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Joseph McGuire

The surface tension kinetic and steady state behavior exhibited by mixtures of selected surfactants with a recombinant Factor VIII (rFVIII) were evaluated with automated interfacial tensiometry. In particular, fresh solutions of rFVIII with different concentrations of Tween 80, sodium dodecylsulfate (SDS), or dodecyltrimethylammonium bromide (DTAB) were prepared and their surface tension decrease monitored continuously by the DuNuoy ring and Wilhelmy Plate methods. The range of surfactant concentrations studied included several orders of magnitude, with values below and above the critical micelle concentration (CMC). The steady state behavior of rFVIII-Tween mixtures was adequately described by current theory for mixtures of random chain protein molecules with small surfactants at equilibrium. However this theory did not clearly explain the behavior of rFVIII-SDS and rFVIII-DTAB mixtures. Rather, the strong protein-surfactant association resulting in higher surface activities than that observed for pure surfactant or protein alone at each concentration, with full displacement of protein from the interface at high surfactant concentration. Analysis of protein-surfactant surface tension kinetics using a simple first-order rate equation produced two rate constants(K_1 and K_2) in each case, presumably reflecting initial adsorption and subsequent rearrangement (including protein-surfactant dissociation) at the interface. The values of these rate constants differed for each rFVIII-surfactant mixture, in a manner consistent with surface tension data.

Surfactant Interactions with Recombinant Factor VIII at the Air/Water interface.

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I want to give this thesis as a gift to my parents, for their love and support.

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CHAPTER 1
INTRODUCTION

Protein behavior at interfaces is extremely important in many aspects of biotechnology and pharmaceutical industry. For example, bioseparation and purification of proteins by chromatography and foam fractionation methods involve competitive adsorption of proteins and enzymes at solid-liquid and gas-liquid interfaces.

Protein adsorption is the first step in the biological response to materials. Protein adsorption can influence the biological response in at lease two general but not entirely independent ways. First, adsorbed protein can expose receptors or ligand and participate in one or more of the myriad "lock-and-key" biochemical reactions, especially those involved in signal transduction. Second, protein-surface-water interactions are governed by purely kinetic/thermodynamic rules, for a given surface chemistry/energy immersed in water, the free energy of adsorption is solely dependent on solute molecular characteristics rather than biological potential of that solute. Amphilicity (interaction energetic with water) is paramount among these solute characteristics, governing both sign and magnitude of the free energy change occurring upon adsorption. The linkage between these two different aspects of protein adsorption lies in the fact that interfacial energetic controls the amount of

protein adsorbed and biochemistry influences activity of protein in the adsorbed state.

Factor VIII (FVIII) deficiency (hemophilia A) cause prolonged and potentially fatal bleeding after trauma or surgery, joint and muscle hemorrhages, and easy bruising. Replacement with FVIII concentrates derived from donated blood has been the mainstay of treatment. Recombinant ("genetically engineered") factor VIII was launched in the UK in 1994, with the aim of further reducing the risk of viral infection. In medical use, recombinant factor VIII (rFVIII) adsorption at all kinds of surfaces, such as dose vials, columns, etc., is remarkably problematic. The adsorbed protein at surfaces lost their activity. This costs millions of dollars for the production corporation. So the key question is to study the behavior at rFVIII at surfaces, find the way to reduce the adsorption, introduce surfactant to investigate the effect of reducing the adsorption of rFVIII. Surfactants are amphiphilic molecules consisting of a hydrophilic head-group and a hydrophobic tail. The solubility of surfactants in water is low since hydrocarbon-water contacts are unfavorable. The introduction of surfactant molecules into water requires a local ordering of the water molecules that surround the hydrocarbon tails. However, in the formation of micelles, the unfavorable hydrocarbon-water contacts are minimized and the entropy of the system is increased. This phenomenon is called the hydrophobic effect.

The surfaces of materials of almost any type that come into contact with protein-containing solutions tend to become quickly occupied by proteins. Issues surrounding this well-known problem contributing to the subject of much research, as the resulting interfacial layers can lead to profound alterations in the properties of the material-fluid interface, affecting material performance in a number of applications in biotechnology and medicine. But in addition, the loss of protein function or activity through adsorption and surface-induced structural alteration is itself a significant problem in several contexts, among them formulation and administration of therapeutic proteins. The main objective of this study is to investigate the mechanism of rFVIII adsorption at interfaces, with a view toward quantifying surface chemical effects on binding and structural change. In this way, we will able to provide useful direction for minimizing the "adsorption loss" of rFVIII that occurs during solubilization and administration of a given formulation, as well as minimizing any surface-induced loss that occurs during the manufacturing process. The mechanism of protein stabilization by surfactants is unclear and is the theme of this research project. By comparing three different kinds of surfactants based in their charged groups, the interaction of rFVIII with non-ionic surfactant (Tween 80), anionic surfactant (SDS) and cationic surfactant (DTAB) at the air/water interface is investigated by surface tension kinetics, both the time dependence and steady state model was studied at different concentrations of surfactants, by which the stabilizing effect of surfactants were derived. Rate constant analysis was used to confirm this conclusion from surface tensiometry study.

Chapter 2
LITERATURE REVIEW

The role of proteins at interfaces is recognized as a key factor in controlling the function of many biological systems. For example, protein adsorption is the first stage of the body's response to an implanted biomaterial, leading to cell adhesion and inflammation or thrombus formation. Therefore, the control of protein adsorption at interfaces is an important step towards resolving the issue of biomaterials compatibility. The study of interaction between protein and low molecular weight surfactants has importance in several biotechnological disciplines. For example, the protein-surfactant interaction controls colloidal stability in foods and pharmaceuticals, is made use of in SDS-polyacrylamide electrophoresis and controls detergency efficiency. The "equilibrium" in systems containing ionic surfactants and proteins has been investigated extensively, but the molecular mechanisms of the interactions involved are still not well understood.

2.1 Protein at interfaces

The problem of protein used in pharmaceutical industries to be avoided is "surface denaturation" of water-soluble proteins. When a molecule with this structure reached the surface, there is a strong tendency for the hydrocarbon parts of the protein molecule to cover the surface, and this is accomplished by unfolding the protein molecule to form a flat sheet. To prevent surface denaturation, a number of immobilization methods have been developed, for example, covalent linking and polymer matrix immobilization. Gidalevitz and Huang *et al.* (1998) concluded that

the spreading of a lipid monolayer at the aqueous solution surface before protein adsorption injection does not prevent proteins from unfolding, and cross linking of proteins results in intact enzyme layer at the subphase surface. They used a specular X-ray reflectivity to study the structural integrity of water soluble enzymes of glucose oxidase (GOx), alcohol dehydrogenase, and urease monolayers at the air-aqueous interface. They concluded that one way of preventing proteins from unfolding at the air-water interface is to create artificially a hydrophilic boundary at that interface, the uniform layer of the polar head-groups penetrating the water interface should prevent the hydrocarbon parts of the protein molecule from reaching the surface and then unfolding to a flat sheet. A monolayer of dibehenoylphosphatidyl choline (DBPC) phospholipids was deposited on the PBS buffered (PH=7.4) aqueous surface, after compression X-ray reflectivity measurements were carried out. Then solution of glucose oxidase was injected underneath the monolayer, the surface tension dropped almost 25% immediately after the protein injection, indicating the adsorption of protein molecules at the interface. The monolayer was then recompressed and left for 2 hr to let the system reach equilibrium. X-ray reflectivity measurements of the DBPC/GOx system were carried out under the same conditions as for the pure DBPC monolayer. The results of XR measurements did not suggest the presence of any glucose oxidase at the interface. Another way to prevent the surface denaturation is to crosslink them covalently to make their unfolding more difficult. The crosslinked layers of glucose oxidase and urease at the air-water interface were examined with X-ray reflectivity. The thickness of both the glucose oxidase and urease layers are close to the real

protein dimensions in three-dimesional crystals. The XR data show that covalent crosslinking prevents (or partially prevents) the unfolding of the proteins molecules at the air-water interface.

Bieze, Pereira and Theodoly *et al.* (2000) study the surface rheological properties of β-casein and bovine serum albumin (BSA) adsorbed at the air/water interface. β-casein, a flexible protein, was used at a very low concentration 0.001wt%, at the air/water interface, proteins in bulk solution adsorb to the interface and drop the surface tension of the air bubble from 72mN/m to 51mN/m. Beverung *et al.* (1996) stated that when the data are plotted on a logarithmic scale, it can be divided into three regimes, the first regime is called the lag time, the fewer the proteins in the bulk solution, the longer the lag time. The second regime is a rapid slope downwards, this is when the protein molecules are going through conformational rearrangement at the interface and rearranging themselves at the interface. Once most of the protein molecules in the bulk solution have gone through rearrangement at the interface the decrease in tension slows down, this is the beginning of the third regime, the protein molecules rearrange in a more tightly packed configuration due to interaction with neighboring protein molecules leading to irreversible interfacial gel formation. In the surface rheology experiments, the β-casein is purely elastic and BSA, a globular protein, has both an elastic and viscous component when it is approximately neutrally charged. BSA ix forming a viscoelastic gel, then the surface tension versus the natural log of the surface area was plotted it appeared to be elliptical; this suggested that BSA was producing a

purely viscous gel at the air/water interface. The surface chemistry of the protein and the surface determine the residence time due to the initial interaction energy. The dynamics or denaturability of the protein itself, together with its residence time, probably controls the surface denaturability of the protein.

Protein adsorption is an intricate process, resulting from different kinds of interactions between the various components present in the system. Creighton stated (1984) that it only occurs spontaneously if $\Delta_{ads}G = (\Delta_{ads}H - T\Delta_{ads}S) > 0$. For water-soluble proteins, of which the three-dimensional structure has been solved, it has been shown that the polar and apolar residues are more or less evenly distributed over the surface of the molecule. Folding of polypeptide chain into the compact, relatively inflexible architecture of a globular protein molecule involves a substantial loss of conformational entropy. For most globular proteins, 40%-70% of the polypeptide chain participates either in an α-helix or β-sheet structure. They summarized the interactions that determine the structure of a protein molecule in an aqueous environment.

Privalov *et al.* (1979) comparing the compact globular, native state with the completely unfolded, denatured state, a negative value for the Gibbs energy of unfolding, $\Delta_{\text{compact-unfolded}}G$, implies that the compact state is more stable than the unfolded state, and *vice versa*. Hydrophobic dehydration and changes in the rotational freedom of the polypeptide chain, both involving large entropic effects, are the main competing factors. The existence of a highly structured, compact

protein molecule in an aqueous environment demonstrates the constructive power of chaos: the ordered protein structure is preferred because of increased disorder in the surrounding water. As a result of the compensating contributions, the native structure of globular proteins in aqueous solution is only marginally stable. Changes in conditions, such as pH, temperature, etc. or the environment, such as introducing an interface, may alter the conformation of the protein.

In the adsorbed state, at one side of the protein molecule, the aqueous environment is replaced by the sorbent material, intra-molecular hydrophobic interaction is less important as a structure stabilizing factor. Hydrophobic parts in the dissolved protein that tend to be buried in the interior of the molecule may become exposed to the sorbent surface without making contact with water. Such a structure change would involve a loss of intra-molecular hydrophobic bonds. Because hydrophobic interactions in the interior of the protein molecule support the formation of α-helix and β-sheet, a reduction of such hydrophobic interactions may cause a decrease on those secondary structure, which, in turn, leads to increased rotational freedom and hence, an increase in the conformational entropy of the protein molecule.

The behavior of proteins at biological and synthetic interfaces is often characterized by a strong history dependence caused by long relaxation times or irreversible transitions. Calonder and Tie *et al.* (2001) use multi-step kinetic experiments in which the *ith* step is an exposure of a SiO_2 surface to a flowing

fibronectin or cytochrome c solution of concentration C_i for a time t_i and measure the protein adsorption by optical waveguide light mode spectroscopy (OWLS). They observed that the rate of adsorption onto surfaces containing equal amounts of adsorbed protein can be greatly enhanced if the adsorbed layer is prepared by multiple adsorption steps, and this history dependence can be attributed to the irreversible formation of clusters or ordered domains.

Protein packing at the interface can give valuable information to protein adsorption behavior. Gerstein and Chothia *et al.* (1996) determined the packing efficiency at the protein-water interface by calculating the volumes of atoms on the protein surface and nearby water molecules in 22 crystal structures. They found that an atom on the protein surface occupies, on average, a volume ≈7% larger than on atom of equivalent chemical type in the protein core. Larger volumes result from voids between atoms and thus imply a looser or less efficient packing. Furthermore, the volumes of individual atoms are not related to their chemical type but rather to their structural location, more exposed atoms have larger volumes.

Dynamic surface tension measurement is a very useful technique to study the protein adsorption. Ybert and Meglio *et al.* (1998) presented new data on the adsorption of bovine serum albumin (BSA) at the free water interface using pendant drop method and an accurate description of the initial stage of adsorption. They observed the lag time of protein adsorption, during which the surface pressure is

about zero, there is no energy barrier to adsorption, which means that the dynamics is dominated by the transport in the bulk.

2.2 Surfactants at Interfaces

Purcell Lu *et al.* (1998) studies the surface excess of sodium dodecyl sulfate (SDS) in aqueous solution of SDS and the polymer poly(vinylpyrrolidone) (PVP), the surface excess was measured as a function of SDS and PVP concentrations using neutron reflection. Below the critical aggregation concentration (CAC) the adsorption of SDS is increased by the presence of PVP, this indicating that the two components interact cooperatively at the surface. Between the CAC and the critical micelle concentration (CMC) of the surfactant there is a slight depletion of SDS from the surface. They observed the surface tension data of pure SDS, the curve is in reasonable agreement with measurements on SDS by Mysels et.al (1986), from 1mM to 6mM, below CMC, the surface tension decreases with time and the steady state value decreases with increased SDS concentration.

Pawelec and Socnowski *et al.* (2000) focus on experimental determination of dynamic mechanical properties of the air-water interface during compression in the presence of DPPC monolayer, a single-phospholipid model of the pulmonary surfactant, and the influence of CTAB present in the aqueous subphase on the surface with DPPC. It was shown that the interface with adsorbed surfactants specifically reacted to deformation, mainly due to kinetics of structural reorganization of molecules in the monolayer but also due to the mass change.

Bergstrom *et. al* (1999) investigated the geometrical structure of pure SDS and DTAB micelles using small-angle neutron scattering (SANS). Both SDS and DTAB micelles appeared to be disk-like in pure D_2O and the corresponding data were best fitted with a model for oblate ellipsoids of revolution with half axes a=12.0 Å, b=20.3 Å ([SDS]=1.0 wt.%) and a=12.4 Å, b=21.6 Å ([DTAB]=1.0 wt.%) Woodward (1995) analyzed surface tension as a function of time of Tween 80, their results showed at five seconds the surface tension was 42.5mN/m and it decreased to 38.1mN/m at 175 seconds.

2.3 Protein-Surfactants Interactions

2.3.1 Protein-Surfactant Interaction in Solution

Tanford (1980) stated that proteins and surfactants mixtures might interact with each other in bulk solution involving the formation of surfactant-protein complexes which have different properties from those of the pure protein. The binding of surfactant to protein will reduce the concentration of free surfactant molecules available for interaction with the protein at the interface which may show up as an apparent increase in the CMC. This is consistent with our results of rFVIII-Tween 80 mixtures.

Tanford (1980) stated that ionic surfactants are known to interact with proteins in solution, this interaction is usually stronger for SDS than for cationic surfactants. But nonionic surfactants are known to generally interact poorly with soluble proteins. Otzen *et al.* (2002) found the 102-residue monomeric protein S6

unfolds in the anionic SDS above the CMC, with unfolding rates varying according to two different modes. Spherical micelles lead to saturation kinetics in unfolding (mode 1), while cylindrical micelles prevalent at higher SDS concentrations induce a power-law dependent increase in the unfolding rate (mode 2). Furthermore, unfolding does not occur in mode 2 in the cationic surfactant LTAB, which is unable to form cylindrical micelles. A strong retardation of unfolding occurs at higher LTAB concentrations, possibly due to the formation of dead-end protein-surfactant complexes.

Tanford (1980) concluded three types of protein-surfactant interactions in solution: 1) Binding of surfactant by electrostatic or hydrophobic interactions to specific sites in the protein, 2) Cooperative adsorption of surfactant to the protein without gross conformational changes and 3) Cooperative binding to the protein followed by conformational changes. The changes 1)-3) can occur in the same system when the surfactant concentration is increased. The conformational changes in case 3) involve changes in the secondary structure. Several models for the protein-surfactant complexes have been suggested by Reynolds and Tanford *et al.* (1970) as rigid rod, Shirahama *et al.* (1974) as pearl and necklace and Lundahl *et al.* (1986) as flexible helix. In the cooperative region, above the critical association concentration (CAC), the interaction is mainly of hydrophobic character.

Deo, Jockusch and Ottaviani *et al.* (2003) use surface tension measurement to study the polymer-surfactant interactions in aqueous solutions of a

hydrophobically modified polymer, poly-(maleic acid/octyl vinyl ether) (PMAOVE), with SDS. The addition of SDS to the PMAOVE solution causes a change of the surface tension in two stages. At 2mM SDS, a sharp decrease of the surface tension occurred, indicating the formation of mixed micelles of SDS and hydrophobic groups of PMAOVE followed by a gradual decrease with SDS concentrations. At approximately 20mM SDS, the surface tension reached a value similar to that of SDS micelle solutions and remained constant with further increase in the SDS concentration, suggesting the coexistence of mixed micelles and pure SDS micelles in the solution.

Nozaki and Reynolds *et al.* (1974) studied the interaction of a cationic detergent with bovine serum albumin, they found at higher detergent concentrations the cationic detergent resembles sodium dodecyl sulfate, it binds cooperatively to serum albumin and to other proteins with accompanying gross denaturation to form extended rod-like complexes. Their results show the binding at low detergent concentration for bovine serum albumin and oralbumin in 0.015 M phosphate buffer, pH 5.6. Noncooperative binding to discrete site of native serum albumin is observed, followed by a highly cooperative association at higher detergent concentration. Only the cooperative interaction is observed for ovalbumin. The binding constant is derived from this treatment. Quite similar results were obtained in 0.03 M Tris chloride buffer, pH 8.1, it yielding the same number of sites but a higher association constant. It is evident that the sites for binding of the cationic detergent are smaller in number and weak in affinity, cationic detergent also seems to have a marked pH

dependence. Addition evidence states that the onset of micelle formation prevents reaching saturation in the cooperative binding.

