AN ABSTRACT OF THE DISSERTATION OF

Omkar Joshi for the degree of Doctor of Philosophy in Chemical Engineering presented on December 5, 2005.
Title: Interaction of Recombinant Factor VIII and the Nonionic Surfactant Tween 80 at Interfaces

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Dr. Joseph McGuire

The role of the nonionic surfactant Tween 80 on the behavior of the therapeutic recombinant protein Factor VIII (rFVIII) was investigated at solid/liquid and air/water interfaces. In order to provide a model system to compare results obtained for the complicated rFVIII – Tween system, a well-characterized globular protein lysozyme was used. The experimental scheme involved the introduction of the protein and Tween to the adsorption substrate in different manners, either lysozyme – Tween together or in sequence as lysozyme followed by Tween or vice versa. It was observed that the addition of Tween together with lysozyme reduced the amounts adsorbed at hydrophobic surfaces, while no such reduction was observed on hydrophilic surfaces. A high Tween concentration was required to effect the removal of the lysozyme molecules from the hydrophobic surface and Tween was not effective in removing lysozyme from the hydrophilic surface at any concentration. These results suggest that the Tween – surface interaction is important in determining lysozyme adsorption. Similar observations were made for the rFVIII – Tween system at hydrophobic and hydrophilic silica interfaces. In this case, the presence of interfacial and solution Tween together resulted in complete

prevention of rFVIII adsorption. Electrostatic forces were observed to be play an important role in rFVIII adsorption. The rFVIII – Tween interactions at solid interfaces were also evaluated using intrinsic fluorescence and biological activity measurements. Results obtained with respect rFVIII adsorbed mass, and structure or biological activity change upon adsorption, were evaluated in parallel. This parallel evaluation suggested that rFVIII adsorption on hydrophilic, negatively charged surfaces is likely to be highly ordered and oriented in a manner that retains the solvent accessibility of the active sites in rFVIII. On the other hand, rFVIII may adsorb to hydrophobic surfaces in different orientations, with a likelihood of surface induced unfolding. rFVIII – Tween interaction at the air/water interface was investigated separately. Surface tension data recorded for rFVIII – Tween mixtures suggested that Tween dominated the air/water interface as the Tween concentration was increased. Reduced interface-induced unfolding was observed at high Tween concentrations. These results were also thought to contribute to the reduction in rFVIII aggregation typically observed as a result of exposure to the air/water interface.

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Interaction of Recombinant Factor VIII and the Nonionic Surfactant Tween 80 at Interfaces

by

Omkar Joshi

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APPROVED: Redacted for privacy

Major Professor, representing Chemical Engineering

Redacted for privacy

Head of the Department of Chemical Engineering

Redacted for privacy

Dean of the Graduate School

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

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Omkar Joshi, Author

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This work is a tribute to a great professor, a true friend and an exceptional man I have had the privilege of working with during my graduate studies. Joe McGuire has been, and will always remain, a great source of inspiration in my professional and personal life. I would not have been at this stage of my academic career of achieving my Ph.D. degree if it were not for Joe. Quite literally. After completing my Masters, also with Joe, I had made up my mind on pursuing a career in the biotech industry and not continuing with my Ph.D. After close to 5 months in the job wilderness, I was offered a position by a company in Seattle. At the same time, Joe was awarded a research contract by Bayer HealthCare. Joe offered me a chance to work on the Bayer project, and over a period of 8 days, convinced me to continue graduate school and turn down the job offer. It was a hard decision for me to make at that time, especially because I had got an opening after trying relentlessly for what felt like forever. But I was comforted by the knowledge that I would always have the backing of my advisor in ensuring that my Ph.D. would be successful and productive. It turned out that the decision was an inspired one. I had truly the most wonderful time while working on the Bayer project. I had the opportunity of doing research which was fast-paced, was industrially significant, and was a lot of fun at the same time. After working on the project at Bayer as an intern, I transitioned into a full time position and have now been with Bayer for the last half year. The company in Seattle shut operations and laid off all employees six months after my brief brush with it.

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CONTRIBUTION OF AUTHORS

Liping Chu performed the surface tension measurements on Tween 80 and Factor VIII –Tween 80 solutions described in Chapter 4. Dr. D.Q. Wang of Bayer HealthCare provided practical perspectives on rFVIII manufacture and was involved in the work related to Chapters 3 and 4.

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CHAPTER 1

INTRODUCTION

The advent of modern biotechnology, particularly recombinant technology, has led to the commercial production of an increasing number of protein therapeutics. Typically, the protein drugs are produced in small quantities and are extremely high value products. A prime example of such a protein is the recombinant coagulation Factor VIII (rFVIII). Blood coagulation consists of a series of enzymatic steps, in other words the "coagulation cascade". Factor VIII is a part of the coagulation cascade and is involved in the activation of Factor X. A functional deficiency in Factor VIII leads to haemophilia, a congenital bleeding disorder that occurs in approximately 1 in 10,000 males. rFVIII is used in Factor VIII replacement therapy used as a treatment of haemophilia. The amount of Factor VIII circulating in human plasma is minute, typically about 200-250 nanograms per ml plasma. This low amount makes biotechnological manufacture and replacement therapy feasible (Shwartz 1992). It is estimated that the total amount of Factor VIII required to treat all haemophiliacs in the world is only 500 grams a year. But at the same time, the long series of complicated steps involved in the manufacture of rFVIII means that the unit cost of rFVIII is very high. In this background, the loss of even small quantities of rFVIII as a result of surface adsorption during production, packaging and administration becomes especially relevant. The following chapters encapsulate the work done to better understand the adsorption phenomena of

proteins in relation to the various interfaces that proteins are exposed to and the presence of nonionic surfactant Tween 80 which is often added to therapeutic protein formulations.

Chapter 2 is a study of globular protein lysozyme adsorption at hydrophobic and hydrophilic silica/water interfaces and the effect of Tween concentration and method of addition on lysozyme adsorption. The main motivation for studying lysozyme adsorption before exploring the more complex protein rFVIII was to generate a solid framework for the consistent interpretation of results obtained with rFVIII. The protein, the interface, and the protein solution are the three important parameters that determine protein adsorption. We introduce the proteins (rFVIII and lysozyme) to different interfaces and change the solution composition by varying the Tween concentration. Lysozyme and rFVIII are very different proteins, especially in terms of complexity and size with lysozyme having a molecular weight which is about 20 times less than that of rFVIII. Comparing results obtained for lysozyme and rFVIII with a similar experimental design allows us to evaluate the effect of the protein characteristics on adsorption

Chapter 3 is an investigation of rFVIII adsorption at solid/liquid interfaces in relation to Tween concentration, method of addition and the surface characteristics of the solid/liquid interface. We simultaneously investigate the mass, biological activity, and the structure upon adsorption of rFVIII. This enables us to shed light on the driving forces relevant in rFVIII adsorption, and the possible conformations/orientations adopted by rFVIII at different interfaces. The separate roles of the protein, the surfactant and the surfactant-protein complex in adsorption are also explored.

Chapter 4 explores rFVIII adsorption at air/liquid interfaces and the subsequent change in structure, biological activity and aggregation behavior. The

air/liquid interface is considered as a model hydrophobic interface. A separate and focused investigation of the rFVIII behavior in response to exposure to the air/water interface is warranted by the possibility of molecular penetration and the likelihood of molecules exposed to the interface returning to solution, which is unique for such interfaces.

INTERACTIONS OF LYSOZYME WITH NONIONIC SURFACTANT TWEEN 80 AT HYDROPHOBIC AND HYDROPHILIC SILICA INTERFACES

Omkar Joshi and Joseph McGuire†

Department of Chemical Engineering, Oregon State University, Corvallis OR 97331

† Corresponding author Address102 Gleeson Hall, Oregon State University, Corvallis OR 97331 Email: mcguirej@engr.orst.edu

CHAPTER 2

INTERACTIONS OF LYSOZYME WITH NONIONIC SURFACTANT TWEEN 80 AT HYDROPHOBIC AND HYDROPHILIC SILICA INTERFACES

Abstract

Surfactants are known to modulate the interfacial behavior of proteins. Here, we describe the interaction of a model globular protein lysozyme with nonionic surfactant Tween 80 at solid/water interfaces. Circular dichroism (CD) spectroscopy revealed no significant alteration in lysozyme secondary structure with the addition of Tween 80 up to a concentration of 80 ppm, suggesting that the direct protein-surfactant interaction was rather weak. Hydrophilic and silanized, hydrophobic silica surfaces were used as adsorption substrates and lysozyme adsorption was monitored by ellipsometry. The sequence of lysozyme-Tween addition was varied in order to identify the separate roles of protein, surfactant and the protein-surfactant complex in the interfacial phenomena of the lysozyme-Tween system. Lysozyme-Tween co-adsorption at the hydrophobic surface resulted in a reduction in amount of protein adsorbed with increase in Tween concentration. No such reduction was observed on the hydrophilic surface. In addition, a Tween pre-coat prevented lysozyme adsorption on the hydrophobic surface but such a pre-coat was completely ineffective in lowering adsorption on the hydrophilic surface. These differences in Tween action on surfaces of different wettability was attributed to the difference in Tween binding strengths at these surfaces. Tween was able to adsorb strongly on the hydrophobic surface, and the interfacial Tween prevented or reduced lysozyme adsorption. The lysozyme adsorption kinetics on hydrophobic surfaces pre-coated with Tween, with or without Tween being present in solution appeared to be very

similar. This underlined the importance of interfacial Tween and the Tween-surface interaction in determining lysozyme adsorption.

2.1 Introduction

Proteins are surface active and are known to preferentially locate at interfaces. The surface activity of proteins derives from their polymeric structure and amphipathic nature (Horbett 1982). Multiple contacts with the surface may form due to the large size of protein molecules, leading to strong binding. Proteins are also known to undergo conformational changes upon adsorption, which are attributed to the tendency for nonpolar residues to be internalized. In fact, the unfolding of proteins at interfaces results in an entropic gain and is recognized as one of the driving forces of protein adsorption (Norde 1991, Norde 1998). Another theory proposes that the "bound water", or the hydration layer in the near vicinity of the surface with which the protein molecules interact, controls protein adsorption (Krishnan 2005). Resistance to the displacement of protein molecules from the surface is hypothesized to depend on the affinity of water for the surface. This implies that hydrophilic surfaces adsorbed only limited amounts of protein, whereas protein molecules can readily replace the bound water from hydrophobic surfaces and adsorb in large amounts. Various modes of binding with a wide array of surfaces are possible due to the presence of polar, charged and nonpolar amino acid side chains in proteins. Thus interfaces of any type that come into contact with proteins become rapidly coated by proteins. Proteins at interfaces are important in many diverse areas; prominent examples being biomaterials engineering, modern biotechnology and food processing. In the biotechnology industry in particular, protein adsorption is a major concern in upstream bioreactors, downstream packaging and administration to the end

user. Protein adsorption results in not only the loss of material but also the biological activity of the therapeutic protein drug due to surface induced denaturation.

Surfactants adsorb readily at most interfaces. In addition, surfactant molecules associate with one another in solution in order to minimize the contact of the hydrophobic chains with water. Surfactants also interact with proteins in solution to form protein-surfactant complexes (Reynolds 1976). The protein-surfactant complex may have different interfacial behavior compared to pure protein or pure surfactant. Formation of such complexes also reduces the concentration of free surfactant molecules in solution which may increase the critical micellar concentration (CMC, Arnebrant 1995). Surfactants are known to modulate the interfacial behavior of proteins. The interaction of surfactants and proteins upon adsorption, either competitive or sequential, may result in a difference in net protein adsorbed at an interface as compared to adsorption from a pure protein solution. Published results (Arnebrant 1995) suggest that adsorption from a protein-surfactant solution may result in one of three outcomes: complete hindrance, reduced amounts, or increased amounts of protein adsorption. Complete hindrance is attributed to the faster diffusion of the smaller surfactant molecules (as compared to protein molecules) and the adsorbed surfactant layer sterically preventing protein adsorption. Reduced and increased amounts are usually attributed to the formation of surfactant-protein complexes with reduced or increased surface activity, respectively. Sequential introduction of surfactants after protein adsorption may result in removal of adsorbed protein molecules. This may be due to the formation of surfactant-protein complexes and the subsequent solubilization of these complexes, and/or due to replacement of adsorbed protein molecules by surfactant molecules on account of the (possibly) stronger surfactant-interface interaction. The removal of adsorbed protein molecules mediated by surfactant depends on the protein, surfactant and surface properties

among other factors. McGuire et al (1995) report that the dimethyl triammonium bromide-mediated elution of structural stability mutants of bacteriophage T4 lysozyme increases with increase in the stability of the protein. The difference in the protein elutability mediated by anionic, cationic and nonionic surfactants corresponds with the strength of binding to protein in solution (Arnebrant 1995). Nonionic surfactants which are known to bind rather weakly to proteins are not as effective in removing adsorbed protein molecules from the interface. Elwing et al (1989) studied the elutability of proteins adsorbed on wettability gradient surfaces. The authors note that nonionic surfactants do not affect the amount adsorbed on the hydrophilic surface but have a considerable effect on the amount adsorbed on the hydrophobic surface, presumably because of the difference in surfactant binding strength at the interface.

2.2 Materials and Methods

2.2.1 Protein, surfactant and buffers

Lysozyme, from chicken egg white, was purchased from Sigma (Lot number 051K7028). Phosphate buffer (PB, 0.01 M) at pH 7 was prepared by dissolving 1.08 g sodium phosphate dibasic (Sigma) and 0.53 g of sodium phosphate monobasic (Mallinckrodt) in 1 L distilled deionized water (DDW). Lysozyme was dissolved in filtered (0.2 μm Pall filter) phosphate buffer at a concentration of 5 mg/ml. This solution was used as protein stock solution for all adsorption experiments. Protein solutions were prepared fresh each day. Tween 80 (J.T. Baker) was dissolved in DDW to obtain concentrated stock solutions at 10,000 and 50,000 ppm. The Tween stock solutions were aliquoted into 2 ml Eppendorf vials and frozen at -80 °C and then thawed just before use.

2.2.2 Preparation of hydrophilic and hydrophobic adsorption substrates

Silicon (Si) wafers (crystal grade, type N, boron doped, orientation 1-0-0, thickness 525 ± 18 μm, resistivity 0.01-0.02 ohm-cm) were purchased from WaferNet Inc. Silicon wafers were oxidized by placing in a furnace at 1 atm and 1000 °C for 18 minutes to obtain an oxide film thickness of 300 Å (Podhipleux 1998). Wafers were cut into 1 × 3 cm plates using a tungsten pen, and rinsed with acetone to remove dust and organic residues. Plates were further cleaned using a standard acid/base cleaning procedure (Klintstorm 1992). The plates were first immersed in a 10 ml mixture of NH_4OH: H_2O_2: H_2O (1:1:5 volume ratio) and heated at 80 °C in a water bath for 10 minutes. Silica plates were then rinsed in copious amounts of DDW, transferred to a 10 ml mixture of HCl: H_2O_2: H_2O (1:1:5 volume ratio) and heated at 80 °C for 10 minutes. The plates were rinsed with DDW again and dried under a flow of nitrogen. At this stage, the silica plates have hydrophilic surface characteristics, as evidenced by a water contact angle of 0 – 10 °. Contact angle measurements were obtained using a video-based FTA 32 (First Ten Angstroms) instrument. Hydrophilic plates were stored in ethanol until further use.

For preparing hydrophobic silica plates, the nitrogen-dried plates were transferred to a silanizing solution containing 1% dichlorodimethyl silane in xylene, and kept for 1 hour. The plates were then rinsed sequentially with xylene, acetone and ethanol. The silanization procedure rendered the silica plates hydrophobic, as evidenced by a water contact angle of 90 – 100 °. The hydrophobic plates were stored in ethanol until further use.

2.2.3 Evaluation of lysozyme secondary structure

The circular dichroism spectra of a protein contain information about its secondary structure (Vermeer 1998). The CD experiments at Tween 80 concentrations of

0, 8, 20 and 80 ppm were performed to evaluate the effect of Tween 80 on the structure of lysozyme in solution and to investigate whether Tween modifies the structure of the lysozyme molecule in any way. The stock lysozyme solution was diluted to 0.5 mg/ml by adding filtered phosphate buffer. Intermediate Tween stocks at 200, 500 and 2,000 ppm were prepared from the 10,000 ppm Tween stock. A small volume (20 µl) of the intermediate stock solutions was added to 0.5 ml lysozyme solution at 0.5 mg/ml to obtain 8, 20 and 80 ppm Tween-containing solutions.

The CD spectra were obtained using a J-720 UV Spectrum spectropolarimeter (JASCO). All experiments were carried out at 25 °C. A cylindrical cuvette with a 100 µm pathlength was used. The CD spectra were recorded with every 1 nm increment in wavelength, starting at 290 nm and ending at 182 nm. In order to increase the signal-to-noise ratio, six scans were recorded for each sample and then averaged. The CD spectra of protein-free Tween-containing buffer were subtracted from the lysozyme CD spectra in every case.

2.2.4 Evaluation of lysozyme adsorption kinetics

Lysozyme adsorption kinetics were studied by *in situ* ellipsometry. An ellipsometer measures the change in the state of polarization upon the reflection of a laser beam. The effect of reflection is characterized by the angles Δ, defined as the change in phase, and Ψ, the arctangent of the factor by which the amplitude ratio changes, with reference to the two component plane waves into which the electric field oscillation is resolved (Archer 1968). Ellipsometry allows the analysis of protein adsorption on solid, specular surfaces in real time and *in situ* in biologically relevant aqueous media (Elwing 1998). An automatic ellipsometer (L-104SA, Gaertner Scientific Corp.) with a 1 mW He-Ne light source was used. The angle of reflection was set equal to the angle of incidence at

70 °. Each silica plate (hydrophobic and hydrophilic) was suspended in a trapezoidal fused quartz cuvette (Hellma) which was equipped with a magnetic stir-bar and filtered phosphate buffer was added. The optical properties (Δ and Ψ) of the laser beam reflected from the bare surface were measured for 30 minutes, at 15-second intervals. Lysozyme, Tween, mixed lysozyme-Tween or buffer solutions were then injected into/flowed through the cuvette in a manner described in the following sections. The adsorbed protein/surfactant layer changes the optical properties of the reflected laser beam which can then be related to the adsorbed mass. A one-film-model ellipsometry program (Krisdhasima 1992) was used for the calculation of adsorbed mass. The program uses the values of the ratio between molar mass and molar reflectivity (M/A) and partial specific volume (V) of the adsorbing species. The M/A and V used for lysozyme were 3.841 g/ml and 0.761 ml/g, respectively. As explained by McGuire et al (1995) protein-specific values of V and M/A were used to determine the adsorbed mass in both the presence and absence of surfactant, as it is not possible to assign a correct value to these parameters for mixed, protein/surfactant films. This approach does not influence any of the trends observed in these experiments.

The experimental scheme involved the introduction of Tween together with (co-adsorption), after (sequential) and before the introduction of the protein (pre-coat). For adsorption on hydrophobic silica, different Tween concentrations (0, 8, 20 and 80 ppm) were used in each case. For adsorption on hydrophilic silica, experiments were performed without Tween (0 ppm) or in the presence of Tween at a "high" concentration (80 ppm). This scheme was devised to help determine the optimum concentration of Tween to obtain a desired effect as well as to identify the mechanism governing Tween-protein interactions at interfaces.

2.2.4.1 Tween - lysozyme co-adsorption

Tween 80 concentrated stock solutions at 10,000 and 50,000 ppm were diluted to obtain intermediate Tween solutions at 2,000, 5,000, and 20,000 ppm. Lysozyme solutions (0.5 ml, 5 mg/ml) were taken in 2 ml Eppendorf vials and 20 μl of intermediate Tween solutions were added to obtain 80, 200, and 800 ppm Tween-containing protein samples. These samples were vortexed briefly to ensure mixing. At the start of an experiment, 4.5 ml filtered PB was added to the cuvette. A (hydrophobic or hydrophilic) silica plate was then suspended in the cuvette and bare surface Ψ and Δ were monitored for 30 minutes. Lysozyme-Tween solutions (0.5 ml) were then added to the cuvette to obtain a final protein concentration of 0.5 mg/ml and a Tween concentration of 0, 8, 20 or 80 ppm for hydrophobic and, 0 or 80 ppm for hydrophilic silica. Adsorption was monitored for 30 minutes. The sample was then rinsed by flowing phosphate buffer through the cuvette, at a flow rate of 30 ml/min for 5 minutes. Buffer elution was monitored for a further 25 minutes.

2.2.4.2 Lysozyme – Tween sequential adsorption

Tween-free lysozyme solutions were added to a cuvette containing hydrophobic or hydrophilic silica and kinetic adsorption data were recorded for 30 minutes. After this, the surface was rinsed with phosphate buffer at a flow rate of 30 ml/min and elution was monitored for a further 25 minutes. Tween 80 solutions at 80, 200 and 800 ppm were obtained by diluting concentrated Tween stock solutions. Tween solutions (0.5 ml) were then added such that the final Tween concentrations reached 8, 20 or 80 ppm. Tween was allowed to contact the surface (and the surface-adsorbed protein) for 15 minutes, after which the sample was rinsed with buffer. Data was recorded for a further 25 minutes after rinse.

2.2.4.3 Tween – lysozyme co-adsorption at a surface pre-coated with Tween

In this case, 4.4 ml filtered PB was added to the trapezoidal cuvette. One-tenth (0.1) ml Tween sample was then added such that the final Tween concentration in the cuvette after the addition of protein was 8, 20 and 80 ppm. Tween adsorption was monitored for 45 minutes, after which time lysozyme solution (0.5 ml) was added to the cuvette to obtain a final protein concentration of 0.5 mg/ml. Adsorption of surfactant-protein mixture (i.e. the lysozyme which was added and the Tween that was already present in the cuvette) was monitored for 30 minutes. The plate was then rinsed with buffer for 5 minutes and buffer elution data was recorded for a further 25 minutes.

2.2.4.4 Lysozyme adsorption at a surface pre-coated with Tween

The procedure followed was similar to 2.2.4.3 up to the point where protein is added. Here, the sample was rinsed with phosphate buffer for 5 minutes and elution followed for a further 25 minutes. After this, a 0.5 ml lysozyme solution was added to the cuvette to obtain a final protein concentration of 0.5 mg/ml. Adsorption was monitored for 30 minutes. The sample was then rinsed with phosphate buffer for 5 minutes and buffer elution data was recorded for a further 25 minutes.

2.3 Results and Discussion

2.3.1 Lysozyme secondary structure as a function of Tween concentration

There are numerous reports (Leffers 2004, Bisaglia 2005, Manning 2004) which describe the effect of surfactants on the structure of proteins in solution. In particular, ionic surfactants such as sodium dodecyl sulfate (SDS) and dimethyl

triammonium bromide (DTAB) are reported to have a denaturing effect on the structure of proteins. Tween 80 is a nonionic surfactant. Nonionic surfactants are known to be much weaker than ionic surfactants, and are not generally expected to alter protein secondary structure in solution.

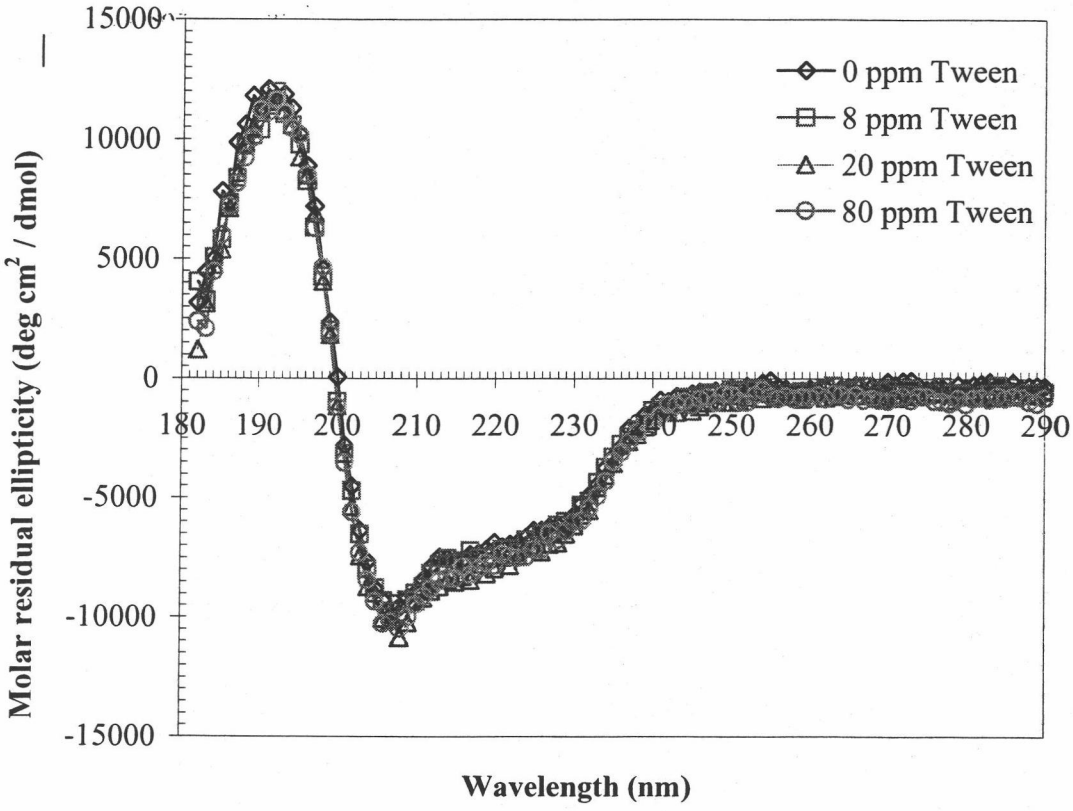

Figure 2.1 Circular dichroism spectra of lysozyme in the presence of Tween 80

The CD spectra of lysozyme in the presence of 0, 8, 20 and 80 ppm Tween are shown in Figure 2.1. In all cases, a minimum in the molar residual ellipticity was observed around 208 nm which is typical for a protein which has high α-helix content (Bloemendal 1995). The lysozyme spectra at different concentrations of Tween were essentially identical, which suggests that increasing Tween concentration did not induce

any secondary structure change in lysozyme. For the purpose of the secondary structure content analysis, lysozyme was considered to consist of 129 amino acids, and have a molecular weight of 14,300 Da. However, these findings do not preclude the possibility that Tween binds with lysozyme to form a surfactant-protein complex. It is likely however that the strength of this binding is not strong enough to effect any detectable change in secondary structure. Hillgren et al (2002) have noted a similar behavior in the case of Tween 80 interaction with lactate dehydrogenase (LDH). Pyrene fluorescence measurements were carried out to get an indication of the local polarity in the Tween-LDH system. A hydrophobic interaction between LDH and Tween was observed at or below the Tween CMC, but this interaction was not strong enough to denature the protein. While considering the Tween-lysozyme system, our results definitely rule out the possibility that a "new" lysozyme species with completely different structural characteristics is generated as a result of Tween addition. The fact that we are essentially dealing with a protein that is not substantially altered upon interaction with surfactant allows for a more direct interpretation of the adsorption kinetics described in the subsequent sections.

2.3.2 Lysozyme adsorption kinetics as a function of Tween concentration and method of addition

2.3.2.1 Tween – lysozyme co-adsorption on hydrophobic silica

The adsorption kinetics of lysozyme in the presence of 0, 8, 20 and 80 ppm Tween on hydrophobic silica are shown in Figure 2.2. The lysozyme adsorption kinetics in the absence of Tween were comparable to results obtained by Lee (1999) under

similar experimental conditions. Lee (1999) reported adsorbed amounts of 0.23 μg/cm^2 and 0.18 μg/cm^2 at the end of a 60-minute adsorption and 15-minute buffer elution cycle, respectively. A reduction in adsorption with an increase in Tween concentration was observed. Also, reduced amounts remained after rinse. These observations suggest two possible mechanisms, Tween diffuses to the surface faster owing to its smaller size as compared with lysozyme and thereby prevents lysozyme adsorption, and/or Tween forms complexes with lysozyme which have a reduced surface activity. These mechanisms are usually associated with protein-surfactant systems that display a reduction in protein adsorption when surfactant is added.

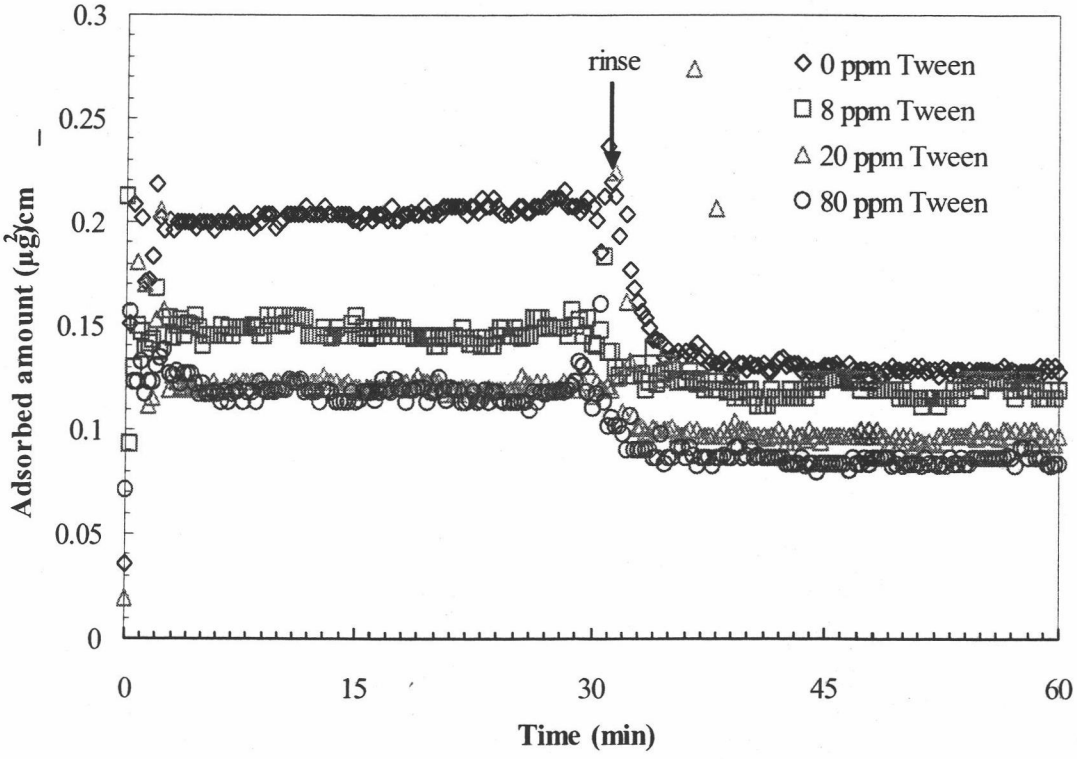

Figure 2.2 Co-adsorption of lysozyme on hydrophobic silica with Tween at concentrations of 0, 8, 20 and 80 ppm

The adsorption kinetics for the lysozyme-Tween solutions at 80 ppm Tween were comparable to that for pure Tween adsorption (Figure 2.6), suggesting that Tween may be dominating the interfacial process at high concentrations. There appeared to be a correlation between the amount adsorbed and the Tween CMC in a single component solution in pure water (13-15 ppm). Only minor further reduction in the amount adsorbed after 30 minutes (ads. cycle) and amount adsorbed after 60 minutes (des. cycle) was observed once the Tween concentrations exceeded the CMC. This observation is illustrated in Figure 2.3.

