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Effect of urea on the conformational behavior of poly(*N*-isopropylacrylamide)

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Abstract The effect of urea on the conformational behavior of poly(*N*-isopropylacrylamide) (PNIPAM) in dilute aqueous solution has been investigated using fluorescence spectroscopy, fluorescence quenching and fluorescence anisotropy measurements via pyrene (Py) probe and acenaphthylene (ACE) label studies. It was demonstrated that urea promotes the partitioning of the hydrophobic probe, Py, towards the bulk aqueous phase at temperatures above the lower critical solution temperature (LCST) of the polymer due to swelling of the compact coil conformation. However, the compact coil structure of the polymer at temperatures greater than its LCST is not completely destroyed, even for urea concentrations up to 3 M, at which the phase transition is hardly observed. As expected, urea has little effect on the conformational behavior of PNIPAM at temperatures below its LCST.

Key words Poly(*N*-isopropylacrylamide) · Urea · Pyrene · Fluorescence technique · Conformation

Introduction

Poly(*N*-isopropylacrylamide) (PNIPAM) is a well known "intelligent" polymer. Its aqueous solution shows a lower critical solution temperature (LCST); i.e., when heated above this temperature, the solution turns opaque [1–3]. A similar phenomenon can be observed when the polymer is occluded in glutaraldehyde-crosslinked chitosan hydrogels [4]. The phase transition behavior of PNIPAM in aqueous solution and in hydrogels has been attributed to the formation of polymeric aggregates above its LCST and dissociation of the aggregates below that temperature. Light scattering and various fluorescence technique studies have revealed that an increase in temperature induces substantial changes of the conformation of the polymer [see, for example, refs. 1–3]. Below the LCST the polymer molecules are dissolved as extended coils and above the temperature they collapse into small compact globules. However, the LCST of the polymer can be altered by either co-polymerization of isopropylacrylamide with other monomers (hydrophobic or hydrophilic monomers) or by introduction of surfactants into the polymer solution [5, 6]. The interest in the alteration of the LCST of the polymer is due to various reasons, for instance, many biological applications must be performed at 37 °C. Therefore, polymers or polymer networks with an LCST greater than 37 °C might be more suitable. In fact, several routes including hydrophilic modification and addition of surfactants such as sodium dodecyl sulfate have been explored to modify the behavior of the polymer [6, 7]. In a recent investigation we have been able to prevent the aggregation by the addition of a small amount of urea [8].

Urea and its derivatives are well known denaturants of proteins, converting a protein molecule from the native, specifically folded compact shape to a conformation approaching that of a random coil [9, 10]. Urea also increases the critical micelle concentration of ionic and non-ionic surfactant solutions [11, 12]. It is widely used as a modifier of the aqueous solvent to study hydrophobic interactions in protein and micelle solutions. In fact, urea and its derivative, guanidine hydrochloride, have been used as denaturants for quantitative

studies on the stability of enzymes. From such studies it is generally possible to obtain an estimate of the conformational stability, $\Delta G_D(H_2O)$, of the protein, i.e., how much more stable the globular native conformation of the protein is than its unfolded, denatured conformation. In addition, from this kind of study, inferences can be drawn about the structure of the protein. For example, when the denatured curve has more than one step, this generally indicates that the protein has more than one hydrophobic domain and thereby it is possible to estimate the relative stability of the different domains.

The aim of the current work is to investigate the details of the effect of urea on the conformational behavior of PNIPAM. For this purpose, we have prepared acenaphthylene (ACE) labeled PNIPAM and studied the effect of urea by means of various fluorescence techniques.

Material and methods

Materials

Pyrene (Py) was purchased from ACROS and purified by extraction with ethanol in a Soxhlet extractor. The crystals collected from the extraction are purified Py. *N*-Isopropylacrylamide (NIPAM) was synthesized using acryl chloride and isopropylamide by the method described in the literature [13, 14]. The purification and characterization of the monomer have been reported earlier [4]. Urea (analytical grade from Xi' an Chemicals) was recrystallized twice from a mixture of ethanol and water (1:1, v:v). The purity of urea was checked by measuring the absorbence of a 5 M solution in a 1-cm cell at 260 nm. The absorbence was less than 0.1 indicating that the urea was free of heavy metals, cyanate and biuret [15]. α,α′-Azobisisobutyronitrile (AIBN) was recrystallized from ethanol prior to use. Acenaphthylene (ACE) (Aldrich-85%) was purified by triple recrystallization from ethanol followed by sublimation. Dioxane (analytical grade from Xi' an Chemicals) was used without further purification. Water used in this work was de-ionized and then doubly distilled.

