Probing Shear Thinning Effects on IgG Molecules at the Air-water Interface via Viscosity and Rheological Measurements

By

Camille Gleason

Submitted to the graduate degree program in Pharmaceutical Chemistry and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science

_____

Chairperson – Russ Middaugh

_____

Chanel K. Yee

_____

David Volkin

Date Defended: 12/04/2013

The Thesis Committee for Camille Gleason

Certifies that this is the approved version of the following thesis:

Probing Shear Thinning Effect on IgG Molecules at the Air-water Interface via Viscosity and Rheological Measurements

_____

Chairperson Russ Middaugh

Date approved: 12/04/2013

Abstract:

Shear thinning behavior, observed in shear viscosity tests of IgG therapeutic molecules, may lead to inaccurate reporting of apparent viscosity, depending on the severity of the shear thinning behavior. To determine whether shear thinning is an intrinsic bulk property or an interfacial phenomenon, shear thinning behavior was tested as a function of bulk concentration with IgG1 and IgG2 molecules. Indeed if shear thinning is a bulk property, then the higher the concentration, the greater the shear thinning effect. The 70 mg/ml and 0.007 mg/ml samples showed the least shear thinning in comparison to 0.7 mg/ml concentrations of both the IgG1 and IgG2 molecules. This suggests that high bulk concentration did not produce the greatest shear thinning effects; therefore bulk properties do not contribute to the shear thinning effect. To test the sensitivity of the IgG molecules at the air-water interface, the surface area to volume ratio (SA:V) of samples exposed to the air-water interface were varied by the measuring system. The measuring systems used were: Cone and Plate 25 mm (CP 25), Cone and Plate 50 mm (CP 50) and Double Gap (DG). Their respective SA:V exposed to air were: 155 m^{-1}, 108 m^{-1}, and 33 m^{-1} with an Anton Paar MCR 302 rheometer. For both IgG1 and IgG2 molecules, the measuring systems with the highest SA:V ratios produced the most dramatic shear thinning effects. IgG1 molecules were more sensitive to changes in SA:V but IgG2 molecules showed higher magnitude shear thinning effects. To further probe the behavior of the IgG molecules at the air-water interface, interfacial oscillatory rheology with a Bicone (BC) was performed at a constant stress and strain (1 Hz, 1 %). The IgG molecules showed solid behavior (G'_i) at 0.7 mg/ml over a 22 hour period. At 70 mg/ml

and 0.007 mg/ml, liquid behavior (G''_i) was dominant for both molecules. In fact, at 70 mg/ml G'_i was not detected by the rheometer. Furthermore, the addition of polysorbate 20 (PS20) minimized shear thinning behavior as well as G'_i. IgG1 had a G'_i/G''_i crossover point over half an hour after testing while IgG2 had a crossover point seconds into the test. This suggests different film formation behavior for IgG1 and IgG2. The slow continuous growth of G'_i over 22 hours suggests the formation of multi-layer's of mAbs beneath the air-water interfacial monolayer.[4] To orthogonally verify the presence of IgG molecules at the air-water interface, interfacial tension (IFT) was also measured at the 0.7 mg/ml IgG molecule concentrations and in the respective IgG buffers. For both IgG molecules, IFT was reduced relative to the buffers.

Acknowledgements

Chanel Yee, Aylin Vance, Peter Masatani, Hong Shen, and Russ Middaugh

Amgen and University of Kansas

Table of Contents

Introduction:

Importance of Understanding IgG Properties

In the past, most protein therapeutics were formulated at low concentrations. However, more recently protein formulations and newly developed antibody therapeutics are required to be formulated at high concentrations because they need to be administered at high doses. The challenge of developing high concentration immunoglobulin (IgG) formulations presents unique problems to the pharmaceutical industry. The IgG therapies required at high doses for efficacy are often in the range of several mg/kg and are dosed at home by the patients themselves.[5] With a limiting syringe volume of <1.5 mL, the therapeutic IgG would ideally need to exceed 100 mg/ml, producing a high concentration IgG liquid formulation that is often viscous and can be difficult to inject. In addition, the large resistance (viscosity) posed by the viscous IgG material is often a manufacturing challenge due to the high resistive forces exerted during ultrafiltration and diafiltration (UFDF) that can foul lines and filters. Similar viscous forces are problematic with pre-filled syringes and auto-injectors which can malfunction or stall at the injection site causing excessive pain to the patient when certain expulsion threshold limits are exceeded.[6] In the manufacturing process of a therapeutic drug, monitoring attributes such as viscosity and other important rheological properties, i.e., the storage modulus G' and the loss modulus G", etc., of the IgG formulation are imperative to achieve desirable physical properties of the final drug product. The rheological properties of an IgG drug product can be indicative of changes in the bulk state as a function of storage, processing, formulation or delivery system. Because rheological measurement does not require dilution, the actual concentrated solution properties can be monitored as a function of the

shear or oscillatory force applied. This is particularly beneficial because most of the techniques used to characterize protein therapeutics require dilution and thus cannot directly monitor relevant phenomena such as phase separation, gelation and particulation. Qualitative rheological measurements can be used as a biophysical technique to monitor viscous IgG therapeutics.[7]

The Problem – Data Inconsistency from different measuring systems

Commonly used systems for testing IgG molecule shear viscosity is the cone and plate (CP) and the double gap (DG) system attachments to the torque rheometer. Viscosity data is used to calculate resistive forces of increasingly viscous monoclonal antibody (mAb) solutions which are critical to manufacturing and syringe dosing.[6] The resistance to flow of the IgG solution is measured and converted to a viscosity measurement. Below is an illustration, Illustration 1, for the CP and DG measuring system. The blue area represents the bulk liquid being probed in each measuring system while the red brackets indicate liquid portions of the sample exposed to air. Note the larger air-water interface in the CP compared to the DG.

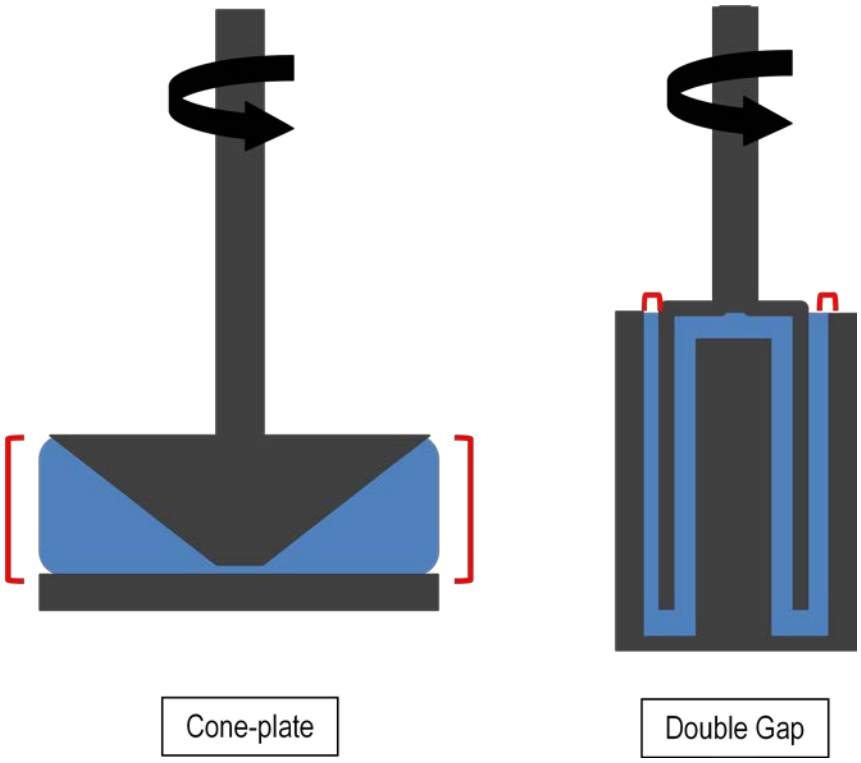

Illustration 1 – The cross section of the CP (left) and DG (right) measuring systems are pictured above. The grey represents the measuring system while the blue region represents the sample being measured. The red brackets highlight the exposed surface area to air for the particular measuring system.

Depending on the measuring system used, IgG viscosity profiles often appeared non-Newtonian in the low shear region. The viscosity profile, at the lower shear rate range, appears to be very viscous and as shear rate increases the viscosity plateaus. This type of viscosity profile is called the shear thinning effect. The shear thinning effect is an initial resistance to the low forces (low shear rate) which are immediately followed by flow (an exponential reduction of viscosity). Depicted below, in Illustration 2, is a diagram of two viscosity profiles of an IgG formulation: one the result of using a CP (left) and the other (right) a result of using a DG.

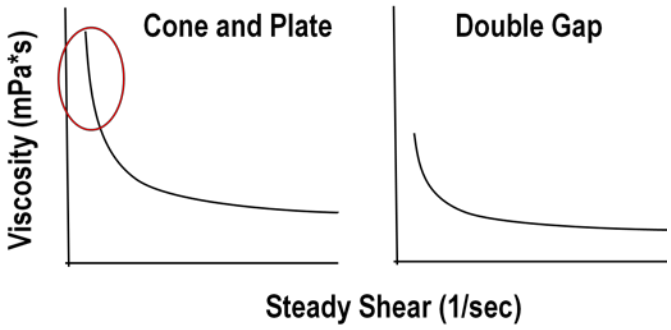

Illustration 2 – Data inconsistency on the viscosity profile is shown for a typical IgG using different measuring systems: CP (left) and DG (right).

The viscosity profile produced by the CP shows shear thinning (indicated by the red circle) while the viscosity profile run by the DG shows reduced shear thinning behavior. In some instances, an antibody product in a shear viscosity test never quite reaches steady state (plateau) due to the extensive shear thinning behavior. The presence of inconsistent shear thinning viscosity profiles from the same IgG formulation, simply by using different measuring systems, are misleading as to the true shear viscosity value. The shear thinning effect observed in some IgG formulations may either be an artifact of the measuring systems or indeed, reflect a characteristic flow behavior of the IgG molecule. The inconsistency observed in the viscosity profile could also be a mere difference in the degree of detection sensitivity varied from one measuring system to another.

The hypothesis

Since discrepancies found in viscosity profile patterns can make the interpretation of rheological measurements of IgG formulations difficult, it is critical to the formulation

scientist to identify what is causing these discrepancies to gain more insight into the physical properties of the formulations. To investigate the hypothesis that shear thinning effects are an artifact of the measuring system, we attempt to examine the two commonly used systems, CP and DG, in more detail. As shown in Illustration 1, the significant differences between the two systems are: (1) the testing volume and, (2) the size of molecular exposure to the air-water interface. Also shown in Illustration 1, the SA:V ratio of the sample exposed to the air-water interface is greater for CP. In other words, more molecules are exposed to the interface during measurement in the CP configuration than the DG counterpart. With air, a very hydrophobic medium, how do IgG molecules behave at the air-water interface? Is shear thinning, shown in Illustration 2, merely a concentration- related bulk behavior? Or can the difference in molecular exposure, and the subsequent molecular re-organization, at the interface account for discrepancies in viscosity profiles?

