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Thermo- and pH-sensitive polymers containing cholic acid derivatives

A. Benrebouh, D. Avoce, X.X. Zhu*

Département de chimie, Université de Montréal, C.P. 6128, Succursale Centre-ville, Montreal, Québec, Canada H3C 3J7

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Abstract

New copolymers were prepared by free radical polymerization in solution from *N*-isopropylacrylamide and methacrylate monomers derived from cholic acid with ethylene glycol and oligo(ethylene glycol) spacers. These copolymers contained 1–5 mol% of the methacrylate derivatives of cholic acid. The lower critical solution temperature (LCST) of poly(*N*-isopropylacrylamide) in water was modified by small amounts of cholic acid-bearing comonomers since the bile acid residues tend to induce the aggregation of the polymers. In addition to the thermosensitivity, the copolymers also showed a response to pH changes when the carboxylic acid group of cholic acid was liberated by a selective hydrolysis of the ester protecting group. The addition of salt lowered the LCST of the polymers while the addition of surfactants raised it. The effects of a common surfactant, sodium dodecylsulphate, and that of a bile salt, sodium cholate, were compared. The chemical compositions, molecular weights and glass transition temperatures of the polymers have been determined. The glass transition temperatures of the copolymers were found to vary with their chemical compositions and the lengths of the spacer group.

Keywords: Cholic acid; Poly(*N*-isopropylacrylamide); Thermosensitive polymers

1. Introduction

Bile acids are biological compounds synthesized in the liver, stored in the gall bladder and released for lipid digestion in the gastro-intestinal tract [1]. Their amphiphilicity, rigid structure and acid–base properties make them useful building blocks in a variety of applications [2–5]. Various polymers have been made with bile acid residues on the main chain [6–9], as pendant groups [9–16] and as chain-end groups [17]. Because of the biological origin of bile acids, their inclusion in polymers could lead to better biocompatibility when the materials are used for biomedical applications. We also reported recently that the incorporation of oligo(ethylene glycol) spacers in the methacrylate monomers made from cholic acid resulted in improved hydrophilicity of the polymers [18]. In this study, the same monomers have been used to copolymerize with *N*-isopropylacrylamide (NIPAM) to obtain thermosensitive polymers with response to pH changes and other stimuli.

In fact, much has been learned on the thermosensitivity of poly(*N*-isopropylacrylamide) (PNIPAM) and related polymers [19–31]. These polymers can form hydrogen bonds with water but when temperature rises above a certain lower critical solution temperature (LCST), a disorder is created in the solution leading to the disruption of hydrogen bonds and, eventually, the hydrophobic aggregation of the polymer chains. The turbidity of the solution can be visualized as the precipitation of the polymer occurs. Therefore, the LCST can be easily determined by turbidimetry or by differential scanning calorimetry (DSC) [27]. The LCST of the polymers depends on the chemical compositions of both the polymer and the aqueous medium. The hydrophobic substituents on the polymers tend to lower its LCST while the hydrophilic substituents raise it [19,26,31]. The presence of hydrophobic groups can induce the aggregation of the polymers [20–22,28]. Additives in the solution also affect considerably the LCST [23,24]. When the additive is compatible with only one of the two phases (such as a salt), the LCST decreases whereas when it is compatible with both phases (such as a surfactant), the LCST increases. It is also known that the salt-induced change in LCST of the polymers depends on the chemical structure of the added salt [23]. The pH of the solution also influences the LCST value depending on the degree of protonation or deprotonation of the polymers [30]. Since the thermosensitive polymers display a strong physical and chemical modification in response to a relatively weak stimulus, they are sometimes called 'intelligent polymers' [29]. This important property

* Corresponding author. Tel.: +1-514-343-6733; fax: +1-514-343-7586.
E-mail address: julian.zhu@umontreal.ca (X.X. Zhu).

