AN ABSTRACT OF THE THESIS OF

Krisdhasima Viwat for the degree of Doctor of Philosophy in Chemical Engineering presented on December 11, 1991.

Title: β-Lactoglobulin Adsorption Equilibrium and Kinetics at Silanized Silica Surfaces

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The adsorption equilibrium and kinetic behavior exhibited by β-lactoglobulin at silanized silicon surfaces of varying hydrophobicity were examined using ellipsometry. Adsorption equilibrium results were used to construct adsorption isotherms; the adsorbed mass of β-lactoglobulin was observed to increase with increasing surface hydrophobicity, within a defined range of hydrophobicity. Adsorption kinetics recorded for β-lactoglobulin on each surface were compared to the kinetic behavior predicted by a simple model for protein adsorption. The model described the data well in all cases, enabling interpretation of the kinetic behavior in terms of contact surface hydrophobicity influences on rate constants affecting protein attachment and unfolding at the interface. In particular, both experimental and simulation results seem to be in support of a hypothesis that rate constants defining protein arrival and conversion to an irreversibly adsorbed
state increase with increasing surface hydrophobicity, while the rate constant defining desorption of protein from a reversibly adsorbed state decreases with increasing surface hydrophobicity. Contact surface hydrophobicity was quantified using contact angle analysis to determine the polar component of the work required to remove water from unit area of surface. Quantitative consideration of possible mass transfer influences on the observed adsorption rates supports the notion that the experiments were not conducted in a transport-limited regime; i.e., true kinetics were measured.
β-Lactoglobulin Adsorption Equilibrium and Kinetics at Silanized Silica Surfaces

by

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NOMENCLATURE

\[ a_i \]
\[ b \]
\[ C \]
\[ DDS \]
\[ D \]
\[ d_2 \]
\[ d_j \]
\[ E_p \]
\[ E_s \]
\[ k \]
\[ k_1 \]
\[ k_4 \]
\[ M \]
\[ n_i \]
\[ p \]
\[ R_p \]
\[ R_s \]
\[ R^p \]
\[ R^s \]
\[ r_{ij}^p \]
\[ r_{ij}^s \]
\[ r_i \]
\[ s \]
\[ s_l \]
\[ t \]
\[ W_a \]
\[ W_p \]
\[ W_{p,\text{water}} \]
\[ \beta_p \]
\[ \beta_s \]
\[ \Gamma \]
\[ \gamma_s \]
\[ \gamma_{sd} \]
\[ \gamma_{sp} \]
\[ \gamma_{SL} \]
\[ \gamma_{L} \]
\[ \gamma_{L,d} \]
\[ \gamma_{L,p} \]
\[ \gamma_{L}\text{-polar or non-dispersive component of liquid surface tension, mN/m} \]

Coefficient in equation (5.9); \( i=1-5 \)

Coefficient (ordinate intercept) in equation (3.1.14), mJ/m²

Protein concentration, mg/L

Dichlorodimethylsilane

Diffusion coefficient, cm²/s

Adsorbed film thickness, nm

Imaginary part of \( d_2 \)

Amplitude of incident light beam in plane \( p \)

Amplitude of incident light beam in plane \( s \)

Coefficient (slope) in equation (3.1.14)

Adsorption rate constant, L mg⁻¹min⁻¹

Desorption rate constant, min⁻¹

Molecular weight, daltons

Refractive index in phase \( i \)

Plane of incident light

Amplitude of reflected light beam in plane \( p \)

Amplitude of reflected light beam in plane \( s \)

Total refraction coefficient in plane \( p \)

Total refraction coefficient in plane \( s \)

Refraction coefficient at interface between phase \( i \) and \( j \), in plane \( p \)

Refraction coefficient at interface between phase \( i \) and \( j \), in plane \( s \)

Coefficient in the exponent argument of equation (5.9)

Plane normal to the plane of incident light

Conformational change rate constant, min⁻¹

Time, min

Work of adhesion, mJ/m²

Polar or non-dispersive component of the work of adhesion, mJ/m²

Polar component of the work of adhesion between a solid surface and water, mJ/m²

Phase angle of the light beam in plane \( p \)

Phase angle of the light beam in plane \( s \)

Adsorbed mass, μg/cm²

Solid surface energy, mJ/m²

Dispersive component of solid surface energy, mJ/m²

Polar or non-dispersive component of solid surface energy, mJ/m²

Solid-liquid interfacial tension, mN/m

Liquid surface tension, mN/m

Dispersive component of liquid surface tension, mN/m

Polar or non-dispersive component of liquid surface tension, mN/m
\( \gamma_{L, \text{water}} \)  
- Polar or non-dispersive component of the surface tension of water, mN/m

\( \Delta \)  
- The change in phase of the light, degrees

\( \theta \)  
- Contact angle (in equation (3.3.1)), degrees

\( \theta \)  
- Total fractional surface coverage of protein (in the Results and Discussion section)

\( \theta_1 \)  
- Fractional surface coverage of reversibly adsorbed protein

\( \theta_2 \)  
- Fractional surface coverage of irreversibly adsorbed protein

\( \lambda \)  
- Wavelength, nm

\( \rho \)  
- Ratio of parallel and normal reflection coefficients

\( \phi_1 \)  
- Angle of incident light

\( \phi_2 \)  
- Angle of refraction

\( \Psi \)  
- The arctangent of the factor by which the amplitude ratio changes
1. INTRODUCTION

The study of protein adsorption at solid-liquid interfaces has important applications to many fields in basic research, medicine, and industry. The adsorption behavior is not only dependent on the nature of the protein itself but also on the characteristics of the contact surface, the solution and their interdependency.

Many clinical researchers have investigated the behavior of blood components at interfaces. Baier (1978) found that blood protein, usually fibrinogen, adsorbs immediately to non-physiologic material prior to the adhesion of platelets and white blood cells. In fact, blood platelets arrive continuously, but adhesion was not observed to occur until the proteinaceous layer attained a sufficient thickness. Pre-adsorption of protein on a surface can affect cell growth and spreading as well. For example, Schakenraad et al. (1986) investigated the influence of surface energy on growth and spreading of human fibroblasts in the presence and absence of serum proteins. They found that cell growth in the presence of serum proteins did not differ significantly among various surfaces. In contrast, cell growth was observed to be different among the surfaces in the absence of serum proteins. Tosteson and Corpe (1975) discovered that adhesion of marine
Chlorella vulgaris to glass can be enhanced by the presence of bio-polymers, composed of protein and carbohydrate, in the medium solution. One might conclude that macromolecules in the medium adsorb to form organic films, and these films appear to be obligatory precursors to subsequent adhesion of both living and dead particulate matter, such as bacteria, cells, or larval organisms (Baier, 1984).

Fouling of heat exchange surfaces in the dairy industry causes many problems, including those associated with hydraulic and thermal disturbances that occur during processing. Cleaning operations have to be carried out in order to bring the heat exchange surface back to its original state (Lalande, 1985). Serum proteins in milk, particularly β-lactoglobulin, are important components in the formation of deposits (De Wit, 1981). Protein adsorption also plays an important role in separation and purification processes, especially in membrane fractionation and chromatographic techniques. For example, conditioning of a chromatographic adsorbent to prevent protein loss during purification is periodically done (Blakesley et al, 1975). Additionally, HPLC columns can be treated with protein to prevent further protein adsorption while still retaining their capacity to separate low molecular weight material (Lesins, 1988).

A thorough understanding of the mechanisms associated with the aforementioned applications involving protein adsorption has not yet been established due to the complex
interactions that take place between macromolecules and contact surfaces, and among macromolecules in solution. An enhanced understanding of single protein activity at solid-liquid interfaces should improve our understanding of these types of problems and provide better direction in our attempt to address them.
2. LITERATURE REVIEW

2.1 Surface Induced Conformational Changes

Comparisons of denaturation energetics and adsorption energetics suggest that adsorption-induced conformational changes are highly probable (Dillman and Miller, 1973; Andrade et al., 1984). Conformational changes occur to minimize interfacial free energy. Additional noncovalent bonds between the protein and surface can lead to a more "spread" configuration among protein molecules, and can assist displacement of those less tightly bound. Although there have been many hypotheses associated with adsorption-induced conformational changes in protein, little direct data is available. However, there is a large amount of experimental evidence supporting the concept that proteins exist in multiple states on a surface.

Morrissey and Stromberg (1974) used infrared difference spectroscopy to study protein conformational states adopted on silica particles. This method allows deduction of conformational state by providing information on the fraction of carbonyl groups actually bound to the surface. Their findings suggest that with respect to fibrinogen, more significant protein-surface hydrogen bonding is experienced at low solution concentrations relative to high solution concentrations. They also found that spectra for prothrombin and bovine serum albumin were unaffected by solution
concentration, indicating that the internal bonding of serum albumin and prothrombin is sufficient to prevent changes in their structure while adsorbed.

Andrade et al. (1984) used intrinsic ultraviolet total internal reflection fluorescence (UV TIRF) spectroscopy to monitor the conformational changes experienced by human plasma fibronectin on hydrophobic and hydrophilic silica. Intrinsic UV TIRF spectroscopy follows adsorption by providing indirect information on the local environment of tryptophan residues. A disadvantage of this method, however, is that UV-photochemical changes may be observed and interpreted as surface-induced. They reported that protein adsorbed on hydrophobic and hydrophilic surfaces showed fluorescence maxima at 326 and 321 nm, respectively, where the maximum of fresh protein in solution is 321 nm. These results indicate that the adsorbed human plasma fibronectin on the hydrophobic surface had undergone some surface conformational change.

Another method that has lent support to the multiple states theory involves comparison of the isotherms obtained from single-step and successive addition of protein. Jönsson et al. (1987) used this method to investigate the adsorption behavior of secretory fibronectin (HFN) and immunoglobulin G(IgG). They found differences between the single step- and the successive-addition isotherms for each protein adsorbed on hydrophobic silica. These results were taken to indicate virtual irreversibility of the adsorption process due to
surface-dependent conformational changes occurring over time. For hydrophilic silica, the successive-addition isotherm of HFN approached that obtained by single step-addition; at longer times they were observed to eventually coincide. They suggested that HFN adsorbed to hydrophilic silica tends to undergo less conformational changes compared to HFN adsorbed to hydrophobic silica. Recently, similar experiments were done by Wahlgren and Arnebrant (1990). They studied the adsorption of β-lactoglobulin onto pre-adsorbed (from a 0.1% β-lactoglobulin solution) silica, methylated silica, and polysulfone. The results indicate that the mass adsorbed from 12% protein solution onto both preadsorbed methylated silica and preadsorbed polysulfone surfaces have values in the same range as adsorption from a 0.1% β-lactoglobulin solution to each bare surface.

Elwing et al. (1988) used ellipsometry to study conformational changes experienced by the protein complement factor 3 on hydrophilic and hydrophobic silicon surfaces. They observed a larger adsorbed mass on hydrophobic surfaces than on hydrophilic surfaces. This is because protein molecules are assumed, in general, to change conformation to a greater extent on hydrophobic surfaces relative to hydrophilic surfaces. This is due to the presence of hydrophobic interactions between the solid surface and hydrophobic "pockets" in the protein molecule. This interaction gives the molecule an extended structure, covering
a relatively large area of the surface. The repulsive force normally acting between native protein molecules is probably decreased for conformationally changed molecules adsorbed on a hydrophobic surface (Elwing et al., 1988). This means that one should expect a greater number of adsorbed molecules per unit area on a hydrophobic surface. On a hydrophilic surface the interaction will, in general, be quite different. Forces acting between the surface and the molecule may be smaller in magnitude, and since the resulting conformational change will be smaller, a larger repulsive force will be present between the molecules. Therefore, the packing of adsorbed molecules will not be as close as that on a hydrophobic surface, and, in general, a smaller number of adsorbed molecules will be found on a hydrophilic surface, with each molecule occupying a smaller area on the surface.

