1

Protein Adsorption to Biomaterials

David Richard Schmidt, Heather Waldeck, and Weiyuan John Kao,

Within milliseconds after biomaterials come in contact with a biological fluid such as blood, proteins begin to adhere to the surface through a process known as protein adsorption. Protein adsorption is initially strongly influenced by protein diffusion, but protein affinity for the surface becomes critically important and, over time, higher-affinity proteins can be replaced by lower-affinity proteins in a dynamic process. By the time cells arrive, the material surface has already been coated in a monolayer of proteins; hence, the host cells do not "see" the material but "see" instead a dynamic layer of proteins. Multiple parameters influence protein adsorption to a substrate surface including the chemical and physical properties of both the protein and the material surface, as well as the presence of other proteins on the surface.

Many methods have been developed in the last several decades to study protein adsorption to biomaterial surfaces. These new techniques provide information about the type and conformation of adsorbed proteins from multicomponent solutions such as blood serum. Nanomaterials as well as functional group immobilization and novel, stimuli-sensitive polymer surfaces have provided new alternatives for the study and modulation of protein adsorption, with insight into the mechanisms underlying protein adsorption and subsequent cell adhesion. However, a molecular-level understanding of all aspects of protein adsorption is still incomplete. The future of this field, however, is bright as new technologies offer great promise for further elucidation of protein adsorption.

Abbreviations

AFM	Atomic force microscopy
ATR-FTIR	Attenuated total reflectance-Fourier transform infrared spectroscopy
ELISA	Enzyme-linked immunosorbent assay
FTIR	Fourier transform infrared spectroscopy
HA	Hydroxyapatite
IR	Infrared

D.R. Schmidt, H. Waldeck, and W.J. Kao • School of Pharmacy, University of Wisconsin-Madison, Madison, WI, 53705, USA; Department of Biomedical Engineering, College of Engineering, University of Wisconsin-Madison, Madison, WI, 53705, USA

D.A. Puleo and R. Bizios (eds.), *Biological Interactions on Materials Surfaces*,
DOI 10.1007/978-0-387-98161-1_1,

MALDI-ToF/MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
PEG	Polyethylene glycol
PEO	Polyethylene oxide
pI	Isoelectric point
PLGA	Poly(lactic-*co*-glycolic acid)
PLLA	Poly(L-lactic acid)
PNIPAAm	Poly(*N*-isopropylacrylamide)
RGD	Arginine–glycine–aspartic acid
SAM	Self-assembled monolayer
SEIRA	Surface-enhanced infrared absorption
SEM	Scanning electron microscopy
SPR	Surface plasmon resonance
STM	Scanning tunneling microscopy
ToF-SIMS	Time-of-flight secondary ion mass spectrometry
XPS	X-ray photoelectron spectroscopy
2D	Two dimensional
3D	Three dimensional

1.1. Introduction

The host response to a biomaterial is critical in the determination of the success of biomedical implants. This response is in turn dictated by the function of cells that respond to the implant; however, one of the most important aspects of such events is that the cells of the body do not interact directly with the implant material itself but with proteins bound to the surface of the implant material. Almost immediately upon coming into contact with blood, a biomedical implant is coated with serum proteins in a process known as protein adsorption. "Adsorption" means adherence of a molecule to the surface of a solid and should not be confused with the term "absorption," which indicates that the molecule is brought into the solid. Consequently, once cells finally get to the surface they no longer "see" the biomaterial surface itself but instead "see" a dynamic coating of proteins [1–3].

The nature and activity (that is, the ability to interact with cells and other biological molecules) of these proteins once they are adsorbed dictates the initial cellular, and subsequent, host responses. Cells interact with proteins through direct binding to receptors on the cell membrane but do not have receptors for a material surface alone without a coating of proteins. Consequently, the presence of proteins allows cells to adhere to surfaces; the presence of increased amounts of protein may lead to increased cell adhesion, but this is not always the case [1–3].

In subsequent sections of this chapter, the underlying concepts of protein adsorption will be discussed in greater detail, followed by a presentation of more recent discoveries in the field of protein adsorption and future directions for this critical area for biomaterials.

1.2. Fundamentals of Protein Adsorption

1.2.1. General Concepts

Before addressing the underlying properties that influence protein adsorption at solid surface interfaces and specific protein examples, it is important to discuss the concepts relating to protein adsorption in some detail. To begin with, protein adsorption to a biomaterial

is influenced by the bulk concentration of the protein in solution. The practical application of this concept is that higher concentration of a single protein in a liquid solution surrounding a material generally leads to more protein on the material surface. However, this phenomenon gets considerably more complicated when more than one type of protein is present in solution because, in this case, more than one protein becomes involved. This phenomenon will be discussed later in the section "The Monolayer Model and Protein–Protein Interactions". Another important aspect in protein/material interactions is the movement of the protein in solution, which is principally governed by the rate of diffusion. In turn, the rate of diffusion is dictated primarily by the size of the protein. Faster (that is, smaller) proteins tend to get to the surface first and adsorb onto that surface before slower moving (that is, bulkier/bigger) proteins arrive.

Yet another important aspect, and perhaps the most important one for long-term considerations, is the affinity of a protein for the substrate surface. Affinity of a protein for a surface describes how likely that protein will adsorb and how strongly it will adhere to the substrate surface. Compared with proteins with a lower affinity, proteins with a higher affinity for a material are more likely to adsorb and stay on the substrate surface. This interaction can vary tremendously depending on the protein and the material surface. Proteins that adsorb to a material form a number of bonds on that surface; proteins with high affinity have more and/or stronger bonds while proteins with low affinity tend to have weaker and/or a smaller number of such bonds [1, 2, 4].

Taking these three aspects together, namely, protein concentration, rate of diffusion, and affinity, one can come to the conclusion that, in a multicomponent solution, proteins are in competition for a surface. Arriving at the surface first, however, does not necessarily mean that a protein will stay there permanently. Proteins adsorbed on a material surface replace each other over time in a process called the Vroman effect, named for the well-known scientist (also poet and painter) who initially discovered and studied this phenomenon. The bonds between a protein and a material surface established during protein adsorption are not completely static; they can be broken and reformed randomly over and over. However, a single protein is highly unlikely to desorb (the process opposite of adsorption) off a material surface because that event requires that all bonds between the protein and surface are broken simultaneously. In contrast, another protein may form bonds with the sites that have become available as bonds are broken between an already adsorbed protein and the material surface. If the new protein has higher affinity with surface sites, this protein will take over the specific site from the lower-affinity protein (Figure 1.1). Over time, the higher-affinity protein can replace the previously preadsorbed, lower-affinity protein; thus, a dynamic protein layer that changes over time is formed on the material surface [1, 2, 4].

1.2.2. Protein Properties

Overview of Relevant Protein Properties

Once a protein arrives at a material surface, it interacts with that surface through intramolecular bonds, such as hydrophobic interactions (nonpolar domains of the protein molecule avoid polar regions of the material surface or vice versa in a process referred to as the hydrophobic effect); ionic bonds (bonds between positive and negative charges); and charge transfer (a stabilizing charge is transferred between two molecules). The prevalence of these types of bonds between proteins and material surfaces is strongly influenced by the properties of individual proteins, which are summarized in Table 1.1. Protein size influences protein adsorption because it affects the rate of protein diffusion; the size of a protein molecule also partially determines the affinity of the protein. Larger proteins may have more binding sites for interaction with regions on the material surface and thus they may adsorb readily.

