CELL–MATERIAL INTERACTIONS

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I. Introduction

The nature of cell adhesion to substrate materials has a tremendous effect on cell function and tissue development. Signaling cascades initiated by cell adhesion have the ability to regulate a variety of events, including embryogenesis, tissue differentiation, and cell migration (Koenig and Grainger, 2002; Longhurst and Jennings, 1998). Signaling via receptor–ligand interactions provides the cell with vital information about its

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extracellular environment. Coordinated cellular responses to these signals enable cells to adapt their function to their specific environment.

Understanding how cells interact with a substrate is crucial in the development of functional biomaterials. The field of tissue engineering requires the creation of materials that are capable of directing tissue development, and one approach in achieving this control is through the manipulation of biological interactions between cell and material. A striking example of how cell–surface interactions influence cell function is the *in vitro* culture of mammary epithelial cells on tissue culture polystyrene (TCPS) vs laminin-coated surfaces. *In vivo*, epithelial cells are found as a monolayer on a basal lamina. When cultured on untreated TCPS *in vitro*, these cells lose their normal cuboidal morphology and ability to secrete milk proteins. However, upon addition of laminin to the surface, normal function and morphology are retained (Horwitz, 1997). Thus, in order to design and select appropriate polymeric scaffolds for tissue engineering, understanding the influence of the polymer surface chemistry on cell viability, growth, and function is critical.

Investigation of methods to control cell–material interactions is required in order to achieve the goal of developing ideal biomaterials and implants that can elicit specific, timely, and desirable responses from surrounding tissues. Study of cell–substrate interactions may also aid in analyzing differences between cell behavior *in vivo* vs *in vitro*. While *in vitro* experiments of cell–substrate interactions cannot reproduce the complex cascade of events and cellular responses that occur *in vivo*, they enable controlled, quantifiable experimental characterization that is difficult to obtain *in vivo*. Although this chapter discusses only the effects of receptor and ligand properties on cell–substrate interactions, these interactions may also be influenced by numerous co-factors such as cytokines and growth factors, thus greatly increasing their complexity. Because most cell–substrate interactions are mediated by biological molecules, a review of how cells adhere to their native extracellular matrix is first required.

II. Cell Surface Receptors and their Ligands

Whether on natural or synthetic materials, cell adhesion is mediated by protein interactions with cell surface receptors. There are several classes of cell surface receptors, and this chapter will discuss integrins, selectins, and immunoglobulins. These receptors bind their ligands with high affinity and specificity, and while each receptor family regulates separate cellular functions, there is some overlap between families. Each of these receptor families possesses a characteristic molecular structure, with every receptor

normally consisting of an extracellular, transmembranous, and intracellular domain; this structure is illustrated in Fig. 1. The transmembrane region of receptors is generally hydrophobic and is roughly 6–8 nm in length, or approximately the thickness of the membrane (Hammer and Tirrell, 1996). The cell membrane itself is heterogeneous in composition and behaves like a fluid at physiological temperatures, where the viscosity of the membrane lipids can be 100–1000 times that of water, thus allowing receptor diffusion laterally within the membrane (Bussell *et al.*, 1995a,b; Hammer and Tirrell, 1996). The membrane contains a two-dimensional suspension of proteins, where the area fraction of the membrane occupied by receptors may reach 0.4 (Hammer and Tirrell, 1996). Both proteins and lipids diffuse in the plane of the membrane, where the lipid diffusivity is approximately 10^{-8} cm^2/s, and receptor proteins exhibit a wide diffusivity range from 10^{-11} to 10^{-8} cm^2/s. The diffusion of receptors within a membrane is influenced by several factors, including hydrodynamic and

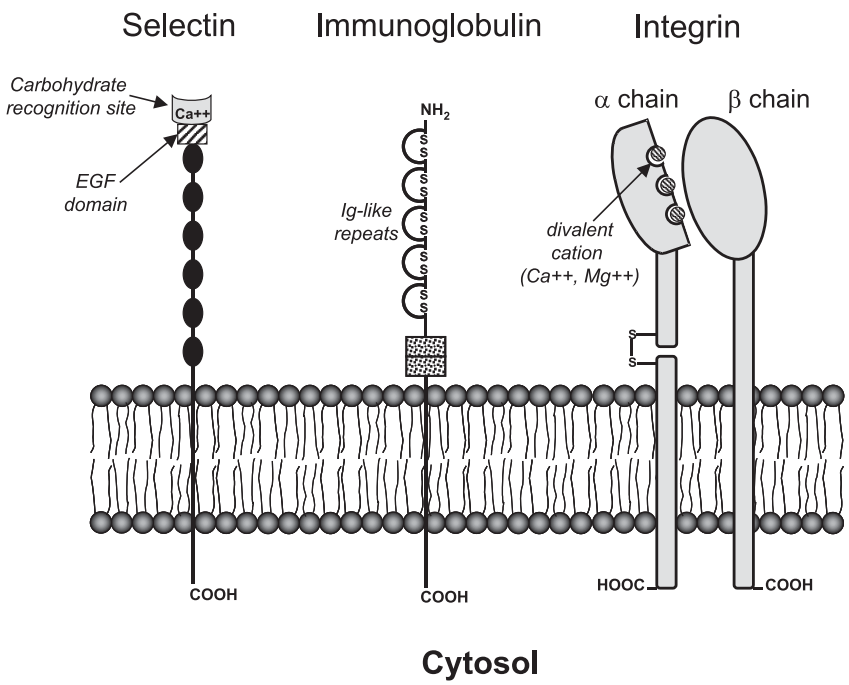

FIG. 1. Interaction of cells with the ECM is mediated by ligand binding to cell surface receptors, such as selectins, immunoglobulins, and integrins. Receptor–ligand binding allows cells to respond to changes in their extracellular environment. The general structure of these three types of receptors is illustrated here.

thermodynamic interactions between proteins, as well as obstruction or binding by cytoskeletal elements, all of which may reduce the receptor diffusivity (Bussell *et al*., 1995b).

This lateral mobility within the membrane is important because it participates in the regulation of receptor binding to ligands. Receptor diffusivity determines the rate at which receptors can find each other, thereby determining the transport-limited rate of binding. Under purely diffusive mechanisms, the rate of receptor collision, k_+ can be estimated according to the equation:

$$k_+ = 2\pi Ds \tag{1}$$

where D is the sum of the lateral diffusivity of both interacting proteins and s is the radius of the encounter complex, or the distance at which the receptor and ligand are close enough to react (Bell, 1978). Because most proteins recognize and react with each other very rapidly, a diffusion-limited reaction rate is normal for many receptor interactions.

Most cellular interactions with the extracellular matrix (ECM) occur via integrins (Hynes, 2002). However, with the design of novel biomaterials in mind, the presence of other cell surface receptors such as proteoglycans and immunoglobulins may be exploited in order to create materials that elucidate a desired cellular response. Thus, while these receptor families are not typically highly involved in cell–substrate binding, their existence provides the bioengineer with more possibilities to achieve alteration of cell behavior via biomaterial modification.

A. Integrins

Integrins are the principal receptors on mammalian cells for binding most extracellular matrix proteins, including collagen, laminin, and fibronectin (Giancotti and Ruoslahti, 1999; Juliano, 2002). The integrin superfamily is comprised of homologous transmembrane linker proteins that mediate both cell–surface and cell–cell interactions. Each integrin receptor consists of a noncovalently assembled heterodimer of one α- and one β-subunit (Fig. 1; Longhurst and Jennings, 1998). In general, both subunits are *N*-glycosylated proteins with a large extracellular domain, a single hydrophobic transmembrane region, and a short cytoplasmic domain. There exist $\sim$18 different α-subunits and 8 β-subunits, forming 24 different heterodimers (Hynes, 2002; van der Flier and Sonnenberg, 2001). Integrins differ from other cell surface receptors, such as those that bind to hormones and other soluble signaling molecules, in both their

binding affinity and concentration. Integrins bind their ligand with a relatively low affinity ($K_a = 10^6$–10^9 liters/mole), and are present at 10- to 100-fold higher concentration on the cell surface (Alberts *et al.*, 1994). This arrangement allows cells to weakly bind to a large number of matrix molecules, thus enabling it to explore its environment without losing attachment to it.

Integrins mediate cell adhesion through binding to a diverse array of ligands. These ligands include ECM proteins such as collagen, plasma proteins such as fibrinogen, and transmembrane immunoglobulins such as ICAM-1 (Longhurst and Jennings, 1998; Plow *et al.*, 2000). The majority of ligands for integrin receptors are found as short peptide sequences within extracellular matrix proteins. The binding site in integrins for extracellular matrix ligands is composed of short regions in the N-termini of both subunits, with ligand specificity determined by the particular combination of α- and β-subunits (van der Flier and Sonnenberg, 2001). There are ~12 integrins that bind fibronectin and 7 that bind laminin (Plow *et al.*, 2000; van der Flier and Sonnenberg, 2001). Many integrins can bind to more than one ligand, and several individual ligands can bind to more than one integrin. The most widely recognized and characterized ligand peptide motif is the RGD (Arginine–Glycine–Aspartic acid) sequence found in a variety of ECM proteins (Pierschbacher and Ruoslahti, 1984; Ruoslahti, 1996). Integrin recognition of this ligand occurs in almost all cell types and acts to promote their adhesion. However, some adhesive peptide sequences exhibit more cell selectivity, thereby encouraging the adhesion of only specific cell types. One example of this is REDV (Arginine–Glutamine–Aspartic acid–Valine), which is derived from the III-CS domain of human plasma fibronectin and has been shown to induce attachment and spreading of endothelial cells, but not fibroblasts, vascular smooth muscle cells, or platelets (Hubbell *et al.*, 1991). Table I lists the major adhesive peptide sequences within several ECM proteins, as well as their receptors.

