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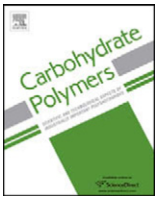

New anionic amphiphilic thermosensitive pullulan derivatives

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ABSTRACT

New anionic amphiphilic thermosensitive pullulan derivatives have been synthesized through reaction of carboxymethylpullulan (CMP) with two Jeffamine® differing by their molecular weight (Jeff M2005 and Jeff M600). The structure of the derivatives was confirmed through FTIR and ^{1}H NMR spectra. Their physico-chemical behavior has been studied through viscometric, fluorescence measurements, which evidenced the amphiphilic, thermosensitive character of these derivatives. The physico chemical characteristics of the synthesized pullulan derivatives depend on the Jeffamine used and on the degree of substitution with Jeffamine units introduced.

1. Introduction

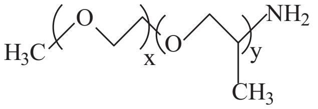
Amphiphilic polymers have been received great attention over the last years. They are formed from both hydrophilic and hydrophobic segments; the characteristics of the polymers (nature of the support, distribution and the nature of the hydrophobic groups, charge ratio) determine their hydrophilic/hydrophobic balance (HLB). In aqueous solutions the hydrophobic segments form intra- and/or intermolecular associations, depending on the hydrophobicity of the hydrophobic units and the concentration range, leading to the formation of aggregates or of a physical network. The formation of hydrogel nanoparticles by the self-assembly of hydrophobized polysaccharides has received much interest with respect to various applications in biotechnology, pharmacy and medicine; the formation of reversible physical networks is important in their application as tensioactive agents or thickeners.

The most studied amphiphilic pullulan is the cholesteryl substituted derivative synthesized by Sunamoto team (Akiyoshi, Nagai, Nishikawa, & Sunamoto, 1992) which forms nanoparticles in water by self-aggregation (Akioshi & Sunamoto, 1996). These nanoparticles can include through hydrophobic forces various biological active substances as: drugs (Akioshi, Taniguchi, Fukui, & Sunamoto, 1996), proteins (Ayame, Morimoto, & Akiyoshi, 2008) (BSA, Akiyoshi et al., 1996; insulin, Akiyoshi et al., 1998). Alkyl substituted pullulan derivatives of various nature, lengths and/or substitution degree with alkyl chains were studied and their associative behavior was reported (Bataille, Huguet, Muller, Mocanu, & Carpov, 1997; Duval, LeCerf, Picton, & Muller, 2001; Glinel, Huguet, & Muller, 1999; Simon, Dugast, LeCerf, Picton, & Muller, 2003; Souguir, Roudesli, About-Jaudet, LeCerf, & Picton, 2007). Synthesis and characterization of thermosensitive pullulan nanoparticles were also reported (Deguchi, Akiyoshi, & Sunamoto, 1994).

The study presents the synthesis and characterization of new anionic pullulan derivatives which contain, linked through amidic bounds, two kinds of Jeffamine units of various molecular weights; taking into account that the Jeffamines contain polyoxyalkyleneamines (polyethylene oxide, polypropylene oxide) backbone with thermoassociative properties, is to be expected that the polysaccharide–Jeffamine derivative possess also amphiphilic and thermosensible characteristics. Physico-chemical characterization of the Jeffamine–carboxymethylpullulan derivatives evidences their polyelectrolyte, amphiphilic and thermosensitive character.

2. Materials and methods

- Carboxymethylpullulan (CMP) synthesized in laboratory, as described (Mocanu, Mihai, Picton, LeCerf, & Muller, 2002).
- Jeffamine M-600 (Fluka) LCST = 59 °C at 10 g L^{-1} in water; M-2005 (Hunstman) LCST = 25 °C at 10 g L^{-1} in water.



M -600 PPO/PEO ratio:9/1

M-2005 PPO/PEO ratio:29/6

- N,N′-dicyclohexyl carbodiimide (DCCI) (Fluka), dimethylaminopyridine (DMAPy), DMSO.

