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Characterization of Chitosan/Hyaluronan Complex Coacervates Assembled by Varying Polymers Weight Ratio and Chitosan Physical-Chemical Composition

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Abstract: Herein, we synthesized and characterized polysaccharide-based complex coacervates starting from two water-soluble biopolymers, i.e., hydrochloride chitosans and sodium hyaluronan. We used chitosans encompassing a range of molecular weights from 30,000 to 400,000 and showing different fraction of acetylated units (i.e., $F_A = 0.16, 0.46$, and 0.63). This set of chitosans was mixed with a low molecular weight hyaluronan to promote electrostatic interactions. Resulting colloids were analyzed in terms of size, polydispersity and surface charge by Dynamic Light Scattering. The weight ratio between the two polyelectrolytes was studied as additional parameter influencing the liquid-liquid phase separation. Main results include the following: the polymers weight ratio was fundamental in dictating the colloids surface charge, whereas chitosan physical-chemical features influenced the dimension and homogeneity of colloids. This contribution presents additional understanding of the complex coacervation between these two oppositely charged polysaccharides, with the potential translation of present system in food and biomedical sectors.

Keywords: chitosan; hyaluronan; complex coacervation; molecular weight; acetylation degree; colloidal system

1. Introduction

The molecular constituents of food colloids are proteins, polysaccharides and lipids. Dynamic and structural features of the dissimilar phases related with food systems and the various interactions with guest constituents represent important parameters for food processing covering huge amounts of applications [1–5].

Among different polymers, the use of chitosans in food applications has gained increasing attention in recent years [6–10]. Chitosans are biopolymers obtained mainly from the deacetylation of chitin. They are composed of two building β -1→4 linked sugars, i.e., glucosamine, GlcNH_2 (deacetylated, D unit) and *N*-acetyl-glucosamine, GlcNAc (acetylated, A unit) randomly or partly blockwise distributed along the polymer chain. The fraction of A-type unit, i.e., F_A , defines the acetylation degree of chitosans, which is of pivotal importance in modulating polysaccharide properties such as linear charge density, solubility and susceptibility to enzymatic degradation [11]. Chitosans are low toxic, non-immunogenic and they could represent a vehicle for the delivery of drug molecules [12,13]. These biopolymers are extremely versatile, and can be assembled in different macro/nano-systems as hydrogels, scaffolds and hydrocolloids [14–18]. Furthermore, chitosans can be chemically modified to synthesize derivatives showing improved solubility at neutral pH [14,19–25].

Complex coacervation represents a liquid-liquid phase separation in which a polymer-rich phase is in equilibrium with a polymer-poor phase [26,27]. In this context, the polycation chitosan may electrostatically interact with negatively charged macromolecules such as hyaluronan for assembling colloids to be used as carriers of payloads in different fields of application, ranging from food to pharmaceutical sectors [28–38].

The goal of this work is to investigate on some key experimental variables affecting complex coacervation of chitosans when mixed with a low molecular weight hyaluronan. Hyaluronan was selected as polyanion due to its biological relevance as main component of extracellular matrix and the ability to form colloids when mixed with chitosans. In more detail, the role played by polymers weight ratio and chitosan physical-chemical features, such as F_A and molecular weight, in forming colloids is here disclosed. In our previous contribution, we identified a structure–function relationship between chitosan acetylation degree and immune cells response, showing that instable (heterogeneous) but not stable (homogeneous) colloids manifested a pro-inflammatory activity towards human macrophages [39]. Hence, it results of paramount importance understanding factors that could influence complex coacervation of these two polyelectrolytes. By systematically varying chitosan molecular weight, acetylation degree, and polymer mixing, herein, we propose an in-depth characterization of the resulting colloids.

