Synthesis and Characterization of a Hydrophobically Modified Copolymer of *N*-Isopropylacrylamide and Glycinyl Acrylamide

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Introduction

Over the past 20 years, the synthesis and solution properties of polymers responsive to external stimuli have been the focus of many studies, in view of their potential applications in drug delivery,[1] diagnostics,[2] separations,[3] and robotics.[4] Polymers that can respond differently to more than one stimulus are of particular interest when designing multifunctional devices. Poly(*N*-isopropylacrylamide) (PNIPAM) in water exhibits well-characterized phase changes. It undergoes a temperature-induced collapse from an extended coil into a globule, a transition revealed on the macroscopic scale by a sudden increase in turbidity of a PNIPAM solution, as its temperature exceeds 32 °C[5] (known as the cloud point or lower critical solution temperature, LCST). Polymers bearing carboxylic acid functional groups, such as poly(acrylic acid), are pH-sensitive, as they adopt a coiled conformation in solutions of low pH, where the carboxylic acid groups are protonated, and an extended conformation in solutions of high pH, where the negatively charged carboxylates undergo strong electrostatic repulsion. Graft and random copolymers of *N*-isopropylacrylamide (NIPAM) and acrylic acid (AA) have been shown to be responsive to both pH and temperature.[6−8]

We have prepared a new type of pH- and temperature-sensitive polymer that possesses not only temperature-responsive groups (NIPAM) and pH-responsive groups (glycine) but also hydrophobic groups consisting of a pyrene group linked to a secondary amide nitrogen also carrying an octadecyl chain. This copolymer, PNIPAM−Gly−Py (Figure 1), is a highly effective modulator of the release of dyes entrapped in liposomes.[9] Moreover, it improves the efficiency of cytoplasmic delivery of liposome-encapsulated dyes.[10] The preparation and chemical characterization of this polymer and of a copolymer of NIPAM and glycine acrylamide (PNIPAM−Gly, Figure 1) are described in this note.

Experimental Section

Materials. Water was deionized with a Barnstead NANOpure water-purification system. Dioxane and tetrahydrofuran (THF) were distilled from sodium under nitrogen. 4-(1-Pyrenyl)butyric acid, octadecylamine, carbonyldiimidazole, and *N*,*N*-azobis(isobutyronitrile) (AIBN) were obtained from Aldrich Chemical Co., Inc. *N*-(Acryloxy)succinimide (NASI) was obtained form Acros Chemicals. Di-*tert*-butylcresol and glycine ethyl ester hydrochloride were purchased from Sigma. *N*-Isopropylamine (NIPAM) was a gift from Kohjin Co. Ltd. (Lot H021225). It was recrystallized from a toluene:hexane (1:1, v/v) mixture. Thin-layer chromatography (TLC) was performed with silica plates (Merck) eluted with CH_2Cl_2/MeOH (9/1 v/v). *N*-[4-(1-Pyrenyl)butyl]-*N*-*n*-octadecylacrylamide (**2**) was prepared as described previously.[11]

Instrumentation. ^{1}H 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. 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.