Parker and Song *et al.* (1992) found the formation of ordered structure (alpha-helix or beta-sheet) in certain peptides is known to be induced by interaction with SDS micelles. The SDS-induced structures formed by these peptides are amphiphilic, having both a hydrophobic and a hydrophilic face. SDS induces helical folding in a wide variety of non-helical proteins.

2.3.2 Protein-Surfactant Interaction at Interfaces

Andrade (1985) indicated that the conformational state of an adsorbed protein may differ from that of a bulk protein, a phenomenon called surface denaturation. Green, Hopkinson and Jones (1998) studied the process of thermal denaturation for adsorbed globular hen egg lysozyme and BSA and made a direct comparison with thermal denaturation in the bulk. FTIR spectra was used to investigate the adsorbed protein layers, it appears that the effect of adsorption at an interface is broadly similar for both BSA and lysozyme. Both proteins begin to aggregate at lower temperatures, but at lower rates, than when in bulk solution. BSA is a less rigid molecule than lysozyme and is not as stable; it unfolds almost completely upon adsorption, the process of surface unfolding differs between the two molecules, occurring mostly during heating for lysozyme and during adsorption for BSA. During adsorption, the secondary structure of lysozyme changes suggesting an increase in β-sheet formation and the existence of aggregation at room temperature. In the bulk state the heating of both lysozyme and BSA resulted in

heat induced intermolecular association via two discrete and sequential transitions. The protein molecules initially undergo an unfolding transition at around 50 ^{0}C, and then at around 70 ^{0}C intermolecular association commences leading to aggregation. The onset of intermolecular association and aggregation is a rather sharp transition and there is an intermediate state where the protein is unfolded but remains with its native secondary structure largely intact and is not yet susceptible to the formation of intermolecular associations. In contrast to the bulk phase, when adsorbed at an interface discrete unfolding and aggregation transitions of the protein were not observed. Some partial unfolding of the proteins occurs during adsorption and thus the initial structures upon initiation of heating differed significantly from that of their native structure in the bulk state. During adsorption, changes in the secondary structure of lysozyme were also observed suggesting that the protein molecules already undergo some intermolecular association as well as unfolding even under ambient temperatures. The character of the adsorbing interface affects the extent and rate of conformational rearrangements of the adsorbing protein. At a hard, hydrophilic interface, obtained by coating the ATR crystal with PMMA, there was very little structural change during adsorption; a liquid-like hydrophobic interface by an EPDM coating, facilitated considerable protein unfolding and secondary structural changes during adsorption, while a hard hydrophobic interface, polystyrene, provide an intermediate situation.

Dickinson *et al.* (1998) indicated that the two extreme types of mechanisms by which surfactants remove protein from the interfaces is first, the solubilization

mechanism, desorption of protein from the surfaces arises ad a result of protein solubilization into the aqueous phase by water-soluble surfactant which forms a protein-surfactant complex, the surfactant does not itself have to adsorb at the surface, but it must interact strongly with the adsorbed protein. Second, the replacement mechanism, competitive displacement of adsorbed protein arises because the surfactant-surface interaction is stronger than the interaction between the protein (or protein-surfactant complex) and the surface, the surfactant does not actually have to interact with the protein, but it must bind to the surface. In practice, the actual mechanism will tend to lie between these extremes, with competitive adsorption involving ionic surfactants mainly proceeding *via* a solubilization mechanism, and that involving non-ionic surfactants mainly proceeding *via* replacement.

When the concentration of a surfactant is much lower than its critical micelle concentration (CMC), the surfactant molecules lie flat at the air/water interface [Porter, 1994]. As the concentration is increased, more molecules adsorb to this interface, such that the surface concentration remains linearly proportional to the bulk concentration [Tanford, 1973]. This crowding phenomena force the surfactant molecules to order themselves such that the hydrophilic groups are oriented towards the bulk water and the hydrocarbon chains are pointed towards the air or hydrophobic solid. At sufficiently high surfactant concentrations (at or above the CMC), there is an oriented monolayer of surfactant molecules and maximum surfactant adsorption at the interface. This surface saturation is responsible for the

sharp decrease in slope observed in experimental plots of surface properties versus surfactant bulk concentration. Surfactant micelles are formed in the bulk phase when the concentration of the surfactant is above the CMC. It is thus evident that a range of surfactant concentrations showing different surfactant association structures could influence protein adsorption at an interface [porter, 1994].

Decreased protein adsorption at interfaces in the presence of non-ionic surfactants can be attributed to the surface activity of non-ionic surfactants and, in some case, direct interactions between the surfactant and protein molecules (Dickinson, 1998, Bam *et al.*, 1998, Miller *et al.*, 2000). In mixed protein/surfactant systems in which the surfactant binds to hydrophobic regions of the proteins, the protein is less surface-active than it would be in a solution containing no non-ionic surfactant. This explains the increase in surface tension relative to that of the pure protein solution that can be observed at extremely low surfactant concentrations (Randolph and Jones, 2002). Adsorption of surfactants and protein molecules in the mixed system to an interface is competitive. Non-ionic surfactants bind tighter than proteins or protein-surfactant complexes at interface [Dickinson, (1998)].

It is apparent that the presence of a surfactant would considerably affect the way in which a protein interacts with an interface, adsorption from mixtures of protein and surfactant is affected by the protein-surfactant interaction and by the concentration of each portion. Green and Su *et al.* (2001) studied adsorption from the surfactant/protein mixtures to the air/water interface and investigate how the

surface concentration of protein and surfactant varies with the interaction in bulk solution. Surface temsiometry allows a direct quantification of the amount of each component in the mixed surface layer. The surface tension profile was compared between the pure SDS and SDS/lysozyme mixtures. Despite the general trend that the surfactants decrease the surface tension and the formation of a more surface-active complex, they also observed the occurrence of a peak around the SDS concentration of 1mM, it indicates in increasing solubility of the surface aggregates. And the CMC for the complex is lowered compared with pure SDS, the opposite trend indicated the aggregation of the surfactant is likely to be facilitated by the association of polypeptide fragments, this is consistent with that the surface tension above the CMC in the mixed system is lower than that of the pure surfactant, they assume above the CMC the system contains both surfactant-rich and surfactant-lean aggregates and that there is no formation of pure surfactant aggregates.

Green and Su *et al.* (2001) used neutron reflectivity to investigate the interaction between the cationic surfactant dodecyltrimethylammonium bromide (C_{12}TAB) and preadsorbed lysozyme layers at the hydrophilic silica-water interface. The effect on the adsorbed protein layer structure upon addition of a range of surfactant concentrations, from 0.2 to 14mM, was determined and the surface excesses of both protein and surfactant within the adsorbed layers calculated. It was found that upon increasing surfactant concentration the protein was gradually replaced by the surfactant at the interface. Protein removal over the low surfactant concentration range was accompanied by little surfactant adsorption, showing that

desorption was likely to be caused by the formation of highly soluble protein/surfactant complex. At the high surfactant concentration, protein removal was driven by the interplay of the interactions involving the protein, surfactant, and substrate. This result together with previous measurements using SDS suggests that for surfactants with the same alkyl chain length the fraction of protein removal is dictated by the nature of the surfactant head groups.

There was not so much previous study on rFVIII with surfactants, Fatouros and Sjostrom *et al.* (2000) investigate the influence of temperature, pH and ionic interactions on the structural stability and surface adsorption of a recombinant factor VIII product, rFVIII SQ. The stability of rFVIII SQ was followed by measuring the biological activity (VIII:C), by circular dichroism (CD) and by the surface tension measurement using pedant drop method. The results show that the surface tension decreased exponentially with time. Nonionic surfactants such as polysorbate 80 and polysorbate 20 protected VIII:C to an equally high degree against surface adsorption at both glass and air interfaces. They also used as a protein stabilizing agent, albumin was less effective, but it is possible that this is because it is a protein itself and may have been affected by the agitation. The albumin molecule could be depleted from the solution by adsorption at the continuously formed air interface and subsequent denaturation and precipitation, thereby losing its protective effect. This conclusion consists with our results from IAsys. The presence of rFVIII SQ decreased the surface tension of pure buffer, they indicated that at elevated temperatures (above 20 ^{0}C), the conformational flexibility of the protein increases,

thereby exposing hydrophobic domains/amino acids that increase the driving force towards the hydrophobic interface.

2.4 Hemophilia A and recombinant Factor VIII

Hemophilia A is an X-linked recessive disorder which is caused by a deficiency of factor VIII. Intact FVIII complex, usually purified from the blood, consists of two distinct gene products: factor VIII and multiple copies of von Willebrand factor (vWF). This complex display a molecular mass ranging from 1-2 million Dalton, of which up to 15% is carbohydrate. Persons suffering from hemophilia A exhibit markedly reduced levels of (or complete absence of) factor VIII complex in their blood. About one in 10,000 males are born with a defect in the factor VIII complex and there are approximately 25,000 hemophiliac subjects currently resident in the USA. Hemophilia A affects 10-20 per 1,000,000 male. (Schwartz et al., 1990)

Recombinant Factor VIII (rFVIII) is the antihemophilic factor for A, which is obtained using recombinant DNA technology. With this technology, pure protein is synthesized in the laboratory instead of being extracted from blood. Recombinant FVIII is free from the viruses that may be present in plasma derived antihemophilic factor preparations. They are also free from other plasma proteins responsible for immunosuppressant effects and allergic reactions.

Lenting et al. (1998) reported the structure of Factor in plasma, Factor VIII protein exists as a metal ion complex of 200 kDa heavy chain and 80 kDa light

chain doublet and interacts with vWF secreted from vascular endothelial cells. The mature factor VIII protein contains 2332 amino acids arranged in six domains, namely A1 (residues 1-336), A2 (372-710), B (741-1648), A3 (1896-2019), C1 (2020-2172), C2 (2173-2332). The A-domains are surrounded by acidic regions, (A1, A2, A3) occur twice in the heavy chain and once in the light chain, have homology to ceruloplasmin, a copper binding plasma protein. The light chain also contains a single B-domain, and two C-domains along with the A-domain. There are seven disulphide bridges in the mature molecule along with some free Cysteine residues. Factor VIII contains 25 consensus sequences that allow N-linked glycosylation, 19 of which are located in the B-domain. The entire molecule is highly glycosylated.

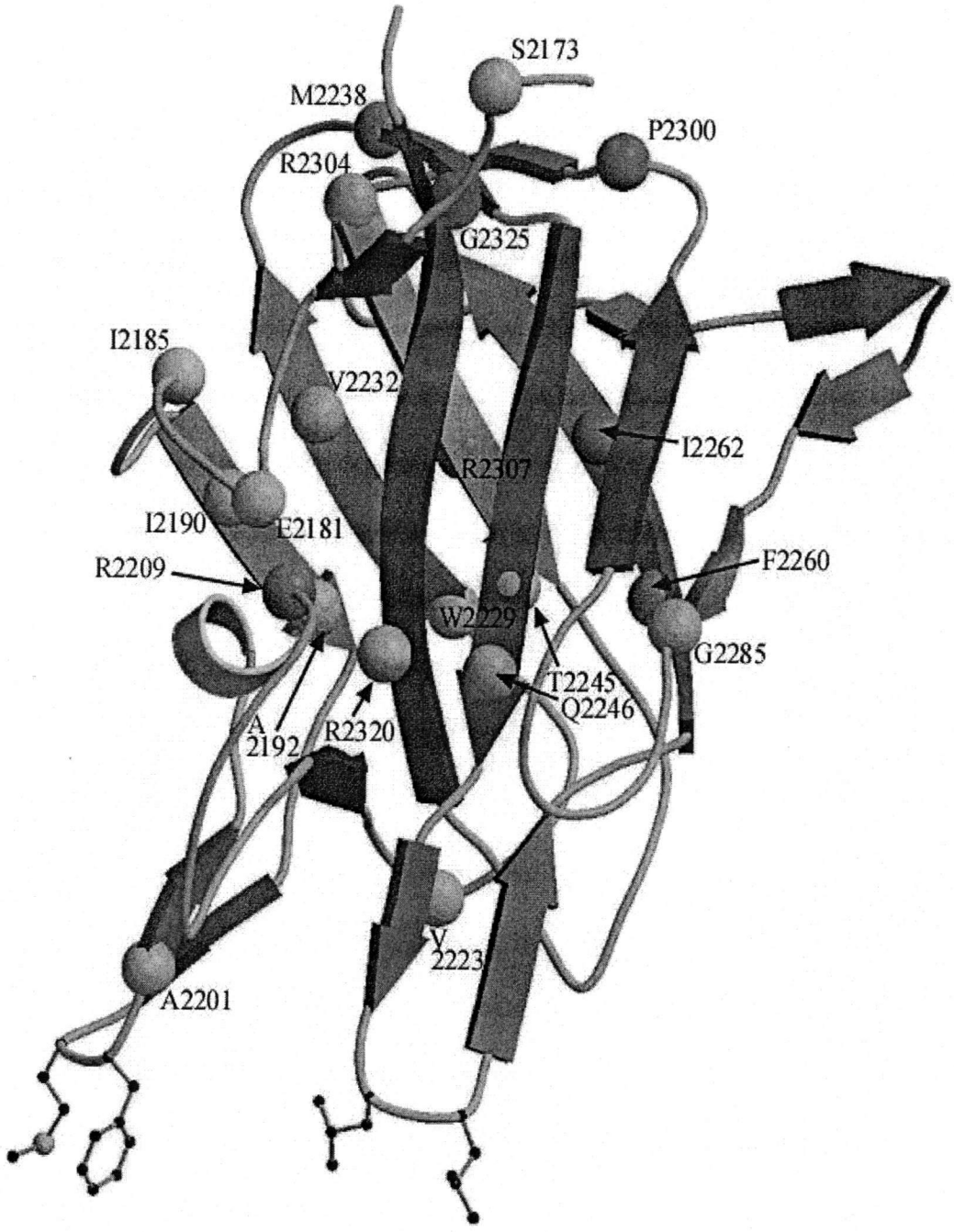

Figure 2.1 Three-dimensional structure of Factor VIII. The positions in the protein fold that are found to be mutated in patients with hemophilia A are shown by spheres. Dark spheres are sites that display severe defects in clotting when mutated; light spheres are sites that display milder defects. The atoms at the bottom of the protein are amino acids thought to embed themselves into exposed membranes at sites of blood-vessel damage. Lenting et al (1998)

Chapter 3
MATERIALS AND METHODS

3.1 Materials

The recombinant Factor VIII used this thesis was provided by Bayer corporation (Bayer Pharmaceuticals Corporation, Berkeley, CA) in a dry carbon dioxide package, it was stored in a -80 ^{0}C freezer. In order to avoid the denaturation of protein when it is melted, we thawed the frozen rFVIII only once, then divided it into 5 ml sterile Cryovial vials (Fisherbrand, Pittsburgh, PA). Only one is taken form the freezer and let it thawed in ice when used.

All surfactants used are of analytical reagent grade. Sodium Dodecyl Sulfate (SDS) (L-4390, Lot 101K0035) and Dodecyltrimethylammonium bromide (DTAB) (D-8638, Lot 52H0711) were purchased from Sigma Chemical Co. (St. Louis, MO). SDS and DTAB are both commonly used ingredient in detergents. Polyoxyethylenesorbitan Monooleate (Tween 80) (P-8074, Lot 121K0041) was also purchased from Sigma Chemiclal Co. (St. Louis, MO). Distilled deionized water (DDW) was produced by MEGA-PURE® 3A WATER STILL system (Barnstead Thermolyne Corporation, Dubuque, IA). Sodium phosphate monobasic NaH_2PO_4 (Lot 7892 KVXK) and sodium phosphate dibasic Heptahydrate $Na_2HPO_4 \cdot 7H_2O$ (Lot 7914 V33596) were both purchased from Mallinckrodt Baker, Inc. (Paris, KY). Buffer solutions (PH=7.0) were prepared by mixing 1.08g $Na_2HPO_4 \cdot 7H_2O$ and 0.55g NaH_2PO_4 dissolved in 1L distilled, deionized water. Sodium azide (EM Science, Cherry Hill, N. J.), used as an antimicrobial agent, was

added to the solution at a concentration of 0.02% (mass per volume) prior to mixing. Factor VIII was diluted by phosphate buffer (PH 7.0). Both buffer and protein solutions were filtered using 0.45 μm filter (Millipore Corp., Bedford, MA) prior to use to remove undissolved materials and other impurities.

3.2 Surface/Interfacial Tension Theory

Dynamic surface tension is measured by FTÅ T10 Tensiometer, which was purchased from First Ten Ångstroms (Portsmouth, VA). Surface tension is a measurement of the cohesive energy present at an interface. The molecules of a liquid attract each other. The interactions of a molecule in the bulk of a liquid are balanced by an equal attractive force in all directions. Molecules on the surface of a liquid experience an imbalance of forces as indicated below.

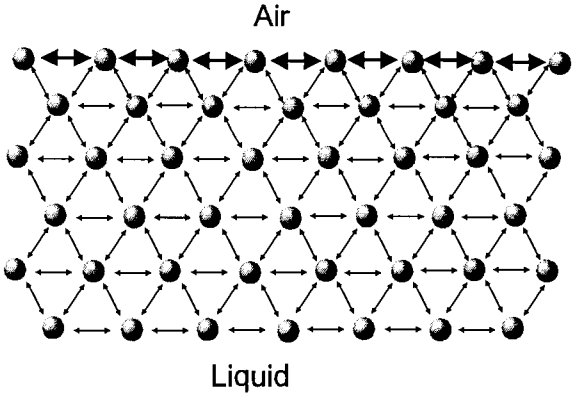

Figure 3.1 Water molecules in bulk solution and at the air/water interface

The net effect of this situation is the presence of free energy at the surface. The excess energy is called surface free energy and can be quantified as a

measurement of energy/area. It is also possible to describe this situation as having a line tension or surface tension which is quantified as a force/length measurement. The common units for surface tension are dynes/cm or mN/m. These units are equivalent. Polar liquids, such as water, have strong intermolecular interactions and thus high surface tensions. Any factor which decreases the strength of this interaction will lower surface tension. [Adamson, 1975]

In these experiments a probe is hung on a balance and brought into contact with the liquid interface tested. The forces experienced by the balance as the probe interacts with the surface of the liquid can be used to calculate surface tension. The forces present in this situation depend on the following factors: size and shape of the probe, contact angle of the liquid/solid interaction and surface tension of the liquid. The size and shape of the probe are easily controlled. The contact angle is controlled to be zero (complete wetting). This is achieved by using probes with high surface energies. FTÅ probes are made of a platinum/iridium alloy which ensures complete wetting and easy and reliable cleaning.

