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REVIEW

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Bridging the Gap Between Physicochemistry and Interpretation Prevalent in Cell−Surface Interactions

Evan A. Dubiel,[‡,§] Yves Martin,[‡,§] and Patrick Vermette*[,‡,§]

[‡]Laboratoire de bio-ingénierie et de biophysique de l'Université de Sherbrooke, Department of Chemical and Biotechnological Engineering, Université de Sherbrooke, 2500 Boulevard de l'Université, Sherbrooke, Québec, Canada J1K 2R1

[§]Research Centre on Aging, Institut universitaire de gériatrie de Sherbrooke, 1036 rue Belvédère Sud, Sherbrooke, Québec, Canada J1H 4C4

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1. INTRODUCTION

Biomaterials science and tissue engineering involve the study and design of materials capable of eliciting a desired and controllable response from cells and biological environments. Research efforts studying the processes at interfaces between engineered synthetic materials and biological environments have exploded in the past two decades.[1] Such research applies to the design of surfaces for medical implantation ranging from dental implants,[2−4] contact lenses,[5−7] to drug delivery[8−10] and the growth of tissue substitutes in scaffolds.[11−13] Particular emphasis is placed on engineering surfaces that would rapidly promote the integration of an implant (e.g., artificial joints) into the host to reduce healing time.[14] In conjunction, many efforts have also focused on developing surfaces for the prevention of inflammation and thrombosis of implanted tissue and foreign materials.[15−19] These studies highlight only some of the research in biomaterials science and tissue engineering. Within biomaterials science, tissue engineering, and the numerous other subdisciplines that have emerged over the past two decades are cell−material and cell−surface interactions.

Many studies involving cell−surface and cell−material interactions focus solely on an end result after almost randomly chosen periods of times and culture conditions. Less emphasis is placed on understanding the mechanisms that influence cell behavior on a surface. As a result, there is an abundance of literature on information regarding the behavior and response of

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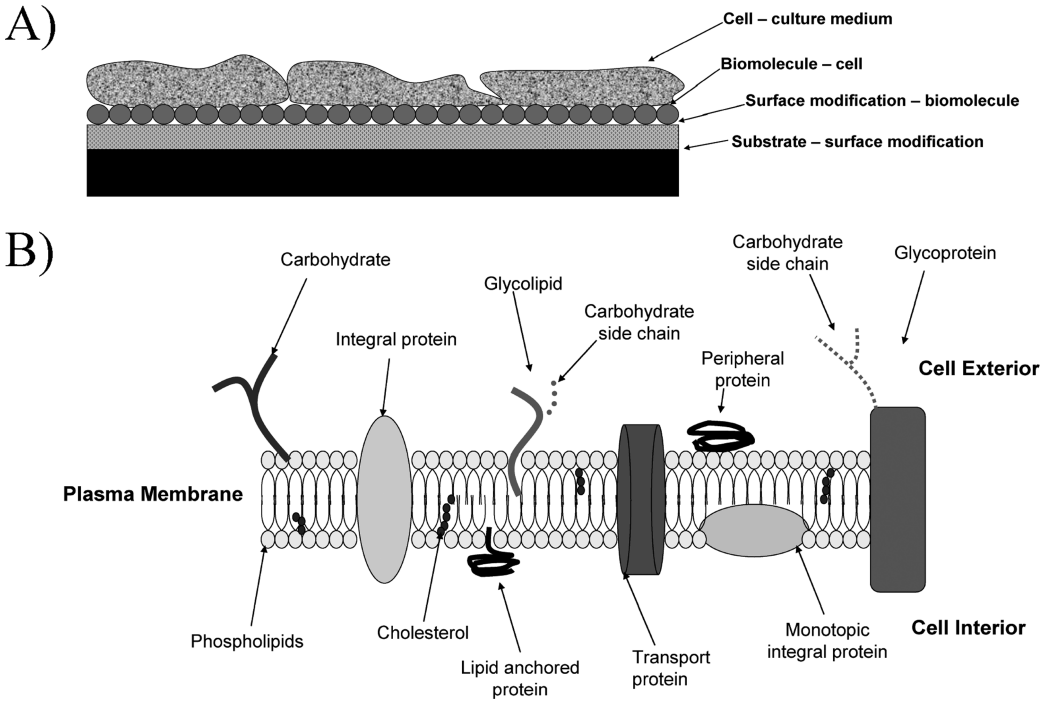

Figure 1. (A) Traditional cell−surface experimental setup. (B) Cross section of generic eukaryotic cell surface (i.e., plasma membrane). Many of the components portray a localized net electric charge. Figures not drawn to scale.

cells toward surfaces but limited understanding of the events and conditions that factor into such behavior. One of the aims of this article is to outline the influence surface and intermolecular forces have on the response and behavior of cells on surfaces. Particularly, emphasis is placed on the influence of electrostatic and van der Waals forces, hydrophobic interactions, hydrogen bonding, and steric repulsion.

A traditional cell−surface colloidal system generally consists of four interfaces: (i) the substrate−surface modification, (ii) the surface modification−biomolecule, (iii) the biomolecule−cell, and (iv) the cell−culture medium. As shown in Figure 1A, the system is typically engineered in a "bottom-up" fashion. For example, a substrate is modified by means of self-assembled monolayers (SAMs) or plasma polymerization. Subsequently, proteins, extracellular matrix (ECM) components, or other biomolecules, such as growth factors, are adsorbed to the surface via chemisorption or physisorption, followed by cell seeding in culture media. Although almost all studies employ such a colloidal system, its relevance to the in vivo reality is a credible issue. As observations suggest, the traditional cell culture approach is not necessarily representative of the architecture, mechanical forces, and mass transport properties of a physiological environment. As such, large efforts should be made in biomaterials and tissue engineering to design in vitro systems that further represent the physiological in vivo reality. Although advances have been made and some studies have in actual fact elicited a desired cell response, much research is still required to truly understand cell−surface and cell−material interactions to significantly advance not only molecular biology but also biomaterials and tissue engineering research.

Many studies have been performed on cell adhesion and responses to surfaces using a variety of phenotypes and surface modifications.[20−30] As shown in Table 1, results of previous studies on cell adhesion and responses to surfaces always vary somewhat between subsequent studies. The differences in results can be a consequence of varying phenotypes, surface modification, and/or a variety of other differences in experimental methods. For example, both Webb et al.[30] and Keselowsky et al.[24] studied osteoblast adhesion to amine (NH_2)- and methyl (CH_3)-bearing surfaces. Webb et al. found osteoblast activity (adhesion and migration) followed $CH_3 > NH_2$, whereas Keselowsky et al. found the opposite. Between the two studies there are many differences in the experimental processes. Webb et al.[30] modified substrates with organosilanes, whereas Keselowsky et al.[24] used organothiol SAMs. Webb et al.[30] did not cover the modified surface with proteins, whereas Keselowsky et al.[24] coated the organothiol-modified surface with fibronectin (Fn). Both conclude that cell

Table 1. Cell−Surface Studies

phenotype	modification	ref	relative level of adherence/behavior
fibroblast	organosilane + SM[a]	21	$NH_2 \approx COOH > CH_3 = OH$
	organothiol + PM[b]	25	TCPS[c] > CH_3 > COOH
	commercial TCPS	22	UPS[d] > TCPS
	organosilane + SM	29	$NH_2 > CH_3$
	commercial TCPS + PM	27	Primaria > TCPS
endothelial	commercial TCPS + PM	27	Primaria > TCPS
	organosilane + PM	26	NH_2 > glass
	organosilane	23	alkylamine > arylalkyamine
osteoblast	organosilane	30	$CH_3 > NH_2$
	organothiol + PM	24	$NH_2 > CH_3$
leukocyte	organothiol	20	CH_3 > OH > COOH
	organothiol + PM	28	OH > COOH > CH_3
	organothiol	28	CH_3 > COOH > OH

[a] SM = serum modification. [b] PM = protein modification. [c] TCPS = tissue culture polystyrene. [d] UPS = untreated polystyrene.

