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Stabilization of chitosan/hyaluronan colloidal polyelectrolyte complexes in physiological conditions

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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.

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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°

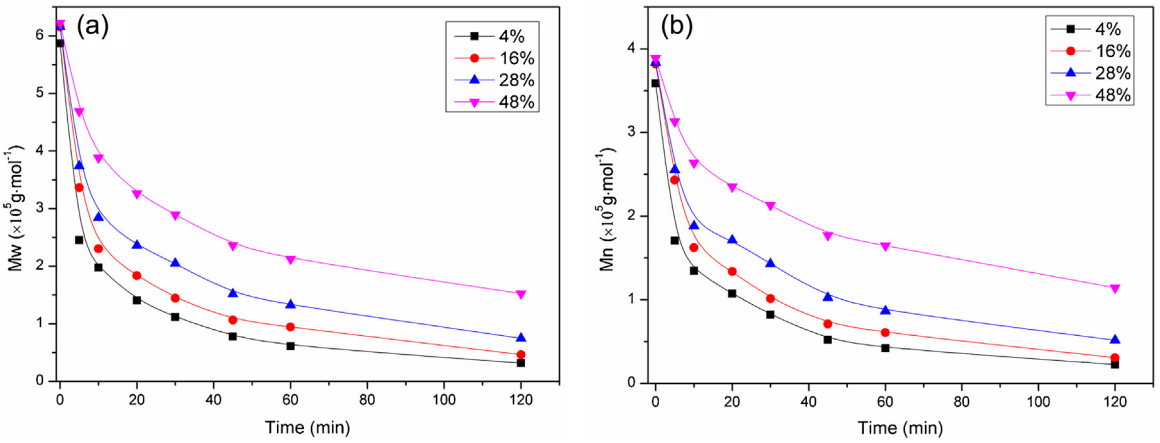

Fig. 1. The hydrolysis kinetic curves of 4 kinds DA of chitosans: (a) weight-average molar mass of chitosan, (b) number-average molar mass of chitosan.

scattering angle). The self-correlation function was expanded in a power series (Cumulants method). The polydispersity value provided by the software is a dimensionless value defined by $\mu_2/(\Gamma)^2$, where μ_2 is the second cumulant of the correlation function and (Γ) is the average decay rate. All measurements were performed at least in triplicate with 10 measurements each at 25 °C.

Zeta potentials were derived from electrophoretic mobility measurements using Smoluchowski's equation. Electrophoretic mobilities (μE) of the particles were determined at 25 °C with the Malvern Nanosizer SZ. μE was expressed as the average of 10 measurements with a relative error of 5%.

2.2.4. Transmission electron microscopy (TEM)

The morphology of chitosan–HYA particles were examined using transmission electron microscopy (TEM, Philips CM120). A droplet of 0.1% (v/v) washed nanoparticles dispersion was deposited onto formvar-coated copper grids. Excess solution was carefully blotted off using filter paper and air-dried at room temperature (24 h) for imaging.

2.2.5. Evaluation of colloidal stability

The colloidal stabilities of 0.05% and 0.1% (w/v) PECs dispersions was monitored over time at +4 °C, 20 °C, or 37 °C. The average particles diameters and the zeta potentials were measured with the Malvern Nanosizer SZ at 25 °C.

3. Results and discussion

3.1. Physico-chemical properties of chitosan and hyaluronan

3.1.1. Chitosan

To study the influence of charge density and chain length on the formation of polyelectrolyte complexes, chitosans of various degrees of acetylation and molar masses were prepared. Prior to synthesize different M_w of chitosan, four different kinds of degree of acetylation (DA) (4, 16, 28, and 48%) of high M_w of chitosans ($M_w \sim 6 \times 10^5$ g mol^{-1}) were hydrolyzed, and the hydrolysis kinetic curves of chitosans were reported in Fig. 1.

The DA of chitosan greatly impacted the hydrolysis kinetics based on weight-average and number-average molar masses of chitosans. As seen in Fig. 1, on increasing DA, the hydrolysis kinetics decreased, for a fixed nitrite/glucosamine units molar ratio of 0.1. At reaction completion, the obtained molar masses increased with DA. This is consistent with the mechanism the nitrous deamination that only takes place at glucosamine residues. Hence, when the density of these residues decreased, with increasing DAs, the number of cleavage sites decreased, thus the molar masses of the fragments were higher. Fig. 1 shows that chitosans with controlled molar masses and DAs could be obtained and the various samples used in this work are described in Table 1.

3.1.2. Hyaluronan

To obtain HYAs of lower molar masses than the initial sample ($M_w = 1.96 \times 10^6$ g mol^{-1}), we selected the ultrasound assisted depolymerization described by Miyazaki, Yomota, and Okada, 2001 and Polexe and Delair (2013). The depolymerization kinetics, reported in Fig. S1 (in Supplementary data), depicted a rapid decrease in molar masses with time associate with a concomitant sharp increase in polydispersity index (I_p) in the early stage of the reaction, then, followed a slower kinetics with a gradual decrease in I_p, as the sonication time was prolonged. After 12 h of sonication, a molar mass of 2.64×10^4 g mol^{-1} of HYA was obtained. For the PEC formulation, two molar masses of HYA obtained by sonicating 3 and 6 h were used: 5.90×10^4 g mol^{-1} ($I_p = 1.192$), and 3.90×10^4 g mol^{-1} ($I_p = 1.163$).

