6-1-2012

Protein Aggregation Through Acoustic Cavitation

John A. Giarratano

University of Denver

Follow this and additional works at: https://digitalcommons.du.edu/etd

Part of the Pharmacy and Pharmaceutical Sciences Commons

Recommended Citation
https://digitalcommons.du.edu/etd/240

This Thesis is brought to you for free and open access by the Graduate Studies at Digital Commons @ DU. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of Digital Commons @ DU. For more information, please contact jennifer.cox@du.edu,dig-commons@du.edu.
Protein Aggregation Through Acoustic Cavitation

Abstract
Therapeutic proteins represent an essential piece of a health management plan for diseases such as diabetes, cancer, hemophilia, Crohn's Disease, and myocardial infarction. These proteins, however, must be maintained in their correct, biologically active conformation throughout processing, transportation, and delivery. This requirement poses serious engineering challenges because of a protein's susceptibility to thermodynamic instabilities resulting from the weak bonds driving the tertiary structure of the molecule. A particularly problematic type of protein degradation is aggregation. Administration of aggregated proteins, a particularly problematic degradation form, can have dire consequences, including blocking a patient's responsiveness to therapy, inducing immunogenicity, and even anaphylactic shock and death. Normal shipping and delivery methodologies are suspected of causing protein aggregation after the normal quality control process has been completed. This work investigates the effect of acoustic cavitation on protein aggregation as a function of impurity level, gas-liquid surface to value ratios, protein concentration, solution viscosity, density, surface tension, and nebulization time. A 0.2M and pH of 4.2 Glycine buffer solution was utilized with IVIg protein at 0.5, 1.0, 5.0, and 10.0 mg/ml and 20ºC. Protein aggregates were characterized using Microflow imaging and NanoSight tracking analysis. Transient cavitation and formation of radicals was monitored using classical iodine assays. Higher protein aggregation is observed in solutions that initially contain greater amounts of impurities or have a larger contact area with the gas interface. Aggregate production in hyper clean solutions, with no gas-liquid interface, initially increases with protein concentration, but eventually decreases at high concentrations. In contrast, aggregation rates in hyper clean solution with a gas-liquid interface continue to fall with increasing protein concentration. The size of the particulate in these two conditions suggests different degradation pathways. The small sizes when a gas interface is available are likely a result of the large area over which the process takes place. The effect of concentration is actually an effect of diffusion or availability for proteins at the surface. The large sizes found in conditions with no gas interface suggest a much more concentrated process consistent with an intense energy release at a single location. Moreover, monitoring of the formation of I$_3^-$ from iodine as a function of nebulization time shows increasing production or radicals. All this supports the hypothesis that ultrasonic pressure waves in protein solutions cause transient cavitation which upon bubble implosion release hydroxyl radicals that can attack the protein in solution. In this circumstance, a rise in viscosity at higher protein concentration inhibits cavitation by elevating the lowest pressure region based on a specified pressure drop.

Document Type
Thesis

Degree Name
M.S.

Department
Bioengineering

First Advisor
Corinne S. Lengsfeld, Ph.D.

Second Advisor
Joseph Angleson
Third Advisor
Michelle Zeles-Hahn

Keywords
Aggregation, Cavitation, Nebulization, Protein

Subject Categories
Medicine and Health Sciences | Pharmacy and Pharmaceutical Sciences

Publication Statement
Copyright is held by the author. User is responsible for all copyright compliance.

This thesis is available at Digital Commons @ DU: https://digitalcommons.du.edu/etd/240
PROTEIN AGGREGATION THROUGH ACOUSTIC CAVITATION

A Thesis

Presented to

the Faculty of Engineering and Computer Science

University of Denver

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

John A. Giarratano

June 2012

Advisor: Corinne S. Lengsfeld
ABSTRACT

Therapeutic proteins represent an essential piece of a health management plan for diseases such as diabetes, cancer, hemophilia, Crohn’s Disease, and myocardial infarction. These proteins, however, must be maintained in their correct, biologically active conformation throughout processing, transportation, and delivery. This requirement poses serious engineering challenges because of a protein’s susceptibility to thermodynamic instabilities resulting from the weak bonds driving the tertiary structure of the molecule. A particularly problematic type of protein degradation is aggregation. Administration of aggregated proteins, a particularly problematic degradation form, can have dire consequences, including blocking a patient’s responsiveness to therapy, inducing immunogenicity, and even anaphylactic shock and death. Normal shipping and delivery methodologies are suspected of causing protein aggregation after the normal quality control process has been completed. This work investigates the effect of acoustic cavitation on protein aggregation as a function of impurity level, gas-liquid surface to value ratios, protein concentration, solution viscosity, density, surface tension, and nebulization time. A 0.2M and pH of 4.2 Glycine buffer solution was utilized with IVIg protein at 0.5, 1.0, 5.0, and 10.0 mg/ml and 20°C. Protein aggregates were characterized using Microflow imaging and NanoSight tracking analysis. Transient cavitation and formation of radicals was monitored using classical iodine assays. Higher protein aggregation is observed in solutions that initially contain greater amounts of impurities or
have a larger contact area with the gas interface. Aggregate production in hyper clean solutions, with no gas-liquid interface, initially increases with protein concentration, but eventually decreases at high concentrations.

In contrast, aggregation rates in hyper clean solution with a gas-liquid interface continue to fall with increasing protein concentration. The size of the particulate in these two conditions suggests different degradation pathways. The small sizes when a gas interface is available are likely a result of the large area over which the process takes place. The effect of concentration is actually an effect of diffusion or availability for proteins at the surface. The large sizes found in conditions with no gas interface suggest a much more concentrated process consistent with an intense energy release at a single location. Moreover, monitoring of the formation of I$_3^-$ from iodine as a function of nebulization time shows increasing production or radicals. All this supports the hypothesis that ultrasonic pressure waves in protein solutions cause transient cavitation which upon bubble implosion release hydroxyl radicals that can attack the protein in solution. In this circumstance, a rise in viscosity at higher protein concentration inhibits cavitation by elevating the lowest pressure region based on a specified pressure drop.
Acknowledgements

I’d like to thank my wife and family for putting up with me over the course of this research. I would also like to acknowledge that this project was made possible by my graduate advisor, Dr. Corinne Lengsfeld, my lab group and the Denver University Engineering Department, Dr. Sadler in the Biology Department, and our friends in the Carpenter Lab group at the University of Colorado, School of Pharmaceutical Sciences.
Table of Contents

Chapter One: Motivation ................................................................................................................... 1
Introduction ........................................................................................................................................ 1
Protein Degradation ......................................................................................................................... 1
  Protein Aggregation ...................................................................................................................... 2
Cavitation .......................................................................................................................................... 3
  Acoustic Cavitation ...................................................................................................................... 4
  Ultrasonic Nebulization .............................................................................................................. 5
Aggregate Characterization ............................................................................................................... 6
  Micro-flow Imaging .................................................................................................................... 6
Nanosight Tracking Analysis ............................................................................................................ 8
Intravenous Immunoglobulin ........................................................................................................... 9
Outline ............................................................................................................................................. 10

Chapter Two: Materials and Methods ............................................................................................ 11
Materials .......................................................................................................................................... 12
Sample Preparation ......................................................................................................................... 12
  Ultracentrifugation ..................................................................................................................... 13
Methods .......................................................................................................................................... 14
  Ultrasonic Nebulizer ................................................................................................................... 14
  Viscosity ....................................................................................................................................... 15
  Density .......................................................................................................................................... 16
Surface Tension ................................................................................................................................. 17
UV-Spectroscopy ............................................................................................................................. 19
Micro-Flow Imaging ......................................................................................................................... 20
NanoSight Tracking Analysis ........................................................................................................... 22
Headspace Dependence .................................................................................................................. 23
Concentration Dependence ............................................................................................................ 25
Nebulization Time Dependence ....................................................................................................... 26
Volume Dependence ....................................................................................................................... 26
Analysis Plan .................................................................................................................................... 27

Chapter Three: Results .................................................................................................................... 28
Viscosity .......................................................................................................................................... 28
Density .......................................................................................................................................... 30
Surface Tension ............................................................................................................................... 30
Formation of Triiodide Ions (I\textsubscript{3}) ................................................................................... 31
Headspace Dependence .................................................................................................................. 35
Time Dependence ........................................................................................................................... 37
Volume Dependence ....................................................................................................................... 40
Concentration Dependence ............................................................................................................ 42

Chapter Four: Discussion .................................................................................................................. 47
Gas - Liquid Interface ....................................................................................................................... 47
TABLE OF FIGURES

Figure 1: Visual schematic of stable and transient cavitation.................................4

Figure 2: Representative graph of data gathered in the μm range by MFI...................8

Figure 3: Representative graph of data gathered in the nm range by NTA...............9

Figure 4: MABISMist™ II Ultrasonic Nebulizer Model 40-270-000.......................15

Figure 5: A: A digital photograph, representative of photographs taken in triplicate at each IVIg concentration. B: A magnified image of the meniscus and right triangle to calculate the angle of meniscus...................................................18

Figure 6: MFI image from current data compared with one from previous publication [14]........................................................................................................................................21

Figure 7: Still image of viewing frame during NTA recording.............................23

Figure 8: Photographic profile of experimental set up during A: No-Space run. B: Small-Space run. C: Large-Space run.................................................................25

Figure 9: IVIg absolute viscosity as a function of protein concentration for sample concentrations......................................................................................................................29

