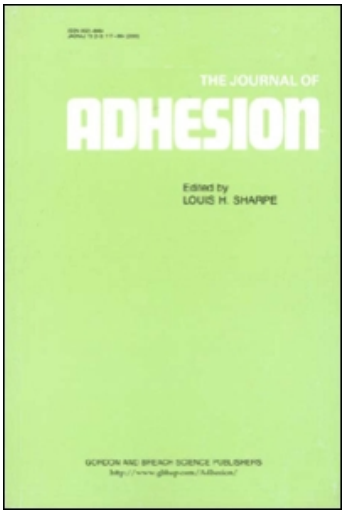

The Journal of Adhesion

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713453635

The Presence of Adsorbed Proteins on Particles Increases Aggregated Particle Sedimentation, as Measured by a Light Scattering Technique

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To cite this Article McKeon, K. D. and Love, B. J.(2008) 'The Presence of Adsorbed Proteins on Particles Increases Aggregated Particle Sedimentation, as Measured by a Light Scattering Technique', The Journal of Adhesion, 84: 7, 664 — 674

To link to this Article: DOI: 10.1080/00218460802255541

URL: http://dx.doi.org/10.1080/00218460802255541

The Journal of Adhesion, 84:664–674, 2008

ISSN: 0021-8464 print/1545-5823 online
DOI: 10.1080/00218460802255541

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Protein conformational changes are often induced when bound to surfaces and can modulate colloidal stability of protein coated particles in dispersion. We evaluated bovine serum albumin (BSA) adsorbed onto polystyrene particles at room temperature. A z-axis translating laser light scattering device (ZATLLS) measured the sedimentation velocity of protein-coated particles tracking aggregation characteristics compared with non-coated ones. Sedimentation velocities of particles moving in the dispersion, and the resulting viscosity and density of the residual solution following sedimentation determined aggregate size in the dispersion using Stoke's law. Our experiments objectively show that albumin-coated polystyrene forms aggregates. Interestingly, coating particles with protein slows the sedimentation velocity which should correspond to a more dispersed system, but it leads to higher aggregate sizes due to the larger influence of proteins in solution raising solution viscosity. Protein-bound particles were observed to fall out of solution in a more controlled and steady manner compared with uncoated particles.

Keywords: BSA; Dispersion; Light scattering; Particle aggregation; Sedimentation

INTRODUCTION

Inorganic and organic surfaces may be modified by attaching or adsorbing either peptide chains or proteins for protective coatings,

Received 9 November 2007; in final form 21 May 2000.
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cellular adhesion, or biocompatibility [1]. Protein interactions have been evaluated within a wide range of materials including brush-forming polymers [2], hydrogels [3], drug delivery systems [4], stabilization within colloidal dispersions [5], and interactions within food products such as wine [6]. When proteins are subjected to different chemical and cellular environments, both reversible and irreversible protein conformational changes arise, and protein misfolding can result. Chemical environmental changes (pH, ionic strength, surface energy) can increase the driving force for protein aggregation and affect dispersion stability [7,8]. Aggregation of specific proteins has also been implicated in cell death occurring in neurological diseases such as Alzheimer's, Parkinson's, Huntington's, and other amyloid diseases [7,9–11]. Overall, there is a growing interest to understand how proteins in particle dispersions affect aggregation, binding, and stability.

Several scattering-based tools have measured particle aggregation, including circular dichroism (CD), Raman and infrared spectroscopy, small-angle neutron scattering, and fluorescence [11,12]. Light scattering is a common method used in aggregation studies and the two types are static (SLS) and dynamic (DLS). SLS measurements focus on the regulation of molecular mass and size. DLS measures size variation of dispersed particulates and is the type we are interested in for this experiment [11,13]. Two devices that employ DLS are spectrofluorimetry and analytical centrifugation. Both require scrupulously clean glassware and fluid filtering to reduce the inadvertent scattering potential of dust particles. These can cause their own scattering if the index of refraction difference between fluid and particle is sufficiently large [11,13]. Sedimentation yields a driving force to separate particles of one density from a dispersion composed of a solvent of a subtly different density [14]. The relationship between driving force and particle size is given by the Stokes-Einstein equation which can infer the average size of the aggregates formed for a specific set of experimental conditions [9,12]. Our adaptation of this experiment uses particle sedimentation under normal gravitational force to allow random protein interactions over a longer period of time to simulate ones occurring in real time.

