3

Protein-Surface Interactions

3.1 INTRODUCTION

The behavior of proteins at surfaces plays a vital role in determining the nature of the tissue-implant interface. Adsorbed proteins affect blood coagulation, complement activation, and bacterial and cell adhesion. Furthermore, adsorbed proteins can influence biomaterial surface properties and degradation. This chapter presents the basic principles of protein adsorption in terms of protein and surface characteristics that affect the behavior of proteins at solid surfaces.

3.2 IMPORTANT PROTEIN AND SURFACE PROPERTIES

The properties of both the protein and the surface with which the biomolecule is interacting influence interfacial behavior (Fig. 3.1). Tables 3.1 and Table 3.2 list important protein and surface properties, respectively.

3.2.1 Protein Properties

The properties of proteins that influence surface activity are related to the primary structure of the protein, meaning that the sequence of amino acids affects protein-surface interactions. Larger molecules are likely to interact with surfaces because they are able to contact the surface at more sites (Fig. 3.2). For example, an albumin molecule (67 kDa) forms about 77 contacts with a silica substrate, and fibrinogen (340 kDa) forms about 703 contacts per molecule. Size, however, is not the sole determinant, because hemoglobin (65 kDa) exhibits greater surface activity than the much larger fibrinogen.

Because of their hydrophilicity, charged amino acids are generally located on the outside of proteins and are readily available to interact with surfaces. Consequently, the charge, as well as the distribution of charge on the protein surface, can greatly influence protein adsorption. As with size, however, charge is not the only determinant. Interestingly, proteins often show greater surface activity near their isoelectric point (the pH at which the molecule exhibits zero

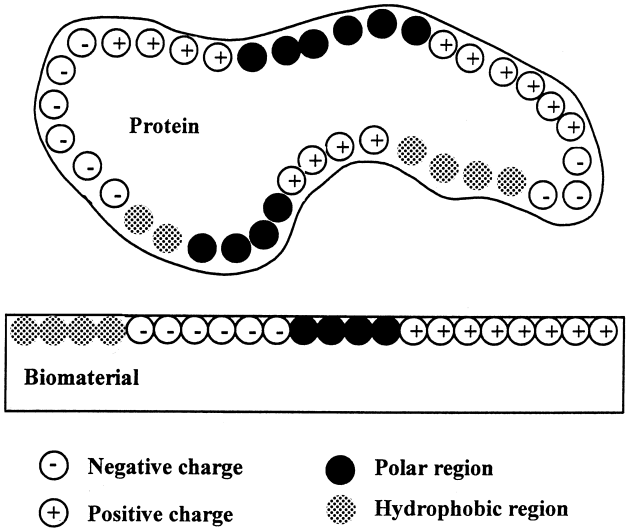

Figure 3.1. Cartoon illustrating the importance of both molecular and substrate properties in determining protein-surface interactions.

TABLE 3.1. Properties of Proteins That Affect Their Interaction With Surfaces

Property	Effect
Size	Larger molecules can have more sites of contact with the surface
Charge	Molecules near their isoelectric point generally adsorb more readily
Structure	
Stability	Less stable proteins, such as those with less intramolecular cross-linking, can unfold to a greater extent and form more contact points with the surface
Unfolding rate	Molecules that rapidly unfold can form contacts with the surface more quickly

TABLE 3.2. Properties of Surfaces That Affect Their Interaction With Proteins

Feature	Effect
Topography	Greater texture exposes more surface area for interaction with proteins
Composition	Chemical makeup of a surface will determine the types of intermolecular forces governing interaction with proteins
Hydrophobicity	Hydrophobic surfaces tend to bind more protein
Heterogeneity	Nonuniformity of surface characteristics results in domains that can interact differently with proteins
Potential	Surface potential will influence the distribution of ions in solution and interaction with proteins

