High-Throughput Studies of the Effects of Polymer Structure and Solution Components on the Phase Separation of Thermoresponsive Polymers

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ABSTRACT: This paper describes a high-throughput method for characterization of the temperature-dependent folding of poly(*N*-isopropylacrylamide), PNIPAM, using dark field microscopy to measure this thermoresponsive polymer's lower critical solution temperature (LCST). The effect of ionic solution components (halide and alkali metal ions) on the polymer precipitation temperature follows the Hofmeister series whereas solution isotopic effects using D_2O give rise to a roughly linear increase in the LCST with the mole fraction of D_2O added. The polymer structure itself was also varied through the synthesis of *N*-alkylacrylamide copolymers. It was found that replacement of the isopropyl *N*-alkyl pendant groups of PNIPAM with varying amounts of *N*-*n*-propyl groups results in a monotonic decrease in the LCST. Repeated analyses of the same sample and of separately prepared samples show that this type of analysis is quite precise and that investigations of subtle effects of polymer microstructure and solvation are feasible even when the difference in LCST temperatures is <1 °C. Such effects may be difficult to study by other methods.

Introduction

Thermoresponsive polymers such as poly(*N*-alkylacrylamide)s undergo folding that results in a dramatic decrease in their solubility above a lower critical solution temperature (LCST).[1,2] The phenomena seen with these synthetic polymers mirror the cold denaturation processes of peptides and proteins.[3,4] Although there exists a level of uncertainty about the exact mechanism of polymer folding in both the synthetic and biological cases,[5,6] the release of hydrophobic hydration water is believed to play a crucial role in the process. At low temperature, water molecules bind to hydrophobic residues of the polymer in a low-entropy configuration. At temperatures above the LCST, the ordered water molecules are released into the bulk solution, increasing the system's entropy and thereby allowing the endothermic desorption of the water to be coupled into a net exergonic process.

Current methods for studying LCST phenomena in thermoresponsive polymers include light scattering, UV−vis absorption measurements, differential scanning calorimetry, and infrared spectroscopy.[7−12] Most of these techniques, however, suffer from low-throughput capabilities and require relatively large amounts of polymer for analysis. These factors limit the ability to probe the myriad of subtle solvent and polymer structure effects that presumably would have to be part of any complete study designed to elucidate the molecular details of the folding mechanism. This is unfortunate because such information obtained for structurally diverse synthetic polymers like poly(*N*-alkylacrylamide) copolymers might be more broadly applicable to the understanding of related processes such as the cold denaturation of proteins. To help remedy this situation, we have designed and built a linear temperature gradient microfluidic device in combination with a dark field optical microscopy setup for obtaining all temperature readings in an LCST measurement simultaneously as a function of position rather than sequentially as a function of time.[13] The process works by exploiting the fact that when a thermoresponsive polymer folds and aggregates above its LCST, the solution becomes cloudy, leading to a readout of the phase transition temperature by the position on the temperature gradient of the abrupt change in the amount of light scattered. The need for no more than 2 μL of total solution in order to make a measurement is an especially advantageous feature of this method. Moreover, as shown below, we can couple the use of this device with pool-split syntheses of libraries of poly(*N*-alkylacrylamide)s.[14] Using such syntheses, we can compare poly(*N*-alkylacrylamide) copolymers whose *N*-alkyl substituent is varied but whose degree of polymerization and polydispersity are identical. Such syntheses provide us with a way to probe very similar polymers without facing ambiguities due to synthesis variabilities inherent in typical radical addition polymerizations.