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response and behavior is dictated by the surface chemistry of the modified substrate. Although such a conclusion is logical and correct, because of the differences in experimental methods, it is next to impossible to draw a comprehensive understanding of the surface mechanisms that govern osteoblast response and behavior. One can only draw conclusions based on the specific surface composition employed, as these researchers have done. Although these studies suggest a useful strategy for understanding and modulating cell behavior, they do not provide insight into the specific mechanisms that govern such behavior, nor strategies to control and modulate such behavior for biomedical applications. Limitations such as these just described can be extended to nearly all (including the few presented in Table 1) biomaterials and tissue engineering related studies. The result is a large quantity of literature with limited basis of general comprehensive knowledge and understanding.

In a recent keynote address,[31] Nerem categorized biomaterials and tissue engineering research over the last two decades. The 1990s were classified as "the go go years" in which it was seen as a potential scientific revolution and interest and research exploded. The years 2000−2005 were categorized as "the sobering years" as researchers began to realize the enormous problems and complexities associated with such research. The year 2005 to present is categorized as "back to the future" as some researchers are now focusing on overcoming the many hurdles to retain the potential that research in biomaterials, tissue engineering, and other related disciplines was believed to have in the 1990s.[31] We agree with Nerem's categorization and hope this article helps advanced the science to the potential it was initially believed to have.

We begin with presenting biological aspects. The cell surface (i.e., the plasma membrane) is briefly discussed, followed by cellular adhesion of the cell surface to ligands. Physical aspects are then presented. Fundamental interactions including Coulomb, van der Waals, double-layer, hydrophobic, H-bonding, and steric repulsion are introduced, and their relevance to biology is discussed. An extensive discussion on systems for studying cell−material interactions follows. Finally, surface chemistry is presented because it is essential to biomaterials science. Particular emphasis is placed on SAMs and plasma polymerization modifications to enhance chemical activity, along with their advantages and disadvantages with respect to surface uniformity and stability. The design of low-fouling surfaces is also discussed.

Depending on the scientific background of the reader, sections of this article may appear trivial. However, the primary aim of this review is to collectively present literature relevant to cell−material and cell−surface interactions from all three fundamental scientific disciplines to present the reader with a wide spectrum of perspectives of such literature, subsequently directing future research considerations and interpretation in the direction of general comprehension of cell behavior on surfaces. We believe that fundamental understanding is necessary to identify and design surfaces, materials, and systems to elicit and control a desired cell response.

This article covers a large timeline of scientific literature, as this was necessary to provide a full picture of the concepts behind cell−material interactions. Most studies directly related to cell−material interactions that are reviewed in our article have largely been published in the last 20 years. At the same time, we also review the fundamental concepts of surface science that are necessary to better understand and appreciate such interactions, some of which originated nearly a century ago.

2. CELLULAR ADHESION TO SURFACES

2.1. Cell Surface

Generally, the cell surface can be depicted as shown in Figure 1B. The plasma membrane (i.e., cell surface) is selective in the reactions that are allowed to proceed and the molecules that are allowed to penetrate. The plasma membrane mainly consists of a lipid bilayer composed of phospholipids. Scattered throughout the lipid bilayer are membrane proteins and carbohydrates (generically known as polysaccharides).

The plasma membrane is a nonrigid body. Plasma membrane structure is largely represented using the Fluid-Mosaic model proposed by Singer and Nicolson.[32] The Fluid-Mosaic model utilized many previous studies in its formulation[33] and is continuously being redefined. In the Fluid-Mosaic model, lipids and proteins behave like a fluid and for all intensive purposes are free to move around, in a diffusive manner, within the two-dimensional structure of the plasma membrane.[34,35] The lipid component of the cell surface gives the cell its fluidlike properties. The three primary classes of lipids composing the cell membrane are phospholipids, glycolipids, and cholesterol. The most abundant lipids are the phospholipids. Glycolipids are lipids with a carbohydrate side chain. Cholesterols are located within the interior of the plasma membrane. Carboxyl groups close to the polar head of the phospholipids interact with hydroxyl groups of the cholesterol via H-bonding. The result is increased spatial separation of adjacent polar head groups of the phospholipids. During interaction between a plasma membrane protein (i.e., a receptor) with another ligand, there is an abundance of cholesterol within the vicinity of the membrane protein relative to the plasma membrane as a whole. This further isolates the membrane protein from the rest of the plasma membrane, aiding in the ligand−receptor interaction. The formed structure is known as a lipid raft.

The two main functions of membrane proteins are to provide communication between the cell and its external environment (i.e., cell signaling) and to regulate the flow of substances into and out of the cell. A membrane protein (i.e., a cell receptor) interacts with a signaling protein (i.e., a ligand). This is the first in a series of events that dictate subsequent cell function and behavior. Transport proteins are membrane proteins that regulate the flow of substances in and out of the cell. To name a few, the flow of substances can include the intake of nutrients or the removal of waste from the cell.

There are many different types of membrane proteins. Classification is dependent on the structure and the manner in which it is integrated to the plasma membrane. For example, membrane proteins containing carbohydrate side chains are called glycoproteins and those that do not penetrate the plasma membrane are known as peripheral membrane proteins. Peripheral membrane proteins are anchored to the plasma membrane via weak electrostatic interactions and H-bonding. Proteins that are covalently bound to the lipid molecules within the plasma membrane are known as lipid-anchored membrane proteins.

The majority of membrane proteins are categorized as integral membrane proteins. Integrins and cadherins are classified as integral membrane proteins. Integral membrane proteins possess hydrophobic regions that interact with the hydrophobic interior of the plasma membrane (i.e., the tails of the phospholipids). As such, there is a high affinity of the protein for the plasma membrane, making integral membrane proteins the most difficult to isolate from the plasma membrane. Integral membrane

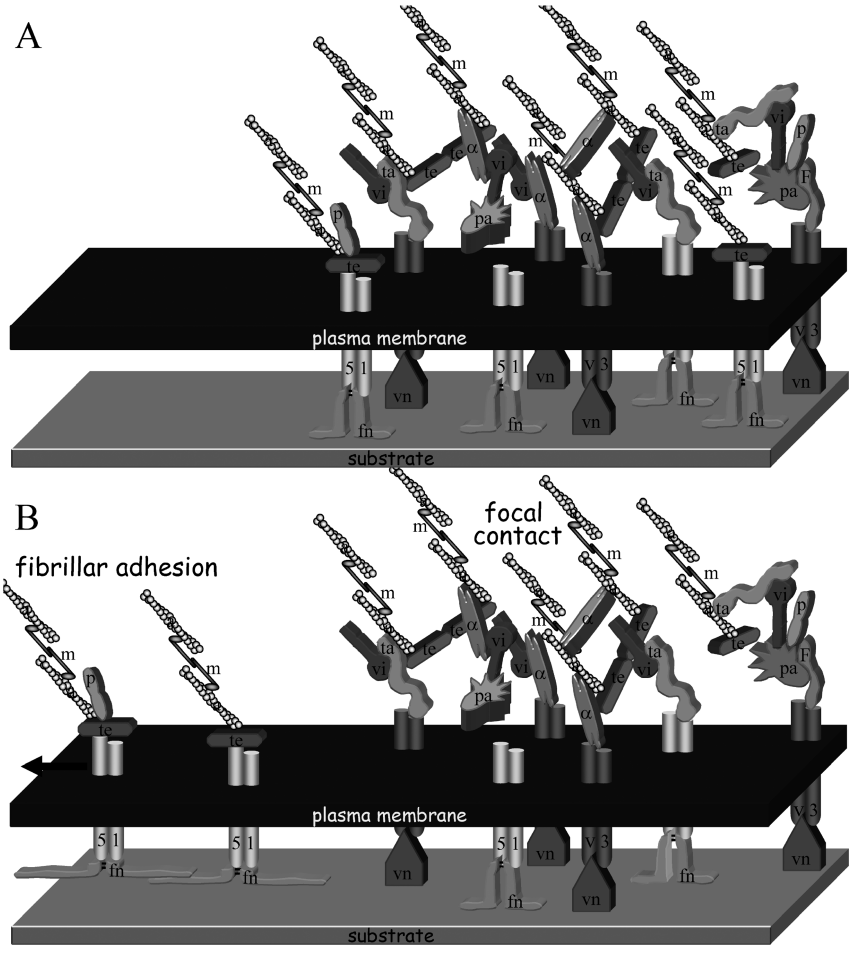

Figure 2. Structure and dynamics of cellular adhesion to surfaces. (A) Cellular adhesions initially contain both α5β1/Fn and αvβ3/Vn integrin−ECM adhesions. (B) A contractile force provided by myosin II translocates the α5β1/Fn centripetally toward the cell center to form a fibrillar adhesion, leaving behind a focal contact. Abbreviations: a = actin, α = α-actinin, m = myosin II, p = parvin/actopaxin, pa = paxillin, ta = talin, te = tensin, and vi = vinculin. Reproduced with permission from ref 45. Copyright 2001 The Company of Biologists.

