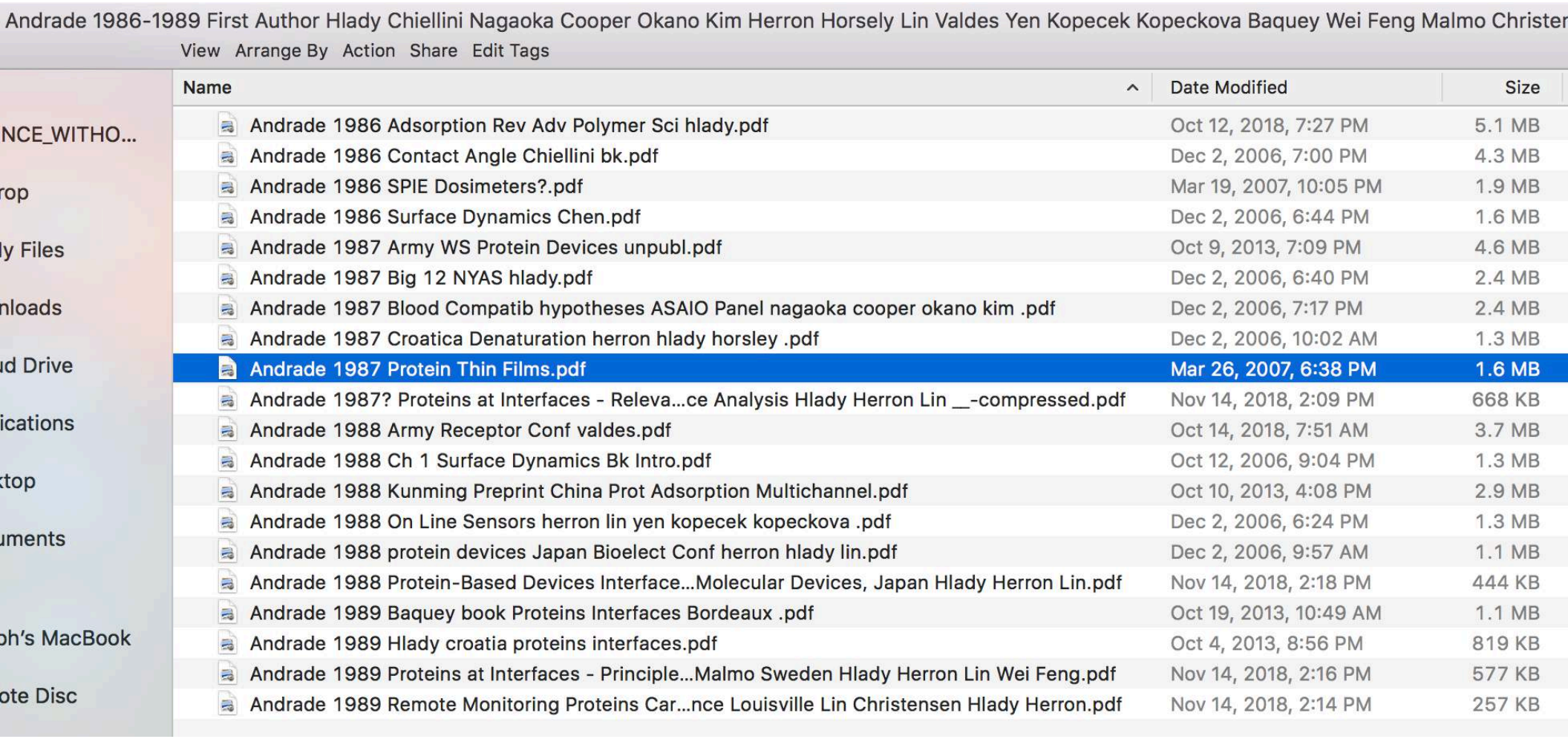
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Protein Adsorption and Materials Biocompatibility: A Tutorial Review and Suggested Hypotheses

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A comprehensive review of protein adsorption at solid-liquid interfaces is presented, including a brief review of protein structure and the principles of protein adsorption. Adsorption-based biocompatibility hypotheses and correlations are discussed, including surface charge, interface energetics, passivation, protein-resistant surfaces, and the role of adsorbed immunoglobulins and complement. New methods for the study of protein adsorption are discussed, including total internal reflection techniques (absorbance, fluorescence, and Raman) and ellipsometry. Qualitative "rules of thumb" of protein adsorption are also presented.

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Advances in Polymer Science 79

1 First Observable Event?

It is commonly stated that the first readily observable event at the interface between a material and a biological fluid is protein or macromolecule adsorption. Clearly other interactions precede protein adsorption: water adsorption and possibly absorption (hydration effects), ion bonding and electrical double layer formation, and the adsorption and absorption of low molecular weight solutes — such as amino acids. The protein adsorption event must result in major perturbation of the interfacial boundary layer which initially consists of water, ions, and other solutes.

Body fluids have a rich and complex composition, permitting a wide range of interactions and competitive processes.

The great majority of the available work has focused on blood/plasma/serum applications. In recent years the interaction of tear components with contact lenses have become very important.

Much of the early work is reviewed by Brash and Lyman [1] and by Vroman [2]. Protein adsorption is a very old field — the earliest studies probably being monomolecular film observations using Langmuir-Blodgett troughs [3]. A comprehensive monograph which reviews protein adsorption is now available [4].

Early observations showed that blood clotted faster in clean glass tubes than in siliconized glass. When Mr. Hageman's blood failed to clot *in vitro*, it was speculated that a "Hageman Factor" was responsible for the *in vitro* "activation" of blood coagulation (see Ref. [2] for a delightful account).

Early observations, particularly the work of Vroman [2, 5], showed that proteins adsorb on practically any surface. Vroman first showed [5a] that adsorption of protein on a hydrophilic surface rendered it less hydrophilic and also that protein adsorption on a hydrophobic surface rendered it hydrophilic.

Knowledge and understanding of protein structure and properties in the 1950's was rapidly evolving. The unique secondary, tertiary, and even quaternary structures of proteins were becoming understood [6–8] and the delicateness of protein three-dimensional conformation was recognized, including the possibility for "denaturation" at liquid/air and solid/liquid interfaces [2, 3].

The fact that Hageman Factor, a plasma protein, circulated free in blood without inducing coagulation, but when exposed to glass or silicate clay surfaces did induce coagulation, led to a unique and novel hypothesis: changes in conformation induced by the adsorption of Hageman Factor result in enzymatic activity which initiates a sequence of reactions leading to fibrin formation. Although interface-induced denaturation of proteins at liquid/air interfaces was well-known, the interest in *in vitro* blood handling and the early development of medical devices provided an impetus for understanding and controlling surface-induced coagulation.

At about the same time, the field of protein separation and purification was undergoing rapid development. The introduction of materials for protein chromatography, such as cross-linked dextran, agarose, and polyacrylamide, provided a means to study protein-surface interactions, as well as to dramatically advance knowledge in protein biochemistry.

2 Protein Structure

The treatment and understanding of protein adsorption requires familiarity with modern concepts of protein structure and function, such as provided in Ref. [6–8]. A concise review is available in Chapter 1 of Ref. [4].

Proteins are biological macromolecules constructed for specific and unique functions. They are high molecular weight polyamides produced by the specific co-polymerization of up to 20 different amino acids. The amino acid composition, called the primary structure, is generally unique and specific to each particular protein. The hydrogen bonding characteristics of the polyamide bond in the backbone of proteins result in various secondary structures, such as the well-known α-helix and β-sheet. Intramolecular associations, including ionic interactions, salt bridges, hydrophobic interactions, hydrogen bonding, and covalent disulfide bonds, result in a unique tertiary structure for each polypeptide chain. Finally, two or more polypeptide chains, each with its own primary, secondary, and tertiary structure, can associate to form a multi-chain quaternary structure. Most proteins contain short, carbohydrate sequences off the main polypeptide chain and are therefore called glycoproteins.

The fundamental principles of protein structure and function are available in modern biochemistry textbooks [6–7]. The current edition of Stryer is particularly recommended [7]. The books by Dickerson and Geis [6, 9] are excellent tutorials on the fundamental principles of protein structure. The modern text by Schulz and Schirmer [8], *Principles of Protein Structure*, is outstanding. In addition, modern physical

Table 1. Minimal protein information required for adsorption studies

Quantity	Method	Significance/Information
Molecular Weight	Light scattering, Osmometry, Gel permeation chromatography	Size, shape, mass of molecule.
Size & Shape	Light scattering sedimentation	Size, shape, mass of molecule.
Amino Acid Composition	Amino acid analysis	Basic.
Electrophoretic Mobility	Gel electrophoresis	Related to net charge.
Isoelectric Point	Isoelectric focusing	Number of charged groups.
UV-Visible Absorption Spectrum	Absorption spectroscopy	Tyrosine-Tryptophan environments, presence of absorbing ligands or impurities.[a]
Fluorescence Spectrum	Fluorescence spectroscopy	Tryptophan environment, presence of fluorescent ligands or impurities.[b]
CD Spectrum	Circular Dichroism	Secondary structure, α-helix, β-sheet, . . .
Subunits	SDS-gel electrophoresis	Quaternary structure components & their molecular weight.
Subunits & —S—S—	Gel electrophoresis in mercaptoethanol	Tertiary structure — breaks —S—S— bonds.
Solubility — Salting Out	Salting out fractionation	Solubility under different solvent conditions, pH, ionic strength, hydrophobic interactions.

[a] For example, heme in myoglobin & hemoglobin;
[b] For example, bilirubin in albumin

biochemistry texts, in particular Freifelder [10] and Cantor and Schimmel [11], are essential to the understanding of protein structure, function, and interfacial behavior. Bull's *Physical Biochemistry* is still an outstanding introduction to the subject [12]. Walton's books on biopolymers are also highly recommended [13, 14], as is Tanford's book [15].

Table 1 lists those characteristics of a protein which are normally considered essential for a minimal characterization. Most of the information desired is available for practically all proteins which may be of interest to the biomaterials investigator. Generally, all of this information is available in the literature, and the investigator need only to check for purity, homogeneity, and perhaps activity of the protein preparation.

Table 2. Additional protein information desired for adsorption studies

Quantity	Method	Information/Significance
Molecular Vibrations	Raman and infrared spectroscopy	Secondary & tertiary structure.
Thermal Denaturation	Raman, CD, or fluorescence	Conformation (secondary/tertiary) & structure as f (temperature).
Monolayer Properties	Langmuir trough or pendant drop	Air/water interfacial properties, π-A curves, can deduce closepacked monolayer dimensions.
Binding Properties	Equilibrium dialysis for lower molecular weight ligands — affinity chromatography for others	Ligand binding characteristics, such as Ca^{++} binding to fibrinogen, and prothrombin, fatty acid and bilirubin binding to albumin, heparin binding to antithrombin III and fibronectin.
Amino Acid Sequence	Usually cleavage fragments plus sequencing	Sequence important in structure and function.
3-D Structure	X-ray diffraction	Complete stereo-chemistry of tertiary or quaternary structure.
Enzymatic or Specific Biochemical Activity	Substrate turnover, antibody binding, ligand binding	Is protein (enzyme) functional? Does it bind its specific ligands?

For a complete adsorption analysis, the amino acid sequence and secondary, tertiary, and quaternary structure of the protein should be known (Table 2). This is usually only available if a complete X-ray diffraction analysis has been done. Some information on ligand-binding characteristics of the protein should be available: Does is bind low molecular weight solutes? Does it have specific macromolecular binding characteristics, etc.? For example, the physical properties of albumin are different depending on its fatty acid and bilirubin composition. The properties of hemoglobin are different if its heme ligand is in the deoxy or oxy form or in the met (Fe^{+3}) form [9]. The activity of many proteins is enhanced or potentiated when bound to appropriate ligands. For example, heparin binding to antithrombin III greatly increases the binding of thrombin. All such information is useful in predicting and interpreting protein interactions with surfaces.

The general solubility characteristics of the protein, including its behavior in different pH and ionic strength environments, its behavior in urea solutions, its behavior

Table 3. Major Non-Covalent Forces and Interactions Important in the Organization and Stabilization of Protein Structure in Aqueous Solutions [8,16,17]

Interaction	Description	Directional?	Effect of Increasing		Binding energy (kcal/mol)	Free energy change $H_2O \rightarrow EtOH$ (kcal/mol)
			Temperature	Ionic Strength or Dielectric Constant		
Ionic	Coulombic interaction due to opposite charges.	None — spherical symmetry	Decreases	Decreases	−5	−1
H-bonding	A donor-acceptor effect involving the H-atom of the donor — essentially an ionic interaction. Water participates in H-bonds.	Yes, linear bond lengths ~3 Å; bond energies ~ 3 to 6 kcal/mol	Decreases	Decreases	$-COO^-\cdots N^+H_3-$ −3 $-NH\cdots O=C-$	
Charge-Transfer (Donor-Acceptor)	In water this is mainly due to π — π interactions. Trp interaction strongest as it is strong electron donor.	Yes — analogous to H-bonding	Decreases	Increases		
Hydrophobic or Entropic	Water cannot interact with apolar groups via H-bonding, resulting in water adjacent to such apolar groups being more organized or more ordered. If such apolar groups come together, some ordered water is released, increasing entropy — hence apolar groups tend to "interact." Hydrophobic interactions are particularly strong in aqueous solutions.	None	Increases	Increases; effect is ion-specific		−2.4 (phe)

in solutions containing small amounts of methanol, ethanol, or glycerol, and related information on solution characteristics are all important in interpreting and predicting interactions at interfaces.

The fundamental classes of forces and interactions important in protein structure and function are summarized in Table 3. The four general classes are: (1) ionic or electrostatic interaction, due to the attraction or repulsion of two or more groups carrying a net charge; (2) hydrophobic interaction, a largely entropically driven process due primarily to water structure effects adjacent to hydrophobic interfaces; (3) hydrogen bonding, a unique type of dipole/dipole interaction which results in interaction energies comparable to very weak covalent bonds; (4) and other interactions, primarily those of a charge-transfer or partial electron donor/acceptor type, which in aqueous solutions are often dominated by π — π interactions. It is important to point out that all of these interactions take place in an aqueous medium, and that the hydrogen bonding and dielectric properties of water play a very major role (see Ref. [8,16,17] for details).

There has been considerable effort on the prediction of secondary and tertiary structures of protein from the amino acid sequence using computeraided minimal potential energy calculations [8]. The question as to how a primary amino acid sequence begins to produce secondary and super-secondary structures and fold into its equilibrium tertiary structure and functional domains is a very active field of structural biochemistry. A related problem is the mechanism by which a protein unfolds or denatures [18–20] which is of fundamental interest in the protein adsorption process.

A polypeptide chain in water has a specific pattern of polar and non-polar groups. There is a tendency to minimize the surface area between non-polar groups and the aqueous phase. This can be treated in terms of a surface-free energy argument or in terms of the classical hydrophobic interaction argument. The non-polar groups tend to be excluded from the surface of the polypeptide globule with the highly polar and charged amino acids on the surface interacting strongly with water. There are some regions on the surface which are non-polar, and there are some regions in the interior which contain polar groups, either through hydrogen bonding or salt bridge associations. About 90% of all internal polar groups form hydrogen bonds. The solvent entropy (hydrophobic interaction) effect contributes significantly to overall protein structural stability. The intramolecular interactions are highly efficient and effective.

Disulfide bonds confer additional stability. A frequently encountered structural component is the sequence —Cys—Cys— with both residues forming disulfide bonds with other cystines. This is a useful architectural unit and forms the basis for linking three different chain segments in close proximity. This structure is found in serum albumin.

The interior of the protein has a very high packing density, comparable to that in the protein crystal. The average packing density of a protein is about 0.75, which is incredibly high when one considers that a close-packed structure of spheres has a packing density of 0.74. Thus the packing density of proteins in terms of volume utilization is even more efficient than most close packed metals [8]. One often hears the criticism that the structure of a protein in the crystal must be very different than in the solution. This is not the case, as the solution packing of a protein is essentially identical to that in the crystal. It is important to note that active centers in reactive sites of proteins tend to be much more loosely packed than the rest of the molecule,

thereby providing the increased flexibility required for chemical reactions and recognition processes.

Careful study of the structure of available proteins suggests regions of amino acid homology and structural homology in very different protein molecules. This has led to the concept of the structural and functional domain. One can consider large globular proteins as being made up of a set of functional domains, a more important structural concept than the chain composition of the protein. Active sites are often located at the interface between two structural domains. One can, therefore, consider proteins as being modular structures with the structural or functional domains as the modules. A good example is the immunoglobulin molecule shown in Fig. 1 (an IgG).

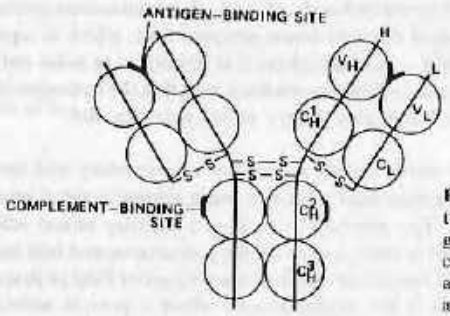


Fig. 1. Structure of an IgG molecule showing the light (L) and heavy (H) chains, the antigen-binding sites (in the Fab regions), the complement-binding site (in the Fc region), and the constant (C) and variable (V) amino acid regions (from Ref. [8])

There are both functional and structural domains. A functional domain may consist of one or more structural domains. A functional domain is a functionally autonomous region of the molecule. In the case of the IgG, the Fab portion is the functional antigen recognition domain while the Fc region is an effector domain. Structural domains are geometrically separate.

Knowing the binding characteristics of the various domains in the molecule allows the investigator to begin to deduce certain types of adsorption behavior. For example, it is known that human plasma fibronectin contains several heparin binding sites, with pK's in the 8–9 range and with a high, positive charge density. It would be reasonable to suspect that when fibronectin interacts with negatively-charged surfaces it may do so through such binding sites, just as it does with heparin.

The large globular proteins of blood plasma are monomers — that is, they consist of only one chain or a set of covalently linked chains. An oligomer is an aggregate of monomers whereby the aggregate is held together by non-covalent bonds. Most intracellular proteins are oligomers; proteins in blood plasma are large monomers, generally consisting of several funtional domains.

A structural domain can be defined as a region of locally high electron density separated by regions of low electron density or clefts in the electron density map. Amino acid residues that are far apart along the chain tend to be far apart in the three-dimensional structure. The concept of neighborhood correlation suggests, perhaps based on kinetic folding considerations, that those amino acids which are close together in a sequence tend to stay close together in the three-dimensional structure. Such neighborhood correlation is probably a consequence of the chain-folding process.

The domains are probably those pieces of a chain which fold independently. Most globular proteins have domains of 100–150 residues and are roughly 25 Å in diameter.

Five different classes of structural domains have been identified, based on the secondary structural makeup [8]. Structural symmetry is often evident, particularly in aggregate or oligomeric proteins. The more contacts, the more stable the interaction.

Depending on the fit, the number and strength of contacts, and other factors, a monomer-oligomer equilibrium will exist. For example, in the case of Concanavalin A the tetramer is in equilibrium with dimer and monomer under normal conditions. In the case of insulin the monomer, dimer, and hexamer are all in equilibrium. In hemoglobin the dimer and tetramer are in equilibrium. In the case of the adsorption of such proteins, one must not only know what is the aggregation state of the protein in solution, but must be able to deduce the adsorbed state. Do the molecules adsorb as dimers [21]? Do they adsorb as dimers and then associate as tetramers or hexamers on the surface, etc.?

Some of the free-energy considerations used to probe protein oligimerization and aggregation are also applicable to adsorption. Protein-protein interactions usually involve surface complimentarity, which can provide significant specificity. The specificity of a surface is determined by its shape, its pattern of hydrogen-bond donors and acceptors, its pattern of charges, and even its pattern of hydrophobic patches. Based on immuno globulin studies, a 100 Å^2 surface can form on the order of a thousand patterns showing different binding specificities [8].

An important question is how the linear, amino acid sequence spontaneously folds into the three-dimensional structure of the native form. There has been considerable work on the folding process, as well as on the unfolding or denaturation process. It is possible to take a protein in its native form, and by subtly changing its microenvironment (pH, ionic strength, temperature, addition of denaturants such as urea, addition of other ions, etc.), induce the protein to undergo a conformational change. The energetics of that change can be measured. In a number of such studies, summarized briefly in Table 4, one can conclude that the native state is only marginally stable and is in equilibrium with a variety of other states under normal conditions. One can write the equilibrium equation N ⇆ D. The total free energy change in going from the

Table 4. Estimates of the stability of globular proteins under physiological conditions*

Protein	Denaturant	$\Delta G_D^{H_2O}$ (kcal/mol)
Lysozyme	GdnHCl or Urea	9.5
	T-pH	10.0
Ribonuclease	GdnHCl or Urea	8.5
	T-pH	6.0
Myoglobin	pH	14.0
α-Chymotrypsin	T-pH	14.0
Immunoglobulin light chain (Wes) domain	GdnHCl	5.5

* (from Ref. [20], p. 38)

native (N) state to one of the available denatured (D) states is only in the vicinity of 10 kcal/mole for an average globular protein of 150 residues [20]. This is an energy corresponding to only two to five hydrogen bonds per molecule! It is clear that protein conformations, therefore, are very fragile and truly only marginally stable. For example, the burying of just one surface tryptophan residue provides an energy of 3.4 kcal/mole. Thus, when a protein finds itself in the vicinity of an interface, its microenvironment is altered, and clearly its equilibrium will be altered, probably altering the protein conformation in the adsorbed state.

In vitro folding and denaturation studies have established that the process occurs in the time span of 0.1 to 100 seconds. It is commonly assumed that there exist folding nuclei — that is local regions of alpha helices which are stable and which function as nuclei for additional folding or organization [8]. This provides a domain, therefore, with strong local neighborhood correlation. It is expected that there are various folding pathways and intermediate structures which are used during the folding process. Some of the theoretical methods which are now being applied to the simulation of folding processes may, in modified form, have application to the simulation of the adsorption process.

A protein and its associated water molecules constitute an extremely small thermodynamic system subjected to the continual Brownian motion or buffeting of the solvent molecules [22]. Such transient changes result in fluctuations in the local thermodynamic properties of the system. A protein with a molecular weight of 25000 experiences internal energy fluctuations of about 40 kcal/mole [22]. This is much larger than the energy changes involved in ligand-induced conformational transitions. These thermal fluctuations can result in volume changes which provide transitory cavities or channels in the protein. These dynamic fluctuations are a consequence of the thermodynamic uncertainty of small systems. They exist within a single macromolecule and are not correlated with similar fluctuations in other molecules. It is clear from these thermodynamic fluctuations that conformational mobility must be expected. It is now known from NMR studies that even such large and rigid structures as the planar aromatic amino acid side-chains can flip in and out of the protein surface [23].

Proteins are therefore dynamic, flexible objects whose physical and chemical properties are dominated, not only by their conformation, but also by the continual changes in conformation which are a consequence of their microscopic size [22]. The marginal stability of most protein conformations suggests that processes at one point in the protein might well have an effect on a portion of the molecule far removed.

The process of ligand binding to a protein may also involve conformational change. The process might be viewed as follows [22]:

Freely diffusing ligand approaches the protein. Assume that the ligand binding site is not a rigid structural feature, but involves transitory conformations. If the protein is in a transitory "open" conformation, the ligand may diffuse in and bind. The binding interactions may now stabilize a conformational state which is optimum for ligand-protein interaction, a state which previously, and in the absence of ligands, would be unstable.

One can envision a conformational fluctuation spectrum with the native state being a mean conformation. Since that mean conformation is only marginally stable, it is clear that any local microenvironmental change, such as a solid surface, may well stabilize other conformations.

In order to begin to understand and appreciate how a protein may interact with a surface, it is important to be able to "see" the protein in three dimensions. This usually requires the use of models which, in the case of proteins, are terribly cumbersome and expensive. In many institutions molecular models have been replaced by three-dimensional molecular computer graphics. There are some 70 major molecular graphics installations throughout the world which have the capabilities of imaging large macromolecular structures in three dimensions.

Ideally, one should be able to predict a three-dimensional structure from the amino acid sequence. There has been considerable activity along these lines. Current methods can predict the structural class of a protein or domain. Although about two-thirds of all residues in a polypeptide chain can be assigned to the correct secondary structure, the three-dimensional structure cannot be predicted.

If one independently knows the three-dimensional structure, for example, from X-ray crystallography, i.e., all of the atoms are identified and their atomic coordinates known, then this information can be fed into a computer and the three-dimensional structure displayed. Such coordinates and related information are available for over a hundred proteins from the Protein Data Bank (Brookhaven National Laboratory, Brookhaven, New York). The structure can be displayed as a wire or stick model or as a space-filled model. With space-filled models, one sees only the outer surface of the protein. Using one of a number of algorithms for defining a protein surface [27] and by color coding the different amino acid residues or atom types present on the surface, one can quickly "see" which regions of the molecule are hydrophobic, positively or negatively charged, non-charged but polar, and other features.

Feldmann and others have developed a series of teaching tools for macromolecular structure using color-coded molecular graphics-derived images [24–28]. Connolly and others have improved upon this approach by clearly showing which portions of the protein surface are indeed accessible to a water molecule, as opposed to those portions which are inaccessible, such as in clefts, etc [27]. Images based on the Connolly methods directly show those regions of the protein surface which can be expected to interact with other molecules. It provides immediate, comprehensible information about steric complimentarity.

Another major advantage of molecular graphics is that it allows real time manipulation of several interacting molecules while quantitatively monitoring the stereochemistry and even the interaction energies. These methods are only beginning to be used for studies of interactions between molecules and only in the most preliminary way for the simulation of protein adsorption [29].

It is recommended that any reader seriously interested in protein adsorption obtain *Teaching Aids for Macromolecular Structures* [28], which is commercially available for about $20.00. These aids clearly show the dramatic potential of surface protein structural visualization for the development of hypotheses of protein-surface interactions.

How can all this protein information help our understanding of the adsorption process? Figure 2 is a schematic and idealized view of a single protein interacting with a single, well-characterized solid surface. Assume that the solid surface is well-characterized [30]. Presume that we know precisely the location of hydrophobic regions

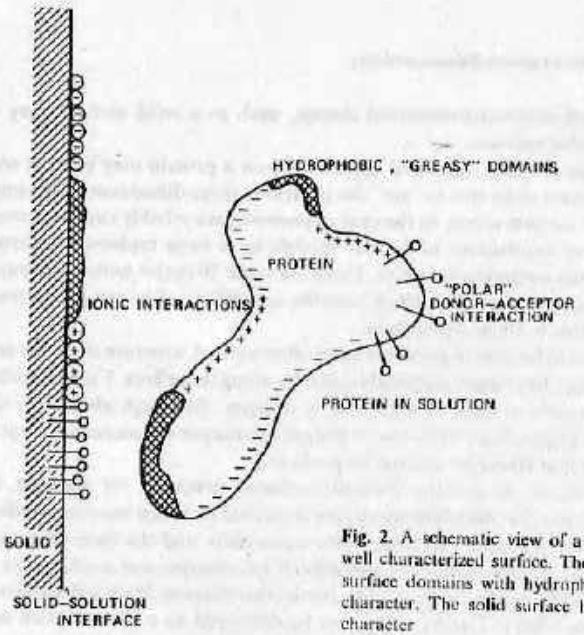


Fig. 2. A schematic view of a protein interacting with a well characterized surface. The protein has a number of surface domains with hydrophobic, charged, and polar character. The solid surface has a similar domain-like character

on the surface, the location of positively and negatively charged regions, neutral hydrophilic regions, etc.; i.e., the solid surface properties are thoroughly mapped on a submicroscopic level. We also presume that we have comparable information for the protein; we know its three-dimensional surface structure, the distribution of functional groups, and related properties. We now begin to allow the protein to approach the solid surface (by molecular graphics?) and sample the variety of potential interaction orientations. Clearly, one or more of these interaction orientations will tend to dominate on certain areas of the surface, depending on the interactions present.

It is clear, given the protein and solid surface in Fig. 2, that the protein can interact with the solid surface in a variety of different ways, depending on the particular orientation by which it approaches and the overall binding energetics. Such mental gymnastics, aided by three-dimensional graphics and at least crude estimates of interaction-free energies, should aid in the formulation of definitive hypotheses and in the explanation of data [29].

3 Mass Transport

All adsorption and desorption processes depend on transport of solute to and from the interface. There are basically four major transport mechanisms (Fig. 3):

a) diffusion;
b) thermal convection;

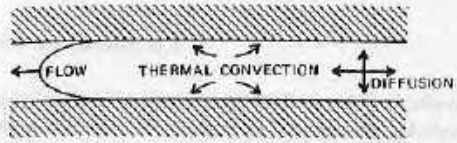


Fig. 3. The parabolic flow profile in a thin wall channel. In addition to flow, mass transport can occur by molecular diffusion and by thermal convention

c) flow (commonly called convective transport);
d) coupled transport, such as convective-diffusion processes.

Turbulent or stirred solutions may incorporate all of the processes noted.

Assume that a freshly prepared interface, such as an air/solution interface in a Langmuir trough, is optimally thermostated to minimize temperature gradients and thus minimize thermal convection. Assume there is no energy barrier to adsorption during the initial stages — every molecule which hits the interface sticks and adsorbs. All molecules near the interface will be rapidly adsorbed, resulting in a depletion of solute in the volume adjacent to the interface, called the sublayer. This concentration gradient drives diffusion from the bulk of the solution towards the depleted sublayer. Under these conditions, the rate of adsorption is equal to the rate of diffusion, dn/dt [31]:

$$\frac{dn}{dt} = C_0 \left(\frac{D}{\pi t}\right)^{1/2} \tag{1}$$

where n = number of molecules;
C_0 = bulk solution concentration;
D = diffusion coefficient;
t = time.

Thus the *rate* of adsorption is proportional to $D^{1/2}$ and $t^{-1/2}$ (Fig. 4).

Integrating gives the total number of molecules, n, adsorbed at the elapsed time (t):

$$n = 2C_0 \left(\frac{Dt}{\pi}\right)^{1/2} \tag{2}$$

The total surface concentration at time, t, is proportional to $D^{1/2}$ and $t^{1/2}$ (Fig. 4). D for proteins is in the range of 10^{-6} to 10^{-7} cm^2/s.

All fluid interfaces contain an undisturbed layer of solution adjacent to the interface. Mass transport in this boundary layer occurs only by diffusion. The thickness of the boundary layer depends on temperature, stirring, and the interface itself. It is up to 0.1 cm thick in unstirred systems and approaches 10^{-3} cm in vigorously stirred systems [5, 31, 32].

Once the interface is partially saturated with adsorbed solute molecules, then the rate of adsorption falls below the rate of diffusion, suggesting an energy barrier to adsorption.

Molecules at or near the interface may diffuse back into the bulk solution, particularly if the free energy of adsorption is not very high. Mass transport equations which account for back diffusion are available [5, 31, 32].

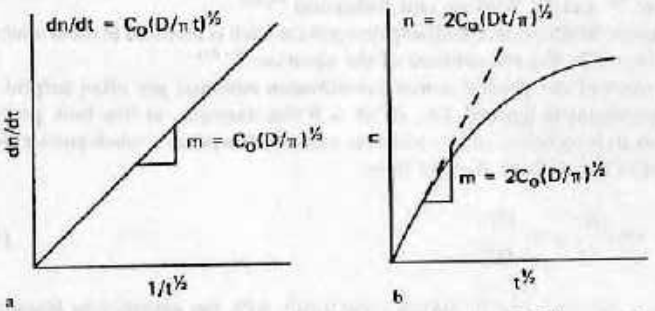


Fig. 4a and b. Plotting of diffusion-limited adsorption data (see Ref. [33]). **a.** rate of adsorption, dn/dt, is proportional to $t^{-1/2}$; **b.** the total amount adsorbed, n, in the initial stages is proportional to $t^{1/2}$

Adsorbed molecules may diffuse laterally at the interface. Although surface diffusion is well-known in classical surface chemistry [33], data on adsorbed macromolecules is sparse. Burghardt and Axelrod [34]; and Michaeli et al. [35] have both demonstrated rapid interface diffusion of adsorbed albumin.

In many situations, the system is primed with a buffer solution which is displaced by the protein solution of interest (Fig. 5a). Assuming constant, laminar established flow, the velocity (V) in a rectangular flow channel of width (w), thickness (b), and length (l), where $b \ll w$ has a characteristic parabolic profile, given by [36]

$$V = 6\bar{V}\left(\frac{y}{b} - \left(\frac{y}{b}\right)^2\right) \tag{3}$$

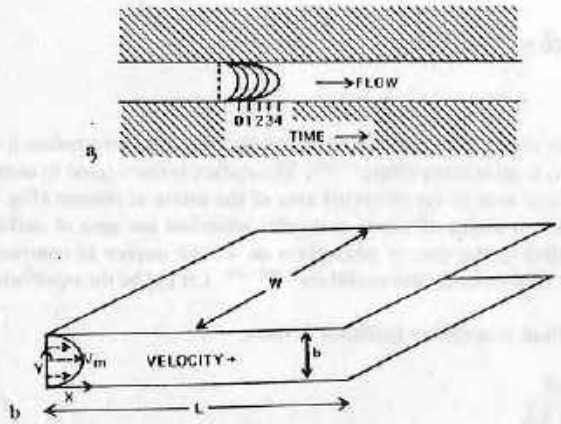


Fig. 5a. The development of the concentration profile due to a plug of protein solution entering a buffer-primed, thin plate flow channel. Note that a "bullet-shaped" concentration profile develops with time (assuming no diffusion); **b.** the geometry and coordinate system used in the convection-diffusion treatment

where $\bar{V}$ is the average velocity and y is the y coordinate (see Fig. 5b).
The volumetric flow rate, $Q = A\bar{V}$, where A is the cross sectional area of the channel, i.e., $A = b \cdot w$, therefore,

$$V = \frac{6Q}{b^2 w} y\left(1 - \frac{y}{b}\right) \tag{4}$$

It can be shown that the wall shear rate, γ, is

$$\gamma = \frac{6Q}{b^2 w} \tag{5}$$

thus

$$V = \gamma y\left(1 - \frac{y}{b}\right) \tag{6}$$

which is the equation for the parabola shown in Fig. 5.

Physically, the shear rate, γ, is the change in fluid velocity over a change in distance, dV/dy, or the velocity gradient. The units of γ are

$$\frac{\text{cm/s}}{\text{cm}} \text{ or } \text{s}^{-1}$$

The shear stress is also of interest. Shear stress (τ) for Newtonian fluids is linearly related to γ,

$$\tau = \mu\gamma \tag{7}$$

where μ is the viscosity.

Note that the velocity at the wall ($y = 0$ and $y = b$) is zero, meaning nothing moves at the wall, according to classical fluid mechanics.

Now imagine that a protein solution enters the flow chamber at time $t = 0$. The protein solution (of uniform concentration and flow velocity) begins displacing the buffer solution (Fig. 5a). Given a parabolic velocity profile, Fig. 5b shows the development of the concentration profile in the cell at various times after entrance. A "bullet-shaped" concentration profile develops. This is easily observed using a dye solution or blood. No protein reaches the surface by convective flow alone. Protein is transported to the interface by diffusion.

The flow system can be designed to produce mixing which minimizes concentration profiles. Such processes are discussed in any basic fluid mechanics or flow injection analysis text book [36, 37].

A complete treatment of mass transport to interfaces requires combining convective (flow) and diffusion processes [36–38] as the molecules present in a flowing stream are transported by flow (convection) and by diffusion simultaneously. Fortunately, this mass transport problem is well-treated [38], particularly in the chemical engineering literature. Robertson [39, 40] and Leonard [41] are chemical engineers who have been

very productive in the area of protein adsorption – they and their students have presented and used convective diffusion treatments extensively.

For the flow direction and coordinate system given in Fig. 5b, and assuming diffusion perpendicular to the solid-liquid interface, the general convective diffusion equation in Cartesian coordinates is [38, 40]:

$$\frac{\partial C}{\partial t} + V(y)\frac{\partial C}{\partial x} = D\frac{\partial^2 C}{\partial y^2} \tag{8}$$

where

$$V(y) = \gamma y\left(1 - \frac{y}{b}\right) \tag{9}$$

C = concentration,
t = time,
D = diffusion coefficient.

The origin of the coordinate system is the entrance to the flow chamber (far left of Fig. 5b). Assuming there is no adsorption at the interface, the boundary conditions are [40, 42]:

at $t = 0$, $C = 0$ for all y, $x > 0$;
at $x = 0$, $C = C_0$ for all y, $t > 0$;
at $y = b/z$, $C = 0$ for $t < x/Vm$;
$C = C_0$ for $t \geq x/Vm$;
at $y = 0$, $\partial C/\partial y = 0$ for all $x > 0$, $t > 0$.

The equation can be solved using various numerical methods.

As the protein solution moves into and through the rectangular flow channel, a parabolic concentration profile develops (Fig. 5a). Diffusion washes out the flow profile, eventually equalizing the concentration at C_0.

In the absence of diffusion, the injected material would never reach the wall due to the laminar nature of the flow. In the presence of diffusion, the concentration at the wall rises with time in an S-curve fashion. Lok generated a dimensionless equation which defines the time course of diffusion [40]:

$$\tau = t\gamma^{2/3}\frac{D^{1/3}}{l^{2/3}} \tag{10}$$

where l is the distance from the entrance to the midpoint of the flow chamber and where $\tau = 3$ to reach a plateau value (i.e. the concentration at the wall is C_0). Solving for t, we find that

$$t = \tau\frac{l^{2/3}}{\gamma^{2/3}D^{1/3}} \tag{11}$$

Using the typical values $D = 4.0 \times 10^{-7}$ cm^2/s for gamma globulin, $l = 2.5$ cm, $\gamma = 47.4$ s^{-1} for a volume flow rate of 2.0 ml/min and $\tau = 3$ to reach equilibrium,

the diffusion time is 57 seconds from the time the newly injected fluid enters the cell.

The study of adsorption or desorption kinetics in the first minute or so must take full account of convection-diffusion processes. Fortunately this has been well-modeled by Lok, et al. [40] and by Watkins and Robertson [39, 42].

In any adsorption situation, the adsorption process itself is involved in the boundary conditions, thus affecting the solution of the equation [39–41].

Limiting cases of the general convective-diffusion equation are often helpful. If the time dependence is ignored, i.e., $\partial C/\partial t = 0$ (for example, at low bulk protein concentration, at long times, and/or when the rate of adsorption is much greater than the transport to the surface), then we have

$$V(y)\frac{\partial C}{\partial x} = D\frac{\partial^2 C}{\partial y^2} \tag{12}$$

This simpler expression can be solved numerically with the appropriate boundary conditions. This is the so-called transport limited or Leveque case [38, 41].

Another limiting case is when $V = 0$ (no flow), then we get the common diffusion expression

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial y^2} \tag{13}$$

Techniques which allow one to monitor the boundary layer as a function of time, such as total internal reflection fluorescence (TIRF) spectroscopy [4, 43], permit a quantitative evaluation of interfacial mass transport processes using, for example, fluorescently-tagged macromolecules which do not adsorb, such as fluorescein-labeled dextran [40].

Vroman [44] has recently demonstrated the great importance of volume and concentration in limiting interfacial transport and thus in influencing the adsorption process (see also Sect. 4.6).

4 Protein Adsorption Principles

4.1 Background

The classical Langmuir theory for gas adsorption can be applied to adsorption from solution, if the solution is sufficiently dilute [45, 46]. The surface is considered to consist of sites of about the same area as the projected area of the solute of interest (Fig. 6).

Let $\bar{\nu}_s$ be the number or moles of solute molecules adsorbed per area of surface. The subscript on $\bar{\nu}$ refers to the case of adsorption on a *solid surface* as contrasted with $\bar{\nu}$ used in the case of protein-ligand equilibria [4, 47–49]. Let [A] be the equilibrium solute concentration.

The classical Langmuir adsorption isotherm is then

$$\bar{\nu}_s = \frac{kA}{1 + kA} \tag{14}$$

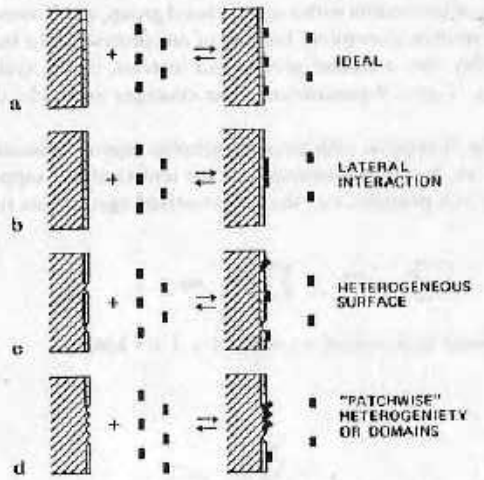


Fig. 6a–d. Schematic view of adsorption from solution onto smooth, planar surfaces where the surface "sites" are considered to have the same area as the projected area of the solute of interest. a. Top, the ideal (Langmuir) case; b. clustering of adsorbed solute due to attractive lateral interactions or positive cooperativity; c. heterogeneous surface, i.e., two sets of binding sites; d. "patchwise heterogeneity" or surface domains of different adsorptive properties

which assumes:

1. only one molecule can be adsorbed per site (commonly called the monolayer assumption);
2. only one type of site is present (homogeneous surface);
3. the adsorption of one molecule does not affect the adsorption energy of other molecules (no lateral interactions or cooperativity);
4. only one adsorbing species is present (no competitive adsorption);
5. dilute solution; and
6. reversible adsorption.

These assumptions are identical to those in the ideal theory of multiple equilibria used to analyze low molecular weight ligand binding to proteins [4, 47–49]. Figure 7 shows how these assumptions can be considered in more refined treatments of adsorption from solution [45].

The case of the heterogeneous surface (Fig. 6) is treated as

$$\bar{\nu}_s = \sum_i \left(\frac{k_i A}{1 + k_i A} \right) \qquad (15)$$

The case of patchwise heterogeneity versus "homogeneous" heterogeneity cannot be distinguished by adsorption data using low molecular weight solutes [45]. The case of lateral interactions can also be treated using assumed functions for the lateral interaction energy (Figs. 6 and 7) [45].

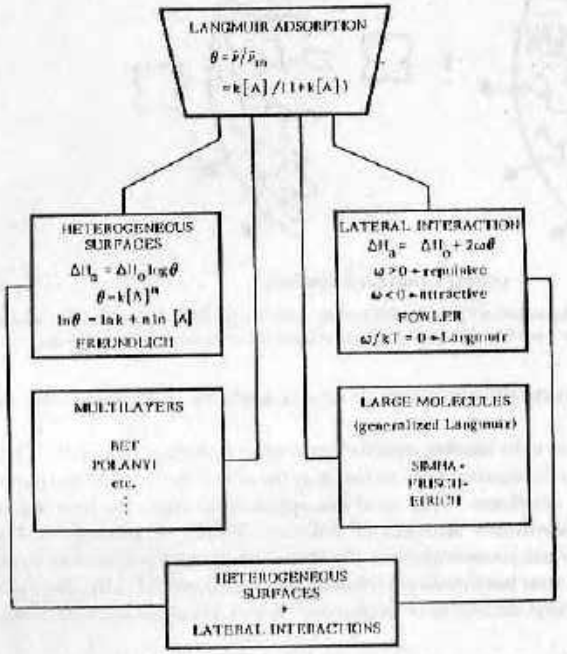


Fig. 7. The relationships between the different models of adsorption. The various assumptions involved in the classical Langmuir treatment can be considered explicitly in other, more complex models (see Ref. [45] for further details)

The adsorption theories and protein-ligand equilibria theories are formally identical. If one simply treats the protein molecule as a heterogeneous surface (two or more classes of binding sites), interacting with identical low molecular weight ligands, the equations are identical. The assumptions involved are generally identical — for example, one ligand per site, which is the same as one adsorbed molecule/site or the monolayer assumption of Langmuir adsorption. Lateral interaction in the Langmuir treatment becomes cooperativity in the ligand equilibria treatment; heterogeneous surfaces are the same as two or more classes of binding sites; etc.

There is a class of adsorption or ligand equilibria data which is of particular importance and relevance to our discussion — the literature of protein chromatography. Proteins can be separated by ion exchange, hydrophobic, and charge-transfer processes on suitable chromatographic supports. Generally, a support is used which has very low interactions with protein (k is very low). A common one is cross-linked agarose. The support is then derivatized with the proper type and density of binding site (immobilized ligand). The ligand may be a carboxylic acid group, a quaternary ammonium group, a charge-transfer (electron donor or electron acceptor) group, or a hydrophobic group. We shall only consider the hydrophobic case as an example here. The underivatized agarose is a high water content gel which consists mainly

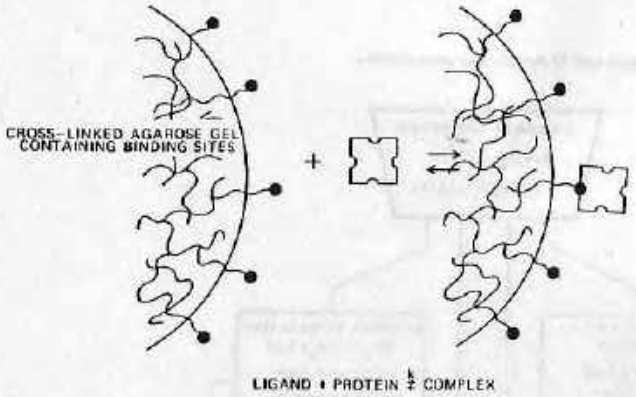


Fig. 8. Schematic of a hypothetical protein interacting with a crosslinked agarose gel containing certain bonding sites. The bonding sites on the gel are considered to be immobilized ligands

of —OH groups, which are considered to have a negligible interaction with most proteins in water.

Consider Fig. 8 where the binding equilibrium is expressed diagramatically. Clearly the binding groups on the agarose bead surface play the same role as ligand in a multiple equilibria treatment. Jennissen [50] has used this approach to study the interaction of protein with alkyl-substituted agaroses of different degrees of substitution (alkyl group densities or surface concentrations). His data [51] suggests a cooperative binding process, where about four butyl residues (binding sites) are involved in the adsorption. In the case of the methyl derivative of Sepharose[1], 6 to 7 methyl groups are required for adsorption [51].

The usual adsorption experiment consists of a fixed surface and a variable protein concentration, thus, the "ligand" concentration is constant and the protein concentration is varied. The expressions can be derived in terms of [P] rather than [A], where [P] is the equilibrium protein concentration [4]:

$$\bar{\nu} = \frac{\text{moles bound protein}}{\text{total moles ligand}} = \frac{[PA]}{[A_T] = [PA] + [A]} \tag{16}$$

For n identical, non-interacting sites

$$\bar{\nu} = \frac{nk\,[P]}{1 + k\,[P]} \tag{17}$$

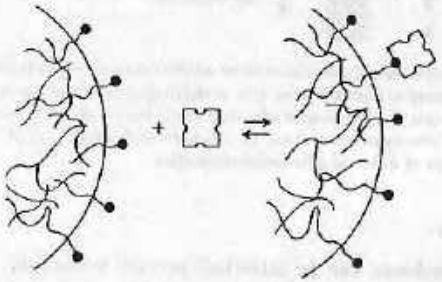
Due to steric considerations, n will generally be a lower number than in the free ligand case. For the adsorption of proteins on a solid surface, the total ligand concentration is usually represented as the actual surface area of the adsorbent. In studies utilizing cross-linked gel chromatographic adsorbents, ligand concentration is often given as moles/ml packed gel. Units of [k] are reciprocal concentration units.

[1] Sepharose is the commercial cross-linked agarose produced by Pharmacia, Inc.

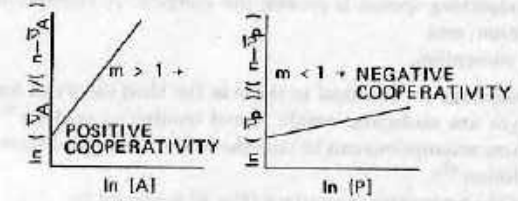
Jennissen's data [50, 51] shows that in terms of ligands bound (for example, butyl groups), the process is positively cooperative. Binding of protein to the first butyl group increases the probability of interaction with a second butyl group, etc. However, in terms of protein binding or protein adsorption, binding of one protein to the butyl groups decreases the probability that a second protein can interact, or the system exhibits negative cooperativity. Figure 9 summarizes these concepts in simple, diagrammatic form.

The adsorption or interaction of proteins with chromatographic supports discussed in the previous section made one overriding assumption: the underivatized support does not significantly interact with proteins; i.e., the underivatized agarose has such

a $\bar{\nu}_p$ = (moles bound A) ÷ (moles total protein) = (nk[A]) ÷ (1 + k[A])



b $\bar{\nu}_p$ = (moles bound P) ÷ (total moles A) = (nk[P]) ÷ (1 + k[P])



c

Fig. 9a. Top: Protein — soluble ligand classical binding equilibria and definition of $\bar{\nu}$ as moles bound ligand/moles total protein; b. Center: Protein adsorption — binding equilibria now treated in terms of soluble protein concentration; $\bar{\nu}$ now defined as moles bound protein/moles total immobilized ligand. c. Bottom: Hill plots for each case, demonstrating that positive cooperativity with respect to binding of *ligand* is negative cooperativity with respect to binding (adsorption) of protein

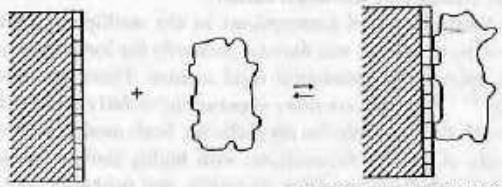


Fig. 10. Typical adsorbent surfaces can be considered to have very high binding site ("ligand") densities, resulting in multivalent interactions with adsorbed protein. If the multivalent interactions are of sufficient number and energy, the adsorptive interactions is "irreversible"

a low interaction energy per site with proteins that for all practical purposes that interaction is negligible. This is not the case with most other surfaces with which proteins come in contact. Using our earlier definition of surface site from Fig. 6, Fig. 10 schematically shows what happens when a typical surface of very high site density interacts with a protein. This is the case with hydrophobic surfaces such as, for example, polyethylene, polypropylene, polystyrene, and related biomedical polymers. One can consider every monomer unit exposed at the surface in such polymers to be a surface site interacting with the hydrophobic domains or patches on a protein by strong hydrophobic interactions. A large number of such interactions per individual protein molecule results in irreversible adsorption. This is, of course, also experienced in conventional protein adsorption chromatography, whether by hydrophobic or ionic (electrostatic) mechanisms. When the site density on the chromatographic support is too high, the protein is irreversibly bound. Sometimes this condition can be overcome by changing the elution medium. In the case of ion exchange chromatography, one would go to a higher ionic strength eluant, which would decrease the magnitude of the electrostatic interactions. Under these new ionic strength conditions, that protein would then be reversibly bound and could be eluted. The same is true of irreversible hydrophobic adsorption. If one elutes with an eluant which destroys or decreases the magnitude of the hydrophobic interaction, then what was an irreversible process is reversible in the new solution environment [52]. Most studies of protein adsorption onto solid surfaces in the literature are under irreversible or at best semi-reversible conditions.

Ideally, protein adsorption experiments should be conducted on surfaces of low binding site density so that the interaction remains reversible and can be analyzed by multiple equilibria and related models. This will then allow the estimation of the number of interaction sites and information on binding constants and interaction energies. This is not only useful for the characterization of the surface, but for the characterization of the protein itself, i.e., the accessible regions on the protein surface which are involved in the adsorption process. There are cases and conditions in which proteins can be studied on hydrophobic surfaces in a reversible fashion — for example, proteins which appear to be very weakly hydrophobic or at the bottom of the scale in hydrophobic chromatography experiments [52]. Albumin is highly hydrophobic and near the top of the hydrophobic chromatography scale and would be expected to adsorb irreversibly to most low energy surfaces. On highly hydrophilic surfaces,

such as clean, wettable quartz, it is known that some proteins adsorb in a highly reversible fashion, including albumin [53]. Here, the hydrophobic interactions are probably minimal, and the major adsorptive interaction is probably ionic.

The most useful information on the interactions of proteins with surfaces will come from studies analogous to those of protein chromatography, where well-characterized and understood proteins are studied with well-characterized surfaces of known functional group type and density. The information obtained is then analyzable in such a way as to deduce interaction site densities and interaction energies. Only with such data in hand will we be able to begin to quantitatively treat and understand protein adsorption.

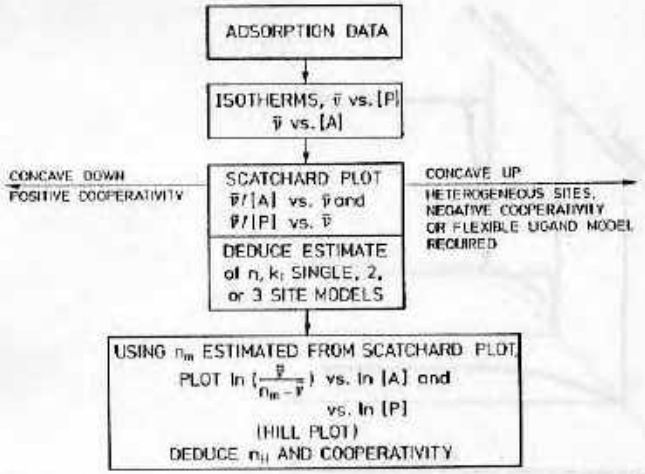


Fig. 11. Scheme for the analysis of protein adsorption data using the methods of multiple equilibria of proteins

Given the assumptions and limitations outlined in this section and elsewhere [41], conventional adsorption isotherm data can by analyzed in terms of multiple equilibria models. The general scheme is outlined in Fig. 11. First, the adsorption data is taken with sufficient care to get a wide range of points over a wide range of concentrations, preferably as both a function of protein solution concentration and immobilized ligand concentration, as outlined by Weber [54, 55]. In many cases, the ligand surface concentration will be kept constant or will be largely unknown. The adsorption isotherm is then plotted. It is then most convenient to perform a Scatchard plot [41]; if the plot is approximately linear, an estimate of n and k can be derived. If the plot has a strong concavity, then one can use the approximation techniques to derive a set of n and k for two different assumed site populations. The shape and concavity of the Scatchard plot immediately indicates several things. If it is concave-down, one can assume that this is due primarily to a positive cooperativity process. For example, the papers by Jennissen make such conclusions [50, 51]. If it is concave-up, one can assume two or more populations, i.e., heterogeneous sites. One can also assume

negative cooperativity. The concavity may be due to the fact that one is using a flexible ligand, and a flexible ligand model is needed to analyze such data [56]. However, from the Scatchard plot or from the original isotherm, one can generally obtain an estimate of the upper value for n. Using this value for n, one can perform a Hill plot, i.e. log $\bar{\nu}$/n-$\bar{\nu}$ versus log [P] [4,48,57]. From the slope of the Hill plot one can deduce some measure of cooperativity.

For many proteins and many surfaces, the adsorption will be essentially irreversible, which will result in a Scatchard plot with a shape which is not easily analyzed. The models and methods presented here are not adequate for treating such data. It is important under such conditions to redo the adsorption experiment, preferably on a modified surface of lower binding site density or energy. Figure 12 outlines in schematic form what one might expect if this is done, although no such data are at present available in the literature.

