AN ABSTRACT OF THE THESIS OF

Warren D. Gray for the degree of Honors Baccalaureate of Science in Chemical Engineering presented on 27 May 2009. Title: Surfactant Effects on Adsorption of Recombinant Factor VIII (rFVIII) at the Air-Water Interface.

Abstract Approved: _____
Joseph McGuire

The primary goal was to characterize the adsorption of a recombinant therapeutic protein in the presence of selected surfactant species at the air-water interface. For this purpose, dynamic interfacial tensiometry was used to determine the surface tension kinetics exhibited by protein solutions containing either Tween 80 or the poly[ethylene oxide]-poly[propylene oxide]-poly[ethylene oxide] triblock copolymer surfactant, Pluronic® F68. Tween 80 is widely used in downstream processing and formulation, while F68 is widely used upstream in cell culture for its protective effect on mammalian cells. Very low protein concentrations were used in order to represent conditions consistent with cell culture operations. The apparent effect of each surfactant on protein adsorption was determined to be strongly dependent on surfactant concentration. While F68 appeared to be more effective at reducing protein adsorption at extremely low surfactant concentrations, Tween 80 appeared to be much more effective at higher surfactant concentration. Conclusions point to the opportunity to optimize surfactant use in upstream and downstream processing in biotechnology, where surfactant design or selection might be based on ensuring protein stability and activity retention by simultaneous use of surfactants which act according to complementary stabilization mechanisms.

Key Words: protein, surfactant, interface, adsorption

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Surfactant Effects on Adsorption of Recombinant Factor VIII (rFVIII) at the Air-Water Interface

by

Warren D. Gray

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Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Warren D. Gray, Author

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To a young undergraduate, the Honors Thesis and all that it entails can be incredibly daunting. One might say that the stars aligned when I found myself working in Dr. Joseph McGuire's lab; I could not have asked for a more ideal arrangement. I would like to thank Dr. McGuire for making what could have been an arduous and dry process something enticing, rewarding, and a living part of me. His intellect, wit, and imagination-invoking teaching skills imbued in me a real desire to work for the greatest results. I am proud to call him my mentor and friend. Undoubtedly, his influence will continue to guide my educational and career paths.

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TABLE OF CONTENTS

LIST OF FIGURES

Page

DEDICATION

To my parents, who never fail to believe in me.

INTRODUCTION

Hemophilia is a genetic blood disorder wherein the clotting cascade, which is the body's response to a vascular wound, is impaired, resulting in severe blood loss for relatively minor injuries. It is especially prevelant in men: one in 5000 males in the United States have hemophilia (National Hemophilia Foundation, 2006). The cause of the extended bleeding is due to errors in the bodily production of proteins in the clotting cascade, specifically the malformed protein Factor VIII (FVIII). The medical advances of the day have provided hemophiliac patients with a solution: the industrial production of recombinant FVIII (rFVIII) from genetically engineered cells (such as from kidney cells of baby hamsters) in bioreactors. However, the production of rFVIII is complex and the annual worldwide production is on the order of several hundred grams. The cost for weekly doses of rFVIII for patients is between $60,000 and $150,000 annually, and complications such as major surgeries or HIV/AIDS treatment can increase the cost exponentially (National Hemophilia Foundation, 2006). It is suspected that the yield of rFVIII is reduced (and therefore cost increased) due in part to issues during production, specifically the adsorption and subsequent irreversible denaturing of rFVIII to air-water and solid-water interfaces. If a molecule adsorbs to interfaces, it is termed a surface active agent (or surfactant). One method of protein adsorption prevention is to introduce other surface active agents which will compete with rFVIII for interface adsorption. The purpose of this project is to investigate the use of two surfactants, Pluronic® F68 and Tween80, to prevent rFVIII adsorption at the air-water interface. Data analysis from this project will justify further investigation, and it is hoped that eventually conclusions

garnered from this lab will be implemented in the pharmaceutical industry to reduce the cost for hemophiliac patients, as well as recipients of other recombinant proteins.

BACKGROUND

The polar nature of water molecules causes them to interact with one another, forming hydrogen bonds. Other molecules within an aqueous solution interact with each other and water molecules by forming hydrogen bonds and ionic bonds. However, a nonpolar molecule fails to form hydrogen bonds with water (possibly forming other bonds), and this interaction is less favorable than if water molecules were free to interact with themselves. The water molecules around the nonpolar/hydrophobic molecules become organized and in an effort to reduce this order, the system will encourage nonpolar molecules to be placed together, thereby freeing the ordered water molecules to interact freely with the water molecules in bulk. This tendency for the hydrophobic molecules to gather together in order to allow the water molecules movement and interaction with each other is called the "hydrophobic effect" (Jeremy M. Berg, The Hydrophobic Effect, 2007).

This phenomenon occurs due to thermodynamics. The thermodynamic term "entropy" refers to the degree of disorder in a system, and the second law of thermodynamics states that "the total entropy of a system plus that of its surroundings always increases." This explains the hydrophobic effect because when water molecules depart from hydrophobic molecules, they are more free to interact with other water molecules (the disorder is greater) than if they are interacting with the nonpolar molecules (Jeremy M. Berg, The Laws of Thermodynamics Govern the Behavior of Biochemical Systems, 2007).

Proteins are polymers consisting of amino acids connected by peptide bonds. Each amino acid contains a side chain with varying properties, such as hydrophobicity, hydrophilicity, ionizability, and size. The combination of these characteristics give

protein regions certain abilities, which is how proteins are specialized to perform certain functions, such as an enzyme, structure, signal, etc. The hydrophobic regions of FVIII can utilize the hydrophobic effect when the protein exists in natural conditions. However, problems arise when it occurs in foreign environments, such as in the production of rFVIII.

