EFFECT OF HOFMEISTER IONS

ON THERMODYNAMICS OF COMPLEX COACERVATION BETWEEN HYALURONIC ACID AND CHITOSAN

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Abstract

Franz Hofmeister's research in 1888 led to the discovery of the Hofmeister series, an ion series the effects of which on the behavior of aqueous protein solutions were found to be significant. Other biomacromolecules also had their stability in solution determined by the presence of Hofmeister series. With respect to thermodynamics, this work seeks to understand the impact of this series on the complexation and coacervation of hyaluronic acid (HA) with chitosan (CHI) at three distinct pHs (3.25, 5.25, and 6.25) and two different molecular weights (HA, 1200 kDa & 199 kDa). While light microscopy images were used to confirm that the HA/CHI mixtures led to coacervates and not just precipitate particles, turbidimetric titration experiments were used to optimize the conditions affecting coacervation such as salt type, pH and concentration of buffering agent and polyelectrolyte. It has been determined that isothermal titration calorimetry is useful for comprehending the thermodynamics of coacervation. Our results indicate the validity of the direct Hofmeister effect for cations and the reverse Hofmeister effect for anions. Furthermore, the salt screening effect is readily evident since the interaction between the two polyelectrolytes is strongest when salt is absent. Additionally, it was found that as pH was increased, there was a stronger interaction between the two macromolecules.
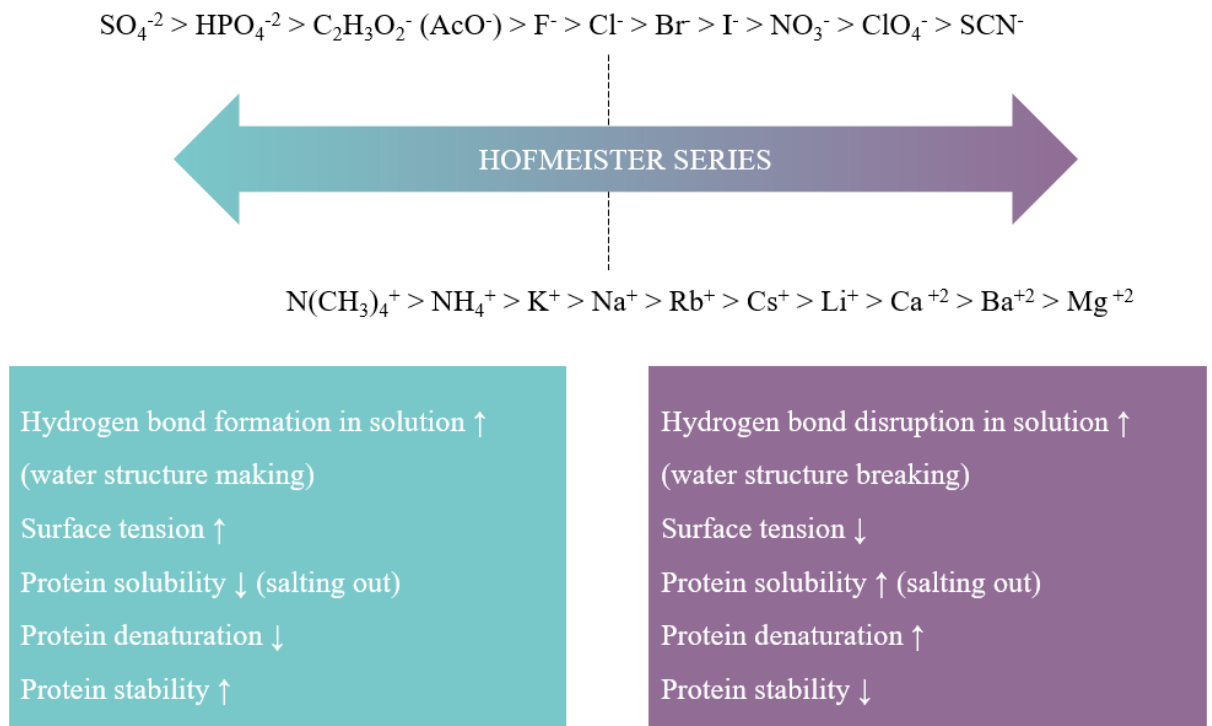
Introduction

Complex coacervation, a liquid-liquid phase separation in aqueous media, happens when two or more macromolecules with opposing charges associate with each other. Ionic

biomacromolecules such proteins, ionic polysaccharides, RNA, and DNA are the most often favored examples of the macromolecules that make up the complex coacervates (Aumiller et al., 2014). The sandcastle worm *Phragmatopoma Californica*, for instance, has been found to secrete proteins rich in polar amino acids which bind foreign mineral particles together through complex coacervation (Zhao et al., 2005). Meanwhile, application of coacervates for the encapsulation of small substances, RNA, DNA, proteins, and other biomaterials has been the subject of several investigations(LWJ Holleman et al., n.d.; Turgeon et al., 2007). Recently, coacervate droplets from ATP-cationic protein mixtures were even used to develop protocells that experience active growth (Nakashima et al., 2021).

It has been demonstrated that coacervation is affected by several factors including temperature, pH, ionic strength, the concentration and molecular mass of polymers, the charge density of polyelectrolytes, and the mixture's stoichiometry (Kayitmazer et al., 2015). Electrostatic interaction of oppositely charged macromolecules coupled with the entropy gain brought on by the release of the counterions leads to the coacervation process (ben Messaoud et al., 2018; Kayitmazer, 2017; Ou & Muthukumar, 2006)

The factors described above affect how the phase separation develops, how stable the coacervation is, and how the characteristics of the coacervates are determined. Another factor of the ionic environment is under investigation in this study. Based on the hydration of ions and proteins and the in-solution behaviors of proteins according to their polarity and size, the Hofmeister series is a useful, qualitative list of ions (Mazzini & Craig, 2017). The list takes its name after Franz Hofmeister who investigated how cations and anions affected the solubility of proteins(Hofmeister, 1888).

