

Amphiphilic Fluorescent Copolymers Nucleotides Interactions

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Received 4 May 2006; accepted 10 July 2006

DOI 10.1002/app.25244

Published online 11 May 2007 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: New hydrophobic monomer *N*-methacryloyl-*N'*- α -naphthylthiourea (MANTU) was synthesized and copolymerized with hydrophilic *N*-isopropylacrylamide by free radical copolymerization. And *N,N*-dimethylacrylamide was introduced to adjust the lower critical solution temperature of the resulting copolymers. Photophysics and the effect of pH of copolymer in aqueous solutions were studied by intrinsic fluorescence observations. The self-aggregation behavior was investigated by transmission electron microscopy. The amphiphilic polymers with fluo-

rescent behavior were confirmed to be thermo- and pH-sensitive. The interaction of the tercopolymer with adenosine 5'-monophosphate and adenosine 5'-triphosphate was described. Different interactions of nucleotides with the copolymer were found and possible mechanisms were discussed. © 2007 Wiley Periodicals, Inc. *J Appl Polym Sci* 105: 2532–2539, 2007

Key words: poly(*N*-isopropylacrylamide); amphiphilic copolymer; thermosensitivity; pH-sensitivity; nucleotide

INTRODUCTION

Thermosensitive copolymers and polymeric micelles, especially copolymers based on *N*-isopropylacrylamide (NIPAAm), are one of the most important drug delivery carriers.^{1–6} They exhibit a lower critical solution temperature (LCST) in aqueous media, below which PNIPAAm segments are fully hydrated with an extensive chain conformation and above which they are extensively dehydrated and compact. Through the control of temperature, hydrophobic drugs can be encapsulated or released. During the circulation in body fluids, drug-loaded micelles will be subjected not only to temperature variations, but also to the variations of concentrations of salts and pH values.

Many studies investigated the salt effect on the phase transition behaviors of NIPAAm copolymers.^{7–9} Studies showed that the amphiphilic copolymers were more sensitive to salt than the hydrophilic polymers, and the hydrophobic structures played a significant role in controlling the phase transition behaviors. However, few studies have been carried out to investigate pH effects on the phase transition behaviors of nonionic NIPAAm copolymers, and those studies showed that the effects of near neutral pH were negli-

gible.⁷ While the pH values of human body fluids radically change from 0.9 to 1.5 of stomach juice to ~8.8 of pancreatic juice, our previous study indicated that the copolymer of (*p*-methacrylamido)acetophenone thiosemicarbazone and NIPAAm showed a comparatively narrow pH-sensitivity (pH = 6.5–8.5).¹⁰ The pH-sensitive polymers (water-insoluble at low pH, water-soluble at high pH) are of particular interest, because the release rate of the drug can be triggered by the pH of the environment and be used to provide about constant release rates of basic drugs with strongly pH-dependent solubility.^{11,12}

In this article we report our studies on the effects of a wide range of pH on NIPAAm copolymer properties by fluorescent spectroscopy and transmission electron microscopy. Results indicated that amphiphilic copolymer showed different self-assembly behavior as the pH changed. This presentation provides important information for the design and synthesis of the thermosensitive delivery carriers for hydrophobic drugs.^{4,13}

On the other hand, nucleotide sensing and quantification by means of luminescent probes represent an important target in supramolecular chemistry because of their many biological and biomedical implications.¹⁴ The interaction of the tercopolymer of NIPAAm with *N,N*-dimethylacrylamide and *N*-methacryloyl-*N'*- α -naphthylthiourea, with adenosine 5'-monophosphate (AMP) and adenosine 5'-triphosphate (ATP) is described and we found the potential molecular recognition of this receptor for ATP in aqueous solution.

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Contract grant sponsor: National Natural Science Foundation of China; contract grant number: 20474044.

EXPERIMENTAL

Material

N-isopropylacrylamide (NIPAAm; Acros Organics; 99%) was recrystallized from mixtures of toluene and hexane (1/2 by volume). *N,N*-dimethylacrylamide (DMAAm; Fluka Chemie; >98.0%) was distilled before use. KSCN (Shanghai Chemical Reagents Co., AR) was recrystallized from mixtures of ethanol and water (1/1 by volume). α -Naphthylamine (Shanghai Chemical Reagents Co.) was used as received. *N,N'*-azobisisobutyronitrile (AIBN; Shanghai Chemical Reagents Co.) was recrystallized from methanol before use. Tetrahydrofuran (THF) and Acetonitrile (Shanghai Chemical Reagents Co.) were dried and distilled over Na before use. Methacryloyl chloride was prepared by refluxing a mixture of thionyl chloride and methacrylic acid, followed by distillation. Adenosine 5'-monophosphate disodium salt, hexahydrate (Na₂AMP·6H₂O; ultra pure grade) and adenosine 5'-triphosphate disodium salt, trihydrate (Na₂H₂ATP 3H₂O; ultra pure grade) were purchased from Amresco, USA. All the other reagents used are all of analytical grade.