Integrin receptors are always present on the cell surface, although they often must be activated in order to bind a ligand (Hynes, 2002). Integrin expression does not necessarily increase with enhanced integrin-mediated adhesion, indicating the existence of other pathways by which the cell creates signals to change the affinity or avidity of the integrins. Activation of other surface receptors may induce an "inside-out" signaling cascade that results in conformational changes in integrins, altering their ability to bind ligands, a property known as receptor affinity (Bennett, 1998; van der Flier and Sonnenberg, 2001). Affinity is a measure of the binding strength between a single ligand and a single receptor; increases in integrin affinity mean that ligand binding can occur at lower ligand

TABLE I
ADHESIVE PEPTIDE SEQUENCES WITHIN ECM PROTEINS AND THEIR RECEPTORS (FOR REVIEW, SEE YAMADA, 1991)

ECM protein	Adhesive peptide sequence	Major receptor(s)
Fibronectin	RGDS	$\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$, $\alpha_{IIb}\beta_3$, $\alpha_3\beta_1$, $\alpha_5\beta_1$
	LDV	$\alpha_4\beta_1$, $\alpha_4\beta_7$
	REDV	$\alpha_4\beta_1$
	PHSRN	Synergistic for $\alpha_5\beta_1$ binding
Laminin	YIGSR	67-kDa binding protein
	PDGSR	Unknown
	LRGDN	$\alpha_v\beta_3$, $\alpha_5\beta_1$
	IKVAV	110-kDa binding protein
	LRE	Unknown
	IKLLI	$\alpha_3\beta_1$ and cell–surface heparan sulfate
Vitronectin	RGDV	$\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_{IIb}\beta_3$
Collagen I	RGDT	$\alpha_v\beta_3$
	DGEA	$\alpha_2\beta_1$
Fibrinogen	RGDS	$\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
	RGDF	$\alpha_{IIb}\beta_3$
	KQAGDV	$\alpha_{IIb}\beta_3$

Amino acid abbreviations: A = Arginine; D = Aspartic acid; E = Glutamate; F = Phenylalanine; G = Glycine; H = Histidine; I = Isoleucine; K = Lysine; L = Leucine; N = Asparagine; P = Proline; Q = glutamine; R = Arginine; S = Serine; T = Threonine; V = Valine; Y = Tyrosine.

concentrations. This occurrence may be due to changes in the rates of dissociation or association of the ligand as a result of agonist-induced conformational changes in the integrin itself. Modulation of integrin avidity can increase the number of integrin–ligand interactions, thereby strengthening cell adhesion. This behavior may occur via an agonist-induced rearrangement or clustering of integrins on the cell surface that is meant to increase the number of random encounters between integrin and ligand. As the cytoplasmic tail of the β-subunit is associated with the cell cytoskeleton, these changes in avidity likely occur due to a reorganization of cytoskeletal structures in response to other agonists or signals; it has been shown that Ca^{2+} induces the organization of the $\alpha_L\beta_2$ integrin into clusters that enhance the avidity of $\alpha_L\beta_2$-ligand binding interactions (Bennett, 1998; Binnerts *et al.*, 1996). The concepts of integrin affinity and avidity and their effects on cell adhesion are illustrated in Fig. 2. Recent evidence also indicates that conformational activation and lateral clustering of integrins are closely and inextricably coupled (Li *et al.*, 2003).

Just as the cytoplasmic domain of integrins is essential for transmitting intracellular signals that change integrin activity to achieve "inside-out"

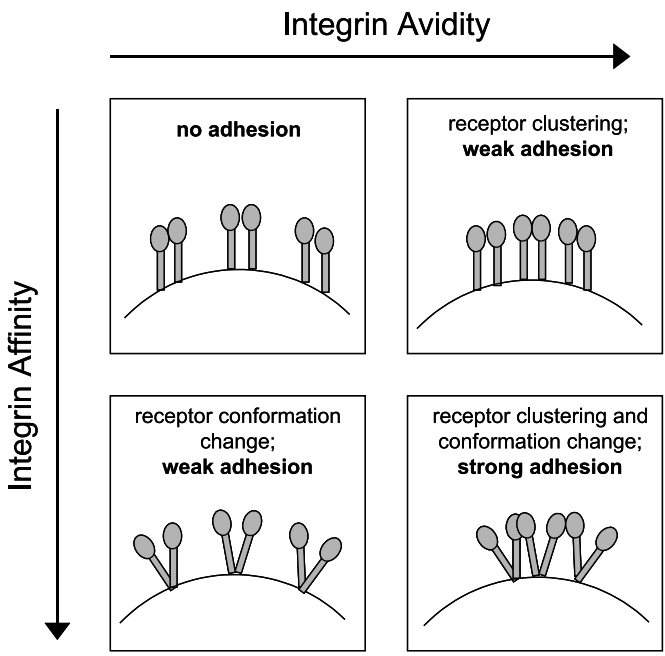

FIG. 2. Activation of cell surface receptors can initiate "inside-out" signaling, whereby the cell may alter its presentation of integrins. Integrin affinity and avidity may be modulated by a variety of agonists, resulting in alteration of ligand binding.

signaling, they are also required for communication in the opposite direction. This "outside-in" signaling allows cells to gain information about their extracellular environment through integrin–ligand binding and then transmit that information to the cytoplasm so that the cell function can appropriately respond to its surroundings. The implications of this signaling will be discussed later, in Section III.A; however, clearly, cell interactions with both synthetic and natural matrices are bi-directional and dynamic.

B. PROTEOGLYCANS AND SELECTINS

All eucaryotic cells possess a carbohydrate-rich coating known as the glycocalyx, depicted in Fig. 3. Membrane proteins may be decorated or masked by these carbohydrates, which occur as oligosaccharide side chains covalently bound to membrane proteins or lipids, and as polysaccharide chains of integral membrane proteoglycans (Alberts *et al.*, 1994).

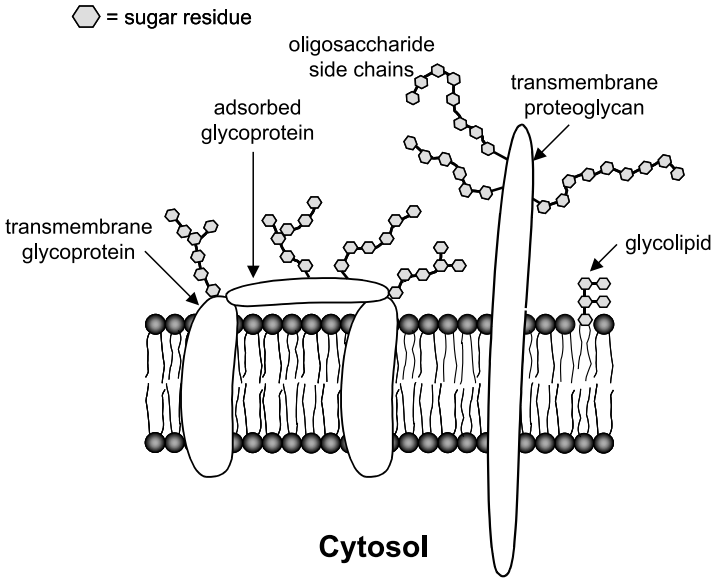

FIG. 3. Both adsorbed and transmembrane glycoproteins contribute to the formation of the cell glycocalyx. Once thought to function purely as a protective layer, cell surface oligosaccharides also participate in specific cell-recognition processes.

In addition to covalently bound saccharides, the glycocalyx also contains adsorbed glycoproteins and proteoglycans that have been secreted into the extracellular space.

It was originally believed that the sole function of the glycocalyx was to protect the cell against mechanical and chemical damage and to keep foreign objects and other cells at a distance. While this function of the glycocalyx is still valid, it has been discovered that these cell surface oligosaccharides also play a role in specific cell-recognition processes (Bertozzi and Kiessling, 2001; Sampson *et al.*, 2001). The oligosaccharides are composed of a diverse array of sugars, often branched, and covalently bound in a variety of linkages. This arrangement of diverse, exposed saccharides is well suited for mediating specific cell–cell adhesion processes, such as those occurring in sperm–egg interactions or inflammatory responses. The repulsive steric barrier created by the glycocalyx also functions to eliminate nonspecific adhesion while permitting specific interactions (Lauffenberger and Linderman, 1993). This means that the length of other cell surface adhesion molecules will play an important role, as the receptors must reach beyond the glycocalyx to bind to ligands.

The ligands for carbohydrate binding are known as lectins, which are often presented as components of the selectin family of cell–cell adhesion molecules. Selectins contain a carbohydrate-binding lectin domain connected to an epidermal growth factor-like motif, followed by a number of complement regulatory protein-like repeats, a transmembrane region, and a short cytoplasmic tail (Fig. 1; Furie and Furie, 1995). They bind to sialylated glycans, such as sialyl Lewis X, in a calcium-dependent manner (Juliano, 2002), and little is known about their association with the cell cytoskeleton. The selectin family consists of P–selectin, found in the α-granules of platelets and in the Weibel-Palade bodies of endothelial cells, E–selectin, which is expressed only by activated endothelial cells, and L–selectin, which is constitutively expressed on leukocytes (Mousa, 1998). The lectins have relatively low affinity for their carbohydrate ligands, and both the association and dissociation of the oligosaccharide with the lectin occurs very rapidly, resulting in only transient adhesion. Selectin binding is most prevalent in the bloodstream, where they are best known as the receptors that mediate the rolling on the blood vessel wall and subsequent extravasation of leukocytes and other inflammation-related molecules (McEver, 1995). The expression and function of selectins is tightly regulated such that they are only activated at specific times (Juliano, 2002). While primarily important in cell–cell interactions, this chemistry provides another opportunity to exploit and control cell surface interactions.

Membrane bound proteoglycans bind with low specificity relative to integrins. In addition to binding lectins, proteoglycans exhibit charge-mediated binding to ECM molecules. The glycosaminoglycann (GAG) blocks of proteoglycans are sulfated, resulting in a net negative charge that enables them to interact with clustered positive charges on proteins such as fibronectin. A higher degree of GAG sulfation causes tighter binding to these proteins. The positively charged protein regions may be described by the binding sequence, XBBXBX, where X is a hydrophobic amino acid and B is lysine or arginine (Cardin and Weintraub, 1989). Proteoglycan binding sequences of this nature have been identified in fibronectin, vitronectin, and laminin (West and Hubbell, 1997).