Bioreactors producing rFVIII contain aqueous nutrient broth and are sparged with air to provide oxygen for the growth of genetically engineered cells. The air molecules within the bubbles and at the air gap form a hydrophobic surface at the air-water interface. Additionally, the metal molecules of the bioreactor and auxiliary parts form a hydrophobic surface at the solid-water interface. The bioreactor systems contain rFVIII proteins which are affected by the hydrophobic effect: in an effort to increase the entropy of the system by freeing the water molecules in unfavorable interactions with the hydrophobic regions, the proteins will adsorb to the air-water and solid-water interfaces (Figure 1). Unfortunately, the new environment in which the proteins are placed encourage the unfolding of the rFVIII, which destroys the viability of the protein.

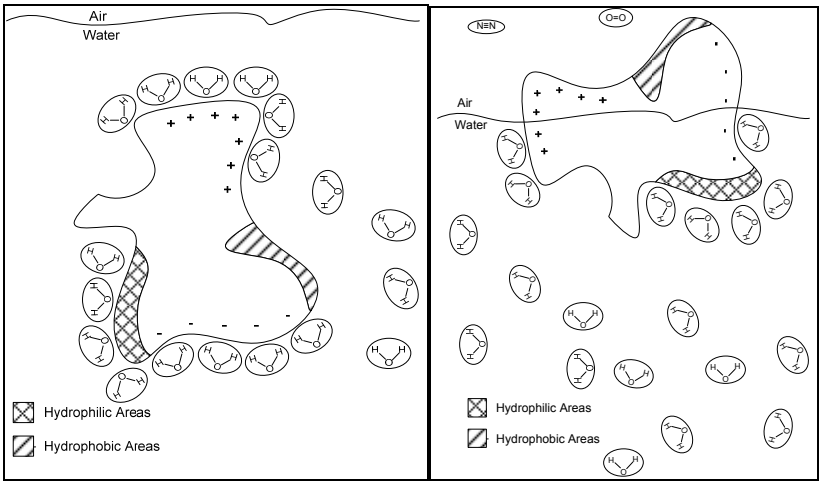

Figure 1: Representation of energetically-driven protein adsorption to air-water interface. Adsorption of protein increases the entropy in the bulk solution.

The introduction of additional surfactants implies that there may be competition among the species for interfacial adsorption. For example, a surfactant may have a greater adsorption rate constant, which would occupy the interface and prevent rFVIII adsorption. If the surfactant has greater affinity for the interface than rFVIII, then it will be less likely to leave the interface to make room for rFVIII. This further prevents rFVIII adsorption. Another scenario is when surfactants and rFVIII aggregate to form complexes. In such cases, the hydrophobic regions of the protein and surfactants may interact, thereby reducing the exposed hydrophobic regions of the complex. When that happens, the water molecules in the bulk solution no longer interact with the now-hidden hydrophobic regions, and the system will not energetically favor the adsorption of the protein to the interface. In this project, Pluronic® F68 and Tween80 are two surfactants investigated for their potential in preventing rFVIII adsorption at the air water interface. They are FDA-approved and are currently used in various parts of rFVIII production.

Directly measuring the amount of adsorbed surfactant or rFVIII is difficult. However, it is possible to measure adsorption indirectly by examining the surface tension of the air-water interface. As surfactants (including rFVIII) adsorb, the molecules interfere with the ordered structure of the water molecules at the interface. The interface is destabilized and as a result, the surface tension decreases. Therefore, interfacial surfactant adsorption is directly proportional to observed surface tension depression. Tensiometry is the method of measuring surface tension, and a tensiometer is an apparatus used to measure surface tension.

A tensiometer inserts a probe into the air-water interface, after which a force is applied upward to pull the probe from the surface. A liquid film connects the probe to the

surface, and the tensiometer increases the force on the probe. The probe is pulled farther from the interface and eventually the counter force of the film reaches a maximum when the film circumference at the interface decreases. Current surface tension of the measured interface can be calculated from

$$F = p * \gamma * \cos(\theta), \tag{1}$$

where p is the wetted perimeter of the probe and θ is the contact angle of liquid on the probe (John Davies, 1996). The probes used are prepared so that they are assumed to exhibit contact angles equal to 0^o. Therefore, only the "wetted" perimeter of the probe and the maximum measured force are required to calculate the surface tension at the air-water interface.

Protein-surfactant interactions at liquid-air and liquid-solid interfaces are complex and depend highly on protein and surfactant concentrations and behavior. Ideally, if the protein concentration of a solution is kept constant and surfactant concentration is increased, five distinct regions of steady state surface tension depression can be observed (Omkar Joshi, 2008). A theoretical plot of this type, along with the protein-surfactant behavior which gives rise to each region, is presented in Figure 2. In dilute protein solutions (such as those being studied this project) the five regions of this plot are harder to observe. However, the shapes of the plots, when compared to the standard plot shown in Figure 2, can still give a good indication of the protein-surfactant interactions taking place.

