Fluorescence Studies of Hydrophobically Modified Poly(*N*-isopropylacrylamides)

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Received May 24, 1990; Revised Manuscript Received August 28, 1990

ABSTRACT: Copolymers of *N*-isopropylacrylamide (NIPAM) and *N*-alkylacrylamides have been prepared by free-radical copolymerization in dioxane using azobis(isobutyronitrile) as the initiator. The synthesis and characterization of copolymers with decyl-, tetradecyl-, and octadecyl-substituted acrylamides (NIPAM to *N*-alkylacrylamide molar ratios in the copolymers 100:1 and 200:1) are reported here. Two fluorescently labeled copolymers were prepared by copolymerization of NIPAM and *N*-[4-(1-pyrenyl)butyl]-*N*-octadecylacrylamide (4) in 200:1 and 400:1 molar ratios. The solution properties of the polymers have been studied in water as a function of polymer concentration and temperature. Cloud-point determinations and experiments with three fluorescent probes, pyrene (Py), perylene (Per), and 1,6-diphenyl-1,3,5-hexatriene (DPH), reveal the existence of polymeric micelles below the lower critical solution temperature (LCST) in aqueous solutions of the C_{14} and the C_{18} copolymers but not of the C_{10} copolymers. The polymeric micelles are disrupted at the LCST. Above the LCST the hydrophobic substituents are distributed randomly among collapsed and aggregated PNIPAM chains. Fluorescence studies of the pyrene-labeled copolymers confirm the presence of hydrophobic microdomains below the LCST and their disruption at the LCST.

Introduction

Amphiphilic polymers contain hydrophobic and hydrophilic segments in the same molecule. Such a composition tends to lead to a schizophrenic behavior, itself the cause of unique solution properties, especially in water. As a consequence they have found applications in a variety of areas,[1] from thickeners in food, rheology-controlling substances in coating fluids and latex-based paints,[2] to additives in enhanced oil recovery or water treatment.[3] The physical properties of amphiphilic polymers depend to a large extent on their chemical composition. Also they are extremely sensitive to the relative amount of hydrophobic to hydrophilic moieties. Amphiphilic polymers compositions encompass all copolymer types. Structures attainable are limited only by the imagination of the polymer scientists and their skills in polymer synthesis. Amphiphilic copolymers may include the hydrophobic group in their main chain, as for example in polyionenes,[4] or as side chains. Examples of the latter structures include *n*-alkyl-grafted cellulose ethers and acylated polysaccharides,[5] copolymers of maleic anhydride and either *n*-alkyl vinyl ethers[6] or *n*-alkylethylene,[7] copolymers of acrylamide and *n*-alkylacrylamides,[8] quaternized alkylpolyethyleneimines,[9] or poly(amino acids) carrying alkyl side chains.[10]

In the area of the life sciences too, synthetic amphiphilic polymers have found unique applications. The property sought after here is the capability of the hydrophobic substituents to interact specifically with lipophilic structures, such as lipid bilayer membranes. The hydrophobic groups become the "anchoring points" of the polymers on model membranes.[11] Once the polymer is safely anchored to a membrane, it can serve as a modulator if it responds by some physical change to an external stimulus. Elegant systems using light pulses or changes in pH as external triggers have been described for model membrane systems.[12] The application of heat as an external stimulus has not been explored in these systems. This article describes the preparation and properties of functional amphiphilic polymers designed to act as heat-sensitive modulators of membranes. The selection of a suitable polymer was influenced by a report in a related field of the use of heat to control flow through ultrathin nylon capsules.[13] These capsules were surface-grafted with poly(*N*-isopropylacrylamide) (PNIPAM). The grafted polymer acted as a thermoreversible valve, blocking the escape of permeants from the capsule above a critical temperature. This macroscopic behavior was attributed to a heat induced collapse of individual macromolecular chains. Another macroscopic phenomenon attributed to the collapse of a polymer chain is the thermoreversible phase separation exhibited by many hydrophobic water-soluble polymers. Indeed PNIPAM exhibits this phenomenon: it is extremely soluble in water at room temperature, but it precipitates from the solution at 32 °C.[14] This temperature is convenient for working with membranes. In addition the synthesis of PNIPAM can be modified easily to allow the incorporation of hydrophobic anchor groups.

Therefore copolymers of *N*-isopropylacrylamide (NIPAM) and *n*-alkylacrylamides were chosen as promising candidates for membrane-anchored thermosensitive polymers. A series of such copolymers has been prepared. They differ in the length of the alkyl chain (C_{10}, C_{14}, and C_{18}) and in the number of *n*-alkylacrylamides per NIPAM units (1:100 and 1:200 in molar units). Two fluorescently labeled amphiphilic copolymers were prepared by copolymerization of NIPAM and *N*-[4-(1-pyrenyl)butyl]-*N*-*n*-octadecylacrylamide in 200:1 and 400:1 molar ratios (Figure 1). The synthesis, characterization, and solution properties of these polymers are reported here. Special emphasis will be given to results obtained from fluorescence techniques, including polarity measurements with the pyrene (Py) probe, local viscosity determinations based on fluorescence depolarization measurements with perylene (Per) and 1,6-diphenyl-1,3,5-hexatriene (DPH), and the monitoring of the fluorescence of the pyrenyl group in the case of the labeled polymers.[15]

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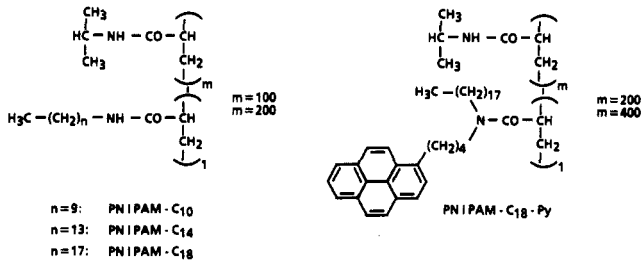

Figure 1. Chemical structures of the amphiphilic polymers described in this study.

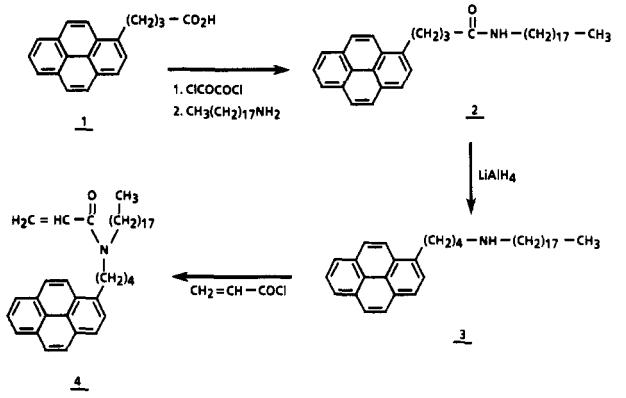

Figure 2. Synthetic scheme for the preparation of the *N*-[4-(1-pyrenyl)butyl]-*N*-*n*-octadecylacrylamide.

