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CHARACTERIZATION OF THE ADHESIVE INTERACTIONS BETWEEN CELLS AND BIOMATERIALS

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7.1 INTRODUCTION

Cellular adhesion is critical for many cellular functions, including spreading, proliferation, and migration. The interactions between cells and their environment are mediated by adhesion receptors located on the cell surface. Adhesive interactions can take place between cells or between a cell and the surrounding extracellular matrix (ECM). Adhesion receptors are responsible for both types of interactions. Biomaterials have been introduced as a means to facilitate cell adhesion and infiltration during the repair or replacement of damaged or diseased tissues. For biomaterials to successfully act as an alternative ECM, the interactions between cells and biomaterials must mimic the adhesive interactions in native tissue. Therefore, the control and optimization of adhesive interactions is an important aspect of material fabrication. Cell–material interactions can be regulated through material design and processing. This chapter focuses on the adhesion receptors responsible for the interactions that occur within native tissue, current biomaterial fabrication methods that attempt to mimic these interactions for tissue engineering applications, and measurement techniques that investigate cell–substrate and cell–cell adhesion strength.

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7.2 ADHESION RECEPTORS IN NATIVE TISSUE

Cells form connections with the ECM as well as with each other through adhesion receptors that are present on the surface of the cell. There are three major classes of adhesion receptors: integrins, cadherins, and members of the immunoglobulin (Ig) family. Although the exact functions of each class of receptor may vary, many adhesion receptors share some common properties, including the formation of receptor clusters after binding with an extracellular ligand and the formation of connections to the underlying cytoskeleton.

7.2.1 Integrins

Integrins are a superfamily of cell adhesion receptors that exist as 24 distinct transmembrane αβ heterodimers,[1] which can be found in Table 7.1. Currently, there are 18α and 8β subunits identified, which associate through noncovalent interactions.[2] The term *integrin* originates from the importance of such receptors at maintaining the "integrity" of the cytoskeleton. Integrins primarily interact with

TABLE 7.1 Integrin Heterodimers and Their Extracellular Matrix Binding Sites

β Subunit	α Subunit	ECM Binding Site
β_1	α_1	Laminin, collagen (GFOGER)
	α_2	Collagen (GFOGER), laminin, E-cadherin
	α_3	Laminin
	α_4	VCAM-1, ICAM-4, fibronectin
	α_5	Fibronectin (RGD)
	α_6	Laminin
	α_7	Laminin
	α_8	Fibronectin (RGD)
	α_9	VCAM-1
	α_{10}	Collagen (GFOGER), laminin
	α_{11}	Collagen (GFOGER)
	α_v	Fibronectin (RGD)
β_2	α_D	ICAM, VCAM-1, fibronectin, fibrinogen
	α_L	ICAM, ICAM-2, ICAM-4
	α_M	ICAM, ICAM-4, fibrinogen
	α_X	ICAM, ICAM-4, fibrinogen, collagen
β_3	α_v	Fibrinogen, fibronectin (RGD), ICAM-4
	α_{IIb}	Fibrinogen, fibronectin (RGD)
β_4	α_6	Laminin
β_5	α_v	Vitronectin (RGD)
β_6	α_v	Fibronectin (RGD)
β_7	α_4	VCAM-1, fibronectin
	α_E	E-cadherin
β_8	α_v	Vitronectin (RGD)

ECM ligands but also have the ability to interact with cell surface ligands. Integrins serve as a connection between the extracellular environment, where they bind to a ligand or adjacent cell surface, and the intracellular environment, where they bind to the cytoskeleton. Individual integrins may bind to multiple ligands, and multiple integrins can share the same ligand.[3] Integrin activation results in alterations of cell behavior (e.g., adhesion, proliferation, shape, survival or apoptosis, motility, gene expression, differentiation).[1] Because of the importance of integrin activation on cell function, biomaterials can be designed to mimic integrin interactions and achieve specific cell functions.

The function of integrins as transmembrane links between their extracellular connections and the cytoskeletal elements within the cell often plays an important role in mechanosensing. With the exception of $\alpha 6\beta 4$, which links to the intermediate filaments of the cytoskeleton, most integrins form intracellular connections with the actin cytoskeleton.[2] This anchoring function of integrins plays an important role in several cell functions, including blocking apoptosis and triggering the progression of the cell cycle.

Current research has supported the function of integrins in mechanotransduction, indicating that integrin activation and initiation of downstream signaling pathways can result in multiple cellular responses, including ECM remodeling, differentiation, and survival signaling. In cardiomyocytes, hemodynamic overload results in stimulation of cell growth and survival signaling.[4] Because of the stretch resulting from hemodynamic overload, integrin binding domains on the ECM become exposed, triggering integrin activation and the initiation of downstream signaling. Similarly, intracellular integrin activation can occur through the deformation of the underlying cytoskeleton because of stress.[5] Structural alterations of the actin-filamin cytoskeleton expose binding sites for the β tails of integrins, causing activation and stimulation of downstream signaling pathways.