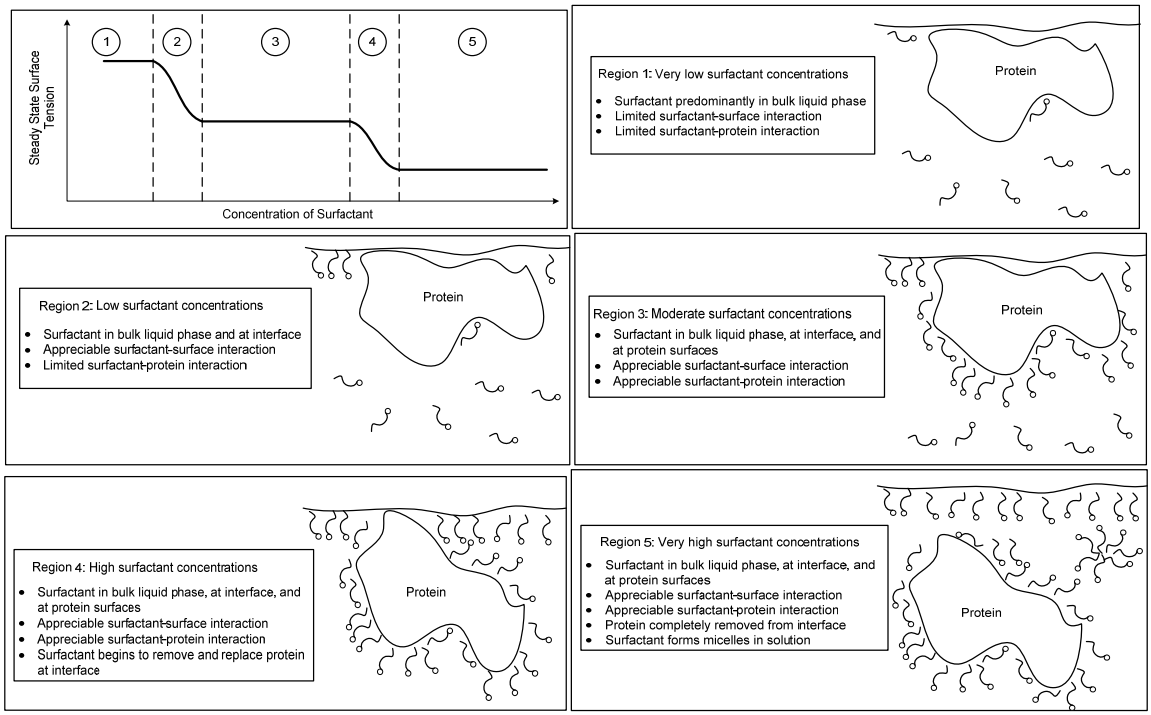

Figure 2: theoretical correlation of steady state surface tension depression values of protein-surfactant solutions. Actual results may vary based on protein and surfactant behavior and concentrations.

In addition to surfactant concentration, individual surfactant structure has a major impact on protein-surfactant interaction and surfactant-surface interactions. Two important surfactants used in recombinant protein production are Tween 80 and Pluronic® F68; the structures of these surfactants are presented in Figure 3.

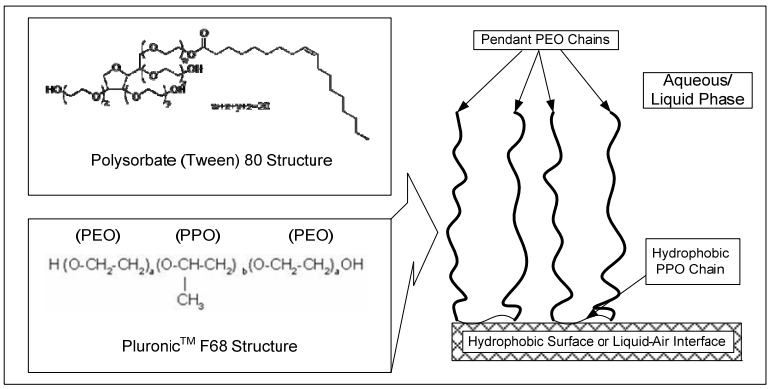

Figure 3: Structures of Tween80 and Pluronic® F68 compared; Tween 80 is much smaller and has shorter chains than F68. F68 in aqueous solution is also pictured.

The structures of these two surfactants differ in several ways. Tween80 is composed of a single chain which is considerably shorter than the chains of F68. In addition, Pluronic® F68 is a triblock copolymer of hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO) chains. F68 contains a PPO chain weight of roughly 1600 g/mol, and PEO chains which make up 80% of the surfactant weight. The molecular weight of F68 is 8400 g/mol, where the much smaller Tween 80 has a molecular weight of 1300 g/mol. These masses, in addition to the shape of the polymers, alter the surfactant's behavior which helps tailor each for specific purposes.

EXPERIMENTAL PROCEDURES

Fibrinogen solution preparation

To prepare the fibrinogen solutions used during this project, enough freeze-dried human plasma fibrinogen[1] was combined with 10 mL high-performance liquid chromatography (HPLC) grade ultrapure water to create a 1.0 mg/mL incubation stock solution. Initially, the HPLC water was pre-warmed to 37 °C prior to introducing the solid fibrinogen. Using an incubator[2], this solution was incubated at 37 °C on an 8.1 rpm rotating tube clamp (known as a "rotisserie") until all of the protein dissolved. While handling the protein solution, effort was made not to shake or disturb the solution, as doing so could cause bubbling of the surface which results in increased protein adsorption and denaturing. Serial dilutions of this incubation stock solution, each in 10 mL of HPLC water at 21 °C, were then made to achieve the desired test solution concentrations. The volume of these final solutions was required to be at least 10 mL due to the physical limitations of the tensiometry equipment. For incubation and test solution preparation, 15 mL sterile Falcon tubes[3] were used. Freeze-dried fibrinogen has a maximum solubility of 6 mg/mL, so our desired stock solution of 1 mg/mL was judged to present no solubility problems (Shih-Jeh Wu, 1999). That assumption was correct, as we were able to repeatedly prepare solutions of 1 mg/mL concentration without precipitation of protein.

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[1] Item F3879, Sigma-Aldrich, St. Louis, MO. Reported to be 59% protein (97% clottable), with the remainder sodium citrate and sodium chloride.
[2] Model 1565, VWR International, West Chester, PA
[3] Model 35-2097, Becton Dickinson Labware, Franklin Lakes, NJ

Fibrinogen concentration trials

To analyze the effect of bulk protein concentration on surface adsorption, a 1.0 mg/mL fibrinogen incubation stock solution was incubated at 37 °C, as previously described, for 1 hour. An appropriately sized aliquot of this solution was then diluted with 10 mL of HPLC water, to create a 0.10 mg/mL test stock in a 15 mL Falcon tube and slowly rotated several times to disperse the protein. This test stock was then quickly transferred to a tensiometry vessel and surface tension measurements were begun using the "ring method". These measurements continued until the surface tension settled to a final steady state value (as indicated by a nearly horizontal surface tension vs. time relationship). This process was repeated with test solutions of 0.075, 0.050, 0.01, 0.005 mg/mL.