The mathematical interpretation of the force measurements depends on the shape of the probe used. Two types of probes are commonly used, the Du Nouy Ring and the Wilhelmy Plate. Both are available from FTÅ. In our experiments, we use both Du Nouy Ring and Wilhelmy plate, the result show no more than 0.5mN/m difference.

Du Nouy ring: This method utilizes the interaction of a platinum ring with the surface being tested. The ring is submerged below the interface and subsequently raised upwards. As the ring moves upwards it raises a meniscus of the liquid. Eventually this meniscus tears from the ring returns to its original position. The process is shown in the diagram below:

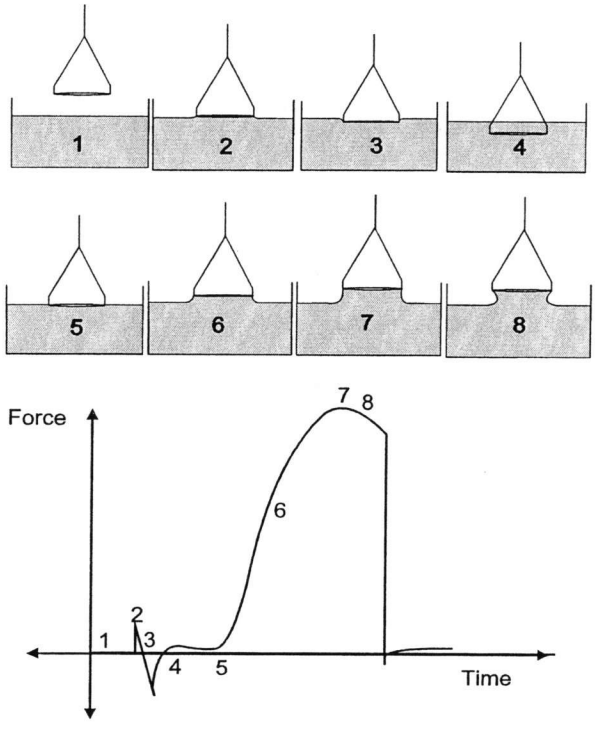

Figure 3.2 Measurement of surface tension by Du Nouy ring.

The ring is above the surface and the force is zeroed. 2. The ring hits the interface and there is a slight positive force because of the adhesive force between the ring and the interface. 3. The ring must be pushed through the interface (due to its interfacial tension) which causes a small negative force. 4. The ring breaks through the interface and a small positive force is measured from the supporting wires of the ring. 5. When lifted through the interface the measured force starts to increased. 6. The force keeps increasing until, 7, the maximum force is reached. 8. After the maximum

there is a small decrease in the force until the lamella breaks. (Krüss, 1992)

The calculation of surface or interfacial tension by this technique is based on the measurement of this maximum force. The measurement of surface and interfacial tension as performed by the T10 tensiometer is based on force measurements of the interaction of a probe with the surface or interface of two fluids. (Lunkenheimer, 1989)

Wilhelmy Plate: This method utilizes the interaction of a platinum plate with the surface being tested. The calculations for this technique are based on the geometry of a fully wetted plate just in contact with, but not submerged in, the heavy phase. In this method the position of the probe relative to the surface is significant. As the surface is brought into contact with the probe the T10 will notice this event by the change in forces it experiences. It will register the height at which this occurs as the 'zero depth of immersion'. The plate will then be wetted to a set depth to ensure that there is indeed complete wetting of the plate (zero contact angles). When the plate is later returned to the zero depth of immersion, the force it registers can be used to calculate surface tension.

3.3 Measurement of Surface Tension Kinetics

All the experiments were carried out at a controlled room temperature ($22\,^{0}C$). First, the frozen rFVIII (-80 ^{0}C freezer) was thawed, the rFVIII provided by Bayer Corporation is at high concentration 677.3IU/ml and 625.9IU/ml, which

were provided by Bayer in 2002 and 2003 separately. 1 mg rFVIII contains 4712 IU. Then rFVIII was diluted to the desired concentration C_p ($28\ \mu g/ml$) with 0.01 M sodium phosphate buffer, PH 7.0. For each experiment, the volume required for the small vessel is 20 ml, the concentration of rFVIII and all kinds of surfactants in the final 20 solution should be as precise as possible, so based on calculation, the volume of each part is indicated below:

1. rFVIII& Tween 80

rFVIII (stock concentration 677.3 IU/ml), the rFVIII should be diluted 5.13 times, Tween 80 (stock concentration 0.2 mM), the volume of Tween 80 added in the final solution X(ml) is calculated from 0.2X/20=Y [Y(mM) is concentration needed in sample]

Total volume of sample 20ml, so the volume of high concentration added to the vessel is 3.9ml, the volume of distilled, doionized water is 20-3.9-X ml.

2. rFVIII & SDS

rFVIII (stock concentration 625.9 IU/ml), the rFVIII should be diluted 4.74 times. SDS (stock concentration 50 mM), the volume of SDS added in the final solution $X(ml)$ is calculated from $\frac{50X}{20} = Y$ [Y(mM) is concentration needed in sample]

Total volume of sample is 20ml, so the volume of high concentration added to the vessel is 4.22 ml, the volume of distilled, doionized water is 20-4.22-X ml.

3. rFVIII&DTAB

DTAB (stock concentration 100mM), the volume of DTAB added in the final solution $X(ml)$ is calculated from $\frac{100X}{20} = Y$ [Y (mM) is concentration needed in sample]

Total volume of sample is 20ml, so the volume of high concentration added to the vessel is 4.22 ml, the volume of distilled, doionized water is 20-4.22-X ml.

After rFVIII is thawed, in order to eliminate the contaminants, it was filtered through a 0.2μm filter prior to measurement, and then it was added to the vessel. The target concentration of Tween 80 Polyoxyethylene Sorbitan Monooleate (polyoxyethylenesorbitan monooleat) we need is 0.002-0.012mM, at a 0.002mM interval and 0.02-0.08mM, at a 0.02mM interval. The reported CMC (critical micelle concentration) is 0.012mM. SDS is a kind of negatively charged, strong surfactants, the molecular weight is 288.4 g/mole, the target concentration we need is 0.005, 0.05,0.5,1,2,3,4,5,6,7,8,9mM, the CMC is 8mM. DTAB is kind of positively charged, strong surfactants, the molecular weight is 308.3 g/mole, the CMC is 15mM, the target concentration we need is 0.005, 0.05, 0.5, 2, 5, 10, 15mM.

The protein solution was gently stirred with a tiny magnetic stirring bar for about 1 minute. The vessel was placed on the stage of the automated tensiometer, the ring or the plate was placed on the hook. The surfactant was added at the

beginning of the measurement, this is necessary to get the real starting point of the protein and surfactant mixture. Between each experiment, the platinum ring or plate was carefully rinsed with deionized, distilled water and 100% ethanol followed by flaming in the oxidizing portion of the flame of a propane burner until the ring or plate was "red" hot. Before each experiment, we tested the surface tension of water using the cleaned ring or plate to validate its cleanliness and the valued it measured is convincible by observing the surface tension around 72mN/m.

Surfactant	MW (g/mole)	CMC(mM)	Stock Concentration(mM)	Charge
Tween 80	1310	0.012	0.2	no
SDS	288.4	8	50	+
DTAB	308.3	15	100	-

Table 3.1 Properties of the three kinds of surfactants used in this paper.

Chapter 4
RESULTS AND DISCUSSION

4.1 Surface Tension Kinetics of Surfactants

4.1.1 Surface Tension kinetics of Tween 80

The kinetic surface tension for Tween 80 at each concentration is shown in Figure 4.1a. It is apparent that the surface tension of Tween 80 decreased with time and approached a steady state after different length of time. The time it needs to approach the steady state dependents on the concentration of Tween. The dynamic surface tension also dependents on the concentration of Tween and time. The $\gamma(c,t)$ values decrease with increasing time, and at the same time, the kinetic surface tension is decreased at higher concentration. The kinetic surface tension begins with a fast decrease followed by slow approach to the steady state. For the concentration below the CMC, the surface tension decreased slowly. When the concentration was above the CMC, the surface tension decreased at a greater rate and more rapidly attained a steady state. From Figure 4.1b, at concentrations below the CMC, the steady state value of surface tension is different for each concentration, and the higher the concentration, the lower the surface tension at steady state; at concentrations above the CMC, the steady state value of surface tension all approached the same for each concentration.

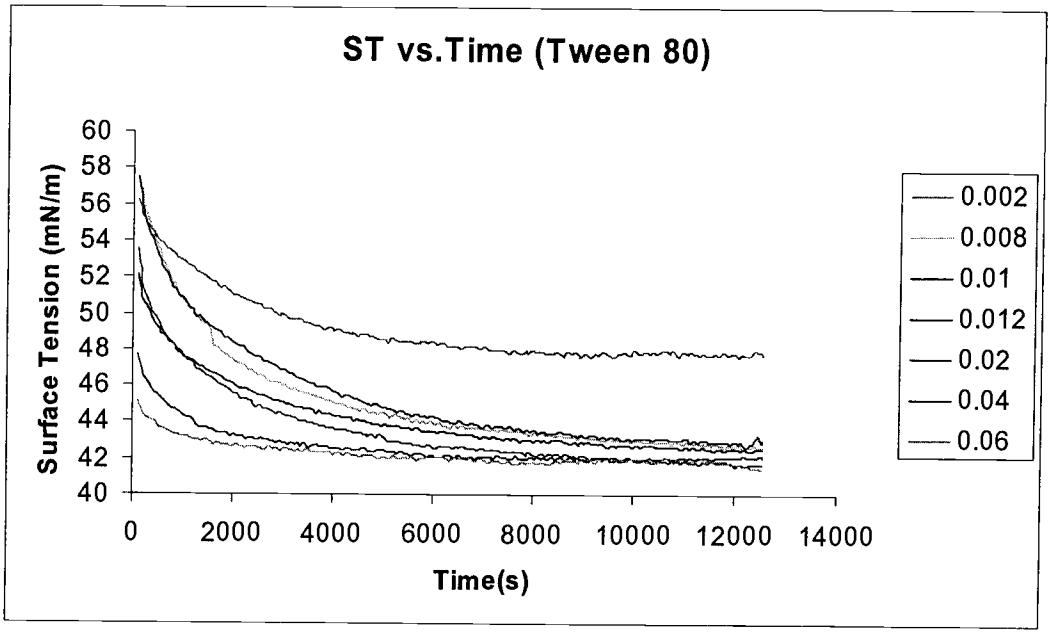

Figure 4.1a Surface Tension as a function of time at different concentrations of Tween 80

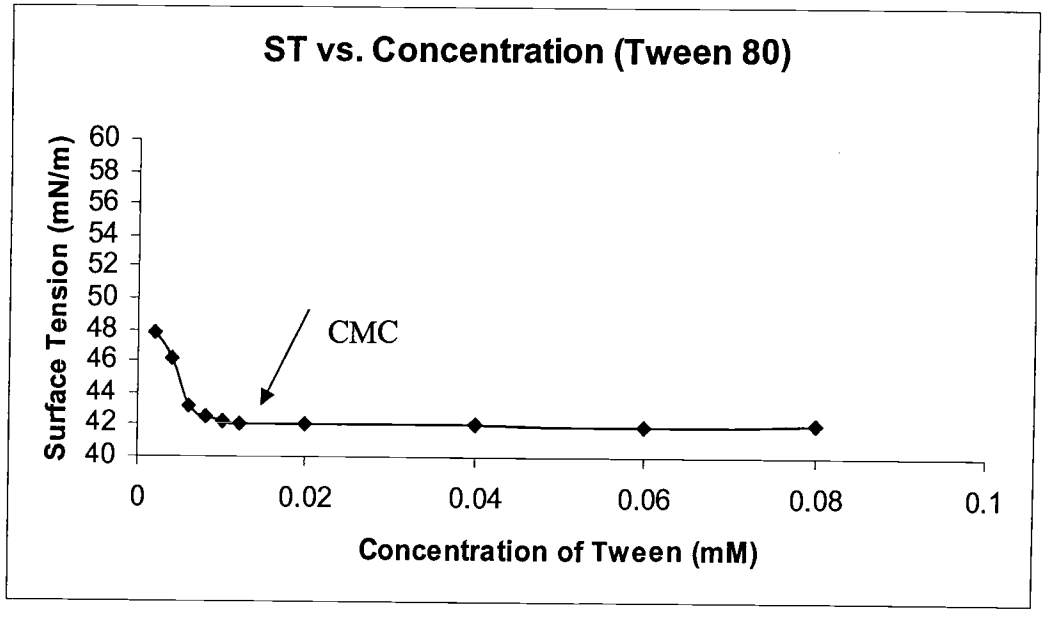

Figure 4.1b Surface tension at steady state as a function of the concentration of Tween

4.1.2 Surface Tension kinetics of SDS

The dynamic surface tension versus time at different concentrations of SDS is shown in Figure 4.2a. Even after short time, the $\gamma(c,t)$ values measured are lower than 72.8mN/m that determined for pure water, and $\gamma(c,t)$ are proportional to the bulk surfactant concentration. As seen in Figure 4.2a all curves look like a straight line, so we can conclude from the plot that SDS exhibits extremely fast adsorption kinetics at all concentrations. There is obvious initial slope and steady state. For SDS, at all concentrations, it reached the steady state at more rapid rate than Tween. And the steady state value decreased with increasing concentration of SDS until around the CMC, where the steady state values are similar which is shown in Figure 4.2b.

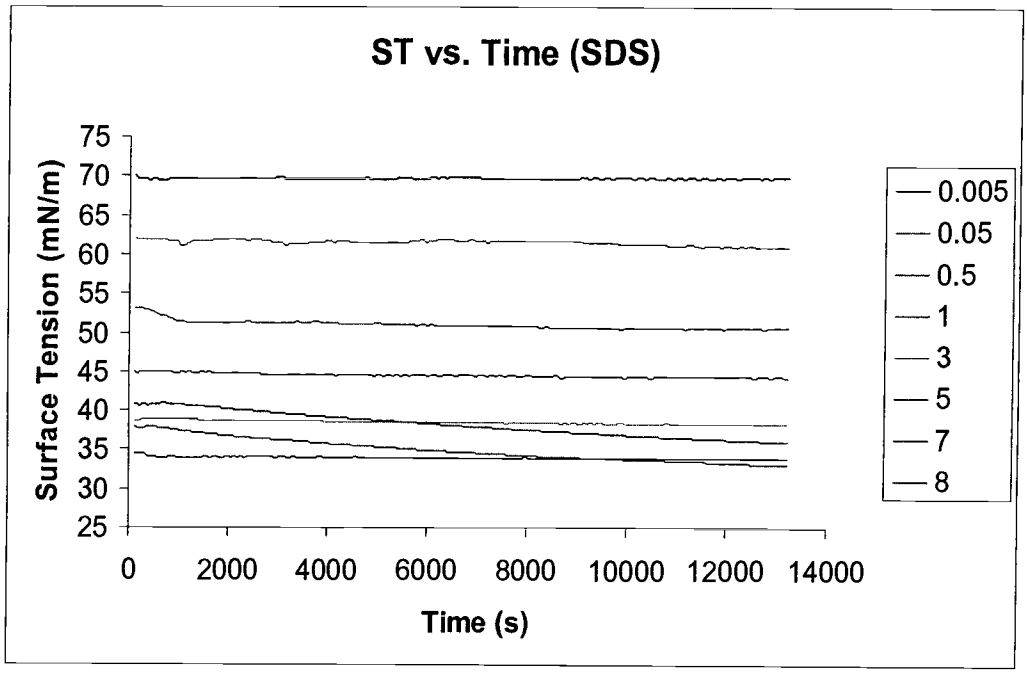

Figure 4.2a Surface Tension as a function of time at different concentrations of SDS

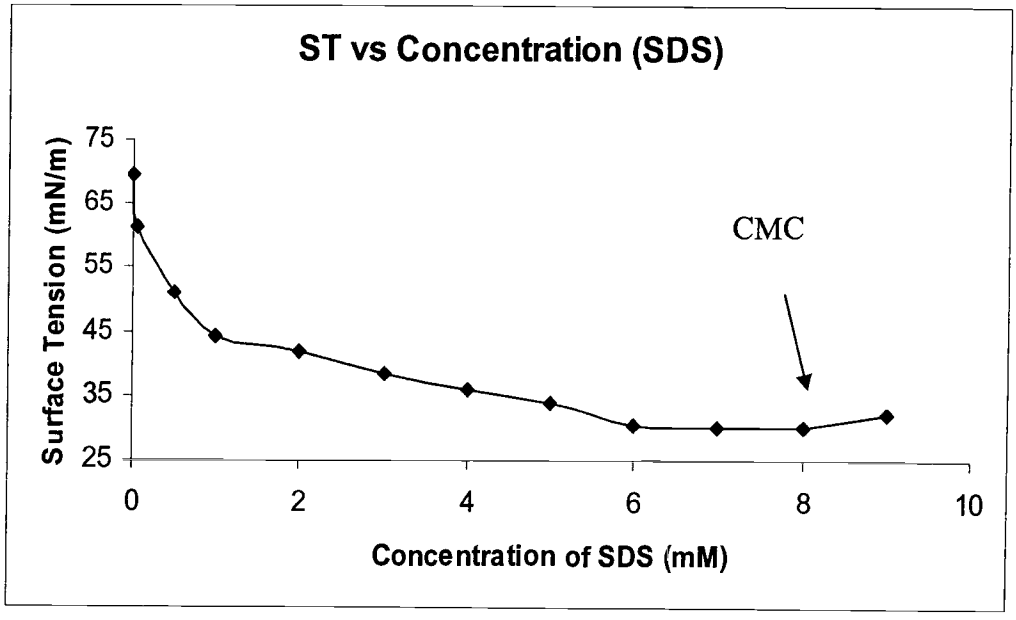

Figure 4.2b Surface tension at steady state as a function of the concentration of SDS

4.1.3 Surface Tension kinetics of DTAB

The dynamic interfacial tension versus time at different concentrations of DTAB is shown in Figure 4.3a. Even after short time, the $\gamma(c,t)$ values measured are lower than 72.8mN/m that determined for pure water, and $\gamma(c,t)$ are proportional to the bulk surfactant concentration. DTAB, although exhibits extremely fast adsorption kinetics at all concentrations below the CMC, when compared with SDS, the rate of adsorption to the interface is shower than SDS as there shows a slope at the beginning. The surface tension at the steady state is proportional to the concentration of DTAB below the CMC, it decreased when the concentration is increased; while around the CMC, the surface tension at the steady state is the same for all concentration around the CMC which is shown in Figure 4.3b.