The effects of the Hofmeister series are mostly explained by more specific interactions between ions and proteins, and between ions and water molecules in proximity to proteins, albeit the exact process is not fully understood(Fu & Schlenoff, 2016; Ou & Muthukumar, 2006). These ions have the ability to change how the molecules in the solution behave by supporting or inhibiting the hydrogen bonds between the water molecules (Moelbert et al., 2004). The hydrophobic forces may predominate as a result of the presence of the kosmotropic ions (left hand side in Figure 1), increasing the interaction between the solute particles. On the other hand, the chaotropic ions (right hand side in Figure 1) can promote a more favorable contact between the solvent molecules and the particles (the biomacromolecules under study), increasing the solubility.

$$SO_4^{-2} > HPO_4^{-2} > C_2H_3O_2^- (AcO^-) > F^- > Cl^- > Br^- > I^- > NO_3^- > ClO_4^- > SCN^-$$

HOFMEISTER SERIES

$$N(CH_3)_4^+ > NH_4^+ > K^+ > Na^+ > Rb^+ > Cs^+ > Li^+ > Ca^{+2} > Ba^{+2} > Mg^{+2}$$

Hydrogen bond formation in solution ↑ (water structure making) Surface tension ↑ Protein solubility ↓ (salting out) Protein denaturation ↓ Protein stability ↑	Hydrogen bond disruption in solution ↑ (water structure breaking) Surface tension ↓ Protein solubility ↑ (salting out) Protein denaturation ↑ Protein stability ↓

Figure 1. Effect of Hofmeister series ions on proteins. (Bockris & Saluja, 1972; Collins & Washabaugh, 1985; Hasted, 1973; Marcus, 1996; Mason et al., 2010; Mazzini & Craig, 2017; Sadeghi & Jahani, 2012; Zhang & Cremer, 2006)

Based on liquid-liquid phase separation as a consequence of aggregation of lysozyme, Zhang and Cremer presented the reverse Hofmeister effect at low salt concentrations and the direct Hofmeister effect at salt concentrations greater than about 200-300 mM(Zhang & Cremer, 2009). The presence of these two series were explained by pointing out that the anions with bigger volumes (ClO_4^- and SCN^-) were better at shielding, and could interact with positively charged lysozyme sites due to their lower tendency for hydration. Additionally, it was suggested that positively charged macromolecules which were previously functioning under the reverse Hofmeister effect at low salt concentrations would be exposed to the direct Hofmeister effect as a result of an increase in the concentration of the chaotropic anions. Mason et al. observed a similar behavior on the liquid-liquid phase separation of humanized IgG2 (M_w: ~148 kDa, pI = 7.2), experiencing the reverse effect at low concentrations of salt and the direct effect at high concentrations of salt (Mason et al., 2010).

The effect of this series has lately been studied not just for proteins but also for other biomacromolecules and natural or synthetic polymers. Li et al. examined the coacervation of

amphoteric diallyl dimethylammonium chloride and sodium styrene sulfonate copolymer (Li et al., 2018). Regarding the effect of the Hofmeister series on coacervation of this polyampholyte, they found that sulfate, phosphate, and acetate ions did not inhibit the coacervate formation. This was contrary to expectations because these ions belong to the class of kosmotropes that reduce polymer solubility. Meanwhile, Perry et al. (Perry et al., 2014)examined the effect of Hofmeister series on coacervation of poly (acrylic acid sodium salt) (pAA) and poly (allylamine hydrochloride) (pAH), using turbidimetric titrations. They concluded that the salting-in effect caused the rate of coacervation to increase at salt concentrations lower than 75 mM for all salts employed in the research. Although addition of salt to the medium enhanced the solubility of polyelectrolytes, the stability of the phase separation was disturbed. Nevertheless, the ions studied displayed behavior of direct Hofmeister series with respect to the coacervation of pAA and pAH.

A quantitative and thermodynamic method that can be employed specifically for the detection of interactions between biological molecules is known as isothermal titration calorimetry (ITC) (Pierce et al., 1999). By determining the enthalpy of mixing of bovine serum albumin (BSA) in various salt solutions, Janc et al. observed the effect of reverse Hofmeister series on the system (Janc et al., 2018). Combining Raman spectroscopy and ITC studies, Fu and Schlenoff investigated the complex coacervation of poly (diallyl dimethylammonium chloride) (PDADMAC) and poly (styrene sulfonate, sodium salt) (Fu & Schlenoff, 2016). The results indicated that the ions that support structure of water, aka kosmotropes, provided an endothermic contribution while enhancing the hydrogen bonds and reducing the ΔH_{PEC} (enthalpy of polyelectrolyte complexation). Comparison of ΔH_{PEC} versus $-T\Delta S_{PEC}$ values revealed that entropy was responsible for the complexation of these two polyelectrolytes. Another study investigated the interaction between integration host factor, an *E. coli* protein and H' of DNA, and determined the binding enthalpy by using ITC (vander Meulen et al., 2008). However, no relationship with the Hofmeister series could be concluded with the salt ions studied.

In the literature, only protein/protein, protein/polyelectrolyte, and synthetic polyelectrolyte/polyelectrolyte systems were examined for the influence of Hofmeister series on coacervation. The effect of the Hofmeister series on the coacervation between two physiologically-derived oppositely charged polyelectrolytes with relatively large persistence lengths, which are semi-flexible range, however, has not been studied in the literature to the

best of our knowledge. Coacervation conditions between anionic hyaluronic acid (HA, persistence length of 4 nm (Hayashi et al., 1995) and cationic chitosan (CHI, persistence length of 6.5 nm (Berth et al., 2002) were investigated in a prior work (Kayitmazer et al., 2015). However, in the study, the sole salt component employed was NaCl. Another research (Karabiyik Acar et al., 2018), employed HA-CHI coacervation to create scaffolds using NaCl and $MgCl_2$ salts, with only two different salt types being examined. But to fully comprehend the impact of the Hofmeister series, more salts must be investigated.

The primary objective of this study is to investigate how the Hofmeister series affects HA-CHI coacervation in terms of thermodynamics using ITC. Since HA and CHI are biocompatible and biodegradable polymers and may be employed as tissue scaffolds constructed of HA-CHI coacervates, the knowledge acquired from this effort will be crucial for biomaterials applications.

