

COMPLEX COACERVATION BETWEEN TWO OPPOSITELY
CHARGED AND SEMI-FLEXIBLE BIOPOLYMERS

by

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To my beloved son, Deniz Ali

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ABSTRACT

COMPLEX COACERVATION BETWEEN TWO OPPOSITELY CHARGED AND SEMI-FLEXIBLE BIOPOLYMERS

Complex coacervation is the spontaneous liquid-liquid phase separation between two oppositely charged macromolecules. Complex coacervation has been used in various applications such as food industry, pharmaceutical industry, cosmetics, biotechnology, and tissue engineering. The first chapter of this thesis includes the examination of thermodynamic characteristics of the interaction between two biopolymers, hyaluronic acid (HA) and chitosan (CHI) under different conditions such as ionic strength, pH, temperature, and molecular weight by using Isothermal titration calorimetry (ITC). The results showed that dominant driving force for HA-CHI complexation was the entropy gain due to counterion release, as indicated by the higher contribution of $T\Delta S_{IP}$ values to ΔG than enthalpy for all conditions. In second chapter of this thesis, meso-structure of complex coacervation, protein/polyelectrolyte (HA-BSA) and polyelectrolyte/polyelectrolyte (HA-CHI) coacervate systems were compared by using small angle neutron scattering (SANS) and noticed that α (power-law exponent) values for both systems depend on the pH due to charge density of polyelectrolytes and protein. Furthermore, radius of gyration of complexes is higher compared to the free protein and polyelectrolyte solutions. In third chapter of this thesis includes the effect of concentration and different salts of Hofmeister series on complex coacervation of HA-CHI and CHI-BSA systems. Turbidity allowed us to determine critical salt concentration where the turbidity started to show a sharp increase. While some salts' effect on the formation of complex coacervation resulted in opposite behavior, some of them showed direct behavior of Hofmeister series.

ÖZET

ZIT YÜKLÜ İKİ-YARI ESNEK BİYOPOLİMERİN KOMPLEKS KOAKSERVASYONU

Kompleks koaservasyon, zıt yüklü iki makromolekül arasında kendiliğinden sıvı-sıvı faz ayrılmasıdır. Karmaşık koaservasyon, gıda endüstrisi, ilaç endüstrisi, kozmetik, biyoteknoloji ve doku mühendisliği gibi uygulama alanlarında da kullanılmaktadır. Bu tezin ilk bölümü zıt yüklü iki biyopolimer olan hyaluronik asit (HA) ve kitosan (CHI) arasındaki etkileşimin iyonik kuvvet, pH, sıcaklık ve moleküler ağırlık gibi farklı koşullar altında termodinamik özelliklerinin izotermal titrasyon kalorimetresi (ITC) kullanılarak incelenmesini içermektedir. HA-CHI kompleksleşmesi için baskın itici gücün farklı koşullar altında, karışık iyon salınımından kaynaklanan $T\Delta S_{IP}$ değerlerinin ΔG 'ye entalpiden daha yüksek katkısı sağlamasından dolayı, entropi olduğu gözlenmiştir. İkinci bölümde protein/polielektrolit (HA-BSA) ve polielektrolit/polielektrolit (HA-CHI) kompleks koaservat sistemlerinin mezo-yapısı, farklı pH ve iyonik kuvvet altında küçük açılı nötron saçılımı (SANS) kullanılarak karşılaştırılmıştır. Her iki sistem için elde edilen α (kuvvet kanunu üssü) değerlerinin, polielektrolitlerin ve proteinin yük yoğunluğundan dolayı pH'a bağlı olduğunu farkedilmiştir. Ayrıca koaservatların dönme yarıçapı, R_g , protein ve polielektrolit çözeltilerine kıyasla daha yüksektir. Tezin üçüncü bölümünde Hofmeister serisinin farklı tuzlarının ve konsantrasyonlarının HA-CHI ve CHI-BSA sistemlerinin kompleks koaservasyonu üzerindeki etkisi yer almaktadır. Türbidimetri ile bulanıklığın keskin bir artış göstermeye başladığı kritik tuz konsantrasyonunu belirlenmiştir. Bazı tuzların kompleks koaservasyon oluşumuna etkisi zıt davranışla sonuçlanırken, bazıları doğrudan Hofmeister serisinin davranışını gösterdi.

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LIST OF SYMBOLS

C_{inj}	Concentration of the solution in the syringe
I	Ionic strength
I_{total}	Total ionic strength of the medium
K_a	Binding constant
M_t	Concentration of the titrant in the cell that is diluted after the injection
M_t^o	The initial concentration of the material within the cell at the start of the experiment.
n	Stoichiometry
n_{coac}	The stoichiometry for the coacervation step
n_{IP}	The stoichiometry for the ion pairing step
Q_{coac}	The amount heat of second step
Q_{IP}	Molar heat of the ion pairing step
T	Temperature (K)
V_o	Volume of the cell
X_t	Concentration of the material injected by the syringe in the cell
X_t^o	Hypothetical concentration of the material injected by the syringe in the cell
α	Degree of ionization of hyaluronic acid
β	Degree of ionization of chitosan
ΔG	Gibb's free energy
ΔH	Enthalpy change
ΔH_{coac}	Enthalpy of polyelectrolyte coacervation
ΔH_{IP}	Enthalpy of ion-pairing
ΔQ_{coac}	The change in heat in the coacervation step
ΔQ_{IP}	The change in heat between an injection and a subsequent.

ΔS	Entropy change
Θ_{coac}	The fraction of sites involved in coacervation
% T	Transmittance
100 - % T	Turbidity



LIST OF ACRONYMS/ABBREVIATIONS

BSA	Bovine serum albumin
DD	Degrees of deacetylation
DNA	Deoxyribonucleic acid
ITC	Isothermal titration Calorimetry
LM	Light Microscopy
pAH	poly (allylamine hydrochloride)
PDADMAC	poly (diallyldimethylammonium chloride)
RNA	Ribonucleic acid
SANS	Small Angle Neutron Scattering
UV-VIS	Ultraviolet and visible

1. INTRODUCTION

1.1. What is Coacervation?

Complex coacervation is defined as the liquid-liquid phase separation of oppositely charged macroions in solution. In this phase separation the liquid phase (rich in macromolecule content) with a high density and viscosity is called “coacervate” (Figure 1.1). The phase with low density and poor in terms of macromolecules is defined as the supernatant [1–5]. Bungenberg de Jong et al. [6] invented the term “coacervate” for the viscous liquid at the bottom phase which is the polyelectrolyte-rich phase in equilibrium with the top phase which is called the dilute “polyelectrolyte-deficient” phase (Figure 1.2). After this study, Oparin mentioned that the first life was formed in coacervate drops via emphasizing its similarities with protocell [7].

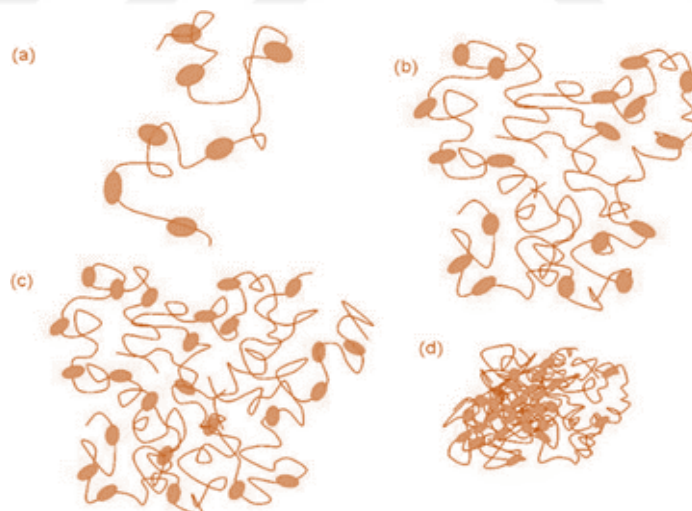


Figure 1.1. Schematic representation of coacervate formation. (a) Complexation with intra-polymer interaction, (b) formation of soluble state (c) intermediate state of interpolymer complexation, (d) coacervate solution with polymer rich and poor phase together. (Reproduced from Ref.[8] with permission from The Royal Society of Chemistry).

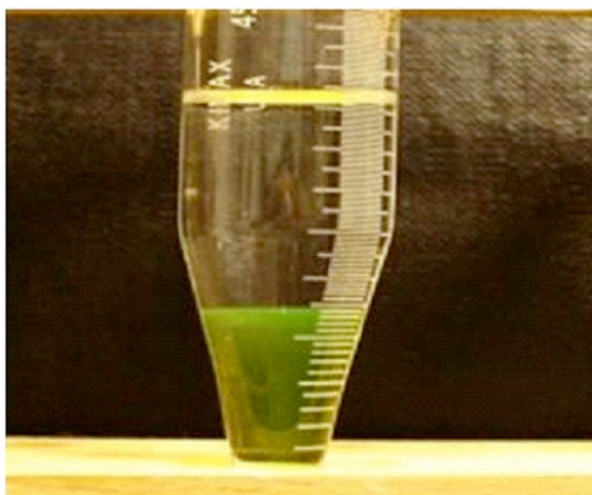


Figure 1.2. Coacervate supernatant phase obtained after centrifugation of BSA-F (bovine serum albumin labeled with fluorescein isothiocyanate) +poly(diallyldimethylammonium chloride) (PDADMAC) mixture at pH 9.5 and $I = 0.1 \text{ M NaCl}$ (Adapted from [9], Copyright (2011) with permission from Elsevier).

Coacervation phenomenon can take place between various macromolecules such as proteins [10–12], dendrimers [13], micelles [14,15], inorganic nanoparticles [16,17], nucleotides [18] or other polyelectrolytes and surfactants [19-20]. Coacervation is used in several application areas such as purification of monoclonal antibody-type proteins [21,22], encapsulation of enzymes to ensure their stability and activity [23], biomedical adhesion in tissue engineering [24,25], and biomimicry of membraneless organelles and protocells [26].

Coacervates differ macroscopically from precipitates and flocs: Precipitation is solid-liquid phase separation, in which the solid phase is rich macro-ion phase with crystalline properties. The flocs are formed by solid-liquid phase separation, flocs remain suspended in the solution longer due to the poor macro-ion solid phase (Figure 1.3).

When comparing polyelectrolyte hydrogels and coacervates, while hydrogels contain more than 5% polymer to get gel form, composition of polymers and protein solutions in coacervates goes up to at most 0.1% by weight [12].

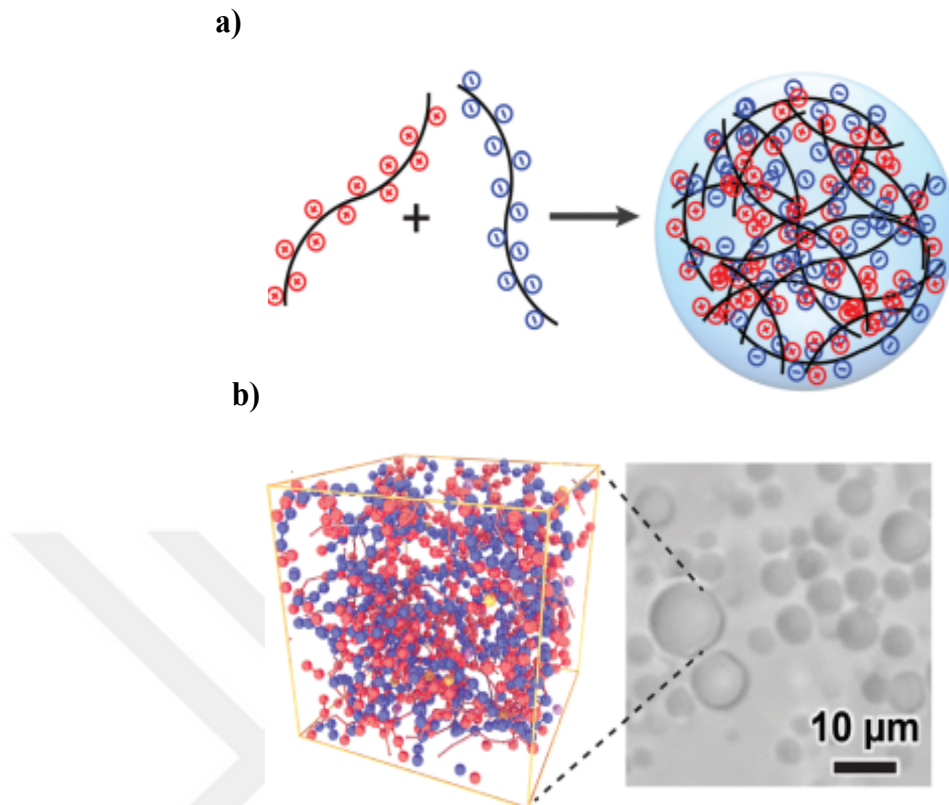


Figure 1.3. (a) Schematic demonstration of the complex coacervation between two oppositely charged linear polymers. (b) Optical microscope images of the “coacervate” phase in the form of droplets dispersed in the supernatant phase (Adapted from Ref. [27] with permission from The Royal Society of Chemistry).

1.2. Coacervate Types

1.2.1. Simple and Complex Coacervation

Simple coacervation is a phase separation process which is occurred upon the addition of a non-solvent or a change in environmental conditions (e.g., temperature, pH, or ionic strength) by a single polyelectrolyte in a solution [28]. On the other hand, complex coacervation is initiated by electrostatic interactions between macroions and by entropy gain due to release of counterions. Manaf et al. [29] focused on the encapsulation efficiency and the release rate of citronella essential oil from simple coacervation with gum Arabic. They also investigated complex coacervation between gum Arabic and gelatin and compared

simple coacervation with complex coacervation. They found that while encapsulation efficiency was comparable, the release rate was slower for complex coacervation.

1.2.2. Polyelectrolyte-Colloid Systems

Polyelectrolyte-colloid coacervation is a specific type of complex coacervation, which stands out by maintaining the original structure and properties of the colloid. One of the general features observed in both polyelectrolyte-colloid and polyelectrolyte-polyelectrolyte systems is the formation of soluble complexes before coacervation (Figure 1.4). The release of counterions is a significant factor in both polyelectrolyte-polyelectrolyte and polyelectrolyte-colloid coacervation, impacting the entropy of the system [9].

The coacervation of polyelectrolyte-colloid systems are affected by several parameters various factors such as the linear charge density of the polymer (ξ), the surface charge density of the colloid, the ionic strength (I), and other variables like the molecular weight of the polymer. These parameters play a significant role in determining the occurrence of coacervation [9].

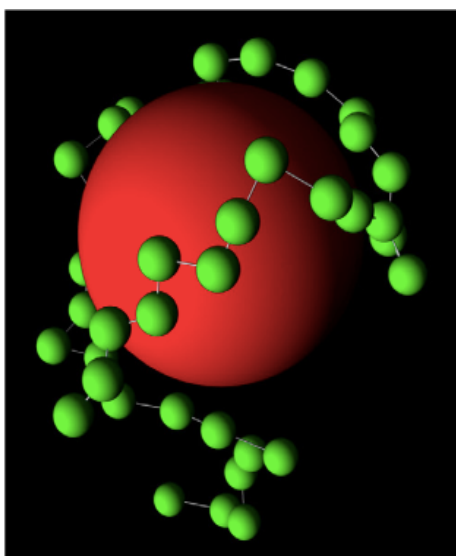


Figure 1.4. A schematic illustration of polyelectrolyte-colloid systems (Adapted from [9] Copyright (2011) with permission from Elsevier).

1.2.3. Protein-Polyelectrolyte Systems

The interaction between proteins and oppositely charged polyelectrolytes is exemplified by the strong binding of basic proteins, such as histones, to DNA. This well-known interaction showcases how proteins interact with oppositely charged polyelectrolytes. In fact, protein and polyelectrolyte do not even need to be oppositely charged for the formation of soluble complexes. Since proteins have charge patches on the surface (Figure 1.5), complexes can form between proteins and polyelectrolytes with the same sign of net charge. For instance, a protein can electrostatically interact with a negatively charged polyanion at regions where there is a positive charge patch even if net charge is negative [30].

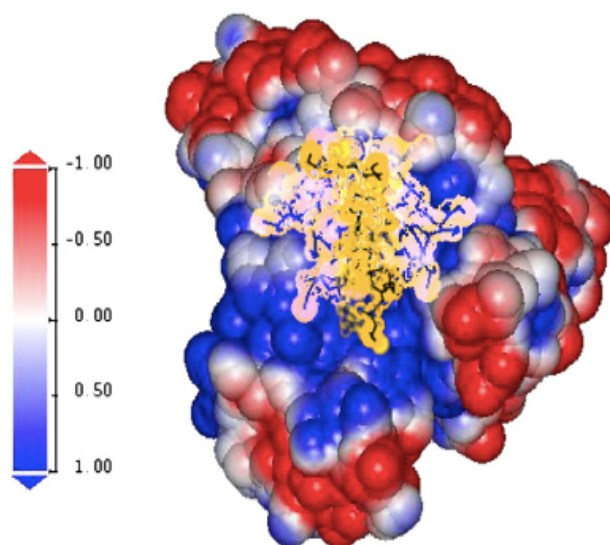


Figure 1.5. Conformational freedom of charged decamer at its binding site on the protein, for pH 6.0 and $I=10$ mM. Decamer is represented by orange atoms and black backbone. Red and blue correspond to negative and positive protein potentials, respectively (Adapted from [9] Copyright (2011) with permission from Elsevier).

The characteristics and dynamics of protein-polyelectrolyte complexes can be influenced by factors such as length of the polyelectrolyte chains and ratio of charges between the protein and the polyelectrolyte. Cousin et al. [31] focus on the structure of

complexes formed between proteins (lysozyme) and negatively charged polyelectrolytes (PSSNa) using small-angle neutron scattering (SANS) (Figure 1.6). While high protein ratio and long polyelectrolyte chain result in gel-like samples due to the cross-linking by lysozyme at high protein/polyelectrolyte ratios, short polyelectrolyte chain exhibit liquid-like behavior. On the other hand, samples containing low protein/polyelectrolyte ratios and long polyelectrolyte chains behaved as liquids on a macroscopic level due to the unfolding of lysozyme as a result of hydrophobic interactions with PSSNa [31].

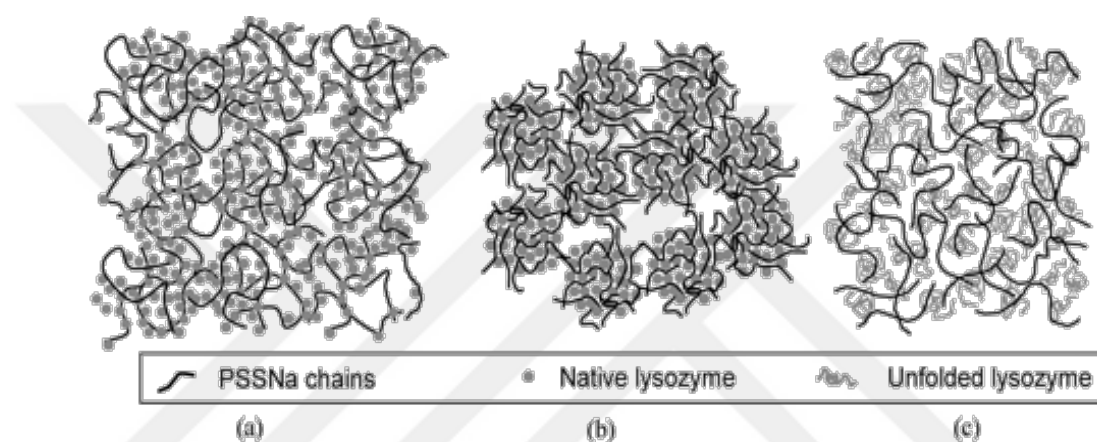


Figure 1.6. Pictures of the different structures of lysozyme PSSNa complexes as suggested by SANS in excess of polyelectrolyte negative charge (a) high protein ratio (low charge excess), long chains; (b) high protein ratio (low charge excess), short chains; (c) high polyelectrolyte ratio (large charge excess) (Reprinted with permission from [31] Copyright (2005) American Chemical Society)

1.2.4. Polyelectrolyte-Polyelectrolyte Systems

Polyelectrolyte-polyelectrolyte coacervation occurs between polyanions and polycations, and results in two separate phases: a phase rich in polymers (coacervate) and a phase with a lower concentration of polymers. The features of the coacervate phase depend on the structure of PEs and can be affected by various factors such as pH, ionic strength, molecular weight of polyelectrolyte, polyelectrolyte concentration, charge density, charge ratio and temperature [1], [32–34]. The recent focus on PE-PE systems stems from their significance in diverse biological scenarios, such as intracellular organization [35–38].

1.3. Affecting Parameters of Coacervation Systems

The coacervation process and the characteristics of coacervates can be affected by various factors such as ionic strength (salt concentration), pH, molecular weight, charge density, charge ratio, and temperature.

These conditions play a significant role in shaping the behavior and properties of coacervates. In the studies conducted between proteins and polyelectrolytes, in addition to these parameters, the electrical charge anisotropy of the protein, the flexibility of the polyelectrolyte, the molecular weight of the protein and the temperature of the environment were also found as important factors [12]. The formation of coacervates, which are dense liquid phases, is strongly influenced by the salt concentration or ionic strength of the system. Chollakup et al. conducted a study using a PAA/PAH system to examine the impact of ionic strength on coacervation [39]. They found that increasing the concentration of NaCl has an influence on coacervate formation. Coacervation was not observed at lower salt concentrations while using a low concentration of polyelectrolytes. However, higher concentration of polyelectrolytes resulted in precipitation. Higher salt concentrations can prevent or dissolve complex coacervates by screening the electrostatic interactions between the oppositely charged polyelectrolytes (Figure 1.7).

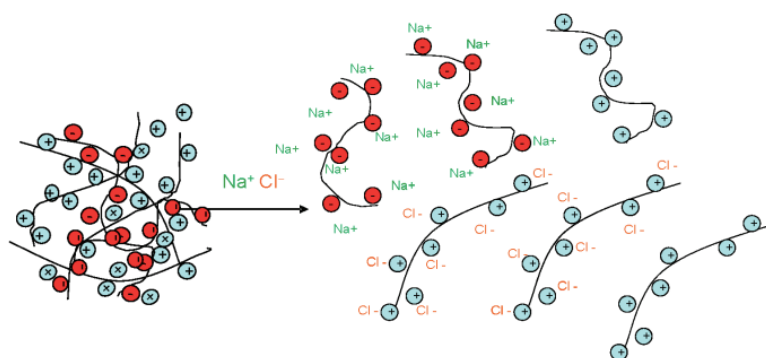


Figure 1.7. Schematic presentation of the screening of interacting oppositely charged groups by added salt (Reprinted with permission from [39] Copyright (2010) American Chemical Society)

The phase separation behavior of oppositely charged biopolymers, such as HA and CHI, is influenced by the charge ratio between them [40]. Light microscopy observations reveal that at lower charge ratios, spherical coacervate droplets are formed, while higher charge ratios lead to the formation of precipitates (Figure 1.8). The presence of NaCl can result in the coexistence of coacervate droplets and precipitates. The mismatch in charge spacing between HA and CHI chains affects the dominant phase-separated species, with precipitates being favored at higher charge ratios [40].

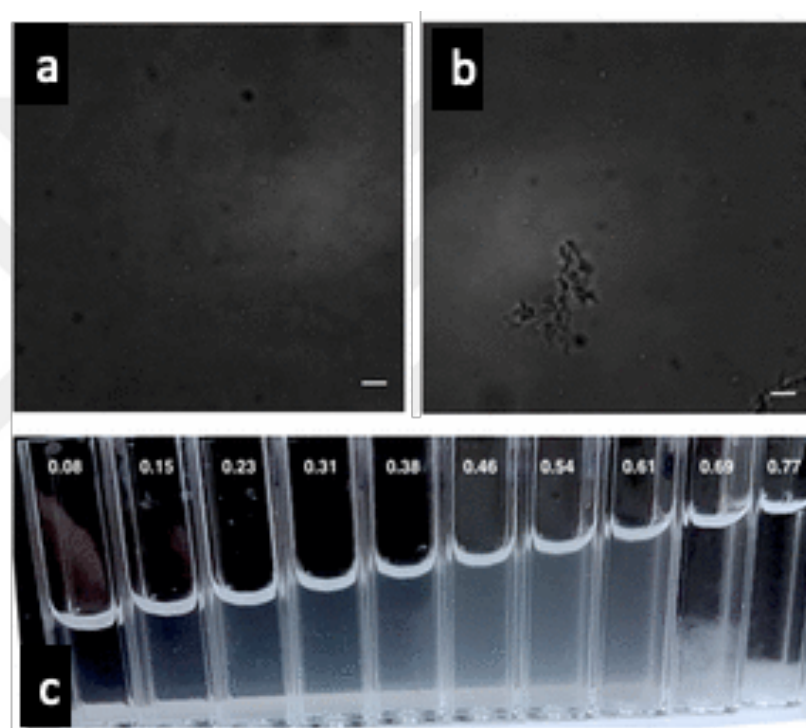


Figure 1.8. The optical microscopy images depict the HA-CHI mixtures. **(a)** shows a coacervate suspension with a charge ratio ($[-]/[+]$) of 0.31 **(b)** precipitation formed at ($[-]/[+]$) = 0.77 **(c)** The visual appearance of the effect of charge ratio on HA-CHI mixtures at pH 6.25 and ionic strength of 300mM (Reprinted with permission from [40] Copyright (2018) American Chemical Society).

Dubin et al. used phase contrast microscopy, DLS and turbidity methods to determine the important pH points in the phase transitions of complex coacervation process between bovine serum albumin (BSA) and poly(dimethyldiallylammonium chloride) (PDADMAC) (Figure 1.9) [41]. These transitions can be characterized by specific pH values such as p_{Hc}

which represents the beginning of soluble complex formation, pH_ϕ which marks the onset of coacervate formation and pH_p indicates the point at which precipitation begins. (Figure 1.10) [1].

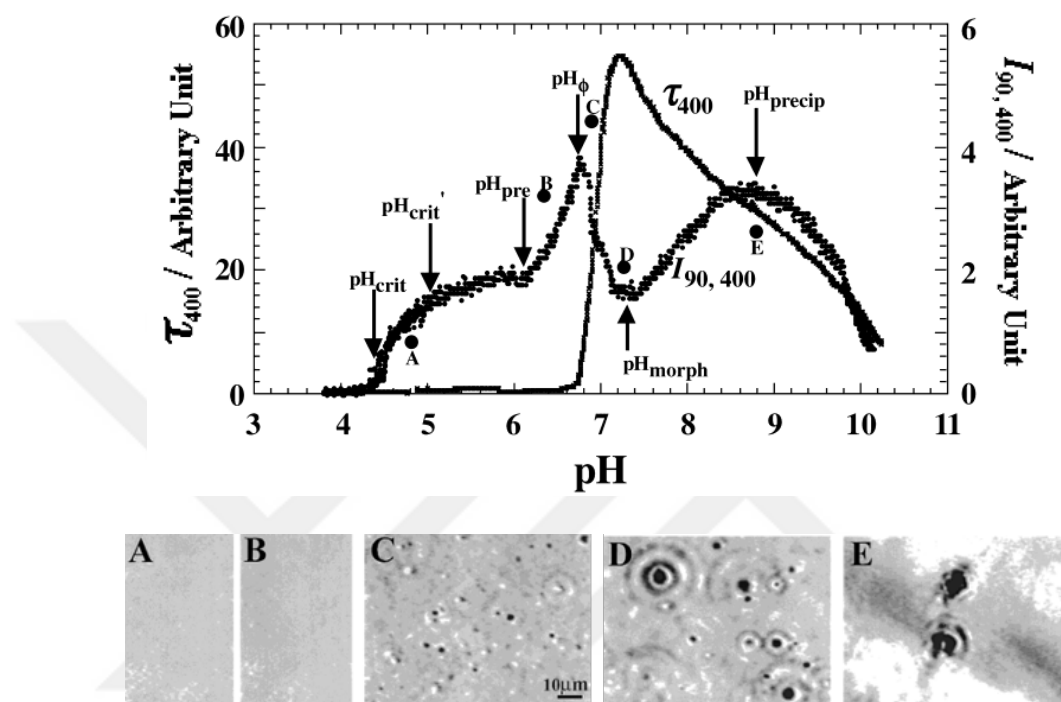


Figure 1.9. The pH profile shows the typical variations in turbidity (τ) and scattering intensity of BSA and PDADMAC complexation in 100mM NaCl, observed at 25 °C. The phase contrast microscope images A-E, correspond to specific points along the pH profile (Reprinted with permission from [41] Copyright (2000) American Chemical Society).

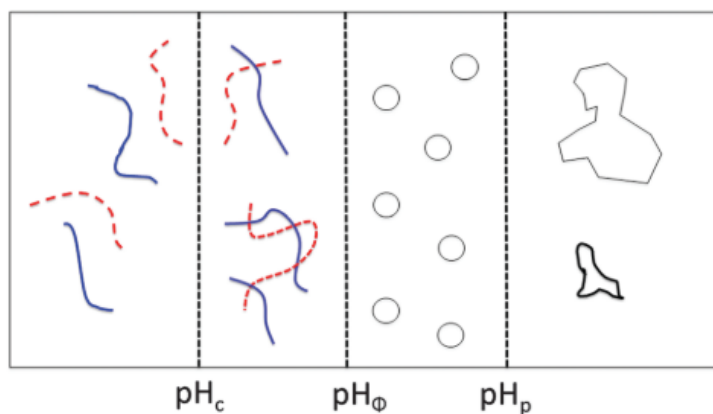


Figure 1.10. The schematic diagram depicts the various regions of phase separation at different pH levels (Permission of Ref from [1] from The Royal Society of Chemistry).

1.4. Driving forces of Coacervate Formation

There are two driving forces that enable complex coacervation to occur. One of them is enthalpic contributions caused by electrostatic causes and the other and more effective than the first one is entropic contributions caused by the export of small ions (counter-ions) [42].

Priftis et. al [43] mentioned that formation of complex coacervation of polyelectrolyte-polyelectrolyte systems included two steps: the first one is called ion pairing and the second one is complex coacervation. In their study, they noticed that high salt concentration affects both steps in a negative way and enthalpy and entropy values decrease as a result of coacervation providing reduction of forces. Priftis and coworkers also studied the interaction between poly(ethyleneimine) and poly(D,L-glutamic acid) or poly(D,L-aspartic acid) and observed that high salt concentration has a negative effect on complex coacervation. The reason is the screening of polyelectrolyte charges by the salt ions which limits the intermolecular electrostatic interactions. Priftis and coworkers also observed that PEC formation is affected by temperature when salt is added into the solution [42].

2. THERMODYNAMIC INVESTIGATION OF HYALURONIC ACID-CHITOSAN COMPLEX COACERVATION BY ISOTHERMAL TITRATION CALORIMETRY

2.1. Introduction

The two most used techniques to study complex coacervation are turbidimetric titrations and optical microscopy. Although turbidimetric titrations contribute to answering qualitative questions about the effect of some important properties (i.e. pH, salt concentration and temperature), thermodynamic analysis is necessary to obtain information on whether the complexation is enthalpic or entropic.

In this respect, isothermal titration calorimetry (ITC) allows to investigate the interaction of oppositely charged macromolecules and get significant information about parameters mentioned above during their formation of complex coacervates.

The ITC device (Figure 2.1) measures the changes in enthalpy (ΔH), entropy (ΔS), Gibbs free energy (ΔG), binding constant (K), stoichiometry (n : number of sites for binding of ligand for each macromolecule).



Figure 2.1. Photograph of the ITC instrument in our laboratory.

Enthalpy changes in the system can arise from interactions between electrostatic charges. Entropy changes can result from changes in the conformation of macromolecules, hydrophobic interactions and/or solvent effects.

In an ITC experiment, the titrant, as a ligand, is introduced with a syringe into a macromolecule which is in the sample cell at constant temperature (Figure 2.2). In the meantime, heat (ΔH) can be given to the environment or absorbed from the environment due to any interaction/bonding occurred. As shown in Figure 2.2, the power value (in microcalories per second or microJoules per second) that ensures that the alteration of temperature between the sample cell which includes a macromolecule as mentioned above and the reference cell that contains water or buffer solution is zero, ($\Delta T = 0$) is recorded by the ITC.

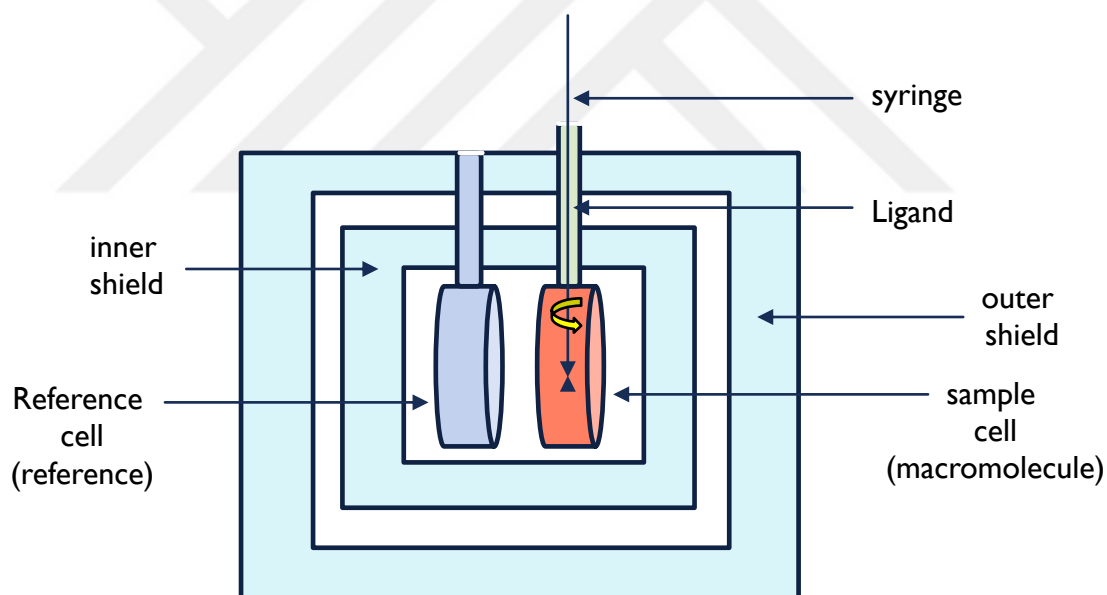


Figure 2.2. Schematic diagram of adiabatic jacket in an ITC instrument including sample cell and reference cell.

The area under each peak gives off heat after each injection. As the amount of non-binding ligand decreases, the length of the peaks decreases until the saturation point is reached. Once the full saturation point has been reached, the added titrants now begin to give peaks of similar size (Figure 2.3). The data of the control experiment, in which there is no molecule other than the solvent in the sample cell, are subtracted from the data of the

experiment in which the sample cell of the macromolecule is located, and correction is made according to the effect of this dilution.

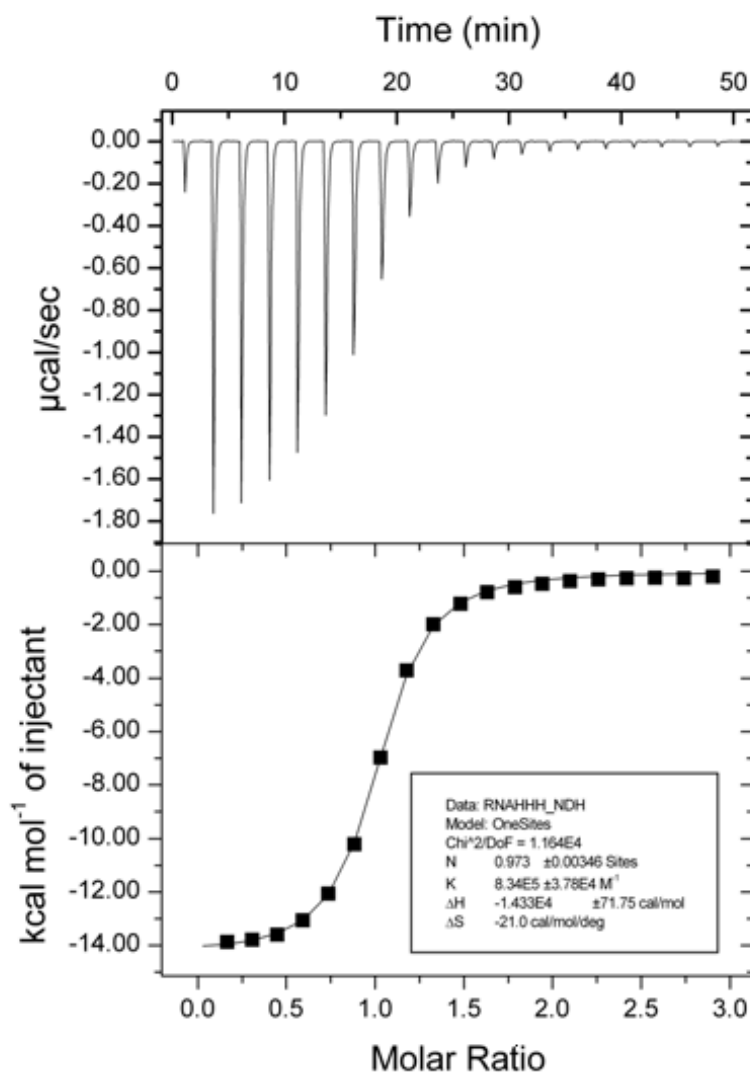


Figure 2.3. Upper panel represents an experimental thermogram acquired from ITC instrument. Each peak represents heat release by ligand injection into sample cell which includes macromolecule. Lower panel shows the obtained thermodynamic parameters (N, K, ΔH) from an ITC experiment by reading the isotherm plot.