Figure 10: Log of IVIg absolute viscosity as a function of log of protein concentration.29

Figure 11: Surface tension as a function of IVIg protein concentration as calculated through experimental capillary action.........................................................31

Figure 12: UV-Spectrum of I_{3^-} absorption as a function of time.......................33

Figure 13: UV-Spectrum of I_{3^-} absorption as a function of time.......................34

Figure 14: Triiodide ion (I_{3^-}) as a function of nebulization time as determined by UV-Spec for I_{3^-} absorbance.................................................................35

Figure 15: Concentration of IVIg particles as a function of head space in the nebulizer as calculated using MFI.................................................................36

Figure 16: Concentration of IVIg particles as a function of head space in the nebulizer as calculated using NTA.................................................................37

Figure 17: Particle concentration as a function of nebulization time using MFI........38
Figure 18: Particle concentration as a function of nebulization time using NTA……39
Figure 19: Particle concentration as a function of time using MFI……………………40
Figure 20: Particle concentration as a function of sample volume using MFI……………41
Figure 21: Particle concentration as a function of sample volume using MFI…………42
Figure 22: Particle concentration as a function of sample concentration using MFI……43
Figure 23: Particle concentration as a function of sample concentration using NTA…44
Figure 24: Particle concentration after 15 minutes of nebulization as a function of IVIg solution concentration with a small head space…………………………………….45
Figure 25: Particle Concentration after 15 minutes of nebulization as a function of IVIg solution concentration with a small head space as analysed using NTA…………46
Figure 26: A: Nebulizer set-up with nozzle for drug delivery. B: Nebulizer set-up during small-space headsplace dependance experiment…………………………………48
Figure 27: Triiodide ion concentration (blue) and total particle concentration (red) as a function of nebulization time. ……………………………………………………………51
TABLE OF TABLES

Table 1: Density as a function of concentration for IVIg protein as determined by a mass divided by volume relationship .................................................................30
CHAPTER ONE: MOTIVATION

Introduction

Protein therapeutics are a valuable clinical tool in the fight against diseases and conditions such as cancer, diabetes, hemophilia, Crohn’s Disease, and myocardial infraction [1]. The number of commercially available protein-based products in the US has gone from three in the late 1980s to nearly 150 today [2]. Physical degradation of proteins has become a subject of considerable interest within the pharmaceutical industry as the number of therapeutic protein products has increased in recent years. This interest is driven by the fact that in order for protein therapeutics to be effective, they must remain in their correct, biological active state throughout production, transportation, and delivery to patients. The ability for a therapeutic protein to demonstrate human health benefits, as well as become an economically viable, FDA approved drug, hinges on relatively weak molecular stability and high susceptibility to degradation [2].

Protein Degradation

Processes that modify covalent bonds within a protein, such as deamidation, oxidation, and disulfide bond shuffling, are known as chemical degradation. In this type of protein degradation, new chemical entities are generated through the making and breaking of covalent bonds. Conversely, physical degradation involves protein instabilities that lead to changes in physical state without change in chemical composition.
These instabilities can lead to undesirable phenomena including protein unfolding, adsorption to surfaces, and aggregation. For the purpose of this research, all references to protein degradation will be in the context of physical degradation.

Protein Aggregation

For protein therapeutics to work, proteins involved must be folded into three dimensional globular structures known as the native state [3]. The loss of native protein structure, resulting in a fully or partially unfolded state, can lead to the formation of aggregates held together by hydrophobic attraction. This phenomenon occurs when specific subunits of one molecule are ‘attracted’ to specific subunits of another, interacting in an intermolecular manner [5]. Continued propagation leads to the formation of large aggregates. Conformational stability of a protein’s native state relies on many factors such as amino acid sequence, pH, temperature, and concentration [5]. In high concentrations, there is a thermodynamic competition between a protein’s properly folded native state and aggregation due to partially folded intermediates [1-3,5].

Protein aggregation within pharmaceuticals is a subject of heightened interest for the bio-pharma industry because of possible adverse effects upon administration to patients [1-5]. Studies have shown that aggregated therapeutics can cause blocking of responsiveness to therapy and onset of immunogenicity [4-7]. Aggregation often happens in bioprocessing during cell culture, purification, formulation, and fill finish operations [7]. Because of this, the FDA has guidelines for the size of particles allowable for finished drug products. However, since the aggregated state has a lower free energy than the native state, it has been shown that environmental factors during storage, shipping and delivery can cause aggregation. It has also been suggested that aggregates
smaller than the FDA’s requirements could be responsible for immunogenic reactions [9]. For these reasons, it is necessary for pharmaceutical researchers to understand the cause and mechanism of therapeutic protein aggregation.

**Cavitation**

Cavitation is the formation and collapse of cavities in incompressible liquids [29]. In 1917, Lord Rayleigh predicted enormous local temperatures and pressures at the location of the collapse. This collapse of bubbles, caused by cavitation, produces intense local heating and high pressures with very short lifetimes [29]. Cavitation can take place anywhere the local pressure in a liquid drops below the vapor pressure and a cavity or bubble is formed. Well known examples of this are propellers rotating through a liquid where bubbles can be seen forming behind the trailing edge of the prop blade. This process can occur in many bioprocessing steps such as pumping, mixing, and post filling processes; post filling processes can include over agitation of a vial or ultrasonic nebulization for pulmonary drug delivery. Although it is recognized that no single mechanism for the aggregation of proteins exists, it is well understood that all of the possible mechanisms must involve thermodynamic energy needed to drive proteins from their native state [2,5]. Many potentially damaging conditions including air-water interface, turbulent vortexing, freeze-thaw, extreme temperature, and chemical degradation have been heavily studied by pharmaceutical researchers [8]. However, despite parallels between the thermodynamic effect of bubble collapse and the known causes of protein aggregation, very little has been written about cavitation as a mechanism for aggregation in protein therapeutics.
Acoustic Cavitation

Acoustic cavitation is the formation, growth, and implosive collapse of bubbles in liquids irradiated with high-intensity ultrasound [29]. Two types of acoustic cavitation exist: stable and transient. Stable cavities grow or shrink and then oscillate about some equilibrium size, often existing for many acoustic cycles. Transient cavities exist for only a few cycles until the forcing frequency is near its resonance; Figure (1). These bubbles can expand to several orders of magnitude larger than their original size before imploding. For the duration of this paper, any statements involving cavitation will be in reference to acoustic cavitation.

Figure 1. Visual schematic of stable and transient cavitation. Above: Bubble forms and oscillates through many acoustic cycles during stable cavitation. Below: Bubble forms and grows until implosion during transient cavitation.
Suslick, et al. (1999) was able to show that the collapse of bubbles in a multi-bubble cavitation field produces hot spots with effective temperatures of ca. 5000 K, pressures of ca. 1000 atm, and heating and cooling rates above $10^{10}$ Ks$^{-1}$. Experiments where it would be possible to isolate and study these extreme conditions in the presence of proteins could lead to a better understanding of cavitation as a mechanism for protein aggregation. We hypothesize that protein therapeutic exposure to cavitation alone will not only result in the formation of aggregates, but by varying parameters such as nebulization time, sample volume, solution properties, and concentration, methods of mitigation for protein degradation by cavitation will be identified.

**Ultrasonic Nebulization**

An ultrasonic nebulizer is a device that uses piezoelectric effect to generate high-frequency acoustic energy, which forms aerosol droplets by cavitation [22]. These devices are used for the pulmonary delivery of therapeutic proteins for diseases such as cystic fibrosis. Most nebulizers on the market today are small, portable, and inexpensive machines, making them a perfect model system to study the effects of cavitation on protein therapeutics. Pressure waves transmitted through a liquid volume alternatively expand and compress until tension forces on nucleation sites are sufficient to vaporize small volumes of liquid [29]. In water, the thermal degradation of water molecules leads to the formation of hydrogen atoms and hydroxyl radicals. Studies have shown that DNA can serve as a nucleation site for bubble formation [20]. By that rationale, the existence of protein aggregates acting as nucleation sites within a fluid volume would only add to propagation of aggregates due to energy transfer from increased cavitation. Thus if the liquid volume within an ultrasonic nebulizer could be isolated, its contents post
nebulization would be a representative sample of protein therapeutics exposed to cavitation in the absence of outside environmental factors.

**Aggregate Characterization**

Several well-known studies have been conducted showing adverse effects of protein aggregates in the micron as well as nanometer range. In some cases, these aggregates are below the particle size limits for acceptable protein formulations imposed by the FDA [9]. Due to known issues of protein immunogenicity in aggregated protein formulations, it is important to characterize size, size distribution, and aggregate concentration. This process is not trivial as protein aggregates are typically very heterogeneous, with sizes ranging from a few nanometers to several micrometers. Small aggregates in the nm range have recently been implicated as the cause of immunogenicity; therefore, it is important to characterize particles at a very small scale [9]. Until recently, this task was almost impossible due to the limitations of particle counting instrumentation. Particle characterization for this experiment utilized two relatively new technologies which take advantage of optical techniques and innovative software to get accurate particle size distributions.