However, adsorption behavior may be different if it occurs at other interfaces besides solid/liquid. Feijter et al. (1978) studied the adsorption of various proteins at air-water interfaces, and proposed that conformational changes are related to the availability of adsorption sites on the contact surface. This means that the adsorbed molecules will have more space to extend their structure in the case of greater number of available adsorption sites. They observed that the thickness of an adsorbed $\kappa$-casein film decreased while its adsorbed mass increased for an incubation time greater than four hours. This indicates that the adsorbed $\kappa$-casein spreads
out on the surface, with the time scale of the process being on the order of several hours. This effect was observed at bulk concentrations at or below 0.0001% by weight but not at higher protein concentrations. They suggested that the extent to which adsorbed \( \kappa \)-casein molecules change their conformation depends on the number of molecules adsorbed at the surface. For low bulk concentrations, where the surface is not completely occupied, conformational rearrangement of the adsorbed molecules is considerable; at high bulk concentrations, where the surface is completely occupied, the shape of the adsorbed molecules is hardly affected.

Soderquist and Walton (1980) used circular dichroism to study conformational characteristics of plasma proteins desorbed from polymer surfaces. They found that the desorbed molecules were changed in structure compared to their structures prior to adsorption. The desorption process itself depends upon the incubation time of the protein with the substrate: the longer the incubation time, the slower the desorption rate. They explained that each molecule on the surface undergoes a structural transition as a function of time that occurs in the direction of optimizing protein/surface interaction. They also proposed that the probability of desorption decreases with an increase in the period of incubation, and that protein slowly adsorbs more or less irreversibly.
Horbett and Brash (1987) reviewed a large body of evidence which supports the existence of multiple states of adsorbed protein. Some strong evidence includes numerous observations indicating the presence of weakly and tightly bound proteins: for example, rinsing a surface after protein contact does not remove all of the protein, yet some of the remaining protein is removable during a second, longer buffer rinse. Also, decrease in SDS elutability of proteins from an adsorbed layer are observed as the protein-surface contact time increases.

2.2 Bilayer Adsorption

Many workers reported that protein can adsorb onto a surface in more than one layer. Arnebrant et al. (1985) studied adsorption of β-lactoglobulin and ovalbumin on hydrophilic and hydrophobic chromium surfaces using ellipsometry and potential measurements. On clean hydrophilic surfaces the ellipsometric results showed that a thick, highly hydrated layer is obtained, which can be partially removed by aqueous buffer rinsing. Changes in the electrode potential, simultaneously measured relative to a standard calomel electrode during both adsorption and rinsing, were also observed for this surface. The results suggest that the protein adopts a bilayer formation on the surface, with the bottom layer unfolded and attached by strong polar bonds to the surface, and not removed by rinsing. Rinsing experiments
showed that the upper protein layer is loosely attached, which indicates that the outer layer has a structure closer to that of the native state. They described this adsorption behavior in terms of surface induced conformational changes and charge interactions between the protein molecule and surface as follows.

In a protein molecule, there are always some polar amino acid side chains that can interact strongly with a surface, even if both the protein and surface are negatively charged. Such binding is expected to result in unfolding of the protein, and irreversible adsorption. The consequence of this would be the probable exposure of hydrophobic loops into aqueous solution; adsorption of a second protein layer can therefore reduce the interfacial free energy. In the case of a negatively charged metal surface, it should be expected that positive groups such as the N-terminal and lysine residues bind with direct contacts to the surface. Such charge compensation may explain why the electrical effects of this bottom monolayer appear relatively weak, compared with the upper layer. Ionic interaction, in addition to hydrophobic interaction between the top and bottom layers, can link groups in protein layers. Thus, in the case of a negatively charged surface in contact with protein molecules with a negative net charge, the upper layer will tend to orient its positive residues downwards and the negatively charged side-chains outward from the surface. Due to the bulkiness of the protein
molecule, a stronger dipolar effect might therefore be expected for the upper layer. In the case of protein exposed to a hydrophobic metal surface, the values of the thickness and of the refractive index are consistent with the formation of a monolayer.

Bilayer adsorption is not only detected by ellipsometry, but also by other methods such as fluorescence spectroscopy. Walton and Maenpa (1979) used fluorescence spectroscopy to study the behavior of bovine serum albumin adsorption on particles of copolypeptide. They found that the change in fluorescence is not only due to adsorbed protein, but also to loosely bound protein in close proximity to the surface. The concentration of loosely attached molecules is at least as high as that of strongly attached molecules.

Bilayer adsorption seems to depend upon substrate surface properties, but other evidence reveals that increased polymerization of protein causes formation of a second layer. Arnebrant and Nylander (1988) used ellipsometry to study the effect of divalent metal ions (zinc and calcium) on the adsorption of insulin on chromium and titanium surfaces. Self-association of insulin molecules in aqueous solution to form oligomer units is influenced by specific interactions with divalent metal ions. They observed that the adsorbed mass of hexamer insulin per unit area was comparable to the calculated value for a hexagonal close-packed monolayer of hexamers. However, adsorbed mass was greater when the protein
in solution was mainly in the form of dihexamers. This higher adsorbed amount may be interpreted as the formation of a second layer, which can be removed easily by rinsing.

2.3 pH Effects

The charge of a protein molecule depends upon the difference between the solution pH and the isoelectric point of the protein, at which its net charge is zero. For pH values greater than the isoelectric point the net charge of the protein is negative, while for lesser values the net charge is positive. Many workers have studied the effect of pH on adsorption and in general have shown that maximum adsorption occurs or near the isoelectric point. A protein in solution is more extended when its net charge is greater and more globular when its net charge is smaller. The configuration has major effects on the amount of protein adsorbed, since a more globular molecule would require fewer sites for adsorption than a more extended molecule. Therefore, the likelihood of adsorption is greater for a molecule with a more globular configuration (Lee and Ruckenstein, 1988). There are also repulsive interactions between adsorbed, charged molecules that hinder further adsorption. At the isoelectric point, this intermolecular electrostatic repulsion is minimized.

The degree to which pH actually affects protein adsorption is determined to a large extent by the
conformational stability of the particular protein molecule. Norde and Lyklema (1978) observed that plateau amounts of adsorbed protein are virtually independent of pH for bovine pancreas ribonuclease (RNase), whereas those for human plasma albumin (HPA) vary by as much as a factor of two. Relative to RNase, HPA exhibits a high degree of conformational adaptability, which allows this protein to change its structure as conditions in the solution change. The changes in structure for HPA are reflected in the observed changes in adsorbed mass onto a given surface as a function of pH. Conversely, the lack of change in the RNase protein structure in response to changing pH leads to the adsorbed mass being independent of changing solution conditions.

Changing solution pH can result in desorption of protein as well. Bagchi and Birnbaum (1981) studied the effect of pH on adsorption and desorption of goat and rabbit immunoglobulin G. They observed that both adsorption and desorption, upon pH cycling, is not reversible. Changing the pH from 4.0 or 10.0 to 7.8 does not cause the adsorbed mass to reach the same amount obtained uniquely at pH 7.8. They suggested that since protein adsorption takes place through multicontact points, complete desorption is energetically less favorable than adsorption. Adsorption can be achieved by a single contact, but desorption must be accompanied by the breaking of all contact points.
2.4 Ionic Strength Effects

The extent to which ionic strength affects protein adsorption is a function of the role electrostatics plays in the adsorption driving force. At low ionic strength the charges of the protein molecules act fully to bring about both a greater contribution of electrostatic forces to the total interaction, and more extended protein conformation, both of which decrease the adsorbed mass (Lee and Ruckenstein, 1988). At higher ionic strength, the surface charge of the protein molecules becomes increasingly shielded, resulting in reduced repulsive double-layer interaction between molecules and in a more globular configuration (Baghi and Birnbaum, 1981; Lee and Ruckenstein, 1988). Therefore, a greater amount of protein would be expected to adsorb. Previous work has generally shown an increase in the amount of protein adsorbed as ionic strength increases (Baghi and Birnbaum, 1981; Jönsson et al., 1987; Soderquist and Walton, 1980; Lee and Ruckenstein, 1988), but decreases in adsorbed amount have also been observed (Jönsson et al, 1987; Soderquist and Walton, 1980).

The effect of ionic strength on adsorbed mass is best described with reference to other factors. Luey et al. (1991) suggested that the ionic strength (NaCl concentration) influences on adsorbed mass may be related to both solid surface properties and protein stability in solution. They observed that increased ionic strength shields the electrostatic repulsion between a negatively charged protein
molecule and a hydrophilic surface, thereby increasing adsorbed mass. On the other hand, they also observed that as the ionic strength increases, adsorbed mass was observed to decrease on a hydrophobic surface. In this case, it is possible that the protein molecule stabilized its conformation by incorporation of ions into its structure. Electrostatic interactions are certainly not the only important interactions that take place during protein adsorption, and such varying observations for the adsorbed amount as a function of ionic strength may also involve ionic strength-dependent conformational changes of adsorbed protein (Jönsson et al., 1987).

2.5 Adsorption Kinetics

Many experimental observations have indicated that a major portion of the final adsorbed amount had been adsorbed within the first few minutes (Andrade et al., 1984; Soderquist and Walton, 1980). Soderquist and Walton (1980) used spectrophotometer to monitor the kinetics of protein adsorption. They proposed that there are three distinct processes contributing to the kinetics of uptake of protein on polymeric surfaces. First, rapid and reversible adsorption of the proteins occurs in a short period of time. Up to 50 - 60% surface coverage there is a random arrangement of adsorbed molecules, but at about this level some form of surface transition occurs that is probably in the direction of surface
ordering, thereby allowing further protein uptake. Second, each molecule on the surface undergoes a structural transition as a function of time that occurs in the direction of optimizing protein/surface interaction. Third, the probability of desorption decreases with an increase in the period of incubation, and protein slowly adsorbs more or less irreversibly.

Lundström et al. (1985) presented a dynamic model of protein adsorption on solid surfaces. The model, describing the fractional surface coverage of adsorbed molecules as a function of time, is based on the assumptions that a protein molecule may change conformation after adsorption and may desorb from the surface. Additionally, the rate of changing conformation was assumed to be dependent upon the availability of adsorption sites. The model was mathematically manipulated to obtain the steady-state fractional surface coverages; however, an expression describing the kinetic behavior cannot be obtained analytically. The model was compared with the case of Langmuir-like adsorption of a molecule in two different orientations, and the case of irreversible adsorption. Plateau values of isotherms constructed with the Langmuir model were reached at lower concentrations than were those constructed with the Lundström model.

There are also experimental observations that indicate protein molecules are exchanged on a solid surface with protein from the solution. Elwing et al. (1987a,b) used
specific antibodies to detect the exchange reactions of adsorbed protein antigens on wettability gradient plates. They observed that exchange reactions take place more readily on hydrophilic than on hydrophobic surfaces. They also pointed out that their method is indirect, and precautions should therefore be taken in the interpretation of these results. The results obtained were probably influenced by several factors, including the amount of specific antibodies in the antibody preparation, and the number of antigen determinants involved in the binding reaction. Recently, Lundström and Elwing (1990) presented a slightly modified and expanded version of an earlier model (Lundström, 1985) to allow for bulk-surface exchange reactions among proteins in one- and two-component solutions. The paper featured manipulation of the equations describing the fractional surface coverage of protein in specific states, and simulations of total surface coverage as a function of equilibrium concentration, and as a function of time. Due to the complexity of the model, involving several intermediate states of adsorbed protein, an expression describing the kinetic behavior of molecules in each state cannot be obtained analytically. However, the model can be used to numerically simulate the change in fractional surface coverage of protein in any specific state as a function of time by assuming values for pertinent rate constants.
Currently, there is no adequate experimental methodology to directly monitor these fractional surface coverages. Because of this limitation, a more simplified model that can be statistically compared with the available data is needed.

2.6 Mass Transfer in Protein Adsorption

In considering the kinetics of any interfacial process, the question of transport versus reaction control must be addressed. Protein adsorption at the interface is controlled not only by the intrinsic kinetic rate which is a function of protein solution and surface properties, but also by the rate of transport of protein molecules from the bulk solution across the concentration boundary layer near the interface. These transport phenomena are characterized by diffusion as well as hydrodynamics in a flow system. The diffusion limitation will exist as long as there is a significant concentration gradient near the solid surface (Wojciechowski and Brash, 1990).