Lastly, monosaccharides may also participate in cell adhesion. Most commonly, galactose or lactose binds to the asialoglycoprotein receptor on hepatocytes (Geffen and Speiss, 1992). *In vivo*, this interaction serves to clear protein aggregates from the circulation. Regarding biomaterials synthesis, however, this mechanism has been exploited to create materials that induce hepatocyte-specific adhesion or enable liver-specific drug delivery (Cho *et al.*, 2001a; Gutsche *et al.*, 1994; Kobayashi *et al.*, 1994a).

C. Immunoglobulins

The immunoglobulin (Ig) superfamily of receptors contains over 100 different molecules, and participates primarily in the mediation of cell–cell interactions (Buck, 1992). These receptors contain one or more Ig-like domains that are characteristic of antibody molecules (Fig. 1), and they function in a wide variety of cell types in which they are involved in many varied biological processes (Juliano, 2002). While Ig binding does initiate cytoplasmic signals, relatively little is known about the interactions of Ig receptors with cytoplasmic proteins. The most recognizable members of the Ig receptor family are ICAMs (intercellular adhesion molecules), VCAMs (vascular cell adhesion molecules), NCAM (neural cell adhesion molecule), and CD44s. This wide array of receptors is capable of binding in various manners; they commonly exhibit homophilic binding with identical Ig receptors, or they may bind to other different Ig receptors, nonIg family molecules such as integrins, and even some ECM components (Buck, 1992; Horton, 1996). NCAM is expressed on a variety of cell types, including most nerve cells, and binds cells together via a calcium-independent homophilic interaction. The binding interactions of NCAM are fairly versatile, however, as it may also bind to heparan sulfate and collagen (Brummendorf and Rathjen, 1996). Neural glial cell adhesion molecule (NgCAM) also exhibits a complex interaction pattern, binding to at least six different proteins including laminin and two integrin receptors responsible for fibronectin and vitronectin interactions (Brummendorf and Rathjen, 1996). Thus, even if an Ig receptor does not interact directly with the ECM, it can still regulate adhesion to the ECM substrate by signaling through the intermediate integrin receptor. These interactions of Ig receptors with ECM molecules and integrins have been shown to be important in events such as neurite outgrowth (Brummendorf and Rathjen, 1996).

Lastly, the CD44 receptors form a subgroup of the Ig family that is expressed on almost all cell types. CD44 is commonly overexpressed in cancer cells (Lesley *et al.*, 1993) and assists in cell metastasis, indicating that the CD44–ligand interaction plays an important role in stimulating cell migration (Bartolazzi *et al.*, 1994; Catterall *et al.*, 1995). The principal ligand for CD44 is hyaluronic acid (HA), a glycosaminoglycan found in the ECM of many tissues, although CD44 may also bind to several other ECM proteins, including collagen, fibronectin, laminin, and osteopontin (Goodison *et al.*, 1999). While all CD44 isoforms have the capability to bind HA, CD44 may be found in an active, an inducible, or an inactive state with respect to HA binding. CD44 appears to play a prominent role during embryogenesis, and it is involved primarily in the maintenance of

3D organ and tissue structure (Goodison *et al*., 1999). Binding of CD44 to hyaluronic acid mediates a multitude of cell functions, including cell aggregation, proliferation, migration, angiogenesis, and gene expression (Turley *et al*., 2002). Clearly, this degree of regulation makes hyaluronic acid-based chemistries attractive for many tissue engineering applications (Baier-Leach *et al*., 2003).

D. Anti-adhesive Matrix Molecules

While a lack of cell adhesion will not promote cell survival or proliferation, there are several physiological processes that depend upon cell detachment from the ECM. Examples of this necessary de-adhesion can be found in normal development and tissue homeostasis, as anti-adhesive molecules cause different levels of de-adhesion ranging from complete cell detachment to the localized detachment required for cell migration. Investigation of these anti-adhesive molecules is important, as their use in biomaterials may provide a means to generate matrices that are conducive to cell migration by mimicking the adhesive/anti-adhesive nature of the native ECM.

In vivo, the ECM is composed of both adhesive and anti-adhesive components which interact both with cells and with each other in a complex manner. The principal molecules falling under the anti-adhesive classification are the tenascins, thrombospondins (TSPs), and secreted protein acidic and rich in cysteine (SPARC) (Sage and Bornstein, 1991; Sage, 2001). Several cell surface molecules, such as integrins, syndecans, and CD36, can serve as receptors of anti-adhesive ECM molecules (Orend and Chiquet-Ehrismann, 2000). Cells can exhibit varied adhesion responses to individual matrix components versus combinations of the same molecules. For instance, expression of metalloproteinases by synovial fibroblasts occurred when cultured on mixtures of tenascin-C and fibronectin, but not when either protein was presented alone (Tremble *et al*., 1994). Additionally, while these anti-adhesive proteins may antagonize the proadhesive activities of other matrix proteins, they have also been found to promote integrin-mediated cell adhesion under certain circumstances (Orend and Chiquet-Ehrismann, 2000).

Anti-adhesive molecules affect cell adhesion by changing the arrangement of cytoskeletal proteins and by altering signaling cascades. Specifically, tenascin-C, TSP-1, and SPARC all downregulate the actin stress fiber system and disrupt focal adhesions when added in solution to spread cells (Murphy-Ullrich, 2001; Orend and Chiquet-Ehrismann, 2000). Reorganization of the actin cytoskeleton is a major pathway by which

anti-adhesive molecules modulate cell adhesion. Altering the state of actin polymerization has a great effect upon cell shape, and can also influence gene and protein expression profiles (Bissell *et al.*, 1999; Orend and Chiquet-Ehrismann, 2000). Several studies have explored the link between cell morphology and function, and this topic will be discussed later in the chapter (Section III.B).

III. Integrin–Ligand Binding and Signal Transduction

Integrin–ligand binding provides a critical pathway by which cells can explore and examine their extracellular environment. This interaction initiates signaling cascades that essentially control cell behavior, and these signals may change with cell substrate identity or conformation. Various ligand properties can affect cell signal transduction processes, including ligand surface concentration, strength of receptor–ligand adhesion, degree of receptor occupancy by ligand, and ligand affinity (Hammer and Tirrell, 1996). This relationship between substrate or ligand properties and cell function is illustrated in Fig. 4.

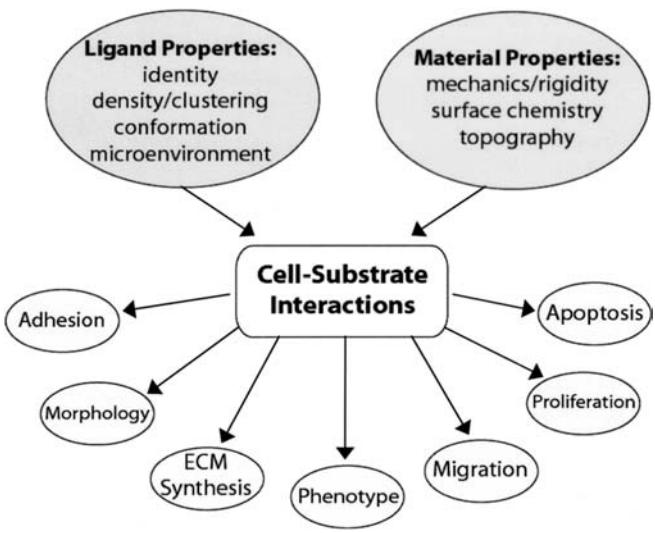

FIG. 4. Alterations in either ligand or material properties may alter the nature of cell–substrate interactions. These interactions, in turn, impact many aspects of cell function.

A. Focal Adhesions

The function of integrins is not solely to act as cell surface receptors for ECM ligands, but also to mediate interactions between the cell cytoskeleton and the ECM, thereby initiating signaling cascades in response to the extracellular environment. Thus, the integrins are transmembrane linkers, with the intracellular domain indirectly connected to bundles of actin filaments. In a 2D *in vitro* culture of fibroblasts, most of the cell is separated from the substrate by a >50 nm gap. In some areas, however, clusters of adhesion sites form focal contacts, decreasing this gap to 10–15 nm and causing very strong cell attachment (Alberts *et al.*, 1994; Burridge *et al.*, 1988).

Focal adhesions are integrin-based structures that mediate strong cell–substrate adhesion and enable signal transduction between the ECM and cell cytoplasm (Boudreau and Jones, 1999; Wehrle-Haller and Imhof, 2002; Zamir and Geiger, 2001). The action of integrin–ligand binding initiates a sequence of events involving cytoplasmic attachment proteins that enables the integrin to link indirectly to actin filaments in the cell cortex. Following integrin–ligand binding, the cytoplasmic tail of the β-chain associates with cytoskeletal proteins, inducing the localized clustering of talin, vinculin, paxillin, and tensin, which participate in integrin linkage to actin filaments (Boudreau and Jones, 1999; Burridge *et al.*, 1988). This assembly and organization of actin filaments promotes more integrin clustering, which acts as a positive feedback system, as the ligand binding and cytoskeletal organization are further enhanced (Giancotti and Ruoslahti, 1999). The result of this sequence of events is the formation of aggregates of ECM proteins, integrins, and cytoskeletal proteins on either side of the cell membrane, and these components comprise the focal adhesion. This transmembrane attachment of ECM to the cytoskeleton via integrins is required for robust cell–substrate and cell–cell adhesions. In the absence of this anchorage, the attachment site may be ripped out of the cell (Alberts *et al.*, 1994). This cytoskeletal connection is also necessary for the formation of focal adhesions. Cells that have been modified to lack the cytoplasmic domain of the β-chain are still capable of binding ligands, but not able to cluster at focal contacts (Solowska *et al.*, 1989).

