicated that the tetraphenylphosphonium borate ion interacted with acids and hydrogens, no inertia site was observed for the tetraphenylphosphonium ion. This suggests that the overall interaction rates do not provide specific values within the site. When ion trafficking in the collagenous macromolecule was examined with ATR-FTIR, an increase in the salt peaks was observed for both ions, and both ions were weakened by interaction with the macromolecule. Using the results from this experiment as a reference, we demonstrated that the inertia of the tetraphenylphosphonium ion is due to a nonspecific interaction at the site. In conclusion, this thesis has explained from a Mult instrumental perspective how hydrophobic cations can collaborate with weakly hydrated anions and modulate the macromolecule, and in addition, the correlation between the hydration patterns of hydrophobic ions such as tetraphenylene borate-phosphonium and their effects on the macromolecule.

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Appendix A: Gaussian Fits of MCR-Raman Spectra

The hydration spectrum for tetraphenyl borate (TPB^-) and tetraphenyl phosphonium (TPP^+), as described in Section 3.2.1 and shown in figure 34, are shown. The dangling -OH stretch for tetraphenyl borate was observed at 3578 cm^{-1} and for tetraphenyl phosphonium at 3641 cm^{-1} . Although Dor Ben Amotz also showed the dangling -OH stretch for tetraphenyl arsonium at 3641 cm^{-1} ,⁹⁷ the dangling -OH shoulder for tetraphenyl phosphonium is visible, as shown in figure 34, and is not as clear as the dangling -OH in the related study. To confirm that the observed shoulder truly belongs to the dangling -OH peak, the MCR-treated spectra were first fitted with Gaussian functions. Figure 39 shows the fitted MCR-Raman spectra for both salts. The MCR-Raman spectrum for tetraphenyl phosphonium was fitted to three Gaussian functions at 3330 , 3485 , and 3641 cm^{-1} . The dangling-OH observed in Figure 34 is similar to that reported in the literature⁹⁷ and has the same wavenumber. On the other hand, for tetraphenyl borate, the entire spectrum was fitted with three Gaussian fits centered at 3280 cm^{-1} , 3440 cm^{-1} , and 3578 cm^{-1} . As shown in figure 34 and reference⁹⁷, the peak assigned to dangling-OH is at 3578 cm^{-1} .

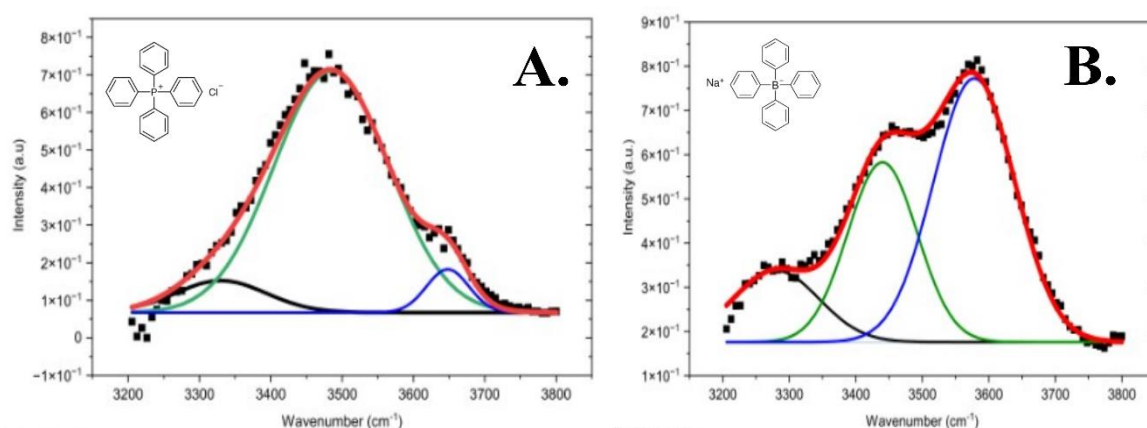


Figure 39. Gaussian fits of the MCR-Raman hydration spectra of tetraphenyl borate (TPB⁻) and tetraphenyl phosphonium (TPP⁺). **A)** For TPP⁺, the spectrum was decomposed into three Gaussian components at 3330, 3485, and 3641 cm⁻¹, confirming the presence of a dangling –OH feature at 3641 cm⁻¹. **B)** For TPB⁻, the spectrum was fitted with three Gaussian peaks centered at 3280, 3440, and 3578 cm⁻¹, where the band at 3578 cm⁻¹ corresponds to the dangling –OH vibration.