Integrin activation as controlled through substrate stiffness has recently been shown to play a role in both osteogenic differentiation and tumor progression. The differentiation of mesenchymal stem cells into osteoblasts varied with the stiffness of the matrix, resulting in greater differentiation on stiffer substrates.[6] Additionally, a similar correlation was found for $\alpha 2$ integrin expression, indicating that this integrin subunit may play a role in transmitting mechanical signals into downstream signals for differentiation. This hypothesis was confirmed through a knockdown of $\alpha 2$ by siRNA that resulted in a downregulation of osteogenic differentiation.[6] The integrin $\alpha 5\beta 1$, which is important in the formation and remodeling of the fibronectin network of the ECM, has been shown to play a role in matrix stiffening and tumor progression. Increased matrix stiffening as a result of integrin activation was shown to accelerate tumor metastasis.[7] Understanding the mechanisms of tumor progression is critical for developing methods of prevention or treatment.

The role of integrins in mechanotransduction should be exploited in order to initiate or inhibit downstream signaling in response to integrin activation from mechanical stress. Biomaterial design, specifically material properties and three-dimensional structure, should address ways to promote integrin activation in situations when activation can lead to positive effects such as cell survival or

differentiation but also address means to inhibit integrin activation. Inhibition of integrin responses to mechanical changes that occur during the progression of cancer could have significant therapeutic implications. Nanoparticles that contain integrin-like particles within their membranes could bind to ligands on the ECM and prevent ECM ligand binding to integrins on the cell membrane, inhibiting integrin activation and the associated downstream effects. Additional studies on the signaling pathways involved in cancer progression may also reveal how to counteract integrin activation intracellularly. If there is an antagonistic signaling pathway that can be activated to stop downstream signaling or reverse ECM remodeling, therapeutic mechanisms could be designed to target the activation of those antagonist receptors.

Integrin molecules have been shown to cluster upon activation, particularly as a result of binding to a component of the ECM. Integrin clustering triggers the formation of focal adhesions, which are complexes that can transmit mechanical and regulatory signals. Focal adhesions are critical for several types of downstream signaling, including tyrosine phosphorylation, cellular pH elevation, enhanced phosphatidylinositol-4,5-biphosphate (PIP2) synthesis, and activation of the mitogen-activated protein kinase (MAPK) cascade, among others.[3] Focal adhesions provide a signaling platform that can mediate several subsequent reactions to ligand binding at once because of the proximity of activated integrins and their downstream effectors. Current research has implicated abnormalities in focal adhesion formation in several disease states, including some types of cancer, rheumatoid arthritis, and cardiovascular disease.[8] The assembly and disassembly of focal adhesions is not fully understood, but Rho GTPase is thought to play a crucial role.[3,9] The formation of focal adhesions is an important aspect of mechanosensing and it is generally thought that adhesive interactions that result from focal adhesions are stronger than those that are formed by a single ligand-receptor binding event. Engineering biomaterials to contain functional signaling platforms that promote focal adhesion formation could provide a means to study and control the signaling of cells *in vitro*.

In addition to being regulated by ligand binding, integrin function can be controlled intracellularly. For many integrins, the active state is not constitutive. These integrins exist on the cell surface in an "off" or inactive state in which no ligand binding or downstream signaling can occur until there is activation by an intracellular signal.[2] Platelet activation is an example of this type of integrin regulation. To be capable of binding to fibrinogen, von Willebrand factor (vWF), and fibronectin, the integrin $\alpha IIb\beta 3$ must be internally activated. Inside-out activation can occur through several different routes, including through thrombin, adenosine diphosphate (ADP), or epinephrine signaling, which function through G protein–coupled receptors; through signaling, which occurs through the vWF receptor; or through collagen signaling, which occurs through the collagen receptor and the integrin $\alpha 2\beta 1$.[2] Recently, it has been shown that the cytoskeletal protein α-actinin plays an important role in the "inside-out" signaling that activates the platelet ligand $\alpha IIb\beta 3$.[10] An understanding of integrin activation in platelets could lead to the development of drugs and biomaterials that can help initiate clotting from both the inside and the outside.

Some integrin–ligand pairings are more common than others, and specific sequences that appear frequently have been identified. Among the most common of these is the arginine-glycine-aspartic acid (RGD) sequence. Approximately one-third of integrins have binding sites for the RGD tripeptide, which can be found on many ECM proteins, including fibronectin, vitronectin, fibrinogen, and the latency-associated peptide (LAP) complex part of inactive transforming growth factor β. Although the RGD sequence is not readily exposed by collagen or laminin, there are cases in which denaturation or cleavage of these proteins results in exposure of the RGD sequence and subsequent integrin binding.[11] The RGD-binding integrins include all five of the αV integrins, two β1 integrins, and the αIIbβ3 integrin. RGD binding integrins can bind a large number of ECM and soluble vascular ligands. The ligands that contain the specific tripeptide active site bind with the integrins through an identical atomic basis.[12]

The affinity of integrins to the RGD sequence has been exploited extensively in tissue engineering research and therapy development. Recently, nanocarriers with RGD tethering on the surface have been shown to use the integrin–ligand specificity to target tumors that are rich with RGD-binding integrins.[13] Optimization of this drug delivery vehicle to increase the specificity, targeting, and loading efficiency of the nanocarrier can have a significant therapeutic impact. RGD peptides have also been shown to positively influence the differentiation of mesenchymal stem cells into articular chondrocytes[14] as well as the development of functional cardiac tissue from neonatal cardiac cells.[15]

Similar to the RGD sequence, the tripeptide leucine-aspartic acid-valine (LDV) is a common ligand among a group of integrins. LDV is an acidic motif that is functionally related to RGD and is suggested to bind to integrin receptors in a similar fashion.[16] LDV is present on fibronectin, and a related sequence is present on vascular cell adhesion molecule 1 (VCAM-1). The β2 integrins as well as α4β1, α9β1, and α4β7[9,11,16] contain a binding site for the LDV ligand.

