# Insight into the Effect of Methylated Urea on the Phase Transition of Aqueous Solutions of Poly(*N*-isopropylacrylamide) by Microcalorimetry: Hydrogen Bonding and van der Waals Interactions

**Yating Gao,[1] Jinxian Yang,[1] Haiyan Fan,[1] Yanwei Ding,[1] Xiaodong Ye[1,2]**

[1]Department of Chemical Physics, Hefei National Laboratory for Physical Sciences at the Microscale, University of Science and Technology of China, Hefei, Anhui, 230026, China
[2]CAS Key Laboratory of Soft Matter Chemistry, University of Science and Technology of China, Hefei, Anhui, 230026, China
Correspondence to: X. Ye; (E-mail: xdye@ustc.edu.cn)

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**ABSTRACT**: The effect of different functional groups of methylated urea on the phase transition of poly(*N*-isopropylacrylamide) (PNIPAM) aqueous solutions has been studied by a high-sensitivity differential scanning calorimetry. The results reveal that with the addition of osmolytes with N—H groups, the enthalpy change increases with the number of DSC cycles, presumably due to the gradual formation of hydrogen bonds with dehydrated C═O groups of PNIPAM at high temperature. Moreover, with the addition of tetramethylurea (TMU) without hydrogen bond donor groups, the enthalpy change of PNIPAM solution remains unchanged with the number of DSC cycles and decreases with the TMU concentration, suggesting that the van der Waals interactions between TMU and isopropyl groups of PNIPAM and the weakening of hydrophobic interactions between isopropyl groups play a dominant role in the effect of TMU on the phase transition of PNIPAM. © 2016 Wiley Periodicals, Inc. J. Polym. Sci., Part B: Polym. Phys. **2016**, *54*, 1145–1151

**KEYWORDS**: additives; differential scanning calorimetry (DSC); polyamides; stimuli-sensitive polymers; water-soluble polymers

**INTRODUCTION** Much attention has focused on the denaturation of proteins by urea.[1–9] Despite extensive studies, the molecular mechanism of the urea-induced denaturation of proteins remains to be elucidated. Two distinct mechanisms have been proposed: one is the "indirect mechanism," in which urea changes the structure of water molecules and makes hydrophobic groups more easily solvated[1,3,10,11]; and the other is the "direct mechanism," in which urea interacts with the backbone and/or side groups of proteins via hydrogen-bonding interactions, other electrostatic interactions and van der Waals attractions, thus causing the proteins to denature.[2,4,6–8,12] For example, the work of Berne's group reveals that the dispersion interaction between urea molecules and the backbone and side chains of a mutant lysozyme is stronger than the interaction for water using molecular dynamic simulations and the study supports the direct mechanism.[7] Zhou et al. further studied the urea-induced denaturations of five protein/peptide systems by performing large scale molecular dynamics simulations and suggested the "dispersion interaction-driven" mechanism should be common for all proteins.[9] Other studies show that the direct hydrogen bonds formed between urea and protein/peptide backbones is the main reason for the denaturation.[4,8] Pielak et al. used pressure perturbation calorimetry and found that there is no correlation between a solute's impact on water structure and its effect on the protein stability and suggested that other hypotheses based on preferential interaction and excluded volume should be considered.[5]

Poly(*N*-isopropylacrylamide) (PNIPAM), a mimic for the cold-denatured proteins due to its simpler structure and thermally sensitive property which is soluble in water at low temperature and insoluble at higher temperature above ~32 °C,[13] has received much attention.[14–47] A variety of techniques have been used to study the phase transition of aqueous solutions of PNIPAM, such as laser light scattering,[14–20] fluorescence spectroscopy,[21–23] turbidimetry,[24–26] nuclear magnetic resonance,[27–29] Raman spectroscopy,[30–32] infrared spectroscopy,[33–36] and differential scanning calorimetry (DSC).[37–46] The effect of urea on the phase transition of PNIPAM was also studied by fluorescence spectroscopy,[48] atomic force microscopy-based single molecule force spectroscopy[49,50] and Fourier Transform Infrared Spectroscopy (FTIR).[51] For example, Sagle et al. suggested that urea may interact directly with PNIPAM by forming hydrogen bonds with amide groups of PNIPAM in a bivalent manner

Additional Supporting Information may be found in the online version of this article.