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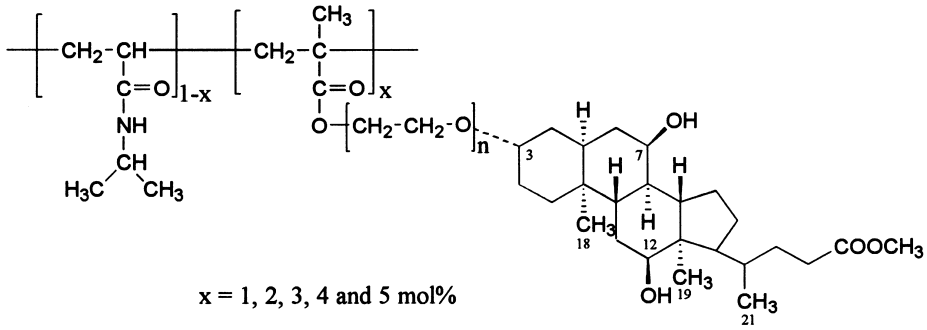

Fig. 1. General structure of the copolymers of NIPAM and the methacrylate monomers derived from cholic acid (ME(EG)$_n$CAME, where $n = 0$, 1, 2 and 4).

makes them useful in industrial, medical and biotechnological applications [28–34].

2. Experimental

2.1. Material and instruments

Most of the chemicals used were purchased from Aldrich and used as received, including cholic acid, benzenesulfonyl chloride, NIPAM, sodium dodecylsulphate (SDS), sodium cholate (NaC), ethylene glycol (EG), diethylene glycol, tetraethylene glycol, triethylamine (NEt_3) and pyridine. Methacryloyl chloride was freshly prepared from methacrylic acid and benzoyl chloride, both from Aldrich. Pyrene and 2,2′-azo-bis(isobutyronitrile) (AIBN) were purchased from Eastman Kodak (Rochester, NY, USA) and were recrystallized prior to use. Tetrahydrofuran (THF) was dried and redistilled with sodium before use.

The NMR spectra of the monomers and polymers were recorded on Bruker AMX-300 and ARX-400 operating at 300.0 and 400.0 MHz, respectively, for ^{1}H. The solvents used were deuterated chloroform and dimethylsulfoxyde (DMSO-d_6). The relaxation delay was fixed at 10 s and 32 scans were accumulated. Mass spectrometry was done for the monomers on an MS 50 TC TA (Kritos) apparatus.

The number- and weight-average molecular weights (M_n and M_w) of the polymers, relative to polystyrene standards, were determined by size exclusion chromatography (SEC) on a Waters SEC system with a flow rate of 1 ml/min at 33°C. A 0.25 wt% polymer solution in THF was injected into a set of three ultra styragel columns with a nominal porosity of 1000, 500 and 100 Å, using THF as the eluent. Differential scanning calorimetry (DSC) traces were obtained on a DSC 2910 calorimeter (TA Instruments, New Castel, DE, USA) with a heating rate at 10°C/min for the glass transition temperatures. The LCST was also determined on a CARY 1 BIO UV–visible spectrophotometer coupled to a temperature controller at a wavelength of 500 nm. The heating rate was set at 0.5°C/min. Elemental analysis (EA) of the copolymers was done on an EA 1108 CHN Fisons instrument. Prior to the analysis, the polymer samples were precipitated from their THF solutions by the addition of ether, and the samples were dried in a vacuum oven at 40°C for 48 h.

2.2. Preparation of monomers and copolymers

The methacrylate monomers containing oligo(ethylene glycol) spacers of varying lengths and bile acid residues were synthesized as reported previously [18]. One of the monomers contained no EG spacer ($n = 0$) while the others contained a spacer with one, two and four EG units. These monomers are generally represented by ME(EG)$_n$CAME ($n = 0$, 1, 2 and 4) in the following text and their general structure is shown in Fig. 1 as one of the repeating units in the copolymers.

For each of the monomers derived from cholic acid, five copolymers were synthesized (Fig. 1) containing 1, 2, 3, 4 and 5 mol% of the cholic acid-bearing monomers. All of them were prepared as described in the following procedure. NIPAM (0.044 mol) and 1–5 mol% of the cholic acid-bearing monomer [ME(EG)$_n$CAME] were dissolved in 30 ml of dried THF. AIBN (1 mol%) was added to the mixture, which was then degassed. The temperature was raised gradually during 2 h to reach 68°C, and stirred for 24 h. The mixture was then cooled down to room temperature and the solvent was evaporated. The residue was dissolved in 10 ml of THF and precipitated in 100 ml of diethylether. The copolymer was recovered by filtration and dried under vacuum. The conversion rate obtained for all copolymers varied from 95 to 97%.