Proteins are large molecules, and can include a number of side chains. Diffusion coefficients may vary widely among proteins depending on their concentrations and the electrostatic condition of the solution (Cussler, 1984). This will, more or less, affect the transport-controlled adsorption rate.

Many simulations of protein adsorption have been performed by combining adsorption kinetic and mass transport
equations. Iordanski et al. (1983) combined a kinetic model, allowing for both reversibly and irreversibly bound fractions, with static diffusion equations for which the thickness of the concentration boundary layer was estimated from that of the hydrodynamic boundary layer. According to their kinetic model, it is questionable that the adsorption rate of native protein from solution is independent of the number of adsorption sites occupied by the irreversibly bound fraction. In any event, they found that a three-fold increase in the diffusion coefficient from $2 \times 10^{-7}$ to $6 \times 10^{-7}$ cm$^2$/s resulted in about a 33% decrease in the time required to reach adsorption equilibrium. Increasing the diffusion coefficient beyond $6 \times 10^{-7}$ to $10^{-6}$ cm$^2$/s only slightly decreased the time required to reach equilibrium.

Lok et al. (1983) interpreted their experimental data with reference to a transport model. They found that a time-independent solution, or the so called Lévêque solution, agreed well with their data at low concentrations. At high concentrations, however, the time dependent solution was shown to be superior. They explained that deviation from the Lévêque solution at high concentration is due to limitations associated with the time-independent transport model, and not influenced by kinetic factors. They concluded that the initial adsorption rate of protein molecules onto a solid surface is transport-limited at both low and high concentration. This indicates that the rate of protein
binding to the surface is faster than the rate of protein transport from solution to the surface. Thus the rate of adsorption is equal to the rate of diffusion, \( \frac{d\Gamma}{dt} \):

\[
\frac{d\Gamma}{dt} = MC(D/\pi t)^{1/2}
\] (2.1)

where \( \Gamma \) = adsorbed mass, \( M \) = molecular weight of protein, \( C \) = bulk solution concentration, \( D \) = diffusion coefficient of protein, and \( t \) = time. Integrating equation (2.1) gives the adsorbed mass, \( \Gamma \) at any time:

\[
\Gamma = 2MC(Dt/\pi)^{1/2}
\] (2.2)

Equation (2.2) shows that adsorbed mass is proportional to \( t^{1/2} \). If the adsorption is entirely transport limited, the diffusion coefficient of the adsorbing protein can be estimated. According to equation (2.2), the slope of a plot of \( \Gamma \) vs. \( t^{1/2} \) is proportional to \( D^{1/2} \). In several protein adsorption systems (Iwamoto et al., 1985; Van Dulm and Norde, 1983; Dass et al., 1987; Hlady et al., 1986; and Wojciechowski et al., 1986), \( \Gamma \) was observed to vary linearly with \( t^{1/2} \) during the initial stages of adsorption but the calculated diffusion coefficient was found to be significantly less than the actual value for that protein. Wojciechowski and Brash (1990) suggested that such a process was still entirely diffusion-limited; however, the actual diffusion coefficient was depressed to some extent. On the other hand, Young et al. (1987) suggested that the adsorption of \( \alpha_1 \)-macroglobulin, when \( \Gamma \) is proportional to \( t^{1/2} \), is not diffusion-controlled. The apparent diffusion coefficients are \( 0.5 \times 10^{-7} \) and \( 0.2 \times 10^{-7} \) cm\(^2\)/s from bulk
concentrations of 2.5 and 5.0 µg/ml, respectively. This concentration dependence of the initial adsorption rate suggests that the intrinsic adsorption kinetics may be dominating the adsorption. In any event, the diffusion limitation will contribute to the adsorption process as long as there is a significant concentration gradient near the wall (Wojciechowski and Brash, 1990). Giroux et al. (1990) used FTIR/ATR as a tool to study human fibronectin adsorption. They suggested that data collected at high bulk protein concentration represented neither diffusion nor reaction controlled conditions over the duration of an experiment. This is in agreement with Wojciechowski and Brash (1990) in which there is a range of conditions over which both the rate of binding and the rate of diffusion apparently control adsorption kinetics. Giroux et al. (1990) modeled protein adsorption kinetics by numerically solving the diffusion equation with five different kinetic rate expressions used as a boundary condition. They found that the model incorporating "dual layer" kinetics adequately described the features of the data. However, it is not clear whether true multilayer adsorption was observed because the calculated value of adsorbed mass for a monolayer is substantially higher than the value they found experimentally.
3. THEORY

3.1 Evaluation of Solid Surface Properties

From a thermodynamic standpoint, the extent of protein adsorption on a solid material would be influenced only by surface energies, i.e., the surface tensions of the contact material, liquid medium and adsorbing protein molecules. However, absolute solid surface energies are usually difficult to measure, so much effort has been dedicated to measurement of surface properties related to solid surface energy ($\gamma_s$). To date there is no standard method for contact surface characterization, i.e., measurement of $\gamma_s$. Often, materials are characterized under conditions that are not representative of the environment in which they must perform, i.e., an aqueous environment.

Contact angle measurements are the basis for one of the most sensitive, yet simple techniques for describing surface energetics and thermodynamics. Contact angle data have been used extensively to evaluate solid surface properties related to surface energy. The derivation of these techniques and their development are discussed in most surface chemistry texts (Aveyard and Haydon, 1973; Hiemenz, 1986). Following is brief review of basic contact angle principles, with a description of the method chosen for contact surface characterization in this work.
3.1.1 The Contact Angle

The contact angle, $\theta$, formed when a drop of liquid contacts a solid surface is shown in Figure 3.1. The subscripts, $S$, $L$ and $SL$ refer to the solid-vapor, liquid-vapor and solid-liquid interfaces respectively. The contact angle formed by a sessile drop on a given surface is routinely measured with a contact angle goniometer. This instrument consists of a light source, illuminating a stage on which the liquid drop/solid system rests, and a telescope. The drop is viewed through the telescope; cross hairs in the telescope and a 360° scale around the eyepiece enable accurate measurement of the contact angle.

Young's equation (a force balance) written in its simplest form for the drop of liquid at equilibrium on a homogeneous, plane surface is

$$\gamma_S = \gamma_{SL} + \gamma_L \cos \theta \quad (3.1.1)$$

The Dupré equation (an energy balance) defines the work of adhesion, $W_s$, required to part the liquid from the solid,

$$W_s = \gamma_S + \gamma_L - \gamma_{SL} \quad (3.1.2)$$

The quantity $\gamma_{SL}$ cannot be directly measured experimentally. Therefore, the Young and Dupré equations are combined to yield the following relationship for the work of adhesion between the solid and the liquid,

$$W_s = \gamma_L (1 + \cos \theta) \quad (3.1.3)$$

Rigorously, equation (3.1.3) applies only to a system at equilibrium. Consequently, the liquid must be saturated with
Figure 3.1 The Contact Angle
the solid, and the vapor and homogeneous solid surface must be at adsorption equilibrium. This is not often the case, and values of $\theta$ for a given system may vary widely. Additionally, it should be noted that direct application of equation (3.1.3) to common engineering materials may yield misleading results, as these surfaces exhibit energetic heterogeneities and other surface irregularities.

Several methods are available for the determination of $\theta$. Depending on the nature of the solid and test liquid(s) used, and the environment in which the solid surface behavior will actually be monitored, some methods result in more useful relationships than others.

The most common methods used to identify differences in surface constitution for materials used in biological fluid contact are sessile drop techniques. These are classified as advancing angle techniques, since during drop formation and contact, the liquid advances along a part of the surface with which it has not previously been equilibrated. Alternatively, the line of solid-liquid contact may occur at a position on the solid that has previously been immersed in the test liquid, i.e., the liquid recedes or is pulled back across the part of the surface with which it has reached equilibrium. Obtained this way, $\theta$ is referred to as the receding contact angle.
3.1.2 Polar and Dispersive Contributions to Surface Tension

To more quantitatively treat contact angle data, Fawkes (1964) introduced the London dispersion force contribution to the surface free energy, $\gamma^d$. Liquid surface tension may then be expressed as

$$\gamma_L = \gamma_L^p + \gamma_L^d$$

(3.1.4)

where superscripts $p$ and $d$ refer to polar and dispersive force components. The polar contribution to surface tension represents the sum of all forces that may be referred to as nondispersive (e.g., hydrogen bonding, electrostatic and dipole-dipole interactions). At the interface between any liquid and another in which the intermolecular attraction is entirely due to London dispersion forces, the only appreciable interfacial interactions in which these liquids take part are the London dispersion forces. Moreover, Fowkes (1964) showed that the geometric mean of the dispersion force attractions should predict the magnitude of the interaction between dissimilar materials. Therefore, if only dispersive interactions are present at a liquid-liquid interface, the effect of interfacial attraction on the tension in the interface can be predicted by the geometric mean of the dispersion force components of the surface tension of two liquids 1 and 2 as $(\gamma_1^d \gamma_2^d)^{1/2}$. The tension in the interfacial region of liquid 1 is then $\gamma_1 - (\gamma_1^d \gamma_2^d)^{1/2}$, and that in the interfacial region of liquid 2 is $\gamma_2 - (\gamma_1^d \gamma_2^d)^{1/2}$. The interfacial tension is the sum of the tensions in both regions and is
given by
\[ \gamma_{12} = \gamma_1 + \gamma_2 - 2(\gamma_1 \gamma_2)^{1/2} \] (3.1.5)

By analogy with equation (3.1.2) (the Dupré equation), the energy required per unit area to separate the liquid-liquid interface is expressed as
\[ W_a = \gamma_1 + \gamma_2 - \gamma_{12} \] (3.1.6)

and, since \( W_a \) may also be divided into dispersive and polar components, i.e., \( W_a = W_a^d + W_a^p \) (Fowkes, 1972), then
\[ W_a^d = 2(\gamma_1 \gamma_2)^{1/2} \] (3.1.7)

Applying equation (3.1.7) to liquid contact with a solid surface; liquid-solid interface, the expression for \( W_a \), where subscripts "1" and "2" are replaced by "L" and "S" respectively, can then be rewritten as
\[ W_a = \gamma_L(1 + \cos \theta) = 2(\gamma_L \gamma_S)^{1/2} + W_a^p \] (3.1.8)

The value of the polar contribution to the work of adhesion depends upon the polar character of both the solid and the liquid that are in contact, i.e.,
\[ W_a^p = f(\gamma_S^p, \gamma_L^p) \] (3.1.9)

However, there is no way of calculating \( \gamma_S^p \) directly, and the functional representation of equation (3.1.9) is not known. In hope that development of an expression for \( W_a^p \) would contribute to a sound theoretical basis for calculation of \( \gamma_S \), effort has been dedicated to providing direction for identification of \( W_a^p \) and its use in theoretically sound surface characterization. To obtain \( W_a^p \) according to equation (3.1.8), the dispersive components of both liquid (\( \gamma_L^d \)) and
solid surface energy \((\gamma_s^d)\) must be previously determined. Following is a brief discussion of the evaluation of \(\gamma_L^d\) and \(\gamma_s^d\).

For a given solid-liquid contact, if the surface tension of either the solid or the liquid has only a dispersive component (i.e., \(\gamma_s^p\) or \(\gamma_L^p = 0\)), then the interaction between the two can be entirely attributed to dispersive forces (Fowkes, 1964; 1972). For this case equation (3.1.8) may be written

\[
W_d = \gamma_L(1 + \cos \theta) = 2(\gamma_L^d \gamma_s^d)^{1/2} = W_s^d
\]

(3.1.10)
i.e., the work of adhesion is totally due to dispersive interactions. Rearranging,

\[
\gamma_L^d = \gamma_L^2(1 + \cos \theta)^2/4\gamma_s^d
\]

(3.1.11)
\(\gamma_L\) and \(\theta\) are readily measurable. Consequently, given a solid surface of known \(\gamma_s^d\) with which no polar interactions may take place, the dispersive component of liquid surface tension, \(\gamma_L^d\), may be found with a single contact angle measurement. Obviously, evaluation of \(\gamma_s^d\) requires knowledge of \(\gamma_L^d\). Fortunately, a number of liquids exist (particularly hydrocarbons) whose surface tension is totally due to dispersive forces. For these liquids, \(\gamma_L = \gamma_L^d\), where \(\gamma_L\) may be measured by conventional methods. A different rearrangement of equation (3.1.10) yields

\[
\cos \theta = 2(\gamma_L^d \gamma_s^d)^{1/2}/\gamma_L - 1
\]

(3.1.12)
Choosing a series of nonpolar liquids, for any solid a plot of \(\cos \theta\) versus \((\gamma_L^d)^{1/2}/\gamma_L\) \((=1/\gamma_L^{1/2})\) should be a straight line with
slope \(2(\gamma^d_s)^{1/2}\) intercepting the ordinate at -1. Armed with a "diagnostic solid" for which \(\gamma_s = \gamma_s^d\) (e.g., paraffin), test liquids exhibiting both polar and nonpolar character may be characterized with use of equation (3.1.11).

