Controlled Association of Amphiphilic Polymers in Water: Thermosensitive Nanoparticles Formed by Self-Assembly of Hydrophobically Modified Pullulans and Poly(*N*-isopropylacrylamides)

Kazunari Akiyoshi,* Eui-Chul Kang, Satoshi Kurumada, and Junzo Sunamoto

Department of Synthetic Chemistry & Biological Chemistry, Graduate School of Engineering, Kyoto University, Sakyo-ku, Yoshida Hommachi, Kyoto 606-8501, Japan

Tania Principi and Françoise M. Winnik*

Department of Chemistry, McMaster University, 1280 Main St. W, Hamilton, Ontario L8S 4MI, Canada

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ABSTRACT: Thermoresponsive hydrogel nanoparticles were prepared by self-assembly of two different hydrophobically modified polymers, namely a cholesterol-bearing pullulan (CHP) and a copolymer of *N*-isopropylacrylamide (NIPAM) and *N*-[4-(1-pyrenyl)butyl]-*N*-*n*-octadecylacrylamide] (PNIPAM-C_{18}Py). The interactions between CHP and PNIPAM-C_{18}Py were investigated by fluorescence spectroscopy, dynamic light scattering, and size exclusion chromatography. After ultrasonication of a mixture of CHP and PNIPAM-C_{18}Py (5:1 by weight) at 25 °C, monodisperse nanoparticles (Dh = 45 nm) were obtained, consisting of self-assembly of the two polymers associated via their hydrophobic moieties. Evidence from fluorescence and dynamic light scattering demonstrated that, above 32 °C, the lower critical solution temperature (LCST) of PNIPAM-C_{18}Py, the colloidal mixed nanoparticles increase in diameter (from 47 to 160 nm), but no macroscopic aggregation could be detected. This phenomenon was thermoreversible: upon cooling the particles recovered their original diameter.

Introduction

The concept of supramolecular assembly has led to new methodologies for the design of functional nanostructures based on the controlled association of hydrophobically modified (HM) polymers in water. Such polymers consist usually of a water-soluble main chain carrying a small number of hydrophobic groups. The hydrophobic substituents associate and form hydrophobic domains with several transient cross-links connecting polymer chains. The solution properties of HM-polymers have been studied extensively in relation to their applications as thickeners in food and as rheological modifiers in latex-based paints,[1,2] as well as with respect to their biotechnological and pharmaceutical applications.[1,3] Most studies focus on the association of one specific polymer in water. There are relatively few investigations of the assembly of HM-polymers having different chemical composition. A recent study of mixed systems consisting of amphiphilic polyelectrolytes and neutral HM-polymers bearing the same hydrophobic groups indicates that association of different polymers can take place under propitious circumstances,[4] but in most cases, it seems, polymers of different chemical constitution tend to segregate in solution. One method to generate stable hybrid systems involves the preparation of interpenetrating polymer networks (IPN) hydrogels.[5] IPNs are mixed polymeric systems held together mainly by the permanent entanglement of two or more cross-linked networks.[6−8] IPN hydrogels are generally prepared by a sequential synthesis, in which a secondary gel is synthesized in the presence of a cross-linker within a preformed chemically cross-linked gel. The physical properties of such gels depend on their phase morphology, a parameter that often has proved to be difficult to control.

We report here the preparation and properties of functional hydrogel nanoparticles consisting of different polymers constructed on the nanometer scale by the self-assembly in water of hydrophobic groups linked to two neutral HM-polymers: HM-pullulans and HM-poly(*N*-isopropylacrylamides) (HM-PNIPAM). Cholesterol-bearing pullulans (CHP) have been studied in detail by Akiyoshi and Sunamoto.[9−11] Using a battery of physical measurements, they demonstrated that CHP's in water form spherical monodisperse nanoparticles made up of a small number of self-aggregated CHP molecules. The nanoparticles have a hydrogellike structure, in which the pullulan main chains are cross-linked noncovalently via associating cholesteryl moieties. The nature of the association of CHP's in water depends on the solution concentration. In the dilute regime, distinct nanoparticles form predominantly. As the polymer concentration exceeds approximately 2% (w/w), the onset of the semidilute regime for this polymer, the viscosity of CHP solutions increases suddenly,[12] and as the concentration exceeds 3.5% (w/w), macrogels form via interconnection of many CHP nanoparticles. The association and dissociation of CHP can be induced by external triggers, such as the addition of β-cyclodextrin and its guest molecules such as adamantanecarboxylic acid.[13] The CHP nanoparticles also act as hosts for hydrophobic molecules and various soluble proteins.[14,15]

