

Some Background Concepts

THOMAS A. HORBETT, BUDDY D. RATNER, JEFF M. SCHAKENRAAD, AND FREDERICK J. SCHOEN

3.1 BACKGROUND CONCEPTS

Buddy D. Ratner

Much of the richness of biomaterials science lies in its interdisciplinary nature. The two pillars of fundamental knowledge that support the structure that is biomaterials science are *materials science*, introduced in section 2, and the *biological-medical sciences*, introduced here. Complete introductory texts and a large body of specialized knowledge dealing with each of the chapters in this section, are available. However, these four chapters present sufficient background material so that a reader might reasonably follow the arguments presented later in this volume on biological interactions, biocompatibility, material performance and clinical performance.

In as short a time as can be measured after implantation in a living system (< 1 second), proteins are already observed on biomaterial surfaces. In seconds to minutes, a monolayer of protein adsorbs to most surfaces. The protein adsorption event occurs well before cells arrive at the surface. Therefore, cells see primarily a protein layer, rather than the actual surface of the biomaterial. Since cells respond specifically to proteins, this interfacial protein film may be the event that controls subsequent bioreaction to implants. Protein adsorption is also of concern for biosensors, immunoassays, array diagnostics, marine fouling and a host of other phenomena. Protein adsorption concepts are introduced in Chapter 3.2.

After proteins adsorb, cells arrive at an implant surface propelled by diffusive, convective or active (locomotion) mechanisms. The cells can adhere, release active compounds, recruit other cells, grow in size, replicate and die. These processes often occur in response to the proteins on the surface. Cell processes lead to responses (some desirable and some undesirable) that physicians and patients observe with implants. Cell processes at artificial surfaces are also integral to the unwanted buildup of marine organisms on ships, bacterial biofilms on implants and the useful growth of cells in bioreactors used to manufacture biochemicals. Cells at surfaces are discussed in Chapter 3.3.

After cells arrive and interact at implant surfaces, they may differentiate, multiply, communicate with other cell types and organize themselves into tissues comprised of one or more cell types. Cells secrete extracellular matrix (ECM) molecules

that fill the spaces between cells and serve as attachment structures for proteins and cells. The processes of angiogenesis (small blood vessel formation) and vasculogenesis (formation of larger blood vessels) are critical to provide this new tissue with nutrition and remove wastes. Finally, these tissues have distinctive reactions to irritation and injury. The development, organization and response to injury of tissues must be understood to appreciate the interplay between synthetic materials and tissues. Tissue structure and organization is reviewed in Chapter 3.4.

Finally, cells and tissues respond to mechanical forces. Two samples made of the same material, one a triangle shape and the other a disk, implanted in soft tissue, will show different healing reactions with considerably more fibrous reaction at the asperities of the triangle than along the circumference of the circle. Blood cells and the endothelial lining of the blood stream show distinctly different behaviors depending upon whether they are exposed to high or low shear forces. In recent years, much has been learned about the way external mechanical forces are transduced at cell surfaces into chemical signals that, in turn, direct cytoskeleton formation (or dissolution) and influence the nucleus of the cell to up- and down-regulate genes and messenger RNAs. Chapter 3.5 overviews mechanical effects on blood cells.

3.2 THE ROLE OF ADSORBED PROTEINS IN TISSUE RESPONSE TO BIOMATERIALS

Thomas A. Horbett

INTRODUCTION

The replacement of injured or diseased tissues with devices made from materials that are not of biologic origin is the central approach in current biomaterials science and clinical practice. The prevalence of this approach is due largely to the fact that these materials are not attacked by the immune system, unlike donor tissues or organs. This fundamental difference arises

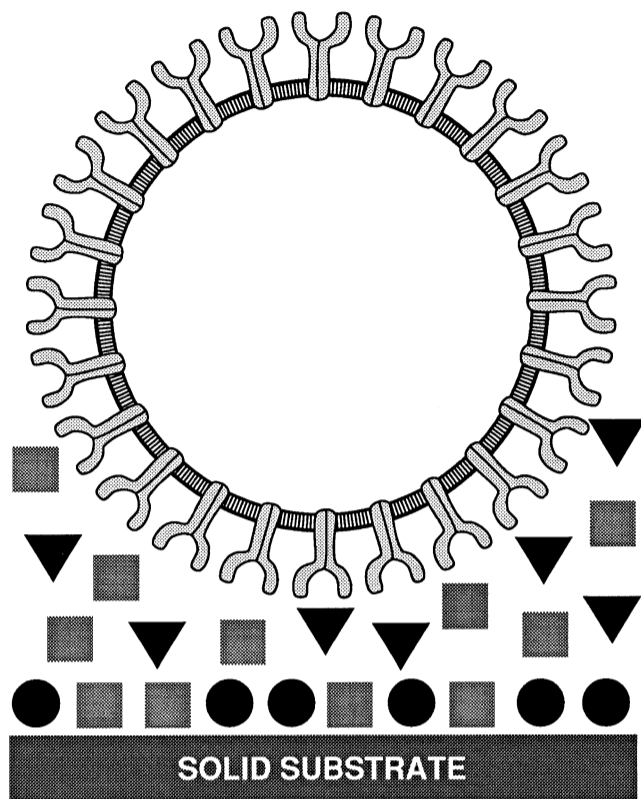


FIG. 1. Cell interactions with foreign surfaces are mediated by integrin receptors with adsorbed adhesion proteins that sometimes change their biological activity when they adsorb. The cell is shown as a circular space with a bilayer membrane in which the adhesion receptor protein molecules (the slingshot-shaped objects) are partly embedded. The proteins in the extracellular fluid are represented by circles, squares, and triangles. The receptor proteins recognize and cause the cell to adhere only to the surface-bound form of one protein, the one represented by a solid circle. The bulk phase of this same adhesion protein is represented by a triangle, indicating that the solution and solid phase forms of this same protein have a different biological activity. The figure is schematic and not to scale. (From Horbett, 1996.)

from the presence of immunologically recognizable biologic motifs on donor tissue and their absence on synthetic materials.

Nonetheless, there are other types of biological responses to implanted biomaterials that often impair their usefulness, including the clotting of blood and the foreign body reaction. Clearly, the body does recognize and respond to biomaterials. The basis for these reactions is the adsorption of adhesion proteins to the surface of the biomaterials that are recognized by the integrin receptors present on most cells. The adsorption of adhesion proteins to the biomaterial converts it into a biologically recognizable material, as illustrated in Fig. 1.