Materials

The high molecular weight sodium salt of hyaluronic acid (HA, molecular weight of 1.2 MDa as determined by viscosity experiments) was kindly donated as a gift by Dr. Kazuyuki Miyazawa from Shiseido Co. (Yokohama, Japan). The low molecular weight sodium hyaluronate (HA, molecular weight of 199 kDa as determined by viscosity experiments, Lot Number: 026564) was purchased from Lifecore Biomedical (Chaska, MN, USA). Chitosan HCl (CHI, Molecular weight 396 kDa, determined from gel permeation chromatography, Product Code: 54039) with 83% degree of deacetylation (DDA) was purchased from Heppe Medical Chitosan GmbH (Halle, Germany). NaCl (Product Code: 106404), KCl (Product Code: 104936), and $NaOAc.3H_2O$ (Product Code: 106267) were purchased from Merck (Darmstadt, Germany). Citric acid (99%, Product Code: C0759) and 2-(N-Morpholino)ethanesulfonic acid hydrate (MES hydrate, >99.5%, Product Code: M8250) were purchased from Sigma Aldrich (Massachusetts, USA). $NaNO_3$ (Product Code: 481757), and $MgCl_2.6H_2O$ (Product Code: 459337) were purchased from Carlo Erba (Milano, Italy). Research grade $CaCl_2.2H_2O$ was purchased from VWR Chemicals (Product Code: 22317.260) (Ohio, USA) and ISOLAB Chemicals (Product Code: 909.026) (Wertheim, Germany). Methanol used for ITC washing (liquid chromatography grade, Product Code: 947.047.2501), research-grade 2.00, 4.00, 7.00, and 10.00 buffer solutions used for pH meter calibration (Product Codes: 908.B02, 908.B04, 908.B07 and 908.B10, respectively) and 1 N and 0.1 N NaOH & HCl solutions used for pH

adjustments (Product Codes: 969.20V, 969.22V, 932.13V, and 932.15V, respectively) were purchased from ISOLAB Chemicals (Wertheim, Germany). All solutions were filtered using 0.45 µm cellulose acetate membrane filters (Product Code: 56133345, 56253345) from Labmarker (Istanbul, Turkey). The semipermeable membranes, snakeskin tubes of MWCO 10000 g/mol and 3500 g/mol (product code: 68100 and 680035, respectively) were purchased from Thermo Scientific™ (Massachusetts, USA). Isopropyl alcohol (Product Code: TK.090250.02501) used for slide and coverslip cleaning was purchased from ISOLAB Chemicals (Wertheim, Germany). Nitrogen gas (99.999% purity) used for the same purpose was purchased from Linde Gaz (Dublin, Ireland).

Methods

Dialysis for Counterion Exchange

In Milli-Q water, 75 mL of polyelectrolyte solutions containing either 0.5 mg/mL HA or 0.5 mg/mL CHI were prepared. Separately, again in Milli-Q water, 0.1 M solutions of the salts containing the ions to be exchanged with were prepared. The semipermeable membranes were then submerged in 5 L of salt solutions after being filled with the polymer solutions. Polymers were dialyzed sequentially for 24 hours against three batches of the produced salt solutions. Then, three days of dialysis against Milli-Q water followed with the water being changed every 24 hours.

Through the SEM-EDS analyses (FEI-Philips XL30 Environmental Scanning Electron Microscope with Field Emission Gun (equipped with Energy Dispersive X-ray Analysis Unit, EDAX) conducted in the Advanced Technologies Research and Development Center of Bogazici University, the replacement of the counterions was verified.

Turbidimetric Titration

MES or citric acid and the corresponding salt were first dissolved in a volumetric flask to obtain buffered salt solutions. The pH values were then adjusted by adding basic or acid solutions (1 N or 0.1 N NaOH or 1 N or 0.1 N HCl or Glacial AcOH for NaOAc condition) to get the necessary pH value for the experiment (6.25 ± 0.5, 5.25 ± 0.5, or 3.25 ± 0.5). Then, in these salt solutions, the HA and CHI polymers were individually dissolved. The solutions with the conditions described in Table 1 were mixed for two hours to prepare well-dissolved solutions of 0.5 or 0.1 mg/mL CHI and 0.5 mg/mL HA.

Table 1. Concentrations of each salt used in the experiments with different experimental conditions.

Salt Type	Concentration of salt (mM)	I_{total} of the medium with 5 mM concentrated MES or citric acid as the buffering agents
NaCl	47	50 mM
KCl	47	50 mM
CaCl$_2$	15.67	50 mM
MgCl$_2$	15.67	50 mM
NaNO$_3$	47	50 mM
NaOAc	47	50 mM

The HA solution was titrated into the CHI solution. Both the titrant and the titrate had exactly 10 times the volumes used in the ITC experiments). Using a colorimeter (PC950, Brinkmann, USA), the titrated solution's percent transmittance (% T) was continually monitored and converted to % Turbidity (100 - % T). The titration intervals and total number of additions in the titrations were kept the same as the experiments with the ITC.

Light Microscopy

Samples of HA/CHI mixtures with molar ratios of 0.16, 0.35, and 0.52 were taken from a concurrent experiment of turbidimetric titration. Images were captured with an inverted light microscope (CTR6000, Leica, USA). Particle size analysis and monitoring of precipitates and coacervates were done using these images. The Adobe Photoshop (Version 22.1.20169.0) application was used to analyze particle size and type. First, three areas on the images were arbitrarily marked. By converting pixels to millimeters, the size of each particle was determined using the ruler tool and noted on the picture. The particle was classified as a coacervate if it was spherical and a precipitate if it was not. It was also checked whether measurements on every angle gave the same size. Then the number of coacervate droplets were calculated and converted into percentages using the Equation (1) and compiled in Table 2:

$$\% \ coacervates = \frac{(number \ of \ coacervates)}{(number \ of \ coacervates + number \ of \ precipitates)} \times 100 \quad (1)$$

Isothermal Titration Calorimetry (ITC) Experiments with Dialysis and Stocked-Solution Method

The polymer solutions were prepared in the same way as for the experiments of turbidimetric titration, except they had four times the concentrations (2.0 mg/mL HA and 0.4 mg/mL CHI). All other concentrations of materials were identical to those used in the turbidimetric titration studies.

Thermo Scientific™ (Massachusetts, USA) semi-permeable membranes with a molecular weight cut-off limit of 10 kDa were used to dialyze the polymer solutions, replenishing the buffered salt solutions every three hours over the course of five batches. The goal of this procedure is to eliminate the heat from buffer mismatching as a result of the dilution of the buffered salt solution ions. Following dialysis, polyelectrolyte solutions were diluted using the last batch of the buffered salt solution to 0.1 mg/mL (CHI) and 0.5 mg/mL (HA), respectively, and mixed for two hours. All solutions were then filtered through cellulose acetate membranes with 0.45 μm pore sizes (Labmarker, Istanbul, Turkey). All solutions to be used in the ITC instrument were degassed using the Thermovac degasser (Malvern, England) prior to the experiments. Since precipitation was observed when CHI solution was added to HA solution (Kayitmazer et al., 2015), HA solution is titrated into CHI solution in the sample cell using the ITC instrument (ITC200, Malvern, England) with 0.1 mg/ml CHI & 0.5 mg/ml HA dissolved in the buffered salt solution of NaCl at pH of 5.25 and I_{total} of 50 mM. Room temperature was used for the experiments. Every experiment was carried out for a minimum of four times.

