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Polyelectrolyte complexes via desalting mixtures of hyaluronic acid and chitosan—Physicochemical study and structural analysis

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ABSTRACT

Polyelectrolyte complexes (PECs) were prepared from Chitosan (CS) and Hyaluronic Acid (HYA) homogeneous mixtures of aqueous solutions. The method consisted of preparing a homogeneous mixture of the two polysaccharides via charge screening at high salt concentrations. Then, the mixture was dialyzed, leading to the controlled self-assembly of the two polyelectrolytes. Critical parameters like the chitosan degree of acetylation (*DA*) and molar mass (*Mw*), the residual salt concentration and the molar charge ratio $r = n_{NH3^+}\ (CS)/n_{COO^-}\ (HYA)$ accounted for the transition from homogeneous aqueous solutions to colloidal suspensions ($r = 0.1$) or gel coacervates ($r = 0.5$). The influence of the *DA* and *Mw* of CS was evaluated by visual observations, light scattering and rheological measurements. For low values of *r*, Small Angle X-ray Scattering (SAXS) experiments revealed that the HYA nanostructure was weakly affected by the presence of PECs. On the contrary, the structure was impacted when increasing *r*, revealing a heterogeneous aggregate morphology with ladder-like chain interactions.

1. Introduction

Polysaccharide biomaterials have a wide potential of applications in health sciences, for drug delivery (Hamman, 2010; Luo & Wang, 2014) or tissue engineering (Li, Ramay, Hauch, Xiao, & Zhang, 2005; Tan, Chu, Payne, & Marra, 2009; Yamane et al., 2005) because polysaccharides are generally regarded as safe, biocompatible and exhibit a variety of suitable properties for biomedical applications. Polyelectrolytes are polymers bearing ionizable groups. These groups dissociate in aqueous solutions leading to charged polymer chains and counterions, dispersed or nanostructured in the solution. This specificity of polysaccharide polyelectolytes has been widely used since they can form complexes with salts (Hori, Winans, & Irvine, 2009), proteins (Water et al., 2014) or other polyelectrolytes (Coimbra et al., 2011; Delair, 2011; Li et al., 2005; Wu et al., 2007).

Among them, chitosan (CS) receives a growing attention, as the only cationic and naturally occurring polysaccharide at acidic pH ($pKa = 6.3–6.7$). It is obtained from the partial deacetylation of chitin, its parent polymer extracted from the exoskeleton of crustaceans, endoskeleton of cephalopods or cell walls of fungi. Chitosan is a copolysaccharide of *N*-acetyl-D-glucosamine and D-glucosamine. It is defined by its degree of acetylation (*DA*) corresponding to the molar fraction of acetylated residues and is known for its bioactivity, biodegradability and mucoadhesion as well as a low toxicity. It has been associated to negatively charged polysaccharides like alginates (Li et al., 2005), carboxymethyl cellulose (Chen & Fan, 2007), hyaluronic acid (Al-Qadi, Alatorre-Meda, Zaghloul, Taboada, & Remunán-López, 2013; Tan et al., 2009), dextran sulfate (Drogoz, David, Rochas, Domard, & Delair, 2007), heparin (Costalat, Alcouffe, David, & Delair, 2015; Costalat, David, & Delair, 2014), to form a wide variety of biomaterials such as nanoparticles (Costalat, David et al., 2014; Delair, 2011), scaffolds (Coimbra et al., 2011), fibers (Desorme et al., 2013; Ma et al., 2012), hydrogels (Martínez-Ruvalcaba, Chornet, & Rodrigue, 2007), *etc.*

Hyaluronic acid (HYA) is an anionic polysaccharide at pH values above its $pK_A = 2.9$. Its repeat disaccharide unit is composed of D-glucuronic acid and *N*-acetyl-D-glucosamine, linked by β-1,4 and β-1,3 glycosidic bonds. HYA is widely present in the extracellular matrix of various tissues of the human body, such as in skin, cartilage, synovia and vitreous humor. HYA exhibits some interesting features such as anti-inflammatory properties (Foschi et al.,

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1990) and plays an important role in tissue regeneration (Chen & Abatangelo, 1999). Due to the outstanding properties of each polysaccharide, biomaterials containing hyaluronic acid and chitosan are actually widely studied. Recently, Kaderli et al. published a study about the efficacy of HYA-CS hydrogel for osteoarthritis therapy on rabbit model which provided promising results (Kaderli et al., 2015).

Polyelectrolyte complexes are generally formed by mixing aqueous solutions of two oppositely charged polymers and the entropic gain generated by the liberation of the counterions drives the association reaction. The main limitation when associating HYA and CS is the spontaneous association leading to precipitates. Hence, there is a need to control the complexation to develop materials with high potential for biomedical applications.

For this purpose, we investigated a controlled complexation between HYA and CS by slowing down the process of association. In particular, we used the dialysis method developed in our team to induce and control the self-assembly of the two polyelectrolytes (Costalat, Alcouffe, David, & Delair, 2014; Costalat et al., 2015; Costalat, David, et al., 2014). In the case of polysaccharides, additional interchain interactions like hydrophobic interactions and/or H-bonding may play a role. Complexation depends on various parameters either intrinsic, like the polymer charge density (Dautzenberg, 1997; Webster, Huglin, & Robb, 1997) and chain stiffness (Narambuena, Leiva, Chávez-Páez, & Pérez, 2010), or extrinsic such as the molar charge ratio (Márquez-Beltrán et al., 2012), pH (Kayitmazer, Koksal, & Iyilik, 2015), temperature (Chollakup, Smitthipong, Eisenbach, & Tirrell, 2010), ionic strength (Webster et al., 1997) and the polymer concentration (Schatz, Domard, Viton, Pichot, & Delair, 2004). Macroscopically, polyelectrolyte complexes can be divided into three categories: soluble complexes (Dautzenberg, 1997; Kabanov & Zezin, 1984), colloidal suspensions (Delair, 2011; Schatz et al., 2004) and biphasic systems ranging from precipitates (Chollakup et al., 2010) to coacervates (de Kruif, Weinbreck, & de Vries, 2004; Water et al., 2014) composed of a complex-rich phase and a polymer-poor phase. Recently, Kayitmazer et al. (2015) published an in-depth study of the complex coacervation between hyaluronic acid and chitosan, in the case of rather diluted systems and HYA chain lengths with *Mw* between 50 kg mol^{-1} and 750 kg mol^{-1}.