The interaction of adhesion receptors with adhesion proteins thus constitutes a major cellular recognition system for biomaterials. Therefore, the role of adsorbed adhesion proteins in mediating cellular interactions with biomaterials will be the primary focus of this chapter. Examples illustrating the ability of adsorbed adhesion proteins to influence cellular interactions with foreign materials are presented first. Then, some of the major physicochemical characteristics of

protein adsorption are illustrated and discussed, including rapid kinetics, monolayer adsorption, and competitive adsorption. Molecular spreading events related to the conformational stability of the protein are presented at some length as background for a section on how the biological activity of adsorbed adhesion proteins is affected by the substrate. The final section summarizes the principles underlying the role of adsorbed proteins in mediating platelet response to biomaterials as an illustrative case study representative of many other types of cellular responses.

This chapter includes material that is discussed in greater detail in several previous review articles by the author (Horbett, 1993, 1994, 1999; Horbett and Brash, 1995). Those articles also give citations to all the work discussed here.

EXAMPLES OF THE EFFECTS OF ADHESION PROTEINS ON CELLULAR INTERACTIONS WITH MATERIALS

Protein adsorption to materials can be performed with a single protein, typically in a buffer solution, or from complex, multiprotein solutions such as blood plasma that can contain hundreds of proteins. Single proteins in buffer can be used to model fundamental aspects of protein adsorption or to study biological reactions to one protein. Adsorption from complex media approximates the reaction observed from implanted biomaterials. Examples of both types of adsorption are presented.

The Effects of PreadSORption with Purified Adhesion Proteins

PreadSORption of certain kinds of proteins onto a solid substrate such as a tissue culture polystyrene greatly increases its adhesiveness to many kinds of cells, and such proteins are called adhesion proteins. The increased adhesiveness is because many cells have receptors on their cell membrane that bind specifically to these specialized proteins. The adhesion receptors are called integrins. For example, fibronectin preadsorption greatly increases adhesion of fibroblasts, whereas albumin preadsorption prevents it. Experiments of this type have been done with a wide variety of cells and adhesion proteins, with basically similar results. Adhesion proteins also promote the flattening out or spreading of the cell onto the surface. A specific example is provided by measuring the percentage of attached cells that spread on surfaces pretreated with increasing concentrations of fibronectin. Spreading is only about 5% in the absence of fibronectin, but increases to nearly 100% as the fibronectin concentrations in the preadsorption solution are increased from 0.03 to 3 $\mu\text{g/ml}$.

Another example of the effect of fibronectin is shown in Fig. 2, which also contrasts it with the effects of the non-adhesive protein immunoglobulin G. As shown in the figure, the adhesion of the fibroblast-like 3T3 cells to a series of polymers and copolymers of 2-hydroxyethyl methacrylate (HEMA) and ethyl methacrylate (EMA) varies, being much less on the hydrophilic polyHEMA-rich surfaces than on the hydrophobic polyEMA-rich surfaces. The preadsorption of the surfaces

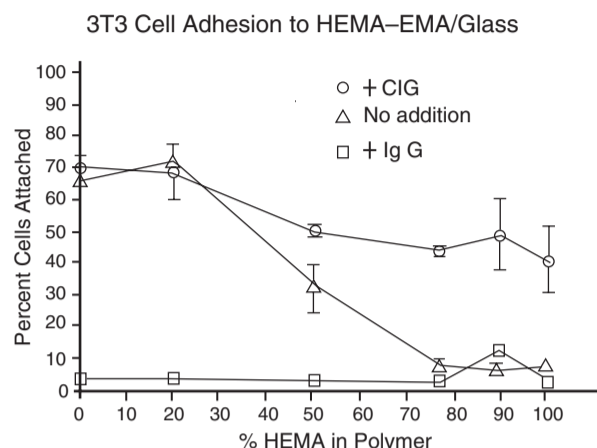


FIG. 2. 3T3 cell adhesion to HEMA-EMA copolymers: effect of preadsorption with fibronectin (designated CIG in the figure) or immunoglobulin G. Unpublished data from the author's laboratory.

with immunoglobulin G greatly reduces the adhesion of the cells to all the surfaces. In contrast, surfaces preadsorbed with fibronectin (designated CIG in the figure) are all fairly adhesive, much more so than the IgG preadsorbed surfaces or to the HEMA-rich surfaces not preadsorbed with fibronectin.

Preadsorption of adhesion proteins also affects cell interactions with surfaces studied under *in vivo* conditions. For example, platelet deposition onto polymeric arteriovenous shunts in dogs is greatly increased when fibrinogen or fibronectin are preadsorbed to the surfaces.

Depletion Studies

Although adsorption of purified adhesion proteins to a surface is one way to see their effect on cell adhesion, as presented in the preceding section, it does not mimic very well what occurs with implanted biomaterials. This is because implants are exposed to complex mixtures of proteins such as plasma or serum, so the adhesion protein must compete with many others for adsorption to the biomaterial. In that condition, despite its presence in the bulk phase, a given adhesion protein may really play little or no role. It is possible that very little of the adhesion protein may adsorb to the surface, as it is outcompeted by other proteins for the limited surface sites. Thus, a more biologically relevant way to understand the role of an adhesion protein in reactions to implants is to study the effect of their selective depletion from the complex mixture. The observations presented in this section and the articles they are based on are presented in greater detail in a review by the author (Horbett, 1999).

Selective depletion means that only one of the proteins is removed from the mixture at a time, by immunoadsorption chromatography, by use of plasma from mutant individuals lacking the adhesion protein of interest, or by selective enzymatic degradation. Thus, the more important role of adsorbed vitronectin as opposed to fibronectin in mediating attachment and spreading of cells on many surfaces has emerged from

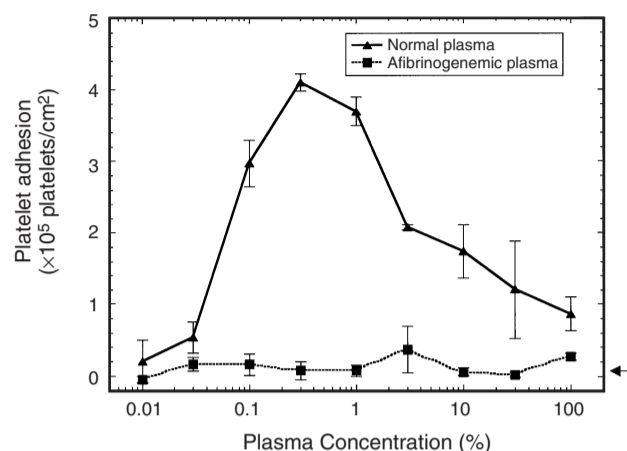


FIG. 3. Platelet adhesion to Immulon preadsorbed with normal and afibrinogenemic plasma. Platelet adhesion to Immulon I preadsorbed with normal (triangles) or afibrinogenemic plasma (squares). The solid line represents the platelet adhesion to Immulon I preadsorbed with a series of dilutions of normal plasma, whereas the dotted line represents the platelet adhesion to Immulon I preadsorbed with a series of dilutions of afibrinogenemic plasma. The arrow at lower right corner indicates platelet adhesion to Immulon I preadsorbed with 2% BSA only. Source: Fig. 4 in Tsai and Horbett (1999).

immunoadsorption studies. Several studies illustrate the important role that adsorbed fibrinogen plays in the adhesion of platelets, neutrophils, and macrophages.