There are several application areas of ITC: 1) protein-protein interactions including antigen-antibody interaction [44], 2) protein-small ligand interactions such as nucleotides, mono- and oligosaccharides, cofactors, fatty acids, detergents, drugs [45–49], 3) interaction of protein and peptides with metals [50], 4) interaction of protein and nucleic acids [51], 5)

interaction of polymers and nanoparticles [52], 6) interaction of nucleic acids and small ligands [53], 7) protein-polysaccharides interaction [54], formation of micelles [55,56], 8) phase transports [57], 9) interaction of oligosaccharides and organic molecules [58].

Although enthalpy changes (binding heat) are obtained directly from the ITC measurements, the values of ΔS and ΔG are obtained by a model [59]. According to this model it is assumed that the ligand can bind to one or more but similar binding sites. This model is used in ITC analysis software (eg. Microcal Origin). This software converts raw calorimetry data to a binding isotherm, which finds the binding stoichiometry and binding constants from the binding isotherm (Figure 2.3). ΔG is obtained from the equations $\Delta G = -k.T.\ln K$. Entropy (ΔS) can also be calculated with the Gibbs free energy equation ($\Delta G = \Delta H - T\Delta S$). The ΔH values are directly related to the raw data as described above, and the stoichiometry and binding constant obtained from the binding isotherm is dependent on a model. But the assumptions accompanying this model that come with the software are not suitable for every system. For instance, in studies of the interaction and complexation between proteins and polyelectrolytes systems, the ligand is protein. The numerous regions on the polyelectrolyte that bind with proteins can affect each other in a variety of cooperative and non-cooperative ways. Hence, instead of the model in software, another model 'overlapping bond region' model [60] which indicates proteins as ligands and polyelectrolytes as a dimensional and homogeneous structure has been used [61,62].

Girard et al. [63] studied beta-lactoglobulin and pectin system (with high and low methoxyl contents) and applied the 'overlapping binding site model' [60] instead of two binding sites and the other models introduced with the instrument software. With overlapping binding site model, they obtained higher stoichiometry and lower binding constant results. They also found that enthalpy for soluble and intra-polymeric complexes in the same run is significant and for soluble and inter-polymer complexes both entropy and enthalpy are significant [63].

In a coacervation between two oppositely charged polyelectrolytes, it is much more difficult to find reliable ΔG and K values by fitting obtained curve with ΔH data, because the formation of soluble complexes may have different thermodynamics than coacervation. Particularly in calorimetry data, the experimental results may indicate multiple mechanisms.

Tirrell and coworkers developed a two-step binding model in their study of the thermodynamics of poly (L-ornithine hydrobromide) (PO) / poly (L-glutamic acid) (PGlu) coacervation between these two polypeptides [43] (Figure 2.4).

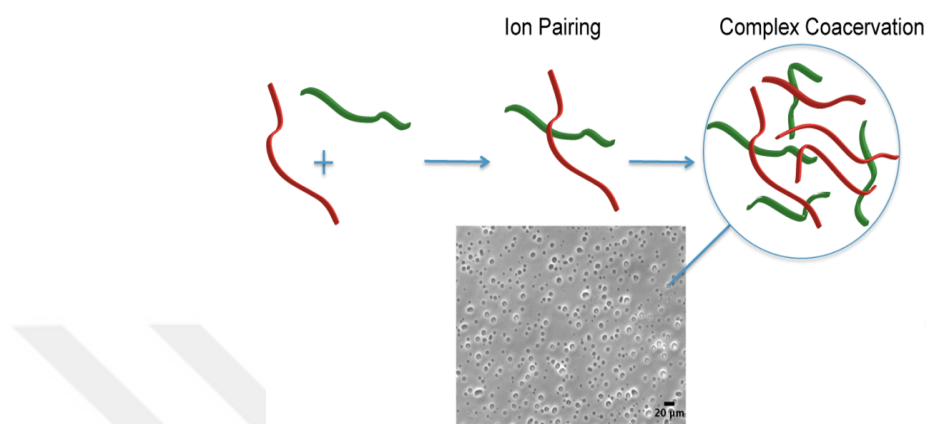


Figure 2.4. Scheme of the two-step binding of polyelectrolytes. The optical microscopy image of PO/Pglu complex coacervation) (Reprinted with permission from [43] Copyright (2012) American Chemical Society)

According to this model, the first step analyzes the thermodynamics of formation of soluble complexes, and the second step examines the thermodynamics of the conversion of soluble complexes to coacervates.

Ball et al. found that with endothermic values of coacervation between protein (bovine serum albumin, BSA) and polyelectrolytes (polyallylamine hydrochloride, PAH) driven force of this system is entropy due to the release of counterions [62]. Feng et al. also obtained endothermic values from interaction of weakly charged (pH-dependent), polyelectrolyte; poly(vinyl amine) (PVAm) and carboxymethyl cellulose (CMC). This means that polyelectrolyte complexation occurs with contribution of entropy gain [64].

However, most of the systems consisting of protein and polysaccharide found complexation based on enthalpy which means exothermic process occurred [59],[65]. Girard et al. investigated the interaction between β -Lactoglobulin-pectin complexes. They found that the enthalpy changes arised “from the interaction between β -lg and pectin, conformational changes, and aggregation of molecules” [59].

Interactions between biomaterials [66], synthetic polymers and biopolymers [8], biomacromolecules and vesicles [62], and polymers and surfactants [67,68] have also been investigated with ITC instrument.

Priftis et al. found that formation of coacervation from oppositely charged polypeptides poly(ethyleneimine) with poly(D,L-glutamic acid) or poly(D,L-aspartic acid) has similar results with other examples given above which is entropically driven via counterion release [42]. They also observed that numerous conditions such as salt, pH, temperature have significant effects on thermodynamic results obtained from complex coacervation of this system [42].

Priftis et al. also used a modified two-step ITC model to investigate complex coacervation of synthetic polyelectrolytes; poly(L-ornithine hydrobromide) (PO)/poly(L-glutamic acid sodium salt) (Pglu), and observed a complexation thermodynamics driven by entropy. In the model they used for the titration curve, there are two parameters which are adjustable: ion pairing (ΔQ_{IP}) and coacervation (ΔQ_{coac}) [43]. While ion pairing is related to the electrostatic interaction of opposite charges, the coacervation corresponds to the coacervate formation step. Through these two parameters, the total heat exchange for each injection ΔQ_{total} is obtained ($\Delta Q_{total} = \Delta Q_{IP} + \Delta Q_{coac}$). While the total heat change is shown as black squares, the heat changes of the ion pairing and coacervate phase are shown as dashed red and dotted green lines, respectively in Figure 2.5 [42].

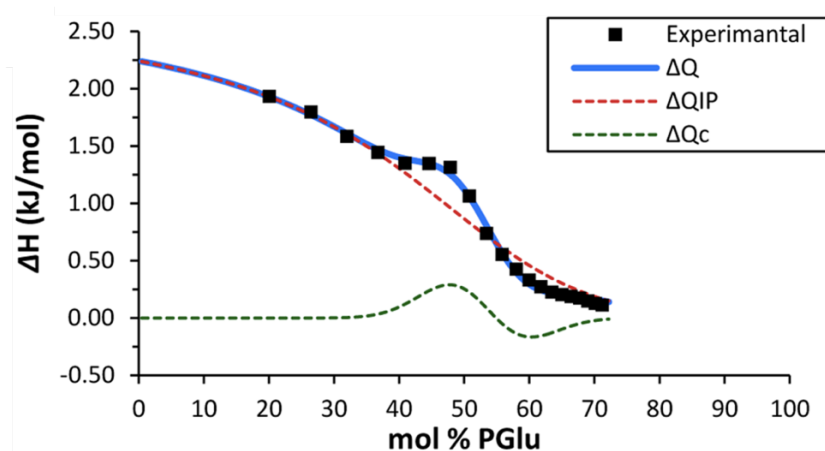


Figure 2.5. Fitted curve by using the modified two-step model of complex coacervation of poly(L-ornithine hydrobromide) (PO)/poly(L-glutamic acid sodium salt) (Pglu) (Reprinted with permission from [43] Copyright (2012) American Chemical Society).

ΔG_{IP} is calculated with obtaining K_a values from the fitted graph. Furthermore, they also calculated entropy changes by Gibbs free energy equation ($\Delta G_{IP} = \Delta H_{IP} - T\Delta S_{IP}$).

According to the above literature review on coacervation and the thermodynamic analysis of coacervation, the subject of this chapter is the investigation of thermodynamic features of the complex coacervation of semi-flexible biopolyelectrolytes with opposite charges using ITC instrument. Semi-flexibility means that the persistence length of the polymer is neither as high as actin filaments (17.7 μm) nor as low (up to 2 nm) as vinyl polymers [69]. Hyaluronic acid (HA), a polyanion, and chitosan (CHI), a polycation, these two polyelectrolytes are semi-flexible (Figure 2.6). HA has 4 nm intrinsic persistence length and CHI has 6.5 nm [70,71].

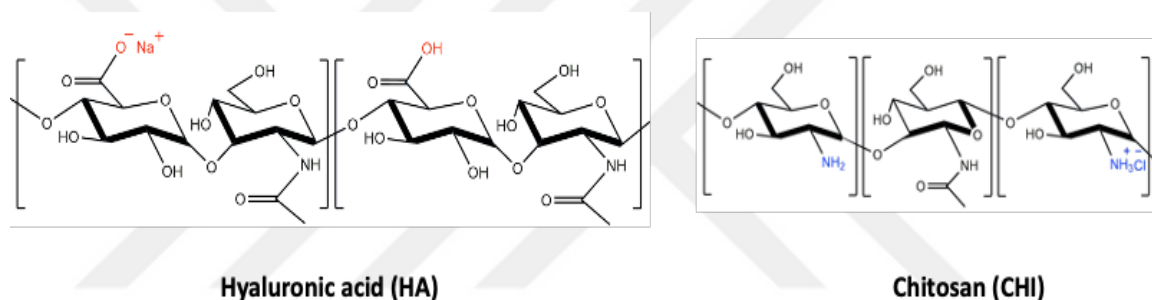


Figure 2.6. Chemical structures of Hyaluronic Acid (HA) and Chitosan (CHI).

These two polysaccharides have also been used in many fields such as tissue engineering [40] and drug delivery [72]. Chitosan contains N-acetyl glucosamine and D-glucosamine monosaccharides linked by $\beta(1\rightarrow4)$ bonds and is formed as a result of the treatment of chitin in alkaline conditions. The deacetylation degree (DD) of chitosan is directly related to the charge density of this polymer [73]. HA is a natural linear biopolyelectrolyte which is found extensively in the connective tissue and extracellular matrix, bonded with $\beta(1\rightarrow4)$ and $\beta(1\rightarrow3)$ of D-glucuronic acid and N-acetyl glucosamine monosaccharides [71]. The distance between two successive charges is 1.3 nm when HA completely loses its protons and 0.6 nm and CHI is completely deacetylated form in acidic environment [1].

2.2. Aim of Study

While coacervation between proteins and strong (pH-independent) polyelectrolytes [74], oppositely charged and weak (pH-dependent) polyelectrolytes [64] and oppositely charged polypeptides [43] has been found entropically driven (when the $T\Delta S$ value is higher than the ΔH value), most of protein and polysaccharide systems [61], [65] undergo an enthalpy-based complexation. (i.e. exothermic signaling).

The unknowns in coacervation between HA and CHI are influenced by the thermodynamic properties of the coacervation parameters such as the molecular weight of the polymers, the ratio of the opposite charges of the polymers, the ionic strength, pH, and temperature of the system. Coacervation between polydiallyldimethylammonium chloride, which is one of the strong polyelectrolytes, and sodium polyacrylate, has been attributed to the release of counterions due to low enthalpy results with positive values and high entropy results with positive values [75]. Since in the HA-CHI system there is only interaction between the carboxyl groups in HA and the amine groups in CHI, in this study, instead of multiple-binding model, a developed two-step model was used where the first step will describe the intramolecular structure which is formed via soluble complexes, and the second one will describe the insoluble inter-polymer complexation (coacervation).

Although Kayitmazer et al. determined the factors which affect the phase separation between HA-CHI with various techniques such as light microscopy, turbidity, and zeta potential experiments [1] a thermodynamic investigation is needed to obtain information on the mechanism that leads to figure out the consequences of parameters including pH, ionic strength, temperature and molecular weight of the polymers, on the thermodynamic properties of the coacervation. In the literature, HA-CHI coacervation was not examined thermodynamically. Therefore, this study work may answer the question whether the coacervation is based on entropy driven or enthalpy. The thermodynamic evaluation of HA-CHI coacervation will also increase the application areas of these coacervates.

2.3. Experimental Section

2.3.1. Materials

Dried sodium hyaluronate (HA) samples that are purified from a bacterial fermentation process of *Streptococcus pyogenes* were purchased from Lifecore Biomedical (Chaska, MN, USA) with molecular weights (Mw) of 51, 150, 234.4 (measured with SEC-MALLS) and 752 kDa (found by intrinsic viscosity). Chitosan chloride (Protasan UP CL 113) with 83 % degrees of deacetylation (DD) and Mw of 80 kDa (determined by viscosity) was provided from Novamatrix (Sandvika, Norway). The source of chitosan (CHI) is Atlantic shrimp. The polymers were pharmaceutical and injectable grade. All polymer solutions were filtered with 0.45 μm cellulose acetate membranes (Sartorius Stedim Biotech) before ITC experiments. NaCl and CH_3COONa were purchased from Sigma-Aldrich (Darmstadt, Germany). CH_3COOH (acetic acid) were purchased from VWR (Radnor, Pennsylvania, USA). HCl and NaOH were purchased from Merck (Darmstadt, Germany). Milli-Q water (Millipore Inc., Milford, MA, USA) was employed throughout all the experiments.

2.3.2. Methods

2.3.2.5. Potentiometric Titration Experiments of Chitosan and Hyaluronic Acid.

Potentiometric titration experiments were done to determine degree of ionization of CHI and HA in 0.15M NaCl under inert gas (nitrogen). Control experiments (polymer-free) were also done in 0.15M NaCl solution. The concentration of CHI and HA was 0.5 mg.ml⁻¹ and 0.1mg.ml⁻¹, respectively. The pH of CHI, HA and salt solutions were adjusted with 0.05 N, 0.1 N and 1 N HCl and 0.05 N, 0.1 N and 1 N NaOH. The initial pH of CHI and HA solutions were fixed to 1.5 and 7.5, respectively.

The pH of solutions was determined by using a Thermo Orion-3 pH meter equipped with a VWR, Symphony – 14002-780 electrode and a Beckmann ATC temperature probe. pKa of CHI and HA were determined using the same method described in our previous study [1]. Degree of ionization of the polyelectrolytes was calculated from the modified Henderson–Hasselbalch equations.

2.3.2.2. Optical Light Microscopy Analysis. A drop of sample with different molar ratio of HA to CHI was put on a glass slide and covered with a microslide. Light microscopy images were recorded using a Leica DMI 6000B Inverted Microscope equipped with a DFC295 camera (Wetzlar, Germany). Samples were then visualized at a magnification of 20x. Image analysis was done using the software of Leica Application Suite, Version 3.8.0.

2.3.2.3. Zeta potential and Scattering Intensity Measurements. The zeta potential and scattering intensity (count rate) measurements for different molar ratio of HA-CHI ranging from 0.03 to 0.53 were performed using a ZetaSizer NanoZS (Malvern, UK) instrument, which uses the laser Doppler electrophoresis technique. All experiments were done in pH 4.0 acetate buffer with $I = 0.15 \text{ M}$ at 25°C and all samples were filtered using $0.45 \mu\text{m}$ cellulose acetate membrane filters (Sartorius A.G., Germany). Samples were loaded into folded capillary cells (DTS1060) for zeta measurements and disposable cells for size measurements. Measurements were repeated at least three times. The electrophoretic mobility of the complexes was converted into a ζ -potential by the Malvern software using the Smoluchowski equation [76].

2.3.2.4. Isothermal Titration Calorimetry (ITC). Isothermal titration calorimetry experiments were performed with a MicroCal ITC200 system (Malvern Instruments, Malvern, UK). All polymer samples were prepared by dissolving in 100 mM acetate buffer and the desired ionic strength was obtained by adding NaCl. Samples were dialyzed by diafiltration through 10 kDa MWCO cellulose acetate centrifugal filters (Pall Corporation) to avoid buffer mismatching. All samples were degassed for 10 minutes before ITC experiments to eliminate bubble formation in the cell and syringe. The ITC experiment was designed as 20 injections which were 240 seconds apart from each other.

Injection volumes were $0.4 \mu\text{L}$ for the first and $2 \mu\text{L}$ for the following 19 injections of 7.5 mM HA (in order to use two-step model for fitting, the concentration of 51 kDa HA was increased from 7.5mM to 15mM to obtain more data in the low charge ratio region) solution into the cell containing 3 mM CHI solution at constant 310 rpm stirring. The reference cell was filled with Milli-Q water. For all conditions evaluated by isothermal titration calorimetry, CHI was in the cell as the polycation, and HA was injected as the polyanion. Reverse titration was not done since in our previous work we observed that titration of CHI

solution into HA solution resulted in precipitation [1]. Heat of dilution was obtained by titration of HA solution into the cell which contained only the buffer solution without CHI. Then heat of dilution for HA into buffer/salt solution was subtracted from heat of binding for HA into CHI titration. The heat of dilution for buffer/salt solution into CHI titration was not subtracted since it had negligible values. All experiments were repeated at least three times. Averages and standard deviations are reported for each experimental condition. (see Appendix A for detailed raw data of heat of binding for HA into CHI titration and heat of dilution for HA into buffer/salt solution).

2.3.2.5. ITC Data Analysis. We followed the data analysis method by Priftis et al. [43] which is a two-step model to analyze the data obtained from the ITC as a result of the complex coacervation between HA and CHI. The first step, which is called ‘ion coupling/pairing’ [43] is related to soluble complexation via electrostatic interactions between polyelectrolyte chains.

Molar heat of the ion pairing step (Q_{IP}) which has enthalpy change (ΔH_{IP}) is determined as

$$Q_{IP} = \frac{n_{IP}M_t\Delta H_{IP}V_o}{2C_{inj}\Delta V} \left[1 + \frac{X_t}{n_{IP}M_t} + \frac{1}{K_a n_{IP}M_t} - \sqrt{\left(1 + \frac{X_t}{n_{IP}M_t} + \frac{1}{K_a n_{IP}M_t}\right)^2 - \frac{4X_t}{n_{IP}M_t}} \right], \quad (2.1)$$

where ΔH_{IP} is the change in enthalpy as a result of ion pairing, n_{IP} is the stoichiometry of X binding to M (X and M are hyaluronic acid and chitosan in our system, respectively), V_o is the volume of the cell (taken as 200 μ l for MicroCal ITC200), ΔV is the volume of HA injected into the cell, C_{inj} is the concentration of the injectant (HA), K_a the is binding constant. X_t is the concentration of HA in the cell following its injection by the syringe described as

$$X_t = \frac{C_{inj}}{V_o} \left(1 - \frac{\Delta V}{2V_o}\right). \quad (2.2)$$

M_t is the concentration of CHI in the cell subsequent to titration with HA is expressed as

$$M_t = M_t^o \left(\frac{1 - \frac{\Delta V}{2V_o}}{1 + \frac{\Delta V}{2V_o}} \right). \quad (2.3)$$

Hence, the change in heat (ΔQ_{IP}) between an injection and a subsequent one is determined through the following expression:

$$\Delta Q_{IP}(i) = Q_{IP}(i) - Q_{IP}(i-1) + \frac{\Delta V}{V_o} \left(\frac{Q_{IP}(i) - Q_{IP}(i-1)}{2} \right). \quad (2.4)$$

At the second step, which corresponds to liquid-liquid phase separation, a.k.a., complex coacervation the assumption of the model is that the amount of heat (Q_{coac}) added to the system is correlated to the amount of polymer in the coacervate. This relationship is expressed by the following expression:

$$Q_{coac} = \frac{n_{coac} \Theta_{coac} M_t \Delta H_{coac} V_o}{C_{nj} \Delta V}, \quad (2.5)$$

where n_{coac} is the stoichiometry for the coacervation step and ΔH_{coac} is the change in enthalpy as a result of coacervation. The Θ_{coac} is pragmatically defined by a Gaussian curve which is described by the following expression:

$$\Theta_{coac} = \exp \left(-\frac{(f - f_{coac})^2}{a_{coac}^2} \right). \quad (2.6)$$

f and f_{coac} are expressed with the following relationships, i.e. $f = X_t / (X_t + M_t)$ and $f_{coac} = \frac{n_{coac}}{1 + n_{coac}}$. Here, α_{coac} is an empirical parameter which is related to the width of the gaussian curve that describes the heat of coacervation [77]. The heat change in the coacervation step of the model is calculated by determining the difference between the titration steps:

$$\Delta Q_{coac}(i) = Q_{coac}(i) - Q_{coac}(i-1) + \frac{\Delta V}{V_o} \left(\frac{Q_{coac}(i) - Q_{coac}(i-1)}{2} \right). \quad (2.7)$$

The total heat is calculated by summing up ΔQ_{IP} (Equation (2.4)) and ΔQ_{coac} (Equation (2.7)). The calculated total heat is then subtracted from the experimental heat to find the error for each injection by the following equation:

$$Total\ Error = \sum (\Delta H_{total}^{model} - \Delta H_{total}^{raw})^2. \quad (2.8)$$

The total of square of errors are minimized with nonlinear least squares curve fitting via Excel Solver after providing initial guesses for the following six parameters: n_{IP} , ΔH_{IP} , n_{coac} , ΔH_{coac} , K_a and α .

Finally, binding constant (K_a) is used to determine the Gibbs free energy (ΔG_{IP}) of ion pairing ($\Delta G = -R \cdot T \cdot \ln(K_a)$). Afterwards, entropy change (ΔS_{IP}) of ion pairing step can be calculated from the following expression: $\Delta G_{IP} = \Delta H_{IP} - T \Delta S_{IP}$.

2.4. Results and Discussion

We performed ITC data analysis with Microsoft Excel using the modified two-step model. Through the Solver program in Excel, we tried to provide data fitting via least-squared error minimization by using six adjustable parameters (ΔH_{IP} , K_a , n_{IP} , ΔH_{coac} , α , n_{coac}) in the model. For this error minimization of Excel Solver, it was necessary to give estimated values to these six parameters. The estimated values for the parameters n_{IP} , n_{coac} , ΔH_{coac} , ΔH_{IP} , and α were given considering the raw (experimental) data. ΔH_{IP} values obtained were generally small. However, considering that the HA-CHI system comes together with non-specific interactions, the reason for the low ΔH_{IP} values can be understood. In general, the ΔH_{coac} value was close to zero. The reason for this can be the reduction of water release ent (the release of counter-ions concentrated on polyelectrolytes, such as Na^+ and Cl^- , into the environment, followed by the release of water molecules that hydrate them) in coacervation step due to the hydrogen bonding of the hydroxyl ($-\text{OH}$) groups in the HA and CHI biopolymers with the water molecules in the environment. The ΔH_{coac} value may have been close to zero because there was no water release (the amount of water in the coacervation phase remained the same as before the coacervation) (Figure 2.7).

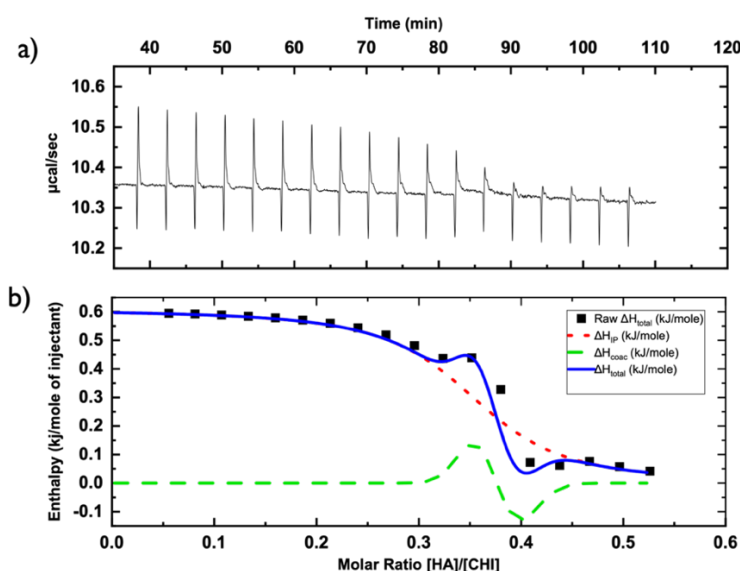


Figure 2.7. (a) Real-time thermogram of ITC experiment and (b) Enthalpy data (squares) obtained from the ITC instrument, fitted enthalpy curves of ion pairing (red dash line) and coacervation (green dash line) steps and total enthalpy (blue line) curves by injection of HA to CHI at pH 4.5 in 100 mM acetate buffer with 0.15 M total ionic strength (I).

It is also seen that $-T.\Delta S$ values, instead of ΔH_{total} , contribute to the change in Gibbs free energy (ΔG). This result shows that the complex coacervation between HA and CHI is mostly entropically driven rather than electrostatic interactions.

Molar ratio of anionic HA to cationic CHI ($[HA]/[CHI]$) affects the phase separation of HA-CHI system. Figure 2.8a visually demonstrates how the HA-CHI mixture changes from a transparent solution to opaque suspension as $[HA]/[CHI]$ increases at pH 4.00 and $I_{total} = 0.15$ M.

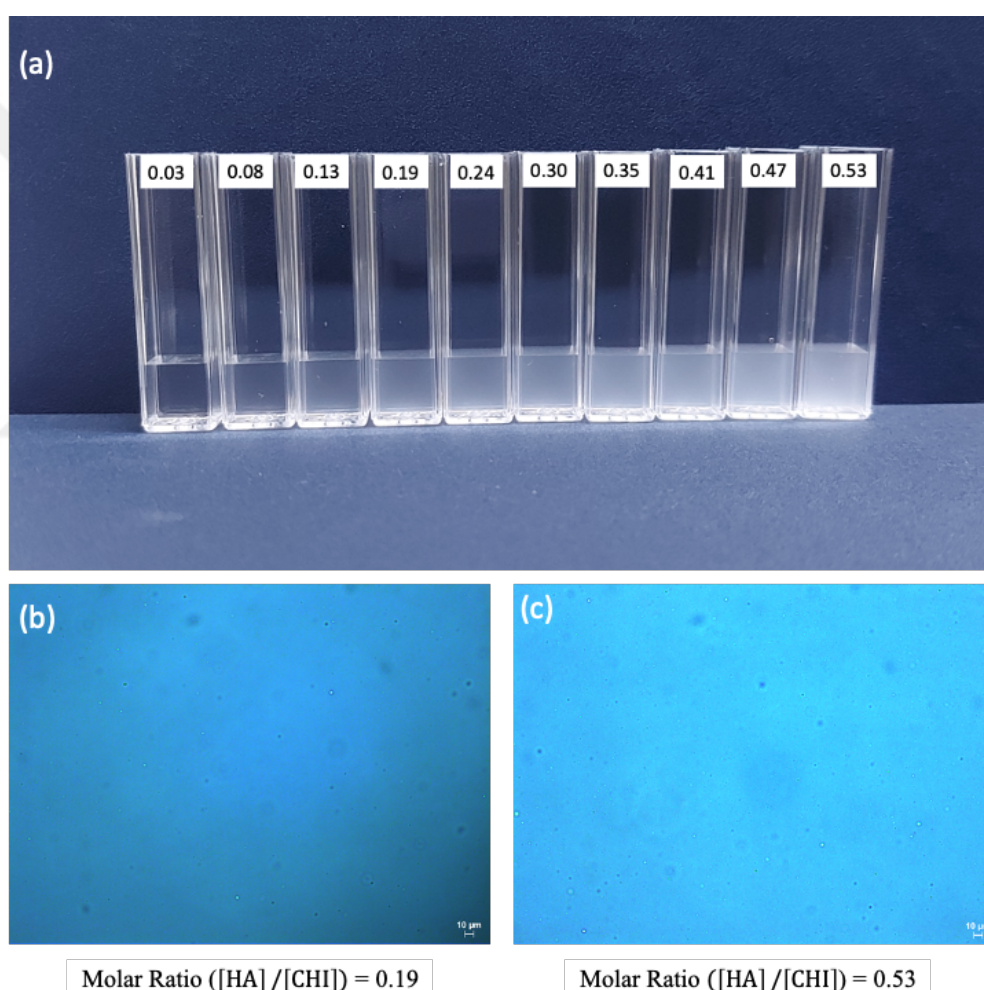


Figure 2.8. (a) HA-CHI mixture solutions with different molar ratio ($[HA]/[CHI]$), optical light microscopy images of HA-CHI mixture (b) at $[HA]/[CHI] = 0.19$, (c) $[HA]/[CHI] = 0.53$ at pH 4.0 acetate- buffer/NaCl solution with $I_{total} = 150\text{mM}$. Scale bar is $10\text{ }\mu\text{m}$ for light microscopy images.

Meanwhile, light microscopy images in Figure 2.8b shows that coacervate droplets are even present at low charge ratios such as 0.19 and the number of droplets increases as molar ratio goes to 0.53 (see Appendix A for light microscopy images for all conditions). $[HA]/[CHI]$ can be converted to the charge ratio of negative (deprotonated carboxylic acid) to positive (protonated amine) groups expressed as

$$\frac{[-]}{[+]} = \frac{\frac{m_{HA} \cdot \alpha_{HA}}{MW_{HA}}}{\frac{m_{CHI} \cdot \beta_{CHI} \cdot DD}{MW_{CHI}}}, \quad (2.9)$$

where MW_{HA} and MW_{CHI} correspond to the monomer molecular weight of HA and CHI, respectively, while m_{HA} is the mass of HA, m_{CHI} is the mass of CHI. DD is the degree of deacetylation for CHI. α_{HA} and β_{CHI} are the degrees of ionization values for HA and CHI, respectively.

For example, $[HA]/[CHI] = 0.19$ and 0.53 corresponds to the charge ratios ($[-]/[+]$) of 0.015 and 0.11 , respectively. Diameter of droplets is $2.1 \pm 0.48 \mu m$ and $2.4 \pm 0.7 \mu m$ for $[HA]/[CHI] = 0.19$ and 0.53 , respectively. Meanwhile, number of these microdroplets were observed to increase by 7-folds with an increase in molar ratio from 0.19 to 0.53 .

Figure 2.9a represents zeta potential, scattering light intensity, and ITC curves for the titration of HA into CHI at pH 4.0 acetate-buffer/NaCl with $I_{total} = 0.15$ M. Zeta potentials decrease with molar ratio ($[HA]/[CHI]$). However, zeta potential values are all positive till at least $[HA]/[CHI] = 0.525$ (Figure 2.9a) indicating that HA-CHI coacervation is non-stoichiometric, which was previously [1] attributed to three reasons: (a) charge spacing for HA and CHI is not equivalent, (b) HA and CHI, which are semi-flexible polyelectrolytes, mostly maintain their conformational chain entropy upon complexation as opposed to flexible chains such as vinyl-based polyelectrolytes, (c) upon binding, pK_a of HA and CHI are affected by each other; the former becomes more acidic while the latter becomes more basic.

Meanwhile, intensity of scattered light in HA-CHI mixtures (Figure 2.9b) increases slightly at the inflection point of the ITC curve; i.e. at $[HA]/[CHI] = 0.275$. This small increase in scattering intensity and visual transition from transparent to opaque suspension can be explained by the increase in the number of coacervate droplets. The constant size of

the droplets can then be attributed to non-zero zeta potentials which limit the growth of these droplets.

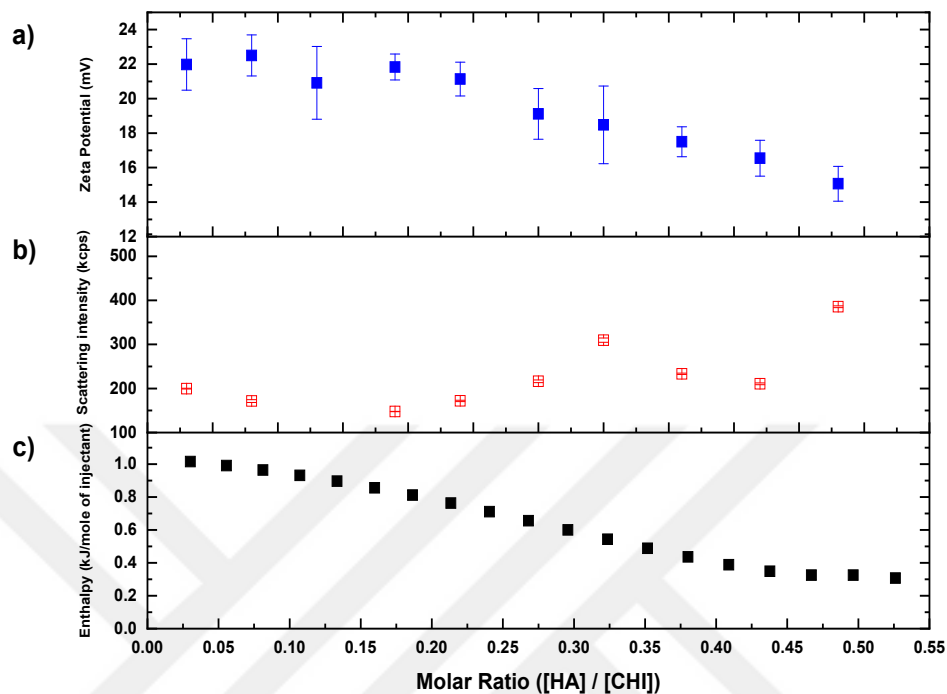


Figure 2.9. (a) Zeta potentials at different molar ratios ($[HA]/[CHI]$), (b) scattering light intensity (kcps: kilocounts per second) values at different molar ratios ($[HA]/[CHI]$), and (c) enthalpy of injection of HA to CHI at pH 4.0 acetate-buffer/NaCl solution with $I_{\text{total}} = 0.15 \text{ M}$, 25°C .

2.4.1. Influence of Ionic Strength (I) on the Thermodynamics of Complex Coacervation

We examined the effect of ionic strength (I) on HA-CHI coacervation by doing ITC experiments at pH = 4.00 in different ionic strengths ranging from 0.05 M to 0.5 M in acetate-buffer/NaCl solution. Table 1 show that enthalpy values for ion-pairing (ΔH_{IP}) are in the 0.2-1.0 kJ/mole range while that of coacervation are in the 0.02-0.3 kJ/mole range depending on the ionic strength. Such small values for enthalpy were also observed for poly(diallyldimethyl ammonium chloride)/poly(sodium acrylate)[75], poly(ethylene imine)/anionic polypeptide [42], poly(glutamate)/poly(glycine-co-lysine)[77] pairs and polyampholytes of methacrylic acid and N-(3-aminopropyl) methacrylamide hydrochloride [78], indicating the non-specific nature of interactions between HA and CHI. The

endothermic values for both steps suggested that complexation and phase separation of HA-CHI system separation were not driven by enthalpy.

For both ion-pairing/complexation and coacervation steps, increasing the ionic strength resulted in a decrease of enthalpy values. This decrease can be explained by the charge screening effect of salt, which might point to electrostatic nature of interactions between HA and CHI. On the other hand, comparison of ΔH_{IP} with $T\Delta S_{IP}$ values (Table 1) indicates that entropy has a stronger contribution to ΔG than enthalpy has at all ionic strengths. Thus, entropy gain due to counterion release seems to be the dominant driving force for HA-CHI complexation. As the counterions are released as a result of electrostatic self-assembly of anionic HA and cationic CHI, the volume occupied by each counterion becomes greater which leads to the increase in entropy [79]. Our results are consistent with other systems where dominance of counterion release entropy was shown directly by small angle neutron scattering for complexation of lysozyme-polystyrene sulfonate [80], by conductivity for cationic lipid-DNA complexation [81], and indirectly by isothermal titration calorimetry for anionic polypeptide-cationic polypeptide interactions [77]. On another note, it is generally expected that configurational entropy of a polyelectrolyte decreases upon complexation. However, this reduction in entropy would be less for a semi-flexible polyelectrolyte which would not be as compact as a flexible chain would be in the complex state. Therefore, the entropy increase associated with counterion release contributes more to the free energy than the loss of configurational entropies of the HA and CHI semi-flexible chains.

