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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 ($Me_6$TREN) 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.