Chapter 1: Introduction

1.1 Protein Folding

Proteins are macromolecules found in every cell that regulate many biological processes. A large diversity exists in the protein family because of the many varied functions each protein can carry out; however, these are constructed from only 20 different amino acids. Each protein has a unique sequence and structure accomplished in four phases [1-3]. The sequence of amino acids is called the primary structure and describes the protein backbone [1-3]. This amino acid chain is flexible, and many different spatial arrangements can be formed [2]. In the secondary structure, the amino acid chain forms either an α-helix or β-sheet, making an unconstrained arrangement and allowing hydrogen bonding [1-3]. The tertiary structure allows for distant segments to come into contact with each other as the three dimensional (3-D) folding transpires [1-4]. If the protein requires multiple subunits to be effective, then either copies of the same polypeptide chain or different ones merge together to form the quaternary structure [1-3]. All four folding processes are depicted in Figure 1 [1]. Although the active site of a protein may only use 10% or fewer of the amino acid residues, the rest of the amino acids are necessary for making the correct spatial conformation [2].

Figure 1: A schematic diagram representing the four steps in the protein folding process by Lehninger et al. [1].
Once the protein has formed its physical architecture, it is in a reactive and stable conformation called the native state [1, 2]. Native structures are highly sensitive to their surrounding environment [2, 5-8]. For example, changes in temperature, pH, ionic concentration, or surface energy can cause the protein to unfold [2, 5-8]. Protein denaturing breaks down the quaternary, tertiary, and secondary structures, but leaves the primary one undamaged [2]. However, if the denaturing stays within a limited range and the original conditions are restored, this process is reversible and the native structure will reform [2, 5]. If the denaturing conditions are outside the stable range, then a misfolded structure is induced (Figure 2) [2, 5]. It is estimated that 30-50% of all proteins are either misproduced or misfolded; therefore, the body developed molecules to oversee the protein folding procedure [2, 4, 8]. Chaperone molecules assist protein folding, guide the protein through the appropriate pathway, and guard against any influences that could lead to an improper structure [2, 4]. These helpful molecules are non-specific so each one can help many different proteins with the folding process [2]. Since misfolded proteins are more likely to aggregate, the proper 3-D configuration of the protein is crucial [2, 4].

![Figure 2: A native state protein unfolds after a denaturing agent is added to the system. This can result in either the protein misfolding or returning to the native state conformation.](image-url)
Protein folding, both native state and misfolded, is determined by the forces within the polypeptide chain, water, and other molecules in the surrounding environment [9]. Although chaperone molecules decrease the amount of misfolded proteins, misfolding still occurs. This increase in internal energy of the protein compared to the native one creates a driving force that leads to aggregation [2, 4]. Protein aggregation has been linked to many diseases including Alzheimer’s, Huntington’s, and other amyloid-based diseases. [2, 4, 5, 9-12]. These diseases are characterized by insoluble protein aggregates embedded in different regions of the brain [4, 9, 10, 13]. Aggregation also occurs during the processing, formulation, or storage of proteins in medical and research facilities [8, 9]. The Food and Drug Agency is concerned that these protein aggregates could lead to either a harmful immunological reaction or impede protein research [4, 8, 9].

1.2 Protein Adsorption

Adsorption of proteins onto a surface is used in numerous disciplines including biology, medicine, and biotechnology [14]. This adsorption can stabilize the surface by adding more repulsive steric forces and reducing the attractive van der Waals forces [15]. Some examples of protein interaction experiments include enzyme-linked immunoassays (ELISA), medical device coatings such as biochips or biosensors, drug delivery, extracellular matrix protein scaffolding, micropatterning, brush forming polymers, hydrogels, and stabilization of colloidal dispersions and food products such as wine [14, 16-28]. The orientation of the protein is important in the accuracy of these analyses and in the proper transport of molecules or devices [14]. Methods for measuring the amount of protein adsorption are radiotracers, quartz crystal microbalance (QCM), ELISAs, total internal reflection fluorescence (TIRF), circular dichroism, infrared spectroscopy, neutron reflection, and atomic force microscopy (AFM) [14, 18, 19].

The level of protein adsorbed at room temperature is estimated to be on the order of several milligrams per square meter; however, surface characteristics such as size, surface parameters, and curvature of the colloidal particle, and the adsorptive conditions play a defining role in this process [14, 18, 29-31]. The protein characteristics, namely the charge, size, stability
of the structure, amino acid composition, and steric conformation, all determine how and if the protein will adsorb and its subsequent actions [14, 29, 32]. Adsorption is often an irreversible process since the protein conformation is changed during this procedure [14, 18]. One possible explanation is that hydrophobic parts of the protein reassemble to interact with hydrophobic areas of the adsorption surface and results in multiple site connections [14]. The change in protein conformation from adsorption could lead to a higher level of aggregation.

