Solution Properties of Hydrophobically Modified Copolymers of *N*-Isopropylacrylamide and *N*-Glycine Acrylamide: A Study by Microcalorimetry and Fluorescence Spectroscopy

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Received November 10, 1999; Revised Manuscript Received February 16, 2000

ABSTRACT: The temperature- and pH-induced coil−globule transition has been studied in dilute aqueous solutions for different copolymers of *N*-isopropylacrylamide (NIPAM) and *N*-glycine acrylamide (Gly) using turbidimetry, scanning microcalorimetry, fluorescence spectroscopy, and dynamic light scattering. The four different samples prepared are a copolymer of NIPAM and Gly (PNIPAM−Gly), a copolymer of NIPAM, Gly, and *N*-(1-pyrenyl)methylacrylamide (PNIPAM−Gly−Py), and their hydrophobically modified (HM) derivatives, namely a copolymer of NIPAM, Gly, and *N*-(*n*-octadecylacrylamide) (PNIPAM−Gly−C_{18}) and a copolymer of NIPAM, Gly, and *N*-[4-(1-pyrenyl)butyl]-*N*-*n*-octadecylacrylamide (PNIPAM−Gly−C_{18}Py). Polymeric micelles 16 ± 2 nm in diameter were detected in cold solutions of the hydrophobically modified polymers. All polymers underwent pH-dependent phase separation upon heating. Endotherms with enthalpies on the order of the strength of hydrogen bonds were observed at temperatures concurring, in the case of PNIPAM−Gly and PNIPAM−Gly−Py, with the transition temperatures detected by classical cloud-point measurements. Discrepancies between the two values were detected in the case of the hydrophobically modified polymers. Evidence from fluorescence spectroscopy, corroborated by dynamic light scattering and microcalorimetry data, suggests that the pH- or temperature-stimulated coil to globule collapse of the polymer main chain does not trigger the disruption of the hydrophobic core of HM-polymer micelles.

Introduction

It is a well-known fact that a large number of aqueous polymer solutions exhibit heat-induced phase separation. The macroscopic phenomenon is the same for all polymers: a clear solution suddenly becomes "milky" when it is heated to a specific temperature. On a molecular level, though, quite distinct mechanisms can be operative for different classes of polymers. Poly(*N*-isopropylacrylamide) (PNIPAM) in water undergoes heat-induced phase transition at about 31 °C.[1] Below this temperature, the dissolution of the polymer in water is favored as a result of hydrogen bonding between water molecules and the polymer *N*-isopropyl groups. The water molecules take specific orientations to form these hydrogen bonds, leading to a negative entropic contribution to the free energy (ΔG) of solution. An increase in solution temperature triggers the disruption of hydrogen bonds formed between water molecules and the polymer. When the solution reaches a temperature (cloud point or lower critical solution temperature, LCST) for which the entropic component exceeds the enthalpic contribution to ΔG of solution ($\Delta G > 0$), polymer precipitation occurs. A nice feature of the phase transition of PNIPAM is that the actual temperature of the transition can be finely tuned through the incorporation of comonomers within the NIPAM backbone. For example, copolymers incorporating units bearing pH-sensitive functional groups, such as carboxylic acids or tertiary amines, possess a pH-sensitive cloud point in aqueous solutions. Examples include graft and random copolymers of NIPAM and acrylic acid,[2−5] copolymers of NIPAM and monomers with amino acid side chains,[6,7] or terpolymers of NIPAM, butyl methacrylate, and (*N*,*N*-diethylamino)ethyl methacrylate.[8] Like PNIPAM itself, these copolymers do not aggregate in water if their solutions are kept below their pH-dependent cloud point.

Others monomers often incorporated along the PNIPAM backbone are *N*-alkylacrylamides with alkyl groups chains ranging from dodecyl to hexadecyl. Such copolymers, known as hydrophobically modified (HM) copolymers, form micellar assemblies in water at all temperatures.[9,10] Below the cloud point, the polymeric micelles, consisting of hydrophobic microdomains surrounded by a corona of hydrated PNIPAM chains, are kept apart from each other. As the micellar solution reaches its cloud point, the polymer chains collapse, triggering aggregation of individual micelles and subsequent macroscopic phase separation. We reported recently the synthesis of a hydrophobically modified copolymer of NIPAM and a pH-sensitive comonomer, *N*-glycine acrylamide.[11] Preliminary experimental results based on fluorescence spectroscopy and cloud-point determinations led us to conclude that this copolymer (PNIPAM−Gly−C_{18}Py, Figure 1) forms micellar aggregates in water below the solution cloud point and that the phase separation temperature depends on pH and ionic strength. Thus, this polymer possesses two of the key properties of responsive polymers, and indeed we demonstrated that complexes of this copolymer and liposomes may be used as temperature- or pH-responsive delivery agents.

To gain further control over the solution properties of this copolymer, we conducted a series of experiments designed, on the one hand, to assess via fluorescence spectroscopy and dynamic light scattering the fate of the hydrophobic microdomains during phase transition

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10.1021/ma9919054 CCC: $19.00
Published on Web 03/31/2000

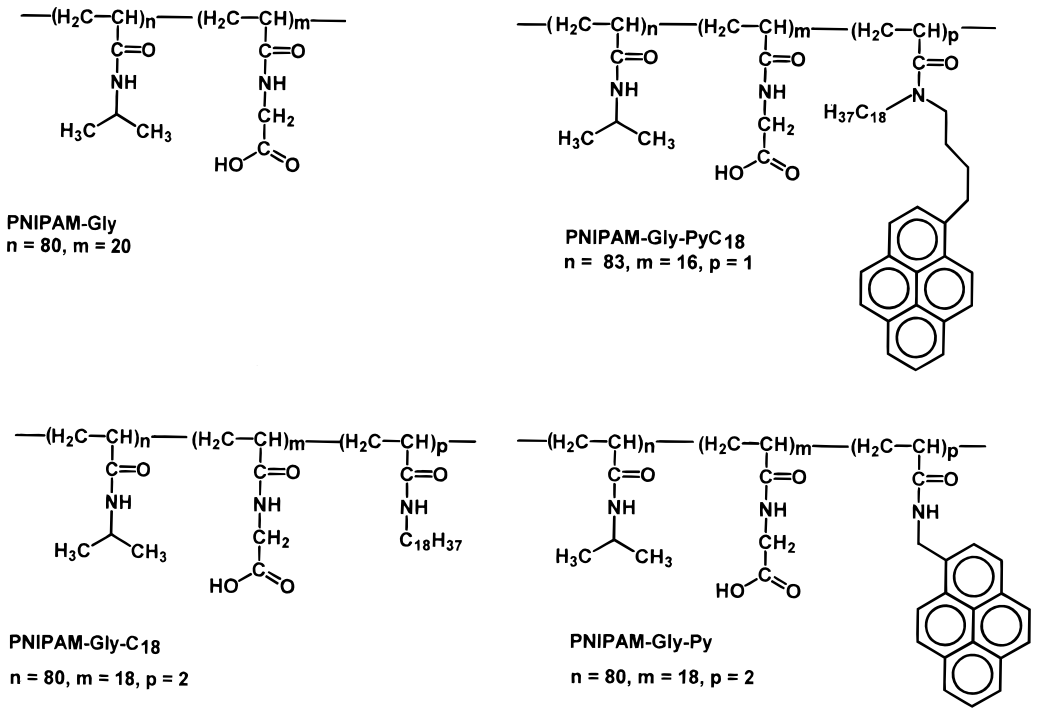

Figure 1. Structure of the polymers used in this study.