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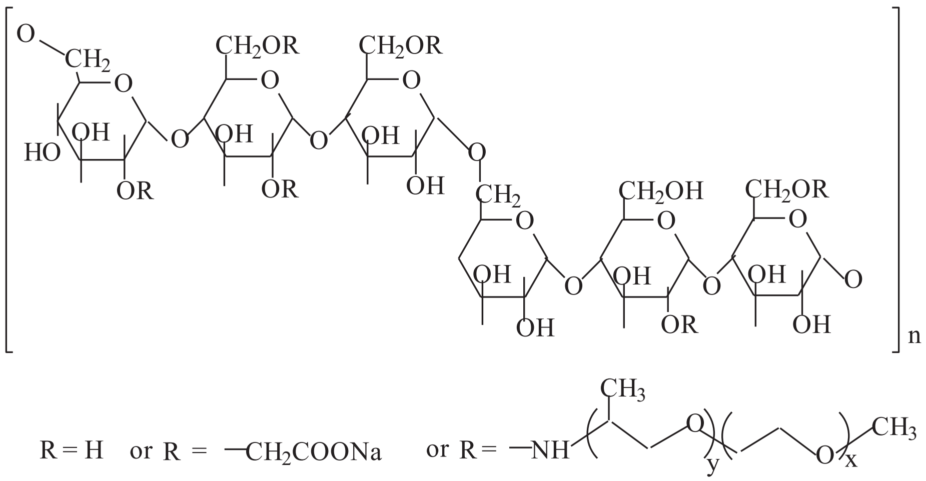

Scheme 1.

3. Methods

Synthesis of Jeffamine-carboxymethylpullulan derivatives (CMP–Jeff). 1 g (4.54 mmoles) CMP powder in H$^+$ form (freshly dried by freeze-drying and then at vacuum 24 h at room temperature) was swollen in 100 mL DMSO freshly distilled for 24 h at 20 °C; then, 0.1 g (0.454 mmoles) DCCI in 5 mL DMSO was added and the reaction mixture was stirred for 2 h; 0.91 g (0.454 mmoles) of Jeff M-2005 and 0.006 g (0.05 mmoles) DMAPy were added and the reaction was continued for 48 h at 20 °C. After that, reaction mixture was precipitated from acetone, filtered, dissolved in 10 mL NaOH solution 0.5N, dialyzed against deionized water and dried by freeze-drying. The other syntheses were performed in the same conditions, but using various molar ratio Jeff/CMP.

The degree of substitution (DS) with Jeffamine units was established through conductimetric titrations (through the difference towards the initial ion exchange capacity with COOH groups) and verified through N total measurements (Shimadzu TOC-V CSN Total organic carbon/Nitrogen analyzer apparatus) and ^{1}H-NMR spectroscopy by peaks integration (BRUKER AVANCE DRX 400 spectrometer, solvent: D_2O, temperature 20 °C).

3.1. Molecular weight

The absolute average molecular weights and molecular weight distribution were determined at 25 °C by coupling on-line a size exclusion chromatography (SEC), a multi-angle laser light scattering (MALLS) and a differential refractive index detector (DRI).

$LiNO_3$ 0.1 M used as carrier, was filtered through 0.1 μm filter unit (Millipore), carefully degassed (DGU-20A3 Shimadzu, Japan), eluted at 0.5 mL min^{-1} flow rate (LC10Ai Shimadzu, Japan). 100 μL of sample solution was injected with an automatic injector (SIL-20A Shimadzu, Japan).

The SEC line consisted of an OHPAK SB-G guard column as protection and two OHPAK SB 804 and 806 HQ columns (Shodex Showa Denko K.K., Japan) in series. The column packing is a polyhydroxymethylmethacrylate gel.

The MALLS photometer, a DAWN-EOS from Wyatt Technology Inc. (Santa Barbara, USA) is filled with a K5 cell and a Ga-As laser (λ = 690 nm). The collected data were analyzed using the Astra V-5.3.4 software package. The concentration of each eluted fraction has been determined with the DRI (RID 10A Shimadzu, Japan) according to the known values of dn/dC (0.141 mL g^{-1} for CMP and derivatives). The SEC/MALLS/DRI technique has been described elsewhere (Dulong et al., 2004).

Viscometric measurements were performed with an Ubbelhode capillary (0a, K = 0.005094) temperature: 20 °C.

Fluorimetric measurements were performed using pyrene 10^{-6} M as fluorescent probe with a Perkin Elmer fluorimeter; excitation: 335 nm; emission: I1: 373 nm; I3: 383 nm (Henni-Silhadi et al., 2008).

4. Results and discussion

The chemical structure of Jeff–CMP derivatives is presented in Scheme 1.