**Micro-flow Imaging**

It has been common practice for years in pharmaceutical manufacturing to complete visible and sub-visible particulate testing of therapeutics to ensure their safety and suitability for clinical use [9]. Currently, the FDA has guidelines for acceptable numbers of particles >10 and >25 µm. However, as previously explained, it may be important to measure the concentration of protein aggregates to at least one, maybe two
orders of magnitude smaller to avoid potential immunogenic response. Also, since the shape of protein aggregates are variable and “fibril-like” in nature, it is important to use a counting device that does not rely on spherical and opaque particles for accurate counts [10, 12-15]. In order to achieve particle characterization from 2 µm and larger, Micro-Flow Imaging (MFI™) is utilized. By capturing digital images of particles suspended in a fluid as they pass through a sensing zone, MFI is able to automatically analyze a sample and provide a digital archive of parameters such as equivalent circular diameter (ECD), Feret diameter, aspect ratio, circularity, and intensity [13-17]. With the added benefits of instrument automation, high sensitivity, a broad size range, and requiring only a limited amount of sample for analysis, MFI offers advantages that cannot be matched by manual microscopy [13].

MFI’s software exports particle size and concentration data in the form of an excel spread sheet that can be easily formatted into graphs for interpretation. Figure (2) is a particle size vs. particle concentration graph that is representative of the data gathered during these experiments.
Figure 2. Representative graph of data gathered in the µm range by MFI. Particle concentration as a function of particle size for samples analyzed before nebulization pre-neb (blue) and nebulized for 3 (red), 7 (green), 11 (purple) and 15 (light blue) minutes respectively.

Nanosight Tracking Analysis

Recent reviews in protein aggregation have outlined the importance of analyzing sub visible particles in the nanometer range [9]. Although standards have been set in the µm range, it has been shown that particles down to the 0.1 µm size could be responsible for immunogenic response in patients. Following current standards and ignoring sub visible particles could result in adverse events in patients after commercialization, even in situations where the product was deemed safe and effective in clinical trials [9]. This research intends to analyze samples for particles in the submicron range with the use of Nanoparticle Tracking Analysis (NTA™), which is an innovative system for sizing particles from about 30-1000 nm. Combining laser light scattering microscopy with a charge-coupled CCD camera, NTA enables the visualization and recording of
nanoparticles in solution. This allows the NTA software to identify and track individual nanoparticles moving under Brownian motion [11,12].

Nanosight’s software exports particle size and concentration data in the form of an Excel spreadsheet that can be easily formatted into graphs for interpretation. Figure (3) is a particle size vs. particle concentration graph that is representative of the data gathered during these experiments.

![Particle Size vs. Particle Concentration for Headspace Dependence using Nanosight](image)

**Figure 3.** Representative graph of data gathered in the nm range by NTA. Particle Concentration as a function of particle size for samples analyzed before nebulization (pre-Neb), and after 15 minutes of nebulization with nebulizer set up for No-Space (red), Small-Space (green) and Big-Space (purple).

**Intravenous Immunoglobulin**

In the following experiments, intravenous immunoglobulin (IVIg) will be used as a model proteinaceous compound. For over 25 years, IVIg has been used in the treatment of autoimmune and inflammatory diseases. It consists of IgG obtained from pools of plasma of several thousand healthy blood donors [24]. Previous studies have indicated
nebulization IVIg as a good means of drug delivery for infants [43]. However, IVIg works through many complex mechanisms that modulate the activation and neutralization of B and T lymphocytes and pathogenic autoantibodies. Given this mechanism of action, it can be assumed that aggregated IVIg will interact with the immune system and that aggregation of IVIg has the potential for adverse immunogenic response. Thus, IVIg is a well suited model proteinaceous compound for these experiments.

**Outline**

This thesis will characterize protein aggregation by means of ultrasonic nebulization to determine the impact of viscosity, density, surface tension, gas contact area, impurities, and exposure time on protein aggregation. Our aim is to provide evidence that transient cavitation induces protein aggregation in therapeutics, and through investigation of fluid properties, suggest strategies for mitigation of aggregate formation. We hypothesize that ultrasonic nebulizers induce transient cavitation, and that proteins nucleate this cavitation much like plasmid DNA. This will be done through classical iodine assays and altering variables such as concentration, sample volume and nebulization time. Finally, solution properties such as viscosity, density and surface tension will be collected to determine if they can be changed in order to shut down cavitation.
CHAPTER TWO: MATERIALS AND METHODS

Intravenous immunoglobulin (IVIg) is a protein drug product prepared from pools of blood plasma from at least 3000, but up to 100,000 individuals. It is comprised of a broad range of immune antibodies directed to pathogens and foreign antigens, and has been reported to have a beneficial effect on scores of immune mediated diseases [24]. IVIg protein was obtained from a pharmacy in the form of the drug product Gamunex (Talecris Biotherapeutics, Durham, NC), stored in 1.0 ml aliquots at 100 mg/ml concentration and used as a model proteinaceous compound for aggregation. Four protein concentrations were chosen (0.5, 1.0, 5.0 & 10.0 mg/ml) that represent dosages used in IVIg therapeutic administration. Fluid property characterization was done with a model CVO (Bohlin Instruments, Inc, UK) viscometer to determine viscosity at each concentration. Surface tension was determined using capillary action techniques. Formation of triiodide ions (I₃⁻) formed by the oxidation of potassium iodide (KI) during nebulization was analyzed using a USB-ISS-UV/VIS (Ocean Optics, Inc., Dunedin, FL) UV-Spectrometer. Dilutions of 10.0 mg/ml were purified with ultracentrifugation using an Optima™ LE-80K Ultracentrifuge (Beckman Coulter, USA). Particle matter such as protein aggregates created during nebulization of IVIg protein were analyzed at the μm size using Micro-flow Imaging™ (MFI) model DPA 4100 (Brightwell Technologies Inc., Ottawa, Canada). Sub-micron particle characterization was performed using a
Nanoparticle Tracking Analysis (NTA), Nanosight model LM20 (Nanosight, Ltd., Salisbury, UK).

**Materials**

Deionized water (DI) from Milli-Q integral ultrapure water system (EMD Millipore, Billerica, MA); sodium hydroxide (NaOH), hydrochloric acid (HCl), glycine and potassium iodide (KI) (Sigma-Aldrich®, St. Louis, MO); 0.22 µm MILLEX®GP sterile syringe filters (Millipore, Co., Billerica, MA); Hellmanex (Hellma®, Plainview, NY); IVIg protein from drug product Gamunex (Talecris Biotherapeutics, Durham, NC).

**Sample Preparation**

First, buffer was made from glycine powder mixed with ultrapure DI water from a Milli-Q to a 2.0M buffer stock solution and titrated to pH 4.2 using HCl and NaOH. When needed, the 2.0M glycine buffer was diluted using DI water to 0.2M and re-titrated to pH 4.2 due to the acetic nature of DI water. Sterile 0.22 µm syringe filters were used along with a 15 ml syringe to filter all buffers for storage, and before use. Buffer was always stored in a refrigerator at 5°C when not in use. 1.0 ml aliquots of 100.0 mg/ml IVIg protein were obtained for use as the model proteinaceous compound for this experiment. Concentrated 100.0 mg/ml IVIg was pipetted into 9.0 ml of the 0.2M glycine buffer to make 10.0 ml of 10.0 mg/ml IVIg solution. This pre-centrifuged solution was then loaded into an ultracentrifuge and spun down to remove all particles >100 nm, which is larger than native IVIg proteins. After ultracentrifugation, the purified IVIg solution was diluted into the three other concentrations (0.5, 1.0 & 5.0 mg/ml) as needed for the experiments using filtered 0.2M glycine buffer. Particle composition of glycine
buffer was tested before every dilution and experiment using MFI and NTA. All samples were colorless, free of visible particles and possessed viscosity ranging from that of water to several times water depending on concentration. When not in use, all samples were stored in a refrigerator at 5°C. No sample was stored for longer than 72 hours before use. All experiments were conducted at room temperature (18-25°C). Samples removed from the refrigerator were left out at room temperature for ~10 min in order to normalize temperature and reduce any air bubbles in the solution.

Ultracentrifugation

In order to purify the samples before nebulization and avoid adverse effects of filters on the protein mixture, ultracentrifugation was used to dispose of particles >100 nm in size within the solution. It is important to ensure the solution is ultrapure because existing particles are known to act as nucleation sites for nonnative protein aggregation and therefore could create variability within samples [1]. To do this, a rough estimation was calculated using Stokes Law (Equation 1) in order to determine the settling velocity of small particles within the solution where \( V_g \) is sedimentation velocity, \( d \) is particle diameter, \( \rho_p \) is particle density, \( \rho_l \) is liquid density, \( \mu \) is viscosity of liquid, and \( G \) is gravitational acceleration.

**Stokes Law of Sedimentation**

\[
V_g = \frac{d^2 (\rho_p - \rho_l)}{18 \mu \times G} \quad [1]
\]

This estimation is ‘rough’ in that assumptions must be made regarding the shape and density of the particles being assessed. In order for Stokes Law to work, particles
must be assumed to be spherical. It is thus determined that the settling velocity for 100 nm particles is \( \sim 4.92 \text{ cm/hr} \). In order to facilitate all the particles settling at the bottom of a 30 ml test tube, the samples were spun at 112,000g for three hours, which corresponds to 25K RPM using a SW-28 rotor. After samples were placed inside the sample tubes and attached to the rotor, the centrifuge was vacuum sealed and kept at 5°C for the duration of centrifugation.