and, on the other hand, to determine via microcalorimetry the thermodynamic characteristics of the phenomenon. It is common to determine the temperature of the heat-induced phase transition of PNIPAM solutions in water by simple detection of changes in solution turbidity, either visually or spectrophotometrically. This approach is suitable for well-behaved systems, but often it is complicated by variations in the size of the precipitated aggregate and by partial settling of precipitates.[12,13] Since several polymer solutions studied here did not have sharply defined cloud points, we examined all the solutions by microcalorimetry, a more sensitive method.[14] This technique not only can provide the transition temperature and the thermodynamic parameters associated with a phase change but also can give a measure of the cooperativity of the transition.[15] Thus, Tiktopulo and co-workers have reported that the collapse of PNIPAM in water is not an "all-or-non transition", but that it proceeds by the independent collapse of polymer chains of ca. 90 units.[16,17]

Fluorescence spectroscopy and dynamic light scattering studies were used to probe the properties of the hydrophobic microdomains. In fluorescence experiments, we take advantage of the dual emission of the pyrene-labeled copolymer PNIPAM−Gly−C_{18}Py. In aqueous solutions, this polymer exhibits an emission spectrum consisting of two components: a "pyrene monomer" emission due to the emission of spatially isolated excited pyrenes and a "pyrene excimer" emission resulting from the emission of an excited-state complex formed between two pyrene chromophores. High excimer intensity, relative to the monomer emission intensity, suggests that pyrene groups are in close proximity, incorporated within hydrophobic microdomains. A pH- or temperature-induced decrease in excimer emission and concomitant increase in monomer intensity signals the disruption of these hydrophobic microdomains.

In this work, cloud-point determinations, microcalorimetry, dynamic light scattering, and fluorescence spectroscopy have been used to analyze the association behavior in water of PNIPAM−Gly−C_{18}Py and to study the dependence of the aggregation process on the ionic strength, pH, and temperature of the solution. Several model copolymers were studied as well, to allow an independent assessment of (1) the influence of hydrophobic modification and (2) the incorporation of a pH-sensitive comonomer. These model compounds are copolymers of (1) NIPAM and *N*-glycine acrylamide (PNIPAM−Gly), (2) NIPAM, *N*-glycine acrylamide and *N*-(1-methylpyrenyl)acrylamide (PNIPAM−Gly−Py), and (3) NIPAM, *N*-glycine acrylamide, and *N*-*n*-octadecylacrylamide (PNIPAM−Gly−C_{18}) (Figure 1). The preparation and characterization of these copolymers are presented in the first section. Results from microcalorimetry, together with data gathered from dynamic light scattering measurements and fluorescence spectroscopy, are discussed in terms of the pH- and temperature-dependent formation and disruption of micellar assemblies of the various NIPAM copolymers prepared.

Experimental Section

Materials. Water was deionized with a Barnstead NANOpure water purification system. Reagent grade solvents (Caledon) were used without further purification, except for tetrahydrofuran (THF), which was distilled from sodium under nitrogen, and dichloromethane, which was dried over $MgSO_4$ (BDH). 1-Pyrenylmethylamine hydrochloride, acryloyl chloride, and *n*-octadecylamine were obtained from Aldrich Chemical Co. Inc. *N*,*N*-Azobis(isobutyronitrile) (AIBN) was purchased from Spectrum Chemicals. *N*-Acryloxysuccinimide (NASI) and *N*-isopropylacrylamide (NIPAM) were obtained form Acros Chemicals. Isopropylamine and glycine ethyl ester hydrochloride were purchased from Sigma. *N*-*n*-octadecylacrylamide was prepared as previously described.[18] NIPAM was recrystallized from a toluene:hexane (1:1, v/v) mixture. Isopropylamine was purified by distillation. Buffers were prepared from 0.1 M citric acid and 0.1 M sodium hydroxide solutions, except if otherwise stated. Ionic strength was adjusted by addition of NaCl (0.1 M). The copolymers PNIPAM−Gly and PNIPAM−Gly−C_{18}Py were prepared as described previously.[11]

Instrumentation. Proton NMR spectra were recorded on Bruker 200 and 500 MHz spectrometers. Infrared spectra were

recorded on a BioRad FTS-40 spectrometer. UV spectra were measured with a Hewlett-Packard 8452A photodiode array spectrometer, equipped with a Hewlett-Packard 89090A temperature controller. Potentiometric titrations were performed using a Tanager Scientific Systems 8901 dual pH meter and titrimeter. Solution viscosities were determined with an Ubbelohde viscometer with solutions of the polymers in THF kept at 27 °C. Gel permeation chromatography (GPC) measurements were performed with a Waters 590 programmable HPLC system (eluent 0.1 M $NaNO_3$, flow rate of 0.7 mL/min), Ultrahydrogel columns (Waters) equipped with a Waters 486 UV detector, and a Waters 410 differential refractometer. Dynamic light scattering was performed on a Brookhaven BI9000 AT instrument equipped with an argon laser ($\lambda = 514$ nm, scattering angle 90°). The measurements were performed at 20 °C using polymer solutions (0.1 g L^{-1}) equilibrated at room temperature for 24 h and filtered through a 0.45 μm membrane prior to measurements. Data were analyzed using the software provided by the manufacturer (CONTIN calculations).

Fluorescence Measurements. Fluorescence spectra were recorded on a SPEX Industries Fluorolog 212 spectrometer equipped with a GRAMS/32 data analysis system. Temperature control of the samples was achieved using a water-jacketed cell holder connected to a Neslab circulating bath. The temperature of the sample fluid was measured with a thermocouple immersed in a water-filled cuvette placed in one of the four cell holders in the sample compartment. The slits setting ranged from 0.5 to 2.5 nm (emission) and 1.0 to 2.0 nm (excitation) depending on the chromophore concentration. The excitation wavelength was 346 nm, unless otherwise stated. Excitation spectra were monitored at 378 nm (monomer emission) and 485 nm (excimer emission). Samples for spectroscopic analysis were prepared from stock solutions (5.0 g L^{-1}). Ionic strength was adjusted by the addition of NaCl (0.1 M). Solutions in water were not degassed. Solutions in methanol were degassed by vigorous purging (1 min) with methanol-saturated argon. The pyrene excimer-to-monomer ratio (I_E/I_M) was calculated by taking the ratio of the intensity (peak height) at 480 nm to the intensity at half sum of the intensities at 378 and 397 nm.

