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Hyaluronic acid and alginate covalent nanogels by template cross-linking in polyion complex micelle nanoreactors

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ABSTRACT

Hyaluronic acid (HA) and alginate (AL) covalent nanogels cross-linked with L-lysine ethyl ester were prepared by template chemical cross-linking of the polysaccharide in polyion complex micelle (PIC) nanoreactors. By using this method we were able to prepare HA and AL nanogels without organic solvents. PICs were prepared by using poly(ethylene oxide)-block-poly[(3-acrylamidopropyl)-trimethylammonium chloride] (PEO-*b*-PAMPTMA) or poly[(*N*-isopropylacrylamide)-block-PAMPTMA] (PNIPAAM-*b*-PAMPTMA). Only PNIPAAM-*b*-PAMPTMA block copolymers allowed to prepare PIC with small and controlled size. Short polysaccharide chains (X_n = 50 and 63 for AL and HA, respectively, where X_n is the number of monosaccharidic units present in the polysaccharide) where used to optimize PIC formation. The remarkable difference in charge density and rigidity of HA and AL did not have a significant influence on the formation of PICs. PICs with small size (diameter of about 50–80 nm) and low polydispersity were obtained up to 5 mg/mL of polymer. After cross-linking with L-lysine ethyl ester, the nanoreactors were dissociated by adding NaCl. The nanogels were easily purified and isolated by dialysis. The dissociation of the nanoreactors and the formation of the nanogels were confirmed by ^{1}H NMR, DLS, TEM and ζ-potential measurements. The size of the smallest nanogels in solution in the swollen state was 50–70 nm in presence of salt and 80–100 nm in water.

1. Introduction

Nanogels are hydrophilic three dimensional polymer networks which vary in size from a few nanometers to 1000 nm having interesting features such as the deployment in areas of the body not easily accessible after intravenous injection, intracellular drug delivery and large surface area that allow easy multivalent conjugations and very rapidly response to environmental stimuli. (Jayakumar, Nair, Rejinold, Maya, & Nair, 2012; Motornov, Roiter, Tokarev, & Minko, 2010; Nayak & Lyon, 2005; Oh, Drumright, Siegwart, & Matyjaszewski, 2008; Peppas, Hilt, Khademhosseini, & Langer, 2006; Seo, Lee, Jung, & Na, 2012).

Rigorous control over the size of these nanocolloids is essential for their *in vivo* biodistribution to reach target tissues and organs but also to evade the reticuloendothelial system (RES) and to be cleared from the bloodstream by renal filtration, reducing risk of accumulation (Gao, Xu, Philbert, & Kopelman, 2007).

Chemical cross-linked nanogels are usually obtained by inverse emulsion polymerization (Landfester, 2006; Moya-Ortega, Alvarez-Lorenzo, Sigurdsson, Concheiro & Loftsson, 2012) and precipitation polymerization (Pelton, 2000) and recently also by liposomal template (Hong, Vreeland, dePaoli Lacerda, Locascio, Gaitan, & Raghavan, 2008; Kazakov, Kaholek, Teraoka, & Levon, 2002; Van Thienen, Raemdonck, Demeester, & de Smedt, 2007) or inverse mini-emulsion (Oh, Tang, Gao, Tsarevsky, & Matyjaszewski, 2006a) controlled radical polymerization (CRP).

Among negatively charged polysaccharides, hyaluronic acid (HA) and alginate (AL) are the most interesting in the field of biomedical applications.

HA is a naturally occurring polysaccharide present in the extracellular matrix and in synovial fluids. Owing to its biocompatibility and biodegradability, since HA can specifically bind to various cancer cells that over-express CD44, it has been extensively investigated for biomedical applications such as tissue engineering, drug delivery and molecular imaging (Lee, Mok, Lee, Oh, & Park, 2007; Ossipov, 2010; Xu, Jha, Harrington, Farach-Carson, & Jia, 2012; Yang, Kootala, Hilborn, & Ossipov, 2011).

AL is an anionic biopolymer consisting of linear chains of α-L-glucuronic acid and β-D-mannuronic acid with properties such as a high degree of aqueous solubility, a tendency for gelation in proper condition with high porosity of the resulting gels, biocompatibility, and non-toxicity (Draget & Skjak-Braek, 2011; Guo,

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Zhang, Jiang, Cao, Ding, & Jiang, 2007; Hamidi, Azadi, & Rafiei, 2008; Lee & Mooney, 2012; Park & Lee, 2011).

The preparation of HA and AL nanogels usually gives rise to the formation of particles with size larger than 100 nm (Hong et al., 2008; Lee et al., 2007; Yang et al., 2011; Zhao et al., 2011). Aiming to the preparation of covalently cross-linked nanogels with size below 100 nm, in this paper we report the use of polyion complex micelles (PICs) as nanoreactors for template cross-linking of anionic polysaccharides. PICs are formed by self-assembly of oppositely charged polyelectrolyte homopolymers or block copolymers (Cohen Stuart, Besseling, & Fokkink, 1998; Kabanov, Bronich, Kabanov, Yu, & Eisenberg, 1996; Kataoka, Togawa, Harada, Yasugi, Matsumoto, & Katayose, 1996; Maggi, Ciccarelli, Diociaiuti, Casciardi, & Masci, 2011; Van der Burgh, de Keizer, & Cohen Stuart, 2004; Voets, de Keizer, & Cohen Stuart, 2009). Better control of the size and polydispersity of PICs is usually obtained using double hydrophilic block copolymers consisting of neutral and ionogenic blocks, in which the neutral block will form the shell of PIC micelles.