proteins also possess hydrophilic regions by which they interact with the external aqueous environment of the cell. Some integral membrane proteins protrude from only one side of the cell, known as integral monotopic proteins, whereas others span the entire thickness of the plasma membrane, known as transmembrane integral proteins.

This discussion has been overly simplified for the purpose of brevity. We have only discussed the plasma membrane as a barrier that defines the boundary of a cell and its external environment as that is the most relevant for this article. However, the plasma membrane also acts as a boundary for the separation of organelles in the intracellular compartment. For detailed discussion on the overall context of the plasma membrane and more detail into its structure, the reader is referred to widely used biology texts by Lodish et al.[36] and Becker et al.[37] The interactions (H-bonding and hydrophobic) mentioned here are discussed in detail in section 3.2.

2.2. Cellular Adhesion

It is generally believed within the scientific community that cells adhere to surfaces through an intermediate interaction between an extracellular matrix (ECM) protein and a surface. ECM proteins are introduced to the surface by two ways. In one way, cells synthesize and secrete ECM proteins such as Fn and vitronectin (Vn) to promote adherence.[38] In the other way, proteins are preadsorbed to the surface independently prior to the introduction of cells to the surface. Subsequently, when cells are introduced to a protein-coated surface, less protein synthesis via the cells is necessary.

Cellular adhesion is a dynamic process that involves many different proteins.[39,40] Adherence of cells to surfaces is achieved through an interaction between adhesive ligands on the ECM proteins and receptors on the cell surface. This interaction is mediated by integrins.[41] Integrins are heterodimeric transmembrane glycoproteins that consist of α- and β-subunits.[42] While we discuss only integrin cell receptors in this article, we note to the reader that there are other known types of cell receptors. In general, most cell receptor molecules fall into five categories: immunoglobins, integrins, selectins, mucins, and cadherins. For a concise review on the different types of cell receptors, the reader is referred to literature by Pierres et al.[43]

The two main classifications of cellular adhesions are focal contacts and fibrillar adhesions. Focal adhesion contacts are flat, elongated structures usually located at the cell periphery, which involve the cytoskeletal proteins tensin, vinculin, α-actinin, and talin as well as signaling proteins including focal-adhesion-kinase (FAK) and paxillin. Fibrillar adhesions are more centrally located and involve the cytoskeletal protein tensin.[44]

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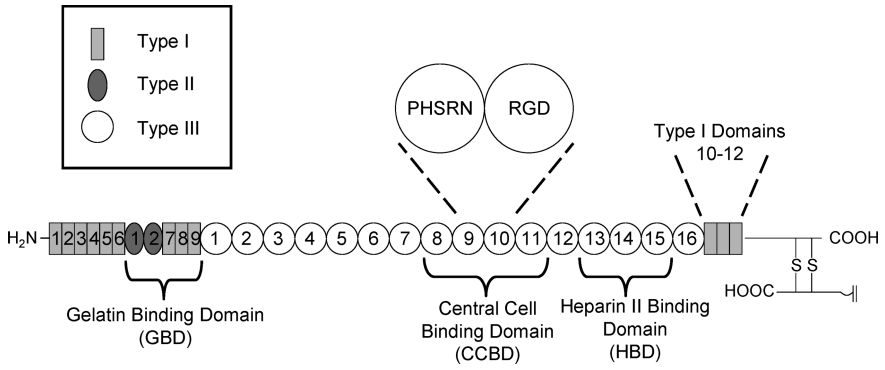

Figure 3. Primary structure of fibronectin (Swiss Institute of Bioinformatics, ExPASy Proteomics Server, http://www.expasy.org/cgi-bin/protparam?P02751, accessed on July 28, 2010). There are two nearly identical mirror segments of ~220−230 kDa joined by two disulfide bonds.

The dynamics of cellular adhesion are hypothetically illustrated in an informative commentary by Zamir and Geiger.[45] α5β1 and its interaction with Fn along with the αVβ3 integrin appear to be the most studied complexes in conjunction with cellular adhesion. As shown in Figure 2, adhesions initially contain both α5β1 and αVβ3 integrins. Colocalization of α5/αV subunits appears early during adhesion; then over time, these α-subunits segregate.[46] Moreover, experiments have shown that β3 integrins remain with the initial adhesions while β1 undergoes translocation.[47] A contractile force provided by the protein myosin II translocates the α5β1 along with tensin centripetally toward the interior of the cell and forms a fibrillar adhesion in a process known as fibrillogenesis. In the same manner as the α5 and β1 integrin subunits, Fn has been shown to translocate during cellular adhesion.[48] Furthermore, covalent linking of Fn to a substrate can impede fibrillogenesis.[49] On the other hand, αVβ3 does not translocate even though the same contractile force is present, leaving what is referred to as a focal adhesion contact.[45,50] Depending on the substrate properties and composition, focal contacts alone or both focal contacts and fibrillar adhesions may be present in cellular adhesions.[49,51]

It should be mentioned, as was done in the studies just highlighted, that there are likely other integrin and ECM complexes involved in cellular adhesion. Most integrin receptors have the ability to bind to multiple adhesive ligands. In addition, an adhesive ligand can bind with multiple integrin receptors.[52] For a concise review on integin α- and β-subunits and their associated ligand receptors along with insight into the resultant signaling and cell function, we refer the reader to articles by Hynes.[42,53]

2.3. Protein Mediation Between Cells and Surfaces

As already mentioned, receptors on the cell surface interact with the adhesive ligands of the ECM proteins that are adsorbed to the underlying substrate to form adhesions (i.e., focal and fibrillar structures). The availability of such adhesive ligands for interaction with cells is dependent on interactions between the ECM protein and the underlying substrate. As a result of such interactions, ECM proteins will undergo conformational and orientation changes upon adsorption to a substrate. The nature of the interaction mechanisms (i.e., surface and intermolecular forces) involved is the next subject of this article.

Proteins are nonrigid bodies. Thus, while the primary structure remains intact upon interaction with a surface, the secondary, tertiary, and quaternary structures may undergo spatial changes from their natural conformation. This is known as a conformational change. An orientation is the axial and azimuthal angles of the protein with respect to an arbitrary axis. The resultant conformation and orientation of a protein due to interactions with a surface has a direct impact on which particular ligands are available for interactions with cell surface receptors, thus directly influencing the behavior of cells on the surface.

Table 2. Cellular Attachment to Fibronectin Domains[a]

phenotype	CCBD	HBD	GBD	ref
human bone	+	+	−	61
bovine corneal epithelial	+	−	−	61
endothelial	+	+	n/a	65
osteocarcinoma	+	+	n/a	64
monocyte	n/a	n/a	+	66

[a] n/a = not available because the domain of Fn in the literature was not studied.

