

Protein Aggregation during Ultrasonic Nebulization

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Introduction

Protein therapeutics are a valuable clinical tool in the fight against diseases and conditions such as cancer, diabetes, hemophilia and myocardial infarction [2]. The number of commercially available protein-based products in the US has gone from three in the late 1980s; to nearly 150 today [1]. Physical degradation of proteins has become a subject of considerable interest within the pharmaceutical industry as the number of therapeutic proteins 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. Because protein conformation stability is based largely on relatively weak molecular at the tertiary level, these molecules can be susceptible to several degradation pathways [1].

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 [1]. 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.

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 proteins native state relies on many factors such as amino acid sequence, pH, temperature and concentration [5]. In high concentration, there is a kinetic 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 [4-6]. Aggregation often happens in bioprocessing during cell culture, purification, formulation and fill finish operations [6]. For this reason 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 [8]. For these reasons it is necessary for pharmaceutical researchers to understand the cause and mechanism of therapeutic protein aggregation.

Cavitation is the formation and collapse of cavities in incompressible liquids. In 1917, the father of cavitation, 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 [16]. 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 and mixing as well as post filling processes such as 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 exist, it is well understood that all of them must involve thermodynamic energy needed to drive proteins from their native state [2,5]. Many potentially damaging conditions such as air-water interface, turbulent vortexing, freeze-thaw, extreme temperature and chemical degradation have been heavily studied by pharmaceutical researchers. 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 is the formation, growth and implosive collapse of bubbles in liquids irradiated with high-intensity ultrasound [16]. Two types of acoustic cavitation exist: stable and transient. Stable cavities grow 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. These bubbles can expand to several orders of magnitude larger than their original size before imploding. Suslick, et al. was able to show that the collapse of bubbles in a multi bubble cavitation field produces hot spots with effective temperatures of ca. 5000K, pressures of ca. 1000atm, 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, viscosity and concentration, methods of mitigation for protein degradation by cavitation will be identified.

An ultrasonic nebulizer is a device that uses piezoelectric effect to generate high-frequency acoustic energy, which forms aerosol droplets by cavitation [17]. 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 [16]. 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 [15]. 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.

Due to known issues of protein immunogenicity in aggregated protein formulations, it is important to characterize size, size distribution and aggregate concentration [8]. This process is not trivial as protein aggregates are typically very heterogeneous, with sizes ranging from a few nanometers to several micro meters. Small aggregates in the micron range have recently been implicated as the cause of immunogenicity, therefore it is important to characterize particles at a very small scale.

In the following experiments, intravenous immunoglobulin (IVIg) will be used as a model proteinaceous compound. IVIg has been used in the treatment of primary and secondary antibody deficiencies, more specifically autoimmune and inflammatory diseases, for over 25 years. It consists of normal IgG obtained from pools of plasma of several thousand healthy blood donors [12]. 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.

This paper will characterize protein aggregation by means of ultrasonic nebulization to determine the impact of viscosity, density, gas contact area, impurities and exposure time on protein aggregation. The identification of driving mechanisms will enable the development of mitigation strategies for a variety of atomization systems.

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 [12]. 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. Formation of triiodide ions (I_3^-) 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 Device, 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 (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. This 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 centrifuged 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 NanoSight. 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 being used for an experiment. 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. To do this, a rough estimation was calculated using the Stokes Law in order to determine the settling velocity of small particles within the solution. This estimation is 'rough' in that assumptions must be made regarding the shape and density of the particles being assessed. Thus, it was determined that the settling velocity for 100 nm particles is ~4.92 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. The centrifuge was vacuum sealed and kept at 5°C for the duration of centrifugation.

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 and uses the piezoelectric effect to generate high-frequency acoustic energy which generates aerosol droplets by cavitation [13]. The particles created by this nebulizer are in the range of 3-9 µm as measured by Phase Doppler Particle Analyser [14], however for 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 internal DI water was also changed out between runs due to a temperature increase after use.

Viscometer

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 ml samples of each concentration were pipetted into the cup and run for each analysis. All concentrations were run 12 times and an average viscosity was calculated using an average of all the runs.

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 [15], it is known that triiodide

ion is formed by the oxidation of KI. In order to test for this phenomenon in the presence of IVIg protein, 1.0 ml of 200 mM KI was added to 9.0 ml of both 1.0 mg/ml and 10.0 mg/ml concentrations. 8.0 ml samples were then placed into the sample cup and nebulized on high for 15 min. The nebulizer was stopped at briefly at 3, 6, 9, 12, and 15 minutes in order to remove a sample volume of 200 μ l 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. During triiodide analysis, purified glycine buffer and IVIg protein were used as the blank so only the iodine spectrum would show.

As expected, triiodide ions could not be detected for either concentration of IVIg. However, it was hypothesized that IVIg itself was a scavenger of free iodine ions, and therefore an abundance of KI was necessary in order to form detectable concentrations of triiodide. To overcome this problem, 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.

