

ELUCIDATION OF THE DENATURATION MECHANISM OF UREA ON MACROMOLECULES IN AQUEOUS MEDIUM

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MACROMOLECULES IN AQUEOUS MEDIUM

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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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The urea molecule is a well-known denaturant for a wide range of macromolecules. To date, there is no unified molecular-level explanation for urea-induced denaturation of all macromolecules. As a result, considerable effort has been directed toward this subject in recent years, because osmolyte protein interactions are of central interest and have implications ranging from polymer physics to cell biology. Detailed urea denaturation mechanisms, focusing on the entropically driven formation of urea clouds, urea induced cross-linking mechanism, and osmolyte clouding around macromolecules, have been proposed in the literature. However, no agreement has been reached on the molecular machinery of urea denaturation. In this thesis, the urea-induced solubility changes of macromolecules in aqueous solutions were investigated by utilizing the lower critical solution temperature (LCST) of poly (*N* - isopropylacrylamide) (PNIPAM) and poly (*N,N*-diethylacrylamide) (PDEA) as a function of urea concentration up to 6.0 M. Due to the lack of suitable probing methods for the collapsed state of interested macromolecules, a temperature-controlled ATR – FTIR spectroscopy based method was developed to explore the interaction between urea and the collapsed state of macromolecule. LCST measurements revealed that the solubility of PDEA increases with increasing urea concentrations, whereas PNIPAM salts-out from the solution monotonically, despite the fact that both polymers have similar molecular structures. Temperature gradient ATR-FTIR measurements were carried out to further investigate this discrepancy. First, no favourable urea accumulation towards the collapsed form of macromolecules was observed up to 6.0

M urea, which puts doubt on the urea clouding mechanism. Moreover, at elevated (> 3.0 M) urea concentrations, the collapsed form of the PNIPAM and PDEA accumulated towards the cooler parts of temperature gradient in solution, indicating the preferred form of the macromolecule is the soluble (uncollapsed) form. These surprising results indicate that both PNIPAM and PDEA prefers the soluble form at elevated urea concentrations above the LCST. As a molecular mechanism, the urea molecules act as a "glue" between the amide groups of PNIPAM, bringing intra- and inter-molecular parts of the macromolecule into close proximity, and act as a collapsed form of macromolecules. Apparently, such a mechanism is not valid for the PDEA. Our findings shed new light not only on aqueous phase phenomena, but also on the aggregated (collapsed) phase of macromolecules in aqueous medium.

Keywords: Urea, PNIPAM, PDEA, Denaturation Mechanism, Lower Critical Solution Temperature (LCST), ATR – FTIR spectroscopy.

ÖZET

ÜRENİN SULU ORTAMDA BULUNAN MAKROMOLEKÜLLER ÜZERİNDEKİ DENATÜRASYON MEKANİZMASININ AYDINLATILMASI

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Üre çeşitli makromoleküller için iyi bilinen bir denatürant molekülüdür. Bugüne kadar, tüm makromoleküllerin üre kaynaklı denatürasyonu için moleküler seviyede detaylı genel bir açıklama bulunmamaktadır. Bu eksikliğin sonucu olarak ve ozmolit protein etkileşimlerinin polimer fiziklerinden hücre biyolojisine kadar çeşitli alanlarda merkezi öneme sahip olduğundan son yıllarda bu konuyu açıklamak için ciddi çaba gösterilmiştir. Literatürde, üre bulutlarının entropik olarak yönlendirilen oluşumuna, üre kaynaklı çapraz bağlama mekanizmasına ve makromoleküller etrafında ozmolit bulutlanmasına odaklanan ayrıntılı üre denatürasyon mekanizmaları önerilmiştir. Ancak, üre denatürasyonunun moleküler mekanizması üzerinde bir mutabakata varılamamıştır. Bu tez çalışmasında, sulu çözeltilerdeki makromoleküllerin üreden kaynaklanan çözünürlük değişiklikleri, poli (*N*-izopropilakrilamid) (PNIPAM) ve poli (*N*, *N*-dietilakrilamid) (PDEA) alt kritik çözelti sıcaklığı kullanılarak 6.0 molar üre konsantrasyonuna kadar incelenmiştir. İstenen makromoleküllerin üre ile çökmüş durumunu çalışabilecek uygun ölçüm tekniklerinin bulunmaması nedeniyle, çökmüş makromolekül - üre etkileşimlerini çalışabilecek sıcaklık kontrollü ATR-FTIR spektroskopisi tabanlı bir yöntem geliştirilmiştir. Alt kritik çözelti sıcaklığı ölçümleri ile üre konsantrasyonu artarken PDEA'nın çözünürlüğünün arttığı ancak moleküler yapıları çok benzer olmasına karşın PNIPAM'ın monoton bir şekilde çözünürlüğün azaldığı gösterilmiştir. Bu farklılığı daha da araştırmak için sıcaklık gradyanlı ATR-FTIR ölçümleri gerçekleştirilmiştir. İlk olarak, 6.0 M'ye kadar olan ürenin çökmüş makromoleküllere birikmesi gözlenmemiştir ve bu da ürenin bulutlanma mekanizması hakkında şüphe uyandırmaktadır. Ayrıca, yüksek (> 3.0 M) üre konsantrasyonlarında,

PNIPAM ve PDEA'nın çökmüş formu, çözelti içindeki sıcaklık gradyanının daha soğuk kısımlarına doğru birikerek, makromolekölün tercih edilen formunun çözünür (çökmemiş) form olduğunu göstermektedir. Bu şaşırtıcı sonuçlar, hem PNIPAM hem de PDEA'nın alt kritik solüsyon sıcaklığının üstünde bulunan ve yüksek üre konsantrasyonlarında çözünür formu tercih ettiğini görülmüştür. Moleküler mekanizma olarak, üre molekülleri, PNIPAM'nin amit grupları arasında "yapıştırıcı" görevi görmesi ve makromoleküllerin intra- ve inter- moleküler kısımlarını birbirlerine yaklaştırması ve bu sayede makromolekülleri çökmüş gibi davranmasını sağlamaktadır. Ancak bu mekanizma PDEA makromolekülünde etkili olmamaktadır. Bulgularımız sadece sulu faz olgularına değil, aynı zamanda sulu ortamda makromoleküllerin çökmüş fazına ışık tutmaktadır.

Anahtar kelimeler: Üre, PNIPAM, PDEA, Denatürasyon Mekanizması, Düşük Kritik Çözelti Sıcaklığı (DKÇS), ATR – FTIR spektroskopisi.

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Figure 3.38 ATR-FTIR bottom heating measurement results of PNIPAM up to 6.0 M 1,3-Dimethylurea (shown with red squares) above the LCST (55 °C) as a function of 1,3-Dimethylurea concentration. The y axis of the graph indicates the intensity of 1,3-Dimethylurea over the intensity of 1,3-Dimethylurea without polymer at 1175 cm⁻¹ in H₂O. The standard deviations of PNIPAM up to 6.0 M 1,3-Dimethylurea for 4.0 M, 5.0 M, and 6.0 M 1,3-Dimethylurea are shown with error bars.....79

Chapter 1 – Introduction

1.1 Effect of Ions and Osmolytes to Biological Functions

Osmolytes are small organic molecules that are omnipresent in all organisms (e.g., animals, plants, bacteria, etc.), and significant evolution regarding their properties to these organisms has been observed in nature [1]. Osmolytes can be classified as polyhydric alcohols (polyols), amino acids and their derivatives, and combination of methylamines and urea in terms of their chemical structure. These types of osmolytes have been found in almost every water-stressed organism [1, 2]. It was shown that utilizing organic osmolyte systems have two major advantages: [1] i) compatibility of them with macromolecular structure and function at high or variable (or both) osmolyte concentrations, and ii) requirement of significantly less protein modification to function for them in concentrated intracellular solutions.

Regarding the effects of osmolytes on proteins and other (bio)macromolecules in aqueous environment, that can be categorized as “stabilizers”, in which these osmolytes are functioned as increasing the stability of proteins and/or (bio)macromolecules in aqueous solutions, and favours the folded state (or native state, N) compared to the unfolded state (U) of the interested protein for the equilibrium of $U \text{ (unfolded)} \rightleftharpoons N \text{ (folded)}$. The other subgroup of osmolytes acts as “denaturants”, where the function of these osmolytes is opposite to the stabilizers and they denature proteins and/or (bio)macromolecules in aqueous solutions, and favour the U state compared to N state for the interested proteins [2, 4]. Stabilizer, and denaturant osmolytes can be seen in part A of the Fig 1.1. The more well-known examples of stabilizer and destabilizer are the ions of Hofmeister series shown in part B of the Fig. 1.1. Note that the stabilizing (salting-out) ions and stabilizer osmolytes have similar influence on proteins and (bio)macromolecules. Additionally, a similarity can also be seen for destabilizing (salting-in) ions and denaturant osmolytes. Although detailed molecular mechanisms of ion-macromolecule interactions are reported for the Hofmeister ions, [5-10] the molecular level details of how osmolytes influence the aqueous medium, and macromolecules in the aqueous solution is still not fully known. Achieving molecular level detailed mechanisms of osmolytes affecting the stability of proteins and (bio)macromolecules in aqueous medium against several factors (e.g., temperature, pH, humidity, concentration, osmotic pressure etc.) is quite important. This is simply due to the natural presence of osmolytes in the cytosol and extracellular fluid of numerous organisms; fungi, protozoa, multicellular algae, vascular plants,

insects, cartilaginous fishes, amphibians, and even mammals including humans [1, 2] As a consequence, it is vital to investigate osmolytes and their behaviour in full-scale, since a variety of protein-related disorders are shown to cause by protein misfolding, protein aggregation (e.g., Alzheimer, and Parkinson) or conformational abnormalities, which can play an important role in the pathogenesis of amyloid-related diseases and pose a significant barrier to the production and development of protein therapies [1, 3, 11-14]

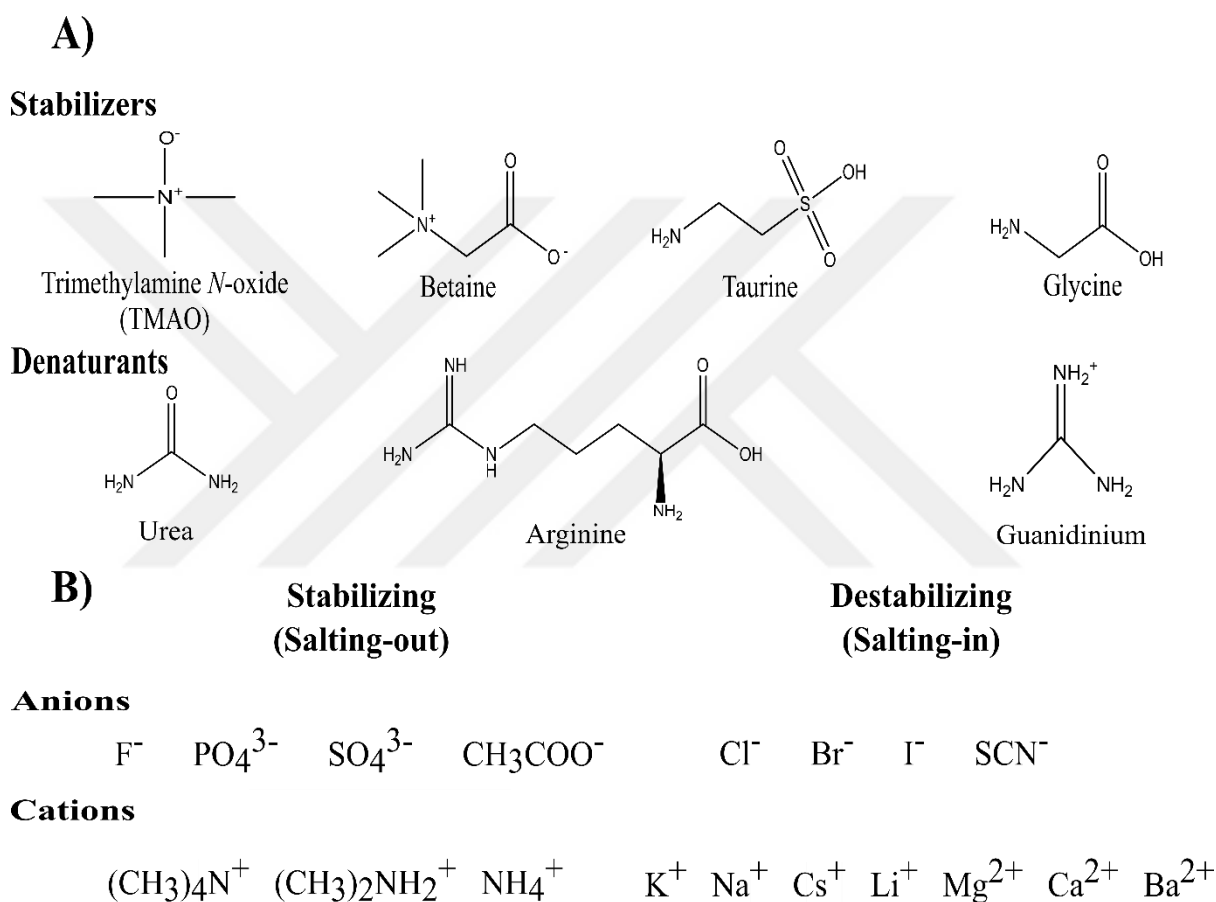


Figure 1.1 A) Few examples of stabilizers and denaturants that can affect macromolecular structure. The anionic and cationic sequences of the Hofmeister series. **B)** The anionic and cationic sequences of the Hofmeister series. Inspired from Ref [1].

In the following part, firstly, the Hofmeister series will be explained to demonstrate its impact to the observed effects as “salting-out” and “salting-in” agents to proteins and several other (bio)macromolecules, in which the former ones have similar effects to the interested macromolecules in aqueous medium as “stabilizers” of a subgroup of osmolytes, and latter ones have similar effects to the same molecules in the same conditions as “denaturants” of a subgroup of osmolytes. Later, guanidinium ion (Gnd^+), a well-known denaturant, will be discussed to explore its effect as denaturant by considering selected studies from the literature to further comprehend its molecular mechanism for the observed effects at several different systems. Accordingly, a section was added to explain the effect of examples of other osmolytes on proteins. Namely, trimethylamine *N*-oxide (TMAO) is discussed to demonstrate what is known about the molecular mechanisms of the stabilizing function compared to other stabilizing osmolytes. Furthermore, the nonadditive effects in osmolyte systems were discussed. When a stabilizer (TMAO), and a denaturant (urea) are present at the same time in aqueous medium, their effect is different from their individual effects to the aqueous solution and the macromolecules. The next subject covered is the comprehensive discussion of the influence of the urea molecule to reveal what is known about the urea molecule in the literature as a denaturant. Finally, the proposed urea-induced denaturation mechanisms are discussed by using poly (*N* - isopropyl acrylamide) (PNIPAM), which is a well-known thermoresponsive macromolecule that has been used extensively for mimicking proteins to study protein denaturation, is pointed out to illustrate the current understanding of the denaturation mechanisms.

1.1.1 Ions (The Hofmeister Series)

In 1888, Franz Hofmeister investigated the solubility of egg-white proteins in various aqueous salt solutions at different salt identity and concentrations and revealed an ion specific trend. The main outcomes of this investigation were the origin of the observed effects that was related with thermodynamics, but not kinetics over the timescale of the experiment, and the ability of particular salts to cause protein aggregation in the medium was in accordance with the nature of salts in an ion specific manner [15]–[17]. With these observations, the hypothesis of Franz Hofmeister was the capability of adsorption of water for each ion dictated its behaviour that with the consideration of this idea, classification of the interested salts was done accordingly as kosmotropes (which are known as strongly hydrated, or “water structure makers”), and chaotropes (which are known as weakly hydrated, or “water structure breakers”), in which nowadays this is known as classical Hofmeister series for anions, and it can be seen in Fig. 2 [15], [16], [18]. In compliance with this series, kosmotropes (ions with green background) demonstrates “salting-out” behaviour in solution that they lead to aggregation of macromolecules in the medium by decreasing the solubility and increasing the stability of the proteins, whereas chaotropes (ions with red background) demonstrates “salting-in” behaviour in solution that they lead to solubilize macromolecules in the medium by increasing the solubility and decreasing the stability of the proteins structure [17], [19].

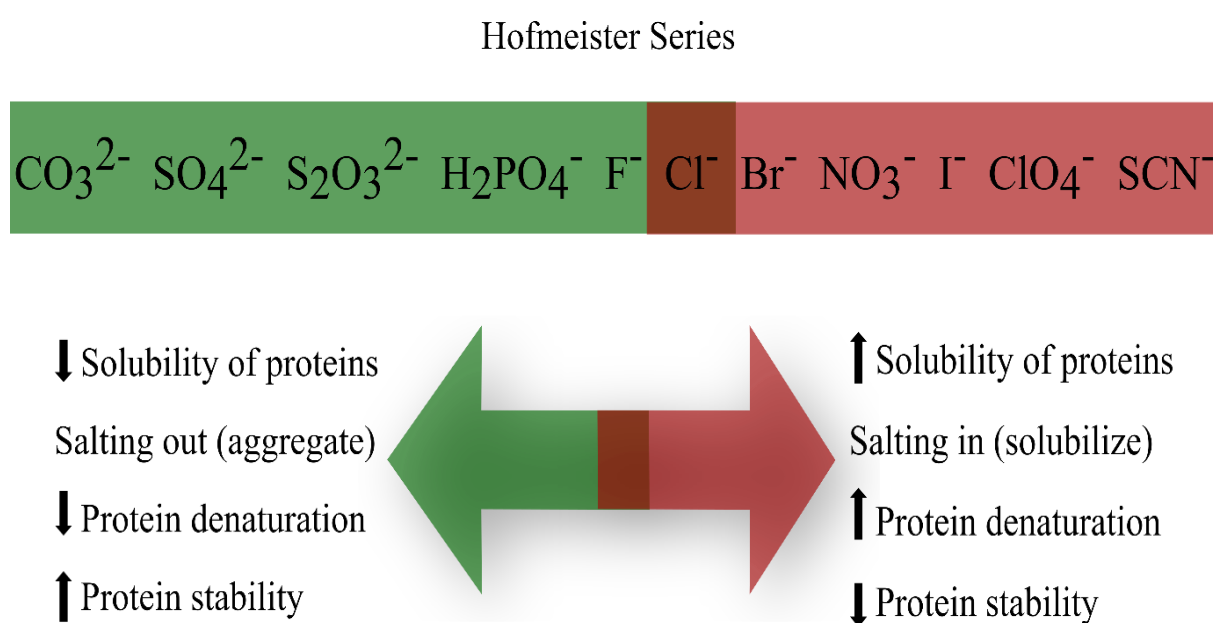


Figure 1.2 The Hofmeister series for anions. (Inspired from ref. [19])

The Hofmeister series have recently received a lot of attention owing to their importance in a variety of fields that enzyme activity [20]–[22], protein stability [23, 24], and protein-protein interactions [24, 25], proteins in aqueous solutions are only one of many instances of the physical behaviour obeying the Hofmeister series. Although Hofmeister effects for macromolecules in aqueous solution are common, the molecular-level processes by which ions function are still being studied [19] To investigate the effects of the Hofmeister series to the macromolecules in aqueous medium, time-resolved and thermodynamic studies [26, 27] of water molecules in salt solutions have been performed that these studies demonstrated the change in the bulk water structure is not a major component of the Hofmeister effect.

In line with the work of Bakker and coworkers, they used femtosecond pump-probe spectroscopy to measure the orientational time for water molecules in salt solution of Cl^- , Br^- , I^- , ClO_4^- or SO_4^{2-} salts. It was found that the dynamics of the water molecules were much slower in the first hydration shell of the anions compared to the bulk water, yet the structure of the bulk water was not affected by presence of concentrated (even at 6M) both kosmotropic (SO_4^{2-}), and chaotropic (ClO_4^-) anions, therefore the conclusion regarding to their work was ions do not participate long-ranged interactions with the bulk water as “structure maker” or “structure breaker” [26]. Note that this view is also challenged by recent elastic second harmonic scattering measurements of salts. It was shown that presence of ions generates a non- ion specific long-range water - water correlation in aqueous solution [28]–[30]

The same topic was also investigated by Pielak and his coworkers via pressure perturbation calorimetry to investigate the effect of stabilizers and denaturants on proteins (which included several sugars, guanidinium ion and urea, as well) by probing the sign of derivative of the partial molar heat capacity with respect to the pressure at constant temperature to assign the effect is a solute maker (< 0) or breaker (> 0) of the water structure. This is because heat is required to break the structure and thus lessening the structure decreases the heat capacity of the solution, that makes C_p inversely proportional to P at constant T , and vice versa for the structure breakers [27]. As a result of their investigation, there was no direct correlation between the type of solute that was used (stabilizer and denaturant) and the effect of structure making or breaking to the bulk water, thus it was shown not only via spectroscopic measurements but also via studying thermodynamics that the solutes do not act as a water structure makers nor water structure breakers for the bulk water [26], [27]

Finally, Cremer and his coworkers utilized surface selective vibrational sum frequency spectroscopy (VSFS), which can be used conveniently to examine both interfacial water structure and ordering of the monolayer, and the Hofmeister effect was exemplified in an octadecylammonium monolayer spread on salt solutions [31]. The tendency of any given anion to introduce gauche defects into the fatty amine film at constant temperature and pressure was highlighted due to the sensitivity of VSFS to the alkyl chain conformation of the monolayer, and the Hofmeister series was followed to the anion's capacity to disturb the alkyl chain region. [31]. This effect was linked strongly with the dispersion forces for determining the effects of anions, yet there was less obvious involvement of the anions in the interfacial water structure and hydrogen bonding [31].

With all these studies into the consideration, it can be stated that the kosmotropes, and chaotropes do not make or break water structures, respectively as depicted by the Hofmeister series for the anions. Instead, in order to further understand this subject with molecular level details, models based on direct ion-macromolecule interactions as well as interactions with water molecules in the initial hydration shell of the macromolecule were proposed [19] that these models were shown to be valid for the hydrophobic collapse of poly (*N* - isopropylacrylamide) (abbreviated as PNIPAM) [32], a well-known thermoresponsive polymer that collapses upon heating in aqueous solutions [33, 34], and its molecular structure can be seen in Fig. 3A. This was done by observing the lower critical solution temperature (abbreviated as LCST) behaviour of PNIPAM as a function of 11 different sodium salts to explore the Hofmeister series, since the hydrophobic collapse of polymers (and also proteins) are important for mimicking protein folding. Moreover, the LCST behaviour of PNIPAM, to a degree, is similar to the cold denaturation of proteins [32] By considering the sodium salts, while the capacity of certain anions (CO_3^{2-} , SO_4^{2-} , $\text{S}_2\text{O}_3^{2-}$, and H_2PO_4^-) were reducing the LCST typically followed the Hofmeister series, study of solvent isotope effects and variations in LCST with ion concentration and identity revealed that various processes were at work [32] The phase transition of PNIPAM was shown to associate with the hydration entropy of the anion in solutions with sufficient concentrations of strongly hydrated anions (CO_3^{2-} , SO_4^{2-} , $\text{S}_2\text{O}_3^{2-}$, and H_2PO_4^-) whereas weakly hydrated anions (I^- , ClO_4^- , and SCN^-) were salted out by surface tension effects and enhancement of the hydration through direct ion binding was displayed [32].

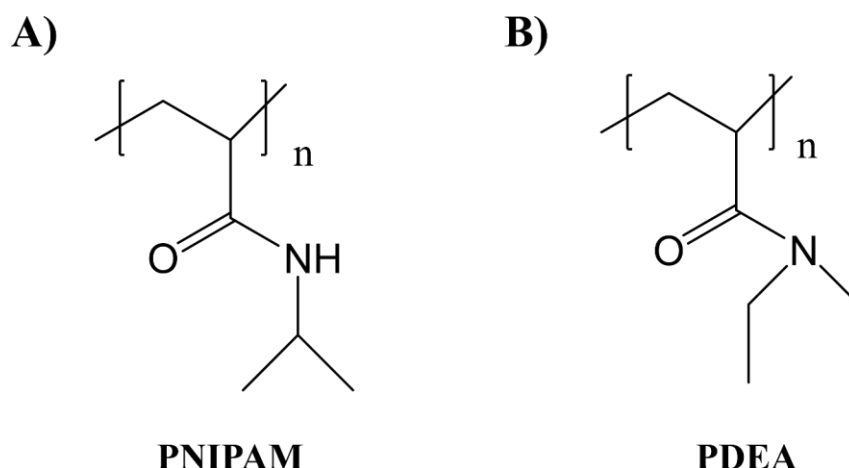


Figure 1.3 **A)** Representation of the molecular structure of poly (*N* - isopropylacrylamide), which is abbreviated as PNIPAM. **B)** Representation of the molecular structure of poly (*N, N* - diethylacrylamide), which is abbreviated as PDEA.

In the literature, the effects of anionic Hofmeister series were investigated not only using PNIPAM with its LCST behaviour at several molecular weights [35], and its monomer alongside other relatively small molecules (e.g., *N*-methylacetamide (NMA)) [36], but also other thermoresponsive polymers (e.g., Poly (*N, N* – Diethylacrylamide), abbreviated as PDEA) [37], and even elastin-like polypeptides (e.g., abbreviated as ELPs, more specifically ELP V₅-120 or ELP V₅A₂G₃-120 more hydrophilic one) [38] that with these investigation, the significance of hydrophilicity/hydrophobicity of functional groups of the interested macromolecules, chemical environment in aqueous solutions, as well as specific ion effects (kosmotropes vs. chaotropes) for the explanation of observed anionic Hofmeister effect was further comprehended.

For specific ion effects, the effect of thiocyanate anion (SCN⁻, a well-known chaotrope) were examined in the literature at air/water interfaces in water [39], and also very recently by using polyethylene oxide oligomers and with different molecular weights of polymers [40] that for other kosmotropic and chaotropic anions, the investigation of their specific effects to interfacial water structure near to PNIPAM [41], and bovine serum albumin (BSA) [42], alongside several elastin-like polypeptides (ELPs) [43] were also utilized for the same purpose.

Regarding the explanation of the effects of the observed Hofmeister anionic series for various molecules that has been stated above, a simple theory was also tried to be created to explain the Hofmeister series by Jun Xie et al. that by considering the surface functional groups of the protein that interacts with salt ions from the solution and including the ion pairing into

the thermodynamic model, authors were able to qualitatively explain several experimental and computational observations on the Hofmeister series; since the latter allowed the counter ion effects (considering both cationic and anionic contributions, which was mostly neglected for the consideration of the effects of Hofmeister series of the salts) [44] However, as stated in the paper of Pavel Jungwirth, which opposes the work of Jun Xie et al. by simply pointing out the deficiencies for explaining the Hofmeister series, many simplifications were done by the authors for their model to be followed easily; such as, only the backbone was considered but not the effects of side chains for the proteins that both sides play comparable roles in the Hofmeister series, or the hydrogen bonding and ion pairing were demonstrated simply that only the C=O groups had importance regarding the interactions of ions with the backbone of the proteins whereas the effects of N-H groups almost completely neglected [45] By considering this opposition with the factors that had been stated in the work of Pavel Jungwirth [45], one might be able to state as the direct Hofmeister anionic series cannot be explained with ease or with a rather simplistic approach for all interested molecules.

Aside from the direct Hofmeister series for anions, reverse of the same series was also observed for several systems that include the interfacial water structure of the interface of negatively charged hydrophilic, negatively charged hydrophobic, and positively charged hydrophilic aqueous/oxides by using vibrational sum frequency spectroscopy (VSFS), [46] triglycine [47], and also lysozyme [48], as well. For triglycine, it was studied by nuclear magnetic resonance (NMR) spectroscopy, and molecular dynamics (MD) simulations that by changing the charge of the uncapped N-terminus of the interested macromolecule from negatively charged to positively charged, the authors were able to observe the reversal of the Hofmeister anionic series (from direct Hofmeister series to reversed), and concluded as the chemical and physical characteristics of peptides and proteins play an important role in ion specific actions, subsequently this work provided a molecular justification of Hofmeister ordering for anions. For the latter one, it was found out that protein aggregation in salt solutions was discovered to follow two unique Hofmeister series depending on concentration of salt that at low salt concentrations, where the process was driven by the association of anions with the positively charged lysozyme surface, and the LCST followed an inverse Hofmeister series, in which inversion of the Hofmeister series might have a correlation between the size and hydration thermodynamics of the anions [47] Conversely, at higher salt concentrations, a direct Hofmeister series with a correlation of the polarizability of anions was found for the liquid-liquid phase transition that eventually these findings imply that positively charged

macromolecular systems should exhibit inverse Hofmeister behaviour only at low salt concentrations and then return to a straight Hofmeister series as salt concentration increases [48].

The rationalisation of the Hofmeister series can be done not just only for the anions, but also for the cations that even though the cationic Hofmeister series studies much less compared to the anionic Hofmeister series, the general trend for several cations of the Hofmeister series still can be depicted and shown in Fig 1.4.

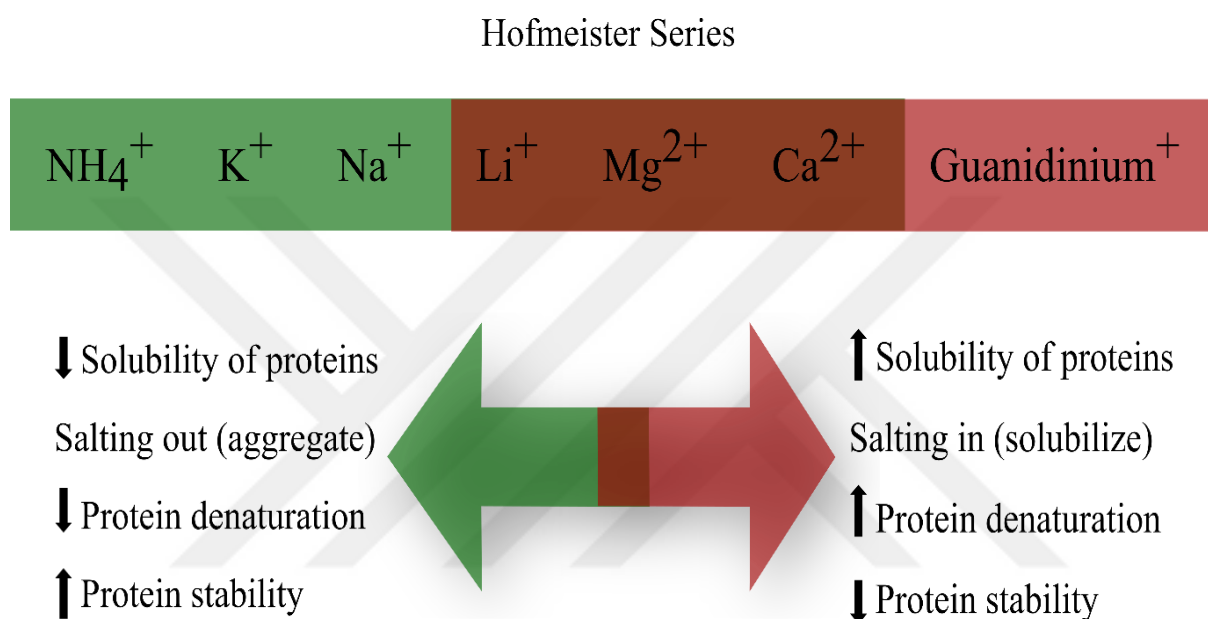


Figure 1.4 The Hofmeister series for some common cations. Inspired from ref. [49].

The investigation of the cationic Hofmeister series was done in the literature, in a way, recently that the primary focuses for exploring this series were accomplished by considering various systems, which includes: interfacial water structure on the surfaces of fused quartz and titanium dioxide (TiO_2) by probing (VSFS) [46], weak carboxylic acids [49], benzene [50], via MD simulations, butyramide for mimicking the backbone of a protein with Fourier Transform Infrared Spectroscopy (FTIR) alongside VSFS [51], PNIPAM with LCST measurements, and MD simulations [52], and also elastin-like polypeptides (ELPs) [53] that the specific ion effects were also considered for the same purpose by focusing on various monovalent cations, in which ion pairing can be observed in aqueous solutions, with dimethyl phosphate anion [54], as well as iodide ion for PNIPAM [55].

Lastly, regarding the Hofmeister series, the nonadditivity, combination of the effect of weakly hydrated (chaotropes), and strongly hydrated (kosmotropes) mixture of two salts in aqueous solutions [56], was explored very recently in the literature. It was reported as it could potentially affect the coil-globule transition of PNIPAM (in which the salts are Na_2SO_4 , and NaI that the concentration of former kept constant and the concentration of latter was increased up to certain point (0.1 M)) [56]–[58]. This kind of effect could also be observed not just for anions but also cations, too (this time the anions were only SO_4^{2-} , and I^- that four different alkali metal ions were investigated, namely Li^+ , Na^+ , K^+ , and Cs^+ that the LCST measurements were done with the usage of these salts in aqueous solutions for PNIPAM) [59] .



1.1.2 Osmolytes

Guanidinium ion (Gnd^+) is one of the most well-known osmolytes, alongside urea molecule, that can denature various proteins, in which it has been investigated in the literature for more than 60 years [60]–[63]. The representation of molecular structure of the guanidinium ion (and for other common osmolytes) can be seen in Fig. 1.5. Earlier investigations regarding the denaturant effects of Gnd^+ , together with urea molecule, could be observed on several protein mimicking molecules and also proteins, such as: acetyltetraglycine ethyl ester (ATGEE), and carbobenzoxyglycine derivatives [60], α -Chymotrypsin, and β -Lactoglobulin [61], cytochrome c [63], ribonuclease [61], [62], and lysozyme [61]–[63] that for each one of these studies demonstrated the overall better denaturant capability of Gnd^+ compared to urea. The experimental results regarding these studies were obtained by using primarily solubility data of interested proteins with addition of these osmolytes into medium as a function of molarity, temperature, etc., plotting denaturation curves as a result of fluorescence spectroscopy (as an example), and eventually calculating apparent binding constants to have an information about conformational free energy change ($\Delta G_{\text{H}_2\text{O}}$) between native and denaturated states of interested proteins, and also enthalpy and entropy individually with the addition of Gnd^+ , urea molecule or any other osmolytes [60]–[63]. Even though several attempts were made to explain the observed denaturant effect of Gnd^+ to proteins in the light of obtained results from these investigations by using “simple binding model” to indicate that there is a direct interaction between the osmolyte and protein, or possible indirect interactions (e.g., hydrophobic effects or interactions between osmolyte and aqueous medium) [60]–[63], these attempts unfortunately could not provide molecular level explanation for the interested topic during that time period. With the improvement of research tools over time (especially from 21st century), by using MD simulations [64]–[66], thermodynamic measurements (LCST) [66], and spectroscopy (improved versions of NMR, [64, 66] FTIR, [66] etc.), the molecular mechanism behind the denaturant effect of Gnd^+ was explored in depth.

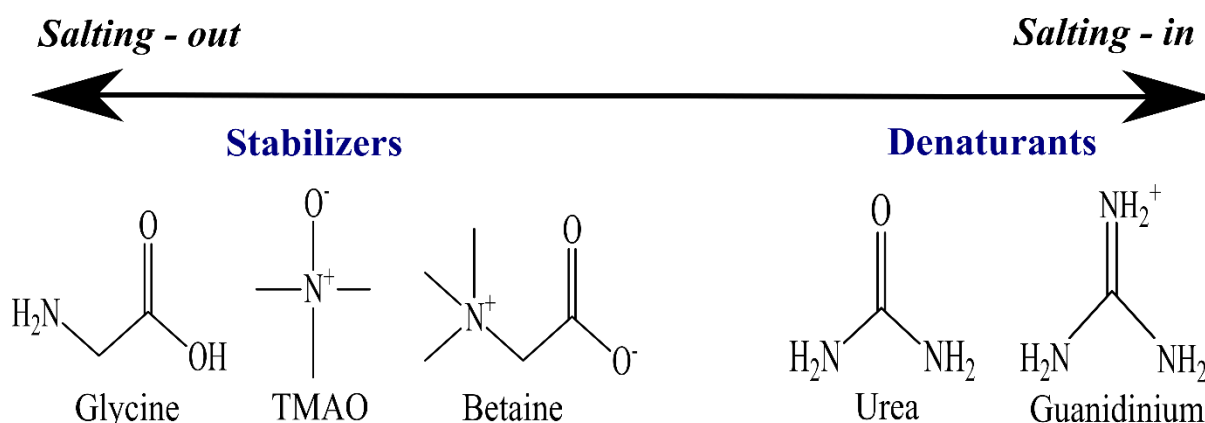


Figure 1.5 The rank order of common osmolytes typically found for neutral and negatively charged macromolecules [68]. (Inspired from ref. [68])

By using circular dichroism (CD), differential scanning calorimetry (DSC), NMR and MD simulations, denaturation of the Trp-cage miniprotein by urea and guanidinium was investigated by Heyda et al. that two-state unfolding process was determined from the experiments, even though each experimental technique was sensitive to different aspects of the denaturation process [64] DCS provided information about gross thermodynamic changes that within the temperature interval (from 10 to 120 Celsius degrees), calorimetric curves induced already by 0.05 M of denaturants, CD gave information regarding over global changes in the alpha helical content that guanidinium was two times more effective as a denaturant with respect to the urea for the unwinding of the α -helix, whereas NMR shed light on per residue changes in electronic environment that no intermediate was detected for the unfolding of the Trp-cage miniprotein by urea and guanidinium [64] Microsecond MD simulations with femto-second time resolution were able to explain the molecular mechanism of Trp-cage unfolding in a detailed manner, in which at 2.0 M concentration of the denaturants, the unfolding was observed [64] In overall, it was stated as urea was slightly better denaturant compared to guanidinium by considering the results of MD simulations (but DC results stated the opposite) [64] Even though there were differences regarding the interaction of urea and guanidinium with several peptide moieties (since urea has propensity for the backbone, whereas guanidinium tends to interact with side chains that have different polarities and charges), in general the destabilizing effect of urea and guanidinium to Trp-cage was similar [64].

