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Amphiphilic and thermosensitive copolymers based on pullulan and Jeffamine®: Synthesis, characterization and physicochemical properties

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ABSTRACT

The synthesis of thermosensitive copolymers based on pullulan and polyether amine was performed in water using a water-soluble carbodiimide and N-hydroxysuccinimide as activators. Jeffamine® M2005 was chosen as a polyether to impart thermosensitive character to the copolymer. Pullulan was modified into carboxymethylpullulan, to bring carboxylate groups to the polysaccharide so as to further the grafting reaction. The copolymers were characterized by FT-IR, ^{1}H NMR spectroscopy and molecular weights measurements (by SEC coupled with MALS/DRI/Viscometer lines). The thermosensitive behaviour of CMP-g-M2005 copolymers was studied by fluorescence spectroscopy of pyrene, by rheometry and microDSC measurements. The sol–gel transition temperature was found dependent on the solvent, the grafting degree of M2005 and the concentration of the copolymer. For example it was 35 °C in water, 28 °C in acid buffer (0.1 M, pH 5.4) and 26 °C in saline phosphate buffer (0.15 M, pH 7.4) for a grafting degree of 0.20 at a concentration of 5 wt%.

1. Introduction

The development of drug delivery systems based on biocompatible and biodegradable polysaccharides has become of great interest for the scientific community (Bhattarai, Gunn, & Zhang, 2010; Chung & Park, 2009). Appropriate polysaccharides, such as dextran or pullulan, already studied for biomedical applications (Nishikawa, Akiyoshi, & Sunamoto, 1996; Xi et al., 1996), are good candidates to achieve a stimuli-responsive matrix (Hoare & Kohane, 2008). With such systems, drugs can be delivered by controlled means to the desired site of drug action. Nevertheless, polysaccharides often need to be chemically modified to improve the drug loading, particularly in the case of hydrophobic drugs (Akiyoshi, Deguchi, Moriguchi, Yamaguchi, & Sunamoto, 1993; Henni et al., 2005; Henni-Silhadi et al., 2007; Simon, Dugast, Le Cerf, Picton, & Muller, 2003). For example, polysaccharides were used by Gref, Rodrigues, and Couvreur (2002) to prepare novel amphiphilic copolymers based on dextran and polyesters for biomedical applications. Also, pH-sensitive pullulan acetate was used as a delivery system to release indomethacin (Na, Jeong, & Lee, 1997). Similarly, targeted anti-cancer drug delivery was studied by Na and Bae (2002) with nano-particles self-assembled from pullulan acetate-sulfadimethoxine conjugates.

Thermosensitive polymers exhibit a transition at a temperature defined as the lower critical solution temperature (LCST). Under the LCST, the polymer is soluble and then precipitates at its LCST. In some cases, the thermosensitive polymers exhibit a sol–gel transition. In this context, the use of formulations able to form hydrogels at body temperature appears to be very attractive since these formulations are liquid at room temperature (easily injectable, indeed) and get gellified after administration into the body. Thus, the viscoelastic properties of the formulation ensure long residence times and controlled delivery of the active molecule to the desired site of drug action. For this reason, thermosensitive amphiphilic copolymers, such as poloxamers (poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) PEO–PPO–PEO block copolymers), have been widely used in the biomedical field. These copolymers are water-soluble at room temperature and precipitate or undergo a sol–gel transition (according to the PEO/PPO ratio, concentration and molecular weight of the copolymers) at a higher temperature due to hydrophobic interactions occurring under these conditions. However, these copolymers are not of great interest when used alone, as they exhibit low mechanical properties and short residence times of the loaded drug because of the rapid dissolution of the gels after local administration (Nanjawade, Manvi, & Manjappa, 2007). To improve the mechanical properties of such copolymers, new approaches are considered, i.e., blending polyethers with polysaccharides (Mayol, Quaglia, Borzacchiello, Ambrosio, & La Rotonda, 2008) or grafting polyethers to polysaccharides (Cho, Park, Jeong, & Yoo, 2009; Karakasyan, Lack, Brunel, Maingault,

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& Hourdet, 2008; Mocanu et al., 2011a; Mocanu, Mihai, Dulong, Picton, & Lecerf, 2011b; Park et al., 2009).

The latter procedure has been chosen for this study and pullulan was used as a biocompatible and biodegradable polysaccharide to prepare copolymers grafted with polyether amine to obtain thermosensitive and amphiphilic copolymers. Pullulan is a water-soluble polysaccharide produced by *Aureobasidium pullulans*, its biological activity and chemical properties being adjustable by chemical modification (Shingel, 2004). Its linear flexible chains are formed from α-1,4-linked glucose units, which are included in α-1,6-linked maltotriose units. Various amphiphilic pullulan derivatives have been prepared in our laboratory, for example, carboxymethylpullulans grafted with alkyl chains were synthesized (Bataille, Huguet, Muller, Mocanu, & Carpov, 1997). These hydrophobically modified carboxymethylpullulans (HMCMPs) were shown to develop spontaneous inter- and/or intramolecular interactions in aqueous solution, depending on the content and length of the grafted chains (Duval-Terrié et al., 2003; Duval-Terrié, Huguet, & Muller, 2003). These interactions lead to the formation of hydrophobic micro-domains able to retain hydrophobic drugs.

Secondly, amphiphilic and pH-sensitive hydrogels were successfully synthesized in our laboratory with various polysaccharides – pullulan (Dulong, Mocanu, & Le Cerf, 2007; Mocanu, Mihai, Lecerf, Picton, & Dulong, 2007), alginate (Colinet, Dulong, Hamaide, Le Cerf, & Picton, 2009), hyaluronan (David et al., 2004) – and their amphiphilic and drug release properties having been demonstrated. More recently Mocanu et al. (2011a,b) synthesized in organic solvent (DMSO) thermosensitive copolymers based on poloxamer or Jeffamine®.

In this work, Jeffamine® was the polyether amine used to synthesize the thermosensitive copolymers, and namely M2005 produced by Hunstman (Netherlands). M2005 is a block copolymer based on poly(propylene oxide), PPO, and poly(ethylene oxide), PEO, containing a primary amino group attached to the end of the PPO block and having a PO/EO ratio of 29/6. PPO-b–PEO copolymers have already been used in biological studies, such as gene delivery systems and showed good cytocompatibility with different cells (Peddada, Harris, Devore, & Roth, 2009).

Here, we report the synthesis of carboxymethylpullulan grafted with M2005 (CMP-g-M2005 copolymers) in water. The copolymers have been characterized by FTIR- and ^{1}H NMR-spectroscopy, and their absolute average molecular weights have been measured at 25 °C by coupling on-line a size-exclusion chromatograph (SEC), a multi-angle light scattering system (MALS), a viscometer and a differential refractive index detector (DRI). The thermosensitive behaviour has been studied as a function of the grafting degree of M2005, and in different solvent, i.e. in water, in citrate/phosphate buffer pH 5.4 to simulate the vaginal fluid for possible local applications and in saline phosphate buffer pH 7.4 to simulate the blood fluid conditions for possible injection. The thermosensitive properties of the copolymers have been studied by fluorescence spectroscopy of pyrene, rheometry and microDSC.

