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Insight into the effect mechanism of urea-induced protein denaturation by dielectric spectroscopy

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Dielectric relaxation spectroscopy was applied to study how urea affects the phase transition of a thermosensitive polymer, poly(*N*-isopropylacrylamide) (PNIPAM), which has been widely used as a protein model. It was found that there is a pronounced relaxation near 10 GHz for the ternary system of PNIPAM in urea aqueous solution. The temperature dependence of dielectric parameters indicates that urea can reduce the lower critical solution temperature (LCST) of PNIPAM, *i.e.*, stabilize the globule state of PNIPAM and collapse the PNIPAM chains. Based on our results, the interaction mechanism of urea on the conformational transition of PNIPAM was presented: urea replaces water molecules directly bonding with PNIPAM and acts as the bridging agent for the adjacent side chains of PNIPAM. Accordingly, the mechanism with which urea denatures protein was deduced. In addition, it is worth mentioning that, from the temperature dependence of the dielectric parameters obtained in the presence of urea, an interesting phenomenon was found in which the effect of urea on PNIPAM seems to take 2 M as a unit. This result may be the reason why urea and TMAO exit marine fishes at a specific ratio of 2 : 1.

1. Introduction

Urea is one of the indispensable osmotic agents for marine elasmobranchii (sharks, skates and rays), holocephalans and coelacanths, because they need urea to maintain their *vivo* osmotic balance so that they can adapt to the harsh environment of high pressure, high salinity, *etc.*[1–4] However, urea is also well-known as a kind of protein denaturant.[5,6] Therefore, the interaction between protein and urea is an interesting issue.

In fact, researchers began to pay attention to the study of urea-induced protein denaturation as early as 1900. However, the molecular mechanism has not yet been well understood.[1,5–10] At present, the main controversies in this field fall into two aspects: one is the indirect mechanism,[6,7] which argues that urea modifies the tetrahedral network structure of water and makes the hydrophobic parts of protein become more favorably solvated; the other is the direct mechanism,[9,10] which postulates that urea interacts directly with the protein *via* hydrogen bonds. Among them, Chen *et al.*[6] made a conclusion that urea affected the solvation of proteins by changing the solvent environment according to the orientation flipping study of interfacial urea and water molecules with sum frequency resonance spectroscopy. Su *et al.*[10] studied the effects of urea on several non-restrained polyamino acids as well as their dimers by all-atom molecular dynamics simulations, and they concluded that urea denatures protein through a direct mechanism where urea binds favorably to the large non-polar side chains. Lindgren *et al.*[8] compared the interaction energies between water and urea with chymotrypsin inhibitor 2 by the molecular dynamics simulation. They suggested that compared to water, the energetically more favorable interaction between protein and urea may act as a driving force which promotes protein to unfold itself by increasing its solvent accessible surface area.

Although a large number of experiments and theoretical studies have been done, the mechanism of urea-induced protein denaturation still remains obscure. The main impediment is attributed to the complexity of protein structure.[9] To solve this problem, researchers have found some polymers which have similar properties to protein. Among them, a typical protein model, poly(*N*-isopropylacrylamide) (PNIPAM), which not only contains an amide group similar to the characteristic functional groups of peptide, but also experiences a phase transition resembling the denaturation process of proteins, has been given massive focus.[1,7,9,11–13] Up to now, a variety of techniques have been applied to research the effect of urea on the phase transition of PNIPAM, such as FTIR,[9] gel filtration chromatography,[9] molecular dynamics simulation,[1,10] DSC,[14] ellipsometry,[1] NMR,[13] *etc.* For example, Sagle *et al.*[9] explored the interaction between urea and PNIPAM by FTIR and gel filtration chromatography, and compared it with methylated urea. They proposed that urea decreases the lower critical solution temperature (LCST) of PNIPAM, namely stabilizing the globule state of PNIPAM through hydrogen bonding directly with the amide groups of PNIPAM. By means of molecular dynamics simulations, Pica *et al.*[7] gave a rational explanation for the effect of urea on the phase transition of PNIPAM from the perspective of Gibbs

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energy trade-off stemming from several aspects such as solvent-excluded volume, PNIPAM–solvent energetic attraction and PNIPAM conformational change. Through a comparative study of the effect of urea on the phase transition of PNIPAM and PDEA by ^{1}H MAS NMR, PGSE diffusion and NOESY experiments, Feng *et al.*[13] concluded that the existence of amide hydrogen has an important effect on the phase transition of these polymers. In the work of Gao *et al.*,[14] the influence of urea on the phase transition of PNIPAM was explained from the perspective of the relevant thermodynamic information, such as enthalpy change, obtained by differential scanning calorimetry.