Cloud-Point Determinations. Cloud points were determined by spectrophotometric detection of the changes in turbidity ($\lambda = 600$ nm) of aqueous polymer solutions (1.0 g L^{-1}) heated at a constant rate (0.5 °C min^{-1}) in a magnetically stirred UV cell. The value reported is the temperature corresponding to a decrease of 20% of the solution transmittance.

Calorimetric Measurements. Calorimetric measurements were performed on a NANO differential scanning calorimeter N-DSC (Calorimetry Sciences Corp.) at an external pressure of 3.0 atm. The cell volume was 0.368 mL. The heating rate was 1.0 °C min^{-1}, unless other wise specified.

Syntheses. a. Synthesis of *N*-Acryloylglycine Ethyl Ester (NAGEE). Acryloyl chloride (2.33 mL, 28.6 mmol) was added dropwise over a period of 1 h to a solution of glycine ethyl ester (4.0 g, 28.6 mmol) and triethylamine (8 mL) in dichloromethane (200 mL) kept under nitrogen at 5 °C. At the end of the addition the reaction mixture was brought to room temperature and stirred overnight. The mixture was filtered to remove triethylamine hydrochloride. It was washed with water and brine and dried over $MgSO_4$. Evaporation of the solvent yielded an oil, which solidified upon standing at room temperature (3.8 g, 89%). The solid was recrystallized from benzene/hexane (1/1 v/v). ^{1}H NMR ($CDCl_3$): δ 1.30 (t, 3H, J = 7.1 Hz, OCH_2CH_3), 4.13 (d, 2H, J = 5.1 Hz, CH_2), 4.24 (q, 2H, J = 7.1 Hz, OCH_2CH_3), 5.70 (dd, 1H, J = 1.9, 9.7 Hz, *CH*H=CH−), 6.18 (dd, 1H, J = 9.7, 17.0 Hz, CH_2=C*H*), 6.26 (br, 1H, N*H*), 6.34 (dd, 1H, J = 1.6, 17.0 Hz, CH*H*=CH−). ^{13}C NMR ($CDCl_3$): δ 126.2 (C=C), 130.0 (C=C), 165.8 (C=O), 169.5 (C=O). MS (CI): m/z 158 (M + H, 100%).

b. Preparation of PNIPAM−Gly−Py. The polymer was obtained by postmodification of a copolymer (PNIPAM−Gly−NASI) of NIPAM (0.75 mol), NAGEE (0.20 mol), and *N*-acryloylsuccinimide (0.05 mol) prepared as described previously.[11] The copolymer PNIPAM−Gly−NASI (1.66 g) was dissolved in THF (20 mL). 1-Pyrenylmethylamine, hydrochloride (0.046 g, 0.17 mmol), and triethylamine (0.023 mL, 0.17 mmol) were added to the solution. The reaction mixture was kept at room temperature under nitrogen for 20 h. Isopropylamine (0.1 mL) was added to the mixture, which was stirred for an additional 2 h. The polymer was isolated by precipitation into dry diethyl ether. It was purified by repeated precipitations from THF into diethyl ether (1.48 g). The resulting polymer (1.38 g) was dissolved in THF/H_2O (4/1 v/v, 40 mL). Sodium hydroxide (0.1 g, 2.5 mmol) dissolved in THF/H_2O (4/1 v/v, 10 mL) was added to this solution. The solution was kept at room temperature for 15 h. Then the pH of the solution was adjusted to 3.6 with 0.1 N HCl. The solvent was concentrated until the polymer separated as an oily material. This was dissolved in THF and the resulting solution was added dropwise to vigorously stirred diethyl ether to recover the polymer by precipitation. The polymer was purified by further reprecipitation and dried in high vacuo for a few hours (1.35 g). GPC was used to determine the molecular weight of the polymer. The colums were calibrated with poly(ethylene oxide) standards. Through the use in tandem of a UV detector and a refractive index detector it was ascertained that PNIPAM−Gly−Py is not contaminated with low molecular weight UV-absorbing impurities and that all the chromophores are bound covalently to the polymer. The glycine content was obtained by titration of the carboxylic acids, using stepwise additions of HCl to a solution of fully ionized PNIPAM−Gly−Py. The amount of pyrene incorporation (1.2×10^{-4} mol of Py g^{-1} or 1 pyrene chromophore for every 71 NIPAM units) was determined from UV absorption data of polymer solutions in THF.

FT-IR (KBr, cm^{-1}): 3368 (NH stretch), 3072 (vinylic CH stretch), 2973 (CH_3 asym), 2873 (CH_3 sym), 1735 [**O**=**C**−(OH)], 1653 (**O**=**C**(NH) stretch), 860 (aromatic C−H out-of-plane bending). UV (methanol): λ_{max} 342 nm. ^{1}H NMR ($CDCl_3$): δ 1.13 (br, 3H, CH_3), 1.65−2.20 (m, backbone CH_2−CH), 3.99 (br, 1H, NHC**H**$(CH_3)_2$), 6.0−7.0 (br, NH), and 8.0−8.5 (m, aromatic H).

c. PNIPAM−Gly−C_{18}. NIPAM (4.5 g, 40 mmol), NAGEE (1.41 g, 9 mmol), and *N*-*n*-octadecylacrylamide (0.324 g, 1.0 mmol) were dissolved in dry THF (80 mL), and the solution was degassed with N_2 for 15 min. The solution was heated to 65 °C. AIBN (39 mg, 0.24 mmol) was added to initiate the polymerization. After 16 h at 65 °C, the mixture was cooled to room temperature. It was concentrated, and the polymer (5.5 g, 87%) was isolated by precipitation into diethyl ether. It was purified by two reprecipitations from THF into diethyl ether. The purified polymer (1.5 g) was dissolved in THF/water (4/1 v/v, 100 mL). A solution of sodium hydroxide (0.18 g, 4.5 mmol) in THF/water (4/1 v/v, 30 mL) was added to the polymer solution. The reaction mixture was kept at room temperature for 15 h. Then the pH of the solution was adjusted to 4.0 by dropwise addition of HCl (0.1 N). The solution was concentrated to yield a solid which was dissolved in THF. The polymer was isolated by precipitation into diethyl ether (1.3 g, 95%). It was further purified by repeated reprecipitations from THF into diethyl ether. FTIR (KBr, cm^{-1}): 3324 (NH stretch), 2877 (CH_3 sym), 1732 [**O**=**C**(OH)−], 1651 [(**O**=**C**(NH)], 1216 (C−O stretch).

