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Association states of multisensitive smart polysaccharide–*block*-polyetheramine copolymers

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ABSTRACT

The water soluble properties of different copolymers based on pullulan–*block*-polyetheramine have been deeply studied. The polyetheramine group (PEA) corresponding to a propylene oxyde/ethylene oxyde ratio (PO/EO) of 29/6, is condensed, via amine link, to pullulans with various chain lengths. Different polysaccharide/PEA (PS/PEA) ratio copolymers have been investigated through macroscopic (cloud point, enthalpy) and mesoscopic scale approaches (critical aggregation concentration (CAC), mean number average hydrodynamic diameter (Dh) or aggregation number (N_{ag})). These systems are both pH and/or thermo-sensitive. Finally, it seems that three states can describe such systems (i) isolated copolymers (unimers) below the CAC, (ii) water soluble aggregates above the CAC and whatever the PS/PEA ratio is due to hydrophobic interactions and (iii) non-soluble aggregates (above the critical temperature) due to dehydration of polyetheramine groups but only if PS/PEA ratio is low enough.

1. Introduction

Amphiphilic polymer systems (Strauss & Jackson, 1951) and their self-assembly in aqueous media have attracted a lot of scientific interests thanks to their variety of applications such as surfactants (polysoaps) (Martínez, González, Porras, & Gutiérrez, 2005) as enhanced rheological agents (associative polymers) (Charpentier-Valenza, Merle, Mocanu, Picton, & Muller, 2005) or drug vector (Endres, Beck-Broichsitter, Samsonova, Renette, & Kissel, 2011). Therefore, such systems are also largely used in several fields (pharmaceuticals, paints and coatings, cosmetics, etc.) (Henni-Silhadi et al., 2007). Among the amphiphilic system used, copolymers based on biopolymers seem to be very promising. More precisely, polysaccharides are particularly interesting due to their expected biocompatibility and low toxicity (Qiu, Feng, Wu, Zhang, & Zhuo, 2009) and as well as their recoverable origin. Several polysaccharides (such as dextran, alginate, chitosan, pullulan or glucuronoxylan) have already been hydrophobically modified by grafting hydrophobic groups (alkyl, polycaprolactone and so on) (Colinet, Dulong, Hamaide, Le Cerf, & Picton, 2009; Dulong, Mocanu, Picton, & Le Cerf, 2012; Ebringerova, Srokova, Talaba, Kacurakova, & Hromadkova, 1998; Esquenet & Bulher, 2001; Rouzes, Durand, Leonard, & Dellacherie, 2002). These modifications result in very attractive physicochemical properties in aqueous media, which are mainly due to intra- and/or intermolecular associations leading to more or less aggregated structures susceptible to be used as hydrophobic clusters for drug delivery (Henni et al., 2005). Such systems also permit rheological or interfacial control (Charpentier-Valenza et al., 2005; Colinet et al., 2009; Henni et al., 2005; Simon, Le Cerf, Picton, & Muller, 2002).

In order to obtain a better control of the associative properties, more interests have been focused last years on the development of smart polymers which are also called sensitive polymers. Further, they exhibit strong modification of their physicochemical properties when a suitable stimulus is varied on a very narrow domain (Liu, Cellesi, Tirelli, & Saunders, 2009; Weiss & Laschewsky, 2012). Stimuli as ionic strength, pH (Ichikawa & Fukumori, 2000), applied to associative polymers in aqueous media, appear of great interest opening a large way in new applications in cosmetics or biomedical domains (Schatz & Lecommandoux, 2010). Thermosensitivity of water-soluble polymer is generally obtained by chemically modification with specific low critical solubility temperature (LCST) groups (Durand & Hourdet, 2000; Karakasyan, Lack, Brunel, Maingault, & Hourdet, 2008; Mocanu, Mihaï, Dulong, Picton,

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& Le Cerf, 2012; Tizzotti et al., 2010). At low temperature in aqueous media, such LCST polymers lead to an homogenous solution but heating promotes phase separation appearing up to a critical temperature called cloud point (CP). The minimum value of CP in the phase diagram, is more precisely called LCST (Durand & Hourdet, 2000).

Polyetheramine (PEA) as methoxypoly(oxyethylene/oxypropylene)-2-propylamine (Jeffamine®, commercialized by Hunstman) is one of these LCST system families. In our group, we have already worked on a specific mono-functional polyetheramine named Jeffamine®-M2005 composed by propylene oxide (PO) and ethylene oxide (EO) unit with a PO/EO ratio of about 29/6. Such interesting system exhibits a cloud point temperature of about 25 °C in pure water at 10 g L^{-1} (Mocanu, Mihaï, Dulong, Picton, & Le Cerf, 2011). Interestingly thermosensitive polysaccharides can be synthesized by grafting such LCST groups onto anionic polysaccharide as carboxymethylpullulan (CMP) and the resulting physicochemical properties are studied in aqueous solution (Esquenet & Bulher, 2001). Most of the studied thermosensitive systems reported in the literature concern grafted polymers. That is why we have proposed recently a new approach based on an original synthesis leading to block copolymers (Belbekhouche, Ali, Dulong, Picton, & Le Cerf, 2011). In this work, we have reported the coupling of a polysaccharide (i.e. pullulan) with various lengths with the Jeffamine®-M2005 resulting in an homologous series of copolymers with a secondary amine link leading to a pH dependant cationic character (pK_a ~9.5). Preliminary results evidence that the physicochemical properties of such copolymers are directly linked to the HLB (hydrophilic/lipophilic balance), given by the polysaccharide/polyetheramine (PS/PEA) ratio. However, in this study it has also been highlighted the importance of the environmental medium (pH and temperature). Significantly, the thermosensitive properties present peculiar interest since the macroscopic thermal transition occurs near the ambient temperature. Indeed, previously size exclusion chromatography/multi angle laser light scattering/differential refractive index (SEC/MALS/DRI) and absorbance measurements clearly displayed the copolymers association depending on both temperature and pH parameters for all studied systems. Controlling physicochemical property of copolymer by temperature and pH parameters have triggered interest mainly due to the fact that these parameters can be varied in biological and chemical systems. By introducing an ionizable backbone in a neutral system, the resulting copolymer become reacting to either an increase or decrease in the pH. The copolymer ability to be monitored by both temperature and pH leads to an additional control over the copolymer properties (Dimitrov, Trzebicka, Müller, Dworak, & Tsvetanov, 2007). In the present work, the physicochemical properties of the above-cited multisensitive macromolecular amphiphilic systems have been studied deeply with a focused attention on the influence of pH, temperature, PS/PEA ratio (HLB) and copolymer concentration.