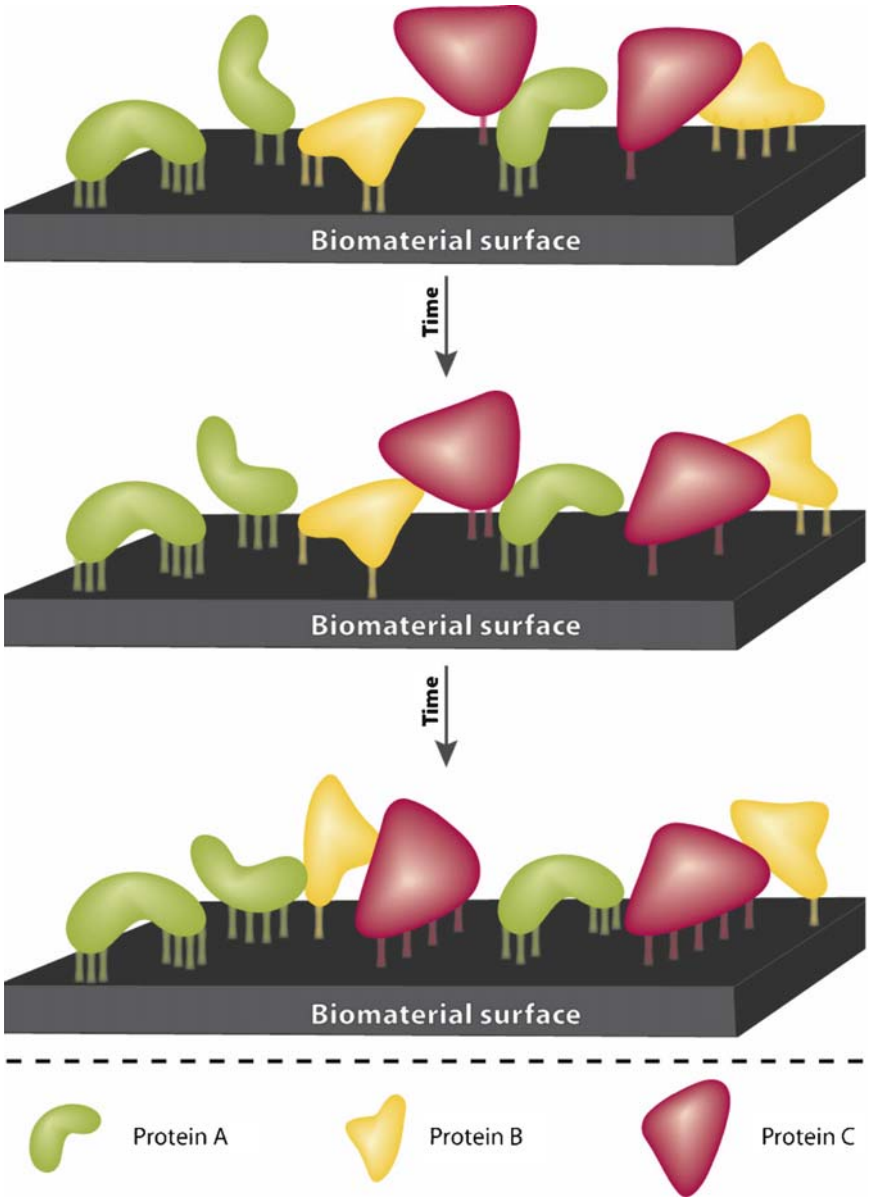

Figure 1.1. Schematic of the sequential adsorption of proteins as described by the Vroman effect. Initially, many protein molecules in various conformations are adsorbed onto the biomaterial surface. On the left portion of all three frames are two proteins A (green) in different conformations, which change over time. In the center of the figure, different proteins B (yellow) with multiple bonds are replaced over time by a larger, higher-affinity protein C (red) that arrived later on the substrate surface.

A number of other factors come into play when considering the underlying properties of the amino acid constituents of proteins. Since proteins consist of sequences of amino acids, their properties affect the adsorption properties of protein molecules. Amino acids may be charged (depending on the pH of their environment), and thus be more polar;

Table 1.1. Protein properties that affect protein adsorption on material substrates [1, 2] (adapted from [1]).

Protein property	Description
Hydrophilicity/hydrophobicity	Since more hydrophilic side chains are present on the outside of a protein molecule in aqueous media, they may interact with substrate surfaces. Polar (hydrophilic) domains tend to adsorb to polar material surfaces, while hydrophobic domains tend to adsorb to hydrophobic material surfaces
Size	In a multicomponent solution, smaller proteins diffuse more quickly and arrive at the substrate surface faster. Smaller proteins, however, form fewer contact points with the material surface than larger proteins
Charge	Charged proteins preferentially adsorb on substrate surfaces of the opposite charge. On most surfaces, however, proteins adsorb the most at their p*I* where they are at neutral charge
Structural stability/rigidity	Proteins that are less structurally stable exhibit greater unfolding upon adsorption onto a material surface and form more contact points/bonds. Proteins that unfold quickly form bonds with the surface before other proteins from a multicomponent solution arrive at that surface

such amino acids are more hydrophilic because they are attracted to the polar water molecules in their environment. These charged hydrophilic amino acids then have a tendency to be on the outside of a protein and thus can interact preferentially with the polar regions of the material surface. Positively charged domains preferentially adsorb to negatively charged surface regions and vice versa; however, this scheme of interactions is not the dominant process for adsorption because many other aspects influence the protein adsorption process. Generally, proteins tend to adsorb more readily near their isoelectric point (p*I*; that is, when the environmental pH is such that the protein has no net charge). This adsorption event was theorized to be due to reduced electrostatic repulsion with other proteins on the material surface. Hydrophilic domains tend to preferentially interact with hydrophilic surface regions and vice versa. Hydrophobic domains tend to be buried within the three-dimensional (3D) structure when proteins are in solution; however, these domains may become exposed during unfolding while the protein interacts with a material surface. Depending on the extent of conformation of the protein, pertinent amino acid sequences may not be completely hidden within the protein structure [1, 2, 4].

