Protein Adsorption and Inactivation on Surfaces. Influence of Heterogeneities

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Contents

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I. Synopsis

The causes of protein heterogeneity during adsorption on different types of surfaces are examined. The analysis includes surfaces for adsorption, monolayer adsorption, thermodynamics, parameters that influence adsorption, models that incorporate heterogeneity either in the solute or of the surface sites, and also a time-varying conformational change of the adsorbed protein. Some techniques that help provide estimates of the qualitative nature of protein adsorption such as ellipsometry, total internal reflection fluorescence, protein fluorescence, and circular dichroism are presented. Wherever possible, the influence of heterogeneity on the qualitative and quantitative aspects of proteins adsorbed to different surfaces is delineated. Appropriate examples throughout highlight the importance of considering heterogeneity more seriously in analyzing modeling and experimental results in order to obtain more correct and novel physical insights into the protein adsorption process.

II. Introduction

The adsorption of proteins occurs at different types of interfaces, and the initial layers of proteins adsorbed significantly affect the process occurring at these interfaces. Interfacial interactions are important in the bioseparation of proteins and other biological macromolecules of interest. Other areas of interest where these interactions are important are biomedical applications of artificial devices, biosensors (Rechnitz, 1987), immunoassays (Gribnau et al., 1986), and drug delivery systems (Davis et al., 1984).

Over the years, significant attention has been paid to the determination of the quantitative aspects of protein adsorption to different surfaces. This effort was justified; however, in addition to the amount of protein adsorbed, the biological consequence of proteins at solid surfaces often depends on the nature and the state of the adsorbed layer. In particular, information about the protein conformation and orientation or, more precisely, time-dependent protein structural changes in the adsorbed layer is urgently required. The lack of information about how proteins are organized has hindered the delineation of the role of the interface in protein adsorption studies in spite of 3 decades of research.

Elgersma et al. (1990) have recently emphasized that a shortcoming of several studies is that an insufficient number of variables is studied. Some of the parameters that may influence protein–surface interactions include electrostatic interactions (Elgersma et al., 1990; Clark et al., 1988, 1989), pH (Bagchi and Birnbaum, 1981; Sonderquist and Walton, 1980), negatively charged surfaces (Norde and Lyklema, 1978a,b; Norde, 1983), surface charge (Hlady and Füredi-Milhofer, 1979), coadsorption of low molecular weight ions (Elgersma et al., 1990, hydrophobicity, and isoelectric point (Abramson, 1942). Other factors that may also influence protein adsorption onto surfaces include intermolecular forces between adsorbed molecules, solvent–solvent interactions, strength of functional group bonds, chemistry of the solid surface, topology, and morphology.

Proteins in solution diffuse to the interface. At the interface some of the conformational and hydration energy of the protein is lost. This is thermodynamically favorable (MacRitchie, 1978). Initially, at low protein concentrations the rate of adsorption is diffusion controlled. But after some time, especially at high

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surface protein concentrations, there is an activation energy barrier to adsorption (Graham and Phillips, 1979), which may involve electrostatic, steric, and osmotic effects close to the interfacial or surface layers. Then, the ability of protein molecules to interpenetrate and create space in the existing film and to rearrange at the surface is rate-determining.

Initially, Joly (1965) suggested that enzymes adsorbed at gas–liquid interfaces are generally present in an unfolded partially active or inactive state as a more or less rigid film. Graham and Phillips (1979) then stated that the capacity of proteins to unfold at an interface depends very much on the conformational stability of flexible segments of the protein molecule. Precipitation of protein also occurs at the interface. Agitation of protein solutions may induce a coagulation of the protein at the interface under certain circumstances. Coagulation occurs when the two-dimensional solubility limit is surpassed as a result of interface compression. Interfaces are primarily responsible for protein inactivation as highlighted by the experiments of Virkar et al. (1979). Using a partially-filled disk reactor these authors noted that shear-associated damage can be severe, but it arises when gas–liquid interfaces are present. Then, the replenishment of the interface associated with intense shear causes interfacial denaturation. Dunnill (1983) suggests that this, rather than shear alone, is the explanation for much loss of protein structure and enzyme activity in pumps (Virkar et al., 1979) and in centrifuges and ultrafiltration systems where air is entrained. Virkar et al. (1979) further noted that a decrease in the air–liquid interfacial area by completely filling up the reactor vessel minimized the enzyme inactivation. Similar observations may be made with liquid–liquid (for example, present in liquid–liquid extraction systems) or liquid–solid (for example, high-pressure liquid chromatographic separations) systems.

When proteins are adsorbed at interfaces, they undergo a change from their globular conformation to an extended chain conformation (MacRitchie, 1987). A significant conformational change results in the surface denaturation of the protein. Recognize that proteins in vivo may be changing their conformations continually. Since not all of these changes are irreversible, they all do not lead to activity changes of the proteins. The term denaturation is perhaps restricted to significant conformational changes in proteins. This is never applied to polymers. For example, as the energy environment changes, the molecular conformations of polymers may change. The analysis of protein adsorption at surfaces including mechanisms, kinetics, time-dependent conformational changes, etc. is a difficult process. The adsorption of proteins/enzymes at the interface is a complex phenomenon that involves the following steps that occur simultaneously (Aptel et al., 1987): (a) Transport to the interfaces by diffusion or diffusion/convection. Mixing and shearing action would generally enhance this step. (b) Adsorption/desorption at the interface, described by an interfacial chemical reaction and its related kinetic adsorption and desorption mechanisms. (c) Structural alterations of molecules in contact with the interface, and at higher occupancy, interactions with other adsorbed molecules. (d) Adsorption competition between molecules of different nature or molecular weight. Lok et al. (1983a) correctly pointed out that the factors that influence protein adsorption onto surfaces include intrinsic protein adsorption kinetics, chemical equilibrium between surface-adsorbed protein and free solution proteins, and flow of protein past the adsorbing surface. They also hint that the conformation of proteins in the adsorbed layer may be an important factor.

The possible effects of a given surface on a given protein (mixture) would include, among others, permanent or reversible adsorption with or without concomitant denaturation or conformational changes, preferential adsorption of specific proteins, and changes in the microenvironment of enzymes.

Example 1. A brief description of adsorption and inactivation of proteins on glass surfaces.

The adsorption of proteins on the surface of glass is well-known in many areas of biochemistry (Silman and Katchalski, 1966; Hummel and Anderson, 1965; Bull, 1965). For example, fibronectin and laminin (cell spreading–promoting glycoproteins) bind strongly to glass (Barnes, 1984). Fibronectin is a surface-active protein and readily denatures at solid–liquid interfaces. This is the primary reason for its efficient adsorption at both hydrophobic and hydrophilic surfaces. Fibronectin, however, appears to adsorb more on hydrophobic than on hydrophilic surfaces primarily due to a higher conformational change on hydrophobic surfaces (Grinell and Feld, 1982).

More detailed protein adsorption followed by subsequent denaturation studies are required to delineate the mechanisms involved at the interface. This is of primary importance since it is the initial protein layer(s) that mediate and control further interactions at the interface. For example, the adhesion of blood platelets to glass surfaces is known in the field of clinical chemistry, and blood platelets adhere more on surfaces coated with fibrinogen (Zucker and Broman, 1969). The amounts of proteins adsorbed on the surface of glassware has a significant effect on the quantitative analysis of very small amounts of protein in the case of radioimmunoassay or enzyme immunoassay (Rosselin et al., 1966). Adsorption of proteins on a glass surface is not a specific phenomenon but rather a general phenomenon which is usually neglected because of the low surface area of typical glassware. Though adsorption of proteins to glass is important, especially in clinical studies, one really needs to know more about the adsorption of proteins in general, and blood proteins in particular, to different polymeric surfaces that have significant biomedical usage.

An area of considerable interest where interfacial interactions are exceedingly important is in biomedical applications of artificial devices. A wide number of clinically important implants and devices exist. Some (for example, catheters) may only contact the blood once, and for a relatively short time; others (for example, kidney dialyzers) may be exposed to blood for hours, while tissue implants (for example, heart valves) will hopefully last for the lifetime of the patient (Hoffman, 1982). Leonard et al. (1987) have emphasized that even though the basic properties of an ideal blood-compatible material cannot be agreed upon, it should, in principle, be effective until the lifetime of an individual. This is not surprising since different materials should, in general, be rather specific for different types of usage. Thus, an understanding of the rapid adsorption of

plasma proteins when blood contacts an artificial surface or foreign material (Baeir and Dutton, 1969; Vroman and Adams, 1969) is of importance.

Example 2. A brief description of adsorption of blood proteins to different "artifical" surfaces.

A major disadvantage in the use of blood contacting "foreign" materials is the formation of a thrombus at the blood–polymer interface. Thrombosis involves a series of events beginning with the deposition of a protein layer at the blood–polymer interface. The formation of this protein layer is followed by the adherence of platelets, fibrin, and possibly leukocytes (Young et al., 1982; Ihlenfeld et al., 1979). Further deposition with possible concurrent entrapment of erythrocytes and other formed blood elements in a fibrin network constitutes thrombus formation. The growth of this thrombus eventually results in partial or total blockage if the thrombus is not sheared off or otherwise released from the surface (Sharma et al., 1982). An understanding of the physics and chemistry of the initial layer of protein adsorption cannot be over emphasized.

The surface-induced coagulation of blood is a critical factor in the design and application of most devices for use with the cardiovascular system. The clotting time of blood is dependent upon the material with which it is in contact. It is important to study the adsorption behavior of proteins that are a major component of plasma such as albumin, γ-globulin, and fibrinogen in relation to the antithrombogenicity of polymer materials. The possible effects of a given surface on a protein (mixture) would include, among others, permanent or reversible adsorption with or without concomitant denaturation or conformational changes, preferential adsorption of specific proteins, and changes in the microenvironment of enzymes. Since the adsorption of proteins on a surface depends on both the protein and the surface, it is important to characterize the nature of both the protein sample and the surface. How homogeneous or heterogeneous are each of these? Does the heterogeneity affect the adsorption and further properties, and by how much?

Adsorption of proteins at solid–liquid interfaces has been reviewed in the literature (MacRitchie, 1978; Norde, 1986; Hlady and Andrade, 1986; Lundstrom et al., 1987). Not much information is presented in a concise manner regarding the heterogeneity of either the protein adsorbate or of the surface, and the subsequent effects of this heterogeneity on denaturation of the adsorbed protein on the surface. Also, although much is known about the quantitative nature of protein adsorption on different surfaces, there are still apparently no rigorous mathematical theories that describe protein adsorption on different surfaces, particularly the qualitative aspects. The studies presented together in this paper should provide a judicious framework within which one can compare the status of one's work and hopefully stimulate work in appropriate directions in the future. The studies presented should be viewed only as appropriate examples, since surely more studies are available in the literature that would help further delineate or reinforce the ideas presented below or even perhaps elucidate other ideas or factors of importance. One of the major intents of the paper is to focus on heterogeneity in adsorption and its subsequent effects on protein denaturation. This is important since as indicated earlier, the initial adsorbed protein layer mediates or controls the adsorption of further layers.

III. Adsorption of Proteins

A. Surfaces for Protein Adsorption

Adsorption of blood proteins has been done on different glass and polymeric surfaces. Some recent examples are polyethylene tubing, silicone rubber tubing, plasticized poly(vinyl chloride) tubing, and a segmented polyether urethane urea tubing (Young et al., 1988); poly(vinyl chloride) (PVC), a copolymer of methacrylic acid/methacrylate (PMA), and surface-grafted poly(ethylene oxide) (PEO) films; (Golander and Kiss, 1988); silica with two different surface energies (Johnsson et al., 1987); silicon or glass plates (Elwing et al., 1987); and polymeric surfaces with varying surface properties and functionalities which are polydimethylsiloxane (PDMS), polydiphenylsiloxane (PDϕS), polycyanopropylmethylsiloxane (PCPMS), poly(methylmethacrylate) (PMMA), poly(styrene sulfonate) (PSS), and other polymeric materials (Young, 1984).

