ROLE OF DOMAIN UNFOLDING AND SILICONE OIL-WATER INTERFACE IN
AGGREGATION OF A MONOCLONAL ANTIBODY

by

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ABSTRACT

Monoclonal antibodies (mAbs) are an important class of therapeutic protein drugs that have been developed by pharmaceutical companies to treat major diseases. However, aggregation of mAbs is of major concern for pharmaceutical industry because it can affect product efficacy and can lead to immune response in patients. Reported incidences of immune response in patients range from 3-100%. Monoclonal antibodies fall under the category of multi-domain proteins where each domain contributes to the overall stability of the protein. In this work we found that partial unfolding of a monoclonal antibody resulted in protein aggregation. Partially unfolded protein species were formed when protein was incubated in sub-denaturing concentrations of Gdn.HCl and they had perturbed tertiary structure but native-like secondary structure. Further, partially unfolded protein species had unfolded C\textsubscript{H}2 domains.

In this work, we also investigated the tendency of a monoclonal antibody to form an interfacial gel upon adsorption to the silicone oil-water interface. Moreover, we also found that rupture of the interfacial gel can result in aggregation and particle formation in the bulk solution. Inclusion of surfactants in protein formulation can presumably inhibit interfacial gel formation and this resulted in reduced aggregation due to interfacial gel rupture. Lastly, we demonstrated that particles formed from interfacial gel rupture have a
net neutral zeta potential making them colloidally unstable. Thus, over time, particles can agglomerate when incubated quiescently to form more particles.

The form and content of this abstract are approved. I recommend its publication.

Approved: John F. Carpenter
DEDICATION

This work is dedicated to my parents and family members who encouraged me to pursue my dream. To my friends who became family through this journey.
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CHAPTER I

INTRODUCTION

Proteins: structure, function and the process of protein folding

Proteins are biological macromolecules that carry out most of the biochemical processes in the body and constitute the majority of cellular structures.1 Proteins are made up of 20 different amino acids that are arranged in a linear chain and joined together by peptide bonds between the carboxyl group of one amino acid and the amino group of the following amino acid residue. In order to perform their function, proteins must fold into complex three-dimensional (3D) conformation.1 The overall structure of the protein could further be divided into repeating local secondary structures such as alpha-helix, beta sheet that are stabilized by hydrogen bonds.1

The first 3D structure of the protein myoglobin was solved in 1958 by John Kendrew and colleagues using x-ray analysis.2 Since then, structures of various proteins have been investigated and significant amount of information has been obtained regarding protein folding. Previous study showed that there is relation between sequence of amino acids of a protein and structure and thus the overall structure of the protein is dictated by the amino acid sequence.1 In 1973, a report suggested that protein folding is a hierarchical process with rapid initial formation of protein secondary structures (either alpha helix or beta sheet).3 The initial structure formed in this process is referred to as molten globule state or partially folded state of the protein and is an intermediate between denatured and native state of the protein.3

In 1980s, protein folding was considered to be the sum of many different small interactions such as hydrogen bonds, van der waals forces, electrostatic interactions and hydrophobic interactions. However, later studies showed that hydrophobic interactions
are the dominating forces driving protein folding. Various studies have investigated the energy landscape of protein folding and have concluded that protein folding process is a funnel shaped energy landscape with many high energy states and a few low energy states as shown in figure 1.4

Figure 1: Energy landscape diagram showing protein's degrees of freedom. Energy landscape diagram showing protein’s degrees of freedom. The figure shows the pathways a protein can take to attain the lowest energy native (N) state. (a) Energy landscape diagram for a fast folding protein, (b) rugged energy landscape with kinetic traps, (c) energy landscape for a protein in which folding is dominated by diffusional conformational search, (d) landscape where a protein has to pass through an intermediate. figure from reference 4.4

The funnel shaped energy landscape indicates heterogeneous nature of the protein folding process in order to achieve native state.4 Proper folding of polypeptide into its native conformation is a prerequisite for the molecule to perform its biological function.

Proteins as therapeutic drugs

Protein molecules folded into native state can also have therapeutic significance. A large number and variety of protein molecules can be found in nature. Protein molecules can range from polypeptide chain of a few amino acids to thousands of amino acids. These protein molecules that are found naturally in every living cell can also be synthesized in vitro to perform various functions. With the advent of biotechnology, a lot of protein molecules are being designed and developed to perform variety of functions.
Specially, the field of pharmaceutical sciences has been revolutionized with protein molecules being developed as therapeutic drugs. Protein molecules when used for therapeutic purposes as often termed as biopharmaceuticals or biologics. These protein biologics can be used to treat many conditions ranging from wrinkles and snake-bites to deadly diseases such as cancer. Other examples of protein therapeutics are human growth hormone for patients with hormone deficiency, insulin for patients suffering from diabetes. Biologics are also being developed to treat diseases such as anemia, cystic fibrosis and various forms of cancer.

In the past decade, there has been a shift in the type of biologics being developed by pharmaceutical companies. The focus has shifted towards monoclonal antibody drugs that are more specific and effective in nature to treat various diseases. Protein therapeutic drugs that are approved by the FDA for treatment of several diseases have increased over a period of time. Over the last decade, there has been an influx of monoclonal antibody drugs targeted to treat variety of diseases. The first monoclonal antibody (mAb) product was approved in 1986 and since then the number of approved monoclonal antibodies (mAbs) per year is increasing. As of November 2014, there are forty-seven monoclonal antibody products that are approved and marketed in the United States and Europe. These protein molecules are an important class of drugs because they are very specific in action. Also, mAbs can be conjugated with other therapeutically relevant molecule for delivery to the target site. Of the many advantages that mAbs offer for therapeutic purposes, there is a disadvantage associated with it being immunogenic making the patients intolerant towards the drug.
Antibody molecules have a complex 3D structure. Each molecule comprise of 4 polypeptide chains (2 heavy chains and 2 light chains) that come together to form a Y-shaped molecule. They are multidomain protein molecules with an approximate molecular weight of 150 kDa. The variable (V) regions of both heavy and light chains approximately consist of the first 110 amino acids and form the antigen-binding (Fab) region of the protein, whereas the remaining amino acids constitute the constant (C) regions forming the Fc region of the protein. The secondary structure of the molecule is formed as the polypeptide chains form anti-parallel β-sheets constituting about 70% of the protein’s secondary structure.

Monoclonal antibody molecules comprise of 12 individual domains. For a multidomain protein, it has been shown that each individual domain contributes to the overall stability of the protein. Further, the least stable of domain of the protein dictates the stability and drives the aggregation of the protein.

![Figure 2: Linear schematic of a monoclonal antibody molecule. Taken from reference 6.](image)

**Protein stability and aggregation**

It is important to study aggregation of protein molecules because aggregation has been implicated in numerous diseases such as Alzheimer’s disease, Parkinson’s disease...
and systemic amyloidosis.\textsuperscript{12,13} It is also important to study protein aggregation while developing therapeutic protein products. During development of a protein molecule as a therapeutic drug, aggregation may be encountered during all stages of processing including purification, filling, storage and even during administration to patients.\textsuperscript{14,15}

There is great concern about aggregates in therapeutic products, because administration of protein aggregates into patients may cause adverse reactions including unwanted immune response and anaphylactic shock.\textsuperscript{16}

In order to minimize aggregate levels in drug products, it is important to understand the mechanism of protein aggregation and come up with strategies to minimize it.

Protein aggregation can be described as generally irreversible assembly process from initially native individual protein molecules into oligomers of protein molecules with nonnative structures.\textsuperscript{17} Protein aggregation behavior such as aggregation rate, type of aggregates is dependent on the protein molecule and the environment to which they are subjected.\textsuperscript{18} There are several factors that can determine protein stability and aggregation such as temperature, pH, cosolutes, preservatives and surfactants.\textsuperscript{17} Temperature at which the protein is stored is an important stability parameter because extreme temperatures can lead to protein unfolding and can cause aggregation.\textsuperscript{17} The thermodynamic stability of the protein is characterized by free energy of unfolding ($\Delta G_{\text{unf}}$).\textsuperscript{17} Thus, $\Delta G_{\text{unf}}$ is negative at extreme temperatures (i.e. high temperatures, 50-100°C and low temperatures of less than 10°C). At extreme temperatures, protein molecules undergo unfolding resulting in physical degradation and promoting aggregation. Temperature can also affect reaction kinetics between protein molecules as temperature can increase the kinetic energy of
molecules and thus increases the probability of collisions between them. In this manner, the rate of protein aggregation is increased at higher temperatures. Similarly, pH of the solution dictates overall charge of the protein and can influence aggregation. Protein molecules are stable at a narrow pH range away from the isoelectric point (pI) of the protein and become unstable if the pH is outside of this range. The solution pH dictates the type (positive or negative) and total charge on the protein molecule.\textsuperscript{17} The overall charge on the protein can influence electrostatic interactions between protein molecules. If the net charge on the protein is neutral (which is the case at isoelectric point), then it can lead to agglomeration of protein molecules.\textsuperscript{17} On the other hand, extreme pH conditions can lead to protein unfolding and aggregation. At extreme pH, there is increased charge repulsion within the protein molecule that destabilizes folded protein conformation because the folded protein has greater charge density than the unfolded protein.\textsuperscript{17} Many protein molecules like ribonuclease A, interleukin-1β have been shown to undergo degradation at extreme pH values.\textsuperscript{19,20} The charge on the protein molecule can also influence colloidal stability of protein molecules. When proteins are highly charged, repulsive interactions between molecules makes them colloidally stable. On the other hand, if the pH is close to isoelectric point of the protein, the net neutral charge on protein molecule can make protein-protein interactions attractive and can lead to assembly of molecules into aggregates.

Presence of excipients in the protein solution can also affect protein stability. According to the wyman linkage theory, differential binding of ligand to a protein in a two-state equilibrium, will shift the equilibrium towards the state with greater binding.\textsuperscript{17} For example, the native conformation of acidic fibroblast growth factor binds polyanions
and this binding shifts the equilibrium between the native state and unfolded states to favor the native state. The same mechanism applies for weakly interacting solutes such as sucrose, trehalose, and mannitol. Thus, many cosolutes that stabilize the protein (e.g. Sucrose, trehalose etc.) and the ones which destabilize the protein (e.g. Urea, GdnHCl) perform function through this mechanism. Protein stabilizing cosolutes such as sucrose and trehalose are preferentially excluded from the surface of the protein molecule and the degree of exclusion is proportional to the solvent exposed surface area of the protein molecule. In this process, cosolute molecules are depleted around the surface of the protein and water is enriched around the protein surface. We can interpret this phenomenon as negative binding of cosolutes to protein molecules. During the process of unfolding, protein surface area is increased leading to greater degree of preferential exclusion. In other words, there is greater negative binding of cosolute to unfolded state. Thus, the net effect is to favor the native state of the protein. The same phenomenon can also be explained by Lechatelier’s principle which states that a thermodynamic system tends to minimize an unfavorable change. So, during preferential exclusion, the protein chemical potential increases. Since the unfolded states have greater surface area and more preferential exclusion, protein native state with reduced surface area with lower preferential exclusion are favored over more solvent exposed states. The net result of this entire process is that free energy of unfolding is increased in the presence of cosolutes that are preferentially excluded such as sucrose and trehalose. In studies carried out on protein molecules including rhIFN-γ and rhGCSF, sucrose was preferentially excluded from the surface of the protein contributing to increase in thermodynamic stability and decreasing the aggregation rate. In figure 2, we observe that rhGCSF is
stabilized in the presence of sucrose.\textsuperscript{22} Surfactants are another component of a protein formulation that inhibit surface induced aggregation by binding to interfaces and thus inhibiting protein from interacting with surfaces.\textsuperscript{17}

Figure 3: Reaction coordinate diagram of rhGCSF aggregation in PBS at pH 7. N* is the transition state species, A\textsubscript{2} is the dimeric aggregation intermediate. Dotted arrows illustrate, relative to protein native state (N), shifts in the free energies of unfolded state (U) and N* when sucrose is added.\textsuperscript{17,22} Taken from reference 17.

Colloidal stability of protein molecules also plays an important role in protein aggregation besides conformational stability. Protein molecules can be assumed as colloidal particles that can assemble into higher molecular weight aggregates. Henceforth, it is important to have an understanding of protein-protein interactions and come up with strategies to stabilize them. B\textsubscript{22} is a thermodynamic solution parameter that directly quantifies overall protein-protein interactions on the molecular level which include hard sphere, electrostatic, van-der Waals and other short range interactions.\textsuperscript{17} Static light scattering is a powerful tool to study protein-protein interactions in solution. Static light scattering can be used to investigate the osmotic second virial coefficient (B\textsubscript{22}). The osmotic second virial coefficient (B\textsubscript{22}) is generally normalized with B\textsubscript{22}\textsuperscript{HS}, where B\textsubscript{22}\textsuperscript{HS}
denotes steric-only or hard sphere value of the osmotic second virial coefficient.\textsuperscript{24} When \( B_{22}/B_{22}^{\text{HS}} \) values are greater than 1, it indicates overall repulsive interactions between protein molecules whereas \( B_{22}/B_{22}^{\text{HS}} \) values less than 1 indicates attractive protein-protein interactions.\textsuperscript{17,22,24} Several factors can influence protein-protein interactions e.g. pH, cosolutes. As mentioned above, pH dictates the overall protein charge and thus protein-protein interactions. Similarly, cosolutes such as salt can influence protein-protein interactions by shielding electrostatic interactions between protein molecules. The DLVO theory predicts that the protein surface charge affects the distribution of ions in its surroundings and results in increased concentration of counter ions close to the surface, establishing an electric double layer around the protein molecule.\textsuperscript{25} For two isocharged protein molecules, the interactions are repulsive due to an osmotic repulsive force which arises when the electric double layers overlap as the local concentrations of ions in the double layer is greater than the ion concentration in the bulk.\textsuperscript{17,25} Also, the range of the force is dependent on the thickness of the electric double layer around the molecule which decreases with increasing ionic strength of the formulation.\textsuperscript{25} The influence of pH and ionic strength affecting protein stability has been seen in many studies. For example, rhGCSF at pH 3.5 is highly charged and electrostatic repulsions are stronger at pH 3.5 than at pH 7 that is close to the isoelectric point of the protein.\textsuperscript{22} Further, increasing the ionic strength of solution by addition of salt decreases \( B_{22} \) values as electrostatic repulsions are shielded.\textsuperscript{22}

**Sub-visible particles as components of protein aggregates**

Quantification of protein aggregates is based on mass percentage of the total protein sample and aggregates are usually characterized as either soluble or insoluble in
nature. However, sub-visible particles which are constituents of the protein aggregates but they do not constitute a sufficient mass fraction of the protein population to be quantified based on protein mass in form of particles or by loss of monomeric protein. These sub-visible particles can be quantified and characterized by counting their number in various size ranges and are important to study because of immunogenic properties. These particles range in size ranges from 0.1-100μm and can contain thousands of protein molecules assembled together. Monitoring sub-visible particles is a sensitive technique for detecting and quantifying protein aggregates and for improving product quality. They can also provide insight in protein aggregation pathway as described below.

These particles species in nanometer and micron sized range can be intrinsic particles e.g. non protein material but originating from formulation components such as silicone oil, glass particles, rubber from stoppers. These particles have the tendency to interact with protein depending on formulation conditions. Otherwise, these particles can be inherent particles originating from protein itself because of protein assembly. Several techniques are available to characterize these particles ranging from light obscuration, flow imaging, resonant mass measurement etc. Many of these have been utilized for sub-visible particles analysis in this thesis work.

**Partially unfolded protein species and their role in aggregation**

As mentioned above, protein conformation plays a significant role in driving aggregation. Earlier, it was believed that fully unfolded protein molecules react to form aggregates. However, in several more recent studies it was observed that aggregates can form from partially unfolded protein molecules, which have perturbed tertiary
structure but native-like secondary structure\textsuperscript{32}. And some studies have documented that these partially unfolded species are constituents of the native state ensemble of substates.\textsuperscript{39} Partially unfolded states exist in variety of structures and are often intermediates in the protein folding process.\textsuperscript{40,41} Further, these aggregation prone molecules are at extremely low levels and minor changes in the solution environment of the protein can shift the population towards large increase in aggregation prone molecules. For example, earlier studies on therapeutic proteins have shown how changes in solution pH\textsuperscript{30} or addition of antimicrobial preservative such as benzyl alcohol\textsuperscript{42-46} can stimulate large increases in aggregation rate by causing relatively modest shifts in the protein population towards partially unfolded species.