Dielectric spectroscopy, which is sensitive to interactions among molecules and polarity changes in various systems, can tell us a lot of information closely related to substance nature; for example, polarization, interaction and the spatial structure and so forth. Thus, it has been widely used in a variety of systems.[15] Previously, many researchers, including our group, applied dielectric spectra to study the micro mechanism of the conformational transition of PNIPAM[11,16–20] and the interaction between urea and water.[21–23] For example, Ono *et al.*[19] showed that the phase transition of PNIPAM is dominated by the dehydration of PNIPAM through analyzing the temperature dependence of both the dielectric relaxation strength and the hydration number of the PNIPAM monomer. Recently, Agieienko *et al.*[23] reported the effective hydration number of urea and its dipole moment in urea aqueous solution obtained by dielectric spectroscopy and non-covalent analysis and concluded that water molecules might form double hydrogen bonds with urea molecules. Nakano *et al.*[24] studied the phase transition of PNIPAM in protic solvents and aprotic solvents by dielectric spectroscopy. Their analysis of the two relaxation processes respectively showed that the density of hydrogen-bonding sites in the PNIPAM solution dominated the relaxation process of solvent molecules and the dynamics of PNIPAM chains were closely related to the type of solvent. Füllbrandt *et al.*[11,25,26] have made a large contribution to the phase transition of PNIPAM microgels and linear PNIPAM chains by dielectric spectroscopy. They not only obtained the dependency of the phase transition of PNIPAM on the concentration and crosslinking density of PNIPAM, but also analyzed the dynamics of linear PNIPAM in water. In recent years, our group has also done a lot of research on the phase transition of PNIPAM.[18,20,27–30] For instance, in the most recent work,[27] the structure and dynamics of the PNIPAM chain and the solvent unit that are involved in the solvation of PNIPAM were revealed, which may provide some new insight into the cononsolvency phenomenon. In addition, through dielectric spectroscopy, we have also studied the influence of charged groups on the structure and volume phase transition of PNIPAM.[20] Overall, it is obvious that dielectric spectroscopy has striking potential to explain the influence of urea on the conformation of PNIPAM and its microscopic mechanism.

In this work, to provide an answer for the mechanism of urea-induced protein denaturation, high frequency dielectric measurements in the range of 500 MHz–40 GHz were carried out on PNIPAM aqueous solution with different urea concentrations at temperature from 17.8 °C to 39.9 °C. Based on the obtained dielectric parameters, the microscopic mechanism of urea on the phase transition of PNIPAM was validated and how urea denatures protein at the molecular level was answered. Unexpectedly, it was found that the special role of urea and protein might be the reason why trimethylamine *N*-oxide (TMAO) and urea exist in marine elasmobranchii at a specific ratio of 1 : 2.

2. Experimental section

2.1 Materials

Linear PNIPAM (M_w = 30 000 g mol^{-1}) and urea were bought from ALDRICH and Beijing Chemical Works, respectively. Twice distilled water was used during the experiment. The molecular formulas of both PNIPAM and urea are shown in Fig. 1. Urea aqueous solution was obtained by dissolving urea crystals in deionized water. PNIPAM urea aqueous solution was gained by adding an equal amount of PNIPAM in the urea aqueous solution at various concentrations. The concentration of PNIPAM in this work is 10 mg mL^{-1}.

2.2 Dielectric measurements

Dielectric measurements were performed in the frequency range from 500 MHz to 40 GHz by an Agilent E8363C PNA series network analyzer (Agilent Technologies, made in America) equipped with an Agilent 85070E open-ended coaxial probe (Agilent Technologies, made in America). The experimental temperature range is 17.8–39.9 °C. The temperature is controlled by a thermostat (DC-0506) with a temperature stability of 0.05 K.

After calibration according to the recommended procedures from Agilent, the probe terminal was put into the reagent bottle filled with samples, and the network analyzer provides the real and imaginary parts of permittivity as a function of frequency, which is automatically calculated by the built-in measurement system. Each dielectric measurement can be completed in a matter of seconds.