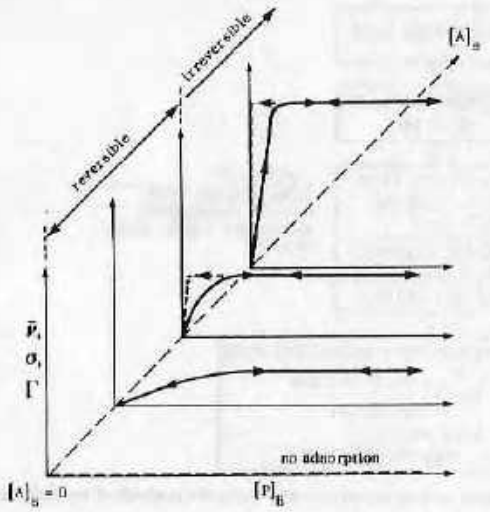


Fig. 12. Hypothetical 3-D plot of protein adsorption isotherm (Γ plotted against $[P]_a$) as a function of surface ligand concentration, $[A]_s$. Note that the system is reversible only up to a critical $[A]_s$ and then behaves irreversibly for higher ligand surface concentrations. The right arrows (→) denote adsorption; the left-facing ones (←), desorption

Assume that we have a surface — for example, a substituted agarose analogous to the chromatography supports discussed earlier — in which the ligand surface density is allowed to vary. Further assume that each of these different supports of different ligand group density is studied with respect to different protein solution concentrations, and the amount adsorbed is determined. One then has two sets of isotherms, basically $\bar{\nu}$ vs. [P], as a function of ligand group density or $\bar{\nu}$ vs. [A], i.e., ligand group density or concentration, as a function of [P]. These can be plotted in a three-dimensional form as indicated in Fig. 11 and analyzed by the methods described herein. This has been described briefly by Weber [55]. Note from Fig. 12 that we are assuming

that as the ligand group density [A] increases, the adsorption isotherm becomes irreversible, which is no longer within the domain of analysis of the models and methods presented here. However, the adsorption behavior can be shifted to a reversible domain if the solution conditions are changed as discussed earlier.

There are some inherent limitations and assumptions in the multiple equilibria treatment. One is the fact that the treatment was devised primarily for ion interactions with proteins, that is small, spherically, symmetric rigid solutes. There are models available for non-rigid solutes [55–59], which are more appropriate to fatty acid binding to proteins, particularly through the hydrophobic alkyl chains. Such models would be more appropriate to the study of protein interactions with highly mobile surfaces, such as those of elastomers and low glass transition materials, and probably even the substituted agaroses. Finally, both the rigid solute and the soft solute models [55–59] assume that the protein is conformationally rigid, although the rigid model has been treated in terms of allosteric phenomena or conformational coupling of the binding process [60]. In most cases of particular interest, the protein cannot be assumed to be conformationally rigid, and further methods, models and means of obtaining data will be required in order to interpret and understand such processes. The allosteric treatments of conventional rigid ligand-protein equilibria [60], coupled with the treatments of mobile ligand-rigid protein equilibria [56,57] should allow such problems to be approached. The methodologies and discussion in this section, therefore, are but a first step in the analysis of protein interactions at solid-liquid interfaces.

4.2 Thermodynamics

What is the driving force for protein adsorption? Is the adsorption driven by overall energetic (enthalpic) interactions or does the entropic contribution prevail? Do both entropic and enthalpic contributions play a major part in the adsorption process, the extent of each depending on the particular protein and surface in question? An illuminating thermodynamic analysis given by Norde and Lyklema [62,66] for the adsorption of two different globular proteins (human serum albumin, HSA, and bovine pancreatic ribonuclease, RNase) on polystyrene latices will be presented. We believe this analysis has general validity.

Two parts of the adsorption isotherm can be distinguished: the initial part and the plateau region. At very low protein solution concentration, the adsorbed amounts are so low that lateral interaction between protein molecules can be neglected. This part of the isotherm (or its initial slope) reflect only the interaction between protein and adsorbent. In the case of HSA adsorption on negatively-charged polystyrene latices at pH = i.e.p., the adsorption was found to be independent of temperature, T, while at pH greater or less than the i.e.p., adsorption increases with increasing temperature [68]. The positive values of $(\partial\Gamma/\partial T)$ suggest that the process is enthalpically unfavorable and is entropically driven. The more the pH differs from the i.e.p., the larger $(\partial\Gamma/\partial T)$ becomes, implying that unfolding of HSA on the surface is a process which is not favored enthalpically.

At the plateau region direct calorimetric measurements of $\Delta_{ads}H$ (adsorption enthalpy) showed that for a number of protein-surface combinations there is a part of the pH range where $\Delta_{ads}H > 0$. Since $\Delta_{ads}G < 0$ for the process to occur, $T\Delta_{ads}S$

> 0. The positive sign of the entropy change indicates that there are conditions where the process of adsorption is entropically favorable, if not driven solely by positive $\Delta_{ads}S$. For rigid proteins like RNase, unfolding is opposed by strong intramolecular enthalpic forces. If the RNase molecule cannot gain entropy by adsorption or cannot find other enthalpy sources, it can not and does not adsorb. This was found in the case of interactions between positive-charged haematite and RNase; no adsorption took place due to electrostatic repulsion [68]. Equal signs of the surface charges and the protein charge does not necessarily imply an absence of adsorption, however. It was found in a number of cases that negatively-charged HSA adsorbs on negatively-charged surfaces with its negative groups oriented toward the surface. This was due to counterions incorporated in the adsorbed protein layer between the surface and protein molecule providing an electrostatic bridging mechanism [69]. It is an important part of the adsorption process since it contributes both to $\Delta_{ads}H$ and $\Delta_{ads}S$.

Following are the contributions to enthalpy and entropy of protein adsorption that have to be considered, according to Norde and Lyklema [61,62]:

1. Hydrophobic dehydration results from bonding of the protein's hydrophobic patches to the hydrophobic regions on the adsorbent. The enthalpic part of this interaction is small; the entropy change is positive. Hydrophobic dehydration is relatively unimportant for hydrophilic surfaces and/or rigid hydrophilic proteins.
2. Overall electrostatic interactions depend on the surface charge and protein charge, both of which are usually functions of pH and solution ionic content. It is a more decisive parameter for rigid protein because of the ion-incorporation and possible conformational changes of flexible proteins.
3. Protein conformational changes contribute positively both to the enthalpy and entropy of adsorption. Such contributions are pH-dependent.
4. Ion incorporation has two important aspects: a) due to the transport of ions from solution to the adsorbed protein layer, contributions to enthalpy and entropy are usually negative; b) in the case of charge redistribution in the electrical double layer, the ion contribution to the enthalpic forces depends on the protein-surface charge difference, pH, and solution ionic content. Its entropic contribution is positive.
5. Overall, Van der Waals interactions are relatively insignificant due to the similar Hamaker constant of proteins and water.
6. Specific binding may also play an important role and its contributions to both enthalpy and entropy of the adsorption depend on the specific protein-surface combination.

Apparently, no single factor can be used to predict the process of adsorption; there are always several different properties of protein and adsorbent that determine the protein-surface interaction. As a summary, the following general guidelines can be given:

1. The affinity of a negatively-charged surface for a given protein increases if the surface is more hydrophobic or/and has smaller electrokinetic potential.
2. The affinity of protein towards a given negatively-charged surface increases the more hydrophobic the protein is and/or the smaller the amount of negatively-charged parts of protein occupy the inner region of the adsorbed protein layer.

3. Adsorption is enhanced by higher concentration and valency and smaller chaotropicity of the indifferent ions.
4. The adsorption process is often entropically driven with the gain in entropy arising from dehydration of the adsorbent surface and structural rearrangements inside the protein molecule (the state of hydration and field overlap changes inside the adsorbed protein included).

4.3 Kinetics

The kinetics of single protein adsorption have been discussed and modeled by several groups [70–73]. The models are summarized in Figs. 13a–f. These models assume the process is surface reaction limited, i.e. protein transport to the interface is not rate limiting. The nomenclature is:

C_0: bulk solution concentration [g/l]
$C_s(t)$: adsorbed surface concentration at time t [kg/m^2]
$(C_s)_\infty$: plateau surface concentration at long t [kg/m^2]
k_i: rate constants [min^{-1} or l/g min]
n_i: number of molecules in state i per unit area of surface
a_i: surface area fraction occupied by molecules in state i
n_s: total number of adsorbed molecules per unit area of surface

Figure 13a shows basic Langmuir reversible adsorption, discussed earlier.

The Lundström model [70] is given in Fig. 13b. He assumes that protein adsorbs with a rate constant k_a into State 1. Upon adsorption, some of the adsorbed proteins in State 1 (native) may conformationally change (via rate constant k_r) to State 2 (denatured). Letting n_1 and n_2 be the number of molecules per unit area in States 1 and 2, and a_1 and a_2 be the area fractions occupied in each state, he says (noting that the unoccupied area fraction $= (1 - a_1n_1 - a_2n_2)$ that [70]:

$$\frac{dn_1}{dt} = (k_aC_0 - k_rn_1)(1 - a_1n_1 - a_2n_2) \tag{18}$$

The build-up of proteins in State 2 is given by

$$\frac{dn_2}{dt} = k_rn_1(1 - a_1n_1 - a_2n_2) \tag{19}$$

Lundström shows that [70]:

$$n_2 = \frac{k_aC_0}{k_r}\ln\left(\frac{k_aC_0}{k_aC_0 - k_rn_1}\right) - n_1 \tag{20}$$

He goes on to determine relations for a_2/a_1 at steady state (assuming monolayer coverage) and for $n_1 + n_2$, given experimentally by radioisotope or ellipsometry studies.

The Lundström model allowing for desorption is given in Fig. 13c, although it has not been treated in the literature.

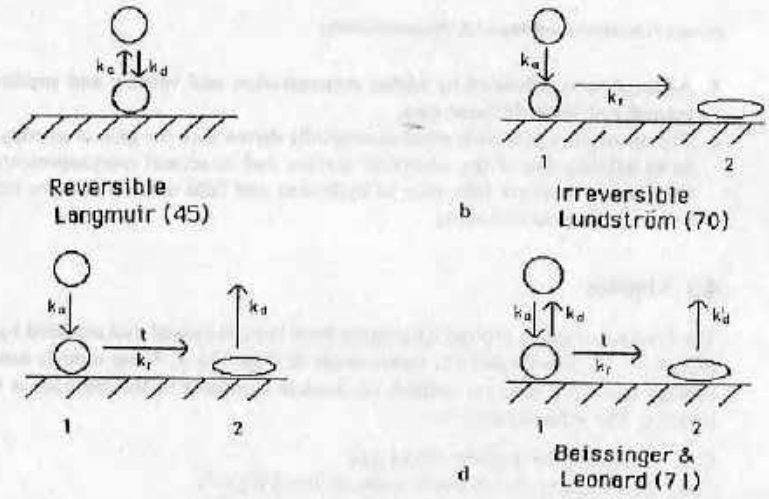


Fig. 13. Most of the kinetic models which might be applicable to protein adsorption (see Refs. [70–73]); k is rate constant, subscript a and d are adsorption and desorption respectively, 1 and 2 are adsorption states — usually native and denatured

Beissinger's and Leonard's model (Fig. 13d) accounts for desorption of both native and denatured adsorbed species (States 1 and 2, respectively). They used the classical Langmuir-Hinshelwood model for catalytic reactions (the surface is the catalyst for conformational change or denaturation of the adsorbed protein), which assumes equilibrium at a steady state between adsorbed and solution molecules. They show:

$$\frac{da_1}{dt} = C_0 k_a(1 - a_1 - a_2) - a_1 k_r(1 - a_1 - a_2) - a_1 k_d \tag{21}$$

$$\frac{da_2}{dt} = a_1 k_r(1 - a_1 - a_2) - k'_d a_2 \tag{22}$$

$$n_s = n_1 + n_2 \tag{23}$$

The equations were best fit to albumin on quartz adsorption data by a nonlinear regression method to obtain values of k_a, k_d, k'_d, k_r, and n_s. They then extended the model to consider the competitive adsorption of two proteins [71].

Soderquist and Walton [72] added time dependence of the surface reaction to the model. They also allowed for readsorption of desorbed material (Fig. 13e). This model in principle takes into account the time dependence of the conformational change of adsorbed protein. They consider three distinct processes or states:

1. Rapid and reversible adsorption reaching a pseudo-equilibrium during the first minute;
2. Surface-induced change in conformation which optimizes protein-surface interaction and decreases the probability for desorption;
3. At $t \to \infty$, adsorbed material is fully denatured and if desorbed can not readsorb; desorption from State 3 is irreversible.

Process 1: Rate of adsorption, $\frac{dn_1}{dt}$, is proportional to solution concentration, C_0, but desorbed material readsorbs at a different rate.

$$R_{ads} = (k_a C_0(t_0) + k'_a C_1(t_1) + \ldots) A = A \Sigma k_a^{(i)} C_i(t_i) \tag{24}$$

where i refers to solution species which had been on surface for t_i; A is available surface area; k'_a is the rate constant for species i.

Process 2 is difficult to model.

Process 3: The desorption rate is much slower, particularly after long contact times. They assume that fully denatured protein does *not* desorb.

$$R_{des} = \Sigma k'_d (C_s)_i (t_i) \tag{25}$$

where $(C_s)_i$ is the adsorbed concentration which has resided for a period t_i on the

surface. If we consider material incubated for a definite time, t, and all desorbed material is continuously removed, then

$$R_{des} = k_d C_s(t) \tag{26}$$

which can be written in the form

$$R_{des} = k_d C_s(1 + k_r t^{n-1}) \tag{27}$$

where k_r is the rate constant for conformational change of the adsorbed protein.

Combining (24) and (27) by noting that at steady state $R_{ad} = R_{des}$, they get

$$A \Sigma k_a^{(i)} C_i(t_i) = k_d C_s - (1 + k_r t^{n-1}) \tag{28}$$

at $t \to 0$, this reduces to the Langmuir equation for reversible adsorption; as $t \to \infty$, the process becomes fully irreversible (see Fig. 19 which will be discussed later). The model reconciles essentially all of the physical evidence according to Soderquist and Walton [72], although several of its assumptions will be questioned later.

Sevastianov et al. [73, 74] have developed a model which considers the effect of surface heterogeniety on the adsorption process. They define "centers of irreversible adsorption", labeled P, and "centers of irreversible desorption", labeled D. They argue, in agreement with Soderquist and Walton, that desorbed material is conformationally altered and thus cannot readsorb — hence desorption is irreversible. The results of this model are given as Fig. 14, taken from Ref. [73]. The model also includes the case where adsorption may be transport limited. The model fits commonly observed adsorption data, including the "overshoot" phenomenon (Fig. 14, top) (discussed in Ref. [72]) to be discussed later.

Although these models are ambitious, complex, and tend to fit experimental data

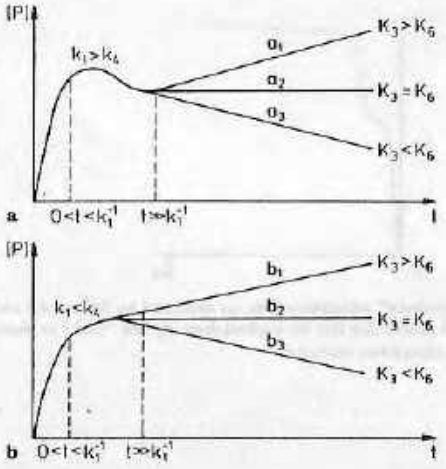


Fig. 14a and b. Results of the Sevastianov, et al. kinetic model for protein adsorption [73, 74]: k_1 is an adsorption rate constant [s^{-1}]; k_2, k_4, k_5 are "velocity" constants in [M/s]; and K_3 and K_6 are transport constants in [kg/M^2-s]. Curves a_1, a_2, a_3 (top) are for $k_1 > k_4$, $C_1 > 0$, $C_2 < 0$; curves b_1, b_2, b_3 (bottom) are for $k_1 < k_4$ and for C_1, $C_2 > 0$ (from Ref. [73])

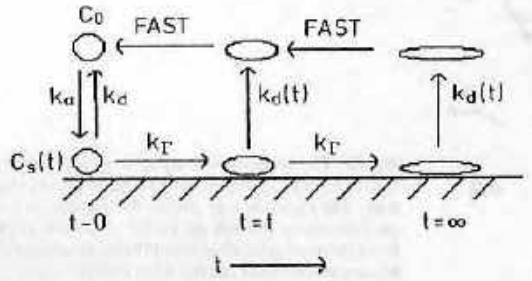


Fig. 15. Suggested general kinetic model for protein adsorption in the absence of any covalent bond formation or disruption. Any protein desorbed in a denatured state is assumed to rapidly renature in solution. If the surface is heterogeneous, then two or more such scenarios can be formulated, with appropriate account of the area fractions of each type of surface present

reasonably well, in many cases, some of the assumptions are not reasonable. Although proteins do indeed undergo time-dependent conformational changes on a surface, if those proteins desorb, they probably "renature" in solution, becoming indistinguishable from proteins in bulk solution. Protein denaturation is generally reversible *unless* covalent bonds have been formed or disrupted, which could happen if the surface is catalytically active or if proteolytic enzyme activity is present.

Therefore, a more general kinetic model is suggested in Fig. 15, based on the pioneering studies reviewed in Fig. 13. This is essentially a simplified form of the Soderquist and Walton [72] treatment, except we have assumed that any protein desorbed in a denatured state rapidly renatures in solution. This model could be combined with two or more classes of surface sites (heterogeneous surfaces) to treat more complex materials. To our knowledge, no modeling or data fitting to such a model is presently available.

4.4 Conformation Effects

We have already established that the protein adsorption process may result in significant conformational changes. In addition to adsorbed amounts and rates, the orientation and conformation of the adsorbed protein are critical (Fig. 16). Conformation refers to the secondary (α-helix, β-sheet), tertiary, and quaternary structures.

Certain orientations may make a specific site on the protein inaccessible to ligand, substrate, or antigen. For example, consider the adsorptive immobilization of a specific IgG for a solid-phase immunoassay. The procedure will be optimal if the Fab domains are free to bind antigen (Figs. 1 and 16).

If the protein's structure is changed due to the adsorption process and/or the new local micro-environment, then it is said to be fully or partially "denatured", meaning that its properties are no longer those of the native protein. Conformational changes can occur due to ligand binding (for example, hemoglobin), substrate binding (many enzymes), heparin binding (antithrombin III), and surface binding (Hageman Factor, fibronectin, albumin, etc.). Although there have been many hypotheses relating to protein adsorption conformational effects, there is little direct data available.

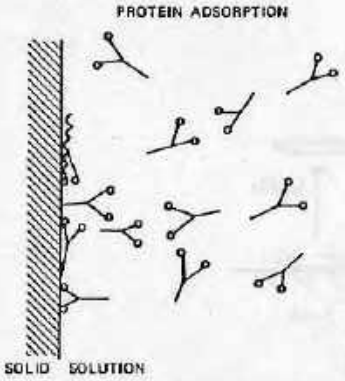


Fig. 16. The adsorption of an IgG molecule (shown for convenience as Y-shaped) may result in several orientations. The molecule may adsorb through one or both of the Fab ends or through the Fc tail – or lie on its "side". In addition to such *orientation* effects, the molecule may be conformationally altered or *denatured*

The surface itself may also be conformationally altered or "denatured", due to its interaction with the protein, particularly polymer surfaces which tend to relax or change in response to new environments [30].

We have already established that proteins are highly dynamic structures, which are constantly "sampling" different conformations due to local thermodynamic fluctuations. Some such transient conformations can be stabilized in new micro-environments, such as at a solid-liquid interface.

Many studies of proteins at air-solution interfaces have indirectly established that the adsorbed proteins undergo detectable conformational changes. Similar studies at solid-liquid interfaces are few. We review here only several key studies.

Morrissey [53] used transmission infrared spectroscopy to study protein adsorption onto silica particles in a heavy water (D_2O) buffer. By observing the shift in the amide I absorption band, he could deduce the fraction of protein carbonyl groups involved in bonding to the silica surface. He found that bovine IgG had a bound fraction of 0.20 at low bulk solution concentrations, but only about 0.02 at high solution concentrations. However, neither prothrombin nor bovine serum albumin exhibited a change in bound fraction with concentration. Parallel experiments with flat silica plates using ellipsometry showed that the IgG-adsorbed layers had an optical thickness of 140 Å and a surface concentration of 1.7 mg/m² at low bulk solution concentration — in concentrated solutions the surface amount was 3.4 mg/m² with a thickness of 320 Å (Fig. 17).

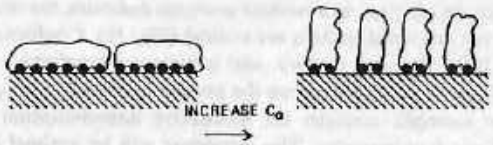


Fig. 17. At low solution concentration, the protein has no neighbors on the surface and thus can optimally adapt to the surface, maximizing the number of binding interactions. At high solution concentration, any one adsorbed protein is immediately surrounded by neighbors, minimizing the probability that it can conformationally adapt to the interface. This behavior leads to the differences in adsorbed amount and adsorbed protein thickness (determined by ellipsometry), as discussed in the text

Morrissey suggested that at low solution concentrations the adsorbed protein has sufficient time and "elbow room" to accommodate to its new microenvironment by conformational change resulting in significant hydrogen bonding to the silica surface. At high solution concentrations, the collision frequency with the surface is so high that an adsorbed protein has neither the time nor the room to optimize its interaction with the surface. This general interpretation is common in the air/solution field as well. Air/water studies show that the protein only needs to get a sufficient "foothold" on the surface to minimize the probability for desorption [3]. Once attached to the surface, the rest of the protein is "dragged" to the interface, thereby optimizing the interaction. The foothold need only be roughly 100–200 Å², in contrast to the cross sectional areas of typical proteins, 1000–10000 Å². Morrissey's high bulk concentration case may represent a minimum foothold.

Walton and his students have also pioneered the study of protein conformational changes, using fluorescence and circular dichroic spectroscopies [72, 75, 76]. Transmission CD spectra of Hageman Factor, adsorbed on a stack of quartz plates, suggested conformational changes upon adsorption. CD spectra of proteins eluted after different contact times with the surface show that the degree of conformational change is directly related to the contact time for periods of up to 10 days. It is now generally accepted that protein conformational change can be a rather slow process.

Brash has also used CD to study eluted proteins and finds large changes in α-helix content of fibrinogen, perhaps due to enzymatic fragmentation produced by the surface-induced activation of plasminogen to plasmin [77, 78].

Transmission fluorescence studies of adsorbed Hageman Factor show changes, which can be interpreted in terms of conformational and other micro-environmental effects [76], although such studies must consider substrate effects on fluorescence [75].

Adsorption may lead to an increase or decrease in titrable groups. Titration data can thus be interpreted in terms of conformational changes.

Soderquist and Walton [72] showed an "overshoot" in adsorbed amount as a function of time and a kink or inflection in the isotherm at about half saturation (Fig. 18, see also Fig. 14). They proposed:

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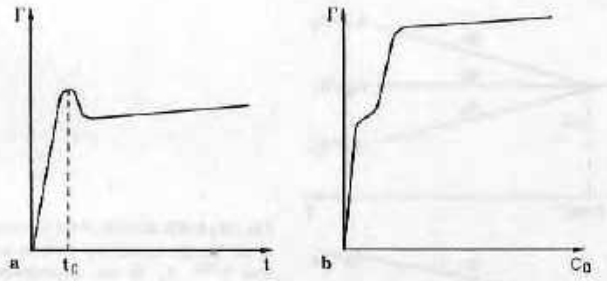
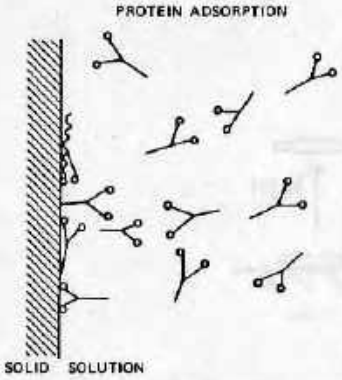


Fig. 18 a. A schematic of kinetic "overshoot" adsorption data, as discussed by Soderquist and Walton [72], Van Dulm and Norde [65], and others. See text for explanations. b. The "kink" at about half-saturation often observed in protein adsorption isotherms



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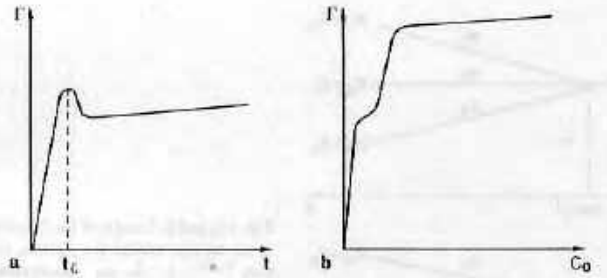

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equilibrium is present. The protein is adsorbed in a random arrangement at coverages less than 50%.
2. At coverages greater than 50%, surface transitions and ordering may develop which lead to more efficient packing and in increase in adsorbed amount, hence the kink in the isotherm.
3. Given sufficient time, adsorbed proteins undergo conformational changes which lead to increased surface interaction. During this process, proteins less optimally adsorbed undergo desorption, hence the overshoot in the time curve.
4. The desorption rate decreases with increasing residence time.
5. Desorbed protein may be permanently denatured, suggested by CD results.

Although this last point may not be general, the others are now accepted as rough "rules of thumb" for protein adsorption, though clearly each protein-surface-solution system is unique and may not exhibit all of the features noted.

Van Dulm and Norde [65] (in a study of human plasma albumin on negatively-charged polystyrene latices) showed a fast initial adsorption followed by desorption, probably due to conformational change of the adsorbed albumin, which induces the release of leass tightly bound protein. This result was observed at pH 4, where the albumin has a net positive charge and was not observed at pH 7.4, where it is highly negative.

The attenuated total reflection (ATR) Fourier transform infrared spectroscopic (FT-IR) studies of Gendreu, Jakobsen, and others [79] have the potential for direct determination of conformational changes during the adsorption process due to shifts in the infrared absorption bands. Sakurai et al. [80,81], have used ATR-FTIR, as well as CD, to probe conformational changes upon adsorption.

Total internal reflection fluorescence (TIRF) spectroscopy has recently been applied by several groups [4,39–42] and complete reviews are now available [4,42]. The method can easily follow the kinetics of adsorption, using proteins labeled with extrinsic fluors, such as fluoroscein or rhodamine. The intrinsic UV fluorescence of tryptophan (Trp) can be used to follow adsorption. The UV approach has the advantage that the tryptophan fluorescence is sensitive to the local micro-environment and no label is required. The major disadvantage of the UV method is the UV photochemical changes which occur, although such changes can be minimized by working at low light levels.

The intrinsic UV fluorescence of proteins is dominated by the tryptophan indole rings. The absorption maximum is 280–290 nm with the fluorescence maximum ranging from 315–355 nm, depending on the local environment of the indole side-chains. Quantum yields range from 0.04 to 0.50; 0.10 is a common value. As the local environment polarity or dielectric constant increases, the fluorescence maximum shifts up to 355 nm, such as for an indole ring in water or buffer. Trp moieties in highly hydrophobic environments fluoresce at 315–320 nm. Thus the fluorescence emission maximum (and the quantum yield) provide indirect information as to the local environment of the Trp fluors.

Although a number of proteins of interest (human serum albumin, for example) contain a single Trp, most contain two or more. Thus the spectrum observed is the sum of all active Trp fluors, making it difficult to deduce the local environment of each fluor. Nevertheless, the UV fluorescence emission spectrum is useful in deducing orientation and/or conformation changes upon adsorption.

Fibronectin (Fn) adsorption from 0.05 mg/ml solution showed very different adsorption kinetics on hydrophobic and hydrophilic surfaces. The data for static and flow adsorption and desorption have been reported [82]. The quantity of interest here is the fluorescence maximum, which for Fn on hydrophilic silica is identical to that in bulk solution, suggesting no major conformational change upon adsorption. The Fn-silica surface interactions probably involve the charged groups on the surface of the molecule, most likely the highly positively-charged heparin binding regions of the molecule (pK = 8–9).

Fn adsorbed on hydrophobic silica, however, fluoresces at 326, suggesting a slight denaturation of the molecule. Fn interactions with the hydrophobic surface may involve some of the apolar residues in the protein interior, suggesting a partial denaturation. Clearly, studies on surfaces of a range of charge, polarity, and apolar character would be of interest.

Bovine albumin has been studied to date via intrinsic TIRF only on hydrophilic quartz [92,159]. The fluorescence maximum (1 mg/ml BSA in PBS) is 342 nm and shifts to 333 nm upon adsorption. These results suggest that the adsorption of BSA onto silica changes the conformation of the molecule such that the two Trp fluors are in a more hydrophobic environment.

Studies using radio-iodinated proteins in the TIRF apparatus permit a direct measurement of the amount adsorbed, thus allowing one to deduce if the quantum yield is also changed on adsorption [84].

Hydrophobic chromatography studies of proteins intramolecularly crosslinked to minimize unfolding suggest that ". . . the effective hydrophobicity of proteins is due in part to the extent to which buried hydrophobic residues are exposed by protein unfolding" (Ref. [83], p. 97).

Both Vroman [44] and Chuang [85–87] have shown that specific antibodies can be used as probes of adsorbed protein orientation or conformation. Chuang showed that fibrinogen adsorbed on Cuprophane surfaces could be measured quantitatively, using a ^{125}I-labeled antifibrinogen-IgG [85], however fibrinogen adsorbed on polyvinyl chloride ". . . was not readily accessible for reaction with ^{125}I-antifibrinogen-IgG."

Clearly specific antibodies, and particularly monoclonal antibodies, may be very useful in probing the properties of adsorbed proteins. Specific antibodies have been used to probe the structure of antigens in solution [88]. Consider the adsorption of a simple protein with a small number of reasonably well-defined epitopes (surface sites with antibody binding activity), as in Fig. 19. Clearly epitopes E and A are not accessible for binding, while B, C, and D would be sterically accessible. One could also envision a conformational change upon adsorption which produces an epitope

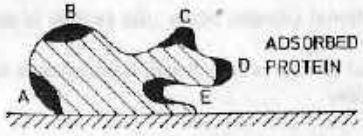

Fig. 19. Schematic of a protein with five different antigenic sites (epitopes). Each epitope may have one or more specific monoclonal antibodies. A set of such antibodies can be used to probe which epitopes are accessible or not, allowing the investigator to deduce the orientation of the adsorbed protein

normally unavailable. Antibodies to such "hidden" epitopes may be generated if the protein is first denatured in solution to expose the normally hidden site.

It is important to note that antibodies are very large molecules (Fig. 1) and may be sterically unable to bind to an adsorbed protein, even if the proper epitopes are indeed exposed to the solution (Fig. 19).

Chuang and coworkers observed that a specific antibody to thrombin showed very low reactivity with prothrombin in solution but very high reactivity with adsorbed prothrombin, suggesting that adsorbed prothrombin exposes a new binding site equivalent to one of the thrombin epitopes [87]. The reactivity of adsorbed prothrombin for the thrombin antibody was surface specific, suggesting that adsorbed prothrombin has different orientations and/or conformations on the surfaces examined. After a thorough study, Chuang concluded [87], "The data from this study appear to demonstrate that adsorption of prothrombin to artificial surfaces, such as PVC, had induced some conformational changes of the macromolecule that resembled antigenically the domain(s) of adsorbed thrombin . . . Monoclonal antibodies specific either to prothrombin or thrombin may be useful to pinpoint the exact domain(s) involved in such changes."

Chuang further cautions that "In solid phase immunoassay, it is generally assumed that antigens adsorbed to a surface, such as polystyrene microtiter dishes, will react with specific antibody in a manner similar to that antigen-antibody reaction in solutions such as occur in immune precipitation. However, our evidence and others [89] seem to point out that data obtained from solid phase immunoassays should be interpreted with caution since adsorption of a nonantigen to a polymer surface could render it immunoreactive to previously unreactive antibodies."

Clearly a set of monoclonal antibodies may help elucidate the nature of adsorbed protein orientation and conformation. Such studies are in progress by several groups.

4.5 Desorption, Exchange, Hysteresis

The adsorption of macromolecules is rarely an equilibrium process. Just as the properties of synthetic polymers are often dependent on non-equilibrium processes and relaxation phenomena [30], so do the properties of adsorbed proteins depend on time, metastable states, and hysteresis processes.

As the adsorption site density (Fig. 12) or total free energy of adsorption increases, one moves from the realm of "reversibility" to that of "irreversibility." As proteins can undergo conformational and orientational changes on a surface, they can optimize their interfacial interactions so as to provide the maximum free energy of adsorption. Such conformational alterations are relatively slow and hence very time-dependent.

Soderquist and Walton [72] showed that the desorption rate is a function of residence time. The adsorption process can be characterized in three stages:

1. Short times where adsorption is reversible; presumably little or no time is available for conformational changes.
2. At longer times where slow conformational changes occur, the process is semi-reversible and desorption occurs very slowly.
3. At long times where the conformational change is completed, adsorption is now irreversible, and desorption is improbable.

Those proteins which do not undergo any significant conformational change at a particular interface may not show any significant time-dependence.

Soderquist and Walton proposed that the kinetic "overshoot" (Fig. 18a) and the kink at around half-saturation (Fig. 18b) often observed in protein adsorption studies may be due to time-dependent, surface-induced transitions. The conformational changes probably result in some unfolding and an increased number of protein sites contacting the surface. Conformationally changed protein requires greater surface area — those proteins further along in the conformational change process "consume" surface at the expense of their neighbors, which are less tightly adhered to the surface. Therefore, the amount of protein adsorbed can go through a maximum as noted in Fig. 18. Van Dulm and Norde have made similar observations [65].

The isotherm ("equilibrium") case follows a similar argument (Fig. 18b). At low C_0, the surface is not saturated, and the adsorbed proteins are randomly oriented on the surface. At greater than 50% coverage, lateral interactions among neighbors become important, possibly producing an ordering of the adsorbed proteins, which can be thought of as a surface phase transition. The more ordered adsorbed layer requires less area per molecule, hence the surface has "room" for more proteins to adsorb — thus the kink in the isotherm (Fig. 18b). Direct evidence of two-dimensional protein crystallization and ordering is now available for the case of antibodies deposited on haptenated phospholipid monolayers [90].

Soderquist and Walton's [72] model of the protein adsorption process (discussed earlier, Fig. 13) reduces to the classical Langmuir (small-molecule) adsorption as $t \to 0$. As $t \to \infty$, their model predicts that adsorption is irreversible. A model which reduces to Langmuir adsorption at short contact time and to irreversible adsorption at very

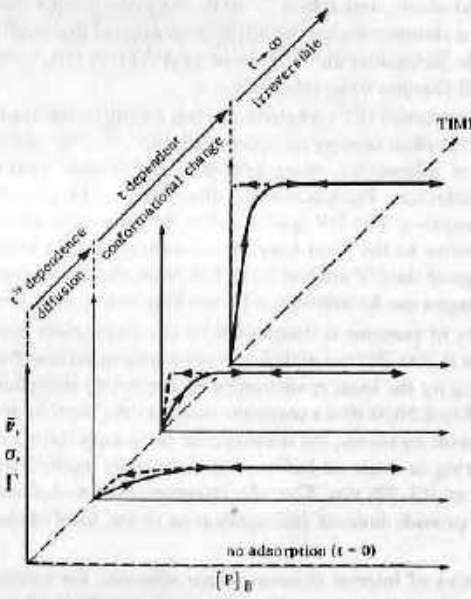

Fig. 20. Schematic adsorption "isotherms" with a constant surface site concentration ([A]$_s$ in Fig. 12 is here constant), but with adsorption time as a variable. At very short times, adsorption is diffusion controlled. At short times, the protein has insufficient time to conformationally adjust to the interface, thus adsorption can be reversible and of the Langmuir type. At longer times, conformational adjustments begin leading to the commonly observed semi-or ir-reversible behavior of protein adsorption. Other nomenclature same as Fig. 12

long contact times is certainly consistent with much of the data in the literature. Walton and Koltisko [91] have examined both protein stability and surface-induced, time-dependent conformational changes. Such treatments deserve to be more fully developed and investigated.

The tendency for an adsorbed protein to undergo conformational change is protein- and interface-specific, as well as time-dependent. Total internal reflection fluorescence (TIRF) studies on IgG adsorption and desorption on hydrophobic and hydrophilic surfaces as a function of residence time show clearly both the time-dependence, as well as the surface-dependence, of desorption [92].

Figure 20 summarizes the situation in schematic form. Assuming a constant surface binding site concentration (fixed surface properties), we examine the amount bound as a function of time and bulk solution concentration. At very short times and particularly at low bulk concentrations, adsorption is diffusion-controlled and shows a $t^{1/2}$ dependence. At longer times the sluggish conformational changes begin to become important. A significant hysteresis now begins to appear on the adsorption-desorption isotherm. At long contact times the conformational adjustments are complete, adsorption-free energy is maximized, and adsorption is irreversible, producing maximum hysteresis.

Clearly, proteins with a high concentration of binding sites on their surfaces may adsorb in the proper orientation, resulting in a multipoint attachment and high adsorption-free energy, even without any conformational adjustment. Thus, hysteresis can be present in some systems even at very short contact times. Proteins with very stable tertiary or quaternary structures may not show significant time-dependence due to the low probability for conformational change.

It is commonly observed that protein desorption can be very slow or even non-existant, but protein exchange can be rapid. This "anomaly" has been pointed out many times [93, 94]. Fortunately Jennissen's studies [95-97] and the arguments and work discussed in this chapter lead to a reasonable explanation.

Consider the cartoon in Fig. 21. Imagine some adsorbed proteins with multiple

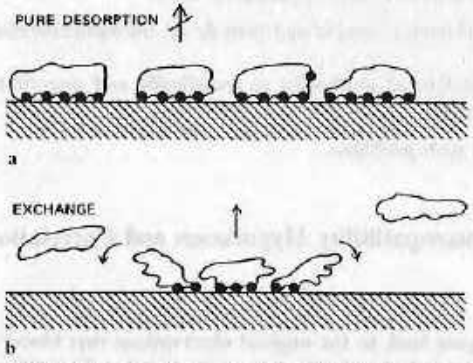

Fig. 21a. Cartoon showing the improbility of desorption for a protein adsorbed by 4 or 5 binding sites; b. exchange of adsorbed protein with protein in the bulk solution is probable, however, due to the fact that as one foot releases, a foot from a different protein may attach. See text for further discussion. Explanation due to Jennissen [95-97]

binding sites. Call each protein binding site a "foot". Statistically, a protein foot may lift off every now and then. Clearly, all feet must be lifted off in order for the protein to desorb. This is statistically improbable, thus desorption cannot occur. We say the protein is irreversibly bound. Exchange is a different matter. Now we have proteins present in the local environment, diffusing to and colliding with the surface. If one of these proteins should statistically place a foot on the space vacated by a "desorbing" foot of an adsorbed protein, then a "competition" is present. If the new protein then puts several feet down and becomes anchored, it may indeed induce the removal of the first protein. Therefore, one protein can, in essence, be lifted off by a number of proteins adsorbing and attempting to accommodate with the surface. This is a very schematic and simplistic explanation, but it appears to at least qualitatively explain the situation.

The adsorption of polydisperse synthetic polymer shows similar behavior in that the average molecular weight of the adsorbed polymer increases with time — high molecular weight material is more tightly bound (more "feet" attached), thus inducing desorption of the low molecular weight molecules (see Fig. 22).

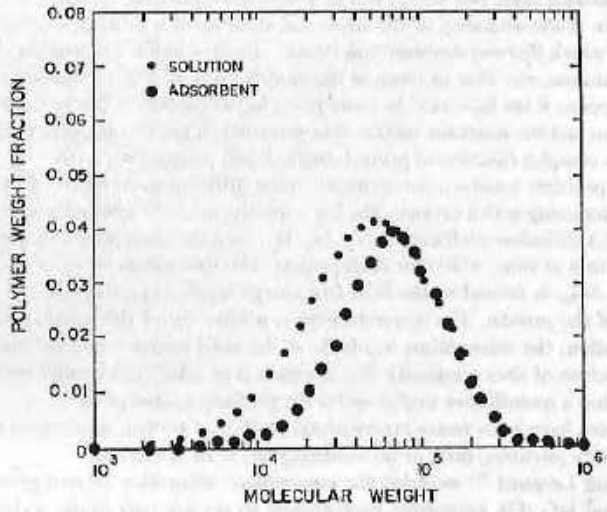

Fig. 22. Molecular weight effect of synthetic polymer adsorption. Molecular weight (MW) distribution of poly(vinyl chloride) in solution and in the adsorbed layer at equilibrium. Note that the adsorbed material has a higher average MW than the bulk solution (from Ref. [100], p. 120)

This is probably the basis of the complex protein exchange processes observed by Vroman and coworkers [98]. The more feet and energy per foot (total adsorption-free energy), the lower the probability that exchange can occur. Hence in a competitive adsorption process, one finds that those molecules present in the highest amounts and of smaller size bind first (due to diffusion and collision arguments) and are then exchanged by the more strongly adsorbing components. Vroman and Adams have shown [98] for plasma proteins adsorbing on glass and certain metal oxides that the

exchange hierarchy is: albumin < gamma globulin < fibrinogen < fibronectin < Factor XII (Hageman Factor) < high molecular weight kininogen (HMWK) (albumin adsorbs first).

4.6 Competitive Adsorption — the "Vroman Effect"

The adsorption of protein from single component solutions is qualitatively understood, although a quantitative understanding and models or theories with predictive character are not yet available. *If* the structure and solution properties of the protein are known and *if* the solid-buffer interface properties are known, then by careful examination of the "surface" of the protein (ideally via molecular computer graphics), we can indeed predict what orientation of the protein is "preferred" on that particular surface.

Recently, considerable progress has been made on the calculation of electrostatic and hydrophobic interactions in biochemical systems [189–191]. We can expect such calculations to become common for protein-solid surface interactions. Thus we can expect approximate values for the adsorption energy in selected systems to appear in the near future. The problem of time-dependent conformational adaptation of the protein to the surface (and vice versa) will be much more difficult. Initially, we will have to resort to crude measures of the structural stability of a protein, such as the temperature at which thermal denaturation occurs, the urea molar concentration for solution denaturation, etc. One or more of the models given in Fig. 13 should apply.

But what happens if we have two or more proteins in solution? Clearly there will be a competition and the resultant surface concentration of the two proteins at some time, t, will be a complex function of protein, surface, and solvent properties.

The general problem involves, for example, three different proteins (A, B, C) in solution and interacting with a surface. The key variables are bulk solution concentration (C_A, C_B, C_C), diffusion coefficients (D_A, D_B, D_C), and the adsorption free energy, which is a function of time, $\Delta G(t)$ for each protein. The free energy of adsorption at initial contact, ΔG_0, is related to the final free energy state, ΔG_∞, through the "denaturability" of the protein. The denaturability is a function of the stability of the protein in solution, the interactions available at the solid surface, and the surface occupancy (fraction of sites occupied). The problem is to take these qualitative concepts and develop a quantitative model useful for predicting adsorption.

Although there have been many experimental studies of protein adsorption from binary and trinary mixtures, little or no modeling has been attempted.

Beissinger and Leonard [71] modeled the competitive adsorption of two proteins, albumin (A) and IgG (G), permitting each protein to occupy part of the surface in two different states, and also allowing for desorption of each protein from each of its two allowed states. The model uses 12 adjustable parameters, making it difficult to apply for predictive purposes.

Adsorption from plasma is considerably more complex, as literally hundreds of different proteins are present [99] — all competing for the surface.

Vroman has shown by antibody methods that plasma interactions with solid surfaces result in a hierarchial adsorption process [98]. The high concentration proteins dominate the surface at short times due to the higher collision rates. As time passes

various exchange processes occur and proteins with higher surface affinities dominate the surface. Finally at very long times only the highest affinity proteins are present on the surface, even if their bulk solution concentration is very low.

This effect is well-known in synthetic polymer adsorption and results in low molecular weight species (fast diffusion) adsorbed initially, but the high molecular weight fractions are preferentially adsorbed at very long times (higher adsorption free energy) (see Fig. 22).

Adams, et al. [101] used a curved disc on a flat surface to study the effect of solution volume at constant surface area on competitive plasma protein adsorption. Although the experiment was qualitative, it elegantly demonstrated the importance of exchange, bulk solution concentration, and surface-volume ratio on competitive adsorption [98].

Recently Horbett [102] and Brash and ten Hove [103] have quantitatively demonstrated the "Vroman effect" in a series of experiments studying competitive adsorption of fibrinogen, albumin, IgG, and hemoglobin from diluted plasma.

The adsorption values at 5 minutes onto glass and polyethylene as a function of plasma dilution are given in Fig. 23. Fibrinogen adsorption on glass is maximal at about 1.5% plasma. From 0 to 1.5% plasma, a "typical" protein adsorption isotherm is observed. On polyethylene, the result for fibrinogen is similar. Albumin adsorbs in high amounts on the hydrophobic polyethylene surface showing a "normal" concentration dependence. The kinetics of fibrinogen adsorption show clearly that fibrinogen does indeed adsorb at the high concentration, but is removed or exchanged within a minute or so — exactly what Vroman observed many years ago.

Other workers have observed concentration-dependent competitive adsorption, including Grinnell and Feld [89] (fibronectin), and Breemhaar et al. [104] (fibrinogen, IgG, albumin).

What are these high affinity plasma components which compete so effectively for certain surfaces? Vroman suggests high molecular weight kininogen (HMWK), based on studies with HMWK-deficient plasma [98]. Breemhar et al. [104] suggest it could be a lipoprotein. Another explanation may lie in Brash's observation [77,78] that plasminogen can be activated by contact with glass to plasmin, which can then degrade adsorbed fibrinogen. Clearly there are many possibilities.

Clearly studies are needed which monitor the adsorbed protein population from plasma as a function of time. Only two methods come to mind:

1. Elute all adsorbed material from a sample and then do an ultrasensitive electrophoretic separation;
2. Use a large set of specific labeled antibodies to specifically and quantitatively bind with the adsorbed proteins.

Both approaches are fraught with problems.

5 Adsorption Based Biocompatibility Hypotheses and Correlations

5.1 Surface Charge

The surface charge concept goes back to the original observations that blood clots more rapidly in a glass tube than in hydrophobic glass or plastic tubes. This difference

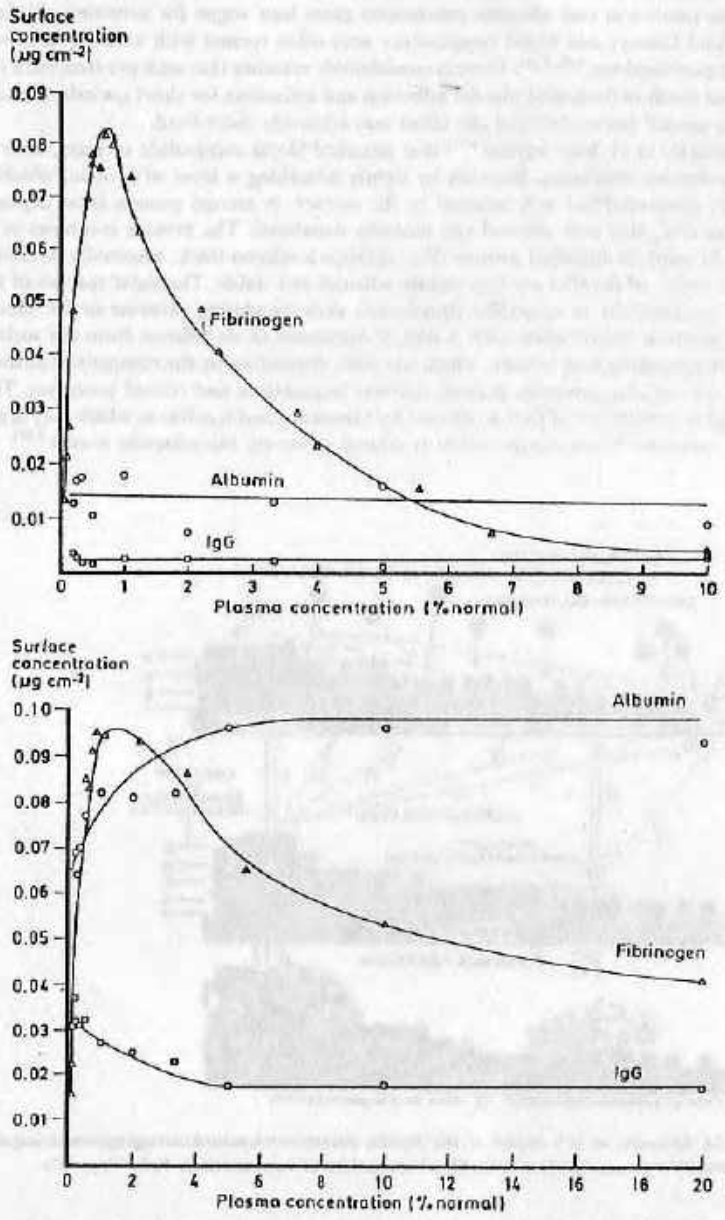

Fig. 23. Brash and ten Hove's results on the adsorption of three plasma proteins from diluted plasma as a function of total plasma concentration. Up: adsorption on glass showing a maximum adsorption of fibrinogen at about 1% plasma; Down: adsorption on polyethylene; plasma was diluted with isotonic Tris, pH 7.35. Adsorption time was 5 minutes (reprinted from Ref. [103])

was attributed to the negative surface charge of glass and is still quoted in hematology textbooks as the mechanism for surface-induced activation of coagulation. With the discovery of Hageman Factor and the intrinsic contact activation mechanism of coagulation, the negative surface charge theory was given some credibility. It was shown that Hageman Factor could indeed bind to negatively-charged surfaces: quartz, glass, and various silicate minerals. The counter theory argued that negatively-charged surfaces were more blood compatible because heparin, a common anti-coagulant, was highly negatively charged. Indeed, early studies by Lovelock and Porterfield [105] on the sulfonation of polystyrene to produce sulfonic acid groups analogous to those on heparin showed that such surface increased static blood coagulation times.

Sawyer's pioneering studies to measure the surface potential of the vascular surface by electrokinetic methods demonstrated that the vascular surface was negatively charged [106]. The mechanism suggested is that plasma proteins are generally negatively charged and therefore are repelled from a negatively-charged surface. This simplistic approach was very satisfying and dominated the blood compatibility field for several decades, despite the contradiction that Hageman Factor was known to be activated by negatively-charged surfaces.

It is now known that negatively-charged proteins are not repelled from negatively charged surfaces for a variety of reasons. One is that although a protein may exhibit a negative charge it may have localized regions or domains where negative charge is not present, where positive charge may be present, or where no charge is present. We demonstrated earlier in this chapter that proteins can adsorb to surfaces by a variety of interaction mechanisms. It is important to note that under normal physiologic conditions, the negative charge is screened by counterions beyond about 10 Å. By the time a protein is within 10 Å of the surface, specific intermolecular interactions are already dominant and gross electrostatic repulsion is not a significant effect.

Clearly, proteins can adhere to surfaces by electrostatic mechanisms, particularly at low ionic strength where the electrostatic field of the surface and the protein is much more extended. Indeed, this is the basis of ion exchange chromatography, so widely used for the separation, purification, and characterization of proteins. However, by the time one reaches the 0.15 M salt concentration of the physiologic environment, general electrostatic processes are no longer dominant.

5.2 Interfacial Energetics

With the wide availability of synthetic plastics in the late 50's and early 60's, there was considerable interest in relating the surface properties of plastics to their blood interactions. The only method of measuring a surface property was via wettability, i.e., contact angle measurements. Zisman and coworkers developed the critical surface tension concept [107], which permitted researchers to obtain empirical measures of the surface energy of polymeric materials. This approach was widely applied by Baier and eventually led to the hypothesis that surfaces with a critical surface tension in the range of 20–25 dyne/cm have optimal blood compatibility [108]. Such surfaces do indeed adsorb proteins. Baier argues that the critical surface tension of passive adsorbed protein films, and indeed of the vascular intima itself, is in the range of

20–25 dynes/cm. No satisfying mechanism has been proposed for this correlation. One suggestion is that the apolar component of the surface tension of water is 22 dynes/cm, within the range postulated by Baier.

An important hypothesis related to interface energetics is that of Nyilas, who said that the free energy of adsorption basically drives conformational change [132]. He probed this approach by measuring the enthalpy of adsorption using micro calorimetry and attempted to relate surfaces with low heats of protein adsorption with increased blood compatibility.

Shortly after the development of the critical surface tension concept Fowkes [109] and Girifalco and Good [110] developed means to estimate the surface-free energy and the surface-free energy components of solids by judicious application of probe liquids for contact angle studies and by intermolecular interaction approximations. These developments led Lyman and others to attempt to correlate the surface-free energy of polymers with their blood compatibility [111]. Lyman argued that as the surface-free energy increases, it increases the probability for protein binding and activation, such as by the Hageman Factor mechanism, and thereby decreases blood compatibility.

Lyman's ideas were extended by Andrade who argued that the governing parameter is not the surface-free energy of the polymer, but the interfacial-free energy of the polymer-water interface [112]. He argued that as the solid side of the interface begins to look more and more like water in energetic terms, there is a decreased driving force for protein adsorption and denaturation. This approach provided a semi-quantitative rationale for the rapidly developing interest in hydrogels as blood compatible surfaces.

With the development of neutral hydrophilic methacrylates in Prague, originally for contact lens applications in the early 60's [113], considerable interest was generated in the application of these materials in the cardiovasular environment. The qualitative argument was that such soft, water-rich surfaces must be relatively non-traumatic to proteins and cells. The development of neutral hydrophilic polysaccharide-based particles for protein chromatography in the late 60's provided evidence that such surfaces do indeed show minimal binding of proteins.

Basically, the interfacial-free energy hypothesis said that as the interfacial-free energy goes to zero, the driving force for protein adsorption goes to zero, and adsorption cannot occur [112]. If adsorption cannot occur, no mechanism is present by which to activate coagulation, and therefore the surface is blood compatible. Unfortunately, the contact angle methods and approximations for deducing interfacial free energy could not discriminate between materials ranging from about 40% water, such as poly(hydroxyethyl methacrylate), to over 95% water, such as the lightly cross-linked agaroses [114]. Thus the hypothesis could not be rigorously tested. Also, the highly hydrophilic surfaces required were not always stable or longlived in the cardiovascular environment.

5.3 Protein Passivation

In the mid to late 60's, considerable interest in the pre-adsorption of proteins evolved. The basic idea was that if one could saturate the surface with a layer of proteins, then the surface would not be available for the binding and activation of Hageman Factor

or other contact activation proteins. Basically, albumin-treated surfaces were resistant to platelet adhesion, while other proteins — especially fibrinogen — promoted adhesion. This work has been well reviewed recently [115–117, 192].

The result was that albumin passivation came into vogue for sometime. In fact, artificial kidneys and blood oxygenators were often treated with albumin solutions prior to clinical use [118, 119]. There is considerable evidence that such pre-treatment did indeed result in decreased platelet adhesion and activation for short periods, perhaps up to several hours, but that the effect was relatively short-lived.

Matsuda et al. have argued [127] that practical blood compatible surfaces, such as the polyether urethanes, function by tightly adsorbing a layer of protein, which is highly denatured but well adhered to the surface. A second protein layer deposits on the first, also well adhered and partially denatured. The process continues as in Fig. 24 until an adsorbed protein film, perhaps a micron thick, eventually develops. Inner layers of the film are very tightly adhered and stable. The outer reaches of the film are basically in reversible equilibrium with circulating proteins in the blood. The problem occurs when such a film de-laminates or de-adheres from the surface thereby exposing bare surface, which can then, depending on the competitive adsorption and cellular processes present, activate coagulation and related processes. This model is reminiscent of that developed by Moncanin and Kaelble in which they argue that optimum blood compatibility is related to strong bio-adhesion events [128].

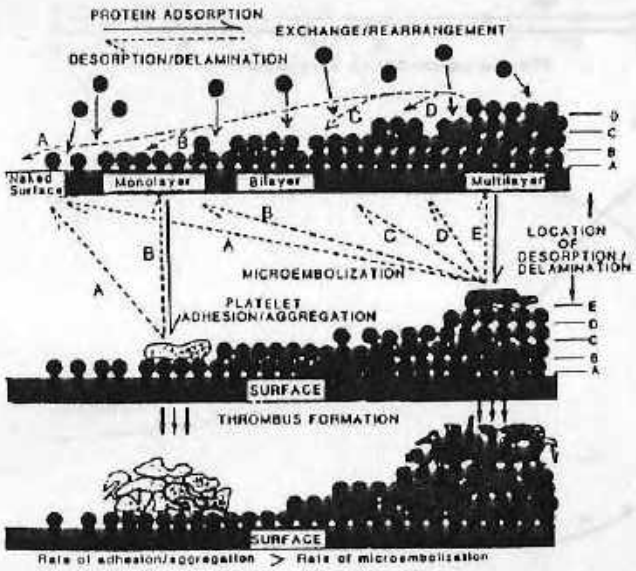

Fig. 24. Matsuda, et al.'s model of the protein adsorption/denaturation/aggregation/desorption/delamination process involved in the blood interactions of materials (from Ref. [127], p. 357)

5.4 Protein Resistent or Repulsive Surfaces

A current hypothesis, which is receiving considerable attention, is that one can indeed produce a surface which actively repels proteins and other macromolecules [123, 124, 133]. The basic idea is presented in Fig. 25, which shows that a neutral hydrophilic polymer, which exhibits considerable mobility or dynamics in the aqueous phase, can actively repel macromolecules from the interface by steric exclusion and interface entropy methods. This method has been well-known and applied in the field of colloid stability for many years [120]. The most effective polymer appears to be polyethylene oxide, probably because of its very high chain mobility and only modest hydrogen bonding tendencies [121–125].