In this paper we will report the preparation of nanogels of HA and AL by using PIC micelles as nanoreactors. From the micellar point of view, it is interesting to compare the behavior of HA and AL because AL has a charge density which is twice that of HA. PIC micelles with a coacervate core containing the polysaccharide have been prepared by using block copolymers with opposite charge made with poly(ethylene oxide)-*block*-poly[(3-acrylamidopropyl)-trimethylammonium chloride] (PEO-*b*-PAMPTMA) and poly(*N*-isopropylacrylamide)-*block*-poly[(3-acrylamidopropyl)-trimethylammonium chloride] (PNIPAAM-*b*-PAMPTMA). This method allows the preparation of small polysaccharide nanogels without using organic solvents. Formation of PIC micelles was studied as a function of polyelectrolyte chain length and ionic strength and PIC micelles were used as nanoreactors for cross-linking of HA and AL with L-lysine ethyl ester in the presence of a water soluble carbodiimide. Finally, in order to obtain the free nanogels, nanoreactors were dissociated by increasing the ionic strength.

2. Materials and methods

2.1. Materials

Alginate was a commercial sample provided by Fluka with a fraction of guluronic acid $F_G = 0.40$, average molecular weights $M_w = 3.2 \times 10^5$, $M_n = 1.8 \times 10^5$, $M_\eta = 2.5 \times 10^5$ and polidispersity $I = 1.8$. Hyaluronic acid sodium salt, $M_w = 2.7 \times 10^5$, $M_n = 1.4 \times 10^5$, $M_\eta = 2.0 \times 10^5$ and polidispersity $I = 1.9$ was kindly provided by Fidia Advanced Biopolymers (FAB) srl Abano Terme, Padua, Italy. respectively. Tris(2-dimethylaminoethyl)amine (Me_6TREN) was synthesized as previously described (Xia, Gaynor, & Matyjaszewski, 1998). Poly(ethylene oxide) methyl ether with number average molecular weight 5000 (PEO114-OH, $M_w/M_n = 1.03$) from Aldrich was used as received without purification. CuCl from Fluka was washed with acetic acid followed by methanol to remove impurities. $CuCl_2$ from Fluka was used as received. (3-Acrylamidopropyl)-trimethylammonium chloride (AMPTMA, 75% w/v in water, $d = 1.12$ g/mL) from Aldrich was used as received. *N*-isopropylacrylamide (NIPAAM, Aldrich) was recrystallized from hexane and dried under vacuum prior to use. Alginate (Fluka, 68% of guluronic acid) and hyaluronic acid (Sigma–Aldrich) were used as received. L-Lysine ethyl ester (LYS), ethyl 2-chloropropionate (ECP, Aldrich) and all other reagents were used as received.

2.1.1. Polysaccharides hydrolysis

HA and AL molecular weight was reduced by acid hydrolysis with HCl. To a 5% solution of the polysaccharide, 37% (w/w) HCl was added to obtain a concentration of HCl of 0.1 M for HA and up to pH 3.5 for AL. The lower concentration of HCl in the hydrolysis of alginate was necessary to avoid alginate precipitation. The solutions were then kept to 80 °C for the time necessary to obtain the target molecular weight, as determined by gel permeation chromatography (GPC). When the target molecular weight was reached, the solution was neutralized with 1 M NaOH. The polysaccharide was finally dialyzed with distilled water and isolated by lyophilization. An alginate sodium salt sample with $M_n = 0.99 \times 10^4$, $M_w = 1.65 \times 10^4$, number average degree of polymerization ($X_n = 50$) was obtained while HA had $M_n = 1.32 \times 10^4$, $M_w = 1.91 \times 10^4$, $X_n = 63$. The samples will be named AL_{50} and HA_{63}, respectively, were the suffixes represent the number of monosaccharide residues in the polysaccharide.

2.1.2. Preparation of PEO-b-PAMPTMA and PNIPAAM-b-PAMPTMA

The block copolymers were prepared by ATRP as already described (De Santis, Ladogana, Diociaiuti, & Masci, 2010; Patrizi, Diociaiuti, Capitani, & Masci, 2009). The following block copolymers were prepared: $PNIPAAM_{110}$-*b*-$PAMPTMA_{60}$, $PNIPAAM_{100}$-*b*-$PAMPTMA_{30}$ and PEO_{114}-*b*-$PAMPTMA_{50}$, where the numbers represent the X_n of the polymer segment. Polydispersity of the block copolymers were below 1.3.

2.1.3. Preparation of polyion complex micelles (PIC)

To prepare 1 mg/mL PIC solution of PEO_{114}-*b*-$PAMPTMA_{50}$ or $PNIPAAM_x$-*b*-$PAMPTMA_y$ with alginate or hyaluronic acid, the polysaccharide and the block copolymer were separately dissolved in distilled water (1 mg/mL). As an example, 20 mg of $PNIPAAM_{110}$-*b*-$PAMPTMA_{60}$ (0.0483 mmol of AMPTMA residues) were dissolved in 20 ml of distilled water and 9.56 mg of AL_{50} (0.0483 mmol of anionic monosaccharide residues) were dissolved in 9.56 ml of distilled water. The solutions were left to stand overnight at room temperature to achieve complete dissolution. PIC micelles were obtained by quickly mixing the solutions at room temperature with vigorous stirring. The mixture was left to equilibrate until a stable light scattered intensity was obtained. The mixing fraction $f_+ = n_+/(n_+ + n_-)$, with n_+ and n_- being the moles of positive and negative charges in solution, respectively, was fixed to 0.5, in order to obtain complete charge neutralization. PICs will be named $N_yA_xAL_n$, $N_yA_xHA_m$, $E_{114}A_{50}AL_n$ or $E_{114}A_{50}HA_m$, were *N* is PNIPAAM, *E* is PEO and the suffixes represent the value of X_n of the polymer chain.