Although RGD-binding integrins can recognize the RGD sequences that are exposed when collagen is degraded or cleaved, another specific amino acid sequence can be recognized by integrins when collagen structure is intact. The glycine-phenylalanine-hydroxyproline-glycine-glutamic acid-arginine (GFOGER) sequence exists on triple helical collagens. The sequence is recognized by a group of collagen-binding integrins, including α2β1, an important integrin in hemostasis. It is speculated that the GFOGER sequence is exposed once per microfibril unit of collagen.[11] The proximity of the ligand sequences on a microfibril of collagen promotes integrin clustering and focal adhesion formation, the importance of which has already been discussed.

Similar to collagen, binding sequences on laminin are only recognized by RGD-binding integrins if the ECM protein has been disrupted. The integrin binding sequence tyrosine-isoleucine-glycine-serine-arginine (YIGSR) has been discovered as the minimum sequence necessary to promote binding and adhesion between integrin receptors and epithelial cells on intact laminin.[17] YIGSR is found to be highly active in epithelial cells yet much less active in chondrocytes, osteoblasts, and fibroblasts.

7.2.2 Cadherins

Cadherins are a superfamily of glycoproteins that function to mediate cell-to-cell adhesions. Most cadherins are composed of an extracellular domain that sets up interactions among neighboring cells, a transmembrane domain, and a cytoplasmic domain, which is often linked to the elements of the cytoskeleton.[18] Cadherins are calcium-dependent molecules, and the calcium-binding domain is conserved throughout the various types of cadherins within the superfamily.[19] Because of the involvement of cell-to-cell adhesion in numerous cellular processes, cadherins have been found to contribute to cell signaling, recognition, and sorting in addition to cell adhesion. Alterations to normal cadherin function have been linked to several diseases, particularly cancer.

The cadherin superfamily can be divided into at least six subclasses. The oldest and most well understood are the classical cadherins: E-, N-, and P-cadherins as well as VE-cadherin. Classical cadherins are single spanning transmembrane proteins that primarily function in the formation of adherens junctions. Adherens junctions are typically located on epithelial cells and are formed by the interaction of classical cadherins.[20] E-cadherin is primarily associated with adherens junctions, but similar structures exist in a variety of epithelial cell types; for example, in squamous epithelial cells, both E- and P-cadherin independently form adherens junctions.[21] The cytoplasmic domain of adherens junctions bind to β-catenin or plakoglobin, which in turn bind to α-catenin. α-Catenin links the cadherin–catenin complex to the actin cytoskeleton either through direct binding to actin or indirect binding to vinculin, ZO-1, or α-actinin, which leads to actin binding.[20] Evidence has shown that lateral clustering of cadherins occurs in the formation of adherens junctions and that the redistribution of cadherin binding sites is a means to regulate cell adhesion as well as stimulate a stronger adhesion between cells.[22]

The classical cadherins also play a vital role in development. It has been shown that N-cadherin functions in neural development, including retina development and the formation of neural nodes and neural networks. Recently, attempts to mimic the N-cadherin structure present during neural development has shown promising results, including induction of the differentiation of neural stem cells and desirable cell–cell interaction.[23]

Desmosomal cadherins function in the formation of desmosomes and are one of the few types of cadherins that bind to the intermediate filaments of the cytoskeleton rather than actin. Desmocollin and desmoglein are the two subfamilies of the desmosomal cadherins. Tissues that undergo mechanical stress, such as the epidermis and the myocardium, are rich in desmosomes.[19] Structurally similar to adherens junctions, desmosomes link to the intermediate filaments of the cytoskeleton. Desmosomes are the result of a heterotropic interaction between one desmocollin and one desmoglein cadherin. The cytoplasmic domains of desmosomes directly link to plakoglobin, which binds to a second intermediate protein, desmoplakin.[24] Desmoplakin forms the connection between the cadherin complex and keratin intermediate filaments.[25] Similar to adherens junctions, evidence indicates that desmosomal cadherins cluster to form desmosomes.[26] Desmosome expression

has been found not only to be tissue specific but also cellularly specific within a single tissue, such as in different strata in the same stratified epithelial tissue.[27] Because of this specificity, it is hypothesized that desmosomes may play a role in epithelial tissue differentiation.

Other subclasses of the cadherin superfamily include the protocadherins, the 7TM-cadherins, the T-cadherins, and the FAT family of cadherins. Protocadherins are a very large family of cadherins that exhibit moderate adhesive activity. The major subfamilies of the protocadherins are μ-protocadherin and CNR-cadherin. Although the exact function of these cadherins is still unclear, it is hypothesized that they play a role in the development of the nervous system.[19] The 7TM-cadherins are a family of membrane proteins that contain seven transmembrane segments and function similarly to G protein–coupled receptors and have a large impact on cell adhesion related signaling. The Flamingo cadherin is one of the better studied 7TM-cadherins, and it is thought to have an important role in establishing the polarity of the cell.[19]

T-cadherins are the only type of cadherins that have no transmembrane or cytoplasmic domains. Rather, the T-cadherin is linked to the membrane through a glycosylphosphatidylinositol (GPI) anchor. GPI-anchored proteins are thought to be more densely located within lipid raft domains, which are known signaling platforms. Research in cardiomyocytes suggested that T-cadherins may also be located in lipid rafts and therefore function in cell signaling.[19,28] Cadherins in the FAT family have very large extracellular domains and are most highly expressed by proliferating cells that are undergoing development rather than in adult tissues. This observation has led to speculation that FAT cadherins have functions that are beyond cell–cell adhesion and are more closely related to cell migration and maturation during morphogenesis.[19]

Similar to the research that has been done to exploit integrin–ligand interactions for tissue engineering, the cell–cell interactions and downstream effects that result from cadherin activation could be of interest in many applications, including stem cell differentiation studies and engineering of epithelial and endothelial layers. Biomaterial design should consider the inclusion of cadherin-like particles on the surface to promote cell attachment and necessary interaction between cells.