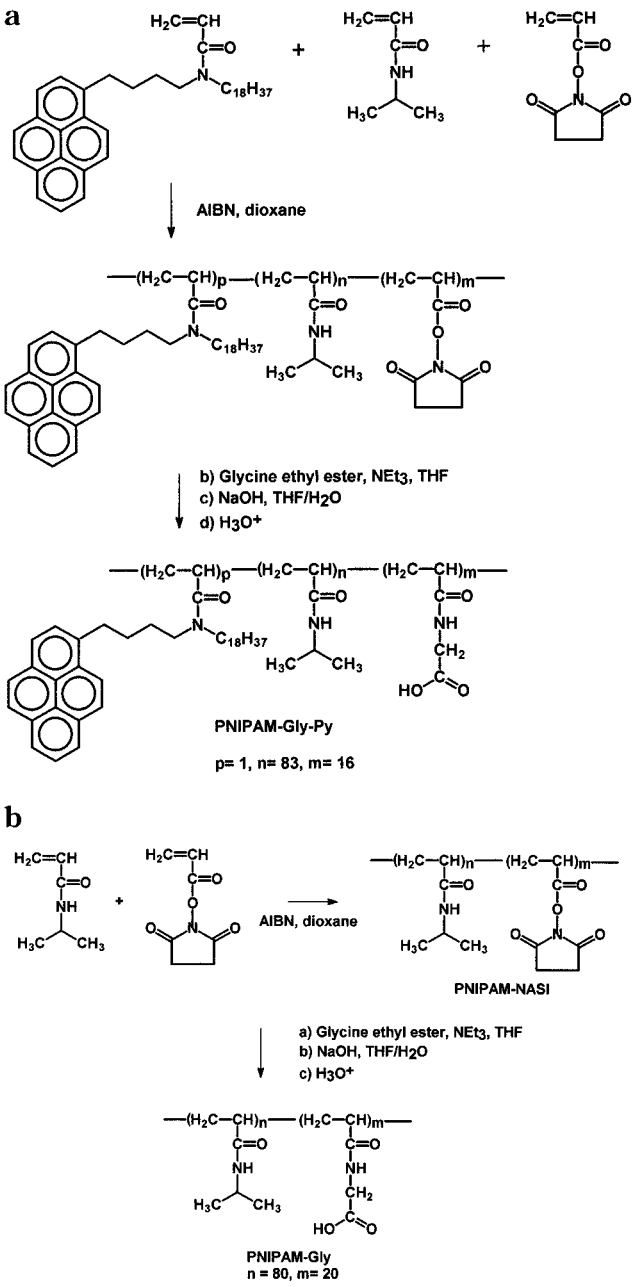

Figure 1. Synthetic scheme for the preparation of PNIPAM−Gly−Py (a) and PNIPAM−Gly (b).

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Dynamic light scattering was performed on a Brookhaven BI9000 AT instrument equipped with an argon laser ($\lambda = 514$ nm; scattering angle, 90°). Fluorescence spectra were recorded on a SPEX Industries Fluorolog 212 spectrometer equipped with a DM3000F data analysis system. The slits were set at 1.0 nm (emission) and 1.0 nm (excitation). The excitation wavelength was 330 nm.

Synthesis. Copolymerizations of *N*-Isopropylacrylamide, *N*-(Acryloxy)succinimide, and *N*-[4-(1-Pyrenyl)-butyl]-*N*-*n*-octadecylacrylamide. NIPAM (1.81 g, 16 mmol), NASI (0.48 g, 2.8 mmol), and **2** (0.11 g, 0.02 mmol) were dissolved in dry dioxane (40 mL) and degassed with N_2 for 15 min. The mixture was heated to 75 °C. AIBN (0.04 g) was added to initiate the polymerization. After 15 h, the mixture was cooled to room temperature. The polymer was isolated by precipitation into diethyl ether and purified by precipitations from dioxane into diethyl ether (800 mL). The white, fluffy copolymer (PNIPAM−NASI−Py, 1.85 g) was dried in vacuo for 2 h at room temperature. ^{1}H NMR ($CDCl_3$) [200 MHz] δ: 1.12 (br, $-CHCH_2CH-$, $-CH_3$, $(CH_2)_{15}$), 1.67 (br, $-CH_2CHCH_2-$, $-NCH_2CH_2-$), 1.93−2.20 (br, $-NCH_2CH_2-CH_2-$, $-N(CH_2)_2CH_2CH_2-$Py), 3.09−3.15 (br, $COCH_2CH_2CO$), 4.46−4.52 (br, $-CHNH-$, Py$-CH_2$, NCH_2), 5.78−6.95 (br, NH). FT IR (KBr pellet) (cm^{-1}): 3435.6 (N−H stretch), 3314.4 (interpolymeric H bonding), 3070.6 (vinylic, C−H stretch), 2933.2 (CH_3 stretch), 2875.6 (CH_2 stretch), 1737.4 (OC−N−CO), 1652.4 (CO stretch), 1459.4 (CH_2 scissors mode).