3.2. Formation of polyelectrolyte complexes

3.2.1. Degree of polymerization of polyelectrolytes and degree of acetylation of chitosan

Chitosans of low, medium, high molar mass with DAs ranging from 4% to 48% were complexed with two M_w of HYA,

Table 1
Physicochemical characteristics of chitosans determined by ^{1}H NMR (DA) and SEC (weight-average molar mass M_w, number-average molar mass M_n and polydispersity index I_p).

DA (%)	$M_w \times 10^5$ (g/mol)	$M_n \times 10^5$ (g/mol)	I_p
4	5.86 ± 0.09	3.59 ± 0.10	1.63 ± 0.05
	3.65 ± 0.04	2.36 ± 0.04	1.54 ± 0.03
	1.86 ± 0.03	1.23 ± 0.03	1.51 ± 0.04
	0.88 ± 0.01	0.60 ± 0.02	1.49 ± 0.05
	0.21 ± 0.01	0.16 ± 0.01	1.34 ± 0.10
16	6.15 ± 0.10	3.82 ± 0.11	1.61 ± 0.05
	3.69 ± 0.05	2.41 ± 0.05	1.53 ± 0.04
	2.15 ± 0.03	1.41 ± 0.04	1.52 ± 0.05
	1.14 ± 0.02	0.72 ± 0.02	1.59 ± 0.05
	0.43 ± 0.01	0.31 ± 0.01	1.40 ± 0.05
28	6.16 ± 0.08	3.84 ± 0.13	1.60 ± 0.06
	3.18 ± 0.05	2.18 ± 0.04	1.45 ± 0.04
	1.79 ± 0.02	1.30 ± 0.01	1.38 ± 0.02
	0.92 ± 0.01	0.63 ± 0.01	1.47 ± 0.04
48	6.22 ± 0.16	3.89 ± 0.09	1.60 ± 0.05
	2.65 ± 0.04	1.56 ± 0.05	1.70 ± 0.06
	1.48 ± 0.02	1.08 ± 0.01	1.37 ± 0.02
	0.76 ± 0.01	0.64 ± 0.02	1.18 ± 0.04
	0.15 ± 0.02	0.13 ± 0.02	1.17 ± 0.02

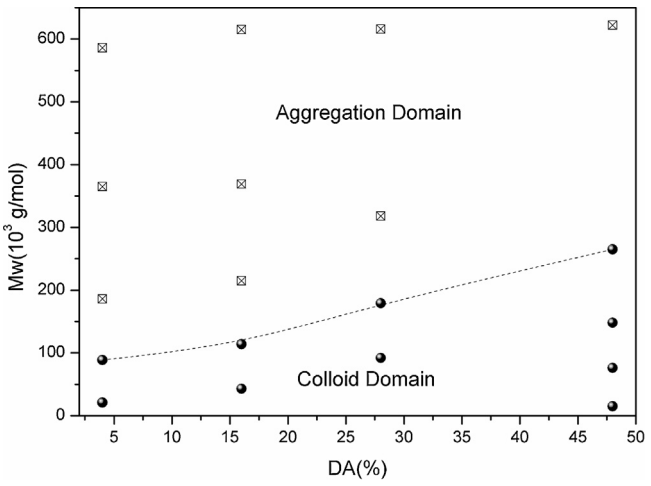

Fig. 2. Particles formation available diagram in terms of the molar mass of chitosan as a function of DA at polymer concentration of 0.1% and pH 4: (⊠) aggregates; (●) colloidal particles.

5.90×10^4 g mol^{-1} and 3.90×10^4 g mol^{-1}. An initial molar concentration in glucosidic residues of 6.0×10^{-3} M ($\sim 10^{-3}$ g mL^{-1}) was used in this part of experiment and the added HYA was set to the same concentration.

High molar masses of chitosan could not sufficiently disentangle to form particles, hence the observed aggregates. The formation of fairly isodisperse particles required low and medium molar mass chitosans (around 2×10^5 g mol^{-1} and lower) as shown in the particle formation diagram, Fig. 2. The domain available for the formation of colloidal particles was limited by a critical upper value of M_w, which increased with the DA of chitosan, at constant polymer concentration of 0.1% and pH 4. As DA increased, hydrophobic interactions compensated or even predominated over electrostatic interactions. Consequently, various physico-chemical parameters of chitosan solutions were impacted such the second virial coefficient, the radius of gyration etc. (Schatz et al., 2003).

The influence of the M_w of chitosan (0.1%, w/v, pH4) and HYA (0.1%, w/v) on the average diameters of PECs-based particles was investigated. Various M_w of chitosan, with DAs ranging from 4% to 48% were complexed with HYA samples of M_w of 39 and 59 kg mol^{-1} (Fig. S2). We observed an increase in the average PECs particle sizes with increasing chitosan M_w. The DAs of chitosans had no pronounced impact on particle sizes, which remained in the 225–300 nm range for chitosans of related M_w (88, 114, 92 and 76 kg mol^{-1}) (Fig. S2). At constant DA, the variations of HYA M_w did not alter particle sizes, though the two HYA M_w were close to one another. Further investigation of the impact of the molar mass of HYA required the sonication of the initial HYA for 1, 2, 3, 6, and 12 h and then complexing with chitosan (0.1% and 0.2%, w/v, DA48%, M_w 1.48×10^5 g mol^{-1}, pH 4). The results were reported in Fig. 3.

A decrease in the average particles sizes and PDI was concomitant with the decrease in M_w of HYA till 6 h of sonication time. A slight increase was observed with 12 h of sonication of HYA which could be attributed to an increase in the M_w polydispersity. Therefore, the optimum M_w of HYA was 39 kg mol^{-1} (with the sonication time of 6 h) to achieve the best control over particle size and size distribution.