With the usage of FTIR spectroscopy, LCST measurements, MD simulations and thermodynamic modelling, mechanisms related to effects of guanidinium (Gnd^+) to the collapsed and uncollapsed states of elastin-like polypeptide (ELP, which consisted of 120 repeating pentameric units of Val-Pro-Gly-Val-Gly (VPGVG_{120}) and it is an uncharged

thermoresponsive polymer) were investigated by Okur and his coworkers [66] According to the findings, by changing the anion pair of the Gnd^+ , the action of cation varied that the depletion of Gnd^+ was observed from the ELP/water interface upon pairing with SO_4^{2-} (a salting out anion) in the solution; in which, this process led to the stabilization of the collapsed state of the ELP via excluded volume effect and the strong partition from the ELP/water interface was detected for the anion, accordingly [66] For $\text{Gnd}^+ - \text{SCN}^-$ ion pairing (where the anion is a well-known salting in anion and binds to ELP strongly), two different effects were observed for affecting the collapsed and uncollapsed states of ELP at two concentration regimes that at low concentrations, the collapsed state of ELP was favoured owing to cross-linking of the cation to the polymer via inter and intramolecular interactions, where the partition of the cation was also assisted with SCN^- , whereas for higher concentrations ($> 1.5 \text{ M GndSCN}$); the uncollapsed state of ELP was stabilized due to prevention of cross-linking binding since there were considerable amount of the anion and cation that partitioned to surface of the polymer [66] Lastly, for GndCl , the uncollapsed state of ELP was favoured at all tested concentrations of salts that it interacted with an intermediate fashion with ELP [66] The obtained results gave a precise, molecular-level mechanistic understanding of how changing the anion effects the stability of polypeptides in three unique physical regimes, and it also assists for comprehending why guanidinium salts may be such effective and diverse protein denaturants [66].

From Fig. 1.5, it can be seen that Gnd^+ is on the right-hand side of the chart, meaning that it is a denaturant, yet osmolytes can also be classified as “stabilizer”, as well. Trimethylamine *N*-oxide (TMAO) belongs to that classification, and it is a well-known stabilizer for proteins and several other (bio)macromolecules, in which its molecular structure can be seen on Fig. 1.5. Regarding the stabilizing effect of TMAO, various studies were done for exploring the mechanism behind it in the literature recently that one of the most important foundations with this subject was observing the complex formation of TMAO in aqueous solutions. Bonn and his coworkers used broadband dielectric spectroscopy and femtosecond mid-infrared spectroscopy, for investigating the aqueous solutions of trimethylamine-*N*-oxide (TMAO). [67] According to this study, slower rotational dynamics were observed for water molecules that were interacting with hydrophobic parts of TMAO molecules, and interactions of water molecules were found to be stronger with hydrophilic N-O group of TMAO molecules, which lead to formation of stable TMAO-water complexes ($\text{TMAO} \cdot 2\text{H}_2\text{O}$ / $\text{TMAO} \cdot 3\text{H}_2\text{O}$) that life span of these complexes was found to be more than 50 ps (extremely long) [67] Because of these observations and spectroscopic findings regarding to O-D stretching vibrations, it was

stated by authors as TMAO stabilizes proteins via an indirect mechanism, since it can form relatively stronger and long-lived complexes with water (TMAO.2H₂O/TMAO.3H₂O) and this leads to proteins to be dehydrated (thus, intact secondary and/or tertiary structure for proteins) compared to other amphiphilic molecules such as; tetramethylurea (TMU) and *t*-butyl alcohol (TBA), in which they are known as destabilizers [67].

The molecular orientation of TMAO in aqueous medium at water/hydrophobic interfaces was also investigated in the literature to acquire information regarding the hydrophobicity/hydrophilicity of parts of TMAO (methyl groups vs. NO moiety, etc.) that this was accomplished by using vibrational sum frequency spectroscopy (VSFS) in the work of Sagle et al. [68] For this purpose, the octadecyltrichlorosilane (OTS)/water interface was examined, in which it was considered as a representation of an aqueous solution that contacts directly with a hydrophobic medium, and methyl groups of TMAO directed away from the OTS and into the aqueous phase according to the measurements [68] According to the authors, the highly hydrophilic nature of methyl groups linked to a strongly electron-withdrawing atom, such as quaternary nitrogen, might explain this orientation that further investigations at the air/water interface revealed a high degree of TMAO alignment, although dangling OH from water was still present even at 5 M TMAO [68] Furthermore, the addition of this osmolyte generated moderate increase to the surface tension of the interface, suggesting that this species was relatively depleted at the interface compared to the bulk solution, in which these findings could potentially have implications to effect of TMAO on proteins as a stabilizer [68]. The depletion of the osmolyte from hydrophobic/aqueous interfaces could occur owing to the significant hydration required for the methyl groups, and also the oxide moiety [68]. With the consideration of entropy, stabilization of the folded and native conformations of proteins should be assisted with depletion effects, even though orientational effects might have a role for this case, too [68].

Elucidation of the mechanism for stabilizing effect of TMAO, betaine, and glycine (well-known stabilizers, can be seen in Fig. 5) to macromolecules was done recently by Liao et al. that the hydrophobic collapse of an elastin-like polypeptide (ELP) was examined by observing the effects of these osmolytes [69]. The stabilization of the collapsed conformation of the ELP was detected with the addition of each interested osmolyte, whereas the LCST was decreased linearly as a function of osmolyte concentration [69] The investigation of the surface tension at the air/water interface demonstrated that both betaine and glycine increased the surface tension, yet TMAO affected oppositely [69]. By considering the obtained results of

atomically precise molecular dynamics (MD) simulations, the accumulation of TMAO at the polymer/water interface was observed modestly, however there were severe depletions of glycine and betaine from the same interface [69]. The authors conducted further FTIR measurements to evaluate the effect of each osmolyte to the water structure of bulk water for identifying alternative mechanisms regarding the effects of osmolytes, and according to the obtained data, the OH stretch of the IR spectra for TMAO red shifted that the mechanism behind it was very sensitive to the protonation state of the NO moiety [69]. Similar observation was also done for glycine, but there was no effect for betaine; which indicates that there was no correlation between the observed effect of osmolytes to the hydrophobic collapse of the ELP and the effects of osmolytes to appeared OH spectrum of IR spectra for each [69]. Lastly, the results of MD simulations showed that the perturbation of the water structure was the least from TMAO, and the most from glycine that these findings indicated that the stabilization of the collapsed state of the ELP occurs via different mechanism for TMAO compared to other two osmolytes and it is believed by authors as stabilization of proteins with TMAO takes place as a surfactant on the diverse surfaces of folded proteins [69].

One of the interesting features about TMAO is that it can be found in many marine organisms, such as cartilaginous fishes (e.g., sharks and rays), in which TMAO can be utilized for counteracting the denaturing effects of urea for these fishes [1], [70]. The counteraction of TMAO against urea for osmotic pressure on these animals kept attention in the literature that several studies about this subject was done to understand molecular mechanism for urea, TMAO, and urea + TMAO in aqueous medium with various molecules. By using dielectric spectroscopy in the regime from 100 MHz to 1.6 THz, the interaction between urea, TMAO and water was investigated by Hunger et al. that TMAO.3H₂O complexes could be observed for all tested urea concentrations and there was not any significant hydrogen bonding between urea and TMAO [71]. For equal amounts of urea and TMAO in a ternary solution, an anomalous nonlinear increase was observed for the solute reorientation times for both urea and TMAO, even though lacking a direct interaction between urea and TMAO [71]. Because of this finding, it was stated from the authors as counteraction of TMAO in the presence of urea is not happening due to a direct interaction between them, instead water mediated interaction between urea and TMAO was stated as an indicative for the nonlinear increase [71]. A correlation between the nonlinear increase of the reorientation and change in viscosity was observed that the hydrogen bond structure of the ternary solution was enhanced in a cooperative manner with urea and TMAO [71]. Therefore, both TMAO and urea leads to formation of enhanced

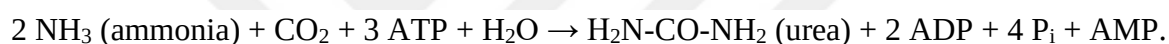
hydrogen bond network owing to balancing the defects in the hydrogen-bonded structure of water from TMAO (which has three hydrogen bond donor sites and a hydrogen bond acceptor site) and urea (which has four hydrogen atoms to be used as hydrogen bond donor) [71] Thus, it was concluded by authors as the rationalization of the mechanistic functioning of TMAO as osmoprotectant can be done by considering both anisotropic distribution of hydrogen-bond donor and acceptor sites at urea and TMAO, and the formation of strong complexes (TMAO.3H₂O) due to the interaction between TMAO and the water molecules [71].

The same subject was also investigated by using ab initio molecular dynamics simulations (AIMD), polarization-resolved femtosecond infrared pump-probe spectroscopy and nuclear magnetic resonance spectroscopy (NMR), the hydrophobic interaction between TMAO and urea was shown by Bonn and his coworkers, where there is no direct hydrogen bonding between them in aqueous solutions [72]. In accordance with their findings from this investigation, due to the stronger hydrogen bonding between TMAO and the water molecules with respect to the weaker hydrogen bonding between urea and the water molecules, the unfavourable interactions between TMAO and urea could be associated via the methyl groups of TMAO [72]. Thus, there is no replacement of hydrogen bonded water molecules to TMAO by urea that the compensatory osmotic effect of TMAO and urea can be rationalized by considering their interactions with the water molecules separately [72].

Similar results were also obtained by Nico F.A. van der Vegt on his several studies that with the usage of MD simulations, he investigated the same systems by using several amino acid monomers, and glycine [73], neopentane [74], and 15-residue polyalanine chain, together with R2 – fragment of Tau protein [75]. The general obtained results from these studies and many others can be stated as there was no favourable direct interaction between urea and TMAO, whereas the interaction between TMAO and water molecules led to overall observed stabilizer effect of TMAO in urea + TMAO solutions indirectly [58], [73]–[76].

1.2 Urea as Denaturing Agent

Urea is a universally well-known osmolyte as denaturant to numerous proteins (e.g., chymotrypsin, Trp-cage miniprotein, trypsin, ubiquitin, ovalbumin, human placental cystatin, etc.) [77]–[83] that it has been employed in laboratories to aforementioned types of proteins and several more to explore the urea-induced denaturation mechanism, and it is known in the literature as denaturation of the proteins occurs by unravelling native structure of the proteins with urea via destabilization of internal, and non-covalent interactions. Even though urea is utilized unnaturally by researches to investigate the denaturation mechanism in their researches, in nature, urea is produced by mammals, including humans, that can be considered as primary producers of urea, and it also found in the urine of some amphibians, alongside some fishes [84] The production of urea for mammalians occurs due to the urea cycle, where urea is converted from ammonia that the overall chemical reaction for this conversion can be shown as: [84]



The investigation of the denaturant property of urea was proceeded almost more than 90 years ago that primarily osmotic pressure measurements were employed with variance of the estimation for the molecular weights of interested proteins (e.g., serum albumin, gliadin, serum globulin, excelsin etc.) by using urea at different conditions (e.g., being near or far away from the isoelectronic point (PI) of the interested protein, variation of the temperature, several concentrations for urea, etc.) [85]–[89] Alongside these investigations, the utilization of solubility measurements [90], circular dichroism (CD) [91], as well as rudimentary NMR [92], were also done subsequently starting from 1960's, in which the effect of urea as denaturant to several proteins was attempted to be comprehended in the literature. Even though there are numerous studies regarding this subject, agreement remains elusive for the urea induced denaturation mechanism that two primary lines of reasoning have been offered in the literature, and summarized in Fig. 1.6. According to the "indirect mechanism," urea increases easy solvation of hydrophobic groups by modifying water structure that this mechanism was primarily proposed from transfer tests that revealed hydrocarbons to be more soluble in aqueous urea of the work of Frank et al. [93] and has since been challenged by further research [27], [94]–[96]. The opposite point of view links the denaturation to the protein's "direct" interactions with urea, also known as "direct mechanism" [60], [63], [97]–[99]. There is disagreement within the direct contact model about the type and degree of the interactions of

urea with the polar/apolar side chains and the peptide backbone, as well as the function of hydrogen bonding.

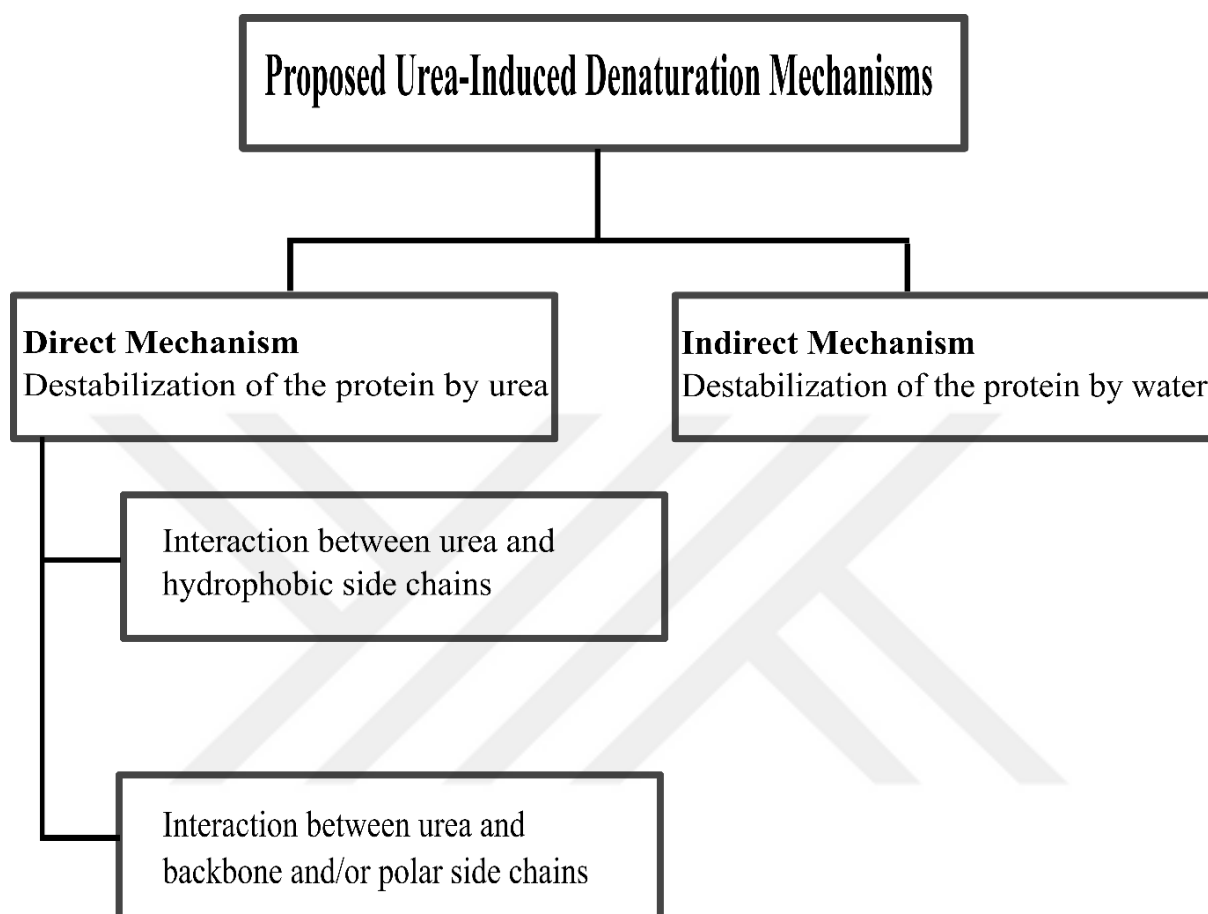


Figure 1.6 The representation of proposed urea-induced denaturation mechanisms in the literature.

Nowadays, with the improvement of surface specific techniques, the investigation of the urea-induced denaturation mechanism can be done with molecular level details. For instance, by using protein mimicking polymer (namely, poly(*N*-isopropylacrylamide), abbreviated as PNIPAM, a well-known thermoresponsive polymer that collapses upon heating in aqueous solutions [33], [34]), in which it has LCST behaviour that can be considered as cold denaturation/renaturation of proteins [32]. With the usage of PNIPAM, the investigation of the interested topic was done by several authors in the literature recently. In the next section (Section 1.3), explanations of the several studies regarding urea-induced denaturation by using PNIPAM in a detailed manner are displayed.

1.3 Urea Denaturation Mechanisms in the Literature

In the literature, several investigations of the urea-induced polymer collapse mechanism can be found that the effect of urea to the collapse mechanism of polymers are investigated by using molecular dynamics (MD) simulations [100], [101], theoretical calculations [102], NMR [103], dynamic light scattering (DLS) [104], and FTIR spectroscopy [105]. Ropero et al. studied PNIPAM by using molecular dynamics (MD) simulation, where the main topic addressed in their work was why urea stabilizes the folded (globular) configuration of PNIPAM in aqueous solution. It was also observed in their earlier studies that this question could not be answered properly by solely analyzing structural data from molecular simulations [100]. The PNIPAM coil-to-globule collapse transition that resulted in a swollen globular form in both systems, pure water and 5.8 M urea, with the amide groups retaining the majority of their hydrogen bonds with water (or in other words, hydration of the amide groups was observed for before and after folding) [100]. As a result, it was stated from the authors as, a direct hydrogen bonding mechanism involving the amide group was unlikely to sustain the folded form of PNIPAM in aqueous urea solution' and this finding was confirmed further by a comparison of the electrostatic energy distributions of urea and water molecules in the FSS (first solvation shell) and the bulk solvent [100]. There were differences in the structural polymer properties of the unfolded and folded states in water and urea solution that these changes, yet, had no influence on the solvent (urea, water) binding energy for those states at temperatures above and below the unfolding-folding transition temperature [100]. It was discovered that PNIPAM was preferentially solvated by urea in both folded and unfolded forms due to favorable urea-PNIPAM van der Waals (VdW) dispersion interactions, and the preferential urea solvation was more significant at the isopropyl group, resulting in the formation of urea clouds around the PNIPAM chain [100]. Because urea had favorable interactions with the polymer, it was logical to assume that (according to the authors) urea moves the folding equilibrium to the unfolded state in order to increase the solvent exposed surface area, yet the opposite was observed during the research that the formation of urea clouds caused by favorable enthalpic urea-PNIPAM interactions resulted in an overcompensating unfavorable entropy, which shifts the folding equilibrium to a folded state with a reduced exposed surface area [100].

Similarly, van der Vegt et al. proposed an osmolyte clouding mechanism as a result of their molecular dynamics (MD) simulations, in which folding causes a decrease in osmolyte accumulation, where this process is entropically driven that fluctuation of attractive interactions between solute and solvent is responsible for the hydrophobic interaction, and these attractive solute-solvent interactions are related to concentration fluctuations of the solvation shell of the combined solutes [101]. According to the osmolyte clouding mechanism, it can lead to a decrease of the lower collapse temperature, if the change in the polymer-solvent energy of the transition from extended state to coiled state is not significant that an illustration of this mechanism can be done by considering the potential mean forces between two neopentane molecules (as a sample molecule) in pure water and in 6.9 M urea solution from the previous study of the same author [101], [106]. With the consideration of the obtained results from this study, despite the fact that urea improves water solubility of neopentanes, and functions as a protein denaturant at this concentration, it strengthens the hydrophobic interaction of the two neopentane molecules [106]. Moreover, the contribution of cavity fluctuations to the potential mean forces in water, and 6.9 M urea is comparable that the crucial difference is due to fluctuating attractive solute-solvent interactions, which contribute to a greater increase in entropy in 6.9 M urea solution when the hydrophobes are in contact [106]. This process is weaker than depletion stabilization of hydrophobic contacts due to energy-entropy compensating effects, and it was examined that it causes unanticipated urea effects in hydrophobic folding equilibria (namely for thermoresponsive polymers, PNIPAM and PDEA) [101], [106].

The investigation of the urea-induced denaturation of proteins by Sagale et al. via direct binding mechanism was done by using a thermoresponsive polymer, PNIPAM, spectroscopically that Fourier transform infrared (FTIR) spectroscopy, and also Stokes radius measurements were utilized to supplement thermodynamic studies of hydrophobic collapse of PNIPAM [105]. The lower critical solution temperature (LCST) of the macromolecule was discovered to decrease as urea was added to the solution (up to 7.0 M). [105] As a result, urea promoted in the hydrophobic collapse of PNIPAM [105]. Furthermore, these thermodynamic measurements were significantly associated with amide I band data, indicating that the drop in the LCST was caused by direct hydrogen bonding of urea to the polymer's amide moieties, in which the obtained results distinctly implied this hydrogen bonding via observation of a new peak around 1652 cm^{-1} and increased intensity of this peak with increasing the concentration of urea, alongside the single peak of the amide I band around 1625 cm^{-1} [105]. Also, the

hydrogen bonding was discovered to be very cooperative, consistent with a cross-linking (bivalent binding) mechanism that Stokes radius measurements below the LCST of PNIPAM using gel filtration chromatography verified cross-linking [105]. In Fig. 1.7, representations of the studies of each described urea denaturation mechanisms can be seen.

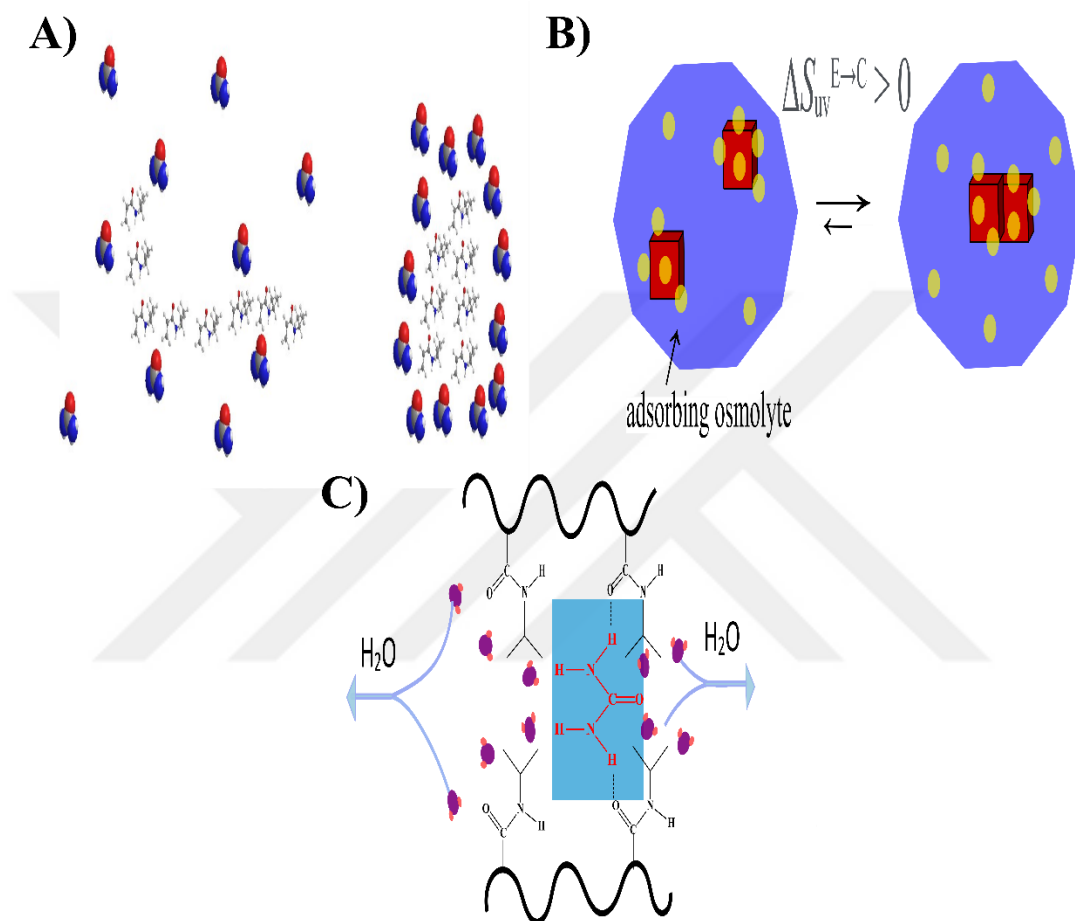


Figure 1.7 The schematic representations of proposed urea denaturation mechanisms from Ref. [101], [102], [106]. **A)** part of the figure (urea clouding mechanism) is inspired from [101], **B)** part of the figure (osmolyte clouding mechanism) is inspired from [102], and **C)** part of the figure (cross-linking mechanism) is inspired from [106], respectively.

Even though there are several studies regarding the urea-induced collapse mechanism that can be found in the literature, the consensus on the molecular machinery of urea denaturation is yet to be achieved. In this thesis, the urea-induced solubility changes of macromolecules in aqueous solutions were explored by exploiting the lower critical solution temperature (LCST) of poly (*N* - isopropylacrylamide) (PNIPAM) and poly (*N*, *N* -diethyl acrylamide) (PDEA) at various concentration of urea (up to 6.0 M, with 0.5 M increments). The LCST measurements of both macromolecules pointed out that the solubility of PDEA increases with increasing the urea concentration, whereas PNIPAM has a “salting-out” behaviour under the same conditions, albeit the molecular structures of these macromolecules are similar to each other. In contemplation of investigating the interaction between urea and the collapsed state of the macromolecules, a temperature-controlled ATR - FTIR based method was developed. The results of ATR - FTIR measurements indicated that both PNIPAM and PDEA favour the unfolded structure above the LCST evident by the depletion of macromolecules from the warmer parts of the aqueous medium, without any favourable urea accumulation towards the collapsed form of macromolecules for all tested urea concentrations. This finding was contrary to the proposed urea clouding mechanism in the literature. By utilizing the temperature-controlled ATR - FTIR based method, the behaviour of these macromolecules was further investigated, and accumulation of both PNIPAM and PDEA was observed towards the cooler parts of the solution at elevated (> 3.0 M) urea concentrations. This observation was also evidence again for soluble macromolecule conditions. These findings suggest that urea molecules act as a "glue" between the amide groups of PNIPAM, bringing intra- and inter-molecular parts of the macromolecule together, even though this mechanism might not be pertinent to PDEA macromolecule.

Chapter 2 – Materials and Methods

2.1 Materials

Poly(*N*-isopropylacrylamide) (PNIPAM) (Polymer Source, Inc., 118.500 g/mol), poly(*N*-isopropylacrylamide) (PNIPAM) (Sigma Aldrich, 85.000 g/mol), poly(*N,N*-diethylacrylamide) (PDEA) (Polymer Source, Inc., 55.000 g/mol), urea (Sigma Aldrich, 99.0-100.0% purity), 1,1-Dimethylurea (Sigma Aldrich, 99% purity), 1,3-Dimethylurea (Sigma Aldrich, $\geq 95\%$ purity), 1,3-Dimethylurea (Sigma Aldrich, $\geq 99\%$ purity), 1,1,3,3-Tetramethylurea (tetramethylurea) (Alfa Aesar, 99% purity), deuterated urea (urea- d_4) (Sigma Aldrich, 98% D), sodium thiocyanate (NaSCN) (Sigma Aldrich, $\geq 98.0\%$ purity), sodium sulfate (Na_2SO_4) (Sigma Aldrich, $\geq 99.0\%$ purity), H_2O (Millipore Nanopure system, 18.2 M Ω cm), deuterium oxide (D_2O) (Eurisotop, 99.90% D) were used without any further purification.

2.2 Sample Preparation

2.2.1 Lyophilization of Polymer Samples

In order to perform LCST and ATR-FTIR measurements, the exact mass of the polymer sample must be known and the polymer sample must be as pure as possible. Hence, lyophilization of polymer samples is required. For this purpose, primary preparation of polymer stock solutions with 20 mg/mL concentrations in 10 mL volumetric flasks was done by measuring appropriate mass of solid polymer and dissolving with deionized water. Prepared stock solutions were stored in the refrigerator overnight to make sure that the polymer (PNIPAM and/or PDEA) is dissolved completely and vortexed 1 - 2 times during this time to assist dissolution. Then, preparation of samples in Eppendorf tubes was done by transferring appropriate volume aliquots of stock solution into Eppendorf tubes with a micropipette. For LCST measurements, 25 μL of the 20 mg/mL stock solution and 75 μL of deionized water were used to prepare samples in Eppendorf tubes, where the final Eppendorf sample would be 100 μL solution of 5 mg/mL and for ATR-FTIR measurements, 100 μL solution of the 20 mg/mL stock solution was used to prepare samples in Eppendorf tubes. Transfer of the aliquot to the bottom of the Eppendorf tube was done as cleanly as possible; since solution droplets on the walls of the Eppendorf tube would leave solid residue during the vacuum-drying. The Eppendorf tubes were sealed with punctured lids, where the holes on the lids were necessary for allowing the vacuum-caused drying out to actually occur during the lyophilization step. For

each set of samples in Eppendorf tubes, separate punctured lids were used to lyophilize different polymers and polymer sources for avoiding contamination (e.g., a separate set of punctured lids were used for PNIPAM/Sigma Aldrich, PDEA/Polymer Source and PNIPAM/Polymer Source). Next, the Eppendorf tubes were submerged into liquid nitrogen to freeze the samples around 5 minutes before proceeding to vacuum-drying in the desiccator. Later, the Eppendorf tubes were removed from the liquid nitrogen and were placed upright inside a box quickly for avoiding any melting of the samples while exposed to room conditions. The box of samples was placed inside the desiccator and the samples were vacuum dried overnight. Lyophilization (also known as freeze-drying) is a process in which water and other contaminants (e.g., volatile impurities, monomers etc.) are removed from the sample after it is frozen and placed under vacuum, allowing the sample to be sublimated [107]. Therefore, lyophilized samples result in identical Eppendorf tube polymer samples of known mass, which can then easily be dissolved/diluted to obtain the desired solutions for LCST and ATR-FTIR measurements. After completion of the vacuum-drying, the samples were removed from the desiccator and were used for LCST and/or ATR-FTIR measurements, accordingly. The setup for the vacuum-drying in the desiccator of the lyophilization of polymer samples can be seen in Fig. 2.1.



Figure 2.1 The setup for the vacuum-drying in the desiccator (on the right) of the lyophilization of polymer samples with a vacuum pump (on the left).

2.2.2 Preparation of Stock Solutions of Osmolytes and Salts

For the preparation of stock solutions of urea, urea-d₄, 1,1-Dimethylurea, 1,3-Dimethylurea, deuterated 1,3-Dimethylurea, tetramethylurea, sodium thiocyanate, and sodium sulphate, appropriate masses of each compound were weighted into 10 mL volumetric flask and were dissolved in deionized water or in D₂O to obtain specific concentrations for the stock solutions and lower concentrations were obtained by serial dilution with deionized water or D₂O.

2.3 Methods

2.3.1 Lower Critical Solution Temperature (LCST) Measurements

LCST measurements were performed with the Stanford Research Systems (SRS) OptiMelt instrument, MeltView MPA100. OptiMelt is used primarily to determine the melting points of chemical substances, with microprocessor-controlled temperature ranging (the temperature range is 10-400 °C), which can be utilized to acquire data of melting point with ± 0.1 °C accuracy, and a built-in digital camera, which provides real time images of the interested samples over the temperature range that a phase transition during heating can be observed [108]. The LCST values of the interested samples were done by observing the starting temperature of an abrupt intensity change of light over the temperature range. In Fig. 2.2, an exemplary graph for determining the LCST value of an interested sample can be seen.

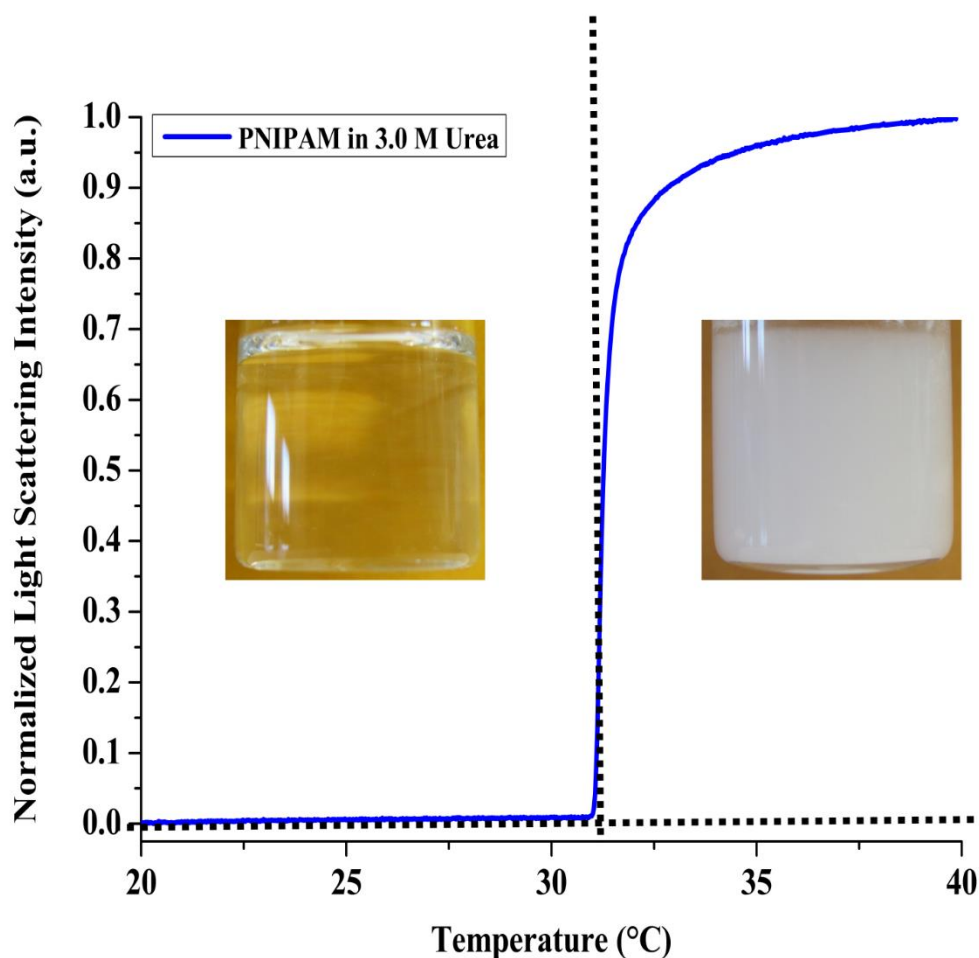


Figure 2.2 The graph of normalized scattering light intensity (a.u.) versus temperature (°C) for PNIPAM in 3.0 M urea. The image on the left represents the unfolded (or uncollapsed) state of PNIPAM (below the LCST) and the image on the right represents the folded (or collapsed) state of PNIPAM (above the LCST). The dashed lines were placed on both axes onto the graph to indicate the starting point of the intensity change of light over the temperature range.

For the LCST measurements, 100 μL of the interested samples (lyophilized 5 mg/mL of PNIPAM or PDEA in deionized water, in D_2O , in urea solutions and its derivatives (alongside urea- d_4 solution) and in NaSCN and Na_2SO_4 salt solutions) were injected into three of rectangular soda lime glass one end sealed capillary tubes (from Paul Marienfeld GmbH & Co. KG with dimensions of 80 mm x 1.50 mm x 1.20 mm (length x height x width)) by using disposable syringes (from Iso-Lab, 2 mL). During injection of the interested samples into the capillary tubes, the temperature of the environment, and the tools should be maintained below the LCST of the interested samples as much as is feasible to avoid a phase transition for the interested sample before measuring the LCST value with OptiMelt. Therefore, to adjust the temperature of the environment, capillary tubes and syringes were placed into an ice bath. The

LCST measurements were preceded by adjusting start and finish temperatures accordingly and heating rate was set up as 1.0 °C/min for each interested sample. Light scattering intensity from the samples was monitored with a high-resolution camera of the OptiMelt and LCST value of the interested sample was determined as the starting temperature where the light scattering intensity was changed sharply for the interested sample over a wide range of temperature. A representative example of results of LCST measurement can be seen in Fig. 2.3.