Synthesis

KSCN (17.05 g, 0.175 mmol) was dissolved in 150 mL of anhydrous acetonitrile and the solution was then cooled by ice-salt bath. Under stirring, 14.4 mL (0.15 mol) of methacryloyl chloride was added dropwise into it. And then the reaction was allowed to stand for 5 h at room temperature. The precipitated KCl was removed by filtration. α -Naphthylamine (18.61 g, 0.13 mol) was dissolved in 60 mL of acetonitrile and added dropwise into the filtrate at room temperature. The system turned to yellow from dark red and began to precipitate greatly. After being filtrated and washed with water, the yellow precipitate was vacuum-dried. Then the solid was dissolved in acetone and subsequently a large excess of cold water was added to give crystalline *N*-methacryloyl-*N'*- α -naphthylthiourea (MANTU). Yield, 75%; mp, 135–137°C. FTIR (KBr, cm⁻¹): 3400, 3183, 3048, 3004, 1340, 1678, 1523, 1184, 772. ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 12.53 and 8.88 (1H \times 2, each s, two NH), 7.19–7.93 (7H, ArH), 5.99 and 5.67 (1H \times 2, each m, CH₂=), 2.05 (3H, s, -CH₃). Elemental Anal. Calcd for C₁₅H₁₄N₂O₂S (%): C, 66.64; H, 5.22; N, 10.36; S, 11.86. Found (%): C, 66.59; H, 5.30; N, 10.28; S, 11.78. ESI-MS (in CH₃CN): *m/z* (RI): 271 (M⁺ + 1, 100).

Copolymerization

In a typical synthesis, 1.13 g (10 mmol) of NIPAAm and 0.0541 g (0.2 mmol) of hydrophobic monomer MANTU were dissolved into 20 mL of dry THF. The solution was then purged with nitrogen under stir-

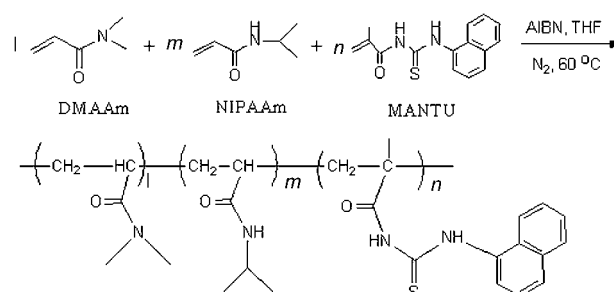
ring, and initiator AIBN (at 0.9% mol relative to the monomers feed) was then added by syringe. After heating to 60°C, the reaction was run for 24 h under a nitrogen atmosphere. After this time, most of THF was removed under reduced pressure and the solution was poured into a great amount of cold diethyl ether to precipitate the polymer. The collected polymer was reprecipitated three times by diethyl ether from THF and vacuum-dried at 40°C for 48 h to get a dicopolymer (Polymer 1). Yield, 70%.

Tercopolymers were synthesized similarly by adding the third monomer, DMAAm (0.495 g, 5.0 mmol), for Polymer 2 and (0.297 g, 3.0 mmol) for Polymer 3. Yield, 87.5 and 83.2%, respectively. IR (KBr, cm⁻¹): 3439, 3288, 3073, 2972, 2934, 2871, 1631, 1545, 1459, 1402, 1388, 1367, 1259, 1153, 1057, 620. ¹H-NMR (300 MHz, CDCl₃): δ (ppm): 4.0 (-CH(CH₃)₂), 7.0 (ArH), 1.0 (-CH(CH₃)₂), 1.2–2.5 (b, protons from main chain), 2.9 (-N(CH₃)₂). The general synthetic outline for polymerization is shown in Scheme 1.

Characterization

¹H-NMR spectra were recorded in CDCl₃ on a Varian Mercury VX-300 MHz spectrometer (USA). Fourier transform infrared (FTIR) spectra were obtained using a Nicolet 670 FTIR spectrometer (USA) and the samples were ground and pressed into KBr pellets for analysis. Elemental analysis was conducted on a Flash EA 1112 series elemental autoanalyzer (Italy). Mass spectra were obtained on an LCQ-Advantage electrospray ionization-mass spectrometer (ESI-MS) (Finnigan, England). Melting points were measured on a Reichert 7905 melting-point apparatus (uncorrected).