3.2.2. *Effect of the charge ratio (R)*

The charge molar ratio represents the excess of one polymer versus the other one, the higher the n^+/n^- ratio, the greater the excess in chitosan. As seen in Fig. 4, particle sizes increased with increasing R, three folds for the highest DA and 2 folds for all the other ones. The increase in size with R can be attributed to the presence of increasing excess of non-complexed polymer segments at the interface and/or also to a less dense complex core containing more uncomplexed ammonium groups. Interestingly, this behavior was also observed in Fig. 5 for two concentrations of polyelectrolytes and Fig. 6 for various pH values of the particle formation medium.

3.2.3. *Influence of the polyelectrolyte concentration on particles sizes*

Polyelectrolyte concentrations higher than 0.2% (w/v) lead to aggregated materials (data not shown). For concentrations of 0.2% (w/v) and lower, fairly isodisperse particles were obtained. PECs formed using concentration of 0.1% (w/v) for both HYA (M_w 3.90×10^4) and chitosan lead to smaller particles than at 0.2% irrespective of the charge ratio (Fig. 5).

3.2.4. *Influence of pH of polymer solutions on particles sizes*

The impact on particle sizes of the pH of the complexation medium was investigated for DAs 4, 16, 28 and 48% and for increasing R ratios. The screened pH values were 4.0, 4.5, 5.0 and 5.5 in order to ensure protonation of chitosan. As seen in Fig. 6, an increase in pH of the chitosan solution (HYA being solubilized in water) led to a decrease in the particle hydrodynamic radii at most ratios. On increasing the pH, the concomitant reduction of the charge density of chitosan will induce a decrease in the macromolecule stiffness,

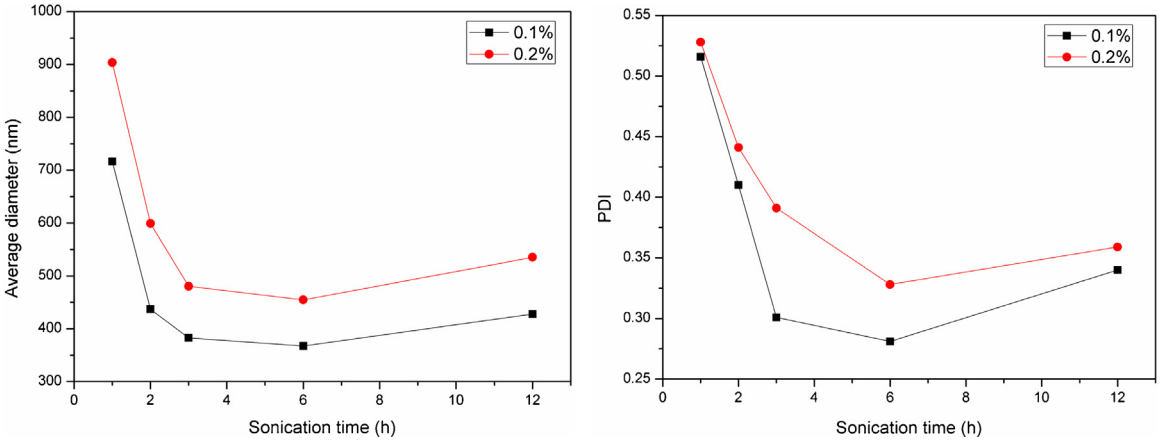

Fig. 3. Influence of sonication time of HYA (0.1 and 0.2%, w/v) on average sizes and polydispersity index (PDI) of PECs. Conditions: (■) chitosan (0.1%, w/v, DA 48%, pH4, M_w 1.48×10^5) + HYA (0.1%, w/v, with different sonication time); (●) chitosan (0.2%, w/v, DA 48%, pH4, M_w 1.48×10^5) + HYA (0.2%, w/v, with different sonication time).

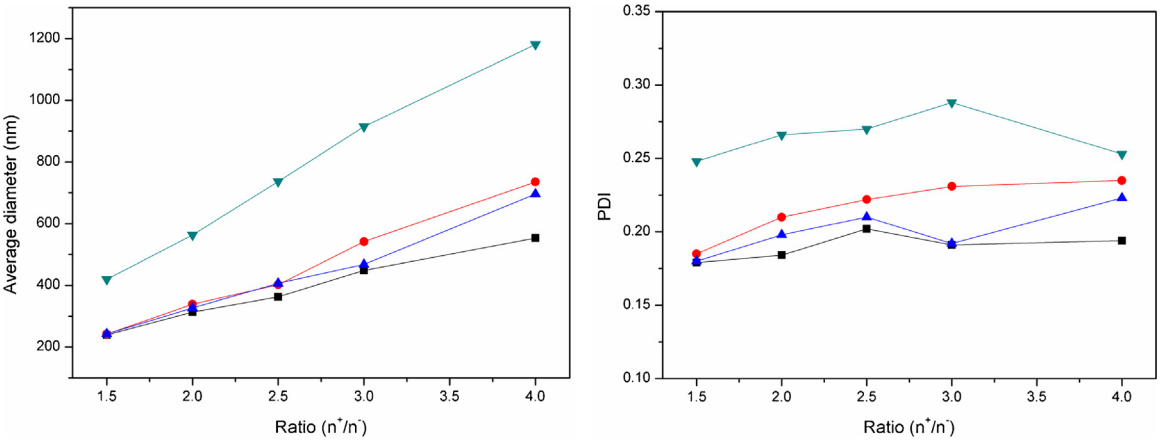

Fig. 4. Influence of the charge ratio R on average sizes and polydispersity index (PDI) of PECs particles. Conditions: HYA (0.2% (w/v), M_w 3.90×10^4), chitosan: 0.2% (w/v), pH 5: (■) chitosan (DA4%, M_w 8.86×10^4); (●) chitosan (DA 16%, M_w 1.14×10^5); (▲) chitosan (DA 28%, M_w 9.20×10^4); (▼) chitosan (DA 48%, M_w 1.48×10^5).

as a result of the decreased repulsive electrostatic interactions. Therefore, the conformational adaptation required for the charge matching should be easier, thus leading to more compact complexes.

