Thermodynamics of Biomacromolecular Interactions in Aqueous Solutions

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Dorota Roberts

School of Chemical Engineering and Analytical Science
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ABSTRACT

An understanding of the interactions between polyelectrolytes and proteins is vital to determine structure and functionality of materials constructed of these two components. Possible applications for the protein-polyelectrolyte composites are ranging from materials used to deliver drugs to the methods of protein stabilisation for storage of therapeutics, biosensors, and encapsulation of medicines for triggered release. The binding of globular proteins to the polyelectrolyte chains can prevent undesired protein aggregation and may help to extend the shelf-life of the protein-containing food. The aim of this project is to study the mechanism of non-covalent binding between proteins and polyelectrolytes, responsiveness of the protein-polyelectrolyte composites to external stimuli such as changing pH, presence of salt of different types and concentrations or influence of enzyme on the integrity of protein-polyelectrolyte multilayer film.

Our study was focused on the effects of different mono- and multivalent salts on binding affinity between a negatively charged polyelectrolyte - poly(styrene sulfonate) PSS and bovine serum albumin BSA or myoglobin. The complex formation between these polymers was examined using the static light scattering (SLS), turbidimetric and potentiometric titrations, differential scanning calorimetry (DSC) and theoretical studies based on molecular dynamics simulations. We established that the inter- and intramolecular interactions between proteins and polyelectrolytes are primarily driven by the electrostatic forces at the conditions when the polymers are in low ionic strength solutions and attractive or repulsive relations are based upon the charge density and its distribution. When proteins are interacting with polyelectrolytes in solutions of high ionic strength the electrostatic interactions are screened by the salt originated co-ions. In these conditions there is a competition between salting-out effect on proteins leading to protein aggregation or protein-polyelectrolyte complex formation, which can prevent undesired protein-protein association. The forces driving the attractive interactions at high ionic strength are of non-electrostatic origin, these are mainly hydrophobic forces. The computer simulation study shows that more flexible polyanionic chains are stronger binders to the positive patches on protein surface than these of a more rigid backbone. Also a total energy of binding depends on a sum of electrostatic and non-electrostatic energies.

The formation of multilayers composed of a protein and a polyelectrolyte, where components were: poly-L-lysine – a positively charged homopolypeptide and polygalacturonic acid - a polysaccharide was examined using a quartz crystal microbalance with dissipation monitoring. A 10 and 11 layer film, deposited on the charged surface, exhibited the linear growth pattern for first 5 layers and exponential growth for a flowing 5 (or 6) layers. The influence of pectinase enzyme on digesting the polygalacturonic acid component of the multilayer was most effective for 1 AU/mL concentration of pectinase. After the enzyme was applied the multilayer film was fully disintegrated within the period of 20 minutes for pectinase at 1 AU/mL and the time of disintegration was extended to 120 minutes for pectinase at 0.1 AU/mL.

Silk fibroin aqueous solutions were tested rheologically for their structural properties involving the existence of fibroin aggregates. We examined the process of ageing of fibroin solutions and solid-liquid transformations taking place within the fluid. The transitions between viscous and elastic behaviour of the fibroin’s semi-dilute solutions were initiated by strain, shear frequency and temperature. We highlighted that the irreversible change in secondary structure of the silk fibroin in aqueous solutions are taking place after the 48 hour period of time since the preparation of protein fluids. We recommend that further processing of silk fibroin such as electrospinning should be completed within the 48 hour after dissolution.
DECLARATION

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Signature: ............................................................
Name of author: Dorota Roberts
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CHAPTER 1

INTRODUCTION

The behaviour of protein-polyelectrolyte mixtures in aqueous salt solutions has been studied intensively over the last few decades. Recent scientific interest in protein-polyelectrolyte materials is driven by an underlying need for improvement in human health and comfort and also for human therapeutic requirements. Research on protein-polyelectrolyte structure is most beneficial for pharmaceutical applications, for example responsive delivery systems for protein therapeutics, enzyme immobilisation in biosensors. Researchers are focused on nano-scaled to submicroscopic structures with controllable and repeatable properties that respond to environmental conditions. These materials known as multilayer thin films are classified as nano-composites. Polyelectrolyte multilayers are attractive to use for a wide range of applications. For example, composites produced by the multilayer deposition of oppositely charged macromolecules can be used as materials for food processing and preservation; for extending the shelf-life of medications; in pharmaceutical slow-release products; and in the stabilisation and storage of proteins such as insulin.

Polyelectrolyte multilayer films were introduced in the early 1990’s (Decher 1991) as thin structures of consecutive polyanions and polycations organized in a multilayer structure. The weak physical bonding of each pair of oppositely charged groups is multiplied by a large number of binding points creating a strong connection between layers. Multilayer properties can be modified to meet the needs of specific requirements. Knowing the response of the multilayer composite to the external stimuli can help in designing responsive materials.
Polyelectrolyte multilayers are non-rigid thin films with tunable properties such as permeability, wall thickness or integrity, which can be adjusted by altering the solution pH, salt concentration or temperature etc. The responsiveness of the multilayer depends on the properties of its components, such as the polyelectrolyte stiffness, charge density, or if the component is a protein, on the charge distribution and the native protein conformation. The binding mechanism between the components of a multilayer film can provide insight into multilayer structural and functional properties. A multilayer composite can be engineered by either allowing its self-assembly until equilibrium is achieved or by applying controlled-assembly of multilayer components, where equilibrium is prevented by kinetic interference.

Much research on polyelectrolyte multilayers has involved two synthetic polyelectrolyte chains carrying opposite charges of the same or various charge densities (Glinel 2007, v. Klitzing 2004, Sukhishvilli 2005). These systems are now well established and described in recent literature (Decher 2003). Nevertheless, polyelectrolyte multilayer behaviour is not fully understood, especially in more complicated systems, such as where polymers are collapsed or for those systems composed of macroions containing acidic and basic groups in which case small changes of pH can trigger electrosatically driven binding and complex formation. These more complex arrangements are illustrated in interactions between polyelectrolytes and proteins.

The focus of this work is to determine and understand the factors that control protein-polyelectrolyte binding. In this case, the size of the protein and its native structure determine the stoichiometry of the macromolecular interactions. The distribution of cationic and anionic groups, on protein surfaces leads to charged patches, which control repulsive or attractive interactions with polyelectrolytes.

We applied various techniques of material characterisation to provide an in-depth study of polyelectrolyte – protein interactions. Static light scattering (SLS) experiments were used to characterize the self-interactions between proteins or between polyions, the latter provides insight into polyion conformation. In addition SLS was used to measure the sizes of small soluble protein-polyion complexes. Turbidimetric titrations were carried out to monitor formation of large complexes and phase separation as a function of pH, ionic strength and time. Potentiometric titrations were used to determine acid dissociation constants ($pK_a$) of protein groups in the presence and absence of polyelectrolytes, which gave additional insight into the protein-polyion binding mechanism. Further on, we demonstrated and characterised the process of multilayer formation. A silica crystal was used as a charged substrate in the quartz crystal microbalance with dissipation, which is used to monitor rheological properties of the thin films which are then related to multilayer growth and thickness.

To aid our understanding of protein-polyion binding mechanisms, we employed a computational study of one protein and one polyelectrolyte simulated using the molecular dynamics simulation software GROMACS (GROningen MACHine for Chemical Simulations). The model system composed of a polyelectrolyte chain, represented by a necklace of charged hard spheres, and a fully atomistic illustration of a protein where the electrostatic potential around the protein was controlled by changing the pH and ionic strength. This work complements the experimental
studies of polyelectrolyte – protein interactions. The computer simulations gave us insight into the binding mechanism and effects of polyelectrolyte properties such as stiffness, charge density and protein properties such as charge distribution. We applied Lennard-Jones potential and a distance dependant dielectric to monitor short and long-range electrostatic interactions and represent solute - solvent conditions. Within this model, we investigated the effects of polion charge density and stiffness on the binding strength and mechanism.

A final part of this thesis also includes a brief study of a semi-dilute silk fibroin aqueous solution in which we examined secondary and tertiary structure transitions in the protein under applied shear, changing temperature, ageing of the solution and shear frequency. The main objective was to understand the triggers of the silk fibroin self-aggregation process, leading to the formation of larger agglomerates and eventually liquid crystal separation. This knowledge could be used to establish suitable conditions for the process of electrospinning of silk fibroin nano-fibres for tissue engineering.

1.1. Thesis outline and research objectives

This research project was carried out to investigate the interactions between proteins and polyelectrolytes in aqueous salt solutions. Understanding the intra and intermolecular interactions is a key aspect in controlling the behaviour of interest in protein-polyelectrolyte systems. This research project used analytical techniques for macromolecular solution characterization and theoretical simulations based on molecular dynamics. The key objectives of the work are outlined below.

The thesis is composed of 7 chapters in which we examine the behaviour of protein-polyelectrolyte or protein only solutions and the origin of inter and intramolecular interactions. Chapter 2 is a review covering interactions between proteins and polyelectrolytes in aqueous solutions. The initial part of the chapter provides an introduction to the behaviour of charged polymers in aqueous solutions of dilute regime. We then provide a literature review on what is known about the protein-polyelectrolyte complex formation and various factors affecting this process. Next, we describe protein structure and mechanisms of their self-aggregations on supramolecular scale followed by a section on specific ion (Hofmeister) effects in colloidal systems. To conclude chapter 2, we explain the foundations of nano-composite material fabrication based on protein-polyelectrolyte interactions. The nano-composite is constructed using a technique of layer-by-layer assembly, where a final product is a multilayer thin film – a structure which is highly sensitive to the external stimuli such as pH, temperature or salt concentration in surrounding fluid.

In chapter 3, we study protein-polyelectrolyte complex formation using a combination of experimental approaches including turbidity, static light scattering and potentiometric titrations. The measurements are done as a function of pH, ionic strength, and salt type to provide
insights into the electrostatic interactions. Particular emphasis is placed on measuring weak protein-polyion interactions, which leads to the formation of small complexes, detected here by using either static light scattering or potentiometric titrations. Most previous studies have characterised interactions in terms of turbidimetric titrations, which can only be used to detect the formation of large complexes that probably occur due to inter-complex interactions. These might not be representative of the interactions between proteins and polions. In this chapter, we establish the link between turbidimetric titrations and formation of soluble complexes in aqueous solutions containing poly(styrene sulfonate) PSS and bovine serum albumin BSA – a large globular protein. In chapter 3 we also report our findings for complex formation between PSS and myoglobin, a protein that undergoes a pH dependent aggregation in solutions near to its isoelectric point at low ionic strength.

Chapter 4 contains the molecular dynamics simulation results investigating protein-polyelectrolyte complex formation. The focus of this chapter lays in exploring the mechanism behind polyion and protein binding on a one-to-one basis. Here we examined binding affinities for a range of solution conditions corresponding to changes of pH and ionic strength. Additionally we tested the effects of polyion chain stiffness on binding to the protein. The model was composed of one all-atomistic representation of human serum albumin (HSA) and a coarse-grained model for the negatively charged polyion. The simulations of a one-to-one macromolecule give us an insight into the preferable binding conditions for polyelectrolyte and protein. We target binding patches on the solvent accessible surface area of the protein, for their ability to sustain physical connection with polion chains of various flexibilities. The purpose of the theoretical part of this work is to support experimental research and our understanding of different aspects of binding mechanism at the initial level of intermolecular interactions.

In chapter 5, we provide findings for the formation of multilayer films constructed using poly-L-lysine (homopolymer) and polygalacturonic acid (PGA), the character of the multilayer growth and the dynamics of the film disintegration by enzymatic catalysis. The work presented in this chapter was published as a part of scientific journal article in Carbohydrate Polymers volume 84 (2011) pages 960–969, the title of the article was: ‘Enzymatic degradation of poly-L-lysine-polygalacturonic acid multilayers’ and the authors were: Marta Westwood (Institute of Food Research), Dorota Roberts (School of Chemical Engineering and Analytical Science) and Roger Parker (Institute of Food Research). We aim to exploit organised structures such as multilayer films to explore the possibilities of improvement in process control of stabilising proteins, by incorporating them into a composite. Knowing the response of a protein-polyelectrolyte multilayer to pH, resistance for enzyme digestion, ionic strength, type of salt used as agent screening long-range electrostatic would aid in creating composites such as optical and electrochemical materials, biomedical devices, surface modification, biosensors, dye capsules and photoreaction inhibitors. Research on protein-polyelectrolyte structure is most beneficial for pharmaceutical applications, for example responsive delivery systems for protein
therapeutics, enzyme immobilization in biosensors. Knowing the response of the multilayer composite to the external stimuli can help in designing these medical advances.

In chapter 6, we examine the effects of arginine on protein – protein interactions and on protein aggregation and its inhibition. Here we study the following proteins: myoglobin – a single chain and medium size protein containing a heme group, insulin – a small spherical hormone protein composed of two polypeptide chains and poly-L-arginine – a homopolypeptide able to penetrate cell walls and enhance the delivery of drugs. An insight into protein aggregation was motivated by needs of understanding protein-protein complex formation and methods for inhibition of unwanted self-assembly, which is a common issue in bioprocessing. Proteins contain many different aminoacid groups and are likely to develop both – positive and negative – charges within one macromolecule, causing attractive electrostatic interactions towards other macromolecules of the same kind. Therefore, we test ways of prevention that spontaneous self-assembly though introducing polyelectrolyte stabiliser preserving proteins in controlled agglomerations, which are easy to disassemble through external stimuli.

Finally, chapter 7 contains experimental research on the dynamic properties of silk fibroin, a large elongated block copolymer protein extracted from silk fibre. We investigated the rheological properties and the ageing of fibroin aqueous solutions to establish suitable conditions for further processing of the silk fibroin involving fabrication of scaffold biomaterial for tissue engineering.
Understanding the relationship between physico-chemical properties of proteins and their ability to form complexes with synthetic polyelectrolytes through non-covalent bonds is essential for food and pharmaceutical industries. In this work, we are focused on the examination of the mechanism of complex formation between proteins and polyelectrolytes for application in therapeutics, protein stabilisation, food preservation and extending its shelf-life. We consider synthetic polyelectrolytes and those of a natural origin with elongated geometry, the effect of small ions on polyelectrolytes and polyampholytes - which are mainly proteins of their native spherical geometry. An important factor in these systems are; the electrostatic interactions between charged groups and charged patches on protein surfaces, dependence on the polyion properties such as charge density, stiffness, chemical structure, tacticity, hydrophobicity. These features of local and global structure of the protein-polyelectrolyte complexes are still poorly understood.

Recent and current research on protein-polyelectrolyte interactions, the mechanism of complex formation between proteins and polyelectrolytes and methods to influence the structure of such associates is reviewed in this chapter.

In the first part of this chapter, we present a brief introduction to the behaviour of polyelectrolytes in aqueous solutions. Next, we describe the theoretical and experimental studies on protein-polyelectrolyte interactions leading to self-assembly of these particles. This is followed by the description of protein structure and their ability to interact with charged particles of low molecular weight. After that, we review the specific ion effects of mono- and multivalent salt on macromolecules in aqueous solutions. Finally, we explore the methods of controlled self-assembly of the protein-polyelectrolyte multilayer thin films. The formation of multilayer composites was established only recently and it is a subject of great interest in many areas of scientific research.
2.1. Polyelectrolyte interactions in dilute and semi-dilute solutions

Polyelectrolytes are polymers or copolymers, which carry inoizable groups and become charged when releasing small ions, which often are called counterions, into the surrounding solvent. Polyelectrolyte behaviour has a complex dependence on the distribution of the charge carried along the chain or the chemical composition, and to a lesser extent the molecular weight, geometry or taciticy of the macromolecule. In the case of non-charged polymers, molecular weight, length or network of polymeric macromolecule are considered to have a major influence on the behaviour of a neutral polymer in solution. The existence of any charges in the bulk solution surrounding the macromolecule play a much smaller role in determining the physical properties of the such polymers.

The difference between dilute and semi-dilute polyelectrolyte solution properties is determined by the strength of inter- and intramolecular interactions. In a semi-dilute regime, the chains overlap and intermolecular interactions play a major role in influencing the fluid properties. In dilute solutions, polyelectrolyte chains are dispersed and intramolecular forces control the solution behaviour. The crossover between dilute and semi-dilute solutions occurs at much lower concentrations of the polyelectrolyte than that in neutral polymeric solutions. The transition between the regimes can be observed through the relationship between the viscosity of the solution and polyion concentration.

2.1.1. Charged macromolecules in infinite dilution

For a charged macromolecule at infinite dilution, the interactions involve only one macroion and the co-ions present in the bulk solution. In the majority of the theoretical approaches to modelling polyelectrolyte solutions, the model usually ignores the atomistic representation of polymer and the solvent by replacing the charged groups or monomers of polymeric chain with a necklace of spheres carrying assigned charges, either positive (polycation) or negative (polyanion), respectively. The presence of solvent is replaced by a continuum usually by using a fixed dielectric constant. A total potential energy of the polyion is a sum of potentials of covalent and non-covalent interactions, where chemical bonding is given by harmonic potential of a defined monomer bond length. For non-chemical bonding the energies defining the interactions between macromolecules are: a Yukawa interaction for the screened Coulomb potential between charged monomers, and the Lennard-Jones 6-12 potential \( V_{6-12} \) for the short-range interactions, which are usually described by:
\[ V_{ij, LJ} = 4 \varepsilon_{ij} \left[ \left( \frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left( \frac{\sigma_{ij}}{r_{ij}} \right)^{6} \right] \]  

where \( r_{ij} \) is the distance between the particles given by:

\[ r_{ij} = |r_i - r_j| \]  

\[ \varepsilon_{ij} \] is the depth of the potential at the local minimum (potential well):

\[ \varepsilon_{ij} = \sqrt{\varepsilon_i \varepsilon_j} \]  

and \( \sigma_{ij} \) is the finite distance at which the inter-particle potential is zero

\[ \sigma_{ij} = \frac{1}{2} (\sigma_i + \sigma_j) \]  

The screened Coulomb potential describing long-range electrostatic interactions is given by:

\[ V_{ij, Coul} = \frac{\varepsilon_{ij} e^2}{4 \pi \varepsilon_0 r_{ij}} \exp(-kr_{ij}) \]
where $z_i$ and $z_j$ are particle charges dispersed in a solvent of the relative dielectric permittivity $\varepsilon_r$, $\varepsilon_0$ is the permittivity in vacuum, and $\kappa^{-1}$ is the Debye screening length.

2.1.2. Aspects of screening the electrostatic potential

In aqueous salt solutions, a polyelectrolyte will be surrounded by an ionic cloud containing an excess of counterions. In these conditions electrostatic interactions between the polyelectrolyte charged groups are screened. Consequently, the long-range repulsive interactions between charged beads of polyion are weakened exponentially with the increasing distance between them. Increasing salt concentrations leads to increased screening which can cause structural changes to the polyelectrolyte. At very low salt concentrations, the electrostatic repulsive interactions are long-ranged and can cause the polyelectrolyte to behave like a rod. In the opposite condition, when there is a high concentration of salt ions, the chain is flexible and takes the form of a spherical, globule-like conformation. The effect of salt concentration is not explicitly included in the description of total potential energy of the polyelectrolyte chain (section 2.1.1), but their influence is included in the Debye screening length $\kappa^{-1}$ which can be expressed by:

$$\kappa^{-1} = \frac{1}{\sqrt{8\pi l_B N_A I}}$$  

(2.6)

where: $N_A$ is the Avogadro number, $I$ is an ionic strength of a solution and is a function of the concentration of ions given by:

$$I = \frac{1}{2} \sum_{i=1}^{n} c_i z_i^2$$  

(2.7)

$c_i$ is the molar concentration of ion $i$ [mol/L] and $z_i$ is a charge number of the ion.

and $l_B$ is the Bjerrum length of the medium given by:

$$l_B = \frac{e^2}{4\pi\varepsilon_0\varepsilon_r k_B T}$$  

(2.8)

where $T$ is the temperature [K], and $k_B$ is Boltzmann constant. For water at room temperature $l_B$ equals 0.7 nm.
2.1.3. Polyelectrolyte complexes

In aqueous solutions containing positively and negatively charged polynomials, a polyelectrolyte complex is formed through the non-covalent link between oppositely charged groups. This process is affected by the charge density of each polion. The polynomials can be divided into 'weak' and 'strong' polyanions (polyacids) or polycations (polybases). This classification is based on the number of charged groups within the polion chain. A strong polyelectrolyte is one that dissociates completely in wide range of pH (its acid dissociation constant $pK_a$ is in very low or very high pH) and all its groups along the chain become fully charged. The example of such strong polyelectrolyte is poly(styrene sulfonate) PSS. A weak polyelectrolyte has its $pK_a$ within pH range between 2 and 10; this implies that such polion will be only partly dissociated in the intermediate pH region. The example of weak polyelectrolyte is poly(acrylic acid) PAA.

In the case of complexation of polycations with weak polycacids, the composition and the structure of the polyelectrolyte complexes obtained depend on: pH, ionic strength of the bulk solution, the degree of neutralization of the polycacid, the proximity of pH to the polycacid $pK_a$, the hydrophobicity of polions, and the concentration of the complex ingredients. As the complex formation progresses its stability increases with the degree of polymerisation, or the number of charges along one polymer chain (Tsuchida 1994).

The stages of complex formation are shown in Figure 2.2. The onset of complexation involves a one-to-one interaction between oppositely charged polions. Following this, small complexes can undergo conformational changes due to intra-complex forces.

![Diagram of the aggregation of polyelectrolyte complexes](Figure 2.2 Diagram of the aggregation of polyelectrolyte complexes (Tsuchida 1994))
Finally, the complexes aggregate together to form large structures such as: networks, entanglements, fibrils, lamellas or spherical objects with organised networks and randomly entangled phases.

2.2. Polyelectrolyte – protein interactions

Complex formation between synthetic polyelectrolytes and globular proteins has increasingly been drawing the interest of researchers. The possible applications for materials constructed of proteins and polyelectrolytes are widespread. Some example applications include protein separation (Morawetz 1952, Wang 1996), bioactive sensors (Decher 1998) and for enzyme immobilization (Margolin 1995, Ivniova 2003). There are many techniques, which are used to monitor growth of protein-polyelectrolyte complexes. A recent review by Cooper (2005) describes microscopic, light scattering, spectroscopic, calorimetric and reflectometric methods that can be applied to study these interactions. In particular, light scattering and turbidimetric titrations have been used extensively for detection of the onset and growth of the protein-polyelectrolyte complexes (Wang 1996, Dubin 1996, Kayitmazer 2003, Giger 2008). A key issue in understanding the protein-polyelectrolyte interactions is to examine what factors control the binding and the conformational changes to the protein upon binding, which in turn control the reversibility of the interaction and whether or not the protein retains its native activity. These factors include protein properties such as size, polarity, and charge distribution; polion properties such as charge density, stiffness, hydrophobicity, and chemical composition. A few studies on protein-polyelectrolyte interactions also examined a number of bound protein globules to one polyelectrolyte chain (Gao 1997, Girard 2003).

Within this work we distinguish two major categories of non-covalent binding depending on strength of attractive interactions between polyion and protein. First is a weak interaction where the polyanion binds to small, multiple positive patches on the protein surface without affecting the native conformation of the protein. The second type of attractive interactions is described as a strong binding, where the polyanion binds to a single, large positive patch on the protein surface. These two types of binding were indicated in a few previous studies on protein-polyelectrolyte binding, for example: Sedlák and Antalík (1998), Seyrek (2003) and Fan (2009). The weak binding is a key issue in terms of understanding the behaviour of polyelectrolytes in solutions with proteins because the mechanism of this type of binding allows the complex formation even on the ‘wrong side’ of the protein’s isoelectric point. These aspects of protein-polyion interactions point towards the charge density as the most important driving force of the protein-polyelectrolyte complex formation. Further we explore how the properties of the polyelectrolyte affect the binding affinities.

The polion charge density, stiffness and hydrophobicity are key factors controlling protein-polyelectrolyte interactions. For instance Hattori and Dubin (2000), examined the binding between β-lactoglobulin (BLG) and poly(styrenesulfonate) (PSS) or poly(2-acrylamido-2-
methylpropanesulfonate) (PAMPS). PSS and PAMPS are strong polyanions with similar linear charge densities but different bare persistence length, which are equal to 1.2 nm and to 2.4 nm for PSS and for PAMPS, respectively. The bare persistence length is measured in the limit of infinite ionic strength and is taken as a measure of the intrinsic polymer flexibility. The conclusions of the study indicated that more flexible polyelectrolyte chains – like PSS – are able to bind more promptly to the protein than stiffer chains such as PAMPS. The intrinsic binding constants for PSS were reported to be much greater for PSS than for PAMPS. However, the binding might also be controlled by hydrophobic interactions as the hydrophobicity of PSS is greater than that of PAMPS. This is due to the existence of phenyl residues near the PSS backbone, when PAMPS has polar amide residues near the vinyl groups. Hattori and Dubin stated that hydrophobicity of the polyanion refers to the accessibility of the polymer's alkyl chain. The effect of hydrophobicity of the polyanion chain on binding to BLG - which is a hydrophobic protein - suggests that BLG has a greater binding affinity to more hydrophobic polyelectrolytes. This trend was also confirmed in a study by Gao and Dubin (1999), where the interactions between BLG and a number of alternating copolymers of maleic acid and alkyl vinyl ethers were determined. A minimum alkyl chain length of three or four methylenes was required for significant hydrophobic interactions to occur. This property was also confirmed for interactions between BSA and hydrophobically modified polyions (Porcar 1999). However, the binding affinity between BSA and PSS appear to be stronger than the one for BLG-PSS in the same pH and ionic strength conditions, this may be due to greater hydrophobicity of BSA than BLG (Tanford 1979).

A follow up study, compared binding of BLG to PSS with the binding to poly(vinylsulfate) (PVS), which is a non-hydrophobic polyanion (Hattori and Dubin, 2005). BLG binding to PSS is stronger as reflected by a higher value of the intrinsic binding constant for BSA and PSS than that of BLG and PVS. Because PVS is also a flexible polyanion with a similar bare persistence length to PSS, this dependence implies that polyanion hydrophobicity has a strong effect on binding.

Protein binding to polyelectrolytes was also examined in detail by Cooper and co-workers (2006) using turbidimetric titrations and light scattering techniques complemented with molecular modelling. The role of polyanion properties such as chain stiffness and charge mobility were isolated from studies of hyaluronic acid (HA), pectin and poly(acrylic acid) (PAA) polyions. The polyions are divided into two categories, where the weak polyacids are considered as polyions with mobile charge (annealed) and the strong polyions with fixed charge (quenched). The charge sequence distribution of the polyanion influences the binding to bovine serum albumin BSA and micelles. Binding affinity increases with polyanion chain flexibility and charge mobility. Additionally, the binding of the polyanions to BSA was strongly dependant on ionic strength. At low ionic strength the complex formation occurred through binding between the polyanion chains and a single positive patch and at high ionic strength the binding involved multiple smaller positive patches. Binding affinity is predominantly influenced by chain flexibility and charge mobility, while the complex formation is less sensitive to polyanion charge density. Also the charge density is reported to be dependant on the colloid charge heterogeneity,
conformational properties of the complex structure and ionic strength. The effects of increasing charge density sometimes weakens the binding due to repulsions within the polyanion macromolecule when forming condensed states on the protein. Cooper also examined polyanions charge sequence distribution, using co-polymers with different content of ionic residues, where the polyions ability to bind to BSA was tested against low and high content of negatively charged groups.

Another study on the effects of polyelectrolyte stiffness on binding to oppositely charged colloid particles was carried out by Kayitmazer et al. (2003). Mixed cationic/nonionic micelles and protein serum albumin were examined for their binding abilities with a flexible chain of hyaluronic acid (HA), or with a stiffer chain formed by a copolymer of acrylamidomethylpropanesulfonate (AMPS) and acrylamide (AAm). These polyions have a similar linear charge density at neutral pH. For serum albumin, interactions were examined for binding to AMPS-AAm copolymers of higher charge densities than in case of micelles, and to heparin - a highly charged and flexible biopolyelectrolyte. The binding between micelles or protein to polyelectrolytes was weaker for stiffer polyion chains; this tendency was more evident at higher ionic strengths as in these conditions the chain stiffness is subject to the differences in bare persistence length. In the case of binding to serum albumin the flexible polyion chains were able to adopt configurations upon binding to positive patches, which allowed the chain to minimise the repulsive electrostatic forces originating from negatively charged patches on the protein surface.

2.2.1. Relationship between protein conformation and binding

The general qualitative theory of protein structural stability during the process of protein adsorption on charged polystyrene latices was first described by Norde and Lyklema (1978). The findings describe structural rearrangement in the human plasma albumin and bovine pancreas ribonuclease macromolecule, dehydration of the charged lattice surface, redistribution of the protein charged groups at the interface and polyelectrolyte surface, and the protein polarity. The degree of structural adjustment was found to be dependant on pH and temperature. In the pH region near to the protein isoelectric point, the changes were minimal, and the heterogeneity of the charge distribution on the proteins surface is at its peak. Additionally, these factors were reported to make a primary contribution to the behaviour of protein in adsorption to polyelectrolytes. Proteins are less likely to unfold when interacting with immobilised polionys versus soluble ones because the rigidity of the polyion backbone is lower than the charged surfaces.

Polyelectrolytes in aqueous solutions may exhibit behaviour recognised as ‘weak’ or ‘strong’ binding to proteins, which is linked to the impact of the polyion binding on the protein’s native conformation. Sedlák and Antalík (1998) indentified polyanions that cause and do not cause conformational changes on the native protein structure upon binding. For instance, complexes
formed between two different polyanions: poly(vinyl sulfate) (PVS) and poly(4-styrene-sulfonate) (PSS) and Cytochrome c (cyt c) were linked to conformational changes in the hydrophobic interior of cyt c globule partial unfolding and denaturation of the protein.

Ivniova et al. (2003) also studied model proteins, examining their capability of forming water-soluble complexes with synthetic polyanions. The study described lysozyme, chymotrypsinogen or glyceraldehyde-3-phosphate dehydrogenase complexing with sodium poly(methacrylate) (PMA), sodium poly(acrylate) (PAA), sodium poly(anetholsulfonate) (PAS), and potassium poly(vinylsulfate) (PVS). Differential scanning calorimetry (DSC) thermograms showed that these polyanions noticeably reduced the temperature of protein denaturation (melting temperature). The most severe denaturation effect was observed for lysozyme when complexed with poly(styrenesulfonate) (PSS), in which case the melting peak was not observed at all, as the protein was already denatured through interactions with PSS alone. The shift in melting temperature increased according to the following order: PMA < PVS < PAA < PAS < PSS. Upon decomposition of the protein-polyelectrolyte complex, the melting temperature of the native protein was recovered for proteins complexing to PMA, PVS or PAA. For proteins complexed with either PAS or PSS complexes the denaturation (melting) temperature of the native protein was not recovered upon decomposing the complex. This implies that binding of PAS and PSS affect the protein secondary and tertiary structure. The range of temperatures examined by Ivinova and co-workers suggests that protein-polyelectrolyte interactions in solutions result in the effective denaturation of the majority of model proteins only when a certain critical temperature is achieved.

These studies by Sedlák (1998) and Ivniova (2003) define the cases where no conformational changes were observed and also the cases where it was observed. The conformational changes of the proteins are associated with strong polyelectrolyte binders and a pH dependence on binding, even in concentrated salt solutions, whereas weak polyelectrolyte binding is screened at high salt concentrations.

The assessment of protein-polyelectrolyte behaviour in solution was also carried out to establish the proportion of cumbic and non-cumbic (hydrophobic and H-bonding) binding interactions that affected the conformation of cyt c. It was concluded that polyanions can cause conformational changes in the core of protein globules through non-cumbic interactions. However, only partial denaturation occurs as the protein is transformed into a semi-unfolded state, often termed a molten globule, where helices are still preserved (de Jongh 1992).

2.2.2. \( \text{pK}_{a} \)s of protein residues in presence of polyelectrolytes

Perturbation in the acid dissociation constant \( \text{pK}_{a} \) of the ionisable groups depends upon the change during a transition between native and denatured states or upon protein complexation with polyelectrolytes. The average protonation state of the protein can be determined experimentally by pH titration. Titration curves were extensively studied by Tanford and co-
workers (Roxby 1971, Nozaki 1967). Their study included titration curves of denatured (random coil) and native conformations of protein. The titration curves for unfolded proteins are predictable using random coil models to describe the proteins. However, $pK_a$s of ionisable groups are perturbed in the presence of strong intramolecular interactions which occur in natively folded proteins. If the presence of polyelectrolyte is predominant it is likely that it would partly or fully unfold the protein. The pH titration curves presented by Haynes (1994) of hen egg-white lysozyme in the presence of 0.1 M potassium chloride were compared to those obtained by Tanford and Roxby (1972) in studies of lysozyme with 6 M guanidinium chloride and 0.1 M potassium chloride, as shown in Figure 2.3. There was a pronounced difference between the protonation states between folded and unfolded lysozyme. The titration curves indicate that the $pK_a$s values of polypeptide groups in pH region from 2 to 5 differ substantially from the corresponding $pK_a$s in the native conformation of lysozyme.

![Figure 2.3 pH titration curve of lysozyme in 0.1 M KCl (solid line) and of lysozyme in 0.1 M KCl with 6 M GuHCl (Haynes 1994)](image)

Experiments and theory suggest that the $pK_a$s of the ionisable protein groups are linked to their local environment, such as the presence of neighbouring polar or non-polar groups and the hydrogen bonding environment, and are dependant on the local electrostatic potential on the protein solvent accessible surface area. Lyklema and Norde found that the fraction of protonated basic residues on a protein in solution is greater than on the same protein adsorbed onto the charged polystyrene surface (Norde 1978). This result indicates that the complexation of the protein with polyelectrolyte influences the protonation state of the protein.
2.2.3. Kinetics of complex formation

In work presented by Stroupe and co-workers (1977), the kinetics of association and dissociation of the progesterone-binding globulin to progesterone steroid (polyion) was measured by fluorimetry. The maximal rate of binding in all tested conditions occurs near the physiological pH and there is a decreasing affinity when pH is either much higher or lower than the physiological pH of 7. At the physiological pH the steroid-binding site was available due to the local amino acid residues integrity. The complex formation was examined at equilibrium and under non-equilibrium conditions. The complexation behaviour is pH dependant in both cases. At pH 7.4 one of the amino acid groups, which was not contributing in binding to progesterone, became deprotonated, as the equilibrium was more rapid for that group than that of the steroid with its binding site.