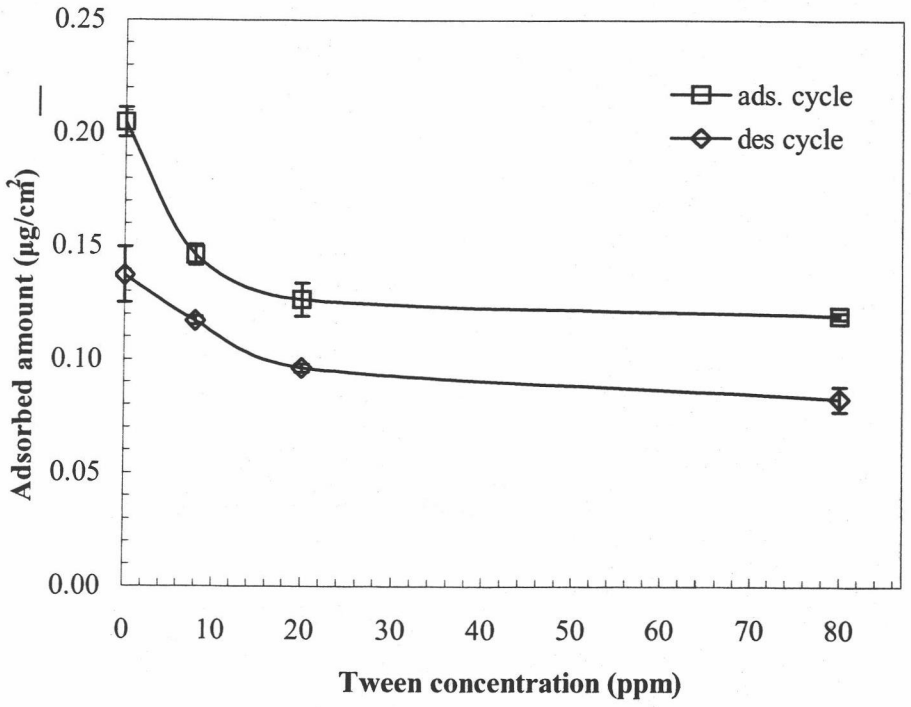

Figure 2.3 Adsorbed amounts on hydrophobic silica after co-adsorption of lysozyme and Tween, and after buffer elution

2.3.2.2 Lysozyme-Tween sequential adsorption on hydrophobic silica

Lysozyme adsorption kinetics with the introduction of Tween at a concentration of 8, 20 or 80 ppm after the lysozyme buffer elution step are shown in Figure 2.4. The first 60 minutes of the plots were essentially identical, since they constitute the same experimental condition up to that point i.e. adsorption of 0.5 mg/ml lysozyme followed by a rinse with buffer. Tween solutions at different concentrations were then introduced. It is important to note that there was no lysozyme remaining in solution at this stage, since the protein solution was replaced with buffer during the rinsing procedure. Also, the interfacial lysozyme is relatively stably bound considering that these are the protein molecules which have resisted buffer elution. When Tween solutions at 8 and 20 ppm were added, adsorbed mass was observed to increase, consistent with Tween adsorption at the empty sites on the surface and/or on the adsorbed protein layer. After rinsing, adsorption values were essentially identical to those before Tween addition suggesting that at these concentrations the Tween was loosely held and had no apparent effect on the amount of protein adsorbed.

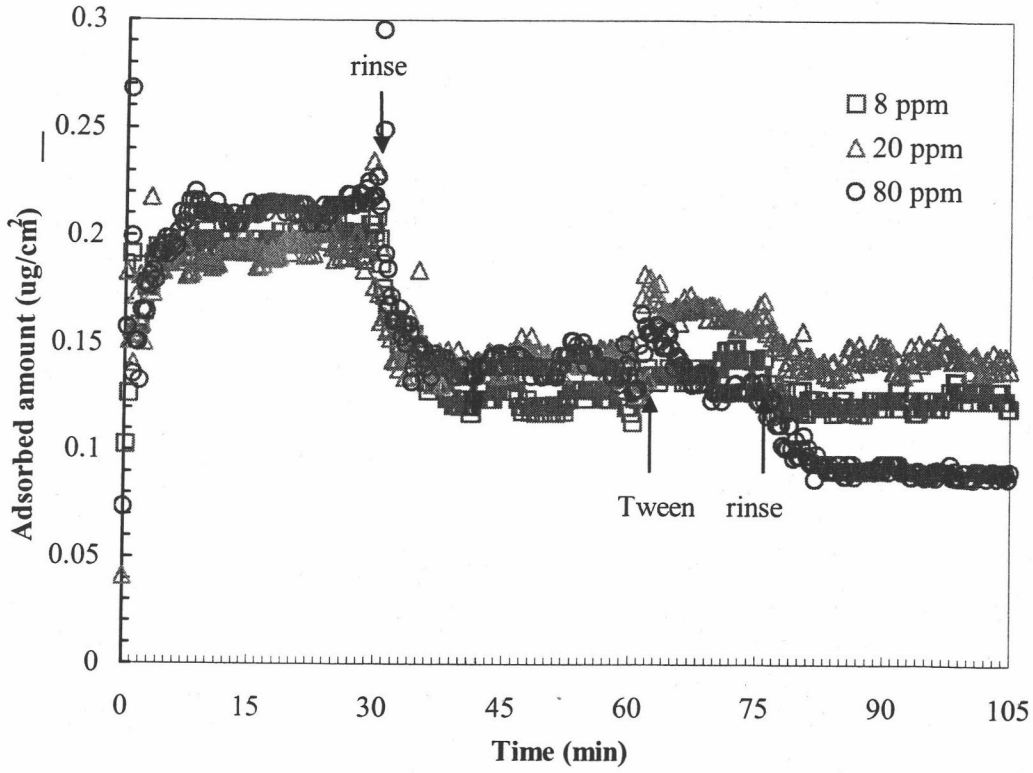

Figure 2.4 Lysozyme adsorption on hydrophobic silica followed by the addition of Tween

At "high" Tween concentrations (80 ppm), a decreasing trend in the kinetic plot was observed immediately following Tween adsorption suggesting that a removal of adsorbed protein molecules occured. Upon rinse, the amount adsorbed was lesser than that before Tween addition, and was consistent with pure Tween adsorption. This suggests that the adsorbed lysozyme molecules may have been replaced by Tween when Tween was present at high concentrations. The fact that an initial increase in adsorbed amount upon Tween introduction was observed at all concentrations including 80 ppm suggests that Tween may require binding to the protein layer in order to effect removal.

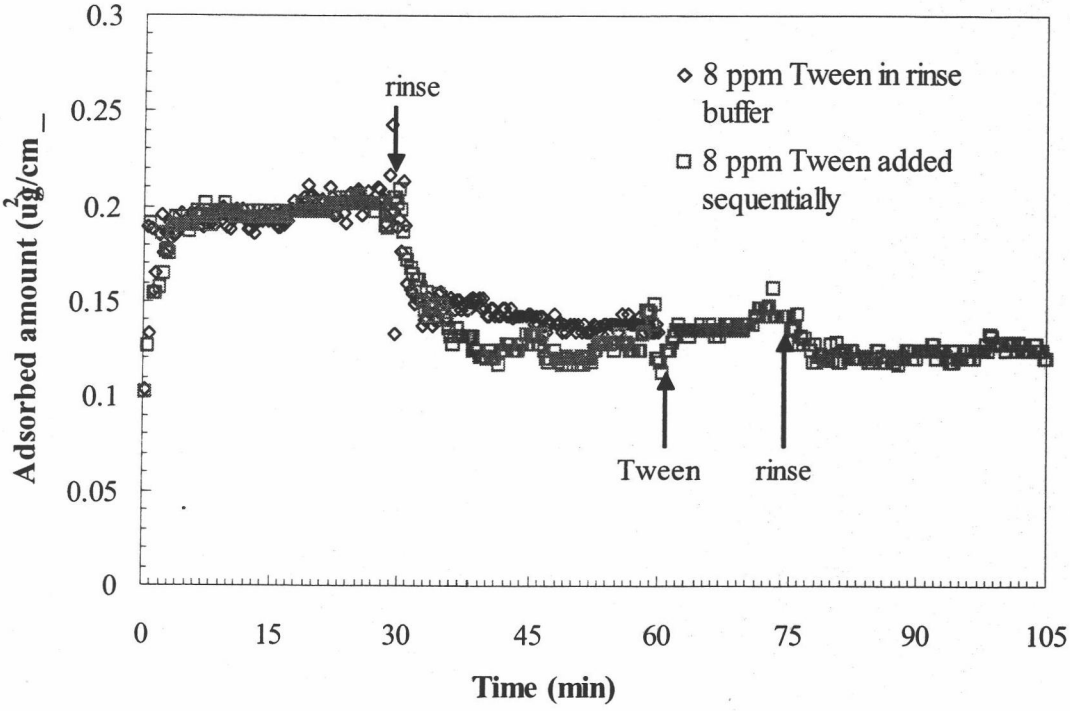

Figure 2.5 Comparison of Tween sequential addition: in rinse buffer and after lysozyme buffer elution

Figure 2.5 depicts a special case in which 8 ppm Tween was introduced in the rinse buffer during elution. Lysozyme adsorption kinetics for this case are compared with those obtained when there was no Tween in the rinse buffer, but 8 ppm Tween was added after the buffer elution cycle. Comparison between the two plots showed that elution kinetics for the first 5 minutes were indistinguishable. But after this time, the rate of elution appeared to decrease for the case where Tween was added to the rinse buffer. This was attributed to two mechanisms which may be occurring simultaneously, lysozyme elution and concomitant Tween adsorption at the empty sites. This reasoning was supported by the observation that the adsorbed amount after 60 minutes for the "Tween in rinse" case was consistent with the case when Tween is added after completion of the

elution cycle. While the kind of adsorption kinetic data shown in Figure 2.5 generate valuable information, interpretation of the adsorption/desorption phenomena of mixed protein-surfactant systems is further complicated due to the simultaneous occurrence of separate mechanisms. We believe that the tests presented in Figure 2.4 are more effective at evaluating the surfactant-mediated elution of lysozyme.

2.3.2.3 Lysozyme-Tween co-adsorption on hydrophobic silica pre-coated with Tween

Results depicted in Figure 2.6 pertain to the case where Tween was allowed to adsorb at the hydrophobic interface first, followed by the addition of lysozyme, and elution with buffer.

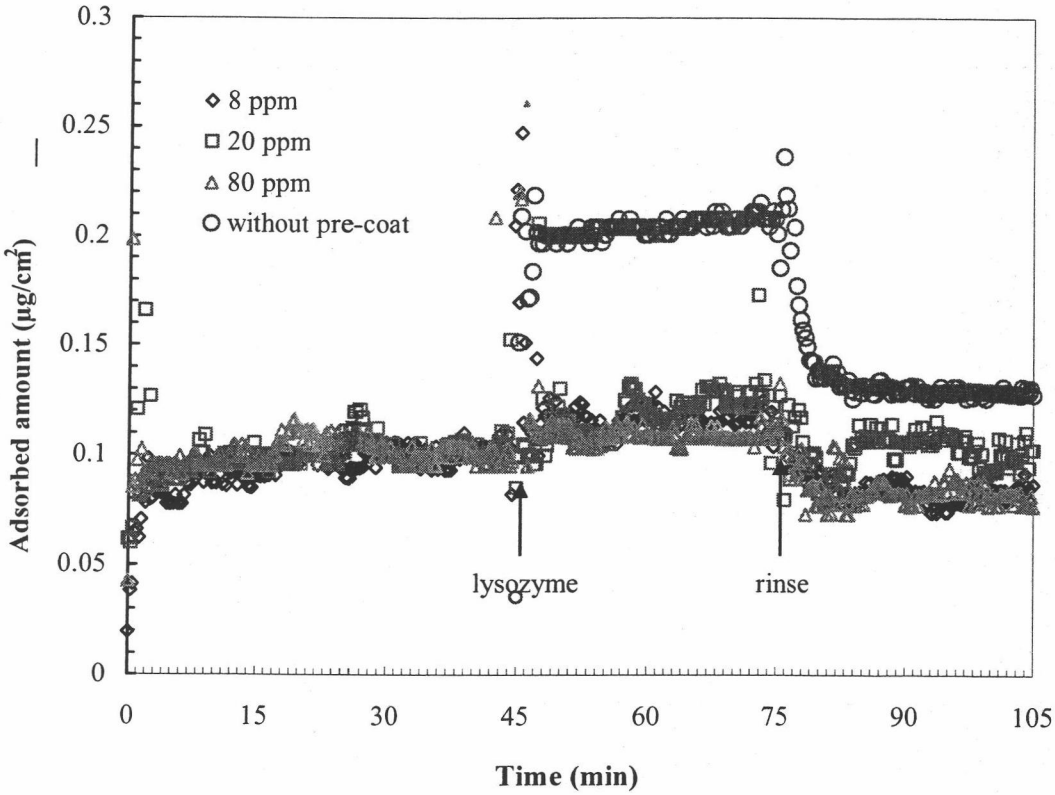

Figure 2.6 Lysozyme-Tween co-adsorption on hydrophobic silica pre-coated with Tween

There was no competition for the surface during the initial adsorption phase; consequently, Tween molecules were expected to form a tightly packed layer at the interface. In addition, Tween molecules were present in solution when lysozyme was added. It was therefore not surprising that very little lysozyme adsorption at the Tween pre-coated surface was observed. The difference in lysozyme adsorption was clearly seen when compared with the case where no Tween pre-coat was present. Note that the zero time for the "without pre-coat" plot is shifted ahead to correspond with the time point at which lysozyme was introduced for the other plots. The results indicate that lysozyme was unable to replace Tween adsorbed at a hydrophobic interface to any appreciable extent, especially when excess Tween was present in solution. Also, the lysozyme adsorbed along with the loosely held Tween was apparently rinsed away and the adsorbed amounts reached final values which were consistent with pure Tween kinetics. There was no apparent difference in adsorption behavior at surfaces pre-coated with 8, 20 or 80 ppm Tween suggesting that any Tween concentration higher than 8 ppm may be sufficient to saturate the interface and result in reduction of lysozyme adsorption.

2.3.2.4 Lysozyme adsorption on hydrophobic silica pre-coated with Tween

In order to more directly isolate the contribution of interfacial Tween in the observed reduction in lysozyme adsorption, a rinsing step was introduced after the formation of a Tween pre-coat prior to the addition of lysozyme. This step removed Tween present in solution and ensured that the Tween molecules remaining were resistant to elution. Figure 2.7 displays lysozyme adsorption on hydrophobic silica pre-coated with a stable Tween layer as described above.

A large fraction of Tween molecules remained adsorbed even after the hydrophobic surface was rinsed with buffer. This apparent irreversibility is contrary to the behavior of other surfactants such as SDS and DTAB, the adsorption of which is generally completely reversible upon buffer dilution. Some lysozyme adsorption was noted on the Tween layer. The amount adsorbed was comparable to the previous case in which lysozyme-Tween were co-adsorbed on to a Tween pre-coated surface. This suggests that the reduction in protein adsorption at a Tween pre-coated surface was a consequence of the presence of interfacial Tween. Conversely this suggests that the faster Tween diffusion to the interface may play a more crucial role than the formation of lysozyme – Tween complexes. But contrary to the previous case, when the surface was rinsed following lysozyme adsorption (105 minutes), no desorption was observed. When lysozyme adsorbed on Tween pre-coat which had not been rinsed and when there was Tween present in solution as well, it is likely that the protein adsorbed loosely on to the Tween layer. But when the Tween layer was rinsed and the lysozyme added, lysozyme possibly occupied the empty sites on the surface and even replaced some of the Tween. Due to the direct interaction with the surface, the lysozyme molecules will likely be rather tightly bound. This hypothesis is supported by the almost complete lack of lysozyme elution observed in Figure 2.7.

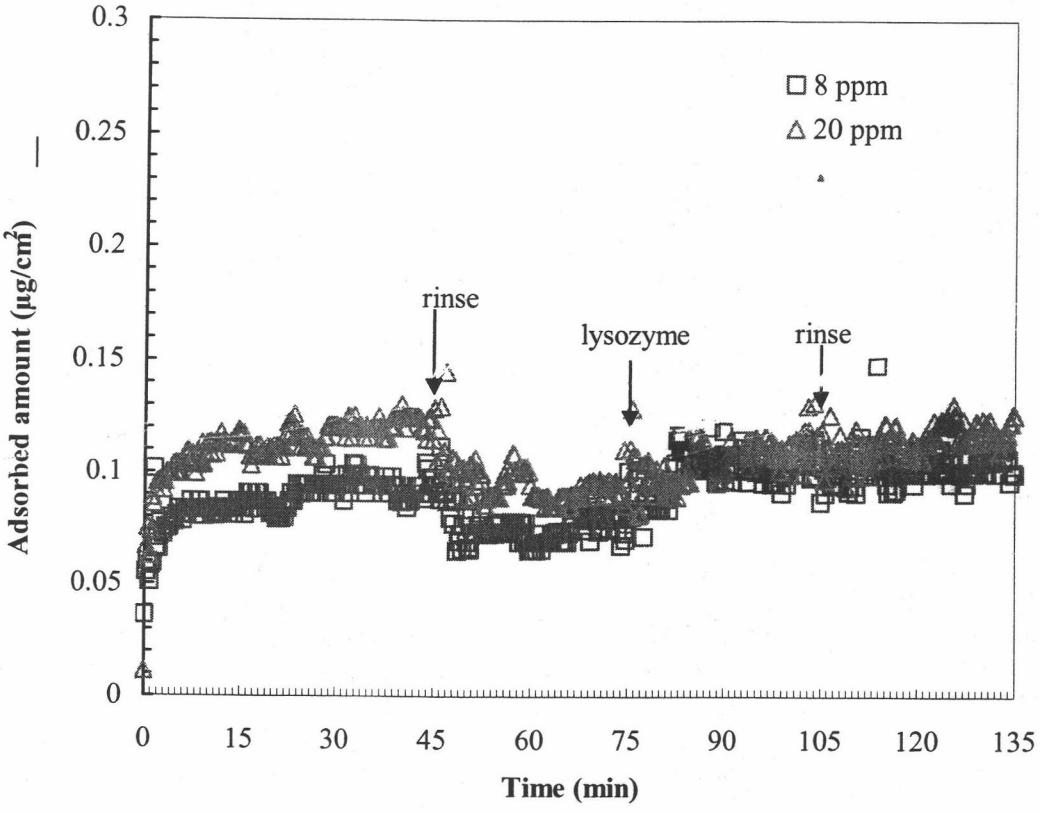

Figure 2.7 Lysozyme adsorption on hydrophobic silica pre-coated with Tween

2.3.2.5 Identity of the adsorbed layer

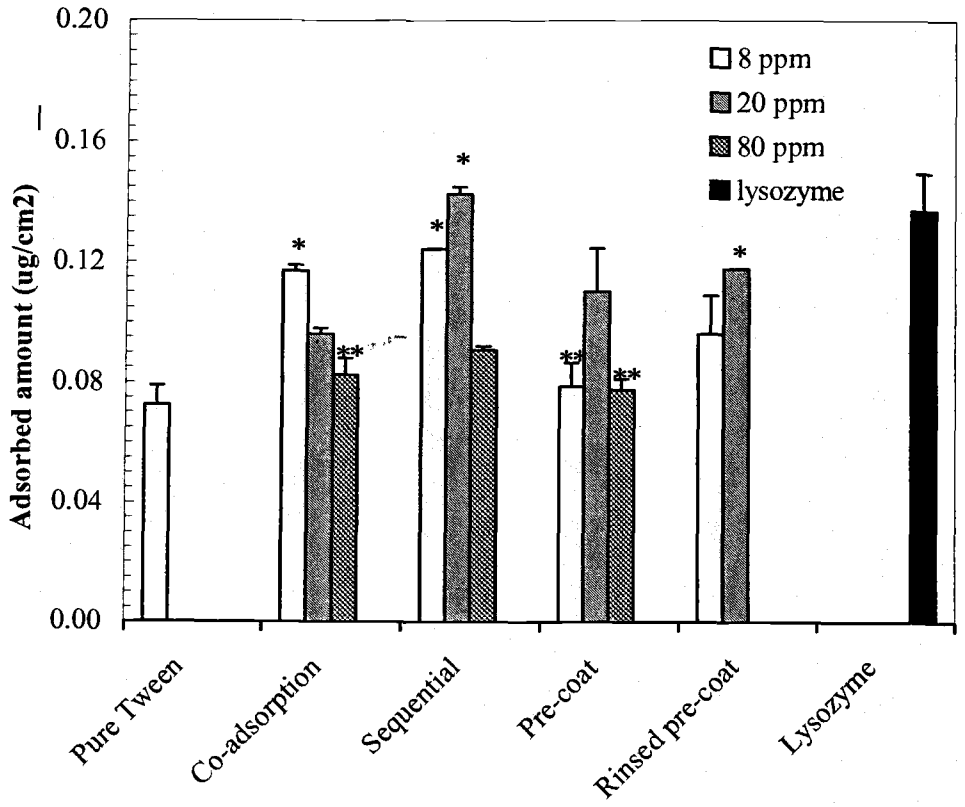

Figure 2.8 Post-elution adsorbed amounts on hydrophobic silica based on the order of lysozyme/Tween addition
Legends: * Not significantly dissimilar to lysozyme, significantly dissimilar to Tween; ** Significantly dissimilar to lysozyme, not significantly dissimilar to Tween

Lysozyme and Tween are added to the adsorbing system by different methods as described previously. The challenge involved in interpreting ellipsometry results with multi-component systems is that the technique detects the total adsorbed mass and does not differentiate between individual adsorbing species. We propose to use the final rest values of adsorbed amounts at the end of the final elution step as a semi-quantitative indicator of adsorbed layer identity. In every case the experiment was ended by performing a final elution step. The final elution step is expected to remove the loosely

held protein/surfactant molecules. The post-elution adsorption values were calculated as an average of the last of the 12 data points (in other words, the last 3 minutes), and are plotted in Figure 2.8. Single ANOVA analysis was then performed with a p-value less than 0.05 considered significant. Data was compared to pure Tween and pure lysozyme adsorption values. The identity of the adsorbed layer can then be considered to be lysozyme, if the adsorbed amount was significantly dissimilar to pure Tween but not significantly dissimilar to lysozyme. Results indicate that the final adsorbed layer probably consisted of mostly lysozyme for co-adsorption with 8 ppm Tween, sequential adsorption with 8 and 20 ppm Tween. On the other hand, the final adsorbed layer probably consisted of mostly Tween for co-adsorption with 80 ppm Tween, sequential adsorption with 80 ppm Tween and co-adsorption on a surface pre-coated with 8 and 80 ppm Tween. The results for co-adsorption on a surface pre-coated with 20 ppm Tween were somewhat surprising, in so much that they do not conform to the trend observed for co-adsorption on surfaces pre-coated with 8 and 80 ppm Tween. The final layers for surfaces pre-coated with Tween and rinsed prior to lysozyme adsorption appeared to consist of lysozyme or of both lysozyme and Tween. This underlined the importance of having Tween in solution from a complete lysozyme repulsion standpoint.

2.3.2.6 Comparison of lysozyme-Tween co-adsorption on hydrophobic and hydrophilic silica

Figure 2.9 depicts the lysozyme-Tween co-adsorption kinetics on hydrophobic and hydrophilic silica. As discussed previously, there was a large reduction in the net amount adsorbed when 80 ppm Tween was added to lysozyme during adsorption to hydrophobic silica.

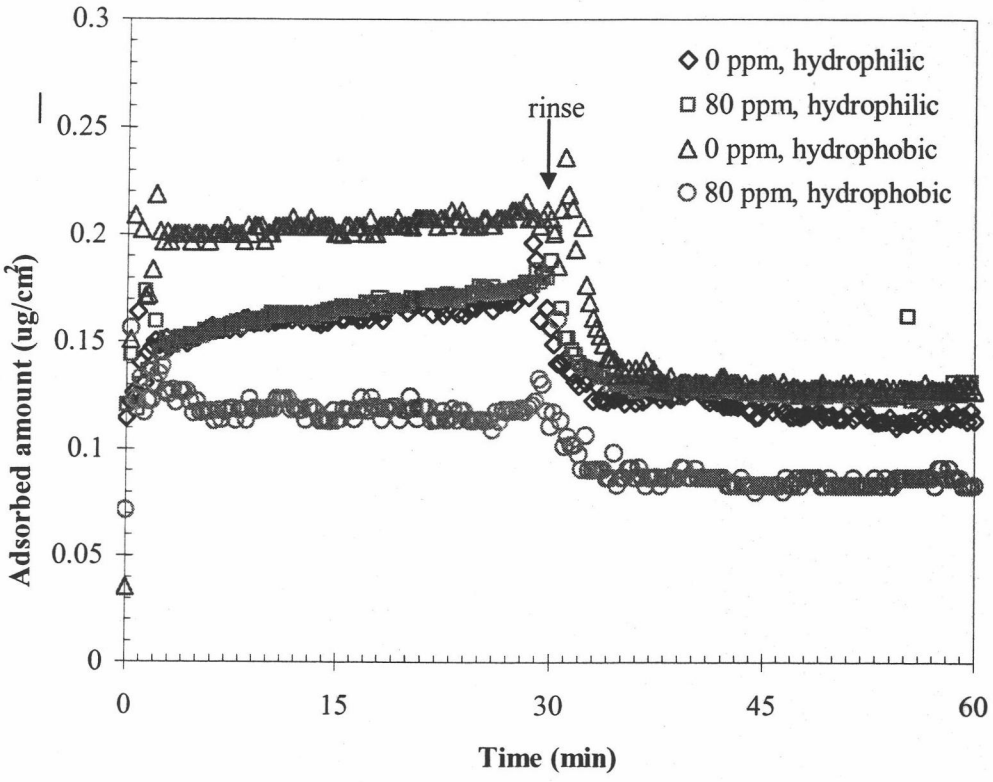

Figure 2.9 Comparison of lysozyme-Tween co-adsorption on hydrophobic and hydrophilic silica

For adsorption on hydrophobic silica, the co-adsorption of lysozyme-80 ppm Tween resulted in adsorption kinetics which closely corresponded with those for pure Tween adsorption. No similar reduction in adsorbed amount was observed when 80 ppm Tween was added to lysozyme during adsorption to hydrophilic silica. In fact, there appeared to be no difference between the adsorption kinetics of lysozyme on hydrophilic silica at 0 and 80 ppm Tween. The dynamics of the protein-surfactant interaction in solution should, in theory, remain the same when adsorption occurs in the presence of a hydrophilic or a hydrophobic surface. So the observed differences in adsorption behavior at different hydrophobic and hydrophilic surfaces have to be attributed to the protein/surfactant – surface interaction. Tween is a nonionic surfactant which adsorbs at

an interface only on account of its (Tween's) amphiphilicity. The hydrophobic surface is energetically more dissimilar to the aqueous environment than is a hydrophilic surface. Therefore, it is likely that the Tween-hydrophilic surface bond is relatively weak compared to the Tween-hydrophobic surface bond. For adsorption to a hydrophobic or a hydrophilic surface, it is expected that Tween diffuses to the surface faster, owing to its smaller size in comparison with lysozyme. But even if the Tween molecules were able to reach the hydrophilic surface first, there was no apparent effect on the subsequent lysozyme adsorption. The results obtained suggest that lysozyme is more successful at replacing the Tween at hydrophilic surfaces due to the weaker Tween-surface interaction.

2.3.2.7 Comparison of lysozyme-Tween co-adsorption on hydrophilic and hydrophobic surfaces pre-coated with Tween

Figure 2.10 shows the comparison of lysozyme-Tween co-adsorption kinetics on hydrophobic and hydrophilic silica pre-coated by the adsorption of 80 ppm Tween. It was observed that Tween adsorbed in reduced amounts on hydrophilic as compared to hydrophobic silica. This supports the theory that Tween has a greater affinity for hydrophobic than hydrophilic surfaces. In the experimental protocol followed here, Tween was allowed to reach the interface first without any competition with the protein. When lysozyme was added, the protein apparently replaced Tween from the hydrophilic surface and adsorbed to the same extent as on bare hydrophilic silica. In fact a comparison of adsorption kinetics on Tween pre-coated and bare hydrophilic silica revealed no difference in the lysozyme adsorption or elution behavior. This suggests that lysozyme completely replaced Tween, and the fact that Tween had reached the surface first was irrelevant. This underlines the relative importance of adsorbing species – surface interaction over the lysozyme – Tween interaction in solution in the adsorption process.