Jeon et al. (1991) have very recently indicated that a large number of studies have been done to minimize protein adsorption to different surfaces. This is important in such diverse areas as chromatographic supports, blood-contacting devices, immunoassays, etc. An excellent protein-resistant surface is poly(ethylene oxide) (PEO). Jeon et al. (1991) indicate that between two adsorbed PEO surfaces in a good aqueous solvent repulsion forces develop at certain separation distances due to a steric repulsion phenomenon. The protein-resistant character is probably caused by a steric stabilization effect. Jeon et al. (1991) recently analyzed theoretically the protein-resistance character of PEO chains terminally attached to a hydrophobic solid substrate. These authors state that the good protein resistance properties of PEO are related to the fact that its refractive index is the lowest among the water-soluble synthetic polymers, resulting in a low van der Waals interaction with the protein. Finally, the van der Waals attraction is small compared to the steric repulsion.

In a later study on the effect of protein size on protein–surface interactions in the presence of poly(ethylene oxide), Jeon and Andrade (1991) determined the PEO surface density conditions for optimal protein resistance. These authors noted that for small proteins ($R \sim 20$ Å), D should be small (~10 Å), and for large proteins ($R \sim 60$–80 Å), D should be larger (~15 Å). Here D is the average distance between end-attached PEO chains, and R is the protein radius. Jeon and Andrade (1991) emphasize that these results evolve from the trade-offs between steric repulsion and the assumed weak hydrophobic interaction between the PEO layer and the protein.

Some surfaces have been precoated to attain desired characteristics. Albumin is the most abundant plasma protein. Albumin molecules in the native state are well-known not to be included in thrombus formation and platelet aggregation. Therefore, good thromboresistance of a polymer could be achieved by the selective adsorption of albumin onto a polymeric material. Sato et al. (1987) noted that albumin-precoating on controlled porous glass regulates the thrombin inactivation.

These authors suggest that albumin protects the thrombin from self-hydrolysis. It is possible that the thromboresistance of the albumin-precoated surface is due to the reduced activation of prothrombin to thrombin.

It has been shown by researchers (Absolom et al., 1984; Lee and Kim, 1974) that the surface properties, and more specifically, the surface tension, of various potential cardiovascular implant materials is related to the protein adsorption to those surfaces. Absolom et al. (1987) recently utilized the sedimentation volume (V_{sed}) method to characterize the surface tension of protein-precoated polymer particles. In this technique the protein-coated polymer surfaces are never exposed to an air interface. Various pairs of liquids (with differing surface tensions) are mixed. A fixed mass of a given polymer powder is suspended in a constant volume of the liquid mixtures. Some liquid mixtures so prepared have lower and some higher surface tensions than the suspended polymer particles. A maximum in V_{sed} occurred when the surface tension of the suspending liquid was equal to that of the particles. Vargha-Butler et al. (1985) have utilized this method to characterize the surface properties of different polymer particles. The position of the V_{sed} maxima, and hence the surface tension, γ_{PV}, of the particles was found to depend on polymer surface tension as well as on the type and bulk concentration of the coating protein solution.

Experiments were performed with the following powders: polytetrafluoroethylene (PTEE), poly(vinyl chloride) (PVC), and nylon-6,6. The serum proteins used for precoating were human serum albumin, human serum immunoglobulin G, and human fibrinogen. These authors indicate that at high bulk concentrations the nature of the underlying substrate materials are entirely masked. The advantage of this method is that it is inexpensive and versatile. It can be applied to investigations involving, in general, any type of particulate material. It does not rely on the use of sophisticated optical equipment that has to be calibrated and the results carefully analyzed. Also, the sedimentation volume method does not require the use of substrates having any specific properties. The only disadvantage or limitation of this method is that it can be applied only to surfaces which can be obtained in particulate form. Considering the advantages and the simplicity in the usage of the method, it is anticipated that this method will be used more frequently in the future by different workers, especially if one is looking for a nonoptical method of surface characterization.

Example 3. A brief analysis of blood protein adsorption from a mixture of proteins.

Lahav (1987) recently investigated the adsorption of thrombospondin, fibrinogen, and fibronectin.Two of these were in solution and one was surface adsorbed. All binding assays were performed in 7-mm diameter flat-bottom wells made of polystyrene. All of these proteins form part of the blood coagulation process (Lahav et al., 1982; Leung, 1984) and have been shown to interact with each other when one of them is adsorbed to a surface (Lahav et al., 1984; Leung and Nachman, 1982). Lahav (1987) concludes that in general multicomponent systems in which multiple binding can take place could show a complex pattern of interactions. Since Lahav's study (1987) presents a more realistic picture of (blood) protein interactions at surfaces, such studies should be more emphasized in the future. As indicated by Lahav (1987) and as is to be anticipated these studies will be difficult due to the complex interactions involved. Nevertheless, they are necessary if one wants appropriate physical insights into blood protein adsorption on surfaces under "actual" circumstances. As a matter of fact, there are a large number of proteins in blood that would significantly affect interactions amongst themselves and with the surface during the adsorption process. The complex pattern of protein interactions with the interface should lead to an increasing heterogeneity in adsorption.

The surfaces for protein adsorption and the amounts of protein adsorbed needs to be better characterized. The heterogeneity of the surface will significantly influence adsorption and subsequent reactions occurring on the surface. The nature of the amount of protein adsorbed is also of significance. Does a monolayer of protein adsorbed molecules exist? Or, do we also have a second layer of protein adsorption? What is the structure of the adsorbed protein molecules on the surface? The next couple of sections begin to address this problem.

B. Monolayer Adsorption

Proteins are intrinsically surface active and tend to concentrate at surfaces. The chemical composition of the surface plays a major role in protein adsorption. Norde and Lyklema (1979) indicate that the mechanism of protein adsorption to surfaces is rather complex. This involves the attachment of different segments of one and the same protein molecule to the sorbent surface so that the molar Gibbs energy of adsorption usually attains large values. These segments usually consist of a number of amino acid residues. Shirahama et al. (1990) indicate that in a solution containing a mixture of proteins, the interface will initially accommodate the protein molecules that have the largest diffusion rate coefficient, and are most abundantly present in the solution. These initially adsorbed protein molecules may be displaced by other protein molecules that have a higher affinity. Shirahama et al. (1990) emphasize that the final composition of the adsorbed layer at a given interface is determined by the concentration of the various kinds of proteins in the solution, the intrinsic adsorption affinities, and the possibilities that the proteins have to desorb.

Researchers in the past have successfully modeled the adsorption behavior of adsorbates in many cases, using the Langmuir model (which was originally developed for gases) even though it does not conform with theory. Rudzinski et al. (1983) indicate that other appropriate "liquid" counterparts of the "gaseous" empirical isotherm equations have been developed. These include counterparts of the Freundlich (Rudzinski and co-workers, 1973, 1974, 1981; Dabrowski and Jaroniec, 1979a,b, 1980a,b), and the Dubinin–Radushkevich (Oscik et al., 1976; Jaroniec and co-workers, 1978, 1980, 1981), and Toth (Jaroniec and Derylo, 1981) empirical equations. Rudzinski et al. (1983) emphasize that the application of these empirical equations to correlate experimental data in solution adsorption has not been accompanied by a sufficient care about the limitations in their applicability. For example, these

empirical equations do not reduce correctly to Henry's law at sufficiently low concentrations of one of the components of a liquid mixture. This contradicts rigorous thermodynamic predictions. Nevertheless, these studies with these known constraints have provided some "restricted" physical insights into the adsorption of adsorbates on different surfaces. It may be suggested that these results can be of value for interpreting the adsorption of proteins.

Rudzinski et al. (1983) indicate that in many adsorption systems the structure of the solid/solution interface/system is adequately represented by a monolayer adsorption system. In these types of systems the equilibrium bulk solution exhibits small or moderate departures from an ideal solution behavior. In these systems the protein layer directly in contact with the surface is held more firmly than the second and following layers.

Recently, investigators are paying increasing attention to the fact that structural variations might be taking place (Jaroniec et al., 1983; Cuypers et al., 1987). Lundstrom (1985) utilized the Freundlich isotherm to describe a protein-adsorption model that includes more than one orientation or conformation of the adsorbed protein. This study allowed the determination of important parameters that influence protein adsorption on biomaterials. The different conformational states of the protein would require similar energies. Perhaps, a most probable conformational state, with fluctuations about it obtained from statistical thermodynamics is more appropriate. Nevertheless, it is, therefore, of importance to study not only the quantitative but also the qualitative aspects of protein adsorption to surfaces. The following study of comparative protein adsorption in model systems is a good example.

Example 4. Protein adsorption on surfaces with quantitative as well as qualitative features.

Shirahama et al. (1990) have studied the adsorption of lysozyme, ribonuclease, and α-lactalbumin on hydrophilic silica and on hydrophobic polystyrene-coated silica, which are both negatively charged. These authors monitored the adsorption process by reflectometry and by streaming potential measurements. Reflectometry provided a quantitative measure of the protein adsorbed, and streaming potential measurements provide qualitative information of the protein adsorbed (composition of the adsorbed layer). The authors emphasize that both sequential and competitive adsorption from flowing solutions never led to adsorbed amounts that exceed values corresponding to monolayer coverage (that is, 1–2 mg/m^2). It is this *flowing* condition that prevents association between two proteins which then constrains the adsorption to monolayer coverage. These proteins have similar molar mass, (globular) size, and therefore, diffusion coefficient. Thus, the effects of molecular size and diffusion coefficient on the adsorption preference are practically negligible. The proteins do, however, differ in their isoelectric point, hydrophobicity, and stability.

Shirahama et al. (1990) conclude that at the hydrophilic surface (SiO_2) the adsorption is largely determined by electrostatic interaction. This is because of the following: (a) The protein amount adsorbed from single-protein solutions increases with increasing charge contrast between the protein and the adsorbent surface. (b) Sequential adsorption occurs only if the second protein has a more favorable electrostatic interaction with the adsorbent surface. (c) The final composition of the adsorbed layer essentially consists of the protein that has the most favorable electrostatic interaction with the adsorbent. (d) The authors emphasize that a remarkable feature of their studies for all three proteins is that the initial adsorption rates are not significantly affected by the nature of the surface, whereas at the later stages of the process, the curves for $\Gamma(t)$ at the hydrophobic and hydrophilic surfaces do differ markedly. At the later stages of the adsorption process, the surface becomes crowded with protein molecules. Further variations in $\Gamma(t)$ are due primarily to orientation and conformational effects of the preadsorbed molecules. This would lead to an increasing heterogeneity of the adsorbed protein on the surface.

These authors also indicate that at the hydrophobic surface (PS/SiO_2) electrostatic interactions have some effect, but they definitely do not dominate the adsorption process.

More studies like the analysis by Shirahama et al. (1990) are required that provide information on both the quantitative as well as the qualitative aspects of protein adsorption on surfaces. Although not much information on the qualitative characterization of protein adsorption is available in the literature, the quantitative aspects of protein adsorption to surfaces has frequently been studied.

McNally and Zografi (1990) have recently proposed a model in which the molecules can exist in three regions: (a) the bulk solution, (b) the surface (air–water interface), and (c) the subsurface, a region several molecular diameters below the surface. The subsurface region is in between the interface and the bulk solution, wherein the solute is readily depleted. The authors utilized the diffusion-controlled model:

$$\frac{\Gamma}{c} = 2\left(\frac{Dt}{\Pi}\right)^{1/2} \tag{1}$$

to describe the initial adsorption kinetics of hydroxypropyl cellulose (HPC) and hydroxyethyl cellulose (HEC). Here Γ is the concentration of the adsorbate molecules, c is the concentration of the molecules in solution, D is the diffusion coefficient of the molecules in solution, and t is the time. The initial adsorption is described by a surface depleted of adsorbed solute molecules in which molecules will instantly move from the subsurface to the surface, thus leaving a zero concentration at the subsurface. This causes a diffusion-controlled gradient between the bulk solution and the subsurface. It is this rate of diffusion that should govern the overall kinetics of adsorption (Langmuir and Schaefer, 1937). The diffusion-controlled equation given above implies that a good straight-line relationship can be obtained for the initial time period. After a while, the plots become more nonlinear which is caused by the buildup of an energy barrier to adsorption on the surface as well as to bulk diffusion.