2. Materials and methods

2.1. Materials

Pullulan was purchased from Hayashibara Biochemical Laboratory (Japan). Jeffamine M2005 was kindly provided by Huntsman. 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride (EDC) was purchased from Sigma–Aldrich (France), and N-hydroxysuccinimide (NHS) and sodium chloroacetate from Acros Organics (France). Finally, sodium chloride (NaCl), sodium hydroxide (NaOH), hydrochloric acid (HCl), citric acid and sodium hydrogenophosphate (Na_2HPO_4) were purchased from VWR (France). Water was purified with the milli-Q water reagent system (Millipore, USA). All compounds were used without further purification.

Citrate/phosphate buffer (0.1 M, pH 5.4) was prepared by adding 44.6 mL of 0.5 M Na_2HPO_4 and 17.7 mL of 0.5 M citric acid into a 200 mL gauged flask, adjusted with milli-Q water (ionic strength 0.1 M). Saline phosphate buffer (0.15 M, pH 7.4) was prepared by dissolving phosphate buffer saline tablets (from Sigma, France) in 200 mL of milli-Q water (ionic strength 0.15 M).

2.2. Synthesis of CMP-g-M2005

CMP was synthesized beforehand by the reaction of pullulan with sodium chloroacetate in the presence of NaOH by a method previously described (Duval-Terrié et al., 2003). The CMP obtained had a substitution degree of 0.8 in carboxylate groups (determined by conductimetric titration), i.e. 0.8 of the 3 hydroxyl functions of anhydroglucose unit (AGU) were transformed into carboxylate groups.

The synthesis of CMP-g-M2005 was performed at 4 °C to avoid the collapse of M2005. For example, 1 g of CMP was dissolved in 50 mL of milli-Q water. Then a cold solution of M2005 (2 g in 25 mL of milli-Q water) at a pH adjusted to 4.7 with 1 M HCl was added. During the reaction, the concentration of CMP was 12.5 g L^{-1} and the concentration of M2005 was 25 g L^{-1}. The EDC/NHS mixture (dissolved in 1 mL of milli-Q water) was added and the reaction was allowed to proceed at 4 °C under stirring for 24 h. At the end of the reaction, CMP-g-M2005 was purified by dialysis (MWCO cut off 12–14 000 g mol^{-1}, Sodipro, France) against milli-Q water/ethanol (66/33, v/v) during two days to remove unreacted M2005, and then against different solvents to remove unreacted EDC and to recover the sodium carboxylate groups – 0.1 M NaOH (for 1 h), pure water (for one night), 0.1 M HCl (for 1 h), pure water (for one night), 0.1 M NaOH (for 1 h), and finally, pure water again (for one night). At the end of the dialysis steps, the product was filtered and lyophilized. To improve the purification and completely remove unreacted Jeffamine®, the lyophilized product was added to 250 mL acetone and kept under stirring for 24 h, then filtered and dried under vacuum at 40 °C. The mass yield was of 90%.

2.3. Characterization of CMP-g-M2005

2.3.1. Infrared spectroscopy

The infrared spectroscopy was performed on a spectrometer AVATAR 360 FT-IR, NICOLET (Thermo Electron Corporation, USA). The samples were analyzed by transmission from 800 to 4000 cm^{-1}.

2.3.2. NMR spectroscopy

^{1}H NMR spectra of the copolymers were performed with Bruker Avance 400 spectrometer at 400 MHz in D_2O.

2.3.3. Fluorescence spectroscopy

Fluorescence measurements were carried out with a Fluoromax-4 Spectrofluorimeter (Horiba Jobin Yvon, France) equipped with a Xenon lamp. Pyrene was used as a fluorescent probe. The solutions of pyrene were prepared as follows: a stock solution (10^{-3} M) was prepared in acetone and stored at 5 °C. A 100 μL aliquot of this solution was introduced into a 250 mL gauged flask and the solvent was evaporated. After the evaporation of acetone, the gauged flask was filled with milli-Q water and its contents were stirred for 24 h; the final pyrene concentration was of 4×10^{-7} M. This solution was then used to prepare the samples of CMP-g-M2005 with different concentrations of the copolymer at 20 °C. The fluorescence emission spectra (350–450 nm) were performed at an excitation wavelength of 332 nm. The emission

spectra of pyrene showed vibronic bands at $\lambda_1 = 372$ nm (the first band, intensity I_1) and at $\lambda_3 = 382$ nm (the third band, intensity I_3) – in all graphs, the I_1/I_3 ratio values represent the average of 5 measurements, the uncertainty being of ±0.1.

2.3.4. SEC/MALS/DRI/Viscometer

The absolute average molecular weights and molecular weight distribution were determined at 25 °C by coupling on-line a size-exclusion chromatograph (SEC), a multi-angle light scattering system (MALS), a viscometer and a differential refractive index detector (DRI). 0.1 M $LiNO_3$, used as carrier, was filtered through a 0.1 μm filter unit (Millipore, USA), carefully degassed (DGU-20A3 Shimadzu, Japan), and eluted at a 0.5 mL min^{-1} flow rate (LC10Ai Shimadzu, Japan). 100 μL of a 0.45 μm filtrated sample solution (at 2.2 g L^{-1}) were injected with an automatic injector (SIL-20A Shimadzu, Japan). The SEC line consisted of an OHPAK SB-G guard column for protection and two OHPAK SB 804 and 806 HQ columns (Shodex Showa Denko K.K., Japan) in series. The column packing was a polyhydroxymethylmetacrylate gel. The MALS photometer, a DAWN-EOS from Wyatt Technology Inc. (Santa Barbara, USA) was provided with a K5 cell and a Ga-As laser ($\lambda = 690$ nm). The viscometer was a ViscoStar II from Wyatt Technology Inc. (Santa Barbara, USA). The whole collected data (light scattering, DRI and viscosity) were analyzed using the Astra V-5.3.4 software package. Molar masses were obtained with the Zimm order 1 method. The concentration of each eluted fraction was determined with DRI (RID 10A Shimadzu, Japan) according to the known values of dn/dC (0.141 mL g^{-1} for CMP and derivatives).

According to the hypothesis of spherical shape of studied colloids, the Einstein–Simha equation gives the R_h from intrinsic viscosity (1)

$$R_h = \left(\frac{3M[\eta]}{4\pi\upsilon Na}\right)^{1/3} \quad (1)$$

where M is the molar mass, $[\eta]$ is the intrinsic viscosity, υ is a parameter which takes the value of 2.5 for spherical supposed particle, Na is the Avogadro number.

All data allow us the determination of Mark–Houwink (MH) relation (Eq. (2), where K and a represent the characteristic parameters of MH relation), the R_g vs M relation (Eq. (3)) where b represent a characteristic parameter and finally the ratio $g = R_g/R_h$

$$[\eta] = KM^a \quad (2)$$

$$R_g = K'M^b \quad (3)$$

2.3.5. Rheological measurements

Rheological measurements were performed in aqueous media (pure water or citrate/phosphate buffer solution (0.1 M, pH 5.4) or saline phosphate buffer (0.15 M, pH 7.4)) within the 2.5–10 wt% range of copolymer concentration, with an AR2000 Rheometer from TA Instrument (U.K.), using standard-size double concentric cylinder geometry. Oscillation procedures were generally performed at the frequency of 1 Hz and at 1 Pa stress sweep, between 5 and 70 °C (or 50 °C) at a rate of 0.1; 0.5 and/or 1 °C min^{-1}, in heating and cooling steps. A solvent trap was used to prevent any evaporation during the measurement. The analyses of the results were performed with Rheology Advantage Instrument Control AR V3.0.0 software. The linearity domain of the copolymer solutions was checked by performing a stress sweep procedure, at 1 Hz between 0.01 and 10 Pa at 20 °C.