The FTIR spectra of Jeff–CMP derivatives confirmed their structure through the presence of the characteristic band of amide I (C=O) at 1655 cm^{-1}, amide II (C–N–H) group at 1599 cm^{-1} and of carboxylic group at 1721 cm^{-1}.

^{1}H NMR spectrum of Jeff–CMP derivative presents, besides the characteristic peaks due to polysaccharide and Jeff protons at 3.5–4.5 ppm, a peak corresponding to methyl protons of PPO units at 1.12 ppm and of methoxy protons at 3.4 ppm. The ratio was taken between the anomeric proton of glucopyranosic unit at 5.4 ppm and the methyl protons of Jeff at 1.12 ppm (Fig. 1).

By using various molar ratios Jeff–CMP for the two Jeff mentioned, many CMP–Jeff conjugates were synthesized, whose characteristics are presented in Table 1.

A good agreement between the values of DS determined from conductimetric titration, N analyses (with Shimadzu TOC-V CSN Total organic carbon/Nitrogen analyzer apparatus) and ^{1}H NMR spectra was observed.

The hydrophilic CMP precursor and both higher modified samples were analyzed using SEC/MALLS/DRI methods. The results are reported in Table 2.

CMP shows a classical polydispersity for natural polymers. Concerning the CMP-J600: 0.4 sample, the data are quite similar to the precursor. On the other hand we obtain higher molecular weights for CMP-J2005: 0.39 sample. This increase in molecular weight and notably for the weight average molecular weight should be explained by the formation of few aggregates between polymeric chains. More information about the conformation can be obtained from the slope of Mw versus Rg log–log plot. A value of 0.65 is obtained for the CMP in good agreement with a random coil flexible conformation (Table 2). For both modified CMP, the slope is lower. Isolated chains are more compact due to intramolecular interactions. These data confirm the lower viscosity for the modified CMP.

Viscosity measurements (performed in NaCl 0.1N solution, to screening of charges), presented lower specific viscosity values of the CMP-J2005 derivatives than those of the CMP precursor

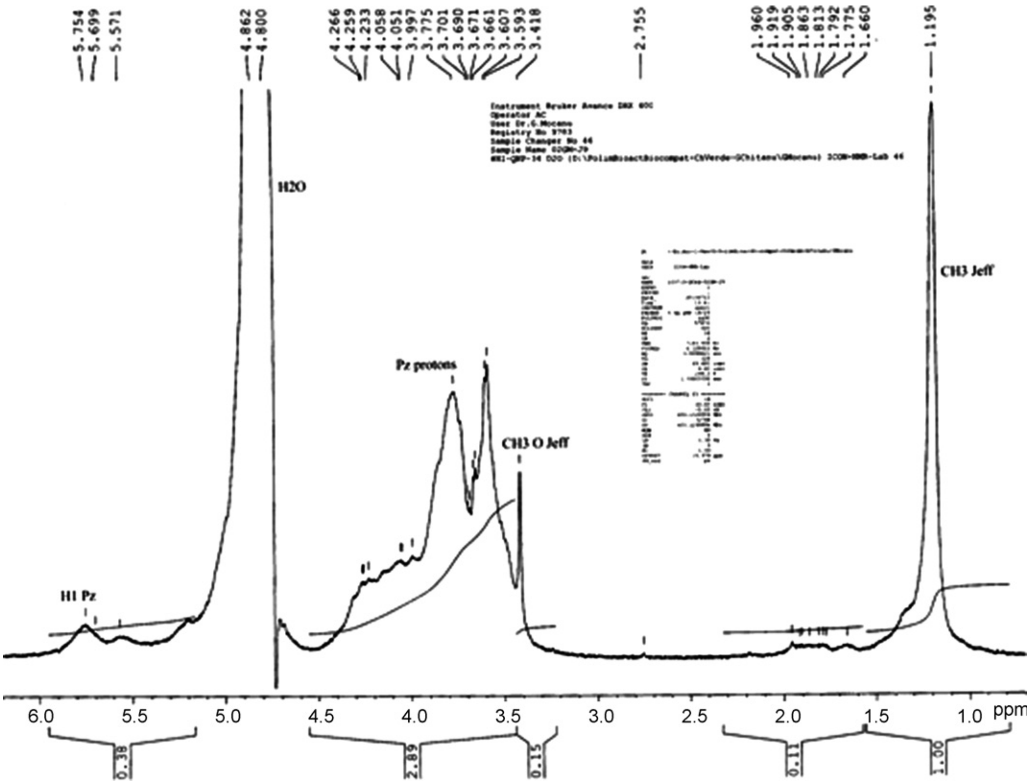

Fig. 1. ^1H NMR spectrum of CMP-J600: 0.2.