Beissinger and Leonard (1980) proposed a model for albumin adsorption in which the adsorbed molecule can exist in one of two adsorbed states, θ_1 and θ_2. Albumin molecules in solution near the surface are assumed to be able to adsorb in state 1. In state 1, they can either desorb or enter state 2. At state 2, only desorption of the albumin molecule is possible, and state 2 is only accessible from state 1. The parameters in the model do have physical significance. For

example, the rate constant for desorption from state 1 is larger than the rate constant for desorption from state 2. Thus, the molecules adsorbed in the state 2 are much more tightly held than those desorbed in state 1. The model formulation gives an indication of heterogeneity of adsorption sites on the surface and a heterogeneity in the adsorbed molecules on the surface. Beissinger and Leonard (1980) extended the model to also include the competitive adsorption of albumin and γ-globulin.

An "exchange" reaction often takes place in protein adsorption wherein the protein molecules may desorb from the surface in the presence of other protein molecules. In an "exchange" reaction, a second molecule gradually replaces the originally adsorbed one (Jennisen, 1978, 1981) and the original bonds are broken one by one. If the number of binding sites to the surface are large, an exchange reaction is an improbable process. If the exchanged molecules are of the same kind as the originally adsorbed ones, the total free energy has not changed after the completed exchange reaction, but for molecules of different kinds, a lower total free energy of the second molecule when it binds to the surface may be a thermodynamic force for the exchange reaction. This "exchange" reaction would also contribute toward the heterogeneity of the protein in the adsorbed layer.

Another interesting observation is that when a protein molecule resides on a surface long enough, it forms all its possible bonds with a surface and this may be the reason for the conformation change of the molecule. This might lead to stronger bonds formed with the surface with increasing time. This makes the exchange reaction and also spontaneous desorption more difficult. An increased residence time of adsorbed molecules on the surface would also increase the heterogeneity of the adsorbed molecules on the surface. Proteins are known to be heterogeneous. Heterogeneity refers to the property of certain highly purified proteins to show molecular heterogeneity when scrutinized by high-resolution immunochemical, biochemical, or biophysical techniques (Wang et al., 1975; Eisen, 1980). Some examples of molecular heterogeneity include intramolecular disulfide interchange in serum albumin (Sogami et al., 1969), hypothesized enzymatic nicking prior, perhaps, to isolation of concanavalin A (Wang et al., 1971), or adventitious modification (for example, deamidation of a few asparagine and glutamine residues and limited proteolytic attack on one or a few peptide linkages) during purification or subsequent manipulation (or both). Colvin et al. (1954) and Haurowitz (1950) have suggested that heterogeneity is an inherent property of proteins, and that even with a high degree of purification, the protein will represent a population of variable but closely related members. Besides, the energies for protein adsorption on a surface need not necessarily be homogeneous; in fact, it is reasonable to assume a "distribution" in energies for protein adsorption. Thus, there can be heterogeneities in proteins and of adsorbents. It would be of interest to develop a parameter of heterogeneity like "dispersion" or a "standard deviation" and relate it to the residence time effect. This should shed physical insights into the time-dependent conformational changes occurring at the interface.

Lundstrom and Elwing (1990) very recently presented a simple mathematical model where some kinetic parameters of interest were defined for the understanding of protein-exchange reactions on a solid surface. They also considered the residence time effect. The authors emphasize that when a protein molecule is reversibly adsorbed on a surface, it can be exchanged by another molecule of the same or another kind with two major phenomena occurring. There is a time-dependent composition change of the adsorbed protein layer and the possible occurrence of conformationally changed molecules in solution. These authors also noted the existence of four different states on the surface. Heterogeneity should also be explicitly incorporated in their model to provide better physical insights into time-dependent conformational changes occurring at the surface.

Lundstrom and Elwing (1990) do indicate the existence of three types of surfaces for protein adsorption. The first type is when the exchange reaction and reversible adsorption takes place with small conformational changes because of a short residence time of the protein on the surface. Heterogeneity in this case would be relatively small. The second type is when only the exchange reaction occurs. The residence time is longer than the first type which leads to conformational changes of the adsorbed molecule. The surface will keep releasing conformationally changed molecules into the solution. These conformationally changed molecules in solution may also adsorb on the surface. This would contribute to increasing heterogeneity of molecules both in solution and on the surface. Such a surface may also cause both surface-oriented biological phenomena and unwanted effects away from the surface. The third type is when at least one kind of protein molecule is irreversibly adsorbed on the surface. In this case, the surface is constantly covered with adsorbed protein that undergoes time-dependent conformational changes on the surface. The parameters of heterogeneity (i.e., "standard deviation" or "dispersion") may or may not be related for the protein molecules in solution and at the surface.

Tan and Martic (1990) have noted that, because of their multifunctionalities, protein molecules can exist in several conformational states (Horbett and Brash, 1987). The free energy change required to go from one structure to another is several kcal/Gmol which corresponds to the dissociation of a few hydrogen bonds. Similarly, due to the flexible and dynamic fluctuations of the protein molecules similar rearrangements are possible on the surface. Thus, the driving force for adsorption is entropic resulting from dehydration owing to the hydrophobic interaction between proteins and the surface. Also, the unfolding of the protein as it adapts to its new environment must be considered. The conformational state of the adsorbed protein can significantly affect its biological function. Tan and Martic (1990) emphasize that though conformational changes of adsorbed proteins have been studied by spectroscopic (Horbett and Brash, 1987; Andrade, 1985) and immunochemical methods (Elwing et al., 1988), direct measurements of protein structural changes in situ on polymer particles are difficult to apply. Tan and Martic (1990) thus attempted to characterize the

adsorbed protein conformational states as well as the desorbed protein.

Finally, some comments should be made with regard to the desorption/exchange process for proteins adsorbed on a surface. Since the protein molecule attaches itself to the adsorbent surface by different segments (which usually consist of a number of amino acid residues), the molar Gibbs free energy of adsorption may attain large values. Thus, adsorbed proteins are difficult to remove even by diluting the solution. However, Shirahama et al. (1990) point that if the solution contains a displacer or other protein whose molecules have an affinity for the adsorbent, then any desorbing segment can be replaced by another. Simply speaking, desorption of the molecule is now virtually an exchange process, and since $\Delta G_{exchange} \ll \Delta G_{desorption}$, Shirahama et al. (1990) indicate that this process is much more likely. Since thermodynamics plays an important role in protein adsorption, it is analyzed to some extent in the next section.

C. Thermodynamics

Mesteri et al. (1984) and Partyka et al. (1986) utilized the calorimetric method to analyze the adsorption mechanism and the structure of nonionic surfactant films on a hydrophilic silica surface by using the differential molar enthalpies of adsorption (ΔH_d^a). ΔH_d^a is exothermic at lower degrees of coverage, θ, and at a higher θ, ΔH_d^a becomes endothermic. The molar enthalpy of adsorption reaches an endothermic minimum around $\theta \approx 0.5$, and subsequently when the coverage ratio approaches unity, the enthalpy of adsorption comes close to zero. These authors (Mesteri et al., 1984; Partyka et al., 1986) noted that the longer the polar chain, the greater the endothermic values. Also, if the protein chain increases, ΔH_d^a changes from exothermic to endothermic at higher degrees of coverage. Mesteri et al. (1984) and Partyka et al. (1986) indicate that an increase in adsorption with decreasing temperature is in agreement with an exothermic process. Furthermore, the simultaneous decrease in the enthalpies (in absolute values) is certainly due to a heterogeneous surface site distribution, the most energetic sites being the first to be occupied.

Moacanin and Kaelble (1977) suggested that a material's thrombogenic response to blood is influenced partially by the polar and dispersive components of a material's surface free energy. The surface free energy of the interface between the liquid and a biomaterial surface, γ_{sl} is a measure of the imbalance of forces at the interface. The larger the value of γ_{sl}, the greater the imbalance in forces.

Young et al. (1988) analyzed the adsorption of α_2-macroglobulin on polyether urethane urea, polyethylene, silicone rubber, and plasticized polyvinyl rubber at different bulk protein concentrations. The binding strength of the four biomaterials for protein decreases in the order silicone rubber > polyethylene > plasticized poly(vinyl chloride) > polyether urethane urea. The polyethylene and silicone rubber were the most hydrophobic and the polyether urethane urea was the least hydrophobic biomaterial. Protein affinity was found to be the highest for silicone rubber and polyethylene and the lowest for the polyethylene urethane urea. Note that the biomaterial surface-water free energy also decreases in this same order. This supports the theory that a material with a higher dispersion component and a low polar component of the surface energy (that is, the hydrophobic material) adsorbs protein films more strongly than a biomaterial with a lower dispersion component (Moacanin and Kaelble, 1977). Finally, the lowest binding strength between the biomaterial and the protein is because the polar and the dispersive components of the biomaterial exactly match those of the protein.

MacRitchie (1985, 1987) has analyzed the thermodynamics of protein adsorption at interfaces. Proteins in solution diffuse to the interface. Proteins on adsorption at the air–water interface, undergo a change from their globular configuration in solution to an extended chain structure. On energetic grounds, it is expected that the polypeptide backbone lies in the plane of the surface with the polar and nonpolar side chains directed toward and away from the aqueous phase, respectively. MacRitchie (1987) further indicates that when a protein molecule adsorbs, interfaces of low free energy replace an area of high surface free energy. The polar side chains are in water and the nonpolar side chains in air. The lowering of the free energy is the driving force and gives rise to the unfolding of the molecule at the surface.

Norde and co-workers (1979, 1986) indicate that the change of entropy upon adsorption is an important source of information if the nonconformational and conformational contributions can be separated. Upon adsorption, a conformational change takes place toward a configuration of higher affinity. With time and structural modifications, the protein attaches itself to the surface by different segments. These structural changes, though minuscule, contribute toward the adsorption free energy and increasing degrees of heterogeneity of protein adsorbed at the interface. Norde et al. (1986) indicate that desorption requires a higher free energy than the free energy for initial binding. Thus, the desorption isotherm shows a hysteresis curve and does not follow (or coincides with) the adsorption curve. This degree of hysteresis is lower for molecules with a rigid molecular structure. It is further anticipated that molecules that exhibit a higher degree of hysteresis in the adsorption/desorption curves would exhibit a greater degree of heterogeneity of conformational states at the interface. Also, longer residence times of the protein at the surface would increase the degree of hysteresis for flexible molecular structure proteins.

Norde et al. (1986) analyzed thermodynamically the adsorption of HPA on hydrophobic and hydrophilic oxide surfaces. These authors indicate that for protein adsorption at the hydrophobic oxide surfaces that have the same charge sign as the protein molecules, the entropy gain must originate from the protein molecule itself. This can occur either from the dehydration of hydrophobic patches, or from structural changes, or both. The authors assume that the helix content in the adsorbed state is comparable to that calculated from the desorbed material. Then, the entropy increase from the loss of α-helix content largely compensates for the positive heat of adsorption, ΔH_{ads}. Protein adsorption on a hydrophilic surface having the same charge sign

as the protein proceeds simultaneously by virtue of structure changes in the protein molecules.

Lee and Ruckenstein (1988) studied the adsorption of bovine serum albumin onto polymeric surfaces of different hydrophilicities. These authors proposed an improved explanation regarding the thermodynamic driving force for protein adsorption. These authors proposed that there are two positive entropic contributions (a) an entropy gain due to the dehydration of the protein surface and (b) an entropy gain due to adsorption. This according to Lee and Ruckenstein (1988) involves the attachment of a part of the protein molecule, and the dangling of the protein in the solution as loops and tails. Just a simple adsorption of the protein on the surface would, however, yield a negative entropy due to losses of degrees of freedom. There are also two enthalpic effects: (1) a positive one associated with dehydration, and (2) a negative one due to interactions with the solid. The total entropic effect dominates and therefore protein adsorption is entropically driven.

D. Parameters That Influence Adsorption

There are various parameters that influence protein adsorption. These include electrostatic interactions, isoelectric point, pH, negative charged surfaces, surface charge, coadsorption of low molecular ions, intermolecular forces between adsorbed molecules, solute–solvent interactions, strength of functional grcup bonds, chemistry of solid surface, morphology, and topology. The effects of some of these parameters on protein adsorption is presented below.

