Solution Properties of Phosphorylcholine-Based Hydrophobically Modified Polybetaines in Water and Mixed Solvents†

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ABSTRACT: Fluorescently labeled amphiphilic polybetaines have been prepared, and their dilute solution properties in water, chloroform, methanol, and mixed organic solvents have been investigated by fluorescence spectroscopy, dynamic light scattering, and ^{1}H NMR spectroscopy. The copolymer chains consisted of *N*-isopropylacrylamide and *N*-phosphorylcholine-*N*′-ethylenedioxybis(ethyl)acrylamide units in ∼1/1 molar ratio, as well as from 3 to 10 mol % hydrophobic units, such as *N*-*n*-(octadecyl)acrylamide, *N*-(1*H*,1*H*-perfluoro-*n*-octyl)acrylamide, or *N*-[(1-pyrenyl)-4-butyl]-*N*-(*n*-octadecyl)acrylamide. In water, the polybetaines associate in multichain aggregates ranging in size from 170 to 220 nm. The assembly of the polymers in water is triggered by two cumulative effects: (1) hydrophobic interactions between the hydrocarbon or fluorocarbon chains and (2) ion pair formation between the phosphorylcholine groups. The polymers do not associate in methanol, but chain aggregation occurs in chloroform, where the phosphorylcholine groups form clusters stabilized by water structuration around these highly polar groups.

Introduction

Amphiphilic polymers in aqueous environment form well-defined nanosized assemblies, such as spheres, capsules, ultrathin films, or structured hydrogels and give access to different length scales, levels of interaction, and time scales. The association process is reversible: the assemblies may disintegrate upon dilution or changes in environmental conditions, such as temperature, ionic strength, or pH. Given the richness of amphiphilic polymer chemistry, it can be applied to build a panoply of novel responsive delivery systems, bioreactors, or diagnostic tools. Indeed, polymers have long been used in experimental drug delivery systems to provide controlled release of small organic compounds or macromolecules. Nonetheless, decades after their first description, only a very small number of polymer-based controlled delivery systems are approved for use in humans. In many cases, biodistribution studies show that polymeric micelles are quickly scavenged by the phagocyte system after intravenous administration, their capture presumed to be triggered by the adsorption of plasma proteins onto their surface. It has been known for several decades that poly(ethylene glycol) (PEG) chains are exceptionally effective in preventing protein adsorption, and this property has been fully exploited in the design of drug delivery systems. In the early 1970s, a biomimetic strategy led Nakaya and co-workers to design polymers incorporating in their structures phosphorylcholine (PC) moieties.[1] The underlying hypothesis of their work was that surfaces incorporating PC-polymers would inhibit protein adsorption, in much the same way as do cell membranes which display a high PC concentration in their outer surface. Indeed, PC-polymers have been shown to impart enhanced biocompatibility and hemocompatibility to surfaces onto which they are coated.[2−5] Currently, solutions, hydrogels,[6] and nanoparticles[7] composed of PC-polymers are evaluated as delivery vehicles in cosmetics[8,9] and pharmaceutical formulations.[10]

It may surprise that despite their outstanding properties, PC-polymers have received much less attention than PEG-based materials. A factor hampering widespread use of PC-polymers lies in the fact that the monomer employed in the synthesis of most PC-polymers, 2-methacryloyloxyethyl phosphorylcholine (MPC),[11−13] is not readily available. It is of limited stability and is difficult to purify. We reported recently an alternate method leading to PC-polymers using a procedure whereby PC groups are linked to a polymer chain via reductive amination of phosphorylcholine glyceraldehyde by primary amine groups attached to the polymer.[14] The synthesis, which proceeds with high overall yield and provides flexibility in polymer architecture and composition, was applied to the preparation of copolymers of *N*-isopropylacrylamide (NIPAM) and *N*-(phosphorylcholine)-*N*′-(ethylenedioxy)bis(ethyl)-acrylamide (NPCAM) (PNIPAM-PC) and several terpolymers, consisting of NIPAM units, NPCAM units, and hydrophobic units, such as *N*-(*n*-octadecyl)acrylamide (PNIPAM-PC-C_{18}), *N*-(1*H*,1*H*-perfluoro-*n*-octyl)-acrylamide, (PNIPAM-PC-F), or *N*-[(1-pyrenyl)-4-butyl]-*N*-(*n*-octadecyl)acrylamide (PNIPAM-PC-C_{18}Py) (Figure 1). A preliminary investigation based on room temperature ^{1}H NMR spectroscopy revealed that PC-polymers bearing hydrophobic substituents form micellar structures in water which, we assumed, consist of hydrophobic clusters insulated from the aqueous environment by the PC-units,[14] which, as they are zwitterions, give rise to permanent dipoles able to associate in water via ion pair formation.[15,16]

In this work, a combination of fluorescence spectroscopy, ^{1}H NMR spectroscopy, dynamic light scattering, and surface tension measurements was used to study the solution properties of PNIPAM-PC and its hydrophobically modified derivatives. Our objectives were to

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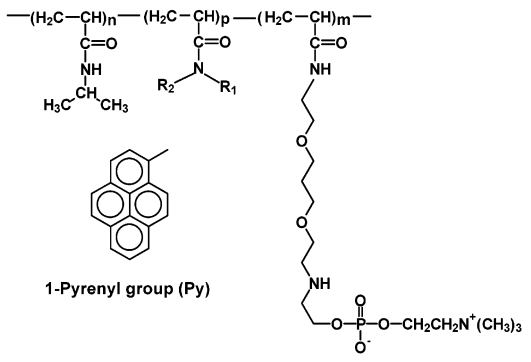

PNIPAM-PC:	n = 50, m = 50, p = 0	
PNIPAM-PC-C_{18}:	R_1 = H	$R_2 = C_{18}H_{37}$
PNIPAM-PC-C_{18}Py:	$R_1 = (CH_2)_4$Py	$R_2 = C_{18}H_{37}$
PNIPAM-PC-F:	R_1 = H	$R_2 = CH_2$-$(CF_2)_6$ CF_3

Figure 1. Chemical structure and composition of the polymers studied.

assess the role of extrinsic parameters, such as solution concentration and temperature, as well as intrinsic parameters, such as the chemical nature of the hydrophobic groups and their incorporation level, on the conformation of HM-PC polymers and on the relative extent of intra- and interchain interactions. As PNIPAM-PC derivatives are soluble in nonaqueous solvents, experiments were conducted also in methanol, chloroform, and mixed solvents to compare features of the nanoassemblies formed by the polymers in various environments.