7.2.3 Immunoglobulins

Immunoglobulins are a superfamily of membrane proteins that share a common domain referred to as the Ig fold motif.[29] Igs have been found to have an important role in the activation and regulation of the immune system because immune cells must be nonadherent when circulating the blood and lymph but become adherent when migrating through tissue.[30]

There are three main subfamilies of Igs, which function similarly in different tissue types. The intracellular cell adhesion molecule (ICAM) family are type I transmembrane glycoproteins that contain two to nine Ig domains. ICAM-1 is expressed constitutively in venular endothelial cells and some leukocytes and can be stimulated by cytokines.[29] ICAM-1 can serve as a ligand to some integrins, which

is an important part of immune system activation. ICAM-1 has recently been used as a way to attract stem cells to an area of injury on the endothelium. By coating the surface of mesenchymal stem cells with antibodies to ICAM-1, Ko et al.[31] were able to successfully target the interaction between the stem cells and the endothelium. ICAM-2 is expressed constitutively on platelets and endothelial cells but unlike ICAM-1 is not affected by cytokines. ICAM-4 is expressed on erythrocytes.

VCAM is a transmembrane protein that contains six or seven extracellular Ig domains. Similar to ICAM-1, VCAM can also function as a ligand for integrins and is responsive to cytokines.[29] VCAM can be expressed on both vascular and nonvascular cells and is an important mediator in some cell signaling pathways. Junctional adhesion molecules (JAMs) are also type I transmembrane proteins that contain two extracellular Ig domains and are found in the tight junctions of endothelial and epithelial cells.[32] JAM proteins are known to form homodimers and have an important role in the trafficking of leukocytes.

Research in biomaterial development for tissue engineering applications aims to mimic the native adhesive interactions that are mediated by integrins, cadherins, and immunoglobulins. Controlling the interactions between cells and the underlying substrate offers a means to control the downstream effects of cell adhesion, which includes cell spreading, proliferation, and migration. In the following section, modifications that intend to optimize these interactions are discussed.

7.3 OPTIMIZATION OF CELLULAR ADHESION THROUGH BIOMATERIAL MODIFICATION

Degradable polymeric scaffolds are typically used *in vitro* and *in vivo* in the field of tissue engineering and serve as a temporary matrix that can be seeded with cells to promote healing, proliferation, and differentiation at an injury site. Polymeric scaffolds must meet certain criteria before being used: they must have degradation, mechanical, adhesive, and biocompatible properties that will result in proper healing and regeneration of tissue at the implant site.[33] Some of the commonly used natural and synthetic polymers are discussed briefly in this chapter, but for more thorough information on the properties these materials, please see Tables 7.2 and 7.3 for a list of materials as well as suggested references for further information.

Several factors play a role in how cells adhere and respond to biomaterials. On a basic level, the hydrophilicity of a material has an effect on cell adhesion. In a study completed by Schakenraad et al.,[34] several commonly used polymers were tested, and the results showed that those with a higher degree of hydrophilicity better supported cell adhesion than those that were hydrophobic. Although cells may prefer a hydrophilic polymer *in vitro*, biomaterials always exist *in vivo* in the presence of a protein solution. In the native environment, cells rarely interact with biomaterials directly but instead interact with an adsorbed protein layer on the surface of a material. Protein adsorption is also related to the hydrophilicity of a material. Highly hydrophilic materials resist adsorption and therefore resist cell adhesion *in vivo*.

TABLE 7.2 Commonly Investigated Natural Biomaterials for Tissue Engineering Applications

Material	Key Reviews	Current Research
Polysaccharides		
Agarose	33	76–78
Alginate	51	52–54
Hyaluronic acid	79	80–82
Chitosan	83	84–86
Polypeptides		
Collagen	87	88–91
Gelatin	92	93–95
Silk	96	97–99

Poly(ethylene glycol) (PEG) is an example of a hydrophilic synthetic polymer that resists protein adsorption. This quality is exploited for applications in which cell adhesion is not ideal. As a linear chain, PEG has poor overall material properties, but as a network, its properties are greatly improved. PEG is nondegradable *in vivo*, but this shortcoming can be overcome by copolymerization. Copolymerization with degradable moieties such as lactic acid has been shown to result in degradation of these modified PEG scaffolds. In such scaffolds, the bioactive moieties degrade rapidly, breaking apart the PEG polymer into monomer degradation products.[35] Although the degradation of modified PEG varies based on the material chemistry, average rates tend to reach 100% degradation after the first month after implantation.[35] Modifications and copolymerization of PEG is common for tissue engineering applications. For example, a modified PEG hydrogel has recently been used as an injectable scaffold for cartilage tissue engineering.[36] Copolymerized scaffolds

TABLE 7.3 Commonly Investigated Synthetic Biomaterials for Tissue Engineering Applications