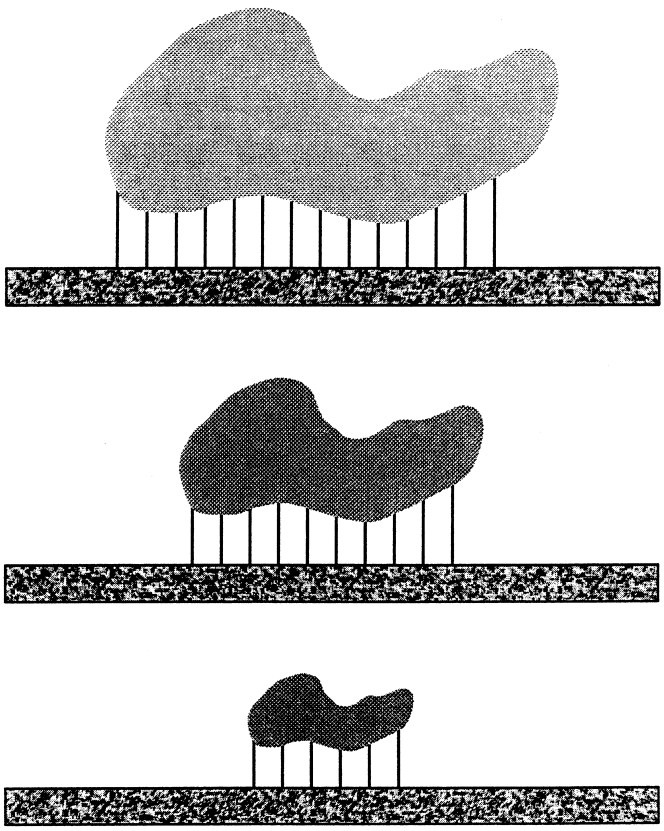

Figure 3.2. Effect of protein size on interaction with a surface.

charge, denoted pI). Although at first thought this might seem odd, two effects can explain the observation. First, consider that protein molecules do not interact with the surface in isolation. For example, 1 ml of a solution containing 1 μg of a 50-kDa protein will have approximately 10^{13} molecules. With so many molecules in solution, not only can they interact with the surface, but the molecules can also interact with each other (lateral interactions). At the isoelectric point, reduced electrostatic repulsion between uncharged adsorbing molecules can allow more protein to bind. A second explanation relates to alterations in protein structure because of changes in the charge of amino acids (see Chapter 2). If the conformation is altered, different amino acids could be exposed on the surface of the protein, which could consequently change the way the molecule binds to the substrate.

Properties related to unfolding of the protein also affect adsorption. Unfolding of a protein is likely to expose more sites (points) for protein-surface contact (Fig. 3.3). Therefore, factors related to a greater extent or rate of unfolding can result in greater surface activity. Less stable proteins or those with less intramolecular cross-linking are likely to unfold more or faster. For example,

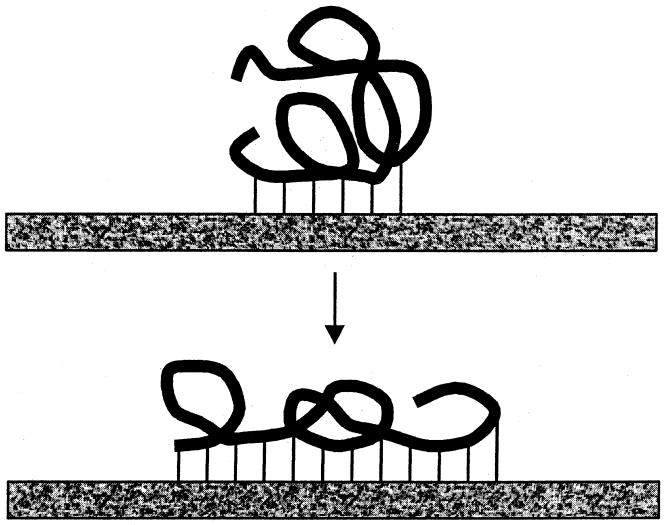

Figure 3.3. Effect of protein unfolding on interaction with a surface.

substitution of hydrophobic valine for glutamic acid in hemoglobin makes hemoglobin S (see Chapter 2, Section 2.2) less stable. The destabilized protein is consequently less soluble and results in fibrous precipitates that distort red blood cells.

The amphipathic nature of proteins, with their polar, nonpolar, and charged amino acids, also contributes to surface activity (Fig. 3.1). Although hydrophilic polar and charged amino acids are generally located on the exterior of the molecule and hydrophobic residues on the interior, this is not absolute. Thus hydrophobic amino acids might be available on the protein surface for interaction with substrates. Additionally, unfolding of proteins can expose hydrophobic regions and allow interaction with the surface.

3.2.2 Surface Properties

The properties of biomaterial surfaces that influence interaction with proteins are similar to those for proteins. In discussing surface properties, they are frequently grouped in three categories: geometric, chemical, and electrical. Substrates with more topographical features will expose more surface area for possible interaction with proteins. For example, surfaces with grooves or pores have greater surface area compared with smooth surfaces. Other surface features, such as machine marks introduced during processing, provide additional sites for protein interaction.

The surface chemical composition will determine which functional species are available for interaction with biomolecules. The oxidized (passivated) surface of a metallic biomaterial exposes metal and oxygen ions. Similarly, ceramic, and

some glass, surfaces comprise metal and nonmetal ions. A variety of functional species, such as amino, carbonyl, carboxyl, and aromatic groups, can be present on the surface of polymeric biomaterials. Depending on which species are exposed, biomolecules (or even particular regions of the molecule) may have different affinities for various surfaces. For example, hydrophobic surfaces tend to bind more protein as well as binding it more tenaciously.