With test liquids of known \(\gamma_L, \gamma_L^d,\) and \(\gamma_L^p,\) and with methodology for evaluation of \(\gamma_s^d,\) evaluation of solid surface influences on \(W_p^s\) should be possible according to equation (3.1.9). Rearranging equation (3.1.8) gives

\[
W_p^s = \gamma_L(1 + \cos \theta) - 2(\gamma_L^d\gamma_s^d)^{1/2}
\] (3.1.13)

Equation (3.1.13) can be used to evaluate \(W_p^s\) for each test liquid put in contact with a given material. Having evaluated \(\gamma_L^p\) for each test liquid (where \(\gamma_L^p = \gamma_L - \gamma_L^d\)), a plot of \(W_p^s\) versus \(\gamma_L^p\) can be constructed. The relationship between \(W_p^s\) and \(\gamma_L^p\) is usually linear, with the values of slope and intercept suggested to be unique for each material.

The value of the polar component of the work of adhesion between any solid surface and water \((W_p^s,\text{water})\) provides an index of surface hydrophobicity (McGuire, 1990). \(W_p^s,\text{water}\) can be calculated as the following:

\[
W_p^s,\text{water} = k(\gamma_p^L,\text{water}) + b
\] (3.1.14)

where \(k\) and \(b\) are the slope and intercept, respectively, of a plot of \(W_p^s\) versus \(\gamma_L^p.\) Since \(W_p^s,\text{water}\) can be determined from the equation above, actual contact angle data for water need not be used. This avoids rather serious problems associated with using pure water as a diagnostic liquid (Andrade, 1985).
3.2 Ellipsometry

The ellipsometer is used to determine the thickness and refractive index of thin films by measuring changes in the state of polarized (laser) light reflected from the sample surface. The technique may be applied to any substratum-film combination that provides reasonably specular reflection of the incident light beam. The measurement of the effect of reflection on the state of polarization of light (ellipsometry) is also referred to as reflection polarimetry or polarimetric spectroscopy (Archer, 1968). These measurements may be used to yield the optical constants of the substratum or the thickness and refractive index of a film covering the substratum, and the technique can be applied to surface films with thicknesses ranging from those corresponding to partial monoatomic coverage up to several microns. In situ ellipsometry (dynamic ellipsometry, automatic ellipsometry, auto gain ellipsometry) is used to continuously monitor the thickness and refractive index of a film as it grows.

Ellipsometric measurements involve illuminating the surface of a sample with monochromatic light having a known, controllable state of polarization and then analyzing the polarization state of the reflected light. The monochromatic light source is a low-power, helium-neon laser normally having a beam wavelength of 6328 Å. The beam is passed through a polarizer where its state of polarization is converted from
circular to linear before striking the sample surface. This constant intensity, linearly polarized beam is then converted to one of circular polarization if a quarter-wave compensator is inserted in the optical path. The light reflected from the sample surface, with its polarization altered by the optical properties of the sample, passes through a rotating analyzer prism, and is sensed by a photodetector. The photodetector converts the light energy into an electric current proportional to the intensity of the reflected light passing through the analyzer. The measured optical properties of each adsorbed film are used to determine its refractive index and thickness; these film properties are then used to estimate adsorbed mass on the surface.

The state of polarization is defined by particular phase and amplitude relationships between the two component plane waves into which the electric field oscillation is resolved. One wave, designated p, is in the plane of incidence and the other, designated s, is normal to the plane of incidence. If the p and s components are in phase, the wave is said the be plane (linearly) polarized. A difference in phase (other than 180°) corresponds to elliptical polarization. In general, reflection causes a change in the relative phases of the p and s waves and a change in the ratio of their amplitudes. Reflected light is characterized by the angle Δ, defined as the change in phase, and the angle Ψ, the arctangent of the factor by which the amplitude ratio changes. If the
amplitudes of the incident and reflected beams are designated \( E \) and \( R \), respectively, and the phase angles are designated \( \beta \), then the angles \( \Delta \) and \( \Psi \) can be expressed as follows

\[
\Delta = (\beta_p - \beta_s)_{\text{reflected}} - (\beta_p - \beta_s)_{\text{incident}} \tag{3.2.1}
\]

and

\[
\Psi = \arctan[(R_p/R_s)(E_p/E_p)] \tag{3.2.2}
\]

Figure 3.2 depicts a typical system for ellipsometric study consisting of a film of refractive index \( n_2 \) and thickness \( d \) on a reflecting substrate of index \( n_3 \) immersed in a medium of index \( n_1 \). The values of the indices \( n_2 \) and \( n_3 \) may be complex numbers (if they absorb light to any degree), but \( n_1 \) will be treated as a real number in the following development (McCrackin, 1963).

Considering light incident (at angle \( \phi_1 \)) at the boundary between the immersion medium and film, the cosine of the angle of refraction can be written as

\[
\cos \phi_2 = \sqrt{1 - \left(1 - \left(\frac{n_1}{n_2}\sin \phi_1\right)^2\right)} \tag{3.2.3}
\]

To make sense of ellipsometric data, the relationship between \( \Delta \) and \( \Psi \) and the properties of the reflecting system must be known. The relationship is developed with the Fresnel reflection coefficients, which represent the ratio of the electric field vector, \( R \), of the reflected wave to that, \( E \), of the incident wave. For the isotropic system of Figure 3.2, the parallel (p) and normal (s) reflection coefficients for light incident at the immersion medium-film interface are

\[
r_{12}^p = \frac{n_2 \cos \phi_1 - n_1 \cos \phi_2}{n_2 \cos \phi_1 + n_1 \cos \phi_2} \tag{3.2.4}
\]
Figure 3.2 Reflection from a Film-covered Surface
and
\[ r_{12}^s = \frac{n_1 \cos \phi_1 - n_2 \cos \phi_2}{n_1 \cos \phi_1 + n_2 \cos \phi_2} \] (3.2.5)

Similarly, the reflection coefficients \( r_{23}^p \) and \( r_{23}^s \) can be developed.

The total reflection coefficients, \( R^p \) and \( R^s \), which include the contributions of reflections from lower boundaries are given by
\[ R^p = \frac{r_{12}^p + r_{23}^p \exp D}{1 + r_{12}^p r_{23}^p \exp D} \] (3.2.6)
and
\[ R^s = \frac{r_{12}^s + r_{23}^s \exp D}{1 + r_{12}^s r_{23}^s \exp D} \] (3.2.7)

where \( \cos \phi_3 \) values (required for calculation of these coefficients) are given by an expression similar to equation (3.2.3), and
\[ D = -4\pi j n_2 (\cos \phi_2) d_2 / \lambda \] (3.2.8)

where \( \lambda \) is the wavelength of light used, \( j = (-1)^{1/2} \), and \( d_2 \) is the film thickness. The ratio of the parallel and normal reflection coefficients is defined as \( \rho \), where
\[ \rho = \frac{R^p}{R^s} \] (3.2.9)

This ratio may be expressed in terms of \( \Delta \) and \( \Psi \) as
\[ \rho = \tan(\Psi) \exp(j\Delta) \] (3.2.10)

Finally, the complex refractive index of the reflecting substratum can be calculated from
\[ n_3 = n_1 (\tan \phi_0 [1 - 4\rho (\sin^2 \phi_0) / (\rho + 1)^2]^{1/2} \] (3.2.11)

Equation (3.2.11) must be solved for the substrate prior to studying adsorption onto that substratum. Fortunately, it can be solved directly with acquisition of \( \Psi \) and \( \Delta \) for the clean,
bare substratum surface (i.e., \(d_2 = 0\)).

Several methods are available for determining the thickness and refractive index of the adsorbed film; however, it is most efficient to solve the preceding equations directly. Substituting equations (3.2.6), (3.2.7), and (3.2.9) into (3.2.10) and rearranging gives a quadratic expression of the form

\[ C_1(\exp D)^2 + C_2(\exp D) + C_3 = 0 \]  

(3.2.12)

where \(C_1\), \(C_2\), and \(C_3\) are complex functions of the refractive indices, angle of incidence, \(\Psi\) and \(\Delta\). If the refractive index of the film is known, two solutions of \(\exp D\) (hence, \(d_2\)) may be calculated. Since the coefficients are complex, calculated values of film thickness should be expected to be complex as well. However, the correct film thickness must be a real number as it represents a real quantity; therefore, the solution of equation (3.2.12) that yields a real film thickness is the correct solution. In practice, experimental errors result in both solutions yielding complex values of \(d_2\). In such cases, the solution with the smallest imaginary component is chosen as the correct solution, and the imaginary component itself provides a relative measure of error. The real portion of \(d_2\) is then used to compute \(\Delta\) and \(\Psi\) by equations (3.2.6) through (3.2.10). Of course, since the imaginary component of \(d_2\) \((d_j)\) was dropped, these values will differ from the experimental angles by amounts \(\delta \Delta\) and \(\delta \Psi\), and \(d_j\), \(\delta \Delta\) and \(\delta \Psi\) are all measures of experimental error. For the
results to be valid, however, $\delta\Delta$ and $\delta\Psi$ must be within the limits of the experimental error incurred in actually determining $\Psi$ and $\Delta$; this is a more direct determination of the validity of an experiment than is the magnitude of $d_i$. 
4. MATERIALS AND METHODS

4.1 Protein Solution Preparation

β-Lactoglobulin from bovine milk, purchased from Sigma Chemical Co. (St. Louis, MO), was carefully weighed (Mettler Model AE 240, Mettler Instrument Corp., Hightstown, NJ) and dissolved in a phosphate buffer solution. The protein solution was stirred for 20 min, then use either directly in an experiment or after dilution. Buffer solutions were prepared by titrating a solution of 0.01 M sodium phosphate monobasic monohydrate (NaH₂PO₄·H₂O) and 0.01 M sodium phosphate dibasic (Na₂HPO₄). Sodium azide (NaN₃), used as an anti-microbial agent, was also added to the solutions at a concentration of 0.02% (mass per volume) prior to the titration to pH 7.00. Both buffer and protein solutions used in the kinetic studies were filtered (0.22 μm type GV, Millipore Corp., Bedford, MA) prior to injection into a fused quartz, trapezoid cuvette (Hellma Cells., Germany).

4.2 Surface Preparation

All surfaces were prepared from a single type of silicon (Si) wafer (hyperpure, type N, phosphorous doped, plane 1-0-0) purchased from Wacker Siltronic Corp. (Portland, OR). First, the Si wafers were cut into small plates of approximately 1x2 cm using a tungsten pen. They were subsequently treated to exhibit hydrophilic or hydrophobic surfaces.
Hydrophilic Treatment

The following treatment was slightly modified from the method as described in the literature (Jönsson et al., 1982). Each small Si plate was placed into a test tube and 5 mL of the mixture NH$_4$OH:H$_2$O$_2$:H$_2$O (1:1:5) was added to the tube followed by heating to 80°C in a water bath for 15 min. The Si plates were then rinsed with 20 mL of distilled-deionized water (Corning Mega pure system$^{\text{TM}}$, Corning, NY) followed by immersion in 5 mL HCl:H$_2$O$_2$:H$_2$O (1:1:5) for 15 min at 80°C. Each plate was then rinsed with 30 mL distilled-deionized water. In order to maintain some stability in the hydrophilicity of the surface, each Si plate was stored in 20 mL of 50% ethanol/water solution.