2.3.6. MicroDSC

Calorimetric measurements were performed with a MicroDSC evoIII (Setaram, France). Standard Hastelloy cells were used, the same quantity of solution (exactly 500 ± 1 mg) being introduced in both reference (milli-Q water) and measurement cells. Thermograms were obtained using heating and cooling cycles (between 5 and 70 °C, at a rate of 0.5 °C min^{-1}). An isothermal step (10 min at 5 °C) was used to equilibrate the system before the heating cycle. Transition enthalpies were calculated by integrating the peaks.

Table 1
Experimental conditions of grafting reaction of M2005 to CMP and experimental grafting rates; molar ratio $n_{EDC}/n_{M2005} = 3$.

Reaction	Samples	n_{EDC}/n_{NHS} [a]	τ_{th}	τ_{exp} [b]	Grafting efficiency (%)
1	CMP-g-M2005-0.13	3	0.28	0.13	46
2	CMP-g-M2005-0.20	26	0.28	0.20	71
3	CMP-g-M2005-0.21	26	0.28	0.21	75

[a] Molar ratios.
[b] Determined by ^{1}H NMR $\tau_{exp} = \frac{I_{CH_3}(c)}{3 \times I_{Hanom}(b)}$.

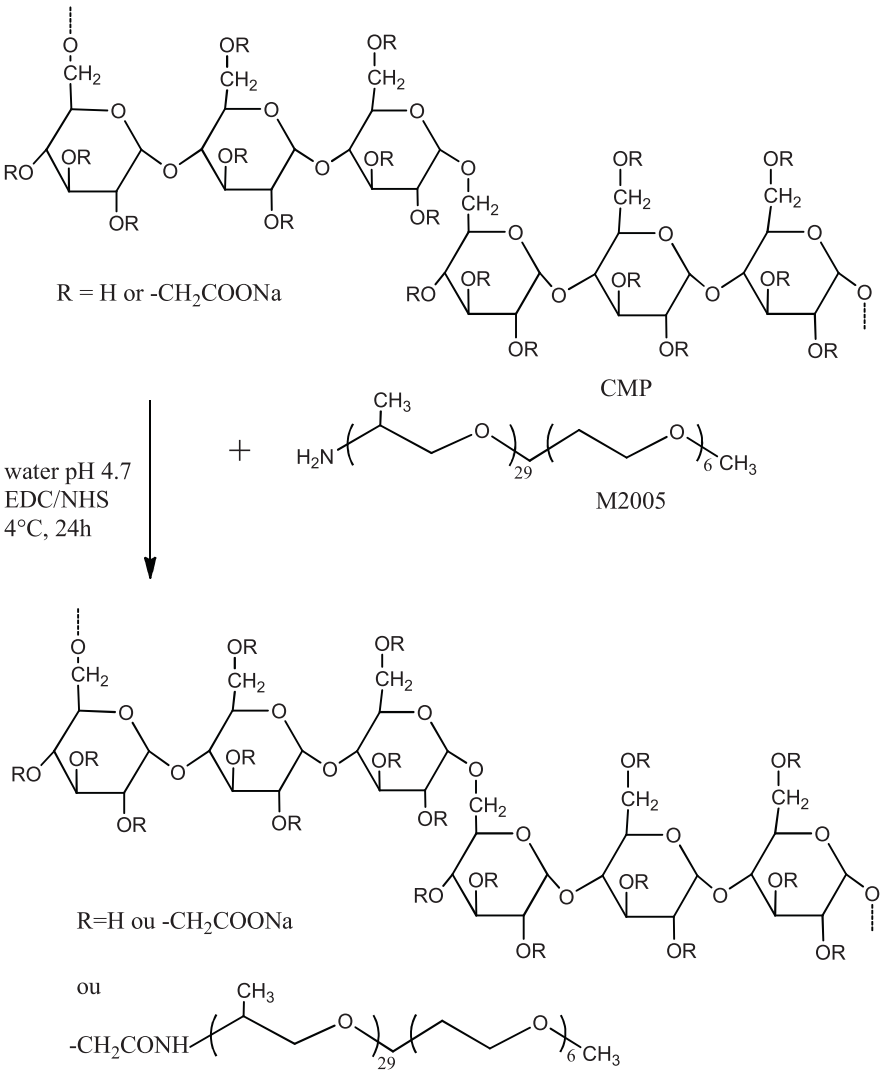
3. Results and discussion

3.1. Synthesis of CMP-g-M2005

The coupling reaction between amino groups and carboxylate functions is often activated with carbodiimide and has been frequently reported in literature (Drotleff et al., 2004). For water-soluble polysaccharides, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride (EDC), a water-soluble carbodiimide, is a good candidate. Many works have reported the chemical modification of polysaccharides using EDC. The reaction of the amino groups with carboxylate functions in the presence of EDC occurs at acidic pH. The acidic pH conditions are necessary to protonate nitrogen atoms of carbodiimide. At pH 4.7, nitrogen atoms of carbodiimide are sufficiently protonated, polysaccharide is under its carboxylate form (RCOONa), in equilibrium with its acidic form (RCOOH), and the amino group is sufficiently nucleophilic. Thus, carbodiimide reacts with carboxylate groups to form an unstable intermediate O-acylurea, which reacts with N-hydroxysuccinimide (NHS) to form a more stable activated ester (Staros, Wright, & Swingle, 1986). Then, the amino group of Jeffamine® reacts on activated ester to form an amide linkage by a nucleophilic attack. In the absence of nucleophiles, a rearrangement reaction of the O-acylurea occurs to give a more stable N-acylurea through an intramolecular acyl transfer (Kuo, Swann, & Prestwich, 1991).

In this work, the synthesis of CMP-g-M2005 (Scheme 1) was performed in water – an economical and non-polluting type of synthesis – at 4 °C in the presence of EDC and NHS as activators and at weak acidic pH (4.7). The temperature was maintained at 4 °C during all the reaction time, to avoid M2005 precipitation. The low reaction temperature implied slow kinetics, which is the reason for the reaction time being of 24 h.

Different experimental conditions were tested by varying the molar ratio of EDC over NHS and at fixed molar ratio of EDC over carboxylate groups (0.8). The theoretical grafting rate (τ_{th}) was fixed at 0.28. This rate is molar one, meaning that, 0.28 carboxylate groups per AGU are grafted. As seen in Table 1, the experimental grafting rates (τ_{exp}, determined by ^{1}H NMR) were always lower than the theoretical ones and depended on the EDC/NHS ratios. For reactions (2) and (3), better yields in grafting rates (71 and 75%) were obtained with lower quantities of NHS, as compared to reaction (1) (46%). Secondly, reproducibility of the reaction was checked with reactions (2) and (3), where the same grafting rate was obtained under the same experimental conditions. So, it appears that the best experimental conditions have been those applied to reactions (2) and (3) (EDC/M2005 molar ratio of 3; EDC/COONa molar ratio of 0.8; EDC/NHS molar ratio of 26), where the efficiency of the grafting



Scheme 1. Grafting reaction of Jeffamine® M2005 to CMP.

reaction has been of 71 and 75%. In the following of the study, the samples of reactions (2) and (3) showed the same physicochemical behaviour and have been denoted CMP-g-M2005-0.20.