Polymerization

ACE labeled PNIPAM (PNIPAM/ACE) was synthesized by radical polymerization in dioxane solution initiated with AIBN. A 30 mg sample of AIBN, dissolved in a small amount of dioxane was added to 25 mL of an oxygen-free solution of NIPAM (20 wt %) and ACE (0.2 wt %) in dioxane. After 20 h polymerization at 70 °C, the mixture was cooled and the solvent was evaporated within a fume hood. The residue was dissolved in tetrahydrofuran (THF) and precipitated with *n*-hexane. The crude polymer was further purified by 3-fold precipitation with diethyl ether from a methanolic solution and then vacuum dried at 60 °C. The final yield is 3.2 g. The viscosity average molecular weight of the polymer was determined in THF according to a literature method [16]. It was 1.9×10^5. The LCST of the polymer in aqueous phase is 33 °C (temperature uncorrected).

Polymer solutions

Steady-state fluorescence measurements of polymer solutions of 10^{-3} wt % (except that for fluorescence anisotropy measurements) were performed using a quartz cuvette of path length 1 cm. PNIPAM solutions were prepared from a stock solution (0.1 wt %), which was prepared freshly every day. The working solution of PNIPAM containing urea was prepared as follows. A known amount of urea was added to a 50 mL volumetric flask. To this solution, 40 mL of water were added to dissolve the urea, then 0.5 mL PNIPAM stock solution was added with shaking and finally the solution was made up to 50 mL with water.

Probe studies

For the experiments involving occlusion of organic probe molecules into the water-soluble polymer solutions, the probe, Py was initially dissolved in diethyl ether to obtain a stock solution of known concentration (ca. 10^{-3} M). This solution was then diluted to 10^{-5} M. Twenty μL of the stock solution (10^{-5} M) of the probe were injected into a l0 mL volumetric flask. The ether was evaporated at room temperature overnight. Subsequently, ACE-labeled polymer solution (10^{-3} wt %) containing a known amount of urea was added to the flask. To ensure solubilization and equilibration, the polymer/probe solutions were sonicated for 20 min and equilibrated for more than 12 h.

Fluorescence quenching experiment

Nitromethane has been used as a quencher in this study. A stock solution of 1.08 M in methanol was prepared before use. The quencher solution was added to 3 mL of PNIPAM/ACE solution held in 1 cm quartz cuvette using a 5–50 μL micro-pipette. The polymer solution containing nitromethane was shaken for at least ten minutes after each addition of the quencher solution and before spectroscopic measurements to allow equilibration.

Analytical methods

All fluorescence spectra were recorded on a Perkin-Elmer LS 50B luminescence spectrometer. Fluorescence anisotropy measurements were performed on the same apparatus using the polarization accessory. This arrangement allows estimation of I_{vv}, I_{vh}, I_{hh} and I_{hv}, where I_{vv} and I_{vh} stand for the fluorescence intensities observed parallel and perpendicular, respectively, to the plane of vertically polarized excitation. Similarly, I_{hv}, and I_{hh} are the fluorescence intensities measured perpendicular and parallel, respectively, to the plane of horizontally polarized excitation. The instrumental correction factor, G, and the fluorescence anisotropy, r, can be calculated by using Eqs. 1 and 2, respectively.

$$r = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \quad (1)$$

where

$$G = I_{hv}/I_{hh} \quad (2)$$

All measurements and calculations were carried out automatically by the system. Each measurement was repeated at least 10 times and was performed using a quartz cuvette with a path length of 1 cm.

Results and discussion

Pyrene probe study

In studies of the aqueous solution behavior of polyelectrolytes and polysoaps, Py is a widely used fluorescence

probe because of its unique photophysical properties. It is a typical hydrophobic probe and its solubility in water is quite low (3×10^{-7} M) [17]. The profile of its fluorescence emission spectrum is very sensitive to the polarity of its microenvironment. In particularr, the ratio of the intensity of the third peak to that of the first peak (I_3/I_1) of the emission of Py is a measure of the polarity of its microenvironment. The lower the ratio, the more polar is the environment of the probe [18, 19]. This is the basis of the well-known Py scale for solvent polarity.

