Carbohydrate Polymers 119 (2015) 149–158

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Stabilization of chitosan/hyaluronan colloidal polyelectrolyte complexes in physiological conditions

Danjun Wu, Thierry Delair*

Ingénierie des Matériaux Polymères, UMR CNRS 5223, Université Claude Bernard Lyon 1, 15 Bd. André Latarjet, Bât. Polytech, 69622 Villeurbanne Cedex, France

ARTICLE INFO

Article history:
Received 18 July 2014
Received in revised form
18 November 2014
Accepted 19 November 2014
Available online 26 November 2014

Keywords:
Chitosan
Hyaluronan
Polyelectrolyte complexes
Stabilization
Physiological conditions
Nanoparticles

ABSTRACT

Polyelectrolyte complexes (PECs) between hyaluronan (HYA) and chitosan were obtained by the one-shot addition of default amounts of polyanion to an excess of polycation. The impact of intrinsic parameters (degree of polymerization and degree of acetylation) and extrinsic parameters (charge mixing ratio, the concentration and pH of polyelectrolyte solutions) on particle sizes and polydispersity were investigated. The PECs maintained their colloidal stability when stored in water. To preserve the colloidal stability at physiological salt concentration and pH, biological nontoxic metallic Zn(II) was added either post or during the formation of the particles. Dynamic light scattering results showed the PEC particle sizes in phosphate buffer saline remained constant and displayed a good stability at room temperature for at least 35 days, irrespective of the stabilization process by Zn(II). These results open promising prospects for the zinc cation stabilized chitosan–HYA PECs as efficient and safe tools for drug delivery.

1. Introduction

Polyelectrolyte complexes (PECs) are formed by Coulomb interactions between oppositely charged polyelectrolytes (Dautzenberg & Kriz, 2003; Muzzarelli, Stanic, Gobbi, Tosi, & Muzzarelli, 2004). PECs are formed using water as a solvent, via a simple process to implement such as the one-shot addition (Schatz, Domard, Viton, Pichot, & Delair, 2004), therefore, this elaboration strategy is quite promising for manufacturing safe materials for life sciences applications, such as gene (Strand, Danielsen, Christensen, & Vårum, 2005; Duceppe & Tabrizian, 2009) or drug delivery (Sarmento et al., 2006; Muzzarelli, Greco, Busilacchi, Sollazzo, & Gigante, 2012).

However, PECs suffer from a lack of stability in physiological conditions due to the presence of electrolytes and of pH values responsible for a decrease in the charge density of polyions. The effect of electrolytes was well described by Dautzenberg and Kriz (2003). According to this paper, complexes from weak polyelectrolytes redissolve at a critical salt concentration and those from strong polyelectrolytes irreversibly precipitate. On a practical stand point, colloidal PECs can remain stable for various periods of time if stored in water (De la Fuente, Seijo, & Alonso, 2008; Parajó, D'Angelo, Welle, Garcia-fuentes, & Alonso, 2010) for chitosan–hyaluronan (HYA) complexes or in low salt concentration. In a fairly detailed investigation, Umerska et al. (2012) found that chitosan–HYA particles could be stored at room temperature for 3 to 4 weeks, in water. Similarly chitosan–poly-γ-glutamic acid colloids, either positively or negatively charged, were stable up to 6 weeks, in deionized water (Lin et al., 2005). Many formulations of chitosan–dextran sulphate PECs colloids were no longer stable in the presence of saline (Weber et al., 2010).

Neutral to high pHs, but also low values, can be deleterious to colloidal PECs stability. In these conditions the charge density of one of the polyelectrolytes is too low, or even nil, to maintain the complex integrity. For instance, when the pH was adjusted in the interval 3–6.4, Sæther, Holme, Maurstad, Smidsrød, and Stokke (2008) observed that the chitosan–alginate particle diameter remained constant. But, at pH 7.0 the diameter increased about 50 times. Chitosan–chondroitine sulphate nanoparticles remained stable up to 7 days in water, but the diameters increased 5 to 8 folds after storing for 30 min in, respectively, 0.9% sodium chloride and phosphate buffer saline (PBS) (Hansson et al., 2012). chitosan–HYA particles dramatically increased in size right after dilution in PBS (Parajó et al., 2010) and so did particles using poly(γ-glutamic acid) as polyanion (Lin et al., 2007). To prevent the deprotonation of

Abbreviations: PECs, polyelectrolyte complexes; DA, degree of acetylation; HYA, hyaluronan sodium salt.