MFI

Measurements of particles 2 – 40 μ m 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 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 unnebulized, filtered glycine buffer. Counts from the controls are reported, but not subtracted from the final particle counts for all other samples.

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 sat 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 was created. The internal volume was thus exposed to the smallest amount of gas/liquid interface and agitation within the sample appeared to be negligible.

Small-Space: An 8 ml sample of 1.0 mg/ml IVIg was placed into the sample cup and the nebulizer was set up so that there was a 1 cm space between the internal reservoir volume and a lid for a 50 ml conical tube that fit snugly above the sample cup. The glass plug was placed on top of the lid for weight so that the jet did not displace the lid. Vapor particles were thus allowed to form within the headspace volume and recollect inside the internal reservoir.

Large-Space: An 8 ml sample of 1.0 mg/ml IVIg was placed into the sample cup and the nebulizer was set up with 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 experimental setup allowed for the greatest amount of gas/liquid interface.

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, 5.0 and 10.0 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 for particle concentration at the size range.

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. A 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 for time dependent aggregation experiments. 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, due to sample volumes required for immediate analysis using the MFI.

Volume 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. This sample was then further diluted to 1.0 mg/ml and used for all volume dependence experiments. Volumes of 3, 5 and 8 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, the 3 and 5 ml volumes were required to run twice. Volumes for the second run were added to the first before samples were analyzed.

Results and Discussion

Viscosity for IVIg concentrations between 1 & 10 mg/ml were tested using a model CVO Bohlin Instruments Viscometer. Measurements were taken 12 times for each concentration then averaged. The distribution of viscosity by concentration is shown in Figure 1. Error bars represent the standard deviation of measurements for each concentration. The change in absolute viscosity varies as expected as a linear relationship between the log of concentration and log of viscosity.

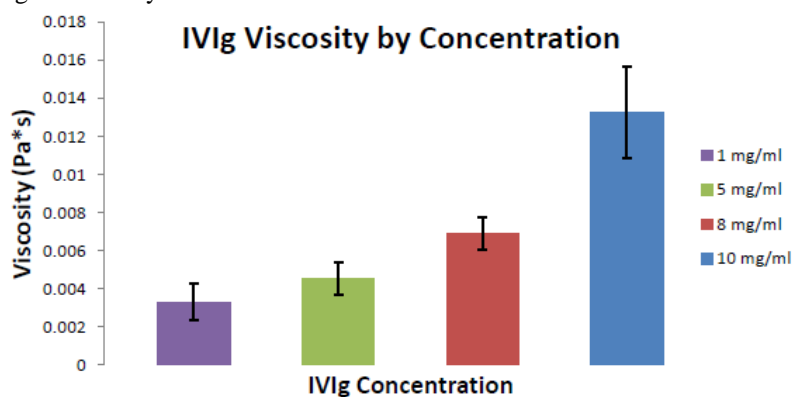


Figure 1. IVIg absolute viscosity as a function of protein concentration. Error bar represents plus and minus a single standard deviation

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 understood that minor energy input in to protein solutions with a gas-liquid interface can permanently alter the protein conformation resulting in aggregation [1-3]. Great care was taken in the current study to ensure the results of aggregation were solely a result of cavitation and was not complicated by the presents of a gas-liquid interface. Figure 2 demonstrates that the number of aggregated particles 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. It can be seen that the concentration of aggregates increase significantly when the any atomizing jet formation is allowed. The results presented for the remainder of this work will be for nebulizer geometries with no head space.

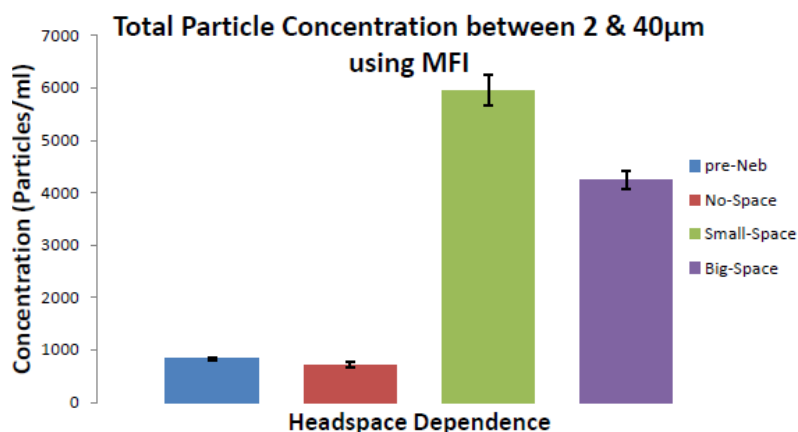


Figure 2. Concentration of IVIg particles as a function of head space in the nebulizer. Error bar represents plus and minus a single standard deviation