3. Result and discussion

3.1 Dielectric behavior of PNIPAM urea aqueous solution

Fig. 2a and b show the temperature dependence of dielectric spectra in three-dimensional representations for a urea–water binary system and PNIPAM–urea–water ternary system. From Fig. 2a and b, it can be seen that the two systems both exhibit an obvious relaxation around 10^{10} Hz, and the relaxation in Fig. 2b seems to drop down near T = 32.5 °C. To clearly express the relation between the relaxation and temperature, the temperature dependent relaxation frequencies (f) and maximum dielectric loss (ε_{max}'') for the binary and ternary systems are shown in Fig. 2c

PNIPAM Urea

Fig. 1 Structure graph for the two samples in our experiment.

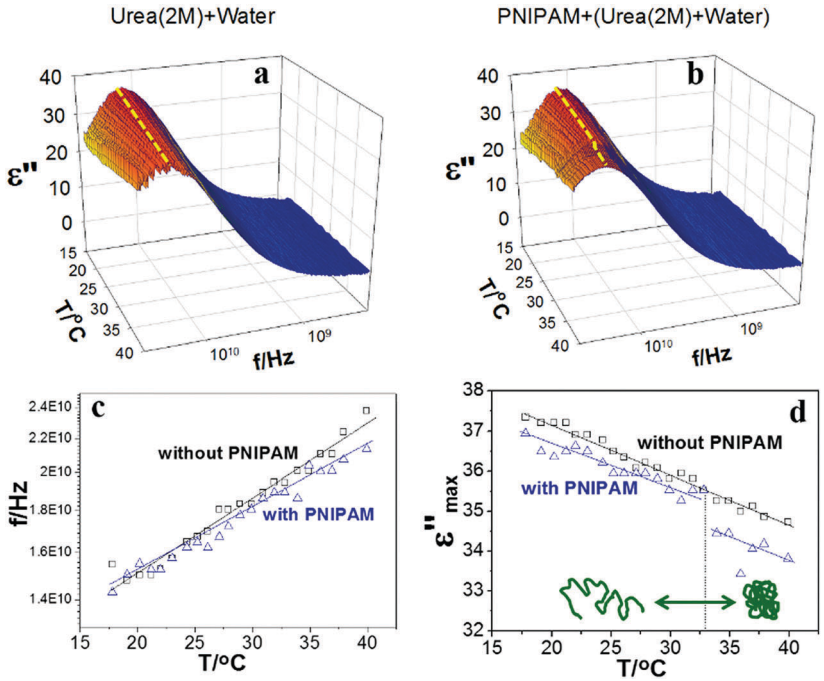

Fig. 2 Specific dielectric spectroscopy 3D graphs for urea (2 M) aqueous solution (a) and PNIPAM (10 mg mL^{-1}) in urea (2 M) aqueous solution (b). The temperature dependent characteristic frequency (f) (c) and the maximum dielectric loss (ε_{max}'') (d) for the urea (2 M) aqueous solution with or without PNIPAM (10 mg mL^{-1}). The yellow dotted lines are the peak of the dielectric loss.

and d, respectively. From Fig. 2c and d, it can be seen that the two systems have no significant difference in the relaxation frequency which rises with the increase of temperature; while, referring to the dielectric loss, different from the system without PNIPAM, ε_{max}'' of the PNIPAM urea solution is lower at near 32.5 °C. This is reasonable for the mutation of temperature dependent dielectric spectra at 32.5 °C because it is close to the reported LCST of PNIPAM which relates to the coil–globule transition. That phenomenon has been observed in many systems, including PNIPAM aqueous solution[19,31,32] and other gel systems.[33,34]

3.2 Effect of urea on the LCST of PNIPAM

To determine whether the presence of urea affects the phase transition of PNIPAM, or whether the dielectric spectrum can directly reflect the influence, it is necessary to get the dielectric parameters of the dielectric spectra. Therefore, the Cole–Cole equation (eqn (1)) was used to fit the experimental data.

$$\varepsilon^* = \varepsilon_h + \sum_{i=n} \frac{\Delta\varepsilon_i}{1 + (j\omega\tau)^\beta} \quad (1)$$

where ε^* is the complex permittivity, $\Delta\varepsilon_i (= \varepsilon_l - \varepsilon_h)$ is the dielectric increment of the ith relaxation (ε_l and ε_h represent the permittivity at low frequency and high frequency, respectively), $j = (-1)^{1/2}$, ω is the angular frequency, τ is the relaxation time, and β is the parameter indicating the distribution of relaxation time.