For the current study, as expected, the profile of the fluorescence emission spectrum of the probe, Py, is dependent upon the temperature of the polymer solution as evidenced by the temperature dependence of the ratio of I_3/I_1, for the PNIPAM/Py system in the absence of urea (Fig. 1). Clearly, the ratio at high temperature is obviously greater than that at low temperature. As an example, the small figure inserted in Fig. 1 shows the temperature dependence of the fluorescence emission spectrum of Py occluded in PNIPAM solution with no urea. This is in accordance with the statement that the polymer adopts a relatively compact coil conformation at high temperature and a loose coil conformation at low temperature. Introduction of urea affects the fluorescence emission property of the probe. The ratio I_3/I_1 decreases with increasing urea concentration at temperature above 34 °C (Fig. 1), whereas there is no significant change at temperatures below 33 °C. The effect of urea on the temperature dependence of the profile of the probe in PNIPAM solution is summarized in Fig. 1. The changes in the ratio I_3/I_1 may be understood in terms of the conformational changes induced in the polymer.

PNIPAM adopts a compact coil conformation at temperatures above its LCST, characterized by larger I_3/I_1 values, indicating that more probe molecules are partitioned in the hydrophobic polymer-rich phase. The addition of urea will disrupt the intramolecular hydrophobic interaction. Consequently, the compact coil conformation would become swollen and some probe molecules would enter into the aqueous phase. The average microenvironment of the probe becomes more polar and thereby the ratio I_3/I_1 decreases with increasing urea concentration. At temperatures below the LCST, the polymer adopts a loose coil conformation and has no ability to dissolve water-insoluble bulky probes. In this case, addition of urea will have little effect upon the conformation of the polymer. Therefore, the profiles of the probe do not change very much with the addition of urea.

Reference to Fig. 1 further reveals that for the system with 0.45 M of urea the ratio I_3/I_1 increases gradually from 0.64 at 24 °C to 0.69 at 44 °C and the transition in the ratio is hardly observed, a similar result to that obtained from a reference system with no polymer [20]. These observations might be an indication of the repartitioning of the probe towards the bulk aqueous phase, suggesting unfolding of the polymer coil. Furthermore, with increasing urea concentration at temperatures greater than 32 °C, the I_3/I_1 value gradually approaches that of the reference system with no polymer (see Fig. 1), indicating that the hydrophobic microdomains within the polymer break down gradually. It can

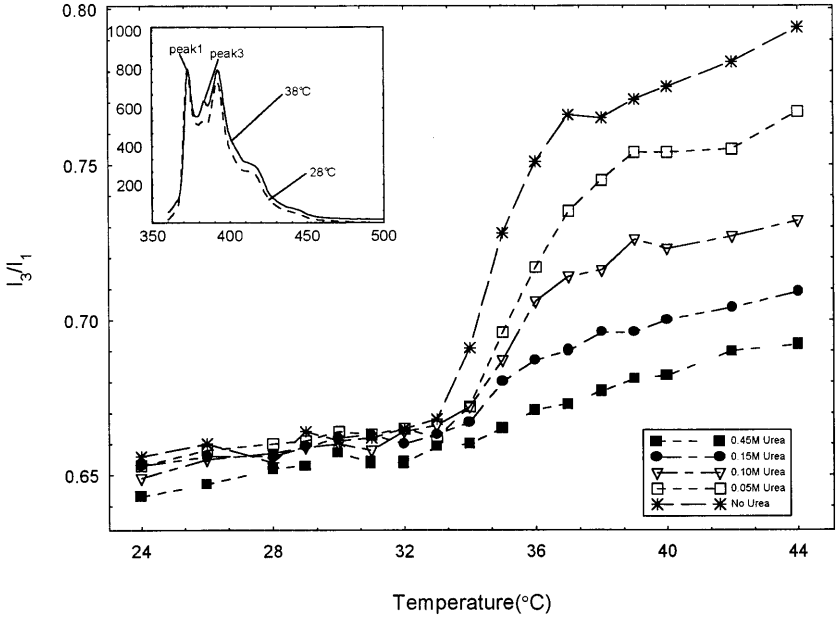

Fig. 1 I_3/I_1 ratio for the fluorescence emission of pyrene (2×10^{-7} M) dispersed in a series of PNIPAM solutions (10^{-3} wt %) with various concentrations of urea as functions of temperature. The insert shows the fluorescence emission spectrum of pyrene at two different temperatures

be also noted that the conformational transition becomes more diffuse with increasing urea concentration. Therefore, at high temperature and in the presence of a large amount of urea, the polymer conformation might be a loose coil and/or a loose coil with some submolecular hydrophobic microdomains, which might be too small to compartment the bulky hydrophobic probe molecules.