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 was used for all of the measurements. Concentration of I_3^- was determined using the Beer-Lambert Law. Figure 3 shows concentration of triiodide over time, calculated by solving the aforementioned equation at each time point. It is observed that the concentration of triiodide increases with nebulization, suggesting that radicals are being formed by transient cavitation as a result of nebulization. The concentration should increase with time as more cavitation events occur releasing more radicals into solution to carry out the necessary triiodide reaction. The reason the concentration plateaus is because the available iodine in solution is likely exhausted. Figure 2 also plots the number of particles measured using the MFI as a function of nebulization time. The increase in triiodides exhibits the same trend as the particle concentration with the exception particle concentration continues to increase in time even after the triiodide concentration plateaus.

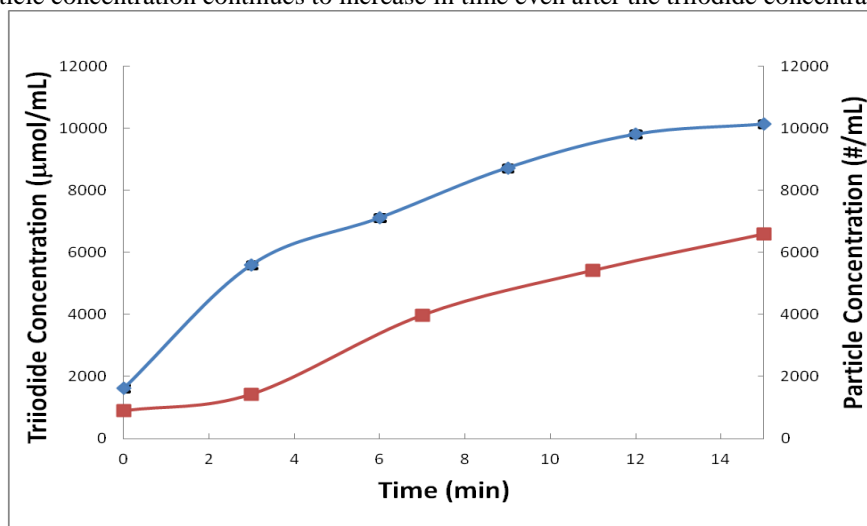


Figure 3. Triiodide ion concentration (blue) and protein aggregate concentration between 2 and 4 μm as a function of nebulization time.

The rate and intensity of cavitation is a function of the amount of energy put in to the acoustic wave, frequency of excitation, vapour pressure of the fluid and fluid density. One could envision a method of equalizing the energy per mass placed into each sample by making small adjustments of the sample volume. However, as the acoustic waves are focused into the center of sample reservoir, the complexity of wave focusing and reflection off all surfaces complicates one's ability to understand how to alter sample volume to maintain the energy per mass. Particle generation as a function of sample volume was measured to identify a range where decreasing volume and increasing particle generation for a single solution concentration could be observed. The normal operating sample volume was 8 ml and the volume could be safely decreased to 5ml causing increase particle generation rate or increasing cavitation, Figure 4. Density was measured as a function of IVIg concentration from 0.5 to 10 mg/ml; only 2% change was observed (data not shown).

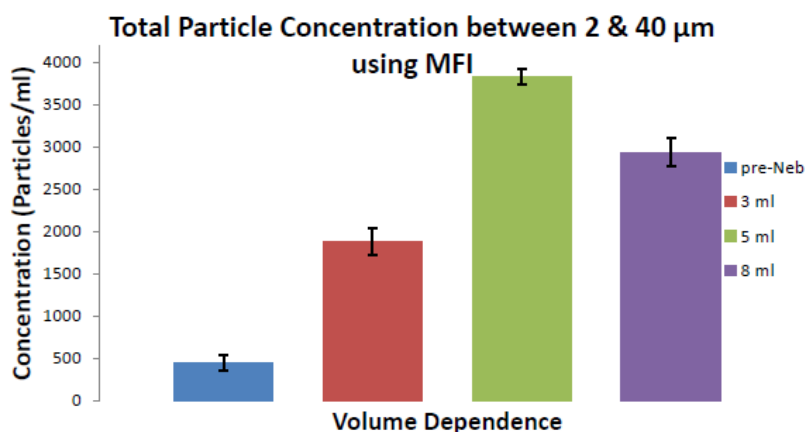


Figure 4. Particle concentration as a function of sample volume. Error bar represents plus and minus a single standard deviation