Results and Discussion

Preparation of the Polymers. The pyrene-labeled copolymer PNIPAM−Gly−Py was prepared by postmodification of a copolymer of NIPAM, *N*-acrylamidoglycine ethyl ester (NAGEE) and *N*-acryloxysuccinimide (NASI) in THF, which was treated, first, with 1-pyrenylmethylamine to introduce the pyrene chromophore through an amide link and, second, with a mild base to hydrolyze the ester groups of the protected glycine residues (Figure 2). The hydrophobically modified copolymer PNIPAM−Gly−C_{18} was obtained by copolymerization of NIPAM, NAGEE, and *N*-(*n*-octadecyl)acrylamide, followed by deprotection of the carboxyl-

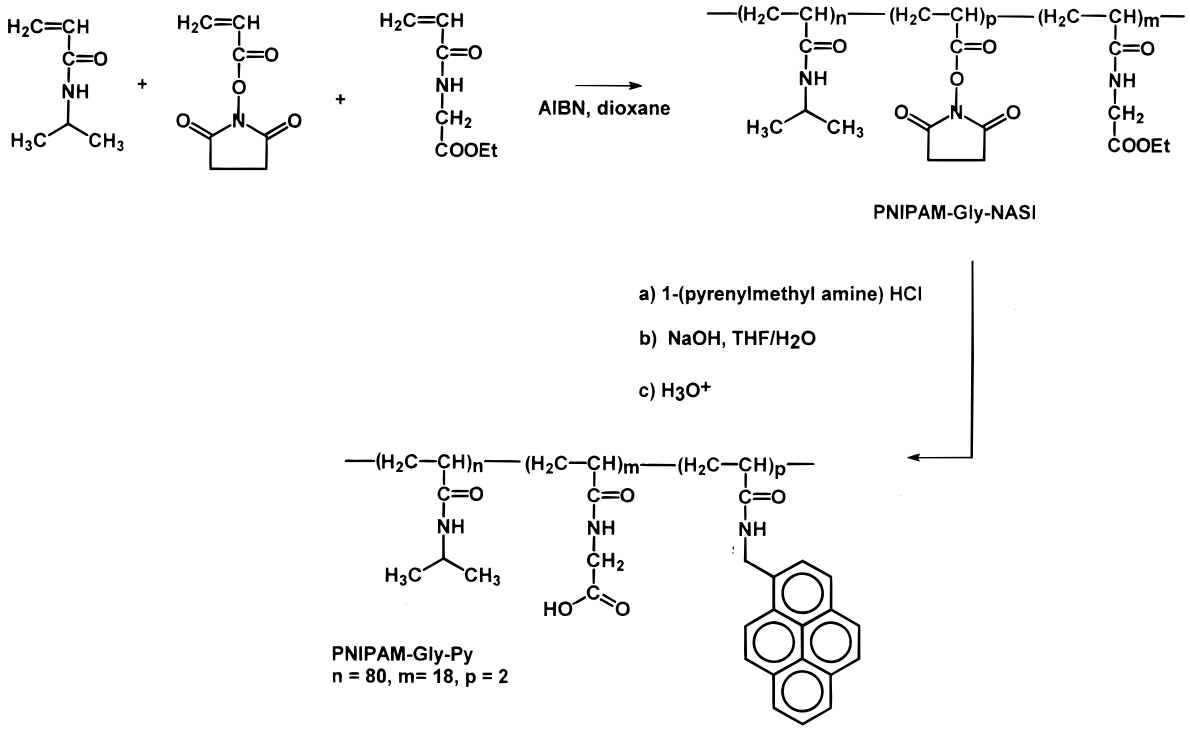

Figure 2. Synthetic scheme for the preparation of PNIPAM−Gly−Py.

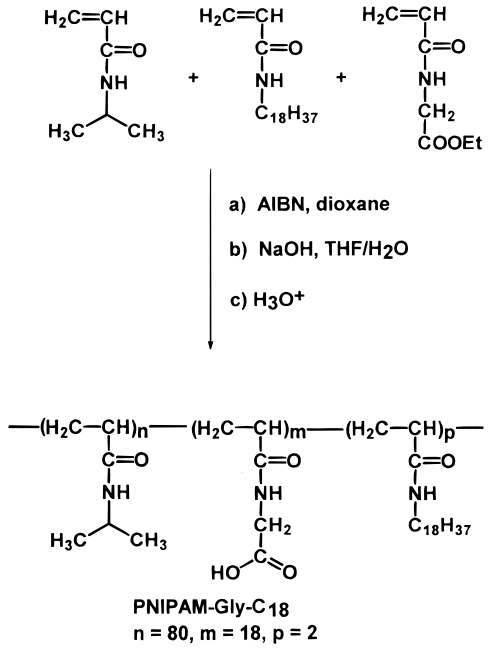

Figure 3. Synthetic scheme for the preparation of PNIPAM−Gly−C_{18}.

ic acid moieties (Figure 3). In both procedures, the polymerization proceeded in homogeneous solution, yielding polymers with a random distribution of the comonomers, since it has been demonstrated previously that the acrylamides NIPAM, NASI, and NAGEE have very similar reactivity ratios. The monomer feed ratio in all syntheses was chosen to yield copolymers having approximately the same glycine content (20 mol %). To ascertain that the actual polymer composition was indeed the desired one, the polymers were analyzed either in the protected ethyl ester form or in their final form, as described below and summarized in Table 1.

The exact glycine content of the copolymers was determined by titration of the carboxylic acid groups, using stepwise additions of HCl to a solution of fully ionized copolymers. These values were corroborated by analysis of the ^{1}H NMR spectra of the copolymers in the ester form. Use was made of the areas under the signal at 4.17 ppm, attributed to the resonance of the glycine ester unit methylene protons and the signal at 3.99 ppm, attributed to the resonance of the NIPAM residue methine proton. The level of pyrene incorporation (1.2×10^{-4} mol of Py g^{-1}) was obtained from UV absorption data of a polymer solution in MeOH, using 1-(pyrenyl)methylamine ($\epsilon_{342} = 32\,800$) as reference. This value corresponds to the composition of copolymers bearing approximately 1 pyrene chromophore for every 71 NIPAM units. The level of octadecyl substitution of PNIPAM−Gly−C_{18} was determined from the ^{1}H NMR spectrum of a polymer solution in $CDCl_3$, using the relative areas of the broad singlet at 3.77 ppm (NIPAM methine proton) and the triplet at 0.86 ppm (terminal methyl protons of $C_{18}H_{37}$).