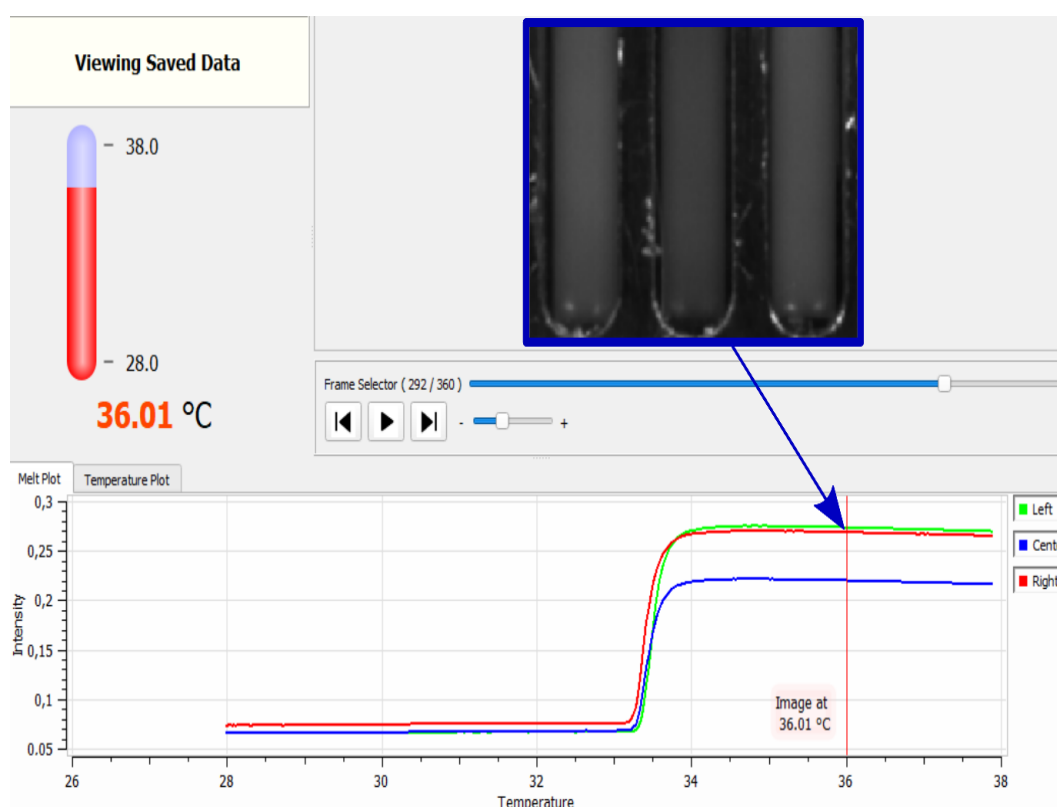


Figure 2.3 The results of the LCST measurement of lyophilized 5 mg/mL of PNIPAM in 3.0 M urea for three samples (shown with green, blue, and red). The folded (or collapsed) state of PNIPAM can be seen on the digital camera image around 36 °C (indicated dark blue rectangle, and arrow, above the LCST).

2.3.2 Attenuated Total Reflectance – Fourier Transform Infrared Spectroscopy (ATR – FTIR) Measurements

Attenuated Total Reflectance – Fourier Transform Infrared Spectroscopy (ATR – FTIR) measurements were done by using a Brüker ALPHA II compact FT-IR spectrometer equipped with a platinum attenuated total reflection (ATR) setup containing a monolithic diamond crystal and tightly soldered into tungsten carbide. For ATR – FTIR top heating measurements, an external heating device was used, which consists mainly of a heating compartment (where the temperature can be adjusted up to 75 °C), a resistor and a copper metal plate. The setup measurement parameters for data acquisition were as follows: 2 cm⁻¹ resolution, 32 scans for both sample scan time and also background scan time, data were saved from 4000 cm⁻¹ to 400 cm⁻¹ and temperature was adjusted to 45 °C or 50 °C (unless otherwise is stated) for the ATR – FTIR bottom heating measurements, whereas the temperature of external heating device was adjusted to 55°C (unless otherwise is stated) for the ATR – FTIR top heating measurements. At least 30 minutes were required for adjustment of the intended temperature before starting the measurements. Background spectra were taken initially and then sample spectra were taken accordingly. The samples were investigated in the order of: H₂O (or D₂O), polymer (PNIPAM or PDEA) in H₂O (or in D₂O), from 1.0 M to 6.0 M urea solution (urea-d₄ solution, as well as solutions of other urea derivatives and sodium salts) and polymer (PNIPAM or PDEA) in specific concentration of urea solution (urea-d₄ solution, as well as solutions of other urea derivatives and sodium salts). For each interested sample, 50 µL (unless otherwise is stated) were used for the measurements and spectra were taken after 0 minutes (immediately after placing on top of the ATR crystal), 3 minutes, 5 minutes and 10 minutes. During the ATR – FTIR bottom heating measurements, the sample droplet was covered by using a screw cap of centrifuge tubes (from Iso-Lab, 50 mL), in which edges of the screw cap were covered with Parafilm®. The experimental setup of ATR – FTIR measurements can be seen in Fig. 2.4.

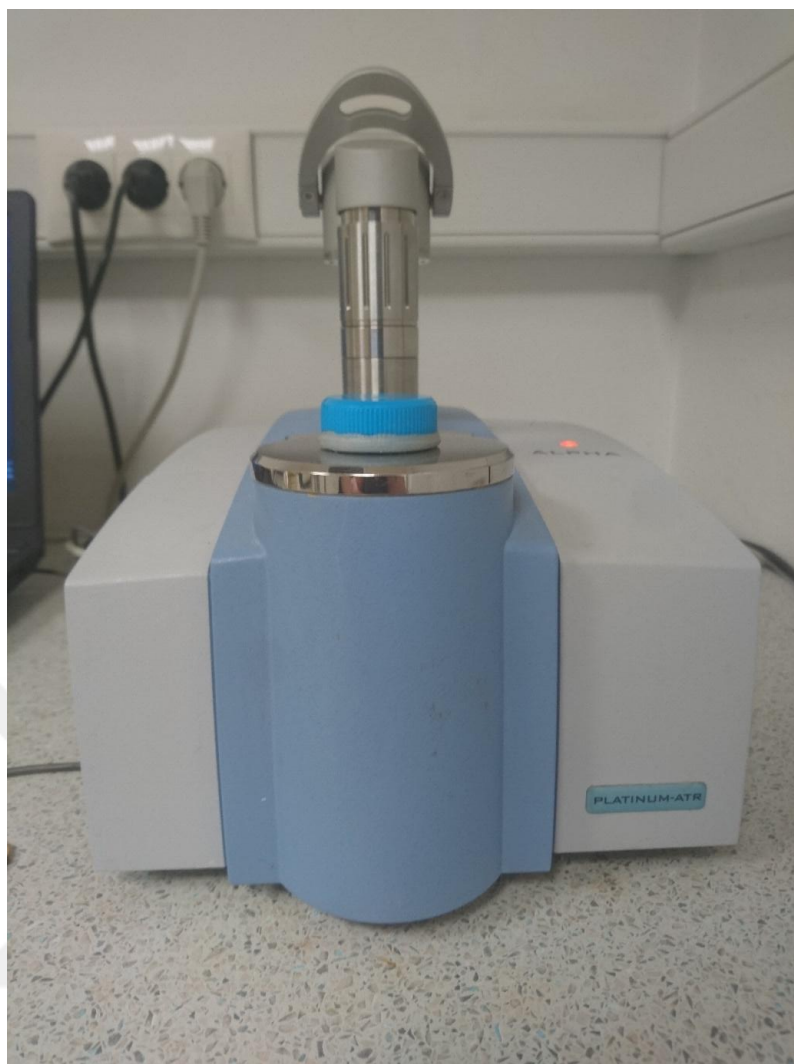


Figure 2.4 The experimental setup of ATR – FTIR measurements. Bruker ALPHA II compact FT-IR spectrometer equipped with a platinum attenuated total reflection (ATR) setup containing a monolithic diamond crystal and tightly soldered into tungsten carbide was used alongside a screw cap of centrifuge tubes (from Iso-Lab, 50 mL), in which edges of the screw cap was covered with Parafilm to cover the sample droplets.

Chapter 3 – Results and Discussion

3.1 An ATR – FTIR based Method Development for Studying Collapsed State of Macromolecules

3.1.1 Probing the Collapsed State of Macromolecules by using ATR – FTIR Spectroscopy

The elucidation of macromolecules in their different forms, such as the aqueous two phases of proteins, uncollapsed and collapsed forms of thermoresponsive polymers, is a necessity to achieve a multiperspectivity on a molecular level picture of macromolecules behaviour in aqueous medium. In this thesis, it is needed to explore osmolyte – macromolecule interactions. Although numerous experimental techniques have been utilized to investigate the soluble phases of macromolecules such as NMR [109], [110], Raman [111], [112], FTIR [113], MD simulations [114], Differential Scanning Calorimetry (DSC) [115], [116] etc., the same techniques mostly fail to provide any useful information for the collapsed form of macromolecules, since investigation of the heterogeneous solution is quite demanding. As a consequence, building a new method is essential to be able to probe the collapsed or least soluble forms of aqueous macromolecules.

In order to achieve examining the collapsed state of macromolecules above their lower critical solution temperature (LCST), an experimental method was developed, which enables to measure various macromolecules in different aqueous environments at considerable temperature ranges, possibly with small-scaled quantities of each molecule. All of these criteria can be met by utilizing temperature-controlled ATR-FTIR, where the scheme of experiment for ATR – FTIR measurements of a macromolecule solution droplet at different temperatures (below and above the LCST) that corresponds to viable investigation of both uncollapsed and collapsed states of the macromolecules can be seen in Fig. 3.1.

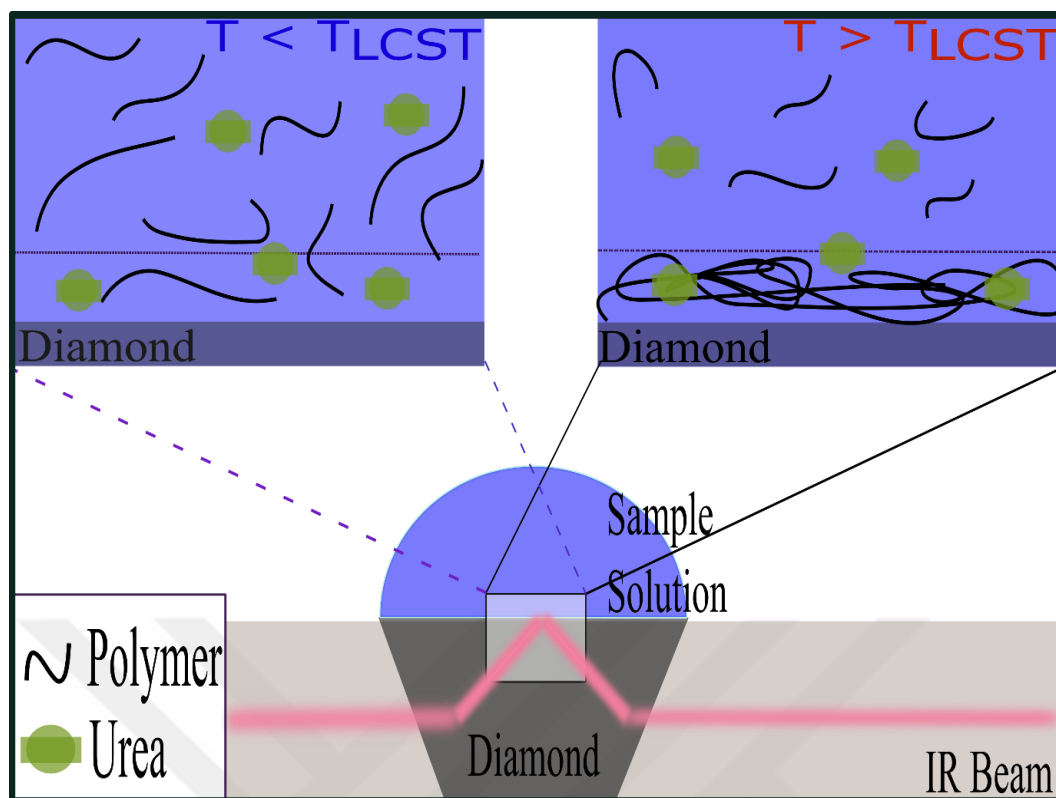


Figure 3.1 Experimental scheme showing an aqueous solution droplet (50 μ L) placed onto the diamond ATR crystal. The upper left insert represents an initially homogeneous polymer solution (below the LCST), whereas the upper right insert represents the collapsed state of polymer which is formed above the LCST.

ATR – FTIR is a unique technique for probing macromolecules and proteins in various aqueous environments (e.g., in H_2O and/or in D_2O , in various concentrations of urea solutions and its derivatives or their deuterated versions, urea- d_4 solutions, etc.) with relatively small sample volume requirements (unless otherwise is stated, volume of the sample droplet for each measurement was only 50 μ L), since it provides a real time information regarding the interested samples below and above the LCST (shown in upper left and right parts of Figure 3.1, respectively) by adjusting temperature correspondingly on top of the ATR crystal. Nevertheless, in order to utilize ATR – FTIR spectroscopy for probing the protein rich phase of proteins or collapsed states of macromolecules predominantly above their LCSTs a number of experimental conditions and parameters need to be controlled. Namely, the influence of

- i. Exposure of the droplets to ambient air,
- ii. Temperature to the aqueous sample, and
- iii. The time duration of measurement, as well as
- iv. The volume of the sample droplet were explored.

In the first set of ATR-FTIR measurements a 20 mg/mL of PNIPAM in H₂O is measured at 4 °C (below the LCST), and at 45 °C (above the LCST). These two spectra can be seen in A part of Fig. 3.2, and zoomed-in version of them in between 1200-1800 cm⁻¹ in part B. There are 6 major spectral features can be seen in between 1200 – 1800 cm⁻¹, in which these are the peak around 1285 cm⁻¹ (which is attributed to amide III of PNIPAM), the peaks at 1370 cm⁻¹, and at 1390 cm⁻¹ (which are antisymmetric deformations of -C(CH₃)₂ of PNIPAM), the peak around 1465 cm⁻¹ (which is symmetric deformation of -C(CH₃)₂ of PNIPAM), the peak around 1560 cm⁻¹ (which is attributed to amide II of PNIPAM), and the peak around 1650 cm⁻¹ (which is attributed to amide I of PNIPAM). [117] It is worth noting that PNIPAM related bands for the sample at 45 °C are more enhanced compared to the one at 4 °C. This is a first indication that PNIPAM collapses to the surface of an ATR crystal, and supports the idea shown in Figure 3.1.

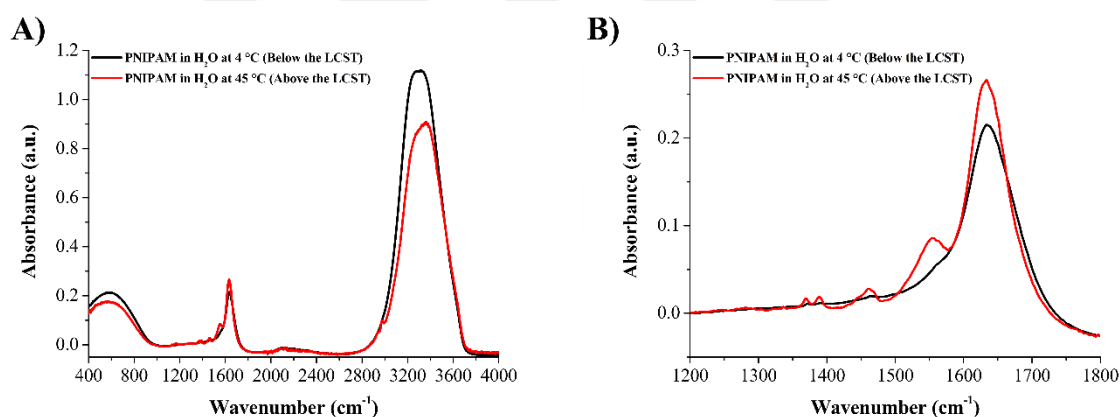


Figure 3.2 **A)** The overall ATR – FTIR spectra of PNIPAM (20 mg/mL) in H₂O at 4 °C (below the LCST), and at 45 °C (above the LCST). **B)** The ATR – FTIR spectra of PNIPAM (20 mg/mL) in H₂O at 4 °C (below the LCST), and at 45 °C (above the LCST) focused in between 1200– 1800 cm⁻¹. The ATR – FTIR spectra of each sample is colour coded separately, and the colour coding for each one of them is displayed in the inset.

During the investigation of the collapsed state of the polymers with ATR – FTIR measurements above the LCST, it was important to understand the effect of penetration depth to the samples for attenuated total reflection geometry, since evanescent wave, which occurs owing to the interaction between IR beam and sample droplet across an evanescent field, cannot extend into the sample droplet entirely and it is well-known as the penetration depth of the diamond ATR crystal is only up to 2.0 μm [66], [118]. The penetration depth can be affected by several parameters that includes the wavelength of incident light used during measurements in vacuum, the refractive indices of diamond ATR crystal and also the sample droplet, and the incidence angle. Calculation of the penetration depth can be done by using the formula: [66]

$$d_p = \frac{\lambda}{2\pi n_1 \sqrt{\theta - \left(\frac{n_1}{n_2}\right)^2}} \quad \text{eqn. 3.1}$$

Where d_p denotes the penetration depth, λ is the wavelength of the incident light in vacuum that is used during measurements, n_1 and n_2 are the refractive indices of the diamond ATR crystal and the sample droplet, respectively and lastly, θ is the incidence angle. Since λ , n_1 , and θ remained constant during the measurements and 2π is a constant itself, only one parameter could possibly affect the penetration depth, which is the refractive index of the sample droplet that was interested (n_2).

Since the prediction shown in Fig. 3.1 seems valid, above-mentioned experimental conditions and parameters are controlled to find the best conditions to perform experiments for the collapsed form of the macromolecules.

Among the adjustments about utilization of ATR – FTIR spectroscopy, exposure of the same droplets to ambient air was especially important to consider for generating the ATR – FTIR based method for the investigation of each interested sample, since any exposure from air conditions (e.g., humidity) for the sample droplets during the measurements could be potentially problematic for deuterated samples (e.g., hydrogen versus deuterium exchange (H – D exchange) on the sample droplets over time) that would provide incorrect results from the measurements. With the intention of examining the possible effects of the exposure of the same droplets to ambient air (i.), and other previously stated subjects on the interested samples throughout the research (ii., iii., and iv.), control measurements were performed in advance to generate the ATR – FTIR based method for investigating the collapsed state of interested macromolecules above their LCSTs accordingly.

Firstly, the ATR – FTIR spectra of 50 μL of D_2O sample droplet was taken at 30 $^\circ\text{C}$ by letting the sample droplet be exposed to ambient air over time. The ATR – FTIR spectra of this sample can be seen in Fig. 3.3. The general peak assignment of the spectra in Fig. 3.3 can be done as follows, the bending mode of D_2O can be observed around 1180 cm^{-1} , the bending mode of HOD can be observed around 1450 cm^{-1} , the O-D stretch of HOD can be observed around 2495 cm^{-1} , and the O-H stretch of HOD can be observed around 3415 cm^{-1} [119]. The sample droplet retained its volumes even after 30 minutes that it dried after 40 minutes., As might be expected, the H – D exchange was observed upon time by letting the sample droplet to be exposed to ambient air (except for D_2O fresh, and D_2O after 40 minutes samples) that the significant increase of intensity of the O-H stretching of HOD, and the decrease of intensity of the O-D stretching of HOD were observed.

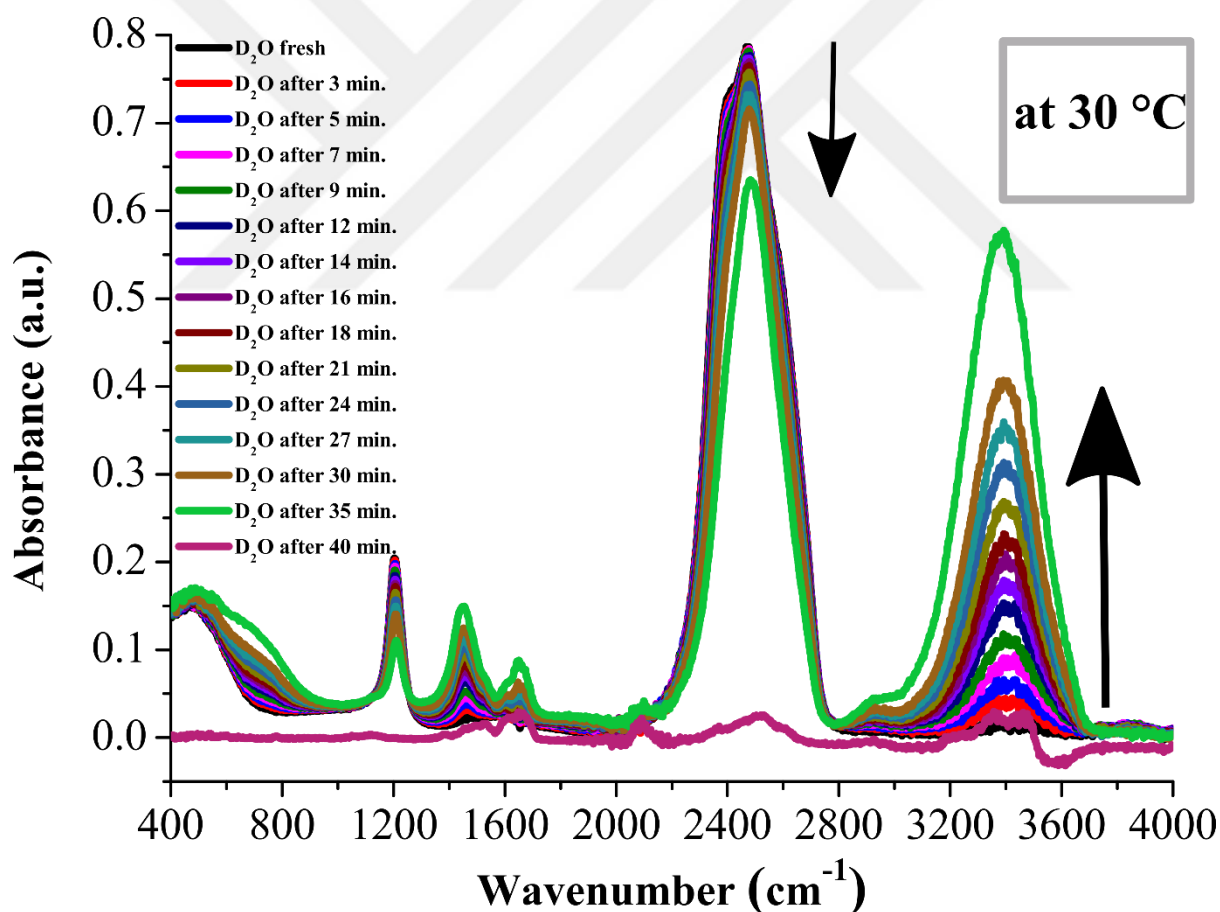


Figure 3.3 The ATR – FTIR spectra of the 50 μL of D_2O sample droplet at 30 $^\circ\text{C}$ with the exposure of the sample droplet to ambient air. The arrow directions show the evaluation of each spectrum as a function of time.

Next, the ATR – FTIR spectra of 50 μL PNIPAM and PDEA in D_2O sample droplets were measured at 30 $^\circ\text{C}$ by letting the sample droplets be exposed to ambient air over time. The ATR – FTIR spectra of PNIPAM in D_2O , and PDEA in D_2O can be seen in Fig. 3.4, and Fig 3.5, respectively. Both sample droplets retained their volumes even after 30 minutes. For the 50 μL of PDEA in D_2O of sample droplet, it almost completely dried after 40 minutes, yet much longer time was necessary to dry for the dried 50 μL of PNIPAM in D_2O of sample droplet (the sample droplet did not dry completely even after 72 minutes). For both sample droplets, H – D exchange could still be observed on the ATR – FTIR spectra for the the 50 μL of both PNIPAM and PDEA in D_2O of sample droplets that even though the overall spectra are more complex compared to the spectra in Fig. 3.3, the increase of intensity of the O-H stretching of HOD, and the decrease of intensity of the O-D stretching of HOD were still observed for both sample droplets distinctly.

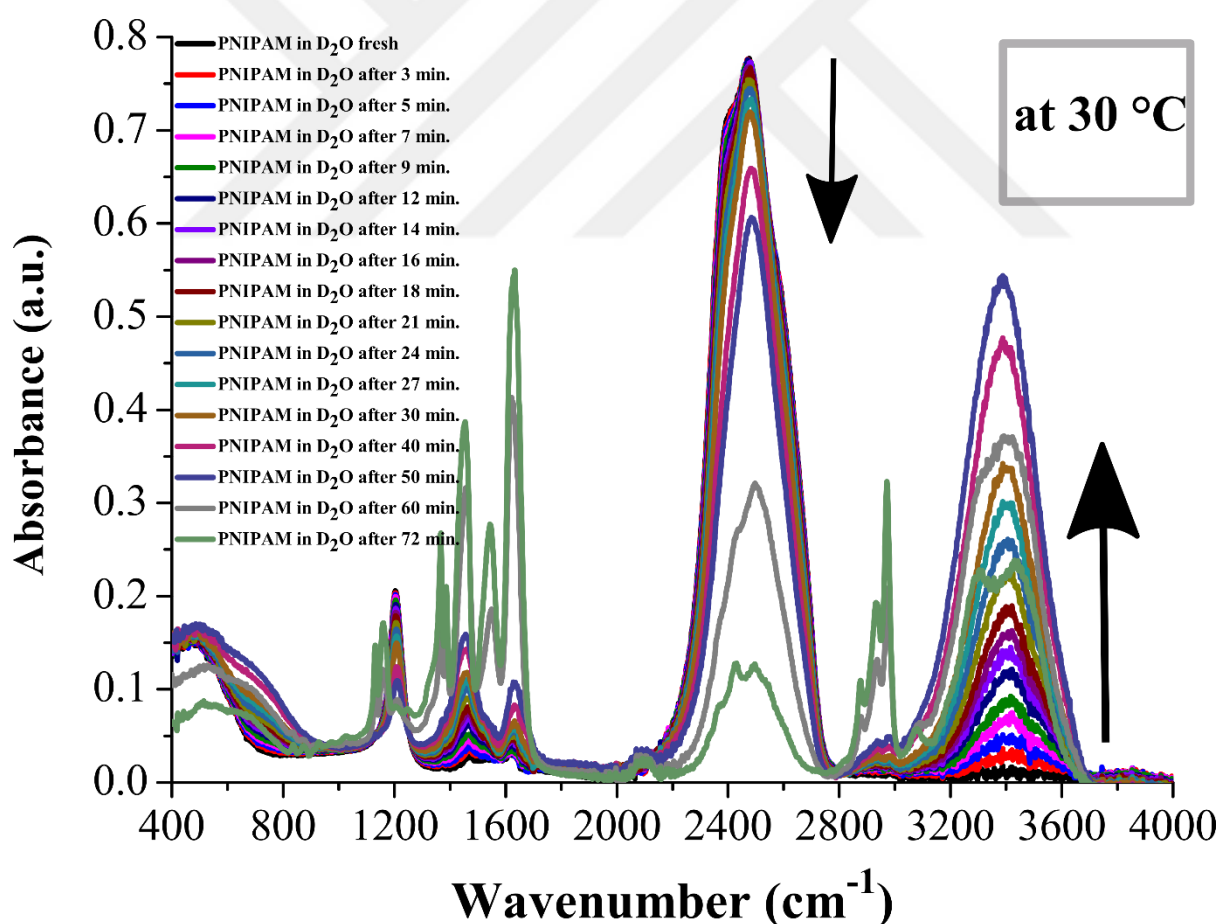


Figure 3.4 The ATR – FTIR spectra of the 50 μL of PNIPAM in D_2O sample droplet at 30 $^\circ\text{C}$ with the exposure of the sample droplet to ambient air. The arrow directions show the evaluation of each spectrum as a function of time.

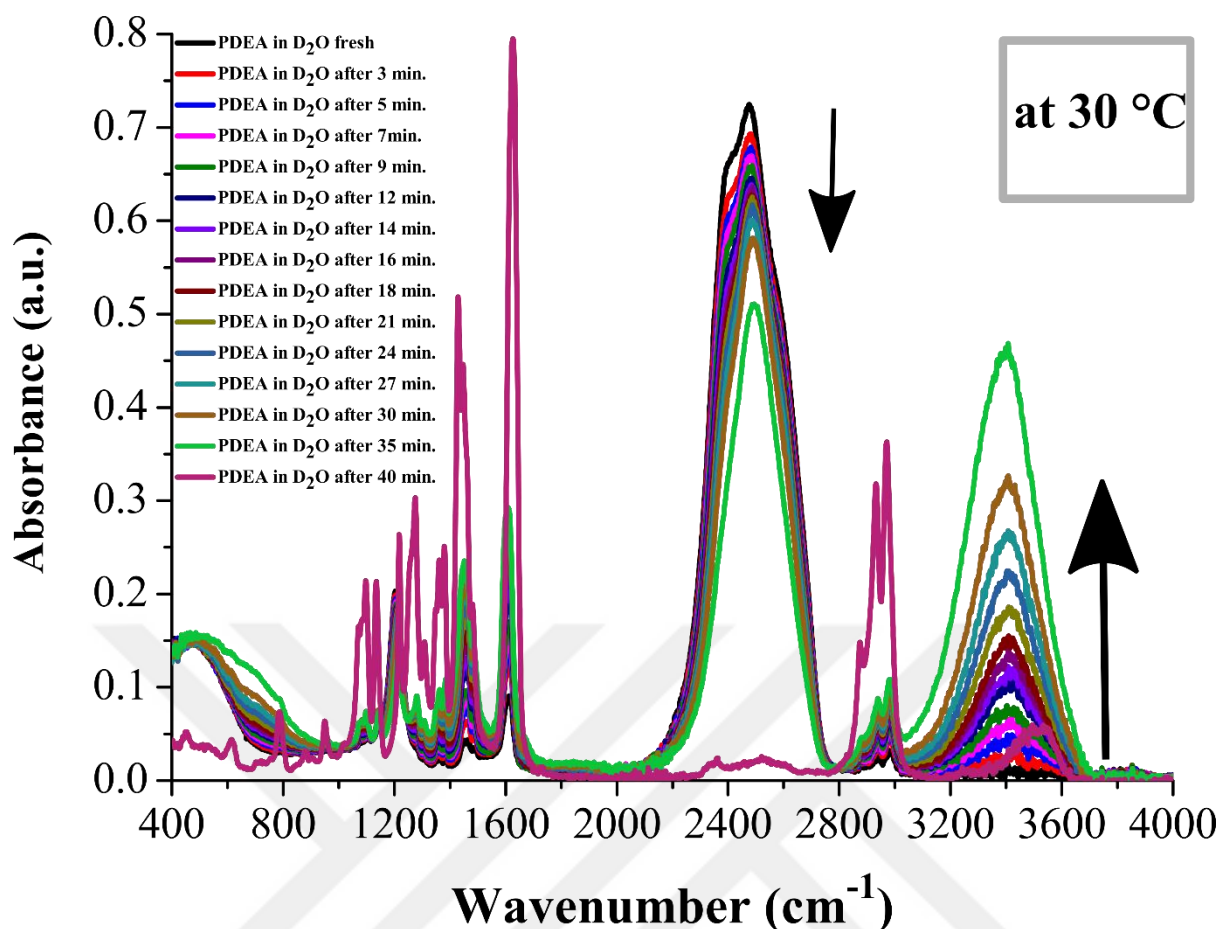


Figure 3.5 The ATR – FTIR spectra of the 50 μL of PDEA in D_2O sample droplet at $30\text{ }^\circ\text{C}$ with the exposure of the sample droplet to ambient air. The arrow directions show the evaluation of each spectrum as a function of time.

By considering the spectra of D_2O , PNIPAM in D_2O , and PDEA in D_2O sample droplets, the H-D exchange susceptibility of each sample droplet can be observed distinctly that in order to prevent this situation, isolation of the sample droplets was done by using a screw cap. The ATR – FTIR spectra of 50 μL of D_2O sample droplet was taken at $30\text{ }^\circ\text{C}$ by isolating the sample droplet with screw cap over time, and its spectra can be seen in Fig. 3.6. Interestingly, the sample droplet was able to retain its volume at least 120 minutes without drying and H – D exchange for the sample droplet was much less compared to the the ATR – FTIR spectra of 50 μL of D_2O sample droplet was taken at $30\text{ }^\circ\text{C}$ with the exposure of the sample droplet to ambient air over time (in Fig. 3.3). Isolation of the sample droplet from ambient air for the ATR – FTIR measurements affected the obtained ATR – FTIR spectra directly, which can be understood with ease by comparing the amount of time required, and also the intensity of the H – D exchange on the sample droplet over time in between Fig. 3.3, and Fig. 3.6.

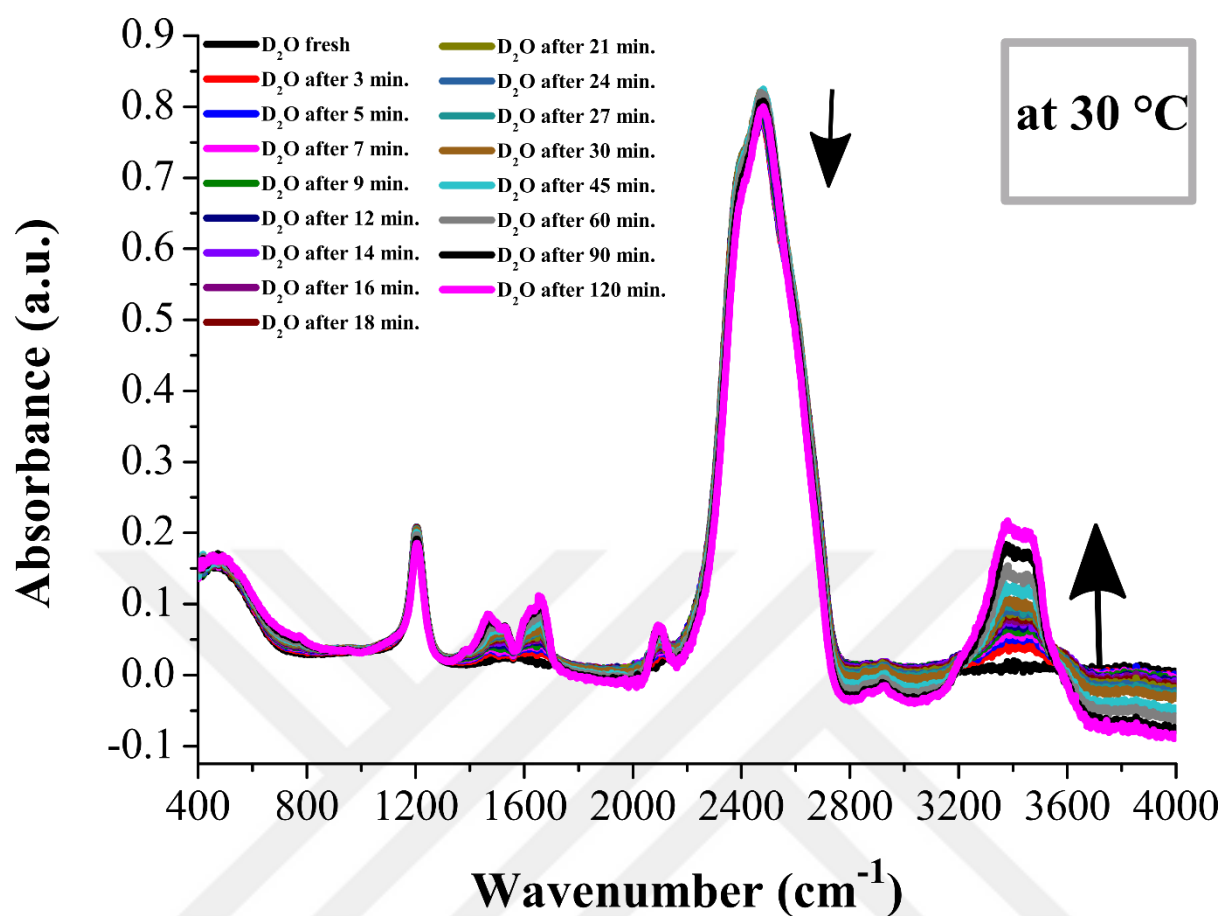


Figure 3.6 The ATR – FTIR spectra of the 50 μL D_2O sample droplet at $30\text{ }^\circ\text{C}$ with the isolation of the sample droplet. The arrow directions show the evaluation of each spectrum as a function of time.

Later, the ATR – FTIR spectra of 50 μL PNIPAM and PDEA in D_2O sample droplets were taken at $30\text{ }^\circ\text{C}$ by isolating the sample droplets with screw cap over time, and their spectra can be seen in Fig. 3.7, and Fig. 3.8, respectively. Similar to the 50 μL of D_2O sample droplet, both of these two sample droplets were also able to retain their volumes for at least 120 minutes without drying. Strikingly, H – D exchanges for both 50 μL PNIPAM and PDEA in D_2O sample droplets were much less compared to the obtained ATR – FTIR spectra of the same sample droplets by letting them be exposed to ambient air (Fig. 3.4, and Fig. 3.5).