The effects of removal of fibronectin or vitronectin or both proteins from serum on the adhesion of endothelial cells depends on surface chemistry. On tissue culture polystyrene (TCPS), fibronectin removal has little effect, whereas vitronectin removal greatly reduces adhesion. The results clearly show the primary role of adsorbed vitronectin in supporting endothelial cell adhesion to TCPS. In contrast, adhesion to a surface modified by ammonia in a glow discharge requires fibronectin, since removal of that protein greatly reduces adhesion to this surface, while vitronectin removal has little effect. However, the results for TCPS are more typical, i.e., on most surfaces studied by this method it appears that vitronectin, not fibronectin, is the primary agent responsible for cell adhesion.

Platelet adhesion to surfaces preadsorbed with plasma deficient in fibrinogen is much less than to the same surface preadsorbed with normal plasma, as illustrated in Fig. 3. Most of the adhesion supporting activity can be restored to fibrinogen deficient plasma by addition of normal levels of exogenous fibrinogen. In contrast, removal of fibronectin or vitronectin or von Willebrand's factor from plasma has little effect on platelet adhesion (not shown), even though these other plasma proteins act as adhesion proteins when adsorbed as single proteins to surfaces. It appears that too little of these other proteins adsorbs from plasma to make much difference, i.e., competition from fibrinogen and other proteins keeps their surface concentration too low and so their removal has no effect.

When mice are depleted of fibrinogen by treatment with an enzyme that degrades it, the adhesion of neutrophils and macrophages to a polymer implanted in their peritoneal cavity

is greatly reduced. The fibrinogen depleted animals exhibited near normal neutrophil and macrophage adhesion to the implants if the implants are preadsorbed with fibrinogen. These studies illustrate the power of the depletion method very well. Previously, it had been thought that either complement or IgG would be the main adhesion proteins for neutrophils and monocytes because of the presence of receptors on these cells that bind these proteins. Instead, it appears that an integrin receptor for fibrinogen (CD11b/CD18, also known as Mac-1) plays a major role, at least during the initial or acute phase of the foreign body response in the mouse peritoneal cavity.

Inhibition of Receptor Activity with Antibodies

Another way to show the role of adhesion proteins in cell interactions with biomaterials is to add specific inhibitors of their function. Adhesion proteins cause cell adhesion by binding to integrin receptors that specifically recognize the adhesion protein. One way to inhibit this reaction is to add an antibody that binds to the receptor, blocking access to the adhesion protein. Examples of this approach are now presented.

Platelet-receptor-mediated interactions appear to be the primary mechanism of platelet interaction *in vivo* with certain vascular grafts because platelet deposition is largely inhibited by antibodies to the glycoprotein III/IIIa receptor. *In vitro* platelet adhesion to surfaces preadsorbed with blood plasma is also inhibited by anti-glycoprotein IIb/IIIa in a dose-dependent manner, as illustrated in Fig. 4. In this study, samples of the polyurethane Biomer were preadsorbed with plasma and then exposed to platelets in an albumin containing buffered saline suspension that had increasing amounts of the antibody. As shown in the figure, adhesion declined to very low values when high concentrations of the anti-integrin antibody were present.

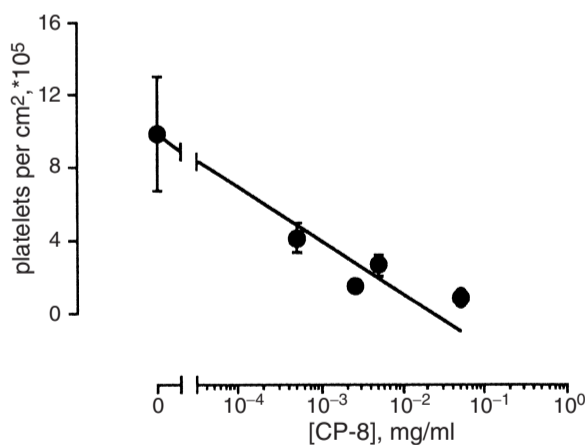


FIG. 4. Effect of anti-IIb/IIIa antibody on platelet adhesion to Biomer preadsorbed with plasma. Adhesion of platelets incubated in monoclonal antibody LJ-cp8 (monovalent Fab' fragment directed against the glycoprotein (GP) IIb/IIIa complex to Biomer. Substrates were contacted with 1.0% plasma for 2 hr, then with washed, antibody-treated platelets for 2 hr. From: Chinn, Horbett, and Ratner (1991).

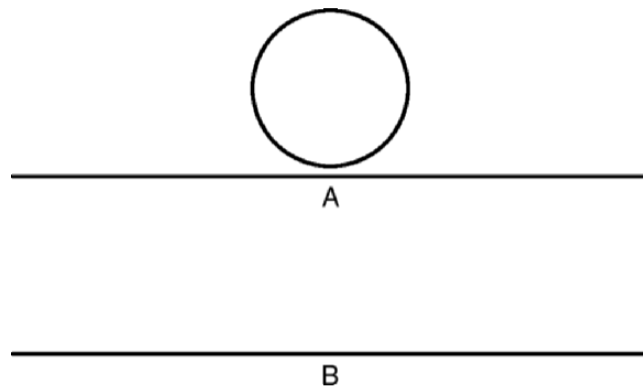


FIG. 5. The conversion of nonwetting polystyrene surface (top panel) into one completely wettable by water (bottom panel) is due to the adsorption of proteins.

THE ADSORPTION BEHAVIOR OF PROTEINS AT SOLID/LIQUID INTERFACES

Adsorption Transforms the Interface

Figure 5 illustrates an experiment that is performed by the author to demonstrate the adsorption of proteins to surfaces. As illustrated in part A, a water droplet placed on the surface of an unused polystyrene cell culture dish is easily visible because it beads up, i.e., the contact angle between the droplet and the polystyrene surface is very high because of the water-repellant, hydrophobic nature of polystyrene. If a cell were placed on a polystyrene dish instead of the water droplet, it also would encounter a very nonwetting surface. Part B of the figure illustrates the results of placing a water droplet on the surface of a polystyrene dish that had first been exposed to a protein solution for a short time and then rinsed extensively with water. As illustrated, no water droplet can be seen on this surface, reflecting the fact that in this case the added drop of water completely spread out over the surface of the preadsorbed dish. This happens because the water in part B was not able to interact with the polystyrene surface, because the surface had become coated with a layer of the hydrophilic protein adsorbate. Similarly, cells that come into contact with surfaces adsorbed with proteins do not directly "see" the substrate, but instead they interact with the intervening protein adsorbate.