It also can be noted that the LCST does not change with increasing urea concentration, indicating that addition of urea has little effect upon the delicate balance between hydrophilic and hydrophobic interactions within the polymer. This observation is in support of the indirect mechanism explaining the effect of urea in which urea acts only at the solvent level, altering the structure of water in a way that facilitates the dissolution of the hydrophobic species.

To further confirm the tentative conclusion described above, a series of fluorescence quenching experiments were conducted. This is because quenching measurement can reveal the accessibility of a fluorophore to a quencher, and the localization of a fluorophore either covalently bound to a polymer chain or dispersed in a polymer solution. In the current work, nitromethane was chosen as a quencher due to its hydrophilicity and charge neutrality.

In the probe's spectrum, the intensity at 380 nm was chosen to stand for the fluorescence intensity. The data were treated by means of the Stern-Volmer equation (Eq. 3) [21].

$$I^{\circ}/I = 1 + k_{\mathrm{q}}\tau^{\circ}[Q] \tag{3}$$

Here I° and τ° are the fluorescence intensity and lifetime in the absence of the quencher, respectively, and k_{q} is the bimolecular quenching constant for the diffusion-controlled quenching process. The fluorescence lifetime data used in the present work were measured in Professor Soutar's Laboratory with an Edinburgh Instruments 199 spectrometer. The details of the measurements may be found in [20]. The results are listed in Table 1. Before making any further consideration of the data listed in the table, it is to be noted that urea modifies the viscosity of an aqueous solution significantly and solution viscosity is one of the major factors affecting the quenching efficiency. Therefore, compensation must be made for the variation in the viscosity of the system under study.

Table 1 Bimolecular quenching constants derived from fluorescence intensity data, k_{q} (I), for pyrene (2×10^{-7} M) dispersed in the PNIPAM solution (10^{-3} wt %) at 40 °C. Nitromethane was used as a quencher ($\lambda_{\mathrm{ex}}/\lambda_{\mathrm{em}}$ = 340/380 nm)

Urea (M)	τ° (ns)	η (cp)	k_{q}(I) ($\mathrm{M}^{-1}\ \mathrm{s}^{-1}$)	k'_{q}(I) ($\mathrm{M}^{-1}\ \mathrm{s}^{-1}$)
0	130.2	0.8896	0.7×10^9	0.6×10^9
0.45	137.4	0.8976	2.3×10^9	2.1×10^9

It is known that the fluorescence quenching efficiency is determined by the collision frequency of a fluorophore with a quencher as given by Eq. 4.

$$Z = k_{\mathrm{q}}[Q] \tag{4}$$

Here k_{q} stands for the same as that in Eq. 3. It may be calculated using the Smoluchowski equation (Eq. 5) [22].

$$k_{\mathrm{q}} = \frac{4\pi RD\mathrm{N}}{1000} \tag{5}$$

$$= \frac{4\pi\mathrm{N}}{1000}\left(R_{\mathrm{f}} + R_{\mathrm{q}}\right)\left(D_{\mathrm{f}} + D_{\mathrm{q}}\right) \tag{6}$$

where R is the collision radius, D is the sum of the diffusion coefficients of the fluorophore (D_{f}) and quencher (D_{q}), and N is Avogadro's number. The diffusion constant may be calculated according to the Stokes-Einstein law (Eq. 7) [23]:

$$D = \frac{kT}{6\pi\eta R} \tag{7}$$

where k is the Boltzman constant, η is the viscosity, and R the radius of the fluorophore or quencher.