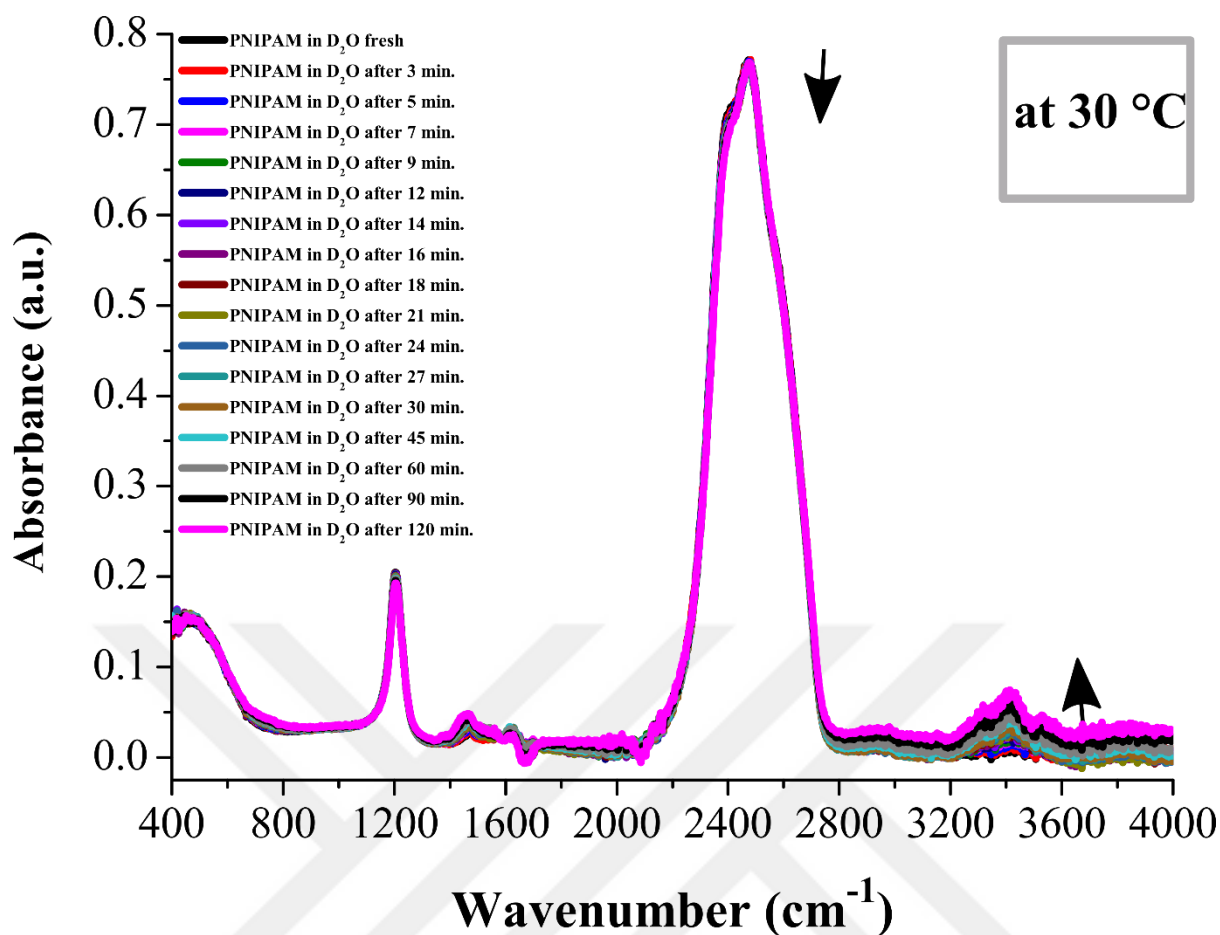


Figure 3.7 The ATR – FTIR spectra of the 50 μL of PNIPAM in D₂O sample droplet at 30 °C with the isolation of the sample droplet. The arrow directions show the evaluation of each spectrum as a function of time.

These observations clearly demonstrated that the isolation of the sample droplets from ambient air must be done for the ATR – FTIR measurements in order to obtain the ATR – FTIR spectra, in which exposure from the air could be prevented much more effectively.

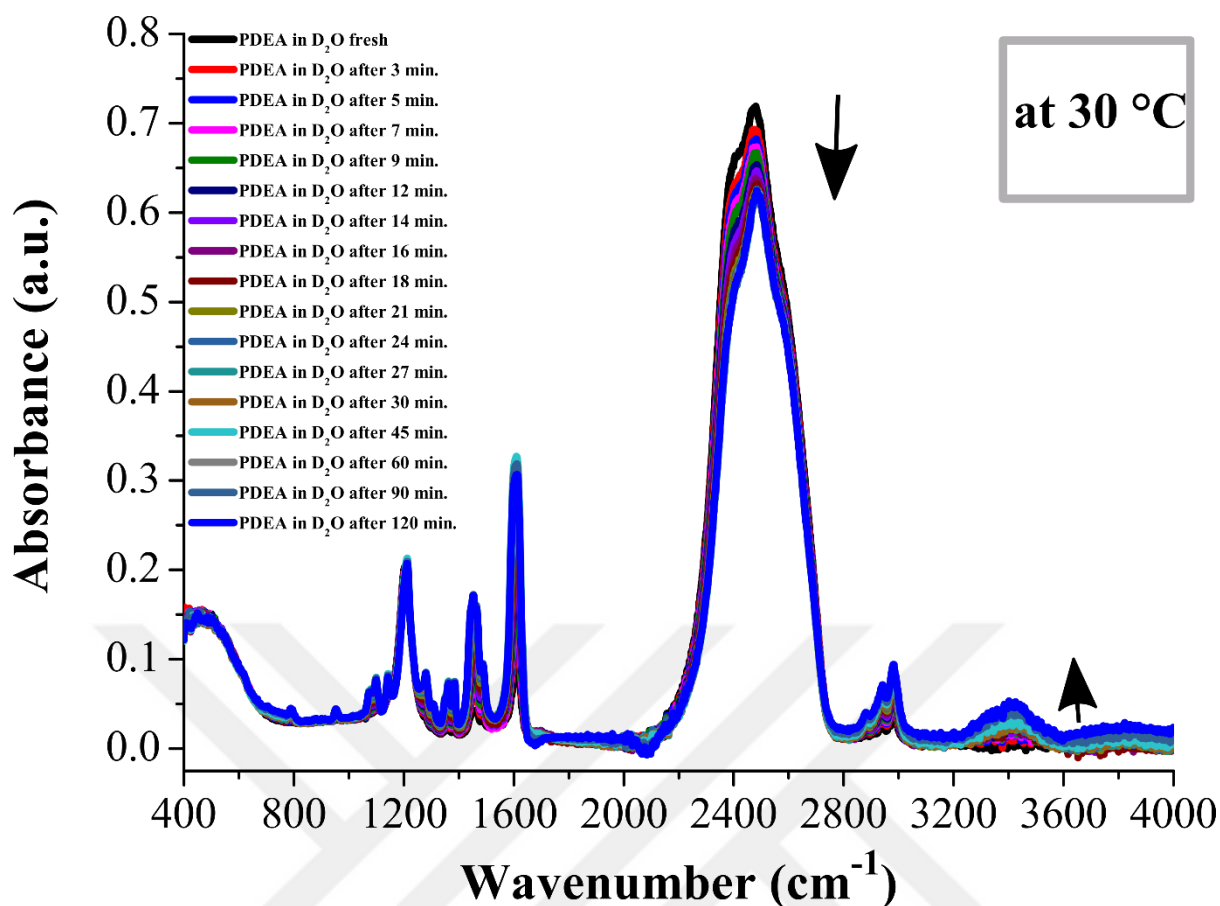


Figure 3.8 The ATR – FTIR spectra of the 50 μL of PDEA in D_2O sample droplet at $30\text{ }^\circ\text{C}$ with the isolation of the sample droplet. The arrow directions show the evaluation of each spectrum as a function of time.

To illustrate the significance of the insulation for the sample droplets throughout the measurements against H-D exchange, the ATR – FTIR spectra of both polymers in D_2O at $30\text{ }^\circ\text{C}$ with exposure to ambient air, and with isolation from ambient air after 30 minutes for each are plotted in Fig. 3.9. The H-D exchange for the sample droplets that were exposed to ambient air was observed much more pronounced relative to the sample droplets that were isolated from ambient air (for comparison, one can examine the relative intensities of O-D, and O-H stretches for PNIPAM (black vs. red), and PDEA (blue vs. pink) around 2495 cm^{-1} , and 3415 cm^{-1} , respectively).

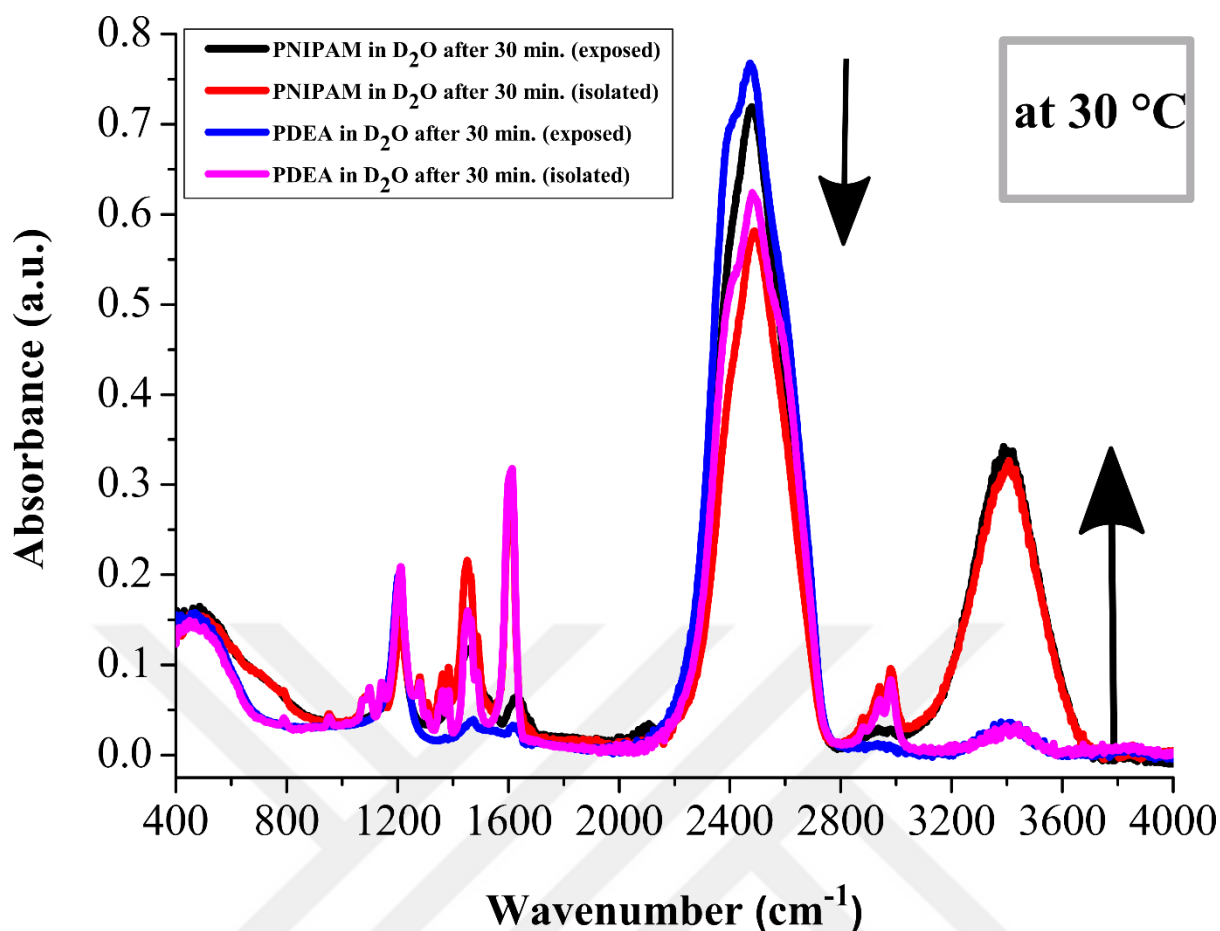


Figure 3.9 The ATR – FTIR spectra of the 50 μL of PNIPAM in D₂O sample droplet at 30 °C with exposure to ambient air (shown with black), the 50 μL of PNIPAM in D₂O sample droplet at 30 °C with isolation from ambient air (shown with red), the 50 μL of PDEA in D₂O sample droplet at 30 °C with exposure to ambient air (shown with blue), and the 50 μL of PDEA in D₂O sample droplet at 30 °C with isolation from ambient air (shown with pink). The arrow directions show the evaluation of each spectrum as a function of time.

Next, it was taken into consideration that for probing the collapsed states of interested macromolecules in sample droplets, temperature needed to be increased (more than 30 °C was required). Consequently, examination of the effect of temperature to the aqueous sample (ii.) was done by increasing the temperature to 45 °C for each measurement for comparison of the ATR – FTIR spectra of interested sample droplets at 30 °C (below the LCST for both polymers), and at 45 °C (above the LCST for both polymers). The ATR – FTIR spectra of 50 μL of D₂O sample droplet with isolation from ambient air at 45 °C can be seen in Fig 3.10. As can be expected, this time the 50 μL of D₂O of sample droplet retained its volume only up to 30 minutes, which is much less compared to the 50 μL of D₂O of sample droplet at 30 °C with isolation of the sample droplet from ambient air (more than 120 minutes, Fig. 3.6).

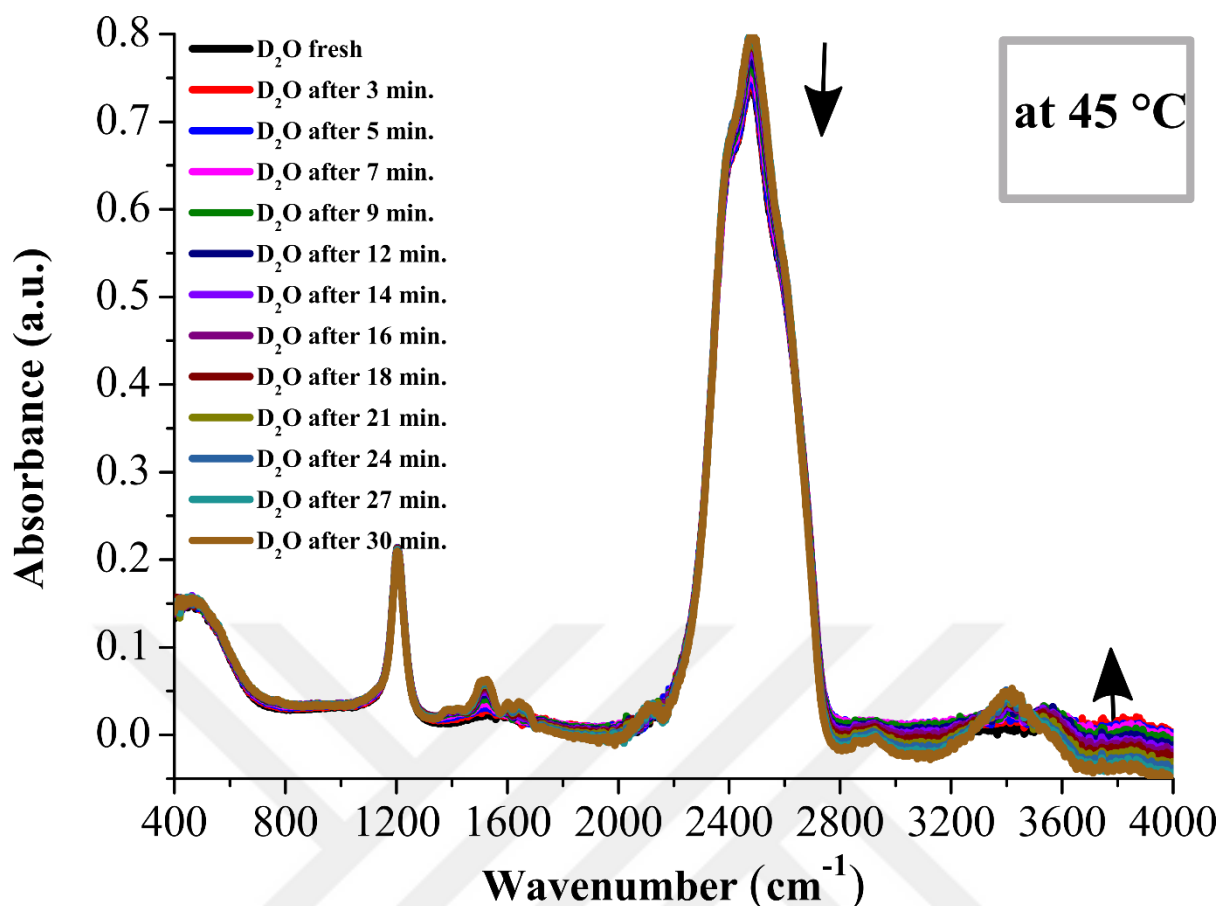


Figure 3.10 The ATR – FTIR spectra of the 50 μL D_2O sample droplet at 45 $^\circ\text{C}$ with the isolation of the sample droplet. The arrow directions show the evaluation of each spectrum as a function of time.

Next, the ATR – FTIR spectra of 50 μL PNIPAM and PDEA in D_2O sample droplets were taken at 45 $^\circ\text{C}$ with isolation of the sample droplets from ambient air over time, and their ATR – FTIR spectra can be seen in Fig. 3.11, and Fig. 3.12, respectively. Both of these samples were able to retain their volumes for at least 27 minutes without drying (yet the 50 μL of PNIPAM in D_2O sample droplet dried after 30 minutes). Once again, H – D exchanges for these samples were much less in comparison to the same sample droplets with exposure to ambient air (Fig. 3.6, and Fig. 3.7).

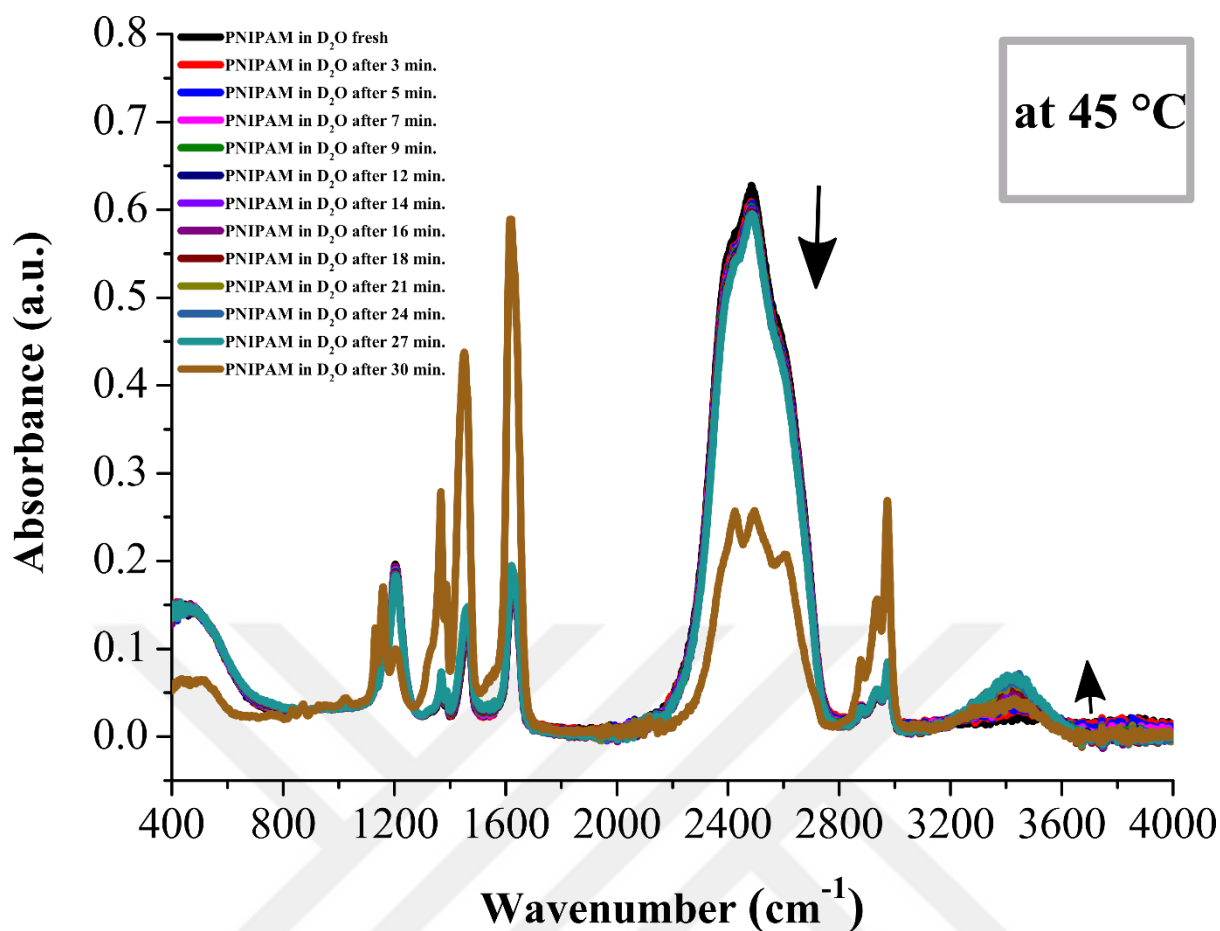


Figure 3.11 The ATR – FTIR spectra of the 50 μL of PNIPAM in D_2O sample droplet at 45 °C with the isolation of the sample droplet. The arrow directions show the evaluation of each spectrum as a function of time.

With the consideration of the obtained ATR – FTIR spectra at 45 °C for each interested sample droplets with their isolation from ambient air, it can be distinctly stated as adjustment of the temperature to above the LCST for each polymer did not create any obstacle for investigation, especially within the first 10 minutes throughout the measurements. These observations enabled to check another parameter (e.g., effect of temperature to the aqueous sample, or ii.) for the ATR – FTIR based method that this method can be utilized for two different temperature regimes for the interested polymers, namely, below the LCST, and above the LCST.

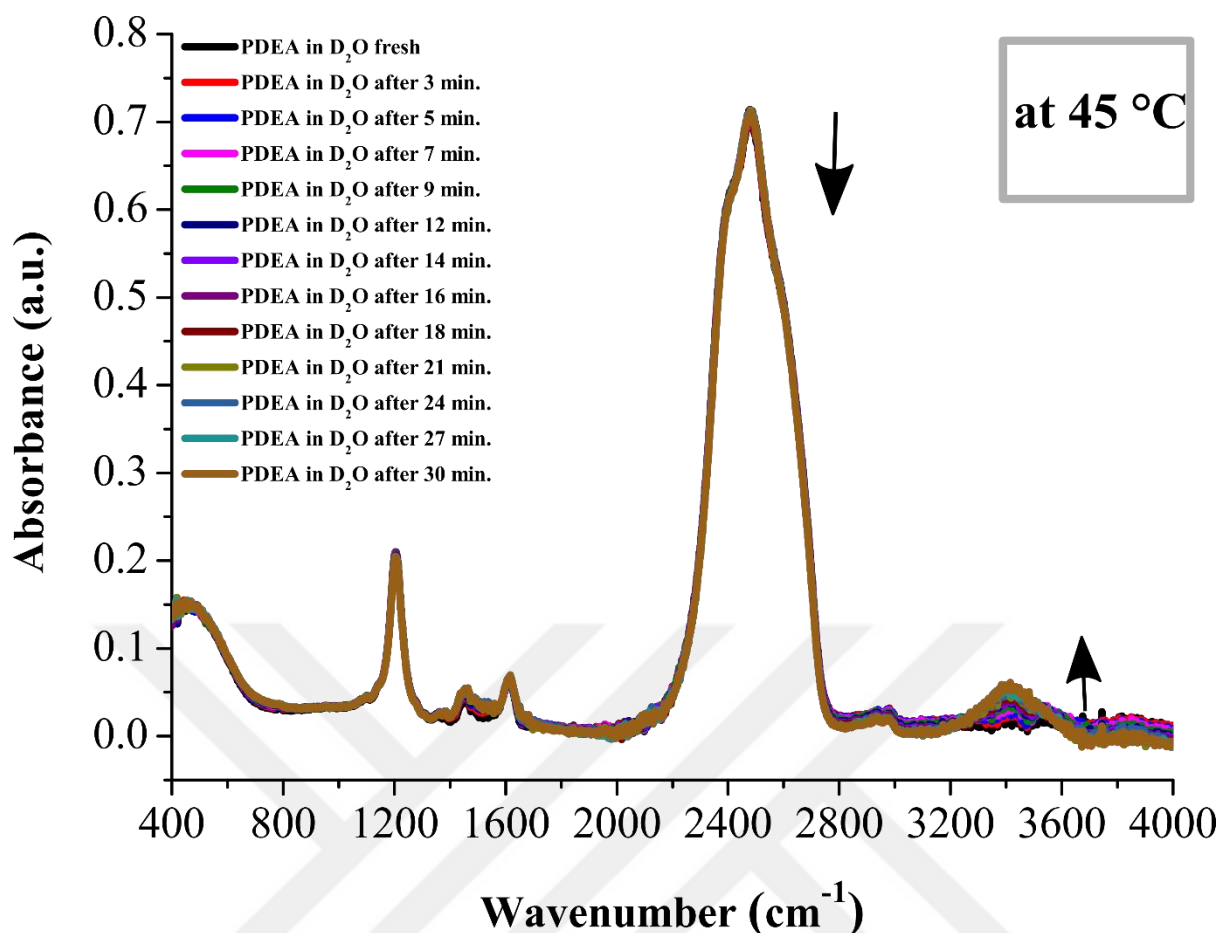


Figure 3.12 The ATR – FTIR spectra of the 50 μL of PDEA in D_2O sample droplet at $45\text{ }^\circ\text{C}$ with the isolation of the sample droplet. The arrow direction shows the evaluation of each spectrum as a function of time.

The time duration of measurement (iii.) was also another important parameter to generate the ATR – FTIR based method, since the H-D exchange for the interested sample droplets was governed with kinetics. For the investigation of this parameter, ATR – FTIR measurements were done for both polymers in H_2O at $45\text{ }^\circ\text{C}$. The overall ATR – FTIR spectra for PNIPAM in H_2O at $45\text{ }^\circ\text{C}$ with isolation from ambient air can be seen in A part of Fig. 3.13, and in B part of the same figure demonstrates the peaks of the macromolecule that is known as side chain deformation in between $1370\text{--}1390\text{ cm}^{-1}$ [117].

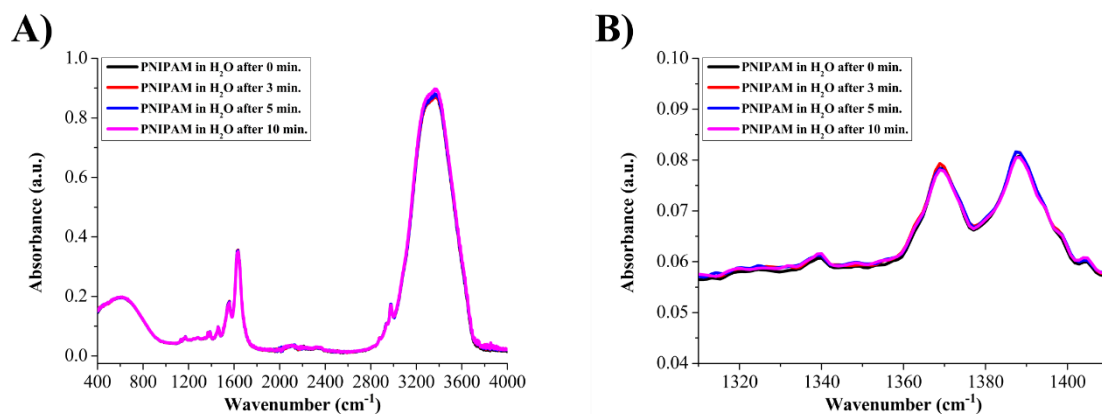


Figure 3.13 **A)** The ATR – FTIR spectra of the 50 μL of PNIPAM in H_2O sample droplet after 0 min., 3 min., 5 min., and 10 min., respectively, at 45 $^{\circ}\text{C}$ with isolation from ambient air. The colour coding for each sample can be seen on the inset of the figure. **B)** The ATR – FTIR spectra of the 50 μL of PNIPAM in H_2O sample droplet after 0 min., 3 min., 5 min., and 10 min., respectively, at 45 $^{\circ}\text{C}$ with isolation from ambient air that focused in region between 1310 – 1410 cm^{-1} . The colour coding for each sample can be seen on the inset of the figure.

As it can be seen from Fig. 3.13, there is almost no change observed on the overall spectra of PNIPAM in H_2O at 45 $^{\circ}\text{C}$ with isolation from ambient air that this was also valid for the intensity of peaks of the polymer that corresponds to side chain deformation. The overall ATR – FTIR spectra for PNIPAM in H_2O at 45 $^{\circ}\text{C}$ with isolation from ambient air can be seen in A part of Fig. 3.14, and in B part of the same figure demonstrates the peaks of the macromolecule that is known as side chain deformation in between 1360-1380 cm^{-1} [120].

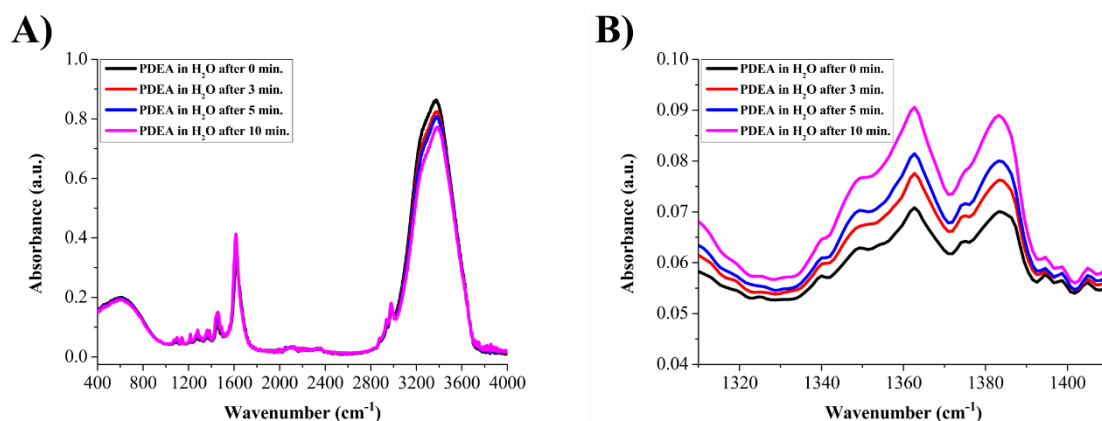


Figure 3.14 **A)** The ATR – FTIR spectra of the 50 μL of PDEA in H_2O sample droplet after 0 min., 3 min., 5 min., and 10 min., respectively, at 45 $^{\circ}\text{C}$ with isolation from ambient air. The colour coding for each sample can be seen on the inset of the figure. **B)** The ATR – FTIR spectra of the 50 μL of PDEA in H_2O sample droplet after 0 min., 3 min., 5 min., and 10 min., respectively, at 45 $^{\circ}\text{C}$ with isolation from ambient air that focused in the region between 1310 – 1410 cm^{-1} . The colour coding for each sample can be seen on the inset of the figure.

From Fig. 3.14, it can be seen that there is only little difference regarding the intensity of the overall spectra at various times, which can be seen more clearly in the B part of Fig 26. However, the intensity difference for side chain deformation of PDEA differs at most only 0.02 a.u. (comparing the intensity of the obtained spectra after 0 minutes/or fresh, and after 10 minutes). Therefore, the ATR – FTIR spectra after 3 minutes were predominantly used further analysis for the research. The main reason for this preference was related primarily with the exposure of the interested sample droplet to the environment (e.g., H/D exchange for deuterated samples). Since any effect from the environment to the sample droplets regarding their collapses would be decreased by choosing the obtained ATR – FTIR spectra of the sample droplets after some time (e.g., after 10 minutes, the sample droplet could be significantly exposed to the environment even with the isolation of the sampled droplets from ambient air that can be seen in previous figures).

Last but not the least, a final set of control measurements were done for the last adjustment of generating the ATR – FTIR based method to check the effect of the volume of the sample droplet (iv.). Its observation was done by using the ATR – FTIR spectra of 200 μL of D_2O of sample droplet, in which the spectra was taken at 30 $^{\circ}\text{C}$ with exposure to ambient air over time. The spectra of this sample can be seen in Fig. 3.15. As can be expected, the 200 μL of D_2O of the sample droplet retained its volume for a much longer time (more than 60 minutes) compared to the 50 μL of D_2O of the sample droplet in the same conditions (less than 40 minutes, see Fig. 3.3). Similar to previous sample droplets of D_2O , H – D exchange was observed once again on the ATR – FTIR spectra for the 200 μL of D_2O of sample droplet in a much more pronounced extent (especially after 60 minutes). For previous measurements, 50 μL was the volume for the interested sample droplets regarding the ATR – FTIR measurements at specific concentrations of polymers (20 mg/mL). Since the obtained spectra of the interested sample droplets after 3 minutes were taken into account for analyses, these measurements in Fig. 3.15 might be utilized as an indicator for other measurements to investigate higher concentrations of the interested macromolecules over a wider range of time period by using increased volume for the sample droplet. The isolation of the sample droplet for examining the interested macromolecules with increased volume of the sample droplet by using this ATR – FTIR based method would possibly provide a longer timespan regarding the investigation.

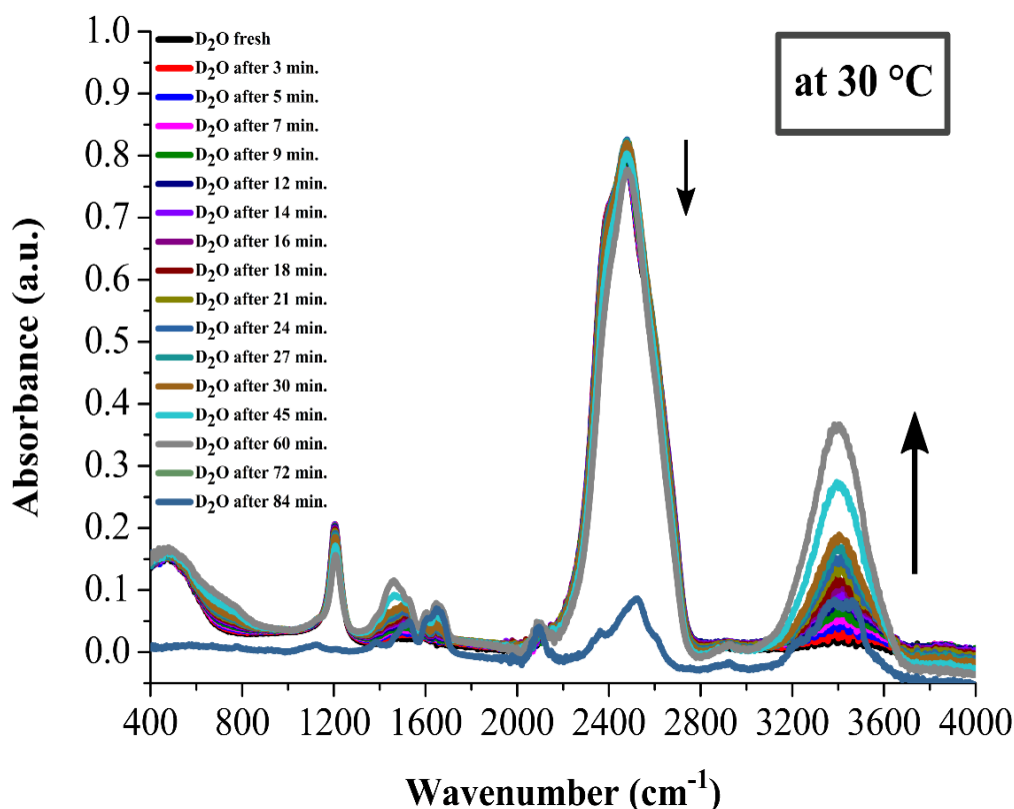


Figure 3.15 The ATR – FTIR spectra of the 200 µL of D₂O sample droplet at 30 °C with exposure to ambient air over time. The arrow directions show the evaluation of each spectrum as a function of time.

With all these considerations regarding the generation of the ATR – FTIR based method to investigate the collapsed states of PNIPAM or PDEA above the LCST, the ATR – FTIR measurements were done by isolating the sample droplet with screw cap during the measurement, the temperature was adjusted to 45 °C (unless otherwise is stated, above the LCST) for each measurement, the obtained spectra from measurements after 3 minutes were considered for further analysis, and the volume of the sample droplets was 50 µL throughout the whole ATR – FTIR measurements.

In order to interpret the obtained spectra for the collapsed state of polymer(s) in specific concentrations of urea solution (along with other urea derivatives and sodium salts) above the LCST, straightforward mathematical formulations were generated by using the spectra of H₂O (or D₂O), polymer (PNIPAM or PDEA) in H₂O (or in D₂O), and from 1.0 M to 6.0 M urea solution (urea-d₄ solution, as well as solutions of other urea derivatives and sodium salts), separately. For instance, to interpret the obtained spectra of ATR – FTIR bottom heating

measurement of PNIPAM in 6.0 M urea solution in H₂O at 50 °C (above the LCST), generation of the mathematical formula was done by using the spectra of H₂O, PNIPAM in H₂O, and 6.0 M urea. In this case, firstly, the spectrum of PNIPAM in H₂O was multiplied by a coefficient with the aim of matching the intensity of this spectrum with the PNIPAM in 6.0 M urea solution in H₂O at specific peak positions (namely, methylene deformation modes of PNIPAM at around 1370 and 1390 cm⁻¹ [121], [122]), in which there were not any overlapping peaks could be observed due to H₂O and 6.0 M urea. Later, similar to PNIPAM in H₂O, the same procedure was also applied to the spectrum of 6.0 M urea that its spectrum was multiplied with a coefficient for matching its intensity with the PNIPAM in 6.0 M urea solution in H₂O at specific peak positions (namely, bending mode of –NH₂ group of urea around 1160 cm⁻¹ and symmetric stretch of –CN group of urea around 1460 cm⁻¹ [123]), in which there were not any overlapping peaks could be observed due to H₂O and PNIPAM. Finally, the generation of the mathematical formulation for the PNIPAM in 6.0 M urea solution in H₂O was done by adding the multiplied versions of PNIPAM in H₂O and 6.0 M urea with coefficients and subtracting the spectrum of H₂O (without using any coefficient for the spectrum of H₂O) from this addition accordingly. The mathematical formulations were generated not just for the obtained spectra of ATR – FTIR bottom heating measurements above the LCST, but for the obtained spectra of ATR – FTIR top heating measurements above the LCST, as well.