The temperature dependence of the dielectric increment for PNIPAM aqueous solution with different concentrations of urea is shown in Fig. 3. From Fig. 3, under the same concentration of urea, the dielectric increment presents a downward trend with an increase of temperature. However, there is a palpable inflection point at a certain temperature (namely the LCST of PNIPAM) which shifts to lower temperature with an increase of urea concentration, *i.e.* urea can decrease the LCST of PNIPAM. It is also interesting that the urea concentration dependence of LCST is not fully linear, but has an inflection at around 4 M (shown in the inset in Fig. 3), which will be explained in detail later.

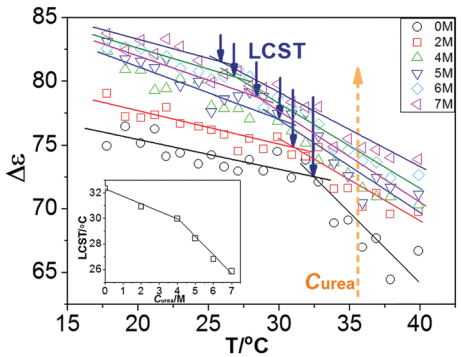

Fig. 3 Relation between dielectric increment and temperature in the presence of urea at different concentrations (the inset is the concentration dependent diagram for LCST).

3.3 Mechanism analysis

In order to analyze the dielectric spectra measured in the experiment, the HN function (eqn (2)) was employed to fit the experimental spectra.

$$\varepsilon^* = \varepsilon_h + \sum_{i=n} \frac{\Delta\varepsilon_i}{[1 + (j\omega\tau)^\alpha]^\beta} \quad (2)$$

where ε^* is the complex permittivity, $\Delta\varepsilon_i(=\varepsilon_l-\varepsilon_h)$is the dielectric increment of the ith relaxation (ε_l and ε_h represent the permittivity at low frequency and high frequency, respectively), $j=(-1)^{1/2}$, ω is the angular frequency, τ is the relaxation time, and α and β are the parameters indicating the distribution of relaxation time.

It is important to determine the number and type of relaxation for fitting. For example, using high-frequency dielectric relaxation spectroscopy, Krakovský *et al.*[35] investigated nanophase-separated structures of epoxy-based hydrogels. By fitting, it was concluded that the dielectric spectra were superposed by four sub-relaxations; namely, Maxwell Wagner Sillars (MWS) polarization, epoxy network dipole, bound water and free water. In a study by Hunger *et al.*,[36] the observed dielectric spectra were attributed to three sub-relaxation superpositions, namely solute relaxation, bulk water relaxation and fast water relaxation. In this current work, for a multicomponent system such as PNIPAM urea aqueous solution, the apparent relaxation is actually contributed by a number of components. To further explain the effect mechanism of urea on PNIPAM, combined with relevant reports,[35,37–41] peak processing has been done on the ternary system of PNIPAM urea aqueous solution. Fig. 4 represents the result of peak splitting of the ternary system after fitting with the HN function (eqn (2)). It can be seen that the dielectric spectrum of the PNIPAM, urea and water ternary system contains four sub-relaxations (relaxation frequencies are 22.0 GHz, 11.5 GHz, 8.5 GHz, and 0.21 GHz respectively; because the obtained α and β are close to 1, all the sub-relaxations here are attributed to Debye-type relaxations).

3.3.1 Why the ratio is 2 : 1 for urea and TMAO in marine elasmobranchii. Fig. 5 shows the temperature dependence of the dielectric increment and frequency of each sub-relaxation. It is obvious that both parameters of each relaxation inflect at the LCST of the corresponding PNIPAM urea aqueous solution. That is to say, the properties of other substances in the system also changed with the addition of urea. The specific details of this finding will be explained in the next section.

Interestingly, it can be seen from Fig. 5 that the effect of urea on each relaxation seems to increase in units of 2 M. This reminds us of literature which found that urea and TMAO exist at a specific ratio of 2 : 1 in many of marine elasmobranchii.[2,36,42]

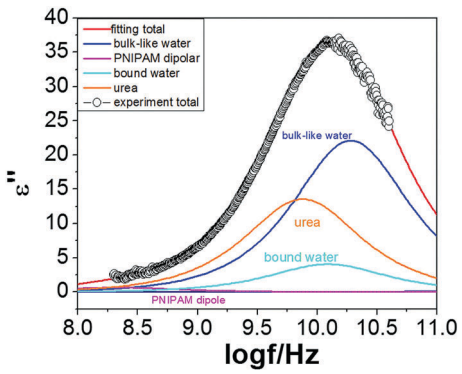

Fig. 4 Peak processing example for PNIPAM (10 mg mL^{-1}) urea (4 M) aqueous solution at room temperature. (Hollow circles – experimental data, red – overall relaxation obtained by fitting, blue – bulk-like water, cyan – bound water, orange – urea, magenta – PNIPAM dipole.)