For a multidomain protein like monoclonal antibody, the overall process of unfolding and aggregation is complicated. It has been shown that in a multidomain protein, unfolding of a single domain can lead to aggregation. For example, Souillac et al. have shown that for an IgG1 molecule, the observed aggregates were due to association of C\textsubscript{H}3 domains.\textsuperscript{47} Several other studies on antibody molecules have shown that C\textsubscript{H}2 domain is involved in the process of aggregation.\textsuperscript{48,49}

In chapter II, we are investigating partial unfolding of a monoclonal antibody in sub-denaturing concentrations of guanidine hydrochloride (GdnHCl). In GdnHCl solutions at concentrations from 1.2-1.6M, the monoclonal antibody was partially unfolded. As demonstrated by fluorescence and circular dichroism spectroscopy, the partially unfolded state of the antibody had perturbed tertiary structure but retained native secondary structure. When these partially unfolded species were subjected to isothermal incubation over a period of 11 days, soluble aggregates were observed as observed using
size exclusion chromatography (SEC), analytical ultracentrifugation (AUC) and dynamic light scattering (DLS). Over the entire concentration range (0-2.0 M) of GdnHCl, protein-protein interactions were attractive, as quantified by negative osmotic second virial coefficients measured with static light scattering. However, aggregates were observed only in those concentrations that led to partial unfolding of the protein. Differential scanning calorimetry (DSC) studies showed that the antibody’s $C_{H2}$ domains were unfolded in antibody molecules that had been incubated in 1.2 M and higher concentrations of GdnHCl. These results suggest that unfolding of the $C_{H2}$ domains leads to aggregation. In this study, we established that both conformational and colloidal stability of the protein are important while studying aggregation. Further, both components of the protein stability or either one of them could be the dominating factor in driving protein aggregation.

**Protein interactions with interfaces**

Adsorption of protein molecules to interfaces is a problem during protein formulation and processing. Proteins are amphiphilic molecules leading to surface activity similar to surfactants. Adsorption of protein to interfaces can reduce protein stability by influencing the structure. The extent of conformational change depends on the type of interface with which protein is interacting and also characteristics of the protein molecule as shown in figure 3.
Adsorption of protein molecules to interfaces is of major concern to the pharmaceutical industry. Pharmaceutical proteins encounter various interfaces throughout their development as they go through purification, filling, freeze-thaw, transportation, storage and delivery to patients\textsuperscript{52-57}. These interfaces such as air-water interface and silicone oil-water interface are relatively hydrophobic in nature resulting in adsorption of protein molecules\textsuperscript{58}. Upon adsorption, protein molecules form a viscoelastic gel at the interface\textsuperscript{59,60}. The entire process from adsorption to formation of a viscoelastic gel by protein can be divided into three regimes\textsuperscript{50,61}. The first regime (I) is a lag phase and this period is primarily dependent on the diffusion of the protein to the interface. During the second regime (II), the interfacial tension drops, indicating accelerated adsorption of the protein to the interface. Towards the end of this period, a monolayer of the protein is formed covering the interface and intermolecular interaction between protein molecules start dominating. During the third regime (III), there is a slight decrease in interfacial tension where a multilayer is formed resulting in a gel like network and giving a viscoelastic behavior to the adsorbed layer. The multilayer gel like network is stabilized.
by intermolecular interactions at the interface.\textsuperscript{50,61} When this adsorbed protein is agitated, it can result in aggregation and particle formation in the bulk solution.\textsuperscript{55}

Silicone oil-water interface is one of the common interfaces encountered by therapeutic proteins during storage.\textsuperscript{62} Silicone oil is widely used as a lubricant in storage containers such as pre-filled syringes and vial stoppers.\textsuperscript{62} The mechanical properties of silicone oil provide an advantage for its use as lubricant on glass. It spreads easily on glass and it has high compressibility. Further, silicone oil as a polymer is resistance to oxidation and it has decreased surface reactivity which makes it suitable for long life applications. For many of the glass syringes, the application of silicone oil is carried out by spraying an emulsion of oil in water on the inner surface of the syringe.\textsuperscript{63,64} An emulsion of silicone oil in water consists of oil droplets suspended in water. Since there is immiscibility of oil and water the interfacial characteristics are affected and there is polarization and orientation of water dipoles at the oil-water interface. As a result negative charge is developed around the oil droplet. The electronegativity of the oil-water interface is attributed to a concentration difference between $\text{H}_3\text{O}^+$ and $\text{OH}^-$ ions contained in the water layer located at the interface.\textsuperscript{65}

However, in pre-filled syringes, formation of protein particles has been linked to the presence of silicone oil.\textsuperscript{62,66} In order to shed light on the mechanism by which silicone oil-water interface can induce protein aggregation, it is important to understand the interaction of protein molecules with that interface. Previous studies carried out on monoclonal antibody molecules have demonstrated that they undergo conformational change upon adsorption to the silicone oil-water interface resulting in aggregation.\textsuperscript{54,67} Further all these studies have demonstrated that protein tends to aggregate in the presence
of silicone oil. Thus, it is important to understand the mechanism behind silicone oil-induced protein aggregation and come up with strategies to minimize it.

An efficient way to minimize adsorption of protein molecules to interfaces like silicone oil-water interface is by using surfactants in protein formulation. Surfactants tend to inhibit adsorption of protein to the interface and thus prevent interfacial induced protein aggregation. PS 20 and PS 80 are the most common nonionic surfactants currently used in biopharmaceutical formulations.

In chapter III, we are investigating the tendency of a monoclonal antibody to form a viscoelastic gel upon adsorption to the silicone oil-water interface. The process of interfacial gel formation by proteins can be monitored by using an interfacial rheometer. In this study, we established that a monoclonal antibody upon adsorption to the silicone oil-water interface forms an interfacial viscoelastic gel. Further, we showed that when a mechanical stress is applied to this gel layer by rupturing the interface, it results in particle formation and aggregation in the bulk solution. As a control, when there was no perturbation of the interface, we did not observe any aggregates in the bulk solution. We also tested the ability of surfactants such as PS20 and PS80 to inhibit surface induced aggregation. We showed that presence of PS 20 and PS 80 in the formulation reduced aggregation and particle formation presumably by inhibiting interfacial gel formation.

In chapter IV, we are investigating the effect of ionic strength on interfacial viscoelastic gel formed at the silicone oil-water interface. Subsequently, we are also investigating the role of colloidal instability of sub-visible particles formed from interfacial gel rupture on further particle growth. Increasing ionic strength did not have a
significant effect on the interfacial viscoelastic gel formed at the silicone oil-water interface. Further, in this study, we established that the sub-visible particles formed from rupture of interfacial viscoelastic gel had a net neutral zeta potential indicating that the particles which are mix of protein-silicone oil were colloidally unstable. When these particles were incubated quiescently, it led to further particle growth over a period of 13 days.

A thorough understanding of the mechanism driving aggregation and particle formations can assist in coming up with strategies to minimize this form of degradation in protein therapeutics.
CHAPTER II

PARTIAL UNFOLDING OF A MONOCLONAL ANTIBODY: ROLE OF A SINGLE DOMAIN IN DRIVING PROTEIN AGGREGATION

Introduction

Protein aggregation is implicated in numerous diseases such as Alzheimer’s disease, Parkinson’s disease and systemic amyloidosis. Control of protein aggregation is also important for the development of therapeutic protein products. During development of protein drugs, aggregation may be encountered during all phases of processing such as purification, shipping, storage, and even during administration to patients. There is great concern about aggregates in therapeutic products, because administration of protein aggregates into patients may cause adverse reactions such as unwanted immune response and anaphylactic shock.

Thus, it is critical that aggregate levels in therapeutic protein products are controlled and minimized. In turn, it is important to understand the mechanisms for protein aggregation and to develop strategies to reduce this form of degradation. Key in these efforts is identifying the species in a population of protein molecules that are prone to react to form aggregates.

Early studies led to the proposal that fully unfolded protein molecules react to form aggregates. However, in several more recent studies it was observed that aggregates can form from partially unfolded protein molecules, which have perturbed tertiary structure but native-like secondary structure. And some studies have

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documented that these partially unfolded species are constituents of the native state ensemble of sub-states.\textsuperscript{39}

Therefore, even under conditions that thermodynamically favor the native state, protein aggregates can form. Furthermore, the aggregation-prone protein molecules are typically at extremely low levels\textsuperscript{80,81} so that even modest increases in the absolute concentrations of these reactive species can substantially increase aggregation rates. For example, earlier studies on therapeutic proteins have shown how changes in solution pH\textsuperscript{30} or addition of antimicrobial preservative such as benzyl alcohol\textsuperscript{42-46} can stimulate large increases in aggregation rate by causing relatively modest shifts in the protein population towards partially unfolded species.

Most studies showing that partial unfolding leads to aggregation have focused on relatively small, single domain proteins like apomyoglobin\textsuperscript{82}, RNase A\textsuperscript{83} and cytochrome c.\textsuperscript{42} Aggregation of small therapeutic proteins like GCSF\textsuperscript{46,84} and rhIL-1ra\textsuperscript{44,45,85} have also been shown to occur via partial unfolding.

Multidomain proteins may also aggregate as a result of unfolding of one or more domains. For example, Souillac et al. have shown that for an IgG1 molecule, the observed aggregates were due to association of C\textsubscript{H}3 domains.\textsuperscript{47} Several other studies on antibody molecules have shown that C\textsubscript{H}2 domain is involved in the process of aggregation.\textsuperscript{48,49} Other works carried out on multi-domain proteins, which are not antibody molecules, have shown that the least stable domain of the molecule is involved in the aggregation process.\textsuperscript{10,86,87} Oligomerization of multi-domain proteins has also been shown to occur via domain swapping\textsuperscript{88}, in which one protein molecule exchanges a domain with an identical molecule, forming an oligomer.
Physical instability of mAb is of great concern in the field of pharmaceutical biotechnology. Recently, it has been shown that exposure to low pH or high ionic strength can stimulate aggregation of monoclonal antibodies by favoring formation of partially unfolded protein molecules.\textsuperscript{54,89,90} Studies of antibody aggregation under such pharmaceutically relevant stresses also have tried to identify specific domains in the protein molecule that unfold and are responsible for aggregation. For instance, Kim et al. showed that F_{ab} unfolding caused by low pH or high salt concentration led to aggregation of an IgG1 molecule.\textsuperscript{24} On the other hand, Majumdar et al. showed that aggregation caused by high concentrations of salts was mediated by C_{H2} instability.\textsuperscript{49}

Addition of chaotropes has long been used to perturb protein structure in order to study populations of partially- and fully-unfolded molecules.\textsuperscript{91-93} Furthermore, some studies have shown that partially unfolded species populated in low concentrations of denaturant readily aggregate.\textsuperscript{94,95,96} In contrast, it is commonly observed that proteins do not aggregate in solutions that contain denaturant concentrations that result in complete unfolding. For example, proteins purified from bacterial inclusion bodies are routinely unfolded and solubilized in concentrated GdnHCl\textsuperscript{97,98}, and do not aggregate until refolding is initiated by reducing the denaturant concentration.\textsuperscript{99,100}

Protein aggregation also has been shown to be controlled by protein-protein interaction energetics, which is function of protein conformation and solvents.\textsuperscript{10,17,22} Static light scattering is a powerful tool to study protein-protein interactions in solution. Static light scattering can be used to investigate the osmotic second virial coefficient (B_{22}), which is a measure of strength of net protein-protein interactions. A positive value of B_{22} indicates repulsive forces between protein molecules while a negative value
indicates attractive interactions.\textsuperscript{101,102} Protein-protein interactions can be affected by solution conditions such as pH, ionic strength, sometimes independent of effects on conformational stability.\textsuperscript{17}

In our study, we used various concentrations of GdnHCl to alter protein conformation in a model mAb, such that the protein structure ranged from native to partially- to fully-unfolded. We found that at intermediate concentrations of GdnHCl, only certain domains of the protein were perturbed, whereas the overall secondary structure was not altered. Our hypothesis was that partially-unfolded species of a mAb (formed at low concentrations of GdnHCl) would aggregate, whereas native, folded mAb or fully-unfolded mAb molecules (found at high denaturant concentrations) would not. Furthermore, we measured protein-protein interaction energetics as a function of GdnHCl concentration to probe the importance of colloidal stability in denaturant-induced protein aggregation.

To test our hypothesis, we determined the equilibrium unfolding curve for the mAb. Further, we used circular dichroism (CD), fluorescence spectroscopy, limited proteolysis, analytical ultracentrifugation, and differential scanning calorimetry to characterize the protein’s structure and to identify the domains perturbed by GdnHCl. In addition, we used light scattering to determine protein-protein interaction energetics of the mAb in various concentrations of GdnHCl. Finally, protein aggregation was quantified with size exclusion chromatography, dynamic light scattering and analytical ultracentrifugation.
Experimental procedures

Materials

Purified monoclonal antibody (mAb) was provided by MedImmune (Gaithersburg, MD) in a lyophilized form. The lyophilized material was reconstituted with 2.2 mL of water for injection (WFI) to obtain 50 mg/mL protein in 10 mM Histidine, 6% trehalose, 2% arginine, 0.025% PS80 at pH 6.0. USP grade reagents such as 2-(N-morpholino)ethanesulfonic acid (MES) and guanidine hydrochloride (GdnHCl) were purchased from Fisher Scientific (Fair Lawn, NJ). Unless otherwise indicated, deionized MilliQ® water was used to prepare all solutions. Lyophilization vials (3 mL) and caps were purchased from West Pharmaceutical (Lionville, PA). Cuvettes used for circular dichroism (CD) and fluorescence spectroscopy were purchased from Starna cells (Atascadero, CA).

Protein sample preparation

The reconstituted protein concentration was determined using an Agilent® 8540 spectrophotometer (Santa Clara, CA) using an extinction coefficient of 1.45 mL/mg-cm at 280 nm. The concentration of stock protein solution was estimated to be 50 mg/mL. For experiments, the stock protein solution was diluted into different concentrations of GdnHCl to give the desired final denaturant concentration and a final protein concentration of 1 mg/mL. A stock solution of 7.0 M GdnHCl in 10 mM MES (pH 6.0) was used. Stock GdnHCl concentration was determined using refractive index measurements.103

Antibody unfolding curve
Far-UV CD spectra were collected for protein equilibrated in different concentrations of GdnHCl using Chirascanplus spectrometer (Applied Photophysics, UK) and 1 mm path-length cuvette. Protein at 1.0 mg/mL was allowed to equilibrate in several concentrations of GdnHCl at 37°C for one day. The CD signal at 218 nm was monitored as a function of GdnHCl concentration to obtain the unfolding curve. Triplicate samples were analyzed for each concentration of GdnHCl and one scan per sample was collected.

**Fluorescence and circular dichroism (CD) spectroscopy**

The mAb, at a concentration of 1 mg/mL, was incubated in 0-2.0 M GdnHCl solutions at 0.2 M increments. Triplicate samples were incubated at 37°C and were analyzed using fluorescence and CD spectroscopy on day 0, 1, 6 and 11 of incubation. For each sample, one spectrum was recorded.

Intrinsic tryptophan fluorescence spectra were acquired using Photon Technology International (PTI) spectrofluorometer (Lawrenceville, NJ). Tryptophan (Trp) excitation was carried out using 295 nm wavelength and emission spectra were collected from 300 to 400 nm with 1 nm/s data collection rate and 1 s integration time. The slit widths for Trp excitation and emission were 4 nm and 1 nm respectively. The maximum tryptophan fluorescence peak position was obtained by calculating the first derivative of the emission spectrum. We also calculated the tryptophan fluorescence center of mass by integrating the emission spectrum.\textsuperscript{104} Far-UV CD spectra were collected for incubated protein samples using Chirascanplus spectrometer (Applied Photophysics, UK) in 1 mm path-length cuvette. For each time point of incubation, the CD signal at 218 nm was plotted as a function of GdnHCl concentration.
Static light scattering

We used Brookhaven light scattering system (Brookhaven Instruments Corporation, Holtsville, NY) to obtain static light scattering (SLS) measurements. Protein samples were prepared at concentrations ranging from 0.5-5.0 mg/mL in various concentrations of GdnHCl and the scattering intensity was measured at 90°. Triplicate samples were prepared for each condition, and scattering intensity was acquired for each sample replicate. All buffers were filtered using 0.02 µm Anotop 25 syringe filters (Whatman International Ltd). The relationship used to determine B$_{22}$ is derived from the virial expansion of the ideal osmotic pressure equation:

\[
\frac{Kc}{R_{90}} = \frac{1}{M} + 2B_{22}c \quad \text{(Eq. 1)}
\]

where c is the protein concentration (g/mL), K is the optical density constant (mL mol g$^{-2}$ cm$^{-1}$), M is the protein molecular weight (g/mol), R$_{90}$ is the excess Rayleigh ratio at 90° (cm), and B$_{22}$ is the second osmotic virial coefficient (mL mol g$^{-2}$). B$_{22}$ values determined from Equation 1 were scaled by the theoretical value of the hard sphere (HS) second virial coefficient to obtain $b_2^* = (B_{22}/B_{22}^{HS})-1$ where $B_{22}^{HS} = (2/3)\pi d^3$ and d is the effective HS diameter for a monomer. As an estimate of the HS diameter, the hydrodynamic diameter (11.4 nm) was determined using dynamic light scattering (DLS) as described below.