2.1.4. Dissociation of the PIC micelles by addition of NaCl

PIC micelles were dissociated by adding a 2 M NaCl solution. The solutions were allowed to stand for at least 10 min before measurements, obtaining a stable reading. PICs dissociation as a function of NaCl concentration was followed by DLS and by ^{1}H NMR.

2.1.5. Cross-linking of PIC micelles

L-Lysine ethyl ester dihydrochloride (LYS) was used as a cross-linker in presence of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide(NHS) as catalyst (Tomihata & Ikada, 1997). As an example, to the solution of $N_{110}A_{60}AL_{50}$ PIC prepared as described before, we added 3 ml of a solution containing 23.88 mg (0.0966 mmol) of LYS, 9.25 mg (0.0483 mmol) of EDC and 5.56 mg (0.0483 mmol) of NHS in order to obtain a molar ratio [LYS]:[EDC]:[NHS]:[COOH] = 2:1:1:1. The pH was adjusted at about 6.0 by adding HCl and the cross-linking was carried out at 25 °C under gentle stirring in a shaking water bath for 4 days maintaining the pH at 6.0 by adding an HCl solution.

2.1.6. Dissociation of the cross-linked nanoreactors by addition of NaCl

The nanogels obtained after cross-linking but still inside the nanoreactors were released by adding a concentrated solution (2 M) of NaCl. Measurements were performed 10 min after each addition of NaCl. This time was adequate to obtain a stable measure. After addition of NaCl, the solution containing the nanogels and free the PNIPAAM-*b*-PAMPTMA chains was dialyzed against 0.4 M NaCl and then against distilled water in dialysis tube with 50.000 Dalton cut-off (Float-A-Lyzer G2, Spectrum). The residual solution in the dialysis tube was lyophilized. The lyophilized material was dissolved in D_2O for ^1H NMR and in distilled water for DLS.

2.2. Methods

2.2.1. GPC

Molecular weight distributions were obtained using a GPC system equipped with a LabFlow 4000 HPLC pump, TSK-GEL α-3000 (30 cm × 7.8 mm ID, 7 μm) column and a Shimadzu RID-10A refractive index detector (Shimadzu, Kyoto, Japan) thermostated at 30 ± 0.1 °C. The columns were thermostated at 25 ± 0.2 °C. Lactose (1 mg/mL) was used as volume marker. The concentration of the polymeric solutions was 1 mg/mL. The injection volume was 50 μL. Before the injection, all the samples were filtered with 0.45 μm nylon filters (Millipore).

Alginate and hyaluronic acid were analyzed by eluting with NaCl 0.2 M at a flow rate of 0.8 mL/min. Monodisperse pullulan standards were used for calibration. Molecular weights were obtained using the universal calibration method.

2.2.2. ^1H NMR

^1H NMR spectra were recorded at 298 K on a Varian XL-300 spectrometer operating at 300 MHz. ^1H NMR spectra of PIC micelles and nanogels were obtained preparing 5 mg/mL solutions in D_2O. Dissociation of PICs and nanoreactors was carried out by adding a 2 M NaCl solution prepared in D_2O.

2.2.3. Dynamic light scattering (DLS)

DLS data were obtained with a Brookhaven Instruments Corp. BI-200SM goniometer equipped with a BI-9000AT digital correlator using a solid-state laser (125 mW, λ = 532 nm). If not otherwise stated, measurements of scattered light were made at a scattering angle θ of 90°. The temperature of the copolymer solution was controlled with accuracy of 0.1 °C. All samples were prepared by filtering PIC solutions with a 0.45 μm Millipore filter (Durapore) into a clean scintillation vial. Experiments were carried out at 25 °C. The solution was allowed to equilibrate until stable scattered intensity was obtained. Experiment duration was in the range of 5–20 min. Cumulant analysis or CONTIN were used to fit the data to obtain the *z*-average hydrodynamic diameter (D_h). The data reported were calculated as the average of three different measures carried out preparing three different PICs and nanogels batches for each sample. The uncertainty reported in the tables was calculated as standard deviation of these three measurements.

2.2.4. Energy filtered-transmission electron microscopy (EF-TEM)

The samples were observed in a Zeiss EM902 Transmission Electron Microscope (TEM), operating at 80 keV and equipped with an "in-column" electron energy filter. The filter was settled to collect only elastic electrons ($\Delta E = 0$), with the result to enhance image contrast and resolution due to the elimination of the inelastic electrons in the image formation (reduction of the chromatic aberration). Briefly, a droplet of nanogel solution (1 mg/mL) was deposited onto 400 mesh copper grid covered with a very thin (about 20 nm) amorphous carbon film. The excess of liquid was removed by placing the grid onto a piece of filter paper. Finally, the grid was dried at room temperature. Samples were observed staining with 2% w/v phosphotungstic acid (PTA) buffered at pH 7.3.