Clearly, the relationship between k_{q} and viscosity may be obtained by introduction of Eq. 7 into Eq. 6.

$$k_{\mathrm{q}} = \frac{4\pi\mathrm{N}}{1000}\left(R_{\mathrm{f}} + R_{\mathrm{q}}\right)\left(\frac{1}{R_{\mathrm{f}}} + \frac{1}{R_{\mathrm{q}}}\right)\frac{\mathrm{k}T}{6\pi\eta} \tag{8}$$

Eq. 8 shows that for a truly diffusion-controlled fluorescence quenching process and at constant temperature the bimolecular quenching constant should be inversely proportional to the solvent viscosity. If a specific state of a system, at which the solvent viscosity is 1 cps, is chosen as a reference state, the effect of viscosity at a given temperature upon bimolecular quenching constant can be reduced using Eq. 9:

$$k'_{\mathrm{q}} = k_{\mathrm{q}}\eta \tag{9}$$

where k'_{q} is the viscosity reduced bimolecular quenching constant and k_{q} is the measured bimolecular quenching constant. The viscosity data for aqueous solvent containing different concentrations of urea were kindly supplied by Dr. Kirpach [24]. According to Eq. 9, the viscosity reduced bimolecular quenching constants were calculated and are also listed in Table 1. With reference to the table, it is apparent that, at 40 °C, the viscosity reduced bimolecular quenching constant, k'_{q}, for the polymer system with 0.45 M of urea is more than two times greater than that for the polymer system with no urea. The difference in the quenching efficiency for the two systems with and without urea may be observed more directly by comparing Figs. 2a, b (note: the scale is

different). Since urea was found to have little effect upon the quenching efficiency of nitromethane for Py in the reference system with no polymer, the increase in k'_q upon addition of urea can be attributed to the effective disruption of the hydrophobic microdomains within the polymer coil. With the disruption of the domains, the polymer coils start to expand or reorganize, for example, breaking or partially breaking into even smaller submolecular microdomains. Therefore, the ability of the polymer to solubilize water-insoluble organic compounds decreases. Consequently, partition of Py in the micro-multiphase of the system shifts to the aqueous phase which is more accessible to the water-soluble quencher, nitromethane.

It should be noted that conclusions from probe studies must be drawn with care. This is because the probe studies are complicated by the nature of the probe employed. For the current study, Py has a sufficiently large solubility in water to allow the fluorescence from the probe in the bulk aqueous phase to make a significant contribution to the overall fluorescence. In addition, urea may modify the property of water, making it a better solvent for non-polar organic molecules or groups [25]. Therefore, repartitioning of Py towards the bulk aqueous phase upon addition of urea may be a result of both the conformational change of the polymer and the modification of water quality due to the addition of urea. Clearly, at this instance, any results from probe, particularly Py, studies must be treated cautiously. Considering the reasons discussed above, it is absolutely necessary to supplement the study by fluorescence labeling techniques.