Herein the effects of solvent components and polymer structure variance on the folding of PNIPAM and related polymers are explored using our newly developed techniques. First, the ability of halide and alkali ions to affect the LCST of PNIPAM was systematically studied, and the results are compared with the Hofmeister series. Second, solution isotopic effects on the LCST were measured with various mole ratios of D_2O:H_2O. The results indicate a roughly linear relationship between the mole fraction of D_2O and the LCST of a poly(*N*-alkylacrylamide).[15] The precision of this technique for LCST determination was evaluated using repetitive analyses of the same polymer sample and of separately prepared but otherwise identical polymers. Finally, we have used a series of isomeric poly(*N*-alkylacrylamide)s to show the subtle effects of polymer structures on the macromolecular folding processes. These studies used

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LCST measurements of a series of poly(*N*-*n*-propylacrylamide-*co*-*N*-isopropylacrylamide) samples of identical molecular weight that were isomeric by virtue of having 1-propyl or 2-propyl substituents on the amide nitrogens of the polymer. A linear relationship between the mole fraction of the more hydrophobic poly(*N*-*n*-propylacrylamide) and the LCST temperature was found with the more hydrophilic poly(*N*-isopropylacrylamide) having a 5.4 °C higher LCST.

Experimental Section

Light Scattering. Light scattering experiments were performed with a Brookhaven Instruments BI-200SM goniometer using a Melles Griot HeNe laser. Analysis of M_w values from light scattering experiments were carried out using Brookhaven Instruments Zimm Plot software.

Poly(*N*-acryloxysuccinimide) (PNASI). This polymer was prepared following a literature procedure.[16] A solution of *N*-acryloxysuccinimide (15.00 g, 89 mmol) in 600 mL of dry benzene was degassed and heated to 60 °C under a positive pressure of N_2. A degassed solution of 2,2′-azobis(isobutyronitrile) (90 mg, 0.55 mmol) in 20 mL of dry benzene was then added to the monomer solution via a forced siphon. After stirring for 36 h the heat was removed, and the mixture was allowed to cool to room temperature under a positive pressure of N_2. The white precipitate was filtered and washed with fresh dry benzene, freshly distilled THF, and finally anhydrous ether. The material was then dried for 18 h under vacuum. The dry white powder so obtained was then triturated under N_2 with 400 mL of freshly distilled THF for 3 days. The fine white powder was then filtered, washed with anhydrous ether, and dried under vacuum to yield 14.17 g (95%) of the pure polymer: ^{1}H NMR ($CDCl_3$) δ 3.15 (bs, 1H), 2.80 (bs, 4H), 2.10 (bs, 2H).

Synthesis of Homo- and Copolymers of *N*-Alkylacrylamides 1, 2, 4, 5, or 6 from Poly(*N*-acryloxysuccinimide) 3. Homopolymers were prepared using a single amine (in these cases either 1-aminopropane or 2-aminopropane) using a 5-fold excess of the amine during aminolysis of the active ester PNASI.[14,16] In the case of copolymers, the similar 5-fold molar excess of amines was used, but the molar ratio of the two amines was varied. Because of the differences in nucleophilicity of the primary vs secondary amine ($NH_2CH_2CH_2CH_3$ vs $CH_3CH(NH_2)CH_3$), the ratio of *N*-*n*-propyl/*N*-isopropyl groups incorporated into the product copolymer was not the same as the ratio of the amines added. In the specific case of copolymers **4**−**6**, an initial 2:1 mole:mole ratio of $[n\text{-PrNH}_2]_0/[i\text{-PrNH}_2]_0$ produced a 6:1 ratio of *n*-Pr/*i*-Pr groups in the PNNPAM-*c*-PNIPAM product; a 1:1.5 mole:mole ratio of $[n\text{-PrNH}_2]_0/[i\text{-PrNH}_2]_0$ produced a 2:1 ratio of *n*-Pr/*i*-Pr groups in the PNNPAM-*c*-PNIPAM product; and a 1:4 mole:mole ratio of $[n\text{-PrNH}_2]_0/[i\text{-PrNH}_2]_0$ produced a 1:1 ratio of *n*-Pr/*i*-Pr groups in the PNNPAM-*c*-PNIPAM product. This result is consistent with the idea that the secondary amine is less nucleophilic than the primary amine.