To selectively hydrolyze the methyl ester protecting group on the carboxylic acid group of cholic acid, 0.1 g of copolymer was dissolved in 4 ml of methanol. An aqueous solution of lithium hydroxide (0.1 N, 1 ml) was added to the mixture and stirred at room temperature for 24 h. Methanol was then evaporated and a 2 N hydrochloric acid solution was added dropwise until the pH reached 2. The white precipitate formed was poured in diethylether to collect the hydrolyzed copolymer, which was then filtered, washed several times with chloroform and dried.

Table 1
Chemical composition, molecular weights and glass transition temperatures of the copolymers

Copolymers	ME(EG)$_n$CAME (mol%)			M_w	M_w/M_n	T_g ((°C)
	In the feed	Experimental				
		^{1}H NMR	EA			
poly[NIPAM-*co*-MECAME] $n=0$	1	0.89	0.91	26,300	2.52	130.3
	2	1.75	1.78	20,400	2.09	130.7
	3	2.87	2.69	17,000	2.07	131.3
	4	3.78	3.55	23,800	2.26	131.7
	5	4.91	4.61	28,200	2.37	136.1
poly[NIPAM-*co*-ME(EG)$_1$CAME] $n=1$	1	0.94	0.95	19,300	1.99	124.9
	2	1.89	1.91	19,600	2.09	126.4
	3	2.82	2.86	20,700	2.14	129.5
	4	3.91	3.81	19,200	2.06	128.8
	5	4.88	4.76	24,100	2.09	131.8
poly[NIPAM-*co*-ME(EG)$_2$CAME] $n=2$	1	0.89	0.98	18,900	2.02	123.4
	2	1.75	1.91	20,900	1.84	121.6
	3	2.87	2.86	22,900	2.10	119.8
	4	3.89	3.82	20,000	2.08	119.8
	5	4.83	4.71	23,000	2.14	117.1
poly[NIPAM-*co*-ME(EG)$_4$CAME] $n=4$	1	1.02	0.95	23,600	2.26	122.5
	2	1.81	1.86	25,900	2.10	119.4
	3	2.77	2.82	27,000	2.24	112.8
	4	3.81	3.73	24,600	2.30	101.7
	5	4.77	4.66	23,500	1.45	99.5

2.3. Fluorescence studies

For fluorescence studies, stock solutions of the surfactant (50 mM) in a 1.7 μM pyrene solution in Millipore water were first prepared. Two polymers were used to study the polymer aggregation, PNIPAM and the copolymer of NIPAM with 3 mol% of the metacrylic derivative of cholic acid methyl ester with a diethylene glycol spacer [ME(EG)$_2$-CAME]. To study the micellization of the surfactants, samples were prepared by dilution of the stock solution of the respective surfactant (sodium dodecyl sulfate (SDS) or sodium cholate (NaC)) containing pyrene (1.7 μM) to the desired concentrations by the use of a 0.01 wt% polymer solution containing 1.7 μM pyrene.

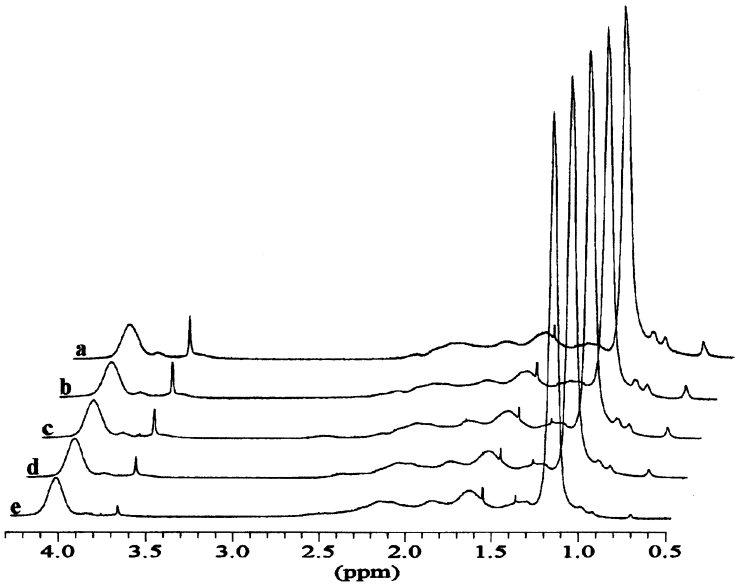

Fig. 2. ^{1}H NMR spectra of the copolymers poly[NIPAM-*co*-ME(EG)$_1$CAME] in $CDCl_3$. The molar percentage of the comonomer ME(EG)$_1$CAME is: (a) 5%; (b) 4%; (c) 3%; (d) 2%; and (e) 1%.