As seen from Table 2.1, both enthalpy and entropy for the ion-pairing step decrease with ionic strength (I). For higher ionic strength $I > 0.3M$, the enthalpy values for the second step, a.k.a. the coacervate phase, approached zero and no significant increase or decrease was observed. The decrease of entropy with salt concentration can be explained with the extent of the release of counterions. At lower ionic strengths, a higher number of counterions are released from the associating polyelectrolytes of HA and CHI due to the higher volume of released counterions, as explained above. However, at higher salt concentrations, the volume increase per each released counterion is limited, which limits the entropy increase. Also, the osmotic pressure within polyelectrolytes and the bulk solution would be the same, which inhibit or limit the release of counterions. This inverse relationship between salt

concentration and osmotic pressure for polyelectrolytes was also experimentally shown for poly(styrene sulfonate) (PSS) solutions [82].

The reduction in entropy with salt concentration was also observed by computational modeling studies of highly charged polyelectrolytes [83]. Although HA and CHI are usually considered as weakly charged polymers, the chains at pH = 4.00 are 89 ± 1.8 % charged (Figure 2.10) and fully charged, respectively. Ou & Muthukumar [84] have attributed the suppression of entropy gain in the presence of salt to the variance in osmotic pressure due to the higher concentration of free counterions in the solution. Calorimetry experiments to investigate interaction of cationic poly(ethylene oxide)-g-polyethylenimine with DNA also revealed a drop in entropy upon a ten-fold increase in salt concentration[84] have demonstrated lowering of both enthalpy and entropy when salt concentration was systematically increased from 0 mM to 800 mM for poly(L-ornithine hydrobromide)/poly(L-glutamic acid) system.

The stoichiometry and binding constant also decrease notably, within error limits, with salt concentration as a result of screening of electrostatic interaction. This reduction of n and K points can be explained by the dissociation of the polyelectrolyte complex, which is expected from weakly charged polyelectrolytes such as HA and CHI [34].

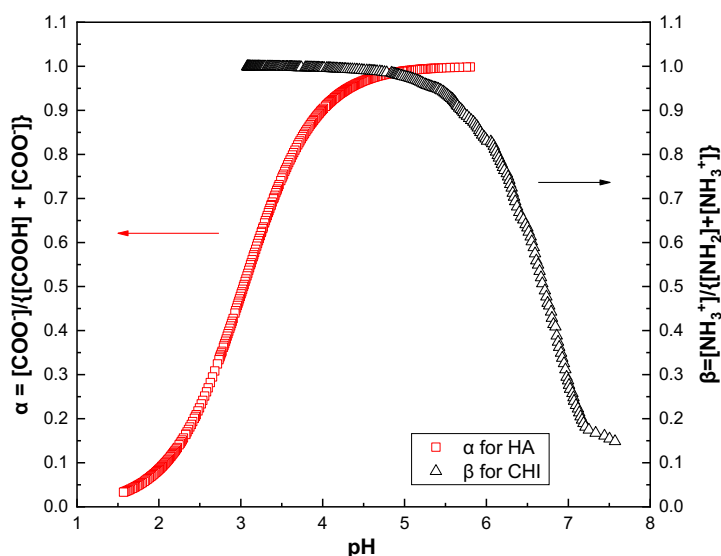


Figure 2.10. Potentiometric titration curves of CHI (80 kDa) and HA (234.4 kDa) in 0.15M NaCl.

Table 2.1. Thermodynamic properties of HA (234.4 kDa)/CHI (80 kDa) mixtures in 100 mM acetate buffer of pH = 4.0 with added NaCl at different total ionic strengths (I, M).

Temperature 22 °C. $C_{\text{initial}}^{\text{HA}} = 7.5 \text{ mM}$, $C_{\text{initial}}^{\text{CHI}} = 3.0 \text{ mM}$.

Ionic Strength (I_{total}, M)	n_{IP}	n_{coac}	ΔH_{IP} (kJ/mol)	ΔH_{coac} (kJ/mol)	$T\Delta S_{\text{IP}}$ (kJ/mol)	K_a (L/mmol)
0.05	0.4	0.6	0.9	0.1	15	274
0.10	0.3	0.4	0.9	0.1	12	96
0.15	0.2	0.7	0.7	0.1	11	67
0.30	0.2	0.3	0.5	0.0	10	52
0.50	0.2	0.5	0.2	0.0	10	44

2.4.2. The Effect of the pH of the Medium on the Thermodynamics of Complex Coacervation

pH is a determining factor in complexation and coacervation of weakly charged macroions. Carboxyl groups of HA and amine groups of CHI are weak acids and bases, respectively. Depending on the pH, the degree of ionization of these polyelectrolytes varies, which concurrently changes their linear charge density. Therefore, we did ITC experiments at different pH values ranging from 3.76 to 5.76 in 150mM acetate-buffer/NaCl solution to determine the effect of pH on complex coacervation.

At low pH between 3.76 and 4.50, enthalpy for ion pairing (ΔH_{IP}) is slightly endothermic and has values less than 1.0 kJ/mole (Table 2.2). For $\text{pH} \geq 5.0$, ΔH_{IP} switches from endothermic to exothermic region and reaches to -2.9 kJ/mole at pH 5.76. This transition can be attributed to increasing electrostatic interaction between HA and CHI with

pH; i.e. at pH 3.76, HA and CHI are $82 \pm 6\%$ and 100% charged, respectively, while at pH 5.76, HA and CHI are 100% and $77 \pm 1\%$ charged, respectively. Considering that HA approximately has a charge spacing that is twice that of CHI, charges of HA and CHI are more complementary to each other at higher pHs, leading to stronger interaction. Exothermic behavior is then expected if an analogy were made between formation of covalent bonds and attractive electrostatic interactions; i.e. as energy is released to form covalent bonds, stronger electrostatic self-assembly between HA and CHI at higher pHs leads to an exothermic process.

At all pHs, entropy ($T\Delta S$) has a stronger contribution to ΔG than enthalpy (ΔH) of either ion pairing or coacervation steps. This result confirms that counterion release is dominant in HA-CHI complexation and coacervation. Our data is in agreement with alginate in poly(diallyldimethylammonium chloride (PDADMAC) and reverse titration [32] which showed higher contribution from entropy than enthalpy at pHs between 4 and 10.

Table 2.2. Thermodynamic properties of HA (234.4 kDa)/CHI (80 kDa) mixtures in 100 mM acetate buffer of with added NaCl at 0.15 M ionic strengths (I) with different pH values. Temperature 25°C. $C_{\text{initial}}^{\text{HA}} = 7.5 \text{ mM}$, $C_{\text{initial}}^{\text{CHI}} = 3.0 \text{ mM}$.

pH	n_{IP}	n_{coac}	ΔH_{IP} (kJ/mol)	ΔH_{coac} (kJ/mol)	$T\Delta S_{\text{IP}}$ (kJ/mol)	K_a (L/mmol)
3.76	0.3	0.6	+0.7	0.2	12	90
4.00	0.4	0.5	+0.9	0.0	10	45
4.50	0.3	0.4	+0.6	0.0	9	32
5.00	0.4	0.3	-0.2	0.0	12	119
5.50	0.3	0.6	-1.8	0.0	14	620
5.76	0.2	0.9	-2.9	-0.4	11	318

2.4.3. Influence of the Molecular Weight of Hyaluronic Acid on the Thermodynamics of Complex Coacervation

In addition to the key physicochemical parameters such as ionic strength, pH and temperature, our previous studies [1] have indicated that higher chain length of the polymers would result in a more extended coacervation region. This was confirmed with a lower pH for the onset of coacervation of the polymer with the higher molecular weight. Here, we determined all endothermic values for the enthalpy of the ion pairing step, which increased with molecular weight of HA (Table 2.3). A similar behavior has been reported in poly(sodium acrylate) (PAA) titration into PDADMAC [76] where molecular weight of PAA was increased from 2kDa to 100 kDa, which corresponded to ΔH_{IP} of +3.1 and +4.0 kJ/mole, respectively. Likewise, titration of poly(glutamic acid) into poly(L-ornithine hydrobromide) (PO) also resulted in higher ΔH_{IP} values when degree of polymerization of both polyelectrolytes was raised from 100 to 400; i.e. ΔH_{IP} of +3.0 and +3.2 kJ/mole, respectively. Priftis et al. has attributed this endothermic increase with chain length to the “cooperativity of electrostatic interactions”. Meanwhile, in all these cases included ours $T\Delta S$ is one order of magnitude higher than ΔH_{IP} , which again corresponds to a stronger influence of counterion release for the HA-CHI system. Lastly, although an entropy loss is expected for longer chains when the polymer is transferred from a free state to a more frozen state in the coacervates, that entropy loss is insignificant at pHs far away from the onset of coacervation; i.e. pH of titration was 4.0 while $pH\phi = 2.3$.

Table 2.3. Thermodynamic properties of HA CHI mixtures in 100 mM acetate buffer of pH = 4.0 with added NaCl at 0.15M ionic strength (I) with different molecular weights (kDa) of HA Temperature 25 °C $C_{initial}^{HA} = 7.5$ mM, $C_{initial}^{CHI} = 3.0$ mM.

Molecular Weight of HA (kDa)	n_{IP}	n_{coac}	ΔH_{IP} (kJ/mol)	ΔH_{coac} (kJ/mol)	$T\Delta S_{IP}$ (kJ/mol)	K_a (L/mmol)
51	0.4	2.7	0.6	0.0	9	33
150	0.1	0.6	0.9	0.0	11	50

Table 2.4. Thermodynamic properties of HA CHI mixtures in 100 mM acetate buffer of pH = 4.0 with added NaCl at 0.15M ionic strength (I) with different molecular weights (kDa) of HA Temperature 25 °C $C_{\text{initial}}^{\text{HA}} = 7.5 \text{ mM}$, $C_{\text{initial}}^{\text{CHI}} = 3.0 \text{ mM}$ (cont.).

234.4	0.4	0.6	0.9	0.0	10	42
752	0.1	0.8	1.2	0.1	11	54

2.4.4. Influence of Temperature on the Thermodynamics of Complex Coacervation

While HA has a highly hydrophilic nature because of its hydroxyl groups, chitosan with DD of 83% possesses acetyl groups which give the cationic polymer a slightly hydrophobic character. At $I_{\text{total}} = 0.150 \text{ M}$, the electrostatic interactions may be screened enough to give a more prominent role for hydrophobic interactions between acetyl groups. Thus, we examined the effect of temperature on the thermodynamics of HA-CHI mixtures to obtain more apparent evidence for the presence of hydrophobic interactions between CHI chains. As it is well-known, strength of hydrophobic interactions tends to increase with temperature. This leads to aggregation between acetyl groups in the chitosan chains. Then a lower number of CHI chains will be available to interact with HA, which would lead to positive enthalpy ΔH_{IP} values. Also, it was reported that CHI solutions experience an increase in viscosity with temperature [85]. It should be noted that this decrease is more prominent at higher CHI concentrations such as the ones within the coacervate droplets. Lowering of viscosity at higher temperatures would then indicate “unfolding/expansion” of this polymer and exposing its hydrophobic regions. Although the linear decrease in intrinsic viscosity with temperature was interpreted as such that CHI did not undergo any conformational changes [86] Nielsen and co-workers have pointed out that the changes in solubility of chitosan solutions with temperature might be a result of conformational transition of chitosan at high temperatures [87]. We hypothesize that such a transition to a more “open” structure would lead to aggregation of acetyl groups in the chitosan, possibly in a limited extent that it would not be affecting the intrinsic viscosity. Which would also reinforce expulsion of a higher number of water molecules from the HA-CHI complexes, contributing to higher $T\Delta S_{\text{IP}}$ than ΔH_{IP} (Table 2.4).

Table 2.5. Thermodynamic properties of HA (234.4 kDa)/CHI (80 kDa) mixtures in 100 mM acetate buffer of pH = 4.0 with added NaCl at 0.15M ionic strength (I) with different temperature values °C. $C_{\text{initial}}^{\text{HA}} = 7.5 \text{ mM}$, $C_{\text{initial}}^{\text{CHI}} = 3.0 \text{ mM}$.

Temperature (° C)	n_{IP}	n_{coac}	ΔH_{IP} (kJ/mol)	ΔH_{coac} (kJ/mol)	$T\Delta S_{\text{IP}}$ (kJ/mol)	K_a (L/mmol)
15	0.3	0.5	+0.4	0.1	12	128
22	0.2	0.7	+0.7	0.1	11	67
25	0.4	0.5	+0.9	0.0	10	45
37	0.2	0.4	+0.7	0.1	11	46
45	0.4	0.4	+0.8	0.0	11	45

2.5. Conclusion

In this chapter, we investigated the thermodynamic properties of a complex coacervation formed between two weak and semiflexible biopolymers, CHI and HA, under varying conditions such as salt strength, pH, temperature, and molecular weight using ITC instrument. As well as ITC, zeta potential and scattering intensity analysis provided insights into the net charge and size distribution of the complex coacervates. Light microscopy allowed visualization of the coacervates, and their morphology was found to depend on the conditions of the system. A two-step model was used to analyze the ITC data of the complex coacervation between HA and CHI. The first step being ‘ion coupling/pairing’ [43] which results of electrostatic interactions between polyelectrolyte chains. The second step involves the formation of a thermodynamically stable phase, known as complex coacervates, due to the entropy gain from counterion release. Changes in the molar ratio of anionic HA to cationic CHI have an impact on the separation of the HA-CHI system which can be observed through microscopic analysis even at low charge ratios, with increasing number of droplets and size as the molar ratio rises. Meanwhile, zeta potentials decrease with increasing molar ratio of HA to CHI but remain positive until at least $[\text{HA}]/[\text{CHI}] = 0.525$, indicating non-

stoichiometric coacervation of HA-CHI as a contribution of three results mentioned in result and discussion part.

The effect of ionic strength on coacervation of HA and CHI was investigated through experiments conducted at different ionic strengths. Results showed that the small enthalpy values for ion-pairing and coacervation, suggesting that interactions between HA and CHI are non-specific. Both complexation and phase separation were found to be endothermic processes, indicating that they were not driven by enthalpy. Increasing the ionic strength decreases the enthalpy values for both ion-pairing/complexation and coacervation steps which shows an electrostatic nature of the interactions between HA and CHI. Entropy has a stronger contribution to the free energy than enthalpy for all ionic strengths, and the entropy gain due to counterion release is the dominant driving force for HA-CHI complexation. Configurational entropy of a polyelectrolyte is expected to decrease upon complexation, but the entropy increase associated with counterion release contributes more to the free energy than the loss of configurational entropies of the semi-flexible chains. Furthermore, the suppression of entropy gain in the presence of salt has been attributed to the higher concentration of free counterions in the solution, which leads to a variance in osmotic pressure. Calorimetry experiments have also shown. the stoichiometry and binding constant decrease with an increase in salt concentration due to the screening of electrostatic interactions. The decrease in stoichiometry and binding constant can be explained by the dissociation of the weakly charged polyelectrolyte complexes.

The degree of ionization and charge density of weakly charged macroions, such as HA and CHI, depend on pH. ITC experiments were performed at different pH values to determine the effect of pH on complex coacervation. The enthalpy for ion pairing (ΔH_{IP}) was found to be slightly endothermic at low pH and switched to exothermic at $pH \geq 5.0$ due to increasing electrostatic interactions between HA and CHI with pH. At all pH values, entropy ($T\Delta S$) was found to have a stronger contribution to ΔG than enthalpy (ΔH) for both ion pairing and coacervation steps, indicating that counterion release is dominant in complexation and coacervation. These findings are consistent with previous studies on alginate in PDADMAC [32].

Chitosan has a slightly hydrophobic nature due to its acetyl groups, which can cause it to interact with other chitosan chains in a hydrophobic manner. At higher temperatures, these

interactions become stronger, leading to the aggregation of acetyl groups in chitosan chains. This transition leads to a more open structure and causes the expulsion of water molecules from HA-CHI complexes, resulting in a higher $T\Delta S_{IP}$ than ΔH_{IP} .

We investigated the coacervation behavior of chitosan and hyaluronic acid polymers of different molecular weights. The higher molecular weight of HA resulted in a higher enthalpy of ion-pairing, which is consistent with previous studies in other polyelectrolyte systems. The increase in endothermic values with chain length can be attributed to the cooperativity of electrostatic interactions. However, the contribution of counterion release was still found to be dominant, as reflected by the higher $T\Delta S$ values than ΔH_{IP} . The entropy loss for longer chains was found to be insignificant at pHs far from the onset of coacervation.

This study elucidates that various factor such as ionic strength, pH, temperature, and molecular weight that can influence the thermodynamic properties of complex coacervates of HA and CHI and understand the behavior of this weak semi-flexible bio-polyelectrolytes through the complexation. This knowledge can be used for various potential application areas in the future.

3. DETERMINATION OF THE DIFFERENCES BETWEEN PROTEIN-POLYELECTROLYTE AND POLYELECTROLYTE-POLYELECTROLYTE COACERVATES BY SMALL ANGLE NEUTRON SCATTERING (SANS)

3.1. Introduction

Scientific research on coacervates is significant for both basic research and technological applications. However, it is particularly essential for basic research to understand non-specific interactions in biological structures. Membraneless organelles such as p granules [88] and nucleoli [89] are assumed to result from a complex coacervate-type liquid-liquid phase separation due to their surface tension properties and rearrangement ability with liquid-like behavior. Furthermore, studies on the effects of media variables including pH, temperature, and ionic strength on coacervates may provide information about the intracellular phase separations by excellent mimic property of these droplet structures.

Another important biological point of coacervates is that although there is 10-20% protein in the coacervate phase (by weight) as a result of protein-polyelectrolyte phase separation, protection of their secondary [90] and tertiary structures [91] is similar to macromolecular crowding which has effects on coacervation formation, observed during intracellular phase separation [92]. Taylor et al. observed that phase separation containing proteins and RNA is provided by crowding which reduced the amount of protein necessary for phase separation [93]

Coacervates have found wide usage areas in technology such as medical adhesives [94] and tissue scaffolds [40],[95] for various cell types in tissue engineering, as encapsulant of flavors in the food industry [96] and as a microencapsulation platform in various other industries [97]. The formation of complex coacervates is induced by the solution conditions (pH and ionic strength) and the release of counterions of the polyelectrolytes.

Meso-structure is crucial for the recognition of coacervates as a more effective microencapsulation method [8]. The reason for this is that the substances entrapped in the coacervates can diffuse easily in the coacervate environment, whether it is a reactant or a product. The most important factor that can affect this diffusion is the meso-structure of the coacervate which can be quantified by the macro- and micro-viscosity of the coacervate. For example, the macroviscosities of bovine serum albumin (BSA)-polydiallyldimethylammonium chloride (PDADMAC) coacervates were found as 0.23-13.6 Pa.s [98] and microviscosities as 0.01-0.06 Pa.s [8]. These values are way more higher than the dynamic viscosity of water which is 8.9×10^{-4} Pa.s. When the polymer concentration in the coacervate was close to the entanglement concentration of the polymer, the dynamic viscosity for PDADMAC was found to be 0.05 Pa.s [99] which is too dense to allow free diffusion. The importance of the meso-structure is evident at this point, so that the encapsulated molecule can be easily released. In structures with very high dynamic viscosity of the coacervate structure, it can be difficult for the encapsulated molecule to diffuse. In this respect, it is important to optimize coacervation to use them as an effective microencapsulation method.

In this perspective, various analysis methods have been used to elucidate the coacervates. When small angle neutron scattering experiments (SANS) are performed for the sodium polystyrene sulfate (PSSNa)/lysozyme system, it is found that the structure of the coacervates is not a completely entangled network, but regions with high neutron scattering have dense aggregates of proteins and polymers, and low neutron scattering is dilute with the protein or polymer [31]. It has been suggested that it is composed of regions with low density. The parameters that change in these different structures are the charge ratio of the polyelectrolyte to the polymer and the molecular weight of the polymer. Other studies investigating the structure of coacervates have used small-angle X-ray scattering (SAXS), rheology, and cryogenic-transmission electron microscopy (Cryo-TEM) as well as SANS experiments [9],[100]. In the literature, it was found that the linear charge density [101], molecular weight and flexibility [102] of the polyelectrolytes, and the pH and ionic strength of the medium [103] also affect the structure of the coacervates.

Another unknown factor regarding the structure of complex coacervates is the effect of the macroion's surface curvature on the coacervate structure. In order to understand the effect of surface curvature on the coacervate structure, a polyelectrolyte is used for one of

the macroions that make up the coacervate, while an oppositely charged globular protein with surface curvature is used for the other (Polyelectrolyte-Protein system). In order to understand the effect of surface curvature, an oppositely charged polyelectrolyte with a relatively more linear structure can be selected instead of the protein used in this system (Polyelectrolyte-Polyelectrolyte system).

In the hypothesis we put forward with this chapter; we suggest that the meso-structures of coacervates containing protein with a higher degree of curvature than polyelectrolytes will have a more diffuse network. This expectation may arise from two different reasons: The first reason is that the polyelectrolyte bypasses the region with the same charge sign on the protein surface (the positive or negative charge condition for both the protein and the polyelectrolyte) due to the curvature property of the protein, causing a weaker binding with the proteins and resulting a more dispersed structure. The other possible reason is that when using a polyelectrolyte rather than a protein, entropic penalty, of the restriction of freedom of the polyelectrolyte, will be greater than for protein used due to the curvature feature. Therefore, with this research, we plan to study which cause will be more important in determining the structure of complex coacervation.

In this chapter, we compare coacervates consisting of polyelectrolyte-polyelectrolyte (PE-PE) and coacervates consisting of polyelectrolyte-protein (PE-Protein) interaction. The first system has hyaluronic acid (HA), as an anionic polyelectrolyte, and chitosan (CHI), as a cationic polyelectrolyte. HA and CHI were chosen because these two polyelectrolytes are sought after products in tissue engineering [40], being biocompatible and biodegradable [104], [105].

Although we have not done experiments within the scope of tissue engineering in this study, our long-term aim is that the coacervates formed should be able to degrade when used as tissue scaffolds. Therefore, it must be fully understood the structural factors so that degradation of coacervates can be controlled following cell microencapsulation.

Kayitmazer et al. [102] studied chitosan-BSA coacervates with the SANS technique and observed that their structures are voluminous and more viscous rather than solid-like. However, in the literature, there is not any small angle neutron scattering (SANS) technique that has been applied to HA-CHI as a PE-PE system and HA-BSA as a PE-protein system

to investigate the effect of surface curvature on the coacervate structure and to compare these two systems.

Small angle neutron scattering (SANS) is a very powerful technique that provides an understanding to the structures at nano- and meso-scale. In a SANS experiment, a beam of neutrons is directed towards a sample which can be at a different state such as an aqueous solution, a solid, or a crystal [106].

In a simple SANS experiment, elastic scattered neutrons are collected, and the scattering intensity (I) are measured at different scattering angles (θ) or scattering vectors (q). This information provides the scattering pattern which can give valuable information about the size, shape, and interactions of the sample's constituents. In this technique, applied neutron is highly penetrated and non-destructive for a wide range of materials including biological macromolecules, polymers, colloids, nanoparticles, and complexes.

3.2. Aim of Study

Membraneless organelles such as P granules [88] and nucleoli [89] are assumed to result from a complex coacervate-type liquid-liquid phase separation due to their surface tension properties and rearrangement ability with liquid-like behavior. Furthermore, studies on the effects of media variables including pH, temperature, and ionic strength on coacervates may provide information about the intracellular phase separations by excellent mimic property of these droplet structures.

Another important biological point of coacervates is similarity between protection of secondary [90] and tertiary structures of protein [92] and macromolecular crowding which has effects on coacervation formation, observed during intracellular phase separation [92].

Meso-structure is another crucial factor for the recognition of coacervates as a more effective microencapsulation method [8]. Another unknown factor regarding the structure of complex coacervates is the effect of the macroion's surface curvature on the coacervate structure.

In this chapter, we compare coacervates consisting of polyelectrolyte-polyelectrolyte (PE-PE) and coacervates consisting of polyelectrolyte-protein (PE-Protein) to understand the effect of surface curvature. The first system has hyaluronic acid (HA), as an anionic polyelectrolyte, and chitosan (CHI), as a cationic polyelectrolyte. HA and CHI were chosen because in the literature, there is not any small angle neutron scattering (SANS) technique has been applied to HA-CHI as a PE-PE system and HA-BSA as a PE-protein system to investigate the effect of surface curvature on the coacervate structure and to compare these two systems.

3.3. Experimental Section

3.3.1. Materials

Sodium hyaluronic acid (HA) (MW 950 kDa) was a gift from Dr. Kazuyuki Miyazawa (Shiseido Co., Yokohama, Japan) with nominal molecular weight (MW) of 950 kDa. Chitosan chloride that has 83% DD and MW of 396 kDa was obtained from Heppe Medical Chitosan “HMC⁺” company (Halle, Germany). BSA was obtained from Sigma (Suitable as diluent in ELISA applications, initial fractionation by heat shock). NaCl was bought from Sigma-Aldrich and Merck. HCl and NaOH were obtained from Merck. Milli-Q water (Millipore, Milford, MA, USA) was used for preparing protein, polymer and salt solutions.

3.3.2. Methods

3.3.2.1. Preparation of Coacervate Solutions. Optimum conditions were investigated for complex coacervation of HA-CHI and HA-BSA. All polymer solutions were prepared in NaCl (50mM, 100mM, 150mM, 200mM, 250mM and 300mM). Two methods are applied to get coacervation from HA-CHI and HA-BSA systems. In the first method, for HA-BSA system; polymer solutions were adjusted to pH 2.80, 3.00 and 4.09 before mixing HA and BSA at equal volumes (weight ratio (r) of BSA to HA is 10). In the second method, polymer solutions were mixed at equal volumes and then adjusted to pH 2.80, 3.00 and 4.09. For HA-CHI system, these two methods were applied, and polymer solutions were adjusted to pH

2.80, 3.00, 4.00, 4.09 and 5.00. However, polymer solutions were mixed at different volumes to get the desired charge ratio of HA to CHI (Table 3.1 and Table 3.2).

Coacervate yield was calculated for each polymer mixture solutions by the following equation:

$$\text{Coacervate yield} = \frac{\text{Mass of the coacervate phase after supernatant is discarded}}{\text{Mixture mass after pH adjustment}} \times 100. \quad (3.1)$$

The mixed polymers which were turbid was transferred into the weighed centrifuge tubes and centrifuged for 25 minutes at 5000 rpm for three times until phase separation was observed. After phase separation was observed, supernatant was discarded and coacervate phase weight was measured to calculate coacervate yield. Fresh samples were prepared under the conditions determined by light microscopy and the yield was calculated. Determination of coacervate yield was important to find out how much sample should be prepared for small angle neutron scattering (SANS) experiments.

The complex coacervates was generally obtained by mixing the respective macromolecules, centrifuging them one or more times at 4000-5000 rpm and discarding the supernatant. Apart from the complex coacervates, HA, CHI and BSA alone as the control samples was prepared and measured at the pH and ionic strength of the preparation of the coacervate.

Table 3.1. Coacervation experiment conditions for BSA/HA system.

#	pH	r	NaCl (mM)	HA (mg/mL) (final)	BSA (mg/mL) (final)	HA volume (mL)	BSA volume (mL)	Result	Yield (%)
1	3.00	10	50	0.025	0.25	6	6	Coacervation	0.2
2	3.00	10	50	0.05	0.5	8	8	Precipitation	N/A
3	4.00	10	50	0.025	0.25	8	8	Precipitation	N/A
4	5.00	10	50	0.025	0.25	8	8	No coacervation	N/A
5	3.00	10	100	0.025	0.25	8	8	Precipitation	N/A

Table 3.2. Coacervation experiment conditions for BSA/HA system cont.).

6	4.00	10	100	0.025	0.25	8	8	Precipitation	N/A
7	3.00	10	300	0.025	0.25	8	8	No coacervation	N/A
8	4.00	10	300	0.025	0.25	8	8	No coacervation	N/A
9	3.00	5	50	0.05	0.25	8	8	Precipitation	N/A
10	4.00	5	50	0.05	0.25	8	8	Precipitation	N/A
11	2.80 (1 st Method)	10	250	0.05	0.5	8	8	Coacervation	0.4
12	2.80 (2 nd Method)	10	250	0.05	0.5	8	8	Coacervation	
13	4.09(1 st Method)	10	100	0.05	0.5	8	8	Precipitation	N/A
14	4.09(2 nd Method)	10	100	0.05	0.5	8	8	Precipitation	N/A
15	4.09(1 st Method)	10	150	0.03	0.3	8	8	Precipitation	N/A
16	4.09 (2 nd Method)	10	150	0.03	0.3	8	8	Precipitation	N/A
17	4.09 (1 st Method)	10	200	0.04	0.4	6	6	Coacervation	0.3
18	4.09 (2 nd Method)	10	200	0.04	0.4	6	6	Coacervation	0.4

Table 3.2. Coacervation experiment conditions for HA-CHI system.

#	pH	Charge ratio [-]/[+]	NaCl (mM)	HA (mg/mL) (final)	CHI (mg/mL) (final)	HA volume (mL)	CHI volume (mL)	Result	Yield (%)
1	2.00	0.33	50	0.25	0.25	8	8	coacervation and precipitation	N/A
2	3.00	0.33	50	0.25	0.25	8	8	coacervation	0.1
3	4.00	0.55	50	0.25	0.25	8	8	coacervation	N/A
4	5.00	0.66	50	0.25	0.25	8	8	coacervation	N/A

Table 3.2. Coacervation experiment conditions for HA-CHI system (cont.).

5	6.00	0.82	50	0.25	0.25	8	8	precipitation	N/A
6	3.00	0.33	100	0.25	0.25	8	8	coacervation	N/A
7	4.00	0.55	100	0.25	0.25	8	8	coacervation	N/A
8	3.00	0.11	50	0.165	0.5	8	8	coacervation	N/A
9	4.00	0.18	50	0.165	0.5	8	8	coacervation	N/A
10	2.80 (1 st Method)	0.15	250	0.05	0.25	8	8	coacervation	N/A
11	2.80 (2 nd Method)	0.15	250	0.05	0.25	8	8	coacervation	11
12	2.80 (1 st Method)	0.33	250	0.25	0.25	8	8	coacervation and precipitation	N/A
13	2.80 (2 nd Method)	0.33	250	0.25	0.25	8	8	coacervation and precipitation	N/A
14	2.80 (1 st Method)	0.20	250	0.190	0.310	8	13	coacervation	0.4
15	2.80 (1 st Method)	0.26	250	0.222	0.278	8	10	coacervation	0.3
16	4.09 (1 st Method)	0.21	200	0.136	0.364	6	16	coacervation	0.1

3.3.2.2. Sample preparation for SANS analysis. After optimization studies, coacervation yields were elevated for SANS experiments (Table 3.3 and 3.4). All polymer solutions were prepared in NaCl solutions with different ionic strength (50 mM, 200 mM and 250 mM). Two methods are applied to reach coacervation with HA-CHI and HA-BSA systems. In the first method, for HA-BSA system; pH of polymer solutions was adjusted to pH 2.80, 3.00 or 4.09 before mixing HA and BSA in equal volumes (weight ratio (r) of BSA to HA was 10). In the second method, polymer solutions were mixed at equal volumes, and then pH was adjusted to 2.80, 3.00 or 4.09. For HA-CHI system, these two methods of sample preparation were also applied, and pH of HA and CHI were adjusted to pH 2.80, 3.00 or 4.09. Polymer solutions were mixed at different volumes to get the desired charge ratios of HA to CHI.

Small Angle Neutron Scattering (SANS) experiments were performed on the NGB30 SANS instrument at the NIST Center for Neutron Research (Gaithersburg, MD, US). Samples were prepared in D₂O buffer and loaded into demountable titanium cells with 1 mm pathlength. A 9-position sample stage was used to hold the titanium cells during measurements. Three different sample-to-detector distances were used (1.3 m, 4 m and 13 m with lens optics) to cover a q-range from 0.004 Å⁻¹ to 0.5 Å⁻¹. The incident neutron wavelength (λ) was 6 Å for 1.3 m and 4 m detector distances, while λ was set to 8.4 Å for 13 m position. SANS data reduction was performed using NCNR SANS reduction macros in IgorPro software package [107].

Table 3.3. Coacervation experiment conditions of HA-CHI system for SANS experiments.

pH	Charge ratio [-]/[+]	NaCl (mM)	HA (mg/mL) (initial)	CHI (mg/mL) (initial)	HA volume (mL)	CHI volume (mL)
3.00 ± 0.05	0.33	50	0.5	0.5	8	8
2.80 ± 0.05	0.20	250	0.5	0.5	8	13
4.09 ± 0.05	0.21	200	0.5	0.5	6	16

Table 3.4. Coacervation experiment conditions of HA-BSA system for SANS experiments.

pH	NaCl (mM)	HA (mg/mL) (initial)	BSA (mg/mL) (initial)	HA volume (mL)	BSA volume (mL)
3.00 ± 0.05	50	0.05	0.5	6	6
2.80 ± 0.05	250	0.1	1	8	8
4.09 ± 0.05	200	0.08	0.8	6	6

3.3.2.3. Optical Light Microscopy Experiments. A small amount of sample was inserted on a microslide. A Leica DMI 6000B Inverted Microscope with a DFC295 camera was used to get optical microscopy images of HA-CHI complex coacervate droplets. 20x magnification of microscopy helped to visualize the samples. Leica Application Suite, Version 3.8.0 software was performed for image analysis.

3.3.2.4. Turbidity vs. pH profile for the HA /CHI and HA/BSA systems at different ionic strength and polymer concentration. Effects of ionic strength and pH on HA-CHI complexation were studied by turbidimetry. Samples were stirred during taking turbidity measurements by a colorimeter. MW of HA was 950 kDa and MW of CHI with 83% DD was 396 kDa. These experiments were done with a 2 cm path length fiber optic probe colorimeter (Brinkmann PC 950) with a filter of 420 nm.

Turbidity (τ) is written as $100-T\%$, in which T is the transmittance. HA, BSA and CHI were dissolved in solutions of different NaCl concentration (50mM, 200mM and 250mM). The polymer solutions of HA-CHI solutions were brought to pH 1.5 before mixing. On the other hand, BSA and HA were brought to pH 7.0 before mixing. Increasing and decreasing of the pH is performed by using 1 N and 0.1 N NaOH and HCl. For comparing polyelectrolyte-polyelectrolyte coacervation with polyelectrolyte-protein coacervation, formation conditions of maximum coacervation (salt concentration, polymer concentration and pH) are chosen. Therefore, polymer concentrations are different for both systems (HA-

CHI and HA-BSA). (see Appendix A for turbidity graphs of HA-BSA and HA-CHI systems for all conditions)

3.3.2.5. Potentiometric Titration of Chitosan and Hyaluronic Acid in Different Salt Concentration. Potentiometric titration experiments were performed to figure out ionization degree of CHI and HA in 50 mM NaCl under inert nitrogen gas. Control experiments (polymer-free) were also done in 50 mM NaCl. Concentrations of polyelectrolyte solutions of CHI and HA were 0.5 mg. ml⁻¹. The pH of CHI, HA and salt solutions were adjusted with 0.05 N, 0.1 N and 1 N HCl and 0.05 N, 0.1 N and 1 N NaOH. The initial pH of CHI and HA solutions were fixed around 1.5 and 7.5, respectively. The pH of solutions was checked with a Thermo Orion-3 pH meter that has a VWR, Symphony – 14002-780 electrode and a Beckmann ATC temperature probe. pK_a and ionization degree of CHI and HA were determined using the same method in our previous study [1].

3.4. Result and Discussion

3.4.1. Turbidity and Potentiometric Measurements and Light Microscopy

In order to compare the complex coacervation formed by PE-PE systems with PE-protein pairs, turbidimetry analysis and light microscopy (LM) experiments were performed under the conditions used in SANS experiments. In the turbidimetry experiments performed at different salt concentrations, turbidity increase (100-%T) was not observed at higher pH values than the isoelectric point of BSA (pI = 4.9), as seen in Figure 3.1 for the HA-BSA system. This is because the net charge of BSA molecule in this region is negatively charged as HA molecule (Figure 3.2). The fact that both molecules are negatively charged, in other words, there are no oppositely charged molecules, is proof of this, as coacervation does not occur for each salt condition. Another observed situation is that as the salt concentration increases, turbidity increment started, in other words, the formation of coacervation and subsequent precipitation occurred at higher pH values. The reason for this is due to the screening effect. As the salt concentration increases, the intermolecular electrostatic interaction decreases. Therefore, interaction is observed at lower pH (where the amount of

BSA positive charge increases) (Figure 3.1) and then precipitation occurs at lower pH. Light microscopy images have showed the coacervate droplets and any precipitation did not occur at fixed pH values for PE-protein system (Figure 3.3, 3.5 and 3.6). Diameter of droplets is $6.0 \pm 0.29 \mu\text{m}$ at pH 2.8, $8.7 \pm 0.31 \mu\text{m}$ at pH 3.00 and $5.1 \pm 0.48 \mu\text{m}$ at pH 4.09.