by use of FTIR and Stokes radius measurements.[51] Rodríguez-Ropero and van der Vegt showed that urea molecules can preferentially interact with the hydrophobic isopropyl groups of the PNIPAM chains via van der Waals dispersion interactions on the basis of molecular dynamics simulations.[52,53]

Among all used techniques, DSC has long been used to study the phase transition of aqueous solutions of proteins and PNIPAM[37–46,54–59] and a single narrow endothermic peak was found in the phase transition of aqueous solutions of PNIPAM.[42] Cho et al. suggested that there are at least two different thermal processes during the phase transition using temperature modulated differential scanning calorimetry.[40] In a previous paper, we used DSC to study the influence of urea on the phase transition of aqueous solutions of PNIPAM and found that the enthalpy change ($\Delta H_{heating}$) in the first heating process decreases with increasing of the concentration of urea, while increases with the number of heating-cooling cycles for a certain concentration of urea.[46] Our results indicate that the total enthalpy change during the phase transition might be contributed by two parts: one is endothermic part related to the apolar groups ($\Delta H_a$), and the other is related to the polar group ($\Delta H_p$) which is exothermic.[46] However, the mechanism of the interaction between PNIPAM and urea has not been fully solved. For example, which functional groups are involved in the formation of hydrogen bonds with PNIPAM, N—H group or C═O group of urea, or both?

In this study, we extend this investigation to five small molecules, including 1,1-dimethylurea (1,1-DMU), 1,3-dimethylurea (1,3-DMU), tetramethylurea (TMU), *n*-butanol and *n*-butylamine, to study the effect of different functional groups on the phase transition of aqueous solutions of PNIPAM. Among these five molecules, 1,1-DMU and 1,3-DMU have both N—H group and C═O group and are more hydrophobic than urea. TMU has no N—H group, but it has C═O group which can serve as hydrogen bond acceptor. Both *n*-butanol and *n*-butylamine only have hydrogen bond donor groups. The structures of urea and methylated urea are shown in Figure 1. Our results show that in the first heating process, the enthalpy change of the PNIPAM solution decreases with increasing of the hydrophobicity of methylated urea, presumably due to the stronger van der Waals interaction between methylated urea with more methyl groups and hydrophobic isopropyl groups of PNIPAM. Moreover, in the presence of osmolytes with hydrogen bond donor groups, such as N—H groups, the enthalpy change of the PNIPAM solution increases with the number of heating-cooling DSC cycles, suggesting the gradual formation of hydrogen bonds between PNIPAM and osmolytes. Furthermore, in the presence of TMU, the enthalpy change remains a constant with the number of heating-cooling cycles, indicating that the water molecules binding to hydrophilic amino groups (N—H) and the van der Waals interaction between TMU and isopropyl groups remain unperturbed during the heating-cooling cycles. Our results also suggest that in the presence of TMU, the van der Waals interaction between TMU and isopropyl groups of PNIPAM and the weakening of hydrophobic interactions between isopropyl groups play a dominant role in the influence of TMU on the phase transition of aqueous solution of PNIPAM.

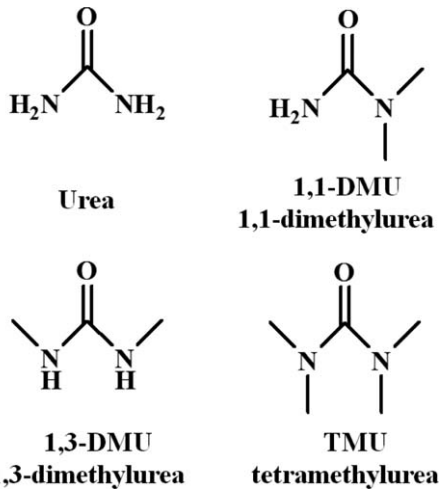


**FIGURE 1** Chemical structures of urea and methylated urea.

## EXPERIMENTAL

### Materials

*N*-isopropylacrylamide (NIPAM, Aldrich, 97%) was recrystallized three times from a mixture of benzene and *n*-hexane. 4,4'-azobis(isobutyronitrile) (AIBN) was recrystallized three times from ethanol. 1,1-Dimethylurea (1,1-DMU, 98.0%) was purchased from J & K Chemical. 1,3-Dimethylurea (1,3-DMU, 98.0%) and tetramethylurea (TMU, 99.0%) were purchased from TCI. *n*-Butanol (99.7%) and *n*-butylamine (99.0%) were purchased from Aladdin. Urea (99.0%) was purchased from Sinopharm. All chemicals were used as received unless otherwise specified. Water with a resistivity of 18.2 MΩ cm used in all experiments was purified by filtration through a Millipore Gradient system after distillation.