Temperature Dependence of the Properties of the Polymers in Aqueous Solution. Cloud-Point Determinations. All the NIPAM/Gly copolymers were soluble in water at or below room temperature, independent of the pH of the solutions. However, depending on pH, their aqueous solutions became turbid when heated above their cloud point. This temperature was determined for polymer solutions of various pH by the simple spectrophotometric method based on the detection of changes in a solution transmittance at a wavelength of light absorbed by neither the solvent nor the solute ($\lambda = 600$ nm). A series of plots of the changes in turbidity of solutions of PNIPAM−Gly and PNIPAM−Gly−C_{18}Py as a function of temperature are presented in Figure 4, for solutions of pH ranging from 2.0 to 5.85. The pH of the solutions was adjusted through the use of citric acid buffers; the ionic strength (0.1 M NaCl) was kept constant throughout. The drop in transmittance as a function of temperature is very sharp in the case of acidic solutions, when the carboxylic groups of

Table 1. Physical Properties and Composition of the Polymers

polymer	composition (mol %)[b] NIPAM	gly	C_{18}	pyrene content[c] (mol g^{-1})	M_n	M_w (M_w/M_n)
PNIPAM−Gly[a]	80	20			30 000	77 000 (2.5)
PNIPAM−Gly−Py	78	17		1.2×10^{-4}	23 000	
PNIPAM−Gly−C_{18}	78	19	3		22 000	
PNIPAM−Gly−C_{18}Py[a]	83	19	1	9.4×10^{-5}	25 000	54 000 (2.2)

[a] From ref 11. [b] From analysis of the ^{1}H NMR spectra of the corresponding copolymers of NIPAM and NAGEE (see text). [c] From UV absorbance spectra.

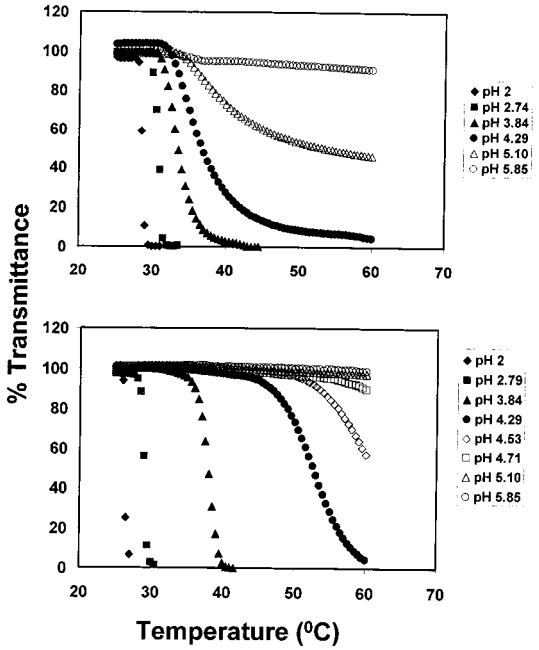

Figure 4. Changes in transmittance (λ = 600 nm) as a function of temperature for solutions of PNIPAM−Gly (top) and PNIPAM−Gly−C_{18}Py (bottom) of pH 2.0, 2.74, 3.84, 4.29, 5.10, and 5.85; citric acid buffers, [NaCl] = 0.1 M.

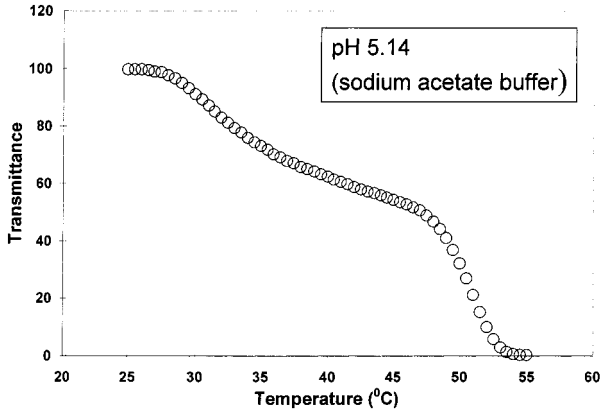

Figure 5. Changes in transmittance (λ = 600 nm) as a function of temperature for a solution of PNIPAM−Gly, pH = 5.14, sodium acetate buffer, [NaCl] = 0.1 M.

the glycine residues are fully protonated (pH 2.00 and 2.74). In this pH range, all the copolymer solutions have a similar cloud point (28 ± 0.5 °C, [NaCl] = 0.1 M). This value is slightly lower than the cloud point of PNIPAM (31 °C), indicating a slight increase in the hydrophobicity of the copolymers in their protonated form compared to the NIPAM homopolymer.

As the pH of the polymer solutions increases, and consequently the glycine groups are progressively deprotonated, the decrease in transmittance takes place over an increasingly broader temperature range, until a pH is reached for which the solution retains its clarity over the entire temperature range scanned (20−60 °C). The polymer is then fully ionized, and it remains soluble in water at all temperatures. Figure 5 displays a transmittance/temperature plot recorded when a solution of PNIPAM−Gly in an acetate buffer (pH: 5.14) was heated through its cloud point. The temperature dependence of solution transmittance is different from that recorded for a citric buffer solution of nearly identical pH. It seems to display two transitions, a situation that reflects the dependence of the cloud point of PNIPAM not only on the presence and concentration of salt but also on the chemical nature of the salt, in particular the anion structure.[19,20] The trends have been correlated with the viscosity *B* coefficient of ions, a measure of ion−water interaction.[21] For example, the chloride ion, which has a slightly negative *B* coefficient (−0.007), is a "salting-out" anion and functions as a structure maker for water. It strengthens hydrophobic interactions among the NIPAM acrylamide residues and promotes the formation of globular structures of the polymer.

The cloud points of copolymers exhibit a marked dependence on ionic strength. For example, in the case of PNIPAM−Gly−C_{18} in NaCl solutions of pH 2.0, when the polymer is fully protonated, the cloud point decreases linearly from 28 °C ([NaCl] = 0.1 M) to 16 °C ([NaCl] = 1.5 M). The same trend in transition temperature has been reported as part of a study of the cloud point of PNIPAM.[22] The cloud points of PNIPAM−Gly−C_{18} solutions of pH 4.0, where the glycine residues are partly ionized, also decrease with increasing [NaCl].

It is interesting to note that the presence of hydrophobic substituents, *n*-octadecyl or *N*-1-[(pyrenyl)butyl]-*N*-*n*-octadecyl moieties, does not affect significantly the cloud points of the respective glycine copolymers in their protonated forms (Figure 4). This was observed also in the case of HM-PNIPAM carrying a few molar percent of octadecyl groups.[18] However, large differences in cloud points between the PNIPAM/Gly copolymers and their hydrophobically modified analogues were recorded in solutions of pH 4−5 (Table 2), in which the copolymers exist in a partially deprotonated form. We note however that these cloud points were determined on the basis of turbidity measurements of solutions that did not exhibit a sharp decrease in transmittance upon heating past the cloud point (Figure 4, top). Arbitrarily throughout this study, we took as the cloud point of a solution the temperature corresponding to a decrease of 20% of the sample transmittance at 600 nm. This value may not reflect the true transition temperature when the drop in transmittance occurs gradually. To establish if indeed the discrepancies between glycine/NIPAM copolymers and their hydrophobically modified analogues are an artifact of the measurement technique, we conducted in parallel and with the same polymer solutions a microcalorimetry study of the heat-induced phase transitions.