Experimental Section

Materials. Analytical grade solvents (Merck, Riedel de Haen) were used without further purification. Thin-layer chromatography was done with silica plates (Merck). Spots were detected either with a UV lamp or by spraying the plates with a bromocresol blue solution (Merck) and with Hanes–Isherwood reagent.[16] Water was purified with a Millipore Milli-Q System. Triethylamine was distilled from Na under N_2 before use. All other chemicals were used as received. Pyrenebutyric acid, oxalyl chloride, di-*tert*-butylcresol, and tetradecylamine were purchased from Aldrich Chemicals; decylamine and $LiAlH_4$ from Fluka, octadecylamine from Riedel de Haen, acryloyl chloride, and AIBN from Merck, and NIPAM from Eastman Kodak Chemicals.

Monomer Synthesis. *N*-*n*-Alkylacrylamides. *n*-Decyl-, *n*-tetradecyl-, and *n*-octadecylacrylamide were prepared by acylation of the respective amine with acryloyl chloride according to the following general procedure: To a solution of *n*-alkylamine (0.1 mol), triethylamine (0.12 mol), and di-*tert*-butylcresol (50 mg), to inhibit free-radical polymerization of the monomers, in CH_2Cl_2 (300 mL) heated at 40 °C under N_2, acryloyl chloride (0.11 mol) was added slowly with a syringe. After stirring for 2 h, the reaction mixture was cooled to room temperature. It was washed with 0.1 N HCl, brine, and saturated $H_2O/NaHCO_3$/NaCl and dried with $MgSO_4$. The solvent was removed in vacuum. The crude products were purified by recrystallization from acetone (single spot on TLC ($CHCl_3$/MeOH 20:1)). The isolated yields ranged from 70 to 80%: mp *n*-decylacrylamide 43 °C (reported value 45–46 °C);[17] *n*-tetradecylacrylamide 61.5–62 °C; *n*-octadecylacrylamide 73–73.5 °C (reported value 74 °C).[17] NMR and IR spectra confirmed the assigned structures.

***N*-[4-(1-Pyrenyl)butyl]-*N*-*n*-octadecylacrylamide (4, Figure 2).** A solution of 4-(1-pyrenyl)butyric acid (1, 2.5 g, 8.7 mmol) in a mixture of dry benzene (50 mL) and DMF (0.5 mL) was refluxed with oxalyl chloride (2 mL, 22 mmol) for 2 h and then was stirred overnight at room temperature.[18] After removal of all volatiles under vacuum, the crude acid chloride was dissolved in CH_2Cl_2 (100 mL). To the solution cooled to 0 °C a solution of *n*-octadecylamine (2.3 g, 8.7 mmol) and triethylamine (1.4 mL, 10 mmol) in CH_2Cl_2 (50 mL) was added dropwise. The reaction mixture was stirred overnight at room temperature. Evaporation of the solvent and crystallization from ethanol gave the crude amide 2 (4.0 g, 7.4 mmol, 85%). Without further purification 2 (3.5 g, 6.5 mmol) suspended in THF (100 mL) was added dropwise to a suspension of $LiAlH_4$ (1.7 g, 45 mmol) in THF (200 mL). The reaction mixture was stirred overnight at 50 °C. It was then cooled to 0 °C. Water was added carefully to destroy the excess hydride. THF was removed in vacuum. The residual aqueous phase was extracted three times with diethyl ether. The ether phase was dried over $MgSO_4$. Evaporation of the solvent yielded 3 (2.65 g, 5.0 mmol, 75%). This material (2.1 g, 4 mmol), triethylamine (0.8 mL, 5.7 mmol), and di-*tert*-butylcresol (20 mg) were dissolved in CH_2Cl_2 (80 mL). Acryloyl chloride (0.4 mL, 5 mmol) was added dropwise to the solution kept at room temperature. The mixture was stirred for 2 h. It was then washed with 0.1 N HCl, saturated $H_2O/NaHCO_3$, and H_2O and dried with $MgSO_4$. Evaporation of the solvent under vacuum gave 4, which was purified by flash chromatography (silica gel, eluted with petroleum ether/ethyl acetate 6:1). The purified product 4 (1.4 g, 2.4 mmol, 60%) showed one spot on TLC; mp 56–57 °C; UV (MeOH) 341 nm (ϵ 34 900). NMR and IR spectra confirmed the structural assignments.

Polymerizations. *N*-Isopropylacrylamide–*N*-*n*-Alkylacrylamide Copolymers. NIPAM (20 mmol), an *N*-*n*-alkylacrylamide (0.2 or 0.1 mmol), or *N*-[4-(1-pyrenyl)butyl]-*N*-*n*-octadecylacrylamide (0.1 or 0.05 mmol) and azobis(isobutyronitrile) (AIBN) (0.1 mmol) were dissolved in dioxane (40 mL, freshly distilled from sodium under N_2). The solution was degassed by bubbling with N_2 (10 min). It was heated to 60 °C for 2 h; then it was cooled to room temperature. The polymers were recovered by precipitation in diethyl ether (800 mL). They were dissolved in dioxane (40 mL) and reprecipitated in diethyl ether (800 mL). The dried polymers were dissolved in water. The solutions were filtered. The polymers were isolated by lyophilization of this solution. They were analyzed and used without further purification.

Physical properties and composition of the samples are compiled in Table I.

Instrumentation. UV spectra were recorded with a Hewlett-Packard 8480A diode array spectrometer. Temperature-controlled experiments were done with a Hewlett-Packard 89100A temperature control accessory consisting of a digitally controlled thermoelectrically heating and cooling cell holder with sample stirring capability and programmed temperature ramping (heating rate 0.5 °C min^{-1}). The temperature of the sample fluid was measured with a Hewlett-Packard 89102A Teflon-coated temperature-sensing probe immersed in the sample fluid. Dilute solution viscosities were determined from THF solutions (3 g L^{-1}) at 27 °C with a Ubbelohde viscometer (Schott).

Fluorescence Measurements. Steady-state fluorescence spectra were recorded on a SPEX Fluorolog 212 spectrometer equipped with a DM3000F data system. The temperature of the water-jacketed cell holder was controlled with a Neslab circulating bath connected to a Neslab MTP-6 programmer. The temperature of the sample fluid was measured with a thermocouple immersed in the sample fluid. Excitation spectra were measured in the ratio mode. Emission spectra were not corrected. For measurements of the I_1/I_3 ratio of the pyrene emission the excitation slit widths were set at 3.6 (excitation) and 0.9 nm (emission). The excitation wavelength was 336 nm. For measurements of spectra of pyrene-labeled samples the excitation wavelength was set at 330 nm. Slit widths ranging from 1.8 to 3.6 nm were chosen, depending on the chromophore concentration. The excimer-to-monomer ratios were calculated by taking the ratio of the emission intensity at 480 nm to the half-sum of the emission intensities at 379 and 399 nm. For measurements at different temperatures samples were heated with a rate of 0.2 °C min^{-1}.