Here, we show that the intrinsic parameters of chitosan like the *DA* and the *Mw*, as well as the residual salt concentration led to the formation of materials of different nanostructures. These nanostructures were investigated by Static Light Scattering and Small Angle X-ray Scattering (SLS and SAXS) and they were compared with results obtained in our team for chitosan-heparin and chitosan-dextran sulfate systems.

2. Experimental

2.1. Materials

Sodium hyaluronate produced by fermentation of *Streptococcus Equi* was purchased from HTL-biotechnology (France) with a molar mass $Mw \approx 1000$ kg mol^{-1} measured by SEC-MALLS. Chitosan obtained from shrimp shell chitin with a medium molar mass and low Degree of Acetylation was purchased from Mahtani chitosan Pvt. Ltd. India (batch type 243, $DA \approx 1\%$ measured by ^{1}H NMR, $Mw = 150$ kg mol^{-1} and dispersity $Đ = Mw/Mn = 1.9$ measured by SEC-MALLS). The chitosan raw material was further purified by dissolution at 0.5% (w/v) in diluted acetic acid aqueous solution and by filtering the resulting solution on successive cellulose Millipore membranes with decreasing porosity ranging from 3 μm to 0.22 μm, allowing the elimination of all insolubles. The purified chitosan was precipitated with a 37% ammonium hydroxide solution. Repeated washings with deionized water were necessary to remove the excess of ammonia and recover neutral pH. Then, chitosan was freeze-dried.

Table 1
Macromolecular characterization of homologous series of chitosans. The degree of acetylation (DA) was obtained by ^{1}H NMR; The molar mass (Mw) and dispersity (*Đ*) by SEC-MALLS.

DA (%)	*Mw* (kg mol^{-1})	*Đ*
1	56 ± 3	1.5 ± 0.2
	85 ± 5	1.7 ± 0.2
	150 ± 8	1.9 ± 0.2
5	55 ± 3	1.5 ± 0.2
	75 ± 4	1.6 ± 0.2
	160 ± 8	1.6 ± 0.2
14	28 ± 1	1.4 ± 0.1
	100 ± 5	1.7 ± 0.2
	160 ± 8	1.9 ± 0.2
28	19 ± 1	1.3 ± 0.1
	107 ± 5	1.6 ± 0.2
	170 ± 9	1.9 ± 0.2
49	17 ± 1	1.3 ± 0.1
	120 ± 6	1.2 ± 0.1
	185 ± 9	1.6 ± 0.2

The purified chitosan was *N*-reacetylated in controlled conditions in order to obtain a homogeneous series of samples of different *DAs*. Chitosan was dissolved in a water/1,2-propanediol mixture (1/1 (v/v)). Then, acetic anhydride was dissolved in propanediol and added dropwise. The propanediol minimized the hydrolysis of acetic anhydride. These homogenous conditions allow a statistical distribution of *N*-acetyl residues within the chains with no *O*-acetylation (Vachoud, Zydowicz, & Domard, 1997). The *N*-reacetylated chitosan was isolated by precipitation with aqueous ammonia. Washings were achieved by centrifugation and/or dialysis with deionized water.

The *N*-reacetylated chitosans were finally depolymerized in controlled conditions by nitrous deamination to produce chitosans with lower molar masses. Chitosan was dissolved at 0.5% (w/v) in a 0.2 M acetic acid/0.15 M sodium acetate buffer. A 1 g L^{-1} sodium nitrite aqueous solution was added to chitosan solutions to obtain a nitrite/glucosamine units molar ratio of 0.1 (for *DAs* from 1% to 15%), and 0.2 (for *DAs* from 30% to 50%) to optimize the nitrous deamination reaction kinetics. The reaction was performed under high mechanical stirring for different times in order to reach the desired molar mass. Low molar mass chitosans were recovered by precipitation with aqueous ammonia, then washed repeatedly with deionized water until neutrality and finally freeze-dried.

2.2. Methods

2.2.1. Chitosan characterization

The degree of acetylation was calculated by the Hirai et al. method by ^{1}H NMR spectroscopy on a Brucker Avance III 400 MHz 5 mm at 300 K (Hirai, Odani, & Nakajima, 1991).

The weight average molar mass *Mw* and the dispersity (*Đ*) were measured by size exclusion chromatography (2500 and 6000 PW TSK gel columns from Tosohaas) coupled online with a differential refractometer (Wyatt Optilab T-rEx) and a multi angle laser light scattering detector (Wyatt Dawn EOS) operating at $\lambda = 633$ nm. A degassed 0.2 M acetic acid/0.15 M ammonium acetate buffer with a pH 4.5 was used as the eluent. The flow rate was maintained at 0.5 mL/min. The refractive index increments (dn/dc) were indicated independently for each degree of acetylation according to a previous study (Schatz, Viton, Delair, Pichot, & Domard, 2003). The physicochemical parameters of the chitosan samples are reported in Table 1.