2. Materials and Methods

2.1. Materials

Hydrochloride chitosans (CHs) showing different viscosity average molecular weight, \overline{M}_v , (determined by viscometry) and different fraction of acetylated units, F_A , (determined by $^1\text{H-NMR}$) were kindly provided by Novamatrix/FMC Biopolymer (Sandvika, Norway) and by the late Prof. Kjell Morten Vårum (NTNU, Trondheim, Norway). The physical-chemical features of CHs are reported in Table 1. Sodium hyaluronan (HA), ($\overline{M}_v = 90,000$, Bioibérica S.A.) was kindly provided by Sigea Srl (Trieste, Italy). Sodium tripolyphosphate pentabasic (TPP $\geq 98\%$) was from Sigma-Aldrich Co. (St. Louis, MO). Deionized water was used in all experiments.

Table 1. Fraction of acetylated units (F_A), intrinsic viscosity, $[\eta]$, viscosity average molecular weight, \overline{M}_v , molecular weight of chitosan repetitive unit, $MW_{r.u.}$, and viscosity average degree of polymerization, \overline{DP}_v , of hydrochloride chitosans used for the synthesis of complex coacervates. \overline{M}_v is calculated using the following Mark-Houwink-Sakurada parameters: $K = 8.43 \times 10^{-3} \text{ mL/g}$ and $a = 0.92$. [40]

F_A	$[\eta]$ (mL/g)	\overline{M}_v	$MW_{r.u.}$ (g/mol)	\overline{DP}_v
0.16	110	30,000	198	152
	681	220,000		1111
	1026	340,000		1717
0.46	340	100,000	200	500
	650	210,000		1050
	920	300,000		1500
0.63	300	90,000	>201	448
	550	170,000		846
	950	310,000		1542

2.2. Preparation of Complex Coacervates

The synthesis of colloids was performed according to a previously reported procedure [36]. Briefly, polymers and TPP were solubilized in deionized water at a concentration equal to 0.6 mg/mL for chitosans, 1.25 mg/mL for sodium hyaluronan and 0.5 mg/mL for TPP. After complete solubilization, 150 μL of TPP solution were added dropwise to 3 mL of hyaluronate solution under stirring. Solutions were filtered through 0.22 μm filters (Biosigma, Italy) and stored at room temperature until use. In a

5 mL beaker 500 μ L of the HA-TPP solution were added to 0.5 or 2.0 mL of chitosan solutions under stirring to allow the formation of colloids at different CH/HA weight ratio, i.e., equal to 1:2 and 2:1, respectively. Solutions were kept under stirring for 10 min and left at rest for 20 min before being analyzed. The final pH of solutions was in the range 4–6.5.

2.3. Physical-Chemical Characterization of Complex Coacervates

Dynamic Light Scattering (DLS) Analyses

Formulations were investigated by means of dynamic light scattering (DLS) on a Zetasizer Nano ZS with 173° detection optics (Malvern Instruments) to evaluate their size (hydrodynamic diameter), PolyDispersity Index (PDI) and surface charge (ζ -potential). Each formulation was analyzed at least in triplicate at $T = 25^\circ\text{C}$ after dilution 1:10 *v/v* in deionized filtered water using disposable cuvettes. The size was expressed as the Z-average hydrodynamic diameter, obtained by a cumulative analysis of the correlation function using the viscosity and refractive index of water in the calculations. ζ -potential was determined via the laser Doppler velocimetry (LDV) technique.

3. Results and Discussion

Characterization of complex coacervates at different chitosan physical-chemical composition and polymers weight ratio. We mixed chitosans showing different chemical composition and mass with a low molecular weight hyaluronan in order to build a large set of colloids (Figure 1). Upon injection of the polyanion-TPP solution, liquid-liquid phase separation (complex coacervation) occurred following to the formation of (i) low energy interactions, as well as hydrogen, electrostatic and hydrophobic between the two biopolymers [33,36], and (ii) the entropy gain due to the release of counterions and water molecules from polyelectrolytes. Preliminary (qualitative) considerations have been drawn on the basis of visual observations: aggregates were detected to the naked eye in the case of formulations composed of high molecular weight or high acetylated chitosans (Table 2). Therefore, we can conclude that low chitosan acetylation degree, as well as medium-to-low molecular weight, are essential in promoting an efficient complex coacervation event. Conversely, chitosan-to-hyaluronan weight ratio plays a marginal role.