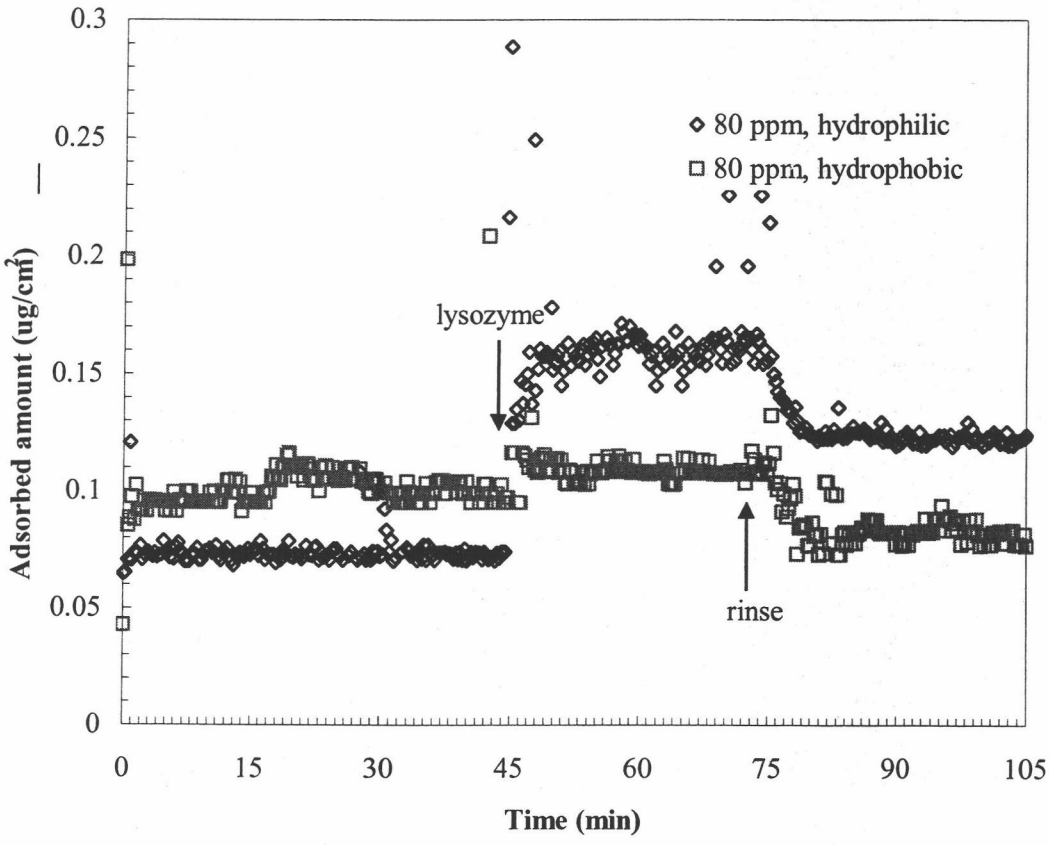

Figure 2.10 Lysozyme – Tween co-adsorption on hydrophobic and hydrophilic surfaces pre-coated with 80 ppm Tween

2.3.2.8 Comparison of lysozyme-Tween sequential adsorption on hydrophilic and hydrophobic silica

Figure 2.11 presents results for the case where 80 ppm Tween was added sequentially following lysozyme adsorption on and subsequent elution from hydrophilic and hydrophobic silica. It was noted earlier that when Tween was added in such a manner in the presence of hydrophobic silica, Tween was apparently able to remove the adsorbed protein layer and the final adsorbed mass after rinse was comparable to that for pure Tween. No such effect was seen in the presence of hydrophilic silica. There was a small

increase in adsorbed amount upon the introduction of Tween. The adsorbed amount dropped back to the value before Tween addition when the hydrophilic surface was rinsed. This observation is consistent with previous cases, where no "Tween effect" was observed probably as a result of the weak Tween-hydrophilic surface interaction. The results for the sequential Tween introduction experiments suggest that a strong Tween-surface interaction is required to effect protein removal. This in turn suggests that the replacement mechanism in which surfactant is able to remove adsorbed protein molecules on account of strong surfactant-surface affinity may be more relevant here than the solubilization mechanism

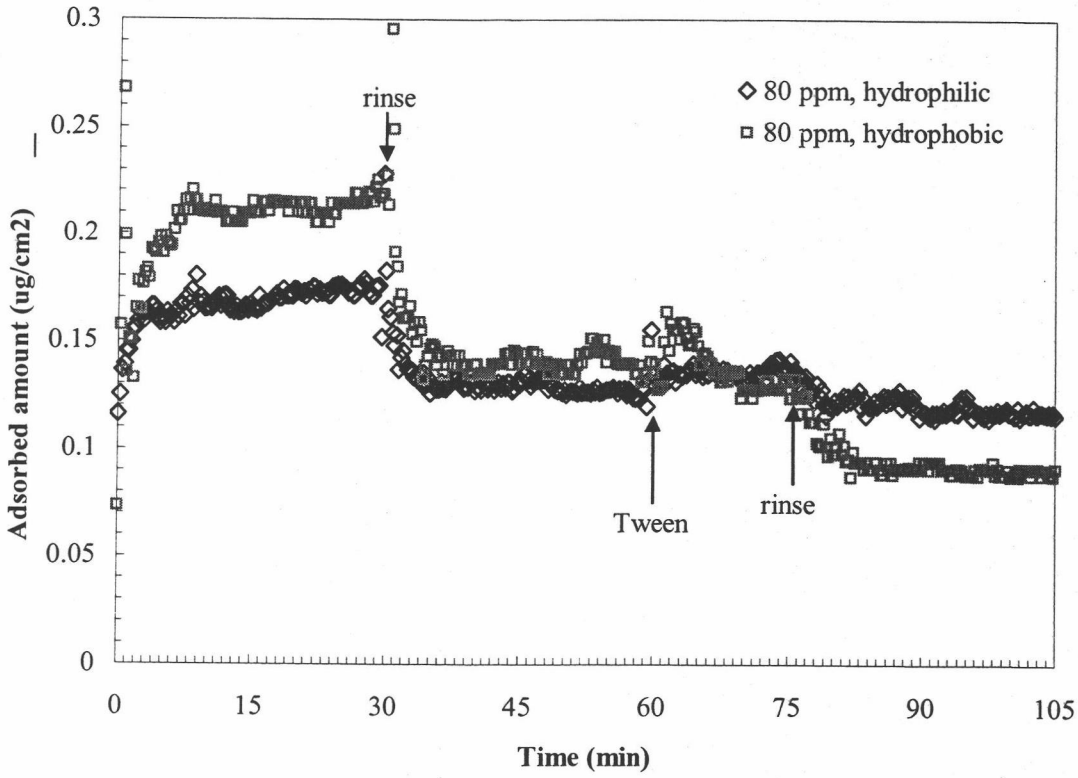

Figure 2.11 Lysozyme adsorption kinetics on hydrophobic and hydrophilic silica when 80 ppm Tween was added after lysozyme elution

2.4 Conclusions

The addition of Tween decreased lysozyme adsorption on hydrophobic silica, and the reduction in adsorbed amounts increased with the Tween concentration. Separate studies involving the formation of an interfacial Tween layer prior to lysozyme adsorption revealed the importance of interfacial Tween in reducing lysozyme adsorption. A comparison of adsorption kinetics on hydrophilic and hydrophobic silica surfaces provided an opportunity to compare the protein/surfactant – surface and protein – surfactant solution interaction and their relevance to the lysozyme adsorption processes. Contrary to the observations made in regard to the hydrophobic surface, the amount of lysozyme adsorbed at the hydrophilic silica surface did not change as the Tween concentration was increased. This suggested that strong Tween – surface binding is necessary to prevent lysozyme adsorption. This claim was supported by the results for Tween pre-coat on hydrophilic surface. In this case, lysozyme readily replaced Tween and adsorbed in amounts identical to those without Tween pre-coat. Data described in this work suggest that Tween-surface interaction may play a more crucial role than Tween-lysozyme interaction in solution. Accordingly, when adsorption occurs from a mixed lysozyme/Tween system, the faster diffusion of Tween to the interface is likely to contribute dominantly to the reduction in adsorption. Protein replacement at the interface by Tween may govern the removal of adsorbed lysozyme molecules when Tween is sequentially introduced at a high concentration.

2.5 References

Archer, J. 1968. *Manual on Ellipsometry*. Gaertner Scientific Corporation.

Arnebrant, T., and M. C. Wahlgren. 1995. Protein-surfactant interaction at solid surfaces in T. A. Horbett and J. L. Brash, eds. *Proteins at Interfaces II: Fundamentals and Applications*. American Chemical Society, Washington D.C.

Bisaglia, M., I. Tessari, L. Pinato, M. Bellanda, S. Giraudo, M. Fasano, E. Bergantino, L. Bubacco, and S. Mammi. 2005. A topological model of the interaction between a-synuclein and sodium dodecyl sulfate micelles. *Biochemistry* 44: 329-339.

Bloemendal, M., and W. C. J. Jr. 1995. Structural information on proteins from circular dichroism spectroscopy possibilities and limitations. *Pharm Biotechnol.* 7: 65-100.

Elwing, H. 1998. Protein adsorption and ellipsometry in biomaterial research. *Biomaterials* 19: 397-406.

Elwing, H., A. Askendal, and I. Lundstrom. 1989. Desorption of fibrinogen and g-globulin from solid surfaces induced by a nonionic detergent. *Journal of Colloid and Interface Science* 128: 296-300.

Hillgren, A., H. Evetsson, and M. Alden. 2002. Interaction between Lactate Dehydrogenase and Tween 80 in aqueous solution. *Pharmaceutical Research* 19: 504-510.

Horbett, T. A. 1982. Protein adsorption on biomaterials. Pages 233-244. *Biomaterials: Interfacial phenomena and applications.*

Klintstrom, S. W. 1992. Ellipsometry and wettability gradient surfaces. Pages 115. *Department of Physics and Measurement Technology*. Linkoping University.

Krisdhasima, V., J. McGuire, and R. Sproull. 1992. A one-film-model ellispometry program for the simultaneous calculation of protein film thickness and refractive index. *Surface and Interface Analysis* 18: 453-456.

Krishnan, A., Y.-H. Liu, P. Cha, D. Allara, and E. A. Vogler. 2005. Scaled interfacial activity of proteins at a hydrophobic solid/aqueous-buffer interface. *Journal of Biomedical Materials Research Part A* 75: 445-457.

Lee, W.-K. 1999. Kinetic modeling of the adsorption of structural stability mutants of bacteriophage T4 lysozyme at solid-water interfaces. Pages 276. *Bioresource Engineering*. Oregon State University, Corvallis.

Leffers, K.-W., J. Schell, K. Jansen, R. Lucassen, T. Kaimann, L. Nagel-Steger, J. Tatzelt, and D. Riesner. 2004. The structural transition of the prion protein into its pathogenic conformation is induced by unmasking hydrophobic sites. *Journal of Molecular Biology* 344: 839-853.

Manning, M., and W. Colon. 2004. Structural basis of protein kinetic stability: Resistance to sodium dodecyl sulfate suggests a central role for rigidity and a bias towards b-sheet structure. *Biochemistry* 43: 11248-11254.

McGuire, J., M. C. Wahlgren, and T. Arnebrant. 1995. Structural stability effects on the adsorption and dodecyltrimethylammonium bromide-mediated elutability of bacteriophage T4 lysozyme at silica surfaces. *Journal of Colloid and Interface Science* 170: 182-192.

Norde, W., and J. Lyklema. 1991. Why proteins prefer interfaces. *Journal of Biomaterial Science Polymer Edition* 2: 183-202.

Norde, W., and T. Zoungrana. 1998. Surface-induced changes in the structure and activity of enzymes physically immobilized at solid/liquid interfaces. *Biotechnology and Applied Biochemistry* 28: 133-143.

Podhipleux, N. 1998. Adsorption of the wild type and a synthetic structural stability variant of bacteriophage T4 lysozyme. Pages 124. *Bioresource Engineering.* Oregon State University, Corvallis.

Reynolds, J. A., and C. Tanford. 1976. Determination of molecular weight of the protein moiety in protein-detergent complexes without direct knowledge of detergent binding. *Proc. Natl. Acad. Sci. USA* 73: 4467-4470.

Vermeer, A. W. P., M. G. E. G. Bremer, and W. Norde. 1998. Structural changes of IgG induced by heat treatment and by adsorption onto a hydrophobic Teflon surface studied by circular dichroism. *Biochimica et Biophysica Acta* 1425: 1-12.

INTERACTIONS OF RECOMBINANT FACTOR VIII WITH NONIONIC SURFACTANT TWEEN 80 AT SOLID/WATER INTERFACES

Omkar Joshi[1], D.Q. Wang[2] and Joseph McGuire[1,3]

1. Department of Chemical Engineering, Oregon State University, Corvallis, OR 97331

2. Process Sciences, Bayer HealthCare, Berkeley, CA 94701

3. Corresponding author. Address102 Gleeson Hall, Oregon State University, Corvallis OR 97331 Email: mcguirej@engr.orst.edu

CHAPTER 3

INTERACTIONS OF RECOMBINANT FACTOR VIII WITH NONIONIC SURFACTANT TWEEN 80 AT SOLID/WATER INTERFACES

Abstract

The adsorbed mass of rFVIII and corresponding structure and activity upon exposure to hydrophobic or hydrophilic solid/water interfaces bearing positive or negative charge is evaluated. The simultaneous evaluation of adsorbed mass, structure and biological activity on surfaces displaying varied surface characteristics enables us to postulate an interfacial model involving possible driving forces relevant in rFVIII adsorption and the likely orientations adopted by rFVIII at the surface. The effect of nonionic surfactant Tween 80 was also studied. In particular, the Tween concentration was varied from 8 ppm to 80 ppm (200 ppm in certain cases) and the method of Tween addition was varied such that Tween reached the interface prior to, in competition with, or following rFVIII. Tween at high concentrations was effective in reducing rFVIII adsorption at hydrophobic interfaces but had no such effect at hydrophilic interfaces. Results suggest that a strong Tween – surface interaction which occurs at hydrophobic surfaces may be relevant in the observed reduction in rFVIII adsorption. The comparison of rFVIII adsorbed mass from solutions at different ionic strengths suggests that electrostatic forces may be relevant in determining the amounts of rFVIII adsorbed. The rFVIII tertiary structure was changed upon exposure to hydrophobic nanoparticle surfaces, but the structure remained largely comparable to native rFVIII upon exposure to hydrophilic surfaces. The biological activity of rFVIII was reduced when the protein was exposed to hydrophobic surfaces at low Tween concentration. In general, the structure and activity were better protected when the Tween concentration was increased to 80 ppm.

This was attributed to a greater fraction of rFVIII molecules remaining unadsorbed and/or Tween preventing rFVIII from unfolding at the surface due to steric constraints.

3.1 Introduction

Factor VIII is a high-molecular-weight (280 kDa) multidomain protein that is an essential blood coagulation factor. Factor VIII has a domain structure of A1-A2-B-A3-C1-C2, in which the heavy chain is composed of A1, A2 and B domains and the light chain is composed of the A3, C1 and C2 domains (Derrick 2004). FVIII exists as a heterodimer of the heavy and light chain and is associated with 50-fold excess von Willebrand Factor (vWF) when present in plasma (Kaufman 1992). In the blood coagulation cascade, FVIII serves as a cofactor for factor IXa in the activation of factor X to factor Xa (Curtis 1994). FVIII is first cleaved by thrombin to the active form, Factor VIIIa which is a heterotrimer consisting of the A1 domain, the A2 domain and the light chain (Gilbert 1998). The thrombin proteolysis of FVIII to FVIIIa thus results in the removal of the B domain (Bardelle 1993). The A2 and A3 domains interact with Factor IXa while the C-terminal domain of the C2 domain interacts with vWF and, upon activation, with phosphatidyl-L-serine-containing membranes. A functional deficiency in FVIII causes hemophilia A, a congenital bleeding disorder.

There are relatively few reports on the structure of the full-length FVIII molecule. The heavy and light chains are held together by metal ion-dependent and hydrophobic interactions (Fay 2005). Sudhakar and Fay (1996) evaluated the hydrophobic sites on the surface of FVIII and FVIIIa using intrinsic and extrinsic probe fluorescence. An identical binding and emission pattern for fluorescent probe bisanilinonapthalsulfonic acid of FVIII and FVIIIa, which lacks the B-domain, suggested that the B domain does

not contain any hydrophobic sites on the surface. Sudhakar and Fay (1996) identified two hydrophobic sites each on the isolated heavy and light chain. The authors further proposed that one hydrophobic site each on the heavy and light chain are retained on the surface of reconstituted FVIII while the other two hydrophobic sites participate in intersubunit association. Fowler et al (1990) used electron microscopy to study the domain structure of Factor V and FVIII. Each Factor V and FVIII molecule contained a large globular domain 12-14 nm in diameter. A single tail up to 50 nm was also often resolved in preparations containing a high-molecular weight heavy chain. Fowler et al present a molecular model of FVIII in which the A and C domains constitute the globular head and the connecting B domain is represented by a two-stranded tail. Stoilova-McPhie (2002) used the method of 2-dimensional crystallization of B domain-lacking FVIII onto phospholipid monolayers followed by electron microscopy and crystallography to solve the structure of such FVIII. The domain arrangement displayed A3 domains in close association with the C domains near the membrane surface. Four C2 loops are embedded within the lipid monolayer. The C1 domain is nearly perpendicular to C2, with the C1 long axis almost parallel to the membrane. Looking towards the membrane, A1 appears to fully covering C1, while C2 is partially overlapped by the A2 domain of the adjacent molecule.

Historically, Factor VIII was derived from human plasma. The risk of pathogen transmission and the limited availability of plasma have led to the development of a recombinant Factor VIII (Jiang 2002). rFVIII is the largest molecule ever successfully cloned by genetic engineering techniques and is the largest and most complex protein currently manufactured (Boedeker 2001). The rFVIII molecule is sensitive to both chemical and physical degradation. The degradation involves changes in higher order structure and may be caused by a number of pathways including aggregation, precipitation or adsorption onto surfaces (Wang 2003). Surface adsorption of rFVIII is rapid

(DiMichele 1996). Approximately 50% of rFVIII product may be lost due to adsorption just during sterile filtering (Osterberg 1997). In many studies involving rFVIII, the activity loss of rFVIII is the only indicator for adsorption. This paper addresses the rFVIII adsorption issue with three indicators: adsorbed mass, higher order structure and biological activity. These indicators are complementary to each other and the assimilation of all information obtained facilitates a comprehensive understanding of rFVIII adsorption.

In the past, human serum albumin (HSA) was added to rFVIII solutions to prevent adsorption during manufacture and packaging. This practice has been discontinued due to the risk of infectious agents originating from HSA. Nonionic surfactant Tween 80 is now added as a replacement for HSA since surfactants are generally effective in reducing protein adsorption. Fatouros et al (2000) have reported that the agitation induced denaturation of rFVIII is significantly reduced upon addition of 0.2 mg/ml Tween 80 or Tween 20. Recently, our laboratory completed a two-year study of the surface tension kinetics of Tween-rFVIII mixtures at the air/water interface (Chu 2005). In this work, we present rFVIII adsorption on solid interfaces, particularly the effect of Tween concentration and method of addition and the effect of the surface characteristics of the solid/liquid interface on the adsorbed mass and the corresponding structure and activity loss experienced by FVIII.

3.2 Materials and Methods

3.2.1 Protein, surfactant and buffers

The recombinant Factor VIII (rFVIII) used in this work was a gift from Bayer HealthCare (Berkeley, CA). The rFVIII used in ellipsometry experiments was obtained in a frozen liquid formulation at a concentration of 533 μg/ml. The protein was

formulated in 0.02 M 3-(N-Morpholino)propanesulfonic acid hemisodium salt (MOPS, Sigma), 220 mM NaCl, 25 mM $CaCl_2$, 1 % sucrose and 80 ppm Tween 80. The frozen rFVIII solution was thawed and aliquoted in 2 ml Eppendorf vials. The Eppendorf vials were then placed in a -80 °C freezer and were thawed just prior to use. The MOPS buffer used for incubation and rinsing during ellipsometry experiments was prepared without any Tween. Tween 80 (J.T. Baker) was dissolved in distilled, deionized water to obtain concentrated stock solutions at 10,000 and 50,000 ppm. The Tween stock solutions were aliquoted into 2 ml Eppendorf vials and frozen at -80 °C and then thawed just before use.

The rFVIII used in the fluorescence and activity assays was formulated in the KG-2 buffer consisting of 30 mM NaCl, 2.5 mM $CaCl_2$, 22 g/l glycine, 3.1 g/L L-histidine and 10 g/L sucrose at pH 6.8. The buffer excipients were provided by Bayer HealthCare. The protein solution contained approximately 100 µg/ml rFVIII and 20 ppm Tween.

3.2.2 Preparation of hydrophilic and hydrophobic silica adsorption substrates

Silicon (Si) wafers (crystal grade, type N, boron doped, orientation 1-0-0, thickness 525 ± 18 µm, resistivity 0.01-0.02 ohm-cm) were purchased from WaferNet Inc. Silicon wafers were oxidized by placing in a furnace at 1 atm and 1000 °C for 18 minutes to obtain an oxide film thickness of 300 Å (Podhipleux 1998). Wafers were cut into 1 × 3 cm plates using a tungsten pen, and rinsed with acetone to remove dust and organic residues. Plates were further cleaned using a standard acid/base cleaning procedure (Welin-Klinstorm 1992). The plates were first immersed in a 10 ml mixture of NH_4OH: H_2O_2: H_2O (1:1:5 volume ratio) and heated at 80 °C in a water bath for 10 minutes. Silica plates were then rinsed in copious amounts of DDW, transferred to a 10 ml mixture of HCl: H_2O_2: H_2O (1:1:5 volume ratio) and heated at 80 °C for 10 minutes. The plates were

rinsed with DDW again and dried under a flow of nitrogen. At this stage, the silica plates have hydrophilic surface characteristics, as evidenced by a water contact angle of 0 – 10 °. Contact angle measurements were obtained using a video-based FTA 32 (First Ten Angstroms) instrument. Hydrophilic plates were stored in ethanol until further use.

For preparing hydrophobic silica plates, the nitrogen-dried plates were transferred to a silanizing solution containing 1% dichlorodimethyl silane in xylene, and kept for 1 hour. The plates were then rinsed sequentially with xylene, acetone and ethanol. The silanization procedure rendered the silica plates hydrophobic, as evidenced by a water contact angle of 90 – 100 °. The hydrophobic plates were stored in ethanol until further use.

3.2.3 Evaluation of the surface charge of silica adsorption substrates

Silica wafers were cut into 3.8 × 6.35 cm plates and cleaned and silanized using the procedure described previously to produce hydrophilic and hydrophobic adsorption substrates. The surface charge was measured using the EKA ElectroKinetic Analyzer (Anton Paar) instrument which measures the streaming potential. The MOPS buffer, with no Tween and diluted 20-fold with DDW, was used as the electrolyte solution. The calculated zeta potentials for the hydrophilic and hydrophobic silica plates were -37.95 mV and -29.47 mV, respectively.

3.2.4 Nanoparticles conforming to different surface characteristics

Nanoparticles provide a large surface area for interfacial processes to take place while remaining dispersed due to their colloidal nature. Thus, nanoparticles enable the use of solution phase assays to study phenomena occurring on a solid substrate. Nanoparticles conforming to four distinct surface characteristics were obtained and used

as supplied. Table 3.1 describes the properties of the various nanoparticles used. The sizes of the nanoparticles were sufficiently larger than rFVIII to eliminate any particle curvature effects.

Table 3.1 Properties of nanoparticles used in structure and activity tests

Description	Surface characteristics		Mean Diameter (nm)	Supplier
	Wettability	Charge		
Sulfated white polystyrene latex	Hydrophobic	Negative	75	Interfacial Dynamics Corp.
Amidine white polystyrene latex	Hydrophobic	Positive	78	Interfacial Dynamics Corp.
Nyacol ® 9950 silica	Hydrophilic	Negative	100	EKA Chemicals
Bindzil ® CAT 80 alumina-coated silica	Hydrophilic	Positive	40	EKA Chemicals

3.2.5 Evaluation of rFVIII secondary structure as a function of Tween concentration

The circular dichroism (CD) spectra of a protein contain information about its secondary structure (Bloemendal 1995). The CD experiments were performed to evaluate the effect of Tween on rFVIII secondary structure and investigate whether Tween alters the rFVIII molecule in any way. The solution contained 533 μg/ml protein formulated in the MOPS buffer and 80 ppm Tween. Small volumes of concentrated Tween stock were added to prepare solutions containing 160 and 280 ppm Tween. It was

not possible to study rFVIII at a Tween concentration lower than 80 ppm without simultaneously diluting the protein.

CD spectra were obtained using the J-720 UV Spectrum spectropolarimeter (JASCO). All experiments were carried out at 25 °C. The cuvette used had a 100 μm pathlength and was cylindrical in shape. CD spectra were recorded with every 1 nm increment in wavelength, starting at 300 nm and ending at 194 nm. In order to increase the signal-to-noise ratio, six scans were recorded for each sample and then averaged. The CD spectra of protein-free Tween-containing buffer were subtracted from the rFVIII CD spectra in every case.

3.2.6 Evaluation of rFVIII adsorption kinetics

Factor VIII adsorption kinetics were studied with *in situ* ellipsometry. An ellipsometer measures the change in the state of polarization upon the reflection of a laser beam. The effect of reflection is characterized by the angles Δ, defined as the change in phase, and Ψ, the arctangent of the factor by which the amplitude ratio changes, between the two component plane waves into which the electric field oscillation is resolved (Archer 1968). Ellipsometry allows the analysis of protein adsorption on solid, specular surfaces in real time and *in situ* in biologically relevant aqueous media (Elwing 1998). An automatic ellipsometer (L-104SA, Gaertner Scientific Corp.) with a 1 mW He-Ne light source was used. The angle of reflection was set equal to the angle of incidence at 70 °. Each silica plate (hydrophobic and hydrophilic) was suspended in a trapezoidal fused quartz cuvette (Hellma) which was equipped with a magnetic stir-bar and filtered MOPS buffer was added. The optical properties (Δ and Ψ) of the laser beam reflected from the bare surface were measured for 30 minutes, at 15-second intervals. Factor VIII, Tween, mixed rFVIII-Tween or buffer solutions were then injected into/flowed through the

cuvette in a manner described in the following sections. The adsorbed protein/surfactant layer changes the optical properties of the reflected laser beam which can then be related to the adsorbed mass. A one-film-model ellipsometry program (Krisdhasima 1992) was used for the calculation of adsorbed mass. The program uses the values of the ratio between molar mass and molar reflectivity (M/A) and partial specific volume (V) of the adsorbing species. It is difficult to assign these values for rFVIII, since rFVIII is a highly glycosylated protein. In the absence of accurate values specifically for rFVIII, the values for a model globular protein, lysozyme, were used instead. Lysozyme was selected to enable easier comparison of results to a recently published article from our group (ref) that used a similar experimental scheme with lysozyme and Tween. The M/A and V used for lysozyme were 3.841 g/ml and 0.761 ml/g, respectively. As explained by McGuire et al (1995) protein-specific values of V and M/A were used to determine the adsorbed mass in both the presence and absence of surfactant, as it is not possible to assign a correct value to these parameters for mixed, protein/surfactant films. This approach does not influence any of the trends observed in these experiments.

The experimental scheme involved the introduction of Tween together with (co-adsorption), after (sequential) and before the introduction of the protein (pre-coat). For adsorption on hydrophobic silica, different Tween concentrations (8, 28 and 88 ppm) were used in each case. For adsorption on hydrophilic silica, experiments were performed in the presence of Tween at low (8 ppm) or high (88 ppm) concentrations. It must be noted that rFVIII adsorption kinetics with no Tween could not be recorded since the protein was obtained in a liquid formulation containing 80 ppm added Tween and was used without any further modification. Thus the lowest Tween concentration studied was 8 ppm, obtained by the 10-fold dilution of the protein sample during the ellipsometry procedure.

3.2.6.1 Tween - rFVIII co-adsorption

Tween 80 concentrated stock solutions at 10,000 and 50,000 ppm were diluted to obtain intermediate Tween solutions at 2,000, 5,000, and 20,000 ppm. Frozen rFVIII vials were thawed by holding in a 37 °C water bath. Twenty (20) µl of intermediate Tween solutions were added to obtain 200 and 800 ppm Tween-containing protein samples. These samples were vortexed briefly to ensure mixing. At the start of an experiment, 4.5 ml filtered formulation buffer was added to the cuvette. A (hydrophobic or hydrophilic) silica plate was then suspended in the cuvette and bare surface Ψ and Δ were monitored for 30 minutes. FVIII-Tween solutions (0.5 ml) were then added to the cuvette to obtain a final protein concentration of 0.053 mg/ml and a Tween concentration of 8, 28 and 88 ppm for hydrophobic and 8 and 88 ppm for hydrophilic silica. Adsorption was monitored for 30 minutes. The sample was then rinsed by flowing buffer through the cuvette, at a flow rate of 30 ml/min for 5 minutes. Buffer elution was monitored for a further 25 minutes.

3.2.6.2 rFVIII – Tween sequential adsorption

rFVIII solutions at a Tween concentration of 8 ppm were added to a cuvette containing hydrophobic or hydrophilic silica and kinetic adsorption data was recorded for 30 minutes. After this, the surface was rinsed with buffer at a flow rate of 30 ml/min and buffer elution was monitored for a further 25 minutes. Tween 80 solutions at 80, 200, 800 and 2000 ppm were obtained by diluting concentrated Tween stock solutions. Tween solutions (0.5 ml) were now added such that the final Tween concentrations were 8, 20 and 80 ppm. Tween was allowed to contact the surface (and the surface-adsorbed

protein) for 15 minutes, after which the sample was rinsed with buffer. Data was recorded for a further 25 minutes after rinse.

3.2.6.3 Tween – rFVIII co-adsorption at a surface pre-coated with Tween

In this case, 4.4 ml filtered PB was added to the trapezoidal cuvette. One-tenth (0.1) ml Tween sample was then added such that the final Tween concentration in the cuvette after the addition of protein was 8, 20 and 80 ppm. Tween adsorption was monitored for 45 minutes, after which time a 0.5 ml rFVIII solution was added to the cuvette to obtain a final protein concentration of 0.053 mg/ml. Adsorption of surfactant-protein mixture (i.e. the rFVIII which was added and the Tween that was already present in the cuvette) was monitored for 30 minutes. The plate was then rinsed with buffer for 5 minutes and buffer elution data was recorded for a further 25 minutes.

3.2.6.4 rFVIII adsorption at a surface pre-coated with Tween

The procedure followed was similar to the preceding case up to the point where protein is added. Here, the sample was rinsed with buffer for 5 minutes and buffer elution followed for a further 25 minutes. After this, a 0.5 ml rFVIII solution was added to the cuvette to obtain a final protein concentration of 0.053 mg/ml. Adsorption was monitored for 30 minutes. The sample was then rinsed with buffer for 5 minutes and buffer elution data was recorded for a further 25 minutes.

3.2.7 Evaluation of rFVIII tertiary structure

The rFVIII tertiary structure, in solution and on adsorption to colloidal particles, was studied using fluorescence emission spectroscopy (Koutsopoulos 2005, Natale 2005). Tryptophan is a well documented intrinsic fluorophore (Lakowicz 1999).

rFVIII has 37 tryptophan residues (Vehar 1984). There are several well documented results that relate tryptophan fluorescence with the protein tertiary structure. The tryptophan fluorescence depends on the local environment of the amino acid residues within the protein molecule. Analysis of fluorescence emission spectra often involves the calculation of the wavelength at which the fluorescence intensity is at a maximum (λ_{max}). If the λ_{max} of tryptophan spectra within the protein dissolved in an aqueous medium is shifted to shorter wavelength in relation to the λ_{max} of free tryptophan in water, the tryptophan must be internal and in a nonpolar environment (Friefelder 1976).

The rFVIII sample contained approximately 100 μg/ml protein and 20 ppm Tween and was formulated in the KG-2 buffer. The sample was diluted with KG-2 buffer containing no Tween so that the final Tween concentration was 8 ppm. A small volume of concentrated Tween stock solution was added to 2 ml rFVIII samples to obtain 20 and 80 ppm Tween – containing solutions. The volume of each nanoparticle suspension added was determined based on the specific surface area of each suspension and the molecular dimensions of rFVIII. The longest axis of the rFVIII molecule is reported to be 14 nm (Fowler 1990). One rFVIII molecule was assumed to occupy a 14 x 14 nm area on the surface. The area required for the adsorption of all molecules was calculated by taking the product of area occupied by one molecule and the number of rFVIII molecules in the 2-ml sample volume. A 50% excess area was provided in each case to account for molecular proximity effects and to ensure that rFVIII molecules had sufficient surface area for adsorption. The volume of nanoparticle suspension can then be easily estimated by dividing the area required by the specific surface area of the suspension. The sulfate and amidine polystyrene lattices were obtained as 8% and 4% (g/100ml) suspensions respectively and were used as supplied. The silica and alumina-coated silica nanoparticles, obtained in the form of 50% and 42% suspensions, were diluted 10-fold using KG-2

buffer just prior to use. The volume of suspension added corresponded to the same surface area provided in each case. The samples were then equilibrated overnight by placing on a tube rotator. Protein-free buffer samples were prepared in an analogous manner. After overnight equilibration, samples were transferred to a quartz cuvette and tested for internal fluorescence. Emission spectra of rFVIII and rFVIII adsorbed on nanoparticles was obtained using the PTI fluorometer (QuantaMaster, Photon Technology International). The excitation wavelength was set at 295 nm to selectively excite the tryptophan residues within rFVIII. Emission spectra were recorded at 1 nm increments from 305 to 405 nm. The excitation and emission slit widths were set at 0.25 and 2.0 mm, respectively. Three scans were recorded and averaged in each case in order to increase the signal-to-noise ratio. The data was corrected for protein-free background. Each experiment was performed in triplicate. In order to estimate the wavelength at which maximum fluorescence emission (λ_{max}) occurred, the background-corrected data were differentiated using the FeliX32 software supplied by the fluorometer vendor. The wavelength at which the differentiated curve intersected the x-axis was noted and reported as λ_{max}.