* Corresponding author. Tel.: +33 4 72 44 85 87; fax: +33 4 72 43 85 87.
E-mail address: Thierry.Delair@univ-lyon1.fr (T. Delair).

http://dx.doi.org/10.1016/j.carbpol.2014.11.042
0144-8617/

chitosan and maintain constant the charge density on the polymer, the primary amino group of chitosan was quaternarized. Thus, the authors preserved the colloidal stability for 48 h in PBS (Bal et al., 2010). Trimethyl chitosan (TMC)/α-galactosidase A (α-GAL) enzyme complexes were stable for 4 days in a low salt concentration buffer such as 10 mM HEPES pH7.5 (Giannotti, Esteban, Oliva, García-parajo, & Sanz, 2011). Verheul et al. (2011) investigated the colloidal stability of trimethyl chitosan (TMC)/HYA complexes in physiological buffers. They observed a large increase in size after 30 min of incubation in 150 mmol L^{-1} NaCl. The authors suppressed this deleterious effect of salt by modifying the original polymers into thiolated derivatives. Covalently stabilized nanoparticles were formed by cross-linking thiolated TMC and thiolated HYA via the formation of disulfide bonds.

Steric stabilization can also be another means to increase the colloidal stability of nano-scaled PECs in physiological conditions. Qi et al. observed a good conservation of the colloidal properties of chitosan–BSA (bovine serum albumin) nanocomplexes on storing for one month in PBS. These results were due to the covalent conjugation on the protein of non ionic dextran chains serving as non-ionic stabilizers (Qi, Yao, He, Yu, & Huang, 2010). Van der Lubben et al. (2003) observed 3 month stability at +4 °C or room temperature in PBS (pH 7.3) of 1% (w/v) suspensions of chitosan microparticles obtained by ionic gelation with sodium sulfate. But this result should be attributed to the non ionic stabilizer Tween® 80, a polyoxyethylene sorbitan sodium monooleate, used during the elaboration process. Recently, our group demonstrated that chitosan–dextran sulphate colloidal complexes were stable in PBS, on condition chitosan, the major component, had a high degree of acetylation (DA around 50%). Indeed, the colloids were sterically stabilized, since chitosan no longer behaved like a polyelectrolyte. (Weber et al., 2010; Costalat, David, & Delair, 2014).

Hence, the colloidal stability of PECs in physiological conditions is far from being fully addressed and this work is devoted to the elaboration of chitosan–HYA nanoPECs with improved stability in physiological media and to the establishment of their high potential of applications as bioactive (macro) molecule delivery systems in nanomedicine.

2. Materials and methods

2.1. Materials

Chitosan was provided by Mahtani Chitosan PVT, Ltd., India, batch 114 (degree of acetylation (DA) ~ 4%, average molar mass (M_w) ~ 5.8×10^5 g mol^{-1}). Prior to use, the polymers were purified as follows: dissolution in acetic acid aqueous solution, filtration through Millipore membranes of decreasing porosity (from 3 to 0.45 μm), precipitation with an ammonium solution, rinsing with deionized water until neutrality, and lyophilization.

Purified high M_w chitosan was *N*-acetylated in homogeneous media at different DAs with acetic anhydride. The reaction was performed in a hydro-alcoholic mixture according to the procedure previously described by Vachoud, Zydowicz, and Domard (1997). After re-acetylation, chitosan solutions were neutralized, rinsed with deionized water, and then lyophilized.