Steady-state fluorescence spectra were recorded at room temperature on a Perkin–Elmer LS 50B spectrophotofluorimeter using narrow (0.2 mm) slits. An excitation at 336 nm was used and the emission spectrum was recorded from 350 to 450 nm at 90 nm/min. I_3/I_1 ratio was calculated by taking the ratio of the maximum peak intensity at 384 nm to that at 372 nm.

2.4. Thermosensitivity tests

The LCSTs of all the copolymers were measured by turbidity experiments. The inflection point of the turbidity curves was taken for the value of the LCST of the polymers. To test the effects of added salts and surfactants, the copolymer containing 3 mol% of ME(EG)$_4$CAME was used. The polymer concentration was at 5% w/v in distilled water. The concentration of sodium chloride was varied from 0 to 20 M. The pH of the copolymer solution in a phosphate buffer (0.05 M, pH = 6.8) was adjusted by adding drops of an alkali solution (1 N NaOH) or hydrochloric acid. Two surfactants, SDS and NaC, were used in the experiments. Their concentrations were varied from 0 to 60 mM.

3. Results and discussion

3.1. Characterization of the copolymers

The copolymerization was easy to perform and the yields obtained were quite high (95–97%). The molecular weights of the polymers and their distributions were consistent (Table 1). They were not optimized for the purpose in this study. The chemical compositions of the copolymers were determined with both ^{1}H NMR and EA. As shown in Fig. 2, the intensities of the NMR signals at 0.71, 0.92 and 0.99 ppm, corresponding to the CH_3 groups at positions 18, 19 and 21 of cholic acid, respectively, all increased with the increasing molar fraction of the cholic acid-bearing comonomer. This is also true for the methyl ester protons at 3.66 ppm. For NIPAM in the copolymers, two different signals were found at 1.14 and 4.02 ppm, corresponding to the six CH_3 protons and one CH proton, respectively, of the isopropyl group. The chemical composition of the copolymers was obtained by integrating the assigned signals. The results in Table 1 show a good correlation between the monomer compositions in the feed before polymerization and the experimental values of the resulting copolymers. The results are in good agreement with those obtained from EA of the nitrogen content in the copolymer (Table 1), since only NIPAM contains nitrogen.

The methyl ester of cholic acid was used in the synthesis because of the higher solubility of the resulting monomers. To remove selectively the methyl ester protecting groups of the cholic acid residues on the copolymers, hydrolysis was performed under mild conditions to avoid the cleavage of the cholic acid residue from the polymer chain. Dayal et al. [35] showed that at room temperature LiOH was as efficient as NaOH in the hydrolysis of the methyl ester of bile acids in a mixture of methanol and water. Therefore, LiOH was used for the hydrolysis since the copolymers were soluble in the mixture of water and methanol. The success of the selective hydrolysis was evidenced by the disappearance of the methyl ester proton signal at 3.66 ppm in the NMR spectrum, while the other proton signals of the cholic acid residue remained unchanged (Fig. 3). This is consistent with the results of the selective hydrolysis that have been reported previously [13,15,18].

All the copolymers have shown a single glass transition temperature (T_g) between the T_g values of the corresponding homopolymers (Table 1), an indication of the formation of statistically random copolymers. In all the copolymers, the molar fractions of the comonomers ME(EG)$_n$CAME were low, ranging from 1 to 5 mol%, but their effects on the T_g were quite significant. The rigidity of the MECAME comonomer (without the EG spacer) increases the T_g of the copolymers of NIPAM. However, the incorporation of a flexible spacer tends to lower the T_g value. This is especially pronounced with the increasing length of the (EG)$_n$ spacer. In the case of $n = 4$, the T_g values of the coplymers are even lower than that of PNIPAM.