The molecular weights and polydispersities of the polymers were estimated by size exclusion chromatography (SEC) analysis using an Agilent 1100 HPLC system (USA) equipped with a refractive index detector and a Plgel column (5 μ m, 300 mm \times 7.5 mm). THF was used as eluent at a flow-rate of 1.0 mL/min. Polystyrene standard with a narrow distribution was used to generate a calibration curve. Agilent GPC-Addon Rev.A.02.02 was used for data acquisition and analysis.



Scheme 1 Synthesis route of amphiphilic tercopolymer.

TABLE I
Results of Polymerization and Characterization

Polymer	<i>l</i> : <i>m</i> : <i>n</i> , feed ratio ^a	<i>l</i> : <i>m</i> : <i>n</i> ^b	\bar{M}_n^c (g mol ⁻¹)	\bar{M}_w/\bar{M}_n^c	Conv. (%)
1	0 : 100 : 2	0 : 100 : 2	218,000	1.18	70.0
2	50 : 100 : 2	76 : 100 : 2.5	145,000	1.57	87.5
3	30 : 100 : 2	40 : 100 : 2.5	229,000	1.24	83.2

^a Feed molar ratio of monomer DMAAm : NIPAAm : MANTU. The amount of initiator AIBN was 0.9 mol % of total monomers.

^b Estimated from ¹H-NMR spectra based on the peak integral ratios of the dimethyl protons of DMAAm segments [$-\text{N}(\text{CH}_3)_2$, $\delta = 2.9$], the methine protons of NIPAAm segments [$-\text{CH}(\text{CH}_3)_2$, $\delta = 4.0$], the hydrophobic group protons from MANTU in the range of 6.2–8.6.

^c Determined by GPC.

LCST determination

The LCST values of thermosensitive (co)polymers were estimated by cloud points (cp) measurements, which were done visually by following the variation of the turbidity of polymeric aqueous solutions with temperature. Polymeric aqueous solution (1.0 wt %) in a sample tube was immersed in a thermostated cell with a circulating water bath. The heating rate was regulated around 0.1°C min⁻¹ and the cp was defined as the temperature at which the solution started to turn cloudy. The reproducibility of the determination was $\pm 0.5^\circ\text{C}$.

TEM observation

The size and morphology of polymer particles in aqueous solutions were determined by a JEM-100CXII

transmission electron microscope (TEM; Hitachi, Japan). 0.5 mL dilute polymer solutions (5 mg/mL) with the addition of 5 μL of HCl (10^{-2} mol/L) or NaOH (10^{-2} mol/L) were sonicated, then applied, respectively, onto formvar-membrane-coated copper grids and dried at 20°C (below the LCST) to form a thin film for observation.

Luminescence studies of copolymer in water

The self-aggregation of copolymer in water was fluorometrically investigated. The fluorometric measurements were recorded on a Shimadzu RF-5301PC spectrometer (Shimadzu, Japan). The slit settings were 5 or 10 nm, and emission spectra were monitored with an excitation wavelength of 330 nm (λ_{ex}).

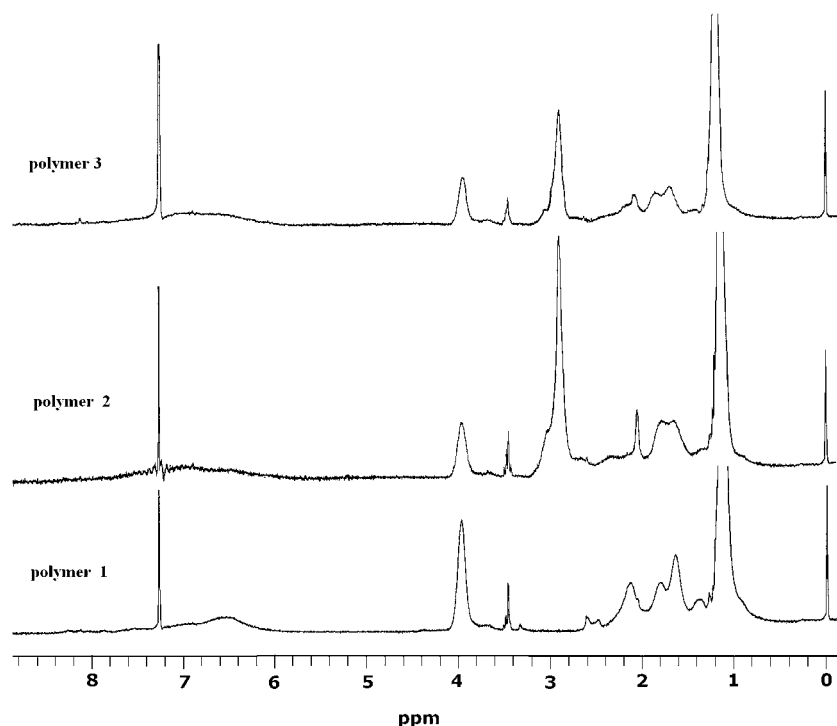


Figure 1 ¹H-NMR spectra of Polymer 1, 2, and 3 in CDCl₃, around 3.4 ppm derived from residual diethyl ether.