Hydrophobically modified poly(*N*-isopropylacrylamides) (HM-PNIPAM) also form polymeric micelles in water, but their properties are quite different from those of HM-pullulans.[16−19] HM-PNIPAM micelles consist of hydrophobic domains surrounded by hydrophilic poly-

* Corresponding authors. K. Akiyoshi: phone (81) 75 753 5637, Fax (81) 75 753 5912, e-mail g54112@sakura.kudpc.kyoto-u.ac.jp. F. M. Winnik: phone (905) 525 9140, ext 23497; Fax (905) 540 1310, e-mail winnikf@mcmaster.ca.

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mer main chains. They exist in extremely dilute solutions, and they always involve several polymer chains. They are stable indefinitely in cold water, but they are severely disrupted when their aqueous solutions are heated above a critical temperature, at which the hydrophilic PNIPAM chains collapse and the hydrophobic groups are accommodated, mostly as isolated entities, within the separated PNIPAM-rich phase.[20] Poly(*N*-isopropylacrylamide) itself does not associate in cold water, but its aqueous solutions undergo thermoreversible phase separation at a critical temperature (31 °C).[21] The phase separation mechanism of PNIPAM in water has been shown to take place in two steps:[22] (1) the collapse of individual polymer chains from a highly hydrated extended coil into a globule and (2) the aggregation of the globules triggering macroscopic phase separation. In the case of HM-PNIPAM's, the phase transition temperature depends modestly on the structure of the hydrophobic group, its location along the chain, and its level of incorporation. The hydrophobic substituent of the HM-PNIPAM's employed in this study is the *N*-[4-(1-pyrenyl)butyl]-*N*-*n*-octadecylacrylamide group. Pyrene was employed as a fluorescence label to monitor the changes in the structure of the hydrophobic core of HM-PNIPAM micelles via the relative intensity of the pyrene monomer emission and pyrene excimer emission.[23]

We preliminarily reported interactions between different hydrophobized polymers such as hydrophobically modified pullulans and poly(*N*-isopropylacrylamides) in water.[24] This study describes in detail the formation of hybrid thermoresponsive nanoparticles by self-assembly of a cholesteryl group-bearing pullulan (CHP) and a pyrene-labeled HM-PNIPAM (PNIPAM-C_{18}Py) (Figure 1). Evidence for the existence of mixed micelles was gathered from fluorescence spectroscopy and size exclusion chromatography experiments carried out in mixed solutions. Further information on the size of the hybrid nanoparticles and their sensitivity to changes in solution temperature was obtained from dynamic light scattering studies.

Experimental Section

Materials. Water was purified with a Millipore Milli-Q System (Kyoto University) or a Barnstead NANOpure water purification system (McMaster University). Analytical grade solvents were used without purification. The HM-pullulan sample, CHP-108-0.9, was synthesized as reported previously from a pullulan of molecular weight 108 000. [9,10] It contains 0.9 cholesterol groups per 100 glucose units. The HM-PNIPAM sample, PNIPAM-C_{18}Py, was prepared as previously described.[16] It has a viscosity average molecular weight of 380 000 and contains on average one *N*-[4-(1-pyrenyl)butyl]-*N*-*n*-octadecylacrylamide group per 400 NIPAM units.