In addition to providing stable anchorage sites for cell–substrate interactions, focal adhesions can also serve as pathways for signal transduction. Integrin-mediated cell adhesion initiates a number of biochemical events, including cytoplasmic alkalinization due to activation of a Na^+–H^+ antiporter, increases in intracellular calcium, activation of tyrosine kinases, protein tyrosine phosphatases, and lipid kinases, and changes in gene expression (Bennett, 1998; Romer, 1995). Signaling

molecules congregate at focal adhesions, suggesting that these contacts serve as foci for "outside-in" signaling cascades. For instance, several protein kinases are localized to focal adhesions, and there is evidence that their activity changes with substrate composition (Koenig and Grainger, 2002). Because protein kinases participate in phosphorylation of various intra- and extracellular proteins, they are thus capable of significantly affecting cell survival and function in response to the specific ECM environment.

It is well known that cell adhesion, and by consequence, cell signaling, depends upon the molecular identity of the ECM on which the cell is anchored. Different cell types exhibit preferential binding to some ECM components over others. However, the physical state of the ECM may also govern the formation of focal adhesions and modulation of cell function. Fibroblasts cultured on either covalently immobilized fibronectin (FN) or substrates coated with FN form different types of adhesion complexes (Katz *et al.*, 2000). The FN did not vary in conformation or density; only its physical state was altered. Cells on nonimmobilized FN are capable of rearranging the ECM in fibrils, resulting in "fibrillar adhesions" which contain low levels of focal adhesion components such as paxillin, vinculin, and tyrosine-phosphorylated proteins, and bind primarily through the $\alpha_5\beta_1$ integrin. Yet, cells on immobilized FN displayed traditional focal contacts bound through the $\alpha_v\beta_3$ integrin and contained high amounts of cytoskeletal proteins normally associated with focal adhesion formation. These differences in the adhesion of a single cell type to a single ECM component illustrate the complexity of cell–substrate interactions, and imply that the deformability or rigidity of the ECM regulates local tension at adhesion sites, thus activating signaling such as local tyrosine phosphorylation.

However, it must be noted that our current understanding of focal adhesions is based primarily upon 2D *in vitro* studies, and we know very little about their 3D *in vivo* counterparts. Cell behavior exhibits differences even between 2D and 3D *in vitro* cultures. For example, fibroblasts cultured on flat surfaces exhibit an artificial polarity between the upper and lower surfaces of these normally nonpolar cells, yet when cultured in 3D collagen matrices, their morphology and migration patterns change significantly (Elsdale and Bard, 1972; Friedl and Brocker, 2000). Cell-derived 3D matrices have been used to examine how *in vivo* cell–substrate adhesion varies from the traditional planar environment normally used *in vitro*. Significant differences have been illustrated between 3D-matrix interactions relative to 2D substrates, thus challenging the use of traditional tissue culture conditions for understanding *in vivo* structure, function, and signaling of cell adhesions (Cukierman *et al.*, 2001). *In vivo* 3D-matrix

interactions not only display enhanced biological activity and narrowed integrin usage, but also differ in structure, localization, and function from 2D *in vitro* adhesions. Additionally, it was found that neither a 3D collagen gel fabricated *in vitro* nor a flattened, 2D cell-derived matrix could mimic the results obtained using the 3D cell-derived matrix (Cukierman *et al.*, 2001). This observation illustrates that both the biological composition and the 3D quality of these matrices are integral in creating the *in vivo* cell adhesions.

B. Effect of Cell Adhesion and Shape on Cell Function

Alterations in ECM–integrin interactions may cause changes in both cell shape and function (Boudreau and Jones, 1999; Folkman and Moscona, 1978). The intracellular signaling pathway activated by integrin binding has recently been implicated as playing a role in ECM-dependent changes in cell shape. Both the magnitude and duration of expression of signaling molecules, such as mitogen-activated protein kinases (MAPK), are affected by integrin binding (Boudreau and Jones, 1999), and alterations in MAPK expression often correlate with changes in cell morphology. The nature of these intracellular signals initiated by integrin–ligand binding ultimately controls cell function. As will be discussed in this section, cell–ECM interactions can determine cell fate, with respect to differentiation, apoptosis, and migration; these relationships are summarized in Fig. 4.

1. Differentiation

The mechanism by which cells switch between proliferative and differentiated phenotypes is important in creating systems that control tissue development. Numerous examples of how the ECM influences cell differentiation can be found *in vivo*; one such example was described in this chapter's introduction, where variations in substrate material were shown to have drastic effects on mammary epithelial cell function (Horwitz, 1997). Several studies have examined the relationship between cell shape and proliferation, where a proliferative phenotype is indicative of a de-differentiated state. Increased spreading of both fibroblasts and endothelial cells proportionally stimulated DNA synthesis, and thus progression through the cell cycle (Folkman and Moscona, 1978). Substrate adhesivity and cell confluence were used to modulate cell shape, resulting in rounder cells synthesizing significantly less DNA than spread cells. Another study

was performed in which cell spreading was increased without increasing the area of cell–substrate contacts (Chen *et al.*, 1997), and this resulted in the same finding, where more highly spread cells displayed increased proliferation. Lastly, photolithographic methods were used to pattern adhesive islands of various sizes to further explore the relationship between hepatocyte spreading and function (Singhvi *et al.*, 1994). When cells are restricted to a minimum spread area, they exhibit more differentiation markers than spread cells.

One cell-type that exhibits drastic switches between growth and differentiation is the hepatocyte. Numerous *in vitro* studies have been performed to investigate whether this alternation between differentiation and growth may be controlled via culture conditions or substrate materials. Some studies have concluded that differentiation can be controlled through the type of ECM presented (Caron, 1990; Sudhakaran *et al.*, 1986; Tomomura *et al.*, 1987), while others have implicated cell–cell interactions (Ben Ze'ev *et al.*, 1988; Bissell *et al.*, 1987), cell shape (Ben Ze'ev *et al.*, 1988; Bissell *et al.*, 1987), matrix rigidity (Opas, 1989), or presence of a 3D matrix environment (Dunn *et al.*, 1989) as key in this modulation. To clarify these results, a study was performed in which cell spreading, ECM composition, and ECM geometry were controlled, enabling the analysis of how ECM molecules switch hepatocytes between growth and differentiation (Mooney *et al.*, 1992). The degree of cell spreading on varying densities of several ECM coatings was identified as a critical determinant in cells switching to a differentiated state. Lower ECM coating densities induced a rounded cell morphology and inhibited proliferation while promoting expression of differentiation markers. Cells on higher density ECM coatings were significantly more spread and exhibited increased proliferation with decreased differentiation. These results also verified that neither cell–cell contact nor 3D culture was necessary to induce hepatocyte proliferation, as had been suggested by other studies.

The conformation of adsorbed proteins is also important in mediating cell differentiation, and implicates specific integrin receptors as playing crucial roles in this event (García *et al.*, 1999). Fibronectin conformation was varied via adsorption to different substrate materials. Differences in FN conformation altered the presentation of the $\alpha_5\beta_1$ integrin binding site in FN, and presentation of this integrin controlled switching between proliferative and differentiated phenotypes in myoblasts. Differentiation of myoblasts occurred with the highest degree of $\alpha_5\beta_1$binding and was completely inhibited by an antibody to α_5 (García *et al.*, 1999). In a similar set of experiments, osteoblasts exhibited enhanced expression of several differentiation markers when cultured upon FN whose conformation allowed for the greatest presentation of the $\alpha_5\beta_1$ integrin binding site

(Stephansson *et al.*, 2002). This modulation of differentiation is specific to the α_5 integrin, as antibodies to α_v did not inhibit differentiation (Stephansson *et al.*, 2002).

The ability to understand the mechanism behind cellular differentiation is important not only to developmental biologists, but also to tissue engineers. Tissue engineering first requires the *in vitro* expansion of a cell population, followed by the seeding of these cells within a scaffold to form a functional tissue replacement. Thus, it is evident that knowledge of how to switch these cells from growth to differentiation is of paramount importance in this application. By employing our knowledge of how this phenotypic switch is modulated, we can tailor biomaterials design in order to promote cell growth or differentiation.

2. *Apoptosis*

Apoptosis, or programmed cell death, can occur following disruption of the ECM by pharmacologic or genetic mechanisms (Boudreau *et al.*, 1995; Stupack and Cheresh, 2002). While apoptosis is necessary for proper tissue development, inappropriately triggered cell death can detrimentally affect tissue function. The survival of many cell types requires integrin-mediated adhesion to the ECM (Ruoslahti and Reed, 1994; Stupack and Cheresh, 2002), and when deprived of extracellular signals, nearly all cell types are susceptible to apoptosis (Meredith and Schwartz, 1997). It has even been suggested that apoptosis is a default pathway that cells enter in the absence of extracellular signals that instruct them otherwise (Raff, 1992). Cells exhibit varying sensitivities to apoptosis, such that no single treatment can effect apoptosis protection for all cell types. Death by apoptosis has been inhibited by exposing cells in suspension to immobilized integrin antibodies (Meredith *et al.*, 1993). These results indicate a role for integrin signaling in the adhesion-dependent control of apoptosis. To further explore the relationship between cell adhesion and apoptosis, a nonadhesive substrate was patterned with small, closely spaced circular adhesive islands (3–5 μm island size), and the extent of cell spreading across these islands was controlled by changing the spacing between them (Chen *et al.*, 1997). In this manner, the authors were able to keep the cell–substrate contact area constant while changing the area of the cell itself (i.e., the same number of adhesive islands per cell, but increased distance between these islands increases cell spreading). The results implicated cell shape as a critical determinant in apoptosis, as increased cell spreading caused decreased apoptosis, with cell–ECM contact area remaining constant. This relationship between cell shape and apoptosis was consistent, although cells cultured with ligands for the β_1 receptor were more sensitive to apoptosis

than those cultured with the $\alpha_v\beta_3$ ligands, indicating the ability of different adhesion receptors to convey distinct death signals.