A large number of studies[2,42–45] show that TMAO, as a protein stabilizer, can counteract the urea-induced denaturation of protein, and the counteraction is most effective when the ratio of urea and TMAO reaches to 2 : 1. There may be some inextricable link between why the ratio is 2 : 1 for urea and TMAO in marine elasmobranchii and our results (Fig. 5). As we know, in order to adapt to the harsh conditions of high pressure and high salinity, marine organisms usually accumulate large amounts of urea *in vivo*, while, on the other hand, urea can denature proteins. To deal with this contradictory issue, marine organisms ingeniously accumulate another type of osmolyte, such as TMAO or betaine, so as to counteract the denaturation of urea.[42,45] According to Fig. 5, the effect of urea on PNIPAM seems to increase with a 2 M increment. Let's make a hypothesis. Considering PNIPAM as a model of protein, here we use protein instead of PNIPAM. Suppose that in order to adapt to the harsh environment of high pressure and high salt, fish need to amass 2 M urea *in vivo*; meanwhile, 2 M urea can denature 1 M protein (from Fig. 5), namely destroy the protein function and influence the life of fish. Accordingly, it is desiderate to accumulate some substances to overcome the destructive effect of urea on protein. As biological evolution, due to their stabilization of protein, methylamine substances such as TMAO gradually accumulate *in vivo* in fish. In addition, it has been confirmed that the counteraction of TMAO to urea can be the most effective when the ratio of urea and TMAO reaches 2 : 1.[42,43] Thus, to stabilize 1 M protein and prevent it from being denatured by urea, at least 1 M methylamine substance such as TMAO is needed to accumulate in fish. On these grounds, it is easy to understand why the special ratio of urea and TMAO in marine organisms is 2 : 1.

3.3.2 Interaction mechanism of PNIPAM and urea. As is known to us, the conformation transition of PNIPAM in water is closely related to the solvation of water.[19] When the temperature is below the LCST (32 °C), most of the polar amide groups of PNIPAM are hydrated.[46] Such hydration enables PNIPAM to maintain a stretched coil in water. When the temperature is higher than the LCST, the water molecules bonded with PNIPAM are released, and PNIPAM forms a collapsed spherical state *via* intramolecular hydrogen bonding.[13] That is, the different phase transitions arise from specific polymer–solvent interactions.[24,47] Then, for the PNIPAM urea aqueous solution, besides the interaction of water molecules with PNIPAM, there are also urea molecules. Contrasting the ^{1}H MAS NMR of the two similar thermosensitive hydrogels (PNIPAM and PDEA), Wang *et al.*[13] emphasized that amino hydrogen on the side chains of PNIPAM plays a significant role and the direct hydrogen bonding between urea and PNIPAM is the main reason for the effect of urea on PNIPAM. Zangi *et al.*[48] compared the conformation behavior of a polymer chain purely composed of hydrophobic groups in 7 M urea aqueous solution and water solution by molecular dynamics simulation. The result shows that the attractive dispersion interaction between urea and the protein side chains and backbone favorably competes with that of water and this may be the major cause of urea-induced denaturation. Thus, the indirect mechanism,