ITC Data Analysis

The method of summing (Fu & Schlenoff, 2016; Niskanen et al., 2021; Yang et al., 2020) is used to obtain the enthalpy of complexation values with lower standard deviation values. First, the average enthalpy of the last 15 of 20 titrations was calculated for the buffer solution titration into buffer solution (BiB) and buffer solution titration into chitosan solution (BiC) experiments, separately; i.e. $\Delta H_{BiB_{avg}}$ and $\Delta H_{BiC_{avg}}$, respectively. Second, the average enthalpy values of the titration of HA solution into CHI solution (HiC) and the HA solution into buffer solution (HiB) were calculated separately; i.e. $\Delta H_{HiC_{avg}}$ and $\Delta H_{HiB_{avg}}$, respectively. Third, the total enthalpy of the dilution was subtracted from the enthalpy of HA to CHI titration to calculate the enthalpy of complexation (ΔH_c) as given in the Equation (2). For every experimental condition, the standard deviation of enthalpy for three different runs was calculated.

$$\Delta H_c = \Delta H_{HiC_{avg}} - \left(\Delta H_{HiB_{avg}} + \Delta H_{BiB_{avg}} + \Delta H_{BiC_{avg}} \right) \qquad (2)$$

Results And Discussion

To solely investigate the effect of a certain ion, the original counterion of the polymer was exchanged with that certain ion. For example, to understand the effect of acetate ions, Cl^- which is the original counterion of CHI, was exchanged with CH_3COO^-. On the other hand, to determine the effect of Mg^{+2} ions, Na^+ which is the original counterion of HA, was exchanged with Mg^{+2}. The replacement of ions was confirmed with EDAX (Figures SI 1 – 7).

Experimental conditions were selected after consideration of various factors. For example, experiments were done at pH values less than 6.5 since chitosan is known to precipitate at higher pH (Qin et al., 2006). Thus, pH values of 6.25, 5.25 and 3.25 were chosen for this study. To understand the effect of Hofmeister series on HA/CHI coacervation, the following points were considered when determining the type of salt ions: 1) Salts that are water-soluble at concentrations in the range of 0 - 0.8 M were used, 2) HA-CHI mixture should not precipitate in the solution of the salt used, 3) salts with low toxicity considering the future possible biomedical applications of coacervates.

Buffers are widely used in the experiments done using ITC (Alatorre-Meda et al., 2010; Al-Qadi et al., 2013, 2016; Bharadwaj et al., 2006; Bončina et al., 2010; Courtois & Berret, 2010; Flanagan et al., 2015; Harnsilawat et al., 2006; Jelesarov & Bosshard, 1999; Vitorazi et al., 2014). Good's buffers are used especially in biological systems and are found to be "ideal" for biological research (Taha et al., 2014). Thus, MES is chosen for experiments done at pH 6.25 since the pK_a of this buffer is 6.15 at 20°C (Good et al., 1966). To ensure that the buffering agent was effective throughout the experiment, change of pH at different mole ratios of HA to CHI was recorded. As seen in Figure 2, the pH value remained in the desired range. 9:1 salt:buffering agent molar ratio was maintained for all experiments to minimize the energetical effects that could arise from the dilution of the buffering agent in ITC. In the optimization experiments done with 0.1 mg/mL CHI and 0.5 mg/mL HA, precipitation was observed for higher molar ratios of HA/CHI mixtures in NaCl & $MgCl_2$ salts at $I_{total} \geq 100$ mM (Figures SI: 8 – 11). Therefore, we did the rest of the experiments at total ionic strength of 50 mM.
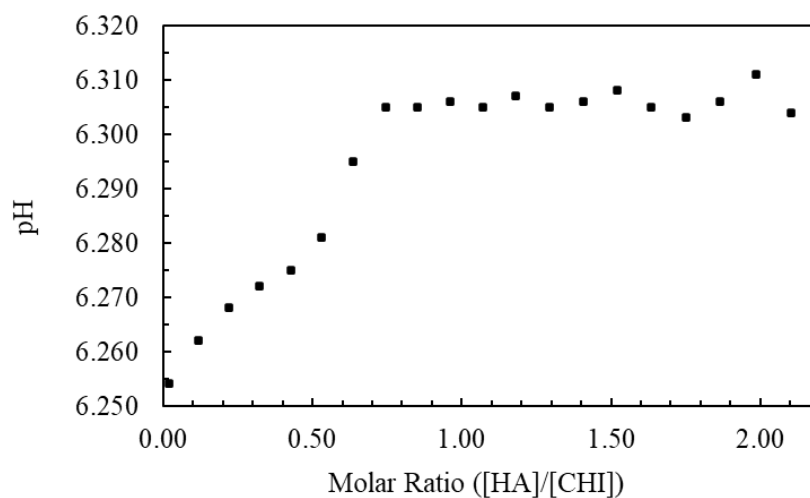
Figure 2. Change of pH with molar ratio of HA to CHI prepared from solutions of initial concentrations of 0.3 mM CHI & 3.0 mM HA dissolved in buffered salt solution at pH of 6.25 ± 0.05, I_{total} of 50 mM (1:9 mole ratio of MES:NaCl).