The impact of the pH reduction was more marked for the lower DAs than for the higher ones. If we set $R = 2$, the particle size decrease was approximately 4 folds for DAs 4% and 16% but only 2 folds for DAs 28% and 48%. This is consistent with the loss of the polyelectrolyte character of chitosan with increasing DAs as reported in the law of behavior of chitosan solutions by Schatz et al. (2003) and Lamarque, Lucas, Viton, and Domard (2005). However, it is worth noting that at pH 5.5, the colloidal PECs were prone to flocculation, related to the low charge density of the chitosan amino groups. At low charge density, the collapse of the

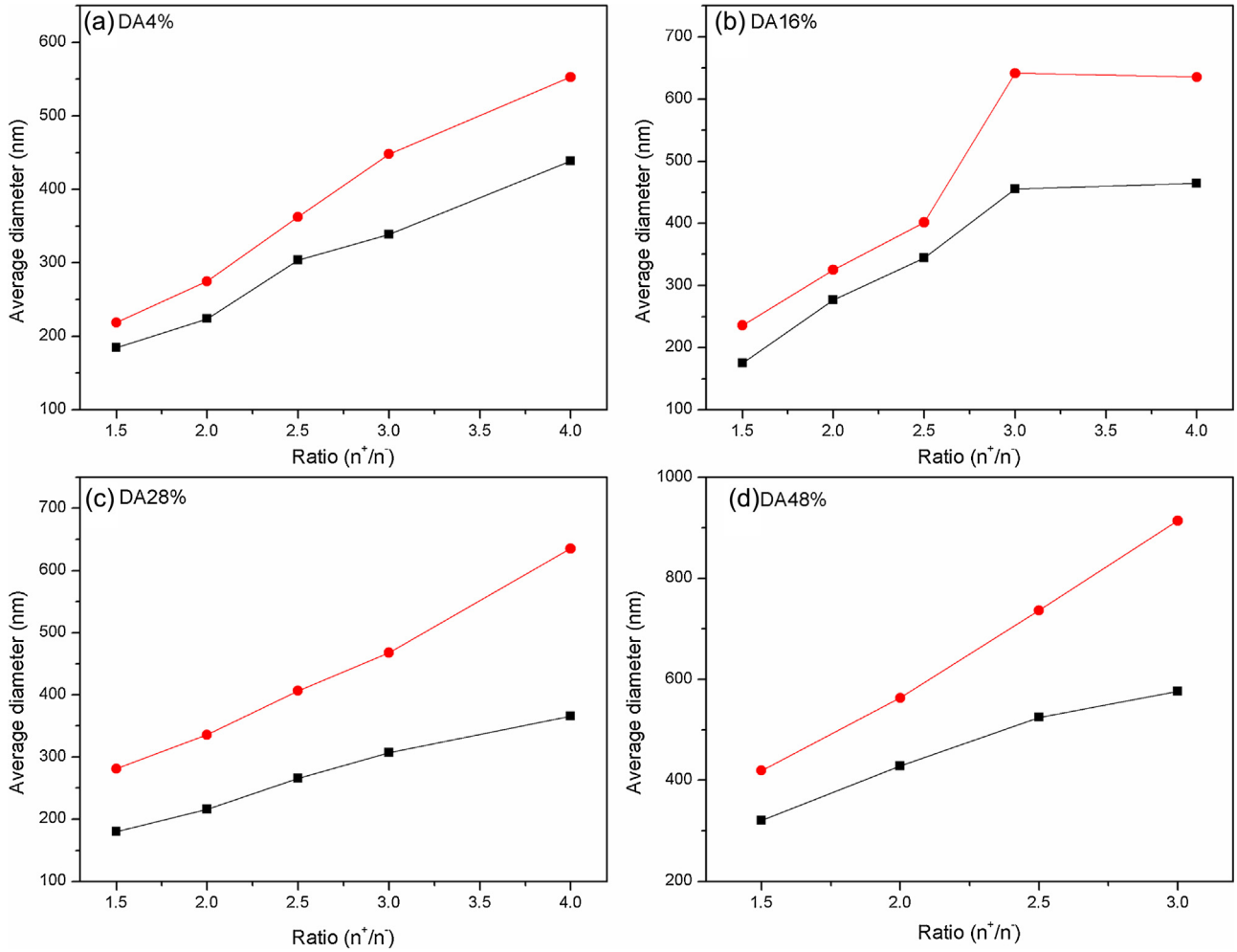

Fig. 5. Average sizes of PEC particles as a function of the charge ratio at two concentrations: (■) chitosan (0.1%, w/v, pH5), HYA (0.1%, w/v); (●) chitosan (0.2%, w/v), HYA (0.2%, w/v). Conditions: chitosan (a) (DA 4%, M_w 8.86×10^4); (b) (DA 16%, M_w 1.14×10^5); (c) (DA 28%, M_w 9.20×10^4); (d) (DA 48%, M_w 1.48×10^5).