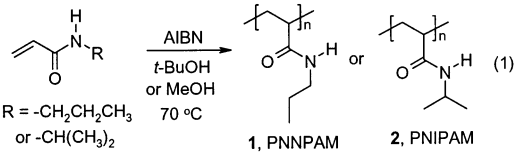
The synthesis of poly(*N*-*n*-propylacrylamide-*c*-*N*-isopropylacrylamide) with a 6:1 mole ratio of *n*-propyl:isopropyl groups is representative of the procedure used to synthesize all polymers. A solution of isopropylamine (0.4 mL, 5 mmol) and *n*-propylamine (0.8 mL, 10 mmol) in 5 mL of dry DMF was added to a solution of poly(*N*-acryloxysuccinimide) (500 mg, 3 mequiv NASI) in 10 mL of dry DMF. Within 10 s a large amount of precipitate formed, and the slurry was stoppered and stirred for 18 h. The mixture was filtered, and the supernatant was precipitated into 700 mL of ether. After stirring for a few minutes, the fine white precipitate, which initially forms aggregates, evolved to large particles. After allowing this suspension to settle for 10 min, the solution was decanted and 500 mL of fresh ether was added. After stirring the resulting suspension for 10 min, the crude product was isolated by filtration and dried. The crude product was then redissolved in 10 mL of THF and reprecipitated using 125 mL of hexanes. This process was repeated three times, yielding 220 mg (66%) of the pure polymer **4**. ^{1}H NMR ($CDCl_3$): δ 6.80 (bs, 7H), 4.00 (bs, 1H), 3.15 (bs, 12H), 2.60−1.25 (bm, 37H), 1.80 (bs, 6H), 0.90 (bs, 18H).

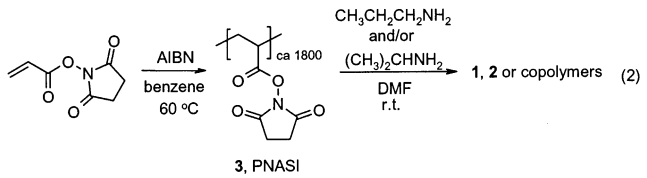
Fabrication of Temperature Gradient Device. Fabrication of the temperature gradient device has been previously decribed.[13,17,18] Briefly, two $^1/_8$ in. wide hollow square brass tubes (K&S Engineering, Chicago, IL) were laid in parallel and separated by 3 mm stints made of glass slides. The two brass tubes were then fixed into position using clamps and placed under a dark-field condenser in an inverted microscope (Nikon, Eclipse TE 2000-U). To establish the temperature gradient, hot and cold antifreeze solutions were circulated through individual brass tubes using standard water bath circulators (Fisher Scientific, Pittsburgh, PA). A coverglass was laid on top of two brass tubes by applying vacuum grease, and the linear tempearture gradient was confirmed by taking temperature measurements at various points perpendicular to the copper tubes with a type E thermocouple.

LCST Measurement of Thermoresponsive Polymers. To measure the LCSTs of thermoresponsive polymers, rectangular borosilicate capillary tubes (Vitrocom, Mountain Lakes, NJ) with a high aspect ratio (100 μm × 1 mm × 2 cm) were used as sample containers. Polymer solutions were introduced into the tubes through capillary action and were subsequently sealed with vacuum grease before being laid parallel to the temperature gradient. Polymer clouding was imaged through a dark-field condenser using a CCD camera. Two standard polymer solutions, PNIPAM 10 mg/mL in water (LCST 30.2 °C) and PNIPAM 10 mg/mL in 0.7 M KCl (LCST 26.0 °C), were used as internal temperature standards to determine the temperature gradient for every experiment. Clouding curves of polymers were plotted from line scans of scattering intensity drawn along the temperature gradient where pixel positions were converted to temperatures according to the gradient obtained in the reference samples (cf. Figure 2). The LCST was defined as the onset point in the clouding curve.