Rapid Adsorption Kinetics and Irreversibility

The kinetics of adsorption of proteins to solid surfaces typically consists of a very rapid initial phase, followed by a slower phase upon approach to the steady-state value. Initially, proteins adsorb as quickly as they arrive at the largely empty surface. In this phase, adsorption is linear when plotted against $\text{time}^{1/2}$, characteristic of a diffusion-controlled process. In the later, slower phase, it is more difficult for the arriving proteins to find and fit into an empty spot on the surface.

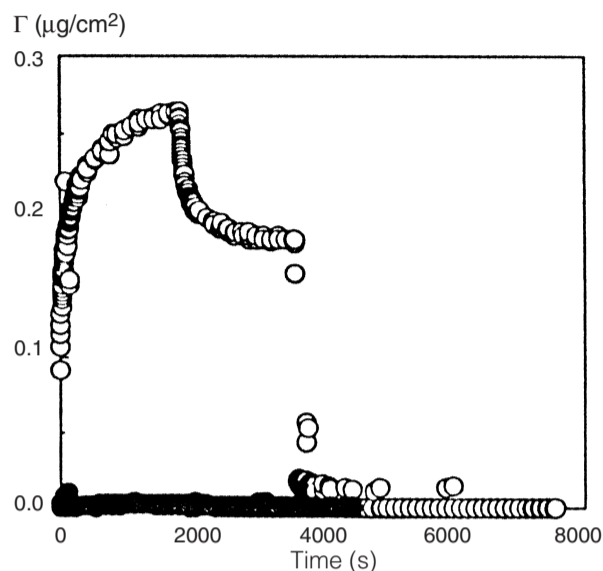


FIG. 6. The adsorption kinetics of lysozyme to a silica surface as studied with ellipsometry. The adsorbed amount versus time for adsorption of lysozyme to silica followed by buffer rinsing after 1800 sec, addition of surfactant (SDS) after 3600 sec, and a final rinse with buffer after 5400 sec (open circles). Adsorption from a mixture of the protein and surfactant for 1800 sec followed by rinsing is also included (closed circles). The experiments were carried out at 25°C in 0.01 M phosphate buffer, 0.15 M NaCl, pH 7. From Arnebrandt and Wahlgen (1995).

Figure 6 shows the time course of adsorption of lysozyme on silica measured with a high-speed, automated ellipsometer

capable of very rapid measurements. At the earliest measurement time, less than a second into the study, the adsorption has reached almost half of the steady-state value. At 2000 sec, the protein solution was replaced with a buffer, resulting in some removal of loosely bound protein, but the adsorption stabilizes and would have remained at this value for much longer than shown, due to the tight, irreversible binding. At 4000 sec, a solution of the detergent sodium dodecyl sulfate (SDS) was infused, leading to complete removal of the protein. Thus, this experiment illustrates the rapid adsorption of proteins. It also illustrates that most of the adsorbed protein is irreversibly bound, as indicated by the fact that washing the surface with buffer does not remove the protein. The adsorbed protein is only removed when a strong surfactant (SDS in this example) is used. All these features are characteristic of protein adsorption to solid surfaces.

The Monolayer Model

The existence of a close packed monolayer of adsorbed protein is suggested by studies with single protein solutions in which a saturation effect can often be observed in the adsorption isotherm (Fig. 7). Adsorption to surfaces exposed to different concentrations of protein until steady state adsorption is achieved (2 hours or more) increases steeply at low bulk-phase concentrations but typically reaches a plateau or saturation value at higher bulk concentrations. Usually, the plateau value falls within the range expected for a close-packed monolayer of protein (about 0.1 to 0.5 $\mu\text{g}/\text{cm}^2$, depending on the diameter and orientation assumed for the protein).

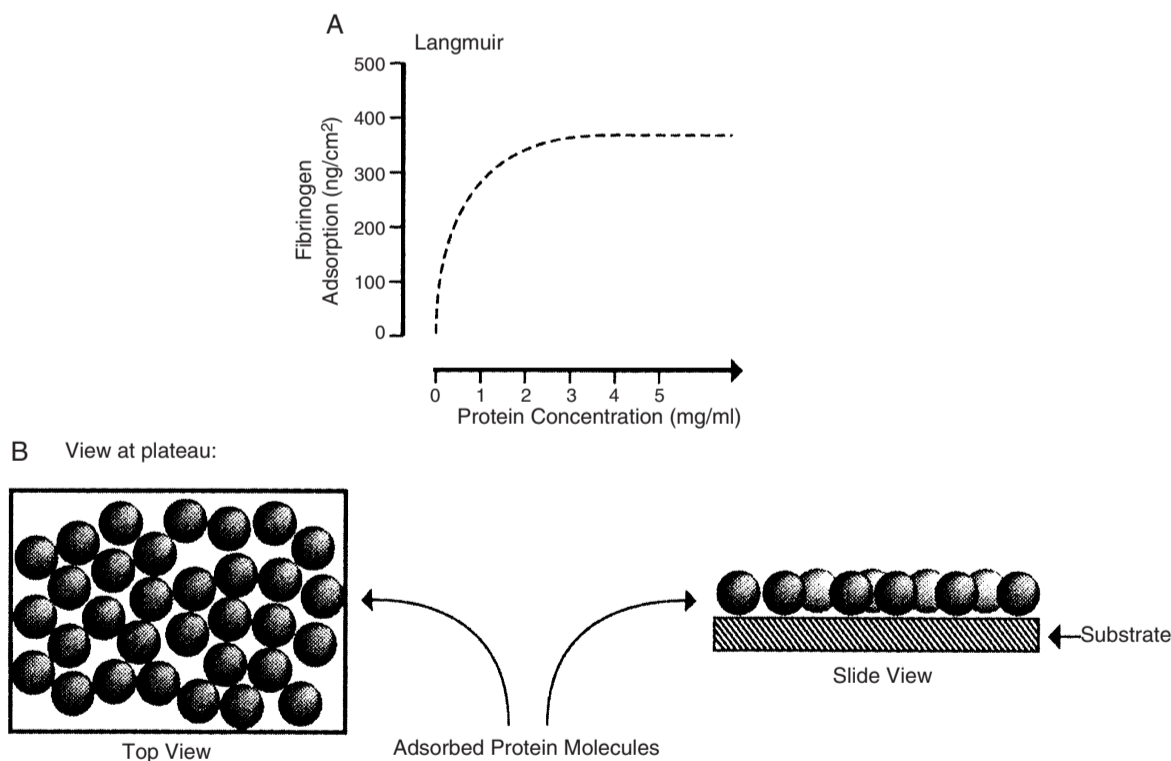


FIG. 7. Adsorption isotherms and the monolayer concept. From Horbett (1996).