Silanization of Silicon Surfaces

The hydrophilic Si plates were rinsed with 40 mL distilled-deionized water, then dried with N$_2$. The surfaces were then stored in a desiccator for 24 hours. Dried hydrophilic Si plates were then treated to be hydrophobic by immersion in a stirred solution of dichlorodimethylsilane (DDS, Aldrich Chemical Co., Inc., Milwaukee, WI) in xylene for 1 hour. The degree of silanization was controlled by varying the concentration of DDS. The concentrations used in this work ranged from 0.0 to 1.0% DDS in xylene. Finally, the silanized silicon surfaces were sequentially rinsed in 100 mL xylene, acetone, then ethyl alcohol. The plates were dried in a desiccator, and their pertinent surface characteristics (Ψ,
evaluated using ellipsometry and contact angle analysis.

4.3 Surface Characterization

The hydrophilic-hydrophobic balance of each surface was determined using contact angle analysis according to equations (3.1.11) through (3.1.14). The contact angle formed when a drop of liquid is brought into contact with a solid surface was measured with a contact angle goniometer (Ramé-Hart, Inc., Mountain Lakes, NJ) equipped with an environmental chamber. The plate is placed in the environmental chamber which is maintained at about 25°C; water-saturated conditions are ensured by the presence of cotton wool saturated with warm water.

The diagnostic liquids used included a series of ethanol/water solutions (5, 10, 20, 30, 40, and 50% by volume), 1-bromonaphthalene (C_{10}H_{7}Br) and diiodomethane (CH_{2}I_{2}). The liquid surface tension ($\gamma_L$) of each diagnostic liquid was measured by the ring method with a DuNoüy tensiometer (CSC Scientific Co., Inc., Fairfax, VA). Measurements were performed at approximately 25°C and were reproduced to within 0.1 mN/m.

4.4 Adsorption Equilibrium

Seven surfaces exhibiting different extents of silanization (prepared using concentrations of 1, 0.2, 0.04,
0.01, 0.005, 0.0025, and 0.0% by volume of DDS in xylene) were used in the equilibrium study. The optical properties ($\Psi_s, \Delta_s$) of cleaned Si plates were measured using an ellipsometer (Model L104 SA, Gaertner Scientific Corp., Chicago, IL). The measurements were taken at six different regions on each surface. Protein solutions of seven different concentrations (100, 200, 300, 500, 700, 1000, and 1500 mg/L) were prepared by dilution from a stock solution of 1500 mg/L.

Cleaned, characterized surfaces were placed in 30 mL beakers and immersed in 20 mL protein solution: contact was maintained for 8 hours in a water-bath maintained at room temperature (approximately 26°C). Surfaces were then carefully removed from the solutions, rinsed by immersing in 500 mL distilled-deionized water three times, and kept in a desiccator for 24 hours before measuring adsorbed film properties. Ellipsometric measurements were taken at eight different regions on each surface. The data ($\Psi$ and $\Delta$) were stored on a floppy disk. The computer program described in Appendix A was used to import the data from the disk and determine the refractive index and thickness of each adsorbed film which, in turn, were used to calculated the adsorbed mass of protein on each surface.

4.5 Adsorption Kinetics

Five surfaces exhibiting different extents of silanization were prepared using concentration of 1, 0.1,
0.05, 0.025, and 0.01% by volume of DDS in xylene. Silanized, bare surfaces were placed into a trapezoid cuvette designed in our laboratory (see Figure 4.1). The ellipsometer sample stage was then adjusted to obtain a maximum in reflected light intensity. Thirty milliliters of filtered buffer solution was then injected into the cuvette. The surface was left to equilibrate with the buffer for 1 hour. Fine adjustments of the stage were conducted in parallel with ellipsometric measurement of bare surface optical constants to obtain a steady value of \( \Psi_s \) and \( \Delta_s \). Final measurements of bare surface properties were then recorded. The buffer solution was carefully removed from the cuvette and replaced with 30 mL of filtered protein solution. The values of \( \Psi \) and \( \Delta \) were ellipsometrically measured and recorded every 30 seconds for 8 hours under static conditions, i.e., no stirring and no flow. Recorded values of \( \Psi \) and \( \Delta \) were used in the computer program to determine film refractive index, thickness, and the adsorbed mass of protein on each surface according to procedure described in Appendix A.
Figure 4.1 The Trapezoid Cuvette
5. RESULTS AND DISCUSSION

5.1 Adsorption Equilibrium

Hydrophobic interactions contribute to protein adsorption by elevating the driving force for protein molecules from aqueous solution to make contact with a solid surface (Lee and Kim, 1974; Lee and Ruckenstein, 1988). Hydrophobic surfaces have a low affinity for water molecules; the protein will change its conformation to reduce contact of its hydrophobic regions with the aqueous environment by either "burying" hydrophobic regions internally or associating them with similar regions on the solid surface.

Contact angle analysis was used to determine the hydrophobic character of the solid surfaces studied in this work. Contact angle data recorded for a number of diagnostic liquids contacting each surface was used to determine the slope and intercept defining the linear relationship between $W_p$ and $\gamma_l^p$. The values of slope and intercept determined for each surface, as shown in Appendix E, were subsequently used to calculate the value of the polar component of the work of adhesion between each surface and water ($W_{p,\text{water}}$), i.e., the work required to part water molecules from unit area of surface. The hydrophobic character of a surface decreases with increasing $W_{p,\text{water}}$.

The surfaces used in this study were silanized silicon surfaces, where methyl groups are covalently bound to the
silicon atoms by a comparatively strong force. The density of methyl groups on the surface can be varied by changing the degree of silanization. A higher density of methyl groups on a given surface will cause that surface to exhibit a more hydrophobic character. Values of $W_{sp,water}$ recorded for each silanized silicon surface are listed in Appendix E.

Adsorption isotherms were constructed for $\beta$-lactoglobulin by contacting it at seven concentrations with each of the seven silanized silicon surfaces. Applying the Lorentz-Lorenz relationship (Appendix A), the adsorbed mass of protein on each surface was calculated from its film thickness and refractive index.

Adsorption isotherms constructed for the seven different surfaces are shown in Figures 5.1-3. The isotherms for silicon prepared with 1.0 and 0.2% DDS, shown in Figure 5.1, almost coincide. This indicates that increasing the surface hydrophobicity from that resulting from preparation with 0.2% DDS ($W_{sp,water} = 10.27 \text{ mJ/m}^2$) to that corresponding to treatment with 1% DDS ($W_{sp,water} = 2.94 \text{ mJ/m}^2$) was not observed to affect the equilibrium adsorbed mass in the concentration range studied. The protein apparently "sees" a similar distribution of methyl groups for each surface; presumably each of these surfaces is "saturated" with methyl groups. For surfaces prepared with DDS in a range between 0.005% ($W_{sp,water} = 41.8 \text{ mJ/m}^2$) and 0.2% ($W_{sp,water} = 7.0 \text{ mJ/m}^2$), Figure 5.2 shows that adsorbed mass increased substantially with surface
Figure 5.1 Adsorption Isotherms Constructed for Surfaces Treated with 1.0 and 0.2% DDS

Δ - 1% DDS, ○ - 0.2% DDS
Figure 5.2 Adsorption Isotherms Constructed for Surface Treated with 0.2, 0.04, 0.01, and 0.005% DDS
Figure 5.3 Adsorption Isotherms Constructed for Surface Treated with 0.005, 0.0025, and 0.0% DDS

Δ - 0.005% DDS, ○ - 0.0025% DDS, and □ - 0.0% DDS
hydrophobicity, indicating that protein adsorption is enhanced with increasing surface hydrophobicity. Hydrophobic interaction contributes to the increase in the entropic driving force that accompanies adsorption of protein from solution. Entropy is increased due to freedom gained by water molecules resulting from partial dehydration of the solid surface and the protein "surface", and in part due to structural changes that occur in the less rigid, partially dehydrated protein molecule. Finally, it is interesting to note that adsorbed mass was not affected by a further decrease in surface hydrophobicity, attained by surface preparation with DDS concentrations at 0.005, 0.0025, and 0.0%, as shown in Figure 5.3. Apparently, below some critical extent of surface methylation, the protein did not "see" a difference in chemistry among the surfaces. The population of methyl groups was perhaps not dense enough to affect the magnitude of the entropic driving force for protein adsorption on any of these surfaces.

The plateau value in adsorbed mass was estimated for each isotherm by fitting the data to a Langmuir-type equation as described in Appendix B. A plot of the plateau value estimated for each surface versus \( W_{\text{water}}^p \) is shown in Figure 5.4. The result shown in Figure 5.4 agrees with phenomena discussed with reference to Figures 5.1-3. Plateau values in equilibrium adsorbed mass were observed to decrease with decreasing degree of methylation (from 0.2% to 0.005% DDS),
Figure 5.4 Effect of Surface Hydrophobicity on Plateau Values Attained in Equilibrium Adsorbed Mass
or increasing \( W_{\text{water}} \) (from 7.0 to 41.8 mJ/m\(^2\)). Plateau values in adsorbed mass were similar for surfaces treated with 1% and 0.2% DDS, and were observed to level off for (hydrophilic) surfaces treated with 0.005% to 0.0% DDS.

5.2 Adsorption Kinetics

Lundström (1985) proposed a mechanism for reversible protein adsorption. The mechanism allows for two forms of adsorbed protein, resulting in two types of fractional surface coverage: a "native" and "denatured" form. These forms of protein can also be desorbed into the bulk solution. One unique character of this model is that each form of adsorbed molecule covers a different surface area. Currently, there exists no direct experimental evidence providing knowledge of the ratio of the surface area covered by the native form to that of the denatured form. The model for protein adsorption adapted from this mechanism is complicated, and an analytical solution of the change in the fractional surface coverages as a function of time cannot be obtained.

Recently, Lundström and Elwing (1990) presented a more complex model for protein adsorption. The model allows for several forms of adsorbed molecules, as well as a "self exchange" feature, where adsorbed molecules can be replaced by similar molecules from the bulk solution. They also solved for a case allowing for exchange between dissimilar molecules. Due to complexity of these models, only numerical solutions
can be obtained, allowing for simulation studies to be performed with selected relative values of pertinent rate constants. It is not very meaningful to use one of these models to compare with the type of experimental data obtained by current methodology because the fractional surface coverage in the specific stages cannot be measured with certainty. Thus the rate constants governing different parts of the mechanism cannot be determined.

A mechanism for irreversible protein adsorption can be proposed as consisting of two steps. In step 1, corresponding to short contact time, the protein molecule reversibly adsorbs to the surface, with its adopted surface conformation closely approximating its native form. In step 2, a surfaced-induced conformational change takes place in which the reversibly adsorbed molecule is changed to an irreversibly adsorbed form. These steps are illustrated in the simple mechanism of protein adsorption sketched in Figure 5.5. In the present development, it is reasonable to assume that the final stage of protein adsorption is, more or less, irreversible because the desorption rate decreases with increasing contact time (Andrade et al., 1984; Brash et al., 1969; Brash and Horbett, 1987). In the first step of the proposed mechanism, protein molecules are allowed to desorb into the bulk solution, which is simply an indirect way of incorporating the self exchange feature of the previous model (Lundström and Elwing, 1990).
Figure 5.5 Proposed Mechanism of Protein Adsorption
Neglecting the influence of diffusion, an expression for adsorbed mass as a function of time, protein concentration and reaction rate constants can be derived by first writing equations describing the time-dependent fractional surface coverage of protein in each of the two states shown, one reversibly adsorbed ($\theta_1$) and one irreversibly adsorbed ($\theta_2$), as follows:

\[
d\theta_1/dt = k_1C(1-\theta_1-\theta_2) - (k_1 + s_1)\theta_1 \tag{5.1}
\]

and

\[
d\theta_2/dt = s_1\theta_1 \tag{5.2}
\]

Also, at any time, the total surface coverage is described by

\[
\theta = \theta_1 + \theta_2 \tag{5.3}
\]