The results of the turbidimetric titration experiments with different cations of salts with Cl⁻ at pH of 6.25 are given in Figure 3. At higher molar ratios of [HA]/[CHI], the order of the turbidity values for the cations was $Mg^{+2} > Ca^{+2} > K^+ > Na^+$. This indicates that the yield of coacervation also follows this order because formation of a higher number and/or larger size of complexes should lead to higher total scattering intensity, and consequently larger turbidity values. This order matches well the direct Hofmeister series effect aside from the observation that K^+ appears before Na^+. For cations at pH = 6.25, an increase in turbidity was observed when switching from monovalent to divalent ions. This increase indicates that divalent ions promote polyelectrolyte complexation and coacervation. The results at I_{total} = 50 mM are in agreement with Perry et al. (Perry et al., 2014), who observed that at low ionic strength values, phase separation between poly(acrylic acid) and poly(allylamine) at pH = 6.5 was more favorable in salts with divalent ions than monovalent ones. Unfortunately, turbidimetric titrations of HA to CHI in different salt anions (NaX⁻, X⁻ being NO_3^- or OAc⁻) cannot be reported since CHI was not dissolved in 1:9 MES:NaX solutions.
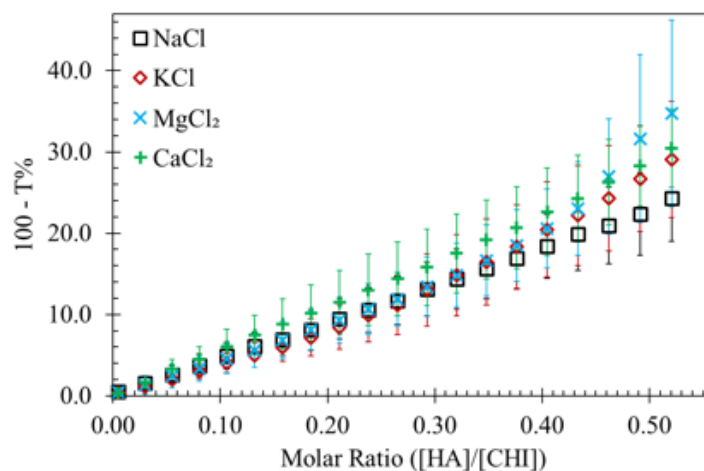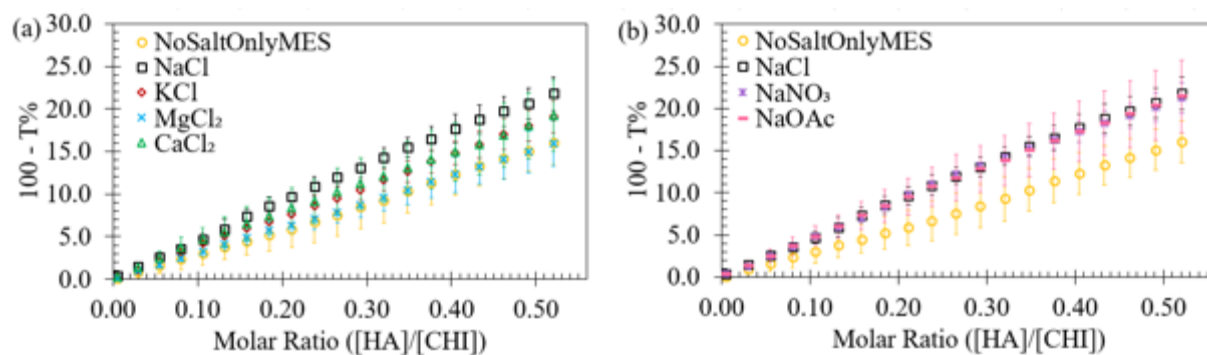
Figure 3. Plot of turbidity (100 - T%) vs. molar ratio ([HA]/[CHI]) in MES buffered salt solution of pH of 6.25 ± 0.05 at I_{total} = 50 mM. Initial concentrations of CHI and HA (1200 kDa) are 0.1 mg/ml and 0.5 mg/mL, respectively.