In addition to specific amino acid sequences, proteins have shapes and structures that may also influence their adsorption on material surfaces. “Soft” proteins, that is, proteins with lower than average thermodynamic stability, less internal bonding, and/or crosslinks (such as disulfide bonds), tend to adsorb more easily than “hard” proteins, which have a higher level of thermodynamic stability compared with “soft” proteins. “Relatively” softer proteins tend to adsorb strongly to surfaces due to the easier unfolding of protein molecules that have lower levels of thermodynamic stability [1, 2]. As a protein unfolds, more domains may be revealed and be available for subsequent interactions with the material surface. The structure of a protein may be crucial to its adsorption because specific binding sites may only be exposed when the protein molecules are in a specific conformation. A protein with specific activity in bulk solution may lose its activity once it undergoes a conformational change

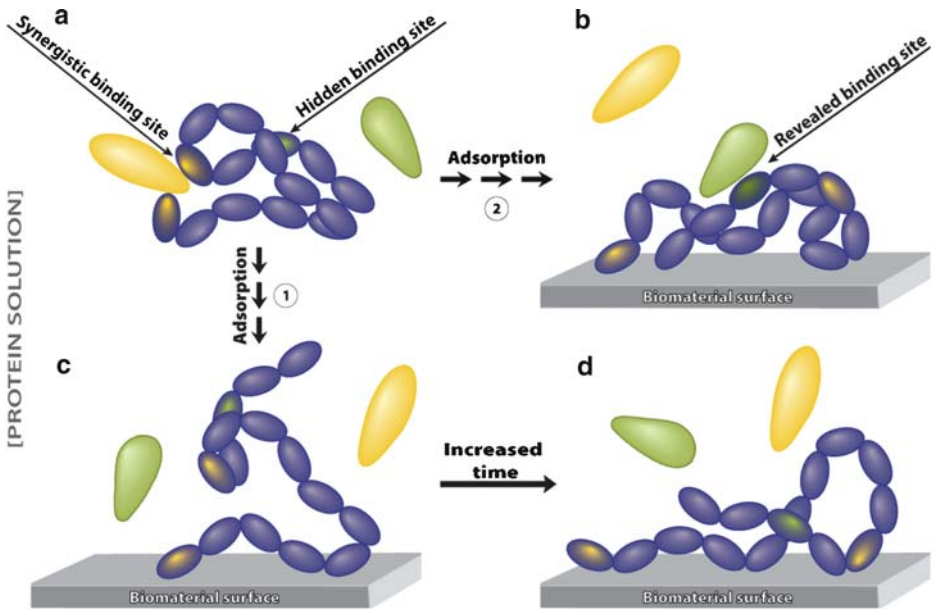

Figure 1.2. Schematic depiction of protein conformational changes upon adsorption of proteins on material surfaces. The protein in this illustration has a binding site that requires a specific structure of two regions of the molecule (A). Once the protein is adsorbed, these conformational epitopes are no longer functional because the two regions are far apart (C). Alternatively, the protein may have a hidden biding site that is revealed but becomes available for binding to another molecule once the protein has unfolded upon adsorption on the material surface (B). Over time, the adsorbed protein may continue to unfold, thereby exposing additional binding sites (D).

upon adsorption to a material surface; pertinent protein unfolding and changes in protein activity may occur over some period of time (Figure 1.2) [1, 2, 4].

The Monolayer Model and Protein–Protein Interactions

Adsorption of a specific protein on a substrate surface is also affected by the presence of other proteins in the solution. Excepting coagulation, most blood serum proteins do not adhere to one another on a surface. Exceptions to this rule occur because the conformation of the adsorbed protein may expose new binding sites for subsequent protein–protein interactions; this process is hypothesized to occur when extracellular matrix proteins adsorb on material surfaces. Since most proteins do not adsorb in a conformationally favorable fashion to mediate subsequent protein–protein binding, protein–protein bonds reflect a small percentage of the total protein layer compared with protein–surface bonds. Consequently, monolayers and near-monolayers are the rule in protein adsorption on material surfaces [1, 2, 4, 5].

The presence of other proteins on a material surface may induce lateral interactions between adsorbed protein molecules. Many proteins (such as enzymes) have specific binding sites that need to be accessible for effective function; moreover, the presence of other proteins on the material surface can sterically hinder target proteins from interacting with their respective binding sites and thus affect bioactive function. Additionally, proteins adsorbing

on a material surface may unfold and thus expose previously hidden bioactive epitopes; however, the presence of other proteins on a material surface can sterically contain this unfolding process, rendering respective epitopes unavailable for binding. Lateral protein interactions become more apparent in situations involving increases from a lower to greater concentration of protein in the bulk solution; in this case, an increased number of proteins on the material surface may interact with one another [1, 2, 4, 5].

1.2.3. Surface Properties

Overview of Material Surface Properties Relevant to Protein Adsorption

The properties of individual proteins are important in protein adsorption, but are only half the story. The material surface itself is another determinant of the quantity, type, conformation, and function of proteins that preferentially adsorb there. Material properties of surfaces that affect protein adsorption are listed in Table 1.2. It should be noted that, even before proteins adsorb onto a material surface, water molecules from the aqueous biological environment interact with the material. In the case of hydrophobic materials, a "shell" of water molecules forms in which these molecules interact with each other more than with the hydrophobic surface. One hypothesis postulates that these surface-surrounding water molecules represent a fairly ordered scenario with a decreased level of entropy; disruption of this layer with proteins is energetically favorable due to a concomitant increase in entropy. This increase in entropy is the primary motivating force behind protein adsorption to material surfaces that are hydrophobic. Consequently, enhanced protein adsorption and conformational change are observed on hydrophobic surfaces. Greater conformational change may also lead to a greater loss of protein activity in cases that require the protein to be in its native state. In contrast, since more water molecules can form hydrogen bonds directly on hydrophilic surfaces, competition between water molecules and proteins results in decreased protein adsorption and conformational change. Moreover, material surfaces may have a distribution of charge. Since like charges tend to repel one another, opposite charges promote adsorption and ionic bonding of proteins on such substrates. For example, since the majority of blood serum proteins are negatively charged, a net negative charge on a material surface may reduce adsorption of these proteins. This outcome, however, is complicated because proteins contain amino acids that are themselves either positively or negative charged; for this reason, even if a protein is net negatively charged, there may still be positively charged domains on that molecule.

Table 1.2. Surface properties that affect protein adsorption on material substrates [1, 2] (adapted from [1]).

Surface property	Description
Hydrophilicity/hydrophobicity	Hydrophobic surfaces tend to adsorb more proteins, while hydrophilic surfaces tend to resist protein adsorption
Charge	Opposite charges between the surface and protein promote increased protein adsorption, while like charges tend to reduce protein adsorption
Topography	Increased surface roughness and topological features provide increased material surface area for protein adsorption
Chemistry	The chemical composition of a substrate surface dictates the types of bonds between protein and material surface

Topographical features on a biomaterial surface may also strongly influence protein adsorption. Increased surface roughness provides more surface area for protein adsorption onto the biomaterial surface; this event may lead to a net increase in protein adsorption. Such protein adsorption outcomes may be achieved either intentionally (by incorporating material surface physical modifications such as grooves, etc., during manufacturing) or unintentionally (through creation of surface defects and flaws during the manufacturing processes). Surface defects of micrometer size can accommodate thousands to millions of blood serum proteins [1, 2, 6, 7].

Effect of Material Surface Functional Groups on Protein Adsorption

The chemical properties of a material surface can strongly influence adsorption of proteins and, through these proteins, subsequent adhesion of various cell types. Material surface chemistry and topography affect cell functions via the conformation and bioactivity of the adsorbed proteins. Examples of material surface functional groups and their observed effects on proteins and cells are listed in Table 1.3. It should be noted though that these are generalized observations and may vary depending on experimental conditions and the presence of a multicomponent protein solution. Since "protein surface concentration" is not the same as "protein surface activity," higher protein concentration at the material surface does not necessarily mean that these surfaces are more bioactive [8].