2. Materials and methods

2.1. Materials

2.1.1. Copolymer controlled synthesis

The controlled synthesis of an homologous copolymer series, pullulan–*block*-polyetheramine, was reported in the previous manuscript (Belbekhouche et al., 2011), together with their chemical characterization and some preliminary results on their associative behavior depending on both pH and temperature. Briefly, the synthesis strategy is based on a coupling reaction between the reducing sugar of the polysaccharide and the amino-terminated polyetheramine. The last glucose unit of pullulan chain is in thermodynamic equilibrium between closed and opened form. Consequently, each polysaccharide chain reacts with only one amino terminated polyetheramine chain, resulting in only linear copolymer (Fig. 1). Conventional reductive amination of carbonyl compounds needs the presence of reducing agent such as hydride (Brown, 1961). In the present synthesis, sodium cyanoborohydride ($NaCNBH_3$, a highly selective reducing agent, for reductive amination of aldehydes and ketones) has been chosen to couple the two blocks (Borch, Bernstein, & Dupont Durst, 1971). One of the encountered problem is that unreacted pullulan remains after reaction. The quantity of unreacted pullulan has been quantified around 30% in the optimum synthesis condition. However, to study the physicochemical property of these systems in the optimal condition, unreacted pullulan was successfully removed by cation exchange resin ($SO_3{}^-$), which permits to retain the copolymer in its cationic form. This purification method has already been used to purify polysaccharide (Yalin, Yuanjiang, & Cuirong, 2005).

Hereafter, the abbreviations of the homologous copolymers series will be "P (Mn)–*block*-PO_{29}–EO_6", where P is used for pullulan, Mn is the pullulan number molar mass. In these serie the polysaccharide/polyetheramine (PS/PEA) ratio is characteristic of the hydrophilic/lipophilic balance HLB. Their characteristics are given in Table 1.

Our previous results (Belbekhouche et al., 2011) have clearly demonstrated the aggregation tendency in dilute regime at pH 9 and at 15 °C. Logically, the aggregation number (N_{ag}) increased when HLB decreased. But in acidic media, the aggregation was strongly diminished (polyetheramine pK_a is near 9.2 ± 0.1 and copolymer pK_a is near 9.5 ± 0.1) due to the effect of electrostatic repulsions which inhibit association.

2.1.2. Other reagents

Dimethyl sulfoxide (DMSO), sodium hydroxide (NaOH), hydrochloric acid (HCl) were purchased from Acros. Dialysis tubing (SpectraPore) was purchased from Fisher Scientific. Pyrene and cetylpyridinium chloride were purchased from Sigma–Aldrich. All reagents were used without further purification. Water was purified with the Milli-Q reagent system (Millipore).

2.2. Methods

2.2.1. Surface tension measurements

The equilibrium surface tension of the aqueous solutions was measured according to the Wilhelmy plate method (Krüss K12 tensiometer, Germany). The platinum plate was cleaned before each measurement by immersion in deionised water followed by heating in a flame. The surface tension, γ (mN m^{-1}), was measured under controlled temperatures as a function of concentration to determine the critical aggregation concentration (CAC). The measure time was 15 min, which was sufficient to reach the equilibrium state (uncertainty ±1 mN m^{-1}).

2.2.2. Absorbance measurement

The cloud point temperature (CP) of aqueous copolymer solution (prepared in Milli-Q at a controlled pH) was detected by absorbance measurements at 400 nm, using a PerkinElmer Lambda 7UV/vis spectrophotometer (USA) at the inflection point of the curve. The temperature of the solution was raised at a constant rate of 0.5 °C min^{-1}. All temperature transition values were repeatable and reproducible (±0.1 °C).

2.2.3. Differential scanning microcalorimetry (μDSC)

Measurements were performed with a μDSC3 Evo from Setaram (France) from 5 to 50 °C (scanning rate 0.5 °C min^{-1}) on copolymer solution. The volume of the sample cells was around 0.5 mL; equilibrated with a reference filled with the same weight of solvent.

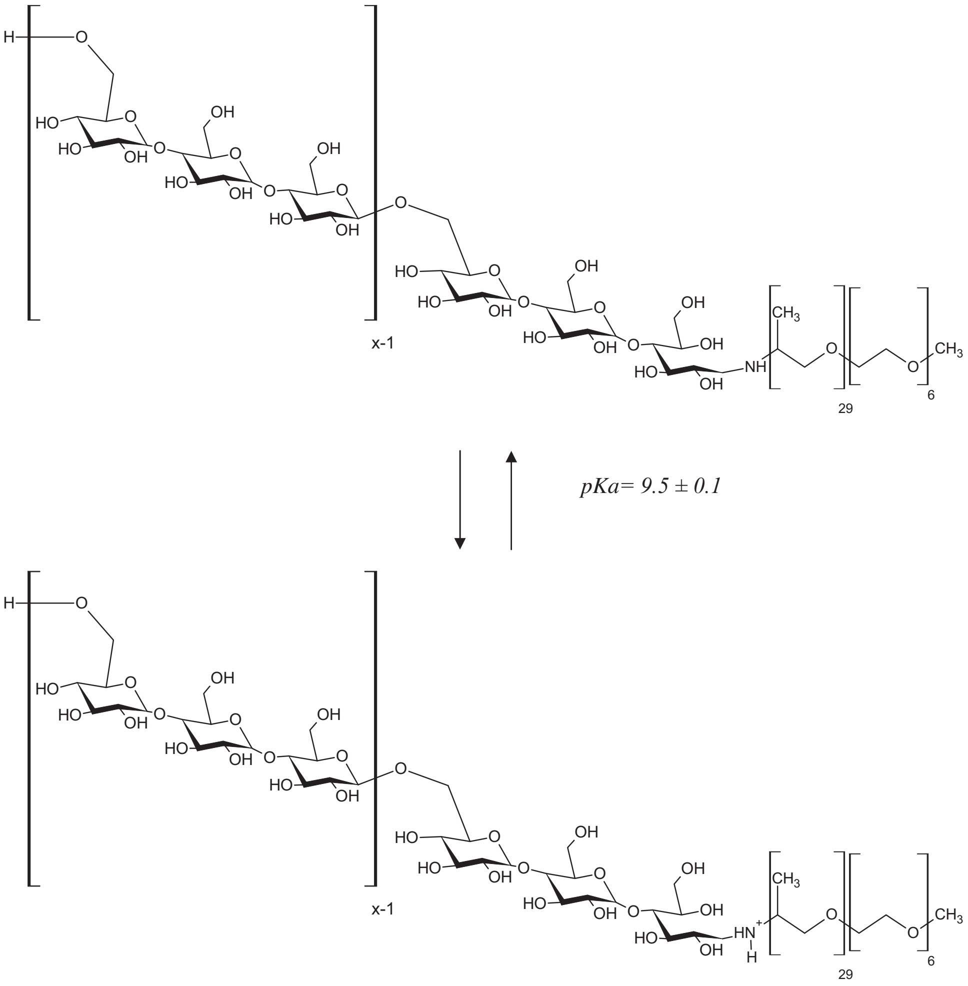

Fig. 1. Chemical structure of pullulan–*block*-PO_{29}–EO_6.