Fluorescence anisotropies (r) were determined with a SPEX 212 spectrometer equipped with two Glan-Thompson polarizers in the L-format configuration and a SPEX 1935A autopolarization accessory. The monochromator slits were set at 9 nm. The excitation wavelengths were 360 and 413 nm; the emission wavelengths were 430 and 444 nm for DPH and Per, respectively. The fluorescence anisotropy was calculated from the relationship[19] $r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where $G = I_{VH}/I_{HH}$ is an instrumental correction factor and I_{VV}, I_{VH}, I_{HV}, and I_{HH} refer to the resultant emission intensities polarized in the vertical or

Table I
Physical Properties of the Polymers

polym	NIPAM:C_n:Py composition[a]	$[\eta]$, mL g^{-1}	M_v[c]	M_n[d]	M_w[d]	M_w/M_n[d]	LCST, °C
PNIPAM	1:0:0	39.0	360 000	19 000	31 000	1.63	31.8
PNIPAM-C_{10}/200	240:1:0	39.9	370 000	19 000	31 000	1.67	30.2
PNIPAM-C_{10}/100	114:1:0	39.5	360 000	24 000	41 000	1.72	29.4
PNIPAM-C_{14}/200	220:1:0	40.3	370 000	26 000	43 000	1.64	28.8
PNIPAM-C_{14}/100	108:1:0	40.5	380 000	22 000	37 000	1.68	23.2
PNIPAM-C_{18}/200	240:1:0	39.7	370 000	17 000	27 000	1.64	30.3
PNIPAM-C_{18}/100	126:1:0	39.7	370 000	23 000	38 000	1.66	25.6
PNIPAM-C_{18}Py/400	435 (365[b]):1:1	40.7	380 000	20 000	35 000	1.73	31.7
PNIPAM-C_{18}Py/200	206 (181[b]):1:1	41.3	390 000	23 000	38 000	1.66	30.6

[a] By ^{1}H NMR. [b] By UV. [c] From $[\eta] = 9.59 \times 10^{-3} M_n^{0.65}$. [d] By GPC (relative to polystyrene).

horizontal detection planes (second subindex) upon excitation with either vertically or horizontally polarized light (first subindex).

Fluorescence lifetimes were measured with a LS-1 instrument from Photon Technology Int., Inc. (London, Ontario, Canada), equipped with a thyratron-gated N_2 lamp and a proprietary analog stroboscopic optical boxcar detection system. Samples were excited at 337 nm. Detection wavelengths were set at 395 nm for pyrene probe experiments and at 376 and 480 nm for the pyrene label monomer and excimer emissions, respectively. The temperature of the water-jacketed cell holder was controlled with a Neslab circulating bath.

Samples for Spectroscopic Analysis. Polymer stock solutions (alkylated copolymers 5 g L^{-1}; labeled copolymers 1 g L^{-1}) were prepared. They were kept at 5 °C to ensure complete dissolution of the polymers. Aliquots of the stock solutions were diluted to the desired concentration. It was verified that the spectral properties of the solutions were constant over a 2-week period of time. Solutions in methanol were degassed by vigorous bubbling with solvent-saturated argon for 1 min. For the steady-state measurements solutions in water were not degassed, since it was determined that for aqueous solutions the fluorescence intensities of pyrene or PNIPAM-C_{18}-Py were not affected significantly by degassing. Samples for fluorescence lifetime measurements were degassed by a 20-min gentle bubbling of argon. Samples for experiments with pyrene as a probe were prepared by mixing stock solutions of polymer (5 g L^{-1}) with water saturated with pyrene (ca. 6×10^{-7} M pyrene). Samples for fluorescence anisotropy measurements were prepared by adding a concentrated solution of the probe in an organic solvent (2 μL, [DPH] = 2.1×10^{-3} M in THF, [Per] = 4×10^{-4} M in acetone) to an aqueous polymer solution (2.5 mL). Liposomes were prepared by a 2-min sonication (40 W, Branson Sonifier) of DMPC in water (1 g L^{-1}) and filtration through a 0.22-μm filter.

Cloud-Point Measurements. Cloud points were determined by spectrophotometric detection of the changes in turbidity of solutions heated at a constant rate in a magnetically stirred UV cell, as described previously.[20]

Results

Synthesis and Characterization of *N*-Isopropylacrylamide–*N*-*n*-Alkylacrylamide Copolymers. Poly(*N*-isopropylacrylamide) and the *n*-alkyl copolymers were prepared by free-radical polymerization in organic medium. Identical synthetic procedures were followed to prepare all copolymers. The experimental conditions were chosen to favor statistical growth of the polymers and to achieve control of the copolymer compositions by the initial monomer feed ratios. The reactions were carried out to an approximate conversion of 60% in dioxane, which is a solvent for the monomers, homopolymers, and copolymers. All the monomers were *N*-alkylacrylamides, and hence they were expected to have similar reactivity ratios. The polymers were purified by repeated precipitations of dioxane solutions into diethyl ether, a nonsolvent for PNIPAM but a good solvent of all the monomers. The purity of the polymers was assessed by TLC and GPC.

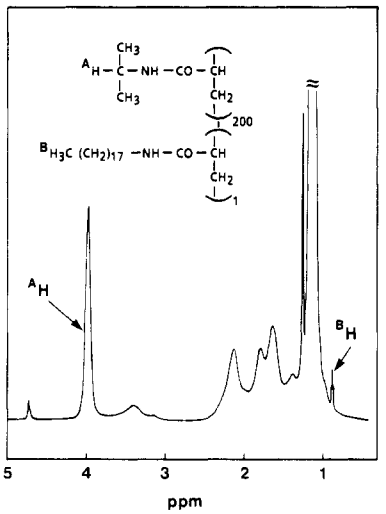

Figure 3. ^{1}H NMR spectrum of PNIPAM-C_{18}/100 in $CDCl_3$ after D_2O exchange.

The absence of *n*-alkyl monomer in the purified polymers was ascertained by analytical TLC, using either a Hanes–Isherwood solution to detect the alkyl groups[16] or UV irradiation to detect pyrene chromophores.

Molecular weights were estimated from the intrinsic viscosities[21] of polymer solutions in THF, using the relationship $[\eta] = 9.59 \times 10^{-3} \bar{M}_n^{0.65}$ established by Fujishige for PNIPAM.[22] Here it is assumed to be valid also for the NIPAM-*n*-alkylacrylamide copolymers (Table I). Molecular weights and molecular weight distributions were calculated as well from GPC measurements run in THF and calibrated with polystyrene standards (Table I). Both techniques confirm that the molecular weight characteristics of the copolymers are independent of their chemical composition.