Experimental Section

Materials. Water was deionized using a Milli-Q water purification system (Millipore). Dioxane was distilled from sodium under nitrogen. L-Glycerylphosphorylcholine, cadmium dichloride was obtained from Sigma Chemical Co. *N*-Isopropylacrylamide, obtained from Aldrich Chemical Co., was recrystallized from acetone/heptane 2/3 v/v. *N,N*-Azobis(isobutyronitrile), trifluoroacetic acid, diethylene glycol, sodium periodate, tetranitrobenzenesulfonic acid (TNBS), and sodium cyanoborohydride were purchased from Aldrich Chemical Co. The ion-exchange resins AG 501-X8 (D) and DOWEX 2X8-400 were obtained from BioRad Laboratories and Supelco, respectively. *N*-(*n*-Octadecyl)acrylamide,[17] *N*-[(1-pyrenyl)-4-butyl]-*N*-(*n*-octadecyl)acrylamide,[17] *N*-(1*H*,1*H*-perfluorooctyl)acrylamide,[18] *N*-BOC-*N*′-(ethylenedioxy)bis(ethylacrylamide),[19] and PNIPAM-NHBOC[19] were prepared as previously described. All solvents were reagent grade and used as received. Spectra/Pore membranes (Spectrum) were employed for dialysis.

Instrumentation. NMR spectra were recorded on a Bruker ARX-400 400 MHz spectrometer. Infrared spectra were recorded on a Bomem Hartmann & Braun IR spectrometer fitted with a Bomem Grams/32 data analysis software. UV/vis spectra were measured with a Hewlett-Packard 8452A photodiode array spectrometer. Gel permeation chromatography (GPC) measurements were performed with a Waters 590 programmable HPLC system equipped with a Waters 486 UV detector and a Waters 410 differential refractometer. Styrogel columns (Waters) eluted with THF (flow rate 0.7 mL min^{-1}) and calibrated with polystyrene standards were used to determine the molecular weights of NBOC protected polymers (see below). Ultrahydrogel columns (Waters) eluted with 0.1 M $NaNO_3$ (0.7 mL min^{-1}) and calibrated with pullulan standards were used to determine the molecular weight of PNIPAM-PC. Dynamic light scattering was performed on a Brookhaven BI9000 AT instrument equipped with an argon laser (λ = 514 nm, scattering angle 90°). The measurements were performed at 20 °C using polymer solutions (1.0 g L^{-1}) equilibrated at room temperature for 24 h and filtered through a 0.45 μm membrane prior to the measurements. Data were analyzed using the software provided by the manufacturer (CONTIN calculations).

Fluorescence Measurements. Steady-state 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. All measurements were carried out at 25 °C. The slits setting were 0.5 mm (excitation) and 2.0 mm (emission) for experiments using PNIPAM-PC-C_{18}Py or 1.0 mm (excitation) and 0.25 mm (emission) for measurements with pyrene (probe). Emission spectra were recorded with an excitation wavelength of 346 nm (PNIPAM-PC-C_{18}Py) or 333 nm (Pyrene probe). Excitation spectra of aqueous PNIPAM-PC-C_{18}Py were measured in the ratio mode. They were monitored at 378 nm (monomer emission) and 476 nm (excimer emission). Samples for analysis, prepared from stock solutions of PNIPAM-PC-C_{18}Py (5.0 g L^{-1}), had a polymer concentration of 0.01 g L^{-1}. Solutions were kept in the dark at 5 °C for 12 h prior to measurements. 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 half sum of the intensities at 380 and 400 nm.

Fluorescence lifetimes were measured on a Fluorolog-Tau-3 multifrequency phase modulation fluorimeter (Jobin-Yvon Horiba Inc.). The excitation light from a 450 W xenon lamp was modulated with a Pockels cell. Phase and modulation values were determined relative to a glycogen aqueous solution. The excitation wavelength was set at 346 nm. Pyrene monomer and excimer emissions were monitored at 376 and 476 nm, respectively. The frequency of the analyzing light was chosen in the range of 0.1−100 MHz. All measurements were carried out at 25 °C. Data were analyzed with the Datamax Spectroscopy software based on GRAMS/32 from Galactic Ind. Data were fit to a multiexponential decay law, $F(t) = \sum a_i e^{-t/\tau_i}$, where a_i and τ_i are the preexponential factors and the lifetime of the ith component, respectively. The goodness of the fit was determined by the χ^2 value and examination of the residuals. The preexponential factors a_i are related to the observed fractional intensity contribution f_i by the relation $f_i = a_i\tau_i/\sum_j a_j\tau_j$. The average lifetime $\langle\tau\rangle$ was calculated from $\langle\tau\rangle = f_1\tau_1 + f_2\tau_2$.

Surface Tension Determinations. The surface tension of aqueous polymer solutions was measured with a DCAT11 tensiometer (Future Digital Scientific) equipped with a Wilhelmy plate. Polymer solutions of various concentrations were prepared by dilution of a stock solution (5.0 g L^{-1}). They were kept at room temperature for 24 h prior to analysis. Surface tension values were recorded until the standard deviation between 50 consecutive values was less than 0.02 mN m^{-1}.

Synthesis. Synthesis of Phosphorylcholine Glyceraldehyde. A solution of l-α-glycerophosphorylcholine, $CdCl_2$ (10.53 g 23.9 mmol), in water (200 mL) was treated at room temperature for 30 min with a sufficient amount of resin AG 501-X8(D) (40.0 g) to remove all chloride ions, as detected by a silver nitrate solution (0.1 M in 1 N HNO_3). The resin was separated by filtration. The filtrate was cooled in an ice/water bath. A solution of sodium periodate (10.23 g, 47.8 mmol) in water (10 mL) was added dropwise to the cold solution. The reaction mixture was stirred at 0 °C for 5 h. The reaction mixture was concentrated in vacuo to yield a white solid, which was then triturated in methanol. The insoluble material was removed by filtration. Concentration of the filtrate in vacuo yielded phosphorylcholine glyceraldehyde, as a colorless liquid (5.76 g, 99%). Surprisingly, the ^{1}H NMR spectrum of the product in D_2O did not present the expected low field signal characteristic of an aldehydic proton (Figure 2). The aldehyde exists only in the hydrated form, as evidenced by the occurrence in the ^{1}H NMR spectrum of a triplet at δ 5.19 ppm (J = 4.8 Hz) attributed to the resonance of the acetal proton. ^{13}C NMR of (CD_3OD, δ): 53.68 [$(CH_3)_3N^+-$), 59.49 ($-CH_2N^+$), 66.40 ($N^+(CH_3)_3-CH_2-\mathbf{C}H_2-OP$), 67.73 [$(OMe)_2CH-\mathbf{C}H_2-OP$], 96.65 ppm [$(OMe)_2\mathbf{C}H-CH_2-OP$). ^{31}P NMR ($CD_3OD$, δ):