The analysis was done using the Setaram software. μDSC measurements were repeatable and reproducible (±0.1 °C).

2.2.4. Size measurements

Particle size was measured using quasi elastic light scattering (QELS) with a Zetasizer 4700 Malvern Instruments, Brookheaven Instruments Corporation (USA) using a 630 nm Ar–Ne laser at a fixed scattering angle of 90° and at controlled temperature. The intensity–intensity time correction function was analyzed by the cumulative method. Each measurement was repeated eight times. The mean number average hydrodynamic diameter (Dh) was calculated from the diffusion measurement using the Stokes–Einstein equation (accuracy less than 2%).

2.2.5. Fluorescence spectroscopy

2.2.5.1. Critical aggregation concentration (CAC) determination. Fluorescence measurements were carried out with a Fluoromax-4 Spectrofluorimeter (Horiba Jobin Yvon, France) equipped with a xenon lamp. In these experiments, a pyrene stock solution (10^{-3} mol L^{-1}) was prepared in acetone. A 400 μL aliquot of this solution was introduced into empty vials and the solvent was evaporated under vacuum. After evaporation, the vials were filled with 1 L of water and gently stirred for 24 h. This solution was then used to prepare the sample with different concentration of copolymer in aqueous solution at pH 2 or 12 was prepared. The mix was stirred for 24 h to ensure the incorporation of the molecular probe into possibly existing polymer hydrophobic domains.

Table 1
Chemical structure, polysaccharide/polyetheramine (PS/PEA) ratio, theoretical copolymer molar mass, aggregate copolymer number molar mass at pH 9 and aggregate number (N_{ag}) at 25 °C of the pullulan–*block*-PO_{29}–EO_6 (Belbekhouche et al., 2011).

	PS/PEA ratio (w/w)	$Mn_{theo.\ copolymer}$ (g mol^{-1})[a]	$Mn_{ag.\ copolymer}$ (g mol^{-1})[b]	x[c]	N_{ag}[d]
P37000–*block*-PO_{29}–EO_6	95/5	39,000	250,000	73	6
P18000–*block*-PO_{29}–EO_6	90/10	20,000	140,000	35	7
P7100–*block*-PO_{29}–EO_6	78/22	9000	430,000	13	47
P2300–*block*-PO_{29}–EO_6	54/46	4300	370,000	4	83
P504–*block*-PO_{29}–EO_6	20/80	2500	210,000	1	80

[a] Theoretical copolymer average number molar mass.

[b] Aggregate copolymer average number molar mass at pH 9 and 15 °C from SEC/MALS/DRI measurement. Polymer concentration is between 5 and 10 g L^{-1} according to the pullulan molar mass.

[c] Number of maltotriose repeat unit.

[d] Aggregation number estimated.

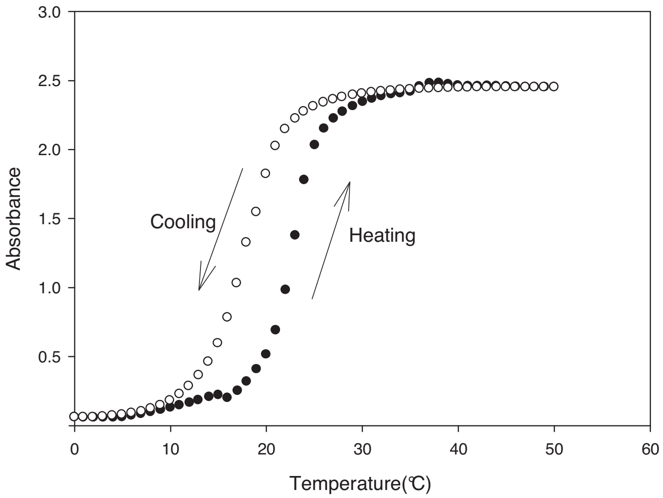

Fig. 2. Temperature transition hysteresis curves of P2300–*block*-PO_{29}–EO_6 at $20\,g\,L^{-1}$ (pH 12) 0.5 °C min^{-1}.

The final pyrene concentration was $4.10^{-7}\,mol\,L^{-1}$. At this low concentration, no excimer band due to the interaction of an excited state pyrene with a ground state pyrene was observed (Amiji, 1995; Fischer et al., 1998; Petit-Agnely, Iliopoulos, & Zana, 2000). Such a low concentration was chosen to minimize the influence of pyrene on the formation and/or the stability of hydrophobic domains. All samples were excited at 332 nm and the emission spectra of pyrene showed 5 vibronic peaks notably at I_1 = 372 nm (intensity I_1) and I_3 = 382 nm (intensity I_3). The ratios I_1/I_3 (accuracy less than 5%) were then calculated leading to a measure of the polarity of pyrene micro-environment (Kalyanasundaram & Thomas, 1977).

2.2.5.2. Aggregation number (N_{ag}) determination. Aggregation number was estimated by using the steady-state fluorescence quenching technique. Cetylpyridinium chloride was used as quencher (Akiyoshi, Deguchi, Tajima, Nishikawa, & Sunamoto, 1997). The steady-state quenching data in a micro heterogeneous system such as an aqueous aggregation solution did not fit in the simple Stern–Volmer kinetics (Stern & Volmer, 1919), but fitted in the quenching kinetics (Akiyama et al., 2007; Stern & Volmer, 1919; Turro & Yekta, 1978), Eq. (1).

$$N_{\text{ag}} = \frac{\ln(I_0/I)}{[\text{Quencher}]}([\text{aggregate copolymer}] - \text{CAC}) \qquad (1)$$

where I and I_0 are fluorescence intensity in the presence or absence of a quencher, [Quencher] is the quencher concentration, and [aggregate copolymer] is the copolymer self aggregate concentration. Plotting $\ln(I_0/I)$ versus [Quencher] gives a straight line. From the slope, the copolymer aggregation number, N_{ag}, could be calculated (accuracy less than 5%).