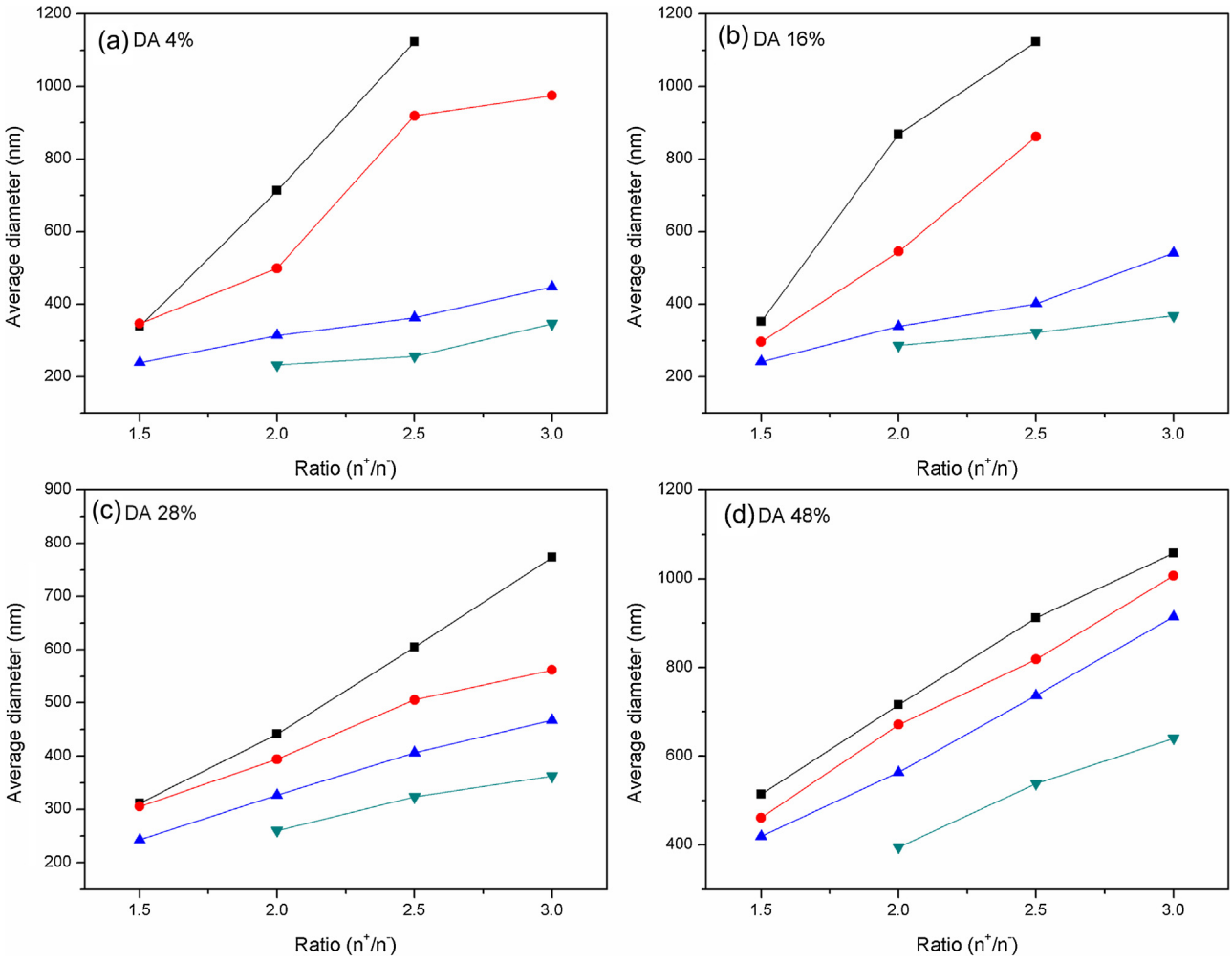

Fig. 6. Average sizes of PECs as a function of charge ratio at different pH of chitosan solutions: (■) pH4; (●) pH4.5; (▲) pH5; (▼) pH5.5. Conditions: chitosan (0.2%, w/v) and HYA (0.2%, w/v, M_w 3.90×10^4); (a) chitosan (DA4%, M_w 8.86×10^4); (b) chitosan (DA16%, M_w 1.14×10^5); (c) chitosan (DA28%, M_w 9.20×10^4); (d) chitosan (DA48%, M_w 1.48×10^5).

polysaccharide chains via hydrogen bonding, led to a reduction in colloidal stability.

To have a more in-depth understanding the pH effect, the pH of the HYA solution was adjusted to match that of the chitosan solution. As seen from Fig. S3, the pH adjustment of HYA solution, induced a significant particle size reduction only at pH 4 for both investigated DAs of 16 and 28%. For other pH, the influence was limited. These experiments confirmed the decrease in particle sizes with increasing pH observed above.

3.2.5. Formation of particles: Optimal conditions

Regarding the production of PEC-based particles, the influences of all the studied parameters on the size and polydispersity of particles are summarized in Table 2.

From all previous results, it can be stated that the formation of small and isodisperse particles requires the following conditions: low molar mass of chitosan (below 200 kg mol^{-1}), low molar mass of HYA (around 39 kg mol^{-1}), relatively low ratio of n^+/n^- (1.5–2), relatively higher pH of chitosan solution (pH ~ 5), and low concentration of polyelectrolytes (0.1%, w/v). Following these optimal conditions, colloidal PECs with 0.1% (w/v) of chitosan (DA = 48%, $M_w = 1.50 \times 10^4$, pH 5) and of HYA (M_w 3.90×10^4) $R_{n+/n-} = 2$ displayed an average diameter 169 ± 3.1 nm and a polydispersity index about 0.05 ± 0.01.