The chemical composition of the copolymers was determined from a careful analysis of the ^{1}H NMR spectra of $CDCl_3$ solutions before and after D_2O exchange. The ratios of isopropyl to *n*-alkyl groups were calculated from the area of the singlet at 4.01 ppm due to the resonance of the C-2 proton of the isopropyl groups and the area of the triplet centered at 0.9 ppm, attributed to the terminal methyl protons of the alkyl chains (Figure 3). The values calculated from the spectra recorded before and after D_2O exchange were very similar in most cases. The mean values from the two spectra are reported in Table I for all the copolymers. In all cases the molar ratios of isopropyl to alkyl groups determined by NMR exceeded the initial monomer feed ratios by about 10%. In the case of the pyrene-labeled polymers PNIPAM-C_{18}Py/200 and PNIPAM-C_{18}Py/400, the chemical composition was determined also from the UV absorption of the pyrene chromophore, assuming that the extinction coefficient of

pyrene in the polymers is identical with that of the monomer (4). The UV-based chemical composition was found to be slightly lower than the initial monomer feed molar ratio (Table I). In the following discussions the polymer compositions corresponding to the monomer feed ratios will be used. Therefore, assuming a degree of polymerization of about 3300, there are on average 33, 16, and 8 hydrophobic groups in the copolymers with NIPAM to n-alkylacrylamide molar ratios of 100:1, 200:1, and 400:1, respectively.

Solution Properties of the Amphiphilic PNIPAM Samples in Water. Lower Critical Solution Temperature. All copolymers were soluble in water at or below room temperature. As expected the aqueous solutions became turbid when heated, signaling the occurrence of an LCST. In their classic study of the LCST of water-soluble polymers, Taylor and Cerankowsi proposed as a general rule that the LCST should decrease with increasing hydrophobicity of the polymer.[23] Therefore the LCST of amphiphilic PNIPAM was expected to decrease with increasing n-alkyl chain length and, for a given chain length, with increasing n-alkylacrylamide content. Indeed a depression of the LCST was observed for copolymers containing C_{10} and C_{14} alkyl chains (Table I). Surprisingly, this trend is reversed in the C_{18}-containing copolymers: they have an LCST lower than PNIPAM but higher than the C_{14}-containing copolymers. Solutions of the pyrene-labeled C_{18} copolymers have LCST values close to that of PNIPAM (Table I). This unusual trend suggests that in solutions of the C_{18}-substituted copolymers the alkyl chains are not exposed to water but rather form a micellar structure protected from the water and therefore do not make a hydrophobic contribution to the LCST.

Micropolarity. Fluorescence experiments with hydrophobic probes were conducted in order (1) to confirm the presence of hydrophobic microdomains in aqueous solutions of the amphiphilic PNIPAM and (2) to detect changes in the polarity and rigidity of these hydrophobic microdomains as a function of alkyl chain length and concentration. In one set of experiments trace amounts (ca. 6×10^{-7} M) of pyrene were added to the aqueous solutions. Changes in the fine structure of the pyrene emission were monitored. Specifically the ratio I_1/I_3 of the intensity of the (0,0) band (I_1) to that of the (0,2) band (I_3) of the emission was measured under various conditions. This ratio is used routinely in the study of micellar structures in water.[24] It takes a high value in polar media (I_1/I_3 = 1.81 in water). It decreases with decreasing polarity (I_1/I_3 = 1.12 for pyrene solubilized in sodium dodecyl sulfate (SDS) micelles). In aqueous solutions of the C_{14}- and C_{18}-PNIPAM amphiphiles the I_1/I_3 ratio decreased rapidly with increasing polymer concentration (Figure 4). In the case of the PNIPAM-C_{10}/200 the ratio remained constant (1.80) over the entire polymer concentration range probed, a behavior similar to that of PNIPAM. In this case as well no significant change in I_1/I_3 was observed, compared to water, even at high polymer concentrations. This result implies that pyrene is not solublized by either PNIPAM or the C_{10}-PNIPAM copolymers. In solutions of these polymers pyrene resides mostly in an aqueous environment. The temperature dependence of the ratio I_1/I_3 was monitored for solutions of each copolymer (0.5 g L^{-1}). In all cases I_1/I_3 reached a limiting value, between 1.2 and 1.4, in solutions heated above their LCST (Figure 5). These values are in good agreement with results reported recently by Schild and Tirrell for PNIPAM and its copolymers with hexadecylacrylamide.[25]

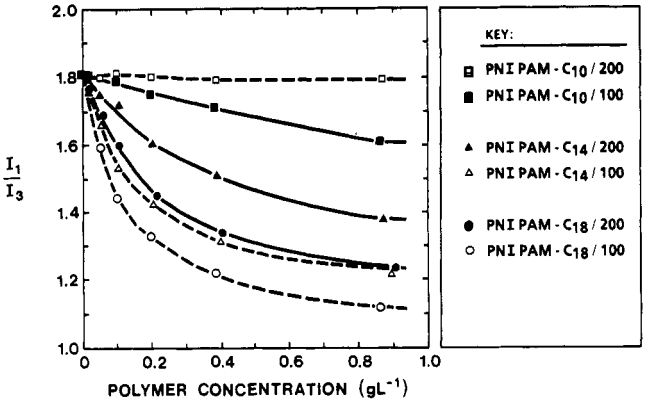

Figure 4. Plot of the changes in the ratio I_1/I_3 for pyrene in aqueous solutions of amphiphilic poly(N-isopropylacrylamides) as a function of polymer concentration (15 °C, λ_{exc} = 336 nm).

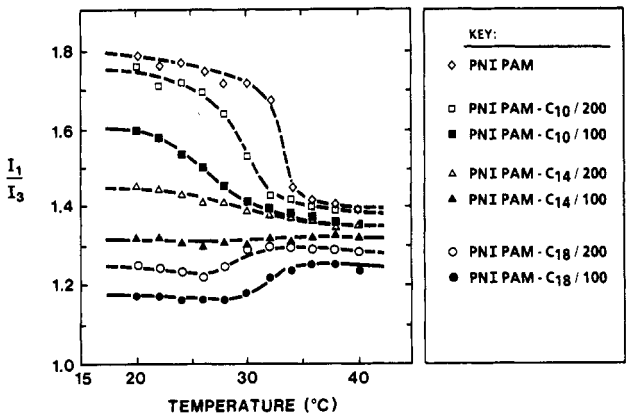

Figure 5. Plot of the changes in the ratio I_1/I_3 for pyrene in aqueous solutions of amphiphilic poly(N-isopropylacrylamides) as a function of solution temperature (polymer concentration: 0.5 g L^{-1}, λ_{exc} = 336 nm).

