COMPLEXATION OF SEMI-FLEXIBLE BIOPOLYELECTROLYTES

by

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COMPLEXATION OF SEMI-FLEXIBLE BIOPOLYELECTROLYES

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To my dear family

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ABSTRACT

COMPLEXATION OF SEMI-FLEXIBLE BIOPOLYELECTROLYTES

Complex formation and phase separation of two oppositely charged biopolyelectrolyte solutions were investigated by using turbidimetry. In our study, we have chosen to work with solutions of hyaluronic acid (HA), a polyanionic polysaccharide, and chitosan (CHI), a polycationic polysaccharide. There are many factors that affect the phase separation and the complexation of HA with CHI such as pH and ionic strength of the medium, polyacid/polybase charge ratio, molecular weight (chain length), and degree of deacetylation (DD) of chitosan. After mixing of these two polyelectrolyte solutions, the mixture became either clear or turbid, and formed flocculants, precipitates or coacervates. Among visual observation, turbidimetry helped us determine the onset of phase separation either at a certain pH, pH_{φ}, or at a certain critical ionic strength, I_c. Aside from the complexation studies, potentiometric titrations were done to determine degree of ionizations of HA and CHI. In order to understand and differentiate between coacervation and precipitation, optical light microscopy was used.

ÖZET

YARI ESNEK BİYOPOLİELEKTROLİTLERİN KOMPLEKSLEŞMELERİ

Zıt yüklü iki polielektrolit solisyonunun kompleks oluşumları ve faz ayrımları türbidimetri (bulanıklık ölçümü) ile incelenmiştir. Çalışmamızda, polianyonik polisakkarit olarak hiyalüronik asit (HA) solisyonunu ve polikatyonik polisakkarit olarak kitosan (CHI) solisyonu seçilmiştir. HA ve CHI'nın faz ayrımlarını ve kompleksleşmelerini etkileyen birçok faktör vardır. Ortamın pH`ı ve tuz derişimi, poliasit/polibaz yük oranı, polielektrolitlerin moleküler ağırlığı (polimer zincir uzunluğu), ve kitosanın deasetilasyon derecesi bunlardan bazılarıdır. Bu iki polielektrolit çözeltisinin karıştırdıktan sonra, karışım berrak ya da bulanık olabilir veya karışımda topaklaşma, çöküntü veya koaservasyon oluşumu olabilir. Görsel gözlemlerin yanısıra, türbiditimetrik titrasyonlar bize faz ayrımının başlangıcı olan pH'ı, pH_{φ}, veya kritik tuz derişimini (I_c) belirlemede yardımcı oldu. Kompleks oluşumunun yanı sıra, HA ve CHI'nın iyonlaşma derecelerini belirlemek için potensiyometrik titrasyonlar da yapıldı. Koaservasyon ve çöküntü ayrımını daha iyi anlayabilmek için optik ışık mikroskobu kullanıldı.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iv

ABSTRACT v

ÖZET vi

LIST OF FIGURES ix

LIST OF TABLES xii

LIST OF SYMBOLS xiii

LIST OF ACRONYMS/ABBREVIATIONS xv

1. INTRODUCTION 1

1.1. Polyelectrolytes 1

1.2. Interaction of PEs with Oppositely charged Macromolecules 3

1.3. Complex Coacervation 7

1.4. Hyaluronic Acid 9

1.5. Chitosan 10

1.6. Semi-Flexibility of HA and CHI 12

1.7. Literature Examples for HA/CHI Systems 12

2. OBJECTIVES OF THE PROJECT 14

3. EXPERIMENTAL 15

3.1. Materials 15

3.2. Potentiometric Titrations 15

3.3. Turbidity Measurements 16

3.3.1. Effect of ionic strength and total polymer concentration 16

3.3.2. Type-1 titrations 16

3.3.3. Charge ratio of HA/CHI 17

3.4. Optic Light Microscope Imaging 17

4. RESULTS AND DISCUSSION 18

4.1. Degree of Deprotonation of Hyaluronic Acid and Chitosan.....18
4.2. Effect of pH on the Complexation of HA and CHI.....21
4.3. Effect of Ionic Strength on Complexation of HA and CHI 25
4.4. Effect of Total Polymer Concentration on Complexation of HA and CHI 29
4.5. Effect of Chain Length on Complexation of HA and CHI.....30
4.6. Effect of DD% of CHI on Complexation of HA and CHI.....31
4.7. Effect of Polyacid/Polybase Charge Ratio on the Complexation of HA and CHI ..32
5. CONCLUSIONS AND FUTURE WORK 36
APPENDIX: CALCULATION OF CHARGE RATIO OF HA/CHI 38
REFERENCES 41

LIST OF FIGURES

Figure 1.1. Some polyelectrolyte examples (a) polystyrene sulfonate (PSS), (b) poly(dimethyldiallylammonium chloride) (PDADMAC), (c) heparin, (d) polyallylamine hydrochloride (PAM), (e) polyacrylic acid (PAA). 1

Figure 1.2. Complexation mechanism of oppositely charged polyelectrolytes. 6

Figure 1.3. Process of the complex formation of SDS/TX-100 and PDADMAC as the charge density of the micelles increases. Darker circles indicate the higher charge density of the micelles. 7

Figure 1.4. Photographs of coacervates which encapsulate soil drops, taken by optical microscopy. (Left) Coacervates from alpha gliadin/arabic gum, c_p=10g/L, pH 3.5, (right) microcapsules obtained from pea globulin/arabic gum, at pH 3.5. 8

Figure 1.5. Complex coacervation of synthetic glue protein analogue A) At pH 6, B,C) At pH 7.4, D) Complex coacervates under water without dissolving. 8

Figure 1.6. Ion exchange chromatography analysis of BLG-A and BLG-B composition in different phases. Blue line: supernatant, red line coacervates. 9

Figure 1.7. Molecular structure of Hyaluronic acid.....10

Figure 1.8. Molecular structure of Chitosan. 11

Figure 1.9. General mechanism for deacetylation of chitin.....11

Figure 4.1. ΔV vs. pH titration graph for HA (Mw=234.4 kDa), pKa is 2.82. 19

Figure 4.2. ΔV vs. pH titration graph for CHI (Mw=260 kDa, DD% = 83%), pKa is 6.39.....19

Figure 4.3. Combined pH titration profiles for HA (234.4 kDa) and CHI (260 kDa). 20

Figure 4.4. Turbidity vs. pH titration graph at different I (M), total polymer concentration is (cp) = 0.5 mg/ml. 21

Figure 4.5. Turbidity (τ) vs. pH titration graph for determining pH_{φ} of HA/CHI, Mw_{HA}=234.4 kDa, Mw_{CHI}= 260 kDa. 22

Figure 4.6. pH_{φ} vs. I for HA(234.4 kDa)/CHI(260 kDa), c_p = 0.5 mg/mL 23

Figure 4.7. Turbidity vs. pH titration graph for HA(150 kDa)/CHI(260 kDa). Total polymer concentration of solution (c_p) = 0.5 mg/ml. 23

Figure 4.8. Turbidity vs. pH titration graph for HA(234.4 kDa)/CHI(260 kDa) at different temperatures, c_p = 0.5 mg/mL and I = 0.3 M. 24

Figure 4.9. Turbidity vs. I (M) at different ionic strength, total polymer concentration (c_p) = 0.5 mg/mL. 27

Figure 4.10. Critical ionic strength (I_c) vs. pH titration and Debye length (κ^{-1}) vs. pH titration for HA(234.4 kDa)/CHI(260 kDa) system, c_p = 0.5 mg/mL. 28

Figure 4.11. Turbidity vs. I (M) at different temperature, total polymer concentration (c_p) = 0.5 mg/mL. 29

Figure 4.12. Turbidity vs. pH titration graph for HA/CHI. Mw of CHI is constant and Mw of HA samples are different. C_p = 0.5 mg/mL and I = 0.30 M. 30

Figure 4.13. Turbidity vs. pH titration graph for HA/CHI systems at different DD% of CHI. C_p = 0.5 mg/mL and I = 0.30 M 31

Figure 4.14. Turbidity vs. charge ratio of HA/CHI systems, at pH = 3, c_p = 0.5 mg/mL and I = 0.30 M. 33

Figure 4.15. HA/CHI samples at different charge ratios of HA/CHI at pH 3.0 and Cp=0.5 mg/mL. a-d: Charge ratio from 0.1 to 3.0 before centrifugation. e: after

centrifugation of the sample with charge ratio of 0.22: white coacervate at the bottom of the cell.....34

Figure 4.16. Optic Light microscope image complex coacervates of HA and CHI, 1:1 weight ratio. Red circles show complex coacervates.....35

Figure 4.17. Optical Light microscope image for precipitates of HA and CHI, 1:1 weight ratio. 35

LIST OF TABLES

Table 4.1. pKa values of HA were determined by potentiometric titration. 18

Table 4.2. DD%, Mw and pH values for different chitosan samples.....32

LIST OF SYMBOLS

b	Distance Between Two Neighboring Charge Spaces
c_p	Total Polymer Concentration
^0C	Degree of Celsius
DD%	Degree of Deacetylation
F_{el}	Helmholtz Free Energy
G_{el}	Gibbs Free Energy
I_c	Critical Ionic Strength
kDa	KiloDalton
L	Contour length
L_p	Persistence length
mg/mL	Milligram per milliliter
nm	Nanometer
P	Pressure
pH_c	Onset pH For Soluble Complex Formation
pH_φ	Onset pH for Insoluble Complex Formation
pK_a	Acid Dissociation Constant
pK_b	Base Dissociation Constant
S_{el}	Electrostatic Entropy
U_{el}	Electrostatic Energy

α	Degree of ionization for Hyaluronic Acid
β	Degree of deprotonation for Chitosan
κ	Debye-Huckel Parameter
κ^{-1}	Debye Length
λ_b	Bjerrum Length
μm	Micrometer
ξ	Linear Charge Density
ρ	Overall Charge Density
σ_c	Critical Colloid Surface Charge Density
τ	Turbidity
$\vec{\nabla}\psi$	Gradient of The Electrostatic Potential

LIST OF ACRONYMS/ABBREVIATIONS

BLG	β-lactoglobulin
BSA	Bovine Serum Albumin
CHI	Chitosan
GAGs	Glycosaminoglycans
HA	Hyaluronic Acid
HCl	Hydrochloric Acid
HM-HEC(-)	Hydrophobically Modified Anionic Hydroxyethylcellulose
NaCl	Sodium Chloride
$NaNO_3$	Sodium Nitrate
NaOH	Sodium Hydroxide
PAA	Polyacrylic Acid
PAM	Polyallylamine
PAsp	Poly(L-Aspartic Acid)
PDADMAC	Poly(Dimetyhyldiallylammonium Chloride)
PDMAEMA	Poly(Dimethylaminoethyl Methacrylate)
PE	Polyelectrolyte
PEC	Polyelectrolyte Complex
PGlu	P(L-Glutamic Acid)
PHis	Poly(L-Histidine) (Phis)
Plys	P(L-Lysine)

PO	Poly(L-Ornithine)
P(NIPAM)	Poly(*N*-Isopropylacrylamide)
PSS	Polystyrene Sulfate
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscope
TX-100	Triton X-100

1. INTRODUCTION

1.1. Polyelectrolytes

Polyelectrolytes (PEs) are polymers containing negatively or positively charged repeating units on their backbones or pendant groups and small molecular counterions for maintenance of electroneutrality [1]. PEs may contain both anionic or cationic groups which are classified as polyampholytes; e.g. proteins. PEs are mainly of biological, semi synthetic or synthetic origin. The polyphosphates and polysilicates are examples to inorganic PEs. Given below are some PEs examples:

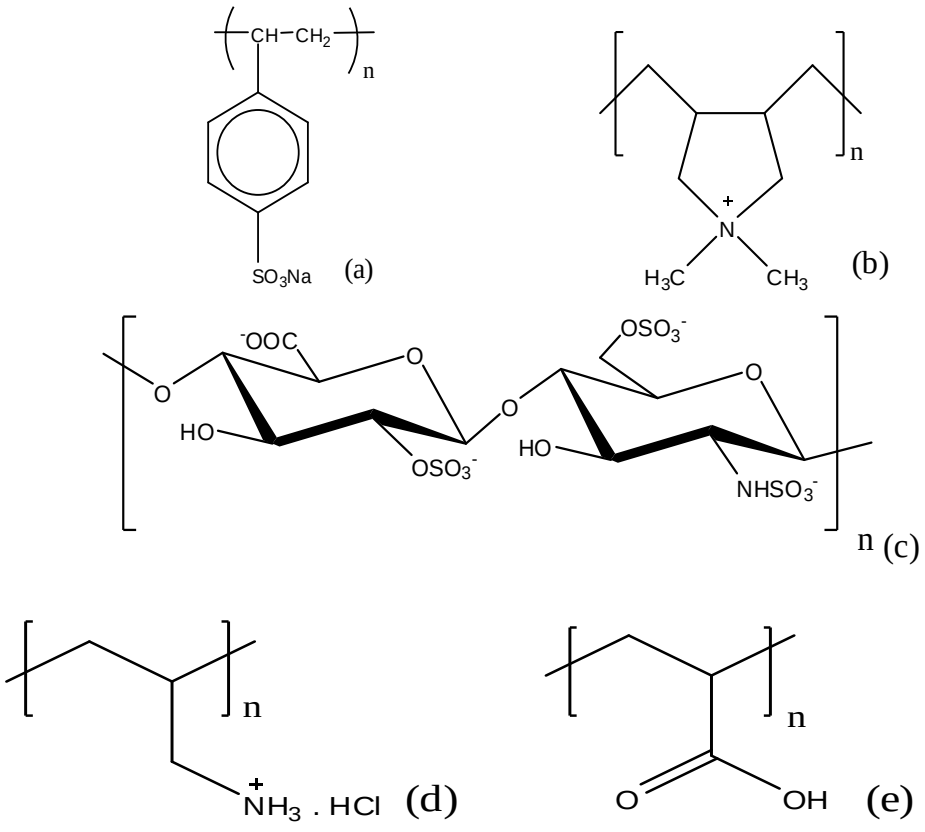

Figure 1.1. Some polyelectrolyte examples (a) polystyrene sulfonate (PSS), (b) poly(dimethyldiallylammonium chloride) (PDADMAC), (c) heparin, (d) polyallylamine hydrochloride (PAM), (e) polyacrylic acid (PAA).