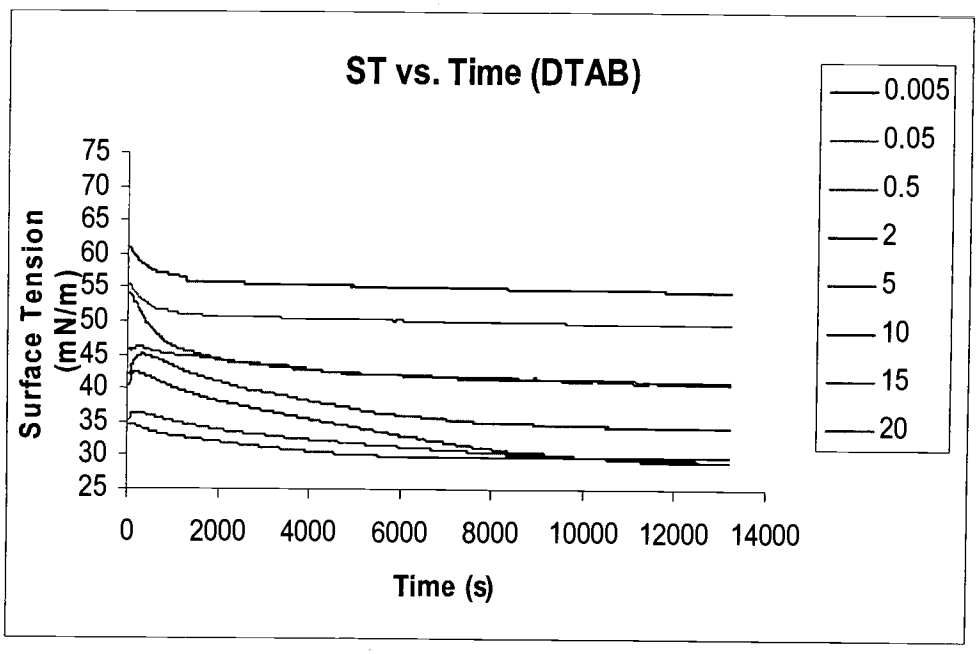

Figure 4.3a Surface Tension as a function of time at different concentrations of DTAB

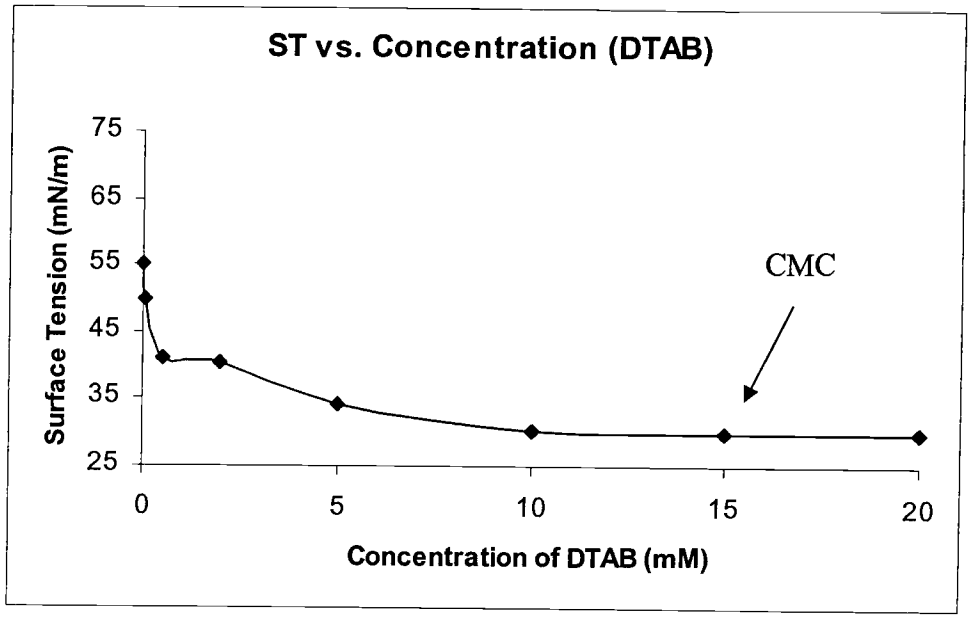

Figure 4.3b Surface tension at steady state as a function of the concentration of DTAB

4.2 Surface Tension Kinetics of Protein with Surfactants

In this session, we will discuss the adsorption kinetics of rFVIII/surfactant solution to the air/water interface from interfacial tension data, and compare the results among the non-ionic, anionic and cationic surfactants.

4.2.1 *Surface Tension Kinetics of Recombinant Factor VIII with Tween 80*

Figure 4.4 illustrates the surface tension changes versus time of rFVIII formulation with different concentration of Tween 80. Figure 4.5 shows the surface tension of low concentration (28μg/ml) rFVIII, it also decreased with time and finally after about half an hour stays at the steady state. The steady state value is about 47.5mN/m. Compared these two graphs, we can visualized that with increased Tween 80 concentration in the rFVIII formulation, the steady state value is decreased corresponds to Tween concentration. The relationship between steady state interfacial tension and added Tween 80 concentration for rFVIII-Tween mixtures is shown in Figure 4.6. The plot was equilibrium interfacial tension for mixed solution against Tween concentration on the logarithm scale. We visualized five regions that consist with the theory that described for ideal circumstances, with random chain molecules and small non-ionic surfactants at equilibrium. At very low Tween concentrations, region I, below 0.004mM, the surface tension at steady state is 47mN/m, since pure rFVIII reach steady state value at 47.5mN/m, so the interfacial adsorption behavior is governed by pure rFVIII; then the surface tension decreased with increasing Tween concentration in region II, 0.004mM to 0.01mM,

for low concentration of rFVIII, even at steady state, the surface is not fully occupied by rFVIII, there are still "empty site" at the air/water interface, Tween molecules may occupy these "empty site" and leads to decrease of surface tension, as well as the formation of surface active, rFVIII-Tween complexes; then a "level off' was observed in region III, 0.01mM to 0.02mM, in this concentration range, there is no change in the component at the air/water interface due to the same surface tension, the hypothesis is made that it is energetically favorable for Tween to bind to rFVIII but not to the air/water interface. Interestingly, the CMC for pure Tween in distilled deionized water 0.012mM maybe exceeded in this range. In region IV, the interfacial tension decreased again with increasing Tween concentration up to about 0.042mM, this due to Tween replaced rFVIII from the interface continually until it reach region V, rFVIII was completely displaced by Tween from the interface beyond 0.042mM Tween. After this decrease, further increases in Tween concentration have no effect on interfacial tension change, and the CMC for rFVIII –Tween system is determined as the concentration defining the transition between regions IV and V, about 0.042mM to 0.046mM in the tests. Compared with the CMC for pure Tween, we observed increased CMC for rFVIII and Tween mixtures, this due to mixtures of rFVIII and Tween in solution, the binding of Tween to rFVIII will reduce the available Tween molecules for interaction with rFVIII at the interface.

rFVIII provided by Bayer already contains 5ppm (or 0.0038mM) Tween 80 when diluted to 131.9IU/ml. So in order to know the effect of Tween 80, it is

necessary to compare the kinetic data recorded for rFVIII/Tween complex with that for Tween in the absence of rFVIII at the concentration 0.004 higher than the complex system. Figure 4.7a-4.7d shows the surface tension vs. time profile which contains the same percentage of Tween at each graph. No appreciable difference observed in the steady state value of interfacial tension demonstrated by rFVIII/Tween mixtures and by Tween alone, once the Tween concentration reached about 0.014mM, suggesting that the steady state interfacial behavior is governed by Tween at concentrations well below that defining the transition between regions IV and V. So we conclude that above a Tween concentration about 0.014mM, the equilibrium interfacial tension appears to be dominated by Tween, and Tween itself exhibits faster adsorption kinetics compared with rFVIII.

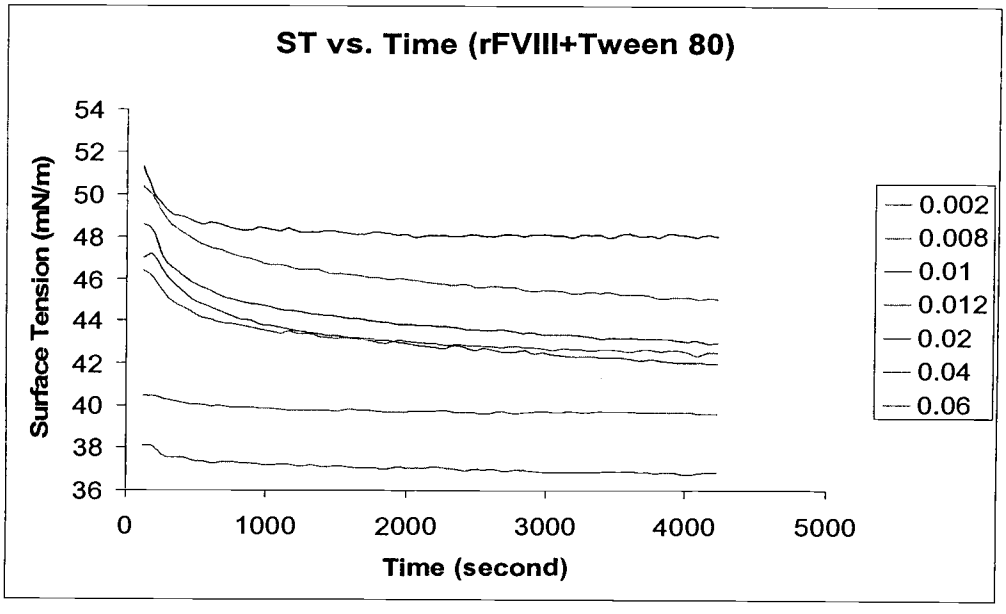

Figure 4.4 Surface Tension as a function of time at different concentrations of rFVIII/Tween 80 mixture

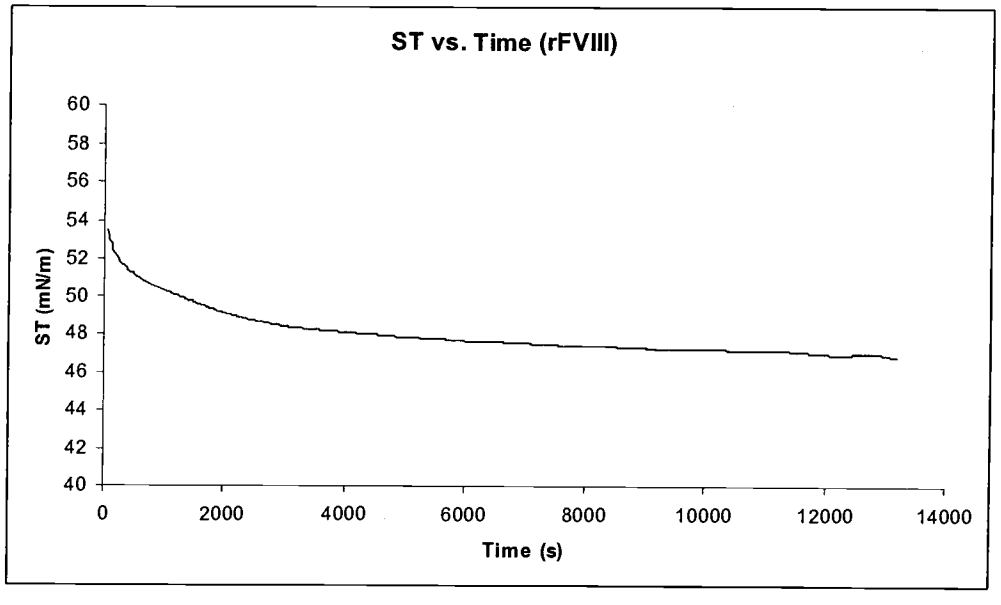

Figure 4.5 Surface Tension as a function of time for pure rFVIII (28 μg/ml)

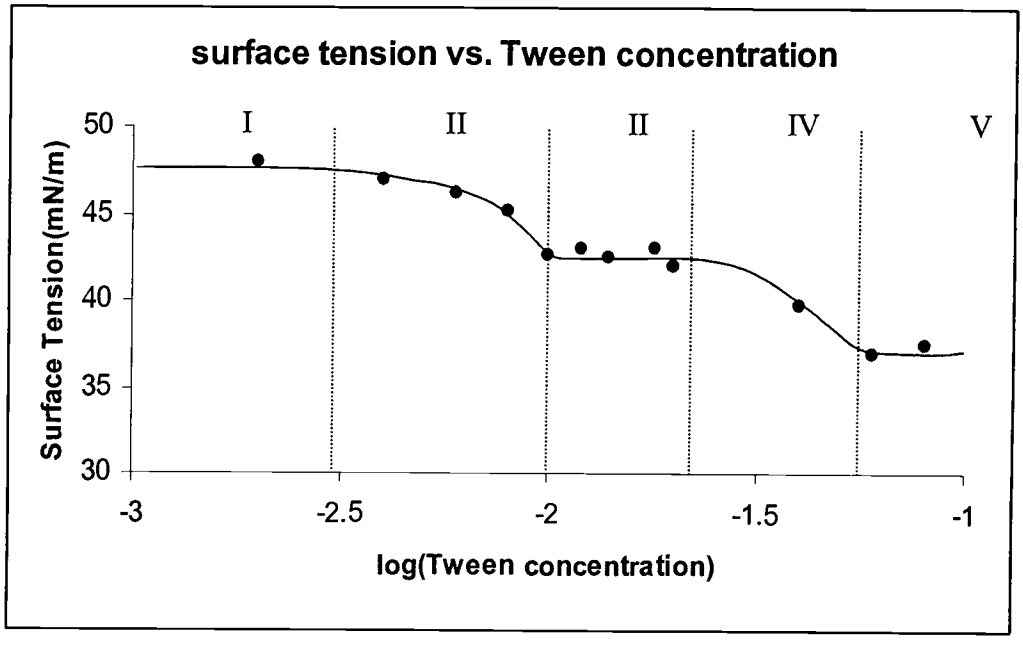

Figure 4.6 Surface Tension as a function of Tween concentration on the logarithm scale.

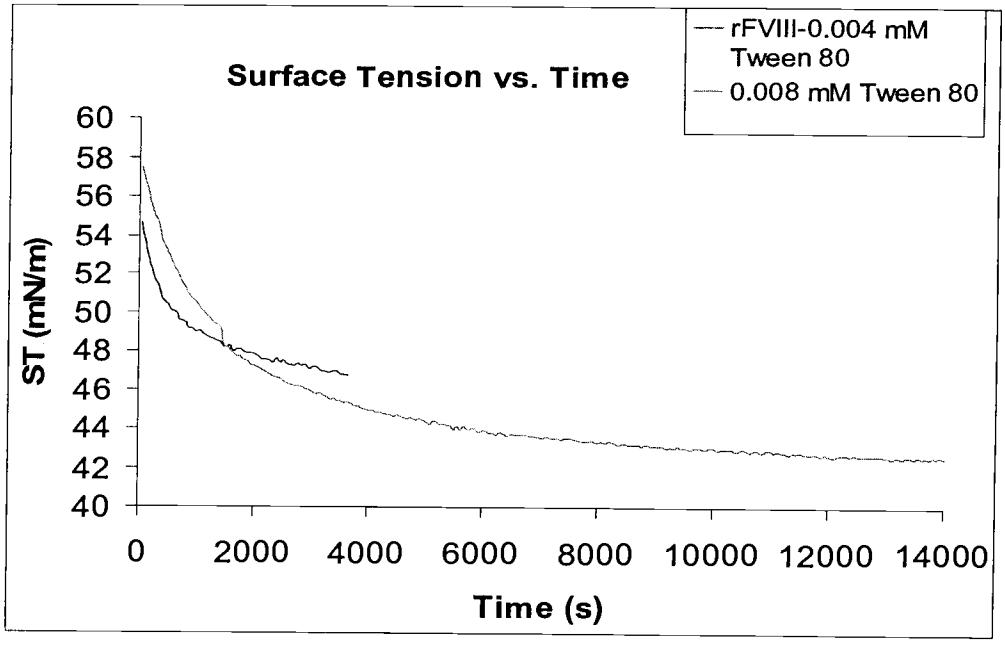

Figure 4.7a Surface Tension comparisons between rFVIII/Tween mixture and Tween with 0.008mM Tween in formulation

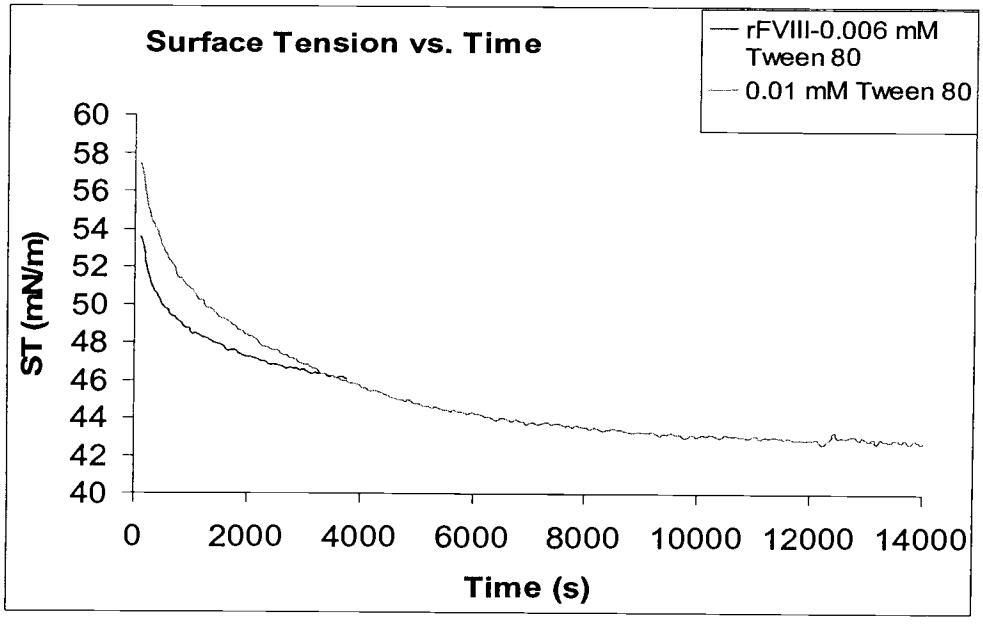

Figure 4.7b Surface Tension comparisons between rFVIII/Tween mixture and Tween with 0.01mM Tween in formulation

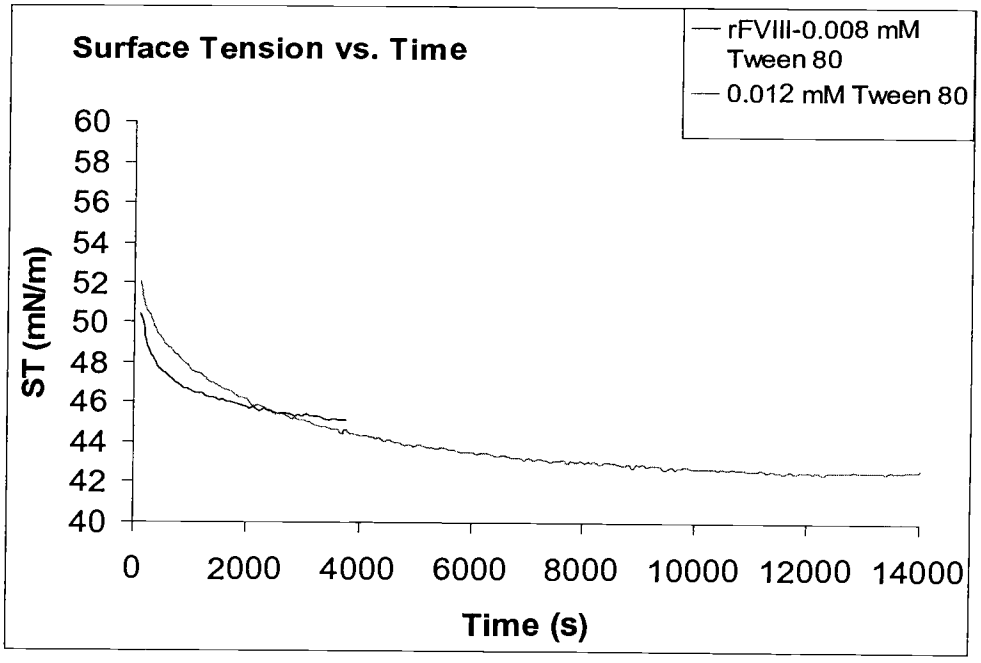

Figure 4.7c Surface Tension comparisons between rFVIII/Tween mixture and Tween with 0.012mM Tween in formulation.