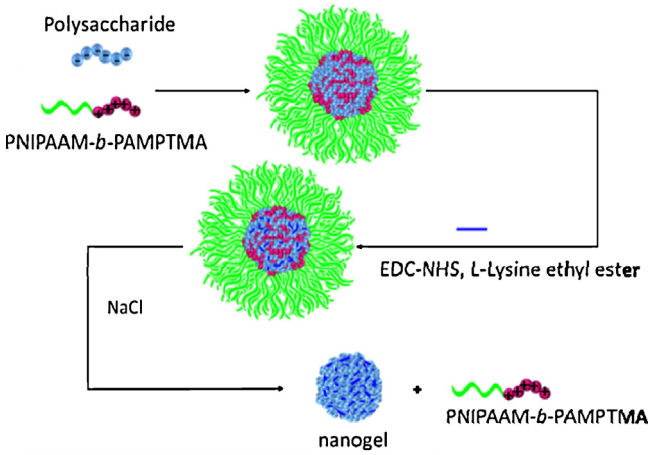

Fig. 1. Schematic representation of PIC micelle formation, cross-linking and dissociation of the nanoreactor.

2.2.5. ζ-Potential measurements

The particle electrophoretic mobility was measured by means of laser micro electrophoresis technique in a thermostated cell (±0.2 °C) using a Malvern Zeta-master instrument (Malvern, UK). Since the particle size is much larger than the Debye screening length, the ζ-potential was calculated from the measured electrophoretic mobility u by means of the Henry relationship

$$\zeta = \frac{3\eta u}{2\varepsilon_0 \varepsilon_m f(K_D R)}$$

where ε_m and η are the permittivity and the viscosity of the aqueous phase, ε_0 is the dielectric constant of free space, and $f(K_D R)$ is a function (Robert J. Hunter – "Foundations of Colloid Science" – Claredon Press, Oxford, 1987) which depends upon the inverse of the Debye screening length K_D and the particle radius R.

3. Results and discussion

3.1. Preparation of polyion complex micelles

The scheme of the preparation of PIC micelles and nanogels is reported in Fig. 1 and is based on the similar work we carried out with chitosan (Maggi et al., 2011). PIC micelles are expected to behave as lyophobic colloids (Verwey, 1947) because kinetic aspects like exchange or addition reactions and structural rearrangements could be important.

Therefore, to avoid inaccurate particle characterization, all size and shape data were determined 48 h after complexation. In any case, size and shape were measured at different time after preparation. We did not observe significant differences between measures carried out after 1 h and 48 h after PIC preparation. Only a slight decrease of the polydispersity index was observed. The size remains unchanged also after 2 months indicating that the micelles are very stable.

HA, AL, PEO, PNIPAAM and PAMPTMA length was chosen taking into account the important role of these parameters in PIC micelles stabilization, preventing the formation of macroscopic aggregates and precipitation (Cohen Stuart et al., 1998; Kabanov et al., 1996; Van der Burgh et al., 2004). Therefore, the length of the polysaccharide chains was chosen similar to the length of

Table 1
D_h of PIC micelles prepared in water. C_{PIC} = 1 mg/mL, f_+ = 0.5, T = 25 °C.

Samples	D_h (nm)[a]	μ_2/Γ^2
$E_{114}A_{50}AL_{50}$	320 ± 15	0.35
$E_{114}A_{50}HA_{63}$	350 ± 14	0.41
$N_{100}A_{30}HA_{63}$	62 ± 4	0.12
$N_{110}A_{60}HA_{63}$	67 ± 5	0.17
$N_{100}A_{30}AL_{50}$	56 ± 5	0.14

[a] The uncertainty was calculated as standard deviation (SD) (n = 3).

Table 2
D_h of PIC micelles prepared at different NaCl concentrations. C_{PIC} = 1 mg/mL, f_+ = 0.5, T = 25 °C.

	D_h (nm)		
	NaCl 0.01 M	NaCl 0.05 M	NaCl 0.1 M
$N_{100}A_{30}HA_{63}$	74 ± 7	78 ± 6	197 ± 13
$N_{110}A_{60}HA_{63}$	72 ± 6	83 ± 7	200 ± 12
$N_{100}A_{30}AL_{50}$	75 ± 5	80 ± 6	215 ± 12
$N_{110}A_{60}AL_{50}$	85 ± 5	87 ± 5	213 ± 14

the cationic block and the neutral block was chosen significantly longer than the charged polymers. AL and HA with number average degree of polymerization X_n of 50 and 63 (AL_{50} and HA_{63}) and the block copolymers $PNIPAAM_{110}$-*b*-$PAMPTMA_{60}$, $PNIPAAM_{100}$-*b*-$PAMPTMA_{30}$ and PEO_{114}-*b*-$PAMPTMA_{50}$ were used to prepare the PIC micelles that will be named $N_yA_xAL_n$, $N_yA_xHA_m$, $E_{114}A_{50}AL_n$ or $E_{114}A_{50}HA_m$, were *N* is PNIPAAM, *E* is PEO and the numbers represent the values of X_n of the polymer chain.

The final concentration of the PIC micelles (C_{PIC}) was 1 mg/mL and the mixing fraction, f_+, was kept constant at f_+ = 0.5. Because of the complete ionization of the quaternary ammonium ions of PAMPTMA, PICs were prepared in distilled water at pH 7, having the carboxylate groups of the polysaccharides mostly charged in order to maximize the interpolyelectrolyte interactions.