### Sample Preparation

PNIPAM was prepared by free radical polymerization with AIBN as the initiator, the details have been described previously.[17,37,45] After polymerization, the PNIPAM sample was further fractionated by a dissolution/precipitation process in a mixture of dry acetone and dry hexane at room temperature. To characterize the weight-average molar mass ($M_w$) and the z-averaged root-mean-square radius of gyration ($\langle R_g \rangle$) of PNIPAM fraction, a commercial laser light scattering (LLS) spectrometer (ALV/DLS/SLS-5022F) equipped with a multi-$\tau$ digital time correlation (ALV5000) and a cylindrical 22-mW He-Ne laser ($\lambda_0 = 632$ nm, UNIPHASE) as the light source was used here. The $M_w$ and the $\langle R_g \rangle$ are 9.3 $\times$ $10^5$ g/mol and 44.5 nm, respectively. The polydispersity index ($M_w/M_n$) is $\sim$ 1.24 estimated from the relative width $\mu_2/<\Gamma>^2$ of the line-width distribution measured in dynamic LLS by using $M_w/M_n \sim 1 + 4\mu_2/<\Gamma>^2$. The data obtained by LLS was shown in Supporting Information Figures S1 and S2.

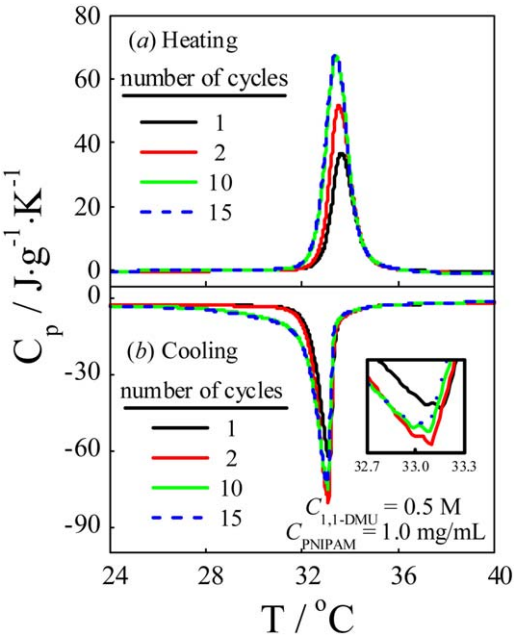


**FIGURE 2** DSC traces of aqueous solution of PNIPAM containing 0.5 M 1,1-DMU at different heating-cooling DSC cycles (cycles 1, 2, 10, 15), where the concentration of PNIPAM ($C_{PNIPAM}$) was kept at 1.0 mg/mL. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

### DSC Measurements

Phase transition of aqueous solutions of PNIPAM with different osmolytes were measured by a high-sensitivity differential scanning calorimetry (nano-DSC model, TA instrument incorporation, USA) with a corresponding solution without PNIPAM as reference at a constant pressure of 3 atm. The scanning rate was 1.00 °C/min at the temperature range from 5 °C to 60 °C. The concentration of PNIPAM in each sample solution was 1.0 mg/mL, and the concentration of osmolyte was ranged from 0.5 to 2.0 mol/L. The details of the DSC process have been described previously.[46] Briefly, each sample was first heated from 5 °C to 60 °C and then cooled down from 60 °C to 5 °C. To check the reversibility of the phase transition, 15 repeat times were carried continuously on each DSC measurement. Baselines were measured at the same scanning rate from 5 °C to 60 °C using the osmolyte aqueous solution with equal concentration in the sample cell and the reference cell. The transition temperature ($T_t$) and enthalpy change ($\Delta H$) during phase transition was regarded as the maximum and the integration of the peak, respectively. The errors for the transition temperature and enthalpy are within ± 0.04 °C and ± 0.7 J·g$^{-1}$, respectively.