2.2.4. Phase separation of protein-polyelectrolyte complexes

Protein-polyelectrolyte complexes can be classified as soluble or insoluble if a precipitate can be detected. Another, closely related, classification describes a reversible and irreversible complex formation, depending on whether environmental changes can reverse complex formation resulting in the separation of polyelectrolyte macromolecules from proteins without affecting their native conformational structure. Phase separation of protein-polyelectrolyte complexes is of continuous interest for researchers. Well-controlled, selective protein separation can be applied in both the pharmaceutical and food industries. The phase separation behaviour is controlled by electrostatically driven interactions between complexes, which depend upon the salt concentration. At low ionic strength, the precipitate is more likely to be composed of an amorphous material as the protein-polyion interactions are strong and tightly organised. In higher ionic strength solutions, the charges on both the protein and the polyelectrolyte are screened by the counterions. The strong, long-range electrostatics are shielded and the solvent can freely penetrate the aggregates, resulting in the formation of a second liquid phase or coacervate (Mattison 1995). The phase behaviour can be monitored through light scattering and turbidimetry in dilute macromolecular solutions and by rheometry to study semi-diluted macromolecular solutions.

Bohidar and Dubin (Bohidar 2005) studied the dynamics of coacervate formation between bovine serum albumin (BSA) and poly(diallyldimethylammonium chloride) (PDADMAC). The coacervates were first described by Bungenberg de Jong (1930, 1949) as clusters of complexed macromolecules held together by hydrophobic forces. The assemblies are composed of dense fluid phase containing aggregates of highly concentrated complexes in equilibrium, where the surrounding - less dense - second fluid phase contains non-aggregated macromolecules and low concentration of complexes. Bohidar and Dubin monitored the process of coacervation by
dynamic light scattering and rheological measurements. The study showed that protein
dissociation in the coacervate phase was fast and independent of polyelectrolyte molecular
weight. The coacervate formation was reported to be one or two orders of magnitude slower
than diffusion of the titrated protein into the system and independent of the ionic strength.
Rheologically tested viscoelastic behaviour also indicated a fragile network of coacervates, with
solid-like characteristics at low strain but re-forming after breakage by shear. Understanding of
the coacervate behaviour and formation of boundaries between two liquid phases can give an
insight into protein-polyelectrolyte interactions. Bohidar and Dubin described coacervation as a
process following the formation of soluble protein-polyelectrolyte complexes, this occurs when
the net charge of the soluble complexes approaches zero. The complex net charge depends on
the charge of a colloidal particle, such as protein, and a number of the particles bound to
polyion chain. The latter depends on both protein-polyelectrolyte stoichiometry and the protein-
polyelectrolyte binding constant. Coacervation may be enhanced by changes in size of either
protein or the polyelectrolyte.

2.2.5. Computer simulation studies of protein-polyelectrolyte interactions

There are simulation studies for understanding the factors that control protein-polyelectrolyte
interactions and the subsequent complex formation. Carlsson and co-workers (2001) examined
the complexation between negatively charged polyelectrolyte and a hard sphere with embedded
charges whose locations were chosen to mimic the charge distribution of lysozyme. Monte
Carlo simulations indicated that complex formation was dependant on the charged state of the
protein sphere. As a consequence, the binding sensitivity depended on the pH and ionic
strength, which influence the range and strength of electrostatic interactions. The complexation
is weaker at low ionic strength with the exception being when the protein has a very high
positive net charge, in which case the affinity for the protein-polyelectrolyte binding is enhanced.
Also, the pH dependant complex formation between protein and polyelectrolyte required the
existence of non-electrostatic forces, in addition to electrostatic attractions, for the protein-
polyion binding to occur. Carlsson indicated that the distribution of the polyion beads on the
protein surface is inhomogeneous and the polymer backbone contracted upon binding.
In another study by Tian (2010), molecular dynamics simulations were used to examine the
interactions of a polyelectrolyte chain with a charged dendritic particle. The influence of the
stiffness of polymer chain on the binding affinity with dendrimer was examined. Changing the
size and persistence length of the polyelectrolyte chain affected the complexation between the
macromolecules. When the size of polyion chain increases and its shape changes from oblate
to prolate there is a transformation in chain confirmation, upon binding with dendrimer, from coil-
like to U-shape or V-shape and further to rod-like as the stiffness of polymer is increased. The
polyion-dendrimer interactions were dependant on the bending energy of the chain and the
intermolecular electrostatic attraction; for a very stiff polyelectrolyte backbone the elastic energy
increases, which reduces the polion ability to collapse onto charged dendrimer. Tian’s results also indicated that it is possible to design a polyelectrolyte chain rigid enough to allow a pH and/or ionic strength triggered release of the polion from the complex with the dendrimer.

2.3. Structure of proteins

The atomic crystal coordinate structures of individual proteins are available at the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank, which provides a vital resource for studying biological macromolecules (www.rcsb.org). The structures obtained from the protein data bank for visualisation of studied proteins in this work include that of myoglobin 1WLA (Maurus 1997), insulin 2A3G (Smith 2005) and human serum albumin 1AO6 (Sugio 1999). The atomic models of the proteins included in this study were used to visualise primary, secondary and tertiary structures and calculate the electrostatic potential on the surface of the proteins. In this section we define in more detail the structure of the protein and the method of calculating the solvent accessible surface area. We then summarise the environmental aspects that affect protein-protein interactions.

2.3.1. General concepts of protein structure

The basic classification of protein structure is considered at four levels. The primary structure is related to the sequence of amino acids connected by covalent bonds. Secondary structure is determined by the local three dimensional conformations of polypeptide chains, which is controlled by hydrogen bonding, hydrophobic and van der Waals interactions. The most common forms are the α-helix and β-sheet. An α-helix is constructed by the hydrogen bonding between carbonyl oxygen (C=O) and the amine group (N-H) along the polypeptide backbone. These hydrogen bonds lead to the formation of a dipole moment on the α-helix, where the carboxyl end is negative and amino end is positive. A β-sheet is formed by β-strands, which are fully extended parts of chains incorporated into a β-sheet. The β-sheets are significantly different to α-helices. The hydrogen bonds between the amine and carbonyl oxygens along the backbone support the stability of the β-sheets. The interactions that form β-sheets occur between groups on separate strands, of the distant parts of the same macromolecule (in the primary sequence) or different macromolecular chains (in secondary sequence). As a consequence, β-sheets can form from either intra- or intermolecular arrangements. Tertiary structure describes the 3-dimensional structure of the whole macromolecule and describes how the different regions of secondary structure are arranged relative to each other. The tertiary structure of many proteins is spherical (often described as globular) with an irregular surface. These structural units may develop into domains of several polypeptide chains, which can interact with each other to form larger assemblies, which is termed the quaternary structure.
Proteins in solution have acidic and basic residues, which can have negative or positive charges. The density and distribution of the charges is determined by the protein’s individual peptide composition. The pH conditions determine the ionization state of acidic or basic groups as determined by the logarithmic measure of the acid dissociation constant $pK_a$. Most groups have $pK_a$ values in the approximate range of 2 to 12 in water. The $pK_a$ depends on the local environment of the ionisable group such as the polarity or the local electrostatic potential conditions i.e. ionic strength. When the globular protein is in its native state the ionisation of the groups can only occur on the exterior surface, as the inner area is stabilised by the presence of the salt bridges, hydrophobic interactions or hydrogen bonds. Therefore, the charged state of whole protein is determined by the number of positively and negatively charged residues on the solvent accessible surface area. In a condition when net charge of a protein macromolecule equals zero, the positive and negative charges compensate each other. This pH condition is known as an isoelectric point $pI$. The isoelectric point depends on unique composition of amino acid groups which create the protein macromolecule. At the pH near the $pI$ value the distribution of charges on the protein surface facilitates the protein-protein attraction leading to self aggregation. This property was reported by Giger and Dubin in their study on aggregation of insulin (Giger 2008), where the investigation was focused on so called ‘isoelectric precipitation’ of insulin at low ionic strength and the effect of heparin – a biopolymer composed of disaccharide units of uronic acid and glucosamine – on suppression of insulin’s self aggregation. The isoelectric precipitation of insulin was examined using turbidimetric titrations and computer visualisation techniques. The results of Giner and Dubin’s work suggested that interprotein electrostatic attractions increase when pH equals $pI$ at low ionic strength, this dependency is in the contrary to a widespread view that electrostatic forces between protein macromolecules are minimised near the isoelectric point. The effect of added heparin to the solutions containing insulin suggested that the presence of the biopolyelectrolyte inhibits self-aggregation of insulin when pH is near $pI$. Addition of heparin causes non-covalent binding between insulin globules and the extended, polyanionic heparin chain, and in fact there is a number of insulin macromolecules connecting with one heparin chain. This allows creation of small but stable insulin/heparin complexes, the increase in turbidity was minor for insulin solutions containing heparin in comparison with insulin-only solutions. Giner and Dubin also report that the addition of heparin to the solution containing aggregated insulin can help to break up the aggregates what is illustrated by the decrease in turbidity. The findings support the conclusion that the aggregation of dimeric insulin may be primarily driven by the electrostatic interactions.
2.3.2. Solvent accessible surface area

In this work, we are mainly concerned with the proteins in their native state, which interact with each other or with polyions. These interactions are predominantly controlled by the groups on the protein surface, which can be characterised in different ways. The protein surface can be defined using an approach proposed by Lee and Richards (Lee 1971), in which every atom of the protein is considered as a sphere with a size given by appropriate van der Waals radius. The spheres of bonded atoms are overlapping. The atoms on the surface of the protein globule form a complicated, irregular surface area. Detailed illustration of this concept is shown in Figure 2.4. The boundary defined by the atom spheres is known as the van der Waals surface.

![Figure 2.4 Illustration of Lee and Richards approach](image)

The solvent accessible surface area is determined from the path of the centre of a solvent molecule sphere rolled over the entire protein surface. The solvent sphere cannot penetrate all small interstitial spaces between atoms of the protein molecule. Consequently the solvent accessible surface area looks as if it is smoother than the van der Waals surface.

2.3.3. Environmental aspects influencing protein-protein interactions

In this work, we examine the protein-protein interactions in aqueous solutions, since our interest lies in environmental conditions based on biological systems. The understanding of the behaviour of protein-protein systems can improve the control over the processes of crystallisation and dissolution of proteins and help to inhibit their self-association and aggregation during storage. Additionally, the mechanism of protein aggregation may provide vital information about the origin of diseases.
Protein-protein interactions depend on electrostatic, hydrophobic and hydrophilic, van der Waals, hydrogen-bonding, solvation and dispersion forces (Israelachvili 1991). The ‘length’ or their range depends on the environmental conditions of the medium in which the protein particles are dissolved. Water has a high dielectric constant, which leads to reduced electrostatic interactions and larger attractive dispersion forces. Furthermore increasing salt concentration leads to a reduction in the range of electrostatic interactions through the screening effect described in section 2.1.2. The mechanism for salt-specific effects is shown in Figure 2.5, where in the region marked green, dissolved ions can preferentially interact with the protein surface creating a salting-in effect or protein-protein repulsion, whereas preferential exclusion interactions between salt ions and the protein surface leads to a salting-out effect (or protein-protein attraction), which is greatly enhanced at high salt concentrations (greater than 0.5 M). The salting-in behaviour is due to preferential interactions between proteins and salt, which have an electrostatic origin arising from the net charge and large dipole moment of the protein surface. Because of this, the solubility of protein raises with increasing salt concentration (white region in Figure 2.5). On the other hand the rapid decrease of solubility of the protein is caused by salting-out behaviour, which results in the formation of domains of several protein particles surrounded by small ions. This mechanism leads to protein precipitation. The general concept of protein salt-induced precipitation was recently reviewed by Curtis et al. (1998, 2002).
2.4. Hofmeister series

The first systematic study on specific ion effects was initiated at the end of the 19th century at the University of Prague, the study was published as a series of articles under the title: ‘About the science of the effect of salts’ where Franz Hofmeister and his student S. Lewith (Hofmeister 1887, Lewith 1887) explained the regularities in precipitation of the proteins under the influence of salts and the connection of these effects with the behaviour of salt ions, beyond the influence of the ion valency. Hofmeister and co-workers (Limbeck 1888, Hofmeister 1890, Hofmeister 1891, Münzer 1898) proved that there is an influence of ions beyond that of their valency. In addition, they established that specific ion effects could be correlated with the water-withdrawing capability of the ions. The specific ion effects were examined in different systems such as precipitating of molecules such as proteins, collagen, colloidal ferric oxide and sodium oleate. For instance, sodium chloride has a stronger colloid precipitating ability than potassium chloride when in water solution. The Hofmeister series ranks the ions according to their salting-out effectiveness for globular proteins. In Figure 2.6, we present the complete order of anions and cations in the ‘Hofmeister series’.

In addition to the salting-out effects of different ions, Hofmeister examined the cause of changes in osmotic pressure between different salt solutions at the same salt concentration. He found that, the osmotic pressure of a given volume of salt solution varies from the pressure within a volume of a gas containing the same number of molecules. The origin of the difference is due to salt dissociation in the aqueous solution, and also is related to the water withdrawing ability of the ions. The research on salts in aqueous solutions was also described by Poiseuille (1847), who reported the increase or decrease of viscosity of aqueous solutions containing some salts, which was linked to specific ion effects. This matter was also examined at the beginning of the twentieth century by Jones and Dole, who defined the Jones-Dole viscosity B coefficients, which linked the viscosity and the entropy of salt dilution in water, which in turn corresponds to the affinity of the ion for water molecule. The effect of salt on the Jones-Dole coefficients gave the terms for ‘water-structure maker’ and ‘water-structure breaker’, which were established by Frank and Evans (Jones 1929, Cox 1934, Frank 1945). Specific ion effects have also been observed in the area of the gels swelling, colloids zeta potentials, sols viscosity, and the heats of hydration of ions.
Figure 2.6 The order of anions and cations in Hofmeister series (Kunz 2010) and the effects of ions on proteins in aqueous solutions

In more recent studies, the attention on specific ion effects includes studies of simple aqueous solutions and also more complicated mixtures such as solutions of macroions and their complexes. Studies include ion interactions with flat, charged surfaces, and much more heterogeneous surfaces such as protein particles. Furthermore, the nucleophilic or electrophilic character of ions is often studied as well as the softness or hardness of the ions. All these aspects of the specific ion effects are subject to experimental (Koelsch 2007) as well as theoretical examinations (Curtis 2006).

Koelsch and co-workers discuss the aspects of ions-water interactions at the charged interfaces in terms of the classical approach of the Poisson-Boltzmann theory and its simplified assumptions about univalent ions that are close to the charged interfaces. Basing on the broad review of recent experimental research it is highlighted that there are major differences between behaviour of ions of the same valence, but different type. Specific ion effects can be found in many research disciplines related to biological and chemical systems, where the pronounced
differences upon ion behaviour in relation to charged mono- and multilayers, micelles, dispersions are reported.

In an article by Curtis and Lue (2006) it was highlighted that protein-protein interactions are strongly correlated with protein-salt interactions. The interactions are determined by the forces of preferential exclusion or binding of the salt ion to the protein macromolecule. The exclusion and binding is linked to the position of the ion in the Hofmeister series. In order to capture these effects using numerical approaches, the discrete nature of water needs to be taken into account, which is not possible using continuum models.

2.5. Polyelectrolyte multilayers

There are a variety of possible applications for polyelectrolyte multilayer composites such as optical and electrochemical materials, biomedical devices, surface modification, biosensors, dye capsules and photoreaction inhibitors. Research on protein-polyelectrolyte structures is most beneficial for pharmaceutical applications, for example responsive delivery systems for protein therapeutics, and for enzyme immobilization in biosensors. There are a variety of already existing applications for the polyelectrolyte multilayer composites such as biomedical devices (US Patent 7566746 2009), surface modification (Jones 2006), biosensors, dye or drug encapsulation systems and photoreaction inhibitors (Prevot 2006). Knowing the response of the multilayer composite to the external stimuli can help in designing these medical advances.

Polyelectrolyte multilayers were introduced in the early 1990s (Decher 1991, 1992). The multilayer structures consist of alternating layers of oppositely charged macromolecules. The layer of adsorbed material shows a higher concentration of polymer than bulk solution further away from the interface. Therefore, a transition between dilute and semi-dilute polyelectrolyte concentration regimes is observed when polyelectrolytes are adsorbed on the charged surface through electrostatic attraction between charges across the substrate and opposite charges on the macromolecules dissolved in fluid surrounding interface. The electrostatic forces play a major role in stabilising the multilayer wall. The relatively weak interactions between each pair of oppositely charged functional groups are multiplied by a large number of binding points creating a strong link between the layers. Multilayer properties such as film permeability, triggered decomposition or changes of the film thickness can be modified to meet the need of the application. Understanding the intra- and intermolecular interactions affecting a multilayer, constructed of protein and polyelectrolyte, and determining the correlation between composite organization and the complex formation mechanism between protein and polion in solution is a key objective of this work. The ultimate goal is to improve process control in fabrication of protein-polyelectrolyte multilayers and determine optimal protein binding conditions.
2.5.1. Layer-by-Layer assembly of polyelectrolyte composite

The polyelectrolyte multilayers are created using a layer-by-layer (l-b-l) technique, shown in Figure 2.7, which can be used to form nanocomposites made of many different components in a single device, which allows controlled assembly of polyelectrolytes, proteins, microcapsules, colloids or biological cells. In contrast to the spontaneous self-assembly of oppositely charged molecules, the l-b-l method gives the possibility to control the composite architecture through changing the device properties.

Figure 2.7 Schematic layer-by-layer assembly of polyelectrolytes

The adsorption of a film layer starts on the negatively or positively charged surface of substrate, such as a silica plate, which is dipped in a solution containing a polyanion (or a polycation for negatively charged plate). In the diagram shown in Figure 2.8, initial deposition begins with polyanions, which are attracted via electrostatic forces to create a monolayer. The adsorbed film is then washed using solvent to remove any excess of macromolecules, leaving only fully adsorbed polyanion molecules (step 2). Step 3 includes adsorbing polycation, which is then washed with solvent in step 4. This straightforward sequence is then repeated to create a simple two-component multilayer structure.

The chemical composition of polyelectrolytes plays a major role in the design of a thin multilayer film as various polyelectrolytes may have different properties such as charge density, hydrophobicity, stiffness or polydispersity all of which can impact upon the multilayer formation and structure. By tuning these properties, the process of constructing multilayer films can be modified according to required composite architecture. It has been established by Rubinstein et al. (1996) that the charge density on the polyion chains has a major impact on the physicochemical properties of the polyelectrolyte solutions. Likewise, a study by Glinel et al. (2007) showed that changing charge densities of the multilayer components affects the intermolecular interactions within the constructed films, causing either collapse or swelling of the composite. Furthermore, the polyion charge density controls the multilayer growth mechanism.
The polyelectrolyte multilayer composites built with synthetic polyelectrolytes have been extensively studied. The most recognizable systems are poly(styrene sulfonate) (PSS)/poly(diallyldimethyl ammonium chloride) (PDADMAC) and poly(styrene sulfonate) (PSS)/poly(allylamine hydrochloride) (PAH) (Fery 2001, Antipov 2002). These polyelectrolyte composites were examined for their responsiveness to temperature, pH changes (Steitz 2002, Prevot 2006, Glinel 2007), and the influence of visible and ultraviolet light (Sukhishvilli 2005). While synthetic systems are well studied, less is known about the interactions between two charged biomacromolecules or between a synthetic polyelectrolyte and biopolyelectrolyte (Zhang 2007, Mutka 2008).

2.5.2. Multilayer film growth

There are different forms of multilayer growth. The first type of growth is termed linear, as the change in mass or thickness upon each deposition step is a constant for each pair of adsorbed polyelectrolytes. Integrated structures characterized by linear growth have been studied for their respective individual layer profiles, charge stoichiometry, surface charge distribution, presence of counterion and layer interpenetration. The best known examples of linear growth are multilayers composed of PSS/PAH or PSS/PDADMAC. A study by Ladam et al. (2000) introduces a zone model to describe simple, flexible and highly charged homopolyelectrolyte multilayer films, in which three distinctive zones of various structural properties were introduced.
Zone (I) consists of one or few layers located in direct contact with the substrate. These layers (or layer) are affected by the surface properties of the substrate and tend to form thinner deposits than those observed in other parts of the film. Zone (III) corresponds to the part of the multilayer exposed to the solvent, layers in this zone are influenced by the interface to an environmental fluid. Zone (II) occurs between zones I and III; the layers in this zone are not influenced by other media such as counterions. This zone represents the majority of the multilayer film, where polyelectrolytes are capable of forming 1:1 stoichiometric complexes. In this zone, charges are fully compensating each other creating a zwitterionic composition, where total net charge equals zero even though the atoms carry a formal partial charge and small gradients of excess charge are neutralized by the traces of counterions. Zone II is considered physicochemically metastable, and the local properties of the film are constant in this region. Zones (I) and (III) may contain a total net charge due to imbalance caused by the charge on the surface of the substrate or the presence of large number of the counterions in the fluid surrounding the newly formed architecture. The ideal multilayer model (also described as ‘the zone model’) assumes that zone (I) and zone (III) are formed first at interfaces (with either substrate or environmental fluid) and then after they reach their final form, the core - zone (II) is built, which then increases with each deposition step. In addition, there is a finite number of deposition steps when the zone model theory can be applied. If the number of layers is very large then layers are strongly overlapping each other and zones are indistinguishable (Decher 2003).

The other type of multilayer build-up mechanism occurs when mass and thickness of composite rises exponentially with the number of deposition steps. An exponential growth is developed when the components of multilayer film differ considerably in their charge density and as a result the ability of oppositely charged polylons to interact with each other is diverse from the system where polyelectrolyte charges compensate each other. The classic example of exponential growth was published by Porcel et al. (2006), where the multilayer growth mechanism was studied using a system composed of hyaluronic acid and poly-L-lysine (HA/PLL). With this system, during deposition HA does not penetrate the inner layers of the composite and is only adsorbed on the interface. However, during the deposition of poly-L-lysine, the polycation interacts with the outer HA layer and a certain fraction of unbound PLL is able to diffuse through the entire film network. Following this process, the composite consists of bound and unbound PLL chains. The presence of diffused PLL may induce interpolyion repulsion throughout the structure and swelling within a newly formed HA layer leading to the exponential growth. However, the two types of multilayer growth described here reflect only ideal systems; such a simple categorisation is insufficient as most multilayer build-ups will exhibit both types of growth depending on buffer/solvent composition, pH, tacticity of the contributing polymer chains, charge density and distribution, and the number of adsorbed layers.
2.5.3. The effect of ionic strength and type of salt

Ionic strength has a strong influence on the thickness of the multilayer wall. The addition of salt to the aqueous solution of polyion can control layer width with ångström precision (Decher 1992). Increasing salt concentration results in a rise of the polyelectrolyte layer thickness. The effect of ionic strength varies for different polyions and the type of salt used according to the ‘Hofmeister series’ (Kunz 2004). The thickness of the single polyelectrolyte layer increases when changing the ions following the order: \( \text{Li}^+ < \text{Na}^+ < \text{K}^+ \) for the cations and \( \text{F}^- < \text{Cl}^- < \text{Br}^- < \text{I}^- \), \( \text{NO}_3^- < \text{SCN}^- \) for the anions. The effect of anions is greater than cations due to their dimensions and larger difference in their polarizability. Some aspects of the ‘Hofmeister series’ was explained in section 2.4.

2.5.4. pH-responsive multilayers

The multilayer system studied by Glinel et al. (2007) shows a multilayer build-up composed of poly(styrene sulfonate) PSS and poly(allylamine hydrochloride) (PAH). PSS behaves as a

\[ \text{Figure 2.9 PSS/PAH multilayer response for change of pH (Glinel 2007)} \]
strong polyanion in solutions over a wide range of pH, whereas PAH is a weak polycation, for which the charge density varies with acidity of the aqueous solution. In multilayers prepared at low pH the charge density on the polyelectrolytes compensate each other forming tight multilayer walls as shown in Figure 2.9. When the composite is exposed to rising pH, there is a rapid decrease in the positively charged groups of PAH. In these pH conditions the multilayer wall has an excess of negative charge, which causes repulsive interactions between neighbouring, uncompensated charges and swelling of the multilayer wall.

2.5.5. Thermo-responsive multilayers

Thermo-responsive polyelectrolytes change conformation at a characteristic temperature called the lower critical solution temperature (LCST). These polyelectrolytes are water soluble at temperatures below the LCST, forming random coil conformations. When temperature rises above the LCST, molecules collapse to globular structures due to hydrophobic interactions (Sukhishvilli 2005). This structural change is illustrated in Figure 2.10, where the red and blue colours represent hydrophilic and hydrophobic groups respectively.

Temperature-responsive materials allow control of surface wetting, adhesion properties and controlled release mechanisms. There are several aspects affecting thermo-responsiveness of multilayer composites. The most important are the presence of excess charge after assembly and the intermolecular association of polyelectrolyte units which provide thermal response (Okhapin 2003).
2.5.6. Conclusions

Composites of oppositely charged polyelectrolytes and proteins have great potential to be used to produce environmentally responsive structures for the encapsulation and then delivery of active ingredients in pharmaceutical and food preservation applications. The control of charge density and charge distribution within the structure can be used in designing of ‘smart’ materials. The self-assembly multilayer components can be monitored though many experimental techniques such as surface plasmon resonance (SPR), quartz crystal microbalance with dissipation (QCMD), reflectometry or elipsometry. These instruments allow sufficient characterisation of the mass and thickness of the deposited material, and characteristics of the material response to different environmental conditions such as an enzymatic degradation, pH and temperature change, presence of magnetic field and many other stimuli.
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CHAPTER 3

PROTEIN – POLY Electrolyte INTERACTIONS

3.1. Abstract

Determining inter- and intra-molecular relations between polyions in aqueous solutions helps to develop the understanding of self-assembly mechanisms or build controlled assembly structures. However, delineating the factors controlling these interactions is difficult. Interactions depend upon polyion charge density, stiffness, and hydrophobicity as well as on protein charge distribution. This study is focused on determining the factors controlling the interactions between a negatively charged polyelectrolyte - poly(styrene sulfonate) (PSS) with either myoglobin or bovine serum albumin (BSA). The protein-polyelectrolyte complex formation is probed as a function of ionic strength, salt type and pH using turbidimetric and potentiometric titration experiments, static light scattering, and Micro DSC experiments. Emphasis is placed on studying specific ion effects, first on the behavior of polyion-only and protein-only solutions in terms of the osmotic second virial coefficient. Turbidity and light scattering experiments of the mixture are then used to measure the effect of ion type on the pH at the onset of protein-polyion complexation. Our experimental system is based on poly(styrene sulfonate) PSS with either bovine serum albumin BSA or myoglobin. We observed that the PSS behavior does not depend on specific ion type for monovalent salts, but including divalent cations leads to a reduced size of the polyion. Further, solutions of divalent cations lead to increased repulsion between proteins in the polyion-free solution. Interestingly, we find strong specific ion effects on protein-polyelectrolyte interactions. The BSA-PSS interactions, leading to onset of complex formation, are stronger in solutions of LiCl than of NaCl. Further, the pH at which solutions go turbid, terms the \( p\Delta \) increases with decreasing LiCl and NaCl concentration, in contrast to studies with MgCl\(_2\) where increasing ionic strength leads to elevated \( p\Delta \) due to screening of attractive electrostatic interactions. A key outcome is that we correlated the initial onset of complex formation as measured by static light scattering with the values of the \( p\Delta \), the latter which reflects the formation of large visible structures. Our experimental research also included an investigation on the effects of mono- and divalent cations on the behavior of myoglobin-PSS solutions. We established that divalent cations cause the onset of the soluble complexes formation at very high pH values. For the NaCl solutions, the myoglobin-PSS system shows a decrease in the \( p\Delta \) with increasing NaCl concentration. However, the formation of small complexes between myoglobin and PSS as characterized in terms of potentiometric titrations occurs more readily with decreasing ionic strength as expected for interactions stabilized by electrostatic forces.
3.2. Introduction

Interactions of charged polymer chains with charged colloidal particles such as proteins in aqueous solution have been researched extensively in the last several decades. The interest in these systems is based on a principal difference between neutral polymer chains dispersed in solvent and the polymer chains that become charged in solution. For the neutral polymers the scale of intermolecular interactions is much smaller than the scale used to describe the physical properties of the polymers such as molecular weight, number of mer units or length of the chain etc. when the polyelectrolyte macromolecules exhibit a much more complex behaviour in solution than neutral macromolecules. This fundamental difference in behaviour between neutral and charged polymer chains in solution shows that the theories describing interactions of neutral polymers (Doi 1986, De Gennes 1979) cannot be employed to the polyelectrolytes.

Recent experimental work in the field of protein-polyelectrolyte interactions was concentrated on the formation of organised structures – multilayers – composed of strong or weak polyelectrolytes with proteins such as bovine serum albumin (BSA), where the reflectometry technique was used to monitor the multilayer formation (Kovačević 2006). Also Westwood et al. (ib 2011) studied a protein-polysaccharide system in organised thin film structures, where quartz crystal microbalance with dissipation, dual polarisation interferometry and atomic force microscopy were applied to determine thickness, density and morphology of poly-L-lysine-polygalacturonic acid multilayer composites and their degradation under the influence of enzyme. The protein-polypelectrolyte structures were reviewed in terms of polyelectrolyte structure in article by Wittemann (2006), where proteins were tested for their interactions with linear polyelectrolytes and spherical polyelectrolyte brushes in a range of concentrations of added electrolyte and pH. However studies on organized protein-polyelectrolyte structures rely on a fundamental understanding of the interactions between proteins and polyelectrolytes. The mechanism of binding of one-to-one or many protein molecules to one polyelectrolyte chain is not well understood as the process is controlled by hydrophobicity and stiffness of the polion chain, charge density and distribution on either protein or the polyelectrolyte. Not only the structural aspects of the interactions driving formation of the protein-polyelectrolyte complexes are critical to understand and control the protein-polyelectrolyte binding, also the range of the interactions is important. The medium-range interactions (up to 10 nm) drive binding of polyanions to the proteins of a negative net charge, commonly referred to as binding on ‘the wrong side’ of isoelectric point or ‘patch binding’. In this case a polyanion can form electrostatic bonds with multiple, small positive patches on the protein surface even if the majority of the protein surface carries the same charge as the polion (Park 1992). The local short-range interactions are more evident in solutions of high ionic strength when the repulsive electrostatic forces are screened by the presence of co-ions. In solutions of very low ionic strength (<0.01 M) interactions are those of long range (10-1000 nm). In such conditions, binding of the polyanion to a net negative protein is not possible (or polycation to a net positive protein) because the
Electrostatic repulsions are too strong to allow the macromolecules to interact at close separation. The binding is only possible when the protein is near to its isoelectric point and has a large well defined positive patch on the surface.

Our main objective for undertaking this study is to improve upon the fundamental knowledge about the mechanisms of protein-polyelectrolyte complex formation, to explore the effects of external stimulants on the interactions between the macromolecules, and to link this knowledge to controlling the fabrication of materials formed by protein-polyelectrolyte systems. The possible applications of this study range from drug delivery systems (Malmsten 2002), food technology (Westwood (a) 2011), biosensors (Franchina 1999) and novel stabilization methods for therapeutic proteins during processing and formulation (Giger 2008). We probe the contributions of electrostatic interactions (long-range), hydrophobic forces and excluded volume effects (short-range) occurring between proteins and polyelectrolytes by monitoring how they depend on salt concentration and salt type (according to the ‘Hofmeister series’) and pH. The components in the tested arrangement are: a strong polyanion, which has a fixed charge in majority of pH range and two globular proteins, which carry both types of charge with its pH dependant distribution and an electrolyte of various valence and species. There is a rising interest in functioning and environmental stimuli of such interactions.

In our study we are focused on determining the effects of ionic strength and type of salt, on the inter- and intramolecular interactions present in aqueous solution between poly(styrene sulfonate) (PSS) and bovine serum albumin or myoglobin. To examine our system, we used light scattering techniques such as static light scattering and turbidimetric titrations, micro differential scanning calorimetry and pH titrations. These techniques were also used by Harnsilawat (2006) to describe a protein- polysaccharide interaction between β-lactoglobulin and sodium alginate. Of particular interest was to examine the early onset of soluble complex formation using static light scattering and pH titrations and to correlate this step with the formation of larger complexes which can be monitored using turbidity. This will allow us to determine whether turbidity can be used to characterise the initial interaction between protein and polyelectrolyte as changes in turbidity correspond to the formation of large aggregates, which would arise from inter-complex interactions as opposed to initial formation of small protein-polyion complexes. We chose to study the interactions with a polyanion (PSS) which is smaller than those typically used in previous studies. This allowed us to use static light scattering to probe the formation of soluble complexes between protein and polyelectrolyte.

Further on in this chapter, we describe the method of sample preparation, and the fundamentals of experimental techniques such as static light scattering, potentiometric and turbidimetric titrations and micro differential scanning calorimetry. Then we present our results and discuss the findings in terms of other recent studies in the area of protein-polyelectrolyte interactions.
3.3. Methodology and sample preparation

3.3.1. Static light scattering (SLS)

Static light scattering is a technique frequently used to characterise the behaviour of macromolecules in solution in terms of intermolecular interactions, the weight average molecular weight, size and configuration of the macromolecules. In this work we applied the SLS measurements to determine the value of the osmotic second virial coefficient $A_2$, and the weight-average molecular weight $M_w$ of the solutions containing a polyelectrolyte or a protein minus that of the solvent.