**Methods**

**Ultrasonic Nebulizer**

A MABISMist™ II Ultrasonic Nebulizer Model 40-270-000 (MABIS Healthcare, Inc., Lake Forest, IL) was chosen as the device to induce cavitation during this experiment. This nebulizer operates at a frequency of 2.5 MHz, 10 watts of power and uses the piezoelectric effect to generate high-frequency acoustic energy which generates aerosol droplets by cavitation [23]. The particles created by this nebulizer are in the range of 3-9 µm as measured by Phase Doppler Particle Analyzer [20], however for the majority of this experiment, impaction of the impinging jet is such that secondary droplet formation does not occur and the sample volume is never converted to aerosol. All experiments, except those testing volume dependencies, were carried out according to manufactures specifications with a maximum sample volume of 8 ml. All experiments, except those testing time dependencies, were run for a total of 15 min at the high setting.

Sample cups used to hold the sample volume were rinsed thoroughly with ultrapure DI water followed by 15 minutes of nebulization on high with filtered glycine buffer to remove any further particles through sonication. DI water was also used to rinse
the sample cup between successive runs. To avoid particle accumulation between runs, the glass plug, rubber gasket and rim of the nebulizer were wiped down with 90% IPA and allowed to dry before further use. The DI water reservoir was also changed out between runs due to a 15° C temperature increase after use. A photographic diagram is displayed in Figure (4).

![Diagram of interface between glass plug, sample volume and water reservoir.](image)

**Figure 4.** Left: MABISMist™ II Ultrasonic Nebulizer Model 40-270-000. Right: Diagram of interface between glass plug, sample volume and water reservoir.

**Viscosity**

Samples of IVIg were diluted from 100 mg/ml to concentrations of 1.0, 5.0, 8.0 and 10.0 mg/ml in order to get a representative distribution of viscosities between 0 (Water) and 10.0 mg/ml. All samples are run on a model CVO Bohlin Instruments Viscometer with a C14 ‘cup and bob’ coaxial cylinders. The bob is made out of Titanium and the Cup stainless steel. 2.0 ml samples of each concentration were pipetted into the cup and run for each analysis. The dynamic viscosity (η) for each run is calculated using an Arrhenius function (Equation 2) built into the software of the
viscometer where \((c)\) is pre-exponential factor, \((k)\) is the exponential constant, and \((t)\) is the absolute temperature.

Arrhenius Function

\[
\eta = ce^{k/t} \quad \text{[2]}
\]

All concentrations were run 12 times and an average viscosity was calculated using an average of all the runs.

Density

Samples of IVIg were diluted from 100 mg/ml to concentrations of 0.5, 1.0, 5.0 and 10.0 mg/ml. A weigh boat was placed into the A&D model GR-202 (A&D®, Ablingdon, UK) analytical balance and the reading was zeroed. A volume of 5.0 milliliters of each concentration was loaded into the weigh boat one at a time with a pipette. The balance was allowed to stabilize and mass measurements were recorded for each concentration. This process was repeated five times for ultrapure DI water and each concentration. The density at each concentration was found by solving the density equation (Equation 3) where \((\rho)\) is the density of the fluid in g/cm\(^3\), \((M)\) is the mass in grams, and \((V)\) is the volume in ml.

Density Equation

\[
\rho = \frac{M}{V} \quad \text{[3]}
\]
Surface Tension

Samples of IVIg were diluted from 100 mg/ml to concentrations of 0.5, 1.0, 5.0 and 10.0 mg/ml. Each concentration, along with ultrapure DI water was pipetted into cuvettes and labeled by their respective IVIg concentration. All cuvettes were filled completely so no meniscus was observed at the top edge. The cuvette was brought to the glass capillary tube suspended from a fixed anchor in order to ensure that the angle with respect to gravity and position of the capillary tube within the liquid volume remained unchanged. The depth of placement was also maintained from test to test. Digital photographs were taken of the cuvette to accurately measure the contact angle and the resulting height ($\Delta H$) of the liquid volume within the capillary. This was done three times for each concentration and DI water. Between runs the capillary and cuvette were thoroughly washed using DI water and dried using dry, filtered house air. The capillary was tapped to ensure no air bubbles stopped the wicking of the fluid into the tube. Measurements were made from the lowest to highest concentration to reduce the impact of residual impurities.

The photographs were then uploaded onto a PC and normalized to actual size so $\Delta H$ could be determined by measuring with a ruler. Using the known value of 7.28E-2 N/m for the surface tension of DI water at 20° C, the inner diameter of the capillary tube was determined. Rough estimations for the angle of the meniscus ($\Theta$) were determined by magnifying the photograph of the meniscus inside the capillary tube and sketching a right triangle with $\Theta$ at the edge where the meniscus meets the sides of the glass capillary. By measuring the lengths of the opposite side and hypotenuse, the inverse sine of $\Theta$ was
taken for all concentrations. Finally, surface tension (T) was calculated by solving the equation for capillary action (Equation 4) where ΔH is the height of the liquid volume within the capillary tube, ρ is the experimentally measured density, r is the radius of the capillary tube, g is the acceleration due to gravity, and Θ is the angle of the meniscus.

Capillary Action

\[ ΔH = \frac{2T\cosΘ}{ρrg} \]  

[ 4 ]

Figure (5) shows an example of a representative photograph taken of a cuvette and capillary tube, as well as a zoomed in image of a meniscus within a capillary tube. A right triangle is drawn in and labeled with the opposite side (0), hypotenuse (h) and angle of meniscus (Θ).

Figure 5. A: A digital photograph, representative of photographs taken in triplicate at each IVIg concentration. B: A magnified image of the meniscus and right triangle to calculate the angle of meniscus.
UV-Spectroscopy

A 100 mg/ml sample of IVIg was diluted to 1.0 and 10.0 mg/ml concentrations. Based on previous work using this well-established method to quantitatively monitor transient cavitation [19], it is known that triiodide ions are formed by the oxidation of KI. Initial experiments at traditional KI concentrations yielded no results, because IVIg is a good scavenger of $I^-$ or the intermediates. Therefore to test for radical formation in the in the presence of IVIg protein, an elevated concentration was required. The optimal concentration was determined through a set of experiments accessing the signal to noise ratio.

1.0 ml of the initial concentration of 200 mM KI was added to 9.0 ml of both 1.0 mg/ml and 10.0 mg/ml protein solutions. 8.0 ml samples were then placed into the sample cup and nebulized on high for 15 min. The nebulizer was stopped briefly at 3, 6, 9, 12, and 15 minutes in order to remove a sample volume of 200 µl from the sample cup. This sample was then diluted to a total volume of 1.0 ml and placed in a plastic cuvette with a 0.4 cm path length and immediately analyzed using a USB-ISS-UV/VIS (Ocean Optics, Inc., Dunedin, FL) UV-Spectrometer. The UV-Spectrometer was tested for operational integrity using known UV spectral identities for proteins and KI [31]. During triiodide analysis, purified glycine buffer and IVIg protein were used as the blank so only the iodine spectrum were visible.

Under these conditions no triiodide ions were detected for either concentration of IVIg. Others have found that IVIg itself was a scavenger of free radicals (private conversations with the Randolph Lab), and therefore an abundance of KI might be
necessary in order to form detectable concentrations of triiodide. Several concentrations of KI were attempted with the best results near saturation. A solution of saturated KI (8.675 M) was made. Again, 1.0 ml of this new KI solution was added to 9.0 ml of both 1.0 and 10.0 mg/ml IVIg and immediately analyzed for triiodide ions. Absorbance values were measured and recorded at 10 nm increments for 300 – 400 nm wavelengths. Absorbance values at the 350 nm peak were used to solve the Beer-Lambert relationship and calculate concentrations of triiodide ion.

Concentration of $I_3^-$ was determined using the Beer-Lambert Law (Equation 5) where $A$ is absorbance at 350 nm, $e$ is molar absorptivity, $b$ is the path length of the cuvette used, and $c$ is the concentration of solution. The known molar absorptivity of $I_3^-$ at 350 nm is 2.32E4 L/mol*cm [34]. Path length of the cuvettes used is 0.4 cm.

Beer-Lambert Law of Absorbance

$$A = ebc$$  \[5\]

Micro-Flow Imaging

Measurements of particles 2 – 40 µm in size were performed using a Brightwell Technologies Inc. MFI model DPA4100 located at the University of Colorado, Anschutz Medical Campus. Prior to each sample analysis, filtered glycine buffer was flushed through the flow cell, and analyzed to ensure a clean baseline before particle sample analysis. All samples were allowed to sit at room temperature for at least 10 minutes after sample preparation to remove any air bubbles that could be picked up during analysis by the MFI. Using a pipette, a 1.0 ml volume was loaded into the flow cell dock and drawn into the system at a rate of 0.22 ml/min. During each run, 0.1 ml of fluid was
allowed to purge through before particle analysis began and a total of 0.8 ml of fluid was analyzed. For each sample, a total of three runs were conducted and averaged for further analysis. A negative control was conducted using unprocessed, filtered glycine buffer. Counts from the controls are reported, but not subtracted from the final particle counts for all other samples. Between sample analyses, the flow cell was cleaned by purging 1.0 ml of Hellmanex (Hellma®, Plainview, NY) and finally flushed with ultrapure DI water until cleanliness of the flow cell was ensured.

Images of protein aggregates larger than 20 µm were captured during analysis and referenced against images from literature. Although protein particles are highly heterogeneous in shape, common features of large ribbon-like aggregates can be distinguished as shown in Figure (6).