Size exclusion chromatography (SEC)

On day 0, 1, 6 and 11, SEC analysis of triplicate mAb samples that had been incubated at 37 °C was performed to determine monomer and soluble aggregate levels. A Tosoh TSKgel G3000SW xl was used, and protein in the eluate was quantified using absorbance at 280 nm. Prior to loading the sample in a given concentration of GdnHCl,
the column was equilibrated with one column volume of that particular concentration of GdnHCl in 0.1 M Na₂SO₄, 0.1 M Na₂HPO₄, pH 6.8, which was also used as the mobile phase at a flow rate of 1 mL/minute. Percent recoveries of soluble protein and percent soluble aggregates were calculated by normalizing against total peak area of chromatograms for day 0 samples. Only monomeric mAb was detected by SEC in day 0 samples.

**Dynamic light scattering to determine mAb hydrodynamic diameter**

On day 0, 1, 6 and 11, DLS analysis of triplicate mAb samples that had been incubated at 37 °C was performed to determine the hydrodynamic diameter of the mAb using a Zeta-sizer Nano ZS (Malvern, U.K). Hydrodynamic diameters were calculated from measured diffusion coefficients and values of solution viscosity using the Stokes-Einstein equation:

\[
D = \frac{k_B T}{6\pi \eta r}
\]  
(Eq. 2)

where \(k_B\) is Boltzmann’s constant, \(T\) is absolute temperature, \(\eta\) is viscosity of solvent containing GdnHCl and \(r\) is the hydrodynamic radius. Cumulant analysis was performed for each sample. Viscosities as a function of GdnHCl were estimated using the public domain software program SEDNTERP program.

**Analytical ultracentrifugation (AUC)**

On day 0, 1, 6 and 11, AUC analysis of mAb samples that had been incubated at 37°C was performed using sedimentation velocity using a Beckman XL-A analytical ultracentrifuge equipped with absorbance optics. Samples prepared in each concentration of GdnHCl were sedimented at 25°C at rotor speed of 40,000 rpm and data collected at 294 nm. The raw data collected from sedimentation velocity experiments were analyzed
using software program SEDFIT. The meniscus position was allowed to vary as a fitted parameter, and the cell bottom position was fixed at 7.2 cm. The frictional ratio ($f/f_0 = 1.5$) and mAb partial specific volume ($\nu = 0.727$ L/kg) suggested by Arthur et al. (65) were used. Buffer densities and viscosities for each concentrations of GdnHCl were calculated using the public domain software program SEDNTERP program. The sedimentation coefficient values of monomer were corrected to standard conditions of water at 20°C using the formula:

$$s_{20,w} = s_{T,B} \left( \frac{\eta_{T,B}}{\eta_{20}} \right) \left( \frac{1 - \nu \rho_{20}}{1 - \nu \rho_{T,B}} \right)$$  \hspace{1cm} (Eq. 3)

where $T$ and $B$ denote the values at the temperature and under the buffer conditions of the experiment, and index 20,w indicates standard conditions.

Ellman’s reagent test and quantification of covalent aggregates

To ascertain if the mAb had any free cysteines that might play a role in aggregate formation, we checked for reactivity of Ellman’s reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB) with monoclonal antibody as a function of GdnHCl concentration. Briefly, 2 mM of Ellman’s reagent was added to 30 μM protein samples on day 0 and day 1. Absorbance at 412 nm was measured using Agilent® 8540 spectrophotometer (Santa Clara, CA). The presence of covalent aggregates was quantified using SDS-PAGE gel. For each time point, mAb samples incubated at 37°C in 1, 1.2, 1.4, 1.6, 1.8 or 2 M GdnHCl were run on SDS-PAGE gel. The intensity of each band corresponding to covalent aggregate was calculated using Quantity One software on a Biorad Gel Doc XR+ instrument.

Proteinase K assay and SDS-PAGE
In order to characterize partially unfolded protein species, a proteinase K assay
was conducted for mAb samples incubated in 0.6, 1.0, 1.2, 1.4, 1.6, 1.8 and 2 M
GdnHCl. The supplier of the Proteinase K has shown that the enzyme maintains activity
in GdnHCl solutions at concentrations up to 3 M GdnHCl (5 PRIME, Gaithersburg,
MD.). Proteinase K (20 ng) in 5 µL was added to 50 µL of a 1 mg/mL solution of mAb.
The samples were incubated at 37°C for 1 hr. After incubation, proteins in the samples
were precipitated by addition of 12 µL of trichloroacetic acid (TCA). After 10 minutes
of incubation on ice, the samples were centrifuged at 14,100 g for 5 minutes. Supernatant
was removed from the centrifuged sample leaving the protein pellet intact. The pellet was
washed with 200 µL of ice-cold acetone and the tubes were centrifuged at 14,100 g for 5
minutes. Pellets were washed 2x with ice-cold acetone and dried in 95°C heat block for 5
– 10 minutes. The tubes were removed and 40 µL of 10% SDS and 10 µL of 4X loading
dye were added to each sample pellet. The samples were boiled for 5 minutes in 95°C
heat block. The samples were centrifuged at 14,100 g for 5 minutes before loading 15 µL
of each sample on SDS-PAGE gel. As a control, non-digested mAb was subjected to
TCA precipitation protocol to observe the effect of TCA on protein. The SDS-PAGE gel
was stained with Coomasie blue stain and washed prior to obtaining the gel image.

**Differential scanning calorimetry (DSC)**

The stability and domain unfolding of mAb in various concentrations of GdnHCl
was determined using a VP-capillary differential scanning calorimeter (MicroCal,
Northampton, MA). Samples were mixed and held at room temperature, and DSC
analysis was performed within 30 minutes of sample preparation. Protein samples at 1
mg/mL were analyzed over a temperature range of 10-90°C at a scan rate of 90°C/h with
a 15 min pre-scan thermo stating. For each GdnHCl concentration, a buffer baseline was obtained and protein thermograms were obtained after subtracting the buffer baseline using origin software (Originlab Corporation, Northampton, MA). For each concentration of GdnHCl tested, three vials of protein solution were prepared and analyzed.

Results

Far-UV CD spectrum and unfolding curve for monoclonal antibody (mAb)

To examine the mAb secondary structure, far-UV CD spectra (Fig. 5A) for mAb at 1 mg/mL were obtained. A minimum was observed at 218 nm, likely dominated by contributions from $\beta$-sheet structure.$^{112,113}$ Mean residue ellipticity at 218 nm was plotted as a function of GdnHCl concentration (Fig. 5B), which showed an onset of protein unfolding at 1.8 M GdnHCl.

Spectroscopic characterization of partially unfolded protein species

To obtain insight into protein conformation prior to aggregation, intrinsic tryptophan (Trp) fluorescence and CD spectra were obtained on the day of preparation (day 0) and after 24 hours of incubation at 37°C.

The wavelengths for maximum tryptophan fluorescence ($\lambda_{\text{max}}$) were plotted as a function of GdnHCl concentration (Fig. 6A). It is important to note that this mAb contains 22 Trp residues and thus the Trp fluorescence reflects an average over all fluorescing Trp residues. In the absence of GdnHCl, $\lambda_{\text{max}}$ for the native mAb occurred around 330 nm. This indicates that most of the fluorescing tryptophans are buried in the hydrophobic interior of the protein.$^{114}$ In solutions containing 0-0.8 M GdnHCl, $\lambda_{\text{max}}$ did not shift appreciably, but it increased by ca. 5 nm at GdnHCl concentrations ranging from 1.2-1.6 M, indicating a moderate perturbation in the mAb tertiary structure. At day 0, $\lambda_{\text{max}}$
was around 335 nm for samples containing 1.2-2.0 M GdnHCl. On day 1, $\lambda_{\text{max}}$ values measured in samples containing 0-1.6 M GdnHCl were very similar to those observed on day 0, but $\lambda_{\text{max}}$ increased to 350 nm at GdnHCl concentrations of 1.8 and 2.0 M. This increase in $\lambda_{\text{max}}$ reflects an average increase in the exposure of fluorescing Trp residues to an aqueous environment, and is thus indicative of a major perturbation of tertiary structure.  

We also calculated the tryptophan fluorescence center of mass for the same set of samples (Fig. 6B). The trend observed was similar to that for the maximum tryptophan fluorescence wavelengths plotted in fig 6A. However, due to the asymmetrical peak shape, this analysis resulted in an apparent 10 nm red shift in the reported peak position.

In an attempt to obtain further insights into changes in tertiary structure, near UV-CD spectroscopy was also used to analyze the same set of samples. However, unlike fluorescence spectra, near UV-CD spectra did not show changes (data not shown) indicative of tertiary structure perturbations until GdnHCl was at concentrations at which there were also secondary structure alterations.

To study secondary structure, mean residue ellipticities at 218 nm determined from far-UV CD spectra were plotted as a function of GdnHCl (Fig. 6C). On both days 0 and 1, only a small decrease in signal intensity was observed as up to 0.4 M GdnHCl was added to mAb samples, with no further intensity changes in day 0 samples with up to 2.0 M GdnHCl. In contrast, after a day of incubation, there was a dramatic decrease in intensity at 1.8 and 2.0 M GdnHCl (Fig. 6C) indicating perturbation of the mAb secondary structure.
On both day 0 and 1, tertiary structure of mAb was perturbed in the presence of 1.2, 1.4 and 1.6 M GdnHCl (Fig. 6A), but secondary structure appeared to be largely unaffected (Fig. 6C). However, in 1.8 and 2.0 M GdnHCl on day 1, concomitant, large perturbations of both tertiary and secondary structure were observed. It important to note that protein aggregation was not observed for any of the samples on day 0 and day 1 (see below). Thus, the spectroscopic results reflect alteration in protein structure prior to any aggregation.

Figure 5: (A) Far-UV CD spectrum for mAb at 1 mg/ml. The CD signal at 218 nm was followed as a function of GdnHCl concentration to obtain denaturation curve for mAb. (B) Mean residue molar ellipticity for signal at 218 nm as a function of GdnHCl concentration. Data points represent the mean ± SD for triplicate samples. Error bars for certain data point are smaller than symbols.
Figure 6: (A) Maximum tryptophan fluorescence wavelength for mAb on day 0 (closed circles) and day 1 (open circles). (B) Tryptophan center of mass for mAb on day 0 (closed circles) and day 1 (open circles). (C) CD signal as observed for mAb on day 0 (closed circles) and day 1 (open circles). Data points represent the mean ± SD for triplicate samples. Error bars for certain data point are smaller than symbols.
Protein-protein interactions

$B_{22}$ values normalized by the hard sphere value for the mAb are shown as a function of GdnHCl in Fig. 7. For protein incubated in 0-2.0 M, the normalized $B_{22}$ values are negative, indicating that the protein-protein interactions are attractive in these concentration ranges.\textsuperscript{89}

Figure 7: The second osmotic virial coefficient $B_{22}$, normalized by the $B_{22}$ value for a hard sphere ($B_{22}/B_{22}^{\text{HS}} - 1$) gives value of $b_2^*$ as a function of GdnHCl for mAb. Data points represent the mean ± SD for triplicate samples. Error bars for certain data point are smaller than symbols.

Characterization of soluble aggregates

*Size exclusion chromatography (SEC)* -

mAb samples incubated in 0-2.0 M GdnHCl at 37°C over a period of 11 days were analyzed for soluble protein and high molecular weight species by SEC (Fig. 8). There were no significant losses of soluble protein during the incubation period (Fig. 8A). No soluble aggregates were observed by SEC on day 0 and day 1 (Fig. 8B). After 6 days of incubation at 37°C, high molecular weight species were observed in samples containing 1.2, 1.4, 1.6 and 1.8 M GdnHCl (Fig. 8B), with additional increases seen by
day 11. In contrast, no aggregates were detected for mAb samples in 0-1.0 M and 2.0 M GdnHCl, even after 11 days of incubation.

*Dynamic light scattering (DLS)* -

To further characterize the soluble aggregates, dynamic light scattering was used to measure mAb hydrodynamic diameters in samples after incubation at 37°C (Fig. 9). On day 0, before aggregates were detected by size exclusion chromatography, the hydrodynamic diameter of mAb in 0-2.0 M GdnHCl was ca. 11 nm. On day 1, the average hydrodynamic diameter increased to ca. 15 nm in solutions containing 1.4 M and greater concentrations of GdnHCl. This increase may have resulted from unfolding of the mAb and/or formation of small amount of aggregates. Consistent with results from SEC analysis that showed formation of soluble aggregates, after 6 days of incubation the measured hydrodynamic diameter increased dramatically in samples incubated in 1.2 to 2.0 M GdnHCl, with a maximum of about 28 nm observed in samples incubated in 1.4 and 1.6 M GdnHCl. A similar trend was observed on day 11.

*Sedimentation velocity analytical ultracentrifugation (SV-AUC)* -

mAb samples incubated in 0-2.0 M GdnHCl were analyzed by AUC to determine the levels of high molecular weight species (Fig. 10). The AUC results showed trends identical to those observed with SEC and DLS.
Figure 8: (A) Percent soluble protein recovery as determined by size exclusion chromatography (SEC) relative to day 0. (B) % high molecular weight species observed using size exclusion chromatography relative to day 0. Open circles represent day 1 samples, closed squares represent day 6 samples and open squares represent day 11 samples. Data points represent the mean ± SD for triplicate samples. Error bars for certain data point are smaller than symbols.

Figure 9: Average size of soluble protein as observed using dynamic light scattering (DLS). Closed circles represent day 0 samples, Open circles represent day 1 samples, closed squares represent day 6 samples and open squares represent day 11 samples. Data points represent the mean ± SD for triplicate samples. Error bars for certain data point are smaller than symbols.
Figure 10: Percent aggregates as observed using analytical ultracentrifugation (AUC). Closed circles represent day 0 samples, open circles represent day 1 samples, closed squares represent day 6 samples and open squares represent day 11 samples.

*Far-UV CD spectroscopy of incubated mAb samples -*

Far-UV CD spectroscopy was used to analyze changes in the secondary structure of mAb upon formation of soluble aggregates in incubated samples (Fig. 11). After 6 and 11 days, mean residue ellipticity measured at 218 nm became more negative for samples incubated in 1.4 M and 1.6 M GdnHCl, reflecting the characteristic intermolecular β-sheet structure that is typical of protein aggregates. In samples incubated in 1.8 and 2.0 M GdnHCl, mAb unfolding occurred, as evidenced by less negative ellipticity values.
Figure 11: Mean residue ellipticity at 218 nm for incubated samples. Closed circles represent day 0 samples, open circles represent day 1 samples, closed squares represent day 6 samples and open squares represent day 11 samples. Data points represent the mean ± SD for triplicate samples. Error bars for certain data point are smaller than symbols.

Percent covalent aggregates by SDS-PAGE analysis -

The total percentage of protein that formed covalent aggregates was quantified by SDS-PAGE analysis (Fig. 12). Protein in 1.4 M and greater concentrations of GdnHCl showed covalent aggregates. However, these covalent aggregates were only a fraction of the total amount of aggregates observed for these samples (Figs. 8, 10 and 12).

We also tested for the presence of free cysteines in the mAb using Ellman’s reagent.\textsuperscript{117} The Ellman’s reagent test did not show the presence of free thiols (data not shown); we suggest that covalent aggregates were formed from disulfide shuffling within existing aggregates.
Figure 12: Percent covalent aggregates for mAb as a function of GdnHCl concentration. Solid squares represent day 0 samples, solid triangles represent day 0.5 samples, solid inverted triangles represent day 1 samples, solid diamonds represent day 1.5 samples, solid circles represent day 2 samples, open squares represent day 3 samples, open triangles represent day 4 samples, open inverted triangles represent day 6 samples, and open diamonds represent day 11 samples.

Proteinase K assay -

Proteinase K assay was used to determine proteolytic digestion pattern for mAb in various concentrations of GdnHCl on days 0 and 1 (Fig. 13). The band at ~ 50 kDa corresponds to the mAb’s heavy chain and the band at ~ 25 kDa corresponds to the light chain. For samples digested by proteinase K on day 0, the intensity of the band for the heavy chain decreased with increasing concentration of GdnHCl, and the band disappeared entirely in samples containing 1.0-2.0 M. In contrast, the presence of GdnHCl across the entire range tested (0-2.0 M) did not appear to affect the intensity of the band for the light chain. For day 1 samples, the heavy chain band exhibited a similar trend, but in samples containing 1.6-2.0 M GdnHCl, the light chain band was greatly diminished.

It should be pointed out that TCA precipitation of the mAb in the absence of GdnHCl or proteinase K led to formation of covalent aggregates (lane 2). In contrast, no
such aggregates were observed when the protein was analyzed by SDS-PAGE in absence of TCA precipitation (Fig. 13C).

Figure 13: SDS-PAGE for day 0 (A) and day 1 (B) samples following proteinase K digestion. M represents molecular weight standards and number corresponds to particular concentration of GdnHCl. Samples marked with ‘+’ are digested samples and ‘–’ are the non-digested samples. (C) mAb samples in non-reduced (NR) and reduced (R) forms without TCA precipitation.
Hydrodynamic behavior of protein in 0-2.0M GdnHCl -

Sedimentation velocity analytical ultracentrifugation (SV-AUC) was used to determine sedimentation coefficients corrected for standard conditions ($s_{20,w}$) for the mAb in 0-2.0 M GdnHCl (Table 1). In 0 M GdnHCl, the mAb had a sedimentation coefficient of 7.3 S both on day 0 and day 1. The value of sedimentation coefficient decreased with increasing concentrations of GdnHCl. This trend is apparent on day 0 as well as day 1.