On a microscopic scale, biomaterial surfaces can be inhomogeneous. Patches, or domains, of different functionality can exist on biomaterial surfaces, and these patches can interact differently with biomolecules. For example, many metallic biomaterials contain at least two different phases, such as the α- and β-phases in Ti-6Al-4V. Not only can the different phases behave differently when interacting with biomolecules, but grain boundaries behave differently than do grain interiors. In polymers, segregation resulting from folding of macromolecular chains can give microstructural domains. Depending on the chemical species present within the various domains, proteins will have different affinities for the patches.

The surface potential influences the structure and composition of the electrolyte solution adjacent to the biomaterial. Counterions are attracted to the surface, and normally isotropically distributed water molecules become ordered. The combined effects of water ions, molecules, and net surface potential will determine whether interaction with biomolecules is enhanced or hindered.

3.3 ADSORPTION AND DESORPTION

Adsorption is the process whereby molecules adhere to solid surfaces. Protein-surface interactions result in high local concentrations of the protein, reaching concentrations up to 1,000 times higher than in the bulk solution. As discussed in other chapters, this accumulation of protein, and especially accumulation of certain proteins, on biomaterial surfaces plays a critical role in determining the fate of the tissue-implant interface.

In addition to the protein and surface properties described above, adsorption also depends on the availability of molecules for interaction with the substrate. Molecules can be brought to the surface by one or more of four major transport mechanisms: 1) diffusion, 2) thermal convection, 3) flow, and 4) coupled transport, such as the combination of convection and diffusion. Variables such as concentration, velocity, and molecular size are important in determining the arrival of protein molecules at a surface. As an example, consider the effects of diffusion. Simple diffusion is described by the following equation:

$$\frac{\delta C}{\delta t} = D\frac{\delta^2 C}{\delta x^2} \tag{3.1}$$

where C is concentration, D is the diffusion coefficient, and x is distance. At short times and under conditions in which the rate of adsorption equals the rate

of diffusion

$$\frac{\mathrm{d}n}{\mathrm{d}t} = C_0\left(\frac{D}{\pi t}\right)^{1/2} \tag{3.2}$$

where n is the surface concentration of protein, C_0 is the bulk concentration of protein, and t is time. *Equation 3.2* shows that a higher bulk concentration and/or higher diffusion coefficient (which is inversely related to molecular size) result in a larger number of molecules arriving at the surface. Under conditions in which convection is also present, resulting in convective diffusion, the treatment becomes more complex and depends on the geometry of the interface. For flow in a thin channel:

$$\frac{\delta C}{\delta t} + V(y)\frac{\delta C}{\delta x} = D\frac{\delta^2 C}{\delta y^2} \tag{3.3}$$

where

$$V(y) = \gamma y\left(1 - \frac{y}{b}\right) \tag{3.4}$$

and V is the velocity of flow, x is the distance down the channel, y is the location within the height of the channel, γ is the wall shear rate, and b is the height of the channel. After applying the pertinent boundary conditions, *Equation 3.4* must be solved with numerical methods.

Once present at the surface, protein molecules can interact with the substrate via intermolecular forces, such as ionic bonding, hydrophobic interactions, and charge-transfer interactions. In contrast to its importance in stabilizing protein structure, hydrogen bonding does not play a major role in protein-surface interactions. Because water is good at forming hydrogen bonds, it is as likely to form hydrogen bonds with a surface as would amino acids in the protein molecule. Exactly which intermolecular forces govern protein-surface interaction will depend on the particular protein and surface (Fig. 3.1).

Even with a solution containing a single type of protein, the layer of adsorbed protein is likely to be heterogeneous. As molecules adsorb to a clean surface, there are few limitations on their interaction with the substrate, and each molecule can form many contacts with the surface (Fig. 3.4). As the surface becomes occupied, however, less surface is available for adsorption of subsequent protein molecules. Consequently, molecules in different orientations might be able to bind to the surface, even though fewer protein-substrate contacts are made (Fig. 3.4). Different orientations can also allow the protein to avoid or minimize repulsive interactions with previously bound biomolecules. In addition to reasons of available contact area, proteins can exist on the surface in different orienta-