Reaction of PNIPAM−NASI−Py with Glycine Ethyl Ester Hydrochloride. Triethylamine (0.4 mL, 2.9 mmol) and glycine ethyl ester hydrochloride (0.21 g, 1.5 mmol) were added consecutively to a solution of PNIPAM−NASI−Py (1.51 g) in dry THF (50 mL). The mixture was stirred at room temperature for 18 h under N_2. Distilled isopropylamine (1.0 mL, 0.01 mmol) was added to quench the excess succinimide groups. The resulting mixture was stirred for 2 h. The polymer was isolated by precipitation into diethyl ether and purified by two precipitations from THF into diethyl ether (1.51 g, white powder). ^{1}H NMR ($CDCl_3$) [500 MHz] δ: 0.85 (t, CH_3), 1.12 (br, $(CH_2)_{15}$ + CH_3), 1.34 (d, CH_3), 1.62 (br, 6H, $CHCH_2CH-$ + $-NCH_2CH_2-$), 1.78 (br, $-CH_2CHCH_2-$), 2.15 (br, $-NCH_2CH_2CH_2-$, $-N(CH_2)_2CH_2CH_2$Py), 2.64 (s, $-NHCH_2$), 3.96 (br, $CHNH$, Py$-CH_2$, $-NCH_2$), 4.15 (br, $COOCH_2CH_3$), 7.83−8.23 (m, CH_2, Py). FT IR (KBr pellet) (cm^{-1}): 3438.3 (N−H stretch), 3307.4 (interpolymeric H bonding), 3057.9 (vinylic, C−H stretch), 2972.3 (CH_3 stretch), 2876.1 (CH_2 stretch), 1655.7 (CO stretch), 1460.0 (CH_2 scissors mode).

Deprotection of PNIPAM−Gly Ester−Py. Sodium hydroxide (0.09 g, 2.2 mmol) dissolved in THF/H_2O (4/1 v/v, 10 mL) was added to a solution of PNIPAM−Gly ester−Py (1.12 g) in THF/H_2O (4/1 v/v, 50 mL). The solution was kept at room temperature for 15 h. Then the pH of the solution was adjusted to 3.35 with 0.1 N HCl. The solvent was concentrated until a yellow oil separated from the aqueous phase. The aqueous phase was decanted, and the remaining oil was dissolved in THF (5 mL). This polymer was isolated by precipitation into diethyl ether (800 mL) and dried in vacuo for 2 h, yielding PNIPAM−Gly−Py as a white powder (0.73 g). ^{1}H NMR ($CDCl_3$) [500 MHz] δ: 0.86 (t, CH_3), 1.12 (br, $(CH_2)_{15}$, CH_3), 1.62 (br, $-CHCH_2CH_2$, $-NCH_2CH_2$), 1.80 (br, $-CH_2CHCH_2$), 2.05 (br, $-NCH_2CH_2CH_2-$, $-N(CH_2)_2CH_2CH_2-$Py), 3.98 (br, $CHNH$, Py$-CH_2-$, $-NCH_2$), 6.31 (br, NH), 7.83−8.23 (m, CH_2, Py). FT IR (KBr pellet) (cm^{-1}): 3435.5 (N−H stretch), 3309.9 (interpolymeric H bonding), 3073.3 (vinylic, C−H stretch), 2973.2 (CH_3 stretch), 2876.1 (CH_2 stretch), 1649.9 (CO stretch), 1459.5 (CH_2 scissors mode).

