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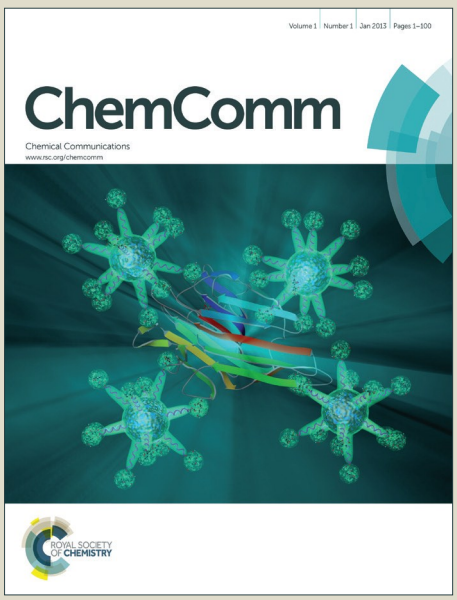

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Disclosing the nature of thermo-responsiveness of poly(*N*-isopropyl acrylamide)-based polymeric micelles: aggregation or fusion?

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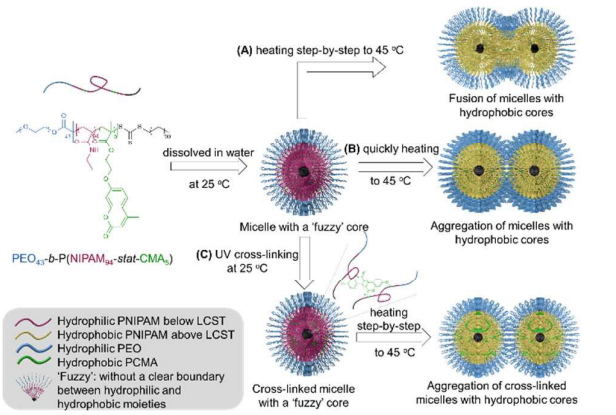
The apparent size increase of poly(*N*-isopropyl acrylamide) (PNIPAM)-based polymeric micelles upon heating was usually ascribed to their volume growth or aggregation in aqueous solution. Herein we designed a photo-cross-linkable PNIPAM-based copolymer and proposed another thermo-responsive behaviour—fusion, which is disclosed by transmission electron microscopy (TEM) after *in situ* fixing morphologies at desired temperatures.

Thermally responsive polymeric nanostructures such as micelles, vesicles, and hydrogels, *etc*. have attracted enormous attention over past decades.[1-10] Among them, PNIPAM-based polymeric micelles were intensively studied. This is because PNIPAM and its derivatives have a sharp transition through lower critical solution temperature (LCST) and versatility in terms of copolymer architecture variation, and may be applied in a wide range of fields such as controlled drug delivery.[11-13]

However, thermally responsive polymeric nanostructures still confront some important problems. One of which is the controversial observations upon heating similar polymeric nanoparticles through their transition temperatures.[14] In most cases, the nature of thermal responsiveness has been regarded as the volume change,[15] aggregation and morphological transition.[16-18] However, there are still questions about the volume change: (1) what is the origin of the mass when the volume of the micelle significantly increases upon heating? (2) How is the mass squeezed during the volume shrinkage process upon cooling?

Two answer the above questions, it is necessary to disclose the nature of thermal responsiveness of polymeric nanostructures. Various techniques were employed, such as nuclear magnetic resonance (NMR),[19, 20] Fourier transform infrared spectroscopy (FTIR),[21-23] dynamic light scattering (DLS),[24] *etc*. Also, some computational and simulation work were performed to reveal the thermodynamic behaviour.[25, 26] However, these techniques only provide with indirect evidence. Usually, cryogenic transmission electron microscopy (cryo-TEM) is a powerful tool for visualizing the original morphologies of most soft nanostructures in solution.[15] Unfortunately, one may worry about whether the cryo-TEM provides direct evidence of the original morphology of thermo-responsive polymeric nanostructures in solution because the sample preparation process involves significant temperature drop which may eventually induce a morphological change.