Material	Key Reviews	Current Research
Polyesters		
Poly(glycolic) acid	100	101–103
Poly(L-lactic) acid	104	105,106
Poly(D,L-lactic acid-*co*-glycolic acid)	107	47–50
Poly(ε-caprolactone)	108	109–111
Poly(propylene fumarate)	112	113–115
Polyorthoester	116	
Other		
Polyanhydrides	117	118,119
Polyphosphazenes	120	121–123
Polycarbonates	100	124,125
Poly(ethylene glycol)/poly(ethylene oxide)	126	36,37,127,128
Polyurethane	129	130,131

composed of an alginate–PEG combination have shown promise in islet of Langerhans encapsulation, with the double cross-linking properties of the scaffold allowing for better scaffold stability.[37]

The adsorption of ECM proteins onto the surface of biomaterials is one of the simplest means to improve cell attachment to a biomaterial and can be increased by using the use of culture medium that contains serum. In general, the major components of serum are albumin, vitronectin, and fibronectin. Protein absorption on a biomaterial surface as a mediator of cell adhesion has been demonstrated extensively in the literature.[38–41] A study conducted using a hydrophobic self-assembled monolayer was incubated in a fibronectin solution before cell seeding and showed that because of to the fibronectin adsorption, fibroblasts were able to strongly adhere to the scaffold and maintain adhesion under applied shear stress conditions.[42] In addition to serum, a more specific protein solution can be used to pretreat a tissue engineering scaffold to promote cell adhesion. Use of a specific protein solution adds an additional layer of control or targeting and can result in interactions between the cell and the material, which can in turn mediate desired downstream effects on cell behavior.

One method of optimizing cell adhesion to a tissue engineering scaffold is to incorporate adhesion motifs within the scaffold composition. As mentioned earlier, known adhesive domains such as RGD and YIGSR are present within the ECM and promote adhesion in the native environment via integrin binding. Interactions between the cell and one of these domains can promote anchorage, migration, and signal pathway activation, which in turn mediates numerous intracellular reactions.

To exploit these adhesive domains for tissue engineering applications, short bioadhesive peptides have been tethered onto the surface of synthetic and natural polymers. Molecules are typically tethered through the use of PEG or poly(ethylene oxide) polymer spacers so that the bioactive molecule can be presented to a cell.[43,44]

Bioadhesive peptides can be derived from natural or synthetic sources, each with advantages and disadvantages. Naturally derived bioadhesive peptides have been successfully used in biomimetic material studies but are very difficult to isolate and purify, especially while maintaining functionality. Because of this shortcoming, synthetic bioadhesive peptides are commonly fabricated and used for biomimetic applications.[45]

The inclusion of bioadhesive peptides has shown significant enhancement of cellular activities.[44,46] Peptides are typically tethered in a random yet spatially uniform manner across the surface of the biomaterial. Recently, studies have shown that if the peptides are arranged within clusters, the cellular response is increased.[46] As discussed earlier, integrin clustering occurs during adhesive interactions to promote stronger or multiple simultaneous downstream effects. In a study investigating how cell adhesion with an orthopedic implant can be used to promote better tissue integration, Petrie et al[46] showed that clustering of bioadhesive ligands on the surface of the implant upregulated osteogenic signaling and differentiation of human mesenchymal stem cells.

Polyesters are a commonly used type of synthetic material (see Table 7.3) that typically undergoes hydrolytic degradation. Poly(glycolic acid) (PGA), poly(L-lactic acid) (PLLA), and poly(D,L-lactic acid-*co*-glycolic acid) (PLGA) are polyesters and

are among the most widely used synthetic polymers.[33] PLGA is a copolymer of PLA and PGA. The copolymer is amorphous and exhibits a faster degradation rate and lesser mechanical strength than PLLA alone depending on processing. PLGA undergoes bulk degradation as a result of ester hydrolysis, the rate of which can be controlled by altering the ratio of PLA and PGA in the copolymer. PLGA has been used extensively and has been shown to support new tissue growth in several bone tissue engineering applications.[47–49] PLGA has also been shown to promote restoration of function when seeded with neural stem cells *in vivo*.[37,50]

Polyesters are typically nonadhesive materials, but their material properties offer ideal conditions for many tissue engineering materials such as smooth muscle cell culture. To overcome the adhesion limitation, Ilagan and Amsden[44] tethered RGD sequences to the surface of the polyester material through the use of a PEG spacer. The results of the study showed that inclusion of the bioadhesive peptides significantly improved cell adhesion and proliferation.

In addition to the use of a polymer spacer, nanopatterning is another means to tether bioadhesive peptides to the biomaterial surface. Fabrication is achieved through a number of techniques, including self-assembly, self-assembling monolayers, stamping, and nanoprinting.[45] Alginate is a natural polymer that is commonly used for nanopatterning studies. Alginate forms a hydrogel when exposed to divalent ions, such as calcium. Gelling is easily reversed by sequestering ions through the use of a chelating agent.[51] Alginate offers limited cell attachment without modification, although this has been shown to be ideal for the culture of hepatocytes, resulting in native cluster formation and albumin production *in vitro*.[52] Alginate has also been used in studies with fibroblasts, in which the cells were found to maintain their function for a prolonged period of time within an alginate sponge.[53] Similarly, chondrocytes have been found to maintain their native phenotype more effectively in the three-dimensional environment provided by alginate.[54]

Because of the inert nature of alginate, it is a popular candidate for modification by nanopatterning the surface with tethered bioadhesive peptides. In a study conducted by Comisar et al.,[55] an alginate hydrogel was coupled with RGD, which was nanopatterned into "high-density islands." Results showed that the pattern of the islands elicited different cellular responses. Whereas focal adhesion kinase (FAK) phosphorylation, an important marker for focal adhesion formation and cell spreading, was most responsive to closely patterned islands, osteogenic differentiation occurred when islands were farther spread apart.