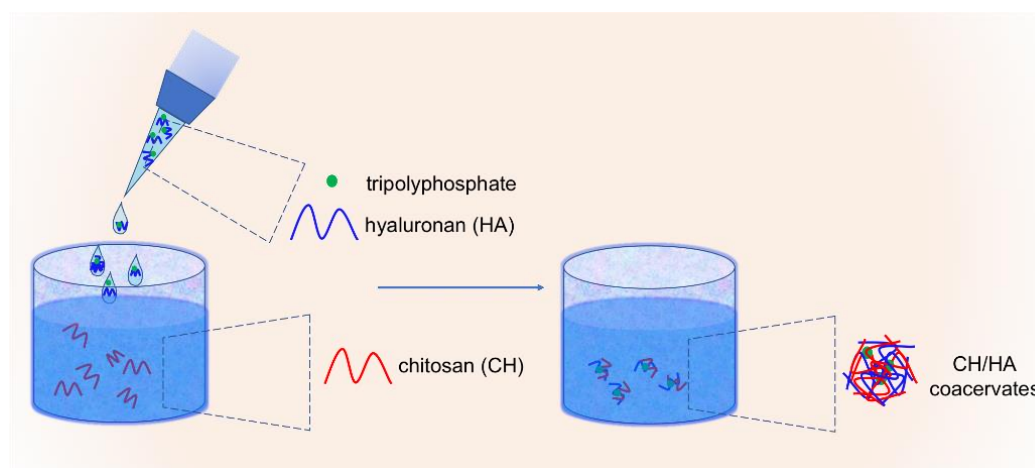


Figure 1. Sketchy representation of complex coacervation process between chitosan (the polycation) and hyaluronan (the polyanion). Tripolyphosphate serves as “adjuvant” cross-linker for chitosan. Electrostatic interactions—together with gain of entropy due to counterions and water release from polyelectrolytes—drive complex coacervates formation.

Table 2. Characterization of CH/HA complex coacervates after dilution 1:10 (v/v) in deionized water. Colloids were assembled using chitosans with different fraction of acetylated units ($F_A = 0.16, 0.46$ and 0.63) and different viscosity average molecular weights (\overline{M}_v). Polymers weight ratio and aggregation at time zero of the resulting formulations are reported.

F_A	\overline{M}_v	CH:HA Weight Ratio	Notes
0.16	30,000	1:2	no aggregation
	220,000	2:1	no aggregation
		1:2	no aggregation
		2:1	no aggregation
	340,000	1:2	no aggregation
		2:1	limited aggregation
0.46	100,000	1:2	no aggregation
	210,000	2:1	no aggregation
		1:2	no aggregation
		2:1	no aggregation
	300,000	1:2	no aggregation
		2:1	limited aggregation
0.63	90,000	1:2	no aggregation
	170,000	2:1	no aggregation
		1:2	limited aggregation
		2:1	no aggregation
	310,000	1:2	no aggregation
		2:1	limited aggregation

Physical properties (i.e., dimensions, polydispersity and surface charge) of colloids at different chemical composition or polymers weight ratio were investigated by a DLS analysis. A different behavior of colloids composed of F_A 0.16 chitosan and different CH/HA weight ratio was detected as a function of chitosan molecular weight (Figure 2a). In general terms, in the case of CH/HA 1:2 weight ratio colloids dimensions were almost independent on chitosan molecular weight. Overall, our results are in line with previous findings, wherein a 1:1 CH/HA weight ratio and a medium molecular weight chitosan were considered in the analysis [39]. Conversely, the dimension of colloids increased abruptly by increasing chitosan chain length for CH/HA 2:1 weight ratio up to the formation of micrometric colloids. A further increment of chitosan degree of polymerization was accompanied by a decrease of hydrodynamic diameter. Concerning colloids composed of medium acetylated chitosan, i.e., $F_A = 0.46$, no apparent influence of CH/HA weight ratio was detected (Figure 2b) up to chitosan molecular weight of 210,000. Upon further increasing the degree of polymerization, CH/HA 1:2 colloids manifested an increment of dimensions at a variance with CH/HA 2:1 counterparts. For $F_A = 0.63$ chitosans, dimensions of both types of colloids resulted to be independent on chitosan molecular weight up to $\overline{M}_v = 170,000$ (Figure 2c), whereas an increment of hydrodynamic diameter was spotted for $\overline{M}_v = 310,000$ chitosan.