The adsorption values from complex protein mixtures also typically are in the monolayer range. For example, the sum total of the amount of adsorption of the three major proteins in plasma (albumin, IgG, and fibrinogen) on the HEMA-EMA series of surfaces is also in the range of 0.1 to 0.5 $\mu\text{g}/\text{cm}^2$. In addition, the fact that competition exists between proteins for adsorption to a surface (see next section) also indicates the existence of limited sites. Thus, when a monolayer is the limit, there must be some selection for which proteins are present in the adsorbed film. It should be noted that well-defined plateaus are not always observed, but instead adsorption rises much more slowly at higher bulk-phase concentrations than at low concentrations, i.e., Freundlich isotherms do occur.

Competitive Adsorption of Proteins to Surfaces from Protein Mixtures

Adsorption from mixtures of proteins is selective, leading to enrichment of the surface phase in certain proteins. In this context, enrichment means that the fraction of the total mass of the adsorbed protein layer corresponding to a given protein is often much higher than the fraction of this protein in the bulk phase mixture from which it adsorbed. Since the solid can accommodate only a small fraction of the total protein present in the bulk phase, and proteins vary greatly in their affinity for surfaces, some adsorbed proteins are present in greater amounts than others. Studies of surfaces exposed to plasma have shown that many different proteins are present in the adsorbed film.

The competitive phenomena underlying differential enrichment from multi protein mixtures are most clearly illustrated in binary mixtures of proteins. Figure 8 has three separate curves in it, which overlap at the high and low ends. These curves represent the typical outcome of binary-mixture studies, but for three different conditions. For example, when a radio-labeled protein such as fibrinogen ("A" in the figure) is mixed with various amounts of an unlabeled protein such as albumin ("B" in the figure), the adsorption of fibrinogen ("A") always declines when sufficiently high amounts of albumin ("B") are present. However, the amount of competing protein needed to inhibit the adsorption of the labeled protein is different in each curve. This is meant to illustrate that, for a given pair of competing proteins, the competition curves will be different if

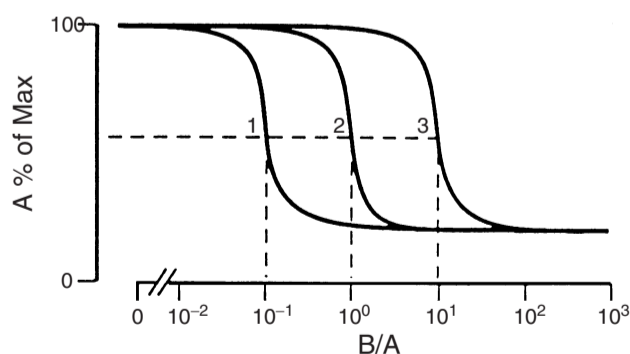


FIG. 8. Competitive adsorption of two proteins from a mixture. From Horbett (1993).

the surfaces they are competing for are different. In addition, if the surfaces are kept the same, but the competition of different pairs of proteins are studied, the curves will differ because the ability of proteins to compete for surface sites is quite different for different proteins.

For example, inhibition of fibrinogen adsorption to polyethylene requires a roughly 10-fold excess by weight of lower-affinity competing proteins such as albumin, but is effectively inhibited by the higher-affinity protein hemoglobin even when hemoglobin is present at only 10% of the mass of fibrinogen. However, the amounts needed for this inhibition will vary with the surfaces. Affinity variation is thus a major principle determining the outcome of competitive adsorption processes.

Examples of surface enrichment from complex protein mixtures are readily available. Although fibrinogen is only the third most concentrated protein in plasma, after IgG and albumin, biomaterials exposed to plasma are enriched in fibrinogen in the adsorbed phase. Hemoglobin is present in very low concentrations in plasma (0.01 mg/ml or less), but it is still adsorbed in amounts similar to the more predominant proteins because of its high surface affinity. Albumin, a lower-affinity protein, presents a counterexample. Albumin concentration in plasma is much higher than fibrinogen, yet the surface concentration of albumin adsorbed from plasma is typically about the same as fibrinogen. The high concentration of albumin in the plasma drives it onto the surface according to the law of mass action. Similarly, fibrinogen adsorption is higher from plasmas that contain higher concentrations of fibrinogen. Thus, mass concentration in the bulk phase is the second major factor determining competitive adsorption behavior.

The adsorption of fibrinogen from plasma exhibits some unusual behavior. On some surfaces, fibrinogen adsorption is maximal at intermediate dilutions of plasma (see example in Fig. 9A). In addition, fibrinogen adsorption from full-strength or moderately diluted plasma is higher at very early adsorption times than at later times (example shown in Fig. 9B). These are examples of the Vroman effect for fibrinogen. This phenomenon is a clear example of the unique effects of competitive adsorption on both the steady-state and the transient composition of the adsorbed layer that forms from plasma. The Vroman effect appears to involve displacement of initially adsorbed fibrinogen by later-arriving, more surface-active plasma proteins, especially high-molecular-weight kininogen, and transitions in the adsorbed fibrinogen that make it less displaceable with adsorption time (reviewed in Slack and Horbett, 1995).

MOLECULAR SPREADING EVENTS: CONFORMATIONAL AND BIOLOGICAL CHANGES IN ADSORBED PROTEINS

Proteins that adsorb to solid surfaces can undergo conformational changes because of the relatively low structural stability of proteins and the tendency to unfold to allow further bond formation with the surface. Conformational changes can be detected with many types of physicochemical methods and also by measuring changes in the biological activity of the adsorbed proteins.