PEs can be obtained by ionizing uncharged polymers e.g. polystyrene sulfonate and polyacrylic acid, or by polymerizing ionic monomers e.g. poly(diallyldimethylammonium chloride) (PDADMAC), or by isolation and chemical modification of natural polymers such as cationic starch, heparin and gelatin.

PEs can be classified according to their dissociation strength in aqueous solution as strong or weak PEs. Strong PEs are ionized or dissociated completely when they are dissolved in the aqueous solution throughout the majority of the pH range. In contrast, weak PEs are partially dissociated at the intermediate pH range in their aqueous solutions, so they have dissociation constant; pK_a and pK_b. pH and ionic strength of the solution affect the dissociation of weak PEs.

PEs have been used in a wide range of application areas in different fields of industry due to their intrinsic properties. Some part of industrial areas where PEs have been used are given below:

- Paper industry
- Wastewater treatment
- Medicine and pharmacy
- Petrol industry
- Chemical industry
- Cosmetics
- Food industry

Cationic starch [2] is used as one of the water-soluble polymeric retention aids in the paper industry which is used to prepare a uniformly formed paper web with anionic short fibers and fillers.

N,N-dimethylaminopropylacrylamide (DMAPAA) is a cationic polyelectrolyte and it is used as high molar mass polymeric flocculant agents which can catch a large number of oppositely charged colloids, for dewatering of inorganic sludge [3]. Co-polymerization of DMAPAA with N-isopropylacrylamide (poly(NIPAM) makes cationic thermosensitive flocculants.

Hydrophobically modified anionic hydroxyethylcellulose (HM-HEC(-)) [4] shows higher viscosity at low polymer concentrations. Addition of small amount of surfactant such as sodium dodecyl sulfate (SDS) increases the viscosity and it becomes a good candidate for polymer flooding in enhanced oil recovery.

1.2. Interaction of PEs with Oppositely charged Macromolecules

Through electrostatic interactions, PEs can form complexes with oppositely charged macromolecules such as proteins [5-6], dendrimers [7], surfactant micelles [8], inorganic nanoparticles [9] and polyelectrolytes [10-11] in aqueous solutions. Complexes can show different phase behavior and can be in the different forms from soluble complexes to coacervates and flocculate, or precipitates depending on many factors which are given below:

- Ionic strength (salt concentration)
- pH
- Polyelectrolyte concentration
- Molecular weight of polyelectrolytes
- Stoichiometry (polyacid/polybase charge ratio)
- Temperature
- Chain flexibility
- Shear
- Intrinsic properties of macromolecules e.g. degree of deacetylation, degree of sulfonation, location of functional groups

Complexation of PEs with oppositely charged macromolecules is an entropy- and electrostatic energy-driven process. During the complex formation, small counterions of PEs are released and are not confined any more to the backbone of the polyelectrolyte. Figure 1.2. simply illustrates the general mechanism of oppositely charged PEC formation. Freedom of the chain conformation of the polyelectrolytes is neglected in this figure. Release of counterions results in an increase in entropy due to higher degrees of

conformational freedom for the counter ions when unbound from the polyelectrolyte. Spruijt *et al.* [12] have studied the complex formation of oppositely charged macromolecules and PEs by theoretical calculations and experiments. They used the free energy of the electrical double layer theory which is developed by Voorn and Overbeek [13-14]. The electrostatic free energy, which is considered as Hemholtz free energy (F_{el}), is a thermodynamic quantity (Equation 1.1) at constant volume. Gibbs free energy (G_{el}) (Equation 1.2) is used when volume of the solution affects the electrostatic interactions.

$$\Delta F_{el} = \Delta U_{el} - T\Delta S_{el} \tag{1.1}$$

$$\Delta G_{el} = \Delta U_{el} - T\Delta S + P\Delta V \tag{1.2}$$

U_{el} is the electrostatic energy of the system and is defined as in Equation 1.3. Electrostatic interaction with water ions and ions on PEs backbone is neglected for calculating U_{el}. ΔS_{el} is the entropy change. Chain conformation of the PEs is an important factor for ΔS_{el} but chain conformation effect is neglected, and only entropy change due to counterion release is considered.

$$U_{el} = \frac{1}{2}\int_V \rho(\vec{r})\psi(\vec{r})dV = \frac{\varepsilon_r \varepsilon_o}{2}\int_V (\vec{\nabla}\psi^2)dV \tag{1.3}$$

where $\rho(\vec{r})$ is the overall charge density and $\vec{\nabla}\psi$ is the gradient of the electrostatic potential and it is influenced by the salt concentration, and pH of the solution.

Complex formation of polyelectrolytes with oppositely charged colloids such as proteins, micelles and dendrimers has been investigated by Dubin and his co-workers. [8,15] As notified in their papers, electrostatically-driven complex formation is affected by different variables. Theoretical approximations [16-19] have shown the relationship

between some of these variables as Equation 1.4. σ_c is the critical colloid surface charge density, ξ is the linear charge density and κ is the Debye-Huckel parameter.

$$\sigma_c \, \xi^a \sim \kappa^b \tag{1.4}$$

$$\xi \approx \frac{\lambda_b}{b} \tag{1.5}$$

a and b may be empirical scaling parameters or may be derived theoretically [15]. κ^{-1} is the Debye length and it is related with root square of ionic strength of the solution [8]. ξ [20-21] is linearly related with λ_b, which is Bjerrum length and nonlinear related with b, which is defined as the distance between neighboring charged monomers and is affected by many factors such as the pH and degree of ionization of PEs.

Complex formation of oppositely charged polyelectrolytes can be described as in Equation 1.6, replacing σ_c with another ξ and they are also related with the Debye-Huckel parameter.

$$\xi^a \, \xi^b \sim \kappa^c \tag{1.6}$$

Linear charge densities of weak polyelectrolytes are influenced by pH, ionic strength, intrinsic factors such degree of deacetylation for Chitosan, or degree of sulfonation for sulfonated polyelectrolytes and degree of ionization due to changes in the charge space (b) on the polyelectrolyte backbone.

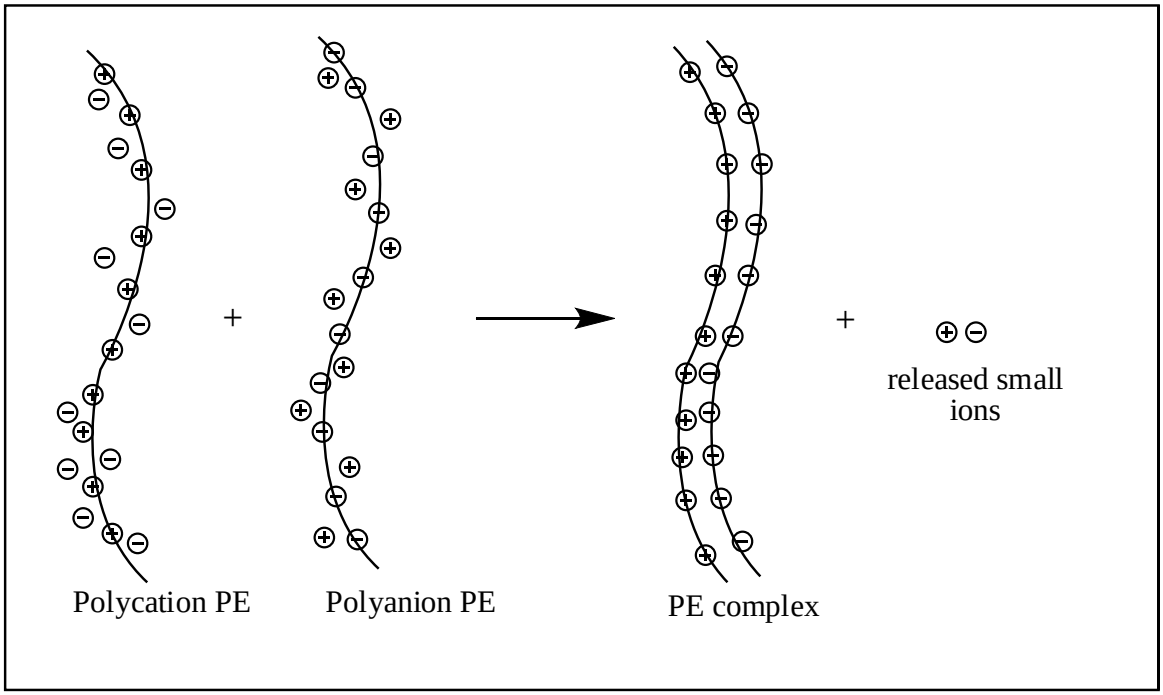

Figure 1.2. Complexation mechanism of oppositely charged polyelectrolytes.

Wang *et al.* [22] investigated complexation of PDADMAC, a strong cationic polyelectrolyte with anionic/nonionic mixed micelles of sodium dodecyl sulfate/Triton X-100 (SDS/TX-100) at different ionic strengths. At low surface charge densities of micelles, complexes formed with PDADMAC were soluble due to low electrostatic interaction strength between these oppositely charged macroions. As the charge density of the micelles increased, complexes changed from a state of coacervates to flocculates and precipitates. Flocculation is a solid-liquid phase separation, where solids are suspended in the solution. Further aggregation of flocculates leads to precipitation of complexes. Figure 1.2 shows clearly the process of the complexation of the micelles and PDADMAC from soluble complexes via coacervates to precipitates.

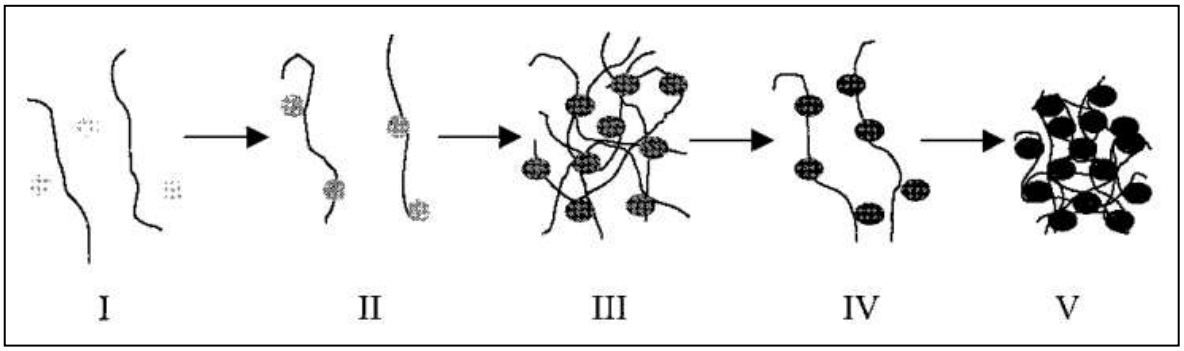

Figure 1.3. Process of the complex formation of SDS/TX-100 and PDADMAC as the charge density of the micelles increases. Darker circles indicate the higher charge density of the micelles [22].

PDADMAC-BSA (bovine serum albumin) is an example for complexation of PE-protein systems. Dubin *et al.* [23] investigated the complex formation and phase behavior of the PDADMAC and bovine serum albumin (BSA) at different conditions such as ionic strength, pH, protein/polyelectrolyte stoichiometry by using turbidimetric titrations. They determined the critical conditions from soluble complexes to coacervates for PDADMAC/BSA systems.

1.3. Complex Coacervation

Complex coacervation is a liquid-liquid phase separation of oppositely charged macromolecules. Electrostatic interactions between macroions and entropy gain by release of counter ions are driving forces for complex coacervation [24]. Complex coacervation occurs when the net charge of the solution is zero. Coacervation was first observed and described by Bungenberg de Jong who studied phase behavior of Arabic gum (a weak cationic polyelectrolyte) /natural gelatin (a weak anionic polyelectrolyte) system [25]. Polymer-rich and viscous phase was described as the coacervate and dilute phase as the polymer-deficient phase, respectively. Complex coacervation provides denser and viscous solutions without starting the sample preparation from concentrated samples as in the hydrogels.

Complex coacervation has widespread application areas including protein purification [26], microencapsulation of drugs, flavors, and oil [27-28], stabilization of processed food [29], long-life aromatization for textiles [30] and wastewater treatment for determination of identity of elements in water such as arsenic [31]. Stewart and his co-workers [32] have mimicked adhesive proteins of sandcastle glue to produce medical adhesives by utilizing complex coacervates of synthetic polyelectrolytes, Figure 1.5.

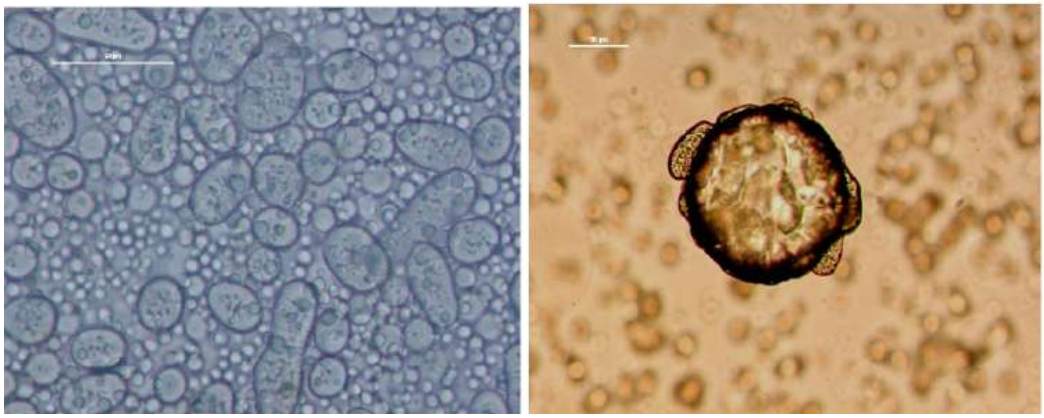

Figure 1.4. Photographs of coacervates which encapsulate soil drops, taken by optical microscopy. (Left) Coacervates from alpha gliadin/arabic gum, c_p=10g/L, pH 3.5, (right) microcapsules obtained from pea globulin/arabic gum, at pH 3.5 [27].