3. Results and discussion

3.1. Macroscopic scale

In our previous study (Belbekhouche et al., 2011), we have evidenced a critical temperature transition by spectroscopy. Such transition appears clearly reversible as shown by the nice hysteresis obtained in Fig. 2 after heating–cooling process at 0.5 °C min^{-1} for P2300–*block*-PO_{29}–EO_6 at $20\,g\,L^{-1}$ (pH 12). This hysteresis may be explained as the time needed to recover a good hydration state during cooling, return after the strong overlapping of PO groups in a shrunken state occurring above the thermal transition (Karakasyan et al., 2008). Such phenomenon, already observed, was mainly function of the scanning temperature rate (Fettaka et al., 2011). However, such transitions occur only for systems presenting short pullulan length (PS/PEA ≤ 78/22). Above that, solutions remain clear even at high temperature in the studied experimental condition. These first results, obtained from a macroscopic point, lead us to deeply investigate the behavior of these systems as a function of both pH (pH 2 and 12) and temperature. We have plotted the cloud point temperature for each system manifesting such transition (i.e. for PS/PEA ratio ≤ 78/22 (w/w)) as a function of temperature and for pH 12 and 2 in Fig. 3a and b respectively.

Like other LCST systems, e.g. poly(N-isopropylacrylamide) (PNIPAM), the studied LCST polyetheramine precipitates above the cloud point temperature (Jeong, Kim, & Bae, 2002). Such precipitation phenomenon, caused by a strong aggregation, is due to PO association groups for which the aqueous hydration is limited at high temperature. Due to the smallest amount of EO compared to PO (EO/PO ratio 6/29) in this copolymer, EO may not be efficient to provide steric stability. Generally, addition of hydrophilic group incorporated to LCST polymer is well known to affect phase transition (Chen & Hoffman, 1995; Dong & Hoffman, 1991). Effectively the phase diagram of pullulan–*block*-PO_{29}–EO_6 (Fig. 3) evidences higher CP than those obtained for EO_6–PO_{29}–NH_2 alone. The hydrophilic character of the polysaccharide limits the association of LCST groups (Durand & Hourdet, 2000). Consequently, the association (cloud point) occurs for larger temperature as the pullulan length increases. For the two highest PS/PEA ratios, with the highest hydrophilic character, no cloud point could be measured in the employed experimental condition. The same tendency is observed by Mizoguchi, Ida, Matsuyama, and Yamamoto (2010) who modified a LCST polymer (i.e. the PNIPAM) by a hydrophilic block (i.e. polyacrylic acid). As expected, CP is logically smaller for copolymers in neutral form (i.e. pH 12) than in ionic one (i.e. pH 2). These results are in agreement with our previous data from SEC/MALS/DRI measurements (Belbekhouche et al., 2011). Above copolymer concentration of 1 or 2% (respectively for pH 12 and 2), CP does not change noticeably in the concentration studied range. No decantation of the studied solutions has been observed above the cloud point at pH 2 (i.e. ionic form of copolymers), like what is observed for alkaline condition (i.e. neutral character) in the studied experimental condition. This may be due to the electrostatic repulsions which inhibit thermal association of polyetheramine block leading to sterical stabilization of aggregated structures. For copolymers that have displayed a macroscopic transition by absorbance measurements (i.e. P7100–*block*-PO_{29}–EO_6, P2300–*block*-PO_{29}–EO_6 and P504–*block*-PO_{29}–EO_6) a clear macroscopic transition has also well been shown by μDSC measurement whatever the pH (2 or 12) is. For such systems the enthalpy per PO units has been plotted as a function of polysaccharide content (Fig. 4). ΔH values for EO_6–PO_{29}–NH_2 is found respectively about 8 and 6 kJ mol^{-1} per PO unit at pH 12 and pH 2. Such results agree well with those obtained by Karakasyan et al. (2008), pointing out the influence of ionic charge on the association process. ΔH decreases continuously when pullulan length increases, i.e. when the HLB increases. Similar results have been obtained on PEO–PPO–PEO copolymers (Beezer et al., 1994). Hydrophilic blocks linked to PPO block prevent the phase separation process (Tiera et al., 2005). As for absorbance measurement, any transition has been evidenced by μDSC measurement for the highest PS/PEA ratio (i.e. P18000–*block*-PO_{29}–EO_6 and P37000–*block*-PO_{29}–EO_6) even if these systems have shown aggregation behavior highlighted by our previous SEC/MALS/DRI measurement (Belbekhouche et al., 2011). These results may indicate that the evidencing of aggregation depends on the scale of observation as previously reported by Fettaka et al. (2011). For this reason it has been decided to study pullulan–*block*-PO_{29}–EO_6 systems at mesoscopic scales.