Our lab has created a new way to infer aggregation from sedimentation velocity using a z-axis translating laser light scattering (ZATLLS) system. This device consists of a moving stage attached to two spiral columns, a laser and detector system, motor, and magnetic counter (Figure 1). Although solutions are typically filtered to remove dust for DLS measurement, the solutions used in this experiment were not. Since the particles used were so large compared with dust, this

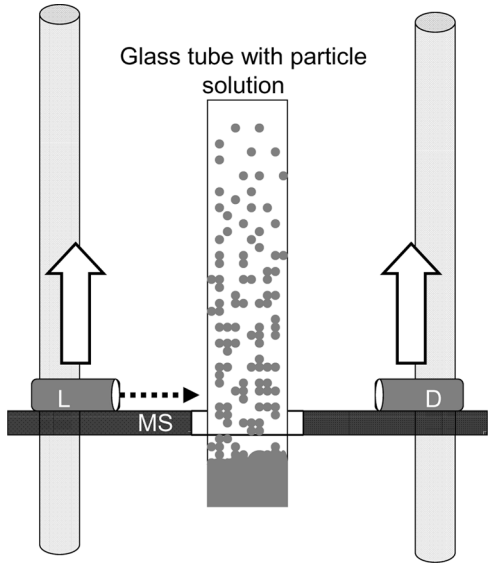

FIGURE 1 A schematic diagram of the ZATLLS device with the moving stage (MS), laser (L), and detector (D) represented. Once the rectangular glass tube containing the particle dispersion is placed within the device, the MS can translate along the tube collecting height and voltage data for each time interval.

was considered a secondary effect. A rectangular glass tube containing the protein-coated particle solution is inserted and recording can commence. The stage distance is powered by the motor and the distance traveled is tracked by the magnetic counter [9]. The ZATLLS device has already proven effective when measuring sedimentation velocity of glass spheres in aqueous solutions [15], low- and high-density particles in organic resins [16], and transglutaminase-activated bovine serum albumin (BSA) on polystyrene particles [9].

Our success in evaluating specific biochemical phenomena using enzyme inhibition suggested that other studies on more general protein-protein interactions might also be useful. We used BSA, a molecular transporter that controls the movement of many molecules including proteins around the body [17–19]. BSA has also been used as a blocking protein, preventing other molecules from attaching to a particular surface, in many experiments including micropatterning, enzyme-linked immunosorbant assays (ELISAs), and protein covered biochips [20–22]. Although BSA has some aggregation potential, especially after denaturing, the objective here was to measure the effect of a BSA coating on polystyrene particles [7,12,23], specifically

to determine whether protein-protein interactions arise in this blocking protein compared with dispersions containing the particles alone. Aggregate size was inferred using the measured sedimentation rate and the physical properties of the residue after sedimentation.

EXPERIMENTAL PROCEDURES

ZATLLS

Polystyrene (PS) particles (poly(styrene with 2% divinylbenzene)) were purchased from PolySciences (Warrington, PA, USA) and used as received. BSA was purchased from Sigma (St. Louis, MO, USA) in powdered form and used as received. The range of particle sizes given by the manufacturer is from 37–74 microns. 0.5 g of PS particles was dispersed in 20 ml of 0.1 M borate buffer (pH 8.5). The solution was vortexed and then centrifuged to recover the particles. Then 15 ml of a 10 mg/ml BSA-borate buffer solution was added and incubated overnight at room temperature. The solution was re-centrifuged to isolate protein-bound PS particles. Next, 25 ml of a 16 v/v% glycerol-water solution, a neutrally buoyant solution, was added to disperse the protein-coated particles. If no protein was being measured then, after the first centrifugation, 25 ml of the 16 v/v% glycerol-water solution was added to the PS particles for use as a control.