Results and Discussion

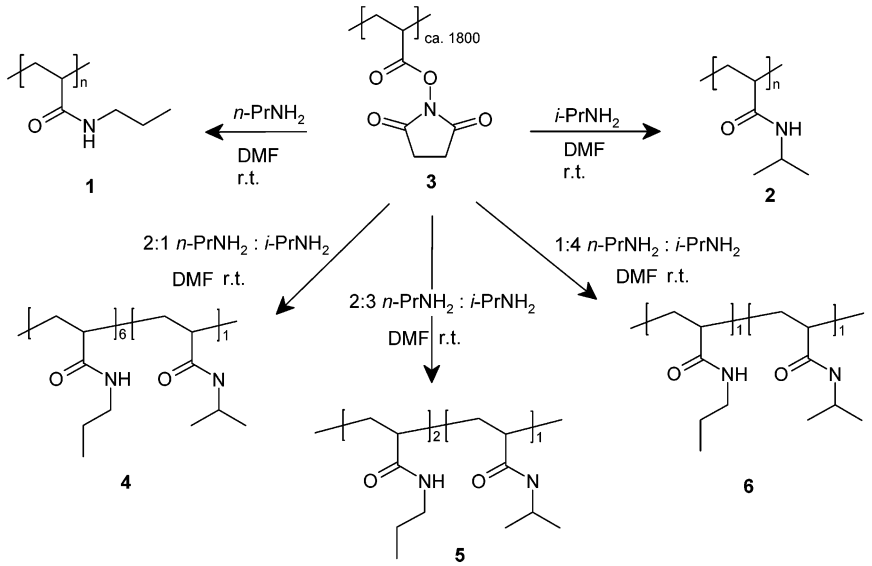
Polymer Synthesis and Characterization. The poly(*N*-alkylacrylamide)s used in this study were prepared either by polymerization of the monomers (eq 1) or by derivatizing a polymer containing an activated ester (eq 2). Homopolymers of poly(*N*-*n*-propylacrylamide) (PNNPAM) and poly(*N*-isopropylacrylamide)





(PNIPAM) were prepared by both routes. Copolymers of poly(*N*-*n*-propylacrylamide) and poly(*N*-isopropylacrylamide) (PNNPAM-*c*-PNIPAM) were all prepared by aminolysis of the active ester polymer poly(*N*-acryloxysuccinimide) (PNASI) (eq 2). The steric bulk of isopropylamine diminished its rate of substitution for the active ester by a factor of 3 relative to that of the straight-chain *n*-propylamine; therefore, the ratio of amines incorporated into the polymer was not the same as the ratio of amines added. This did not pose a problem since the ratio of amides on the copolymers could be determined easily by ^{1}H NMR spectroscopy.

Scheme 1. Synthesis of an Isomeric Library of Poly(*N*-propylacrylamide)s Containing *N*-*n*-Propyl, *N*-Isopropyl, or Mixtures of *N*-*n*-Propyl and *N*-Isopropyl Pendant Alkyl Groups



The advantage of using reaction 2 is shown in the synthesis in Scheme 1 where a library of structurally isomeric copolymers was prepared that differ only in their mole fraction of $-CH_2CH_2CH_3$ vs $-CH(CH_3)_2$ groups. In terms of this paper, the use of the common starting active ester polymer **3** has significant advantages and some possible disadvantages. The major advantages are that polymers derived from **3** using mixtures of isomeric amines have the same molecular weight, degree of polymerization, and polydispersity. The fact that the product copolymers had an LCST that was linearly dependent on the mole fraction of the different amide alkyl groups (vide infra) also suggests (although does not prove) that these products are random copolymers. This synthesis also offers advantages in that more structurally diverse libraries are also potentially available. There are, however, some potential problems with using **3** as a common precursor. Specifically, since we can measure the LCST with significant precision, variations of polymer composition from run to run due to adventitious hydrolysis of <1% of the active ester groups or due to incorporation of trace amounts of other groups from impurities in the amines can occur. The presence of <1% of an impurity cannot be detected by NMR spectroscopy and could lead to superficially similar samples with small differences in LCST values. For example, in one sample where only a small excess of $CH_3CH_2CH_2NH_2$ relative to **3** was used, the PNNPAM product had a higher LCST, an effect we attributed to the presence of some $-CO_2H$ groups (from hydrolysis of the NASI groups upon workup).[19,20] However, in our experience the differences seen for LCST values of the "same" poly(*N*-alkylacrylamide) derived from different samples of **3** using excess amine are no greater than the differences in LCST values for different samples of **2** prepared in separate conventional polymerizations.