To provide a specific example of various ligands influencing cell behavior, let us consider Fn. Fn is a dimer glycoprotein of ~450 kDa found in the ECM and blood. It reacts with many biomolecules and plays a wide variety of roles in physiological processes such as embryogenesis, angiogenesis, and wound healing.[54−58]

Plasma and cellular Fn are similar but vary slightly. We assume the structure of plasma and cellular Fn as identical for the purpose of brevity. For differences in the structure of plasma and cellular Fn, we refer the reader to an article by Hayashi and Yamada.[59] Fn has a primary structure as shown in Figure 3. There are two similar polypeptide chains of ~225 kDa each. Each chain has a slight variation in amino acid sequence.[60] There is no absolute general consensus on the specific functions of each of the three domains presented in Figure 3 with respect to cellular function. Various phenotypes respond differently to the domains of Fn, particularly, the gelatin binding domain (GBD) and heparin II binding domain (HBD). Such differences are a result of different phenotypes having different cell surface receptors. Whereas one phenotype may have a cell receptor specific for a particular domain of Fn, another phenotype may lack a receptor with such specificity. For example, as shown in Table 2, human bone cells show attachment to the HBD whereas bovine corneal epithelial cells do not.[61] The central cell binding domain (CCBD) is the primary ligand believed to be responsible for cellular adhesion to Fn. Within this domain (see Figure 3) is the 9th−10th type III repeat, which contains the proline-histidine-serine-arginine-asparagine (PHSRN) synergy site and the adhesive tripeptide arginine-glycine-aspartic acid (RGD) sequence.[62]

The PHSRN synergy site is not responsible for adhesion alone. Rather, in unison with the RGD site, cellular adhesive affinity is enhanced.[63] In general, the HBD produces a modest (if any) effect on cellular adhesion alone.[61] However, together with the CCBD, cells show enhanced migration and proliferation in the presence of the HBD.[64,65] As shown in Table 2, the GBD does not contribute to cellular adhesion for both human bone and bovine corneal epithelial cells.[61] However, for monocytes, the GBD domain is believed to play a role in cellular adhesion.[66] We note that there are many other domains of Fn that we do not include here, again for the purpose of brevity. The reader is referred to a review by Hynes and Yamada for identification and the biological properties of such domains.[67]

A single ligand has multiple influences on a single phenotype, and varying phenotypes have different responses to single ligands. More importantly, because protein conformation and orientation influences which adhesive ligands are available for interactions with cells, we explicitly see how protein conformation and orientation directly influence cell behavior. Because properties of the substrate can influence protein conformation and orientation, the substrate can have a direct influence on cell behavior.

It is important to note that the information outlined in the previous paragraph has limitations. Many of the studies presented drew conclusions from either utilizing synthetic domain fragments of Fn or through the blocking of particular domains via antibodies. The complete absence of certain domains as a result of using domain fragments likely has drastic effects on cell behavior. Cellular function is rarely the result of one ligand on a protein. Rather it is a result of simultaneous cell receptor interactions with multiple surface ligands. For example, it is believed that GBD stimulates fibrillogenesis in fibroblast adhesion but is not a direct contributor to cell adhesion. Rather, fibrillogenesis is stimulated through a cooperative mechanism of the cell surface receptor transglutaminase with both the CCBD and GBD.[68] In conclusion, while synthetic fragments of Fn may give some information regarding the role of particular ligands, such results should be interpreted with caution because, in a physiological environment, such synthetic fragments might not exist. Furthermore, even when not using synthetic Fn fragments, one still likely has Fn fragments and even contaminants within their system. Fn is usually purified from blood plasma using chromatography. Regardless of purification technique, the end product contains trace amounts of contaminants such as proteolytic fragments, fibrinogen, and plasma gelatinase.[69−71] Thus, all results should be interpreted with caution as some may not be completely physiologically representative.

The orientation and conformation of surface-adsorbed Fn determines which particular ligands (CCBD, GBD, and HBD) are available for potential interactions with cell receptors, ultimately eliciting cell function. The conformation of Fn is dependent on parameters such as the type of Fn (e.g., plasma, cellular) and the conditions of the solution environment (i.e., pH, salt presence, etc.).[72] For the case of surface adsorption, the properties of the surface and/or conjugation mode have an influence on Fn conformation.[73,74] Regardless, depending on such parameters, Fn takes a compact or extended conformation. In a compact form, the RGD ligand is not accessible.[72] As such, this conformation is not favorable for cellular adhesion. Alternatively, an extended conformation better exposing the adhesive ligands is more favorable for cellular adhesion.

For all intensive purposes, a particular conformation/orientation of Fn or any proteins is the resultant of a superposition of protein−substrate and protein−protein interactions.[75] In the initial stages of adsorption, protein−substrate interactions are of most relevance. As the surface coverage increases, interactions between neighboring surface protein−protein interactions also become relevant. These interactions are dependent on the properties of the substrate and the protein. We will now address the physical nature of such interactions.

3. INTERACTIONS IN CELLULAR ADHESION

Often, little emphasis is placed on the surface when studying cell−material interactions. Regularly, the unrestricted word "immobilized" is used to describe a biomolecule on a surface, essentially marginalizing the importance of the chemical or biochemical surface modification employed prior to cell seeding. However, the modification is in fact the precedence of cell behavior on a surface. There are many different genres of interactions that can occur in cell−material studies. Interactions that are of particular importance are those at and within the vicinity of the four interfaces of the colloidal system (shown earlier in Figure 1A). To describe a molecule that is immobilized onto a surface, the term adsorption universally applies. Chemisorption (i.e., sharing/exchange of electrons) and physisorption (i.e., no sharing/exchange of electrons) are the two generic interactions that occur in cell−surface studies. Within these genres are many different subgenres of surface and intermolecular interactions, not all of which are completely understood.

The interactions between cells, biomolecules, and surfaces are very complex. Such complexity arises from the dynamics inherent in physiological and biological systems. Often multiple genres and subgenres of interactions are involved in a cell−surface system, none of which occurs at the same time. Furthermore, such interactions are not necessarily additive in space as there is a dependence on the properties of the molecule (i.e., polarizability, dielectric permittivity, etc.) and its surroundings (i.e., nearest-neighbor distance, solvents present, etc.).[76]

The thermodynamics of biological systems are very complex. Biological systems are not necessarily even approaching thermodynamic equilibrium. Numerous liquid−liquid and liquid−solid phase boundaries are involved, to name only two. Thermodynamic analysis of such interfaces usually assumes flatness and homogeneity. However, such assumptions are unrealistic as biological interfaces can be significantly curved and are rather heterogeneous. Nevertheless, some insight can be gained from thermodynamic analysis that utilizes such assumptions. We refer the reader to a concise review by Lyklema for such a discussion on the thermodynamics of biological interfaces.[77]

In this section, we briefly introduce the intermolecular and surface interactions (i.e., chemisorption and physisorption) relevant in cell−surface science. For comprehensive treatment of such concepts, the reader is referred to excellent texts by Israelachvili[78] and Hunter.[79] In addition, Leckband and Israelachvili[80] and Baszkin and Norde[81] provide excellent reviews on the same concepts with a special emphasis placed on biology. Throughout this section, we refer to such works.

3.1. Chemical Interactions

Physics, chemistry, and bulk materials science generally use the terms covalent or chemical interaction/bond to describe the interaction between two molecules that share/exchange charge. In surface science, the term chemisorption is used to describe the same principle for the more specific case of when a molecule is bound to a surface under such properties. Typical features of

chemisorption are high affinity with the surface and uniform directional bonding of the adsorbate (relative to one another) throughout the surface.[78] Often, one will also encounter the term "grafted" to describe molecules chemisorbed to a surface.

A common form of chemisorption employed in surface science is amide bonding via carbodiimide chemistry. The surface can be composed of chemically available COOH groups from SAMs,[82] carboxymethyldextran (CMD) grafting,[17] or glutaric anahydride.[73] With the help of catalysts, biomolecules are then exposed to the surface in a buffer solution and become immobilized through the formation of amide bonds between available NH_2 groups in the biomolecules and the available COOH groups on the surface. Another type of chemisorption is employing a free sulfur atom in the biomolecule for interaction with a maleimide functional surface, thereby forming maleimide chemisorption.[73,74,83,84] It must be taken into account whether the chemical moieties are available in the biomolecule to be chemisorbed. For example, Fn has ~75 lysine residues with available NH_2 functionalities for chemisorption. Although Fn has 65 cysteine residues (a sulfur containing amino acid), all but two are available for maleimide chemistry.[73] The others are already employed for disulfide bonding in the tertiary structure. The RGD motif is commercially available in the form of glycine-arginine-glycine-aspartic acid-serine (GRGDS), the tail amino acids present for the purpose of chemical grafting, whether it is chemisorption to a surface or another application.