Therefore, conventional TEM where no obvious temperature variation during sample preparation may be a better choice than cryo-TEM for investigating the real morphology of thermally responsive soft nanostructures. The key is still how to keep their original morphology in solution.[27-33] Cross-linking the nanostructure in solution is a good choice to keep the original morphology. For example, Chen *et al.* prepared a series of vesicles with subtle nanostructures visualized by TEM upon *in situ* sol-gel reactions in the vesicle membranes.[30, 31] Zhao *et al.* introduced photo-cross-linking moieties in block copolymers.[27, 29]



Scheme 1 Schematic illustration of the fusion and aggregation structures upon heating polymeric micelles. All samples were photo-cross-linked before TEM study. Blue: PEO; Yellow: hydrophobic PNIPAM; Green: PCMA; Purple: 'fuzzy' core of the micelle composed of hydrophilic PNIPAM and hydrophobic PCMA.

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In this study, we reveal another thermally induced size increase (fusion of micelles) by TEM upon *in situ* fixing their structure in solution. As shown in Scheme 1, a thermally responsive block copolymer, poly(ethylene oxide)-*block*-poly[*N*-isopropyl acrylamide-*stat*-7-(2-methacryloyloxyethoxy)-4-methylcoumarin] [(PEO-*b*-P(NIPAM-*stat*-CMA)], was directly dissolved in water to form the micelle with a 'fuzzy' core at room temperature. Hydrophilic PEO chains form the coronas of the micelle. PNIPAM is thermally responsive while PCMA is photo-cross-linkable by UV radiation within minutes.[34] The P(NIPAM-*stat*-CMA) block forms the 'fuzzy' core, which indicates there is no clear boundary between the hydrophobic and hydrophilic moieties.[35, 36] Upon step-by-step heating the aqueous solution above the LCST of PNIPAM (*e.g.*, 45 °C), the *un-cross-linked* micelles will end up in a fusional mode (route A). When the equilibrating time is shorter (route B) or the chain mobility in the micelle core becomes less (route C), only the aggregation of micelles occurs during the heating process.

Just to be clear, the cross-linking procedure of coumarin moieties was utilized in this research with two different purposes at two different temperatures. Cross-linking at 45 °C is simply for fixing the morphology of nanostructures for better TEM study. Similarly, at 25 °C, the UV cross-linking also fixes the original micellar structure in solution for comparing the thermally responsive behaviour of these cross-linked micelles with un-cross-linked micelles.

The PEO-*b*-P(NIPAM-*stat*-CMA) block copolymer was synthesized through a typical reversible addition-fragmentation chain transfer (RAFT) polymerization (see Scheme S1 in the electronic supplementary information, ESI†). The chemical structures of as-prepared PEO-based macro chain transfer agent (macro-CTA) trithiolcarbonate, the monomer 7-(2-methacryloyloxyethoxy)-4-methylcoumarin (CMA), and the obtained PEO_{43}-*b*-P($NIPAM_{94}$-*stat*-CMA_5) block copolymer were confirmed by ^{1}H NMR spectra in $CDCl_3$ (Fig. S1 - Fig. S3, ESI†).

At 25 °C, while PNIPAM is still water-soluble, the block copolymer can be directly dissolved into deionized (DI) water at 1.0 mg/mL to form micelles of 38.9 nm (see Fig. S4, ESI†) as a result of the hydrophobic coumarin moieties and the end group effect.[37, 38] Therefore, the thermo-responsive behaviour of the PEO_{43}-*b*-P($NIPAM_{94}$-*stat*-CMA_5) block copolymer in water was first evaluated by ^{1}H NMR.

Fig. 1 showed the ^{1}H NMR spectra in D_2O at different temperatures. The signals labelled "b" and "c" are associated with the methylene and methyl protons of the thermal responsive PNIPAM. From 25 to 45 °C, the peak intensity of PNIPAM decreased dramatically, indicating reduced mobility and solvation degree. Then, the thermal behaviour of the *un-cross-linked* micelle solution was characterized by DLS. The micelles solution was heated step-by-step from 25 °C to 45 °C with 20 min of equilibrating time at every 2 °C interval. This prolonged equilibrating time made it possible for the thermally sensitive PNIPAM chains to reach the equilibrium state. Fig. 2 revealed the size variation during the heating process. The hydrodynamic diameter (D_h) increased to 74.0 nm with a final PDI of 0.048 at 45 °C. During this process, the size change occurred mainly from 29 °C to 31 °C, corresponding to the LCST of PNIPAM around 32 °C. The corresponding size distributions and the scattered intensity were presented in Fig. S5-Fig. S6, ESI†. A heating-cooling cycle was repeated for 3 times (Fig S7, ESI†), confirming a reversible and repeatable process.