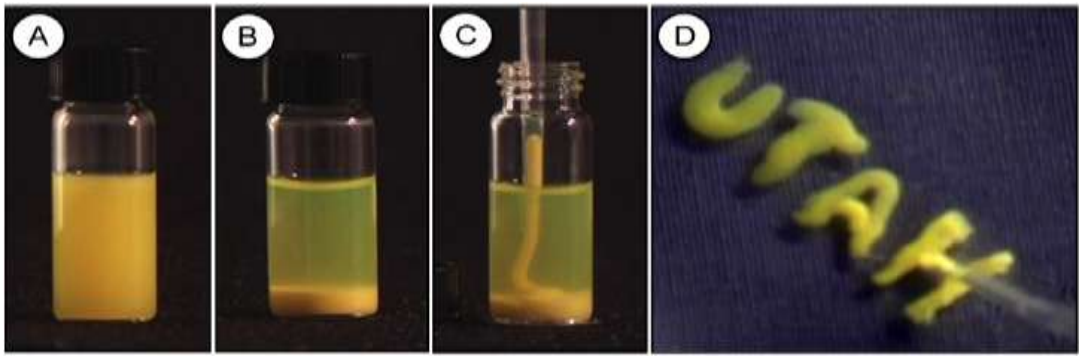

Figure 1.5. Complex coacervation of synthetic glue protein analogue A) At pH 6, B,C) At pH 7.4, D) Complex coacervates under water without dissolving [32].

Two isoforms of β-lactoglobulin (BLG) are separated through coacervation with cationic polyelectrolyte PDADMAC based on the charge anisotropy between BLG-A and BLG-B at mild conditions [26]. BLG-A has more negative charge than BLG-B so, its binding affinity with PDADMAC is stronger than BLG-B. In Figure 1.6, liquid-liquid phase separation is observed. According to anion exchange chromatography, the amount of BLG-A is higher in the content of the coacervates phase (polymer dense phase)

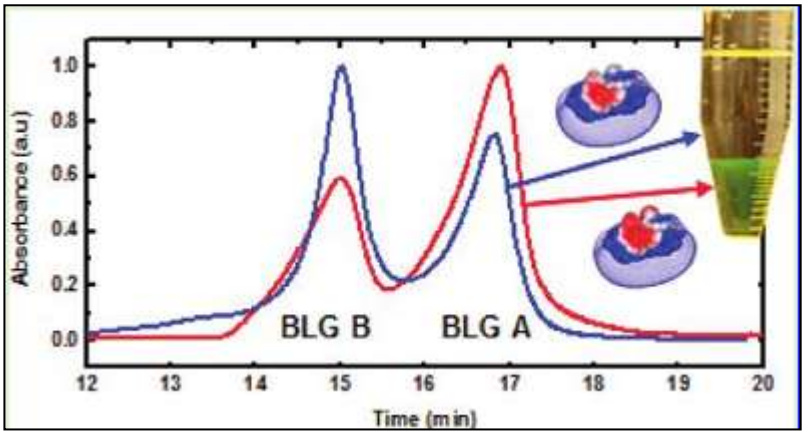

Figure 1.6. Ion exchange chromatography analysis of BLG-A and BLG-B composition in different phases. Blue line: supernatant, red line coacervates [26].

1.4. Hyaluronic Acid

Hyaluronic acid (HA) (Figure 1.7) is an anionic linear biopolyelectrolyte with disaccharide repeating units of N-acetyl glucosamine and glucuronic acid connected by β(1-4) and β(1-3) glycosidic bonds. HA is one of the members of glycosaminoglycans(GAG). Since HA does not contain any sulfate groups in its molecular structure, it is differentiated from other members of GAGs. HA is present abundantly in the skin, eye and it is a component of synovial fluid and extracellular matrix. HA acts as lubricant and shock absorber in the joints. HA is produced by isolating from bovine vitreous humor, rooster combs [33], and genetically modified bacteria such as streptococcus [34].

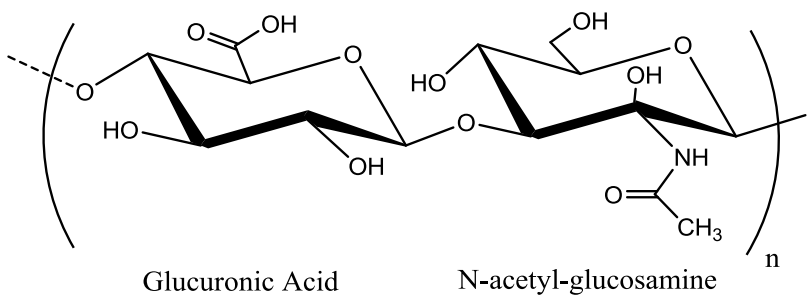

Figure 1.7. Molecular structure of Hyaluronic acid.

Biocompatibility, non-immunogenicity, and biodegradability lead to usage of hyaluronic acid for extensive range of biomedical applications such as wound healing [35], dermal filling reagent [36] and cell proliferation [37]. In addition, HA is recognized by CD44 which is a cell surface receptor and it is known as cell adhesion molecule so HA has been investigated for enhancing the drug delivery [38], and directing anticancer drugs to tumor cells which are "over-expressing CD44 receptors [39].

1.5. Chitosan

Chitosan (CHI) (Figure 1.8) is a cationic linear biopolysaccharide which is a cationic copolymer of β (1-4) linked N-acetyl glucosamine and D-glucosamine. CHI does not exist naturally, it is a deacetylated form of chitin. Chitin is deacetylated with strong base hydrolysis by homogeneous [40] or heterogeneous [41] treatments. General reaction scheme for deacetylation of chitin to obtain chitosan is given in Figure 1.7. Degree of deacetylation (DD%) is an important factor for physical and chemical properties of CHI such as solubility and chemical reactivity.

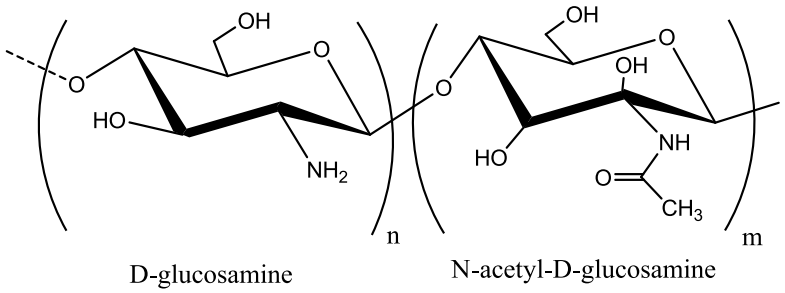

Figure 1.8.Molecular structure of Chitosan.

Chitosan has attracted interest in the field of biomaterials due to its excellent intrinsic properties such as biocompatibility, biodegradability and non-toxicity [42]. CHI can be used in drug delivery [43], gene transfer [44-45], surface coating [46] and hydrogels [47-48].

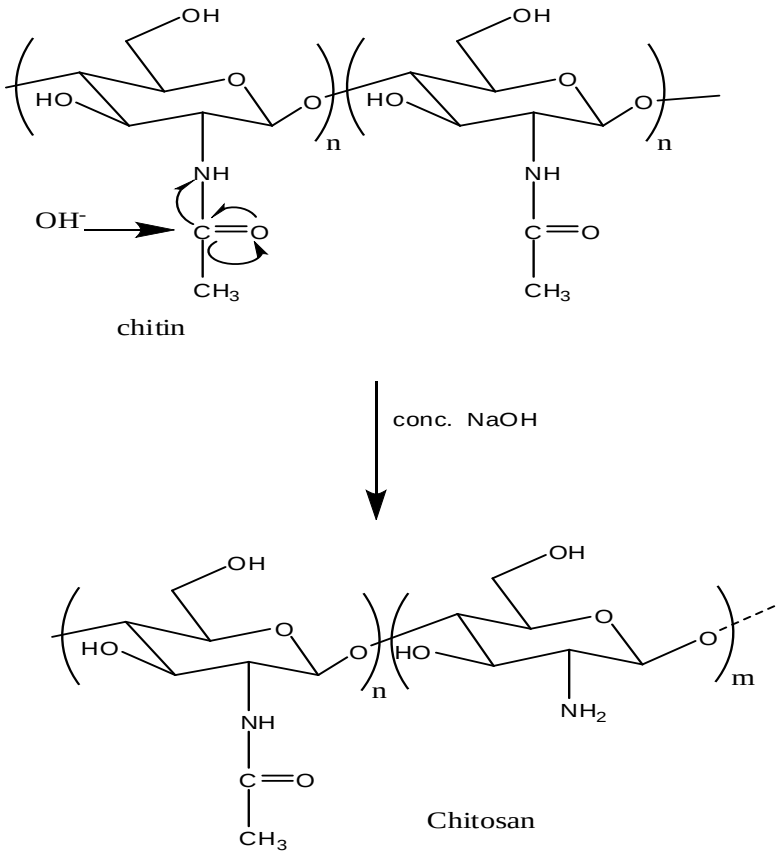

Figure 1.9. General mechanism for deacetylation of chitin.

1.6. Semi-Flexibility of HA and CHI

There are many theory and models to define and describe the stiffness of polymer chains in the solution. Krotky and Parod [49] suggested a model called as worm-like chain model for semiflexible polymers in the solution. Wormlike chains have polymer conformation in a broad band between Gaussian coils and rigid rods [50]. These two limits are depending on the ratio of the contour length/persistence length (L/L_p) and they are given below:

If $L/L_p >> 1$ ⟶ Gaussion coil limit

If $L/L_p << 1$ ⟶ Rigid rod limit

Persistence length (L_p) of HA is reported to range from 4 to 9 nm in the literature [51-54] and L_p of CHI ranges from 4.6 to 40 nm as reported in the literature [55- 60]. L_p is influenced by some factors such as type of solvent, charge density of polymer, ionic strength and DD% of CHI. That's why, these polyelectrolytes can be considered as semi-flexible.

1.7. Literature Examples for HA/CHI Systems

HA and CHI solutions can form complexes because of the electrostatic interactions between them. Many research groups have investigated and studied their complexation and possible biomedical applications of HA and CHI mixtures. HA and CHI nanoparticles can be used for drug delivery such as heparin loaded HA-CHI nanoparticles for asthma treatment [61], gene delivery for disease treatment [62] and gene transfection [63]. HA-CHI based polyelectrolyte complexes in the form of hydrogel, microspheres and sponges can be employed for wound healing [64], tissue engineering [65], scaffold [66] or drug releasing systems [67]. HA-CHI based films can be used for cell-proliferation[68], or modification of biomaterial surfaces [69]. For preparation of such biomaterials and polyelectrolyte complexes, various techniques are utilized such as hydrolysis [64], carbodiimide chemistry [70], electronic spraying [69], solvent evaporation [67] and lyophilization of HA-CHI mixtures [64]. However, to the best of our knowledge, there isn't any study where complex coacervates are obtained from HA and CHI. In addition,

complex coacervation of HA and CHI has not been used for microencapsulation for drug delivery or gene delivery in treatment of diseases.

2. OBJECTIVES OF THE PROJECT

Hyaluronic acid (HA) and Chitosan (CHI) have been used in a broad variety of industrial and biomedical applications and new possible applications have been investigated due to their preferential extensive biochemical properties. They form polyelectrolyte complexes in aqueous solutions because of electrostatic interactions. Phase separation of HA and CHI complexes from solvent can be in the form of liquid-liquid (complex coacervation) or solid-liquid. To the best of our knowledge, this is the first work where complex coacervates are obtained from the HA/CHI system rather than precipitates or microgels.. In addition, phase behavior of HA/CHI system in different conditions such as pH, ionic strength, chain length and polyacid/polybase stoichiometric ratio have not been investigated in detail by any other study. Optimization of these parameters will be essential for application of HA/CHI coacervates.

The aim of this project is investigating the phase separation and determining the formation conditions for complex coacevation of HA and CHI as a function of pH, ionic strength, total polymer concentration, molecular weight (chain length), degree of deacetylation (DD%) of CHI, and polyacid/polybase charge ratio (stoichiometric ratio) by performing turbidimetric titrations. The difference between complex coacervation and precipitation of HA/CHI system is characterized by optical light microscopy. Hence, this project is the first study to investigate such conditions for phase separation of HA/CHI system.

3. EXPERIMENTAL

3.1. Materials

Dried sodium hyaluronate (HA) samples were purchased from Lifecore Biomedical (Chaska, MN, USA). Mass average molecular weights (M_m) of the HA were given as 51, 150, 234.4 and 752 kDa by the manufacturer. Chitosan samples with various degrees of deacetylation (DD%) and M_w (37% - 332.4 kDa, 54% - 365.1 kDa , 58% - 123.5 kDa, 76% - 345.6 kDa, 99% - 210 kDa) were kindly provided by Sabina Strand (Norwegian University of Science and Technology, Trondheim, Norway***).*** Chitosan chloride with 83 %DD and M_w of 260 kDa was purchased from Novamatrix (Sandvika, Norway). The polymers were used as received. All solutions are filtered with 0.22 and 0.45μm cellulose acetate membranes (Sartorius Stedim Biotech). NaCl was purchased from Sigma-Aldrich and Merck. HCl and NaOH were purchased from Merck. Milli-Q water (Millipore, Milford, MA, USA) was used throughout the experiments.

3.2. Potentiometric Titrations

Potentiometric titrations were done to determine the degree of ionization of HA and CHI in aqueous medium under inert gas (Argon or nitrogen). A pH-meter (Thermo Orion-3), pH electrode (VWR, Symphony – 14002-780) and temperature probe (Beckmann ATC) were employed for potentiometric titrations. All polymer and blank (polymer-free) solutions were dissolved in 0.5 M NaCl solution. Concentration of the polymer solutions was 0.1 mg/mL. pH of the solutions were adjusted by using 0.1-1 N HCl and 0.1-1 N NaOH. Initial pH of the HA solution was adjusted to pH 1.5 while pH of the CHI was adjusted to pH 2. pH of the polymer and blank (polymer-free) solutions was increased from lower pH to higher pH by addition of 0.1 N NaOH with a microburette (Gilmont Instrument Co., Barrington, IL, USA). pK_a of HA and CHI corresponded to the pH at the midpoint of $\Delta V = V_{polymer} - V_{blank}$ (V: Volume of added NaOH into solutions). The degree

of ionization is calculated with the modified Handerson - Hasselbach Equation [71] (α and β are the degree of ionization of HA and CHI, respectively):

$$pK_a = pH + \log\left(\frac{1-\alpha}{\alpha}\right) \quad (3.1)$$

$$\alpha + \beta = 1 \quad (3.2)$$

3.3. Turbidity Measurements

Turbidimetric titration experiments were performed with a 2 cm path length fiber optic colorimeter probe (Brinkmann PC 950) at 420 nm and UV/Visible spectrophotometer (Shimadzu UV-Pharmaspec 1700). Turbidity (τ) is defined by 100-T%, where T corresponds to the transmittance. pH of the solutions were measured with a pH meter (Thermo Orion 3-Star) and a pH electrode (VWR pHASE combination electrode). Effect of ionic strength, pH, total polymer concentration, Mw of polyelectrolytes, DD% of CHI and charge ratio of HA/CHI on HA/CHI complexation was investigated with turbidimetric titrations. Experiments for different parameter were repeated at least one more time.

