

Chapter 4: Thermal Denaturing and its Influence on the Self-Aggregation Potential of Bovine Serum Albumin Using Laser Light Scattering

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4.1 Abstract

Protein misfolding can be induced by many factors; however, a period exists where the misfolding can potentially reverse. Temperature was used to induce both reversible and irreversible structural changes on bovine serum albumin (BSA) before coating onto polystyrene particles. Sedimentation velocity of the BSA-coated particles was evaluated using a z-axis laser light scattering instrument. Approximately the same sedimentation velocities were measured for experiments with reversibly and irreversibly denatured BSA. Solution viscosity, density, and the sedimentation velocity were measured and Stoke's law calculated the average aggregate size. Different aggregate sizes were found for the reversibly and irreversibly changed BSA-coated particles due to a large difference in solution viscosity. The average aggregate size for reversibly induced changes overlapped ranges found in previous experiments at room temperature. Irreversibly denatured BSA showed a 67% increase in average aggregate size compared to the reversibly denatured one. We showed that conformational modifications, induced by denaturing of BSA at a high temperature before adsorption, led to a higher level of protein-particle aggregation.

4.2 Introduction

The protein native state is both reactive and stable; however, it is highly susceptible to changes in the environment [1-5]. Changes in temperature, pH, ionic concentration, or surface energy all cause the protein to unfold and become inactive [1-5]. A limited stability range exists and if equilibrium is restored, this structural change is reversible [1, 2]. If the denaturing conditions fall outside this limit, a misfolded structure is produced [1, 2]. These denaturing conditions raise the internal energy of the protein and trigger the driving force to aggregate [2, 6]. Alzheimer's, Huntington's, and other amyloid based diseases are some neurodegenerative diseases where protein aggregation occurs [1, 2, 6-10]. Insoluble protein aggregates called plaques are found in the brain and are believed to be involved in disease progression [6-8, 11].

Adsorption of a protein onto a surface has also been shown to denature the protein irreversibly [12, 13]. The surrounding environment and surface features for example, size, surface condition, and curvature, all regulate adsorption of the protein onto the target area [12-15]. The charge, size, stability, amino acid composition, and steric structure of the protein also influence protein adsorption [12, 14, 16]. Protein adsorption is used in many experimental procedures including enzyme-linked immunoassays (ELISA), biochip and biosensor coatings, and drug delivery [12, 13, 17-19] This change in protein structure from adsorption could also trigger aggregation.

Numerous techniques are used to measure aggregation but we used laser light scattering since particle size can be determined [7]. Both static light scattering (SLS) and dynamic light scattering (DLS) systems use a laser directed at a solution with a detector to measure light transmission. Although DLS is able to differentiate particle size, most machines require clean glassware and filtering of the sample since dust can cause scattering [7, 20]. Our lab built a DLS instrument called z-axis laser light scattering (ZATLLS) where a laser and detector system are mounted on a stage that transverses the solution as height and voltage values are recorded [8-10, 21, 22]. Larger particles, in this case polystyrene, have the desired protein adsorbed on to them so that the scattering of dust is a smaller contribution. ZATLLS has been used to measure the sedimentation velocities of both low- and high-density particles in organic resins, glass spheres in aqueous solutions, bovine serum albumin (BSA) on polystyrene particles, transglutaminase activated BSA on polystyrene particles, and human serum albumin (HSA) on

polystyrene particles [8-10, 21, 22]. Sedimentation uses a difference in density between the particles and the solvent for separation [23].

Albumin is an abundant, heart-shaped plasma protein found in many mammals and regulates metabolism, normalizes blood pH, and carries many molecules around the body [13, 24-28]. It is used as a blocking and modeling protein as structural modifications are easily induced allowing it to adsorb onto most surfaces [8, 12, 13, 17-19, 29-31]. The denaturation process is key in understanding protein stability and this paper focuses on the aggregation of thermally induced reversible and irreversible changes in BSA [32]. Prior work indicates that these thermal changes are reversible if heated to temperatures below 50°C and irreversible if above 61°C [33-35]. We chose to denature BSA before adsorbing onto polystyrene particles and then measure the aggregation potential. We expect that reversibly transformed BSA will show similar results to previous work and more aggregation would be found in irreversibly modified BSA.