## RESULTS AND DISCUSSION

Figure 2 shows DSC thermograms for 1.0 mg/mL PNIPAM aqueous solution containing 0.5 M 1,1-DMU at four different heating-cooling DSC cycles (circles 1, 2, 10, 15). As a control, we also carried DSC measurements on an aqueous PNIPAM solution with no 1,1-DMU and obtained reproducible DSC thermograms, as shown in Supporting Information Figure S3. Figure 2 shows that the phase transition of aqueous solution of PNIPAM in the presence of 1,1-DMU is irreversible and the enthalpy change increases with the number of DSC cycles, indicating the gradual formation of hydrogen bonds between 1,1-DMU and hydrophilic carbonyl groups (C═O) of PNIPAM at high temperature, which will be discussed in details in the following sections. Moreover, the transition temperature during the cooling process is lower than that during the heating process because of the intra and/or interchain hydrogen-bonding interaction in aggregated state.[34,42,43,60,61] Furthermore, a bimodal transition during the cooling process is observed, as shown in the insert of Figure 2, presumably due to the breaking of hydrogen-bonding formed at high temperature and the dissociation of the hydrophobic groups, respectively.[34,42] Note that only one peak exists during the cooling process when the concentration of 1,1-DMU increases to 2.0 M and the number of DSC cycles increases to 14 (not shown), indicating the decrease in the number of the intra and/or interchain hydrogen bonds between PNIPAM repeating units.

Figure 3 shows that the enthalpy change in the first heating process decreases as the concentrations of the two kinds of dimethylurea (1,1-DMU and 1,3-DMU) increase from 0 to 2.0 M, reflecting that more dimethylurea molecules bind to the hydrophobic isopropyl groups and thus causing the decrease in the number of water molecules around hydrophobic isopropyl groups. Stumpe and Grubmüller stated that urea preferentially interacts with nonpolar residues of almost all amino acids on the basis of molecular dynamics simulations.[6] Berne

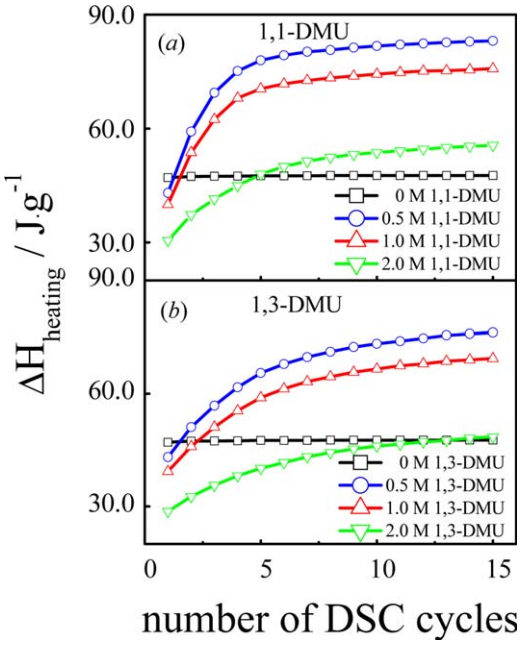


**FIGURE 3** The influence of number of heating-cooling DSC cycles on the enthalpy change ($\Delta H_{heating}$) of 1.0 mg/mL PNIPAM aqueous solutions with different concentrations of 1,1-DMU (*a*) and 1,3-DMU (*b*) in the heating processes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