Elgersma et al. (1990) studied the effect of electrostatic contributions on the adsorption of monomeric BSA on polystyrene lattices. They investigated the influence of surface charge on the latex. These authors showed that BSA adsorption occurs spontaneously even when the protein has the same charge sign as the sorbent. The isoelectric point of bovine serum albumin is 4.7–5.0, and for both the negatively charged lattices, the initial slopes decrease with increasing pH. These authors are unclear why the negatively charged BSA molecule has a high affinity for the negatively charged polystyrene surface. They do indicate that analyzing this particular problem on the basis of electrostatic interaction alone is not enough.

Researchers have observed (Bagchi and Birnbaum, 1981; Sonderquist and Walton, 1980; Morrisey and Stomberg, 1974) a maximum in the amount of protein adsorbed with pH, and indicate that it is due to the decrease in conformational stability of the protein with increasing net charge on the molecule. This results in a greater tendency for structural rearrangements of the adsorbing molecules which create a larger surface area per molecule and cause a small amount of protein to be adsorbed. These structural rearrangements on the surface would contribute to the microheterogeneity of proteins adsorbed on the surface. Furthermore, at pH values away from the isoelectric point of the protein, there is an increased electrostatic repulsion between adsorbed molecules which leads to a smaller amount of adsorbed protein. This increased electrostatic repulsion would also increase the microheterogeneity of the adsorbed protein molecules. Elgersma et al. (1990) do indicate that maximum adsorption around the isoelectric point is found with BSA adsorbed on negatively charged lattices. Furthermore, maximum protein adsorption around the isoelectric potential has been reported for albumin, immuno-γ-globulins, fibrinogen, hemoglobin, and gelatin, but for conformationally stable proteins like cytochrome *c* and RNase no such maximum in adsorption is observed. One may anticipate that lower degrees of microheterogeneity to be observed for conformationally stable proteins than for proteins that do not exhibit this conformational stability.

Clark et al. (1988, 1989) and Poole et al. (1984) analyzed the adsorption of BSA at the air–water interface. They noted that the addition of polycationic lysozyme to polyanionic bovine serum albumin at neutral pH extends the range of solution conditions under which stable foams are produced with individual proteins. Electrostatic interactions stabilized the multiprotein complex at the interface. Clark et al. (1989) further showed that extensive aggregation of the protein (presumably of electrostatic and/or hydrophobic origin) is occurring at the two film surface, resulting in the formation of a gel-like network. Any such process would presumably be facilitated by the partial unfolding of BSA that occurs following adsorption at the air–water interface. This partial unfolding and aggregation of the protein would lead to an increased microheterogeneity of the adsorbed protein at the interface.

Abramson (1942) noted that horse serum albumin adsorbed on negatively charged quartz and colloidal particles at its isoelectric point. At a pH of 4.8 a maximum of adsorption occurs. Norde and Lyklema (1978a,b) too noted that human serum albumin exhibits a maximum in adsorption on negatively charged polystyrene lattices. Norde (1983) further noted that in adsorbed BSA the average position of the carboxyl group is relatively close to the sorbent, probably because of nonelectrostatic interactions. Positive ions from the solution may be incorporated in the contact region between the protein and the surface to prevent an accumulation of net negative charge (van Dulm et al., 1981).

It has been found that maximum protein adsorption as a function of pH is not determined by the isoelectric potential of the protein but rather the protein and the particle together. This is shown for the albumin–polystyrene latex system, immunoglobulin on polystyrene system, and also for other systems with similar properties (Elgersma et al., 1990).

Elgersma et al. (1990) indicate that the adsorption of protein involves co-adsorption of low-molecular weight ions to screen any excess potentials that may develop in the contact region between the protein and the charged latex surface, due to the tendency of the protein to expose certain groups to the latex. Therefore, the analysis of co-adsorption of low molecular weight ions is important in studying the protein adsorption process.

Moyer and Gorin (1940) studied the competitive adsorption behavior of albumin and γ-globulin on the surfaces of quartz and collodian particles. These authors noted that these proteins hardly adsorbed on each other after a sample had been coated with one protein and then exposed to another, although one protein may replace another at the surface. Also, the nature of the surface influences the adsorption process

in which the hydrophilic protein adsorbed more readily to more hydrophilic surfaces and vice versa. One may reasonably anticipate the competitive behavior to increase the microheterogeneity of the adsorbed protein on the surface.

Kochwa et al. (1949) studied the sequential and simultaneous adsorption of albumin, γ-globulin, and fibrinogen on artificial surfaces. These authors noted that when a polyurethane surface is first exposed to unlabeled protein and then to labeled protein, the prior exposure was always found to decrease the uptake of the labeled protein over that observed for labeled protein on a virgin surface. γ-Globulin blocked the sequential application of labeled albumin by 27%, and albumin blocked labeled γ-globulin by 46%. In the sequential studies, the surface was exposed first to one single protein solution and then to a second solution containing only the other protein. In the simultaneous studies, a surface was exposed to a solution containing a mixture of both proteins. These authors observed that for the sequential experiments, the amount and kind of adsorption depended on the sequence of exposure. The main observation for the simultaneous (competitive) adsorption process is that there is a large reduction in the adsorbed amount of one component in the presence of the other as compared to the single component adsorption of either.

We have briefly analyzed some of the parameters that influence protein adsorption on different surfaces. Rudzinski et al. (1983) indicate that the protein adsorption model is complicated by surface heterogeneity or energetic heterogeneity of the surface sites. This fact has often been brought out in the literature (Zchuchovitskii, 1957; Hansen and Mai, 1957; Delmas and Patterson, 1960; Siskova and Erdos, 1960a,b; Coltharp and Hackerman, 1973a,b; Everett, 1964, 1965). Initially, Rudzinski and co-workers (Rudzinski et al., 1973, 1974; Oscik et al., 1976, 1984; Dabrowski and Jaroniec, 1979a, 1980a) attempted a quantitative description of solution adsorption on solid surfaces. These authors applied the method of the Stieltjes transform, utilized earlier by Sips (1948, 1950), to describe the gaseous adsorption on actual heterogeneous solid surfaces. The authors noted that the Sips' theoretical results on gaseous adsorption could be easily modified to solution adsorption by a simple transformation of variables. However, later on Rudzinski et al. (1983) indicated that the method of Stieltjes transform cannot be applied in the case when molecules of the liquid mixture may have different cross-sectional areas (or heterogeneity) on the solid surface. This surface heterogeneity of the adsorbed protein molecules (or, in a more general sense, the biological macromolecule) may arise either due to the energetic heterogeneity of the surface sites, to a heterogeneity of the molecule in question in solution, or to a combination of the above or other reasons. The influence of heterogeneity on protein adsorption and on reactions on the surface are examined in the following section.

IV. Heterogeneity in Protein Adsorption

The surfaces for protein adsorption need to be better characterized. The heterogeneity of the surface will significantly influence adsorption and subsequent reactions occurring on the surface. The heterogeneity of the surface will also influence the rate and extent of protein denaturation on the surface. It would be of interest to develop a measure of heterogeneity of the surface and then be able to relate it to the extent of protein denaturation or conformational changes that occur upon protein adsorption at the surface. Such studies exhibit the potential to provide novel insights into the nature of protein adsorption on different surfaces. Norde and Lyklema (1979) have emphasized that a detailed analysis of structural rearrangements of proteins adsorbed on surfaces has eluded investigators. They estimated and analyzed the structural contributions of the adsorbed proteins to thermodynamic functions. In a recent review Horbett (1987) has mentioned the importance of different processes that lead to protein structural rearrangements or conformational changes on adsorption at a surface. This author indicated a continuously changing conformational state of the protein adsorbed on the surface. In a further study of fibrinogen adsorption from plasma onto polyethylene and glass, Slack and Horbett (1988) indicate that fibrinogen is initially adsorbed onto the surface. Later on, surface-active proteins like high-molecular-weight kininogen (HMWK) are primarily responsible for the displacement of the fibrinogen (not tightly bound) to the surface. This displacement phenomenon would also contribute to the heterogeneity of the protein adsorbed on a surface. A better understanding of the processes that interact with the protein would shed novel insights into controlling the heterogeneity of the protein adsorbed on the surface. In this section we will examine the influence on protein adsorption at interfaces of (a) heterogeneity in solute(s), (b) heterogeneity in the surfaces, (c) models incorporating heterogeneity, and (d) the implications of this heterogeneity on protein adsorption and the mediation of further reactions on the surface.

The application of mathematical distributions of proteins adsorbed on surfaces is a complex problem. The application of mathematical distributions of proteins adsorbed on surfaces is not only a more realistic approach to the actual situation but also it presents a novel technique to gain valuable physical insights into the protein adsorption process and into the influence on subsequent adsorbed protein-mediated reactions occurring on the surface. The approach using distributions would provide a knowledge of the time-dependent compositions and conformation of proteins in the adsorbed layer on the surface.

In a relevant though not similar study Malhotra and Sadana (1987a,b) assumed a continuous normal distribution of the thermal activation energy for deactivation, and using this developed a simple mathematical model to find the activity–time trajectories for a microheterogeneous enzyme. Using this model, these authors were able to show a time-dependent change in the activity and composition of the enzyme. This composition change was revealed as a change in the width and in the mean of the distribution of the activation energies of deactivation for the enzyme. Malhotra and Sadana (1989) further analyzed the influence of intra-particle distribution on the deactivation characteristics of microheterogeneous enzymes, and noted that intra-particle diffusion effects alleviated the influence of microheterogeneity on the deactivation

characteristics of an enzyme exhibiting first-order kinetics of deactivation. Lundstrom and Elwing (1990) in their analysis of simple kinetic models for protein exchange reactions on solid surfaces also noted that it would be of interest to analyze the possible influence of diffusional limitations on the initial coverage of molecules in the different states. Finally, Malhotra and Sadana (1990) analyzed the role of the initial state distribution on the first-order deactivation of microheterogeneous enzyme samples. Their analysis primarily showed that very detailed deactivation data is necessary to distinguish between different distributions of deactivation activation energies in enzymes.

There is apparently not much information available in the literature regarding the heterogeneity of adsorbates on different surfaces. This is a complex process, especially when differences in molecular sizes between the components of a solution exist. Jaroniec et al. (1983) presented a model of multi-solute adsorption from dilute aqueous solutions involving energetic heterogeneity of the solid and differences in the molecular sizes of the solutes. These authors determined the parameters characterizing energetic heterogeneity of the solid and the ratio of the molecular sizes of the two arbitrary solutes. The authors also assumed negligible effects of association and dissociation in both phases. Jaroniec (1981) also proposed an equilibrium constant for an adsorbate that involved a symmetrical quasi-distribution of adsorption sites and inequality of molecular sizes of both solutes. Using the Jaroniec model (1981), Jaroniec et al. (1983) were able to show that for some systems the effects connected with differences in molecular sizes of solutes play a more important role than the heterogeneity effect. However, for systems where the molecular size ratio of the two solutes is close to one, then the heterogeneity effects are dominant.

Up until now, the most advanced treatments of heterogeneous adsorption from solutions composed of molecules of different sizes have been by Jaroniec et al. (1983), Dabrowski (1983), and Rudzinski et al. (1983). Jaroniec et al. (1983) and Dabrowski (1983) adopted a rather kinetic approach for the derivation of the adsorption isotherm, while the Rudzinski et al. (1983) isotherm was derived by means of the condensation approximation method. Other studies on the adsorption on energetically heterogeneous surfaces are also available (Borowko and Jaroniec, 1983; Nikitas, 1985).

Very recently, Nikitas (1989) developed a simple mathematical method which makes possible the development of isotherms for adsorption from dilute solutions composed of molecules with different sizes starting from isotherms based on the equality of the molecular sizes of the components. The treatment was restricted to random heterogeneous surfaces. This method was able to extend the Temkin and Langmuir–Freundlich isotherms to include size effects. This author utilized three distribution functions of partial surface coverage on sites, V_i, with adsorption energy, U_i. A uniform distribution generated the generalized Temkin isotherm valid for solvent and solute molecules of equal size. This author defined a heterogeneity factor, $\lambda = U_o/(kT)$ where U_o is the mean adsorption energy, k is a constant, and T is temperature. This factor describes the width of the adsorption energy distribution, and thus it increases with increasing heterogeneity of the adsorbent. Nikitas (1989) noted that the extension of the Temkin isotherm to include size effects resulted in a complicated expression for the isotherm. The exponential distribution generates, as a first approximation, the Freundlich isotherm. Finally, a quasi-Gaussian distribution was related to the Langmuir–Freundlich isotherm. Here, the degree of surface heterogeneity is expressed by the parameter c which ranges from 0 to 1 as one passes from heterogeneous to homogeneous surfaces.