With the optimization of the ATR- FTIR based method by checking several possible sources that might affect the investigation of the collapsed states of interested macromolecules above the LCST, application of the ATR – FTIR based method was primarily done on PNIPAM and PDEA with using two salts from the Hofmeister series, namely NaSCN and Na₂SO₄, which are well-known chaotropic and kosmotropic salts that solubilizes and aggregates proteins, respectively. [124] The main reasons for choosing these two salts for the investigation were essentially the well-characterized effects of each one of the salts onto several systems that can be found in the literature, including lysozyme [48], triglycine [125], and elastin-like polypeptides (ELPs, (VPGVG)₁₂₀) [43], and relative ease to purchase each salt, in which then they could be used facilely to demonstrate the collapse of the interested macromolecules in these salt solutions on top of the ATR crystal as depicted on the right-hand side of the Fig. 3.1 that further details can be seen in the next section (Section 3.1.2).

3.1.2 Testing the New Method to Investigate the Collapsed States of Macromolecules in NaSCN and Na₂SO₄ Salt Solutions

Sodium thiocyanate (NaSCN) and sodium sulfate (Na₂SO₄) are well-known salts of the anionic Hofmeister series. The SCN⁻ is a chaotropic anion that is weakly hydrated and has a “salting – in” behaviour. Whereas, SO₄²⁻ is found on the other side of the anionic Hofmeister series that is a well-known “kosmotropic anion” and strongly hydrated and has a “salting – out” behaviour [15], [32]. Their interaction with macromolecules is distinctly different as well. Weakly hydrated anions (SCN⁻) can predominantly bind directly (e.g., direct binding of anion to amide group of PNIPAM), and strongly hydrated anions (SO₄²⁻) mostly influence the macromolecule indirectly (e.g., excluded volume effect) [32]. By employing the salts of these two distinct Hofmeister anions, the collapsed states of PNIPAM and PDEA were investigated above the LCST to demonstrate the potential of the new ATR – FTIR based method. For each ATR – FTIR measurements, 50 µl of sample droplet was utilized and measured at 35 °C, which was above the LCST for both PNIPAM in H₂O, and PNIPAM in several concentrations of the salt. [32] The results for NaSCN with PNIPAM and PDEA can be seen in Fig. 3.16, Fig. 3.17, in Fig. 3.18, and Fig. 3.19, respectively.

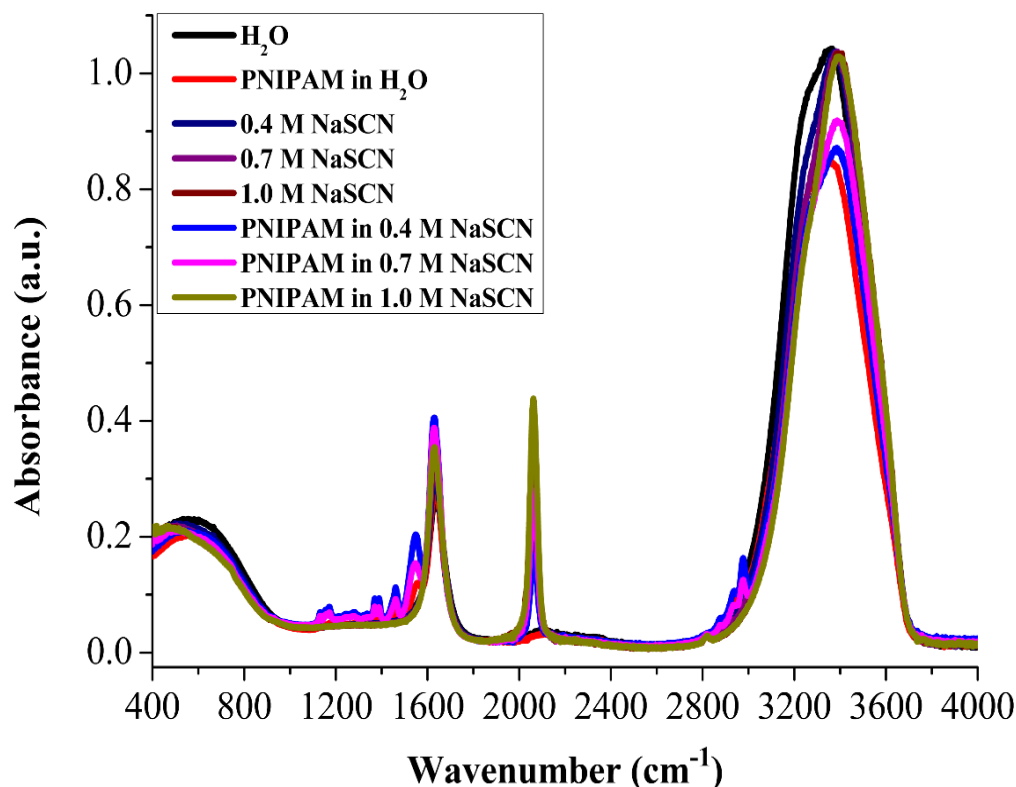


Figure 3.16 The ATR – FTIR spectra of H₂O, PNIPAM (20 mg/mL) in H₂O, x M NaSCN and PNIPAM (20 mg/mL) in x M NaSCN (where x = 0.4, 0.7, and 1.0, respectively) at 35 °C. The ATR – FTIR spectra of each substance is colour coded separately, and the colour coding for each one of them is displayed in the inset.

The spectra for NaSCN concentrations (at 0.4 M, 0.7 M, and 1.0 M) in the presence and absence of PNIPAM were acquired and can be seen in Fig. 3.16. Four distinct spectral regions can be seen. The 400 - 1000 cm⁻¹ is the libration modes of water, 1200 - 1800 cm⁻¹ is the amide bands of the polymer, the fingerprint CH bands along with the water bending mode can be seen. Moreover, 2000 - 2200 cm⁻¹ is the CN stretching of SCN⁻ anion and 2800 - 3700 cm⁻¹ is the region for CH and OH stretch modes. In order to capture the polymer behaviour in the presence of salt, one can focus on the amide region. Figure 3.17 plots the PNIPAM spectra in various NaSCN concentrations. The collapsed state of PNIPAM as a function of NaSCN concentration above the LCST was investigated by observing the intensity change of side chain deformation bands of the macromolecule around 1370 and 1390 cm⁻¹ (deformation of -C(CH₃)₂). Note that the same trend can be seen with other macromolecule related spectral

features.

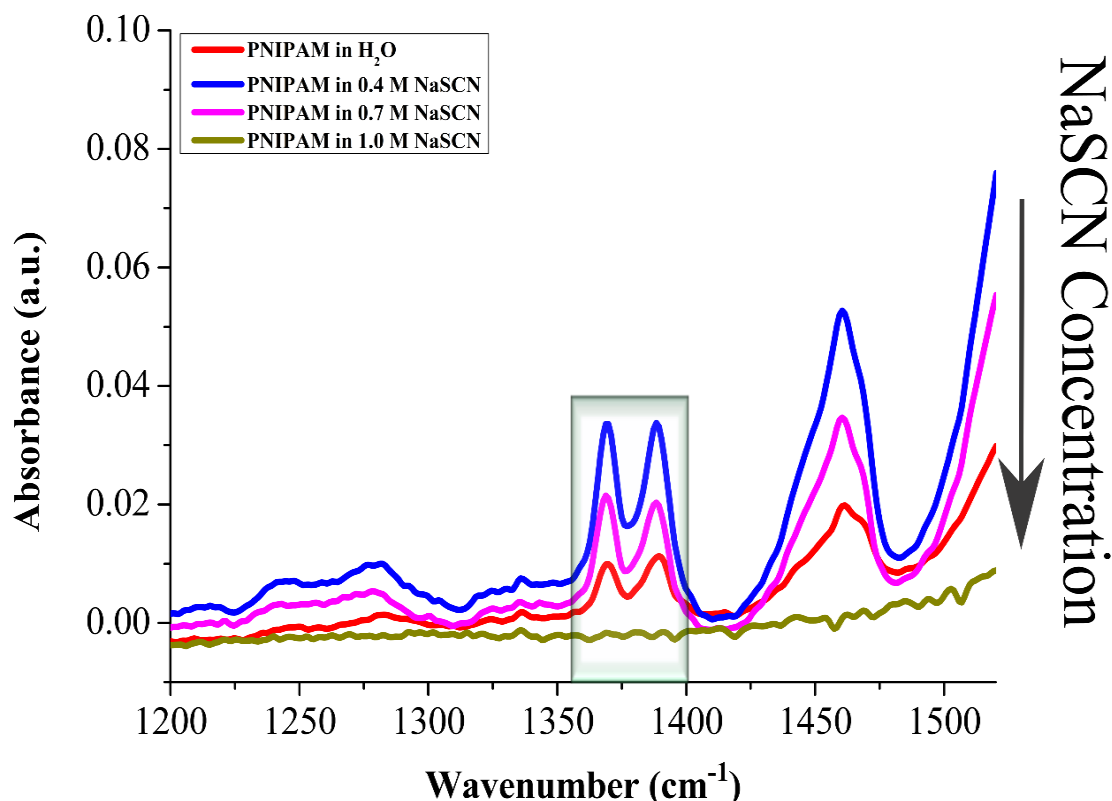


Figure 3.17 The ATR – FTIR spectra of PNIPAM (20 mg/mL) in H₂O, and PNIPAM (20 mg/mL) in x M NaSCN (where x = 0.4, 0.7, and 1.0, respectively) at 35 °C in between 1240 – 1550 cm⁻¹. The ATR – FTIR spectra of each substance is colour coded separately, and the colour coding for each one of them is displayed in the inset. The shadowed black coloured rectangle is displayed to indicate the intensity change of the peak for the side chain deformation of PNIPAM around 1370 and 1390 cm⁻¹, and the direction of black arrow indicates the increasing concentration of NaSCN.

The other spectral features that can be seen in Fig. 3.17 can be assigned as follows; symmetric deformation of -C(CH₃)₂ of PNIPAM around 1465 cm⁻¹, antisymmetric deformation of -C(CH₃)₂ of PNIPAM around 1370, and 1390 cm⁻¹, and lastly the peak around 1285 cm⁻¹ is attributed to amide III of PNIPAM [117]. By considering the intensity change of the peaks of PNIPAM around 1370, and 1390 cm⁻¹ for each sample, it can be clearly observed that the intensity of the interested peaks decreases with increasing the NaSCN concentration. The sample in the absence of salt may not be directly comparable due to the low ionic strength of the solution in deionized water compared to other samples. Overall, the decreasing trend can be observed correspondingly (this is especially the case from 0.4 M to 1.0 M NaSCN). This observation of the collapsed state of PNIPAM as a function of NaSCN salt above the LCST

could be stated as an expected behaviour for macromolecular collapse, since SCN⁻ is a well-known chaotropic anion that tend to solubilize macromolecules with increasing concentration of the anion and this behaviour of anion to the collapse state of PNIPAM was distinctly shown with ATR – FTIR based method. In order to further test this method, the investigation of the collapsed state of PDEA as a function of the same salt above the LCST was also performed. The ATR – FTIR spectra of the macromolecule and others can be seen in Fig. 3.18.

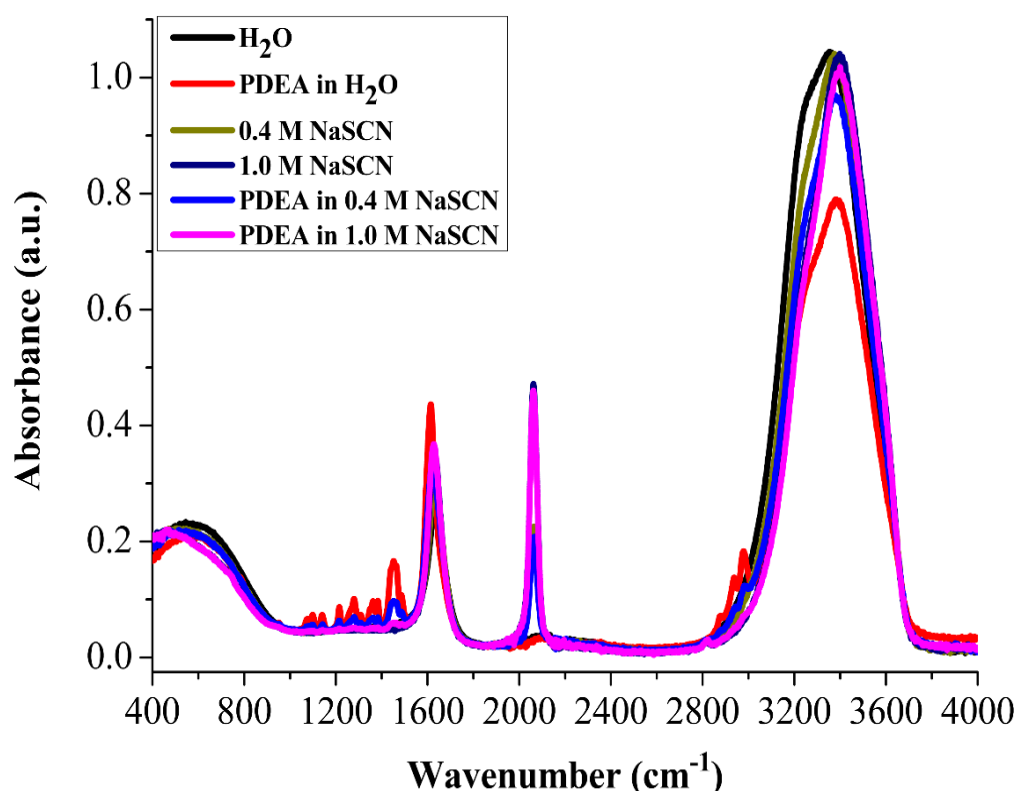


Figure 3.18 The ATR – FTIR spectra of H₂O, PDEA (20 mg/mL) in H₂O, y M NaSCN and PDEA (20 mg/mL) in y M NaSCN (where y = 0.4, and 1.0, respectively) at 35 °C. The ATR – FTIR spectra of each substance is colour coded separately, and the colour coding for each one of them is displayed in the inset.

The same experimental parameters that were used for PNIPAM in NaSCN were also utilized for ATR-FTIR measurements of PDEA. Again, the same four distinct spectral regions can be seen in figure 3.18. The collapsed state of PDEA as a function of NaSCN concentration above the LCST was investigated by monitoring the intensity change of the peak for the side chain deformation of the macromolecule around 1360 and 1380 cm⁻¹ that can be seen in Fig. 3.19 [120].

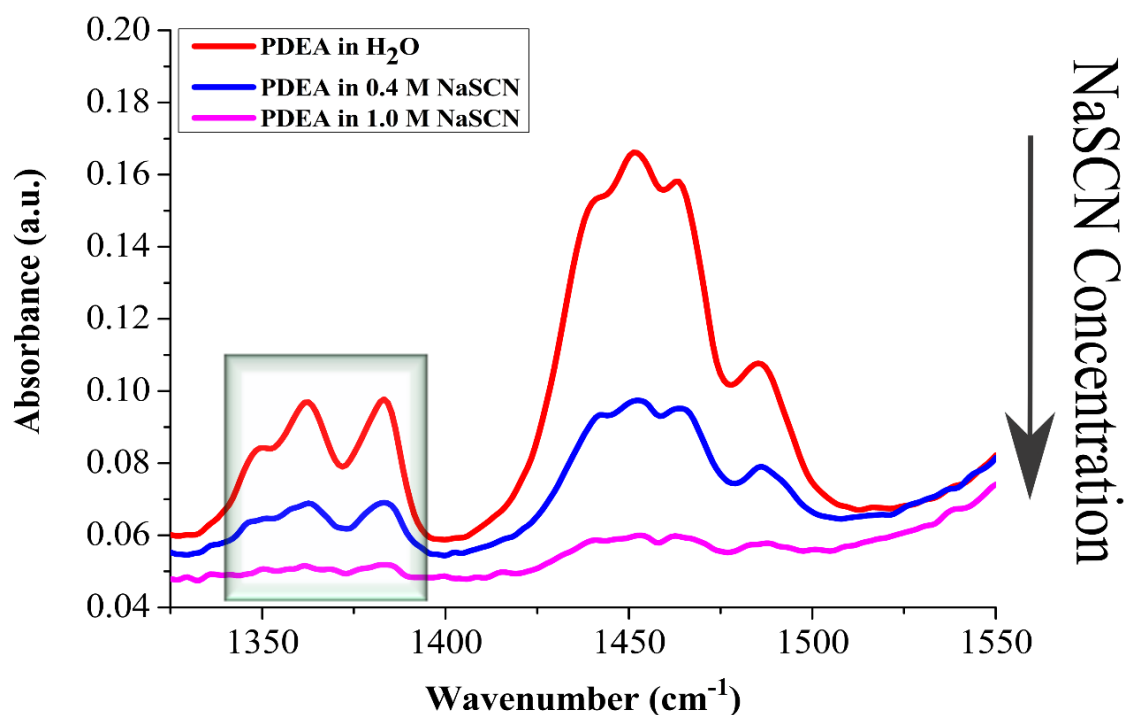


Figure 3.19 The ATR – FTIR spectra of PDEA (20 mg/mL) in H₂O, and PDEA (20 mg/mL) in y M NaSCN (where $x = 0.4$, and 1.0 , respectively) at 35 °C in between 1325 – 1550 cm⁻¹. The ATR – FTIR spectra of each substance is colour coded separately, and the colour coding for each one of them is displayed in the inset. The shadowed black coloured rectangle is displayed to indicate the intensity change of the peak for the side chain deformation of PDEA around 1360 and 1380 cm⁻¹, and the direction of black arrow indicates the increasing concentration of NaSCN.

The peak assignment of Fig. 3.19 can be done according to [120] that namely, deformation of -CH group of PDEA is observed in between 1300-1500 cm⁻¹, and the peak intensity of PDEA around 1360 and 1380 cm⁻¹ is decreasing as increasing the concentration of NaSCN above the LCST, comparably observed for PNIPAM, as well. Evidently, by considering the observed effects of NaSCN as chaotropic salt that solubilizes the interested macromolecules with increased concentrations, it can be clearly stated that the ATR – FTIR based method is capable to investigate the collapse mechanism of not just only PNIPAM but also PDEA as a function of NaSCN concentration above the LCST. Still, in order to further validate the application of the method to the collapse state of the macromolecules above the LCST, another salt of the Hofmeister series, Na₂SO₄ (a well-known salt as “kosmotrope”), was employed to explore the expected behaviour of PNIPAM and PDEA as a function of Na₂SO₄ concentration above the LCST should be an increase of the peak intensity for each macromolecule with increasing the concentration of Na₂SO₄, since the salt is known as

“salting-out” agent that aggregates the interested proteins and/or macromolecules in aqueous medium [32]. The investigation of PNIPAM at several concentrations of Na_2SO_4 above the LCST by ATR – FTIR spectroscopy can be seen in Fig. 3.20.

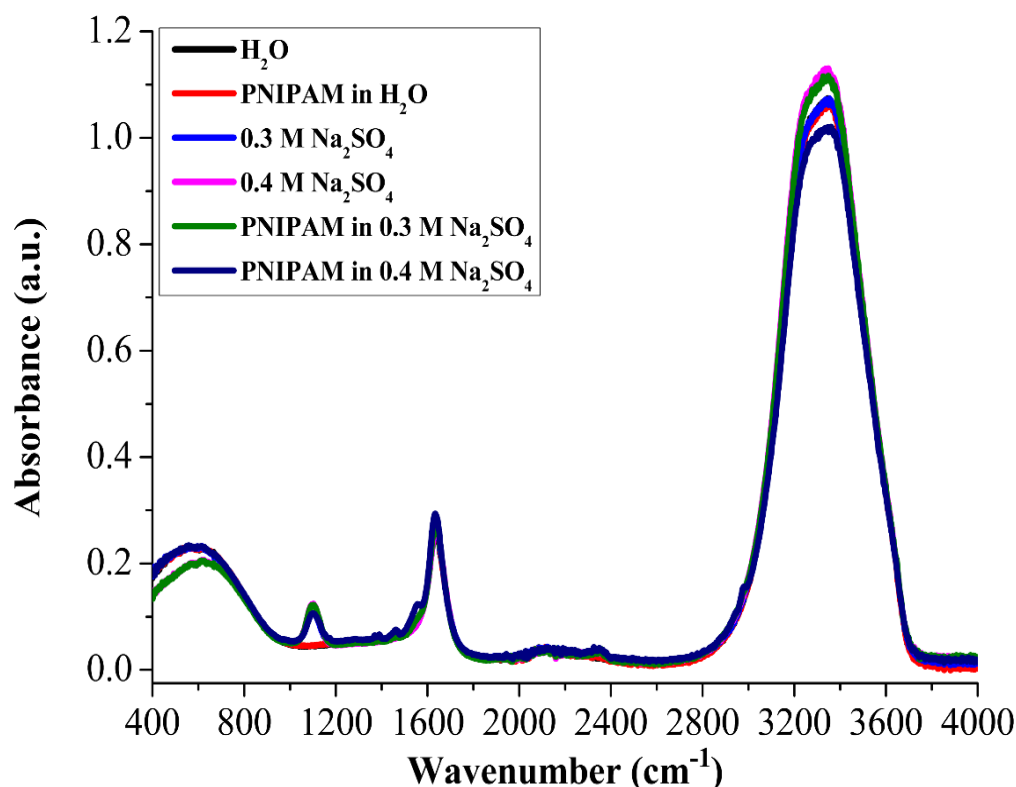


Figure 3.20 The ATR – FTIR spectra of H_2O , PNIPAM (20 mg/mL) in H_2O , x M Na_2SO_4 , and PNIPAM (20 mg/mL) in x M Na_2SO_4 (where $x = 0.3$, and 0.4 , respectively) at 35°C . The ATR – FTIR spectra of each substance is colour coded separately, and the colour coding for each one of them is displayed in the inset.

Similar to the measurements of NaSCN containing samples, ATR – FTIR measurements were performed with the same sample volume ($50\ \mu\text{l}$) and at the same temperature (35°C), that is above the LCST of PNIPAM in all solutions [32]. The collapsed state of PNIPAM as a function of Na_2SO_4 concentration above the LCST was investigated by observing the intensity change of the peak for the side chain deformation of the macromolecule around 1370 and $1390\ \text{cm}^{-1}$ (deformation of $-\text{C}(\text{CH}_3)_2$) that can be seen in Fig. 3.21.

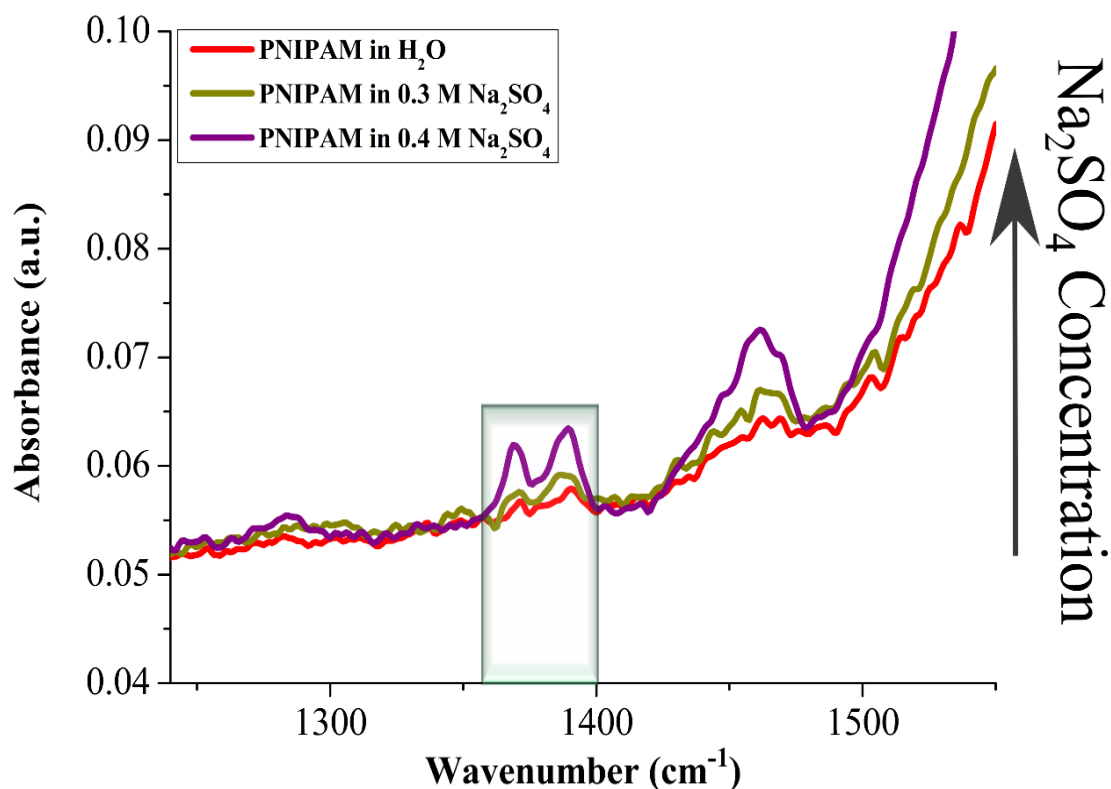


Figure 3.21 The ATR – FTIR spectra of PNIPAM (20 mg/mL) in H₂O, and PNIPAM (20 mg/mL) in x M Na₂SO₄ (where x = 0.3 and 0.4, respectively) at 35 °C in between 1240 – 1550 cm⁻¹. The ATR – FTIR spectra of each substance is colour coded separately, and the colour coding for each one of them is displayed in the inset. The shadowed black coloured rectangle is displayed to indicate the intensity change of the peak for the side chain deformation of PNIPAM around 1370 and 1390 cm⁻¹, and the direction of black arrow indicates the increasing concentration of Na₂SO₄.

The peak assignment of PNIPAM related bands in Fig. 3.21 is the same with Fig. 3.17 [117]. It is clear that the PNIPAM related vibrational features (especially the bands at 1370 and 1390 cm⁻¹) enhance with increasing Na₂SO₄ concentrations. According to the Fig. 3.21, it can be stated that Na₂SO₄ distinctly aggregates PNIPAM above the LCST that leads to further collapse of the macromolecules, especially when the concentration of the salt is 0.4 M. To further test the method, the same type of ATR – FTIR measurements were also done for PDEA by using the same salt and same conditions. The overall ATR – FTIR spectra of the measurements for PDEA can be seen in Fig. 3.22.

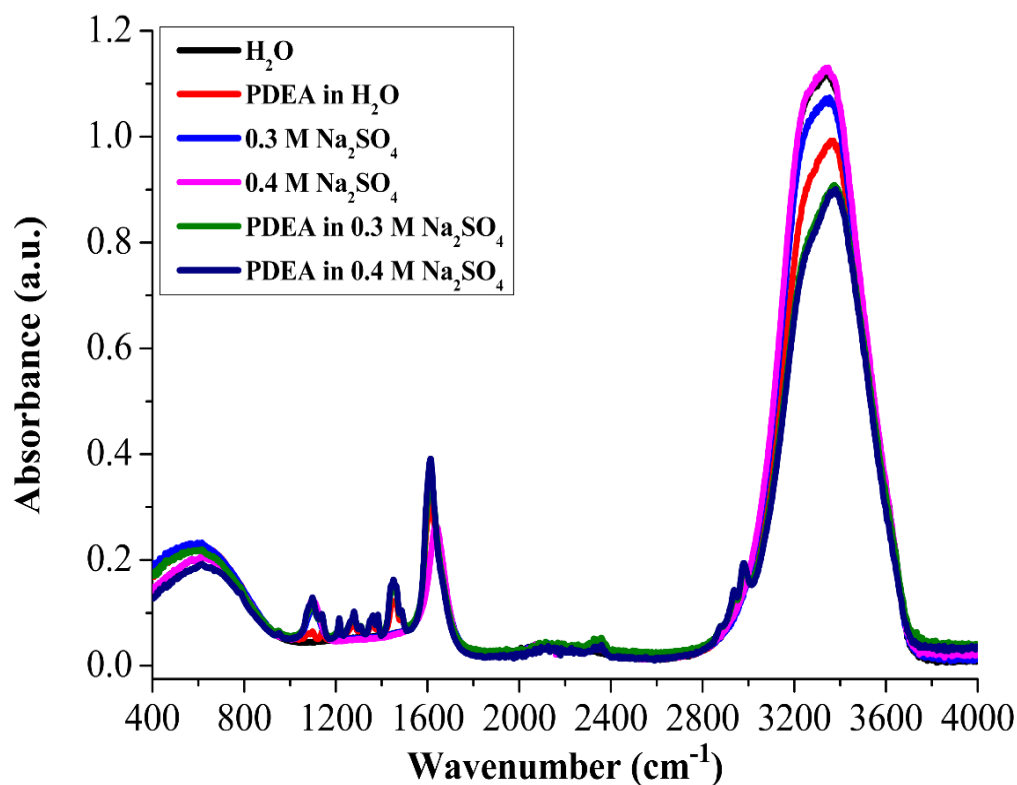


Figure 3.22 The ATR – FTIR spectra of H₂O, PDEA (20 mg/mL) in H₂O, y M Na₂SO₄ and PDEA (20 mg/mL) in y M Na₂SO₄ (where $x = 0.3$, and 0.4 , respectively) at 35 °C. The ATR – FTIR spectra of each substance is colour coded separately, and the colour coding for each one of them is displayed in the inset.

The collapsed state of PDEA as a function of Na₂SO₄ concentration above the LCST was also investigated by observing the intensity change of the peak for the side chain deformation of the macromolecule around 1360 and 1380 cm⁻¹ that can be seen in Fig. 3.23.

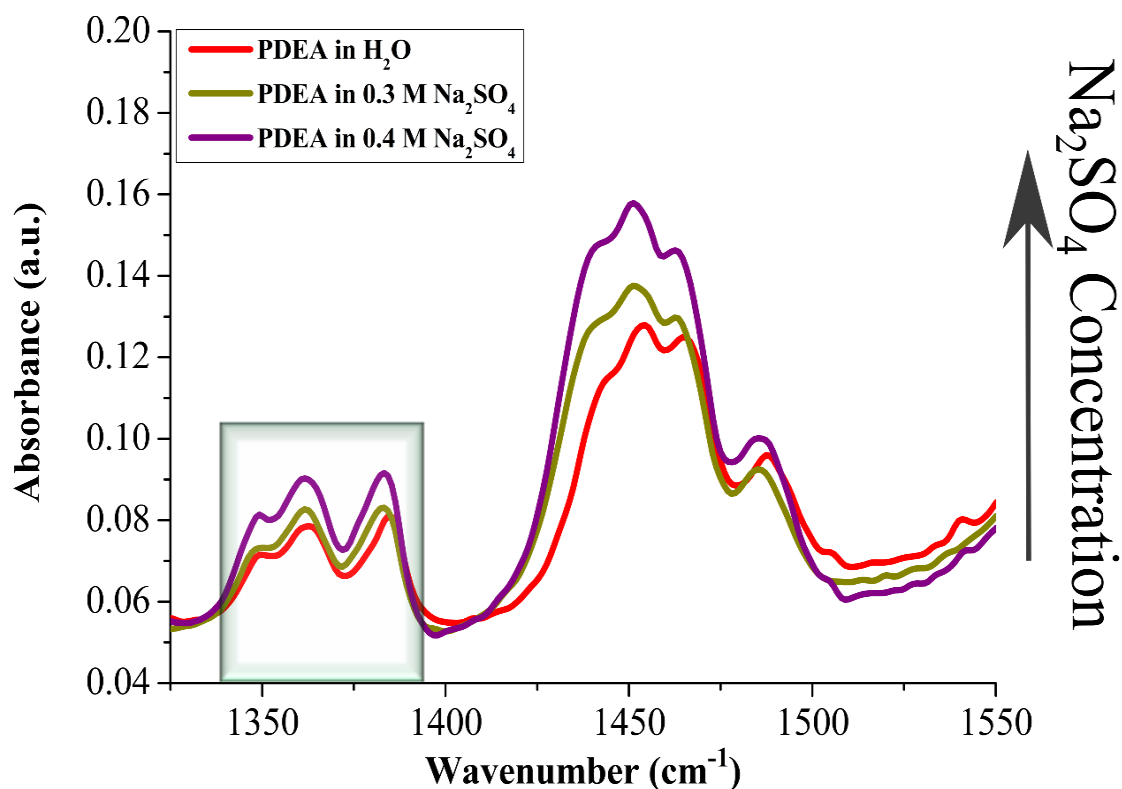


Figure 3.23 The ATR – FTIR spectra of PDEA (20 mg/mL) in H₂O, and PDEA (20 mg/mL) in y M Na₂SO₄ (where y = 0.3 and 0.4, respectively) at 35 °C in between 1325 – 1550 cm⁻¹. The ATR – FTIR spectra of each substance is colour coded separately, and the colour coding for each one of them is displayed in the inset. The shadowed black coloured rectangle is displayed to indicate the intensity change of the peak for the side chain deformation of PDEA around 1360 and 1380 cm⁻¹, and the direction of black arrow indicates the increasing concentration of Na₂SO₄.

The peak assignment of PDEA related bands in Fig. 3.23 is the same with Fig. 3.19. [120] The peak intensity of PDEA around 1360 and 1380 cm⁻¹ is increasing while increasing the concentration of Na₂SO₄ above the LCST, once again comparably observed for PNIPAM, as well. Clearly, by considering the observed effects of Na₂SO₄ as kosmotropic salt that aggregates the interested macromolecules more with increased concentrations. Overall, the ATR – FTIR based method is capable of investigating the collapse form of the macromolecules for both polymers with “salting-in” and “salting-out” agents above the LCST. Even though our ATR – FTIR based method seems valid to probe interested macromolecules in their collapsed form, the ATR-FTIR based considerations should be ruled-out. The change of the penetration depth due to refractive index change of the solution as a function of cosolvent concentration for each sample droplet of both PNIPAM and PDEA was required to be done before

interpreting the collapse form of each macromolecule above the LCST qualitatively, as well as quantitatively before performing the ATR – FTIR measurements. Consequently, the refractive index change induced penetration depth was examined for interested sample droplets that the details regarding this topic can be seen in the next section (Section 3.1.3).

3.1.3 Refractive Index of the Solution

As mentioned in Section 3.1.1, refractive index of the sample droplet, n_2 , could affect the penetration depth. In order to examine the effect of the refractive index of the sample droplet, a graph of linear fit for the urea solutions (from 1.0 M to 6.0 M) from ATR – FTIR bottom heating measurements above the LCST was plotted for the band of symmetric stretch of -CN group of urea at 1460 cm^{-1} in Figure 3.24. In accordance with the obtained graph, there is a linear correlation between absorbance, and urea concentration to some extent at low concentration regime of urea for the peak intensity corresponding at specific peak position. However, at elevated concentrations of urea, offset of the linearity for the graph can be observed (specifically the case at 6.0 M urea). Nevertheless, the R^2 value for the linear fit is equal to 0.986 that it can be considered as an indication for a reliable correlation throughout the investigated urea concentrations at around 1460 cm^{-1} . Furthermore, this demonstrates that the ATR – FTIR spectra of the interested urea solutions are only barely affected from the refractive index of the solution.

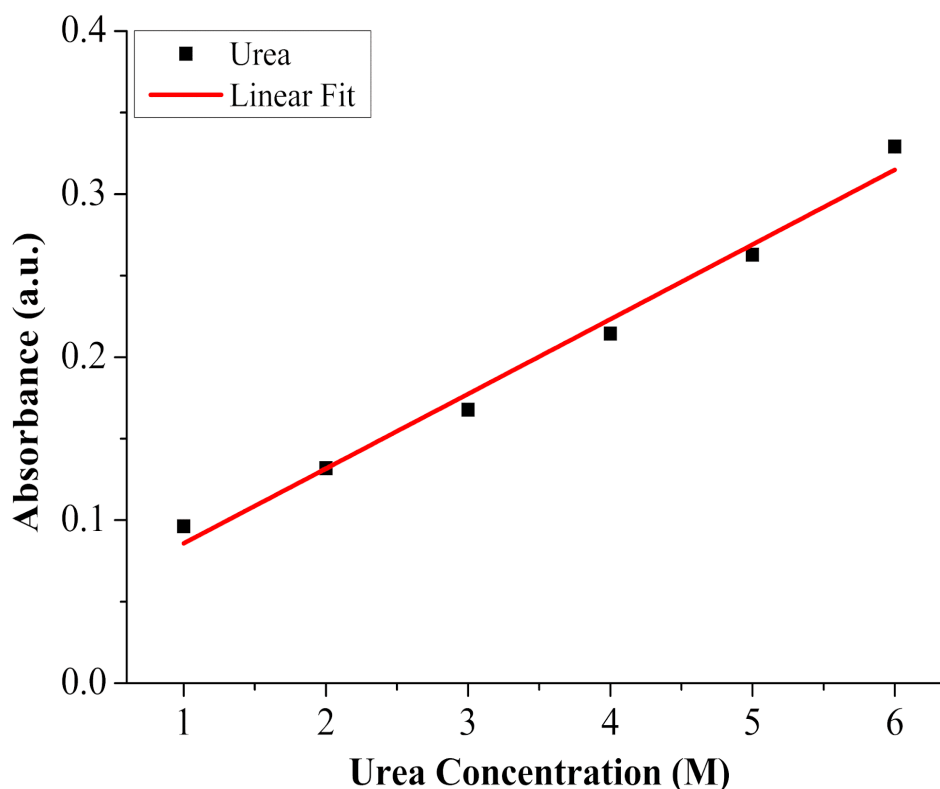


Figure 3.24 The graph of linear fit analysis for urea solutions from 1.0 M to 6.0 M at around 1460 cm^{-1} ($R^2 = 0.98596$).