The effect of pH on turbidity of HA/CHI mixtures at different mole ratios was also examined with different types of salt (Figure 4 & 5). pH 5.25 was chosen since this pH is within the region where the buffering agent MES is still effective. For the experiments at pH 3.25, citric acid was used since its pK_{a_1} is 3.1. At pH 3.25 and 5.25, the maximum turbidity values at the highest [HA]/[CHI] in different cations of salt with Cl$^-$ did not follow the Hofmeister series. For example, the maximum turbidity order at the highest mole ratio for cations is $Na^+ < Mg^{+2} < K^+ < Ca^{+2}$ at pH = 5.25 and $Mg^{+2} < K^+ = Ca^{+2} < Na^+$ at pH = 3.25. The lower the pH, the more complex the order becomes. On the other hand, all the anions had similar turbidities at constant pH, as shown in the Figures 4 and 5. This result indicates that the Hofmeister series did not have any effect on the coacervation of the HA/CHI system at pHs of 5.25 and 3.25 at I_{total} of 50 mM.
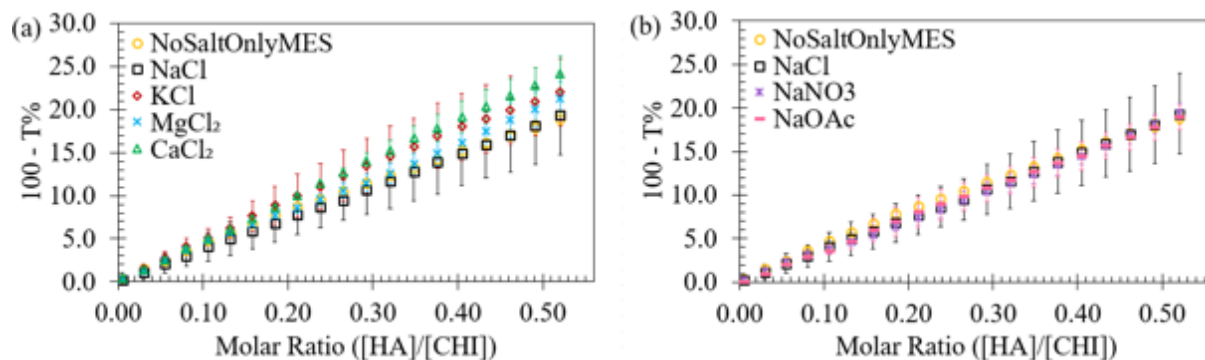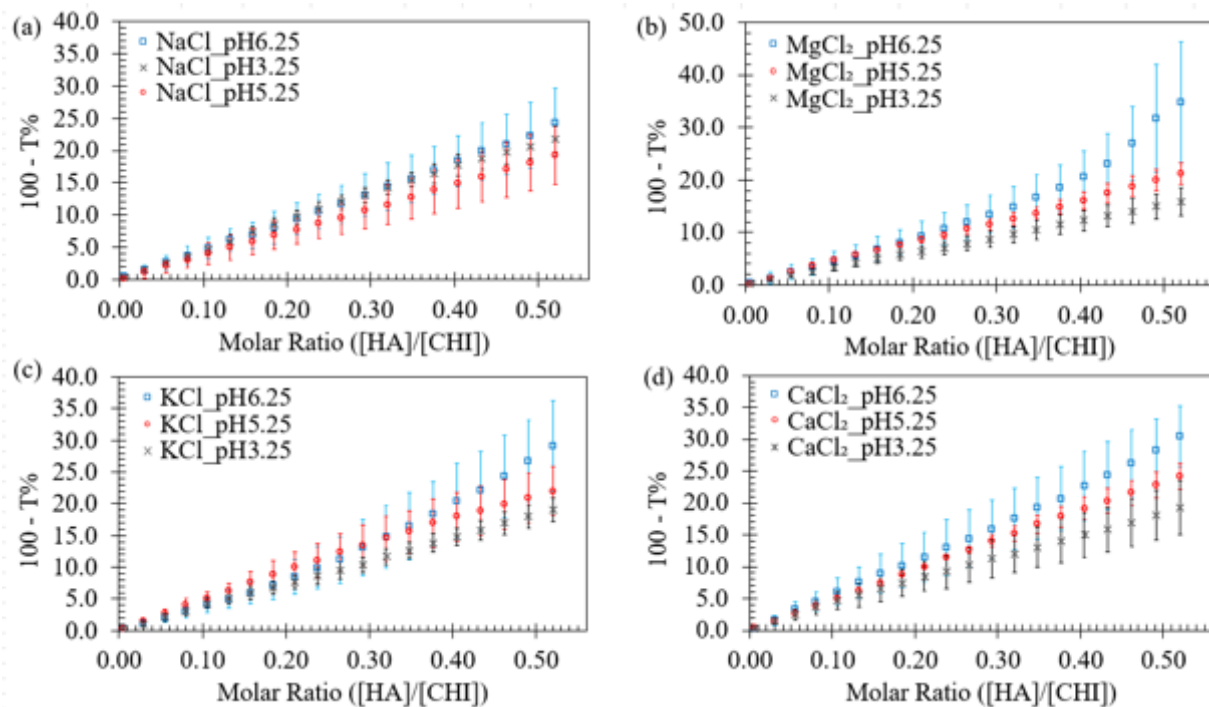
Figure 4. Plot of turbidity (100 – T%) vs. molar ratio ([HA]/[CHI]) in MES buffered salt solutions with (a) all the cations, (b) all the anions at pH = 5.25 ± 0.05 and I_{total} of 50 mM. Initial concentrations of CHI and HA (1200 kDa) are 0.1 mg/ml and 0.5 mg/mL, respectively.
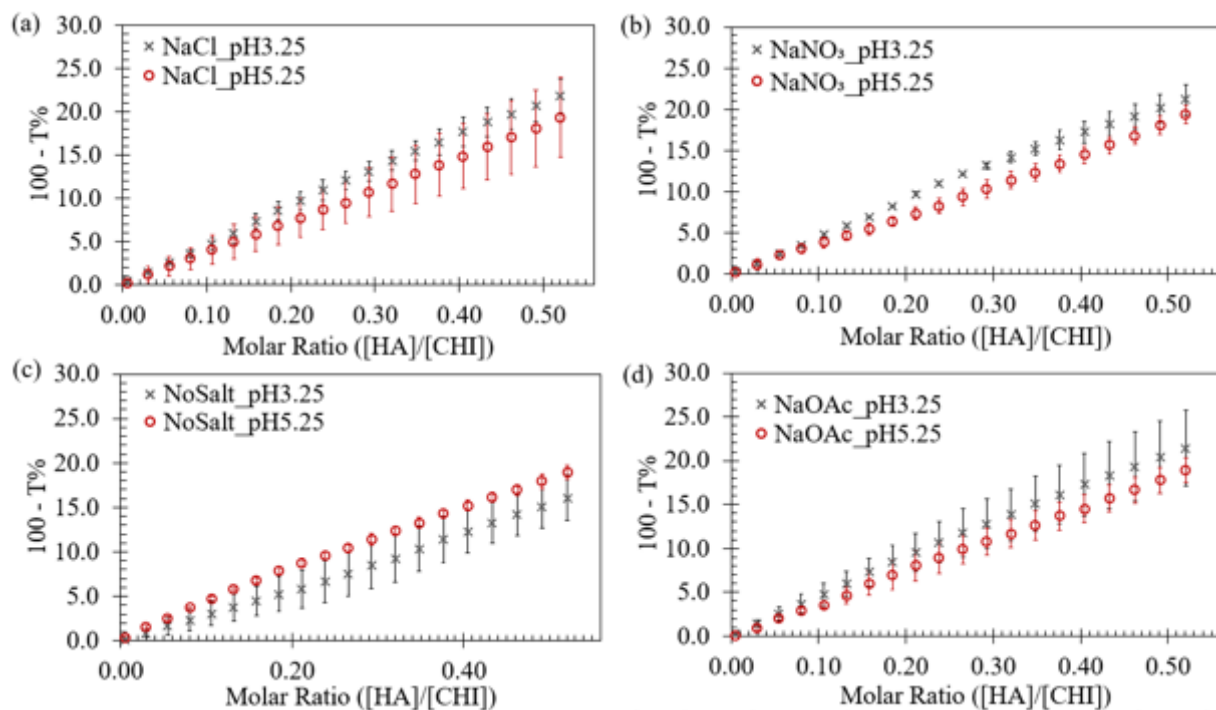

Figure 5. Plot of turbidity (100 - % T) vs. molar ratio ([HA]/[CHI]) in citric acid buffered salt solutions with (a) all the cations, (b) all the anions at pH = 3.25 ± 0.05 and I_{total} of 50 mM. Initial concentrations of CHI and HA (1200 kDa) are 0.1 mg/ml and 0.5 mg/mL, respectively.

As can be seen in Figure 6, for all the cations, turbidity increased as the pH increased from 3.25 to 6.25. For the anions, the opposite trend is observed (Figure 7). For the case of OAc^-, this result can be attributed to the basicity of this anion. Here, the contribution of counterion release to the increase in the entropy, and consequent favoring of coacervation should be emphasized. It should also be noted that the acidity of the medium increases the tendency of the weakly basic anions to be released from the vicinity of the polyelectrolyte to the bulk. Therefore, in acetate containing salt solutions, coacervation is enhanced in the more acidic medium.

Figure 6. Plots of turbidity (100 – T%) vs. molar ratio ([HA]/[CHI]) at different pHs for (a) NaCl, (b) MgCl$_2$, (c) KCl, (d) CaCl$_2$ salts. All experiments were done using initial concentrations of 0.1 mg/mL CHI & 0.5 mg/ml HA dissolved in the buffered salt solutions of I$_{total}$ of 50 mM.