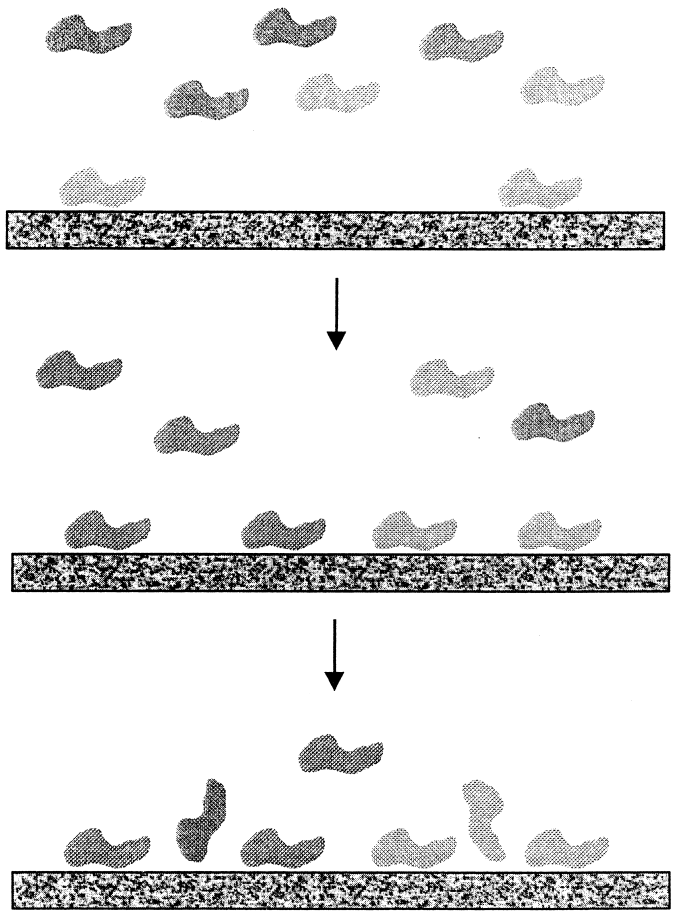

Figure 3.4. Effect of surface occupancy on adsorption of subsequent protein molecules. [Adapted from Horbett, T.A., and Brash, J.L. Proteins at interfaces: Current issues and future prospects, *Proteins at Interfaces: Physiochemical and Biochemical Studies*, J.L. Brash and T.A. Horbett (eds.), American Chemical Society, Washington, D.C., 1987, pp. 1–33]

tional states because of the heterogeneity of both the protein molecule and surface (Fig. 3.5); different orientations may be needed to bring complementary functionalities on the surface and protein into close proximity. For example, amphipathic proteins, with their polar, nonpolar, and charged amino acids, can interact differently with the biomaterial's various microstructural features, which have distinct structural and chemical properties.

The different orientations of adsorbed protein molecules not only affect the amount of protein bound to the surface but also have functional significance. Consider an enzyme or an adhesive protein, such as fibronectin, adsorbing to a biomaterial. Depending on the orientation of the molecules, the active site needed for catalytic activity of the enzyme could be inaccessible, either because it is interacting with the surface or because access to the active site is prevented

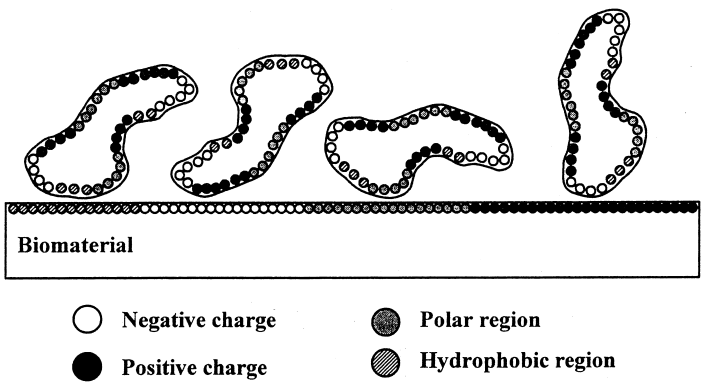

Figure 3.5. Different orientations of adsorbed protein molecules resulting from heterogeneity of both the protein and the surface.

by adjacent molecules (steric hindrance). Similarly, if the RGD-containing domains of fibronectin (Fig. 2.19) are not available for interaction with cells, the protein may not be able to support cell attachment.

Desorption is the reverse of adsorption; molecules previously bound to a surface detach and return to the bulk phase. For desorption to occur, all contacts between protein and surface must be simultaneously broken (Fig. 3.6). Although well characterized for small molecules such as gases, desorption of proteins is slow or nonexistent. Unless dramatic changes are made in the interfacial environment, such as increased ionic strength, lowered pH, and use of chaotropic agents or detergents, protein adsorption is largely irreversible because of the requirement of simultaneous dissociation of all interactions between molecule and surface. The difficulty or improbability of simultaneous disruption