Fluorescence Lifetimes. The ratio I_1/I_3 is a measure of the polarity averaged over all solubilization sites. It cannot provide site specific information, unless the location of pyrene is known. Such information can be obtained from lifetime measurements. Changes in Py fluorescence lifetimes with temperature were determined in solutions of the C_{10}-, C_{14}-, and C_{18}-PNIPAM amphiphiles. For solutions kept below their LCST the decay curves of Py fluorescence cold not be fit to a single-exponential law. Since the pyrene concentration was too low for excimer formation, as confirmed by steady-state measurements, this deviation from a monoexponential decay law is diagnostic of heterogeneity of pyrene environments. Py lifetimes in two control systems, in water and in SDS micelles, followed single-exponential decay laws with lifetimes of 200 and 340 ns, respectively. In solutions of PNIPAM and its amphiphilic copolymers, Py lifetimes were fit to a biexponential decay law (Table II). In all cases one lifetime was identical with that of Py in water. The second component was much shorter (ca. 40 ns) in solutions of PNIPAM and C_{14}-PNIPAM or much longer (ca. 360 ns) in solutions of C_{14}- and C_{18}-PNIPAM. These components are attributed to the emission of Py preferentially solubilized in a polymeric environment. That the lifetime of Py in PNIPAM and C_{10}-PNIPAM is much shorter than in water reveals that Py fluorescence is quenched by the amide groups, in accord with observations by Thomas on the quenching of Py emission by acrylamide and polyacrylamides.[26] On the other hand, when Py is solubilized by the C_{14}- and C_{18}-PNIPAM amphiphiles, it experiences an environment similar to that provided by surfactant micelles. In solutions heated above their LCST, the Py

Table II
Fluorescence Lifetimes of Pyrene in Aqueous Solutions of Amphiphilic PNIPAM Solutions[a]

sample	τ_1, ns	a_1	f_1[b]	τ_2, ns	a_2	f_2[b]	$\langle\tau\rangle$,[c] ns
PNIPAM	44	0.27	0.07	200	0.73	0.92	188
P-C_{10}/100	37	0.55	0.19	200	0.45	0.81	169
P-C_{14}/100	200	0.50	0.36	354	0.50	0.64	298
P-C_{18}/100	200	0.61	0.46	370	0.39	0.54	290
SDS[d]	340		1				
Py in water[e]	200		1				

[a] Temperature 20 °C; polymer concentration 0.5 g L^{-1}. [b] f_i fractional intensity, $f_i = a_i\tau_i/\sum a_i\tau_i$. [c] $\langle\tau\rangle$ average lifetime, $\langle\tau\rangle = \sum a_i\tau_i^2/\sum a_i\tau_i$. [d] [SDS] above the critical micelle concentration. [e] Saturated solution.

Table III
Fluorescence Anisotropy of Perylene and 1,6-Diphenyl-1,3,5-hexatriene (DPH) in Amphiphilic PNIPAM Solutions[a]

polym	DPH[b]	perylene[c]	polym	DPH[b]	perylene[c]
P-C_{14}/200	0.27	0.14	P-C_{18}/100	0.28	0.15
P-C_{14}/100	0.28	0.15	P-C_{18}Py/200		0.23
P-C_{18}/200	0.29	0.14	P-C_{18}Py/400		0.23

[a] Temperature 20 °C. [b] Polymer concentration: 2 g L^{-1}. [c] Polymer concentration: 1 g L^{-1}.

decay obeyed a single-exponential decay law, with $\tau = 220 \pm 10$ ns for all three amphiphilic copolymers.

Microviscosity. The viscosity of the environment within a micelle or any such hydrophobic microdomain, termed microviscosity, can in principle be determined from the depolarization of fluorescence of probes specifically solubilized in such domains. In this study 1,6-diphenyl-1,3,5-hexatriene (DPH) and perylene (Per) were chosen as fluorophores, since their use as microviscosity probes is well documented. Anisotropy values (r) measured for DPH and Per in aqueous solutions of the C_{14}- and C_{18}-PNIPAM samples are listed in Table III. They do not depend on the chain length or on the level of substitution of alkyl chains. They are markedly different from the values of the same probes in SDS micelles ($r = 0.070$, DPH, reported value 0.070)[27] or in hexadecyltrimethylammonium bromide micelles ($r = 0.026$, Per, reported value 0.038),[28] but for DPH they fall in an range intermediate between liposomes in the solid phase ($r = 0.31$ in DMPC at 20 °C) and in the fluid phase ($r = 0.17$ in DMPC at 27 °C). Also, they are of the same magnitude as those reported for DPH in aqueous solutions of related polymeric amphiphiles: poly(ethylene-*co*-maleic acid), poly(1-decene-*co*-maleic acid), and poly(1-octadecene-*co*-maleic acid), 0.187, 0.225 and 0.273, respectively.[27] Anisotropies could not be measured in solutions of PNIPAM and of the C_{10}-PNIPAM copolymers. The intensities of the emission were too low, indicating that the probes were not solubilized by the polymer. This observation is consistent with the pyrene fluorescence data already presented.

Spectroscopic Properties of the Pyrene-Labeled Amphiphilic PNIPAM Samples in Aqueous and Methanolic Solutions at Room Temperature. In methanolic solutions PNIPAM-C_{18}Py/200 and PNIPAM-C_{18}Py/400 exhibit an emission due to locally excited pyrene chromophores (intensity I_M, pyrene "monomer" emission) with the (0,0) band located at 376 nm. In water, at 20 °C, both polymers show a strong, broad, and featureless emission centered at 480 nm, in addition to the pyrene monomer emission (Figure 6). This emission (intensity I_E) originates from pyrene excimers. Identical excitation spectra were obtained for emissions monitored at 376 and 480 nm, and their maxima correspond to the UV absorption spectra. Therefore both the monomer and excimer

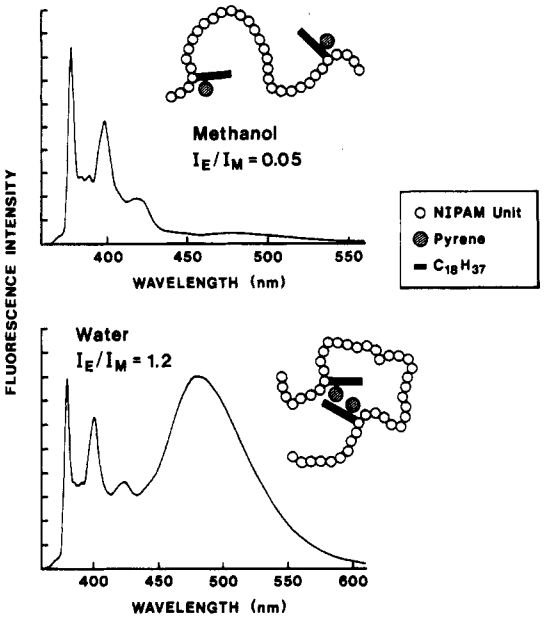

Figure 6. Fluorescence spectra of PNIPAM-C_{18}Py/200 (a) in methanol and (b) in water (polymer concentration 40 ppm, λ_{exc} = 330 nm, 15 °C).

emissions originate from excited isolated pyrene chromophores.[29] The dynamic nature of the excimer was confirmed by fluorescence decay measurements. The excimer time-dependent profiles showed a growing-in component ($\tau \approx 20$ ns) and a complex decaying component ($\langle\tau\rangle \approx 110$ ns, Table IV) for polymer solutions in water and in methanol.