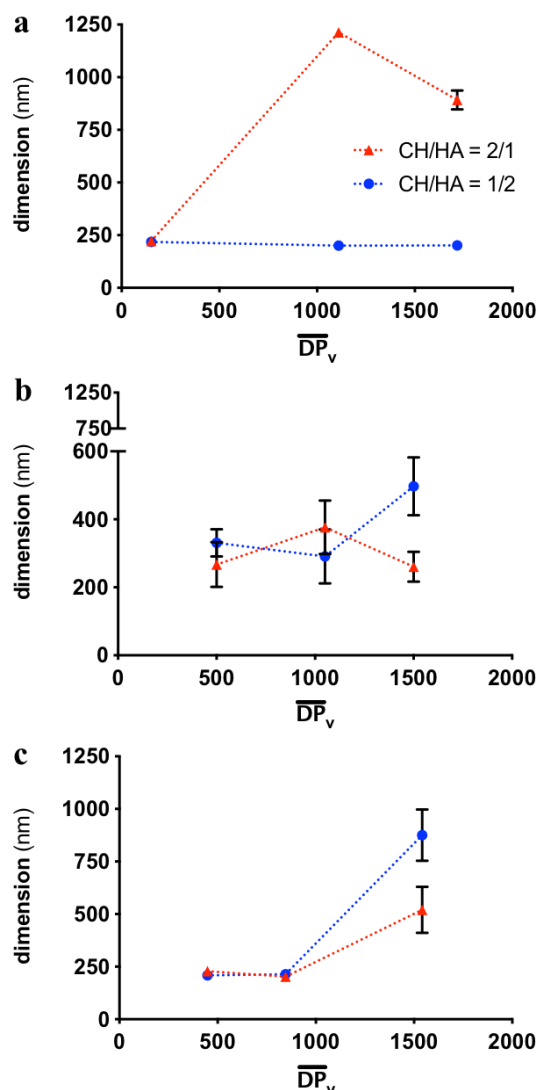


Figure 2. Hydrodynamic diameter of CH/HA complex coacervates showing different polymers weight ratio (CH:HA = 1:2 and 2:1). Colloids were assembled using chitosans with fraction of acetylated units (F_A) of 0.16 (a), 0.46 (b) and 0.63 (c), respectively. Data are reported as a function of chitosan average degree of polymerization, i.e., \overline{DP}_v . Colloids were analyzed by DLS after dilution 1:10 (v/v) in deionized water. Data are means (\pm SD) of at least three measurements.

Further considerations have been made with respect to the polydispersity index (PDI) (Figure 3). For F_A 0.16 chitosan, a correlation between dimensional homogeneity and chitosan molecular weight emerged for the two types of polymers weight ratio analyzed (Figure 3a). In more detail, by using a weight ratio equal to 1:2, PDI values were independent on chitosan molecular weight and lower than 0.2. These results are in line with what previously reported for CH/HA of 1:1 and medium molecular weight chitosan-based colloids [39]. In the case of CH/HA 2:1, PDI values (linearly) increased as a function of chitosan molecular weight, suggesting a correlation between dimensional dispersity and polymer chain length. In the case of colloids fabricated with high acetylated chitosans, i.e., $F_A = 0.46$ and 0.63 - none straightforward correlation between polymers weight ratio and PDI was detected (Figure 3b,c). All in all, PDI values ≥ 0.2 were recorded for all these formulations, suggesting the formation of more heterogeneous colloids with respect to lower acetylated and low molecular weight chitosan-based counterparts.