The rectangular glass column containing the solution was then placed vertically into the ZATLLS device (Figure 1). The time interval between each run and scanning length were controlled by user defined inputs in LabVIEW (National Instruments, Austin, TX, USA). The time interval between scans worked out to 7 minutes [9]. Dispersion as a function of location and time was then measured repeatedly as the particles settled. After each experiment, the clarified solution was decanted to recover the fluid for later pychnometry and viscosity measurements to determine whether desorbed protein affected solution properties. Experiments typically took several hours which was perceived not to be long enough for protein detachment from particles or bacterial growth to occur.

Viscosity and Density

After decanting, the solution was allowed to further settle to remove any spurious PS particles that still remained and aliquots were taken for viscosity and density measurements. An AR-G2 rheometer (TA Instruments, DE, USA) with a 60 mm cone geometry was used to measure the viscosity of the decanted solution. The density of the

solution was measured with a DA-300M density meter (Mettler-Toledo, Inc., Columbus, OH, USA).

RESULTS

Experiments were conducted to compare the sedimentation velocity of both BSA-coated particles and the neat PS particles. Raw data was collected in labVIEW and any outlying measurements were eliminated using a smoothing routine. This was performed by comparing the recorded voltages for each height interval and then averaging the similar numbers for a final voltage value. Figure 2 shows representative graphs of BSA-coated particles and neat PS particles with the amount of transmitted light through the glass tube as a function of height over time. With increased settling time, more light penetrated through the glass tube and was sensed by the detector. The horizontal lines depicted on these graphs represent arbitrarily picked isovoltage values which were used to track the settling of particles in solution over time, much like a calibration curve. For example, in Figure 2(a) time scan 7 minutes crosses the isovoltage value 0.33 v at an approximate height of 17.09 cm as shown by the circle on the graph. Even with the smoothing process the data were still noisy and there was some subjectivity in establishing where each crossing occurred. Once these are analyzed, velocity curves can be generated for each individual isovoltage.

Figure 3 shows four isovoltage curves for both (a) BSA-coated particles and (b) the control particles devoid of protein. Linear trend lines were fitted to each isovoltage curve and the velocity was equal to the slope. In each experiment, the four velocity values were then averaged together for a final sedimentation velocity. The average aggregate size, D, for each run is inferred using Stoke's law [9,12]:

$$\mathrm{D}^2 = \frac{\upsilon \mathrm{b} 3\eta}{4\Delta\rho \mathrm{g}},$$

where υ is the measured sedimentation velocity, b is a dimensionless variable associated with laminar flow of spherical particles in the creeping flow regime, η is the solution viscosity, $\Delta\rho$ is the density difference between the polystyrene particles, and g is the gravitational constant. The range for the sedimentation velocities, viscosity, density, and the inferred aggregate size range for a single experiment are shown in Table 1. Data that fell more than one standard deviation from the mean particle size were excluded. Several experiments were run and replicate results were found.