Others experimental conditions with different grafting rates (0.14 or 0.41) and different molar ratios of EDC over carboxylate groups (0.4 instead of 0.8) and different molar ratios of EDC over NHS (1 or 3) have been tested. But in these conditions, the grafting efficiency was very low (lower than 40%) and we did not show the results of the corresponding copolymers.

Particular attention was paid to the purification procedure, to ensure the elimination of unreacted Jeffamine®, which could modify the physicochemical properties of the copolymers. For example, M2005 traces could lead to the appearance of a cloud point of the copolymer solutions upon increasing temperature. The chosen purification procedure seemed to be efficient as no cloud point appeared between 15 and 60 °C, when the CMP-g-M2005 copolymers were solubilized in water and then heated. On the other hand, the copolymer solutions underwent a sol–gel transition, depending on the concentration range. The successive dialysis steps against NaOH/water/HCl/water/NaOH/water were necessary to remove unreacted EDC, or more precisely, EDU (N-ethyl-N′-(dimethylaminopropyl)urea – the hydrolyzed form of EDC). Without these purification steps, the NMR spectra of the copolymers showed the presence of EDU (spectra not shown), retained by the electrostatic interactions between carboxylate groups and the quaternary amine group of EDU.

FTIR spectra of the CMP-g-M2005 copolymers (Fig. 1) evidenced the efficiency of the reaction by the presence of the characteristic band of amide (C=O) at 1650 cm^{-1} and the presence

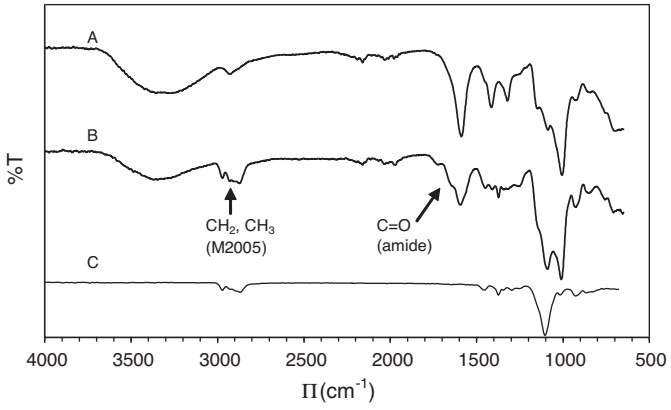


Fig. 1. FTIR spectrum of CMP-g-M2005 copolymer (B) and precursors CMP (A) and M2005 (C).

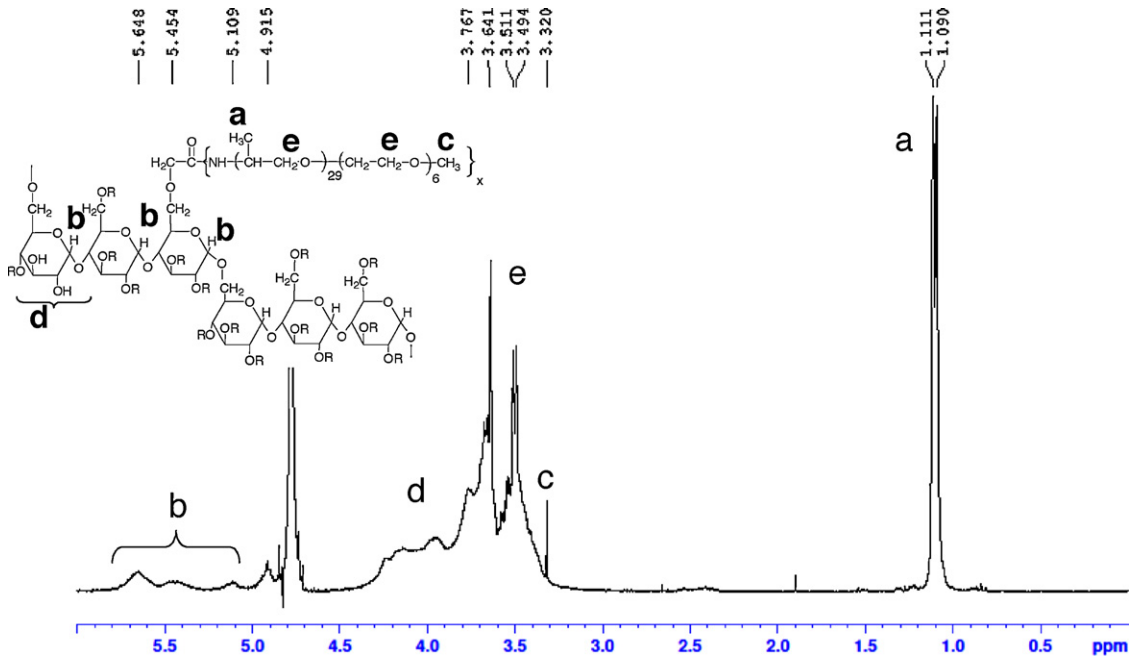


Fig. 2. ^{1}H NMR spectrum of CMP-g-M2005-0.20.

of the characteristic bands of Jeffamine® M2005: at 2875 cm^{-1} and 2970 cm^{-1} (for the stretching vibrations of C–H_2 and C–H_3 groups, respectively), at 1450 cm^{-1} and 1370 cm^{-1} (for the bending vibrations of C–H_2 and C–H_3 groups, respectively), at 1090 cm^{-1} (for the ether C–O–C band of Jeffamine® M2005) and at 1010 cm^{-1} (for the ether C–O–C band of CMP).

^{1}H NMR spectra (Fig. 2) of the CMP-g-M2005 copolymers showed the presence of the characteristic peaks of Jeffamine® M2005 (CH and CH_2 protons of PO and EO units at 3.5–3.7 ppm; methyl protons of PO units at 1.14 ppm; methoxy protons of M2005, CH_3–O, at 3.32 ppm) and the characteristic peaks of CMP (CH and CH_2 protons at 3.5–4.5 ppm; 2 anomeric protons H_{1-4} at 5.5–5.7 ppm and one anomeric proton H_{1-6} at 5.0–5.2 ppm (Glinel, Sauvage, Oulyadi, & Huguet, 2000). The integration of the characteristic peaks of M2005 (methoxy protons at 3.37 ppm) and CMP (anomeric proton at 5.0–5.7 ppm) allowed the calculation of the experimental grafting degrees (τ_{exp}) of M2005 for each sample. The results are given in Table 1.

3.2. Characterization of copolymers

The copolymers have been characterized at 25 °C in 0.1 M $LiNO_3$ at 2.2 g L^{-1} by SEC/MALLS/DRI/Visco. The mass recovery of analyzed samples, regarding to integration peak and injected quantity thanks to the known value of d*n*/d*C*, was about 70%. Molar masses distributions with both Light Scattering (LS) and DRI profiles are reported in Fig. 3 for all the studied samples. The whole results are compiled in Table 2. We can remark a noticeable increase of molar masses when the amount of M2005 increases on the CMP precursor. This is partly due to the increase of the repetition unit molar mass (M_0) with grafting rate. The calculated DPn (i.e. M_n/M_0) does not change between CMP and CMP-g-M2005-0.13 and is just slightly increased for CMP-g-M2005-0.20. This evidences that the main part of the particles remains isolated and that any degradation occurs during the grafting reaction. Nevertheless, we can notice a slight increase of both M_w and I_p when grafting amount increase. Considering that M_w is strongly sensible to the presence of aggregates, we can argue that aggregates, even in very few quantities, are also present in the solution.