Table 1. Sedimentation coefficient for monomer of mAb as a function of GdnHCl on day 0 and 1 of incubation. Note: hydrodynamic size of mAb is time-dependent at higher concentrations of GdnHCl.

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DSC to observe mAb domain unfolding transitions -

DSC analysis was used to obtain information about domain unfolding in 0-2.0 M GdnHCl solutions. The native protein showed three thermal unfolding transitions ($T_m$) (Fig. 14). The first transition ($T_{m,1}$) observed at around 48.9°C can be attributed to unfolding of the $C_H^2$ domain. The second and third transitions ($T_{m,2}$ and $T_{m,3}$) observed at around 72.1°C and at 84.5°C can be attributed to $F_{ab}$ region and $C_H^3$ regions of the mAb, respectively. In 1.4 M GdnHCl, the first transition ($T_{m,1}$) was not detected, suggesting that $C_H^2$ domain was unfolded prior to heating. Also, the other two transitions ($T_{m,2}$ and $T_{m,3}$) shifted to lower temperatures, indicating that the presence of 1.4 M GdnHCl reduced the stability of the $C_H^3$ and Fab domains.

In solutions containing 0-2.0 M GdnHCl, $T_{m,2}$ and $T_{m,3}$ values decreased with increasing concentrations of GdnHCl. For $T_{m,1}$ a decrease was observed from 0 to 1.0 M GdnHCl, but no transition could be detected in 1.2-2.0 M GdnHCl.

Figure 14: DSC thermograms for native mAb (solid black line) and mAb in the presence of 1.4 M GdnHCl (dashed black line). The native mAb shows three transitions marked as $T_{m,1}$, $T_{m,2}$ and $T_{m,3}$.
Table 2. Thermal unfolding transitions for mAb in sub-denaturing concentrations of GdnHCl as measured by DSC. ND signifies that the transition (T_{m,1}) was not observed for that particular sample. Each number represents mean transition temperature (T_m) and the standard deviation of triplicate measurements.

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<th>T_{m3} (°C)</th>
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Discussion

Partially unfolded protein species as a constituent of protein ensemble - Hydrogen-deuterium exchange and NMR relaxation studies have revealed significant conformational heterogeneity of protein molecules under native state conditions.\textsuperscript{81,118,119} The ensemble of native protein molecules has access to diverse conformations, which may be critical for biological function.\textsuperscript{120} Also included in the native state ensemble may be partially unfolded protein species that are prone to aggregation.\textsuperscript{118,120}
The levels of these partially unfolded states (and hence the resulting rates of aggregation) can be modified within the native state ensemble by changes in the solution conditions. For example, the presence of low concentrations of GdnHCl results in accumulation of partially unfolded protein molecules that tend to aggregate. We found that the large, multi-domain mAb became partially unfolded with unfolding of C\textsubscript{H}2 domain at low concentrations of GdnHCl, which in turn resulted in aggregation.

Conformational and colloidal instability govern protein aggregation - Protein aggregation can be controlled by both the conformational state of a protein, and the energetics of protein-protein intermolecular interactions. Early studies of protein aggregation focused on the role of protein conformation in the aggregation process. Many reports have shown that protein aggregation proceeds via assembly of partially unfolded protein molecules. These species can be populated under a variety of conditions, including elevated temperatures, pH extremes, exposure to interfaces, presence of low levels of chemical denaturants and freeze-thawing.

For large proteins that have multiple domains, the analysis of overall protein stability is a complicated process because each individual domain contributes to stability. It has been suggested that the stability of least stable protein domain will dominate the conformational stability of protein towards aggregation. Also, the reversibility of unfolded regions of multi-domain protein may be unfavorable, often leading to aggregation. Further, the overall protein structure is stabilized by both inter and intradomain interactions.

The mAb investigated in the current study consists of twelve domains. Because partially unfolded molecules of this protein are involved in aggregation, we expected that
the least stable domain will unfold at lower concentrations of GdnHCl than other domains and lead to aggregation. It has been shown that mutations in this particular mAb reduced only the stability of C_{H2} domain and led to increased aggregation compared to the wildtype protein.\textsuperscript{11} Similarly, in our DSC study, we observed that the unfolding transition corresponding to C_{H2} domain was not detected for protein in 1.2 M and higher concentrations of GdnHCl (Fig. 14 and table 2). Thus, it can be suggested that unfolding of C_{H2} domain of mAb resulted in partially unfolded species for this protein, which in turn were prone to aggregation. Other studies carried out on monoclonal antibody have also reported that C_{H2} domain can play a causal role in aggregation.\textsuperscript{48,49,122,129}

We also found that the S value obtained from sedimentation velocity experiments was inversely proportional to hydrodynamic radius of the protein. As the concentration of denaturant increased, the S value became smaller, signifying a larger hydrodynamic radius of protein (Table 1). We also observe that the S value for protein in 1.2 - 1.6 M GdnHCl is intermediate for that of native protein and that observed in 2.0 M GdnHCl. This observation further supports the conclusion that protein in 1.2 - 1.6 M GdnHCl was partially unfolded.

Partially-unfolded protein species have structurally perturbed regions and that are more susceptible to proteolytic cleavage than the structured regions.\textsuperscript{130} Studies have shown that proteolytic enzymes can be used as probes of the structure and dynamics of partially folded states of protein and that the residual native structure of the protein molecule can be sufficient to prevent extensive proteolysis.\textsuperscript{131} Some reports have depicted that altering solution conditions to increase the population of unfolded molecules results in increased proteolysis.\textsuperscript{110} For example, Latypov et al. showed using
proteinase K that in 0 M urea, IL-1ra was resistant to proteolysis and only 25% molecules were digested. However, 90% of IL-1ra molecules were digested in 5 M urea.\textsuperscript{111} We found that the mAb was more susceptible to proteolysis in 2 M GdnHCl (Fig. 13B) than in buffer alone, because in the denaturant the protein molecules were mostly present in an unfolded state. However, the susceptibility of partially unfolded species in 1.2-1.6 M GdnHCl to proteolysis was intermediate to that of folded and unfolded state. Overall, the heavy chain was more susceptible to proteolysis than the light chain of this mAb as observed from Fig 13. For samples digested by proteinase K on Day 0, the intensity of the band for the heavy chain decreased with increasing concentration of GdnHCl, and the band disappeared entirely in samples containing 1.0-2.0 M. In contrast, the presence of GdnHCl across the entire range tested (0-2.0 M) did not appear to affect the intensity of the band for the light chain. A similar trend was observed for day 1 samples as well, with the light chain getting digested further for samples containing 1.6-2.0 M GdnHCl. This trend indicates unfolding of protein in higher concentrations of GdnHCl.

Colloidal instability relates to intermolecular interactions leading to aggregation. Under solution conditions where protein molecules experience net attractive interaction energies, assembly into aggregated species is favored. Under these conditions, there may or may not be an associated change in protein conformation.\textsuperscript{17} Earlier studies have shown that protein – protein interactions are most attractive at low concentrations of GdnHCl.\textsuperscript{132,133} For some proteins, colloidal instabilities control rates of aggregation.\textsuperscript{22} In our study, we found that colloidal stability was not a dominating factor in the overall process of aggregation. Despite the b$_2^*$ being negative for protein in 0-2.0 M GdnHCl, no aggregates were observed for mAb samples incubated in 0-1.0 M GdnHCl (Fig. 7).
Aggregates were only observed for protein in 1.2 M and higher concentrations of GdnHCl where tertiary structure was perturbed (Fig. 6) and led to C\textsubscript{H2} domain unfolding (Fig. 14). Thus, with this particular mAb that conformational perturbations caused by GdnHCl that lead to aggregation do not also cause a change in colloidal stability. Thus, conformation stability is dominant over colloidal stability in governing the aggregation process for the mAb.

Characterization of mAb aggregates was investigated using CD spectroscopy. Increased level of intermolecular β-sheet is a common feature of protein aggregates\textsuperscript{29}. The structural transition of native protein to form nonnative intermolecular β-sheet structures can occur regardless of the initial secondary structural composition.\textsuperscript{17} For instance, aggregation of protein molecules like rhIFN-γ and prion proteins results in intermolecular β-sheet structure concomitant with a significant loss of α-helix.\textsuperscript{23,134} Although secondary structure of native mAb molecules is about 70% β-sheet \textsuperscript{6}, aggregation of the mAb used in this study (Fig. 11) and earlier research resulted in an increase in β-sheet content.\textsuperscript{135} The high β-sheet content may also reflect the retention of intact, native-like domains of the mAb molecules within the aggregate.\textsuperscript{88,136} Overall it appears that the aggregation-prone, partially unfolded molecules retained native-like β-sheet secondary structure based on CD spectroscopy (Fig. 6A), despite having an unfolded C\textsubscript{H2} domain. And upon aggregate formation there was an increase in non-native β-sheet due to additional structural perturbation as a result of aggregation.

We also investigated the potential for the aggregates of the mAb to have covalent intermolecular contacts. We found that there were no free thiols in this mAb under different concentrations of GdnHCl. However, upon further incubation intermolecular
disulfide bonds were formed, making the aggregates irreversible. These results suggest that in addition to structural perturbation occurring initially upon aggregate formation, there are structural changes in the mAb molecules in the aggregate that allow non-native intermolecular disulfide bonds to form. Such formation of covalent links between protein molecules in aggregates has been observed for other proteins.\textsuperscript{137-139}

**Conclusion**

We observed that the $b_2^*$ values were negative for the mAb molecules over the entire range of 0 to 2 M GdnHCl. However, aggregation was only observed for samples at 1.2M and higher concentrations of GdnHCl. Therefore, colloidal stability does not control aggregation of this mAb. Rather, at 1.2M and higher concentrations of the chaotrope, the $\text{C}_\text{H}2$ domains of the mAb were unfolded resulting in a population of partially unfolded, aggregation-prone protein molecules. Thus, for this mAb, conformational perturbation predominately controlled rates of aggregation.
CHAPTER III

GELATION OF A MONOCLONAL ANTIBODY AT THE SILICONE OIL-WATER INTERFACE AND SUBSEQUENT RUPTURE OF THE INTERFACIAL GEL RESULTS IN AGGREGATION AND PARTICLE FORMATION

Introduction

Pharmaceutical proteins encounter various interfaces during manufacturing and storage as they go through purification, filling, freeze-thaw, transportation, storage and delivery to patients. Exposure of protein solutions to interfaces often results in protein aggregates and particles in the bulk solution. Protein molecules readily adsorb to many interfaces, such as the relatively hydrophobic, air-water and silicone oil-water interfaces. Upon adsorption to interfaces, proteins have potential to unfold, aggregate and form viscoelastic gel layers. The protein gel formed can be stabilized by a variety of noncovalent interactions between protein molecules including hydrogen bonding and electrostatic interactions.

Recently, Rudiuk et al. showed that rupturing the gel layer of protein formed at the air-water interface of an IgG solution resulted in the release of protein aggregates from the interface into the bulk solution. This model is of interest because it suggests a mechanistic explanation of interface-induced aggregation related to perturbation of protein gel layers during mechanical stress such as agitation. Furthermore, they showed that the presence of surfactants in the IgG formulation reduced the amount of aggregation that was detected. Our study is an extension of the work done by Rudiuk et

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Nonionic surfactants, commonly polysorbate 20 and polysorbate 80, are often used as stabilizers in protein formulations in order to reduce aggregation.\textsuperscript{126,147} One mechanism by which they may inhibit aggregation is through competitive adsorption to interfaces, due to their higher adsorption energies per unit area than protein molecules.\textsuperscript{51} Above the critical micelle concentration (CMC), polysorbates can saturate hydrophobic interfaces and thus be most effective at inhibiting interfacial adsorption of protein molecules.\textsuperscript{148,149} This observation is consistent with many biologics formulations containing surfactant concentrations above their CMC level. However, some formulations contain surfactants below their CMC level, and a few studies have shown polysorbates can confer significant protection to protein against surface-induced aggregation even below their CMC concentrations.\textsuperscript{141,148,150} In some cases this was through partial competitive adsorption. In other cases, surfactant molecules bound to the native protein molecules at stoichiometric ratios, and the resulting complexes were resistant to aggregation.\textsuperscript{124,151,152}

The silicone oil-water interface is commonly encountered by therapeutic proteins during storage in drug product containers.\textsuperscript{62} Silicone oil is widely used as a lubricant for the plunger in pre-filled syringes, and for stoppers for glass vials.\textsuperscript{62} In pre-filled syringes, formation of protein particles has been linked to the presence of silicone oil,\textsuperscript{54,62,66,153} and conformational changes have been observed in a number of monoclonal antibodies upon adsorption to the silicone oil-water interface.\textsuperscript{54,67,154} Proteins have been shown to form gels at other oil-water (e.g., coconut oil-water) interfaces but this phenomenon has not yet
been characterized at the silicone oil-water interface.\textsuperscript{155} We hypothesize that formation of protein gels at the silicone oil-water interface occurs rapidly and that such gel formation contributes to protein aggregation and particle formation.

Interfacial gel formation by proteins can be monitored by interfacial shear rheology measurements.\textsuperscript{70,71} The data obtained from these experiments can provide important insights into the formation of interfacial layers and can help characterize viscoelastic materials.\textsuperscript{145} In one approach, an oscillatory electromagnetic force is applied to a magnetized rod located on an interface of interest, and the resulting frequency-dependent interfacial deformation is measured.\textsuperscript{156} Interfacial gel formation by protein molecules adsorbed at the interface can be determined by comparing the shear elastic (storage) and viscous (loss) moduli.\textsuperscript{56} In the current study, we used interfacial shear rheology to determine the concentration-dependent time required for a monoclonal antibody (mAb) to adsorb and form a viscoelastic gel at the silicone oil-water interface.

We also hypothesized that rupture of the interfacial protein gel formed at the silicone oil-water interface would result in mAb aggregates in the bulk aqueous phase, similar to the results observed by Rudiuk et al.\textsuperscript{146}. We used micro flow imaging (MFI) and resonance mass measurement (RMM) to characterize the concentrations and sizes of sub-visible particles. We also used size exclusion chromatography (SEC) to observe loss of mAb monomers and formation of soluble aggregates. Further, we tested the effectiveness of PS 20 and PS 80 at inhibiting aggregation induced by exposure of the mAb to the silicone oil-water interface.

The model of interfacial gel rupture presented here is applicable to therapeutic proteins that are stored in prefilled syringes or vials with siliconized stoppers and are
exposed to the silicone oil-water interface during their shelf life. Perturbation of the protein gel layer has been shown as the major cause of protein aggregation in various studies such as rotation of prefilled syringes\textsuperscript{153} and agitation of protein formulations.\textsuperscript{56} However, the complexity of those models makes a mechanistic understanding difficult. The major benefit of this model is the direct study of mechanical rupture of the interfacial gel formed at the silicone oil-water interface. Conversely, the simplistic experimental setup is a significant limitation of this study. The mechanical impact on the protein solution is unlikely to directly relate to real world stresses. Therefore, this model should be used in conjunction with other models to fully understand the phenomena of aggregate formation resulting from the interaction of protein, interfaces, and agitation.

**Materials and Methods**

**Materials**

Purified mAb was provided by MedImmune (Gaithersburg, MD) in a lyophilized formulation. The lyophilized material was reconstituted with 2.2 mL of water for injection (WFI) to obtain 50 mg/mL mAb in 10 mM histidine, 6% (wt/vol) trehalose, 2% (wt/vol) arginine and 0.025% (wt/vol) PS 80 at pH 6.0. Following reconstitution, the mAb solution was dialyzed against 10 mM histidine buffer at pH 6.0. The stock protein solution was then diluted by a factor of 50 to obtain the 1 mg/mL solution used in the studies. Dialysis is not a robust method to remove PS 80 from solution, however the dilution step resulted in a maximum PS 80 concentration of 0.0005% (wt/vol). It is assumed this low concentration of PS has a minimal impact. This was the starting material for the experiments carried out below and used as the control during the polysorbate study.
USP grade reagents such as L-histidine, silicone oil (50 cst) were purchased from Fisher Scientific (Fair Lawn, NJ). Silicone oil (1000 cst) used to study rupture of interfacial gels was of medical grade and purchased from Dow Corning (Midland, MI). Unless otherwise indicated, deionized MilliQ® water was used to prepare all solutions. Lyophilization vials (2 mL) and caps were purchased from West Pharmaceutical (Lionville, PA). The rotating mixer was purchased from Appropriate Technical Resources (Laurel, MD).