To deeply understand the phenomenon in the DLS studies, conventional TEM was employed to investigate the morphology by *in situ* photo-cross-linking in solution. After step-by-step heating to 45 °C, the micelle solution was exposed to UV light for 5 min at 45 °C to fix the morphology of micelles for TEM characterization. The related photodimerization process was presented in Scheme S2, ESI†. The cross-linking degree was calculated to be 53.6 % by the variation of the characteristic peak of coumarin at 320 nm *via* UV-vis spectroscopy. To prepare TEM samples, the pre-heated TEM copper grids loaded with the micelle samples were placed in a drying oven at 45 °C to minimize the influence of temperature change on the morphology of thermally responsive micelles. TEM images revealed that upon heating to 45 °C, the micelles formed fusional structure consisting of two or several individual

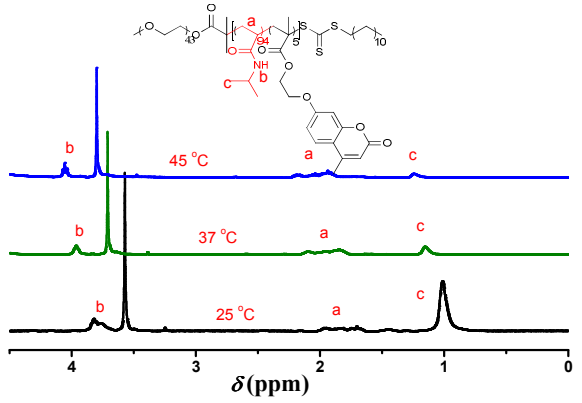

Fig. 1 ^{1}H NMR spectra of PEO_{43}-*b*-P($NIPAM_{94}$-*stat*-CMA_5) micelles in D_2O at 25, 37 and 45 °C. $C_{polymer}$ = 10.0 mg/mL. Samples were equilibrated for 30 min before characterization. The signals of PNIPAM weakened as temperature increase. The migration of chemical shift to lower fields was caused by the variation of resonance signal of inner standard at higher temperature.

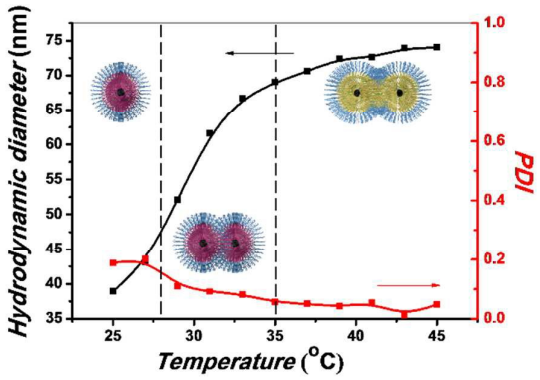

Fig. 2 Size increase and corresponding interpretation of un-cross-linked polymer micelles upon step-by-step heating to 45 °C. $C_{polymer}$ = 1.0 mg/mL. Blue: PEO; Yellow: hydrophobic PNIPAM; Green: PCMA; Purple: 'fuzzy' core of the micelle composed of hydrophilic PNIPAM and hydrophobic PCMA.