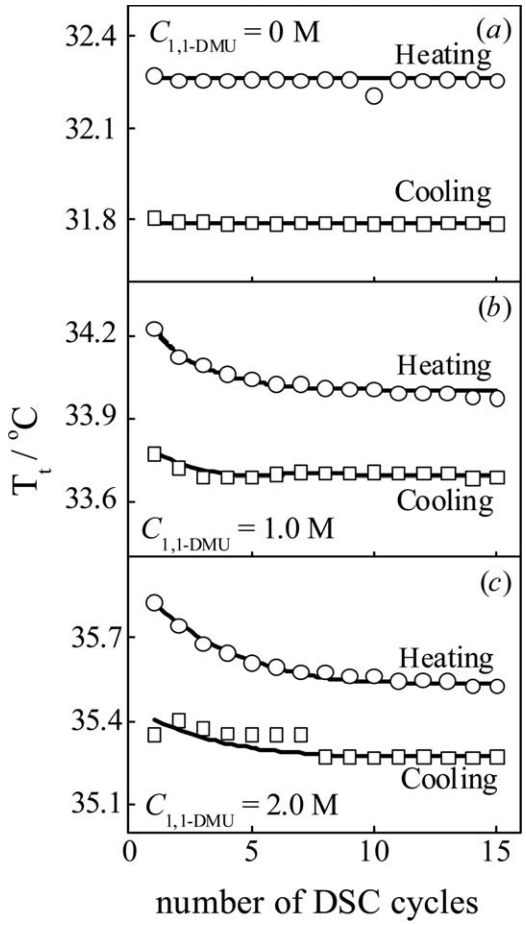


**FIGURE 4** The influence of the number of heating-cooling DSC cycles on the transition temperature ($T_t$) of aqueous solutions of PNIPAM with different amounts of 1,1-DMU. The concentration of PNIPAM ($C_{PNIPAM}$) was kept at 1.0 mg/mL and 1,1-DMU concentration ($C_{1,1\text{-DMU}}$) were 0 M (*a*), 1.0 M (*b*) and 2.0 M (*c*), respectively.

et al.[7] and van der Vegt et al.[52,53] also stated that urea can preferentially accumulate in the first solvation shell of protein and PNIPAM via van der Waals dispersion interactions. Therefore, both of 1,1-DMU and 1,3-DMU may interact stronger with isopropyl groups than urea because of their higher molecular weights.

Many reports have shown that the dissociation of water molecules around the hydrophobic isopropyl groups of PNIPAM is the main reason for phase transition of aqueous solutions of PNIPAM at temperature higher than its transition temperature,[34,38,39,55] and the number of water molecules dehydrated from each repeating unit of PNIPAM could affect the heat during the phase transition.[62–64] Note that the number of hydrated water molecules per PNIPAM repeating unit is ~11 when the temperature is below the transition temperature.[64–68] Two or three water molecules form hydrogen bonds with amide groups of PNIPAM, and the rest water molecules are around the hydrophobic isopropyl groups.[35,67] So with the increasing of the concentration of dimethylurea, the number of water molecules around hydrophobic isopropyl groups gradually decrease, and thus making the total enthalpy change decrease. Moreover, for a certain concentration of dimethylurea, the enthalpy change increases with the increasing of the number of DSC cycles. As suggested in our previous study,[46] the heat related to the apolar group ($\Delta H_a$) and that related to the polar amide groups ($\Delta H_p$) during the phase transition of PNIPAM are endothermic and exothermic, respectively. During the phase transition of PNIPAM, more dimethylurea molecules may form hydrogen bonds with dehydrated amide groups, reflecting the larger possibility to form hydrogen bonds with dehydrated amide groups than the hydrated one and/or due to the dehydration of nearby isopropyl groups during the phase transition. The absolute value of $\Delta H_p$ decreases to ~0 after ~10 DSC heating-cooling cycles because the replacement of dimethylurea molecules by water molecules at 5 °C needs longer time, presumably due to the steric hindance of PNIPAM segments as PNIPAM aggregates may behave like a "gel" when the temperature sudden decreases to 5 °C.[34,60] While $\Delta H_a$ remains nearly unchanged, thus the total enthalpy change increases. Our results shows that the formation of hydrogen bonds and van der Waals interactions between dimethylurea and PNIPAM should be taken into account during the phase transition of aqueous solutions of PNIPAM in the presence of dimethylurea, indicating that these two forces may also be considered during the dimethylurea-induced denaturation of proteins.[54,56] Furthermore, Figure 3 shows that the enthalpy change with the addition of 1,3-DMU is close to that with the addition of 1.1-DMU during the first heating process, presumably due to the similar van der Waals interaction with isopropyl groups. However, 1,3-DMU may have a weaker ability to form hydrogen bonds with C═O groups because of the steric hindance of methyl groups, so both the increasing rate of enthalpy change and the maximum enthalpy change of PNIPAM solution with the addition of 1,3-DMU is smaller than that with the addition of 1,1-DMU for the same concentration.