3.3.1. Effect of ionic strength and total polymer concentration

HA and CHI at different concentrations (0.1, 0.25, 0.5 and 1.0 mg/mL) were dissolved in 5 mM NaCl stock solution. pH of the solutions were adjusted to pH 2.3, 3, 4.5 and 5.2 before HA and CHI mixed at equal volume. Weight ratio of HA/CHI was 1:1. Ionic strength of the HA-CHI solutions was increased with gradual addition of 4 M NaCl solution. Ionic strength at the maximum turbidity was recorded as the critical ionic strength for HA-CHI solutions.

3.3.2. Type-1 titrations

HA and CHI of 0.5 mg/mL were dissolved in 0.3 M NaCl solution. After mixing HA and CHI at 1:1 weight ratio, Type-1 titrations [72] in which pH of solution is changed by

addition of acid or base, for HA-CHI systems were initiated at pH 2 and pH was increased with 1 N and 0.1 N NaOH, respectively. pH_{φ} was defined as the pH where noticeable turbidity changes were observed.

3.3.3. Charge ratio of HA/CHI

Turbidity measurements were carried out with UV/Visible spectrophotometer (Shimadzu UV-Pharmaspec 1700) at 420 nm. Molecular weight of HA was 234.4 kDa and Mw of CHI with DD % 83% was 260 kDa. Both polyelectrolyte solutions were dissolved in 0.3 M NaCl. Before HA and CHI solutions were mixed at various charge ratios of HA/CHI, pH of HA and CHI solutions were adjusted to pH 2.3 and 3, respectively. Calculation of charge ratio of HA/CHI is given in the Appendix.

3.4. Optic Light Microscope Imaging

Optic light microscopy imaging is used to distinguish complex coacervates from precipitates and to better understand the complexation of HA/CHI. Optic light microscopy (Leica DMI 6000B Inverted Microscope) at the Physics Department was used to carry out the imaging experiments. HA and CHI were mixed at the desired charge ratio, ionic strength and total polymer concentration before imaging.

4. RESULTS AND DISCUSSION

4.1. Degree of Deprotonation of Hyaluronic Acid and Chitosan

Hyaluronic acid (HA) and Chitosan (CHI) are weak acid and base, respectively. Their dissociation constant (pKa) values were determined with potentiometric titrations. Degree of deprotonation of HA and CHI, α and β, were calculated by using the modified Handerson - Hasselbach Equation

. Calculating the degree of deprotonation of HA and CHI is important for determining the charge ratio of HA/CHI and determining the charge density of polyelectrolyte chains of HA and CHI.

In Figure 4.1, ΔV(ml) is given as a function of pH for HA (Mw =234.4 kDa) and pKa of HA corresponds to the midpoint of ΔV. pKa in 0.5 M NaCl for HA (Mw =234.4 kDa) is 2.8. pKa of HA in 0.01 $NaNO_3$ is noted as 2.9 in the literature [21]. pKa of HA is in the acidic range due to the carboxylic acid groups in its molecular structure.

In Table 4.1, pKa values of HA for different mass average molecular weights (MW) are given. As seen in the table, pKa values depend on Mw. As Mw of HA increases, pKa's become higher due to less effective ion-exchange between protons and Na^+ ions as a result of HA's increasing chain length.

Table 4.1. pKa values of HA were determined by potentiometric titration.

Polyelectrolyte	pKa
HA (Mw: 51 kDa) [73]	2.39±0.02
HA (Mw: 150 kDa) [73]	2.48±0.02
HA (Mw: 234.4 kDa)	2.82±0.01

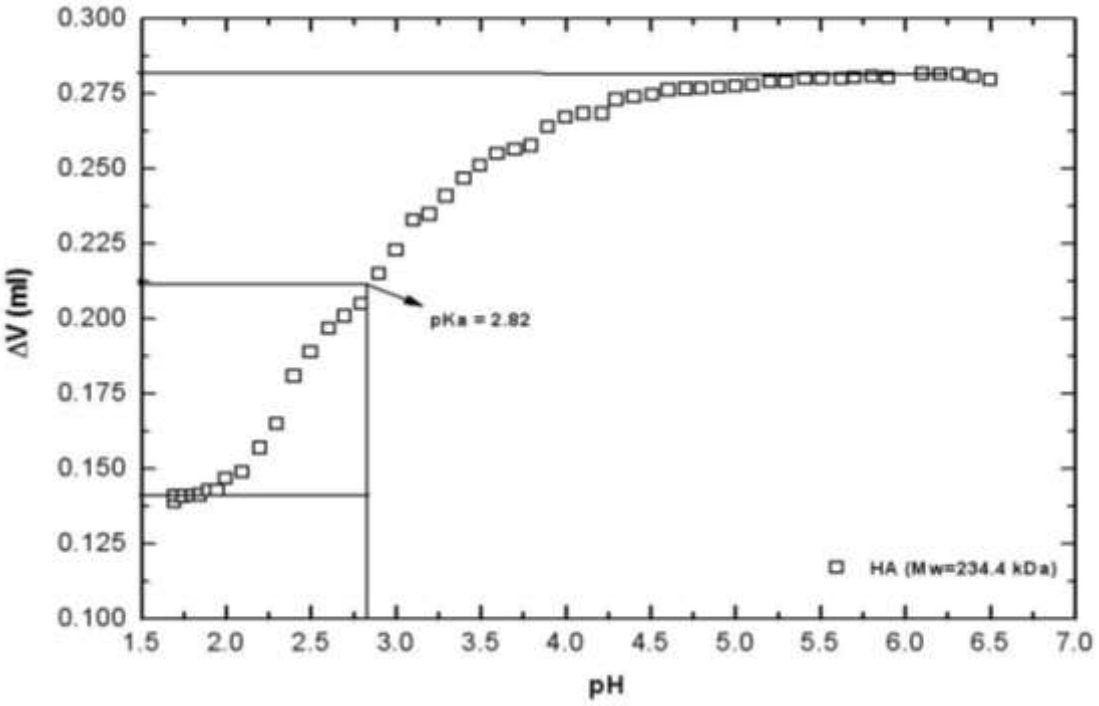

Figure 4.1. ΔV vs. pH titration graph for HA (Mw=234.4 kDa), pKa is 2.82.

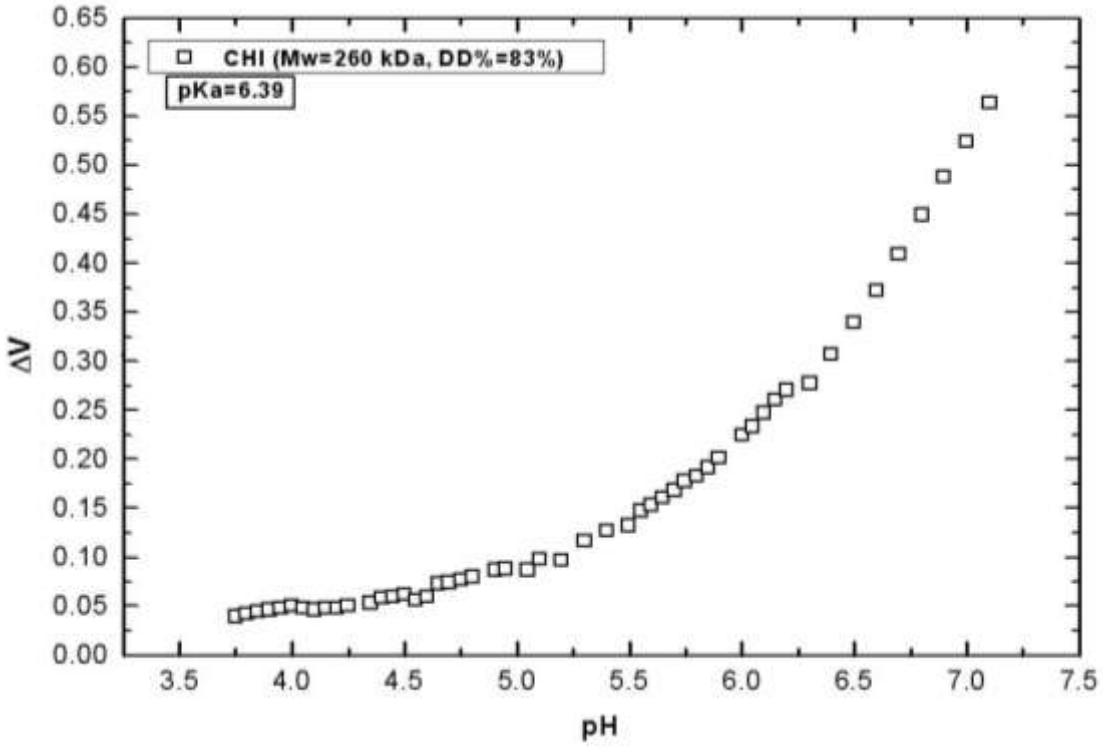

Figure 4.2. ΔV vs. pH titration graph for CHI (Mw=260 kDa, DD% = 83%), pKa is 6.39.

In Figure 4.2, ΔV(ml) is given as a function of pH for CHI (Mw =260 kDa, DD%= 83%). pKa of CHI is found as 6.39 in 0.5 M NaCl and it is consistent with the literature [74-75]. pKa of CHI is near to basic range because it has amine groups in its molecular structure .

Figure 4.3 combines pH titration profiles for HA (234.4 kDa) and CHI (260 kDa). As clearly seen in the Figure 4.3, degree of ionization (deprotonation) of HA (α) increases as pH increases up to pH 4.5 and then it becomes constant to 1. Therefore, charge density of HA which is pH dependent like charge density of CHI, explicitly increases up to pH 4.5. However, degree of ionization (degree of deprotonation) (β) is close to 1 until pH 4.5 and then it decreases as pH increases. Thus, at pH 4.5, higher yield is expected from phase separation. Potentiometric titration for CHI was terminated at that pH since CHI precipitated above pH 7.0. Thus, beta at pH 7.0 was assumed to go to zero.

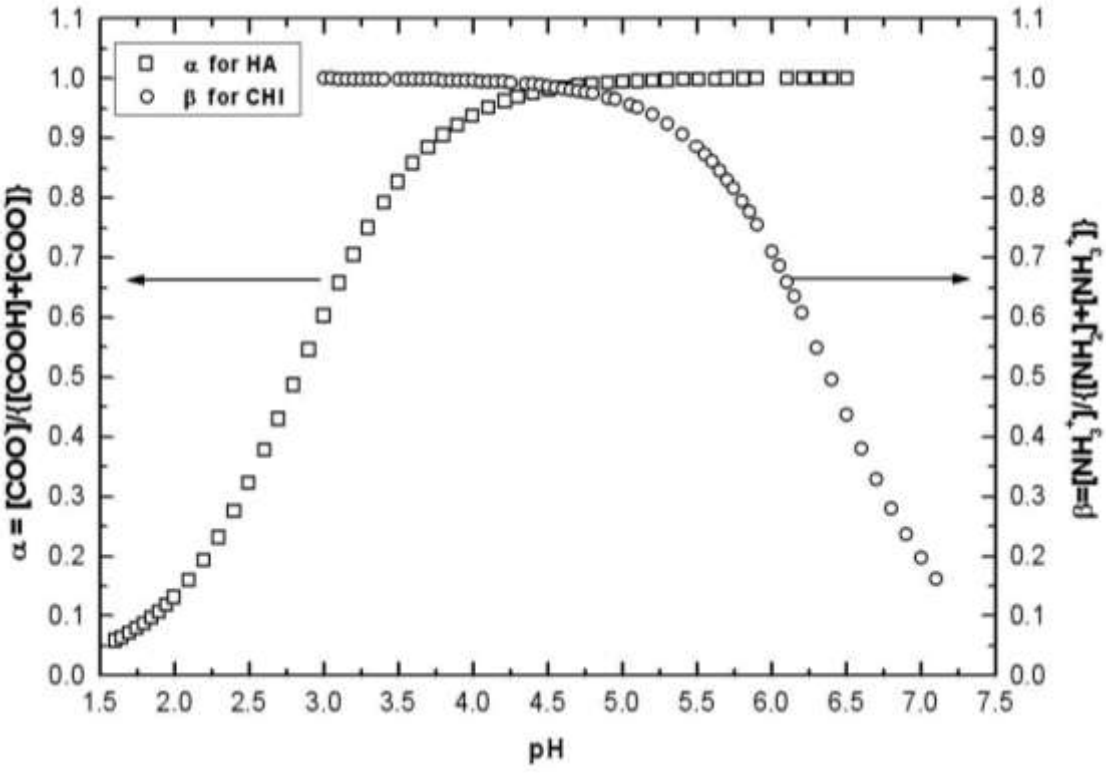

Figure 4.3. Combined pH titration profiles for HA (234.4 kDa) and CHI (260 kDa).