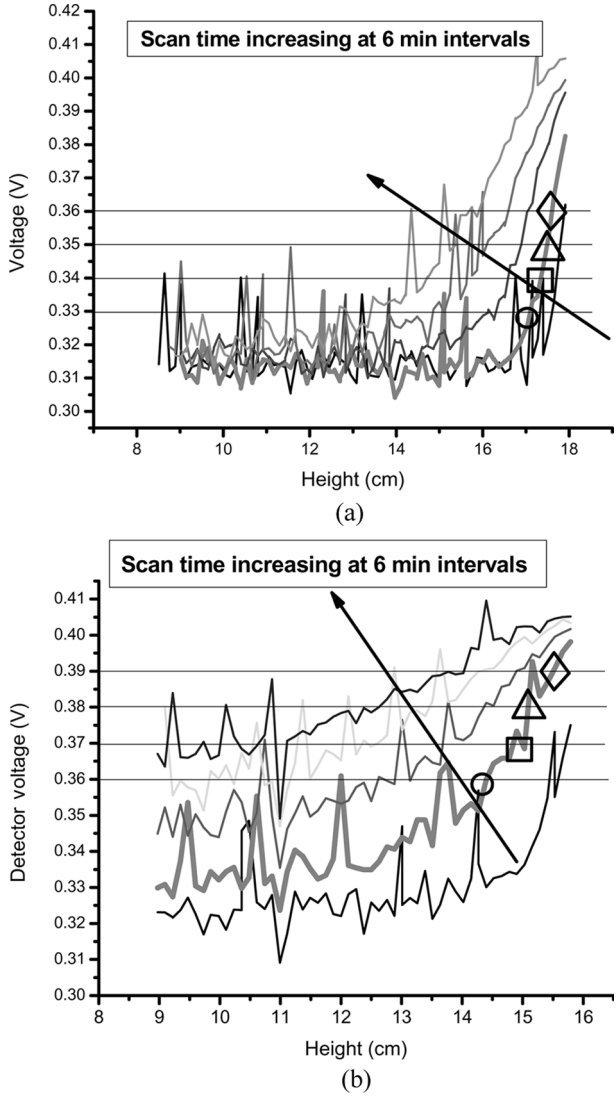

FIGURE 2 Sedimentation graphs are shown for single experiments of (a) BSA-coated polystyrene particles and (b) the neat particles in the 16 v/v% glycerol/water solution. The four horizontal lines on each graph represent the designated isovoltage markers. These markers were used to find the height for each scan to determine sedimentation velocity. The highlighted black shapes represent the height values recorded for the second sedimentation traversal as it passes by each of the isovoltage markers.

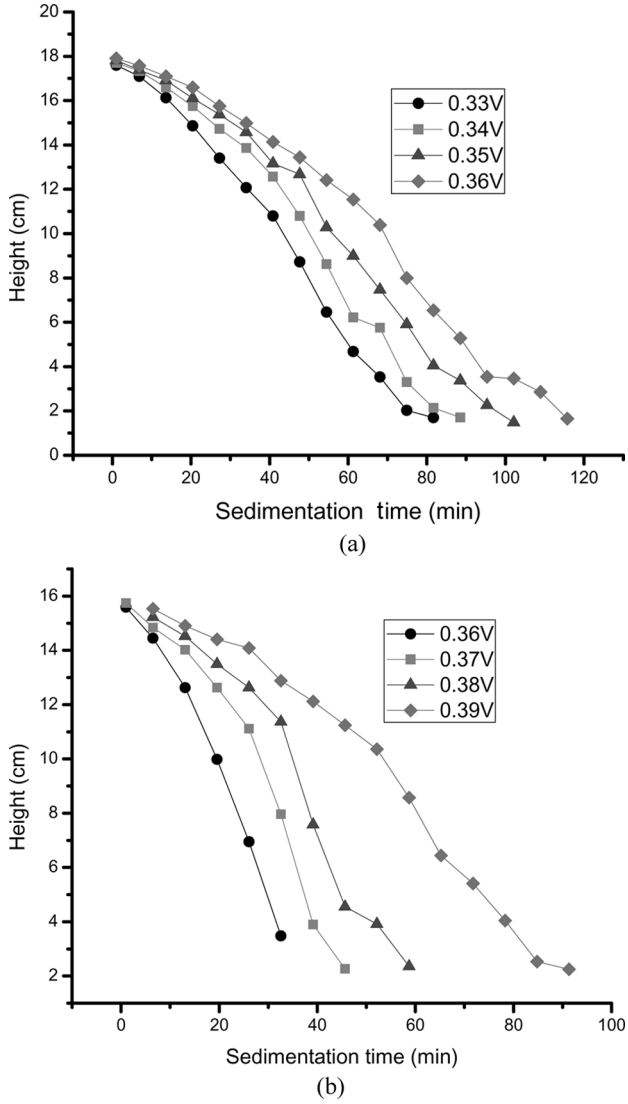

FIGURE 3 Four curves are plotted using the data gathered from the isovoltage markers in Figure 2. Single runs of (a) BSA-coated particles and (b) the control are shown. A least squares fit is applied to each isovoltage curve to determine the sedimentation velocity range. The non-coated particles display a wider range of velocities compared with the more consistent BSA-coated ones due to the non-settling of some particles.