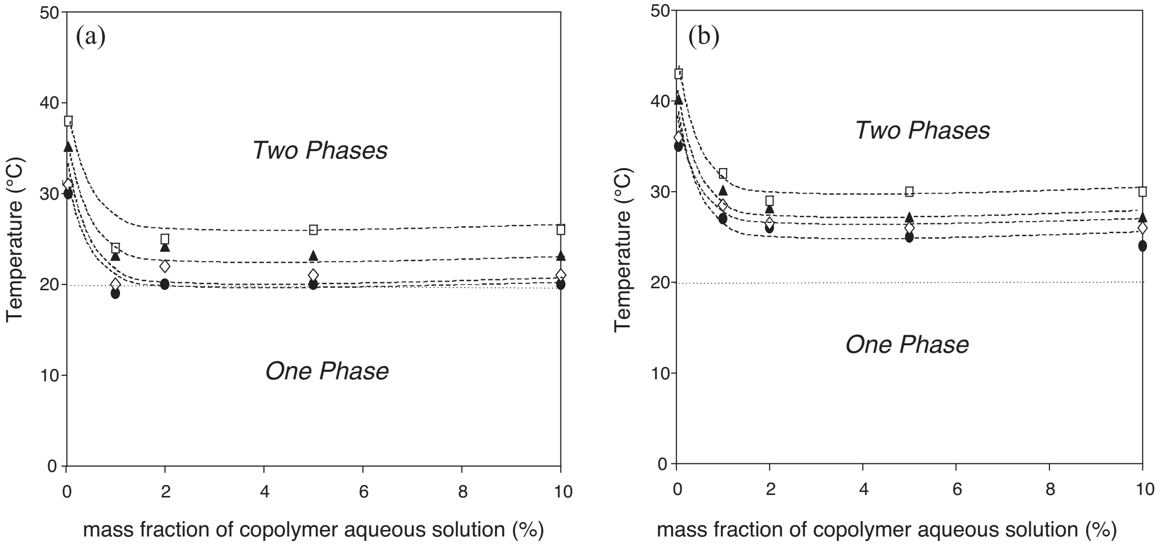

Fig. 3. Phase diagram temperature as a function of mass fraction of copolymers (● EO_6–PO_{29}–NH_2, ◇ P504–*block*-PO_{29}–EO_6, ▲ P2300–*block*-PO_{29}–EO_6 and □ P7100–*block*-PO_{29}–EO_6) in aqueous solution at pH 12 (a) and pH 2 (b).

3.2. Mesoscopic scale

In order to investigate deeply the present systems, we propose to follow their behavior at lower scale than macroscopic one (i.e. mesoscopic) notably in order to determine the CAC, the Dh, or the N_{ag} evolution for all systems depending on concentration, temperature, pH and/or HLB.

3.2.1. Critical aggregation concentration

CACs have been determined by two distinct methods: tensiometry and pyrene fluorescence (I_1/I_3) as shown in Fig. 5 for the systems with the highest PS/PEA ratio without macroscopic aggregation behavior (i.e. P37000–*block*-PO_{29}–EO_6).

The intensity ratio of pyrene emission I_1/I_3 is a characteristic data of the polarity around pyrene (Goddard, Turro, Kuo, & Ananthapadmanabhan, 1985). The decrease of I_1/I_3 indicates the formation of hydrophobic clusters expected to occur with the aggregation of amphiphilic polymers (Henni et al., 2005). CAC values are determined at the inflexion point of I_1/I_3 fall down versus log C (Damas et al., 2001) but are also investigated using tensiometric measurements. As for the determination of critical micellar concentration (CMC), conducted classically for surfactant, the plot of γ_{eq} versus the logarithm of concentration can be used. CAC is then obtained when the surface tension becomes independent of the copolymers concentration. As observed from Fig. 5, P37000–*block*-PO_{29}–EO_6 well evidences a CAC which is quite similar whatever the used method is. Therefore, all systems are studied through such techniques at 20 °C and for pH 2 and 12 (Table 2). Our results well show a good agreement between both approaches.

Fluorescence method is preferred because it is more sensitive and more suitable for study at low (5 °C) and high temperature (55 °C). First of all, it is clearly demonstrated that all the studied copolymers evidence a CAC for low concentrations, even for the more hydrophilic ones (i.e. P18000–*block*-PO_{29}–EO_6 and P37000–*block*-PO_{29}–EO_6) for which any macroscopic transitions have been evidenced. These results are relevant since they show that the scale of observation is of primary importance. Secondly, it appears that CAC occurs for each system even below the macroscopic temperature transition. Amphiphilic characteristics can modify the structural organization before the cloud point. In fact three levels of organization can be considered: (i) isolated molecules or ‘unimer’ (before the CAC), (ii) soluble aggregates with apolar clusters (above the CAC), (iii) strong insoluble aggregates

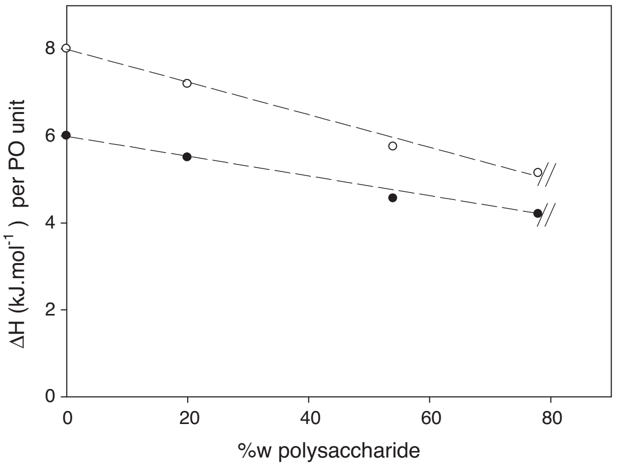

Fig. 4. Transition enthalpy per PO units as a function of massic percentage of polysaccharide contained in pullulan–*block*-PO_{29}–EO_6 at ● pH 2 and ○ pH 12, 20 g L^{-1}.

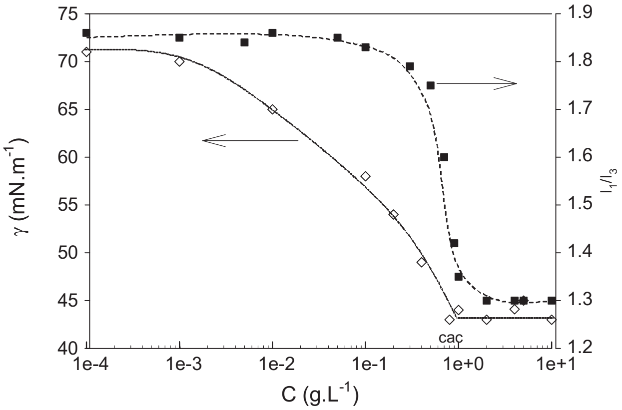

Fig. 5. Variation of fluorescence characteristics of pyrene (■) and surface tension (◇) as a function of P37000–*block*-PO_{29}–EO_6 concentration at pH 2 and 20 °C.

Table 2
CAC values of pullulan–*block*-PO_{29}–EO_6 determined by tensiometric and fluorescence methods at pH 2 and 12 in aqueous solution at 20 °C.

Samples	CAC ($g\,L^{-1}$)			
	Estimated by tensiometric method		Estimated by fluorescence method	
	pH 2	pH 12	pH 2	pH 12
P504–*block*-PO_{29}–BO_6	0.45	0.06	0.43	0.10
P2300–*block*-PO_{29}–EO_6	0.51	0.15	0.48	0.15
P7100–*block*-PO_{29}–EO_6	0.54	0.18	0.60	0.20
P18000–*block*-PO_{29}–EO_6	0.58	0.31	0.64	0.31
P37000–*block*-PO_{29}–EO_6	0.60	0.36	0.75	0.40

(above the cloud point) are not observed for the most hydrophilic copolymers.