3.2. Fluorescence studies of the aggregation

The steady-state fluorescence spectrum of pyrene has five vibronic bands. The first vibronic band (I_1) near 372 nm shows enhanced fluorescence intensities in a more polar environment while the third vibronic band (I_3) near 384 nm remains insensitive to the change of environment. Therefore, a plot of the ratio of the third vibronic bands to the first (I_3/I_1) should exhibit an inflection point at the critical aggregation

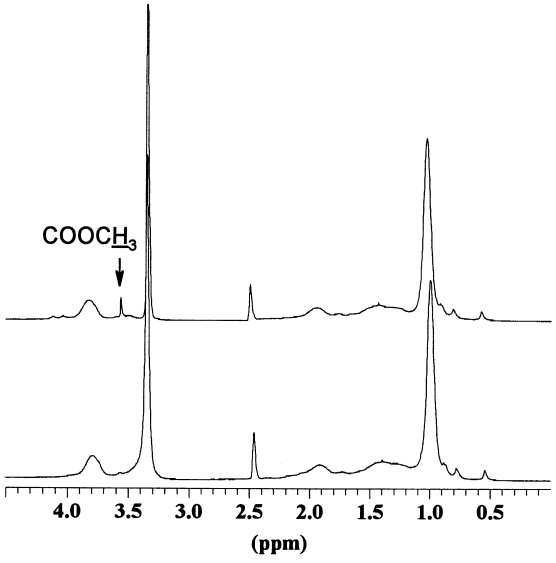

Fig. 3. ^{1}H NMR spectra of poly[NIPAM-*co*-ME(EG)$_4$CAME] containing 5 mol% of the comonomer ME(EG)$_4$CAME in DMSO showing selective hydrolysis of the methyl ester group on the cholic acid residues before (top) and after (bottom) hydrolysis.

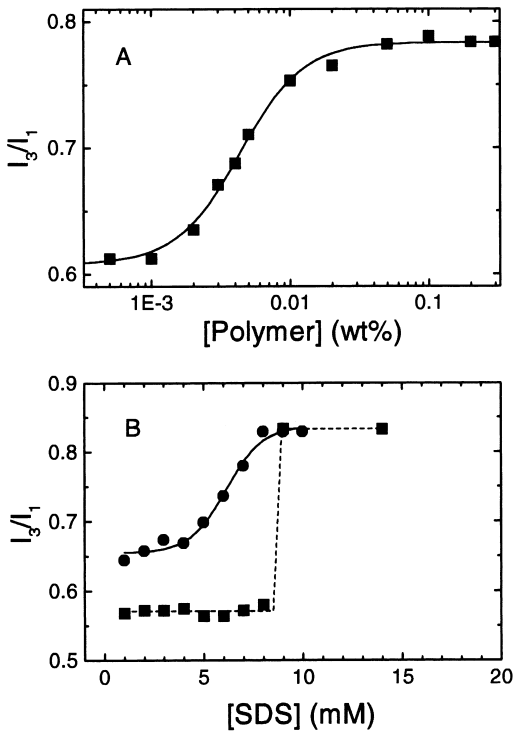

Fig. 4. (A) The I_3/I_1 ratio of the pyrene fluorescence spectra plotted as a function of the polymer concentration showing the aggregation of one of the copolymers, poly[NIPAM-*co*-ME(EG)$_2$CAME] containing 3 mol% of the comonomer ME(EG)$_2$CAME. (B) The effect of an added copolymer on the micellization of SDS in aqueous solutions. SDS alone without any polymer (squares) and SDS with 8×10^{-4} wt% poly[NIPAM-*co*-ME(EG)$_2$-CAME] containing 3 mol% ME(EG)$_2$CAME (circles).

concentration (CAC), when the probe pyrene experiences a change from a hydrophilic environment to a hydrophobic environment in the core of the aggregates [36]. Fluorescence experiments were carried out to determine the CAC of the copolymer that contains 3 mol% of ME(EG)$_2$CAME. Since the pyrene concentration is very low (1.7 μM), the effect of pyrene on the aggregation process should be negligible.

Fig. 4A shows the variation of the I_3/I_1 ratio of pyrene as a function of polymer concentration. The CAC can be clearly observed for the copolymer. The aggregation starts at a very low polymer concentration, ca. 1.5×10^{-3} wt% (the onset of the change in the ratio of I_3/I_1). The middle inflection point is at ca. 4×10^{-3} wt%. However, for the same concentration range, no aggregation for PNIPAM took place since no inflection point was observed.