Models for adsorption for solute in solid–solution adsorption systems are simple but are complicated by the energetic heterogeneity of the solid surface sites. Everett (1964) and Delmas and Patterson (1960) brought attention to the importance of surface heterogeneity in solution adsorption on solid surfaces. As indicated earlier, Rudzinski et al. (1973) tried to quantitatively describe solution adsorption on heterogeneous solid surfaces by applying the method of Stieltjes transform used by Sips (1948) to describe gaseous adsorption on an actual heterogeneous surface. They showed that Sips' theoretical results on gaseous adsorption can easily be applied to protein adsorption from solution by a simple transformation of variables. The technique does have two limitations, however. The equation does not reduce correctly to Henry's law at significantly low concentration of one of the components in the liquid mixture. Also, the method of Stieltjes transform cannot be used when the molecules of the liquid mixture have different cross-sectional areas on the solid surface. In their earlier studies, Rudzinski et al. (1973) did recognize the limitations of their analysis and did try to remove these two limitations. Later, Rudzinski et al. (1983) developed a general isotherm which approached the problem of surface heterogeneity in adsorption from a binary liquid mixture on an actual solid surface. This isotherm correctly showed the transition from the Dubinin–Radushkevich and Freundlich isotherm equations to Henry's law. It is generalized by taking into account the different cross-sectional areas of the adsorbed molecules. Their technique correctly took into account the different cross-sectional areas of the adsorbed molecules.

The Nikitas method (1989) is now utilized to develop isotherms for heterogeneous adsorption from dilute solutions involving differences in molecular sizes of components. Nikitas (1989) developed new adsorption isotherms from dilute solutions consisting of different size molecules, starting from isotherms based on the equality of the molecular sizes of the components.

The partial adsorption isotherm for protein or other biological macromolecular adsorption on a random heterogeneous surface may be expressed as

$$\frac{\alpha_i}{1-\alpha_i} = K \exp\left(\frac{E_{ads}}{kT}\right) \tag{2}$$

if size effects could be neglected, and

$$K = \frac{\gamma_s \delta x_A}{\gamma_a} \tag{3}$$

Besides, the solution is dilute enough so that the activity coefficient of the protein molecule in solution is unity. α_i is the partial surface coverage on sites with adsorption energy E_{ads}, δ is the adsorption equilibrium constant, x_A is the molar fraction of the adsorbate in the bulk

solution, and γ_s and γ_a are the surface activity coefficients of the adsorbate (protein or other biological macromolecule) and the solvent, respectively. Nikitas (1989) emphasizes that the activity coefficients γ_a and γ_s depend upon the surface coverage, α over the whole adsorption layer, while they are independent of the adsorption energy, E_{ads}.

Adsorbate size effects may be included in eq 2 by modifying it to yield

$$\frac{\alpha_i}{(1-\alpha_i)^m} = K \exp\left(\frac{E_{ads}}{kT}\right) \tag{4}$$

where m is a size ratio parameter equal to the ratio σ_a/σ_s of the areas covered at the adsorption layer by a solute, s_a, and a solvent, s_s, molecule. In this case:

$$K = \frac{{\gamma_s}^m \delta x_A}{\gamma_a} \tag{5}$$

Next, Nikitas (1989) considers the expression:

$$\frac{\alpha_i}{b-\alpha_i} = K \exp\left(\frac{E_{ads}}{kT}\right) \tag{6}$$

which bears physical meaning when the parameter b equals unity. The heterogeneity is incorporated in the analysis by including a distribution function $\chi(E_{ads})$, and the total surface coverage is obtained by a weighted-average expression. The total surface coverage is given by

$$\begin{aligned}\frac{\alpha}{b} &= \int_{-\infty}^{\infty} \frac{1}{b}\alpha_i \chi(E_{ads})\, dE_{ads} \\ &= \int_{-\infty}^{\infty} \frac{K \exp(E_{ads}/kT)}{1 + K \exp(E_{ads}/kT)} \chi(E_{ads})\, dE_{ads} \\ &= h(K)\end{aligned} \tag{7}$$

The equilibrium constant is given by

$$K = \Phi\left(\frac{\alpha_i}{b}\right) \tag{8}$$

where

$$\int_{-\infty}^{\infty} \frac{\alpha_i}{b-\alpha_i} \chi(E_{ads})\, dE_{ads} = c\Phi\left(\frac{\phi}{b}\right) \tag{9}$$

and c is a constant.

The Nikitas (1989) expression of an isotherm for adsorption from dilute solutions composed of different size adsorbate molecules and same-size solvent molecules on a random heterogeneous surface is given by

$$c_1 x_A = \frac{\gamma_A}{{\gamma_s}^m} \frac{d^n \phi(\alpha/b)}{db^n}\Big|_{b=1} \tag{10}$$

where c_1 is a constant. Once again, it may be suggested that these results can be useful for interpretating the adsorption of proteins.

Example 5. Applications of heterogeneous adsorption of solutes from dilute solutions.

Nikitas (1989) indicates that the analytical expressions obtained from eq 10 are dependent on the distribution function $\chi(E_{ads})$. Let us consider a uniform distribution function and a quasi-Gaussian distribution (Sips, 1948).

The uniform distribution function is given by (Nikitas, 1989):

$$\chi(E_{ads}) = \begin{cases} 0, & E_{ads} < E_{ads,m} - E_{ads,o},\ E_{ads} > E_{ads,m} + E_o \\ \frac{1}{2}E_{ads,o} & E_{ads,m} - E_{ads,o} \le E_{ads} \le E_{ads,m} + E_o \end{cases} \tag{11}$$

Young and Crowell (1962) and Nikitas (1988) indicate that the above uniform distribution function generates a generalized Temkin isotherm valid for solvent and solute molecules of equal size:

$$\alpha = \frac{kT}{2E_{ads,o}} \ln \frac{1 + K\left[\frac{(E_{ads,m} + E_{ads,o})}{kT}\right]}{1 + K\left[\frac{(E_{ads,m} - E_{ads,o})}{kT}\right]} \tag{12}$$

where K is given by eq 3.

From eq 7 and 11 one obtains:

$$\Phi\left(\frac{\alpha}{b}\right) = \frac{1 - \exp\left(\frac{2\lambda\alpha}{b}\right)}{\exp\left(\frac{2\lambda\alpha}{b} - \lambda\right) - \exp(\lambda)} \exp\left(\frac{E_{ads,m}}{kT}\right) \tag{13}$$

where $\lambda = E_{ads,o}/kT$ is the heterogeneity factor. Nikitas (1989) indicates that this heterogeneity factor describes the width of the adsorption energy distribution. This factor, thus, increases with an increase in the heterogeneity of the adsorbent.

Equation 13 yields the following adsorption isotherms:

$$c_1 x_A = \frac{2\lambda \exp(2\alpha\lambda)[\exp(\lambda) - \exp(-\lambda)]\gamma_a}{[\exp(2\lambda\alpha - \lambda) - \exp(\lambda)]^2 {\lambda_s}^2} \tag{14}$$

and

$$\begin{aligned}c_1 x_A = {} & 2\lambda\alpha \exp(2\lambda\alpha)[(1-\lambda\alpha)e^{2\lambda\alpha-\lambda} - \\ & (1+\lambda\alpha)\exp(\lambda)][\exp(\lambda) - \exp(-\lambda)]/[\exp(2\lambda\alpha - \lambda) - \\ & \exp(\lambda)]^3 \left(\frac{\gamma_a}{\gamma_s}\right)^3\end{aligned} \tag{15}$$

for $m = 2$ and 3, respectively. Note the "similarity" in the two expressions. Nikitas (1989) emphasizes that the inclusion of size effects utilizing the Temkin isotherm results in a complicated expression. It is of interest to note that, as the heterogeneity parameter $\lambda \to 0$, eq 13 and 14 reduce to the corresponding expression valid for homogeneous surfaces, and is given by

$$c_1 x_A = \frac{\alpha}{(1-\alpha)^m} \frac{\gamma_a}{{\gamma_s}^m} \tag{16}$$

Sips (1948) indicates that the quasi-Gaussian distribution:

$$\begin{aligned}\chi(E_{ads}) = {} & \frac{1}{kT} \sin(\pi h) \exp\left[\frac{h(E_{ads,m} - E)}{kT}\right] / 1 + 2\cos \\ & (\pi h) \exp\left[\frac{h(E_{ads,m} - E)}{kT}\right] + \exp\left[\frac{2h(E_{ads,m} - E)}{kT}\right]\end{aligned} \tag{17}$$

is related to the Langmuir–Freundlich isotherm:

$$\left(\frac{\alpha}{1-\alpha}\right)^{1/c} = K x_A \tag{18}$$

Nikitas (1989) indicates that the degree of surface heterogeneity is given by the parameter c which goes from 0 to 1 as we go from heterogeneous to homogeneous surfaces.

The function $\phi(\alpha/b)$ is given by

$$\phi\left(\frac{\alpha}{b}\right) = c_1\left(\frac{\alpha}{b-\alpha}\right)^{1/c} \quad (19)$$

Then, the extension of eq 18 to include size effects may be written as

$$c_1 x_A = \frac{\alpha^{1/c}}{(1-\alpha)^{(m-1)+1/c}} \frac{\gamma_a}{\gamma_s^m} \quad (20)$$

As expected, eq 20 reduces to eq 16 when $c = 1$ for homogeneous surfaces.

Other studies on the effect of heterogeneity are also available. Jaroniec et al. (1983) presented a simple equation for multi-solute adsorption from dilute aqueous solutions on solids. Their proposed model provided a simple relationship between amount adsorbed and the concentrations of the two arbitrary solutes. From their model, these authors could obtain a parameter that characterized the energetic heterogeneity of the solid and the ratio of molecular sizes of two arbitrary solutes. The assumptions made by Jaroniec et al. (1983) include (i) monolayer adsorption, (ii) differences in molecular sizes of the solutes, (iii) ideality in the adsorption space and in the bulk solution, (iv) energetically heterogeneous solid, and finally (v) the effects of association and dissociation in both phases are neglected. Jaroniec et al. (1983) successfully applied their model to the adsorption of different phenols (2,4-dichlorophenol, *p*-nitrophenol, etc.) in dilute solution on activated carbon at 293 K. These authors noted that the effects connected with differences in molecular sizes of solutes are considerably greater than heterogeneity effects. It would be of interest to extrapolate these studies to the adsorption of proteins and other biological macromolecules of interest to appropriate surfaces/interfaces.

Corsel et al. (1986) analyzed the adsorption and desorption of prothrombin, albumin, and fibrinogen to phospholipid bilayers by ellipsometry. Adsorption of proteins to biological membranes is of importance in many physiological processes, and is of significance especially in blood coagulation. In this case, the final product is a blood clot of polymerized fibrin that is formed after the splitting of circulating fibrinogen by thrombin. Corsel et al. (1986) indicated that there were complications during their measurements of the adsorption, k_{on}, and desorption, k_{off}, rate constants. This was due to the presence of different classes of binding sites. Note that it has been shown that the values of k_{off} and k_{on} are generally dependent on the surface concentration (Γ) of the protein (Cuypers et al., 1987). Kop et al. (1989) indicate that sorption rate constants should therefore preferably be measured at "initial values", that is, at values of low surface coverage. Equilibrium measurements of the binding of prothrombin to dioleoylphosphatidylserine (DOPS) have demonstrated a biphasic adsorption with sites characterized by an equilibrium constant, K_d ($= k_{off}/k_{on}$) equal to 6×10^{-10} M, $\Gamma_{max} = 0.26$ μg/cm^2, and for the "low-affinity" sites $K_d = 10^{-7}$ M, $\Gamma_{max} = 0.12$ μg/cm^2. Here Γ_{max} represents the adsorbed quantity of prothrombin (in this case) at maximal surface coverage (Kop et al., 1984). Corsel et al. (1986) obtained similar results for the "same" system except that Γ_{max} in both cases was equal to 0.18 μg/cm^2. They explained this shift by the use of a more physiological calcium concentration of 1.5 mM in their studies compared to 10 mM utilized by Kop et al. (1984). Corsel et al. (1986) indicate that, in the absence of specific biological binding sites, protein adsorption to the phospholipid bilayers would include secondary changes in the surface interactions of protein molecules. These secondary changes would then lead to a heterogeneity of the protein adsorbed on the surface.