The influence of a number of surface functional groups on protein adsorption has been studied. Methyl ($–CH_3$), nonpolar, hydrophobic groups tightly bind fibrinogen (a protein involved in blood clotting), and immunoglobin IgG, a family of antibody proteins involved in the immune response. *In vivo*, the $–CH_3$ group induces high recruitment of inflammatory cells to the material surface, likely due to the high adsorption of IgG on material surfaces containing $–CH_3$. Hydroxyl groups –OH increase the hydrophilicity of a material surface and, thus, reduce the affinity of plasma proteins there. The –OH group induces changes in the conformation of fibronectin, thus exposing adhesive domains for cell "focal" (tight mechanical links) adhesion. Amine groups ($–NH_2$) are polar and, thus, hydrophilic; these groups are also

Table 1.3. The effect of material surface functional groups on proteins and cells (adapted from [8]).

Functional group	Properties	Effect on proteins and cells
$–CH_3$	Neutral; hydrophobic	Has high affinity/binding with fibrinogen; binds strongly with IgG; promotes increased leukocyte adhesion and phagocyte migration
–OH	Neutral; hydrophilic	Has decreased affinity for plasma proteins; induces exposure of cell adhesive domains on fibronectin; increases differentiation of osteoblasts
$–NH_2$	Positive; hydrophilic	Has high affinity for fibronectin; promotes increased myoblast proliferation; promotes differentiation of osteoblasts; promotes increased endothelial cell proliferation
–COOH	Negative; hydrophilic	Has increased affinity for fibronectin and albumin

Note: These are generalized observations and may vary depending on experimental conditions and the presence of other proteins in solution.

positively charged in blood serum and most other solutions in which the solvent is water. The $–NH_2$ group binds strongly to fibronectin and induces several responses from various cells (Table 1.3). Moreover, $–NH_2$ also triggers acute inflammatory reactions *in vivo*. Carboxyl (–COOH) groups are negatively charged in blood serum and other aqueous protein solutions, are hydrophilic, and interact preferentially with fibronectin and albumin [8, 9].

Multiple functional groups, such as the ones mentioned earlier in this section, can be found on the same material surface. In the case of mixed –OH and $–CH_3$ groups, hydrophobic and hydrophilic influences are effectively in competition with each other. In this case, as the surface concentration of –OH groups is increased, protein adsorption decreases (likely due to the increased hydrophilicity associated with increased –OH groups) [9]. In the case of mixed $–NH_2$ and –COOH functional groups, platelet adhesion was the lowest when these two functional groups were mixed in equal molar fractions making the net charge of the surface approximately neutral [8, 9]. It should be noted that, over time, the presence of water and other molecules in the surrounding environment may modify these material surface groups [8, 9].

Nonfouling Surfaces

While hydrophobic surfaces promote increased protein adsorption, highly hydrophilic surfaces are characterized by decreased protein adsorption. The phenomenon of decreased protein adsorption is known as nonfouling. Since serum protein adsorption on the surfaces of cardiovascular biomaterials is associated with blood clotting and inflammatory/immunological responses, a strategy in biomaterials research and applications is to use materials that inhibit serum protein adsorption. Although polysaccharides can also be used for such applications, by far the most widely used material is polyethylene glycol (PEG; also known as polyethylene oxide [PEO] depending on its molecular weight), as well as its derivatives and copolymers. PEG, a very hydrophilic material surface, was chosen and is used in numerous drug delivery, biomaterials, and tissue engineering applications primarily because of its low level of protein adsorption. The unique ability of PEG to greatly limit protein adsorption is due to the highly hydrophilic nature of its polymer coils. Once PEG comes into contact with water, these hydrophilic coils become surrounded by water molecules and, thus, get highly hydrated. When a protein arrives at the PEG surface, the adsorption process requires compression of these hydrated coils. Since this process requires energy (because water molecules must be removed), it is thermodynamically unfavorable. However, some small amount of protein still adsorbs and, given enough exposure time, protein adsorption may occur, but to a smaller degree [10, 11].

A recent example of protein adsorption research on polyethylene glycol involved the use of a plasma deposited PEG-like polymer called tetraethylene glycol dimethyl ether (tetraglyme $[CH_3O(CH_2CH_2O)_4CH_3]$). Tetraglyme-coated surfaces resist protein adsorption *in vitro*; these surfaces, however, still induced a foreign body reaction *in vivo*, most probably due to monocyte (a critical inflammatory/immune leukocyte) activation on the tetraglyme surface. Although resistant to other serum proteins, the blood complement factor C3 adsorbs to tetraglyme in significant amounts (approximately 10 ng/cm^2) [12]. Monocytes may interact with, and bind to, complement factors in the blood. Monocyte adhesion to tetraglyme was considerably reduced when C3-depleted serum (instead of normal serum) was used, suggesting that monocyte binding to tetraglyme is primarily mediated by monocytes binding to adsorbed C3. This result provides an explanation for the reported foreign body reaction to PEG-coated surfaces *in vivo* because significant C3 adsorption in such cases may be inducing adhesion and subsequent activation of monocytes [12]. In certain cases, monocyte adhesion to PEG hydrogels is comparable to that observed *in vitro* on tissue culture polystyrene culture plates, potentially due to the same C3-related mechanism [13].

1.2.4. Environmental Effects

The environment in which protein adsorption occurs can also affect the extent of, and conformation during, such interactions. Temperature can have a significant impact on protein adsorption; temperatures significantly above room temperature (for example, 80°C) can increase the amount of protein that is adsorbed. However, very high temperatures may lead to denaturation of proteins and loss of important bioactivity. The temperature at which temperature-driven denaturation of proteins (and thus changes in bioactivity and adsorption) occurs can vary considerably for different types of proteins due to differences in protein stability. Temperature-driven denaturation of proteins, particularly those with cysteine residues, can lead to protein aggregation on material surfaces. Since many published studies on protein adsorption were conducted at room temperature (22°C), for this reason, the difference between room and *in vivo* temperature (37°C) should be considered when analyzing the results of studies on protein adsorption.

Environmental pH can also affect protein adsorption because changes in the charge of both the material surface and the protein molecule may lead to variations in electrostatic interactions. Adsorption of a single protein is the highest at the p*I* but is reduced at other pH conditions. Decreases in protein adsorption due to either increases or decreases in the pH away from the p*I* lead to increased repulsion between like-charged protein molecules.

Lastly, the presence of detergents/surfactants (that is, molecules that are both hydrophilic and hydrophobic) can alter protein adsorption to a material surface by competing with proteins for adsorption sites or by binding directly to proteins. Adsorption of proteins in the presence of surfactants has not been well studied yet [4, 14].

1.2.5. Adsorption of Serum Proteins

There are more than 150 proteins in human blood serum; many of them have been used in studies of protein adsorption. These studies provided information regarding the sequence of protein adsorption on many material surfaces including glass, metal oxide

Table 1.4. Select human blood serum proteins and their main biological functions.