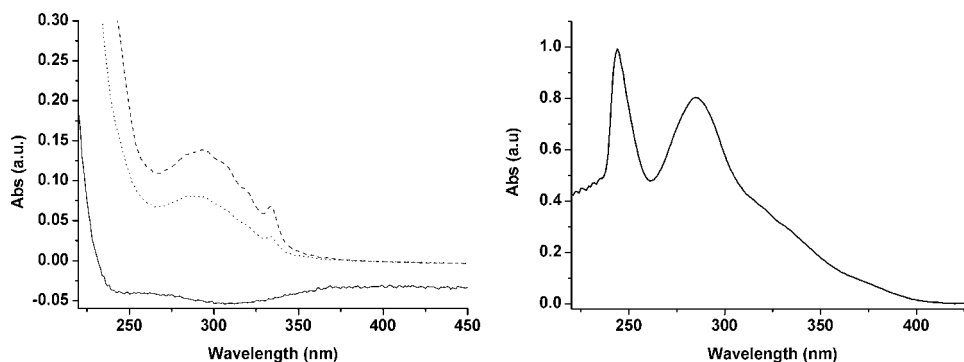


Figure 2 UV-vis absorption spectra of polymeric aqueous solutions (left, 10^{-4} g/mL): 1 (dash), 2 (dot), and PNIPAAm homopolymer (solid) for comparison; spectrum of monomer MANTU in CHCl_3 (right, 5×10^{-5} mol/L).

The luminescence studies were carried out in water at about 20°C , which was below the LCST of copolymer. Copolymer solution of water (3.5 mL; 10^{-4} g/mL) was continuously titrated by hydrochloric acid (10^{-2} mol/L) or sodium hydroxide (10^{-2} mol/L) solution. From each run, relative fluorescence intensity as a function of the volume of added H^+ or OH^- was recorded. The pH values of the solutions were measured by a pXSJ-216 ionanalyzer (REX[®], Shanghai, China).

Interactions of copolymer with nucleotides

Solution of Copolymer 3 (3.5 mL; 5×10^{-5} g/mL in 0.05 mol/L Tris-HCl buffer, pH = 7.4) was continuously titrated by a solution of ATP (0.114 mol/L in 0.05 mol/L Tris-HCl buffer, pH = 7.4) or AMP (0.114 mol/L in 0.05 mol/L Tris-HCl buffer, pH = 7.4). Fluorescence spectra were recorded at the excitation wavelength of 330 nm (λ_{ex}).

RESULTS AND DISCUSSION

Polymer synthesis and characterization

Hydrophilic monomer NIPAAm was radically copolymerized with hydrophobic comonomer MANTU. Scheme 1 shows the general synthetic outline for polymerization. $^1\text{H-NMR}$, GPC, UV-vis absorption, FTIR, and fluorescence spectroscopies were used to characterize the polymers. The compositions and molecular weights of the polymers are summarized in Table I.

The $^1\text{H-NMR}$ spectra of Copolymer 1, 2, and 3 are shown in Figure 1. The characteristic peaks at 4.8–6.0 ppm corresponding to the vinyl groups of monomers disappeared completely. And those derived from the protons of MANTU hydrophobic group around 6.2–8.6 ppm emerged. The main difference of $^1\text{H-NMR}$ peaks between the binary Copolymer 1 and the tercopolymer (Polymers 2 and 3) appears at about 2.9 ppm, which belongs to the *N*-dimethyl groups of DMAAm. Figure 2 shows the UV-vis absorption of Polymers 1

and 2, homopolymer PNIPAAm, and the monomer MANTU. The UV-vis of Polymer 3 is similar to that of Polymer 2. The Polymers (1 and 2) display the absorption at about 280 and 330 nm, due to the E_2 and B vibrating band of naphthyl group respectively, which are analogous to the monomer absorption system, while NIPAAm homopolymer displays no absorption at that range of wavelength. Because of the introduction of fluorescent naphthyl groups, the amphiphilic copolymer shows a specific fluorescence in aqueous solutions with an excitation maximum at about 330 nm and an emission maximum at about 375 nm (Fig. 3).