Intrinsic viscosity decreases with grafting amount of M2005, indicating a possible densification of the polymer coils due to intramolecular association (Simon et al., 2003) between polyether groups. This is confirmed by chromatograms (Fig. 3), which show that for a same elution volume (and thus, for a same hydrodynamic volume) the copolymers present higher molar masses than the CMP precursor. This associative behaviour leads to a more globular conformation of the polymer coil. This is well confirmed with the decrease of Mark–Houwink exponent (i.e. exponent *a*) and R_g vs *M* exponent (i.e. exponent b) when the amount of M2005 increases. Finally the shape factor ρ (i.e. R_g/R_h) well agrees with an expensed coil for the CMP precursor ($\rho \sim 1.5$). It also agrees with a more compact conformation for copolymers since ρ strongly decreases with associative behaviour toward 1 for the most important amount of M2005. This behaviour has been already evidenced by Liang, Zhou, Song, Zaitsev, & Chu (1999) on thermosensitive copolymers of poly(N-isopropylacrylamide) and poly(ethylene oxide) or PNIPAM–POE. Thus, CMP-g-M2005 copolymers clearly evidence an amphiphilic character at 25 °C for polymer concentration of 2.2 g L^{-1} and in salt media (i.e. $LiNO_3$ 0.1 M). These results seems indicating that the thermal transition may occur in such conditions at molecular scale.

Table 2
Results of SEC/MALS/DRI/Viscometer analysis (at 25 °C, in $LiNO_3$ 0.1 M and at 2.2 g L^{-1} for polymer concentration).

	CMP	CMP-g-M2005-0.13	CMP-g-M2005-0.20
M_n (g mol^{-1})	130 000	270 000	370 000
M_w (g mol^{-1})	230 000	560 000	815 000
DPn calc.	575	570	611
$I_p = M_w/M_n$	1.8	2.0	2.2
R_g (nm)	26	28	34
R_h (nm)[a]	18	23	34
$[\eta]$ mL g^{-1}	192	180	164
K from MH (mL g^{-1})	2.03×10^{-2}	2.34×10^{-2}	5.12×10^{-2}
a from MH	0.74	0.69	0.61
b from R_g vs *M*	0.67	0.52	0.32
$\rho = R_g/R_h$	1.45	1.22	1.0

[a] From viscosity measurements.

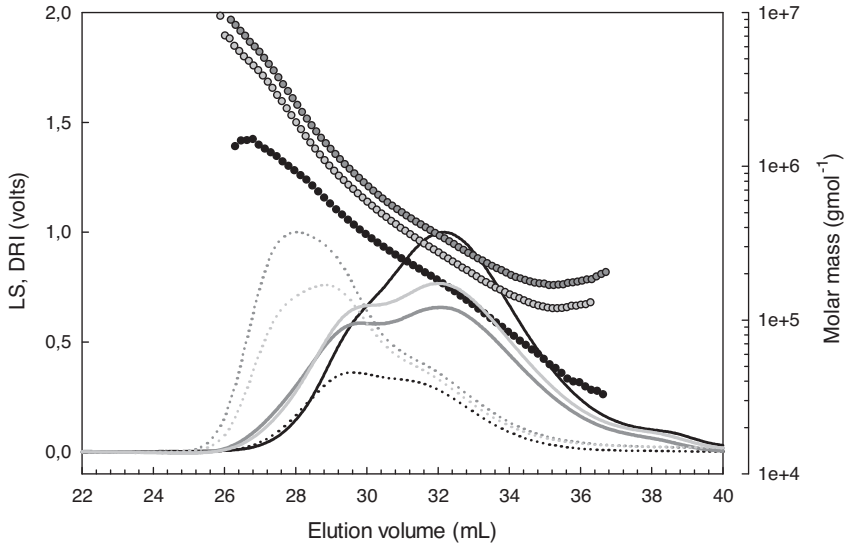


Fig. 3. Molar mass distributions of pullulan derivatives ($LiNO_3$ 0.1M); full lines: DRI; dotted lines: LS; CMP: black, CMP-g-M2005-13: light grey, CMP-g-M2005-20: dark grey.

3.3. Thermosensitive character

3.3.1. Fluorescence spectroscopy

Thermo associative transition (i.e. hydrophobic cluster formation) of copolymers can be followed by pyrene fluorescence spectroscopy. It is known that the change in the I_1/I_3 ratio, corresponding to the intensity of the first (I_1 = 372 nm) and the third (I_3 = 383 nm) vibronic peaks, is sensitive to the polarity of the pyrene micro-environment (Chen, Yu, Cheng, Yu, & Cheung, 2006; Wilhelm et al., 1991). High values of the I_1/I_3 ratio (about 1.8 in water) evidence a polar micro-environment of pyrene, whereas lower values of I_1/I_3 ratio indicate a more apolar micro-environment. Fig. 4 gives the variations of the I_1/I_3 ratio (pyrene at 4×10^{-7} M) for the most grafted copolymer (i.e. CMP-gM2005-0.20) in pure water as a function of temperature in both dilute and semi-dilute regime of concentration, respectively 1 g L^{-1} and 50 g L^{-1}. For dilute domain of copolymer concentration (i.e. isolated molecules), one can notice a clear decrease of I_1/I_3 ratio with increasing temperature indicating that pyrene micro-environment becomes apolar. This can be correlated with a critical aggregation Temperature (CAT) that can be estimated at about 35 °C corresponding to the establishment of hydrophobic associations leading to the elaboration of hydrophobic clusters (Henni-Silhadi et al., 2007). In the semi-dilute domain of copolymer concentration (i.e. 50 g L^{-1}), the transition in I_1/I_3 evolution can be observed at about 35 °C, the same temperature than for dilute regime. Nevertheless the I_1/I_3 ratio of concentrated system is significantly lower than for dilute regime at low temperature (1.3 at 10 °C). This result well indicates that a thermo sensitive behaviour occurs for grafted copolymers. It also evidences that hydrophobic association may also occur even below the critical temperature as seen for the most concentrated system.

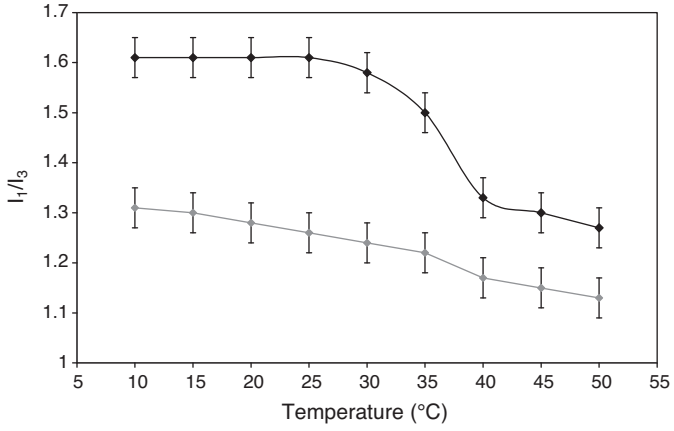


Fig. 4. Intensities ratio (I_1/I_3) of the vibronic bands of pyrene in pure water as a function of temperature of CMP-g-M2005-0.20 at 1 g L^{-1} (♦) and 50 g L^{-1} (◆).