Table 1
Chemical characteristics of CMP–Jeff derivatives synthesized.

Sample	Jeffamine	Initial molar ratio Jeff/CMP	Ion exchange capacity, meq/g	DS with COOH groups	DS^*_{Jeff}		
					As difference from initial DS_{COOH}	From N analyses	From ^1H RMN
CMP	–	–	3.23	0.71	–	–	–
CMP-J2005: 0.03	M 2005	0.1/1	3.09	0.68	0.03	0.03	
CMP-J2005: 0.13	M 2005	0.2/1	2.64	0.58	0.13	0.10	
CMP-J2005: 0.19	M 2005	0.3/1	2.40	0.52	0.19	0.20	
CMP-J2005: 0.39	M 2005	0.5/1	1.50	0.32	0.39	0.35	
CMP-J600: 0.1	M 600	0.2/1	2.73	0.60	0.1	0.09	0.08
CMP-J600: 0.2	M 600	0.4/1	2.40	0.51	0.2	0.2	0.18
CMP-J600: 0.4	M 600	0.8/1	1.50	0.31	0.4	0.4	0.32

DS^*_{Jeff} – the number of glucopyranosic OH substituted with Jeff units.

(Fig. 2). These data suggest the existence of hydrophobic preponderant intramolecular interactions between the Jeff M-2005 units linked on the polysaccharide backbone; a decrease of hydrodynamic radius of the polymeric coil occurs, the polymeric chains become more compact, with the formation of more compact aggregates. This phenomenon induces the formation of hydrophobic clusters that could be of great interest for the elaboration of controlled release matrices of hydrophobic drugs.

Table 2
Characteristics of CMP and higher modified samples from SEC/MALLS/DRI.

	Mn ($g\,mol^{-1}$)	Mw ($g\,mol^{-1}$)	Ip	d(Rg)/dMw
CMP	190,000	330,000	1.7	0.65+/−0.05
CMP-J600: 0.4	240,000	360,000	1.5	0.45+/−0.05
CMP-J2005: 0.39	320,000	660,000	2.1	0.40+/−0.05

By using Huggins equation, in dilute regime, $[\eta]$ and k' constants were obtained, presented in Table 3.

$$\frac{\eta_{sp}}{C} = [\eta] + k_H[\eta]^2 C$$

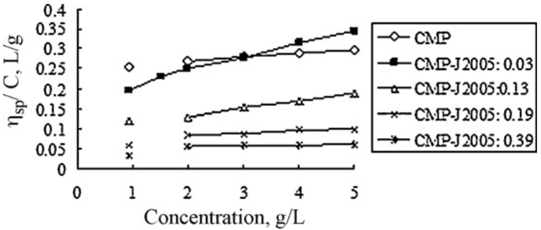

Fig. 2. Reduced viscosities of CMP-J2005 derivatives; temperature: 20 °C; 0.1 M NaCl.

Table 3
$[\eta]$ values and Huggins constants for CMP–Jeff derivatives at 20 °C in NaCl 0.1 M.

Sample	$[\eta]$ L g^{-1}	k_H
CMP	0.24	0.2
CMP-J2005: 0.03	0.17	1.1
CMP-J2005: 0.13	0.10	1.8
CMP-J2005: 19	0.06	2.6
CMP-J2005: 0.39	0.04	4.3
CMP-J600: 0.1	0.20	0.2
CMP-J600: 0.2	0.18	0.6
CMP-J600: 0.4	0.11	2.3

where C – solution concentration; k_H represents Huggins constants and depends on the nature of polymer/polymer interactions in solution; this constant gives also informations about interactions between polymer chain and the solvent used. Values ranging between 0.3 and 0.8 are attributed to a random coil polymer conformation, while larger than 0.8 values correspond to a rigid polymer backbone and can be the result of the intermolecular associations.

The value of k_H, about 0.2 for CMP, is in agreement with the usual value for a polymer in good solvent. On the other hand, the Huggins' coefficient for modified samples increases with increasing DS. This suggest the apparition of polymer/polymer interactions via hydrophobic forces, which becomes stronger than the polymer/solvent interactions; the hydrophobic chains tend to group together in order to limit the contact with water. With a DS of 0.2, the copolymer with M2005 is more affected than one's with M600. The aggregation trend is depending on the length of Jeff.