4.2.2 Recombinant Factor VIII with SDS

The equilibrium surface tensions are plotted versus C_s in Figure 4.8 and 4.9 (on the logarithm scale). At low concentrations, from 0.005mM to 5mM, the surface tensions of the mixed solutions are well below the pure SDS solutions, and we didn't observe the trend that they will emerge even after sufficient long time. At 0.005mM and 0.05mM, the rFVIII/SDS complex attained their equilibrium at around 39mN/m; at the concentration range from 0.5mM to 5mM of SDS, the complex system attained their equilibrium around 32mN/m. The steady state value are much lower than pure rFVIII adsorption to the air/water interface which is 47mN/m, this means the interface is covered by SDS or rFVIII-SDS complex, and rFVIII-SDS complex has higher affinity to the interface than pure SDS. There are strong interactions between SDS and rFVIII at the surface even at very low SDS concentrations. At higher concentration of SDS, these bind to the protein in a highly cooperative adsorption, which is characteristic for hydrophobic interaction. The binding of SDS molecules might change the conformation of rFVIII. When SDS concentration is increased over 6mM, different adsorption kinetics is observed. In the concentration range from 6mM to 9mM, initially, the surface tension of rFVIII/SDS solution is lower than SDS solution at the same length of time, as the adsorption process is going on, the two surface tension versus time curves is merged together, the surface tension of rFVIII/SDS solution approaches that of pure SDS solutions, indicating that the interfacial layer probably contains barely only SDS

molecules. We also observed the time that when the two curves merge is dependent on the SDS concentration. These can be explained by Figure 4.10. The air/water interface is covered by rFVIII-SDS complex, so the surface tension of mixed solution is initially below that of pure SDS, rFVIII-SDS has higher priority to adsorb to the interface over SDS, but eventually SDS molecules replace the rFVIII/SDS complex from the interface and achieved the pure monolayer of SDS at the interface.

Ionic surfactant, such as SDS, may cause unfolding of the secondary or tertiary structure of proteins. This may due to the binding of the ionic SDS head-group to proteins. In figure 4.10, the surface tension at steady state for each SDS concentration as a function of the logarithm of the surfactant concentration displays three characteristic regions for rFVIII/SDS system, with increasing SDS concentrations, there are: (I) electrostatic binding, (II) cooperative binding, and (III) saturation. [Jones (1975)]

At low SDS concentrations, below 0.5mM, the binding between rFVIII and SDS is dominated by electrostatic force. As more surfactants are introduced into solution, the cooperative binding may occur, characterized by the sharp decrease in surface tension with increasing surfactant concentration. In this concentration range, the binding affinity increases as more SDS molecules are bound to rFVIII. And the formation of micelle-like structure of rFVIII/SDS may take place, which is a cooperative process. It has been suggested that the protein may undergoes unfolding

in the cooperative binding region. When SDS concentration is further increased, the surface tension is "level off", means that the system has already attained saturation, during this range, rFVIII may fully unfolded and SDS molecules bound equally on the unfolded polypeptide chain.

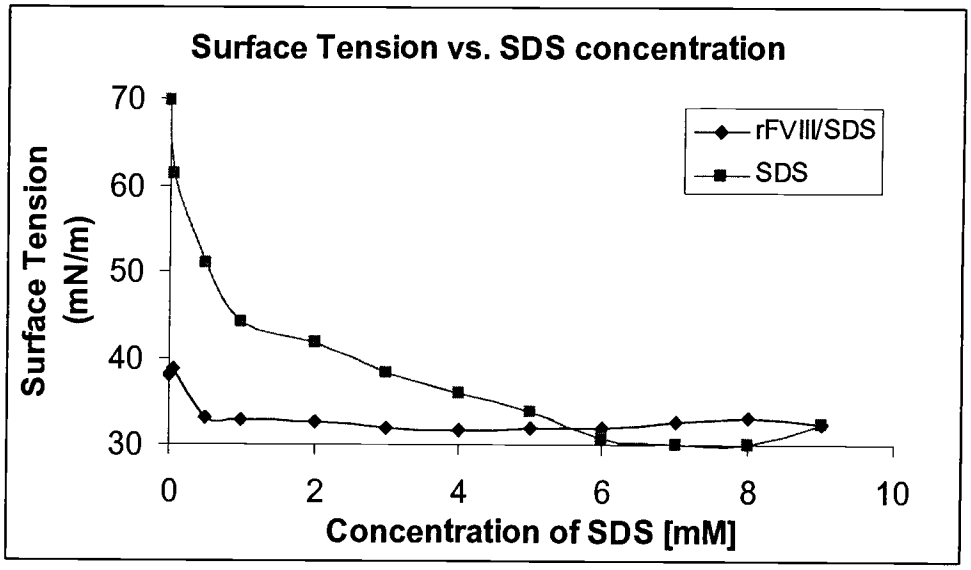

Figure 4.8 Surface tension vs. SDS concentration, comparison between rFVIII/SDS mixture and SDS

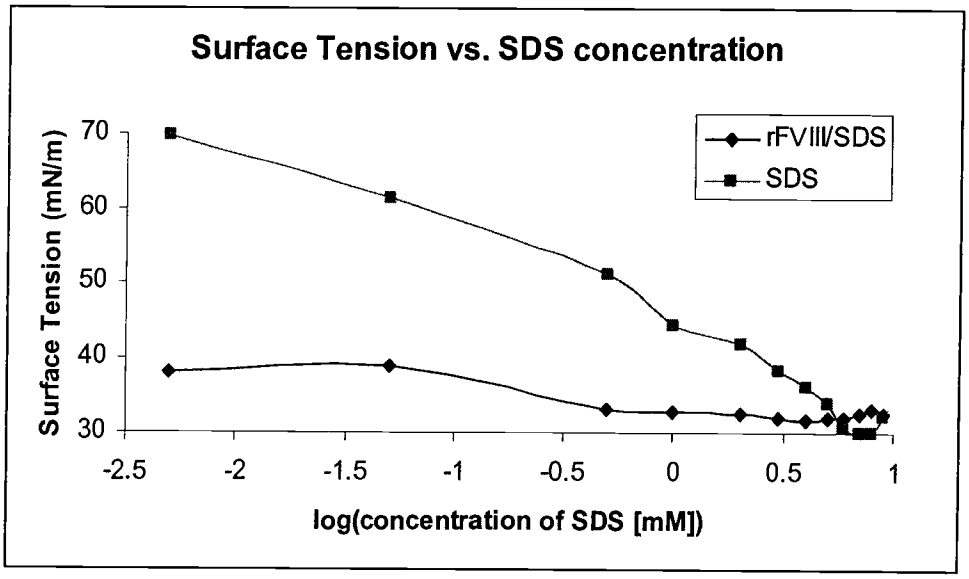

Figure 4.9 Surface tension vs. SDS concentration, comparison between rFVIII/SDS mixture and SDS on the logarithm scale.

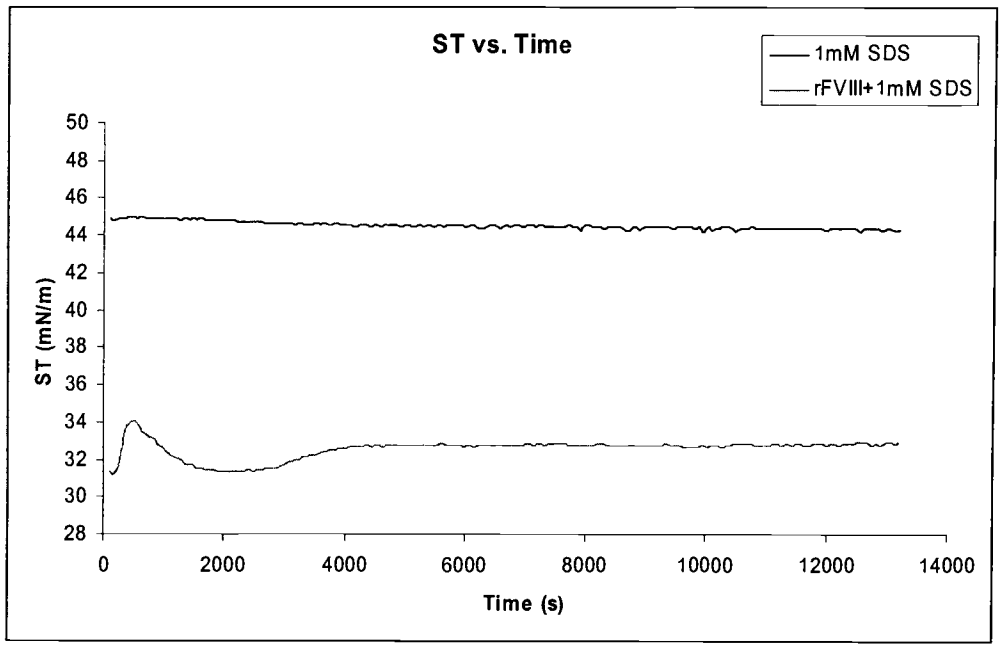

Figure 4.10a Surface Tension comparisons between rFVIII/SDS mixtures and SDS with 1mM of SDS in formulation

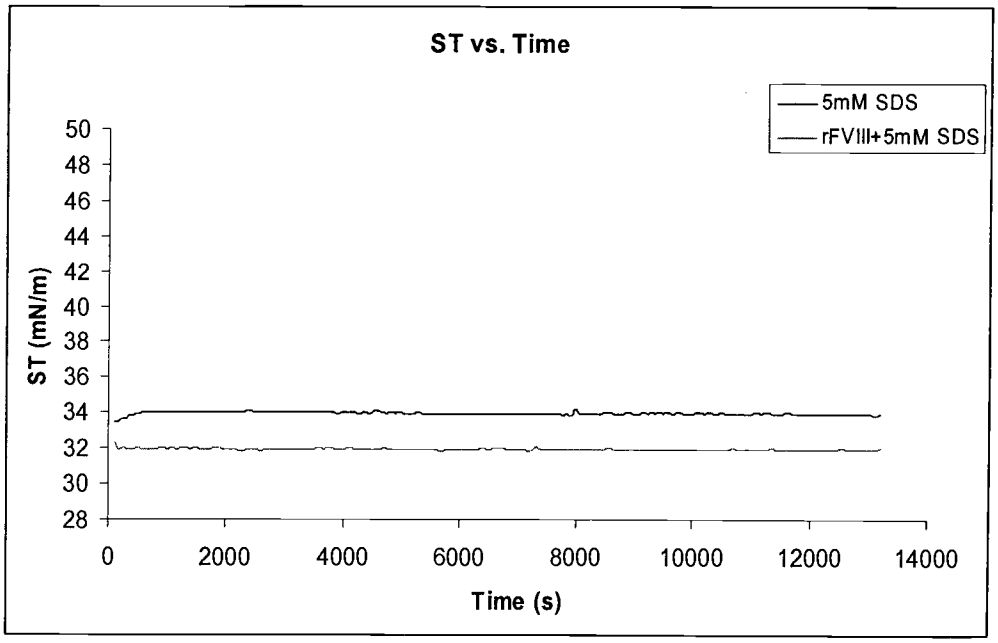

Figure 4.10b Surface Tension comparisons between rFVIII/SDS mixtures and SDS with 5mM of SDS in formulation

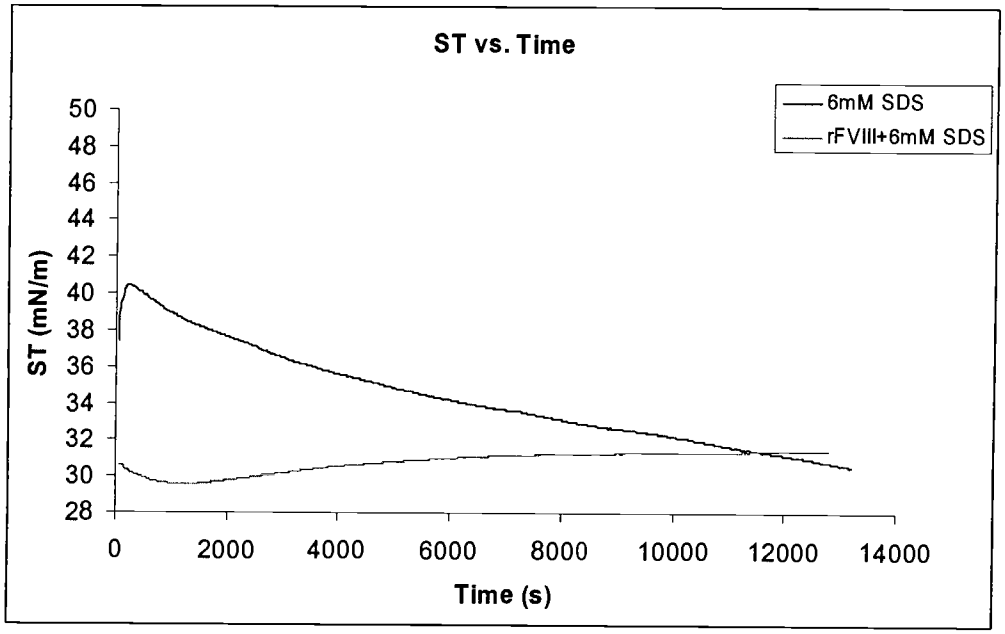

Figure 4.10c Surface Tension comparisons between rFVIII/SDS mixtures and SDS with 6mM of SDS in formulation

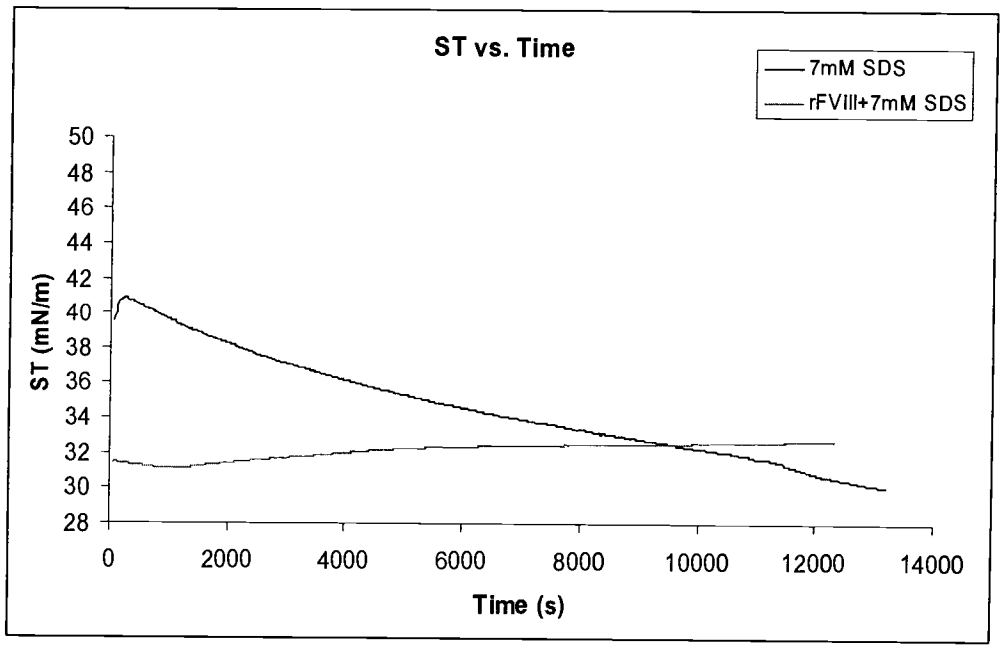

Figure 4.10d Surface Tension comparisons between rFVIII/SDS mixtures and SDS with 7mM of SDS in formulation

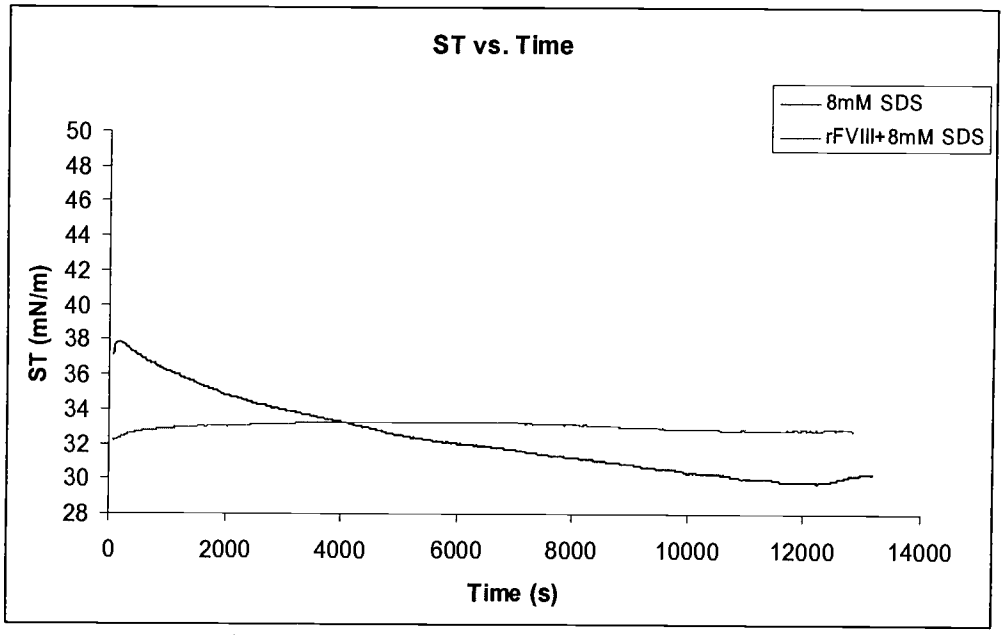

Figure 4.10e Surface Tension comparisons between rFVIII/SDS mixtures and SDS with 8mM of SDS in formulation

4.2.3 Recombinant Factor VIII with DTAB

The adsorption kinetics of the pure surfactant and protein/surfactant mixed solutions were studied while varying the DTAB concentration. The equilibrium surface tensions are plotted versus C_s in Figure 4.11 and 4.12 (on the logarithm scale). Surface tensions of the mixed solutions are also well below the pure DTAB solutions, underlining strong interactions between DTAB and rFVIII at the surface even at very low DTAB concentrations. As a cationic surfactant, DTAB expressed strong interaction with rFVIII at the air/water interface, and the strong interaction resembles that with SDS. At higher concentration of DTAB at 5mM, the surface tension approaches that of pure DTAB solutions, indicating that the interfacial layer probably contains barely only DTAB molecules.