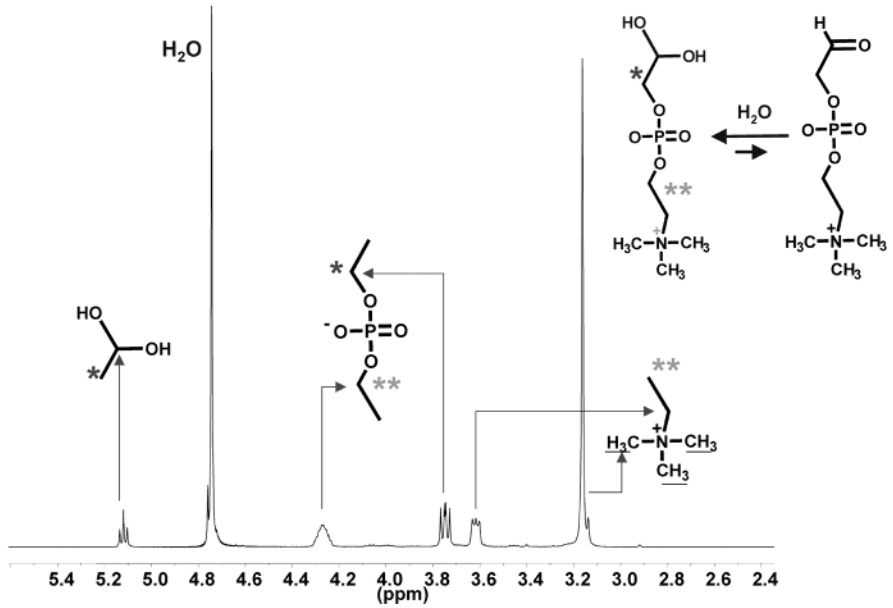

Figure 2. ^{1}H NMR spectrum of phosphorylcholine glyceraldehyde in D_2O indicating the structural assignments.

Table 1. Composition and Characteristics of the Polymers Prepared

polymer	NIPAM[a] (mol %)	PC[b] (residual NH_2)[c] (mol %)	$C_{18}H_{37}$[a] (mol %)	Py[d] (mol %)	$C_8F_{15}H_2$[e] (mol %)	diameter[f] (nm)
PNIPAM-PC	49.6	50.4 (0)				20, 172
PNIPAM-PC-C_{18}(5)	52.6	42.6 (1)	4.8			18, 178
PNIPAM-PC-C_{18}(10)	42.5	48.8 (2)	8.7			15, 73
PNIPAM-PC-C_{18}(15)	44.8	41.4 (0)	13.8			*g*
PNIPAM-PC-C_{18}Py	45.1	52.0 (6)	2.9	2.9		192
PNIPAM-PC-F	47.6	47.6 (3)			4.8	25, 212

[a] From ^{1}H NMR measurements. [b] From phosphate colorimetric test. [c] From $-NH_2$ colorimetric test. [d] From UV absorbance measurement. [e] From quantitative ^{19}F NMR measurement. [f] From DLS measurements of polymer solutions in water, polymer concentration 1.0 g L^{-1}. [g] This polymer is not soluble in water.

0.85 ppm. FTIR (NaCl pellet, v): 3391.4 (br, s, OH), 1650 (s) 1228 (s, P=O), 1086 (s, P−O−), 1086 (s, −P−O−CH) 972.3 cm^{-1} (s, $(CH_3)_3N^+$).

Preparation of the Polymers. All copolymers were prepared by the same three-step procedure, exemplified below for the synthesis of PNIPAM-PC-C_{18}Py.

Copolymerization of *N*-Isopropylacrylamide (NIPAM), *N*-[(1-Pyrenyl)-4-butyl]-*N*-*n*-octadecylacrylamide, and *N*-BOC-*N*′-(ethylenedioxy)bis(ethylacrylamide). A solution of NIPAM (0.26 g, 2.32 mmol), *N*-[(1-pyrenyl)-4-butyl]-*N*-*n*-octadecylacrylamide (0.13 g, 0.232 mmol), and *N*-BOC-*N*′-(ethylenedioxy)bis(ethylacrylamide) (0.7 g, 2.32 mmol) in dioxane (15 mL) was degassed for 30 min by vigorous bubbling of nitrogen. The mixture was heated to 60 °C. AIBN (1.9 mg, 1.12×10^{-2} mmol) was added at once. The polymerization mixture was stirred at 60 °C for 16 h. It was cooled to room temperature. The solvent was removed by evaporation. The polymer, PNIPAM-NHBOC-C_{18}Py, was purified by two precipitations from THF into hexane and dried in vacuo (yield: 80%). M_w 35 500; M_w/M_n 1.7.

Deprotection of PNIPAM-NHBOC-C_{18}Py. Trifluoroacetic acid (4.07 g, 35.7 mmol, 20 equiv per BOC equiv) was added to a solution of the copolymer (0.8 g) in dichloromethane cooled in an ice/water bath. The reaction mixture was stirred for 24 h. As the reaction proceeded, several aliquots of methanol were added to keep the reaction mixture homogeneous. The solvent was evaporared. The polymer, PNIPAM-NH_3^+-C_{18},CF_3COO^-, was purified by two precipitations from THF into diethyl ether/hexane 1/1 v/v and dried in vacuo (yield: ∼100%).

Preparation of PNIPAM-PC-C_{18}Py. Dowex 2X8-400 (4.0 g) was added to a solution of PNIPAM-NH_3^+-C_{18}Py (0.7 g) in water/ethanol (75 mL, 2/1 v/v). The mixture was stirred at room temperature for 1 h. The resin was separated by filtration, and the filtrate was concentrated in vacuo to yield PNIPAM-NH_2-C_{18}Py. A solution of phosphorylcholine glyceraldehyde (1.02 g, 3.90 mmol) in methanol (25 mL) was added to a solution of PNIPAM-NH_2-C_{18}Py in methanol (35 mL). The solution was stirred at room temperature for 3.5 h. A solution of sodium cyanoborohydride (0.25 g, 3.9 mmol) in methanol (15 mL) was added dropwise to the reaction mixture cooled to 0 °C. At the end of the addition the reaction mixture was warmed to room temperature and stirred for 19 h. The polymer was purified by dialysis against distilled water for 3 days. It was isolated by lyophilization. ^{1}H NMR (D_2O, δ): 0.91 (br, $\mathbf{H_3}C-C_{17}H_{34}$), 1.15 (br, $(\mathbf{H_3}C)_2-CH-NH-$), 1.30 (br, $C\mathbf{H_2}$, octadecyl group), 1.5−2.2 (br, $C\mathbf{H_2}-C\mathbf{H}$, main chain), 2.36 (br, $C\mathbf{H_2}$, PC group), 2.75−2.85 (br, $C\mathbf{H_2}-NH$, PC group), 3.22 (s, $(C\mathbf{H_3})_3N^+-$), 3.37 (br, $-C\mathbf{H_2}-N^+$), 3.55−3.80 (br m, $-C\mathbf{H_2}-O$, ethylenedioxy group), 3.89 (br, $-NH-C\mathbf{H}(CH_3)_2$), 4.01 (br, $-C\mathbf{H_2}-O-P$), 4.31 ppm (br, $C\mathbf{H_2}-O-P$). ^{31}P NMR (CD_3OD, δ): 1.75 ppm. ^{13}C NMR (CD_3OD, δ): 21.86 ($-NH-CH(\mathbf{C}H_3)_2$), 29.84 ($-\mathbf{C}H_2-$, octadecyl group), 41.51 [$-NH-\mathbf{C}H(CH_3)_2$], 52.69 [$(\mathbf{C}H_3)_3N^+-$], 70.43 ppm ($-\mathbf{C}H_2-O-$, ethylenedioxy group). UV (methanol): 342 nm. The level of pyrene incorporation was calculated from the absorbance of a polymer solution in methanol using *N*-[(1-pyrenyl)-4-butyl]-*N*-(*n*-octadecyl)-amine (ϵ = 34 900 mol^{-1} dm^3 cm^{-1} at 342 nm)[17] as a model compound.