4.3 Materials and Methods

4.3.1 Solution Preparation

Polystyrene (PS) particles (poly(styrene with 2% divinylbenzene)) with a size range of 37-74 μm were purchased to be used as received from PolySciences (Warrington, PA). BSA in powder form was purchased from Sigma (St. Louis, MO) and also used as received. BSA 10 mg/ml solutions were heated to either 46°C or 76°C in a water bath. After 10 minutes, the solutions were removed and cooled to room temperature [36]. Twenty mL of 0.1M borate buffer (pH 8.5) was added to 0.5 g PS particles and centrifuged for recovery. The denatured and cooled BSA solution was added and shaken overnight at room temperature. The protein-coated PS particles were later retrieved by centrifugation. Finally, a 16% (v/v) glycerol-water solution acts as a relatively neutrally buoyant solution to disperse the BSA-coated particles [8-10].

4.3.2 ZATLLS

A rectangular glass column containing the BSA-coated particle solution was positioned vertically in the ZATLLS instrument. Time interval and scan length were inputted by the user into a LabVIEW program (National Instruments, Austin, TX, USA) with each experiment lasting

approximately three hours. Voltage was recorded as a function of height for each scan. As the particles settled, the clarified regions allowed more light to reach the detector so that sedimentation velocity could be measured [8-10].

4.3.3 Viscosity and Density

After each experiment, the clarified solution was saved for density and viscosity measurements. Viscosity was measured using an AR-G2 rheometer (TA Instruments, DE, USA) with a 60 mm cone geometry. A DE-40 pycnometer (Mettler-Toledo, Inc., Columbus, OH, USA) was used to find solution density [8-10].

4.4 Results

Sedimentation velocities were found for BSA heated to 46°C or 76°C prior to adsorption onto polystyrene particles. The labVIEW program logged voltage and height data during each scan but only data in the upward direction was utilized. Noise in the recording was smoothed in Microsoft Excel by comparing and averaging the voltage values at each height for each time interval. Representative graphs showing voltage as a function of time for BSA heated to either 46°C or 76°C for one experiment are shown in figure 1. Arbitrary voltage markers, 0.34 V, 0.35 V, 0.36 V, and 0.37 V, were used to find the height where each scan crossed. These values were then plotted to make a sedimentation velocity graph (Figure 2). This process is demonstrated using the corresponding shapes of the four curves in the velocity graph to outline values on the first scan in figure 1 [8-10].

Using a least squares fit, linear trend lines were fitted to Figure 2 to find the slope of each isovoltage curve. These slopes were averaged together to determine the final sedimentation velocity in each experiment. Once values for sedimentation velocity, v , solution viscosity, η , the dimensionless creeping flow variable, b , the density difference, $\Delta\rho$, between the particle, 1.0500 g/cm³, and the solution, and the gravitational constant, g , were found, Stoke's law,

$$D^2 = \frac{vb3\eta}{4\Delta\rho g}$$

was utilized to calculate the average aggregate size, D [8-10]. Average values for sedimentation velocity, density, viscosity, and aggregate size are shown in table 1; however, experimental data falling outside one standard deviation from the mean aggregate size was excluded. Welch's t-test compared the average aggregate sizes of BSA heated to 46°C (n=4) and 76°C (n=4). The difference between the two was determined to be significant with $p < 0.01$.

4.5 Discussion

In this study, BSA was reversibly and irreversibly denatured before adsorption onto polystyrene particles. Similar voltage vs. height graphs were generated for both denaturation temperatures (Figure 1). Although nearly identical sedimentation velocities were found, vastly different aggregate values were calculated for the denaturing conditions. The average particle size for BSA after exposure to 46°C was $104 \pm 13 \mu\text{m}$ while BSA heated to 76°C was $155 \pm 16 \mu\text{m}$, a 67% increase. This difference in aggregate size is attributed to the almost 50% difference in average viscosity of the clarified residual solutions from the different denaturing procedures. The higher viscosity measured from the 76°C heated BSA experiments caused the particles to settle slower due to the large amount of protein desorption.