The relaxation rates (Γ) measured at different scattering angles plotted as a function of the square of the scattering vector (q^2) gave a straight line passing through the origin which means that the relaxation modes are virtually diffusive and the PIC micelles are spherical in shape and polydispersity is low (Xu, Winnik, Hallett, Riess, & Croucher, 1991).

The *z*-average hydrodynamic diameters (D_h) obtained by dynamic light scattering (DLS) are reported in Table 1.

Even though well defined PICs were obtained with chitosan and block copolymers with a PEO neutral block (Maggi et al., 2011), we were not able to obtain PIC with controlled size using PEO-*b*-PAMPTMA with HA and AL. As can be seen in Table 1, nanoparticles with size higher than 300 nm were obtained. The polydispersity index (μ_2/Γ^2) was high (0.35 and 0.41), meaning that highly polydisperse in size PICs were formed. Besides, after one or two days a precipitate was formed, confirming that the nanoparticles were not stable. We do not have an explanation for this behavior, but we can reasonably suppose that when the positive charge of PAMPTMA is neutralized, interactions between PAMPTMA and PEO take place, giving rise to the formation of uncontrolled size and unstable PICs.

However, by using a PNIPAAM neutral block, well defined small PICs were obtained (Table 1) with size ranging from 56 to 77 nm. This size is similar to that obtained preparing chitosan PICs (Maggi et al., 2011).

Unexpectedly, the PNIPAAM neutral block works better than PEO block does. The polydispersity index (μ_2/Γ^2) of the nanoparticles was always between 0.10 and 0.20 indicating that polydispersity of the nanoparticles is low. The size of the micelles seems to depend slightly on the length of the PAMPTMA block. As expected, small PICs were obtained with a short PMPTMA block. The size of the PICs are similar to those obtained by other authors (Kataoka et al., 1996; Maggi et al., 2011; Van der Burgh et al., 2004) and are close to the limit of the maximum diameter of the micelles calculated assuming PNIPAAM and PAMPTMA chains in their fully extended conformation (65 nm for $PNIPAAM_{100}$-*b*-$PAMPTMA_{30}$ and 85 nm for $PNIPAAM_{110}$-*b*-$PAMPTMA_{60}$). This is indicative of the formation of core-shell PIC micelles without mixing of core and shell chains, but the high values close to the limit of the contour length lead us to suppose that a significantly swelled coacervate core could be formed. This will be discussed again later in the ^{1}H NMR section. It is interesting to note that there are not significant differences in the size of the PICs formed with HA and AL despite the meaningful difference of charge density of the two polysaccharides, with AL having a charge density which is twice that of HA. We also succeeded in preparing PICs with small size at concentration of 5 mg/mL, as already obtained in our preceding work with chitosan (Maggi et al., 2011). It is worthwhile to underline that the preparation of PIC of controlled size at relatively high concentration of polysaccharides is not an easy task (Mincheva et al., 2009a, 2009b) and is an important result for the preparation of significant amounts of nanogels. We think that this can be due to the complete degree of ionization of both the polyelectrolyte involved in the coacervate formation and to the optimization of the length of all polymer chains.

PIC micelles were also prepared at different ionic strength in presence of 0.01, 0.02 and 0.05 M NaCl (Table 2). It can be seen that D_h increases remarkably by increasing the ionic strength of the solution, especially at 0.1 M, in agreement with the results of other authors (Dautzenberg & Rother, 2004; Harada & Kataoka, 1997; Oh, Bronich, Bromberg, Hatton, & Kabanov, 2006b; Park, Akiyama, Yamasaki, & Kataoka, 2006; Solomatin, Bronich, Bargar, Eisenberg, Kabanov, & Kabanov, 2003; Voets et al., 2009; Yuan, Yamasaki, Harada, & Kataoka, 2005). The values obtained at this ionic strength are considerably higher than the maximum value calculated by means of the contour length of the polymers. Different hypotheses can be formulated to explain this behavior. The decrease of the ionic interactions between the oppositely charged polymers could cause a significant swelling of the core. Alternatively, the formation of these large nanoparticles could be explained by the formation of micellar aggregates or mixed core PICs in which charged and neutral polymers coexist. These results suggest that preferably small well defined PICs have to be prepared in water without added salt or with a very small amount of salt.

^{1}H NMR spectrum of $N_{110}A_{60}AL_{50}$ PIC micelles at 25 °C in D_2O, C_{PIC} = 5 mg/mL is reported in Fig. 2. Significant increase of the width of the signals of PAMPTMA block and alginate was observed as compared to those of the PNIPAAM block, *e.g.*, the signal *d* at about 3 ppm of the protons of the methyl groups of the quaternary ammonium ion with respect to the signal *h* of the methyl groups of PNIPAAM. This observation suggests that PIC micelles with an interpolyelectrolyte core and a PNIPAAM shell were formed, inducing a decrease of the mobility of PAMPTMA and alginate chains. We have to underline that we did not observe a significant decrease of the integral of the signals of PAMPTMA as compared to PNIPAAM as usually is observed for PIC formation. This means that the coacervate core of the PIC reasonably maintains a significant degree of hydration that permits a residual mobility of the charged chains.

This is in agreement with the results of the DLS measurements that demonstrated that the size of the PIC is close to the limit imposed by the contour length of the block copolymer. This could be useful for the reticulation of polysaccharide chains because could allow easier penetration of the cross-linker inside the coacervate core.