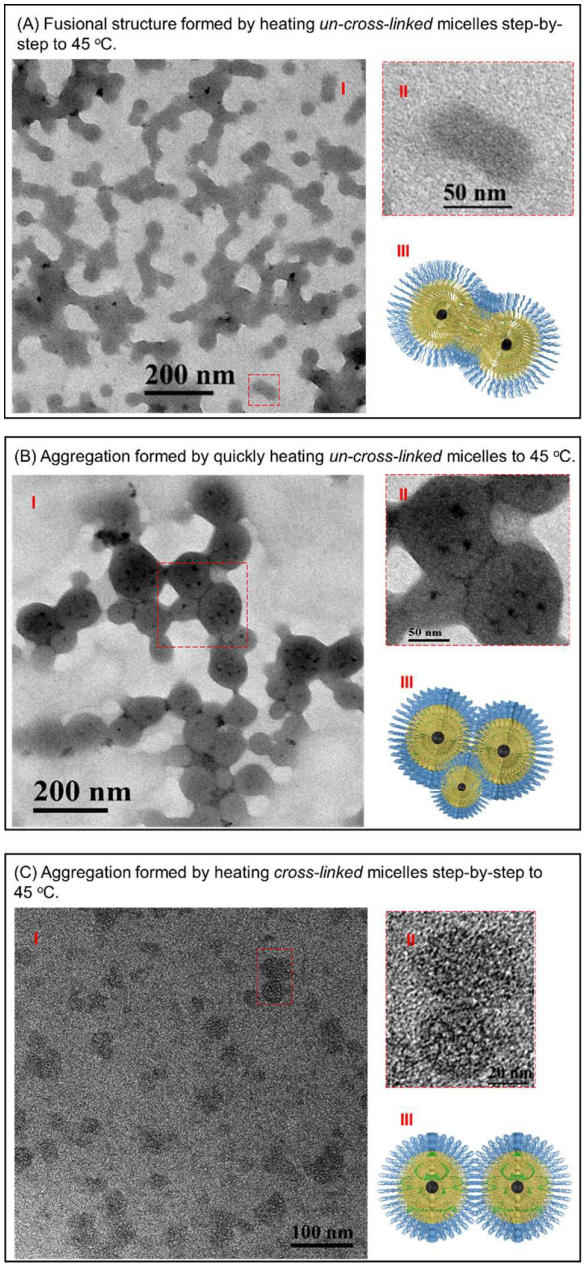

Fig. 3 TEM images of thermo-responsive polymeric micelles with different structures upon heating. Blue: PEO; Yellow: hydrophobic PNIPAM; Green: PCMA.

micelles without clear boundaries (Fig. 3A). Also, some unpaired micelles (*ca.* 40.8 ± 4.8 nm) co-existed with the fusional structure (Fig. S8, ESI†). This phenomenon is unique in the PNIPAM-based micelles and it is interpreted that the prolonged equilibrating time below LCST accelerated the movement and collision of individual micelles, thus resulting in more chance to encounter, tangle and cross mutually. When the temperature was above the LCST, tangled hydrophilic chains became water-insoluble so as to create a hydrophobic environment nearby. Under these circumstances, solution concentration, equilibrating time and chain mobility may be key points for the formation of fusional structures.

To testify the above hypothesis, a series of micelles solutions with different initial concentrations were applied with the same step-by-step heating process. The corresponding size increases were listed in Fig. S9, ESI†. When the concentration was as low as 0.1 mg/mL, the micelles solution merely had a size change (7.0 %), indicating a concentration-dependent thermal behaviour. The relatively low concentration diminished the aggregation and fusion.

On the other hand, PNIPAM is known to have a fast and fully reversible coil-to-globule transition at its LCST, which lasts as short as hundreds of seconds.[18] While in our case, the prolonged equilibrating time is critical to the fusional structure. The micelle solution (1.0 mg/mL) was then directly immersed in water bath at 45 °C, which was far above the LCST of PNIPAM. The original light blue solution turned into white immediately. After equilibrating for 20 min as well, the white solution was first studied by DLS. The results at different initial concentrations were listed in Table 1.

As shown in Table 1, after quickly elevating the temperature to 45 °C, the diameter of polymer micelles had a higher proportion of increment. For example, at 1.0 mg/mL, the D_h reached 139.0 nm. In contrast, the D_h is only 74.0 nm in the step-by-step process. This phenomenon is also highly concentration-dependent because a higher initial concentration leads to a larger final size. TEM study (Fig. 3B and Fig. S10, ESI†) revealed a large scale of aggregation and clear boundaries between single micelles. The slowly tangling and crossing process will no longer exist so that the fusional process was hindered.