Fig. 4B shows the effect of the copolymer on the micellization of SDS. It is clear that the concentration at which micellization occurs decreases when the copolymer is added in the solution, even in very small quantities. For example, the addition of 8×10^{-4} wt% of the copolymer decreases the CMC of SDS from 8.5 to ca. 6 mM. The CMC of NaC was determined to be ca. 13.5 mM [37] but the addition of similar amounts of the same copolymer did not cause any significant change of its CMC (data not shown). It is to be noted that we have to keep the concentration of the added copolymer well below the onset of its own CAC.

3.3. LCST measurements

3.3.1. Effect of chemical composition

The transmittance of UV–visible light was measured as a function of temperature. A few examples are shown in Fig. 5. At low temperature, the solution was transparent

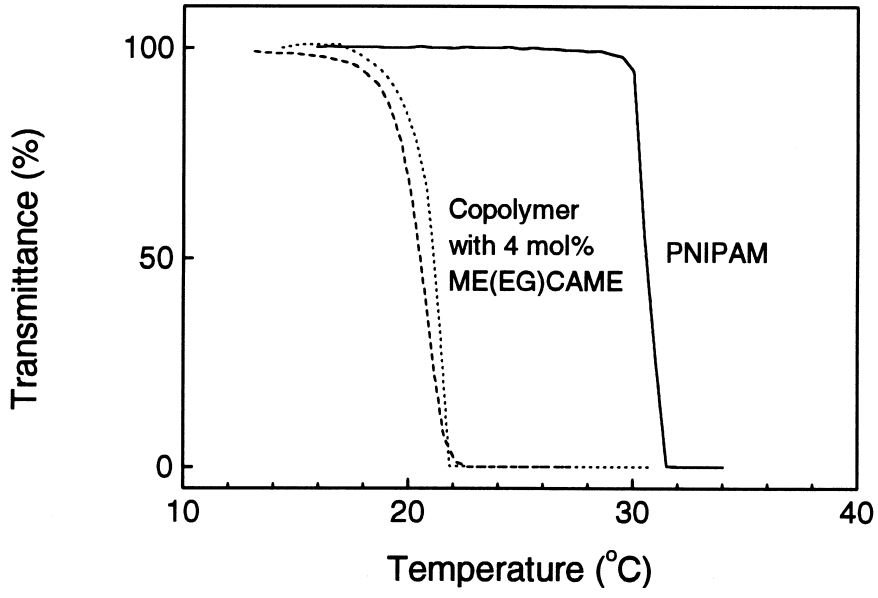

Fig. 5. LCST of the aqueous solutions of the homopolymer PNIPAM (solid line) and the copolymer poly[NIPAM-*co*-ME(EG)$_4$CAME] containing 4 mol% of the comonomer ME(EG)$_4$CAME before (dashes) and after (dots) hydrolysis measured at 500 nm wavelength on a UV–visible spectrophotometer. Heating rate was set at 0.5°C/min.

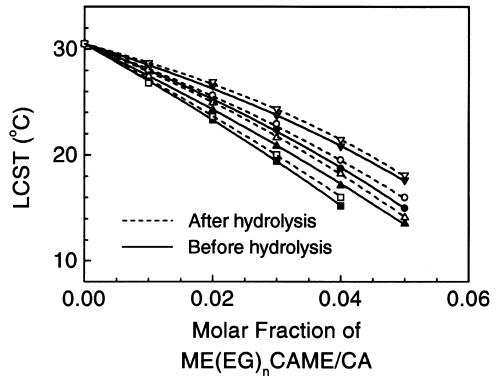

Fig. 6. LCST of copolymers before (closed symbols, solid line) and after (open symbols, dashes) hydrolysis as a function of the molar fraction of the comonomer derived from cholic acid. Spacer length is expressed by the number of EG units: $n = 0$ (squares); $n = 1$ (upward triangles); $n = 2$ (circles); and $n = 4$ (downward triangles).

and the transmittance of the light was high. While the temperature rose, the polymer started to aggregate and phase separation occurred. The solution became cloudy and the transmittance decreased rapidly as the solution became opaque. Fig. 5 shows the typical narrow temperature range during which this phase separation occurred. In the presence of cholic acid residues, the aggregation of the polymers became easier, leading to lower LCST values of the copolymers as shown in the example of the copolymer containing 4 mol% of $ME(EG)_4CAME$. The liberation of the carboxylic acid groups of the cholic acid residue by selective hydrolysis raised the LCST value slightly (Fig. 5) since it increased the hydrophilicity of the polymer. It is to be noted that the carboxylic acid groups in this case were still in the protonated form.