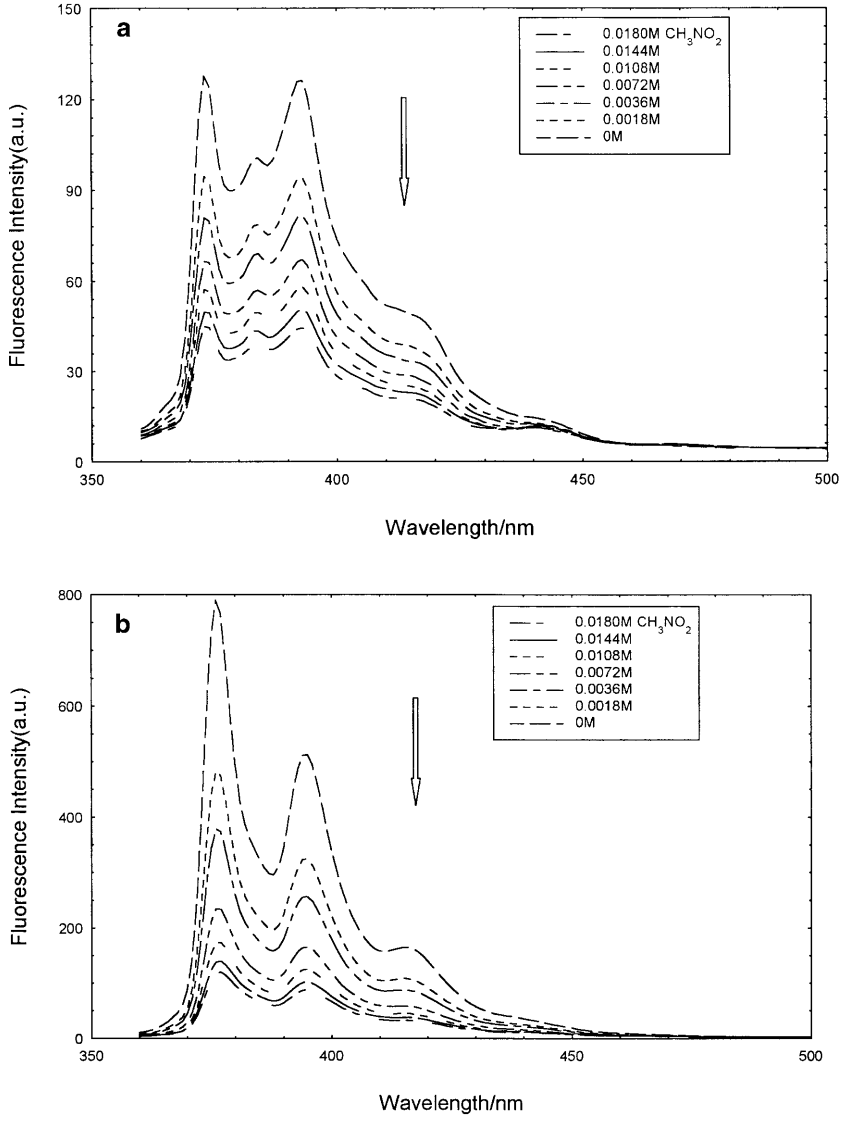

Fig. 2 Steady-state fluorescence emission spectrum of pyrene dispersed in the aqueous solution of PNIPAM (10^{-3} wt %) in the presence of various concentrations of nitromethane ($\lambda_{ex} = 340$ nm). (*a*) No urea, (*b*) 0.45 M urea

Fluorescence quenching via labeling techniques

The accessibility of a fluorescent label affixed to a polymer chain can be estimated by fluorescence quenching experiments. In principle, information regarding polymer conformation and details such as coil compactness, charge density and coil size may be assessed through the efficiency of quenching by a given low molar mass species [26]. In the current studies of ACE-labeled PNIPAM in dilute aqueous solutions, again nitromethane was employed as quencher. The k_q value for each system was calculated according to Eq. 3. The corresponding viscosity reduced k_q values were taken from Eq. 9. It is to be noted that all the bimolecular quenching constants were from the fluorescence intensity data. The results are listed in Table 2. In most cases, the Stern-Volmer plots are good straight lines (Fig. 3).

Examination of the quenching data reveals that, at temperatures below the LCST of the polymer, the bimolecular quenching constant for the polymer system with no urea is about 4.4×10^9 s^{-1} M^{-1}, a value typical of that expected for a diffusion-controlled quenching process of fluorescently labeled polymer in dilute aqueous solution. In this respect, the quenching efficiency for the ACE labeled PNIPAM using nitromethane as a quencher is similar to that observed for poly(*N,N′*-dimethylacrylamide) and poly(acrylic acid) in dilute aqueous solution in which the polymers adopt extended coil conformations within a wide pH range [27, 28], but is much more efficient than that shown for the hypercoiled form of poly(methylacrylic acid) [26]. These observations indicate that the PNIPAM/ACE adopts a loosely coiled conformation in dilute aqueous solution below its LCST. This result is in support of those from other studies using different techniques. The increase in urea concentration has little effect upon the k_q value, indicating that addition of urea may not alter the polymer conformation very much at a temperatures below the LCST of the polymer. At temperatures above the LCST and in the absence of urea, the viscosity reduced bimolecular quenching constant, k'_q for the PNIPAM/ACE system is only 1.1×10^9 s^{-1} M^{-1}, which is about three times lower than that of the same system at 22 °C, suggesting that the polymer might adopt a quite compact coil conformation at temperatures above its LCST. It is rather surprising that addition of urea at high temperature has little effect upon the k'_q value, indicating that the label may be still trapped in some hydrophobic microdomains even for urea concentration up to 3 M. In other words, the polymer conformation does not change as much as would be expected from probe studies. As mentioned earlier, the repartitioning of Py towards the bulk aqueous phase may be due to several reasons. One of them is the conformational change of the polymer. However, the conformational change may not be a simple homogeneous process. At high temperature, the loose coil conformation may consist of a number of sub-molecular hydrophobic microdomains. These domains might be too small to compartment the bulky hydrophobic probe molecules, but the label might still be trapped in it because the label is small in size and is a part of the polymer chain. Therefore, it is not difficult to understand that the quenching efficiency from the probe studies is significantly greater than that from the label studies. A similar result has been reported by Braud and coworkers [29] in the studies of the effect of urea upon the conformational behavior of poly(methylacrylic acid). They discovered that the compact coil conformation of poly(methylacrylic acid) is not completely destroyed even for urea concentrations up to 8 M. In addition, the result described above is further supported by fluorescence anisotropy studies.