Thermosensitive properties of copolymers

The polymer synthesized in this study contains the NIPAAm moiety as its hydrophilic segments and thus the polymer is thermosensitive. The LCST value of PNIPAAm homopolymer is about 32°C in pure water. In this study the LCST values of Polymers 1, 2, and 3

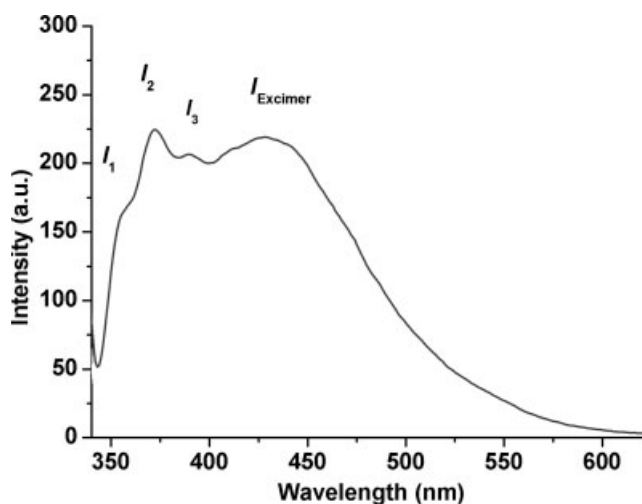


Figure 3 Fluorescence emission spectra of Polymer 3 solution (Tris-HCl, pH = 7.4) at the concentration of 10^{-4} g/mL at 20°C excited at 330 nm.

are 25.0, 49.0, and 38.0°C, respectively. For Polymer 1, because of the introduction of hydrophobic segments, the hydrophobic effects become stronger and the polymer chains tend to collapse; thus the polymers become less soluble in water and their LCSTs decrease.^{7,15,16} On the contrary, for Polymers 2 and 3 the hydrophilic segments of DMAAm introduced make the hydrophobic effects become weaker and the LCSTs of 2 and 3 increase to 49.0 and 38.0°C, respectively, which are higher than those of PNIPAAm homopolymer. Polymer 2 contains more DMAAm segments than does Polymer 3, and so its hydrophilic/hydrophobic ratio is higher and its LCST is higher than that of Polymer 3. In the following studies, Polymer 3 was selected as a typical sample because its LCST is close to body temperature.

Photophysics of tercopolymer in aqueous solutions

The synthesized copolymers show a specific fluorescence behavior in aqueous solutions with an excitation maximum at about 330 nm and an emission maximum at 375 nm. Figure 3 shows the typical fluorescent spectroscopy of copolymer in Tris-HCl solution at pH = 7.4. I_1 , I_2 , and I_3 belong to the monomer emission band, and I_2 is the maximum emission peak; thus the maximum emission wavelength is 375 nm (λ_{EM}). The broad peak ($I_{Excimer}$) at about 430 nm without fine structures belongs to the excimer, which is formed from the ground state and excited-state naphthyl groups.^{17,18}

Photophysics and the effect of pH of copolymer in aqueous solutions were studied by intrinsic fluorescence observations. Figure 4(a) shows the fluorescence intensity of Polymer 3 upon the addition of hydrochloric acid (pH = 3.1–7.0). With the decrease of the pH of the polymer solution, the monomer emission (I_1 , I_2 , and I_3) intensity of fluorescence decreased, while the excimer emission ($I_{Excimer}$) increased with the continuous addition of acid. The relative intensity of excimer emission can be characterized by the excimer-to-monomer intensity ratio (I_E/I_2), which was obtained by measuring the intensities of the monomer emission at 375 nm and the excimer emission maximum at 430 nm.¹⁹ The ratio of I_E/I_2 increased from 0.97 to 1.73. Figure 4(b) shows the fluorescence intensity of Polymer 3 upon the addition of sodium hydroxide (pH = 7.0–10.6). With the increase of the pH of the polymer solution, both the monomer emission intensity and the excimer emission intensity decreased. The ratio of I_E/I_2 decreased from 0.97 to 0.96. This interesting fluorescence responsive behavior can be attributed to the pH-sensitivity characteristic of copolymers. The triggering of this pH-sensitivity may be attributed to the hydrophilic segments (due to the variation of the amide–water hydrogen bonding), or the hydrophobic