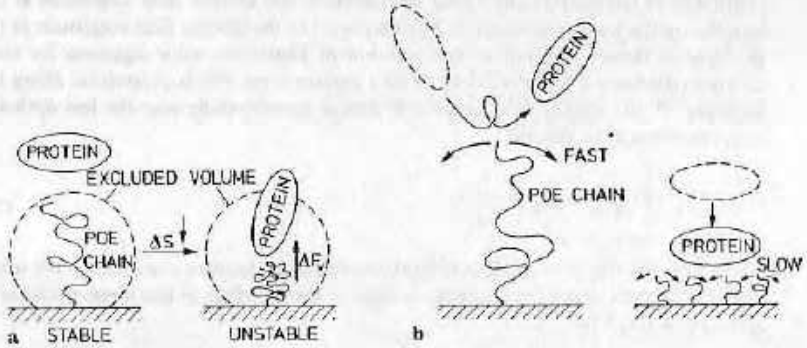

Fig. 25a and b. A protein resistant surface based on the steric repulsion argument commonly used in the colloid stability field [120]. The interaction between a polyethylene oxide grafted surface and a protein solution is shown. **a.** suggests an excluded volume or steric repulsion mechanism; **b.** the surface dynamics or polymer chain motion mechanism (from Ref. [133])

Merrill and Salzman have developed PEO soft segment polyurethanes and indeed have demonstrated minimal adsorption of blood proteins and minimal platelet adhesion on such surfaces [121]. Nagaoka et al. have studied various methacrylate copolymers with PEO side-chains of varying lengths and showed a direct correlation between minimization of platelet adhesion with increased PEO side-chain length and surface mobility [123, 133].

Gregonis et al. have shown that PEO bound to quartz surfaces greatly minimizes the adsorption of proteins from plasma and from singly component protein solutions [122]. Bell and coworkers at Los Alamos have developed a theory of cell-cell adhesion based on the steric repulsion characteristics of the hydrophilic macromolecules present on cell surfaces [124].

5.5 Immunoglobulins and Complement

Within the last ten years or so there has been considerable interest in the activation of the Complement system by surface-induced processes. Immunoglobulin of the IgG or IgM class, adsorbed at a surface with the right orientation and spatial ordering

and distribution, can activate Complement [125]. Certain hemodialyzer membranes, particularly of the cellulose type, are known to activate Complement via the alternate pathway, resulting in a transient, white cell depletion in the early stages of hemodialysis [125]. Sevastianov and Tseytlina recently reported the Complement activation properties of a variety of biomedical polymers [126]. There is therefore considerable interest on the adsorption of Complement components C1 and C3, as well as IgG, on surfaces, not only on cardiovascular materials, but also on intraocular lens implants, and at other sites of demonstrated Complement activity.

5.6 Other

Hoffman[129] and Baier [130] have reviewed most of the hypotheses and mechanisms suggested for blood compatibility in general and for the role of protein adsorption in particular. The safest statement one can make is that protein adsorption is indeed important in the blood compatibility process, in the compatibility of soft contact lenses, in the stability and acceptance of intraocular lenses, in the soft tissue foreign body reaction [131], and in virtually all situations where solid surfaces come into contact with physiologic environments.

It is also safe to say that, because of the great complexity of proteins, even of the simplest, most well-characterized proteins, such as insulin, lysozyme, and myoglobin, and because of the very wide range of proteins present in most physiologic environments, very simplistic hypotheses and mechanisms are generally not very applicable.

6 New Methods

6.1 Background

Techniques and methods for the study of protein adsorption have been well reviewed [47]. It is now generally recognized that it is not necessarily the type and amount of protein present at the surface which is most important, but rather the orientation and conformational state of those proteins. At present it is virtually impossible to predict the specific conformation of an adsorbed protein at a particular interface. The techniques used in the determination of protein conformation in solution or in the solid state do not usually apply to adsorbed proteins. Hence, the difference between adsorbed and bulk solution protein conformation has to be inferred indirectly.

Protein adsorption studies are performed either on high surface area material dispersed in a liquid phase containing dissolved protein, or on low surface area material, often flat, which is in contact with protein solution. Both approaches complement each other and can provide valuable information about adsorbed protein layers.

In the case of adsorbents with high surface area, changes in protein bulk solution concentration before and after adsorption are usually large enough for independent determination of the amount of protein adsorbed, either via solution depletion meas-

urements or directly at the surface after the solid phase has been separated from the solution phase. Such studies are usually performed using various colorimetric methods of quantitative protein analysis or by measuring the radioactivity of proteins labelled with ^{125}I, ^{14}C, or ^{3}H. In some cases, labelling *per se* cause changes in protein adsorbability to a certain surface [134], producing different surface concentrations of labeled and nonlabeled protein and erroneous results. Similar problems apply also to protein labeled with fluorophores, such as fluorescein, rhodamine-B, and other dyes.

Due to the importance of the blood coagulation process, the adsorption of protein is often studied in buffer solutions at conditions similar to "physiological" (pH = 7.4, 0.15 M NaCl, 37 C). Analysis of the adsorption process starts with the protein surface concentration presented as a function of equilibrium protein solution concentration, i.e., the adsorption isotherm. Most protein adsorption isotherms display a well-defined plateau in the dilute concentration range ($C_b < 1$ mg ml^{-1}). The adsorbed amount is usually up to the equivalent of a close packed monolayer, indicating an absence of multilayer adsorption. The adsorption isotherm is often claimed to be a "Langmuirian type", although the premises of Langmuir adsorption are rarely fullfilled, almost never investigated (with the exception of dilution effects), nor experimentally confirmed in the protein adsorption. The adsorbed amount usually changes only slightly with dilution, indicating apparent irreversibility [135]. However, it has been shown that proteins on the surface can exchange both with the proteins from the solution [136], as well as laterally with the neighboring protein molecules via surface diffusion [137]. The shape of the adsorption isotherm depends on experimental parameters; it can provide crude information about protein-adsorbent interaction. The initial isotherm slope reflects the affinity of protein towards the surface; the (apparent) adsorption isotherm plateau values can be related to the molecular dimensions of the adsorbed protein molecule. In some cases, the adsorption isotherms were found to display kinks which are thought to reveal distinct steps of interfacial protein rearrangement as the concentration of protein in the solution increases [138–140]. All of these effects were discussed earlier (Sect. 4).

In most cases, the adsorbing surface is tacitly assumed to be completely inert and nonresponsive to protein attachment. This assumption may be valid for surfaces of crystalline material but in the case of "hairy" polymer surfaces, changes in the polymer surface conformation due to the presence of adsorbed protein may be expected.

The major advantage of protein adsorption studies on high surface area materials is that changes of some extensive properties which accompany the process of adsorption are large enough to be directly measured: heat of adsorption through microcalorimetry [141], uptake or release of small ions by a combination of electrokinetic methods and titration [142], thickness of adsorbed layer or an increase of the volume fraction of solid phase by a hydrodynamic method like viscometry [143]. Chromatographic-like analysis can also be applied to protein adsorption [144].

For many areas of interest the most valuable information describing the interactions between a protein and a surface is conformational change of the adsorbing protein molecule as it passes from solution to the interface. Low surface area samples in combination with some form of spectroscopic method are generally used in the evaluation of protein conformation. Recent advances in this area warrant a more detailed description of the experimental approaches.

6.2 Spectroscopy by Evanescent Surface Waves [92, 154, 155]

As a rule these methods are based on the concept of evanescent surface waves caused by total internal reflection at a solid/liquid interface. In order to understand the so-called "evanescent" spectroscopy, a simplified theory of total internal reflection is given below. More complete treatments can be found in various specialized monographs and reviews [145–147].

An electromagnetic wave reflection at an interface forms a standing wave, due to superposition of the incident and reflected waves. This also occurs in the case of total internal reflection, where the standing wave forms in the optically more dense medium (medium 1). The nature of the standing wave is a function of both the optically more dense medium and the less optically dense medium (medium 2). In the familiar case of metallic reflection, the standing wave is aligned with its node at the reflecting metallic surface. In the case of the interface between two dielectric media, the electric field amplitude right at the interface, but in the denser medium, has a nonzero value. Since the boundary conditions for electromagnetic wave reflection do not allow any discontinuity in tangential field across the interface, the electric field amplitude at the interface in the less dense medium 2 will be equal to the electric field amplitude at the interface in denser medium 1. The solution of Maxwell's wave equation for total internal reflection shows the existence of a surface wave which propagates along the interface [148]. Its electric field amplitude decays exponentially into the less optically dense medium 2. In general,

$$E^t = E^{t,0} \exp\left(-\frac{z}{d_p}\right) \tag{29}$$

where E^t is the transmitted electric field amplitude at distance z normal to the interface; $E^{t,0}$ is the electric field amplitude right at the interface in less dense medium 2; and d_p is defined by:

$$\frac{1}{d_p} = \left(\frac{2\pi}{\lambda_2}\right)\left[\left(\frac{n_1}{n_2}\right)^2 \sin^2\theta_i - 1\right]^{1/2} \tag{30}$$

where n_1 and n_2 are refractive indices of the optically more dense and less dense media, respectively; λ_2 is the wavelength of the electromagnetic wave in medium 2; and θ_i is the angle of incidence of the electromagnetic wave measured from the interfacial normal. One can arbitrarily define d_p as the distance from the interface where the electric field amplitude decreases to e^{-1} of its interfacial value (often called the "depth of penetration of the evanescent wave"), i.e. $\exp(-z/d_p) = \exp(-1)$. An this case it can be shown that:

$$d_p = \lambda/2\pi(n_1^2 \sin^2\theta_i - n_2^2)^{1/2} \tag{31}$$

The magnitude of $E^{t,0}$ can be calculated from Fresnel's law. If the electric field amplitude as a function of distance z is expressed per unit of incident electric field amplitude, $E^{i,0}$, of the perpendicular polarized electromagnetic wave, then:

$$E_1^s = (E_1^{s,0}/E_1^{i,0}) \exp(-z/d_p) = (2\cos\theta_i)/[1-(n_2/n_1)^2]^{1/2} \exp(-z/d_p) \quad (31)$$

From an experimental point of view it is important to recognize that the profile of $(E_1^s)^2$ as a function of z is proportional to the profile of the intensity of electromagnetic radiation in the proximity of the interface in medium 2. Such a profile will determine the "surface sensitivity" of the evanescent wave; the "depth of penetration" is smaller if:
— there is greater mismatch between the refractive indices of two media,
— the wavelength of electromagnetic radiation is shorter, and
— the incident angle of electromagnetic wave is closer to its critical value.

Thus, choosing the experimental parameters one can design a sensing profile to a particular need. It has to be mentioned that in this simplified derivation of evanescent spectroscopy theory two implicit assumptions were made:
1. Both media are nonabsorbing, only real refractive indices were being used,[1] and
2. Any species adsorbing to the interface is not distinguished as an optically separate layer.

What is even more relevant to the present subject is that a thin dielectric layer of polymer can be situated between two media without grossly distorting the optical nature of the interface, i.e. total internal reflection would occur as if no polymer layer is present even if its refractive index is unmatched to both media. Such a layer will, however, affect the intensity of the evanescent wave and particularly its "depth of penetration."

One can distinguish between methods in which absorption of the evanescent surface wave in different wavelength regions is measured (these are often called "attenuated total reflection" methods), and methods which use the evanescent wave to excite other spectroscopic phenomena, like fluorescence and Raman scattering or light scattering. As the methods of conventional fluorescence spectroscopy have been shown to be exceptionally successful in studies of proteins and other biopolymers, their "evanescent" surface-sensitive counterparts will be reviewed first.

6.3 Total Internal Reflection Fluorescence (TIRF)

TIRF at solid/liquid interfaces was introduced by Hirschfeld [149]. Although this first use of TIRF was the study of bulk dissolved fluorescein in the vicinity of a fused silica-electrolyte interface, a number of advantages over the conventional transmission technique were demonstrated:

1. The intensity-concentration relationship was linear up to concentrations a hundred-fold higher;
2. Multiple total reflections increased the sensitivity of TIRF; and
3. Adsorption of dye to the surface increased the local concentration, enhancing the TIRF sensitivity.

[1] In this sense, the name "total internal reflection" is a misnomer since depending on the extent of the absorption of medium 2, there will be an electromagnetic energy "leakage" across the interface making rigorous critical angle definition not applicable. However, for weakly absorbing medium 2 the concept of total internal reflection is useful. The reader is referred to Ref. 146 and 147 for the derivations of evanescent wave equations for absorbing multilayered interfaces.

Further application involved collected of fluorescence from dansyl-labeled bovine serum albumin via TIRF optics [150], TIRF-immunoassay for specific dye-labeled antibodies binding from the solution to an antigen-coated surface [151], and a "virometer" — a optical sensor for viruses treated with a fluorescent probe bound to the virus nucleic acid [152, 153].

In TIRF protein adsorption experiments, it is desirable to correlate the intensity of excited fluorescence with excess protein concentration at the interface. Such an adsorbed layer is often in equilibrium with bulk-nonadsorbed protein molecules which are also situated inside the "evanescent volume" and thus contributing to the overall fluorescence. Various calibration schemes were proposed, using external nonadsorbing standards [40, 154], internal standard in a form of protein solution together with a type of evanescent energy distribution calculation [154], and independent calibration of protein surface excess [155]. Once the collected fluorescence intensity is correlated with the amount of adsorbed protein, TIRF can be applied in the study of various interactions between surface and protein.

Two different sources of fluorescence are possible:

1. Proteins containing amino acids like tryptophan and tyrosine which intrinsically fluoresce upon excitation in the ultraviolet range can be employed, hence, the name total internal reflection intrinsic fluorescence-TIRIF, or
2. Protein can be covalently labeled with an extrinsic fluor like fluorescein, rhodamine, etc.

Both approaches have distinct advantages and disadvantages: the first approach provides the possibility of recording intrinsic protein fluorescence emission and excitation spectra, which in turn can provide information about conformational changes in adsorbed proteins. It is also directly comparable with the methods of conventional fluorescence spectroscopy of proteins in solution. An interesting combination of TIRF and ^{125}I-labeled protein γ-detection was recently developed for determination of both the amount of protein and its fluorescence quantum yield in the adsorbed state [26]. It was shown recently that with appropriate apparatus the intrinsic fluorescence lifetimes of adsorbed protein can be determined [27]. Some proteins may show very weak fluorescence due to low quantum yield, have only a small number of tryptophanyl and/or tyrosinyl residues per molecule, and may be particulary photosensitive and unstable upon exposure to ultraviolet light [92].

The extrinsic probe approach is more suitable to competitive protein adsorption studies and to kinetic studies, provided that protein labeling by an extrinsic fluor does not influence protein adsorbability. The uptake of fluorescein-labeled albumin, γ-globulin and fibrinogen onto silicone rubber coated surfaces has been followed as a function of time and flow rate [39]; it was demonstrated that adsorption was diffusion limited [40]. A combination of total internal reflection with either fluorescence photobleaching recovery (TIR/FRP) or fluorescence correlation spectroscopy was described [137, 155, 157]. With this variation of TIRF, it is possible to determine surface desorption rates and surface diffusion coefficients without unnecessary perturbation of chemical equilibrium, like bulk protein concentration changes or changes in the composition of the buffer solution. Energy transfer between multi-labeled BSA (donor/acceptor pairs: dansyl/eosin and 4-chloro-7-nitro-2,1,3-benzoxadiazole/rhodamine) has found to decrease upon the adsorption of BSA, as detected by TIRF [158],

a feature that can be interpreted as a conformational change of adsorbed BSA. Fluorescent probes, used in probing membrane protein properties, have recently been employed in the evaluation of conformational changes upon adsorption [159].

Fluorescence from labeled adsorbed protein has also been excited with the evanescent surface wave created by integrated optics. Both optical fiber [160] and flat rectangular waveguides [93] have been used. Interesting use of optical fiber as a remote protein sensor was demonstated; the excitation light was sent down the fiber whose tip was immersed in protein solution, evanescently excited fluorescence was collected by the same fiber and delivered to a scanning monochromator [160].

Changing the incident excitation beam angle and/or the angle of fluorescence observation provides other interesting information: a fluorophore concentration profile from the interface to distances which are within an order of magnitude of the wavelength used [161]. In the case of adsorbed protein, variable angle total internal reflection fluorescence (VATIRF) would not only give as a result an average thickness of the protein layer but its spatial distribution.

In conclusion, TIRF promises to be exceedingly useful in the study of protein-substrate interactions. It gives *in situ*, possibly remote, real-time information about protein adsorption-desorption parameters, conformational changes upon adsorption and hopefully, nanosecond time-resolved fluorescence lifetime information about adsorbed proteins [156].

6.4 Absorbance Spectroscopy of Adsorbed Proteins

Both conventional transmission absorbance spectroscopy and its evanescent counterpart (often called attenuated total reflection (ATR) spectroscopy) are based on measuring the light characteristics after it has propagated through the sample. Such techniques are classified according to the wavelength of the light used. Ultraviolet wavelengths are absorbed by all proteins. This feature has been used in the transmission absorbance mode in the evaluation of molar absorptivity of β-lactoglobulin films on the surfaces of quartz plates [162]. The absorption of visible light by rhodamine-BSA adsorbed to a number of stacked quartz plates was measured as a mean to calibrate extrinsic fluor TIRF experiments [155]. The use of attenuated total reflection both in ultraviolet and visible wavelength range for protein adsorption studies has to our knowledge not been reported, although a UV-TIR absorbance study of synthetic polymer adsorption has been reported [163].

ATR spectroscopy in the infrared has been used extensively in protein adsorption studies. Transmission IR spectra of a protein contain a wealth of conformational information. ATR-IR spectroscopy has been used to study protein adsorption from whole, flowing blood *ex vivo* [164]. Fourier transform (FT) infrared spectra (ATR-FTIR) can be collected each 5-10 seconds [165], thus making kinetic study of protein adsorption by IR possible [166]. Interaction of protein with soft contact lens materials has been studied by ATR-FTIR [167]. The ATR-IR method suffers from problems similar to TIRF; there is no direct quantitation of the amount of protein adsorbed, although a scheme similar to the one used for intrinsic TIRF has been proposed [168]; the "depth of penetration" is usually much larger than in any other evanescent method, i.e. up to 1000 nm; water absorbs strongly in the infrared and can overwhelm the protein signal, even with spectral subtraction applied.

6.5 Raman Spectroscopy of Adsorbed Proteins

Raman spectroscopy is another powerful method used for the study of solution protein conformation. Its application at the solid/liquid interface is conceivable [169], but to our knowledge it has not been applied to protein adsorption on polymer surfaces. One of the problems is that most proteins have a small Raman scattering cross-section. In order to enhance the weak Raman scattering signal, use of an evanescent streak propagating through a thin polymer waveguide layer with adsorbed protein has been demonstrated [93]. Total internal reflection optics were used to induce Raman scattering of 5 μm thick dry BSA films on a sapphire internal reflection element [170]. Another possibility to enhance the signal is to induce resonance Raman scattering of a protein absorption band; in the visible wavelength range this would apply mainly to proteins containing heme in their structures. Two other ways of enhancing the Raman scattering signal from surfaces were devised: use of surface plasmon excitation at the surfaces of thin silver films [171] and surface enhanced Raman scattering (SERS) by silver island films and finely dispersed silver colloids [172]. The Raman spectra of 75 Å thin phospholipid monolayers has been obtained by the first method [173]. SERS has been used to induce resonance Raman spectra from cytochrome-c and myoglobin adsorbed at a silver electrode [174]. The full potential of Raman scattering techniques applied to the adsorption of proteins has yet to be exploited.

6.6 Ellipsometry

Ellipsometry is another powerful tool in solid/liquid interface analysis. While evanescent spectroscopy has the capability of sensing the adsorbed protein molecules by interaction with the evanescent surface wave, ellipsometry "sees" a protein layer on the reflecting surface as a distinct optical medium. Ellipsometry is based on the calculation of the optical properties of the reflecting surface, given the change in the state of the polarization of the reflected light. Once the optical properties of the bare surface are known, the ellipsometric analysis can be applied to any dielectric film deposited on the surface. The exact relations between wavelength, optical constants, thickness and incident angle were obtained in exact form in the 19th century, but they could not be solved in the closed form. Early work on ellipsometry used only approximate equations, neglecting the higher order terms of the Taylor expansions of the Drude equations. The introduction of computers made routine analysis of the equations readily available. The use of ellipsometry in protein-surface interaction studies advanced rapidly after Trurnit [175, 176] introduced an automated recording ellipsometer and measured the activity of the proteolytic enzyme, chymotrypsin, at a solid/liquid interface. Bovine serum albumin was deposited on the solid phase and depending on the condition used (ionic strength and pH) chymotrypsin was either removed or adsorbed to the albumin layer.

Studies of the role of protein-surface interactions in blood coagulation were done by Vroman [56]. The plasma proteins were adsorbed onto various hydrophilic or hydrophobic surfaces. Vroman showed that fibrinogen was an important component of the plasma protein layer adsorbed to the solid/liquid interface.

A complete review of the early work applying ellipsometry to biomedical problems is available [177].

The surface of silicon has optimum optical properties and the adsorption of proteins

was followed on both hydrophobic and hydrophilic silicon surfaces [178, 179]. A fibronectin adsorption study showed, for example, that the adsorption is partially reversible on the hydrophilic surface, but much less so on the hydrophobic surface [179]. Interaction of antibodies with preadsorbed fibronectin layers suggest that fibronectin adsorbs in different orientation or conformation on the two surfaces [179].

Ellipsometry can follow the interactions between two types of biological macromolecules, the first of those two bound physically to the surface, the other acting from the solution. The binding of concanavalin A to adsorbed mannan [180] and of cholera toxin to adsorbed ganglioside [183] are examples. The adsorption of complement factors to an antibody-coated surface was monitored by ellipsometry and a modification of the same method was used for quantification of migration inhibition of human polymorphonuclear leucocytes [182]. Interaction of proteins and cells with affinity ligands covalently coupled to silicon surfaces has been also studied [183].

Recent development in the application of ellipsometry to protein/substrate interaction studies is due to the introduction of the "dynamic ellipsometer", capable of recording both analyser and polariser positions automatically with time, which significantly improves the time resolution of the method [184]. The Lorentz-Lorenz equation is usually applied in order to calculate the mass of adsorbed proteins [185]. In particular, molecular weight, molar refractivity and partial specific volume are needed to calculate adsorbed mass from the experimentally determined thickness and refractive index of deposited film. While in most of the previous studies the assumption was made that the refractive index of the adsorbed layer was constant, the experiments done by Cuypers showed that such an assumption is not justified [186]. The refractive indices of protein layers varied as a rule with the time of adsorption [187]. So far, no general rules emerged about the course of refractive index changes. The refractive index of fibrinogen adsorbed on hydrophilic chromium oxide surface, for example, remained almost constant ($n = 1.4$) while the thickness increased to 12 nm. On the contrary, the refractive index of fibrinogen adsorbing on hydrophobic chromium was found to change significantly with time of adsorption; on the onset ($t < 150$ s) it was 1.8, only to drop to 1.48 while the thickness of the layer continued to increase to 7 nm. From this point on the fibrinogen layer became optically denser and thinner. Final values for the fibrinogen layer deposited from 10 μg/ml solution (0.01 M Tris-HCl buffer, pH = 7.0) onto hydrophobic chromium surface were: thickness, $d = 3.5$ nm, refractive index, $n = 1.72$. Such changes directly reflect some conformational changes and packing or ordering of the adsorbing protein. It is, however, difficult to explain such a high value of refractive index of the protein layer. It was reported that the refractive indices of prothrombin layers adsorbed on two phospholipids (di-C14:0 phosphatidylserine and di-C18:0 phosphatidylserine) were 1.46 and 1.90, respectively [185]. In order to account for this discrepancy, the authors speculated that refractive index values higher than the values for the pure protein reflected an interaction between the protein and the adsorbing surface and that in order to compensate for higher refractive indices of the protein film, a decrease of the refractive index of adsorbing surface has to be introduced. This assumption cannot be proved experimentally, but it was supported by finding that the adsorption of protein onto more swollen phospholipid layers, (i.e. onto a layer with a lower refractive index) resulted in higher refractive indices of protein film [187]. Clearly, more detailed modeling and computer simulation studies would be helpful.

7 Some "Rules of Thumb" (See also Sect. 4.2)

7.1 Area Required For Initial Adsorption

At an air/water interface, the two-dimensional interfacial pressure (Π) can be easily monitored using an instrumented Langmuir trough. The initial adsorption rate at a clean surface is simply the rate of diffusion

$$\frac{d\Gamma}{dt} = K_a C_0 \tag{32}$$

where K_a is the adsorption rate constant. Assume that the interface is partially occupied and the interfacial pressure is Π. "In order for a molecule to adsorb, it must compress molecules already adsorbed against the interfacial pressure Π to create an area of interface, ΔA, equal to that required for the molecule to move into (Ref. [3], p. 289)."

The work required is $\int_0^{\Delta A} \Pi dA$ or $\Pi \Delta A$ if Π is approximately constant. Then

$$\frac{d\Gamma}{dt} = K_a C_0 \exp\left(\frac{-\Pi \Delta A}{kT}\right) , \tag{33}$$

where k is Boltzman's constant, T is absolute temperature, and no desorption has been assumed (valid for $\Pi < 15$ mN/m). Equation 33 can be expressed as:

$$\ln\left(\frac{d\Gamma}{dt}\right) = \ln (K_a C_0) - \frac{\Pi \Delta A}{kT} \tag{34}$$

A simple linear plot of the data allows ΔA to be obtained. The results of a set of experiments (Table 5) are surprising. ΔA is independent of the size or molecular weight of the protein. Although the cross-sections of the proteins studied range from ~1000 to 10,000 A^2, ΔA is nearly constant at 100 to 200 A^2. Conclusion: ". . . *only a small portion of the protein molecule needs to enter the interface in order for adsorption to then proceed spontaneously* (Ref. [3], p. 290)." It is as if only a small "foothold" or "handhold" is required to stabilize the molecule against desorption. Now firmly planted at the interface, the molecule can optimize its interfacial interactions by time-dependent orientation and perhaps conformational changes. The size of the "foot" is obviously relevant to the exchange discussion in Sect. 4.5.

Table 5. Values of ΔA for various proteins (from Ref. [3])

Protein	Concentration ($g\,l^{-1}$)	ΔA (A^2)	Molecular Weight
Myosin	0.03	145	600000
Human γ-globulin	0.01	130	180000
Human albumin	0.02	100	70000
Ovalbumin	0.03	175	44000
Lysozyme	0.01	100	15000

An alternative interpretation of the data is that ΔA is proportional to the number of water molecules per protein participating in the adsorption and denaturation process and is related to water activity at the interface.

7.2 Electrical Potential Barrier

A similar experiment to that noted above can be performed, but now let the interface be populated by a molecular layer at constant Π and known interface electrical potential. A molecule adsorbing at such an interface must do work against the electrical potential barrier, as well as against the interfacial pressure. We get

$$\frac{dn}{dt} = K_a C_0 \exp - \left(\frac{(\Pi \Delta A + q\Psi)}{kT} \right) \tag{35}$$

where q is the charge on the adsorbing molecule and Ψ is the interface electrical potential (3). Table 6 and Fig. 26 present data on lysozyme adsorption into different monolayers. Clearly the *electrostatic effects are very significant.*

Table 6. Relative initial rates of adsorption of lysozyme into different monolayers[a]

Monolayer	Rate	ζ-Potential (mV)
Cephalin	34.5	−68.1
Polyglutamic acid	13.2	−51.5
Pepsin	5.4	−16.7
Serum albumin	4.5	−14.1
Octadecanol	5.0	Not measured
Trypsin	3.0	+3.2
Lysozyme	(1.0)	+12.2
Polylysine	0.3	+38.5

pH 6.5, I.S. = 0.01, charge on lysozyme ≃ +9 units.
[a] from Ref. 3

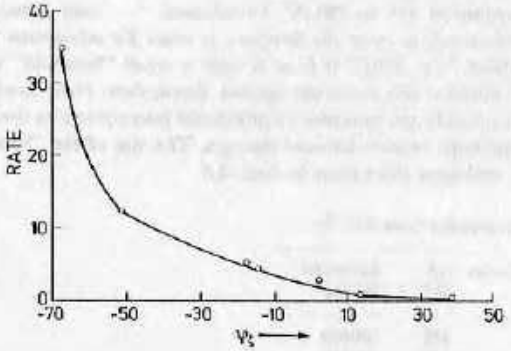

Fig. 26. Plot of the data of Table 6 showing rate of lysozyme adsorption at air/water monolayers of different zeta potentials. Refer to Eq. (35) and text for details (from Ref. [3])

Because of the large size of the protein and the ΔA required for initial adsorption, only a small region of the molecule need participate in the initial stage of the process.

It is well-known that *protein adsorption tends to be at a maximum at the isoelectric point* because the protein has zero net charge. Under such conditions, electrostatic barriers to adsorption are minimized.

Protein adsorption is dependent on the nature and concentration of the electrolyte. Norde and Lyklema have shown [61] that adsorption generally increases with decreasing charge on the protein and on the surface, although counterion effects can often override this generalization.

7.3 Hydrophobicity

The now classic studies by Norde and Lyklema and their detailed thermodynamic analysis (Sect. 4.2) have established that the *interaction between a protein and a surface increases with increasing hydrophobicity of the surface and increases with increasing hydrophobicity of the protein.* "Desorption from hydrophobic surface usually does not occur whereas proteins can often be removed from hydrophilic surfaces by exposure to extreme pH, high ionic strength, or by extensive rinsing [61]."

7.4 Diffusion and Mass Transport

Proteins have a low diffusivity: $D \sim 2$ to 6×10^{-7} cm^2/s. Therefore, the *initial stages of adsorption are generally diffusion limited* and the amount adsorbed is proportional to $t^{1/2}$. If the adsorption rate, dn/dt is:

1. $\sim 1/t^{1/2}$, diffusion is rate limiting;
2. $> 1/t^{1/2}$, then convection or other mass transport effect is present;
3. $< 1/t^{1/2}$, the adsorption is reaction limited; i.e., there are energy barriers to the adsorption process.

7.5 Time

As *contact or residence time increases, the protein tends to orientationally and conformationally adjust to the interface*, leading to stronger bonding, and greater irreversibility of adsorption.

7.6 Surface

Surface adsorption site energy and density are very important. Most *biomaterial surfaces have very high site densities*, making it difficult to study the mechanisms governing adsorption. Low site density surfaces are available. Heterogeneous surfaces, such as block copolymers and polymer blends, may have very unique adsorption properties. If one of the phases or domains tends to dominate the surface, it may act as a homogenous surface. If both phases are present on the surface, then two or more

very different classes of adsorption sites will be present. Protein adsorption on each of the two phases can be very different [80, 81].

7.7 Protein

The protein's intrinsic properties (size, molecular weight, 3-D structure, surface site density, conformational stability) are all very important and must be fully characterized and understood in order to interpret adsorption data.

8 Summary/Conclusions

The general principles of protein adsorption are beginning to be identified and understood. New techniques and methods are now available which, together with well-established methods, allow one to thoroughly probe the adsorption process.

In the past, the solid surface and the solid/solution interface were often poorly characterized and poorly understood. A range of new concepts and tools are now available for the study of solid/solution interfaces [30]. A new monograph on protein adsorption is now available [41]. An international conference on protein and polyelectrolyte adsorption was recently held and proceedings will soon be available [188].

Protein surface structure and conformational dynamics are now much better understood. In the near future, we can expect extensive application of computer molecular graphics to better visualize and understand protein-surface interactions.

Very qualitatively — protein adsorption is roughly understood. The challenge now is to take what we know about proteins, surfaces, and adsorption and to begin to quantitatively model the protein adsorption process.

The future looks bright!

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ANDR-26

CONTACT ANGLE ANALYSIS OF BIOMEDICAL POLYMERS: FROM AIR TO WATER TO ELECTROLYTES

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I. The Early Years Prior to 1960

The surface characterization of biomedical materials prior to the early 1960's consisted primarily of relatively qualitative observations as to whether surfaces were hydrophobic or hydrophilic (1). Contact angle techniques were well-known and were widely applied in industry, and general correlations had evolved between blood interactions in glass as opposed to siliconized glass tubes. Vroman was just beginning his studies on protein interactions with surfaces (2). There was considerable interest during this time period on the interaction of cells in culture with solid substrates, and there was some attempt to correlate and quantitate that interaction through contact angle measurements (3).

II. The 1960's

The situation improved considerably with the development of Zisman's critical surface tension (γ_c) concept in which the advancing contact angle of a series of probe liquids was measured, the data plotted as indicated in Figure 1, extrapolated to cosine θ=1, and the intercept identified as the critical surface tension for wetting. This concept is very well-known and is described in all basic textbooks and reviews on this subject (4,5), therefore will not be discussed further.

Detailed studies with a variety of model compounds, primarily Langmuir/Blodgett monolayers, allowed Zisman and coworkers to relate the critical surface tension to the nature of the functional group or groups present in the surface (5). It was clearly documented (using carefully prepared control surfaces) that the advancing contact angle was highly reproducible and that the surfaces showed minimum contact angle hysteresis. This method was applied by Dr. Robert Baier, who was a research fellow in Zisman's laboratory, to the biomedical materials problem in the late 60's and early 70's (6). The availability of a reproducible and reasonably precise measure of surface properties provided by the critical surface tension encouraged many investigators to attempt to develop correlations between that variable and various biological responses, including blood coagulation, cell adhesion, in vitro cell culture, platelet interactions, and protein adsorption.

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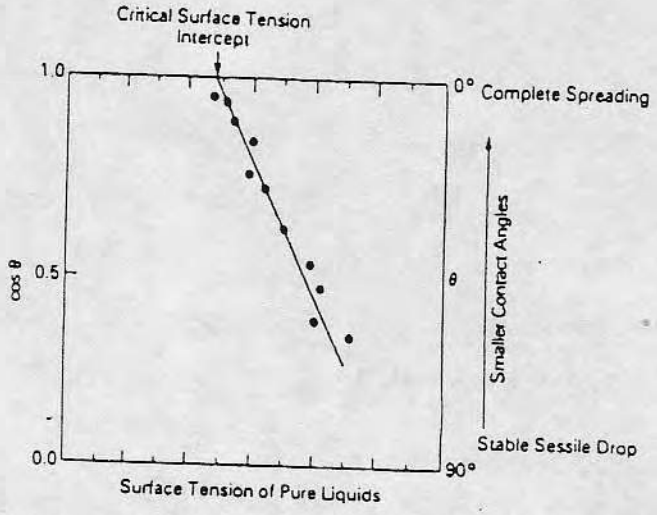

FIGURE 1: A typical Zisman γ_c plot. The cosines of the contact angle for a range of pure liquids on a given solid are plotted against the liquid surface tensions. The critical surface tension is given by the intercept at $\cos \theta = 1$ and is defined as the surface tension of that liquid which would just totally spread on the solid surface. This is an empirical measure related to the surface free energy of the solid and is called the critical surface tension for wetting of that particular solid.

At about the time Zisman was refining the γ_c concept, Fowkes, Good, and coworkers were developing means to estimate surface and interfacial energetics using approximations of the work of adhesion at interfaces based in part, on intermolecular force considerations (7,8). The interactions at interfaces were basically considered to be due to two sources: London dispersion interactions and everything else generally called polar interactions. Fowkes showed that the London dispersion interactions could be deduced for various liquids and approximated for interfaces, using the geometric mean hypothesis commonly used in intermolecular forces (7) (Figure 2).

Simultaneously, Robert Good and coworkers were calculating interfacial interactions from first principles, using the expressions available for intermolecular forces (9). Given appropriate summation and/or integration and assuming intermolecular force additivity among molecules, expressions were developed which basically complimented the Fowkes treatment. Using Good and coworkers methods, it was possible to deduce the surface-free energy of polymer surfaces to compare or correlate that with the experimentally derived critical surface tension and generally to begin to develop a mechanistic understanding of the nature of interfacial processes and biological systems.

Lyman applied the methods of Fowkes and Good to the problem of coagulation and platelet adhesion on polymer surfaces and drew a correlation between the surface free energy of a polymer and its propensity to activate the coagulation of blood (10). Lyman's pioneering work stimulated a great deal of interest in relating the surface properties of polymers to their blood responses.

It is important to point out that all through this period, the contact angle data used was derived by advancing contact angle measurements of a series of highly purified model liquids (6,11).

A number of individuals extended the Fowkes geometric mean approximation for dispersion interactions to nondispersive or polar interactions (12), even though Fowkes clearly warned that such an approximation for polar interactions was not warranted and probably inaccurate. Nevertheless, many workers in the basic polymer surface science community (12) and in the biomedical community (13,14) showed that by such an approximation one could deduce not only the dispersion component of the surface energy or the surface tension of the solid, but its polar component as well. Given these components, one could approximate the interfacial-free energy, including its polar and dispersion component for a set of interfaces (10-14). There is considerable activity even today in estimating dispersion and polar components at interfaces and attempting to relate them to biological events. It is particularly pronounced in the field of bacterial adhesion and in the area of dental materials.

III. The Hydrogel Years

With the invention and development of hydrogels for soft contact lens application by Wichterle and Lim in Prague (15) and with the success and growth of the soft contact lens industry, considerable attention was focused on the role of water and hydrophilicity on biological

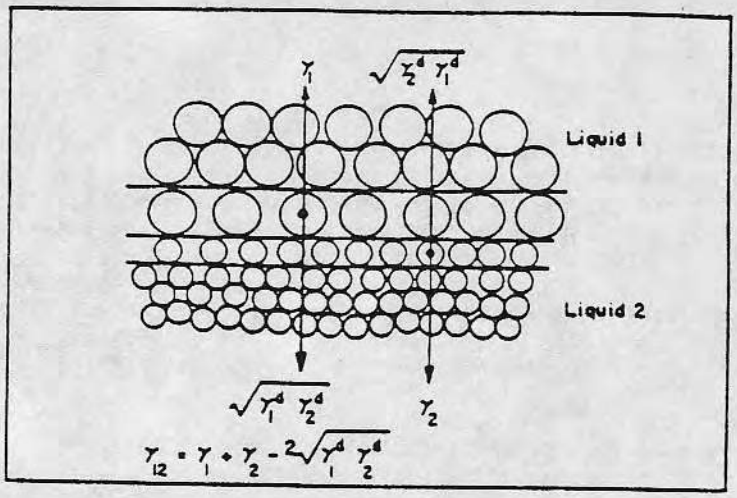

FIGURE 2: Fowkes' model of an interface showing the overall surface tensions (surface-free energies) γ_1 and γ_2 and the geometric mean approximations to the work of adhesion, $\sqrt{\gamma_1^d \gamma_2^d}$ (from Ref. 7).

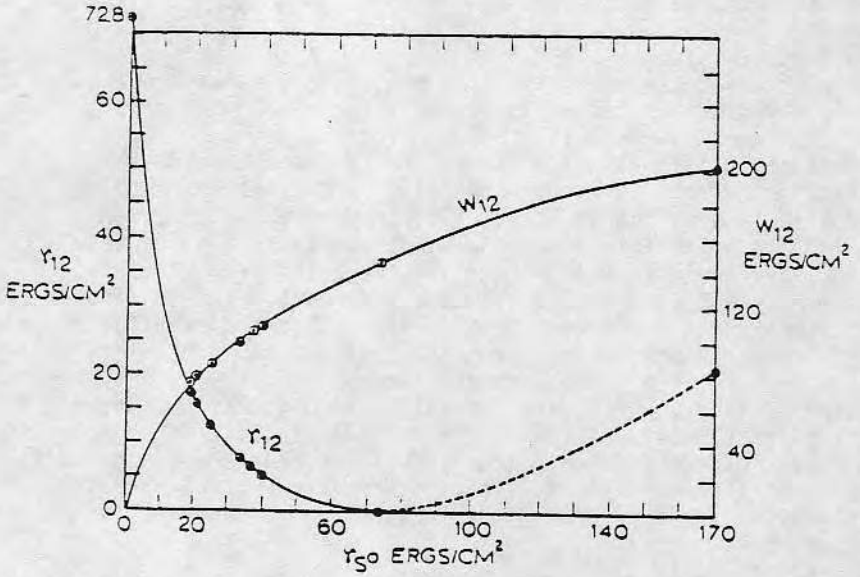

FIGURE 3: Plot of interfacial-free energy, γ_{12}, and work of adhesion, W_{12}, in water as a function of the surface-free energy, $\gamma_s o$, of various solid surfaces (from Ref. 14).

interactions. There were debates as to whether or not the surface of the endothelium was indeed hydrophilic or hydrophobic (6,16,17). There was considerable effort spent on the development of hydrophilic coatings and grafts for polymers. Radiation grafting of hydrophilic monomers to various substrates was a very active field.

Holly and Refojo observed that advancing water contact angles on these highly hydrophilic surfaces showed that the surfaces were hydrophobic, whereas receding water angles indeed showed that they were hydrophilic (18). Clearly the materials were hydrophilic under water, but outside of water some of the polymers appeared to be hydrophobic. This anomaly was explained by Holly and Refojo in terms of the restructuring or reorientation of polymer surfaces in response to their local environment, such as to minimize their interfacial energy (18,19).

In the early 70's, Andrade argued that the critical surface variable for biocompatibility is not the critical surface tension nor the surface-free energy, but rather the interfacial free energy of the polymer against water and showed that highly hydrophilic materials have very low interfacial-free energies (14,20) (Figure 3).

Although this was difficult to demonstrate experimentally, at about the same time W. C. Hamilton had developed a simple technique for measuring the interfacial energetics of polymers by the use of an octane droplet introduced at the polymer water interface (21). Because the surface tension of octane was exactly the same as the dispersion component of the surface tension of water (21.8 dynes/cm), it allowed a number of cancellations in the equations to occur, greatly simplifying the treatment and permitting one to deduce rather straight forwardly the interface energetics (22). (Holly and Refojo were performing similar experiments at about the same time.) Andrade and coworkers applied this method to attempt to deduce the interfacial energetics at gel-water interfaces, showing that indeed within the errors and uncertainties of the methods that the gel water interface has as low an interfacial-free energy as could be measured (20,22-23).

There is now considerable interest in measuring both advancing and receding water contact angles (24). It is well-known that the advancing angle tends to represent or sense the hydrophobic character of the surface, while the receding angle tends to sense primarily its hydrophilic character, and thus both angles are highly useful in getting a fuller understanding of the surface properties.

It has also become evident in the last ten years or so that polymer surfaces can be highly dynamic and can indeed show very different surfaces in air or vacuum as opposed to underwater. The question of the dynamics of polymer surfaces has been recently reviewed (19) and is the subject of a major symposium in June, 1986 (25).

IV. Where do we stand?

There are a number of major, unresolved problems:

1. We have no good way of estimating the polar interactions present at polymer surfaces. For the last ten years or so, Fowkes has treated these interactions as electron donor/acceptor or partial acid/base interactions and showed quite conclusively that, at least in nonaqueous systems, classical dipole/dipole interactions are relatively unimportant if partial acid/base character is present. He has demonstrated contact angle methods of probing these effects and has shown that IR analysis of polymer surfaces is probably the most useful

and direct way to characterize hydrogen bonding and electron donor/acceptor tendencies (26). No one has to my knowledge seriously applied these concepts to the study of biomedical polymer interactions with water and electrolyte solutions.

2. Although we have means to measure advancing and receding angles, the dynamics of such angles, and their hysteresis, it is somewhat difficult to interpret the results. This is because contact angle hysteresis is due to a number of different sources (Table 1). Although water is the solvent of choice due to its biological relevance, water is a difficult liquid to use for contact angle measurements because of its very small molecular volume; it readily penetrates into solid surfaces and is therefore not an inert probe of the surface energetics (27). The role of water penetration into the surface, subsequent water-induced plasticization of the surface region, and finally the inherent surface dynamics of the polymer chains and side chains all influence the contact angle results. The use of larger probe liquids, such as ethylene glycol, glycerol, and perhaps others may minimize the penetration effect (27). The surface dynamics may be partially sorted out by making measurements as a function of temperature. The dynamics can be probed in the absence of hydrophilicity by, for example, the use of model polymers with different alkyl side chain lengths (28,29). Molecular surface dynamics can in principle be probed directly by NMR methods (30).

Although considerable progress was made going from non-aqueous probe liquids to water, water is not the physiologic environment. The physiologic environment is a buffered ionic solution. Preliminary measurements of ionic surfaces with sodium chloride solutions of different ionic strengths suggest that we can no longer continue to ignore the role of ions in the interface energetics of biomedical polymers (31,32). Indeed it has been known for many years from electrokinetic measurements, using streaming potential and related techniques, that neutral hydrophobic polymers are highly negatively-charged based on electrokinetic measurements (33). This was considered an anamoly for a time, but it is now generally understood to result from ion adsorption at the polymer electrolyte solution interface (34). The adsorbed layer of ions basically sets up an interface potential, which is what the electrokinetic method measures. So even for "neutral" polymers, contact angle measurements in ionic solutions may be desirable. They are mandatory for polymers which we know are charged, such as the various sulfate and sulfonic acid containing polymers and surface films with synthetic heparin activity (32,35).

V. What do we know?

We have some current correlations. We know that the dispersion and polar contributions to the surface energy of a polymer and the consequent interfacial-free energies against water do indeed correlate with protein adsorption, cell adhesion, and coagulation time measurements, albeit not always in the direction in which we initially expected (29,36-38). We know that there are also correlations with γ_c and bioadhesion and related phenomena. Baier has postulated that optimum biocompatibility occurs in the γ_c range of about 25 ± 5 dynes/cm (6) because this is the range of minimum bioadhesion, which would be interpreted as minimum protein adsorption or minimum cell adhesion. Although this hypothesis is highly controversial, there is data in the literature to support it, and many groups are following that line of reasoning.

TABLE 1 (Ref. 24)
Sources of Contact Angle Hysteresis

General assumption	Specific assumption	Effect on hysteresis	Time dependent
Surface is smooth	Surface must be smooth at the 0.1 to 0.5 μ level	$\Delta\theta$ increases with increasing roughness (θ_{adv} increases and θ_{rec} decreases with increasing roughness)	No
Surface is heterogeneous	Surface must be homogeneous at the 0.1 μ level and above	θ_{adv} dependent on low-energy phase; θ_{rec} dependent on high-energy phase	No
Surface is nondeformable	Modulus of elasticity in surface $> \sim 3 \times 10^5$ dyn/cm^2	Not known	Yes—due to surface deformation/relaxation effects
Wetting liquid does not penetrate surface	Liquid molecular volume > 60-70 cc/g-mole	Increased liquid penetration leads to increased hysteresis	Yes—due mainly to diffusion
Surface does not reorient	Reorientation time $\gg$ time of measurement	Increased tendency to orient leads to increased hysteresis	Yes
Surface immobile, therefore, surface entropy is constant	Configurational entropy independent of local environment	Unknown—but probably increase in hysteresis as surface mobility increases	Yes

VI. What do we need to consider?

Consider a hypothetical protein adsorption experiment (Figure 4). Figure 4a shows the polymer surface in air. We assume that this is a highly mobile polymer surface with both polar and apolar constituents. In air, it is an apolar material with its surface-free energy minimized. The material is introduced into water or electrolyte solution. It quickly restructures its surface and rearranges to show its polar constituent to the aqueous phase, thereby minimizing the interfacial-free energy (19). A protein diffuses by; the protein collides with the surface (Figure 4). Clearly the analysis of that initial contact must be done, respecting the fact that the surface is fully equilibrated in water just prior to and at the moment of contact. The surface the protein "sees" can therefore only be characterized or measured by underwater or receding water contact angle methods (24) (Figure 4b).

In order for the protein to contact the surface, water must be displaced from both the protein contact area as well as the surface contact area. Clearly, it is unlikely for that water to be displaced if it is tightly bound to either the protein or the surface. If that is the case, the protein will simply diffuse away, and the collision would have resulted in no net adsorption. If however, the protein now collides in a different orientation, perhaps one exposing a hydrophobic patch and perhaps if it hits the region of the surface which is partially hydrophobic and from which the surrounding waters can be removed, then there will be a transient interaction or adhesion. If the interaction energy is large enough, the protein will have a residence time at the surface. Given the various principles of protein adsorption (39), a number of things may happen. In light of the new local environment and of the statistics and dynamics of the process, another region or part of the protein may contact the surface leading to a second attachment point or "foot." If the polymer surface is itself in motion, a portion of the polymer surface may statistically approach and contact the protein. Once we have two or three contacts we have a cooperative binding process, and the protein is essentially adsorbed.

Now begins the process of long-term conformational change and accommodation--both of the protein, which is now said to be undergoing "denaturation," and the surface, which is restructuring or modifying in light of its new microenvironment. If the surface is binding to the protein through hydrophobic associations, then it may well be that characterization of the polymer surface in air (through advancing contact angle measurements) is important in estimating the hydrophobic "potential" of the surface. Although those hydrophobic polymer surface residues would not have been exposed during the initial phases of protein contact, they may well be exposed and participating in the interaction later on in the process as the protein and polymer surface maximally accommodate to one another (Figure 4d).

So measurements of polymer surface properties in air are neither good nor bad, just as measurements in water are neither good nor bad. Both are important and both are necessary in order to optimally correlate and understand the processes. In fact, ideally we would like to know the surface relaxation or restructuring time to get an idea as to what is the probability for a surface to accommodate to or "denature" in response to an adsorbed protein.

VII. Conclusions

The surface properties of biomedical polymers are indeed important in the adsorption of proteins and in subsequent biological interactions. It

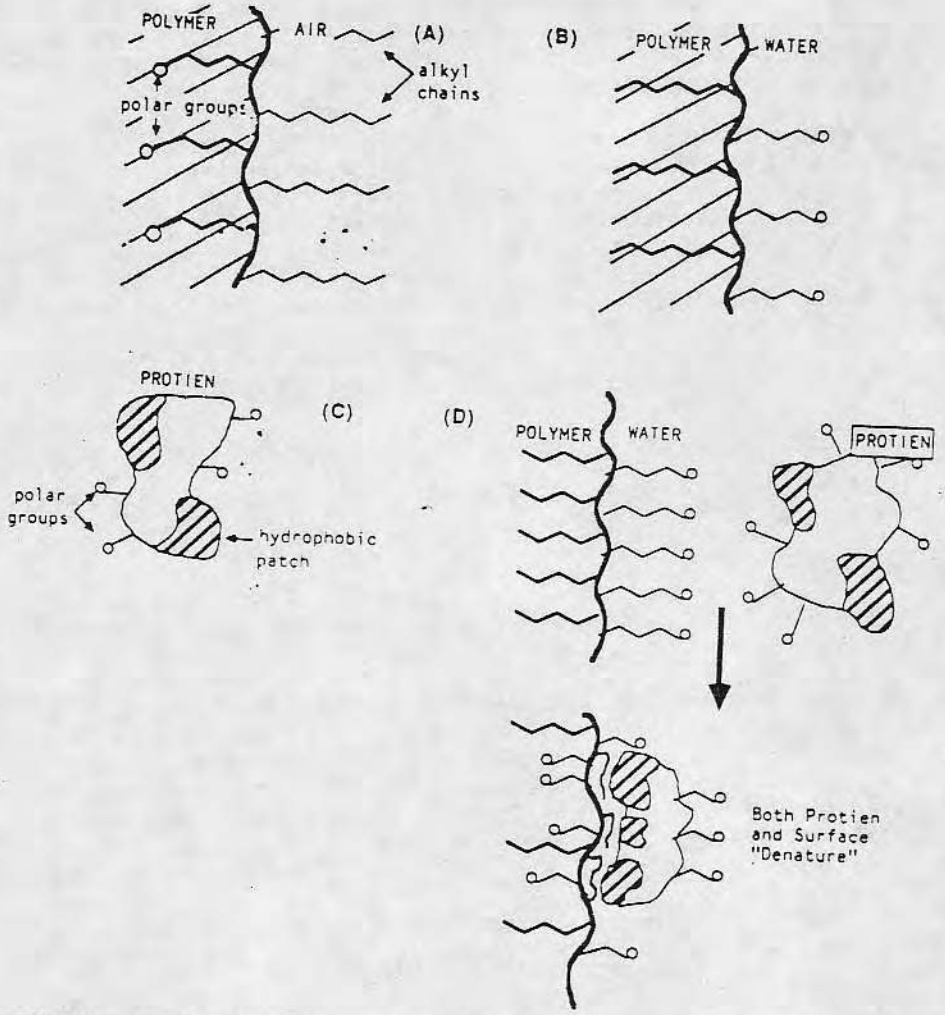

FIGURE 4: A schematic view of a dynamic polymer surface in different environments.

a) in air;

b) in water;

c) a protein molecule in water exhibiting its equilibrium "surface";

d) the polymer-adsorbed protein system with the protein shown interacting mainly via hydrophobic interactions and denatured to expose a third hydrophobic patch to the surface. The polymer surface has restructured to hydrophobically interact with the protein and, where appropriate, hydrophobically interact with the aqueous phase.

is important to characterize polymer surfaces with full awareness that they may be highly dynamic and somewhat amphoteric in character. Measurements should be made under conditions which carefully characterize both the hydrophobic and hydrophilic nature of the surface, which at this stage is best performed by advancing angle measurements to probe the hydrophobic character and under water or receding water measurements which probe the hydrophilic character. These measurements should, if possible, attempt to deduce the time course of surface restructuring or reorientation in going from the hydrophobic to hydrophilic environment and vice versa.

In addition, we must begin to develop standardized methods to probe the partial acid/base or electron donor/acceptor properties of polymer surfaces and their interfaces with aqueous electrolyte solutions. This can in part be done by electrokinetic (zeta potential) methods, as well as perhaps by infrared and Raman spectroscopy, preferably in the electrolyte solution of interest. The dynamics of polymer surfaces can indeed be directly probed by nuclear magnetic resonance and electron spin resonance spectroscopy, as well as perhaps by fluorescence spectroscopy. It is expected that these methods will continue to develop and will begin to be applied by the biomaterials community in the very near future.

IX. Acknowledgements

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Fiber optic immunodetectors: sensors or dosimeters?

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Abstract

Biosensors based on specific interaction of the analyte of interest (ligand) with a biochemical agent (receptor) which specifically and tightly binds the analyte are being developed by many groups, agencies, and institutions using a variety of detection technologies. We are studying fluorescent methods using evanescent wave excitation on the surface of planar and cylindrical (fiber optic) optical waveguides. A relatively low ligand-receptor binding constant results in reversibility but low sensitivity. Most biological receptors bind very strongly and specifically--almost irreversibly--which provides an "irreversible" means of detection. Means to regulate ligand-receptor binding constants are discussed in order to provide semi-reversible, sensitive, specific sensors, rather than "one-shot" detectors or dosimeters.

Introduction

A homogeneous silica optical fiber with its coating and cladding removed will interact with its surroundings via evanescent-wave modes at the interface. Antibody (Ab) or antigen (Ag) molecules can be covalently linked to the silica fiber surface. The immobilized biomolecules will bind complementary antigen or antibody from a surrounding solution. If the bound antigen or antibody is fluorescent, a fluoro-immunoassay can be performed.[1,2] The sensitivity of such a sensor is enhanced in a competitive immunoassay mode, where a fluorescently-labelled antigen or antibody competes for the binding sites on the immobilized biomolecules, thereby providing a competitive binding fluoro-immunoassay. Such sensors have the potential for remote, unattended, pseudo-continuous monitoring of biomolecules, providing that the Ag-Ab complex is reversible and responds rapidly to the bulk Ag (or Ab) level in the solution.[1-3] In order to maximize sensitivity, it is desirable for the Ag-Ab constant to be as high as possible, meaning the off-rate is very slow--hence slow response times. Ideally the Ag-Ab binding constant should be maximal during the analysis and then be very low between analyses. Thus the sensor would function as a dosimeter which can be "zeroed" between measurements, thereby maximizing both sensitivity and "reversibility." Because of our interest in developing fiber optic or optical wave guide immunosensors[1], optical modulation of the binding constant is desired. Several approaches are suggested based on photo-isomerization processes which change the microenvironment of the binding site, which in turn affects the binding constant. The photo isomerization approach is compatible with the goal of developing truly remote, semicontinuous, unattended immunosensors based on optical fibers and wave guides.