Fluorescence anisotropy studies

It can be imagined that the segmental mobility of a polymer in a compact coil conformation would be lower than that in a loose coil conformation. Therefore, comparison of the segmental mobility of a polymer at different conditions should give some reasonable, detailed information regarding the conformation of the polymer. It should be noted, however, that the selection of label and labeling method is crucial for a successful fluorescence anisotropy study. In the current work, ACE was chosen due to the fact that there is no rotation independent of the segment to which the ACE label was tagged.

With reference to the probe study results (see Fig. 1), two urea concentrations were chosen to conduct the anisotropy studies. The results are shown in Fig. 4.

Table 2 Bimolecular quenching constants derived from fluorescence intensity data, $k_q(I)$, for ACE-labeled PNIPAM (10^{-3} wt %) at 40 °C. Nitromethane was used as a quencher ($\lambda_{ex}/\lambda_{em}$ = 290/340 nm)

Temperature (°C)	Urea (M)	$\tau°$ (ns)	η (cp)	$k_q(I)$ (M^{-1} s^{-1})	$k'_q(I)$ (M^{-1} s^{-1})
40	0	19.7	0.8920	1.2×10^9	1.1×10^9
	0.10	20.1	0.8912	1.3×10^9	1.2×10^9
	0.45	21.0	0.8976	1.3×10^9	1.2×10^9
	3.00	23.4	1.0171	1.0×10^9	1.0×10^9
22	0	20.3		4.4×10^9	
	0.10	21.6		5.0×10^9	
	0.45	22.5		5.2×10^9	

Upon examination of the figure, several points about the urea effect upon the conformational behavior of PNIPAM may be drawn:

(i) At temperatures below 31 °C, the *r* value of the ACE-labeled PNIPAM system is small and largely unaffected by the addition of urea, an indication of a loose coil conformation being adopted by the polymer both in the absence and presence of urea.

(ii) At temperature above 31 °C, the *r* values of the two systems are quite different. For the system with no urea, the *r* values are quite large, indicating a compact coil conformation being adopted by the polymer. The *r* value decreases dramatically with addition of urea, indicating that the polymer coil is getting looser. Clearly, the results described in (i) and (ii) are in support of those drawn from Py probe studies. However, the differences are seen to exist if we examine Fig. 4 further.

(iii) The temperature at which the curve starts to increase in Fig. 4 is about two degrees lower than that in Fig. 1. The decrease may be attributed to the labeling effect, which is of a hydrophobic modification of the PNIPAM in nature. Furthermore, for the system with 0.45 M urea, the phase transition occurred at around 32 °C, the LCST of PNIPAM. This demonstrates that PNIPAM/ACE does experience a conformational transition around its LCST even in the presence of 0.45 M