In solving equations (5.1) through (5.3), it will be assumed that the protein molecule occupies the same surface area in states 1 and 2. The equations can then be solved analytically for $\theta_1$ and $\theta_2$:

\[
\theta_1 = c_1 \exp(-r_1t) + c_2 \exp(-r_2t) \tag{5.4}
\]

and

\[
\theta_2 = c_3 \exp(-r_1t) + c_4 \exp(-r_2t) + c_5 \tag{5.5}
\]

where the roots $r_1$ and $r_2$ (both negative numbers) for each equation above are given by

\[
r_1 = 1/2(k_1C + k_1 + s_1) + 1/2[(k_1C + k_1 + s_1)^2 - 4s_1k_1C]^{1/2} \tag{5.6}
\]

and

\[
r_2 = 1/2(k_1C + k_1 + s_1) - 1/2[(k_1C + k_1 + s_1)^2 - 4s_1k_1C]^{1/2} \tag{5.7}
\]
Addition of equations (5.4) and (5.5) yields an expression for \( \theta \) as a function of time:

\[
\theta = \theta_1 + \theta_2 = A_1 \exp(-r_1 t) + A_2 \exp(-r_2 t) + A_3
\]  

(5.8)

The parameter \( A_3 \) corresponds to \( \theta_2 \) at \( t = \infty \), and \( A_1 \) and \( A_2 \) are unknown functions of protein concentration and rate constants. The surface coverages obey the initial condition that at \( t = 0 \), \( \theta_1 = \theta_2 = \theta = 0 \), as well as the final conditions: at \( t = \infty \), \( \theta_1 = 0 \) and \( d\theta_2/dt = 0 \). An expression for total adsorbed mass \( (\Gamma) \) as a function of time can be obtained from equation (5.8) as:

\[
\Gamma = a_1 \exp(-r_1 t) + a_2 \exp(-r_2 t) + a_3
\]  

(5.9)

The parameters \( a_1 \), \( a_2 \), and \( a_3 \) listed in Table 5.1 are the products of \( \Gamma_{\max} \), the equilibrium adsorbed mass, with \( A_1 \), \( A_2 \), and \( A_3 \) respectively. The values of kinetic model parameters averaged from the values listed in Appendix D are shown in Table 5.1.

Ellipsometric measurements of \( \beta \)-lactoglobulin adsorption were performed over a period of eight hours at a protein concentration of 1000 mg/L on each of five silanized silicon surfaces prepared with 1, 0.1, 0.05, 0.025, and 0.01% DDS. The concentration of protein used in these experiments was selected because it is a relatively high value that should minimize diffusional limitations while still allowing for unambiguous ellipsometric measurement. The five modified silicon surfaces were selected based on the results of the equilibrium study.
Table 5.1 Averaged Values of Kinetic Model Parameters
(with reference to the values in Appendix D)

<table>
<thead>
<tr>
<th>% DDS</th>
<th>$W_{s,\text{water}}$</th>
<th>$a_1$</th>
<th>$a_2$</th>
<th>$a_3$</th>
<th>$r_1$</th>
<th>$r_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>2.935</td>
<td>-.104</td>
<td>-.045</td>
<td>.149</td>
<td>1.516</td>
<td>.0125</td>
</tr>
<tr>
<td>0.1</td>
<td>10.265</td>
<td>-.101</td>
<td>-.063</td>
<td>.164</td>
<td>1.976</td>
<td>.0092</td>
</tr>
<tr>
<td>0.05</td>
<td>10.272</td>
<td>-.105</td>
<td>-.053</td>
<td>.158</td>
<td>1.996</td>
<td>.0092</td>
</tr>
<tr>
<td>0.025</td>
<td>25.259</td>
<td>-.111</td>
<td>-.058</td>
<td>.169</td>
<td>2.031</td>
<td>.0088</td>
</tr>
<tr>
<td>0.01</td>
<td>27.03</td>
<td>-.096</td>
<td>-.061</td>
<td>.157</td>
<td>2.180</td>
<td>.0067</td>
</tr>
</tbody>
</table>
Some representative results of those kinetic tests are shown in Figures 5.6-10. Experiments with each type of surface were replicated at least four times to ensure consistency in the technique and to decrease experimental error associated with the results. The data indicate that adsorption on surfaces with greater hydrophobicity reaches a plateau faster than does adsorption to surfaces of less hydrophobic character. This is in agreement with the results of Wahlgren and Arnebrant (1990), where plots of adsorbed mass of β-lactoglobulin on silica and methylated silica surfaces versus time were compared.

Interpretation of the plateau values (parameter $a_3$) in the kinetic plots is not as clear as the results from the adsorption isotherms would indicate, i.e., that β-lactoglobulin adsorbs to a greater extent on hydrophobic surfaces. One might suggest that there is bilayer or partial bilayer formation on increasingly hydrophilic surfaces which is rinsed away before ellipsometric measurement in the equilibrium study. In any event, this unclear result could in part be due to experimental difficulty experienced in maintaining a constant ellipsometric reading for the bare surface properties during the period in which the buffer solution is removed, and the protein solution is injected into the cuvette. However, this inconsistency is believed not to significantly affect the rate of protein adsorption. It is important to note that performing the kinetic study under
Figure 5.6 Adsorption Kinetic Data Recorded on a Silicon Surface Treated with 1% DDS (The fitted line through the data is generated with equation (5.9).)
Figure 5.7 Adsorption Kinetic Data Recorded on a Silicon Surface Treated with 0.1% DDS (The fitted line through the data is generated with equation (5.9).)
Figure 5.8 Adsorption Kinetic Data Recorded on a Silicon Surface Treated with 0.05% DDS (The fitted line through the data is generated with equation (5.9).)
Figure 5.9 Adsorption Kinetic Data Recorded on a Silicon Surface Treated with 0.025% DDS (The fitted line through the data is generated with equation (5.9).)
Figure 5.10 Adsorption Kinetic Data Recorded on a Silicon Surface Treated with 0.01% DDS (The fitted line through the data is generated with equation (5.9).)
static conditions has advantages over conducting the experiments under flow conditions. For example, under flow conditions, problems associated with initial mixing can affect kinetic behavior as well as the plateau values, since protein concentration at the boundary layer is drastically changed during the initial adsorption period.

The pattern of the kinetic data recorded on each type of surface agree very well with the model proposed in equation (5.9). Nonlinear regression performed on adsorption kinetic data fit to equation (5.9) yields estimates of the parameters $a_1$, $a_2$, $a_3$, $r_1$ and $r_2$, as shown in Appendix D. Incorporating the known values for $r_1$ and $r_2$ into equations (5.6) and (5.7) yields two relationships among the unknown values $k_1$, $k_1$, and $s_1$; these relationships correspond to a specific protein-surface interaction. In particular, simple algebraic manipulation of equations (5.6) and (5.7) yields the following two equations with the three unknown rate constants:

\[(r_1 + r_2) = (k_1 C + k_1 + s_1)\]  \hspace{1cm} (5.10)

and

\[r_1 r_2 = s_1 k_1 C\] \hspace{1cm} (5.11)

Without a third relationship among the rate constants, their absolute values remain unknown. Figures 5.11 and 5.12 show plots of averaged values of $r_1$ and $r_2$, as listed in Table 5.1, versus $W_s^p_{water}$ for each surface. The data indicate that as surface hydrophobicity decreases, the magnitude of $r_1$ tends to increase while that of $r_2$ tends to decrease.
Figure 5.11 Effect of Surface Hydrophobicity on Parameter $r_1$
Figure 5.12 Effect of Surface Hydrophobicity on Parameter $r_2$
The parameters $r_1$ and $r_2$ are complex functions of the rate constants, as shown in equations (5.6) and (5.7), and it is therefore difficult to explicitly define the relationship between $r_1$ or $r_2$, and the individual rate constants. However, based on the nature of equation (5.9), a partial explanation of the adsorption behavior can be obtained with reference to Figures 5.11 and 5.12. Due to its smaller magnitude, $r_2$ affects the rate of increase in adsorbed mass, $\Gamma$, for a longer period than does $r_1$: $r_1$ only affects $\Gamma$ at very early times, as $\exp(r_1t)$ rapidly approaches zero as time increases. Based on equation (5.9), with negative $a_1$ and $a_2$, it would take longer for $\Gamma$ to reach a plateau as the magnitude of $r_2$ decreases. This is observed as surface hydrophobicity decreases, in agreement with the behavior of $\Gamma$ seen in Figures 5.6-10. Considering the magnitude of $r_1$ and its behavior as a function of surface hydrophobicity, Figure 5.11 might lead one to expect a delay in the initial adsorption rate with increasing surface hydrophobicity. It should be emphasized here that $r_1$ has little relevance at times corresponding to plateau attainment. In any event, such behavior was not obviously observed in these kinetic experiments.

Parameters $r_1$ and $r_2$, interpreted with reference to equations (5.10) and (5.11), indicate that the magnitude of $(k_1C + k_4 + s_1)$ increases while that of $(s_1k_1C)$ decreases with decreasing surface hydrophobicity, as shown in Figures 5.13 and 5.14. Even though the values of the rate constants cannot
Figure 5.13 Effect of Surface Hydrophobicity on \((r_1 + r_2)\) or \((k_1C + k_s + s_1)\).
Figure 5.14 Effect of Surface Hydrophobicity on $(r_1 \times r_2)$ or $s_i k_i C$
be determined explicitly, the behavior of \((r_1+r_2)\) and \((r_1-r_2)\) as illustrated in Figures 5.13 and 5.14 would lend support to a hypothesis that \(s_i\) and \(k_i\) increase with increasing solid surface hydrophobicity, while the tendency of adsorbed protein to desorb (represented by \(k_i\)) decreases.

Surfaces with more hydrophilic character are expected to carry a net negative charge in these experiments, whereas a lower degree of electrostatic repulsion would be expected at a hydrophobic surface. Thus, a larger repulsive force may be present at a hydrophilic surface. This may, in part, contribute to the reason that \(k_i\) increases, while \(k_i\) decreases as surface hydrophobicity increases. In addition, a favorable hydrophobic interaction may also contribute to this observation.

5.3 Simulation of Protein Adsorption

Numerical simulation is another way to study the relationship between rate constants, adsorbed mass, and fractional surface coverages of protein, i.e., \(\theta\), \(\theta_1\), and \(\theta_2\). Eight simulations were thus designed to investigate effects associated with varying \(s_i\), \(k_iC\), and \(k_i\) in the manner shown in Table 5.2.

Effect of \(s_i\) on Fractional Surface Coverage

The effect of conformational change rate constant \((s_i)\) on the fractional surface coverages \(\theta\), \(\theta_1\), and \(\theta_2\) as functions of
### Table 5.2 Selected Values of Rate Constants Used in the Model Simulation

<table>
<thead>
<tr>
<th>Run #</th>
<th>$s_i$ min$^{-1}$</th>
<th>$k_iC$ min$^{-1}$</th>
<th>$k_i$ min$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.01</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>0.01</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>4</td>
<td>0.1</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>0.01</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>0.01</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>7</td>
<td>0.1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>
time was investigated at "high" and "low" values of $k_1C$ and $k_1$. When the value of $s_1$ is low (0.01), $\theta_2$ reaches a plateau at a slower rate, and $\theta_1$ approaches zero at a slower rate than when the value of $s_1$ is high (0.1). This behavior is consistent at high and low values of $k_1C$ and $k_1$ as shown in pair comparisons of runs 5 and 7, and 6 and 8, in Figure 5.15.

**Effect of $k_1C$ on Fractional Surface Coverage**

The effect of the adsorption rate constant ($k_1C$) on $\theta$, $\theta_1$, and $\theta_2$ as functions of time was studied at low and high values of $k_1C$ and $s_1$. Runs 1 and 5 and runs 4 and 7 are compared in Figure 5.16. Relative to the high value of $k_1C$, at the low value (0.1), both total surface coverage and $\theta_2$ reach their plateau value at a slower rate, and the maximum value of $\theta_1$ is lower, and $\theta_1$ approaches zero at a slower rate than when the value of $k_1C$ is high (1.0). This seems most evident at high values of $s_1$ (0.1).