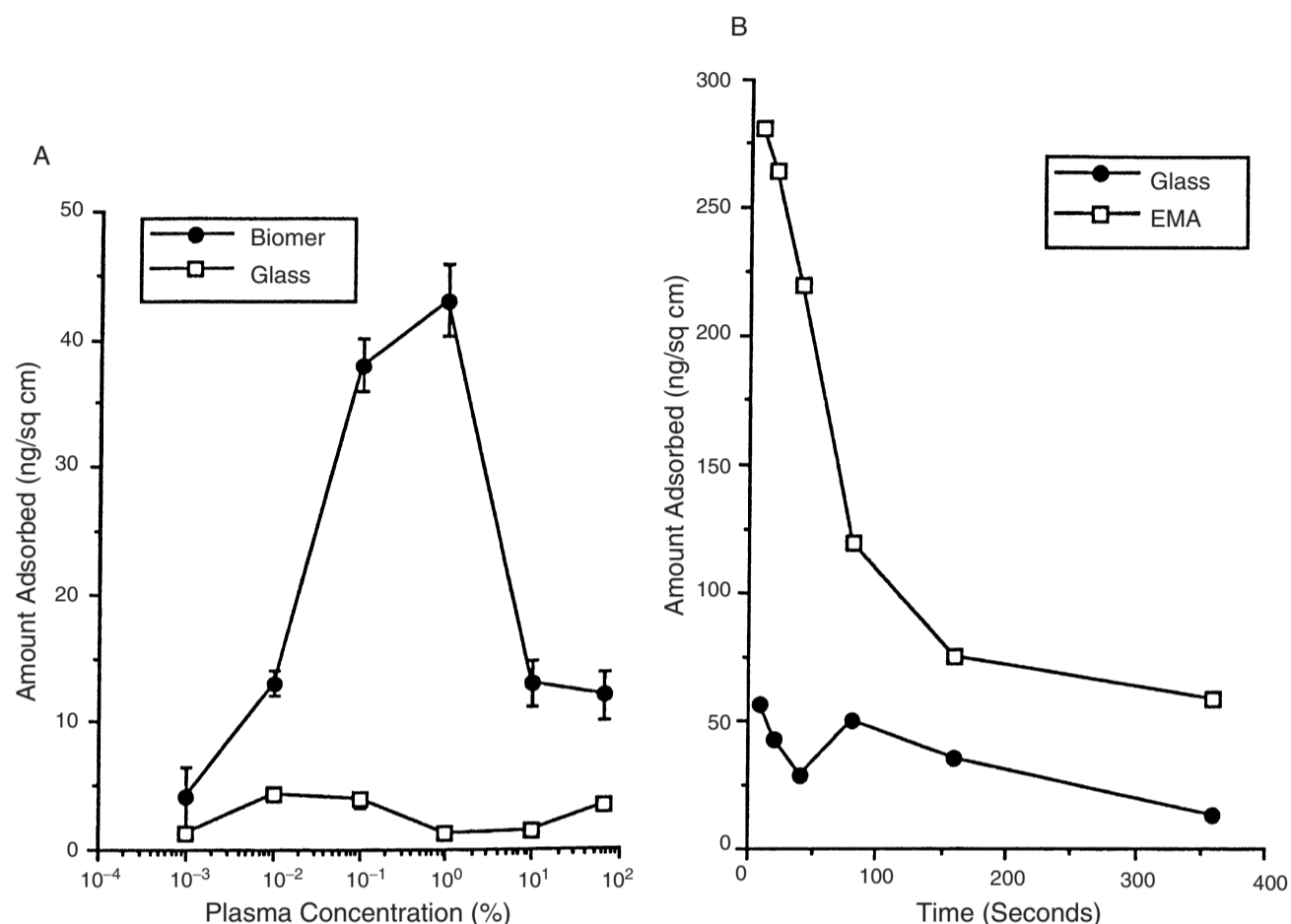


FIG. 9. The Vroman effect. (A) Fibrinogen adsorption to biomer and glass from various concentrations of blood plasma. (B) Time course of fibrinogen adsorption to glass and poly(ethyl methacrylate) (PEMA) from undiluted blood plasma. From: Fig. 1 in Slack and Horbett, (1995).

Physicochemical Studies of Conformational Changes

“Soft” proteins are found to adsorb more readily and more tenaciously than “hard” proteins. In this context, a “soft” protein is one with a low thermodynamic stability, whereas a “hard” protein is more stable to unfolding in solution in response to denaturing conditions such as elevated temperature. This concept and the articles supporting the following discussion are presented in detail elsewhere (Horbett and Brash, 1995).

Comparison of the adsorptive behavior of different proteins to their molecular properties indicates that less stable proteins are more adsorptive. The important role of structural stability in adsorption is also supported by recent studies with engineered mutant proteins with single amino acid substitutions that vary in stability. Lysozyme adsorption at the solid/liquid interface and tryptophan synthase occupation of the air/water interface are greater for less stable mutants.

Several studies with differential scanning calorimetry (DSC) methods seemed to indicate that adsorbed proteins may lose much of their structure, depending on how “soft” they are. Heat is taken up at a certain temperature for proteins in

solution due to unfolding of the native protein at the transition temperature. An absence or reduction of this effect for an adsorbed protein suggests that the adsorbed protein has already undergone the transition, i.e., that it has already unfolded upon adsorption. The transition enthalpy of lysozyme adsorbed to negatively charged polystyrene was much less than for the protein in solution (0–170 kJ/mol for the adsorbed protein versus about 600 kJ/mol for the native protein, depending on the pH). However, for lysozyme adsorbed on hematite, the unfolding enthalpy is only about 20% less than for the native protein, indicating that changes in the enthalpy of unfolding depend on the adsorbing surface. Furthermore, for lactalbumin the heat released is nearly zero when adsorbed on either the polystyrene or the hematite surface, suggesting complete unfolding of lactalbumin on both surfaces. These observations are consistent with the lower stability of lactalbumin in comparison to lysozyme. Several proteins adsorbed to pyrolytic carbon do not show any release of heat at the expected transition temperature, suggesting that pyrolytic carbon induces complete unfolding, a result that is consistent with the tenacious binding of proteins to this surface. It has also been shown that albumin and lysozyme adsorbed to polystyrene

exhibit no unfolding enthalpy, whereas lysozyme adsorbed to a hydrophilic contact lens still exhibits about 50% of the heat released by the native protein. Streptavidin adsorbed to polystyrene displays an unfolding enthalpy that is very similar to that for the native protein in solution, probably because of the greater stability of streptavidin in comparison to lysozyme or albumin.

However, more recent studies of adsorbed proteins by the DSC method in conjunction with other, more direct conformational measurements such as circular dichroism (CD) show that at least some adsorbed proteins that appear to be completely denatured as judged by DSC still have considerable amounts of their native structure as measured by CD. It thus appears that some proteins become somewhat more stable after adsorption, and thus do not show heat release at the normal melting temperature.

The concept of molecular spreading of the adsorbed protein suggested by these observations has been used to explain differences in the amount of IgG adsorbed during stepwise adsorption. When the final concentration of bulk protein is achieved in a series of smaller concentration steps, as opposed to bringing the bulk concentration to its final value in one step, adsorption is smaller. Conformational changes upon adsorption of fibronectin to polystyrene beads and Cytodex microcarrier beads have been detected using electron spin resonance spectroscopy. Many other physicochemical studies are consistent with partial unfolding of the adsorbed proteins (Andrade, 1985; Horbett and Brash, 1995; Lundstrom, 1985).

Biological Changes in Adsorbed Proteins

Although physicochemical studies sometimes suggest complete denaturation of adsorbed proteins, most probes for biological activity suggest the changes are more limited (reviewed with citations in Horbett, 1993). Thus, enzymes retain at least some of their activity in the adsorbed state, especially when the surface are more fully loaded with enzyme. Measurements of enzyme activity or retention times during passage over hydrophobic chromatography matrices have shown that the degree of denaturation is highly dependent on the protein, the length of time the protein has spent on the matrix, the solvent, and other conditions, and is not necessarily complete.