![Image of Protein Aggregate from MFI data.](image1)

![Image of Protein Aggregate from Sharma, et al. 2010.](image2)

**Figure 6.** MFI image from current data compared with one from previous publication [14].

MFI data was collected and interpreted using Excel software. Since we are interested in the total amount of particles within a size range, the integrated sum of particles per ml are reported for each variable respectively. Total particle concentrations per ml were determined by summing the values from 2 to 40 µm for each run.
NanoSight Tracking Analysis

Measurements of particles 1 – 1000 nm in size were performed using a NanoSight, model LM20 (NanoSight, Amesbury, United Kingdom). Samples were introduced to the 0.3 ml sample chamber through a peripheral injection site using disposable lure-slip syringes (National Scientific, Rockwood, TN), until liquid reached the tip of the nozzle on the opposite side. Excess sample remaining in the disposable syringe was injected into the sample chamber until a drop was purged from the nozzle on the opposite side between runs to enable the greatest amount of homogeneity among runs in each sample set. In total, less than 1.0 ml was analyzed for each sample. All measurements were performed at room temperature immediately after samples were nebulized.

The software used for capturing recordings of the protein aggregates and analyzing the data was the NTA 2.0 Build 127. Samples were recorded for 60 or 90 seconds depending on particle concentration with lower concentrations being recorded for the later. Figure (7) shows an example of the viewing frame recorded by NTA software. Sub-micron particles can be seen as white dots with rings around them. Samples were run a total of 5 times and averaged before further analysis.

NTA data were collected and interpreted using Excel software. Since we are interested in the total amount of particles within a size range, the integrated sum of particles per ml are reported for each variable respectively. Total particle concentrations per ml were determined by summing the values from 1 to 1000 nm for each run.
Figure 7. Still image of viewing frame during NTA recording.

Headspace Dependence

No-Space:
An 8 ml sample of 1.0 mg/ml IVIg was placed into the sample cup with the nebulizer set up so the glass plug sits just above the internal reservoir volume level. As the sample was nebulized, the glass plug restricted the jet created by the ultrasonic field such that no vapor particles were created. The internal volume was thus exposed to the smallest amount of gas/liquid interface and agitation within the sample appeared negligible. Figure (8A).

Small-Space:
An 8 ml sample of 1.0 mg/ml IVIg was placed into the sample cup with the nebulizer set up so there was a 1 cm space between the internal reservoir volume and a lid from a 50 ml conical tube that fit perfectly above the sample cup. This geometry
allowed for a total headspace volume of about $7cm^3$. The glass plug was placed on top of the lid for weight so the jet did not displace the lid. Vapor particles were thus allowed to form within the $7cm^3$ volume and recollect inside the internal reservoir. Figure (8B).

Large-Space:

An 8 ml sample of 1.0 mg/ml IVIg was placed into the sample cup with the nebulizer set up so the reservoir of a 50 ml conical tube attached to the sample cup such that the internal reservoir had the entire volume of the sample cup and conical tube to circulate during nebulization. This volume of roughly $70cm^3$ allowed for the greatest amount of gas/liquid interface. Figure (8C).
Concentration Dependence

An initial concentration of 100 mg/ml IVIg was diluted down to 10.0 mg/ml using filtered 0.2 M glycine buffer at pH 4.2 and ultracentrifuged to remove particles >100nm. The purified sample was then further diluted to concentrations of 0.5, 1.0, and 5.0 mg/ml using filtered 0.2 M glycine buffer at pH 4.2. An initial sample of each concentration was tested on the MFI and NanoSight to get a baseline particle count to compare to nebulized samples. 8.0 ml of each concentration was then pipetted into the sample cup of the ultrasonic nebulizer and nebulized at frequency 2.5 MHz for 15 min. Each sample
was immediately analyzed using the MFI and NTA for particle concentration at the µm and nm size range respectively.

**Nebulization Time Dependence**

An initial concentration of 100 mg/ml IVIg was diluted into a concentration of 10.0 mg/ml using filtered 0.2 M glycine buffer at pH 4.2 and ultracentrifuged to remove particles > 100 nm. The sample was then further diluted to 1.0 mg/ml, which was the concentration used for all time dependence experiments. Time points 0, 3, 7, 11, and 15 min were chosen as representative times for time dependent aggregation which we assumed to behave in a classical nucleation and growth manner well represented by an exponential form. 8.0 ml of this sample was then loaded into the sample cup and run for the length of each time point respectively. Each sample was run independently, as all 8.0 ml were required for immediate analysis using the MFI and NTA.

**Volume Dependence**

An initial concentration of 100 mg/ml IVIg was diluted to a concentration of 10.0 mg/ml using filtered 0.2 M glycine buffer at pH 4.2 and ultracentrifuged to remove particles > 100 nm. This sample was then further diluted to 1.0 mg/ml and used for all volume dependence experiments. Volumes of 3.0, 5.0 and 8.0 ml were pipetted into the sample cup and nebulized for 15 minutes on high power. For each volume, the glass plug was lowered to just above the liquid to restrict the impinging jet. In order to obtain enough volume to run the MFI and NTA, the 3.0 and 5.0 ml volumes were required to run twice and the volumes for the second run were added to the first before samples were analyzed.
Analysis Plan

Independent variables in this research are nebulizer geometry, solution concentration, nebulization time and sample volume. Total particle concentrations analyzed by MFI and NTA depend on these independent variables. Statistical analysis was done by two sample, two tailed, paired t-Tests. All data were compared to pre-nebulized controls performed at the beginning of each experiment. In this case, type I errors were controlled by using the Bonferroni correction factor where alpha value ($\alpha$) of 0.05 is divided by the number of variables ($n$) compared to the control (Equation 6). For example, particle concentrations at four solutions concentrations were compared to the control, thus correcting the alpha value ($\alpha_c$) to 0.0125.

Bonferroni Correction Factor

$$\alpha = \frac{0.05}{n} = \alpha_c$$ [6]
CHAPTER THREE: RESULTS

Viscosity

Viscosity for IVIg concentrations between 1 and 10 mg/ml were tested using a model CVO Bohlin Instruments Viscometer. Measurements were taken 12 times for each concentration and averaged. The distribution of viscosity by concentration is shown in Figure (9). From left to right, bars represent 1.0, 5.0, 8.0, and 10.0 mg/ml concentrations of IVIg protein, respectively. Error bars represent the standard deviation of measurements for each concentration. The change in absolute viscosity varies as expected with a linear relationship between the log of concentration and log of viscosity, as seen in Figure (10) [39]. Using a two tailed, paired t-Test, p-values for 5.0, 8.0 and 10.0 mg/ml are 0.005, 1.7E-04 and 7.5E-06 respectively.
**Figure 9.** IVIg absolute viscosity as a function of protein concentration for sample concentrations of 1.0 (blue diamond), 5.0 (red square), 8.0 (green triangle) and 10.0 (blue cross) mg/ml. Error bar represents one standard deviation. Viscosity is significantly different than the solution concentration directly preceding it considering Bonferroni correction factor (*).

**Figure 10.** Log of IVIg absolute viscosity as a function of log of protein concentration for sample concentrations of 1.0 (blue diamond), 5.0 (red square), 8.0 (green triangle) and 10.0 (blue cross) mg/ml.
Density

Density measurements were taken for ultrapure DI water as well as 0.5, 1.0, 5.0, and 10.0 mg/ml concentrations of IVIg by measuring the mass of 5.0 ml of each concentration using an A&D model GR-202 (A&D®, Ablingdon, UK) analytical balance.

The results for each concentration can be seen in Table (1). Dividing the experimentally determined value for the density of water by the known value of 0.9982 g/cm$^3$ from literature [44], this method calculates the density of pure water to within 1.0%. Statistical analysis by two tailed, paired t-Test between each solution concentration and water gives p-values of 0.010, 0.02, 0.07 and 0.09 for 0.5, 1.0, 5.0 and 10.0 mg/ml concentrations respectively.

Table 1. Density as a function of concentration for IVIg protein as determined by a mass divided by volume relationship.

<table>
<thead>
<tr>
<th>IVIg Density by Concentration</th>
<th>0.5</th>
<th>1.0</th>
<th>5.0</th>
<th>10.0</th>
<th>DI H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Volume (ml)</strong></td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td><strong>Mass (g)</strong></td>
<td>4.9878</td>
<td>5.0280</td>
<td>4.9758</td>
<td>4.9683</td>
<td>4.9430</td>
</tr>
<tr>
<td><strong>Density (g/cm$^3$)</strong></td>
<td>0.9976</td>
<td>1.0056</td>
<td>0.9952</td>
<td>0.9937</td>
<td>0.9886</td>
</tr>
<tr>
<td><strong>Standard Deviation</strong></td>
<td>0.0251</td>
<td>0.0444</td>
<td>0.0155</td>
<td>0.0174</td>
<td>0.0151</td>
</tr>
</tbody>
</table>

Surface Tension

Static surface tension measurements were taken for DI water as well as 0.5, 1.0, 5.0, and 10.0 mg/ml concentrations of IVIg protein by determining the capillary action at each concentration and solving for surface tension using experimentally determined density and $\Theta$ of meniscus at 20°C. The experimentally determined value for DI water (0.9886 g/cm$^3$) was used and since it was determined there was no significant difference
in density between concentrations of IVIg, the average of all values (0.9980 g/cm\(^3\)) was used for all concentrations to solve for surface tension. Figure (11) shows the average surface tension for each protein concentration over three trials. Standard deviation was determined by calculating the surface tension for each trial. Statistical comparison between water and solution concentrations by two tailed, paired t-Test gave p-values of 0.04, 0.01, 0.43, and 0.02 for 0.5, 1.0, 5.0 and 10.0 mg/ml respectively.