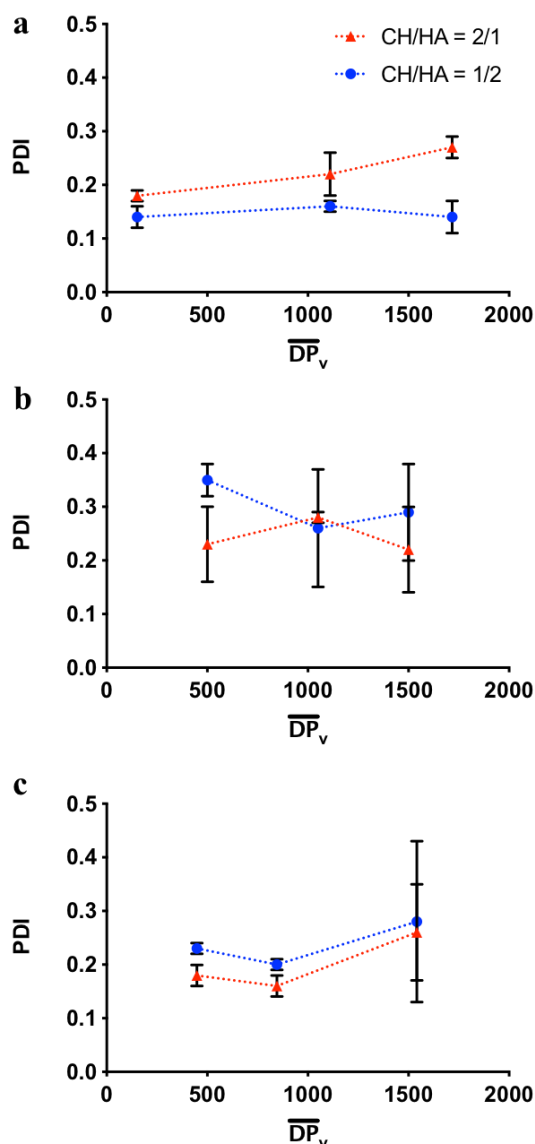


Figure 3. PolyDispersity Index (PDI) of CH/HA complex coacervates showing different polymers weight ratio (CH/HA = 1:2 and 2:1). Colloids were assembled using chitosans with fraction of acetylated units (F_A) of 0.16 (a), 0.46 (b) and 0.63 (c), respectively. Data are reported as a function of the chitosan average degree of polymerization, i.e., \overline{DP}_v . Colloids were analyzed by DLS after dilution 1:10 (v/v) in deionized water. Data are means (\pm SD) of at least three measurements.

Finally, surface charge measurements were undertaken to give further insights onto colloids physical properties. ζ -potential analyses pointed at a different colloids surface charge depending on the weight ratio between chitosan and hyaluronan (Figure 4). As a general consideration, a positive surface charge was detected for chitosan-prevalent colloids (i.e., weight ratio CH/HA = 2:1), whereas a negative surface charge was found for their counterparts (i.e., weight ratio CH/HA = 1:2). This opposite behavior clearly reflects the different polycation/polyanion balance throughout the surface matrix. In the case of $F_A = 0.16$ chitosan, the increment of molecular weight associated with an increase of colloids surface charge, indicating that a greater amount of D-type sugars, i.e., glucosamines, are available at the colloids surface. This typical behavior was detected both for CH/HA 1:2 and 2:1 coacervates (Figure 4a). For $F_A = 0.46$ chitosan, ζ -potential was found to increase up to chitosan $\overline{M}_v = 210,000$, and subsequently decrease for higher chitosan degree of polymerization (Figure 4b). Lastly, an almost independency on chitosan molecular weight was noticed with $F_A = 0.63$ chitosan for both CH/HA 1:2 and 2:1 colloids (Figure 4c).