3.2.8 rFVIII biological activity in the presence of nanoparticles

The rFVIII activity was evaluated using a one-stage clotting assay based on the activated partial thromboplastin time (aPTT, Rosen 2002). Factor VIII acts as a cofactor in the presence of Factor IXa, calcium, and phospholipid in the enzymatic conversion of Factor X to Xa. In this assay, the test samples are incubated at 37°C with a mixture of rFVIII deficient plasma substrate and aPTT reagent. Calcium chloride was then added to the incubated mixture and clotting was initiated. An inverse relationship exists between the time (seconds) it takes for a clot to form and the logarithm of the rFVIII activity. Activity levels for unknown samples were interpolated by comparing the clotting

times of various dilutions of test material with a curve constructed from a series of dilutions of standard material of known activity and were reported in International Units per mL (IU/mL).

In order to evaluate the effect of nanoparticles on the assay, a control experiment was performed in which rFVIII at a final concentration of 1 IU/ml was added to the plasma. Thirty (30) µl of each nanoparticle suspension or buffer was added to the plasma substrate, clotting was initiated by calcium chloride and the time required for clot formation was measured in each case. The tests were performed in triplicate. Results are presented in Table 3.2. The clot times with the addition of the nanoparticles are comparable to those with no nanoparticles added. This indicated that nanoparticles by themselves did not affect the activity assay.

Table 3.2 Control experiment to evaluate effect of nanoparticles on the rFVIII activity assay

Sample	Clot time (seconds) (Mean ± S.D.)
Buffer	36.73 ± 0.70
Negative, hydrophobic nanoparticles	38.00 ± 0.36
Positive, hydrophobic nanoparticles	38.60 ± 0.89
Negative, hydrophilic nanoparticles	39.00 ± 0.56
Positive, hydrophilic nanoparticles	38.80 ± 0.36

The rFVIII samples used for activity testing were prepared as indicated for the fluorescence experiments. However, the samples were not equilibrated overnight

but tested within 6 hours of preparation. Samples were kept at room temperature in the duration prior to actual testing. Samples were tested at least twice.

3.3 Results and Discussion

3.3.1 rFVIII secondary structure as a function of added Tween

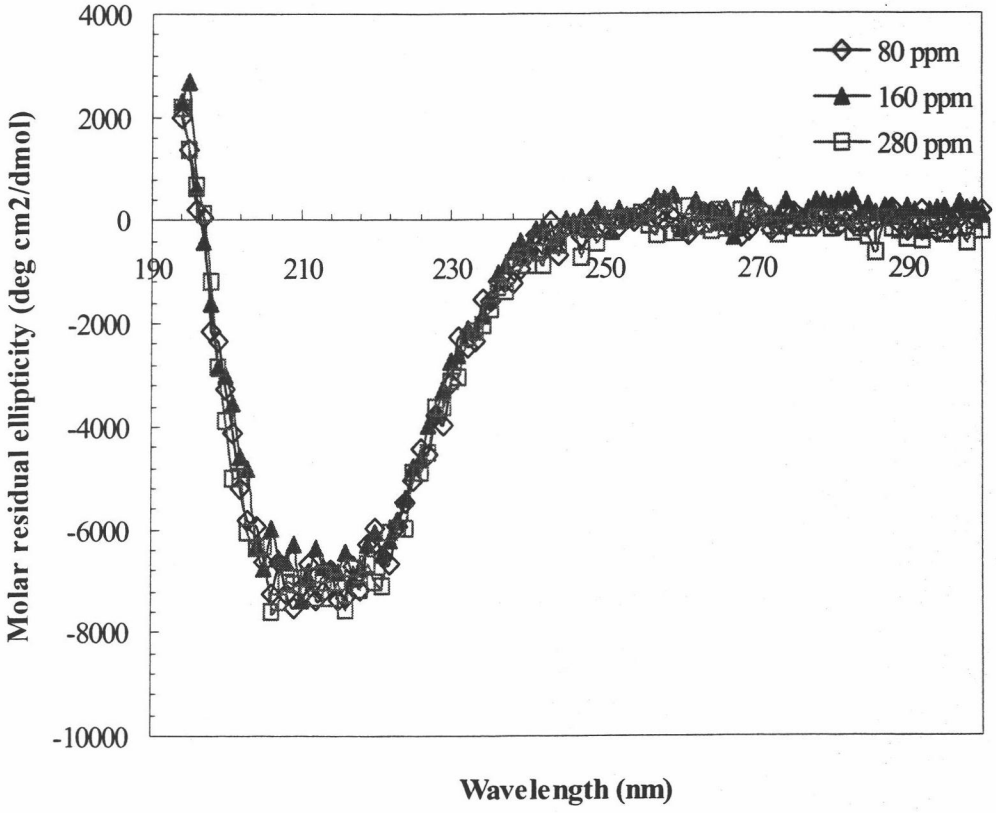

Figure 3.1 Circular dichroism spectra of rFVIII as a function of Tween concentration

The CD spectra of rFVIII at Tween concentrations of 80, 160 and 280 ppm are shown in Figure 3.1. The CD spectra at each Tween concentration were nearly identical which suggests that Tween in the concentration range studied had no effect on the rFVIII secondary structure. It must be noted that Tween concentrations lower than 80

ppm were not evaluated. So we cannot rule out the possibility of structure change as the Tween concentration is increased from a low value to 80 ppm. The CD spectra obtained correspond closely to results published by Grillo (2001), also for rFVIII at low Tween. This suggests that Tween may not alter secondary structure in any concentration range. The CD data was then deconvoluted using the "cdsstr" program described by Johnson (1999). Results are presented in Table 3.2. rFVIII appeared to have a low helical content and a more pronounced β structure. The data compares well with results obtained by Grillo (2001). The breakup of the structural elements at different Tween concentrations was very similar. This provided additional mathematical support to the observation that Tween at concentration ranging from 80 to 280 ppm did not alter rFVIII secondary structure.

Table 3.3 Calculated secondary structure of rFVIII at 80, 160 and 280 ppm Tween

Secondary structure component	Tween concentration		
	80 ppm	160 ppm	280 ppm
α-helix	6	6	8
3/10 helix	4	5	4
extended β-strand	26	28	26
β-turns	14	13	13
polyprolinelike 3/1 helix	9	8	9
others	41	41	41

3.3.2 Factor VIII adsorption kinetics as a function of Tween concentration and method of addition

3.3.2.1 Tween – rFVIII co-adsorption on hydrophobic silica

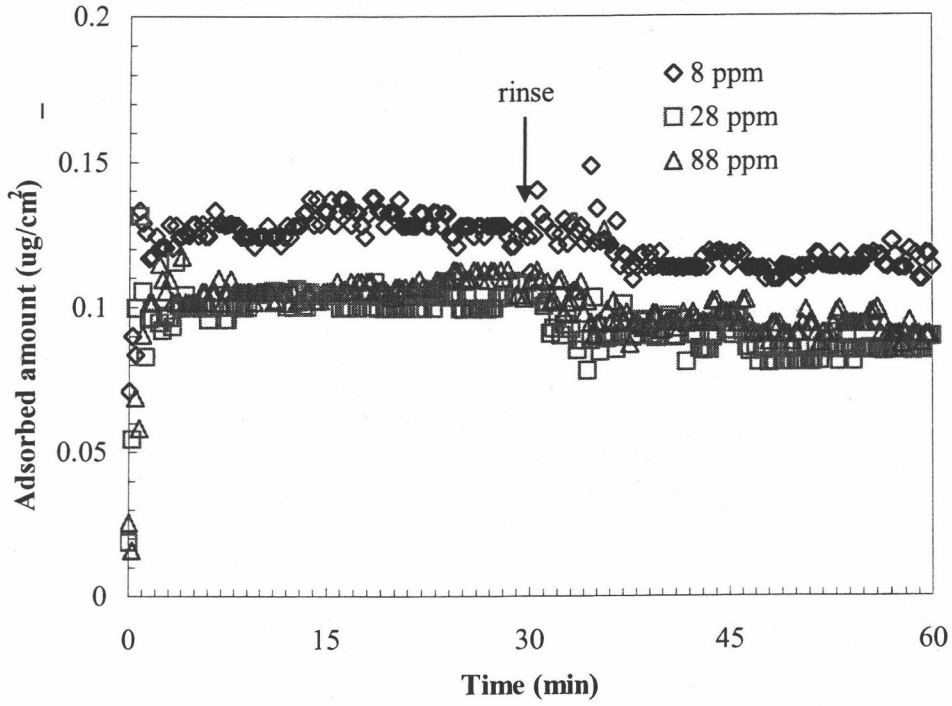

Figure 3.2 Co-adsorption of Factor VIII with Tween at concentrations of 8, 28 and 88 ppm on hydrophobic silica

Figure 3.2 depicts rFVIII adsorption kinetics on hydrophobic silica in the presence of 8, 28 and 88 ppm Tween. The amounts adsorbed after 30 minutes in the presence of 8, 28 and 88 ppm Tween were 0.139 ± 0.021 μg/cm^2, 0.097 ± 0.011 μg/cm^2, 0.109 ± 0.001 μg/cm^2, respectively. The amount adsorbed decreased as the Tween concentration was increased. The amounts remaining after rinse were 0.118 ± 0.004 μg/cm^2, 0.080 ± 0.009 μg/cm^2, 0.094 ± 0.005 μg/cm^2 for 8, 28 and 88 ppm Tween, respectively. These values also decreased as the Tween concentration was increased. The observed reduction in adsorbed amounts is consistent with two possible mechanisms,

Tween diffusing faster to the interface on account of its smaller size in comparison with rFVIII and/or forming a complex with rFVIII with a reduced surface activity. There is some evidence for the formation of rFVIII – Tween complexes as evidenced by the trends in the steady state surface tension value of rFVIII at different Tween concentrations (Chapter 4). A reduction in adsorbed amounts at the hydrophobic surface with an increase in Tween concentration was also observed for lysozyme-Tween co-adsorption (Chapter 2). The molecular weight of lysozyme is 20 times lesser than that of rFVIII. As a result, it is possible that the faster diffusion of Tween may assume greater importance in the observed reduction in rFVIII adsorbed amounts as compared with lysozyme.

The rFVIII – Tween adsorption kinetics at high Tween concentration were consistent with those for Tween by itself (Figure 3.6), suggesting that Tween may dominate the interfacial processes when present at a high concentration. An unambiguous interpretation of co-adsorption results is complicated by the lack of data for rFVIII adsorption alone. It is likely that rFVIII by itself adsorbs in a greater amount than observed upon co-adsorption with 8 ppm Tween.

3.3.2.2 rFVIII – Tween sequential adsorption on hydrophobic silica

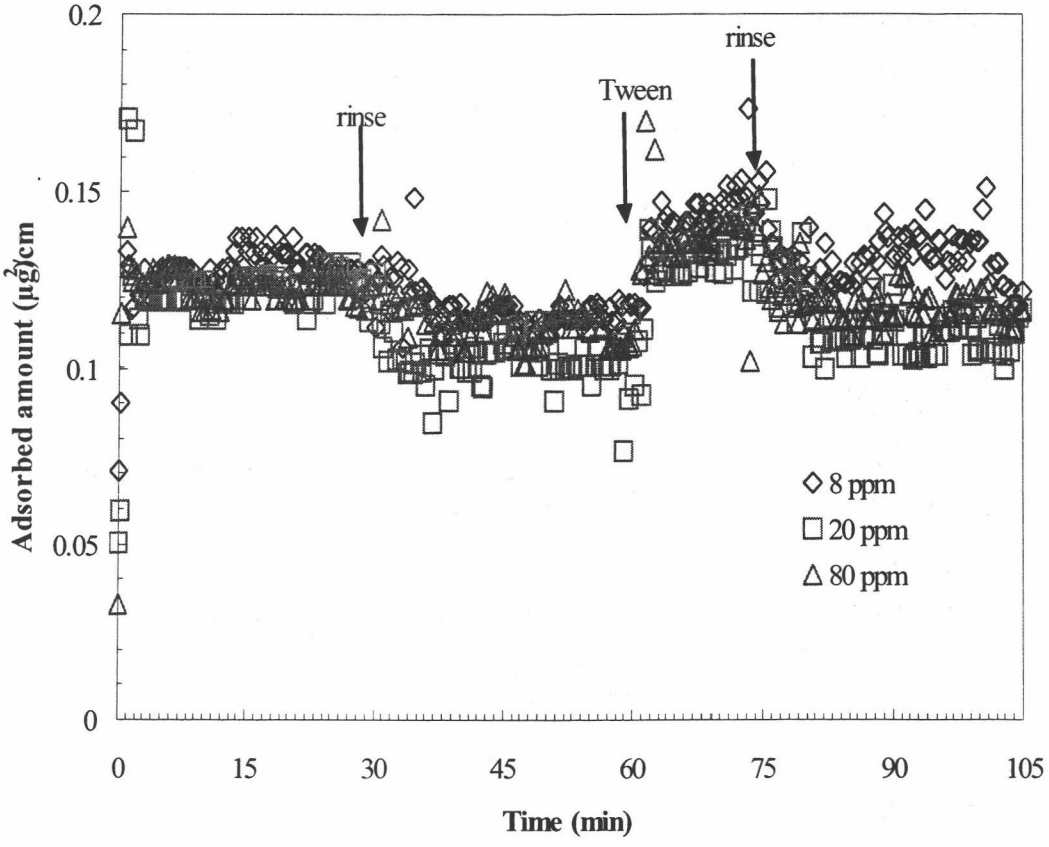

Figure 3.3 rFVIII – Tween sequential adsorption on hydrophobic silica

Figure 3.3 depicts the case where rFVIII and 8 ppm Tween co-adsorption and rinse were followed by the addition of 8, 20 and 80 ppm Tween. The first 60 minutes of the plot were essentially identical for all the three experimental conditions. At the end of 60 minutes, the adsorbed layer was likely composed of stably bound rFVIII and Tween molecules that resisted buffer elution. When Tween was then introduced, an increase in surface concentration was observed. This suggests that Tween was adsorbed at the empty sites on the surface and/or to the layer already present. When the hydrophobic surface was

rinsed after Tween addition, the surface concentration reverted back to values before Tween adsorption. This was consistent with Tween being loosely held at the interface. There was no net decrease in surface concentration after Tween introduction and rinse, as compared to the 60-minute value in Figure 3.3. This was true even when Tween was added at a concentration of 80 ppm, which was contrary to the results obtained with the lysozyme-Tween system (Chapter 2). A net removal of lysozyme was observed when 80 ppm Tween was added. Recombinant FVIII is a much larger molecule than lysozyme. This allows the formation of more contact points with the interface for the rFVIII molecules as compared with lysozyme molecules. Recombinant FVIII adsorption can be expected to be much stronger, which is supported by the lack of reduction in adsorbed amount upon introduction of 80 ppm Tween. In fact, there was no reduction in adsorbed amount even when Tween was introduced at a concentration as high as 200 ppm (data not shown). In conclusion, the introduction of Tween after rFVIII adsorption is unlikely to effect any reduction in surface concentration, irrespective of the Tween concentration. It is also instructive to compare the amounts adsorbed when 8 ppm Tween was introduced together with rFVIII (30-minute value in Figure 3.3) and sequentially (75-minute value in Figure 3.3). The 75-minute value was greater, which suggests that there might be history-dependent molecular rearrangement taking place which enables a more efficient interfacial packing of Tween molecules (Calonder 2001, Joshi 2005)

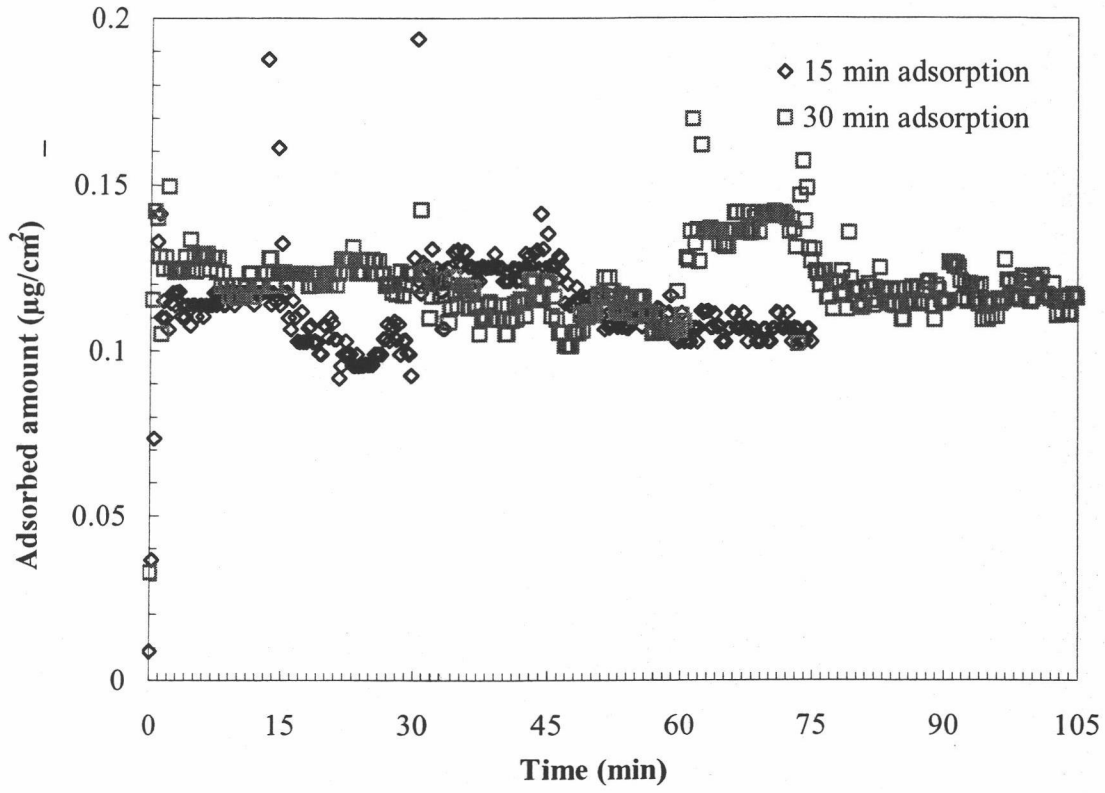

Figure 3.4 Adsorption time effect on the sequential adsorption of rFVIII with 80 ppm Tween on a hydrophobic surface

It is well-recognized that adsorption induces structural changes in the protein molecules and the magnitude of these changes increases as the time scale of adsorption increases. As a result of the surface-induced structural alteration, protein molecules become increasingly tightly bound to the surface. A separate experiment in which the rFVIII adsorption and rinse times were changed to 15 minutes from the original 30 minutes was performed with the objective of generating an adsorbed layer consisting of rFVIII molecules with a lower binding strength. This would then enable us to test whether Tween is successful at replacing the rFVIII (adsorbed with a potentially lower binding strength) as was observed with lysozyme. The results are presented in Figure 3.4. No net reduction in surface concentration upon Tween introduction was apparent even when the

adsorption and rinse times were reduced by half. The calculated values for adsorbing species resisting elution upon buffer dilution, i.e. amount remaining after rinse divided by the amount adsorbed just prior to rinse, were comparable for both 15 and 30-minute adsorption runs. Moreover, these values were rather high, 82-84%, suggesting that rFVIII binds very tightly irrespective of time allowed for adsorption and adsorption-induced structure change. Therefore it was not surprising to find that 80 ppm Tween was unable to replace the rFVIII at the interface.

3.3.2.3 rFVIII – Tween co-adsorption on hydrophobic silica pre-coated with Tween

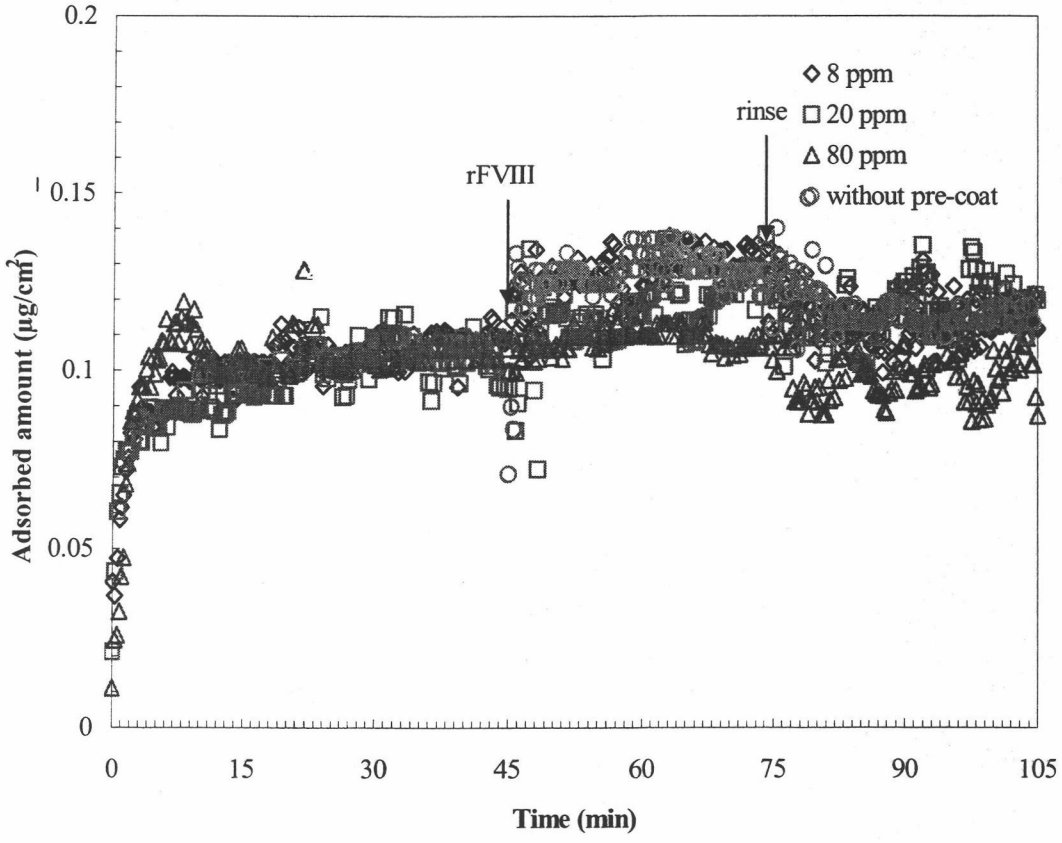

Figure 3.5 rFVIII-Tween co-adsorption on hydrophobic silica pre-coated with Tween

Results for rFVIII – 8 ppm Tween co-adsorption on a hydrophobic interface pre-coated with Tween are presented in Figure 3.5. Results for rFVIII – 8 ppm Tween with no pre-coat are superimposed here beginning at the 45 minute time-point to correspond with protein addition for the pre-coat experiments. Tween adsorbed in comparable amounts when added at 8, 20 and 80 ppm concentrations. The rFVIII sample containing 8 ppm Tween was then added. The solution present in the cuvette contained the Tween added to form the pre-coat. For the 8 ppm pre-coat, the amount adsorbed after protein addition was similar to that with no pre-coat. The data for the 20 ppm pre-coat showed a delayed increase in adsorbed amount to that with no pre-coat. The 80 ppm pre-coat displayed nearly no further increase in adsorbed amount after addition of protein. The comparable amounts adsorbed from solutions containing 8, 20 and 80 ppm Tween suggest similar Tween interfacial concentrations in each case. If the Tween interfacial layers in each case are in similar states of orientation and "packing", the difference in rFVIII adsorption on the Tween layers may then be attributed to the difference in Tween concentration in solution. We have already noted that adsorption kinetics of rFVIII containing 88 ppm Tween were consistent with those for pure Tween (Figure 3.2). When considering co-adsorption, two possible mechanisms were suggested, namely Tween reaching the interface faster or Tween forming a complex with rFVIII which alters the protein surface activity. In the case at hand (Figure 3.5), we have ensured that Tween had no competition for getting to the interface. But here we observed a reduction in adsorbed amount only when 80 ppm Tween was present in solution. This suggests that either the rFVIII – Tween interaction in solution may be important in the observed reduction in adsorption, or Tween experiences a concentration-driven "packing optimization" at the interface which serves to minimize rFVIII adsorption.

3.3.2.4 rFVIII – Tween co-adsorption on hydrophobic silica pre-coated with Tween with no added Tween in rFVIII solution

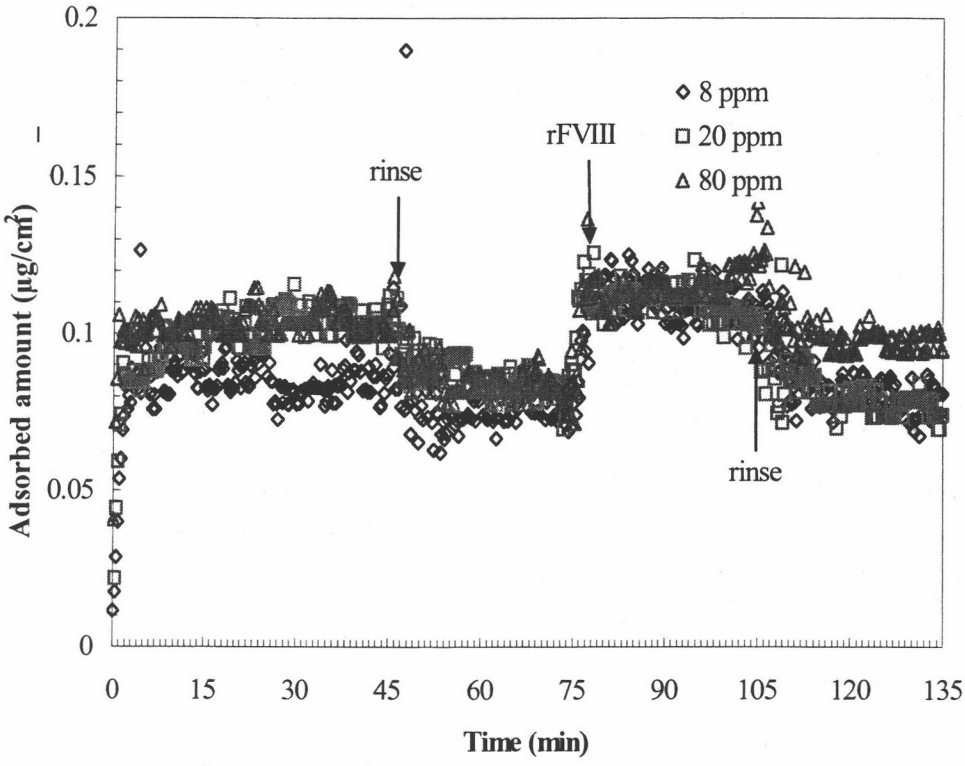

Figure 3.6 rFVIII – Tween co-adsorption on hydrophobic silica pre-coated with Tween (no Tween present in solution)

In order to isolate the contribution of interfacial Tween to the rFVIII adsorption process, hydrophobic silica pre-coated with Tween was rinsed with buffer to remove the free Tween in solution. rFVIII was introduced after this. Results are presented in Figure 3.6. Tween adsorption was not completely reversible upon buffer dilution and on average 82% of the adsorbed Tween resisted elution. This suggests that a tightly bound interfacial Tween layer was formed. However, the interfacial Tween was unable to prevent rFVIII adsorption. When rFVIII together with 8 ppm Tween was added at the 75-minute time-point, an almost instantaneous increase in adsorbed amount was observed.

The adsorption plateaus reached after rFVIII addition were identical, irrespective of the Tween concentration with which the pre-coat was generated. The interfacial Tween was apparently not effective at preventing rFVIII adsorption. As discussed in Chapter 2, when lysozyme was added on a Tween pre-coated hydrophobic surface, there was no further adsorption. These observations suggest that whereas interfacial Tween was important to prevent lysozyme adsorption, a combination of interfacial and solution Tween was important to prevent rFVIII adsorption. The difference in lysozyme and rFVIII behavior may be attributed to the higher surface binding strength exhibited by rFVIII.