At low concentrations of DTAB, rFVIII/DTAB complex are formed at the air/water interface and these complexes are more surface active than DTAB and rFVIII. From the surface tension data, at 0.05mM, 0.5mM and 2mM DTAB, the equilibrium surface tension value are lower then that of pure DTAB at the same concentrations. And we cannot see any trend that the surface tension curves will merge even after several hours. So when the rFVIII/DTAB mixed solution attains steady state, rFVIII is occupied by DTAB and adsorbs at the interface with higher affinity. But at higher DTAB concentrations, from surface tension data, the two curves for our DTAB and rFVIII/DTAB tend to merge together. These can be

explained by Figure 4.13. The rFVIII/DTAB complex is replaced by pure surfactant molecules so surface tensions are the same for the two kinds of solutions.

DTAB is also an ionic surfactant, like SDS, the only difference is their charged group, DTAB may cause unfolding of the secondary or tertiary structure of proteins. This may due to the binding of the cationic DTAB head-group to proteins. In Figure 4.12, the surface tension at steady state for each DTAB concentration as a function of the logarithm of the surfactant concentration displays three characteristic regions for rFVIII/DTAB system, with increasing DTAB concentrations, there are: (I) electrostatic binding, (II) cooperative binding, and (III) saturation. [Jones (1975)]

At low DTAB concentrations, usually below 0.5mM, the binding between rFVIII and DTAB is dominated by electrostatic force. As more surfactants are introduced into solution, the cooperative binding may occur, characterized by the sharp decrease in surface tension with increasing surfactant concentration. In this concentration range, the binding affinity increases as more DTAB molecules are bound to rFVIII. And the formation of micelle-like structure of rFVIII/DTAB may take place, which is a cooperative process. It has been suggested that the rFVIII may undergoes unfolding in the cooperative binding region. When DTAB concentration is further increased, the surface tension is "level off", means that the system has already attained saturation, during this range, rFVIII may fully unfolded and DTAB molecules bound equally on the unfolded polypeptide chain.

Compared with surface tension kinetics between SDS and DTAB, below CMC for both SDS and DTAB, it was found that displacement of rFVIII from the air/water interface by ionic surfactants are similar for SDS and DTAB. But when comparing the surface tension kinetics at the same concentration between SDS and DTAB, rFVIII/SDS system represents higher affinity to the air/water interface than that of rFVIII/DTAB system, indicating by the slightly lower steady state surface tension values. This effect, however, is minimized as increasing surfactant concentrations, that around the CMC of both SDS and DTAB, the surface tension approached the similar value. This due to the increased percentage of surfactant layer adsorbed at the interface and the rFVIII/surfactant complexes are replaced by pure surfactant molecules. SDS has higher binding force with rFVIII than DTAB, but these two ionic surfactant all induced the conformational change of rFVIII, in opposite, Tween 80, non-ionic surfactant, has negligible effect on protein conformational change.

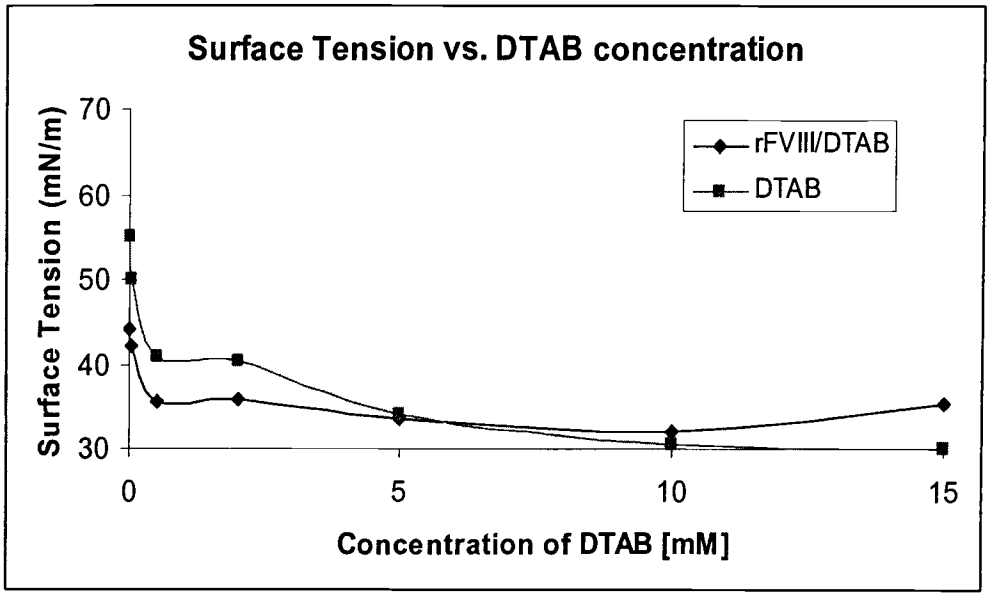

Figure 4.11 Surface tension vs. DTAB concentration, comparison between rFVIII/DTAB mixture and DTAB.

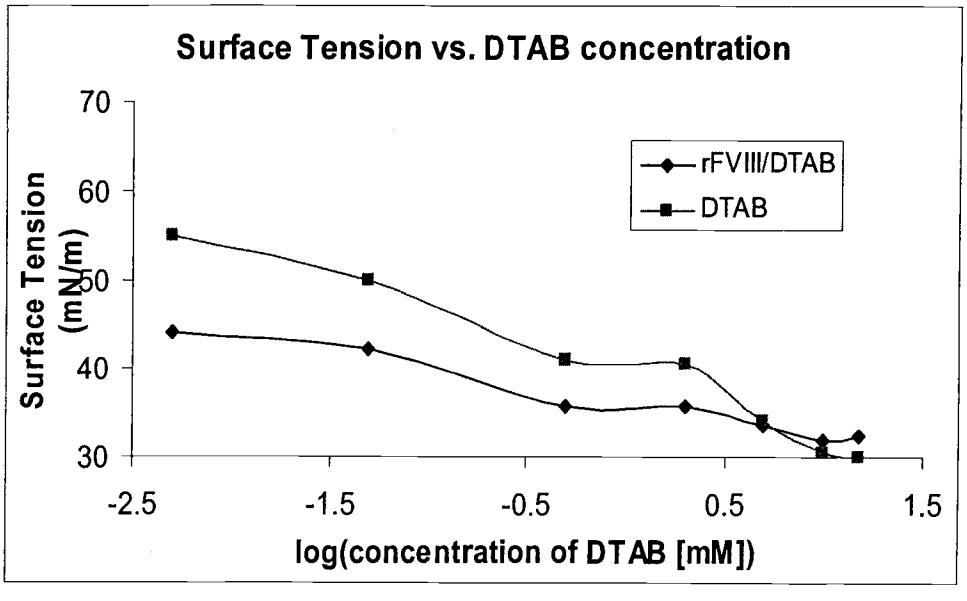

Figure 4.12 Surface tension vs. DTAB concentration, comparison between rFVIII/DTAB mixture and DTAB on the logarithm scale.

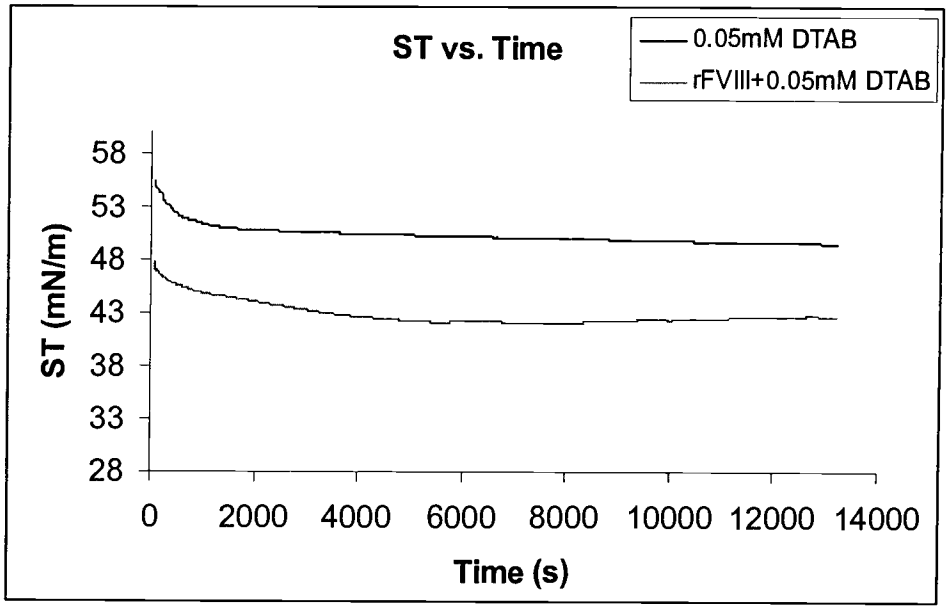

Figure 4.13a Surface Tension comparisons between rFVIII/DTAB mixtures and DTAB with 0.05mM DTAB in formulation

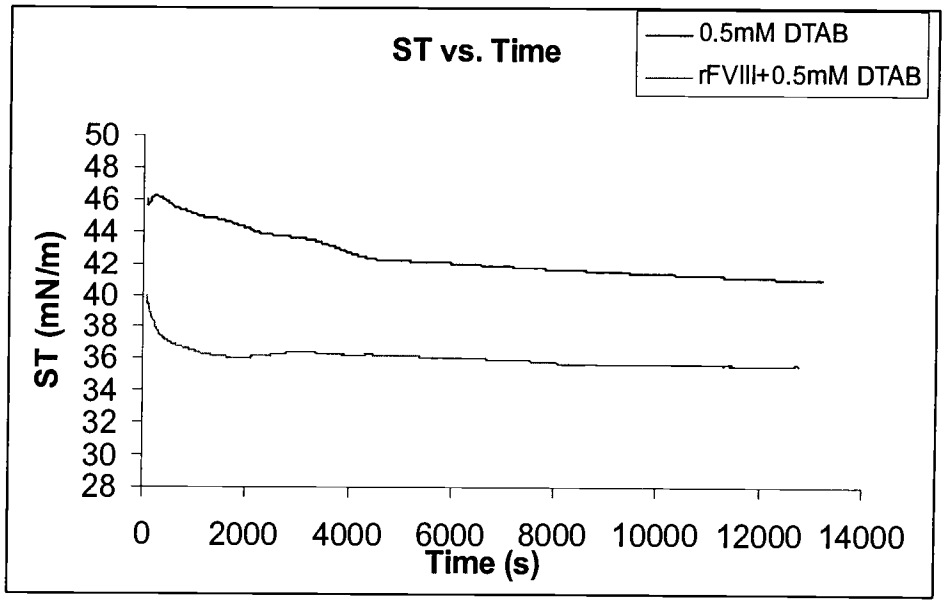

Figure 4.13b Surface Tension comparisons between rFVIII/DTAB mixtures and DTAB with 0.5mM DTAB in formulation

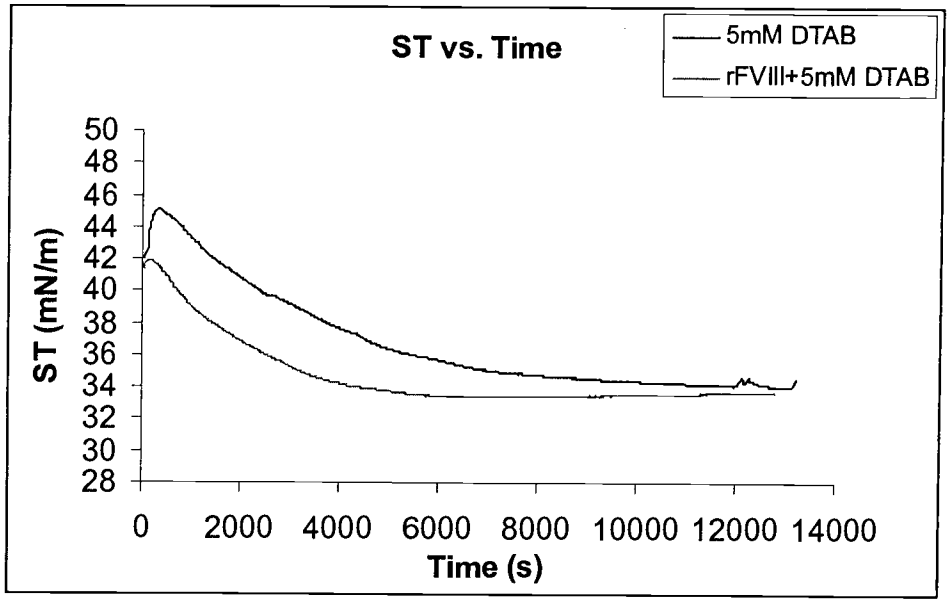

Figure 4.13c Surface Tension comparisons between rFVIII/DTAB mixtures and DTAB with 5mM DTAB in formulation

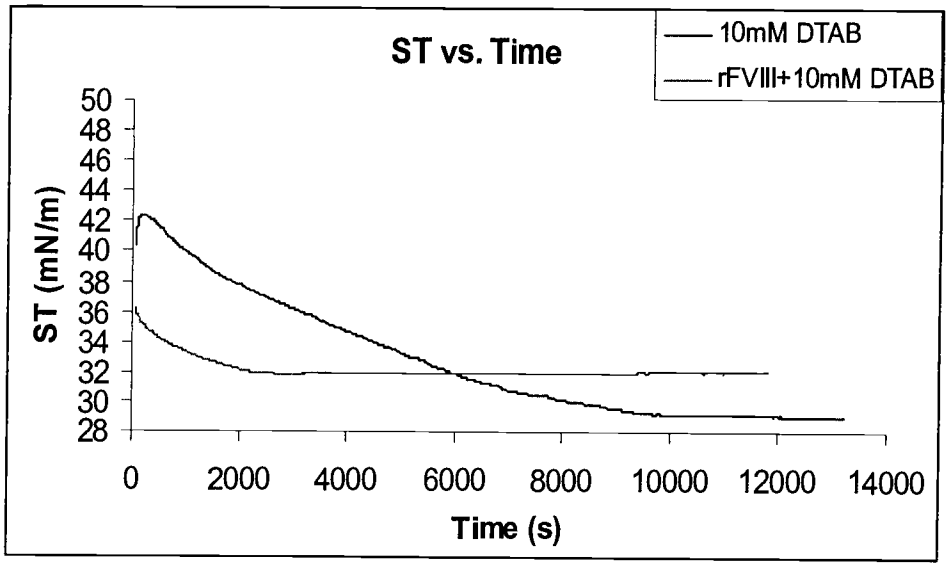

Figure 4.13d Surface Tension comparisons between rFVIII/DTAB mixtures and DTAB with 10mM DTAB in formulation

4.3 Analysis with Reference to the Adsorption Model

The first-order rate constants are estimated with the empirical equation,

$$\ln((\gamma-\gamma_{ss})/(\gamma_0-\gamma_{ss}))=-Kt \qquad [4.1]$$

where γ is the interfacial tension at any time t, γ_{ss} is the equilibrium interfacial tension and γ_0 is the interfacial tension of the pure solvent, which is 72.8mN/m. $K(\text{min}^{-1})$ is the first-order rate constant. As previously observed by Graham and Phillips, a plot of Equation [4.1] usually yields two linear regions with the change of slope that occurred at the time that interfacial tension attains its steady state. The initial rapid decrease, which exists while surface concentration is increasing, reflects the adsorption of substance from the bulk solution to the air/water interface, this corresponds to a first-order rate constant of adsorption (K_1). The second slope, which exists when the surface concentration remains unchanged, is related to rearrangement of the molecules within the interfacial layer, this corresponds to the first-order rate constant of rearrangement (K_2).