When PIC micelles are formed, the increase of the ionic strength *I* should screen electrostatic interactions causing PIC micelles to swell adopting a looser structure and eventually, to dissociate above the so-called critical ionic strength, I_{cr} (Cohen Stuart et al.,

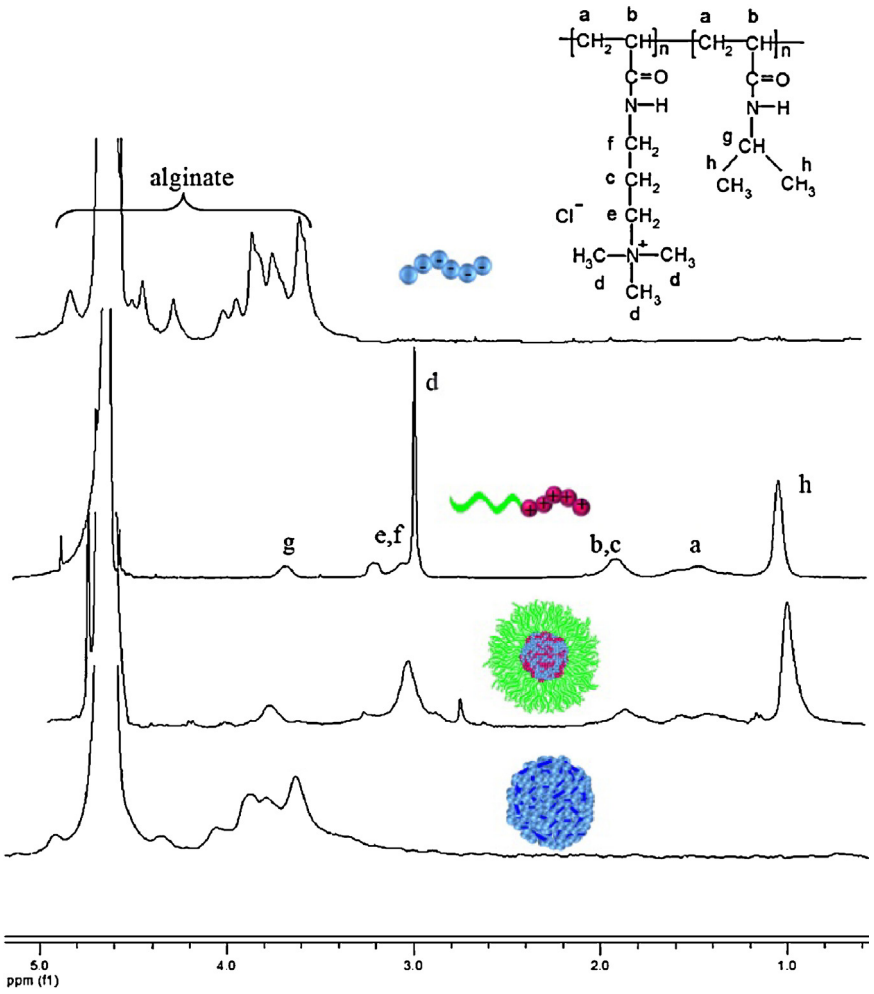

Fig. 2. ^{1}H NMR spectra of (from top to the bottom) AL_{50}, $PNIPAAM_{110}$-*b*-$PAMPTMA_{60}$, PIC $N_{110}A_{60}AL_{50}$ and purified AL_{50} nanogels in D_2O at 25 °C.

1998; Dautzenberg & Rother, 2004; Harada & Kataoka, 1997; Kabanov et al., 1996; Oh et al., 2006b; Park et al., 2006; Solomatin et al., 2003; Voets et al., 2009; Yuan et al., 2005). The effect of the NaCl concentration has been followed by ^{1}H NMR. When NaCl is added, the mobility of the ionic polymers increase as a consequence of the decrease of the electrostatic interactions and the signals of the polyelectrolytes become sharper.

Since, as already discussed before, there is only a change of the height of the signals while the integral remains more or less constant, we studied the dissociation of the PICs by measuring the dependence of the ratio between the height of the signal *h* of the methyl groups of PNIPAAM and the height of the signal *d* of the protons of the methyl groups of the quaternary ammonium ion of PAMPTMA (Fig. 3). The graph obtained for $N_{110}A_{60}AL_{50}$ PIC micelles shows that a significant recovery of mobility of the charged chains is obtained starting from 0.1 to 0.15 M NaCl and that the PICs are completely dissociated already at 0.3 M NaCl. A similar result was obtained for HA PICs. At similar NaCl concentration, a strong decrease of the scattered intensity in DLS experiments was observed and D_h decreases to about 3–5 nm, compatible with the size of free polymer chains.

These I_{cr} values are lower than those reported in the literature and for the PICs of chitosan. This confirms that the interpolyelectrolyte bond between PAMPTMA and the polysaccharides is weak. If we reconsider the results obtained for the formation of PICs at various ionic strength (Table 2) we can now conclude that at 0.1 M NaCl a decrease of the ionic interactions has already occurred and that probably the increase of the size of the PICs are due to significant swelling of the coacervate core.