Why regulate binding constants?

Specific chemical assay in complex mixtures generally depends on the use of specific, high affinity binding agents, such as antibodies, membrane receptors, enzymes, lectins, chelates, etc. Generally the greater the binding constant, the greater the ultimate sensitivity of the assay. Such assays are "one-shot" measurements. In the case of immunoassay[4], one takes a sample, mixes the reagents, makes a reading, then everything is discarded.

There is considerable interest and activity in developing specific chemical sensors, i.e. detectors which respond to changes in concentration of a specific chemical--either continuously or at least semi-continuously.[1-3] Clearly we desire a high binding constant for maximal sensitivity, but we may also require a fast response time to permit continuous or semicontinuous measurements. Some means of decreasing the binding constant between measurements is therefore desirable. Ideally we would like to be able to "zero" the sensor between each measurement and yet make the measurement often enough to have a near continuous readout. Finally, we prefer a sensor with the maximum possible dynamic range. These characteristics are generally mutually exclusive, unless we can regulate the binding constant.

The very high binding constants, which provide these agents with their exquisite sensitivity, usually means that the dissociation rate of the complex is very slow.[5] Therefore, such systems are in reality dosimeters rather than true sensors or function as sensors with very slow time response. In order to reuse such a device, there must be a means to weaken the bond to permit the complex to dissociate in a reasonable time.

In the case of membrane receptors for biochemicals, toxins, and drugs, the ligand-receptor complex is often internalized or otherwise "turned over." New receptors are put in place on the membrane surface.[6] The standard means to dissociate Ag-Ab complexes is to induce a significant conformational change in Ag, Ab, or both, by drastic changes in the local solution environment, such as pH 2-3, pH 11, high concentration of chaotropic salts, high concentrations of agents which diminish hydrophobic interactions, etc.[7] Unfortunately, such treatments often lead to irreversible conformational changes which destroy the specific binding properties.[7] In addition, it is difficult to deliver acid, base, etc., on command to a remote sensor site.

There is a great need for a method which can remove specifically bound ligand without the use of often damaging eluting agents.

The ability to regulate ligand-receptor binding constants would be of major significance to the development of (a) truly effective specific binding sensors with optimum sensitivity, response times, and dynamic range; (b) better molecular separation processes; (c) better understanding and control of a wide range of biological and medical processes.

Our discussion will be directed at the antigen (Ag) - antibody (Ab) interaction and its application in a remote, semi-continuous immunosensor. The concept and technology is applicable to the general case of ligand-receptor interactions and to basically all processes which depend on stereo-selective molecular interactions.

If L denotes the concentration of unbound hapten ligand or antigen; P, the concentration of unoccupied antibody active sites; and PL, the concentration of hapten-active site complex; then the equilibrium binding expression is given by:

$$P + L \underset{}{\overset{Ka}{\rightleftharpoons}} PL \ , \qquad (1)$$

where: $Ka = [PL]/([P][L])$. Ka is defined as the intrinsic association constant for the interaction which is related to the individual association and dissociation constants. Using similar notation, the association and dissociation rates are given by equations (2) and (3) respectively:

$$P + L \overset{k1}{\rightarrow} PL, \quad d[PL]/dt = k1*[P]*[L] \qquad (2)$$

$$P + L \overset{k2}{\leftarrow} PL, \quad d[PL]/dt = -k2*[PL] \qquad (3)$$

where k1 is the second order, biomolecular association rate constant (afferent rate constant), k2 is the first order, unimolecular dissociation rate constant (efferent rate constant), and d[PL]/dt is the time derivative of the concentration of hapten-active site complex. If the interacting system contains only the above three species (P,L, and PL), then both afferent and efferent rates are concurrent, and the entire rate law is given by equation:

$$d[PL]/dt = k1*[P]*[L] - k2*[PL] \qquad (4)$$

Finally, at equilibrium no changes in complex concentration can occur; i.e. $d[PL]/dt = 0$. Therefore:

$$k1*[P]*[L] - k2*[PL] = 0 \qquad (5)$$

$$k1/k2 = [PL]/([P]*[L]) \qquad (6)$$

The right sides of equations (1) and (6) are identical, establishing the desired relation between the equilibrium and individual rate constants:

$$Ka = k1/k2 \qquad (7)$$

The first order dissociation constant (efferent rate constant) is thought to reflect directly the binding strength between hapten and the antibody.

The antibody active sites are 50% saturated when [L] = 1/Ka. Furthermore, if we assume that ligand binding can be accurately measured for active site saturation values between 10% and 90%, then the sensitivity of the immunoassay is about two orders of magnitude in ligand concentration: $0.1/Ka < [L] < 10/Ka$. For example, an immunosensor utilizing a monoclonal antibody with an affinity of $10^8 M^{-1}$ could accurately measure ligand concentrations between 10^{-7} and 10^{-9} molar. This range could be extended by using several monoclonal antibodies with affinities differing by two orders of magnitude. It can also be shown that the affinity of the antibody must be decreased by two orders of magnitude to dissociate 90% of

the ligand. This is well within the range observed with changes in solvent polarity (next section).

Clearly the ability to regulate a binding constant would permit a sensor to function over a much wider range of concentration.

Monoclonal antibodies to the hapten fluorescein have been produced[16] and their association rates and dissociation lifetimes measured[8] by a fluorescence quenching technique[9] (Table 1). The affinities of these antibodies vary over a 650-fold range. Most of this variability arises from the dissociation rate (the dissociation lifetime ranges from 11 to over 5000 seconds), which is thought to reflect directly the binding strength between hapten and the antibody.

Table 1. Kinetic Parameters of Selected Monoclonal Antifluorescyl Antibodies

Clone	Association Rate	Dissociation Lifetime	Affinity
	$M^{-1}\ s^{-1}$	s	M^{-1}
4-4-20	6.28×10^6	5376	3.38×10^{10}
20-19-2	1.28×10^6	454	5.81×10^8
20-20-6	1.08×10^7	38	4.10×10^8
6-10-6	5.33×10^6	14	7.46×10^7
20-4-4	4.67×10^6	11	5.14×10^7

Kinetic experiments were performed at 2^o C. Association rates were determined using a pseudo-first order assay in which unliganded antibodies were added in 10-100 fold excess to 1.0 nM fluorescein. Dissociation lifetimes (reciprocal dissociation rates) were determined using a competitive inhibition assay in which 5-aminofluorescein was added in 10-fold excess to liganded antibodies. Affinity constants were computed from kinetic parameters.

Approaches to binding constant modification and regulation.

Thermodynamic studies of antifluorescyl antibodies[8] have suggested several approaches for altering the affinity of these antibodies. The first approach is temperature perturbation. All three antibodies exhibited negative enthalpies which produced strong temperature dependencies in affinity (Ka) values. Affinities decreased by about 300-fold as temperature was increased over a range of 2^o to 70^o C. The effect was fully reversible over this temperature range. Negative enthalpy changes have been reported for several other anti-hapten antibodies,[10-12] and may be a general feature of antibody reactions. Thus, affinity modulation by temperature perturbation looks quite promising.

Another approach is based on reducing the contribution of the hydrophobic effect to ligand binding. This can be accomplished by decreasing the polarity of the bulk solvent. This approach proved effective for antifluorescyl antibodies, which exhibited negative heat capacities indicative of hydrophobic binding. An experiment, using the 4-4-20 protein, is shown in Figure 1.[13] Liganded samples of clone 4-4-20 were titrated with 2-methyl-2,4-pentanediol (used as a solvent for protein crystalization) over a concentration range of 0-50% (v/v). The affinity of the antibody decreased 1000-fold over this concentration range.[13]

The hydrophobic effect has been implicated in almost all protein-ligand interactions studied to date and is thought to be a generally operative mechanism in these reactions.[14] Thus, controlled use of organic molecules is an effective way to modulate affinities.

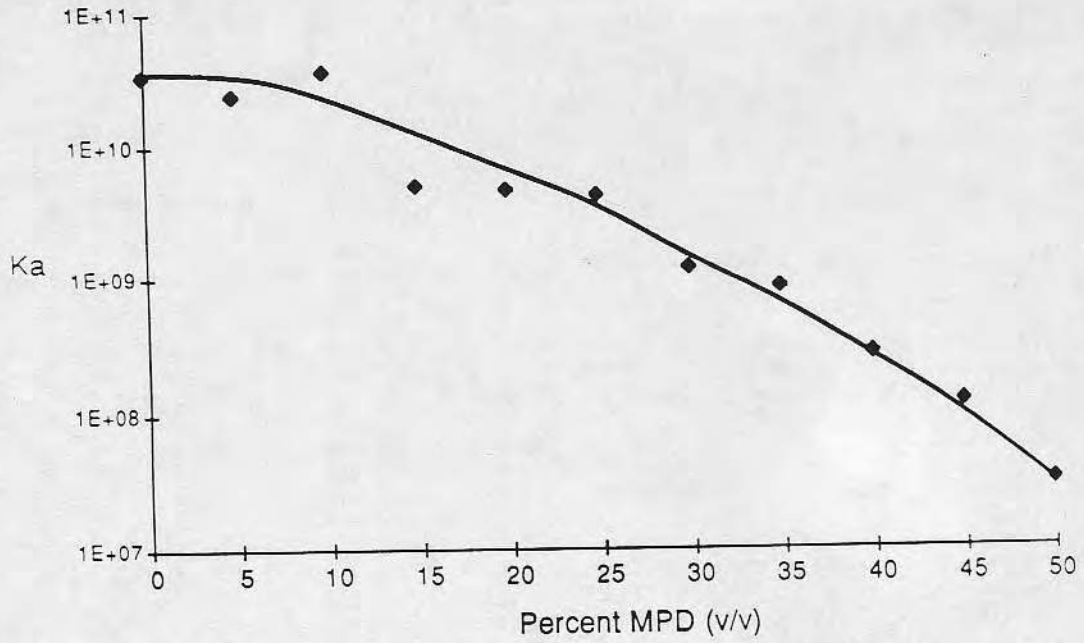

Figure 1. Effects of 2-Methyl-2,4-Pentanediol (MPD) on the affinity (Ka) of 4-4-20. Aliquots of MPD were added to liganded 4-4-20 in 50 mM sodium phosphate buffer (pH 6.8). Preparations were allowed to equilibrate overnight before affinities were determined by fluorescence methodology (from Ref. 13).

In summary, both elevated temperatures and organic molecules which disturb hydrophobic association in water effectively lower the affinities of antifluorescyl antibodies. Both effects appear to be fully reversible during the time course of the experiments (24 hrs.). The long term stabilities of the antibodies under these conditions will have to be investigated further. Comparison of results obtained for the 4-4-20 protein suggested that changing solvent polarity was more effective than temperature perturbation. This was especially true at room temperature. Using the 4-4-20 protein as a "worst case" scenario, changes in affinity of 100- to 1000-fold can be achieved by altering solvent polarity. The dissociation rate is expected to respond in a similar manner to organic solvents. The relation of the rate constants to affinity (Ka) is given by the following relation, where k1 and k2 are the association and dissociation rate constants, respectively:

$$Ka = \frac{k1}{k2} \qquad (9)$$

Increasing concentrations of organic solvents will increase the viscosity of the solvent, which will decrease k1. This effect will only amount to about a two-fold change, so the majority of the decrease in Ka will probably arise from a faster dissociation rate.

Some consideration must be given to the usefulness of antifluorescein as a model system. In our discussion of affinity modulation by temperature perturbation and solvent polarity, we have primarily been concerned with the magnitudes of enthalpy and heat capacity changes. Enthalpy values of about -10 kcal $mole^{-1}$ have been reported for a number of different antibodies.[10-12] This value is certainly close to the average enthalpy of the antifluorescyl antibodies discussed here. Antifluorescyl antibodies also exhibit average heat capacity values. In a compendium of twelve different protein-ligand interactions, Sturtevant[14] found that heat capacity values fell in a range of -100 to -1000 cal K^{-1} $mole^{-1}$, with an average of about -300 cal K^{-1} $mole.^{-1}$ Thus, we believe that the antifluorescein system is a good representative of ligand-binding proteins in general.

How can we do it?

How can we apply the relatively basic data and conclusions just described to the development of means to externally regulate and control ligand-receptor binding? We have considered several approaches. Our discussion of these approaches will focus on the case where the specific model binding agent (antibody, Ab) is immobilized at a solid surface. We also assume for simplicity that the immobilized Ab surface is placed in a flowing, isothermal solution.

It is clear that the dissociation rate constant (k2) can be increased by a factor of 10 if the interfacial temperature could be changed from 20^{o} to 40^{o} C, decreasing the response time

from around 500 sec to nearly 50 sec. Although this approach has potential as there are ways to perturb the interfacial temperature by remote means, we prefer the solution microenvironment approach.

Since Ag-Ab binding is highly stereo specific, a change in the Ab binding site conformation is likely to significantly change the binding constant. The conformation can, of course, be altered by changes in the local solution polarity and hydrophobicity. The conformation may also be regulated by a direct photoisomerization process or indirectly by a photoisomerization process coupled to the active site via an allosteric effect. There are many biological examples of such processes.[15]

The most extensively studied photoisomerization process is that of azobenzene and its derivatives.[16]

Figure 1 demonstrated the dramatic effect of pentanediol on the Ag-Ab association constant. It is well known that local changes in pH, ionic strength, or concentration of agents which alter water structure (such as chaotropic salts, ethylene glycol, pentanediol etc.) can disrupt Ag-Ab complexes.[7,14] Indeed this is a key part of the affinity chromatography process.[17] As relatively high local solution concentration of these agents are required, one immediately has a reagent delivery problem. As our interest is in the development of simple remote sensors, how can one deliver such an agent, remotely, and without plumbing?

Consider Figure 2. Antibody or receptor is covalently immobilized at the solid surface. A synthetic polymer or polypeptide containing azobenzene groups in the side chain (or the main chain) is co-immobilized. The Ab (IgG) dimensions are ~150 Å --the binding sites can be oriented away from the surface as shown. The same approximate dimensions would hold for a membrane receptor, such as the acetylcholine receptor.

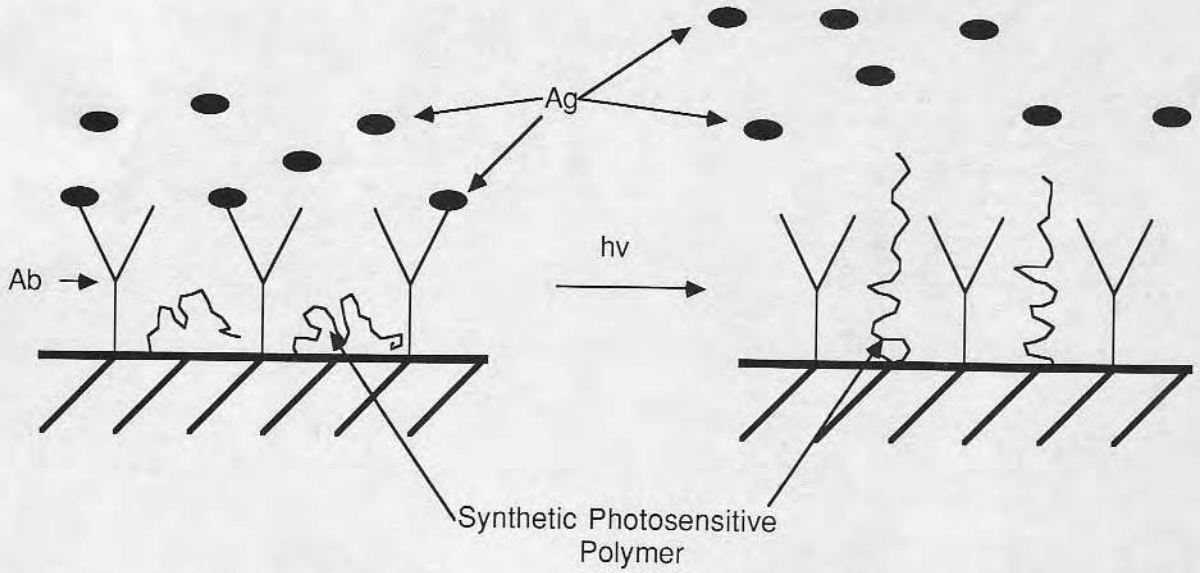

Figure 2. A synthetic photosensitive polymer co-immobilized with an antibody (Ab) at a solid-liquid interface. Light of energy hν induces a conformational change in the synthetic polymer which produces a coil expansion. The expanded polymer changes the solution microenvironment in the vicinity of the Ab binding sites, thus regulating the Ab-Ag binding process.

The trans-cis isomerization of azobenzene-containing polymers can result in significant changes in the shape and size of the polymer molecule. In the case of free molecules in solution, this results in major changes in viscosity--and is called the photoviscosity effect[18]. For a polymer molecule attached to a rigid solid surface, the expansion of the coil results in the molecule extending further into the solution. As the polymer chain expands towards the Fab region, the solution properties around the Fab binding site are greatly altered. If the polymer is similar to pentanediol in general chemical nature, then we would expect results similar to those in Figure 1.

Although the photoviscosity effect is well known in solution studies[18] and has been demonstrated in membranes and gels[18-22], we are unaware of any work on the interfacial application described above (Figure 2). The few available photoviscosity studies have tended to use charged polymers, where photoregulation is achieved via ionization of groups

(via an induced change in ionization constant or pH) which then repel each other and expand the polymer.[23]

There are a variety of ways to induce and regulate the effect, all involving the action of photoisomerizable groups. For stability the photo-active group should be covalently attached to the polymer or protein rather than physically bound or complexed. The photoactive group may be a comonomer which is incorporated into the polymer during the polymerization process. Another approach is to bind or graft the group as a side chain.

We are investigating such possibilities using a variety of synthetic, methacrylate-based hydrophilic polymers. Results will be reported later.

Acknowledgments

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Probing Polymer Surface and Interface Dynamics*

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Polymeric materials generally exhibit various molecular motions and relaxations. Such relaxation processes, which include the glass transition temperature, have significant effects on physical and mechanical behavior. Polymer molecules and segments at surfaces and interfaces also exhibit motions and relaxations. In air or vacuum, such motions 'permit' the surface to restructure to minimize the surface free energy. In aqueous solution, the polymer surface restructures and reorients to optimally interact with the aqueous solvent, thereby minimizing the interfacial free energy. XPS and related high vacuum techniques probe the vacuum-equilibrated surface. The best way to probe the polymer–liquid interface is via dynamic contact angle or wetting methods. A number of issues and concerns are discussed: (1) the size or hierarchy of structures; (2) the time course of surface dynamic processes; (3) theory, modeling, and simulation of surface dynamics; and (4) experimental methods.

INTRODUCTION

The powerful instrumental surface analysis techniques which have been developed over the last 20 years are being increasingly applied for the study of polymer surfaces and interfaces. Generally these methods (such as XPS, Auger electron spectroscopy, SIMS, etc.) require ultra-high vacuum environments. The assumption is generally made (often implicitly rather than explicitly) that the polymer surface is indeed stable and that the results of the analysis are applicable to the non-vacuo environments wherein the polymer surface is usually applied. Such an assumption is often invalid, particularly for polymers used as biomedical devices or in other applications where the polymer surface is exposed to water or other highly polar environments.

Polymer surfaces can be highly mobile. Such surface dynamics permit the interface to restructure or reorient in response to different environments. The effect is particularly pronounced in aqueous solutions, where the polarity of the aqueous phase provides a high interfacial free energy driving force for the migration or orientation of polar phases, blocks, segments, or side chains towards the aqueous phase, thereby minimizing the interfacial free energy. In vacuum, air, or other nonpolar surfaces, the polymer orients its apolar components towards the interface, again minimizing the interfacial free energy. These effects are now becoming well-known and a recent review is available.[1] A conference on the subject was held in June, 1986.[2]

There are a number of issues and concerns (Fig. 1):

The size or hierarchy of structures

These are responsible for polymer surface dynamics, ranging from blocks or domains to small side chain functional groups. The surface dominance of apolar phases, blocks, or domains at the interface with air and vacuum is now well established. Polymers containing very low energy blocks (fluoropolymers and silicones are the best examples) generally exhibit the surface characteristics of the low energy constituent.[3,4]

The elegant and pioneering freeze-etch XPS study by Ratner *et al.* of polyacrylamide or polyhydroxyethyl methacrylate (both highly polar polymers) grafted onto polyethylene or onto polydimethyl siloxane (both highly nonpolar) clearly demonstrated the dominance of the polar phase at the water or ice interface, followed by reorientation and dominance of the nonpolar phase at the vacuum interface.[4,5]

The effect is well documented in blends. It is a common observation that polymers containing even small amounts of silicone exhibit surface properties (in air or vacuum) characteristic of pure silicone materials.[5,6]

Homopolymers or simple copolymers with amphiphilic (both polar and nonpolar character) tend to orient the main chain and side chains in response to their environment in order to minimize the interfacial free energy.[7,11]

One component of surface dynamics which has been virtually ignored, except in the chromatography literature, is the role of intrinsic side chain mobility on the surface properties. A case in point is the alkyl chain dynamics on reversed phase chromatography supports.[12,13] Clearly there should be a surface configurational entropy or excluded volume effect on the surface properties.[14] This is generally ignored in theoretical treatments of interface thermodynamics as, again, the surfaces are generally assumed to be rigid and immobile. We have discussed the effect qualitatively[14] and Jhon *et al.* are treating it theoretically.[15]

In water, long alkyl chains are expected to collapse towards the solid surface due to hydrophobic interactions. This has been discussed recently by van Damme *et al.*,[16] as well as in the chromatography literature.[12,13] Finally neutral hydrophilic chains are expected to be extended and highly mobile in water[17] and collapsed or even substrate 'buried' in air or vacuum.

The time course of such processes (Fig. 2)

How long does it take a surface to adjust or relax to a change in its environment? Relaxation effects in bulk

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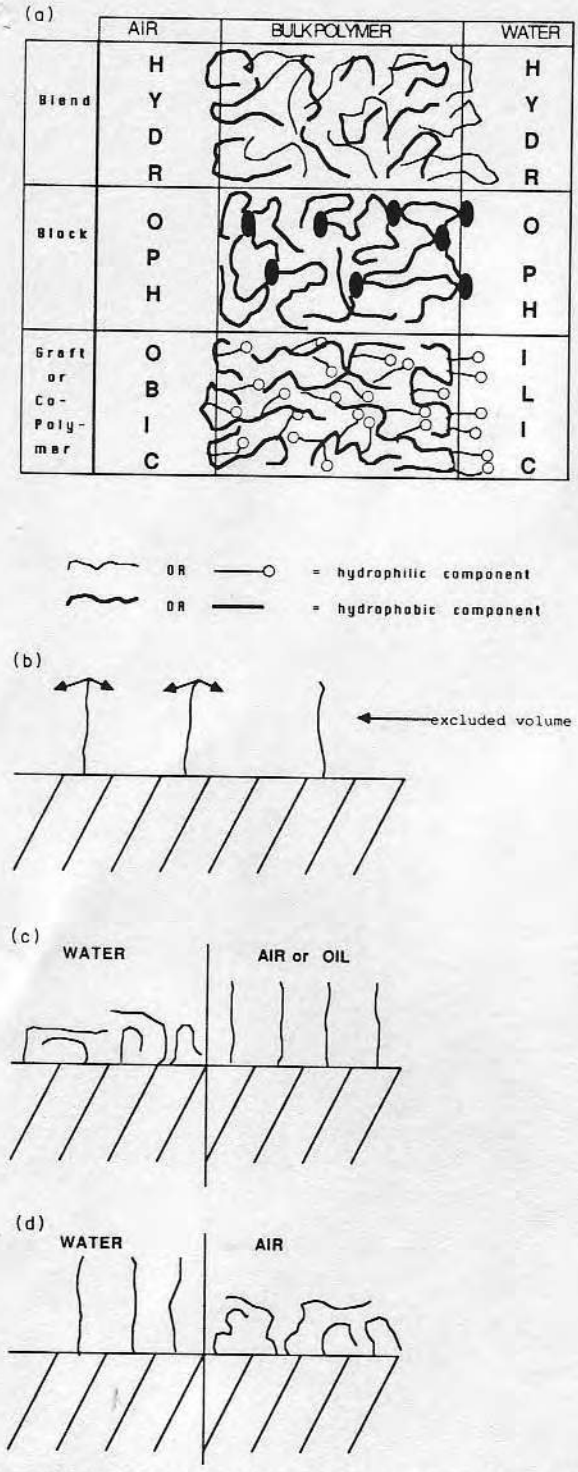

Figure 1.(a) Surface reorientation of hydrophobic and hydrophilic components due to the presence of a low energy or high energy interface. The bold line indicates the hydrophobic component. The —○ indicates a hydrophilic side chain, and the ● indicates a hydrophilic phase or domain. *Blend*: A blend of two homopolymers segregates or orients at the interfaces as shown; the hydrophobic polymer dominates in air or oil, the hydrophilic in water. *Block*: The hydrophilic block dominates at the water interface; the hydrophobic phase dominates in air or oil. *Graft or Co-Polymer*: Again, the hydrophilic component dominates in water. (b) An extended chain or functional group will exhibit motions resulting in a significant configurational entropy component to the interfacial thermodynamics. These effects have not been considered in standard texts on interfacial thermodynamics. (c) Alkyl or other hydrophobic chains at an interface will tend to be extended in air or oil and compacted in water. (d) The opposite to 1(c), where a neutral hydrophilic chain, such as polyethylene oxide, is partially extended in water and partially collapsed in air.

polymers are well-known and form a major subject of inquiry and application in polymer science and engineering.[2] Bulk relaxation transitions, such as the glass transition, side chain rotation, etc. are well-known and generally understood. Although clearly polymer components adjacent to an interface will have different motions and relaxation due to the influence and constraints imposed by the interface, we do expect some relation or, at least, correlation between the bulk relaxation and the relaxations active at the interface.[2,8] Clearly the time course must depend on the intrinsic rigidity of the polymer. In the case of a flexible elastomer or in general at temperatures substantially above the Tg, we can expect the surface accommodation to take place in the seconds to hours range, while highly rigid polymers may require hours, days, or even longer. Very little experimental data are available.[2] Dynamic contact angle methods provide one way of probing such phenomena.[14]

Theory, modeling, and simulation

One can successfully model the energetics and motions of polymer chains in vacuum or in ideal solvents. Such modeling is difficult in polar solvents (water is the most difficult of all) and for polar polymers. Some realistic measure or estimate of polymer solvent interaction or solubility parameters must be available. This is especially difficult in water due to the unique partial acid-base character of water and due to hydrophobic interaction effects. Fowkes and co-workers have tackled the job of estimating partial acid-base properties of polymers using enthalpic heat of solution and IR band shift data (see Ref. 18 for a review). The water problem is being addressed successfully by the protein biochemistry community and estimates of polar and hydrophobic interactions for protein functional groups in water are becoming available.[19] The question of interface motions or dynamics is, of course, complicated by the polar and hydrophobic interactions, the latter which is due primarily to solvent (water) entropy effects.

Experimental methods

We have already argued that XPS, SIMS, and related methods are not too helpful. We feel that three methods are particularly useful:

(a) Contact Angle Dynamics[14]
(b) Interfacial Fluorescence[25,26]
(c) Interfacial NMR[30-33]

Contact angle dynamics. The dynamic electrobalance method for contact angle and surface tension measurement is well suited for the study of contact angle kinetics and hysteresis. We have previously reviewed and described the method.[14] It has been applied by a number of groups to study contact angle and surface dynamics.[2,14,16] The method is suitable for studying the time dependence of the contact angle and thus the time dependence of the interfacial equilibration, although very few data are available in the literature (see Refs 1-2).

There are data on the immersion and emersion velocity dependence on the dynamic contact angle, usually interpreted in terms of liquid viscosity and relaxation

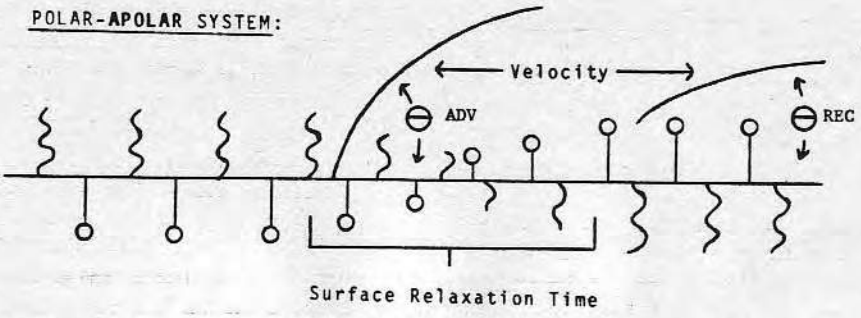

Figure 2. The Holly and Refojo Model[10] of polymer surface reorientation or relaxation. The O—— indicates hydrophilic pendant groups, the ∿∿ indicates hydrophobic pendant groups. The hydrophobic groups orient towards the outer surface in air, minimizing the surface free energy; the hydrophilic groups orient towards the water phase in water, minimizing the interfacial free energy. The measurement will be dependent on the velocity if the surface relaxation or reorientation time is in the same range.

effects.[22] Using a low viscosity liquid, such as water, velocity effects should be dependent on polymer surface relaxation times (Fig. 2). One must keep in mind, however, other mechanisms for change in the angle with time, such as water penetration (absorption) effects. Water absorption can be studied by ^3H_2O measurements.[20]

An effect which we have observed in a preliminary manner is the change in buoyancy slope observed in a Wilhelmy plate hysteresis loop at constant velocity. In a study of the surface properties of poly sulfo-*n*-alkyl methacrylates, we observed significant differences in the advancing and receding slopes, which correlated with alkyl chain length and solution ionic strength.[21] The effect is maximized at low ionic strength (distilled water), attributed to the charged polymer side chains and polymer loops or tails being extended into the aqueous solution, whereas the charged groups would be driven towards the surface in air. The process is also dependent on alkyl chain length and flexibility. Such surfaces are far from simple and clearly are exhibiting strong polyelectrolyte and ionic surfactant effects. They do serve to demonstrate, however, that these effects are significant and can, in principle, be studied by dynamic contact angle methods.

Interfacial fluorescence. Various fluorescent probes are sensitive to interface polarity, interface electrical potential, interfacial pH, and even interface motions.[23] Such probes have been widely used for the study of biological membranes and are beginning to be widely applied for the study of chromatographic supports.[25,26] We have applied a total internal reflection fluorescence (TIRF) method to probe the adsorption of proteins at polymer-water interfaces.[24] Harris and coworkers are studying alkyl-derivatized silica by the TIRF method, using fluorescent probes.[25] If the probe is covalently immobilized at the surface, then one can perform stop-flow kinetic experiments and directly measure the surface relaxation processes via the time course of fluorescence emission.

Nuclear magnetic relaxation (NMR). NMR techniques are the most direct means of obtaining information on interface mobility and dynamics. High surface area particulate systems are used because of the inherently low sensitivity of the technique. Pulsed Fourier transform ^{13}C methods with proton decoupling permit spin-lattice (T_1) and spin-spin (T_2) relaxation times to be deduced. Much of the work has focused on alkyl-derivatized silicas because of their great importance to the chromatography field.[12] Polymer adsorption at silica and polymer latex surfaces has also been examined, particularly polyethylene oxide on silica. These studies permit conclusions as to chain and segment mobility.[12,17,30-33]

CONCLUSIONS

Given sufficient mobility, polymer surfaces will reorient or restructure in response to their local micro-environment to minimize their interfacial-free energy with the surrounding phase.

The interfacial-free energy at a polymer-water interface is a sufficient driving force to cause restructuring of the polymer surface and orientation of the dipolar and other groups, which can directly interact with water towards the aqueous phase. These processes are time-temperature dependent, and correspond to the relaxation characteristics of the polymer; thus, long equilibriation times with water may be required before the effect is maximally manifested.

Even relatively rigid polymers, such as poly(methyl methacrylate), reorient at the polymer-water surface, due to relaxation mechanisms in the surface region which may occur at lower temperatures than in the bulk, perhaps due to surface-induced water plasticization of the interfacial region, and due to segmental side chain motions which are activated at or near room temperature.

In systems containing hydrophilic phases of submicroscopic dimensions, such as common diblock and triblock copolymers, given sufficient mobility, the hydrophilic phase will dominate the interface in water, the hydrophobic phase will dominate in air.

It is suggested that the adsorption of biological and other macromolecules at a polymer-water interface will result in considerable restructuring of the polymer surface in a response to the local microenvironment of the adsorbed macromolecule, as well as the local water and other solution components.

We propose that is is necessary to characterize the surface properties directly at the solid-water interface,

as well as the more commonly and classically performed solid–air or vapor interface, when searching for correlations between the surface properties of polymers and their biological behavior. Further, the polymer–water interfacial properties may need to be characterized as a function of hydration time or after suitable water equilibration.

The surface motions of polymers are probably different from the bulk motions. Few data are available; experimental methods with which to probe surface motions are becoming available and have been discussed.

Polymer surface restructuring effects in response to a surrounding liquid phase are probably most pronounced in aqueous systems due to the unique hydrogen bonding and acid-base characteristics of water.

Finally, these effects are not readily detectable by classical advancing contact angle measurements, including determinations of critical surface tension, nor by x-ray photoelectron spectroscopy or other analysis techniques which primarily probe the solid–vacuum or solid–air interface.

Methods are available for the study of the time dependence of polymer surface relaxations. The most directly useful method is the time-dependent Wilhelmy plate method for measuring contact angle dynamics and hysteresis. Other methods include fluorescence probes, interface vibrational spectroscopy and nuclear magnetic relaxation methods. The latter provides direct information related to surface and interfacial motions, though high surface area particulate samples are generally required for adequate sensitivity.

As data on surface relaxation times and processes become available, we can expect significant progress in the modeling and simulation of such effects and eventual theories and scaling laws[5,27,29] with practical predictive value.

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Chemical Concepts for Ultra Small Electronic Devices
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"Proteins As Engineering Devices"

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Abstract

Proteins are macromolecular polyamides used in biology for a myriad of structural, information storage and processing, detection and reception, transport, chemical, and mechanical functions. There are about 5,000 different individual proteins in biological organisms, about 2,000 of which are enzymes involved in specific chemical transformations. There has been considerable interest and speculation in the application of proteins to non-biological problems, such as information storage, as recognition elements in biosensors, and as structural materials. Indeed, protein fiber systems are well-known (wool, silk) and have unique and special properties which cannot be matched by synthetic macromolecules. However, the structural proteins are among the simplest proteins known. It is clear that most proteins are indeed molecular machines and molecular devices. They have applications in addition to those exploited by biological organisms and processes. Two specific examples will be given:

1. Proteins, in particular antibodies, as recognition elements in biosensors, with particular emphasis on fluoroimmunosensors, using fiber optic and waveguide technologies.

2. Proteins as microactuation devices and even as motors.

In addition, there will be a brief discussion of possible technologies for the preparation of thin films of proteins, their characterization, and their deposition under microarea resolution and computer control.

Reference

J. D. Andrade, "Thin Organic Films of Proteins," Thin Solid Films 152 (1987) 335.

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Proteins as Engineering Machines and Devices*

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Introduction:

Proteins are biological macromolecules with unique architectures and structures. They perform most of the vital functions in biology. There are at least 5,000 different proteins, ranging in size from 10^4 - 10^7 Daltons. Each is a molecular machine with specific characteristics and functions (1). Such functions may include catalytic activity (enzymes), structures, motion, transport, light detection, light emission, recognition, communication, and probably information storage. The discovery, characterization, and modification of proteins is occuring at a very rapid rate. Once rare or exotic proteins can now be produced in quantity, inexpensively, using modern biotechnological processes.

There is no reason why nature's molecular machines can not be used by engineers and materials scientists for non-biological functions. At present some proteins are used for structural/mechanical applications (silk sutures and fabrics) and for specific chemical recognition (antibodies in biosensors).

Engineers and materials scientists generally have no background in biology or biochemistry, thus there is little awareness of the potential application of proteins in engineering. Biomedical engineers have provided several application examples, however, including protein-based wound dressings and artificial skin (2), sutures (3), membranes for chemical separations (4), and even disposable contact lenses (5). Chemical and biochemical engineers have immobilized and applied enzymes in biochemical reactors for a variety of synthetic and bioprocess applications (7). There has been some speculation as to the

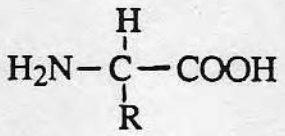
* An expanded version of Ref. 1

possibility of arrays of proteins for computing and information storage application (6). Such "Biochips" possibilities have generated considerable public and even financial interest. There is now some interest in using the self-assembly properties of two dimensional arrays of proteins as an aid in producing nano structures using photolithography and related processes (8).

In this paper I attempt to provide a brief overview of the structure and generic biological applications of proteins. I then suggest a set of examples to attempt to demonstrate the potential application of proteins in non-biomedical areas.

Structure:

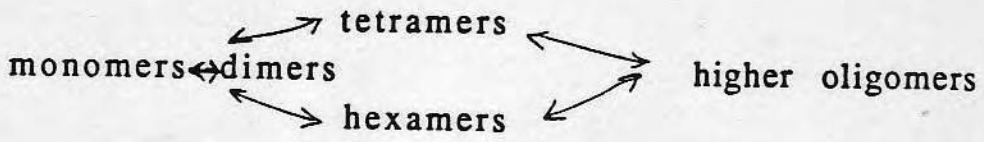
Proteins are complex copolymers of highly specific sequences consisting of some 20 different amino acid "monomers",



where R can be polar, apolar, positively charged or negatively charged (9). Protein molecular weights cover the range of roughly 10^4 - 10^6 Daltons. A particular protein generally has one or more specific functions and is clearly molecularly engineered to carry out that function in an efficient, specific and often unique manner.

The primary structure of a protein is its amino acid sequence which constitutes the primary information and basically controls all properties and functions of the molecule. A secondary level of structure includes local amino acid sequence associations which result in helix, sheet or turn structures. Globular proteins generally have a complex tertiary structure which can often be determined by X-ray diffraction of the crystal (9). Finally, two or more polypeptide chains can associate via their tertiary structures to form a quaternary structure. Nearly 200 proteins have been crystallized and their tertiary and/or quaternary structures determined (10). The atomic coordinates of these proteins are readily available and can be viewed using modern molecular graphics on standard computers and via available software (see the Journal of Molecular Graphics).

Certain proteins can dimerize, oligomerize or even self-assemble:



Insulin dimerizes and at higher concentrations will form hexamers. Hemoglobin commonly exists as the tetramer (11). Albumin also dimerizes and forms higher order oligomers. Fibrinogen, on the action of the activating proteolytic enzyme thrombin, self-associates and polymerizes to form the network that produces blood clotting (12). Tubulin self-assembles to form the microtubules found within most cells (13, 14). Actin and myosin self-associate to form the characteristic filaments of muscle and a variety of other structures (13-16).

The tertiary and quaternary structures of proteins are stabilized by hydrophobic, ionic and hydrogen bonding interactions. However, for most globular proteins the "native" state is only marginally stable and the protein can be easily "denatured", i.e. lose part of its biological activity (17):

native→denatured

$\Delta G \simeq 5-14$ kcal/mole

Proteins are now generally recognized to be dynamic, flexible structures of marginal stability. Changes in pH, temperature, ionic strength, or solute environment can often change the conformation (three-dimensional structure) and biochemical activity of a protein.

Although there is much activity in trying to predict the tertiary and quaternary structure of proteins from their primary amino acid sequences, the predictions are not usually very good (10,18). This is partly because many states are only marginally different in overall free energy so that the "true" equilibrium or native state is difficult to find. It is also because intermolecular force functions in ionic solutions are not well understood, including the classical hydrophobic and electrostatic interactions.

The interaction potential functions which are available, however, can be incorporated into the computer graphics algorithms to yield

three-dimensional images of the constituent atoms and their interaction fields, permitting "docking" or interaction studies between two proteins, between, a protein and a surface, between an enzyme and its substrate etc.

Although large proteins appear to be extremely complex and difficult to understand, considerable progress has been made in deducing a set of "building-block" principles for proteins. Most proteins consist of structural domains, roughly 15-20K Daltons (9). An even smaller structural unit, the module, whose molecular weight is about 5K Daltons, is now being elucidated (19).

Many complex proteins are now known to consist of a variety of repetitive structures, many of which are similar in many different proteins (22).

Programs are now available to help distinguish and predict such structures based on amino acid sequence boundaries (20,21).

Interfaces

The application of proteins as engineering machines and devices often involve using the protein on or coupled to a suitable substrate or matrix. For example, biosensors often use proteins with specific recognition properties covalently attached to electrodes or to optical fibers; enzymes used in biochemical engineering are often immobilized onto particles or fibers.

Proteins at interfaces can be very different from the same proteins in their solution state. The structure and function of proteins can be significantly altered in the new microenvironments presented by solid-liquid or liquid-air interfaces (23). Other concerns include the stability of the immobilized protein - does it change with time? Is it stable in the application environment? Can it be replaced or regenerated after loosing activity?

Most proteins will adsorb at solid-aqueous interfaces to form reasonably compact, generally monomolecular and often irreversible films. A number of recent reviews of this phenomenon are available (23-28). Although no rigorous or quantitative theories of protein adsorption are available, there is a general qualitative understanding of the process (23-28). Several groups are now working with model proteins whose structures are well known and are attempting to

develop methods of simulating and predicting the adsorption process (27, 29-30). Practical uses of adsorbed protein films include diagnostic immunoassay, which uses preadsorbed specific immunoglobulins, and the preadsorption of human plasma albumin to improve the blood compatibility of cardiovascular devices (26).

The Langmuir monolayer trough has been widely used for the study of proteins at air-water interfaces (31, 32, 28) and indeed many of the "rules of thumb" on protein adsorption have been derived from Langmuir trough studies. The transfer of proteins to solid supports by Langmuir-Blodgett methods has been accomplished, although the process is difficult and frequently unpredictable (28, 31). Recently, Uzigiris (33) bound a hapten-specific IgG to a transferred hapten-phospholipid layer and showed that the close-packed IgG layer assumed a hexagonal structure. Uzigiris suggested that the technique may be useful as a means to produce ordered two-dimensional immunoglobulin films.

Giaever and Keese (34) have suggested that an adsorbed protein film, when exposed to UV light in the presence of ozone, will be modified in the exposed areas, resulting in loss of antigenicity and decreased reaction with its specific antibody. This would allow one to prepare protein films with selected, predefined regions of antibody reactivity or lack of reactivity.

Specific proteins are routinely bound to chromatographic supports and surfaces, in general via direct coupling (35, 36) or affinity methods. If the protein has a specific interaction with a particular ligand, that ligand can be immobilized to a solid support (35). Passage of a protein mixture over the immobilized ligand column results in specific binding of the protein to the immobilized ligand surface. This is a popular and commercially important separation, purification and characterization process known as affinity chromatography (36). Often a specific antibody is immobilized for the binding of its specific protein antigen. The complex can be disrupted by an eluting solution of appropriate pH, ionic strength etc. (36). Suitable ligands include antibodies (for antigens), sugars (for certain lectins), heparin (for lipoproteins, fibronectin, etc.), collagen (for fibronectin), etc.

Certain proteins may also self-aggregate and even self-assemble into complex structures (37). The protein tubulin self-associates to

form microtubules (14, 38). Fibrinogen, after appropriate activation, polymerizes to fibrin (12). Many other examples are known (37, 39). A variety of very complex films are also known, such as cell membranes, which include embedded transmembrane proteins and protein complexes, e.g. ion channels and receptors. Several groups are attempting to reconstitute purified receptors in organic thin films, generally lipid bilayers. For example, the acetylcholine receptor is being incorporated into bilayers on appropriate microelectronic devices to produce specific biosensors for nerve agents (72). Proteins can also be ordered in electric, hydrodynamic and gravitational fields, and it is likely that such fields can be used to assist and even to control the ordering in protein films in the near future.

The properties of protein monolayers, except for their chemical interactions, are not well known. Certainly films of proteins containing chromophores (such as hemoglobin [Fe^{2+}heme]) have the expected optical absorption and resonance Raman properties. The refractive indices of protein monolayers are known via ellipsometry, a technique widely used to study protein adsorption, (26, 27, 40). Crude packing characteristics and surface viscosity data are available at the air-water interface from Langmuir trough studies (28). There is very little experience or data on protein conformation (26), mechanical properties,(28) or optical or electronic properties of protein monolayers. Only in the last few years have research groups begun to apply modern surface analytical techniques to characterize protein monolayers (23). The field, therefore, is wide open.

Applications:

Cooperative protein interactions result in functions and behavior reminiscent of non-biological machines and devices. These functions are, in large part, responsible for what we know as life in animal systems. Enzymes can and have been treated as chemical machines (41). In Welch's words (42, 43):

> "Living systems...contain devices of molecular dimensions capable of performing mechanical work.... A molecular energy machine is a single enzyme molecule that cyclically couples energy released by one form of reaction, e.g. ATP, to an otherwise unfavorable reaction, e.g. contraction, without (during the lifetime of its cycle) being exposed to the macroscopic and thermalizing environment ..... Molecular energy machines require some degree of structural

presence of calcium ion, triggers a conformational change which permits a trapped, excited state in a bound chromophore to decay, resulting in photon emission. The bioluminescent properties of these proteins and others are beginning to be extensively used in clinical and analytical chemistry as quantitative and end-point detection methods.

A major application of protein layers or films is for separation and purification, particularly in the chromatography field (36). Immobilized enzymes are widely used in certain biochemical-biotechnology processes (7). Another major "application" is the understanding of adsorption of proteins with respect to the blood and biocompatibility of medical devices (26). There is considerable interest in the development of protein-resistant surfaces, which actively repel proteins, to minimize protein-surface interactions. Such surfaces would have wide application in chromatography, membrane separation processes and medical devices, including contact lenses.

Biosensors are a new area with considerable interest and activity (46). Most biosensors depend on the specific binding characteristics of proteins or other biological molecules for the specificity and sensitivity of the sensor. Immunoglobulins are of particular interest as they can be used to detect their specific antigen with great selectivity and sensitivity (47).

There is some potential for the use of protein films in energy (solar) collection and transfer, such as chlorophyll-containing and electron transfer-redox enzyme protein systems. Light detection and transduction are also of interest, at least as a model system, utilizing proteins such as rhodopsin.

Although there has been considerable discussion of protein-based information storage and even computation, no practical or even defensible concepts or designs have yet involved (48). However, information storage and processing in biology is well know and to some extend, understood (49).

> organization. The transduction process must be insulated from the thermalizing bulk phase. Molecular machines must successfully compete against the thermal environment."

In a casual and simplistic sense, one might consider an enzyme or organized assemblages of enzymes to be molecularly engineered "Maxwell's demons" permitting reactions and processes to occur which cursory analysis would suggest to be contrary to the laws of thermodynamics.

Particurlarly dramatic examples of protein machines, from an engineering point of view, are the various motions and force generation processes so common throughout all biology. The most common and perhaps well-understood example is the interaction of myosin and actin, together with regulation by the proteins tropomyosin and troponin, in the function of muscle. Here we see protein molecules designed with exquisite self-assembly character, coupled with specific binding interactions, highly regulated by accessory proteins and by calcium ions. The end result is a sliding-filament, force-generation mechanism responsible for virtually all muscle motion throughout biology (44, 45).

Enzymatic activity, e.g. (ATPase), is built right into one of the heads of the myosin molecule, and it is indeed ATP hydrolysis via the myosin ATPase activity which provides the fuel for the force generation process.

The self-assembly of α– and β-tubulin to form the ubiquitous intracellular microtubular structures is an interesting example of the use of proteins for structural purposes. The coupling of tubulin structures with the protein dynein, in the case of cilia and flagella, and with the protein kinesin, in the case of intracellular tubules, makes possible the characteristic wave-like motion of cilia and flagella and the conveyor belt linear transport processes now recognized as so important in the interior of living cells. Bacterial flagella, made up of the membrane-anchored protein complex called flagellin, are responsible for rotary motion, which at one time was thought to be inpossible in biology (13-16).

Some proteins function essentially as light sources, e.g. the luciferin-luciferase bioluminescence process. Another interesting example is the calcium-sensitive photoprotein aequorin which in the

Examples:

Two dimensional arrays and patterns.

Many strains of bacteria contain ordered arrays of proteins as the outermost part of their cell surface. These surface or S-layers have been well studied (50). These materials were used by Douglas, et al. (8) as patterning structures for nanometer molecular lithography. The protein patterns, in the range of 5-30 nm periodicity, are attractive tools for producing ion milled patterns on the nanometer level.

A very different application is in the production of fine ultrafiltration membranes. The 2D structure of the S-proteins result in channels of about 3.5 nm diameter (51). The self assembly of other proteins can lead to different structures and presumably different filtration properties. The protein is deposited on a microporous support, upon which it self assembles. The ordered protein film is then stabilized by chemically cross-linking. Such materials are being studied for their ultrafiltration properties and applications (51).

It is likely that other self assembling proteins will find application in the production of nano structures, particularly myosin and tubulin (see earlier discussion).

In addition, carefully ordered 3-D structures can have unique optical properties. For example, the ordered collagen in the cornea (52) and crystallin in the lens (53) provide the transparency and refraction properties so vital for biological vision processes.

Light Detection - Rhodopsin

Rhodopsin is a membrane - spanning protein which is the fundamental detector and transducer of light in most organisms (54). Various rhodopsins are responsible for initial light detection and transduction in the photoreceptor cells of the vision apparatus (55) IT is a hydrophobic (typical of membrane proteins) protein of 41000 D in size. It contains a chromophore, retinal, which undergoes a cis-trans conformational change as part of the light detection/transduction process. Bacteriorhodopsin has been extensively studied, although its

protein and chromophore are not exactly the same as mammalian rhodopsin.

Rhodopsins, although normally water insoluble, can be extracted from membranes by detergents, purified and reconstituted. The protein can not only be incorporated into lipid bilayers by vesicle or Langmuir-Blodgett methods (see ref. 56 for an introduction to these methods), but has also been incorporated into polymerized, semi-rigid bilayer membranes of lipid-like polydiacetylenes (57). The membrane-bound rhodopsin retained chemical, photo chemical and enzymatic properties. The authors concluded: "This functional protein behavior demonstrates that sensitive vertebrate membrane proteins can be usefully incorporated into membrane bilayers that have been modified by polymerization reactions."

Lawrence & Birge have suggested (58) that bacteriorhodopsin may be useful as a high capacity optical memory, possibly as stable dried films on a silica or sapphire support - a so called "Biochip" element.

Other light-sensitive proteins have also been reconstituted. Heckl et al. (59) have shown that the light-harvesting chlorophyll protein complex can be reconstituted into phospholipid monolayers on a solid support. They even regulated the protein concentration and distribution in the monolayer.

It is clear that considerable progress is being made in the development of functional light detection/transduction devices based on proteins and that this may be a useful and important area for continued research and development.

Needs and Opportunities

In order to understand, predict and apply protein films, we must understand the forces and interactions responsible for protein structure and function, as well as their interactions among themselves and other proteins and with solid supports. Although the electrostatic, hydrophobic and hydrogen bonding interactions are qualitatively understood (60-62), better potential functions are needed to model and effectively predict protein structure, folding (63), denaturation (64), and adsorption (26). Direct measurements of such forces and interactions at the protein film-aqueous solution interface are needed,

such as via the methods of Israelchvilli (62) and Klein (65). The simulation and modeling of proteins via computer molecular graphics and intermolecular interaction potentials is in its infancy (66). With better potential functions and with information on the structure of protein films, attempts should be made to develop theories and models for protein interactions and for the film-forming process. Such models will permit the design of structures and means for selfassembly of films with unique, designed properties. The modeling and simulation of film formation and film properties should include the effects of impressed fields.

Methods to characterize protein films, in situ, i.e. underwater, are needed. In spite of the progress made in applying reflection fluorescence (67) and IR (68) methods, more definitive methods are required to probe and characterize packing, orientation and structure of protein films. Perhaps the most promising approach is direct imaging in water using scanning tunneling microscopy and atomic force microscopy (71). Synchotron scattering and diffraction should also be considered. Other means of imaging and characterizing protein films should be encouraged. Fourier transform Raman spectroscopy in the near-IR has great potential for characterizing the conformation and orientation of protein thin films. Methods to characterize and evaluate the properties of protein thin films should be developed, including electronic, magnetic, optical and mechanical properties.

There should be attempts to apply the unique properties of certain proteins in mixed films; for example, embedding receptors in lipid or polymer films for sensor and gating applications (72, 73) and the co-immobilization of antibody and synthetic photoactivable polymers to regulate antigen-antibody binding constants. Optimization of enzyme properties for reactions in adverse environments and modified or synthetic enzymes for new chemical reactions also should be encouraged (74).

Summary

Proteins are a series of polymers with unique and varied structures and properties. They are beautiful examples of macromolecular engineering for specific and unique purposes. The modeling, preparation, characterization and application of protein

films should lead to a variety of very useful and unique applications. Perhaps most importantly, experience and study of proteins will greatly aid and stimulate materials scientists and engineers to engineer better new generations of macromolecules for new and now unknown applications.

As we consider new generations of engineering machines and devices, it would be useful for some attention to be paid to the 5000 proteins available in biology which have been molecularly engineered over 2 x 10^9 years of evolution to provide a wide range of machine- and device-like activities. Undoubtedly, the harnessing of proteins for practical machine- and device-like activities will require far more knowledge and experience with the following: (1) the handling of these molecules: (2) their incorporation and reconstitution into lipid or polymer-like matrices; (3) their modification, possibly by genetic engineering and site-directed mutagenesis technologies, for the enhancement of self-assembly properties; (4) enhancing their stability in non-biologic environments. Most importantly however, we need to begin teaching new generations of engineers about biochemistry and particularly proteins in order to apply proteins in novel and practical ways.

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J Andrade
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Plasma Protein Adsorption: The Big Twelve[a]

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INTRODUCTION

FIGURE 1 presents a protein adsorption complexity spectrum and in schematic form suggests that proteins vary greatly in complexity. The simplest include the insulin dimer, lysozyme, and myoglobin, whose X-ray crystallographic, three-dimensional structures are well-known and whose function is generally well understood. The structures of such proteins can be imaged and manipulated using modern computer graphics algorithms and equipment. Through careful consideration of the surface chemistry of such proteins and by appreciation that the surface chemistry is far from uniform on a protein particle (there are different "faces," each with different interaction potentials with specific surfaces or interfaces), one can develop estimates or hypotheses as to optimum interaction orientations for such model proteins at model interfaces. This approach has been very fruitful in the study of the adsorption of chicken and human lysozyme at model surfaces,[1,2] as well as in studies of the adsorption of myoglobin on model surfaces and the interaction of the adsorbed protein with specific monoclonal antibodies.[3]

Most of the plasma proteins, however, are considerably more complex. Their detailed three-dimensional structures are not known. For example, the plasma immunoglobulins vary greatly, particularly their Fab portions and variable regions, because of their wide and varied specific antigen binding characteristics. Although the structure of fibrinogen is fairly well-known,[4] the detailed three-dimensional structure of even the major domains is not yet available.

The problem increases in complexity if one considers multicomponent solutions, particularly multicomponent solutions of complex proteins, such as serum or plasma and tears, because one now has a variety of proteins all interacting with the surface in a competitive fashion, and interacting with each other, both on the surface and in the bulk solution.