The determination of the LCSTs of the aqueous solutions of the copolymers showed the effect of the $(EG)_n$ spacers. Fig. 6 shows the LCST values for all the copolymers before and after selective hydrolysis as a function of the molar fraction of the cholic acid-bearing monomers. The following features can be observed: (1) By increasing the length of the $(EG)_n$ spacer, the solubility of the copolymer is improved. Therefore, more energy is required to break the hydrogen bonds to cause the aggregation and precipitation of the copolymer, leading to an increased LCST. (2) When the molar fraction of the cholic acid-bearing monomers increases in the copolymer, a gradual decrease in the LCST is observed since they induce the aggregation of the polymers. (3) With selective hydrolysis of the methyl ester groups of the cholic acid residues, the liberated carboxylic acid groups improve the hydrophilicity of the copolymers leading to a small but systematic increase in the LCST values of all the copolymers. It is important to mention that at higher pH, the LCST values of the copolymers in the deprotonated forms were much higher (see Fig. 7B and the discussion below).

3.3.2. Effects of additives on the LCST

To study the effect of additives on the LCST, the aqueous solutions of poly[NIPAM-*co*-$ME(EG)_4CAME$] containing 3 mol% of $ME(EG)_4CAME$, before and after selective hydrolysis, were used for the systematic tests.

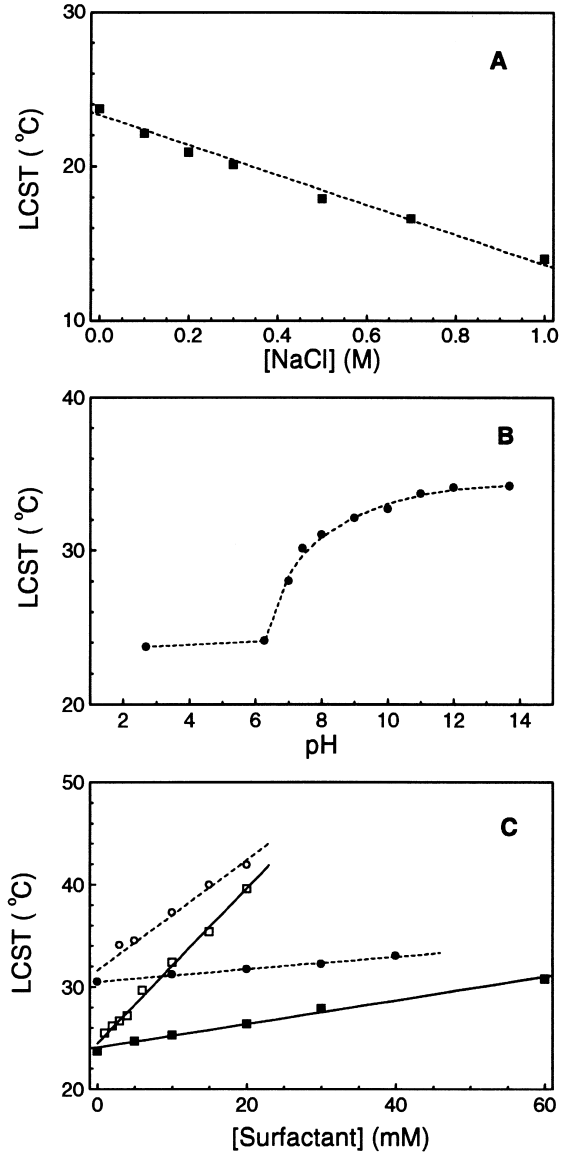

Fig. 7. LCST variation of the copolymer poly[NIPAM-*co*-$ME(EG)_4$CAME] containing 3 mol% of the comonomer $ME(EG)_4CAME$ as a function of the amount of additives. Effects of: (A) sodium chloride salt; (B) the pH of the medium (the copolymer used was in the hydrolyzed form); and (C) the added surfactants for the copolymer (squares) and the homopolymer PNIPAM (circles). The surfactants used include SDS (open symbols) and NaC (closed symbols).