The fact that pyrene excimer emission is very strong for aqueous polymer solutions implies that the pyrene groups are in close spatial proximity. This may be due either to a nonstatistical distribution of the pyrene groups along the polymer contour or to the occurrence of hydrophobic microdomains that bring together pyrene groups attached to monomer units well separated along the polymer chain. It seems unlikely that the pyrene-containing units are clustered as blocks along the polymer chain for the following reasons: (1) the synthetic procedure was chosen to prevent such block formation; (2) when the same polymers are dissolved in good solvents, THF or methanol for example, their emission exhibits little or no contribution from pyrene excimers, contrary to what would be expected to occur if the pyrene groups were attached to neighboring monomer units. Further evidence for the existence of hydrophobic microdomains arises from the following experiments:

Concentration effects: Spectra of PNIPAM-C_{18}Py/400 and PNIPAM-C_{18}Py/200 in water were recorded over a concentration range as wide as possible. The relative amount of excimer emission (intensity I_E) to monomer emission (intensity I_M) was monitored. The ratio was remarkably insensitive to changes in polymer concentration ranging from 25 to 250 ppm ($I_E/I_M = 1.10 \pm 0.02$ for PNIPAM-C_{18}Py/200; $I_E/I_M = 0.75 \pm 0.05$ for PNIPAM-C_{18}Py/400). This fact implies that the overall solution structures do not change once a minimum polymer concentration has been reached. Whether the micelles are intramolecular or involve aggregation of several polymer chains has not been determined unambigously. It is the object of current investigations.

Microviscosity: For determinations of microviscosities in solutions of the labeled polymers only Per could be used, since DPH cannot be excited selectively in the presence of pyrene. Fluorescence anisotropy values for

Table IV
Fluorescence Lifetimes of Pyrene in PNIPAM–C_{18}Py/200 and PNIPAM–C_{18}Py/400[a]

	water				methanol			
	monomer		excimer		monomer		excimer	
polymer	τ, ns	prefactor	τ, ns	prefactor	τ, ns	prefactor	τ, ns	prefactor
P–C_{18}Py/200	$\tau_1 = 32$	$a_1 = 0.69$	$\tau_1 = 21$	$a_1 = -0.26$	$\tau_1 = 44$	$a_1 = 0.15$	$\tau_1 = 23$	$a_1 = -0.70$
	$\tau_2 = 119$	$a_2 = 0.31$	$\tau_2 = 19$	$a_2 = 0.18$	$\tau_2 = 129$	$a_2 = 0.85$	$\tau_2 = 110$	$a_2 = 1.00$
			$\tau_3 = 80$	$a_3 = 0.82$				
	$\langle\tau\rangle = 59$				$\langle\tau\rangle = 124$			
P–C_{18}Py/400	$\tau_1 = 27$	$a_1 = 0.58$	$\tau_1 = 27$	$a_1 = -0.88$	$\tau_1 = 112$	$a_1 = 0.65$		
	$\tau_2 = 107$	$a_2 = 0.42$	$\tau_2 = 29$	$a_2 = 0.47$	$\tau_2 = 205$	$a_2 = 0.35$		
			$\tau_3 = 88$	$a_3 = 0.53$				
	$\langle\tau\rangle = 86$				$\langle\tau\rangle = 144$			

[a] Temperature 20 °C.

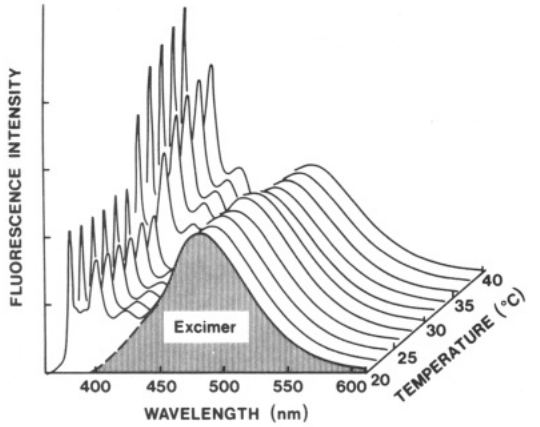

Figure 7. Emission spectra of PNIPAM–C_{18}Py/200 at various temperatures (polymer concentration 38 ppm, λ_{exc} = 330 nm).

Per in both solutions were higher than in the solutions of the corresponding unlabeled amphiphilic polymers, PNIPAM–C_{18}/100 and PNIPAM–C_{18}/200 (Table III). A small bathochromic shift (≈3 nm) of the emission maxima for Per in solutions of the labeled polymers, compared to C_{18}–PNIPAM polymers, may indicate the occurrence of specific interactions between Py and Per. These may contribute toward an increase in Per fluorescence anisotropy. The fluorescence depolarization of the pyrene labels was measured as well. Extremely low values of r were obtained, for both Py monomer and excimer emissions.

Temperature effects: Solutions of PNIPAM–C_{18}Py/200 and PNIPAM–C_{18}Py/400 in water were heated from 20 to 40 °C, and the pyrene fluorescence was monitored. The spectra underwent noticeable changes as the solution temperature reached the LCST. The Py monomer emission increased at the expense of Py excimer emission (Figure 7). The overall fluorescence intensity remained essentially constant over the temperature range probed. The spectroscopic changes were thermoreversible: upon cooling of the polymeric solutions, the Py monomer emission decreased and the Py excimer emission recovered its initial intensity. Excitation spectra remained unaffected by the heating and cooling cycles.

Discussion

Poly(*N*-isopropylacrylamide) and Amphiphilic PNIPAM in Water. The solution properties of PNIPAM in water have been examined by a variety of experimental techniques, including viscosity measurements,[22] osmometry,[14] light scattering,[30] and microcalorimetry.[31] The following description of PNIPAM solutions has emerged. In organic solvents the polymer behaves as a flexible coil, as indicated by Mark–Houwink exponents $\alpha \approx 0.65$ in methanol and THF.[22] In water the polymer becomes more elongated, presumably as a result of hydrogen bonding between the amide groups of the polymer and surrounding water molecules.[31] These interactions trigger the formation of a layer of highly organized water around the polymer chains. From the thermodynamic point of view, the formation of hydrogen bonds between polymer and water contributes favorably to the enthalpy of mixing ($\Delta H_m < 0$) and unfavorably to the entropy of mixing ($\Delta S_m > 0$). As the solution temperature is raised, hydrogen bonds are broken and bound water is released. The relative values of the thermodynamic functions change: the entropic term becomes dominant, such that the free energy of mixing takes a positive value at the LCST. These changes result on a macroscopic scale in the separation of a polymer-rich phase, concomitant, on the molecular level, with a collapse of the polymer chains from expanded coils to a more compact conformation.[30]

The results of the study reported here clearly demonstrate that the properties of PNIPAM can be severely affected by the attachment of very low levels of *N*-alkyl chains to the polymer backbone. One aspect or our results concerns the importance of the size of the pendant alkyl groups in controlling the behavior of amphiphilic co-PNIPAM. This is discussed here with particular emphasis on the thermally induced changes in solution properties.