Previous work investigating plasmid DNAs degradation via transient cavitation discovered that the DNA molecule acted as a nucleation site [15]. The current work also explored this concept by increasing the concentration of IVIg in solution during nebulization, resulting in increased particle counts. This strongly suggests that IVIg protein molecules, like plasmid DNA, act as nucleation sites for cavitation, Figure 5. Notice that the concentration of particles after 15 minutes of nebulization increases approximately monotonically with increasing concentration from 0.5 to 5 mg/ml. These concentrations represent only a small change in fluid viscosity. However, the concentration of particles generated decreases slightly as concentration increases from 5 to 10 mg/ml. This is expected as there is a statistically significant increase in absolute viscosity from one concentration to the other. Probabilistic analysis of computational models of hydrodynamic cavitation show that cavitation ceases at these higher viscosity values for a given pressure variation. It is likely that in these high viscosity solutions the entire sample no longer is exposed to sufficiently high pressure variations to generate transient cavitation. It is important to note that in these studies the concentration of particles between 10 and 1000 nm as measured by the Nanosight (NanoSight, Amesbury, United Kingdom) was unchanged from the control solutions (data not shown). The concentrated nature of a cavitation implosion results in denaturation of all native state IVIg protein within a small volume, leading to larger particle sizes but lower concentrations due to the small volumes involved.

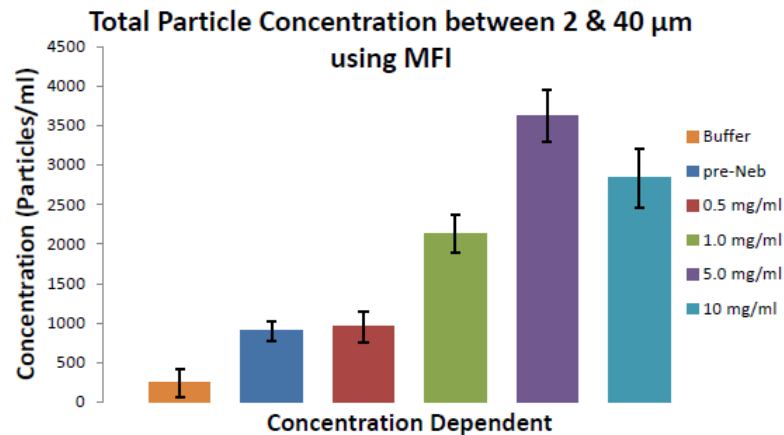


Figure 5. Particle concentration after 15 minutes of nebulization as a function of IVIg solution concentration with no head space thus a function of cavitation only. Error bar represents plus and minus a single standard deviation

Particle concentration generated in nebulization runs where the gas-liquid interface was allowed to exist, Figure 6, shows the opposite trend with solution concentration. The large drop in particle generation from 0.5 to 1.0 mg/ml is likely a result of increase diffusion time to the gas-liquid interface as a result of increased fluid viscosity as well as a surface saturated with IVIg. The exact same results are observed in the nanometer particle size range, but at higher particle concentrations (data not shown). Because the proteins involved in the aggregation process are spread out over a large surface area they nucleate to smaller particle sizes. Also the larger areas involved lead to larger particle counts.

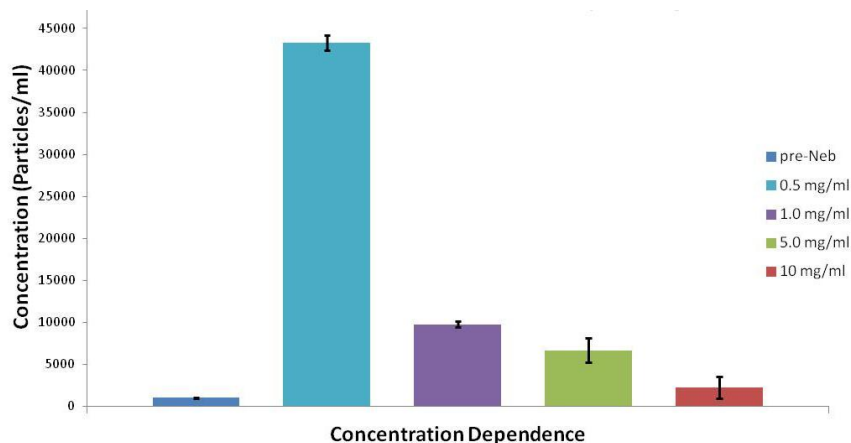


Figure 6. Particle concentration after 15 minutes of nebulization as a function of IVIg solution concentration with a small head space. Error bar represents plus and minus a single standard deviation

Summary and Conclusions

The objective of this work was to assess the impact of ultrasonic nebulization on IVIg aggregation. It was determined that the gas-liquid interface area strongly drives IVIg aggregation in the micron and nanometer particle size range. IVIg was also identified as a nucleation site for transient cavitation. Transient cavitation leads to the aggregation of IVIg molecules in the micrometer size range. Finally, transient cavitation can be mitigated by utilizing larger IVIg concentrations. We hypothesize that concentrations above 20 to 50 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 hydrophobic-hydrophilic surface interactions.

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