1.3 Methods to Measure Protein Aggregation

Techniques used to measure protein aggregation are small-angle neutron scattering or reflection, circular dichroism (CD), Raman and infrared spectroscopy, fluorescence, turbidity, membrane filtration, gel filtration, and laser light scattering [5, 7-9, 19]. Laser light scattering provides a more efficient measure of aggregation since particles scatter light effectively and particle size, shape, and growth can be measured [9]. The measurements are also more precise, sensitive, and acquired relatively quickly [33]. Two types of laser light scattering are static (SLS) and dynamic (DLS), but both use the same setup of a laser aimed at a cell containing an optically clear sample and a detector to capture the light scattered [9]. SLS measures the molecular mass and size whereas DLS evaluates size variation on the material of interest [34]. Analytical centrifugation and spectrofluorimetry both use DLS; however, some conditions must be met for these systems to produce accurate data [34, 35]. Both need very clean glassware and the sample must be filtered as dust will result in incorrect findings [9, 34]. Laser light scattering provides specific and quantitative data by determining the dimensions of the aggregate [9].

1.3.1 Z-axis Translating Laser Light Scattering Device

Our lab has constructed our own DLS system called the z-axis laser light scattering (ZATLLS) device [10-12, 36, 37]. The ZATLLS machine consists of a laser and detector mounted onto a stage. The stage is powered by a motor and able to transverse the suspended rectangular glass column containing the sample (Figure 3) [10-12, 36, 37].
As the solution settles, the amount of light reaching the detector improves. A LabVIEW program records the height and voltage values for each scan. Sedimentation provides a force that separates particles from a solvent by using the differences in density [38]. This system has already been used to measure the sedimentation velocities of both low- and high-density particles in organic resins, glass spheres in aqueous solutions, and transglutaminase activated bovine serum albumin on polystyrene particles [10, 36, 37].

1.3.2 Aggregate Size Calculation

Assuming that both the particles and aggregates are spherically shaped and the solution flow dynamics are such that the particles settle in the creeping flow regime, the aggregate size
may be found by using the solution and particle characteristics (Figure 4) \[11, 12, 36, 39\]. Protein interactions occur under normal gravitational force and for a significant period of time to emulate real time interactions \[11\].

![Diagram of forces on a particle in solution](image)

**Figure 4**: A close up view of the forces on a single particle in solution. The buoyancy force is composed of the solution viscosity \(\eta_s\) and the gravitational force \(g\) multiplied by the density of the solution \(\rho_s\). The downward force consists of the density of the particle \(\rho_p\) and gravity.

Once values for the sedimentation velocity \(v\), solution viscosity \(\eta\), density difference between the particle and solution \(\Delta\rho\), and \(b\), a dimensionless variable associated with creeping flow are acquired, Stoke’s Law can be applied to find the average aggregate size, \(D\) (Equation 1) \[10-12, 36, 37, 39\].

\[
D^2 = \frac{3bv\eta}{4\Delta\rho g}
\]

Equation 1
1.4 Albumin

Albumin is an abundant, heart-shaped globular protein found in many mammals such as humans, cattle, rats, and mice (Figure 5) [40-42].

![Figure 5: The 3-D heart shaped configuration of albumin [41].](image)

Albumin is synthesized in the liver and found in every tissue and secretion within the body [41, 42]. It aids in metabolism, maintains blood pH, and disperses exogenous and endogenous ligands throughout the body [18, 41, 43, 44]. Fatty acids, hematin, bilirubin, cysteine, glutathione, copper, nickel, mercury, silver, and gold are some of the many molecules albumin binds with making it a highly flexible molecule [41, 42]. The structure has three homologous domains connected through 17 disulfide bonds and an overall negative charge [40-43]. Albumin is one of the most studied proteins since it is readily available at low cost, stable, and attaches reversibly to numerous ligands [41]. The pharmaceutical industry is interested in albumin since the distribution, metabolism, and efficacy of many drugs are dependent on this molecule [41, 43].

1.4.1 Functions of Albumin

Albumin readily adsorbs to most surfaces due to conformational changes making it an ideal modeling and blocking protein [10, 14, 17, 18, 20-24, 31, 45-48]. Two of the most common species of serum albumin used in research are bovine (BSA) and human (HSA). Both have been
used in aggregation experiments to model neurodegenerative diseases such as plaque formation of β-amyloid (Aβ) in Alzheimer’s disease [5, 10, 49]. Burguera and Love measured the inhibition effects of creatine on protein aggregation using transglutaminase activated BSA as a model protein for Aβ [10]. In blocking studies, BSA is the species predominantly used over HSA, and it coats a surface to prevent other molecules from binding to it [17, 18, 20, 21, 23, 24, 27, 31, 45-47]. Experiments utilizing this property of albumin are ELISAs, micropatterning, and biochip coatings [17, 20, 21, 23, 24].