Similar studies have investigated how the density and placement of adhesive peptides as well as other bioactive molecules such as proteins and growth factors affects the behavior of cells that are seeded onto modified scaffolds.[43,56,57] Because adhesion receptors cluster upon ligand binding, closely packed patterns of bioactive molecules tend to elicit different cellular responses than those that are more spread out. Nanopattern fabrication can also be achieved through the use of a self-assembled monolayer (SAM). Self-assembled monolayers are typically formed using thiol molecules assembled in a designated pattern onto a substrate such as gold or glass.[58] Bioactive molecules have been successfully attached to the thiol molecules and have been shown to mediate changes in cell behavior such as proliferation and differentiation.[43,57]

In addition to integrin binding domains, cadherins have also been attached to the surfaces of biomaterial scaffolds and have been shown to promote angiogenesis.[45] There are still some inherent limitations with the use of adhesive oligopeptides, however. Synthetic peptides have much lower activity than that of the native ligands and have limited specificity. There are also several conformational differences between the native adhesive domains and the synthetic ones, which can similarly result in lower adhesion activity and specificity.

The physical topography of a biomaterial surface can also influence the adhesion of cells to the surface. The topography of a material has the ability to create strict sites of cell adhesion. Similar to tethering bioadhesive peptides, the creation of sites for cell–material interactions more closely mimics the *in vivo* environment of the tissue. Contact guidance is the ability of cells to spread with directionality, which is often dictated by the topography of the material.[59] Micro- and nanofabrication techniques have been used to exploit native contact guidance.

An example of micro topography used in tissue engineering applications is the use of patterned co-cultures in the creation of tissue-engineered constructs. Patterned co-cultures allow for control over the degree of contact, including cell–cell contact as well as cell–material interactions, and are created through a variety of possible microfabrication techniques, including photolithography, microfluidics, and polydimethylsiloxane (PDMS) stencils.[60] Parallel grooves have also been used to promote a variety of adhesive interactions. Receptor-binding domains can be concentrated on the raised portions of the grooves so the adhesive receptors on cells can better recognize the domains and mediate the formation of focal adhesions. ECM elements can be directed to the parallel grooves of the scaffold, promoting a highly organized cell–ECM environment *in vitro*. In a similar fashion to the parallel groove topography, the capillary network has been mimicked by using fabrication techniques that alter the biomaterial topography. The capillary network was reconstructed using a highly porous elastomer scaffold that contained a parallel array of channels. Neonatal rat heart cells were cultured within these channels and showed better contractile properties after the 8-day study.[61]

The consideration of cell–substrate interactions in biomaterial design has resulted in the successful creation of materials that are able to elicit downstream cellular responses such as differentiation and proliferation. In the following section, methods to quantify cell–substrate interactions are discussed. Quantification of cell adhesion can provide another means to characterize the interactions between cells and an underlying substrate.

7.4 MEASUREMENT OF CELL ADHESION

The adhesion of cells to an underlying substrate can be quantified through the use of cell adhesion assays. In general, cells are allowed to establish adhesive interactions to a substrate of interest and then are exposed to a detachment force. Adhesion assays are often categorized based on the type of force that is applied, resulting in three major categories: micromanipulation, centrifugation, and hydrodynamic shear

stress.[62,63] Currently, the vast majority of cell adhesion measurements are studied in two-dimensional systems. In the future, the field would benefit greatly from the development of quantitative assays that could characterize cell adhesion in a three-dimensional environment because this is a more relevant configuration and representation of the native tissue.

Before the development of assays based on detachment force, "stick and wash" assays were commonly used for the study of cell adhesion.[63,64] In a stick and wash assay, cells were allowed to adhere to a surface and then were simply subjected to washing over the surface with buffer. Although many of the first discoveries involving cell adhesion ligand–receptor interactions were made using this technique, there are inherent limitations. Stick and wash assays had poor reproducibility and applied uneven and unknown detachment forces.[63,64] These limitations led to the development of the measurement techniques discussed next.

7.4.1 Micromanipulation

In micromanipulation techniques, the detachment force can be applied as either a vertical force pulling cells normal to the surface or a shear force, pulling cells tangential to the surface.[62] Micromanipulation covers a range of techniques that include micropipette aspiration, atomic force microscopy (AFM), and laser tweezers.[63] With these techniques, it is possible to collect real-time force-displacement measurements on a single cell and investigate specific interactions between cell adhesion receptors and the substrate.