Blood serum proteins	Concentration in normal human blood (mg/ml)	Major function
Albumin	40	Maintains osmotic pressure; forms complexes with, and thus "carries," other serum molecules
Complement C3	1.6	Participates in complement system activation and function
Hemoglobin	0.01	Transports oxygen
High molecular weight kininogen	0.08	Participates in blood clotting
Immunoglobin (IgG)	15	Participates in the immune response
Fibrinogen	3	Participates in blood clotting
Fibronectin	0.3–0.4	Mediates cell adhesion
Factor XII	0.03	Participates in blood clotting
Vitronectin	0.3	Mediates cell adhesion

surfaces, and polymers such as polyethylene. The most widely studied serum proteins in protein adsorption studies are listed in Table 1.4. Pertinent studies reported in the literature provided evidence that serum protein adsorption on glass follows the sequence: albumin first, followed by IgG, fibrinogen, fibronectin, Factor XII, and high molecular weight kininogen. Since albumin is a small protein, but is present in high concentration in serum, it tends to adsorb first on material substrate surfaces. Albumin, however, has a relatively low affinity and so, over time, it is partially replaced by larger, higher-affinity proteins such as fibrinogen [1, 7].

1.3. Techniques for the Study of Protein Adsorption

Numerous techniques exist for the study of protein adsorption and for the characterization of biomaterial properties pertinent to such processes. This section will focus exclusively on techniques that directly monitor either adsorbing proteins or the adsorbed protein layer on material surfaces. Techniques that indirectly lead to information about protein adsorption (for example measuring and comparing the hydrophobicity of one surface versus another) may allow predictions of relative protein adsorption on those material surfaces but will not be covered in this section. Several of the more important techniques for the analysis of protein adsorption will be highlighted for discussion, and powerful emerging techniques will also be discussed in this section.

1.3.1. Established Techniques for the Study of Protein Adsorption

Various aspects of protein adsorption, including kinetics, thickness of the protein layer, conformation of adsorbed proteins, identity of adsorbed proteins, structure of the adsorbed protein layer, and/or the types of forces operating between the protein and the surface, have been the focus of research activities that either developed new or adapted existing methodologies for such studies. In the study of the chemical composition of adsorbed protein layers, spectroscopy/spectrometry methods have been very useful. Auger electron spectroscopy did not provide useful information on adsorbed proteins because this technique is very destructive to organic matter. On the other hand, X-ray photoelectron spectroscopy (XPS) has been used extensively in protein adsorption studies. In XPS, when a material surface is irradiated by a beam of monochromatic X-rays, photoelectrons are released and are then captured by a detector and analyzed. XPS provides detailed elemental (but not molecular) composition analysis, is relatively nondestructive, and is very useful for single-protein studies; however, this method is very limited for analysis of multicomponent solutions due to its inability to resolve individual proteins in an overly complex spectrum. Secondary ion mass spectrometry (SIMS) and time-of-flight (ToF)-SIMS have become widely used in studies of protein adsorption. ToF-SIMS offers significant advantages in protein resolution over SIMS. In both SIMS and ToF-SIMS, bombardment of the adsorbed protein layer with a focused beam of either ions or atoms results in the emission of secondary particles, which can then be analyzed to provide elemental and molecular information about the material surface. ToF-SIMS is similar to SIMS but includes an additional step. In this case, the secondary ion particles are accelerated through a field-free drift region; heavier ions travel more slowly than lighter ions, allowing for greater separation and increased sensitivity of detection of the ToF-SIMS over the SIMS technique [15–18].

Infrared (IR) spectroscopy has also been used to examine protein adsorption. An IR beam is reflected from a sample and a spectrum can be developed from the IR absorbance to different components of the adsorbed protein. Different chemical bonds have differing vibrational frequencies and thus absorb the IR beam at different frequencies; this property allows for the generation of an IR spectrum "fingerprint," which provides information about the chemical bonds, which, in turn, provide information about the protein conformation and load. IR has limited use in analyzing multicomponent protein solutions because the spectra in these cases can become very complicated and difficult to resolve.

Fourier transform infrared spectroscopy (FTIR) allows for the measurement of all wavelengths at once (rather than one at a time). A more advanced method called attenuated total reflectance (ATR)-FTIR uses total internal reflection of the IR beam back into the detector along the region of contact between the sample and the device. By combining IR with ATR, it is possible to greatly reduce the area of the material surface needed for analysis. Both techniques provide conformational and submolecular information about adsorbed proteins. IR spectroscopy can be used to analyze activity shifts in proteins by measuring the IR spectrum of a protein in one state and then subtracting this IR spectrum from another one obtained in a different (for example, active) state of the same protein, or, in the case of an enzyme, when it acts as a reaction intermediate. This spectrum difference between the two states contains the respective vibrational bands. An additional advantage of this technique is that since all redundant vibrational bands cancel out, the information thus obtained relates to the change in protein functionality only [15, 16, 18–20].

One of the oldest, and most popular, techniques for studying protein adsorption is the use of solute-depletion strategies. In such studies, changes in the bulk concentration of a protein are measured over time as the protein adsorbs onto a material surface. One assumption that must be made in this case is that all loss of protein from the bulk solution is due to its adsorption onto the material surface. The kinetics of protein adsorption onto a specific material surface at constant temperature can then be determined by plotting the change in bulk protein concentration versus time. Another similar technique that has been used extensively is to label proteins in solution with either fluorescent or radioactive probes. In this case, the concentration of adsorbed protein is determined by measuring either fluorescence or radioactivity, respectively, on the material surface. Protein adsorption can also be measured indirectly using immunoassays such as an enzyme-linked immunosorbent assay (ELISA). In an ELISA, an antibody directed against the protein is used to detect the presence of the adsorbed protein. Binding of the antibody to specific portions of the target protein indicates that the adsorbed protein is in a recognizable conformation, at least for that particular antibody. This approach provides information regarding the bioactivity of a protein rather than just confirming and quantifying the presence of proteins on material surfaces [19].

Several techniques have been developed to study the thickness of the adsorbed protein layer on material surfaces. Ellipsometry, a method that utilizes polarized light to nondestructively examine surfaces, is one of the most widely used such techniques. In a standard ellipsometry procedure, polarized light is directed at a surface at an angle, and is then reflected back to a detector. Changes in this reflected polarized light beam provide information about the interface, primarily the thickness of the adsorbed protein layer, which in turn can be directly correlated to the amount of adsorbed protein. Ellipsometry measures protein layer thicknesses in the nanometer range and is highly accurate. Surface plasmon resonance (SPR) is comparable to, and provides similar information as, ellipsometry; SPR, however, depends on excitation of surface plasmons (instead of changes in polarized light). Another method that has some similarities to ellipsometry is "neutron reflectivity," in which a beam of neutrons

directed at the adsorbed protein layer is reflected back (in the form of reflected radiation) and is measured in a detector. The resulting reflected radiation profile provides information not only about the thickness of the adsorbed protein layer but also about the conformation of the adsorbed molecules. Neutron reflectivity is not widely used because of several drawbacks, which include very limited equipment availability, the need for a large specimen surface area, and the risk of making radioactive the material surface tested [15, 21].