FIGURE 1 also includes a complexity axis for the surface or interface. One can consider that the simplest interface available is probably the air-water interface. Much of what we know of protein adsorption and protein behavior at interfaces is taken from Langmuir Blodgett monolayer studies.[5,6] Studies at liquid-liquid, particularly oil-water, interfaces have also provided much information and such interfaces are

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extremely useful model systems.[7,8] One can directly monitor the kinetics of adsorption, the spreading characteristics of the adsorbed proteins, conformational changes, and two-dimensional ordering-structuring by Langmuir Blodgett and interfacial tension methods.[7,8]

The surface of an amorphous, homogenous homopolymer, such as poly(methyl methacrylate) (PMMA) or poly(dimethyl siloxane) (PDMSO), could also be considered relatively simple. One must remember, however, that such surfaces may not be truly rigid, particularly the surfaces of elastomers, such as PDMSO, because of the

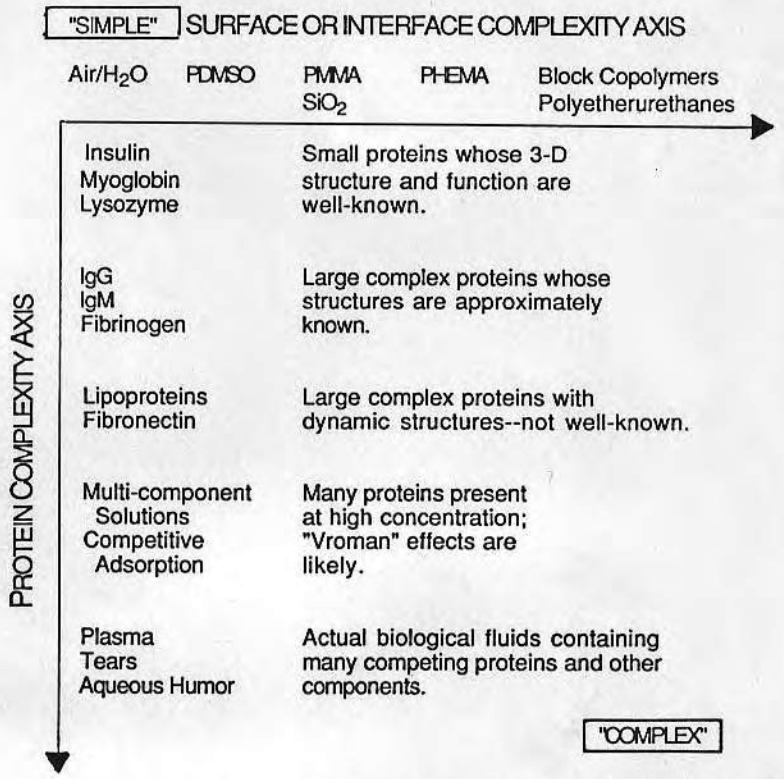

FIGURE 1. A protein adsorption complexity matrix. The upper left represents "simple" systems; the lower right shows highly complex systems. A solid surface complexity axis runs from left to right; the protein complexity axis runs from top to bottom. See text for abbreviations and details.

mobility of polymer chains and segments at room and body temperature. In addition, the intrinsic mobility and dynamics of proteins themselves suggest that very complex interactions and even interpenetrations may occur. Even for a rigid surface, such as PMMA, the amphiphilic nature of the polymer's hydrophilic ester group and the hydrophobic backbone can result in slight water uptake, and even side-chain orientation or reorientation at the polymer-water interface.[9] Virtually all polymer surfaces are dynamic. Methods are available for at least qualitatively characterizing the nature of

the surface in air and underwater and even the dynamics of surface restructuring in going from one environment to another.[9]

As one continues along the material surface complexity axis (FIGURE 1), one eventually gets to such complex surfaces as block copolyetherurethanes, the most widely used materials for cardiac assist devices.[10] Such materials may have two or more phases and interphases present in the bulk structure and at the surface. Bulk and surface morphology are dependent on processing history, as well as on specific polymer chemistry. In addition, there is some evidence that the more hydrophobic phase tends to dominate the surface in air or vacuum, while the more hydrophilic phase tends to dominate it in an aqueous environment.[10] There is also concern that because of the dynamics of such elastomers, particularly due to the mobility of the soft segment phase, the water-equilibrated surface may restructure in order to optimally equilibrate with an adsorbed protein film—that is, the surface in water may change during the protein adsorption process as a result of the protein microenvironment with which it is now trying to equilibrate.

Although the situation in FIGURE 1, particularly at the bottom right, would appear to be quite complex, the situation at the top left is indeed relatively simple and is beginning to become qualitatively, even quantitatively, understood.

Most of what we know about protein adsorption is summarized in FIGURE 2, a general kinetic model for the process, assuming a one component protein solution for simplicity. A protein of bulk concentration, C_o, diffuses to and collides with the interface. At time zero initial contact occurs. If the interaction forces are sufficient, the protein stays on the surface for a certain residence time, probably in the range of milliseconds to seconds. Air-water interface studies have shown that a minimum contact area is required, which probably relates to the magnitude of the hydrophobic

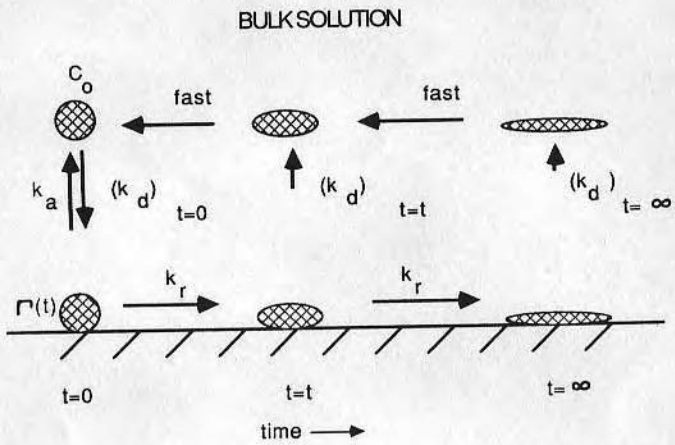

FIGURE 2. A kinetic protein adsorption model based in part on the ideas of Lundstrom, Walton, and Jennissen.[15,21–24] C_o is the bulk protein concentration; Γ is the surface concentration, which is a function of time t; k_a is the on-rate constant and $k_d(t)$ are the off-rate constants, which are a function of contact time (residence time).

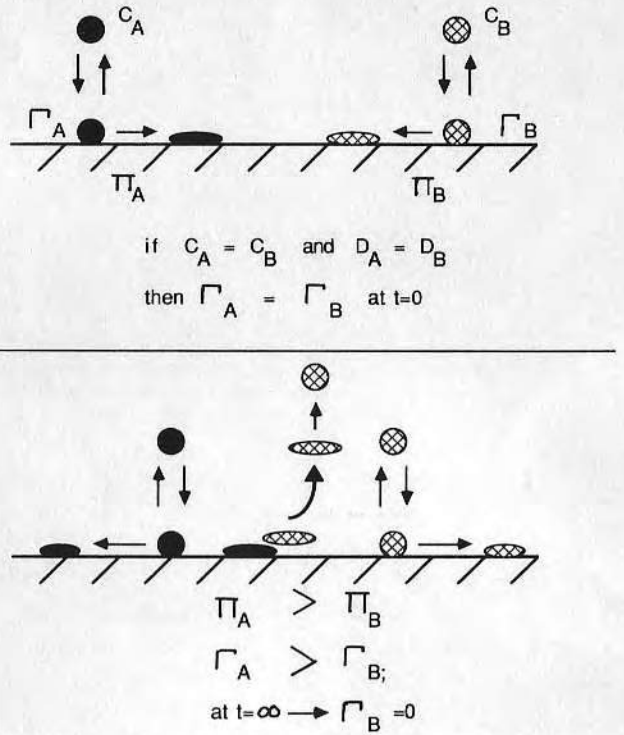

FIGURE 3. The competitive surface spreading hypothesis (from Bagnall.[8]) *Top:* Two proteins, *A* and *B*, have the same bulk concentration and diffusion coefficients and therefore have the same surface concentration (Γ) at $t = 0$ (initial contact). If the spreading pressure (π) of one is greater than the other, then eventually the protein with the larger π will dominate the interface. Here we show that A displaces B, since $\pi_A > \pi_B$.

interaction required for initial stabilization of the protein-surface complex.[3,11] The protein can desorb at this stage; an appropriate desorption rate constant is indicated in FIGURE 2.

While on the surface, the protein may begin a surface denaturation process, which is probably related to its intrinsic conformational lability. This is also related to the fact that globular proteins are only marginally stable,[12] and an energy of 5–15 kcal/mole is sufficient to denature them in normal buffer solutions.[11–13] Therefore, the interactions with the surface, particularly interactions of a hydrophobic nature, significantly affect the solution equilibrium of the protein.[11]

There may be a strong configurational entropy driving force in going from the

globule to a more extended state, particularly if the extended state can be accommodated by maintaining a degree of hydrophobic interaction comparable to that provided by the globular state. Hydrophobic interaction can, of course, be provided, in part, by interactions with a partially hydrophobic surface. Dill has modeled and considered the configurational entropy aspects of globular protein structure,[12] as well as of hydrophobic surfaces containing alkyl chains—such as those commonly used in chromatography.[14]

A distorted or extended protein is generally less soluble in solution; thus, one expects the desorption tendency to decrease, allowing for increased binding and/or surface denaturation. Desorption of the protein is therefore a function of its residence time on the surface. With increasing contact times the probability for desorption decreases, as indicated in FIGURE 2. Assuming that the adsorption process does not result in any covalent bond changes in the protein molecule, the model then also assumes that if a denatured or partially denatured protein does desorb, it rapidly renatures to the equilibrium globular state. This portion of the model is controversial, as there is evidence in the literature that some desorbed proteins are permanently changed[15,16] due to covalent bond changes during the process.

Competitive adsorption in two component systems has been modeled by Cuypers *et al.*[17] His results suggest that the "Vroman effect"[18,19] is predicted by a competitive adsorption model which allows an exponential decrease in the affinity constant with increasing occupancy of the surface, similar to the classical Langmuir treatments of adsorption which incorporate a lateral interaction and variable surface site energy term.[20]

The effect of a surface-induced conformational change has been treated in a preliminary way by the models developed by Lundstrom *et al.*,[21–23] in which two states are considered: the initial state at time = 0, and the "equilibrium" state at long contact times, $t = \infty$. Lundstrom's models, which to date have been published only for single component solutions, nicely predict and explain adsorption behavior of labile globular proteins in single component systems.

FIGURE 3 begins to ask the question of what happens during a competitive adsorption process and is based on the ideas of Bagnall[8] and Jennissen,[24] who showed that the surface denaturation or accommodation process is dependent on the number and type of neighbors. If two different proteins have adsorbed next to each other, one is generally more labile or conformationally adaptable to the interface than the other. We can say that one "spreads" at the interface more effectively than the other. We can consider a spreading constant for such a protein, analogous to the solid-vapor and solid-liquid spreading constants so commonly used in classical surface chemistry.[20] One would expect that the spreading characteristics would be related to the solution denaturability[13] of the protein, and particularly to its behavior at water-air and water-oil interfaces.[5–8] Clearly, the more "surface active" protein would spread more effectively and may displace the other protein from the interface. This is another mechanism by which the Vroman effect can be explained. Clearly, the next step is to develop a model which incorporates the ideas of Cuypers,[17] Lundstrom,[21–23] and Bagnall,[8] but generalized for complex multicomponent systems.

THE BIG TWELVE

Blood plasma, however, consists of far more than one or two proteins. High-resolution, two-dimensional electrophoresis shows over 150 bands from plasma. It has been estimated that a typical cell has over 5000 different proteins! We have chosen to consider only those proteins in plasma that are expected to compete effectively for the interface in the initial collision or contact process, *i.e.*, those which are present at sufficient plasma concentration to be considered major constituents. We have chosen to consider only those proteins generally present in plasma at about 1 mg/ml or higher concentration. The twelve such proteins are shown in FIGURE 4 and described in TABLE 1. FIGURE 4 somewhat schematically separates the proteins by electrophoretic mobility and sedimentation coefficient, *i.e.*, by net charge and size, respectively. Albumin is clearly the major constituent, followed by IgG. The high- and low-density lipoproteins are also major components. The properties of these twelve proteins are summarized briefly and qualitatively in TABLE 1. TABLE 2, based on a simple diffusion limited mass transport view, asks the question, "When does the next protein arrive?"[25] or "Who gets there first?"

Clearly, protein collisions with the surface are dominated by albumin. Seven times as many albumin molecules as IgG molecules strike the surface, and a thousand times more albumin molecules than IgM molecules strike the surface. So it is certainly reasonable and predictable that the surface would be largely populated at or near time zero by albumin, assuming that there is sufficient interaction between albumin and the surface to lead to a reasonable residence time.[26] About one in seven of the collisions with the surface involves IgG. If IgG has a greater affinity or greater spreading pressure at the surface than does albumin, then the spreading IgG molecule may tend to displace some of the preadsorbed albumin from the surface. Thus, although the surface is rich in albumin initially, one may find that the concentration shifts to IgG with increasing time. One could also argue that, although fibrinogen may occupy 1 in 150 or so of the sites at time zero, the sites it does occupy are particularly stable and perhaps it is "irreversibly" adsorbed. Assuming that the affinity constant for albumin is not high, occasionally an albumin molecule will desorb. Although the now vacant surface site is seeing a collision frequency continually dominated by albumin, if a molecule of fibrinogen collides with that site, the adsorptive interaction, with its much higher affinity constant, will be longer lived and more stable than if albumin had collided with the vacant site.

Thus, there are two approaches to the Vroman effect. One can assume no conformational change, but essentially a surface occupancy lateral interaction model, such as that developed by Cuypers,[17] or one can assume a surface denaturation model, such as that being developed by Lundstrom.[21–23] Clearly, both are important, and both are occurring, not only for the Big Twelve discussed in TABLES 1 and 2, but for the other 150 or more protein constituents in plasma.

Nevertheless, we now know enough to begin to develop models, at least for two and three component systems. When those match the excellent, competitive adsorption data now becoming available,[19] we can, using more sophisticated mathematical and computer techniques, extend them to many components and parameters. A key problem is obtaining parameters which reflect or at least relate to the interaction energy at time 0, the surface denaturability, and perhaps the interaction energy at very long times.

We must know the nature of the solid interface. Polyurethanes are probably too complex at this stage for truly fundamental understanding. The best models are the column chromatography supports used for protein separation. Here the highly hydrophilic, nonionic nature of agarose and related matrices provide only very low energy interactions with proteins. Basically, such interactions prove to be weak and highly reversible, and therefore protein mixtures pass through such columns with little or no retention. By derivatizing agarose supports with low-density ionic, hydrophobic, or charge-transfer groups, one now imparts an interaction mechanism. The protein, depending on its structure and repertoire of surface functional groups, interacts with

TABLE 1. Properties of the "Big 12"—Plasma Proteins Whose Concentration is >1 mg/ml[a]

Protein	Plasma Concentration g/l – mg/ml	Plasma Concentration μmol	Molecular Weight	Diffusion Coefficient (10^{-7}cm^2/s)	Electrophoretic Mobility and Net Charge	$CD^{1/2}$	Carbohydrate (%)	Sialic Acid (%)
Albumin	40	600	66,000	6.1	$\alpha_1 - 19$	1500	0	0
IgG	8–17	53–113	150,000	4.0	α_2–γ_3	200	2.9	0.3
		100*						1.6 M/M
LDL	4.0	2	2,000,000	2.0	β	3	—	—
HDL	3	18	170,000	4.6	α_1	39	—	—
α-Macroglobulin	2.7	3.3	725,000	2.4	α_2	5	8.4	1.8
								48 M/M
Fibrinogen	2–3	6–9	340,000	2	$\beta_2 - 26$	11	2.5	0.6
		7.5*						4–8 M/M
Transferrin	2.3	30	77,000	5.0	β_1	67	5.9	1.4
							(2 chains)	variable
							biantennary	4 M/M
α-Antitrypsin	2	40	(51,000)	5.2	α_1	91	14	3.6
			54,000				content variable,	6 M/M
			(45,000)				biantennary	(variable)
Haptoglobins	2.0	20	100,000	1-1 4.7		43	20	5.3
	1.6–3.0	8–15	200,000	1-2 (monomer)	α_2		(in β-chain)	
	1.2–2.6	3–6.6	400,000	2-2			bi- & triantennary	
C3	1.6	9	180,000	4.5	β_2	19	2.7	0.01
IgA	1–4	7–27	150,000	4	α_2–γ_3	30	7.5	1.8
		15*						9 M/M
IgM	0.05–2	0.06–2	900,000	2.6	β_2–γ_3	1.6	11.8	1.3
		1*						42 M/M

[a]Data are from refs. 43 and 44. Numbers used for calculation of $CD^{1/2}$ are indicated by *asterisks*.

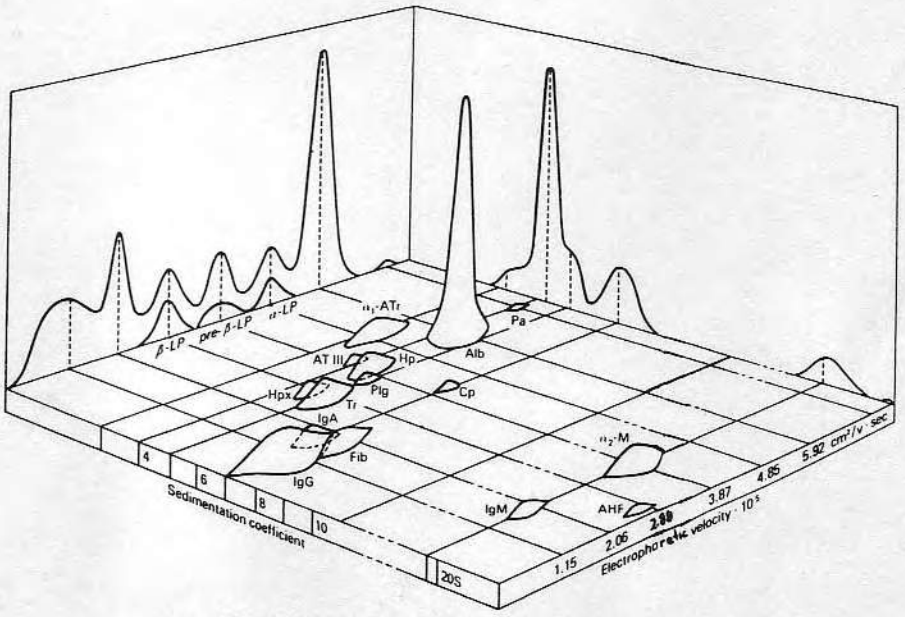

FIGURE 4. The major plasma protein constituents (based on ref. 27).

TABLE 2. Plasma Concentrations (C) and Diffusion Coefficients (D) for the Big 12 in Plasma (Data from TABLE 1)[a]

Protein	C (μmol/l)	D (10^{-7} cm^2/s)	$C\sqrt{D}$
Albumin	~600	6.1	1500
IgG	~100	4.0	200
α-Antitrypsin	~40	5.2	91
Transferrin	~30	5.0	67
Haptoglobin 1-1	~20	4.7	43
HDL	~18	~4.6	39
IgA	~15	4.0	30
Complement 3	~9	4.5	19
Fibrinogen	~7.5	2.0	11
α-Macroglobulin	~3.3	2.4	5
LDL	~2	~2.0	3
IgM	~1	~2.6	1.6

[a]Rate of arrival of protein molecules at the interface $dn/dt = C(D/\pi t)^{1/2}$.

the immobilized ligand on the agarose particle and is "retained." The adsorption is reversible if the interaction energy is not too high and if the number of interaction points per protein molecule is not greater than one. After the appropriate residence time the protein molecule is desorbed and goes on down the column, where it interacts with another ligand and another particle, stays for a moment, is released, and goes on. This process continues, and for a column of particular length, packing or support, and protein concentration and mixture, leads to a residence time or retention time in the column. This retention time is an empirical measure of the interaction energy or the affinity constant and the residence time of each adsorption event.

It is relatively straightforward to rank proteins on the basis of their interaction with hydrophobically derivatized agarose, so-called hydrophobic chromatography supports. This was done a decade ago by Hofstee.[28] One can do the same with cationic and anionic derivatized columns to get a measure of electrostatic interaction. As the hydrophobic and electrostatic interactions are probably the two dominant ones, because most of the hydrogen bonding possibilities are swamped by the 55-M concentration of water in aqueous solutions and even in blood plasma, one can characterize the intermolecular interaction potential for a protein interacting with chromatographic supports.

One must also apply what is known about the structure of the protein. If its three-dimensional structure is known, as discussed earlier for lysozyme, insulin, and myoglobin, then one should do graphics modeling and attempt to predict the optimum orientation of the protein for interaction with certain model surfaces. Structural information is available, even for the big plasma proteins, particularly fibrinogen,[4] where the charge distribution and the different domains and fragments are well-known, the four carbohydrate residues are precisely located, and where one can begin to guess as to what might be the orientation of the molecule on certain types of surfaces.

One possibly important feature of plasma proteins, which has been largely ignored in consideration of their interfacial properties, is the fact that virtually all of them are glycoproteins and contain carbohydrate chains, generally terminated with negatively charged sialic acid residues. Although Kim and coworkers[29] studied the adsorption of desialylated immunoglobulins and fibrinogen many years ago, there has been little consideration of the role of carbohydrates. This is probably because the major

carbohydrate-containing proteins in plasma, which make up half or so of the Big Twelve (TABLE 1), have been largely ignored by the blood compatibility and surface chemistry community.

TABLE 3 briefly summarizes the glycoprotein and sialic acid composition of plasma proteins. The haptoglobins, for which very little adsorption data is available,[32] contain 20% carbohydrate. Unfortunately, the structure of the haptoglobins is not well-known and indeed they constitute a family of proteins rather than being a distinct, homogenous molecule, such as albumin. Two important protease inactivators, α-antitrypsin and α-macroglobulin, also have high glycoprotein contents. Transferrin, an iron-binding protein, is also fairly rich in carbohydrate. The adsorption characteristics of α-macroglobulin and transferrin have been studied by Young and Cooper.[33] There has been virtually no work on haptoglobin adsorption[32] or on antitrypsin, IgM, or complement C3. The extensive study by Young did show that the adsorption of macroglobulin and transferrin is apparently not of very high affinity. Although there could be many reasons for this, it does lead one to suggest that perhaps proteins with high carbohydrate contents, particularly if that carbohydrate is more or less uniformly distributed on the surface of the molecule, might enhance the solubility of the molecule and mask hydrophobic amino acid residues on its surface, thereby decreasing its adsorption at hydrophobic interfaces. Kim's study showed that desialylated fibrinogen did not influence fibrinogen adsorption,[29] probably because the carbohydrate in fibrinogen is located in the coiled-coiled region near the disulfide knot and on the β-chain in the terminal domains. There is apparently no carbohydrate on the γ-chain or the more extended, and probably more surface-active, α-chain. Also, the total carbohydrate in fibrinogen is relatively low.[4]

The role of carbohydrate in adsorption, which we call the "glycoprotein hypothesis," is probably only important for haptoglobin and antitrypsin. The carbohydrate in IgM is localized and not uniformly distributed around the molecule. Macroglobulin has such a unique dimeric structure that its adsorption will probably prove to be dependent on its unique structure and/or shape in solution.

FIGURE 5 illustrates the structure and composition of the plasma lipoproteins. We have included HDL, particularly HDL 3, and LDL 1 and 2 in the Big Twelve previously discussed (TABLE 1). There is evidence from at least two groups that the lipoproteins have unique and important adsorption properties.[35-37] Breemhaar *et al.* have shown preferential adsorption of high-density lipoprotein onto hydrophobic supports, including a demonstration of the Vroman effect with LDL, HDL, and fibrinogen mixtures.[35] We have shown that LDL adsorption from single component solutions increases significantly when going from 20° to 37°C on elastomers, but not on

TABLE 3. Carbohydrate and Sialic Acid Composition of Major Plasma Proteins (from Refs. 30, 31)

Protein	Percent CHO	Percent Sialic Acid
Haptoglobin	~20	5.3
α-Antitrypsin	~14	3.6
IgM	~12	1.3
α-Macroglobulin	~8.4	1.8
Transferrin	~5.9	1.4
IgG	~2.9	0.3
C3	~2.7	0.01
Fibrinogen	~2.5	0.6
Albumin	~0	0

rigid supports.[36] This leads to the hypothesis that the lipid phase transition in LDL, which occurs in the vicinity of 37°C, may be important in controlling the adsorption properties and perhaps the surface denaturability of such proteins. We have also shown that the adsorption of LDL and HDL from single component solutions onto rigid hydrophobic and hydrophilic supports suggests a reaction-limited process for both classes of lipoproteins adsorbed on hydrophobic supports, but adsorption on hydrophilic surfaces is diffusion limited.[37] One could draw the hypothesis that the lipoproteins may therefore be undergoing significant conformational alteration on the hydrophobic supports. Although there is not very much data on lipoproteins, their dynamic structure and their very high lipid content suggests a major role in the plasma adsorption process.

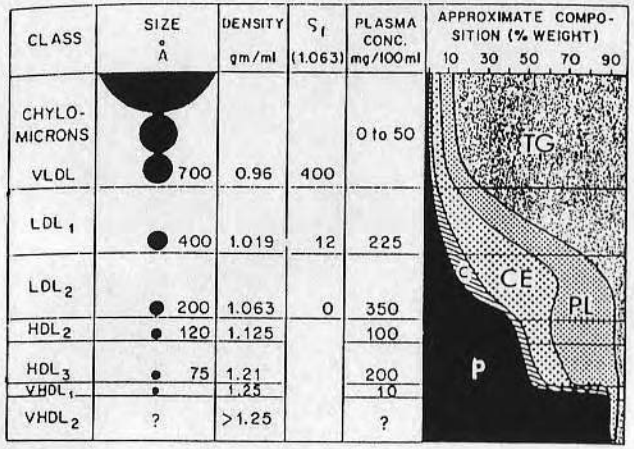

FIGURE 5. Schematic distribution of the major classes of plasma lipoproteins according to their flotation in the analytical ultracentrifuge. *TG* – triglycerides; *PL* – phospholipids; *CE* – cholesterol esters; *C* – cholesterol; *P* – apoprotein (from ref. 34).

SURFACE DENATURABILITY

In addition to the on- and off-rate constants, which can be derived as described above, one should have a measure of the ability of the protein molecule to denature at the interface as a function of time. Such information is very difficult to get at solid-liquid interfaces, although, in principle, some of the surface-sensitive spectroscopic techniques can provide some such information. For example, ATR-FTIR studies of the adsorption of a single component protein from dilute solutions can provide evidence of conformational change as a function of time at the interface.[38] Total internal reflection fluorescence also provides evidence for changes with time at the interface,[39] but such techniques are specialized, difficult to quantitate, and difficult to interpret in terms of actual structural changes. Also, they are substrate limited and

even substrate specific due to the optical properties required for the total internal reflection condition. Some such information is also available via ellipsometry in terms of changes in the refractive index and thickness of the adsorbed layer as a function of contact time (see refs. 17 and 21).

We feel that a more fruitful approach may be to study the behavior of proteins at water-air, water-oil, water-fluorocarbon liquid, and water-siloxane liquid interfaces, using the standard, proven, and inexpensive surface and interfacial tension techniques.[6–8,20] With such techniques, one can measure the spreading pressure through the decrease in surface or interfacial tension of various individual proteins. One can also measure the temperature dependence of interfacial processes. Although this does not relate directly to the interface between a biomedical material and plasma, it helps characterize the interfacial activity of the various protein species of interest. One can develop an empirical parameter and use it as a coefficient or exponent in the appropriate terms in the equations. The problem is to get such data (chromatographic and interfacial activity data) for the proteins of interest. Although some such data is available in the literature, it is generally very protein specific, and there is no compilation of information on the interfacial activity of plasma proteins. If such data were available, the modeling and simulation of the Vroman effect and other complex interfacial processes could actually be straightforward.

CONCLUSIONS

We offer the following conclusions:

1. FIGURE 6 suggests that the early stages of adsorption (very short contact times) are functions of the particular chemistry of the surface, the particular three-dimensional structure and orientation of the protein, and the number of species and their concentration. In addition, adsorption at short contact times is also a function of occupancy and lateral interactions, as modeled so nicely by Cuypers.[17] We suggest that the time-dependent conformational adaptation to the surface, as modeled by Lundstrom,[21–23] is related to the bulk solution denaturation tendencies of the protein, including thermal denaturation, denaturation in urea or guanidinium chloride solutions, and denaturation in solutions of different pH. We further suggest that surface and interfacial tension measurements of proteins at water-air, water-oil, and other water-liquid interfaces will be useful measures of protein surface activity at the solid-water interface.
2. We suggest that lipoproteins are very important in the adsorption process and in the Vroman effect, due in part to their structural and compositional dynamics, particularly on hydrophobic elastomeric surfaces, where one might expect some interpenetration of protein and lipid components with the polymer chains.[36]
3. It is known that the presence of carbohydrates in proteins both masks and protects the proteins from proteolytic attack and helps improve their solubility, as well as providing stabilization of conformation.[30,31] It is therefore reasonable to suggest that proteins with high carbohydrate contents, particularly if the carbohydrate is more or less uniformly distributed over the protein, may show little adsorption or interaction with hydrophobic or with negatively charged supports.
4. At equilibrium adsorption will be dominated by proteins with the following characteristics: large molecular weight and large size, which suggests a large number of potential contact points with the surface; readily denaturable or

conformationally labile, permitting the protein to readily accommodate to the microenvironment of the interface; and low carbohydrate content, or at least significant regions of the molecule which are free of carbohydrate, because of the possible masking effect of carbohydrate at hydrophobic and/or negatively charged surfaces.

Although it is clear that fibrinogen is not necessarily the equilibrium species in plasma, it has the attributes described above. It is low in carbohydrate and what carbohydrate it does have is localized. It is conformationally quite labile and indeed has very high interfacial activity and spreading pressure. It is large and asymmetric and can have very large contact areas. It has a fairly asymmetric charge distribution,

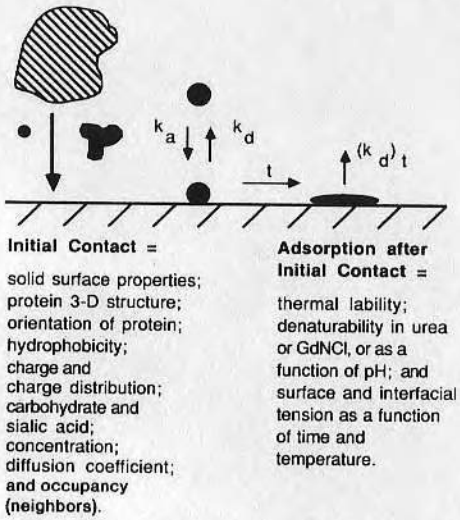

FIGURE 6. The key parameters involved in the initial contact phase of adsorption (left side) and in the time-dependent surface and protein "denaturation" processes (right side).

as well as significant hydrophobicity, so it can interact successfully with a wide variety of interfaces.

CAUTION

We have assumed throughout the discussion that there are no covalent changes imposed on the molecules prior to, during, or after the adsorption process. Clearly, the work of Brash and others demonstrates that covalent bond changes can indeed occur.[16] Certainly plasma has a variety of active proteases and protease inhibitors, which

change in concentration depending on local needs and processes. Clearly the conformational adaptation to the surface which we have described may make a molecule more or less susceptible to proteolysis or to other chemical processes. Indeed, the very act of interacting with certain types of surfaces could, in part, direct covalent chemistries, such as possibly the interaction of C3 with nucleophilic surfaces.[40] The surface activation models, which are being developed by Sefton,[41] Mann,[42] and coworkers, coupled with the modeling and simulation suggested here, will be an important next step in attempting to treat truly practical blood-material interfaces.

SUMMARY

We have discussed the general principles of protein adsorption at solid-liquid interfaces from single component and multicomponent solutions, based on qualitative kinetic models that include mass transport considerations, initial interaction energies, surface-dependent conformational changes, and possible desorption processes. We have surveyed plasma protein components greater than one milligram per milliliter in concentration, which we call "The Big Twelve." We considered their size, concentration, diffusion coefficient, structure and function, and methods of estimating their "surface denaturability" by using bulk solution measures of denaturation and conformational change. We have suggested that the role of the carbohydrate moieties in plasma proteins may have some bearing on their adsorption properties. We further suggest that lipoproteins, because of their lipid phase transition and conformational lability at body temperature, may tend to dominate the adsorption process, particularly on mobile elastomeric polymer surfaces. We suggest that detailed consideration of the structure and characteristics of each of the proteins involved is necessary in order to begin to understand plasma adsorption processes. Detailed characterization and understanding of the solid surface in the aqueous and protein environments are also required.

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Surfaces and Blood Compatibility

Current Hypotheses

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J. D. Andrade: Introduction

Virtually every physical and chemical characteristic of materials has been suggested as being important in blood coagulation and thrombosis. The surface properties that have been suggested as influencing blood interactions have been reviewed by Baier[1] and Hoffman.[2] Andrade and Hlady recently reviewed those hypotheses as they specifically related to protein adsorption.[3] Some of the more well-known and widely investigated hypotheses are given in **Table 1.**[3-17]

We now know that proteins do not interact with surfaces solely on the basis of the net electrostatic charge. Indeed, a protein can adsorb to different surfaces by different mechanisms due to the wide variety of functional groups present on the exterior of all proteins.[4] Because cell interactions with surfaces are generally mediated through adsorbed protein layers, it is doubtful that the old negative surface charge hypotheses are of any general validity.

In principle, the surface and interfacial free energy hypotheses consider all of the various interactions that may be present at interfaces: electrostatic, dipole interactions; hydrogen bonding; and hydrophobic interactions, among others. In practice, the means available to estimate interfacial energetics are very crude and either underrepresent or ignore many of the possible interactions. Fowkes[5] has considered this problem in detail and is developing methods to properly account for the partial acid-base or electron donor-acceptor interactions at interfaces. Although there are some reasonable correlations between interfacial parameters and cell adhesion or platelet deposition,[6,7] we can expect considerable improvment of correlations in the fu-

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Table 1. Surfaces and Blood Compatibility: Classical Hypotheses

Property	Biological Response	Investigator	Ref.
Negative Surface Charge	Plasma protein and cell repulsion	Sawyer	8, 9
Negative Surface Charge	Hageman factor contact activation	many	see 9, 10
Surface Energy	Decreased surface energy provides optimum blood compatibility.	Lyman	see 3, 9, 10
Interfacial Free Energy (IFE)	Decrease in IFE decreases protein adsorption.	Andrade	11
Albumin Surfaces	Albumin adsorption or immobilization results in decreased thrombosis.	many	9, 12
Glycoproteins	Surface adsorbed glycoproteins, esp. fibrinogen, results in platelet adhesion.	Kim	13
Heparin Surfaces	Local heparin release decreases coagulation.	Leninger, Falb, Gott, et al.	9
High Interfacial Energy	Provides heat of adsorption, which drives conformational change and contact activation.	Nyilas	14
Surface Motion	Increased motion leads to decreased adsorption and denaturation.	Nyilas	15
	Increased motion leads to decreased adsorption.	Merrill Nagaoka	16 17

ture, after we have properly accounted for polar interactions at surfaces.

The albumin hypothesis has led to a great deal of work on protein adsorption, pre-adsorption, and immobilization. The effect of albumin adsorption is relatively short-lived, probably due to competitive adsorption processes and the replacement of albumin with other plasma proteins.

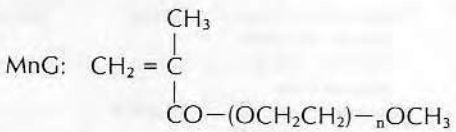
In this article we will discuss several current hypotheses in some detail:

1. Dynamic surface hypotheses—S. Nagaoka *et al.* of Toray Industries and Dept. of Surgery, Okayama University.
2. Protein adsorption and preadsorption—S. L. Cooper, University of Wisconsin.
3. Surface morphology and microstructure—T. Okano, University of Utah.
4. Pharmaceutically active surfaces—S. W. Kim, University of Utah.

Hydrated Dynamic Surfaces

S. NAGAOKA, Y. MORI, H. TANZAWA, Y. KIKUCHI, F. INAGAKI, Y. YOKOTA, AND Y. NOISHIKI

Polymers with poly(ethylene oxide) (PEO) chains were synthesized by the random copolymerization of methoxy poly(ethylene glycol) monomethacrylates (MnG; n, chain length of PEO) with methyl methacrylate (MMA), and by the photoinduced graft copolymerization of MnG to poly(vinyl chloride) (PVC) with photo-sensitive dithiocarbamate groups[17]:



MnG: $CH_2=C(CH_3)-CO-(OCH_2CH_2)_n-OCH_3$

n = 4, 9, 15, 23, 50, 100

The adsorption of blood components onto polymer surfaces with similar chemical states and water contents, but different PEO chain lengths, has been studied by using rabbit blood. **Figure 1** shows the effect of PEO chain length (n) on the adhesion of blood components to the surface of the random copolymers, P(MMA-co-MnG). These copolymers are favorable for study of the interaction between the PEO chain itself and blood components because PEO sidechains randomly distribute along the backbone in these polymers and do not have particular ultra-structures such as microdomains.

It was found that plasma protein adsorption and platelet adhesion onto these polymers significantly decreased with increased PEO chain length, and very few platelets were observed when PEO chain length approached 100.

According to NMR spectroscopy of these polymers in the hydrated state, one of the most essential differences between surfaces with long and short PEO chains was their mobilities. **Figure 2** shows the ^{13}C NMR spectra of the hydrated random copolymers. The line widths of the PEO chains indicate that the chain mobility increases with increasing chain length, and the correlation time (τ_c) of the longest (n = 100) PEO chains was 10^{-10} s, which was close to the value for PEO in free motion, while τ_c of the short PEO chain was 10^{-8} to 10^{-9} s, and indicated restricted motion.

Furthermore, the nature of water in the hydrated polymers has been studied by ^1H NMR spectroscopy. **Figure 3** shows the temperature dependence of ^1H NMR signal intensity of water in the hydrated random copolymers. The water that did not freeze at −60°C is referred to as the "intermediate water," combined and moving with the PEO chains.

Why does the rapid movement of the hydrated long PEO chains existing on the surface reduce the adhesion of blood components onto the surface? **Figure 4** schematically indi-

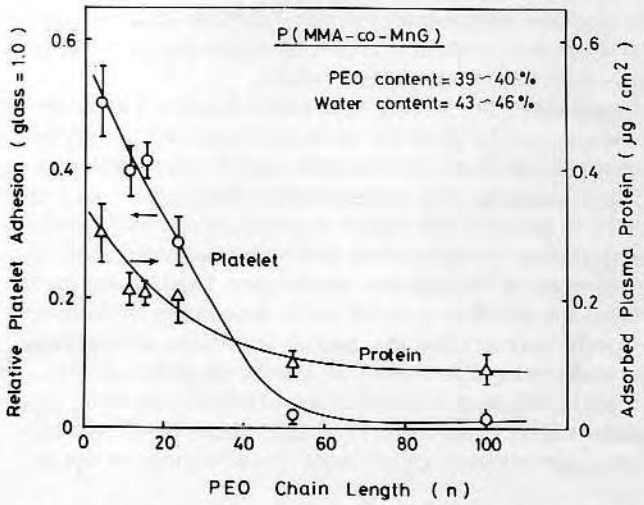


Figure 1. Effect of PEO chain length (n) on the adhesion of platelets and adsorption of plasma proteins onto P(MMA-co-MnG) with similar chemical states and water contents after being exposed to rabbit platelet rich or platelet poor plasma for 3 hours. Mean values and standard deviations (N = 4) are shown. The amount of adherent blood components significantly decreased with an increase in the PEO chain length.

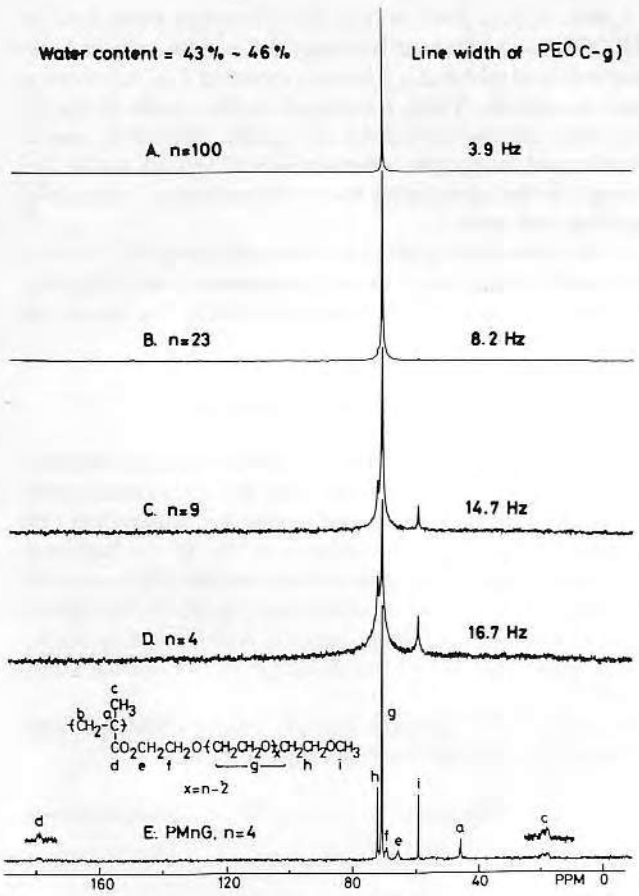


Figure 2. 25 MHz ^{13}C MNR spectra and line widths of P(MMA-co-MnG) measured in water at 27°C: A, n = 100; B, n = 23; C, n = 9; D, n = 4. the longest PEO chain shows the highest mobility.

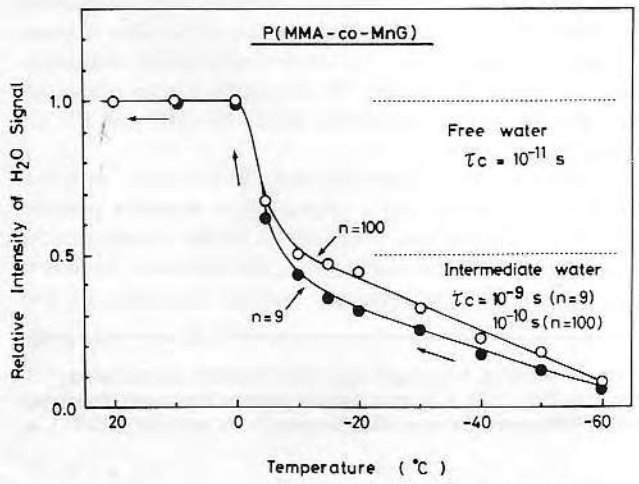


Figure 3. Temperature dependence (increasing curve) of ^1H NMR signal intensity of water in hydrated P(MMA-co-MnG). The water which does not freeze at −60°C is referred to as the "intermediate" water which is combined with the PEO chains.

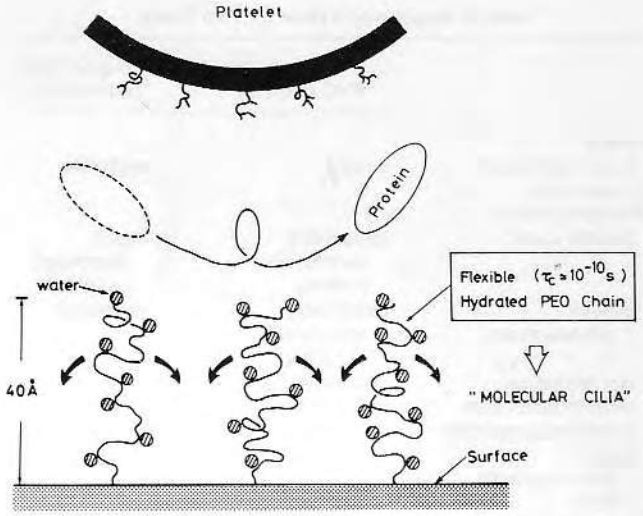


Figure 4. Schematic representation of the interaction between blood components and hydrated PEO chains on the surface. The rapid movement of the long PEO chains influences the micro-hemodynamics at the blood material interface more effectively than do the short PEO chains.

cates our hypothesis. We suppose that the rapid movement of long hydrated PEO chains influences the micro-hemodynamics at the blood-material interface. That is to say, the microstream of water combined with flexible long PEO chains will prevent stagnation and consequent adhesion and denaturation of plasma proteins, which causes platelet adhesion more effectively than does short PEO chains.

As this PEO chain movement on the surface of the material resembles that of the cilia of some micro-organisms, we call these long flexible PEO chains on the surface "molecular cilia." PEO chains were found to be more flexible in the graft copolymers than in the random copolymers, owing to the micro-domain structure of the graft copolymers.

We have carried out *in vivo* and *ex vivo* evaluations of PEO polymers by using the graft copolymer with the longest (n = 100) PEO chains, PVA-g-M100G; **Figure 5** shows its structure and properties. Following implantation of PVC-g-

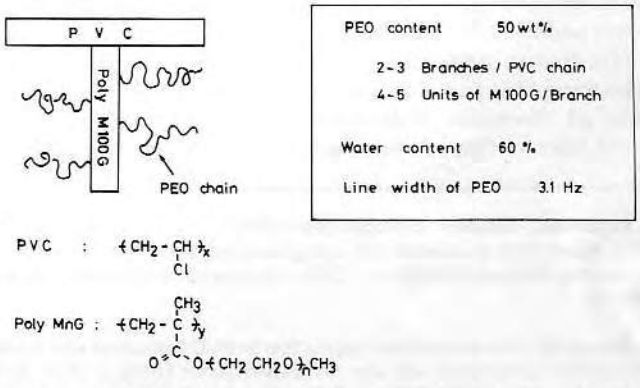


Figure 5. Schematic model and properties of the graft copolymer, PVC-g-M100G.

Table 2. Results of 3 Hour *Ex Vivo* Tests

	PVC (control)	PVC-g-M100G (with PEO)
Surface		
blood component adhesion	severe	negligible
Circulating blood		
platelet count	remarkably decreased (−50%)	slightly decreased (−15%)
platelet adhesiveness	remarkably stimulated (+700%)	no change
clot retraction	decreased (−20%)	no change
recalcification time	shortened (−30%)	no change
platelet aggregability	no change	no change
partial thromboplastin time	no change	no change

M100G into the femoral vein of mongrel dogs, neither platelet adhesion nor fibrin deposition was observed on its surface for up to 72 days. As is well recognized, absence of thrombus formation on the surface is a necessary, but not sufficient, condition for blood compatibility in a wider sense.

Tubes (I.D. 5 mm, length 90 cm) made from PVC-g-M100G were implanted between the jugular vein and carotid artery of rabbits for 3 hours; untreated PVC tubes were used as controls. **Table 2** summarizes the results of the *ex vivo* tests. On the surface of the control PVC tube, severe platelet and leucocyte adhesion was observed, while decreases in the circulating blood components, especially platelets, was seen.

In the case of the graft copolymer with long PEO chains, the amount of adherent blood components was negligible, and almost no deterioration was observed in the circulating blood.

Comment by J. D. Andrade

Clearly, the hydrated dynamic surface approach appears to be very promising. A recent study has considered steric exclusion to be a natural mechanism for minimizing cell adhesion.[18] Recent measurements of the forces between two plates coated with polyethylene oxide (PEO) confirm the active repulsion effect where the adsorbed PEO layers begin to overlap,[19] an effect which is well-known to occur, and is widely applied in the stabilization of colloidal particles.[20]

The effect seems general, feasible, widely applicable, and is deserving of further study and application.

Protein Adsorption and Preadsorption

S. L. COOPER

The effect of preadsorbed proteins on fibrinogen and platelet interactions has been studied by Young *et al.*, using the Wisconsin canine *in vivo* shunt model. In addition to studies on the preadsorption of albumin and fibrinogen, preadsorbed thrombospondin, von Willebrand factor (vWf), fibronectin (FN), transferrin, and other proteins have been evaluated. The work is readily available in a number of published papers.[21-23]

The *in vivo* canine shunt model allows one to measure deposition of iodine-labelled fibrinogen as well as the deposition of chromium 51-labelled platelets as a function of blood contact time. A summary of much of the available data is presented in **Figures 6 and 7. Figure 6A** presents fibrinogen data on PVC surfaces precoated with fibrinogen, von Willebrand factor, and fibronectin, or purified fibrinogen with fibronectin and von Willebrand factor removed. Note the transient peak in the adsorption onto uncoated PVC and the strong enhancing effect of vWf and FN on fibrinogen deposition.

The platelet data (**Figures 6B and 7B**) is similar, with the preadsorbed proteins again appearing to enhance platelet deposition, as is the data in **Figure 7A** for the preadsorption of transferrin; note the significant scale difference between **Figures 6 and 7.** Fibrinogen and platelet deposition on the

Figure 6A. Transient fibrinogen deposition on PVC precoated with purified canine proteins. Fibrinogen deposition (±SEM) on uncoated PVS, (N = 9) and PVC precoated with von Willebrand factor (vWf), ○ (N = 4), fibronectin (FN), □ (N = 5), and partially purified fibrinogen (fibrinogen containing vWf and FN) (FGN+), △ (N = 4), or purified fibrinogen (partially purified fibrinogen further purified to remove FN and vWf) (FGN−), ▲. (N = 2).

Figure 6B. Transient platelet deposition on PVC precoated with purified canine proteins. Platelet deposition (±SEM) on uncoated PVC, × (N = 9) and PVC precoated with von Willebrand factor (vWf), ○ (N = 4), fibronectin (FN), □ (N = 5), and partially purified fibrinogen (fibrinogen containing vWf and FN) (FGN+), △ (N = 4), or purified fibrinogen (partially purified fibrinogen further purified to remove FN and vWf) (FGN−), ▲. (N = 2).

Figure 7A. Same as **Figure 6A,** but surface contains preadsorbed transferrin.

Figure 7B. Same as **Figure 6B,** but surface contains preadsorbed transferrin.

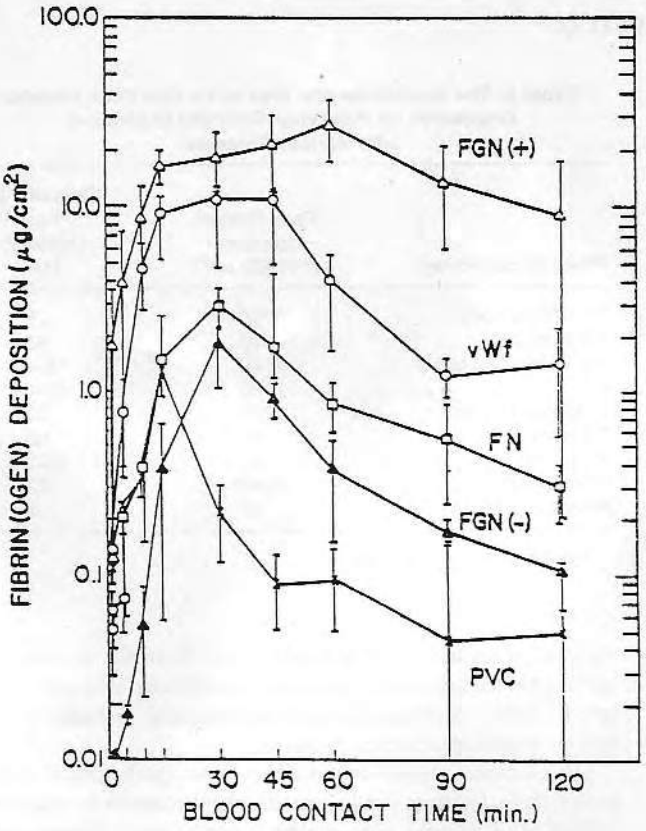


Figure 6A

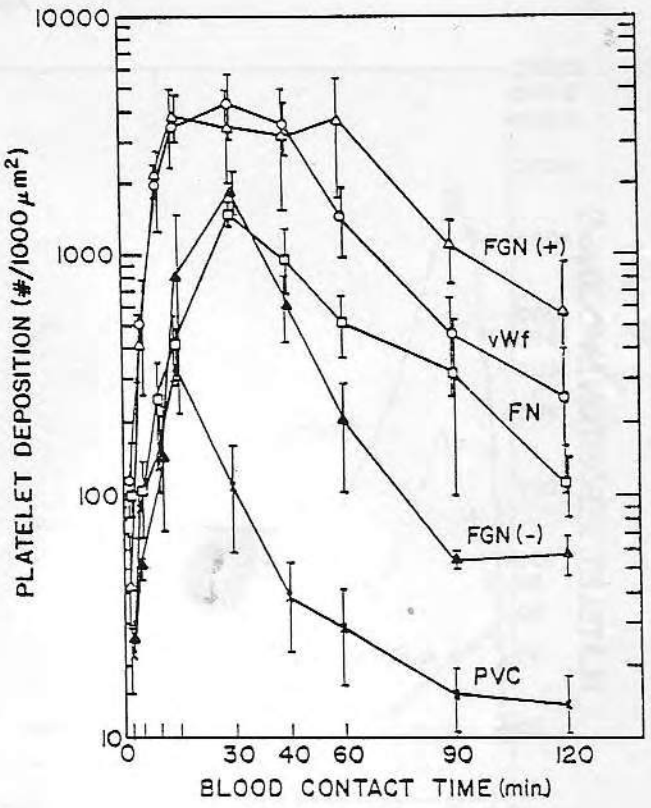


Figure 6B

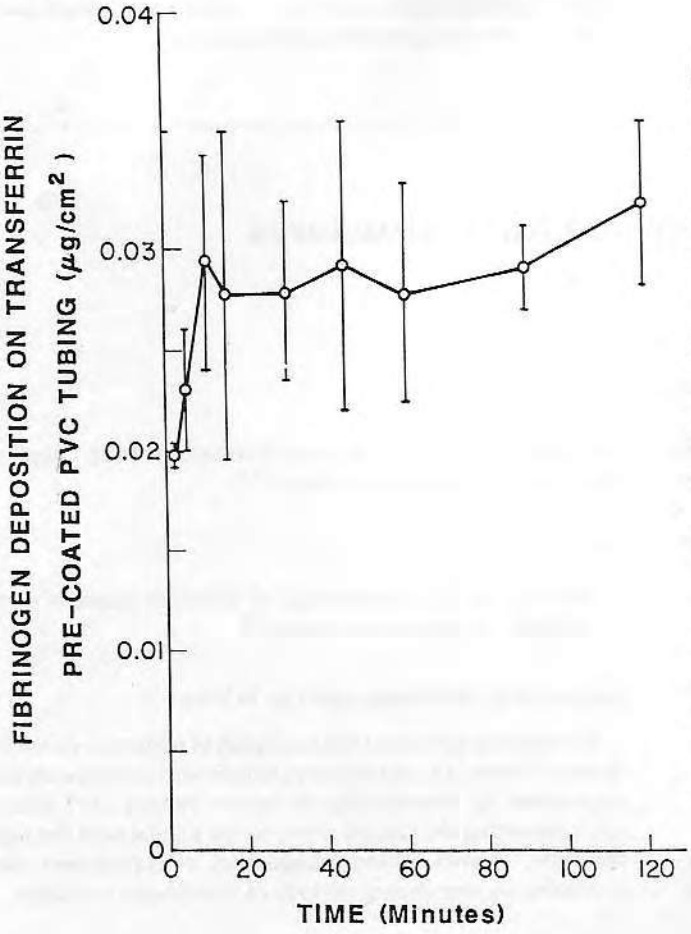


Figure 7A

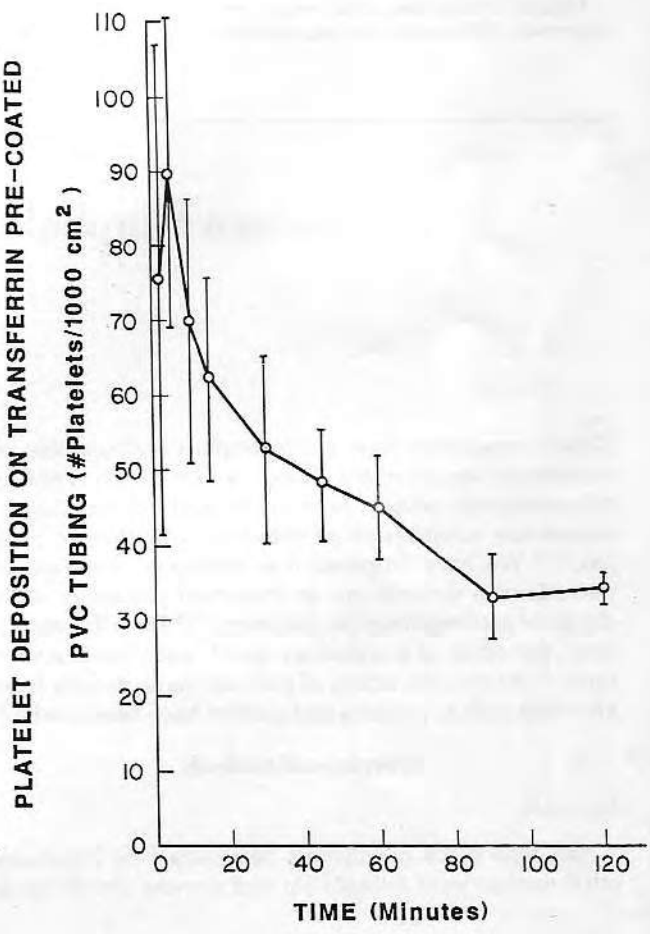


Figure 7B

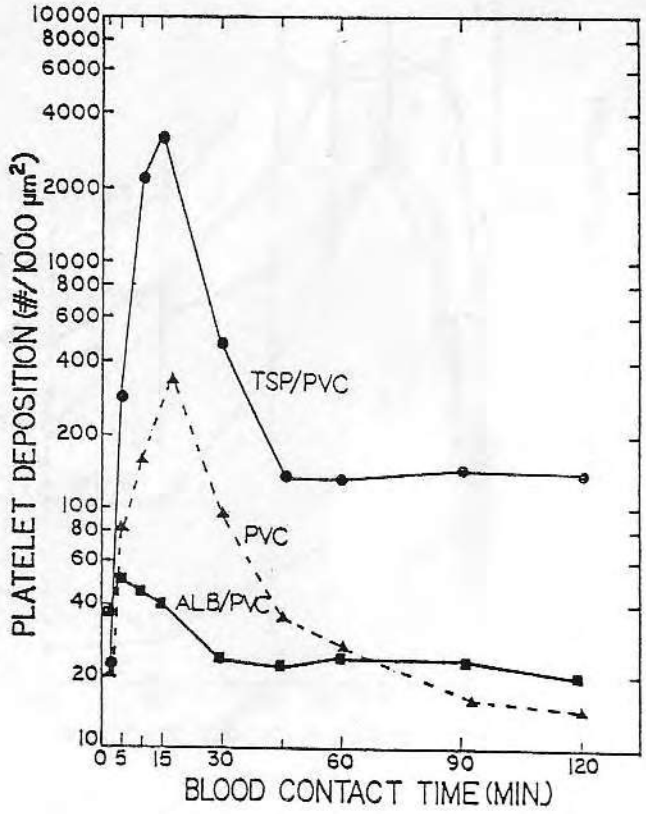


Figure 8. Platelet deposition versus time: uncoated PVC ▲, thrombospondin (TSP)-coated PVC ●, and albumin (ALB)-coated PVC ■.