AFM can be used to evaluate morphologic changes occurring during cell adhesion, adhesion strength measurements, and interaction forces between cells. AFM images are capable of showing cell flattening and spreading, and it is generally accepted that the flatness of a cell designates good adhesion (Fig. 7.1).[65] A typical morphologic change related to adhesion is the appearance of structured stress fibers, indicating the stability of the cells on the underlying substrate. The adhesion strength of cells to an underlying substrate can be quantitatively measured on a single cell level using AFM techniques. The force necessary to laterally displace a cell with the

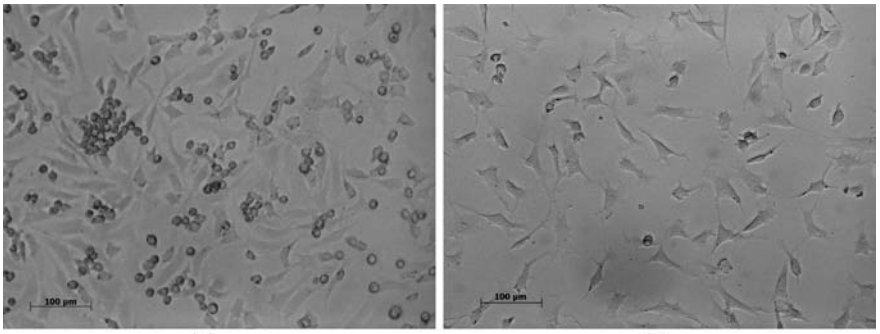

FIGURE 7.1 Morphologic assessment of adhesion.

AFM cantilever can be measured in real time by recording the deflection of a laser beam versus the lateral displacement of the AFM cantilever.[65] In addition to measuring the adhesion strength, the AFM technique can also estimate the timescale over which adhesion occurs and eventually saturates.

As previously mentioned, the interaction between cells is mediated by cell adhesion molecules called cadherins. The interaction forces between cells can also be evaluated at the molecular level using AFM. Single biomolecules can be imaged, and the force necessary to disrupt cell–cell interactions can be characterized.[65]

AFM techniques can have several practical difficulties.[66] The underlying substrate as well as surrounding cells can influence the force measurements that are collected from a single cell. Additionally, the user must be sure that the forces being measured are the attachment forces of the cell and not simply a measure of cell membrane strength. Other practical issues include z-axis restrictions and protein adsorption to the AFM cantilever.

Micropipette aspiration is another type of micromanipulation technique that is capable of measuring the strength with which a single cell or even a single biomolecule on a cell surface is attached to an underlying substrate. The displacement force used by micropipette aspiration is suction pressure, which can be applied tangential or normal to the cell surface[62,67,68] and can be designed in various ways. The micropipette can be designed to simply apply suction pressure onto a cell that is attached to a substrate, resulting in partial or complete removal of the cell from the surface and into the pipette tip. It can be designed so that the cell detaches from the substrate and attaches instead to a bead that is held by suction force at the tip of the probing pipette, or it can be designed such that a cell is freely moving inside one pipette until it attaches to a bead or cell held by a second pipette.[69]

In a study by Athanassiou and Deligianni,[62] vertical (normal) suction forces were applied to individual bone marrow cells that had been allowed to attach to fibronectin. To establish a suction force normal to the cell surface, the tip of the micropipette was bent at a 130-degree angle. The results showed that detachment occurred in phases. First, deformation was observed, without detachment of the cell, followed by a second phase in which detachment was observed as a result of pressure increases. The strength of the adhesion of bone marrow cells to fibronectin was found to increase as the time allowed for cell attachment was increased.

Qin et al.[68] also used micropipette adhesion as a means to quantify the interaction of tenocytes grown *in vitro* to fibronectin and type I collagen modified PLGA. In this study, the suction force of the micropipette was applied tangential to the surface of the cell, although the results of the study were quite similar. With tenocytes, the adhesion strength increased as seeding time increased. In both studies, soluble antibodies were used to disrupt cell adhesion by inhibiting ligand–receptor binding. Both studies showed that inclusion of competitive soluble antibodies decreased cell adhesion strength to the respective substrates.[62,68]

Micromanipulation assays offer a sensitive and quantitative means to investigate cell–substrate and cell–cell interactions at the molecular level. However, these assays are limited to applying small forces and can only be used for individual cell studies in which the seeding time is short. For longer adhesion times or for quantification of a

larger cell population, assays that provide a greater distractive force must be considered.

7.4.2 Centrifugation

For larger cell populations, centrifugation assays offer a simple and reproducible means to quantify cell adhesion. In general, cells are seeded onto a substrate and allowed to adhere for a period of time, typically no longer than 1 h.[63] After adherence, cells are subjected to a perpendicular detachment force generated by spinning at a specified speed in a standard laboratory centrifuge.[63] A schematic of a typical centrifugation assay procedure can be seen in Figure 7.2. The ratio of post-spin cell count to pre-spin cell count results in the adherent fraction of cells at the designated force set by the centrifugal speed.

Centrifugation assays have also been used to quantify differences between initial adhesion and "strengthened" adhesion.[64] Strengthened adhesion is defined as adhesion that occurs while cells are incubated on a substrate. As with the micromanipulation assays, adhesion strength increased with longer seeding times (e.g., 30 vs. 60 min),[64,70] showing that adhesion is time dependent. Reyes and Garcia[70] further modified the centrifugation assay and developed the mean adhesion strength value, which is the force that causes 50% cell detachment, for fibrosarcoma cells seeded on fibronectin coated 96-well plates. Centrifugation assays offer a simple and reproducible method of characterizing biomaterials based on the ability of the material to successfully initiate cell adhesion.

Despite the success of centrifugation assays, there are limitations, including the fact that only one speed can be subjected to cells at a time and that at longer adhesion times (>1 h),[63] the distraction forces generated by the centrifuge are not large enough to displace large cell populations.