3.2.6. Morphology

The morphology of the chitosan–HYA complexes formed in the optimal conditions was examined by TEM (Fig. 7) and the observed sizes were smaller than those obtained by DLS, as the result of the drying process prior TEM observation, while DLS reflected the average hydrated radii of the particles.

3.3. Evaluation of colloidal stability

PECs using optimal conditions had 10–14 days of stability in water solution at room temperature (Fig. S4). Based on our previous work about chitosan–dextran sulphate complexes, PECs using chitosan with M_w around 150 kg mol^{-1} showed better stability than that of using low M_w (13 kg mol^{-1}) of chitosan (Weber et al., 2010). Similarly, colloidal chitosan–HYA PECs using chitosan of M_w 148 kg mol^{-1} featured an increased stability in water for at least one month for the three tested temperatures (4, 20 and 37 °C) as shown in Fig. S5.

As detailed in the introduction of this paper, the conservation of the colloidal stability in physiological conditions is essential for biomedical applications. Colloidal PECs from two weak polyelectrolytes are very sensitive to in their environment, such as ionic strength and pH. As a matter of fact, the dispersions were destabilized upon dilution in PBS for complexes obtained with chitosan at

Table 2
Effect of an increase in various parameters on the properties of PEC particles with an excess of chitosan: (+) increase, (−) decrease, (×) no significant effect.

	M_w of chitosan	M_w of HYA	DA of chitosan	Ratio (n^+/n^-)	pH of chitosan	pH of HYA	Conc. of polyelectrolytes
Size	+	+	×	+	−	×	+
Polydispersity index	+	+	×	+	×	×	+

DA 4%, or completely solubilized when the DA was 48%. Considering these results, there was a need to stabilize our chitosan–HYA colloidal PECs. Tiyaboonchai and Limpeanchob (2007) reported that zinc ions could stabilize chitosan–dextran sulfate and their argument was that the particles were smaller in the presence of zinc sulfate, the particle sizes being measured by dynamic light scattering after dilution of the dispersions in de-ionized water. But in this work, the authors also showed that the particles redissolved in a 10 mM HEPES buffer pH 7.4, hence evidencing a lack of stability. Other reports of literature proved that zinc(II) was coordinated by the oxygen-containing donor groups of HYA in nearly neutral aqueous solution in the pH interval 6.0–6.5 (Burger et al., 2001). Interestingly, as a kind of d^{10} ions, Zn(II) can also adopts a tetracoordinate mood with ligands like chitosan (Ogawa & Oka, 1993; Wang, Du, & Liu, 2004). For the Zn–chitosan complexes, two models had been proposed to elucidate the structure of chitosan and metal ions: pendant pattern, in which Zn(II) was bound to one amino group of chitosan, and bridge pattern, in which Zn(II) bond to two or more amino groups and hydroxyl groups of one or more chitosan chains as a bridge (Wang et al., 2004).

Moreover, zinc has been reported to have a low cytotoxicity and also anti-viral replication properties (Suara & Crowe, 2004; Haraguchi, Sakurai, Hussain, Anner, & Hoshino, 1999). Therefore, we tried to stabilize PECs dispersion by cross-linking the colloidal particles with zinc(II) derivatives. In this study, zinc chloride ($ZnCl_2$) and zinc sulfate ($ZnSO_4$) were added either during or after the formation of the complexes. It should be noted that precipitation was observed when PBS and $ZnCl_2$ or $ZnSO_4$ solutions were mixed, while no aggregation formed when $ZnCl_2$ or $ZnSO_4$ stabilized chitosan–HYA nanoparticles were in PBS.

Prior to the stability study, the turbidity of PECs dispersion with and without zinc ions was measured by UV spectrometer in PBS. To a dispersion of nanoparticles obtained from 0.1% or 0.2% chitosan (DA = 48%, $M_w = 1.48 \times 10^5$ g mol^{-1}, pH = 4) and HYA ($M_w = 3.90 \times 10^4$ g mol^{-1}, pH = 4), different concentrations of $ZnCl_2$ or $ZnSO_4$ were added, followed by the same volume of 2× PBS to the previous mixture. The turbidity of the nanoparticles in PBS (1×) was studied by UV at the wavelength of 500 nm.

The results showed that for $ZnCl_2$ or $ZnSO_4$ concentrations of 1.0–1.5 mM, the turbidity of nanoparticles (0.05% (w/v)) in PBS, was almost similar to the turbidity of nanoparticles in H_2O, corresponding to the horizontal line in the figures (Fig. S6). When the salt concentration reached a critical value, particles aggregated due to the loss of electrostatic stabilization via charge screening. Nanoparticles at 0.1% easily aggregated on adding salt to 3.0 mM. In order to avoid precipitation, the salt concentration was selected at 1.5 mM.

The particle size variations with increasing $ZnCl_2$ or $ZnSO_4$ concentrations were reported in Fig. S7. A contraction in particle size was observed with zinc ion stabilized PECs, suggesting that Zn(II) may act as hardening agent, as proposed by Tiyaboonchai and Limpeanchob (2007) for chitosan–dextran sulfate colloidal PECs. The contraction in size was more important with zinc sulfate than with zinc chloride and that can be correlated to the decrease in zeta potential observed in Fig. 8. This effect will be discussed later in the paper.