Chemisorption is employed in the formation of SAMs of organosulfurs and organosilanes (discussed later in section 5.3). Organosulfur SAMs are chemisorbed to gold surfaces via Au−S covalent bonds. Organosilanes are chemisorbed to glass or other silicon-based material surfaces.

3.2. Physical Interactions

All long-range interactions that do not involve the sharing/exchange of electrons in surface science technically fall under the category of physisorption. In physics, chemistry, and bulk materials science, such interactions are referred to as physical interactions or sometimes physical bonds. Although electrostatics is the nature of nearly all physisorbed interactions, some physical interactions (such as hydrophobic) are not completely understood. There is no universally accepted interaction potential to describe every interaction. In this section, where possible, we describe interactions quantitatively with the interaction potential. The resultant force of an interaction can be found by taking the derivative of the interaction potential with respect the spatial dependence (in this article, the separation distance, r).

We begin with the simplest interaction, that being the *Coulomb interaction*. The interaction potential of the Coulomb interaction is given by

$$w(r) = \frac{1}{4\pi\varepsilon\varepsilon_o}\frac{q_1 q_2}{r} \quad (1)$$

where q_1 and q_2 are the charge magnitudes of the two interacting bodies, ε is the dielectric permittivity of the interacting medium, ε_o is the permittivity of free space, and r is the separation distance. If the two charges have the same polarity (i.e., both negative or positive), the interaction potential becomes positive; thus, the two charges experience a repulsive force. If the two charges have opposite polarities (i.e., one negative and one positive), the interaction potential becomes negative; thus, the two charges experience an attractive force. For all interactions, a negative interaction potential corresponds to an attractive force and a positive interaction potential corresponds to a repulsive force. We also note that, if one takes the derivative of eq 1 with respect to r, the result is *Coulomb's Law*. For simplicity, we present the interaction between two point charges only. For other geometrical configurations, we refer the reader to the classic texts of Griffiths[85] and Jackson.[86]

The Coulomb interaction is relevant in cell−surface science, because surfaces with an associated charge are usually correlated with cell behavior to a charge polarity. Large biomolecules such as proteins and growth factors also portray local charge in their amine (NH_2^+) and carboxyl ($COOH^-$) groups. The net charge of a large biomolecule is dependent on the pH of the solvent and can be estimated by knowing its isoelectric point. The cell surface also has a charge association. For example, hyaluronan is an osteoblast cell surface matrix protein of high negative charge.[87] Thus, hypothetically, an osteoblast cell resists binding to a negatively charged surface (e.g., a COOH−SAM-modified substrate) compared to a positively charged surface (e.g., a NH_2−SAM-modified substrate). For further insight on the role of electrostatic interactions at biological interfaces, the reader is referred to literature by Kleijn and van Leeuwen.[88] The Coulomb interaction is often discussed indirectly in cell−surface literature as cell behavior correlation to substrate charge polarity is often discussed. Although it is correct that a simplistic Coulomb interaction is relevant in a cell−surface system, it is somewhat naïve to believe that it is the sole relevant physical interaction in the overall system.

A well-known and studied interaction is known as the van der Waals (VDW) interaction. The interaction potential is generally given by

$$w(r) = -\frac{C_{VDW}}{r^6} \quad (2)$$

where r is the separation distance and the constant C_{VDW} is dependent on the properties and geometry of the interacting bodies. In 1873, van der Waals proposed the successful "equation of state".[89] The success of van der Waals' equation of state inspired theoretical research on the origin of the forces in a gaseous system. Such origins were originally described quantitatively by Keesom, Debye, and London. Thus, the VDW force has three distinct sources of molecular interaction: (i) The first source is between molecules with permanent dipoles (or more generally multipoles) and is known as the *orientation* or *Keesom interaction*.[90] (ii) The second source occurs when the dipole moment of one molecule induces a dipole moment in another. Such dipole induction is dependent on the polarizability of the molecule whose dipole moment is induced. This is known as the *induction* or *Debye interaction*.[91] (iii) The third interaction source is the result of interaction of the natural dipole moments as a result of an electron orbiting between two molecules. All molecules express such an "instantaneous" dipole moment. This is perhaps the most important interaction of the three VDW contributors because it is the strongest and is always present no matter the molecules interacting. This force is known as the *dispersion* or *London interaction*.[92] Together these three interactions make up the total VDW interaction. More quantitative detail on the individual Keesom, Debye, and London interactions can be found in the texts of Israelachvili[78] and Hunter.[79] Applying the theoretical and conceptual principles of VDW interactions to biological processes is discussed in detail by Israelachvili.[93]

Although one or more of the Keesom, Debye, or London interactions are always present in any interaction, in cell−surface

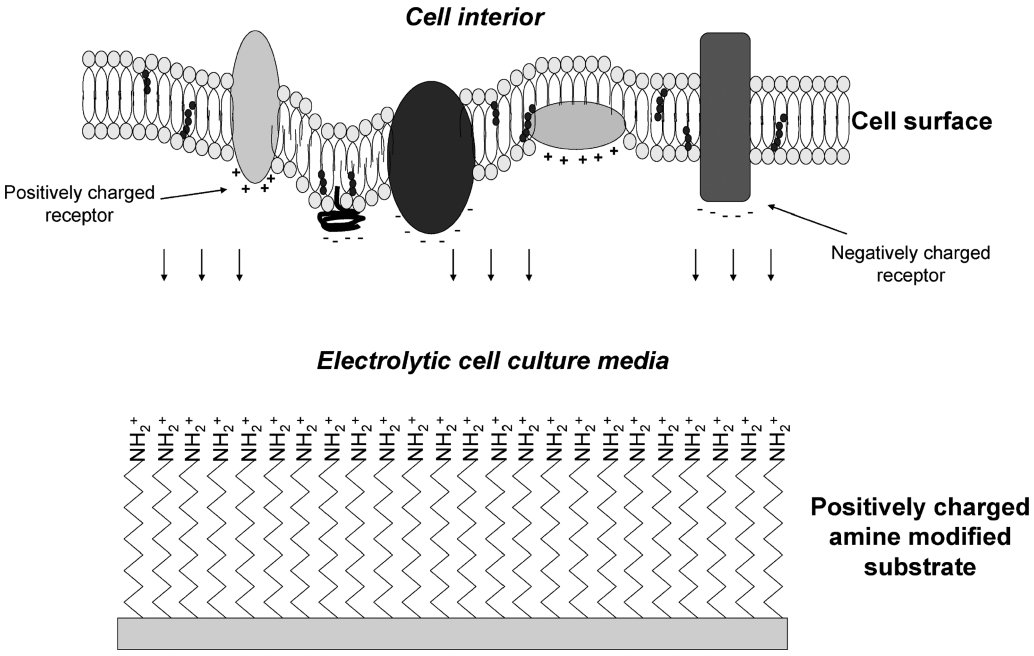

Figure 4. Double-layer-like interaction between a modified substrate bearing a charge and a cell surface with charge-bearing receptors. Figure not drawn to scale. The arrows indicate that the cell is approaching the surface.

research, macroscopic as opposed to microscopic descriptions of the VDW interaction are often more convenient and relevant to cell−material interactions. The VDW interaction between two macroscopic bodies can be approximated using pairwise summation known as *Hamaker theory*.[94] Using *Lifshitz theory*,[95] the Hamaker constant (analogous to C_{VDW}) can be determined for two macroscopic bodies interacting through a medium. The VDW interaction potential and force between various macroscopic geometries in terms of the Hamaker constant are presented by Leckband and Israelachvili.[80]

The interaction between a cell and a surface can intuitively be thought of as the interaction of two different, charged surfaces in an electrolytic solution. Although gravity is the primary force responsible for bringing the cell from the suspension medium to the surface, there is in actual fact another interaction consequent of the electric charge the plasma membrane and modified surface portray in the electrolytic culture medium in which they interact. Such an interaction is generally known as the *double-layer* (DL) *interaction*. The DL interaction results when two charged surfaces approach one another in an electrolyte solution: charges can be attracted, repulsed, or bound to the surfaces. Such an interaction is given quantitatively by

$$w(r) \approx + C_{ES}\, e^{-\kappa r} \qquad (3)$$

where C_{ES} is a constant and the factor κ^{-1} is known as the Debye length. The constant C_{ES} is dependent on the properties (i.e., surface charge density, potential, geometry, etc.) of the surfaces, and the Debye length depends on the properties of the liquid (i.e., solute concentration, type, solution temperature, etc.). C_{ES} can be determined by solving the Poisson−Boltzmann equation or other trying theories.