In agreement with the above presented results, data from Table 2 confirm that aggregation occurs for lower concentration when PS/PEA ratio is low. The role of the pH is also confirmed since the aggregation is limited by cationic repulsion for acidic media. These results are in agreement with those obtained by Istratov, Kautz, Kim, Schubert, and Frey (2003). It can be noticed that above CAC values, I_1/I_3 ratio keeps close to 1.3 whatever the studied system is. This value is slightly higher than the one found for usual surfactants composed by aliphatic chains, where I_1/I_3 ratios keep close to 1–1.1 at temperature ranging above 35 °C (Hierrezuelo, Aguiar, & Carnero Ruiz, 2005). It means that pullulan–*block*-PO_{29}–EO_6 aggregate cores are less hydrophobic than inner cores of molecular surfactants. This is probably due to PO units which are principally located inside the pullulan–*block*-PO_{29}–EO_6 aggregate cores. This result is also pointed out by Bakshi, Sharma, Kaur, Sachar, and Banipal (2006) who studied micelle formation of a LCST polymer, the Pluronic® L64, i.e. $EO_{13}PO_{30}EO_{13}$. For non-thermosensitive copolymers, a slight increase of CAC is usually observed with the temperature due to a better solubility of the system (Khatua, Gupta, & Dey, 2006). However, as presented in Fig. 6, for all studied copolymers, it is clearly observed, a decrease of CAC when temperature is increasing. But this effect is logically more pronounced at pH 12 (i.e. for neutral systems) and for the lower PS/PEA ratio. For copolymers with PS/PEA ratio lower than 78/22, a thermal transition is evidenced above 20 °C. Contrarily, the temperature influence is quite inexistent from PS/PEA ratio of 90/10 (i.e. P18000–*block*-PO_{29}–EO_6 and P37000–*block*-PO_{29}–EO_6). Similar results have also been observed with Pluronic® L64 or $EO_{13}PO_{30}EO_{13}$ (Bakshi et al., 2006). These results agree well with the above macroscopic observations.

3.2.2. Copolymer size

The physicochemical characteristics of the studied systems are also investigated through dynamic light scattering (DLS) measurements at 1 $g\,L^{-1}$ as a function of temperature for both pH 2 and 12 (Fig. 7). In order to obtain sufficient scattering signal, it has not been possible to perform measurements below 1 $g\,L^{-1}$. Consequently, each system has been analyzed above the CAC. Whatever the investigated systems are, the distribution of hydrodynamic diameters appears monomodal. Moreover, copolymer sizes depending on both pH and/or temperature have been found reversible. It has to be noticed that the size of pullulan precursors does not depend on the temperature.

The following results partly confirm our previous observations and conclusions. Systems with high PS/PEA ratios (since 90/10) evidence only a very slight and continuous increase of Dh when temperature grows up. Such systems appear too much hydrophilic to lead to a clear thermosensitive response. On the contrary, systems with lower PS/PEA ratio (i.e. ≤78/22) clearly present a very strong increase of Dh for temperature above 20 °C whatever the pH is. Such increase of the size with the temperature has already been observed for different thermosensitive systems (Feil, Bae,

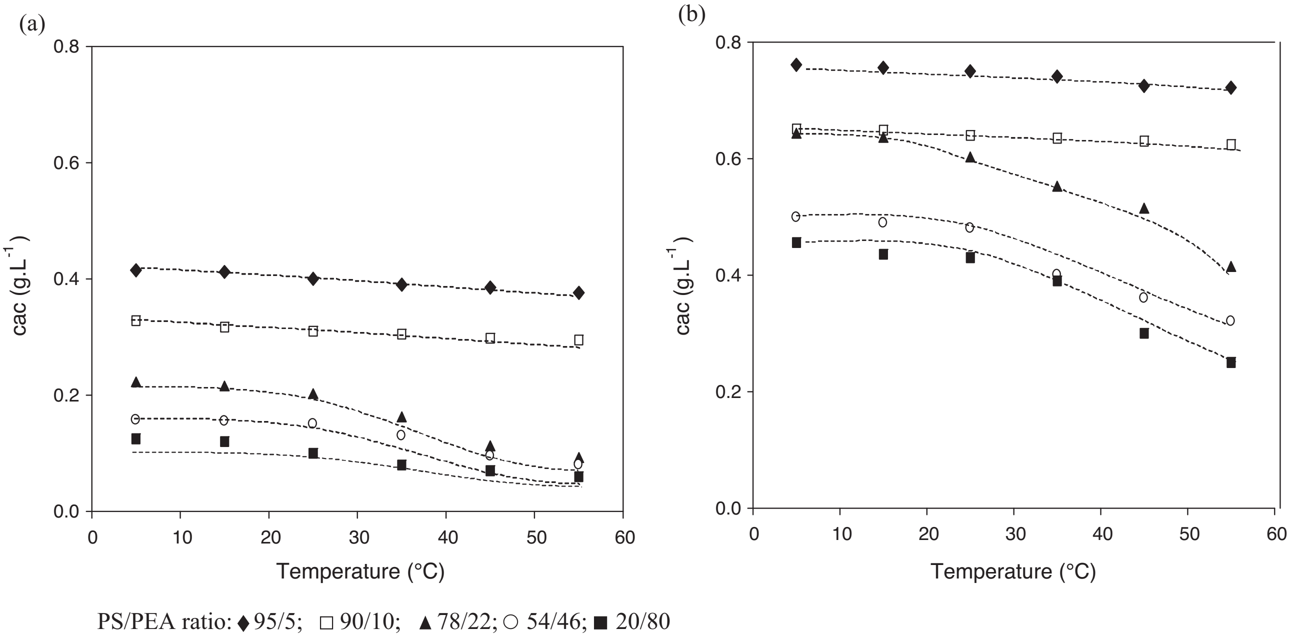

Fig. 6. CAC variation as a function of the temperature at pH 12 (a) and pH 2 (b).