Peak Index	Peak Type	Area Intg	FWHM	Max Height	Center Grvty	Area IntgP
Left	Gaussian	13.20481	150	0.08484	3330	8.70335
Middle	Gaussian	129.9593	190	0.64277	3485	85.65677
Right	Gaussian	8.55689	70	0.11484	3648	5.63988

Table 1: Gaussian fitting parameters for MCR-Raman Spectrum of tetra phenyl phosphonium chloride

Peak Index	Peak Type	Area Intg	FWHM	Max Height	Center Grvty	Area IntgP
Left	Gaussian	22.96822	152.26676	0.16184	3280	13.66703
Middle	Gaussian	53.43322	123.41543	0.40673	3440	31.79496
Right	Gaussian	91.6542	144.33496	0.59664	3578	54.53801

Table 2: Gaussian fitting parameter for MCR-Raman Spectrum of tetra phenyl phosphonium chloride

Appendix B: Subtraction of Water Spectrum from Raw Spectra of Tetraphenyl Borate and Tetraphenyl Phosphonium

The MCR-Raman spectra of tetra phenyl borate and tetra phenyl phosphonium, shown in Figure 34 in Section 3.2.1, are presented. The dangling -OH peaks for each salt appear at 3578 cm^{-1} for tetraphenylborate and 3641 cm^{-1} for tetraphenylphosphonium. In addition to applying the MCR-Raman method, the dangling -OH peaks were also obtained by roughly subtracting the bulk water spectrum from the raw solution Raman spectra without using ALS algorithm. Accordingly, bulk water was subtracted and displayed for both ions, as shown in figure 40. The yellow lines indicate that the dangling -OH peaks are located in the MCR-Raman regions. On the other hand, negative values are observed between 3000 and 3200 cm^{-1} due to excessive subtraction of water. In this context, MCR-Raman also performs bulk water subtraction under the condition that these points are negative, and the spectral area is minimized.

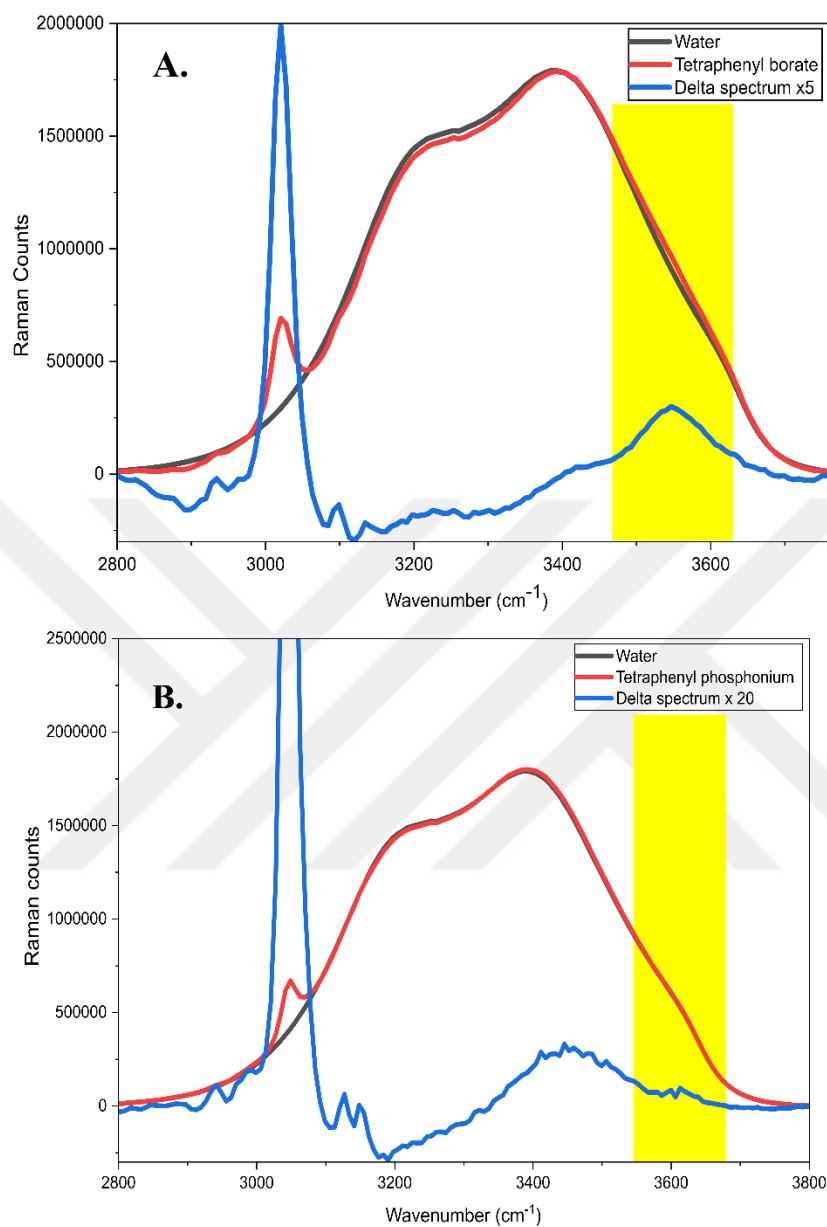


Figure 40. Subtraction of water spectrum from solution spectra of tetra phenyl borate and tetra phenyl phosphonium, highlighting the dangling -OH peaks at 3578 cm^{-1} and 3641 cm^{-1} , respectively. For comparison, bulk water subtraction was also performed on the raw solution Raman spectra, with the resulting features shown in yellow. Negative values between 3000–3200 cm^{-1} arise from excessive water subtraction.