Instrumentation and Techniques. Dynamic light scattering measurements were performed on a Brookhaven BI9000 AT instrument equipped with an argon laser (λ = 514 nm, scattering angle 90°). Data were analyzed using the software provided by the manufacturer (CONTIN calculations). The average diameter of the samples was calculated using the three closest values for each sample. UV spectra were recorded with a Hewlett-Packard 8452A photodiode spectrometer equipped with a Hewlett-Packard 89090A temperature controller. Cloud points were determined spectrophotometrically by changes in turbidity of solutions heated at a constant rate (0.2 °C min^{-1}) in a magnetically stirred UV cell, as described previously.[25] Fluorescence spectra were recorded on a Hitachi F-3010 fluorescence spectrometer. The excitation wavelength was 330 nm, and the slit widths were set at 5.0 nm (excitation) and 3.0 nm (emission). The ratio of the pyrene excimer emission intensity (I_E) to the pyrene monomer emission intensity (I_M) was taken as the ratio of the emission intensity at 479 nm to the half-sum of the emission intensities at 377 and 397 nm. The temperature of the water-jacketed cell holder was controlled with a Neslab circulating bath. Size exclusion chromatography (SEC) was carried out on a chromatography system (Tosoh Ltd. Tokyo) composed of a CCPD dual pump, a CO-8010 column oven, a RI-8010 refractive index detector, and a Chromatocorder 12 data processing system and equipped with a TSK-Gel G4000SWXL column. The eluent was water containing 0.02 wt % NaN_3. The flow rate was set at 0.5 mL/min and at 30 °C. Standard pullulan samples (M_w = 2.37 × 10^4, 4.8 × 10^4, 1.0 × 10^5, 1.86 × 10^5, 3.8 × 10^5, 8.53 × 10^5) (Showa Denko) were used for molecular weight calibration.

Sample Preparation. CHP (12.0 mg) was suspended in water (10.0 mL). The mixture was kept at 50 °C for 24 h to allow the polymer to swell. The resulting suspension was sonicated for 5 min at room temperature (24 °C) with a sonicator probe (UR-200P, Tomy Seiko Co. Ltd, Tokyo, Japan) operated at 40 W. The solution was filtered through a Millipore filter (pore size 0.2 μm). The CHP concentration was determined by the phenol−sulfuric acid method.[9] A stock solution of PNIPAM-C_{18}Py was prepared by adding the polymer (5.0 mg) to water (5.0 mL). The solution was kept for 24 h at 4 °C to allow the polymer to dissolve. Mixed solutions were prepared by adding aliquots of the CHP stock solution (1−9 w/w with respect to PNIPAM-C_{18}Py) to a PNIPAM-C_{18}Py stock solution (final PNIPAM-C_{18}Py concentration 0.1 g L^{-1}). The mixture was kept at 25 °C for 24 h, treated by sonication as described above, and filtered (membrane pore size 0.2 μm) prior to measurements.

Temperature-Controlled Experiments. For fluorescence measurements, a solution of CHP (final concentration 0.5 g L^{-1}) was added to a solution of PNIPAM-C_{18}Py (final concentration 0.1 g L^{-1}). The mixture was sonicated for 5 min, filtered, and placed in a thermostated cell holder in the spectrometer sample compartment. The sample was heated stepwise from 20 to 50 °C. Once brought to a desired temperature within this range, the sample was kept at that temperature for 1 h before measurement. The solutions brought to 50 °C were cooled to 25 °C. Their spectra were recorded at this temperature as a function of time. For dynamic light scattering measurements, mixed solutions prepared under conditions identical to those described for fluorescence studies were placed in the cell compartment of the DLS system and kept at 25 °C for 15 min prior to measurement. The sample was heated stepwise from 25 to 40° C (temperature increment: ΔT = 2 °C from 25 to 29 °C and 36 to 40 °C and ΔT = 1 °C from 29 to 36 °C). Samples were equilibrated at each temperature for 15 min prior to measurements. Measurements were repeated three times at each temperature.

Results and Discussion

Solution Properties of Mixed HM-Pullulan/HM-PNIPAM Systems. The association in water of the two hydrophobically modified polymers was investigated first in solutions kept below the cloud point of HM-PNIPAM. Fluorescence spectroscopy was used to monitor changes in the self-association of HM-PNIPAM in the presence of CHP. The effects of HM-PNIPAM on the characteristics of the CHP nanoparticles were observed by size exclusion chromatography assays and dynamic light scattering studies. Results gathered from these three experimental techniques are reported in turn in the following section.

The photophysical properties of pyrene-labeled hydrophobically modified NIPAM copolymers have been reported in detail by Winnik.[16−18,23,24] Upon excitation at 330 nm, PNIPAM-C_{18}Py in water exhibits an emission due to locally isolated excited pyrene chromophores (intensity I_M, pyrene "monomer" emission) with the (0,0) band located at 377 nm, together with a broad feature-