The reversibility of the thermosensitive character has been demonstrated by cooling the CMP-g-M2005-0.20 copolymer solution (50 g L^{-1}) from 50 to 15, and then to 10 °C, the same I_1/I_3 ratios being obtained compared to the initial heating step (at 15 °C, I_1/I_3 = 1.32 and at 10 °C, I_1/I_3 = 1.30).

Finally, let us notice a specific point. By fluorescence spectroscopy, any association has been observed at 1 g L^{-1} in pure water below 30 °C. In the same time, we have clearly demonstrated an association behaviour (mainly with intramolecular interactions) with SEC/MALS/DRI/Visco analysis for copolymers at 2.2 g L^{-1} in $LiNO_3$ 0.1 M and at 25 °C. These results seem to indicate that 25 °C is above CAT for SEC/MALS analysis and below CAT for fluorescence spectroscopy. This difference should be well explained by the presence of an ionic strength (i.e. $LiNO_3$ 0.1 M) in the eluent of SEC/MALLS line. It is well established that the presence of salt (mainly with polyelectrolyte) lead to a noticeable decrease of the critical temperature (Durand, Hourdet, & Lafuma, 2000; Fettaka et al., 2011).

3.3.2. Rheological study

The rheological measurements have been performed in semi-dilute regime using standard-size double concentric cylinder geometry regarding to the low viscosity of copolymers solutions at a low temperature. Temperature heating ramps in oscillation experiments have been carried out to study the viscoelastic properties of the copolymers (elastic or storage modulus: G', viscous or loss modulus: G'' and complex viscosity: η^*) in the linearity domain and to determine the sol–gel transition temperature. Typical rheological behaviour of copolymer (here CMP-g-M2005-0.20) at 5 wt% in pure water is shown in Fig. 5. At low temperature (i.e. below the transition), the behaviour is clearly viscous with G'' largely higher than G'. When temperature reaches 25 °C, one can notice a strong enhancement of both G' and G'' leading to a cross point of moduli for about 30 °C, then both moduli reach a

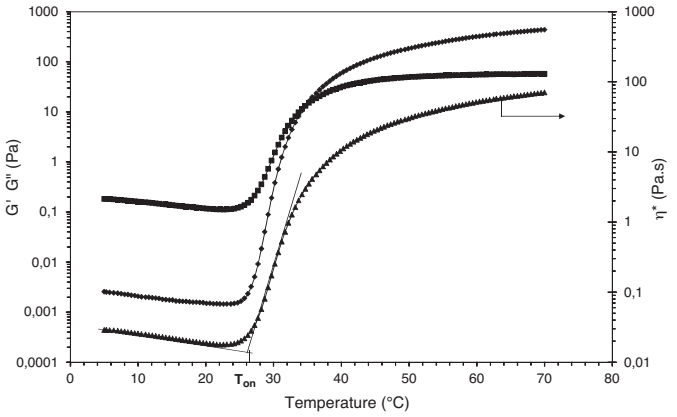


Fig. 5. Rheological profile of CMP-g-M2005-0.20 at 5 wt% in water. Heating ramp: G' (♦), G'' (■) and η^* (▲) vs temperature; oscillation procedure (shear stress 1 Pa, frequency 1 Hz, rate 1 °C min^{-1}).

plateau where G' is largely higher than G'' evidencing a clearly elastic state. During this transition the elastic modulus has taken more than 5 decades. In the same time the complex viscosity is also considerably increased and the onset temperature can be determined as shown in Fig. 5. The synthesized copolymers exhibit an interesting thermo thickening behaviour in the semi-dilute regime of concentration.

Due to their LCST property, the polyether grafted groups self-associate for a critical temperature. Such associations in semi-dilute range of concentration are mainly intermolecular and give rise to a structured liquid. The dynamic of entanglements seems noticeably slowed. We have plot in Fig. 6, the mechanical spectra (i.e. rheological parameters G', G'' and η^* as a function of frequency) for CMP-g-M2005-0.20 below and above the critical temperature respectively at 15 °C and 40 °C. Below the critical association temperature, i.e. 15 °C, the behaviour is typically viscous with G'' largely higher than G' on all the frequency range. Any cross point of the moduli is visible that means that the dynamic of the system is really rapid. Logically, the complex viscosity evidences a Newtonian profile. In this range of temperature, it is clear that any association occur. Consequently, the rheological profile of the copolymer is similar to that of the CMP precursor below the aggregation temperature (results not shown to not surcharge the figure). Above the critical temperature, i.e. 40 °C, the behaviour appears clearly elastic with G' higher than G'' and independent of the frequency. In the same time the complex viscosity exhibits a typical shear-thinning

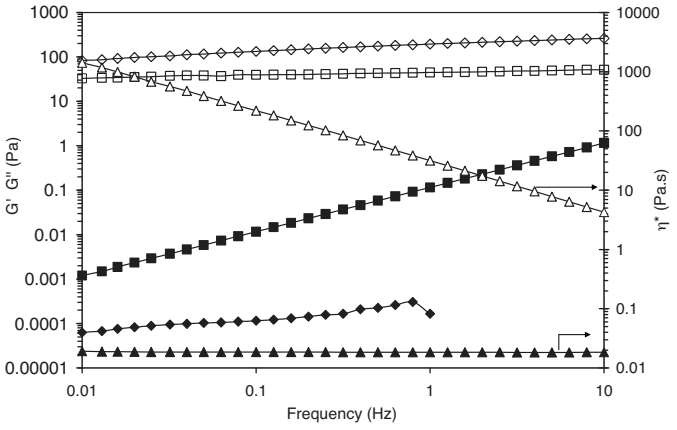


Fig. 6. Frequency dependence of viscoelastic parameters (♦ G', ■ G'', ▲ η^*) of CMP-g-M2005-0.20 in citrate/phosphate buffer 0.1 M pH 5.4 (C = 5 wt%) at 15 °C (full symbol) and 40 °C (empty symbol).

behaviour when elastic character prevails. This result indicates a strongly structured liquid state, sometimes called physically associated hydrogel or pseudo-gel for which the dynamic is quite stopped in the time range of studied frequencies due to intermolecular associations between polyethers grafted groups.

By rheology, the sol–gel transitions have been followed as a function of the amount of grafted polyether (CMP-g-M2005-0.13 and CMP-g-M2005-0.20), of the polymer concentration (between 2.5 and 10 wt%) and of nature of aqueous media. Concerning this last point, 3 different media have been studied: pure water (pH around 4.7–4.8 according to the grafted amount of M2005), citrate/phosphate buffer (0.1 M, pH 5.4) expected to simulate the vaginal pH conditions and finally saline phosphate buffer (0.15 M, pH 7.4) to simulate the blood fluid conditions. The onset temperature (T_{on}, see Fig. 5) corresponds to the beginning of thermoassociations among M2005 groups and T_{gel} (determined at the cross point of G' and G'' (Petit, Bouteiller, Brûlet, Lafuma, & Hourdet, 2007; Winter & Mours, 1997) corresponds to the percolation threshold when the physical network begins to form. Noticeable elastic moduli (G') have been taken at T_{gel} (G_{gel}') and at 50 °C (i.e. on the plateau, noted G_{pl}'). All data are reported in Table 3.