The physical properties and compositions of the polymers are compiled in Table 1.

Colorimetric Determination of the Free Amine Groups.[20] A known amount of polymer (2−4 mg) was dissolved in a solution of excess TNBS (0.02 M) in aqueous sodium hydrogen carbonate (2.0 mL, 20 g L^{-1}, pH 8.2). The solution was kept for 2 h at 40 °C. Hydrochloric acid (3.0 mL, 6 N) was added to the solution, and the resulting mixture was heated at 60 °C for 1.5 h. It was cooled to room temperature and diluted with water (5.0 mL). The amount of TNBS-derivatized

amines in a sample was determined from the absorbance at $\lambda = 345$ nm, using TNBS-derivatized (hydroxy)lysine ($\epsilon_{345\ nm} = 14\ 600$ L mol $^{-1}$cm $^{-1}$)[20] as a reference compound. A solution of TNBS without added polymer was treated under the same conditions. Its absorbance at 345 nm was subtracted from that the TNBS-derivatized polymers to correct for its residual absorbance at this wavelength.

Colorimetric Determination of the Phosphate Content of the Polymers.[21] A magnesium nitrate solution in ethanol (10%, 0.030 mL) was added to an aqueous polymer solution (0.02 mL, 1.0 g L^{-1}). Test tubes containing the samples were heated over a flame to remove the solvent and ash the polymer. They were allowed to cool before the addition of aqueous HCl (0.5 N, 0.30 mL). The capped tubes were heated to 100 °C for 15 min. They were cooled to room temperature. The mixture was then treated with an aqueous ascorbic acid solution (10%, 0.7 mL) and an aqueous solution of ammonium molybdate (0.42% in 1 N H_2SO_4). The resulting mixture was heated at 45 °C for 15 min. The amount of phosphate in a sample was determined from the absorbance of the mixture at $\lambda = 820$ nm using a calibration curve established with KH_2PO_4.

Determination of the Fluorine Content of PNIPAM-PC-F by ^{19}F NMR Spectroscopy. PNIPAM-PC-F (10.0 mg) was dissolved in a solution (1.0 mL) of trifluoroacetic acid (7.5 mg) in a mixture of $CDCl_3$ (14.98 g) and CD_3OD (5.03 g). Quantitative determination of the fluorine content of the polymer was achieved by comparing the area of the signal at δ −78.67 ppm attributed to the resonance of the CF_3 fluorines of trifluoroacetic acid and the area of the signal at δ −84.2 ppm attributed to the resonance of the CF_3 fluorines of the perfluoroalkyl group linked to the polymer. A calibration curve was established under the same conditions using 1*H*,1*H*-perfluorooctylamine (CF_3 signal at δ −84.2 ppm) as standard.

Results and Discussion

Synthesis and Characterization of the Polymers. The synthetic sequence leading to PC-polymers consists of three steps:[14] (1) random free-radical copolymerization in dioxane of NIPAM, *N*-BOC-*N*′-(ethylenedioxy)bis(ethylacrylamide) and, when required, a third acrylamide bearing a hydrophobic substituent,[22] (2) quantitative removal of the BOC protecting group to generate polymer-linked primary amine groups, and (3) reductive amination of phosphorylcholine glyceraldehyde (Figure 2) by the primary amine groups. This sequence was designed after several unsuccessful attempts to link directly L-α-glycerophosphorylcholine to a polymer carrying functional groups which are reactive toward hydroxyl groups, such as isocyanates. We were unable to find reaction conditions under which the *primary* hydroxyl group of L-α-glycerophosphorylcholine reacted to the exclusion of the *secondary* hydroxyl group. Under all circumstances, undesired reactions of the secondary hydroxyl group resulted in some level of cross-linking, yielding gels or insoluble polymers.

Conditions for the preparation of PNIPAM-PC-C_{18}-Py are given in the Experimental Section (see above) as an illustration of the synthetic sequence. All other polymers were obtained by the same approach, starting from various monomer ratios. The composition of the polymers (Table 1) was determined by ^{1}H, ^{19}F, and ^{13}C NMR spectroscopy, UV absorbance, and colorimetric assays. The molecular weights of the protected polymers were determined by GPC calibrated with polystyrene standards. Chromatograms of PC-polymers in aqueous $NaNO_3$ were obtained as well; however, they do not give an accurate estimate of polymer molecular weights, since in aqueous media PC-polymers tend to form aggregates, as described later.

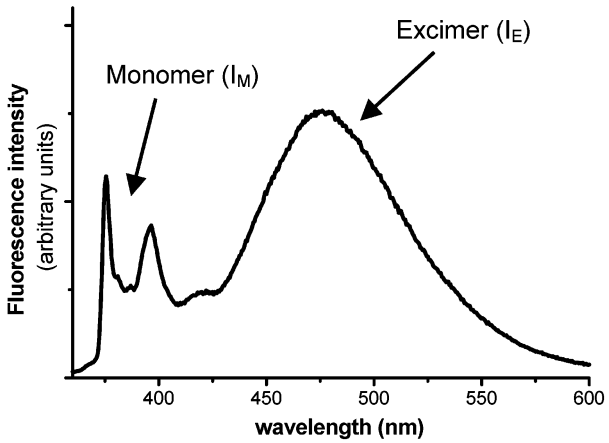

Figure 3. Fluorescence spectrum of a solution in water of PNIPAM-PC-C_{18}Py (polymer concentration 0.01 g L^{-1}, temperature 25 °C, $\lambda_{exc} = 346$ nm).

Polymer Solutions in Water at 25 °C. Fluorescence Studies. The steady-state fluorescence spectrum of a dilute solution of PNIPAM-PC-C_{18}Py in water at 25 °C consists of two contributions (Figure 3): a well-resolved emission, with the (0,0) band located at 378 nm, due to locally isolated excited pyrenes (pyrene monomer emission, intensity I_M) and a strong and featureless emission centered at 480 nm attributed to the emission of pyrene excimers (intensity I_E). Measurements carried out as a function of polymer concentration revealed that the strong excimer emission persisted even in solutions of polymer concentration as low as 2.5×10^{-3} g L^{-1}. The fluorescence lifetime of the pyrene excimer consisted of a growing-in component ($\tau = 5.3$ ns) and a decaying component that could be fit to a single-exponential law ($\tau_E = 60.8$ ns). From these data we conclude that pyrene excimer formation involves dynamic encounter of an excited pyrene group and a ground-state pyrene, in accordance to the mechanism proposed by Birks.[23] The fact that the excimer emission remains strong even from solutions of extremely low pyrene concentration ([Py] $< 3 \times 10^{-7}$ mol L^{-1} for PNIPAM-PC-C_{18}Py $< 2.5 \times 10^{-3}$ g L^{-1}) implies that the chromophores are sequestered within hydrophobic domains where they are kept in close proximity. The temperature dependence of fluorescence spectra of PNIPAM-PC-C_{18}Py aqueous solutions was examined, increasing the temperature from 25 to 70 °C. A modest increase in the intensity of the excimer emission relative to the monomer emission intensity was observed, but the overall features of the emission were not altered upon heating.