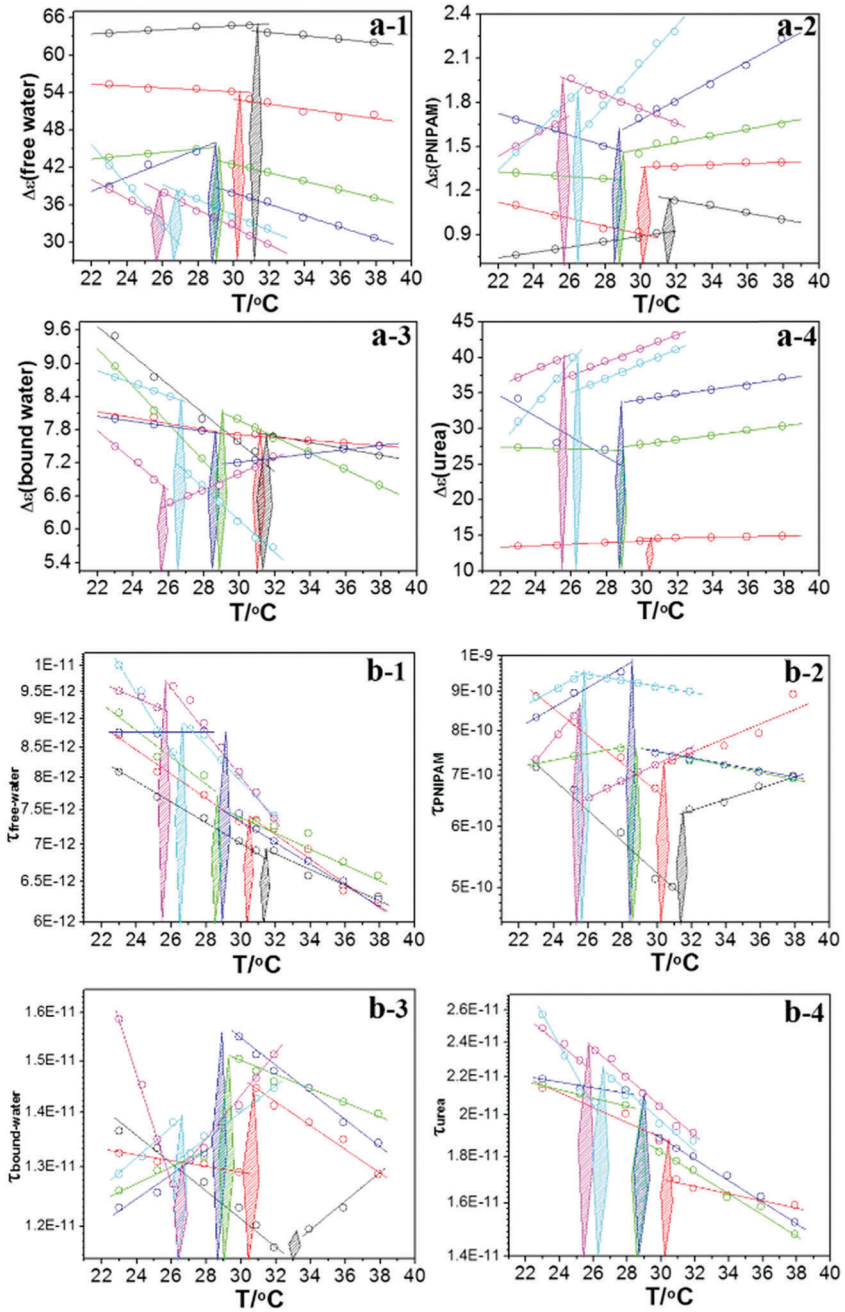

Fig. 5 Temperature dependence of each relaxation dielectric increment (a) and frequency (b). (1 – free water, 2 – PNIPAM dipole, 3 – bound water, and 4 – urea; different colors mean different concentrations of urea: black – 0 M, red – 2 M, green – 4 M, blue – 5 M, cyan – 6 M, and magenta – 7 M.)

urea as a chaotrope, is ruled out. Therefore, in order to make clear the influence of the mechanism of urea on the phase transition of PNIPAM, it is necessary to calculate the number of water molecules and urea molecules bonding with PNIPAM, because the bond number of molecules can reflect the state of PNIPAM in solution to a certain extent.

The dielectric increment of relaxation is related to the dipole moment (μ_i) of the relaxation species in the solution. The Cavell equation,[49] which is compliant with a multicomponent mixture, was used to relate the observed dielectric increments to the molecular properties (*e.g.*, dipole moments of matters μ_i, relaxation concentrations c_i, *etc.*). For the system, the concentrations of free water and free urea were calculated according to eqn (3).

$$\frac{2\varepsilon_l + 1}{\varepsilon_l}\Delta\varepsilon_i = \frac{N_A c_i}{k_B T \varepsilon_0}\mu_i^2 \quad (3)$$

where ε_l is the limiting permittivity at low frequencies, also known as the static permittivity, $\varepsilon_0 (= 8.854187817 \times 10^{-12}\ \mathrm{F\ m^{-1}})$ is the vacuum permittivity, $\Delta\varepsilon_i$, c_i, and μ_i are the dielectric increment, molar concentration, and dipole moment of the ith relaxation, N_A and k_B are respectively the Avogadro and the