In pure water, the more grafted copolymer (i.e. CMP-g-M2005-0.20) exhibits lower sol–gel transition temperatures and higher elastic modulus (both at cross point and at the plateau) than the lower grafted sample (i.e. CMP-g-M2005-0.13). This result is not surprising as the number of potential associations is expected to be higher with higher amount of grafted polyether groups. The effect of polymer concentration has been followed on the CMP-g-M2005-0.20 in pure water (3, 4 and 5 wt%). The pH does not change with the polymer concentration. We can notice only a slight decrease of the transition temperature when polymer concentration increases. In the same time, we can observe a noticeable increase of G'plateau (45 Pa at 3 wt% and 183 Pa at 5 wt%). This result was expected as it fully agrees with results found with classical associatives polymers (Simon et al., 2003). For a same sample and at a same polymer concentration, it appears that the association tendency is sensitively improved (lower transition temperature and higher G') in buffer pH 5.4 compared to pure water (pH 4.8). Even if the pH difference between these two media is not so large, the amount of ionized acidic groups is larger in buffer pH 5.4 than in pure water pH 4.8. Thus, more electrostatic repulsions are expected to occur at pH 5.4 that should diminish the association tendency. We do not observe this effect and moreover our result indicate higher association trend for the higher pH. This phenomenon should be explained by the ionic strength (even low, ionic strength of citrate/phosphate buffer is 0.1 M) brought by the buffer that can lead to a screening effect limiting electrostatic repulsions and by the way, favouring the association's process. This behaviour has been reported previously for similar systems based on polysaccharides (Karakasyan et al., 2008). Besides, the addition of salt is also known to decrease the LCST of thermosensitive polymers due to a salting-out effect (Geever, Nugent, & Higginbotham, 2007; Lee, Song, Jin, & Sohn, 1999; Liu, Wang, Wang, Huang, & He, 2004). The salting-out effect is a combination of several effects, i.e. changes in the water structure in the polymer hydration shell and changes of interactions between polymer and solvent. All of that is fully confirmed when we observe the results in buffer pH 7.4 (ionic strength 0.15 M) for which the transition temperature is largely decreased and elastic modulus extremely increased. Let us notice that in such conditions, the association behaviour is so strong that some heterogeneity appears in the solution leading to difficulties in the rheological measurement. We observed a big difference in elastic moduli of copolymer solutions prepared in buffer pH 5.4 and in buffer pH 7.4 even though the difference in ionic strength was not so high (0.1 M at pH 5.4 and 0.15 M at pH 7.4). We explained this difference by the hypothesis

Table 3
SOL/GEL transition temperature and elastic modulus G' at T_{gel} during the heating step of CMP-g-M2005 copolymers in water, in citrate/phosphate buffer 0.1 M at pH 5.4, or in saline phosphate buffer 0.15 M at pH 7.4. 1 °C min^{-1}.

Samples	Solvent	C wt%	T_{on} (°C)	T_{gel} [a] (°C)	G_{gel}' at T_{gel} (Pa)	G_{pl}' at 50 °C (Pa)
CMP-g-M2005-0.13	Water (pH 4.8)	5	31.3	46.3	8	14
	Buffer pH 5.4	2.5	26.2	49.4	1	1
	Buffer pH 5.4	5	21.0	33.7	9	69
	Buffer pH 5.4	10	16.7	27.3	40	592
CMP-g-M2005-0.20	Water (pH 4.75)	3	27.4	37.3	4	45
	Water (pH 4.75)	4	26.7	36.7	7	78
	Water (pH 4.75)	5	25.7	34.7	13	183
	Buffer pH 5.4	5	20.3	27.8	17	196
	Buffer pH 5.4	10	17.6	25.3	43	1180
	Buffer pH 7.4 (0.1 M)	5	20.3	25.9	6700	>20 000

that the charges are not completely screened at an ionic strength of 0.1 M and the screening is complete at 0.15 M, strengthening the hydrophobic interactions.

To study the reversibility of thermo thickening behaviour, we have conducted rheological measurements (G', G'' and η^*) on the CMP-g-M2005-0.20 as a function of temperature with a heating ramp followed with a cooling ramp at different rate. Three rates have been studied: 0.1, 0.5 and 1 °C min^{-1}. The fastest and slower rate behaviours are plotted in Fig. 7 while the whole results are compiled in Table 4 (T_{gel}, G' at crossing point, ΔT and $\Delta G'$ between heating and cooling ramps).

At 1 °C min^{-1}, a hysteresis in G' profile is observed between heating and cooling step and a difference of about 4 °C in T_{gel} is obtained between both steps. However, no significant differences were observed for G' values (Table 4) demonstrating that the heating and cooling rates only influence the kinetics of association. On the opposite, at 0.1 °C min^{-1} (inset in Fig. 7), a very slight hysteresis is observed in viscoelastic profiles and approximately the same T_{gel} and G' are obtained. For intermediate heating/cooling rate (0.5 °C min^{-1}), the obtained results are logically between those of 1 °C min^{-1} and 0.1 °C min^{-1}. Such inertia in thermo controlled association/disassociation behaviour has been also viewed on thermo sensitive cellulosic derivatives (Fettaka et al., 2011).

3.3.3. MicroDSC

In Fig. 8, we have reported both thermograms (μDSC) and rheological measurements at 0.5 °C min^{-1} for CMP-g-M2005-0.20 (5 wt% in buffer 0.1 M pH 5.4). An endothermic peak is clearly evidenced during the heating ramp for which the onset temperature is not far than that of the rheological transition. It also appears that T_{max} from μDSC and T_{gel} from rheology are not exactly similar. This can evidence that the determination of the transition temperature may depend on the used physicochemical method (i.e. the scale of observed phenomenon, as discuss in a previous study (Fettaka et al., 2011). Confirming the effect of heating/cooling rate see before, we can observe a time lag of T_{max} from μDSC between heating and cooling steps. Thus both methods, i.e. rheological and calorimetric measurements, are appropriate for studying the thermosensitive character of CMP-g-M2005 copolymers.

The transition enthalpy, ΔH, can be calculated by integrating heat flow signal vs temperature. Some authors have demonstrated that the ΔH depends only on poly(propylene oxide) content and not on the poly(ethylene oxide) content (Mitchard et al., 1992; Armstrong et al., 1995). For this reason, we have calculated ΔH in Joule per gram but also in Joule per mole of propylene oxide (ΔH kJ mol^{-1} PO) for our samples, taking into account the grafting degree in M2005 of each sample and assuming that M2005 contains 29 poly(propylene oxide) units. We have also reported the values concerning the Jeffamine® M2005 alone in water (pH 11, indicating its uncharged form). All the results are reported in Table 5.