Interfacial shear rheology measurement

In order to study gelation of the mAb at the silicone oil-water interface, a custom-built interfacial shear rheometer was used as previously described. In this experiment, mAb solution was placed in a glass channel (length x width = 15 cm x 1 cm), which was placed in a glass container. A magnetic rod (diameter x length = 0.06 cm x 2.54 cm), with anodized black and white stripes was inserted in the middle of a 5 cm polytetrafluoroethylene (PTFE) tube (Small-parts.com). The inner diameter of the PTFE tubing was 0.0635 cm. Both ends of the PTFE tubing were sealed with paraffin wax. The magnetic rod assembly was aligned in the middle of a glass channel that contained 40 mL of mAb solution. An aliquot (6mL) of silicone oil (50 cst) was layered on top, in order to cover the entire area of the protein solution in the glass channel. The magnetic rod assembly remained suspended at the silicone oil-water interface.

As previously described, oscillatory forces were applied on the rod by electromagnetic coils placed on each side of the glass channel. Due to the applied force, the magnetic rod moved back and forth, and sheared the silicone oil-water interface. The applied forces were proportional to the difference in currents between the two
electromagnetic coils. Further, this was used to determine the applied stress. A charge-coupled device (CCD) camera was used to track the rod’s motion. The motion was later used to determine the resulting strain. The rheological parameters were calculated using the following equations

\[ G' = \frac{|\sigma|}{|\gamma|} \cos \phi \]  

(4)

and

\[ G'' = \frac{|\sigma|}{|\gamma|} \sin \phi \]  

(5)

Here, G’ and G” are the elastic (storage or solid like) and viscous (loss or liquid like) moduli respectively. In these equations, \( \sigma, \gamma \) and \( \phi \) represent stress, strain and phase angle (the difference between the rod response and the applied force), respectively.

For buffer in the absence of protein, the elastic modulus (G’) is smaller than the viscous modulus (G”). If the added mAb forms a gel at the interface, the initially smaller G’ will surpass G”. Therefore, the interfacial gel transition time can be determined by the crossover time between G’ and G”.

Before each measurement, the glass channel was soaked for 1 hour in a mixture of sulfuric acid and hydrogen peroxide (vol:vol = 2:1) to remove any surface contaminants. Also, this procedure helped to maximize the hydrophilicity of the glass surface and thus ensured that the magnetic rod assembly remained at the center of the glass channel. The surface of magnetic rod assembly was rinsed with ultra-pure water and wiped with 100% ethanol to remove protein or other residue. Then the assembly was placed in a magnetic coil (0.06 Tesla) for 1 hour to magnetize the rod. For buffer solutions containing 10 mM histidine, pH 6.0, the rheometer was calibrated by varying
the frequency of the sinusoidal current applied to the coils (from 0.011 to 4 Hz), resulting in a sinusoidal motion of the magnetic rod that was suspended at the silicone oil-water interface above 40 mL of buffer only. After calibration, a specific volume of solution was removed, taking care not to disturb the suspended magnetic rod assembly. This aliquot was replaced by same volume of mAb solution to obtain different mAb concentrations in the channel. The protein solution was transferred by carefully placing the pipette tip into the aqueous solution phase in the channel and expelling the solution, followed by a single rinsing of the tip. Afterwards, the pipette tip was carefully removed the channel, and then the rheology measurement was started immediately. mAb solutions containing 5, 25 and 50 µg/mL were analyzed. Maintaining a constant volume of solution assured that the interface on which the magnetic rod was suspended would return to its previous height, thus avoiding the need to refocus the CCD camera. During the rheology measurements, three different current amplitudes were analyzed at a constant frequency of 0.125 Hz. The measurement was typically carried out over 8 hours.

**Rupture of interfacial mAb gels formed at the silicone oil-water interface**

In order to study whether rupture of the interfacial protein gel resulted in increased levels of mAb aggregates in the bulk aqueous liquid, 1 mL of mAb at 1 mg/mL in 10 mM histidine, pH 6.0 was added into 2 mL vials and 200 µL of silicone oil (1000 cst) was layered on top of the mAb solution. The concentration of silicone oil used here was higher than that encountered by therapeutic proteins formulated in prefilled syringes. However, this level of oil was required in our studies to form the water-silicone oil interface in the samples. The vials were then closed with a stopper and a 25 G needle (Becton Dickinson and Company) was positioned asymmetrically within the vial, which
was tilted so that the needle would penetrate and recede from the silicone oil-water interface each time the vial was rotated\textsuperscript{146} (Fig. 15). The vials were then placed on a rotating mixer, which was set at 30 rpm. As a control samples, vials containing mAb solution and a silicone oil layer were set up with the needle completely going through the solution such that it would not rupture the interface (Fig. 15). Unlike the test samples, the “no rupture” control samples are in contact with the stopper, which may have an impact on the results. In another control, vials containing mAb solution and a silicone oil layer without a needle were also set up on the rotating mixer. Samples removed from the vials were analyzed on days 0, 1, 3 and 5 using the techniques mentioned below. The protein samples were pulled using a pipette by going through the silicone oil layer. Triplicate vials were prepared and studied for each time point and each interface-rupturing condition.

![Interfacial gel rupture diagram](image)

Figure 15: Interfacial gel rupture, no rupture and no needle setup.
Micro-Flow imaging for particle counting

mAb samples and controls were analyzed for sub-visible particles of sizes greater than or equal to 1µm using microflow imaging (MFI, model # DPA 4100, Protein Simple, Santa Clara, CA). The samples were analyzed on days 0, 1, 3 and 5. The instrument was configured in set-point 3 mode, and a 100 µm flow cell (Part number: 4002-002-001) was used. The total volume of sample dispensed into the flow cell was 0.5 mL, and 0.15 mL of sample was allowed to flow through the cell prior to acquisition of data. Each sample was analyzed once.

Resonance mass measurement (RMM) of particles

Analysis of protein particles and silicone oil droplets was carried out using an Archimedes particle metrology system (Affinity Biosensors, Santa Barbara, CA). A ‘micro’ format resonant mass sensor (channel cross section 8 x 8 µm², resonant frequency 400 kHz) measured particles in a size range 0.2 – 4 µm diameter. For protein particle measurement, a density of 1.4 g/mL was assumed, and for silicone oil droplets, a density value of 0.97 g/mL was used. Triplicate samples were measured for each type of sample on days 0, 1, 3 and 5. ParticleLab software version 1.8.510 was used to obtain particle concentration for different samples. The data were reported as total particle mass for silicone oil and for protein different types of particles detected by the instrument.

Size-exclusion chromatography

mAb samples and controls were analyzed for loss of soluble mAb and soluble high molecular weight species using SEC. A Tosoh TSKgel G3000SW xl column was used. The samples were centrifuged at 14,000g for 10 minutes, and the supernatant was loaded onto the column. Protein in the eluate was quantified using absorbance at 280 nm.
Prior to loading the sample, the column was equilibrated with 0.1 M Na₂SO₄, 0.1 M Na₂HPO₄, pH 6.8, which was also used as the mobile phase at a flow rate of 1 mL/minute. The samples were analyzed on days 0, 1, 3 and 5. Percent recoveries of soluble mAb and percent soluble aggregates were calculated by normalizing against total peak area of chromatograms for day 0 samples. The day 0 samples represent the protein present in the bulk aqueous solution immediately after the silicone oil was layered onto the aqueous phase.

**Rupture of interfacial mAb gels at the silicone oil-water interface in formulations containing surfactants**

mAb formulations (1mg/mL) were prepared that contained PS 20 or PS 80, each at concentrations of 0.01% (w/v) or 0.001% (w/v). The solutions were prepared using aqueous 10% (w/v) solutions of the surfactants purchased from Thermo Scientific (Rockford, IL). Each stock solution was diluted to 0.1% (w/v) by weighing the appropriate amount of polysorbate followed by addition of buffer. The stock of 0.1% (w/v) was used to prepare different mAb formulations. In order to obtain the desired surfactant concentration, the surfactant solution was pipetted directly into the dialyzed mAb solution using a positive displacement pipette. Samples were analyzed by MFI, RMM and SEC (described above) on days 0, 1, 3 and 5.

**Results**

**Interfacial shear rheology**

In figure 16A, it can be observed that the elastic modulus (G’) crossed over the viscous modulus (G’”) within 1 hr as a 50 µg/mL mAb gelled at the silicone oil-water
interface. We also tested other mAb concentrations (Fig. 16B) and observed that gelation time varied inversely with protein concentration.

Rheology measurements were attempted for mAb solutions that contained surfactants. However, due to the low interfacial tension for these solutions, the magnetic rod would not remain suspended at the silicone oil-water interface. Addition of 1 µL of surfactant at 0.001% (w/v) resulted in the magnetic rod dropping into the aqueous solution, even in cases where the protein gel layer was allowed to form overnight before the surfactant was added. Thus, interfacial shear rheology data in the presence of surfactants were not available.

![Figure 16](image)

**Figure 16:** (A) Dynamic interfacial shear moduli as a function of aging time at 50 µg/mL protein concentration. Black open circles represent elastic modulus (G') and Red solid squares represent viscous modulus (G''). (B) Gelation time as a function of protein concentration. Data points represent the mean ± SD for triplicate samples. Error bars for certain data points are smaller than symbols.

**Rupture of interfacial mAb gels at the silicone oil-water interface**

*Micro-flow imaging for sub-visible particles -*

Rupture of the interfacial gel formed at the silicone oil-water interface resulted in a significant increase in sub-visible particles in the samples. Figure 17 A, B and C shows the particle distribution for interfacial gel rupture, no rupture and no needle samples,
respectively, over a period of 5 days. Figure 17D shows the total particle (≥ 1µm) counts for different sample types at different time points. The particle count increased by more than two orders of magnitude for mAb samples in which the interface was ruptured. The control samples when the interface was not ruptured or when no needle was present also showed an increase in sub-visible particles. However, the increase was less than an order of magnitude, much less than that observed for the ruptured samples.

We used microparticle aspect ratio determined by MFI analysis to distinguish between protein and silicone oil particles. The aspect ratio is the ratio of the lengths of a particle’s minor axis to its major axis; its value can range between zero and one. Experimentally, approximately spherical oil droplets are typically observed to have an aspect ratio >0.85 for particles greater than approximately 5 microns. Aspect ratio is not a consistent measure of shape for small particles due to the limited number of pixels in the image. Figure 18 shows the aspect ratio of the particles ≥5µm as detected by MFI for interfacial gel rupture samples as a function of time. Although particle counts increased for all aspect ratios, there was a greater increase in non-spherical particles than spherical ones (Fig. 19). This indicates a greater increase in protein particles than silicone oil droplets. Also, not all the particles detected by MFI appear to be completely proteinaceous or oil droplets; many contained both protein and oil.
Figure 17: Particle distribution results from MFI for (A) Interfacial gel Rupture, (B) No Rupture and (C) No Needle. Black squares represent day 0 samples, red triangles represent day 1 samples, green inverted triangles represent day 3 samples and blue diamonds represent day 5 samples. (D) Total particles greater than or equal to 1 µm as detected by MFI. Data points represent the mean ± SD for triplicate samples. Error bars for certain data points are smaller than symbols.
Figure 18: Aspect ratio of sub-visible particles as detected by MFI for interfacial gel rupture samples. Black squares represent day 0 samples, red triangles represent day 1 samples, green inverted triangles represent day 3 samples and blue diamonds represent day 5 samples. Data points represent the mean ± SD for triplicate samples. Error bars for certain data points are smaller than symbols.
Resonance mass measurement (RMM) for particle analysis -

Repeated rupture by the needle of the silicone oil-water interface resulted in large increases in the protein particle mass (Fig. 20A). In contrast, the protein particle mass detected for control samples (no rupture and no needle samples) remained relatively low and constant during the 5 day (Fig. 20A). Figure 20B shows the total silicone oil particle mass detected for different samples at various experiment time points. The total silicone oil particle mass during incubation of the gel rupture samples. However, for control samples, there was a slight increase in the silicone oil particle mass, detected on day 1, following which it remained constant for day 3 and day 5 samples.

Figure 19: MFI images for one of the interfacial gel rupture samples on day 5.
Size exclusion chromatography -

During incubation with rupture of the silicone oil-water interface there was a time-dependent reduction in soluble mAb, with about 30% loss after 5 days (Fig. 21). In contrast, there was no loss of soluble mAb detected by SEC for control samples (no rupture and no needle), even after 5 days (Fig. 21). Soluble aggregates were not detected in any of the samples (data not shown).

Figure 20: (A) Total protein particle mass and (B) Silicone oil droplet mass as detected by resonance mass measurement for different samples at different time points. Data points represent the mean ± SD for triplicate samples. Error bars for certain data points are smaller than symbols.
Figure 21: Percent soluble protein recovery as determined by size exclusion chromatography (SEC) at different time points. Solid circles represent No Needle samples, open circles represent No Rupture samples and open squares represent Interfacial gel rupture samples. Data points represent the mean ± SD for triplicate samples. Error bars for certain data points are smaller than symbols.

**Effect of surfactants on interfacial gel rupture at the silicone oil-water interface**

*Micro-flow imaging for sub-visible particles -*

mAb formulations containing surfactants above (0.01% PS 20 and PS 80) and below (0.001% PS 20 and PS 80) their CMC levels were subjected to interfacial gel rupture over a period of 5 days. When PS 20 and PS 80 were present at 0.01% (w/v) in the mAb formulation, they provided protection against interface induced aggregation, resulting in reduced number of particles compared to the surfactant free formulation (Fig. 22). When PS 20 and PS 80 were present at low concentration level of 0.001% (w/v), there was still partial inhibition of particle formation, with greater inhibition observed with PS 80 than with PS 20 (Fig. 22). We carried out statistical analysis by performing ANOVA Dunnett’s multiple comparison tests to compare the total particle concentration in samples with PS to the control without surfactant at various time points, defining significant change as \( p \text{ values } \leq 0.05 \). The differences were significant for all surfactant-
containing formulations except for the 0.001% PS 20 formulation, at all tested time points. Figure 23 illustrates some typical MFI images for the gel rupture samples in the presence of surfactants on day 5. As it could be seen from the images, both silicone oil droplets and protein particles were present in the samples, but oil droplets with visible aggregates of protein clumped onto them were not observed.

Figure 22: Total particle concentration greater than or equal to 1 μm as detected by MFI for interfacial gel rupture samples in different formulations at different time points. Data points represent the mean ± SD for triplicate samples. Error bars for certain data point are smaller than symbols.
Figure 23: MFI images of sub-visible particles observed for interfacial gel rupture samples following 5 days for formulations containing (A) 0.001% PS 20, (B) 0.001% PS 80, (C) 0.01% PS 20 and (D) 0.01% PS 80.
Resonance Mass Measurement (RMM) for particle analysis -

Figure 24 A and B shows total protein particle and silicone oil droplet mass for different formulations containing polysorbate over a period of 5 days. The concentration of total protein particle mass measured by RMM (Fig. 24A) showed a good correlation with total particle counts determined using MFI (Fig. 22). Also, all of the formulations showed an increase in silicone oil droplet mass.

Figure 24: (A) Total protein particle mass and (B) Silicone oil droplet mass as detected by RMM for Interfacial gel rupture samples in different formulations at different time points. Data points represent the mean ± SD for triplicate samples. Error bars for certain data point are smaller than symbols.
Size exclusion chromatography -

In interface rupture samples containing 0.001% PS 20 and PS 80, soluble aggregates were detected by SEC (Fig. 25), with lower levels observed with PS 80 than PS 20. In surfactant-free formulations, SEC analysis detected loss of mAb monomer, but no soluble aggregates. mAb formulations containing 0.01% PS 20 or PS 80 had no detectable loss of soluble protein, nor were any soluble aggregates detected.

![](image)

Figure 25: (A) Percent soluble protein recovery as determined by size exclusion chromatography (SEC) at different time points. (B) % high molecular weight species observed using size exclusion chromatography. Black circles represent control formulation, red squares represent 0.01% PS20, green triangles represent 0.01% PS80, blue inverted triangles represent 0.001% PS20 and orange diamonds represent 0.001% PS80. Data points represent the mean ± SD for triplicate samples. Error bars for certain data points are smaller than symbols.

Discussion

The silicone oil-water interface is relatively hydrophobic, and thus proteins such as monoclonal antibodies have a tendency to adsorb to it. We found that the mAb we studied formed a viscoelastic gel at the silicone oil-water interface (Fig. 16). This is consistent with other reports demonstrating that adsorbed proteins may form viscoelastic films at oil-water interfaces. These earlier studies focused on relatively
small, globular proteins like bovine serum albumin, lysozyme, human serum albumin, β-lactoglobulin and β-casein. It has been suggested that interfacial gels of these smaller proteins are stabilized by protein intermolecular interactions at the interface and this mechanism should be applicable to monoclonal antibodies.\textsuperscript{50,61} We found that the rate of viscoelastic gel formation by the mAb on the silicone oil-water interface was protein concentration-dependent (Fig. 16B), consistent with a prior study carried out on β-casein at the air-water interface that showed a direct correlation between protein concentration and time for gel formation.\textsuperscript{145} Extrapolating from Figure 16B to therapeutically relevant protein concentrations (e.g. 50 mg/mL), it can be inferred that there would be nearly instantaneous gelation of mAb at the silicone oil-water interface under those conditions.