Protein Adsorption at the air-water Interface

The hydrophilic hydrogen-bonding tetrahedral nature of water and the hydrophobic properties of air, gives rise to very high surface tension at the air-water interface. Thus, substitution of ordered water at the air-water interface by proteins is energetically favorable due to the comparably lower surface tension of amphiphilic proteins at the air-water interface. The protein effectively acts as a surfactant in water, as more hydrophobic regions of the protein are exposed to air thus disrupting the interfacial water and lowering the surface tension. Given the variable size, composition and flexibility of diverse proteins, each has unique structural characteristics and adsorption kinetics at the air-water interface. Intuitively, the more rigid the protein structure, the more likely the protein fits a

hard-sphere model[4] with little conformational change from the bulk to the interface. Conversely, flexible proteins with fewer energetic barriers are more apt to deform at the air-water interface. Most proteins fall in between these two extremes (hard spheres and linear flexible chains), because they contain complex secondary, tertiary and quaternary structures that vary in accessibility and nature of interactions with the air-water interface[8]. Adsorption often causes changes in the macroscopic properties of an air-water interface. During an adsorption event, molecules located in the vicinity of an interface often behave differently from the bulk, as a result of the molecular restructuring and conformational changes that occur at the interface. As quoted from Vogler, et al.,[4] "*an adsorption phenomenon can be rationalized as protein molecules partitioning from the bulk solution into a 3D interphase separating the bulk from the physical-adsorbent surface, where adsorbed molecules assemble in one or more adsorbed layers with the accumulating concentration much higher than the bulk solution*", as displayed in Illustration 3.

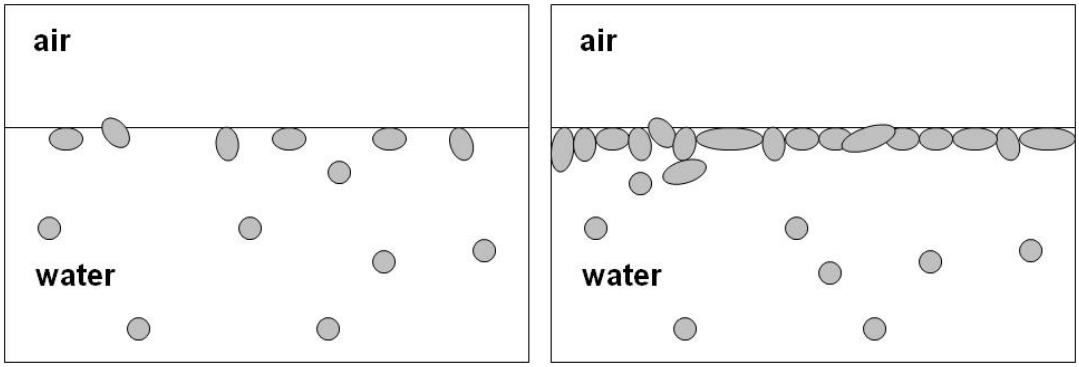

Illustration 3 – The accumulation of protein at an air-water interface. The grey spherical shapes represent protein molecules in bulk, left and at the air-water interface, right.

The authors argued that such an adsorption process was free-energy dependent, which ultimately determines the maximum protein adsorbent capacity within a unit surface area. Monitoring macroscopic properties such as surface tension (free-energy) and surface rheology as a consequence of adsorption behavior, are easily achievable at air-water interfaces.[9] The conformational changes, due to molecular restructuring, within a few atomic adsorbent layers of proteins at the interface would appear much more subtle compared to the macroscopic rheological phenomena, and therefore require much more sophisticated techniques to detect.[9]

Molecular Behavior of Proteins at the Air-water Interface

Protein conformation may vary as a function of bulk concentration and air-water interface area. In the presence of a large interfacial area and low bulk protein concentration, wherein the adsorbent surface is sub-saturated, room is available for proteins to be conformationally altered and expose hydrophobic regions. Conversely, proteins occupying saturated adsorbents (high bulk protein concentration) may displace adsorbed neighbors.[4] Displacing proteins from a saturated adsorbent interface takes energy. Thus, the interplay between lowering the energy by conformational change at the air-water interface versus remaining intact due to the space constraints in tightly packed protein solutions, at least partially determines protein interfacial behavior.

It has been shown that IgG molecules form multi-layers at hydrophobic adsorbent surfaces.[4] The formation of multi-layers is probably due to an initial monolayer formation at the air-water interface. Multi-layer protein adsorption studies suggest that

proteins reorganize at the interface to lower surface energy. While protein molecules at the interface are stabilized in an unfolded or partially unfolded state, molecules from the bulk solution order on to the initial monolayer in turn producing a cascading effect that orders the bulk solution on to a growing three-dimensional (3D) protein layer, also known as the viscoelastic layer. [4]

Characterization of IgG Molecules using Interfacial Rheology

Burgess and colleagues found that IgG molecules exhibited viscoelastic interfacial properties.[10] Note that unlike the IgG therapeutic molecules which are homogenous and highly purified, these molecules were heterogeneous because they were derived from blood which contains a wide variety of IgG molecules. They attempted to correlate temperature, aging, pH and concentration to IgG surface elasticity using interfacial rheology. [10, 11] They found that increasing the temperature from 25°C to 37°C decreased surface elasticity. Thus, the higher kinetic energy at the higher temperature resulted in increased interfacial fluidity. Another property tested on IgG molecules was the effect of aging. Aging can be defined as the static storage time wherein film formation, typically at an interface, takes place. Aging effects of IgG molecules at the interface were conducted to study continuous film formation over the duration of three days. The results suggested that the longest aging time (3 days) produced the greatest surface elasticity while the shortest produced the least. This suggests that IgG molecules at the air-water interface are sensitive to aging effects wherein more aging is positively correlated to increased surface elasticity and less aging is to decreased surface elasticity. The aging study also implied that the IgG film changes over time, possibly as bulk solution IgG molecules interacted with IgG molecules at the interface.[11] An additional

contributing factor familiar to formulation scientists is the effect of pH on interfacial elasticity. Approaching the isoelectric point, the IgG molecules exhibited minimal interfacial elasticity, because the molecules were in their most compact state. Therefore, lateral interactions among molecules were minimized. [10, 11] Conversely, when pH values diverged from the isoelectric point, intermolecular entanglement readily took place increasing surface elasticity.[10] The relationship of IgG molecules at the air-water interface to the formulation pH can be summarized as follows: as the sample pH diverged from the isoelectric point surface elasticity increased, conversely, as the sample approached the isoelectric point surface elasticity decreased. The final parameter to be discussed was the relationship of bulk concentration to surface elasticity of IgG molecules. Several researchers have uncovered a possible dependence of surface elasticity upon bulk concentration but due to the narrow concentration ranges presented, a clear picture of the relationship of concentration to surface elasticity was not apparent.[10, 12] Currently, the concentration ranges tested in the literature fall far below typical high concentrations (>30 mg/ml) in current therapeutic IgG formulations. We are faced with a lack of relevant data from which to draw conclusions as to the relationship of bulk concentration to surface elasticity in the current literature.

Methods used to Measure Bulk and Interfacial Phenomena

Bulk protein properties are often assessed by shear viscosity and oscillatory rheology measurements. Few techniques accurately capture intermolecular interactions of proteins at the air/water interface, among which interfacial rheological measurement and interfacial tension assessment are the two most common methods used to study interfacial phenomena. In particular, interfacial tension assessment by drop shape analysis is often

employed in parallel with rheological techniques to study protein behavior at the air-water interface.

Principles of Shear Viscosity

The shear viscosity (η) is the coefficient that describes the resistance of a fluid to a sliding motion. When conducting shear experiments on an incompressible fluid, the shear stress is directly proportional to the gradient of velocity. The relationship observed between shear stress (force/area) and shear rate (velocity/gap) is called Newton's law of viscosity. In a Newtonian fluid, the shear viscosity remains unchanged regardless of the shear rate. Newtonian fluids are purely viscous because they flow, at a consistent viscosity, regardless of the shear rate of force applied. For non-Newtonian samples, such as those exhibiting a shear thinning effect, a more in depth study of fluid flow is necessary to understand the system. [7]

Principles of Rheology

Rheology is the study of flow and deformation of fluids and is particularly useful in studying non-Newtonian fluids which exhibit viscous and elastic characteristics. When a force is applied to an elastic solid, the shape changes and the deformation is proportional to the applied force. Upon removal of this force the material springs back to its original shape and most of the energy used in producing the deformation is recovered. In contrast, when a force is applied to a liquid, it flows and the flow rate is proportional to the applied force.[13] In rheological terms, a gel is semi-solid and referred to as "viscoleastic" since it exhibits both viscous (liquid) and elastic (solid) characteristics during deformation. By applying small oscillatory strain, the viscoelastic properties of a system can be monitored

with minimum structural damage. G', the storage modulus, describes the elastic energy storage and characterizes the rigidity of a material. G", the loss modulus, describes the viscous energy loss, together G' and G'' describe the resistance of the flow of a viscoelastic material during the shearing in a dynamic rheological test. The term $\tan\delta = G''/G'$ is a measure of the ratio of energy lost to energy stored in a cyclic deformation. When tan $\delta < 1$, the material is more elastic (solid-like); when tan $\delta > 1$, the material is more viscous (liquid-like). A rheological gel point can be defined at the time where G' crosses over with G" (tan $\delta = 1$); it highly depends on the monitoring frequency used.[14, 15] Using rheometric means, we attempt to assess the viscoelastic behavior, namely the gelation crossover point, of IgG molecules at the air-water interface.

Principles of Interfacial Rheology

The rheological forces for protein solutions at the air water interface are often different than the rheological forces found in the bulk solution. Since there are normally attractive interactions between the surface elements, this leads to an increase in the solid interfacial modulus (G'_i) and the liquid interfacial modulus (G''_i) because energy must be expended to overcome these interactions to make the elements flow past one another. For example, the surface of pure water has a significant shear viscosity due to hydrogen bonding between the molecules. The G'_i and G''_i variables measure the solid and liquid parameters of the film formed at an air-water interface. The contribution of bulk rheology to an interfacial rheological measurement is dependent on the Boussinesq number (B_o).