The evaluation of the dispersion stability upon storage was carried out at three temperatures, two concentrations of chitosan–HYA complexes (0.1 and 0.2%, w/v), pre- or post-stabilized by $ZnCl_2$ or $ZnSO_4$. One volume of dispersion was mixed with the same volume of PBS (2×) in order to monitor the particle size evolution in physiological salt and pH conditions (1× PBS) by DLS. Fig. 8 and Fig. S8 show the changes in particles sizes and zeta potentials, respectively, for 0.05% and 0.1% solids, over 35 days of storage at the temperatures of 4, 20 and 37 °C.

As seen from Fig. 8 and Fig. S8, for both particle concentrations, the average sizes of PECs stored in H_2O remained constant whatever the storing temperature and the mode of stabilization. In PBS, the stability of the colloids depended on the storing temperature, the mode of stabilization and the particle concentration. PEC particles post-stabilized with $ZnCl_2$ or $ZnSO_4$ aggregated after 24 h of storing in PBS at 4 °C. Stabilization by $ZnCl_2$ or $ZnSO_4$ during PEC formation ('pre-stabilization') improved the stability of PECs at 4 °C (Fig. 8c and d) for at least one month in PBS. For

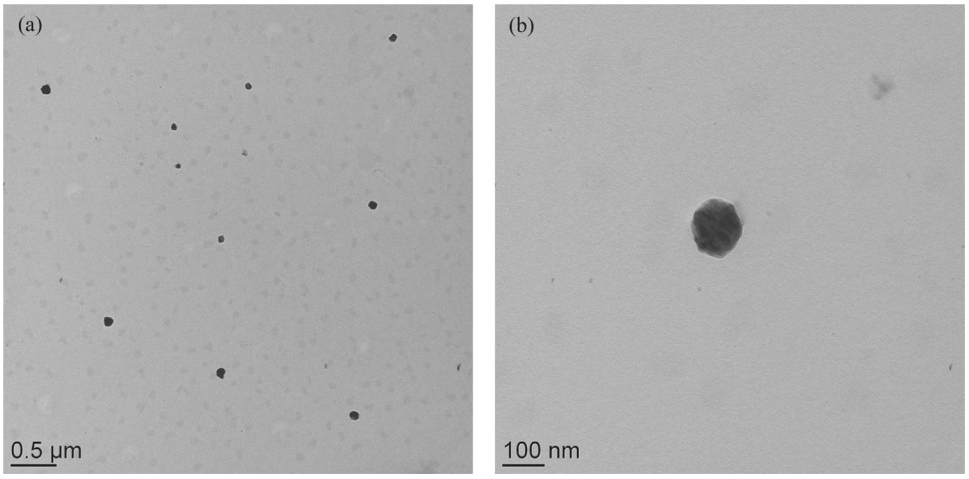

Fig. 7. Transmission electron micrographs of chitosan/HYA nanoparticles based PECs in optical conditions: chitosan (DA = 48%, $M_w = 1.50 \times 10^4$), HYA (M_w 3.90×10^4), $R_{n+/n-} = 2$. (a) The morphology of nanoparticles in the dry state, (b) is the higher magnification of (a).