4.3.1 Rate constant for individual substances

4.3.1.1 Tween 80

The first-order rate constants for Tween 80 are shown in Table 4.1. For Tween 80, a plot of Equation 4.1 usually three linear regions, the initial rapid decrease, which is defined as K_1, represents the penetrating of surfactant molecules to the air/water interface; followed by the second and third slope. The second slope, we defined it as $K_1^{'}$, the pseudo first-rate constant of adsorption, because followed by the initial quick adsorption of surfactant to the interface, there is monomer layer formed at the interface, this may cause a diffusion barrier to the continuous adsorption of surfactant molecules and slowed down the rate of adsorption. Until the interfacial area is totally occupied by surfactant, the slope changed to the third region, which corresponds to the first-order rate constant of rearrangement K_2.During this region, the surface concentration is constant, which related to rearrangement of surfactant molecules within the interfacial layer.

Compared the K_1, $K_1^{'}$ and K_2 at each concentration of Tween 80, at higher Tween concentration, K_1 and $K_1^{'}$ are both increased. Higher K_1 value indicates that Tween molecules adsorb faster to the hydrophobic air/water interface, we attribute this faster adsorption to the increased molecules in the bulk solution. K_2 is related to the stability of the interfacial layer, higher K_2 values indicates the higher mobility

of the Tween monomer layer at the air/water interface. From 0.002mM to 0.06mM, K_2 doesn't show very big difference so the rearrangement of Tween 80 at the air/water interface is not obvious, because Tween is small molecule, not like protein, they cannot exhibit remarkable rearrangement

First-order rate constants K1 and K2 for each concentration of Tween 80			
Concentration of Tween 80 (mM)	K1 (min-1)	K1'(min-1)	K2 (min-1)
0.002	0.42	0.026	0.012
0.004	0.46	0.024	0.01
0.006	0.63	0.023	0.013
0.008	0.85	0.034	0.014
0.01	0.87	0.039	0.0167
0.012	0.72	0.044	0.0169
0.02	0.85	0.057	0.023
0.04	1.017	0.063	0.021
0.06	1.45	0.063	0.02

Table 4.1 First-order rate constants K1 and K2 for each concentration of Tween 80

4.3.1.2 SDS

The first-order rate constants of SDS are shown in Table 4.2. Compared with Tween 80, SDS exhibited higher K_1, and there is no visible $K_1^{'}$ based on the plot by equation 4.1. This may due to that SDS has higher affinity to the air/water interface than Tween. SDS molecules adsorbed immediately after they were introduced in solution and bound tightly on the interface, when the concentration is increased, K_1 is correspondingly increased due to the abundant molecules in

solution to adsorb to the interface. Above 5mM, however, K_1 decreased when the concentration is further increased to 6, 7, 8mM, we hypothesized that the interface is already covered by SDS molecules and there is a diffusion barrier to further adsorption of SDS molecules, it slowed down the adsorption rate. From K_2, there is no obvious concentration dependence; this indicates that small SDS molecules have lower mobility in the interfacial layer.

First-order rate constants K1 and k2 for each concentration of SDS		
Concentration of SDS(mM)	K1(min -1)	K2 (min -1)
0.005	1.49	0.0048
0.05	1.54	0.0024
0.5	1.61	0.012
1	2	0.006
2	2.21	0.006
3	2.29	0.012
4	2.49	0.012
5	2.92	0.013
6	1.78	0.01
7	1.53	0.009
8	1.25	0.0036

Table 4.2 First-order rate constants K1 and K2 for each concentration of SDS

4.3.1.3 DTAB

The first-order rate constants of DTAB are shown in Table 4.3. Compared with Tween 80, DTAB also exhibited higher K_1, because DTAB is potent surfactant which has higher affinity to the air/water interface, but compared between SDS and

DTAB, at the same concentration, SDS shows a slightly higher K_1, and this is consistent with the results from dynamic surface tension data. Small DTAB molecules have lower mobility in the interfacial layer as same as SDS molecules due to the similar K_2.

First-order rate constants K for each concentration of DTAB			
Concentration of DTAB (mM)	K1 (min-1)	K1' (min-1)	K2 (min-1)
0.05	1.148	0.058	0.011
0.5	1.849	-	0.0152
2	0.916	0.058	0.0162
5	1.631	-	0.024
10	1.468	-	0.018
15	2.161	-	0.018

Table 4.3 First-order rate constants K1 and K2 for each concentration of DTAB

4.3.1.4 rFVIII (28μg/ml)

For pure factor VIII (28μg/ml), the first-order rate constant of adsorption is K_1=1.644, first-order rate constant of rearrangement K_2=0.115. Factor VIII has a higher first-order rate constant of adsorption than pure Tween 80, SDS and DTAB. Big protein molecules adsorb faster than the small surfactant molecules. Factor VIII has higher surface affinity than surfactants. Once factor VIII was adsorbed at the air/water interface, due to the long polypeptide chain, the protein molecules represent extraordinary mobility. Factor VIII may undergo conformational changes.

4.3.2 Rate constant for binary mixtures

4.3.2.1 rFVIII/Tween 80

Compared with the first-order rate constant of adsorption of Tween, we find that at each concentration of Tween (Table 4.4), higher K_1 for the protein/surfactant mixture manifests faster adsorption. But compared with rate constant of adsorption of pure protein, the added Tween 80 to the protein solution slowed down the protein adsorption to the interface. From the data that fitted to equation 4.1, there are also three visible linear regions. K_1 increased with concentration of Tween, $K_1^{'}$ didn't show obvious dependence on the concentration of Tween. The magnitude of K_2 indicated that rFVIII/Tween mixtures have negligible mobility on the air/water interface, compared with the K_2 for pure rFVIII adsorption, when Tween is added to rFVIII solution, the interfacial layer exhibits the first-order rate constant of rearrangement about 3 times lower than that of pure rFVIII, this not only indicates that at low concentration of Tween, rFVIII binds to Tween and adsorbs to the interface, and rFVIII has low tendency to unfold; also, the interfacial area tends to be occupied by Tween molecules at higher concentration above 0.04mM.

First-order rate constant for rFVIII/Tween 80 mixtures			
Concentration of Tween 80 (mM)	K1(min-1)	K1'(min-1)	K2(min-1)
0.002	0.576	0.103	0.011
0.004	0.628	0.098	0.046
0.006	0.665	0.09	0.054
0.008	0.857	0.096	0.043
0.01	1.068	0.109	0.044
0.012	0.858	0.097	0.037
0.014	1.225	0.109	0.035
0.016	1.223	0.099	0.043
0.018	1.228	0.104	0.036
0.02	1.002	0.091	0.048
0.04	2.046	0.142	0.049
0.06	1.902	0.158	0.061
0.08	2.932	0.171	0.07

Table 4.4 First-order rate constants for rFVIII/Tween 80 mixtures

4.3.2.2 rFVIII/SDS

The first-order rate constants of rFVIII/SDS mixture, which are shown in Table 4.5, although empirical, confirmed the results from surface tension data. These is no obvious dependence of K_1 on C_{SDS} , even at low concentrations, the large K_1 values mean that immediately after SDS was introduced into rFVIII solution, the adsorption rate is fast, compared with the first-order rate constant of adsorption of rFVIII/Tween, the larger K_1 means that when SDS was introduced into rFVIII solution, it facilities the adsorption of rFVIII to the air/water interface, at low concentration of SDS, 0.005-5mM, from the steady state surface tension data, we concluded that the interfacial layer consists of rFVIII/SDS complex, and this

complex has higher affinity to the air/water interface than pure SDS or rFVIII, but the similar first-order rate constant of adsorption between pure SDS and rFVIII/SDS indicates that they have almost the same speed to adsorb to the air/water interface. From rate constant analysis, we cannot conclude the component of the interfacial layer. For the first-order rate constant of rearrangement K_2, at very dilute SDS concentrations, i.e. 0.005, 0.05mM, the small K_2 which corresponds to rFVIII/Tween complex, we concluded that SDS doesn't induce the unfolding of rFVIII, the binding is dominated by electrostatic force in this region; during the concentration range from 0.5 to 5mM, the increased K_2 means increased mobility of the interfacial layer, which may due to the unfolding of rFVIII polypeptide chain at the air/water interface, this corresponds to the cooperative binding. Then the rate constant of rearrangement decreased again which was similar to that of pure SDS at the same concentrations, this also confirmed the conclusion from surface tensiometry, that from about 5mM to 8mM SDS, the interfacial layer consists of pure SDS molecules, rFVIII/SDS complex was replaced from the air/water interface by SDS.

First-order rate constant for rFVIII/SDS mixture		
Concentration of SDS(mM)	K1(min-1)	K2(min-1)
0.005	1.468	0.0098
0.05	1.752	0.019
0.5	1.83	0.024
1	1.475	0.036
2	2.084	0.048
3	1.566	0.048
4	1.391	0.012
5	2.365	0.012
6	2.32	0.01
7	2.58	0.013
8	2.306	0.01
9	2.715	0.012

Table 4.5 First-order rate constants for rFVIII/SDS mixtures

4.3.2.3 rFVIII/DTAB

The effect of DTAB in rFVIII solution behaves similar to that of SDS, the initial slope - K_1 means the fast adsorption of rFVIII with DTAB to the interface immediately after they were introduce to the solution, the adsorption rate is independent of the concentration of DTAB for there is no obvious relationship between K_1 and C_s. Compared with K_2 with that from pure DTAB, the similar value means that at the interface, rFVIII peptide chains have been displaced by DTAB, DTAB also binds to rFVIII, the strong interaction between rFVIII and DTAB induce unfolding of the peptide chain, but the unfolded chain occupied by DTAB are all in solution.

First-order rate constant for rFVIII/DTAB mixture		
Concentration of DTAB (mM)	K1 (min-1)	k2(min-1)
0.05	1.759	0.035
0.5	2.283	0.012
2	1.529	0.0193
5	1.732	0.032
10	2.486	0.066
15	3.608	0.013

Table 4.6 First-order rate constants for rFVIII/DTAB mixtures

The analysis based on equation 4.1 is empirical, but we still get the information consistent with surface tensiometry data. SDS and DTAB both have stronger interaction with rFVIII compared with Tween.

CHAPTER 5

CONCLUSION

The air/water interface is a classic hydrophobic surface which used to modify protein binding behavior. rFVIII exhibits high percentage of activity loss upon adsorption on biomaterial surfaces. Based on research conducted of rFVIII with three different kinds of surfactant on the air/water interface, and use surface tensiometry to investigate the binding behavior of rFVIII with surfactants, we found Tween 80, which is non-ionic surfactant, can replace rFVIII from the air/water interface, and this replacement mechanism is dependent on the concentration of Tween. SDS and DTAB are both potent surfactants with charged head-groups, the similar replacement mechanism is found, but they may all induce unfolding of rFVIII. And empirical analysis of rate constant is used to confirm these findings. Tween 80, compared with other surfactants, exhibits good stabilizing effect on protein formulation, which can be executed in scaled-up protein production.

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APPENDICES

APPENDIX A

rFVIII Adsorption Kinetic Analysis of IAsys Biosensor Data

Optical biosensors have been widely used for the analysis of both kinetic and equilibrium constant for a variety of protein-protein interactions. The binding of protein or protein-protein complex is monitored in real-time as a change in the refractive index or mass at the biosensor surface. [Schuck (1997); Ramsden (1997); Garland (1996)]

Materials and Methods

All chemicals used are of analytical reagent grade, 0.01M phosphate buffer, pH 7.0, containing 1.08g Na_2HPO_4 and 0.55g NaH_2PO_4 dissolved in 1L distilled, deionized water; Tween 80 and human serum albumin (HSA) were obtained from Sigma Chemical Co. Recombinant Factor VIII was provided by Bayer Corporation (Bayer Pharmaceuticals, Berkeley, CA). 625.9 IU/ml with 1IU corresponds to 4712mg, stored in $-80^0 C$ freezer. When used, rFVIII was thawed in ice and stored in small vials then added to the biosensor wells as quickly as possible, then put back into the refrigerator at $4^0 C$.

The operating principles of IAsys resonant mirror biosensor have been described in detail previously [Cush et al. (1993)]. The instrument detects changes in refractive index occurring within the evanescent field (only a few nanometers from the surface) when protein in solution binds to other molecules, such as ligands,

immobilized onto the sensor surface. The binding can be followed in real-time with instrument producing a plot of response (in arc second) against time. The IAsys instrument is based on a corvette system comprising of a sample well and the resonant mirror sensing surface. The capacity of the single well is from 50-200 μL. With build-in architecture, the block was derivatized as hydrophobic, contact angle 98.2^0.

Before each measurement, a good baseline was achieved by pure phosphate buffer, pH 7.0, recombinant Factor VIII was 28 $\mu g / ml$ in each measurement diluted from 625.9 IU/ml with phosphate buffer. All measurements were conducted by sterile micro-pipette at temperature of 22^0C - 23^0C.

Results and Discussion

Experiments with Tween 80 and rFVIII

The effect of adsorbed ("precoated") Tween on rFVIII adsorption and elution

First, we test pure rFVIII adsorption on hydrophobic surface without added Tween, the result was shown in Fig A1, rFVIII is allowed to adsorb, and the adsorbed layer then rinsed with buffer, we typically find anywhere from 60 to 100% of the adsorbed layer to be resistant to removal, and we will compare this with other experiments. In these tests, we allowed Tween 80 to contact a hydrophobic surface at its CMC (0.012mM), then introduced rFVIII (28 μg/ml) with or without added Tween. rFVIII adsorption was allowed to proceed through attainment of a plateau

(15 or 30 min), after which the sample was "rinsed" (i.e., the cuvette volume was replaced with protein-free, surfactant-free water). In summary, we found that if a solution of rFVIII is introduced to an adsorbed layer of Tween, adsorption is significant but completely removable by rinsing (if the rFVIII solution also includes Tween, at or above 0.012mM). A representative plot illustrating this outcome is shown in Fig. A2. If, on the other hand, a solution of rFVIII is introduced to an adsorbed layer of Tween, and that rFVIII solution does not include added Tween, adsorption is significant and somewhat resistant to elution. A representative plot illustrating this outcome is shown in Fig. 3; just over 40% of the adsorbed rFVIII layer resisted elution in this case.

Experiments with HSA and rFVIII

Our results relevant to evaluation of HSA interactions with rFVIII at interfaces were based on the same kinds of experiments used for evaluation of Tween interactions with rFVIII at interfaces (i.e., the effect of precoating hydrophobic surfaces with HSA on subsequent rFVIII adsorption and elution, and the adsorption and elution kinetics of HSA-rFVIII mixtures). We also studied the effect of contacting an adsorbed layer of rFVIII with HSA, followed by rinsing. Our results are summarized below.

Experiments with HSA at 0.10 mg/ml.

Precoating with HSA (by adsorption from a 0.10 mg/ml solution) followed by rFVIII adsorption typically yielded a resistance to elution of about 50% (see

Fig.A4). An adsorbed layer prepared with a HSA-rFVIII mixture typically brought resistance to elution down slightly, to about 40-50% (Fig.A5).
In separate experiments we followed rFVIII adsorption with introduction of HSA (0.10 mg/ml). Adsorbed mass was observed to decrease immediately in these tests, and upon rinsing, the resistance to elution we recorded (typically about 40-45%) was consistent with that observed for a film formed from a HSA-rFVIII mixture. A representative plot from this type of experiment is shown in Fig. A6.

Experiments with HSA at 1.0 mg/ml.

These results were similar to those just described for HSA at 0.10 mg/ml, but with the resistance to elution of rFVIII being somewhat lower than that recorded above. In particular, precoating with HSA (by adsorption from a 1.0 mg/ml solution) followed by rFVIII adsorption typically yielded a resistance to elution of 40-50% (e.g., Fig. A7). An adsorbed layer prepared with a HSA-rFVIII mixture typically brought resistance to elution down to about 35-40% (e.g., Fig. A8).
In separate experiments we followed rFVIII adsorption with introduction of HSA. Using 10 times more HSA (1.0 as opposed to 0.10 mg/ml), adsorbed mass was observed to decrease immediately as before, but upon rinsing, the resistance to elution we recorded (typically 30-35%) was somewhat lower than that observed for a film formed from a HSA-rFVIII mixture (with HSA at 1.0 mg/ml). A representative plot from this type of experiment is shown in Fig. A9.

Conclusion: From these results, we concluded that maintaining a sufficient amount of Tween in the rFVIII solution apparently allows a high concentration of Tween to stay in direct contact with the surface; rFVIII is thus only loosely held at the interface, not in direct contact with the surface, and readily rinsed away. The data with HSA and rFVIII do indicate that HSA addition reduces the resistance of rFVIII adsorption to these solid surfaces. Moreover, the effect is enhanced with increasing HSA concentration.

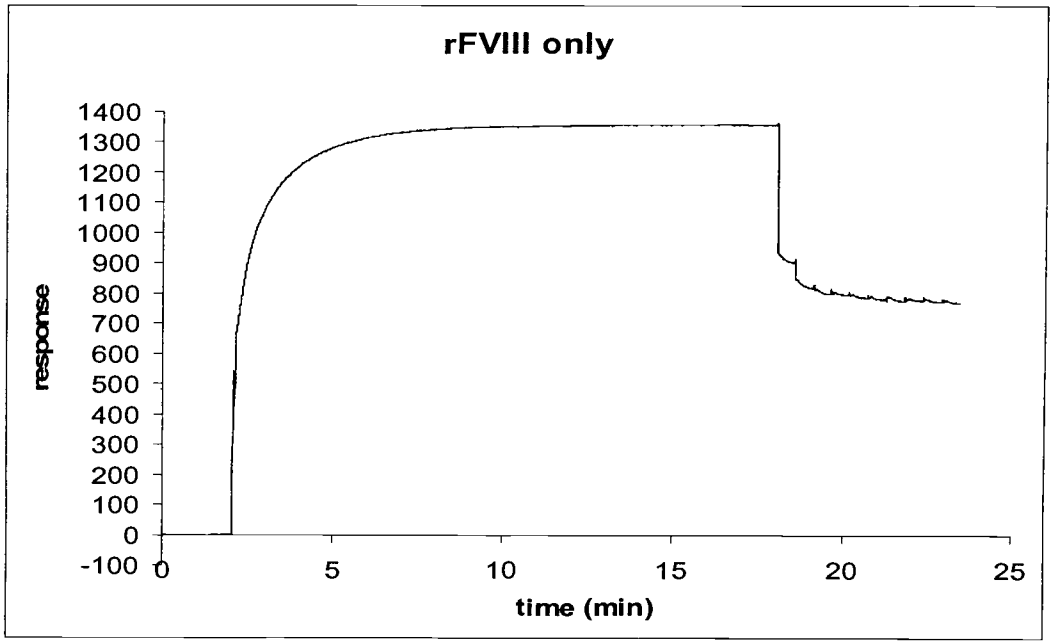

Figure A1 Pure rFVIII adsorption on hydrophobic surface followed by rinsing with phosphate buffer.