**Figure 11.** Surface tension as a function of IVIg protein concentration as calculated through experimental capillary action. Bars represent DI water (blue), 0.5 (red), 1.0 (green), 5.0 (purple) and 10.0 (light blue) mg/ml concentrations. Error bars represent one standard deviation. Surface tension is significantly different than DI water considering Bonferroni correction factor (*).

**Formation of Triiodide Ions (I\(_3^−\))**

Measurements of triiodide ions (I\(_3^−\)) were taken using a USB-ISS-UV/VIS (Ocean Optics, Inc., Dunedin, FL) UV-Spectrometer. A solution of IVIg at 1.0 mg/ml and 8.675M KI was prepared as specified in the methods section. Cavitation forms hydroxyl
radicals (OH) through cavity collapse, creating local regions of high temperature and pressure [33]. The hydroxyl radicals then form hydrogen peroxide (H$_2$O$_2$) that can oxidize the KI solution. The resulting formation of potassium hydroxide (KOH) liberates iodide ions (I$^-$) which later form iodine (I$_2$) and finally react with another free I$^-$ to form I$_3^-$.

\[
H_2O \rightarrow H + OH
\]

\[
OH + OH \rightarrow H_2O_2
\]

\[
2KI + H_2O_2 \rightarrow 2KOH + I_2
\]

\[
I_2 + I^- \rightarrow I_3^-
\]

The absorbance spectrum from 300 – 400 nm at time points every three minutes is shown in Figure (12). The bottom line in blue represents the sample before nebulization and every line after is the UV absorbance spectrum at time 3, 6, 9, 12, and 15 minutes of nebulization exposure. Measurements were taken over this range in wavelengths to display a rise in the peak absorbance at 350 nm. By far the largest increase in absorbance is during the first 3 minutes of nebulization. This is most likely due to this being the time frame where the greatest amounts of iodide ions are available for I$_3^-$ formation.
Figure 12. UV-Spectrum of $I_3^-$ absorption as a function of time for samples taken before nebulization 0 min (blue), 3 (red), 6 (green), 9 (purple), 12 (light blue) and 15 (orange) minutes.

It is a well-known practice by pharmaceutical scientists to measure ‘turbidity’ of a protein solution after storage or upon solution perturbation. Turbidity, also known as opalescence, is the term used to describe the cloudiness or haze in solution [40]. When using a UV-Spectrometer, turbidity depends on the sample path length, protein concentration, and size of the scatterer (protein/aggregates/particles). Therefore, an increase in turbidity for the same pathlength and concentration can occur due to an increase in the size of scatterers, or an increase in concentration of scatterers, or both. This makes an increase in turbidity an indication of an increase in protein aggregation in protein solutions.

Figure (13) shows an example of lower concentration KI (2M vs 8.675M from Figure (12)). In this case, there is not enough free iodine ions to form triiodide in great amounts and as a result, there is not a significant peak at 350 nm as compared with all
other wavelengths. In contrast, as apparent in Figure (12), high concentration of KI results in a very defined peak at 350 nm.

![Graph of UV-Spectrum of I$_3^-$ absorption as a function of time for samples taken before nebulization 0 min (blue) & 15 min (red).](image)

**Figure 13.** UV-Spectrum of I$_3^-$ absorption as a function of time for samples taken before nebulization 0 min (blue) & 15 min (red).

Figure (14) shows concentration of I$_3^-$ over time calculated by solving the Beer-Lambert Law at each time point. It is observed that the concentration of I$_3^-$ increases with nebulization, suggesting that radicals are being formed by transient cavitation as a result of nebulization. The plot indicates behavior reaching a maximum, while radical production should be constantly rising with exposure time. However, the supply of KI to form I$_3^-$ is not infinite and this behavior is an outcome of a limited reactant species.
Figure 14. Triiodide ion (I$_3^-$) as a function of nebulization time as determined by UV-Spec for I$_3^-$ absorbance.

**Headspace Dependence**

An 8.0 ml sample of 1.0 mg/ml IVIg was nebulized on high setting for 15 minutes at three different headspace volumes as described in the methods section. Nebulized samples were immediately analyzed using MFI and NTA. Results from the MFI in Figure (15) demonstrate a statistically significant change in the number of aggregated particles within 2 and 40 µm. Aggregate counts decrease as the available head space in the nebulizer is reduced. This eliminates the formation of the atomizing jet, and eventually eliminating any gas-liquid interface. Although the results from the NTA in Figure (16) exhibit higher measurement uncertainty due to low particle concentrations within 1 and 1000 nm, obvious statistical difference in particle concentration between runs with a gas-liquid interface and those without is maintained. Statistical comparison of MFI data between the pre-nebulized and nebulized samples by two tailed, paired t-Test
gave p-values of 0.03, 0.001, and 9.5E-4 for No-Space, Small-Space and Large-Space nebulizer geometries respectively. Statistical comparison of NTA data between the pre-nebulized and nebulized samples by two tailed, paired t-Test gave p-values of 0.90, 0.01, and 0.007 for No-Space, Small-Space and Large-Space nebulizer geometries respectively. The reader is cautioned in jumping to the conclusion that aggregation is eliminated when headspace is eliminated. When data is observed at a finer scale, differences are seen even with zero liquid-gas interfaces. Except when specified, all results presented for the remainder of this work will be for nebulizer geometries with no head space, thus isolating cavitation as the primary means of protein aggregation.

Figure 15. Concentration of IVIg particles as a function of head space in the nebulizer as calculated using MFI. Bars represent samples analyzed before nebulization pre-Neb (blue), and after 15 minutes of nebulization for nebulizer set up with No-Space (red), Small-Space (green) and Big-Space (purple). Error bars represent one standard deviation. Particle concentration is significantly different from pre nebulized control considering Bonferroni correction factor (*).
Figure 16. Concentration of IVIg particles as a function of head space in the nebulizer as calculated using NTA. Bars represent total concentration of samples analyzed before nebulization pre-Neb (blue), and after 15 minutes of nebulization for nebulizer set up with No-Space (red), Small-Space (green) and Big-Space (purple). Error bars represent one standard deviation. Particle concentration is significantly different from pre-nebulized control considering Bonferroni correction factor (*).

Time Dependence

An 8.0 ml sample of 1.0 mg/ml IVIg was nebulized for time points 3, 7, 11, and 15 minutes. Each time point represents its own individual run, as all 8.0 ml was required for analysis using MFI and NTA. Particle concentrations within 2 and 40 µm increase as nebulization time increases for samples of the same protein concentration when analyzed with MFI, as seen in Figure (17). The concentration of aggregates rises from approximately 1000 to 7000 particles per ml, representing a 7-fold increase. However, it is difficult to draw any conclusions from the NanoSight (1 to 1000nm particle size range) because particle concentrations are below the minimum levels recommended by the manufacture for reliable results, and as such, large standard deviations are observed;
Figure (18). NanoSight tracking analysis (NTA) requires particle concentrations of $10^7 - 10^9$ particles/ml in order to be accurate. Statistical comparison of MFI data between the pre-nebulized and nebulized samples by two tailed, paired t-Test gave p-values of 0.005, 0.001, 0.001 and 0.001 for 3, 7, 11, and 15 minute time points respectively. Statistical comparison of NTA data between the pre-nebulized and nebulized samples by two tailed, paired t-Test gave p-values of 0.05, 0.001, 0.002 and 0.001 for 3, 7, 11, and 15 minute time points respectively.

It is also important to note that each figure representing a different experimental set-up also represents a completely different sample prep. In other words, every figure represents a different ‘lot’ of IVIg that was separately ultracentrifuged, diluted to concentration and stored. Therefore, trends in data, rather than absolute particle concentrations, should be considered when comparing figures to each other.

![Total Particle Concentration within 2 & 40μm using MFI](image)

**Figure 17.** Particle concentration as a function of nebulization time using MFI. Bars represent total particle concentration of samples analyzed before nebulization pre-neb (blue) and nebulized for 3 (red), 7 (green), 11(purple) and 15 (light blue) minutes.
respectively. Particle concentration is significantly different from pre-nebulized control considering Bonferroni correction factor (*).

Figure 18. Particle concentration as a function of nebulization time using NTA. Bars represent total particle concentration of samples analyzed before nebulization pre-neb (blue) and nebulized for 3 (red), 7 (green), 11 (purple) and 15 (light blue) minutes respectively. Error bars represent one standard deviation. Particle concentration is significantly different from pre-nebulized control considering Bonferroni correction factor (*).

Figure (19) shows results from experiments where filtered buffer was nebulized for 15 minutes, followed by IVIg addition to a solution concentration of 1.0 mg/ml. This solution was then allowed to sit in order to compare the rate of aggregation over time for protein that is not exposed to cavitation. These data are compared to particle concentrations as a result protein that is nebulized for 15 minutes. This graph indicates that no significant increase in aggregate formation is taking place over a four hour period due to nucleation alone, indicating that increased particle counts after 15 minutes of nebulization are in fact a product of cavitation.
Figure 19. Particle concentration as a function of time using MFI. Lines represent 1.0 mg/ml IVIg nebulized for time points between 3 & 15 minutes (blue), and IVIg added to buffer that has been nebulized for 15 minutes (red).