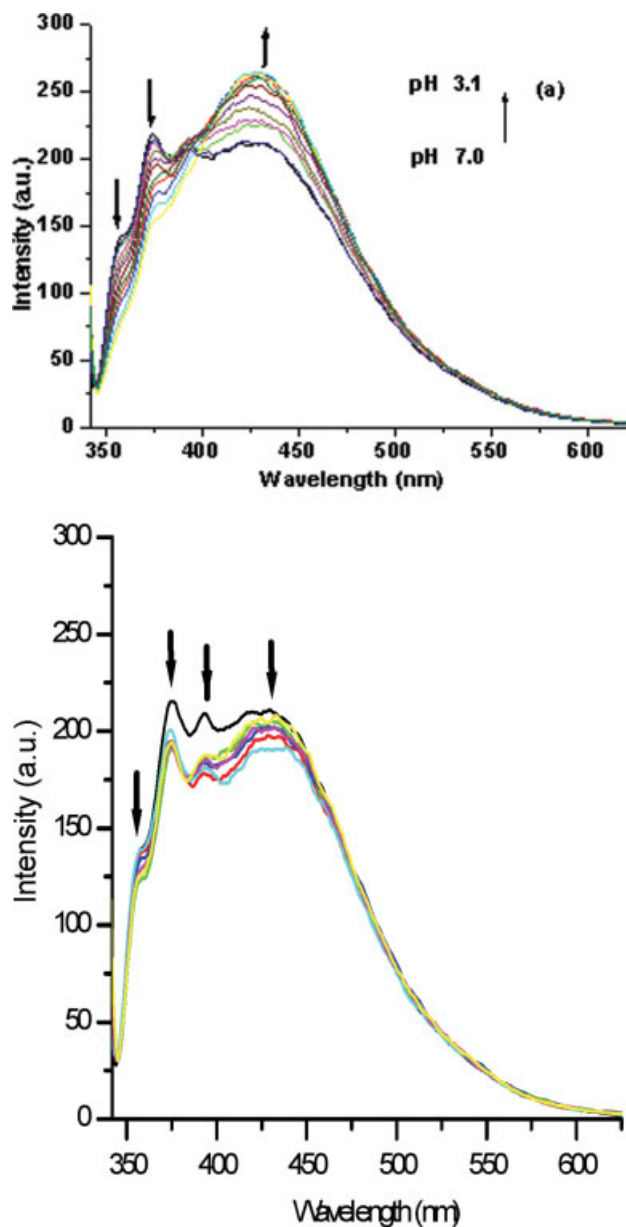


Figure 4 Fluorescence spectroscopy of Polymer 3 aqueous solution (10^{-4} g/mL) upon the addition of HCl (10^{-2} mol/L, 0–290 μ L) (a) and the addition of NaOH (10^{-2} mol/L, 0–150 μ L) (b), respectively, at 20°C excited at 330 nm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

segments (due to the protonation/deprotonation of the thiourea group), or both of them.¹⁰

The addition of acid (pH-decrease) significantly increased the amide–water hydrogen bonding, thereby increasing the solvation of polymer chains, which in turn would increase the water solubility of the polymer. This solubilization created conditions for the more convenient hydrophobic association of naphthyl groups in aqueous solutions. The better associations facilitated the formation of naphthyl excimers, and so the emission intensity of them increased and the corre-

sponding intensity of monomer emission decreased till $\text{pH} = 3.3$, while in the case of pH -increase the amide–water hydrogen bonding was destroyed and the hydrophobic effect enhanced. Thus the polymer chain contracted more and more and made the chromophores closer, thereby probably enhancing self-quenching processes, and decreased slightly the fluorescence intensity of monomer and excimer. But this decrease did not go on after the $\text{pH} = 10.1$. This indicated that the base-sensitivity of Polymer 3 is much more limited than acid-sensitivity, which was analogous to our discussion in Ref. 10.

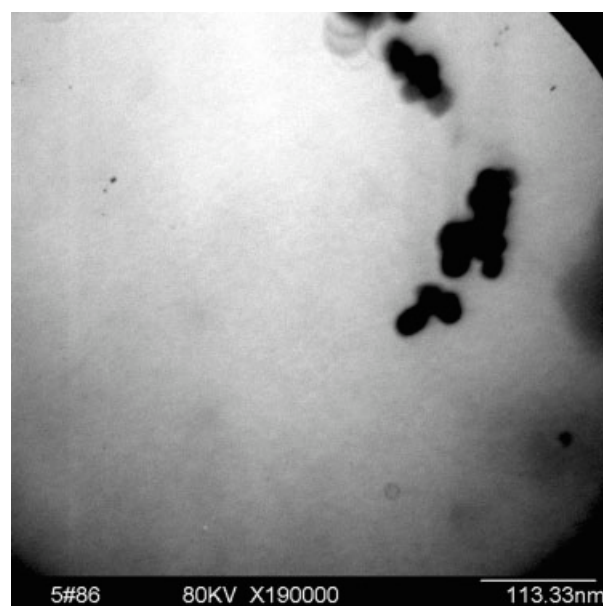
Self-aggregation of tercopolymer in aqueous solutions