Example 6. An explanation of the paradox between concentration-dependent adsorption and lack of desorption in pure buffer.

Case a. The above phenomenon wherein the radiolabeled adsorbed protein does not show net desorption after dilution of the protein solution but readily exchanges with unlabeled protein has been observed for albumin (Brash and Samak, 1978; Cheng et al., 1987) and fibrinogen (Chan and Brash, 1981). Several models have been proposed in the literature to explain these observations; some of those include time-dependent structural changes in the adsorbed protein layer and specific models of the exchange of the bound and unbound protein molecules. These structural changes would lead to an increasing heterogeneity of the adsorbed protein on the surface.

Case b. Kop et al. (1989) analyzed the binding of coagulation factor V to planar phospholipid double layers by ellipsometry. At 20 °C, factor V in buffer solution undergoes a rapid (half-life approximately 25 min) spontaneous denaturation. This destroys the binding capacity of this protein to the phospholipid bilayers. Since the dissociation constant, $K_d = k_{off}/k_{on}$, a decrease in k_{on} leads to overestimations of K_d of several orders of magnitude and an apparently reversible binding isotherm for factor V.

It is of interest to note that in both cases we have time-dependent structural changes–denaturation of the adsorbed protein which leads to an increasing heterogeneity that helps in elucidating the so called "paradox".

The multiple adsorption states exhibited by proteins would yield, in general, a plethora of "different structures" at the interface exhibiting slightly different functionalities. This multiple state of protein adsorption at different sites of the interface should exhibit heterogeneous deactivation behavior at the interface. In any realistic model for protein–enzyme inactivation at interfaces this heterogeneity of adsorption and the subsequent heterogeneity in deactivation should be taken into account. In general, a heterogeneity in an enzyme sample leads to an enhanced stabilization when compared to a homogeneous enzyme (Malhotra and Sadana, 1987a,b). This heterogeneity, as indicated earlier, may be denoted by a distribution in the activation energy for deactivation or in the conformational states. It would be of significant interest to characterize this heterogeneity and distribution in adsorption and get an estimate or a "measure" of this heterogeneity. Then, an analysis could be performed on the overall effect of this heterogeneity on reactions occurring at the interface, protein stability, and properties at the interface.

Horbett and Brash (1987) have recently reviewed the adsorption of proteins to surfaces and emphasize the lack of information on the qualitative state (confor-

mational states) of the protein adsorbed. These authors indicate the importance of predictive techniques to obtain estimates of the quantitative as well as the qualitative nature of the protein adsorbed. Careful and detailed studies are urgently required to describe more clearly the effect or influence of different parameters on protein adsorption at interfaces, and their subsequent effect on the proteins themselves, and on other processes occurring at that interface. Recently, Johnsson et al. (1987) compared the adsorption isotherms for immunoglobulin G (IgG) and secretory fibronectin (HFN) on silica with two different surface free energies by in situ ellipsometry. The results were interpreted as time-dependent conformational changes in the adsorbed protein film, where the degree of changes was dependent on the solid surface free energy. These time-dependent conformational changes of the adsorbed protein molecule lead to heterogeneity in adsorption. Also, the shapes of the adsorption isotherms may depend on the heterogeneity of the protein preparations, interaction between adsorbed molecules, concentration-dependent structural changes in the adsorbed film, or a heterogeneous surface with several types of adsorption "sites" (Bagchi and Birnbaum, 1981; Fair and Jamieson, 1980).

It is, thus, clear that methods are required that both qualitatively and quantitatively analyze the adsorbed state of the protein molecule on different surfaces. It is safe to assume that the adsorbed protein state will be heterogeneous. What is now required are effective experimental techniques that can estimate both the quantity of the protein adsorbed and also the heterogeneity of adsorption. This heterogeneity is one qualitative aspect of protein adsorption. Appropriate models that provide a measure of heterogeneity of protein adsorption are required. From protein adsorption data the experimental technique should also provide a measure of heterogeneity of protein adsorption. Then, the measures of heterogeneity obtained experimentally and by the modeling may be compared. Such comparisons may then lead to better model development or even better experimental techniques that provide more reliable measures of heterogeneity. Clearly, this is an avenue that will provide novel physical insights into the adsorption of proteins on different surfaces. Recognize that as instrumentation advances and becomes more and more sophisticated this aspect of protein heterogeneity on adsorption to different surfaces will become more and more important and prominent. This may well play a significant role in the influence of proteins on reactions occurring at the surface. Thus, it is essential to evaluate or estimate the effect of heterogeneity on protein adsorption. Unfortunately, this aspect has up until now been rather neglected. The next section looks at a few experimental techniques that have been utilized to qualitatively characterize protein adsorption on surfaces. The following section then examines the more recent models that have been utilized to describe protein adsorption on different types of surfaces.

V. Techniques for Qualitative Characterization of Protein Adsorption

Because of their complex (often "patchwise") chemical constitution, proteins may adsorb by different mechanisms on different surfaces. Although it is well-known that physicochemical surface properties strongly affect the protein adsorption, such studies on chemically and morphologically well-characterized surfaces are scarce. Few techniques lend themselves to direct study of the structural properties of proteins at interfaces. The ideal approach should produce quantitative real-time data in situ concerning the amount, activity, and conformation of proteins at the interface. Most approaches are only approximations of this optimum and are generally restricted in their application. We now analyze some of the techniques that have been used to qualitatively characterize protein adsorption on surfaces. These techniques are ellipsometry, total internal reflection fluorescence (TIRF), spectroscopy, immunogold staining technique, and other methods. Other useful techniques such as fluorescence lifetime measurements which elucidate protein dynamics, and ATR-FTIR which analyze surface induced shifts in protein secondary structure are not presented here. In order that these and other techniques are given sufficient importance, they could form the basis of another review article.

A. Ellipsometry

Ellipsometry is an in situ method used to make more quantitative the thickness and refractive indices of adsorbed protein films. In this optical technique the change in state of polarization of light upon reflection from a surface is used to characterize the surface. If proteins are allowed to adsorb to that surface, ellipsometry makes it possible to determine the thickness, the refractive index, and the specific amount of adsorbed molecules. Morrisey et al. (1976) studied adsorbed fibrinogen layers as a function of the surface potential by means of ellipsometry. Changes in compactness as calculated from these parameters were interpreted as indications of conformational changes of the protein. Cuypers et al. (1977) demonstrated the possibility of different protein orientations on hydrophobic versus hydrophilic chromium substrates. Stoner and Srinivasan (1970) measured the thickness and, simultaneously, the interfacial capacitance (that is, surface coverage) for fibrinogen on platinum as a function of the applied potential. It was shown that an attractive electrostatic potential resulted in a flat conformation of the protein adsorbate.

Johnsson et al. (1985) recently compared the adsorption isotherms for immunoglobulin G and secretory fibronectin on silica with different surface free energies by in situ ellipsometry. The isotherms were obtained by either direct or successive addition of the proteins. A significant difference between the direct- and successive-addition isotherms was found for both proteins on hydrophobic silica, whereas the isotherms essentially coincide for the proteins on hydrophilic silica. These authors interpreted their results as time-dependent conformational changes in the adsorbed protein film where the degree of changes was dependent on the solid surface free energy. These changes were most pronounced on hydrophobic silica. For example, secretory fibronectin adsorbed to hydrophilic silica showed less tendency to undergo surface conformational changes as compared to fibronectin adsorbed to hydrophobic silica. Also, at low surface concentration lack of

competition for surface adsorption sites results in a flatter adsorbed conformation while at high surface concentration intermolecular repulsion causes a more extended conformation with fewer surface attachments.

More recently, Golander and Kiss (1988) wanted to correlate the surface functional properties of smooth ESCA-characterized polymer films with their adsorption behavior vis-a-vis some well-known proteins as studied by ellipsometry. These authors used ellipsometry to investigate the adsorption of bovine serum albumin (BSA), γ-globulin (IgG), fibrinogen, and poly-L-lysine (PLL) to silicon wafers, which were surface modified by attaching PVC, a methacrylic acid/methacrylate copolymer (PMA), or PED films all of which were characterized by ESCA. These authors noted that the adsorption of the three plasma proteins and one cationic polyelectrolyte, PLL, is generally lower to the hydrophilic PMA and PEO films than that to the PVC films. This demonstrated the importance of the hydrophobic driving force for protein adsorption. The authors also noted that the chemical constitution of the substrate surface has a significant influence on the course of protein adsorption. For example, the protein isotherms obtained on PVC may be explained by assuming dynamic adsorption models with two adsorption modes, that is, native and denatured molecules having different affinities to the surface. This would also lead to a heterogeneity (as indicated in the earlier sections) of the adsorbed protein states. Of course, it is not only the chemical constitution of the substrate surface that is important in adsorption but also the protein which affects the nature of the interaction. Finally, Golander and Kiss (1988) also noted that a low degree of protein adsorption, $\Gamma < 0.5$ mg/m^2 was observed for surfaces covered with surface-grafted poly(ethylene oxide) (PEO) chains (molecular weight 1900) which were covalently linked by means of terminal CHO groups to surface amino groups.

Up until now quantitative estimates of protein adsorption to surfaces has been made assuming that the surface has a homogeneous composition. This is an idealization since there is no such surface with a homogeneous composition. This aspect and the importance of heterogeneity (either of surface or of protein) has been emphasized throughout this review. Protein adsorption needs to be studied for different surface compositions. The wettability gradient technique developed by Elwing et al. (1987) is one such elegant technique. A surface is prepared on which there is a gradient of a constituent (for example, methyl groups). Then, the adsorption of proteins is quantitatively estimated by ellipsometry along this gradient. The above protein adsorption is also correlated with the degree of wettability using the capillary rise method (Elwing et al., 1987). These authors noted a difference in the amounts of human fibrinogen and γ-globulin adsorbed at the hydrophobic end (0.7 and 0.55 μg/cm^2, respectively) of the gradient and (0.3 μg/cm^2 for both cases) at the hydrophilic end. In between there was a nonlinear decrease. The analysis of human fibrinogen and γ-globulin desorption from the surface indicated that there were qualitative differences. A possible explanation is the varying conformational states of the adsorbed proteins on the surface underscoring the influence of heterogeneity in adsorption.

The technique is rather appealing in that it analyzes the influence of a predetermined and controlled heterogeneous surface on protein adsorption. This, to the best of this author's knowledge is the first study that examines the influence of a heterogeneous surface on protein adsorption. More studies of this type are necessary since they more correctly represent the real-life situation. It would, of course, be better, and more true to the real-life situation if the wettability method could be modified and used not just as a gradient method but more as a technique to evaluate the heterogeneity of a surface that is present in a "random" fashion.

Finally, it should be recognized that ellipsometry and other techniques to be presented (such as total internal reflection fluorescence, protein fluorescence, and circular dichroism) are often suggestive in regard to possible conformational alterations on protein adsorption at different surfaces. More direct methods that, for example, measure activity loss of enzymes on adsorption at surfaces would be beneficial. These techniques are now presented in the following sections.