3.3.2.5 rFVIII – Tween co-adsorption on hydrophilic silica

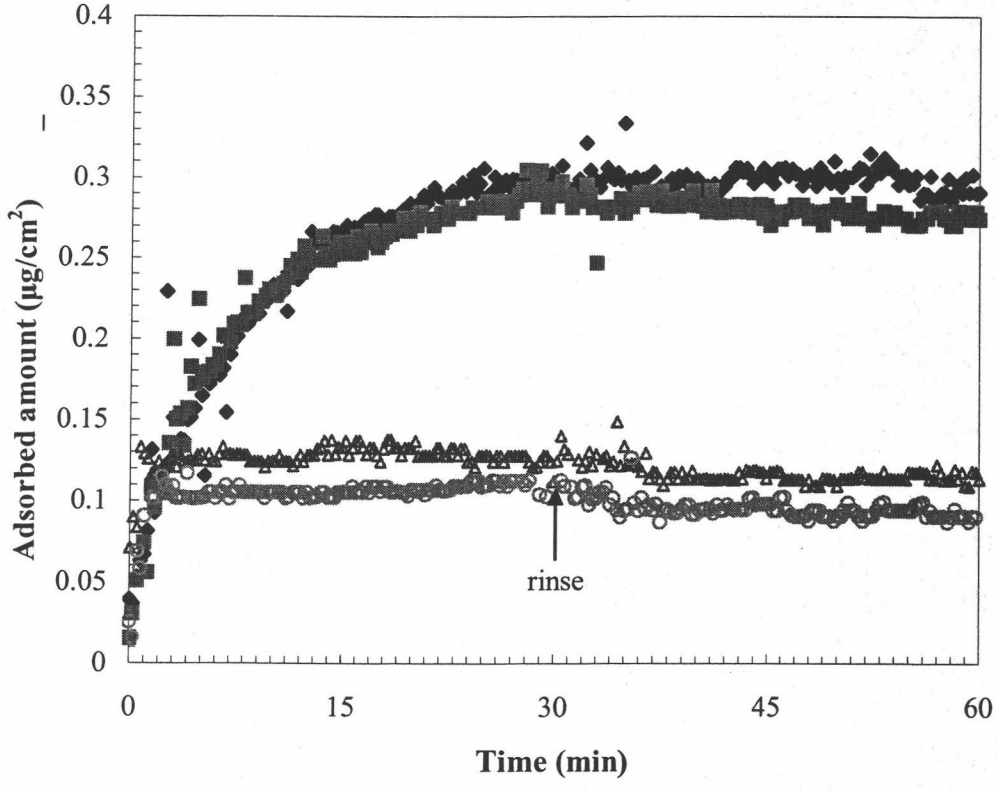

Figure 3.7 rFVIII – Tween co-adsorption on hydrophilic silica (♦: 8 ppm Tween – rFVIII on hydrophilic silica, ■: 88 ppm Tween – rFVIII on hydrophilic silica, Δ: 8 ppm Tween – rFVIII on hydrophobic silica, ○: 88 ppm Tween – rFVIII on hydrophobic silica)

Figure 3.7 depicts a comparison of rFVIII – Tween co-adsorption on hydrophobic and hydrophilic silica at a Tween concentration of 8 and 88 ppm. The amounts adsorbed on hydrophilic silica at both Tween concentration studied were much greater than on hydrophobic silica. It is routinely observed that proteins adsorb in greater amounts on hydrophobic as opposed to hydrophilic surfaces. We have made a similar observation when studying lysozyme adsorption kinetics on hydrophobic and hydrophilic surfaces in Chapter 2. So the behavior of rFVIII on hydrophilic silica was somewhat surprising. As reported in an earlier section, the streaming potential measurements of the silica surfaces revealed that the hydrophilic silica had a higher negative charge density than the hydrophobic silica. In addition, the silanized, hydrophobic silica has methyl groups on the layer closest to the interface. The methyl groups may provide a partial positive character to the outermost boundary of the surface and the negative charge may be buried within. Consequently, the negative charge on the hydrophobic silica is likely to be less solvent accessible. The difference in adsorbed amounts on hydrophobic and hydrophilic silica surfaces suggest that adsorption may be determined by a driving force different than hydrophobic interaction and that electrostatic forces possibly play an important role in rFVIII adsorption. This, in turn, explains the increased adsorption on the hydrophilic, highly negatively charged surface. It is likely that there is a mobile and solvent accessible domain bearing a highly positive charge on the rFVIII molecule, which binds with the negatively charged surface with a high affinity. In fact, the B domain is calculated to have a highly positive charge, and the idea of the B domain being a potential adsorption site is developed further in this work (Section 3.3.3.4).

There was no reduction in adsorbed amount on the hydrophilic silica when the Tween concentration was increased from 8 to 88 ppm. Tween is a nonionic surfactant which adsorbs at an interface only on account of the hydrophobic driving force.

Thereby, Tween binds very weakly on a hydrophilic surface. rFVIII is then able to replace the Tween at the interface. We have discussed the role of solution and interfacial Tween in the rFVIII adsorption process. The nature of rFVIII – Tween complexes will be the same irrespective of the nature of the interface that they are exposed to. But a Tween concentration of 88 ppm had close to no effect on rFVIII adsorption. A likely explanation is that Tween binds in a manner that renders the protein molecule less surface active, but is unable to mask the charges on the surface of the protein molecule.

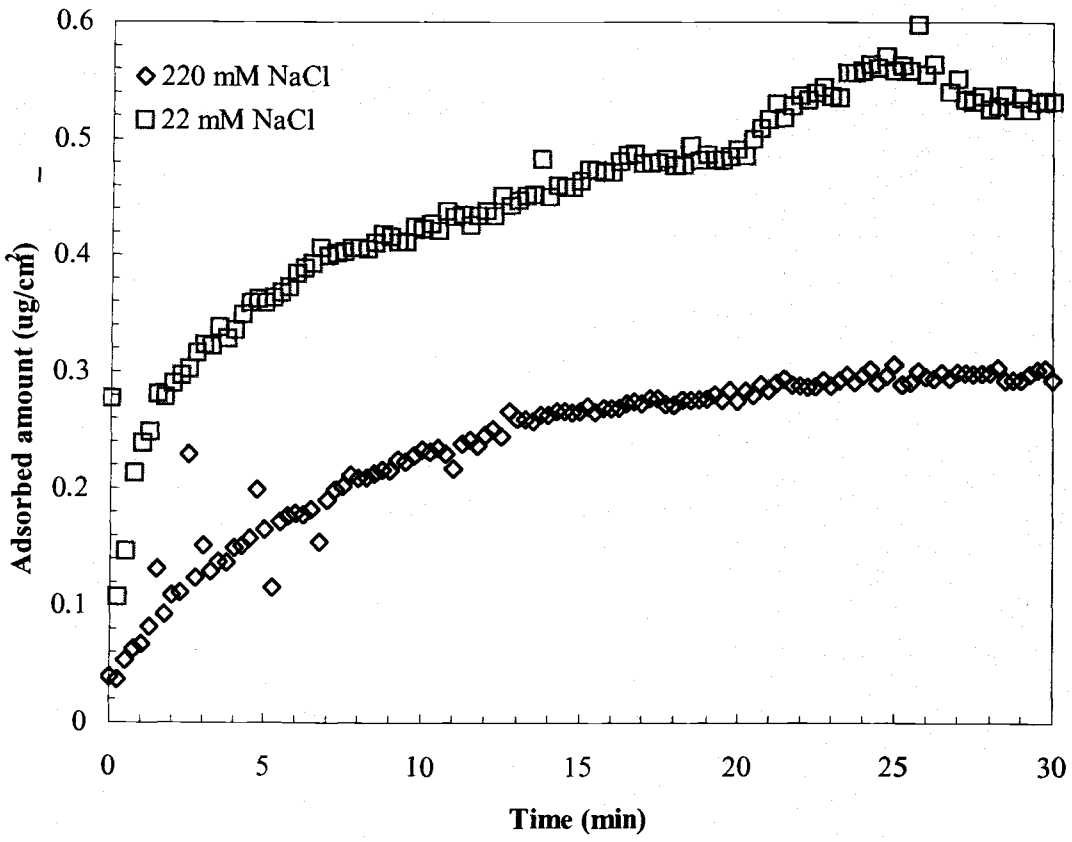

Figure 3.8 Effect of ionic strength on the adsorption of rFVIII – 8 ppm Tween on a hydrophilic surface

In order to test the hypothesis that charge attractions play an important role in rFVIII adsorption, the ionic strength of the buffer was changed. All data reported so far was for rFVIII in a formulation buffer containing 220 mM NaCl and 2.5 mM $CaCl_2$. Results in Figure 3.8 depict a comparison of rFVIII – 8 ppm Tween adsorption from a solution containing 220 mM and ten-fold less i.e. 22 mM NaCl. The concentration of all other buffer components, including $CaCl_2$ was kept constant. An increase in adsorption was observed as the ionic strength was reduced 10-fold. Salt ions shield the electrostatic forces and rFVIII molecules have to approach the surface at a shorter distance for the charge-charge interactions to occur. This in turn decreases the likelihood of an adsorption event. The fact that we see such a large difference in adsorbed amounts with a change in ionic strength is further proof that electrostatic forces play a very important role in rFVIII adsorption.

3.3.2.6 rFVIII – Tween sequential adsorption on hydrophilic silica

A comparison of rFVIII – Tween sequential adsorption kinetics on hydrophobic and hydrophilic silica is presented in Figure 3.9. Almost no removal of rFVIII adsorbed on hydrophilic silica was obtained when the surface was rinsed. Unlike on the hydrophobic surface, there was no discernible increase in adsorbed amount when Tween was introduced following rinsing on the hydrophilic surface. This suggests that rFVIII was adsorbed on the hydrophilic surface in a very tightly packed and possibly highly ordered layer, leaving no space for the subsequent binding of Tween. There was no removal of adsorbed rFVIII following Tween introduction or rinse on the hydrophilic surface. This may be expected since the Tween – hydrophilic surface binding strength is low, and likely to be lower than the strong charge – charge attraction that appears to bind rFVIII to the negatively charged, hydrophilic surface.

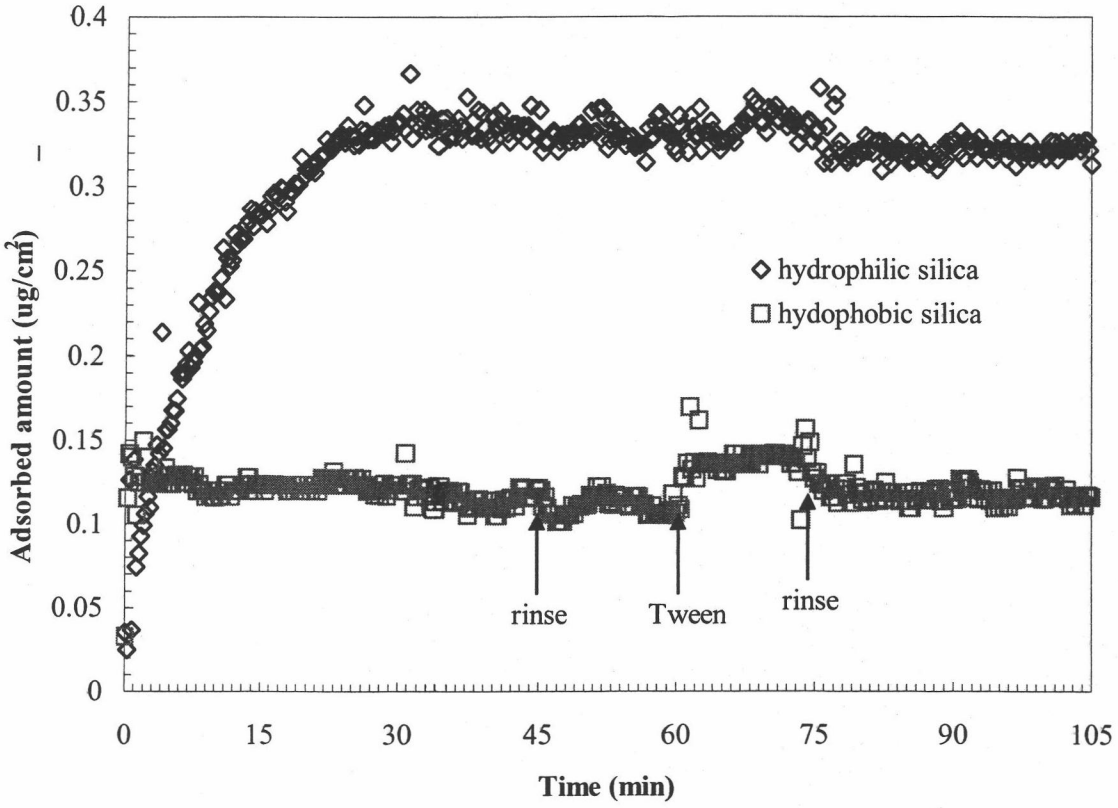

Figure 3.9 Comparison of rFVIII – 80 ppm Tween sequential adsorption on hydrophobic and hydrophilic silica surfaces

3.3.2.7 rFVIII – Tween co-adsorption on hydrophilic silica pre-coated with Tween

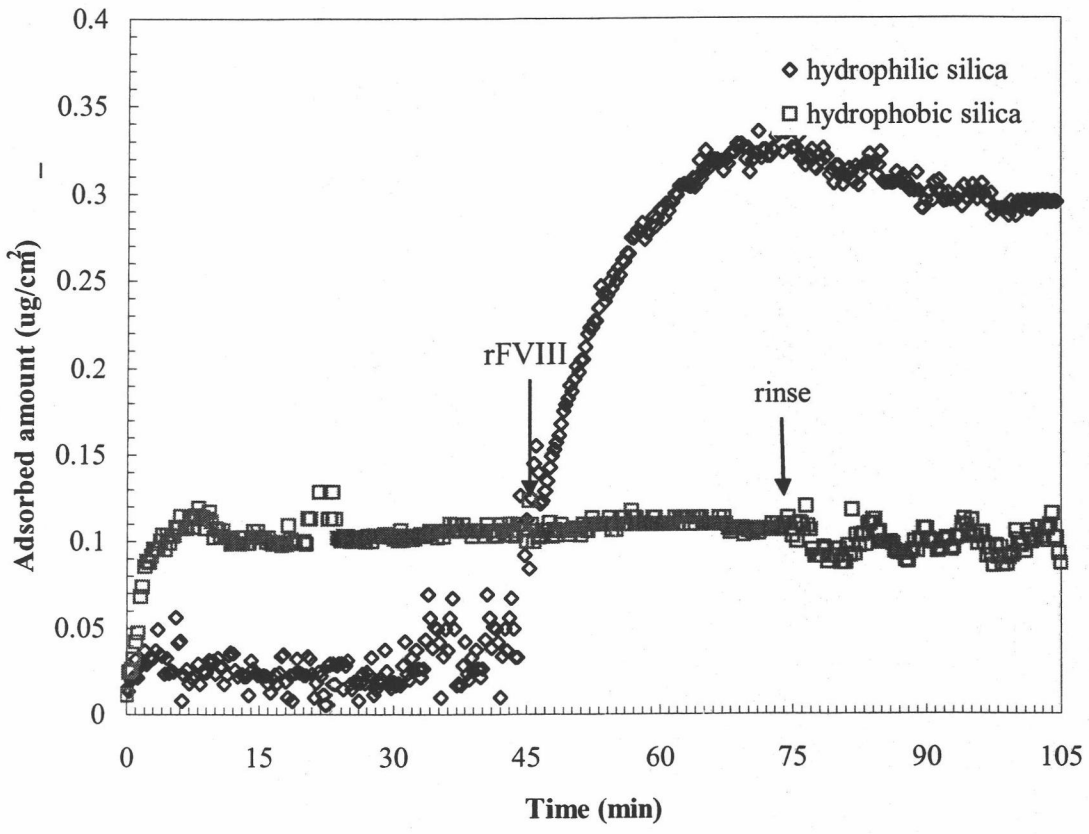

Figure 3.10 Comparison of rFVIII – Tween co-adsorption on hydrophobic and hydrophilic silica pre-coated with Tween

Figure 3.10 shows a comparison of rFVIII – Tween co-adsorption kinetics on hydrophobic and hydrophilic silica pre-coated with a Tween solution at a concentration of 80 ppm. The first 45 minutes represent adsorption of Tween only. Tween adsorbed very weakly to the hydrophilic silica, as evidenced by the low surface concentration (< 0.05 $\mu g/cm^2$) and the unstable nature of the adsorption kinetics. The Tween pre-coat was unable to prevent rFVIII adsorption on hydrophilic silica, and rFVIII adsorbed in amounts similar to those without a Tween pre-coat (Figure 3.7). rFVIII was apparently able to replace the Tween at the hydrophilic interface, even though Tween reached the

hydrophilic interface first. We have now noted that Tween at any concentration and irrespective of the method of addition rFVIII is unable to effect any reduction in rFVIII adsorption at hydrophilic surfaces. This underlines the importance of Tween-surface interaction in rFVIII interfacial phenomena. As noted previously, ellipsometry suggests very little rFVIII adsorption detected on the interfacial Tween layer in the case of hydrophobic silica. This difference in efficacy of the Tween pre-coat on the two surfaces was attributed to the difference in the strength of the Tween-surface binding.

3.3.3 Evaluation of rFVIII tertiary structure

3.3.3.1 Native rFVIII fluorescence spectra at 8, 20 and 80 ppm Tween

Figure 3.11 depicts typical results obtained for rFVIII fluorescence in the presence of Tween at concentrations of 8, 20 and 80 ppm. The fluorescence spectra at different Tween concentrations were nearly identical. The calculated λ_{max} for different Tween concentrations were also nearly identical (Figure 3.12) and statistical analysis revealed no significant difference between groups (p-value > 0.05). This provides additional evidence to the claim that Tween did not alter the structure of the native rFVIII molecule in any way.

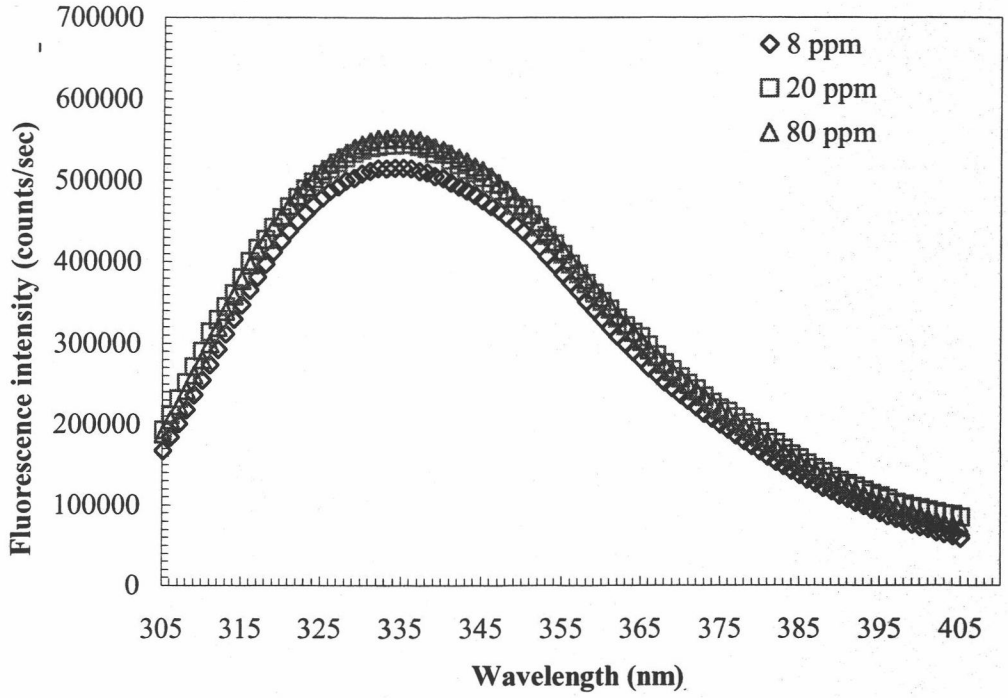

Figure 3.11 Representative plots of rFVIII fluorescence emission spectra at 8, 20 and 80 ppm Tween

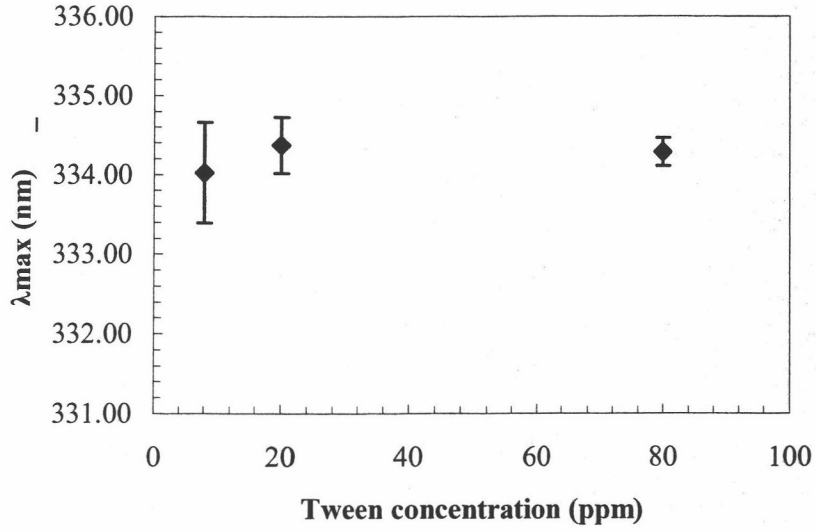

Figure 3.12 λ_{max} for rFVIII samples containing 8, 20 and 80 ppm Tween

3.3.3.2 rFVIII fluorescence in the presence of nanoparticles

Recombinant FVIII fluorescence emission was evaluated in the presence of hydrophobic and hydrophilic nanoparticles that had a positive or negative surface charge. Four types of nanoparticles, each representing a particular surface characteristic, were evaluated. Figures 3.13a through 3.13d depict the typical fluorescence spectra and Figures 3.14a through 3.14d depict the calculated values of λ_{max} in presence of nanoparticles at different Tween concentrations.

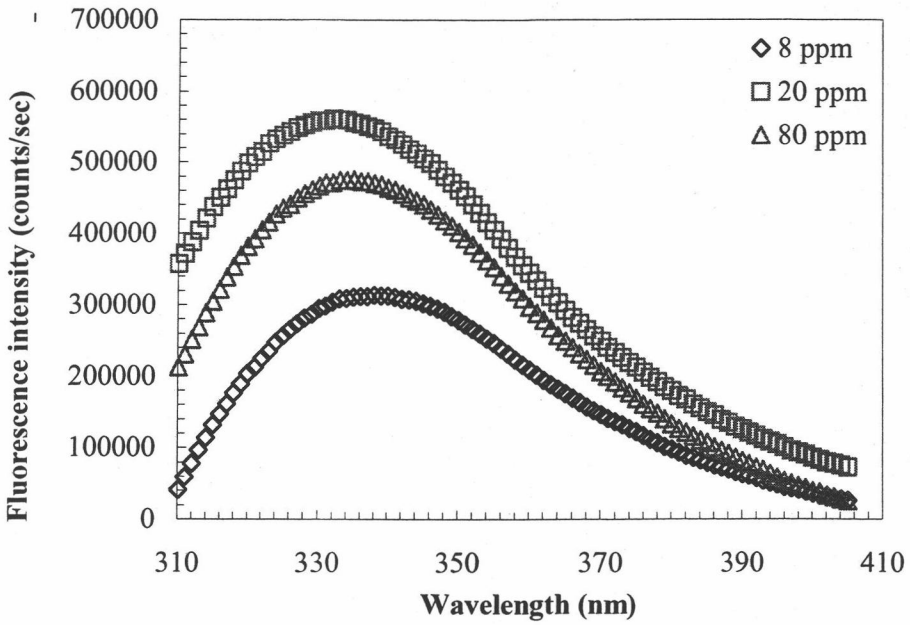

Figure 3.13a rFVIII fluorescence spectra in the presence of negative, hydrophobic nanoparticles

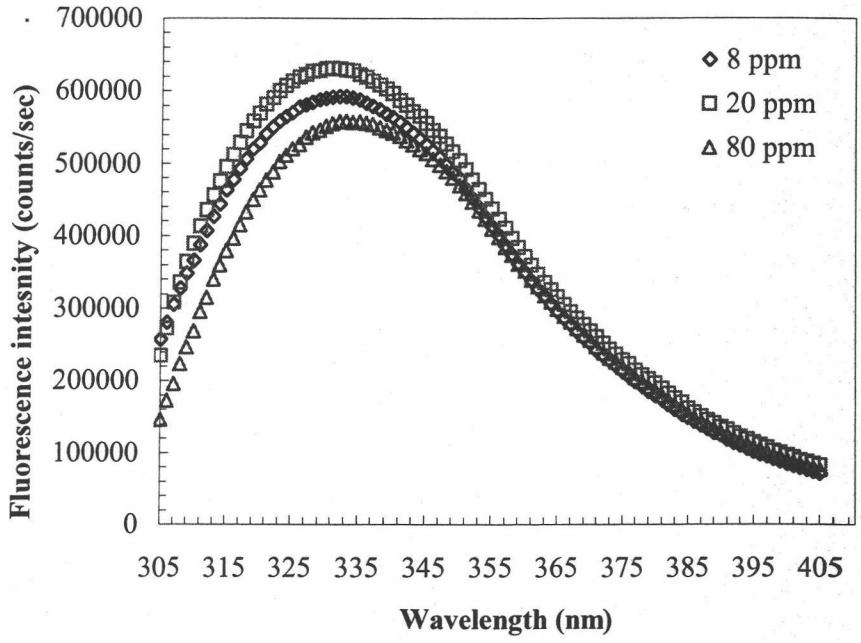

Figure 3.13b rFVIII fluorescence spectra in the presence of positive, hydrophobic nanoparticles

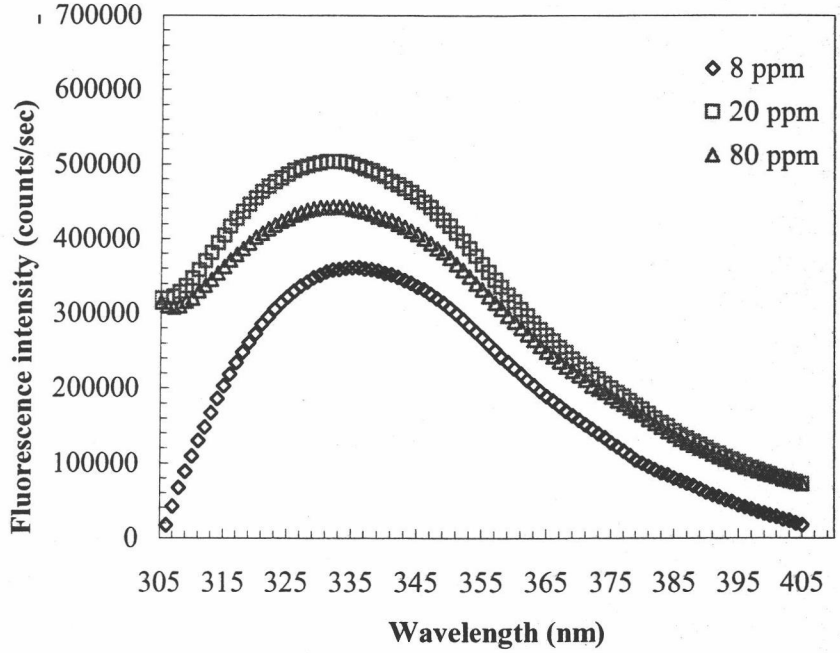

Figure 3.13 c rFVIII fluorescence spectra in the presence of negative, hydrophilic nanoparticles

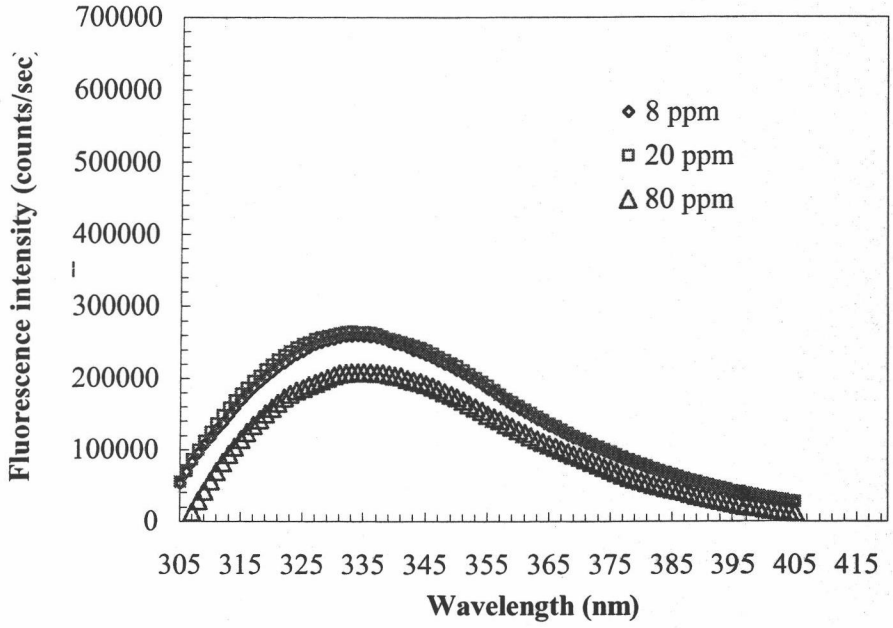

Figure 3.13 d rFVIII fluorescence spectra in the presence of positive, hydrophilic nanoparticles