The SLS measurements were carried out using a DAWN EOS light scattering detector produced by Wyatt Technology, combined with a syringe pump for sample delivery and a computer for data collection and analysis. The instrument contains a laser source of collimated, single frequency vertically polarized light beam, which is directed onto the sample cell surrounded by 18 detectors positioned at different angles. The laser beam is produced perpendicular to the plane in which the intensity and angular dependence of the subsequently scattered light is to be measured. The angles of detection are ranging from 22.5° for first detector to 147° for the last one. In the DAWN EOS instrument, the Ga-As diode is the source of a laser beam with wavelength $\lambda$ equal to 690 nm. The sample was injected at the speed of approximately 40 ml/h using a 2 ml volume syringe equipped with a Whatman filter of pore size equal 0.2 µm. The data was acquired and processed using ASTRA V 5.1.7.3 software.

The measured excess light scattering data is analysed in terms of Zimm plots (Zimm 1948), where the concentration of the analyte is plotted on x-axis and the ratio of $Kc / R_{\theta}$ is plotted on and the Zimm plots are used to calculate the weight-average molecular weight of the particle $M_w$ and the osmotic second virial coefficient $A_2$, which provide information on interactions between two particles in dilute solution. The $A_2$ coefficients were calculated using the following equation:

$$\frac{Kc}{R_{\theta}} = \frac{1}{M_w} + 2A_2c$$

(3.1)

where $R_{\theta}$ is the excess intensity of scattered light at a given angle $\theta$ ($R_{\theta, \text{sample}} - R_{\theta, \text{solvent}}$) (also known as the excess Rayleigh ratio) $c$, $K$ is an optical constant, which is given by:

$$K = \frac{4\pi^2 n_0^2 (dn/dc)^2}{N_A \lambda^4}$$

(3.2)

where $(dn/dc)$ is the refractive index increment, $n_0$ is the refractive index of the solvent, $N_A$ is Avogadro Number ($6.023\times10^{23}$), $\lambda$ is a wavelength of the laser light reaching the detector.
The intercept of the Zimm plot is $1/M_W$ and the value of second virial coefficient $A_2$ is equal to slope of the graph divided by two.

For very small particles such as poly(styrene sulfonate) the angle dependence of scattering is negligible as the characteristic sizes of these macromolecules are in the region of 4 to 5 nm, which is much less than the wavelength of the laser beam, which is 690 nm. For conditions where the size of the scatterer is less than 1/20th the wavelength of the light, the angle dependency is undetectable. The angle dependence of scattered light was measured in the experiments probing the protein-polyelectrolyte complex formation, in these cases, no angle dependence was found therefore was not considered in the data processing.

3.3.2. Turbidimetric titrations

Turbidity is a qualitative measure of the sizes and concentration of particles suspended in solutions. In this method the size and concentration of the particles can be detected through their ability to scatter light. Here, the transmittance of polyelectrolyte solutions is measured while the titrant (or titrator) is added to the solution containing the analyte (or titrand). The turbidity measurement is carried out through the incremental addition of a polyelectrolyte of a known concentration, through the addition of small portions of acid (or base), or by adding a titrant containing a concentrated salt solution. The first type of turbidimetric titrations allows us to determine the transmittance of the tested solution as a function of polyelectrolyte concentration, the second type as a function of pH, and the last type as a function of salt concentration. In this work, we used both turbidimetric methods.

A Brinkmann PC 950 probe colorimeter with the detection wavelength of 490 nm was used to carry out the turbidimetric titrations, as shown in Figure 3.1. The colorimeter was combined with Fisher brand Hydrus 300 pH meter equipped with a combination electrode, where a Fisher Scientific Calomel reference half cell was associated with Ag/AgCl combination half cell.

![Figure 3.1 Schematic diagram of probe colorimeter](image-url)
The colorimeter sends the polychromatic light through the fibre optic light path into the mirror positioned at 2 cm distance from the end of the fibre optic rod. The light is then scattered by the protein particles present in the path length gap, reflected in the mirror and then travels back into the colorimeter through the return light path, where it is filtered to the monochromatic wavelength of 490 nm. The colorimeter detects the transmittance $T$ of the solution, in which the fibre optic probe is immersed. Turbidity of the tested liquids is given by:

$$\text{Turbidity} = 100 - T \%$$

(3.3)

For all the tested samples, we measured a change in turbidity between initial conditions, in which the solutions were prepared, and final stages of the titration. The pH turbidity experiments were carried over the range of pH between 10 and 4, and with solutions containing polyelectrolyte concentrations between 0 to 0.1 g/L. Myoglobin and PSS were prepared in separate bulk solutions of 2 g/L and 0.2 g/L, respectively, whereas BSA and PSS were also prepared in individual bulk solutions of 2 g/L and 0.4 g/L, respectively. For the titrations with respect to pH the experiments were carried out after pH was adjusted to approximately 10 before mixing of PSS and the protein solutions. For each experiment 10 ml of each bulk solution were mixed together in a vessel and stirred slowly. At the beginning of each experiment the transmittance is set to 100 %. Portions of 10 or 100 µL of 0.1 M HCl were titrated into the vessel and the pH and transmittance were recorded after 2 minutes, this period of time was long enough to achieve the equilibrium. The turbidimetric titrations were carried until the large cloudy precipitates appeared, in which case the transmittance readings were not stable due to the sedimentation of the precipitates. In addition, titrations were performed for the same solvent conditions with solutions containing only the protein at the same concentration as used in the protein-PSS titrations.

### 3.3.3. Potentiometric titrations

The potentiometric titration technique was used to establish the effect of PSS concentration and ionic strength on the acid-base equilibria of myoglobin. A range of potentiometric titrations for myoglobin and myoglobin-PSS accompanied by monovalent salt (NaCl) at various ionic strengths were carried out. This gives insight into the pH values where PSS begins to interact with the charged groups on myoglobin. The pH titration experiments were completed within a pH range where PSS behaves like a strong polyanion and myoglobin changes its net charge from negative in solutions at higher pH, to positive in solutions at lower pH. For each set of experiments at a given ionic strength, a titration of a solution containing only myoglobin was performed. Potentiometric titrations (pH titrations) were carried out using a Metrohm meter (model 827 pH lab) combined with a 665 Dosimat Metrohm automatic titrator with an Ag/AgCl combination
probe. All measurements were carried out in a thermostated cell using water bath Julaba F12 under the pressure of argon gas.

The samples tested were composed of myoglobin aqueous solutions prepared at the concentration of 1 g/L in a range of the ionic strengths of 0, 0.01, 0.05 and 0.1 M NaCl. The volume prior to each experiment was set equal to 10 mL and the pH equalled 10. 1 mL volume of PSS solution was then added into the vessel containing the myoglobin solution, resulting in a total analyte volume of 11 mL. The final concentration of PSS in the measuring vessel was $1.43 \times 10^{-5}$ g/L (further described as PSS$_{\text{min}}$) or $5.71 \times 10^{-5}$ g/L (further described as PSS$_{\text{max}}$). The first data point was recorded after 2 minutes from mixing the two solutions of protein and of polyelectrolyte. The potentiometric titration was carried out through the incremental addition of 0.1 M HCl. The portions of acid were 5 or 10 µL and the time between additions was approximately 3 minutes. This allowed achieving the stable pH readings. The titrations were carried out within a range of pH between 10 and 3. In addition, blank titrations were repeated under the same solvent conditions with the same protein concentrations, in which case the PSS addition was replaced with addition of the blank salt solutions. The data recorded was analysed in terms of volume of acid added or myoglobin’s protonation state in function of pH.

3.3.4. Micro differential scanning calorimetry (Micro DSC)

Micro DSC is used to determine transitions in material associated with a heat change. The heat flow measured in this technique can provide information about macromolecular solution transitions such as melting, crystallisation, aggregation or decomposition of the polymers. The Micro DSC technique is often used to detect protein denaturation. The denatured protein has changed structural properties from the native state, which leads to an unfolding enthalpy. The temperature of the transition (melting temperature) is often used to characterise the stability of the protein, as lower melting temperatures correspond to a decrease in stability.

The experiments included in this work were carried out using a SETARAM micro DSC III instrument. At the beginning of the measurement the isothermal equilibration at 20°C was applied for 10 min, then the sample cell and the reference cell were heated from the temperature of 20 to 90°C and the heat flow was set at 1°C/min. The Micro DSC detector recorded the differences in heat effects of the sample and that of the reference. The sample cell contained the protein and polyelectrolyte or the protein only solutions and the reference cell was filled with the corresponding solvent without any macromolecules. We examined the same solvent conditions as used for the potentiometric titrations, except the concentrations of protein and polion were 10 times greater than that used in potentiometric titrations, the ratio myoglobin-PSS was kept at the same as for the potentiometric titrations. The Micro-DSC tests were carried out at pH 6 and 9.
3.4. Results and discussion

The intermolecular behaviour of PSS, BSA, or PSS and BSA in aqueous solutions with mono- and divalent salts was monitored using static light scattering (SLS) and turbidimetric titrations. To determine whether the particles formed attractive or repulsive interactions we analyzed changes of the second osmotic virial coefficient ($A_2$) for solutions containing only one molecule. The differences in weight-average molecular weight ($M_w$) throughout the SLS experiment allowed monitoring the onset of the complex formation in protein-polyelectrolyte mixtures. Changes of transmittance of the liquid samples measured in turbidimetric titrations indicated growth of larger aggregates. Potentiometric titrations were applied to monitor the influence of PSS on myoglobin and examine whether polyanion can inhibit the self-aggregation of the protein. Micro DSC was used to measure the denaturation and melting temperature of PSS-myoglobin complexes, which was used as an indicator of conformational changes to the protein native structure and stability.

3.4.1. Intermolecular interactions of polyelectrolyte or protein in presence of mono or divalent co-ions

The angle independent SLS experiments were performed for aqueous solutions containing PSS in the presence of monovalent (Na$^+$, Li$^+$) and divalent (Mg$^{2+}$) co-ions at pH 7. In each experiment, a minimum of eight PSS samples was used with increasing PSS concentration. Figure 3.2 illustrates examples of Zimm plots for the 90° angle detector obtained for solutions of NaCl at various ionic strengths. Data points are shown for each sample and a linear trendline was fitted to the data obtained for each experiment. The slope of the Zimm plot yields the osmotic second virial coefficient $A_2$ and the intercept is equal to the inverse of an apparent weight-average molecular weight $M_w$. The method of extracting these values is described in section 3.3.1.

In Figure 3.3 is shown a graph of measured $A_2$ values as a function of ionic strengths for PSS solutions containing either: NaCl, LiCl or MgCl$_2$. We established that there is an ionic strength dependence of the second virial coefficient ($A_2$). Within increasing ionic strength from 0.1 M to 0.5 M with monovalent salt, $A_2$ decreases rapidly, whereas for ionic strength above 0.5 M, $A_2$ is insensitive to ionic strength. This behaviour is consistent with studies by Gao and Dubin on polyelectrolyte solutions, where increasing salt concentration screens both intra- and intermolecular electrostatic repulsion of PSS (Gao 1998). The effect of changing from Na$^+$ to Li$^+$ is very small as a similar result is obtained for solutions of 0.5 M or greater. We also established that changing from a monovalent to a divalent salt has a dramatic effect on $A_2$. As shown on Figure 3.3, the value of $A_2$ in a solution of 0.1 M MgCl$_2$ is similar to that obtained in a solution of NaCl at 1 M concentration. Thus magnesium chloride reduces the amount of inter and
intramolecular repulsion on PSS chains at a much smaller ionic strength than that of LiCl or NaCl.

Figure 3.2 Zimm plot obtained for PSS dissolved in aqueous solutions containing 0.1 M NaCl (♦), 0.25 M NaCl (■), 0.5 M NaCl (▲) and 1 M NaCl (■) at pH 7

Figure 3.3 Relationship between the second virial coefficient $A_2$ and ionic strength of solution for mono- and divalent ions, where NaCl (♦), LiCl (▲) and MgCl$_2$ (■)

In Figure 3.4 is shown a plot of apparent weight-average molecular weight $M_w$ of PSS versus ionic strengths of solutions for mono- and divalent ions. The reduction of apparent weight-average molecular weight, observed on Figure 3.4, is due to a decrease in the refractive index
increment $dn/dc$ with an increasing salt concentration. In this work we used a constant $dn/dc$ ratio for PSS solutions equal to 0.1657 ml/g (measured). As a consequence, the decrease of the apparent $M_w$ is illustrating the real change of the $dn/dc$ parameter following addition of salt. This method allows predicting the theoretical value of the refractive index increment ($dn/dc$) at any salt type or concentration by fitting the apparent $M_w$ to the real weight-average molecular weight of PSS. The value of ($dn/dc$) decreases due to the preferential exclusion of salt ions from regions immediately neighbouring polyelectrolyte macromolecules. Thus, in the further work on this project, this method will be used to characterize the interaction between the polyion and the salt.

Further we examine the static light scattering data obtained from measurements of bovine serum albumin solutions in presence of NaCl and LiCl in relation to ionic strength at pH 7, where we test the protein for possible self-aggregation and excluded volume effects caused by increasing salt concentration in pH region where we would expect the onset of BSA-BSA attractive electrostatic interactions. In Figure 3.5 is shown the plots of $A_2$ and $M_w$ of BSA in presence of NaCl and LiCl in relation to ionic strength between 0.01 to 0.15 M. We captured the subtle transition in intermolecular behaviour of BSA in rising ionic strength; this can be observed from changes in values of $A_2$ for solutions in 0.1 and 0.15 M of LiCl, positive $A_2$ suggest that interactions were mainly repulsive in lower ionic strength and changed to slightly attractive in higher ionic strength as the value of $A_2$ falls. The salt type within the same valence seems to have a more evident influence in ionic strength of 0.15 M, where presence of LiCl in BSA solutions can change the character of interactions from repulsive to attractive. In the case of
NaCl, the repulsive character of BSA-BSA interactions is screened with lesser efficiency. Below ionic strength of 0.1 M both salts show no difference in value of $A_2$, this may suggest that specific ion effects within the same ion valence is stronger at higher salt concentrations. This tendency was also observed by Park and Choi (2009) in their study involving measurements of second virial coefficients of BSA aqueous solutions in presence of different salts at wide range of ionic strengths.

The shift from repulsive to attractive interactions is major, however this cannot be observed on the $M_w$ plot, where no major difference in weight-average molecular weight was detected, as the possible increase of $M_w$ is related to existence of irreversible aggregates formation. At pH 7 there are large negatively charged patches on the surface of BSA still present and short-lived attractive interactions in 0.15 M LiCl. To detect if the value of $M_w$ changes throughout pH between 8 and 6 we measured the weight-average molecular weight for solutions with 0.1 M NaCl and 0.1 MgCl$_2$. No major difference in the size of BSA macromolecules was detected and $M_w$ equaled approximately 71,000 Da. This suggests that in the solutions of low ionic strength and wide range of pH BSA is present in its monomeric form.

Figure 3.5 Second virial coefficient ($A_2$) and weight-average molecular weight ($M_w$) of BSA in solutions with LiCl and NaCl, where LiCl at pH 7 (▲) and NaCl at pH 7 (values of $A_2$ Tessier 2002, Vilker 1981) (▲)

3.4.2. Protein-polyelectrolyte (BSA-PSS) complex formation in changing pH and polyelectrolyte concentration – light scattering and turbidimetric analysis

Formation of the protein-polyelectrolyte complexes was monitored using two experimental methods: static light scattering (SLS) and turbidimetric titrations. We chose the same macromolecules – BSA and PSS – as in the introductory study described in section 3.4.1., but here we combined them in the same sample, through this we allowed the protein and polyelectrolyte to interact in chosen conditions. Examining the mixtures of protein with
polyelectrolyte with use of SLS permits to measure weight-average molecular weight of small complexes between one BSA and one PSS macromolecule or slightly bigger BSA-PSS domains. On the other hand, formation and growth of much bigger complexes can be monitored using turbidimetric titration, where the complex-complex interactions can be detected.

In Figures 3.6, 3.7 and 3.8 there are shown the plots of changes in turbidity with respect to pH for the solutions containing 1 g/L of BSA or/and 0.2 g/L of PSS with added salts: NaCl, LiCl and MgCl$_2$ in range of ionic strengths between 0.01 and 0.45 M. The mass (and molecular weight) ratio between interacting BSA and PSS was kept at 1 to 5, respectively. At these concentrations, a clear increase in the turbidity could be observed with decreasing pH. In initial turbidimetric titrations we applied the following concentrations: 1 g/L of BSA and 0.1 g/L of PSS, where mass ratio was at 1 to 10 respectively, but there was no change in turbidity throughout entire range of pHs between 10 and 3.5. This could be due to an insufficient number of PSS molecules to bind to BSA. As a consequence, any formed complexes are of too small size to elevate turbidity of the tested solutions.

For all salt conditions, we conducted a blank titration with BSA in the corresponding salt solution to determine turbidity changes caused by the protein self-aggregation. For all ionic strength conditions and three tested salts we found no significant increase of turbidity in samples containing BSA only. A slight increase in turbidity at low pH (about 4.5) was observed for the samples at 0.45 M salt concentration. The reason for this increase is not clear. An opposite effect was observed in samples where BSA was mixed with PSS; these solutions become turbid with no exception. When pH was lowered by addition of small portions of 0.1 M HCl the net positive charge of BSA increases. The rise of turbidity was caused by the electrostatically driven attraction between the positively charged parts of BSA and the negatively charged PSS molecules.

The results we obtained for BSA and salts titrations showing that there is no self-aggregation into large complexes of BSA in solutions of NaCl, LiCl or MgCl$_2$ over the tested pH range. This is consistent with the finding that the $A_2$ values of BSA remain positive over all pH values for solutions in 0.15 M NaCl, indicating that protein-protein interactions are repulsive (Vilker 1981). On the other hand, we did find that protein-protein interactions are attractive in solutions of LiCl at 0.15 M salt concentration. Still, this is not associated with an increase in turbidity, because the turbidity can only detect large complexes of protein or macroscopic phase separation (i.e precipitation), neither of which occur in the LiCl solutions. The SLS study shown in Figure 3.5 suggests that the self-association is unlikely to be possible as the weight-average molecular weight increases too slowly with decreasing pH to reflect permanent aggregation of BSA. A similar trend was observed in a study of human serum albumin in solutions with caesium chloride (CsCl) – salt containing heavier cation than Na$^+$, Li$^+$ or Mg$^{2+}$, but in case of Cs$^+$ the aggregation effect was much stronger across pH region between 8 and 2 and ionic strengths 0.2 to 0.6 M (Baranov 2004).
Figure 3.6 Turbidity curves for solutions of BSA and BSA-PSS complexes in NaCl at various ionic strengths
Figure 3.7 Turbidity curves for solutions of BSA and BSA-PSS complexes in LiCl at various ionic strengths.
Figure 3.8 Turbidity curves for solutions of BSA and BSA-PSS complexes in MgCl₂ at various ionic strengths
For all pairs of titrations - the BSA only titration and the corresponding one with BSA and PSS at the same salt concentration - we analysed the change of turbidity against pH. In Figure 3.9 is shown the example of titration performed at the same salt conditions and BSA with or without PSS. The pink marked data correspond to the titration of BSA-PSS mixture in 0.01 M NaCl. As the titration progressed from high to low pH, the turbidity rises near pH 5.3. The solution became slightly turbid because the pH 5.3 data point is shifted from the plateau region formed by the data points of turbidity in pH > 5.3. The rise of turbidity at pH 5.3, implies that there are strong attractive complex-complex interactions and the size of protein-polyelectrolyte complexes had reached the detection range of the colorimeter. We identified a characteristic pH of such change in complex size by drawing a straight line across all the points in the plateau and then choosing the last point along that line. The pH at that point is referred to as the \( pH_\phi \), in case of example shown on Figure 3.9 \( pH_\phi \) equals 5.5. An analogous method of defining the characteristic pHs is described by Mattison and Dubin (1995) in studies of BSA – poly(diallyldimethylammonium chloride) PDADMAC interactions with respect to pH and ionic strength. Further decrease of pH below the \( pH_\phi \) led to the formation of very large complexes, which could be visible by the naked eye. These complexes affected turbidity considerably and at pH 4.5 large precipitates were present within the entire volume of the tested solution. We determined the values of \( pH_\phi \), using the method described above, for all salt conditions presented in Figures 3.6, 3.7 and 3.8. The results are presented in Figure 3.10, where \( pH_\phi \) are plotted with respect of changing ionic strength.

\[ \text{Figure 3.9 Example of turbidimetric titration of BSA (♦) and BSA-PSS (♦) for NaCl at ionic strength of 0.01 M NaCl with identified } pH_\phi \]

![Graph showing turbidity change against pH with identified pH_\phi](image-url)
The trends of BSA-PSS phase transitions in low ionic strength vary greatly with respect to the salt type. For the monovalent salts, NaCl and LiCl, with increasing salt concentration, the $pH_\phi$ decreases indicating that the salt ions are screening the macromolecular electrostatic interactions; at higher ionic strength more positive charge on the protein is needed to form complexes with the negatively charged PSS. This result is consistent with the studies by Seyrek (2003) on solutions of BSA and heparin, in which case the $pH_\phi$ decreases with increasing ionic strength over the range of 2 to 150 mM.

There is a major difference in pH values corresponding to phase boundaries between macromolecularly condensed and macromolecularly poor phase for complexes in presence of MgCl$_2$ to either of NaCl or LiCl. The trend in screening of the intermacromolecular attractions in MgCl$_2$ is opposite to monovalent salts. In low ionic strength solutions of MgCl$_2$ (0.1 M) the complex formation was suppressed until pH as low as 4.8, this is considerably below the isoelectric point of BSA defined by Tanford to be equal 5.67 (1955). This result implies that magnesium ions are able to screen the interactions between positively charged BSA and negatively charged PSS to a greater extent than LiCl or NaCl under low ionic strength conditions. This screening ability of magnesium ions is also reflected by $A_2$ data presented earlier, in which case the polion intermolecular electrostatic attractions are much weaker in MgCl$_2$ solutions this may be due magnesium ion ability to collapse the polyanion chain. The collapse could also reduce the accessible binding area of polion. In contrast lithium ions showed the weakest influence on suppressing protein-polyelectrolyte interactions at low ionic strength conditions.
The value of $pH_\phi$ in solutions with 0.01 M LiCl equals 5.72, which is slightly above the reported value of BSA isoelectric point, indicating that BSA has a net negative charge when binding to PSS under these conditions. Such behaviour, where large protein-polyion complexes are formed on the ‘wrong’ side of $pI$ was also reported by Park and Dubin (1992) and by Wang and Dubin (1996) during their study on cationic polyelectrolyte: PDADMAC and the model proteins BSA, $\beta$-lactoglobulin, $\gamma$-globulin, and ribonuclease A. It was suggested by Wang that the phase behaviour in the presence of salt may also depend on the molecular weight of the polyelectrolyte. In our study we tested PSS of 70,000 Da only, however an earlier study on BSA-PSS interactions in solutions with added NaCl by Zhang (2007) showed that characteristic $pH$s, obtained by the same method as we defined our $pH_\phi$s, are much higher for PSS of molecular weight equal to 1,360,000 Da. The data, shown in Figure 3.11, indicate that by reducing the size of the PSS in turns weakens the binding affinity for proteins. The main reason for such large difference in $pH_\phi$s obtained by Zhang and $pH_\phi$s obtained in our study is that the BSA-PSS complexes are much smaller at the onset of complex formation. As a consequence, the observation of binding on the wrong side of $pI$ is much stronger when studying solutions with high molecular weigh polions.

![Figure 3.11 Specific pH values for BSA-PSS phase transitions, where $pH_\phi$s of BSA-PSS in NaCl (□) Zhang (2007)](image)

In our study, we have chosen to examine the small molecular weight polyelectrolyte, because this allows us to determine the early onset of BSA-PSS complex formation at higher $pH$ values than the $pH_\phi$ using the SLS technique. The weight-average molecular weight $M_w$ of the complexes can be obtained from the light scattering data. In Figure 3.12 and 3.13 there are plots illustrating an increase of $M_w$ related to the decrease of $pH$ and in relation to type of salt. In Figure 3.13 is shown the difference in increasing of $M_w$ for BSA-PSS complexes between two monovalent salts with respect to $pH$ and ionic strength. The complexes based on BSA-PSS interactions in NaCl show a minor increase of the average molecular weight with decreasing $pH$ from 8 to 6. This is expected as the protein gains positive charge with decreasing $pH$. Interestingly, through SLS tests, we do find binding to the protein even at $pH$ values much above the $pH_\phi$. This indicates that the turbidity results are not detecting
the early formation of protein-polyion complexes, for the polyions of similar molecular weight as the protein. The $M_w$ corresponding to the pH region between 8 and 6 ranges between 180,000 and 360,000 Da, which suggests that the complexes are, on average, composed of two or three macromolecules. We also performed light scattering on solutions containing only PSS and found a weight average molecular weight of 75,000 Da (Figure 3.4), and for BSA we obtained the average molecular weight of 70,000 Da (Figure 3.5). We expect that the complexes formed in NaCl containing a maximum of one polyion chain with a couple of monomeric BSA globules, and in MgCl$_2$ a maximum of two BSA globules with two or three polyion chains. It is more likely that the complex contains multiple protein monomers. This is because the entire surface of the polyion is available for binding to the protein, whereas only part of the protein surface (in the pH range of 6 to 8) will be available for binding to the polyion.

We excluded the possibility that complexes observed in Figures 3.12 and 3.13 are related to the self-aggregation of BSA monomers. Our SLS measurements for BSA accompanied by NaCl established that at pH 6 and 0.1 M NaCl the value of apparent $M_w$ for BSA does not exceed 75,000 Da, this implies that there is no possibility of one-to-one BSA-BSA complex formation causing the increase of $M_w$ in complexes detected here, for that reason we are certain that the $M_w$ illustrated in Figures 3.12 and 3.13 reflects the complex built of one macromolecule of BSA and one of PSS.

For solutions containing MgCl$_2$, the increase of $M_w$ is much greater when decreasing pH from 8 to 6. Near pH 6 the BSA-PSS complexes have weight average molecular weight of 360,000 Da, which is at least twice as large as the complexes formed in NaCl at the same pH. The difference between BSA-PSS binding in presence of sodium and magnesium ions may be due to the ability of magnesium ion to strengthen the connections between two macromolecules and
actively participate in complex formation, whereas in solutions with sodium ion, the salt is able to screen repulsive interactions between the negatively charged parts of BSA and the polyanion. This would then limit the sizes of the complexes to contain only one polyanion. This does not appear to be the case in solutions of MgCl₂, in which case, it might be possible that a greater part of the protein surface is available for binding polyanions due to screening or binding of magnesium ion to negative parts of the protein surface.

![Figure 3.13 Changes in weight-average molecular weight of BSA-PSS complexes in presence of NaCl and LiCl, where 0.05 M NaCl (△), 0.15 M NaCl (▲), 0.3 M NaCl (▲), 0.05 M LiCl (●) and 0.15 M LiCl (=)](image)

The change of $M_w$ of BSA-PSS complexes within the range of tested pHs shows that the complex formation progressed slowly near pH 7 when near pH 5 the rise in average size of complexes was faster. The value of $M_w$ for complexes in 0.05 M NaCl equals 210,000 Da, in pH 7 and the same salt conditions the $M_w$ was near 160,000 Da. The trend, related to macromolecular interactions in presence of NaCl, demonstrates that lower ionic strength allowed the formation of larger complexes, when higher ionic strength in the BSA-PSS solutions promoted formation of smaller complexes. The difference in $M_w$ between 0.05, 0.15 and 0.3 M NaCl appears to be constant and on average it equals 2,000 Da. The same tendency can be observed for BSA-PSS complexes in solutions with LiCl, apart from that the total size of complexes is larger than these in NaCl. A key finding is that the $M_w$ trends are consistent with the pH trend detected by turbidity measurements, as shown in Figure 3.10. Large BSA-PSS complexes in low ionic strengths could be detected in higher pH region for LiCl than for NaCl. In addition, the $pH_π$ for monovalent salts decreases with increasing ionic strength, which is consistent with the reduction in $M_w$ with raising ionic strength. This relation confirms that in low ionic strength there is a substantial difference in the behaviour of macromolecules even if the associated ions are of the same valence. The decrease of binding with raising ionic strength of either of monovalent salts is due to the screening of the long-range attractive electrostatic interactions in solutions with higher concentration of co-ions. Consequently in higher ionic
strengths the growth of complexes follows the same trend with pH but the complexes are much smaller than those in low ionic strength.

The BSA-PSS structures were studied by Chodankar (2008) using small-angle neutron scattering (SANS) measurements and turbidimetric titrations. The interactions were defined using two types of macromolecular behaviour leading to the phase separation, First liquid-liquid phase separation, where one phase is composed of concentrated macromolecular liquid and other phase consists of dilute macromolecular liquid, and such phase behaviour is referred to as the coacervation. The second type of phase separation involves solid-liquid separation, where the macromolecular material precipitate out of solution. It was observed that complex formation leading to coacervation occurs as the pH is lowered towards isoelectric point of BSA (at 4.7).

The SANS result showed that complexation between BSA and PSS take place near pH 6.5, but not near pH 7.5 in 0.5 M NaCl. However, our SLS test on BSA-PSS, presented in Figure 3.12, showed that even at pH 8 and 0.1 M NaCl there are complexes of a total $M_w$ in the region of 160,000 Da. This implies that even at high pH the BSA macromolecule can form non-covalent connections with PSS. Thus, it may not be so surprising because high molecular weight PSS can form complexes with BSA at pH values around 7.5 (see Figure 3.11).

3.4.3. Protein-polyelectrolyte (Myoglobin-PSS) complex formation – turbidimetric, potentiometric and Micro DSC analysis

In this section we study the complex formation between myoglobin and PSS in aqueous solutions associated with sodium chloride at a range of ionic strengths from 0.01 to 0.45 M. First we present turbidimetric titrations of myoglobin and myoglobin-PSS solutions with added salt. These were performed by adding aliquots of 0.1 M HCl into solutions of 0.1 g/L PSS and 1 g/L myoglobin, which were initially prepared at a basic pH. This was because, in solutions at high pH, myoglobin has a large net negative charge, which prevents the protein from interacting with PSS. Examples of turbidimetric titration curves for solutions of myoglobin at 0.05 and 0.45 M ionic strengths of NaCl are presented in Figures 3.14, 3.15 and 3.16.

In Figure 3.14 we illustrate the changes in turbidity for pairs of turbidimetric titration experiments involving myoglobin and myoglobin-PSS, each with added salt of the same ionic strength. The analysis of each pair allows determining differences in turbidity for myoglobin solution with added PSS or without. For solutions of myoglobin with NaCl of ionic strength below 0.3 M the turbidity increases in pH region near 7.5, which implies that myoglobin undergoes self-aggregation. For solutions of ionic strength 0.3 M NaCl and greater, the salt suppresses the self-aggregation. Most likely, the self-aggregation is driven by attractive electrostatic interactions because increasing salt concentration reduces the aggregation levels.

On the other hand, in low ionic strength solutions where PSS was present, the self-aggregation of myoglobin was suppressed at intermediate pH as reflected by a lower turbidity in the solutions of PSS and myoglobin versus those just containing myoglobin. This trend indicates the polyelectrolyte interacts with myoglobin at pH values as high as 8.5 where myoglobin carries a
net negative charge. The ability of PSS to break up the aggregates of myoglobin is probably related to electrostatic interactions. As mentioned before, the self-aggregation is driven by attractive electrostatic interactions between myoglobin molecules which occur between patches of opposite charge on myoglobin. It is possible that the PSS binds to the positively charged patches on myoglobin thereby preventing the charged patch from forming self-aggregates. Nevertheless, an increase in solution turbidity is observed at lower pH values (approximately 6.5 to 7) in the myoglobin-PSS mixtures. We hypothesize that this increase in turbidity is linked to complex formation between protein and PSS and not self aggregation of myoglobin.

Figure 3.14 Turbidimetric titrations of myoglobin (♦) and myoglobin-PSS (■) for NaCl at ionic strength of 0.01 M, 0.05 M, 0.1 M, 0.15 M, 0.3 M, 0.45 M
Figure 3.15 Turbidity curves for solutions of myoglobin and myoglobin-PSS complexes in NaCl at various ionic strengths.
Figure 3.16 Turbidity curves for solutions of myoglobin and myoglobin-PSS complexes in MgCl2 at various ionic strengths
In solutions at ionic strength greater than 0.3 M of NaCl, myoglobin self-aggregation was not observed in terms of the turbidity measurement. For these salt conditions, we still observed an increase in the turbidity of the solutions containing the mixture of protein and PSS. This increase occurs most likely due to the attractive electrostatic interactions between the negatively charge polyanion and positively charged patches of myoglobin. The finding that the increase in turbidity occurs in the absence of self aggregation provides further support, that the increase observed in low ionic strength solutions is also due to myoglobin-PSS interactions.

The interactions of myoglobin and PSS in solution with MgCl$_2$ followed different pattern than that of solutions in NaCl. The increase in turbidity for PSS-myoglobin solution was almost immediate at the beginning of measurement even at pH as high as 9.5. This increase was not observed in the solutions containing only myoglobin indicating that an interaction between PSS and myoglobin does occur at high pH in the presence of magnesium. This may be due to presence of positively charged divalent magnesium ion, which is more effective at screening the interaction between the polyanion and the negatively charged parts of myoglobin.

In order to investigate the difference between sodium chloride and magnesium chloride further, we used turbidity titrations at constant pH with changing salt concentration on the myoglobin and PSS mixtures as well as on myoglobin only solution. The results are shown in Figures 3.17 and 3.18, where the decrease of turbidity in solutions of 1 g/L myoglobin and of 1 g/L myoglobin with 0.1 g/L PSS was observed upon addition of 100 µL of 4 M NaCl (Figure 3.17) or 4 M MgCl$_2$ (Figure 3.18). The solutions were prepared in 20 mM phosphate buffer to maintain pH equal 7.