The molecular weight of the PNIPAM used in this study and in our earlier report was determined by viscosimetric analysis using literature values of $K = 9.59 \times 10^{-3}$ and $\alpha = 0.65$.[21] After this paper, we found another report listing these values as a range of $K = (2.2-15) \times 10^{-3}$ (mean value being 5.8×10^{-3}) and $\alpha = 0.78 \pm 0.09$.[22] Different exponents of the Mark−Houwink−Sakurai equation in these two reports produce very disparate molecular weights. This has led us to analyze the PNASI-derived PNIPAM polymers molecular weight by static light scattering. Two independent preparations of PNIPAM made by substitution of the active esters of PNASI with isopropylamine were analyzed, and M_w values of 320 000 and 350 000 Da were obtained. These light scattering reports confirm that the PNASI-derived materials are indeed high molecular weight materials and that the M_w values of these polymers are comparable to the M_w values we originally measured by viscometry using the literature K and α values reported by Winnik.[21]

High-Throughput Temperature Gradient Measurements. Until now, LCST measurements have been made consecutively as a function of *time* using a temperature ramp on macroscopic samples. We adapted the linear temperature gradient apparatus (Figure 1) for combinatorial temperature measurements for use in LCST studies as a high throughput alternative to these existing procedures.[13,17,18] The use of a temperature gradient allows data to be collected as a function of *position* instead and makes it possible to obtain multiple

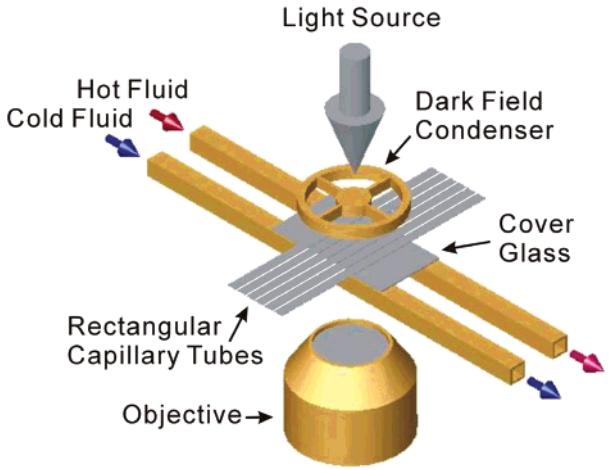


Figure 1. Schematic drawing of a temperature gradient device. Up to 10 rectangular borosilicate capillary tubes were put on the temperature gradient device without noticeable temperature discrepancy across the tubes. Each CCD image fits six capillary tubes simultaneously under a 2× objective.