Changes in the binding of a monoclonal antibody to fragment D of fibrinogen upon fibrinogen adsorption to polystyrene have been attributed to changes in the conformation of fibrinogen after adsorption. Thus, solution-phase fibrinogen does not bind the antibody raised to fragment D, but the surface-adsorbed fibrinogen does, and furthermore, bulk fibrinogen does not compete for the binding of the antibody to the surface-adsorbed fibrinogen. The RIBS (receptor-induced binding site) antibodies are similar: They bind to fibrinogen only after the fibrinogen has bound to either a solid surface or to the platelet IIb/IIIa receptor. The binding of the RIBS antibodies and others that bind to the platelet-binding regions of fibrinogen varies with the length of time after adsorption of the fibrinogen. Platelet adhesion to polymethacrylates has been correlated with the amount of antifibrinogen binding,

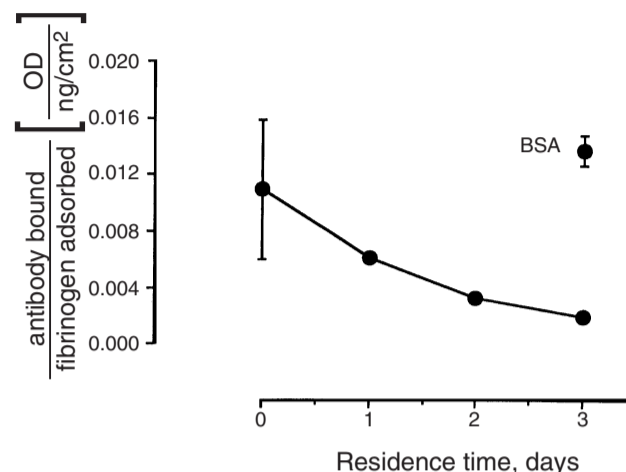


FIG. 10. Transitions in adsorbed fibrinogen. The effect of 3-day residence in buffer or buffered albumin solution upon anti-fibrinogen binding to fibrinogen adsorbed from dilute plasma to biomer is shown. From Fig. 3A in Chinn *et al.* (1992).

suggesting that the adsorbed fibrinogen is in different conformations on the various polymethacrylates.

Fibrinogen undergoes a time-dependent transition after its adsorption to a surface that results in reduced platelet and antibody binding to the adsorbed fibrinogen as well as reduction in the sodium dodecyl sulfate and plasma displaceability, and changes in amide II frequency of the adsorbed fibrinogen. The losses in platelet binding, antibody binding, and SDS elutability are prevented if albumin is included in the storage buffer. An example showing time-dependent losses in antibody binding to fibrinogen and its prevention by albumin is shown in Fig. 10 (from Chinn *et al.*, 1992). Vitronectin also appears to undergo conformational changes upon adsorption that affect its ability to bind heparin and its infrared spectra.

Modulation of the biologic activity of fibronectin has been shown in several studies in which the ability of fibronectin adsorbed to various surfaces to support cell attachment or spreading was found to differ. For example, fibronectin adsorbed to tissue-culture-grade polystyrene was able to support cell attachment and spreading, whereas fibronectin adsorbed to ordinary polystyrene does not support spreading very well unless some albumin is added to the fibronectin solution. Fibronectin adsorbed to self-assembled monolayer films (SAMs) with various functional end groups also varies. On hydrophobic SAMs there is poor cell spreading unless albumin is coadsorbed (albumin "rescuing"). The albumin "rescuing" phenomenon observed for SAMs is similar to the albumin effect on fibronectin's ability to promote cell spreading on polystyrene, and to the effect of albumin addition in preventing losses in platelet adhesion to fibrinogen adsorbed surfaces discussed above. The ability of fibronectin adsorbed to a series of polymers to support the outgrowth of corneal epithelial cells has also been found to vary a great deal, despite the presence of similar amounts of fibronectin on the surfaces.

The effect of albumin addition in enhancing the adhesivity of fibronectin-coated surfaces is opposite of what one

TABLE 1 Principles Underlying the Influence of Adsorbed Plasma Proteins on Platelet Interactions with Biomaterials

1. Synthetic foreign materials acquire bioreactivity only after first interacting with dissolved proteins. The principal means by which the transformation from an inert, nonthrombogenic polymer to a biologically active surface takes place is the interaction of the proteins with the surface that then mediates cell adhesion.
2. Platelets are a major example of why and how adsorbed proteins are influential in cell-biomaterials interactions.
3. Sensitivity of platelets to adsorbed proteins is due to:
 - a. Some proteins in plasma are strongly adhesive for platelets: fibrinogen, fibronectin, vitronectin, and von Willebrand factor.
 - b. Concentrating, localizing, immobilizing effects of adsorbing the proteins at the interface accentuates the receptor-adhesion protein interaction.
 - c. Platelets have receptors (IIb/IIIa and Ib/IX) that bind specifically to a few of the plasma proteins, mediating adhesion.
4. Principles of protein adsorption to biomaterials
 - a. Monolayer adsorption and consequent competition for available adsorption sites means that not all proteins in the plasma phase can be equally represented on the surface.
 - b. Driving forces for adsorption are the intrinsic surface activity and bulk phase concentration of the proteins.
 - c. Surfaces vary in selectivity of adsorption.
 - d. Biological activity of the adsorbed protein varies on different surfaces.

might expect, because the added albumin should reduce the amount of adsorbed fibronectin, as albumin competes for sites on the surfaces. The explanation for the albumin effect is thought to be that by adsorbing along with the fibronectin to the surface, the albumin molecules occupy surface sites near the fibronectin molecules. The adsorbed albumin molecules thus keep the adsorbed fibronectin molecules from undergoing structural changes that they would otherwise do in trying to spread into formerly empty surface sites but cannot do so if albumin molecules fill those sites.

The studies with platelets, fibroblasts, and epithelial cells show that substrate properties somehow modulate the ability of adsorbed proteins to interact with cells. These differences may arise at least in part from differences in the availability of epitopes on adhesive proteins for the cell surface receptor. That is, both the amount of the adsorbed adhesive protein and its “bioreactivity” are actively influenced by the properties of the surface to which it is adsorbed.

THE IMPORTANCE OF ADSORBED PROTEINS IN BIOMATERIALS

Table 1 summarizes the principles underlying the influence of adsorbed proteins in biomaterials used in contact with the blood. All of the principles listed also apply in other environments such as the extravascular spaces, albeit with other proteins and other cell types (e.g., macrophages in the peritoneum adhere via other receptors and other adhesion proteins). The platelets therefore provide a “case study,” and we close this chapter by considering this case.