1.3.2. Emerging Techniques for the Study of Protein Adsorption

Several emerging techniques for the study of protein adsorption offer improvements over the limitations of established techniques. Modern mass spectrometry-based techniques identify proteins from multicomponent solutions and exudates such as blood, serum, tears, and saliva, adsorbed either on biomaterials *in vitro* or on excised devices. These techniques can resolve many and different proteins of a large range of molecular masses. Tandem scanning probe microscopy techniques offer the unmatched ability to image a single protein adsorbed on a material surface. Lastly, new developments in surface IR techniques may offer the opportunity to study changes in protein conformation at the level of a single atomic bond.

The advent of more advanced tandem mass spectrometry techniques improved the sensitivities of ToF-SIMS/SIMS technologies and these techniques have tremendous potential for future studies of protein adsorption on biomaterial surfaces. To improve the resolution of mass spectrometry, researchers have used surface treatments (involving the use of matrix molecules such as nicotinic acid, dihydroxybenzoic acid, and sinapinic acid) to protect the protein sample and improve resolution of individual proteins. Matrix-assisted laser/desorption/ionization (MALDI) time-of-flight mass spectrometry (MALDI-ToF/MS) provides, in theory, unlimited mass resolution and extremely low detection limits. In this method, matrix molecules (such as nicotinic acid, dihydroxybenzoic acid, and sinapinic acid) are added to the adsorbed protein layer prior to irradiation with a pulse laser; the released ions are then carried to a detector for analysis (Figure 1.3). A mass-to-charge ratio for the peptide fragments can then be generated, compared with a database of known protein sequences, and statistically analyzed to determine the likelihood that a particular peptide fragment belongs to a specific protein. This technique can examine a wide mass range but, in practice (and depending on the specific mass spectrometer and equipment setup), the ability to resolve larger proteins can diminish because the large, slower-moving ions do not reach the detector quickly. The cut-off point for decreased sensitivity of larger proteins is highly dependent on the individual mass spectrometer used in the study [15, 22].

Another option is to digest adsorbed proteins with enzymes, blot the resulting solution directly onto a MALDI matrix plate, and analyze the digested proteins using a mass spectrometer. By first digesting the adsorbed protein *in situ*, mass spectrometers with a lower mass range are capable of detecting larger peptide fragments with greater sensitivity. For example, in our lab, adsorption of human and bovine serum on standard tissue culture polystyrene took place for 2, 24, and 96 h and was then analyzed via this digestion method. After washing and removing the nonadsorbed proteins, the adsorbed protein layer was digested with trypsin in 10% NH_4HCO_3 and 2% methanol in a trypsin resuspension buffer. The resultant protein fragments were further separated via reverse phase chromatography followed by MALDI-ToF/MS. The results were compared against the National Center for Biotechnology Information and Expert Protein Analysis System databases and provided evidence that albumin, vitronectin, and complement protein C3 were present on the polystyrene surface [unpublished data].

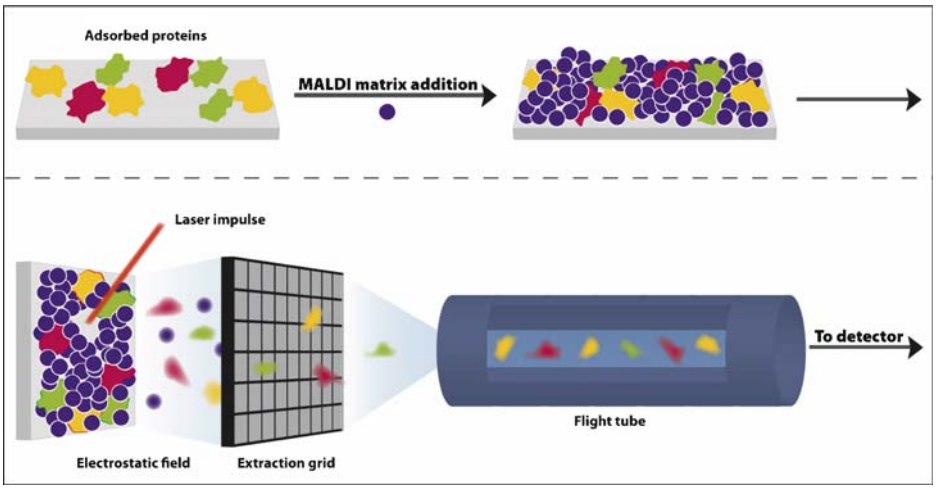

Figure 1.3. Schematic illustration of the major components of the MALDI-ToF/MS technique used for the analysis of proteins adsorbed on a material surface. The MALDI matrix is added to the adsorbed protein sample, which is then bombarded by a pulsed laser. Ion fragments (i.e., ionized peptide fragments from the adsorbed proteins that are generally smaller than the adsorbed protein molecules) are released from the MALDI plate and directed via an electrostatic field into the extraction grid. From there the ions pass though a flight tube to the detector, where proteins and peptide fragments can be analyzed and identified.

MALDI-ToF/MS can also be coupled with protein separation techniques such as two-dimensional (2D) gel electrophoresis to isolate specific bands of proteins from the gel; individual proteins can then be resolved using a mass spectrometer. Potential limitations of MALDI-ToF/MS include the inability to recognize proteins present at very low concentrations and the inability to identify specific proteins when using a MALDI matrix that does not effectively protect the protein sample. The selection of an appropriate MALDI matrix is often a process of trial and error [15, 22]. Future developments involving mass spectrometry will address some of these issues by utilizing tandem equipment strategies and improved protein-processing techniques.

Scanning electron microscopy (SEM), solid-state crystallography, and transmission electron microscopy have also been used to examine the structure of adsorbed proteins; all of these techniques, however, require extensive sample preparation, which may alter either the material surface or the adsorbed protein layer, particularly when the substrate surface interacts with a multicomponent, aqueous protein solution. These methods often require dehydration of the organic sample (which may alter the conformation of proteins), and coating with a conductive substrate (which can sometimes lead to additional artifacts due to changes in the structure of surfaces when dehydrated). Atomic force microscopy (AFM) improves upon other microscopic techniques by allowing imaging of the adsorbed proteins in their native state in nearly physiological conditions and in three dimensions. AFM functions by "feeling" the surface with a very sharp microscale cantilevered tip. More recently, strategies have been developed that combine multiple scanning-probe microscopy techniques to analyze single adsorbed proteins. AFM has been combined with scanning tunneling microscopy (STM) to accurately characterize single adsorbed proteins on conductive substrates. STM functions through measurement of the quantum mechanical tunneling current between

a conductive surface and a very sharp metallic tip. Since the rate of tunneling electrons depends on the distance between the tip and surface, an image can be generated that captures extremely fine changes in height across the scanned surface area. AFM alone provides accurate information about the height of a protein, but STM can provide improved lateral information. Tandem STM/AFM has been used to characterize the morphology of metalloproteins such as azurin [15, 23, 24].

Surface-enhanced infrared absorption spectroscopy (SEIRA), which is an addition to traditional IR spectroscopy, has been applied to study interactions involving surface-immobilized proteins. SEIRA improves on the resolution of traditional IR by amplifying the signal of an adsorbed molecule by approximately two orders of magnitude. In addition to studies on protein recognition, SEIRA has been used to study the adsorption of nucleic acids and DNA. The high level of sensitivity of SEIRA potentially lends the technique to improved analysis of changes in protein functionality over the results obtained by the IR difference method, although it has yet to be used extensively to study adsorbed proteins [20, 25].