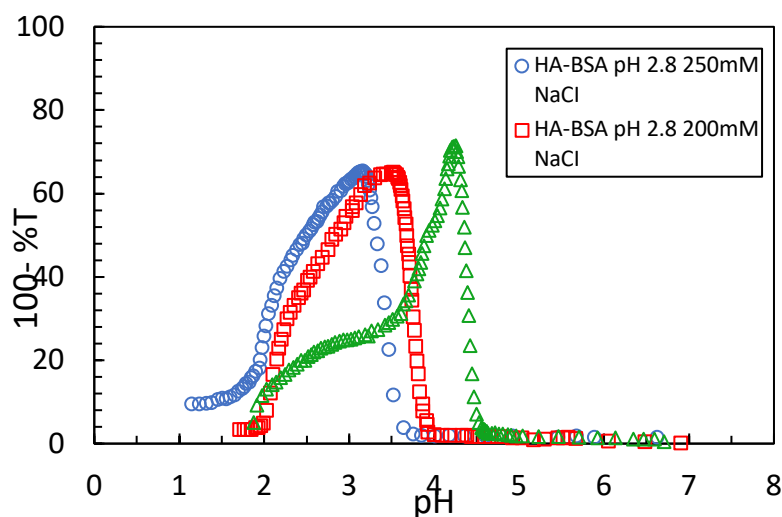


Figure 3.1. Turbidity vs. pH for the HA (950 kDa) /BSA mixture at 50, 200 and 250mM NaCl. pH was decreased gradually from 7.0 to around 1.0.

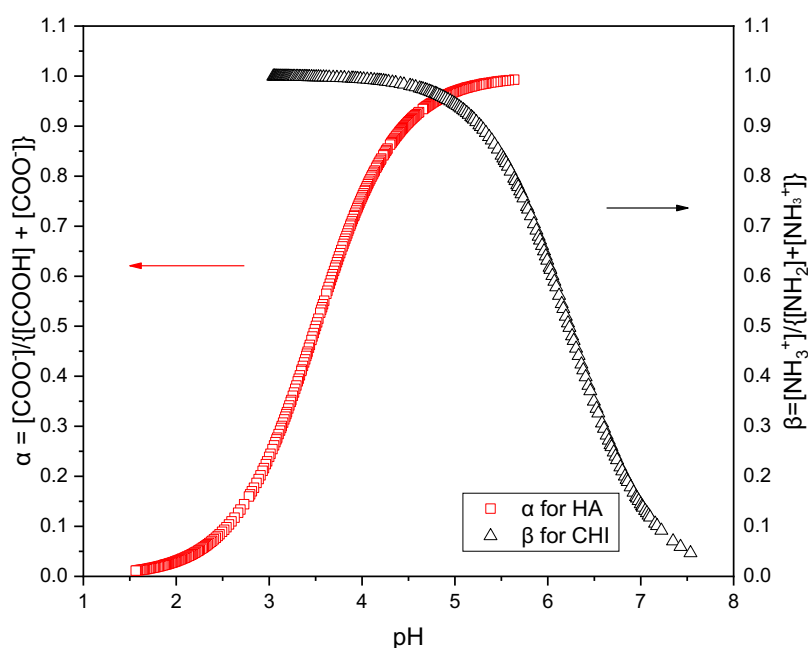


Figure 3.2. Potentiometric titration curves of CHI (396 kDa) and HA (950 kDa) in 0.15M NaCl.

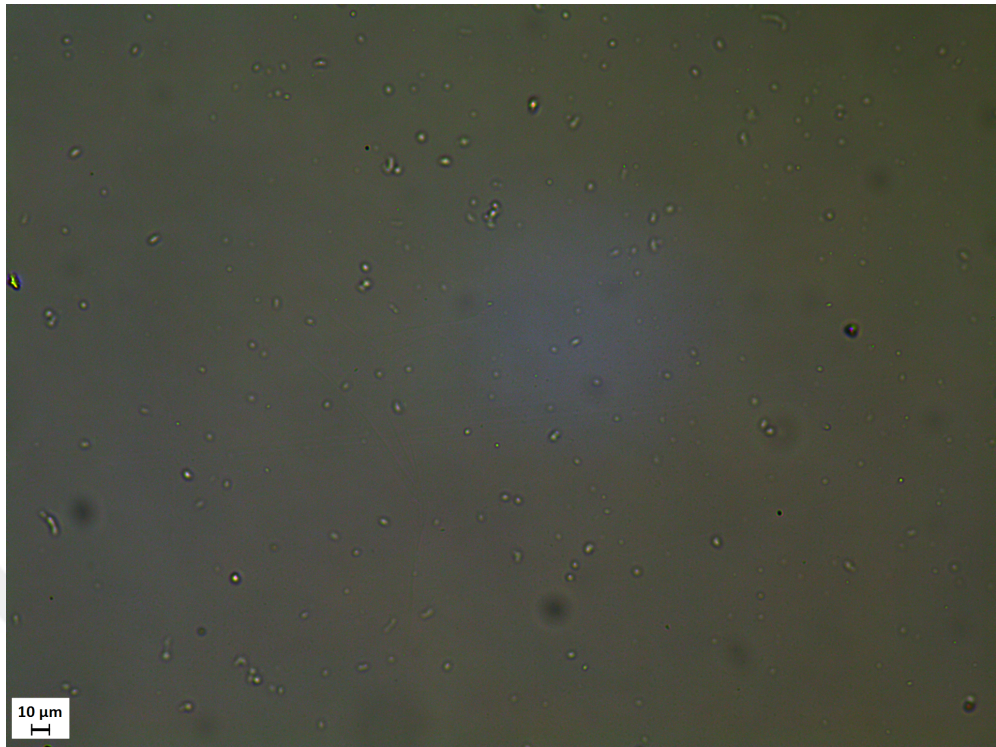


Figure 3.3. Light Microscopy images for HA-BSA system; 20x (pH 4.09, 200mM NaCl).

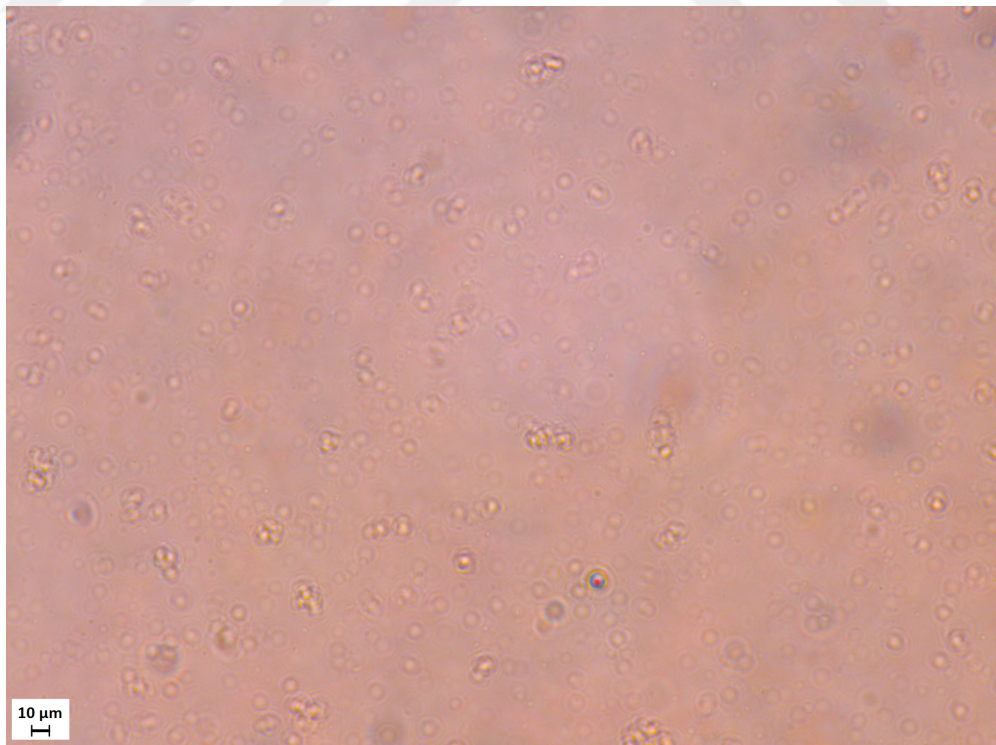


Figure 3.4. Light Microscopy images for HA-BSA system; 20x (pH 3.00, 50mM NaCl).

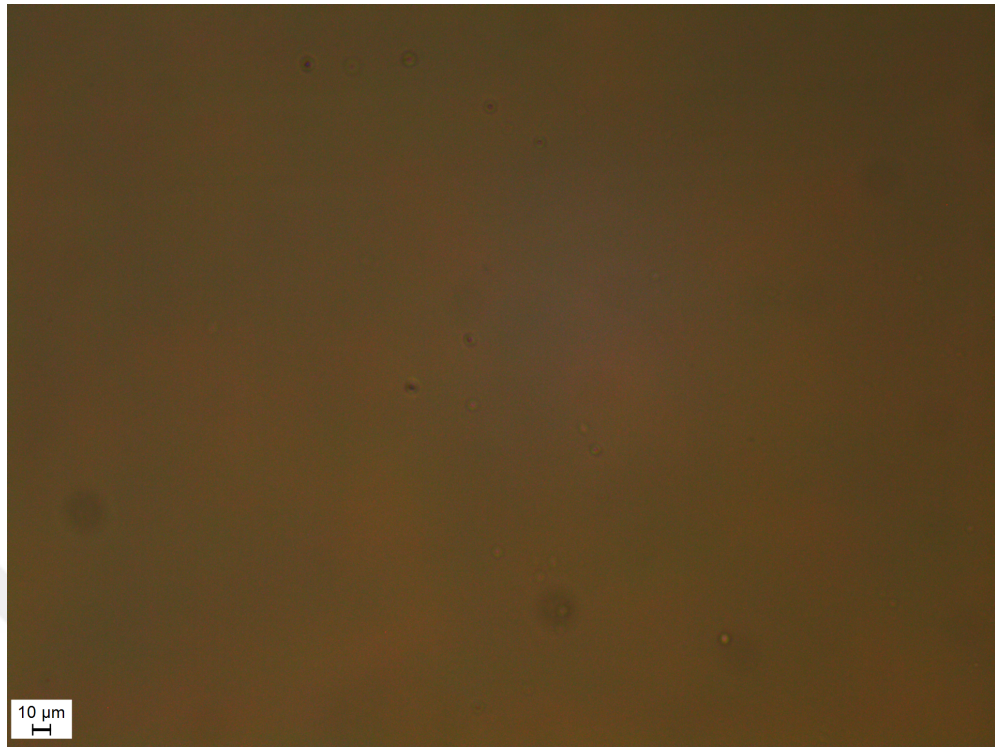


Figure 3.5. Light Microscopy images for HA-BSA system; 20x (pH 2.80, 250mM NaCl).

For the HA-CHI system, it was observed that because of the positive charge of CHI at low pH (Figure 3.2), turbidity started to increase at lower pH values that means complex coacervation occurs around pH 2.0.

In addition, the effect of salt concentration on the pH of the onset of phase separation, pH_{ϕ} values, where coacervation begins, was also observed in this system. It was detected that as the salt concentration increased (increased screening effect), coacervation and precipitation occurred at higher pH values (Figures 3.7).

Light microscopy images have showed the coacervate droplets and any precipitation did not occur at fixed pH values for PE-PE system (Figure 3.7, 3.9 and 3.10). Diameter of droplets is $5.7 \pm 0.35 \mu\text{m}$ at pH 2.8, $6.2 \pm 0.32 \mu\text{m}$ at pH 3.00 and $5.9 \pm 0.45 \mu\text{m}$ at pH 4.09.

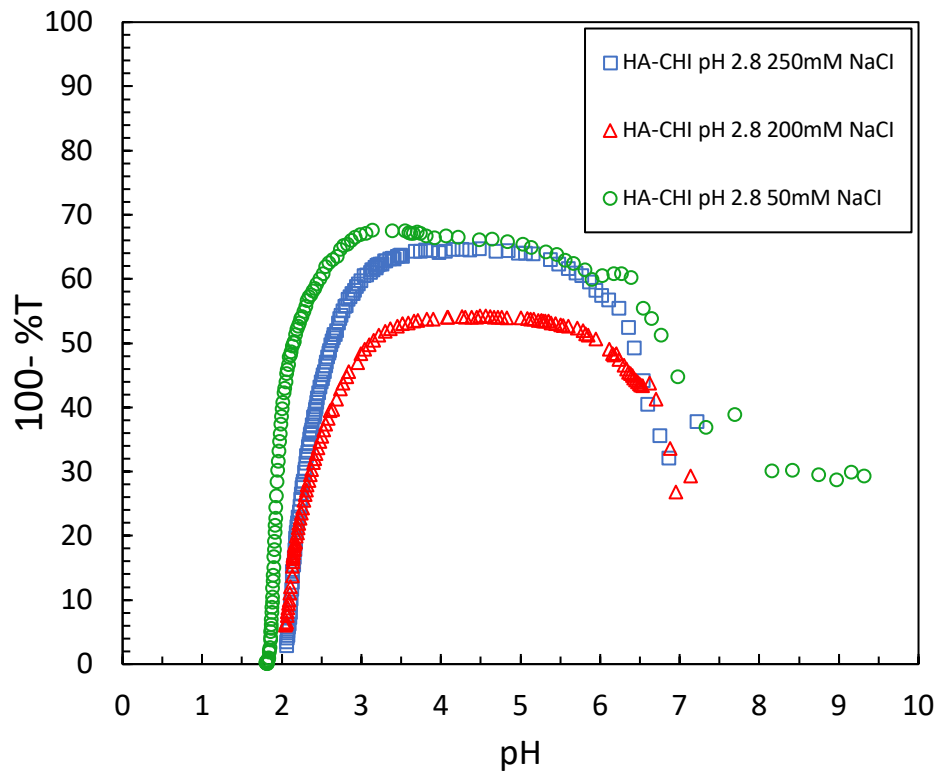


Figure 3.6. Turbidity vs. pH for the HA (950 kDa)/CHI (396kDa) mixture at 50, 200 and 250mM NaCl. pH was increased gradually from 2.0 to around 7.0.

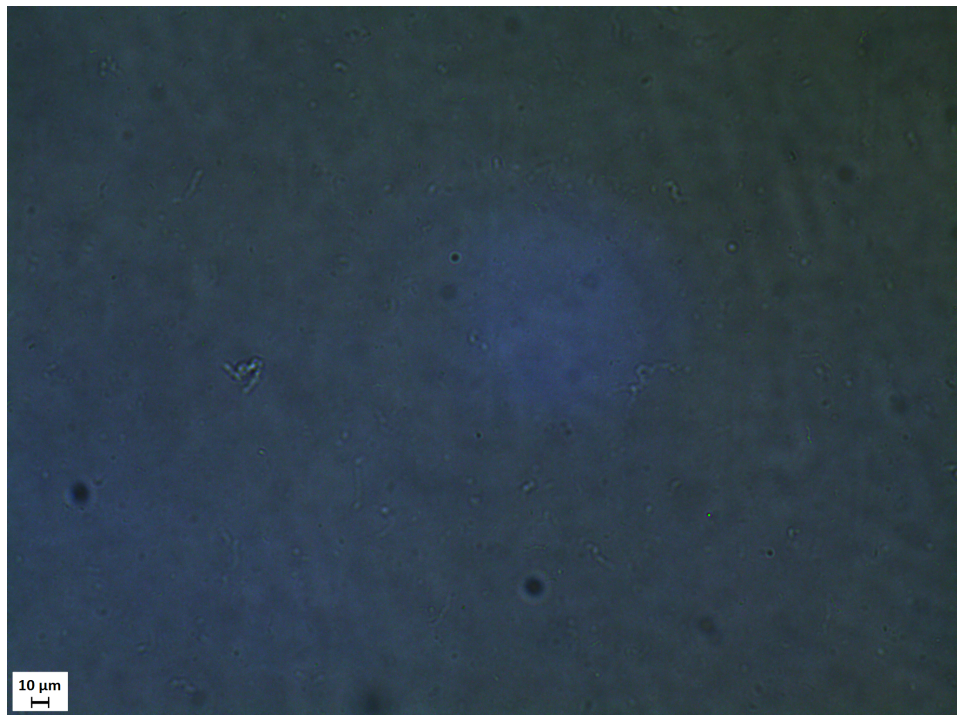


Figure 3.7. Light Microscopy images for HA-CHI system; 20x (pH 4.09, 200mM NaCl).

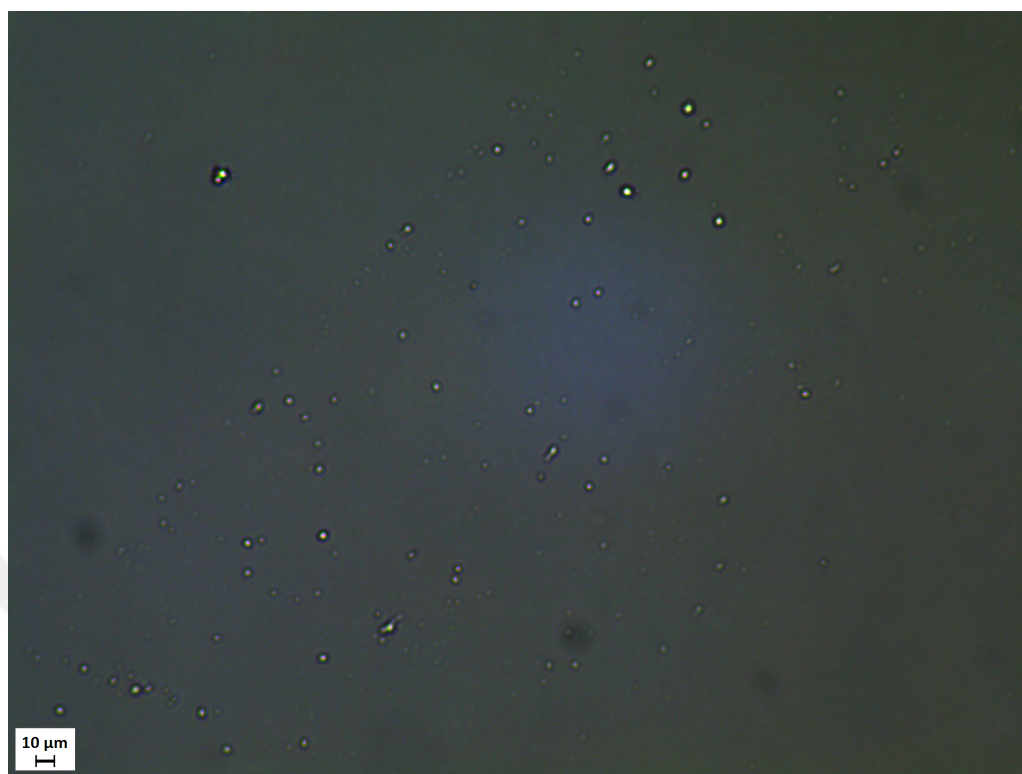


Figure 3.8. Light Microscopy images for HA-CHI system; 20x (pH 2.80, 250 mM NaCl).

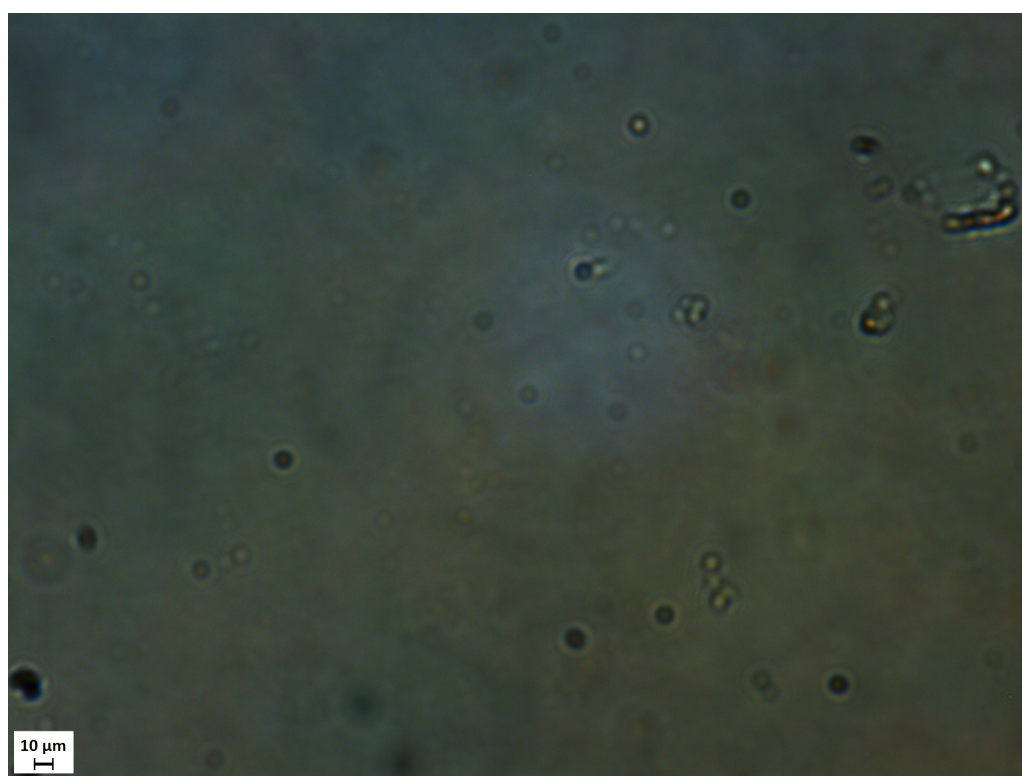


Figure 3.9. Light Microscopy images for HA-CHI system; 20x (pH 3.00, 50 mM NaCl).

3.4.2. Zeta Potential Measurements

Zeta potential measurements were made to examine the charge of complex coacervates between oppositely charged polyelectrolyte-polyelectrolyte and protein-polyelectrolyte systems. Zeta measurements of both systems at pH 2.80, which are among the conditions given in Tables 3.3 and 3.4 above, were carried out at 25°C with Brookhaven 90plus Zeta PALS software device. The values obtained by the zeta potential measurements of HA-CHI and HA-BSA coacervate solutions prepared in 250mM NaCl at pH 2.80 are given in Table 3.5. With the results obtained from the zeta measurements, it was determined that the mobility values were not zero, so it was determined that the interactions were not stoichiometric (1:1 binding ratio). These values are consistent with the results of previous study of Kayitmazer et al. [1] who interpreted the results as follows; pK_a shifts can occur through opposite charge interactions in the HA-CHI mixture. In other words, in the interaction between HA and CHI, basicity of CHI increases with the HA binding, and acidity of HA increases with the CHI binding [1]. Another explanation given is the different charge distance value of polyelectrolytes which does not result in 1:1 binding interaction and results in charge neutralization. Therefore, the electrophoretic mobility values are higher than zero.

Table 3.5. Zeta potential measurement values of HA-CHI and HA-BSA complex coacervate.

Coacervate system	Mobility (μ) ($\times 10^{-8} \text{ m}^2 \text{ s}^{-1} \text{ V}^{-1}$)	Zeta Potential (mV)
HA-CHI	1.33 ± 0.08	17.05 ± 0.97
HA-BSA	-1.13 ± 0.04	-14.47 ± 0.51

Electrostatic interactions are believed to be the primary driving force in coacervate formation of protein-polyelectrolyte systems. Normally, polycations and proteins interact at higher pH values than the protein's isoelectric point, whereas for polyanions this happens at lower pH's. However, Park et al. [108] observed that polyanions interact with proteins at pH higher than the isoelectric pH and they interpreted that heterogeneous charge distribution of

proteins can be the reason. This heterogeneous charge distribution of proteins may hinder their neutralization, which may occur with the interaction of opposite charges. Therefore, the reason why the mobility is less than zero is the effect of the distribution of negative and positive charges in the protein on the net charge of the coacervate.

3.5. Small-Angle Neutron Scattering

Small angle neutron scattering profiles are shown in Figures 3.11, 3.12 and 3.13 for the HA-BSA coacervates and the HA-CHI coacervates, prepared at pH 2.80, 3.00, and 4.09 with different ionic strength (50mM, 200mM and 250mM). The scattering profiles of pure polyelectrolytes (HA and CHI) and protein solutions are significantly different as compared to complex coacervate solutions of HA-CHI and HA-BSA. In our study, we observed a noticeable decrease in the signal at the lower q region for all system.

We tried to fit the scattering data to $I \sim q^{-\alpha}$ and calculate its power-law dependence according to the following Porod-law:

$$I \sim q^{-\alpha}, \quad (3.2)$$

where I is the scattering intensity, q is the scattering vector, and α is the power-law exponent. The power law exponent is evaluated by analysis of the Small-Angle Neutron Scattering (SANS) data after obtaining the slope of the logarithmic plot of the scattering intensity ($I(q)$) versus the scattering vector (q) on a log-log scale. The power law behavior in SANS data is generally observed in the low- q region. This analysis is commonly used to examine the structural feature of various systems, such as macromolecules, complexes, nanoparticles, or aggregates, and provide to characterize their fractal behavior. When the power-law exponent is three ($\alpha=3$), it indicates an object with three-dimension [109].

When interpreting Small Angle Neutron Scattering (SANS) profiles to understand coacervate formation, it is important to take into account the pH and ionic strength parameters that influence the system. Additionally, one should consider that the isoelectric point (pI) of Bovine Serum Albumin (BSA) can be affected by changes in ionic strength. In the study conducted by Salis et al. [110], they investigated the behavior of Bovine Serum Albumin (BSA) at different concentrations and highlighted the shifts in the isoelectric point

(pI) of BSA. They observed that the pI shifted towards lower pH values as the ionic strength increased. The charge characteristics of BSA are influenced by the presence of salts in the solution. Higher ionic strength can lead to stronger screening of the charges on BSA, resulting in a shift in the pI to lower pH values. Interestingly, the study found that at lower salt concentrations, the shift of the pI to the higher values was not as noticeable compared to higher salt concentrations. This suggests that the influence of salt concentration on the pI of BSA is more significant at higher salt concentrations.

When considering the effect of pH in the context of salt concentration, it is important to consider the interaction strength between BSA and other molecules. Although the positive charge of BSA is expected to decrease with increasing pH, the presence of lower salt concentrations can result in higher positive charges at a specific pH value. Therefore, while the positive charge is expected to decrease with increasing pH, when the salt concentration is taken into account, the interaction strength increases due to the fact that the positive charge at pH 3.00 is higher than pH 4.09 due to the lower salt concentration. Another consequence of this is a decrease in intramolecular protein repulsion due to increased positive charge. With all these reasons, we observed a linear decrease in power law dependence (α) for HA-BSA system with increasing interaction strength due to the increase of positive BSA charge. The decrease in the power law exponent (α) caused by the higher negative charge of the protein is consistent with the results reported by Dubin et al. [103].

For the HA-CHI complex coacervation system, an increase in scattering is observed in the low q region as the pH increases (Figure 3.10, 3.12 and 3.13). However, for HA-CHI system, while it was observed an increase in power law dependence (α) with decreasing pH, this effect was not seen at pH 2.80 (Table 3.6). It is important to consider both the charge density of chitosan (CHI) and the concentration of salt in this outcome. As the pH decreases, the ionization degree of CHI decreases (Figure 3.2), resulting in a decrease in its charge density. Additionally, the complexation is adversely affected by the presence of high salt concentration due to the charge screening.

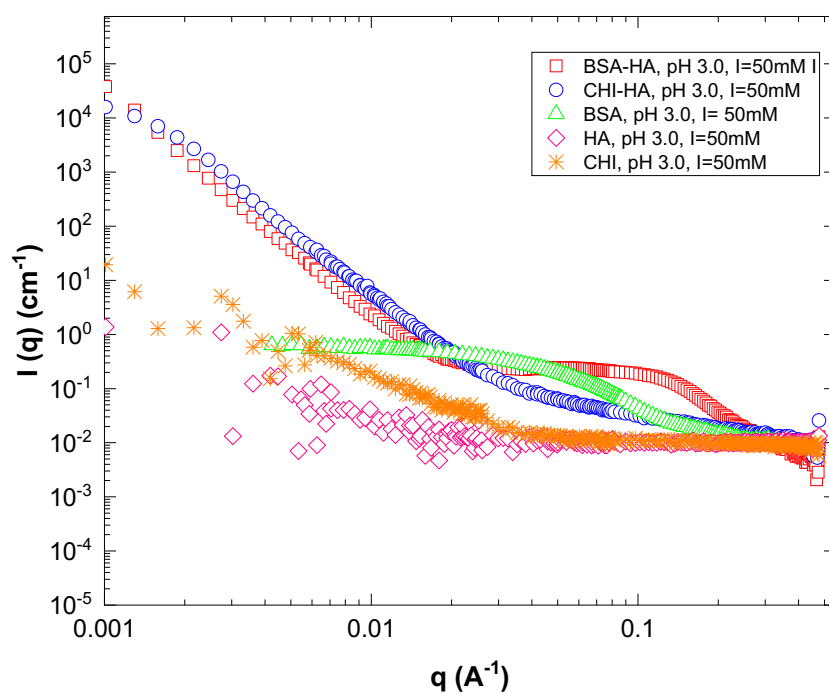


Figure 3.10. Scattering profiles for BSA-HA and HA-CHI coacervates prepared at pH 3.00 ± 0.05 at 50mM (I).

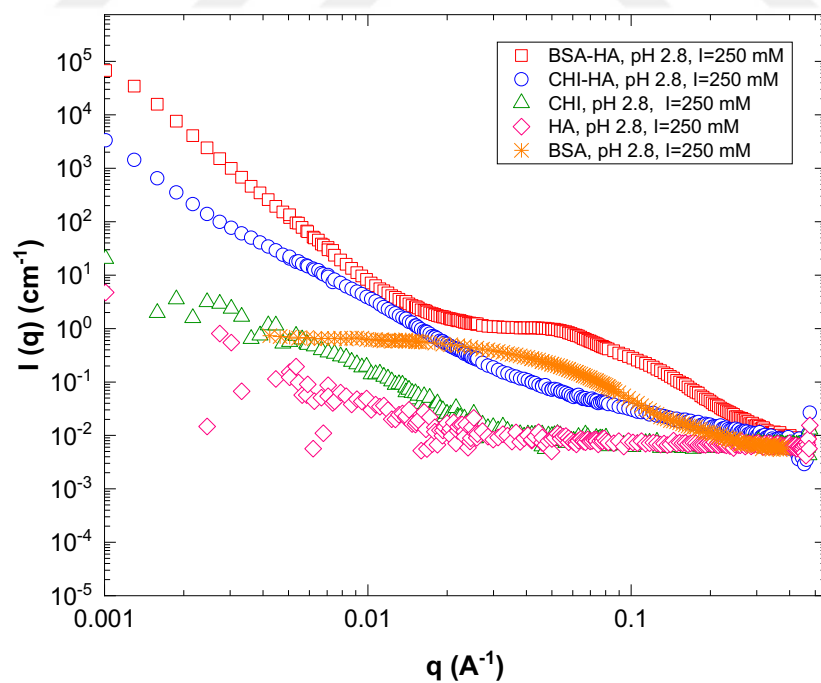


Figure 3.11. Scattering profiles for BSA-HA and HA-CHI coacervates prepared at pH 2.80 ± 0.05 at 250mM (I).

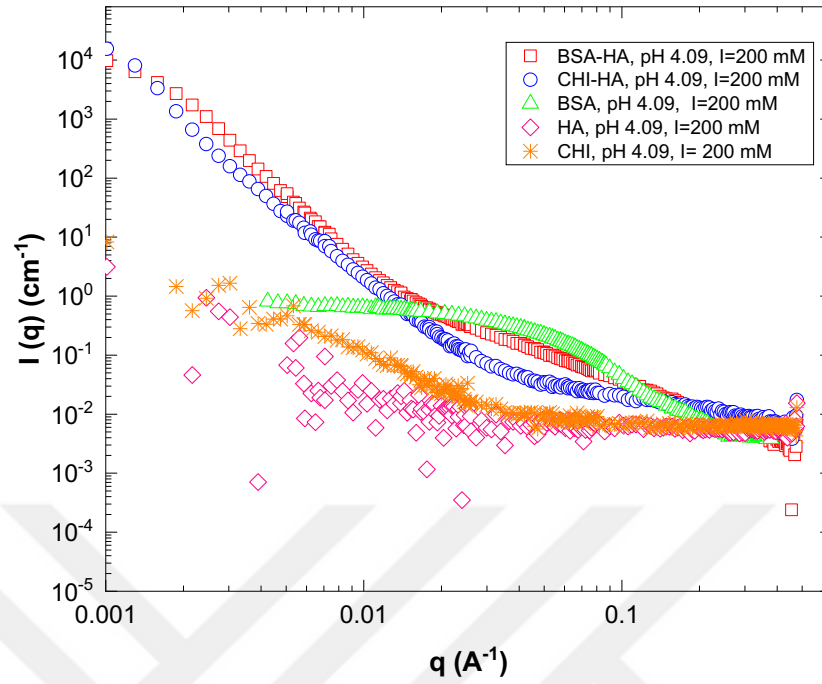


Figure 3.12. Scattering profiles for BSA-HA and HA-CHI coacervates prepared at pH 4.09 $\pm$ 0.05 at 200mM (I)

Table 3.6. Power-Law Dependence (α) for Coacervates at different pH values.

Sample	pH	I (mM)	q range ($\text{\AA}^{-1}$)	corresponding size (nm)	Power-law dependence (α)
BSA-HA	2.80	250	0.0131-0.0208	48-30	3.1
BSA-HA	3.00	50	0.00332-0.0188	209-33	3.7
BSA-HA	4.09	200	0.00563-0.0164	105-39	3.8
HA-CHI	2.80	250	0.005626-0.02991	105-21	2.8
HA-CHI	3.00	50	0.006262-0.03788	105-17	3.8
HA-CHI	4.09	200	0.005338-0.02403	126-26	3.4

The radius of gyration R_g is evaluated by using Guinier-Porod approximation which is derived from Kratky-Porod model

$$\ln(I(q)) \approx \ln(nv^2 \Delta\rho^2) - \frac{1}{3} R_g^2 q^2. \quad (3.2)$$

R_g is obtained from the slope of the linear fit at low q of $\ln(I)$ versus q^2 curve and here $q.R_g$ should be less than 1 ($q.R_g < 1$) if R_g is smaller than 125nm (Figure 3.13).

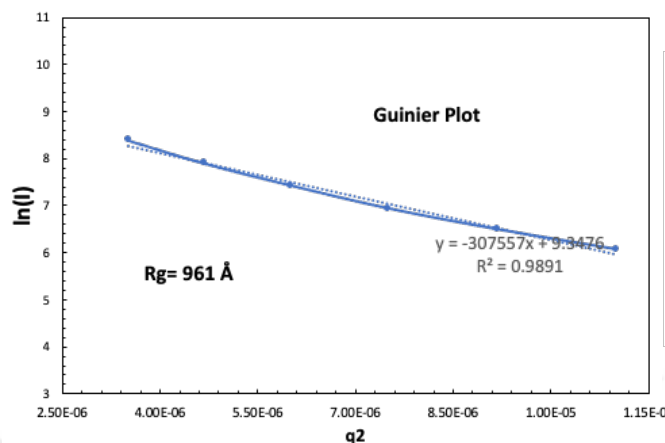


Figure 3.13. Schematic representation of Guinier plot and calculation the Radius of Gyration (R_g).

Radius of gyration for polyelectrolyte-polyelectrolyte and protein-polyelectrolyte system and polyelectrolytes and protein solutions was determined from the Guinier plot for each condition. While BSA and polyelectrolytes solutions have lower R_g values, one can notice that the results indicate that complex coacervate systems exhibit higher R_g values at all pHs. When the results are examined, it is seen in Figure 3.14 that pH was found to significantly influence R_g values in both systems.

For HA-BSA system, increasing pH resulted in decreased R_g in the range from 1233Å to 943Å due to the diminishing the negative charge of the protein (Figure 3.14). These results are consistent with the earlier study reported by Merzougui et al. [111] that they also observed complexation depends on pH values. In the HA-CHI system, increasing pH leads to higher CHI charge density (Figure 3.2), positively affecting coacervate formation and strengthening the electrostatic interaction between CHI and HA. This was confirmed by the increase in R_g values over a specific pH range 2.8 to 4.09 (Figure 3.14). This was confirmed by the increase in R_g values in the range 765Å -1365 Å (Table 3.7).

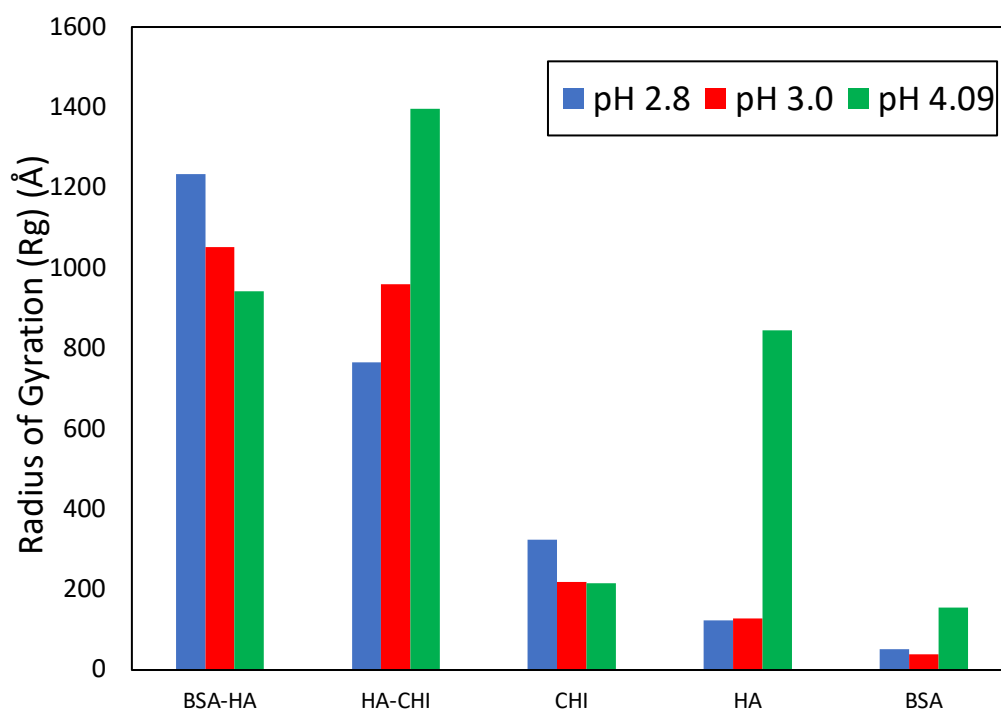


Figure 3.14. The changes in the radius of gyration at different pH and ionic strengths for HA-BSA and HA-CHI complex coacervate systems and free HA, CHI and BSA solutions in NaCl.