The sensitivity of platelet/surface interactions to adsorbed proteins is fundamentally due to the presence of adhesion receptors in the platelet membrane that bind to certain plasma proteins. There are only a few types of proteins in plasma that are bound by the adhesion receptors. The selective adsorption of these proteins to synthetic surfaces, in competition with

the many nonadhesive proteins that also tend to adsorb, is thought to mediate platelet adhesion to these surfaces. However, since the dissolved, plasma-phase adhesion proteins do not bind to adhesion receptors unless the platelets are appropriately stimulated, whereas unstimulated platelets can adhere to adsorbed adhesion proteins, it appears that adsorption of proteins to surfaces accentuates and modulates the adhesion receptor/adhesion protein interaction. The type of surface to which the adhesion protein is adsorbed affects the ability of the protein to support platelet adhesion (Horbett, 1993). The principles that determine protein adsorption to biomaterials include monolayer adsorption, the intrinsic surface activity and bulk concentration of the protein, and the effect of different surfaces on the selectivity of adsorption and biologic activity of the adsorbed protein.

More generally, all proteins are known to have an inherent tendency to deposit very rapidly on surfaces as a tightly bound adsorbate that strongly influences subsequent interactions of many different types of cells with the surfaces. It is therefore thought that the particular properties of surfaces, as well as the specific properties of individual proteins, together determine the organization of the adsorbed protein layer, and that the nature of this layer in turn determines the cellular response to the adsorbed surfaces.

Bibliography

- Andrade, J. D. (1985). Principles of protein adsorption. in *Surface and Interfacial Aspects of Biomedical Polymers*, J. D. Andrade, ed. Plenum Press, New York, pp. 1–80.
- Arnebrandt, T., and Wahlgen, M. (1995). Protein–surfactant interactions at solid surfaces in *Proteins at Interfaces II: Fundamentals and Applications*, ACS Symposium Series 602, T. A. Horbett and J. Brash, eds. American Chemical Society, Washington, D.C., pp. 239–254.
- Chinn, J. A., Horbett, T. A., and Ratner, B. D. (1991). Baboon fibrinogen adsorption and platelet adhesion to polymeric materials. *Thromb. Haemostas.* 65: 608–617.

- Chinn, J. A., Posso, S. E., Horbett, T. A. and Ratner, B. D. (1992). Post-adsorptive transitions in fibrinogen adsorbed to polyurethanes: changes in antibody binding and sodium dodecylsulfate elutability. *J. Biomed. Mater. Res.* **26**: 757–778.
- Horbett, T. A. (1993). Principles underlying the role of adsorbed plasma proteins in blood interactions with foreign materials. *Cardiovasc. Pathol.* **2**: 137S–148S.
- Horbett, T. A. (1994). The role of adsorbed proteins in animal cell adhesion. *Colloid Surf. B: Biointerfaces* **2**: 225–240.
- Horbett, T. A. (1996). Proteins: structure, properties, and adsorption to surfaces. in *Biomaterials Science*, B. D. Ratner, A. S. Hoffman, F. Schoen, and J. E. Lemons, eds. Academic Press, San Diego, pp. 133–141.
- Horbett, T. A. (1999). The role of adsorbed adhesion proteins in cellular recognition of biomaterials. *BMES Bull.* **23**: 5–9.
- Horbett, T. A., and Brash, J. L. (1995). Proteins at interfaces: an overview. in *Proteins at Interfaces II: Fundamentals and Applications*, ACS Symposium Series 602, T. A. Horbett and J. Brash, eds. American Chemical Society, Washington, D.C., pp. 1–25.
- Lundstrom, I. (1985). Models of protein adsorption on solid surfaces. *Prog. Colloid Polymer Sci.* **70**: 76–82.
- Slack, S. M., and Horbett, T. A. (1995). The Vroman effect: a critical review. in *Proteins at Interfaces II: Fundamentals and Applications*, ACS Symposium Series 602, T. A. Horbett and J. Brash, eds. American Chemical Society, Washington, D.C., pp. 112–128.
- Tsai, W.-B., and Horbett, T. A. (1999). The role of fibronectin in platelet adhesion to plasma preadsorbed polystyrene. *J. Biomater. Sci. Polymer Ed.* **10**: 163–181.

3.3 CELLS AND CELL INJURY

Richard N. Mitchell and Frederick J. Schoen

Composed of nucleic acids, proteins, and other large and small molecules, cells constitute the basic structural building blocks of all living matter. They are held together by cell-to-cell junctions to form tissues comprising four general types: epithelium, connective tissue, muscle, and nerve. Organs are assembled from these basic tissues, “glued” together by a largely proteinaceous extracellular matrix (ECM) synthesized by the individual cells. The organs, in turn, perform the various functions required by the intact living organism, including circulation, respiration, digestion, excretion, movement, and reproduction.

A major goal in this and the following chapter is to describe how biological structure is adapted to perform physiologic function. This general and overarching concept extends from cells (and their subcellular constituents) to the organization of tissues and of organs. Beginning with the smallest subunits of cellular organization we will build to progressively more complex systems. In the following chapter, we will extend the concepts of structure–function correlation beyond cells to include extracellular matrix and complex tissues, and will also describe the technologies by which histologists and pathologists examine normal and abnormal tissues. In these chapters, we also provide an introduction to the physiologic responses to environmental stimuli, the mechanisms of cell injury, cell–materials interactions, and the methodologies by which these are all studied.

In this chapter on cells and cell injury and in the following chapter on tissues and the extracellular matrix, we will highlight the following fundamental concepts:

Cells and cell injury:

- General characteristics and functions of cells
- Compartmentalization of regionally specialized function by membranes
- Cellular specialization to facilitate unique functions
- Regulation and coordination of cell function
- The response of cells to injury, including mechanisms of cell death

Tissues and the extracellular matrix:

- The structure and function of the extracellular matrix
- Grouping of cells into tissues
- Integration of tissues into organs
- Remodeling of the extracellular matrix
- The interaction of cells, tissues, and foreign materials
- Basic methods used to study cells and tissues

NORMAL CELL HOUSEKEEPING

In very broad strokes, we will first outline the general organization of a prototypical cell, using it to identify the functional considerations required for maintaining a living cell. We will then revisit each of these structural features to illustrate important concepts in cellular maintenance and response to environment.

Conceptually, cells may be viewed as independent collections of self-replicating enzymes and structural proteins that carry out certain general functions. The most essential cell attributes are:

- Self-replication
- Protection from the environment
- Acquisition of nutrients
- Movement
- Communication
- Catabolism of extrinsic molecules
- Degradation and renewal of senescent intrinsic molecules
- Energy generation

Intracellular constituents exist in a microcosm of water, ions, sugars, and small-molecular-weight molecules called the cytosol or cytoplasm. Within the cytosol is also a source of energy, typically adenosine triphosphate (ATP). Although long conceptualized as a randomly diffusing bag of soluble molecules, the cell is, in fact, a structurally highly ordered and functionally integrated assembly of organelles, cytoskeletal elements, and enzymes.

The cytosol is delimited and protected from the environment by a phospholipid bilayer, the plasma membrane, which permits the cell to maintain cytosolic constituents at concentrations different from those in the surrounding environment. Because of its hydrophobic inner core, the plasma membrane is impermeant to charged and/or large polar molecules; however, it is rendered selectively permeable (i.e., permits