1.4. Recent Advances in Protein Adsorption

1.4.1. Protein Adsorption to Nanomaterials

Nanotechnology has exploded as a research direction in many fields of science; the study of protein adsorption on nanophase materials is no exception. Nanoscale materials, or nanomaterials, are defined as materials that have some component or feature (i.e., grains, surface texture, fibers, particles, etc.) with at least one dimension between 1 and 100 nm. Compared with conventional materials (such as a microphase metal or polymer) nanomaterials exhibit improved properties due to their nanoscale characteristics. Additionally, nanomaterials have vastly increased surface area-to-volume ratios compared with conventional materials [26]. Nanomaterials also better mimic physiological structures as most tissues *in vivo* have nanoscale features because they are assembled from nanoscale units of amino acids, proteins, and lipids, all of which are in the low end of the nanoscale range in terms of size (below 15 nm). In addition, the size of most blood proteins is in the range of 3–15 nm. The characteristic dimension of nanoscale materials is, therefore, nearly the size of, or smaller than, a serum protein molecule [26]. Thus, nanoscale biomaterials may promote protein adsorption, and subsequent cell attachment and function, in ways analogous to those promoted by tissues of the body and, potentially, much more effectively than the results obtained in conjunction with conventional materials. Many material surface properties relevant to protein adsorption can be modulated at the nanoscale level, since properties such as hydrophilicity/hydrophobicity, charge, topography, and chemistry can all greatly affect protein adsorption and conformation. Since such properties of nanoscale substrates vary from those of conventional material substrates, nanomaterials may induce controlled conformations of various proteins and, thus, present multiple and/or different cues and signals to cells.

Several literature reports have characterized protein adsorption to nanomaterials [26–30]. For example, adsorption of lysozyme and cytochrome c (an enzymatic heme molecule) to mesoporous (defined as having pore sizes between 2 and 50 nm) silica increased with pore diameter; specifically, using adsorption isotherms, the highest amount of lysozyme adsorbed increased from 13.4 μmol g^{-1} for the 3.54-nm diameter pores to 35.3 μmol g^{-1} for the 10.98-nm diameter pores with similar concentrations of cytochrome c. Additionally, cytochrome c adsorbed to the mesoporous silicate had greater

bioactivity than the enzyme in solution. Sustained enzyme activity was also observed on a mesoporous carbon support substrate [27]. Additionally, a hydroxyapatite (HA) poly(L-lactic acid) (PLLA) composite made from nanocrystal hydroxyapatite mixed with PLLA promoted increased protein adsorption from fetal bovine serum as a function of increased nano-HA weight percentage content in the range 10–70% [26, 28]. Studies of the adhesion of osteoblasts on nanophase hydroxyapatite, titania, and alumina ceramics reported increased surface concentration of vitronectin, an adhesion protein known to mediate adhesion of osteoblasts [29, 30]. Compared with the respective conventional material, the nanophase ceramics contained many small pores (24–97 nm) where vitronectin may have preferentially adsorbed. Additionally, vitronectin displayed increased conformational unfolding on the nanomaterial (measured via Raman scattering), likely leading to exposure of more cell adhesive domains within this molecule that promoted the observed increased osteoblast adhesion [29, 30].

Researchers have sought to characterize the effect of nanoscale surface features on such phenomena as gross protein adsorption and changes in protein conformation. Recently, fibronectin (a large, cell adhesion molecule) interactions with a poly(lactic-*co*-glycolic acid) (PLGA) surface with nanoscale surface features was imaged for the first time using AFM [26, 31]. Fibronectin at concentrations of 0.5–5 $\mu g\ ml^{-1}$ was adsorbed to PLGA surfaces with 3D spherical "bumps" with sizes of 100, 200, and 500 nm. AFM images revealed small globules of fibronectin present on the 500-nm surface. The fibronectin molecules were more spread and elongated on the 200- and 100-nm surfaces; the greatest initial spreading was observed on the material surface with the 200-nm bumps [31]. This result indicates that there are specific surface feature sizes that promote enhanced spreading of specific protein molecules. It should be noted that since both the 100-nm and 200-nm spherical "bumps" are much larger than the "typical" (5–15 nm) size of proteins, these surface features can support adsorption of multiple protein molecules [26, 31].

1.4.2. Manipulating Protein Adsorption

Besides nanomaterial-directed strategies, researchers have continued to investigate the effect of other material surface properties on protein adsorption. Self-assembled monolayers (SAMs) present specific molecules on a material surface and are used in the study of protein adsorption. For the purpose of studying osteopontin (an extracellular matrix protein involved in inflammation) adsorption, various SAM terminal groups such as hydrophobic ($–CH_3$), hydrophilic but neutral (–OH), positively charged ($–NH_2$), and negatively charged (–COOH) were assembled on a gold material surface. SPR indicated that osteopontin adsorption to the $–NH_2$ surface was in the monolayer range, and comparable to that observed on the -COOH surface. Subsequent interactions of bovine aortic endothelial cells exhibited increased cell spreading on the surfaces tested in the following order: –COOH, $–CH_3$, gold, and $–NH_2$. Although the amount of total protein absorbed was similar on both the –COOH and $–NH_2$ surfaces, cell adhesion and spreading was much higher on the $–NH_2$ surface. This result provides evidence that the conformation and bioactivity of osteopontin on the positively charged surface was more favorable for cell binding. Liu *et al*. [32] speculated that the orientation of osteopontin on the –NH2 surface may have exposed the cell adhesive domain arginine–glycine–aspartic acid (RGD) in an orientation favorable to cell adhesion, while on the –COOH surface, RGD was oriented toward the material surface and not toward the adhering cells. Osteopontin is a very acidic, negatively charged protein that may have bound strongly and in a different orientation to the positively charged $–NH_2$ surface than to the negatively charged –COOH surface [32, 33].

Stimuli-responsive polymers (also known as "smart" polymers, defined as materials that respond to external stimuli by altering a specific property) have been developed to modulate protein adsorption in a dynamic and as-needed fashion. Since protein adsorption on material surfaces may vary tremendously due to hydrophilicity/hydrophobicity properties, switching these properties on a material surface may affect and/or change the adsorbed protein monolayer and, thus, subsequent cell interactions. Poly(*N*-isopropylacrylamide) (PNIPAAm), a thermoresponsive coating, has been used on traditional tissue culture polystyrene and in a microfluidic hotplate. Up to 37°C, the PNIPAAm surface is hydrophobic and promotes protein adsorption; when cooled to room temperature, this polymer undergoes a phase transition and its surface becomes hydrophilic, inducing decreased protein adsorption. PNIPAAm was used in a microfluidic hotplate to induce fast (in less than 1s) protein adsorption/desorption. A different type of stimuli-responsive polymer utilized monolayers with photocleavable 2-nitrobenzyl groups. Bovine serum albumin was adsorbed to this layer, limiting human embryonic kidney cell adhesion due to the inertness of albumin. When this material surface was exposed to ultraviolet light, the 2-nitrobenzyl groups were cleaved and albumin was released; then increased adsorption of fibronectin and human embryonic kidney cell adhesion was observed [34, 35].