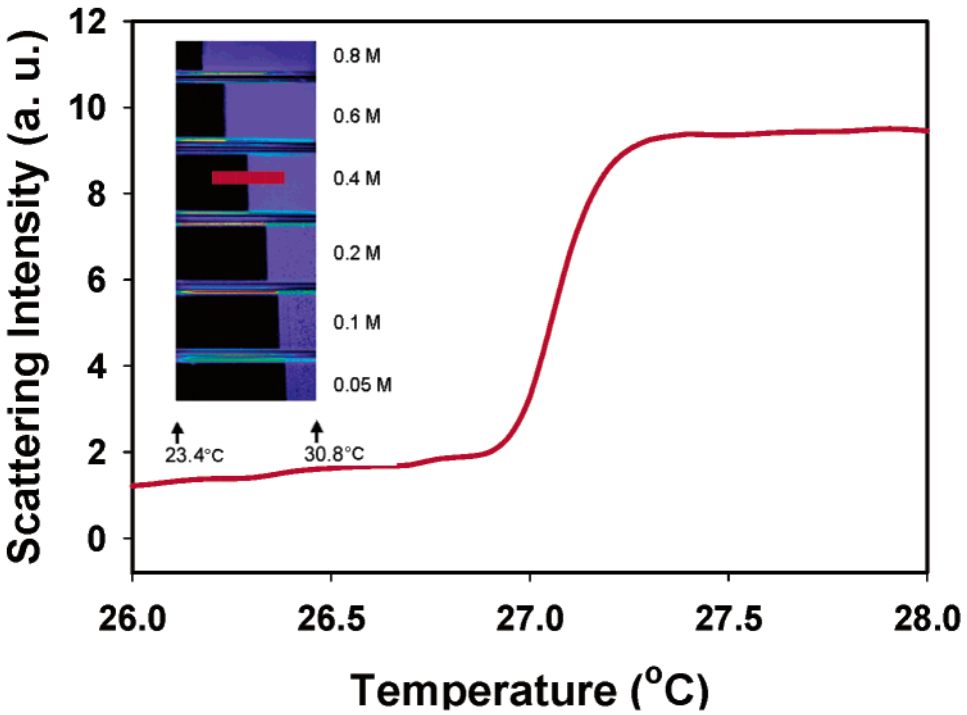


Figure 2. A typical clouding curve for PNIPAM 10 mg/mL in 0.4 M aqueous NaCl. The hot and cold extremes were calibrated with two standard polymer solutions and were 30.8 and 23.4 °C. This curve derives from the red portion of one sample in the inset. Inset: A CCD image of six samples inside square capillary tubes. Solutions of PNIPAM (10 mg/mL) were prepared using NaCl concentrations that ranged from 0.05 to 0.8 M. The precipitant is pseudo-colored blue for clarity.

temperature measurements for multiple samples in a single experiment. The fact that this procedure only requires minute amounts of sample means that the effects of many variables on a material's LCST can be studied more economically with libraries of similar substrates. These high-throughput techniques also provide us with the ability to make very precise relative measurements of the LCST and, hence, facilitate studies of subtle effects of polymer microstructure and environment on a substance's LCST.

In the actual apparatus (Figure 1), dark-field optics were used to image the scattering intensity of the folded and aggregated polymers as a function of position. Six rectangularly shaped borosilicate capillary tubes (100 μm × 1 mm × 2 cm) were placed under a ×2 objective parallel to the temperature gradient. The temperature values along the tubes were determined by measuring the lengthwise position in a CCD camera image. As a demonstration, the tubes were filled with aqueous solutions containing 10 mg/mL of PNIPAM in a series with varying NaCl concentrations. The amount of polymer required in each experiment was only 2 mg, and the 200 μL of solution prepared with this amount of polymer was sufficient to fill dozens of the capillaries described above whose volume was 2 μL each. The phase transition temperature was determined by the onset point of the clouding curve (Figure 2 and inset). As shown in Figure 2, all six positions can be samples of interest. However, in most other experiments we found it useful to place two polymer solutions with known LCSTs at positions 1 and 6. Such solutions served as internal standards and allowed the slope of the temperature gradient to be determined each time an assay was performed and facilitated more precise measurements of the LCST temperature.

Hofmeister Effect. Using the methods described above, LCST measurements of a series of PNIPAM solutions in water were made as a function of ion type and concentration. The results were compared with the known Hofmeister series for salting-in and salting-out effects.[23,24] Halide and alkali metal ions were used as ionic solution components. For each salt, eight solutions with different ion concentrations were prepared. The effects of these solution components on the LCSTs of PNIPAM are plotted as a function of both the particular halide anion (Figure 3) as well as the alkali cation (Figure 4). As can be seen from Figure 3, the ability of a given halide to lower the LCST decreased with its size. Since fluoride has the strongest tendency to form hydrogen bonds with water molecules, less water was available to solvate the macromolecules. According to traditional Hofmeister analysis, this should effectively decrease the number of solvent molecules available to solvate the polymer, thereby making it easier for

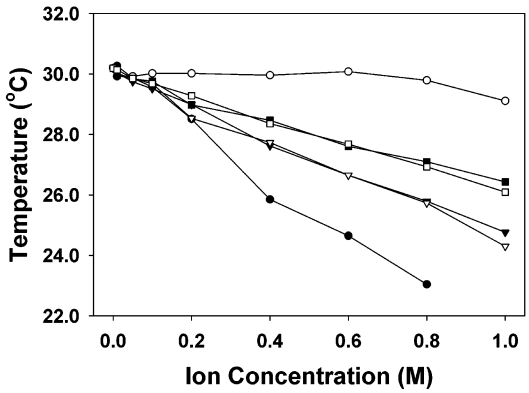


Figure 3. Effect of various sodium and potassium halide solutions on the LCST of PNIPAM. The LCSTs of PNIPAM were measured at eight different concentrations for each sodium or potassium halide (0.01−1.0 M of metal halide in water). The lines were drawn as guides to the eye. ●, KF; ▼, KCl; ■, KBr; ▽, NaCl; □, NaBr; ○, NaI.