The self-aggregation behavior of Polymer 3 in aqueous solution and the effect of pH were explored by TEM observations. Figure 5 shows the transmission electron micrographs of Polymer 3 in aqueous solutions in a base or acid condition, where their pH values are 10.0 and 4.0, respectively. Under base conditions, the polymers showed a spherical morphology with the diameter of about 20 nm, and the micelles had the trend to accumulate together and precipitate at last. That was a very contracted-coil configuration for polymer chains which was from the breaking of the amide–water H-bonding with the increase of pH . On the contrary, with the addition of acid the water solubility of the polymer increased and made good conditions for the originally separated hydrophobic groups to form further assemblies. As shown in Figure 5, the polymers in acid conditions self-aggregated to spindly morphologies with the length of 150–200 nm. TEM observations under acid or base conditions can further confirm the pH -sensitivity of NIPAAm copolymers. The proposed mechanism about this is shown in Figure 5(c).

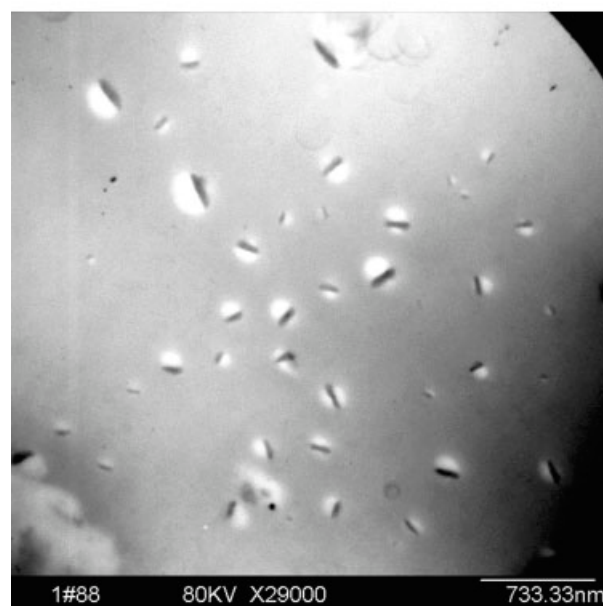
From the self-aggregation of Polymer 3 in acid or base conditions we can conclude that thermosensitive NIPAAm amphiphilic copolymers were also pH -sensitive, especially in acid condition. The main contribution of this pH -sensitivity comes from the H-bonding effects: the amide–water H-bonding's formation or breaking impels the chain extending or chain contracting, respectively.

Interactions with ATP and AMP

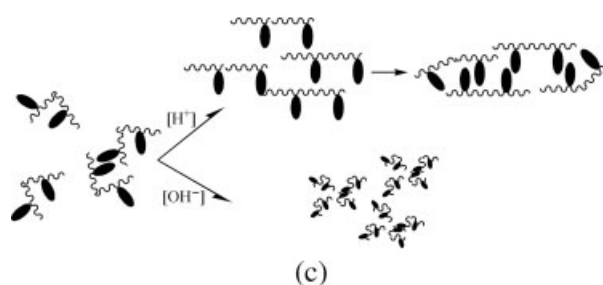
Fluorimetric titrations were conducted in the presence of ATP or AMP sodium salts. The fluorescence spectra are shown in Figure 6. Figure 7 shows the change in fluorescence intensity as the concentration of ATP is increased. In the presence of ATP, monomer emissions (I_1 , I_2 , and I_3) were quenched continuously till the near disappearance, but the excimer emission changed a little. An interpretation for ATP's quench-



(a)



(b)



(c)

Figure 5 TEM photographs of polymer 3 aqueous solutions (0.5 mL, 5 mg/mL) $\text{pH} = 10.0$ (a) and $\text{pH} = 4.0$ (b) aqueous solutions, respectively. Schematic of the proposed mechanism (c).

ing effect could be a π -stacking interaction between the naphthalene unit and the adenine fragment.¹⁴ This interaction can only happen between the ATP and some isolated hydrophobic groups that were localized outside the micelles. So the monomer emissions were fully quenched and the assembled excimers inside micelles were protected and not quenched. Interestingly enough, the interaction of AMP with Polymer 3 did not yield such a quenching effect. In the presence of AMP, both the monomer and the excimer emissions quenched. Probably a different matching between the monophosphate and the thiourea fragment of hydrophobic groups prevented an efficient π -stacking occurring. But the addition of salt increased the "salting-out" effect and made the copolymer chains contract, thereby increasing the nonradiative energy transfer and decreasing fluorescence intensity. These results demonstrate that the amphiphilic tercopolymer inter-