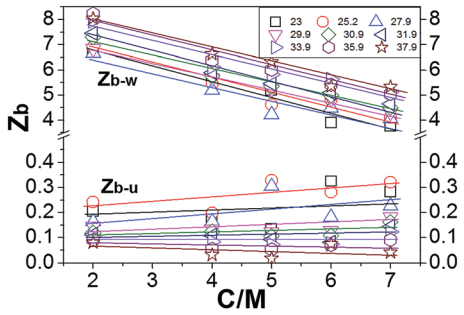

Fig. 6 Concentration dependency graph of $Z_{b\text{-}w}$ and $Z_{b\text{-}u}$ in the experimental temperature range (different colors mean different temperatures).

Boltzmann constant and T is the thermodynamic temperature. Then the bonding numbers of water ($Z_{b\text{-}w}$) and urea ($Z_{b\text{-}u}$) molecules were calculated from eqn (4).

$$Z_{b\text{-}i} = \frac{c_{bound}}{c} = \frac{c_i - c_{free\text{-}i}}{c} \quad (4)$$

where $Z_{b\text{-}i}$ and c_{bound} represent the bonding number and bonding concentration of the i molecules on the side chains of PNIPAM, and $c_{free\text{-}i}$ is the free molecule concentration of the i molecules.

As can be seen from Fig. 6, in the measured temperature range, $Z_{b\text{-}w}$ gradually decreases between 8–3.5 with the increasing concentration of urea. It is worth mentioning that the value of $Z_{b\text{-}w}$ is slightly less than the reported values (11–13)[16,19,50,51] of $Z_{b\text{-}w}$ of PNIPAM in pure water solution. This indicates that the addition of urea urges part of the water molecules that were originally bonding with PNIPAM away from PNIPAM.

$Z_{b\text{-}u}$ calculated by eqn (3) and (4) hardly varies with concentration change. This implies that the change of $Z_{b\text{-}w}$ is not completely caused by the replacement of urea molecules. At low temperature, $Z_{b\text{-}u}$ levels out at around 0.3, that is to say, an average of about three PNIPAM side chains mutually share a urea molecule. Keeping in mind the molecular structure of urea, it can be easily imagined how the urea and PNIPAM interact: the amino hydrogen on both ends of the urea molecule as a hydrogen bond donor forms a hydrogen bond with carbonyl on the PNIPAM side chain, and intermediate carbonyl oxygen acts as a hydrogen bond receptor bond with amide hydrogen on the PNIPAM side chain (Fig. 8b). In that way, urea molecules, as a bridging agent, connect the adjacent side chains of PNIPAM. Also, the bridging effect narrows the distance between the PNIPAM side chains, which squeezes out the weakly bound water molecules that were originally situated among the PNIPAM side chains (shown by the green area in Fig. 8b). Above all, it can be concluded that in addition to the water molecules directly replaced by urea molecules, there are some water molecules that are indirectly affected by the replacement and are also expelled. Accordingly, without these water molecules, the energy required for the collapse of PNIPAM will be reduced, thereby depressing the LCST of PNIPAM. At high temperature, the number of bounded urea molecules is near zero, indicating that the urea molecules also fall off the PNIPAM chains at high temperature, which leads to the formation of hydrogen bonds between the PNIPAM side chains and then collapse.

Furthermore, the hydrodynamic radius R of PNIPAM in urea aqueous solution at different concentrations was obtained according to the Einstein–Stokes equation[52,53] which denotes the relationship between relaxation time and the hydrodynamic radius of macromolecules:

$$f^* = \frac{kT}{8\pi^2\eta R^3} \quad (5)$$

where f^* is the characteristic frequency, and k, R, T and η are the Boltzmann constant, hydrodynamic radius of PNIPAM, experimental temperature and solvent viscosity (refer to the values of η reported in ref. 23).

As can be seen from Fig. 7, compared with R_0, R at $C_{urea} < 4$ M is smaller than R_0, and R at $C_{urea} > 4$ M is greater than R_0. When $C_{urea} < 4$ M, the result that R is smaller than R_0 also confirmed the above analysis of the bond number of molecules. Due to the addition of urea acting as a bridging agent the distance between the side chains is narrowed, which promotes the collapse of PNIPAM, so R is smaller than R_0. With the increase of the concentration of urea, $C_{urea} > 4$ M, the contact probability of urea on the PNIPAM side chains increases. Therefore, in this instance, there is a chance for urea to bond with the side chains that come from different PNIPAM chains, where urea may act as a crosslinking agent to the adjoining chains of PNIPAM (Fig. 8c). The dependence of the PNIPAM hydrodynamic radius R on the urea concentration may proffer an explanation for the inset in Fig. 3.