The pyrene monomer decay was fitted to a double-exponential law ($\langle\tau_M\rangle = 33.4$ ns, $\tau_1 = 10.8$ ns, $a_1 = 0.44$ and $\tau_2 = 38$ ns, $a_2 = 0.56$). The average lifetime is rather short, compared to values usually recorded for aqueous solutions of pyrene-labeled polymers, such as PNIPAM-C_{18}Py, a copolymer of NIPAM and *N*-[(1-pyrenyl)-4-butyl]-*N*-*n*-octadecylacrylamide which does not carry phosphatidylcholine groups ($\langle\tau_M\rangle = 59$ ns).[24] The shortening of the excited pyrene lifetime was attributed at first to the inadvertent presence of an impurity acting as a fluorescence quencher. Exhaustive further purification of the sample by precipitation and dialysis was carried out, but it did not alter the photophysical properties of the aqueous solutions of PNIPAM-PC-C_{18}Py. Control experiments carried out with aqueous solutions of pyrene indicated that the phosphorylcholine

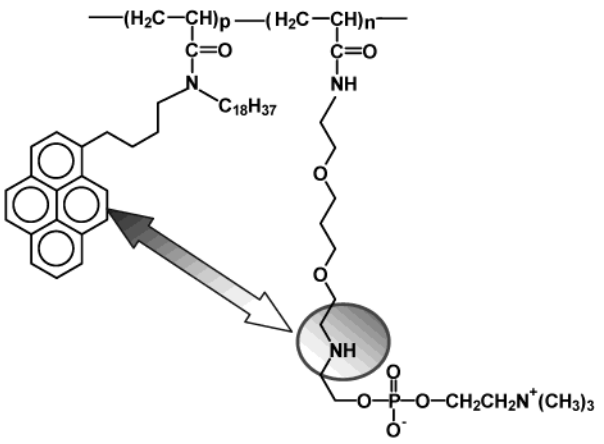

Figure 4. Polymer structural unit that may act as quencher of pyrene emission.

moiety does not quench pyrene fluorescence. We came to suspect that the pyrene emission experiences some level of quenching by polymer-bound secondary amines groups which serve as linkers of the phosphorylcholine group (Figure 4) to the polymer backbone. They are present in high local concentration near the pyrene groups (∼50% of the monomer units of a polymer chain carry a secondary amine). It is known that secondary and tertiary amines can act as quenchers of fluorescence.[25,26] The fact that pyrene emission may be quenched, to a certain extent, by polymer-bound quenchers implies that the polymer chains preserve their flexibility, even when they associate to create hydrophobic domains.

The existence of hydrophobic microdomains in aqueous solutions of the copolymers PNIPAM-PC-C_{18}(5) and PNIPAM-PC-C_{18}(10) was also tested by fluorescence spectroscopy, but since the copolymers do not exhibit inherent fluorescence, pyrene ($\sim 7 \times 10^{-7}$ mol L^{-1}) was added to their solutions as an extrinsic probe. Two spectral features of the probe were monitored as a function of polymer concentration (Figure 5): (a) the ratio I_1/I_3 of the intensity of the (0,0) band (378 nm) to that of the band at 386 nm in the emission spectrum of pyrene;[27] (b) the ratio I_{336}/I_{333} of the intensities at 336 and 333 nm of the pyrene excitation spectrum.[28] Both ratios are indicators of the polarity of the microenvironment sensed by the probe. For example, the ratio I_1/I_3 takes a high value for pyrene in polar media (1.81 in water)[27] and decreases with decreasing polarity of the probe microenvironment (1.12 for pyrene dissolved in sodium dodecyl micelles).[27] The ratio I_{336}/I_{333} displays the opposite trend: it increases with decreasing polarity. In Figure 5, the values of the ratios I_1/I_3 and I_{336}/I_{333} are plotted against polymer concentration (logarithmic scale) for solutions of PNIPAM-PC-C_{18}(5) and PNIPAM-PC-C_{18}(10). The ratios recorded for solutions of concentration lower than 0.02 ± 0.01 g L^{-1} [PNIPAM-C_{18}(10)] or 0.05 ± 0.01 g L^{-1} [PNIPAM-PC−C_{18}(5)] remain constant and indicate a highly polar probe microenvironment. In solutions of higher concentration, the ratio I_1/I_3 decreases while the ratio I_{336}/I_{333} increases, signaling changes in the polarity sensed by the probe. The ratio I_{336}/I_{333} reaches a plateau for solutions of polymer concentration higher than ∼1 g L^{-1}. The variations of I_1/I_3 occur over a broader polymer concentration, the ratio attaining its lowest value (∼1.0) for solutions of polymer concentrations of 5 g L^{-1}, or higher. In summary, the fluorescence probe experiments give unambiguous evidence for the formation, in aqueous solutions of PNIPAM-PC-C_{18}, of hydrophobic microdomains able to solubilize preferentially the hydrophobic pyrene probes. They also provide evidence that, unlike surfactant micellization, the polymer association process does not occur via a cooperative mechanism, as reported previously for a large number of hydrophobically modified polymers.

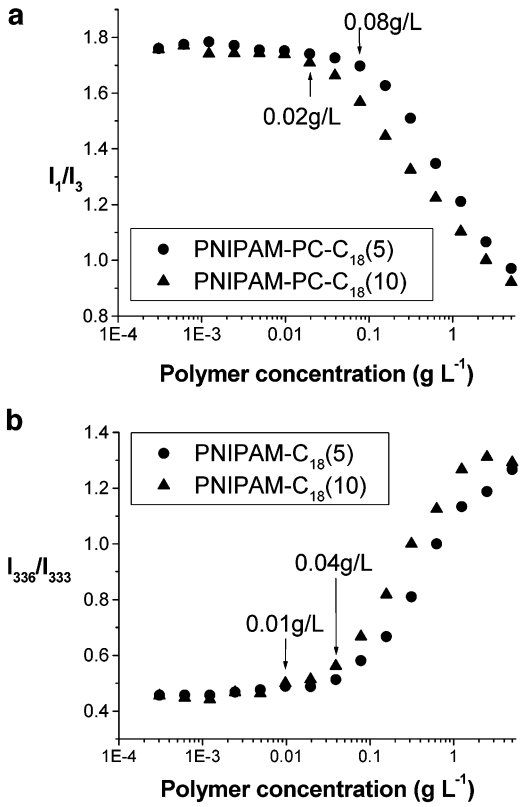

Figure 5. Changes of the intensity ratios I_1/I_3 and I_{336}/I_{333} of pyrene emission in aqueous solutions of PNIPAM-PC-C_{18}(5) (full circle) and of PNIPAM-PC-C_{18}(10) (full triangle) as a function of polymer concentration ([Py] $\sim 7 \times 10^{-7}$ mol L^{-1}, temperature 25 °C, $\lambda_{exc} = 337$ nm).