4.2. Effect of pH on the Complexation of HA and CHI

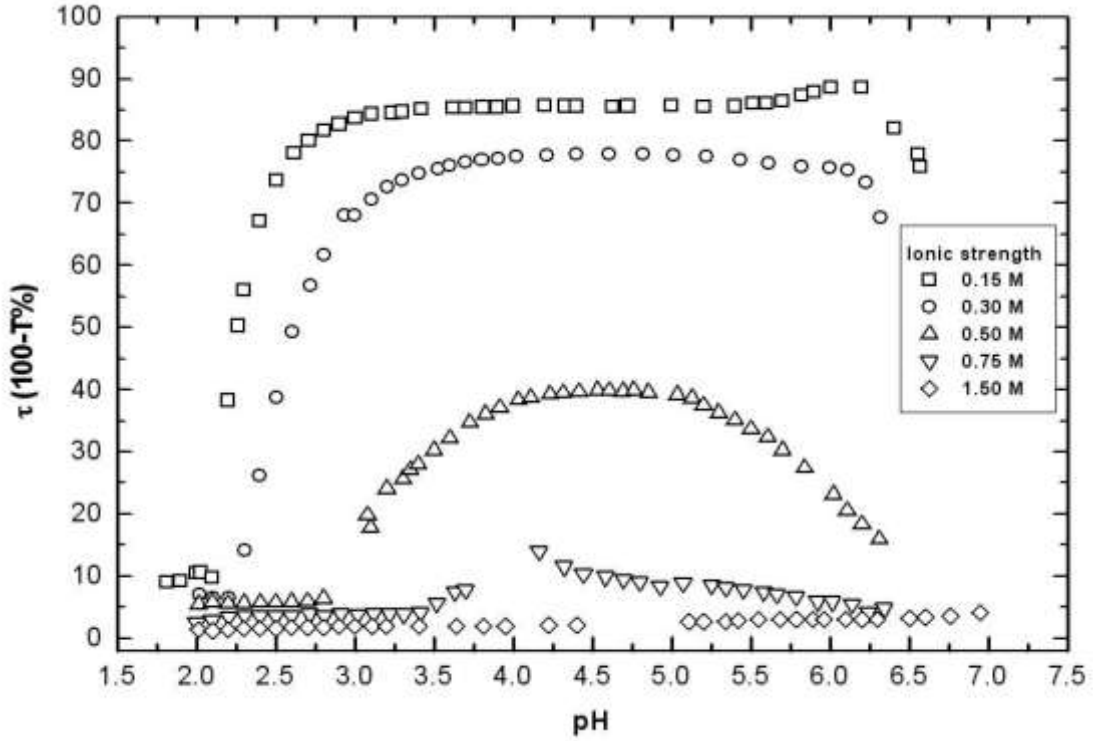

Figure 4.4. Turbidity vs. pH titration graph at different I (M), total polymer concentration is (cp) = 0.5 mg/ml.

pH is one of the most important factors for complexation of weak polyelectrolytes. In Figure 4.4, turbidity (τ) which is defined as 100 – Transmittance % , is studied as a function of pH for Hyaluronic acid/Chitosan (HA/CHI) system at different ionic strengths (I). In these "Type-1" titration experiments, total polymer concentration (c_p) of solutions was 0.5 mg/ml. As seen in the Figure 4.4, phase separation is favored at pH's between pK_a values of HA and CHI due to increasing numbers of chargeable species on HA and CHI. As pH increases, insoluble polyelectrolyte complexes (PEC) take different forms from coacervates to suspended solid particles for I = 0.15 M - 0.75 M NaCl.

Effect of ionic strength on phase separation is seen clearly in Figure 4.5 As ionic strength increases, phase separation is prevented because of screening effects of salt ions.

pH_{φ} which is the pH for the onset of phase separation (determination of pH_{φ} is shown in Figure 4.5) increases with a rise in the ionic strength. In Figure 4.6, pH_{φ}, given as a function of I, is demonstrated to depend linearly on I. As salt concentration (I) of the solution increases, pH_{φ} of the HA/CHI systems gets higher and turbidity gets smaller as shown in Figure 4.4.

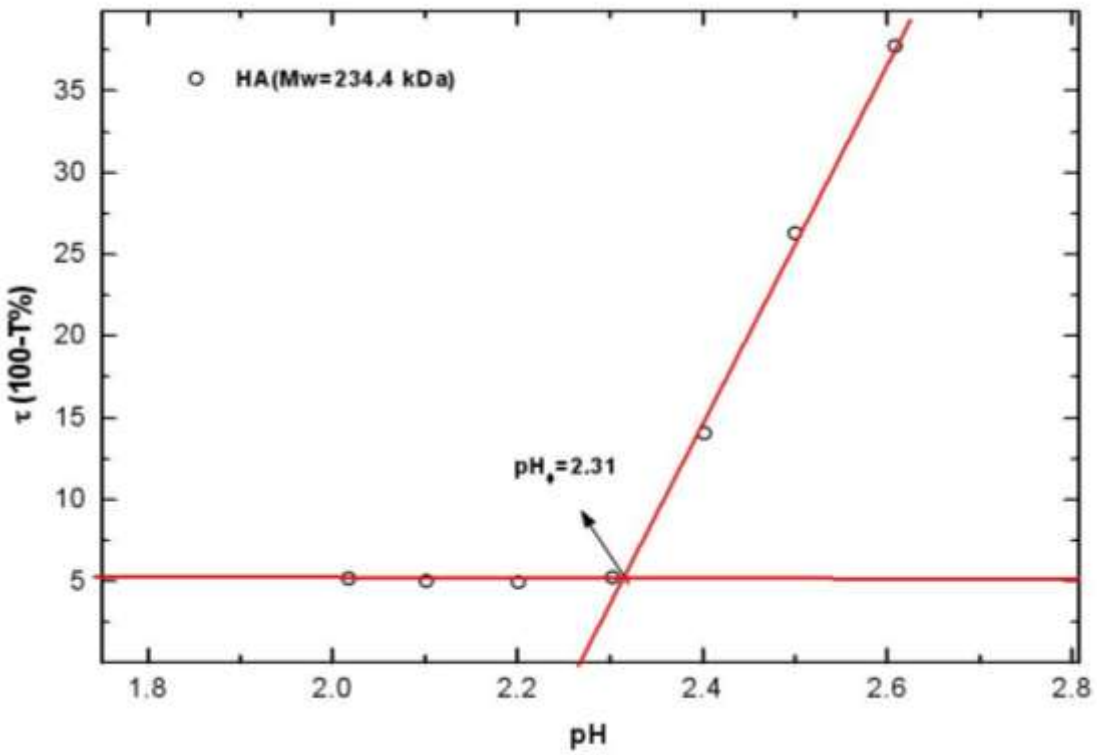

Figure 4.5. Turbidity (τ) vs. pH titration graph for determining pH_{φ} of HA/CHI, Mw_{HA}=234.4 kDa, Mw_{CHI}= 260 kDa.

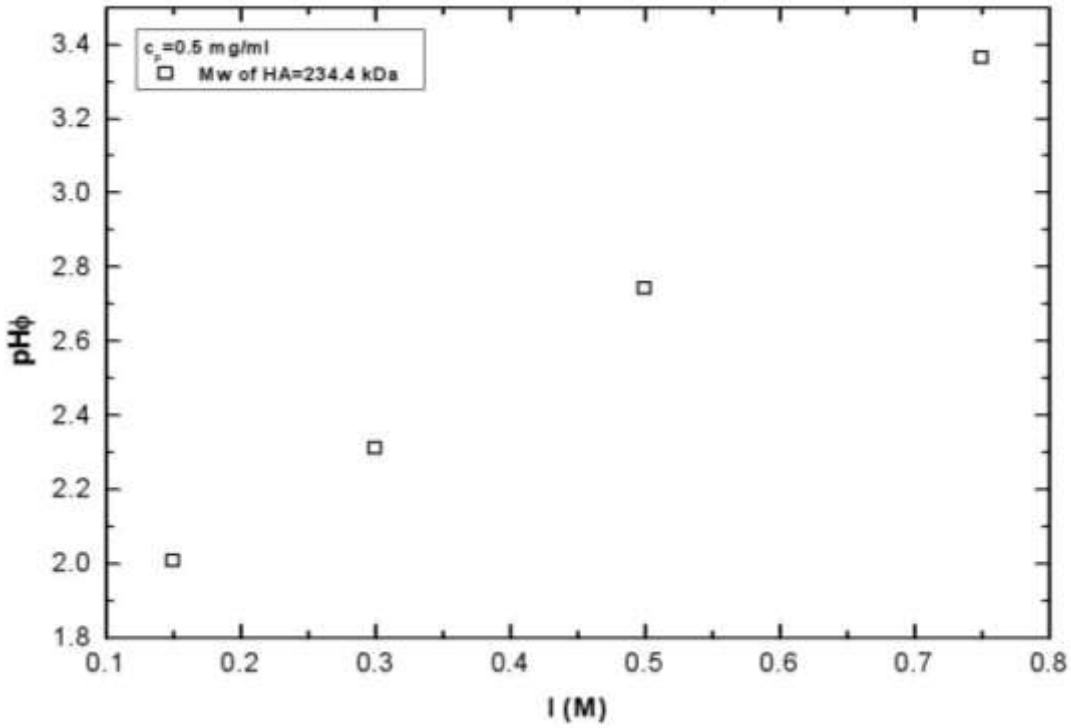

Figure 4.6. pH_{φ} vs. I for HA(234.4 kDa)/CHI(260 kDa), c_p = 0.5 mg/mL .

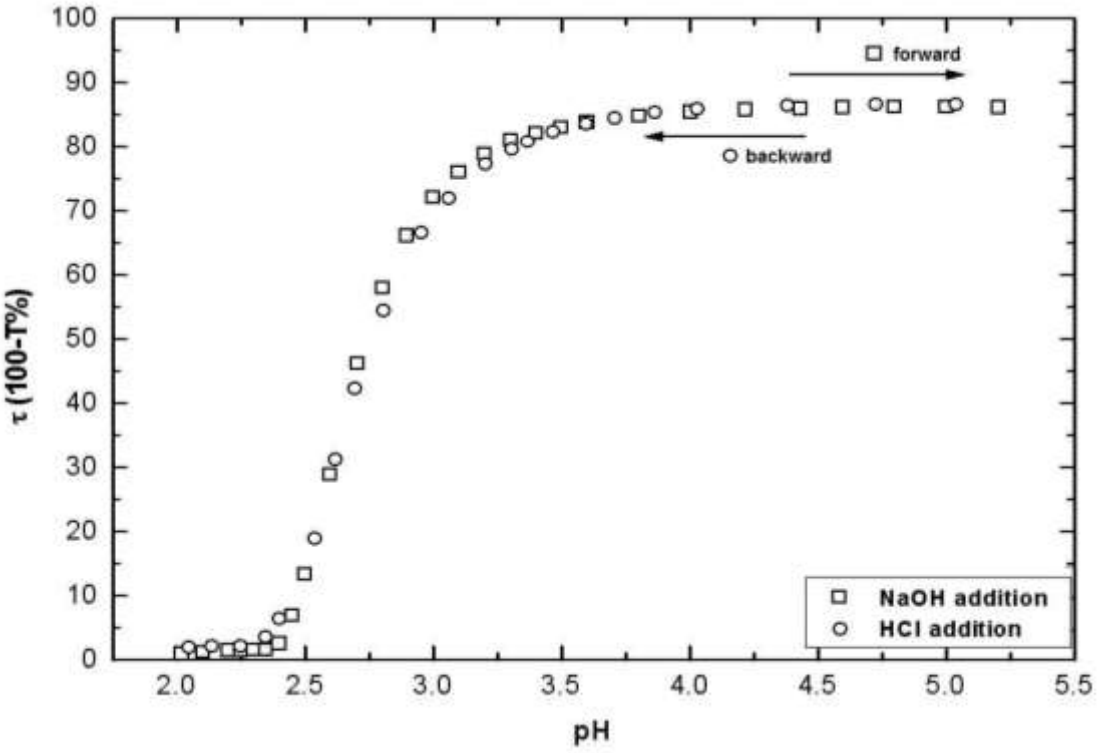

Figure 4.7. Turbidity vs. pH titration graph for HA(150 kDa)/CHI(260 kDa). Total polymer concentration of solution (c_p) = 0.5 mg/ml.

Reversibility of complex coacervation is also investigated with “Type-1” titrations. In these experiments, HA (150 kDa) and CHI (260 kDa) were used. Initially, pH of the mixture was increased with NaOH until pH = 5.21 and then pH of solution was decreased with the addition of HCl until the beginning pH (Figure 4.7) As pH of the solution increases, the appearance of the solution changes from clear to turbid. As pH of solution decreases, appearance of the solution changes from turbid to clear solution at pH < 2.5. Therefore, complex coacervation is reversible as a function of pH.

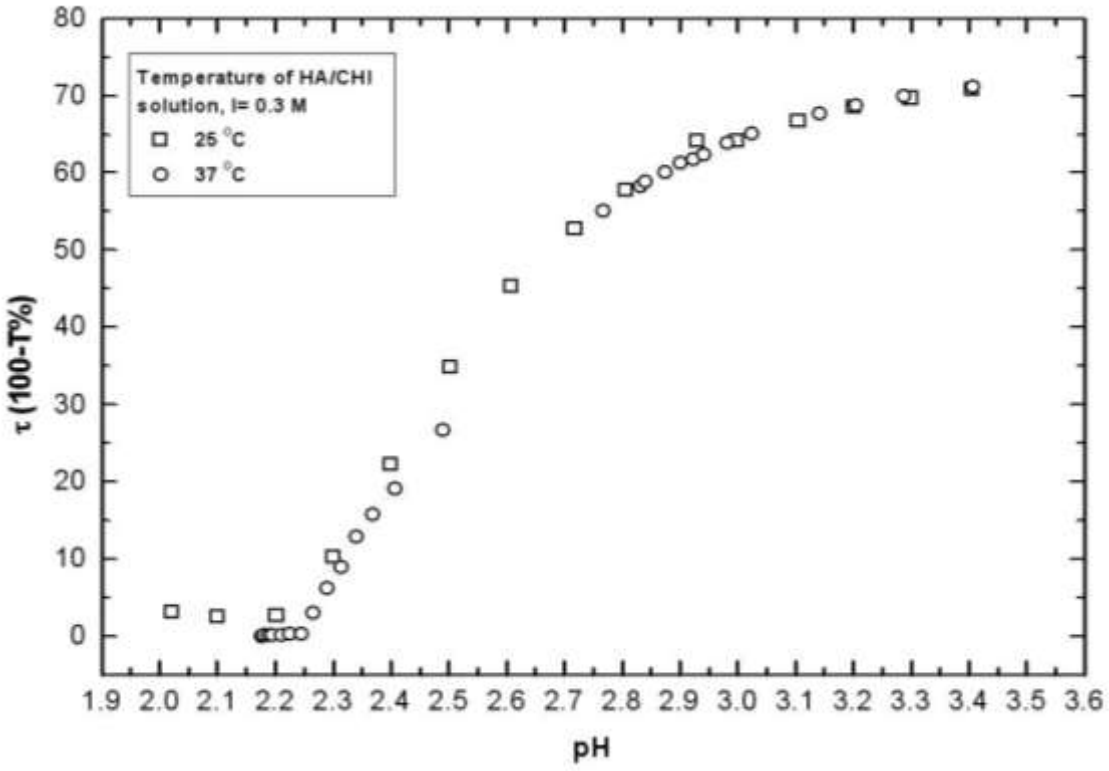

Figure 4.8. Turbidity vs. pH titration graph for HA(234.4 kDa)/CHI(260 kDa) at different temperatures, c_p = 0.5 mg/mL and I = 0.3 M.