Table 3.7. R_g values for HA-BSA and HA-CHI systems at different pHs and ionic strength.

Sample	pH	I (mM NaCl)	R_g (Å)
BSA-HA	2.80	250	1233
BSA-HA	3.00	50	1052
BSA-HA	4.09	200	943
HA-CHI	2.80	250	765
HA-CHI	3.00	50	961
HA-CHI	4.09	200	1396

3.6. Conclusion

In this chapter, we compared coacervates consisting of polyelectrolyte-polyelectrolyte (PE-PE) with coacervates consisting of polyelectrolyte-protein (PE-Protein) to understand the effect of surface curvature. The first system has hyaluronic acid (HA), as an anionic polyelectrolyte, and chitosan (CHI), as a cationic polyelectrolyte. HA and CHI were chosen because in the literature, there is not any small angle neutron scattering (SANS) technique that has been applied to HA-CHI as a PE-PE system and HA-BSA as a PE-protein system to investigate the effect of surface curvature on the coacervate structure and to compare these two systems.

In the turbidimetry experiments performed at different salt concentrations, turbidity increase (%T) was not observed at higher pH values than the isoelectric point of BSA ($pI = 4.9$). This is because the net charge of BSA molecule in this region is negatively charged as HA molecule. The fact that both molecules are negatively charged, in other words, there are no oppositely charged molecules, is proof of this, as coacervation does not occur for each salt condition. Another observed situation is that as the salt concentration increases, turbidity increment started, in other words, coacervation and subsequent precipitation occurred at higher pH values. The reason for this is due to the screening effect. As the salt concentration increases, the intermolecular electrostatic interaction decreases. Therefore, interaction is observed at lower pH and then precipitation occurs at lower pH.

For the HA-CHI system, it was observed that because of the positive charge of CHI at low pH, turbidity started to increase at lower pH values that means complex coacervation occurs around pH 2.0. In addition, the effect of salt concentration on the pH for the onset of phase separation, pH_{ϕ} values, where coacervation begins, was also observed in this system. It was detected that as the salt concentration increased (increased screening effect), coacervation and precipitation occurred at higher pH values.

Zeta potential measurements were made to examine the charge of complex coacervates between oppositely charged polyelectrolyte-polyelectrolyte and protein-polyelectrolyte systems. With the results obtained from the zeta measurements, it was determined that the mobility values were not zero, so it was determined that the interactions were not stoichiometric (1:1 binding ratio). Another explanation given is the different charge distance

value of polyelectrolytes which does not result in 1:1 binding interaction and result in charge neutralization. Therefore, the electrophoretic mobility values are higher than zero.

The power law dependence (α) was determined for both complex coacervation systems of HA-BSA and HA-CHI. In the HA-BSA system, there is a linear decrease in α with increasing interaction strength due to the increase in positive BSA charge. For the HA-CHI system, an increase in scattering is observed in the low q region as pH increases, but the power law dependence is not affected at pH 2.8. This outcome is influenced by the charge density of chitosan (CHI) and the concentration of salt, which impacts complexation formation.

The radius of gyration (R_g) was investigated in polyelectrolyte-polyelectrolyte and protein-polyelectrolyte systems using the Guinier plot. The results revealed that complex coacervate systems exhibited higher R_g values through all pH values, with pH having a significant impact on R_g values in both systems. In the case of the HA-BSA system, increasing pH led to a decrease in R_g due to reduced negative protein charge, while in the HA-CHI system, higher pH resulted in elevated CHI charge density, positively influencing coacervate formation and enhancing the electrostatic interaction between CHI and HA, as confirmed by the increase in R_g values within the specific pH range of 2.8 to 4.09.

4. EFFECT OF DIFFERENT SALT TYPES (HOFMEISTER SERIES) ON PROTEIN- POLYELECTROLYTE AND POLYELECTROLYTE- POLYELECTROLYTE COACERVATES

4.1. Introduction

The Hofmeister series was first studied by Franz Hofmeister in the 1880s and in the 1890s examining the ion effect by conducting studies that showed the precipitation of egg yolk proteins and colloids in the salt media [112]. Hofmeister series is a list of ions which is ordered according to their effect on many experiments such as cutting efficiency of DNA by restriction enzymes [113], charge of globular proteins [114], surface tension of electrolytes [115], and especially protein solubility (“salting-in” or “salting-out”) [116–119].

Anions of the Hofmeister series has commonly been ordered consistent with their “salting-out” effect (kosmotropes that were identified as ‘water structure makers’). They have several beneficial features such as power of stabilization and strong hydration or “salting-in” (chaotropes, as water structure breakers which is assumed to decrease the stabilization of macromolecules and folded proteins) properties of a macromolecule in a solution [120], [121].

Some proteins display reverse Hofmeister behavior due to the net charge of the used protein and the ionic strength of the medium [122], [123]. It should be noted that since the effects of some parameters such as pH, ion concentration, buffers, and temperature, are ignored while creating the ranking of the Hofmeister series, interpretation should be made by considering the lack of these studies [124].

Polyelectrolytes in solution have their counterions for the purpose of charge neutrality. These counterions have a low degree of freedom since they should be within the proximity of the polyelectrolyte according to Gouy-Chapman model. When oppositely charged polyelectrolytes come together to associate into complexes or interpolymer complexes, these counterions are released into the medium. Thus, the counterions gain entropy while their degrees of freedom increase [2], [12], [125–128].

The critical entropic effect is a consequence of counter ion release. This is considered the main reason of that coacervation which is affected by total salt concentration in solution. High salt concentration has a negative effect on entropic driving forces which lead to formation of complex coacervation. So, a decrease in entropic driving forces affects stability of phase separation adversely. On the contrary of high salt concentration's negative impact, low salt concentrations contribute to formation of the coacervation by increase in electrostatic interactions which facilitate available polymer conformations and chain reordering [2],[3], [129–132]. Although there are numerous studies about salt impact on complex coacervation, most of them have used NaCl which is a monovalent salt [39],[42], [129], [133–141].

Salt addition can affect the stability, solubility, and activity of biomacromolecules. Hofmeister series is known as an effect of a salt ion on the stability of a protein [142]. The typical ordering of the anion and cation series of Hofmeister are shown in Figure 4.1.

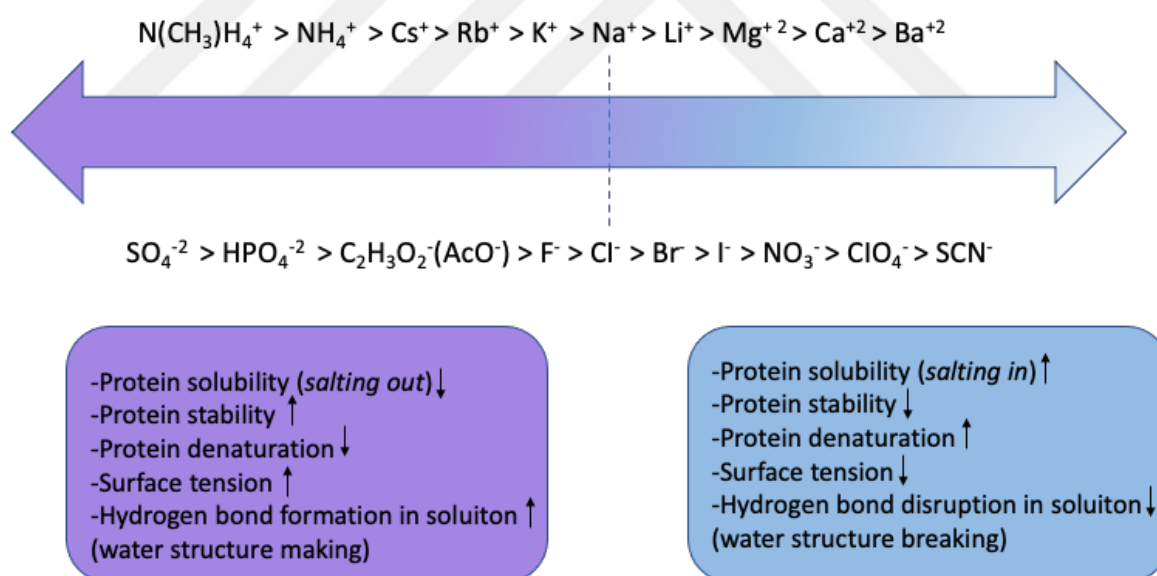


Figure 4.1. The effect of the cationic and anionic Hofmeister series with additional properties, such as “salting-in” and “salting-out”. Adapted from [121] Copyright (2011) with permission from Elsevier) [143].

In literature, there are several studies that investigated polyvalent salts' impacts on the polyelectrolyte complexation. However, precipitate systems have been investigated rather than liquid coacervates [125]. In addition to investigating multivalent salt varieties, the effect

of Hofmeister series on coacervation formation is another significant issue that should be investigated.

Perry and coworkers [3] studied complex coacervation of poly(sodium acrylate) and poly(allylamine hydrochloride) to examine in what way salt type influences when these two polyelectrolytes formed complex coacervation and also investigated whether opposite or correlate behavior of Hofmeister series are displayed. They used a wide range of salt types with different valent values (mono- and divalent) and did turbidimetric titration experiments to figure out of the salt type effect on complexation or coacervate state. As a result of turbidity experiments, the salt concentration is found to have a significant role in coacervate formation. As stated by Perry et al. [3] polyelectrolyte solubility increases via “salting-in” effect with salt addition for all type of salts resulting in favoring complex coacervation and turbidity increase. However, since further addition of salt to the medium, in this case higher than 75mM, a sudden decrease in turbidity value (critical salt concentration) was observed and addition of more salt created a shielding between opposite charges resulted in prevention of coacervates, turning into a clear solution and finally turbidity values reached zero which means no more coacervate structures were present in the solution. They also observed that valency value influences critical salt concentration which means at this value, complex coacervation formation starts decreasing [3].

4.2. Aim of Study

In order to obtain an effective material design, it is necessary to examine each parameter not only for a specific application area, but also for a wider application area. The formation of complex coacervates is induced by the solution conditions (pH and ionic strength) and the release of counterions of the polyelectrolytes. Although there are numerous studies about salt impact on complex coacervation, most of them have used NaCl which is a monovalent salt. In literature, there are several studies that investigated polyvalent salts' impacts on the polyelectrolyte complexation. However, precipitate systems have been investigated rather than liquid coacervates [125]. In this respect, it should be examined that in what way Hofmeister series influences the coacervate formation. While some salts' effects on the formation of complex coacervation resulted in opposite behavior, some of them

showed direct behavior of Hofmeister series. In addition to multivalent salt varieties, the effect of Hofmeister series on coacervation is another significant issue that should be investigated.

In this chapter of thesis, we aimed to investigate the effect of addition of mono and multivalent salts of Hofmeister series (NaCl, KCl, NaNO₃, CaCl₂, MgCl₂, NH₄Cl, NaOAc and Na₂SO₄) on coacervation of oppositely charged polyelectrolytes HA-CHI and CHI-BSA systems and the effects of the concentration of these salts on the formation of coacervate.

It is extremely important to elucidate the structures of protein-polyelectrolyte and polyelectrolyte-polyelectrolyte coacervate systems in applications such as microencapsulation. The studies carried out in this project will contribute to the literature in this sense.

4.3. Experimental Section

4.3.1. Materials

Dried sodium hyaluronic acid (HA) was a gift from Dr. Kazuyuki Miyazawa (Shiseido Co., Ltd., Yokohama, Japan) with nominal molecular weight (MW) of 1200 kDa. Chitosan chloride (CHI) was obtained from Heppe Medical Chitosan “HMC⁺” company (Halle, Germany). Chitosan has 83% Degree of Deacetylation (DD). Molecular weight of CHI is 396 kDa. BSA (Bovine Serum Albumin) was purchased from Sigma (Suitable as diluent in ELISA applications, Initial fractionation by heat shock).

The salts NaCl, Na₂SO₄, NH₄Cl and CH₃COONa.3H₂O were bought from Sigma-Aldrich-Merck. MgCl₂.6H₂O) and NaNO₃ salts were obtained from Carlo Erba. CaCl₂.2H₂O) and KCl were purchased from VWR. Acid and base solutions 0.05, 0.1 and 1N HCl and NaOH were purchased from Merck to adjust the pH of the polymer and salt solutions. All solutions were prepared in Milli-Q water (Millipore, Milford, MA, USA).

4.3.2. Methods

4.3.2.1. Preparation of HA-CHI Coacervate Solutions for Turbidity Experiments with Different Hofmeister Series Salts. Coacervate solutions were prepared with adding solutions in order of CHI, HA polyelectrolytes and salt solution. pH of all solutions (polymer and salt solutions) was adjusted to 6.25 before mixing them. Final concentration of all samples and charge ratio were 1 mM and 0.1735, respectively. Charge ratio is expressed as

$$\frac{[-]}{[+]} = \frac{\frac{m_{HA} \cdot \alpha_{HA}}{MW_{HA}}}{\frac{m_{CHI} \cdot \beta_{CHI} \cdot DD}{MW_{CHI}}}, \quad (4.1)$$

where MW_{HA} and MW_{CHI} correspond to the monomer molecular weight of HA and CHI, respectively, while m_{HA} is the mass of HA, m_{CHI} is the mass of CHI. DD is the degree of deacetylation for CHI. α_{HA} and β_{CHI} are the degrees of ionization values for HA and CHI, respectively.

We tried two different methods for preparation of coacervate suspensions. First, samples were prepared by mixing polycation CHI (590 μ L, 0.5 mg/mL) in Milli-Q water in and addition of the polyanion HA (410 μ L, 0.5 mg/mL) and then the concentrated salt solution (1 mL). Each salt solution was prepared as stock solution (3M) and diluted to desired concentrations to get final concentration (all final salt concentration; 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.75, 1.0 and 1.5 M). However, this method resulted in precipitation. Unfortunately, addition of salt did not help to dissolve the precipitate. Therefore, the second method was followed.

In the second method, coacervation was performed by first polycation CHI (590 μ L) in Milli-Q water addition and second addition was concentrated salt solution (1 mL) and then the polyanion HA (410 μ L) addition as represented in Figure 4.2. All mixed solutions were stirred for 2-3 minutes at room temperature before UV measurements. Turbidity (Turbidity = 100-%T, where T is transmittance) of each mixture was measured with a UV-Vis spectrophotometer at 25 °C (Shimadzu pharmaspace 1700 UV-Vis spectrophotometer, Kyoto, Japan). The turbidity of each concentration was reported as 100-T%. Three measurements were taken during the experiment for all concentrations and all experiments were repeated three times. For UV experiment, same UV disposable cuvette was used for all salt concentrations. After each measurement, the UV cuvette was cleaned using 1.5M salt

solution and rinsed with Milli-Q water several times. Finally, it was rinsed with 2-propanol and then dried with N₂.

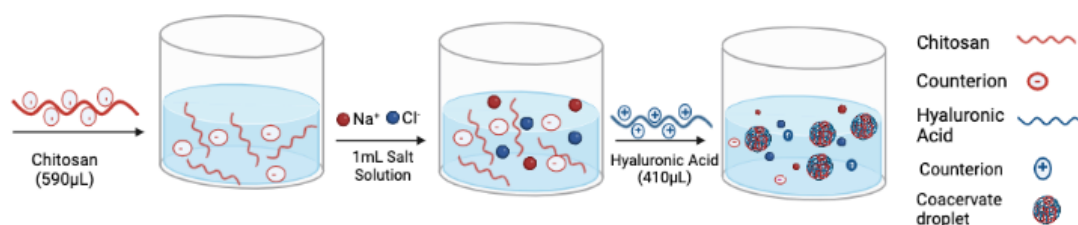


Figure 4.2. Schematic representation of preparation of HA-CHI coacervate solution.

4.3.2.2. Preparation of CHI-BSA Coacervate Solutions for Turbidity Experiments with different Hofmeister Series Salts. BSA and CHI polymer solutions were both prepared at concentrations of 0.5 mg/ml and 0.5 mg/ml. Each salt solution was prepared as stock solution (3 M) and diluted to desired concentrations to get final concentration (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.75, 1.0 and 1.5M). The pH of all solutions (polymer and salt solutions) was adjusted to 6.25 before preparing the complex coacervation solution. Coacervation solution were prepared by mixing order polycation CHI (500 μ L) in Milli-Q water and addition of the concentrated salt solution (1 mL) and then addition of the polyanion BSA (500 μ L) as represented in Figure 4.3.

All mixed coacervate solutions were stirred for 2-3 minutes at room temperature before UV measurements. Turbidity (Turbidity = 100-%T) of each mixture was measured with a UV-Vis spectrophotometer at 25 °C (Shimadzu pharinspace 1700 UV-Vis spectrophotometer, Kyoto, Japan). The turbidity of each concentration was reported as 100-T%. Three measurements were taken during the experiment for all concentrations and all experiments were repeated three times. For UV experiments, same UV disposable cuvette was used for all salt concentrations. After each measurement, the UV cuvette was cleaned using 1.5M salt solution and rinsed with Milli-Q water several times. Finally, it was rinsed with 2-propanol and then dried with N₂.

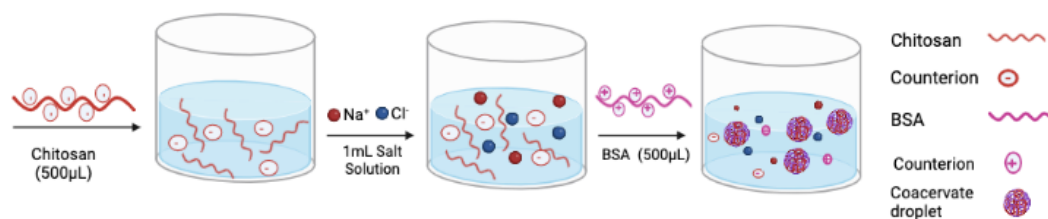


Figure 4.3. Schematic representation of preparation of CHI-BSA coacervate solution.

4.3.2.3. Preparation of Polyelectrolyte-Salt Mixture Solutions for Control Experiments with different Hofmeister Series Salts. Control experiments were conducted to assess the impact of salts from the Hofmeister series on the coacervation process during the preparation of coacervate solutions involving polyelectrolytes (CHI and HA) and a protein (BSA). The purpose of these control experiments was to examine whether the salts caused any aggregation or precipitation of the polyelectrolytes or protein components. HA, CHI and BSA polymer solutions were prepared at concentrations of 0.5 mg/ml and 0.5 mg/ml. Each salt solution was prepared as stock solution (3.0 M) and diluted to desired concentrations to get final concentration (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.75, 1 and 1.5 M). The pH of all solutions (polymer and salt solutions) was adjusted to 6.25 before preparing the mixture solution including polyanion or polycation and salt solution. Polyelectrolyte-salt mixture solutions for CHI were prepared by mixing order CHI (590 µL) in Milli-Q water and addition of the concentrated salt solution (1 mL) and then addition of the Milli-Q water (410 µL) for CHI control experiments as represented in Figure 4.4. For HA, polyelectrolyte- salt mixture solutions were prepared by mixing order Milli-Q water (590µL) and addition of the concentrated salt solution (1 mL) and then addition of the HA (410 µL) as represented in Figure 4.5. For BSA control experiments, polyelectrolyte- salt mixture solutions were prepared by mixing order Milli-Q water (500µL) and addition of the concentrated salt solution (1 mL) and then addition of the BSA (500µL) as represent in Figure 4.6.

All mixed solutions were stirred for 2-3 minutes at room temperature before UV measurement. Turbidity (Turbidity = 100-%T) of each mixture was measured with a UV-Vis spectrophotometer at 25 °C (Shimadzu pharmaspace 1700 UV-Vis spectrophotometer, Kyoto, Japan). The turbidity of each concentration was reported as 100-T%. Three measurements were taken during the experiment for all concentrations and all experiments were repeated three times. For UV experiment, same UV disposable cuvette was used for

all salt concentrations. After each measurement, the UV cuvette was cleaned using 1.5M salt solution (which prepared for the experiment) and rinsed with Milli-Q water several times. Finally, it was rinsed with 2-propanol and then dried with N_2 .

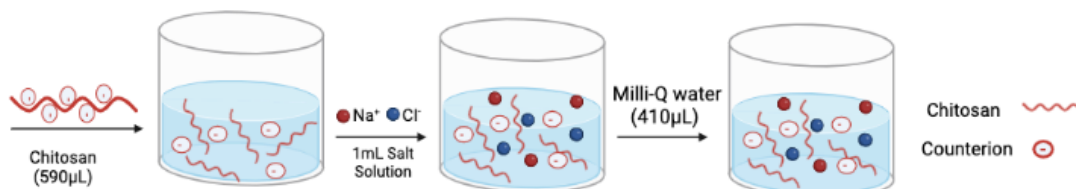


Figure 4.4. Schematic representation of preparation of CHI-Salt mixture solution.

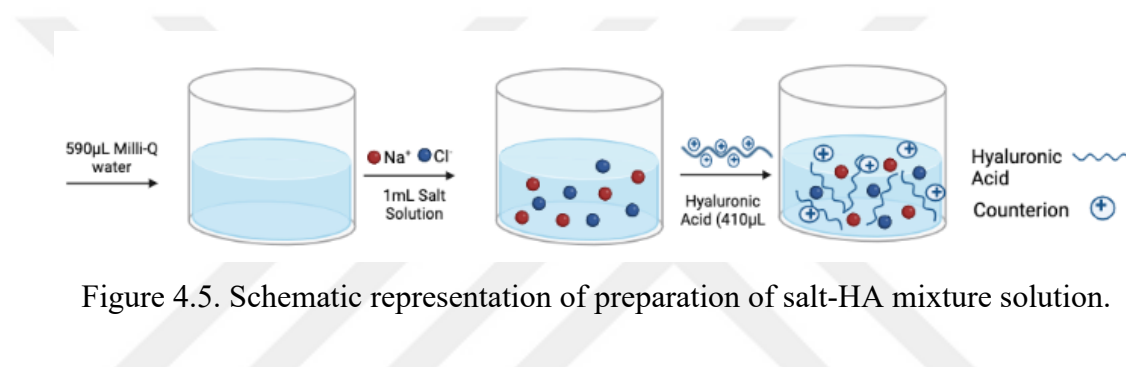


Figure 4.5. Schematic representation of preparation of salt-HA mixture solution.

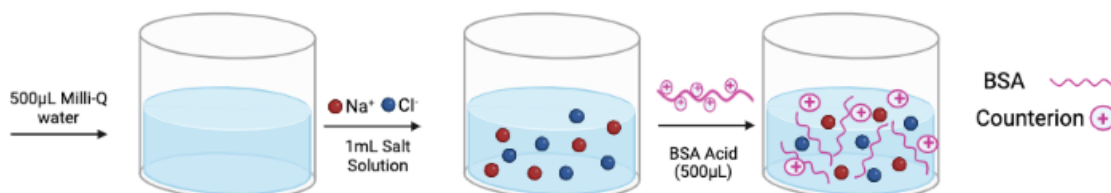


Figure 4.6. Schematic representation of preparation of Salt-BSA mixture solution.

4.4. Result and Discussion

We have investigated the effect of different ($NaCl$, KCl , $NaNO_3$, $CaCl_2$, $MgCl_2$, NH_4Cl , $NaOAc$ and Na_2SO_4) salts of Hofmeister series on complex coacervation formation of HA-CHI and CHI-BSA systems. We further studied the effects of salt concentration on stability of coacervates at fixed pH. As observed in many studies in the literature, there is no generalization that there is a correlation between the salts in the Hofmeister series and complex formation. However, in some complex systems there is a positive correlation with

the Hofmeister series. When interpreting the turbidimetry results obtained for the HA-CHI and CHI-BSA complex coacervation systems we studied, it would be reasonable to interpret the sequencing properties of the salts in the Hofmeister series.

In this perspective, it is observed that there is a positive correlation in turbidities with the results we obtained when the effects of Hofmeister salts on polymer solubility are considered with respect to “salting-in” and “salting-out”. It is stated that the “salting-in” feature increases as one goes to the right in the Hofmeister Series, in other words, there is an increase in polymer solubility. As the solubility of polymers increases, the formation of complex coacervation proceeds in a positive direction. During the formation of complex coacervation, interaction (ion-pairing) occurs between opposite charges of polymers. As the “salting-in” property increases, polymer solubility increases and complex coacervation occurs. According to the turbidity experiments, addition of salt has a positive effect up to a point (critical point or critical salt concentration, Figure 4.7). Further addition of salt ion-pairing between polymers proceeds in the opposite direction and results in a decrease in the turbidity. At this stage, the formation of “soluble complex” begins. If more salt is added, the complex dissolves with the shielding effect of the salt between the opposite charges of the polymers [144].

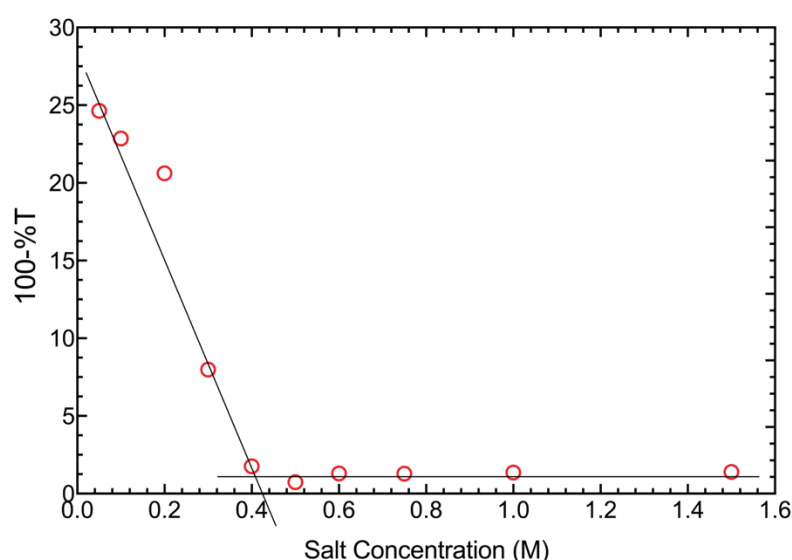


Figure 4.7. An example graph of critical salt concentration determination.

4.4.1. Hofmeister Salts Effects on HA-CHI System

According to the turbidimetry results of complex coacervation systems prepared with reduced “salting-in” properties for cationic salts, the salt concentration range in which coacervation occurs has a wider range. It has been observed that the more the “salting-in” feature increases, proceeds the complex coacervation formation at lower concentrations (Figure 4.8). It was also observed that the results of NaCl and KCl salts were close to each other (Figure 4.8). Similar situations have been observed in the literature [3].

Another property of Hofmeister salts is hydration. Strongly hydrated (cosmotropes) ions reduce the Debye length (the distance between interacting charges) and polymers are allowed to interact with any ion present in the environment. Thus, a decrease occurs in the interaction between polymers [11]. Therefore, this situation was partially observed in the graphs drawn with the data obtained from turbidity measurements of the complex coacervation prepared with anionic salts in the Hofmeister series (Figure 4.9).

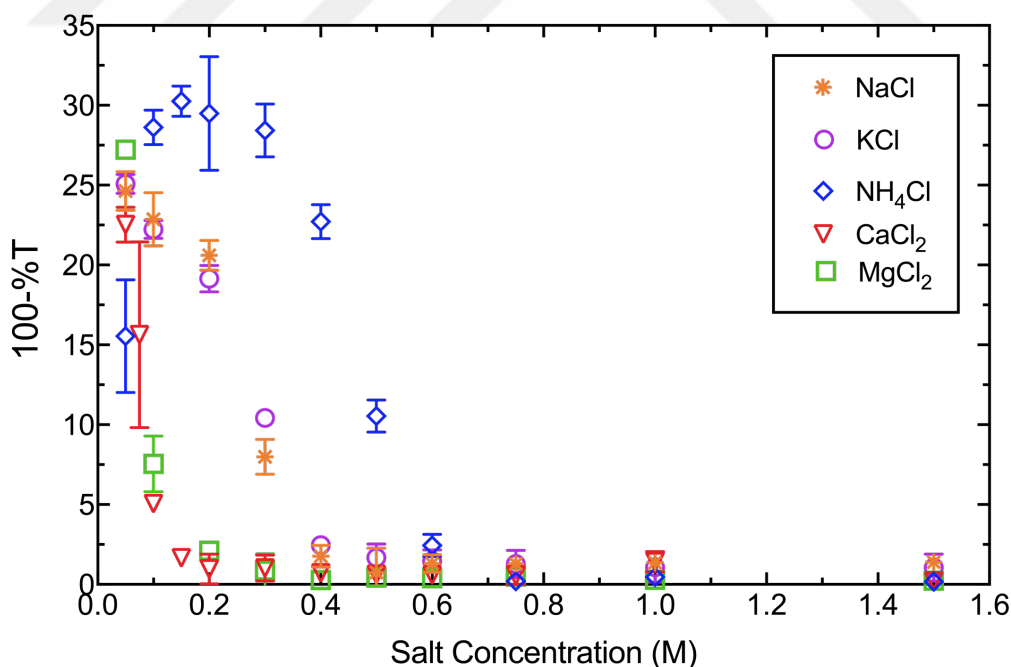


Figure 4.8. Turbidimetry graphs showing the effect of different concentrations of various chlorine (Cl) salts on coacervate formation for the HA-CHI system.

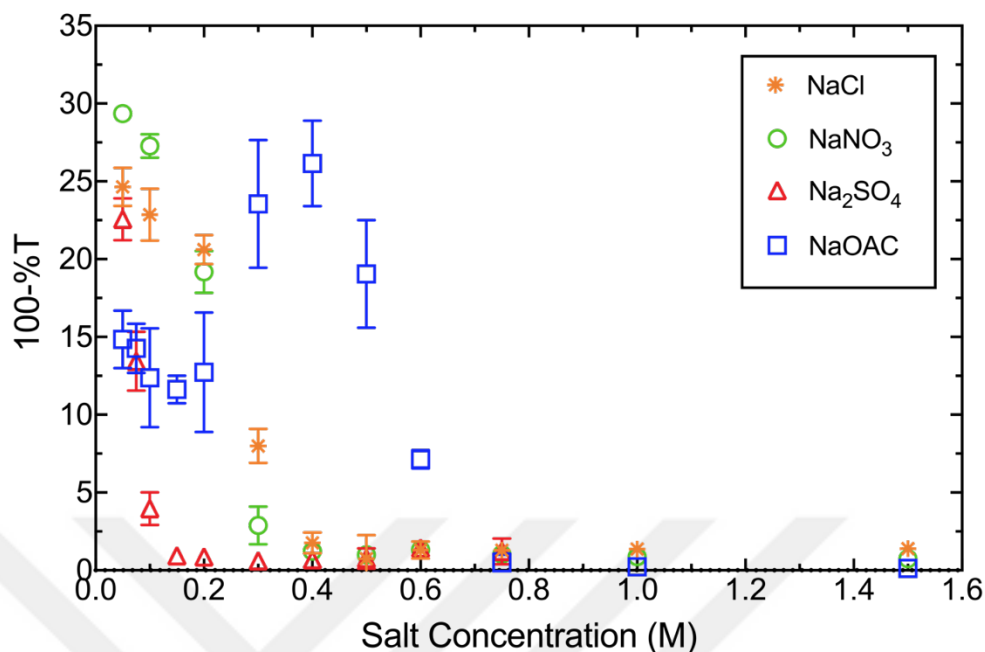


Figure 4.9. Turbidimetry graphs showing the effect of different concentrations of various sodium (Na) salts on coacervate formation for the HA-CHI system.

It was observed that the turbidity values of the complex coacervations prepared with chaotropic anions were slightly higher than those prepared with kosmotropic anions. It is stated above that continued addition of salt during the formation of complex coacervation affected charge interactions between polymers and coacervates began to dissolve. These critical salt concentration values are calculated separately for each salt. It was observed that the critical salt concentration values were lower in strongly hydrated (chaotropic) cationic salts. The reason is that as the chaotropic property increases, the “salting-in” properties increase, and the solubility of the polymer increases and the coacervation reaches the soluble complex form at lower concentrations.

Similar results have been observed in the literature, which supports the results [3]. In anionic salts, the opposite effect (except for Na₂SO₄) was observed than cationic salts. Since sodium acetate (CH₃COONa) salt is a more kosmotropic (strongly hydrated) salt compared to sodium chloride and sodium nitrate salt, it shows “salting-out” feature and reduces polymer solubility. Therefore, higher critical salt concentration values were observed. This increased values for both anionic and cationic salts are given in Table 4.1. It has been observed that divalent salts (CaCl₂, MgCl₂, Na₂SO₄) negatively contribute to the formation of complex coacervation compared to monovalent salts. However, Na₂SO₄ as divalent salt

has lower critical salt concentration. The reasons behind these findings can be attributed to the size of the ions and their charge distribution.

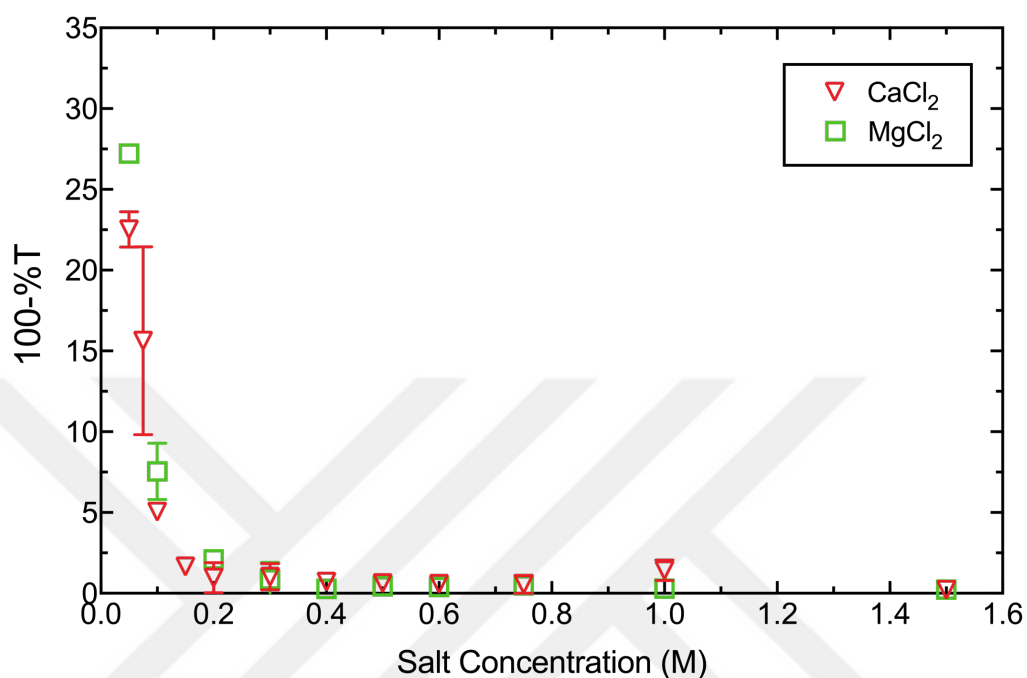


Figure 4.10. Turbidimetry graphs showing the effect of different concentrations of chlorine (Cl) salts with divalent cations on coacervate formation for the HA-CHI system.

Table 4.1. Critical salt concentration values for HA-CHI systems.

Cations	Critical Salt Concentrations (M)	Anions	Critical Salt Concentration (M)
MgCl ₂	0.145 ± 0.02	NaNO ₃	0.34 ± 0.01
CaCl ₂	0.145 ± 0.02	NaCl	0.425 ± 0.02
NaCl	0.425 ± 0.02	CH ₃ COONa	0.68 ± 0.05
KCl	0.47 ± 0.03	Na ₂ SO ₄	0.11 ± 0.03
NH ₄ Cl	0.63 ± 0.02		

4.4.2. Hofmeister Salts Effects on Chitosan and Hyaluronic Acid

The control experiments involving salts from the Hofmeister series were conducted to understand how these ions influence the coacervation process and whether they induce any unwanted aggregation or precipitation of the polyelectrolytes (CHI and HA) or the protein (BSA) components in the coacervate solutions. When examining the turbidity results, it indicates that the addition of Hofmeister salts had no significant effect on the polyelectrolytes (CHI and HA) resulting in no aggregation or precipitation during the coacervation process (Figure 4.11 and 4.12).