Tensiometer operation

For all surface tension measurements, a dynamic tensiometer[4] was used. The tensiometer balance was calibrated with a known mass prior to conducting any experimental runs. The dynamic tensiometer used accommodated both Wilhelmy Plate and Du Nouy Ring type probes. The probes were rinsed using HPLC water and heated until they turned red using a Bunsen burner prior to each experimental run. The vessels used to contain the test solution during tensiometry runs were sterile polystyrene Petri dishes.[5] A new dish was used for each trial to enhance experiment uniformity. To collect and store surface tension data, the manufacturer's software was used. For kinetic runs, continuous sampling was used to measure surface tension repeatedly about every 20

[4] Model T10, First Ten Angstroms, Inc. (FTA), Portsmouth, VA
[5] Model 35-1007, Becton Dickinson Labware, Franklin Lakes, NJ

seconds. For steady state runs, the experimental dish was prepared and left to settle for 2 hours. This dish was then carefully transferred to the tensiometer and 10 sequential surface tension measurements were made in order to determine the final steady state surface tension.

Tensiometry probe determination

Both Wilhelmy Plate and Du Nouy Ring probes were investigated for conducting surface tension measurements. The probes investigated were both platinum-iridium coated.[6] To begin, "ring method" and "plate method" sampling regimes were used, in conjunction with the ring and plate probes respectively, to determine the surface tension of pure water. This was conducted in order to make sure the tensiometer was accurate.

rVIII preparation

In order to prepare required solutions of the therapeutic protein, pre-bottled treatment vials were obtained from our industrial sponsor. After preparing these according to sponsor specifications, the result was 2.5 mL of a solution with 100 IU/mL of active protein; IU or "International Unit" is an arbitrary unit of protein activity agreed upon by doctors and scientists based upon medical trials of the protein being used. This solution also contained several proprietary buffer components and 80 ppm Tween 80. Dilution of this therapeutic protein solution was conducted using a proprietary buffer solution in 15 or 50 mL Falcon tubes[7] depending upon the required volume. Each

[6] Du Nouy Ring: Model 70537, CSC Scientific, Fairfax, VA; Wilhelmy Plate: Type T-107B, CSC Scientific, Fairfax, VA
[7] Model 35-2097, Becton Dickinson Labware, Franklin Lakes, NJ

experimental solution was at least 10 mL, as this was the minimum amount which could be used in the tensiometry apparatus.

Surfactant stock solution preparation

For those experiments which required adding surfactants, two surfactant stocks were created: Tween 80 stock and Pluronic® F68 stock. These were both created to be 1000 ppm initially and added to the experimental solutions as needed. Tween 80 stock was prepared from a pure liquid Tween 80 stock. The 1000 ppm experimental stock was created by diluting the appropriate amount of this pure stock with the proprietary buffer solution in 50 mL Falcon tubes. The F68 stock was created in a similar manner except the pure form was a solid powder which needed to be mixed until dissolved in buffer. Since surfactant coats solid surfaces over time, each of these 1000 ppm stock solutions were remade each day in order to help experimental accuracy. For surfactant/protein studies, protein solutions were prepared as previously described and the appropriate amount of 1000 ppm surfactant stock was added in order to prepare the desired concentration in the experimental solution. For surfactant studies alone, the appropriate 1000 ppm stock solution was diluted, as needed, using proprietary buffer.

RESULTS AND DISCUSSION

Fibrinogen as a model

Fibrinogen was determined to be a model protein due to its availability and functional similarity to the therapeutic protein (Hodges, 2001) (Cussler, 1997). As such, fibrinogen was initially investigated in order to explore general protein behavior and it was economically advantageous to conduct open-ended investigation and fine-tune the necessary laboratory procedures on a relatively inexpensive model before beginning work with the expensive therapeutic protein.

Surface tension dependence on temperature

Upon adding freeze-dried fibrinogen to HPLC-grade water, the mixture was incubated at 37 ^{o}C to mimic physiological settings and encourage the dissolving of protein. Heating the diluted solutions to physiological temperature (37 ^{o}C) was considered as well, but it was realized that the solutions would be subject to conductive heat transfer during tensiometer operation. Literature provides a linear relationship of temperature and surface tension of water.[1] It was found that the surface tension of water at 37 ^{o}C is 69.9 mN/m, but increases over as the solution cools down to 72.8 mN/m at 20 ^{o}C. It was desired that the data reflect surface tension change due only to protein adsorption. Discussion with the sponsor firmed the assumption that the proteins behave similarly in the range of 20 ^{o}C to 37 ^{o}C, so all experimental trial solutions were prepared and run at 20^{o}C. Such protocol minimizes the impact of solution cooling on surface tension change.

Tensiometry probe determination

Visual observations, as well as surface tension data, were collected when determining which probe type to use. Although the two probes were both platinum-iridium coated, their shapes, surface areas, and sampling methods differed. The Wilhelmy Plate, the first probe to be tested, used a "discontinuous" sampling method, in the future referred to as the "plate method". In this type of sampling, the dry probe is lowered to the liquid interface, pushed a certain depth below the interface to "wet" the probe's surface area (roughly 4 mm), drawn back up to the surface and very slowly withdrawn from the liquid phase. At the point where the recorded force is a maximum, the observed force is proportional to the surface tension of the interface. With this value recorded, the plate is pulled upward and completely removed from the interface. With the probe suspended in the air above the tensiometry sample, the tensiometer balance is zeroed and the entire process starts again.

Several aspects of using the Wilhelmy Plate and "plate method" are undesirable when studying protein surface adsorption. First of all, the larger surface area of the Wilhelmy Plate (when compared to the Du Nouy Ring) allows for greater protein adsorption to the surface of the plate. This, in turn, results in visible protein aggregates forming on the plate surface. These aggregates result in uneven wetting of the plate and poor data accuracy. These aggregates also interact with proteins adsorbed to the liquid-air surface to form long protein "tethers" between the plate and the surface. Since the tensiometer zeros its balance between samples, these "tethers" greatly impact the surface tension values recorded by the device. The resulting data, a portion of which is displayed in Figure 4, is inconsistent.