Table 2. Cloud Points and Temperatures of Maximum Heat Capacity (T_{max}) of Aqueous Solutions of *N*-Isopropylacrylamide−*N*-Glycine Acrylamide Copolymers

polymer	pH	cloud point (°C)	T_{max} (°C)
PNIPAM−Gly	1.98	28.3 ± 0.3	
	2.77	30.4 ± 0.4	31.3 ± 0.4
	3.83	32.7 ± 1.7	
	4.25	34.4 ± 1.2	35.0 ± 0.4
	5.10	38.3 ± 2.3	37.4 ± 0.4
PNIPAM−Gly−Py	3.30	29.5 ± 0.2	28.9 ± 0.4
	3.70	38.5 ± 0.4	32.9 ± 0.4
	4.10	43.5 ± 1.5	39.2 ± 0.4
	4.53	54.5 ± 2.4	
PNIPAM−Gly−C_{18}Py	1.98	26.2 ± 0.3	
	2.77	28.6 ± 0.4	28.4 ± 0.4
	3.70	36.7 ± 1.3	36.3 ± 0.4
	4.10	49.0 ± 1.7	40.2 ± 0.4
	4.53	55.6 ± 2.3	

Microcalorimetry Studies of the Heat-Induced Phase Transition of the NIPAM/Gly Copolymers in Aqueous Solution. Figure 6a (top curve) presents the temperature dependence of the partial molar heat capacity, C_p, of PNIPAM−Gly in an aqueous solution of pH 2.77. The phase transition is endothermic with a sharp heat capacity peak. The temperature of maximum heat capacity (31.3 ± 0.4 °C) is slightly higher than the cloud point determined by changes in solution transmittance (30.4 ± 0.4 °C). The heat of transition (ΔH) per NIPAM unit is approximately 0.9 kcal/mol. This transition enthalpy is similar to that registered for the phase separation of PNIPAM solutions measured by us under the same conditions and reported earlier by Fujishige and co-workers.[12] It is consistent with a loss of approximately one hydrogen bond per repeat unit upon phase separation.[23] It can be seen that the heat capacity of PNIPAM−Gly solutions at high temperature is smaller than that of the polymer solutions at low temperature. This decrease has been reported in the case of PNIPAM in water.[16] It indicates that the collapse of the polymer causes a decrease in the number of polymer−water contacts, in analogy with the refolding of proteins upon heat after cold denaturation.[24] This situation is opposite to the more common increase in heat capacity observed during the temperature-induced denaturation of proteins.[15]

The changes with temperature of the molar heat capacity of aqueous solutions of PNIPAM−Gly, PNIPAM−Gly−Py, PNIPAM−Gly−C_{18}, and PNIPAM−Gly−C_{18}Py were recorded under identical conditions for solutions of pH ranging from 2.77 to 5.10 (Figures 6 and 7). Figure 6 summarizes our observations regarding the sensitivity of the LCST to pH of solutions of PNIPAM−Gly and PNIPAM−Gly−C_{18}Py. An increase in pH, or progressive deprotonation of the glycine residues, results in an increase in the transition temperature and a broadening of the endotherm. The heat of the transition per NIPAM unit is unaffected by the changes in pH. The same trends are observed in the endotherms recorded for solutions of PNIPAM−Gly−Py of pH 3.3, 3.7, and 4.1. Thus, the collapse of the copolymers is triggered primarily by the response of the NIPAM units to changes in solution temperature and only mildy affected by the presence of hydrophobic substituents.

We note that broad endotherms (e.g., solutions of pH 4.30 and 5.10) were recorded for solutions that, spectrophotometrically, exhibit sluggish temperature-dependent changes in transmittance (Figure 4). In contrast, the sharp transitions at low pH are observed as a sudden clouding of the solution. Moreover, for solutions of low pH the temperature of the maximum in the endotherm is nearly identical to the cloud point, but for solutions of higher pH there are significant differences between the two values. (Table 2). The two sets of data are strikingly different for solutions of the hydrophobically modified polymer: cloud-point values measured for solutions of pH 4−5 are much higher than the temperatures of the transitions detected by microcalorimetry, which reflect more accurately the thermodynamic phenomena at play in these complex systems. Our observations suggest that one needs to evaluate critically the reliability of cloud-point determinations.

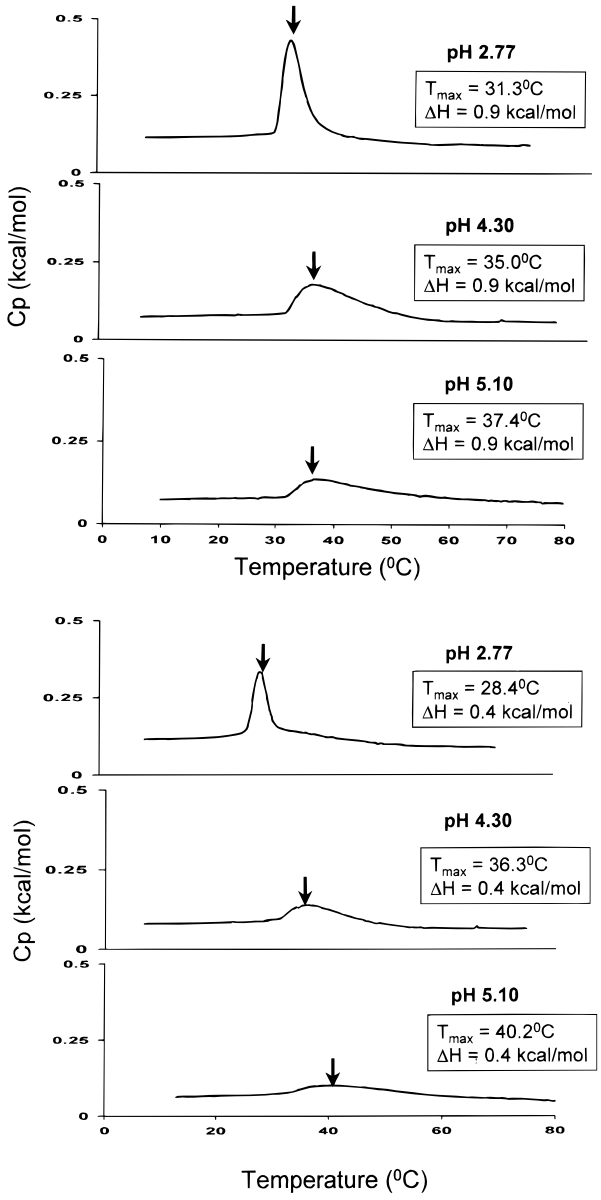

Figure 6. Microcalorimetric endotherms for aqueous solutions of PNIPAM−Gly (upper) and PNIPAM−Gly−C_{18}Py (lower) (1 g L^{-1}) of pH 2.77, 4.30 and 5.10; citric acid buffers, [NaCl] = 0.1 mol L^{-1}; heating rate 1 °C min^{-1}.