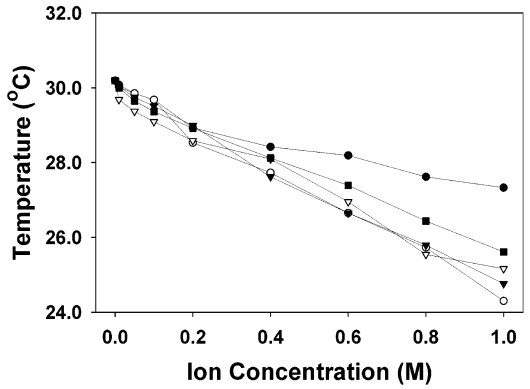


Figure 4. Effect of alkali metal ion variation on the LCST of PNIPAM. The LCSTs of PNIPAM were measured with various alkali metal cations at eight different concentrations (0.01−1.0 M) using chloride as a common anion: ●, LiCl; ○, NaCl; ▼, KCl; ▽, RbCl; ■, CsCl.

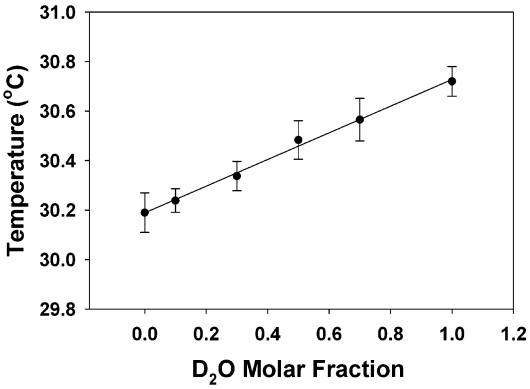


Figure 5. Solvent isotope effects on the LCST of PNIPAM measured using PNIPAM concentrations of 10 mg/mL in a series of solutions with various mole fractions of D_2O contents. The LCST range was from 30.2 to 30.6 °C.

molecules to aggregate or salt-out of solution. On the other hand, the low charge density of the iodide ion should provide the least competition for hydration waters with PNIPAM. Hence, the unfolded state of PNIPAM was stable to a higher temperature at a given halide anion concentration when iodide was present in place of fluoride. While some of these "water organization" effects have recently been called into question and the exact mechanism of action may be controversial,[5] the important point is that the effects observed in our microscopic high-throughput technique are the same as have been seen previously both for PNIPAM solutions and for protein solutions.

The observed effects were more complex for the alkali metal cations as expected (Figure 4). The propensity of cations to lower the LCST increased from lithium to sodium and then decreased from potassium to cesium (Li < Cs < Rb < K < Na). Again, the series obtained here agrees well with the known ability of these ions to salt out proteins and nonelectrolyte solutes like PNIPAM.[23,25,26]

Effects of D_2O Solutions. The effects of hydrogen bonding with solvent water were studied on poly(*N*-isopropylacrylamide (PNIPAM) using varying molar ratios of D_2O and H_2O (Figure 5). As can be seen from the data, the LCST is higher in heavy water than light water. Similar effects have been reported in previous light scattering[27] and pressure perturbation calorimetry[15] measurements using PNIPAM. While the change is relatively small in these experiments, the LCST has a roughly linear relationship with the mole fraction of D_2O. The underlying reasons for this increase in the LCST with D_2O are not precisely known; however, it has been observed that polymer chains in D_2O solutions take on more extended conformations than with H_2O.[27] Furthermore, the amide bonds of the PNIPAM should be relatively well exposed to water below the LCST. Since hydrogen bonding is approximately 5% stronger in D_2O than in H_2O,[28] it is expected that breaking the bonds to the amide moieties would be more enthalpically costly in the former case. This would explain the nearly linear change in LCST with heavy water content. On the other hand, if species such as HOD had more complex interactions with the polymer, one might have expected nonlinear changes instead. Finally, we have also made the isotope measurements with poly(*N*-*n*-propylacrylamide) and found roughly the same behavior. In this case, a ΔT of approximately 0.4 °C was found between pure H_2O and pure D_2O; however, the error bars on the measurements were larger because the scattering cross section of poly(*N*-*n*-propylacrylamide) is roughly half of that for poly(*N*-isopropylacrylamide) at the same concentration.