1.5. Current Limitations and Potential Future Opportunities

Protein adsorption has been studied on many materials and under various conditions. In many of these cases, researchers reported changes in protein conformation and bioactivity upon adsorption; however, the underlying mechanisms correlating material surface chemistry, protein conformation, and protein bioactivity are still not fully understood.

Nanomaterials offer a new, and relatively untapped, opportunity to study these phenomena at the nanoscale. Coupling a wide range of proteins, nanomaterials, and cells with imaging techniques (such as AFM) will provide valuable information regarding specific aspects of these phenomena, potentially leading to improved understanding of protein-surface interactions. In addition to improving current understanding of protein adsorption, nanomaterials may also allow for improved control of protein adsorption (and, thus, subsequent cell adhesion and function pertinent to new tissue formation), leading to the design and formulation of new and improved biomaterials for use in clinical applications.

References

1. Dee KC, Puleo DA, Bizios R. Protein–surface interactions. In: Dee KC, Puleo DA, Bizios R, editors. An introduction to tissue-biomaterial interactions. Hoboken, NJ: John Wiley and Sons, 2002
2. Horbett TA. The role of adsorbed proteins in tissue response to biomaterials. In: Ratner BD, Hoffman AS, Schoen FJ, Lemons JE, editors. Biomaterials science. An introduction to materials in medicine. San Diego: Elsevier Academic Press, 2004, pp. 237–246
3. Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. Semin Immunol 2008;20: 86–100
4. Nakanishi K, Sakiyama T, Imamura K. On the adsorption of proteins on solid surfaces, a common but very complicated phenomenon. J Biosci Bioeng 2001;91(3):233–244
5. Ramsden JJ. Puzzles and paradoxes in protein adsorption. Chem Soc Rev 1995;73–78
6. Kim MS, Khang G, Lee HB. Gradient polymer surfaces for biomedical applications. Prog Polym Sci 2008;33: 138–164

7. Sun S, Yue Y, Hunag X, Meng D. Protein adsorption on blood–contact membranes. J Membr Sci 2003; 222:3–18
8. Thevenot P, Wenjing H, Tang L. Surface chemistry influences implant biocompatibility. Curr Top Med Chem 2008;8:270–280
9. Roach P, Eglin D, Rhode K, Perry CC. Modern biomaterials: a review – bulk properties and implications of surface modifications. J Mater Sci: Mater Med 2007;18:1263–1277
10. Reintjes T, Tessmar J, Gopferich A. Biomimetic polymers to control cell adhesion. J Drug Del Sci Tech 2008;18(1):15–24
11. Ratner BD, Bryant SJ. Biomaterials: where we have been and where we are going. Annu Rev Biomed Eng 2004;6:41–75
12. Mayorga L, Ratner BD, Horbett TA. The role of complement adsorption and activation in monocyte adhesion to ultralow protein adsorption surfaces made by RF plasma deposition of PEO–like tetraethylene glycol dimethyl ether (tetraglyme). World Biomater Congr 2008:1162.
13. Schmidt DR, Kao WJ. Monocyte activation in response to polyethylene glycol hydrogels grafted with RGD and PHSRN separated by interpositional spacers of various lengths. J Biomed Mater Res 2007;83A(3):617–625
14. Miller R, Fainerman VB, Leser ME, Michel M. Kinetics of adsorption of proteins and surfactants. Curr Opin Colloid Interface Sci 2004;9:350–356
15. Merret K, Cornelius RM, McClung WG, Unsworth LD, Sheardown H. Surface analysis methods for characterizing polymeric biomaterials. J Biomater Sci Polym Ed 2002;6:593–621
16. Bhaduri A, Das KP. Proteins at solid water interface – a review. J Dispers Sci Technol 1999;20(4):1097–1123
17. McArthur SL. Applications of XPS in bioengineering. Surf Interface Anal 2006;38:1380–1385
18. Wahlgren M, Arnebrant T. Protein adsorption to solid surfaces. Tibtech 1991;9:201–208
19. Hlady V, Buijs J, Jennissen P. Methods for studying protein adsorption. Methods Enzymol 1999;309(26): 402–429
20. Ataka K, Heberle J. Biochemical applications of surface–enhanced infrared absorption spectroscopy. Anal Bioanal Chem 2007;388:47–54
21. Elwing H. Protein absorption and ellipsometry in biomaterial research. Biomaterials 1998;19:397–406
22. Gallagher WM, Lynch I, Allen LT, Miller I, Penney SC, O'Connor DP, Pennington S, Keenan AK, Dawson KA. Molecular basis of cell–biomaterial interaction: Insights gained from transcriptomic and proteomic studies. Biomaterials 2006;27:5871–5882
23. Silva LP. Imaging proteins with atomic force microscopy: an overview. Curr Protein Pept Sci 2005;6:387–395
24. Bonanni B, Andolfi L, Bizzarri R, Cannistraro S. Functional metalloproteins integrated with conductive substrates: detecting single molecules and sensing individual recognition events. J Phys Chem B 2007;111: 5062–5075
25. Garczarek F, Gerwert K. Integration of layered redox proteins and conductive supports for bioelectronic applications. Agnew Chem Int Ed 2000;39:1180–1218
26. Liu H, Webster TJ. Nanomedicine for implants: a review of studies and necessary experimental tools. Biomaterials 2007;28:354–369
27. Vinu A, Miyahara M, Ariga K. Assemblies of biomaterials in mesoporous media. J Nanosci Nanotechnol 2006;6(6):1510–1532
28. Wie G, Ma PX. Structure and properties of nano–hydroxyapatite/polymer composite scaffolds for bone tissue engineering. Biomaterials 2004;25:4749–4757
29. Webster TJ, Ergun C, Doremus RH, Siegel RW, Bizios R. Specific proteins mediate enhanced osteoblast adhesion on nanophase ceramics. J Biomed Mater Res 2000;51(3):475–483
30. Webster TJ, Schadler LS, Siegel RW, Bizios R. Mechanisms of enhanced osteoblasts adhesion on nanophase alumina involve vitronectin. Tissue Eng 2001;7(3):291.301
31. Miller DC, Haberstroh KM, Webster TJ. PLGA nanometer surface features manipulate fibronectin interactions for improved vascular cell adhesion. J Biomed Mater Res 2007;81A(3):678–684
32. Liu L, Chen S, Giachelli CM, Ratner BD, Jian S. Controlling osteopontin orientation on surfaces to modulate endothelial cell adhesion. J Biomed Mater Res 2005;74:23–31
33. Jandt KD. Evolutions, revolutions and trends in biomaterials science – a perspective. Adv Eng Mater 2007;9(12):1035–1050
34. Mano JF. Stimuli–responsive polymeric systems for biomedical applications. Adv Eng Mater 2008;10(6): 515–527
35. Nakanishi J, Kikuchi Y, Takarada T, Nakayama H, Yamaguchi K, Maeda M. Spatiotemporal control of cell adhesion on a self-assembled monolayer having a photocleavable protecting group. Anal Chim Acta 2006;578:100