For Jeffamine® M2005 solution at 2 wt% in water, the onset temperature corresponds to the beginning of cloud point (determined by visible spectroscopy, not shown here) formation at 17.7 °C. Then the phase transition continues and the maximum of the endotherm occurs at T_{max} = 23.8 °C. The offset temperature, corresponding to the end of the transition, when two phases are present in the solution, can also be determined, in this case T_{off} = 31.5 °C. Thus, the transition phenomenon occurred over a relatively wide temperature range (about 14 °C). The reversibility of the transition was total, because close values of transition enthalpies were obtained

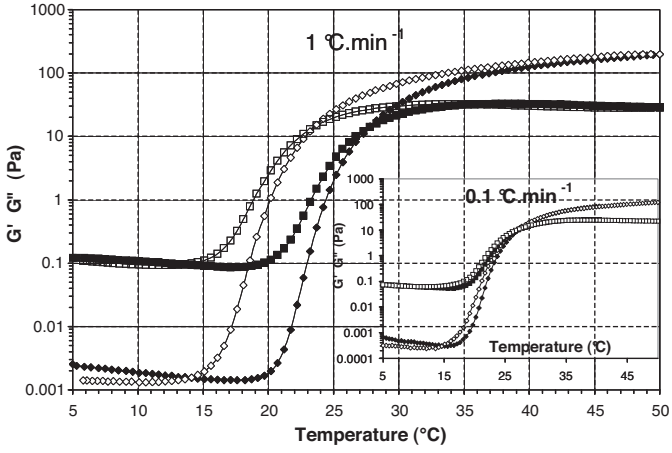


Fig. 7. Viscoelastic profile of CMP-g-M2005-0.20 (5 wt% in water): influence of heating/cooling rate on the SOL/GEL transition temperature; G′ heating step (♦); G'' heating step (■); G′′ cooling step (◊); G'' cooling step (□).

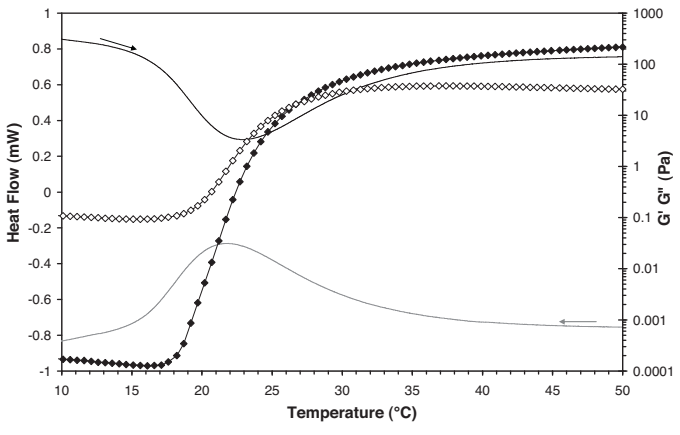


Fig. 8. Thermogram at 0.5 °C min^{-1} (heating step: black line; cooling step grey line), G' (black diamond, ♦) and G'' (white diamond, ◊) vs temperature (heating rate 0.5 °C min^{-1}) of CMP-g-M2005-0.20 (5 wt% in citrate/phosphate buffer 0.1 M at pH 5.4).

Table 4
Effect of heating/cooling rate on the SOL/GEL transition temperature and on G' for samples CMP-g-M2005-0.20 at 5 wt% in citrate/phosphate buffer 0.1 M at pH 5.4.

Rate	0.1 °C min^{-1}			0.5 °C min^{-1}			1 °C min^{-1}		
	Heating[a]	Cooling[b]	ΔT, $\Delta G'$	Heating[a]	Cooling[b]	ΔT, $\Delta G'$	Heating[a]	Cooling[b]	ΔT, $\Delta G'$
T_{gel}	27.3	26.8	0.5	28.1	25.6	2.3	27.7	23.6	4.1
G'	10.3	10.0	0.3	17.1	17.3	0.2	13.8	14.4	0.3

[a] Sol/gel transition temperature (°C) and elastic modulus G' (Pa) at T_{gel} during heating step.
[b] Gel/sol transition temperature (°C) and elastic modulus G' (Pa) at T_{gel} during cooling step.

Table 5
Enthalpy and transition temperature of M2005 and CMP-g-M2005 in pure water or in citrate/phosphate buffer 0.1 M pH 5.4 or saline phosphate buffer 0.15 M pH 7.4 for CMP-g-M2005-0.20. Heating rate 0.5 °C min^{-1}.

Sample	Solvent	C wt%	T_{max} (°C)	T_{on} (°C)	ΔH (J/g)	ΔH (kJ mol^{-1} PO)
M2005 (pH 11)	Water	2	23.8	17.7	2.741	9.3
CMP-g-M2005-0.20	Water	5	29.0	21.9	0.976	2.0
CMP-g-M2005-0.20	Buffer 5.4	5	23.0	16.6	1.485	3.0
CMP-g-M2005-0.20	Buffer 7.4	5	25.2	18.9	1.286	2.6

during the heating step (ΔH = 9.3 kJ mol^{-1} of PO) and the cooling step (ΔH = 9.4 kJ mol^{-1} of PO). But a slight difference in T_{max} (1.3 °C) was observed between the heating and the cooling steps. Our enthalpy value (around 9 kJ mol^{-1} of PO) is comparable with ΔH of pure PPO reported in literature (about 8 kJ mol^{-1} of PO) for poly(propylene oxide) of low molecular weight (<4000 g mol^{-1}).

For aqueous solutions of CMP-g-M2005-0.2 copolymers, we have reported the μDSC data in Table 5 for different media (i.e. the same as studied for rheology). As a confirmation of rheological results, it appears that the transition (T_{max}) is shifted toward lower temperatures as the ionic strength increases (pure water < buffer 5.4 < buffer 7.4). ΔH reduced to PO groups varies between 2 and 3 kJ mol^{-1} PO without any clear tendency regarding the different aqueous media. Considering the accuracy of the result we can consider that the whole enthalpy does not depend on the considered media. Nevertheless, it appears that ΔH is largely lower for copolymers than for pure Jeffamine® M2005. This difference may indicate that the whole PO groups of the copolymers are not totally engaged in the transition; some of them seem to be free and does not participate to the sol–gel transition.

4. Conclusion

Thermosensitive polysaccharides based on pullulan and Jeffamine® M2005 have been synthesized in water using carbodiimide chemistry. Different grafting degrees ($0.13 < \tau < 0.21$) have been obtained by varying the initial quantity of M2005 introduced in the reaction mixture, the highest efficiency of the grafting reaction being of about 75%. FTIR- and NMR-spectroscopy of copolymers showed the efficiency of the reaction. The determination of the absolute average molecular weights of CMP-g-M2005 copolymers at 25 °C by coupling on-line a size-exclusion chromatograph (SEC), a multi-angle light scattering system (MALS), a viscometer and a differential refractive index detector (DRI) clearly evidenced an amphiphilic character at 25 °C for polymer concentration of 2.2 g L^{-1} and in salt media (i.e. $LiNO_3$ 0.1 M). Their structures are more compact than that of the CMP precursor, with mainly intramolecular interactions due to the Jeffamine® grafted groups

The thermosensitive behaviour of CMP-g-M2005 copolymers has been studied by different techniques. We showed that the sol–gel transition temperature can be adjusted by varying the grafting degree of M2005, the concentration and the solvent. Indeed, we have demonstrated by rheological measurements that the sol–gel transition temperature, assimilated to T_{gel} (when $G' = G''$), varies between 25 and 49 °C, depending on the grafting degree, the solvent or the concentration of the copolymer solutions. The sol–gel transition leads to a sharp increase of dynamic moduli (a 5-decade increase in G') and viscosity. We have demonstrated by different methods (rheology and microDSC) that the presence of salts leads to a high decrease in the transition temperature by a salting-out effect and a screening effect of the carboxylate groups promoting the interactions between Jeffamine® grafted groups. The calorimetric results showed that only a part of the Jeffamine® groups are involved in the sol–gel transition.

The hydrogels obtained have hydrophobic clusters and present interesting viscoelastic properties, which makes possible their applications as vaginal drug delivery systems.

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