3. *Migration*

Cell migration across 2D surfaces occurs as a sequence of events. First, the leading edge of the cell protrudes, initiating interactions with the substrate. This step is followed by the contraction of the cell body and finally detachment of the trailing edge. Matrix proteins in the path of migration may force cells to adapt their morphology and/or enzymatically degrade the ECM components via contact-dependent proteolysis or protease secretion (Friedl and Brocker, 2000). The nature of cell–material interactions exhibits a profound effect on a cell's ability to migrate. Both theoretical and experimental evidence indicate that the average speed of cell locomotion exhibits a biphasic dependence on the strength of cell–substrate adhesion (DiMilla *et al*., 1993; Lauffenberger and Horwitz, 1996; Palecek *et al*., 1997; Palecek *et al*., 1999). Maximal cell migration is attained at intermediate cell adhesion strength. Either too weak or too strong adhesion forces induce reduced cell motility. In order to migrate, cells must generate traction forces through surface receptors bound to the ECM. These traction forces are generated by the cytoskeleton at sites of cell adhesion, specifically focal adhesion complexes. Thus, at low adhesiveness, cell–substrate bonds are disrupted by cytoskeletal forces, resulting in the cell's inability to generate the traction required for migration. At high adhesiveness, the cytoskeletal forces are not sufficient to disrupt the strong cell–substrate bonds, also leaving the cell unable to migrate. While integrin-mediated cell motility can be modulated by altering adhesion ligand surface density, cell integrin expression level, or integrin–ligand binding affinity, cell migration speed still ultimately depends upon the cell–substrate adhesion strength (Palecek *et al*., 1997).

Much investigation regarding the effects of cell–substrate interactions on cell migration has focused upon alterations in matrix rigidity. The strength of integrin–cytoskeleton linkages has been found to depend upon the substrate compliance, as cells sense the substrate elasticity and respond by strengthening these linkages proportionally with increases in matrix rigidity (Choquet *et al*., 1997). Cytoskeletal organization is affected by substrate rigidity, as fibroblast actin filaments on stiff substrates are well-defined and distributed throughout the cell, whereas cells on soft materials displayed are extremely fine and localized around the cell periphery (Wong *et al*., 2003). In general, cells cultured on flexible substrates exhibit increased migration rates, with irregular focal adhesions and reduced amounts of phosphotyrosine at adhesion sites (Choquet *et al*., 1997; Katz *et al*., 2000;

Pelham and Wang, 1997). Well-defined, stable focal adhesions are observed in cells cultured on stiff substrates, and this condition is accompanied by decreased cell motility (Pelham and Wang, 1997). The exact mechanism by which cells are able to sense and respond to the mechanics of their substrate has not been completely elucidated, although it is believed that the density and turnover of integrins in focal adhesions sense the elasticity and spacing of extracellular ligands (Wehrle-Haller and Imhof, 2002). The focal adhesion components act as mechanical transducing devices, relaying changes in intra- and extracellular tension into signaling pathways that modify the composition and behavior of focal adhesions, thereby regulating cell contractility and motility (Geiger and Bershadsky, 2001). This establishment of more stable focal adhesions may also lead to the preference of cells to grow on stiff substrates (Lo *et al.*, 2000). This concept of modulating cell adhesion and motility via changes in substrate rigidity has been exploited to synthesize gradient-compliant materials that enable directed cell migration (Wong *et al.*, 2003). Smooth muscle cells were found to migrate toward and accumulate on the stiff regions of the substrate, and the migration speed of cells on the soft regions was significantly higher than that of cells on more rigid areas (Wong *et al.*, 2003). This system illustrates the importance of understanding the mechanisms of focal adhesion regulation of cell function so that control over cell behavior can be achieved in order to synthesize novel materials for use in tissue engineering.

IV. Nonspecific Interactions of Cells with Materials

As discussed earlier in this chapter, cells express numerous types of receptors that mediate interactions with biological substrates. These interactions are specific, and the ligands for the cell receptors may exist as proteins, other receptors, or other biomolecules. Thus far, receptor–ligand interactions have been discussed in the context of cell interactions with components of the natural ECM. Synthetic materials are not intrinsically recognized by cell receptors; instead, their interactions are mediated by protein adsorption to the material, causing cells to interact with synthetic biomaterials via the same ligand–receptor interactions involved in normal binding to the ECM (Elbert and Hubbell, 1996). Even an entirely non biological material interacts with cells through biological pathways, as proteins adsorb to the material, essentially modifying its surface such that the cells recognize it as biological in nature (Castner and Ratner, 2002). In the case of interaction of cells with biomaterials, proteins or other

biological signals may be either adsorbed or intentionally immobilized to the material surface. To control the extent of protein adsorption, material properties such as charge, roughness, and hydrophobicity may be modified. This type of cell–material interaction, which is regulated primarily by protein adsorption and not by the controlled presentation of biological signals, will be discussed as nonspecific cell–material interactions.

A. Dynamics of Protein Adsorption

Proteins play a critical role in regulating cell interactions with both biological and synthetic surfaces. The type and density of proteins presented on a surface are major determinants in cell function. Because proteins are present in all bodily fluids and most cell culture media, it is important to understand the dynamics of protein adsorption to biomaterials. Additionally, protein adsorption to materials impacts the overall performance of biomaterials in several manners, including regulation or inhibition of cell adhesion.

Proteins may adsorb to surfaces for both thermodynamic and electrostatic reasons. The kinetics and thermodynamics of protein adsorption have been reviewed in references Haynes and Norde, (1994); Horbett and Brash, (1995); Ramsden, (2003). The hydrophobic effect is the primary thermodynamic regulator in protein–material interactions, where changes occur in the interaction of water with both the hydrophobic material surface and with hydrophobic amino acid residues on the protein. Specifically, water is poorly bonded to hydrophobic surfaces and forms a more ordered surface layer as it more strongly hydrogen-bonds to itself. Thus, protein adsorption results in an entropic gain as water is released from the hydrophobic material surface and creates a polar surface capable of hydrogen bonding (Hubbell, 1995). Although virtually all surfaces are hydrophobic relative to water, the more hydrophobic materials tend to adsorb larger amounts of protein than hydrophilic ones. Proteins may also interact with surfaces by electrostatic mechanisms. Generally possessing a net negative charge, proteins have a greater tendency to adsorb to cationic surfaces (Horbett and Brash, 1995). However, because proteins contain both positively and negatively charged moieties, they interact to some extent with both cationic and anionic materials. Similarly charged proteins and surfaces may also interact via a soluble multivalent linker of opposite charge, such as Ca^{2+} (Elbert and Hubbell, 1996).

Proteins may adopt an altered conformation upon adsorption to a surface. In solution, hydrophobic sequences within proteins are folded such

that contact with water is minimal. Adsorption on a hydrophobic surface, however, has the ability to cause unfolding of these regions, often resulting in irreversible adsorption of the protein (Kiaei *et al*., 1992, 1995). The conformation of the protein also affects cellular recognition and binding to the material. For example, the nature of a material's surface chemistry has the ability to modulate the conformation of adsorbed fibronectin (FN). Varying surface chemistries results in quantitative differences in the functional presentation of the major integrin-binding domain of FN (Keselowsky *et al*., 2003). Changes in FN conformation can significantly alter the bioavailability of the $\alpha_5\beta_1$ integrin binding site, thereby affecting cell adhesion, proliferation, differentiation, and the composition and localization of integrins in focal adhesion complexes (García *et al*., 1999; Keselowsky *et al*., 2003).

The irreversibility of adsorption of some proteins also emphasizes the importance of understanding the kinetics of the adsorption process. Given a situation where transport of the protein to the material surface is diffusion controlled, Eq. (2) can be used during initial stages of adsorption, where the amount of protein on the surface (Λ) is proportional to the product of the protein concentration in solution (C) and the square roots of protein diffusion coefficient (D) and time (t):

$$\Lambda \propto C(Dt)^{1/2} \tag{2}$$

This relationship allows one to estimate the relative flux of proteins within a multicomponent solution to the material surface. In the case of irreversible protein adsorption, the first protein to arrive stays on the surface permanently, illustrating the importance of understanding the properties of the proteins in solution.

Protein diffusivity, however, is not always the main determinant in the composition of adsorbed protein layers. If this were the case, the composition of the adsorbed protein layer would be the same on different materials exposed to the same solution. This condition is not usually observed (Horbett, 1999), indicating that the affinity of each protein is influenced by the surface chemistry of the biomaterial (Horbett and Brash, 1995). Because proteins differ in affinity for various surface chemistries, the competitive protein adsorption process will also differ, leading to unique protein layer compositions upon different materials. Furthermore, the vast majority of protein adsorption studies have been carried out *in vitro*, assuming that this accurately mimics the *in vivo* environment. However, differences in implant site (i.e., blood-contacting devices vs solid tissue

implants) will result in a different presentation of body proteins, presumably evoking differing cellular responses.

Protein–surface interactions are highly complex, complicating the ability to precisely control the concentration, conformation, and bioactivity of the adsorbed protein. Numerous materials have been modified in order to achieve better control over the adsorption process, thereby enabling better characterization of the material and greater control over cell function. A few of these modifications will be discussed in the following sections.

B. Surface Chemistry

1. Charge

Ionically charged surfaces have long been used to improve cell adhesion upon traditionally nonadhesive substrates. Adhesion and spreading of numerous cell types has been accomplished through the modification of materials to contain charged groups, and positive charges tend to elicit the best cellular response (Davies, 1998). As mentioned earlier, proteins generally possess a net negative charge, thereby leading to the tendency of proteins to adsorb to cationically charged surfaces, such as those created by amine modification.

A variety of methods have been employed to create charged surfaces for the purpose of increasing cell adhesion. These methods include photolithographic patterning (Ito, 1999) or adsorptive coating (Seyfert *et al.*, 1995) of charged chemicals, hydrolytic etching of a polymer surface (McAuslan and Johnson, 1987), or copolymerization with monomers containing ionizable functional groups (Bergethon *et al.*, 1989; van Wachem *et al.*, 1987). Glass slides treated with a variety of polycationic chemicals have been used to promote enhanced leukocyte adhesion (Seyfert *et al.*, 1995). Cell integrity was preserved on all surfaces, and the greatest increase in leukocyte adhesion was observed on materials with the most positive zeta potentials. Studies have examined not only differences in charge identity, but also the effects of charge density on cell behavior (Lee *et al.*, 1997). When both negatively or positively charged molecules were immobilized on surfaces to create a gradient of charge density, both the adhesion and proliferation of chinese hamster ovary (CHO) cells exhibited a biphasic response, with intermediate charge densities causing the greatest increase in both events. This result, however, may be due to changes in surface hydrophilicity with charge density, and not because of the effects of specific functional groups, as CHO cells have previously

been shown to preferentially adhere on moderately hydrophilic surfaces (Lee and Lee, 1993).