Preparation of PNIPAM−Gly Ester. A copolymer of NIPAM and NASI (PNIPAM/NASI, 2.52 g) was prepared as previously described,[12] starting from NIPAM (4.51 g, 40 mmol) and (NASI) (1.02 g, 6.0 mmol). This polymer (0.51 g) was dissolved in THF (15 mL). Triethylamine (0.06 mL, 0.4 mmol) and glycine ethyl ester hydrochloride (0.06 g, 0.4 mmol) were added in turn to the solution. The mixture was stirred at room temperature, under N_2, for 18 h. Isopropylamine (0.5 mL, 0.008 mmol) was added to quench the unreacted hydroxysuccinimide groups, and the mixture was stirred for 2 h. The polymer was isolated by precipitation into hexane (700 mL). It was purified by further precipitations from MeOH (30 mL) into diethyl ether (700 mL). The collected fluffy, white polymer was dried in vacuo at room temperature for 3 h. ^{1}H NMR ($CDCl_3$) [200 MHz] δ: 1.13 (br, CH_3), 1.38 (d, $6CH_3$), 1.66 (br, $-CHCH_2CH-$), 2.15 (br, $2CH_2CHCH_2$), 3.47 (br, CH_2), 3.94 (br, $-NHCH-$, $-NHCH_2-$). FT IR (KBr pellet) (cm^{-1}): 3435.7 (N−H stretch), 3293.1 (H bonded), 3057.6 (vinylic, C−H stretch), 2973.4 (CH_3 stretch), 2877.1 (CH_2 stretch), 2362.0 (CO_2), 1646.2 (CO stretch), 1460.3 (CH_2 scissors mode).

Deprotection of PNIPAM−Gly Ester. The procedure used for the preparation of PNIPAM−Gly−Py was followed, starting with PNIPAM−Gly ester (0.33 g). PNIPAM−Gly was isolated as a white powder (0.07 g). ^{1}H NMR ($CDCl_3$) [500 MHz] δ: 1.12 (br, CH_3), 1.60 (br, $-CHCH_2CH-$), 1.78 (br, $-CH_2CHCH_2-$), 3.47 (s, CH_3, MeOH), 3.98 (br, $-NHCH-$, $-NHCH_2-$), 6.19 (br, NH). FT IR (KBr pellet) (cm^{-1}): 3495.8 (N−H stretch), 3073.9 (vinylic, C−H stretch), 2972.7 (CH_3 stretch), 2875.8 (CH_2 stretch), 1651.8 (CO stretch), 1459.5 (CH_2 scissors mode).

Cloud-Point Determinations. Cloud points were determined by spectrophotometric detection of the changes in transmittance (λ 600 nm) of aqueous polymer solutions (1 g L^{-1}) heated at a constant rate (0.2 °C) in a magnetically stirred UV cell. The value reported is the temperature at which transmittance of the solution decreased by 20% of its value at 25 °C. The following buffers were employed to prepare solutions of controlled pH: pH 2, 0.2 M KH_2PO_4; pH 2.8−4.71, 0.1 M citric acid; pH 5.9, 0.2 M KH_2PO_4; pH 7.4, 0.1 M Tris.[13] All solutions contained 0.1 M NaCl.

Results

The selection of the synthetic sequence employed was dictated on one hand by the need to obtain a random distribution along the polymer chain of the three units, NIPAM, the pyrenyl−octadecyl amide, and glycine and, on the other, by the conflicting solubility properties of the three structural motifs. While NIPAM is soluble in water as well as in several organic solvents, glycine itself is only soluble in water and pyrenyl−octadecyl compounds are soluble only in organic media. Previous work on the copolymerization of NIPAM and amino acid-derived acrylic monomers has taught us that copolymerizations carried out in aqueous medium tend to lead to copolymers with large segments of hydrophilic and hydrophobic units.[14] Therefore, we chose the slightly lengthy scheme depicted in Figure 1.