Solutions below the LCST: *N*-Decyl-containing copolymers do not form organized hydrophobic microdomains in water. They do not solubilize hydrophobic probes. Their properties are almost entirely dictated by the PNIPAM main chain. The situation is quite different in solutions of the tetradecyl- and octadecyl-substituted copolymers. These readily solubilize hydrophobic dyes, as indicated by the following changes, compared to solutions of PNIPAM and C_{10}–PNIPAM: (1) a decrease in I_1/I_3 of the pyrene emission, (2) an increase in pyrene lifetimes, and (3) an enhancement in the fluorescence intensity of Per and DPH. There are however some differences among the four polymers. These are particularly apparent when one compares changes in I_1/I_3, plotted not as a function of polymer concentration but as a function of alkyl group concentration (Figure 8). In solutions of the C_{14} copolymers there is a consistent difference in I_1/I_3 at a given alkyl group concentration: I_1/I_3 is always higher in solutions of PNIPAM–C_{14}/200 than in solutions of PNIPAM–C_{14}/100. The environment sensed by pyrene is more polar in the least-substituted copolymer, implying a clear difference in the extent of hydrophobic structure in the solutions of the two C_{14}-containing polymers. This difference is erased in the C_{18} copolymers. Here the micropolarity sensed by pyrene in the two polymeric solutions is the same for a given alkyl group concentration. Our results corroborate earlier reports on the effects of the size of a pendant alkyl group

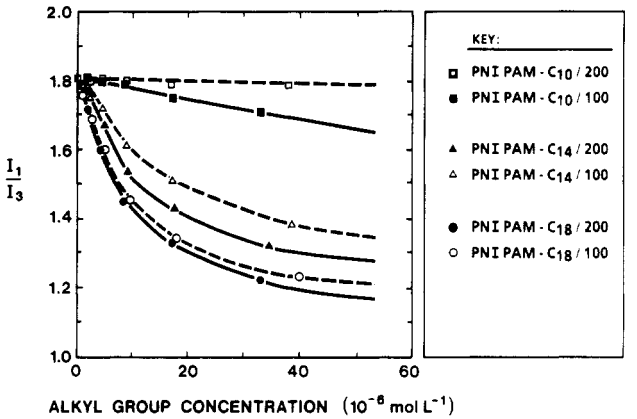

Figure 8. Plot of the changes in the ratio I_1/I_3 for pyrene in aqueous solutions of amphiphilic poly(*N*-isopropylacrylamides) as a function of polymer concentration expressed in alkyl chain concentration (15 °C, λ_{exc} = 336 nm).

on the behavior of amphiphilic copolymers, such as copolymers of maleic anhydride and alkyl vinyl ether[32] and alkyl-substituted polyacrylamides.[8]

Solutions above the LCST: The differences between copolymer solutions as a result of different alkyl group sizes tend to disappear when the solutions are heated above their LCST. Pyrene becomes completely solubilized in the polymer-rich phase which is formed above the cloud point, as seen, for example, by the fact that the pyrene emission decay follows a single-exponential decay law. The micropolarity sensed by pyrene is almost independent of polymer composition. The value of I_1/I_3 is 1.33 ± 0.10 for all polymers, including PNIPAM. Note that this range also includes the value ($I_1/I_3 \approx 1.40$) reported by Schild and Tirrell for a hexadecyl-substituted PNIPAM containing 1.1 mol % alkyl groups.[25] In summary our observations are consistent with the following behavior of the amphiphilic copolymer in water: below the LCST, the C_{14} and C_{18} copolymers form organized micelle-like hydrophobic microdomains. The micelles are disrupted at the LCST. Above the LCST, a polymer-rich phase forms, in which the alkyl chains are distributed randomly among collapsed and aggregated PNIPAM chains.

Pyrene-Labeled Amphiphilic PNIPAM in Water. The chemical structure of the labeled polymers closely matches that of the C_{18}–PNIPAM copolymers described in the previous section. They consist of a PNIPAM main chain that carries at random a small number of hydrophobic substituents. These are not single alkyl chains as in the case of PNIPAM–C_{18} but pairs of hydrophobes composed of a C_{18} alkyl group and a pyrenylbutyl group. It should not be a surprise that the overall solution properties of the labeled polymers closely match those of the corresponding PNIPAM–C_{18} copolymers. For example, they exhibit comparable solubility in water at room temperature. The values of their LCST are identical for a given level of hydrophobe incorporation (Table I). Fluorescence depolarization measurements with perylene indicate the presence of hydrophobic microdomains in cold water. The anisotropy values are slightly higher in the pyrene labeled polymers (Table II). From the available data it is not possible to determine whether this observation reflects an increased rigidity of the microdomains or specific interactions between perylene and pyrene.

This description of the solution properties of PNIPAM–C_{18} is corroborated by the spectroscopy of the pyrene label in solutions of PNIPAM–C_{18}Py. Below the LCST the pyrene groups are confined in hydrophobic microdomains created by the alkyl substituents. They are kept in close

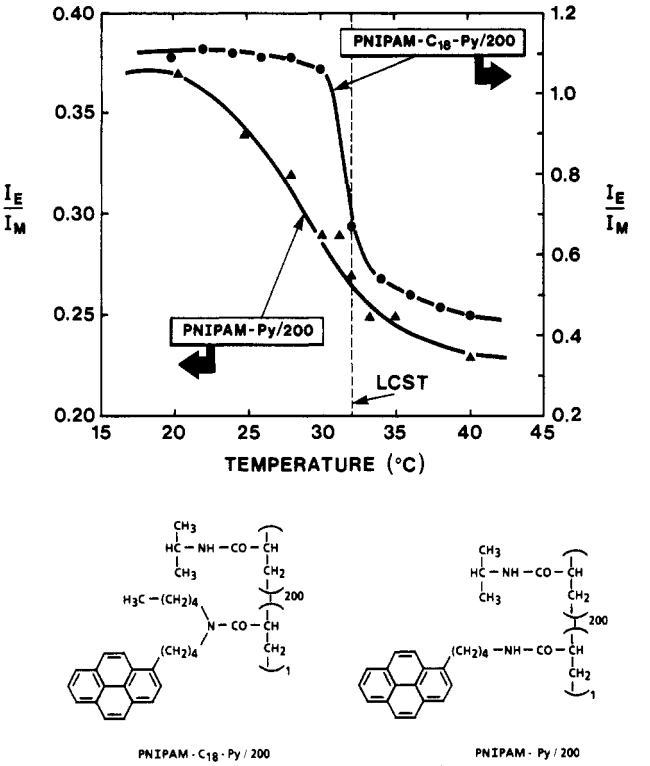

Figure 9. Plot of the changes in ratio I_E/I_M as a function of temperature for pyrene-labeled polymers: (a) PNIPAM–C_{18}Py/200 and (b) PNIPAM–Py/200.

proximity, as confirmed by the strong contribution of excimer emission to the overall pyrene fluorescence. The insensitivity to concentration, above ~ 25 ppm, of the ratio of excimer-to-monomer emission intensity points to the formation of polymeric micelles of a structure unaffected by increasing polymer concentration. When solutions are heated above the LCST, the excimer emission intensity decreases rapidly. The monomer emission increases simultaneously. These changes signal the disruption of the hydrophobic microdomains also observed in PNIPAM–C_{18} solutions. Above the LCST the hydrophobic substituents are distributed randomly in the polymer-rich phase.