Dynamic Light Scattering Studies. Even though PNIPAM exhibits many properties characteristic of amphiphilic polymers, it does not form aggregates in water below the cloud point of the solution. No signal was detected by dynamic light scattering (DLS) analysis of aqueous PNIPAM solutions (20 °C, 0.1−5.0 g L^{-1}). Similarly, no signal was detected by DLS analysis of

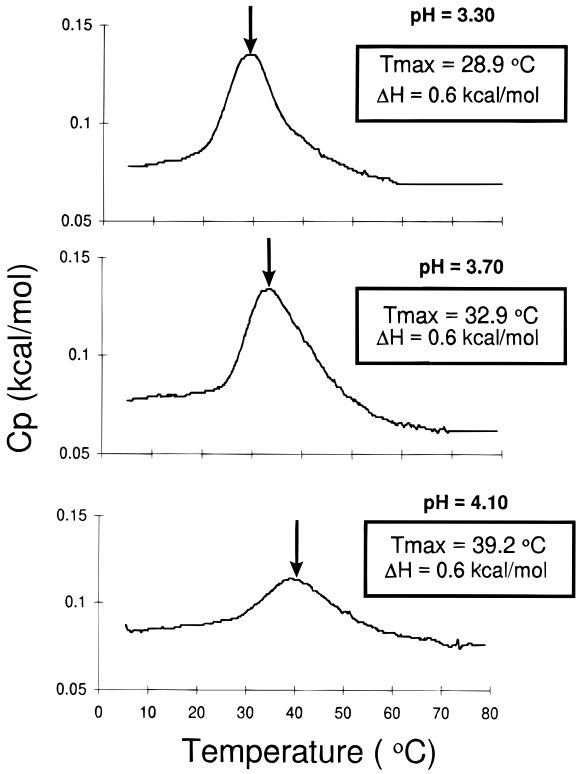

Figure 7. Microcalorimetric endotherms for aqueous solutions of PNIPAM−Gly−Py (1 g L^{-1}) of pH 3.3, 3.7, and 4.10; citric acid buffers, [NaCl] = 0.1 mol L^{-1}; heating rate 1 °C min^{-1}.

aqueous solutions of PNIPAM−Gly and PNIPAM−Gly−Py, even in the case of solutions of low pH, as long as the measurements were carried out below the cloud point of each solution. In contrast, a strong signal was detected when the DLS measurements were performed on aqueous solutions of the hydrophobically modified copolymers (20 °C, 0.1 g L^{-1}, [NaCl] = 0.1 M, pH 2.0−9.0). The effective hydrodynamic diameter of the PNIPAM−Gly−C_{18}Py aggregates was 16 ± 2 nm. The histograms recorded in all cases were broad, a result, we believe, of the broad molecular weight distribution of the polymer. The pH of the solution did not affect the size of the polymeric micelles. Moreover, their size was not affected by changes in salt concentration ([NaCl] = 0.1−2.0 M) of a PNIPAM−Gly−C_{18}Py solution of neutral pH (7.1). Only in solutions near the conditions of ionic strength, pH, and temperature corresponding to the point of macroscopic phase separation did we observe a significant change in the size of the aggregates. For example, in solutions of PNIPAM−Gly−C_{18}Py of pH 2.7 and [NaCl] >1.0 M at 25 °C, the micellar aggregates were much larger, with an average diameter of 115 nm. In summary, the dynamic light scattering studies point to the fact that in aqueous solutions of PNIPAM−Gly−C_{18}Py the structure of the hydrophobic microdomains is determined by the nature of the hydrophobic group and its level of incorporation along the chain. The micellar aggregates are hardly affected by changes in the degree of protonation of the glycine residues. This conclusion may have practical implications in the design of functional systems.

Fluorescence Spectroscopy Studies. The two pyrene-labeled polymers selected for this study carry the label either in close proximity to the polymer backbone (PNIPAM−Gly−Py) or in the vicinity of the hydrophobic side chains (PNIPAM−Gly−C_{18}Py). The

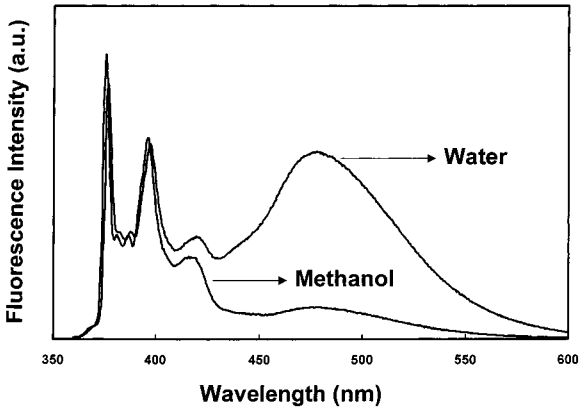

Figure 8. Fluorescence spectra of PNIPAM−Gly−C_{18}Py in water and in methanol; polymer concentration = 0.05 g L^{-1}; λ_{exc} = 341 nm.

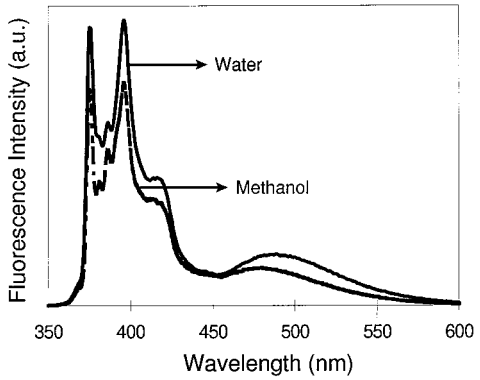

Figure 9. Fluorescence spectra of PNIPAM−Gly−Py in water and in methanol; polymer concentration = 0.05 g L^{-1}; λ_{exc} = 342 nm, methanol; λ_{exc} = 344 nm, water.

labeling sites were chosen to allow us to monitor by fluorescence the influence of pH and temperature conditions, either on the conformation of the polymer backbone (PNIPAM−Gly−Py) or on the structure of the micellar assemblies (PNIPAM−Gly−C_{18}Py). The photophysics of both polymers were studied in methanol, a good solvent for both polymers, and in aqueous solutions of various pH. The emission spectra of both polymers in methanol consist of two contributions (Figures 8 and 9): a structured emission due to locally isolated excited pyrenes (intensity I_M, pyrene "monomer" emission) with the (0,0) band at 377 nm and a weaker broad emission centered at 480 nm, attributed to pyrene excimers (intensity I_E).