Previous studies of the behavior of protein molecules adsorbed to the air-water interface have shown that rupture of protein layers formed at air-water interfaces results in protein aggregates in the bulk liquid phase.\textsuperscript{146} Similarly, in our study we showed that rupture of viscoelastic gel formed at the silicone oil-water interface resulted in particle formation (Fig. 17) that was detected in the aqueous phase. These particle levels increased as the interface was repeatedly ruptured. In samples where the interface was not ruptured, much lower levels of particles were observed. Particle formation in the controls may be due to interface perturbations caused by the agitation alone or interactions with the stopper surface in the “no rupture” case. However, the interface perturbation caused by needle rupture caused a much more significant increase in particle formation. These observations are similar to findings by Rudiuk et al. for their system with the air-water interface.\textsuperscript{146} In another study, it has been shown that the gelled protein aggregates from the interfaces (silicone oil-water and air-water) in prefilled syringes can
get transported into the bulk during agitation.\textsuperscript{153} Taken together the results suggest that rupture of gels of protein molecules formed at interfaces may be a common means of protein particle formation, though studies of a broader range of proteins would be required to confirm this mechanism.

Particles and aggregates formed by rupture of the interfacial gel exhibited irregular, elongated morphologies (Fig. 19), consistent with their formation by rupture of a viscoelastic gel/film. An earlier study carried out on human growth hormone and the mechanical stress applied to it at the air-water interface by bubble aeration also resulted in ribbon-like, elongated structures of insoluble protein.\textsuperscript{166} Particles of similar morphologies are commonly observed after agitation of protein formulations, presumably due to disruption of interfacial gels at air-water and other interfaces. Furthermore, interfacial gel formation and rupture may occur during bioprocessing steps such as transferring or delivering protein solutions through tubing with peristaltic pumps.\textsuperscript{167}

We tested the effects of the presence of PS 20 or PS 80 at different concentrations. When PS 20 or PS 80 were present at higher levels (0.01\% w/v), we saw a decrease in mAb particle formation during incubations with interface rupture, with no loss of monomer or soluble aggregates detected by SEC. At surfactant concentrations above CMC levels, both PS 20 and PS 80 conferred similar protection to the mAb. Addition of PS 80 at sub-CMC levels (0.001\%) resulted in partial inhibition of particle formation, whereas sub CMC levels of PS 20 (0.001\%) did not significantly inhibit particle formation. Presumably, the inhibition of mAb adsorption to the silicone oil-water interface at higher surfactant concentration\textsuperscript{154} prevented interfacial gel formation and resulting particle formation during interfacial rupture. In our experiments, the low
interfacial tensions found in the presence of polysorbates precluded study of the surface rheology of mAb in the presence of surfactants. However, a previous study that used a double wall ring rheometer to explore lysozyme gelation on a coconut oil-water interface showed that PS 80 at a concentrations above its CMC prevented formation of viscoelastic films.\textsuperscript{155}

In our study, we observed soluble aggregates during rupture of the oil-water interface of samples containing 0.001\% PS 20 and PS 80 (Fig. 25). However, no soluble aggregates were detected during rupture of the interface in the samples without surfactant. A similar observation was made in a study of agitation of recombinant Factor XIII by Kreilgaard L. et. al.\textsuperscript{126} They suggested that soluble aggregates were formed and increased in concentration during agitation because the surfactant inhibited the conversion of protein oligomers into insoluble aggregate.\textsuperscript{126} We speculate that a similar mechanism might be applicable to our system in which soluble aggregates were formed during rupture of the oil-water interface in surfactant containing samples.

In our study, we also observed that, at lower concentration (0.001\%), PS 80 was more effective than PS 20 at reducing particle formation. Structurally, the only difference between PS 20 and PS 80 is in their fatty acids, with PS 20 containing primarily lauric acid and PS 80 containing oleic acid.\textsuperscript{149} This makes PS 80 more hydrophobic than PS 20, and confers a lower CMC (0.0017\% for PS80 vs. 0.007\% for PS20). Thus, at a concentration of 0.001\%, the interfacial concentration of PS 80 molecules is expected to be higher than that of PS 20, more effectively hindering the mAb from adsorbing to the interface. In contrast, at higher surfactant concentrations, the interface is expected to be
saturated for both surfactants, and as expected they were equally effective at preventing mAb particle formation and aggregation.

**Conclusion**

Our study showed that the mAb investigated in sub-optimized formulation forms interfacial viscoelastic gel upon adsorption to the silicone oil-water interface. In formulations that are marketed in pre-filled syringes, therapeutic proteins typically are exposed to the silicone oil-water interface for their entire shelf-life. During this period, in sub-optimized formulations we expect that viscoelastic gels could form at the silicone oil-water interface. Further, similar to earlier observations by Rudiuk et al. for the air-water interface we showed that mechanical rupture of the interfacial gel can lead to protein aggregation and particle formation in the bulk aqueous phase. Related mechanical stresses are often imposed on the interface during shipping and handling of protein formulations which may cause the formation of particles and aggregates due to rupture of the interfacial gels. As we have shown here and in earlier studies, the use of surfactants in protein formulations can reduce protein aggregation and particle formation, a phenomenon that may be in part related to the ability of surfactant to interfere with gel formation at interfaces.
CHAPTER IV

COLLOIDAL INSTABILITY OF SUB-VISIBLE PARTICLES FORMED FROM INTERFACIAL GEL RUPTURE AT THE SILICONE OIL-WATER INTERFACE CAN PROMOTE FURTHER PARTICLE GROWTH

Introduction

Aggregation of therapeutic proteins is of great concern in the field of pharmaceutical biotechnology. The presence of protein aggregates in a formulation can lead to decreased product efficacy and may cause adverse reactions in patients such as unwanted immune response and infusion reactions.\textsuperscript{16,168} Aggregates can include soluble oligomers that can be detected by size exclusion chromatography.\textsuperscript{27} Larger aggregates, which are detected by various particle counting and light scattering techniques, are often referred to as particles to differentiate them from smaller oligomeric aggregates.\textsuperscript{27} To minimize the levels of aggregates and particles, it is important to understand the mechanisms for their formation.

Both the conformational and colloidal stability of a protein can affect its propensity to aggregate.\textsuperscript{17} Even perturbations seemingly as minor as small alterations of tertiary structure can promote aggregation and particle formation.\textsuperscript{17,169} Perturbed tertiary structure may reflect shifts in the ensemble of species in the native state towards aggregation-prone partially unfolded protein molecules.\textsuperscript{39,169} Colloidal instability may also contribute to aggregation propensity. The nature and magnitude of protein-protein interactions determines colloidal stability, which can be determined by measuring the osmotic second virial coefficient ($B_{22}$).\textsuperscript{17} The native structure of the protein and the magnitude of protein-protein interactions are influenced by the presence of cosolutes in
the formulation. For example, early studies carried out on rhIFN-γ and rhGCSF demonstrated that the presence of sucrose and sodium chloride affects their conformational and colloidal stabilities. Ions in solution may screen charge-charge interactions between protein molecules. For example, a study on rhGCSF showed that presence of 150 mM NaCl reduced electrostatic repulsion between protein molecules and fostered aggregation.

A further complication in product development is assuring that the protein formulation is compatible with the container/closure system. This issue is particularly challenging for prefilled syringe formulation development. Prefilled syringes are increasingly used as delivery devices because of their associated improved patient compliance, dose accuracy and ease of administration. Prefilled syringes typically must be lubricated with silicone oil to aid in the syringe plunger movement; a disadvantage of prefilled syringes is unwanted interactions of the protein molecules with the silicone oil-water interface, which can cause protein aggregation.

To mitigate the adverse effects of silicone oil-water interface, it is essential to understand the interactions between the protein molecules and this interface. Proteins readily adsorb to silicone oil-water interfaces. Previous studies have shown that such adsorption caused perturbation of tertiary structures of various humanized monoclonal antibodies. Formulation conditions may influence the extent of structural perturbation and the resulting protein aggregation. For instance, Gerhardt et al. showed that the tertiary structure perturbations of a monoclonal antibody were diminished when the ionic strength of the formulation was increased and concomitant agitation-induced aggregation was reduced. However, Britt et al. and Ludwig et al. showed that protein
adsorption to the silicone oil-water interface increased at higher ionic strength. And Basu et al. showed that protein aggregation during agitation in the presence of a silicone oil-water interface was accelerated in the presence of NaCl.

Earlier, we established that proteins adsorbed at silicone oil-water interface form interfacial viscoelastic gels. Rupture of this interfacial gel (e.g., due to application of mechanical forces) results in aggregation and particle formation. In another study, it was shown that mechanical perturbation by an air bubble of protein layers adsorbed to the silicone oil-water interface resulted in particles in the bulk solution. Together, these studies suggest that a common mechanism leading to appearance of particles in bulk solution is formation and subsequent mechanical perturbation of the gelled protein layer at the silicone oil-water interface.

Interfacial-induced aggregation could also be influenced by both conformational and colloidal instability of the protein. Thirumangalathu et al. found that decreased colloidal stability of a monoclonal antibody led to enhanced aggregation when the protein was agitated in the presence of a silicone oil emulsion. Basu et al. showed that addition of 150 mM NaCl reduced colloidal stability of an antibody, and accelerated aggregation during agitation in the presence of silicone oil-coated glass beads. Similarly, Chou et al. found that the presence of 150 mM NaCl decreased colloidal stability of albiinterferon-α2b, leading to increased aggregation upon agitation.

In the current study, we are investigating how particle formation and growth are affected by the colloidal stabilities of both a monoclonal antibody and of particles formed from silicone oil and protein. We modulated the colloidal stability by changing the ionic strength of our protein solution, and we hypothesized that conditions resulting in lower
colloidal stability in the bulk solution would also create stronger interfacial gels at the silicone oil-water interface. Furthermore, we hypothesized that colloidal stability of particles formed by mechanically perturbing the interfacial gels would dictate their subsequent growth and agglomeration kinetics.

**Materials and Methods**

**Materials**

Purified mAb was provided by MedImmune (Gaithersburg, MD) in a lyophilized formulation. The lyophilized material was reconstituted with 2.2 mL of water for injection (WFI) to obtain 50 mg/mL mAb in 10 mM histidine, 6% (wt/vol) trehalose, 2% (wt/vol) arginine and 0.025% (wt/vol) PS 80 at pH 6.0.

USP grade reagents such as L-histidine, NaCl and silicone oil (50 cst) were purchased from Fisher Scientific (Fair Lawn, NJ). Pierce Slide-A-Lyzer cassettes (Thermo Scientific, Rockford, Illinois) with 10 kDa molecular weight cutoff were used for dialyzing protein. Silicone oil (1000 cst) used to study rupture of interfacial gels was of medical grade and purchased from Dow Corning (Midland, MI). Unless otherwise indicated, deionized MilliQ® water was used to prepare all solutions. Lyophilization vials (2 mL) and caps were purchased from West Pharmaceutical (Lionville, PA). The rotating mixer was purchased from Appropriate Technical Resources (Laurel, MD).

**Preparation of protein formulations**

Following reconstitution of the lyophilized formulation with distilled water, the mAb solution was dialyzed into various formulations. The formulations tested for this particular study were 10 mM His and 10 mM His with various NaCl concentrations: 50, 100, 150 and 230 mM. The solution ionic strengths were, respectively, 1.25 mM, 51.25
mM, 101.25 mM, 151.25 mM and 231.25 mM. Each dialyzed protein solution was diluted to a final protein concentration of 1 mg/mL, unless otherwise indicated.

Rupture of interfacial mAb gels formed at the silicone oil-water interface

The experimental setup used to study the effects of NaCl on rupture of the interfacial protein gel on mAb aggregate and particle formation has been described previously. Briefly, 1 mL of protein solution at 1 mg/mL and 200 µL of silicone oil (1000 cst) was layered on top of the mAb solution. Different ionic strength formulations as mentioned above were prepared for interfacial gel rupture and control studies as described previously. However, for this particular study, we carried out two sets of experiments at 15 rpm and 30 rpm, with frequency of mechanical rupture of 0.25Hz and 0.5Hz respectively. Triplicate vials were prepared and studied for each time point and each interface-rupturing condition. Samples were removed from the vials and analyzed on days 0, 1, 2 and 3 using the techniques mentioned below. Therefore, for each treatment type and each time point triplicate samples were analyzed by each of the methods described below.

Micro-Flow imaging for particle counting

Interfacial gel rupture samples and controls were analyzed for sub-visible particles of sizes ≥1µm using microflow imaging (MFI, model # DPA 4100, Protein Simple, Santa Clara, CA). The instrument was configured in set-point 3 mode, and a100 µm flow cell (Part number: 4002-002-001) was used. The total volume of sample dispensed into the flow cell was 0.5 mL, and 0.15 mL of sample was allowed to flow through the cell prior to acquisition of data. Each sample was analyzed once.
Resonance mass measurement (RMM) of particles

Analysis of protein particles and silicone oil droplets was carried out using an Archimedes particle metrology system (Affinity Biosensors, Santa Barbara, CA). A ‘micro’ format resonant mass sensor (channel cross section 8 x 8 µm², resonant frequency 400 kHz) measured particles in a size range 0.2 – 4 µm diameter. For protein particle measurements, a density of 1.4 g/mL was assumed, and for silicone oil droplets a density value of 0.97 g/mL was used. ParticleLab software version 1.8.510 was used to obtain particle concentration for different samples. The data were reported as total particle mass for silicone oil and for protein as detected by the instrument.

Size-exclusion chromatography

Interfacial gel rupture samples and controls were analyzed for loss of soluble mAb and soluble high molecular weight species using SEC. A Tosoh TSKgel G3000SWxl column was used, and protein in the eluate was quantified using absorbance at 280 nm. Prior to loading the sample, the column was equilibrated with 0.1 M Na₂SO₄, 0.1 M Na₂HPO₄, pH 6.8, which was used as the mobile phase at a flow rate of 1 mL/minute. Percent recoveries of soluble mAb and percent soluble aggregates were calculated by normalizing against total peak area of chromatograms for day 0 samples of each formulation condition.

Post rupture-quiescent study to investigate the effect of formed particles

Protein formulations at 1 mg/mL containing no NaCl and the one containing 230 mM NaCl were subjected to interfacial gel rupture at a frequency of 0.25Hz over a period of 1 day as described above. Following day 1 of interfacial gel rupture, the samples were removed from the rotating mixer and allowed to incubate quiescently at room
temperature for the next 13 days. Samples were analyzed for sub-visible particles at various time points over a period of 14 days using MFI, RMM and SEC as described above. For control, samples were incubated quiescently without rupture over a period of 14 days at room temperature. Triplicate samples were prepared and analyzed for each time point.

Zeta potential measurements

A Zeta-sizer Nano ZS (Malvern, U.K) was used to measure the electrophoretic mobility and calculate the zeta potential of mAb in different ionic strength and that of the sub-visible particles arising after 1 day of interfacial gel rupture. A disposable capillary zeta potential cell equipped with gold electrodes (Malvern Instruments Ltd.) was used and each sample type was analyzed in triplicates. Protein formulations containing no NaCl and the one containing 230 mM NaCl were subjected to interfacial gel rupture over a period of 1 day as described above. After 1 day, the samples were centrifuged at 14,000 x g for 10 mins. The supernatant was removed and the pellet was resuspended in either 10 mM His or 10 mM His+230 mM NaCl buffer to obtain zeta potential. As a proof of concept, zeta potential was also obtained for silicone oil emulsion. Silicone oil emulsion was prepared either in water or in 10 mM His buffer by sonicating 5% (v/v) silicone oil in water or buffer at 50% power for 5mins. Following preparation, zeta potential measurements were carried out immediately.

Further, we applied DLVO theory in order to investigate the effect of electrostatics in interaction between protein-silicone oil particles. The interaction potential between two particles can be influenced by both van der Waals attractive and double layer repulsive forces as given in equation 6: \[ \text{\ldots} \]
\[ W(D) = \frac{\varepsilon r_1 r_2 (\psi_1^2 + \psi_2^2)}{4(r_1 + r_2)} \left[ \frac{2\psi_1 \psi_2}{\psi_1^2 + \psi_2^2} \ln \left( \frac{1 + e^{-\kappa D}}{1 - e^{-\kappa D}} \right) + \ln \left( 1 - e^{-2\kappa D} \right) \right] - \frac{A_H r_1 r_2}{6(r_1 r_2) D} \]  

where \( \varepsilon \) is the dielectric constant of water, \( r_i \) is radius of particle \( i \), \( \psi_i \) is surface potential of particle \( i \), \( \kappa \) is inverse debye length, \( D \) is the interparticle separation distance, and \( A_H \) is the Hamaker constant. In this study, we have illustrated interaction potential calculation between two 1µm particles and have assumed the zeta potential as the surface potential of the two particles. The Hamaker constant is approximately 5 kT for proteins.\(^{178,181}\) The Debye length in 10 mM His, pH 6 is about 8.6 nm.\(^{178}\)

**Interfacial shear rheology measurement**

In order to study gelation of the mAb at the silicone oil-water interface as a function of NaCl concentration, a custom-built interfacial shear rheometer was used and instrument calibration, data acquisition method and results analysis approach were as previously described.\(^{56,145,157,177}\)

The buffer calibration file was used for data analysis for protein solution in each formulation condition. For protein analysis on the interfacial rheometer, 40 mL solution of protein at 50 µg/mL was prepared in various NaCl buffers. Then, the rheology measurement was started immediately. The measurement was typically carried out over 20 hours.