The same process was also done for the PNIPAM containing urea samples (from 1.0 M to 6.0 M) and its graph can be seen in Fig. 3.25. The R^2 value for the linear fit is equal to 0.965 that the offset for this figure can be observed more pronouncedly compared to Fig. 3.24. It is not surprising to observe the offset for the interested graph, since there is polymer in each sample solutions, as well as urea molecules, in which both the presence and the concentration of the polymer would eventually affect the refractive index of the sample solutions (n_2). However, the effect of n_2 to sample droplets can still be considered negligible above the LCST for the collapsed state of the tested macromolecules within the concentration regime of urea owing to the obtained rather linear relation between the absorbance, and urea concentration. Note that the presence of macromolecules, although slightly, influence the urea accumulation around the ATR crystal (see Fig 3.28).

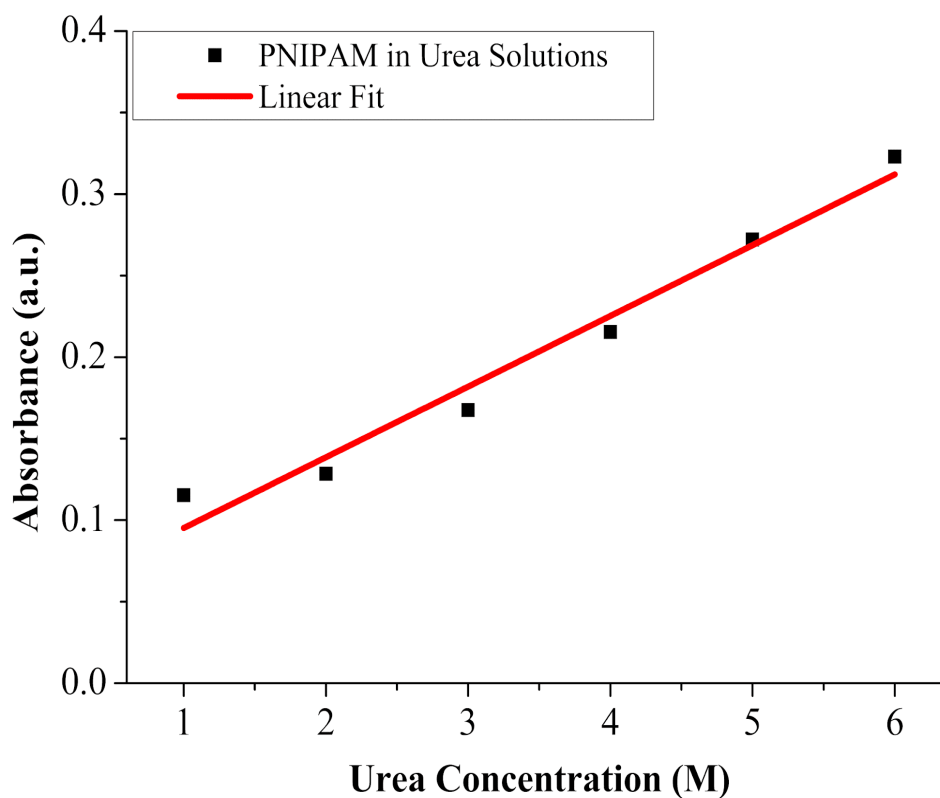


Figure 3.25 The graph of linear fit analysis for PNIPAM in urea solutions from 1.0 M to 6.0 M at around 1460 cm^{-1} ($R^2 = 0.96514$).

By demonstrating the negligible effect of the refractive index of the solution for interested sample droplets of the macromolecules alongside several concentrations of urea (Fig. 3.24, and Fig. 3.25), utilization of the ATR – FTIR based method to the investigation of the collapsed mechanism of the interested macromolecules as a function of urea concentration above the LCST was done accordingly.

3.2 Investigation of the Collapsed States of PNIPAM and PDEA in H₂O (or D₂O) as a Function of Urea (or Urea- d₄) Concentration Above the LCST

In the literature, several researches can be found frequently to examine the soluble (uncollapsed) states of proteins and biomacromolecules with phase transition. In contrast, there is a deficiency regarding the investigation of the collapsed states of the relevant molecules experimentally. Therefore, in order to examine the collapsed states of PNIPAM and PDEA macromolecules, lower critical solution temperatures (LCSTs) of these macromolecules should be determined. Thereby, determining the LCSTs of each macromolecule depending on several urea concentrations will allow the determination of their transition temperature from their uncollased state to their collapsed state. For this purpose, the measurement of LCST values of PNIPAM and PDEA macromolecules depending on the urea concentration can be seen in the next section (Section 3.2.1).

3.2.1 LCST Measurements of PNIPAM and PDEA in H₂O as a Function of Urea Concentration

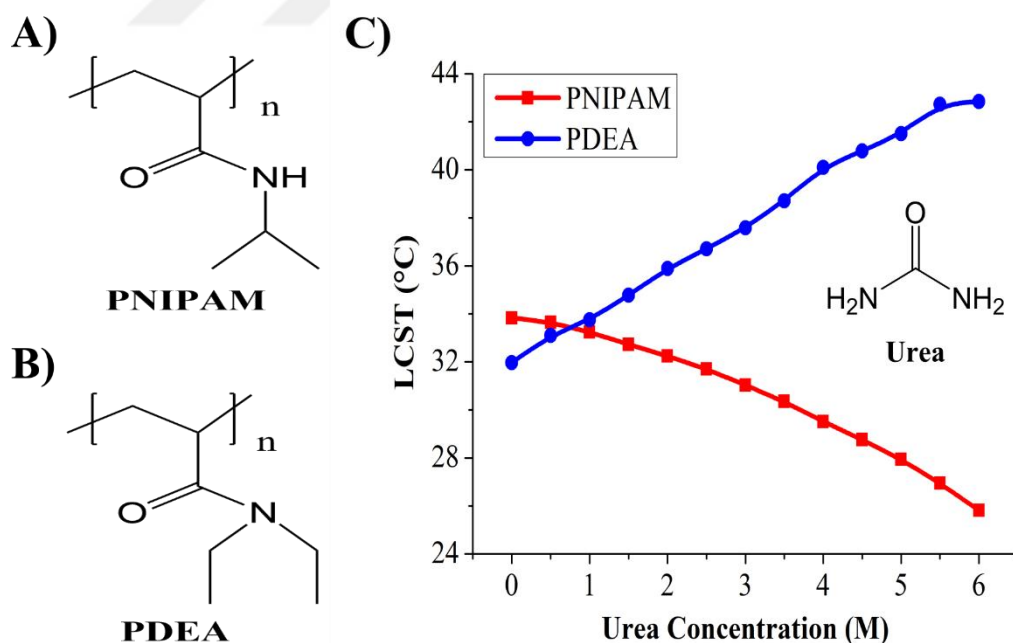


Figure 3.26 **A)** Representation of molecular structure of poly (*N* - isopropylacrylamide) (abbreviated as PNIPAM). **B)** Representation of molecular structure of poly (*N,N* - diethylacrylamide) (abbreviated as PDEA). **C)** LCST measurements of 5 mg/mL PNIPAM and PDEA solutions as a function of urea concentration, and molecular structure of urea is shown in the middle of the graph. All standard deviations were within the data points drawn. Each symbol represents data points from thirteen measurements, and the solid lines are for guiding reader.

First, the LCST measurements of 5 mg/mL PNIPAM and PDEA solutions as a function of urea concentration (with 0.5 M increments, up to 6.0 M) were done C) part of Fig. 3.26). For PDEA, the LCST values (shown with blue circles) display an increasing trend while increasing the concentration of urea, in which it can also be stated as PDEA has “salting-in” behaviour, and this is an expected trend from PDEA since urea is a well-known universally denaturant to numerous macromolecules; therefore, the solubility of interested macromolecule should also increase especially at elevated concentrations of urea (above 3.0 M). For PNIPAM, corresponding trends can also be expected, owing to the similar molecular structure of PNIPAM and PDEA A) and B) parts of Fig. 3.26, respectively). However, the LCST values (shown with red squares) display a decreasing trend while increasing the concentration of urea, in which it can also be stated as PNIPAM has “salting-out” behaviour, even though the molecular structure of these two macromolecules is similar. This intriguing observation of the LCST behaviour of PNIPAM as a function of urea concentration captured our attention to investigate it to a greater extent that despite several researches in the literature regarding not just these two macromolecules (PNIPAM [126], and PDEA [127]), but also their hydrogels, [103] and copolymers [128] that can be found facilely; most of these studies focused on PNIPAM, PDEA, and their derivatives below the LCST, and up to date, there are no experimental results for these macromolecules (especially for PNIPAM) above the LCST. In the direction of investigating PNIPAM and PDEA as a function of urea concentration above the LCST, ATR – FTIR experiments for these two macromolecules at several concentrations of urea above the LCST were conducted. The primary focus of the next section (Section 3.2.2) is examining possible accumulation and/or depletion of urea to/from the collapsed state of the interested macromolecules above the LCST to observe the effect of urea to overall collapse mechanism by using the obtained results from the ATR – FTIR bottom heating measurements to further examine the one of the best-known mechanisms for urea-induced polymer collapse in the literature, “clouding mechanism” by Van der Vegt and his coworkers, [100] accordingly their work states that urea molecules are preferentially binding to the collapse state of PNIPAM.

3.2.2 ATR – FTIR Bottom and Top Heating Measurements for Investigation of the Urea Intensity Above the LCST

For the investigation of the urea-induced collapsed mechanism for the interested macromolecules (specifically for PNIPAM) above the LCST, the ATR – FTIR based method was utilized not just for examining the collapsed states of PNIPAM and PDEA above the LCST, but also urea itself, as well. The fundamental cause regarding this examination was checking one of the proposed mechanism for polymer collapse in the literature, namely “clouding mechanism”, from Francisco Roperro and Nico van der Vegt by using MD simulations that in line with their research, it was proposed as : “Owing to strong van der Waals dispersion interactions with the hydrophobic isopropyl groups, urea molecules preferentially accumulate in the first solvation shell of PNIPAM, resulting in the development of low entropy urea clouds that these clouds act as an entropic driving factor for folding, causing preferential urea binding to the folded state and lowering the lower folding temperature” [100]. Presuming the clouding mechanism is well grounded for the urea-induced collapse mechanism of PNIPAM, one can straightforwardly expect that intensity of urea in PNIPAM for its collapsed state should be higher than the intensity of urea without PNIPAM, since there is a preferential binding between urea and the collapsed state of PNIPAM in the solution. Fortunately, validity of the clouding mechanism can be inspected by using ATR – FTIR based method for investigating the collapsed states of the interested macromolecules above the LCST. Since the mathematical formulation for the interpretation of the obtained ATR – FTIR spectra of the interested macromolecules in H₂O as a function of urea concentration (up to 6.0 M) above the LCST was explained in the last part of the Section 3.1.1, and the obtained results from the ATR – FTIR bottom and top heating measurements for PNIPAM and PDEA with several concentrations of urea in H₂O above the LCST discussed in detail subsequently (Section 3.2.3, and Section 3.2.4, respectively), one can easily extract information regarding the intensity of urea with and without polymer in H₂O at 1160 cm⁻¹, which corresponds to -NH₂ bending mode of urea, [123] from Fig. 3.30, and Fig. 3.32, and further examine whether the clouding mechanism is valid or not. The obtained results for the comparison of the urea intensity with and without the interested macromolecules in H₂O by using ATR – FTIR bottom and top heating measurements above the LCST can be seen in Fig. 3.27, and Fig. 3.28, respectively.

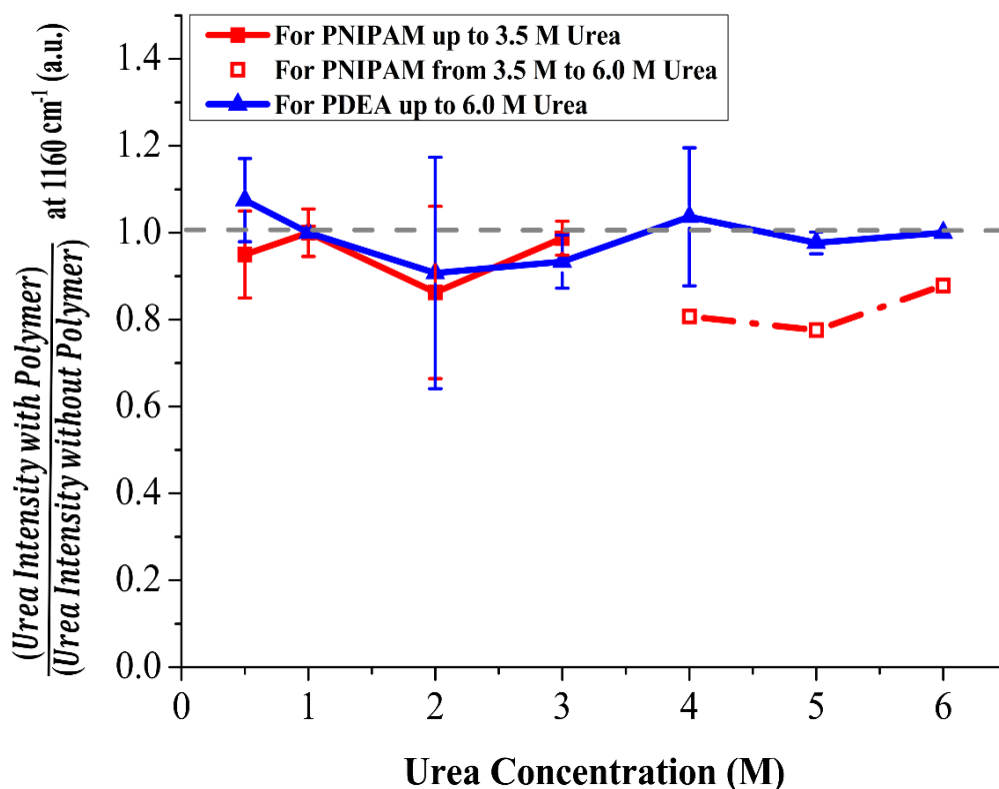


Figure 3.27 ATR-FTIR bottom heating measurement results of PNIPAM up to 3.5 M Urea (shown with red squares), PNIPAM from 3.5 M to 6.0 M Urea (shown with red empty squares), and PDEA up to 6.0 M Urea (shown with blue triangles) above the LCST (45°C and 50 °C) as a function of urea concentration. The y axis of the graph indicates the intensity of urea over the intensity of urea without polymer at 1160 cm^{-1} in H_2O . The standard deviations of PNIPAM up to 3.5 M Urea, and PDEA up to 6.0 M Urea are shown with error bars. The lines are for guiding the reader.

The grey coloured dashed line in Fig. 3.27 indicates the reference point for the obtained results that the urea intensity with polymer in H_2O at 1160 cm^{-1} is equal to the urea intensity with polymer in H_2O at 1160 cm^{-1} . For the PNIPAM up to 3.5 M urea, and PDEA up to 6.0 M urea, the values are around one and do not indicate steep increase or decrease as a function of urea concentration. These results might be interpreted for the collapsed states of PNIPAM less than 4.0 M urea and PDEA up to 6.0 M urea above the LCST as there is no significant accumulation or depletion of urea molecules to the collapsed states of the macromolecules. For the PNIPAM from 3.5 M to 6.0 M urea, even though the values are less than 1, there is almost no, if any, collapsed PNIPAM on top of the ATR crystal to be observed by performing ATR – FTIR bottom heating measurements (especially above 4.0 M urea, see Fig. 3.30 in Section 3.2.3). Consequently, interpretation of this result for checking the validity of the clouding

mechanism would not be reasonable, and therefore, the comparison of the urea intensity with and without the interested macromolecules in H₂O by using ATR – FTIR top heating measurements above the LCST (since with these measurements, the collapsed state of PNIPAM was observed on the ATR crystal even at elevated concentrations of urea, discussed in detail in Section 3.2.4) was also done that the obtained results can be seen in Fig. 3.28.

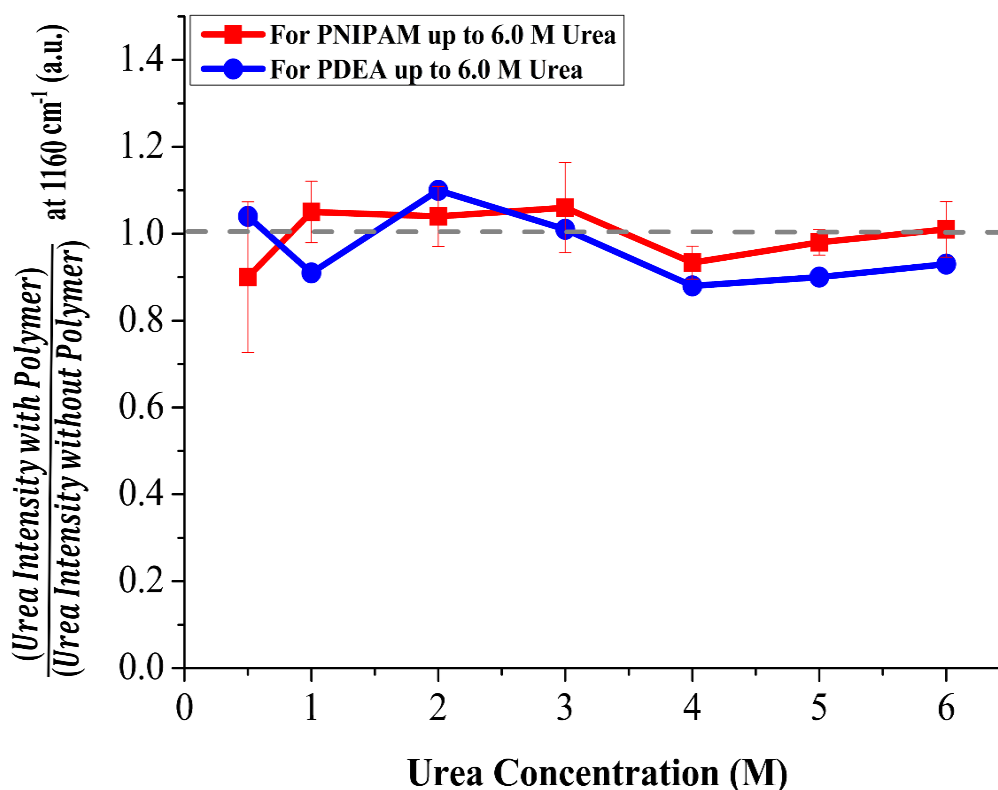


Figure 3.28 ATR-FTIR top heating measurement results of PNIPAM (shown with red squares), and PDEA up to 6.0 M Urea (shown with blue circles) above the LCST (55 °C) as a function of urea concentration. The y axis of the graph indicates the intensity of urea over the intensity of urea without polymer at 1160 cm⁻¹ in H₂O. The standard deviations for PNIPAM are shown with error bars.

Similar to Fig. 3.27, the grey coloured dashed line in Fig. 3.28 indicates the reference point for the obtained results that the urea intensity with polymer in H₂O at 1160 cm⁻¹ is equal to the urea intensity with polymer in H₂O at 1160 cm⁻¹. For all tested urea concentrations, the values are very close to 1, whether PNIPAM or PDEA is used for the investigation. Since there is no significant increase or decrease of the values for each macromolecule, these results distinctly indicate that there should not be a major occurrence of a significant depletion or accumulation of urea molecules during the collapse of PNIPAM and PDEA above the LCST

(even for PNIPAM at and above 4.0 M urea, otherwise it would be displayed in the graph, since the ATR – FTIR based method able to express any intensity change of the interested molecules effectively). With these results, it was interpreted as the urea clouding mechanism should not be the main driving force for collapsing of PNIPAM in H₂O as a function of urea concentration (up to 6.0 M) above the LCST, and the next focus regarding the urea-induced collapse mechanism of PNIPAM and PDEA in H₂O was done by focusing on the intensity of these macromolecules (but not urea directly) in their collapsed states above the LCST with the usage of the ATR – FTIR bottom heating measurements that further details about the subject can be seen in the next section (Section 3.2.3).



3.2.3 ATR – FTIR Bottom Heating Measurements of PNIPAM and PDEA in H₂O as a Function of Urea Concentration Above the LCST

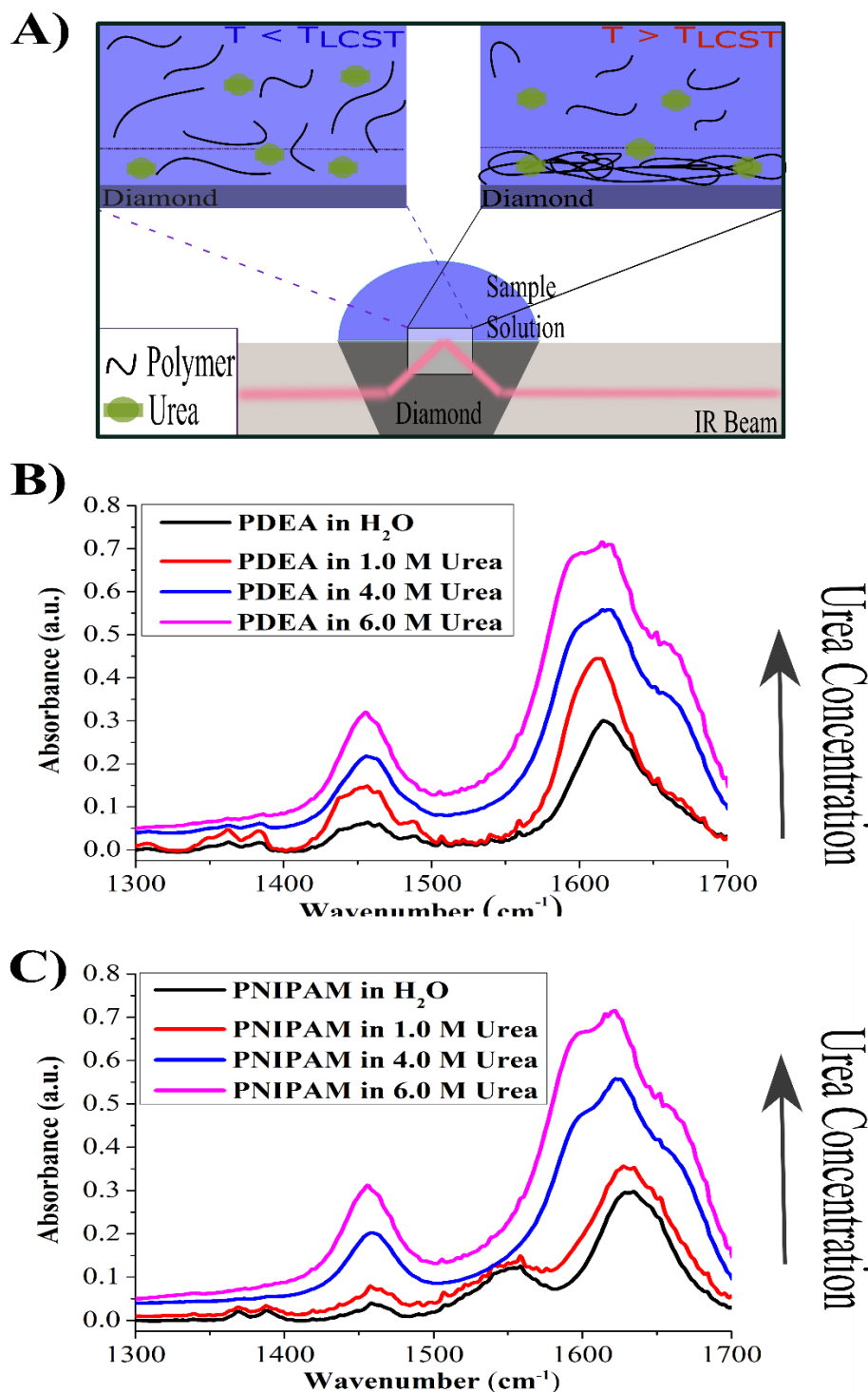


Figure 3.29 **A)** Minimized version of Fig. 3.1 that original figure can be seen in Section 3.1.1. **B)** ATR-FTIR spectra of the collapsed state of the 20 mg/mL PDEA above the LCST (45°C and 50 °C) in H₂O and in the presence of several concentrations of urea (1.0 M, 4.0 M, and 6.0 M respectively). **C)** ATR-FTIR spectra of the collapsed state of the 20 mg/mL PNIPAM above the LCST (45°C and 50 °C) in H₂O and in the presence of several concentrations of urea (1.0 M, 4.0 M, and 6.0 M respectively). For **B)** and **C)** parts of the figure, black lines indicate polymers in H₂O and red, blue and pink lines indicate polymers in 1.0 M, 4.0 M and 6.0 M urea, respectively.

The investigation of the collapsed states of both PNIPAM and PDEA were done by utilizing ATR – FTIR spectroscopy at several concentrations of urea below and predominantly above the LCST A) part of Fig. 3.29 that further details were explained in Section 3.1.1. By using ATR-FTIR spectroscopy, both 20 mg/mL PNIPAM and PDEA in aqueous solutions and also in different concentrations of urea can be studied (B) and C) parts of Fig. 3.29, respectively). For the investigation of collapsed state of these two polymers, specific peaks belong to the urea, namely –NH₂ bending around 1160 cm⁻¹ [123] (not shown here), and -CN symmetric stretching around 1460 cm⁻¹ [123], and to the polymers, namely side chain deformation (for PNIPAM, around 1370 and 1390 cm⁻¹ [121] and for PDEA, around 1360 and 1380 cm⁻¹ [122]) were examined. The peak assignment of other peaks of macromolecules on the interested region (in between 1300-1700 cm⁻¹) can be done as follows: for B) part of Fig. 3.29, specifically PDEA in H₂O, deformation of -CH groups of PDEA can be observed around 1465 cm⁻¹, and the peak around 1635 cm⁻¹ corresponds to amide I region of the macromolecule; whereas for C) part of Fig. 3.29, specifically PNIPAM in H₂O, symmetric deformation of -C(CH₃)₂ of PNIPAM can be observed around 1465 cm⁻¹, and the peaks around 1560 cm⁻¹ alongside 1625 cm⁻¹ correspond to amide II, and amide I regions of the macromolecule, respectively [117], [120]. There are several fingerprints on the overall spectrum that can be used to explore both PNIPAM and PDEA, however due to overlapping peaks arise from urea and/or water molecules with the interested polymers, only the region between 1360-1390 cm⁻¹ (which covers the region of side chain deformations of both PNIPAM and PDEA) was taken into account and considered throughout the research. Interpretation of obtained spectra for both PNIPAM and PDEA was done by taking ratio between the intensity of interested polymer in specific concentration of urea to the intensity of interested polymer in H₂O in considered region of the overall spectra, where further explanation about this process can be found in Section 2.3.2. Consequently, obtained results from ATR-FTIR bottom heating measurements as a function of urea concentration above the LCST for both PNIPAM and PDEA are shown in Figure 3.30.

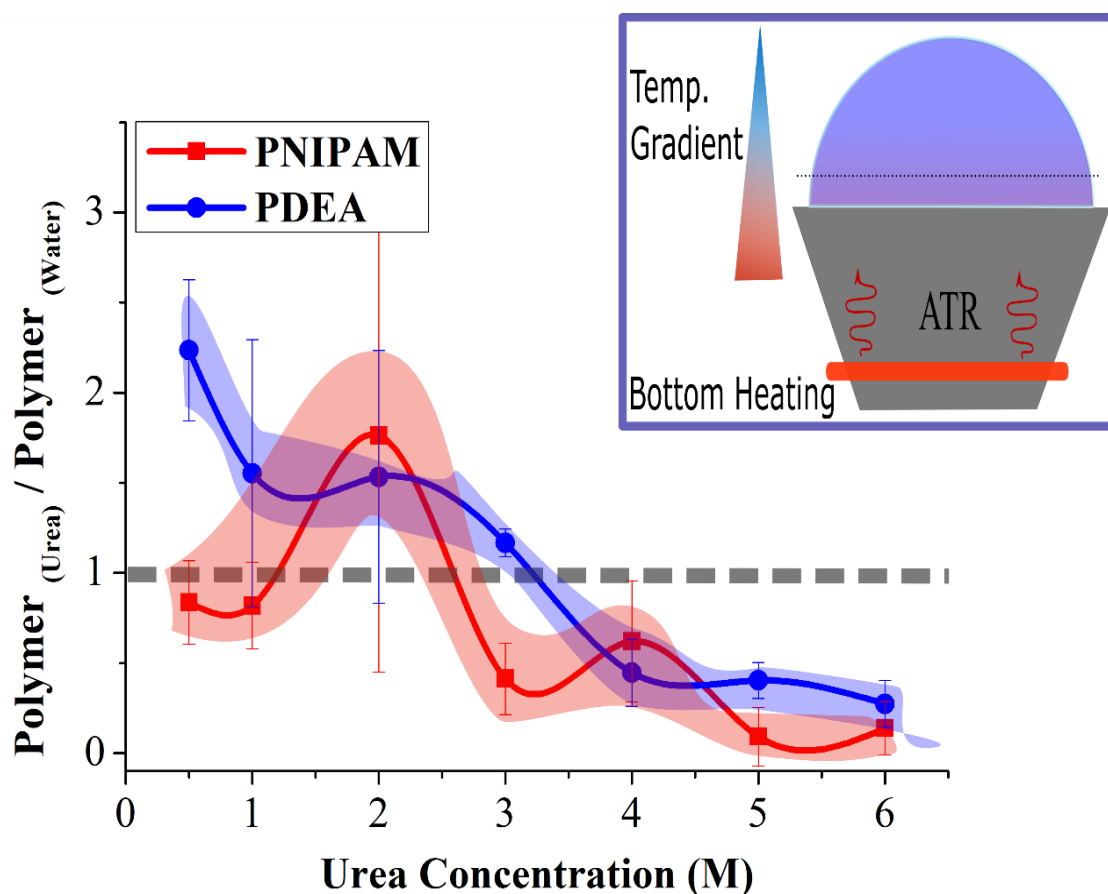


Figure 3.30 ATR-FTIR bottom heating measurement results of PNIPAM and PDEA (20 mg/mL for each polymer) above the LCST (45°C and 50 °C) as a function of urea concentration with an inset of temperature gradient. The y axis of the graph indicates the intensity of polymer for its side chain deformation (for PNIPAM, around 1370 and 1390 cm^{-1} and for PDEA, around 1360 and 1380 cm^{-1}) in urea solution over the intensity of polymer in H_2O for side chain deformation of polymer. Colour coding, shadowing and lines are for the guiding reader.

In the graph of Fig. 3.30, the x axis indicates the concentration of urea and the y axis indicates the ratio between the intensity of polymer in urea over the intensity of polymer in H_2O in considered region of the overall spectra that the value ‘1’ in y axis corresponds to equal intensity of polymer in urea and in water. The aqueous solutions of PNIPAM and PDEA in 0.5 M, 1.0 M, 2.0 M and 3.0 M urea at 45 °C and the same solutions in 4.0 M, 5.0 M and 6.0 M urea at 50 °C were investigated. For PDEA, there is a decrease for its values when the concentration of urea increases and this is once again an expected behaviour to be observed, since urea is a well-known universally denaturant to numerous macromolecules, which is evident by both results of LCST (Fig. 3.26.) and ATR-FTIR bottom heating measurements for the interested macromolecule. Conversely, PNIPAM and PDEA have similar behaviour at relatively low urea concentrations (below 3.0 M), yet this behaviour is changing at elevated

urea concentrations (above 4.0 M). The behaviour of PNIPAM for the results of ATR – FTIR bottom heating measurements is indeed interesting, since the results of the LCST measurements for PNIPAM distinctly indicates that, it is “salting-out” from urea solutions; however, in accordance with ATR-FTIR bottom heating measurements above the LCST, similar to PDEA, PNIPAM is also expelled from the ATR crystal. This is especially the case at 5.0 M and 6.0 M urea, in which there is almost no PNIPAM peak to be observed on ATR crystal. By considering the obtained results from both LCST and ATR – FTIR bottom heating measurements for PNIPAM above the LCST, a collapse mechanism for PNIPAM in urea solutions above the LCST was proposed that the pictorial depiction of this mechanism can be seen in Fig. 3.31.

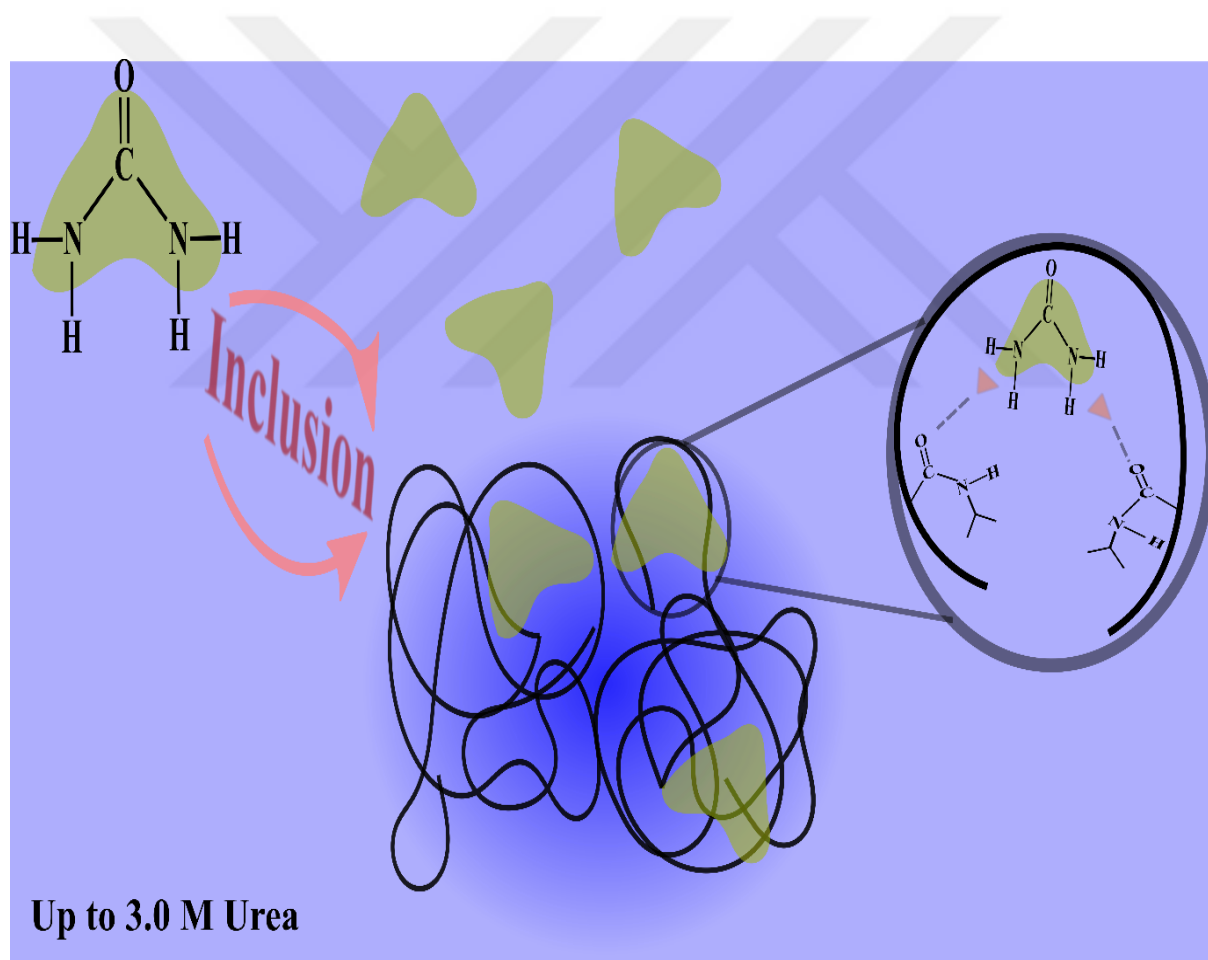


Figure 3.31 Schematic for the facilitation of the collapse mechanism of PNIPAM by urea above the LCST that urea interacts with amide group of PNIPAM in the collapsed state shown with zoomed-in picture.

Intermolecular interactions between urea and amide group of PNIPAM above the LCST could facilitate the observation of the collapsed state for the macromolecule that -NH_2 groups of urea might have significant role regarding this interaction (also shown in zoomed-in picture of Fig. 3.31). A possible explanation for the intriguing behaviour of PNIPAM at elevated concentrations of urea above the LCST case might be related with temperature, since with this kind of set-up configuration for our measurements, there was a formation of small temperature gradient that bottom of the sample droplet was warmer region compared to top of it and the collapsed state of PNIPAM might prefer to migrate to warmer region in the sample droplet. To test this idea, ATR – FTIR spectroscopy was used once more for the same polymers at several concentrations of urea, yet the heating method was switched from bottom to top by using an external heating device. Accordingly, once again there was a formation of small temperature gradient, yet the top of the sample droplet was a warmer region compared to the bottom of it. The obtained results from ATR-FTIR top heating measurements as a function of urea concentration above the LCST for both PNIPAM and PDEA are shown in Fig. 3.32 of the section 3.2.4.