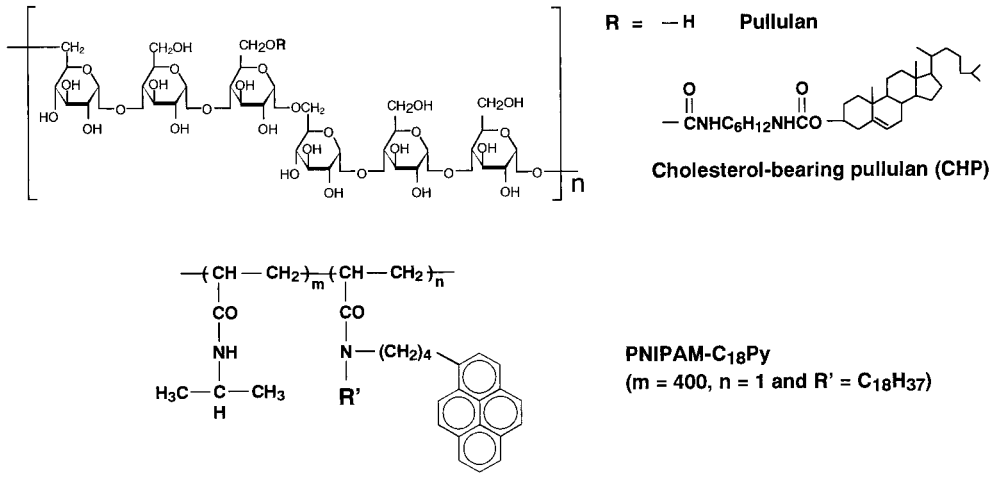

Figure 1. Chemical structure of the polymers used in this study.

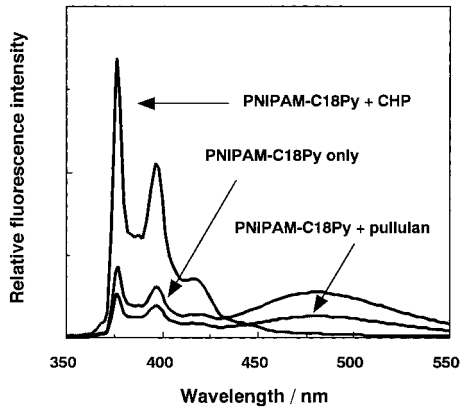

Figure 2. Fluorescence spectra of PNIPAM-C_{18}Py (0.1 g L^{-1}) in water alone and in the presence of either CHP (0.5 g L^{-1}) or pullulan (0.5 g L^{-1}). Temperature = 25 °C; λ_{exc} = 330 nm.

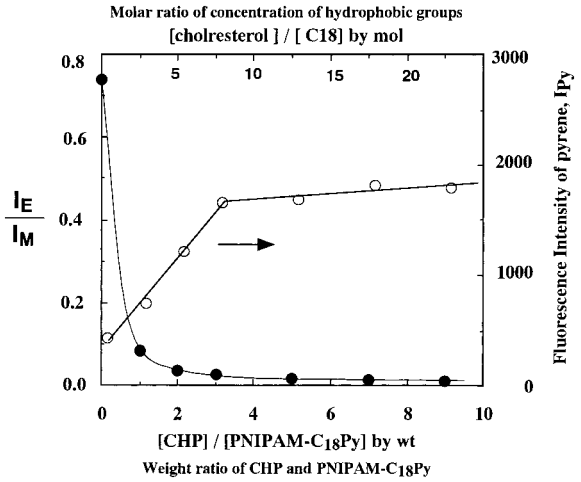

Figure 3. Plots of the changes of the ratio of the pyrene excimer emission intensity (I_E) to the pyrene monomer emission intensity (I_M), I_E/I_M (full circle), and the intensity of pyrene, I_{Py} (open circle), in mixed aqueous solutions of CHP and PNIPAM-C_{18}Py (0.1 g L^{-1}) as a function of the CHP/PNIPAM-C_{18}Py weight ratio. Temperature = 25 °C; λ_{exc} = 330 nm.

less emission centered at 479 nm (intensity I_E) due to pyrene excimers. The strong excimer contribution to the total emission indicates that the pyrene groups reside in close proximity, partly as preformed aggregates, within the hydrophobic microdomains of polymeric micelles.