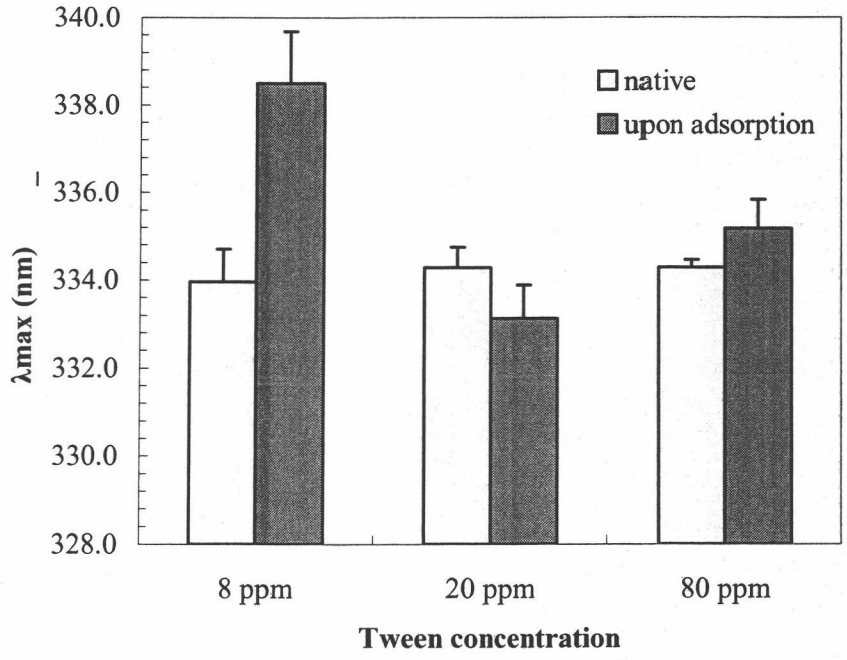

Figure 3.14a λ_{max} in the presence of negative, hydrophobic nanoparticles

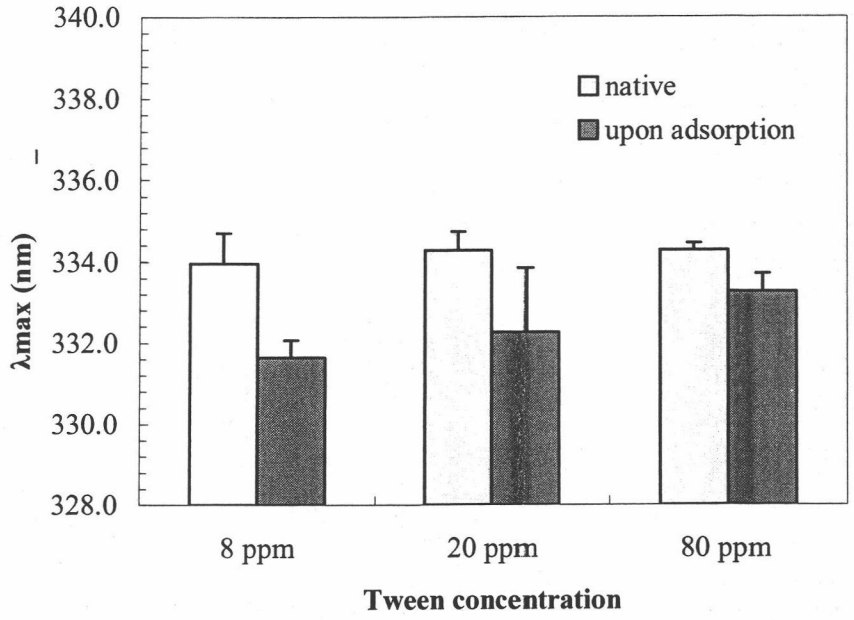

Figure 3.14b λ_{max} in the presence of positive, hydrophobic nanoparticles

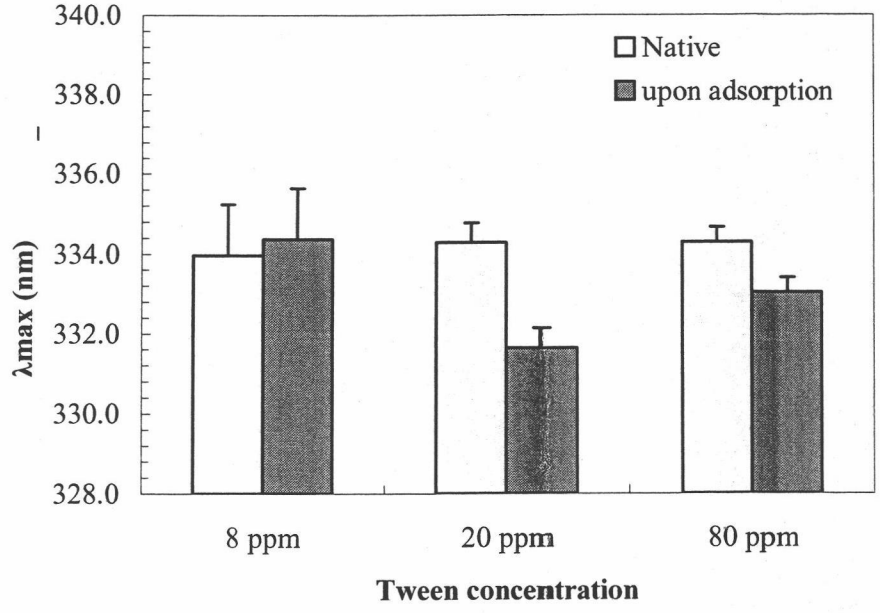

Figure 3.14c λ_{max} in the presence of negative, hydrophilic nanoparticles

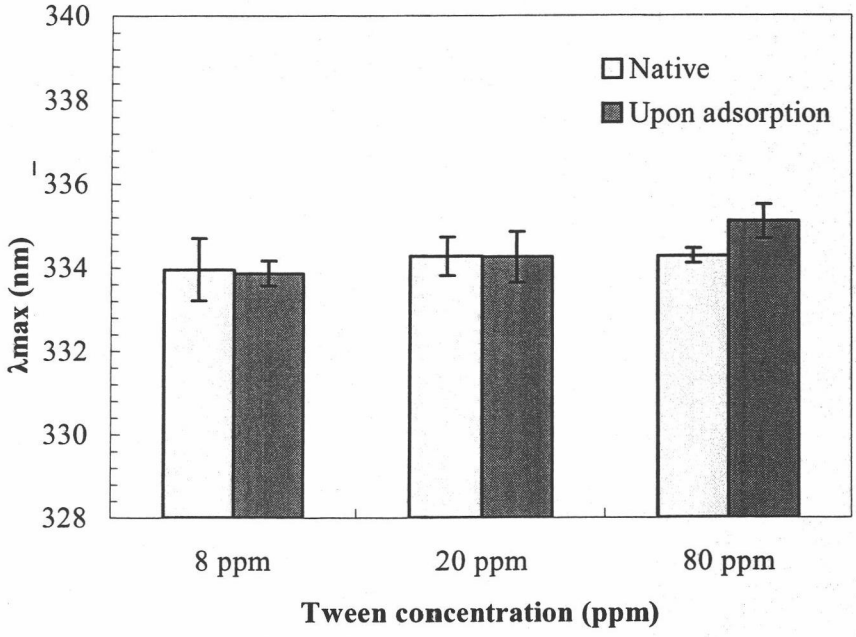

Figure 3.14d λ_{max} in the presence of positive, hydrophilic nanoparticles

The λ_{max} shifted to longer wavelengths (338.5 ± 1.18 nm) as compared to native, when rFVIII was adsorbed on negative, hydrophobic nanoparticles at a low Tween concentration (8 ppm). This suggests that the tertiary structure of rFVIII was significantly altered in a manner that exposes some or all the tryptophan residues to the aqueous environment. As more Tween was added, the λ_{max} upon adsorption approached the native value, suggesting that the addition of Tween prevented unfolding. There are two possible mechanisms which may contribute to this effect. As more Tween is added, there will be an increased competition for surface sites between Tween and rFVIII. More rFVIII is then likely to remain unadsorbed and in a native form, contributing to a λ_{max} value which approaches that for native rFVIII. We also know that Tween adsorbs strongly on a hydrophobic surface. The adsorbed rFVIII molecules may find it increasingly difficult to

unfold due to the steric constraints imposed by the neighboring Tween molecules. On account of this, the structure of the adsorbed rFVIII molecules may be better preserved. When rFVIII was adsorbed on positive, hydrophobic nanoparticles in the presence of low Tween, the λ_{max} shifted to shorter wavelengths (331.6 ± 0.44 nm) as compared to native. Thus, there was some indication of a structure change upon adsorption. But in this case, the tryptophan residues appear to be on average more buried and less exposed to the aqueous environment than the native molecule. This was contrary to the observations made with negative, hydrophobic nanoparticles. We have previously noted that electrostatic forces, i.e. attraction (or repulsion) between the charged surface and the oppositely charged domains in the protein, may play a very important role in the adsorption process. The difference in the direction of the λ_{max} shift on positively and negatively charged hydrophobic surfaces may be attributed to different orientations adopted by the rFVIII in response to the surface charge. As Tween concentration was increased, the λ_{max} upon exposure to positive, hydrophobic nanoparticles approached the native value. This was consistent with the trend observed with negative, hydrophobic particles. Similar mechanisms as discussed for negative, hydrophilic nanoparticles are likely to be responsible for the enhanced structural protection.

When rFVIII was adsorbed on negative, hydrophilic nanoparticles in the presence of low Tween, there was no significant shift in λ_{max} as compared to native. Ellipsometry results on negatively charged silica surfaces indicate that rFVIII adsorbed in large amounts and most probably due to electrostatic attraction. This suggests that rFVIII adsorbed on the negative, hydrophilic nanoparticles as well. But at the same time, adsorption did not appear to induce any unfolding. This may be explained if we consider the formation of a tightly packed and highly ordered layer of rFVIII molecules on the surface. It is likely that the rFVIII molecules orient in a manner that allows the positively

charged domain to face towards the surface. Once this layer is formed, there may not be any space remaining for unfolding. Also, unfolding may not be energetically favorable since that may reduce the electrostatic attractive force. On a negative, ***hydrophobic*** surface, the electrostatic and hydrophobic forces are both relevant. This increases the likelihood of a more random adsorption of rFVIII molecules and in turn explains the loss of rFVIII structure upon adsorption at negative, hydrophobic as compared with the negative, hydrophilic surface. As the Tween concentration was increased to 20 ppm, the λ_{max} appeared to shift to a shorter wavelength. This was also observed with the negative, hydrophobic surface. This trend was somewhat surprising and no explanation for this observation was forthcoming. The λ_{max} in the presence of positive, hydrophilic nanoparticles displayed only small deviations from the native. This was apparent at all Tween concentrations studied. It may be concluded that there is less perturbation in rFVIII tertiary structure when adsorbed on hydrophilic as compared with hydrophobic surfaces.

3.3.3.3 rFVIII activity in the presence of nanoparticles

The biological activity of rFVIII samples containing nanoparticles bearing various surface characteristics was evaluated with the one-stage clotting assay. Control samples with no nanoparticles were evaluated along with every experimental run. Results are presented in Figure 3.15.

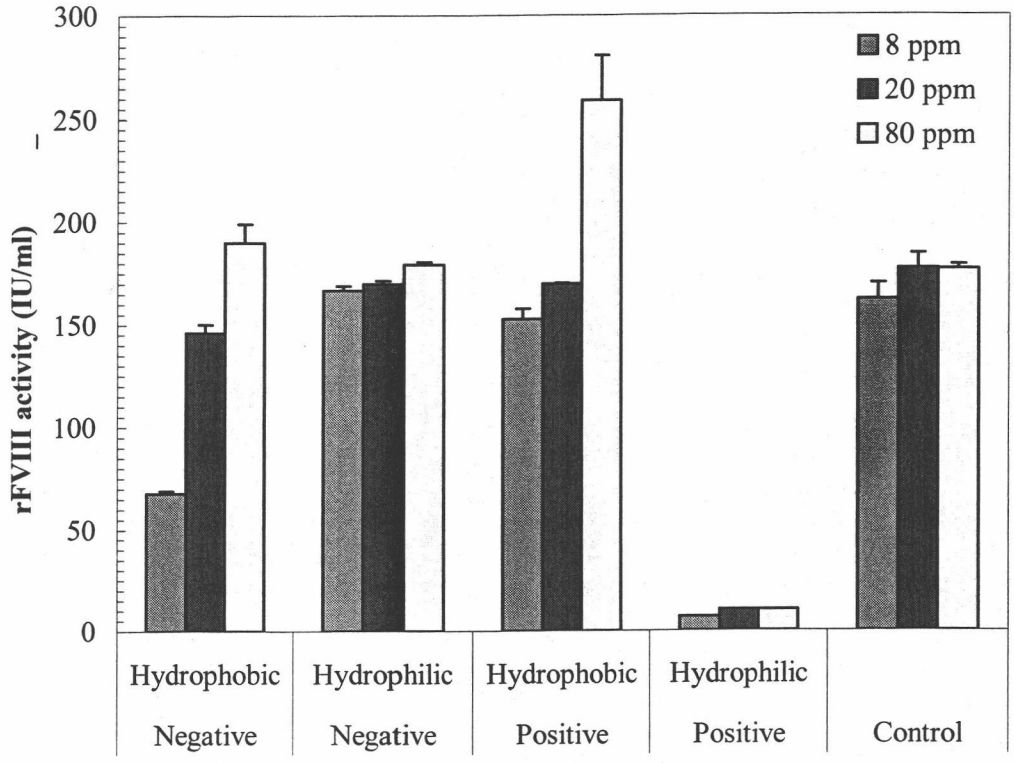

Figure 3.15 rFVIII biological activity in the presence of nanoparticles. Control samples contained no nanoparticles

A large decrease in activity was observed when negative, hydrophobic nanoparticles were added to the rFVIII sample at low Tween concentration. We can correlate this decrease in activity with a significant loss in rFVIII tertiary structure under similar conditions. It is well-known that proteins need to maintain structural integrity to remain biologically active. As the Tween concentration was increased, an increased recovery in biological activity was obtained with the negative, hydrophobic nanoparticles. The improved recovery with increasing Tween concentration was attributed to the better preserved structure and reduced adsorption of rFVIII. The positive, hydrophobic surface caused an activity loss at low Tween, and here too there was an improved recovery with

increasing Tween. The hydrophilic, negative surface caused the least loss in activity at the low Tween concentration. We have noted earlier that rFVIII adsorbed in large amounts at the hydrophilic, negative surface. This suggests that even though the protein molecules were adsorbed, they were still active and in all likelihood the active sites in the protein were not associated with adsorption. An unusually large decrease in activity was noted in the presence of hydrophilic, positive nanoparticles. We suspect that this decrease may be related to the alumina coating present on these particles. Aluminium ions, even when present at a low concentration, have been implicated in the aggregation and inactivation of rFVIII. It is likely that the aluminium ions which are present on the surface of the nanoparticles or likely leached out in solution, caused the large decrease in rFVIII activity.

3.3.3.4 Postulated molecular model for rFVIII interfacial phenomena

Recombinant FVIII adsorption appears to be driven by both electrostatic and hydrophobic forces. Negative surface charge appears to precipitate a large increase in rFVIII adsorption. The overall isoelectric point of rFVIII is reported to be 6.8. This means that the molecule is uncharged at a buffer pH of 6.8. McGuire et al (1995) have demonstrated the importance of charge location and mobility rather than overall charge in the protein adsorption process. We can argue that rFVIII has a domain of high positive charge which strongly interacts with the negatively charged surface. In fact, calculations performed by Trout (personal communication) indicate that the B-domain in rFVIII bears a markedly high positive charge (pI 9.5). The charge calculations are based on the rFVIII primary sequence and do not consider the effect of glycosylation. We hypothesize that rFVIII is likely to adsorb on a negatively charged surface in an orientation that aligns the B-domain towards the surface. Since a particular orientation is preferred due to the charge interactions, the adsorbed layer is likely to be highly ordered and packed. This is depicted

in Figure 3.16 a. The formation of a tightly packed adsorbed layer in turn explains the high adsorbed amounts of rFVIII on hydrophilic, negative surfaces. In spite of large adsorption on such surfaces, good structural protection and high activity recovery was obtained even when a low Tween concentration was used. The B-domain is not required for rFVIII biological activity (Kessler 2005). So the unavailability of the B-domain on account of binding to the surface need not necessarily impact the activity. This is entirely consistent with what we have observed. The tightly packed and highly ordered protein layer does not offer any free space for structural alteration. This may explain the structure protection we have observed on negative, hydrophilic surfaces.

On the other hand, adsorption on a hydrophobic surface is likely to be more random due to the applicability of hydrophobic and electrostatic forces in combination (Figure 3.16 b). Upon adsorption, active sites may no longer remain available to participate in the clotting cascade. This in turn may lead to the observed decrease in rFVIII activity in the presence of hydrophobic nanoparticles. The unordered nature of the adsorbed layer may allow room for unfolding to occur. We have observed this in the tertiary structure results. Tween binding becomes more relevant on the hydrophobic surface as evidence by the stronger Tween-surface interaction on the hydrophobic as compared to the hydrophilic surface. As the Tween concentration is increased, Tween molecules will compete with rFVIII for space at the interface and rapidly fill in the empty sites remaining after rFVIII adsorption. This limits the space available for the surface induced unfolding of rFVIII molecules. We have seen structure preservation when rFVIII was adsorbed on hydrophobic nanoparticles at high Tween concentrations. More rFVIII molecules are also likely to remain unadsorbed at high Tween concentration, which contributes to the improved recovery of biological activity.

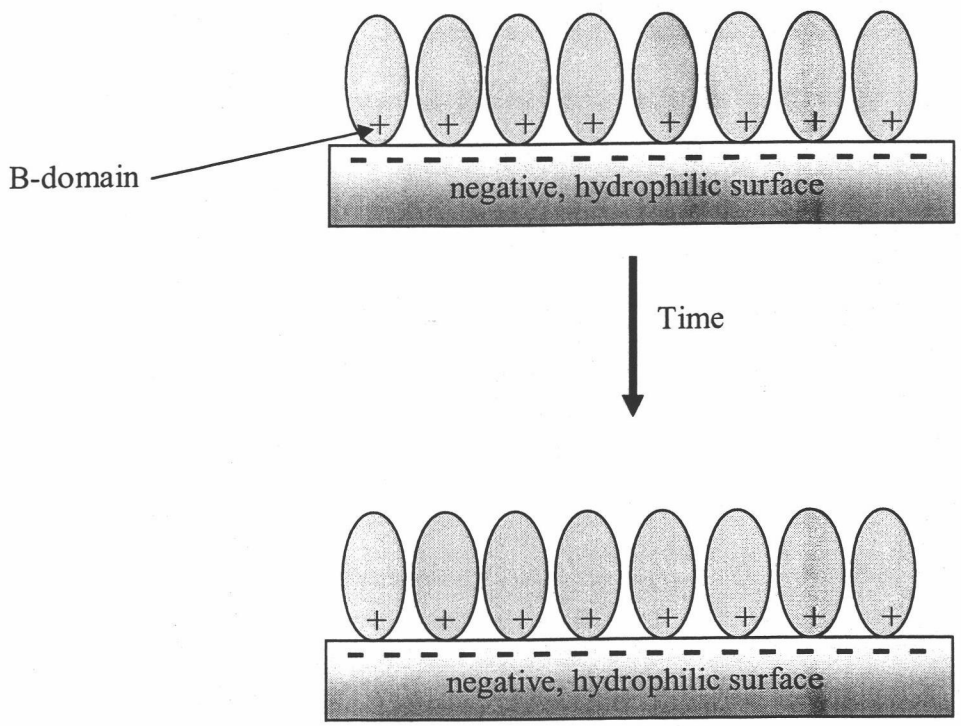

Figure 3.16a Postulated rFVIII adsorption model on negative, hydrophilic surface. The rFVIII molecules are represented by the ellipsoids. Formation of a tightly packed layer of adsorbed rFVIII molecules is depicted, with the positively charged B domain oriented towards the interface. Ellipsometry results suggest that Tween may not be relevant in determining rFVIII adsorption at hydrophilic interfaces, and is not depicted here.

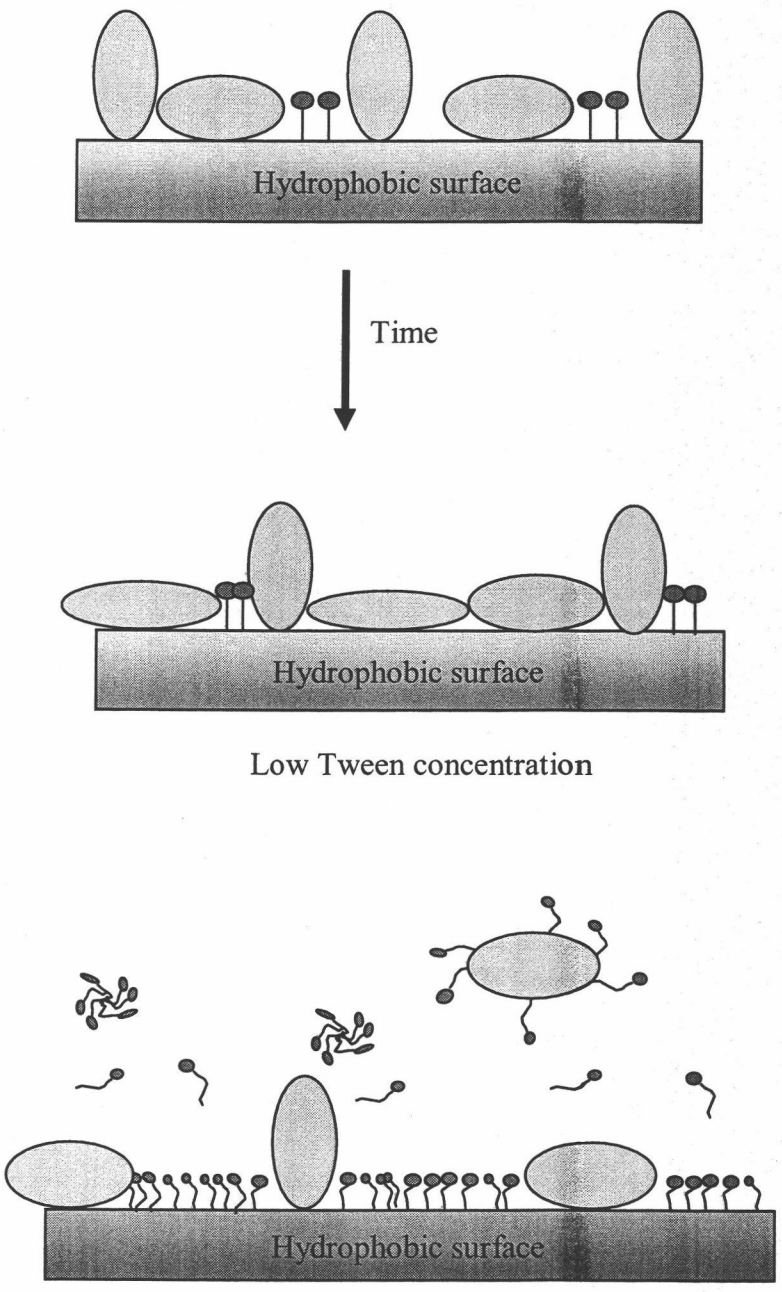

Figure 3.16b Posulated rFVIII adsorption model on hydrophobic surfaces at low and high Tween concentration. rFVIII molecules are represented by the ellipsoids and Tween by the spheres with tails. Structurally altered rFVIII molecules at the interface are depicted by the flattened ellipsoids. The surface-induced unfolding of rFVIII with time is relevant when adsorption occurs from a solution containing a low Tween concentration. Surface-induced unfolding may be prevented by the presence of Tween at high concentrations

3.4 Conclusions

Recombinant FVIII adsorption on hydrophobic and hydrophilic surfaces bearing positive or negative charge was analyzed in terms of adsorbed mass, structure and biological activity. Tween was observed to adsorb to hydrophobic surfaces and was nearly irreversible to buffer elution. Tween was relevant towards determining rFVIII interfacial phenomena at such surfaces. The co-adsorption of rFVIII with Tween at increasing concentrations decreased the amount of rFVIII adsorbed. A combination of a Tween pre-coat and the presence of Tween at a high concentration in solution was required in order to achieve the least rFVIII adsorption on a hydrophobic surface. The tertiary structure of rFVIII was altered in the presence of hydrophobic nanoparticles at low Tween concentrations. The biological activity also dropped under similar conditions. This was attributed to adsorption of rFVIII on hydrophobic surfaces in multiple orientations, in addition to surface-induced unfolding. The structure and activity were better preserved at high Tween concentrations. This was attributed to a reduction in adsorbed amounts and steric repulsion preventing rFVIII adsorption. Electrostatic attraction appeared to govern adsorption on negative, hydrophilic surfaces and rFVIII adsorbed in large amounts on such surfaces. Tween concentration or method of addition did not alter the rFVIII adsorption profile on the hydrophilic surface and this was attributed to the Tween-surface interaction being relatively weak. The hydrophilic surfaces did not induce a large structure or activity loss. This was attributed to the formation of a tightly packed, highly ordered adsorbed layer which did not involve rFVIII active sites in contact with the surface.

3.5 References

Archer, J. 1968. *Manual on Ellipsometry.* Gaertner Scientific Corporation.

Bardelle, C., B. Furie, B. C. Furie, and G. E. Gilbert. 1993. Membrane binding kinetics of Factor VIII indicate a complex binding process. *The Journal of Biological Chemistry* 268: 8815-8824.

Bloemendal, M., and W. C. J. Jr. 1995. Structural information on proteins from circular dichroism spectroscopy possibilities and limitations. *Pharm Biotechnol.* 7: 65-100.

Boedeker, B. G. D. 2001. Production processes of licensed recombinant factor VIII preparations. *Seminars in Thrombosis and Haemostasis* 27: 385-394.

Calonder, C., Y. Tie, and P. R. van Tassel. 2001. History dependence of protein adsorption. *Proceedings of the National Academy of Sciences* 98: 10664-10669.

Chu, L. 2005. Surfactant interactions with recombinant factor VIII at the air/water interface. Pages 79. *Chemical Engineering.* Oregon State University.

Derrick, T. S., R. S. Kashi, M. Durrani, A. Jhingan, and C. R. Middaugh. 2004. Effect of metal cations on the conformation and inactivation of recombinant human Factor VIII. *Journal of Pharmaceutical Sciences* 93: 2549-2557.

DiMichele, D. M., M. E. Lasak, and C. H. Miller. 1996. In vitro factor VIII recovery during the delivery of ultrapure factor VIII concentrate by continuous infusion. *American Journal of Hematology* 51: 99-103.

Elwing, H. 1998. Protein adsorption and ellipsometry in biomaterial research. *Biomaterials* 19: 397-406.

Fatouros, A., and B. Sjostrom. 2000. Recombinant factor VIII SQ - the influence of formulation parameters on structure and surface adsorption. *Internation Journal of Pharmaceutics* 194: 69-79.

Fay, P. J., and P. V. Jenkins. 2005. Mutating factor VIII: lessons from structure to function. *Blood Reviews* 19: 15-27.

Fowler, W. E., P. J. Fay, D. S. Arvan, and V. J. Marder. 1990. Electron microscopy of human factor V and factor VIII: Correlation of morphology with domain structure and localization of factor V activation fragments. *Proc. Natl. Acad. Sci. USA* 87: 7648-7652.

Gilbert, G. E., and A. A. Arena. 1998. Unsaturated phospholipid acyl chains are required to constitute membrane binding sites for Factor VIII. *Biochemistry* 37: 13526-13535.

Grillo, A. O., K.-L. T. Edwards, R. S. Kashi, K. M. Shipley, L. Hu, M. J. Besman, and C. R. Middaugh. 2001. Conformational origin of the aggregation of recombinant human Factor VIII. *Biochemistry* 40: 586-595.

Jiang, R., T. Monroe, R. McGrogers, and P. J. Larson. 2002. Manufacturing challenges in the commercial production of recombinant coagulation Factor VIII. *Haemophilia* 8: 1-5.

Johnson, W. C. 1999. Analyzing protein circular dichroism spectra for accurate secondary structures. *Proteins: Structure, Function and Genetics* 35: 307-312.

Joshi, O. 2005. Fibrinogen adsorption at heparinized silica interfaces: History dependence and protein concentration effects. Pages 98. *Chemical Engineering.* Oregon State University.

Kaufman, R. J. 1992. Biological regulation of Factor VIII activity. 43: 325-339.

Kessler, C. M., J. C. Gill, G. C. White, A. Shapiro, S. Arkin, D. A. Roth, X. Meng, and J. M. Lusher. 2005. B-domain deleted recombinant factor VIII preparations are

bioequivalent to a monoclonal antibody purified plama-derived factor VIII concentrate: a randomized, three-way crossover study. *Haemophilia* 11: 84-91.

Klintstrom, S. W. 1992. Ellipsometry and wettability gradient surfaces. Pages 115. *Department of Physics and Measurement Technology*. Linkoping University.

Koutsopoulos, S., J. van der Oost, and W. Norde. 2005. Structural features of a hyperthermostable endo-beta-1,3-glucanase in solution and adsorbed on "invisible" particles. *Biophysical Journal* 88: 467-474.

Krisdhasima, V., J. McGuire, and R. Sproull. 1992. A one-film-model ellispometry program for the simultaneous calculation of protein film thickness and refractive index. *Surface and Interface Analysis* 18: 453-456.

Lakowizc, J. 1999. Protein fluorescence. Pages 446-485. *Principles of fluorescence spectroscopy*. Kluwer Academic, New York.

McGuire, J., M. C. Wahlgren, and T. Arnebrant. 1995. Structural stability effects on the adsorption and dodecyltrimethylammonium bromide-mediated elutability of bacteriophage T4 lysozyme at silica surfaces. *Journal of Colloid and Interface Science* 170: 182-192.

Natale, P., T. den Blaauwen, C. van der Does, and A. J. M. Driessen. 2005. Conformational state of the SecYEG-bound Sec A probed by a single tryptophan fluorescence spectroscopy. *Biochemistry* 44: 6424-6432.

Osterberg, T., A. Fatouros, and M. Mikaelsson. 1997. Development of a freeze-dried albumin-free formulation of recombinant Factor VIII SQ. *Pharmaceutical Research* 14: 892-898.

Podhipleux, N. 1998. Adsorption of the wild type and a synthetic structural stability variant of bacteriophage T4 lysozyme. Pages 124. *Bioresource Engineering*. Oregon State University, Corvallis.

Podhipleux, N. 1998. Adsorption of the wild type and a synthetic structural stability variant of bacteriophage T4 lysozyme. Pages 124. *Bioresource Engineering*. Oregon State University, Corvallis.

Rosen, S., and M. C. Casoni. 2002. Determination of factor VIII activity in plasma and concentrates: comparison between methods. *American Clinical Laboratory* April: 32-37.

Stoilova-McPhie, S., B. O. Villoutreix, K. Mertens, G. Kemball-Cook, and A. Holzenburg. 2002. 3-Dimensional structure of membrane-bound coagulation factor VIII: modeling of the factor VIII heterodimer within a 3-dimensional density map derived by electron crystallography. *Blood* 99: 1215-1223.

Sudhakar, K., and P. J. Fay. 1996. Exposed hydrophobic sites in Factor VIII and isolated subunits. *The Journal of Biological Chemistry* 271: 23015-23021.

Vehar, G. A., B. Keyt, D. Eaton, H. Rodrigues, D. P. O'Brien, F. Rotblat, H. Oppermann, R. Keck, W. I. Wood, R. N. Harkins, E. G. D. Tuddenham, R. M. Lawn, and D. J. Capon. 1984. Structure of human Factor VIII. *Nature* 312: 337-342.

Wang, W., and D. N. Kelner. 2003. Correlation of rFVIII inactivation with aggregation in solution. *Pharmaceutical Research* 20: 693-700.

RECOMBINANT FACTOR VIII – TWEEN 80 INTERACTIONS AT THE AIR/WATER INTERFACE

Omkar Joshi[1], Liping Chu[1], D.Q. Wang[2] and Joseph McGuire[1,3]

1. Department of Chemical Engineering, Oregon State University, Corvallis, OR 97331

2. Process Sciences, Bayer HealthCare, 800 Dwight Way, Berkeley, CA 94701

3. Corresponding author. Address102 Gleeson Hall, Oregon State University, Corvallis OR 97331 Email: mcguirej@engr.orst.edu

CHAPTER 4

RECOMBINANT FACTOR VIII – TWEEN 80 INTERACTIONS AT THE AIR/WATER INTERFACE

Abstract

The kinetic surface tension data of rFVIII – Tween 80 mixtures were recorded over a range of Tween 80 (Tween) concentrations (8 – 108 ppm) employing interfacial tensiometry. There was no further reduction in steady state values of surface tension of rFVIII –Tween mixtures relative to those of Tween by itself once the Tween concentration was increased beyond 18 ppm. This suggests that 18 ppm may be the critical micelle concentration (CMC) of the binary protein-surfactant system. rFVIII – Tween mixtures were agitated over an extended duration to assure exposure of the system to a large air/water interfacial area. The formation of insoluble aggregates and tertiary structure change were monitored using turbidity measurements and intrinsic fluorescence spectroscopy, respectively. Aggregation was apparent in all rFVIII samples exposed to the air/water interface but the extent of aggregation appeared to be reduced as the Tween concentration was increased. Small changes in rFVIII tertiary structure were also detected at low Tween concentrations, but were not apparent when 80 ppm Tween was present. The biological activity of agitated rFVIII samples was evaluated, and a significant loss in activity was noted at every Tween concentration studied. Improved recovery of activity was obtained with increasing Tween concentration. We hypothesize that Tween at high concentrations reduces the exposure of rFVIII molecules to the hydrophobic air/water interface by preferential adsorption and by the formation of Tween-rFVIII complexes, resulting in a better preserved native rFVIII structure. This in turn leads to reduced aggregation and enhanced activity recovery.