The target polymer, PNIPAM−Gly−Py, was obtained by reaction of glycine ethyl ester with a copolymer of NIPAM, NASI and *N*-4-(1-pyrenyl)-*N*-(*n*-octadecylacrylamide), which itself was obtained by free-radical polymerization in dioxane of the corresponding acrylamides (Figure 1). Reaction of the reactive copolymer with glycine methyl ester and subsequent quenching of residual unreacted *N*-hydroxysuccinimide groups with *N*-isopropylamine led to the ethyl ester derivative of the desired polymer. Evidence that the succinimide sites on the polymer have reacted with the glycine ethyl ester is provided by the disappearance in the ^{1}H NMR spectrum of a broad signal at δ 3.09−3.15, attributed to the resonance of the succinimide methylene protons, together with the appearance of a signal at δ 4.15, assigned to the methylene protons of the ethyl ester moiety. Also, in the IR spectrum, the band at 1737 cm^{-1} due to the imide carbonyl groups is replaced by a band at 1646 cm^{-1}, characteristic of the ester carbonyl stretch. Hydrolysis of the ester group under mild alkaline conditions led to the desired copolymer. Deprotection was confirmed by the loss in the ^{1}H NMR spectrum of the signal at 4.15 ppm. The ^{1}H NMR

Table 1. Physical Properties of the Copolymers Investigated

polymer	M_n	M_w	M_w/M_n	molar ratio NIPAM	molar ratio Gly	molar ratio Py	Gly content, mol g^{-1}	Py content, mol g^{-1}
PNIPAM−Gly−Py	25 000	54 000	2.2	83	16	1	1.2×10^{-3}	9.4×10^{-5}
PNIPAM−Gly	30 000	77 000	2.5	80	20		1.4×10^{-3}	

spectrum of PNIPAM−Gly−Py also exhibits signals characteristic of the NIPAM repeating units, namely, a strong singlet at δ 1.12 and broad signals at δ 1.62 and 1.80 attributed to the methylene and methine protons of the polymer backbone. Evidence for the incorporation of pyrenyl octadecyl groups is given by the presence of a weak triplet at δ 0.86, due to the terminal methyl group of the octadecyl chains and a multiplet (δ 7.83−8.23) attributed to the aromatic protons of the pyrene substituents. A copolymer of NIPAM and glycine−acrylamide, PNIPAM−Gly, was prepared by a similar route, starting with a copolymer of NIPAM and *N*-(acryloxy)succinimide, reacted first with glycine ethyl ester and then with *N*-isopropylamine and finally hydrolyzed under mild alkaline conditions to deprotect the glycine ethyl ester residues.

The physical properties of PNIPAM−Gly−Py and PNIPAM−Gly are listed in Table 1. GPC was used to determine the molecular weight of the polymer, calibrated against poly(ethylene oxide) standards, and 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 (9.4×10^{-5} mol of Py g^{-1} or 1 pyrene chromophore for every 83 NIPAM units) was determined from UV absorption data of polymer solutions in THF, using *N*-[4-(1-pyrenyl)butyl]-*N*-octadecylacrylamide ($\epsilon_{344} = 37\,000$) as the model compound. This result was confirmed by the ^{1}H NMR spectrum of PNIPAM−Gly−Py, using the area of the singlet at 4.01 ppm due to the resonance of the methine proton of the isopropyl groups of the NIPAM units and the area of the triplet centered at 0.86 ppm attributed to the terminal methyl protons of the octadecyl chains

Solution Properties of the Copolymers. Both the hydrophobically modified copolymer, PNIPAM−Gly−Py, and PNIPAM−Gly are soluble in water at or below room temperature, independent of the pH of the solution. However, the solutions become turbid when heated above a critical temperature, signaling the occurrence of a lower critical solution temperature (LCST). As anticipated, the LCST of the NIPAM−Gly copolymers exhibits a pronounced pH dependence. Under acidic conditions, when the carboxylic acid groups are fully protonated, the cloud points of PNIPAM−Gly−Py and PNIPAM−Gly have similar values (ca. 28 °C; see Figure 2). These are slightly lower than the LCST of PNIPAM (31 °C), indicating a slight increase in hydrophobicity of the copolymers.[15] As the pH of the solution increases and the carboxylic acid groups take their ionized forms, the LCST increases and ultimately vanishes for pH values larger than 5 and 8.5 in the case of solutions of PNIPAM−Gly−Py and PNIPAM−Gly, respectively (Figure 2). Contrary to our expectations, the LCST of the hydrophobically modified copolymer was not significantly lower than that of the unmodified analogue,