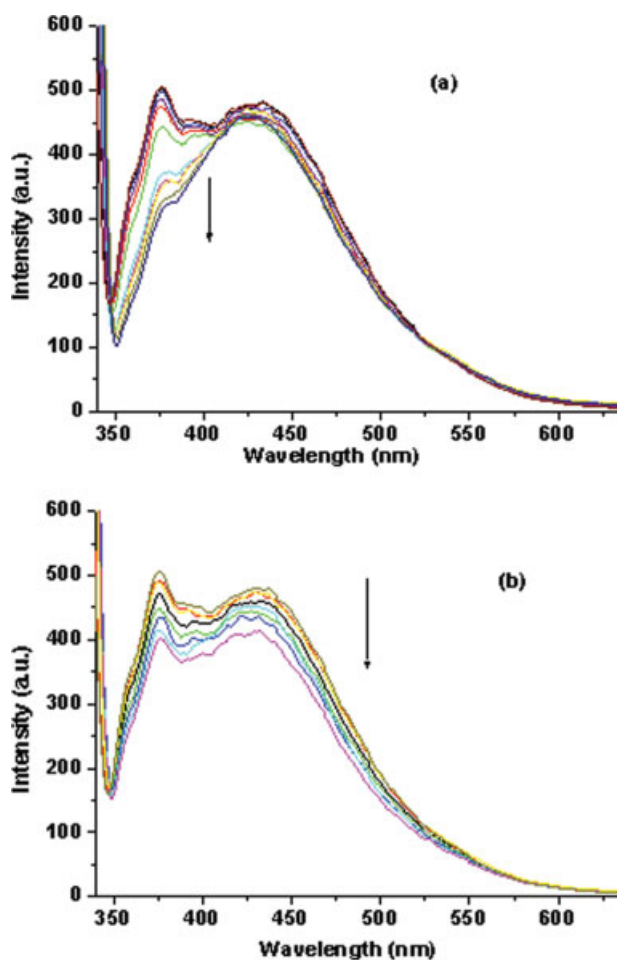


Figure 6 Fluorescence spectroscopy of Copolymer 3 aqueous solution (5×10^{-5} g/mL in 0.05M Tris-HCl, pH = 7.4) upon the addition of ATP (0.114 mol/L, 0–570 μ L) (a) and the addition of AMP (0.114 mol/L, 0–480 μ L) (b), respectively, at 20°C excited at 330 nm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

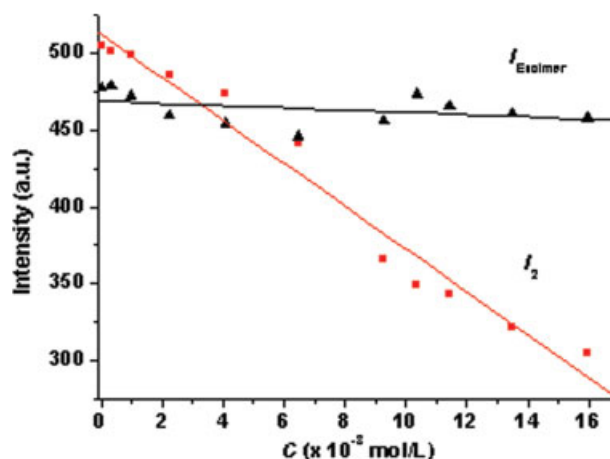


Figure 7 Fluorescence intensity versus the concentration of ATP (mmol/L). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

acts with nucleotides, achieving selectivity for ATP to some degree.

CONCLUSIONS

Amphiphilic, fluorescent and thermoresponsive copolymers were prepared and characterized. The LCST of the polymers can be tuned by the addition of DMAAm. The LCST of Polymer 3 was 38.0°C, which is close to body temperature. The copolymers are also pH-sensitive, as fluorescence emission of Polymer 3 significantly changed upon the addition of acid or base between wide ranges of pH from 3.3 to 10.1. When pH decreased from 7.0 to 3.3, the ratio of I_E/I_2 increased from 0.97 to 1.73. The self-aggregation behavior of the copolymers was confirmed by TEM observation. The copolymer aqueous solution in acid conditions self-aggregated to spindly morphologies with the length of 150–200 nm; in base conditions, a spherical morphology was obtained with the diameter of about 20 nm. The water-soluble copolymer interacts with ATP and monomer emissions (I_1 , I_2 , and I_3) were quenched continuously till the nearly disappearing. These amphiphilic fluorescent copolymers own potential use in the field of thermo- and pH-sensitive, fluorescent responsive material.

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