Dynamic Light Scattering. A dynamic light scattering analysis was carried out with solutions in deionized water (20 °C, 1.0 g L^{-1}) of the PC-polymers in order to confirm, by a different experimental technique, that the polymers form micellar assemblies in water. Indeed, all solutions of sufficiently high concentration (>1.0 g L^{-1}) gave signals that varied in intensity as a function of the structure of the polymer. The distributions of hydrodynamic diameters recorded with all polymers were bimodal, except for solutions of PNIPAM-PC-C_{18}Py which featured a unimodal size distribution, centered at 192 nm (Table 1). In all other cases, the size distribution had two contributions: one peak in the range 15−25 nm, corresponding to individual chains, and a second broader peak which, depending on polymer structure, centered around 170−210 nm and was attributed to larger interchain aggregates. Thus, all polymers, even PNIPAM-PC, assemble in water via, it is believed, ionic interactions between the zwitterions attached to the polymer chains, as reported by Niu[15] and Liaw[16] in their studies of poly(carboxybetaine)s. The absence of isolated PNIPAM-PC-C_{18}Py chains in aqueous solutions of this polymer is noteworthy. It is an indication that even at a low level of incorporation (2.9 mol %) the *N*-[(1-pyrenyl)-4-butyl]-*N*-(octadecyl) moiety

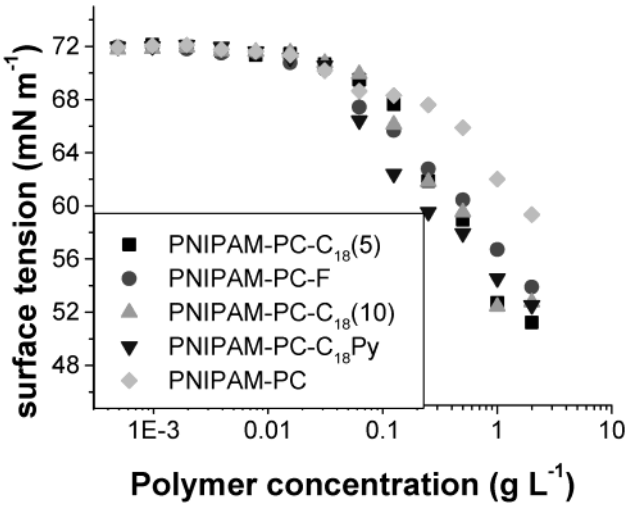

Figure 6. Changes in the surface tension of polymer solutions as a function of polymer concentration (temperature 25 °C).

triggers the formation of stable polymeric micelles, at least when the polymer concentration is sufficiently high. The coexistence of single polymer chains and polymeric assemblies in solutions of PNIPAM-PC-C_{18} may account for the fact that changes in the photophysical parameters I_1/I_3 and I_{336}/I_{333} occur over a broad polymer concentration range, as described in the previous section.

Surface Tension Measurements. All polymers were surface-active above a threshold concentration ($\geq\sim$0.01 g L^{-1}, Figure 6). Differences among the surface activity of the various polymers were minimal. The unmodified polymer, PNIPAM-PC itself, exhibited a mild surface activity, in contrast to poly(methacryloyloxyethyl phosphorylcholine) which does not affect the surface tension of water, even for solution concentrations up to 1 g L^{-1}.[29] We attribute this effect to a contribution of the NIPAM units of the polymer chains, since PNIPAM is known to be surface active.[30] Among the hydrophobically modified PC-polymers, PNIPAM-PC-C_{18}Py was the most effective in reducing the surface tension of water. The surface tension tends to decrease with increasing polymer concentration, without reaching a plateau value, which may be taken as an indication that the PC-polymer aggregates formed in water are polydisperse and that aggregation and adsorption at the air−water interface take place simultaneous.[31] For solutions of the amphiphilic polymers PNIPAM-PC-C_{18}(5) and PNIPAM-PC-C_{18}(10), the concentration corresponding to the onset of decrease in surface tension corresponds closely to the onset of association concentration determined by fluorescence probe measurements (Figure 5).

^{1}H NMR Spectroscopy Studies. In the spectrum of PNIPAM-PC-C_{18}Py in D_2O (Figure 7, top), the signal due to the resonance of the PC trimethylammonium protons (δ 3.22 ppm) is sharp, while the signals due to the main chain protons are broad and lack definition. No signals were detected either at low field, in the 7.0−6.5 ppm range (inset), where the pyrenyl protons are known to resonate, or in the high field spectral domain, where resonances due to the octadecyl protons are expected to occur. Line broadening of ^{1}H NMR signals indicates that the motion of the corresponding protons is restricted. The extensive line broadening of the signals associated with the resonances of the main chain

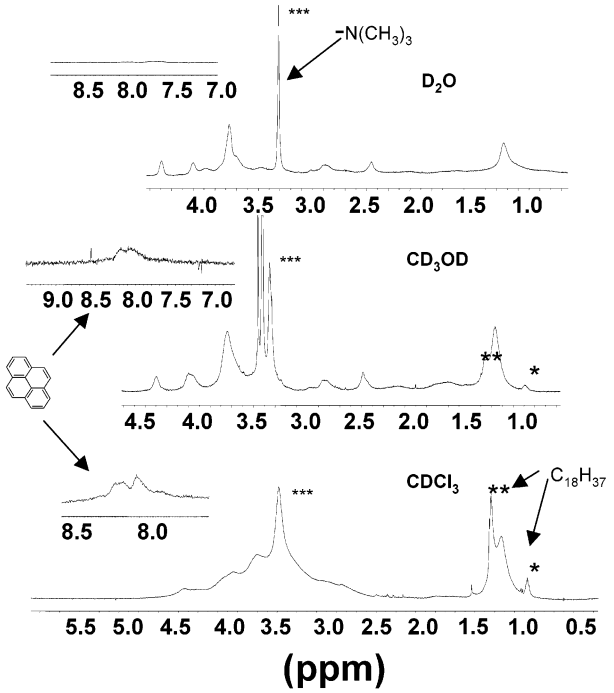

Figure 7. ^{1}H NMR spectra of PNIPAM-PC-C_{18}Py in D_2O, CD_3OD, and $CDCl_3$ (polymer concentration 5.0 g L^{-1}).

protons, of the octadecyl, and of the pyrenyl protons confirms the formation of hydrophobic microdomains where octadecyl and pyrenyl groups associated in close proximity.