In order to quantify the effect of NaCl on elastic modulus, which is an indication of strength of interfacial gel, we used an exponential one phase decay equation to fit the curve of elastic modulus versus time in order to obtain a plateau value. The curve fitting was carried out using graphpad prism (Graphpad software Inc., La Jolla, CA).
Static Light scattering to measure osmotic second virial coefficient ($B_{22}$)

We used a Brookhaven light scattering system (Brookhaven Instruments Corporation, Holtsville, NY) to make static light scattering (SLS) measurements. Protein was dialyzed overnight in different ionic strength formulations. Protein samples were prepared at concentrations ranging from 0.5-5.0 mg/mL in different ionic strength formulations, and the scattering intensity was measured at 90°. Triplicate samples were prepared for each formulation condition, and scattering intensity was acquired for each sample replicate. All buffers were filtered using 0.02 µm Anotop 25 syringe filters (Whatman International Ltd) and the protein solutions were filtered using 0.22 µm syringe filters. The relationship used to determine $B_{22}$ is derived from the virial expansion of the ideal osmotic pressure equation as described previously.$^{105,169}$

$B_{22}$ values were normalized by the theoretical value of the hard sphere (HS) second virial coefficient to obtain $B_{22}/B_{22}^{HS}$ with $B_{22}^{HS}$ given by,

$$B^{HS} = \frac{16 \pi N R^3}{3 M^2} \quad (7)$$

Where N is the Avogadro number, M is molecular weight of protein and R is the hard sphere radius of the protein monomer.$^{182}$ As an estimate of the hard sphere diameter, the hydrodynamic diameter (11.4 nm) was determined using dynamic light scattering (DLS) as described below.

Dynamic light scattering (DLS) to determine the interaction parameter ($k_D$)

DLS measurements were performed at room temperature using a Zeta-sizer Nano ZS (Malvern, U.K). Protein samples were prepared at concentrations ranging from 0.5 – 5.0 mg/ml in the formulations with various concentrations of NaCl. The mutual diffusion coefficient, $D_m$ ($\mu m^2/s$) of the monomeric protein peak was plotted against the protein
concentration (mg/mL) to obtain the interaction parameter, $k_D$ according to the following equation:

$$D_m = D_s (1 + k_D C)$$  \hspace{1cm} (8)

where $D_s$ is the self-diffusion coefficient (the value of $D_m$ at infinite dilution) and $c$ is the protein concentration. The slope of the plot of $D_m$ versus $c$ gives the interaction parameter $k_D$ and intercept would provide $D_s$. The interaction parameter, $k_D$ is used to describe interparticle interactions and colloidal stability of protein molecules according to the following equation:

$$k_D = 2MB_{22} - k_f - 2\nu$$  \hspace{1cm} (9)

where $M$ is the molecular weight, $B_{22}$ is the second virial coefficient, $\nu$ is the protein partial specific volume, and $k_f$ is the first order concentration coefficient in the virial expansion of the frictional coefficient.

**Differential scanning calorimetry (DSC) to assess protein conformational stability**

The conformational stability and domain unfolding of mAb in the absence and presence of 230 mM NaCl was determined using a VP-capillary differential scanning calorimeter (MicroCal, Northampton, MA). Dialyzed protein samples at 1 mg/mL were analyzed over a temperature range of 10-90°C at a scan rate of 90°C/h with a 15 min pre-scan thermo stating. For each buffer concentration, a buffer baseline was obtained and protein thermograms were obtained after subtracting the buffer baseline using origin software (Originlab Corporation, Northampton, MA). For each sample, three vials of protein solution were prepared and analyzed. The mAb in the absence of NaCl shows three transitions $T_{m,1}$, $T_{m,2}$ and $T_{m,3}$. Further, we have analyzed the reversibility of unfolding of the C$_{H2}$ domain for mAb in 10 mM His+230 mMNaCl by heating the
protein samples up to 55°C and cooling it back to 10°C allowing the protein domains to
refold. Then, the sample was heated again over a temperature range of 10-90°C at a scan
rate of 90°C/h with a 15 min pre-scan thermo stating.

Concomitant DLS-Raman spectroscopy to study protein conformation and aggregation

To study the effect of ionic strength on protein conformation, thermal stability
and aggregation, mAb in different NaCl formulations was subjected to heating using an
instrument with combined Raman spectrometer and DLS.184 Once the protein was
dialyzed, the protein concentration was adjusted to 20 mg/mL using the appropriate
formulation. Thermal stability of mAb was tested by heating the protein from 25°C to
90°C at a rate of 1°C/min and with data acquisition at 1°C intervals. The temperature for
onset of aggregation (T onset) was determined as the temperature where the z-average
hydrodynamic size exceeded the base value of hydrodynamic size at initial temperature
by 25%.184

Urea induced protein unfolding curve to compare conformational stability of mAb

In order to compare the conformational stability of the mAb in absence of NaCl
and in the presence of 230 mM NaCl, urea induced protein unfolding curves were
obtained by using the intrinsic tryptophan fluorescence signal of the protein. Protein
samples at 1 mg/mL were prepared in different urea concentrations and equilibrated
overnight at room temperature before obtaining tryptophan fluorescence emission
spectra. Intrinsic tryptophan fluorescence spectra were acquired using Photon
Technology International (PTI) spectrofluorometer (Lawrenceville, NJ). Tryptophan
(Trp). Excitation was carried out at 295 nm, and emission spectra were collected from
300 to 400 nm with 1 nm/s data collection rate and a 1 s integration time. The slit widths
for Trp excitation and emission were 4 and 2 nm, respectively. The normalized maximum tryptophan fluorescence intensity was plotted as a function of urea concentration. We also calculated the tryptophan fluorescence center of mass by integrating the emission spectrum.  

**Statistical analysis**

We did a t-test to compare the samples without NaCl to the samples containing different concentrations of NaCl. The differences across different NaCl formulations were defined significant change as p values ≤ 0.05.

**Results**

**Rupture of interfacial mAb gels at the silicone oil-water interface**

Rupture of the interfacial gel formed at the silicone oil-water interface resulted in a significant increase in sub-visible particles in the bulk solution. In contrast, for all formulations, the control samples when the interface was not ruptured or when no needle was present did not show any substantial increase in the number of particles over various times points (data not shown). When gel rupture was carried out at frequency of 0.5Hz, it resulted in significant amount of aggregation and particle formation across different ionic strength formulations as detected by MFI, RMM and SEC (Fig. 26). No significant difference in aggregation was seen across different ionic strength formulations except for 230 mM NaCl containing formulation that showed slightly higher level of aggregates on a few time points (days 2 and 3) (Fig. 26). Further, a greater increase in the number of particles was detected MFI and RMM on day 3 across different formulation conditions (Figs. 26A and 26B). In order to further investigate the effect of NaCl on aggregation and particle formation due to interfacial gel rupture, we carried out the gel rupture at 0.25Hz
to lower the frequency of mechanical stress. Earlier, when the mechanical stress was at 0.5Hz, there were substantial numbers of particles due to which no consequential effect of NaCl was observed. However, at lower frequency, we observed few more time points that were significantly different in the presence of NaCl (Fig. 27). But, at the end of day 3, the effect of ionic strength is minimal as the particle concentration was not statistically different (Fig. 27A). Nonetheless, at lower frequency of mechanical stress, we observed a greater increase in the number of particles by MFI and RMM on day 3 across different formulation conditions (Figs. 27A and 27B) similar to the higher frequency of mechanical stress.

Post rupture-quiescent study to investigate the effect of formed particles

The sub-visible particles formed after 1 day of rupture of the interfacial gel were allowed to incubate quiescently over a period of next13 days. For both formulations tested, the sub-visible particles increased over next 13 days upon quiescent incubation as detected by MFI and RMM (Figs. 28A and 28B). The rate of increase in the particles was same in both formulations. No loss of soluble protein was detected by SEC for both formulation conditions (Fig. 28D). For control samples, no increase in particles or loss of soluble protein was detected at the end of 14 days. (Figs. 28E-28H).
Figure 26: Interfacial gel rupture study at 30 rpm. (A) Total particle concentration as detected by MFI, (B) Total protein particle mass and (C) Silicone oil droplet mass as detected by RMM, (D) Percent soluble protein recovery as determined by size exclusion chromatography (SEC) for Interfacial gel rupture samples in different formulations at different time points. Data points represent the mean ± SD for triplicate samples. Error bars for certain data points are smaller than symbols. (*) indicates time points with significant difference (p <0.05) as compared to control (10 mM His) samples.
Figure 27: Interfacial gel rupture study at 15 rpm. (A) Total particle concentration as detected by MFI, (B) Total protein particle mass and (C) Silicone oil droplet mass as detected by RMM, (D) Percent soluble protein recovery as determined by size exclusion chromatography (SEC) for Interfacial gel rupture samples in different formulations at different time points. Data points represent the mean ± SD for triplicate samples. Error bars for certain data points are smaller than symbols. (*) indicates time points with significant difference (p <0.05) as compared to control (10 mM His) samples.
Figure 28: (A-D) Interfacial gel rupture and post rupture-quiescent study and (E-H) no rupture-control samples for mAb in 10 mM His (Black bars) and 10 mM His+230 mM NaCl (Blue bars). (A and E) Total particle concentration as detected by MFI, (B and F) Total protein particle mass and (C and G) Silicone oil droplet mass as detected by RMM at different time points. (D and H) Percent soluble protein recovery as determined by size exclusion chromatography (SEC) at different time points. Data points represent the mean ± SD for triplicate samples. Error bars for certain data points are smaller than symbols. Dashed line is used to represent demarcation between rupture and post rupture-quiescent samples. Note: Day 1 samples are interfacial gel rupture samples following which the samples were incubated quiescently over next 13 days.
Zeta potential measurements

Zeta potential values of monomeric mAb across different ionic strength formulations and that of sub-visible particles formed from interfacial gel rupture are shown in table 3. In the absence of sodium chloride, mAb had a zeta potential value of 7.60 ± 0.28 mV. This value decreased upon addition of NaCl in the formulation and the zeta potential value became neutral at higher ionic strength formulation (Table 3). The sub-visible particles formed from interfacial gel rupture had an overall negative charge may be because particles were a mix of protein-silicone oil; nonetheless the charge was almost neutral on these sub-visible particles with no significant effect of NaCl seen on these particles.

Table 3. Zeta potential values for mAb and sub-visible particles from day 1 in different formulation conditions. Each number represents mean zeta potential and the standard deviation of triplicate measurements.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb (10 mM His, pH6)</td>
<td>7.60 ± 0.28</td>
</tr>
<tr>
<td>mAb (10 mM His+50 mM NaCl)</td>
<td>2.92 ± 0.56</td>
</tr>
<tr>
<td>mAb (10 mM His+100 mM NaCl)</td>
<td>0.62 ± 1.35</td>
</tr>
<tr>
<td>mAb (10 mM His+150 mM NaCl)</td>
<td>1.20 ± 1.21</td>
</tr>
<tr>
<td>mAb (10 mM His+230 mM NaCl)</td>
<td>0.45 ± 1.21</td>
</tr>
<tr>
<td>Silicone oil emulsion in water</td>
<td>-30.16 ± 0.37</td>
</tr>
<tr>
<td>Silicone oil emulsion in 10 mM His</td>
<td>-12.5 ± 0.55</td>
</tr>
<tr>
<td>Sub visible particles (10 mM His+230 mM NaCl) resuspended in 10 mM His+230 mM NaCl</td>
<td>-1.21 ± 0.47</td>
</tr>
<tr>
<td>Sub visible particles (10 mM His+230 mM NaCl) resuspended in 10 mM His</td>
<td>-1.14 ± 0.83</td>
</tr>
<tr>
<td>Sub visible particles (10 mM His) resuspended in 10 mM His</td>
<td>-1.69 ± 0.29</td>
</tr>
</tbody>
</table>
Interfacial shear rheology

In figure 29, it can be observed that for all formulations the elastic modulus (G') was higher than the viscous modulus (G'') over the period of measurement and it reached a plateau after some time. The presence of NaCl led to slightly higher values of elastic modulus (G') and thus stronger interfacial gels\(^{185}\) (Table 4). The differences compared to the value for the control sample without NaCl were statistically significant for samples at all NaCl concentration except for the 100 mM NaCl formulation. Overall, there was minimal effect of NaCl on the strength of interfacial viscoelastic gel.

Table 4. Elastic moduli of interfacial gel formed by adsorption of mAb under different conditions. Each number represents the mean and the standard deviation of triplicate measurements. \(p\)-values are a result of statistical t-test performed to compare the plateau value of elastic modulus at each ionic strength to that of the control (10 mM His) sample.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Ionic strength (mM)</th>
<th>Plateau value from one phase decay curve fit of elastic modulus (G') (mN/m)</th>
<th>(p)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM His</td>
<td>1.25</td>
<td>0.127±0.007</td>
<td>control</td>
</tr>
<tr>
<td>10 mM His+50 mM NaCl</td>
<td>51.25</td>
<td>0.159±0.012</td>
<td>0.005</td>
</tr>
<tr>
<td>10 mM His+100 mM NaCl</td>
<td>101.25</td>
<td>0.160±0.037</td>
<td>0.2</td>
</tr>
<tr>
<td>10 mM His+150 mM NaCl</td>
<td>151.25</td>
<td>0.167±0.014</td>
<td>0.0134</td>
</tr>
<tr>
<td>10 mM His+230 mM NaCl</td>
<td>231.25</td>
<td>0.175±0.011</td>
<td>0.003</td>
</tr>
</tbody>
</table>
Figure 29: Dynamic interfacial shear moduli as a function of aging time at 50 µg/mL protein concentration. Black open circles represent elastic modulus (G') and Red solid squares represent viscous modulus (G''). (A) 10 mM His formulation, (B) 10 mM His+50 mM NaCl, (C) 10 mM His+100 mM NaCl, (D) 10 mM His+150 mM NaCl and (E) 10 mM His+230 mM NaCl.
Colloidal stability of protein molecules as a function of NaCl concentration

The protein-protein interactions for mAb are repulsive in the absence of any NaCl as seen from large positive value of $B_{22}/B_{22}^{HS}$ and positive value of $k_D$ (Fig. 30). Addition of slight amount of NaCl decreased the $B_{22}/B_{22}^{HS}$ values with no change seen up to about 50 mM ionic strength. At ionic strength of 101.25 mM and higher, the $B_{22}/B_{22}^{HS}$ were slightly less than 1 indicating attractive protein-protein interactions; nonetheless they were equivalent to that of the hard sphere values. A similar trend was also observed for $k_D$ as a function of ionic strength. The interaction parameter ($k_D$) was positive for mAb in the absence of NaCl and it decreased upon addition of NaCl and became negative for mAb in 30 mM and higher concentrations of NaCl.

![Figure 30](image)

Figure 30: (A) The second osmotic virial coefficient ($B_{22}$) normalized with the $B_{22}$ of hard sphere for mAb as a function of ionic strength. (B) Interaction parameter ($k_D$) values for mAb in different ionic strength. Data points represent the mean ± SD for triplicate samples. Error bars for certain data points are smaller than symbols.
DSC to assess protein conformational stability

DSC analysis was used to obtain information about domain unfolding and to determine the conformational stability of the protein. Figure 31 shows DSC thermograms for the protein in 10 mM His and in the presence of 230 mM NaCl. Three transitions were observed for the protein without NaCl (Table 5). The first transition (T_{m,1}) observed at around 48.5°C can be attributed to unfolding of the C_{H2} domain.\textsuperscript{11,169} The second and third transitions (T_{m,2} and T_{m,3}) observed at around 71.8°C and at 83.1°C can be attributed to unfolding of F_{ab} region and C_{H3} regions of the mAb, respectively.\textsuperscript{11,169} In the presence of 230 mM NaCl, the first two transitions were shifted towards lower temperature and the third transition was not observed due to precipitation of the protein (Fig. 31A and table 5). We wanted to investigate if the shift in first two transitions was due to aggregation that is often irreversible or a decrease in conformational stability of the protein in the presence of 230 mM NaCl. Thus, we carried out unfolding of the C_{H2} domain by heating the samples up to 55°C and then cooling it back to 10°C to allow refolding of the domains. When the protein was heated again up to 90°C, we observed similar DSC thermogram as observed previously signifying reversibility of unfolding of the C_{H2} domain (Fig. 31B).
Table 5. Thermal unfolding transition for mAb in different formulation conditions as measured by DSC. ND signifies that the transition ($T_{m,3}$) was not observed for that particular sample. Each number represents mean transition temperature ($T_m$) and the standard deviation of triplicate measurements.