Protein adsorption is frequently an irreversible process, but the protein can switch between the adsorbed and dissolved states [12, 13, 37]. Since protein characteristics affect protein adsorption, differences between reversible and irreversible changes could exist [12, 14, 16]. Reversible denaturation of BSA displayed an average aggregate size range similar to ranges found in previous experiments conducted at room temperature [9, 10]. A much larger aggregate size is calculated for irreversibly changed BSA. At higher temperatures the percentage of BSA α -helix structure is known to decrease [2, 6]. For example, native state BSA α -helix structure drops from 67% to 44% with protein denaturing occurring at 65°C [33]. Comprehension of denaturation progression is fundamental in understanding protein stability especially in a commonly used protein such as albumin [32].

4.6 Conclusion

The experiments in this study measured sedimentation velocities of reversibly and irreversibly denatured BSA-coated polystyrene particles. Although almost equal sedimentation

velocities were found for BSA heated to 46°C and 76°C, the calculated aggregate sizes varied significantly. This dissimilarity is caused by the considerable difference in the viscosity of solutions measured after each experiment. Solutions of BSA heated to 46°C had a similar aggregation range compared to experiments performed at room temperature showing that this soft denaturing condition was not sufficient to dramatically alter the aggregations response. Irreversible denaturing of BSA had a much larger amount of aggregation due to conformational changes in the protein.

4.7 References

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4.8 Figures and Tables

Table 1: Average sedimentation velocity, density, viscosity, and particle size for BSA heated to 46°C (n=4) and 76°C (n=4) are shown. Any data that fell outside one standard deviation from the particle mean was excluded. Standard deviations for viscosity and density are not shown due to the small differences in measurements.

Figure 1: Representative sedimentation graphs are shown for 46°C BSA-coated polystyrene particles (a) and 76°C BSA-coated polystyrene particles (b). The isovoltage makers, 0.34 V, 0.35 V, 0.36 V, and 0.37 V, are depicted on the graph with horizontal lines. The black shapes on the first scan correspond to the height values used to plot the sedimentation velocity graph in Figure 3.

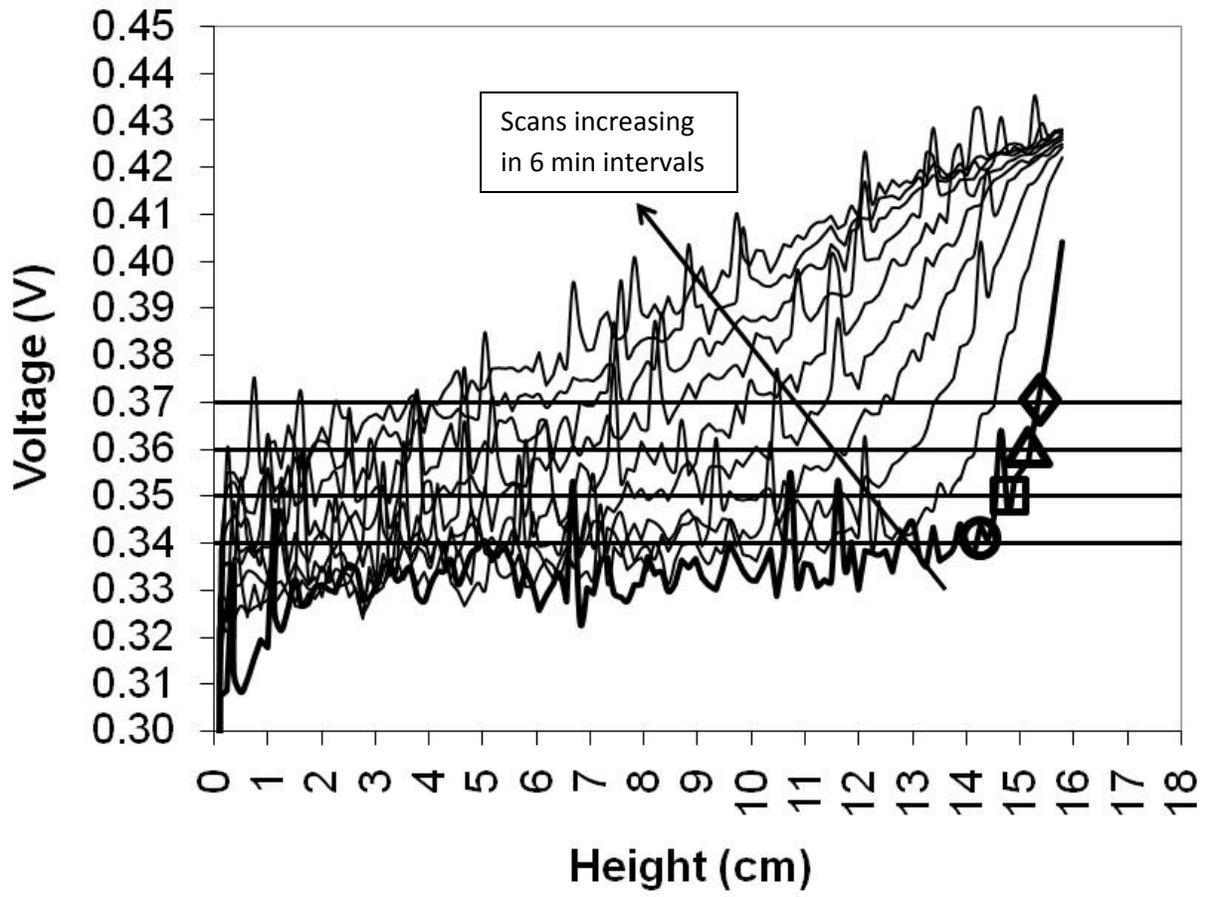
Figure 2: Four curves are shown for 46°C BSA-coated polystyrene particles (a) and 76°C BSA-coated polystyrene particles (b) using values from Figure 2. Linear trend lines were utilized to find the slope of each curve. The four slopes were then averaged together for a final experimental velocity.