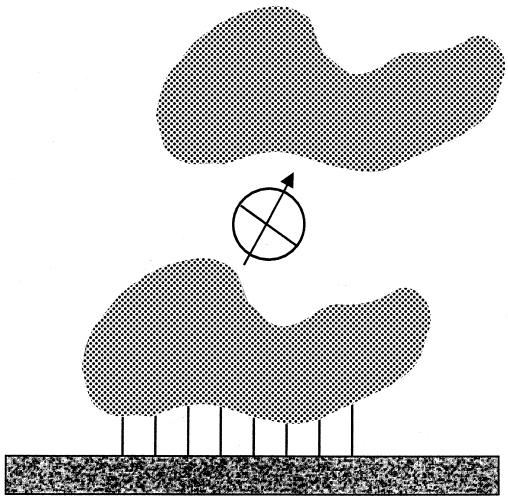

Figure 3.6. Desorption of a protein requires simultaneous breakage of all bonds with a surface.

of all contacts is increased further by large proteins, which can form a greater number of bonds with the surface. Consider the example of fibrinogen adsorption mentioned in Section 3.2.1. For a molecule of fibrinogen to desorb, all 703 contacts with the surface must be broken at the same time. As discussed in Section 3.5, however, adsorbed proteins can be replaced by molecules of the same or a different type of protein.

3.4 CONFORMATIONAL CHANGES

Protein molecules must not be thought of as rigid structures. As discussed in Chapter 2, proteins are flexible chains that have been coiled, folded, and bent to assume a particular conformation (three-dimensional structure). Changes in the microenvironment of the proteins, such as pH and ionic strength, can alter the conformation of the molecule. Likewise, proteins experience structural alterations during interaction with solid surfaces. Their conformation may be changed, but adsorbed proteins generally retain at least some of their biological activity. For example, adsorbed enzymes retain their catalytic properties, and adsorbed antibodies retain their ability to bind antigen, although the level of activity may be diminished.

Two modes of conformational change can occur. First, protein molecules can undergo time-dependent molecular spreading (Fig. 3.7). Initially, the molecule may contact a minimal number of binding sites on the surface by interaction of amino acids on the exterior of the protein. As the length of time the molecule resides on the surface (residence time) increases, the protein may unfold, exposing interior functional groups for interaction with additional binding sites. Overall, this results in a time-dependent increase in the number of contact points between protein and surface. Consequently, desorption becomes less likely as the residence time increases because of the larger number of contacts formed.

Second, altered conformation can result from changes in the bulk solution concentration (Fig. 3.8). At low concentration, abundant surface area is available for each protein molecule. Without near neighbors, molecules can spread to form multiple contacts with the surface. At high bulk concentrations, the amount of surface per molecule decreases and less unfolding can occur, because of adsorbate-adsorbate interactions. Consequently, more protein may be present on the surface, but with each molecule having fewer contacts.

3.5 MULTICOMPONENT SOLUTIONS

Although much of the understanding of protein-surface interactions described above has come from the study of single-protein solutions, adsorption from multicomponent solutions is most relevant to the tissue-implant interface. Body fluids, including blood, tears, and saliva, contain numerous types of biomole-

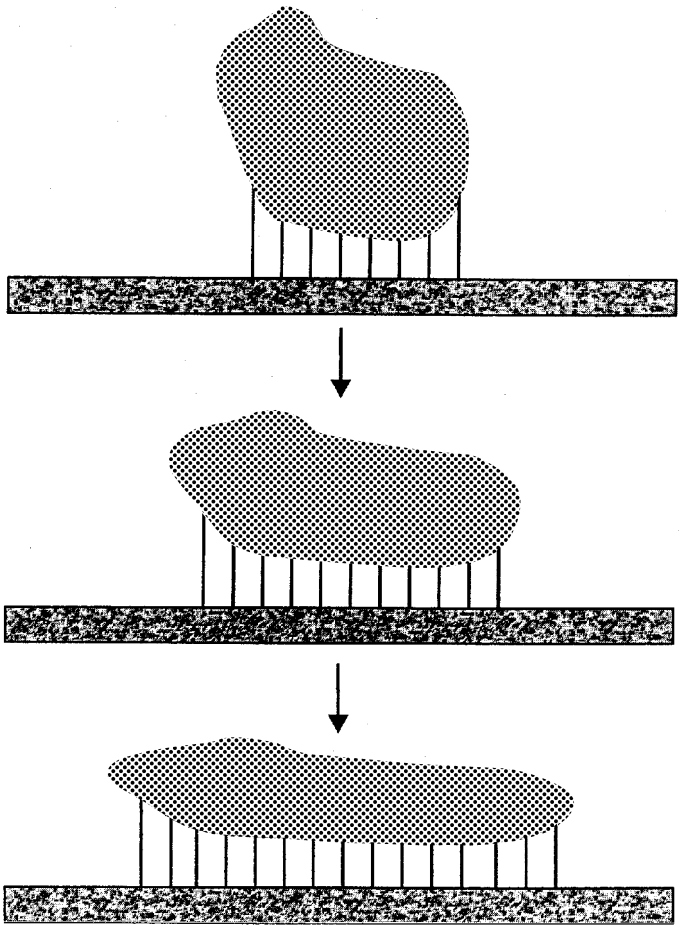

Figure 3.7. Time-dependent molecular spreading of a protein on a surface.

cules. For example, blood contains more than 150 proteins, not to mention lipids, carbohydrates, hormones, etc. Of ultimate importance is knowing which molecules accumulate on a biomaterial surface and how this relates to the bulk composition of the solution.