<table>
<thead>
<tr>
<th>Condition</th>
<th>$T_{m,1}$</th>
<th>$T_{m,2}$</th>
<th>$T_{m,3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM His</td>
<td>48.5 ± 0.2</td>
<td>71.8 ± 0.4</td>
<td>83.1 ± 0.5</td>
</tr>
<tr>
<td>10 mM His+230 mM NaCl</td>
<td>46.0 ± 0.3</td>
<td>69.4 ± 0.5</td>
<td>-ND-</td>
</tr>
</tbody>
</table>

Figure 31: (A) DSC thermograms for mAb in 10 mM His (solid black line) and mAb in 10 mM His+230 mM NaCl (dashed black line). The mAb shows three transitions marked as $T_{m,1}$, $T_{m,2}$ and $T_{m,3}$. (B) DSC thermograms for mAb in 10 mM His+230 mM NaCl. Protein was heated upto 55°C (solid red line) to observe $T_{m,1}$, and then cooled back to 10°C. The same sample was heated again up to 90°C (dashed black line) to check for reversibility of $T_{m,1}$. 
Thermal stability as a function of ionic strength

Figure 32A shows the Raman spectrum for the mAb in 10 mM His. Raman spectral peaks are characteristic markers for protein secondary and tertiary structure. The Raman band 857 cm$^{-1}$ is a tyrosine band position that can give us an indication of tertiary structure of the protein.$^{184,186}$ We followed this signal during heating to monitor changes in tertiary structure; in the presence and absence of 230 mM NaCl (Fig. 32B). The tertiary structure of mAb changed with increase in temperature when no NaCl was present (Fig. 32B). When mAb incubated in other NaCl concentrations was subjected to thermal ramping, the protein precipitated out at around ~65°C and beyond which data could not be analyzed by raman spectrometer (Fig. 32B and 32C, data not shown for other NaCl concentrations). Also, it can be observed that mAb in the presence of 230 mM NaCl showed a change in tertiary structure between 40-50°C which was not present for mAb in the absence of NaCl (Fig. 32B). Figure 7D shows change in Z-average of mAb in 10 mM His and in the presence of 230 mM NaCl. Figure 7E shows the $T_{\text{onset}}$ for mAb as a function of ionic strength. The $T_{\text{onset}}$ decreased upon increasing the ionic strength. Also, the presence of NaCl promoted protein flocculation.
Figure 32: (A) Raman spectrum for mAb in 10 mM His. (B) Tertiary structure marker position for Tyrosine at 857 cm$^{-1}$ as a function of temperature for mAb in 10 mM His (Black open circles) and for mAb in 10 mM His+ 230 mM NaCl (Red open diamonds). (C) Percent beta-sheet structure change as a function of temperature for mAb in 10 mM His (Black open circles) and for mAb in 10 mM His+ 230 mM NaCl (Red open diamonds). (D) Z-average as a function of temperature for mAb in 10 mM His (Black open circles) and for mAb in 10 mM His+ 230 mM NaCl (Red open diamonds). (E) Temperature of onset of aggregation for mAb in different ionic strength as determined from DLS.
Conformational stability measured from urea unfolding curve

Figure 33 shows urea induced protein unfolding curve for mAb obtained by monitoring the protein’s intrinsic tryptophan fluorescence signal. There was a slight decrease in conformational stability of mAb incubated in 230 mM NaCl as seen from slight left shift in the urea unfolding curve. In solutions upto 2 M urea, the tryptophan center of mass did not change in formulations containing no NaCl and 230 mM NaCl. However, a red shift of about 4-5 nm was observed upto 5 M urea again with no difference seen between no NaCl and 230 mM NaCl containing formulation. However, beyond 5 M urea, there was more red shift due to exposure of fluorescing Trp residues to the aqueous environment,¹⁶⁹ and the 230 mM NaCl containing formulation showed a left shift indicating decreased conformational stability.

Figure 33: (A) Tryptophan center of mass and (B) Maximum tryptophan fluorescence intensity for mAb in 10 mM His (black open circles) and in 10 mM His+ 230 mM NaCl (Red open diamonds) as a function of urea concentration. Data points represent the mean ± SD for triplicate samples. Error bars for certain data points are smaller than symbols.
Discussion

Effect of ionic strength on silicone oil induced protein aggregation

Mechanical rupture of protein gels formed at the silicone oil-water interface generated particles that could be detected in the bulk liquid. The effect of ionic strength on the number of particles formed by this mechanical perturbation was minimal, presumably because each rupture event created the same number of particles (Figs. 26 and 27). Higher frequency of gel rupture (i.e., 0.5 vs. 0.25 Hz) resulted in more particles, but after three days of mechanical perturbation of the gelled layer at the silicone oil-water interface, no statistical difference was seen in particle concentrations across different ionic strength formulations at either frequency. In a similar study by Rudiuk et al., light scattering from aggregates produced by a similar mechanical perturbation of antibodies adsorbed at the air-water interface exhibited a linear dependence on the number of mechanical rupture events.\textsuperscript{146}

In our study, we observed a non-linear increase in the number of particles of size $\geq 1\mu m$ on day 3 as compared to days 1 and 2 (Figs. 26 and 27). We postulated that this might have been because of agglomeration of small particles that were below the 1$\mu m$ limit of detection of the microflow imaging instrument. To test this hypothesis, we mechanically ruptured the interfacial gel for one day at 0.25 Hz, and then allowed the samples to incubate quiescently over the next 13 days. We observed an increase in the number of particles detected by MFI and RMM during the quiescent incubation period (Fig. 28). In comparison, samples that had not been subject to the first day of mechanical rupture showed no increase in particles during 14 days of quiescent incubation (Fig 28E-28H). Furthermore, the zeta potentials of particles produced by mechanical rupture were
ca. -1mV (Table 3), suggesting that the suspension of sub-visible particles was colloidally unstable. In the absence of adsorbed protein, suspensions of silicone oil microdroplets in water or 10 mM His solutions exhibit negative zeta potentials (-30mV and -12mV, respectively, see Table 3). At the solution pH of 6.0 employed in this study, our antibody is anticipated to be positively charged, consistent with the measured zeta potential of the monomeric antibody in 10 mM His in the absence of silicone oil (+7.6 mV, see Table 3). Presumably, adsorption of positively charged protein molecules to negatively charged silicone oil effectively neutralizes the surface charge of the particles, resulting in a zeta potential near zero. Electrostatic screening at high ionic strength does not affect this zeta potential, because the charges were already neutralized upon protein adsorption to the silicone oil. Similar charge neutralization upon adsorption of protein to suspension of silicone oil microdroplets has been observed previously. In our study, we have illustrated the activation energy barrier between two micron sized particles having a zeta potential of -1mV using equation 6. The interaction potential was found to be 0 kT between these particles indicating that the activation energy barrier to particle agglomeration was zero. Similarly, in another study, Britt et al. proposed that the near-zero zeta potential of silicone oil microdroplets decreased the activation energy barrier of droplet agglomeration to near zero and thus promoted agglomeration. Consistent with this model of low activation energy barrier of droplet agglomeration, in our study, protein adsorption resulted in colloidally destabilized protein-oil particles (Table 3) which agglomerated over time to yield additional particles large enough to be detected by MFI or RMM. It appears that agglomeration, rather than particle growth by monomer addition was the dominant mechanism responsible for the late appearance of larger particles,
because no loss of soluble protein was detected by SEC at the end of 14 days for rupture-quiescent study. However, a caveat is that the mass of protein loss needed to account for the increase in particles likely below the detection limit for SEC analysis. Further, the increment in particle concentration during quiescent incubation after the initial 1-day period of mechanical rupturing of the gel was similar in both ionic strength formulations (Fig. 28). This effect can be explained by particles having the same neutral charge in the presence and absence of NaCl, as reflected in the zeta potential measurements (Table 3).

**Effect of ionic strength on interfacial viscoelastic protein gel formed at the silicone oil-water interface**

We expected that the ionic strength of the formulation would affect the strength of interfacial viscoelastic gels that is formed at the silicone oil-water interface by influencing protein-protein charge interactions. Other studies have shown that presence of NaCl resulted in stronger interfacial gels (as reflected by higher values of elastic modulus) of heat-denatured beta-lactoglobulin at an oil-water interface.\(^{185,187}\) However, in our study, contrary to our expectation, the effect of added NaCl on the elastic modulus of the gels was minimal (Fig. 29 and table 4). Also, the elastic moduli for beta lactoglobulin gels were much higher\(^ {185}\) (ca. 0.01-0.05 Pa-m, depending on ionic strength) than those that we found for our antibody (ca. 2x10\(^ -4\) Pa-m). We speculate that the weaker gels formed by our antibody may be related to the lower degree of chain entanglement at the interface of our relatively stable antibody compared to the thermally-unfolded beta lactoglobulin.
Effect of ionic strength on conformational and colloidal stability

The conformational and colloidal stability of the protein could influence the interactions between protein molecules in the bulk solution and at the interface.\textsuperscript{17,22,67} Screening by ions such as Na\textsuperscript{+} and Cl\textsuperscript{−} can affect protein-protein electrostatic interactions. The osmotic second virial coefficient ($B_{22}$) provides an indication of protein-protein interactions, including electrostatic interactions, van der Waals, hard-sphere and other short range interactions (e.g. hydrophobic interactions).\textsuperscript{17} Purely steric repulsions are indicated by effective hard sphere value, $B_{22}^{\text{HS}}$, and thus $B_{22}/B_{22}^{\text{HS}} = 1$ indicates a case where nonsteric repulsive interactions roughly balance the attractive interactions.\textsuperscript{89,188} Thus, $B_{22}/B_{22}^{\text{HS}}>1$ indicates repulsive protein-protein interactions and $B_{22}/B_{22}^{\text{HS}}<1$ indicates attractive protein-protein interactions. For two previously studied antibodies, $B_{22}$ decreased with increasing ionic strength.\textsuperscript{25,189} In 10 mM His, pH 6, the mAb under investigation in this study exhibited a $B_{22}/B_{22}^{\text{HS}}$ value of ca. 5.1, indicating repulsive protein-protein interactions (Fig. 30). Addition of 10 mM or greater NaCl decreased the $B_{22}/B_{22}^{\text{HS}}$ value to near 1, indicating that most electrostatic interactions were screened out, leaving only steric protein-protein interactions. Consistent with this conclusion are zeta potential values, which decreased as a function of NaCl concentration (Table 3). Values of $k_D$ showed a similar qualitative trend, with $k_D$ decreasing with increasing ionic strength. Similar trends have been observed in other studies as well.\textsuperscript{25,189-191} As can be observed from equations 8 and 9, $k_D$ could be influenced by hydrodynamic factors and protein partial specific volume, and thus does not purely reflect protein-protein interactions.\textsuperscript{191} The effect of the reduced colloidal stability in the presence of NaCl was
manifested in the flocculation of protein that was observed visually and by DLS during heating studies (Figs. 31 and 32).

Further, the presence of NaCl decreased the conformational stability of the protein as evidenced by decrease in $T_{m,1}$ and $T_{m,2}$ in the presence of 230 mM NaCl (Fig. 31 and table 5). For this mAb, the $C_{H2}$ domain is the least stable domain of the protein. We showed that the unfolding of this domain is reversible in the presence of NaCl (Fig. 31B). The urea unfolding curve also showed that mAb was slightly destabilized in 230 mM NaCl (Fig. 33).

In our gel rupture setup, lower conformational and colloidal stability of the protein did not have a significant role in particle formation as the mechanically-imposed gel rupture dominated the cause of particle formation. However, lower conformational and colloidal stability of the protein at higher ionic strength might play a role when other forms of accelerated stress are applied to the protein. For instance, thermal stress led to early onset of protein unfolding in the presence of NaCl and promoted protein aggregation (Figs. 31 and 32). Similarly, for proteins like albinterferon-α2b and another IgG molecule, lower colloidal stability has been shown to cause more aggregation upon agitation.\textsuperscript{67,150}

**Implications of sub-visible particle colloidal instability and agglomeration**

Commercial protein formulations are designed to be stable over a period of 18-24 months.\textsuperscript{17} Initially, measurable sub-visible particles within a formulation may not be significant in number. However, large quantities of smaller particles may be present. If these particles are colloidally unstable, flocculation may result in the appearance of particles of detectable sizes during the shelf life period. This colloidal instability may be
particularly pronounced in prefilled syringes containing formulations that confer positive charge on protein molecules, such as most commercial monoclonal antibody formulations. Neutralization of the negative charges found on silicone oil droplets upon adsorption of positively charged proteins reduces net charge, lowers zeta potential, decreases colloidal stability, and foster agglomeration.
CHAPTER V

CONCLUSION AND FUTURE RECOMMENDATIONS

Protein aggregation is influenced by both conformational and colloidal stability and both of these parameters could be varied by solution conditions and the interfaces around the protein.

The work in this thesis demonstrated that similar to single domain protein molecules, multi domain proteins like monoclonal antibody can undergo partial unfolding leading to aggregation. The partially unfolded species of a mAb had unfolded C_H2 domains and caused aggregation. The C_H2 domain of the protein was the least stable domain in this multi-domain protein and its instability drove protein aggregation. Other studies carried out on monoclonal antibody have also reported that C_H2 domain can play a causal role in aggregation.\textsuperscript{48,49,122,129} Thus, conformational instability of the protein dictated aggregation profile of the molecule. This finding can help during the development of therapeutic mAb molecules to stabilize individual protein domains and the stability of each individual domain can further help increase the overall stability of the protein. Further work can be carried out in this area by investigating the amino acid residues that are more solvent exposed and dynamic in nature. This would require use of H-D exchange to attribute amino acids that might be the hot-spots in dictating stability and driving aggregation. Identification of least stable domain in a protein and developing strategies to stabilize it can facilitate a more robust protein formulation.

In addition, we also showed that aggregates had covalent intermolecular contacts formed from disulfide shuffling. Supporting work can be carried out by investigating how
protein unfolding leads to solvent exposure of certain amino acids that might be prone to chemical modification. For instance, amino acids such as tyrosine (Tyr) and tryptophan (Trp) are susceptible to oxidation reaction. Exposure of these hydrophobic amino acid residues to solvent can make them amenable to chemical oxidation that can further affect protein stability and aggregation. Future work can be focused on investigation of chemical modification that an unfolded protein can undergo by doing mass spectrometry.

We have shown that besides conformational and colloidal stability of protein in solution being important, protein stability can also be affected by interfaces. In particular, our study was the first study to show that mAb forms an interfacial gel upon adsorption to the silicone oil-water interface. This gel is viscoelastic in nature showcasing both viscous and elastic properties. Additionally, we showed that rupture or perturbation of this interfacial viscoelastic gel results in particles and aggregates in the bulk solution. Interfacial induced aggregation can be minimized by use of surfactants in protein formulation. It is hypothesized and speculated that surfactants inhibit surface induced aggregation by inhibiting interfacial gel formation at the interface. In our study, we were unable to demonstrate and prove this hypothesis because of the mechanical incapability of the interfacial rheometer we used. However, other types of rheometer available in the market can be used to prove and support this hypothesis. Further, more experiments can be carried out by investigating the characteristics of viscoelastic gel formed by different types of protein molecules. It can be hypothesized that a stronger gel would be formed if covalent interactions between protein molecules within the gel are the dominant protein-protein interactions. The measure of the elastic modulus would give us an indication of the strength of the interfacial viscoelastic gel. This work showed that utilizing excipients
in a protein formulation including surfactant can help reduce interfacial induced aggregation.

Furthermore, we have investigated the effect of colloidal properties of protein molecules on the characteristics of the interfacial viscoelastic gel formed at the silicone oil-water interface. Increasing ionic strength did not have a significant effect on the characteristics of the interfacial viscoelastic gel formed at the silicone oil-water interface. Also, we have shown that the sub-visible particles formed from rupture of the interfacial gel are colloidally unstable as they have net neutral charge. The adsorption of protein molecules to silicone oil results in neutralization of charges leading to net neutral zeta potential. These particles have a tendency to agglomerate over a period of time and form more particles. Further work can be carried out in this area but investigating sub-visible particle formation upon compressing and dilation of interfacial gel formed at the silicone oil-water interface. It can be hypothesized that compressing the interfacial gel can lead to particle formation in the bulk solution. Compression and dilation of interfacial viscoelastic gel is another form of mechanical perturbation of the gel. It can be hypothesized that this form of perturbation of gel could further lead to sub-visible particle formation. More work can be carried out by comparing the type of interfacial viscoelastic gels formed at the silicone oil-water interface to the air-water interface. Moreover, a comparison study could also be carried out in evaluating the particle formation due to rupture of the gel formed at silicone oil-water interface with that of the air-water interface.

Overall, the information provided in this thesis shows that proteins are inherently unstable molecules. Further, solution properties govern colloidal and conformational
stability of the protein and these stability parameters could be altered by changing formulation components. Excipients can be utilized in formulation for stabilization of protein molecules. Presence of interfaces could lead to aggregation by affecting conformational and colloidal stability of native protein and sub-visible particles. From a formulation perspective, in order to stabilize the protein against aggregation, solution conditions and interfaces around the protein should be chosen in a manner such that they stabilize the protein conformation and also stabilize protein-protein and particle-particle interactions. Utilization of surfactants in a formulation can reduce interfacial induced aggregation.
REFERENCES