Figure 4(a) shows the influence of number of heating-cooling DSC cycles on the $T_{t,heating}$ and $T_{t,cooling}$ of aqueous solution of PNIPAM with no 1,1-DMU. $T_{t,heating}$ and $T_{t,cooling}$ are independent of the number of the DSC cycles, indicating a reversible phase transition. The difference between $T_{t,heating}$ and $T_{t,cooling}$ is presumably because of the intra and/or interchain hydrogen-bonding interactions of PNIPAM chains formed in the aggregated state. With the addition of 1,1-DMU, the $T_{t,heating}$ during the first heating process increases from 32.26 °C to 34.23 °C and 35.83 °C with concentrations of 1.0 M and 2.0 M, as shown in Figure 4(b,c). The substitution of the hydrogen atom by methyl group (—$CH_3$) makes 1,1-DMU an amphiphilic compound. The accumulation of 1,1-DMU at the aqueous/polymer interface make the surface tension decrease, then make PNIPAM chains more soluble in the water and increase the transition temperature of PNIPAM solution.[69–71] In other words, the presence of 1,1-DMU may weak the hydrophobic interactions between isopropyl groups of PNIPAM chains, which is similar to the phenomena observed in the protein denaturation by Poklar.[54] Moreover, with a certain concentration of 1,1-DMU, as shown in Figure

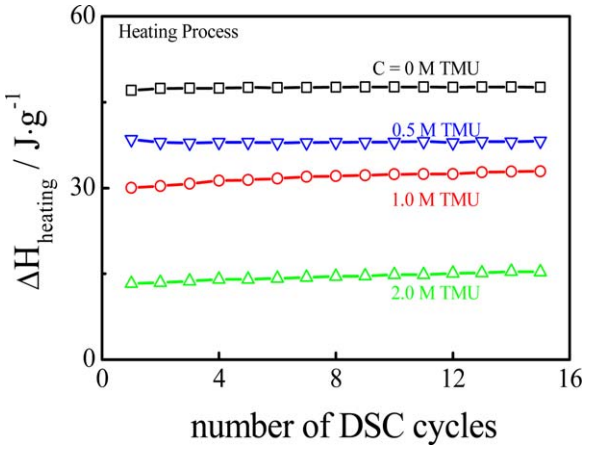


**FIGURE 5** Influence of the number of heating-cooling DSC cycles on enthalpy change ($\Delta H_{heating}$) in the phase transition of aqueous solutions of PNIPAM with different amounts of TMU, where the heating rate was 1.00 °C/min and the concentration of PNIPAM was kept at 1.0 mg/mL. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

4(b) or (c), the $T_{t,heating}$ decreases with the increasing of the number of heating-cooling DSC cycles, indicating more 1,1-DMU molecules bind to amide groups of PNIPAM by hydrogen bonds in a bivalent manner and facilitate the hydrophobic collapse of PNIPAM and suggesting that hydrogen-bonding interactions and van der Waals interactions between 1,1-DMU and PNIPAM have opposite influence on the phase transition. Moreover, the difference between $T_{t,heating}$ and $T_{t,cooling}$ become smaller with the increasing of 1,1-DMU and the number of DSC cycles, presumably due to the decrease in the number of intra and/or interchain hydrogen bonds formed between PNIPAM repeating units during the aggregated state in the presence of 1,1-DMU. The effect of the number of heating-cooling DSC cycles on the $T_{t,heating}$ and $T_{t,cooling}$ of PNIPAM aqueous solution with 1,3-DMU is similar to that of 1,1-DMU (not shown).