B. Total Internal Reflection Fluorescence (TIRF)

TIRF is one technique by which quantitative estimates of protein adsorption from flowing solutions may be obtained. As expected, however, there are difficulties in relating the TIRF signals to the amount of protein adsorbed on the surface. Lok et al. (1983a,b) have attempted to overcome the problems associated with the calibration of the TIRF technique. Several authors have failed to account for all of the factors (Norde et al., 1986; Leveque, 1928; Hsu and Sun, 1988; Langmuir, 1918). Cheng et al. (1987) recently utilized a modified form of the Lok et al. (1983a) method to examine the initial adsorption, desorption, and exchange kinetics of the protein bovine serum albumin (BSA) on six polymer surfaces with widely varying surface properties and functionalities. These authors covalently attached fluorescein isothiocyanate to primary amine groups of BSA. The molar fluorescein-to-BSA ratio was approximately unity. The results indicate that the fluorescence intensity of adsorbed FITC–BSA is proportional to the protein surface concentration for each surface. The initial rate of protein adsorption onto a surface is determined by both transport of protein to the surface and the intrinsic kinetics of adsorption at the surface. This has been described by a convection/diffusion model with appropriate boundary conditions for the channel geometry of the TIRF apparatus (Lok et al., 1983b).

Cheng et al. (1987) recently have shown how bulk solution ionic strength and pH can dramatically affect the fluorescence signal in a TIRF experiment in the absence of any changes in the protein surface concentration. Even the technique and interpretation used by Chen et al. (1987) is not entirely flawless. The TIRF detection point is an approximately 1- × 3-mm oval region in the center of the microscopic slide. The calibration procedure described above relates the fluorescence signal emanating from this small region to the average surface concentration over the entire wetted region of the slide. Cheng et al. (1987) acknowledge some spatial variations but basically they assume the adsorption to be essentially homogeneous with the wetted plate. This is not entirely so since we

do recognize that protein adsorption is heterogeneous, and one should factor this into the calculations. This is especially true if the measure of heterogeneity of protein adsorption is significant. The problem is compounded if a small degree of protein adsorption heterogeneity significantly affects the reactions occurring at the surface which are mediated by the protein adsorbed.

The results of this study show that the initial adsorption of BSA on three of the polymeric surfaces is diffusion limited up to wall shear rates of 4000 s^{-1}. The initial adsorption of BSA on another polymer is diffusion limited at shear rates below about 70 s^{-1} but becomes kinetically controlled at higher shear rates. Studies of the kinetically limited BSA adsorption on this last polymer show that the adsorption process can be described by a kinetic rate expression that is first order in protein concentration. Also, the desorption of adsorbed proteins on five out of the six polymeric surfaces studied is shown to be kinetically limited.

C. Protein Fluorescence and Circular Dichroism

This paper deals with the adsorption of proteins at solid–liquid interfaces—a system that has been most rigorously studied. An example of the adsorption of blood proteins at air–water interfaces is now presented. Clark et al. (1988) utilizing far-UV circular dichroism and intrinsic protein fluorescence compared the spectral properties of resolubilized bovine serum albumin (BSA) with native BSA and interpreted the results in terms of the conformational properties of the proteins. Far-UV circular dichroism spectra reveal only minor changes in the protein secondary structure evidenced by a small reduction in helix content after foaming. The biggest differences in conformation appear to be at the tertiary structure level and are readily detected by intrinsic fluorescence. A major irreversible reduction (>30%) in the intensity of tryptophan emission is reproducibly observed in the foamed sample. The change in conformation induced by foaming does not apparently reflect a change in the state of aggregation of the foamed protein. The native and foamed BSA samples used in the experiments contained similar amounts of oligomer as judged by nondenaturing polyacrylamide gel electrophoresis. Clark et al. (1988) acknowledged that their approach will only allow the observation of irreversible conformational changes that occur as a result of foaming and persist after resolubilization. Nevertheless, they state that their technique has allowed a more thorough study of the nature of these irreversible changes than by fluorescence quenching techniques. They indicate that in the future low-angle X-ray and neutron scattering techniques may be usefully employed in the investigation of the structural properties of adsorbed proteins in situ at the interface. We agree with them on this, and that in the meantime major compromises must be made if preliminary studies are to be made in this field.

Finally, the characterization of possible structural changes of the protein upon surface interaction has been limited to techniques such as presented above and others such as fluorescence lifetime measurements and ATR-FTIR. In general, even with these procedures results can often only be suggestive in regard to possible conformational alterations. Conformational changes of enzymes upon adsorption lead to activity changes which can be measured. Sandwick and Schray (1981) have capitalized on this to analyze the adsorption of four nonblood proteins–enzymes (horse radish peroxidase, alkaline phosphatase, catalase, and β-galactosidase) on to a hydrophobic surface. As expected, a sufficient amount of time ("residence time") should be given to the adsorbed protein to let it attain the different conformational states which are also a measure of the "dispersion" or degree of heterogeneity. This time interval also permits a decrease in the enzyme activity which can be followed. Sandwick and Schray (1981) noted that at high initial concentrations the enzyme tended to adsorb at the "native" conformation, whereas at lower initial concentrations the enzyme tended to adsorb with different conformational states. This seems to indicate that a higher initial enzyme concentration would apparently minimize the "dispersion" in heterogeneity of protein adsorption. The amount of each form present on the surface at any particular instance will be dependent primarily on enzyme solution concentration, but also on other factors such as surface area available, temperature, and solution characteristics (pH and ionic strength).

The next section presents a few of the mathematical models that have been utilized to model protein adsorption studies. None of the models presented incorporate heterogeneity in analyzing the quantitative aspects of protein adsorption. Nevertheless, in accord with the general theme of the paper the examples presented do indicate the presence of heterogeneity in protein adsorption.

VI. Models for Protein Adsorption

A. Nonflowing Cylindrical System (Young et al., 1988)

In a nonflowing cylindrical system, protein transport is a diffusional process (assuming no convection due to thermal or concentration gradients) described by

$$\frac{\partial c}{\partial t} = \frac{1}{r}\frac{\partial}{\partial r}\left(Dr\frac{\partial c}{\partial r}\right) \tag{21}$$

where c is the local protein concentration, t is time, D is the diffusivity of the protein, and r is the radial coordinate from the center of the tube radius, R. For an infinitely long tube c is a function of r and t only, that is, $c = c(r,t)$. The boundary conditions are

$$c(r,0) = c_0 \tag{22}$$

where c_0 is the initial protein concentration. The zero-flux boundary condition at the center of the tube yields

$$\frac{\partial c}{\partial r}(0,t) = 0 \tag{23}$$

The adsorption rate boundary condition at the tubing wall is

$$-D\frac{\partial c}{\partial r}(R,t) = R_{ads}(c,c_s) \tag{24}$$

Here, R_{ads} is the intrinsic adsorption rate constant and is a function of the solution concentration, c, and the surface concentration, c_s. The intrinsic adsorption rate constant is the adsorption rate in the absence of any diffusional limitations. The first case occurs when the

diffusion flux to the surface is much faster than the intrinsic adsorption kinetics. In this case, the adsorption kinetics are not limited by diffusion, and the observed adsorption rate, dc_s/dt, is equal to the intrinsic adsorption rate:

$$dc_s/dt = R_{ads}(c,c_s) \tag{25}$$

The other limiting case occurs when the diffusional flux is much slower than the intrinsic kinetics, and the observed adsorption rate is actually the diffusion rate. In this case, each protein molecule that approaches the surface is immediately adsorbed, and the concentration of soluble protein adjacent to the surface is zero. The boundary condition for this diffusion-limited case is

$$c(R,t) = 0 \tag{26}$$

The solution of eqs 23, 24, and 26 yields the concentration distribution inside the tube:

$$c(r,t) = 2c_o\sum_{n=1}^{\infty}\frac{J_0(\alpha_n(r/R))}{\alpha_n J_1(\alpha_n)}\exp\left(-\alpha_n{}^2\frac{Dt}{R^2}\right) \tag{27}$$

where J_0 and J_1 are the Bessel functions of the first kind of order 0 and 1, respectively, and α_n is the nth zero of J_0. The surface concentration is the time integral of the flux of the protein to the surface:

$$c_s(t) = \int_o^t -D\frac{\partial c}{\partial r}\Big|_{r=R} dt$$

$$= 2c_oR\sum_{n=1}^{\infty}\frac{1}{\alpha_n{}^2}\left[1 - \exp\left(-\alpha_n{}^2\frac{Dt}{R^2}\right)\right] \tag{28}$$

A plot of dimensionless surface concentration $X = c_s/(c_oR)$ against real time for a set of diffusivities may be obtained.

The above is an example of protein adsorption in a cylindrical coordinate system. Let us now examine protein desorption in a Cartesian coordinate system.

B. Protein Convection (Desorption Kinetics) (Cheng et al., 1987)

Consider an absorbing surface with adsorbed protein in equilibrium with a flowing protein solution at concentration c_o. For desorption to occur, the protein solution is replaced by a buffer solution with no protein. At steady state, a concentration boundary layer is established in solution adjacent to the adsorbing surface. Within the region of the concentration boundary layer (where $y \ll b$), a model describing the transport-limited desorption at long times is given by

$$\gamma y\frac{\partial c}{\partial x} = D\frac{\partial^2 c}{\partial y^2} \tag{29}$$

Here x is the direction of flow, y is normal to the polymer surface, γ is the wall shear rate, and b is the thickness of the flow chamber. The boundary conditions are

$$x = 0 \quad c = 0 \text{ for all } y \tag{30}$$

$$y = \infty \quad c = 0 \text{ for all } x \tag{31}$$

$$y = 0 \quad c = c_0 \text{ for all } x > 0 \tag{32}$$

c_o is the solution concentration of protein that is in equilibrium with the surface protein concentration. On solving eq 29 subject to eq 30–32 yields

$$\frac{dc_s}{dt} = \frac{-1}{\Gamma\left(\frac{4}{3}\right)9^{1/3}}\left(\frac{\gamma}{Dx}\right)^{1/3}Dc_o \tag{33}$$

Equation 33 can be used to calculate the expected transport-limited desorption rate given c_s, the surface protein concentration, and c_o, the solution protein concentration in equilibrium with c_s.

C. A Probabilistic Analysis for Protein Adsorption (Hsu and Sun, 1988)

The adsorption of proteins to solid surfaces is often modeled by resorting to the assumptions made by Langmuir (1918) in deriving the adsorption equation. This derivation is based on the mean or averaged behavior of the particles in the system, and so only macroscopic characteristics appear (Boughey et al., 1978; Petersen and Kwei, 1961). A stochastic approach is capable of providing more details about a dynamic system (Stainislaus et al., 1972). Hsu and Sun (1988) adopted a statistical analysis to model the transient behavior of a reversible adsorption of small particles on a solid surface. These authors were able to estimate both the mean and the fluctuating characteristics of the adsorption in a straightforward manner. Though these authors did not give examples of protein adsorption to solid surfaces, their analysis is interesting and should yield valuable insights into protein adsorption on solid surfaces. These authors successfully modeled the deposition of polystyrene particles on nylon fibers (Boughey et al., 1978), the adsorption of CO as a function of time on alumina after preadsorption of water vapor, and the adsorption of hydrogen by $LaNi_5$ (Tanaka et al., 1977).

Example 7. The equations for flowing blood proteins and an artificial or natural surface.

Schaaf and Dejardin (1987) indicate that thermodynamic and structural information may be obtained by the determination of adsorption isotherms and adsorbed layer thicknesses. A "dynamic equilibrium" is definitely established between the flowing blood and the surface. These authors indicate that at least two aspects need to be considered: (a) the rate at which the proteins (or biological macromolecules) become attached to the solid surface when they are in close proximity to the surface, and (b) the diffusive flux from the bulk solution to the depleted interfacial layer.

Diffusion-Controlled Regime. The idealized situation of a surface acting as a perfectly adsorbing barrier was initially considered by Smoluchovski (1916). Herein, any molecule reaching the surface is adsorbed. The appropriate equation and boundary conditions are

$$\frac{\partial c_p(x,t)}{\partial t} = D\frac{\partial^2 c_p(x,t)}{\partial x^2} \tag{34}$$

and

$$c_p(0,t) = 0, t > 0 \tag{35}$$

$$c_p(x,0) = c_{p_o}, x > 0 \tag{36}$$

respectively. Here x is the distance from the interface (or surface), D is the solute (protein or other biological macromolecule) diffusivity, and c_p is the concentration of the protein (or other biological macromolecule). The concentration of the adsorbed protein molecules is given

by

$$c_{p,s}(t) = 2c_{p_o}\left[\frac{Dt}{\pi}\right]^{1/2} \quad (37)$$

The boundary condition eq 35 specifies that the process is completely diffusion controlled.