3.3.3 Interaction mechanism of urea-induced protein denaturation. According to the analysis of the above section, we can conclude that the main reason for urea decreasing the LCST of PNIPAM, namely, urea stabilizing the collapsed state of PNIPAM, is the direct interaction of urea with the amide groups of the PNIPAM side chains. Moreover, as presented in the introduction, the amide side chain of PNIPAM is similar to the peptide group of polypeptides. As is well known, a protein polypeptide chain in its natural state is a helical structure formed by intramolecular hydrogen bonding, like a spiral zipper (Fig. 9a). When urea is added (Fig. 9b), the formation

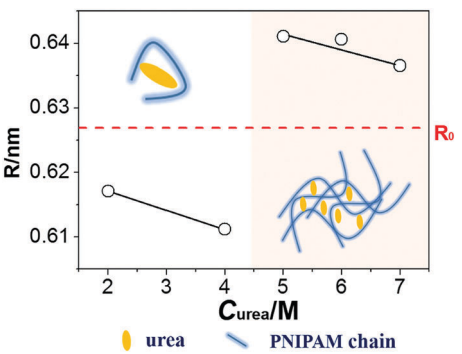

Fig. 7 The dependence of R on urea concentration. (R_0 is the hydraulic radius of PNIPAM in pure water). Insets are sketches of the PNIPAM (blue) and urea (yellow) structures in the two regimes.

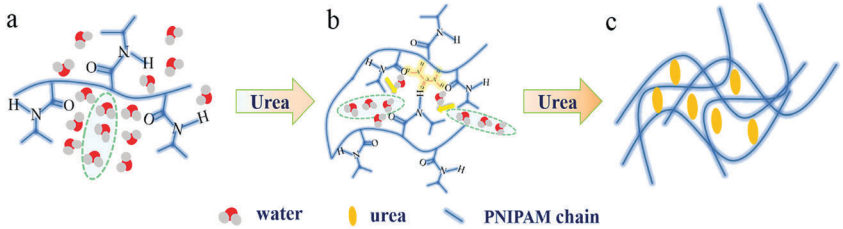

Fig. 8 The interaction mechanism of urea (yellow) on PNIPAM (blue) (a, without urea; b, $C_{urea} < 4$ M; c, $C_{urea} > 4$ M).

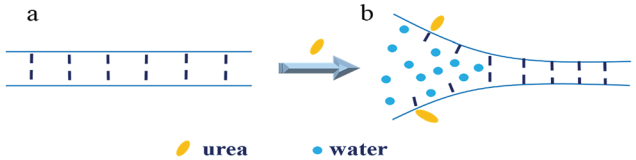

Fig. 9 Mechanism of urea-induced denaturation of protein.

of the hydrogen bond between urea and the peptide group destroys the intramolecular hydrogen bonding of the polypeptide chain, which opens the zipper, and, coupled with the subsequent inburst of surrounding solvent molecules along the opening, jointly promotes the unfolding denaturation of the protein.

4. Conclusion

In this work, the effect of urea on the conformation transition of PNIPAM was investigated by dielectric spectroscopy. In the presence of urea with different concentrations, the temperature dependence of the dielectric parameters proves that urea can reduce the LCST of PNIPAM. The calculated number of bound urea suggests that when urea molecules are added to PNIPAM aqueous solution, they can act as bridging agents between the PNIPAM side chains, which stabilizes the globular state of PNIPAM. In addition, the bridging effect prompts the PNIPAM to release some of the weakly bound water molecules, which decreases the energy needed during PNIPAM collapse, thereby reducing the LCST of PNIPAM.

Based on the protein model, the following mechanism for urea inducing denaturation of protein can be proposed: the hydrogen bond interaction between urea and the peptide groups is a trigger for urea denaturing protein, which opens the entrance for water, and contributes to the unfolding denaturation of protein. In addition, this work also illuminated the reason why the ratio is 2 : 1 for urea and TMAO in marine elasmobranchii. This study provides a new insight into the interaction between urea and protein and proves that dielectric spectroscopy can provide a fundamental reference for research on the unique survival skills of marine organisms.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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