Upon addition of CHP (final concentration, 0.5 g L^{-1}) to a solution of PNIPAM-C_{18}Py (0.1 g L^{-1}) the strong excimer emission all but disappears, while the pyrene monomer emission intensity increases significantly. The ratio of the excimer to monomer emission intensities (I_E/I_M) decreases from a value of 0.74 in the absence of CHP to 0.01 in the presence of CHP (0.5 g L^{-1}). The effect is illustrated in Figure 2 which presents the fluorescence spectrum of PNIPAM-C_{18}Py (0.1 g L^{-1}) in water at 25 °C together with the spectra of this polymer in the presence of CHP and in the presence of unmodified pullulan of molecular weight identical to that of CHP. The CHP-induced decrease in the pyrene excimer emission intensity implies that the pyrene aggregates formed in solutions of pure HM-PNIPAM are disrupted in the presence of CHP, such that the encounter probability of an excited Py and a ground-state Py has become very low. We note that the presence of pullulan with no hydrophobic moieties does not affect appreciably the emission spectrum of PNIPAM-C_{18}Py. Next, we assessed the effects of the relative amount of the two polymers on the fluorescence of PNIPAM-C_{18}Py. The results are presented in terms of the CHP concentration dependence of the ratio I_E/I_M and of I_{Py}, the intensity of the pyrene monomer emission monitored at 377 nm (Figure 3). In these experiments, the concentration of PNIPAM-C_{18}Py was kept constant (0.1 g L^{-1}) and the concentration of CHP was increased from 0 to 0.5 g L^{-1}. The fluorescence properties of PNIPAM-C_{18}Py are very sensitive to the presence of small amounts of CHP. Above a value of 3.0 of the weight ratio of PNIPAM-C_{18}Py to CHP ([CHP]/[PNIPAM-C_{18}Py]), a value corresponding to a 7.5 molar ratio of the hydrophobic groups ([cholesteryl groups of CHP]/[C_{18}Py groups of PNIPAM-C18Py]), the ratio I_E/I_M and the value of I_{Py} level off and remain constant upon further increase of the [CHP]/[PNIPAM] weight ratio. Thus, above this concentration ratio, all the pyrene groups linked to PNIPAM-C_{18}Py are isolated from each other as a result, we believe, of their incorporation into the hydrophobic cholesterol-rich microdomains of the CHP nanoparticles. Further evidence for this association mechanism and for preservation of the HM-pullulan nanoparticle morphology was provided by size exclusion chromatography and DLS experiments.

The association of the two hydrophobically modified polymers was monitored by analytical size exclusion chromatography (SEC) of mixed solutions containing excess CHP ([CHP[/[PNIPAM-C_{18}Py] = 5.0 w/w). Solu-

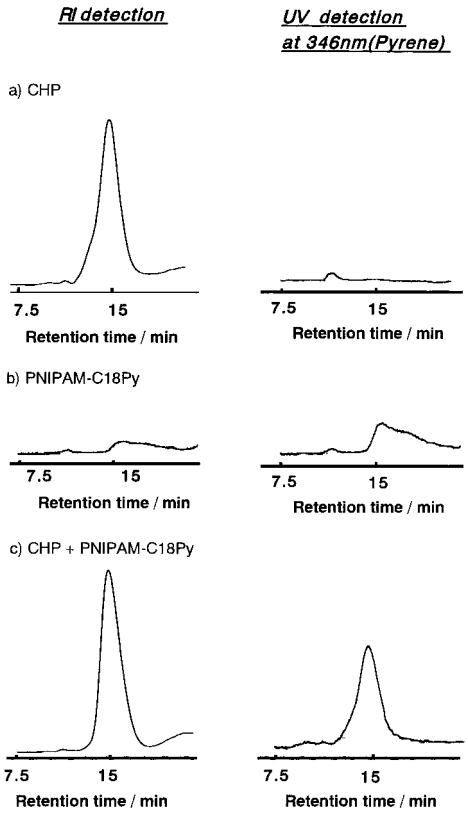

Figure 4. Size exclusion chromatograms of (a) CHP (0.5 g L^{-1}), (b) PNIPAM-C_{18}Py (0.1 g L^{-1}), and (c) a mixture of CHP (0.5 g L^{-1}) and PNIPAM-C_{18}Py (0.1 g L^{-1}). Eluent: 0.02 wt % NaN_3 in water; flow rate: 0.5 mL/min; UV and RI detection.