Kinetic-Controlled Regime. The rate expression for the kinetically-controlled regime contains a dimensional flaw with regard to the fraction of surface covered by the adsorbed molecules in accord with the Langmuirian approach (Schaaf and Dejardin, 1987). Thus, it is not presented here. This is a moot point. Since, in general, when experimental data with regard to protein surfaces is analyzed, the data does not fit either the diffusion-controlled regime process or the kinetically-controlled regime process.

Other complications also arise. Heterogeneities of the surface sites or of the solute molecules themselves also need to be examined to correctly model the more realistic case. Besides, as Schaaf and Dejardin (1987) correctly indicate, and as also pointed out by Collins and Kimball (1949) that the Smoluchovski solution leads to an infinite initial adsorption rate. These authors (Schaaf and Dejardin, 1987) utilized a simple and discrete model to describe material exchange in the vicinity of the interface, and indicated that the boundary condition, eq 35 needs to be modified to obtain physically sound results at very short times. It is apparently critical to characterize relative amounts and the kinetics of adsorption of proteins and other biological macromolecules to surfaces to begin to understand the reactions at the surface. However, other parameters, too, as expected will play a significant role. This is clearly demonstrated in the next example which examines protein adsorption from buffer and plasma onto different copolymers.

Example 8. Some correlations between blood protein adsorption and surface properties.

Grainger et al. (1989) have recently analyzed the influence of substrate hydrophilic–hydrophobic balance on the adsorption of proteins from buffer and plasma using a series of amphiphilic multiblock copolymers composed of poly(ethylene oxide) (PEO) and polystyrene (PS). These authors analyzed the adsorption of albumin, fibrinogen, and immunoglobulin G from single-component buffer, multicomponent buffer, and plasma solution in contact with polymer-coated beads. Initial attempts have been made to correlate protein adsorption and platelet adhesion to polymer surfaces by focusing on the effect of the hydrophobic and hydrophilic balance of constituent chains in amphiphilic polymer surfaces (Yui et al., 1984; Okano et al., 1986; Grainger et al., 1987).

Grainger et al. (1989) comment that a myriad of molecular plasma constituents, including more than 200 proteinaceous components, probably compete to differing degrees in the adsorption process occurring at material interfaces. These authors emphasize that the complex interactions between components in the adsorbed state and in bulk solution, are further perturbed by exchange–desorption influences, and denaturation–renaturation on the surface and in solution. All of these factors, including others, would increase the overall heterogeneity of the adsorbed protein on the surface, thereby further influencing the subsequent reactions occurring in the solution and on the surface. It would be difficult, but not impossible, to include some of these effects in a more realistic model of protein adsorption. Grainger et al. (1989) emphasize the shortcoming of their study by analyzing only three proteins in plasma whereas dozens of proteins are important in blood–surface interactions. Other factors, that increase the heterogeneity of the protein adsorbed besides just the adsorbed protein amounts and kinetics are also important. These parameters may include denaturation, degradation, exchange, etc.

Example 9. A brief description of a technique for measuring protein adsorption wherein the protein molecules are not modified by the introduction of some extrinsic label that might affect the adsorption kinetics.

Norde and Rouwendal (1990) have recently developed the streaming potential technique (in situ monitoring) to study protein adsorption kinetics on the surface of a flow cell. The streaming potential, which is due to the flow past a surface, is given by (Dukhin and Derjaguin, 1974)

$$V_s = \frac{\epsilon\zeta}{\eta c G}\Delta p \quad (38)$$

Here ϵ is the dielectric permittivity, ζ is the zeta potential, η is the viscosity, c is the cell constant, G is the conductance of the cell filled with the solvent, and Δp is the pressure drop. Norde and Rouwendal (1990) indicate that, in general, the adsorption of an (electrically charged) protein influences the ζ of the surface. In their experimental setup of two parallel glass plates, these authors applied a laminar flow of the protein solution.

The analytical solution of the mass-transport limited equation is the Leveque solution (1928) given by

$$\frac{d\Gamma(y)}{dt} = 0.81\left(\frac{\gamma}{yD}\right)^{1/3} Dc \quad (39)$$

Here D is the diffusion coefficient of the protein molecule in solution, γ is the shear rate at the cell wall $(dv/dx|_{x=a} = (a\Delta p/\eta l))$, $2a$ is separation distance between the two parallel plates, l is the length of the parallel plates in the direction of flow, y is distance in the direction of flow, and the x coordinate is perpendicular to flow. Norde and Rouwendal (1990) indicate that eq 39 is applicable only under steady-state conditions with respect to the concentration boundary layer. Also, the diffusion rate of the protein across this layer must be low relative to the rate of interaction of the protein molecule with the cell wall.

The following values were used in eq 38 to determine the streaming potential, $\Delta p = 17 \times 10^4$ N/m^2, $\epsilon = 78.5$, and η equals 8.9×10^{-4} N/m^2 s. For the bare glass buffer solution surface, a ζ potential of about −48 mV has been derived. Norde and Rouwendal (1990) analyzed the adsorption isotherms of myoglobin, ribonuclease, and lysozyme. At pH 7, these authors concluded that myoglobin is isoelectric and ribonuclease and lysozyme are positively charged. The differences in the shapes of the adsorption isotherms, that is the initial slopes (which represents the affinity for adsorption) and the plateau values, may be explained by the differences in the electrical charges between the three proteins that interact with the negatively charged glass surface. Norde and Rouwendal (1990) conclude that the initial adsorption rates of lysozyme, ribonuclease, and myoglobin on the glass surface are transport-limited. This is because the observed effects of wall shear rate and of protein concentration in solution (for low concentrations) on the kinetics of protein adsorption from laminarly flowing solutions is in close agreement with the Leveque convective-diffusion model (Leveque, 1928).

No information was provided by Norde and Rouwendal (1990) on the conformational changes or heterogeneity of the protein in the adsorbed state. This aspect was not incorporated in the model. The next example provides some physical insights into the heterogeneity of the adsorbed protein.

Example 10. A brief description of the behavior of proteins on a molecular scale.

Tilton et al. (1990) have recently analyzed the lateral diffusion of bovine serum albumin adsorbed at the solid–liquid interface (poly(methacrylate) (PPMA) and poly(dimethylsiloxane) (PDMS)) by a combination of total internal reflection fluorescence and fluorescence recovery after pattern photobleaching techniques. Tilton et al. (1990) indicate that lateral mobility, conformation, orientation, and ordering are probably associated in a complex manner. For instance, a conformational–structural change after adsorption may alter the lateral mobility of a protein. This change in the lateral mobility of the adsorbed protein may alter its ability to interact with the protein's nearest neighbors. Tilton et al. (1990) emphasize that the lateral mobility of adsorbed proteins has not been fully characterized, and much of the evidence that supports lateral mobility after adsorption is circumstantial. These authors emphasize that adsorbed proteins do form organized layers, and this may be attributed to lateral mobility (Brash and Lyman, 1969; Dass et al., 1987; Ratner et al., 1981; Fair and Jamieson, 1988).

Tilton et al. (1990) indicate that variations on the fluorescence bleaching technique has most commonly been used to investigate the slow self-diffusion (Eldridge et al., 1980; Schindler et al., 1980; Thompson and Axelrod, 1980). The primary requirement for the technique is that the mobile species bear either an intrinsic fluorescent moiety or a tightly bound extrinsic fluorophore. The rates of molecular transport are determined by creating a gradient of fluorescent and nonfluorescent molecules with a photobleaching pulse of high intensity laser illumination.

Tilton et al. (1990) indicate that the diffusion coefficient is a measure of the dynamics of the adsorbed BSA molecules, and the fractional mobility provides insight into the distribution of dynamical states. The mobile fraction could be verified by an examination of the long-time asymptote of the fluorescence recovery. Fractional mobilities, f, less than unity indicate nonuniformity of the adsorption states of EITC-BSA. Tilton et al. (1990) obtained an f value equal to 0.37 ± 0.05; this indicates that different populations of EITC-BSA (eosin isothiocyanate-labeled bovine serum albumin), characterized by different mobilities, prevail on the PMMA surface. Tilton et al. (1990) indicate that the co-existence of tightly packed adsorbed protein aggregates and isolated adsorbed proteins leads to a nonuniform lateral mobility. Tilton et al. (1990) emphasize that while a distribution of lateral mobilities may be a consequence of an ordering phenomenon, this lateral mobility in itself may be a prerequisite for the formation of such ordered arrangements. Tilton et al. (1990) emphasize that this nonuniformity may also be due to a distribution of BSA conformational states. This would then lead to a heterogeneity of the adsorbed BSA on the EITC surface.

Example 11. An analysis of the influence of surface hydrophobicity on conformational changes of an adsorbed protein.

Lu and Park (1991) have recently analyzed the influence of surface hydrophobicity on the conformational changes of adsorbed fibrinogen. Such studies are essential since a significant amount of attention has been paid to the conformational changes of protein adsorbed on solid surfaces due to the importance of protein conformation on the activity of the adsorbed proteins (Lenk et al., 1989; Kato et al., 1987). Tomikawa et al. (1980) emphasize that the conformational changes of fibrinogen adsorbed on solid surfaces are thought to be responsible for the platelet adhesion to the surface, since the intact fibrinogen in solution does not interact with platelets under the same conditions.

Lu and Park (1991) analyzed the extent of conformational changes of fibrinogen adsorbed on germanium, poly(hydroxyethyl methacrylate), Biomer, and polystyrene surfaces using Fourier transform infrared spectroscopy (FTIR) coupled with attenuated total reflectance (ATR) optics. The authors noted that some α-helical structures were changed into the unordered structures and the content of the β-turns was increased upon the protein adsorption. Basically, these authors noted that the adsorbed fibrinogen underwent a larger degree of conformational changes as the surface hydrophobicity increased.

Lu and Park (1991) underscore the fact that since the analysis of the conformational changes by their weighted-peak shift method is new, it is difficult to conclude at this point that their method quantitates the absolute magnitude of protein conformational changes. Nevertheless, the sum of the weighted peak shifts is expected to correlate with the relative extent of conformational changes. Furthermore, Iwamato et al. (1985) also found that fibronectin experienced greater conformational changes on a more hydrophobic silica surface. Lu and Park (1991) emphasize that when a protein adsorbs on a solid surface with high hydrophobicity, the hydrophobic core is likely to become exposed to the surface due to the hydrophobic interaction. Therefore, the larger conformational changes on more hydrophobic surfaces are understandable. These larger conformational changes on more hydrophobic surfaces would lead to increasing heterogeneities on the surface.

VII. Conclusions

The causes and influence of heterogeneity on initial protein adsorption and the mediation of subsequent reactions on the surface presented provide for a more realistic picture of the adsorption of proteins at the interface. A significant amount of evidence presented (qualitative characterization techniques, modeling studies, energetics of surface sites, etc.) indicates that heterogeneity in protein adsorption does exist. Protein adsorption on surface–interfaces will lead to differing degrees of conformational changes at the interface. These conformational changes will, in most cases, either decrease or increase the rate of subsequent reactions on the surface. It is worthwhile estimating these conformational changes (or qualitative aspects of protein adsorption) by a suitable heterogeneity parameter. This heterogeneity parameter should initially be defined, estimated, and then evaluated as a time-dependent function. Up until now very few models for protein adsorption exist that define an appropriate heterogeneity parameter; what is really required are models that can relate this heterogeneity parameter to experimental results.

Future effort that appropriately incorporates the influence of heterogeneity in protein adsorption studies

and delineates the influence of this heterogeneity (or conformational changes) on the mediation of subsequent reactions at the surface is urgently required to not only shed novel physical insights into the adsorption process but also provide for a more realistic picture of the events occurring at the interface. The introduction of heterogeneity in an analysis of protein adsorption on surfaces and the collection of such data by different investigators should then provide an initial and useful framework for analyzing subsequent protein adsorption studies. This framework should also help build more predictive techniques to analyze not only the quantitative but also the qualitative aspects of protein adsorption.

VIII. References

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