In addition, hydrolysis of the polysaccharide to produce low-molar-mass chitosans was carried out, with a control of the reaction kinetics (Allan & Peyron, 1995). Briefly, the hydrolysis process of chitosan was performed as follows: chitosan samples of various DAs were dissolved at 0.5% (w/v) in an acetic acid/ammonium acetate buffer (0.2 M/0.15 M). A 10 g L^{-1} of sodium nitrite solution was added to the chitosan solution to attain a nitrite/glucosamine units molar ratio of 0.1. The reactions were performed under fast magnetic stirring for various reaction times (5–120 min). Low molar mass chitosans were precipitated with ammonium hydroxide solution and purified by several washing with deionized water until neutrality, and finally lyophilized.

Hyaluronan sodium salt (HYA) with high weight-average molar mass $M_w = 1.96 \times 10^6$ g mol^{-1} was provided from H.T.L. (Javené, France). Low molar mass HYAs were obtained by sonication. The sonication was performed with an ultrasound Sonics Vibra Cell generator (Fisher Scientific Bioblock, France). In brief, HYA solution (1%, w/v) was put in a glass reactor of diameter $\Phi = 3.5$ cm and a maximum liquid height $H_{max} = 7$ cm. It was homogenized by magnetic stirring, and the reactor temperature was kept constant at 25 °C during the experiment thanks to a water circulation in the double walled reactor.

2.2. Methods

2.2.1. Characterization of chitosan and hyaluronan

The degree of acetylation was determined on purified chitosans by ^{1}H NMR spectroscopy (Varian, 500 MHz), according to the method developed by Hirai, Odani, and Nakajima (1991). The water content was determined by thermogravimetric analysis (DuPont Instrument 2950). The weight-average molar mass (M_w) and the polydispersity index (I_p) were measured by an aqueous size exclusion chromatography (SEC) system consisting of a Waters 717 system equipped with a differential refractometer Wyatt Optilab T-rEX ($\lambda = 658$ nm) and interfaced with a multi-angle laser light scattering (MALLS) detector (Wyatt EOS). The separation was carried out on two gel columns (Tosoh TSK PW 2500 and TSK PW 6000). Elution was performed at 22 °C maintaining the flow rate at 0.5 mL min^{-1}, and the degassed 0.2 M acetic acid/0.15 M ammonium acetate buffer with a pH 4.5 was used as the eluent. The samples were prepared at a concentration of 1 mg mL^{-1} and filtered through a 0.45 μm pore-size membrane prior to injection. Refractive index increments (d*n*/d*c*) were determined from a master curve previously established for each degree of acetylation under identical conditions (Schatz, Viton, Delair, Pichot, & Domard, 2003).

For the characterization of HYA, the SEC columns were PL aquagel OH mixed M and PL aquagel OH mixed H (300×7.5 mm, bead diameter: 8 μm). An aqueous buffer (0.066 M Na_2HPO_4/0.066 M KH_2PO_4 (65:35, v:v)) at pH 7.1 was used as the eluent. The d*n*/d*c* was fixed at 0.15.

2.2.2. Polyelectrolyte complex formation

Chitosan was dispersed in Versol® water at 0.1 or 0.2% concentration, taking into account the initial water content. Dissolution was achieved under moderate stirring by adding a stoichiometric amount of acetic acid, with respect to the free amines for each chosen degree of acetylation. Then, the pH of the solutions was adjusted to the desired value (4.0, 4.5, 5.0, and 5.5) with 0.1 M sodium hydroxide or hydrochloric acid. Before use, chitosan solutions were filtered through 0.22 μm pore size Millipore membranes. HYA solutions, at 0.1 or 0.2% concentration, were prepared directly in Versol® water under magnetic stirring.

Colloidal Polyelectrolyte complexes (PECs) were formed in non-stoichiometric conditions at predetermined charge mixing ratios ($R = n^+/n^-$) by a one-shot addition of the polymer solution in default to the polymer in excess under magnetic stirring (1200 rpm) at room temperature. The formed particles dispersions were dispersed in deionized water or PBS (2×) in same volume to obtain 0.05 and 0.1% of colloids, respectively.

2.2.3. Physicochemical characterization of the complex dispersions

Dynamic light scattering measurements of PECs dispersions were carried out using a Malvern Nanosizer SZ equipped with a 5 mW He/Ne laser beam operating at $\lambda = 633$ nm (at 173°