B_o measures the ratio of surface to bulk viscous effects. It can be defined in terms of the interfacial shear viscosity as

$$B_o = \frac{Surface\ Shear\ Viscosity}{Bulk\ viscosity\ \times Length\ Scale}$$

If $B_o \gg 1$, the surface shear viscosity strongly influences the nature of bulk flow near or at the surface. $B_o \gg 1$ also means that the interfacial flow is not coupled to the flow in the bulk. In this case, bulk viscous effects may be neglected.[16] The attractive interactions between surfactant or protein molecules at the interface may be considerably stronger and therefore lead to greatly enhanced interfacial rheology. As described by Murray and Dickinson, "*the developing viscoelasticity of an adsorbed protein film is intimately connected with the conformational changes of the adsorbed protein. These changes may take place quite slowly: depending upon the conditions, it may take several days (or more) before a steady state is reached (if it is ever reached at all!). In this respect the resulting film may be better likened to a very thin film of bulk protein gel – and indeed it behaves in many ways as such.*"[17]

Principles of Tensiometry

Surface (interfacial) tension is a property of the surface of a liquid that allows it to resist an external force. Cohesive forces among liquid molecules are responsible for the phenomenon of surface tension. In the bulk of a liquid, each molecule is pulled equally in every direction by neighboring liquid molecules, resulting in a net force of zero. The molecules at the surface do not have molecules on all sides and therefore are pulled inwards. This creates a net internal pressure, and forces the liquid surface to contract to

the minimal area. This notion is perhaps best exemplified by the shape of a water droplet. In the absence of other forces, including gravity, drops of virtually all liquids would be perfectly spherical. The spherical shape minimizes the necessary "wall tension" associated with the droplet surface according to Laplace's law. Therefore, surface tension is essentially responsible for the ultimate shape of a liquid droplet in the medium in which it is formed, as a collective manifestation of all molecular dynamics and interactions at its interface. [4, 9]

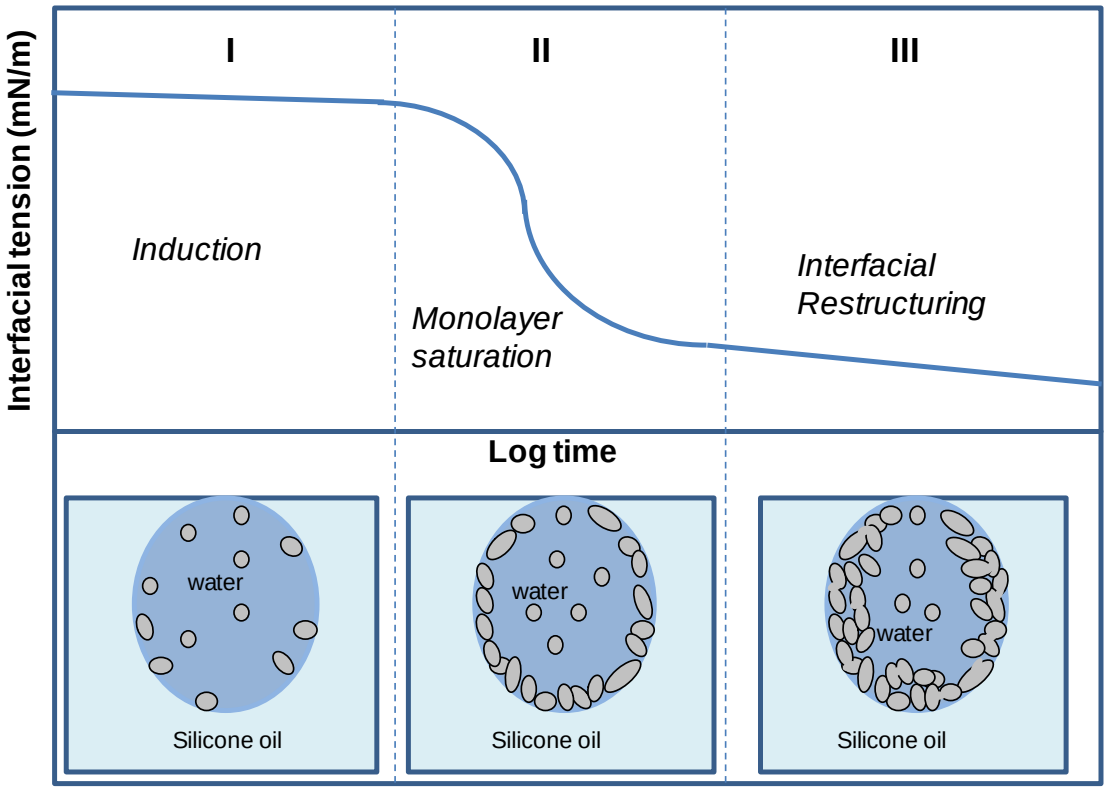

Illustration 4 – A typical protein interfacial tension log time graph illustrating three distinct regimes: induction, monolayer saturation and interfacial restructuring, are shown.

A proposed mechanism in the literature that describes the interfacial molecular behavior, with respect to the IFT profile change, of a protein solution droplet formed in a hydrophobic medium involves three distinct regimes [9]: induction, monolayer formation, and interfacial restructuring, as shown in Illustration 4. During the induction time period, interfacially directed molecular motions via diffusion - molecules present at the interface are structurally indistinguishable from the bulk, hence they do not cause a significant change in IFT. Gradually molecules that are amphiphilic in nature, i.e., protein, surfactants, etc, if available, diffuse to and adsorb at the interface where they undergo necessary conformational changes to alleviate the high surface tension. This can lead to complete or partial unfolding of the adsorbed molecules at the interface and a measureable uptake of such molecules from the bulk. The molecular kinetics of the diffusion process depend on the availability/concentration, size and mobility of such amphiphilic molecules in the bulk, and can be monitored by probing changes in the IFT profile during the monolayer formation process. At the end of this regime, as more molecules diffuse and adsorb to the interface and as a monolayer is formed, the IFT becomes asymptotic and a stable plateau is reached, as shown in Regime II. This asymptotic monolayer saturation regime marks the initiation of the multilayer formation, during which interfacial restructuring occurs as further conformational change and molecular re-orientation take place for the molecules located in the vicinity of the interface, leading to the formation of a viscoelastic layer. This regime usually takes considerably longer times to reach equilibrium because of the slow molecular rearrangements while the viscoelastic layer re-structures as more molecules from the bulk become associated with the molecules adsorbed at the interface.

Materials and Methods:

Sample Preparation:

Both IgG1 and IgG2 molecules were provided by the purification group within Amgen, Inc. The IgG1 sample was provided at a stock solution of 71 mg/ml bulk in 10 mM Sodium Acetate and 9% Sucrose pH 5.0 (A5Su) while the IgG2 sample was provided at a stock solution of 68 mg/ml bulk in 10 mM Sodium Acetate and 5% Sorbitol pH 5.0 (A5S). Both samples were concentrated by the purification group by means of ultrafiltration and diafilitration (UF/DF). Shortly after production both molecules were in long term frozen storage at -80°C and both were defrosted, at 4°C, over a 5 day period. The molecules were then aliquoted in a sterile hood into 50 mL sterile Falcon Centrifuge Tubes (BD Biosciences) to prevent contamination of the sample. The tubes containing the samples were stored at 4°C and covered by aluminum foil to protect from light degradation. An SEC-HPLC assay was run after the samples had been aliquoted (t=0) and again 3 months later (t=3m) to assess whether the sample remained stable under the storage conditions; no appreciable aggregates or clips were observed within that time frame.

All samples tested were diluted within an hour of testing to minimize aging effects. The IgG1 sample was diluted in A5Su buffer while the IgG2 sample was diluted in A5S buffer. Both IgG molecules were diluted by serial dilution in their respective buffers. When testing the ~70 mg/ml samples, they were allowed to come to ambient temperature before testing.

IFT Method:

Dynamic interfacial tension was measured using the pendant drop technique with a Kruss DSA100. A pendant drop was formed using a capillary tip (diameter: 0.51 mm) in air inside a closed box with quartz windows. The drop was illuminated with a white light shining through a diffuser. Image analysis was performed via the software which performed rapid drop image acquisition, edge detection, and fitting of the Young-Laplace equation to determine the interfacial tension. Illustration 5 below shows the experimental set up and the parameters associated with the Young-Laplace equation.

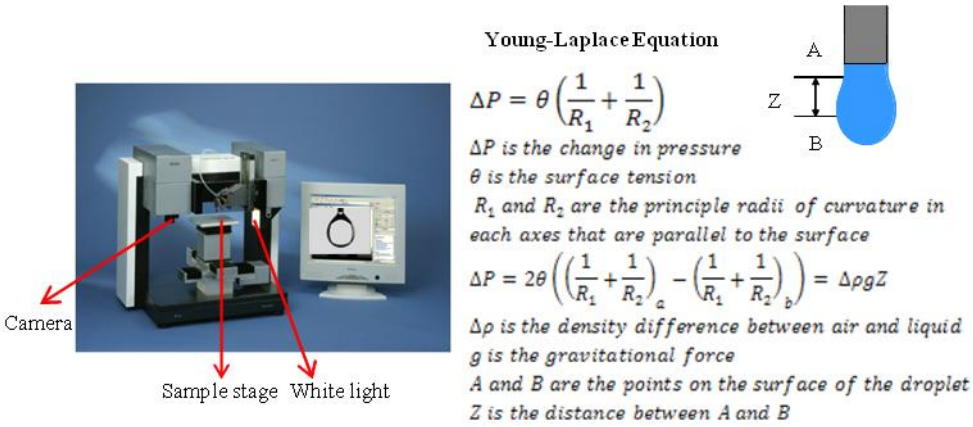

Illustration 5 – The experimental set-up and the method details of measuring the interfacial tension (IFT) of a liquid as a pendant drop are shown.

Each sample was run in duplicate and recorded and analyzed as per the set-up shown in Table 1. All measurements were done at room temperature.

	frames /second	**frames recorded**	**time (sec)**
step 1	1	600	600

step 2	0.2	120	600
step 3	0.02	120	6000
total time		840	7200

Table 1 – The experimental set-up is indicated above.

The densities of 0.7 mg/mL IgG1 and IgG2 in their respective buffers were measured as 1.03387 g/L and 1.02053 g/L using a DMA 4500 (Anton Paar, Richmond VA). The density values were used during analysis to obtain the surface tension.

Bulk Shear Rheology

Rheological measurements were performed on Anton Paar MCR 302 rheometer with variable measuring systems. Two cone and plate (CP) attachments were used in this study. The attachments varied in diameter. The larger CP had a diameter of 50 mm (CP 50) and the smaller a diameter of 25 mm (CP 25). Both CP 50 and CP 25 have an angle of 1°. The sample fill volume of the CP 50 is 630 ul and the sample fill volume of the CP 25 is 110 ul. Illustration 6 shows the CP measuring system.

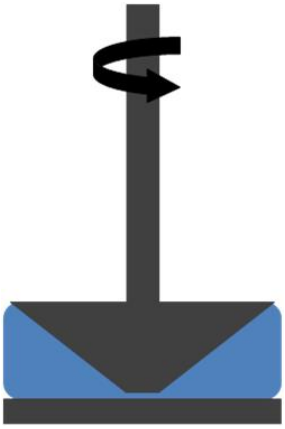

Illustration 6 – The experimental set-up of the cone and plate is shown.

The double gap system was also used as a means of measuring bulk rheology. The DG 26.7 has the following parameters: outer radius1; 13.796 mm, outer radius2; 12.33 mm, gap1; 0.47 mm, Gap2; 0.42 mm and sample volume; 3.62 ml. Similar to the CP system, the DG system measures bulk shear rheology. The DG system is pictured below in Illustrated 7.

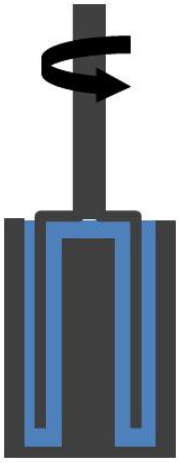

Illustration 7 – The experimental set-up and the DG system is shown.