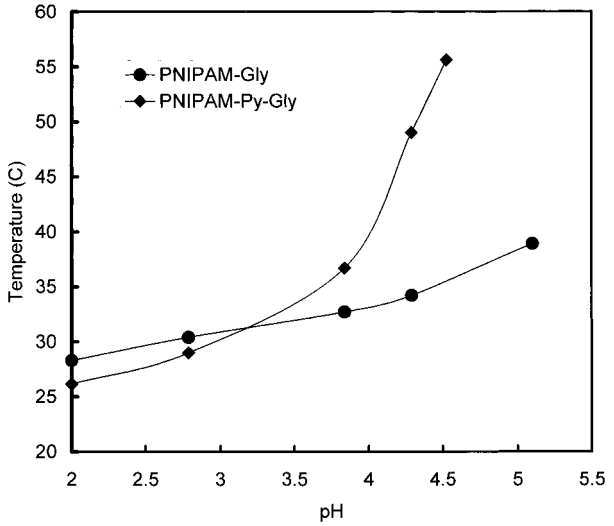

Figure 2. Changes in the cloud point of aqueous PNIPAM−Gly−Py (full diamond) and PNIPAM−Gly (full circle) solutions as a function of pH (polymer concentration, 1 g L^{-1}; 0.1 M NaCl).

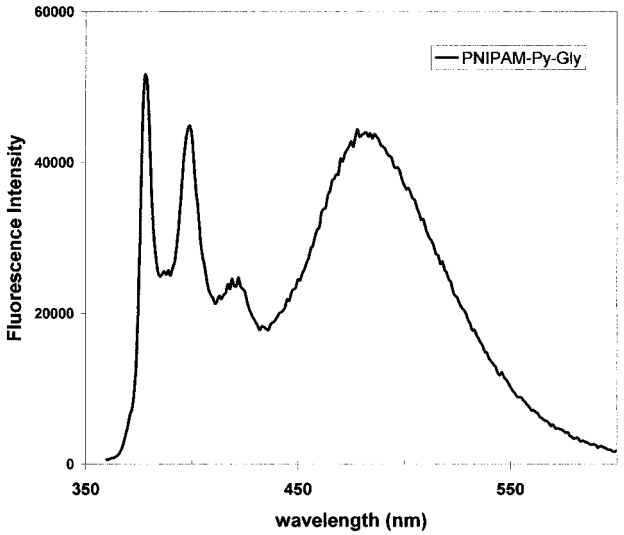

Figure 3. Fluorescence spectrum of PNIPAM−Gly−Py in water (polymer concentration, 0.01 g L^{-1}; λ_{exc} 330 nm).

violating the general rule that the LCST of a copolymer decreases with increasing hydrophobicity.[15] This observation is taken as evidence that the alkyl chains are not exposed to water but rather form the inner core of micellar structures protected from the aqueous environment by the PNIPAM−Gly chains.[16] Quasi-elastic light-scattering measurements confirmed that in acidic solutions, PNIPAM−Gly−Py exists as assemblies 18 nm in diameter (pH 3.0, 0.1 M NaCl), while no micelles are formed in solutions of PNIPAM−Gly, indicating the absence of interchain aggregation, a fact reported also in the case of PNIPAM solutions below their LCST.[17,12]

Further evidence of the formation of polymeric micelles is provided by the fluorescence spectrum of PNIPAM−Gly−Py (Figure 3) in aqueous solution (pH 3, 0.1 M NaCl). The spectrum is dominated by a broad,

featureless emission centered at 490 nm due to pyrene excimers (intensity I_E).[18,19] In addition, there is a contribution from the isolated excited pyrene (monomer emission, intensity I_M), with the [0,0] band located at 378 nm. The fact that the excimer emission is very strong implies that the chromophores are kept in close proximity,[19] incorporated into the core of the polymeric micelles detected by QELS. The influence of pH, temperature, and salt concentration on the solution properties of the polymer is under current investigation, to unravel the relative importance of electrostatic repulsion, hydrogen bonding, and hydrophobic interactions in guiding the formation and disruption of polymeric micelles.

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