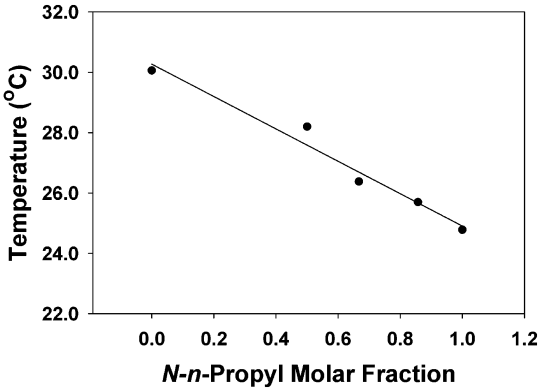


Figure 6. Effect of the identity of the *N*-alkyl substituent on the LCST temperatures for isomeric poly(*N*-alkylacrylamide)s containing varying ratios of isopropyl/*n*-propyl *N*-alkyl groups. The concentration of the poly(*N*-alkylacrylamide)s **1**, **2**, **4**, **5**, or **6** was 10 mg/mL in deionized water.

Effects of Polymer Microstructure. To test the utility of our apparatus for studies of the effects of molecular structure on PNIPAM-like systems, we elected to investigate structurally isomeric polymers by substituting varying amounts of *n*-propyl groups for isopropyl groups on the side chains of PNIPAM (polymers **1**, **2**, **4**, **5**, and **6** in Scheme 1). As noted above, these syntheses were designed so that the homo- and copolymers all had the same degree of polymerization, polydispersity, and molecular weight by preparing all the polymers from the same polymeric intermediate **3** (Scheme 1). LCSTs were measured for each system and the results are shown as a function of the ratio of the *N*-isopropyl to *N*-*n*-propyl substituents (Figure 6). The difference in LCST between PNIPAM and poly(*N*-*n*-propylacrylamide) (PNNPAM) was approximately 5.4 °C, and a monotonic trend can be clearly seen as the ratio of *N*-isopropyl to *N*-*n*-propyl groups was increased. Since the two side chains are very similar, the slightly lower LCST for the *n*-propyl group may reflect a slightly reduced solvent accessible area for the linear molecule vs the branched chain.

Reproducibility of the Measurements. We examined the reproducibility of our measurements of LCST values using both multiple measurements of the same sample and multiple measurements of separately prepared but otherwise identical samples. The values are given in Table 1 in the Supporting Information and indicate precision on the order of ±0.2 °C as well as accuracy on the order of ±0.2 °C for five different trials each of four different samples. Each sample represented a separate synthesis, and the polymers were the product of polymerizations in two different solvents, MeOH and *tert*-BuOH. Syntheses were done in duplicate. A trial was defined as taking a given sample and placing it across the brass tubes. Subsequent trials required repeatedly removing the sample and replacing it across the temperature gradient and remeasuring the LCST. The mean, standard deviation, and standard error for all 20 data points are 24.9 °C, 0.28 °C, and 0.062 °C, respectively. The LCST temperature values measured with this technique are within experimental error of those measured by other techniques.

Conclusion

The linear temperature gradient apparatus for combinatorial temperature measurements described here coupled with a split-pool synthesis of poly(*N*-alkylacrylamide)s is a simple, precise, and high-throughput way to study the effects of polymer microstructure and solution composition of the LCST of thermoresponsive polymers. With polymers like poly(*N*-isopropylacrylamide), effects that produce differences for a polymer's LCST of <0.5 °C can be studied using only a microgram of the polymer in a few microliters of solvent.

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Supporting Information Available: Table of repeated LCST measurements on two different samples of PNNPAM prepared in two different solvents. This material is available free of charge via the Internet at http://pubs.acs.org.

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