The SA:V ratios were calculated for each bulk rheological measuring system and are presented in Table 2 below.

	Volume (mm^3)	Surface Area Exposed to Air (mm^2)	SA:V (mm^{-1})
CP-25	0.11	17.14	155
CP-50	0.63	120.33	108
DG	3.6	68.55	33

Table 2 – The SA:V of the three systems.

The volumes were recorded upon measurement and the surface areas were calculated. For the CP, the tangent the cone angle and the radius of the cone was used to get the height. The height was then multiplied by π and the diameter of the system to get the surface area. For the DG, the equation πr^2 was used to calculate the total area of the outside radius and the inside radius. The resulting areas were subtracted and thus the exposed surface area was calculated.

All bulk shear measurements were taken at 20°C, and the samples were given 5 minutes to equilibrate in the measuring systems. To prevent excessive evaporation, a humidity trap (H-PTD200) connected to the water circulator was used. A shear deformation was imposed on the sample by the cone which is connected to a computer controlled motor. The cone also measures the resistive torque through a torque transducer. This is recorded by the instrument and converted to a viscosity measurement in units of milli pascal

seconds (mPa*s). The samples undergo a logarithmic increase in steady shear starting at $0.1s^{-1}$ up to $1000s^{-1}$ and measurements take about 8 minutes to complete.

Interfacial Shear Rheology

Interfacial shear rheology measurements rely primarily on measuring the rotational motion of a knife-edged bob, disc or ring when placed in the plane of the interface. In this case, the bi-cone (BC) is a concentric dish with a knife edge suspended from an instrument that simultaneously monitors and controls the deflection of the bob on the interface. The interface meniscus is typically positioned at the edge of the bob, with the interface contained in a concentric dish or a second concentric ring. For example, a deflection is applied by the BC on the interface; the damped oscillations of the BC in response to the interface are recorded and analyzed to obtain values of η and G. Illustration 8 shows an intersection of the BC measurement system along with the equation used to derive the interfacial rheology results.

$$\mathbf{T}^{(\theta)} = [(\kappa - \eta)\,\mathrm{div}_\sigma \mathbf{v}^{(\sigma)}]\mathbf{P} + 2\eta\mathbf{D}^{(\sigma)}$$

$\mathbf{T}^{(\theta)}$ *is the Boussinesq surface stress tensor*
$\mathbf{v}^{(\sigma)}$ *is the interfacial velocity vector*
div_σ *is the interfacial divergence operator*
$\mathbf{P}$ *is a projection tensor that transforms every vector into its component tangential to the interface*
η *is the interfacial shear viscosity*
$\mathbf{D}^{(\sigma)}$ *is the interfacial rate of deformation tensor*
κ *is the interfacial dilatational viscosity*

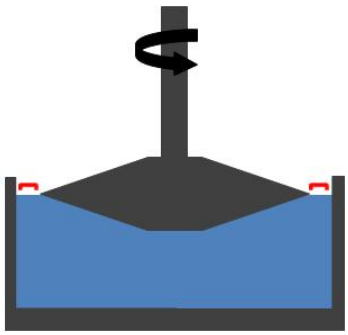

Illustration 8 – The experimental set-up of the Bi-cone is shown along with the Bossinesq-Scriven equation for the surface stress tensor.

Rheological measurements were made with an Anton Paar MCR 302 rheometer. The BC (BC68-5, PN 14340, SN 24908, had the following parameters diameter: 68.223 mm, angle 4.988°, penetration depth 2.211 mm, truncation at 44 um). After diagnostic checks were run on the instrument (normal force was reset, gap was zeroed, inertia checked and motor adjusted) a coffee standard of 0.033 mg/ml was run at 20°C before each interfacial measurement was performed. Instant coffee is rheologically active at the air-water interface and had a G'_i to G''_i crossover in the range of 26-38 mPa within 15 minutes of dissolution. Therefore, it provides a reasonable instrument control. To get the sample at the knife-edge of the BC, the BC is lowered into the cup at a rate of 10um/sec until the normal force exceeds 0.04N at which point the instrument has barely touched the top of the sample and thus stops. The instrument is then lowered a set distance dictated by the distance from the tip of the BC to the knife edge of the BC. This ensures accurate measurement of surface rheology even when sample volumes vary.

After the samples are loaded onto the system, a two minute waiting period is allowed for the liquid to settle. During that time, a solvent trap is placed on top of the cup to prevent evaporation over the next 22 hours.

Results and Discussion:

To better understand if shear thinning behavior in IgG molecules is a bulk property or an interfacial phenomenon, interfacial contributions and bulk contributions to shear thinning were isolated by experiment. Shear viscosity experiments were performed on the bulk IgG molecules as the following parameters were varied: *IgG concentration*, and *degree of IgG exposure to the air-water interface.* Because the viscosity data supported the theory that IgG molecules were affected by the air-water interface, a more in depth analysis of the IgG molecules at the interface was in order. To diminish the contribution from the bulk solution, interfacial rheological assessment of IgG molecules was carried out using the BC. The interfacial molecular behaviors of the IgG molecules were further probed by interfacial tension (IFT) measurements.

Effect of IgG concentration at an air-water interface: a viscosity measurement

If shear thinning behavior is predominantly a bulk effect, then the more molecules present in the bulk solution, the greater the shear thinning effect, due to the increased intermolecular interactions in a crowded solution. Following this logic the higher the concentration, the greater the magnitude of the shear thinning effect. On the contrary, if shear thinning behavior is predominantly an interfacial phenomenon, then the viscosity profile of an IgG molecule will remain unchanged as the bulk concentration changes, because molecules will travel to the interface if they are surface active independent of bulk concentration, provided the size of an air-water interface remains the same. To examine the shear-thinning effect, we have probed the viscosity profile of two IgG

molecules by varying the bulk concentration using a steady shear viscosity measurement, as shown in Figure 1-2. To hold the size of the air-water interface constant, both IgG molecules were tested using the CP-50 measuring system (SA:V = 108 mm^{-1}). The concentration increments used were in the range of 0.007– 70 mg/ml, as detailed below.

The viscosity profile of the IgG1 was monitored for bulk concentration dependent shear thinning behavior and compared against its buffer solution, as shown in Figure 1. The concentration increments of IgG1 used were 0.007, 0.7, and 70 mg/ml. The viscosity profile of the A5Su buffer displays negligible shear thinning with an initial viscosity value of 25 mPa*s at the shear rate of 0.1 sec^{-1}, declining quickly to a final plateau of 1 mPa*s by 1 sec^{-1}. In the low concentration regime, the 0.007 mg/ml IgG1 sample experienced a rather flat viscosity profile pattern and remained flat throughout the entire range of steady shear applied. In the case of the A5Su buffer and 0.007 mg/ml IgG1 sample, the shear thinning effect, if at all present, appears negligible due to the low torque values (viscosity) produced by the sample at the low shear rate range. The 0.7 mg/ml IgG1 sample on the other hand, started out at 280 mPa*s and fell rapidly to 10 mPa*s after the shear rate was increased to 10 sec^{-1}. Thereafter, the viscosity profile declined to a plateau. At this concentration, the IgG1 molecules seemed to exhibit strong resistive forces (viscosity) that decreased with increasing shear rate, producing a marked shear thinning effect. Adding 0.001% PS20 to the 0.7 mg/ml IgG1solution, produced a remarkable similar viscosity profile to that of the A5SU buffer. It is plausible that due to the smaller size of the surfactant molecules, they are much more mobile and readily available to interact with the interface than the IgG molecules. The addition of a surfactant molecule such as PS20 can result in the surface passivation of the surfactant

molecules at the air-water interface, thereby preventing the IgG molecules from migrating into the vicinity of that interface and consequently diminishing the shear thinning effect seen in the 0.7 mg/ml IgG1solution. Indeed, the addition of polysorbate 20 (PS20) to IgG samples can generally eliminate viscous behavior suggesting that a surfactant can replace an IgG at the air-water interface [18]. Thus, the presence of PS20 reduced shear thinning effect in shear viscosity measurements, which suggests the shear thinning effect is dependent on the air-water interface. Understanding the nature of an IgG at the air-water interface may also elucidate some pathways of particle formation.

To our surprise, the 70 mg/ml IgG1 sample produced no discernible shear thinning even though the interface was expected to be saturated with IgG molecules at such a high bulk concentration. This suggests that the exacerbation of the shear thinning effect may not be a function of increasing bulk IgG1 concentration. On the contrary, the lack of such effect may be attributed to crowding of IgG molecules at the interface. Above a certain critical mass, the molecules at the air-water interface are unable to create strong intermolecular interactions that warrant the high viscosities at the low shear rates observed during the shear thinning effect. This suggests that the molecular conformation of IgG molecules at the interface may be affected by steric hindrance and molecular packing depending on the bulk concentration. At high IgG1 concentrations, the individual molecules are crowded at the interface and may have limited space to expose hydrophobic regions despite the energetic gain of reducing surface tension at the air-water interface. The intermolecular interactions are thus limited to the polar exteriors of IgG1 molecules, akin to “hard-sphere” models. This could then impede the shear thinning effect.[8] At certain IgG1 concentrations, such as the 0.7 mg/ml seen here for the IgG1, protein molecules at the

interface assume a more favorable conformational and orientational alignment and foster stronger intermolecular interactions among neighboring molecules as they relax into their optimal configurations with less spatial constraint. The intermolecular interactions and packing at the interface are therefore probably different between the 0.7 mg/ml and 70 mg/ml IgG1 solutions due to the drastic difference in the degree of observed shear thinning effect. Although appreciable shear thinning behavior was not observed in the 0.007 mg/ml IgG1 sample, this could be due in part to the lack of time given to the sample from formulation to measurement. Given time to age, it is possible that the critical mass required at the interface to achieve appreciable shear thinning could be reached.[11]

As shown in Figure 2, IgG2 behavior trended similarly to IgG1 behavior, although at a much larger scale within the lower shear range < 1 sec^{-1}. The viscosity profile of the A5S buffer control at 0.1 sec^{-1} corresponded to a viscosity value of 8 mPa*s. Viscosity continued to decline to 1 mPa*s at 1 sec^{-1} and maintained that viscosity for the remaining duration of the test. The IgG2 buffer, A5S, as well as the 70mg/ml concentration, showed no perceptible shear thinning in the low shear rate region, and remained flat throughout the entire shear range tested. The 70 mg/ml IgG2 viscosity profile was observed to be almost superimposable with that of the buffer solution, as shown in Figure 2. This can be explained as previously discussed in the case of IgG1. The viscosity profile of the 0.007 mg/ml sample produced an initial viscosity value of 95 mPa*s before steadily declining to a plateau as the shear rate was increased to 10 sec^{-1}. This is possibly due to a difference in the aging time required for an appreciable shear thinning effect in the IgG2 vs. the IgG1 sample. Similar to IgG1, IgG2 at 0.7 mg/ml concentration exhibited shear thinning although it was three times greater compared to the IgG1 at the same concentration. The

viscosity profile gradually declined to plateau at 100 sec^{-1}. Adding 0.001% PS20 to the 0.7 mg/ml IgG2 solution did not eliminate the shear-thinning effect. Unlike the IgG1, the initial viscosity value of the IgG2 in the presence of PS20 was 189 mPa*s, nearly one fold higher than the IgG1. The steady state viscosity profile of the IgG2 PS20 containing sample plateaued after 100 sec^{-1}. Based on the data, the presence of PS20 only marginally reduced the shear thinning effect in the 0.7 mg/ml IgG2 sample. PS20 reduces shear thinning in both IgG1 and IgG2 but shear thinning is eliminated in the former but just diminished in the latter, which suggests PS20 interacts in a slightly different manner with the IgG1 compared to the IgG2.