![Figure 3.17 Change in turbidity for solutions of myoglobin-PSS (■) or myoglobin only (♦) with respect to ionic strength of added NaCl](image)
In myoglobin only solutions, the increasing salt concentration lowers turbidity. This effect is most likely due to breaking up myoglobin aggregates, which are forming near pH 7 and low ionic strength. This result is consistent with the previous hypothesis that self-aggregation of myoglobin is driven by attractive electrostatics. There is not much difference between the solutions of sodium chloride and magnesium chloride indicating that the effect is a non-specific electrostatic interaction.

In PSS-myoglobin solutions the effect of added salt causes a slightly smaller decrease in turbidity versus the myoglobin only solutions. This indicates that the predominant effect of salt concentration is breaking up of myoglobin self-aggregates. We propose that the reason for the smaller decrease in turbidity in the mixtures might be due to the lower concentration of myoglobin self-aggregates. This is caused by the presence of PSS and high likelihood that myoglobin-PSS interactions are favoured over myoglobin-myoglobin attractions. Also the presence of PSS in myoglobin solutions at the start within the phosphate only buffer could support breaking up the protein aggregates before any salt was added. This is consistent with results that are illustrated in Figure 3.20, and show a slight decrease in turbidity in a titration where PSS is added to only the myoglobin solution in phosphate buffer.

Most importantly, the comparison of the turbidity result between experiments when NaCl was interacting with macromolecules between the result of experiment when MgCl₂ was used demonstrated that there is not a large difference in the effects of magnesium chloride and in sodium chloride. According to the pH, turbidity titrations of myoglobin-PSS mixtures in magnesium chloride, one might have expected a large increase in turbidity when increasing magnesium chloride concentration as a large turbidity is observed at pH 7 in the pH titrations. This increase is not observed when titrating with magnesium chloride indicating that the pH

Figure 3.18 Change in turbidity for solutions of myoglobin-PSS (■) or myoglobin only (♦) with respect to ionic strength of added MgCl₂
titration is irreversible. Most likely in the solutions at high pH, an irreversible change to myoglobin occurs due to partial unfolding, which leads to the high turbidities at intermediate pH. To define characteristic pH regions for the myoglobin-PSS interactions we used a similar approach as in section 3.4.2., where we analysed BSA-PSS phase behaviour. Apart from identifying \( pH_\phi \) s, we also defined another characteristic pH value - \( pH_c \), which reflects the pH corresponding to decrease of turbidity, where PSS stabilised myoglobin and prevented its self-aggregation. The existence of \( pH_\phi \) s is unique to myoglobin-PSS interactions and it was not noticed in turbidimetric tests of BSA-PSS interactions. In addition, we report a critical pH value for the myoglobin self-aggregation which corresponds to the increase in turbidity in the myoglobin only solution, an effect which was only observed below an ionic strength of 0.3 M. This analysis is only done for the sodium chloride solutions due to the irreversible effects observed at high pH in solutions of magnesium chloride. The results are given in Figure 3.19.

![Figure 3.19 Specific pH values for myoglobin-PSS phase transitions, where \( pH_c \) of myoglobin-PSS in NaCl ( ), \( pH_\phi \) of myoglobin-PSS in NaCl (■), inset plot shows the method for defining for values of \( pH_c \) and \( pH_\phi \) where change in turbidity of myoglobin only solution ( ) and myoglobin-PSS (■)](image)

The phase transitions in solutions containing a mixture of PSS and myoglobin and corresponding to defined \( pH_\phi \) values show increasing trend with rising ionic strength, this is in contrary to what has been observed for BSA and other protein-polyion systems. If the \( pH_\phi \) was controlled by attractive electrostatic interactions between protein and polyion, the value should decrease with increasing ionic strength due to screening the interactions.
We propose that, the difference in mechanism of protein-polycation interactions originates from the existence of oligomeric aggregates of myoglobin even at high pH, where myoglobin is primarily negatively charged, whereas BSA remains in its monomeric form throughout pH between 10 and 5.5. One possibility is that with increasing ionic strength, there is less myoglobin involved in self-aggregation, and consequently more available to interact with the polycation leading to the increase in the $pH_p$ with increasing ionic strength. Similar to the BSA results, we find that the $pH_p$ value is below the isoelectric point of myoglobin ($pI$ equal to 7.2 based on study by Kendrew (1950) and Dumetz (2007)). However, this does not reflect the formation of soluble complexes between protein and polycation. As reflected by the $pH_p$ in Figure 3.19, interactions between myoglobin and PSS do occur at pH values around 8.5 as reflected by the effect of PSS on the aggregation of myoglobin. In addition, in solutions of magnesium chloride, interactions of PSS and myoglobin occur at pH values as high as 9.5. These results indicate that PSS does interact with myoglobin when the protein has a net negative charge.

Next, we probe the complex formation between myoglobin and PSS using turbidimetric measurements at constant pH and titrating a solution of PSS or salt. In the first test we titrate 100 µL portions of 1 g/L PSS into 10 mL of 1 g/L myoglobin solution with added 20 mM phosphate buffer to maintain a constant pH equal 7. As we showed in turbidimetric titrations with respect to pH, presented in Figures 3.15 and 3.16, we expected that at pH 7 the complex formation and growth between myoglobin and PSS will be instant, because in this pH myoglobin’s net charge is positive ($pI$ equals 7.2). The change in turbidity was first detected after the concentration of PSS reached 0.6 mg/L. This indicates that a mass concentration of PSS as low as $8.5714 \times 10^{-9}$ is able to support formation of complexes large enough to be detected through the measurements of turbidity, consequently we conclude that there is a number of myoglobin monomers bound to one polycation chain.

![Figure 3.20 Change in turbidity with respect to concentration of PSS](image-url)
In order to further investigate the interaction of PSS with myoglobin at high pH, we used potentiometric titrations (pH titrations) of aqueous solutions containing myoglobin or myoglobin and PSS followed by Micro DSC thermograms obtained for myoglobin-PSS complexes. The pH titrations start from pH 9.5 and finish in solutions with pH 3.5. An example of a raw titration curve is presented in Figure 3.21, where all titration experiments for myoglobin and NaCl solutions start from basic pH region after which 10 µL of 0.1 M HCl is titrated into the system. This is followed by protonation state $\Delta Z$ of the protein shown in Figure 3.22.

![Figure 3.21](image1.png)

**Figure 3.21 Potentiometric titration curves for myoglobin associated with salt**

![Figure 3.22](image2.png)

**Figure 3.22 Protonation state of myoglobin in changing pH at various ionic strengths**
At pH 9.5, myoglobin has a large net negative charge, which prevents the protein from interacting with itself or polyanion. As acid was added to the solution, the concentration of hydronium ions H$_3$O$^+$ in the solution increased. The excess of hydronium ions could either change the pH of the solution or change the protonation state of protein. Thus, as acid was titrated into the system, the positive charge on the surface of myoglobin increased and eventually the protein was able to interact with the negatively charged patches on the surface of other myoglobin monomers. For the quantitative assessment of the level of protonation on myoglobin’s chain, we calculated the change of protonation state using a mole balance, where hydronium ions are either consumed by the water equilibrium or change the protonation state of myoglobin. Knowing the change in the protonation state of the protein as a function of pH aids in visualizing the binding conditions for myoglobin’s positively charged groups. In addition, we examined the influence of salt on bare myoglobin to illustrate the critical pH values corresponding to the changes of charge density and distribution within the protein in its native state. This initial test helps to assess the possible structural changes caused by the addition of the polyelectrolyte.

We assumed that in solutions at pH near 8.5, myoglobin is structurally in its native state and salt influence at low ionic strengths is minor. Our assumption is based on titration curves of sperm whale metmyoglobin by Breslow (1963). The metmyoglobin solution was titrated from pH 9 to 3 and from pH 3 to 9 in its native and denatured forms. It was found that the number of available protonated groups is minimal at pH equal 8.5 and charge distribution at this pH was the same for native and denatured metmyoglobin. Therefore the titration curves shown in Figure 3.22 (and Figure 3.23) are set to the same charge conditions of myoglobin at pH 8.5, where we assumed that change in protonation state ($\Delta Z$) of myoglobin equals 0 for all salt conditions. The pH curves, shown in Figure 3.22, indicate the dependency on ionic strength of the protonation state of histidine groups, which become available (charged) for titration with acid in solutions at pH approximately equal to 6.8 and for carboxylic groups in solutions with pH approximately equal to 4.4 (Janssen 1972). Increasing salt concentration stabilizes the more charged state of the protein in solutions at low or higher pH. This effect, as shown in Figure 3.22, is most dramatic in solutions up to 0.05 M ionic strength. Above this salt concentration, the effect is screened out. The titration curve in solutions without any salt is significantly different. Most likely, conformational changes of myoglobin have occurred in the absence of salt due to long-ranged intramolecular electrostatic repulsion which is magnified without any salt screening.

Next, we present titration curves in Figure 3.23 obtained for solutions containing both myoglobin and PSS, at two different PSS concentrations, ($PSS_{\text{min}}$ equal to 1.43·10$^{-5}$ g/L and $PSS_{\text{max}}$ equal to 5.71·10$^{-5}$ g/L). The curves correspond to different salt concentrations equal to 0.01 M, (a) 0.05 M (b) and 0.1 M (c). In each case, we find that the titration curves are independent of the presence of PSS in the system for pH values greater than 8. This indicates that the titration curves are insensitive to interactions between the protein and polion if they occur. No interaction is expected at high pH due to the large net negative charge on myoglobin, which
would lead to strong electrostatic repulsion with the negatively charged PSS. However, the turbidity results at low salt concentration do appear to reflect an interaction between protein and PSS at pH values of 8. In solutions, at pH near 7.5 there is a change in ionization behaviour of myoglobin caused by the presence of PSS. More specifically, for each salt solution, we find that the titration curves diverge from each other at pH values around 7.7, 7.3, and 6.7 in solutions of 0.01 M NaCl, 0.05 M NaCl, and 0.10 M NaCl, respectively. This indicates that the PSS changes the $pK_a$ of the ionisable protein amino acids, which are most likely histidine groups as these are the only groups with $pK_a$ values around neutral pH. The perturbed $pK_a$s most likely result from a binding interaction between PSS and myoglobin. Consequently, the pH where the curves diverge reflects a critical pH associated with protein-polyelectrolyte binding. The critical pH values are greater than the $pH_\phi$ and are probably linked to formation of protein-polyion complexes. The dependence of these critical pH values on ionic strength is consistent with an electrostatic binding mechanism as the pH of binding decreases with increasing pH.

![Figure 3.23 Titration curves for myoglobin and PSS, where a) presents the titration in presence of 0.01 M NaCl, b) of 0.05 M NaCl and c) of 0.1 M NaCl, for myoglobin-PSS$_{\text{min}}$ (♦), myoglobin-PSS$_{\text{max}}$ (■) and myoglobin without PSS (▲).](image-url)
The mechanism of binding histidine groups changes depending on PSS concentration. The effect of PSS on the titration curve is less pronounced for solutions with pH near to $pK_a$ values of carboxylic groups. We would expect that binding of the polyelectrolyte to the native state of myoglobin would lead to stabilization of the ionized state of histidine due to the electrostatic interaction with the charged side chains of PSS. However, the results indicate that binding is linked to a decrease in the charged state of the protein at low PSS concentration. This discrepancy appears to indicate that PSS binding leads to conformational changes to myoglobin, an effect which has been observed for other proteins such as lysozyme or cytochrome C in the presence of PSS (Sedlak 1998). The conformational changes could correspond to changes in both the secondary and tertiary structure of myoglobin and changes in the environment of the histidine groups, which could alter their $pK_a$s. This finding is also consistent with study by Zhang (2007), which found that myoglobin binding to PSS was associated with a change in the soret absorption band indicating a change in the heme group conformation. When increasing the PSS concentration, we find that indeed the more highly charged state of the protein is stabilized indicating a greater interaction with PSS. Interestingly, although, it is clear that a greater interaction between myoglobin and PSS is present in the more concentrated PSS solution, the onset of binding appears to be independent of PSS concentration. These effects are also difficult to interpret because changing ionic strength also changes the self-aggregation behaviour of myoglobin which we showed occurs at ionic strengths below 0.3 Molar and in solution at pH values less than 8.

After we examined the effect of PSS concentration and the influence of ionic strength on protonation state of myoglobin, we can conclude that there are two primary effects of salt. Increasing of ionic strength in the solution would favour the charged state of myoglobin as has been observed in titration of myoglobin only solutions (see Figure 3.22). In addition, increasing salt concentration can alter the binding interaction between PSS and myoglobin as observed by the shift in the value of the critical pH observed in the myoglobin-PSS titration curves. The decrease in the critical pH with increasing ionic strength reflects a weaker protein-polyelectrolyte interaction as more positive charge on myoglobin is needed to bind to the polyanion.

Further, we test the influence of PSS concentration on structure of myoglobin to determine if the polyelectrolyte affected the native state of myoglobin through the binding and complex formation. We performed Micro DSC test to analyze possible changes in characteristic temperatures related to denaturation of myoglobin. The ratios of concentrations for myoglobin-PSS$_{\text{min}}$ and myoglobin-PSS$_{\text{max}}$ tests were kept the same as in pH titrations, but by mass it was 10 times greater. All solutions were prepared in 0.1 M NaCl at pH 6 or 9, shown in Figures 3.24 and 3.25, respectively. The results at pH 9 are typical of a melting curve of a native protein except that there is an additional exothermic peak, which occurs at the temperature just below the endothermic peak, which corresponds to the unfolding of myoglobin. The exothermic peak might be related to the presence of the poryphrin ring (heme). The results appear to indicate
that the solution with lower concentration of PSS is more effective at stabilising myoglobin as reflected by an increase in the melting temperature. The non-monotonic behaviour of the
melting temperature with respect to PSS concentration might have the same origin as the non-monotonic effect of PSS concentration on the titration curve. The DSC curves observed at pH 6 do not have a well-defined peak for unfolding of myoglobin. This indicates that the myoglobin is in a partially folded state at intermediate pH. This result is consistent with the large self-aggregation of myoglobin at pH 6 and low ionic strength. Protein aggregation is strongly believed to only occur in the presence of partially folded proteins.

3.5. Conclusions

A key objective of this work was to examine the link between the initial onset of complex formation between protein and polyelectrolyte and the increase in turbidity of protein-polyanion solutions that occurs upon reducing pH, as the latter most likely reflects the interactions between complexes and not the interaction between the protein and the polyanion. For the solutions of BSA, we have used static light scattering to probe the molecular weights of small protein-polyion complexes. We find that the values are well correlated with the $pH_\phi$, that is, with increasing ionic strength the molecular weights decrease indicating weaker interactions which is also consistent with the reduction in the $pH_\phi$, indicating more positive charge on BSA is needed to form the complexes. In addition, a similar correlation is found when changing the salt type from sodium chloride to lithium chloride as lithium chloride appears to be more effective at inducing the BSA-polyion complex formation under low ionic strength conditions. The results with magnesium chloride are significantly different. For these solutions, we find that the $pH_\phi$ values increase with increasing ionic strength. This behaviour is correlated with an increase in the sizes of the protein–polyion complexes formed in solutions above the $pH_\phi$. One possibility is that there is a different mechanism of binding between the protein and PSS in magnesium chloride solutions which leads to the larger complexes. We have hypothesized that the protein has a larger surface for binding to PSS in the magnesium chloride solutions due to the greater screening ability of the magnesium ion. This greater screening ability (which is also reflected by the $A_2$ measurement of the PSS only solutions) could also be the reason why the $pH_\phi$ values in magnesium chloride solutions are lower than those in salts of monovalent cations at low ionic strength.

The results with myoglobin are much more difficult to interpret due to the presence of protein self-aggregation, which is linked to myoglobin unfolding at neutral pH. We find that the PSS is able to inhibit the self-aggregation of myoglobin at pH values around 8.5, which are greater than the estimated $pH_\phi$ values. As with BSA, this indicates that the PSS is forming interactions with myoglobin at pH 2 to 3 pH values of the $pH_\phi$. We also used potentiometric titration as a method for probing the interactions at high pH values. However, these experiments indicated that the interactions of PSS with myoglobin occurred at pH values less than what was observed from the turbidity titrations.
3.6. References


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CHAPTER 4

MOLECULAR DYNAMICS SIMULATIONS OF PROTEIN – POLY ELECTROLYTE COMPLEX FORMATION

4.1. Abstract

The complex formation between protein and polyelectrolyte was examined using molecular dynamics MD. An all-atomistic representation of human serum albumin (HSA) monomer was used and the polyelectrolyte chain was treated as a chain of 60 negatively charged Lennard-Jones spheres. The mechanism of protein-polyelectrolyte interactions and the binding affinities were determined as a function of pH and ionic strength for different amounts of polion stiffness and Lennard-Jones interaction parameters. The binding affinity between polyanion and HSA is greatest at pH 5 for the flexible chain, but this property decreased with increasing rigidity of the polyelectrolyte backbone and with increasing pH. The polion binds to positively charged patches on the surface of HSA at the conditions where the protein net charge is negative. The binding was pH independent for conditions, where Lennard-Jones potential well depth $\varepsilon$ equals 0.5 kJ/mol and binding is pH dependent for $\varepsilon$ in the range of 0.1 to 0.2 kJ/mol. The changes in applied ionic strength influenced long-range electrostatic interactions by screening the charge attractions or repulsions. Both strong and weak polyelectrolyte binders’ behaviour was observed, where the strong binding is defined as the ability to form complexes through polion chain binding to a single large positive patch on protein surface and binding is pH dependant at high salt concentration. The weak binding occurs when polion chain binds to multiple positive patches scattered on protein solvent accessible surface area, and the binding is pH dependant at low salt concentration. Weak binding usually takes place on the ‘wrong side’ of isoelectric point.
4.2. Introduction

Understanding and controlling protein-polyelectrolyte interactions is needed to improve upon the process of protein stabilisation, protein aggregation inhibition, drug delivery and the triggered release of encapsulated material (Yapel 1985, Fiume 1988). There are few experimental studies on complex formation between proteins and polyelectrolytes. The main findings indicate a strong dependence on ionic strength and pH of interactions between proteins and weak polyelectrolytes (Mutka 2008, Seyrek 2003, Mattison 1995), and an increase of binding affinity with chain flexibility (Cooper 2006). Other features influencing protein-polyelectrolyte interactions are the polyelectrolyte charge distribution and density, the polyelectrolyte molecular weight, and protein structural factors such as net charge, charge distribution, and protein size (Cooper 2006). These factors also influence the formation of organised polyelectrolyte structures such as multilayer composites or thin films (Decher 1991, 1992). A good example of how knowledge of protein–polyelectrolyte interactions can help to control the properties of polyelectrolyte multilayer films is given by Sasaki (2008), Moffat (2007) and Westwood (2011). In these studies, polion-containing films were constructed of positively and negatively charged polyelectrolytes or proteins. These films were tested for their responsiveness under influence of external stimuli. The density, distribution of charge and molecular weight of the polion affects the permeability of the polyelectrolyte thin film and its resistance to decomposition. Furthermore, protein-polyelectrolyte binding affinity is stimulated by protein secondary and tertiary structure and forces withholding their conformational arrangement such as hydrophobic interactions, disulfide or hydrogen bridges. The denatured proteins, with unfolded chains, bind to polyelectrolytes by different mechanisms than native proteins (Breslow 1964).

During the past few years, electrostatically driven complex formation between protein and polyelectrolytes has been examined in some detail, where the main interest lies in determining the onset of complexation and then the precipitation of large complexes. The main driving forces of protein-polyelectrolyte binding solutions are the electrostatic interactions that occur between polyelectrolytes and patches of opposite charge on proteins as deduced from the experimental dependence of the protein-polyion complexation on pH and ionic strength. It was observed experimentally that the local interactions between protein patches and polyion chain are important since polyelectrolytes are able to bind to proteins that have the same net charge (Park 1992). Comparing binding affinities of polyions with different chemistry has also indicated that hydrophobic interactions are significant (Sedláček 2009). Seyrek (2003) and co-workers experimental data suggest that complex formation depends strongly on ionic strength, which corresponds to protein-polyelectrolyte affinity and association driven by the presence of the heterogeneous charge at the binding side.

The importance of having charged patches on the protein surface has been highlighted by Seyrek study (2003), in which Delphi, was used to visualize electrostatic potential surfaces for bovine serum albumin BSA, lysozyme, β-lactoglobulin and insulin for various ionic strength and
pH values corresponding to both polyion binding and non-binding conditions. It was found that the anisotropy of electrostatic domains around the protein globule is primarily responsible for the ionic strength and pH dependence on protein-polyelectrolyte binding affinity. Seyrek (2003) also showed that, even if a specific patch on the protein surface causes short-range attractions, the complex formation is still caused primarily by the long-range electrostatic forces. Experimental studies have given key insights into the controlling factors of protein-polyion binding; however, many questions remain with regards to the types of configurations formed during protein-polyion complexation. For example, the mechanism of binding at the molecular scale cannot be fully explored experimentally and consequently the factors controlling the interactions are not fully understood. Accessing this information requires using simulation or theoretical approaches to complement the experimental studies. So far, the majority of theoretical and simulation studies on protein-polyelectrolyte interactions only considered simple models of proteins, such as a hard sphere with embedded charges (Carlsson 2001). In that work, Monte Carlo simulations were used to determine the mechanism of protein-polyelectrolyte complexation. The polyelectrolyte was represented by a sequence of negatively charged hard spheres, and the protein was composed of a hard sphere with embedded pH-dependent individual charges. The positions of charges within the sphere were based on their positions in the crystal structure of lysozyme. The complexation between a negatively charged polymer and the model protein was found to be dependant on the charge status of the protein. A key finding was that complex formation between polyelectrolyte and protein also requires the existence of non-electrostatic interactions.

In our approach, we used molecular dynamics (MD) simulation to determine conditions of the pH-induced binding between polyion and protein. We analysed the mechanism of complex formation between a polyelectrolyte chain of negatively charged beads, and an all-atomistic representation of the protein taken from crystal coordinates and published in the protein data bank (PDB). Our simulations aim to predict by what means the polyelectrolyte binds to a protein; if the polymer chain forms loops or wraps around the protein tightly. Also we examine under what conditions the protein-polyion binding is pH dependent only at low salt concentration and no binding occurs at high salt concentration, and under what conditions pH dependant binding also occurs at high salt concentration. Under what conditions, where electrostatic interactions are not screened by high salt concentration, is it expected that hydrophobic interactions will lead to complex formation (Carlsson 2001).

We choose to study human serum albumin (HSA). HSA has a high conformational stability, a representative composition of amino acid residues and has a similar sequence to bovine serum albumin (BSA), which is commonly used in experimental polyion interaction studies. We assume that all interactions imposed by conditions applied to the model did not cause major conformational changes to HSA. We examined the binding mechanism as a function of controlling factors, such as protein charge distribution, various Lennard–Jones interaction parameters, and polyion stiffness. Particular attention is given to the role of charged patches on the protein surface. The decrease of pH determines the protein charge distribution as controlled
by the $pK_a$ values of the amino acids composing the protein. Formation of positive patches could attract the negatively charged beads of polyelectrolyte.

In the following, we describe the forcefield used by our model. In the initial simulations we examine the effect of pH and ionic strength for various Lennard-Jones interaction parameters. Next, we determine the behaviour of polyanion chain with and without the presence of protein, in a range of bond energy potentials $k$, which controls stiffness of the chain. Finally, we analyse the influence of using a distance dependent dielectric model for describing electrostatic interaction.

4.3. Model

Molecular dynamics was used to study the complex formation between a protein and polyelectrolyte. The model was based on a coarse-grained representation of the polyanion macromolecule and an all-atomistic representation of the protein monomer. The interactions were described using a forcefield based upon the Lennard-Jones potential and the Yukawa (screened Coulomb) potential, to describe non-bonded interactions between protein atoms and polyanion monomers. Bonded interactions were also used to describe the interactions between spheres contained only on the polyanion. The positions of all atoms on the protein were frozen during the simulation.

4.3.1. Total potential energy

The total potential energy for the system $U$ is given by the sum of the energy due to the intermolecular interactions between the polyanion spheres and protein atoms $U_{p,prot}$ and the energy due to the intramolecular interactions between the spheres of the polyanion $U_{pp}$. No intramolecular energy term is needed to describe the interactions between the protein atoms as these are ‘frozen’ during the simulation. The intramolecular energy between polyanion spheres, $U_{pp}$, is given by (4.1):

$$U_{pp} = \sum_{i=1}^{N-1} U_{i,i+1,bond} + \sum_{i=2}^{N-1} U_{i,i,\theta} + \sum_{i=1}^{N-2} \sum_{j=N-i+1}^{N} \left( U_{ij,yuk} + U_{ij,LJ} \right) \quad (4.1)$$

where $U_{i,i+1,bond}$ is the covalent bond energy between sphere $i$ and sphere $i+1$ of the polyanion chain, $U_{i,i,\theta}$ is the covalent bond angle ($\theta$) energy for the angle formed with $i$ at the centre between two spheres within polyanion chain of $N$ beads, where $N_{ext}$ is the number of neighbouring beads excluded from non-covalent interactions. The terms $U_{ij,yuk}$ and $U_{ij,LJ}$ are the screened Yukawa potential to describe electrostatic interactions and the Lennard-Jones potential to describe non-electrostatic interactions, respectively. The energy of intermolecular
interactions \( U_{\text{prot}} \) is given only by the non-bonded interactions between polyanion spheres and protein atoms given by equation 4.2:

\[
U_{\text{prot}} = \sum_{ij} (U_{ij,\text{LJ}} + U_{ij,\text{yuk}})
\]  

(4.2)

The screened Yukawa \( U_{ij,yuk} \) potential is given by:

\[
U_{ij,yuk} = \frac{e^2}{4\pi\epsilon_0\epsilon_r r_{ij}} \exp(-\kappa r_{ij})
\]  

(4.3)

where \( r_{ij} \) is the center-to-center distance between particles \( i \) and \( j \), \( \epsilon_0 \) the permittivity in vacuum, \( \epsilon_r \) the relative dielectric permittivity of water (solvent), and \( \kappa^\prime \) is the Debye screening length given by:

\[
\kappa^{-1} = \sqrt{\frac{\epsilon_0 \epsilon_r \kappa_B T}{2N_A e^2 I}}
\]  

(4.4)

where \( N_A \) is the Avogadro number, \( \kappa_B \) is the Boltzmann constant and \( e \) is the elementary charge. For a temperature \( T \) equal to 298.15 K and \( \epsilon_r \) equal to 80, the Debye screening length \( \kappa^\prime \) is equal to 9.6 Å for an ionic strength \( I \) equal to 0.1 M. In this work, we used two approaches to describe the value for the dielectric constant, \( \epsilon_r \). In the first method, the value is set equal to the bulk dielectric constant of water, 80. In the second approach, we use a distance dependent dielectric, \( \epsilon_r \), equal to 20 \( r_{ij} \) when \( r_{ij} \) is less than 4 nm, and is equal to 80 otherwise. This accounts for exclusion of water at close separations between atoms or spheres and has been used previously to describe the effect of water in protein-based forcefields (Werner 2002, Hauberthür 2003).

The Lennard-Jones potential used to describe the short range steric forces and the longer-ranged non-electrostatic attraction between unbound spheres is given by equation 4.5:

\[
U_{ij,\text{LJ}} = \sum_{i<j} 4\epsilon_{ij} \left[ \left( \frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left( \frac{\sigma_{ij}}{r_{ij}} \right)^{6} \right]
\]  

(4.5)

where \( \sigma_{ij} \) is the separation at which the potential between protein atom \( i \) and polyanion sphere \( j \) is zero, defined as:

\[
\sigma_{ij} = \frac{1}{2}(\sigma_i + \sigma_j)
\]  

(4.6)

and \( \epsilon_{ij} \) is the depth of the potential well, defined as:
\[ e_{ij} = \left( e_i e_j \right)^{0.5} \]  
\[ (4.7) \]

where, \( e_j \) and \( e_i \) are the pure component interaction parameters.

The bond and angle energy are given by harmonic potentials as the following, respectively,

\[ U_{i,i+1}^{bond} = k_{bond} (r_{i,i+1} - r_0)^2 \]  
\[ (4.8) \]

\[ U_{i,\theta}^{\theta} = k_{\theta} (\theta_{i-1,i,i+1} - \theta_0)^2 \]  
\[ (4.9) \]

\( k_{bond} \) is bond force constant, \( r_0 \) is the equilibrium separation of the harmonic bond potential, \( k_{\theta} \) is the angle force constant, \( \theta_{i-1,i,i+1} \) defines angle between \( r_i - r_{i+1} \) and \( r_i - r_{i-1} \), and \( \theta_0 \) is the equilibrium angle of the harmonic angular potential.

4.3.2. Protein

The protein used in our model is human serum albumin HSA (Sugio 1999). The coordinates for the atoms were taken from the pdb structure 1AO6 published in the protein data bank (PDB). Electrostatic surface of HSA was calculated using Adaptive Poisson-Boltzmann Solver (APBS) software (Baker 2001). The \( pK_a \)s of all charged groups were determined using PROPKA3 (Li 2005, Bas 2008, Olson 2011). No partial charges for polar groups are used. Integer values of the charge are used to describe the ionisable groups depending on whether the pH is above or below the \( pK_a \) of the group as described in the next section. As such, the total charge of the protein in the simulation can be calculated from the number of charged groups as listed in Table 4.2 below. The positions of protein atoms are 'frozen' and the protein is placed in the centre of a 35 nm wall length cubic box. Complete sequence of human serum albumin amino acids was published by Meloun at al. (1975). The HSA protein consists of a single polypeptide chain, which contains 585 amino acid residues (Meloun 1972). The molecular weight of human serum albumin calculated from its amino acid composition is 66 500 Da. In the simulations, we only use charged atoms and atoms on the solvent accessible surface of HSA. The charged atoms of the protein and their \( pK_a \)s are presented in Table A (see appendix). The table 4.1 presents distribution of the charges along the amino acid groups according to the pH conditions for the simulations.
Table 4.1: Number of charged amino acids on HSA as calculated using PROPKA3

<table>
<thead>
<tr>
<th>Group/pH</th>
<th>pH 5</th>
<th>pH 6</th>
<th>pH 7</th>
<th>pH 8</th>
<th>pH 9</th>
<th>pH 10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Negative charges</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>59</td>
<td>61</td>
<td>61</td>
<td>61</td>
<td>61</td>
<td>61</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>34</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td><strong>Positive charges</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>23</td>
<td>23</td>
<td>22</td>
</tr>
<tr>
<td>Lysine</td>
<td>58</td>
<td>58</td>
<td>57</td>
<td>53</td>
<td>53</td>
<td>47</td>
</tr>
<tr>
<td>Histidine</td>
<td>10</td>
<td>9</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

4.4. Method

Molecular dynamics simulations were carried out using GROMACS 4 - GROningen MAchine for Chemical Simulations (Berendsen 1995, Lindahl 2001, Hess 2008). Newton’s equations of motion were integrated numerically using the leap-frog algorithm. The time step for data collection of all simulations was set to 0.001 ps (pico seconds) and the total number of time steps for each simulation was 30,000,000. Periodic boundary conditions were used with a cubic box of sides equal to 35 nm and a Nose-Hoover thermostat for temperature control. The reference temperature was 298.15 K and time constant for temperature coupling was set to 0.02 ps. The cut-off for electrostatics and van der Waals interactions was set equal to 10 nm.

For bonded potentials between polyion spheres we considered 60 negatively charged beads with their radius of 0.5 nm, where each Lennard-Jones sphere has an assigned negative charge at -1 and $\text{Nexl}$ equalled 3.

All simulations were performed with a range of ionic strengths between 10 mM and 1000 mM and pH from 5 to 10. The pH determined the charge distribution on the surface of HSA according to the $pK_a$s of ionisable amino acids. When the $pK_a$ is less than the pH, the ionizable group was protonated, and when $\text{pH} < pK_a$ then the ionisable group was deprotonated. The $pK_a$ of each group was calculated using PROPKA3. Only the surface accessible atoms on the protein surface and all the charge protein atoms were included in the simulations. The idea of solvent accessible surface area of proteins was explained in chapter 2, where we described in detail the background behind such concept. The surface accessible atoms were calculated using the Michael Connolly Surface Algorithm (Connolly 1983). The positions of all protein atoms were held constant during the simulation in the centre of 35 nm wall length cubic box. We averaged the results for three different starting positions of the chain for each set of parameters in our simulations.

We also used simulations of only a polynion to investigate the effect of the bond angle potential on the polynion stiffness. In these simulations, a chain of 200 spheres was used in a periodic
box with sides of 100 nm. The same run parameters were used for these simulations as for the protein-polyion simulations. To describe dimensions and compactness of the polyelectrolyte structure, we report values of the radius of gyration $R_g$, which is given by:

$$R_g = \left( \frac{\sum_i m_i \sum_i ||r_i||^2 m_i}{\sum_i m_i} \right)^{\frac{1}{2}}$$

(4.10)

where $m_i$ is the mass of atom $i$ and $r_i$ the position of atom $i$ with respect to the centre of mass of the molecule.

For non-bound energies the finite distance at which the potential between protein atom $i$ and polyion sphere $j$ is zero was calculated for $\sigma_i$ equal to 4 Å for the protein and $\sigma_j$ equal to 10 Å for the polyion. The depth of the potential well was set to 0.1, 0.15, 0.2 and 0.5 kJ/mol. The covalent bond and its angular energies were set to the following values $r_0$ equalled 4.7 Å, $k_{bond}$ equalled 2.4 kJ/mol Å$^2$, $\theta_0$ was 180°, $k_\theta$ equalled to 0.005, 0.05, 0.5, 5, 50 and 500 kJ/mol(°)$^2$.