tions of each polymer alone were analyzed under the same conditions. Through the use in tandem of a refractive index (RI) detector and a UV absorbance detector (detection wavelength: 346 nm), it was possible to detect fractions containing only CHP (RI signal, but no UV signal) and HM-PNIPAM alone or in the presence of CHP (RI and UV response). The chromatogram of a sample of CHP alone presents a single band easily detected by RI changes (Figure 4 a).[8] Elution of a solution of HM-PNIPAM resulted in a weak signal in both RI- and UV-monitored chromatograms (Figure 4b). It may be possible that HM-PNIPAM interacts with the column packing under our experimental conditions which were optimized for the detection of pullulans rather than PNIPAM. Elution of a mixed solution prepared under conditions identical to those used in the preparation of samples for fluorescence studies (CHP, 0.5 g L^{-1}; PNIPAM-C_{18}Py, 0.1 g L^{-1}) gave a single signal in the chromatograms monitored by RI and by UV with an elution volume matching closely that of CHP in the absence of added HM-PNIPAM (Figure 4c). No free PNIPAM-C_{18}Py could be detected. The apparent polydispersity of the aggregate, calculated using a calibration curve of standard pullulans, was estimated to be below 1.2, a value comparable to that determined from the chromatogram of a solution of CHP alone (1.2).

Solutions of CHP in water gave a strong signal in dynamic light scattering measurements. It was confirmed that the nanoparticles were uniform in size with an average diameter of 24 ± 2 nm. A unimodal distribution of particle size was also detected by DLS analysis of a mixed solution ([CHP]/[PNIPAM-C_{18}Py] = 5.0 w/w), but the average size of the colloidal particles (45 ± 4 nm) was significantly larger than the size of CHP particles. It was established previously that the CHP nanoparticles are held together by a few hydrophobic domains consisting of 4−5 associated cholesteryl moieties which act as noncovalent cross-linkers of several pullulan chains.[11] Our DLS results suggest that the PNIPAM-C_{18}Py chains become entangled with CHP chains upon association of their hydrophobic substituents with the cholesteryl groups inside the hydrogel nanoparticle.

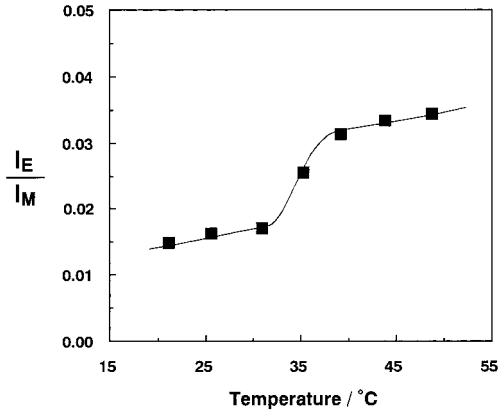

Figure 5. Plot of the changes in the ratio of the pyrene excimer emission intensity (I_E) to the pyrene monomer emission intensity (I_M), I_E/I_M, as a function of temperature for mixed solutions of PNIPAM-C_{18}Py (0.1 g L^{-1}) and CHP (0.5 g L^{-1}). λ_{exc} = 330 nm.

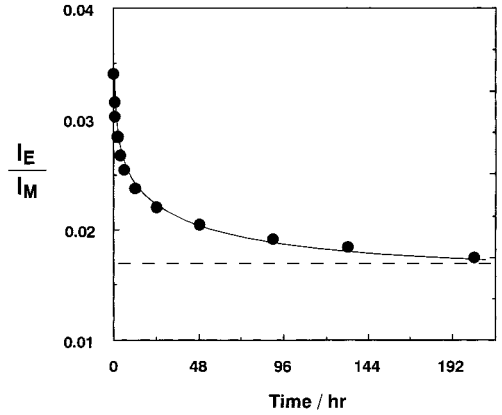

Figure 6. Plot of the changes in the ratio of the pyrene excimer emission intensity (I_E) to the pyrene monomer emission intensity (I_M), I_E/I_M, of mixed solutions of CHP (0.5 g L^{-1}) and PNIPAM-C_{18}Py (0.1 g L^{-1}) as a function of time after cooling from 50 to 25 °C. The dashed line indicates the value of I_E/I_M of the CHP/PNIPAM-C_{18}Py complex in water before heating. λ_{exc} = 330 nm.