Based on the data shown above, the observed shear-thinning effect for both IgGs cannot be proportionally correlated to their bulk concentration. Within a certain concentration range, the shear thinning behavior observed for the IgG1 and IgG2 solutions is exacerbated with increasing bulk concentration. As shown in figure 1-2, the 0.7 mg/ml concentration for both the IgG1 and IgG2 had the steepest decline demonstrating the worst shear thinning effect among all other concentrations tested. Apparently, above a concentration threshold, shear thinning effects disappeared and IgG solutions behaved akin to Newtonian fluids as seen in the 70 mg/ml concentration for both IgG molecules. Molecules at 0.7 mg/ml concentration were apparently not densely packed at the air/water interface in comparison to the 70 mg/ml sample, yet viscosity was at its highest at 0.7 mg/ml in the early shear rate region of the viscosity profiles. This suggests different sample packing at the air-water interface at 0.7 mg/ml compared to 70 mg/ml. It is possible that at 0.7 mg/ml the IgG samples act like flexible chains and expose hydrophobic regions due to the degrees of freedom available to the more dilute system

and can thus form strong intermolecular interactions at the air-water interface, thus heavily contributing to the shear thinning effect. In comparison, the 70 mg/ml samples have limited degrees of freedom due to overcrowding at the interface. At this concentration, molecules act like hard spheres which prevent strong intermolecular interactions from forming thus dramatically limiting the shear thinning effect. It should be noted that the addition of 0.001% PS20 in the IgG2 solution did not completely eliminate the shear thinning effect as it did in the IgG1 viscosity profile. This suggests that PS20 partitions and interacts differently with IgG1 at the air-water interface than with IgG2.

Effect of size of an air-water interface: a viscosity measurement

To examine the relevance of an air-water interface to the shear-thinning effect observed in IgG solutions, we have chosen 0.7 mg/ml concentration for both the IgG1 and IgG2. At this concentration, a shear-thinning effect was observed at its maximum magnitude among all concentrations tested. Different degrees of molecular exposure to the air-water interface can be achieved by varying the surface area per unit volume (SA:V) ratio of IgG solutions to air by employing different measuring systems (CP 25, CP 50, and DG). The higher the SA:V ratio the larger the surface area per unit volume of a liquid solution. The SA:V ratio used in the measuring systems in this study are ranked: CP-25 > CP-50 > DG. If the existence of an interface plays a role in influencing the viscosity data, increasing the SA:V ratio should exacerbate the effect. Conversely if the air-water interface is irrelevant to the measurement then varying SA:V ratio should not affect the viscosity results.

The effect of varying the measuring system, in effect varying the SA:V ratio and its perturbation of shear thinning, will be monitored by a shear viscosity measurement, as shown in Figure 3-4. The viscosity profiles for the A5Su buffer and 0.7 mg/ml of IgG1 measured by CP-25, CP-50 and DG are compared in Figure 3. The viscosity profile of the buffer displays no sign of shear thinning whereas all 0.7 mg/ml samples tested do. IgG1 molecules at 0.7 mg/ml measured with the CP-25 system at 0.1 sec^{-1} produced an initial viscosity value of ~ 900 mPa*s. The same IgG in the DG system at 0.1 sec^{-1} resulted in ~ 80 mPa*s, more than an order of magnitude reduction in viscosity value within the same shear rate regime compared to that of the CP-25 system. The IgG1 shear thinning profile resulting from the CP-50 system fell between that of the CP-25 and the DG systems, which coincided with the same order of SA:V exposure to air (CP-25 > CP-50 > DG). The viscosity profile of IgG1from all three systems started to plateau after the shear rate was above 10 sec^{-1}. Slightly different behavior in the viscosity profile was observed in Figure 4 for IgG2. At 0.1 sec^{-1} IgG2 solutions tested in both CP-25 and CP-50 systems started at ~1400 mPa*s. The viscosity profiles overlap within most of the lower shear range (< 1sec^{-1}), after which the CP-50 sample decreases in viscosity at a slightly higher rate than the CP-25 system counterpart. The pattern difference in the IgG2 profiles between CP-25 and CP-50 is much less prominent compared to the profile for IgG1, suggesting a molecule specific behavioral difference at the air-water interface. The DG profile for IgG2 at 0.1 sec^{-1} corresponds to 200 mPa*s and quickly plateaus after 1 sec^{-1}, exhibiting more than double the initial viscosity value of 63 mPa*s seen for IgG1.

For both IgG1 and IgG2, their respective buffers clearly did not exhibit shear thinning behavior. This indicates that viscous resistance in the low shear regime was so low that

the buffers were acting as Newtonian fluids, suggesting molecular interactions at the interface are similar to behavior to the bulk solution. When comparing buffers to samples containing 0.7 mg/ml IgG molecules, IgG1 and IgG2, follow similar trends in viscosity profiles as molecular exposure increases at the air-water interface. While IgG1 demonstrates different molecular interactions at the interface with increasing exposure to air, closely correlating with the different degree of shear-thinning effect, IgG2 shows minimum difference in molecular alignment and packing between the CP 25 and CP 50 samples as revealed by the viscosity profiles. The greater magnitude of shear-thinning effect and the lack of differentiation between the IgG2 (CP 25 and CP 50) samples seem to suggest that IgG2 is relatively less sensitive to the air-water interface compared to similar IgG1 samples. Rather than following a gradual trend in the change of viscosity profile, just like IgG1, IgG2 seems to be establishing stronger interactions among neighboring molecules as evidenced by the larger magnitude of shear thinning behavior. The lack of sensitivity to the changes in SA:V at the air-water interface may be due in part to the already strong inter-molecular interactions at the interface; the small increase in SA:V exposed thus has little effect on the very strong intermolecular interactions formed with the IgG2. The difference in molecular exposure to air between CP 25 and CP 50 was not big enough to significantly alter such interactions.

Overall, the variability of the shear thinning effect depended on the relative SA:V exposure to air in the given measurement system. This suggests that the shear thinning is an effect highly dependent on the interfacial layer exposed and that shear thinning observed was not an artifact of the measurement system but an effect of the air-water interface.

Effect of IgG concentration at an air-water interface: interfacial rheological measurement

To further probe the molecular behavioral characteristics of the IgG1 and IgG2 at the air-water interface, interfacial rheological tests were conducted at two concentrations: 0.7 mg/ml and 70 mg/ml. Viscoelastic layer formation as a function of concentration at the air-water interface was examined accordingly.

Figure 5 and Figure 6 present the interfacial rheology measurements for the IgG1 and IgG2 samples respectively. To test the kinetic nature of the samples at the air-water interface, G'_i and G''_i were monitored as a function of time while strain and frequency were held constant (1%, 1Hz). To prevent evaporation, the samples were run in a solvent trap at 20°C and tested over a 22 hour period.

Figure 5 displays the interfacial rheology data for the IgG1 sample. The A5Su interfacial rheological profile was dominated by G''_i suggesting the interfacial layer was purely viscous, or liquid-like, in response to the oscillating deformational force applied. This observation correlates well with the A5Su viscosity profiles, as shown in Figure 1, with no shear thinning detected suggesting typical Newtonian behavior. The interfacial rheological profile of the 0.7 mg/ml sample had a G'_i component as well as a G''_i component, characteristics of a viscoelastic layer formed at the air-water interface. The G'_i component remained relatively unchanged over the 22 hour period. The G'_i profile appeared after 32 minutes of oscillatory motion. G'_i continued trending upward until it crossed G''_i at 89 min, the gel point of the system. This suggests a complex viscoelastic

layer was formed gradually at the interface. Upon reaching the gel point, a molecular network was formed following which G'_i increased appreciably and G''_i continued unchanged over the duration of the experiment. As suggested by the data in Figure 3, a larger interface would have accelerated the network formation, and resulted in stronger intra-molecular interactions, and thus a larger shear thinning effect. This data supports what was observed in figure 3, where the shear thinning effect exacerbates with increasing interfacial exposure of the IgG1 molecules. G'_i, upon addition of 0.001% PS20 to the 0.7 mg/ml IgG1 sample, was not detected as the solution exhibited viscous fluid characteristics like the buffer. G''_i dominated the interfacial rheological profile, because the addition of PS20 to 0.7 mg/ml IgG1 made the interfacial layer a predominantly viscous layer. This assessment, of purely viscous behavior, coincides with the viscosity profiles observed for two samples: 0.7 mg/ml IgG1 with PS20 and A52SU buffer. As shown in Figure 1, the addition of PS20 to the IgG1 sample effectively eliminated shear thinning suggesting that its absence is correlated with a predominantly viscous material. The same assessment can be applied to the 70 mg/ml sample as well, in which the lack of shear thinning behavior corresponds well to a viscous liquid with only G''_i apparent.

Figure 6 displays the interfacial rheology data for the IgG2 sample. Similarly, the trend pattern of the interfacial rheological profiles for the A5S buffer, 0.7 mg/ml with 0.001% PS20, and 70 mg/ml samples were dominated by G''_i suggesting the interfacial layer was viscous in response to the oscillating deformational force applied. In Newtonian fluids G'_i is non-existent; suggesting that all samples with contributions from G''_i alone were Newtonian at the air-water interface. All viscosity profiles, save for the 0.7 mg/ml IgG conditions, correlate well with the minimal shear thinning effect observed in figure 2,

suggesting a relatively viscous liquid behavior involving only G''_i shown in figure 6. Just like the IgG1 sample, only the 0.7 mg/ml IgG2 solution contained both G'_i and G''_i in the interfacial rheological profile, characteristic of the formation of a viscoelastic layer. What was unique with the IgG2 sample was that the gel point where G'_i and G''_i cross, appeared early, within a fraction of a minute, compared to IgG1. While the molecular network gradually formed at the air-water interface in the case of IgG1, the molecular interactions of IgG2 molecules occurred rapidly with the elastic component, G'_i, forming faster than its viscous counterpart, G''_i. This is evident when we compare the initial viscosity value between the IgG1 and IgG2 solutions at the 0.7 mg/ml concentration, as shown in figure 3 and 4. The larger the shear thinning detected for IgG2, the stronger the network formation. Moreover, the change in viscosity profile of IgG2 appears less sensitive to the degree of interface exposure. The difference between CP 25 and CP 50 in figure 4 can be explained by the strong resistance of a pre-existing network of the IgG2 molecules established at early times on the interface. At longer times, the pre-existing network gradually experienced conformational changes at the air-water interface, which was evidenced by the continuously evolving G'_i and G''_i throughout the duration of the experiment. Just as suggested by Pathak et al.[3], the two contributing factors found influencing shear thinning effects with IgG molecules are *film formation* at the air-water interface and induced *unstable protein clusters*. Quite possibly, the viscoelastic behavioral differences may be due to different cross linking states of IgG1 vs. IgG2 networks. The increase of G'_i over time can be attributed to the formation of multi-layers of protein beneath the initial monolayer at the air-water interface [19, 20]. It is likely that

the early establishment of inter- and intra-molecular network formation of IgG2 resulted in a different response in the interfacial rheological profile when compared to IgG1.