Preferential attachment of cells has been demonstrated on multiple types of amine-modified surfaces patterned on hydrophobic substrates in the presence of serum. However, because charge and wettability are related properties, it is often difficult to separate the effects of material wettability from those of surface charge. Thus, it is questionable whether observations of cell behavior that involve varying charge density are actually attributable to the cellular response to changes in material hydrophilicity. In one study, use of either a positively charged quaternary amine or an unprotonated amine surface modification resulted in five-fold greater cell attachment over control or negatively charged surfaces (Webb *et al*., 1998). This suggests that the moderate wettability shared by the quaternary amine and amine surfaces is the major factor in determining cell attachment in the presence of serum proteins.

When cells are cultured in serum-free conditions, the cell response to charged surfaces can change dramatically. Cell attachment to quaternary amine surfaces was significantly higher than on all other materials, including the amine surface, which displayed four-fold less cell adhesion than the quaternary amine surface (Webb *et al*., 1998). This result suggests that positively charged functional groups possess an enhanced role in promoting cell adhesion in protein-free environments relative to when proteins are present. The accepted explanation for this phenomenon is that, in the absence of proteins, the negatively charged chondroitin sulfate within the cell glycocalyx exhibits electrostatic interactions with the positive charges upon the material surface. Chondroitinase ABC inhibits the adhesion of cells to normally adhesive amine surfaces, thus affirming the involvement of chondroitin sulfate in mediating the interactions between cells and cationic surfaces in a protein-free environment (Massia and Hubbell, 1992). Furthermore, negatively charged surfaces achieved the lowest levels of cell attachment in the absence of serum, indicating possible repulsion between the anionic cell surface chondroitin sulfate and the material surface. Single cationic amino acids such as arginine and lysine immobilized on polymer substrates were found to support cell adhesion and spreading via strong interactions with cell surface chondroitin sulfate, while interactions with heparan sulfate were weaker (Massia and Hubbell, 1992). There is potential for this interaction between cell–surface glycosaminoglycans and positively charged oligopeptides on adhesion proteins to be exploited for the design of biomaterials. For instance, using materials with specific cationic oligopeptides, it may be possible to achieve greater selectivity with respect to the type of cell–surface glycosaminoglycan or even the cell type.

2. *Hydrophilicity/Hydrophobicity*

As mentioned in the previous section, surface hydrophilicity can be closely linked to surface ionic charge. In general, either extreme in surface hydrophilicity results in decreased cell adhesion and spreading. Extremely hydrophilic surfaces inhibit protein adsorption, leading to inhibited cell adhesion to the substrate. On the other hand, very hydrophobic materials promote irreversible protein adsorption, with the proteins often so denatured such that they are not recognizable by cells (Williams *et al.*, 1995). Several studies have concluded that optimal cell adhesion occurs on materials of moderate hydrophilicity, with water contact angle measurements averaging 40° (van Wachem *et al.*, 1987; Webb *et al.*, 1998). It has been shown that the conformation of adsorbed proteins can be directly controlled by surface wettability through defined alterations in material chemistry (Keselowsky *et al.*, 2003). The central cell-binding domain of fibronectin was kept most intact when adsorbed on hydrophilic surfaces, and essentially destroyed on the most hydrophobic material, supporting previous studies that suggest protein denaturation occurs on highly hydrophobic surfaces. Thus, it is important to note that material hydrophilicity controls not only the amount or type of proteins adsorbed, but also determines the protein conformation. The more preserved protein conformation observed on hydrophilic materials results in enhanced functional presentation of ligands as well as improved cell adhesion (Keselowsky *et al.*, 2003). This specific study demonstrated that well-characterized and controlled alterations in surface chemistry provide a method by which substrate-directed control of adsorbed protein activity may be achieved, with the goal of manipulating integrin binding to elicit a desired cellular response.

When materials are exposed to dilute serum conditions, hydrophilic surfaces promote greater cell attachment, spreading, and cytoskeletal organization relative to hydrophobic surfaces. Enhanced cell spreading has been observed on hydrophilic surfaces in multiple studies (Ruardy *et al.*, 1995; Schakenraad *et al.*, 1986; Webb *et al.*, 1998). Cells on moderately hydrophilic surfaces have a greater cell area, highly organized actin stress fibers, and more focal adhesions than cells cultured on more hydrophobic materials (Webb *et al.*, 1998). Within the various hydrophilic surfaces, alterations in charge and wettability changed cell attachment but neither spreading nor cytoskeletal arrangement. Cytoskeletal organization in particular appears to depend primarily upon general hydrophilicity or hydrophobicity, with little variation between materials within each category (Altankov *et al.*, 1996; Webb *et al.*, 1998).

C. Surface Topography

There are numerous factors involved in cellular responses to the extracellular environment, as many ECM properties are able to prompt cells to activate specific signaling cascades or function in a certain manner. The importance of protein composition and conformation has been discussed earlier in this chapter. Yet, cells also respond to the topography of their substrate material, as the nature of the topography may impart nonspecific biomechanical stimuli (Abrams *et al*., 2002). Study of native basement membranes from a variety of animal sources has demonstrated that these ECM layers possess a complex topography consisting of intertwining fibers mixed with elevations and pores of varying nanoscale dimensions (Abrams *et al*., 2002). The conserved nature of this topography implies that more than just the integrin–ligand interactions are responsible for directing cell behavior.

In the past, most studies have examined surfaces with topographical features on the microscale level, due to limitations in nanoscale fabrication. However, recent advances in creating surfaces with submicrometer surface features have allowed analysis of more biologically relevant features. Nanometer-size topographical features more closely mimic the natural ECM, thus enabling researchers to more accurately recreate a cell's *in vivo* environment.

Surfaces have been fabricated to contain a variety of types of topographical features, examples of which are listed in Table II [reviewed in Abrams *et al*. (2002) and Curtis and Wilkinson (1997)]. The features that have been most extensively investigated with respect to cell function are grooves. Culturing cells on grooved substrates generally results in significant alterations to cell morphology and cytoskeletal arrangement (Braber *et al*., 1998; Brunette, 1986b; Oakley and Brunette, 1993; Oakley and Brunette, 1995). Specifically, cells align along the long axis of the groove, with cytoskeletal elements such as actin and microtubules also organizing themselves parallel to grooves. This cell alignment is dependent upon not only groove width, but also groove depth. The degree of cell orientation increases with greater groove depth and decreases with increasing groove width (Braber *et al*., 1998; Brunette, 1986a; Clark *et al*., 1991). Grooves or ridges that are much wider than the cells tend to have little effect on cell orientation, although cells may align to one edge. When the width is in the order of the size of a cell, the effects on orientation become more pronounced. Multigrooved materials, which consist of a combination of macrogrooves and microgrooves, have been shown to not only control the alignment of cells, but also the orientation of the ECM (Yoshinari *et al*., 2003). Production and alignment of the ECM was

TABLE II
A Summary of Topographical Features and Cell Types that have been Investigated in the Literature

Topographical features	Cell type	Cell functions examined
Cliffs	Astrocytes	Adhesion strength
Cylinders	Cardiomyocytes	Bridging
Fibers	Chondrocytes	Cytoskeletal organization
General roughness	Dermal fibroblasts	Differentiation
Grooves	Dermal keratinocytes	ECM orientation
Nodes	Endothelial cells	ECM production/mineralization
Pits	Epithelial cells	Focal adhesion formation
Pores	Gingival fibroblasts	Migration
Ridges	Gingival keratinocytes	Morphology/elongation
Spheres	Heart fibroblasts	Orientation
Spikes	Leukocytes	Proliferation
Steps	Macrophages	
Waves	Monocytes	
Wells	Neurites	
	Neutrophils	
	Oligodendrocytes	
	Osteoblasts	
	Smooth muscle cells	

For reviews, see Abrams *et al.* (2002); Curtis *et al.* (1995); Flemming *et al.* (1999).

enhanced on multigrooved substrates when compared to microgrooves alone or smooth surfaces. Grooves are also capable of directing cell migration, with deeper grooves again showing greater efficacy. Extension of cells, specifically neurites, has also been stimulated by growth on grooved surfaces (Curtis *et al.*, 1995).

Other features, such as pores or general roughness, have been shown to significantly alter cell behavior. Surfaces with greater texture can encourage cellular differentiation, causing increased mineralization and ECM synthesis by osteoblasts (Groessner-Screiber and Tuan, 1992) or formation of a stratified epidermis by keratinocytes (Pins *et al.*, 2000). Surface roughness also tended to increase fibroblast alignment as well as the migration of vascular and corneal cells in comparison to smooth surfaces (Eisenbarth *et al.*, 1996; Lampin *et al.*, 1997).

Topographical features do not elicit the same response for all cell types tested. For instance, while all fibroblasts align in grooves, this alignment does not occur at all in either neutrophils or keratinocytes (Meyle *et al.*, 1995). Furthermore, when a variety of materials, such as alumina, titania, and hydroxyapatite, were used to create nanophase (< 100 nm grain size) surface roughness, the effects on cell adhesion differed significantly with cell

type. These results may be explained by the preferential adsorption of certain proteins on the nanophase ceramic surfaces; vitronectin preferentially adsorbed to these materials, thereby promoting the selective adhesion of osteoblasts over fibroblasts and endothelial cells (Webster *et al.*, 2000). However, laminin preferentially adsorbs to conventional ceramics, resulting in increased endothelial cell adhesion and decreased attachment of osteoblasts (Webster *et al.*, 2000). The presence of adsorbed proteins may also impact the alignment of cells on grooves. Cells cultured in the presence of serum exhibited greater alignment with topographical patterns than cells cultured in serum-free media (Abrams *et al.*, 2002). The possibility that proteins may preferentially adsorb to groove and ridge boundaries, thereby contributing to the induction of cell alignment, has been proposed (Braber *et al.*, 1998).