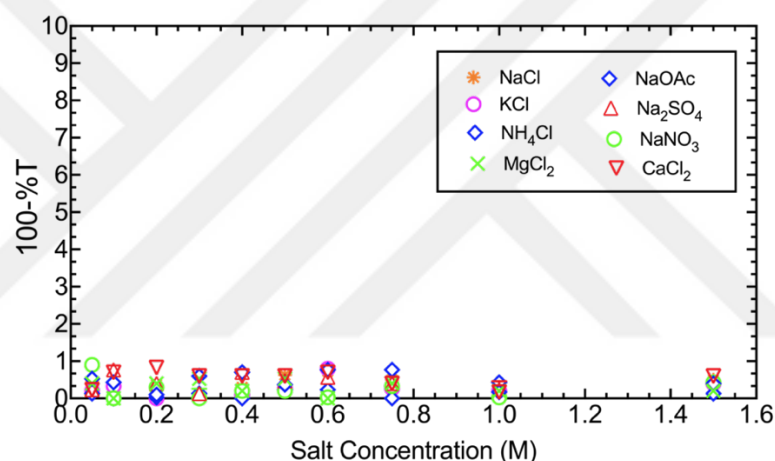


Figure 4.11. Turbidimetry graphs showing the effect of different concentrations of Hofmeister salts on the CHI-Salt mixture solutions.

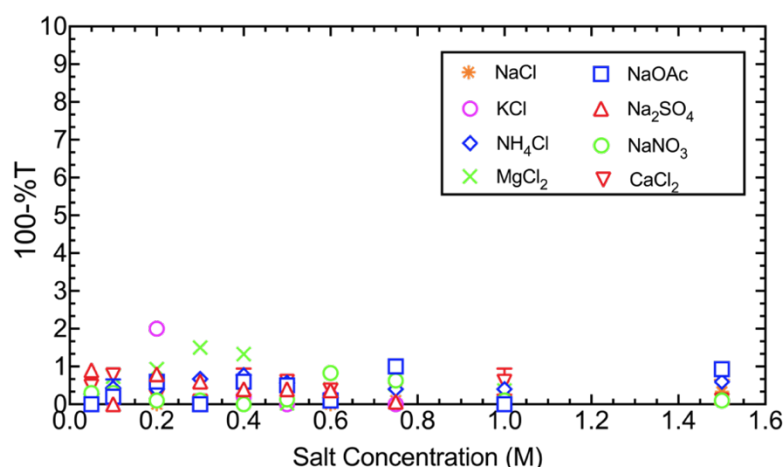


Figure 4.12. Turbidimetry graphs showing the effect of different concentrations of Hofmeister salts on the Salt-HA mixture solutions.

4.4.3. Hofmeister Salts Effects on CHI-BSA System

Effect of different (NaCl, KCl, NaNO₃, CaCl₂, MgCl₂, NH₄Cl, NaOAc and Na₂SO₄) salts of Hofmeister series on complex coacervation formation of CHI-BSA system was investigated. For investigation of salt effect, turbidimetric method was used for CHI-BSA coacervation system at fixed pH 6.25 which is near the biological pH. Higher salt concentration for formation of complex coacervation affected charge interactions between polymers and coacervation began to dissolve (Figure 4.13). Turbidimetry allows to determine the critical salt concentration value where the turbidity started to increase sharply. Critical salt concentration is not solely dependent on ionic strength; there are also other effects including the presence of different types of ions such as divalent or monovalent ions.

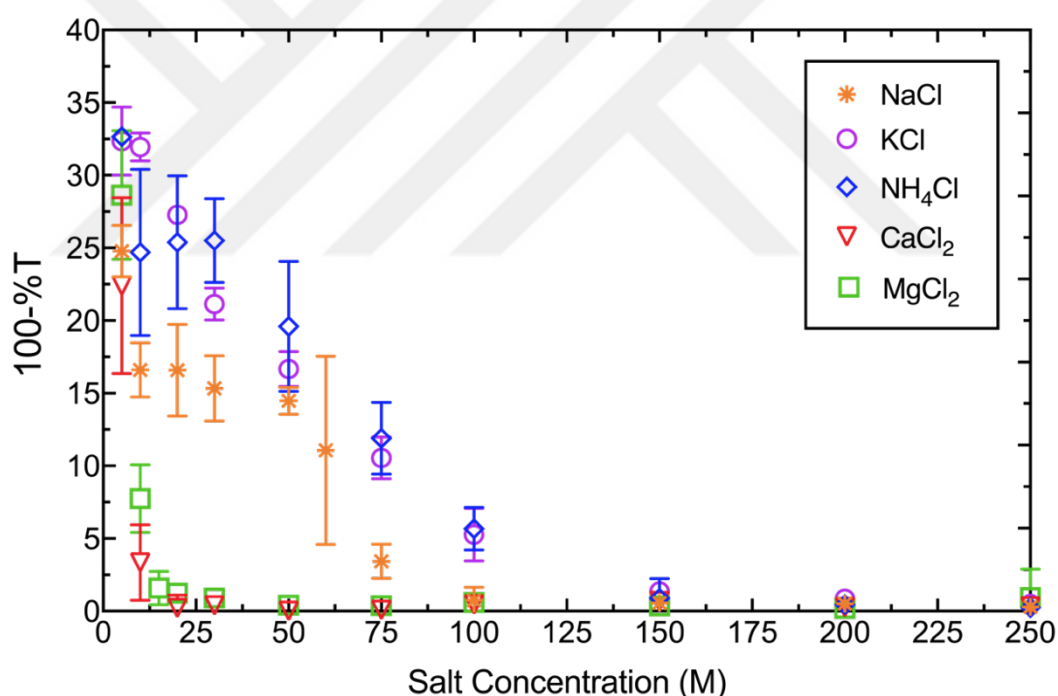


Figure 4.13. Turbidimetry graph showing the effect of different concentrations of various chlorine (Cl) salts on coacervate formation for the CHI-BSA system.

Possibility of conformational change and precipitation of the proteins is higher when protein solutions are prepared with kosmotropic anions due to the high charge density and strong interaction of kosmotropic anions with water molecules [122]. On the other hand, stability of proteins may diminish in chaotropic ions medium since charge density is lower rather than kosmotropic anions. Small kosmotropic ions have smaller polarizability properties [122]. Also, these ions have the ability of holding water of hydration; great

organized hydration shell. On the contrary, large chaotropic ions with greater polarizability remove water hydration quickly. For all these reasons, screening effect of opposite charges in chaotropic ions solutions is greater than kosmotropic ions solutions due to the stronger interaction with polyelectrolytes (Figure 4.14).

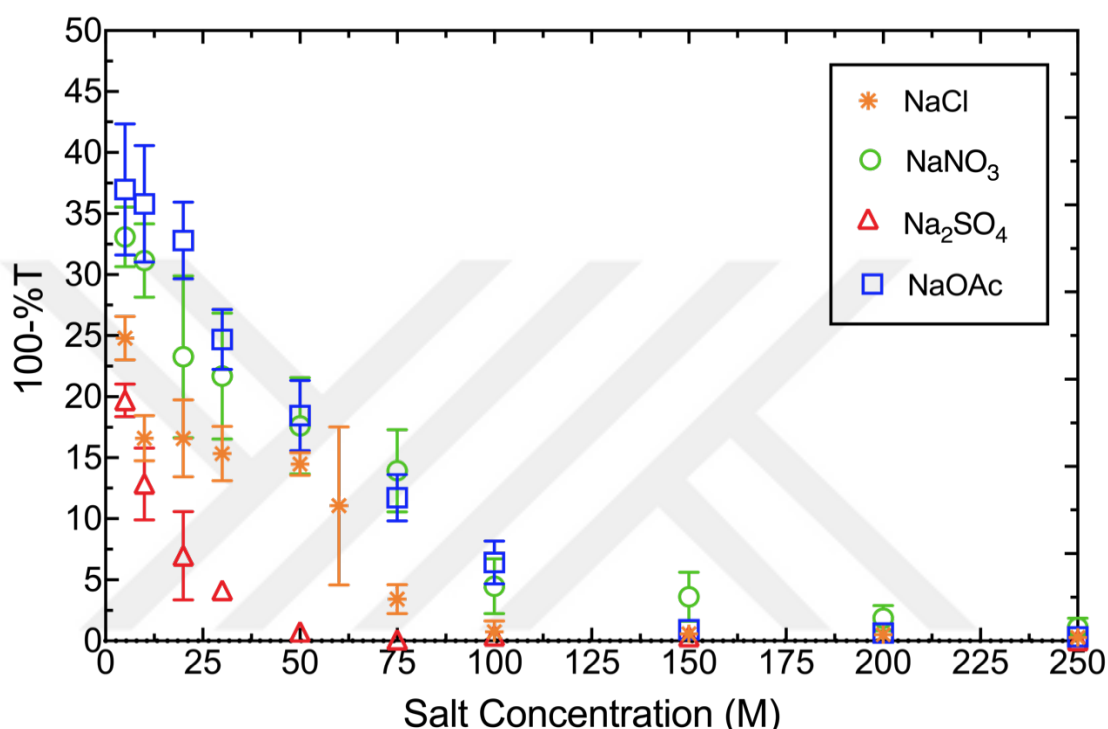


Figure 4.14. Turbidimetry graphs showing the effect of different concentrations of various sodium (Na) salts on coacervate formation for the CHI-BSA system.

In the present work, it was observed that the critical salt concentration values were lower in strongly hydrated (kosmotropes) cationic salts than weakly hydrated cations (Table 4.2). The reason is that the chaotropic ions increase the solubility of the polymer due to the “salting-in” properties. Therefore, coacervation reaches the soluble complex form at lower concentrations. When we focus on the Na salts with different anions, we can see the same correlation between critical salt concentration (Table 4.2) and Hofmeister series. Divalent salts like Na₂SO₄, MgCl₂ and CaCl₂ have inhibitory effect on coacervate formation so they have lower critical salt concentration, the reasons can be explained as the larger size and higher charge of divalent ions provide to a stronger screening effect, reducing coacervate formation and resulting in a lower critical salt concentration (Figure 4.15).

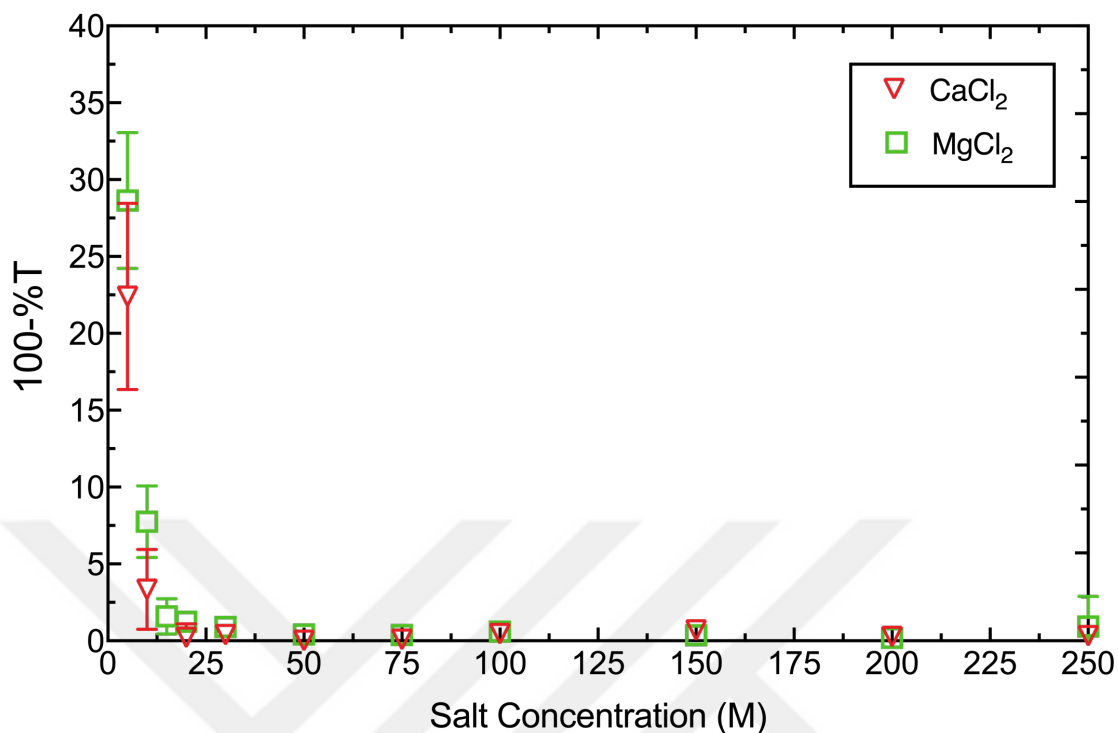


Figure 4.15. Turbidimetry graphs showing the effect of different concentrations of chlorine (Cl) salts with divalent cations on coacervate formation for the CHI-BSA system.

Table 4.2. Critical salt concentration values for CHI-BSA system.

Cations	Critical Salt Concentration (M)	Anions	Critical Salt Concentration (M)
MgCl ₂	0.02 ± 0.01	NaNO ₃	0.14 ± 0.03
CaCl ₂	0.03 ± 0.007	NaCl	0.1 ± 0.04
NaCl	0.1 ± 0.04	CH ₃ COONa	0.12 ± 0.005
KCl	0.14 ± 0.03	Na ₂ SO ₄	0.05 ± 0.01
NH ₄ Cl	0.13 ± 0.004		

4.4.4. Hofmeister Salts Effect on BSA

The control experiments involving salts from the Hofmeister series were conducted to understand how these ions influence the coacervation process and whether they induce any

unwanted aggregation or precipitation of the protein (BSA) component in the coacervate solutions. When examining the turbidity results, it indicates that the addition of Hofmeister salts had no significant effect on the protein (BSA) resulting in no aggregation or precipitation during the coacervation process (Figure 4.16).

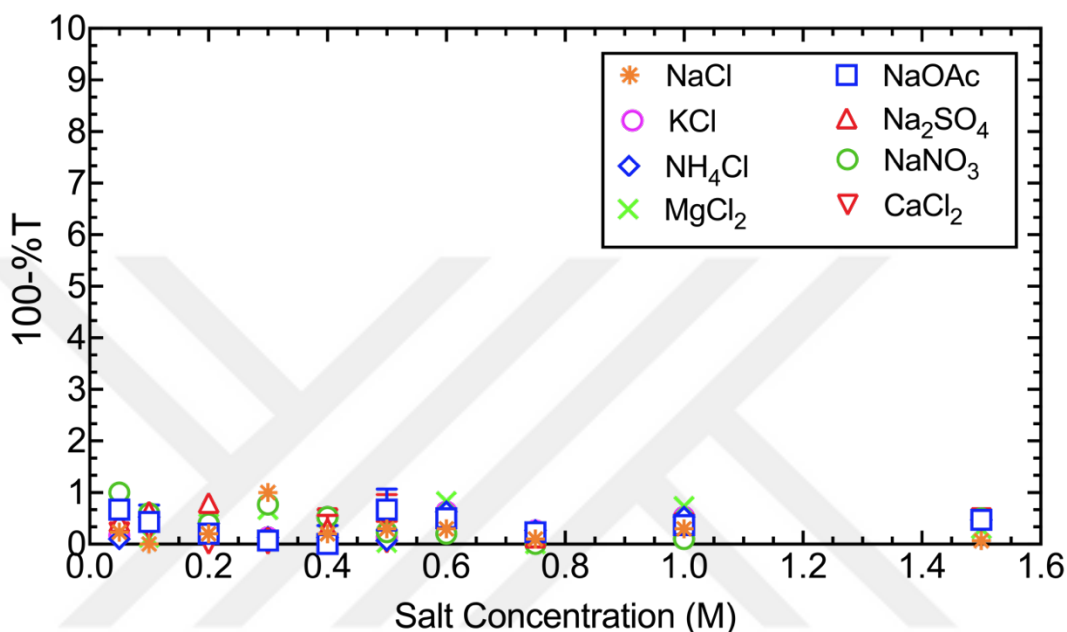


Figure 4.16. Turbidimetry graphs showing the effect of different concentrations of Hofmeister salts on the Salt-BSA mixture solutions.

4.5. Conclusion

In this chapter, we have investigated the effect of different (NaCl, KCl, NaNO₃, CaCl₂, MgCl₂, NH₄Cl, NaOAc and Na₂SO₄) salts of Hofmeister series on complex coacervation of HA-CHI and CHI-BSA systems and the effects of the concentration of these salts on the formation of coacervate.

It is observed that there is a positive correlation in turbidities when the effects of Hofmeister salts on polymer solubility are considered with respect to “salting-in” and “salting-out”. It is stated that the “salting-in” feature increases as one goes to the right in the Hofmeister Series, in other words, there is an increase in polymer solubility. As the solubility of polymers increases, the formation of complex coacervation proceeds in a positive

direction. During the formation of complex coacervation, interaction (ion-pairing) occurs between opposite charges of polymers. As the “salting-in” property increases, polymer solubility increases and complex coacervation occurs. According to the turbidity experiments, addition of salt has a positive effect up to a point (critical point or critical salt concentration). With further addition of salt, ion-pairing between polymers proceeds in the opposite direction and results in a decrease in the turbidity. It has been observed that the more the “salting-in” feature increases, the complex coacervation formation proceeds at lower concentrations.

Another property of Hofmeister salts is hydration. Strongly hydrated (cosmotropes) ions reduce the Debye length (the distance between interacting charges) and polymers are allowed to interact with any ion present in the environment. Thus, a decrease occurs in the interaction between polymers [11]. Therefore, this situation was partially observed in the turbidity graphs of the complex coacervation prepared with anionic salts in the Hofmeister series.

It was observed that the critical salt concentration values were lower in strongly hydrated (chaotropic) cationic salts. The reason is that as the chaotropic property increases, the “salting-in” properties increase, and the solubility of the polymer increases and the coacervation reaches the soluble complex form at lower concentrations. In anionic salts, the opposite effect (except for Na_2SO_4) was observed than cationic salts. Since sodium acetate (CH_3COONa) salt is a more cosmotropic (strongly hydrated) salt compared to sodium chloride and sodium nitrate salt, it shows “salting-in” feature and reduces polymer solubility. Therefore, higher critical salt concentration values were observed. It has been observed that divalent salts (CaCl_2 , MgCl_2 , Na_2SO_4) negatively contribute to the formation of complex coacervation compared to monovalent salts. However, Na_2SO_4 as divalent salt has lower critical salt concentration. The reasons behind these findings can be attributed to the size of the ions and their charge distribution.

It was observed that the critical salt concentration values were lower in strongly hydrated (kosmotropes) cationic salts than weakly hydrated cations. The reason is chaotropic ions increase the solubility of the polymer due to the “salting-in” properties. Therefore, coacervation reaches the soluble complex form at lower concentrations. When we focus on the Na salts with different anions, we can see the same correlation between critical salt

concentration and Hofmeister series. Divalent salts like Na_2SO_4 , MgCl_2 and CaCl_2 have inhibitory effect on coacervate formation so they have lower critical salt concentration, the reasons behind these findings can be attributed to the size of the ions and their charge distribution.



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APPENDIX A: SUPPLEMENTARY DATA

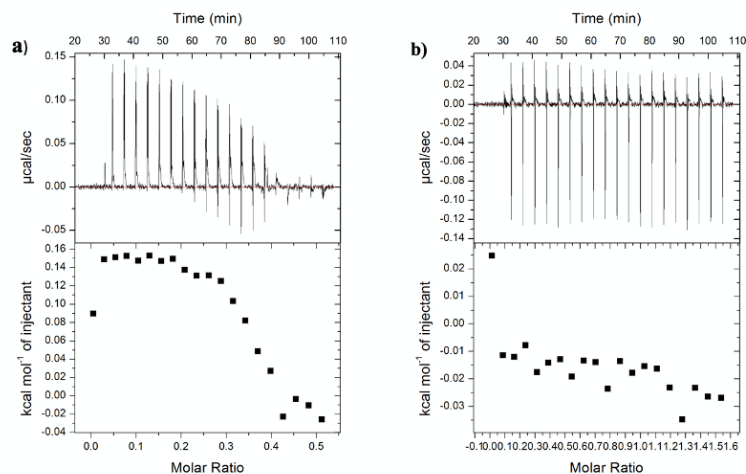


Figure A. 1. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM, 234.4 kDa) and CH (3 mM, 80kDa) (b)The thermogram (upper panel) and enthalpy (lower panel) obtained by titration of HA to acetate buffer at pH 4.0 acetate buffer with 0.05 M *I*, at 22°C.

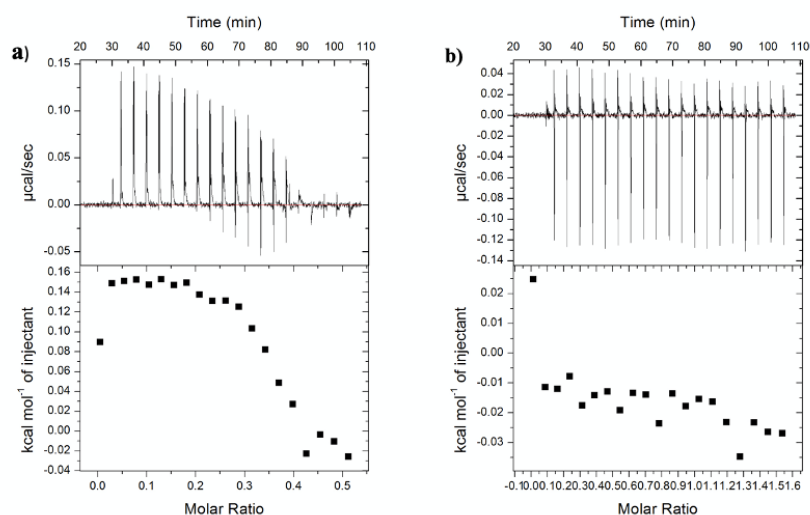


Figure A. 2. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 234.4 kDa) and CH (3 mM 80kDa) (b) The thermogram (upper panel) and enthalpy (lower panel), obtained by titration of HA to acetate buffer at pH 4.0 acetate buffer with 0.1 M *I*, at 22°C.

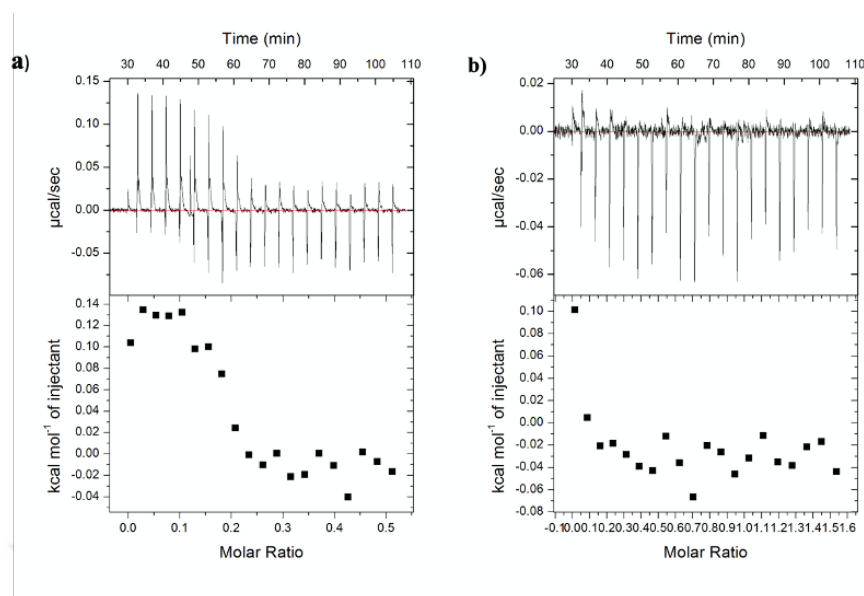


Figure A. 3. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 234.4 kDa) and CH (3 mM 80kDa) (b) The thermogram (upper panel) and enthalpy (lower panel) obtained by titration of HA to acetate buffer at pH 4.0 acetate buffer with 0.15 M I , at 22°C.

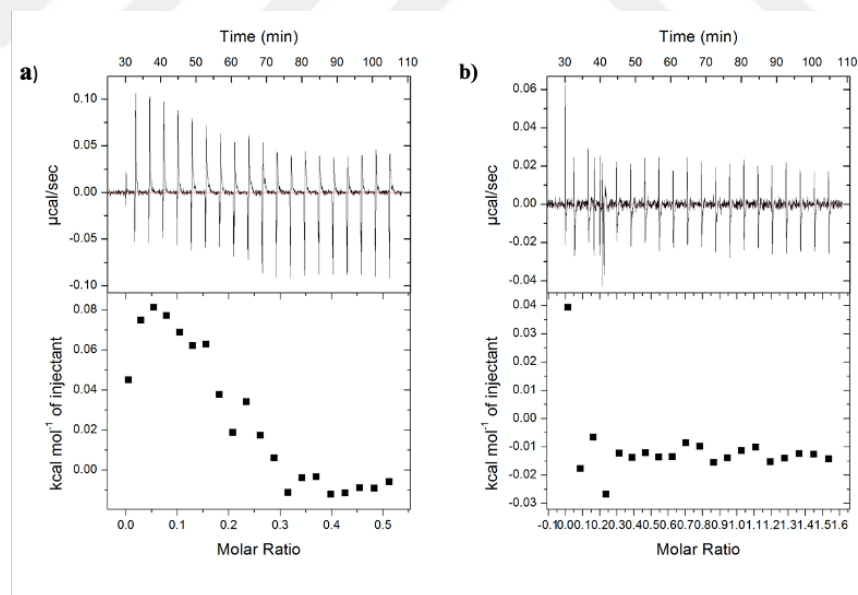


Figure A. 4. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 234.4kDa) and CH (3 mM 80kDa) (b) The thermogram (upper panel) and enthalpy (lower panel), obtained by titration of HA to acetate buffer at pH 4.0 acetate buffer with 0.3 M I , at 22°C

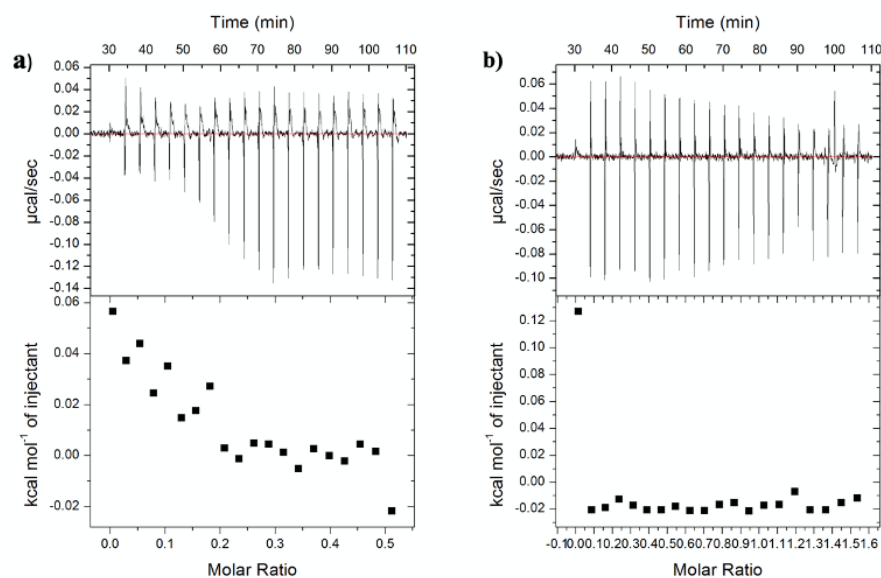


Figure A. 5. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 234.4kDa) and CH (3 mM 80kDa) (b) The thermogram (upper panel) and enthalpy (lower panel) obtained by titration of HA to acetate buffer at pH 4.0 acetate buffer with 0.5 M *I*, at 22°C.

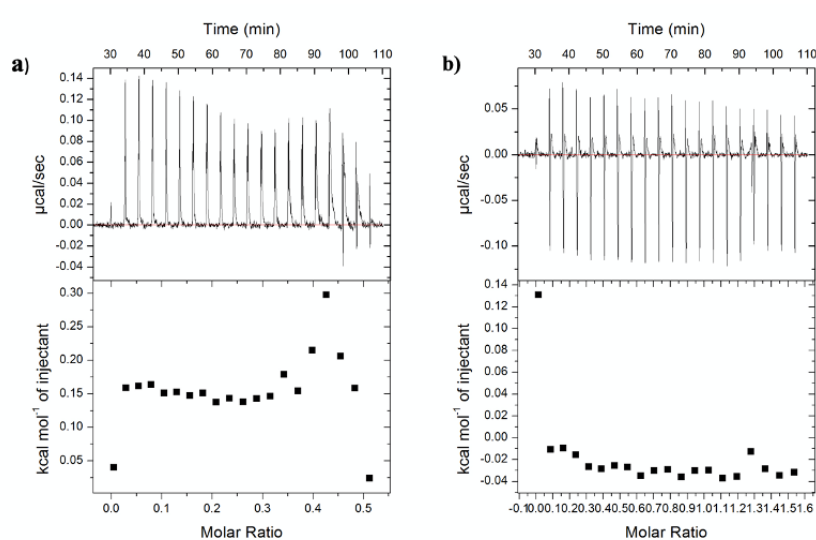


Figure A. 6. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 234.4kDa) and CH (3 mM 80 kDa) (b) The thermogram (upper panel) and enthalpy (lower panel) obtained by titration of HA to acetate buffer at pH 3.76 acetate buffer with 0.15 M *I*, at 25°C.

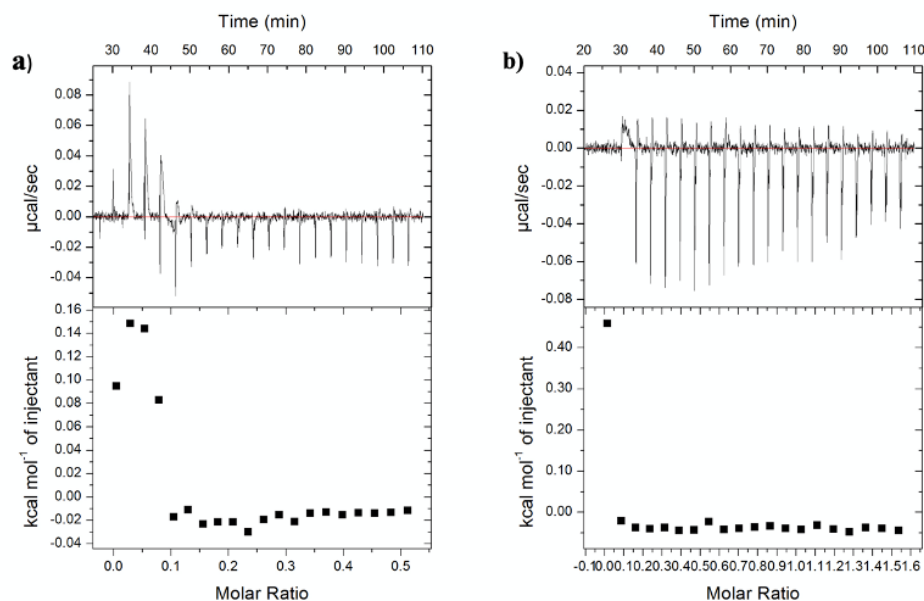


Figure A. 7. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 234.4kDa) and CH (3 mM 80kDa). (b) The thermogram (upper panel) and enthalpy (lower panel), obtained by titration of HA to acetate buffer at pH 4.0 acetate buffer with 0.15 M I , at 25°C.

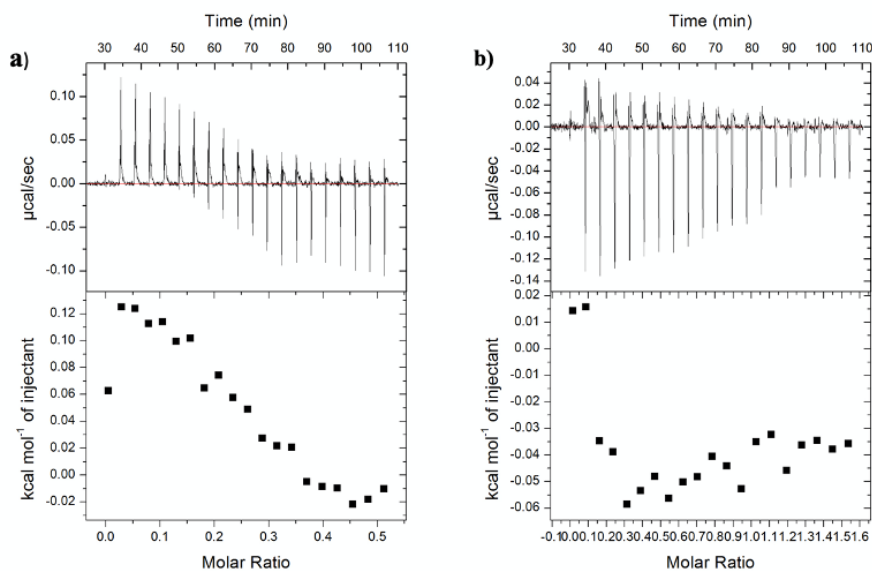


Figure A. 8. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 234.4kDa) and CH (3 mM 80kDa) (b) The thermogram (upper panel) and enthalpy (lower panel), obtained by titration of HA to acetate buffer at pH 4.5 acetate buffer with 0.15 M I , at 25°C.

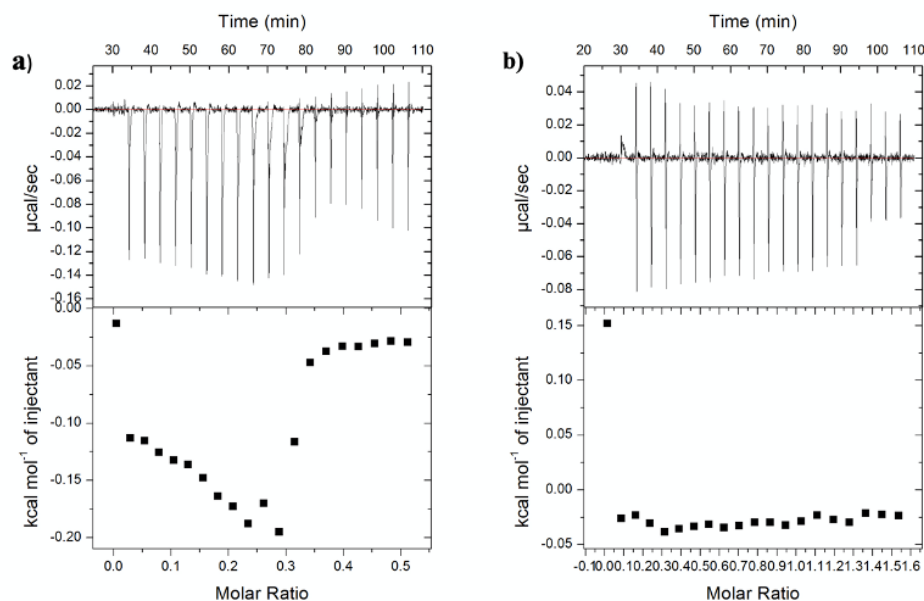


Figure A. 9. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 234.4kDa) and CH (3 mM 80kDa) (b) The thermogram (upper panel) and enthalpy (lower panel) obtained by titration of HA to acetate buffer at pH 5.0 acetate buffer with 0.15 M I , at 25°C.

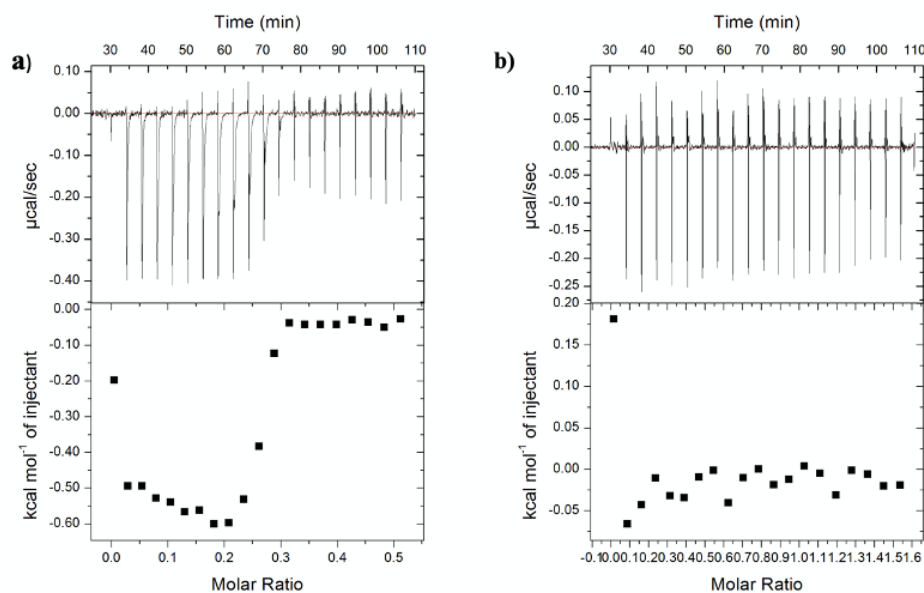


Figure A. 10. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 234.4kDa) and CH (3 mM 80kDa) (b) The thermogram (upper panel) and enthalpy (lower panel) obtained by titration of HA (7.5 mM) to acetate buffer at pH 5.5 acetate buffer with 0.15 M I , at 25°C.