3.3.2.1. Salt effect. As shown in Fig. 7A, a linear decrease of the LCST is observed with the addition of NaCl to the aqueous solution of the copolymer. This decrease was expected since Schild and Tirrell [23] and others [27] reported similar behaviors of PNIPAM. When electrolytes are introduced into the medium, the solvation of the polymer by the water molecules has to compete with the

solvation of the ions. Water molecules interact favorably with the charged entities of the salt in solution to ensure their hydration. The hydrophobic interactions between the segments of the polymer lead to a phase separation at a lower temperature. This is the well-known *salting-out* effect for many macromolecules.

3.3.2.2. pH effect. The copolymers after selective hydrolysis possess free carboxylic acid groups. The protonation of this group depends on the pH of the medium. An increase in the pH leads to a deprotonation of cholic acid, which now becomes ionized and thus more soluble in water. Like the other bile acids, cholic acid is poorly soluble in water in its protonated form ($pK_a = 4.6$) [38] whereas its salt is very soluble [39]. As shown in Fig. 7B, the LCST of the copolymer at high pH values is even higher than the LCST of PNIPAM, although the presence of the salts in the buffer tends to lower the LCST. Fig. 7B shows the response of the thermosensitivity of the copolymer to the pH variation of the solution. In fact, its shape looks very much like an acid–base titration curve. The inflection point of this curve ($pH = 6.9$) may correspond to the neutralization of the cholic acid derivative. Small [40] reported that when sodium cholate was titrated with a hydrochloric acid solution, the complete precipitation of cholic acid occurred at pH 6.5.

3.3.2.3. Surfactant effects. Schild and Tirrell [24] and Winnik et al. [21,22] studied extensively the effect of the surfactant on the LCST of different systems. They reported that the LCST increased with increasing surfactant concentration. This behavior was explained by the solubilization of the polymer in water by the amphiphilic structure of the surfactants. The surfactants isolate the hydrophobic polymer segments from the aqueous environment, thereby raising the LCST.

In this work, we chose to use two surfactants, SDS and NaC, to compare their effects. Shown in Fig. 7C are the changes in LCST as a function of surfactant concentration for the homopolymer PNIPAM and one copolymer poly[NIPAM-*co*-$ME(EG)_4$CAME] containing 3 mol% $ME(EG)_4$CAME. In this concentration range, more or less linear relationships were obtained with both surfactants for both polymers. The difference in the slopes of the lines shows the efficiency of the solubilization with the SDS since a small amount of this surfactant caused a significant increase of the LCST for both polymers. Obviously, the solubilizing power of a sulfate group is much more effective than that of a carboxylate. An interesting feature of Fig. 7C is a comparison of the slopes of the lines for the two different polymers, but with the same surfactant. In both cases, the steeper slopes for the copolymer show the more pronounced effect of the surfactants in the presence of cholic acid residues on the polymer chain. In other words, the cholic acid residues interact favorably with the surfactant molecules and, in doing so, help in the solubilization of the polymer chain by the formation of micelles with the surfactants, causing a steeper increase in the LCST of the aqueous solutions. Nevertheless, SDS is still a better surfactant than NaC. The critical micellar concentration of NaC is much higher than that of SDS [41,42]. Because of its rigidity and bulkiness, NaC is less efficient in the solubilization of the polymers.

4. Conclusions

The incorporation of natural compounds such as bile acids is expected to improve the biocompatibility of polymers. The results show that even a small fraction of cholic acid-bearing monomers can influence the properties of the thermosensitive polymer significantly. The interesting features of the copolymers of NIPAM and the methacrylate derivatives of cholic acid are their response to pH changes and the change of aggregation behavior of the copolymers, in addition to the effect of the oligo(ethylene glycol) spacers. This indicates that the thermosensitivity can be tuned to respond to pH changes of the aqueous media. The effects of the added salt and surfactants are similar to those observed for the homopolymer PNIPAM. These effects have to be taken into account in the molecular design of the thermosensitive materials. Because of the combined sensitivity to both temperature and pH and the inclusion of the biological compounds, the copolymers may be useful in a variety of applications.

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