The $[\eta]$ intrinsic values decrease with length and DS of Jeff, while the constants k_H increase, proving the existence of the interaction forces between hydrophobic groups introduced.

For CMP-J600 substituted derivatives, in dilute solutions, intramolecular hydrophobic interactions can be responsible for their lower specific viscosities, compared with that of the CMP precursor; at higher substitution degrees with M-600 Jeff units, transition between dilute and semi-dilute regime begins at concentrations decreasing with DS increase (Fig. 3).

By using a representation of log η_{sp} versus log c (Utracki & Shima, 1963), the obtained straight lines present intersections which correspond to the transition from the dilute to semi-dilute regime ($C_{crt.}$), therefore to the beginning of chain entanglement (Fig. 3). The $C_{crt.}$ decreases with DS increase with Jeff units on the polysaccharide backbone, which can be explained through the existence of intermolecular hydrophobic interactions.

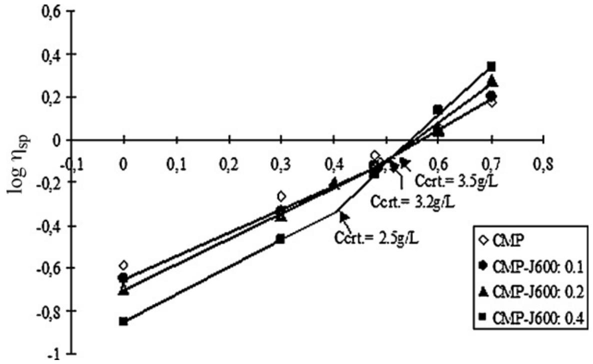

Fig. 3. Utracki and Shima representation (log η_{sp} versus log c) for CMP-J600 derivative.

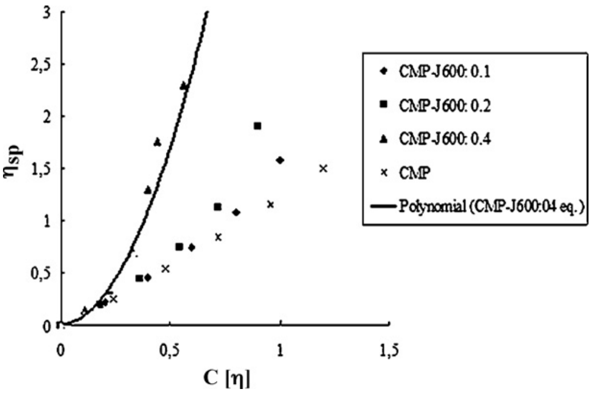

Fig. 4. Specific viscosity of NaCl 0.1 M solution of CMP-J600 derivatives as function of $C[\eta]$.

Rotureau, Dellacherie, and Durand (2005) and Desbrieres (2004) have applied the equation proposed by Matsuoka and Cowman (2002) for associative polymers whose k_H values are much higher than unity.

$$\eta_{sp} = C[\eta](1 + k_1C[\eta] + k_2(C[\eta])^2 + k_3(C[\eta])^3 + \cdots$$

where k_1 is the Huggins constant, $k_2 = (k_1)^2/2!$, $k_3 = (k_1)^3/3!\ldots$

The calculated curve is close to the experimental points and fit the experimental in fairly good way for CMP-J600: 0.4 derivative (CMP-J600: 0.4 eq, Fig. 4).

With DS increasing in Jeff M2005 units, the reduced viscosities decrease for all concentrations (Fig. 5), while for Jeff M600, at lower concentrations the reduced viscosities decrease with DS increase and at higher concentrations the reduced viscosities increase with DS increase (Fig. 5). That means that in semi-dilute domain and higher DS values the hydrophobic intermolecular interactions are prevailing towards the intramolecular ones.

Thermosensitive character of CMP–Jeff conjugates was evidenced through absorbance measurements during temperature variation; the absorbance increases with the temperature for the CMP-J2005 conjugates, while for CMP-J600 ones, do not (Fig. 6). In this case, one can suppose that the amount of this smaller grafting chain is too low to influence the thermosensitive properties of the entire macromolecule.