Figure 7. Plots of turbidity (100 – T%) vs. molar ratio ([HA]/[CHI]) at different pHs for (a) NaCl, (b) NaNO$_3$, (c) No Salt Only MES, (d) NaOAc conditions. All experiments were done using initial concentrations of 0.1 mg/mL CHI & 0.5 mg/mL HA dissolved in the buffered
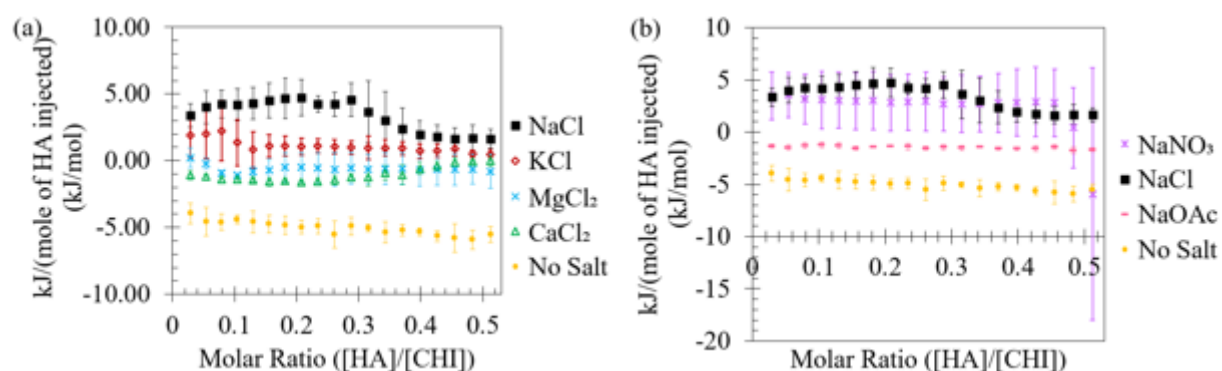
salt solutions of I_{total} of 50 mM. The condition of "No Salt" condition only had 5 mM MES in the medium as solvent.
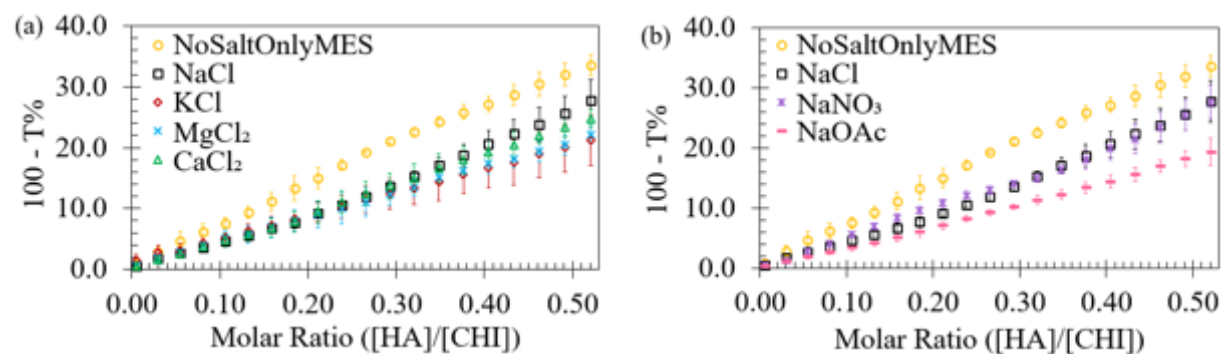
ITC Experiments

In our ITC experiments with 1200 kDa HA, we observed random signals from the thermograms (data not shown). This might be attributed to the near-overlap concentration of 1200 kDa HA in the titration syringe, i.e. 0.5 mg/ml. At overlap concentration greater than or equal to 0.8 mg/ml (Madau et al., 2021), HA chains are known to overlap with each other. We speculate that as HA is titrated into the sample cell, it will be diluted, and the overlapped HA chains will start to dissociate. However, if our four minutes of titration interval were not enough for all the chains to be freed from the overlapping, there would be a chaotic medium where there would be both overlapped and free chains, the latter being available for interaction with CHI. Meanwhile, if the overlapped chains were still able to interact with the CHI in the medium, they would cause higher exothermic contributions than free ones since the overall charge densities of the overlapped form would be higher than their free forms, and molecules with high charge densities release more energy during their interaction with other molecules (Kayitmazer, 2017). In this way, there would be both small and high amounts of energy resulting from overlapped and free HA chains during their interaction with CHI chains. This would be contributing to the appearance of random-like heat graphs. The overlapping concentration value increases as the molecular weight decreases(Snetkov et al., 2020).

Thus, a lower molecular weight HA would help get away from the overlap concentration when used at similar concentrations with 1200 kDa. Considering the discussion above, we decided to use HA with 199 kDa for the ITC experiments. Results of the turbidimetric titrations are given in Figure 9 and the results of the ITC experiments are presented in Figure 10.

Figure 8. Thermograms of ITC experiments: (a) for cations, (b) for anions. All experiments were done using initial concentrations of 0.1 mg/mL CHI & 0.5 mg/mL HA (1200 kDa) dissolved in MES buffered salt solution of pH of 5.25 ± 0.05, I_{total} of 50 mM.

Figure 9. Turbidity (100 – T%) vs. molar ratio ([HA]/[CHI]) curves for (a) all the cations, (b) all the anions. Experiments were done with initial concentrations of 0.1 mg/mL CHI & 0.5 mg/mL HA (199 kDa) dissolved in MES buffered salt solution of pH of 5.25 ± 0.05, I_{total} of 50 mM.
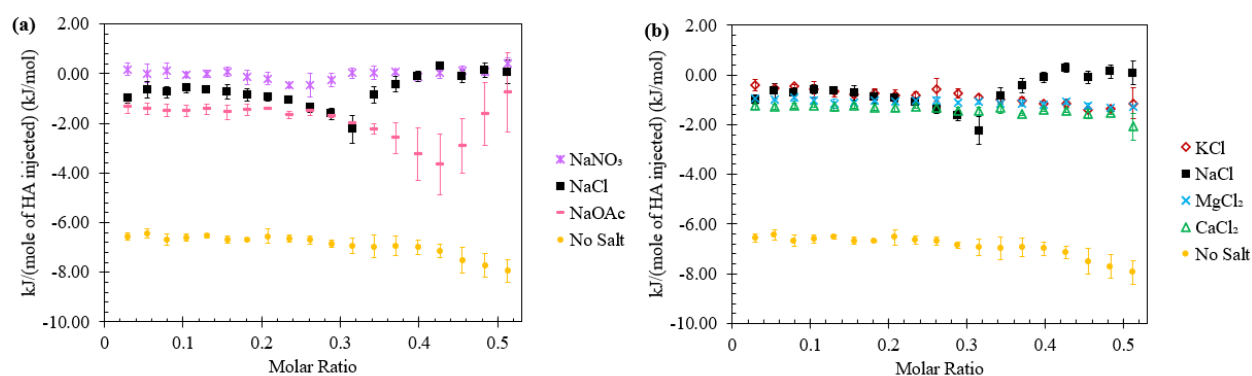
Figure 10. ITC thermograms (a) for anions, (b) for cations. All experiments were done using initial concentrations of 0.1 mg/mL CHI & 0.5 mg/mL HA (199 kDa) dissolved in MES-buffered salt solution of pH of 5.25 ± 0.05, I_{total} of 50 mM.