**Effect of $k_1$ on fraction surface coverage**

The effect of the desorption rate constant ($k_1$) on $\theta$, $\theta_1$, and $\theta_2$ as functions of time was studied at low and high values of $k_1C$ and $s_1$. Runs 2 and 5 and runs 3 and 7 are compared in Figure 5.17. At the lower value of $k_1$, both total surface coverage and $\theta_2$ approach their plateau values faster than do $\theta$ and $\theta_2$ simulated at the higher $k_1$. The maximum value of $\theta_1$ also decreases when the value of $k_1$ is increased, but $\theta_1$ Figure
Figure 5.15 Effect of $s_i$ on Fractional Surface Coverage
($\theta$-dark solid line, $\theta_1$-dashed line, $\theta_2$-solid line)
Figure 5.16 Effect of $k_1C$ on Fractional Surface Coverage
($\theta$-dark solid line, $\theta_1$-dashed line, $\theta_2$-solid line)
Run 2: \( s_1 = 0.01; k_1C = 1.0; \)
and \( k_i = 0.1 \)

Run 5: \( s_1 = 0.01; k_1C = 1.0; \)
and \( k_i = 1.0 \)

Run 3: \( s_1 = 0.1; k_1C = 1.0; \)
and \( k_i = 0.1 \)

Run 7: \( s_1 = 0.1; k_1C = 1.0; \)
and \( k_i = 1.0 \)

Figure 5.17 Effect of \( k_i \) on Fractional Surface Coverage
(\( \theta \)-dark solid line, \( \theta_i \)-dashed line, \( \theta_2 \)-solid line)
approaches zero at a slower rate with increasing $k_1$.

These results are consistent with what one should expect regarding the behavior of $\theta$ and $\theta_2$ in reaching their plateau. If the arrival rate of native protein molecules is faster than the rate that these molecules desorb from the surface (i.e., $k_1 C / k_1$ is large), $\theta$ and $\theta_2$ should reach their plateau faster. Additionally, if the rate of conformational change experienced by the native molecule is increased, it seems clear that the time required for $\theta$ and $\theta_2$ to reach a plateau should decrease.

As surface hydrophobicity increases, adsorbed mass was observed experimentally to reach a plateau in a shorter period of time. As shown in Figure 5.13 and 5.14, the magnitude of $(s_1 k_1 C)$ apparently increased while that of $(k_1 C + k_1 + s_1)$ decreased as surface hydrophobicity was increased. The simulation results support the thought that as $s_1$ and $k_1$ increase, $\theta$ and $\theta_2$ reach plateau faster. Thus the hypothesis that $s_1$ and $k_1$ increase with increasing surface hydrophobicity is completely consistent with results gained experimentally and by simulation.

Finally, one may conclude that the present kinetic model agrees well not only with regard to statistical analysis of the experimental data but also with regard to the simulated kinetic behavior as the rate constants are changed. With trust in the kinetic model, the behavior of reversibly and irreversibly adsorbed protein at solid/water interfaces can probably be monitored successfully with simulation.
5.4 Mass Transfer Limitations

The adsorption of protein to a surface generally involves both transport of protein to the surface followed by binding to that surface. In the case of transport-controlled adsorption, the rate of protein binding to the surface is faster than the rate of protein transport from solution to the surface. Thus, the concentration of protein in the bulk phase will directly affect the rate of adsorption. If the "diffusion to semi-infinite slab" is applied to determine the mass transfer coefficient, the rate of adsorption to the surface can be expressed as equation (2.1), with equation (2.2) predicting the adsorbed mass of protein as a function of time. The period of transport-limited adsorption varies according to protein concentration and protein, solution and surface properties. With careful design of a system to minimize the transport-limited period, true protein adsorption kinetic data can be recorded.

In the present kinetic study, the protein concentration of 1000 mg/L was in part selected to enhance the concentration driving force for transport. The adsorbed mass of β-lactoglobulin recorded experimentally on the most hydrophobic silicon surface, was plotted with that determined from the transport model (equation (2.2)). The result is shown in Figure 5.18 for the first minute. Figure 5.18 provides no indication that the experiment was performed during a transport-limited period. The "kinetic" line, which
Figure 5.18 Comparison of Adsorption Kinetic Model with Transport Model
is generated from the fitted model, yielded a much smaller slope than did the "transport" line. The data point recorded at 1 min is included in Figure 5.18. It is important to note that subsequent data points lie in the same range as the first one; i.e., no data points fall near the transport line.

If adsorption on the most hydrophobic surface is shown not to be controlled by transport, it is extremely unlikely that adsorption on surfaces exhibiting less hydrophobicity can be categorized in this regime as well. The fastest rates of adsorption were observed on the most hydrophobic surfaces. These did not overcome the transport rate; adsorption on the other surfaces should thus be kinetically controlled as well.

The diffusion coefficients of proteins in aqueous solution are usually in the range of $10^6$ to $10^7$, cm$^2$/sec (Andrade, 1985). However, an apparent diffusion coefficient determined from the same experiment used to construct Figure 5.18, calculated according to equation (2.2), is $2.56 \times 10^{11}$ cm$^2$/sec. This value is five orders of magnitude lower than the coefficient for $\beta$-lactoglobulin approximated from data in the literature ($1.55 \times 10^{-6}$ cm$^2$/sec; see Appendix C).

This behavior is also observed in other protein adsorption systems (Young et al., 1987; Van dulm and Norde, 1988; Wojciechowski et al., 1990). In those studies, the actual diffusion coefficient is suggested to be "depressed" to some extent due to surface properties. Currently, there is no theory to support that the diffusion coefficient is surface
dependent, and the "depression" observed in the diffusion coefficient found experimentally is interpreted here as evidence that these experiments were not conducted in a transport-limited regime.
6. CONCLUSIONS

1. Adsorption isotherms constructed for silicon of varying surface hydrophobicities indicate that the adsorbed mass of β-lactoglobulin increases with increasing surface hydrophobicity in the range $7.0 < W_{s, water} < 41.8$ mJ/m$^2$. Outside of this range, the protein molecule apparently did not "see" a difference in chemistry among the surfaces.

2. A simple mechanism of protein adsorption was proposed and the kinetic model based on the mechanism was developed. Even though the mechanism is simple, the significant stages of adsorption have been included. The proposed model agrees very well with experimental results obtained with in situ ellipsometry. The results indicate that adsorption on surfaces with greater hydrophobicity reaches the plateau faster than adsorption on surfaces of less hydrophobic character. The behavior of model parameters obtained from non-linear regression analysis, with changing surface properties supports a hypothesis that the rate constant defining the conformational change ($s_1$), and that defining the initial adsorption ($k_1$) increase with increasing solid surface hydrophobicity, while the desorption rate constant ($k_4$) tends to decrease.
3. Simulations of the proposed protein adsorption kinetic model were conducted. The behavior of fractional surface coverages were monitored through simulation, performed with different sets of rate constants. The simulation results also lend support to the hypothesis associated with the dependence of $s_i$, $k_i$, and $k_i$ on surface hydrophobicity.

4. Simulated results of protein adsorption based solely on a transport-controlled model were used to compared with the data obtained from the experiment performed on the most hydrophobic surface. The comparison indicates that adsorption of protein on all surfaces in this study are not in a transport-limited regime.
7. RECOMMENDATIONS

Since the main purpose of this study was to investigate adsorption behavior of protein where \( \beta \)-lactoglobulin was only one selected protein, it would be instructive to do the same study with other relevant proteins, such as \( \alpha \)-lactalbumin or bovine serum albumin. Even though the behavior of these proteins adsorbed on the silanized silicon surfaces is expected to be different from that of \( \beta \)-lactoglobulin, due to the complexity of the protein molecule, the behavior of these relevant proteins might not be significantly different.

Individual values of \( k_i \), \( k_u \), and \( s_i \) cannot be determined with single protein experiments. Work featuring kinetic tests with very similar proteins, perhaps varying by only one or several amino acids, could presumably be used to relate individual values of \( k_i \), \( k_u \), and \( s_i \) to molecular and surface properties.

The results obtained in this study would be better if the sample surface was stabilized in the cuvette and the cuvette could be positioned and firmly attached to the sample stage. This will substantially decrease the experimental error due to vibration in the laboratory. In addition, the results might also be improved by using the cuvette with stirring device which does not create a significant vibration that might interfere with the ellipsometric readings.
REFERENCES


Baier, R.E., Artificial Organs, 2, 422-426 (1978).


Appendices
APPENDIX A

One Film Model Ellipsometry Program

A computer program was developed to solve the quadratic equation, equation (3.2.12), in such a way that the imaginary component of thickness is minimized, and the difference between calculated $\psi$ and $\Delta$ and observed $\psi$ and $\Delta$ are within the limits of experimental error. Following is a brief treatment of the main program and its subroutine. A flow chart for the program is shown in the schematic of Figure A.1 The algorithm is as follows.

1. The program requests input of the parameters needed to solve the ellipsometry equations. These parameters are the angle of incidence ($\phi_i$), wavelength of light source ($\lambda$), psi and delta of the bare surface ($\Psi_s$ and $\Delta_s$), psi and delta of the film-covered surface ($\Psi$ and $\Delta$), and the lower and upper bounds of allowable film refractive index ($n_{f1}$ and $n_{f2}$), which are generally selected as the refractive index of the medium (air or aqueous solution) and that of the bare substrate (real part only) respectively.

2. The program calculates the real and imaginary parts of bare surface refractive index (the substrate optical constants).

3. The program determines the complex film thickness associated with both the lower and upper values of film refractive index with equation (3.2.12), by calling
subroutine REFRAC and subroutine THICK. This procedure results in four solutions for film thickness in complex form.

4. Subroutine SEARCH is called to determine whether any values of the imaginary part of thickness exist satisfying the "first end criterion", where the error of the imaginary part of film thickness \(d_i\) is less than 0.1. If the first end criterion is not satisfied, this subroutine searches for the first pair of thickness values whose imaginary parts are of opposite sign. If such a pair is found, the average value of the refractive indices associated with this thickness pair is calculated and returned to the main program. If no such pair exists, the program reports "no solution".

5. Subroutine COMPR1 is called to replace either the lower or upper value of refractive index with the average (new) value of refractive index determined in step 4. The rule for replacing the lower or upper bound refractive index \(n_{f1}\) or \(n_{f2}\) is summarized in Table A.1, and is based on the sign of the imaginary part of the film thickness associated with each refractive index. The new refractive index replaces the refractive index associated with a film thickness exhibiting an imaginary part of the same sign as the imaginary part of film thickness associated with the new refractive index. The new lower and upper bound refractive indices (now defining a narrower range) whose associated
thicknesses are of opposite signs in their imaginary parts are sent to the main program.

6. The program calls subroutine REFRAC and subroutine THICK to determine the film thicknesses associated with the lower and upper bound refractive indices as in step 3, then follows step 4.

7. When the first end criterion is satisfied, subroutine COMPR2 is called to verify whether the second end criterion, i.e., $\delta \Psi$ and $\delta \Delta$ are each less than 0.001, is satisfied. If it is, the program reports the final values of film refractive index and thickness. If the second end criterion is not satisfied, the first end criterion is updated and the program returns to step 4.

Subroutine Descriptions

Subroutine REFRAC

This subroutine is called to determine the Fresnel reflection coefficients, the total reflection coefficients, and the ratio of parallel to normal reflection coefficients ($\rho$).

Subroutine THICK

This subroutine is called to solve equation (3.2.12), yielding two values of film thickness, for a given film refractive index.
Subroutine SEARCH

This subroutine establishes whether the first end criterion is satisfied. If it is, the subroutine returns to the main program to verify the second end criterion using subroutine COMPR2. If the "first end criterion" is not satisfied, the subroutine searches for the first pair of thicknesses whose imaginary parts are of opposite sign. The average refractive index will then be calculated from the refractive indices associated with that thickness pair.

Subroutine COMPR1

This subroutine is called to replace either the lower or upper bound refractive index based on the criteria in Table A.1.

Subroutine COMPR2

This subroutine "back calculates" the value of psi and delta based on the refractive index satisfying the first end criterion. The calculated psi and delta are compared with the experimental values to establish whether the second end criterion is satisfied. If it is, the subroutine returns the final film thickness and refractive index to the main program. If the second end criterion is not satisfied, the first end criterion will be updated by decreasing the maximum allowable value of the imaginary part of film thickness in order to "tighten" this end criterion for the next iteration.