4.1 Introduction

Factor VIII is a high-molecular-weight (280 kDa) multidomain protein that is an essential blood coagulation factor. In the blood coagulation cascade, FVIII serves as a cofactor for factor IXa in the activation of factor X to factor Xa (Curtis 1994). Factor VIII has a domain structure of A1-A2-B-A3-C1-C2, in which the heavy chain is composed of A1, A2 and B domains and the light chain is composed of the A3, C1 and C2 domains (Derrick 2004). FVIII exists as metal-ion connected heterodimer of the heavy and light chain and is associated with 50-fold excess von Willebrand Factor (vWF) when present in plasma (Kaufman 1992). A functional deficiency in FVIII causes hemophilia A, a congenital bleeding disorder. Historically, Factor VIII was derived from human plasma. The risk of pathogen transmission and the limited availability of plasma have led to the development of a recombinant Factor VIII (rFVIII, Jiang 2002). Factor VIII is the largest molecule ever successfully cloned by genetic engineering techniques and is the largest and most complex protein currently manufactured (Boedeker 2001). The rFVIII molecule is sensitive to both chemical and physical degradation. The degradation involves changes in higher order structure and may be caused by a number of pathways including aggregation, precipitation or adsorption onto surfaces (Wang 2003). Surface adsorption of FVIII is rapid (DiMichele 1996). Approximately 50% of rFVIII product may be lost due to adsorption just during sterile filtering (Osterberg 1997). Recombinant FVIII is inherently unstable, especially in liquid formulations. Due to the instability of the protein, all rFVIII products currently on the market are in the form of lyophilized powders. The lyophilized products necessitate a reconstitution step, typically performed by the patient. The reconstitution step requires some training on the part of the end user and increases the complexity of infusion. A stable, liquid rFVIII formulation, if developed, will present a

unique advantage in this regard. As discussed previously, interface-induced rFVIII denaturation is a major pathway of rFVIII instability and activity loss. This work provides further insight into rFVIII degradation due to exposure to the air/water interface. This is an active field of research as shown by numerous publications, some of which are summarized here. Fatouros et al (2000) investigated the influence of temperature, pH, ionic interactions and nonionic surfactants on the structural stability and surface adsorption of B-domain deleted rFVIII (BDDrFVIII) using biological activity, circular dichroism and surface tension measurements employing the pendant drop method. The BDDrFVIII samples were agitated by means of a vibrating plate set at 600 rpm for 6 days. Nonionic surfactants Tween 80 and Tween 20 protected BDDrFVIII against activity loss to an equally high degree. Activity recovery was much higher in the presence of these surfactants as compared to control, surfactant-free samples. Surface tension data indicated that the protein adopted more flexible conformation as the temperature was increased. However, the surface tension of BDDrFVIII samples with added nonionic surfactants was not recorded. Near UV CD spectra revealed only minor interactions of the protein with the nonionic surfactants (Tween 20 and Genapol 80) added at a concentration of 200 ppm. Wang and Kelner (2003) studied the stability of rFVIII in solution at different pH in order to identify the mechanisms responsible for rFVIII inactivation under accelerated storage conditions. Size exclusion chromatography – high performance liquid chromatography (SEC-HPLC) analysis revealed that as the content of the native rFVIII peak area dropped with time, there was an increase in the high-molecular-weight (HMW) peak area but no major change in the low-molecular-weight (LMW) peak area. The authors interpreted this aggregation as being the major rFVIII degradation mechanism in the pH range studied (5.2 – 7.0). A linear correspondence was observed between native rFVIII peak area and activity recovery, which suggested that rFVIII aggregation was mainly responsible for

rFVIII inactivation in solution at 40 ° C. SDS-PAGE results showed that the rFVIII aggregation may involve three different mechanisms: disulfide bond formation/exchange, non-reducible crosslinking, and physical interactions. Grillo et al (2001) studied rFVIII aggregate formation employing a number of biophysical techniques including dynamic light scattering, fluorescence and circular dichroism (CD) spectroscopy, Fourier transform infrared (FTIR) spectroscopy and differential scanning calorimetry (DSC). Dynamic light scattering studies revealed that the mean size of rFVIII increased from approximately 12 nm to between 25 and 35 nm after a 7-day incubation at 37 ° C. Only 85% of the original biological activity remained after the 7-day incubation. No significant differences in the CD spectra and the wavelength at which maximum intrinsic fluorescence were observed when the fractionated, native rFVIII sample was compared with the aggregated species. Temperature-induced transitions observed using DSC, intrinsic fluorescence and extrinsic dye (bis-ANS)-binding fluorescence indicated a conformational change in rFVIII that exposed apolar residues to a greater extent. Although there was no alteration in the protein secondary structure, the tertiary structure changed to a limited extent. Grillo et al concluded that fairly minor structural alterations in the rFVIII molecule can result in marked aggregation behavior. Ramani et al (2005) continued the work of Grillo et al and investigated the domains involved in the initiation of aggregation employing CD, SEC, fluorescence and domain specific antibody binding. The ESH 4 antibody which binds to the lipid-binding region, comprised of residues 2303-2332 in the C2 domain of rFVIII, or the 8860 antibody which binds to an epitope in the A2 domain were used as the capture antibodies. In both cases, the ESH 8 antibody which specifically binds to a region in the C2 domain was used as the probe antibody. The antibody binding patterns suggested that the conformation of the C2 domain was altered. It is interesting to note that regions in the C2 domain were also implicated (Gilbert 2002) in phospholipid binding and binding to

von Willebrand Factor (vWF). In particular, Gilbert et al speculated that two hydrophobic spikes (residues 2199-2200 and 2251-2252) may be influential in the adsorption and intermolecular association behavior of native rFVIII. The authors hypothesized that since in plasma the vWF is able to bind to these inherently sticky spikes, the nonspecific interactions are prevented in the body. Surfactants are routinely used as excipients in therapeutic protein formulations. Arakawa and Kito (2000) investigated the mechanism of protein stabilization by surfactants by studying the effect of Tween 80 on the aggregation of bovine serum albumin (BSA). The BSA aggregation was induced via thermal stresses, by subjecting BSA to elevated temperatures such as 50, 60, 70 and 80 °C. Addition of Tween before heating reduced aggregation, and this effect was enhanced with increasing Tween concentrations. The addition of Tween after heating had no effect on aggregate formation. The authors concluded that the observed BSA stabilization upon Tween addition may be preferentially attributed to Tween's role in preventing aggregation over the possibility of Tween significantly stabilizing the native conformation of BSA. Bam et al (1995) explored a novel electron paramagnetic resonance (EPR) technique to evaluate the protein – Tween binding stoichiometry. A specific interaction between the Tween family of surfactants and recombinant human growth hormone (rhGH) was detected with a Tween-protein binding stoichiometry between 2.5-4.1 (Tween-to-protein ratio). Bam et al (1998) further investigated Tween stabilization of rhGH using aggregate formation after agitation, differential scanning calorimetry and injection titration microcalorimetry as indicators. A protective effect was apparent in agitation studies; no effect or even a slight destabilizing effect was noted in DSC and injection titration microcalorimetry experiments. The authors claimed that the protective effect attributed to Tween did not correlate with the Tween CMC but rather the amount of Tween required to bind to the hydrophobic patches on the protein, i.e. the molar Tween:rhGH ratio. They further

hypothesized that the likely mechanism of Tween protection against aggregation was due to Tween sterically blocking the aggregation-prone hydrophobic patches on the protein molecule. On the other hand, Kreilgaard et al (1998) used EPR and CD spectroscopy and found no evidence that Tween 20 binds to either the native state or the folding intermediate of recombinant Factor XIII (rFXIII). But they did find that Tween 20 was useful in reducing the agitation-induced aggregation of rFXIII and that maximum protection occurred at concentrations near the surfactant CMC, irrespective of protein concentration. In this case, competition for the interface between the surfactant and protein was cited as the mechanism responsible for the stabilization of rFXIII. Thus, depending on the specific interaction between the surfactant and the protein, and the surfactant/protein and the interface, either the surfactant-protein complex formation or the interface competition mechanisms may become important.

In this paper, we investigate the effect of Tween and Tween concentration on rFVIII structure, biological activity, and aggregation using a combination of interfacial tension, fluorescence, turbidity and activity measurements.

4.2 Materials and Methods

4.2.1 Protein, surfactant and buffers

The recombinant Factor VIII (rFVIII) used in this work was a gift from Bayer HealthCare (Berkeley, CA). The FVIII was formulated in the KG-2 buffer consisting of 30 mM NaCl, 2.5 mM $CaCl_2$, 22 g/l glycine, 3.1 g/L L-histidine and 10 g/L sucrose at pH 6.8. The rFVIII solutions used in surface tensiometry contained 24.5 μg/ml protein. The rFVIII solutions used in fluorescence spectroscopy and biological activity measurements contained 30 μg/ml protein. The rFVIII solution was kept frozen at -80 °C

and thawed just prior to use. Small volumes of concentrated Tween stock solution were added to the rFVIII samples to obtain samples at various Tween concentrations. Tween 80 was obtained from J.T. Baker, buffer salts were obtained from Sigma and the histidine, glycine and sucrose were provided by Bayer.

4.2.2 Evaluation of rFVIII – Tween surface tension kinetics

The interfacial tension kinetic and steady state behavior exhibited by rFVIII formulations at the air-solution interface were recorded as a function of added Tween concentration using a computer-controlled automatic tensiometer (Model FTÅ T10, First Ten Angstroms, Portsmouth, VA). This instrument measures interfacial tension by the corrected DuNouy ring method, as well as the Wilhelmy plate method. We employed the Wilhelmy plate method in the work described here. In a given experiment, fresh rFVIII solutions were prepared from frozen stock (125 μg/ml rFVIII), Tween 80 was added to yield a desired solution concentration, and interfacial tension kinetic data were recorded over a period of about one hour. All solutions included rFVIII at 24.5 μg/ml, and the concentration of Tween was varied from 8 to 108 ppm. After one hour, the surface tension decrease had slowed appreciably in any given experiment, and we were able to record a good estimate of the steady state value of interfacial tension.

In separate experiments, the interfacial tension kinetic and steady state behavior exhibited by Tween solutions in the absence of rFVIII was recorded as a function of Tween concentration. In a given experiment, Tween 80 was dissolved in water to yield a desired solution concentration (ranging from 5 to 79 ppm), and interfacial tension kinetic data were recorded until a steady state was reached, generally requiring several hours.

4.2.3 Evaluation of insoluble aggregate formation

rFVIII – Tween mixtures were agitated in order to ensure exposure of the sample to a large air/water interfacial area. Recombinant FVIII samples containing 8, 20 and 80 ppm Tween were secured horizontally on a shaker set at 220 ± 20 rpm and agitated over 72 hours at room temperature. Turbidity measurements were employed to monitor insoluble aggregate formation in the rFVIII samples. Turbidity measurements are routinely used to evaluate protein aggregation (Katakam 1997, Maclean 2002). The sample absorbance at 350 nm after agitation for 72 hrs was measured using a Beckman DU-62 spectrophotometer and corrected for protein-free blank sample treated in an analogous manner.

4.2.4 Evaluation of rFVIII tertiary structure upon exposure to the air/solution interface

The exposure of protein molecules to the air/water interface often leads to changes in the higher order structure of the protein. We used intrinsic fluorescence spectroscopy to investigate structural alterations in FVIII, after exposure of rFVIII to the air/water interface induced by extended agitation. Tryptophan is a well documented intrinsic fluorophore (Lakowicz 1999). Recombinant FVIII has 37 tryptophan residues (Vehar 1984). There are several well documented results that relate tryptophan fluorescence with the protein tertiary structure. The fluorescence of tryptophan depends on the local environment of the amino acid residue within the protein molecule. Analysis of fluorescence emission spectra often involves the calculation of the wavelength at which the fluorescence intensity is at a maximum (λ_{max}). If the λ_{max} of tryptophan spectra within the protein dissolved in an aqueous medium is shifted to shorter wavelength in relation to the λ_{max} of free tryptophan in water, the tryptophan must be internal and in a nonpolar environment (Friefelder 1976). Recombinant FVIII samples containing 8, 20 and 80 ppm

Tween were agitated over a period of three days and the fluorescence emission of the samples was tested every 24 hours. This semi-kinetic analysis was designed to enable us to capture the onset of a structure change. Control samples which were not agitated but simply kept at room temperature over the same duration were also analyzed. Emission spectra were obtained using a PTI QuantaMaster fluorometer (Photon Technology International, NJ). The excitation wavelength was set at 295 nm to selectively excite the tryptophan residues within the rFVIII molecule. Emission spectra were recorded at 1 nm increments from 305 to 405 nm at 1 nm/s scan rate. The excitation and emission slit widths were set at 0.25 and 2.0 mm, respectively. Three scans were recorded and averaged in each case in order to increase the signal-to-noise ratio. The data were corrected for protein-free background. Each experiment was performed in triplicate. In order to estimate the wavelength at which maximum fluorescence emission (λ_{max}) occurred, the background-corrected data were differentiated using the FeliX32 software supplied by the PTI. The wavelength at which the first order derivative curve intersected the x-axis was noted and reported as λ_{max}.

4.2.5 Evaluation of the biological activity of rFVIII upon exposure to the air/water interface

The agitated rFVIII samples used in structure analysis were collected in Eppendorf vials and frozen at -80 °C. The frozen samples were thawed later and tested for biological activity using the one-stage clotting assay based on activated partial thromboplastin time (aPTT) (Rosen 2002). Factor VIII acts as a cofactor in the presence of Factor IXa, calcium, and phospholipid in the enzymatic conversion of Factor X to Xa. In this assay, the test samples were incubated at 37°C with a mixture of FVIII deficient plasma substrate and aPTT reagent. Calcium chloride was then added to the incubated

mixture and clotting was initiated. An inverse relationship exists between the time (seconds) it takes for a clot to form and the logarithm of the FVIII activity. Activity levels for unknown samples were interpolated by comparing the clotting times of various dilutions of test material with a curve constructed from a series of dilutions of standard material of known activity and were reported in International Units per mL (IU/mL).

4.3 Results and Discussion

4.3.1 rFVIII-Tween surface tension kinetics

The molecular dynamics contributing to changes in interfacial tension for protein-surfactant mixtures are complex. In ideal circumstances (random chain protein molecules and small, ionic surfactants at equilibrium) the following behavior is expected with increasing surfactant concentration (Dickinson 1989)

I. At very low surfactant concentrations, the interfacial tension is the same as it would be for pure protein.

II. With increasing surfactant concentration the interfacial tension decreases, due to surfactant occupation of "empty sites" at the air-water interface, as well as to formation of surface active, surfactant-protein complexes.

III. At higher surfactant concentrations, interfacial tension is expected to "level off", presumably because it is energetically favorable for surfactant to bind to protein at these concentrations (in this range, the critical micelle concentration (CMC) recorded for the pure surfactant may be exceeded).

IV. Interfacial tension then decreases again with increasing surfactant concentration, a result of complete displacement of protein from the interface by surfactant.

V. Further increases in surfactant concentration have no effect on interfacial tension, and the CMC has been met.

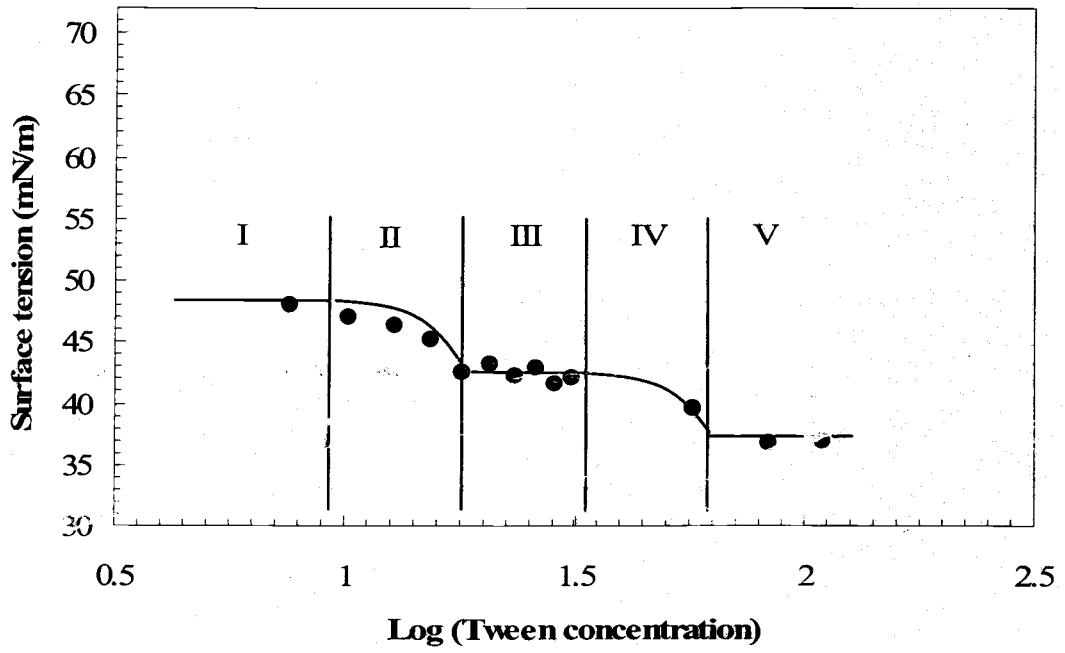

Figure 4.1 Steady state surface tension of rFVIII-Tween mixtures as a function of Tween concentration. The regions 1-V are indicated based on the empirical trends expected for protein-surfactant mixtures as described by Dickinson (1989).

But all of the above relates to an idealized protein-surfactant mixture at equilibrium. While it can constitute a useful reference for interpreting observations with systems of greater complexity, for reasons both theoretical and practical true interfacial equilibrium measurements are not possible for real protein-surfactant mixtures, including rFVIII-Tween. This is due mainly to protein adsorption being an inherently irreversible process, and to measurement uncertainties with origins in the fact that adsorption occurs at the surface of the Wilhelmy plate or DuNouy ring itself. We can at best identify a

"pseudo-equilibrium" state at a given Tween concentration, and this was referred to at the outset as a "steady state" condition.

The relationship between steady state interfacial tension and (added) surfactant concentration for rFVIII-Tween mixtures is shown in Figure 4.1. The data plotted in Figure 4.1 suggest the existence of an interfacial tension vs. surfactant concentration pattern consistent with that described for ideal circumstances (i.e., the five regions identified above). In particular, the CMC of Tween 80 in this rFVIII solution would be determined as the concentration defining the transition between regions IV and V (about 55-60 ppm based on Fig. 4.1). More pertinently, the fact that the data conforms to the empirical 5-region plot suggests the existence of rFVIII – Tween complexes in solution. The precise nature of such complexes, in terms of binding stoichiometry or similar, is not clear at this point.

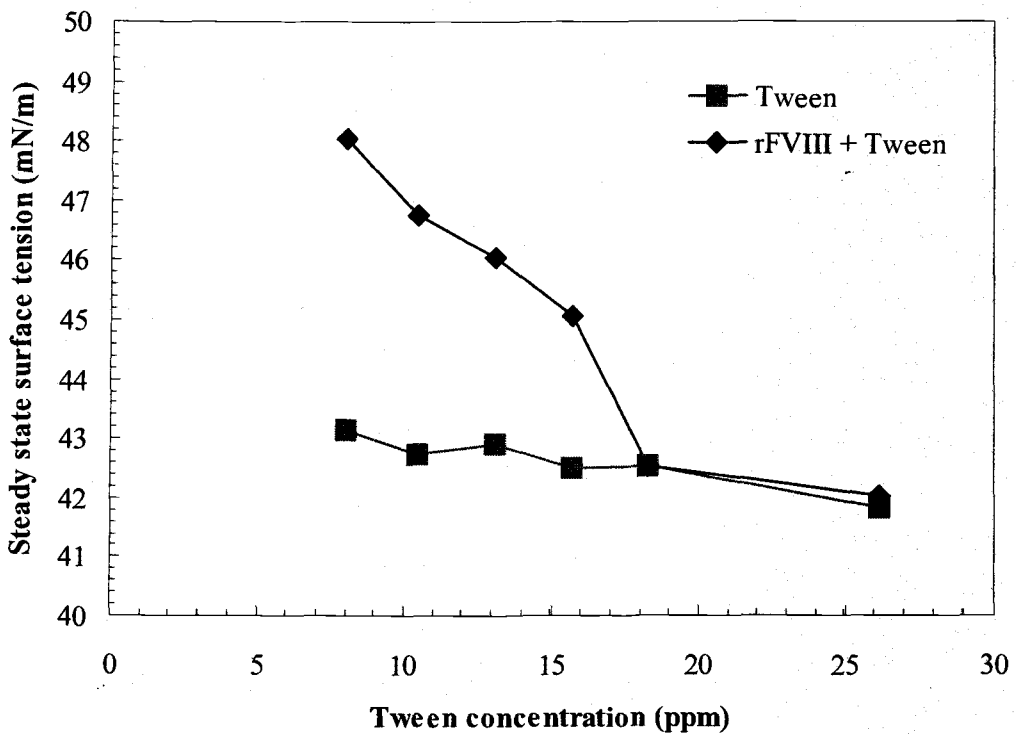

Figure 4.2 Steady state surface tension of rFVIII – Tween mixtures as compared with solutions containing Tween as the only surface active species.

But the premise of surface chemical approaches to identification of the CMC in protein-surfactant mixtures is the determination of the surfactant concentration at which steady state interfacial behavior is governed entirely by surfactant. Comparison of the steady state surface tension data recorded for rFVIII-Tween mixtures with similar data recorded for Tween in the absence of rFVIII, however, suggest that steady state interfacial behavior is governed entirely by surfactant at Tween concentrations well below that defining the transition between regions IV and V. In particular, we recorded no appreciable difference in the steady state value of interfacial tension demonstrated by rFVIII-Tween mixtures and by Tween alone, once the Tween concentration reached about 18 ppm. Representative data illustrating this finding are shown in Figure 4.2. The CMC

of Tween 80 in these rFVIII formulations cannot be unambiguously determined by interfacial tensiometry. But we suggest that above a surfactant concentration of about 18 ppm, the steady state interfacial tension appears to be dominated by surfactant. Changes in the pattern of interfacial tension kinetics observed with increasing Tween concentration beyond this value are consistent with the fact that Tween itself exhibits faster adsorption kinetics with concentration in the range of concentrations studied (Figure 4.3). The time required for the surface tension values to reach a plateau was reduced from about 5 hours for 5 ppm Tween to about 2 hrs for 79 ppm Tween.

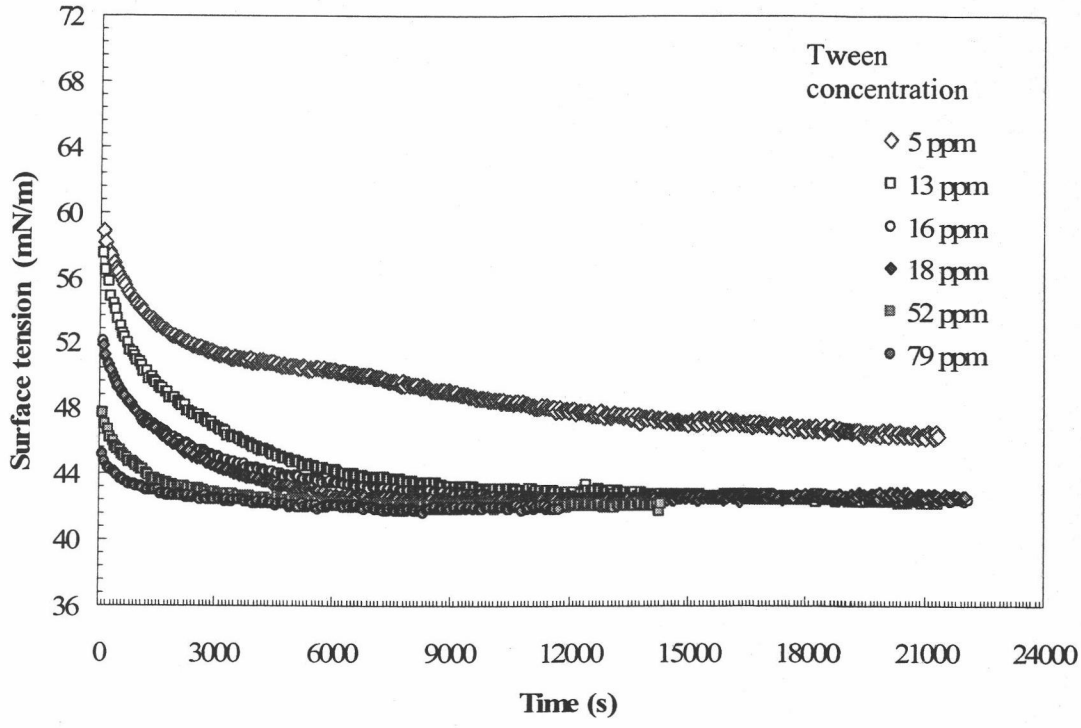

Figure 4.3 Surface tension kinetics exhibited by Tween as a function of Tween concentration

4.3.2 Insoluble aggregate formation

The formation of insoluble aggregates in agitated rFVIII samples containing 20, 50, 80 and 200 ppm Tween was monitored by measuring the absorbance of the samples at 350 nm. Results are plotted in Figure 4.4. The amount of insoluble aggregates formed as result of exposure to the air/water interface decreased as the Tween concentration in the rFVIII sample was increased. Tween is likely to form complexes with rFVIII molecules as the Tween concentration is increased. The presence of Tween surrounding the rFVIII molecule in the form of such complexes may prevent rFVIII molecules from associating with one another, which will reduce the extent of aggregation in the system. No further change in turbidity was observed as the Tween concentration was increased from 80 to 200 ppm. This correlated well with the surface tension data. The rFVIII-Tween surface tension data suggested that Tween may dominate the interface when present in high concentrations. If Tween molecules occupy the interface, rFVIII molecules will be protected from exposure and the resulting damage. This, in turn, may be influential in the observed reduction in insoluble aggregate formation.

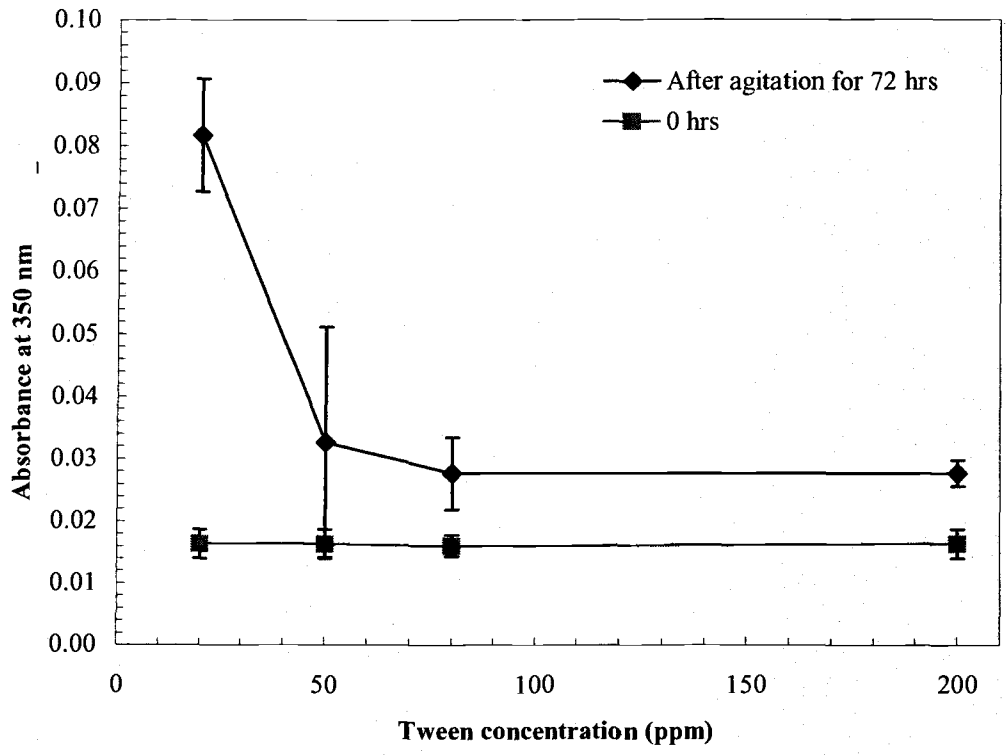

Figure 4.4 Turbidity (Absorbance at 350 nm) data of rFVIII samples exposed to the agitation-induced air/water interface

4.3.2 rFVIII tertiary structure upon exposure to the air/water interface

The wavelengths at maximum fluorescence emission (λ_{max}) of rFVIII samples containing 8, 20 and 80 ppm Tween and agitated for 3 days are presented in Figure 4.5a. The λ_{max} values of control rFVIII samples which were not agitated but simply maintained at room temperature over a period of 3 days are presented in Figure 4.5b.

A small increase in the λ_{max} of agitated samples containing 8 and 20 ppm Tween was observed. The λ_{max} of agitated samples containing 80 ppm Tween did appear to increase but the rate of increase was much less than that seen at low Tween concentrations. In fact, there was no significant difference in the λ_{max} values of the

agitated, 80 ppm Tween-containing rFVIII samples at the 0-day and 3-day time points. Significance was established by performing an ANOVA analysis with the p-value set below 0.05 taken to be significant. These findings suggest that Tween when present at high concentration (80 ppm) protected rFVIII from structural damage induced by exposure to the air/water interface. This may be attributed to Tween being able to occupy a large proportion of the air/water interfacial area generated during agitation which precluded rFVIII from being exposed thereby preventing rFVIII structural change. We have made a similar observation in the case of rFVIII adsorption at the hydrophobic solid/water interface in the presence of 80 ppm Tween (Chapter 3). In that situation, rFVIII adsorption was noted to be reduced with the addition of Tween and the structure of adsorbed rFVIII was also better preserved in the presence of high Tween concentrations. Recombinant FVIII molecules with a perturbed tertiary structure may act as a nucleating seed for intermolecular aggregation events to occur. This is supported by the increased turbidity observed in rFVIII samples containing low Tween. Although it is difficult to say with any certainty whether structural change led to aggregation or vice versa, our results indicate that interface-induced aggregation and structural change are complementary phenomena.