The commonly accepted hydration order of the cations used in this study is $Mg^{+2} > Ca^{+2} > Na^+ > K^+$ (Bockris & Saluja, 1972; Marcus, 2010; Sadeghi & Jahani, 2012; Schlenoff et al., 2008) and the one for the anions used in this study is $OAc^- > Cl^- > NO_3^-$ (Mazzini & Craig, 2017; Patel et al., 2014; Schlenoff et al., 2008; Sullivan et al., 2018; Yang et al., 2020). However, the order of these ions in the series differs in the literature. For example, in some studies, the order of the Mg^{+2} and Ca^{+2} ions are presented in reverse order to the one presented before; i.e. $Ca^{+2} > Mg^{+2}$ (Hyde et al., 2017; Perry et al., 2014). In another study, the hydration

numbers of these divalent ions are found equal (Israelachvili, 2011). The results of our study are in agreement with the divergent orders giving $Ca^{+2} > Mg^{+2} > K^+ > Na^+$. In accordance with the work by Oppermann and Schulz, where the ΔH_c values of the complex formation in the salt solutions with Mg^{+2}, Ca^{+2}, Na^+ and K^+ are found as 2.18 kJ/mole, 2.21 kJ/mole, 1.70 kJ/mole and 1.21 kJ/mole respectively, the enthalpy of the complex formation in the presence of divalent cations are found to be more exothermic than the monovalent cations. (Oppermann & Schulz, 1990). Considering this order, the ITC results in Table 3 can be justified. Since more hydrated ions tend to be expelled more easily from the site of polyelectrolyte complexation (Fu & Schlenoff, 2016), coacervation in the presence of these ions would be more favorable with negative ΔH_c values, i.e. more exothermic interaction between HA and CHI. For example, according to the study, Ac^- and ClO_3^- which are relatively more hydrated anions in the series have more negative ΔH_c of -3.45 & -2.46 kJ/mole than Cl^- and Br^- which are less hydrated anions with ΔH_c of -1.87 and -0.98 kJ/mole. Lastly, our results for the anions are in agreement with the commonly accepted reverse order since the ΔH_c becomes more negative following the hydration order of $OAc^- > Cl^- > NO_3^-$.

Regarding the effect of HA molecular weight on thermodynamics of coacervation, the experiments done with 1200 kDa HA presented the direct Hofmeister Series effect. On the other hand, when the molecular weight of HA was decreased to 199 kDa, the experiments done with K^+ and Mg^{+2} gave the ΔH_c with the smallest values. This can be interpreted such that that the K^+ and Mg^{+2} ions had an inhibiting effect on coacervation as opposed to the expectations.

Table 2. Average complexation enthalpies and the standard deviation values for each salt. All experiments were done using initial concentrations of 0.1 mg/mL CHI & 0.5 mg/mL HA dissolved in MES-buffered salt solution of pH of 5.25 ± 0.05, I_{total} of 50 mM.

Salt	ΔH_c (kJ/mol) (1200 kDa HA)	ΔH_c (kJ/mol) (199 kDa HA)
NaCl	3.20 ± 1.04	-0.93 ± 0.11
KCl	1.12 ± 0.88	-0.84 ± 0.13
MgCl₂	-0.23 ± 0.82	-0.73 ± 0.03
CaCl₂	-1.10 ± 0.14	-1.45 ± 0.03
NaNO₃	2.49 ± 1.90	0.09 ± 0.18
NaOAc	-1.21 ± 0.14	-1.63 ± 0.08
No Salt	-4.84 ± 0.58	-6.70 ± 0.21

Either chaotrope or kosmotrope, aka weakly or strongly hydrated, all ions have the effect of "screening" the charge of the polyelectrolytes after a threshold salt concentration. Perry et al. (Perry et al., 2014) suggested that the ions inhibited the coacervation of pAA and pAH at salt concentrations higher than 75 mM. In our study, the salt screening effect can be observed by both ITC and turbidity experiments. ITC results suggest that the "No Salt" condition, which had only MES buffer solution but no additional salt ions, has the most negative ΔH_c value for titrations of CHI with either HA of 199 kDa or 1200 kDa. In addition, as shown by Figure 9, HA (199kDa)/CHI system in "No Salt" medium had the highest values for turbidity, indicating that the interaction between HA and CHI is the most favored in the absence of salt ions.

When compared to the literature values of the enthalpy of complexation (ΔH_c) of CHI with different macromolecules such as ovalbumin (Xiong et al., 2016), heparin (Ma et al., 2017), chondroitin sulfate (Ma et al., 2017), DNA (Ma et al., 2017), and xanthan (Maurstad et al., 2012), ΔH_c of the HA/CHI interaction is quite small, i.e. closer to zero. A similar result is observed for ΔH_c of HA with other macromolecules. For example, the enthalpy value for the bovine serum albumin/HA and β-lactoglobulin/HA coacervation (Du et al., 2014) is much higher than the ΔH_c of the HA/CHI system. In contrast, the enthalpy of reaction between pAH, poly-L-lysine (pLL), and PDADMAC (Laugel et al., 2006) are much smaller than ΔH_c of the HA/CHI system. The observation that the interaction enthalpies of both HA and CHI with proteins are much higher than the ΔH_c values for HA/CHI interaction can be explained by the relatively lower charge densities of the polysaccharides compared to the proteins (Ma et al., 2017).

Conclusion

In this research, thermodynamics of interaction between negatively charged HA and positively charged CHI is examined with respect to the effect of Hofmeister salts. Turbidimetric titration experiments and light microscopy images suggest no relation to the Hofmeister series, neither in the direct nor in the reverse direction, except for the pH of 6.25 experiments with cations (Mg^{+2}, Ca^{+2}, K^+, and Na^+). At this pH and I_{total} of 50 mM, the salts affect coacervation between CHI and HA by the direct Hofmeister effect.

The results of the ITC experiments propose that the coacervation of the HA/CHI system follows the direct Hofmeister effect through the cations but the reverse Hofmeister effect

through the anions. For the cations, as chaotropic ions increase the solubilities of the polyelectrolytes, these ions increase the probability of the polyelectrolytes to find each other in the solution and form coacervates. Thus, the ΔH_c values in the presence of chaotropic ions such as Mg^{+2} and Ca^{+2} are more negative than the ones in kosmotropic ions such as Na^+ and K^+.

In addition, the effect of salt screening can be observed by both turbidimetric titration and ITC results. Under the condition of "No Salt, Only the Buffering Agent", the turbidity values are the highest, and the ΔH_c values are the most negative, indicating a stronger interaction between these oppositely charged biopolyelectrolytes.

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