The hydrophobic character of CMP–Jeff derivatives was also proved through fluorimetric measurements. The I1/I3 in the pyrene emission spectrum, which is sensitive to the polarity of the microenvironment of the probe, was measured for CMP-J2005 conjugates, as function of concentration and temperature. As can be seen from the data presented in Fig. 7, the I1/I3 decreases as polymer concentration increase, proving the presence of hydrophobic microdomains where pyrene is solubilized. The I1/I3 decrease begins even from dilute solution regime (concentrations lower than 0.1 g/L for CMP-J2005 conjugates with DS ≥ 0.13). A similar hydrophobic association in dilute regime was reported also for alkylated chitosan (Desbrieres, 2004).

In presence of salts (0.1 M NaCl), the I1/I3 in the pyrene emission spectrum decreases, as function of polymer concentration and DS with Jeff units (Fig. 7); this decrease is even more accentuated than in aqueous solutions. Hence, in presence of the salts, the hydrophobic microdomains are also formed, leading to an increase of the pyrene solubilization. This can be explained through two factors: (i) the screening effect of the salts on the ionic charges of CMP conjugates, resulting in a higher flexibility of the polysaccharide chain; in this way, the hydrophobic interactions between the Jeff units of the polymer are favored and the interactions with pyrene are increasing and (ii) the increase in ionic strength of the aqueous solution have as effect a decrease in pyrene water solubility and its transfer in hydrophobic environment (Henni-Silhadi et al., 2008).

The variation of I1/I3 in the pyrene emission spectrum as a function of temperature also evidences the thermosensitive character

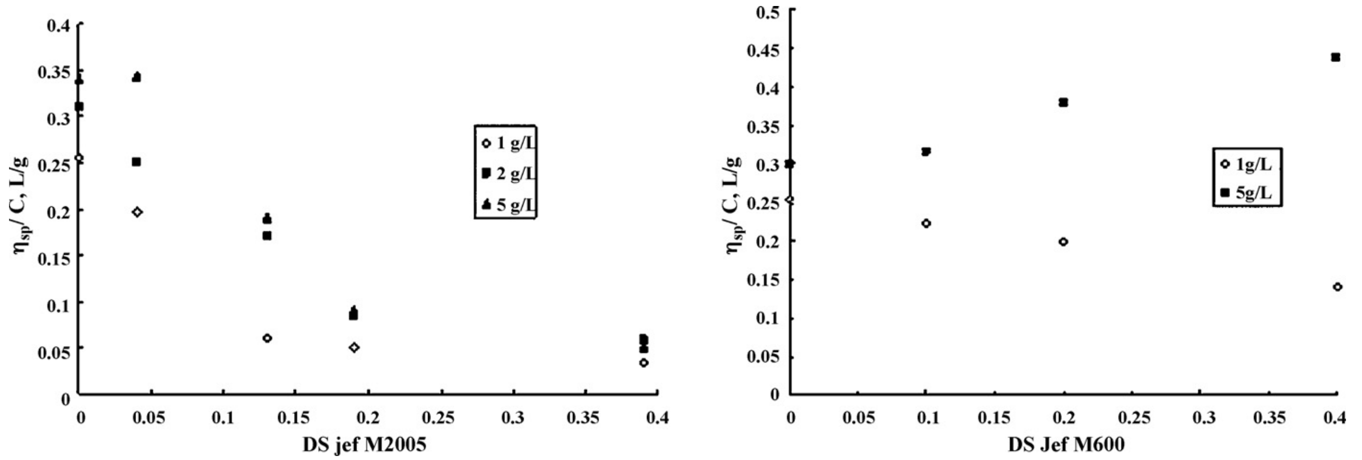

Fig. 5. Influence of DS with Jeff units on reduced viscosities (a): CMP-J2005; (b): CMP-J600.

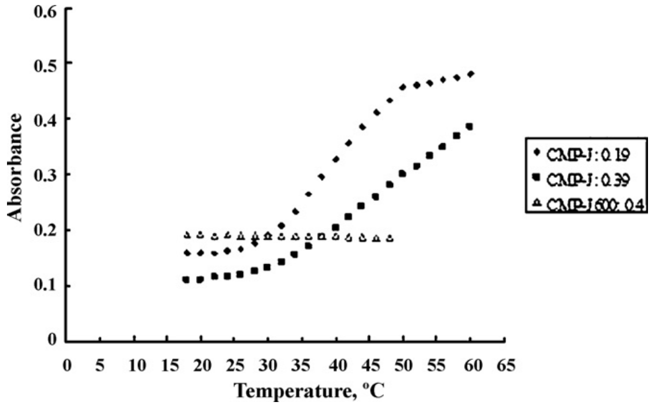

Fig. 6. Variation of absorbance as temperature function; aqueous solutions, concentration 1 g/L.