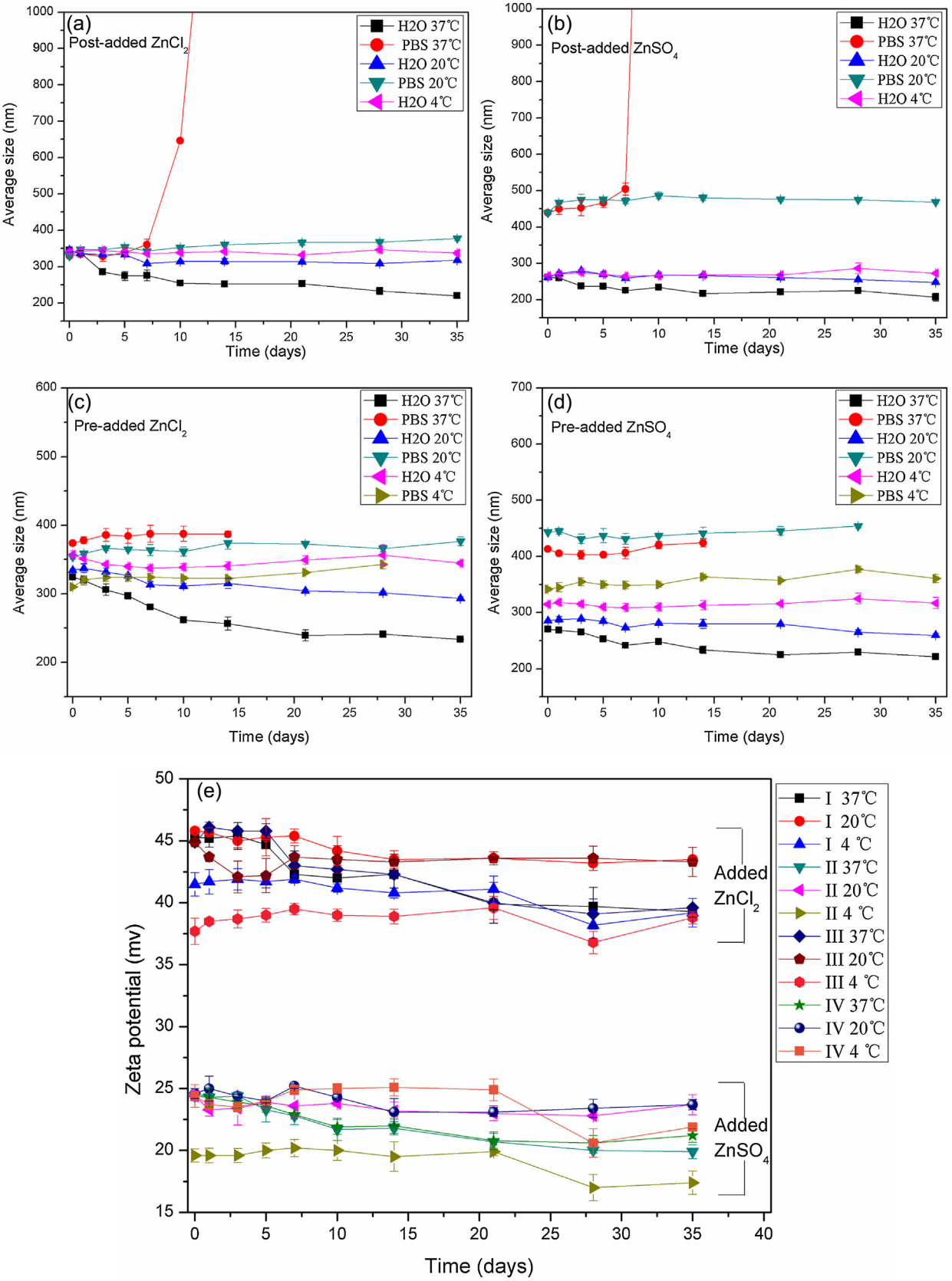

Fig. 8. Stability studies of PECs dispersions (0.05%, w/v) in H_2O and PBS at temperatures of 4, 20 and 37 °C: Average particle size of PECs (a) post-stabilized by $ZnCl_2$, (b) post-stabilized by $ZnSO_4$, (c) pre-stabilized by $ZnCl_2$ (d) pre-stabilized by $ZnSO_4$ (e) Zeta potential of PECs in H_2O ((I) post-stabilized by $ZnCl_2$; (II) post-stabilized by $ZnSO_4$; (III) pre-stabilized by $ZnCl_2$; (IV) pre-stabilized by ZnSO4) (mean ± S. D., $n = 3$).

storing at 20 °C, PECs were stable at low concentration, Fig. 8, irrespective of the nature of the stabilizing salt and of the mode of stabilization, which is quite a good result knowing that, without stabilization, particles dissolved immediately after mixing with PBS. At 0.1% solids, particles remained stable in PBS at room temperature but started to aggregate after 2 weeks of storing at 37 °C. As a general trend, the stabilization during the particle forming process was always more efficient than post-synthesis. For instance, in PBS at 37 °C, the particles sizes of post-stabilized PECs increased after 1 week (Fig. 8a and b) whereas they remained stable for two weeks for the particles stabilized during the particle processing (Fig. 8c and d). Concerning the nature of the stabilizing salt, (compare a with b and c with d in Fig. 8 and Fig. S8), particles stabilized with $ZnCl_2$ were more stable as the average diameter was lower and they aggregated slower than when $ZnSO_4$ was used. This can be attributed to the fact that, the zeta potentials of PECs stabilized by $ZnCl_2$ (no matter the pre- or post-process) were always higher than those obtained after $ZnSO_4$ stabilization (Fig. 8 and Fig. S8e). This difference can arise from the fact that sulfate ions are known to complex chitosan (Van der Lubben et al., 2003) hence neutralizing some of the ammonium charges. This charge neutralization also explains the higher size contraction observed with zinc sulphate in Fig. S7. The mode of stabilization by zinc ions can be attributed to the formation of co-ordinate bonds between electron rich atoms, like nitrogen or oxygen, and the free bonding levels of zinc, ensuring thus a cross-linking of the polysaccharide chains which provided the stabilization. This binding may take place at the nitrogen atoms, and that would account for the fact that the zeta potentials measured for the stabilized particles were systematically lower than without stabilization.

Finally, an essential factor to achieve such a spectacular stabilization of colloidal chitosan–HYA PECs in physiological conditions, was the use of well defined chitosan samples. Particles obtained from chitosan samples with a degree of acetylation lower than 40% flocculated in PBS whereas for DAs above this value, the dispersions maintained their colloidal character in PBS, underlining the essential role of the intrinsic parameters of chitosan over the performances of the nano-PECs. As already noticed by Schatz et al. (2003), the physicochemical properties of chitosan solutions vary with the DA. At DAs around 40%, chitosan can form hydrogen bonds, in particular with water via its numerous *N*-acetyl groups. These hydrogen bonds explain why high DA chitosan remain water soluble at pHs above 7. It also accounts for the stability of the colloids made from these chitosan samples complexed with HYA (this work) or dextran sulfate (Weber et al., 2010).

4. Conclusions

In this work, polyelectrolyte complex (PECs) nanoparticles were obtained by a one-shot addition of default amounts of HYA of various molar masses, to chitosan of different molar masses and degrees of acetylation.

The molar mass of chitosan and HYA had a major impact on the particles formation process. Indeed, fairly isodisperse particles were obtained with low and medium molar mass of chitosans (around 2×10^5 g mol^{-1} and lower) and HYA. The mean diameters of the colloidal PECs proved also dependent on the chitosan to HYA charge ratio R (n^+/n^-), relatively low ratios of n^+/n^- (1.5–2) allowing the formation of small particles.

Colloidal PECs remained stable in water for at least one month at 4, 20 and 37 °C. In physiological salt and pH conditions, particles flocculated and/or re-dissolved. The colloidal stability was greatly improved upon interactions with zinc(II). We succeeded to obtain PECs particles stable in PBS for over 35 days of storing at room temperature.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.11.042.

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