Temperature Effect. Mixed solutions of CHP and PNIPAM-C_{18}Py ([CHP]/[PNIPAM-C_{18}Py] = 5.0 w/w) heated from 25 to 50 °C were monitored by fluorescence spectroscopy and by dynamic light scattering. At 25 °C, the emission of PNIPAM-C_{18}Py/CHP consists mostly of a contribution of the pyrene monomer emission (Figure 2). As the temperature of the mixed solution reaches 31 °C, a temperature which corresponds to the macroscopic phase transition temperature of PNIPAM in water, the contribution of the pyrene excimer undergoes a small, yet significant, increase (Figure 5). This change implies that, on average, the pyrene substituents, located within the CHP hydrophobic cross-linking points, are brought into closer proximity as a result, we postulate, of the contraction of the PNIPAM chains in the mixed aggregates. Upon cooling of the mixed

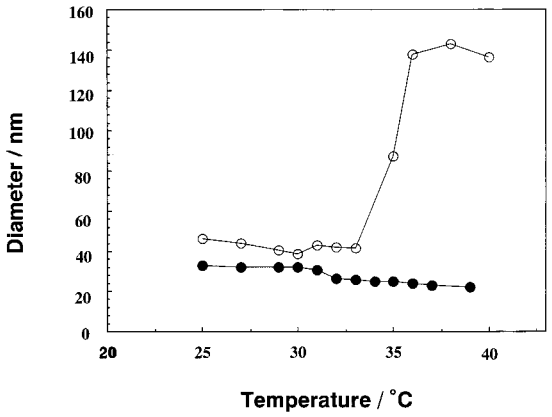

Figure 7. Plot of the changes as a function of temperature of the average diameters of CHP (0.5 g L^{-1}) in water (full circle) and in a mixed CHP (0.5 g L^{-1})/PNIPAM-C_{18}Py (0.1 g L^{-1}) in water (open circle).

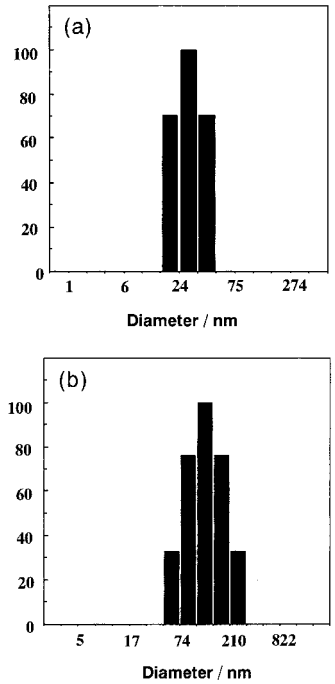

Figure 8. Histograms of the size distributions of the CHP (0.5 g L^{-1})/PNIPAM-C_{18}Py (0.1 g L^{-1}) (a) at 31 °C and (b) at 40 °C.

systems, the original fluorescence spectrum is recovered, albeit only after the solutions are left standing at 25 °C for 3 days. This is shown in Figure 6 which presents the changes with time of the ratio I_E/I_M of CHP/PNIPAM-C_{18}Py solution previously heated to 50 °C.

The thermosensitivity of the mixed systems was also investigated by DLS. The average diameter of the nanoparticles underwent a sudden increase from 47 nm to approximately 150 nm at 32 °C (Figure 7). It should be noted that under the same conditions particles of CHP alone do not exhibit any growth in size (Figure 7). Histograms of the mixed CHP/PNIPAM-C_{18}Py nanoparticles are unimodal at 25 °C as well as at 35 °C (Figure 8). The size change was thermoreversible, but as in the case of the fluorescence properties, it took several days until the particles recovered the initial average diameter (47 nm). Above the LCST several composite CHP/PNIPAM-C_{18}Py nanoparticles aggregate through association between collapsed hydrophobic PNIPAM-rich patches on the surface of the nanoparticles. After cooling of the sample, the aggregates dissociate, as the PNIPAM rehydrate. It is interesting to note that this phenomenon, which occurs within seconds in solutions of PNIPAM, takes several days in the mixed systems surface of the nanoparticles through hydration of PNIPAM.

Conclusion

Thermoresponsive hydrogel nanoparticles have been prepared by the self-assembly of two different hydrophobically modified polymers in water. This mixing of two polymers via association of hydrophobic groups represents a new preparation method of stable functional hydrogel nanoparticles. We reported previously that nanoparticles of hydrophobically modified polysaccharide can be applied in various fields, such as drug carrier systems in medicine[26−29] and protein folding aids or thermal stabilizer of enzymes in biotechnology.[30] It is anticipated that the thermoresponsive nanoparticles prepared in this study are also applicable in these fields, and studies toward this goal are in progress in our laboratories.

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