Table 1

	Sedimentation Velocity ($\mu\text{m/s}$)	Density (g/cm^3)	Viscosity ($\text{mPa}\cdot\text{s}$)	Particle Size (μm)
46°C BSA-coated PS particles (10 mg/ml)	42.08 ± 2.71	1.0358	2.18	104 ± 13
76°C BSA-coated PS particles (10 mg/ml)	41.87 ± 5.25	1.0326	4.78	155 ± 16

Figure 1

a)



b)

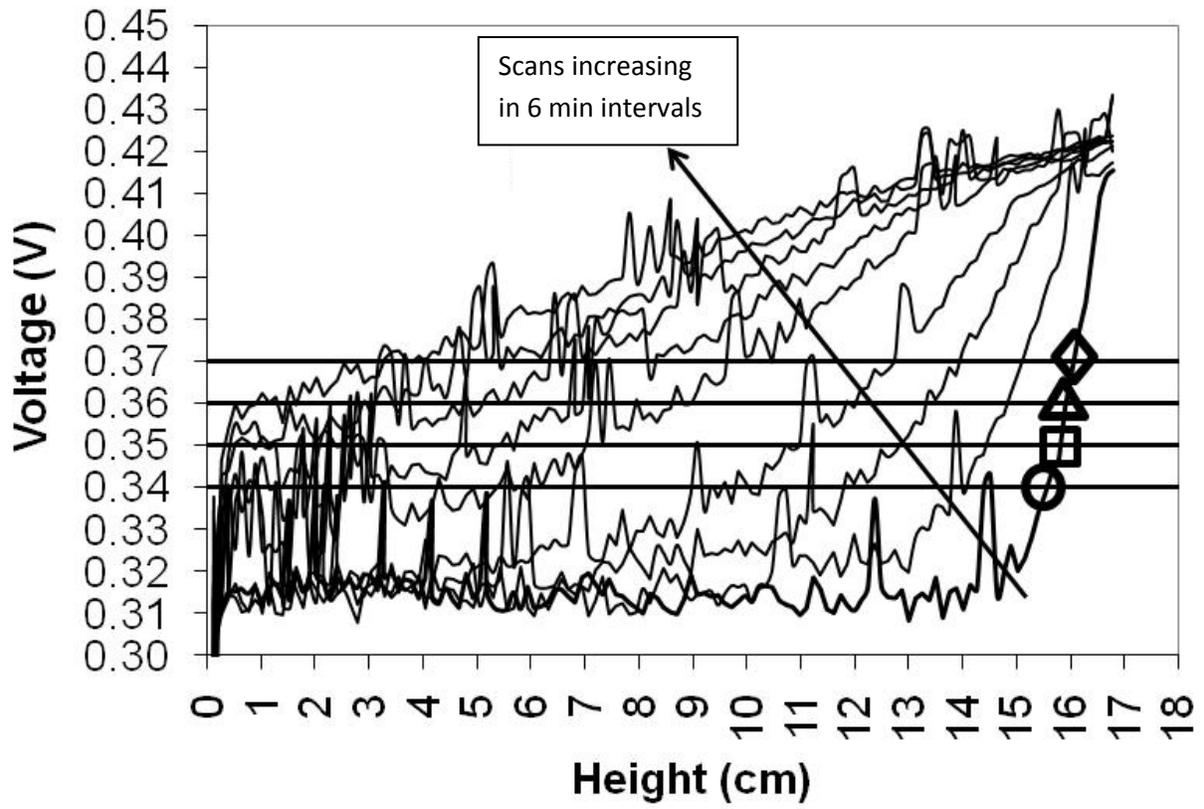
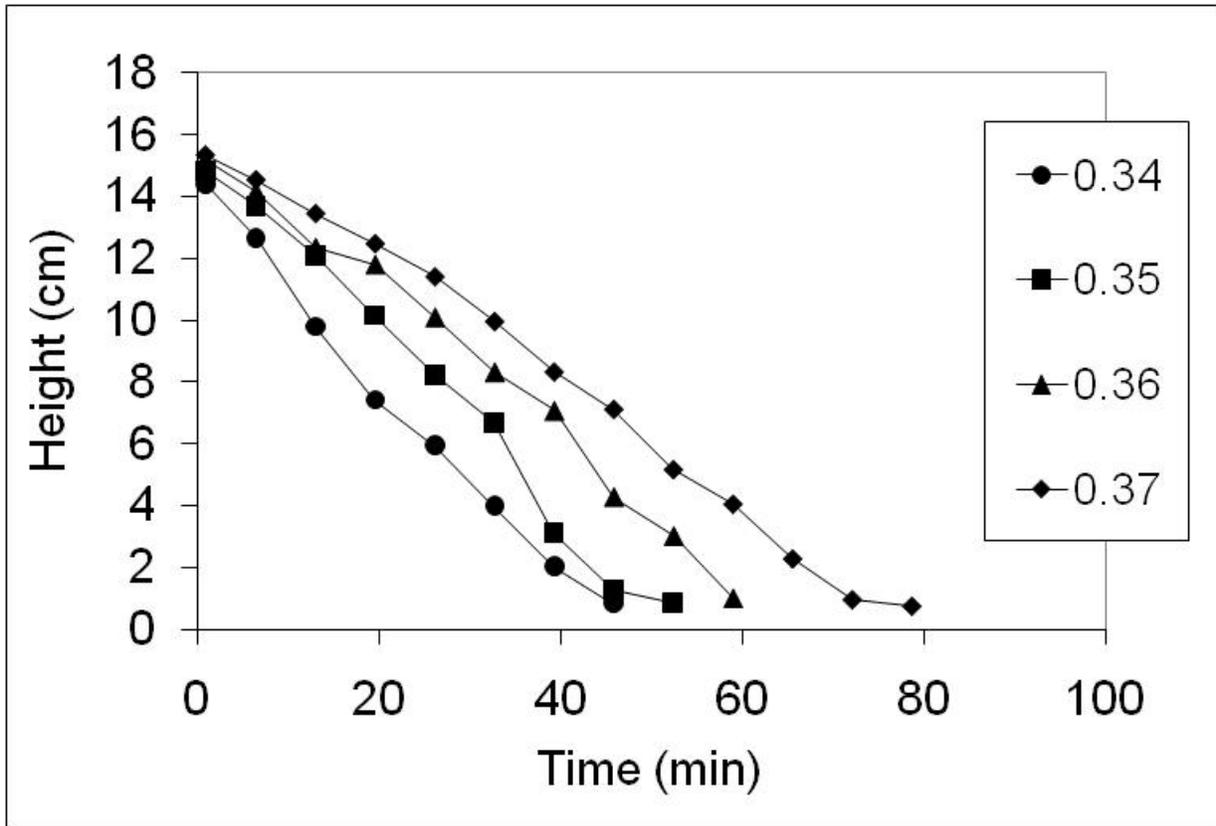


Figure 2

a)



b)