To describe the structure of liquids within our simulations we used a radial distribution function (RDF) $g(r_{ij})$, which is given by:

$$4\pi r^2 g(r_{ij}) = V \sum_i^{N_i} \sum_j^{N_j} P(r)$$

(4.11)

where $V$ is the volume and $P(r)$ is the probability of finding an $i$ protein atom at distance $r$ from a $j$ polyion sphere. The maximal value of $r$ equals $\frac{1}{2}$ size of a periodic boundary, which is 17.5 nm. The minimal value of $r$ equals the sum of radii of polyion sphere (0.5 nm) and protein atom (0.2 nm) as the distance is measured center-to-center, which is 0.7 nm.
4.5. Results and discussion

The protein-polyion binding depends on the electrostatic potential of the protein surface, which, in turn, depends on pH. The pH can be used to determine the distribution of charge groups as described in section 4.4. As the pH decreases, the number of positively charged groups increases and that of negatively charged groups decreases. As such, the net charge of the protein goes from negative to positive with decreasing pH. At the state when the protein carries an equal amount of negative and positive charges, the net charge is zero. The pH at this point is termed the isoelectric point \( (pI) \). A value of \( pI \) is unique for various proteins due to their distinctive composition of charged groups on the solvent accessible surface area. The isoelectric point of bovine serum albumin (BSA) is 5.67 (Tanford 1955) and Tanford suggested that the difference of \( pI \) between HSA and BSA is negligible (there is only one amino acid difference between BSA and HSA). The effect of changing pH on the protein charge electrostatic potential is illustrated in Figure 4.1. There is an increase in the positively charged patch of HSA in the pH range of pH from 6 to 8. The images of HSA globule were generated by molecular visualization software – PyMOL (Delano 2009) with the APBS plug-in (Baker 2001).

\[
\text{pH 6} \quad \text{pH 7} \quad \text{pH 8}
\]

Figure 4.1 Charge distribution on the solvent accessible surface area of HSA for pH 6, pH 7 and pH 8. Red and blue colours corresponding to negatively and positively charged areas, respectively.

The recent study by Zhang examined the size of the positive patch with respect to pH, it was concluded that patch size depends upon pH and the ionic strength of the bulk surrounding the protein globule (Zhang 2007). In this study, we obtained the same dependency with changes of pH; however, the effect of ionic strength on positively charged patch on the protein surface was not examined here. We calculated the net charge of HSA as a function of pH using PROPKA3 (Olsson 2011). The results are presented in Figure 4.2, the major change in charge occurs in the region of pH 10.5 where there is a sudden fall in the number of negative charges.
This transition is related to the acid dissociation constant ($pK_a$) of lysine groups, which at pH 10 are fully protonated. A second major transition is observed in the region of pH around 6.5, where histidine groups are protonated with decreasing pH. Our theoretical prediction, determined by PROPKA3, of isoelectric point for HSA is at 5.79 for the native protein structure. This value of $pI$ is slightly higher to the experimental measurements by Tanford, but still within close proximity. Other measured values of $pI$ for HSA are reported to be 5.0 (Baranov 2004) and 5.5 (Noh 2008). At pH corresponding to an isoelectric point of the protein all histidine groups are positively charged.

4.5.1. Electrostatically driven interactions

Our initial investigation of interactions between HSA and the negatively charged coarse-grained polyelectrolyte chain was focused on tuning the non-covalent potentials between the polyanion and the protein in order to obtain a pH dependent binding. Initial studies focused on using a potential well equal to the value of 0.1 kJ/mol. In these conditions, we examined ionic strength and pH influence on the binding behaviour. For this set of simulations we applied the screened Yukawa potential (Coulomb) without distance dependant dielectric. The Coulomb and Lennard-Jones energies plotted as a function of time from simulations at an ionic strength of 10 mM and pHs 6, 7 and 8 are shown in Figure 4.3. Binding of the polyanion to protein occurs whenever there is a sharp drop in the interaction energies as a function of time. For pH 6, the Lennard-Jones and Coulomb interaction energies are both on the order of approximately -25 kJ/mol,
indicating a complex formation. The Lennard-Jones energy does not reach zero, which indicates a permanent contact of HSA with polyanion beads. At pH 7 the binding is intermittent and, on average, the polion binds to protein on three occasions. The Lennard-Jones energy during binding is approximately -10 kJ/mol, which implies weaker complex formation than observed at pH 6. The electrostatic binding energy remains unchanged for the simulations at pH 6 and 7. This pH change corresponds to a difference in the number of positively charged groups between pH 6 and pH 7 equal to 6, which includes one lysine and five histidine groups (see Table 4.1). The net charge on the protein surface equals to -5 for pH 6 and -11 for pH 7, respectively. This change in the protonation state of the HSA particle alters the binding behaviour as, at a higher pH, the long range electrostatic repulsions are primarily responsible for intermittent character of binding. For simulations at pH 8, there is no protein-polyelectrolyte binding. The binding behaviour can also be described by a plot of the protein atom-polyion sphere pair distribution function $g(r_{ij})$, as shown in Figure 4.4. At pH 6, the radial distribution function has a peak at a separation of $r$ equal to 1 nm. This distance corresponds roughly to the distance of closest approach between a protein atom and polion sphere, which is given by the parameter $\sigma_{ij}$ equal to 0.7 nm. This peak indicates the weaker protein-polyion binding, as the distribution function is reduced at pH 7 relative to that at pH 6. At pH 8, the radial distribution function is close to 0 for $r$ less than 5 nm indicating the exclusion of polion beads from the volume near to the protein surface.

![Figure 4.3](image.png)

Figure 4.3 The Lennard-Jones energy (top) and Coulomb energy (bottom) vs. time for ionic strength of 10 mM across pH 6 (--), 7 (--) and 8 (—)
Figure 4.4 The protein-polyion radial distribution function at the ionic strength of 10 mM for pH 6 (−), 7 (−) and 8 (−).

The results of simulations performed for 500 mM ionic strength for pH values of 6, 7, and 8 are shown in Figure 4.5. At all pH, the binding events are very short lived as the Lennard-Jones and Coulomb energies are close to zero for the entire simulation. The distribution functions, shown in Figure 4.6, also indicate that there is very little protein-polyion binding. The results at 500 mM indicate that the salt screens attractive electrostatic interactions. The complex formation does not occur as the Lennard-Jones energy is too weak to initiate protein-polyion binding at any of the pH investigated.

Figure 4.5 The Lennard-Jones energy (top) and Coulomb energy (bottom) vs. time for ionic strength of 500 mM across pH 6 (−), 7 (−) and 8 (−).
In the above section we demonstrated that the complex formation can be pH dependant when ionic strength is low, which is also confirmed by Seyrek and Dubin et al. (2003). Seyrek showed that at low ionic strength, the pH dependency is demonstrating the behaviour of weak polyelectrolytes binders, where the complexation between BSA and poly(acrylic acid) or heparin was studied. The binding on a ‘wrong’ side of $pI$ and low salt concentration associated with no binding at high salt concentration at the same pH conditions in both cases, leads to the inversion of the salt effect, and in these conditions the maxima of binding occurrence are present when the Debye length extends, at low ionic strength. This appears to be a general consequence caused by unscreened electrostatic attractions, which drive the interactions at low salt concentration.

Next we tested a broader range of ionic strength ranging from 10 mM to 1000 mM to give insight into the screening effect of salt. These simulations were carried out at pH 6 and pH 8 to capture the behaviour at various charge densities on the protein surface. In Figures 4.8 and 4.10 are shown the plots of the radial distribution functions for various ionic strengths for pH 6 and pH 8, respectively. For pH 6 the binding occurs only at the lowest ionic strength (10 mM). With increasing ionic strength, the protein-polyion binding is weakened as the peak in the radial distribution function disappears in all the runs at pH 6 with an ionic strength equal to 100 mM or greater.
At ionic strength higher than 10 mM, the Coulomb potential is approximately zero, with the exception of a few events when the polyion sphere crosses near the protein’s positively charged patch. However this is insufficient to form a stable binding event. These results are in line with data presented in plots in Figure 4.4 for the radial distribution function at pH 6, where there is a peak in the radial distribution function at close separation only. Further, there are data obtained from simulations at pH 8, shown in Figures 4.9 and 4.10, where we present energies associated with interactions and distance between particles at various ionic strength.
When ionic strength is 10 mM, there is a strong pH dependence of binding between protein and polyelectrolyte. At pH 6 the binding is driven mainly by electrostatic interactions (Coulomb) although the Lennard-Jones interactions might also contribute to the complex formation. At pH 7, the polyanion binding is more transient due to electrostatic repulsion arising from the interaction of the polyanion with the net negative charge on the protein, which is equal to -11. At pH 8 and an ionic strength of 10 mM, no binding occurs as the electrostatic repulsion is too strong to allow any binding. In these conditions, the radial distribution function indicates that polyanion spheres are excluded from a region of 5 nm next to the protein surface. This is due to the presence of unscreened long-range electrostatic repulsions between negatively charged beads of polyanion.
and extensive negative charge on the protein surface present at pH 8. This exclusion disappears with increasing ionic strength as the long-range electrostatic interactions become screened. At 1000 mM (and pH 8), there is a noticeable rise in the number of the polyelectrolyte spheres present near HSA surface, as illustrated in Figure 4.10. However, the Lennard-Jones interaction alone is too weak to allow for complex formation at high ionic strength.

The simulation results presented in this section reflect a weak polyelectrolyte binding behaviour. This behaviour was described previously by Sedlák et al. (1998) in which case, pH dependent binding occurs at low salt concentration, and no binding occurs at high salt concentration, where the mechanism of protein-polyelectrolyte complexation was studied through the example of ferricytochrome c binding to two different polyanions — poly(vinylsulfate) and poly(4-styrene-sulfonate) PSS — of different charge distribution and the same charge density. Sedlák and co-workers found that ferricytochrome c formed complexes with poly(vinylsulfate) PVS via cumbic forces, and in these complexes the protein remained in its native state. The interaction with PVS is screened with increasing salt concentration in a similar manner as observed in out simulations. The complex formation with poly(4-styrene-sulfonate) at a low ionic strength and neutral pH resulted in a major change in the protein’s conformation, leading to the exposition of the hydrophobic core of protein. This type of binding occurred even at high salt concentrations. Nevertheless, we were also focused on capturing strong binding, where a pH dependence binding occurs at low ionic strength and at high ionic strength. A good example of this behaviour is observed in interactions of PSS with almost all proteins (especially BSA) (Sedlák 1998). In the next section we examine under what conditions strong binding occurs in the simulations.

4.5.2. Effect of the Lennard-Jones potential

In the following section, we determine the effect of the Lennard-Jones potential on binding between HSA and polyanion. The simulations were carried out for potential well depth $\epsilon$ equal to 0.5 and 0.2 kJ/mol. In section 4.5.1., the value of $\epsilon$ at 0.1 kJ/mol is too low for pH dependant binding to occur at high ionic strength. As a consequence, we studied a range of ionic strengths between 50 mM and 500 mM to determine if the pH dependant binding can occur at all salt concentrations. The energies of non-bound interactions and the radial distribution function for $\epsilon$ equal 0.5 kJ/mol are shown in Figure 4.11. The simulations indicate that at such high value of $\epsilon$, there is no pH dependence on complex formation. At an ionic strength of 100 mM and pH 5, 6, 7 and 8 there is no difference in the radial distribution curve. All simulations, performed at $\epsilon$ equal 0.5 kJ/mol, indicate a very strong protein-polyelectrolyte binding, which is primarily due to the strong Lennard-Jones interactions.
Figure 4.11 The radial distribution function for $\varepsilon$ equal 0.5 kJ/mol at ionic strength of 100 mM for pH 5 (–), pH 6 (–), pH 7 (–) and pH 8 (–). Energies of binding (inset plot) for pH 5 (–) and pH 8 (–), where top graph corresponds to Lennard-Jones and bottom Coulomb energy.

The energies of binding at the above conditions indicate that the electrostatic attraction changes at pH 5 towards a repulsive character at around pH 8, which is expected due to the increased negative charge density on the surface of HSA, as the net charge of the ionisable groups changes from -1 to -18 e (following data presented in Table 4.1). However, this change is insufficient to facilitate pH dependant complexation when $\varepsilon$ equals 0.5 kJ/mol. The Coulomb interaction is too weak compared to the Lennard-Jones energy to cause the electrosatically driven breakup of the complexes even at pH 8, when protein globule carries a total charge equal to -18 e (calculated by PROPKA3). The strength of interactions between polyion and HSA is illustrated in Figure 4.12, which contains a typical snapshot of a protein-polyelectrolyte complex formation from the simulation for $\varepsilon$ equal to 0.5 kJ/mol at pH 8 and ionic strength equal to 100 mM. The images (generated using PyMOL (DeLano 2009)) indicate large patches of negative charge on the surface of HSA with only a few small patches of positively charged areas. The chain binds to the positively charged areas and wraps around the protein tightly even in the presence of repulsive electrostatic interactions. This type of binding is not realistic as the Lennard-Jones attraction is too strong to obtain a pH dependence of the interactions.
Next, simulations with $\varepsilon$ equal to 0.2 were done for a range of ionic strengths between 50 mM and 500 mM. The results are presented in Figure 4.13 for ionic strength 50 mM, Figure 4.14 for 100 mM and Figure 4.15 for 500 mM.

Figure 4.12 A snapshot of the protein-polyelectrolyte simulation at pH 8 for ionic strength 100 mM, $\varepsilon$ equal to 0.5 kJ/mol, where first image shows protein only and second image shows protein and chain. Red and blue colours correspond to negatively and positively charged areas, respectively. Green spheres illustrate polyion’s negatively charged groups.

Figure 4.13 The radial distribution function for $\varepsilon$ equal to 0.2 kJ/mol at ionic strength of 50 mM for pH 5 (--), pH 6 (--), pH 7 (--), and pH 8 (--). Energies of binding (inset plot) for pH 5 (--), and pH 8 (--), where top graph corresponds to Lennard-Jones and bottom to Coulomb energy.
Figure 4.14 The radial distribution function for $\epsilon$ equal 0.2 kJ/mol at ionic strength of 100 mM for pH 5 (−), pH 6 (−), pH 7 (−) and pH 8 (−). Energies of binding (inset plot) for pH 5 (−) and pH 8 (−), where top graph corresponds to Lennard-Jones and bottom to Coulomb energy.

Figure 4.15 The radial distribution function probability of polyion next to HSA for $\epsilon$ equal 0.2 kJ/mol at ionic strength of 500 mM for pH 6 (−), pH 7 (−), pH 8 (−), pH 9 (−) and pH 10 (−). Energies of binding (inset plot) for pH 6 (−) and pH 10 (−), where top graph corresponds to Lennard-Jones and bottom to Coulomb energy.
In simulations with the Lennard-Jones potential well depth $\varepsilon$ equal to value of 0.2 kJ/mol, there is a pH dependence of complex formation between protein and polyelectrolyte at low ionic strength. Small changes to the radial distribution function for changing pH from 5 to 8 are most pronounced at ionic strength of 50 mM as shown in Figure 4.13. With decreasing pH the peak in the radial distribution function decreases indicating weaker interactions between the protein and the polyelectrolyte. In simulations at pH 5, the unscreened attractive interactions, at low ionic strength, cause the polyelectrolyte chain to bind to the protein for the entire simulation time; this can be concluded from examining the energy versus time corresponding to pH 5 of the Lennard-Jones potential plot, as presented in Figure 4.14. Both components of non-bonded interactions make a similar contribution to the attractive interactions at pH 5. Subsequently, at pH 8, the binding is mainly driven by the Lennard-Jones potential as the electrostatics oscillate at around zero energy during the binding. In this case, the polyelectrolyte chain binds weakly to a small positive patch on the protein surface and the binding event is short. The attractive electrostatic energy from the patch binding balances the repulsive electrostatic interactions with the remainder of the negatively charged protein surface. At pH 10 there is no binding event as the Lennard-Jones potential is zero and the RDF indicated no polyelectrolyte spheres near the protein.

In simulations with ionic strength equal to 100 mM, the binding is permanent at pH 8 as reflected by a plot of the Lennard-Jones energy shown in Figure 4.14. A snapshot from this simulation is shown in Figure 4.16.

![Figure 4.16 Snapshot of the protein-polyelectrolyte simulation at pH 8 for ionic strength 100 mM, $\varepsilon$ equal to 0.2 kJ/mol, where first image shows protein only and second image shows protein and chain. Red and blue colours correspond to negatively and positively charged areas, respectively. Green spheres illustrate polyelectrolyte's negatively charged groups](image)

The more permanent binding observed at pH 8 with increasing ionic strength occurs because the higher concentration of salt ions screens electrostatic repulsion between polyanion beads and the net negative charge on the HSA surface, thus the chain remains bound to small positive patches at pH 8, because the Lennard-Jones potential is strong enough to overcome
the salt-weakened electrostatic repulsions. The binding is not as strong as the one observed at the same ionic strength and pH conditions for $\varepsilon$ equal 0.5 kJ/mol as illustrated by the snapshot shown in Figure 4.12. Here the chain is bound tightly across the positively charged surface of HSA, but the ends are loose and detached from the protein. This finding indicates that the electrostatic interactions can repel parts of the polyeion chain.

At high ionic strength (500 mM) the binding occurs in simulations with pH equal to, or less than, 8 as presented in Figure 4.15. In this case, there is no effect of changing pH between 5 and 8 on the binding behaviour. However, with further increase of pH to 9 or 10, no binding occurs. Nevertheless, at high pH there is still a fraction of polyeion spheres near to the protein surface, as reflected by the radial distribution function shown in Figure 4.15. The radial distribution plots are similar to those obtained with $\varepsilon$ equal to 0.1, in which case, the high ionic strength leads to screening of all long-range electrostatic interactions. The short-range electrostatic repulsions still dominate at pH 9 and 10 and prevent a binding event. In Figure 4.17 we present a snapshot of protein-polyion complex formation obtained from a simulation at pH 8 and ionic strength equal to 500 mM and $\varepsilon$ equal 0.2 kJ/mol, which suggests that the binding mechanism is more diffused than that which occurs at low ionic strength: illustrated in Figures 4.12 and 4.16. The binding is much weaker at high ionic strength as the polyeion forms unbound loops in its mid sections near to the protein positively charged patches.

Figure 4.17 Snapshot of the protein-polyelectrolyte simulation at pH 8 for ionic strength 500 mM, $\varepsilon$ equal 0.2 kJ/mol, where first image shows protein only and second image shows protein and chain. Red and blue colours correspond to negatively and positively charged areas, respectively. Green spheres illustrate polyeion’s negatively charged groups

As before, the ends of the polyeion necklace are loose, which implies that the short-range repulsive electrostatic interactions are greater than the Lennard-Jones interactions and can prevent sections of the polyeion from attractive interactions forming with the positive surface of the protein.
Using of the Lennard-Jones potential well depth $\varepsilon$ equal 0.2 kJ/mol in the simulation captures the behaviour of strong polyelectrolyte binders, which is pH dependant at high salt concentration (Mattison 1995, Cooper 2006). In addition, the binding occurs on the ‘wrong’ side of isoelectric point, which has been reported previously by Park et al. (1992). Park and co-workers (1992) examined the induction of protein-polyelectrolyte binding using pH and turbidimetric titrations and quasielastic light scattering. The study included three different proteins; BSA, lysozyme and bovine pancreas ribonuclease complexing with cationic and anionic polyelectrolytes of various charge densities. The authors established that proteins can form associations with polyelectrolytes even if both carry the same net charge. At the very onset of binding the protein-polyelectrolyte complexes were soluble, with progressive pH change the growth of complexes and phase separation were observed. The most important conclusion of the study by Park was that one-to-one binding can be initiated even if the protein carries the same charge as the polion, and it is possible because the complex formation starts with binding to the oppositely charge patches on the protein surface, which defines ‘patch binding’.

In this section, we have shown that by changing the strength of the Lennard-Jones interactions from $\varepsilon$ equal 0.1 to 0.2 kJ/mol, a transition between weak and strong behaviour is observed. This increase in $\varepsilon$ would correspond to an increase in the hydrophobicity of the polion in the real system. The hydrophobicity of polyaninic chains binding to the proteins was recently examined by Sedlák at al. (2009), where the authors tested complex formation between proteins such as chymotrypsinogen A, ribonuclease A, cytochrome c and lysozyme and following polyanions: heparin, poly(vinylsulfate) PVS, poly(4-styrene-sulfonate) PSS and Nafion. The study shown that more hydrophilic polyanion - PVS and heparin interact with proteins primarily through the electrostatic interactions. This type of interactions proved to preserve the properties of proteins at high ionic strength leaving the structural form of globular protein indistinguishable from the one in solutions without the polyanion. Alternatively, interactions of hydrophobic polyanions with proteins, examined by Sedlák and co-workers (2009), have a significantly more destabilizing effect on the proteins.as the thermal tests shown that the hydrophobic polyanions can perturb both tertiary and secondary structures of one of the tested proteins - cytochrome c - even if pH is neutral at room temperature.

4.5.1. Stiffness of the polyelectrolyte backbone

In this section, the influence of the rigidity of polyelectrolyte chain was investigated to establish the effects of polion stiffness on binding affinity to the protein. A range between 0.05 and 500 kJ/mol($\gamma^2$) of $k_\theta$ values was tested in the model, where 0.05 kJ/mol($\gamma^2$) corresponds to the most flexible chain and 500 kJ/mol($\gamma^2$) reflects a rod-like, stiff polion chain. We applied these conditions to simulations at pH 5 and pH 8. The Lennard-Jones potential well depth was equal to 0.1 kJ/mol and the distance dependant dielectric constant equalled 2r. The above values for $\varepsilon$ and $\varepsilon_r$, respectively, were used for all simulations reported in this section. First, we examined
the effect of changing the bond angle potential on the flexibility of the polyelectrolyte chain in simulations where no protein was present. The chain flexibility was analysed from the simulations by determining the radius of gyration and its components at various $k_\theta$ ranging from 0.05 to 500 kJ/mol(°)$^2$. A logarithmic scale plot of the results obtained for an ionic strength equal to 100 mM is presented in Figure 4.18.

![Figure 4.18 Radius of gyration $R_g$ (●) and the components $R_{gx}$ (longest axis) (■), $R_{gy}$ (middle axis) (♦) and $R_{gz}$ (shortest axis) (▲) for polyelectrolyte chains in relationship with angle potential](image)

The radius of gyration ($R_g$) of polyelectrolyte chain rapidly increased with changing of $k_\theta$ from 5 kJ/mol(°)$^2$ and above. Above the cut-off of 5 kJ/mol(°)$^2$, the bond bending potential controls the stiffness of the polyon, whereas below the cut-off the non-bounded interactions between polyon spheres determine the polyon configurations. Additionally, only the two largest components of $R_g$ ($R_{gx}$ and $R_{gy}$) increase with increasing $k_\theta$, whereas the shortest component ($R_{gz}$) only gradually increases. This behaviour reflects the transition from a coil to a rod-like structure.

In order to investigate the role of electrostatic interactions in controlling the polyon structure, we determined the effect on the radius of gyration for a range of ionic strengths between 10 mM and 500 mM. The results are shown in Figure 4.19. The key findings are that there is a slight decrease in $R_g$ with increasing ionic strength for values of $k_\theta$ below the cut-off of 5 kJ/mol(°)$^2$. This reflects the screening of intramolecular repulsive electrostatic interactions.
However the transition from the coil to rod-like behaviour occurs at around a value of $k_\theta$ equal to 5 kJ/mol($\gamma^2$) indicating that the counterion concentration in region between 10 to 500 mM does not significantly influence flexibility. For $k_\theta$ larger than 5 kJ/mol($\gamma^2$), the results at low ionic strength indicate no change in size or shape of the polyion chain, except the $R_g$ at ionic strength of 500 mM differs remarkably from other results presented in Figure 4.19.

For protein-polyion interactions we also examined the effect of stiffness of the polyion backbone. Results of these simulations for ionic strength of 500 mM are shown in Figures 4.20 and 4.21. In Figure 4.20, the pH 5 simulation results are illustrated.
Figure 4.20 The radial distribution function at pH 5 for $k_\theta$ for 0.05 (–), 0.5 (–), 5 (–) 50 (–) and 500 (–) kJ/mol(γ)². Energies of binding (inset plot) for $k_\theta$ of 0.05 (–) 5 (–) and 50 (–) kJ/mol(γ)², where top plot corresponds to Lennard-Jones and bottom plot Coulomb energy.

Figure 4.21 The radial distribution function at pH 8 for $k_\theta$ for 0.05 (–), 0.5 (–), 5 (–) 50 (–) and 500 (–) kJ/mol(γ)². Energies of binding (inset plot) for $k_\theta$ of 0.05 (–) 5 (–) and 50 (–) kJ/mol(γ)², where top plot corresponds to Lennard-Jones and bottom plot Coulomb energy.
In Figure 4.20 and Figure 4.21 are shown the results of protein-polyion simulations to examine the effect of the bond angle potential at an ionic strength of 500 mM for pH values of 5 and 8 for $\varepsilon$ equal to 0.1 kJ/mol. This salt concentration was initially chosen to examine whether or not the pH dependence of “strong” binding could be captured over some range of the bond potential. The data presented in Figure 5.4 indicate that, at pH 5, there is a strong angle potential dependency on the radial distribution function. When the $k_0$ is equal to 0.05, 0.5 and 5 kJ/mol($\gamma^2$), there is a peak in the radial distribution function reflecting the protein-polyion binding. The binding affinity rapidly decreases when the chain becomes more rigid for values of $k_0 > 5$ kJ/mol($\gamma^2$). This value corresponds to the transition from chain to rod-like structure of the polion. For the rigid rod-like chain, the peak in the radial distribution function is reduced, indicating weaker binding than observed for the simulations with $k_0 < 5$ kJ/mol($\gamma^2$). This behaviour is expected for rigid polyelectrolyte chains, as the backbone cannot bend enough to maintain close contact with the charged patches of the protein globule. As a consequence, the binding of the rod-like chain is much more transient than that observed for low $k_0$. Most likely, the attractive electrostatic and the Lennard-Jones interactions are not strong enough to overcome the repulsive electrostatic interactions between the net negative charge of the protein and the polion charge.

The radial distribution functions obtained from simulations at pH 8 and an ionic strength equal to 500 mM are shown in Figure 4.21. Similar behaviour is observed to pH 5, except that the protein-polyion binding appears to be strongest for $k_0$ equal to 0.05 kJ/mol($\gamma^2$). There is no clear trend in the binding affinity with the $k_0$ values greater than 0.05 kJ/mol($\gamma^2$). In each of these runs the binding between protein and polion was transient as observed at pH 5. However, the protein-polyion is weaker at pH 8 than at pH 5 as reflected by a smaller peak in the radial distribution function for the runs with $k_0$ equal to 0.5 kJ/mol($\gamma^2$) and above. Discriminating between the peaks obtained for intermediate values of $k_0$ would require longer runs to obtain equilibrated values. The results indicate that pH dependent binding could be obtained by increasing the stiffness of the polion by making the bond angle potential greater.

4.5.4. Effect of distance dependant dielectric

In this section we study the effects of enhanced electrostatic interactions by using a screened Yukawa potential with a distance dependent dielectric constant. In this case, the dielectric constant is reduced with small separation between two charged spheres to account for the exclusion of water molecules between them. The Lennard-Jones potential well depth was kept constant throughout these simulations and set equal to 0.15 kJ/mol, which is an optimal value of $\varepsilon$ that can capture the pH dependent binding both at low and at high ionic strength. We examined a range of pH and ionic strengths equal to 500 mM and 100 mM. The results for the radial distribution function are presented in Figures 4.22 and 4.23, respectively. The distance dependant dielectric (DDD) model introduced enhanced electrostatics for the interactions
between oppositely charged spheres that are in close proximity to each other to mimic the effect of water exclusion. Therefore, the attractive intermolecular interactions are stronger, at short-range distance, than for the model without a distance dependant dielectric.

![Radial Distribution Function](image)

**Figure 4.22** The radial distribution function for $\epsilon$ equal 0.15 kJ/mol at ionic strength of 500 mM (DDD) for pH 5 (–), pH 6 (–), pH 7 (–), pH 8 (–), pH 9 (–) and pH 10 (–).

Energies of binding (inset plot) for pH 5 (–), pH 8 (–) and pH 10 (–), where top graph corresponds to Lennard-Jones and bottom graph Coulomb energy.

For the simulation carried out using the DDD electrostatic model the protein-polyion binding is only pH dependent when the salt concentration is high. As presented in Figure 4.22 at ionic strength equal to 500 mM and pH 10 the radial distribution function curve indicates polyion spheres are excluded from around the protein. This is also confirmed by examining the non-bonded interaction energies for the simulations at pH 10, which indicate very few binding events. For high ionic strength and values pH equal to 8 or below, the protein-polyion binding is permanent. The Coulomb and Lennard-Jones energies for high ionic strength are significantly greater at pH 8 than those for pH 5 indicating weaker binding at pH 8. For ionic strength at 100 mM the pH dependence of binding affinity is only minor as illustrated by considering the radial distribution functions shown in Figure 4.23. The radial distribution function indicates that binding
Figure 4.23 The radial distribution function for $\xi$ equal 0.15 kJ/mol at ionic strength of 100 mM (DDD) for pH 5 (–), pH 6 (–), pH 7 (–), pH 8 (–), pH 9 (–) and pH 10 (–). Energies of binding (inset plot) for pH 5 (–), pH 8 (–) and pH 10 (–), where top graph corresponds to Lennard-Jones and bottom graph Coulomb energy occurs even at pH 10. This behaviour suggests that the enhanced electrostatic interaction in the DDD model can lead to strong attractive interactions between the polyelectrolyte and protein that overcome the electrostatic repulsion between the large net negative charge on the protein and the negatively charged polyion spheres. The plots of energies presented in Figure 5.24, also indicate that the complex formation for all pH conditions for ionic strength at 100 mM is mainly driven by electrostatics; the coulomb interaction energies at pH 10 is almost as negative as at pH 8.
4.6. Conclusions

In this work, we examined the properties of a system where one human serum albumin monomer was interacting with one negatively charged polyeion using molecular dynamics simulations. Our model system showed that electrostatic interactions played an important role in protein-polyion complex formation. When the protein carries a net negative charge, the polyeion can still bind to a positively charged patch on the protein surface. The electrostatic interaction is not only the driving force behind the complex formation between HSA and polyanion. We found that short-range attractive interactions between polyeion spheres and the protein were needed to capture a realistic mechanism of complexation. For values of $\epsilon$ equal to 0.1 kJ/mol we were able to capture weak polyeion-protein binding, as the binding is pH dependent at low ionic strength. In this case, we found a complexation pH equal to 6 about which corresponds to a net charge of -5 on HSA. At higher salt concentrations, no binding occurs at any pH value, which is consistent with what has been observed experimentally by Cooper (2006). Simulations with a slightly greater $\epsilon$ equal to 0.2 kJ/mol follow behaviour referred to as strong protein-polyion binding in the literature. In this case, pH dependent binding is observed at low and at high ionic strength. This behaviour has been exhibited by PSS binding to proteins such as BSA and $\beta$-lactoglobulin (Park 1992, Gao 1997). Our findings indicate that a slight increase in the strength of the non-electrostatic interactions could lead to the transition between weak and strong binding. This result is consistent with the difference between weak binding polyeions such as poly(vinylsulphate) PVS and strong binding polyeions such as PSS (Sedlák 1998). The latter are more hydrophobic which could be the origin of the increased non-electrostatic attractive interactions.

Finally, we demonstrated that the angle potential applied to model polyelectrolyte particle can support or oppose the binding affinity between HSA and polyelectrolyte. The increasing angle potential enhances the rigidity of the chain and lowers the probability of complex formation.
4.7. References


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5.8. Appendix
Table 4.A: Amino Acid Residues on solvent accessible surface area of HSA generated by APBS.

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CHAPTER 5

THE INFLUENCE OF PECTINASE ENZYME ON THE POLY-L-LYSINE/POLYGALACTURONIC ACID MULTILAYER COMPOSITE

5.1. Abstract

Poly-L-lysine (PLL) and polygalacturonic acid (PGA) multilayer thin films were assembled through layer-by-layer technique and then exposed to an enzyme for determining the dynamics of glycosidic bonds hydrolysis along PGA backbone. The quartz crystal microbalance with dissipation (QCMD) was used to monitor growth and decomposition of the structure. With each deposition step of the polyanion, the mass and thickness of the multilayer increased in constant amounts following linear growth for the first five layers. Exponential growth was observed after depositing the sixth layer. Pectinase was used as the enzyme to digest polygalacturonic acid. Enzyme activity was also determined using dynamic viscosity measurements of PGA aqueous solutions. We established that applying pectinase on multilayer film, which was completed with either PLL or PGA, has the same effect, as the PLL layer does not have a protective ability against pectinase. The addition of pectinase, concentrated to one active unit per milliliter of acetate buffer, leads to a complete disintegration of 10 to 11 layers of PLL/PGA structure.
5.2. Introduction

The possible field of the polyelectrolyte composite applications can be also extended into food processing and preservation. Developing an understanding of the macromolecular interaction between food components, such as polysaccharides and proteins, could provide a fundamental impact on processing food in health-friendly fashion and on chemical digestion of carbohydrates. This research is concentrated on the enzymatic hydrolysis dynamics of pectin in poly-L-lysine/polygalacturonic acid (PLL)/(PGA) multilayer nanocomposite. In addition, we analyse the effect of enzyme on multilayer completed with either of the two components to determine if a film completed with poly-L-lysine would prevent or slow down the enzyme influence on inner parts of the multilayer. Furthermore we study the characteristic interactions between polyelectrolytes constructing the multilayer film, and examine the multilayer growth mechanism, which is associated with the distribution of charges along macromolecules that are available for electrostatic interactions.