TABLE 1 Stokes Analysis of Fluid Dispersion Including the Inferred Particle Size

	Sedimentation velocity-low (μm/s)	Sedimentation velocity-high (μm/s)	Density (g/cm^3)	Viscosity (mPa ∗ s)	Particle size Range (μm)
BSA-coated PS particles (10 mg/ml)	25.53	37.12	1.0377	3.41	114.02–137.48
Neat PS particles (control)	28.43	64.18	1.0325	1.49	66.68–100.18

Values are shown for the sedimentation velocity range, average density, average viscosity, and the calculated particle size range for single experiments of both BSA-coated and non-coated particles. Several experiments were conducted and similar values were recorded for each of the categories. Data that fell outside one standard deviation of the average particle size were excluded.

DISCUSSION

Coating particles with BSA yielded a narrower range in sedimentation velocity compared with the ones measured using control particles devoid of BSA. By comparing the differences in the isovoltage responses, we ascertain that protein-coated particles fall at a relatively constant rate over time. In experiments with uncoated particles, similar to Figure 3(b), higher velocities were computed in each case because only particles that settled were tracked. Some particles remained suspended or attached to the glass tube which increased the range of measured velocities. This is also why the control particle dispersions have a noisier sedimentation response in the scattering mode as compared with dispersions coated with BSA (Figure 2). Even though the non-coated particles possessed a higher velocity, the aggregate size range is smaller owing to differences in solution viscosity and density.

The BSA-coated particle velocity displays a more gradual decline and is smaller than non-coated particles but more consistent. Interestingly, coating particles with BSA yielded larger aggregates compared with control dispersions due to a driving force for protein interactions. The BSA aggregation potential measured here supports the findings of Xu *et al*. [24]. While other proteins may have an even larger driving force for aggregation, BSA has at least some aggregation potential. One theory is that the negative charge on the albumin, −13 at pH 8.5, attracts the more positive areas of adjacent proteins, thus, providing a driving force for aggregation [23,25].

The particle size ranges we calculated in all cases are still larger than the published values by the particle manufacturer; however,

the neat particle size overlaps into the given range. BSA-coated particles had a larger calculated aggregate size due to the higher viscosity value measured. The higher viscosity retarded particle movement causing a slower sedimentation velocity to be recorded even though the protein-coated particles aggregate. Air in the dispersions could also affect the buoyancy of the particles whether protein-coated or not. No effort was made here to regulate dissolved gases in the mixture prior to sedimentation.

Conformation, type, and amount are some characteristics of adsorbed proteins that affect cellular adhesion, proliferation, and differentiation [26]. Charge distribution, size, and stability of the protein are key in the adsorption process; however, particle composition also helps determine the type and amount of protein adsorbed [26,27]. Rezwan *et al*. measured BSA adsorption on several ceramic particles such as alumina, silica, titania, and zirconia. BSA adsorbed most on zirconia even though BSA had to overcome the repulsive electrostatic force [27]. Protein adsorption can also control the attractive forces on particles by increasing repellent forces and decreasing the attractive van der Waals force [28]. In terms of other colloidal dispersion studies, BSA was used in both formation and stabilization of silver and gold nanoparticles alone and as an alloy [29,30]. At small concentrations, BSA has been shown to increase the stability of sodium bis-2-ethylhexyl sulfosuccinate (Aerosol-OT) monolayers when bound [31]. We expected that BSA would actually aid in dispersing polystyrene particles similarly to low molecular weight wine mannoproteins which have been shown to reduce aggregation and stabilize polyphenol based colloidal suspensions [6]. Our work suggests that sedimentation velocity is not the only measurement needed, but that the protein solution characteristics are required as well in order to more accurately determine aggregate size.

CONCLUSIONS

The laser light sedimentation experiments presented here show that BSA coating of polystyrene results in some degree of protein-particle aggregation. BSA-bound particles have a more consistent and controlled sedimentation rate when contrasted with the uncoated particles. The slower sedimentation velocity of the BSA-coated particles is deceiving in terms of aggregation due to the larger solution viscosity which causes the large aggregates to actually settle more slowly. Sedimentation velocity can also measure the effects of other normal or denatured proteins bound on particles of interest. This technique can also be manipulated to vary extensive properties such as solution

pH, ionic concentration, and temperature. Overall, ZATLLS can measure multiple *in vitro* interactions and inhibitory phenomena in an ensemble-based approach.

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