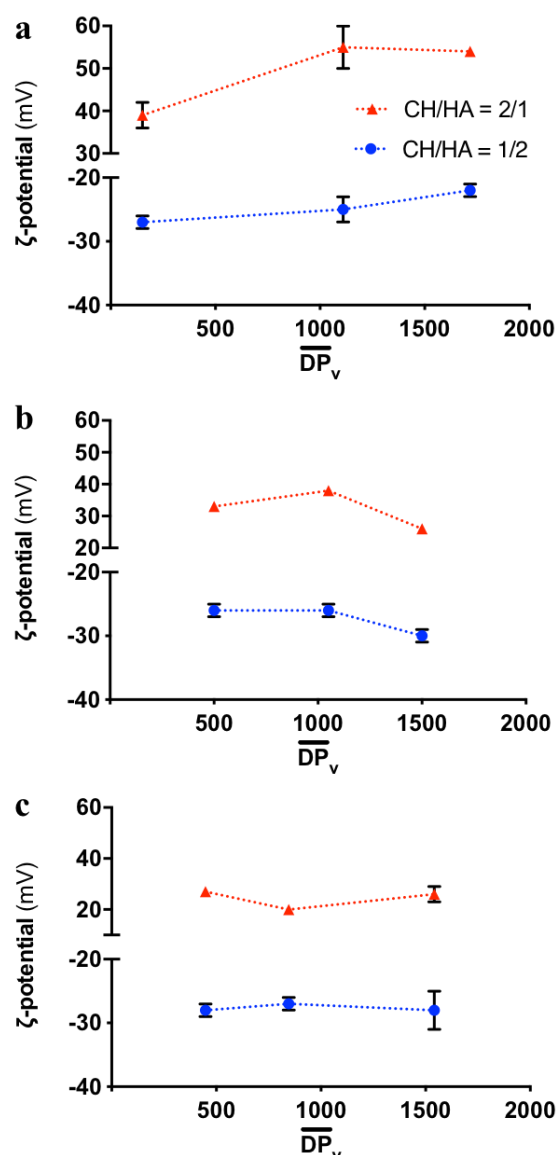


Figure 4. Surface charge, i.e., ζ -potential, of CH/HA complex coacervates showing different polymers weight ratio (CH/HA = 1:2 and 2:1). Colloids were assembled using chitosans with fraction of acetylated units (F_A) of 0.16 (a), 0.46 (b) and 0.63 (c), respectively. Data are reported as a function of chitosan average degree of polymerization, i.e., \overline{DP}_v . Colloids were analyzed by DLS after dilution 1:10 (v/v) in deionized water. Data are means (\pm SD) of at least three measurements.

4. Conclusions

In this work, the complex coacervation between chitosans of varying physical-chemical composition and a low molecular weight hyaluronan has been exploited as method to produce polysaccharide-based colloids. Experimental variables as well as polymers weight ratio, chitosan acetylation degree and molecular weight have been varied to verify the formation and quality of related colloids. The main conclusions arisen from experimental evidences are the following:

- (i) Low acetylation as well as low polycation molecular weight determine a true liquid-liquid phase separation without formation of aggregates; in these conditions, the dilute phase (the supernatant) is in equilibrium with the dense phase (the coacervate);
- (ii) Chitosan-to-hyaluronan mass ratio directs colloids surface charge;
- (iii) Chitosan physical-chemical features influence colloids dimension and homogeneity.

Overall, this contribution provides additional knowledge about the complex coacervation between chitosan and hyaluronan, with the potential translation of related networks in food and biomedical sectors.

Author Contributions: Conceived and designed the experiments: I.D., P.S. Performed the experiments: F.F. Analyzed DLS data: F.F., I.D., E.M., P.S. Wrote the paper: F.F., P.S. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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