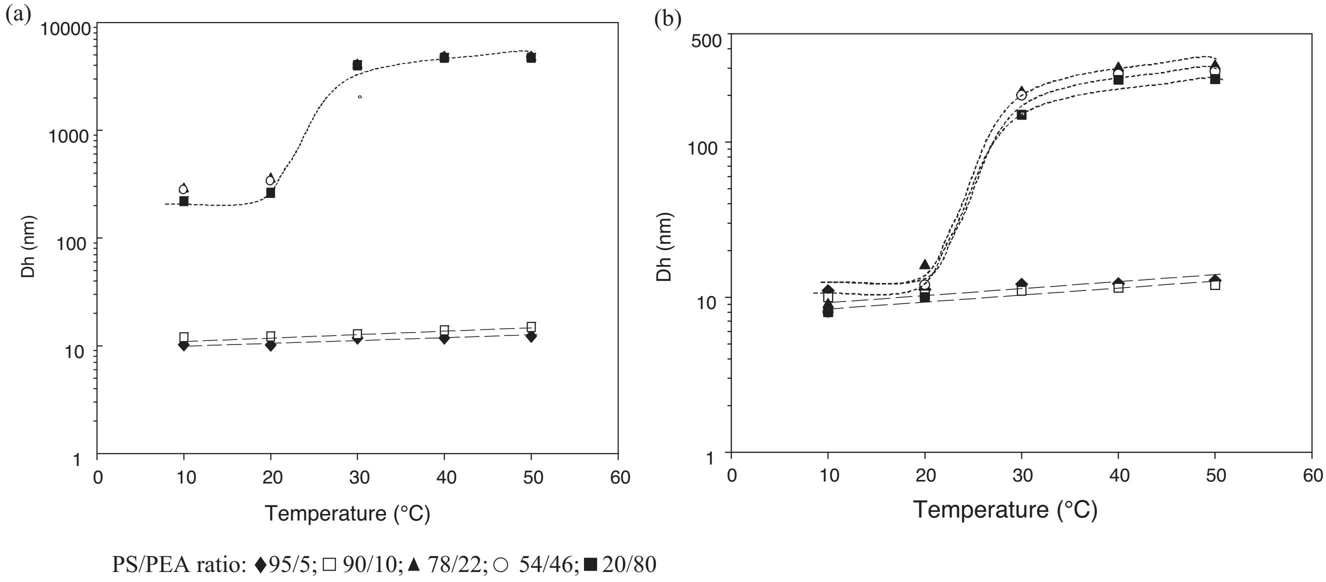

Fig. 7. Size variation as a function of the temperature at pH 12 (a) and pH 2 (b) at 1 g L^{-1}.

Feijen, & Kim, 1993; Tang et al., 2006; Tokuhiro, Amiya, Mamada, & Tanaka, 1991). At pH 12 (i.e. neutral copolymers), the studied systems are already aggregated below the critical temperature (size above 100 nm) and the thermo-association tendency is strongly pronounced leading to very large aggregates, for which the value of the size (above 1000 nm) appears questionable. In agreement, a decantation for higher copolymers concentrations is observed.

In the same time at pH 2, for which pullulan–*block*-PO_{29}–EO_6 systems are cationic, the aggregation before the critical temperature is very limited because of electrostatic repulsions (size about 10 nm). Nevertheless, one can observe a marked thermal transition, leading to higher aggregates size (~200–300 nm) meaning that thermo-association can occur even when electrostatic repulsions are effective but the aggregation stays lower than that observed at pH 12 for neutral copolymer. Consequently, the colloidal size of such aggregates permits to keep the suspension stable in the time. Finally, one can observe that above the thermal transition at pH 2, the size of aggregates depends on the PS/PEA ratio: the larger the PS/PEA ratio, the larger the Dh. This result could appear contradictory with the aggregation trend which is expected to increase with hydrophobic character. However, it is useful to remember that the length of pullulan block largely increases with PS/PEA ratio, meaning that the size of aggregate is not only linked to the aggregation number but also to the size of the ‘unimer’. To clarify this point, some fluorescence measurements has been conducted thanks to quenching approach in order to determine the aggregation number (N_{ag}) of the studied systems.

3.2.3. *Copolymer aggregation number*

An estimation of copolymer aggregation number (N_{ag}) has already been carried out by SEC/MALS/DRI technique (Belbekhouche et al., 2011). In this previous study, it was demonstrated (at a temperature lower than critical temperature) that N_{ag} increased when decreasing the pullulan length in the copolymer. In order to investigate the evolution of this parameter as a function of temperature, pH and PS/PEA ratio, N_{ag} is determined by the steady-state fluorescence quenching technique (Stern & Volmer, 1919). This technique has already been applied for copolymers based on polysaccharide (Akiyama et al., 2007; Turro & Yekta, 1978). The pyrene concentration remains constant (4.10^{-7} mol L^{-1}), the copolymer concentration remains also constant (i.e. 1 g L^{-1}, similar to that used for size measurements) above the CAC, while the quencher concentration increases. It results an inhibition of the pyrene fluorescence.

Table 3 presents N_{ag} values obtained above the CAC by fluorescence confronted to those determined by SEC/MALS/DRI measurement for neutral copolymer below the thermal transition (Belbekhouche et al., 2011). With a very good agreement between both experimental approaches, the results obtained clearly evidence a logical increase of N_{ag} when PS/PEA ratio decreases. Fig. 8 shows the evolution of N_{ag} (by quenching fluorescence) for all studied systems at 1 g L^{-1}, for both pH 2 and 12 as a function of temperature. The results fully confirm the conclusion drawn for the size study. Higher PS/PEA ratio systems (P18000–*block*-PO_{29}–EO_6 and P37000–*block*-PO_{29}–EO_6) do not evidence a noticeable thermal transition but only a continuous and slight increase of N_{ag} with the temperature. This can be explained by the fact that the hydrophilic character is stronger than thermo-response of the Jeffamine® block. For the other systems (from PS/PEA ratio = 78/22 and lower) a strong increase of N_{ag} is evidenced (for temperature about 25 °C) and largely more pronounced for neutral copolymers (i.e. at pH 12) than for ionic ones (i.e. at pH 2). As the pullulan–*block*-PO_{29}–EO_6 copolymer is both thermo- and pH-sensitive, there is a possible competition between thermo-association (in favor of aggregation process) and electrostatic repulsions (in disfavor of aggregation process) depending on the studied experimental condition (Dimitrov et al., 2007). For such cationic systems (pH 2) and above the thermal transition, it logically appears that the lower the PS/PEA ratio, the higher N_{ag} whereas in the same time the lower the HLB, the lower the Dh. This apparent contradiction is easily explained by considering the length of pullulan block (i.e. the size of the ‘unimer’) as discussed previously.

Table 3
N_{ag} of pullulan–*block*-PO_{29}–EO_6 (in neutral form and below thermal transition) obtained by SEC/MALS/DRI and steady-state fluorescence quenching techniques.