Effect of temperature on the phase separation and pH_φ is also investigated because solubility is a temperature-dependent parameter. HA (234.4 kDa) and CHI (260 kDa, 83% DD%) are soluble at both 25±1 ^{0}C and 37±1 ^{0}C . Since 25 ^{0}C is the room temperature and 37 ^{0}C is the body temperature, both temperatures are chosen for the “Type –1“ titration experiments. As seen in Figure 4.8, pH_φ’s at 25°C and 37°C were close to each other; i.e.

2.31 and 2.25, respectively. Hence, an increase in temperature does not have not an important effect on the phase separation. However, additional experiments should be done at a wider temperature range to conclude on the effect of temperature on phase separation of the HA/CHI system.

4.3. Effect of Ionic Strength on Complexation of HA and CHI

Ionic strength is one of the most important influencing factors for complex formation of polyelectrolytes. Figure 4.9 shows the ionic strength dependence of turbidity at pH 3.0, where coacervation was observed upon centrifugation during preliminary measurements. At salt concentrations (I) less than 0.3 ± 0.05 M, turbidity increases with I. This behavior, also observed by Tirrel and his co-workers [76], indicates the "rearrangement of complexes" at low I. Complexes of poly(L-Lysine)/poly(L-Glutamic acid), poly(L-Histidine)/ poly(L-Glutamic acid), poly(L-Ornithine)/ poly(L-Glutamic acid) and poly(L-Lysine)/Poly(L-Aspartic acid) systems also aggregate as coacervates. Tirrel and his co-workers observed that increasing the salt concentration leads to diminishing of PECs and "stoichiometric flocculation". Further increase of I induces dissolution of PECs due to the screening effect of the salt ions. Dautzenberg *et al.* [77] described similar behaviour in their studies with copolymer of PDADMAC and acrylamide/sodium poly(methacrylate).

Critical ionic strength (I_c) is the salt concentration corresponding to the maximum turbidity for turbidity (τ) vs. I plots. As seen in the Figure 4.9, turbidity at different polymer concentrations shows similar behaviors. At pH 3, critical ionic strength of HA/CHI systems is determined as 0.3 ± 0.05 M at different total polymer concentration. At $I<I_c$, turbidity of the solutions increases with salt addition. Increase in turbidity can be evaluated as complex coacervation formation is favored with salt addition. This situation can be explained by using Debye-Huckel parameter, κ which has relationship with ionic strength as shown Equation 4.1 [78] for a univalent salt at 25 ^{0}C where I is in the units of mol/L and κ in nm^{-1}. Debye length (κ^{-1}) is inverse of κ and it has inverse relationship with I as seen in the Figure 4.10.

$$\kappa = 3.281\, I^{1/2} \qquad (4.1)$$

At low ionic strength, Debye length between oppositely charged polyelectrolytes is high. Hence, complex formation of HA and CHI is prevented. As I of the medium is increased, κ^{-1} decreases so complex formation and phase separation increase. At $I > I_c$, small suspended particles were observed in the mixture because complex coacervates aggregate and lead to precipitate formation. Dautzenberg *et al.* [79] attributed this result to the aggregation of polyelectrolyte complexes and named it as flocculation of PECs. Further salt addition leads to appearance of a transparent solution. This result can be explained in two different forms: (i) Further increase of the ionic strength results in redissolution of the complexes due to charge screening of the salt ions. (ii) Formation of a transparent film of HA/CHI system at the bottom of the glass vials leads to observation of the overall solution as a clear solution .

Critical ionic strength (I_c) of HA/CHI system is a pH-related parameter for HA/CHI system as shown in Figure 4.10. Changing the pH of the solutions affects the charge density of HA and CHI due to their weak acid and base behavior, respectively. As mentioned in the potentiometric titration results, degree of deprotonation of HA and CHI changes with the pH. At pH ≤ 4.5, degree of deprotonation of HA increases while number of chargeable units on the polyelectrolyte chain increase. On the other hand, the degree of deprotonation of CHI stays close to 1 at this pH range. Between pH 4 - 4.5, phase separation of HA and CHI is assumed to be much favored, so the required salt concentration for "suppressing the complex coacervation" increases up to pH 4.5. After this pH , I_c of HA/CHI complexation decreases as seen in the Figure 4.9 due to decreasing charge density of CHI.

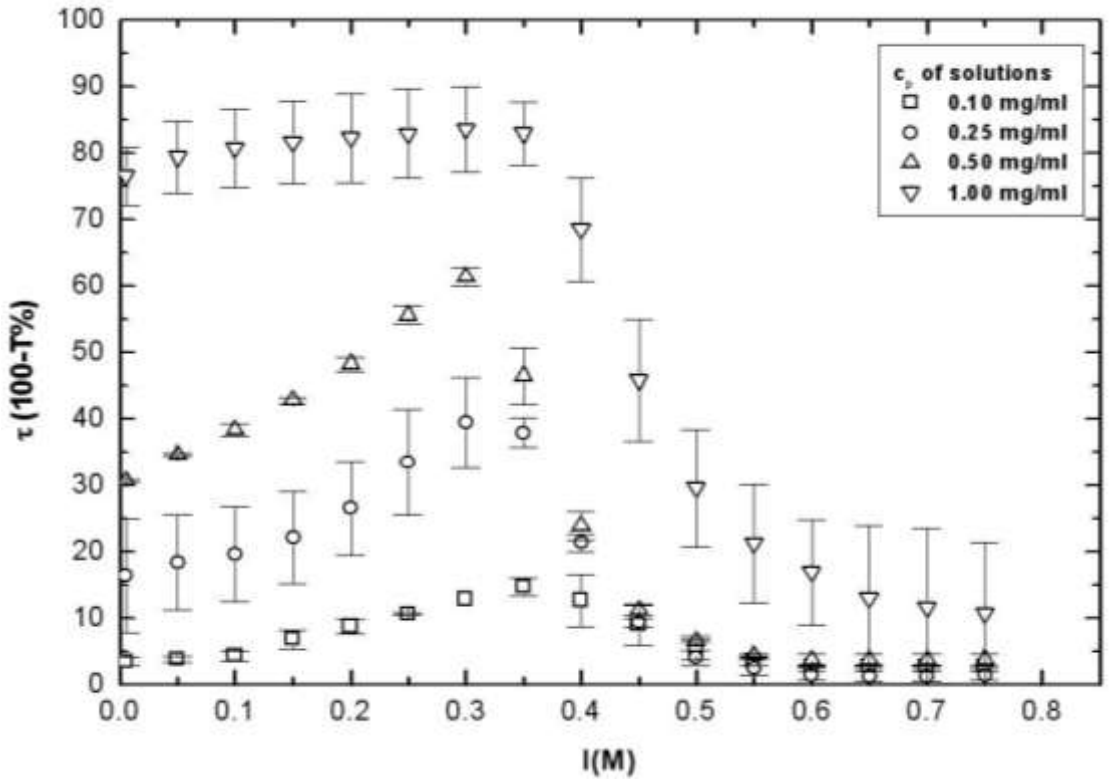

Figure 4.9. Turbidity vs. I (M) at different ionic strength, total polymer concentration (c_p) = 0.5 mg/mL.

Critical ionic strength changes according to the polyelectrolytes chosen. Evan *et al.* [80] has studied the effect of salt concentration on coacervation of polyelectrolyte complexes. For PDMAEMA and PAA, the critical salt concentrations for phase separation were higher than the critical salt concentration of HA and CHI. The monovalent critical salt concentration, $I_{c,}$ is 1.5 M for PDMAEMA/PAA system at pH 6.5. For HA/CHI system, I_c are found as 0.3 M and 0.4 M at pH 3 and pH 4.5, respectively.

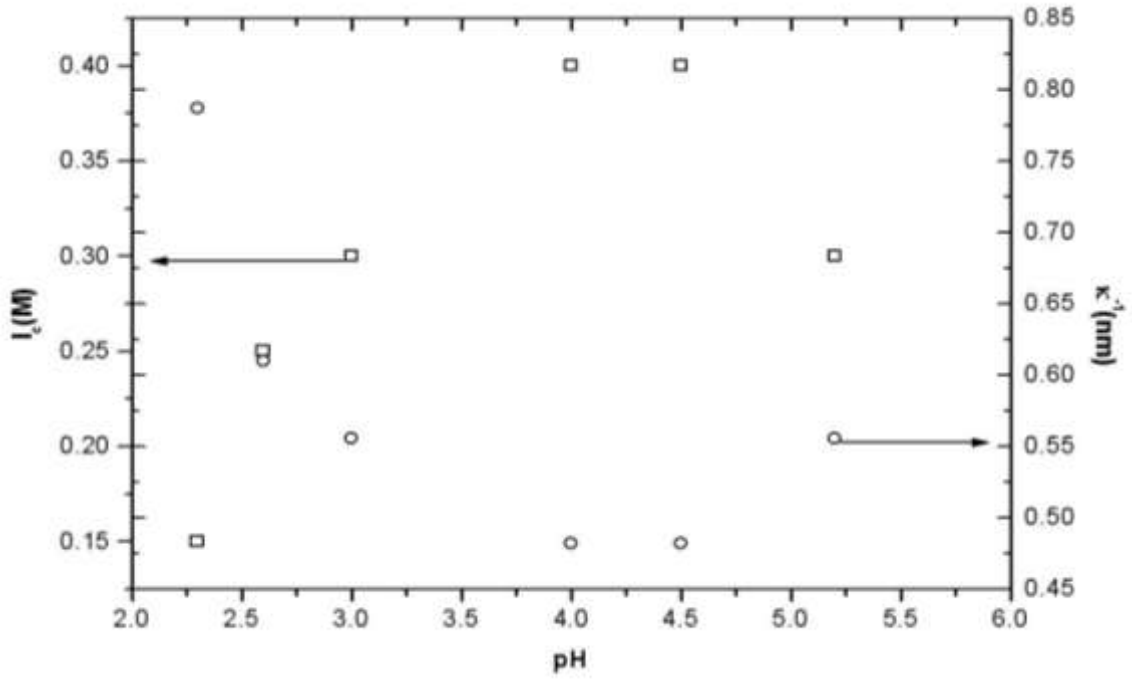

Figure 4.10. Critical ionic strength (I_c) vs. pH titration and Debye length (κ^{-1}) vs. pH titration for HA(234.4 kDa)/CHI(260 kDa) system, c_p = 0.5 mg/mL.

Effects of temperature on the turbidity vs. ionic strength plots have also been examined. Experiments were done at 25 ± 1 ^{0}C and 37 ± 1 ^{0}C and total polymer concentration of solution was 0.5 mg/ml. As seen in the Figure 4.11, similar phase behaviors are notified at both temperatures. I_c of HA/CHI systems at 37 ± 1 ^{0}C is also 0.30±0.05 M. Turbidity of the solution increases at $I \leq I_c$ with salt addition. After critical ionic strength suspended small particles in the solutions were observed and further salt addition leaded to observe clear solution. Therefore, temperature does not have an important effect on the phase separation at this small temperature range. It will be useful to study the HA/CHI system at a wider temperature range to understand the temperature effects on the phase behavior much better.

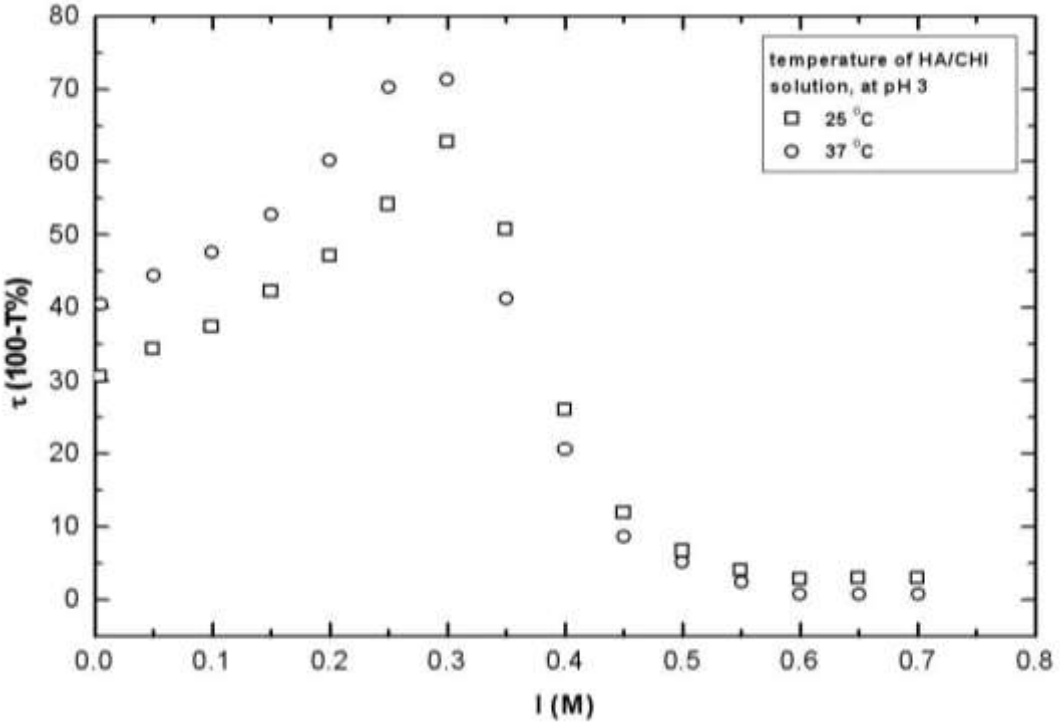

Figure 4.11. Turbidity vs. I (M) at different temperature, total polymer concentration (c_p) = 0.5 mg/mL.