Effect of IgG at an interface: an interfacial tension measurement

Interfacial tension measurements were carried out as an orthogonal technique to the interfacial rheological studies to provide additional insight into the molecular behavior of the IgG1 and IgG2 molecules at the air-water interface. The 0.7mg/ml concentration was chosen since molecular characteristics of shear thinning were most prominent at this concentration for both IgG molecules based on the previous data.

Molecular behavior at the air-water interface of the 0.7mg/ml IgG molecules and their respective buffers were monitored by the change of the respective IFT/log time profile; the data are shown in Figure 7. The A5Su buffer started at 74 mN/m, and in the absence of any amphiphilic molecules, assumed a long induction time of around 93 sec, before a slight drift in IFT was detected. After this period, the IFT decreased only slightly indicating that the interfacial tension was only mildly altered. This is not surprising since the A5Su buffer does not contain amphiphilic molecules that can accumulate at the interface to alleviate the IFT. We suspect that the small decline in IFT is due to impurities from the air accumulating over the 2hrs of the study. In the presence of the IgG1 molecules. The IFT profile starts at 72 mN/m, instead of the original 74 mN/m, suggesting that the protein molecules have readily adsorbed to the air-water interface where they probably underwent conformational change that result in the initial decline of

IFT. Similar findings have been reported in the literature. As described by Lad, et al [19], such adsorption-associated secondary structural changes of protein were observed using infrared spectroscopy with respect to adsorption time (3min – 5hr.). The IFT value for the buffer was found to be ~74 mN/m, indicating that the interface more resembled a layer of primarily water molecules at the air-water interface since the IFT of a water pendant drop in air is about 73 mN/m. Thereafter monolayer formation was progressing gradually, as illustrated in regime II within the duration time of the experiment (~ 10000 s). The re-structuring process, regime III, did not take place within the experimental timeframe. In support of this, only a partial regime II was observed for the IgG1 solutions, indicates that it may take much longer for the 0.7mg/ml solution to reach equilibrium (regime III), if an equilibrium is ever reached.

The interfacial behavioral pattern of the IgG2, also shown in figure 7, reveals different characteristics compared to that of the IgG1. In comparison to its buffer solution, the presence of IgG2 molecules did not immediately serve to stabilize the air-water interface for ~ 85 seconds when the IgG2 solution was exposed to the air. The IFT profile of the IgG2 solution during this period overlaps with that of its buffer solution (A5S). Unlike the IgG1, no relief in IFT was observed during the diffusion period (regime 1). Upon adsorption at the interface, the IgG2 molecules begin to form a monolayer, indicated by regime II, where the IFT profile declines from 73 mN/m to 68 mN/m at the end of the experiment.

Since lower IFT values usually indicate a more stable surface/interface, it seems plausible that at this concentration, IgG1 molecules at the air-water interface assume a more

favorable conformational alignment than the IgG2 molecules, resulting in immediate alleviation of IFT with less spatial constraint at the air-water interface.

This apparent decline in the IFT profile for both IgG molecules occurs at the expense of a certain degree of protein depletion from the bulk. In fact, the larger the overall interface present, the greater the uptake of protein molecules sequestered from the bulk to stabilize the interface. At higher protein concentration, the interface was rapidly saturated with molecules so that molecular alignment was probably sterically hindered, thus giving rise to overall higher IFT. A protein in a saturated solution will have limited degrees of freedom and will thus pay a higher energy penalty to expose hydrophobic regions to lower IFT; therefore the IgG is akin to a “hard sphere” past a certain concentration threshold, because of the constraint on the degrees of freedom. Likewise, at lower protein concentrations, the availability of the protein molecule may be too low at the interface to foster the formation of a complete monolayer. Such is clearly the case in both buffer solutions, resulting in the high IFT penalty.

Conclusion:

Summary of Findings:

The results and discussion presented above have attempted to answer the fundamental questions posed in the introduction:

1. Was shear thinning merely a concentration- related bulk behavior?
2. Or can the difference in molecular exposure, and the subsequent molecular re-organization, at the interface account for discrepancies in viscosity profiles?

To answer the first question of whether shear thinning was directly correlated to bulk behavior, bulk concentration was varied and the effect on shear thinning was monitored on a shear viscosity measurement. The results suggested that shear thinning did not increase as bulk concentration increased arguing against the hypothesis that the observed shear thinning effects were a property of IgG bulk behavior. The shear thinning observed was most dominant in the middle concentration range which suggested strong interfacial behavior at this particular concentration. This middle concentration, because it showed the most dramatic shear thinning effect, was used in subsequent studies to probe the shear thinning effect at the air-water interface.

The second question proposed that IgG molecular exposure to the air-water interface may be responsible for the observed shear thinning effect. To address this question, SA:V at the air-water interface was varied while the shear thinning effect was monitored by shear viscosity measurements. The results revealed a positive correlation between SA:V

exposed to the air-water interface and shear thinning behavior. In other words, the larger the proportion of sample that was exposed to air, the greater the observed shear thinning effect. Distinct differences between IgG1 and IgG2 also became apparent as a function of SA:V exposure. For example, IgG1 was very sensitive to the degree of SA:V exposed while IgG2 was not. Despite the lack of shear thinning sensitivity to SA:V exposed, IgG2 had the greatest shear thinning effect. This surprising difference in the IgG1 and IgG2 shear thinning behavior was further investigated using interfacial rheology.

The examination of the IgG1 and IgG2 behavior at the interface revealed a G'_i and G''_i crossover point often synonymous with a gel formation point. For both IgG molecules, a film was formed at the air-water interface and the G'_i grew at a faster pace than the G''_i over the duration of the experiment. The crossover point was reached almost immediately for IgG2 and took nearly half an hour for IgG1suggesting IgG2 readily forms a strong intermolecular network while IgG1 much more slowly forms a strong intermolecular network.

IgG behavior at the air-water interface was also probed with IFT, to orthogonally verify the observed rheological data. Indeed, both IgG1 and IgG2 lowered the IFT values in regime II compared to the buffer IFT confirming the presence of the IgG molecules at the air-water interface.

At the air-water interface, IgG molecules can be considered to act like hard spheres or flexible chains depending on the bulk IgG concentration. As concentration increases, the energy barrier to displace neighbors and move to a lower energy state increases which in turn prevents IgG molecules from exposing hydrophobic regions to air. Under these

crowded conditions, the IgG molecules mimic the bulk crowded condition. As concentration decreases, the energy barrier to displace neighbors decreases such that these IgG molecules effectively act as surfactants to lower IFT. These conditions provide a monolayer IgG conformation that differs from the bulk IgG's structure. In that event, multi-layer formation may occur ordering the bulk IgG to the monolayer conformation. The initial monolayer may act as a catalyst to changes in the bulk formation such as aggregation, particle formation and gelation.

Though IgG molecules follow similar trends at the air-water interface, they do not have identical behavior at that interface. For the IgG1 molecules, the SA:V exposed to air dramatically affects the observed shear thinning behavior. On the other hand, the IgG2 used here appears insensitive to the degree of SA:V exposed as shown in Figure 4, suggesting the molecules behave differently in response to the air-water interface. Previous studies suggested that protein interfacial behavior is primarily dependent on the size of the protein[4] and was largely independent of individual protein characteristics. In this case, both the IgG1 and IgG2 molecules were approximately 150 kD and although the IgG molecules had similar trends, the distinct IgG molecules proved to act uniquely to the air-water interfaces presented. It cannot yet be concluded, however, that these different properties are subclass specific without more extensive studies of multiple IgGs from each class.

Future Work:

A more in depth study of the effects of formulation changes on interfacial rheology profiles is in order. The aging effect on IgG molecules has not been extensively studied

especially in relation to highly purified and high concentration IgG molecules. Investigations in this area may provide insight into long term storage conditions and the different problems associated with high concentration IgG solutions especially with regard to hydrophobic interfaces such as silicon oil and container surfaces. Furthermore, very high concentrations exceeding 70 mg/ml, have not been investigated and would be of significant value to the pharmaceutical field which often formulates well above 100 mg/ml for commercial IgG therapeutics. Rheological tests, if done with care, can be used in a manner similar to spectroscopy for monitoring material properties and quality control. For such applications a detailed mathematical understanding of rheology may not be necessary.[7]

Biophysical techniques that monitor protein structural changes as a function of film formation would help to characterize multi-layer formation of IgG molecules. A study of protein at the air-water using ER-FTIR showed tertiary structure changes within a few minutes after protein binding to the air-sample interface in the form of non-native anti-parallel beta sheets. After several hours, the spectrum shifted to more ordered beta sheets possibly due to multi-layer formation. The protein layer just below the monolayer at the air-sample interface is presumably more native in structure and thus shifts the average of the ir spectrum to more ordered structure over time [19, 20]. A similar study on IgG molecules using FTIR or Raman methods could prove to be informative and lead to a better understanding of IgG molecules at other hydrophobic interfaces as seen with solid surfaces [21]

Figures

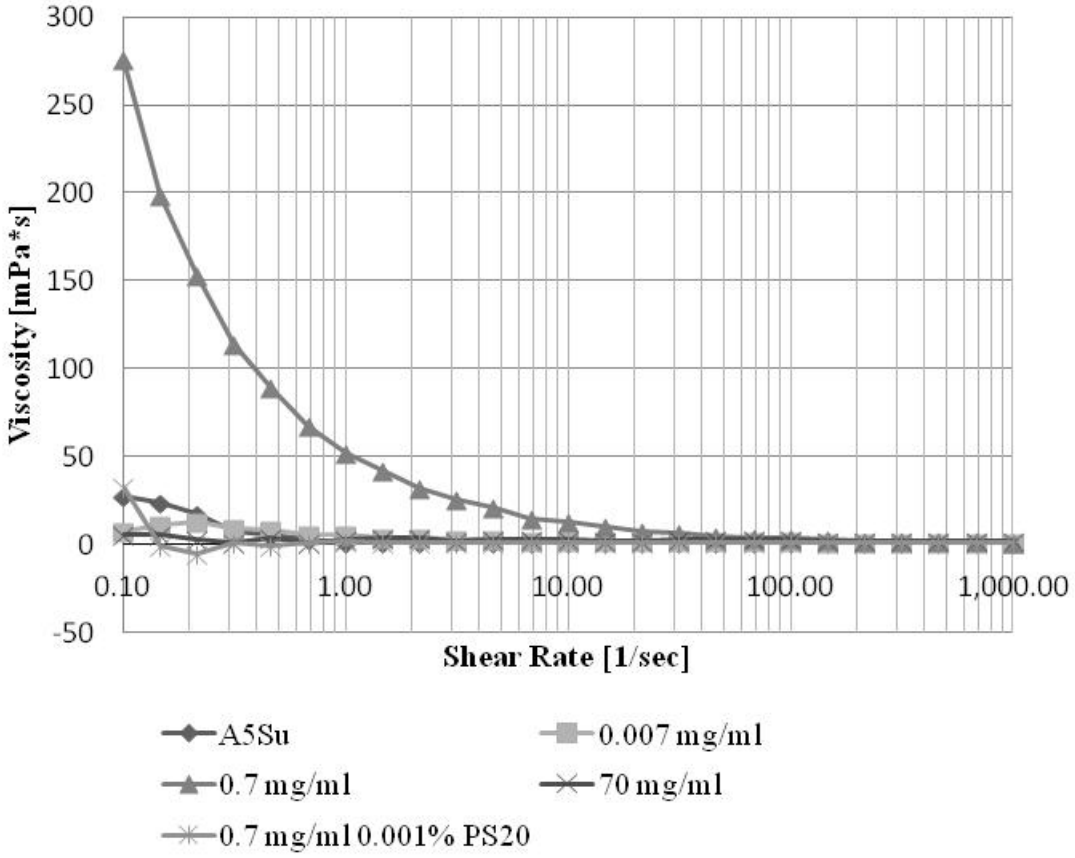

Figure 1 – Viscosity behavior of IgG1 in A5Su at 3 different protein concentrations at 20°C (0.007 – 70 mg/ml) using CP-50 geometry.