Samples	Fluorescence quenching pH 12	SEC/MALS/DRI[a] pH 9
P504–*block*-PO_{29}–EO_6	81 ± 6	80
P2300–*block*-PO_{29}–EO_6	79 ± 5	83
P7100–*block*-PO_{29}–EO_6	45 ± 4	47
P18000–*block*-PO_{29}–EO_6	8 ± 2	7
P37000–*block*-PO_{29}–EO_6	7 ± 1	6

[a] See Belbekhouche et al., 2011.

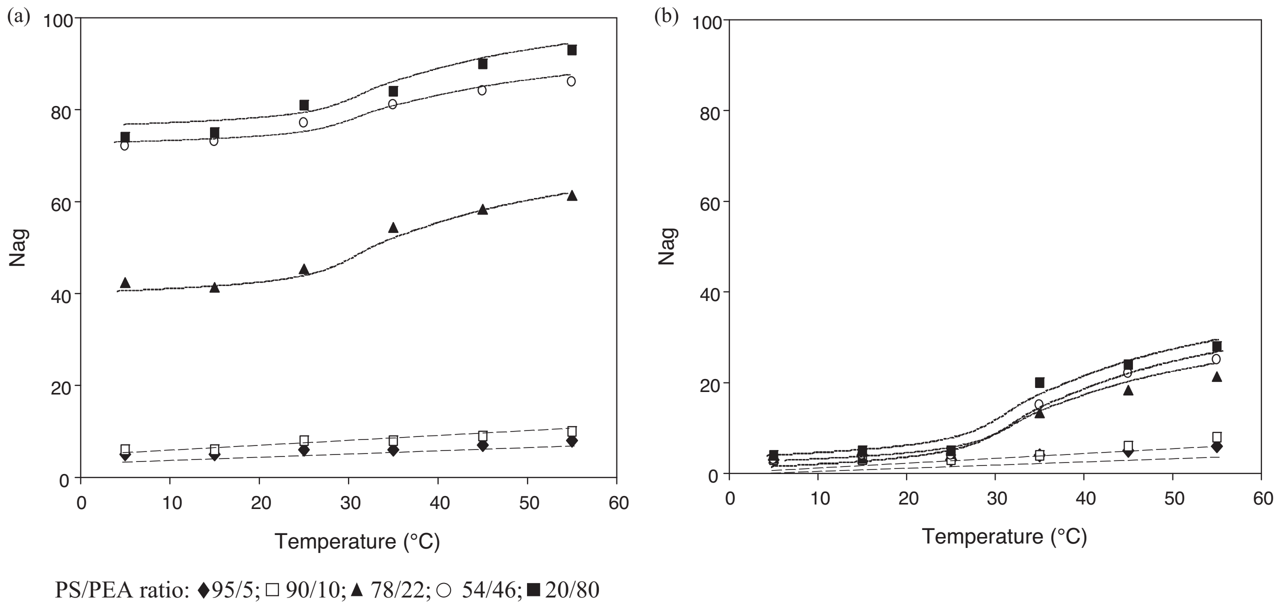

Fig. 8. Copolymer aggregation number variation as a function of temperature by steady-state fluorescence quenching techniques at pH 12 (a) and pH 2 (b) at 1 g L^{-1}.

4. Conclusion

A series of five pullulan–*block*-PO_{29}–EO_6 samples, for which the length of pullulan block varies from 1 maltotriose unit to about 75 (i.e. presenting PS/PEA ratio from 20/80 to 95/5) has been synthesized and characterized in a previous paper (Belbekhouche et al., 2011). The polyetheramine (also called Jeffamine®-M2005) presents LCST properties in aqueous solution. Consequently, these systems are expected to evidence some pH and/or thermo-sensitive properties as polysaccharide and polyetheramine blocks are linked via secondary amine bond (pK_a near 9.5). In this study, we have proposed to deeply study the physicochemical properties of such copolymers in aqueous solution according to hydrophilic/lipophilic balance, copolymer concentration, pH and temperature.

At macroscopic scale, the lower PS/PEA ratio copolymers (i.e. from 20/80 to 78/22) evidence a clear phase transition at a critical temperature (illustrated by both absorbance and μDSC measurements). The phenomenon is fully reversible but with a delay according to the rate of scanning temperatures. That is explained by the dynamic of dehydration/rehydration of the shrunken polyetheramine groups after the thermal transition. The two copolymers with the highest PS/PEA ratios (i.e. 90/10 and 95/5) do not evidence such phase separation since the length of pullulan block is too large and partly prevent the strong thermo-association of polyetheramine groups.

At mesoscopic scale, by fluorescence spectroscopy, tensiometry or dynamic light scattering, CAC, size (Dh) and aggregation number (N_{ag}) have been determined for all of the studied samples, at different pH (2 and 12, i.e. ionic or neutral form) and temperature. Whatever the PS/PEA ratio value, the temperature and the pH are, each system presents a CAC. This is logically lower for less hydrophilic copolymers, neutral character (i.e. pH 12) and/or the highest temperature. As for macroscopic scale, the three samples with lower PS/PEA ratio, also exhibit a clear thermal transition at a mesoscopic scale. However, such transition has not been detected for the two samples with the higher PS/PEA in the studied experimental condition. This behavior has been fully confirmed when following both Dh and N_{ag}.

This clearly indicates that pullulane–*block*-PO_{29}–EO_6 copolymers can exist under three states. Below the CAC, the copolymers are isolated in the solution under ‘unimers’ form. Above the CAC, it can been evidenced some water soluble aggregates due to hydrophobic associations between polyetheramine block. Such interactions seem not to be dependent on temperature, but are favored by low hydrophilic character and neutral character (pH 12) notably. Finally, a third state has been evidenced corresponding to a phase separation and occurring at a critical temperature. This thermo-sensitive behavior seems to concern only the three samples with lower PS/PEA ratio (i.e. P504–*block*-PO_{29}–EO_6, P2300–*block*-PO_{29}–EO_6, P7100–*block*-PO_{29}–EO_6). Above the cloud point temperature, the system is segregated for neutral copolymers (i.e. pH 12) whereas colloidal and stable dispersions are obtained at acidic pH corresponding to ionic copolymers. This is obviously due to electrostatic repulsions which prevent a too strong aggregation of LCST block.

Finally one can consider that polyetheramine block can undergo hydrophobic association (not function of temperature) and in favorable condition some thermo-controlled association due to dehydration if the whole characteristics are not too hydrophilic.

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