3.2.4 ATR – FTIR Top Heating Measurements of PNIPAM and PDEA in H₂O as a Function of Urea Concentration Above the LCST

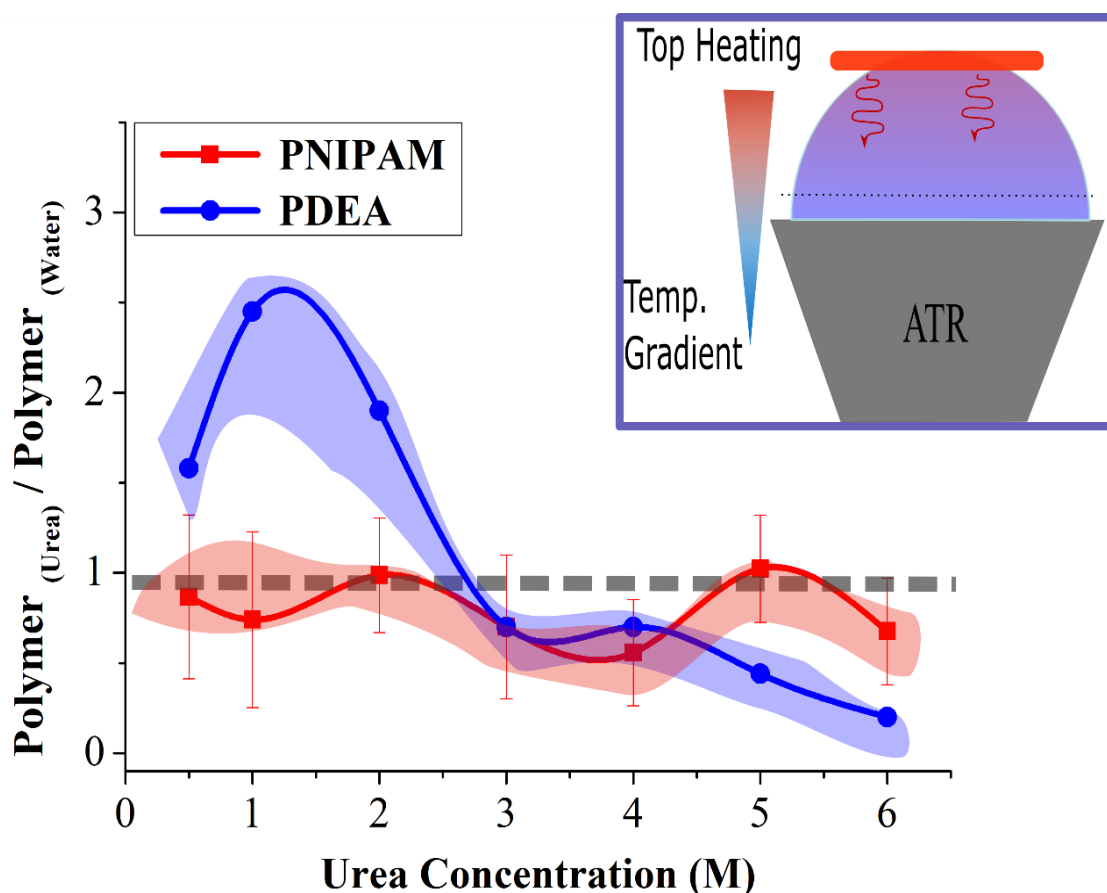


Figure 3.32 ATR-FTIR top heating measurement results of PNIPAM and PDEA (20 mg/mL for each) above the LCST (55°C) as a function of urea concentration with an inset of temperature gradient. The y axis of the graph indicates the intensity of polymer in urea over the intensity of polymer in H₂O for side chain deformation of polymer. Colour coding, shadowing and lines are for the guiding reader.

The aqueous solutions of PNIPAM and PDEA in various concentrations of urea (up to 6M) at 55 °C were investigated. The behaviour of PDEA remains the same compared to the results of ATR-FTIR bottom heating measurements. Strikingly, PNIPAM peaks were observable on ATR crystal even at elevated concentrations of urea, particularly, PNIPAM remained on ATR crystal for all tested urea concentrations. Compared to the results of ATR-FTIR bottom heating measurements, there was a substantial difference for PNIPAM at elevated concentrations of urea (above 3.0 M).

As demonstrated by ATR – FTIR bottom and top heating measurements, PNIPAM and PDEA have different behaviours above 3.0 M urea concentration above the LCST that the main difference regarding their behaviours should be owing to specific interactions between urea molecules and their collapsed states. This can be conjectured by considering not only Fig. 3.28 (where there is no significant urea accumulation/depletion to or from the collapsed state of macromolecules), but also the different amide groups within the macromolecule that would affect the interactions between urea molecules, and collapsed states of the macromolecules. Moreover, the flexibility of the side chains for each macromolecule (e.g., isopropyl group of PNIPAM vs. diethyl group of PDEA) might also be relevant. Another explanation regarding the observed difference for these macromolecules in several urea concentration above the LCST can be done by taking into consideration of bulk solution (e.g., the presence of urea, and water molecules in the bulk alongside their interactions in aqueous medium, but not the surface). However, any possible significant effect from the bulk solution for the collapsed states as a function of urea concentration would be eventually resulted to the same extent for PNIPAM, and PDEA. Thus, it is unlikely that the difference between the behaviours of the collapsed states of these macromolecules would emerge predominantly due to the interactions among urea and water molecules in the bulk solution.

To test whether this intriguing behaviour of PNIPAM above the LCST at elevated concentrations of urea affected by other factors besides temperature specifically, the investigation of PNIPAM was also done in urea- d_4 , and in D_2O as a solvent with an effort to further understand to unravel a solvent effect that might attributed to PNIPAM for its collapsed state at higher urea concentrations above the LCST that details about this investigation can be seen in the next section (Section 3.2.5).

3.2.5 Investigation of the Collapsed State of PNIPAM above the LCST for a Possible Solvent Effect with LCST and ATR – FTIR Bottom Heating Measurements of PNIPAM in D₂O as a Function of d₄-Urea Concentration

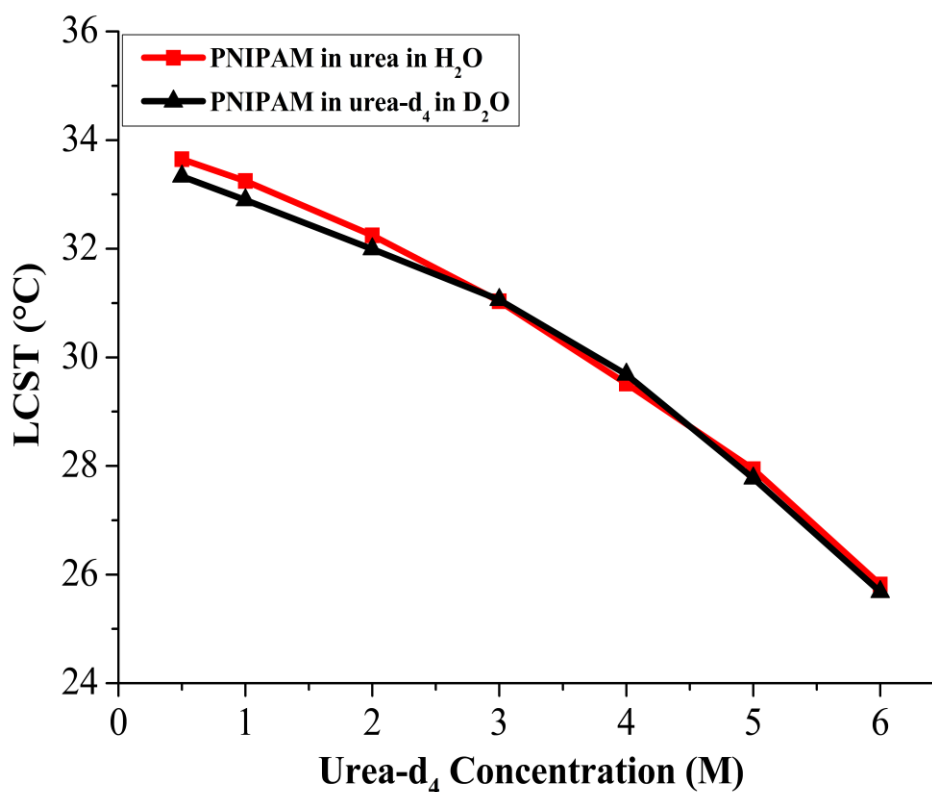


Figure 3.33 LCST measurements of 5 mg/mL PNIPAM solutions as a function of urea-d₄ concentration in D₂O (shown with red squares), in comparison with LCST measurements of 5 mg/mL PNIPAM solutions as function of urea concentration in H₂O (shown with black triangles). Each symbol represents data points from seven measurements of each, and the solid lines are for guiding the reader.

The LCST measurements of 5 mg/mL PNIPAM solutions as a function of urea-d₄ concentration (up to 6.0 M) were done (Fig. 3.33). For PNIPAM, the LCST values (shown with red squares) display once again a decreasing trend while increasing the concentration of urea-d₄, which is similar to the LCST trend of PNIPAM as a function of urea concentration C) part of Fig. 3.26). The difference of the LCST values between PNIPAM in urea solutions and in urea-d₄ solutions can be observed remarkably only when the concentration of the urea or urea-d₄ is 1M that even at this concentration, the LCST difference is around 0.6 °C, and for the other concentrations within the range, the LCST difference is around or less than 0.2°C. It can be observed that PNIPAM is also salting out from the urea-d₄ solutions especially at elevated

concentrations. To investigate this observation in a greater extent, ATR – FTIR bottom heating measurements for PNIPAM as a function of urea- d_4 concentration in D_2O above the LCST were utilized for observing possible any difference between results of the ATR – FTIR bottom heating measurements of PNIPAM as a function of urea concentration in H_2O above the LCST in a qualitative manner. The obtained results can be seen in Fig. 3.34.

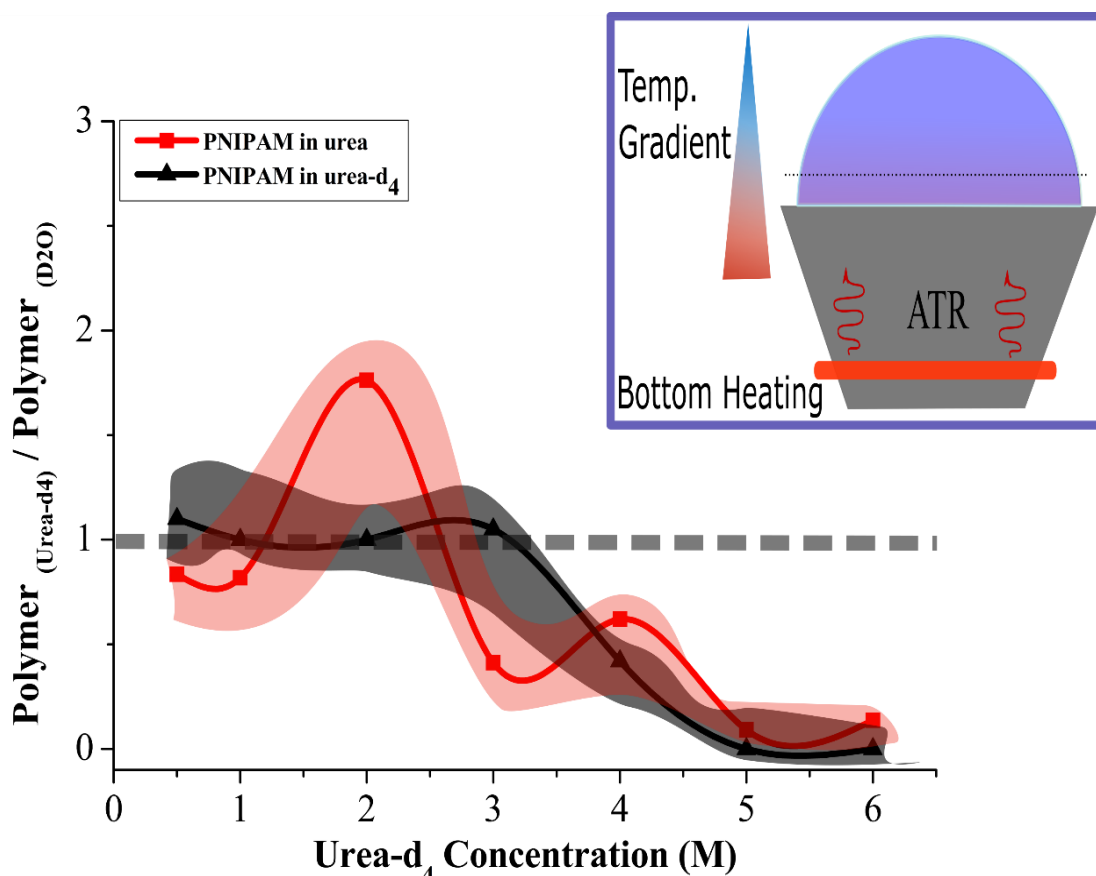


Figure 3.34 ATR-FTIR bottom heating measurement results of PNIPAM (20 mg/mL) above the LCST (50 °C) as a function of urea- d_4 concentration in D_2O (shown with black triangles) in comparison with PNIPAM (20 mg/mL) above the LCST (45 °C and 50 °C) as a function of urea concentration in H_2O (shown with red squares) with an inset of temperature gradient. The y axis of graph indicates the intensity of polymer in urea- d_4 solution over the intensity of polymer in D_2O for side chain deformation of polymer. Colour coding, shadowing and lines are for guiding reader.

Similar to the results of ATR – FTIR bottom heating measurements of PNIPAM as a function of urea above the LCST in H_2O , the macromolecule displays almost identical behaviour below and above 3.0 M that the values are again around 1 for PNIPAM at relatively lower concentrations of urea- d_4 , which indicates PNIPAM is on the ATR crystal; yet at elevated concentrations of urea- d_4 (above 4.0 M), there are almost no PNIPAM peaks to be observed on

the ATR crystal. This observation clearly indicates that changing solvent from H₂O to D₂O does not have an effect on the collapse behaviour of PNIPAM at elevated concentrations of urea above the LCST. Eventually, no difference was found between the results of PNIPAM that were studied in deuterated urea and water versus non-deuterated ones qualitatively, and therefore, it can be stated as it is unlikely that the distinctive behaviour of PNIPAM is due to solvent effect. By considering the obtained results from both LCST and ATR – FTIR bottom and top heating measurements, and displaying that the collapse mechanism should not be affected by the solvent effect (discussed in this section, Section 3.2.5), further inspected version of the collapse mechanism of PNIPAM at elevated concentrations of urea above the LCST can be seen in Fig. 3.35.

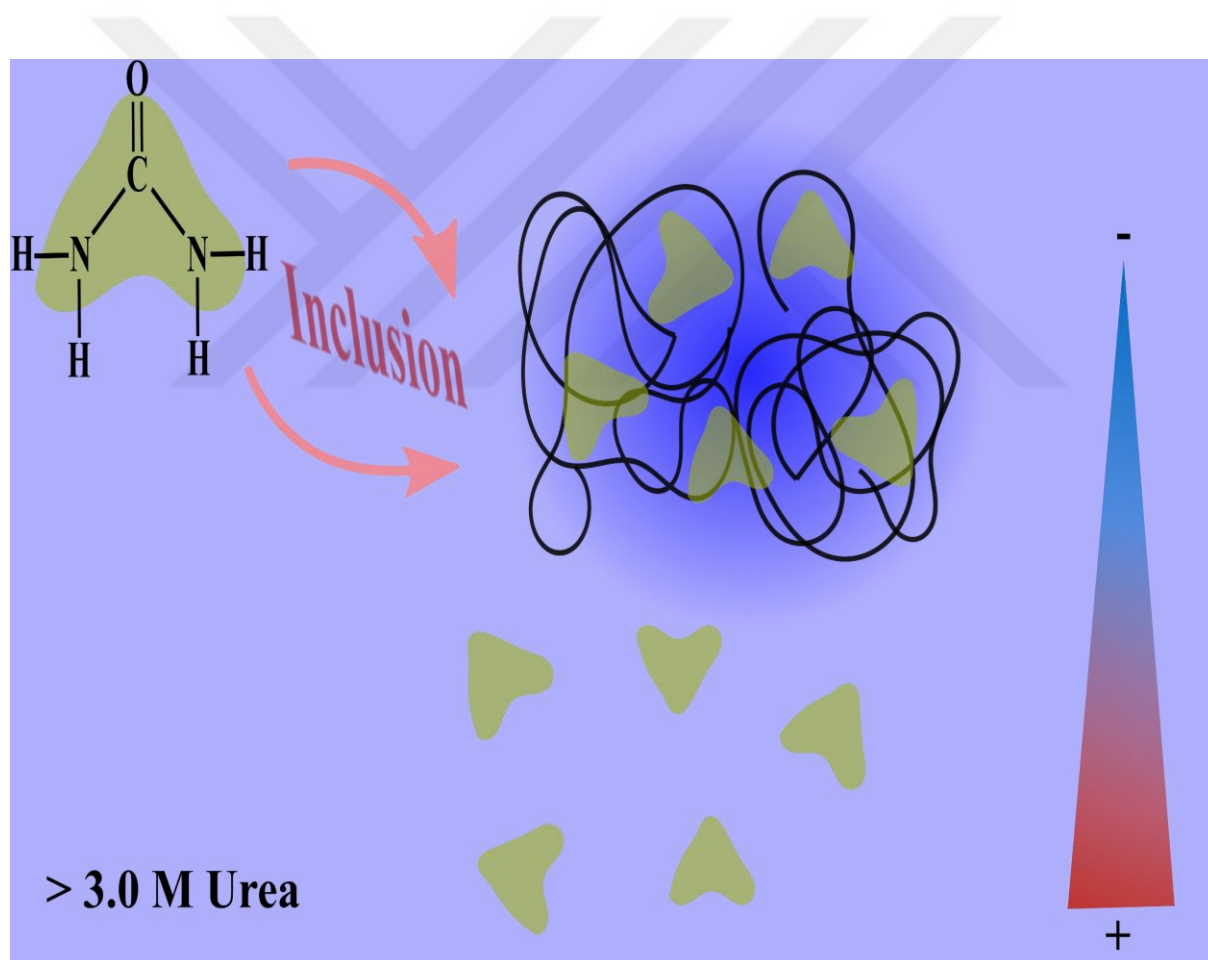


Figure 3.35 Schematic for the further facilitation of the collapse mechanism of PNIPAM by urea (especially above 4.0 M) above the LCST that the collapsed state of macromolecule could be observed pronouncedly.

The collapsed state of PNIPAM could be interpreted as it was further facilitated with elevated concentrations of urea above the LCST evident by the results of ATR – FTIR top heating measurements of PNIPAM that the collapse mechanism was not affected by the presence of deuterated solvent and/or urea. With these observations, in order to investigate both PNIPAM and PDEA to a greater extent, another set of experiments were done by using 1,3-dimethylurea instead of urea to differentiate the proposed mechanism for the collapse of PNIPAM as a function of urea concentration (especially at elevated urea concentrations, where it is used as denaturant for proteins and also other biomacromolecules) above the LCST that any possible, if any, interactions between the urea by its -NH₂ group and amide group of PNIPAM in the collapse state would be proven or disproven. The obtained results of LCST measurements of PNIPAM and PDEA solutions as a function of 1,3-dimethylurea concentration, and ATR-FTIR bottom and top heating measurements of PNIPAM are shown in the parts of Section 3.3.

3.3 Investigation of the Collapsed States of PNIPAM and PDEA in H₂O (or D₂O) as a Function of 1,3-Dimethylurea (or Deuterated 1,3-Dimethylurea) Concentration Above the LCST

3.3.1 LCST Measurements of PNIPAM and PDEA in H₂O as a Function of 1,3-Dimethylurea Concentration

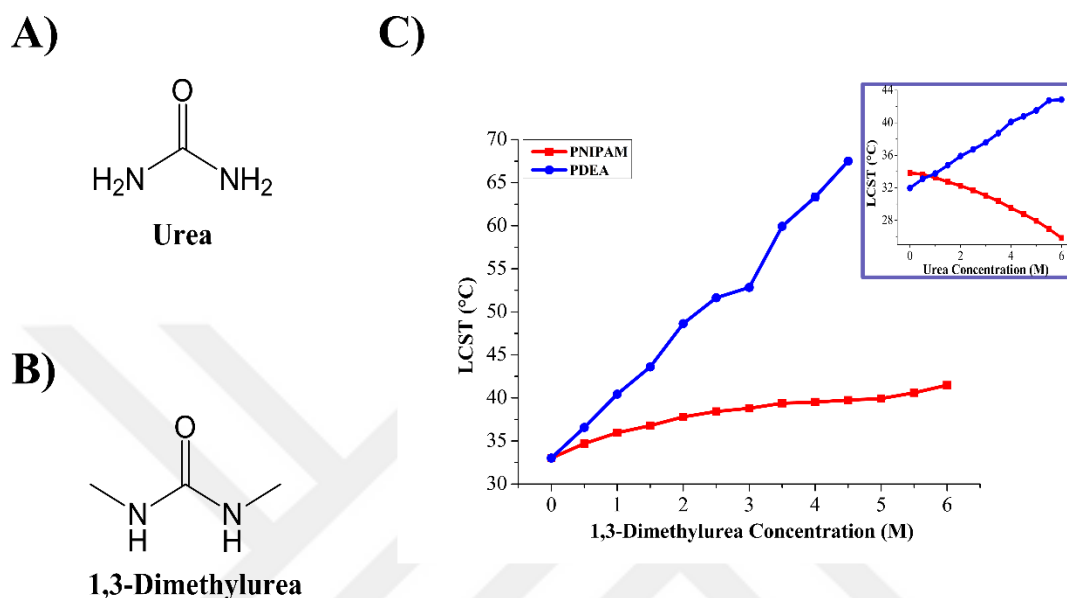


Figure 3.36 A) The molecular structure of urea. B) The molecular structure of 1,3-Dimethylurea. C) LCST measurements of 5 mg/mL PNIPAM and PDEA solutions as a function of 1,3-Dimethylurea concentration with an inset of LCST measurements of 5 mg/mL PNIPAM and PDEA solutions as a function of urea. All standard deviations were within the data points drawn. Each symbol for PDEA represents data points from ten measurements, whereas each symbol for PNIPAM represents data points from thirteen measurements, and the solid lines are for guiding the reader.

First, the LCST measurements of 5 mg/mL PNIPAM and PDEA solutions as a function of 1,3-dimethylurea concentration (with 0.5 M increments, up to 4.5 M for PDEA and up to 6.0 M for PNIPAM) were done (Fig. 3.36). For PDEA, the LCST values (shown with blue circles) display an increasing trend while increasing the concentration of 1,3-dimethylurea and this time for PNIPAM, similar to PDEA, its LCST values (shown with red squares) display an increasing trend, as well. Thus, both PNIPAM and PDEA have ‘‘salting-in’’ behaviour, and 1,3-dimethylurea acts as a denaturant for both of these macromolecules. Due to relatively higher LCST values of PDEA above 3.0 M of 1,3-dimethylurea, ATR-FTIR bottom and top heating measurements were done only for PNIPAM in a complete set. In Section 3.3.2, the results of ATR-FTIR bottom and top heating measurements at elevated concentrations of 1,3-dimethylurea for PNIPAM can be seen.

3.3.2 ATR – FTIR Bottom and Top Heating Measurements of PNIPAM in H₂O as a Function of 1,3-Dimethylurea Concentration at Above LCST

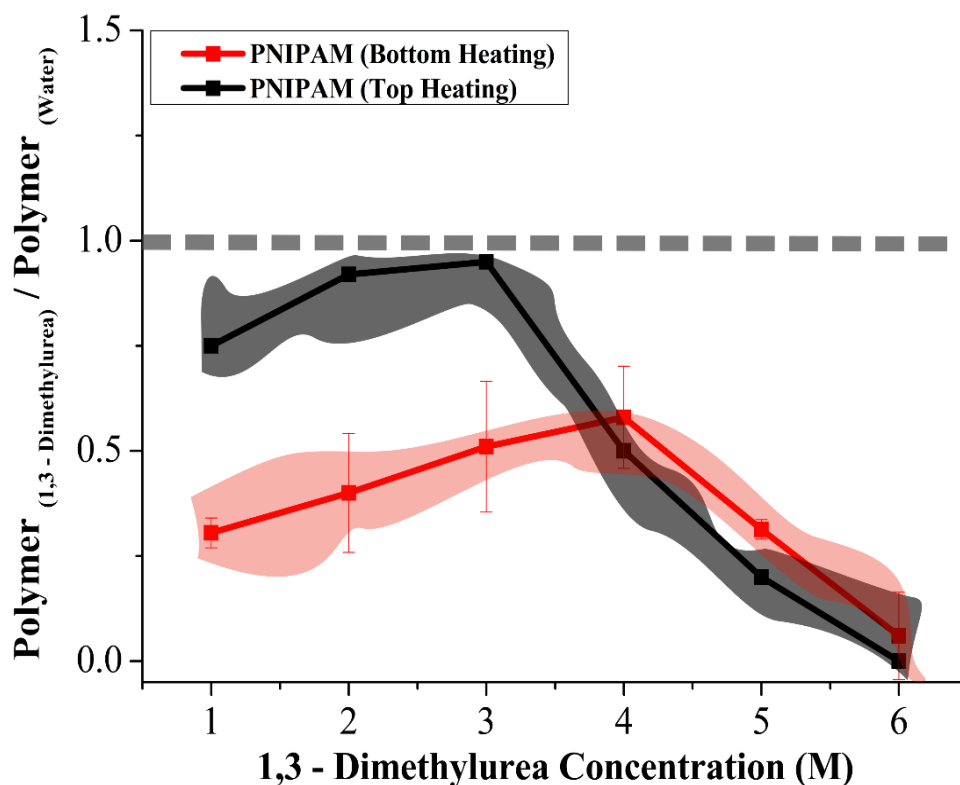


Figure 3.37 ATR-FTIR bottom and top heating measurement results of PNIPAM (20 mg/mL) above the LCST (55°C) as a function of 1,3-Dimethylurea concentration. The y axis of graph indicates the intensity of polymer in 1,3-Dimethylurea solution over the intensity of polymer in H₂O for side chain deformation of polymer. Red data points for the results of ATR-FTIR bottom heating measurements of PNIPAM with standard deviations and black data points for the results of ATR-FTIR top heating measurements of PNIPAM, whereas colour coding, shadowing and the solid lines are for guiding the reader.

For the obtained results of ATR-FTIR bottom and top heating measurements at elevated concentrations of 1,3-dimethylurea (above 4.0 M), it can be seen from Fig. 3.37. that there is little to almost no PNIPAM peak to be observed on ATR crystal; whereas at 3.0 M and below, PNIPAM has “salting-in” behaviour for the results of both measurements, in which this is also evident by the LCST measurements of PNIPAM as a function of 1,3-Dimethylurea, as well. In accordance with these findings, the effect of 1,3-Dimethylurea to the collapse of PNIPAM above the LCST was also investigated, similar to Section 3.2.2, to explore any significant accumulation of 1,3-Dimethylurea throughout the interested concentration regime of the molecule in the sample droplet, owing to the observed salting-in behaviour of PNIPAM from

both LCST and ATR – FTIR bottom and top heating measurements above the LCST. In order to examine this, the ratio between the intensity of 1,3-Dimethylurea with polymer, and the intensity of 1,3-Dimethylurea without polymer at 1175 cm^{-1} , which corresponds to combination of $-\text{CH}_3$ rocking mode, and $-\text{CN}_2$ symmetric stretching of 1,3-Dimethylurea, [129] is calculated and plotted as a function of 1,3-Dimethylurea concentration that the graph can be seen in Fig. 3.38.

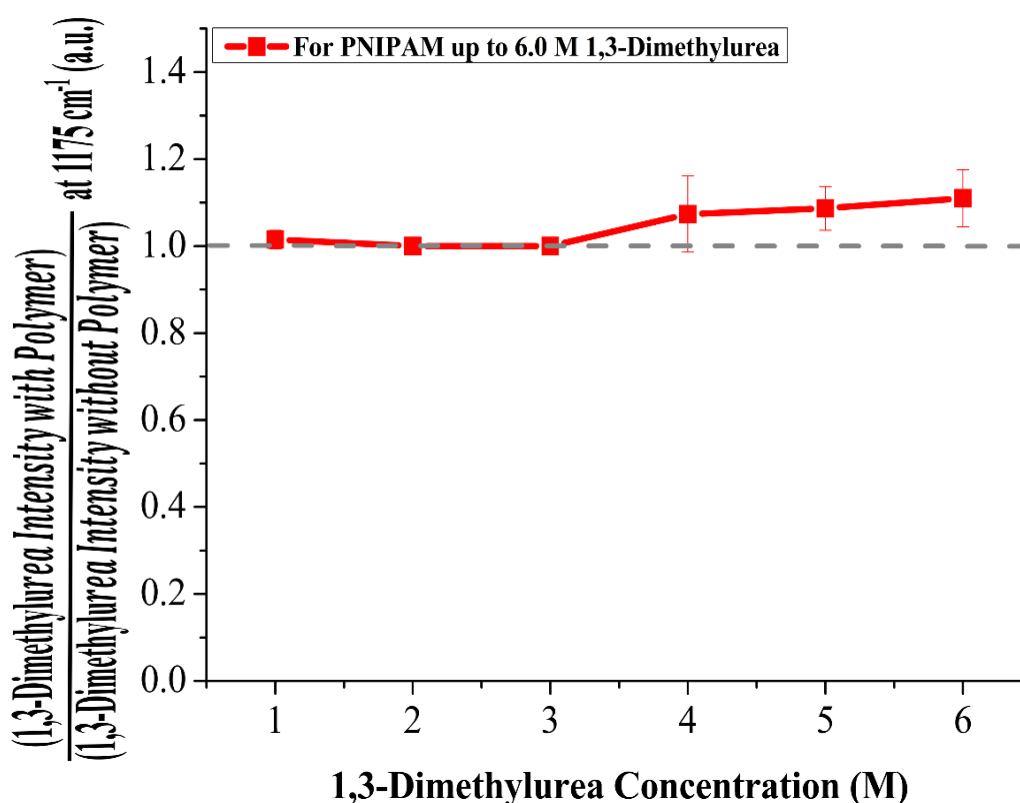


Figure 3.38 ATR-FTIR bottom heating measurement results of PNIPAM up to 6.0 M 1,3-Dimethylurea (shown with red squares) above the LCST (55 °C) as a function of 1,3-Dimethylurea concentration. The y axis of the graph indicates the intensity of 1,3-Dimethylurea over the intensity of 1,3-Dimethylurea without polymer at 1175 cm^{-1} in H_2O . The standard deviations of PNIPAM up to 6.0 M 1,3-Dimethylurea for 4.0 M, 5.0 M, and 6.0 M 1,3-Dimethylurea are shown with error bars.

Similar to Fig. 3.27, and Fig. 3.28, the grey coloured dashed line in Fig. 3.38 indicates the reference point for the obtained results that the urea intensity with polymer in H₂O at 1175 cm⁻¹ is equal to the urea intensity with polymer in H₂O at 1175 cm⁻¹. Within the considered 1,3-Dimethylurea concentrations, the values are very close to 1 (in which, there is only a slight increase regarding the values at and above 4.0 M), for PNIPAM that the absence of a significant accumulation for 1,3-Dimethylurea on the sample droplet up to 6.0 M above the LCST might be interpreted as 1,3-Dimethylurea is indeed a denaturant for PNIPAM compared at tested conditions.

Altogether, it can be stated as PNIPAM has different behaviours in urea and 1,3-dimethylurea solutions above the LCST for its collapse mechanism that PNIPAM is “salting-out” from the solution as a function of urea concentration (especially > 3.0 M, where urea is utilized as denaturant), whereas the macromolecule has “salting-in” behaviour when 1,3-dimethylurea is utilized (similar to urea, especially above 3.0 M). These observations should be interpreted as: the interaction between 1,3-Dimethylurea and amide groups of PNIPAM was diminished with respect to the interaction between the same part of the macromolecule and urea, owing to absence of two H atoms in two -NH₂ groups of urea that are replaced with two methyl groups on 1,3-Dimethylurea. This would eventually render the intermolecular interaction between PNIPAM and urea derivative that could possibly explain the obtained results from Fig. 3.38, and could be utilized to support the collapse mechanism for PNIPAM as a function of urea concentration above the LCST as shown in both Fig. 3.31, and Fig. 3.35 respectively.

Chapter 4 - Conclusion

In this research, the investigation of solubility changes of macromolecules in aqueous medium in the presence of urea was performed by measuring lower critical solution temperatures (LCSTs) of PNIPAM and PDEA at several concentrations of urea (up to 6.0 M). The development of a new method for probing collapsed state of the macromolecules based-on temperature-controlled ATR – FTIR spectroscopy, was accomplished in this thesis. since there is no suitable experimental technique to investigate the collapsed state as well as the interactions of urea molecules with the macromolecules at the collapsed form. Despite having similar molecular structures, according to the presented results of the LCST measurements, the solubility of PDEA gradually increases as a function of urea concentration (up to 6.0 M). In sharp contrast, PNIPAM displayed a monotonic “salting-out” behaviour with increasing urea concentration. To explore this discrepancy further, temperature gradient ATR-FTIR measurements were performed. For this purpose, exposure to ambient air, temperature effect to the aqueous sample, as well as time duration of measurements, and refractive index of the sample solution were investigated individually for checking application of this method to probe the collapsed states. By investigating the specific vibrational fingerprints (1460 cm^{-1} for urea) that belongs to cosolvent urea, and the macromolecules (e.g., 1370 & 1390 cm^{-1} for PNIPAM, and 1360 and 1380 cm^{-1} for PDEA) in aqueous solutions, comparison of the peak intensity within the cosolvent, and within only H_2O for the macromolecules would probe the relative amount of collapsed state on top of the ATR crystal. Regarding the obtained results by using ATR – FTIR based method, up to 6.0 M urea, there was no urea favourable accumulation to the collapsed form of macromolecules, which casts doubt on the widely accepted urea clouding mechanism. A further indication that the preferred form of the macromolecule is the soluble (uncollapsed) form is the accumulation of the collapsed form of the PNIPAM and PDEA toward the cooler portions of the temperature gradient in solution only at elevated ($> 3.0\text{ M}$) urea concentrations. These unexpected results show that both PDEA and PNIPAM prefer the soluble form at the elevated urea concentrations above the LCST, even though PDEA salts-in and PNIPAM appeared to salts-out via LCST measurements. The urea molecules function as a molecular "glue" between the amide groups of PNIPAM as a molecular mechanism by bringing the intra- and intermolecular components of the macromolecule into close proximity. These “urea-glued” macromolecules also act as in the form of collapsed state. This mechanism is apparently only applicable to the PNIPAM macromolecule. Even though there are several

proposed urea denaturation mechanisms for proteins and several other macromolecules in the literature that can be categorized as "direct" and "indirect" mechanisms, more recent findings about this topic indicates that the direct mechanism is predominantly contribute to exploring the urea-induced denaturation mechanism. However, consideration of the indirect mechanism (e.g., hydrophobic effect, interactions between solvent and cosolvent, etc.) should also be considered in order to get the full molecular level picture about the subject. Unfortunately, the investigation of indirect effects is beyond the scope of this thesis. This research provides fresh insight into both the aggregated (collapsed) phase of macromolecules in aqueous medium as well as phenomena that occur in the aqueous solutions.



Chapter 5 - References

- [1] P. H. Yancey, M. E. Clark, S. C. Hand, R. D. Bowlus, and G. N. Somero, "Living with water stress: evolution of osmolyte systems," *Science*, vol. 217, no. 4566, pp. 1214–1222, 1982, doi: 10.1126/SCIENCE.7112124.
- [2] A. Rani and P. Venkatesu, "Changing relations between proteins and osmolytes: a choice of nature," *Physical Chemistry Chemical Physics*, vol. 20, no. 31, pp. 20315–20333, 2018, doi: 10.1039/C8CP02949K.
- [3] T. O. Street, D. W. Bolen, and G. D. Rose, "A molecular mechanism for osmolyte-induced protein stability," *Proceedings of the National Academy of Sciences*, vol. 103, no. 38, pp. 13997–14002, Sep. 2006, doi: 10.1073/pnas.0606236103.
- [4] J. F. Carpenter, B. S. Kendrick, B. S. Chang, M. C. Manning, and T. W. Randolph, "Inhibition of stress-induced aggregation of protein therapeutics," *Methods Enzymol*, vol. 309, pp. 236–255, 1999, doi: 10.1016/S0076-6879(99)09018-7.
- [5] E. Y. Chi, S. Krishnan, T. W. Randolph, and J. F. Carpenter, "Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation," 2003. doi: 10.1023/a:1025771421906.
- [6] H. C. Mahler, W. Friess, U. Grauschopf, and S. Kiese, "Protein aggregation: pathways, induction factors and analysis," *J Pharm Sci*, vol. 98, no. 9, pp. 2909–2934, 2009, doi: 10.1002/JPS.21566.
- [7] W. Wang, "Protein aggregation and its inhibition in biopharmaceutics," *Int J Pharm*, vol. 289, no. 1–2, pp. 1–30, Jan. 2005, doi: 10.1016/J.IJPHARM.2004.11.014.
- [8] M. E. M. Cromwell, E. Hilario, and F. Jacobson, "Protein aggregation and bioprocessing," *AAPS J*, vol. 8, no. 3, p. E572, Sep. 2006, doi: 10.1208/AAPSJ080366.
- [9] A. S. Rosenberg, "Effects of protein aggregates: an immunologic perspective," *AAPS J*, vol. 8, no. 3, p. E501, Aug. 2006, doi: 10.1208/AAPSJ080359.
- [10] M. B. Burg and J. D. Ferraris, "Intracellular organic osmolytes: Function and regulation," *Journal of Biological Chemistry*, vol. 283, no. 12, pp. 7309–7313, Mar. 21, 2008. doi: 10.1074/jbc.R700042200.