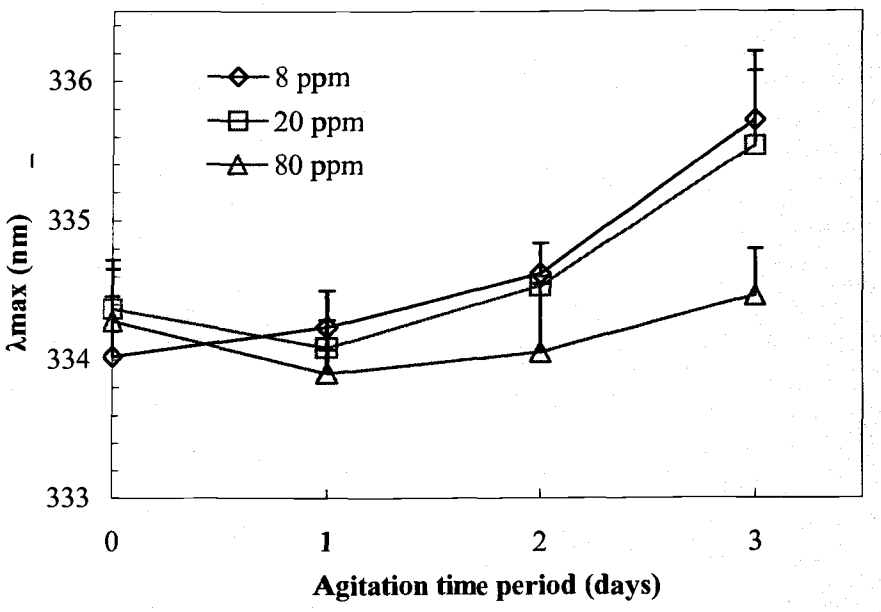

Figure 4.5a Wavelength at maximum fluorescence emission of rFVIII samples exposed to the air/water interface

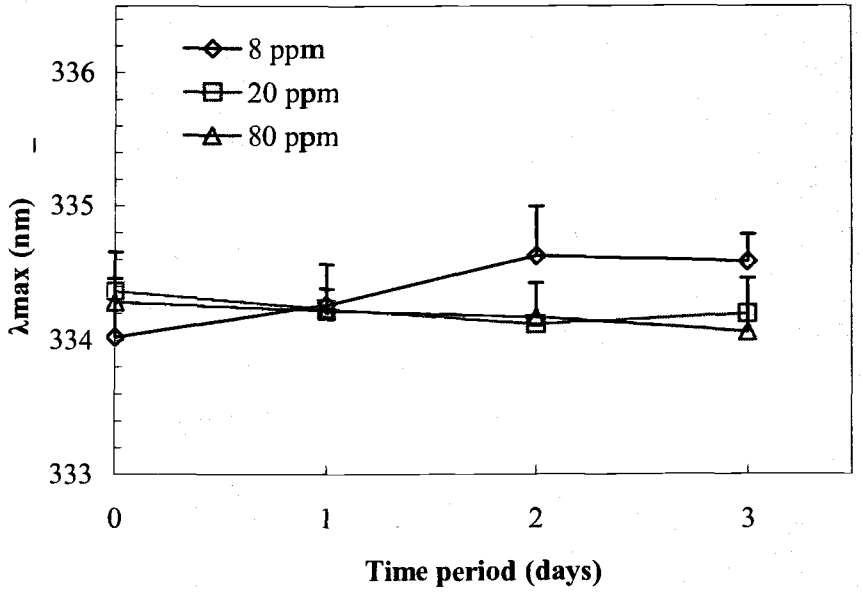

Figure 4.5b Wavelength at maximum fluorescence emission of control rFVIII samples kept at room temperature over the duration of agitation.

The λ_{max} values of control, unagitated samples revealed no significant difference between 0-day and 3-day time points at any Tween concentration (Figure 4.5 b). This suggests that rFVIII did not undergo any major structural change simply because of prolonged storage at room temperature and that the changes in λ_{max} of the agitated samples can be attributed to exposure to the air/water interface. It is important to note that although the discussion throughout this paper focuses on the air/water interface generated by agitation, agitation may also induce other stresses, such as shear. The contribution of shear to any of the observed phenomena cannot be isolated from the interfacial phenomena and is not dealt with further here.

The fluorescence intensity of agitated and unagitated samples was monitored at a wavelength of 334 nm (Figures 4.6 a and b). The fluorescence intensity was normalized with respect to the zero-day value at each Tween concentration. A decrease in fluorescence intensity was observed at all Tween concentrations in both agitated and unagitated samples. Since the rFVIII concentration remained the same, the loss of intensity was attributed to aggregation, and the shielding of the fluorescence emission originating from aggregated molecules. The fluorescence intensity of the unagitated rFVIII sample containing 8 ppm Tween decreased to about 75% after 3 days. A trend was apparent even in the unagitated samples: samples containing higher Tween concentrations displaying 3-day fluorescence intensity values closer to the 0-day values. The rate of decrease in fluorescence intensity was much higher in the agitated samples compared with the unagitated samples. The intensity of the 8 ppm Tween-containing agitated sample dropped to about 46% of the original in 3 days. The rate of intensity loss was much less steep in agitated 80 ppm Tween-containing samples. High Tween

concentrations appeared to prevent rFVIII aggregation in this context as well. This was supportive of the turbidity results described in a previous section.

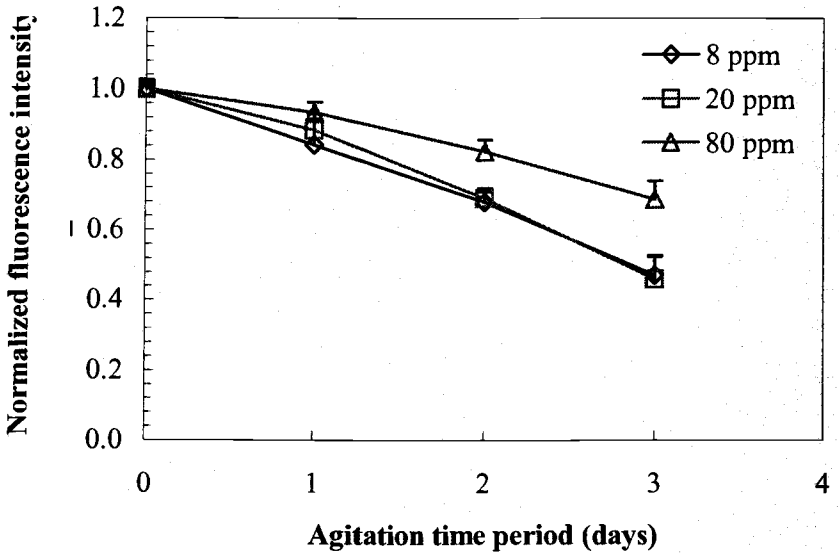

Figure 4.6a Normalized fluorescence intensity at 334 nm for agitated rFVIII samples.

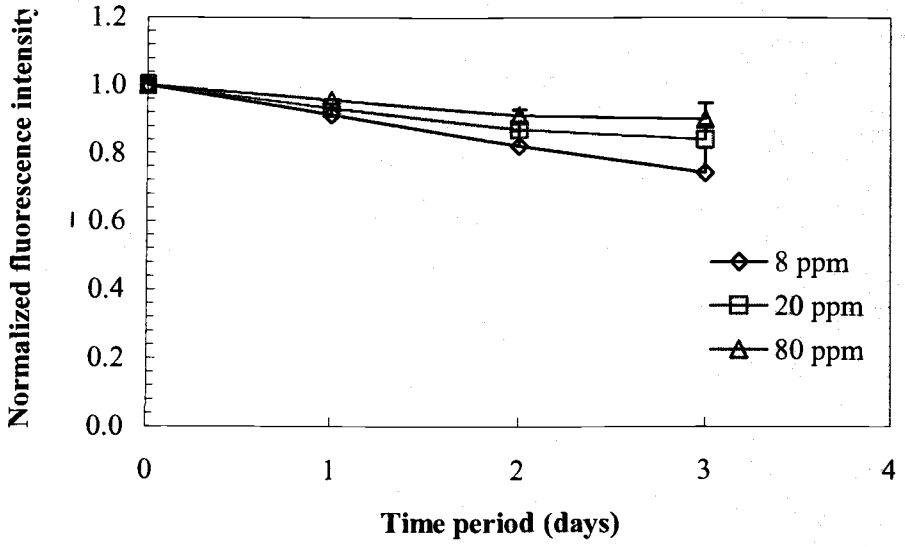

Figure 4.6b Normalized fluorescence intensity at 334 nm for unagitated rFVIII samples

4.3.3 rFVIII biological activity upon exposure to the air/water interface

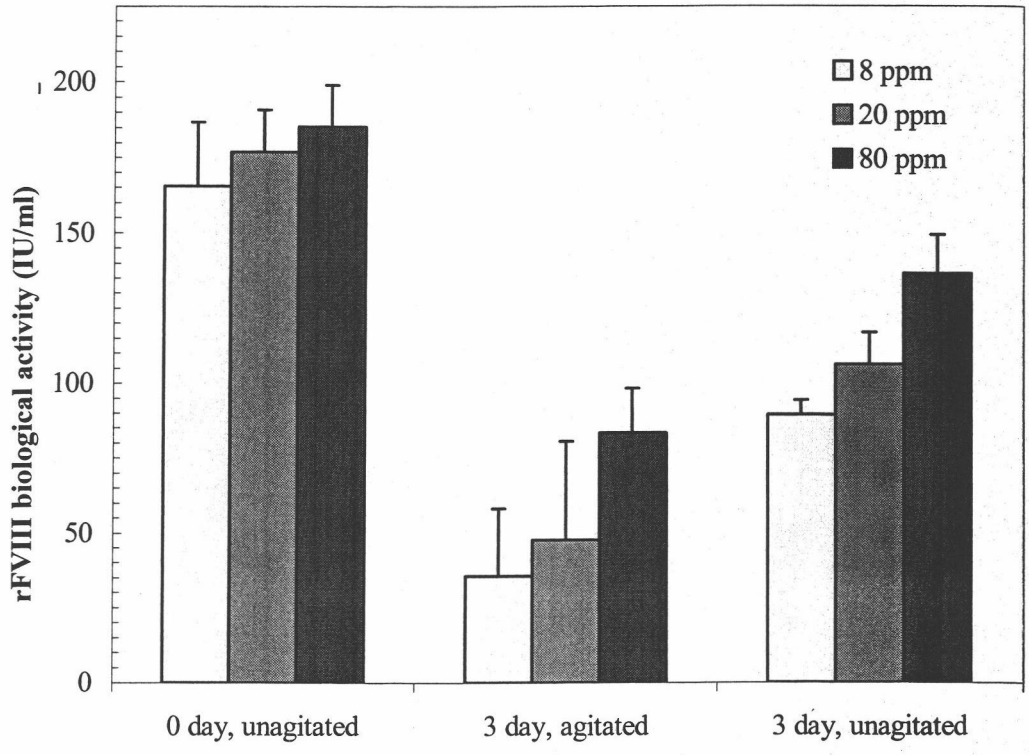

Figure 4.7 Biological activity of 8,20 and 80 ppm Tween-containing rFVIII samples upon exposure to the air/water interface or storage at room temperature for 3 days

The biological activity of rFVIII samples containing 8, 20 and 80 ppm Tween upon agitation for 3 days or upon storage at room temperature over the same duration are presented in Figure 4.7. rFVIII activity was significantly reduced when samples were exposed to the air/water interface. An enhanced recovery in rFVIII activity was obtained as the Tween concentration was increased from 8 to 80 ppm. This was consistent with the improved protection of rFVIII tertiary structure at high Tween concentrations. The biological activity of proteins is often correlated with their native

structure and disruptions in structure may lead to reduction or complete loss of activity. The loss of activity of rFVIII samples containing 8 and 20 ppm Tween upon agitation may be partly attributed to the observed structure change. The activity of unagitated rFVIII samples stored at room temperature over 3 days also were reduced in comparison to the 0-day samples. Since the structure of the unagitated samples was more or less similar to the 0-day samples, it is likely that some other deactivation mechanism may be influential towards the activity loss. Aggregation has often been cited as a physical pathway that leads to a loss in rFVIII activity (Grillo 2001, Ramani 2005). We have observed a reduction in rFVIII aggregation induced by exposure to the air/water interface as the Tween concentration was increased. It is likely that aggregation may also be a factor even when rFVIII is exposed to ambient conditions over an extended period. When considering unagitated rFVIII samples, Tween may become relevant through the formation of Tween-rFVIII complexes, which in turn may help prevent aggregation of rFVIII molecules in solution. Thus the improvement in rFVIII activity recovery in samples containing high Tween concentrations may also be attributed to a reduction in rFVIII aggregate formation.

4.4 Conclusions

The exposure of rFVIII to the air/water interface appeared to induce aggregation. The aggregation may be due to a small change in rFVIII tertiary structure on account of the damage caused by the hydrophobic air/water interface. The extent of aggregation and structure change depended on the amount of Tween 80 that was present in solution. Higher Tween concentrations were beneficial in reducing structure change and aggregation. This was attributed to Tween being able to dominate the interfacial processes, and also form complexes with rFVIII molecules, when present at higher concentrations. Steady state surface tension data supported both Tween interfacial

domination and complex formation at high Tween concentrations. The biological activity of agitated rFVIII samples decreased as compared to the starting value. This decrease in biological activity was likely due to structurally perturbed and/or aggregated rFVIII molecules being less active than the native rFVIII molecules. An improved recovery in biological activity was evident as the Tween concentration was increased, which is in line with the trends encountered with aggregation and structural alteration.

4.5 References

Arakawa, T., and Y. Kita. 2000. Protection of bovine serum albumin from aggregation by Tween 80. *Journal of Pharmaceutical Sciences* 89: 646-651.

Bam, N. B., J. L. Cleland, J. Yang, M. C. Manning, J. F. Carpenter, R. F. Kelley, and T. W. Randolph. 1998. Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions. *Journal of Pharmaceutical Sciences* 87: 1554-1559.

Bam, N. B., T. W. Randolph, and J. L. Cleland. 1995. Stability of protein formulations: Investigation of surfactant effects by a novel EPR spectroscopic technique. *Pharmaceutical Research* 12: 2-11.

Boedeker, B. G. D. 2001. Production processes of licensed recombinant Factor VIII preparations. *Seminars in thrombosis and hemostasis* 27: 385-394.

Curtis, J. E., S. L. Helgerson, E. T. Parker, and P. Lollar. 1994. Isolation and characterization of thrombin-activated human factor VIII. *The Nournal of Biological Chemistry* 269: 6246-51.

Derrick, T. S., R. S. Kashi, M. Durrani, A. Jhingan, and C. R. Middaugh. 2004. Effect of metal cations on the conformation and inactivation of recombinant human Factor VIII. *Journal of Pharmaceutical Sciences* 93: 2549-2557.

Dickinson, E., and C. M. Woskett. 1989. Competitive adsorption between proteins and small-molecule surfactants in food emulsions. *Food Colloids*. Royal Society of Chemistry, Cambridge.

DiMichele, D. M., M. E. Lasak, and C. H. Miller. 1996. In vitro factor VIII recovery during the delivery of ultrapure factor VIII concentrate by continuous infusion. *American Journal of Hematology* 51: 99-103.

Fatouros, A., and B. Sjostrom. 2000. Recombinant factor VIII SQ - the influence of formulation parameters on structure and surface adsorption. *Internation Journal of Pharmaceutics* 194: 69-79.

Gilbert, G. E., R. J. Kaufman, A. A. Arena, H. Miao, and S. W. Pipe. 2002. Four hydrophobic amino acids of the Factor VIII C2 domain are constituents of both the membrane-binding and the von Willebrand Factor-binding motifs. *The Journal of Biological Chemistry* 277: 6374-6381.

Grillo, A. O., K.-L. T. Edwards, R. S. Kashi, K. M. Shipley, L. Hu, M. J. Besman, and C. R. Middaugh. 2001. Conformational origin of the aggregation of recombinant human Factor VIII. *Biochemistry* 40: 586-595.

Jiang, R., T. Monroe, R. McGrogers, and P. J. Larson. 2002. Manufacturing challenges in the commercial production of recombinant coagulation Factor VIII. *Haemophilia* 8: 1-5.

Katakam, M., and A. K. Banga. 1997. Use of poloxamer polymers to stabilize recombinant human growth hormone against various processing stresses. *Pharmaceutical Deceleopment and Technology* 2: 143-149.

Kaufman, R. J. 1992. Biological regulation of Factor VIII activity. 43: 325-339.

Kreilgaard, L., L. S. Jones, T. W. Randolph, S. Frokjaer, J. M. Flink, M. C. Manning, and J. F. Carpenter. 1998. Effect of Tween 20 on freeze-thawing- and agitation - induced aggregation of recombinant human Factor XIII. *Journal of Pharmaceutical Sciences* 87: 1597-1603.

Lakowizc, J. 1999. Protein fluorescence. Pages 446-485. *Principles of fluorescence spectroscopy*. Kluwer Academic, New York.

Maclean, D. S., Q. Qian, and C. R. Middaugh. 2002. Stabilization of proteins by low molecular weight multi-ions. *Journal of Pharmaceutical Sciences* 91: 2220-2229.

Osterberg, T., A. Fatouros, and M. Mikaelsson. 1997. Development of a freeze-dried albumin-free formulation of recombinant Factor VIII SQ. *Pharmaceutical Research* 14: 892-898.

Ramani, K., V. Purohit, S. V. Balasubramanian, and C. R. Middaugh. 2005. Aggregation kinetics of recombinant human FVIII (rFVIII). *Journal of Pharmaceutical Sciences* 94: 2023-2029.

Ramani, K., V. S. Purohit, R. D. Miclea, C. R. Middaugh, and S. V. Balasubramanian. 2005. Lipid binding region (2303-2332) is involved in aggregation of recombinant human FVIII (rFVIII). *Journal of Pharmaceutical Sciences* 94: 1288-1299.

Rosen, S., and M. C. Casoni. 2002. Determination of factor VIII activity in plasma and concentrates: comparison between methods. *American Clinical Laboratory* April: 32-37.

Vehar, G. A., B. Keyt, D. Eaton, H. Rodrigues, D. P. O'Brien, F. Rotblat, H. Oppermann, R. Keck, W. I. Wood, R. N. Harkins, E. G. D. Tuddenham, R. M. Lawn, and D. J. Capon. 1984. Structure of human Factor VIII. *Nature* 312: 337-342.

Wang, W., and D. N. Kelner. 2003. Correlation of rFVIII inactivation with aggregation in solution. *Pharmaceutical Research* 20: 693-700.

CHAPTER 5

GENERAL CONCLUSION

The effect of nonionic surfactant Tween 80 (Tween) on protein adsorption, specifically lysozyme and recombinant Factor VIII at hydrophobic and hydrophilic solid/liquid interfaces bearing positive or negative charge, and at the air/water interface is evaluated. At the solid/liquid interface, the ability of Tween to modulate the protein adsorption behavior appears to depend on the relative strength of protein – surface and surfactant – surface interaction. Tween 80 adsorbed in greater amounts on hydrophobic as compared with hydrophilic solid/liquid interfaces. Consequently, addition of Tween together with, following or prior to protein adsorption at hydrophilic surfaces, had no perceivable impact for either lysozyme or rFVIII. Recombinant FVIII was observed to be more resistant to buffer elution than lysozyme, in other words the rFVIII – surface interaction appears to be stronger than that for lysozyme. As a likely outcome of this difference, Tween was more effective at reducing lysozyme adsorption or removing adsorbed lysozyme than for rFVIII. With reference to rFVIII, electrostatic forces appear to be relevant in adsorption and the positively charged B domain appears to be a likely candidate as an adsorption site at negatively-charged hydrophilic interfaces. This is based on the tertiary structure data pointing to the formation of a tightly packed rFVIII monolayer upon adsorption and a near complete recovery of biological activity upon exposure to negatively charged, hydrophilic nanoparticles. Interfacial tensiometry suggests that Tween dominates the air/liquid interface when the concentration of Tween in rFVIII – Tween mixtures is increased. Tween was also effective at reducing rFVIII aggregation and improving the recovery of the rFVIII biological activity upon exposure to the air/liquid interface.

The manuscripts documented in this dissertation present an efficient model for studying protein – surfactant interaction at interfaces and can be applied to a number of different combinations of therapeutic proteins and surfactants, and such study can be very useful during drug formulation development or process improvement exercises.

BIBLIOGRAPHY

Arakawa, T., and Y. Kita. 2000. Protection of bovine serum albumin from aggregation by Tween 80. *Journal of Pharmaceutical Sciences* 89: 646-651.

Archer, J. 1968. *Manual on Ellipsometry.* Gaertner Scientific Corporation.

Arnebrant, T., and M. C. Wahlgren. 1995. Protein-surfactant interaction at solid surfaces in T. A. Horbett and J. L. Brash, eds. *Proteins at Interfaces II: Fundamentals and Applications.* American Chemical Society, Washington D.C.

Bam, N. B., J. L. Cleland, J. Yang, M. C. Manning, J. F. Carpenter, R. F. Kelley, and T. W. Randolph. 1998. Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions. *Journal of Pharmaceutical Sciences* 87: 1554-1559.

Bam, N. B., T. W. Randolph, and J. L. Cleland. 1995. Stability of protein formulations: Investigation of surfactant effects by a novel EPR spectroscopic technique. *Pharmaceutical Research* 12: 2-11.

Bardelle, C., B. Furie, B. C. Furie, and G. E. Gilbert. 1993. Membrane binding kinetics of Factor VIII indicate a complex binding process. *The Journal of Biological Chemistry* 268: 8815-8824.

Bisaglia, M., I. Tessari, L. Pinato, M. Bellanda, S. Giraudo, M. Fasano, E. Bergantino, L. Bubacco, and S. Mammi. 2005. A topological model of the interaction between a-synuclein and sodium dodecyl sulfate micelles. *Biochemistry* 44: 329-339.

Bloemendal, M., and W. C. J. Jr. 1995. Structural information on proteins from circular dichroism spectroscopy possibilities and limitations. *Pharm Biotechnol.* 7: 65-100.

Boedeker, B. G. D. 2001. Production processes of licensed recombinant Factor VIII preparations. *Seminars in thrombosis and hemostasis* 27: 385-394.

Boedeker, B. G. D. 2001. Production processes of licensed recombinant factor VIII preparations. *Seminars in Thrombosis and Haemostasis* 27: 385-394.

Calonder, C., Y. Tie, and P. R. van Tassel. 2001. History dependence of protein adsorption. *Proceedings of the National Academy of Sciences* 98: 10664-10669.

Chu, L. 2005. Surfactant interactions with recombinant factor VIII at the air/water interface. Pages 79. *Chemical Engineering.* Oregon State University.

Curtis, J. E., S. L. Helgerson, E. T. Parker, and P. Lollar. 1994. Isolation and characterization of thrombin-activated human factor VIII. *The Nournal of Biological Chemistry* 269: 6246-51.

Derrick, T. S., R. S. Kashi, M. Durrani, A. Jhingan, and C. R. Middaugh. 2004. Effect of metal cations on the conformation and inactivation of recombinant human Factor VIII. *Journal of Pharmaceutical Sciences* 93: 2549-2557.

Dickinson, E., and C. M. Woskett. 1989. Competitive adsorption between proteins and small-molecule surfactants in food emulsions. *Food Colloids.* Royal Society of Chemistry, Cambridge.

DiMichele, D. M., M. E. Lasak, and C. H. Miller. 1996. In vitro factor VIII recovery during the delivery of ultrapure factor VIII concentrate by continuous infusion. *American Journal of Hematology* 51: 99-103.

Elwing, H. 1998. Protein adsorption and ellipsometry in biomaterial research. *Biomaterials* 19: 397-406.

Elwing, H., A. Askendal, and I. Lundstrom. 1989. Desorption of fibrinogen and g-globulin from solid surfaces induced by a nonionic detergent. *Journal of Colloid and Interface Science* 128: 296-300.

Fatouros, A., and B. Sjostrom. 2000. Recombinant factor VIII SQ - the influence of formulation parameters on structure and surface adsorption. *Internation Journal of Pharmaceutics* 194: 69-79.

Fay, P. J., and P. V. Jenkins. 2005. Mutating factor VIII: lessons from structure to function. *Blood Reviews* 19: 15-27.

Fowler, W. E., P. J. Fay, D. S. Arvan, and V. J. Marder. 1990. Electron microscopy of human factor V and factor VIII: Correlation of morphology with domain structure and localization of factor V activation fragments. *Proc. Natl. Acad. Sci. USA* 87: 7648-7652.

Gilbert, G. E., and A. A. Arena. 1998. Unsaturated phospholipid acyl chains are required to constitute membrane binding sites for Factor VIII. *Biochemistry* 37: 13526-13535.

Gilbert, G. E., R. J. Kaufman, A. A. Arena, H. Miao, and S. W. Pipe. 2002. Four hydrophobic amino acids of the Factor VIII C2 domain are constituents of both the membrane-binding and the von Willebrand Factor-binding motifs. *The Journal of Biological Chemistry* 277: 6374-6381.

Grillo, A. O., K.-L. T. Edwards, R. S. Kashi, K. M. Shipley, L. Hu, M. J. Besman, and C. R. Middaugh. 2001. Conformational origin of the aggregation of recombinant human Factor VIII. *Biochemistry* 40: 586-595.

Hillgren, A., H. Evetsson, and M. Alden. 2002. Interaction between Lactate Dehydrogenase and Tween 80 in aqueous solution. *Pharmaceutical Research* 19: 504-510.

Horbett, T. A. 1982. Protein adsorption on biomaterials. Pages 233-244. *Biomaterials: Interfacial phenomena and applications.*

Jiang, R., T. Monroe, R. McGrogers, and P. J. Larson. 2002. Manufacturing challenges in the commercial production of recombinant coagulation Factor VIII. *Haemophilia* 8: 1-5.

Johnson, W. C. 1999. Analyzing protein circular dichroism spectra for accurate secondary structures. *Proteins: Structure, Function and Genetics* 35: 307-312.

Joshi, O. 2005. Fibrinogen adsorption at heparinized silica interfaces: History dependence and protein concentration effects. Pages 98. *Chemical Engineering.* Oregon State University.

Katakam, M., and A. K. Banga. 1997. Use of poloxamer polymers to stabilize recombinant human growth hormone against various processing stresses. *Pharmaceutical Deceleopment and Technology* 2: 143-149.

Kaufman, R. J. 1992. Biological regulation of Factor VIII activity. 43: 325-339.

Kessler, C. M., J. C. Gill, G. C. White, A. Shapiro, S. Arkin, D. A. Roth, X. Meng, and J. M. Lusher. 2005. B-domain deleted recombinant factor VIII preparations are bioequivalent to a monoclonal antibody purified plama-derived factor VIII concentrate: a randomized, three-way crossover study. *Haemophilia* 11: 84-91.

Klintstrom, S. W. 1992. Ellipsometry and wettability gradient surfaces. Pages 115. *Department of Physics and Measurement Technology.* Linkoping University.

Koutsopoulos, S., J. van der Oost, and W. Norde. 2005. Structural features of a hyperthermostable endo-beta-1,3-glucanase in solution and adsorbed on "invisible" particles. *Biophysical Journal* 88: 467-474.

Kreilgaard, L., L. S. Jones, T. W. Randolph, S. Frokjaer, J. M. Flink, M. C. Manning, and J. F. Carpenter. 1998. Effect of Tween 20 on freeze-thawing- and agitation - induced aggregation of recombinant human Factor XIII. *Journal of Pharmaceutical Sciences* 87: 1597-1603.

Krisdhasima, V., J. McGuire, and R. Sproull. 1992. A one-film-model ellispometry program for the simultaneous calculation of protein film thickness and refractive index. *Surface and Interface Analysis* 18: 453-456.

Krishnan, A., Y.-H. Liu, P. Cha, D. Allara, and E. A. Vogler. 2005. Scaled interfacial activity of proteins at a hydrophobic solid/aqueous-buffer interface. *Journal of Biomedical Materials Research Part A* 75: 445-457.

Lakowizc, J. 1999. Protein fluorescence. Pages 446-485. *Principles of fluorescence spectroscopy*. Kluwer Academic, New York.

Lee, W.-K. 1999. Kinetic modeling of the adsorption of structural stability mutants of bacteriophage T4 lysozyme at solid-water interfaces. Pages 276. *Bioresource Engineering*. Oregon State University, Corvallis.

Leffers, K.-W., J. Schell, K. Jansen, R. LUcassen, T. Kaimann, L. Nagel-Steger, J. Tatzelt, and D. Riesner. 2004. The structural transition of the prion protein into its pathogenic conformation is induced by unmasking hydrophobic sites. *Journal of Molecular Biology* 344: 839-853.

Maclean, D. S., Q. Qian, and C. R. Middaugh. 2002. Stabilization of proteins by low molecular weight multi-ions. *Journal of Pharmaceutical Sciences* 91: 2220-2229.

Manning, M., and W. Colon. 2004. Structural basis of protein kinetic stability: Resistance to sodium dodecyl sulfate suggests a central role for rigidity and a bias towards b-sheet structure. *Biochemistry* 43: 11248-11254.

McGuire, J., M. C. Wahlgren, and T. Arnebrant. 1995. Structural stability effects on the adsorption and dodecyltrimethylammonium bromide-mediated elutability of bacteriophage T4 lysozyme at silica surfaces. *Journal of Colloid and Interface Science* 170: 182-192.

Natale, P., T. den Blaauwen, C. van der Does, and A. J. M. Driessen. 2005. Conformational state of the SecYEG-bound Sec A probed by a single tryptophan fluorescence spectroscopy. *Biochemistry* 44: 6424-6432.

Norde, W., and J. Lyklema. 1991. Why proteins prefer interfaces. *Journal of Biomaterial Science Polymer Edition* 2: 183-202.

Norde, W., and T. Zoungrana. 1998. Surface-induced changes in the structure and activity of enzymes physically immobilized at solid/liquid interfaces. *Biotechnology and Applied Biochemistry* 28: 133-143.

Osterberg, T., A. Fatouros, and M. Mikaelsson. 1997. Development of a freeze-dried albumin-free formulation of recombinant Factor VIII SQ. *Pharmaceutical Research* 14: 892-898.

Podhipleux, N. 1998. Adsorption of the wild type and a synthetic structural stability variant of bacteriophage T4 lysozyme. Pages 124. *Bioresource Engineering*. Oregon State University, Corvallis.

Ramani, K., V. Purohit, S. V. Balasubramanian, and C. R. Middaugh. 2005. Aggregation kinetics of recombinant human FVIII (rFVIII). *Journal of Pharmaceutical Sciences* 94: 2023-2029.

Ramani, K., V. S. Purohit, R. D. Miclea, C. R. Middaugh, and S. V. Balasubramanian. 2005. Lipid binding region (2303-2332) is involved in aggregation of recombinant human FVIII (rFVIII). *Journal of Pharmaceutical Sciences* 94: 1288-1299.

Reynolds, J. A., and C. Tanford. 1976. Determination of molecular weight of the protein moiety in protein-detergent complexes without direct knowledge of detergent binding. *Proc. Natl. Acad. Sci. USA* 73: 4467-4470.

Rosen, S., and M. C. Casoni. 2002. Determination of factor VIII activity in plasma and concentrates: comparison between methods. *American Clinical Laboratory* April: 32-37.

Schwartz, R., B. Boedeker, D. Schroeder, and D. Hoerlein. 1992. Recombinant-derived blood clotting factor VIII (Kogenate). *Miles Science Journal* 14: 19-25.

Stoilova-McPhie, S., B. O. Villoutreix, K. Mertens, G. Kemball-Cook, and A. Holzenburg. 2002. 3-Dimensional structure of membrane-bound coagulation factor VIII: modeling of the factor VIII heterodimer within a 3-dimensional density map derived by electron crystallography. *Blood* 99: 1215-1223.

Sudhakar, K., and P. J. Fay. 1996. Exposed hydrophobic sites in Factor VIII and isolated subunits. *The Journal of Biological Chemistry* 271: 23015-23021.

Vehar, G. A., B. Keyt, D. Eaton, H. Rodrigues, D. P. O'Brien, F. Rotblat, H. Oppermann, R. Keck, W. I. Wood, R. N. Harkins, E. G. D. Tuddenham, R. M. Lawn, and D. J. Capon. 1984. Structure of human Factor VIII. *Nature* 312: 337-342.

Vermeer, A. W. P., M. G. E. G. Bremer, and W. Norde. 1998. Structural changes of IgG induced by heat treatment and by adsorption onto a hydrophobic Teflon surface studied by circular dichroism. *Biochimica et Biophysica Acta* 1425: 1-12.

Wang, W., and D. N. Kelner. 2003. Correlation of rFVIII inactivation with aggregation in solution. *Pharmaceutical Research* 20: 693-700.