In this study we examined multilayer build-up using positively charged poly-L-lysine (PLL) and polygalacturonic acid (PGA), the latter being a polysaccharide carrying a negative charge. Further, we examined the digestion of the multilayer film by the enzyme pectinase. Poly-L-lysine is a natural preservative for food products such as boiled rice, vegetables, soups and fresh fish for sushi (Hiraki 2003). Additionally, poly-L-lysine is biocompatible and non-toxic to any living cell. For this reason the peptide has biomedical applications, for example in composites, which support primary cell growth, and in biosensors and capsules for drug release (Tryoen-Toth 2002, Richert 2004). Polygalacturonic acid, also known as a pectic acid, is formed by partial hydrolysis of the side groups from pectin chains, which is a polysaccharide responsible for primary cell wall structure and middle lamella of fruits and vegetables. During the process of ripening, pectins are broken down in the hydrolysis of the glycosidic bonds by a group of enzymes recognised as pectinases, which are present in raw fruits. The major industrial applications of pectinase include wine and juice clarification, flavour extraction from the mash, fibre degumming, paper making, and coffee and tea fermentation. The research on enzyme activity and dynamics is needed to understand the mechanistic reasons for diet related diseases caused by modern eating habits and the influence of processed food on our health. A recent study on α-amylase enzyme hydrolysis of starches contained in a multilayer structure (Sasaki 2008) proved that using a quartz crystal microbalance with dissipation (QCMD) is a straightforward tool for the in vitro analysis of multilayer growth and digestion by enzymes. The QCMD allows observation of changes in resonant frequency of the crystal substrate due to deposition of the polyelectrolyte material on its surface. We employed QCMD to monitor poly-L-lysine/polygalacturonic acid multilayer build-up and decomposition. To confirm the result obtained by the QCMD we tested changes of the dynamic viscosity of polygalacturonic acid during a treatment with pectinase.
5.3. Materials and methods

5.3.1. Polyelectrolyte and enzyme solutions

Poly-L-lysine hydrobromide (PLL) was obtained from Sigma-Aldrich (catalogue number P6516) with molecular weight 4,000 to 15,000 Da (by viscosity) and weight average molecular weight of 15,000 Da (by Multi-Angle Laser Light Scattering MALLS). Polygalacturonic acid (PGA) was obtained from Fluka (Sigma-Aldrich catalogue number 81325) with molecular weight of 25,000 to 50,000 Da (literature). The solutions of PLL and PGA were prepared at 0.6 mg/mL in 10 mM sodium phosphate monobasic buffer containing 30 mM of sodium chloride. Galacturonic acid (GA) monohydrate Sigma-Aldrich (catalogue number G2125) molecular weight 212.15 g/mol.

The polyelectrolytes were dissolved in a buffer consisting of 10 mM sodium phosphate monobasic NaH$_2$PO$_4$ from Sigma-Aldrich (catalogue number S0751), 30 mM of sodium chloride NaCl from Sigma-Aldrich (catalogue number S7653) in deionised water at pH 7.

The enzyme used in all experiments consisted of pectinase solution from Aspergillus niger obtained from Sigma-Aldrich (catalogue number P4716) aqueous glycerol solution, 16 units/mg pectinase, 46 mg/mL (Lowry) was dissolved in buffer solution made of 50 mM sodium acetate CH$_3$COONa from BDH Laboratory Supplies (catalogue number 102364Q) in deionised water at pH 4.

5.3.2. Experimental methods

Measurements were carried out using a D300 quartz crystal microbalance with dissipation monitoring (QCMD) (Q-Sense AB, Västra Frölunda, Sweden) with a QAFC 302 axial flow measurement chamber. The adsorbed mass was detected on the surface of a disk-shaped AT-cut piezoelectric quartz crystal positioned between two golden electrodes. An AT-cut crystal is made by cutting along a 35° angle to the y axis of the quartz crystal. In the QCMD instrument the crystal disk is oscillating at a resonant frequency (approximately 5 MHz). The detected decrease of the primary frequency ($\Delta f$) is related to a deposition of mass ($\Delta m$) on the surface of substrate. When the mass distribution is even and the newly formed film structure is elastic (solid-like), then the change of frequency is related to mass adsorption following the Sauerbrey equation (Sauerbrey 1959):

$$\Delta m = -C \frac{\Delta f}{n}$$  \hspace{1cm} (5.1)

where $C$ is the mass sensitivity constant for a 5 MHz crystal, $C = 17.7$ ng/cm$^2$/Hz and $n$ is overtone number, $n = 1, 3, 5,\ldots$ However, a biomaterial structure cannot be considered rigid; its
rheological properties are viscoelastic due to the material flow within structure along with time delay in oscillation between substrate and the surface of adsorbed film. Therefore, the linear dependence of the resonant frequency on mass deposition (the Sauerbrey equation) deviates for biomaterial films - from experimentally observed results (Voinova 2002). To allow more precise examination of viscoelastic material, the quartz crystal microbalance was equipped with a module which measures the dissipation, which can detect loss of energy over the time after the sensor voltage is turned off, what causes the microbalance oscillations to decay. The decay rate is directly related to the viscoelastic properties of the material deposited on the sensor. The QCMD can be used to quantify the dissipation $D$ from the response of the crystal at the resonant frequency of 5 MHz, which is a fundamental frequency, further multiplied by its harmonic overtones ($n$ equal 1,3 and 5) giving frequencies: 15 MHz and 25 MHz. The effect of ‘missing mass’ in the Sauerbrey relation for viscoelastic biofluids can be established by using an appropriate rheological model (Maxwell or Kelvin/Voigt) using relation between dissipated and stored energy in the system, where the rigid part of the film rests on solid substrate and viscoelastic section of the film is exposed to the fluid media. Voinova at el. (2002) provide the following relations for $\Delta f$ and $\Delta D$ which apply only for viscoelastic fluids:

$$\Delta f_{\text{total}} \approx -\frac{\eta_2}{2\pi m_q \delta_2} + \frac{h_1 \rho_1 \omega}{2\pi m_q} \left[ 1 - \frac{2}{\rho_1} \left( \frac{\eta_2}{\delta_2} \right)^2 \frac{G''}{G'^2 + G''^2} \right] \quad (5.2)$$

$$\Delta D \approx \frac{1}{\pi f m_q} \left[ \frac{\eta_2}{\delta_2} + 2 \left( \frac{\eta_2}{\delta_2} \right)^2 \frac{h_1 \omega G''}{G'^2 + G''^2} \right] \quad (5.3)$$

where the index 1 and 2 refer to the thin soft overlayer and the liquid bulk phase, respectively, $\delta_2$ corresponds to a viscous penetration depth of the shear wave in the bulk Newtonian fluid expressed as

$$\delta_2 = \sqrt{-\frac{2\eta_2}{\rho_2 \omega}} \quad (5.4)$$

where $\eta$, and $\rho$ describe viscosity and density of liquid in aqueous solution, respectively, $\omega$ is a circular frequency, $h$ is a film thickness, $m_q$ is a mass of the quartz plate, $G'$ is a storage modulus and $G''$is a loss modulus.

The QCMD response to dissipative viscoelastic multilayer composites can be then modelled using the Maxwell or Voigt models included in QTools software supplied with the QCMD instrument to calculate more accurate mass and thickness of the viscoelastic material. The
QTools uses a simplex algorithm to calculate the minimum of the sum of squares of the scaled errors between the model output and the experimental data corresponding to $\Delta f$ and $\Delta D$ for three frequency overtones. The software requires the values of viscosity $\eta_2$ and density $\rho_1$ of the bulk liquid, which was estimated to be equal 1100 \, [kg/m$^3$] and 1.0 \, [mPas], respectively.

The microbalance chamber was filled with deionised water and thermostated to approximately 37°C for 45 minutes prior to the experiment. The multilayer composites were fabricated by sequential injections of 1.5 mL poly-L-lysine or polygalacturonic acid solutions. The thin monolayers of charged macromolecules were adsorbed on the silica surface of the substrate after each dose of biopolymer solution. Each solution was then left in the microbalance chamber for 4 minutes and after which it was rinsed with 1.5 mL of buffer to remove any excess of free macromolecules in the bulk liquid surrounding the composite. This procedure was repeated until 10 or 11 consecutive layers were formed. The multilayer composites were then treated through introducing pectinase into the measurement chamber where multilayer was deposited onto a silica surface, with 1.5 mL of pectinase enzyme solution, prepared as described in chapter 5.3.1., solutions with pectinase of following concentrations of 0.01, 0.1, or 1 active unit per millilitre [AU/mL] were used. In addition, a negative control of only sodium acetate buffer was used. The film formation was carried out using multiple silica chips to determine repeatability of the experiment. Between each measurement, the silica substrate and microbalance chamber were rinsed thoroughly with deionised water and 2% solution of Hellmanex II alkaline surfactant (Hellma GmbH & Co cat. no. 9-307-010-507). The surfactant was left in the system for 10 minutes and flushed with plenty of distilled water. Afterwards, water was drained and the measurement module was dried out using compressed nitrogen. The silica chip was then treated in an ozon-UV chamber for 10-15 minutes to remove any organic residue from the active surface, rinsed with distilled water and dried with nitrogen.

A TA instruments cone-and-plate rheometer AR2000 was used to carry out the viscosity measurements of polygalacturonic acid solutions containing pectinase. The viscosity assessment was performed using a 60 mm 1° acrylic cone rotating at the shear rate equal 200 s$^{-1}$. The PGA sample was thermostated to the temperature of 37°C. A dose of 100 µL of 5 AU/mL pectinase enzyme was injected after the initial viscosity readings for pure PGA were taken. The viscosity of fluid was monitored for another 60 minutes after the enzyme treatment. The enzyme activity assay was based on the method described by Gomes et al. (Gomes 2009) for an endo-polygalacturonase enzyme.
5.4. Results and discussion

The frequency and dissipation were changing with each deposition step of the material on the silica surface. In Figure 5.1 is shown an example of these changes during multilayer build-up for three overtones, \( n \) equal to 3, 5 or 7. The experimental data corresponding to each overtone are shifted by a constant value from each other for every measurement. Thus the results can be reported using only one of the harmonic overtones; we use the date for \( n \) equal to 3 for a frequency 15 MHz.

![Figure 5.1 QMCD frequency (blue data) and dissipation (red data) changes related to the 10 layer multilayer film composed of PLL/PGA. D3, D5, D7 correspond to dissipation and F3/3, F5/5, F7/7 reflect changes of frequency of harmonic overtones](image)

At the beginning of a multilayer build-up experiment, the frequency and dissipation values are approximately zero as there is no mass adsorbed onto the negatively charged silica chip. Further on, the frequency decreases in the successive steps after each injection of the solution. The first layer was built at the 8\(^{th}\) minute of the experiment using positively charged poly-L-lysine macromolecules; PLL was left to interact in the microbalance chamber for 4 minutes. In the next step, the film was rinsed with buffer solution, which is associated with a minor increase of the frequency. The rinsing was performed to remove unbound polyelectrolyte from the bulk liquid neighboring newly formed layer. The buffer is left in the instrument for 4 minutes. The next step involved an injection of the solution containing negatively charged macromolecules of polygalacturonic acid. This step took place at the 16th minute after measurements started to be recorded. The PGA interacts with the previously deposited PLL as reflected by the sudden drop of frequency and dissipation. The procedure was then repeated until the films consist of 10 or 11 layers. The analysis of the QCMD response for deposition of the material, shown on Figure
5.2a and b, allows us to establish that the layers made of poly-L-lysine and polygalacturonic acid form strong bounds through multiple interactions of electrostatic nature. The electrostatic interactions are a main driving force of multilayer build-up. Secondly, there are shorter range interactions such as dispersion forces and hydrogen bonds (Hammond 2000) between the oppositely charged polyions, which also contribute to multilayer formation.

Figure 5.2 Frequency and dissipation changes for, third harmonic overtone, throughout construction of; a) 10 layer film; b) 11 layer film
The sharp decrease of frequency observed during each deposition step indicates that both PLL and PGA binding affinities to the previously formed layer are high. The material accumulates immediately on the microbalance surface and each layer reached equilibrium within 2 minutes after the material was injected into the QCMD measurement chamber. Furthermore, the frequency of oscillations does not change considerably that when the newly formed layer was rinsed with the buffer solution suggesting the existence of its strong integrity to the top layers of the structure. As the composite build-up progresses, changes in frequency and dissipation observed for the 10th and 11th deposition steps are gradually increased compared to previous steps of adsorption. We consider this phenomenon to be related to major differences between PLL and PGA viscoelastic properties of their monolayer walls. The PLL layers tends to form more compact, solid like structures whereas the PGA layers are softer and more viscous as shown in Figure 5.3. The origin of these properties is suggested in the research of Krzeminski at el. (2006) where the lower molecular weight poly-L-lysine was indicated to have an ability to penetrate and diffuse into a pectin monolayer. Another study by Porcel at el. (2006) using poly-L-lysine and hyaluronic acid indicates the multilayer growth exhibits a similar growth pattern to study of PLL/PGA system.

![Figure 5.3 A relationship between frequency and dissipation at the beginning; △, □ and at the end; ▲, ■; of the film formation](image)

Consequently, we suggest that the PGA adsorbs only on the upper layer of PLL, while PLL disperses into the PGA wall creating an excess of uncompensated positive charge, which then contributes towards larger adsorption of the PGA during the next deposition step and so amplifying the role of PGA in the multilayer structure. Therefore, the viscous character of the composite is predominant near the upper layers of the film, which is evident when analyzing relative changes between frequency and dissipation during deposition of the first bilayer and the final one (Figure 5.3). The frequency and dissipation dependence data are correlated in linear
tendency to the increasing mass and thickness of the multilayer during the first few steps, and after the fifth deposition step this dependency becomes exponential. At the surface of the completed multilayer build-up, the frequency to dissipation ratio, for the PGA deposition step, exhibits a liquid-like behavior. This is associated with a greater decrease of dissipation over frequency change than the ratio of frequency to dissipation for PLL first deposition step, which exhibits only a minor difference between the changes of frequency with decreasing dissipation which is linked to more solid-like structure. Similarly, the final deposition steps of PLL and PGA show an enhanced difference in viscoelastic properties to the first adsorption steps.

The transition from a solid-like to a liquid-like structure within a film formation is strongly associated with the growth character of a multilayer composite. As mentioned in chapter 2.5.1., the increase of thickness and mass associated with each adsorbed layer can be either linear or exponential according to the rheological properties of the examined material, which are measured during QCMD analysis. Therefore in Figure 5.4a and b we present the composite thickness as a function of the number of deposition steps for all structures tested.

For each experiment, the growth is always linear for the first five layers and exponential from the sixth layer onwards. As a result we established that PLL/PGA multilayer structures exhibit a linear - exponential growth for films assembled with at least 10 layers. We applied an enzyme solution (pectinase) to monitor the enzymatic hydrolysis of the polygalacturonic acid embedded into multilayer structure after the completion of the film build-up. The multilayer decomposition was monitored until reaching equilibrium. The rate of breakdown was different for each of the tested concentrations of pectinase, as shown in Figure 5a and b. The oscillating silica surface with completed 10 or 11 layer films reaches the equilibrium frequency of approximately -250 MHz, at which point we applied a solution of pectinase in sodium acetate buffer at pH 4. This pH was chosen because pectinase achieves its optimal activity near the pH 4 (Dinu 2001). The digestion of PGA using pectinase was tested at the following concentrations 0.01, 0.1 and 1 AU/mL. Additionally, we applied pure sodium acetate buffer onto formed multilayers to examine the effect of changing from pH 7 to 4. This procedure was vital to establish if PLL/PGA films are sensitive to buffer solutions at low pH.
Figure 5.4 Increase of multilayer thickness throughout deposition steps for:

a) 10 layer film; b) 11 layer film
Figure 5.5 Dynamics of the enzymatic hydrolysis of polygalacturonic acid composed into;
   a) 10 layer film, b) 11 layer film
We observed an increase of frequency proportional to pectinase concentration following an injection of enzyme into the QCMD measurement cell. Further, there is a negligible rise of the frequency when only acetate buffer was introduced, indicating that the PLL/PGA films are primarily resistant to the lowering of pH from 7 to 4. The PLL/PGA multilayer stability in changing pH and ionic strength has been established during previous research in our group (Moffat 2007). Furthermore, the presence of pectinase causes a distinctive grow of frequency over the time, where 1 AU/mL of enzyme can cause the rise of frequency up to -35 MHz, which is associated with the disintegration of the majority of the PLL/PGA multilayer in a period of 15 minutes. When concentration of pectinase was equal 0.01 and 0.1 AU/mL the dynamics of enzymatic hydrolysis decreased considerably and the decomposition of multilayer was only partial.

The rate of enzymatic hydrolysis was also determined from measurements of dynamic viscosity of 5 mL PGA solution (PGA concentration equal to 10 mg/mL) after application of 35 µL pectinase solution (at 1 AU/mL). The initial viscosity of PGA solution was equal to 0.0016 Pas, as we presented in Figure 5.6. viscosity of solution and molecular weight of PGA decreased after the addition of the enzyme as a result of enzymatic hydrolysis.

The enzyme activity was analyzed following a method described by Gomes at el., where one unit of enzyme activity is defined as the amount of enzyme that reduces the initial viscosity by 50% per minute (Gomes 2009). We established that the average activity of pectinase is equal to 0.0507 units in 100 µL at pH 4 and temperature 37°C. Consequently, in a 5 mL PGA sample tested rheologically we reached 2.535 relative viscosity active units (RVAU). This assay is used to confirm the ability of pectinase to degrade PGA when applied to the multilayer film. The
viscosity of PGA was measured during addition of pectinase at the highest concentration used in the QCMD assessment of enzymatic hydrolysis of PGA.

5.5. Conclusions

Polygalacturonic acid/poly-L-lysine multilayer thin films were constructed and characterized at the average human body temperature of approximately 37°C. The hydrolysis of polygalacturonic acid (PGA) by pectinase was directly monitored by quartz crystal microbalance with dissipation (QCMD). Poly-L-lysine (PLL), used as polycationic macromolecule, was a suitable media for creating electrostatically attractive interactions with PGA, which resulted in a successful multilayer formation of 10 or 11 layer composites. Constructed polyelectrolyte multilayer material exhibited a linear and an exponential growth. Our results indicate that the depositing of poly-L-lysine results in diffusion of PLL into a multilayer structure, which successively leads to an increase of mass and thickness of the composite with each deposition step. Adding pectinase to the multilayer, built of 10 or 11 layers, had the same effects on the integrity of the composite for both types of structure. It was not significant which polyelectrolyte was constructing the top layer. The influence of pectinase concentration proved that 1 AU/mL of pectinase in the environment of polygalacturonic acid is able to digest polygalacturonic acid chains incorporated into the multilayer film, any lower concentration of pectinase tested in this study showed only partial decomposition of the PLL/PGA multilayer.

5.6. Acknowledgements

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CHAPTER 6

PROTEIN – PROTEIN INTERACTIONS. AGGREGATION MECHANISMS AND METHODS OF PROTEIN SELF-ASSEMBLY INHIBITION

6.1. Abstract

Protein aggregation leads to reduced yields throughout bioprocessing, starting from the production of proteins in cells through to manufacturing and during the storage of therapeutics to extending shelf-life of medicines. Despite intensive research on the formation of protein aggregates, many aspects that cause this process are still unknown. The focus of this work is to improve understanding of the protective effects towards protein aggregation of small molecule additives such as arginine, which are often used in bioprocessing.

We investigated the influence of arginine on the formation of protein interactions and aggregation in solutions of insulin, poly-L-arginine and myoglobin. The effects are not regular for all the tested proteins. In the insulin and poly-L-arginine studies, addition of arginine suppressed the spontaneous self-association of protein macromolecules, whereas in the studies on myoglobin, the effect of arginine is minor. The differences may be caused by the mechanism of arginine interactions with macromolecules. We propose that the attractive protein-protein interactions, which originate from non-electrostatic forces, are more likely to be affected by the presence of arginine. The results from experiments involving myoglobin indicate aggregation caused by electrostatic interactions, is likely to remain unaffected by arginine.
6.2. Introduction

Protein behaviour in aqueous solutions and their self-assembly are important issues for progress in the field of biotechnology, genetic and bio engineering and drug discovery. The understanding of protein – protein interactions and environmental conditions controlling the mechanism of protein self-assembly in aqueous solutions are fundamental concepts for determining protein behaviour in solutions *in vivo* and *in vitro*. This knowledge is desired in development of therapeutics, for controlling protein stability during processing, and for extending shelf-life of liquid formulations upon storage. Despite intensive experimental (Wang 2008, Chi 2003) and theoretical (Curtis 2006, Melander 1977) studies on protein structural stability many questions remain unanswered. Of particular interest here is to better understand how protein aggregation can be controlled during bioprocessing by using the small molecule additive - arginine, which is commonly added to protein solutions as a stabilization agent. It was previously reported in literature that arginine can be used to suppress the undesired association of the proteins in solutions (Baynes 2005, Valente 2005, Arakawa 2007, Lyutova 2007 and Hamada 2009). A key challenge is that each protein is structurally diverse and as a consequence, each protein’s behaviour is unique. To address this obstacle, we focused our study on determining the effect of arginine on a few different protein systems with different structures and properties. Our aim was to determine the mechanistic effects of how arginine alters protein-protein interactions and the impact upon aggregation, and to explore methods for inhibition of the protein aggregation.

We selected a few proteins to study as a representative sample. These were: myoglobin - an aggregation-prone globular heme-containing protein, insulin - for its importance as a therapeutic and because insulin exhibits high aggregation propensity under certain solution conditions and poly-L-arginine - in order to isolate how arginine interacts with charged groups on protein surfaces.

In this chapter we present background to each of the chosen proteins and our motivation for studying their self-assembly. Next, we describe the sample preparation and experimental techniques; these include static light scattering, turbidimetric titrations and micro differential scanning calorimetry. In the results and discussion section, we first present the behaviour of zinc-containing and zinc-free insulin in solutions with or without arginine. Then we study the effects of arginine on preventing self-aggregation of myoglobin, which are illustrated by the changes of weight-average molecular weight detected during static light scattering experiments and thermal stability of the protein solutions containing arginine in comparison with the arginine-free myoglobin solutions. Finally, we test solubility of poly-L-arginine in presence of various salts and arginine.
6.3. Materials

6.3.1. Myoglobin

Myoglobin's function in living organisms is involved with the reduction and oxidation reactions taking place in muscles. Also, myoglobin is responsible for lipid oxidation in meat which leads to deterioration of food. Myoglobin is a water-soluble globular protein comprised of a single polypeptide chain containing a prosthetic heme group and approximately 150 amino acids. The molecular weight of myoglobin is between 14,000 to 18,000 Da. The molecular weight varies according to the source of myoglobin; the mammals' myoglobin tends to be larger than the one originated from fish (Chaijan 2007, Yamaguchi 1979, Josepha 2010). It was reported by Faustman (1990) that myoglobin undergoes denaturation in temperature of 40 °C and in extreme alkaline and acidic pHs. Myoglobin is considered to be unstable in aqueous solutions and prone to aggregation. In this work, we used horse heart myoglobin purchased from Sigma-Aldrich (catalogue number: M1882).

6.3.2. Insulin

Insulin is a hormone produced by the pancreas. Its biological function involves processing of glucose dissolved in the blood and regulating metabolic functions of fat and carbohydrates. Other functions of insulin include controlling the production of other proteins. Diabetes is linked to abnormal production of insulin. There are two types of diabetes. Diabetes type one is known as insulin-resistant diabetes, in which case the pancreas produces enough insulin, but insulin does not have the ability to carry out its function in metabolic processes. The second type of diabetes is known as the insulin-dependant diabetes, in which case the pancreas does not produce sufficient amount of insulin to support body function. In the latter case, the patient needs to be treated with insulin injections. There is a growing demand for insulin-based therapeutics on worldwide scale for treating diabetes patients, which involves storage and transportation of the insulin-based drugs.

Insulin consists of two amino acid chains: a 21-residue A chain and 30-residue B chain, which are connected by two disulphate bridges. The molecular weight of insulin is approximately 5,800 Da. The secondary structure of insulin involves a six-molecule assembly into a hexamer with zinc ions holding three dimers together. The positions of zinc ions in the secondary structure of insulin are illustrated in Figure 6.1. The structure of the protein is published in the protein data bank for human insulin 1A10 (Chang 1997) and bovine insulin 2A3G (Smith 2005). The crystallographic data and primary sequence indicate that both types are very similar in structure to the extent that the bovine form has been developed previously as a human therapeutic.
Figure 6.1 Removal of insulin hexamer based on crystallographic structure by Smith (2005), zinc removal illustration by Iwai (2009), where (a) Insulin hexamer, then insulin hexamer broken up into dimeric structures (b) view down the threefold axis to the upper Zn [Zn(u)] and (c) view up the threefold axis to the lower Zn [Zn(l)]
The human (and porcine) insulin differs in sequence from bovine insulin in A chain, where the A8 residue in human insulin is threonine, while in bovine the A8 residue is alanine (Yip 1998). Also, the degradation and binding mechanisms of human and bovine insulin to the insulin receptors are reported to be identical (Kotzke 1995). The insulin used in this work is from bovine pancreas and purchased from Sigma-Aldrich (catalogue number: I4011).

6.3.3. Poly-L-arginine

Poly-L-arginine is a homopolypeptide protein comprised of arginine, which is a basic group with an intrinsic $pK_a$ equal to 12. The secondary structure of poly-L-arginine depends upon composition of the solvent. Under neutral conditions, the peptide forms a random coil. Ion binding occurs in solutions of chaotropic mono-valent anions leading to a conformational change from random coil to $\alpha$-helix, which can also be induced by increasing pH from neutral to alkaline. $\beta$-sheets are formed when heating the sample. Poly-L-arginine is also able to complex with divalent cations through guanidinium groups and peptide nitrogen, which participate as ligands around the copper ions (Tosi 1974). Poly-L-arginine is able to bind to negatively charged cell membrane; proteins that are able to penetrate cell membrane are reported to be rich in poly-L-arginine (Schwieger 2009). This property of poly-L-arginine is vital in the treatment of viral diseases such as HIV where the small molecular drug can be carried though a cell membrane. Poly-L-arginine sulphate purchased from Sigma-Aldrich, with a molecular weight range of 15,000 to 50,000 Da, was used in this research (catalogue number: P7637).

Other materials used in this work included sodium chloride (catalogue number: S7653), magnesium chloride (catalogue number: M8266), lithium chloride (catalogue number: L4408) from Sigma-Aldrich, L-arginine (catalogue number: 371904T), tris buffer (catalogue number: 15504-020) from VWR, potassium chloride (catalogue number: 60132) from Fluka, potassium thiocyanate (catalogue number: P/7240/50) from Fisher Scientific, sodium acetate $\text{CH}_3\text{COONa}$ from BDH Laboratory Supplies (catalogue number 102364Q) and acetic acid from Fisher Scientific (catalogue number AC222140000).

A dialysis membrane of 8,000 Da molecular weight cut-off from Fisher Scientific was used for selected experimental tests on myoglobin and poly-L-arginine (catalogue number: BID-030-030N). Dialysis membrane for insulin was supplied by Sigma with molecular weight cut-off in region of 2,000 Da (catalogue number: D7884).
6.4. Experimental methods

In this section we describe the experimental techniques employed in our study. In section 6.4.1. the method for sample preparation is described, as well as dialysis of the protein solutions. Next we provide the theoretical background of the instrumental techniques carried out to examine the protein-protein interactions. These are static light scattering, turbidimetric titrations and micro differential scanning calorimetry.

6.4.1. Dialysis and sample preparation

The protein solutions used in this work were dialyzed against the appropriate buffer composition to remove small molecule impurities that are included in the commercial formulations. The dialysis membrane was prepared by boiling in pure water for 30 minutes and rinsed thoroughly, repeated twice.

The insulin samples required the process of zinc removal through a two-step dialysis technique. First, insulin was dissolved in acetate buffer of pH 3 and at a protein concentration of 7 g/L. The solution was dialysed against the acetate buffer at pH 3. The acetate buffer was composed of glacial acetic acid (diluted to 0.2 M) with sodium acetate (0.2 M). The sample volume was approximately 20 ml and the total volume of buffer was 3 L. The dialysate was changed 3 to 4 times during the overnight dialysis process. The zinc is removed from the insulin formulation by dialyzing at pH 3 as the histidine residues repel zinc from the insulin hexamer and the protein becomes monomeric. Once the zinc removal was complete, the pH of the insulin solution was adjusted to the required value, and afterwards transferred into a beaker with 3 L of buffer solution at the required composition, and dialysed for another 6 hours. Finally, the insulin solutions were removed from the dialysis bag and used in the experimental testing.

For experiments with myoglobin, a 3 g/L protein solution in 10 mM tris buffer and sodium chloride at ionic strength of 0.15 M was placed in the dialysis tube and sealed. The process of dialysis was carried out in 5 L of the buffer composition for 12 hours at room temperature. The myoglobin samples, prepared according the above method, were tested only in turbidimetric measurements versus time, SLS and Micro DSC. Other experiments involving myoglobin were undialyzed.

The poly-L-arginine solutions were prepared using 10 mM sodium acetate buffer at pH 3. The concentration of poly-L-arginine in tested samples was 0.3 g/L and the samples were prepared using salts of ionic strength of 50 mM NaCl or KCl alone or associated with 50 mM and 1 M Arginine. No dialysis process was applied for preparation of poly-L-arginine solutions.
6.4.2. Static Light Scattering (SLS)

SLS was used to determine the scattering intensity of the protein (or peptide) solution minus that of the solvent. The measured excess light scattering data is analyzed in terms of a Zimm plot (Zimm 1948). The Zimm plots are used to calculate the weight-average molecular weight of the protein, $M_w$, and the osmotic second virial coefficient $A_2$, which provides information about the interaction between a pair of peptides.

In this technique a collimated, single wavelength polarized light beam is directed upon a volume of the solution containing particles or macromolecules.

![Figure 6.2 Static light scattering cell](image)

For the experiments reported here the size of the scatterer is less than 1/20th the wavelength of the light (which is 690 nm), so that there is no angle dependency of the scattered light. As a consequence, in a Zimm plot, the data is plotted as a function of peptide concentration according to:

$$\frac{KC}{R_\theta} = \frac{1}{M_w} + 2A_2c$$  \hspace{1cm} (6.1)

where $R_\theta$ is the excess intensity of scattered light at a given angle $\theta$ ($R_{\text{sample}} - R_{\text{solvent}}$) (also known as the excess Rayleigh ratio), $c$ is the peptide mass concentration, $K$ is an optical constant, which is given by:
\[ \frac{4\pi^2n_0^2(df/dc)^2}{N_A\lambda^4} \]  \hspace{1cm} (6.2)

where \((dn/dc)\) is the refractive index increment of the peptide, \(n_0\) is the refractive index of the solvent, \(N_A\) is Avogadro Number \((6.023 \times 10^{23})\) and \(\lambda\) is the wavelength of the laser light reaching the detector. The intercept of the Zimm plot is \(1/M_N\) and the value of the second virial coefficient \(A_2\) is equal to the slope of the graph divided by two.

Static light scattering (SLS) was carried out using the Wyatt Technology Dawn EOS static light scattering detector combined with ASTRA V 5.1.7.3 software. The samples were injected through the light scattering detector using a syringe pump with an in-line 0.2 µm filter used to remove dust from the sample solution before it entered the detector cell. First the light scattering of the solvent was measured and then the samples were injected in order of increasing concentrations.

6.4.3. Turbidimetric Titrations

The turbidimetric titrations were used to determine the effect of pH or of additive (salt or arginine) concentration on protein aggregation. The protein aggregates are large enough to be detectable through the decrease in transmittance of the incident light due to scattering. In addition, turbidity was monitored as a function of time in experiments to determine the time course of aggregation.

This experimental method was carried out using a Brinkmann PC 950 Probe Colorimeter combined with single wavelength optical probe at 490 nm, as depicted in the schematic diagram shown in Figure 3.1 and described in detail in chapter 3. To monitor changes of pH during this experiment, a Fisherbrand Hydrus 300 pH meter with glass electrode was used. Before each turbidity measurement, the instrument was turned on for 30 min and placed in pure water to equilibrate the output readings. In the titrations of turbidity versus pH, 0.1 M HCl or 0.1 M NaOH were used to adjust pH of the titrated solutions. The output data of the colorimeter is transmittance \(T\), which depends on turbidity by the following relation:

\[ \text{Turbidity} = 100 - T \% \]  \hspace{1cm} (6.3)

The solution to be titrated had a volume of 20 mL and was stirred gently for 10 minutes with the colorimeter already placed in the solution. The colorimeter was set to 100% of transmittance. We monitored a relative change of transmittance between the transmittance of the protein solutions at the beginning of the experiment and after addition of each titrant portion. The readings were taken approximately every 2 minutes after the titrant was added. The volume of the titrant portions varied between 100 µL and 10 µL. For the experiments where turbidity was
measured with respect of time, the titrant was not used, and the measurement begun as soon as the protein was dissolved in the appropriate solvent and stopped after a period of 20 minutes. In all turbidity titrations the samples were kept in constant temperature and slowly stirred.

6.4.4. Micro Differential Scanning Calorimetry

Micro-differential scanning calorimetry measurements were used to monitor structural transitions of proteins upon heating. The thermally initiated aggregation, unfolding or degradation can be detected using Micro DSC. In a Micro DSC experiment, temperature is increased at a set heating rate applied to two chambers containing a sample and a reference. Both chambers are kept at the same temperature. Micro DSC is used to measure the difference of heat flow between a protein sample and the reference, which contains only the solvent at the same mass as the protein sample.

A Setaram III Micro Differential Scanning Calorimeter with combined SetSoft 2000 software was used to carry out the experiments included in this study. Applied heating rate was 1 °C/min. and temperature range was 20 to 90 °C. Before each experiment the sample was equilibrated at 20 °C for 10 min.
6.5. Results and discussion

Static light scattering (SLS) and turbidimetric titrations were used to monitor the behaviour of proteins and determine whether the particles in solutions formed attractive or repulsive interactions. Average-weight molecular weight $M_w$ provided information about the onset of the complex formation and transmittance indicated development of larger irreversibly formed aggregates. Micro DSC was used to measure the melting temperature of myoglobin, which was used as an indicator of myoglobin conformational stability. The conformational stability was correlated with the aggregation behaviour monitored using turbidity.