Although cross-linking techniques provide us with direct insight into the morphology change and better understanding of thermo-responsive mechanisms of nanostructures, the cross-linking techniques themselves may inevitably have certain influences on the thermal behaviour of polymers and their self-assembled nanostructures, regardless of different cross-linking procedures. Therefore, we introduced the cross-linking technique at the beginning at 25 °C to compare different thermal behaviours between the un-cross-linked micelles and cross-linked micelles caused by different chain mobilities.

The directly dissolved micelle solution was placed under UV spot light to cross-link for 3 min. The D_h after photo-cross-linking was 32.9 nm (Fig. S11, ESI†) and the cross-linking degree of the micelle was 42.5 % (up to 56.3 % in 10 min, see Fig. S12 in ESI†). The volume decrease introduced by inter/intra chain dimerization of coumarin in the micelle core was around 39.5 %. On this occasion, the internal PNIPAM micelle core became tighter and the chain movement was, to some extent, restricted. Similarly, a step-by-step heating process was applied to the cross-linked micelles solution. Fig. 4 shows the size increase process upon heating. Started from 29 °C, the volume phase transition process lasted to 33 °C and reached a final D_h of 64.5 nm at 45 °C. Above 35 °C, the diameter decreased continuously, corresponding to the collapsing and shrinkage of PNIPAM chains inside the PEO

coronas. The reversibility of this process was also conducted by 3 cycles (Fig. S13, ESI†). The differences between the final sizes may be attributed to the

Table 1 Size increase after directly immersing the un-cross-linked micelles in a water bath to quickly elevate the temperature to 45 °C [a]

C_{ini} (mg/mL)	25 °C		45 °C		Increase in Dia. (%)
	Dia. (nm)	PDI	Dia. (nm)	PDI	
1.0	38.9	0.188	139.0	0.083	239.0
2.0	38.4	0.129	170.2	0.098	343.2
3.0	37.1	0.109	280.5	0.403	650.0
4.0	39.0	0.156	407.1	0.475	943.8

[a] Samples were equilibrated for 20 min.

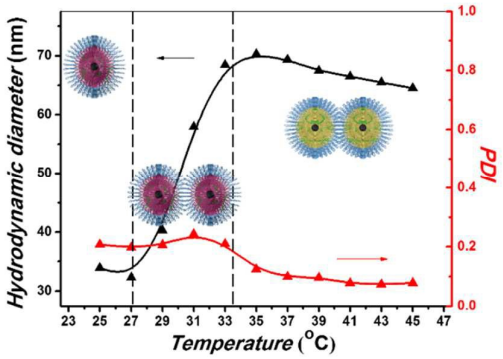

Fig. 4 Size increase and corresponding interpretation of cross-linked polymer micelles upon step-by-step heating to 45 °C. $C_{polymer}$ = 1.0 mg/mL. Blue: PEO; Yellow: PNIPAM; Green: PCMA; Purple: 'fuzzy' core of the micelle composed of hydrophilic PNIPAM and hydrophobic PCMA.

cross-linking procedure, which restricted the motion of block copolymer chains. To further uncover the effects of the UV cross-linking process and reveal the differences between the morphological changes, TEM studies were carried out as well. When the micelles were firstly photo-cross-linked at 25 °C and then heated to 45 °C, no obvious fusion process was observed by TEM (Fig. 3C). The higher temperature only provides the cross-linked micelles with more chance to aggregate but the cross-linked structure limited the chain movement of PNIPAM. Therefore, only the outer interior of PEO corona is mixed (Scheme 1 and Fig. 3C).In summary, a photo-cross-linkable and thermo-responsive diblock copolymer was synthesized to disclose the nature of PNIPAM-based thermal responsiveness of micelles. The facile *in situ* photo-cross-linking at desired temperatures facilitates conventional TEM studies on the thermally responsive nanostructures, revealing that the fusion of micelles is a dominant behaviour when heating the PNIPAM-based micelles step-by-step at higher concentrations. In contrast, the aggregation occurs when quickly heating the micelles, or cross-linking the micelles before heating. These observations provide us with more insights when designing thermally responsive nano-vehicles, especially for popular drug carriers such as polymer micelles.

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