**Volume Dependence**

1.0 mg/ml IVIg was nebulized in 3.0, 5.0 and 8.0 ml volumes. All samples were nebulized for 15 minutes. Based on traditional cavitation theory, more cavitation events are expected as the energy per unit mass is increased. Energy per unit mass can be explored using a constant protein concentration solution with variable sample volumes. In the 2 – 40 µm particle size range as measured by MFI, particle concentrations generated as a result of 15 minutes of continuous nebulization increase as the sample volume was decreased from 8.0 to 5.0 ml, Figure (20). When the sample volume was decreased to 5.0 ml from 8.0 ml, representing a 37.5% change in the energy per unit volume or mass, the particle concentration increased by 30%. This was not the case when the volume was dropped from 5.0 ml to 3.0 ml (representing 40% in volume), where a 49% decrease is observed. Particle concentrations at the nm scale as analyzed by NTA exhibited ‘clean’
or no increase in particle concentration for all conditions with no statistically significant trend in the data; Figure (21). Statistical comparison of MFI data between the pre-nebulized and nebulized samples by two tailed, paired t-Test gave p-values of 0.01, 0.004, and 0.009 for 3.0, 5.0, and 8.0 ml volumes respectively. Statistical comparison of NTA data between the pre-nebulized and nebulized samples by two tailed, paired t-Test gave p-values of 0.73, 0.12, and 0.26 for 3.0, 5.0, and 8.0 ml volumes respectively.

**Figure 20.** Particle concentration as a function of sample volume using MFI. Bars represent total particle concentration for samples analyzed before nebulization pre-neb (blue), and nebulized for 15 minutes at a volumes of 3.0 (red), 5.0 (green), and 8.0 (purple) ml respectively. Error bars represent one standard deviation. Particle concentration is significantly different from pre-nebulized control considering Bonferroni correction factor (*).
**Figure 21.** Particle concentration as a function of sample volume using MFI. Bars represent total particle concentration for samples analyzed before nebulization pre-neb (blue), and nebulized for 15 minutes at a volumes of 3.0 (red), 5.0 (green), and 8.0 (purple) ml respectively. Error bars represent one standard deviation.

### Concentration Dependence

Concentration of IVIg was increased from 0.5 to 10.0 mg/ml and nebulized for 15 minutes. Figure (22) shows results of particle concentrations in the 2 – 40 µm size range as measured by MFI during 15 minute continuous nebulization runs as a function of concentration. The concentration of particles increases approximately monotonically with increasing concentration from 0.5 to 5.0 mg/ml. As protein solution concentration continues to rise above 5.0 mg/ml, the concentration of aggregated protein particles observed appears to decreases although not statistically significant. Particle generation as analyzed by NTA had large error within the sample due to low particle concentration. Statistical comparison of MFI data between the pre-nebulized and nebulized samples by two tailed, paired t-Test gave p-values of 0.14, 0.013, 0.008 and 0.007 for 0.5, 1.0, 5.0 and 10.0 mg/ml concentrations respectively. Statistical comparison of NTA data between
the pre-nebulized and nebulized samples by two tailed, paired t-Test gave p-values of 0.37, 0.04, 0.23 and 0.81 for 0.5, 1.0, 5.0, and 10.0 mg/ml concentrations respectively.

Figure 22. Particle concentration as a function of sample concentration using MFI. Bars represent total particle concentration for samples analyzed before nebulization pre-Neb (blue), as well as nebulized for 15 minutes at concentrations 0.5 (red), 1.0 (green), 5.0 (purple) and 10.0 (light blue) mg/ml respectively. Error bars represent one standard deviation. Particle concentration is significantly different from pre-nebulized control considering Bonferroni correction factor (*).
Figure 23. Particle concentration as a function of sample concentration using NTA. Bars represent total particle concentration for samples analyzed before nebulization buffer (orange) and pre-Neb (blue), as well as nebulized for 15 minutes at concentrations 0.5 (red), 1.0 (green), 5.0 (purple) and 10.0 (light blue) mg/ml respectively. Error bars represent one standard deviation.

The results are not the same when a gas-liquid interface is introduced into the systems; Figure (24) and Figure (25). Small-Space nebulizer geometry was set up as described in the methods section and concentrations of 0.5, 1.0, 5.0 and 10.0 mg/ml IVIg protein was prepared and nebulized for 15 minutes. Unlike the no headspace results, the concentration of aggregated protein measured after processing rises inversely to protein solution concentration in the 2 to 40 μm range as measured by MFI. Moreover the final concentrations represent another order of magnitude increase over the previous results. The concentration of submicron protein aggregates also falls as the solution protein concentration increases. The change is nearly two orders of magnitude, and is at a level in 100s of million per ml; a massive difference from any measurement so far. Statistical comparison of MFI data between the pre-nebulized and nebulized samples by two tailed,
paired t-Test gave p-values of 1.8E-4, 0.49E-4, 0.032 and 0.29 for 0.5, 1.0, 5.0 and 10.0 mg/ml concentrations respectively. Statistical comparison of NTA data between the pre-nebulized and nebulized samples by two tailed, paired t-Test gave p-values of 0.003, 2.3E-4, 0.03 and 0.54 for 0.5, 1.0, 5.0, and 10.0 mg/ml concentrations respectively.

**Figure 24.** Particle concentration after 15 minutes of nebulization as a function of IVIg solution concentration with a small head space. Bars represent pre-nebulized samples of buffer (orange), 1.0 mg/ml IVIg (blue), as well as 10.0 (red), 5.0 (green), 1.0 (purple) and 0.5 (light blue) mg/ml IVIg protein concentration respectively. Error bars represent one standard deviation. Particle concentration is significantly different from pre-nebulized control considering Bonferroni correction factor (*).
Figure 25. Particle Concentration after 15 minutes of nebulization as a function of IVIg solution concentration with a small head space as analysed using NTA. Bars represent IVIg protein concentrations before nebulization pre-Neb (light blue) as well as at 0.5 (blue), 1.0 (red), 5.0 (green) and 10.0 (purple) mg/ml concentrations respectively. Error bars represent one standard deviation. Particle concentration is significantly different from pre-nebulized control considering Bonferroni correction factor (*).
CHAPTER FOUR: DISCUSSION

Gas - Liquid Interface

Proteins are amphiphilic, meaning a single molecule contains both hydrophobic and hydrophilic regions. At liquid–gas interfaces, the proteins will rearrange to expose the hydrophobic regions to the gas phase and hydrophilic regions to the aqueous phase. It is well documented that unstable therapeutic proteins may require only minor energy input to irreversibly alter protein conformations resulting in aggregation [1-5]. Experiments were conducted to verify how to effectively and completely eliminate the contribution of a gas-liquid interface degradation pathway, so the current study was isolating aggregation via cavitation (stable or transient). Figures (15) and (16) demonstrate the number of aggregated particles decrease by approximately an order of magnitude at the micron as well as sub-micron range as the available head space in the nebulizer is reduced. This eliminates the formation of the atomizing jet and eventually eliminates any gas–liquid interface. The concentration of aggregates increases significantly when a gas liquid interface is allowed.

The presence of a gas-liquid interface not only changes the total concentration of protein aggregation, but also changes the influence of solution properties on aggregate formation, especially at the higher concentrations and in the submicron range. A more complete discussion of these results is presented below.
It is interesting to note that the regular operations of nebulizers involve a great amount of gas–liquid interface by virtue of atomization itself. Even in cases where the gas–liquid interface was allowed during this experiment, the available headspace was nowhere near what is involved in drug delivery. Figure (26) shows a side by side comparison of an optional set up for drug delivery (A) and the experimental set up where a gas-liquid interface is allowed (B). Considering the great amount of particle generation, and therefore protein aggregation measured in this experiment, even a cautious investigator would conclude that higher concentrations of protein aggregation would occur after normal nebulization delivery. We acknowledge that the lung is full of macrophage which could likely clean the majority of these problem particles prior to absorption into the blood stream, but these results suggest that a immunogenicity study on lung cells should be conducted to understand the threats of nebulized proteins.

Figure 26. A: Nebulizer set-up with nozzle for drug delivery. Gas interface exists above the liquid volume and through the nebulizer nozzle to the patient. B: Nebulizer set-up
during small-space headspace dependance experiment. Gas interface is impinged by cap and exists only in a 1 cm high, 2.5 cm diameter volume.

**Energy per Mass**

The rate and intensity of cavitation is a function of the amount of energy put into the acoustic wave, frequency of excitation, vapor pressure of the fluid, and fluid density. In addition to altering the surface tension and viscosity, methods for reducing the number of variables influencing the outcomes needed to be addressed. One could envision a method of equalizing the energy per mass placed into each sample regardless of solution density simply by making small adjustments of the sample volume. However, in nebulizers, the acoustic waves are focused into the center of the sample reservoir using a liquid horn. Additionally, the complexity of wave intensity is a function of reflection off all surfaces, especially the gas-liquid interface. This complicates the ability to understand how altering sample volume will impact the average energy per unit mass in the sample volume. Particle generation as a function of sample volume for a single solution density was measured to identify a range where the expected decrease in sample volume resulted in a proportional increase in particle formation. The normal operating sample volume of 8.0 ml could be safely decreased to 5.0 ml, causing increased particle generation rate or increasing cavitation as can be seen in Figure (20). When the sample volume reduced below 5.0 ml, the ability to predict the effect of decreasing volume as increasing energy density is compromised.