4.4. Effect of Total Polymer Concentration on Complexation of HA and CHI

Effect of the total polymer concentration on the formation of PECs was also investigated and it can be seen in Figure 4.9 As the total polymer concentration (C_p) increases, turbidity increases. Macroscopically, precipitates were observed at C_p = 1.0 mg/mL. On the other hand, no precipitates were observed at $c_p \leq 0.5$ mg/mL, where phase separation leads to coacervation. 0.5 mg/mL is the chosen C_p value for further experiments. C_p > 1.0 mg/mL of solutions were not examined because overlap concentration of HA is about 1.0 mg/mL for Mw > 10^6 kDa [81]. Overlap concentration of CHI is found equal to1.05 mg/mL [82].

4.5. Effect of Chain Length on Complexation of HA and CHI

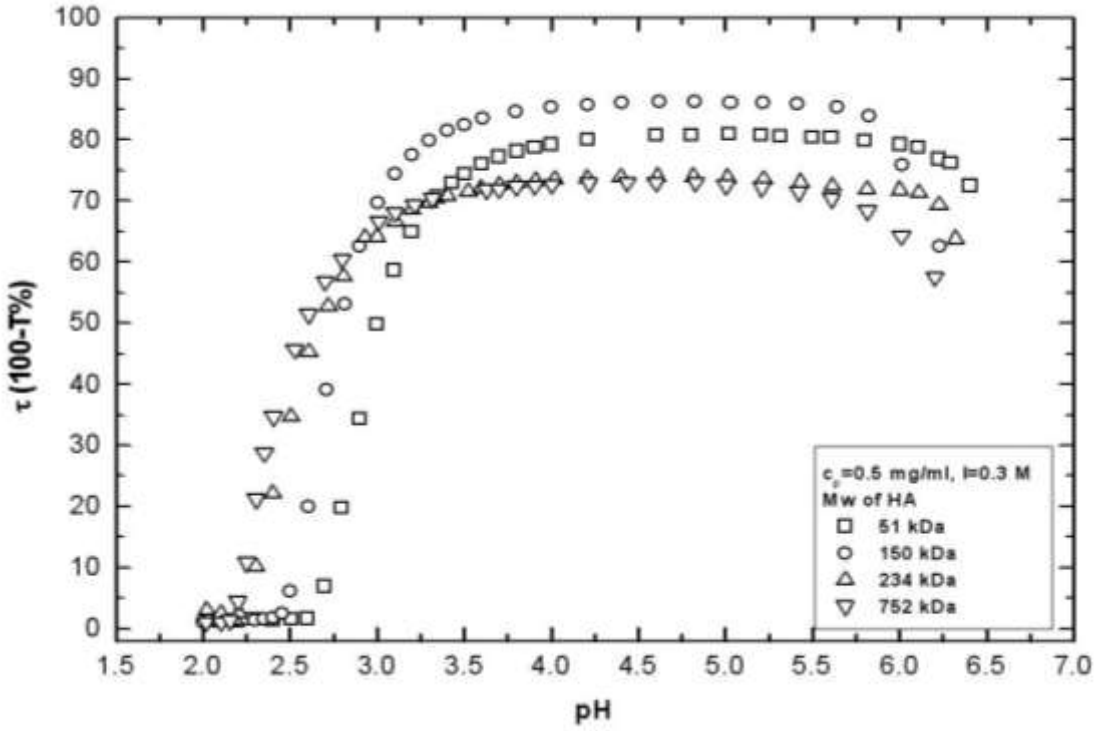

Figure 4.12. Turbidity vs. pH titration graph for HA/CHI. Mw of CHI is constant and Mw of HA samples are different. C_p = 0.5 mg/mL and I = 0.30 M.

Effect of chain length on the complexation of the HA and CHI is investigated by turbidity measurements with HA of different molecular weights. Molecular weight was kept constant for the chitosan. Total polymer concentration of the solutions was 0.5 mg/mL and ionic strength of solutions was 0.30. As seen in Figure 4.12, it is assumed that there is a threshold chain length for pH_φ of HA/CHI complexes. As Mw of HA increases up to 234.4 kDa, pHφ decreases. pH_φ values for HA with 752 kDa and 234.4 kDa are very close to each other. As chain length increases, phase separation increases since the number of the charged units on polyelectrolyte backbone increases.

4.6. Effect of DD% of CHI on Complexation of HA and CHI

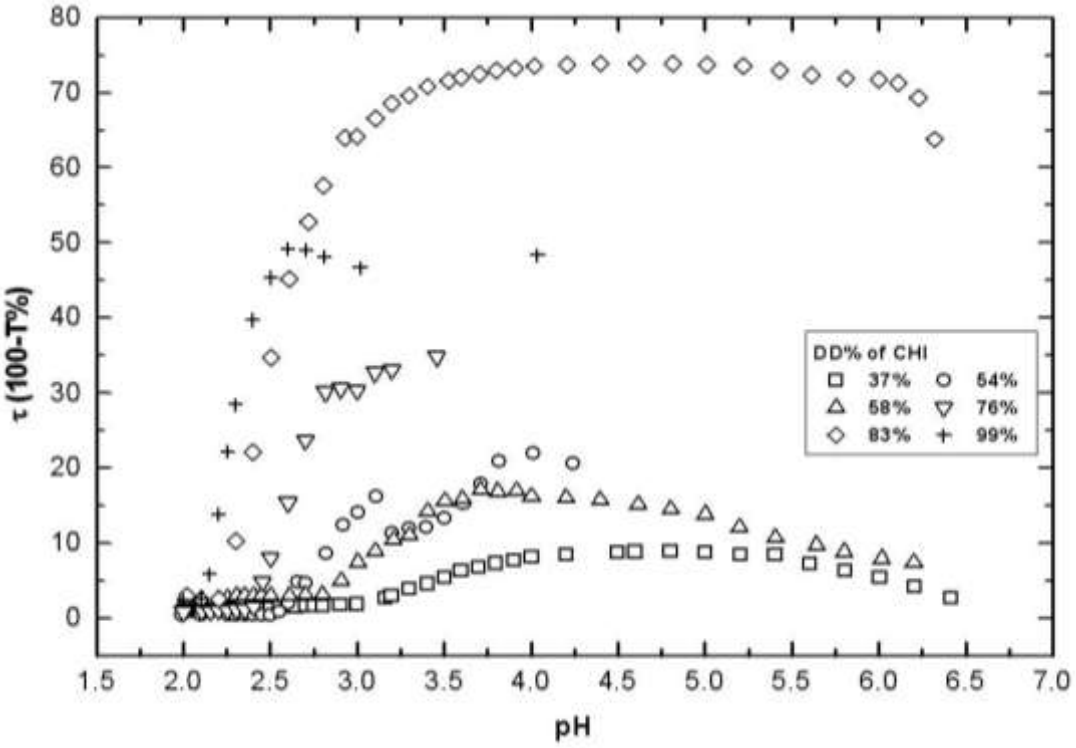

Figure 4.13. Turbidity vs. pH titration graph for HA/CHI systems at different DD% of CHI. C_p = 0.5 mg/mL and I = 0.30 M

Degree of deacetylation (DD%) of chitosan is another important factor for complexation of HA/CHI system. DD% is the percentage of deacetylated units on the whole chitosan polymer chain. As seen clearly in Figure 4.13 and Table 4.2, DD% of CHI changes the pH_φ of the complexes of HA/CHI. In general, as DD % of CHI increases, pHφ decreases. pH_φ of CHI with 54% DD is expected to be higher than pHφ of CHI with 58% DD. However, opposite situation is observed due to the MW difference between the samples. Thus, Mw should be considered when complexation of HA and CHI with similar DD% is considered.

Table 4.2. DD%, Mw and pH values for different chitosan samples

DD % of CHI	Mw of CHI (kDa)	pHφ
37%	332.4	3.06
54 %	365.1	2.54
58 %	123.5	2.80
76 %	345.6	2.39
83 %	260	2.24
99 %	210	2.12

4.7. Effect of Polyacid/Polybase Charge Ratio on the Complexation of HA and CHI

Effect of the charge ratio of HA/CHI (COO^- / NH_3^+) on the complexation and phase behaviour of the HA/CHI system is also investigated. As seen in Figure 4.14 and 4.15 (a-b), τ increases with the charge ratio up to 0.4, where maximum τ is observed. At this charge ratio, only coacervates were observed; i.e. no precipitates or flocculates were observed. The increase in turbidity with charge ratio is expected since the number of available carboxyl groups neutralizing the amine groups is also increasing. For charge ratio of HA/CHI (COO^- / NH_3^+) greater than 0.4, turbidity decreases as charge ratio of HA/CHI (COO^- / NH_3^+) increases and flocculate formation is observed as seen in the Figure 4.11(c-d). Formation of complex coacervates is expected at the charge ratio of HA/CHI (COO^- / NH_3^+) : 1.0, where the number of negatively charged groups at HA and positively charged groups at CHI is equal to each other. However, flocculates and precipitates are observed at this charge ratio. Therefore, it can be concluded that charge neutralization is achieved at lower charge ratios despite the stoichiometry of the charge groups is far from 1:1.

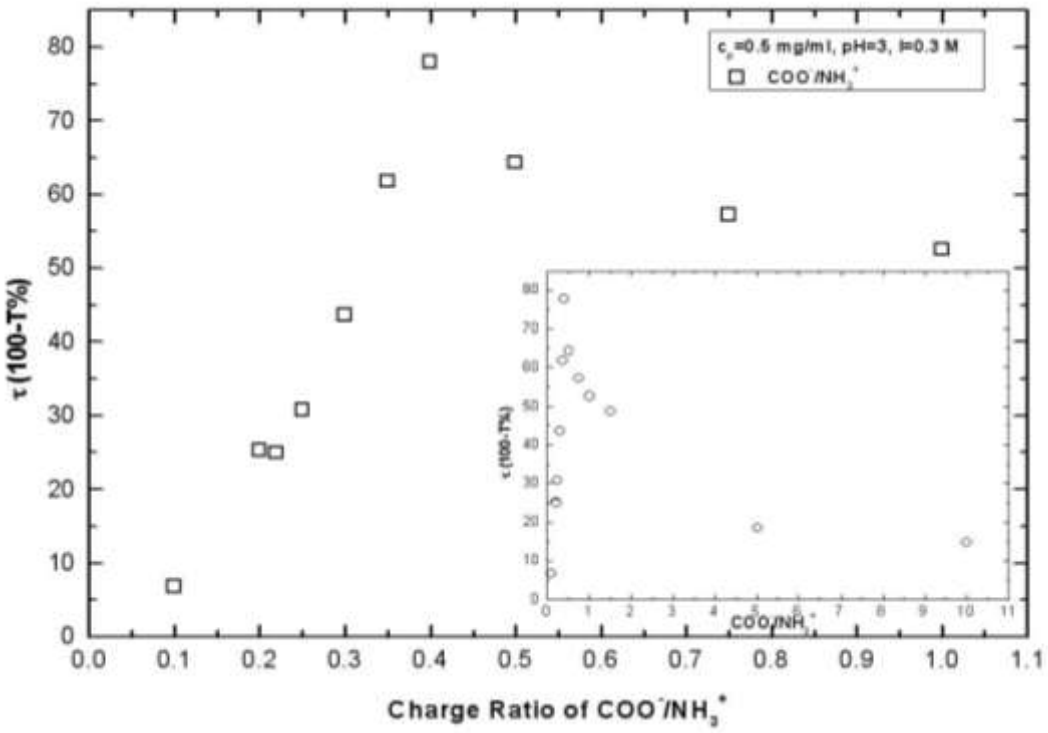

Figure 4.14. Turbidity vs. charge ratio of HA/CHI systems, at pH = 3, c_p = 0.5 mg/mL and I = 0.30 M.

In Figure 4.16 and 4.17, optical light microscopy images for complex coacervates and precipitates are shown for HA/CHI complexes, respectively. During taking the image of complex coacervates, coacervates droplets were moving so fast that images could not be focused. Inside and outside of the red circles in Figure 4.16, very small spheres of coacervates droplets exist. Similar images were taken for PLys/PGlu [76] but their diameters are bigger than coacervates of HA/CHI. Weight ratio for HA/CHI was 1:1 for complex coacervates. At the charge ratio of HA/CHI 0.5, precipitates were observed at the bottom of the vial like Figure 4.17. Optic light microscope images provide us to distinguish between complex coacervates and precipitates.