Figure 5 shows that the enthalpy change of PNIPAM solution decreases with the increasing of the concentration of tetramethylurea (TMU). The $\Delta H_{heating}$ of the PNIPAM solution with a concentration of 0.5 M 1,1-DMU was 43.0 J/g and it became 38.5 J/g with the same concentration of TMU, suggesting a stronger interaction between isopropyl groups and TMU. More important, for a certain concentration of TMU, $\Delta H_{heating}$ remains nearly a constant with the number of heating-cooling DSC cycles, which indicates that TMU may not form hydrogen bonds with PNIPAM. From the structure of TMU, as shown in the Figure 1, we know that TMU cannot serve as a donor to form hydrogen bonds with C═O groups of PNIPAM. Note that Ahmed et al. stated that in the collapsed state of PNIPAM one of the two acceptor hydrogen bonds between C═O group and water is lost and the donor hydrogen bonds of the N—H group and water remains unperturbed, indicating that the hydrogen bonds between the N—H group and water are strong.[35] Gao et al. also mentioned that N—H groups of protein backbones are dominantly hydrogen-bonded with water in the presence of TMU although TMU can serve as proton acceptor for the N—H group.[72] The studies reported by Kumaran and Ramamurthy reveal that TMU does not form hydrogen bonds with the N—H groups of bovine serum albumin (BSA) by use of the fluorescence spectroscopy.[73] Therefore, the hydrogen bond interaction between TMU and PNIPAM has minor influence on the phase transition of aqueous solutions of PNIPAM. On the other hand, hydrophobic interactions are believed to make a major contribution to the conformational stability of globular proteins in aqueous solutions.[74,75] Kumar et al. studied the effect of urea and alkylureas on the stability of the M80-containing Ω-loop of horse cytochrome c and found that TMU can denature the protein more efficiently than urea by weakening the hydrophobic interactions of protein.[76] Thus, our results also show that in the presence of TMU, the van der Waals interaction between TMU and isopropyl groups of PNIPAM and the weakening of hydrophobic interactions between isopropyl groups play a dominant role in the effect of TMU on the phase transition of PNIPAM. Here we suggest that only the addition of osmolytes which serve as hydrogen bond donors can make the enthalpy change of aqueous solutions of PNIPAM increase with the number of heating-cooling DSC cycles. To prove this suggestion, we have further studied the influence of *n*-butanol and *n*-butylamine on the phase transition of aqueous solutions of PNIPAM.

Figure 6 clearly shows that with the addition of osmolytes with N—H groups, the enthalpy change of PNIPAM solution increases with the number of DSC cycles and levels off after several DSC cycles. With the addition of *n*-butanol, the enthalpy change during the first heating process already increases to 85.8 J/g and remains nearly a constant with the number of DSC cycles. Moreover, Figure 6 also shows that the enthalpy change increases faster for osmolytes with higher

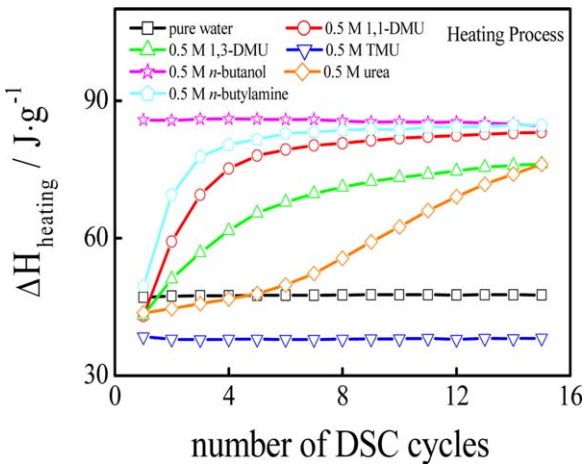


**FIGURE 6** The influence of the number of heating-cooling DSC cycles on enthalpy change ($\Delta H_{heating}$) of aqueous solutions of PNIPAM with different osmolytes during the heating processes. The concentration of PNIPAM ($C_{PNIPAM}$) and concentration of osmolyte ($C_{osmolyte}$) were kept at 1.0 mg/mL and 0.5 M, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

hydrophobicity, indicating the cooperative effect of the hydrophobicity, i.e. the local concentration of osmolytes around PNIPAM increases with hydrophobicity and it is easier for them to form hydrogen bonds with C═O groups of PNIPAM. Thus, our results show that both hydrogen-bonding interactions and van der Waals interactions are important for the influence of osmolytes on the phase transition of aqueous solutions of PNIPAM.

## CONCLUSIONS

In conclusion, the effects of methylated urea on the phase transition of aqueous solutions of PNIPAM have been systematically studied by DSC. With the addition of osmolytes with hydrogen bond donors, the enthalpy change increases with the number of DSC cycles, indicating that the formation of hydrogen bonds between osmolytes and C═O groups of PNIPAM is the main reason for the increase in enthalpy change. Moreover, with the addition of TMU without N—H group, the enthalpy change decreases with the increasing of the concentration and remains nearly a constant with the number of DSC cycles, presumably due to the van der Waals interactions and the replacement of water molecules around the hydrophobic isopropyl groups. Our results show that both hydrogen bonding interaction and van der Waals are important for the effect of osmolytes on the phase transition of aqueous solutions of PNIPAM.

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