When a surface is exposed to a multicomponent solution, certain molecules will be preferentially deposited from the bulk. Furthermore, time-dependent changes in the composition of the adsorbed layer can occur, until a pseudo-steady state is reached. Variables related to both surface activity and availability of biomolecules at the surface contribute to determining the profile of molecules on the surface. Thus the affinity (e.g., size, charge, and conformational stability) and kinetic factors (e.g., concentration and size) described above are important. Because surfaces present a finite amount of area for binding protein, molecules

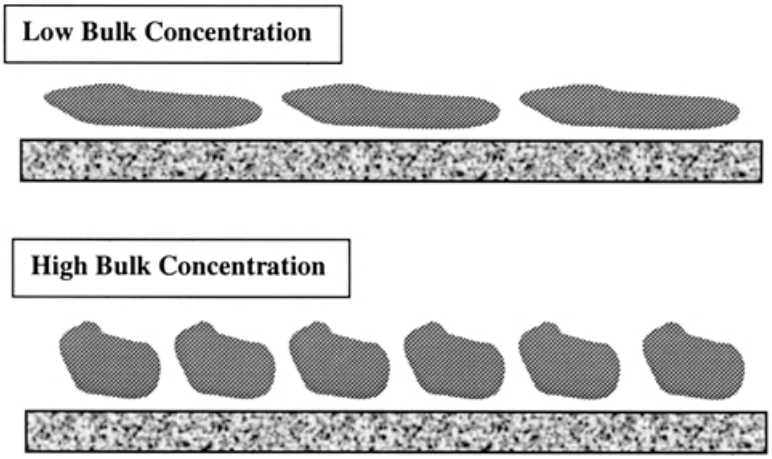

Figure 3.8. Conformational changes depending on the concentration of a protein in the bulk solution.

approaching the surface compete for binding sites, and protein-protein interactions as well as protein-surface interactions are important. In single-protein solutions intermolecular repulsive interactions dominate, but with multicomponent systems attraction between molecules can occur

Considering a simple diffusion-limited situation at the interface, *Eq. 3.2* indicates that molecules present in the bulk solution at high concentration and/or proteins with small size (large diffusion coefficient) will arrive quickly. Although their affinity for the surface may not be optimal, adsorption, even if just temporary, is likely because of their proximity to a "bare" surface with abundant binding sites. With time, molecules having greater affinity for the surface, but with a slower rate of arrival because of lower concentration and/or larger size, approach. The surface, however, may already be occupied by a monolayer of protein. In this case, the only way new molecules can bind to the surface is if previously adsorbed molecules detach. As stated in Section 3.3, pure desorption is rarely observed. Adsorbed molecules can be exchanged, nonetheless. Exchange results from competition for binding sites between the already adsorbed protein and molecules arriving from the bulk solution (Fig. 3.9). As bonds between the adsorbed molecule and the surface are periodically broken, new protein molecules can occupy the binding sites. The first molecule is released from the surface when all of its contacts with the substrate become occupied by the new molecule. Exchange proceeds until the surface is populated with proteins having strong interaction with the substrate. This hierarchical series of collision, adsorption, and exchange processes has been termed "the Vroman effect" (Fig. 3.10).