The variables in investigating cell interactions with topographical features include feature type, fabrication technique, material composition, feature dimensions, feature frequency, and cell type (Abrams *et al.*, 2002). A vast amount of literature is available on this topic, and a good review by Flemming *et al.*, (1999) discusses many more cell–substrate interactions that have not been described here. Combinations of these variables allow for thousands of possibilities, often making it difficult to analyze and compare multiple studies to achieve a general conclusion, as alterations in any of the available parameters may change cell behavior. For instance, while increased surface roughness appears to increase cell adhesion, migration and ECM production, this is not always the case (Anselme *et al.*, 2000), and these varying results may be due to fabrication technique, material composition, or cell type.

V. Controlled Cell–Material Interactions

In designing materials for biological applications, it is often desirable to create systems that enable precise control over cell adhesion and function. This goal is not easily accomplished by relying on adsorbed proteins to provide the appropriate biological cues. Composition and conformation of adsorbed protein layers are difficult to control, resulting in materials that do not exhibit specific regulation of cell behavior. These nonspecifically adsorbed protein layers have even been described as "the enemy" (Castner and Ratner, 2002) which must be defeated in order to develop surfaces that control the conformation and orientation of proteins so that the body will specifically recognize them. As displayed in Table I,

several ECM proteins contain short peptide sequences that are sufficient for cell surface receptor recognition. Incorporation of well-characterized biological moieties, such as these peptide sequences, into biomaterials has enabled investigators to examine the effects of specific biological signals on cell function, and to tailor materials such that they contain a specified amount or spatial distribution of these signals. Materials containing specific biological sequences are able to interact directly with cell surface receptors, and do not require adsorbed proteins to mediate the cell–material interaction.

A. Ideal Surfaces

Controlled presentation of biological signals on a material begins with designing materials to which proteins do not readily adsorb. Given the mechanisms of protein adsorption discussed earlier, such a material would likely be very hydrophilic and uncharged. Various synthetic hydrogel materials meet this description, and it has been shown that protein adsorption on these materials is inhibited. Hydrogels consist of a crosslinked network of water-soluble polymers, where the structure of the water around the polymer hinders protein adsorption, as displacement of this water is energetically unfavorable. Several synthetic polymers have been used in the creation of hydrogels, including poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), polyacrylamide, and poly(hydroxyethyl methacrylate). These materials are all biocompatible, in addition to inhibiting protein adsorption.

The nonadsorptive nature of these materials allows for their modification in order to include specified biological signals. Cells will not adhere to hydrogels without chemical or biological modification of the material. While it may seem that lack of cell adhesion to materials intended for tissue engineering applications would not be a desirable property, this is not necessarily the case. This anti-adhesive property is beneficial, as it allows precise, defined modifications of the material to achieve a specific cellular response without interference by nonspecific cell or protein interactions.

Poly (ethylene glycol) (PEG) has been used to not only create scaffolds, but also to improve the material or molecule hydrophilicity and inhibit protein adsorption on a variety of materials (Elbert and Hubbell, 1996; Tirrell *et al*., 2002). Hydrogels consisting of PEG may be synthesized by modification of PEG to contain photopolymerizable acrylate groups, whose crosslinking to form a hydrogel is initiated by radical formation and propagation. In particular, photopolymerization has been exploited to

make PEG gels under physiological conditions and in the presence of cells (Burdick and Anseth, 2002; Mann *et al*., 2001). A variety of chemistries are available in order to tailor the properties of PEG-containing hydrogels to suit the specific application. For instance, PEG may be combined with other photoactive polymers or monomers to form copolymer hydrogels. Block copolymers of PEG with other monomers may also be synthesized. Specifically, block copolymers of PEG with poly(lactic acid) (PLA) are commonly synthesized to design materials with a wide range of degradation times (Anseth *et al*., 2002; Sawhney *et al*., 1993). Biological molecules such as growth factors (Mann *et al*., 1999) and enzymatically degradable or adhesive peptide sequences (Burdick and Anseth, 2002; Hern and Hubbell, 1998; Mann *et al*., 2001) have also been attached to PEG and shown to retain their bioactivity.

Poly (ethylene glycol) (PEG) has been grafted to numerous materials, including polyurethane and poly(ethylene terephthalate), in order to reduce protein adsorption to the these materials, often for purpose of making them nonthrombogenic (Gombotz *et al*., 1991; Han *et al*., 1993). Longer PEG chains appear to be more effective at blocking protein adsorption, yet it becomes more difficult to achieve high graft densities with increasing PEG molecular weight. However, because PEG molecular weight is not expected to be a major determinant in protein adsorption, the observed molecular weight dependence may, in fact, be due to the inability to generate dense PEG packings with other immobilization schemes, and higher molecular weight PEGs somewhat compensate for this by occupying a larger surface area per attachment site (Elbert and Hubbell, 1996). Modification of surfaces via PEG adsorption has also been investigated. Treatment of surfaces with PEG surfactants has effectively reduced protein adsorption, inhibited platelet adhesion, and prevented white blood cell uptake (Elbert and Hubbell, 1996). Self-assembled monolayers have also been fabricated that contain PEG chains (Lopez *et al*., 1993). Alkane thiols terminated with oligo(ethylene glycols) were successful in virtually eliminating protein adsorption (Prime and Whitesides, 1993). In surfaces modified with PEG brushes, it was concluded that the graft density of the surface-bound polymer chains is the predominant factor in determining material nonadhesiveness (Tirrell *et al*., 2002).

The ability of hydrophilic polymers, specifically PEG, to provide a substrate material that is essentially a blank slate is extremely valuable in both investigations of the nature of cell–substrate interactions as well as in the creation of well-defined biomaterials and tissue engineering scaffolds. In this manner, PEG serves as an ideal material which can be modified to contain numerous biological signals.

B. Adhesion Ligands

1. Integrins

As discussed earlier, cells may interact with substrates via integrins specific for proteins adsorbed upon the material. However, a controlled adhesion environment is difficult to achieve when relying upon protein adsorption to encourage cell adhesion. Furthermore, protein adsorption may not be stable, as the proteins can desorb. To overcome these limitations, whole adhesive proteins and peptide segments of these proteins have been covalently immobilized upon and within biomaterials in order to promote and control cell adhesion (Mann *et al*., 2001; Massia and Hubbell, 1990, 1991; Nuttelman *et al*., 2001). When coupled to a normally non-adhesive material, such as PEG, these proteins can be used to control cell function, density, shape, and cell type adherent upon the material. Control of receptor-mediated cell behavior upon biomaterials requires controlling nonspecific interactions between cells and the material presenting the bioactive ligand; this allows the cell response to be attributed solely to the specific receptor–ligand interaction under study.

Extracellular matrix (ECM) proteins such as collagen and fibronectin have been covalently incorporated into poly(vinyl alcohol) polymer matrices (Kobayashi and Ikada, 1991; Nuttelman *et al*., 2001) and polytetrafluoroethylene vascular grafts to render them cell adhesive (Seeger and Klingman, 1987). Using entire protein structures for covalent immobilization, however, can have several drawbacks. Proteins require relatively mild processing conditions, making it difficult to perform some organic synthesis methods which would be useful for their immobilization. Furthermore, proteins are subject to conformational changes, denaturation, or degradation following incorporation into a synthetic material. Yet, as displayed in Table I, matrix proteins contain short peptide sequences that are sufficient for cell adhesion. Covalent attachment of such sequences to substrates mimics attachment of the whole protein in the sense that it allows the conferral of biological properties to a synthetic polymer, yet it also overcomes several difficulties experienced by using the entire protein molecule. Oligopeptides are less susceptible to denaturation and proteolysis, and may be easier to use in organic syntheses.

Many materials have been modified to contain covalently immobilized adhesive peptide sequences, as summarized in Table III [reviewed in Harbers *et al*. (2002) and West and Hubbell (1997)]. Interaction of cells with peptide-modified surfaces can occur directly, without mediation by adsorbed proteins. Issues that affect selectivity in cellular attachment to peptide-modified surfaces include spacer length, peptide surface

TABLE III
EXAMPLES OF SEVERAL ADHESIVE PEPTIDES THAT HAVE BEEN IMMOBILIZED TO A VARIETY OF SUBSTRATE MATERIALS

Peptide	Material
	Agarose
	Alginate
IKVAV	Collagen
KQAGDV	Glass
PHSRN	Poly(ethylene glycol)
REDV	Poly(ethylene terephthalate)
RGD	Polytetrafluoroethylene
VAPG	Poly(vinyl alcohol)
YIGSR	Polyacrylamide
	Polyurethane

concentration, and the particular peptide sequence that is immobilized. Since the identification of RGD as a ubiquitous peptide sequence that is capable of promoting cell adhesion, this short sequence has been the most widely investigated in terms of biomaterial modification. RGD interacts with a number of integrin receptors and can thus bind to most cell types (Humphries, 1990). Cell response to these peptides is extremely specific, as only a single amino acid change (i.e., RGE instead of RGD) can eliminate cell adhesion and reduce the peptide activity by 100-fold or more (Drumheller and Hubbell, 1994; Hautanen *et al.*, 1989). RGD may also be combined with other fibronectin-derived sequences such as EILDV or PHSRN, the latter effecting a synergistic response (Aota *et al.*, 1994; Komoriya *et al.*, 1991). The extent of cell spreading on RGD-modified materials is determined in part by the RGD concentration, with increasing peptide density resulting in increased attachment and spreading (Burdick and Anseth, 2002; Massia and Hubbell, 1990; 1991; Rezania and Healy, 2000). Attachment of GRGDY to polymer-modified glass substrates demonstrated that a peptide concentration of 10 fmol/cm^2 was sufficient to support fibroblast adhesion and spreading, with clustering of integrins and organization of actin filaments (Massia and Hubbell, 1990; 1991). At a surface density of 1 fmol/cm^2, cells were spread, but did not form focal contacts and exhibited abnormal actin fiber organization.