This computer program is based on McCrackin's calculation
procedure (McCrackin, 1964). McCrackin (1969) also wrote a computer program for ellipsometric computation. His program, however, is somewhat limited when applied to adsorbed protein films. The new program described here uses an algorithm that can accommodate a search for a solution in up to four directions; McCrackin's program can search in only two directions. The main disadvantage of McCrackin's program has to do with fewer number of search directions. If a solution cannot be found in those two directions, that program reports "no solution". For the present program, if the solution cannot be found (in any of four directions) within a range defined by upper- and lower- bound refractive indices, the program requests an input of new limits in film refractive index.
READ $\phi_1, \lambda, \Psi_s, \Delta_s, 
\Psi, \Delta, n_{f1}, n_{f2}$

$n_s, k_s$

LOOP $i = 1, 2$

THICKNESS OF $n_{fi}$

ADJUST 1st END CRITERION

1st END CRITERION
  no
  yes

OPPOSITE SIGN
  no
  yes
  $n_{f,\text{new}}$

THICKNESS OF $n_{f,\text{new}}$

REPLACE $n_{f1}$ OR $n_{f2}$ WITH $n_{f,\text{new}}$

2nd END CRITERION
  yes
  REPORT $n_f, \delta \Psi, \delta \Delta, d$

Figure A.1 Flow Chart of Ellipsometry Program
Table A.1  Rules of Replacing $n_{f1}$ or $n_{f2}$ with $n_{f,new}$

<table>
<thead>
<tr>
<th>Sign of Imaginary Part of Thickness Associated with Each Refractive Index</th>
<th>Replaced Refractive Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n_{f1}(+)$                  $n_{f2}(-)$         $n_{f,new}(+)$</td>
<td>$n_{f1}$</td>
</tr>
<tr>
<td>$n_{f1}(+)$                  $n_{f2}(-)$         $n_{f,new}(-)$</td>
<td>$n_{f2}$</td>
</tr>
<tr>
<td>$n_{f1}(-)$                  $n_{f2}(+)$         $n_{f,new}(+)$</td>
<td>$n_{f2}$</td>
</tr>
<tr>
<td>$n_{f1}(-)$                  $n_{f2}(+)$         $n_{f,new}(-)$</td>
<td>$n_{f1}$</td>
</tr>
</tbody>
</table>
Calculation of the Adsorbed Mass from the Refractive Index and Thickness

Once the refractive index (n) and thickness (d) of a protein film is determined, with knowledge of molar refractivity (A), molecular weight (M), and partial specific volume (V) (needed for the film in buffer solution) of the protein, the mass of adsorbed protein can be calculated. Adsorbed mass of a dried film can be calculated using the Lorentz-Lorenz relationship:

\[ m = 0.1 \, d \left( \frac{M}{A} \right) \frac{(n^2-1)}{(n^2+2)} \]  
\[ (A.1) \]

Cuypers (1983) modified the Lorentz-Lorenz relationship to obtain an expression for determining the adsorbed mass of a film immersed in a buffer solution with a known refractive index \( (n_b) \).

\[ m = 0.3 \, d \, f(1) \, \frac{(n-n_b)}{f(2)} \]  
\[ (A.2) \]

where

\[ f(1) = \frac{(n+n_b)}{[(n^2+2)(n_b^2+2)]} \]  
\[ (A.3) \]

\[ f(2) = \left( \frac{A}{M} \right) - \frac{V(n_b^2-1)}{(n_b^2+2)} \]  
\[ (A.4) \]

For \( \beta \)-lactoglobulin, the ratio of molecular weight to molar refractivity and the partial specific volume were determined as 3.796 and 0.751 respectively (Suttiprasit, 1990).
APPENDIX B

Determination of Plateau Values Obtained in Equilibrium Adsorption Experiments

Equilibrium adsorption results were used to construct adsorption isotherms. The plateau value of each isotherm was statistically determined in order to take all experimental error associated with each isotherm into consideration. The isotherm data were fit to the model:

\[ \Gamma = \Gamma_{\text{max}} \frac{(bC)}{(1 + bC)} \]  \hspace{1cm} (B.1)

where \( \Gamma \) and \( \Gamma_{\text{max}} \) represent the equilibrium adsorbed mass at some protein concentration \( (C) \), and at infinite concentration, respectively; \( b \) is a model parameter. Non-linear regression was used to calculate both \( \Gamma_{\text{max}} \) and \( b \), and their values are listed in Table B.1.
### Table B.1 Equilibrium Adsorption Model Parameters
(in parentheses: standard error of parameters)

<table>
<thead>
<tr>
<th>% DDS</th>
<th>$\Gamma_{\text{max}}$</th>
<th>$b \times 10^3$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.659</td>
<td>1.86</td>
<td>0.955</td>
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<tr>
<td></td>
<td>(0.0890)</td>
<td>(0.0006)</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>0.571</td>
<td>2.8</td>
<td>0.967</td>
</tr>
<tr>
<td></td>
<td>(0.0522)</td>
<td>(0.0007)</td>
<td></td>
</tr>
<tr>
<td>0.04</td>
<td>0.446</td>
<td>3.75</td>
<td>0.951</td>
</tr>
<tr>
<td></td>
<td>(0.0431)</td>
<td>(0.0011)</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>0.437</td>
<td>2.73</td>
<td>0.971</td>
</tr>
<tr>
<td></td>
<td>(0.0372)</td>
<td>(0.0006)</td>
<td></td>
</tr>
<tr>
<td>0.005</td>
<td>0.282</td>
<td>2.91</td>
<td>0.954</td>
</tr>
<tr>
<td></td>
<td>(0.0287)</td>
<td>(0.0008)</td>
<td></td>
</tr>
<tr>
<td>0.0025</td>
<td>0.296</td>
<td>3.25</td>
<td>0.973</td>
</tr>
<tr>
<td></td>
<td>(0.0224)</td>
<td>(0.0007)</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>0.357</td>
<td>1.75</td>
<td>0.976</td>
</tr>
<tr>
<td></td>
<td>(0.0387)</td>
<td>(0.0004)</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX C
Determination of the Diffusion Coefficient of β-Lactoglobulin

The general shapes adopted in solution, and molecular weights of β-lactoglobulin (Pessen et al., 1985) and bovine serum albumin (BSA) (Brown, 1977) molecules are shown below,

β-Lactoglobulin (double spheres)  
MW = 2 x 18,277

Bovine Serum Albumin (prolate ellipsoid)  
MW = 66,267

The calculated molecular volumes of β-lactoglobulin ($V_β$) and BSA ($V_B$) are 47.9 and 130.23 nm$^3$, respectively. Applying Wilke's equation (Bird et al., 1960), the ratio of diffusion coefficients of β-lactoglobulin ($D_β$) to that of BSA ($D_B = 8.5 \times 10^{-7}$ cm$^2$/sec, Lok et al., 1983) can be related to the ratio of their molecular volume as

$$D_β/D_B = (V_β/V_B)^{0.6}$$  \hspace{1cm} (C.1)

Thus, the diffusion coefficient of β-lactoglobulin can be estimated from equation (C.1) as $1.55 \times 10^{-6}$ cm$^2$/sec.
APPENDIX D

Values of the Kinetic Model Parameters

The parameters $a_1$, $a_2$, $r_1$, and $r_2$ are directly determined by non-linear regression analysis of the experimental data fit to the kinetic model. The parameter $a_3$ is $-(a_1 + a_2)$. Values of each of these parameters are shown in Table D.1. The values of standard errors of kinetic model parameters are listed in Table D.2.

Table D.1 Values of the Kinetic Model Parameters

<table>
<thead>
<tr>
<th>Run #</th>
<th>% DDS</th>
<th>$a_1$</th>
<th>$a_2$</th>
<th>$a_3$</th>
<th>$r_1$</th>
<th>$r_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>r214</td>
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<td>-0.0615</td>
<td>0.1829</td>
<td>1.3031</td>
<td>0.0080</td>
</tr>
<tr>
<td>r318b</td>
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<td>-0.0440</td>
<td>0.1523</td>
<td>1.5968</td>
<td>0.0112</td>
</tr>
<tr>
<td>r312</td>
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<td>-0.0453</td>
<td>0.1422</td>
<td>1.6539</td>
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<td>1.5104</td>
<td>0.0178</td>
</tr>
<tr>
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<td>-0.1038</td>
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</tr>
<tr>
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<td>0.0087</td>
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<td>-0.0601</td>
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<tr>
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<tr>
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<td>1.7869</td>
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<tr>
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<td>-0.0627</td>
<td>0.1707</td>
<td>1.8460</td>
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<tr>
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<tr>
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</tr>
<tr>
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<td>-0.1067</td>
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<td>1.7338</td>
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<tr>
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<td>2.0877</td>
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<tr>
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</tr>
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<td>0.0105</td>
</tr>
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</table>
Table D.2  Values of Standard Errors of the Kinetic Model Parameters

<table>
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<tr>
<th>Run #</th>
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<th>$a_1 \times 10^2$</th>
<th>$a_2 \times 10^2$</th>
<th>$a_3 \times 10^2$</th>
<th>$r_1 \times 10^2$</th>
<th>$r_2 \times 10^2$</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.0480</td>
<td>0.0717</td>
<td>7.6931</td>
<td>0.0162</td>
</tr>
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<td>12.1690</td>
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<td>0.0515</td>
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<td>0.0838</td>
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<td>0.0367</td>
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<td>0.0546</td>
<td>12.8040</td>
<td>0.0135</td>
</tr>
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<td>0.0504</td>
<td>0.0458</td>
<td>0.0681</td>
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<tr>
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<td>0.0351</td>
<td>0.0321</td>
<td>0.0476</td>
<td>18.3780</td>
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<td>0.0441</td>
<td>0.0407</td>
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<td>0.0621</td>
<td>32.1371</td>
<td>0.0161</td>
</tr>
<tr>
<td>r526</td>
<td>0.01</td>
<td>0.0393</td>
<td>0.0366</td>
<td>0.0537</td>
<td>27.9146</td>
<td>0.0151</td>
</tr>
</tbody>
</table>
APPENDIX E

Surface Characterization Parameters

The value of the polar component of the work of adhesion between a solid surface and water \( W_{a\text{, water}} \) can be calculated according to:

\[
W_{a\text{, water}} = k(\gamma_{L\text{, water}}) + b
\]

(E.1)

The values of parameters from linear regression of equation (E.1) are tabulated in Table E.1.

<table>
<thead>
<tr>
<th>% DDS</th>
<th>( W_{a\text{, water}} ) mJ/m²</th>
<th>Slope (k)</th>
<th>Intercept (b)</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>2.935</td>
<td>0.191</td>
<td>-4.153</td>
<td>0.51</td>
</tr>
<tr>
<td>0.2</td>
<td>6.980</td>
<td>0.309</td>
<td>-4.482</td>
<td>0.71</td>
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<tr>
<td>0.1</td>
<td>10.265</td>
<td>0.445</td>
<td>-6.252</td>
<td>0.80</td>
</tr>
<tr>
<td>0.05</td>
<td>10.272</td>
<td>0.416</td>
<td>-5.195</td>
<td>0.76</td>
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<tr>
<td>0.04</td>
<td>26.035</td>
<td>1.006</td>
<td>-11.335</td>
<td>0.98</td>
</tr>
<tr>
<td>0.025</td>
<td>25.259</td>
<td>0.983</td>
<td>-11.265</td>
<td>0.97</td>
</tr>
<tr>
<td>0.01</td>
<td>27.030</td>
<td>1.029</td>
<td>-11.171</td>
<td>0.98</td>
</tr>
<tr>
<td>0.005</td>
<td>41.795</td>
<td>1.515</td>
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</tr>
<tr>
<td>0.0025</td>
<td>48.746</td>
<td>1.776</td>
<td>-17.214</td>
<td>1.0</td>
</tr>
<tr>
<td>0.0</td>
<td>54.848</td>
<td>2.012</td>
<td>-19.891</td>
<td>1.0</td>
</tr>
</tbody>
</table>
APPENDIX F

Raw Data of All Experiments

All raw data and associated information in this research is with Dr. J. McGuire in Department of Bioresource Engineering, Gilmore Hall, Oregon State University, Corvallis, OR 97331-3906.
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