We recorded ^{1}H NMR spectra of aqueous amphiphilic PNIPAM-PC samples at several temperatures ranging from 5 to 75 °C. Representative spectra of PNIPAM-PC-C_{18}(5) (polymer concentration: 5 wt %) are shown in Figure 8. As the temperature of the solution was increased, all signals shifted to lower magnetic field, due to the temperature-induced decrease in solution viscosity and corresponding increase in polymer mobility. In Figure 8, the spectra were incremented by 0.5 ppm at each temperature for the sake of clarity. Changes in peak shape took place with increasing solution temperature. Of particular interest are the signals at δ 1.8, 3.4, and 3.8 ppm, attributed to the resonances of the octadecyl methylene protons, the PC trimethylammonium protons, and the methylene groups of the diethylene glycol chain, respectively. We note that the signal due to the diethylene glycol protons of the linker was not altered by changes in solution temperature and can serve as internal standard. The signal due to the octadecyl chain becomes progressively sharper and more intense with increasing temperature, indicating either an increase in chain mobility within the hydrophobic microdomains or heat-induced disintegration of the hydrophobic microdomains. We favor the first explanation, since it is in good agreement with our studies by fluorescence of the temperature dependence of aqueous PNIPAM-PC-C_{18}Py solutions which pointed to the fact that the hydrophobic microdomains are not disrupted upon heating of the solution (see above). The signal due to the PC trimethylammonium protons is unchanged between 25 and 75 °C, but its intensity decreases below 25 °C.

It is interesting to note that the intensity of the signal at 1.0 ppm, attributed to the resonance of the methyl protons of the NIPAM units also increases with increasing temperature, a trend opposite to the spectral

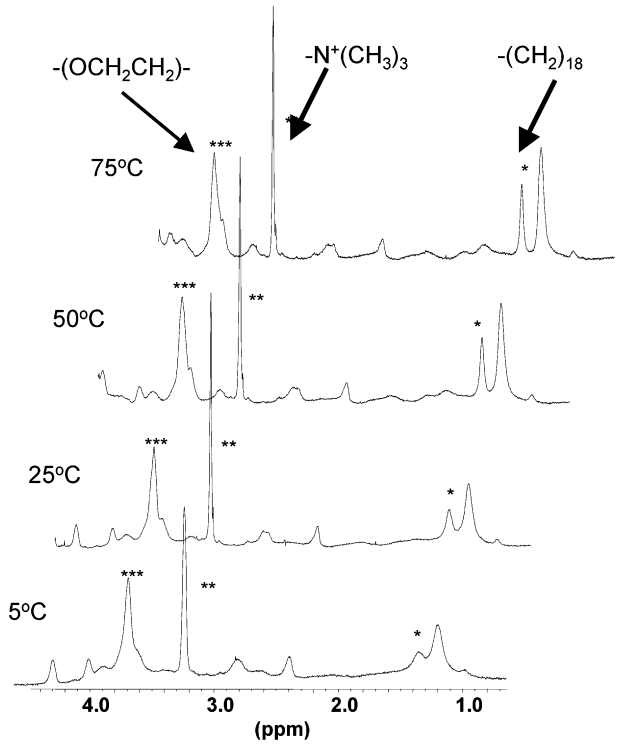

Figure 8. ^{1}H NMR spectra of PNIPAM-PC-C_{18}(5) in D_2O at various temperatures. Arrows indicate signals assigned to the octadecyl methylene protons (*), trimethylammonium methyl protons (**), and ethylene glycol methylene protons (***); polymer concentration 5.0 g L^{-1}. Spectra were shifted upfield by 0.5 ppm with increasing temperature.

changes observed with aqueous solutions of PNIPAM[32] and copolymers of NIPAM and acrylic acid.[33] Aqueous solutions of PNIPAM are thermosensitive, and when the temperature increases above a critical value, the polymer chains collapse and aggregate.[34] This phenomenon is reflected in the ^{1}H NMR spectrum of PNIPAM by a broadening and loss in intensity of the signal at 1.0 ppm. The trend revealed by our temperature studies of PNIPAM-PC-C_{18}(5) would imply that aggregation of NIPAM units in this polymer is prevented at all temperatures, presumably due to the proximity of the highly hydrated PC units.[35]

Polymer Solutions in Nonaqueous Solvents. ^{1}H NMR Spectroscopy Studies. The ^{1}H NMR spectra of solutions of PNIPAM-PC-C_{18}Py in $CDCl_3$, CD_3OD (Figure 7, middle and bottom), contrary to the ^{1}H NMR spectrum of the polymer in water, display signals attributed to resonances of protons of the *N*-[(1-pyrenyl)-4-butyl]-*N*-*n*-octadecyl group, with a triplet at 0. 81 ppm (*), a broad singlet at 1.30 ppm (**) (terminal CH_3 and octadecyl methylene protons), and a broad multiplet between 7.7 and 8.2 ppm (pyrenyl aromatic protons). In the ^{1}H NMR spectrum of the $CDCl_3$ polymer solution, considerable broadening of the signal due to the phosphorylcholine $(CH_3)_3N^+$ protons takes place. This signal also undergoes a downfield shift, compared to the signal observed in the spectrum of the aqueous polymer solution. The signals attributed to the main chain protons, which appear as a broad multiplet between δ 1.4 and 2.2 ppm in the spectrum of a methanolic solution of PNIPAM-PC-C_{18}Py (Figure 3, middle spectrum), are hardly detectable in the spectra of the polymer dissolved in either D_2O or $CDCl_3$, suggesting that the polymer forms aggregates not only in water but also in chloroform.

A study of the ^{1}H NMR spectra of PNIPAM-PC-C_{18}-(5) in $CDCl_3/CD_3OD$ mixed solvents was performed to observe the influence of solvent composition on the conformation and assembly of this polymer in solution (Figure 9). The trimethylammonium methyl protons signal undergoes a slight upfield shift upon slight increase in solvent polarity ($CDCl_3$: δ = 3.30 ppm; $CDCl_3/CD_3OD$ (9/1 v/v): δ = 3.17 ppm). In spectra of polymer solutions in solvents of higher methanol content, the peak retains its chemical shift and becomes sharper. These peak changes, upfield shift and sharpening, with increasing solvent polarity, are indicative of a conformational rearrangement of the polymer in solution. In methanol and in solvents of high polarity, the motions of the polar groups are not restricted, but in solvent of low polarity the mobility of the polar groups is greatly reduced. In pure $CDCl_3$, the polar groups interact with each other, presumably via ion-pair formation among polymer-bound zwitterions. An increase in the intensity of the water signal was observed as methanol-d_4 was added to a $CDCl_3$ solution of PNIPAM-PC-C_{18}(5) (Figure 9). We ascertained that the water content of pure and mixed solvents was low by recording ^{1}H NMR spectra of the solvents in the absence of dissolved polymer. Therefore, the observed rise in water peak intensity in spectra of the polymer in mixed $CD_3OD/CDCl_3$ solutions, compared to a $CDCl_3$ solution, is not due to the inadvertent presence of water in methanol-d_4. It must therefore arise from polymer-bound water molecules that are released into the solvent upon increase of its polarity, an effect which brings further support to the occurrence of a change in polymer conformation with solvent polarity.