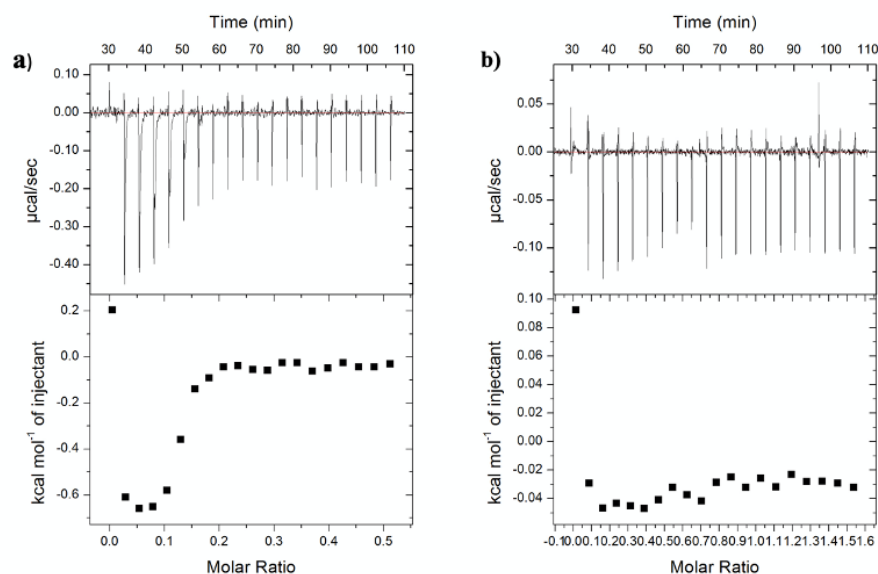


Figure A. 11. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 234.4kDa) and CH (3 mM 80kDa) (b) The thermogram (upper panel) and enthalpy (lower panel), obtained by titration of HA to acetate buffer at pH 5.76 acetate buffer with 0.15 M I , at 25°C.

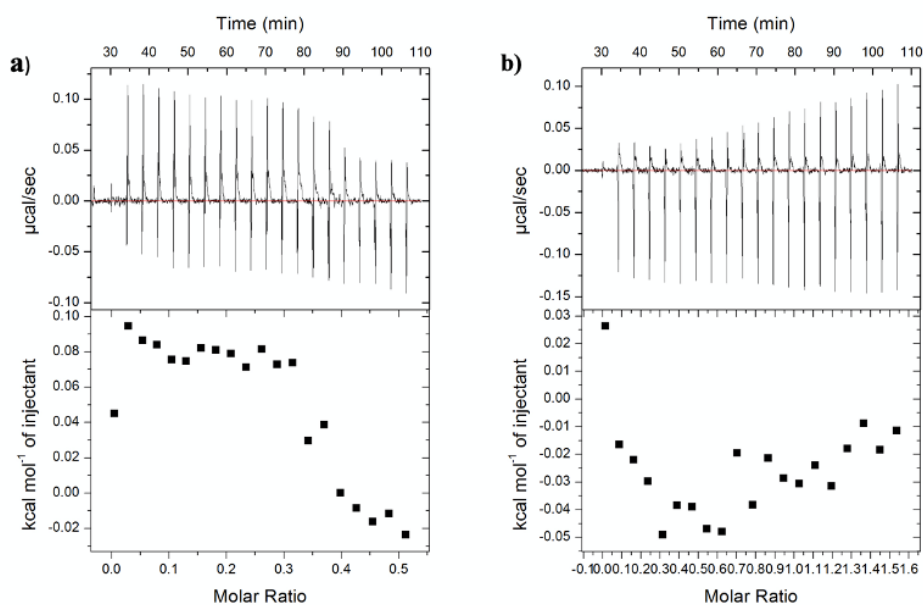


Figure A. 12. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 234.4kDa) and CH (3 mM 80 kDa) (b) The thermogram (upper panel) and enthalpy (lower panel) obtained by titration of HA to acetate buffer at pH 4.0 acetate buffer with 0.15 M I , at 15°C.

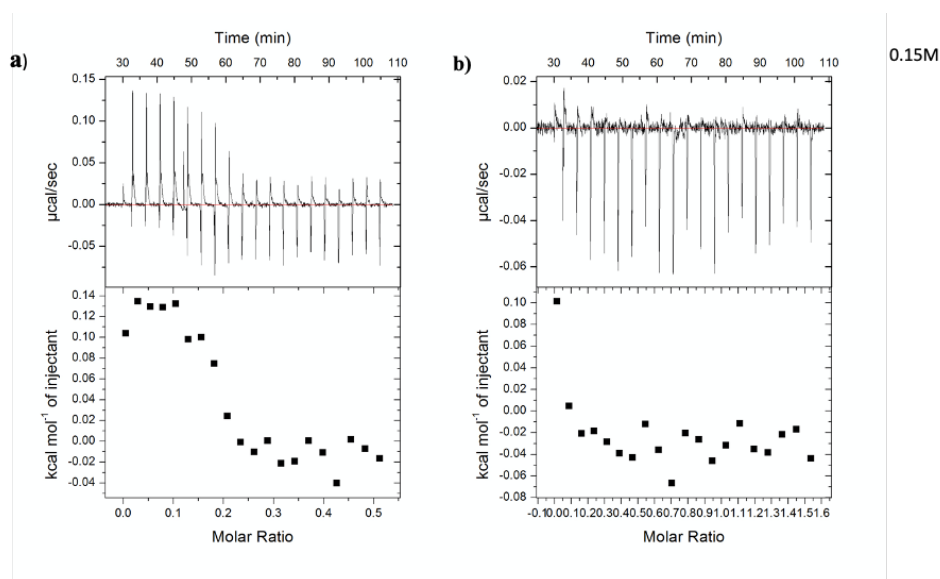


Figure A. 13. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 234.4kDa) and CH (3 mM 80kDa) (b) The thermogram (upper panel) and enthalpy (lower panel) obtained by titration of HA (7.5 mM) to acetate buffer at pH 4.0 acetate buffer with 0.15 M I , at 22°C.

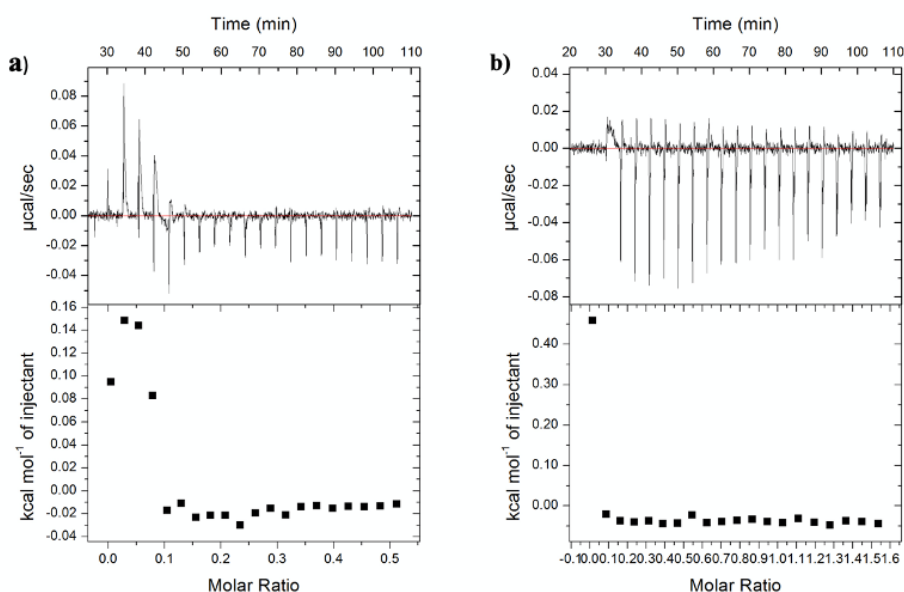


Figure A. 14. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 234.4kDa) and CH (3 mM 80kDa) (b) The thermogram (upper panel) and enthalpy (lower panel) obtained by titration of HA to acetate buffer at pH 4.0 acetate buffer with 0.15 M I , at 25°C.

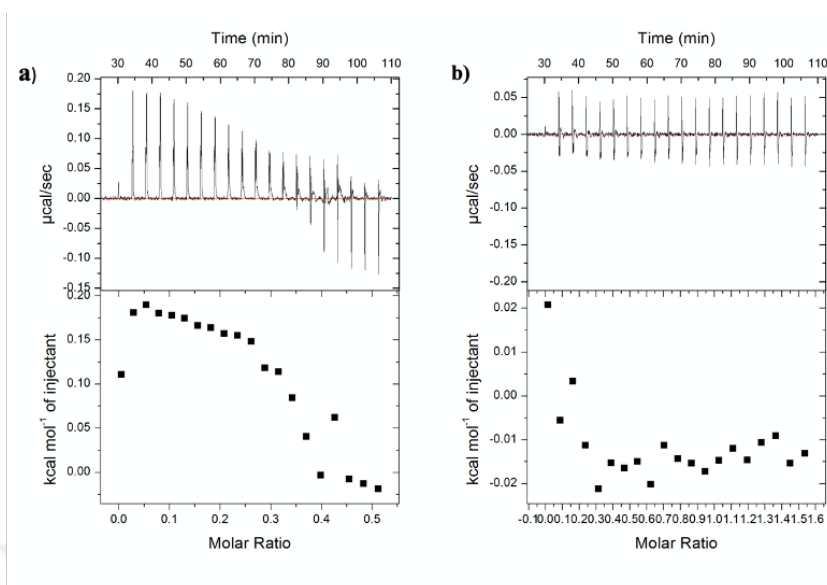


Figure A. 15. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 234.4kDa) and CH (3 mM 80 kDa) (b) The thermogram (upper panel) and enthalpy (lower panel) obtained by titration of HA to acetate buffer at pH 4.0 acetate buffer with 0.15 M I , at 37°C.

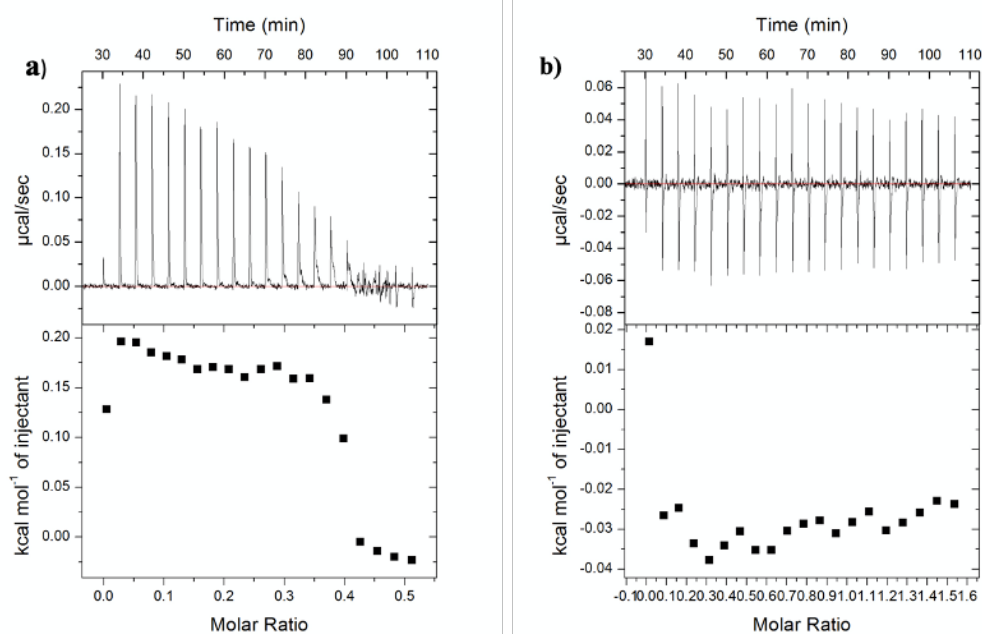


Figure A. 16. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 234.4kDa) and CH (3 mM 80kDa) (b) The thermogram (upper panel) and enthalpy (lower panel) obtained by titration of HA to acetate buffer at pH 4.0 acetate buffer with 0.15 M I , at 45°C.

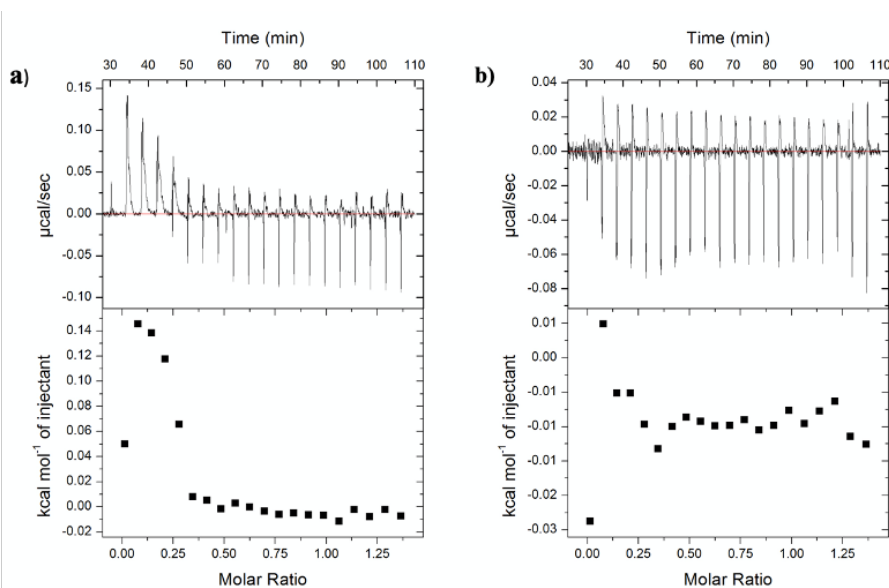


Figure A. 17. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (15 mM 51 kDa) and CH (3 mM 80 kDa) (b) The thermogram (upper panel) and enthalpy (lower panel) obtained by titration of HA (15 mM 51 kDa) to acetate buffer at pH 4.0 acetate buffer with 0.15 M *I*, at 25°C.

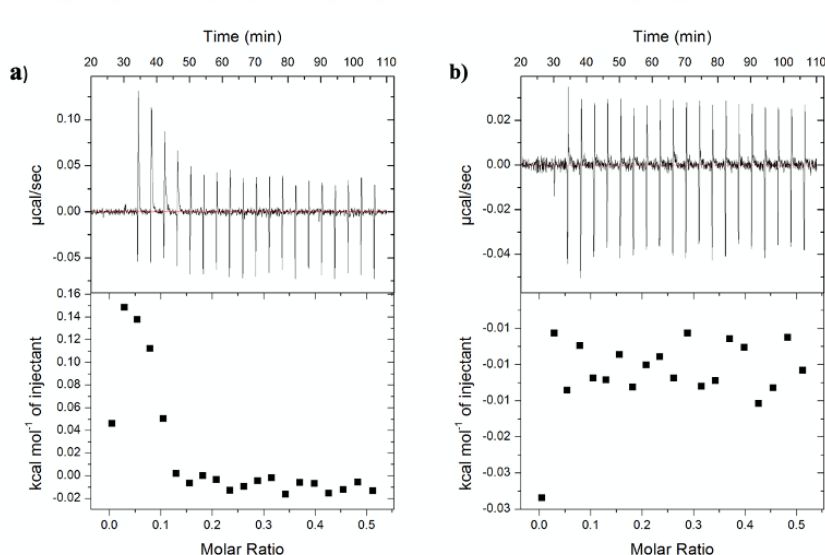


Figure A. 18. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 150 kDa) and CH (3 mM 80 kDa) (b) The thermogram (upper panel) and enthalpy (lower panel) obtained by titration of HA (7.5 mM 150 kDa) to acetate buffer at pH 4.0 acetate buffer with 0.15 M *I*, at 25°C.

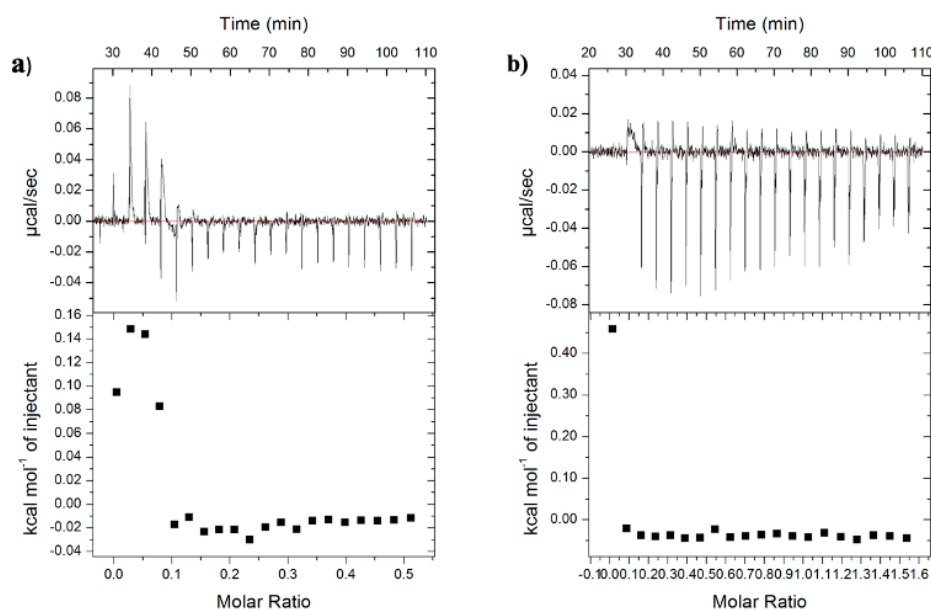


Figure A. 19. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 234.4 kDa) and CH (3 mM 80kDa) (b) The thermogram (upper panel) and enthalpy (lower panel), obtained by titration of HA (7.5 mM 234.4 kDa) to acetate buffer at pH 4.0 acetate buffer with 0.15 M *I*, at 25°C.

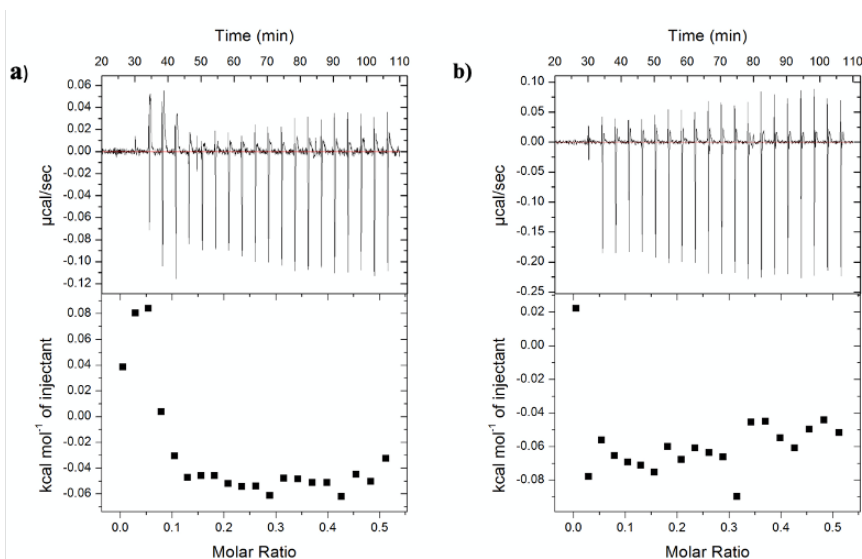


Figure A. 20. (a) Upper panel: ITC thermogram, and lower panel: binding isotherm of HA (7.5 mM 752 kDa) and CH (3 mM 80kDa) (b) The thermogram (upper panel) and enthalpy (lower panel) obtained by titration of HA (7.5 mM 752 kDa) to acetate buffer at pH 4.0 acetate buffer with 0.15 M *I*, at 25°C.

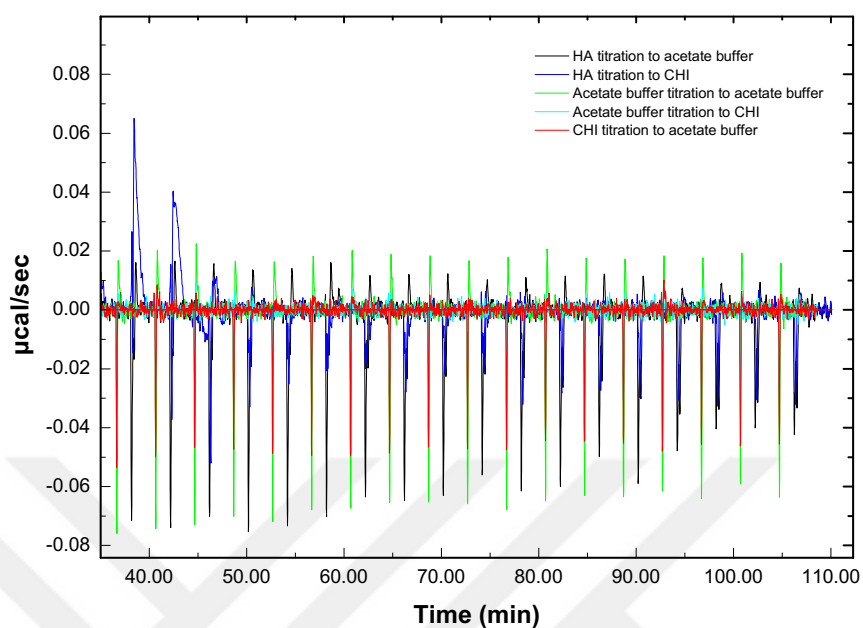


Figure A. 21. Dilution effect raw data of HA, CHI and acetate buffer in 0.15M / NaCl at pH 4.0, 25°C.

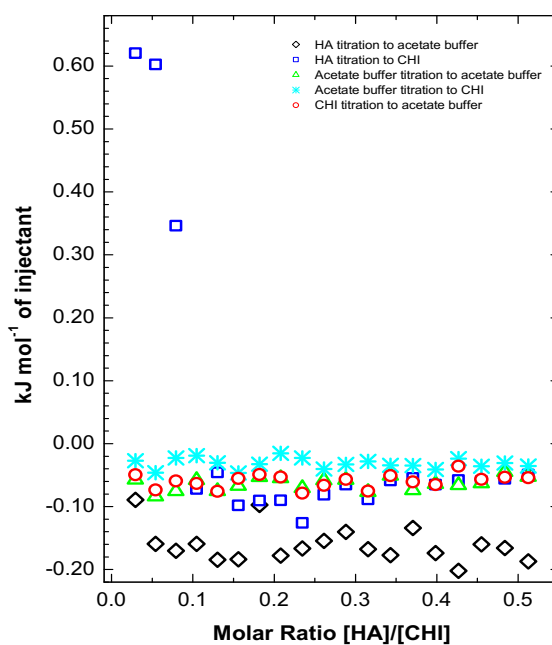


Figure A. 22. Dilution effect thermogram graph of HA, CHI and acetate buffer in 0.15M / NaCl at pH 4.0, 25°C.

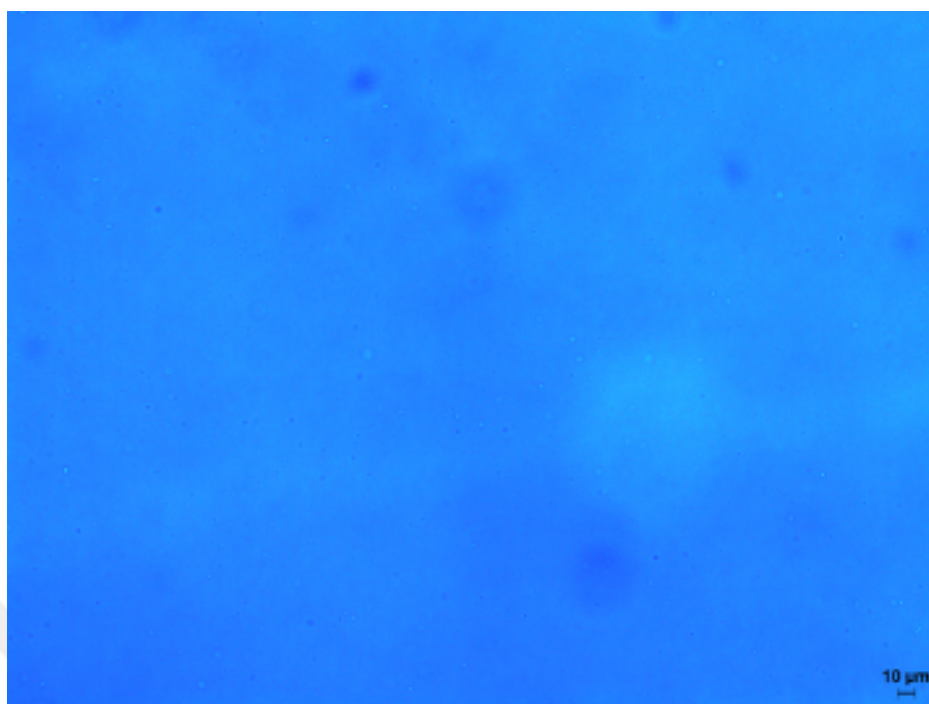


Figure A. 23. Optical light microscopy images of HA-CHI mixture at pH 4.0 acetate buffer solution with 0.05M ionic strength.

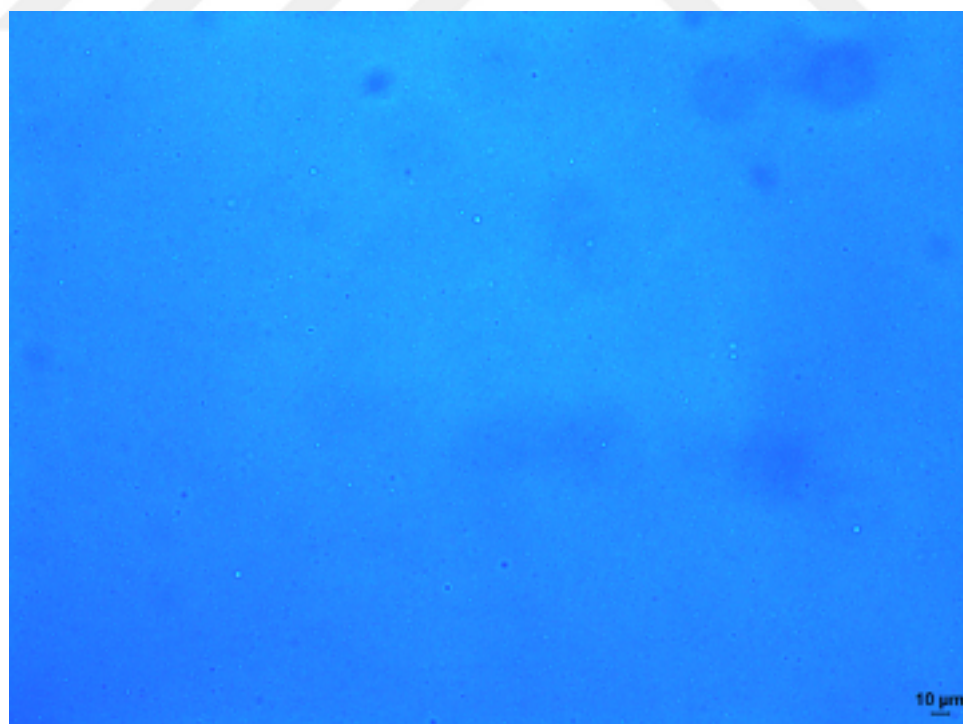


Figure A. 24. Optical light microscopy images of HA-CHI mixture at pH 4.0 acetate buffer solution with 0.1M ionic strength.



Figure A. 25. Optical light microscopy images of HA-CHI mixture at pH 4.0 acetate buffer solution with 0.3M ionic strength.

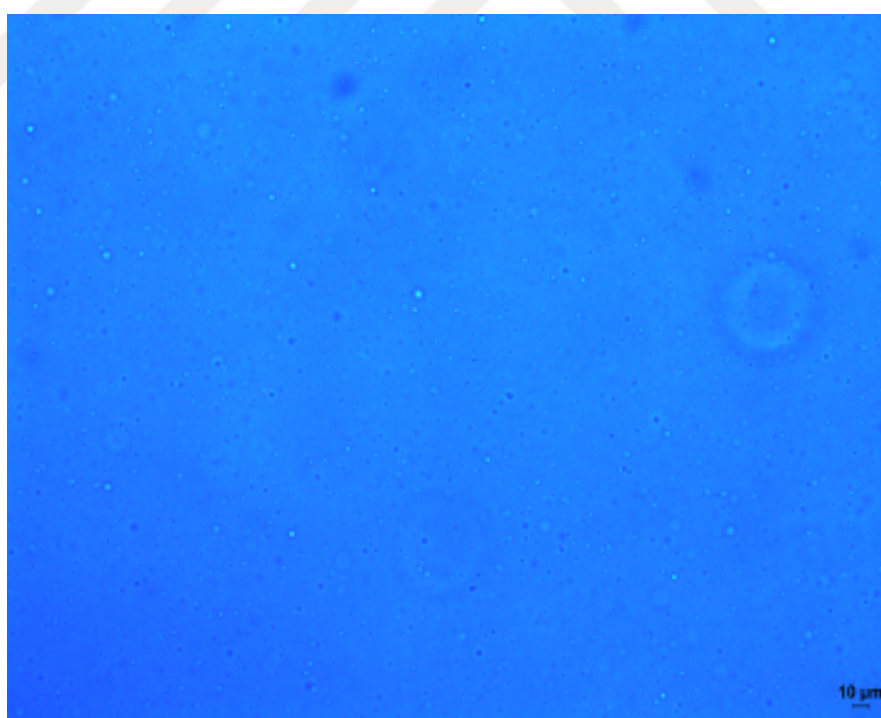


Figure A. 26. Optical light microscopy images of HA-CHI mixture at pH 4.0 acetate buffer solution with 0.5M ionic strength.



Figure A. 27. Optical light microscopy images of HA-CHI mixture at pH 3.76 acetate buffer solution with 0.15M ionic strength.



Figure A. 28. Optical light microscopy images of HA-CHI mixture at pH 4.0 acetate buffer solution with 0.15M ionic strength.

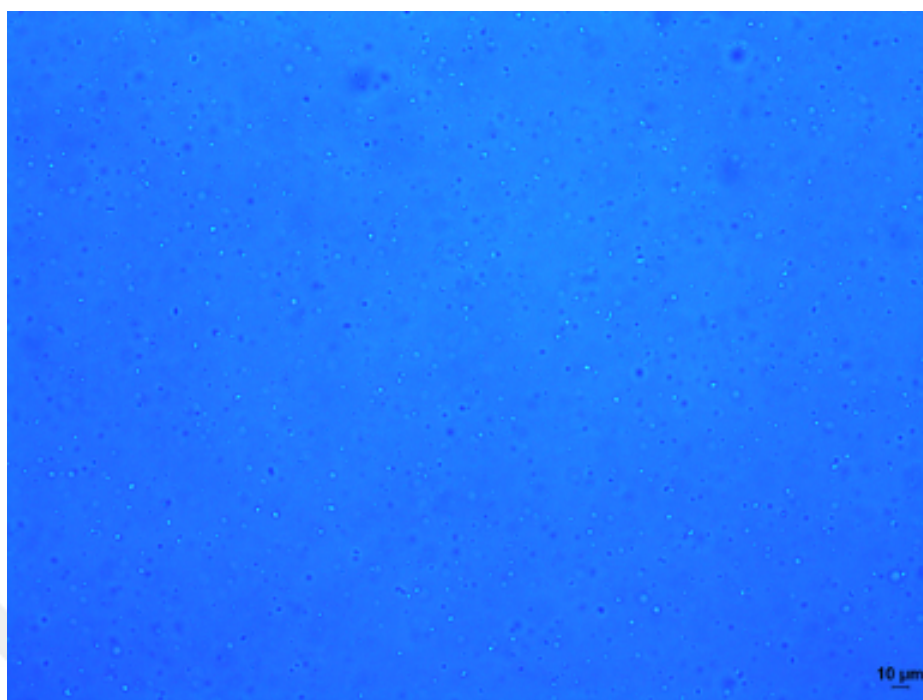


Figure A. 29. Optical light microscopy images of HA-CHI mixture at pH 4.5 acetate buffer solution with 0.15M ionic strength.

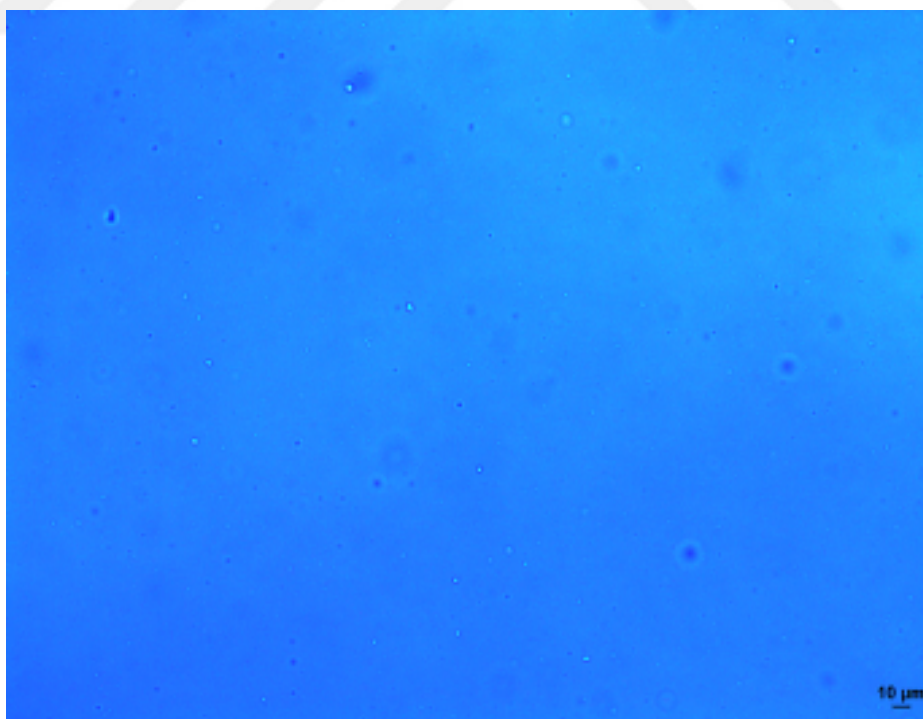


Figure A. 30. Optical light microscopy images of HA-CHI mixture at pH 5.0 acetate buffer solution with 0.15M ionic strength.

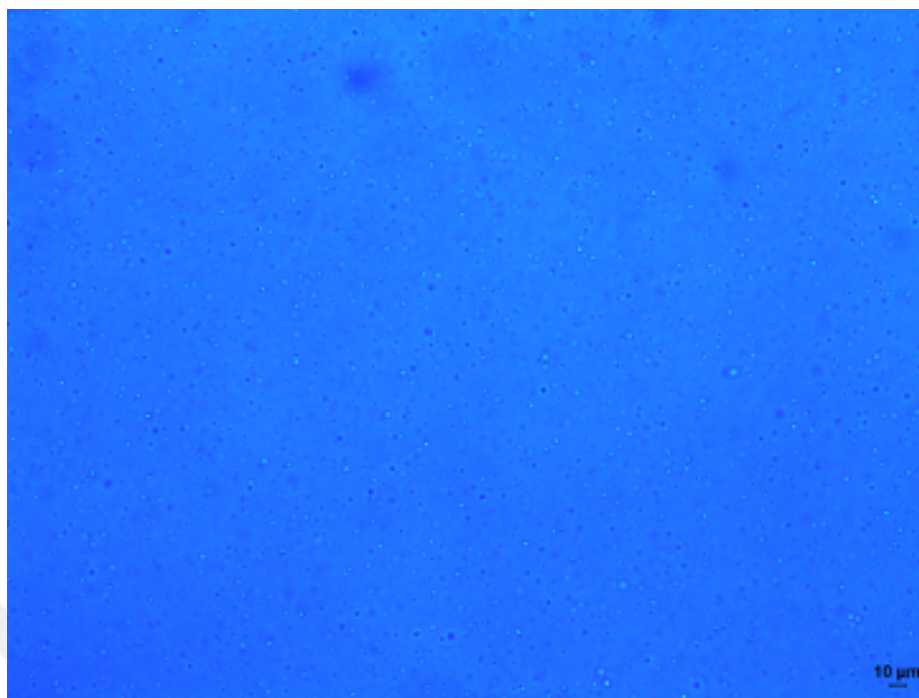


Figure A. 31. Optical light microscopy images of HA-CHI mixture at pH 5.5 acetate buffer solution with 0.15M ionic strength.