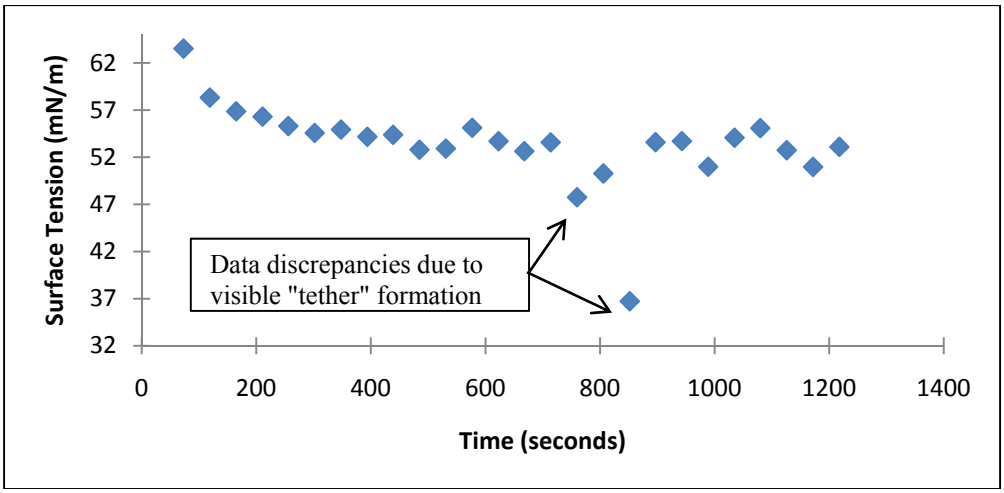

Figure 4: "Tethering" of Wilhelmy Plate. Extremely decreased surface tension data which accompanied visible plate-surface "tether" formation, resulting in incorrect equipment zeroing.

Furthermore, with the "plate method," the protein-coated liquid-air interface is disturbed during every reading which resulted in less uniform protein coating and more "noise" present in the collected data.

Conversely, the Du Nouy Ring utilizes a "continuous," or "ring method," sampling program to recorded surface tension. During this process, the dry ring is initially lowered to the interface, submerged to a pre-specified "wetting depth" (roughly 3 mm), and then pulled upwards toward the interface. The tensiometer records the increasing downward force exerted by the interface as the ring is raised. Once this force reaches a maximum, the tensiometer records this surface tension value and lowers the ring back into the bulk liquid. As opposed to the "plate method," the "ring method" only zeros the tensiometer's balance initially. After the ring is "wetted," it never again breaks the liquid-air interface. This, in addition to greatly reducing the agitation caused by sampling, prevents the visible "tethers" from forming when using the Du Nouy Ring probe. In conjunction with the ring's lower surface area, this method helps to achieve more uniform and consistent data collection.

In addition to considering the sampling methods and complications associated with each probe type, the accuracy of each probe was used to measure the surface tension of pure water. From these experimental runs, it was found that the Wilhelmy Plate was more accurate with pure water (average surface tension of 72.4 mN/m, standard deviation of 0.02 at 21 °C). The Du Nouy Ring yielded fairly good results (average surface tension of 71.4 mN/m, standard deviation of 0.06 at 21 °C) but was not quite as accurate as the Wilhelmy Plate. The ring's results, however, where considered acceptable after observing the complications and poor data quality measured using the plate once protein was introduced to the interface.

Fibrinogen concentration runs

It was hypothesized that different protein concentrations would exhibit different adsorption kinetics. Several solutions were prepared with fibrinogen concentrations of 0.075, 0.050, 0.010, and 0.005 mg/mL to evaluate how protein concentration affects adsorption kinetics. The results of these concentration trials are presented in Figures 5(a) and 5(b).

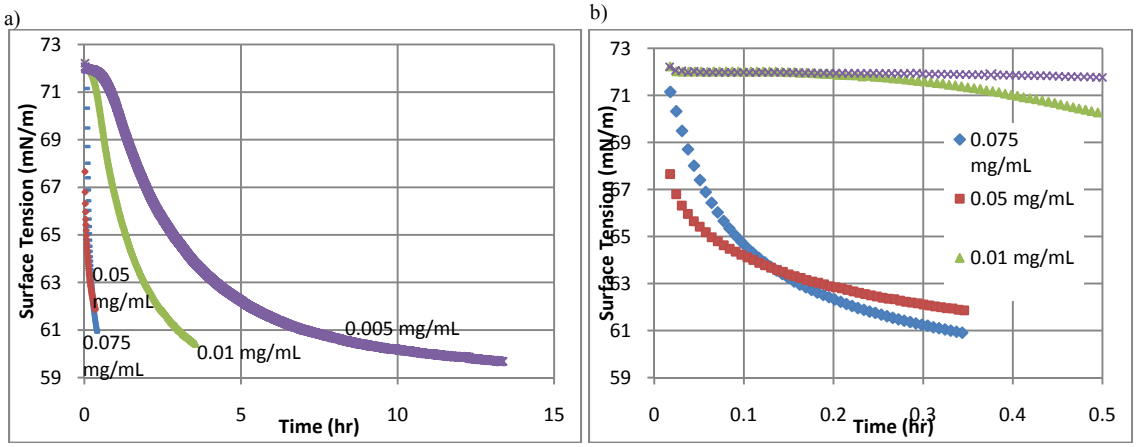

Figure 5: Fibrinogen solutions of differing concentrations exhibit varying adsorption kinetics over a) 14 hours and b) 0.5 hour. Note that overall surface tension drop is independent of concentration. Lower concentrations not only have slower kinetics, but exhibit a lag time before the surface tension begins to drop.