Cell culture media contain a wide variety of solutes such as proteins like albumin, carbohydrates like glucose, dipolar amino acids, and electrolytic salts like NaCl and KCl, all of which influence the Debye length. As mentioned earlier, substrates, biomolecules, and cell surfaces often portray a local net charge. Thus, DL interactions are very relevant in cell−surface science as initial cell−surface or biomolecule−surface interactions can intuitively be thought of as two charged surfaces approaching one another in an electrolyte solution (i.e., culture media or buffer solutions), as shown in Figure 4. This initial interaction likely influences the morphology and behavior of cells on surfaces. Such an interaction is rarely mentioned in cell−surface literature. Analysis is usually performed several hours to days after initial cell seeding to the surface. As mentioned earlier in section 2.2, cell adhesion is a dynamic process. Initial DL-like interactions as just described likely influence the dynamic mechanisms that result in the later observed cellular behavior such as morphology, proliferation, migration, etc. Focus is often placed on observations chosen at a given period of time after cell seeding to the surface, thus often neglecting the dynamic molecular mechanisms that lead to such observations. Some DL interaction potentials and forces for various macroscopic geometries are given by Leckband and Israelachvili.[80] Cells and large biomolecules are nonrigid bodies. Thus, while these interaction potentials for DL in macroscopic geometries are relevant, they would not truly represent a cell−surface DL interaction. This adds complexity to an already complicated system, making a quantitative description challenging.

For surfaces in aqueous solutions, it is common for the DL and VDW interactions to be paired together in what is known as *DLVO* (Derjaguin−Landau−Verwey−Overbeek) *theory*.[96,97] The VDW force is affected very little by changes in the solution or surface environment while the DL force is very sensitive going from attraction to repulsion depending on conditions. Generally, the DL force is of greater magnitude than the VDW force. The VDW force becomes more dominant at smaller separation distances. However, one must also consider that, at smaller separation distances, other forces can become dominant.[80] The fundamental relevance of DL interactions to biological interfaces is further discussed by Kleijn and van Leeuwen.[88]

A force that arises when two surfaces approach one another in a solution is known as the *oscillatory force*. Such a force oscillates

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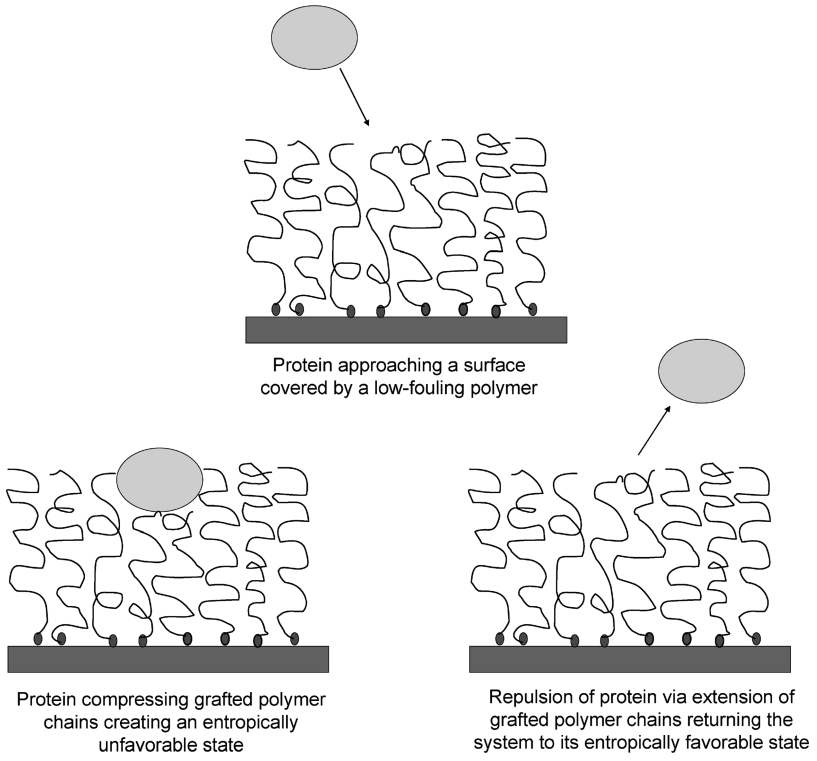

Figure 5. Steric repulsion of proteins from a polymer-grafted surface.

between attractive and repulsive, and the periodicity is dependent on the diameter of the confined molecules between the surfaces. The interaction of the oscillatory force is generally given by

$$w(r) \approx -E_0 \cos\left(\frac{2\pi r}{\delta}\right) e^{-r/\delta} \quad (4)$$

where E_0 is the contact energy and δ is the diameter of the solvent molecules. A requirement for the oscillatory force is a rigid smooth surface. In biology and physiology, cells are nonrigid and large biomolecules are rarely if ever rigid. As such, the oscillatory force should be less relevant in biological systems.[80]

An interaction specific for the presence of hydrogen is known as a *hydrogen bond* (H-bond). The H-bond exists between a hydrogen atom and an electronegative atom or molecule. It pictorially appears to be a VDW−Keesom dipole−dipole interaction. However, quantitatively the affinity of an H-bond is ∼1 order of magnitude higher than an electrostatic VDW−Keesom interaction, yielding it a special name. It has been accepted that there is no exchange/sharing of charge in H-bonding. However, the unique characteristics of H-bonding have them classified by many as quasi-covalent.[78]

The unique behavior of materials in the presence of water has led to the widely studied topic of hydrophobicity. Although the nature of hydrophobic effects and interactions are unknown, their importance in biology and physiology is unprecedented. Hydrophobic materials are usually nonpolar. The first detailed theory of hydrophobicity was the work of Frank and Evans.[98] When a hydrophobic molecule (i.e., solute) is placed in water, water molecules surround the molecule and orient into an ordered structure in the vicinity of the molecule while not losing any of their H-bond sites. Such a phenomenon is not entropically favorable and is known as the *hydrophobic effect*. This is an entropically unfavorable interaction because the water molecules in close vicinity of the solute hydrophobic molecule are well-ordered in comparison to those not in the vicinity. When multiple hydrophobic molecules and/or two hydrophobic surfaces are placed in water, the molecules cluster together and/or the surfaces attract one another. Such interactions are far stronger than if the bodies were not in water (i.e., VDW interactions using Lifshitz theory[99]), yielding it a special name. Such a phenomenon is known as the *hydrophobic interaction*.

Quantitative descriptions of the hydrophobic interaction between surfaces have been developed using force−distance measurements.[100−102] It was found that a hydrophobic interaction generally follows a somewhat exponential decay.[103] We revisit these experiments on the hydrophobic effect later in section 5.6.