Similar observations were reported by Ruiz et al.[36] in a study by ^{1}H NMR spectroscopy of solutions of a copolymer of MPC, methyl acrylate, and methyl methacrylate copolymer in solvents of varying polarity. The decrease in the mobility of PC groups with decreasing solvent polarity was attributed to their aggregation into micellar domains consisting of phosphorylcholine clusters stabilized in chloroform by water structuration around the phosphate groups.[37] These clusters do not exist in methanolic PNIPAM-PC-C_{18} solutions, as implied by the sharpness of the ^{1}H NMR signals corresponding to the PC group.

Fluorescence Spectroscopy Studies. Fluorescence spectra of PNIPAM-PC-C_{18}Py solutions in methanol and chloroform present the same overall features as the emission of this polymer in water, but the pyrene monomer emission is enhanced, compared to the excimer emission, particularly for solutions in chloroform. The ratio I_E/I_M of the excimer emission intensity to the monomer emission intensity decreases from 1.10 in water to 0.55 and 0.12 in methanol and chloroform, respectively. This trend confirms that the hydrophobic microdomains detected in aqueous PNIPAM-PC-C_{18}Py solutions do not form to the same extent in methanol and do not form at all in chloroform. Fluorescence spectra of PNIPAM-PC-C_{18}Py dissolved in mixed $CDCl_3/CD_3OD$ of various composition were measured, and the intensity of pyrene monomer and excimer emission were recorded. Both emission intensities reach a maximum in a mixed solvent containing ~70 vol % $CDCl_3$. Fluorescence spectroscopy and ^{1}H NMR spectroscopy probe events occurring on different time scales; nonetheless,

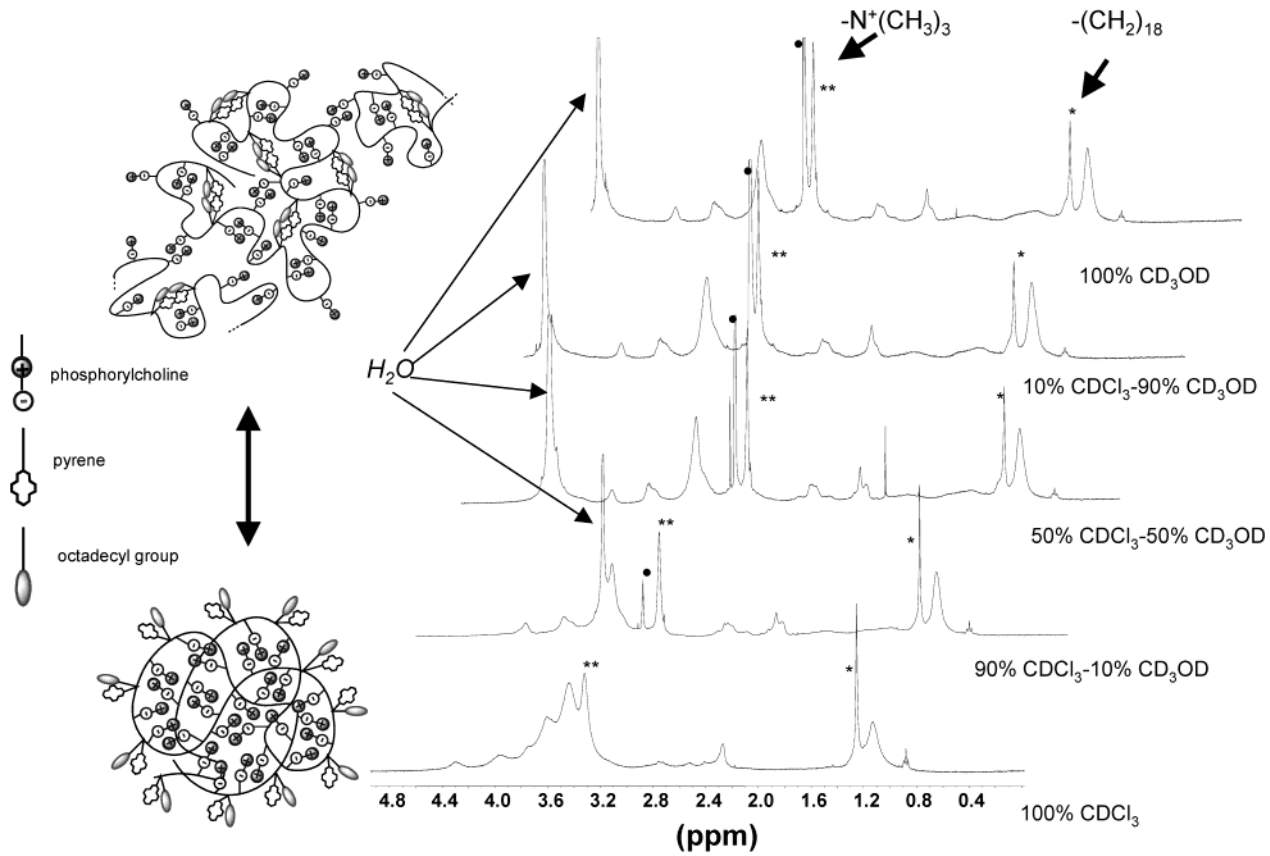

Figure 9. ^{1}H NMR spectra of PNIPAM-C_{18}(5) in $CDCl_3$, CD_3OD, and several mixtures of the two solvents and idealized conformations of the polymer in CD_3OD and $CDCl_3$. The black dot indicates signals attributed to CH_3OH; polymer concentration 5.0 g L^{-1}. Spectra were shifted upfield by 0.5 ppm with increasing CD_3OD content.

they concur in pointing to the fact that the solvent-induced conformational transition occurs in a solvent mixture possessing the optimal balance of polarity and solvating power for the polymer components.[36,38]

Conclusions

Copolymers bearing pendant phosphorylcholine groups and hydrophobic moieties were prepared, and their dilute solutions in water and organic solvents were characterized. The zwitterionic properties of the phosphorylcholine residues control to a a large extent the conformation and association behavior of the copolymers. In the absence of salts, strong ionic interactions among polymeric-linked ion pairs lead to cluster formation not only in water but also in chloroform. Evidence from dynamic light scattering and fluorescence spectroscopy indicates that the ionic interactions cause extensive association of the chains, and that they persist well below the critical overlap concentration. This tendency toward association is enhanced by the presence of the hydrophobic groups, which in water tend to assemble within hydrophobic microdomains. The assembly of the phosphorylcholine polymers in water persists upon increase of the solution temperature, but current work in this laboratory indicates that it is affected drastically by changes in ionic strength or pH as well as by the charge density of the copolymers.

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