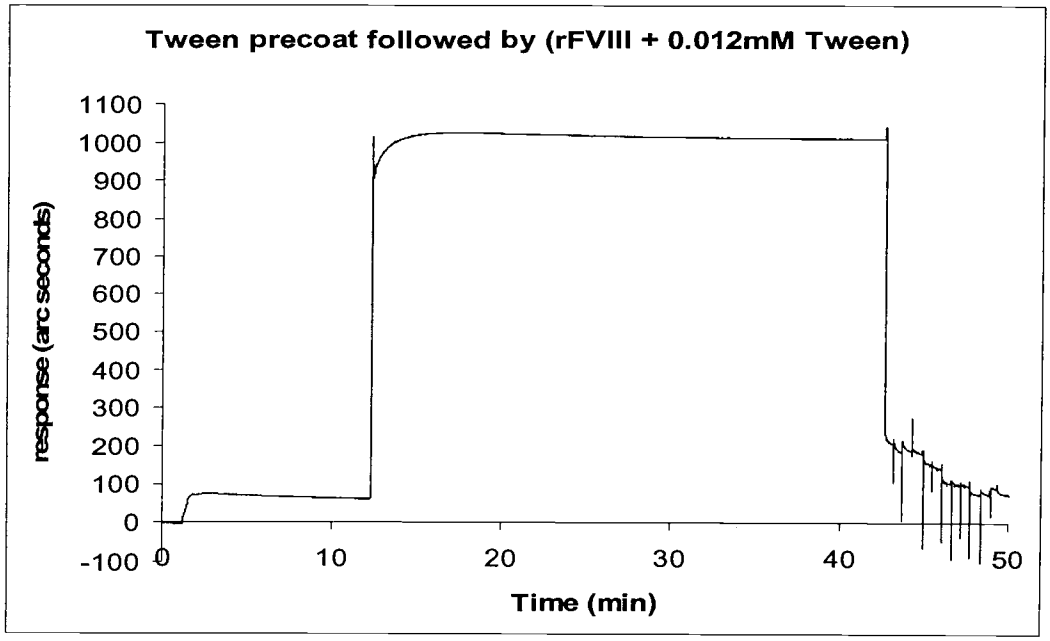

Figure A2 Hydrophobic surface is precoated with 0.012mM Tween followed by adsorption of rFVIII and Tween (0.012mM) mixture.

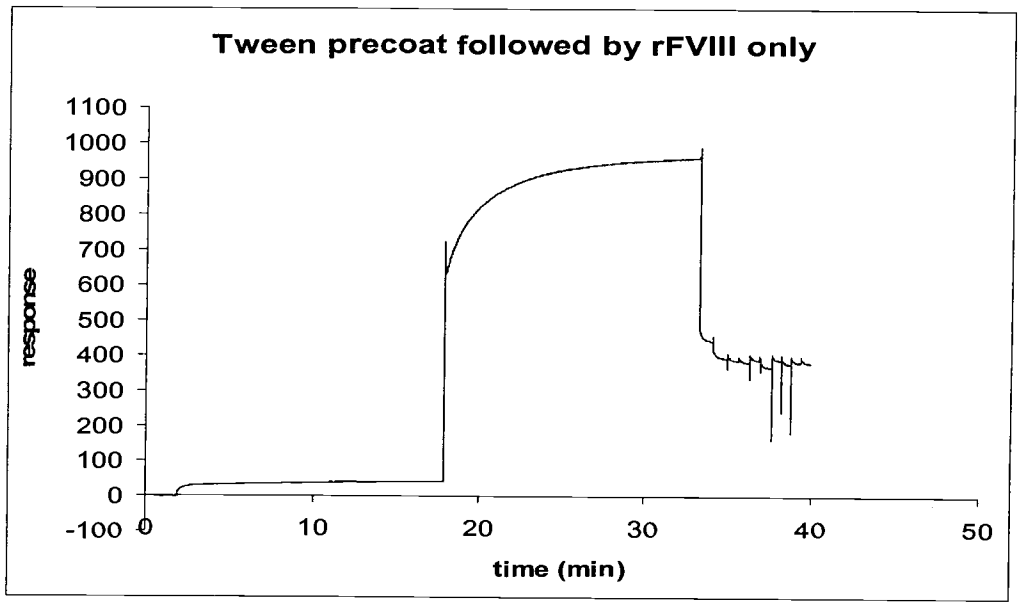

Figure A3 Hydrophobic surface is precoated with 0.012 Tween followed by adsorption of pure rFVIII.

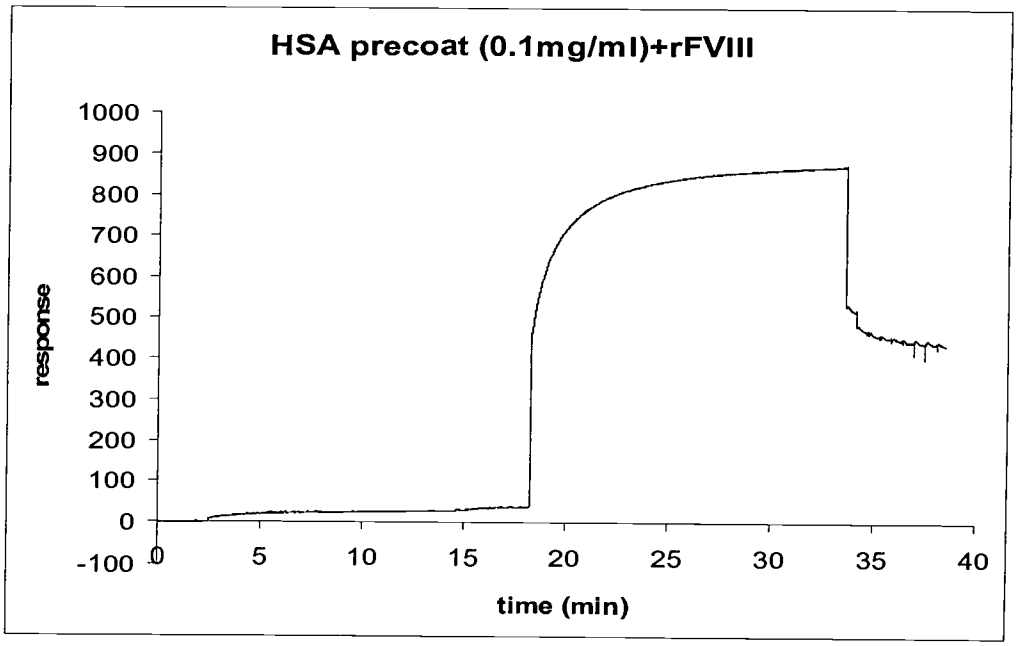

Figure A4 Hydrophobic surface precoated HSA (0.1mg/ml) followed by adsorption of pure rFVIII

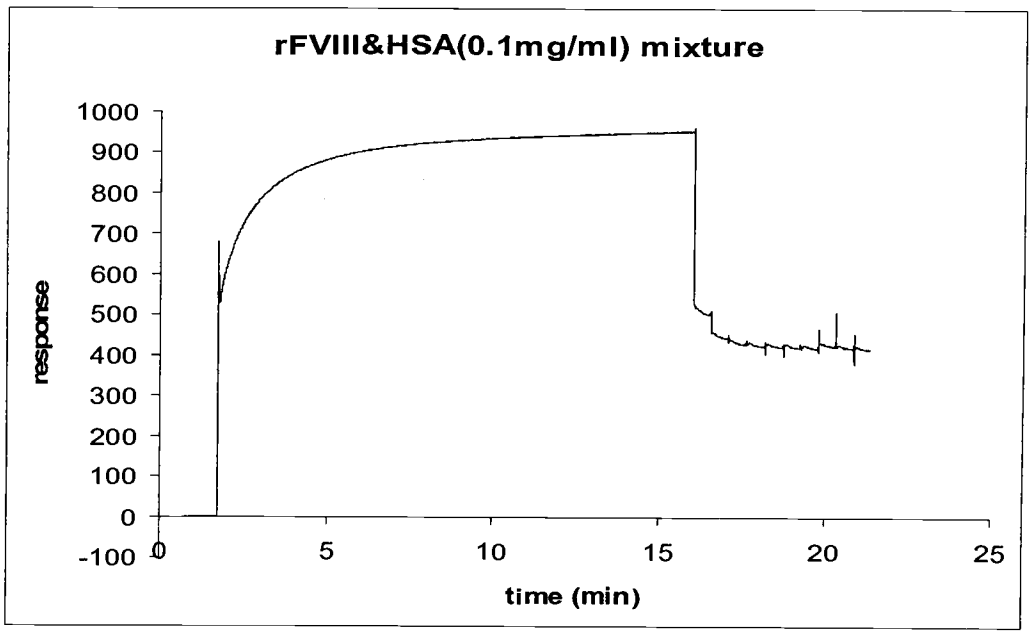

Figure A5 Adsorption of rFVIII and HSA (0.1mg/ml) mixture followed by rinsing with buffer.

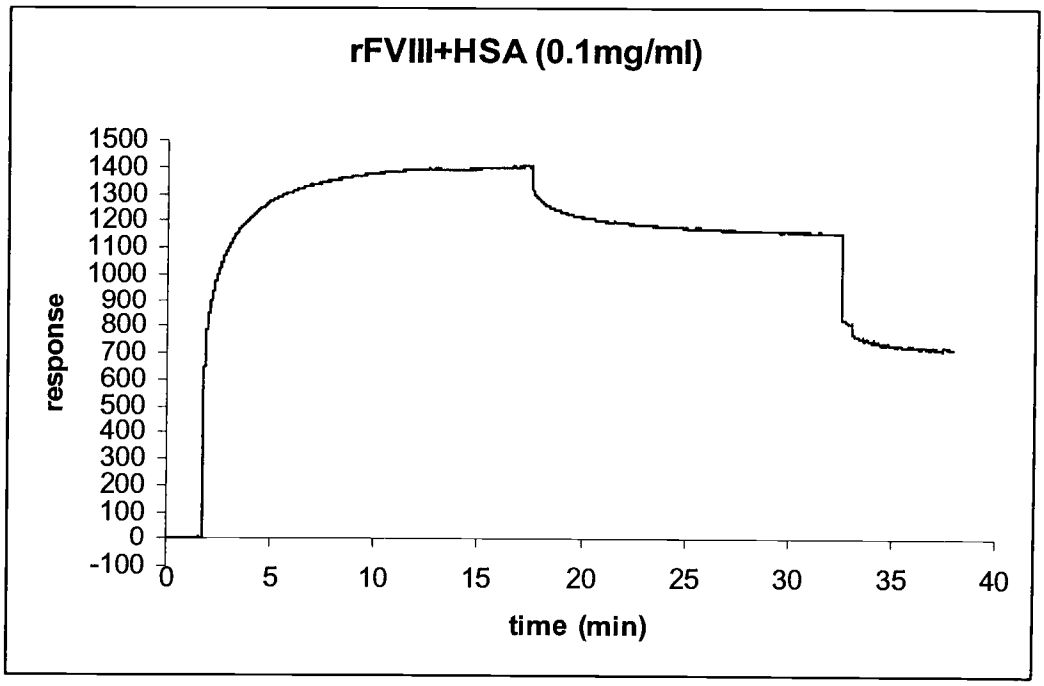

Figure A6 rFVIII adsorption followed by introduction of HSA (0.1mg/ml), then rinse with buffer.

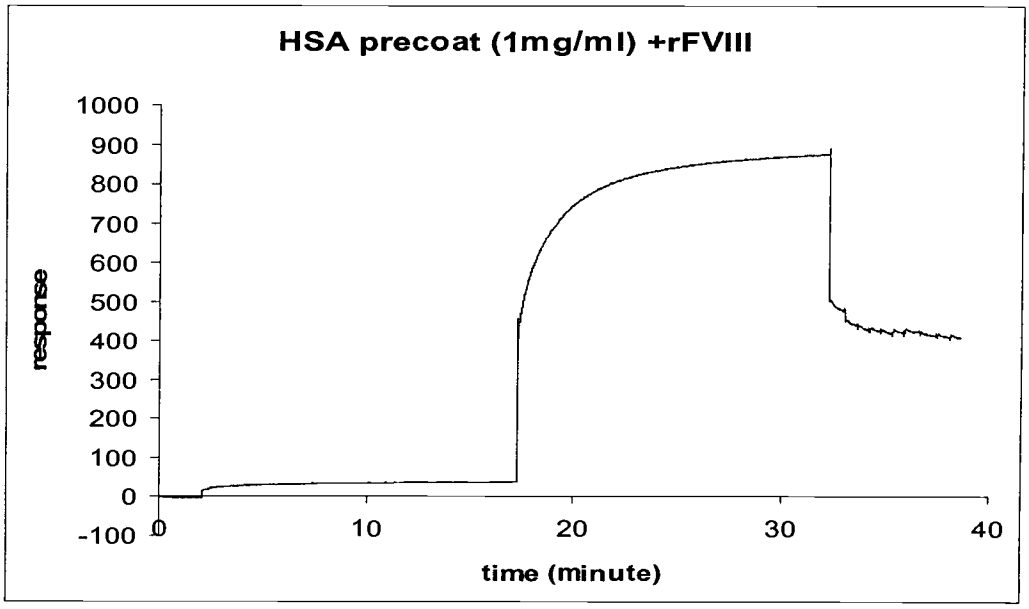

Figure A7 Hydrophobic surface precoated with HSA (1mg/ml) followed by adsorption of pure rFVIII.

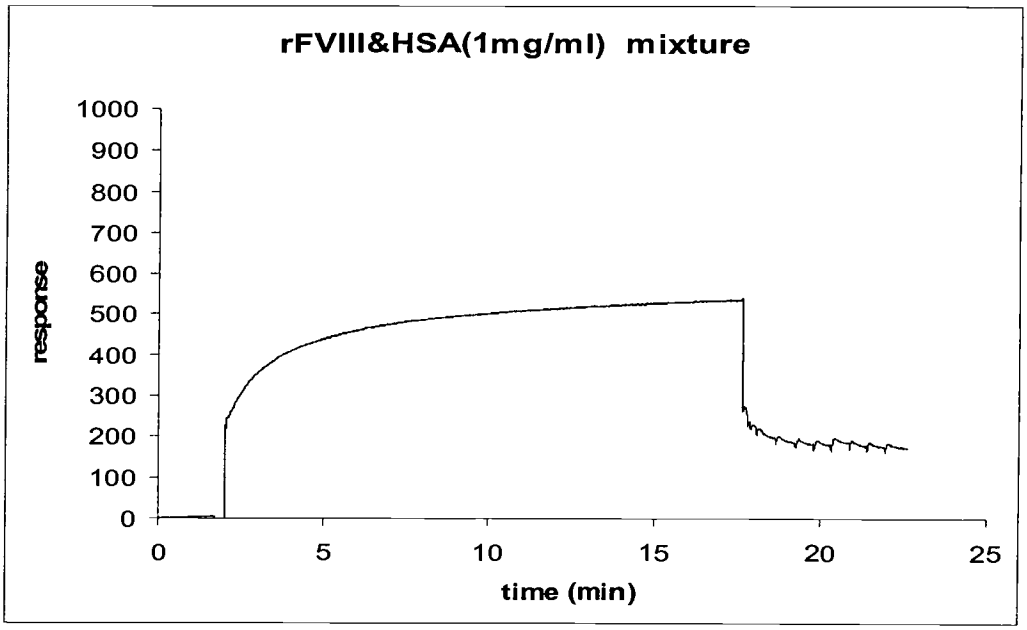

Figure A8 Adsorption of rFVIII and HSA (1mg/ml) mixture followed by rinsing with buffer.

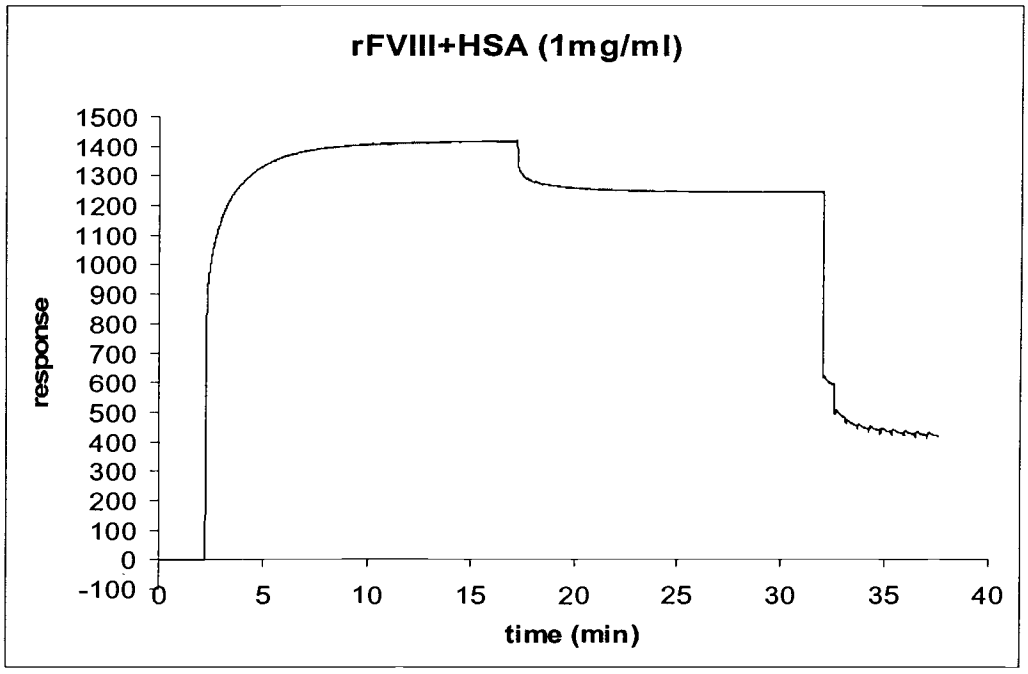

Figure A9 rFVIII adsorption followed by introduction of HSA (1mg/ml), then rinse with buffer.