Table 3. The Magnitude and time of *Ex Vivo* Peak Platelet Deposition on Poly(Vinyl Choride) Precoated with Various Proteins

Preadsorbed Protein	Peak Platelet Deposition (#/1000 μm^2)	Time of Peak Platelet Deposition (min)
Thrombospondin	3390	15
Fibrinogen	2070	30
von Willebrand factor	2100	15–45
IgG	1480	30–60
α_2-Macroglobulin	1220	30
Transferrin	130	10
180kd FN	170	10–20
Fibronectin	2040*	30
Albumin	50	5

* Binds fibrinogen or fibrin.

transferrin surface is remarkably low and similar to that observed for preadsorbed albumin in similar experiments (**Figure 8**). Some of these data are summarized in **Table 3,** together with that of other proteins.

It is clear that preadsorbed albumin surfaces appear to be particularly passive, while preadsorbed transferrin and the 108kd FN fragment also exhibit a moderate passivity with respect to platelet deposition. These data suggest that both the albumin hypothesis and the general hypothesis of the effect of preadsorbed proteins on subsequent blood interactions merit considerable additional study.

Surface Morphology and Microstructures

T. OKANO

Block copolymers having a hydrophilic-hydrophobic microdomain structure are found to exhibit excellent antithrombogenic activity, both *in vivo* and *in vitro,* due to a remarkable suppression of activation of adherent platelets.[24,25] We have proposed that hydrophilic-hydrophobic microdomain surfaces are an important parameter in the design of antithrombogenic polymers.[24,26] From this point of view, the effect of morphology, size,[27] and chemical structure[27,28] on the interaction of polymer surfaces with blood elements such as proteins and platelet have been studied.

Experimental Methods

Materials

ABA type block copolymers constructed by 2-hydroxyethyl methacrylate (HEMA) (A) and styrene (St) (B) or dimethylsiloxane (DMS) (B) were synthesized by a coupling reaction as previously described.[28,29]

Platelet Retention

The number and morphology of adherent platelets were investigated by a column method.[26]

Evaluation of Antithrombogenicity In Vivo

The internal surface of the tube graft of polyester-polyurethane (1.5 mm I.D. and 20 cm in length) was coated with the copolymer by constructing an arterio-venous (AV) shunt, *i.e.,* connecting the carotid artery to the jugular vein through the tube. *In vivo* antithrombogenicity of copolymers was evaluated by monitoring periods of thrombotic occlusion.

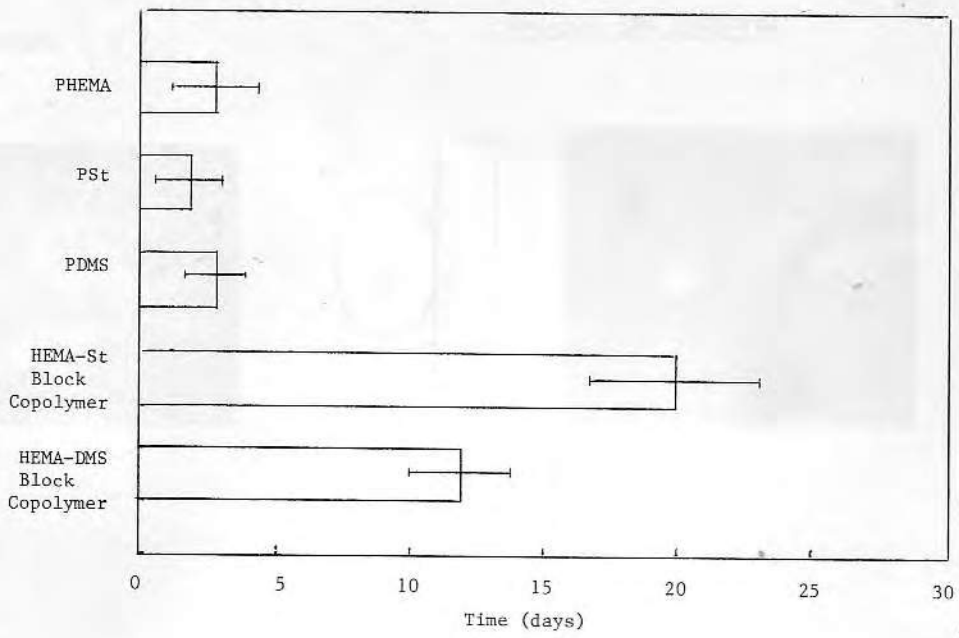


Figure 9. Time to complete occlusion of the tube by thrombus (±SD). Tube size: ID 1.5 mm, 20 cm in length.

Analysis of Adsorbed Protein on Polymer Surfaces

Adsorbed human serum albumin and human γ-globulin were stained with ferritin labelled IgG fractions of rabbit anti-human albumin and rabbit anti-human γ-globulin, respectively, as described in a previous paper.[30]

Results and Discusssion

In Vivo *Antithrombogenic Activity of the Block Copolymers*

In the case of copolymer coated tubes used as an A-V shunt between the carotid artery and jugular vein, blood flow was constant in the range of 25 to 27 ml/sec. When thrombus formed in the tube, blood flow rate decreased in inverse proportion to the growth of the thrombus. *In vivo* antithrombogenicity of copolymers could thus be evaluated by comparing periods of occlusion by thrombosis. Occlusion time was defined as the time at which flow rate decreased to zero. The mean occlusion times of the polymers are shown in **Figure 9,** and were 3 days for PHEMA, 2 days for PSt, and 3 days for PDMS. The block copolymers showed excellent antithrombogenic properties, with the time to occlusion 20 days for HEMA-St block copolymer containing a 0.61 mole fraction of HEMA, and 12 days for HEMA-DMS block copolymer containing a 0.67 mole fraction of HEMA.

The HEMA-St block copolymer containing a 0.61 mole fraction of HEMA exhibited a hydrophilic-hydrophobic microdomain structure in which the morphology was highly ordered alternate lamellae, as previously shown.[25,29] On *in vitro* examination, the surface of the HEMA-St block copolymer was found to have an ability to suppress platelet adhesion and activation[24,25] *i.e.,* shape change and release,[31] as shown in **Figure 10.** This effect does not appear on homopolymer surfaces. The HEMA-DMS block copolymer containing a 0.67 mole fraction of HEMA also formed the hydrophilic-hydrophobic microdomain structure of modified lamellae shown in earlier publications.[28] In the case of HEMA-DMS block copolymer, while platelet adhesion was not decreased, the activation of adherent platelets was remarkably suppressed.[27,28]

Organized Protein Layer Formation on Microdomain Structured Surfaces

The rapid adsorption of plasma proteins is the initial event in a complex series of reactions that occurs when polymeric materials contact blood. This adsorbed protein layer is considered either to accelerate or retard thrombus formation. Therefore, characteristics of the adsorbed protein layer formed on hydrophilic-hydrophobic microdomain surfaces may be important clues to a clear understanding of the role of the microdomain's structure in suppressing the activation of adherent platelets.

In the mono-protein solution system, distribution of adsorbed protein onto hydrophilic-hydrophobic microdomain surfaces of HEMA-St were elucidated by electron microscopy, using the osmium tetroxide fixation technique,[32] which showed that specific adsorption of proteins onto the microdomains had occurred. Albumin selectively adsorbed onto the hydrophilic microdomain, while γ-globulin and fibrinogen were selectively adsorbed onto the hydrophobic microdomain.[32]

In a binary protein solution system, distribution of adsorbed albumin and γ-globulin was investigated by electron microscopy, using ferritin labelled IgG fixation techniques. Adsorbed albumin and γ-globulin formed organized structures corresponding to the microdomain structures of the block copolymer, *e.g.,* albumin selectively adsorbed onto the hydrophobic microdomain.

The organized protein layer formed on the structured microdomain surface is believed to influence the activation of adhering platelets, perhaps by regulation of the distribution of binding sites between platelets and the block copolymer surface.

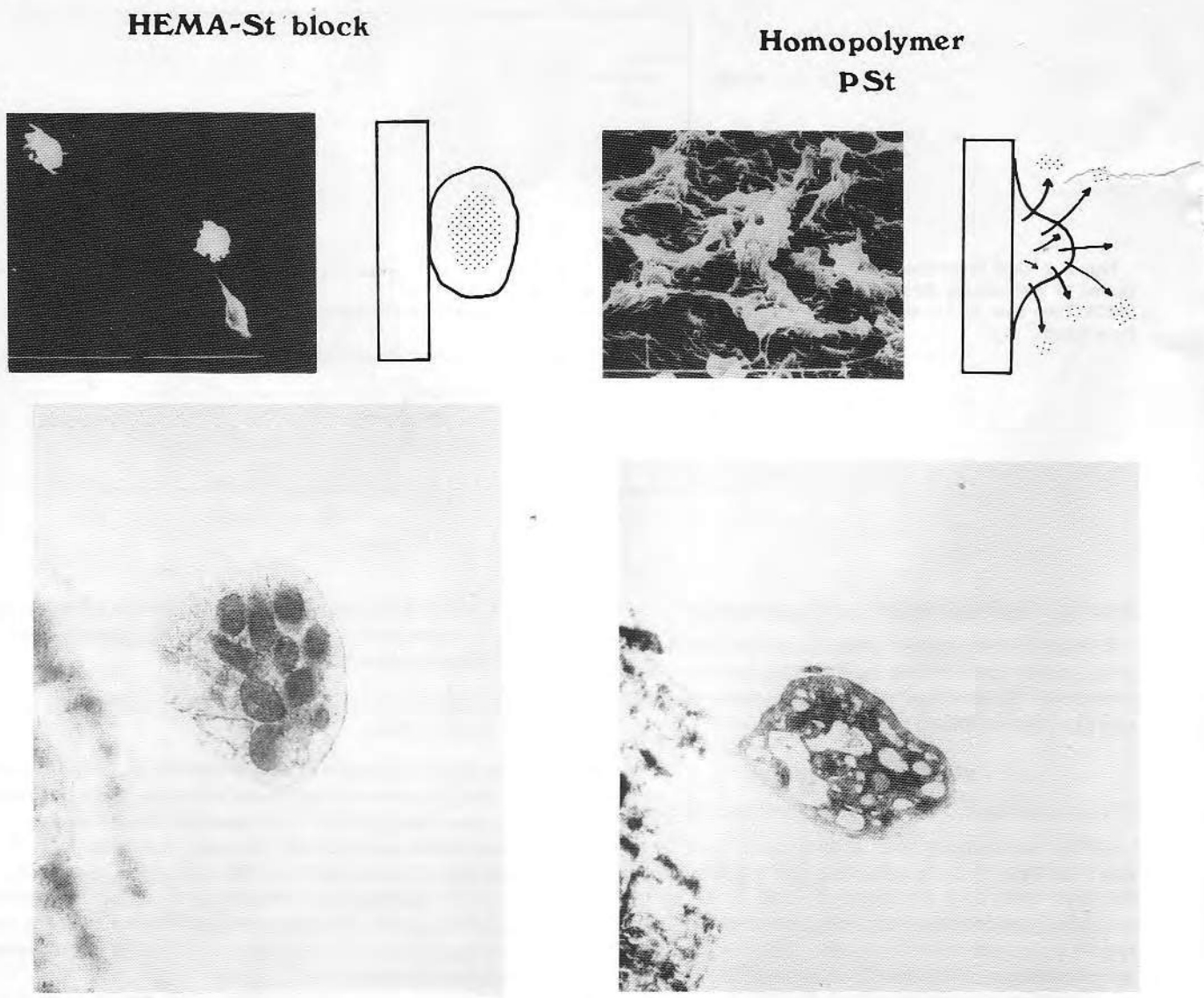


Figure 10. Morphology of Platelets Adherent onto a HEMA-St Block Copolymer Surface (left) and Polystyrene (right).

Pharmaceutically Active Surfaces

S. W. KIM

The development and evaluation of pharmaceutically active surfaces is an area of considerable current interest.[33-38] Among the various antithrombotic agents under investigation are heparin, albumin, prostaglandin (PG), urokinase, and selected conjugates (**Table 4**). These have been evaluated *in vitro* by a number of tests and assays, and some limited *in vivo* testing has also been performed. The active agent can be immobilized on the surface by covalent or other irreversible means, such as physical adsorption mechanisms, or it may be continually released by an appropriate drug delivery strategy. Immobilization of these active agents is dependent upon the exact method of binding to the pol-

ymer support, with particular regard to the role of the spacer type and length on subsequent pharmaceutical activity.

Although many of these approaches are very promising and merit considerable study and probable application, a number of unanswered questions (summarized in **Table 5**) are now being studied.[33-38]

J. D. Andrade: Conclusions

1. The surface dynamics hypothesis has evolved considerably over the past years, and can now be modeled.[18] There is direct experimental evidence[19] for the repulsive force produced by steric exclusion. Some quantitative measure of the actual motion or relaxation times is now available through the work of Nagoaka *et al.* (reported herein), and very preliminary *in vitro* and *in vivo* blood studies suggest that at least PEO surfaces merit considerable additional study.

These surfaces are not inconsistent with the minimum interfacial free engery hypothesis, but there is probably something even more important, which is that such surfaces may indeed show an active repulsion through the entropic steric exclusion mechanism. In order to begin to understand the nature of the process and its biological relevance, we need additional data dealing with protein interaction, stability of such a surface, its adhesion and interaction with platelets and other blood components, and finally more long term *in vivo* blood studies, including turnover rates and emboli generation.

2. The preadsorbed protein hypothesis is clearly very important and, particularly through the efforts of Cooper and his group, is being continually developed and understood. Clearly, the preadsorption of albumin, transferrin, and perhaps other proteins result in a significantly decreased tendency for the adhesion of fibrinogen and platelets. The major question is how do these short-term blood contact responses relate to more long-term behavior.

Table 4. Summary of Pharmaceutically Active Antithrombotic Materials

System	*In Vitro*	*In Vivo*	Comments
Heparin (I) (R)	APTT TT Factor Xa	Dog implants, Rabbit A-V shunts	fibrin ↓ platelet adhesion } → or ↑
PG (I) (R)	platelet aggregation and release	Rabbit A-V shunts	platelet release ↓
Heparin-Albumin (A)	APTT platelet aggregation		fibrin ↓ platelet adhesion } → or ↓
Heparin-PG (R)	APTT platelet adhesion	Rabbit A-V shunts	fibrin ↓ platelet adhesion ↓
Urokinase (I)	fibrinolysis		fibrin ↓

I = immobilized; R = released; A = absorbed; ↑ = increase; ↓ = decrease; → = no change.

Table 5. Unanswered Questions in the Development of Pharmaceutically Active Antithrombotic Surfaces

1-Integrity of Substrate Surface	(I)
2-Stability of Immobilizing Agent	(I)
3-Verification of Spacer Effect	(I)
4-Role of Adsorbed Proteins	(I)
5-Duration of Release	(R)
6-Flow Condition	(R)
7-Dose Effect	(I and R)
8-*In Vitro* and *In Vivo* Correlation	(I and R)

I = Immobilized; R = Released.

3. The work on micromorphologies reported here by Okano has led to a most interesting hypothesis. The main limitation is the use of non-characterized surfaces, particularly in environments and under situations related to cell deposition and adhesion measurements. Considerable surface characterization is required before one can conclude that the effects observed are indeed due to the unique bulk morphologies present in these systems.

4. Pharmaceutically active surfaces have been clearly shown to be effective, and are widely used in certain segments of the clinical-medical device industry today. Extensive work is underway, but much more work into blood-surface interaction is required, particularly of a chronic and long-term nature, before the clinical efficacy of such systems can be determined.

A number of years ago a workshop on this topic was presented[39] which was entitled "Blood-Material Interactions: Twenty Years of Frustration?" Blood-material interaction studies have been ongoing for a very long time, and progress is continually being made. The novel hypotheses presented in this series should greatly augment our understanding of processes, and aid in our development of truly clinically useful blood compatible materials in the near future.

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Simulation of Protein Adsorption. The Denaturation Correlation*

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We suggest means to model and simulate the adsorption of simple proteins at model interfaces. We suggest that molecular computer graphics is a very powerful method with which to study initial contact and interactions of proteins with model surfaces. We present and review kinetic models for protein adsorption and briefly discuss the role of surface-induced conformational change on such models. We suggest that data on the solution denaturation of proteins may be important in estimating protein lability and stability and, together with information on fhe surface tension and interfacial tension behavior of proteins, will help develop hypotheses and correlations with the actual solid/liquid interface behavior.

INTRODUCTION

The modelling and simulation of protein adsorption[1-4], including molecular computer graphics studies of the adsorption of hen and human lisozyme[5,6], have developed rapidly since the Conference presentation.

The simulation of protein adsorption by molecular graphics relies on the X-ray crystallographic coordinates of the protein, readily available in computer-readable format from the Protein Data Bank[7]. These coordinates can be displayed and imaged via a suitable computer and graphics system. Algorithms and software are readily available with which to display the molecule in either stick figure or space filling modes. In both modes, the various amino acids or amino acid sidechains can be color-coded to denote characteristics of interest. We have recently employed a color scheme based on Eisenberg's atomic solvation parameters[8] which was very helpful in visualizing how hen and human lysozyme might interact with a series of model surfaces[6]. This is a very powerful approach and is being extended to the study of other model proteins.

The major limitation of this approach, however, is that, for the time being, the protein has to be treated as a relatively rigid object, interacting with a rigid model surface. The question of conformational adaptation or

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denaturation at the interface has not yet been addressed by such computer modelling methods.

The main advantage and application of the computer structural simulation approach is to deduce the nature of the initial contact and interaction between the protein and the surface. In the case of a relatively rigid protein, such as hen lysozyme, conformational changes upon adsorption may be minimal, and this is probably why the computer predictions and the adsorption data obtained at model interfaces are in reasonable agreement[6].

Most of what we know about protein adsorption is summarized in Figure 1., a general kinetic model for the process, assuming a one component protein solution for simplicity. A protein of bulk concentration, C_o, diffuses to and collides with the interface. At time zero initial contact occurs. If the interaction forces are sufficient, the protein stays on the surface for a certain residence time, probably in the range of milliseconds to seconds. Air/water interface studies have shown that a minimum contact area is required, which probably relates to the magnitude of the hydrophobic interaction required for initial stabilization of the protein/surface complex[9,10]. The protein can desorb at this stage; an appropriate desorption rate constant is indicated in Figure 1.

While on the surface, the protein may begin a surface denaturation process, which is probably related to its intrinsic conformational lability. This is also related to the fact that globular proteins are only marginally stable[11], and an energy of 5—15 kcal/mol is sufficient to denature them in normal buffer solutions[10-12]. Therefore, interactions with the surface, particularly interactions of a hydrophobic nature, can significantly affect the

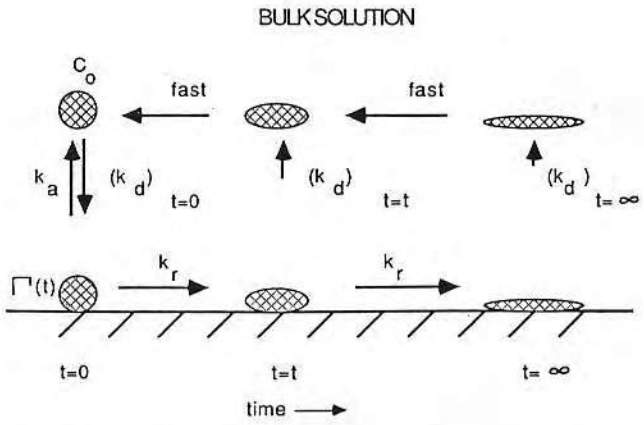

Figure 1. A kinetic protein adsorption model based in part on the ideas of Lundstrom, Walton, and Jennissen[1,14,19,21]. C_o is the bulk protein concentration; Γ is the surface concentration, which is a function of time, t. k_a is the on-rate constant and $k_d(t)$ are the off-rate constants, which are a function of contact time (residence time) (from Ref. 4).

solution equilibrium of the protein[10]. As the interfacial interactions may be very different from the interaction in solution, the solution lability may not be important in many adsorption situations.

There may be a strong configurational entropy driving force in going from the globule to a more extended state, particularly if the extended state can be accommodated by maintaining a degree of hydrophobic interaction comparable to that provided by the globular state. Hydrophobic interaction can be provided, in part, by interactions with a partially hydrophobic surface. Dill has modelled and considered the configurational entropy aspects of globular protein structure[11], as well as of hydrophobic surfaces containing alkyl chains, such as commonly used in chromatography[13].

With increasing contact times, the probability for desorption decreases, as indicated in Figure 1. Assuming that the adsorption process does not result in any covalent bond changes in the protein molecule[14], then the model assumes that if a denatured or partially denatured protein does desorb, it rapidly renatures to the equilibrium globular state.

Competitive adsorption in two component systems has been modelled by Cuypers[2]. His results suggest that the »Vroman effect«[16,17] is predicted by a competitive adsorption model which allows an exponential decrease in the affinity constant with increasing occupancy of the surface, similar to the classical Langmuir treatments of adsorption which incorporate a lateral interaction and variable surface site energy term[18].

The effect of a surface-induced conformational change has been treated in a preliminary way by the models developed by Lundstom et al.[1,19], in which two states are considered — the initial state at time = 0, and the »equilibrium« state at long contact times, $t = \infty$. Lundstrom's models, which to date have been published only for single component solutions, nicely model the adsorption behavior of labile globular proteins in single component systems.

Figure 2. begins to ask the question of what happens during a competitive adsorption process and is based on the ideas of Bagnall[20] and Jennissen[21], who showed that the surface denaturation or accommodation process is dependent on the number and type of neighbors. If two different proteins have adsorbed next to each other, one is generally more labile or conformationally adaptable to the interface than the other. We can say that one »spreads« at the interface more effectively than the other. We can consider a spreading constant for such a protein, analogous to the solid/vapor and solid/liquid spreading constants so commonly used in classical surface chemistry[18]. One would expect that the spreading characteristics would be related to the solution denaturability[12] of the protein, and particularly to its behavior at water/air and water/oil interfaces[9,20,22,23]. Clearly, the more »surface active« protein would spread more effectively and may displace the other protein from the interface. This is another mechanism by which the Vroman effect[16] can be explained. Clearly, the next step is to develop a model which incorporates the ideas of Cuypers[2], Lundstrom[1,19], and Bagnall[20], but generalized for complex multi-component systems.

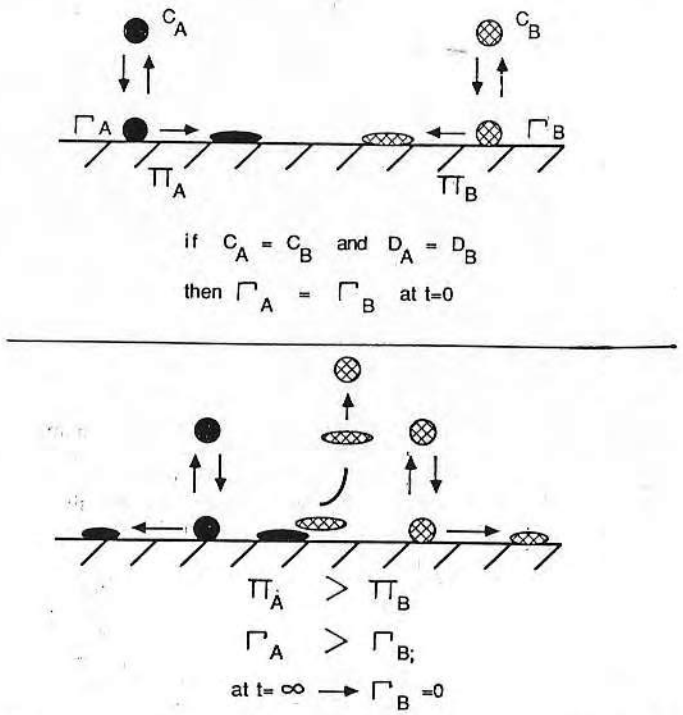

Figure 2. The competitive surface spreading hypothesis of Bagnall[29]. Top: Two proteins, A and B, have the same bulk concentration and diffusion coefficients and, therefore, have the same surface concentration (Γ) at $t = 0$ (initial contact). If the spreading pressure (π) of one is greater than the other, then eventually the protein with the large π will dominate the interface. Here we show, since $\pi_A > \pi_B$ that A displaces B (from Ref. 4).

SURFACE DENATURABILITY

In addition to the on- and off-rate constants described in Figure 1., one should have a measure of the ability of the protein molecule to denature at the interface as a function of time. Such information is very difficult to obtain at solid/liquid interfaces, although, in principle, some of the surface sensitive spectroscopic techniques can provide some such information. For example, ATR-FTIR studies of the adsorption of a single component protein from dilute solutions can provide evidence of conformational change as a function of time at the interface[24]. Total internal reflection fluorescence (TIRF) also provides evidence for changes with time at the interface[25,26], but such techniques are specialized, difficult to quantitate, and difficult to interpret in terms of actual structural changes. Also, they are substrate-limited and even substrate-specific due to the optical properties required for the total internal reflection condition. Some such information is also available via ellipsometry

in terms of changes in the refractive index and thickness of the adsorbed layer as a function of contact time (see References 2, 19).

Another approach is the use of monoclonal antibodies to probe the conformation of adsorbed proteins. Such studies do indeed suggest that epitopes, which are normally masked in solution, can be made accessable upon adsorption at certain interfaces[28,29]. A recent review of conformational changes upon adsorption is available[30]. A number of recent papers in the chromatography literature have appeared which clearly document surface induced conformational changes or denaturation upon adsorption to chromatographic supports[31-34].

As suggested earlier, the intrinsic solution stability of a protein is probably very important in understanding its adsorption behavior. There is a wealth of information in the solution biochemistry literature on the denaturation of proteins. The intensive activity on protein folding and on protein unfolding and refolding has provided an enormous data base which may be helpful to the protein adsorption community[35-39]. Proteins can be denatured as a result of major changes in a number of solution parameters, including temperature, pH, urea concentration, quanidinium chloride concentration, and low molecular weight surfactants. In the case of thermal denaturation, detailed thermodynamic analysis and modelling of the data is available[37].

Through modern specialized instrumentation, it is now possible to follow the denaturation process in real time by monitoring a number of conformationally sensitive parameters simultaneously[39]. It is clear from a brief perusal of this literature that some proteins are very robust and require major changes in solution conditions before they denature. Other proteins are quite labile and denature readily. Although the process of solution denaturation and denaturation at a solid/liquid interface is very different, one would expect some correlation between the surface denaturability of a protein and its intrinsic stability in aqueous solutions, as suggested in Figure 3.

We feel that another useful approach is to study the behavior of proteins at water/air, water/oil, water/fluorocarbon liquid, and water/siloxane liquid interfaces, using standard, proven, and inexpensive surface and interfacial tension techniques[18,20,22,23]. With such techniques, one can measure the spreading pressure through the decrease in surface or interfacial tension of various individual proteins. One can also measure the temperature dependence of interfacial processes. Although this doesn't relate directly to the interface between biomedical material and plasma, it helps characterize the interfacial activity of the various protein species of interest. One can develop an empirical parameter and use it as a coefficient or exponent in the appropriate terms in the equations. The problem is to get such data (chromatographic and interfacial activity data) for the proteins of interest. Although some such data is available in the literature, it is limited, and there is no compilation of information on the interfacial activity of plasma proteins. If such data were available, the modelling and simulation of protein interfacial processes might actually be straightforward.

Our previous argument that interfacial denaturation should be related to solution denaturation is suggested from results available in the protein surface tension and protein monolayer literature. Lysozyme has been particu-

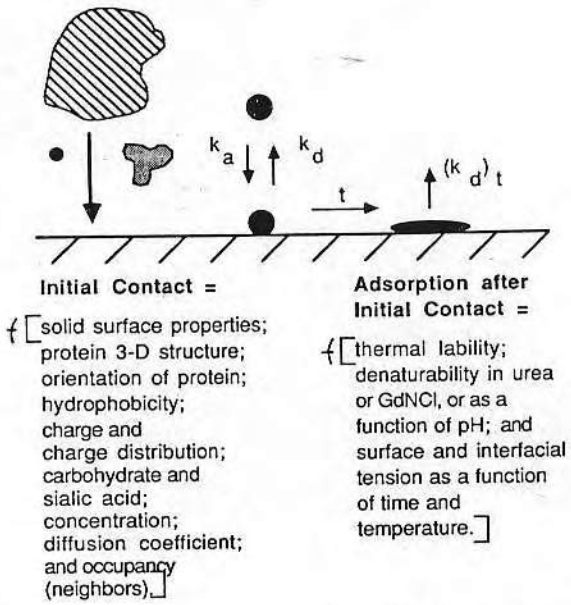

Figure 3. Suggested parameters involved in the initial contact phase of adsorption (left side) and in the time-dependent surface and protein »denaturation« processes (right side) (from Ref. 4).

larly well-studied. Early work has indicated that lysozyme is difficult to spread and unfolds very slowly at interfaces, perhaps due to its rigid structure[40,41]. The air/water interface techniques allow one to measure total adsorption and surface tension independently. The amount adsorbed can reach a steady state value while the surface tension or the spreading pressure is continuing to change significantly, demonstrating the slow rate determining nature of the conformational changes occurring at the interface[41]. One can measure surface and interfacial tension as a function of temperature and in different solution environments, such as various pH, urea, and guanidinium chloride conditions. In this way one can begin to correlate the lability or denaturability of a molecule in solution with its air/water interface or water/oil interface behavior[20,23,42]. The correlation is not necessarily obvious or straightforward, however, as Arnebrant et al.[43] in a preliminary study found a correlation between the adsorption of casein on a hydrophobic chrome surface and its surface tension reduction at the air/water interface, but the maxima in each case were not directly correlated.

Deyme et al.[44,45] have used the method to study collagen adsorption in competition with albumin and fibrinogen, measuring both adsorption and surface and interfacial tension changes separately and dynamically. The methodology, therefore, has great potential for studying competitive adsorption processes.

We cannot expect surface tension studies to mimic solid/liquid interface studies. Generally the solids of interest are rigid and immobile, whereas the air/water and water/oil interfaces are highly mobile and dynamic. Nevertheless, surface and interfacial tension studies are straightforward, easy to perform, relatively easy to interpret, and should be helpful in helping us understand and predict the general interfacial behavior of proteins.

MODEL SYSTEMS

We are now expanding our efforts with hen and human lysozyme as model proteins[5,6] to a larger group of proteins whose crystallographic structures are known and which therefore can be studied by molecular computer graphics techniques. In addition, this set of proteins will be studied for their solution denaturation characteristics, as well as for their surface tension behavior at various air/solution interfaces. Eventually the same set will be studied for their solid/liquid adsorption properties, using a series of model solid supports based primarily on commercially available hydrophobic, ion exchange, and change-transfer chromatographic matrices. Given such data and the models and concepts presented in Figures 1—3., together with the theoretical models and treatments previously cited, we are confident that the general predictive understanding of the adsorption of small, simple, globular, single-domain proteins[47] will be within reach. Indeed, Keshavarz and Nakai have already shown a good correlation between interfacial tension at the oil/protein solution interface and hydrophobicity as measured by retention on hydrophobic chromatography supports[46].

CONCLUSIONS

Figure 3. suggests that the early stages of adsorption (very short contact times) are functions of the particular chemistry of the surface, the particular three-dimensional structure and orientation of the protein, and the number of species and their concentration. In addition, adsorption at short contact times is also a function of occupancy and lateral interactions. We suggest that the time dependent conformational adaptation to the surface is related to the bulk solution denaturation tendencies of the protein, including thermal denaturation, denaturation in urea and guanidinium chloride solutions, and denaturation in solutions of different pH. We further suggest that surface and interfacial tension measurements of proteins at water/air, water/oil, and other water/liquid interfaces will be useful measures of protein surface activity at the solid/water interface.

CAUTION

We have assumed throughout the discussion that there are no covalent changes imposed on the molecules prior to, during, or after the adsorption process. Clearly, the work of Brash and others demonstrates that covalent bond changes can indeed occur[16]. Certainly plasma has a variety of active proteases and protease inhibitors, which change in concentration depending on local needs and processes. Clearly, the conformational adaptation to the surface which we have described may make a molecule more or less susceptible to proteolysis or to other chemical processes. Indeed, the very act of interacting with certain types of surfaces could control direct covalent

chemistries, such as possibly the interaction of Complement-C3 with nucleophilic surfaces[48]. The surface activation models, which are being developed by Sefton[49], Mann[50], and coworkers, coupled with the modelling and simulation suggested here, will be an important next step in attempting to treat truly practical protein-material interfaces.

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SAŽETAK

Simulacija adsorpcije proteina. Korelacija s denaturacijom

J. D. Andrade, J. Herron. V. Hlady i D. Horsley

Predložen je način modeliranja i simuliranja procesa adsorpcije jednostavnih proteina na modelnim površinama. Vrlo je korisno pritom istraživati početni kontakt i međusobno djelovanje proteina i modelne površine s pomoću molekulske računalske grafike. Dan je pregled kinetičkih modela adsorpcije proteina i ukratko je razmotrena uloga konformacijskih promjena proteina uzrokovanih prisutnošću površine. Ističe se da podaci o denaturaciji proteina u otopinama mogu biti izuzetno važni pri ocjenjivanju stabilnosti i labilnosti proteina na površinama. Ti parametri, kombinirani s površinskom napetosti i utjecajem proteina na međupovršinsku napetost, pomažu pri razvijanju hipoteza o adsorpciji proteina i koreliranju tih hipoteza sa stvarnim ponašanjem proteina na međupovršinama krute i tekuće faze.

Molecular Engineering of Ultrathin Polymeric Films

P. STROEVE AND E. FRANSES (EDITORS)

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THIN ORGANIC FILMS OF PROTEINS*

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Biological applications of protein films, machines and devices are briefly reviewed. A brief discussion of protein structure and properties is given. Needs and opportunities in the field include the following: (a) better potential functions to model and predict protein structure, denaturation and adsorption and the assembly of protein films and devices; (b) better methods to characterize protein films, particularly two-dimensional structures and ordering; (c) better means to assemble and stabilize protein machines, such as in lipid or polymer films; (d) means to modify protein stability, binding and enzymatic properties. About 5000 different proteins exist, each "designed" for a specific function: they can serve to stimulate and aid scientists and engineers in the design and development of new generations of molecular machines and devices.

1. INTRODUCTION

Nature uses biomacromolecules for a wide range of functions. The composition and structure of these systems have been optimized over 2×10^9 years of evolution. Such molecules can be used for non-biological applications and can serve as models to help stimulate and guide current and future activities in polymer science and molecular engineering.

The major classes of biomacromolecules are proteins, polysaccharides and polynucleic acids[1]. Lipid aggregates, such as membranes and vesicles, could also be considered. Various combinations include lipoproteins (lipid–protein aggregates), glycoproteins (proteins containing oligosaccharide or even polysaccharide components) and others. Polysaccharides perform important mechanical, physical, biochemical and related functions. Polynucleic acids (deoxyribonucleic acid and ribonucleic acid) are key information storage and transfer molecules[2]. However, because of space and time constraints, we will discuss only proteins.

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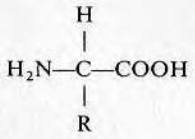
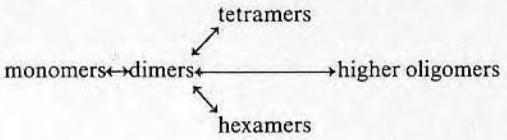
Proteins are complex copolymers of highly specific sequences consisting of some 20 different amino acid "monomers":



(1)

where R can be polar, apolar, positively charged or negatively charged[3]. Protein molecular weights cover the range of roughly 10^4–10^6 Daltons. A particular protein generally has one or more specific functions and is clearly molecularly engineered to carry out that function in an efficient, specific and often unique manner. There are approximately 5000 different proteins in animal cells, about 2000 of which are enzymes. Table I lists some of the functions of proteins and gives representative examples. Most of these functions and applications are discussed briefly in any basic biochemistry textbook[1].

The *primary* structure of a protein is its amino acid sequence which constitutes the primary information and basically controls all properties and functions of the molecule. A *secondary* level of structure includes local amino acid sequence associations which result in helix, sheet or turn structures. Globular proteins generally have a complex *tertiary* structure which can often be determined by X-ray diffraction of proteins in the crystalline state[3]. Finally, two or more polypeptide chains can associate via their tertiary structures to form a *quaternary* structure. Nearly 200 proteins have been crystallized and their tertiary and/or quaternary structures determined[3,17]. The atomic coordinates of these proteins are readily available and can be viewed using modern molecular graphics on standard computers and via available software (see the *Journal of Molecular Graphics*). Certain proteins can dimerize, oligomerize or even self-assemble:



(2)

Insulin dimerizes and at higher concentrations will form hexamers. Hemoglobin commonly exists as the tetramer[1]. Albumin also dimerizes and forms higher order oligomers. Fibrinogen, on the action of the activating proteolytic enzyme thrombin, self-associates and polymerizes to form the network that produces blood clotting[18]. Tubulin self-assembles to form the microtubules found within most cells[8,19]. Actin and myosin self-associate to form the characteristic filaments of muscle and a variety of other structures[7–9,19].

The tertiary and quaternary structures of proteins are stabilized by hydrophobic, ionic and hydrogen bonding interactions. However, for most globular proteins the "native" state is only marginally stable and the protein can be easily "denatured", *i.e.* lose part of its biological activity[20]:

$$\text{native} \longleftrightarrow \text{denatured} \qquad \Delta G \approx 5\text{–}14 \text{ kcal mol}^{-1} \tag{3}$$

Proteins are now generally recognized to be dynamic, flexible structures of marginal stability. Changes in pH, temperature, ionic strength, or solute environment can often change the conformation (three-dimensional structure) and biochemical activity of a protein.

Although there is much activity in trying to predict the tertiary and quaternary structure of proteins from their primary amino acid sequences, the predictions are not usually very good[17,21]. This is partly because many states are only marginally different in overall free energy so that the "true" equilibrium or native state is difficult to find. It is also because intermolecular force functions in ionic solutions are not well understood, including the classical hydrophobic and electrostatic interactions.

The interaction potential functions which are available, however, can be incorporated into the computer graphics algorithms to yield three-dimensional images of the constituent atoms and their interaction fields, permitting "docking" or interaction studies between two proteins, between a protein and a surface, between an enzyme and its substrate etc.

TABLE I

SOME OF THE FUNCTIONS OF PROTEINS[1–4]

Function	*Examples*	*Tissue*	*Specific references*
Structural	Collagen	Bone	1,4
	Silk	Spider web, silk worm	
	Fibrinogen	Blood clot	
Motion	Myosin–actin	Muscle	5,6
	Tubulin–dynein	Cilia, flagella	7
	Tubulin–kinesin	Intracellular tubules	8,9
	Flagellin	Bacterial flagella	4
Optical properties			
Transparency	Collagen–crystallin	Cornea, lens	4
Bioluminescence	Aequorin–luciferase	Worms, fish, etc.	
Recognition	Immunoglobulins	Many	10,11,12
	Lectins	Plants	10,11,12
Catalysis	Enzymes	All	1,13,14
Transport	Hemoglobin (O_2)	Red cells	1
	Transferrin (Fe)	Many	
	Lipoproteins (lipids)	Many	
Toxicity	Toxin peptides	Certain snakes, snails etc.	1,3
Regulation	Insulin	Pancreas	1
		Blood	
Transduction	Rhodopsin (light)	Retina	12,15,16
	Receptors	Membranes	

2. THIN PROTEIN FILMS

Most proteins will adsorb at solid–aqueous interfaces to form reasonably compact, generally monomolecular and often irreversible films. A number of recent reviews of this phenomenon are available[22,23]. Although no rigorous or quantitative theories of protein adsorption are available, there is a general qualitative understanding of the process[24,25]. Several groups are now working with model proteins whose structures are well known and are attempting to develop methods of simulating and predicting the adsorption process[26–28]. Practical uses of adsorbed protein films include diagnostic immunoassay, which uses preadsorbed specific immunoglobulins, and the preadsorption of human plasma albumin to improve the blood compatibility of cardiovascular devices[24].

The Langmuir monolayer trough has been widely used for the study of proteins at air–water interfaces[23,29,30] and indeed many of the "rules of thumb" on protein adsorption have been derived from Langmuir trough studies[22,24]. The transfer of many proteins to solid supports by Langmuir–Blodgett methods has been accomplished, although the process is difficult and frequently unpredictable[23,30]. Recently, Uzigiris[31] bound a hapten-specific IgG to a transferred hapten–phospholipid layer and showed that the close-packed IgG layer assumed a hexagonal structure. Uzigiris suggested that the technique may be useful as a means to produce ordered two-dimensional immunoglobulin films.

Giaever and Keese[32] have suggested that an adsorbed protein film, when exposed to UV light in the presence of ozone, will be modified in the exposed areas, resulting in loss of antigenicity and decreased reaction with its specific antibody. This would allow one to prepare protein films with selected, predefined regions of antibody reactivity or lack of reactivity.

Specific proteins are routinely bound to chromatographic supports and surfaces, in general via direct coupling[33,34] or affinity methods. If the protein has a specific interaction with a particular ligand, that ligand can be immobilized to a solid support[33]. Passage of a protein mixture over the immobilized ligand column results in specific binding of the protein to the immobilized ligand surface. This is a popular and commercially important separation, purification and characterization process known as affinity chromatography[34]. Often a specific antibody is immobilized for the binding of its specific protein antigen. The complex can be disrupted by an eluting solution of appropriate pH, ionic strength etc.[34] Suitable ligands include antibodies (for antigens), sugars (for certain lectins), heparin (for lipoproteins, fibronectin etc.), collagen (for fibronectin) etc.

Certain proteins may also self-aggregate and even self-assemble into complex structures[35]. The protein tubulin self-associates to form microtubules[19,36]. Fibrinogen, after appropriate activation, polymerizes to fibrin[18]. Many other examples are known[35,37]. A variety of very complex films are also known, such as cell membranes, which include embedded transmembrane proteins and protein complexes, *e.g.* ion channels and receptors. Several groups are attempting to reconstitute purified receptors in organic thin films, generally lipid bilayers. For example, the acetylcholine receptor is being incorporated into bilayers on appropriate microelectronic devices to produce specific biosensors for nerve agents[10]. Proteins can also be

ordered in electric, hydrodynamic and gravitational fields[38], and it is likely that such fields can be used to assist and even to control the ordering in protein films in the near future.

3. PROPERTIES

The properties of protein monolayers, except for their chemical interactions, are not well known. Certainly films of proteins containing chromophores (such as hemoglobin [Fe^{2+}heme]) have the expected optical absorption and resonance Raman properties. The refractive indices of protein monolayers are known via ellipsometry, a technique widely used to study protein adsorption[24,25,39]. Crude packing characteristics and surface viscosity data are available at the air–water interface from Langmuir trough studies[23]. There is very little experience or data on protein conformation[24], mechanical properties[23], or optical or electronic properties of protein monolayers. Only in the last few years have research groups begun to apply modern surface analytical techniques to characterize protein monolayers (see ref. 24). The field, therefore, is wide open.

4. APPLICATIONS

The major application of protein layers or films is for separation and purification, particularly in the chromatography field[34]. Immobilized enzymes are widely used in certain biochemical–biotechnology processes[40]. Another major "application" is the understanding of adsorption of proteins with respect to the blood and biocompatibility of medical devices[24]. There is considerable interest in the development of protein-resistant surfaces, which actively repel proteins, to minimize protein–surface interactions[24]. Such surfaces would have wide application in chromatography, membrane separation processes and medical devices, including contact lenses.

Biosensors are a new area with considerable interest and activity[11]. Most biosensors depend on the specific binding characteristics of proteins or other biological molecules for the specificity and sensitivity of the sensor. Immunoglobulins are of particular interest as they can be used to detect their specific antigen with great selectivity and sensitivity[11].

There is some potential for the use of protein films in energy (solar) collection and transfer, such as chlorophyll-containing and electron transfer–redox enzyme protein systems. Light detection and transduction are also of interest, at least as a model system, utilizing proteins such as rhodopsin. Light emission (bioluminescence) via the luciferase–luciferin or aequorin systems is of great interest, especially for immunoassay applications[11].

Although there has been considerable discussion of protein-based information storage and even computation, no practical or even defensible concepts or designs have yet evolved[41]. However, information storage and processing in biology is well known and to some extent understood[2].

5. PROTEIN MACHINES AND DEVICES

Cooperative protein interactions result in functions and behavior reminiscent of non-biological machines and devices. The functions listed in Table I, together with combinations and enhancements of those functions, are in large part responsible for what we know as life in animal systems. Enzymes can and have been treated as chemical machines[42]. In Welch's words[13,14]

> "Living systems ... contain devices of molecular dimensions capable of performing mechanical work .... A molecular energy machine is a single enzyme molecule that cyclically couples energy released by one form of reaction, *e.g.* ATP, to an otherwise unfavorable reaction, *e.g.* contraction, without (during the lifetime of its cycle) being exposed to the macroscopic and thermalizing environment .... Molecular energy machines require some degree of structural organization. The transduction process must be insulated from the thermalizing bulk phase. Molecular machines must successfully compete against the thermal environment."

In a casual and simplistic sense, one might consider an enzyme or organized assemblages of enzymes to be molecularly engineered "Maxwell's demons", permitting reactions and processes to occur which cursory analysis would suggest to be contrary to the laws of thermodynamics.

Particularly dramatic examples of protein machines, from an engineering point of view, are the various motions and force generation processes so common throughout all biology. The most common and perhaps well-understood example is the interaction of myosin and actin, together with regulation by the proteins tropomyosin and troponin, in the function of muscle. Here we see protein molecules designed with exquisite self-assembly character, coupled with specific binding interactions, highly regulated by accessory proteins and by calcium ions. The end result is a sliding-filament, force-generation mechanism responsible for virtually all muscle and other linear motion throughout biology[5,6].

Enzymatic activity, *e.g.* adenosine triphosphatase (ATPase), is built right into one of the heads of the myosin molecule, and it is indeed ATP hydrolysis via the myosin ATPase activity which provides the fuel for the force generation process.

The self-assembly of α- and β-tubulin to form the ubiquitous intracellular microtubular structures is an interesting example of the use of proteins for structural purposes. The coupling of tubulin structures with the protein dynein, in the case of cilia and flagella, and with the protein kinesin, in the case of intracellular tubules, makes possible the characteristic wave-like motion of cilia and flagella and the conveyor belt linear transport processes now recognized as so important in the interior of living cells. Bacterial flagella, made up of the membrane-anchored protein complex called flagellin, are responsible for rotary motion, which at one time was thought to be impossible in biology[4,5,7–9].

Some proteins function essentially as primitive light sources, *e.g.* the luciferin–luciferase bioluminescence process. Another interesting example is the calcium-sensitive photoprotein aequorin which, in the presence of calcium ion, triggers a conformational change which permits a trapped, excited state in a bound chromophore to decay, resulting in photon emission. The bioluminescent properties

of these proteins and others are beginning to be extensively used in clinical and analytical chemistry as quantitative and end-point detection methods.

Rhodopsin, the basis of the light transduction mechanism responsible for vision in most of the animal kingdom, is another membrane-bound protein–chromophore complex[12,15,16]. A wide range and variety of membrane receptors, consisting of either individual proteins or protein complexes, are known. These receptors are responsible for a variety of recognition as well as membrane gating and transport processes[12].

It is clear that, as we consider the invention, study and application of synthetic organic and polymeric thin films, it would be useful for some attention to be paid to some 5000 proteins available in biology which have been molecularly engineered over 2×10^9 years of evolution to provide a wide range of machine- and device-like activities. Undoubtedly, the harnessing of proteins for practical machine- and device-like activities will require far more knowledge and experience with the following: (1) the handling of these molecules; (2) their incorporation and reconstitution into lipid or polymer-like matrices; (3) their modification, possibly by genetic engineering and site-directed mutagenesis technologies, for the enhancement of self-assembly properties; (4) enhancing their stability in non-biological environments.

6. NEEDS AND OPPORTUNITIES

In order to understand, predict and apply protein films, we must understand the forces and interactions responsible for protein structure and function, as well as their interactions among themselves and other proteins and with solid supports. Although the electrostatic, hydrophobic and hydrogen bonding interactions are qualitatively understood[43–45], better potential functions are needed to model and predict effectively protein structure, folding[21], denaturation[20], and adsorption[24]. Direct measurements of such forces and interactions at the protein film–aqueous solution interface are needed, such as via the methods of Israelchvilli[45] and Klein[46]. The simulation and modeling of proteins via computer molecular graphics and intermolecular interaction potentials is in its infancy[47]. With better potential functions and with information on the structure of protein films, attempts should be made to develop theories and models for protein interactions and the film-forming process. Such models will permit the design of structures and means for self-assembly of films with unique, designed properties. The modeling and simulation of film formation and film properties should include the effects of impressed fields.

Methods to characterize protein films *in situ*, *i.e.* underwater, are needed. In spite of the progress made in applying reflection fluorescence[28] and IR[48] methods, more definitive methods are required to probe and characterize packing, orientation and structure of protein films. Perhaps the most promising approach is direct imaging in water using scanning tunneling microscopy[49,50]. Synchotron scattering and diffraction should also be considered. Other means of imaging and characterizing protein films should be encouraged. Fourier transform Raman spectroscopy in the near-IR has great potential for characterizing the conformation and orientation of protein thin films. Methods to characterize and evaluate the properties of protein

thin films should be developed, including electronic, magnetic, optical and mechanical properties.

There should be attempts to apply the unique properties of certain proteins in mixed films; for example, embedding receptors in lipid or polymer films for sensor and gating applications[10,12], and the co-immobilization of antibody and synthetic photoactivable polymers to regulate antigen–antibody binding constants[12,51]. Optimization of enzyme properties for reactions in adverse environments and modified or synthetic enzymes for new chemical reactions also should be encouraged[52].

7. SUMMARY

Proteins are a series of polymers with unique and varied structures and properties. They are beautiful examples of macromolecular engineering for specific and unique purposes. The modeling, preparation, characterization and application of protein films should lead to a variety of very useful and unique applications. Perhaps most importantly, experience and study of proteins will greatly aid and stimulate materials scientists and engineers to engineer better new generations of macromolecules for new and now unknown applications.

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PROTEINS AT INTERFACES: PRINCIPLES RELEVANT TO SURFACE ANALYSIS

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Proteins are nature's molecular machines. There is considerable interest and activity in applying and adapting proteins for the development and application of biomolecular devices and machines [1]. Such applications require the handling and processing of proteins, including their concentration, ordering, and assembly at interfaces. The current principles and understanding of proteins at interfaces are presented and reviewed, including current theories and hypotheses. Progress in the design and application of defined surfaces and materials for the study and precise assembly of protein films is also discussed, particularly gradient and patterned surfaces. We also present a multi-variate approach to the parameterization of surface properties.

Proteins are biological macromolecules [2,3] consisting of some 20 or more monomer types (amino acids) condensed into one or more chains (polypeptide or primary sequences) via amide (-NH-CO) bonds. Many proteins also contain covalent cross links, generally of the -S-S-type, as well as short carbohydrate chains (glycoproteins). Proteins range in size from several thousand (10^3) to tens of millions (10^7) Daltons. A particular protein normally exhibits one or more of the following functions: catalysis (enzymes), mechanical properties (fibrous, structural proteins), chemical recognition (antibodies), chemico-mechanical transduction (muscle and motion proteins), chemical regulation (many), transport (ion channels), optical properties (luciferases, rhodopsins), electron and charge transfer, etc. The structure of globular proteins is generally only marginally stable, partially unfolding to a "denatured" state with a free energy input or change of only 5-15 k cal/mole. Large proteins generally consist of several structural and functional domains. Complex proteins are now believed to consist of domain "building blocks."

Practically all proteins are polymeric surfactants in aqueous solution. Their constituent amino acids have hydrophobic and hydrophilic (neutral, positive, or negative) characters. Hydrophobic interactions are very important in their globular stability and in their interfacial activity. It is now generally accepted that the placement of proteins at interfaces, usually via adsorption from solution, results in a conformation and orientation different from that under "normal" physiologic conditions. Such interface microenvironment -- induced altered states can result in "abnormal" or unexpected biochemical and biophysical properties. Several well known examples are the interface-induced activation of blood coagulation, activation of the complement defense system, activation of inflammatory processes, and enzyme inactivation.

PROTEIN ADSORPTION

A number of recent conference proceedings and an edited monograph are now available [4-6], as well as several comprehensive reviews [7-9]. The general principles and hypotheses now accepted and being tested are shown in Figure 1.

Each protein has its own distinctive and individual "surface" chemistry, produced by the outer shell of amino acids and carbohydrate which interface with the liquid medium [7,11] (Figure 1a).

Diffusion and convective transport results in a collision rate between proteins in solution and the interfaces present [7,10] (Figure 1b).

Although protein molecules collide with the interface in many different possible orientations, one specific orientation will probably result in the most stable adsorption, with hydrophobic surface patches oriented towards hydrophobic surfaces, anionic protein pateches oriented toward cationic surfaces, etc. (Figure 1c). Domain and mosaic surfaces can be expected to have rich and complex interactions with the domains and mosaics on the surface of the protein.