Recent evidence, however, implies that ligand spacing, rather than concentration, is critical in determining the extent of cell adhesion (Griffith and Lopina, 1998). In a system where ligands were tethered to allow for independent variation of ligand concentration and spacing, cell spreading occurred as a function of both ligand concentration and tether

length; spreading was enabled when it was possible for three adjacent ligands to assume positions corresponding to spacing in a high-affinity trivalent branched ligand (Griffith and Lopina, 1998). Many changes in the micro- or nano-environment of immobilized peptides have been shown to exhibit an effect on the nature of cell adhesion (Dori *et al.*, 2000; Houseman and Mrksich, 2001; Maheshwari *et al.*, 2000; Tirrell *et al.*, 2002). For example, peptides presented in clusters of 9 peptides/molecule or higher on materials such as albumin (Danilov and Juliano, 1989) or star-configured PEG (Maheshwari *et al.*, 2000) induce adhesion that is comparable to native matrix proteins and encourage formation of actin stress fibers, whereas presentation of single peptides results in poor cell spreading. Presentation of nonclustered RGD also significantly inhibits fibroblast migration, even when grafted at the same density as clustered ligands (Maheshwari *et al.*, 2000). By using the PEG star molecules, the number of peptides per star and the relative density of stars with and without peptides can be altered to control both the density and spatial arrangement of peptides on a 50 nm length scale (Maheshwari *et al.*, 2000). Other research has demonstrated the necessity of a ligand spacer arm in order to achieve ligand-specific cell adhesion (Hern and Hubbell, 1998). In this study, RGD immobilized with no spacer arm did not mediate ligand-specific cell adhesion and spreading compared to RGD immobilized with a spacer arm consisting of PEG, MW 3400. The reason given for this result was that RGD with no spacer is sterically unavailable; however, this occurrence could also be due to the ability of ligands on a flexible spacer arm to form clusters. Another variable in peptide immobilization is the amino acid conformation, which can be altered by immobilizing the peptide by either its carboxyl end, amino end, or as a looped sequence. Such changes in the nature of peptide immobilization can drastically alter the cellular response (Pakalns *et al.*, 1999). Peptide recognition by cellular integrins may also be controlled by altering the length of surrounding molecules, such as oligo(ethylene glycol) groups (Dori *et al.*, 2000; Houseman and Mrksich, 2001), thus selectively masking the peptides.

Utilization of cell-specific peptide sequences in biomaterials enables the selective adhesion of certain cell types, even in the presence of a mixture of many cell types. As mentioned earlier, REDV promotes the adhesion of endothelial cells, but not other vascular cell types (Hubbell *et al.*, 1991). This selectivity has great potential for endothelialization of vascular devices, where the growth of an endothelial cells, but not fibroblasts or smooth muscle cells, is desired. Another peptide sequence, KRSR, has been shown to selectively promote the adhesion of osteoblasts, which is useful in the rational design of better dental and orthopedic biomaterials (Dee *et al.*, 1998).

These immobilized peptide sequences are capable of controlling aspects of cell behavior that are influenced by cell adhesion and spreading, such as proliferation, differentiation, migration, and extracellular matrix production. As discussed earlier, cell migration can be altered by changing the ligand density, as a high ligand density may restrict cell motility. Matrix mineralization by osteoblasts was also enhanced by higher ligand densities compared to lower ligand concentrations (Rezania and Healy, 2000). However, enhanced cell adhesion and spreading may also be accompanied by decreased cell function. Extracellular matrix production by smooth muscle and endothelial cells was greatest on substrates that were the least cell adhesive, while cells on highly adhesive surfaces displayed significantly decreased ECM production (Mann *et al*., 1999). This result suggests a role of adhesion-mediated signaling events in the regulation of ECM synthesis. As matrix production is a critical part of creating engineered tissues, this study emphasizes the importance of tailoring materials to contain the optimal concentration of ligands such that the desired cellular response is effected.

2. *Proteoglycans/Selectins*

Relative to integrins, interactions of proteoglycans with their ligands have not been widely employed in the design of biomaterials that regulate cell adhesion or function. This lack of investigation is likely due to the fact that these interactions primarily participate in cell–cell contacts, thereby not playing a large role in *in vivo* cell adhesion. However, a few groups have demonstrated that proteoglycan or selectin interactions with ligands can be exploited to create unique biomaterials (Eniola and Hammer, 2003 Ozaki *et al*., 1993). Additionally, investigation of chemical approaches for the development of synthetic glycoconjugate mimics is an active field of study (Bertozzi and Kiessling, 2001; Marcaurelle and Bertozzi, 1999). The chemical synthesis of molecules such as glycopeptides is challenging, but the ability to generate defined glycoproteins on cell surfaces would have a tremendous impact on biological investigation of cells and their interactions with substrates. Research in this area also involves the design of materials that mimic the cell glycocalyx. Such substrates have been synthesized using oligosaccharide surfactant polymers in order to provide a biomimetic surface that suppresses protein adsorption (Holland *et al*., 1998).

Hepatocytes are a cell-type, of particular interest in tissue engineering due to the regenerative capacity of the liver and the quantity of waiting liver transplant recipients, for which there are too few available organ donors. While the asialoglycoprotein receptor on hepatocytes does not

usually promote cell adhesion *in vivo*, monosaccharide asialoglycoprotein ligands have been employed as adhesive ligands in order to induce hepatocyte adhesion to polymer surfaces. Specifically, *N*-acetyllactosamine and *N*-acetylglucosamine have been immobilized on polymer surfaces and found to selectively encourage the adhesion of mammalian and aviane hepatocytes, respectively (Gutsche *et al*., 1994; Kobayashi *et al*., 1994a). When a galactose ligand was bound to particles, it was found that high saccharide surface densities led to internalization of the particles by hepatocytes, which occurs *in vivo* with biological molecules possessing the appropriate monosaccharide ligand (Adachi *et al*., 1994). Lower ligand densities prevented particle internalization and encouraged cell attachment to the particle. Microparticles have also been modified with monosaccharides for the purpose of targeted drug delivery (Cho *et al*., 2001a,b). These drug delivery carriers possess an immobilized saccharide such as galactose, which then localizes to hepatocytes through its unique interactions with asialoglycoprotein.

The use of lectins on biomaterial surfaces may also provide a means of achieving cell-selective adhesion. The lectin Ulex europaeus I (UEA I) has a high affinity for endothelial cell surface glycoproteins. Covalent immobilization of UEA I on poly(ethylene terephthalate) resulted in a 100-fold increase in endothelial cell attachment, while adhesion of monocytes, smooth muscle cells, and fibroblasts was decreased (Ozaki *et al*., 1993). Endothelialization of vascular grafts and other blood-contacting devices is highly important in maintaining a nonthrombogenic implant, and these results indicate that UEA I may be a useful ligand in achieving this goal of selectively encouraging endothelialization *in vivo*. Furthermore, this study demonstrates the potential of lectin–carbohydrate interactions for biomaterial modifications.

Most material modifications with biological molecules involve alteration of a polymeric biomaterial such that it may be recognized by cells. A novel alternative to this scheme is the formation of biomimetic polymeric cells that recognize native tissues as their substrates. As discussed earlier, selectins mediate the rolling of inflammatory cells such as leukocytes on the vascular endothelium and their subsequent extravasation. Acute inflammatory indicators, such as histamine or thrombin, induce increased expression of P–selectin, thereby facilitating leukocyte binding. Chronic inflammation is accompanied by expression of E–selectin. The interaction of selectins with their carbohydrate ligands is transient, yet highly specific. Furthermore, selectin expression is carefully regulated and localized, making selectins excellent candidates for participation in targeted drug delivery (Juliano, 2002).

One group has modified the surfaces of degradable microspheres loaded with an anti-inflammatory drug to contain sialyl-Lewis^x (sLe^x), a sialylated fucosylated carbohydrate which mediates rolling on selectins (Eniola and Hammer, 2003). These sLe^x-modified microspheres specifically interacted with P-selectin-coated surfaces and exhibited similar interactions with the selectin surface as those observed *in vivo* between neutrophils and activated endothelial cells. The rolling velocity of the polymeric cells was controlled via changes in the sLe^x density or wall shear stress. Increasing sLe^x site density decreased rolling velocity, as an increase in ligand density results in the formation of more sLe^x–selectin bonds, thereby slowing down the rolling. Because this method delivers anti-inflammatory drugs via the same route used during *in vivo* recruitment of inflammatory molecules, it displays several advantages over conventional anti-inflammatory drug administration. This system enables localized, sustained delivery of a therapeutic molecule, thereby bypassing the transport limitations and gastrointestinal side effects associated with traditional drug therapy. Additionally, the chemistry used to attach the sLe^x ligands can be extended to develop several different artificial polymeric cell types, including lymphocytes, bone marrow cells, and macrophages.

VI. Conclusion

In order to synthesize rational biomaterials, it is necessary to understand the interactions between the cell and its external environment, specifically materials. Knowledge of how cells interact with native proteins and matrices contributes to the synthesis of biologically recognizable materials. These cell–matrix interactions may be regulated by protein adsorption to materials, as well as by various material physical properties such as surface charge, hydrophilicity, and topographical features. Additionally, materials may also be designed to display only specific biological molecules, such as certain cell adhesion peptides. These modifications create a controlled environment in which cell behavior can be regulated and examined to achieve the desired response. Aspects of cell function, such as differentiation, apoptosis, proliferation, and extracellular matrix production, may be regulated by the expression of specific adhesion molecules, as well as by their density and spatial organization, thus simulating the clustering that occurs upon attachment to native ECM proteins. Modification of materials with biological sequences also allows the creation of biomimetic materials that capture essential features of native matrices, but whose composition is well-defined and characterized.

Exploitation of our knowledge of cell–material interactions can allow the bioengineer to rationally design appropriate materials for applications such as tissue engineering.

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