Aqueous solutions of the two polymers have quite distinct fluorescence properties. The overall fluorescence intensity of aqueous solutions of PNIPAM−Gly−C_{18}Py (0.01 g L^{-1}) is much weaker that that of polymer solutions of identical concentration in methanol. Moreover, the excimer emission is strongly enhanced, compared to the monomer emission (Figure 8). This observation implies that, in water, the chromophores are kept in close proximity, even in a very dilute polymer solution. Under these circumstances, pyrene is known to undergo self-quenching and to show a strong propensity toward excimer emission.[25] As reported in the case of hydrophobically modified pyrene-labeled PNIPAM, a predominant excimer emission confirms the presence of polymeric micelles which sequester the pyrene groups within their hydrophobic microdomains. We note that solutions of PNIPAM−Gly−Py, the corresponding pyrene-labeled NIPAM/Gly copolymer devoid of *n*-octadecyl

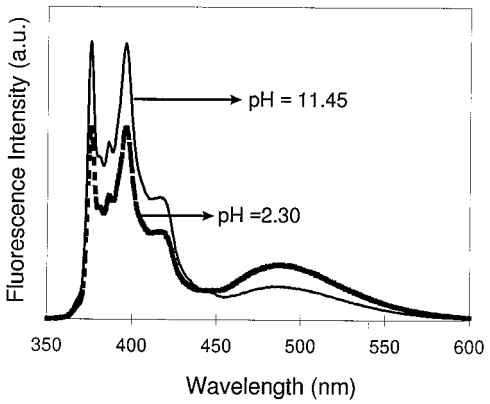

Figure 10. Fluorescence spectra of PNIPAM−Gly−Py in aqueous solutions of pH 2.30 and 11.45; polymer concentration = 0.05 g L^{-1}, [NaCl] = 0.1 M; λ_{exc} = 344 nm.

chains, do not follow the same trend: the pyrene monomer emission prevails, whether the polymer is dissolved in water or in methanol (Figure 9). A further spectroscopic point needs to be clarified. It concerns the mechanism of excimer formation: is it a dynamic excimer, or does it originate from preformed ground-state pyrene aggregates, a frequent occurrence in aqueous solutions of pyrene-labeled polymers?[25] A comparison of the UV absorption spectra and the excitation spectra monitored for the excimer and the monomer emissions[25] of polymer solutions in methanol leads to the conclusion that in this situation pyrene excimers form under dynamic conditions, in accordance with the mechanism originally proposed by Birks.[26] In contrast, for polymer solutions in water, the pyrene excimer originates from preformed pyrene aggregates, independent of the site of labeling. These conclusions apply to solutions of PNIPAM−Gly−Py as well as PNIPAM−Gly−C_{18}Py.

Next, we measured the emission of pyrene from labeled polymer solutions of pH ranging from 2 to 10. All solutions were prepared in citric acid buffers, matching closely the conditions used in the calorimetry measurements and cloud-point determinations, although it was necessary to record fluorescence spectra from solutions of lower polymer concentration (0.05 or 0.1 g L^{-1}, PNIPAM−Gly−Py, and 0.05 g L^{-1}, PNIPAM−Gly−C_{18}Py) to avoid inner filter effects related to excessive pyrene absorbance. The fluorescence spectrum of aqueous PNIPAM−Gly−Py was affected by changes of pH in the range (3.5−5.0) that encompasses the pK_a of the polymer (Figure 10). The overall features of the spectrum were maintained, but the pyrene monomer emission intensity increased at the expense of the pyrene excimer emission intensity. The effect is illustrated in Figure 11, where we plot the changes with pH of the ratio I_E/I_M calculated from the spectra of aqueous solutions of PNIPAM−Gly−Py. The ratio I_E/I_M decreases sharply in a narrow pH range, the midpoint of the transition (pH = 4.2) corresponding approximately to the pK_a of the polymer. It indicates that, as the solution reaches the critical pH corresponding to the sharp transition in the I_E/I_M curve, the electrostatic repulsive forces between the carboxylate substituents overcome the hydrophobic interactions that keep the pyrene groups in close proximity in more acidic solutions. A similar behavior was reported also in the case of pyrene-labeled poly(acrylic acid).[27,28] This pattern, however, was not followed by the variations with pH of the ratio I_E/I_M of the hydrophobically modified

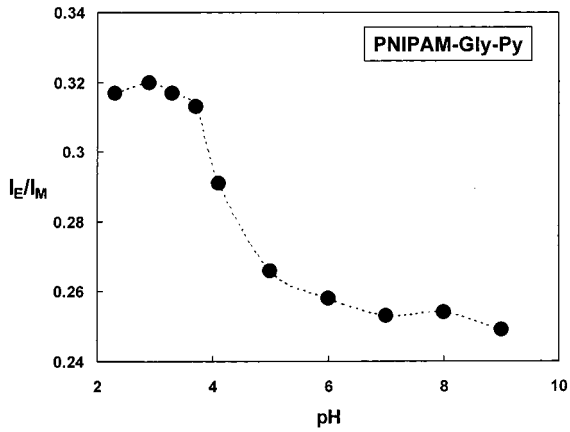

Figure 11. Plot of the ratio of pyrene excimer to monomer emission intensities (I_E/I_M) for 0.1 M NaCl solutions of PNIPAM−Gly−Py as a function of the solution pH; polymer concentration = 0.05 g L^{-1}; λ_{exc} = 344 nm.

polymer PNIPAM−Gly−C_{18}Py. The ratio remained constant, independent of pH ranging from 2.5 to 8.0. Thus, deprotonation of the carboxylic acid and polymer chain expansion cannot overcome the hydrophobic forces responsible for the formation of polymeric micelles. These retain their integrity, a conclusion that brings strength to our interpretation of the microcalorimetry and dynamic light scattering results.

Conclusions

The present work has presented the preparation and characterization of solution properties of four related copolymers of NIPAM and glycine acrylamide. Two polymers carried a few octadecyl groups attached at random along the copolymer main chain. The solution properties of the copolymers and of their hydrophobically modified counterparts were investigated by fluorescence spectroscopy, microcalorimetry, and dynamic light scattering with special emphasis on the effects of solution temperature, pH, and ionic strength on the assembly of the copolymers in water. The hydrophobically modified copolymers form polymeric micelles approximately 16 nm in diameter. The most important conclusion of our study is that, while the polymer main chain is responsive to changes in solution temperature and pH, the hydrophobic microdomains seem unaffected by the collapse or expansion of the main chain. This unique property may prove to be of practical importance when designing a responsive delivery agent based on polymeric micelles, an area of considerable current interest.

Acknowledgment. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada. Ms. T. Principi thanks McMaster University for a Dean of Sciences summer research fellowship.

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MA9919054