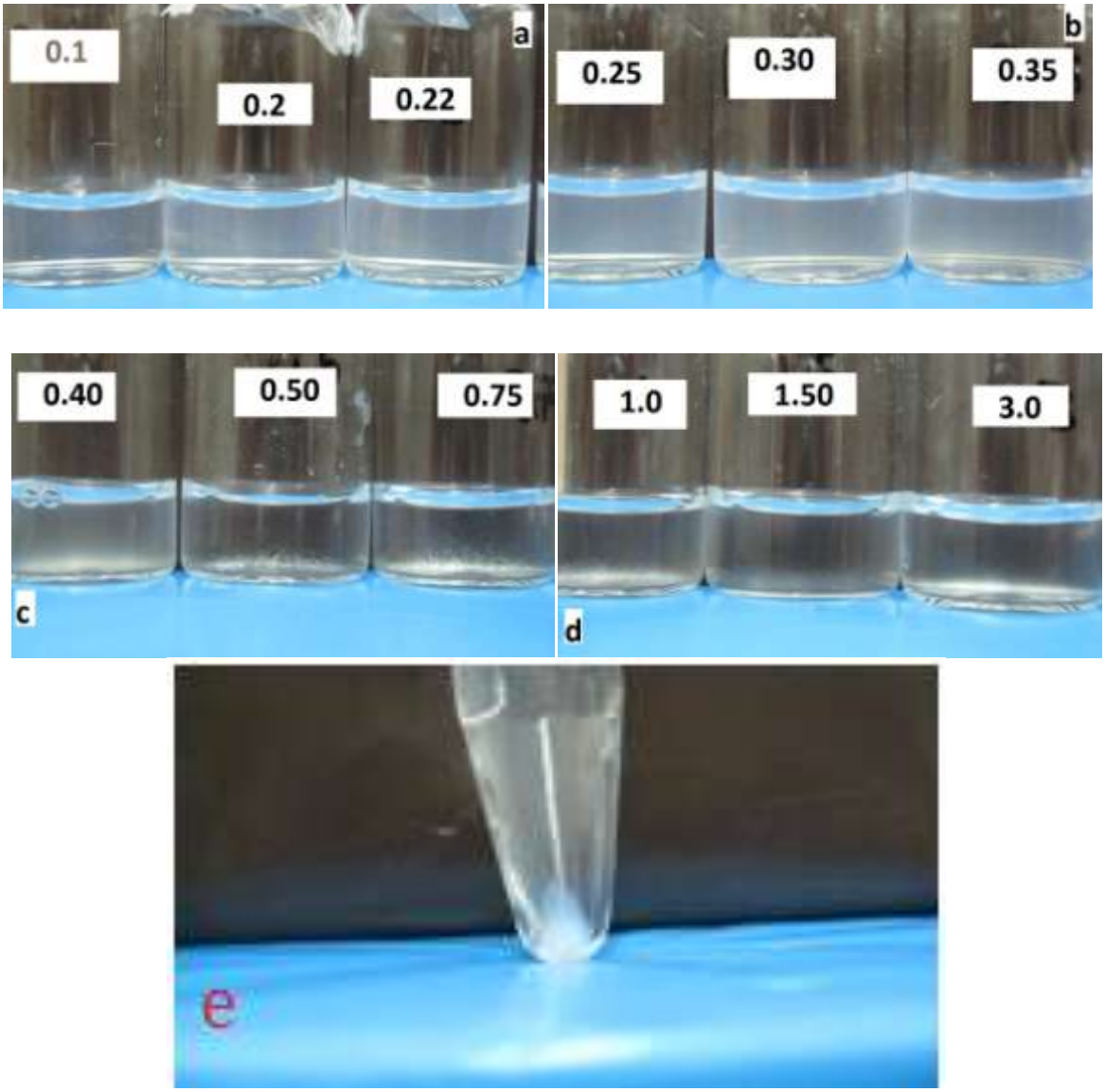

Figure 4.15. HA/CHI samples at different charge ratios of HA/CHI at pH 3.0 and Cp=0.5 mg/mL. a-d: Charge ratio from 0.1 to 3.0 before centrifugation. e: after centrifugation of the sample with charge ratio of 0.22: white coacervate at the bottom of the cell.

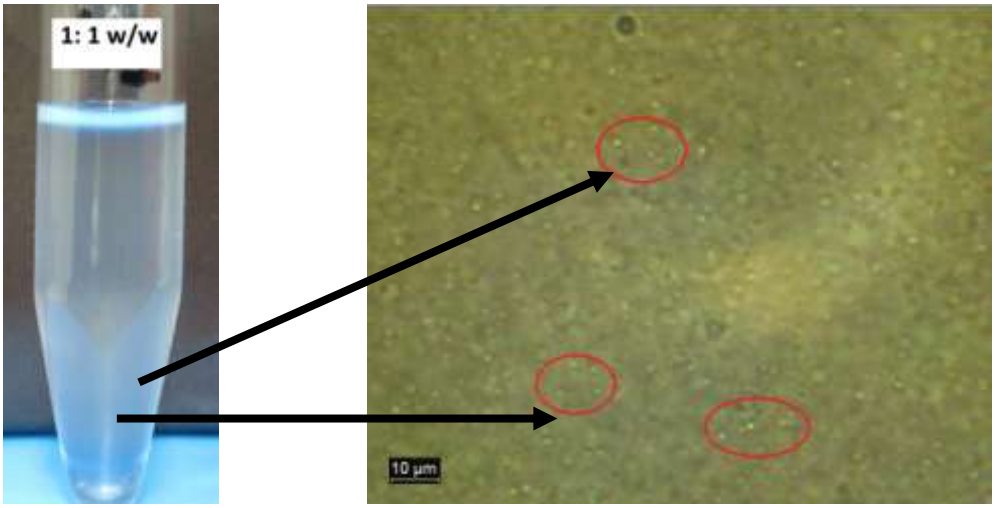

Figure 4.16. Optic Light microscope image complex coacervates of HA and CHI, 1:1 weight ratio. Red circles show complex coacervates.

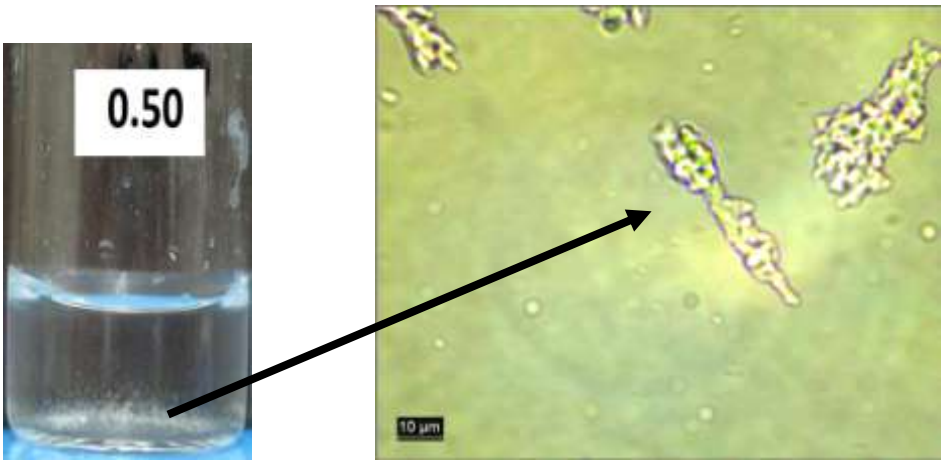

Figure 4.17. Optical Light microscope image for precipitates of HA and CHI, 1:1 weight ratio.

5. CONCLUSIONS AND FUTURE WORK

Complexation and phase behaviors of oppositely charged semi-flexible biopolyelectrolytes, namely hyaluronic acid and chitosan have been investigated. HA/CHI complexes can be in the form of clear solution, complex coacervates, flocculates or precipitates. There are many factors affecting the complexation and phase separation of HA/CHI systems such as pH of the solutions, ionic strength, total polymer concentration, chain length (Mw of polyelectrolytes), degree of deacetylation of CHI, and charge ratio of HA/CHI. All these factors are examined with turbidimetric titrations. In order to understand and distinguish coacervation and precipitation better, optical light microscopy was used.

Phase separation of HA/CHI systems at different conditions can be concluded as below:

- pKa of the HA (234.4 kDa) is 2.82 and pKa of CHI (260 kDa) is 6.39. As chain length increases, pKa values increases.
- Phase separation is favored at pH's between pK_a values of HA and CHI due to increasing numbers of chargeable species on HA and CHI.
- PECs take different forms from coacervates to flocculates as pH increases.
- Complex coacervation is favored for $I < I_c$ due to reaarrangement of the macroions and entropy increase arising from the release of counter ions. After critical I_c, further salt addition leads to redissolution of complexes.
- At body temperature similar phase behaviours were observed in ionic strength changes experiments.
- Increase in total polymer concentration leads to higher amounts of PEC formation.
- As Mw of polyelectrolytes increases, phase separation is favored more.
- As DD% increases, pH_φ decreases, phase separation is favored.
- Charge neutralization for complex coacervation is reached at low charge ratio of HA/CHI (COO^- / NH_3^+) instead of 1:1 charge ratio. As charge ratio of HA/CHI

(COO^{-} / NH_3^{+}) increases, phase separation in the form of flocculation and precipitation were observed.

- Light microscopy images provides us to differentitate between complex coacervates and precipitates.

Possible future works are listed below:

- Effects of temperature on phase separation and complex formation should be investigated at wide temperature range.
- Type -1 titration can be made by taking data at smaller increments to observe the pH_c, which is the onset pH of soluble complexes of the HA/CHI system.
- More optical light microscope images should be taken in order to understand complex coacervates of HA/CHI system much better for different samples at different conditions
- Other characterization techniques such as SEM and fluorescent labeling may be applied to understand complexation and phase behaviors of HA/CHI system.
- Complexation and phase separation of different anionic PE with CHI and different cationic PE with HA can be investigated in order to compare complexation behaviors of HA and CHI.
- Complex coacervates of HA and CHI can be studied in vitro and in vivo conditions.
- We suggest complex coacervates of HA/CHI can be used (i) as scaffold for cell proliferation or (ii) for drug delivery or as gene delivery vector when microencapsulating drug or plasmid DNA.

APPENDIX: CALCULATION OF CHARGE RATIO OF HA/CHI

Equations for charge ratio calculation are given below:

Number of repeat units in 1 mol of HA = $\frac{Mw\ of\ HA\ (\frac{g}{mol})}{Mw\ of\ repeated\ unit\ in\ HA} * N_A \left(\frac{1}{mol}\right)$ (A.1)

Number of repeat units in 1 mol of CHI = $\frac{Mw\ of\ CHI\ (\frac{g}{mol})}{Mw\ of\ repeated\ unit\ in\ CHI} * N_A \left(\frac{1}{mol}\right)$ (A.2)

Number of charged units in 1 mol of HA = $\frac{Mw\ of\ HA\ (\frac{g}{mol})}{Mw\ of\ repeated\ unit\ in\ HA} * N_A \left(\frac{1}{mol}\right) * \alpha_{HA}$ (A.3)

Number of charged units in 1 mol of CHI = $\frac{Mw\ of\ CHI\ (\frac{g}{mol})}{Mw\ of\ repeated\ unit\ in\ CHI} * N_A \left(\frac{1}{mol}\right) * \beta_{CHI} * DD_{CHI}$ (A.4)

Calculation of the number of charged units in 1 mol of HA and CHI is given above. The number of moles of HA and CHI should be known to calculate the charge ratio of HA/CHI.

For the calculations, the total number of charge is assumed to be equal to 1.

$$X_{COO^-} + X_{NH^+_3} = 1 \quad \text{(A.5)}$$

$$X_{NH_3^+} = 1 - X_{COO^-} \quad \text{(A.6)}$$

where X_{COO-} is total number of COO^- charges in the mixture, and X_{NH3+} is the total number of NH_3^+ charges in the mixture.

$$X_{COO^-} = n_{HA}\ (\text{mol})^* \text{ Number of charged units in 1 mol of HA } (\text{mol}^{-1}) \quad \text{(A.7)}$$

$$X_{NH_3^+} = n_{NH_3^+}\ (\text{mol})^* \text{ number of charged units in 1 mol of CHI } (\text{mol}^{-1}) \quad \text{(A.8)}$$

$$\frac{X_{COO^-}}{1 - X_{COO^-}} = \frac{\text{nHA (mol)} * \text{number of charged units in 1 mol of HA } (\text{mol}^{-1})}{n_{CHI} * (mol)\ \text{number of charged units in 1 mol of CHI (mol}-1)} \quad \text{(A.9)}$$

n_{CHI} is derived from Equation (A.9):

$$n_{CHI} = \frac{n_{HA}\ (\text{mol}) * \text{number of charged units in 1 mol of HA } (\text{mol}^{-1}) * (1 - X_{COO-})}{X_{COO-} * \text{number of charged units in 1 mol of CHI (mol}-1)} \quad \text{(A.10)}$$

$p = \frac{\text{number of charged units in 1 mol of HA } (\text{mol}^{-1}) * (1 - X_{COO-})}{X_{COO-} * \text{number of charged units in 1 mol of CHI (mol}-1)}$ as derived from Equation A.10.

$$n_{HA} * Mw_{HA} + n_{CHI} * Mw_{CHI} = \text{total mass} \quad \text{(A.11)}$$

$$n_{HA} * Mw_{HA} + n_{HA} * p * Mw_{CHI} = \text{total mass} \quad \text{(A.12)}$$

let's derive the n_{HA} from Equation (A.12):

$$n_{HA} = \frac{Total\ mass}{MwHA + (p * MwCHI)} \quad \text{(A.13)}$$

In summary, the following quantities can be calculated: (i) the charge ratio of HA/CHI, (b) number of moles of HA and CHI in the solution, and (iii) volume that should be taken from each of the HA and CHI solutions. A sample calculation is given below:

Total polymer concentration (c_p) of the HA/CHI mixture is 0.5 mg/mL while total volume of the mixture is 5 mL. The volumes of 0.5 mg/mL HA and 0.5 mg/mL CHI solutions needed to obtain a charge ratio of HA/CHI of 0.25 at pH 3.0 are calculated as follows: (Mw_{HA} = 234.4 kDa, Mw_{CHI} = 260 kDa, DD of CHI = 0.83, α_{HA} = 0.602, β_{CHI} = 1 at pH 3.0)

Total mass of solute = 0.5 mg/mL * 5 mL = 2.5 mg = 0.0025 g

Number of charged units in 1 mol of HA = $\frac{234400(\frac{g}{mol})}{401.3\ g/mol} * N_A\left(\frac{1}{mol}\right) * 0.602$ = 351.629*N_A

Number of charged units in 1 mol of CHI = $\frac{260000\ (\frac{g}{mol})}{196.808\ g/mol} * N_A\left(\frac{1}{mol}\right) * 1 * 0.83$ =1096.50* N_A

$$\frac{X_{COO^-}}{1-X_{COO^-}} = 0.25 \rightarrow \quad X_{COO^-} = 0.2$$

Let's calculate p = $\frac{351.629\ (\mathrm{mol}^{-1}) * \mathrm{N_A} * (0.8)}{0.2 * 1096.50\ (\mathrm{mol}-1) * \mathrm{N_A}}$ = 1.283

n_{HA} = $\frac{0.0025\ g}{234400\ (\mathrm{g/mol}) + (1.283 * 260000\ (\frac{\mathrm{g}}{\mathrm{mol}}))}$ = 4.402*10^{-9} mol

n_{CHI} = 4.402*10^{-9} mol * 1.283 = 5.647*10^{-9} mol so

M_{HA} in solution = 4.402*10^{-9} mol * 234400 (g/mol) = 1.032*10^{-3} g = 1.032 mg

M_{CHI} in solution = 5.647*10^{-9} mol * 260000 (g/mol) = 1.468*10^{-3} g = 1.468 mg

V_{HA} in solution = $\frac{1.032\ mg}{0.5\ mg/mL}$ = 2.064 mL

V_{CHI} in solution = $\frac{1.468\ mg}{0.5\ mg/mL}$ = 2. 936 mL

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