- [11] S. R. Trevino, J. M. Scholtz, and C. N. Pace, "Measuring and increasing protein solubility," *J Pharm Sci*, vol. 97, no. 10, pp. 4155–4166, 2008, doi: 10.1002/JPS.21327.
- [12] J. Jaspe and S. J. Hagen, "Do Protein Molecules Unfold in a Simple Shear Flow?," *Biophys J*, vol. 91, no. 9, p. 3415, Nov. 2006, doi: 10.1529/BIOPHYSJ.106.089367.
- [13] J. Brown, "Introduction to protein structure. By C Branden and J Tooze. pp 302. garland publishing, New York. 1991 ISBN 0–8513–0270–3 (pbk)," *Biochem Educ*, vol. 20, no. 2, pp. 121–122, Apr. 1992, doi: 10.1016/0307-4412(92)90129-A.
- [14] A. M. Lesk, *Introduction to protein science: architecture, function, and genomics*. Oxford ; New York: Oxford University Press, 2010.
- [15] F. Hofmeister, "Zur lehre von der wirkung der salze," *archiv für experimentelle pathologie und pharmakologie 1888 24:4*, vol. 24, no. 4, pp. 247–260, 1888, doi: 10.1007/BF01918191.
- [16] P. Jungwirth and P. S. Cremer, "Beyond Hofmeister," *Nature Chemistry 2014 6:4*, vol. 6, no. 4, pp. 261–263, Mar. 2014, doi: 10.1038/nchem.1899.
- [17] K. P. Gregory, G. R. Elliott, H. Robertson, A. Kumar, E. J. Wanless, G. B. Webber, V. S. J. Craig, G. G. Andersson, and A.J. Page, "Understanding specific ion effects and the Hofmeister series," *Physical Chemistry Chemical Physics*, vol. 24, no. 21, pp. 12682–12718, 2022, doi: 10.1039/D2CP00847E.
- [18] W. Kunz, J. Henle, and B. W. Ninham, "'Zur Lehre von der Wirkung der Salze' (about the science of the effect of salts): Franz Hofmeister's historical papers," *Curr Opin Colloid Interface Sci*, vol. 9, no. 1–2, pp. 19–37, Aug. 2004, doi: 10.1016/J.COCIS.2004.05.005.
- [19] Y. Zhang and P. Cremer, "Interactions between macromolecules and ions: the Hofmeister series," *Curr Opin Chem Biol*, vol. 10, no. 6, pp. 658–663, Dec. 2006, doi: 10.1016/j.cbpa.2006.09.020.
- [20] M. C. Pinna, A. Salis, M. Monduzzi, and B. W. Ninham, "Hofmeister series: The hydrolytic activity of *Aspergillus niger* lipase depends on specific anion effects," *Journal of Physical Chemistry B*, vol. 109, no. 12, pp. 5406–5408, Mar. 2005, doi: 10.1021/JP050574W.

- [21] P. Bauduin, F. Nohmie, D. Touraud, R. Neueder, W. Kunz, and B. W. Ninham, "Hofmeister specific-ion effects on enzyme activity and buffer pH: Horseradish peroxidase in citrate buffer," *J Mol Liq*, vol. 123, no. 1, pp. 14–19, Jan. 2006, doi: 10.1016/J.MOLLIQ.2005.03.003.
- [22] L. Vrbka, P. Jungwirth, P. Bauduin, D. Touraud, and W. Kunz, "Specific ion effects at protein surfaces: a molecular dynamics study of bovine pancreatic trypsin inhibitor and horseradish peroxidase in selected salt solutions," *J Phys Chem B*, vol. 110, no. 13, pp. 7036–7043, May 2006, doi: 10.1021/JP0567624.
- [23] J. M. Broering and A. S. Bommarius, "Evaluation of Hofmeister Effects on the Kinetic Stability of Proteins," *The Journal of Physical Chemistry B* 2005, 109 (43), 20612–20619 doi: 10.1021/JP053618.
- [24] R. Perez-Jimenez, R. Godoy-Ruiz, B. Ibarra-Molero, and J. M. Sanchez-Ruiz, "The Efficiency of Different Salts to Screen Charge Interactions in Proteins: A Hofmeister Effect?," *Biophys J*, vol. 86, no. 4, p. 2414, 2004, doi: 10.1016/S0006-3495(04)74298-8.
- [25] R. A. Curtis and L. Lue, "A molecular approach to bioseparations: Protein-protein and protein-salt interactions," *Chem Eng Sci*, vol. 61, no. 3, pp. 907–923, 2006, doi: 10.1016/J.CES.2005.04.007.
- [26] A. W. Omta, M. F. Kropman, S. Woutersen, and H. J. Bakker, "Negligible effect of ions on the hydrogen-bond structure in liquid water," *Science*, vol. 301, no. 5631, pp. 347–349, Jul. 2003, doi: 10.1126/SCIENCE.1084801.
- [27] J. D. Batchelor, A. Olteanu, A. Tripathy, and G. J. Pielak, "Impact of protein denaturants and stabilizers on water structure," *J Am Chem Soc*, vol. 126, no. 7, pp. 1958–1961, Feb. 2004, doi: 10.1021/JA039335H.
- [28] Y. Chen, H. I. Okur, N. Gomopoulos, C. M. Romero, P. S. Cremer, P. B. Petersen, G. Tocci, D. M. Wilkins, C. Liang, M. Ceriotti, and S. Roke, "Electrolytes induce long-range orientational order and free energy changes in the H-bond network of bulk water," *Sci Adv*, vol. 2, no. 4, Apr. 2016, doi: 10.1126/SCIADV.1501891.
- [29] Y. Chen, H. I. Okur, C. Liang, and S. Roke, "Orientational ordering of water in extended hydration shells of cations is ion-specific and is correlated directly with viscosity and

- hydration free energy,” *Physical Chemistry Chemical Physics*, vol. 19, no. 36, pp. 24678–24688, Sep. 2017, doi: 10.1039/C7CP03395H.
- [30] Y. Chen, N. Dupertuis, H. I. Okur, and S. Roke, “Temperature dependence of water-water and ion-water correlations in bulk water and electrolyte solutions probed by femtosecond elastic second harmonic scattering,” *J Chem Phys*, vol. 148, no. 22, p. 222835, Apr. 2018, doi: 10.1063/1.5023343.
- [31] M. C. Gurau, S. M. Lim, E. T. Castellana, F. Albertorio, S. Kataoka, and P. S. Cremer, “On the mechanism of the Hofmeister effect,” *J Am Chem Soc*, vol. 126, no. 34, pp. 10522–10523, Sep. 2004, doi: 10.1021/JA047715C.
- [32] Y. Zhang, S. Furyk, D. E. Bergbreiter, and P. S. Cremer, “Specific ion effects on the water solubility of macromolecules: PNIPAM and the Hofmeister series,” *J Am Chem Soc*, vol. 127, no. 41, pp. 14505–14510, Oct. 2005, doi: 10.1021/JA0546424.
- [33] H. G. Schild and D. A. Tirrell, “Microcalorimetric detection of lower critical solution temperatures in aqueous polymer solutions,” *Journal of Physical Chemistry®*, vol. 94, no. 10, pp. 4352–4356, 1990, doi: 10.1021/J100373A088.
- [34] R. Freitag and F. Garret-Flaudy, “Salt effects on the thermoprecipitation of poly-(N-isopropylacrylamide) oligomers from aqueous solution,” *Langmuir*, vol. 18, no. 9, pp. 3434–3440, Apr. 2002, doi: 10.1021/LA0106440.
- [35] Y. Zhang, S. Furyk, L. B. Sagle, Y. Cho, D. E. Bergbreiter, and P. S. Cremer, “Effects of hofmeister anions on the LCST of PNIPAM as a function of molecular weight,” *J Phys Chem C Nanomater Interfaces*, vol. 111, no. 25, pp. 8916–8924, Jun. 2007, doi: 10.1021/JP0690603.
- [36] E. A. Algaer and N. F. A. van der Vegt, “Hofmeister ion interactions with model amide compounds,” *Journal of Physical Chemistry B*, vol. 115, no. 46, pp. 13781–13787, Nov. 2011, doi: 10.1021/JP208583W.
- [37] K. B. Rembert, H. I. Okur, C. Hilty, and P. S. Cremer, “An NH moiety is not required for anion binding to amides in aqueous solution,” *Langmuir*, vol. 31, no. 11, pp. 3459–3464, Mar. 2015, doi: 10.1021/ACS.LANGMUIR.5B00127.

- [38] Y. Cho, Y. Zhang, T. Christensen, L. B. Sagle, A. Chilkoti, and P. S. Cremer, “Effects of Hofmeister anions on the phase transition temperature of elastin-like polypeptides,” *J Phys Chem B*, vol. 112, no. 44, pp. 13765–13771, Nov. 2008, doi: 10.1021/JP8062977.
- [39] P. B. Petersen, R. J. Saykally, M. Mucha, and P. Jungwirth, “Enhanced concentration of polarizable anions at the liquid water surface: SHG spectroscopy and MD simulations of sodium thiocyanide,” *Journal of Physical Chemistry B*, vol. 109, no. 21, pp. 10915–10921, Jun. 2005, doi: 10.1021/JP050864C.
- [40] B. A. Rogers, H. I. Okur, C. Yan, T. Yang, J. Heyda, and P. S. Cremer, “Weakly hydrated anions bind to polymers but not monomers in aqueous solutions,” *Nat Chem*, vol. 14, no. 1, pp. 40–45, Jan. 2022, doi: 10.1038/S41557-021-00805-Z.
- [41] X. Chen, T. Yang, S. Kataoka, and P. S. Cremer, “Specific ion effects on interfacial water structure near macromolecules,” *J Am Chem Soc*, vol. 129, no. 40, pp. 12272–12279, Oct. 2007, doi: 10.1021/JA073869R.
- [42] X. Chen, S. C. Flores, S. M. Lim, Y. Zhang, J. Kherb, and P. S. Cremer, “Specific anion effects on water structure adjacent to protein monolayers,” *Langmuir*, vol. 26, no. 21, pp. 16447–16454, Nov. 2010, doi: 10.1021/LA1015862.
- [43] K. B. Rembert, J. Paterová, J. Heyda, C. Hilty, P. Jungwirth, and P. S. Cremer, “Molecular mechanisms of ion-specific effects on proteins,” *J Am Chem Soc*, vol. 134, no. 24, pp. 10039–10046, Jun. 2012, doi: 10.1021/JA301297G.
- [44] W. J. Xie and Y. Q. Gao, “A simple theory for the hofmeister series,” *Journal of Physical Chemistry Letters*, vol. 4, no. 24, pp. 4247–4252, Dec. 2013, doi: 10.1021/JZ402072G.
- [45] P. Jungwirth, “Hofmeister series of ions: A simple theory of a not so simple reality,” *Journal of Physical Chemistry Letters*, vol. 4, no. 24, pp. 4258–4259, Dec. 2013, doi: 10.1021/JZ402369U.
- [46] S. C. Flores, J. Kherb, N. Konelick, X. Chen, and P. S. Cremer, “The effects of hofmeister cations at negatively charged hydrophilic surfaces,” *Journal of Physical Chemistry C*, vol. 116, no. 9, pp. 5730–5734, Mar. 2012, doi: 10.1021/JP210791J.
- [47] J. Paterová, K. B. Rembert, J. Heyda, Y. Kurra, H. I. Okur, W. R. Liu, C. Hilty, P. S. Cremer, and P. Jungwirth, “Reversal of the hofmeister series: Specific ion effects on

- peptides,” *Journal of Physical Chemistry B*, vol. 117, no. 27, pp. 8150–8158, Jul. 2013, doi: 10.1021/JP405683S.
- [48] Y. Zhang and P. S. Cremer, “The inverse and direct Hofmeister series for lysozyme,” *Proceedings of the National Academy of Sciences*, vol. 106, no. 36, pp. 15249–15253, Sep. 2009, doi: 10.1073/pnas.0907616106.
- [49] B. Hess and N. F. A. van der Vegt, “Cation specific binding with protein surface charges,” *Proceedings of the National Academy of Sciences*, vol. 106, no. 32, pp. 13296–13300, Aug. 2009, doi: 10.1073/pnas.0902904106.
- [50] P. Ganguly, T. Hajari, and N. F. A. van der Vegt, “Molecular simulation study on hofmeister cations and the aqueous solubility of benzene,” *Journal of Physical Chemistry B*, vol. 118, no. 20, pp. 5331–5339, May 2014, doi: 10.1021/JP5011154.
- [51] H. I. Okur, J. Kherb, and P. S. Cremer, “Cations bind only weakly to amides in aqueous solutions,” *J Am Chem Soc*, vol. 135, no. 13, pp. 5062–5067, Apr. 2013, doi: 10.1021/JA3119256.
- [52] E. E. Bruce, H. I. Okur, S. Stegmaier, C. I. Drexler, B. A. Rogers, N. F. A. van der Vegt, S. Roke, and P. S. Cremer, “Molecular mechanism for the interactions of hofmeister cations with macromolecules in aqueous solution,” *J Am Chem Soc*, vol. 142, no. 45, pp. 19094–19100, Nov. 2020, doi: 10.1021/JACS.0C07214.
- [53] J. Kherb, S. C. Flores, and P. S. Cremer, “Role of carboxylate side chains in the cation hofmeister series,” *Journal of Physical Chemistry B*, vol. 116, no. 25, pp. 7389–7397, Jun. 2012, doi: 10.1021/JP212243C.
- [54] P. Ganguly, P. Schravendijk, B. Hess, and N. F. A. van der Vegt, “Ion pairing in aqueous electrolyte solutions with biologically relevant anions,” *Journal of Physical Chemistry B*, vol. 115, no. 13, pp. 3734–3739, Apr. 2011, doi: 10.1021/JP201150Q.
- [55] C. Dalgicdir and N. F. A. van der Vegt, “Improved temperature behavior of PNIPAM in water with a modified OPLS model,” *Journal of Physical Chemistry B*, vol. 123, no. 17, pp. 3875–3883, May 2019, doi: 10.1021/ACS.JPCB.9B01644.
- [56] E. E. Bruce, P. T. Bui, B. A. Rogers, P. S. Cremer, and N. F. A. van der Vegt, “Nonadditive ion effects drive both collapse and swelling of thermoresponsive polymers in water,” *J. Am. Chem. Soc*, vol. 141, p. 35, 2019, doi: 10.1021/jacs.9b00295.

- [57] Y. Zhao, S. Bharadwaj, and N. F. A. van der Vegt, "Nonadditive ion effects on the coil-globule equilibrium of PNIPAM: a computer simulation study," *Physical Chemistry Chemical Physics*, vol. 24, no. 17, pp. 10346–10355, May 2022, doi: 10.1039/D2CP00057A.
- [58] E. E. Bruce, P. T. Bui, B. A. Rogers, P. S. Cremer, and N. F. A. van der Vegt, "Nonadditive ion effects drive both collapse and swelling of thermoresponsive polymers in water," *J Am Chem Soc*, vol. 141, no. 16, pp. 6609–6616, Apr. 2019, doi: 10.1021/JACS.9B00295.
- [59] P. T. Bui and P. S. Cremer, "Cation identity affects nonadditivity in salt mixtures containing iodide and sulfate," *J Solution Chem*, vol. 50, no. 11–12, pp. 1443–1456, Dec. 2021, doi: 10.1007/S10953-021-01125-Z.
- [60] D. R. Robinson and W. P. Jencks, "The effect of compounds of the urea-guanidinium class on the activity coefficient of acetyltetraglycine ethyl ester and related compounds," *J Am Chem Soc*, vol. 87, no. 11, pp. 2462–2470, 1965, doi: 10.1021/JA01089A028/.
- [61] R. F. Greene and C. N. Pace, "Urea and guanidine hydrochloride denaturation of ribonuclease, lysozyme, α -chymotrypsin, and b-lactoglobulin," *Journal of Biological Chemistry*, vol. 249, no. 17, pp. 5388–5393, Sep. 1974, doi: 10.1016/S0021-9258(20)79739-5.
- [62] C. N. Pace, "Determination and analysis of urea and guanidine hydrochloride denaturation curves," *Methods Enzymol*, vol. 131, no. C, pp. 266–280, Jan. 1986, doi: 10.1016/0076-6879(86)31045-0.
- [63] G. I. Makhatadze and P. L. Privalov, "Protein interactions with urea and guanidinium chloride," *J Mol Biol*, vol. 226, no. 2, pp. 491–505, Jul. 1992, doi: 10.1016/0022-2836(92)90963-K.
- [64] J. Heyda, M. Kozisek, L. Bednarova, G. Thompson, J. Konvalinka, J. Vondrasek, and P. Jungwirth, "Urea and guanidinium induced denaturation of a Trp-cage miniprotein," *Journal of Physical Chemistry B*, vol. 115, no. 28, pp. 8910–8924, Jul. 2011, doi: 10.1021/JP200790H.
- [65] P. E. Mason, E. Wernersson, and P. Jungwirth, "Accurate description of aqueous carbonate ions: An effective polarization model verified by neutron scattering," *Journal*

- of *Physical Chemistry B*, vol. 116, no. 28, pp. 8145–8153, Jul. 2012, doi: 10.1021/JP3008267.
- [66] J. Heyda, H. I. Okur, J. Hladilkova, K. B. Rembert, W. Hunn, T. Yang, J. Dzubiella, P. Jungwirth, and P. S. Cremer, “Guanidinium can both cause and prevent the hydrophobic collapse of biomacromolecules,” *J Am Chem Soc*, vol. 139, no. 2, pp. 863–870, Jan. 2017, doi: 10.1021/JACS.6B11082.
- [67] J. Hunger, K. J. Tielrooij, R. Buchner, M. Bonn, and H. J. Bakker, “Complex formation in aqueous trimethylamine-N-oxide (TMAO) solutions,” *J Phys Chem B*, vol. 116, no. 16, pp. 4783–4795, Apr. 2012, doi: 10.1021/JP212542Q.
- [68] L. B. Sagle, K. Cimatu, V. A. Litosh, Y. Liu, S. C. Flores, X. Chen, B. Yu, and P. S. Cremer, “Methyl groups of trimethylamine N-oxide orient away from hydrophobic interfaces,” *J Am Chem Soc*, vol. 133, no. 46, pp. 18707–18712, Nov. 2011, doi: 10.1021/JA205106E.
- [69] Y.-T. Liao, A. C. Manson, M. R. DeLyser, W. G. Noid, and P. S. Cremer, “Trimethylamine *N*-oxide stabilizes proteins via a distinct mechanism compared with betaine and glycine,” *Proceedings of the National Academy of Sciences*, vol. 114, no. 10, pp. 2479–2484, Mar. 2017, doi: 10.1073/pnas.1614609114.
- [70] P. H. Yancey and G. N. Somero, “Counteraction of urea destabilization of protein structure by methylamine osmoregulatory compounds of elasmobranch fishes,” *Biochemical Journal*, vol. 183, no. 2, p. 317, Nov. 1979, doi: 10.1042/BJ1830317.
- [71] J. Hunger, N. Ottosson, K. Mazur, M. Bonn, and H. J. Bakker, “Water-mediated interactions between trimethylamine-N-oxide and urea,” *Physical Chemistry Chemical Physics*, vol. 17, no. 1, pp. 298–306, Dec. 2014, doi: 10.1039/C4CP02709D.
- [72] W. J. Xie, S. Cha, T. Ohto, W. Mizukami, Y. Mao, M. Wagner, M. Bonn, J. Hunger, and Y. Nagata, “Large hydrogen-bond mismatch between TMAO and urea promotes their hydrophobic association,” *Chem*, vol. 4, no. 11, pp. 2615–2627, Nov. 2018, doi: 10.1016/J.CHEMPR.2018.08.020.
- [73] P. Ganguly, T. Hajari, J. E. Shea, and N. F. A. van der Vegt, “Mutual exclusion of urea and trimethylamine N-oxide from amino acids in mixed solvent environment,” *J Phys Chem Lett*, vol. 6, no. 4, pp. 581–585, Feb. 2015, doi: 10.1021/JZ502634K.

- [74] P. Ganguly, N. F. A. van der Vegt, and J.-E. Shea, "Hydrophobic association in mixed urea-TMAO solutions," *J Phys Chem Lett*, vol. 7, no. 15, pp. 3052–3059, Aug. 2016, doi: 10.1021/acs.jpclett.6b01344.
- [75] P. Ganguly, P. Boserman, N. F. A. van der Vegt, and J. E. Shea, "Trimethylamine N-oxide counteracts urea denaturation by inhibiting protein-urea preferential interaction," *J Am Chem Soc*, vol. 140, no. 1, pp. 483–492, Jan. 2018, doi: 10.1021/JACS.7B11695.
- [76] P. Ganguly, J. Polák, N. F. A. van der Vegt, J. Heyda, and J. E. Shea, "Protein stability in TMAO and mixed urea-TMAO solutions," *Journal of Physical Chemistry B*, vol. 124, no. 29, pp. 6181–6197, Jul. 2020, doi: 10.1021/ACS.JPCB.0C04357.
- [77] B. J. Bennion and V. Daggett, "The molecular basis for the chemical denaturation of proteins by urea," *Proceedings of the National Academy of Sciences*, vol. 100, no. 9, pp. 5142–5147, Apr. 2003, doi: 10.1073/pnas.0930122100.
- [78] D. R. Canchi, D. Paschek, and A. E. Garcia, "Equilibrium study of protein denaturation by urea," *J Am Chem Soc*, vol. 132, no. 7, pp. 2338–2344, Mar. 2010, doi: 10.1021/JA909348C.
- [79] K. Martinek, V. S. Goldmacher, A. M. Klibanov, and I. v. Berezin, "Denaturing agents (urea, acrylamide) protect enzymes against irreversible thermoinactivation: A study with native and immobilized α -chymotrypsin and trypsin," *FEBS Lett*, vol. 51, no. 1–2, pp. 152–155, Mar. 1975, doi: 10.1016/0014-5793(75)80874-X.
- [80] A. Das and C. Mukhopadhyay, "Urea-mediated protein denaturation: a consensus view," *J Phys Chem B*, vol. 113, no. 38, pp. 12816–12824, Sep. 2009, doi: 10.1021/JP906350S.
- [81] P. J. Rossky, "Protein denaturation by urea: Slash and bond," *Proceedings of the National Academy of Sciences*, vol. 105, no. 44, pp. 16825–16826, Nov. 2008, doi: 10.1073/pnas.0809224105.
- [82] F. G. Hopkins, "Denaturation of proteins by urea and related substances.," vol. 126, no. 3174, pp. 328–330, 1930, doi: 10.1038/126328A0.
- [83] F. Rashid, S. Sharma, and B. Bano, "Comparison of Guanidine Hydrochloride (GdnHCl) and Urea Denaturation on Inactivation and Unfolding of Human Placental Cystatin (HPC)," *The Protein Journal 2005 24:5*, vol. 24, no. 5, pp. 283–292, Jul. 2005, doi: 10.1007/S10930-005-6749-5.

- [84] “41.5: Nitrogenous Wastes - Nitrogenous Waste in Terrestrial Animals- The Urea Cycle - Biology LibreTexts.” [https://bio.libretexts.org/Bookshelves/Introductory_and_General_Biology/Book%3A_General_Biology_\(Boundless\)/41%3A_Osmotic_Regulation_and_the_Excretory_System/41.05%3A_Nitrogenous_Wastes_-_Nitrogenous_Waste_in_Terrestrial_Animals-_The_Urea_Cycle](https://bio.libretexts.org/Bookshelves/Introductory_and_General_Biology/Book%3A_General_Biology_(Boundless)/41%3A_Osmotic_Regulation_and_the_Excretory_System/41.05%3A_Nitrogenous_Wastes_-_Nitrogenous_Waste_in_Terrestrial_Animals-_The_Urea_Cycle) (accessed Aug. 16, 2022).
- [85] H. A. Barker, “The refractivity of heat-denaturated egg albumin,” *Journal of Biological Chemistry*, vol. 104, no. 3, pp. 667–673, Mar. 1934, doi: 10.1016/S0021-9258(18)75742-6.
- [86] N. F. Burk, “Osmotic pressure, molecular weight, and stability of serum albumin,” *Journal of Biological Chemistry*, vol. 98, no. 1, pp. 353–377, Oct. 1932, doi: 10.1016/S0021-9258(18)76168-1.
- [87] N. F. Burk, “Osmotic pressure, molecular weight, and stability of gliadin,” *Journal of Biological Chemistry*, vol. 124, no. 1, pp. 49–70, Jun. 1938, doi: 10.1016/S0021-9258(18)74070-2.
- [88] N. F. Burk, “Osmotic pressure, molecular weight, and stability of serum globulin,” *Journal of Biological Chemistry*, vol. 121, no. 2, pp. 373–402, Nov. 1937, doi: 10.1016/S0021-9258(18)74271-3.
- [89] N. F. Burk, “Osmotic pressure, molecular weight, and stability of amandin and excelsin and certain other proteins,” *Journal of Biological Chemistry*, vol. 120, no. 1, pp. 63–83, Aug. 1937, doi: 10.1016/S0021-9258(18)74362-7.
- [90] D. B. Wetlaufer, S. K. Malik, L. Stoller, and R. L. Coffin, “Nonpolar group participation in the denaturation of proteins by urea and guanidinium salts. Model compound studies,” *J Am Chem Soc*, vol. 86, no. 3, pp. 508–514, Feb. 1964, doi: 10.1021/JA01057A045/.
- [91] K. P. Barnes, J. R. Warren, and J. A. Gordon, “Effect of urea on the circular dichroism of lysozyme,” *Journal of Biological Chemistry*, vol. 247, no. 6, pp. 1708–1712, Mar. 1972, doi: 10.1016/s0021-9258(19)45534-8.
- [92] E. G. Finer, F. Franks, and M. J. Tait, “Nuclear magnetic resonance studies of aqueous urea solutions,” *J Am Chem Soc*, vol. 94, no. 13, pp. 4424–4429, Jun. 1972, doi: 10.1021/JA00768A004.

- [93] H. S. Krank and F. Franks, "Structural approach to the solvent power of water for hydrocarbons; urea as a structure breaker," *J Chem Phys*, vol. 48, no. 10, p. 4746, Sep. 2003, doi: 10.1063/1.1668057.
- [94] Y. L. A. Rezus and H. J. Bakker, "Effect of urea on the structural dynamics of water," *Proc Natl Acad Sci U S A*, vol. 103, no. 49, p. 18417, Dec. 2006, doi: 10.1073/PNAS.0606538103.
- [95] H. Kokubo and B. M. Pettitt, "Preferential solvation in urea solutions at different concentrations: properties from simulation studies," *J Phys Chem B*, vol. 111, no. 19, pp. 5233–5242, May 2007, doi: 10.1021/jp067659x.
- [96] L. Hua, R. Zhou, D. Thirumalai C, and B. J. Berne, "Urea denaturation by stronger dispersion interactions with proteins than water implies a 2-stage unfolding," *Proc Natl Acad Sci U S A*, vol. 105, no. 44, pp. 16928–16933, Nov. 2008, doi: 10.1073/PNAS.0808427105.
- [97] M. C. Stumpe and H. Grubmüller, "Interaction of urea with amino acids: Implications for urea-induced protein denaturation," *J Am Chem Soc*, vol. 129, no. 51, pp. 16126–16131, Dec. 2007, doi: 10.1021/JA076216J.
- [98] R. Zangi, M. Hagen, and B. J. Berne, "Effect of ions on the hydrophobic interaction between two plates," *J Am Chem Soc*, vol. 129, no. 15, pp. 4678–4686, Apr. 2007, doi: 10.1021/JA068305M.
- [99] H. G. Schild and D. A. Tirrell, "Microcalorimetric detection of lower critical solution temperatures in aqueous polymer solutions," *Journal of Physical Chemistry®*, vol. 94, no. 10, pp. 4352–4356, 1990, doi: 10.1021/J100373A088.
- [100] F. Rodríguez-Ropero and N. F. A. van der Vegt, "Direct osmolyte-macromolecule interactions confer entropic stability to folded states," *Journal of Physical Chemistry B*, vol. 118, no. 26, pp. 7327–7334, Jul. 2014, doi: 10.1021/JP504065E.
- [101] N. F. A. van der Vegt and D. Nayar, "The hydrophobic effect and the role of cosolvents," *Journal of Physical Chemistry B*, vol. 121, no. 43, pp. 9986–9998, Nov. 2017, doi: 10.1021/ACS.JPCB.7B06453.

- [102] A. Pica and G. Graziano, “Why does urea have a different effect on the collapse temperature of PDEAM and PNIPAM?,” *J Mol Liq*, vol. 285, pp. 204–212, Jul. 2019, doi: 10.1016/j.molliq.2019.04.071.
- [103] J. Wang, B. Liu, G. Ru, J. Bai, and J. Feng, “Effect of urea on phase transition of poly(*N*-isopropylacrylamide) and Poly(*N,N*-diethylacrylamide) hydrogels: A clue for urea-Induced denaturation,” *Macromolecules*, vol. 49, no. 1, pp. 234–243, Jan. 2016, doi: 10.1021/acs.macromol.5b01949.
- [104] P. Narang and P. Venkatesu, “New endeavours involving the cooperative behaviour of TMAO and urea towards the globular state of poly(*N*-isopropylacrylamide),” *RSC Adv*, vol. 7, no. 54, pp. 34023–34033, 2017, doi: 10.1039/C7RA05120D.
- [105] L. B. Sagle, Y. Zhang, V. A. Litosh, X. Chen, Y. Cho, and P. S. Cremer, “Investigating the hydrogen-bonding model of urea denaturation,” *J Am Chem Soc*, vol. 131, no. 26, pp. 9304–9310, Jul. 2009, doi: 10.1021/JA9016057.
- [106] N. F. A. van der Vegt, M. E. Lee, D. Trzesniak, and W. F. van Gunsteren, “Enthalpy-entropy compensation in the effects of urea on hydrophobic interactions,” *Journal of Physical Chemistry B*, vol. 110, no. 26, pp. 12852–12855, Jul. 2006, doi: 10.1021/JP062426R.
- [107] “Lyophilization of Parenteral (7/93) | FDA.” <https://www.fda.gov/inspections-compliance-enforcement-and-criminal-investigations/inspection-guides/lyophilization-parenteral-793> (accessed Aug. 16, 2022).
- [108] “Melting Point Apparatus - OptiMelt MPA100.” <https://www.thinksrs.com/products/MPA100.htm> (accessed Aug. 16, 2022).
- [109] J. Spěváček, “NMR investigations of phase transition in aqueous polymer solutions and gels,” *Curr Opin Colloid Interface Sci*, vol. 14, no. 3, pp. 184–191, Jun. 2009, doi: 10.1016/J.COCIS.2008.10.003.
- [110] T. Tanaka, T. Amiya, A. Mamada, and T. Tokuhiro, “NMR study of poly(*N*-isopropylacrylamide) gels near phase transition,” *Macromolecules*, vol. 24, no. 10, pp. 2936–2943, May 1991, doi: 10.1021/MA00010A046.

- [111] W. L. Peticolas, “Application of Raman spectroscopy to biological macromolecules,” *Biochimie*, vol. 57, no. 4, pp. 417–428, Jun. 1975, doi: 10.1016/S0300-9084(75)80328-2.
- [112] G. Pezzotti, “Raman spectroscopy in cell biology and microbiology,” *Journal of Raman Spectroscopy*, vol. 52, no. 12, pp. 2348–2443, Dec. 2021, doi: 10.1002/JRS.6204.
- [113] M. C. Popescu, C. Vasile, and O. Craciunescu, “Structural analysis of some soluble elastins by means of FT-IR and 2D IR correlation spectroscopy,” *Biopolymers*, vol. 93, no. 12, pp. 1072–1084, Dec. 2010, doi: 10.1002/BIP.21524.
- [114] S. Hossain, A. Kabedev, A. Parrow, C. A. S. Bergström, and P. Larsson, “Molecular simulation as a computational pharmaceutics tool to predict drug solubility, solubilization processes and partitioning,” *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 137, p. 46, Apr. 2019, doi: 10.1016/J.EJPB.2019.02.007.
- [115] A. Mathers, F. Hassouna, M. Klajmon, and M. Fulem, “Comparative study of DSC-based protocols for API-polymer solubility determination,” *Mol Pharm*, vol. 18, no. 4, pp. 1742–1757, Apr. 2021, doi: 10.1021/ACS.MOLPHARMACEUT.0C01232.
- [116] E. Prenner and M. Chiu, “Differential scanning calorimetry: An invaluable tool for a detailed thermodynamic characterization of macromolecules and their interactions,” *J Pharm Bioallied Sci*, vol. 3, no. 1, p. 39, 2011, doi: 10.4103/0975-7406.76463.
- [117] Y. Maeda, T. Higuchi, and I. Ikeda, “Change in hydration state during the coil-globule transition of aqueous solutions of poly(*af*-isopropylacrylamide) as evidenced by FTIR spectroscopy,” *Langmuir*, vol. 16, no. 19, pp. 7503–7509, Sep. 2000, doi: 10.1021/LA0001575.
- [118] “ATR FTIR Measurement Optimization | JASCO.” <https://jascoinc.com/learning-center/theory/spectroscopy/basics-of-atr-ftir/> (accessed Aug. 17, 2022).
- [119] G. E. Walrafen, “Raman and infrared spectral investigations of water structure,” in *The Physics and Physical Chemistry of Water*, Boston, MA: Springer New York, 1972, pp. 151–214. doi: 10.1007/978-1-4684-8334-5_5.
- [120] Y. Maeda, T. Nakamura, and I. Ikeda, “Change in solvation of poly(*N,N*-diethylacrylamide) during phase transition in aqueous solutions as observed by IR

- spectroscopy,” *Macromolecules*, vol. 35, no. 27, pp. 10172–10177, Dec. 2002, doi: 10.1021/MA020945W.
- [121] M. H. Futscher, M. Philipp, P. Müller-Buschbaum, and A. Schulte, “The role of backbone hydration of poly(*N*-isopropylacrylamide) across the volume phase transition compared to its monomer,” *Scientific Reports* 2017 7:1, vol. 7, no. 1, pp. 1–10, Dec. 2017, doi: 10.1038/s41598-017-17272-7.
- [122] B. Sun, P. Wu, and Y. Lin, “Structure analysis of poly(*N*-isopropylacrylamide) using near-infrared spectroscopy and generalized two-dimensional correlation infrared spectroscopy,” *Applied Spectroscopy*, Vol. 61, Issue 7, pp. 765–771, vol. 61, no. 7, pp. 765–771, Jul. 2007, doi: 10.1366/000370207781393271.
- [123] J. Grdadolnik and Y. Maréchal, “Urea and urea–water solutions—an infrared study,” *J Mol Struct*, vol. 615, no. 1–3, pp. 177–189, Sep. 2002, doi: 10.1016/S0022-2860(02)00214-4.
- [124] B. Kang, H. Tang, Z. Zhao, and S. Song, “Hofmeister series: Insights of ion specificity from amphiphilic assembly and interface property,” *ACS Omega*, vol. 5, no. 12, pp. 6229–6239, Mar. 2020, doi: 10.1021/acsomega.0c00237.
- [125] J. Hladílková, J. Heyda, K. B. Rembert, H. I. Okur, Y. Kurra, W. R. Liu, C. Hilty, P. S. Cremer, and P. Jungwirth, “Effects of end-group termination on salting-out constants for triglycine,” *J Phys Chem Lett*, vol. 4, no. 23, pp. 4069–4073, Dec. 2013, doi: 10.1021/jz4022238.
- [126] H. Mao, C. Li, Y. Zhang, D. E. Bergbreiter, and P. S. Cremer, “Measuring LCSTs by novel temperature gradient methods: Evidence for intermolecular interactions in mixed polymer solutions,” *J Am Chem Soc*, vol. 125, no. 10, pp. 2850–2851, Mar. 2003, doi: 10.1021/JA029691K.
- [127] D. G. Lessard, M. Ousalem, X. X. Zhu, A. Eisenberg, and P. J. Carreau, “Study of the phase transition of poly(*N,N*-diethylacrylamide) in water by rheology and dynamic light scattering,” *J Polym Sci B Polym Phys*, vol. 41, no. 14, pp. 1627–1637, Jul. 2003, doi: 10.1002/POLB.10517.

- [128] K. Jain, R. Vedarajan, M. Watanabe, M. Ishikiriya, and N. Matsumi, “Tunable LCST behavior of poly(*N*-isopropylacrylamide/ionic liquid) copolymers,” *Polym Chem*, vol. 6, no. 38, pp. 6819–6825, Sep. 2015, doi: 10.1039/C5PY00998G.
- [129] J. Mink, L. Hajba, I. Papai, J. Mihaly, C. Nemeth, M. Y. Skripkin, and M. Sandström, “Vibrational spectroscopic and theoretical studies of urea derivatives with biochemical Interest: *N,N'* -Dimethylurea, *N,N,N',N'*- Tetramethylurea, and *N,N'* -Dimethylpropyleneurea,” *Appl Spectrosc Rev*, vol. 45, no. 4, pp. 274–326, Jun. 2010, doi: 10.1080/05704928.2010.483670.