7.4.3 Hydrodynamic Shear Stress

Flow systems have been developed to apply a wide range of shear detachment forces to large adherent cell populations and are generally considered a more reliable adhesion measurement system.[63] Hydrodynamic shear stress assays are classified according to the geometry of the flow responsible for generating the shear detachment

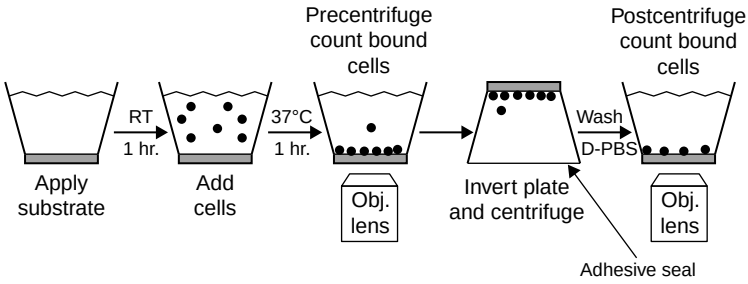

FIGURE 7.2 Schematic of a centrifugation assay procedure.

force. There are three basic flow cell configurations: the parallel plate, the rotating disc, and radial flow between parallel disks. Of these geometries, the parallel plate has been extensively studied, specifically in combination with microscopy.[63] The parallel plate configuration permits the observation of attachment and detachment throughout the assay and has been used frequently in the characterization of leukocyte–endothelial cell adhesion events.[71,72] A typical parallel plate flow cell is designed so that (1) flow is laminar (Reynolds number <2300) and controlled through the use of a syringe pump and (2) the entrance length is minimized so that entry effects can be neglected, and flow can be considered fully developed and parabolic.[72]

The rotating disk geometry results in forces that vary linearly with radial distance, which is an advantage because it can subject a large cell population to a range of detachment forces in one experiment. Common types of rotating disks are the single spinning disk and the small-gap parallel disk viscometer.[63] The rotating disk should only be used for low rotational speeds because increasing the speed results in a greater degree of unsteady and invalidated flow rates. Garcia et al.[73] investigated the use of the single spinning disk configuration on fibronectin-mediated osteosarcoma adhesion to bioactive glass. For a given rotational speed and laminar flow, velocity, temperature, and concentration, boundary layer thickness can be considered constant.[74] The spinning disk was shown to produce reproducible results that demonstrated that cell detachment increased with shear force.[73]

Similar to rotating disk configurations, radial flow systems can also generate a range of shear stresses. In this case, surface shear stress decreases with radial distance. Through the use of immunoglobulins, this geometry has been used to characterize ligand–receptor interactions over a range of forces.[75]

Although hydrodynamic shear stress assays provide a reliable and reproducible means for quantifying cell adhesion *in vitro*, there are some limitations related to the measurement of shear stress. In general, the adhesion strength is reported as a shear stress with units of force per area. Even though this is a useful measure for the investigation of adhesion strength, the net force that is applied is not simply the shear stress but includes parameters such as hydrodynamic drag and torque.[63] Therefore, results of such assays must be carefully examined. There are several practical difficulties of hydrodynamic shear stress assays, including complications with the system setup, preventing the inclusion of air bubbles in the stream of flow, and preventing nonlaminar flow through the chamber.[66]

All of these adhesion assay techniques have advantages and disadvantages, making it clear that there is no perfect solution when it comes to quantifying cell adhesion. A measurement system must be chosen based on the cell system in place and the desired results. Results of adhesion assays must be taken as relative to the cell population and the particular experiment and not as an absolute measure.

7.5 CONCLUSIONS

Adhesion receptors function to modulate cell behavior in a variety of ways, and these functions are desirable to incorporate into the design of biomaterials that will be used for tissue engineering applications. A common method is the tethering of integrin

binding domains such as RGD, LDV, GFOGER, and YIGSR onto the surface of the biomaterial to promote cell adhesion. The inclusion of adhesion receptor ligands into biomaterials enhances cell adhesion and can potentially mediate a desired intracellular reaction to ligand binding.

Research involving the modification of biomaterials to optimize cellular adhesion has made strides including bioactive molecules on the material surface to control cell phenotype and induce changes such as increased spreading, proliferation, and differentiation. Patterning techniques have allowed for control over adhesive domain inclusion on the surface of biomaterials at the micro- and nanoscale. Optimizing biomaterial design to mimic the native adhesive interactions of a cell population provides a method for controlling downstream cell responses and could have significant impacts for therapeutic applications.

The quantification of cell adhesion through the use of a detachment force offers a means to further characterize and optimize cell–cell and cell–substrate interactions. Results of such assays can be used as feedback for biomaterial designs and encourage further manipulation of biomaterials to achieve desired levels of cell adhesion.

By controlling the adhesive interactions through biomaterial design, future studies can focus on the exploration and characterization of the intracellular mechanisms by which the cell response occurs after activation of adhesion receptors. Better understanding of the exact signaling mechanisms could provide invaluable information on cellular development and the progression of disease within a cell. This information could be used in the development of replacement tissues and drug delivery devices to treat diseases such as cancer. Finally, to make the leap from the benchtop to the clinic, the scalability and stability of bioactive scaffolds should be addressed in order to produce an efficient means for applying such devices for tissue replacement and therapeutic interventions.

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DISCLAIMER

The items identified by brand name are the choice of the authors and are not to be construed as conveying an official endorsement by the U.S. Food and Drug Administration.

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