3.2. Cross-linking of HA and AL inside the nanoreactor

Cross-linking of PIC was carried out with L-lysine ethyl ester (LYS) in presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) (Tomihata & Ikada, 1997). This allowed us to perform the reaction

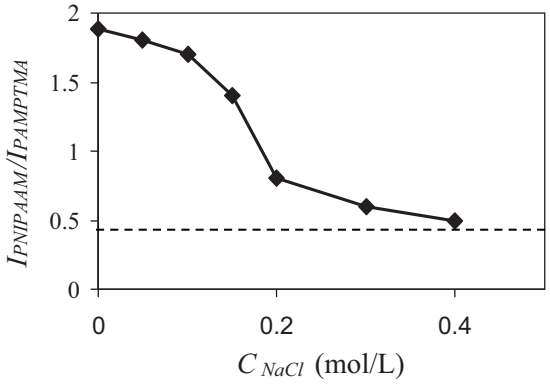

Fig. 3. NaCl dependence of the ratio of the height of PNIPAAM and PAMPTMA signals for $N_{110}A_{60}AL_{50}$ PIC micelles in D_2O at 25 °C. C_p = 5 mg/mL, f_+ = 0.5.

Table 3
D_h of PICs, PICs after reticulation, nanogels after dissociation of the nanoreactor with NaCl and nanogels in water after purification. C_{PIC} = 1 mg/mL, f_+ = 0.5, T = 25 °C. [LYS]:[EDC]:[NHS]:[COOH] = 2:1:1:1.

	D_h (nm)			
	PIC	PIC_{ret}	PIC_{ret} + 0.4 M NaCl	Nanogel in water
$N_{100}A_{30}HA_{63}$	62 ± 4	67 ± 7	55 ± 10	83 ± 12
$N_{110}A_{60}HA_{63}$	67 ± 6	80 ± 8	70 ± 12	91 ± 14
$N_{100}A_{30}AL_{50}$	56 ± 5	78 ± 6	59 ± 11	95 ± 15
$N_{110}A_{60}AL_{50}$	77 ± 5	90 ± 8	65 ± 12	101 ± 16

in mild conditions without affecting the stability of PIC and using a biocompatible cross-linker. The reticulation was carried out at 25 °C with reagents molar ratio [LYS]:[EDC]:[NHS]:[COOH] = 2:1:1:1. The high ratio between the cross-linker and the carboxyl groups was chosen because of the expected difficulty of access to the polysaccharide in the core of the PIC and the relatively low reactivity in this kind of reaction. A blank experiment was carried out by cross-linking a solution containing only 1 mg/mL of AL or HA. The solution became opaque after about 24 h. In this stage nanoparticles with size of 400–800 nm were observed by DLS. After 48 h a precipitate was formed. When PICs were cross-linked, the solutions remained slightly opaque after 48 h. DLS measurements made on the solution during cross-linking showed a small increase of D_h that however remains below 100 nm. The increase of D_h could be due to several factors. Since as a consequence of the preliminary reactions of EDC with the carboxyl groups some negative charges of the polysaccharides could be lost before cross-linking and could not be converted in cross-links because of side reactions, interpolyelectrolyte interactions decrease and the PICs could partially swell. Furthermore, partial intermicellar reaction during cross-linking could lead to the formation of a small amount of bigger aggregates. We can not discriminate between these two possibilities. Anyway, we did not observe the formation of nanoparticles with diameter of 100s nanometers or the formation of precipitate as in the blank experiment, meaning that the polysaccharide is confined inside the core of the nanoreactor and that PNIPAAM chains of the corona effectively screen the core. The segregation of the polysaccharide inside the nanoreactor is therefore essential to obtain nanogels of controlled size.

3.3. Release of the nanogels by dissociation of the nanoreactors

As already described for the PIC micelles, the ionic strength was increased to decrease electrostatic interactions between the polysaccharide and PAMPTMA in order to release the nanogels by dissociating the nanoreactors. Taking into account the results obtained for the dissociation of PICs, we added NaCl to the PIC solutions up to 0.4 M concentration. D_h measured by DLS are reported in Table 3. All the nanogels obtained after dissociation of the nanoreactors are smaller than the reticulated PIC but are similar to the starting PIC. The size of the nanogels is determined by the size of the coacervate core of the PIC and by the swelling that takes place after the release of the block copolymer that is related to the degree of cross-linking. The number of chemical cross-links is certainly lower than the number of physical ionic cross-links that were present in the PIC. Therefore it is reasonable that after release from the nanoreactor the nanogels swells to a size that is higher than the size of the coacervate core that can be speculated to be in the range 10–20 nm.

To obtain purified nanogels, the dissociated cross-linked nanoreactors (nanogels + PNIPAAM-*b*-PAMPTMA + NaCl) were dialyzed first against 0.4 M NaCl and then against distilled water using dialysis tube with 50,000 Dalton cut-off that should retain the nanogels while the PNIPAAM-*b*-PAMPTMA copolymer should be dialyzed. The lyophilized materials were analyzed by ^{1}H NMR, DLS and TEM. In the ^{1}H NMR spectrum of alginate nanogels reported in Fig. 2 the signals of the block copolymer completely disappeared, confirming that they were removed by dialysis. The only residual signals are due to alginate. These signals are quite broad, as expected for alginate chains with severely restricted motion due to cross-linking.