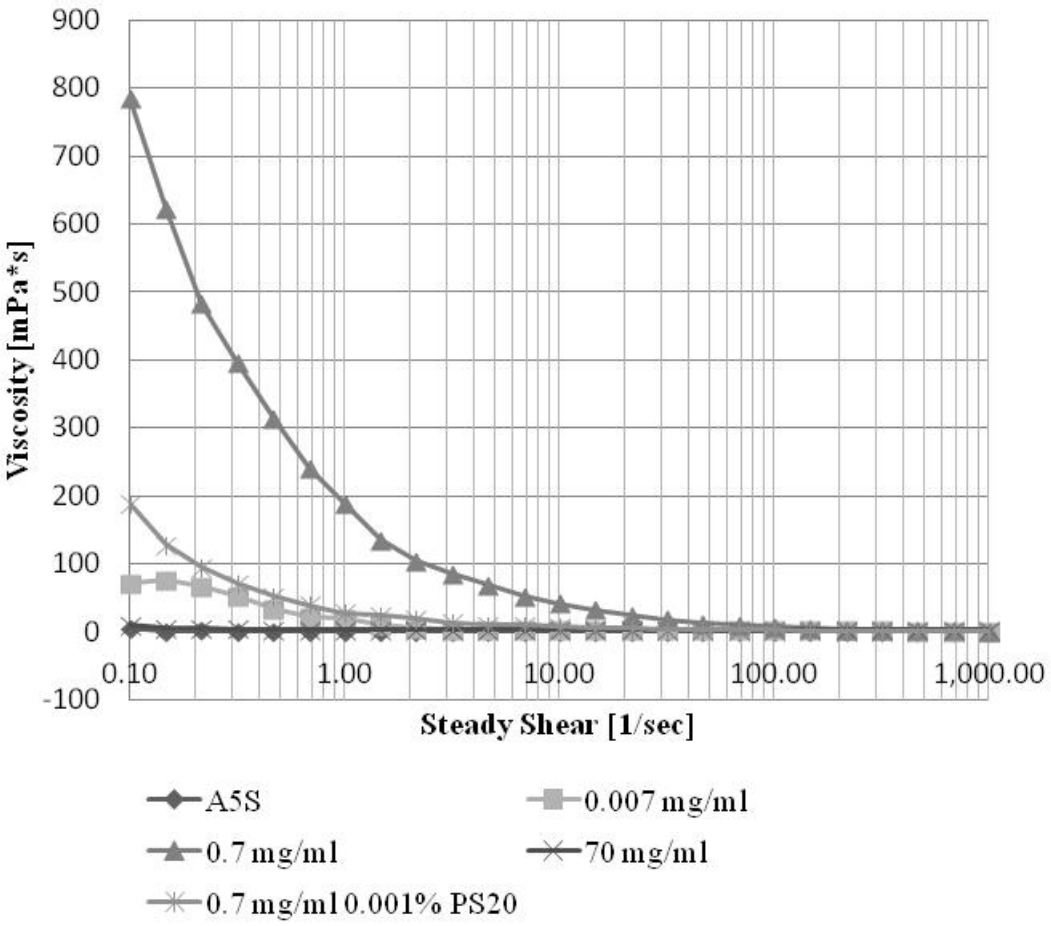

Figure 2 – Viscosity behavior at varying concentrations of IgG2 in A5S at 20°C (0.007 – 70 mg/ml) using CP-50 geometry.

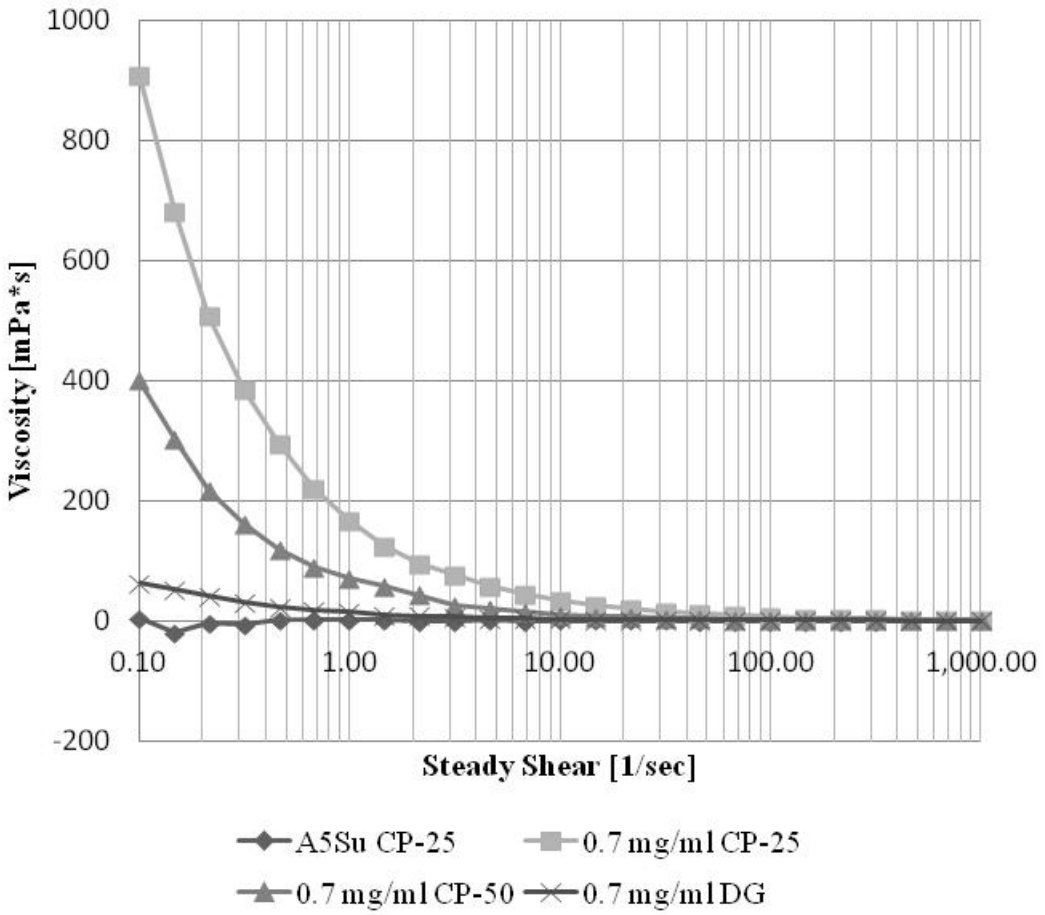

Figure 3 – Viscosity profile of 0.7 mg/ml IgG1 in A5Su at 20°C. Surface Area to Volume ratio exposed to air increases with geometry in the following order CP-25 > CP-50 > DG.

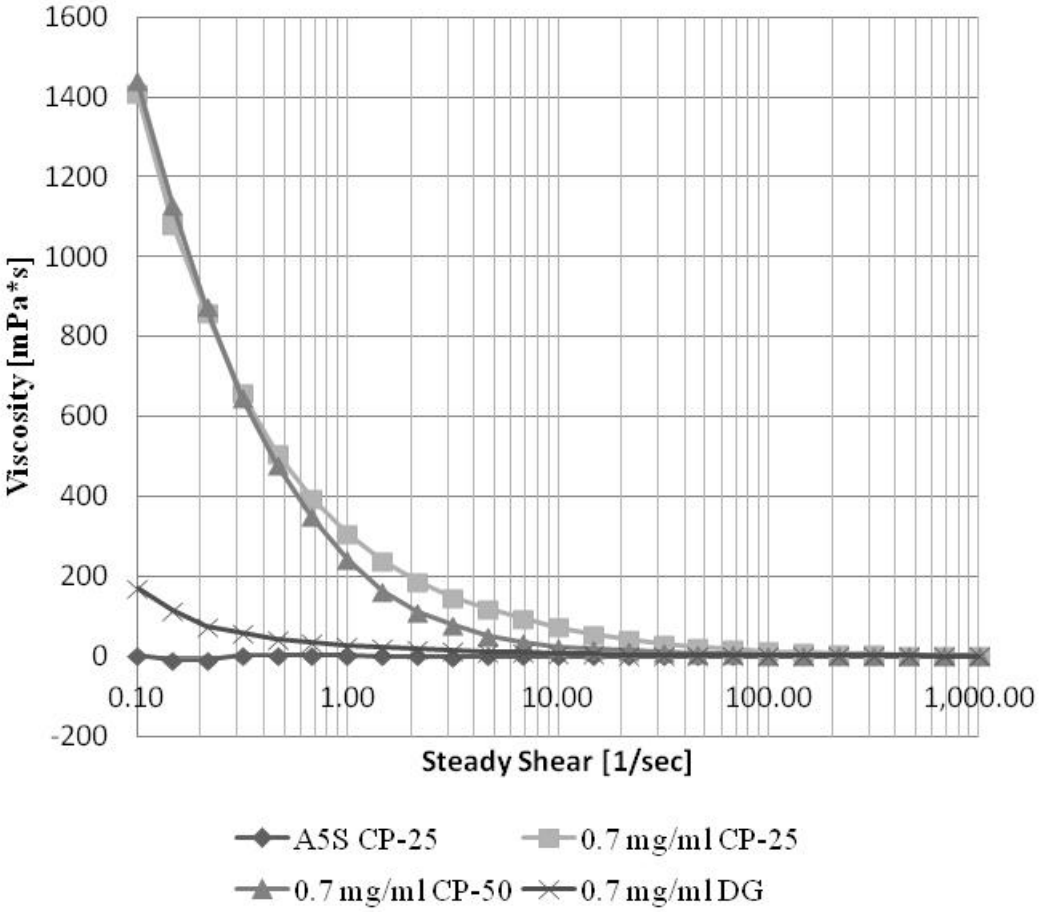

Figure 4 – Viscosity profile of 0.7 mg/ml IgG2 in A5S at 20°C. Surface Area to Volume ratio exposed to air increases with geometry in the following order CP-25 > CP-50 > DG.