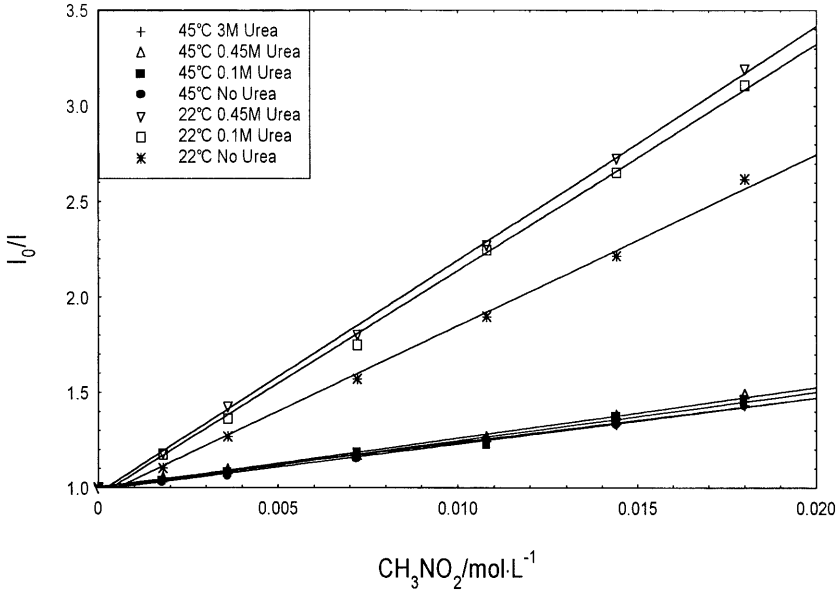

Fig. 3 Stern-Volmer plots for a series of ACE-labeled PNIPAM solutions (10^{-3} wt %) with various concentrations of urea at two different temperatures. Nitromethane was used as a quencher. ($\lambda_{ex}/\lambda_{em}$ = 290/340 nm)

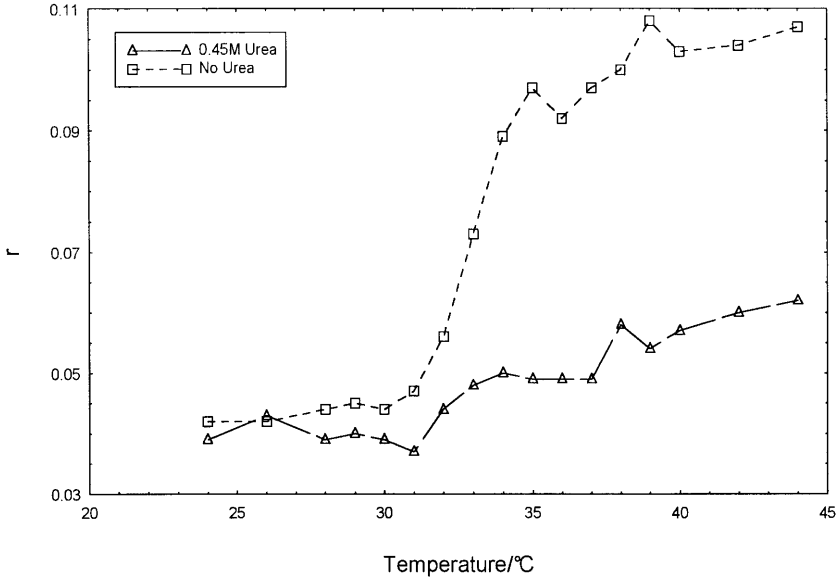

Fig. 4 Fluorescence anisotropy for two ACE-labeled PNIPAM solutions (10^{-2} wt %) with different concentrations of urea as a function of temperature ($\lambda_{ex}/\lambda_{em}$ = 290/340 nm)

urea, a conclusion different from that from fluorescence spectroscopy studies (see Fig. 1), in which the transition in the I_3/I_1 value for the PNIPAM system with 0.45 M of urea almost disappeared. This observation may be understood by consideration of the result from fluorescence quenching studies via the ACE label. In the PNIPAM/ACE system with urea, the ACE label may be still trapped in some sub-molecular hydrophobic microdomains and thereby the segmental motion sensed by the label is significantly slower than that of a polymer chain in a simple homogeneous loose coil conformation.

Conclusions

The following conclusions may be drawn:

(i) Urea promotes the partitioning of the hydrophobic probe, Py towards the bulk aqueous phase at temperatures greater than the LCST of PNIPAM, which can be a result of unfolding of the compact coil conformation of the polymer and/or modification of solvent quality towards the probe. Therefore, care must be taken when drawing conclusions about polymer conformations from probe studies.

(ii) Pyrene probe (I_3/I_1, measurement and fluorescence quenching) and ACE label (fluorescence quenching and anisotropy) studies have demonstrated that the addition of urea to the PNIPAM solution at temperatures greater than the LCST of the polymer results in a more open, water-swollen coil structure.

(iii) The effect of urea upon the conformational behavior of PNIPAM is complex. Fluorescence quenching, particularly anisotropy studies via ACE-labeled PNIPAM supports the view that the addition of urea at temperatures greater than the LCST of the polymer increases the openness of the polymer coil, but the opening process may not be a simple and homogeneous one. In the polymer system at high temperatures with no urea, a polymer molecule may adopt a simple compact coil conformation. With addition of urea, the compact coil may break down into several sub-molecular hydrophobic microdomains. The results from Py probe studies may not support this conclusion due to the complex nature of the partition of Py between the bulk aqueous phase and the polymer-rich phase.

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