Despite numerous experimental and theoretical studies on hydrophobic interactions, its nature and a general quantitative interpretation still remain elusive.[99] It has been suggested that the hydrophobic interaction is a special type of VDW interaction that is a result of the unique dielectric properties of water.[78] Despite the vast unknowns on the origin of hydrophobic interactions, without question these interactions play an important role in biology and physiology due to the presence of water in such systems. They are believed to be responsible for stabilizing biomolecules such as DNA and proteins and are always implied to be involved in the adhesion of cells to surfaces.[104]

A common term that arises in science is *hydrophilicity*. Such a term arises because many molecules are highly soluble in water (as opposed to nonsoluble or hydrophobic) and are thus coined "hydrophilic". Hydrophilic molecules are believed to have a disordering effect (i.e., entropically favorable), the opposite of hydrophobic molecules. However, one must note that no such hydrophilic interaction exists.[78] Rather, hydrophilic matter in

water has a weak hydrophobic interaction in comparison to hydrophobic matter. Hydrophilicity is not a polar opposite characteristic to hydrophobicity as some seem to imply.

The presence of long polymeric and large molecules in biological and physiological systems results in a very significant repulsive force known as a *steric interaction*. There is no single interaction potential that can quantitatively describe steric interactions. As discussed later in section 5.7, the steric interaction potential is dependent on the conformation and density of the polymeric material. Furthermore, the interaction potential is an approximation as opposed to an exact expression. Discussions doubting the validity of such approximations still remain. The magnitude of steric interactions is dependent on polymer density and the molecular weight of the body undergoing interaction.

The nature of steric repulsion is due to entropically unfavorable compression of polymer chains or large molecules upon contact with another large molecule. As shown in Figure 5, the system is compressed due to the encroachment of a large molecule. For the system to return to its entropically favorable state, the polymer chain is extended, repulsing the large molecule. When polymeric materials such as dextran derivatives or poly(ethylene glycol) (PEG) are chemisorbed to surfaces at an optimal density, the magnitude of steric repulsion can in fact prevent not only protein adsorption but also cellular adhesion. Thermal fluctuation in physiological and biological systems also can lead to oscillatory motion of polymer brushes, enhancing repulsion. Steric repulsion is discussed in more detail later in this article (section 5.7) as it pertains to low-fouling surfaces (i.e., little to no nonspecific protein adsorption).

The interactions discussed thus far in this section are vital to protein adsorption. As discussed earlier in section 2 of this article, protein adsorption also plays an influential role in cellular adhesion. For a discussion on physical interactions and protein adsorption, the reader is referred to a series of literature composed by Horbett, Brash, and Hoffmann.[105−107] Very briefly, it is believed that proteins adsorb with a high affinity to hydrophobic surfaces resulting in unfolding of the protein (i.e., drastic conformational change) at the surface followed by the release of water from the interface. Cationic proteins bind to anionic surfaces and vice versa. Thus, attractive DLVO and hydrophobic interactions are the primary forces responsible for the adsorption of proteins to surfaces.[75,108]

An interaction between biological molecules that is somewhat contradictory is the *ligand−receptor* (LR) interaction. The short range, high affinity, well-ordered geometric structure, and specificity of this interaction make it unique from other types of physical interactions. The most studied LR interaction is that between biotin and streptavidin and/or avidin.[109] Biotin−avidin/streptavidin and other LR interactions involve electrostatic, VDW, H-bonding, and hydrophobic interactions in sequence or simultaneously. LR interactions also have a finite life span. The complexity of LR interactions is vast. However, these are perhaps the frontier between physical (i.e., VDW, DL, etc.) interactions and biological function (e.g., diseases). For example, apoptosis and proliferation of cells is regulated by LR interactions. Dysfunction of such LR interactions can result in hyperproliferation, often leading to cancer.[110] For a thorough review of the physics of LR interactions in both a theoretical and experimental context, the reader is referred to an article by Bongrand.[111]

Many of these interactions can be present in a biological system.[76] It is difficult to draw the interaction potential of a force

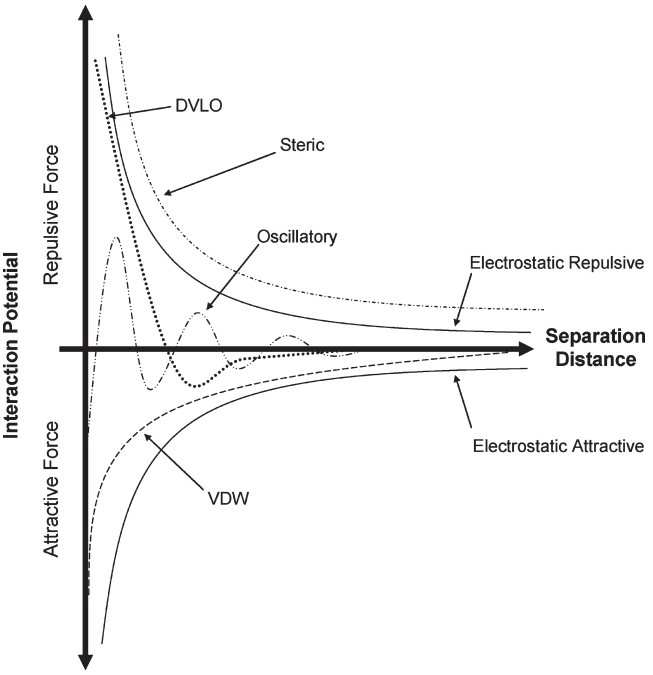

Figure 6. Interaction potentials of surface and intermolecular forces in biological systems. While there is no universal interaction potential to describe the steric interaction, it is generally semiquantitatively represented as shown.[76,80]

in a biological system for reasons mentioned in section 3. To give the reader a semiquantitative representation of these forces, Figure 6 shows the interaction potential of some forces as a function of separation distance.

In summary, this section has shown that cell adhesion is a dynamic complex process involving an array of physical interactions, both specific and nonspecific, occurring at varying stages during cellular adhesion. Furthermore, the nature of some of these interactions remains unknown. It is difficult to describe macroscopic phenomena such as cell adhesion and subsequent behavior from a molecular perspective. Some attempts have been made and are still in the works to unify molecular and macroscopic phenomena associated with cell adhesion. For a discussion on this, the reader is referred to an article by Simson and Sackmann.[112] Briefly, it appears that the elasticity of the cell membrane is the parameter that largely dictates cell adhesion. The interactions of elastic lipid bilayers that comprise the cell membrane with similar or other types of surfaces are described as undulation forces (first coined by Helfrich[113]).

3.3. Interaction Measurements

There are many experimental methods that exist to measure such forces just discussed. Some include surface force apparatus (SFA), atomic force microscopy (AFM), optical tweezers, osmotic pressure measurement, micropipet aspiration, and shear flow detachment measurements, to name a few. For the appropriation of a particular experimental method, the reader is referred to articles by Leckband and colleagues.[80,114] Unfortunately, these techniques are in vitro and are not representative of a physiological environment. Nevertheless, valuable information can be obtained when employing one or more of these techniques.

The two most widely used techniques in characterizing biological forces are the SFA and AFM. The SFA is a very powerful tool for measuring the force between two surfaces at a

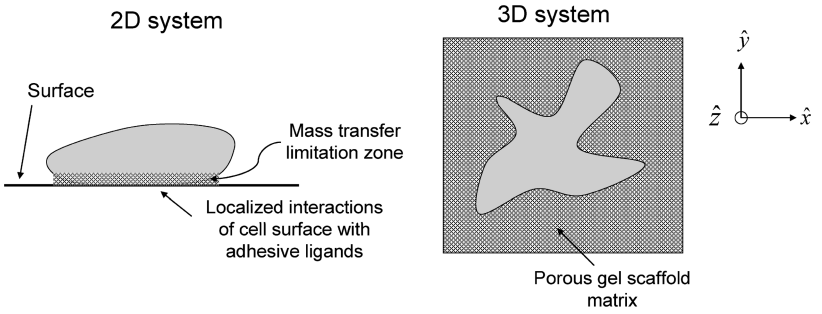

Figure 7. Key conceptual differences between 2D and 3D culture systems.

known separation distance. Modifications by Israelachvili and Adams[115] to the original design of Tabor and Winterton[116] extended the capability of the SFA to liquid environments, making SFA a very powerful instrument in measuring biological forces. The two surfaces can be tailored to meet the specific interests of the user. Force is measured via the deflection of a spring and distance is measured via optical interferometry. SFA can measure the electrostatic profiles (albeit local not global) of proteins and the force−distance profiles between cell membrane receptors and proteins.[117,118] The AFM was developed by Binnig et al. in 1986.[119] The ability of the AFM to achieve high-resolution topographic images and force measurements at the nanometric scale in air and particularly liquids makes it an attractive tool in biology.[120] Force interactions between a molecule and a surface are achievable. A molecule is immobilized onto the cantilever of the AFM; the cantilever is then brought toward the surface. The deflection (measured with a laser) of the cantilever is then used to profile the interaction between the molecule of interest and the surface of interest. Interactions as weak as a single H-bond have been reported.[121]

Valuable information has been acquired using AFM and SFA to measure interactions in biological systems. Such experiments provide insight into the underlying mechanisms and dynamics of cell−surface interactions that are not achievable using static and fortuitous labeling of cells on modified surfaces.