If the collision rate is very high and the interface is populated with protein very quickly, the proteins may not show extensive time-dependent denaturation processes (Figure 1d), although there may be some adjustments in packing, ordering, and lateral interactions [4-7].

If the surface is not highly populated, the adsorbed protein may denature and/or spread at the interface, altering its conformation and orientation to optimally adjust to its new microenvironment (Figure 1d). Such events may result in the

expulsion of less optimally oriented or bound proteins from the interface, as the more optimally oriented or bound protein spreads at the interface (Figure 1e). The tendency for the protein to denature at the interface is related to its intrinsic stability, including the number of disulfide bonds [12].

The presence of two or more different proteins in the solution will result in competitive adsorption processes; with that protein which can most optimally bind and accommodate at the interface tending to displace its less optimally bound neighbors (Figure 1e,f). Thus, a complex hierarchy of adsorbed protein types and amounts can develop with time, related to solution concentration, size, collision rates, interface affinity, and denaturation tendencies. This behavior is now called the "Vroman Effect" [12,13].

It is possible to control and regulate protein adsorption, in part, by modifying the surface or interface or by modifying the protein, such as with steric exclusion modifiers [14,15] (Figure 1g).

Materials and interfaces with a micro-heterogeniety of the same size as the structural domains or building blocks in proteins probably have a particularly rich and complex set of adsorption properties (Figure 1h).

These principles permit biomaterials and surface scientists to consider which surface preparation, modification, and characterization information is required for useful products utilizing proteins or involving protein interactions.

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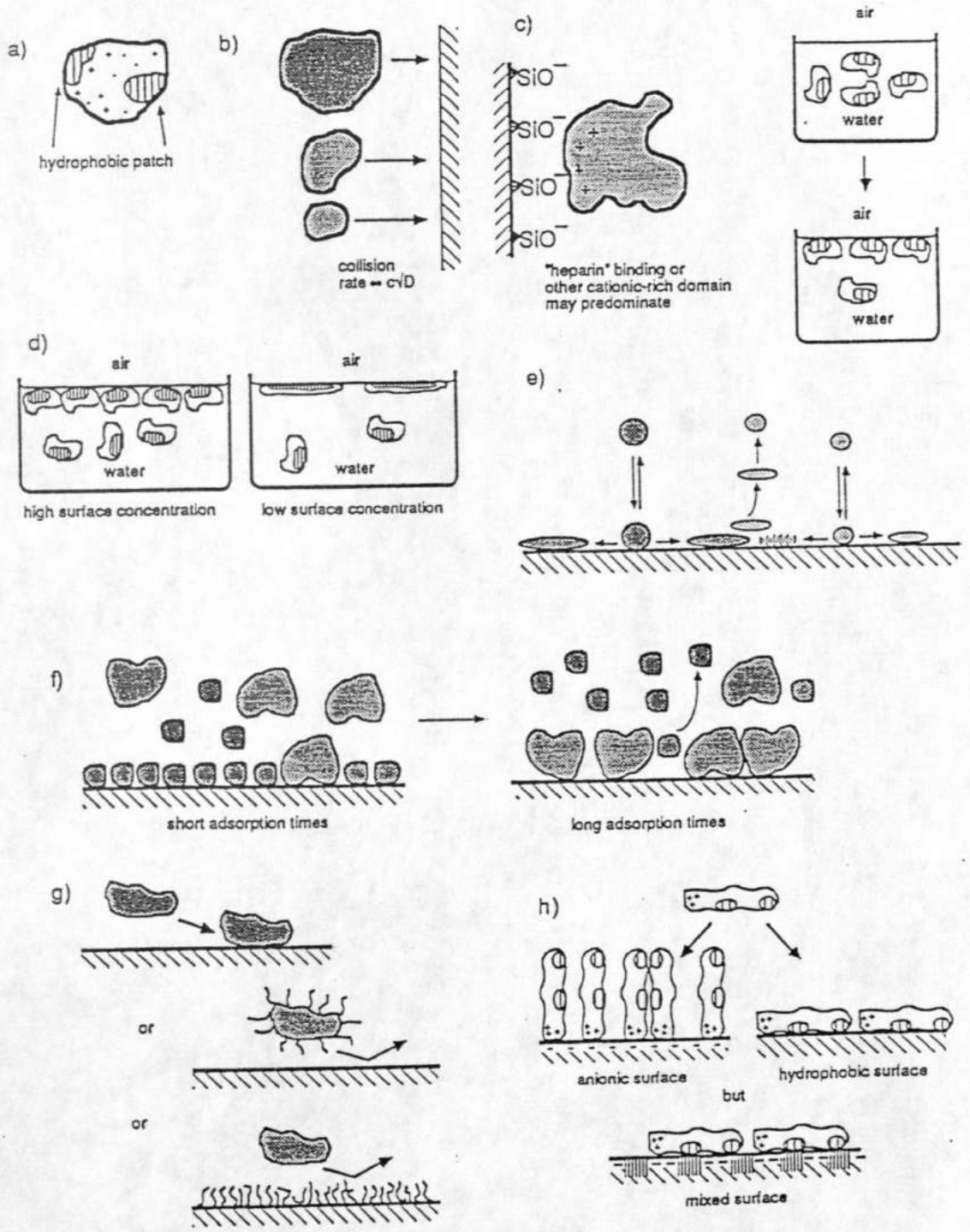

Figure 1. Summary of Current Principles and Hypotheses of Protein Adsorption -- see text for details.

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J Andrade

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RESEARCH,
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THIRD ANNUAL CONFERENCE ON RECEPTOR-BASED BIOSENSORS

Edited by James J. Valdes, Ph.D.
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RESEARCH DIRECTORATE

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the research and development stage and is now in engineering for product configuration for the doctor's office. I've tried to get them to share their fiber coating techniques which they've developed but I don't think they've ever shared that information. On special cases they've said if you send us the reagents, we'll coat them for you but we won't tell you what we do. Their reporting CV's for commercial and diagnostic range from less than the five percent range.

Glass: Certainly less than ten percent.

Patton: Nonspecific absorption on any type of glass surface is a problem, particularly a wave guide in which you are interested in what's happening optically on the surface. I do believe that the nonspecific absorption problem can be solved.

Q: Does anything in this technology preclude the employment of the flow cell incorporating the capillary you described in the flow cell?

Glass: No. That's how we do it. We do it also strictly with capillary filling. All of the work that I showed was done with the flow cell.

Patton: I'd like to introduce Professor Joe Andrade who's dean of the college of engineering at the University of Utah.

Andrade: I would like to continue the same line of discussion and address the question of how one might take the sensor that was just described and try to make a continuous and possibly even a remote device out of it. You might want to subtitle the talk "Some Unsolved Problems and Concerns in the Development of Receptor-Based Optical Biosensors." Our group's goal is remote semicontinuous optical biosensors based on fluoroimmunoassays, not disposable one-shot devices. There is considerable interest in the biotechnology and biochemical engineering business for an on-line process control of biochemical processes. There's some interest in waste and water treatment water monitoring, and considerable interest in medicine and diagnostics. It's possible that this can be done in an in vivo implanted sensor mode and biomedical research as well. There is some interest in the defense establishment in continuous on-line monitoring. Our group's basic science goals are protein biochemistry and how that couples with surface and interface chemistry, with optics and spectroscopy to probe that. Polymer chemistry in surfactants are used to modify it. Our engineering goals are to employ proteins as engineering machines and devices.

The area that's been the most developed in the general engineering community is sensors, biosensors in particular. How does one detect and monitor one of the proteins at an interface, in a reproducible quantitative manner? Almost any labelling process can change the physical chemical characteristics. About five years ago we were starting to look at probes of proteins at interfaces, and decided to would look at the intrinsic fluorescence which is present in most proteins, primarily via the tryptophan amino acid moiety. That absorbs in the UV and fluoresces a little closer into the visible, with a respectable quantum yield of about ten percent. Most proteins of interest are intrinsically labelled with a UV fluor. There's

considerable information available from fluorescence in addition to the intensity and spectral information. From fluorescent and depolarization information, fluorescent lifetimes can be deduced and one can measure energy transfer and use specific probes to change some of these other parameters. There's a wide number of information channels to fluorescence. We use a dove tail prism in the research apparatus and a fiber optic device in the analytical and engineering apparatus. Our device is equipped with a flow cell because we want to control the hydrodynamics and be able to follow the mass transport diffusion limitations and related considerations. On the other side of the flow channel, a gamma detector is mounted so we can use labelled proteins and use the radiolabelled signal as a calibration. The total internal reflections fluorescent signal is similar to the buffer background. As one injects protein solution into the cell, one sees a component of the bulk solution. The evanescent wave and the visible wave penetrate on the order of 2000 angstroms, and in the UV about 1000 angstroms. The protein's dimensions are of the order of about 100 angstroms. You see what might be called a boundary layer or evanescent volume component.

Protein absorption at an interface is kinetically a relatively slow process and takes on the order of minutes to hours to reach saturation. This is a non-specific binding process. At the end of that process you remove the protein solution, the bulk signal disappears, and you can see if the absorbed layer is stable. Is it reversible or irreversible? Normally, you find some component of various populations, some of which will be removed or desorbed into the buffer. The interface optics are very well known, particularly if you have a system where the evanescent modes are well defined. In the total internal reflection prism geometry, there's a single well defined mode. The electric field is decaying exponentially into the low refractor index phase. The fluorescence being emitted is the integral under that and involves a quantum yield, the absorption coefficient of the fluor, concentration of the fluor, the proportion of the fluorescence emission which is collected, the square of the electric field strength, and so forth. One can rigorously quantitate that, if you assume a concentration profile of the interface. We assume a step concentration profile which Myron Block indicated was due to normal absorption or specific binding processes. The protein is concentrated at the interface by a factor of as much as a thousand or more over its concentration in the bulk, and then we assume a uniform bulk concentration profile. You can ratio those two profiles, make a major assumption that the quantum yields of the protein in the layer at the interface and in the bulk are the same. That's a bad assumption in many cases, particularly for the intrinsic fluor. There's another problem in the ultraviolet, which is scattering due to the imperfection of the optics. The optics are never 100 percent perfect, not even an optical fiber, or the fiber solution interface, and so there is a component which does get scattered into the far field. That component propagates into the cell and generates fluorescence from the bulk solution, so the optical trick of evanescence allows you to separate the bound from the bulk through the fact that the evanescent wave is constrained or contained at the interface. There can be some excitation of the bulk through this scattering phenomenon. In the ultraviolet, that is a particular problem because scattering occurs as

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the inverse of the wave length, so when we use ultraviolet detection for fundamental studies, we have a scattering problem which is an order of magnitude greater than when we're in the visible. If you plot the fluorescence as a function of concentration of something with a very high absorption coefficient, you find that the scatter signal rapidly plateaus due to inner filter effect. You never see an inner filter effect because it's essentially linear all the way to saturation or precipitation of the solution. By playing that standardization game, one can sort out the scatter component from the evanescent component, which allows very rigorous quantitation even in the ultraviolet. The advantage of working in the ultraviolet has to do with the interfacial biochemistry to understand what is happening with antibody labelling and antibody immobilization with the kinetics of antigen binding.

It was clear to us about four or five years ago that, if we could detect one protein interacting with and interfacing with another, we could also detect two. That led to our interest in applying total internal reflection fluorescence which was patented by Hirchfeld in the 60's as an analytical tool in immuno assays. One can do fluoroimmuno assay in the ultraviolet; it's not particularly recommended, but certainly antibodies are UV fluorescent and most of the protein antigens of interest are also UV fluorescent. The count rates are low, UV light sources and UV detectors are still a problem, but the biggest problem is that you fry the immobilized component after significant excitation time in any sort of oxygen containing solution. That's not amenable to our long range goal of a continuous or semicontinuous remote sensor. The modes of operation of such a sensor have already been described. There are a whole variety of ways to do it, whether a saturation type assay or competitive binding assay, one can monitor antigen, antibody, and so forth. I'm going to focus on immobilized antibody for antigen or hapten detection. One of the big concerns is how to immobilize the antibody. In conventional, one-shot diagnostic immunoassay, that's often done by physical absorption onto a polystyrene or other support. That can work very well. We've looked at IGG physical absorption on dimethyl dichloro silanized quartz, on unmodified or hydrophilic quartz and on amino propyl silanized quartz. Our experience is that amino silane is a very bad support on which to immobilize and leads to fairly significant changes. The DDS is not too bad, as it is a sort of a model hydrophobic surface which one can covalently immobilize. We do it through a preabsorption of albumin followed by a glutaraldehyde cross-linking of the albumin and a glutaraldehyde coupling and cross-linking of the antibody. We've also tried glutaraldehyde coupled directly to an APS and cross-linked the antibody that way, and we've also played with protein A in order to try to orient the antibody. The model systems that we've used are an anti-digoxin system, a monoclonal to digoxin, and a commercial goat IGG to human IGG antigen. The binding constants in solution have not changed dramatically on binding or immobilization to a glutaraldehyde support or on physical absorption to a DDS support. In some cases there can be a significant change in the binding constant, but not in these model systems with which we've chosen to work.

We are interested in the fiber optic sensor approach and we use a large fiber with a silica core, where the cladding has been stripped in the sensing region. There are really three fundamental problems in making such a technology suitable for remote continuous use. It's

clear that fluorescent reagent is required. There are ways to get around that, but they all have some problems. How do you deliver a fluorescent reagent for a continuous remote sensor? One could have a plumbing system or perhaps a liposomal kind of system, to deliver reagent remotely. We've chosen to approach it from a photochemical point of view by using nitroaromatic linkages which are intrinsically photolabile. They break when they absorb protons of 350 to 360 nanometers, so we're coupling those linkages into a polymer. This polymer is actually a hydropropylmethylacrylamide derivative which has been used extensively as a drug carrier for cancer treatment in experimental animals. One is a side chain to the basic polymer structure, another side chain contains a nitroaromatic group with a reactive amine, which can then combine the fluorescein labelled antigen or antibody. That seems to work reasonably well in bulk solution. The project now is focusing on trying to make a gel which can be interfaced with the optical fiber in such a way that a pulse of 316 nanometer light down the fiber will then release a pulse of fluorescently labelled reagent. The more serious problem is: are we running a sensor or a dosimeter? Most of what's been discussed during the meeting really involves dosimeters. If we pull out the stops on sensitivity and use systems with high binding constants, then for all practical purposes that binding is irreversible. It clearly can be displaced and so made "reversible", but it takes rigorous conditions or much patience in terms of response time to do that. It's both an advantage and a disadvantage of antigen-antibody systems. The on-rate constant, forming the antigen-antibody complex, and the off-rate constant are such that you get a high binding constant. You want a very high binding constant because that gives you a high sensitivity, but it also means that the off-rate is very slow which means that the response time is very slow. We would like to cut that down and have a short response time, but that is at the expense of the binding constant which means the sensitivity drops.

The ideal system is one with a high binding constant during the measurement and a very low binding constant between the measurement. We'd like to be able to use it as a dosimeter during the measurement, and then rezero it between measurements. Some of you have genetically engineered systems which may be able to do that. Can we change the local environment around the antibody to change its binding thermodynamics? We've chosen to look at three approaches to that, but one is going to be very hard, one will be relatively easy, and the other, the most interesting one, will take a few years. One approach is to run the appropriate solution through an antigen-antibody column, and if the solutions are appropriate, you can displace the bond. You do that by changing the conformation of the antigen, the antibody or both, or changing the nature of the media so the antigen antibody interaction is significantly modified. That's a solute approach; you can do it through change in pH, through change in the hydrophobic nature of the hydrophobic structure breaking agents or others. We would like to have a reversible solute in order to avoid plumbing. You can do it by plumbing; simply plumb the system and wash it out with the appropriate elute, but we'd like to avoid that in a truly remote sensor. Our approach is to make a macromolecular switch which, under light, will induce a photoconformational change. We try to get a coil expansion, which basically takes a molecule and pops it out so it's co-

immobilized with the antibody, but under light it expands and now is essentially ruining the surrounding solution. It's a concentrated solute, but when it's finished we can pull it out of the way by simply pulling off the light or exposing to light of a different wave length to change the conformation. Our idea is to have a solute which we can direct into or out of the binding region. The model system we've chosen to work with for this is a series of monoclonal antibodies to fluorine. It's probably the most widely used dye in fluoroimmunoassays. The other good aspect of using this as the hapten is that the fluorescence of fluorescein quenches when it binds to its specific antibody.

It's very easy to do an on- and off-rate assay and to measure the antigen-antibody thermodynamics with this system. The binding constant of different monoclonals changes at different rates with temperature, so we can chose the right binding constant for that particular experiment. Since you have the temperature-dependent data, you can extract the thermodynamics from that. The entropy-enthalpy compensation process leads to the free energy being relatively constant until you get to temperatures at which the molecule begins to irreversibly denature. You can expand that free energy curve and show that there are some significant changes in free energy with temperature. One good aspect of this system is that you can not only measure the overall constant but also the off-rate constant or the dissociation lifetime. If we were operating at about 10° C, it would take about 5000 seconds for this thing to dissociate or reach a level of equilibrium with a change in antigen concentration or fluorescein concentration. At body temperature, that's down to the order of sixty or seventy seconds. It's clear that we can change a dissociation lifetime by changing temperature, at least in many antibody classes. How do you change temperature at the interface without frying the solution? We haven't done this yet with an evanescent wave. Coming in with a near IR beam, water has a fairly strong absorption at about 1.4 to 1.5 microns, and a much stronger absorption at 2. There are good, portable light sources available in that region. The solute approach is an interesting one if we look at the log of the binding constant as a function of the concentration of a particular solute. In this case it's methylpentanediol which is commonly used as a crystallization solvent by x-ray crystallographers. With methylpentanediol the change in the solute concentration drops the binding constant in a substantial manner by up to three orders of magnitude. If we could have a solute similar to that incorporated into a polymer which could be photo changeable, then we might have something. The hydroxypropylmethylacrylamide monomer worked slightly better, it went up to about eight percent, where the other went up to about 50 percent solute. In this particular case, by adding about eight percent weight by volume of the monomer, we dropped the binding constant by an order of magnitude. It does appear feasible from an antigen- antibody thermodynamic point of view to do what we've suggested here. We can try to design a polymer which can be immobilized or co-immobilized at the interface together with the antibody, and have a region which can expand under optical illumination, and as that expands, it drives a concentrated solute into the binding region. The solute is reusable.

The photoconformational system we've chosen, azobenzene is found in literature going back to about the early 1940's. Azobenzene

is essentially nonpolar with no dipole moment in its normal ground or trans state, but when excited with 350 to 360 nanometers it pops into the cis form which has a fairly substantial dipole moment. Polypeptides with azobenzene side chains can undergo significant changes in the alpha helix content as a result of this change in polarity of the side chains under optical stimulation. It's a copolymer approach, and again we used hydropropylmethylacrylamide to give us the water solubility and the general compatibility, and an azobenzene derived hydroxymethylacrylamide monomer to give us this photo conformational property. A small amount of monomer with a reactive side chain allows us to couple to the surface or to antibody or antibody fragments. We started out with methacrylic acid because the change in the polarity of this group due to the trans-cis isomerization changes the pK of this group, which changes the degree of the ionization, which changes the coil size through a polyelectrolyte coil expansion argument. We do see changes in the pK and in the degree of ionization as a result of the photoisomerization, but that's not sufficient to this point in the design of the copolymer to get a significant coil expansion because the methacrylate backbone is extremely hydrophobic. Its hydrophobicity is sufficient to overcome that. We're now moving to an acrylic acid system for the charge component and other components to try to minimize that hydrophobic backbone. We're fairly confident that it will work eventually. It's also obvious that a random copolymer is not likely to work in practice. We're going to have to go to an asymmetric polymer. It's essentially a block system, where we have an immobilization block, the coil expansion photoconformation block and the solute delivery component.

A couple of other projects at which we're looking are of some interest. Light sources which minimize the fluorescence background of serum have a strong use in continuous remote sensors for use in blood and in medical application. It's clear that if you excite much below 500 nanometers you pick up a significant fluorescence from serum. What we've done is, rather than go to the phycobilic proteins in red LED's, we're playing with the new generation of helium neon lasers. A green helium neon which puts out about 543, and relatively inexpensively one could pick up a milliwatt or so at 543, and the rhodamine dyes which have many similarities to the fluorescein in some respects, absorb and emit very nicely as a result of that excitation. That's a direction in which we're going on the sensor side. Another unresolved problem that's been alluded to is this whole question of nonspecific binding. There are many possibilities for nonspecific binding. First of all, there are always parts of the interface that are unmodified with protein, in the case of the classical silane reaction, there are pieces of the interface which may be unsilanized, and one can get physical and nonspecific binding to the support. The antibody or antigen or hapten that you're immobilizing is being immobilized through some chemistry. Dr. Patton referred to a urethane-like linkage in his talk. All of those will become involved in nonspecific binding to various extents, depending on what A, B, C, and D, are in the media. There's nonspecific binding to the antibody itself, because the immobilization results in a slight change in conformation because it's present in a different microenvironment than it was out in the bulk solution. There can be nonspecific binding to the antibody or cross-reactivity with other material.

Our group has been interested in biocompatibility in the blood environment for about twenty years, and we've been very interested in the whole question of protein resistance surfaces; that is, how do you keep proteins off an interface? The only approach that we've come across that seems to be general, nonspecific, and works, is to immobilize polyethylene oxide to the surface and other neutral hydrophilic polymers. Polyethylene oxide appears to be unique. The argument is an excluded volume steric exclusion argument. PEO is used extensively in a colloid field to sterically stabilize colloidal dispersions, and that's one of the reasons that it works. A protein, in order to absorb at such a surface, has to compress or minimize this excluded volume. You might think of compromising the configuration entropy of the PEO chain. That is an undesirable process unless there is a strong interaction between the protein and that particular surface. That's a good nonspecific repulsive technique. We're now immobilizing our antibodies through PEO tethers, so that at least the PEO tether will be reasonably resistant to nonspecific binding. Then we're trying to quench or cover the rest of the surface in and around the antibody with low molecular weight PEO chains, to minimize any other nonspecific binding.

Some of the surface chemistry and biocompatibility concerns we're addressing, then, are fluorescent reagent delivery, remotely under optical control, modulation of antigen-antibody binding constants both thermally and through photoconformational changing, or photoexpansion of an appropriately designed polymer to be co-immobilized with an antibody. The question of optimizing antibody orientation is immobilization, and our approach to that is through polyethylene oxide to get the antibody away from the interface using a fully hydrophilic tether. In essence, to keep it in a microenvironment as similar to that as bulk solution as possible. We're working on minimizing nonspecific binding through steric exclusion arguments, largely using polyethylene oxide approaches, and the model systems that we're working with at the IGG, anti-IGG polyclonal. A digoxin, anti-digoxin monoclonal system was provided by the University of Utah and more recently fluorescein, anti-fluorescein system from Illinois and Utah. We're moving into sensors for coagulation proteins, again sponsored by NIH, and the systems we've chosen to work with initially are thrombin and anti-thrombin 3 and their appropriate monoclonals.

Block: Your work with the ~~Greeley~~ GreN He-Ne source looks very interesting. Just for comparison, all we're dumping on our system is a couple microwatts. You get up to a milliwatt. I think you'll win the prize for the lowest detection level before we get there, because you'd have factor of a thousand immediately, so you'd be able to get to hundreds of molecules easily.

Andrade: We haven't attempted to do any optical optimization as you have, and so our numerical apertures and these things are all suboptimal.

Q: You mentioned the polyethylene oxide tethers. How do you attach the polyethylene oxide chains to the solid surface?

Andrade: There are a variety of ways one can do that. Most of what we've been doing involves the aminopropylsilanization of silica and in using that amino group for other coupling reactions, so the PEO can be derivatized. You can even buy diamino PEO. We are producing PEO with two different functional groups, one with different groups on each end so we can be sure to minimize looping effect.

Q: Are you using a silane?

Andrade: As far as the activation of the silica surface, yes.

Eldefrawi: I would like to make a follow up comment on the sources and ways to get the light. Another approach may be active fibers, which are getting very popular. They're beginning to emerge as commercial products. Your technology has an active fiber that you can pan in one wave length and you can get another wave length.

Andrade: You mean these fibers containing a built in fluor?

Eldefrawi: Terbium, yes.

Q: You mentioned that you're going to cover the rationale leading to the photoexpandable fibers. Could you elaborate on that?

Andrade: There's an old effect in the polymer field called the photo viscosity effect. If you take methacrylic acid or acrylic acid systems, basically polyelectrolytes, and heavily derivatize them with azobenzene derivatives, you can show the viscosity changes dramatically in the presence of light. You can also, in the presence or absence of light, titrate and get the pK of the systems. There's no question that in the appropriately designed polymer that happens in solution.

Q: I could explain a change in viscosity as a change in electronic configuration leading to a change in hydrogen bonding, for instance.

Andrade: Yes, but they're getting viscosity changes of various significant amounts.

Q: You spoke about data where a binding constant enthalpy was a function of temperature. Maybe it's something peculiar to the biochemistry, but chemistry enthalpies don't change with temperature typically. Free energies do by the change in the entropy, component but not the enthalpy.

Andrade: Most of it is based on an analysis that has gone through a whole variety of ligand receptor binding systems.

Q: What that implies is that the bond strength changes with temperature. I don't know too many examples of that.

Andrade: A number of classical bond types in biochemistry do change with temperature.

Patton: Thank you very much for your paper. From where this conference was two years ago, we've come a long way. As I look back on the day, it sounds like wonderful things and exciting things are happening in specific areas: Transducer technology, optical waveguide technology, new solid-state chemfets, and inductive devices. In the big advances in the individual components of what one needs to make a biosensor transducer, technology is moving along. In areas where we know what the receptors are and how they work, many exciting things are happening. We are back to the basic question--how much do we know about membrane receptors themselves? Do we know enough to be able to bring them in, make them up with transducers, and expect the same kind of success that we've had with other receptors like antibodies and enzymes? That's where the question is. From what I've seen in the past two or three years, there has been real progress in both areas. The papers I heard yesterday were encouraging and as a chemist even I could understand part of it. More of the mechanisms of how the receptors are operating seem to be understood. I think this technology definitely has a bright future.

Valdes: I agree that, in the past couple of years this field has come a long way. The first biosensor conference was very small and was limited to contractors. When we first designed the program it had very limited backing at CRDEC, but the program is ranked as one of the top Army research priorities.

POLYMER SURFACE DYNAMICS

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POLYMER SURFACE AND INTERFACE DYNAMICS: AN INTRODUCTION

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ABSTRACT

A brief review of polymer surface motions is presented. Methods of probing polymer transitions and relaxations are discussed, with particular emphasis placed on techniques useful for studying surface motions. Surface motions and relaxations have been demonstrated by inverse gas chromatography and contact angle data. The basic conclusion is that polymer surface motions do occur, resulting in relaxation and re-equilibration of polymer surfaces in response to different environments. Polymer surfaces are highly sensitive to processing and fabrication conditions and relax or re-equilibrate in the use environment. This effect is particularly dramatic in the case of an aqueous environment in which the interaction of the polymer with water provides a strong driving force for reducing interfacial tension by reorientation of polar surface groups to optimally interact with the aqueous phase. In attempting to draw correlations between biological interactions and polymer surface properties, it is important to be aware of the fact that the surface underwater may be, and generally is, very different from the surface in air or in other characterization environments.

INTRODUCTION[1,2]

Classical surface chemistry assumes that solid surfaces are rigid, immobile, and at equilibrium. These assumptions allow one to probe adsorption and wetting or contact angle processes purely from the point of view of the liquid phase because one assumes that the solid phase does not in any way respond, reorient, or otherwise change in the different liquid environments. Although such assumptions may be partially correct for truly rigid solids, they are generally inappropriate for polymers.

Polymer structures and properties are, in general, time and temperature dependent.[3,4] Because of the relatively large size and high molecular weights of synthetic polymer molecules, most polymeric solids rarely achieve true equilibrium. Solid polymers are, therefore, inherently nonequilibrium structures and as such exhibit a range of relaxation times and properties under normal conditions and in response to changing environments. This situation is well-known in the area of bulk polymer properties, but has been largely neglected or ignored in polymer surface chemistry and physics. There is now considerable evidence that the surface

properties of polymers are also time, temperature, and environment dependent.

Transition and relaxation phenomena in solid polymers are treated in practically all polymer science and polymer materials textbooks.[3,4]

The powerful instrumental surface analysis techniques which have been developed over the last 20 years are being increasingly applied for the study of polymer surfaces and interfaces. Generally these methods (such as X-ray photoelectron spectroscopy or XPS, Auger electron spectroscopy, secondary ion mass spectrometry or SIMS, etc.) require ultra-high vacuum environments. The assumption is generally made (often implicitly rather than explicitly) that the polymer surface is indeed stable and that the results of the analysis are applicable to the non-vacuo environments wherein the polymer surface is usually applied. Such an assumption is often invalid, particularly for polymers used as biomedical devices or in other applications where the polymer surface is exposed to water or other highly polar environments.[2]

Surface dynamics permit the interface to restructure or reorient in response to different environments. The effect is particularly pronounced in aqueous solutions, where the polarity of the aqueous phase provides a high interfacial-free energy driving force for the migration or orientation of polar phases, blocks, segments, or side chains towards the aqueous phase, thereby minimizing the interfacial-free energy. In vacuum, air, or other nonpolar surfaces, the polymer orients its apolar components towards the interface, again minimizing the interfacial-free energy.

A number of hypotheses and suggestions relating polymer surface motions to blood and biocompatibility have been reviewed.[1]

Polymer relaxation has been defined by North[5] as "a time dependent return to equilibrium of the system which has recently experienced a change in the constraints acting upon it." If one in any way changes or perturbs the polymer system, the polymer will respond, i.e. relax, to achieve a new state which is closer to equilibrium with the new environment or situation. Relaxation refers to a time-dependent change. One can also speak of a transition, which is a temperature-dependent change.[6]

We can consider that molecular motions have a characteristic frequency. A natural frequency is determined by the temperature as well as the moments of inertia of the participating segments. Micro-Brownian motion of large segments of the polymer chain become possible above the glass transition temperature. Parts of the chain, perhaps of the order of 10 monomer units, can move in a sort of cooperative fashion. The time and temperature characteristics of these motions are, of course, directly interrelated. At a high temperature, a polymer segment may be able to move or respond to a stimulus which is applied for only a very short period of time. At lower temperatures, the polymer does not have the capacity to respond to the stimulus unless it is held for a longer period of time. Thus, if we wait long enough, motions and responses to environments can occur, in principle, at any temperature.[3-8]

In addition to the relatively large cooperative motions of the main chain, there are a variety of other motions present in synthetic polymers. For example, the rotation of a side chain about a carbon-carbon bond, sometimes called the beta relaxation or beta transition, is particularly easy to see in acrylate and methacrylate systems, which have ester-linked side chains. In the case of the methacrylates, these motions are activated in the vicinity of room temperature, whereas the main chain glass transition temperature for poly(methyl methacrylate) is in the vicinity of 130°C.

Elastomeric materials have glass transitions considerably below room temperature. For example, the glass transition of poly(dimethylsiloxane) is around -130°C. Thus at room temperature and at 37°C, this polymer is nearly 170°C above its glass transition temperature. The polymer segments are in motion; it is a highly flexible, open structure which, of course, strongly influences its elasticity characteristics.

The polyurethane materials commonly used for cardiac assist devices and total artificial hearts are two-phase block copolymer systems with one of the blocks, the so-called soft segment, having a glass transition considerably below room temperature, thus providing the elasticity for the material. The other block, the so-called hard segment, normally has a transition temperture considerably above room temperature, which provides rigid pinning points and is largely responsible for the strength of these elastomers.[9]

The size or hierarchy of structures ranging from blocks or domains to small side chain functional groups is responsible for polymer surface dynamics. The surface dominance of apolar phases, blocks, or domains at the interface with air and vacuum is now well established. Polymers containing very low energy blocks (fluoropolymers and silicones are the best examples) generally exhibit the surface characteristics of the low energy constituent.[10,11]

The elegant and pioneering freeze-etch XPS study by Ratner et al. of polyacrylamide or polyhydroxyethyl methacrylate (both highly polar polymers) grafted onto polyethylene or onto polydimethyl siloxane (both highly nonpolar) clearly demonstrated the dominance of the polar phase at the water or ice interface, followed by reorientation and dominance of the nonpolar phase at the vacuum interface.[11,12]

The effect is well documented in blends. It is a common observation that polymers containing even small amounts of silicone exhibit surface properties (in air or vacuum) characteristic of pure silicone materials.[11,13]

Homopolymers or simple copolymers with amphiphilic (both polar and nonpolar character) tend to orient the main chain and side chains in response to their environment in order to minimize the interfacial-free energy.[14-16]

PROBING SURFACE MOTIONS

One would intuitively expect that polymer molecules in the vicinity of the surface or interface would exhibit motions and relaxations different from the motions observed in the bulk, due to the different interfacial environment and due to scaling and boundary constraints. Many techniques are available with which to probe such motions. One is inverse gas chromatography.

A second approach is to use classical measures of polymer transitions and relaxations in highly filled polymers, where a significant proportion of the total polymer molecules are adjacent or close to a solid interface. A third method is to directly measure the wetting properties and surface energetics of polymers as a function of time and temperature. These will be briefly discussed in order.

A number of important techniques are applied in this volume. Lavielle, Harris et al., Owen et al., Tingey et al., Lee et al., and Park and Andrade apply contact angle methods to the problem. Chaney and Barth, Ratner and Yong, and Tingey et al. utilize X-ray photoelectron spectroscopy (XPS). Harris et al. also show how electroosmosis can be used to probe interfacial dynamics.

Inverse GC

Kessaissia et al.[17] have shown that transitions can be observed with short alkyl chains chemically attached to silica supports. They used argon, nitrogen, and methane to probe alkyl-derivatized silica. Alkyl chains grafted to the polymer at a density of two alkyl chains per 100 /showed several transitions, demonstrating the sensitivity of this technique to what must be relatively local, short-range motions and relaxations.

Schreiber and coworkers have shown that inverse GC measurements of polymer films prepared from different solvents show different retention times. The retention times for PMMA at room temperature were a function of the nature of the solvent from which the film was prepared, whereas no such effect was noted for polystyrene. They suggest that the different casting solvents provide chain conformations in solutions which result in different surface conformations in the solid state. They further note

> "...that for any polymer only a single equilibrium surface structure can obtain; when a nonequilibrium surface condition is produced, slow but significant time-dependent variations in film properties are to be expected as the equilibrium condition is sought and attained."[18]

Relatively subtle changes in the surface properties result in different retention times as detected by inverse GC. More recent studies from the same group further document

> "...the ability of polar-group-containing polymers to adopt various chain conformations at and near interfaces, these conformations reflecting interactions between polymer and solvents and between polymer and substrate."[20]

They further show that

> "...the surface conformation of polymer chains is such as to diminish or enrich the surface concentration of polar moleties, depending on whether the polymer is in contact with polar or non-polar media."[19]

Filled Systems

The study of highly filled polymer systems allows one to measure the relaxations and transitions by classical methods, that is by mechanical and dielectric spectroscopies and by thermal methods. Although the literature is still somewhat controversial, it is expected that the fact that the polymer is at a rigid interface, such as a silica filler, must, in principle, constrain its motions and decrease its allowable degrees of freedom, influencing the glass transition and other transition temperatures. Lipatov emphasized the non-specific, rigid surface effect;[21] a direct interaction effect is treated by Howard and Shanks[22] and by Yim et al.[23] A correlation was observed between the shift in T_g and the polymer-filler interaction energy.[23]

Measurements of transitions in highly filled polymers are highly sensitive to the preparation and thermal history of the polymers.[24] In fact, it is important to note that since most solid polymers are nonequilibrium structures, subtle changes in thermal history and preparation conditions may dramatically affect the internal structure and, therefore, the transitions observed. This is already noted in the case of the casting solvent effect studies by Schreiber and Croucher.[18]

Nuclear Magnetic Relaxation (NMR)

NMR techniques are the most direct means of obtaining information on interface mobility and dynamics. High surface area particulate systems are used because of the inherently low sensitivity of the technique. Pulsed Fourier transform ^{13}C methods with proton decoupling permit spin-lattice (T_1) and spin-spin (T_2) relaxation times to be deduced. Much of the work has focused on alkyl-derivatized silicas because of their great importance to the chromatography field.[25] Polymer adsorption at silica and polymer latex surfaces has also been examined, particularly polyethylene oxide on silica. These studies permit conclusions as to chain and segment mobility.[25-30] NMR methods have been used for the study of alkyl-modified chromatographic supports, leading to important conclusions regarding the dynamics of alkyl chain surfaces.[25,31,32]

The use of contact angle dynamics and hysteresis to probe polymer surface dynamics has already been reviewed [1,2] and is discussed in other chapters in this book.

The Time Course of Such Processes[2]

How long does it take a surface to adjust or relax to a change in its environment? Relaxation effects in bulk polymers are well-known and form a major subject of inquiry and application in polymer science and engineering. Bulk relaxation transitions, such as the glass transition, side chain rotation, etc. are well-known and generally understood. Although clearly polymer components adjacent to an interface will have different motions and relaxation due to the influence and constraints imposed by the interface, we do expect some relation or, at least, correlation between the bulk relaxation and the relaxations active at the interface.[33] Clearly the time course must depend on the intrinsic rigidity of the polymer. In the case of a flexible elastomer or in general at temperatures substantially above the T_g, we can expect the surface accommodation to take place in the seconds to hours range, while highly rigid polymers may require hours, days, or even longer. Very little experimental data are available.

CONCLUSIONS

Given sufficient mobility, polymer surfaces will reorient or restructure in response to their local micro-environment to minimize their interfacial-free energy with the surrounding phase. The interfacial-free energy at a polymer-water interface is a sufficient driving force to cause restructuring of the polymer surface and orientation of the dipolar and other groups, which can directly interact with water, towards the aqueous phase. These processes are time-temperature dependent, and correspond to the relaxation characteristics of the polymer; thus, long equilibration times with water may be required before the effect is maximally manifested.

Even relatively rigid polymers, such as poly(methyl methacrylate), reorient at the polymer-water surface, due to relaxation mechanisms in the surface region which may occur at lower temperatures than in the bulk, perhaps due to surface-induced water plasticization of the interfacial

region, and due to segmental side chain motions which are activated at or near room temperature.

In systems containing hydrophilic phases of submicroscopic dimensions, such as common diblock and triblock copolymers, given sufficient mobility, the hydrophilic phase will dominate the interface in water, the hydrophobic phase will dominate in air.

It is suggested that the adsorption of biological and other macromolecules at a polymer-water interface will result in considerable restructuring of the polymer surface in a response to the local microenvironment of the adsorbed macromolecule, as well as the local water and other solution components.

We propose that it is necessary to characterize the surface properties directly at the solid-water interface, as well as the more commonly and classically performed solid-air or vapor interface, when searching for correlations between the surface properties of polymers and their biological behavior. Further, the polymer-water interfacial properties may need to be characterized as a function of hydration time or after suitable water equilibration.

Methods are available for the study of the time dependence of polymer surface relaxations. The most directly useful method is the time-dependent Wilhelmy plate method for measuring contact angle dynamics and hysteresis. Other methods include fluorescence probes, interface vibrational spectroscopy and nuclear magnetic relaxation methods. The latter provides direct information related to surface and interfacial motions, though high surface area particulate samples are generally required for adequate sensitivity.

As data on surface relaxation times and processes become available, we can expect significant progress in the modeling and simulation of such effects and eventual theories and scaling laws with practical predictive value.

Polymer surface restructuring effects in response to a surrounding liquid phase are probably most pronounced in aqueous systems due to the unique hydrogen bonding and acid-base characteristics of water.

Finally, these effects are not readily detectable by classical advancing contact angle measurements, including determinations of critical surface tension, nor by X-ray photoelectron spectroscopy or other analysis techniques which primarily probe the solid-vacuum or solid-air interface.

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10

Competitive Adsorption of Plasma Proteins: A Multi-Channel Approach

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Blood plasma consists of at least 60 and perhaps several hundred different proteins, of which only about 40 have been studied and characterized in some detail (1). Modern quantitative two-dimensional gel electrophoresis (isoelectric focusing and SDS-polyacrylamide gel) of plasma results in at least 600 spots, representing proteins and protein components (2). Proteins are complex macromolecules which are highly surface active. They readily adsorb and concentrate at interfaces by a variety of mechanisms (3-5). There has been much interest and activity in the study of plasma protein adsorption on biomedical polymers in the hope of establishing a correlation between adsorption and the long term blood compatibility of cardiovascular devices (4, 5). Recently, due to an increasinging awareness of the work of Vroman, et al. (6), there has been considerable interest in the competitive adsorption of plasma proteins (4). Brash & Horbett (4) have recently coined the term, the "Vroman Effect", to refer to the competitive adsorption behavior of proteins. There has been some limited success in modeling the Vroman Effect (7, 8). The actual sequence and heirarchy of plasma protein interactions with a surface appear to be dependent on:

1. The particular chemical nature of the polymer surface;
2. The dynamics of the polymer surface (9);
3. The unique structure and surface properties of each of the proteins involved (5);
4. The stability and denaturation properties of each of the proteins involved (10);
5. The liquid medium (pH, ionic strength, temperature, ions, etc.

Our group is addressing these topics.

The nature of the solid surface is characterized by contact angle measurements, x-ray photoelectron spectroscopy (XPS), and inverse gas chromatography (IGC) (see ref. 11). IGC is helpful in obtaining a measure of the partial acid and/or base character of the surface.

Surface dynamics is harder to quantitatively characterize (9). The surface reorientation and restructuring which occurs in going from the air to the aqueous solution environment can be probed via freeze-etch XPS (12), contact angle hysteresis (9, 11), inverse liquid chromatography, and ATR-FT-IR (13). The question that is very difficult to address is how does the polymer surface respond to the adsorbing protein? We have no good way to examine this question, although total internal reflection IR and fluorescence methods offer some hope.

The structure of many proteins is well known and understood (14), especially those proteins whose complete three dimensional structures are known via x-ray crystallography. Using modern computers and molecular graphics programs (15), one can "image" the protein and consider how it may adsorb on a particular surface (3-5, 16). Most plasma proteins are large, globular proteins whose 3-D structures are not known. Our approach to this problem is to use the domain concept of protein structure (14) and to consider the adsorption of plasma proteins in terms of the surface and interfacial properties of their constituent structural domains (7). This approach requires a major commitment to the study and understanding of the structure of each of the plasma proteins of interest.

The stability and denaturation properties of proteins can be assessed by a variety of methods (10). We are examining a set of small, globular proteins of known 3-D structure with the goal of correlating their adsorption properties with structure and denaturation characteristics (17). A multi domain protein often exhibits unique denaturation behavior for each of its component domains. Unfortunately it is not possible even for small, "simple" proteins, to model or simulate denaturation (unfolding) using computer graphics, although progress is being made in the understanding, prediction, and simulation of folding and unfolding of proteins (18).

Even if one knows the structure and dynamics of surfaces and of the proteins, how can one study the competitive adsorption of plasma proteins? This is normally done by radio labelling one protein and then studying its adsorption from plasma (19). This approach is expensive, time consuming, and only practical for examining a small number of interacting proteins. Another approach is to use specific antibodies to probe the concentration and nature of the adsorbed proteins (6, 20). There are many assumptions and problems involved with this method, and it is also impractical if one wishes to look at many different proteins at the same time.

We are beginning to evaluate 2-D gel electrophoresis as a method to measure the concentration of all proteins in plasma as a function of exposure time to polymer surfaces of high surface area. Protein adsorption results in a depletion or decrease of the protein concentration in the solution . The protein solution is sampled as a function of time, and each sample is analyzed by 2-D gel electrophoresis. The different gels are examined using computer methods to determine the solution concentration as a function of time for each of the proteins detected. We have high hopes and expectations for this method.

One aspect of plasma protein adsorption that is often ignored is that the surface-induced enzyme activation which is involved in the activation of coagulation and complement may also result in proteolysis of other proteins. Thus, one expects to see "new" bands appearing and increasing with increasing surface contact. The 2-D gel electrophoresis method permits such processes to be detected and examined.

The study and understanding of plasma protein adsorption is complex and fascinating. Each protein is a unique molecular machine, designed and manufactured for a particular function (21). We must know and understand each of those many different machines if we ever expect to understand. . . and thus to modify and control. . .plasma protein adsorption.

Our work is supported in part by grants from the U.S. National Science Foundation, Army Research Office, National Heart, Lung, and Blood Institute, and by the Center for Biopolymers at Interfaces at the University of Utah.

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Comment by Vert on ion/polyelectrolyte effects: — buffer effects?
Hamsy on Carbon / Baier / reversibility of adsorption
Berenberg Tg Effects

Andrade

PREPRINTS

KUNMING INTERNATIONAL SYMPOSIUM ON POLYMERIC BIOMATERIALS

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May, 3 (Tuesday)

--Morning Session (8:30-11:50 AM)--

8:30-8:40 Opening Address--X. D. Feng

Cochairmen: X. D. Feng, R. W. Lenz

8:40-9:15 A. S. Hoffman (01)

Thermally Reversible Polymers and Hydrogels for Therapeutics,Diagnostics and Bioseparations

or(02) Biomedical Applications of Plasma Polymerization: Two Case Studies: The Small Diameter Vascular Graft and Non-Fouling Surfaces

9:15-9:50 A. Nakajima (03)

Polyaminoacids as Biomaterials

9:50-10:25 A. Bantjes (04)

Specific Sorption on Polymeric Materials for Patient Treatment and for the Isolation of Valuable Plasmacomponents

10:25-10:40 Tea Break

Cochairmen: B. L. He, . S. Hoffman

10:40-11:15 R. W. Lenz (05)

Preparation and Application of Bacterial Polyesters as Biomaterials

11:15-11:50 S. W. Kim (06)

Surface Modification of Polyurethanes for Improvement of Blood Compatibility

--Afternoon Session (2:00-5:20 PM)--

Sesstion A

Cochairmen: S. A. Barenberg, R. X. Zhuo

2:00-2:20 T. Akaike (21)

Molecular Design of Synthetic Immuno-Adsorbent T- and B-Lymphocytes Separation

or (22) Development and New Application of Hepatocyte-Specific Materials

2:20-2:40 P. I. Lee (23)

Contr led Release From Glassy Hydrogels

2:40—3:00 Y. T. Yu (24)

Studies on new Carrier and Method for Immobilization of Yeast Cells

3:00—3:20 D. J. Chen (25)

A New Amphiphilic Network and Its Biomedical Behaviour

3:20—3:40 Tea Break

Cochairmen: S. C. Lin, R. Barbucci

3:40—4:00 B. Jansen (26)

Modification of Polymers for the Prevention of Foreign-Body Infections

4:00—4:20 Y. H. Ou (27)

Modification of Proteins with Polyethylene Glycol

4:20—4:40 Z. T. Wang (28)

Synthesis and Characterization of DVE-Co-MA Polymeric Derivatives of Cis-Platinum Complexes

4:40—5:00 A. C. Albertsson (83)

Degradable Elastic Block Copolymers as Biomaterials

5:00—5:20 Y. Zhang (29)

Polymerization of β-Monosubstituted-β-Proprolactones Using Trialkylaluminum-Water Catalytic Systems and Polymer Characterization

Section B

Cochairmen: J. D. Andrade, Y. T. Yu

2:00—2:20 P. Giusti (32)

Transport Properties of Composite Polymeric Membranes for artificial Organs

2:20—2:40 L. T. Liao (33)

Polymethylmethacrylate (PMMA)Membrane Dialyzer is Good for Reusage

2:40—3:00 H. Liu (34)

The Preparation and Application of Bead Polyacrylamide Immobilized Cell

3:00—3:20 Z. H. Sun (35)

Studies on Functional Polymeric Microspheres. Magnetic Albumin Microsphere ——A New Seperation System in Radioimmunoassay

3:20—3:40 Tea break

Cochairmen: A. N. Cranin, H. Zou

3:40—4:00 K. Takagi (36)

Urokinase-Immobilized Antithrombogenic Catheters

4:00－4:20　Y. X. Li (37)

Biodegradable Block Copolymers for Controlled Release System

4:20－4:40　R. N. Xu (38)

Studies on the Properties of N-acylchitosan Membranes

4:40－5:00　D. X. Sun (39)

Study on the Changes of Heparin Elution Rate of Heparin-PVA Gel

5:00－5:20　T. Shiomi (82)

Binding of Heparin and Urease to Poly(ethylene-co-vinyl alcohol) Membrane

May 4 (Wednesday)

－－Morning Session (8:30－11:40 AM)－－

Cochairmen: A. Nakajima , A. Bantjes

8:30－9:05　Y. Sakurai (07)

Microphase Separated Copolymers as Biomaterials

9:05－9:40　J. Feijen (08)

Development of Polymer-Drug Conjugates

9:40－10:15　H. Tanzawa (09)

New Microspheres for Diagnostic Examination

10:15－10:30　Tea break

Cochairmen: S. W. Kim , J. Feijen

10:30－11:05　J. D. Andrade (10)

Competitive Adsorption of Plasma Proteins: A Multi-Channel Approach

11:05－11:40　C. G. Pitt (11)

Functionalized Polymers for Self-Regulated Drug Delivery

－－Afternoon Session (2:00－5:00 PM)－－

Section A

Cochairmen: C. G. Pitt, P. I. Lee

2:00－2:20　F. Schue (40)

Realisation of a Bioartificial Pancreas using a New Performant Asymetric Membrane

2:20－2:40　Y. S. Sun (41)

The Structures and Properties of Soft Corea Contact Lens Materials

2:40－3:00　C. D. Shu (42)

The in vivo Studies of the Immuno-Isolated Membrane

3:00—3:20 X. Y. Li (43)

Studies on the Initial Processes of Lipid Peroxidation

3:20—3:40 Tea Break

Cochairmen: F. Schue, S. C. Lin

3:40—4:00 I. Yamashita (44)

Development of Biomedical Polyurethanes Based on Triblock-Copolyether or -Polydimethylsiloxane Containing PEO Chain

4:00—4:20 D. J. Chen (45)

Microphase Separation Structure of Tertiary Amine Containing Polyurethane and Its Ionic Polyblends

4:20—4:40 C. H. Li (46)

Surface Modification of Carboxyl - Containing Segmented Polyurethanes

4:40—5:00 M. R. Lu (47)

Synthesis of [Gln^{10}] - Conotoxin GIA

Section B

Cochairmen: H. Zou and B. Jansen

2:00—2:20 T. Yagi (48)

Surface Fluorination with Inorganic Fluorides in Glow Discharge

2:20—2:40 R. P. Xu (49)

A Porous Biomaterial in Spermicide

2:40—3:00 S. C. Qian (50)

A New, Uncoated, Adsorptive Resin(NK-107) in Treatment of Severe Hypnotics Intoxication——Animal Experiment and Clinical Application

3:00—3:20 Z. F. Li (51)

Study on Mixed and Blended Polyether-Urethane-Urea Containing Polytetramethylene Glycol and Polyethylene Glycol Segments

3:20—3:40 Tea break

Cochainrmen: I. Yamashita, C. H. Sun

3:40—4:00 A. N. Cranin (52)

Animal Experimentation with a Polymeric Bone Substitute Material

or (53) A Polymeric Bone Replacement Material in Human Oral and Maxillofacial Surgery

4:00—4:20 H. Zou (54)

A New Type of Contact Lens

or (55) The Study of Contact Lenses Materials

4:20—4:40 M. Xue (56)

The Method of Evaluation on the Biological Properties of the Medical Biological Material (Non-Direct-Contact-Blood)

4:40—5:00 M. Xue (57a)

Assessment of Biocompatibility of Implant for Surgery in Animal

or (57b)Some Experimental Biocompatibility Studies of Medical 1102 Silicon Rubber

May 5 (Thursday)

——Morning Session (8:30--12:00 AM)——

Cochairmen: Y. Sakurai and H. Tanzawa

8:30—9:05 M. Vert (12)

Bioresorbable Polyesters for Temporary Therapeutic Applications

9:05—9:40 S. A. Barenberg (13)

Polymers for Biomedical Applications: An Overview of Opportunities, Requirements, Needs and Unsolved Problems

or (14) Is it Real or is it an Artifact?

9:40—10:00 Tea break

Cochairmen: R. X. Zhuo, S. C. Lin

10:00—10:20 Y. T. Yu (15)

An Oral Adsorbent for Urea Removal in Uremia

10:20—10:40 S. T. Wang (16)

Studies on the Adsorption and Separation Capacities of a New Adsorbent (AAS) for Phenylalanine and Tyrosine

10:40—11:00 S. C. Lin (17)

Synthetic Studies on Blood Compatible Biomaterials Synthesis of Silicone/Polyether-b-Polyurea with Improving Antithrombogenicity

11:00—11:20 X. M. Deng (18)

Reaction of Polymeric Quaternary Ammonium Carboxylate with 2 - Bromo Carboxylic Acid: Synthesis by Coupling of Steroid to Spacer Group of Polyglutamic Acid Derivatives

11:20—11:40 R. X. Zhuo (20a)

New Polyphosphates Containing Both 5-Fluorouracil and Nitrogen Mustard or (20b) Synthesis and Antitumor Activity of Polyphosphates Containing Both Nucleic Acid Base and Phosphonoformic Acid Ethyl Ester

——Afternoon Session (2:00—5:00 PM)——

Section A

Cochairmen: M. Vert, X. M. Deng

2:00—2:20 R. Barbucci (58)

Heparin Complexing Polymers to Improve Blood Compatibility of the Biomedical Materials

2:20—2:40 J. Sheng (59)

The Studies of Optical Properties on Modified Medical Nuture Latex Rubber

2:40—3:00 Y. K. He (60)

Surface properties and Viscosity Behaviour of Polysulfoalkyl Methacrylates

3:00—3:20 W. Chen (61)

Biodegradable Polymers 1. Synthesis and Characterization of Poly (Ethylene Oxide)/Poly(Ethylene Trans,1,4-Cyclohexane Dicarboxylate) Copolymers

3:20—3:40 Tea break

Cochairmen: Y. L. Cheng, A. C. Albertsson

3:40—4:00 B. L. He (68)

Immobilization of Aminoacylase from Aspergillus Orgzae on Macroporous Polymers

4:00—4:20 B. L. He (69)

Synthesis and Biological Activities of a Series of New Antagonists of Luteinizing Hormone Releasing Hormone

4:20—4:40 J. L. Brash (70)

Protein Interactions With Biomaterials Following Contact with Plasma and Blood

4:40—5:00 C. A. Homsy (80)

Effect of Synthetic Hydroxylapatite on Tissue Ingrowth Into a Soft, Porous Matrix

or (81) Endoprosthesis Stabilization with a Soft,Porous Stem Coating: History, Biomechanics and clinical Use

Section B

Cochairmen: T. Akaike, Y. Z. Sun

2:00—2:20 K. Nakamae (62)

Intraocular Implant Materials-Biodegradation and Proliferation of Fibroblast-Like Cells on IOL

2:20—2:40 T. F. Xi (63)

Study on Synthetic Evaluating Hemocompatibility of Biomaterials by Multiparameter Testing

2:40—3:00 J. Sun (64)

A Study of Biological Evaluating Methods and Their Relationship on Biomaterials

3:00—3:20 X. Y. Wu (65a)

Experimental Studies on Microleakage of Four Kinds of Restorative Materials in Vitro

3:20—3:40 Tea break

Cochairmen: K. Nakamae and Y. T. Yu

3:40—4:00 X. Y. Wu (65b)

The Histological Effects of Composite Resin Materials on the Pulps of Monkey Teeth

4:00—4:20 I. Kaetsu (67)

Implantable Drug Delivery Polymeric Needles for Subcutaneous Therapies

4:20—4:40 C. Q. Zheng (66)

Plasma Modification on Polyurethane Surface to Improve Bloodcompatibility

4:40—5:00 R. X. Zhuo (72)

Synthesis and Antitumor Activity of 5-Fluorouracil-N'-Carbonyl Oligopetides

On-line sensors for coagulation proteins: concept and progress report

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The assessment of blood damage and of the activation of the coagulation, complement and/or inflammatory systems by cardiovascular and extracorporeal devices is difficult at best. Immunoassay methods are now available for the measurement of many of the proteins, enzymes and peptides involved in coagulation, thrombosis, complement and inflammation. We present a long-range project and plan to develop an array of remote, on-line, semicontinuous immunosensors for selected coagulation proteins, based on fluoroimmunoassay principles. The free/bound separation step is performed optically. Excitation of fluorescence is performed via an evanescent wave produced by total internal reflection and waveguide optics. Fluorescence emission is collected only in the near field. Means to deliver fluorescently-labelled reagent and to modify the antigen–antibody binding constant are presented and discussed. The results of non-specific binding, plasma–blood fluorescence, and blood compatibility are also discussed.