As expected, as fibrinogen concentration decreased, the rate at which surface tension dropped decreased. The two solutions of lowest concentration exhibited a "lag time" before the surface tension began to drop. A "lag" period occurs when proteins diffuse to the interface, but then rearrange before actually penetrating the interface. This phenomenon is not fully understood in literature, but several theories have been developed to explain this. "Lag time" is expressed in dilute solutions rather than more concentrated solutions because there are fewer proteins to penetrate and so their lag time is amplified. Furthermore, in a highly concentrated system, many more proteins diffuse to the interface and may force the proteins into the interface before they can fully rearrange. Perhaps the most interesting finding was that the surface tension drop was independent of fibrinogen concentration; the final surface tension readings for all runs were within 2 mN/m. The solutions of higher concentrations in Figure 5(a) appear to have not reached the same surface tension as the dilute concentrations, but it is expected that the surface tension drop would be the same if they were allowed to adsorb for the same period of time.

rFVIII concentration runs

After completing concentration trials to study the kinetics of a target protein, a similar procedure was used to determine the effect of the recombinant protein concentration on surface tension depression. Protein concentrations of 1, 2, 5, and 10 IU/mL were run in a buffer solution, with similar trends as seen in the fibrinogen trials (Figure 5). The greatest difference was that steady state values did not converge, as they did with fibrinogen. Additionally, the recombinant protein stock solution contained

surfactant which the fibrinogen did not. These results are presented in Figure 6. These results could be because the recombinant protein concentrations were significantly less than the fibrinogen concentrations, warranting different behaviors. The activity of the 1 IU/mL solution was of special interest, as that is the concentration generally experienced in upstream bioreactor production. The steady state surface tension depression in that case was 57 mN/m for protein alone.

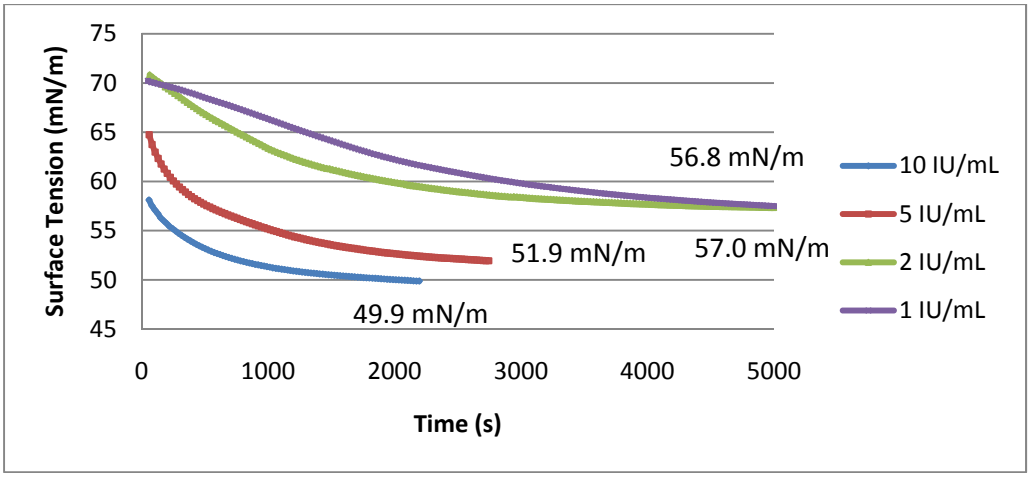

Figure 6: rFVIII solutions of differing concentrations exhibit varying adsorption kinetics over time. Note that overall surface tension drop is independent of concentration for 1 and 2 IU/mL but not at higher concentrations; this is expected to be the result of the presence of surfactant in the protein stock.

Surfactant kinetics

Tween 80 is a surfactant used in the final production line of the therapeutic protein and a small amount is included in the final, freeze dried product. Other surfactants are used in upstream production, such as F68. The kinetics of both surfactants alone in buffer were examined (Figure 7).