6.5.1. Effects of salts and arginine on aggregation of insulin

The aggregation of insulin was monitored using turbidimetric titrations and static light scattering experiments. First the solubility of the native form of insulin (zinc–containing) was measured using a turbidimetric titration as a function of pH. The titration plots are presented in Figures 6.3 and 6.4 where the data correspond to the titrations of a 20 mL of 7 g/L insulin solution containing 50 mM NaCl and dissolved in tris buffer at pH 9. The first titration was performed by adding between 20 or 100 µL titrants of 0.1 M HCl to decrease the pH from 9 to 3. Then, a second titration was performed by adding in titrants of 0.1 M NaOH to raise the pH from 3 to 9. As it can be observed from Figure 6.3, insulin is insoluble when initially dissolved at high pH; this is demonstrated by high turbidity readings. In order to solubilise insulin, pH needs to be lowered to a value below 4. When the pH is then raised again, the turbidity remains relatively low indicating that insulin can maintain its soluble form even at high pH.

In case of titrations from pH 3 to 9 (red data) our trends in changes of turbidity are very similar to the result obtained by Giger and Dubin (2008). Even if the change in turbidity seems to be less significant compared to the titrations from pH 9 to 3 we found a local maximum in pH around 5.5 for both 10 mM and for 50 mM. The exception in similarity between Giger’s and our results is that Giger’s maximum for the titrations at ionic strength of 10 mM is slightly lower, this might be related to the time taken to do the titration. However, our local maximum for the turbidity equals 5.5 and is more realistic as it occurs closer to the $pI$ of insulin. This is expected as repulsive electrostatic interactions are minimised at $pI$ while the local attractive electrostatics is present. Additionally, our change in turbidity, for the titrations from low to high pH, is greater at 10 mM than at 50 mM, consequently these results are consistent with what would be expected when increasing the salt concentration from 10 mM to 50 mM, since the attractive electrostatics at the $pI$ are screened more efficiently by salt at higher concentration.
For the titration from pH 9 to 3 it is clear that the protein does not fully solubilise until the pH is lowered to the region of 3.3. This may be because the hexameric form of insulin is assembled at high pH in the presence of zinc and consequently the existence of hexameric form of insulin could be the precursor to the higher forms of aggregates. When the pH is lowered, insulin loses the zinc ion, which leads to the formation of monomeric insulin. The histidine residues in the insulin chains are charged positively at low pH and can not bind zinc.

The maximum turbidity, in the experiment where the pH 3 to 9 titration is carried out in 50 mM ionic strength, could be noticed in pH region of 4.3 to 5.2, which is not corresponding to the isoelectric point reported in literature to be within a region of pH 5.55 to 5.6 (Wintersteiner 1993). This may indicate that the force causing that self-association of insulin hexamers in high pH is not primarily of the electrostatic origin, as it was shown by Giger and Dubin (2008). In contrast to Giger’s work, who obtained maximal aggregation of insulin at low ionic strength, the total change in turbidity in our experiments is lower for titration from pH 9 to 3 at ionic strength of 10 mM than that of 50 mM NaCl. This trend is also against the electrostatic origin of the attractive interactions at low ionic strength and in-line with the study by Yip et al. (1998). Yip indicated that this difference in origin behind the insulin hexamer self-assembly may be caused by the minor differences in behaviour between human (or porcine) insulin and bovine insulin.
Titrations from pH 3 to 9 (red data) indicated that once the zinc is removed in low pH and the insulin aggregates are broken up, the small rise in turbidity is likely to be based on electrostatic interactions because it is corresponding to pH region nearest to $pI$ of insulin. In case of the titration of hexameric insulin solutions (pH 9 to 3 - blue data), the attractive interactions existing in high pH prevented the protein full dissolution until the pH was lowered to 3.5, in these titrations the electrostatically-based aggregation near $pI$ was not observed.

Further, we examined the effect of arginine on aggregation of undialyzed insulin using turbidimetric titrations with respect to pH, in which case solutions of different buffers containing 1 g/L of insulin were titrated with 0.1 M NaOH from pH 3 to 9. The buffers included (1) 0.2 M acetic acid ($\text{CH}_3\text{COOH}$), (2) 0.2 M acetic acid and 100 mM of arginine and (3) 0.2 M acetic acid, 100 mM of arginine and 100 mM of NaCl. The turbidimetric titration curves for these experiments are shown in Figure 6.5. The gathered data suggest that insulin in the presence of acetic acid is aggregation prone, and the sharp rise of turbidity has its onset at pH 4.5. Most likely the aggregation occurs due to reducing the net charge on insulin when increasing pH towards the isoelectric point of 5.5. This in turn leads to a reduction in repulsive electrostatics, which could prevent aggregation. The addition of arginine and arginine with NaCl eliminates the majority of attractive interactions between insulin molecules throughout the whole range of pH values. However there is a slight increase of turbidity level across the curve corresponding to titration of insulin with acetic acid, arginine and sodium chloride in comparison with the curve representing insulin in solution with acetic acid and arginine only. Salt induced attraction can be
either due to enhancing the hydrophobic interactions between proteins or it could be due to the screening of repulsive electrostatic interactions (Curtis et al 2002).

![Graph showing turbidity changes with pH](image)

Figure 6.5 Turbidimetric titration curves with respect to pH of insulin solutions for insulin with 0.2 M acetic acid (■), with 0.2 M acetic acid and 100 mM arginine (■), and with 0.2 M acetic acid, 100 mM arginine and 100 mM NaCl (■).

Next, we examine the ageing of undialyzed 1 g/L insulin solutions in the presence of 20 mM arginine and 10 mM NaCl and in the presence of 10 mM NaCl both at pH 5.5. This pH was chosen for the proximity to the isoelectric point of insulin, where electrostatic interactions could facilitate the aggregation (Wintersteiner 1933). As reported earlier (in Figure 6.4) turbidity of insulin solutions in 10 mM NaCl should increase in solutions at pH 5.5. To obtain the insulin solution for this experiment, insulin was first dissolved in the corresponding buffer at pH 3.5 and then the pH was adjusted to pH 5.5. Turbidity readings were taken every minute for 30 minutes. The results are shown in Figure 6.6, where it is clear that the addition of arginine leads to stabilisation of insulin in the solution and suppression of the self-association. The aggregation of insulin is not fully prevented as the change on turbidity, equal to 2% of the initial value, is observed during first 12 minutes of insulin ageing in solutions with arginine and NaCl. After that time the aggregates are not changing in size which is illustrated by the plateau region from 12 minute onwards.

For the solutions of insulin and NaCl, an equilibrium plateau of the turbidity begins to occur after 25 to 28 minutes. However the total change in turbidity is much greater than that for insulin with arginine. Our finding should be contrasted to the study by Giger (2008), where an increase of turbidity was only observed in the first 30 seconds for solutions of insulin in 10 mM NaCl at pH 5. Also the total change in turbidity observed by Giger is much lower than the one reported here.
The differences may be due to the slightly lower pH in the study by Giger, the various levels of zinc contamination in the samples and the slightly different origin of insulin, which is from a bovine source in our study versus porcine origin in Giger’s work (2008).

![Change in turbidity vs. time](image)

**Figure 6.6** Aggregation of insulin in solutions at pH 5.5 versus time, for insulin with 20 mM arginine and 10 mM NaCl (■), and insulin with 10 mM NaCl (■)

The effect of arginine on insulin behaviour occurs in solutions within pH 3 to 9 at very low concentrations of arginine, and the suppression of insulin self-assembly is magnified near to the isoelectric point when arginine is added. More understanding of the mechanism of interactions between proteins and arginine can be found in a recent study by Arakawa (2007), where it was proposed that the protective effects of arginine were linked to arginine binding to histidine groups. In order to examine this effect further we studied the effect on insulin interactions of zinc removal in which case histidine groups on insulin are freed up from the zinc binding. The aggregation of zinc-containing insulin and zinc-free insulin was probed with static light scattering experiments for solutions with or without arginine. The results of these experiments showing the change in weight-average molecular weight versus pH are shown in Figure 6.7. The concentration of insulin is 1 g/L and concentration of arginine is 100 mM.
The samples of zinc-containing insulin exhibit a clear trend where $M_w$ increases with rising pH. At pH 7 the $M_w$ equals approximately 31,000 Da, which suggests that all of the insulin is in the hexameric form. The study by Mil thrope (1977) reports that at pH 7, the native, zinc-containing insulin solutions consist of a mixture of hexameric (80%) and monomeric (20%) structures. In such conditions the weight-average molecular weight would be equal 29,000 Da, which is in good agreement to our findings. In the presence of arginine, it was not possible to control the pH and we only studied solutions at pH above and below pH 7. In this case, the weight average molecular weight was less than that expected for a hexamer and at pH between 9 and 10; the measurements indicate the average insulin oligomeric state is a dimer. At pH 5, it is clear that arginine suppresses the hexamer formation of insulin. It is possible that arginine binds to histidine groups and prevents them from binding zinc which is needed for the hexamer to form. As we concluded before, the rising of pH allows zinc ions to form the hexameric insulin if there is no presence of arginine. Therefore, we established that addition of arginine can prevent from formation of larger domains of insulin in the solutions. Still, the presence of zinc in the solution with insulin could lead to the formation of dimeric forms of insulin as the values of $M_w$ at pH 9 and 10 (Figure 6.7) suggest that insulin’s molecular weight is approximately double of the monomeric value. It is possible that some zinc ions can still bind to insulin but the majority of attractive interactions between insulin active site and zinc are screened by arginine.

Further we investigated zinc-free insulin samples which were prepared using the dialysis method described in section 6.4.1. The static light scattering tests were taken at pH 4 and pH 8 for 7 g/L insulin solutions in acetate buffer and (1) 100 mM NaCl, (2) 100 mM arginine, (3) 100 mM arginine and 100 mM NaCl and (4) no other additive. In Table 6.1 we show the weight-average molecular weight data obtained for the above samples.
In a study by Attri et al. (2010), the authors found that zinc-free insulin undergoes a pH-dependent isodesmic association which is strongest at pH 7.2 and is also observed at pH values as low as 3 and as high as 9. We find a similar behaviour here in the low ionic strength buffer where the insulin forms high molecular weight aggregates both at pH 4 and at pH 8. In solution at pH 8, increasing salt concentration to 100 mM appears to lower the level of aggregation. However, the most dramatic effect is observed when adding arginine, which leads to reducing the molecular weight of insulin to the dimer value.

The effect of arginine on the aggregation behaviour at pH 8 is in-line with turbidimetric study presented in Figure 6.5, where the addition of arginine lowered the aggregation propensity at high pH. These results imply that arginine might have a similar effect on the insulin monomer and on the insulin hexamer. In contrast, in solutions at low pH, the zinc-containing insulin does not aggregate. In solutions at pH 4 the values of $M_w$ for all tested samples are reported to be higher than these for pH 8 and the effect of arginine on insulin aggregation is less pronounced in solutions at pH 4. This phenomenon is also confirmed in turbidimetric titrations illustrated in Figure 6.5, which shows that the major aggregation of insulin starts at pH 4.5, below that pH region the turbidity of arginine-containing or arginine-free samples is similar. Thus, one possibility is that arginine only interacts with the deprotonated forms of histidine which might be present at pH 8, but not at pH 4.

Table 6.1: Weight-average molecular weight for zinc-free insulin solutions for pH 4 and 8.

<table>
<thead>
<tr>
<th>Solution</th>
<th>$C_{\text{insulin}}$ [g/L]</th>
<th>$M_w$ [Da]</th>
<th>No. of monomeric insulin units in domain</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH 8</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>0.71</td>
<td>27,424</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>0.36</td>
<td>34,709</td>
<td>6.1</td>
</tr>
<tr>
<td>100 mM arginine</td>
<td>0.72</td>
<td>14,122</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>0.36</td>
<td>10,363</td>
<td>1.8</td>
</tr>
<tr>
<td>100 mM arginine</td>
<td>0.75</td>
<td>12,607</td>
<td>2.2</td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>0.37</td>
<td>9,888</td>
<td>1.7</td>
</tr>
<tr>
<td>Buffer</td>
<td>0.67</td>
<td>20,731</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>0.34</td>
<td>210,647</td>
<td>36.7</td>
</tr>
<tr>
<td><strong>pH 4</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>1.02</td>
<td>35,665</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>1.96</td>
<td>34,876</td>
<td>6.1</td>
</tr>
<tr>
<td>100 mM arginine</td>
<td>1.11</td>
<td>25,857</td>
<td>4.5</td>
</tr>
<tr>
<td>100 mM arginine</td>
<td>1.80</td>
<td>39,112</td>
<td>6.8</td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>1.07</td>
<td>29,034</td>
<td>5.1</td>
</tr>
<tr>
<td>Buffer</td>
<td>1.67</td>
<td>18,920</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>48,241</td>
<td>8.4</td>
</tr>
</tbody>
</table>
6.5.2. Aggregation of myoglobin in presence of sodium chloride and arginine

To determine the nature of interactions causing myoglobin molecules to form aggregates we examined the change in turbidity for myoglobin solutions for a range of ionic strengths for sodium chloride. At this point we again show the results for turbidimetric titration of myoglobin, which were presented earlier in chapter 3, but on this occasion we take a different angle in discussing them. The initial concentration of myoglobin in these tests was 1 g/L with a volume of 20 mL. The solutions were then titrated by adding 0.1 M HCl to lower the pH from 10 to 4. In Figure 6.8 the data are shown for following turbidity measurements at ionic strengths of 0.01, 0.05, 0.1, 0.15, 0.3 and 0.45 M NaCl.

![Graph showing changes in turbidity for myoglobin in presence of no salt, 0.01 M, 0.05 M, 0.1 M, 0.15 M, 0.3 M and 0.45 M NaCl.](image)

**Figure 6.8 Changes in turbidity for myoglobin in presence of no salt (○), 0.01 M (■), 0.05 M (■), 0.1 M (■), 0.15 M (■), 0.3 M (■) and 0.45 M (■) NaCl**

The turbidity of myoglobin in aqueous solutions without NaCl begins to increase in solutions at pH 9, whereas when the salt is present at low ionic strength (less than 0.15 M) the turbidity does not begin to increase until pH 8. In high ionic strength (greater than 0.15 M) solutions there is no change in turbidity of the protein solutions until pH reaches 5.5. This tendency suggests that aggregation of myoglobin predominantly depends on ionic strength and on pH. The maximum in turbidity occurs near pH 6, which is slightly shifted from the expected rise of turbidity caused by the electrostatic forces near the isoelectric point. For horse heart myoglobin the \( pI \) equals 7.2, which indicates that the driving force of the aggregation, at high ionic strength, is related to the removal of the intermolecular repulsive or attractive electrostatic interactions between patches of opposite charge through the screening by the increasing salt concentration.
The finding that aggregation decreases with increasing ionic strength hints that we observed the salting-out effect supporting screening of the long-range attractive electrostatic interactions, the absence of turbidity increase near isoelectric point at high salt concentration supports this result.

For ionic strength of 0.3 and 0.45 M, we observed negative turbidity readings in pH region below 5.5. This is due to the presence of large chunks of myoglobin precipitate, which sedimented on the bottom of the sample container even when stirred. In turbidimetric titrations of myoglobin in solution of lower ionic strengths the precipitate was much smaller and floated freely within whole volume of the sample.

Static light scattering measurements were performed for a range of concentrations of myoglobin solutions, the most concentrated sample contained 3 g/L of the protein and others were 2.4, 1.8, 1.2 and 0.6 g/L. The character of interactions between myoglobin macromolecules were monitored at pH 6, 7 and 8 and two different ionic strengths of NaCl: 0.15 and 0.3 M, these ionic strengths were chosen because very little turbidity was observed under these conditions (Figure 6.8). We present the results for the average molecular weight of freshly prepared myoglobin solutions in presence of NaCl in Table 6.2. The increase of ionic strength from 0.15 to 0.3 M shows that the myoglobin-myoglobin interactions changed its character towards more repulsive at higher salt concentration than at low salt concentration. This is also reflected in the average molecular weight obtained from light scattering, where at 0.15 M of sodium chloride the molecular weight is, on average, higher than that when myoglobin is in the solution with 0.3 M of NaCl. This implies that at pH 8 and ionic strength of 0.3 M myoglobin is in its monomeric form as the average $M_w$ is near the molecular weight of the monomer of horse heart myoglobin reported in literature at 16951 Da (Zaia 1992). At lower salt concentration – 0.15 M – and pH 7 and 8, it is likely that myoglobin’s intermolecular electrostatic interactions are responsible for the aggregation, because the majority of macromolecules appear to be bound in dimeric domains since the average $M_w$ equals nearly twice as the $M_w$ of the myoglobin monomer. It was not possible to achieve an accurate $M_w$ measurement at pH 6, because aggregation occurred too readily and stable SLS readings could not be obtained.

Table 6.2: Effect of ionic strength and pH on $M_w$ for solutions at pH 8 and 3 g/L myoglobin.

<table>
<thead>
<tr>
<th>I [M]</th>
<th>pH</th>
<th>$M_w$ [Da]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 NaCl</td>
<td>8</td>
<td>19359</td>
</tr>
<tr>
<td>0.15 NaCl</td>
<td>8</td>
<td>23050</td>
</tr>
<tr>
<td>0.15 NaCl</td>
<td>7</td>
<td>23171</td>
</tr>
<tr>
<td>0.15 NaCl</td>
<td>6</td>
<td>aggregation</td>
</tr>
</tbody>
</table>
When myoglobin was dissolved in solutions of higher salt concentration – 0.3 M – the salt was able to screen long-range attractive interactions. Also, the effect of pH change from 7 to 8 at ionic strength of 0.15 M NaCl indicates that pH influence on the average molecular weight of the myoglobin at low salt concentration is insignificant, as myoglobin is aggregation prone even in pH region away from the isoelectric point. The ionic strength dependence on $M_w$ at pH 8 is in-line with data obtained from the turbidimetric titrations presented later in this section and indicates that the initial stages of myoglobin aggregation is caused by the long-range electrostatic attractions.

The aggregation mechanism of myoglobin was also examined in terms of ageing of the myoglobin solutions in presence of NaCl. For such assessment we tested the solution of myoglobin in 0.15 M of NaCl at pH 8, where we measured the average molecular weight of the protein in light scattering experiment. Data related to the spontaneous changes of myoglobin molecular weight in time is shown in Figure 6.10, where the sample was tested in 10 minute intervals. We observed the significant rise of the molecular weight of myoglobin in 0.15 M NaCl, for the fresh sample the average molecular weight was reported to be in the region of 23,300 Da, which corresponds to dimeric domains of myoglobin in minority of the sample volume. When the same sample was tested 10 minutes later, the average molecular weight of the protein was in the region of 25,000 Da, which indicates continues growth of the myoglobin particles in the solution towards domains of the dimeric structure. Further, measurements of the average molecular weight showed that, after 40 minutes from preparation of the myoglobin solution, the protein particles were at 27,000 Da, which indicates that majority of myoglobin molecules formed dimers.

Next, we determined the average molecular weight of myoglobin in freshly prepared solutions containing different amounts of sodium chloride and arginine. The average molecular weight
data gathered for the samples, presented in Table 6.3, signifies that at pH 6 the addition of arginine helps to prevent self-aggregation of the protein and this ability is weaker at pH 8. Additionally, the effect of the concentration of arginine in solutions without NaCl has only a minor influence on the aggregation of myoglobin. In the solutions where arginine and NaCl were used to inhibit the self-association of myoglobin the effect of additives on proteins behaviour is more pronounced. The results indicate that myoglobin aggregation is pH dependant and is best prevented at pH 6 with decreasing effect for pH 7 and 8. Myoglobin is most stable in fresh samples when in the presence of 150 mM of NaCl associated with 150 mM of arginine.

Table 6.3: Average molecular weight determined by SLS.

<table>
<thead>
<tr>
<th>Myoglobin 3 g/L with additives:</th>
<th>pH6</th>
<th>pH7</th>
<th>pH8</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 mM arginine</td>
<td>16602</td>
<td>aggregation</td>
<td>19750</td>
</tr>
<tr>
<td>300 mM arginine</td>
<td>16151</td>
<td>aggregation</td>
<td>24917</td>
</tr>
<tr>
<td>150 mM arg 150 mM NaCl</td>
<td>13008</td>
<td>22340</td>
<td>36941</td>
</tr>
<tr>
<td>300 mM arg 300 mM NaCl</td>
<td>17152</td>
<td>27593</td>
<td>52674</td>
</tr>
</tbody>
</table>

This result shows that arginine is effective at preventing aggregation of myoglobin in pH near 6 as we observed predominantly the monomeric myoglobin at all tested conditions. At pH 7 and 8 the addition of arginine causes even greater aggregation than it would be observed in myoglobin solutions without the peptide.

Further, we examined the effect of arginine on the time-course of protein aggregation for different values of pH using 20 mL of solutions containing myoglobin concentration equal to 2 g/L with 10 mM Tris buffer. The data presented in Figures 6.11, 6.12 and 6.13 correspond to samples containing arginine at concentrations of 150, 300 and 500 mM across pH 6, 7 and 8. The increase in turbidity throughout the time for samples containing 150 mM of arginine is most significant in solutions at pH 6, where the turbidity rises of 0.8 % in 20 minutes. This result indicates that at pH 6 and low concentration of arginine, the aggregation occurs readily. This is not unexpected for that low concentration of arginine as the aggregation of myoglobin is greatest in solutions near pH 6 as indicated by the results shown in Figure 6.9. However, the same concentration of arginine at pH 8 is able to break the aggregates of myoglobin. The existence of aggregates at such high pH was confirmed in our static light scattering tests presented in Table 6.2.
Figure 6.10 Turbidity over time for myoglobin with 150 mM arginine, where pH 6 (▲), pH 7 (■) and pH 8 (♦), pH adjusted by adding 0.1 M NaOH.

Figure 6.11 Turbidity over time for myoglobin with 300 mM arginine, where pH 6 (▲), pH 7 (■) and pH 8 (♦), pH adjusted by adding 0.1 M NaOH.

Figure 6.12 Turbidity over time for myoglobin with 500 mM arginine, where pH 6 (▲), pH 7 (■) and pH 8 (♦), pH adjusted by adding 0.1 M NaOH.
Increasing the arginine concentration in myoglobin solutions to 300 mM appears to stabilise the aggregation of myoglobin in all tested pH conditions. The turbidity change is insignificant at that concentration of arginine. A further increase of arginine concentration to 500 mM shows a tendency to break aggregates at pH 6 as the turbidity decreases with time up to 1.5% in relation to fresh protein sample in just 20 minutes. This result implies that 500 mM of arginine in the solutions of myoglobin has the strongest anti-aggregating influence on the protein. At pH 7 there is no change in turbidity, which suggests that myoglobin is either fully solubilised in these conditions or is aggregated in small domains undetectable by turbidimetric measurements. However, at pH 8, there is a slight increase in turbidity at high arginine concentration when at low arginine concentration turbidity decreases in the same pH condition. This is in-line with the SLS study presented in Table 6.3, as it was shown, in higher pH presence of arginine in the myoglobin solution may accelerate the aggregation.

6.5.3. Thermal stability of myoglobin in aqueous solutions

In this section we test the thermal effects associated with myoglobin in solutions within a wide range of temperatures from 20 to 90 °C to examine structural changes which myoglobin undergoes under the thermal treatment. These tests were carried out to monitor the influence of NaCl and arginine on characteristic temperatures of myoglobin associated with such transitions like denaturation, aggregation and melting. Micro DSC experiments were carried out to analyse the changes in the melting temperature of myoglobin in solutions with 150 mM NaCl, and both NaCl and arginine at ionic strength 150 mM. At the concentration of 3 g/L myoglobin in solutions the expected melting temperature of myoglobin is 79 °C; this is based on data published by Bull (1973). The data obtained based on Micro DSC for the myoglobin solutions is presented in Table 6.4 and Figure 6.14.

Table 6.4: Enthalpy corresponding to identified peaks in DSC thermograms at pH 8.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Temperature [°C]</th>
<th>Enthalpy [J/g]</th>
<th>Thermic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myoglobin</td>
<td>80</td>
<td>0.174</td>
<td>Endo</td>
</tr>
<tr>
<td>Myoglobin + 150 mM NaCl</td>
<td>30</td>
<td>0.029</td>
<td>Endo</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.064</td>
<td>Endo</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.006</td>
<td>Endo</td>
</tr>
<tr>
<td></td>
<td>77</td>
<td>0.035</td>
<td>Endo</td>
</tr>
<tr>
<td>Myoglobin + 150 mM NaCl and 150 mM arginine</td>
<td>60</td>
<td>0.0085</td>
<td>Endo</td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>0.0021</td>
<td>Endo</td>
</tr>
</tbody>
</table>
The enthalpy of the denaturation of myoglobin has an endothermic character and for the native form of the protein it is expected to be in the region of 78 °C (Zhang 2007). At this characteristic temperature, myoglobin loses its native structure through destabilisation of the forces holding the protein globule together. These forces are of electrostatic, hydrophobic or of van der Waals origin and in lower temperatures and optimal pH and ionic strength conditions are able to maintain the original conformation of myoglobin. Under rising temperatures the protein undergoes conformational changes associated with endothermic and exothermic peaks detectable using differential scanning calorimetry. Cooper (1994) characterised the endothermic peaks as the indicators of the denaturation of proteins and exothermic peaks as a sign of irreversible aggregation. In our DSC thermograms we obtained exothermic peaks at approximately 85 °C for all tested samples and numerous endothermic peaks in lower temperatures (Table 6.4). The endothermic peaks correspond to different intermediate stages of the myoglobin unfolding. However, the complete denaturation of myoglobin is most probably related to temperatures in the region immediately prior to the exothermic peak related to the melting temperature. For myoglobin solutions with no addition of salt or arginine, the endothermic peak corresponding to denaturation can be found at the temperature of 80 °C, which is associated with major enthalpy. For myoglobin in the solutions with 150 mM of NaCl this peak is of much weaker enthalpy and can be found at the temperature of 77 °C and there are few other endothermic peaks in lower temperatures. Finally, the solution of myoglobin, 150 mM NaCl and 150 mM arginine shows two small endothermic peaks at the temperatures of 60 °C and at 76 °C. The enthalpy associated with thermal effect of endothermic peaks in solutions.
containing arginine is much smaller than for all other peaks linked to denaturation of myoglobin in solutions with no arginine.

The shift of the endothermic effect in myoglobin samples containing arginine to lower temperatures is similar to what was observed by Zhang et al. (2007) in experiments involving polyelectrolyte interacting with myoglobin. It was reported that endothermic peak at 62 °C emerged after addition of poly(styrene sulfonate) (PSS) to myoglobin solutions at pH 7.4. Zhang concluded that existence of two endothermic peaks simultaneously indicates that unbound myoglobin undergoes denaturation at its typical temperature (approximately 78 °C), and polyelectrolyte-bound myoglobin would demonstrate denaturation peak at lower temperature due to perturbed structure caused by complexation with PSS. This indicates that addition of arginine to the solutions of myoglobin may cause similar effects on native protein structure comparable to the addition of polyelectrolyte.

6.5.4. Solubility of poly-L-arginine in aqueous solutions with salts and arginine

Initial experiments were carried out to determine the effect of changing salt concentration and salt type on the solubility of poly-L-arginine solution. 10 mL samples were prepared at a poly-L-arginine concentration of 1 g/L in salt solutions containing either: magnesium chloride, potassium chloride, or potassium thiocyanate at concentrations of either: 10 mM, 50 mM, or 100 mM at pH 6. The solubility was quantified in terms of the turbidity of the solution; an example of such titration is presented in Figure 6.15, where poly-L-arginine was titrated with 100 µL portions of 4 M KCl. For the systems that appeared cloudy, the corresponding salt solution was titrated into the polypeptide solution until an abrupt change in the turbidity measurement was observed which corresponded to the solution going from cloudy to clear. In case of titration, presented in Figure 6.15, we tested the poly-L-arginine sample using static light scattering at two stages of titration; first – at the abrupt change in the turbidity corresponding to ionic strength equal to 0.23 M of KCl, and second – at ionic strength of 0.4 M KCl. In both cases light scattering confirmed that molecular weight of poly-L-arginine in solutions with minimum 0.23 M KCl equalled 12260 Da, which corresponded to the range of average molecular weight for poly-L-arginine specified by the supplier. The polypeptide concentration at the abrupt change is used as an estimate for the solubility and is given in Table 6.6 below. The main finding is that the poly-L-arginine is insoluble in solutions of low salt concentration except for when the salt is magnesium chloride. This indicates that, even though poly-L-arginine has a large positive charge, the polypeptide is still able to form attractive interactions with itself. With increasing salt concentration, the solubility is increased indicating that the salt reduces the attractive interactions. This behaviour is counter-intuitive to what is expected for a regular polyelectrolyte, in which case increasing salt concentration should screen repulsive electrostatic interactions.
Next we examined how arginine altered the interactions between poly-arginine molecules using a turbidimetric titration with respect to arginine concentration. Poly-L-arginine solutions at concentrations of 1 g/L in either 50 mM of KCl or 50 mM of NaCl were titrated with 1 M solution of arginine. The results are shown in Figure 6.16. In samples where there is no arginine present, the turbidity of the sample is greater than 0. This rise is more pronounced for the solution containing KCl, where the initial turbidity reached 2.7 %, and for NaCl it is approximately 0.8 %. Upon adding the arginine solution to the polypeptide and salt solutions the turbidity decreases. After the concentration of 0.06 mM of arginine was added the turbidity reaches a plateau at 0 %, as shown in Figure 6.15. This volume of arginine solution corresponds to 8.71 mg of arginine present in the solution with 10 mg of poly-L-arginine.
Figure 6.15 Change in turbidity of the poly-L-arginine solutions upon addition of arginine, where:

- 50 mM KCl (■)
- 50 mM NaCl (▲)

Following the weight ratio of arginine to poly-L-arginine in solutions needed to facilitate the dissolution of poly-L-arginine we can calculate that each poly-L-arginine macromolecule (average molecular weight 15,000 Da) requires a minimum 75 molecules of arginine to create the abrupt change of turbidity.

The strong effect of arginine on the turbidity of the poly-L-arginine solution indicates that arginine induces repulsive interactions between the polypeptide molecules. In order to examine this effect further, we have measured the second virial coefficient using static light scattering data on poly-L-arginine solutions in the presence of 50 mM KCl or 50 and 100 mM NaCl, both with addition of 50 mM of arginine. The light scattering plots and table containing $A_2$ and the average molecular weigh are shown in Figure 6.17. There is clear ionic strength dependence in the behaviour of poly-L-arginine in aqueous solutions at pH 4.25, where for NaCl and KCl at low ionic strength the character of intermolecular interactions in poly-L-arginine solutions are more repulsive than these for higher ionic strength of NaCl, as reflected by the large values of second virial coefficients. At the ionic strength of 50 mM NaCl, the $A_2$ equals $0.01088 \text{ mol·ml/g}^2$ which indicates that the repulsive interactions are slightly weaker than these for the 50 mM KCl as the $A_2$ equals $0.0137 \text{ mol·ml/g}^2$. When the concentration of salt increases, the repulsive character of interactions decreases by a magnitude. In the case of NaCl, increasing ionic strength from 50 to 100 mM changes the interactions from strong repulsion to mild repulsion. These data support the conclusion that the stabilising effect of arginine on self-association of poly-L-arginine can be weakened or supported by the addition of salt. Furthermore, poly-L-arginine behaves as a typical polyelectrolyte in arginine solutions, in which case, the interactions become much less repulsive with increasing ionic strength due to screening by salt ions.
Next, we examined the effect of pH on the poly-L-arginine solubility using a turbidimetric titration with respect to pH. A solution of poly-L-arginine 0.3 g/L dissolved in aqueous solutions with 50 mM of NaCl or 50 mM of KCl at pH 4 was prepared. At this pH the polypeptide solution appears clear. The turbidimetric titration experiment was carried out, by addition of 50 to 200 µL of 5 M NaOH. Poly-L-arginine is positively charged at low pH and the \( pK_a \) of the arginine group is equal to 11.72. Near this pH we expect the rise of the turbidity due to the precipitation of the polypeptide when the arginine groups become deprotonated and lose their positive charge. The experimental data obtained from the turbidity titrations are shown in Figure 6.18.

### Table 6.1

<table>
<thead>
<tr>
<th>Salt added</th>
<th>( A_2 ) [mol/ml/g]</th>
<th>( M_w ) [Da]</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 NaCl</td>
<td>0.00184</td>
<td>11952</td>
</tr>
<tr>
<td>50 NaCl</td>
<td>0.01088</td>
<td>15387</td>
</tr>
<tr>
<td>50 KCl</td>
<td>0.01370</td>
<td>16900</td>
</tr>
</tbody>
</table>

![Figure 6.16 Light scattering by poly-L-arginine solutions in presence of 50 mM arginine and 50 mM KCl (●), 50 mM NaCl (■) and 100 mM NaCl (♦), values for \( A_2 \) and \( M_w \).]
Figure 6.17 Turbidimetric titration of poly-L-arginine in solution with salts, where 50 mM KCl (♦) and 50 mM NaCl (■).

The results indicate that the poly-L-arginine is already aggregated at pH 4. As expected, with increasing pH, there is a considerable increase in turbidity that occurs around pH 10 to 11 which is slightly below the $\text{pK}_a$ value. This finding indicates that the polypeptide is charged at neutral pH, even though the polypeptide is relatively insoluble under these conditions (see Table 6.5). This may be indication of that the rise in turbidity near pH 12 is only a relative change indicating the formation of bigger aggregates from existing smaller domains, and salts such as KCl and NaCl do not prevent the formation of the domains in poly-L-arginine solutions.
6.6. Conclusions

Insulin, myoglobin and poly-L-arginine were examined for their self-association mechanism in aqueous solutions. The attempts of stabilisation of protein in their dissolved state were based on the addition of different types of salt at various ionic strengths and a small peptide – arginine. The protein solutions were tested for their light scattering properties using the measurements of turbidity and static light scattering (SLS).