Keywords: Biosensors, biocompatibility, coagulation, proteins, immunoassay, fibre optics, controlled delivery

It is now generally accepted that there are no simple, direct, single-parameter measures of the blood 'compatibility' of materials or devices[1,2]. It is generally accepted that plasma protein adsorption and blood cell adhesion play important, albeit complex and highly dynamic, roles[3,4]. Although some materials have developed a reputation for being more blood tolerable — or less blood incompatible — than others, most, if not all, materials and devices require active pharmacological manipulation of the patient or his blood in order to insure the avoidance of thromboembolism.

It is desirable to be able to directly, remotely and continuously measure those blood parameters indicative of activation of the coagulation, complement and inflammatory systems. Such direct, on-line, multiparameter measurements would permit the physician to closely monitor the state of her patients, to more optimally adjust anticoagulant therapy and regimens, drug dose, etc. Such monitoring would also permit the direct evaluation of the blood compatibility of materials and devices in both experimental and clinical settings and should, therefore, permit more rapid progress in the development and improvement of biomaterials and medical devices.

Fortunately, a variety of immunoassay and chromogenic–fluorogenic enzyme-substrate technologies have become available in recent years to supplant and assist more classical methods for monitoring of the haemostatic condition[5,6]. Monoclonal antibodies are now available for most of the coagulation proteins and their various activation and proteolytic fragments[7]. Test kits are now commercially available for the immunoassay of activation and split products[8].

The concentration range of interest for coagulation proteins is from about 1 mg/ml plasma on the high side to the order of 1 ng/ml plasma on the low side[9]. A rough rule of thumb for immunoassay is $0.1/K_a < [Ag] < 10/K_a$, where K_a is the antigen (Ag)–antibody (Ab) association constant (discussed later) and [Ag] is the Ag solution concentration[21]. Thus antibodies are required with K_a values in the range of 10^5 to 10^{11} M^{-1}, depending on the antigen of interest. This is the typical range for monoclonal antibodies, and suitable monoclonal antibodies and immunoassays are already available for all of the coagulation proteins[9].

We and others have previously shown how proteins can be detected at transparent solid–liquid (S–L) interfaces using total internal reflection fluorescence (TIRF) spectroscopy[3,10–12]. The detection of Ag–Ab reactions at the S–L interface via TIRF has also been demonstrated[13–17], including the use of optical fibre and waveguide geometries[16–19]. Single use, disposable fluoroimmunoassay tests, using internal reflection detection geometries, are under commercial development[17,20]. As the costs of reagents, light sources,

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detectors, readout electronics and optical components are reasonable and competitive, commercial products can be expected in the near future.

CONCEPT AND PROGRESS

There are four key problems in the development of truly remote, on-line, continuous (or at least semicontinuous) blood compatibility sensors based on the TIRF fluoroimmunoassay approach[18,21]:

1. Stability and long-term efficacy of the immobilized Ag or Ab;
2. Remote delivery of fluorescent ligand;
3. Modulation and regulation of the Ag-Ab binding constant, K_a;
4. Long-term blood compatibility.

Ab IMMOBILIZATION

Although there are many possible operating modes for a fluoroimmunosensor[18], we are focusing on the case where Ab is covalently immobilized on the waveguide surface and used to detect circulating Ag or hapten in solution. The problem is to immobilize Ab such that it is efficient and effective, preferably oriented with Ag binding sites sterically accessible to Ag. The Ab, the chemistry by which it is immobilized and the surface to which it is immobilized, must be stable for the design life-time of the sensor. Methods for effective Ab immobilization have been extensively discussed[24-26]. We are using a polyethylene oxide (PEO) tether to a silanized silica surface[26], with the carbohydrate on the Ab as the reacting moiety[27]. The reasons for a PEO tether are discussed later.

If the immobilized Ab is coated or covered with a layer of non-specifically bound protein, fibrin or thrombus, then, of course, the sensor ceases to function — this incompatibility problem will be discussed again later.

If the immobilized Ab is susceptible to protease attack, then, of course, the Ab will be degraded and the sensor will cease to function. Many surfaces are known to generate protease activity as a result of activation of the extrinsic coagulation[30] or complement[31] systems — this, too, is part of the general 'biocompatibility' problem.

FLUORESCENT 'REAGENT' DELIVERY

We have previously suggested that one could develop a label-less fluoroimmunosensor by using the intrinsic UV (tryptophan) fluorescence of Ag and Ab[15]. We have successfully used intrinsic UV fluorescence to monitor the adsorption of proteins at S-L interfaces[10,28]. UV excitation often leads to photochemical changes in the tryptophan side chain, with consequent changes in the fluorescence and chemical properties[28,29] of the Ab. In addition, UV sources are still bulky, UV detection is somewhat difficult, and — most importantly — the UV-excited background fluorescence from blood and related media is quite intense. For these reasons, a useful fluoroimmunosensor will probably have to operate in the green-red-near IR regions of the spectrum, and therefore fluorescently-labelled ligands will be required.

The Ag of interest are not intrinsically fluorescent in the visible. Thus a fluorescent label must be employed and the labelled Ag must be 'delivered' to the remote sensor[18]. Remote delivery can be accomplished using a plumbing system or via continuous, controlled delivery from a remote reservoir. Constant reservoir delivery is used for the KCl reference junction in combination pH electrodes, for example. One can also use other means of insuring controlled, continuous delivery using drug delivery technologies[32]. There are at least several disadvantages to the continuous release of fluorescent-Ag or hapten:

1. A larger reservoir and larger amounts of labelled Ag or hapten are required than would be required in a pulsed or 'on demand' delivery system;
2. Continuous release of a labelled Ag may lead to the generation of an immune response and to circulating Ab, which would greatly diminish the effectiveness of the sensor;
3. The reservoir requirements for continuous delivery would make it difficult to produce truly miniature multichannel sensors.

Rarely does one need truly continuous, reversible measurements. More commonly, one may wish to make a measurement every minute, every ten minutes, every hour, even every day. In principle, on-demand delivery can be provided using electrical, magnetic, thermal or even mechanical methods.

As our approach to remote sensing is optical, we have chosen to develop an on-demand, optically based, reagent delivery technology. Basically the fluorescent-labelled Ag is coupled via a photolabile bond to a polymer matrix. A pulse of light of the proper intensity and wavelength results in bond breakage, providing a pulse of released fluorescent Ag, which then competes with circulating Ag for the Ab binding sites on the sensor surface. The chemical details and progress to date are available[22] and are not discussed further here.

REGULATION OF THE ANTIGEN-ANTIBODY ASSOCIATION CONSTANT

A true sensor is reversible and responds to changes in concentration in a predictable and timely manner — a good example is a pH or specific ion electrode. The beauty of immunoassay is its selectivity and high sensitivity. The high sensitivity is due to high values of K_a — in excess of $10^{12}\ M^{-1}$ in many cases: K_a is the ratio of the on-rate constant to the off-rate constant. High K_a values generally require a small off-rate constant, which means that the response to changes in circulating Ag concentration is very slow[21]. Ideally, we desire K_a to have a value consistent with the concentration requirements of the measurement[21] and have a value consistent with the response time desired. This is only possible if we can regulate or modulate the K_a.

Ag-Ab K_a values can be changed by several orders of magnitude by changes in the solution environment, such as temperature, pH or low molecular weight solutes[23,33,34]. We have demonstrated that the hydroxypropyl methacrylamide (HPMA) monomer in aqueous solution is effective in decreasing the K_a of fluorescein-antifluorescyl monoclonal antibodies by two orders of magnitude[33,34]. Poly(HPMA) is a highly versatile, water soluble, synthetic polymer which has been extensively studied as a polymeric drug carrier[35]. We are preparing copolymers of HPMA, HPMA derivatives containing azobenzene side chains and methacrylic or acrylic acid. The objective is to develop a copolymer whose dimensions change dramatically with light. The increased chain length 'delivers' a 'solute' to the Ab binding region, thus changing the local 'solution' environment and affecting the binding constant[21].

Although there are other ways to decrease the K_a, this immobilized 'solute', photoconformation approach is con-

sistent with the optical needs and requirements of the other technologies involved in sensor development. We could, of course, employ a remote reservior or a plumbing system and simply 'wash' off the bound Ag prior to a new measurement. The arguments for and against such an approach are the same as described above in the section on fluorescent reagent delivery.

There are, of course, many concerns in the study and development of a photoconformationally-based macromolecular switch. These are challenging questions, however, and therefore worthy of serious study. We feel that the ability to regulate, under external control, Ab binding constants is important in its own right.

CHRONIC BLOOD COMPATIBILITY

These sensors require that the optical surface containing the immobilized Ab interface directly with the blood. As our major interest is in the sensing of protein antigens, it is not possible to place a membrane or other barriers between the sensing surface and the blood. The deposition of other plasma proteins, of blood cells or of fibrin will compromise and in all likelihood destroy the proper functioning of the sensor. Although one could consider the use of various anticoagulant delivery systems to help improve blood compatibility, what is really required is a general means of preventing or at least minimizing such non-specific interactions without compromising the specific Ab–Ag binding vital to the sensor's function.

An approach which shows some promise is based on the steric exclusion arguments of colloid stability[36-40] and the unique water solution properties of polyethylene oxide[38] (PEO). We have previously demonstrated the low protein adsorption properties of PEO surfaces[39]. Mori, Nagaoka and Tanzana have shown that PEO-grafted surfaces have unusually low protein and platelet adhesion properties[40] — we have suggested they be called protein-resistant surfaces[3]. Merrill and Salzman recently reviewed much of the experience with PEO as a biomaterial[41]. Davis, *et al.* have shown that PEO-coated microparticles resist recognition by the reticuloendothelial system (RES) and circulate in the blood for extended periods[42].

Enzymes covered with grafted PEO chains resist recognition and can be used for treatment of enzyme-deficiency diseases with greatly decreased risk of immunologic recognition[43]. PEO's steric exclusion, entropic repulsion properties are probably due to its unique stereochemistry and fit to the ice-like structure of water[38], a minimal perturbation of water structure which decreases potential hydrophobic interactions, and minimal effect on the motion and relaxation times of water itself[40].

Although PEO surfaces are expected to exhibit substantial blood compatibility and increasing interaction with proteins and cells, little experience is available. Also, the 'compatibility' of PEO has been treated primarily as an empirical observation, and there has been little effort at trying to optimize and maximize the PEO effect.

Our approach to the blood compatibility problem at present is to design and produce PEO-containing surfaces which are optimized with respect to blood compatibility. Basically, all sensor surfaces will be so treated and the antibody is immobilized via a PEO tether. However, the Ab binding sites and the photoconformationally sensitive macromolecules for K_a regulation must be partially shielded or isolated from the PEO in order to function. Clearly, these are challenging molecular engineering problems which will not be quickly nor easily solved.

ENGINEERING

Assuming all of the interface chemistry and biocompatibility problems are solved, such sensors must be cheap, robust, reliable, and quantitative. Many factors influence the intensity of fluorescence[44], and it is unlikely that intensity alone will be sufficient for a reliable sensor. A number of reference channels will certainly be required. In all probability, a non-trivial quantitation algorithm will be necessary.

CONCLUSIONS

The development of truly remote, continuous high sensitivity and specific immunosensors, capable of functioning reliably in blood or in other biological environments, is still in its early stages. There are a number of important technologies which must be developed. As the work progresses, one can envision the development and eventual production of multichannel biosensors based on microintegrated devices incorporating biochemical, optical and even electronic functions.

This paper has briefly reviewed the general concept and at least one approach to each of the major problems.

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PROTEINS AT INTERFACES: PRINCIPLES RELEVANT TO PROTEIN-BASED DEVICES

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ABSTRACT

Proteins are nature's molecular machines. There is considerable interest and activity in applying and adapting proteins for the development and application of biomolecular devices and machines (1). Such applications require the handling and processing of proteins, including their concentration, ordering, and assembly at interfaces. The current principles and understanding of proteins at interfaces are presented and reviewed, including current theories and hypotheses. Progress in the design and application of defined surfaces and materials for the study and precise assembly of protein films is also discussed, particularly gradient and patterned surfaces.

INTRODUCTION TO PROTEINS (2,3)

Proteins are biological macromolecules consisting of some 20 or more monomer types (amino acids) condensed into one or more chains (polypeptide or primary sequences) via amide (-NH-CO) bonds. Many proteins also contain covalent cross links, generally of the -S-S-type, as well as short carbohydrate chains (glycoproteins). Proteins range in size from several thousand (10^3) to tens of millions (10^7) Daltons. A particular protein normally exhibits one or more of the following functions: catalysis (enzymes), mechanical properties (fibrous, structural proteins), chemical recognition (antibodies), chemico-mechanical transduction (muscle and motion proteins), chemical regulation (many), transport (ion channels), optical properties (luciferases, rhodopsins), electron and charge transfer, etc. The structure of globular proteins is generally only marginally stable, partially unfolding to a "denatured" state with a free energy input or change of only 5-15 k cal/mole. Large proteins generally consist of several structural and functional domains. Complex proteins are now believed to consist of domain "building blocks".

Practically all proteins are polymeric surfactants in aqueous solution. Their constituent amino acids have hydrophobic and hydrophilic (neutral, positive, or negative) character, and hydrophobic interactions are very important in their globular stability and in their interfacial activity. It is now generally accepted that the placement of proteins at interfaces, usually via adsorption from solution, results in a conformation and orientation different from that under "normal" physiologic conditions. Such interface microenvironment -- induced altered states can result in "abnormal" or unexpected biochemical and biophysical properties. Several well known examples are the interface-induced activation of blood coagulation, activation of the complement defense system, activation of inflammatory processes, and enzyme inactivation.

PROTEIN ADSORPTION

A number of recent conference proceedings and an edited monograph are now available (4-6), as well as several comprehensive reviews (7-9). The following general principles and hypotheses are now accepted and being tested (Figure).

1. Each protein has its own distinctive and individual "surface" chemistry produced by the outer shell of amino acids and carbohydrate which interface with the liquid medium (7,11). (Figure a)

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2. Diffusion or convective transport results in a collision rate between proteins in solution and the interfaces present (7,10) (Figure b)

3. Although protein molecules collide with the interface in many different possible orientations, one specific orientation will probably result in the most stable adsorption, with hydrophobic surface patches oriented towards hydrophobic interfaces, anionic patches oriented towards cationic surfaces, etc. (Figure c) Domain and mosaic surfaces can be expected to have rich and complex interactions with the domains and mosaics on the surface of the protein.

4. If the collision rate is very high and the interface is populated with protein very quickly, the proteins may not show extensive time-dependent denaturation processes (Figure d), although there may be some adjustments in packing, ordering, and lateral interactions (4-7).

5. If the surface is not highly populated, the adsorbed protein may denature and/or spread at the interface, altering its conformation and orientation to optimally adjust to its new microenvironment. (Figure d) Such events may result in the expulsion of less optimally oriented or bound proteins from the interface, as the more optimally oriented or bound protein spreads at the interface. (Figure e) The tendancy for the protein to denature at the interface is related to its intrinsic stability, including the number of disulfide bonds (12).

6. The presence of two or more different proteins in the solution will result in competitive adsorption processes; with that protein which can most optimally bind and accomodate at the interface tending to displace its less optimally bound neighbors (Figure e,f) Thus, a complex hierarchy of adsorbed protein types and amounts can develop with time, related to solution concentration, size, collision rates, interface affinity, and denaturation tendencies. This behavior is now called the "Vroman Effect" (12,13).

7. It is possible to control and regulate protein adsorption, in part, by modifying the surface or interface or by modifying the protein, such as with steric exclusion modifiers (14,15). (Figure g)

8. Materials and interfaces with a micro-heterogeniety of the same size as the structural domains or building blocks in proteins probably have a particularly rich and complex set of adsorption properties. (Figure h)

CONCLUSIONS AND PROSPECTS

These general concepts and hypotheses provide some appreciation and qualitative understanding of protein interfacial processes. There is considerable progress on the modeling and simulation of selected interfacial properties by several groups (7,8,16-17). Due to the complexity of the general adsorption process, however, a quantitative, truly predictive model for complex multi-component systems will not be available in the near future.

Even these very qualitative concepts are sufficient to suggest that it should be possible to orient and order proteins at interfaces in order to optimize specific activities or functions, such as for biosensor applications (18). This can be done by modifying and/or controlling the structure of the protein in solution or by appropriate modifications and control of the interface itself. For example, one can now readily produce surfaces with a controlled gradient in properties (19,20). Patterned, micro-heterogeneous surfaces can also be prepared (21). Protein interactions and assembly on such surfaces is likely in the near future and offers exciting possibilities for the design and fabrication of protein-

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based devices (27).

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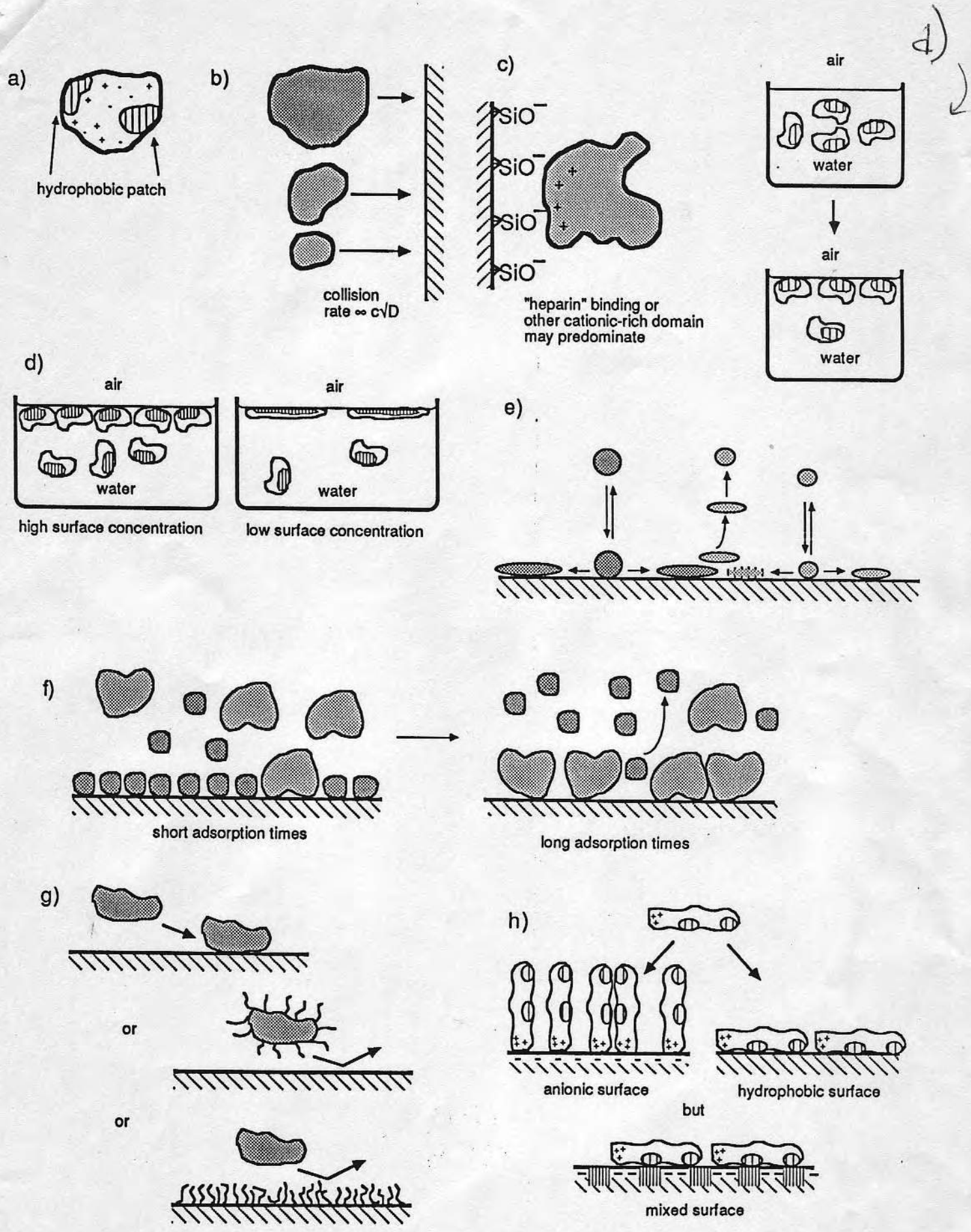
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PROTEINS AT INTERFACES; ISSUES RELEVANT TO HYBRID MEDICAL DEVICES AND ORGANS

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ABSTRACT

The adhesion and growth of cells on solid supports is dependant on cell - solid surface interactions which are in turn controlled by protein - solid surface and protein - cell interactions. The proteins, receptors, and surface chemistries required for cell culture and growth are now known - at least qualitatively. The structure and interfacial properties of vitronectin, fibronectin, laminin, and mussel peptide have been briefly considered. It is suggested that we are now in a position of being able to design - and produce - surfaces which can influence and even control cell adhesion, growth, and viability via surface modification, protein adsorption, and patterning processes; thus, providing a beginning for the design and development of hybrid devices.

INTRODUCTION

Hybrid medical devices often involve one or more cell types on or in suitable materials or supports. In a sense the culture of cells on a dish in the laboratory is the simplest example of a hybrid "device". The dish surface provides mechanical, physical and even chemical requirements for the adhesion, growth and viability of the cells. Different cell lines or types have different requirements for adhesion, spreading, growth, multiplication and general viability. If different cell types have different requirements then it is reasonable to expect that the solid support can be designed to maintain different cell types at different locations on the surface - that is, one could induce a selective ordering or patterning of the cells, a first step in the development of a "synthetic" or hybrid "tissue".

That cells can indeed be patterned by selective modification and control of substrate properties has already been well demonstrated (1-4). There is also work on the development of multi-layer cell-selective structures, particularly in the vascular graft field

(5) - a step toward the design and development of three dimensional hybrid tissues. In this brief paper we consider the role of plasma and serum protein adsorption in cell - solid surface interactions, with emphasis on vitronectin, fibronectin, and laminin.

PROTEIN ADSORPTION:

Historically, the culture of mammalian cells on solid supports has required materials with specific surface chemical treatments and/or a cell culture medium containing fetal bovine serum or other supplements containing a number of proteins and other "factors".

When one pours cell-free cell culture media into a culture dish or support, many interfacial processes and reactions occur (6), including the competitive, time and concentration - dependent adsorption of plasma protein (7). Practically all proteins - including the highly soluble, globular plasma proteins - adsorb at solid/liquid and liquid/air interfaces. The adsorption is dependent on the surface properties of the solid, the surface properties of the protein, the nature of the liquid medium (pH, ionic strength, etc.), the three dimensional structure and general composition of the proteins, the stability or denaturability of the protein and its constituent domains, and the number, size, and concentration of proteins - all of which are "competing" for sites on the surface. These various parameters are now beginning to be systematically addressed by many research groups (7). Although there has been progress in understanding the adsorption of simple proteins (8), the mechanisms and hypotheses for the adsorption of large complex, multi-domain proteins are just beginning to be formulated (7-9). Of particular interest and relevance are the adhesion-promoting substances. Here we consider briefly the glycoproteins vitronectin, fibronectin and laminin and mussel-derived adhesive polyphenolic protein.

Gorbunov and others have proposed that protein-solid interactions in chromatography can be treated in terms of different areas or "patches" on the surface of different interaction potential (8-10). This has been demonstrated quantitatively in the empirical experience that Fab and Fc parts of IgG molecules adsorb differently on different surfaces (11). Most complex proteins are known to consist of a variety of structural and compact domains. Let us briefly treat the surface interactions of the important cell adhesion proteins from this point of view.

VITRONECTIN (VN):

VN (also called serum spreading factor) is the main adhesive protein in routine cell culture media (12). It is an acidic glycoprotein containing 10% carbohydrate, 459 amino acids and exhibiting 2 molecular weights in plasma: 65,000 and 75,000. Its physical properties are known (13-14); it contains at least one free - SH group and has a tendency to multimerize. The study of amino acid sequence, coupled with analysis of

proteolytic fragments, has resulted in proposed models which suggest a number of different structural and functional domains (15-17). Starting from the N-terminal, we have (16):

- Somatomedin B domain - first 44 residues -- probably an "independently folding cysteine-rich domain" (16);
- RGD (Arg - Gly - Asp) - next 3 residues - the cell binding region;
- Long oligosaccharide - containing domain (residues 48-347), rich in proline;
- Highly cationic domain - next 32 residues contain 14 positive and no negative residues -- commonly considered to be the heparin and/or glass binding site;
- Final (C-terminal) 10K dalton domain -- partially cleaved in plasma, accounting for the presence of 65K and 75K dalton forms.

A hydropathic analysis (17) clearly demonstrates the heparin-binding, very hydrophilic, highly basic domain (residues 342-375)(17). There is substantial hydrophobicity on either side of the heparin domain as well as in the central part of the amino acid chain.

It is not surprising, therefore, that VN binds strongly to glass (18). Fibronectin and laminin also bind to glass (18), although VN is far more effective in inducing substrate adhesion in cell culture (12). It may also not be too surprising that VN binds strongly to polymers, and adsorbed more on the more hydrophobic polymers studied (14), even more avidly than fibronectin. VN adsorption kinectics onto polystyrene and oxidized polystyrene are similar -- there is some evidence for a conformational change on adsorption (19). Denatured VN appears to bind heparin more effectively, possibly due to more complete exposure of the heparin binding domain in denatured VN (20,21).

It is perhaps reasonable to suggest that VN is readily denatured upon exposure to solid-liquid interfaces, and that such denaturation is in large part responsible for its ability to adsorb effectively at hydrophobic, hydrophilic, and negatively-charged surfaces. One could say that VN is an extremely surface-active protein. More complete solution denaturation studies, as well as surface and interfacial tension analysis, would help elucidate the mechanisms for its interfacial activity (9).

FIBRONECTIN (FN):

FN, (also called cold insoluble globulin) is a large, 440 K Dalton plasma glycoprotein (30-40mg/100ml, 5% carbohydrate) with a wide variety of functions and binding properties. It is approximately a disulfide bonded dimer (2 x 220K daltons) with an open, flexible structure but consisting of a variety of tightly disulfide cross-linked structural and functional domains, connected by flexible segments. Although FN is much larger and more complex than VN, it is a much better characterized protein and more information is available (22-24). There is insufficient space here to discuss the various domains or

their binding properties. Although most of the domains are fairly well characterized in terms of amino acid sequence and disulfide bonding (22-24), there is little information on the surface activity or non-specific binding attributes of the domains. Although the complete amino acid sequence is available (25), complete hydropathy analysis has apparently not yet been reported. Binding ligands include DNA, cell membrane receptors, heparin, collagen, fibrin, hyaluronic acid, staph aureus, and actin (24,25). The major binding domains are separable by proteolysis and chromatography (26). A very recent study has shown that the 43K dalton collagen-binding domain, which contains 10 biantennary carbohydrate chains, is the most hydrophobic, a surprise in light of the fact that this domain contains 60% of the hydrophilic carbohydrate chains (27).

There have been many studies of the adsorption of FN at solid-liquid interfaces (reviewed briefly in Ref. 28). Basically FN appears to adsorb more on hydrophobic than on hydrophilic surfaces and appears to undergo a greater conformational change on hydrophobic surfaces. The earlier studies by Grinnell and Feld, using anti-FN antibodies (29), also demonstrated these effects. Given the domain structure of FN, one would expect different domains to dominate the adsorption process on different surfaces, however there has been little analysis of existing adsorption data from a structural or function domain perspective.

A thorough analysis of the amino acid sequence hydropathy of the various FN domains, and a comparison with such an analysis for VN, would appear to be in order.

LAMININ:

Laminin (30,31) is the most abundant basement membrane glycoprotein (13% carbohydrate). It is a large (~850K dalton), cross-shaped molecule with three short and one long arms. The molecule consists of three amino acid chains with distinct sequential domains. Laminin binds to a variety of basement membrane components, to glass (14), to cell membrane receptors, and to heparin. The binding activities have been roughly localized to various domains, although the domain characterization of the molecule is in its early stages. Laminin can self-assemble into aggregates with a defined structure (30).

Although Laminin is being used to promote adhesion on cell culture, adsorption data is sparse.

As sequence, structural, and adsorption data develop, it will be interesting to compare Laminin to VN and FN.

MUSSEL POLYPHENOLIC ADHESIVE PROTEIN

Mussel Polyphenolic Adhesive Protein is now being used to treat substrates for enhanced cell attachment and adhesion (32). The commercial name is Cell-Tak (Biopolymers, Inc., Farmington, CT, USA). The protein consists of repeating sequences of hexa- and deca-peptides containing alkyl, amino-alkyl, and phenolic groups. The protein may adsorb primarily via hydrophobic and perhaps hydrogen bonding means, and then adhere to cells via its many lysine residues. A highly random, open solution conformation may facilitate adsorption to a wide variety of surfaces. The material is apparently more effective than laminin or FN with respect to attachment kinetics for several cell types. (product literature).

GRADIENT SURFACES:

It should now be clear, that at least in principle, it is possible to produce surfaces with some selectivity for different proteins. Not only may we be able to prepare surfaces which bind certain proteins, but we should be able to control, at least roughly, the orientation of the adsorbed protein. For example, if our goal is cell adhesion, the VN or FN must be bound such that the cell binding site is accessible and active. At this stage in our understanding of protein-surface interaction, it is not possible to rigorously design a surface with given protein-specific or cell-specific properties. Rather than produce tens or hundreds of individual surfaces of different surface properties for study, it is now possible to prepare surfaces with a continuous gradient in properties (33). Such surfaces have already been very effectively used in the study of protein adsorption and antibody binding by several groups. Using modern multichannel optical detection equipment, it is possible to monitor protein-surface interaction over the entire gradient surface in real time. The study of cell adhesion and culture on gradient surfaces is also underway (4). Such approaches will permit the screening and selection of the surface treatments and/or protein adsorption conditions required for the adhesion and growth of desired cell types.

PATTERNING:

Many years ago, cell adhesion researchers demonstrated that cells in culture would respond to a patterned surface. It is now possible to generate surfaces of defined surface chemistry and geometry by using a variety of processing techniques, including photo-and electron-beam lithography, so widely used in integrated circuit manufacture (34). There has been considerable interest in the growth of nerve cells on geometrically defined surfaces (1). Klebe recently showed that fibronectin could be deposited on a surface in a computer-controlled pattern, resulting in cell adhesion on the fibronectin-patterned surface (2). The combination of patterning, gradient surfaces, and modern

surface modification methods should enable one to design hybrid devices incorporating different cell types and cell layer geometries.

One can envision and predict the development of multi-cellulor structures which could be the precursors of true hybrid organs and devices.

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COLLOQUE
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Vol. 177

Organes Artificiels Hybrides

Concepts et Développement

C. Baquey
B. Dupuy

LES EDITIONS INSERM

PROTEINS AT INTERFACES: PRINCIPLES AND APPLICATIONS

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ABSTRACT

Proteins are nature's molecular machines. There is considerable interest and activity in applying and adapting proteins for the development and application of biomolecular devices and machines (1). Such applications require the handling and processing of proteins, including their concentration, ordering, and assembly at interfaces. Protein interfacial properties are also important in the biocompatibility of medical devices and in protein processing for biotechnology purposes. The current principles and understanding of proteins at interfaces are presented and reviewed, including current theories and hypotheses.

INTRODUCTION TO PROTEINS (2,3)

Proteins range in size from several thousand (10^3) to tens of millions (10^7) Daltons. The structure of globular proteins is generally only marginally stable, partially unfolding to a "denatured" state with a free energy input or change of only 5-15 k cal/mole. Large proteins generally consist of several structural and functional domains. Complex proteins are now believed to consist of domain "building blocks".

Practically all proteins are polymeric surfactants in aqueous solution. Their constituent amino acids have hydrophobic and hydrophilic (neutral, positive, or negative) character, and hydrophobic interactions are very important in their globular stability and in their interfacial activity. It is now generally accepted that the placement of proteins at interfaces, usually via adsorption from solution, results in a conformation and orientation different from that under "normal" physiologic conditions. Such interface microenvironment -- induced altered states can result in "abnormal" or unexpected biochemical and biophysical properties. Several well known examples are the interface-induced activation of blood coagulation, activation of the complement defense system, activation of inflammatory processes, and enzyme inactivation.

PROTEIN ADSORPTION

A number of recent conference proceedings and an edited monograph are now available (4-6), as well as several comprehensive reviews (7-9). The following general principles and hypotheses are now accepted and being tested (Figure).

1. Each protein has its own distinctive and individual "surface"

chemistry produced by the outer shell of amino acids and carbohydrate which interface with the liquid medium (7,11). (Figure a)

2. Diffusion or convective transport results in a collision rate between proteins in solution and the interfaces present (7,10). (Figure b)

3. Although protein molecules collide with the interface in many different possible orientations, one specific orientation will probably result in the most stable adsorption, with hydrophobic surface patches oriented towards hydrophobic interfaces, anionic patches oriented towards cationic surfaces, etc. (Figure c) Domain and mosaic surfaces can be expected to have rich and complex interactions with the domains and mosaics on the surface of the protein.

4. If the collision rate is very high and the interface is populated with protein very quickly, the proteins may not show extensive time-dependent denaturation processes (figure d), although there may be some adjustments in packing, ordering, and lateral interactions (4-7).

5. If the surface is not highly populated, the adsorbed protein may denature and/or spread at the interface, altering its conformation and orientation to optimally adjust to its new microenvironment. (Figure d) Such events may result in the expulsion of less optimally oriented or bound proteins from the interface, as the more optimally oriented or bound protein spreads at the interface. (Figure e) The tendency for the protein to denature at the interface is related to its intrinsic stability, including the number of disulfide bonds (12).

6. The presence of two or more different proteins in the solution will result in competitive adsorption processes; with that protein which can most optimally bind and accommodate at the interface tending to displace its less optimally bound neighbors (figure e,f). Thus, a complex hierarchy of adsorbed protein types and amounts can develop with time, related to solution concentration, size, collision rates, interface affinity, and denaturation tendencies. This behavior is now called the "Vroman Effect" (12,13).

7. It is possible to control and regulate protein adsorption, in part, by modifying the surface or interface or by modifying the protein, such as with steric exclusion modifiers (14,15). (figure g)

8. Materials and interfaces with a micro-heterogeniety of the same size as the structural domains or building blocks in proteins probably have a particularly rich and complex set of adsorption properties. (figure h)

CONCLUSIONS AND PROSPECTS

These general concepts and hypotheses provide some appreciation

and qualitative understanding of protein interfacial processes. There is considerable progress on the modeling and simulation of selected interfacial properties by several groups (7,8,16-17) Due to the complexity of the general adsorption process, however, a quantitative, truly predictive model for complex multi-component systems will not be available in the near future.

Even these very qualitative concepts are sufficient to suggest that it should be possible to orient and order proteins at interfaces in order to optimize specific activities or functions, such as for biosensor applications (18). This can be done by modifying and/or controlling the structure of the protein in solution or by appropriate modifications and control of the interface itself. For example, one can now readily produce surfaces with a controlled gradient in properties (19,20). Patterned, micro-heterogeneous surfaces can also be prepared (21). Protein interactions and assembly on such surfaces is likely in the near future and offers exciting possibilities for the design and fabrication of protein based devices (27).

It is now becoming possible to directly observe proteins on surfaces by scanning tunneling microscopy (STM) (23) and by atomic force microscopy (AFM) (24).

The thrombin-induced polymerization of fibrinogen on a mica surface has now been directly observed by AFM (24).

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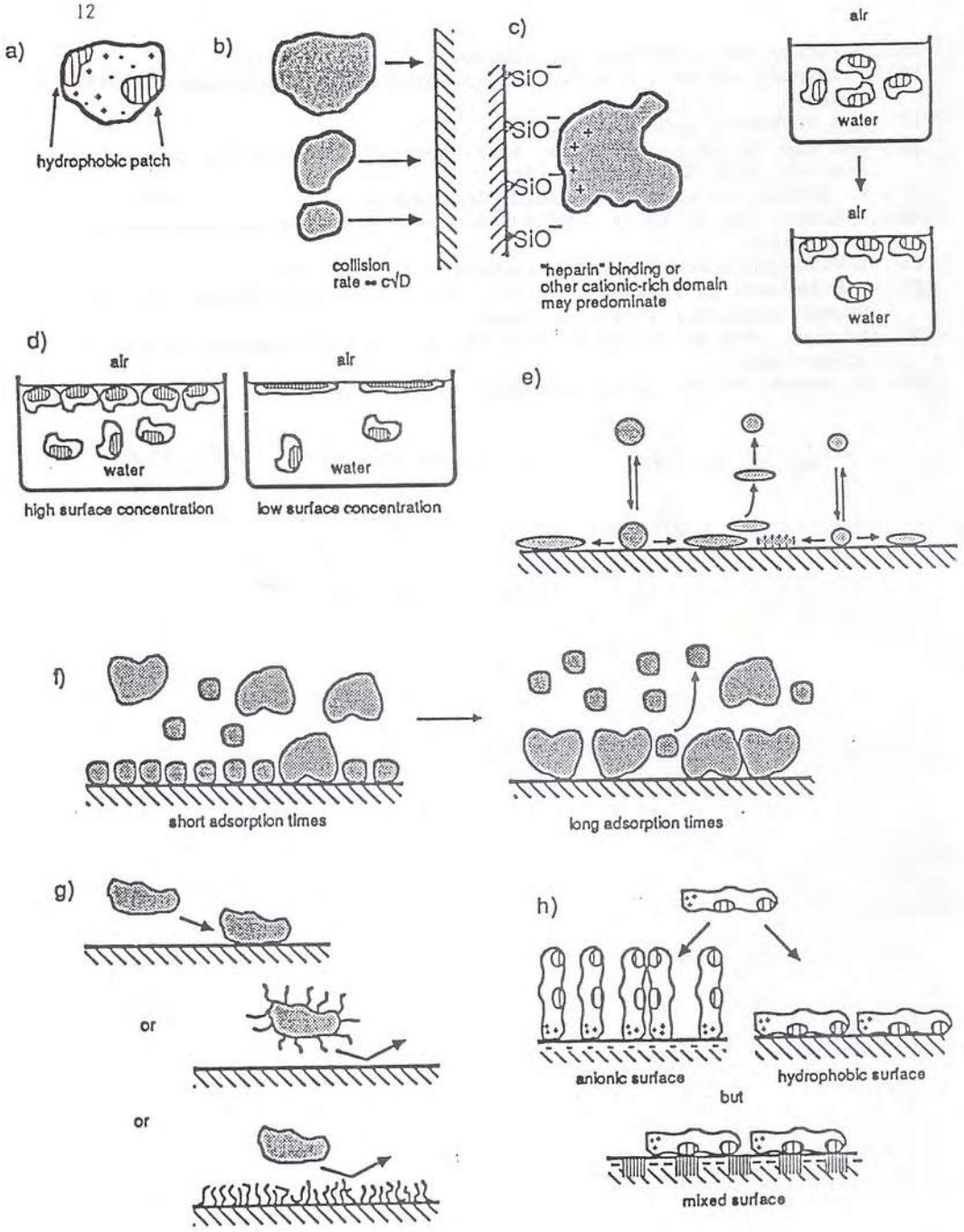
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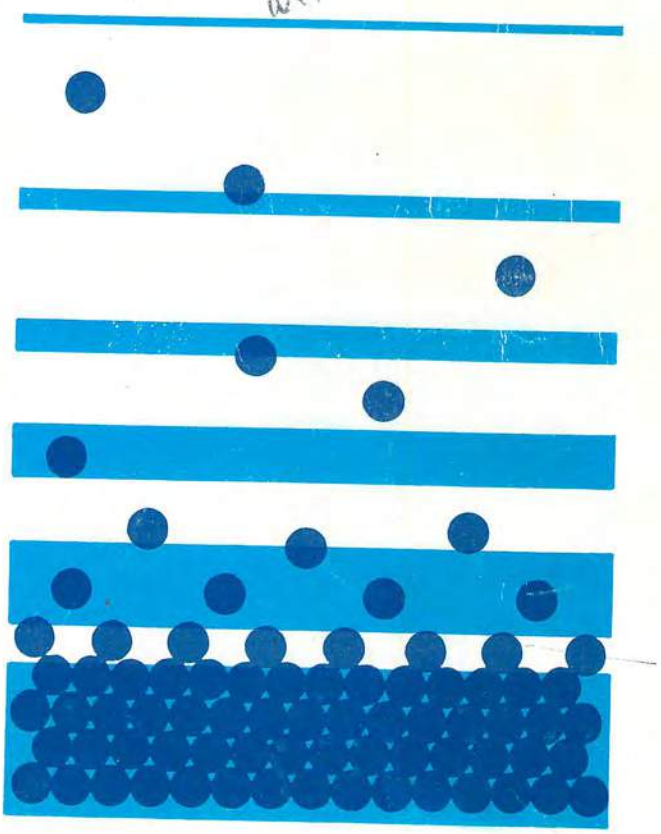
VIII INTERNATIONAL CONFERENCE

CHEMISTRY OF SOLID/LIQUID INTERFACES

RED ISLAND, ROVINJ

22 JUNE - 1 JULY, 1989



"RUĐER BOŠKOVIĆ" INSTITUTE

ZAGREB, YUGOSLAVIA

Disc'n on Ads

rev/irrev denat'n / rev of denat'n

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ASK JACOB KLEIN

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? 2D diffusion - A method. —

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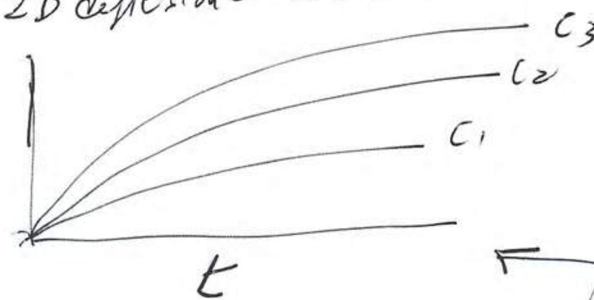

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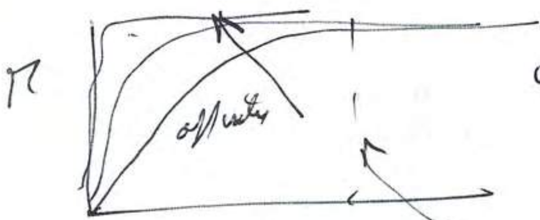

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p.lipid agarose gel bulk soln

E(T) - ask Lyklema ?

Paper: Proteins at Interfaces: Problems & Hypotheses — theory?

BioScience 89, Swedish National Convention of Bioscientists
April 24-26, 1989, Malmo, Sweden

AD ANDR-8

PROTEINS AT INTERFACES: PRINCIPLES AND APPLICATIONS

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ABSTRACT

Proteins are nature's molecular machines. There is considerable interest and activity in applying and adapting proteins for the development and application of biomolecular devices and machines (1). Such applications require the handling and processing of proteins, including their concentration, ordering, and assembly at interfaces. Protein interfacial properties are also important in the biocompatibility of medical devices and in protein processing for biotechnology purposes. The current principles and understanding of proteins at interfaces are presented and reviewed, including current theories and hypotheses.

INTRODUCTION TO PROTEINS (2,3)

Proteins range in size from several thousand (10^3) to tens of millions (10^7) Daltons. The structure of globular proteins is generally only marginally stable, partially unfolding to a "denatured" state with a free energy input or change of only 5-15 k cal/mole. Large proteins generally consist of several structural and functional domains. Complex proteins are now believed to consist of domain "building blocks".

Practically all proteins are polymeric surfactants in aqueous solution. Their constituent amino acids have hydrophobic and hydrophilic (neutral, positive, or negative) character, and hydrophobic interactions are very important in their globular stability and in their interfacial activity. It is now generally accepted that the placement of proteins at interfaces, usually via adsorption from solution, results in a conformation and orientation different from that under "normal" physiologic conditions. Such interface microenvironment -- induced altered states can result in "abnormal" or unexpected biochemical and biophysical properties. Several well known examples are the interface-induced activation of blood coagulation, activation of the complement defense system, activation of inflammatory processes, and enzyme inactivation.

PROTEIN ADSORPTION

A number of recent conference proceedings and an edited monograph are now available (4-6), as well as several comprehensive reviews (7-9). The following general principles and hypotheses are now accepted and being tested (Figure).

1. Each protein has its own distinctive and individual "surface" chemistry produced by the outer shell of amino acids and carbohydrate which interface with the liquid medium (7,11). (Figure a)

2. Diffusion or convective transport results in a collision rate between proteins in solution and the interfaces present (7,10). (Figure b)

3. Although protein molecules collide with the interface in many different possible orientations, one specific orientation will probably result in the most stable adsorption, with hydrophobic surface patches oriented towards hydrophobic interfaces, anionic patches oriented towards cationic surfaces, etc. (Figure c) Domain and mosaic surfaces can be expected to have rich and complex interactions with the domains and mosaics on the surface of the protein.

4. If the collision rate is very high and the interface is populated with protein very quickly, the proteins may not show extensive time-dependent denaturation processes (figure d), although there may be some adjustments in packing, ordering, and lateral interactions (4-7).

5. If the surface is not highly populated, the adsorbed protein may denature and/or spread at the interface, altering its conformation and orientation to optimally adjust to its new microenvironment. (Figure d) Such events may result in the expulsion of less optimally oriented or bound proteins from the interface, as the more optimally oriented or bound protein spreads at the interface. (Figure e) The tendency for the protein to denature at the interface is related to its intrinsic stability, including the number of disulfide bonds (12).

6. The presence of two or more different proteins in the solution will result in competitive adsorption processes; with that protein which can most optimally bind and accommodate at the interface tending to displace its less optimally bound neighbors (figure e,f). Thus, a complex hierarchy of adsorbed protein types and amounts can develop with time, related to solution concentration, size, collision rates, interface affinity, and denaturation tendencies. This behavior is now called the "Vroman Effect" (12,13).

7. It is possible to control and regulate protein adsorption, in part, by modifying the surface or interface or by modifying the protein, such as with steric exclusion modifiers (14,15). (figure g)

8. Materials and interfaces with a micro-heterogeniety of the same size as the structural domains or building blocks in proteins probably have a particularly rich and complex set of adsorption properties. (figure h)

CONCLUSIONS AND PROSPECTS

These general concepts and hypotheses provide some appreciation

and qualitative understanding of protein interfacial processes. There is considerable progress on the modeling and simulation of selected interfacial properties by several groups (7,8,16-17) Due to the complexity of the general adsorption process, however, a quantitative, truly predictive model for complex multi-component systems will not be available in the near future.

Even these very qualitative concepts are sufficient to suggest that it should be possible to orient and order proteins at interfaces in order to optimize specific activities or functions, such as for biosensor applications (18). This can be done by modifying and/or controlling the structure of the protein in solution or by appropriate modifications and control of the interface itself. For example, one can now readily produce surfaces with a controlled gradient in properties (19,20). Patterned, micro-heterogeneous surfaces can also be prepared (21). Protein interactions and assembly on such surfaces is likely in the near future and offers exciting possibilities for the design and fabrication of protein based devices (27).

It is now becoming possible to directly observe proteins on surfaces by scanning tunneling microscopy (STM) (23) and by atomic force microscopy (AFM) (24).

The thrombin-induced polymerization of fibrinogen on a mica surface has now been directly observed by AFM (24).

ACKNOWLEDGEMENTS: Our work is funded in part by the National Heart, Lung, and Blood Institute-National Institutes of Health, the United States Army Research Office, and by the Center for Biopolymers at Interfaces (CBI), a State of Utah Center of Excellence. We thank C.G.Golander, K. Caldwell, H. Elwing, T. Matsuda, W. Pitt, J. Kopecek, J-H Lee, and Y-S Lin for discussions and assistance. V. Hlady thanks the R. Boskovic Institute, Zagreb, Yugoslavia, for a leave of absence.

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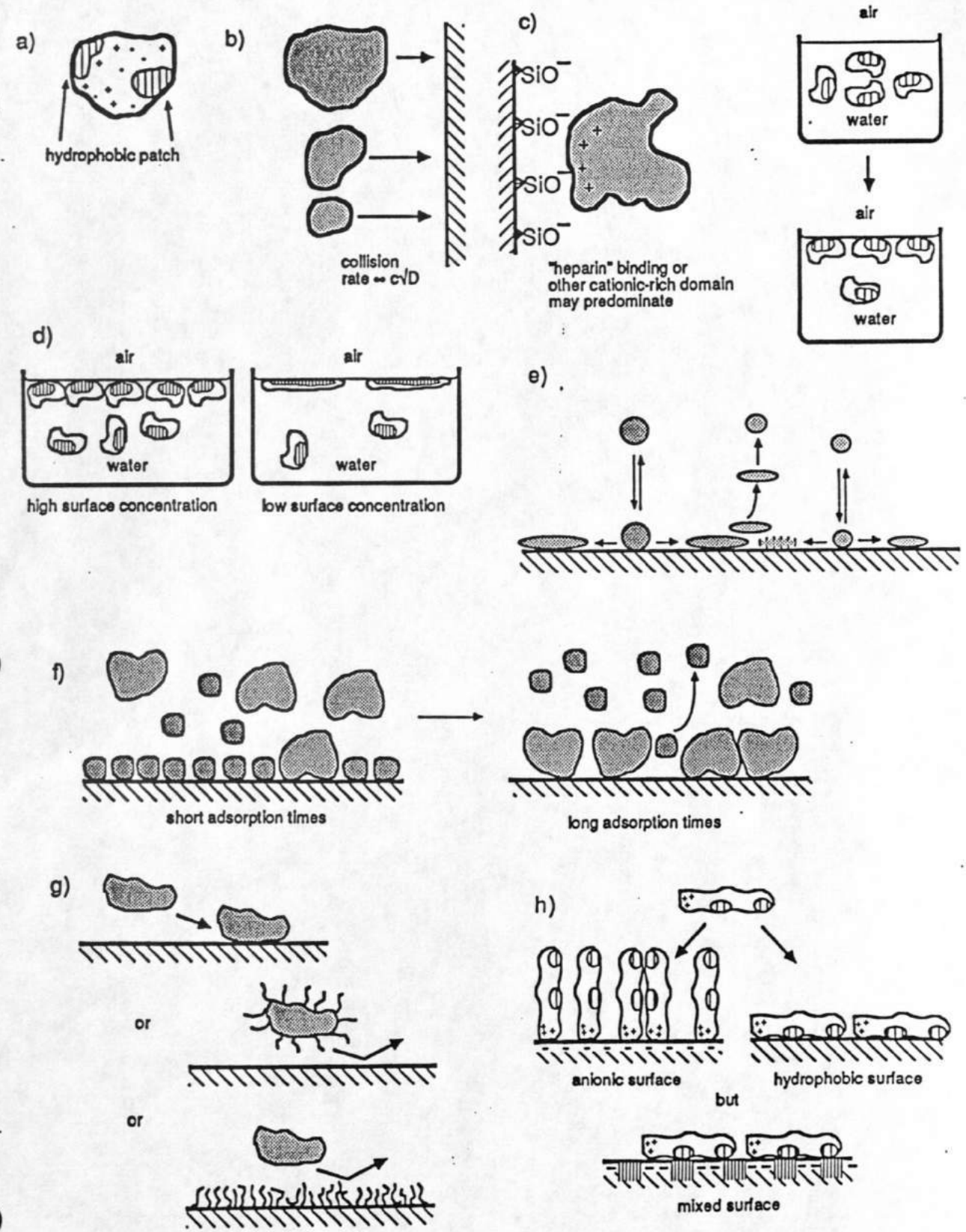
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short adsorption times
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g)
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or
h)
anionic surface
hydrophobic surface
but
mixed surface

AB-ANDR-7
SENSORS '89

Remote Measurement and Monitoring of Plasma Proteins (HL 37046-03)

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University of Utah, Department of Bioengineering
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The blood response to materials and devices is difficult to monitor. It is desirable to monitor the hemostatic condition in animals and patients fitted with in-vivo or ex-vivo devices. Although sensors are available for blood gases, pressure, temperature, and flow - continuous, remote sensors of hemostasis or for specific proteins are not available.

We have been researching the development of immunosensors; based on a fluoroimmunoassay technique for specific proteins, including prothrombin and antithrombin III.(3)

In addition to working with monoclonal Ab to prothrombin (gift of R.G. Mann and W. Church, University of Vermont) and antithrombin III (gift of A. Baquey and J. Caix, University of Bordeaux), we have worked with polyclonals to IgG, monoclonals to diqoxin, and monoclonals to fluoroscein. The latter has served as an excellent model system, because the binding constants and thermodynamics of Ab-hapten binding, including on and off rates, are known (in the bulk solution environment).

Evanescent optical immunosensors have been demonstrated in our laboratory in two different optical geometries: 1) single-reflection TIRF using a collimating lens to collect transmitted fluorescence; and 2) optical fibers using evanescence for both excitation and collection of fluorescence emission.(2,3,6,7,) Raw data obtained in single reflection TIRF is expressed by surface concentration of bound antigen and has the unit of mole/cm^2. With the anti-human IgG Ab system, binding constant approximately 10^8 M^{-1}, we are able to detect surface concentrations of fluorescein-labelled antigen bound to the immobilized antibody on the order of 10^{-14} mole/cm^2. This corresponds to a solution concentration of about 10^{-10} M. The sensitivity can be increased using antibodies with higher binding constants. With the same Ab-Ag system, we can detect 10^{-8} M of Ag in solution using the optical fiber geometry.

We and others now recognize that Ag-Ab binding at an interface is generally orders of magnitude "stronger" than expected, due to a variety of phenomena which we have recently reviewed.(5,8)

This is, of course, a serious problem. We desire that the sensor respond reversibly to changes in analyte concentration. Ab-Ag interactions in solution are indeed reversible and controlled by the overall association constant. We have identified and discussed the key factors responsible for Ag-Ab interface "irreversibility" and have proposed means to address and eliminate each of these key factors.(5,8)

We have demonstrated that the fluorescence background commonly seen in fluoroimmunoassays in blood can be almost completely eliminated by using green Helium-Neon laser excitation (532.8 μm) and tetramethyl rhodamine (TMR) dyes.(2)

We have demonstrated multichannel sensor capabilities using a multichannel polyclonal anti-human albumin sensing plate exposed to fluoroscein-labelled human albumin. The fluorescence was imaged on a two dimensional photometric detector (a charge coupled device-CCD- camera with a resolution of 384 x 576 pixels). This preliminary experiment has readily demonstrated the potential for multichannel remote immunoassay.(7)

We have demonstrated the quantitation of Ag binding by means of appropriate standard solutions and referencing methods using the methods and algorithms developed for our fundamental protein adsorption studies using interfacial fluorescence.

These results and experience have led to the definition of three important tasks, which are the specific aims of the continued work:

1. We propose to immobilize antibody (Ab) and Ab fragments to sensor surfaces by novel means which permit the Ab-antigen (Ag) binding reaction to be reversible, to permit the sensor to respond to changes in circulating protein (Ag) concentration.(1,8) We must also minimize the proteolytic degradation of immobilized Ab by plasma proteases.

2. Fluorescently-labelled Ag must be delivered to the sensing volume to compete with circulating Ag for the Ab binding sites on the sensor surface. As controlled remote delivery of protein Ag can be a problem, we propose to use synthetic small polypeptide epitopes of the Ag of interest. Such small (hexa- to deca-) peptides are readily labelled with fluors and delivered. The delivery mechanism will utilize the temperature-sensitive gel delivery technology of Hoffman and coworkers.

3. A reliable, accurate sensor requires reference and calibration channels. We propose to study and develop multichannel sensors and signal processing methods and algorithms to produce continuous, quantitative monitoring of changing analyte levels. We further propose to produce multi-analyte sensors using a small array of single analyte sensors sharing certain reference and calibration channels.(7)

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