Molecular transport is the primary function of albumin and many drugs have been coupled to it to increase the half-life of the drug in the body. [50-56] For example, angiostatin, a protein that inhibits angiogenesis in tumors, typically has a half-life ranging from 4.8 – 9.6 hours in immunocompetent C57BL/6 mice [54]. The serum concentration at seven days after injection increased from 340 ng/ml to 14 µg/ml when bound to albumin [54]. BSA and HSA have also displayed a protective effect on certain cells [13, 57-61]. Tabernero et al. showed that BSA facilitates brain development by reducing the contact between fatty acids or coenzyme A (CoA) derivatives and neurons [58]. Albumin also increases neuronal survival by increasing the synthesis and release of glutamate within the cells [58]. Bohrmann et al. used in vitro assays to demonstrate that BSA and HSA inhibit aggregation of the Aβ1-40 fragment [49]. Although BSA and HSA have the same 3-D conformation, the sequences are only 76% identical [41]. The differences are in the number and types of amino acid residues, 583 for BSA and 585 for HSA [40, 62]. Therefore, there is a difference in both molecular weight, 66,411 Da for BSA and 66,438 Da for HSA, and in charge, -17 for BSA and -15 for HSA [40].

1.4.2 Adsorption of Albumin

Adsorption is important in many model and blocking studies, but the protein changes conformation once it is attached to a surface [14, 18]. Albumin is considered a “soft” protein as it is likely to adsorb on many surfaces, regardless of any negative forces, because the protein conformation changes so drastically [14, 29, 63]. During adsorption, the albumin conformational structure changes because the number of α-helices decreases compared to the native state [18,
On some surfaces, polystyrene for example, albumin irreversibly changes conformation [63]. However, bound albumin has displayed a stabilization effect on particulates dispersed in a solvent [27, 64]. Deguchi et al. showed that carbon-60 nanoparticle aggregation was suppressed from 1mg/ml through 10 mg/ml HSA solutions therefore stabilizing the carbon nanoparticles [27]. Another example is the increase in stability of the surfactant, sodium bis-2-ethylhexyl sulfosuccinate (AOT), at the air-water interface by the addition of BSA [65]. Albumin displays two opposite effects making it dependent on the composition of the adsorptive layer.

1.4.3 Temperature Induced Denaturing of Albumin

Protein adsorption is considered a dynamic process where the protein switches from the adsorbed to the dissolved state; however, it is not known whether the native structure is reformed after desorption [63]. This denaturation mechanism is essential in understanding protein stability[66]. In this case, we will be focusing on temperature induced denaturation for both reversible and irreversible processes. BSA has been shown to be reversible after thermal denaturation of 50ºC or below [67, 68]. Moriyama et al. showed that the α-helix structure in BSA decreases from 67% to 61% at 45ºC and by 65ºC it has decreased to 44% [67]. Honda et al. also showed an increase in BSA self-aggregation, especially at temperatures above 61ºC over a 20 minute interval, and that the process was irreversible [69]. HSA displays a similar result to BSA since up to 55ºC there is no significant change in the globular structure and above 60 ºC the structure has been irreversibly changed [66, 70]. Michnik et al. found that the reversibility for fatty acid free BSA at 60ºC was 90% compared to 100% for fatty acid free HSA [71]. At 70ºC BSA reversibility had drastically decreased to 67% whereas HSA was still 90% reversible [71]. This difference in stability could be explained by the amino acid differences between the two species.
1.5 Purpose of Study

The overall project was to determine the self-aggregation potential of adsorbed albumin onto polystyrene particles by measuring sedimentation velocity. Three objectives were associated with this goal:

1) Determine whether protein-protein interactions arise in BSA-coated polystyrene particles compared to non-coated particles

BSA is an important blocking protein used to block the available binding area from other molecules. BSA was incubated with polystyrene particles overnight to allow the protein to bind to the particle. The dispersion solution was then placed in the ZATLLS instrument and the particles settled over a three hour time period. The laser light voltages as a function of height were recorded. These values were used to determine the average sedimentation velocity for each experiment. Solution density and viscosity for each run were also measured and utilized to calculate the average aggregate size (Figure 6). As adsorption is known to induce a conformational change in the protein, we theorize that this will increase the level of protein-particle association. This study is shown in chapter 2.

![Figure 6: Adsorption of albumin onto polystyrene (PS) particles before being placed in the ZATLLS device. After the sedimentation velocity experiment, viscosity and density of the recovered solution will be measured.](image_url)
2) *Ascertain the better blocking protein by comparing the self-aggregation between HSA-coated and BSA-coated polystyrene particles*

In this set of experiments, either BSA or HSA was adsorbed onto polystyrene particles. The protein-coated particle solution was placed into the ZATLLS device to measure the sedimentation velocity. Solution characteristics were also measured and used to infer the average aggregate size using Stoke’s Law (Figure 6). The small variations in amino acid length and composition could lead to a difference in stability of the adsorbed protein. This could help determine if one species of albumin is better for blocking studies than the other. These experiments are presented in chapter 3.

3) *Induce both reversible and irreversible denaturing in BSA by temperature before adsorption onto polystyrene particles*

BSA was denatured using thermal exposure in a process similar to the one used by Mitra et al. [70]. Aqueous BSA solutions were heated in a water bath at either 50ºC for reversible denaturing or 70ºC for irreversible denaturing. The solutions were then cooled down to room temperature before allowing protein adsorption onto polystyrene particles (Figure 6). BSA that underwent reversible denaturing should display the same level of aggregation that was previously measured. The irreversible denaturing of BSA is expected to have a larger amount of protein-protein interaction since the protein likely has a different conformational shape than before the adsorption process. The findings are shown in chapter 4.
1.6 References