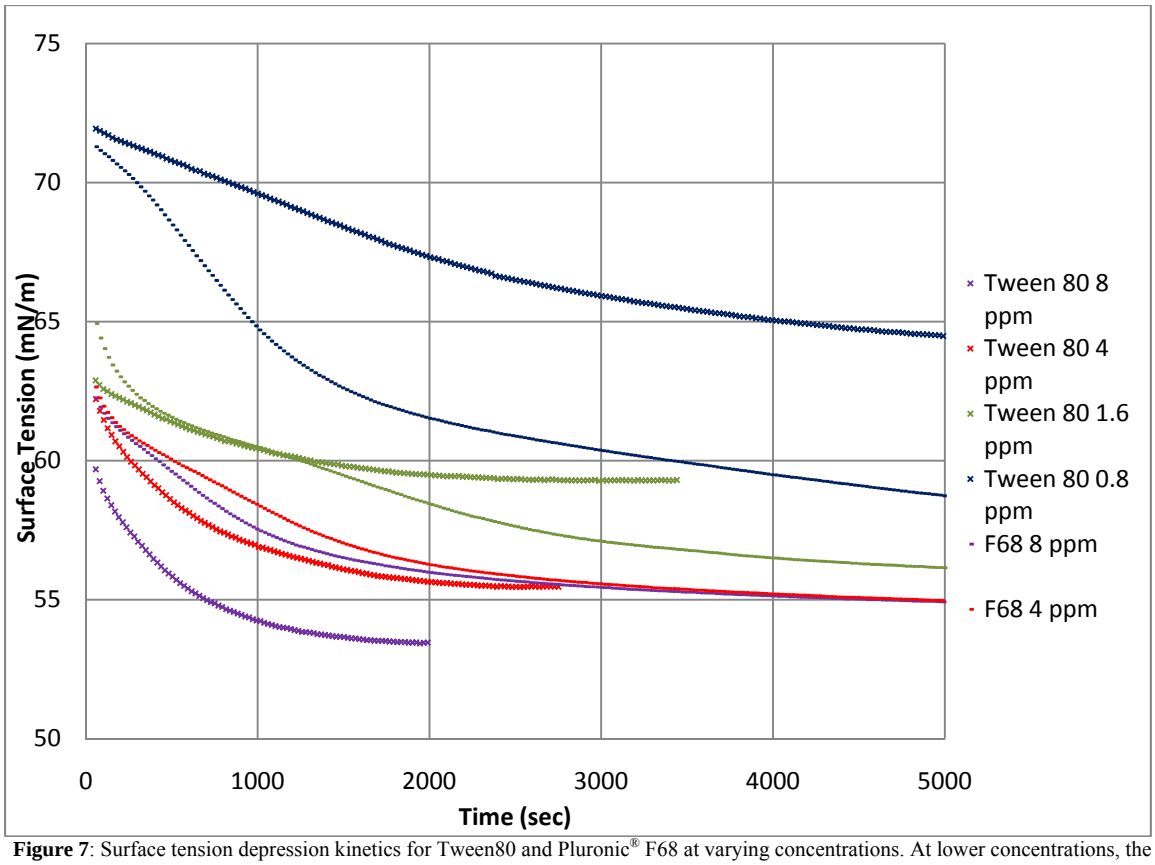

Figure 7: Surface tension depression kinetics for Tween80 and Pluronic® F68 at varying concentrations. At lower concentrations, the two have similar initial kinetics but F68 yields greater surface tension depression. However, at higher concentrations, Tween 80 has faster initial kinetics and greater steady state surface tension depression.

As expected, an increase in surfactant concentration yielded an increase in surface tension depression. At lower surfactant concentration (0.8 ppm), the two surfactants had similar adsorption kinetics initially, but F68 exhibited greater surface activity. However, as the concentration increased, different behaviors became evident. At 4 ppm, the two surfactants acted similarly. At 8 ppm, Tween 80 had greater initial kinetics, and depressed surface tension more at steady state than F68 did.

At the molecular level, Tween 80 is a smaller, globular macromolecule, and will form aggregates with other Tween 80 molecules. However, once these aggregate reach the surface, the molecules easily dissociate and cover the interface. On the other hand, F68 is a much larger, “stringier,” molecules and forms stable aggregates. Once these

aggregate reach the surface, however, the molecules have a harder time dissociating and individually covering the interface. As a result, higher concentrations of F68 (when aggregates are more likely to form) will exhibit less surface activity than would be expected based off of low concentration kinetics.

Steady state surface activity of rFVIII-surfactant solutions

The next step was to examine how the surfactants behave with therapeutic protein in the buffer solution. Adsorption at steady state was of great interest because of its potential correlation to the five region plots mentioned previously (Figure 2). If a plot like this were developed for both surfactants, the effective critical micelle concentration for the system could be determined and used in the development of commercial production. Figure 8 and Figure 9 contain the results.

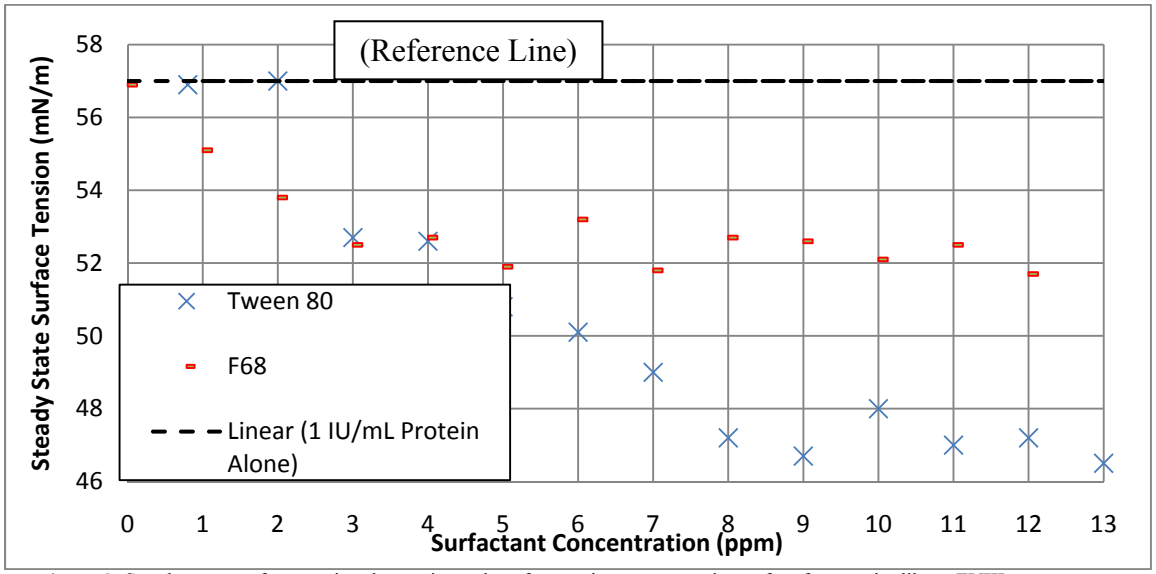

Figure 8: Steady state surface tension depression values for varying concentrations of surfactants in dilute rFVIII solutions. F68 decreases surface tension more at low concentrations but at about 3 ppm Tween 80 starts exhibiting greater surface activity.

These results (of low surfactant concentrations) confirm the findings in Figure 7. At low concentrations (below 3 ppm), F68 has greater surface activity. However, at surfactant concentrations greater than 4 ppm, Tween 80 exhibits greater surface activity at steady state.