Comparison of PNIPAM–C_{18}Py with Other Pyrene-Labeled Water-Soluble Polymers. The spectroscopy of pyrene in aqueous solutions of PNIPAM–C_{18}Py is interpreted easily in terms of photophysical mechanisms well established for pyrene in organic media. It contrasts sharply with the behavior of pyrene attached to other neutral water-soluble polymers, such as (hydroxypropyl)-cellulose,[33] poly(ethylene oxide),[34] and PNIPAM.[35] The major difference is that in all the reported examples of pyrene-labeled water-soluble polymers the pyrene excimers originate from preformed ground-state pyrene aggregates.[36] Such ground-state pyrene dimers or higher aggregates exist only under very special conditions. Their occurrence in aqueous polymeric solutions has been attributed to a gain in free energy of mixing through hydrophobic interactions between the nonpolar pyrene groups. A brief comparison of the spectroscopic properties of PNIPAM–C_{18}Py/200 and PNIPAM–Py/200[35] (Figure 9) is presented here to emphasize the difference in solution properties of two polymers for which the only difference in structure resides in the incorporation of about 16 C_{18} alkyl groups per polymeric chain.

Both polymers consist of a poly(*N*-isopropylacrylamide) main chain labeled with the same amount of pyrene chromophores (1 pyrene/200 monomer units). Aqueous so-

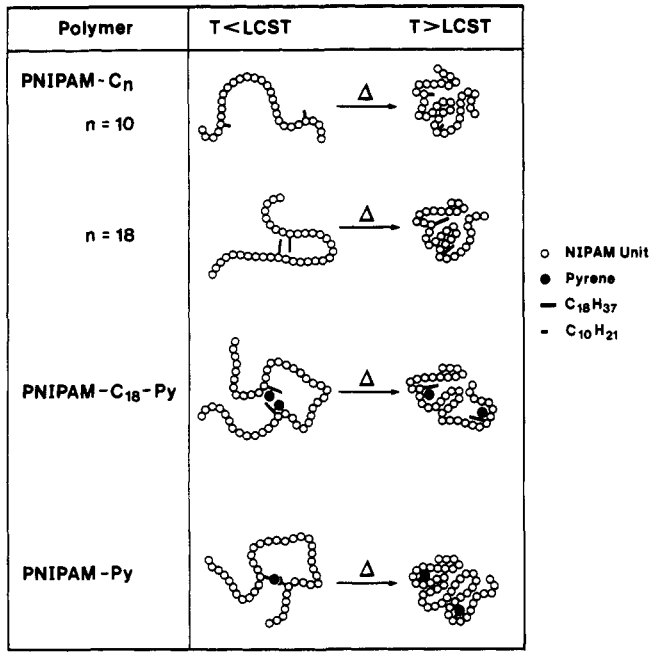

Figure 10. Schematic representation of the thermally induced polymer conformational changes for PNIPAM-C_n, PNIPAM-C_{18}Py, and PNIPAM-Py.

lutions of PNIPAM-Py/200 at room temperature exhibit a pyrene monomer emission and an excimer emission (I_E/I_M = 0.38). This value is lower than in PNIPAM-C_{18}-Py/200 (I_E/I_M = 1.10). Also, the overall pyrene fluorescence intensity from PNIPAM-Py/200 is lower, as a result of a large extent of pyrene self-quenching. Two marked differences between the two polymers are also observed in the thermally-induced spectroscopic changes that occur as the solutions are heated above their LCST. First, in the case of PNIPAM-Py/200, there is a large increase in the total fluorescence intensity, as the temperature exceeds the LCST. Second, the monomer and excimer intensities increase and decrease, respectively, over a wide temperature range below the LCST. This feature is apparent if one compares plots of the thermally induced changes in I_E/I_M for the two polymers (Figure 9). In the case of PNIPAM-Py/200 solutions, heating results in a gradual breaking of the pyrene ground-state aggregates, concomitant with small fluctuations in the polymer conformations. These phenomena are followed at the LCST by a collapse of the polymer chains and intrapolymeric aggregation, resulting in the formation of a phase that provides a hydrophobic environment to the pyrene chromophores. In the case of the amphiphilic PNIPAM-C_{18}Py the decrease in I_E/I_M at the LCST is much more abrupt, indicating that below the LCST the environment sensed by the pyrenes is that of the hydrophobic microdomains. The chromophores do not feel the small changes in the conformation of PNIPAM that occur below the LCST. Our present understanding of the thermally induced changes in aqueous solutions of PNIPAM-C_n PNIPAM-C_{18}Py, and PNIPAM-Py is presented pictorially in Figure 10.

Summary

The properties of a series of PNIPAM amphiphiles have been investigated in aqueous solutions below and above their LCST. This study, which was focused on changes in fluorescence of low molecular weight hydrophobic probes and of pyrene-labeled polymers, has led to the following conclusions: Below the LCST the solution properties of amphiphilic PNIPAM depend on the size of the alkyl group attached: PNIPAM-C_{10} exhibit a behavior typical of PNIPAM, while solutions of PNIPAM-C_{14} and PNIPAM-C_{18} are characterized by the formation of hydrophobic microdomains. Above the LCST, the C_{10}, C_{14}, and C_{18} copolymers form a polymer-rich phase that incorporates the alkyl groups as separate entities. The overall composition of this phase appears to resemble closely that formed by heat-induced precipitation of PNIPAM. The spectroscopy of the pyrene labels attached to C_{18}-PNIPAM is consistent with the presence of hydrophobic microdomains below the LCST, and, above the LCST, of a less hydrophobic phase in which the pyrene chromophores are randomly distributed. The collapse of the PNIPAM chains at the LCST severely disrupts the hydrophobic interactions that exist at room temperature. This effect can be used to great advantage to control thermally the properties of polymers attached to model membranes, as will be described in a forthcoming pub lication.

Acknowledgment. We thank Dr. G. Hamer (XRCC) for his help in measuring and interpreting the NMR spectra, Mr. T. Davidson (XRCC) for GPC measurements, and Miss S. Berg (Universität Mainz) for help in the syntheses. We also thank Professor M. A. Winnik (University of Toronto) for many stimulating discussions as the work was in progress and for his comments on this paper. Financial support for this work was provided in part by the Bundesminister für Forschung and Technologie (H.R. and J.V.). Part of J.V.'s expenses for his stay in Mississauga was provided by an NSERC (Canada) grant to Professor M. A. Winnik.

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