Possible reasons for this could be due to a decreased volume beyond the MABISMist™ II Ultrasonic Nebulizer’s suggested operating volume. Since the acoustic
waves are focused in the center of the sample volume, a parameter that cannot be adjusted, altering the sample volume beyond 5.0 ml could lead to unexpected variability with respect to the acoustic energy field’s focus and reflection off surfaces. Thus, concentration of aggregates for volumes below 5.0 ml may not be a result of the full energy input into the system.

**Increased Aggregation over Time**

Concentration of triiodide as a result of nebulization over time was determined by solving the Beer-Lambert Law for UV-Spec absorbance of IVIg protein in the presence of KI as seen in Figure (14). The concentration increases with time as more cavitation events occur, releasing more radicals into solution to carry out the necessary triiodide reaction. An exception to this was recorded between 12 and 15 minutes of nebulization where the curve seems to plateau or only increase slightly as compared to other time periods. One potential reason for this plateau in concentration is because the available iodide in solution is likely exhausted. The comparably large increase between 0 and 3 minutes of nebulization adds to the validity of this theory since the greatest abundance of free iodide exists during this time.

Figure (17) plots the concentration of particles measured using the MFI as a function of nebulization time. This plot also shows an increase in particle concentration generated over time. By plotting the results of Figure (17) alongside the results of Figure (14), it can be seen that the increase in $I_3^-$ concentration exhibits the same trend as the particle concentration with the exception that particle concentration continues to increase in time even after $I_3^-$ concentration plateaus, Figure (27).
Figure 27. Triiodide ion concentration (blue) and total particle concentration (red) as a function of nebulization time.

This relationship is interesting because the correlation in behavior suggests that IVIg aggregation during nebulization is in fact a product of energy delivered via cavitation within the sample reservoir. The divergence of the two plots after 12 minutes as $I_3^-$ concentration plateaus and particle concentration continues to increase is also expected since particles will continue to aggregate in the presence of existing aggregates while $I_3^-$ can only form as long as there are free iodide available for triiodine formation.

Coupling the correlation between triiodide formation and aggregate concentration as a function of time with the observation that triiodide concentration does not rise in nebulized buffer solutions and that IVIg appears to scavenge radicals necessary to form triiodides from KI, there is strong support for the hypothesis that IVIg acts as a nucleation
site for cavitation under acoustic forcing similar to plasmid DNA. If this is the case, as the concentration rises the number of cavitation sites will rise and higher aggregate production rates will be expected.

This hypothesis is further validated by the observation that higher particle concentrations are reported for experiments with a higher baseline particle concentration in the pre nebulized control. Figure (17) begins with a pre nebulized particle concentration of about 1000 particles/ml, and reports a maximum particle concentration around 6400 particles/ml. Similarly, Figures (20 & 22) begin with a pre nebulized particle concentration around 500 particles/ml, and achieve a maximum particle concentrations of 3800 and 3600 particles/ml respectively. These data can be explained by variability in protein sample preparation and storage. ‘Dirty’ samples yielding higher particle concentrations after nebulization is consistent with hypothesis that proteins can act as a nucleation sight for cavitation.

**Influence of Concentration**

As protein bulk solution concentration is increased from 1.0 to 5.0 mg/ml, there is a clear increase in protein aggregate concentration after 15 minutes of nebulization. This is consistent with the hypothesis that IVIg is acting as a nucleation site for cavitation and aggregating as a result of the process of cavitation. It is important to recognize that under these conditions viscosity changes from 3.3 to 4.5 mPa*s, thus could also contribute to the observed trend. There is no significant difference in experimental values for density and surface tension, while protein concentration is altered by one order of magnitude. It is likely that the change in solution properties (viscosity, surface tension and density) are
less than or equal to the measurement error, leaving only solution concentration as the parameter dominating the observed aggregation behavior.

The exception is the change in trend observed at concentrations higher than 5 mg/ml. Increasing solution protein concentration from 5.0 to 10.0 mg/ml, a one-fold increase, results in a statistically insignificant decrease in aggregate concentration. The change in the solution properties is much different in this range. Surface tension and density are unchanged within experimental uncertainty, while viscosity is almost three times larger. In this case viscosity has damped out the expect rise in aggregate production.

Probabilistic analysis of computational models of hydrodynamic cavitation resulting from a constant driving energy (simulation conducted by Donn Sederstrom for a related project) show that the probability of cavitation decreases, as measured by the magnitude of the lowest pressure region, as viscosity values increase. In fact, during investigations of our exact density and viscosity ranges for the same driving energy, this work demonstrated no cavitation is expected for solution concentration above 25 mg/ml. Physically, for a given system input energy, the frictional losses due to viscous effects consume flow energy that would have otherwise translated into lower pressure regions. In other words, the energy of the acoustic wave is more rapidly diffused over the bulk volume and consumed by frictional or viscous heating, rather than maintaining large pressure fluctuations that generate low pressure regions suitable for cavitation. It is important to note that in these studies, the concentration of particles between 1 & 1000 nm as measured by NTA was unchanged from the control solutions, suggesting that the
nucleation size is above 1000nm. From nucleation and growth theory, one would only anticipate the absence of small particles if the growth rate was high due to an abundance of material in close proximity. The almost singularity nature of a cavitation event is consistent with the above description.

When a gas interface is allowed, measured particle counts increased by 10 fold when analysed at the 2 – 40 µm size using MFI as compared with data from Figure (21). In contrast to aggregation by cavitation alone, micron and submicron aggregates are inversely related to solution protein concentration. The first important observation is that submicron aggregation is occurring, unlike under cavitation dominate conditions. The smaller nucleation size could be a result of a more super saturate process, or because the process is spread out over a larger area and particle growth is limited by diffusion time over a long distance to the nucleation site. The second observation is that the number of submicron particles decreases as a function of solution concentration. Mass diffusivity, by the Stokes-Einstein relationship, is inversely proportional to viscosity [45]. Thus as viscosity increases, the diffusion time over the same distance becomes larger. If the concentration remained the same, a rise in viscosity would lead to more submicron particles as a result of diffusion. However, viscosity is only changing by 38% from 1.0 to 5mg/ml whereas concentration is rising by 500%. In a system dominated by concentration or number of available particles, the growth rate of particles would be expected with increases in the lowest size particle measured. This assumes that the number of protein molecules at the interface is continually rising and does not saturate. This would be true if (1) IVIg was a surface active agent and surface tension continued to
fall with concentration or (2) IVIg was not a surface active agent and the surface tension remained constant as a function of concentration. The latter is observed experimentally. Thus the observed trend would be consistent for an interface driven phenomena. The third observation is that when a gas interface exists, the elimination of cavitation at high viscosity does not drown out the interface driven aggregation process.

**Mitigation strategies**

Based off the data collected in this study, aggregation by cavitation can be mitigated by extremely high concentrations or raising solution viscosities. Extrapolation of the concentration data suggests that solutions above 25 mg/ml would result in aggregate concentrations statistically similar to unprocessed solutions.

Aggregation, primarily driven by a gas-liquid interface, would also be mitigated by very high solution concentrations. However, if the immunogenicity of a protein therapeutic is dominated by the submicron particles, then solution concentrations above 5.0 mg/ml would be suitable. Alternate techniques involving formulations containing surface active agents that preferentially populate the surface could potentially be better.
CHAPTER FIVE: CONCLUSION

The objective of this work was to assess the impact of ultrasonic nebulization on IVIg aggregation. It was determined that the gas-liquid interface can be a strong driving mechanism in IVIg aggregation at the micron and submicron range. IVIg was also identified as a nucleation site for transient cavitation. Transient cavitation aggregates IVIg molecules in the micrometer size range, not at the submicron range. Finally, transient cavitation can be mitigated by utilizing larger IVIg concentrations. Based on the results from this research, we hypothesize concentrations above 25 mg/ml would eliminate particle formation as a result of transient cavitation. These viscosities would also increase the time it takes for IVIg to diffuse to the gas-liquid interface and mitigate aggregation via that mechanism.

Future studies should focus on creating a larger data set with respect to variables such as fluid properties, concentration, volume, portentous compound, and source of cavitation. First, density and surface tension should be reevaluated to obtain statistically significant values between protein concentrations. This would help to refine and reevaluate theories on cavitation induced protein aggregation. Second, a larger range of concentrations should be tested to evaluate if trends in protein aggregate formation from the current research are continued. These concentrations should be representative of those that would be typical for process, shipping, and delivery. Third, volume dependence should be reanalyzed in an attempt to better understand to what extent the
energy per mass relationship plays in cavitation induced protein aggregation. In this case, it might be necessary to find a cavitation source with fewer variables to control than ultrasonic nebulization. Next, many other proteins should be studied for possible immunogenic effects due to cavitation. Protein aggregation due to cavitation using IVIg would suggest this process could take place with other therapeutic proteins. Due to known immunogenic responses to submicron protein aggregation, it would be worthwhile to conduct cavitation mitigation studies on all protein therapeutics before patient administration. Finally, other sources of cavitation should be investigated in order to better mitigate cavitation during bioprocessing. All sources of cavitation should be identified from formulation to delivery in order to avoid adverse effects upon drug treatment.
REFERENCES