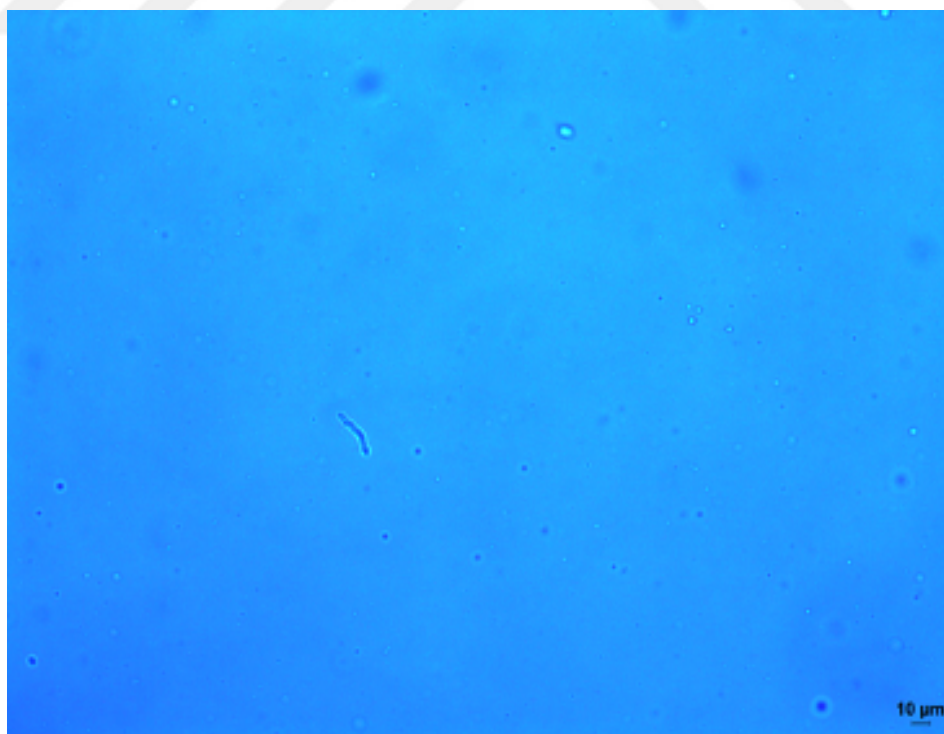


Figure A. 32. Optical light microscopy images of HA-CHI mixture at pH 5.76 acetate buffer solution with 0.15M ionic strength.

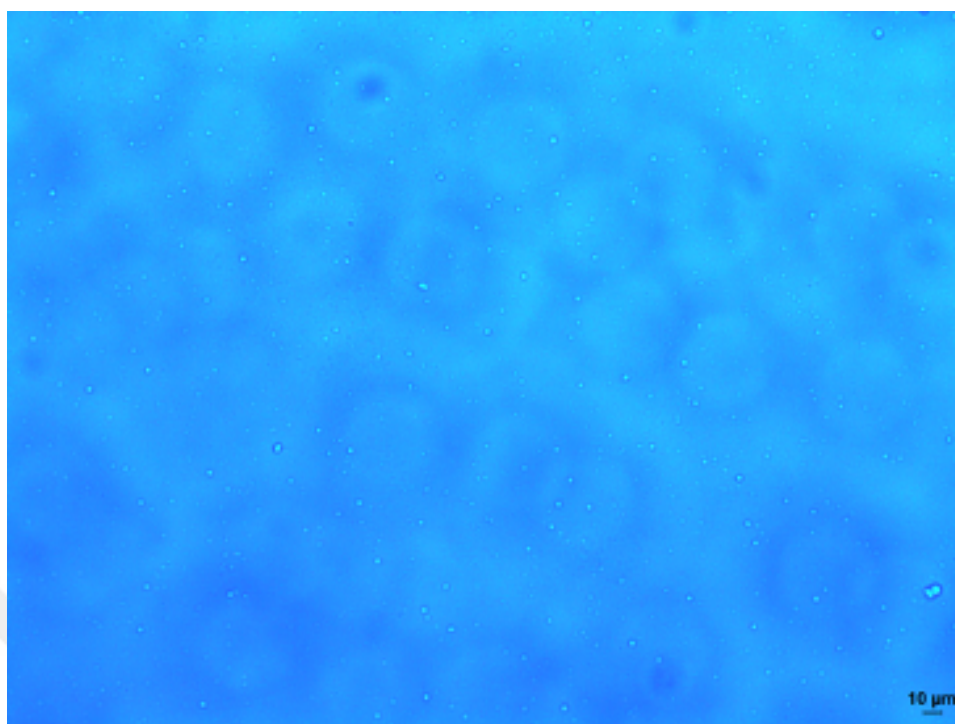


Figure A. 33. Optical light microscopy images of HA-CHI mixture at pH 4.0 acetate buffer solution with 0.15M ionic strength at 15°C.

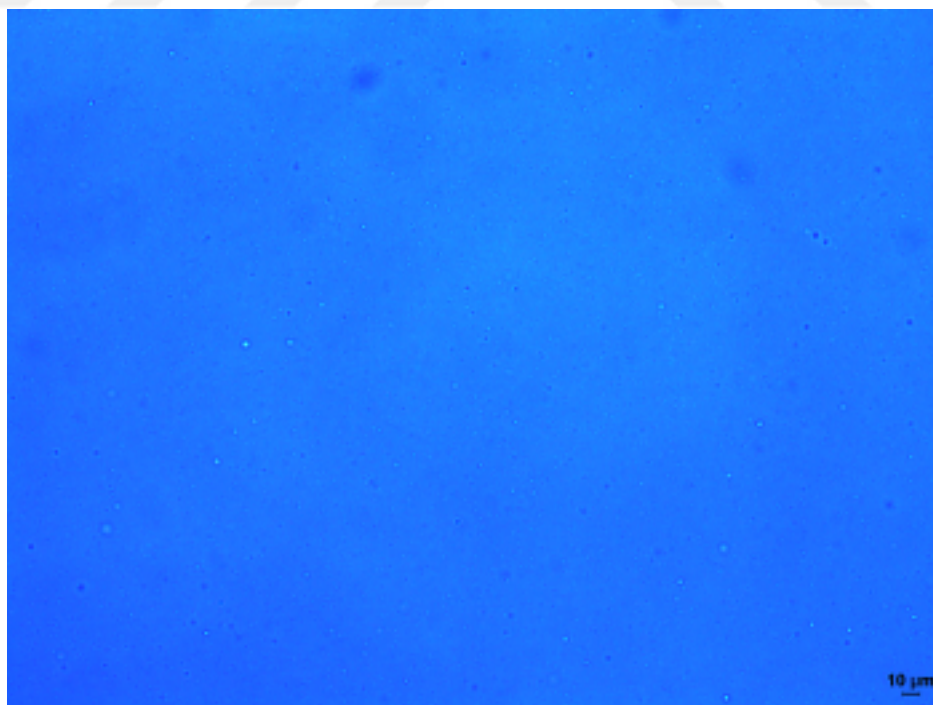


Figure A. 34. Optical light microscopy images of HA-CHI mixture at pH 4.0 acetate buffer solution with 0.15M ionic strength at 22°C.

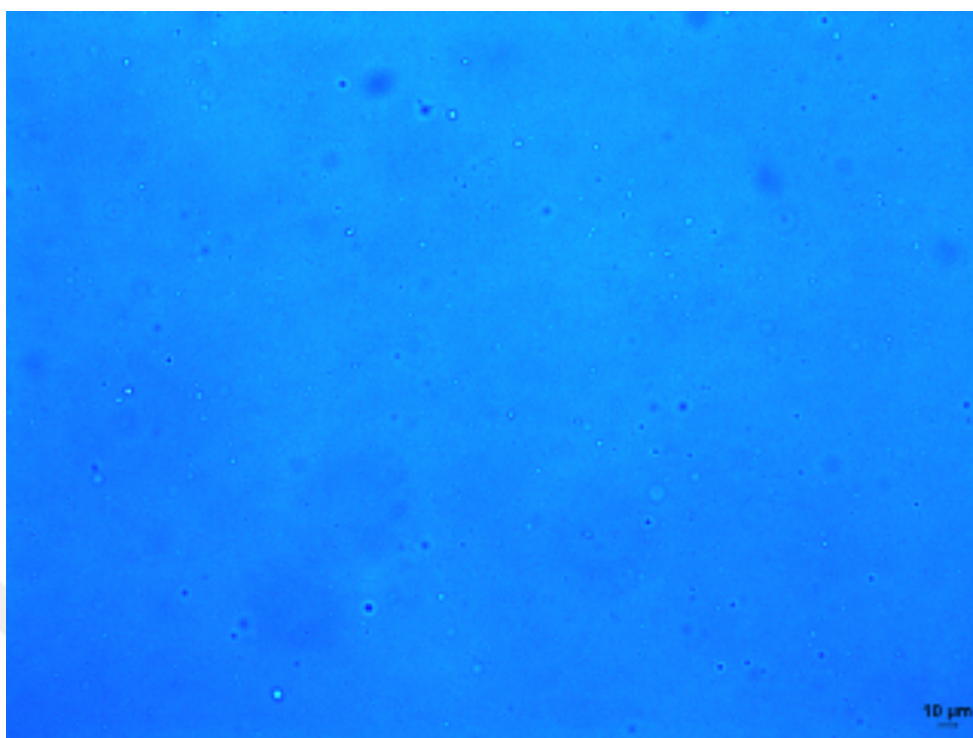


Figure A. 35. Optical light microscopy images of HA-CHI mixture at pH 4.0 acetate buffer solution with 0.15M ionic strength at 25°C.

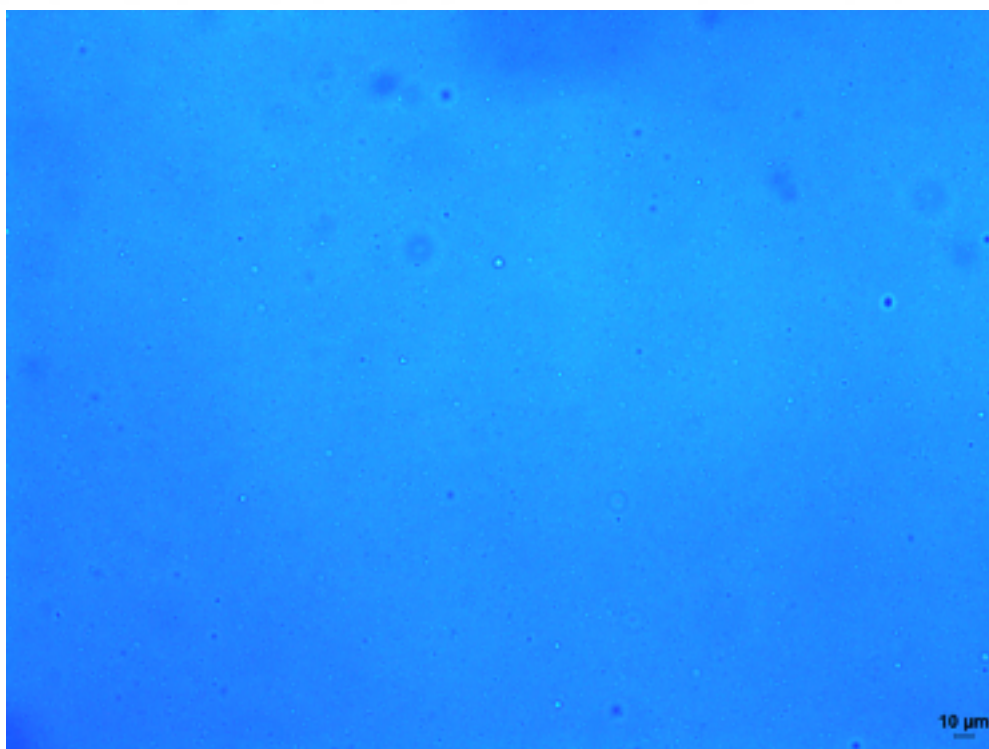


Figure A. 36. Optical light microscopy images of HA-CHI mixture at pH 4.0 acetate buffer solution with 0.15M ionic strength at 37°C.

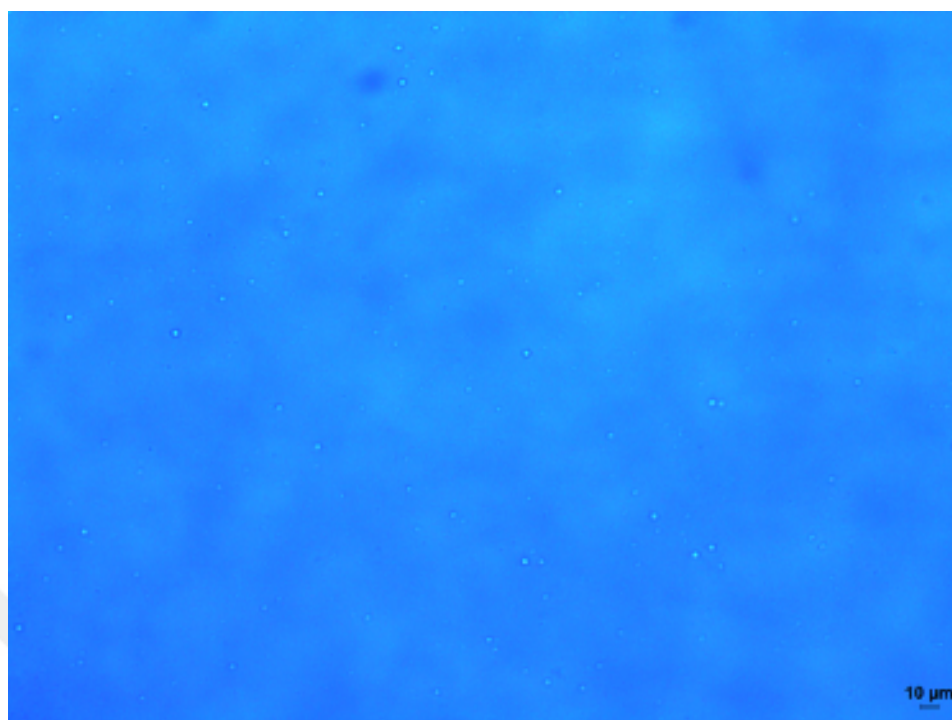


Figure A. 37. Optical light microscopy images of HA-CHI mixture at pH 4.0 acetate buffer solution with 0.15M ionic strength at 45°C.

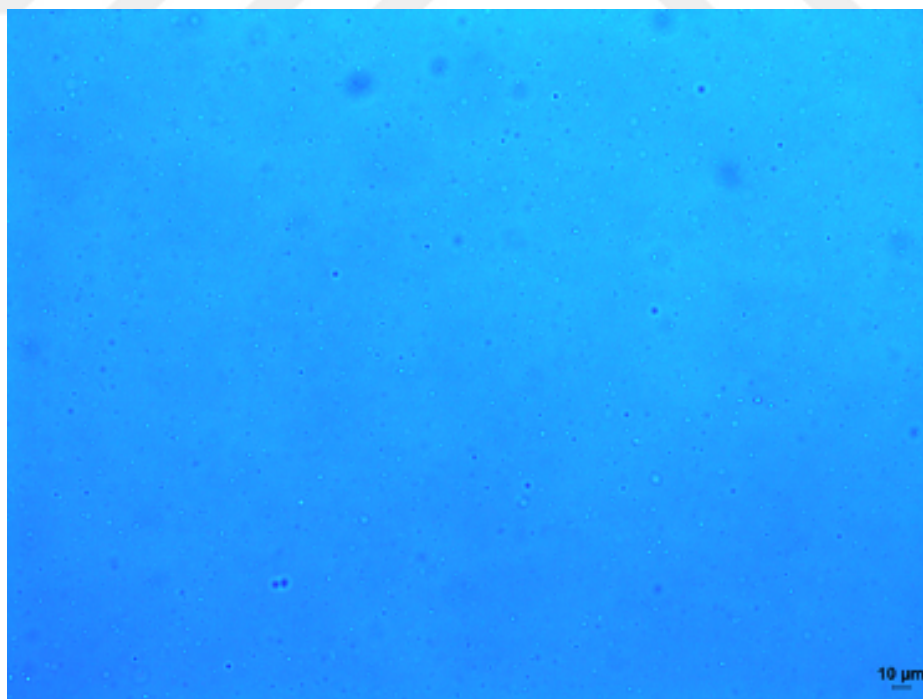


Figure A. 38. Optical light microscopy images of HA (51kDa)-CHI(80kDa) mixture at pH 4.0 acetate buffer solution with 0.15M ionic strength at 25°C.

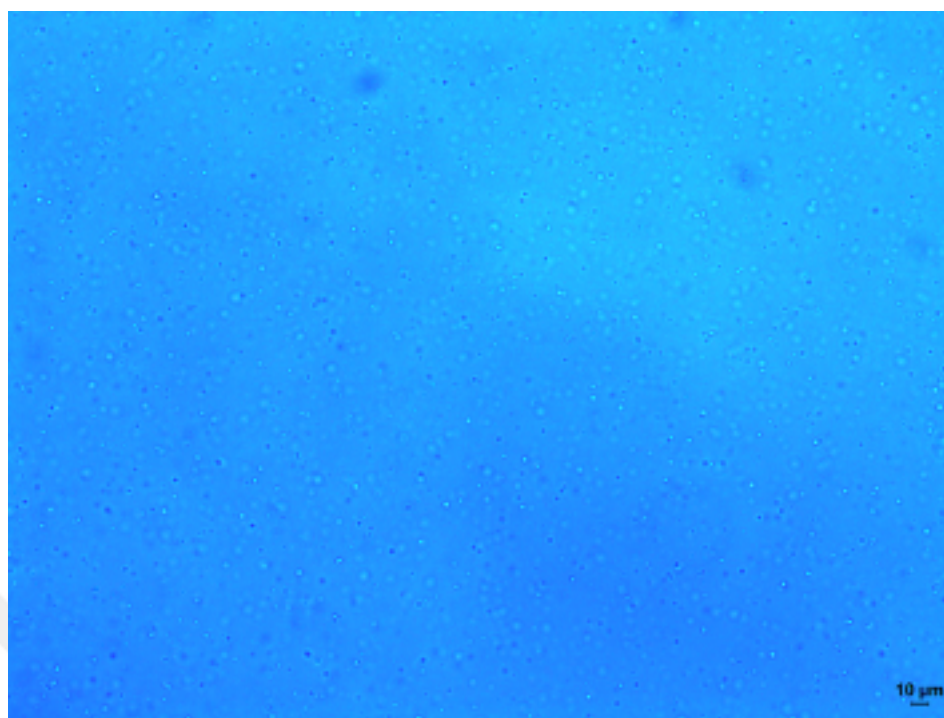


Figure A. 39. Optical light microscopy images of HA (150kDa)-CHI (80kDa) mixture at pH 4.0 acetate buffer solution with 0.15M ionic strength at 25°C.

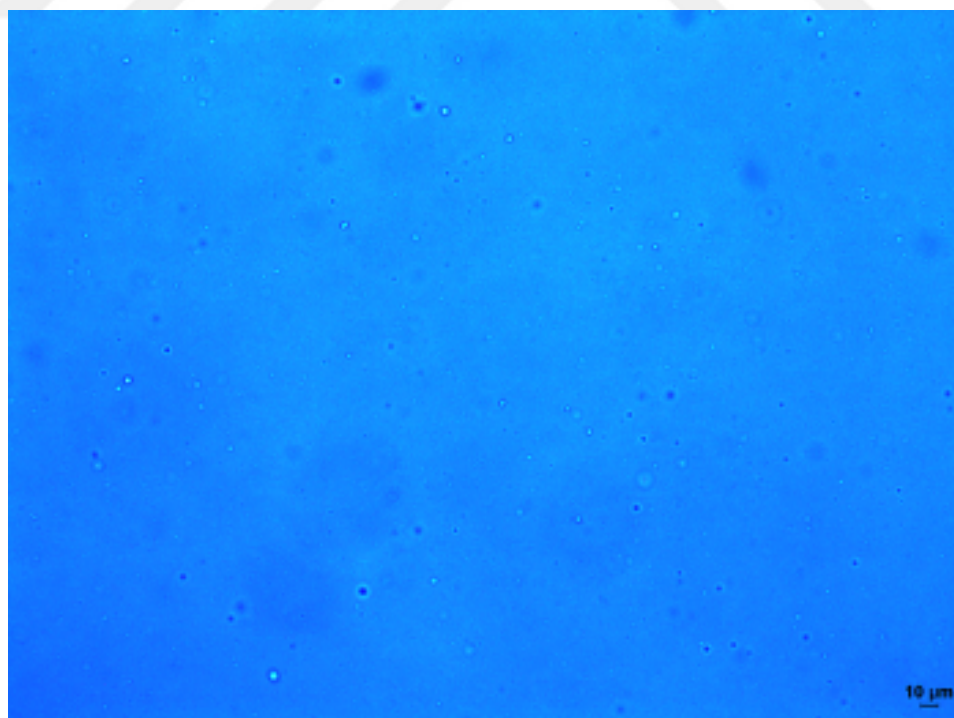


Figure A. 40. Optical light microscopy images of HA (234kDa)-CHI (80kDa) mixture at pH 4.0 acetate buffer solution with 0.15M ionic strength at 25°C.



Figure A. 41. Optical light microscopy images of HA(752kDa)-CHI(80kDa) mixture at pH 4.0 acetate buffer solution with 0.15M ionic strength at 25°C.

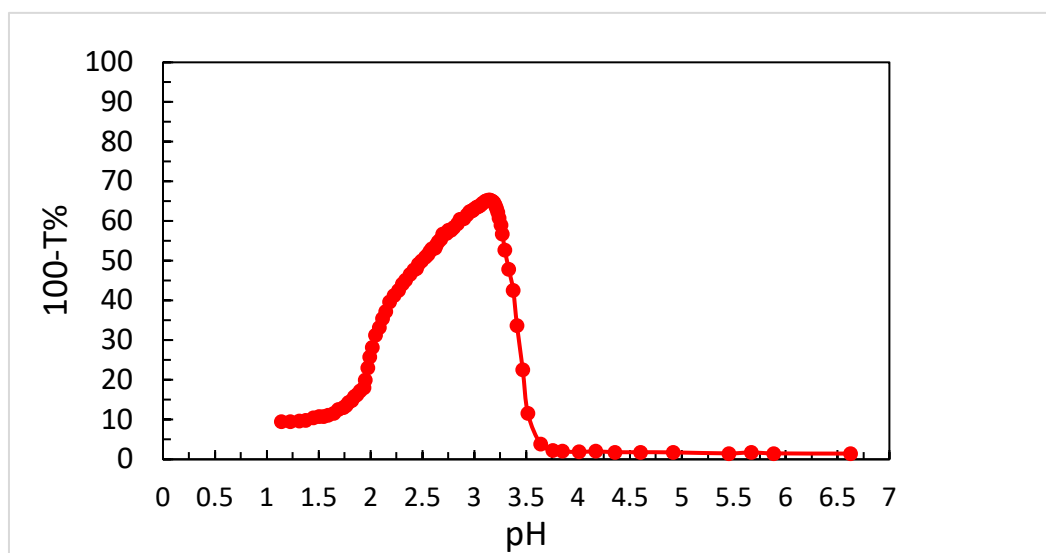


Figure A. 42. Turbidity vs. pH for the HA (950 kDa)/BSA system with final polymer concentration $C_{HA} = 0.05\text{mg/ml}$ and $C_{BSA} = 0.5\text{mg/ml}$ at 250mM NaCl. pH was decreased gradually from 7.0 to around 1.0.

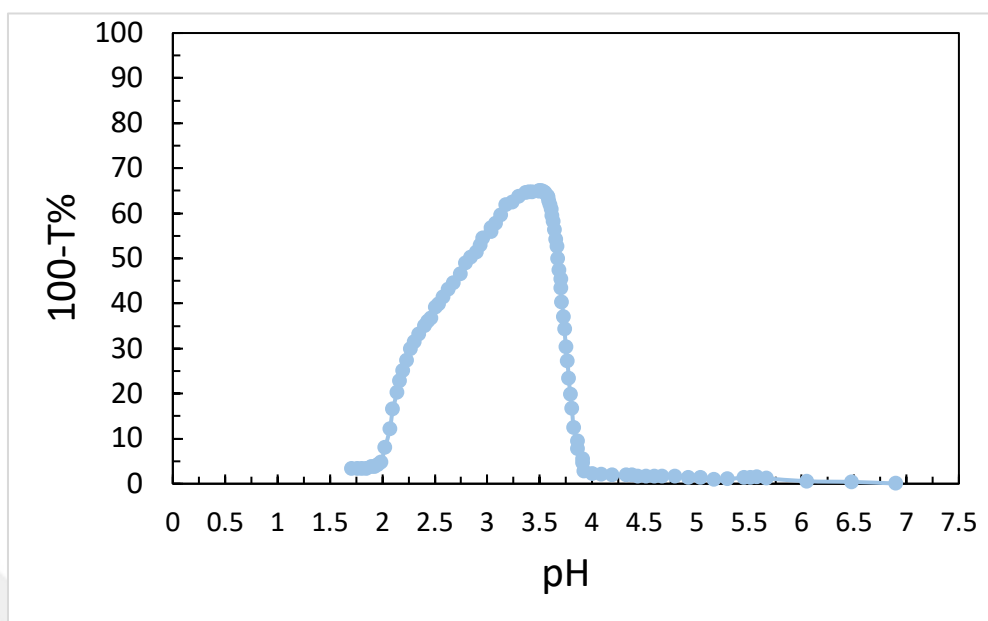


Figure A. 43. Turbidity vs. pH for the HA (950 kDa)/BSA system with final polymer concentration $C_{HA} = 0.04\text{mg/ml}$ and $C_{BSA} = 0.4\text{mg/ml}$ at 200mM NaCl. pH was decreased gradually from 7.0 to around 1.0.

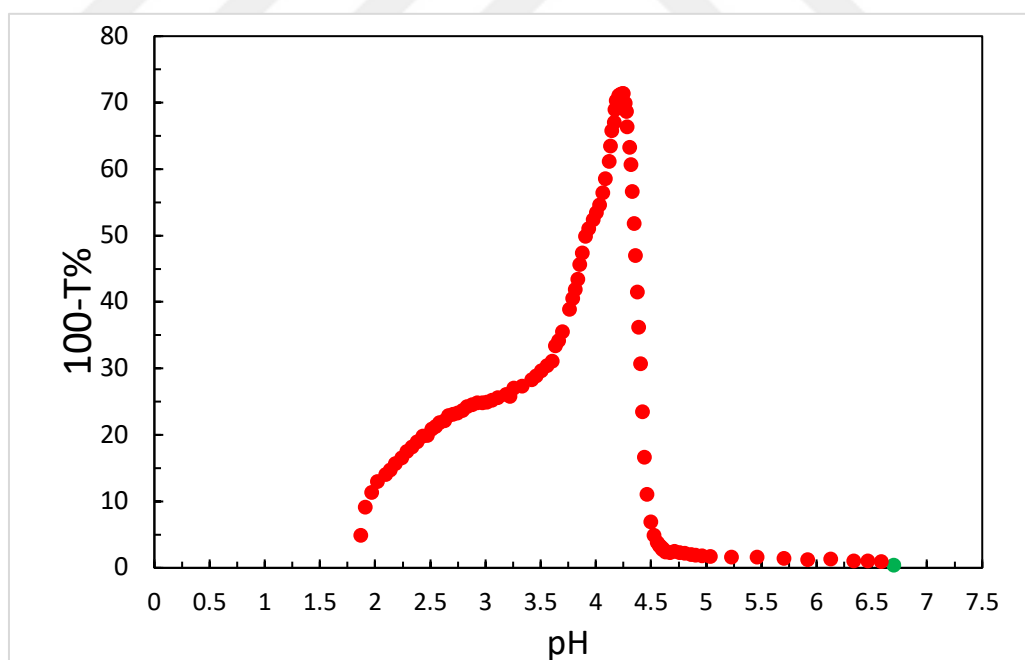


Figure A. 44. Turbidity vs. pH for the HA (950 kDa)/BSA system with final polymer concentration $C_{HA} = 0.025\text{mg/ml}$ and $C_{BSA} = 0.25\text{mg/ml}$ at 50mM NaCl. pH was decreased gradually from 7.0 to around 1.0.

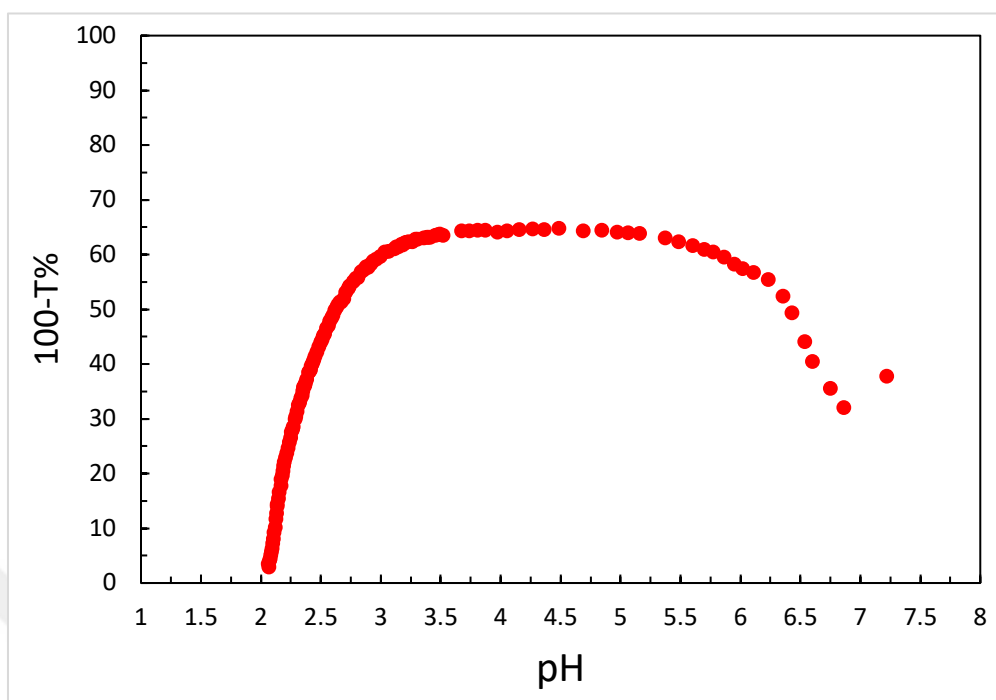


Figure A. 45. Turbidity vs. pH for the HA (950 kDa)/CHI (396kDa) system with final polymer concentration $C_{HA} = 0.19\text{mg/ml}$ and $C_{CHI} = 0.31\text{mg/ml}$ at 250mM NaCl. pH was increased gradually from 2.0 to around 7.0.

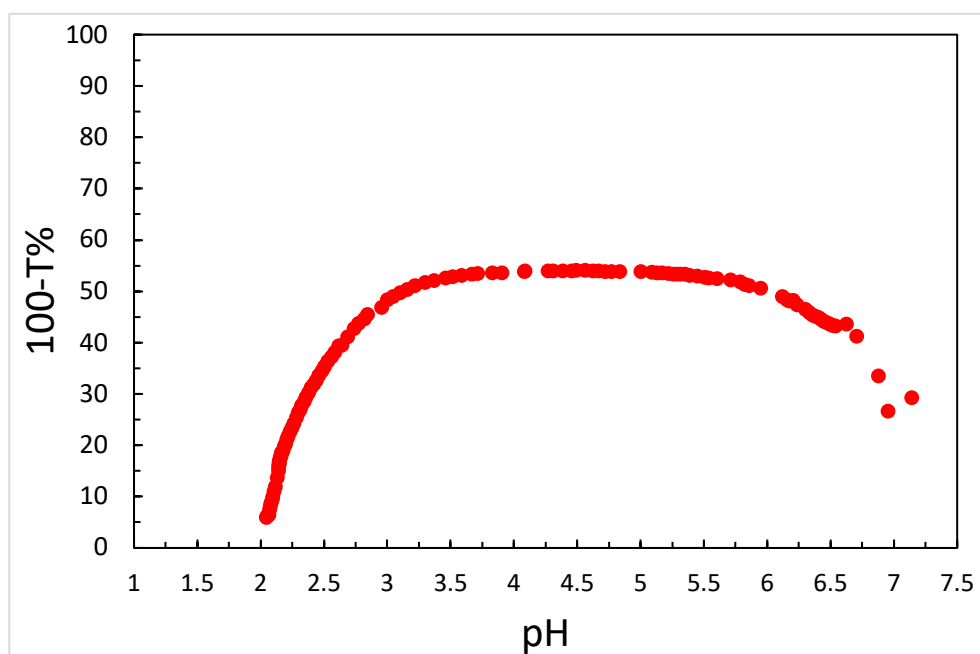


Figure A. 46. Turbidity vs. pH for the HA (950 kDa)/CHI (396kDa) system with final polymer concentration $C_{HA} = 0.136\text{mg/ml}$ and $C_{CHI} = 0.364\text{mg/ml}$ at 200mM NaCl. pH was increased gradually from 2.0 to around 7.0.

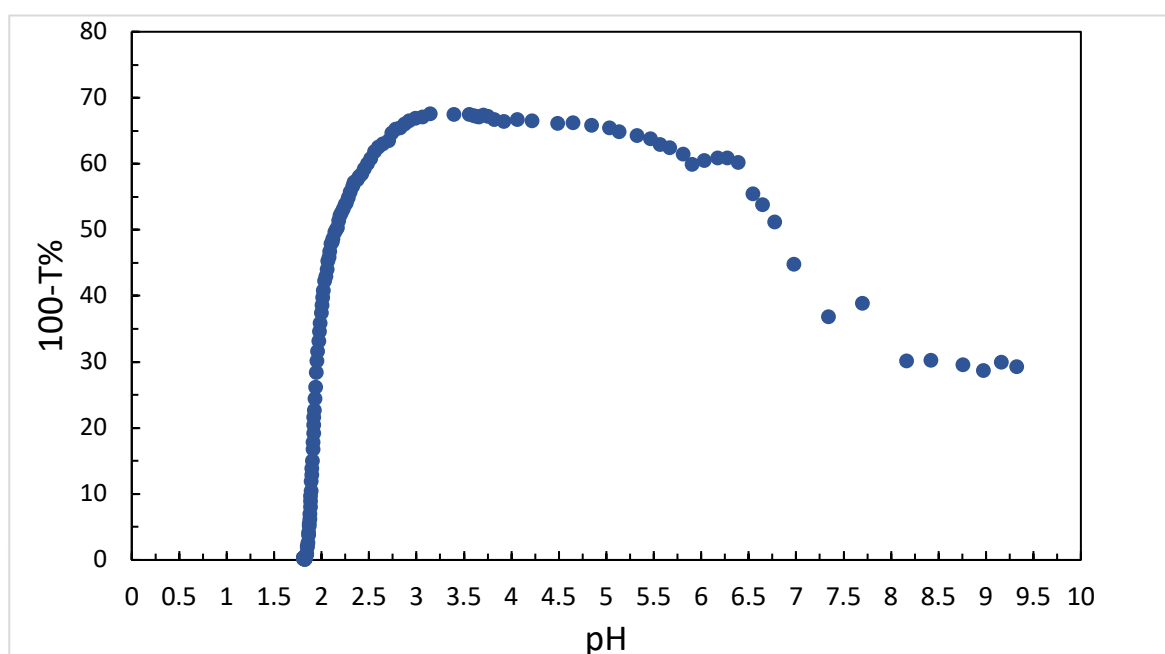


Figure A. 47. Turbidity vs. pH for the HA (950 kDa)/CHI (396kDa) system with final polymer concentration $C_{HA}=0.25\text{mg/ml}$ and $C_{CHI}=0.25\text{mg/ml}$ at 50mM NaCl. pH was increased gradually from 2.0 to around 7.0.

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Figure B. 3. Permission of Ref. [27] from The Royal Society of Chemistry

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Polyelectrolyte-Protein Complexes: Structure and Conformation of Each Specie Revealed by SANS
 Author: Fabrice Cousin, Jérémie Gummel, Diane Ung, et al
 Publication: Langmuir
 Publisher: American Chemical Society
 Date: Oct 1, 2005
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Figure B. 4. Permission of [31] from American Chemical Society



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Phase Behavior and Coacervation of Aqueous Poly(acrylic acid)–Poly(allylamine) Solutions

Author: Rungsima Chollakup, Wirasak Smitthipong, Claus D. Eisenbach, et al

Publication: Macromolecules

Publisher: American Chemical Society

Date: Mar 1, 2010

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Figure B. 5. Permission of [39] from American Chemical Society



Hyaluronic Acid/Chitosan Coacervate-Based Scaffolds

Author: Ozge Karabiyik Acar, A. Basak Kayitmazer, Gamze Torun Kose

Publication: Biomacromolecules

Publisher: American Chemical Society

Date: Apr 1, 2018

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Figure B. 6. Permission of [40] from American Chemical Society

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pH-Induced Coacervation in Complexes of Bovine Serum Albumin and Cationic Polyelectrolytes

Author: K. Kaibara, T. Okazaki, H. B. Bohidar, et al

Publication: Biomacromolecules

Publisher: American Chemical Society

Date: Mar 1, 2000

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Instructor Name	Fatma Akcay Ogur	Expected Presentation Date	2023-09-24

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Thermodynamic Characterization of Polypeptide Complex Coacervation
 Author: Dimitrios Priftis, Nicolas Laugel, Matthew Tirrell
 Publication: Langmuir
 Publisher: American Chemical Society
 Date: Nov 1, 2012
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