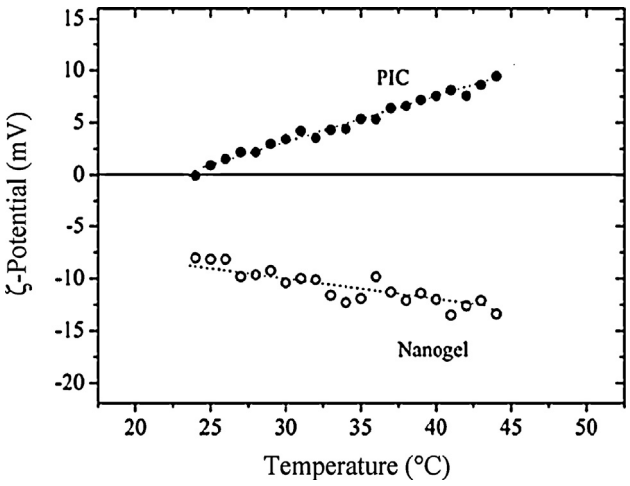

Fig. 4. ζ-Potential as a function of temperature of PIC and purified nanogels of $N_{110}A_{60}AL_{50}$ in water.

The size of the isolated nanogels in distilled water is reported in Table 3. The nanogels have size in the range 80–100 nm. The diameter measured in water is higher than that measured after addition of NaCl to the cross-linked PIC. This can be explained by the shrinking of the nanogels in presence of salt due to electrostatic screening of the repulsion among alginate chains.

ζ-Potential measurements on the nanogels in water were performed to further confirm that the block copolymer was removed. The results are reported in Fig. 4 together with the data obtained for the PIC. At 25 °C the ζ-potential of the PIC is about zero which confirm that the PNIPAAM chains effectively screen the core of the PIC. Instead, the ζ-potential of the purified nanogels in water is negative as expected for nanoparticles having alginate chains on the surface. This confirms that the PNIPAAM-*b*-PAMPTMA chains have been removed from the nanogel. We also measured the ζ-potential as a function of the temperature. From a macroscopic point of view it was surprisingly that by increasing the temperature above the LCST of PNIPAAM no precipitation of the nanoparticles was observed even though the solutions become more turbid and the size of the PIC increased. Above the LCST, as the PNIPAAM chains become hydrophobic, we expected the nanoparticles to become unstable in water giving rise to precipitation. The increase of the value of the ζ-potential probably suggests that when the PNIPAAM chains become hydrophobic and collapse, some PAMPTMA chains are exposed on the external surface of the PICs preventing precipitation.

Direct visualization of the nanogels was obtained by transmission electron microscopy (TEM). Sample preparation and negative staining were made at room temperature with phosphotungstic acid (PTA). Representative TEM micrographs of the purified nanogels are reported in Fig. 5. AL and HA nanogels were spherical in shape and polydispersity is not high. The size in the range 25–50 nm is smaller than the diameter measured by DLS. This is reasonably due to the fact that in the DLS measurements the nanogels are hydrated and swelled in solution while they shrink significantly upon dehydration during TEM sample preparation.

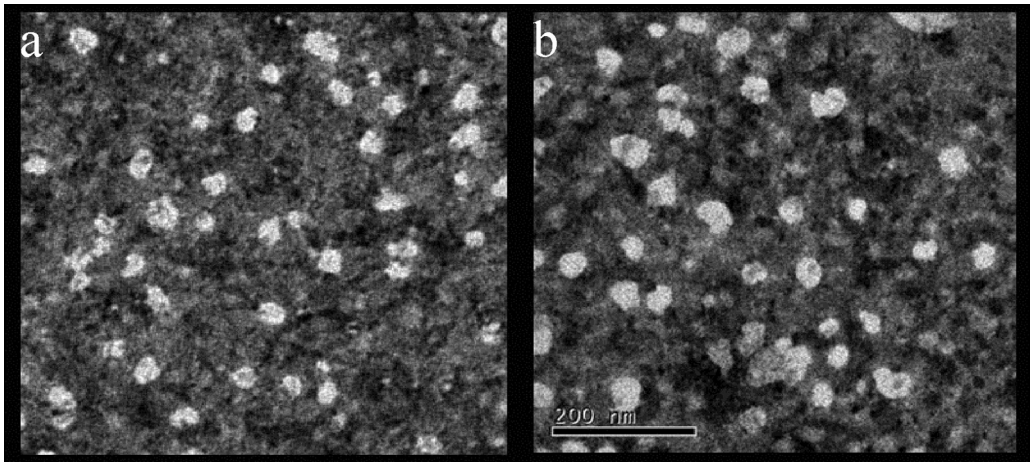

Fig. 5. TEM images of the purified nanogels obtained by $N_{100}A_{30}HA_{63}$ (a) and $N_{100}A_{30}AL_{50}$ (b). Scale bar is 200.

4. Conclusions

In conclusion, alginate and hyaluronic acid covalent nanogels were prepared by template chemical cross-linking of the polysaccharides in polyion complex micelle nanoreactors. Using this procedure we prepared small nanogels (<100 nm) using a biocompatible cross-linker without organic solvents. Small nanoreactors were prepared using PNIPAAM-*b*-PAMPTMA block copolymers while PEO-*b*-PAMPTMA gave rise to the formation of large PICs. Controlled size PICs formation was obtained also at concentration up to 5 mg/mL. This result is of great importance because usually polysaccharide PICs with small size are obtained only at a low polymer concentration. After cross-linking and dissociation of the nanoreactors by adding NaCl small nanogels were obtained with size in solution of 50–70 nm in presence of salt and 80–100 nm in water. The versatility of the method was demonstrated by the use of two polysaccharides significantly different in terms of charge density and rigidity.

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