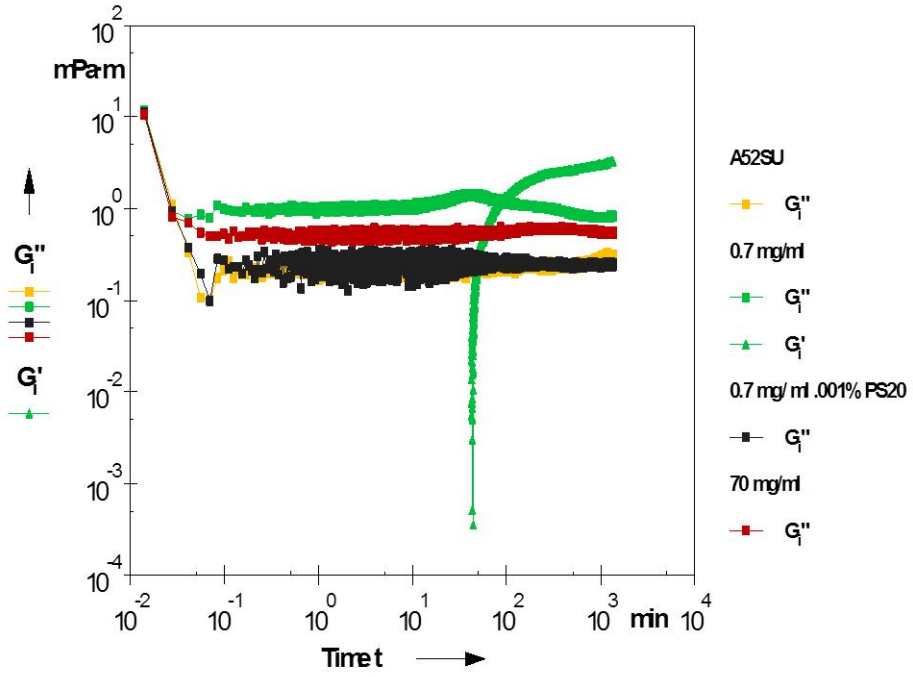

Figure 5 – IgG1 interfacial rheology profile holding stress and strain constant (1%, 1Hz) over a 22 hour period at 20°C. Measurement was performed with a BiCone 65mm in diameter and a solvent trap.

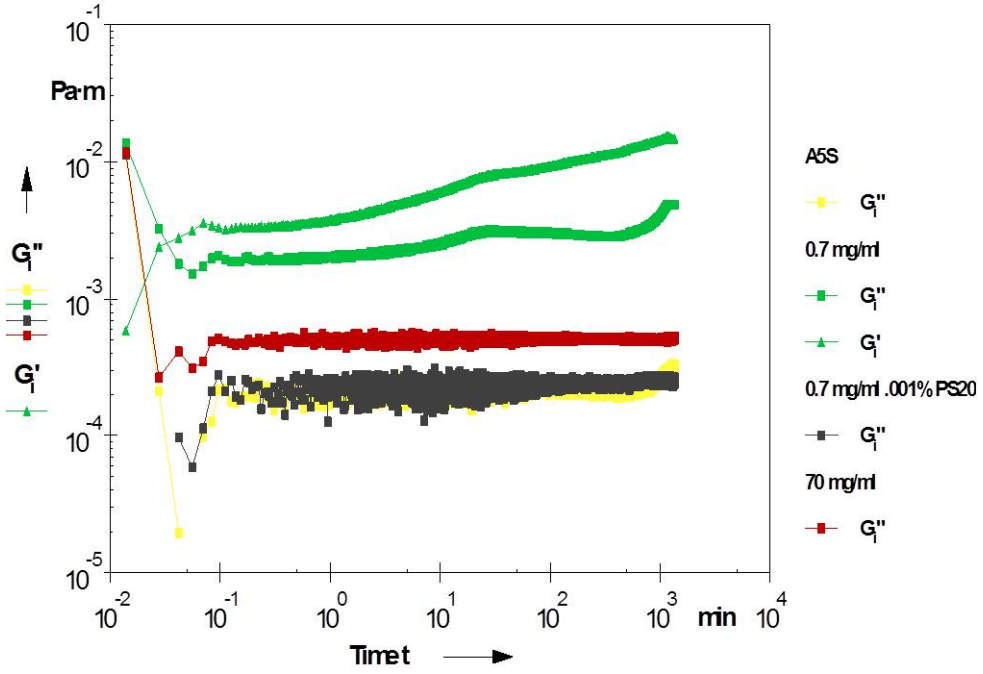

Figure 6 – IgG2 interfacial rheology profile holding stress and strain constant (1Hz, 1%) over a 22 hour period at 20°C. Measurement was performed with a BiCone 65mm in diameter and a solvent trap.

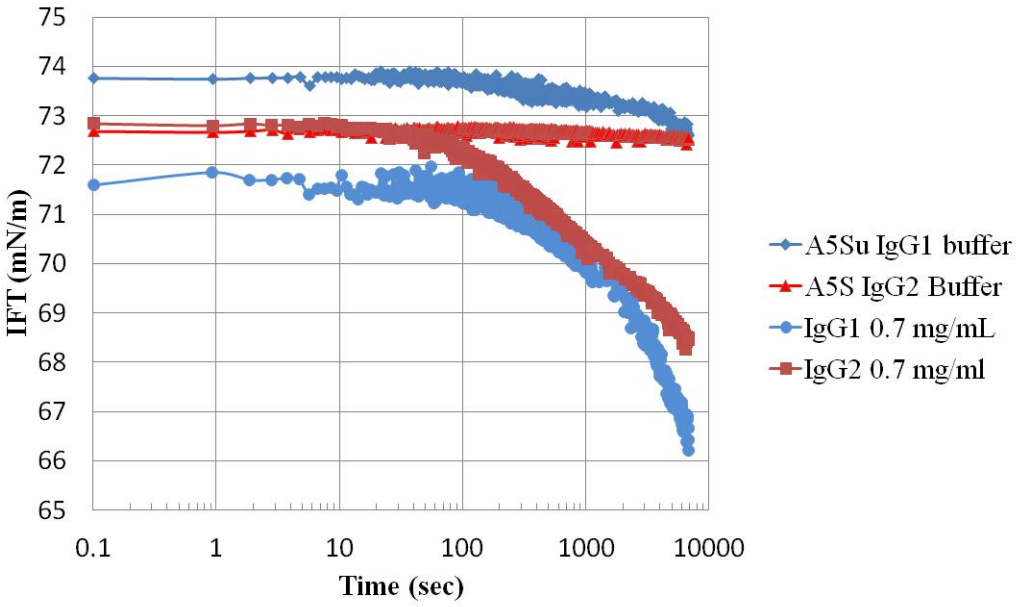

Figure 7 – IgG1 and IgG2 interfacial tension at the air-water interface at 20°C. Samples were formulated at 0.7 mg/ml IgG1 in A5Su and 0.7 mg/ml IgG2 A5S. The respective buffers were used as a control as shown.

References

1. A. Bos, M. and T. van Vliet, *Interfacial rheological properties of adsorbed protein layers and surfactants: a review.* Advances in Colloid and Interface Science, 2001. **91**(3): p. 437-471.

2. Sluzky, V., et al., Kinetics of insulin aggregation in aqueous solutions upon agitation in the presence of hydrophobic surfaces. Proceedings of the National Academy of Sciences, 1991. **88**(21): p. 9377-9381.

3. Pathak, J.A., R.R. Sologuren, and R. Narwal, Do clustering monoclonal antibody solutions really have a concentration dependence of viscosity? Biophys J, 2013. **104**(4): p. 913-23.

4. Vogler, E.A., *Protein adsorption in three dimensions.* Biomaterials, 2012. **33**(5): p. 1201-37.

5. Beck, A., et al., Strategies and challenges for the next generation of therapeutic antibodies. Nature Reviews Immunology, 2010. **10**(5): p. 345-352.

6. Shire, S.J., Z. Shahrokh, and J. Liu, *Challenges in the development of high protein concentration formulations.* Journal of Pharmaceutical Sciences, 2004. **93**(6): p. 1390-1402.

7. Morrison, F.A., *Understanding Rheology*. 2001: Oxford University Press.

8. Chen, P., Molecular Interfacial Phenomena of Polymers And Biopolymers. 2005: Woodhead Pub.

9. Miller, R.a.M., D., *Proteins at liquid interfaces*. 1998, New York; Amsterdam: Elsevier.

10. Burgess, D.J., L. Longo, and J.K. Yoon, *A novel method of assessment of interfacial adsorption of blood proteins.* J Parenter Sci Technol, 1991. **45**(5): p. 239-45.

11. Burgess, D.J., J.K. Yoon, and N.O. Sahin, *A novel method of determination of protein stability.* J Parenter Sci Technol, 1992. **46**(5): p. 150-5.

12. Ariola, F.S., A. Krishnan, and E.A. Vogler, *Interfacial rheology of blood proteins adsorbed to the aqueous-buffer/air interface.* Biomaterials, 2006. **27**(18): p. 3404-12.

13. J. A. Lopes da Silva and Rao, A., Rheological Behavior of Food Gels, in Rheology of Fluid and Semisolid Foods: Principles and Applications: Principles and Applications, A. Rao, Editor. 2010, Springer.

14. A., F.E., Food Biophysics of Protein Gels: A Challenge of Nano and Macroscopic Proportions. Food Biophysics, 2006. **1**(1): p. 41-50.

15. Oakenfull D., P.J., *Protein Gelation*, in *Food Proteins and Their Applications*, S. Damodaran, Editor. 1997, CRC Press. p. 694.

16. Erni, P., P. Fischer, and E.J. Windhab. Rheology of surfactant assemblies at the air/liquid and liquid/liquid interface.

17. Murray, B.S. and E. Dickinson, *Interfacial Rheology and the Dynamic Properties of Adsorbed Films of Food Proteins and Surfactants.* Food Science and Technology International, Tokyo, 1996. **2**(3): p. 131-145.

18. Patapoff, T.W. and O. Esue, Polysorbate 20 prevents the precipitation of a monoclonal antibody during shear. Pharm Dev Technol, 2009. **14**(6): p. 659-64.

19. Lad, M.D., et al., *The adsorbed conformation of globular proteins at the air/water interface.* Physical Chemistry Chemical Physics, 2006. **8**(18): p. 2179-2186.

20. Desfougeres, Y., et al., Strong improvement of interfacial properties can result from slight structural modifications of proteins: the case of native and dry-heated lysozyme. Langmuir, 2011. **27**(24): p. 14947-57.

21. Katzenstein, G.E., Role of conformational changes in the elution of proteins from reversed-phase HPLC columns. Proc. Natl. Acad. Sci. USA., 1986. **83**(12): p. 4268-72.