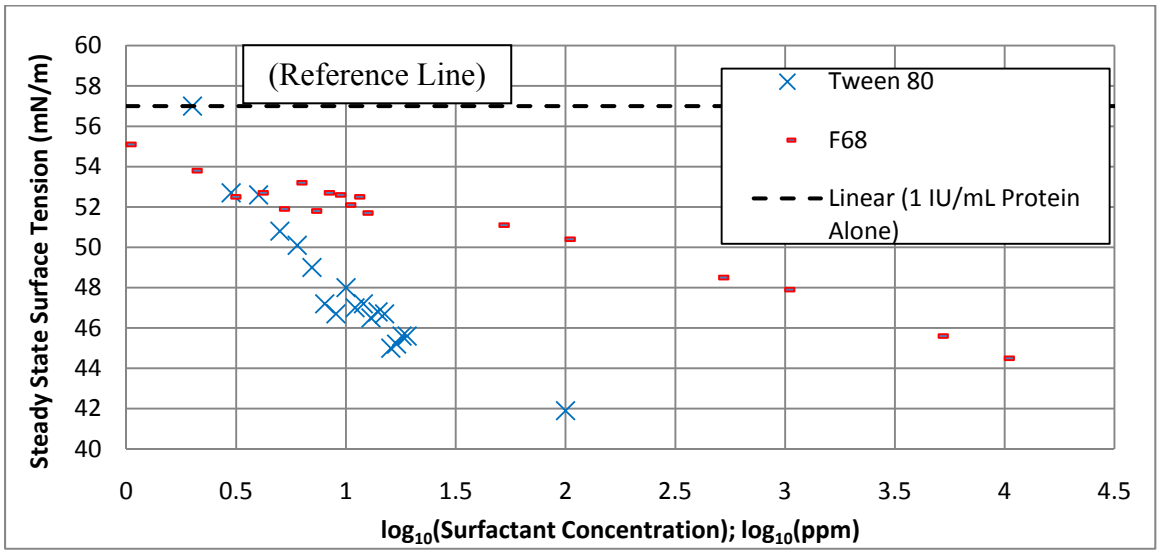

Figure 9: Steady state surface tension depression values for a greater region of surfactant concentrations (in dilute rFVIII solutions). Even at significantly lower concentrations, Tween 80 has greater surface activity than F68 (The activity of 5000 ppm F68 yields 45.6 mN/m, the same yielded by only 19 ppm Tween 80).

The logarithmic scale in Figure 9 allows very high concentrations to be compared to low concentrations. It is interesting to note the superiority of Tween 80 to depress surface tension at higher concentration. For example, Tween 80 at 19 ppm depresses surface tension to a steady state value of 45.6 mN/m. However, F68 must get to a concentration of 5000 ppm before exhibiting this level of surface tension depression. Again, this behavior confirms the findings in Figure 7, that Tween 80 depresses surface tension more than F68 at high concentrations (8 ppm).

Unfortunately, neither Figure 8 nor Figure 9 perfectly resembled the ideal five region plot as described in Figure 4. This could potentially be because the therapeutic protein was at a very low concentration. It could be worthwhile to collect more data

within this range to determine if there are five regions for this system. At the very least, however, these figures are helpful in showing that F68 is significantly less active than Tween 80 at the high levels of surfactants present in commercial processes. Given these findings, it is interesting to note a point of simple economic analysis: Tween 80 is less expensive than F68.[8] However, F68 may provide further benefits to mammalian cells not evaluated in this research, such as cell stabilization.

[8] Biochemicals, Reagents, and Kits for Life Science Research. St. Louis, MO: Sigma-Aldrich Corporation, 2006. Tween 80 $0.02/g vs. $0.11/g Pluronic® F68.

CONCULUSIONS AND PROPOSALS

From these results several conclusions and recommendations can be made. Regarding the two surfactants used, behavior depended upon both surfactant identity and surfactant concentration. The smaller surfactant, Tween 80, was less aggressive in coating hydrophobic interfaces at dilute concentrations. At higher concentrations, Tween 80 forms less stable aggregates which easily dissociate and allow the surfactant to readily coat hydrophobic surfaces, which prevents protein adsorption. In contrast, Pluronic® F68 is a much larger molecule with longer hydrophilic and hydrophobic chains. As solution concentrations of F68 increased, the forming of stable aggregates substantially decreased this surfactant's ability to dissociate and coat hydrophobic surfaces and, theoretically, prevent protein adsorption. These conclusions indicate that, at the high surfactant concentrations present in typical commercial protein production bioreactors, Tween 80 should be investigated as a possible treatment to help prevent protein losses during production.

In addition, several areas for future research in this arena exist. Further studies regarding surfactant prevention of protein adsorption could be conducted; performing studies using scale bioreactors, or sparged columns, would yield a valuable and more complete view of the phenomena which occur in commercial protein production. Additionally, if surfactants continue to be used to prevent protein losses due to surface adsorption, it would be valuable to determine the impact, if any, of surfactant-protein interactions specifically on the activity of the desired proteins. For example, although one surfactant may appear to prevent surface adsorption, if its interaction with proteins causes those proteins to denature or become inactive, that surfactant should be avoided.

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APPENDIX: Sample Calculations

Surface tension calculation (performed automatically by tensiometer software)

$$\text{Recorded Force} = \mathrm{F} = (\mathrm{p})(\gamma)(\cos\theta)$$

$$\text{Recorded Force} = \ 7.96\ \mathrm{mN} = (0.129\ \mathrm{m})(\gamma)(\cos 0)$$

$$\gamma = 61.8\ \mathrm{mN/m}$$

Solution dilution

$$(\mathrm{C_1})(\mathrm{V_1}) = (\mathrm{C_2})(\mathrm{V_2})$$

Where C_1 and C_2 are protein concentration, and V_1 and V_2 are the total liquid volumes, in states 1 and 2 respectively. In this case, C_1 and C_2 are known, and the fresh HPLC water in V_2 is known to be 10 mL. So, assigning the variable V_1 to the required volume of incubation stock to dilute:

$$\left(1.06\,\frac{\mathrm{mg}}{\mathrm{mL}}\right)(\mathrm{V1}\ \ ) = \left(0.075\,\frac{\mathrm{mg}}{\mathrm{mL}}\right)(10\ \mathrm{mL} + \mathrm{V_1})$$

V_1 = 0.761 mL = 761 µL (easily measured using a micropipette)