The tests confirmed that zinc-free insulin is soluble at pH below 4.5 for conditions where there is no additive in the solution. The presence of salt at low ionic strength does not prevent the formation of oligomeric forms of insulin at pH > 4.5, which implies that the aggregation of insulin into the hexameric form through binding to zinc ions may be driven by the interactions of non-electrostatic nature. In the insulin solutions at pH 8 the aggregation depends clearly on addition of arginine, and it was demonstrated that just 20 mM of arginine can suppress the aggregation at pH 5.5 even for zinc-containing insulin solutions.

For the study based on myoglobin, the effect of arginine was evident at pH 7 and 8, as the aggregation was detected through static light scattering as a function of pH, solutions containing arginine and myoglobin at high pHs demonstrated that the addition of arginine supports aggregation at high pH. However, arginine was effective at preventing aggregation of myoglobin at pH 6.

The reason for strong aggregation of myoglobin in low pHs is that the aggregation is driven by partially folded protein, whereas at higher pH the aggregation is relatively weak (when no arginine is added). This is linked to repulsive interactions between negatively charged myoglobin monomers, which at higher pH can maintain their native form. Thus, one possibility is that arginine interacts with partially folded form of myoglobin and makes it less aggregation prone.

Also we demonstrated that the self-association of myoglobin in the solutions with no arginine was strongly dependant on the concentration of salt. This implies that for myoglobin the interactions are mainly driven by the electrostatics.

In solutions with poly-L-arginine the addition of salts has little effect on preventing the self-aggregation of the polypeptide, but the addition of arginine proves that the additive can facilitate the dissolution of poly-L-arginine. One possibility is that arginine binds to poly-L-arginine and removes the intermacromolecular attractions.

The concept of the addition of arginine into the protein solutions to increase their stability and control the aggregation mechanism proves to be correct for the systems where the protein aggregation is not driven by electrostatic interactions. Therefore, we propose that arginine may be more effective for inhibition of aggregation based on hydrophobic forces.
6.7. References


CHAPTER 7

INFLUENCE OF TEMPERATURE AND AGEING ON COAGULATION OF SILK FIBROIN IN AQUEOUS SOLUTION

7.1. Abstract

This work is focused on optimizing the method for the production and purification of aqueous silk fibroin (SF) solution for regenerated fibre spinning process. The aim is to mimic a natural process of spinning silk by silkworm. We investigated the rheological properties of aqueous SF solutions with dynamic rheological tests. This analysis was carried out to determine phase behaviour of SF during the spinning of nanofibre non-woven mats for scaffold materials in tissue engineering. We tested aqueous SF solutions at concentrations between 3 and 12% w/v to establish the viscoelastic properties of the spinning solutions. Freshly prepared (within 48 hours from dissolving) SF solutions exhibited a random coil phase, while the SF self-assembled into aggregates of β-sheets in ‘aged’ solutions. Monitoring the self-assembly and the rheological properties of SF protein dissolved in water under changing deformation, frequency and temperature gives insight into understanding phase transitions during coagulation of silk fibre produced by silkworm.
7.2. Introduction

A consistently reliable biomaterial for tissue engineering has not yet been found, despite the vast progress in material science and engineering. Over the last twenty years, a variety of synthetic polymers have been used successfully in medical applications. However, there are limits in the range of chemical composition, non-degradability or products of the hydrolysis of those polymers. A range of polymers, such as poly(lactic acid) (Kumbar 2008), collagen (Glowacki 2007), chitosan (Jayahumar 2010), alginate (Kuo 2001) or bacterial cellulose (spun into form of fibre by bacteria Acetobacter xylinum) (Svensson 2005) have been developed to form scaffolds for tissue engineering. While those materials offer significant benefits like biocompatibility and non-toxicality, there remains a necessity for a biomaterial, which can be designed and manufactured with a high level of process control over the physical structure, mechanical properties and chemical composition. The scaffold material should be bioactive, biodegradable and biocompatible, in addition to giving physical and chemical signals for cell migration and assembly to three dimensional tissues. The scaffold should provide mechanical integrity for whole tissue structure under physiological stress mainly for pulsatile flow of the microorganisms and cell growth medium.

Throughout the last three decades, interest has risen in using silk-based materials for scaffolds in tissue engineering, biomedical applications, and controlled release systems. Recent study on new methods available for protein purification opened an opportunity for fibroins to provide its best performance as a biomaterial without causing undesired effects that are associated with presence of sericin – a glue-like protein, which holds together the silkworm cocoons. Silk fibroins used for tissue scaffolds need to be free of traces of sericin. Sericin contamination was reported in early tissue engineering research as the source of undesirable immunological responses (Soong 1984). Commonly used biomaterials such as polystyrene and poly(2-hydroxyethyl methacrylate) are no less thrombogenic than silk fibroin biomedical material (Santin 1999). Also, studies on micro and nanostructure and its role in the regeneration of live tissue shows (Kenneth 2008) that repair cells recognise nanometric topologies of fibrous and microporous texture. Those cells are able to create a new extracellular matrix on surfaces that mimic their native environment. For that reason, there is a need for a wide-ranging review of the methods and procedures in all phases of research in designing materials for modern tissue engineering. This recent development indicates silk fibroin as an important resource of natural material, which could become a good biomaterial for tissue engineering.

Silk-based biomaterials are desired in modern material science. The most recent trends in biomedical research aim to imitate the natural environment of artificial tissue through providing the nanostructure of the biomaterial design and produced with high standards and capability to deliver suitable scaffold for tissue regeneration. It has been found that silk fibroin scaffolds improve the effectiveness of, and also provide a lower rate of rejection for body implants (Lawrence 2009).
7.3. Microstructure and morphology of silk fibroin

Silk fibroin obtained from *Bombyx mori* is a natural block copolymer. Equilibrium phase behaviour of block copolymers is controlled by thermodynamic mismatch, including segment interactions, molecular size and composition. A record nine different phases (four cubic, two hexagonal and one lamellar lyotropic liquid crystalline and two micellar solutions) have been reported for a ternary isothermal system of an amphiphilic triblock copolymer and selective solvents (Alexandridis 1998). The silk fibroin block copolymer is composed of hydrophobic blocks with repetitive sequence consisting of short side-chain amino acids, such as glycine and alanine, and hydrophilic blocks with complex sequences that consist of larger side-chain amino acids as well as charged amino acids. The hydrophobic blocks tend to form $\beta$-sheets or crystals through hydrogen bonding and hydrophobic interactions. This structure forms the foundation of the tensile strength of silk fibroin (Simmons 1996). The rigid hydrophobic blocks are joined with less systematic hydrophilic blocks to provide increased elasticity and stiffness of silk fibroins.

The exact procedure of how native silk fibroin solution becomes fibre still remains an area of intensive study. Techniques mimicking the process by silkworms include the spinning of concentrated aqueous solution of silk fibroin into a non-Newtonian liquid crystalline state. In that state, liquid crystals of fibroin are lubricated and stabilised by $\text{H}_2\text{O}$ present in the system. Subsequently, fibroin forms micelle-like structures during phase separation which occurs due to the silk fibroin’s intrinsic hydrophilic–hydrophobic block structure. A hypothesis on self assembling of silk fibroins is presented by Jin and Kaplan (Jin 2003), which reports that the initial production stages of silk fibroin solution involves a gradual increase of silk fibroin concentration and formation of an extended chain structures. This process continues until the hydrophobic blocks start to self-assemble into organised micelles and finally a gel structure. The micelles then aggregate into fibroin globule. At this stage, the protein is in an ordered metastable phase that can sustain enough water content to avoid premature exchange to the $\beta$-sheet structure. The presence of shear throughout the process of spinning maintained by the silkworm’s head movements stimulates the final aggregation of the $\beta$-sheets into crystalline blocks. In the final phases of silk spinning, the silkworm forms a hydrophilic protein (sericin) to join two elementary fibroin filaments and provide an adhesive for cocoon shape structure. Silk fibres spun in such a method are insoluble in the majority solvents such as water, ethanol, dilute acids and alkali.
7.4. Methods of biomaterial fabrication

There are several methods for biomaterial fabrication as listed in Table 7.1 (Wang 2006). This work aims to deliver an insight into methods and a technological approach to prepare a suitable spinning solution for the regenerated fibre non-woven mat based on silk fibroin. Regenerated fibres are produced from natural or synthetic polymers with properties that allow formation of a linear elongated structure. The process of regeneration, or renewal, from the existing shape was introduced to manufacture materials with desired and controlled structural properties. The respectable example and inspiration for the regeneration of silk fibre is a process that takes place in the production of ‘Lyocell’ (also known as Tencel) type fibre, where modified cellulose is processed to form continuous fibre to mimic natural silk (Wachsmann 1997). The regeneration process has brought more control of polymeric material properties and has also opened new fields for ‘smart’ application such as sports clothing with built-in body sensors, temperature and pH indicating textiles or textiles releasing scent.

Table 7.1: Processing of regenerated silk fibroin and related biomedical applications.

<table>
<thead>
<tr>
<th>Material</th>
<th>Processing method</th>
<th>Features</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>film</td>
<td>casting, layer-by-layer deposition</td>
<td>biocompatible, good oxygen and water permeability,</td>
<td>coating materials, wound dressing/skin repair, biosensors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>diverse surface modification options</td>
<td></td>
</tr>
<tr>
<td>hydrogel</td>
<td>sol–gel transition in the presence of acid, ions, and other additives</td>
<td>biocompatible, diverse formulation for gelation, easy delivery (injectable)</td>
<td>guided bone repair, drug release/delivery, cartilage tissue engineering</td>
</tr>
<tr>
<td>non-woven mat</td>
<td>fiber deposition, electrospinning</td>
<td>biocompatible, high strength, diverse surface</td>
<td>guided bone repair, wound dressing/skin repair, tissue engineering</td>
</tr>
<tr>
<td></td>
<td></td>
<td>modification options</td>
<td></td>
</tr>
<tr>
<td>3D porous sponge</td>
<td>salt leaching, gas foaming, freeze drying, freezing and thawing</td>
<td>biocompatible, high porosity (up to 99%) and pore, high strength, diverse surface modification options</td>
<td>bone tissue engineering, cartilage tissue engineering</td>
</tr>
</tbody>
</table>
To regenerate silk fibroin fibre, the physical connections in protein aggregates have to be disintegrated in a good solvent to prevent polymer memory effect. The hydrogen bonding in the structure of silk fibre is strong enough to prevent the dissolution of the protein in the pure water. However, protein easily dissolves in highly concentrated aqueous solutions of chaotropic salts such as LiBr (Khandker 1999). To obtain a salt-free aqueous solution of the protein, the fluid can be dialysed against pure water. A fibroin solution prepared by such technique is soluble in water; nevertheless the protein can still precipitate out of the solution easily. The solvent exchange and dialysis are crucial stages in the production of silk fibroin spinning solution since in this phase the purity and homogeneity of spinning solution is determined.

7.5. Experimental

7.5.1. Materials

The raw fresh silk cocoons were harvested and supplied by a silkworm farm in China. The pupae inside the cocoon were killed by heat source directly after production to avoid damage to the cocoon caused by a hatching silkworm moth. Lithium Bromide (LiBr) was supplied by Sigma Aldrich U.K. and used without any further purification. Sodium Bicarbonate (NaHCO₃) was supplied by Fisher Scientific UK.

The raw cocoons were cut in half and the silkworm pupae removed to avoid unnecessary impurities. This procedure does not occur in industry as cutting cocoons reduces the length of the filament. The purpose of this project was to test material for recreating SF fibre from the solution, for that reason cutting the raw silk fibre and removing the silkworm pupae helps purify the final product without having a major influence to the quality of the filament.

After the cocoons were separated from the pupae remains, the silk fibre was degummed to remove non-fibrous sericin. The degumming procedure is carried out in aqueous solution of a degumming agent, chosen to be sodium bicarbonate (NaHCO₃) following the diagram presented on Figure 7.1. Approximately 10 g cocoons were immersed in 1% w/v solution of sodium bicarbonate in H₂O, which was brought to a boil. The boiling was carried out for 30 minutes and then the fibre was rinsed thoroughly in deionised water. The procedure was repeated once more to increase the amount of sericin removed from the fibre. The intention of adding NaHCO₃ aims to increase pH to the mild alkali value where sericin dissolves better and fibroin remains unharmed.
Wet silk fibres were dried for 24 hours at 95°C until all moisture was removed. Dried silk thread was cut into 1 to 2 mm fragments. The 9.3 M LiBr aqueous solution was used as a dissolving medium for the degummed silk fibroin. The lithium bromide solution was heated to 70°C and stirred slowly. The small portions of SF fragments were added to the 70°C LiBr solution and stirred until fully dissolved. Following dissolution, the solution of silk fibroin was kept at a temperature of 70°C and stirred for a further 1.5 hours. The SF solution was transferred into dialysis membrane and dialysed against pure water for a period of time up to 12 hours to remove the LiBr, the water was changed at least four times during the dialysis step.

### 7.5.2. Methods

The aqueous silk fibroin samples were tested using four different types of rheological measurements: dynamic strain sweep, dynamic frequency sweep, dynamic temperature ramp and steady rate sweep. The viscosity measurements were carried out over a range of 7 concentrations of SF in H₂O between 3 and 12% w/v. A cone-and-plate rheometer TA Instruments ARES was used with Φ = 4 cm cone with angle 2° at a temperature of 20 °C. The 1.5 ml SF samples were transferred onto the plate.

In a Dynamic Strain Sweep (DSS) test a range of sinusoidal deformations (strains) is applied with a constant frequency. This test was used to determine the limits of viscoelasticity and characterise the silk fibroin solutions to examine their non-linear behaviour. Parameters for the DSS test are frequency, temperature and sweep mode.

Dynamic Frequency Sweep (DFS) (strain-controlled) test was used to measure the sample consecutively with selectable frequencies with a constant strain and temperature. The strains for these tests were selected from the dynamic strain sweep test and chosen within the linear viscoelasticity region of the silk fibroin aqueous solution. The period of the testing was dependant on the selected range of frequencies and lay within 0.02 and 20 Hz.
Dynamic Temperature Ramp (DTR) test was used to measure viscoelastic properties of the fluids at temperature ramp rates between 2 and 65°C while the frequency and strain were held constant. Temperature was automatically incremented from lower temperature limits at a rate of 2 degrees per minute. The temperature ramp test was used to examine the aqueous SF fluid behaviour in changing temperature, which allows us to determine the SF processing temperature range, in which the material will not undergo structural changes initiated by temperature only. Additionally, the DTR analysis gave us an insight in temperature driven agglomeration of SF macromolecules, which lead to coagulation and viscoelastic to elasticoviscous material changes.

Steady Rate Sweep (SRS) was used to examine the SF fluid under steady shear deformation for varying magnitudes. The deformation magnitude depended on the range of shear rates, which were chosen to be from 0.001 to 500 s⁻¹. We employed the steady rate sweep to generate flow curves for SF samples in a range of concentrations. The flow curves were created by measurement of the stress and viscosity as a function of shear rate allowing us to characterise the regions where Newtonian fluid underwent a transition to non-Newtonian behaviour of material.

The selection of concentrations was based on the limits of the chosen production method. At concentrations higher than 12% w/v of silk fibroin in water, the viscosity of the samples was too high for any further processing or reliable testing. Also, the concentration of spinning solution inside the silkworm gland is approximately 8 – 9% w/v (Jin 2003), which we aimed to use as the guiding concentration for regenerated SF spinning solution. A pH equal to 6.8 was used for all SF solutions.

According to a study by Xian and Zhengzhong (Xian 2004) a total dialysis time for SF against water or buffer should be 96 hours. However, we established that the silk fibroin is likely to undergo a change in SF secondary structure, and consequently an aggregation, during such a long dialysis process. The ratio between SF volume and volume of water in the dialysis was 1:200. After dialysis was completed we checked the presence of dialysate (LiBr) in final water batch taken from dialysis vessel. This test was done using a 10% w/v water solution of Silver Nitrate (AgNO₃). If the following reaction will occurs:

$$\text{AgNO}_3(\text{aq}) + \text{LiBr}(\text{aq}) \rightarrow \text{AgBr(s)} + \text{LiNO}_3(\text{aq})$$

where, silver bromide (AgBr) precipitates from the solution and forms a cream sediment, which can be detected.

The dynamic frequency sweep test was carried out at five different temperatures to analyse changes of the material’s viscous and elastic response as a function of frequency. The specific five temperatures were obtained by conducting a dynamic temperature ramp (DTR). It was established that, within the range of these temperatures, silk fibroin undergoes physical transitions in its secondary and tertiary structure and it was vital to verify SF rheological behaviour as a function of temperature and frequency. Additionally, several samples were
assessed by carrying out a steady rate sweep (SRS) test to verify the degree of SF deformation as a function of an increasing steady shear rate.

7.6. Results and discussion

7.6.1. Preparation of aqueous silk fibroin solution

Optimising the method for preparation of SF in aqueous solution was focused on minimising the content of solid impurities and producing a homogenous fluid. We found that by removing the remaining pupae, present inside the cocoon, helps to improve the purity of the silk fibroin (SF) after degumming. We observed that sodium bicarbonate (NaHCO₃) – the degumming agent employed for the purpose of this project – may have an influence on the molecular weight of SF, since the rheological tests on samples treated for longer than 1 hour in solution of NaHCO₃ exhibited inconsistency in rheological responses to applied stress. The degumming agent employed for the process should only interact with the silk sericin. The study by Sonthisombat and Speakman (Sonthisombat 2004) suggests that using an enzyme degumming agent for silk fibre provides a controlled degumming process where fibroin remains unaffected and the agent is easily deactivated in other stages of production.

Any interruption into the structure – prior or after degumming – of the native silk such as reducing the length of elementary filament is undesirable in standard industrial processing of silk. However, maintaining the length of silk thread was unnecessary for the purpose of this project, because the structure of filament is disintegrated during the dissolving of the fibroin. For that reason, degummed and dried fibre was cut into small fragments to improve solubility in solution of lithium bromide (LiBr). The cutting also helped to keep the homogeneous character of the SF in solutions containing LiBr and in pure water.

The changes in SF aggregation in non-homogeneous samples observed during initial stages of this study indicate incomplete dissolution of highly entangled fibres maintains the native β-sheets structure, which then assembles leading to formation of liquid crystals at an early stage of the purification. It is possible that entangled fibre areas prevent sufficient amount of solvent to penetrate through and break up the hydrogen bonding in highly ordered β-sheets. During dissolution in LiBr solution the fibroin loses its initial ordered configuration and forms α-helical structure, which should be maintained until the point when the solution undergoes coagulation and crystallisation during electrospinning of the nanofibre mats. When regenerated SF fibre is formed, the SF structure is again ordered in β-sheets, but with a much smaller diameter of the elementary filament, which provides a superior environment for growing neotissue (Gotoh 1998, Inouye 1998).
7.6.2. Rheological Testing

Rheological measurements provided important information about the phase behaviour of the SF aqueous solution and the structural characteristics in terms of fluid behaviour during changes of temperature, shear rate, frequency and strain.

It was established that a SF sample concentration suitable for electrospinning should not be lower than 7% w/v of SF in water. When less material is present in the solution, the aggregation initiated by shear occurs in a small part of the sample. The electrospinning of such solution may result in dropping or spraying instead of formation of a jet leading to a filament. The samples above 7% w/v SF in solution are more suitable for spinning because the dynamic viscosity is sufficient to withstand elongation of the newly spun fibre.

The ageing of SF dissolved in H$_2$O is significant and is influenced by the purity of the sample and the presence of crystal aggregates formed during motion, filtration, or extensive steering. Ageing is an important field of research that is often neglected, however it provides knowledge of spontaneous changes in morphology of the material sample and their effect on further processing.

7.5.2.1. Dynamic Strain Sweep

The relationship between the elastic modulus G' and the viscous modulus G'' to the strain (deformation) was obtained from dynamic strain sweep measurements, which were carried out to find the Newtonian and non-Newtonian rheological behaviour of the silk fibroin solutions. All the SF samples were tested at a temperature of 25 ºC with a frequency of 1 Hz. The strain sweep tests were used to determine the onset of plateau for elastic modulus G’ and viscous modulus G’’ (and dynamic viscosity) in relation to increasing strain as shown in Figure 7.2a. The location of the plateau indicates the range of strains, where each sample exhibits Newtonian fluid behaviour. For strains above the plateau, the sample response for deformation forces is characteristic of non-Newtonian fluid. The values of G' and G'' at the strains below the plateau region reflect background ‘noise’ as the sensitivity of the instrument is weak. The value of strain at the mid range of plateau was taken as a parameter for the dynamic frequency sweep test and dynamic temperature ramp test. All samples were to be tested within the Newtonian fluid regime. The estimated strain, corresponding to the transition between Newtonian and non-Newtonian fluid behaviour, was equal to 5% for the vast majority of tested SF fluid samples. These values were then used as a parameter for the dynamic frequency sweep test.
Figure 7.2 Dynamic Strain Sweep for SF 3 to 12% w/v where: a) presents $G'$ and $G''$ moduli plots vs. strain, for: 3% SF $G'$ (▲), 3% SF $G''$ (△), 5% SF $G'$ (■), 5% SF $G''$ (○), 7% SF $G'$ (×), 7% SF $G''$ (+), 9% SF $G'$ (●), 9% SF $G''$ (●), 12% SF $G'$ (—), 12% SF $G''$ (—); b) presents dynamic viscosity $\eta^*$ vs. strain, for: 3% SF (△), 5% SF (○), 7% SF (×), 9% SF (●), 12% SF (—).
7.5.2.2. Dynamic Frequency Sweep

The plots of G' (storage modulus) and G" (loss modulus) were examined as a function of frequency at the same strain setting. The 5 to 9% w/v SF samples were tested at a range of temperatures between 25 to 45°C. Figure 7.3 shows the increase of G' and G" moduli with rising dynamic frequency for SF 9% w/v at three temperatures; 25, 35 and 45°C. At low values of frequency (~0.01 Hz) all fluids were primarily elastic as the storage modulus G' had a larger value than G". Further rise of the frequency causes the silk fibroin solutions to became more viscous than elastic. This property can be observed when G' and G" plots intersect each other, which corresponds to frequency of 0.02 Hz for SF at 25 and 35°C to 0.05 Hz for SF at 45°C. A second intersection is observed at a frequency of approximately 20 Hz, where the fluid yet again becomes more elastic than viscous.

![Figure 7.3 Dynamic Frequency Sweep for SF 9% w/v, where: G' at 25°C (■), G" at 25°C (□), G' at 35°C (▲), G" at 35°C (Δ), G' at 45°C (●) and G" at 45°C (○)](image)

The elastic fluid behaviour observed at the lowest frequency is due to the physical interactions present when fluid is motionless. We believe that the fresh silk fibroin aqueous solutions are predominantly composed of α-helices. However, within a short time after the dialysis of SF, local electrostatic interactions and hydrogen bridges between and within macromolecules are able to initiate a transition between sol and gel (Matsumoto 2006). After a significant rise of frequency the fibroin becomes more viscous and the loss modulus dominates. We observed that the predominant viscous behaviour is less pronounced for fibroin at 45°C, as higher frequency is required to obtain mainly viscous material. This suggests that SF forms aggregates through
physical interactions more easily at higher temperatures, which was found previously in a study of protein-based polymers (Urry 1998). Once the β-sheets formation begins, water is a poor solvent of silk fibroin and the protein aggregation is inevitable. This leads to phase separation indicated by second G’ and G” crossover. The two points of G’ and G” first and second crossover were examined thoroughly for SF 5, 7 and 9% w/v and shown in Figures 7.4a and b.

Figure 7.4 Structural changes initiated by the frequency in silk fibroin according to concentration and temperature: a) first G’-G” crossover, b) second G’-G” crossover – phase separation

SF concentration shows a major influence on primary and secondary G’-G” crossover. In more concentrated solutions (7 and 9% w/v) the fluid is affected much more by the change of temperature and frequency. Additionally, we observed that the first G’-G” transition (Figure 7.4a) is strongly affected by rising temperature, especially in the range of 30 to 40°C. Furthermore, we established that the aqueous solution of 9% SF shows a greater increase of values of first G’-G” crossover than SF equal 5% with increasing temperature. Additionally, the second G’-G” crossover for SF 9% occurs at much higher values than SF 5%, but rising temperature has only a minor effect on this transition. Further quantitative analyses of the temperature effects on SF and its sol–gel transition were carried out using dynamic temperature ramp.
7.6.2.3. Dynamic Temperature Ramp

The aqueous SF solutions were conditioned at a temperature of 2 °C for 10 minutes, after which tests started with increasing temperatures of 2 degrees per minute within the range of temperatures of 2 to 65 °C. The strain was set at a constant level for each of the samples, the value of which corresponds to data taken from measurements of the dynamic strain sweep test (see section 7.6.2.1.). As previously stated, the value of strain is obtained from the onset of the plateau region in the plot of $G'$ and $G''$ against strain, which corresponds to the Newtonian rheological response on external stress. The frequency for DTR tests was kept constant at 1 Hz, which is insufficient to cause phase separation. The dynamic temperature ramp test was carried out to find out the characteristic changes in the structure of silk caused by increasing temperature. Plots of the DTR test on silk fibroin solutions within the range of temperatures are presented in Figure 7.5a and b.

![Figure 7.5a Dynamic properties of SF vs. rising temperature at constant frequency and strain for 7% SF in aqueous solution. Where: $G'$ (Δ), $G''$ (--) and Tangent Delta (○)](image)

The plots of Figure 7.5a and 7.5b for SF 7% and 9% w/v respectively, show the data for $G'$, $G''$ and Tangent Delta – the phase angle defined as ratio of viscous modulus to elastic modulus – for a range of temperatures, within which SF forms highly ordered, crystalline aggregates. The region of temperatures, where phase transition occurs, begins at 20°C for 9% SF solutions and at 30°C for 7% w/v SF solutions. This suggests that silk fibroin exhibits a significant rheological response to the changing temperature. Analysing the Tan Delta data we established that fluid is
mainly viscous at the onset of the DTR and becomes elastic around 45°C for 7% SF 35°C for 9% w/v SF solutions, where the tendency curve of the Tan Delta data show the inflection point.

Figure 7.5b Dynamic properties of SF vs. rising temperature at constant frequency and strain for 9% SF in aqueous solution. Where: G’ (Δ), G” (→) and Tangent Delta (○)

Conditions where plots (in Figure 7.5b) of G’ and G” overlap correspond to the ordering of the aggregates. It is likely that conditions of low strain and low frequency do not cause structural changes to SF. Consequently, during DTR only rising temperature caused prolonged intersection of the plots G’ and G”, as the sample exhibited Newtonian behaviour in low strain and low frequency. For that reason, the DTR test is the only test which allows the extension of time (shows G’ and G” overlap instead of point of cross-over) during which the material underwent self-assembly and aggregation of fibroin. This highlights the rheological behaviour transitions occurring when silk fibroin, once viscoelastic, becomes elasticoviscous. It is desirable to obtain these conditions during the electrospinning process. The appropriate temperature to carry out the electrospinning should remain between 25 and 45°C to allow slow growth of β-sheet structures. It is preferable to perform the electrospinning in the lower range of these temperatures, because a temperature over 40°C may accelerate coagulation, this then results in crystallisation of the silk fibre occurring too fast before the required elongation is achieved.
7.6.2.4. Steady Rate Sweep

In the steady rate sweep test, a steady shear (deformation) is applied over a range of shear rates. The measurements were taken to determine flow curves for SF solutions. The measured viscosity (shear viscosity) obtained in this experiment corresponds to the internal resistance to flow of the silk fibroin under deformation by shear stress, which provides essential information about the material flow curve. The result of the test performed on three samples of 7 and 9% w/v SF aqueous solution is shown in Figure 7.6.

The measurements were carried out within a range of shear rates between 0.001 and 500 s⁻¹, where the SF rheological behaviour corresponds to that of the region A (lower Newtonian region) where the fluid is Newtonian. This implies that the rate of deformation is too slow to cause changes in entanglements between, nor change in the shape of, the polymer chains. In region A, Brownian motion is able to compensate for any change in flow of the material. Region B corresponds to a transition leading to region C. In region C, polymers exhibit power law reaction to increasing shear rates. Where over the range of shear rates viscosity is almost constant, and will increase as molecular weight falls or molecular weight distribution narrows or polymer concentration decrease. Region D is a transition zone below the upper Newtonian region E (not shown on the plot). In region E, the deformation is too rapid and a further increase of shear rate will produce no further change in silk fibroin chains shape or degree of entanglement.
7.6.2.5. The influence of age on aqueous solution of silk fibroin

This work considers the aging of SF solutions in water over a period up to seven days only, as this is an important problem for solution preparation and processing. SF solutions older than 7 days (168 hours) after they had been dissolved in 9.3 M LiBr are not considered here. We aimed to examine rheological behaviour of the aqueous SF fluid under dynamic flow. The tested samples demonstrated as homogenous nature as possible without pre-ordered β-sheet aggregates. A study by Zainuddin et al. suggested that even at the early stage of dissolution, fibroin may already contain β-strand structure (Zainuddin 2008). The ageing of silk fibroin in aqueous solution was studied to assess the period over which the SF maintains random coil behaviour and also to estimate when the initiation of protein self-assembly take place.

Figure 7.7a The aging effect, strain vs. storage and loss moduli for 7% w/v SF, where: 24 hours $G'$ (▲), 24 hours $G''$ (△), 48 hours $G'$ (■), 48 hours $G''$ (□), 168 hours $G'$ (—), 168 hours $G''$ (―)
The dynamic strain sweep was used to test the effect of ageing on samples of the SF solution as presented in Figure 7.7a and b, where data for 24-hour old sample of SF, 48-hour old sample of SF and 168-hour old sample of SF are shown, respectively. The onset of non-Newtonian behaviour of the SF aqueous solution can be observed at lower strain values for the aged samples when compared to samples freshly made. SF solutions aged for 48 hours and 168 hours exhibited the beginning of non-Newtonian region at a strain of approximately 25%, whereas freshly made samples continue to exhibit Newtonian behaviour up to the final strain value of the experiment (200%). This result indicates that electrospinning of silk fibre should be completed within the first two days after the solution has been prepared. After several days, the protein present in the solution goes through an irreversible physical transition, in which molecular entanglement occurs as protein starts to self-assemble by forming water-insoluble \( \beta \)-sheets (Jin 2003, Matsumoto 2006). At this point silk fibroin is composed of ‘liquid crystals’. When fibroin reaches physical transition often described as sol – gel transition (Matsumoto 2006), the process of electrospinning may be unsuccessful due to difficulties in sufficient elongation of the spinning solution jet.

The ageing of SF was also tested through the dynamic frequency sweep test at 25\(^\circ\)C for the aged samples of SF solution of 7 and 9% w/v the results are shown in Figure 7.8a b.
Figure 7.8 The aging effect, dynamic frequency vs. storage and loss moduli for: a) 7% w/v SF, b) 9% w/v SF; where: 24 hours $G'$ (▲), 24 hours $G''$ (△), 48 hours $G'$ (■), 48 hours $G''$ (□), 168 hours $G'$ (---), 168 hours $G''$ (-)

The transition represented by the first $G'$ – $G''$ crossover occurs in increasing frequencies with the rising age of the samples. The $G'$ and $G''$ plots for the aged samples intersect each other near to a frequency at about 1 Hz compared to that for the non-aged samples at 0.4 Hz. This tendency suggests that the SF undergoes spontaneous gelation, as with increasing age the silk fibroin aqueous solution becomes enriched with $\beta$-sheets (Wang 2008).

In Figures 7.9a and b are shown the results of the dynamic temperature ramp test for SF samples at various ages with increasing temperatures. The age of the SF sample has an influence on the material response to changes in temperature. The aged 7% w/v of SF in water
exhibits the \( G' - G'' \) overlap region, which was characteristic only to a fresh 9% w/v of SF solution. This suggests that aged silk fibroin solutions may undergo a major change in viscoelasticity by becoming more gel-like than freshly prepared aqueous SF solutions. Furthermore, the SF solution \( G' \) shift observed for 9% w/v is even more distinct. In Figure 9a, the storage modulus plot overlaps the plot of loss modulus within range of temperatures from 25 to 58°C. For fresh, 48-hour and 168-hour old samples the \( G' \) and \( G'' \) do not cross. This behaviour suggests that the solution has a mainly elastic nature within all the ranges of temperatures. The major transition of the aged material still takes place within the same range of temperatures as for a recently made sample.

Figure 7.9. Aging effect tested by dynamic temperature ramp on a) 7% SF and b) 9% SF sample; where: 24 hours \( G' \) (▲), 24 hours \( G'' \) (△), 48 hours \( G' \) (■), 48 hours \( G'' \) (□), 168 hours \( G' \) ( – ), 168 hours \( G'' \) ( – )
The investigation of the aging abilities of aqueous SF solution was carried out to understand the intermolecular relations taking place in the sample and also to study the self-assembly ability of the dissolved protein. This section of our research helps estimate the period of time when the SF solution is suitable for electrospinning process and further testing.

7.7. Conclusions

The study included rheological analysis of strain, frequency, temperature, shear rate and most important for rheological study – time.

The process of spinning regenerated silk fibroin fibre has to be carried out in the range of temperatures between 25°C and 50°C, where silk undergoes structural changes, as was established by the dynamic temperature ramp testing. SF solutions present a shear thinning nature under applied shear stress. The concentration of aqueous silk fibroin shows a major influence to dynamic fluid behaviour. A range of SF concentrations tested here showed that the most stable SF samples were those of concentration between 7 and 9% w/v. Also these SF aqueous solutions exhibited the resistance to spontaneous aggregation required for the ageing study. Further, the aged aqueous solutions of SF presented much earlier onset of non-Newtonian fluid behaviour, with respect to increasing strain, than fresh SF solutions, this implies that SF developed areas of aggregated macromolecular domains which may be ordered in β-sheets. Changes in the dynamic frequency vs. shear rate suggest that the SF solutions underwent spontaneous gelation when stored. We recommend that further processing of the aqueous silk fibroin solutions, which require controllable fluid properties of the protein should be completed within 48 hours after the dissolution of SF.

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