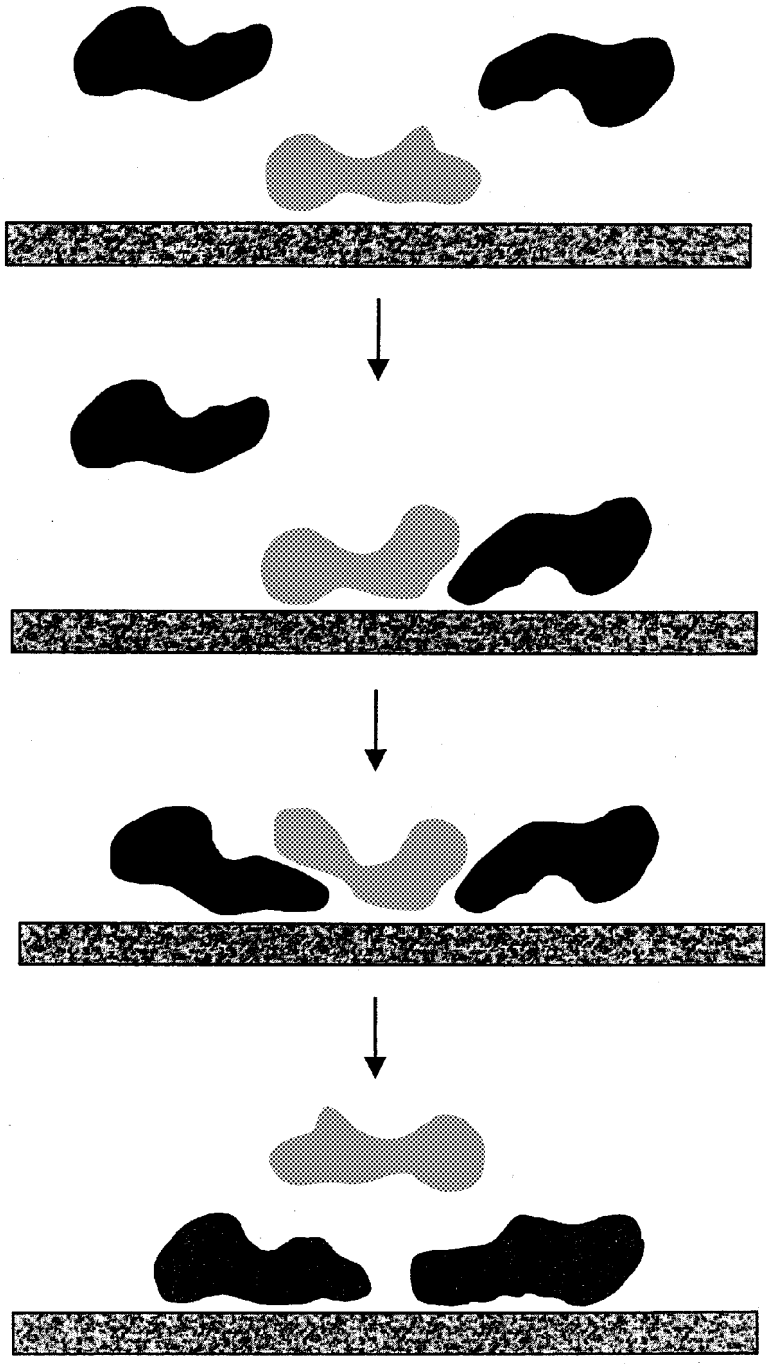

Figure 3.9. Exchange of an adsorbed protein for a different protein. [Adapted from Andrade, J.D., Principles of protein adsorption, *Surface and Interfacial Aspects of Biomedical Polymers*, Vol. 2, J.D. Andrade (ed.), Plenum Press, New York, 1985, pp. 1–80]

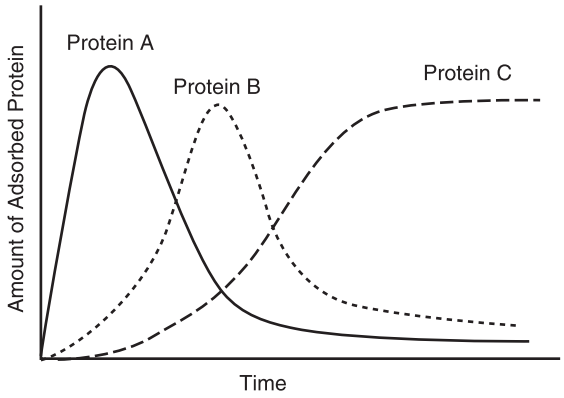

Figure 3.10. Schematic representation of sequential protein exchange on a surface (the Vroman effect).

3.5.1 Example—Blood-Surface Interactions

Blood, with over 150 proteins, serves as a good example to illustrate the events that occur during interaction of a foreign surface with a multicomponent solution. On the basis of mass transport considerations, proteins present at the highest concentration (Table 3.3) will be first to arrive at the surface. The order of availability according to the simplest form of transport, pure diffusion, is shown in Table 3.4. Because of its high concentration and moderate size (diffusion coefficient), albumin dominates initial interactions with the surface. IgG, with its lower concentration and larger size, has a slower rate of arrival at the

TABLE 3.3. Plasma Proteins With the Highest Concentration

Protein	Concentration (mg/ml)	Molecular Weight	Diffusion Coefficient (10^{-7} cm^2/s)
Albumin	40	66,000	6.1
IgG	15	150,000	4.0
α_1-Antitrypsin	3	54,000	5.2
Fibrinogen	3	340,000	2.0
Low-density lipoprotein (LDL)	3	5,000,000	5.4
α_2-Macroglobulin	3	725,000	2.4
Transferrin	2.6	77,000	5.0
IgA	2.3	162,000	3.4
α_2-Haptoglobins	2	100,000	4.7
High-density lipoprotein (HDL)	2	195,000	4.6
Complement 3	1.6	180,000	4.5

Adapted from Andrade, J.D. and Hlady, V. (1987). Plasma protein adsorption: The big twelve, *Ann. NY Acad. Sci.* 516:158–172.