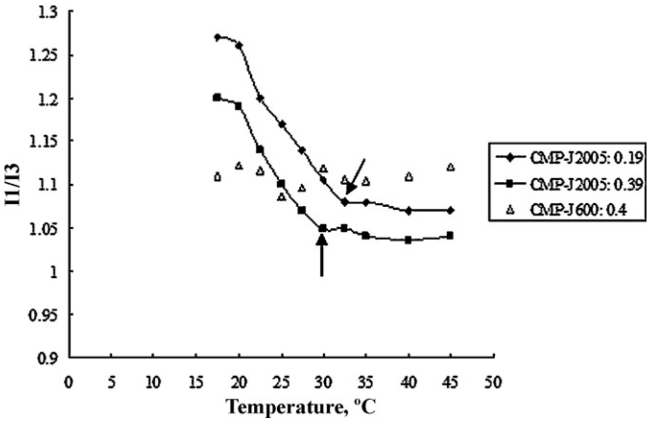

Fig. 8. Variation of I1/I3 ratio in the pyrene emission spectrum as function of temperature for CMP–Jeff derivatives; solvent: water, concentration: 1 g/L.

of the CMP-J2005 conjugates. As can be seen from the data presented in Fig. 8, with temperature increasing, I1/I3 decreases, as function of DS with Jeff M-2005 units. The CMP-J600 conjugate does not present variation of I1/I3, proving again that the Jeff M-600 units do not provide thermosensitive properties to its CMP conjugate. Nevertheless the value of I1/I3 is equal to 1.1, proving an amphiphilic character and the formation of hydrophobic clusters. For CMP-J2005 conjugates one can appreciate the lower critical solution temperatures as: 30 °C for CMP-J2005: 0.39 and 32.5 °C for CMP-J2005: 0.19 derivatives, respectively. The decrease of the I1/I3 as temperature increasing can be explained through the increase of hydrophobic interactions between the Jeff M-2005 units as temperature rise, as mentioned for thermosensitive polymers (Feil, Bae, Feijen, & Kim, 1993); also, heating induces the intra-polymeric aggregation, resulting in the formation of a phase that provides a hydrophobic environment to the pyrene chromophores; pyrene was isolated and sequestered within these hydrophobic microdomain (Liaw, Huang, & Wang, 2002).

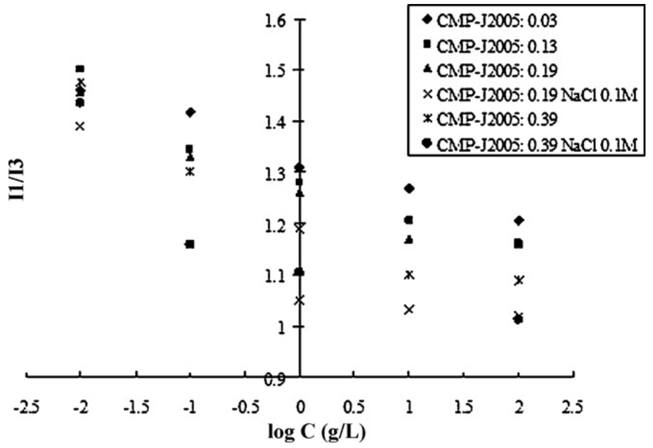

Fig. 7. Variation of I1/I3 ratio in the pyrene emission spectrum for CMP-J2005 derivatives as function of polymer concentration, in aqueous or 0.1 M NaCl solutions; temperature 20 °C.

5. Conclusions

- New anionic thermosensitive pullulan derivatives, containing two kinds of Jeffamine units were synthesized and characterized.
- Jeff M-600 units induce to the obtained CMP derivatives an associative character, due to intramolecular or intermolecular hydrophobic interactions, as concentration polymer function.
- Jeff M-2005 units induce to the obtained CMP derivatives, besides an associative character, due to the preponderantly intramolecular interactions, interesting thermosensitive properties.
- Due to these properties one can envisage interesting potential applications in controlled drug release; further studies are in development to appreciate their performances in this field.

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