TABLE 3.4. Rate of Arrival of the Proteins Listed in Table 3.3, Based on Diffusion-Limited Mass Transport Described by *Equation 1*

Protein	C (μM)	D (10^{-7} cm^2/s)	$C\sqrt{D}$
Albumin	606	6.1	1,497
IgG	100	4	200
α_1-Antitrypsin	56	5.2	127
Transferrin	34	5	76
α_2-Haptoglobins	20	4.7	43
IgA	14	3.4	26
High-density lipoprotein (HDL)	10	4.6	22
Complement 3	9	4.5	19
Fibrinogen	9	2	12
α_2-Macroglobulin	4	2.4	6
Low-density lipoprotein (LDL)	1	5.4	1

Adapted from Andrade, J.D. and Hlady, V. (1987). Plasma protein adsorption: The big twelve, *Ann. NY Acad. Sci.* 516:158–172.

surface. In terms of incidence of protein molecules colliding with the surface, seven times less IgG interacts with the biomaterial than does albumin. Almost 1,500 times less low-density lipoprotein than albumin interacts with the surface. Even though IgG has a slower rate of arrival than does albumin, if its molecules have a greater affinity for the surface, IgG molecules can exchange with bound albumin molecules. Similarly, other proteins with slower rates of arrival sequentially arrive at the surface, and depending on their affinity for the biomaterial, they can replace previously adsorbed molecules. Therefore, fibrinogen, for example, can dominate the surface because of greater affinity, even though its rate of arrival is over 100 times less than that of albumin. The actual hierarchy of blood proteins on surfaces, however, is more complicated than this simplified, diffusion-limited example (Table 3.5). Because of their affinity or additional kinetic factors (e.g., convection), molecules other than those with the highest concentration will also bind to the surface. Adsorption of proteins involved in blood clotting, such as fibrinogen and factor XII (discussed in Chapter 4), has great importance for determining tissue-implant interactions.

TABLE 3.5. Exchange Hierarchy of Plasma Proteins on Glass and Metal Oxide Surfaces

Albumin	Adsorbs First
IgG	↓
Fibrinogen	↓
Fibronectin	↓
Factor XII	↓
High-molecular-weight kininogen	↓

3.6 SUMMARY

- The interaction of proteins with biomaterials is determined by the properties of both the biomolecules and substrate.
- Protein factors that affect their interaction with biomaterials include size, charge, amphipathicity, and structural stability.
- Surface factors that influence their interaction with proteins include topography, charge, chemical composition, and microstructure.
- The rate of arrival of protein molecules at a biomaterial surface also plays a significant role in determining adsorption.
- The multiple states in which proteins exist on surfaces result from effects of orientation, geometric availability of surface area, and conformational changes.
- All protein-surface bonds must be simultaneously broken for a protein molecule to desorb.
- The longer a protein molecule resides on a surface, the less likely it is to be desorbed or exchanged by other molecules.
- In multicomponent solutions, such as real body fluids, proteins compete for surface binding sites, resulting in a series of collision, adsorption, and exchange processes on the biomaterial surface.

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3.8 QUIZ QUESTIONS

1. How does the size of a protein influence its ability to bind to a biomaterial?
2. Explain how the presence of disulfide bonds within a protein influences its ability to adsorb to surfaces.
3. How do adjacent protein molecules affect adsorption in a single component solution? In a multicomponent solution?
4. What factors affect the rate of arrival of protein molecules at a surface?
5. Explain the differences between protein desorption and exchange.
6. Why is pure desorption of a protein unlikely?

3.9 STUDY QUESTIONS/DISCOVERY ACTIVITIES

1. How does the roughness of a biomaterial surface affect protein adsorption? Consider roughness at different scales, that is, macro-, micro-, and nano-roughness.

2. Using *Equation 3.2* and the data shown in Table 3.3, plot the number of molecules of albumin and fibrinogen adsorbing on a surface during the first minute of interaction. How would fluid flow, such as caused by micromotion at the tissue-implant interface, alter adsorption?

3. Why is protein removal more difficult the longer it interacts with a surface? Using the information presented in both Chapters 2 and 3, explain this process, beginning with a protein in solution and ending with an adsorbed molecule that is resistant to removal.

4. Describe the Vroman effect. What is the significance of this phenomenon? What factors influence it?

5. Conduct a literature search on the topic of protein adsorption on biomaterials. Is this an active area of research? What is the focus of the citations you found? Select a recent publication, and summarize it in terms of the concepts presented in this chapter.