4. SYSTEMS FOR STUDYING CELL−MATERIAL INTERACTIONS

4.1. In Vivo Systems

The in vivo environment of cells is far more complex than any controlled 2D or 3D in vitro system. Depending on the tissue type, in vivo cells can have surroundings consisting of other cell phenotypes (e.g., cells from major organs, muscle cells) and ECM molecules. Moreover, the ECM is composed of a restricted number of molecules that have profound effects on the mechanical and chemical environment of cells. The ECM is able to bind soluble growth factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) and thus create molecular gradients that subsequently result in localized interactions with surrounding cells.[122]

The most common ECM molecules are collagens. Other proteins, such as elastin and proteoglycans (such as hyaluronic acid), also contribute in defining mechanical and chemical niches. It is not the aim of this section to summarize ECM and cellular environments of different tissues, although this information is vital when attempting to create representative 3D in vitro culture environments. For example, the study of chondrocytes in vitro necessitates an environment that possesses inelasticity, is highly hydrated, and is sparsely vascularized, and where cells are located at relatively large distances from one another. Hepatic tissue, on the other hand, requires ECM elasticity, high vascularization, and cell aggregation to maintain differentiation. For specific information on different tissue types relative to ECM, vascularization, and cell−cell interactions, the reader is referred to histology texts by Kessel[123] and Stevens and Lowe.[124] Cells in more relevant 3D in vitro culture systems behave much differently than in traditional 2D systems.

4.2. Cells in a Three-Dimensional Environment

The growth of cells in a three-dimensional in vitro environment is not a novel idea in biology. More than 50 years ago,[125] experiments showed differences between the shape of fibroblasts cultured in blood plasma clots relative to the orientation of the fibrous network of the clot, hinting at the importance of the 3D surroundings on cell behavior. Around the same time, mesenchymal cells cultured on glass coverslips showed stress fibers seldom encountered in vivo.[126] Despite these results, 2D systems have been, until recently, the principle vehicle for in vitro cell and biomaterials studies, principally due to their relative ease of use. The current knowledge in cellular physiology, genetics, and proteomics has been influenced in large part by experiments in which cells were artificially grown on flat surfaces, often plastics.

Key conceptual differences between in vitro 2D (i.e., traditional) and 3D cell culture systems are outlined in Figure 7. Traditional 2D cell culture systems consist of adhesive ligands spatially distributed on a surface (e.g., on the $x-z$ plane in Figure 7). This 2D localization of ligands results in adhesive asymmetry of the cellular organism. Furthermore, the rigid nonporous surface upon which the adhesive ligands are supported creates a mass transfer limitation zone. Conversely, in a 3D cell culture system, the adhesive ligands are spatially distributed in all geometric planes. Thus, all areas of the cellular organism are equally exposed to adhesive ligands. Because the adhesive ligands are distributed in a porous gel scaffold, a 3D cell culture system lacks the mass transfer limitation zone (i.e., the cell−substrate interface) of the 2D system.

The emergence of 3D cell culture systems has outlined the importance of many variables that are not considered when culturing cells in 2D. In addition to usual considerations such as nutritive medium composition, cell density, dissolved gas composition, and surface chemistry, variables such as ECM chemical composition and mechanical properties define 3D culture systems. 3D systems can be fabricated from materials derived from in vivo constituents, such as ECM and synthetic materials. Differences in mechanical properties, for example, the elasticity of the support matrix and that of cells, create a boundary. Changes in the flux of metabolites and gases in 3D systems, relative to 2D, also greatly contribute to define cell response.

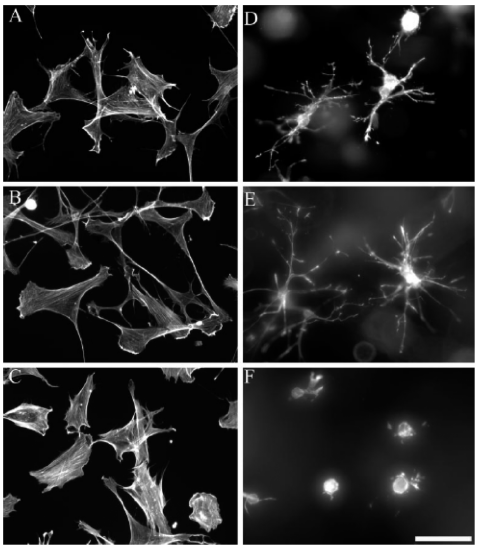

Figure 8. Human fibroblasts project a dendritic network of extensions in collagen matrices but not on collagen-coated coverslips. Fibroblasts were incubated 5 h on collagen-coated surfaces (A−C) or in collagen matrices (D−F). After 1 h, 50 ng/mL PDGF (B and E) or 10 μM LPA (C and F) was added to the incubations. At the end of the incubations, samples were fixed and stained for actin. Bar: 80 μm. Reprinted with permission from ref 128. Copyright 2003 The American Society for Cell Biology.

We first present key results obtained in 3D environments. Then, the important parameters of 3D systems, including the mechanical, biochemical, and transport properties of the matrix scaffold used to support cells, will be discussed. Finally, important culture systems made from natural and synthetic materials will be reviewed, along with reported uses.

4.3. Differences Between Two-Dimensional and Three-Dimensional Cell Culture Systems

A number of experiments have undoubtedly demonstrated dramatic variations in cellular response when culturing cells in a 3D environment compared to 2D flat culture plates. Important differences in key biochemical events, cell shapes, and cell motility have been observed and characterized. Morphological differences often strike the imagination and perhaps best convince the huge gap between cell physiology in 2D and 3D. For example, fibroblasts grown in collagen matrices compared to collagen-coated coverslips present a widely different morphology when exposed to selected biochemical niches, specifically, platelet-derived growth factor (PDGF), known to stimulate dendritic network formation in vivo, and lysophosphatidic acid (LPA), known to cause cellular retraction as shown in Figure 8.[127,128] Moreover, fibroblasts grown in 3D collagen matrices show important differences to growth factor stimulation that impacts the cell-generated contractile forces.[129] Mechanically loaded matrices stimulate the differentiation of fibroblasts into myofibroblasts.[130]

Cell migration in 3D is widely different from distinct focal contacts and stress fibers observed in haptokinetic cell migration in 2D culture systems.[131] Spindle-shaped cells such as fibroblasts, many tumor cells, and endothelial cells constantly reorganize their surrounding ECM through, for example, serin proteases and metalloproteinase-mediated proteolysis. These cells also interact constantly with the ECM through the large number of integrins located on their surface to migrate in 3D systems.[132,133]

Another example is angiogenesis, the formation of new capillaries from existing blood vessels, which is present in various physiological processes whether they be normal, such as embryogenesis and wound healing, or abnormal, such as in developing cancer masses. Large developments have been achieved in understanding important cell−material interactions linked to angiogenesis. However, angiogenesis models in 3D have recently been shown to exemplify the importance of studying cells in the third dimension rather than on flat surfaces.[134,135] The importance of ECM for angiogenesis has been documented for 2D models. Evidence suggests that 3D models yield dramatic differences in cell behavior. Important cellular signaling pathways for angiogenesis, such as mitogen-activated protein (MAP) kinases and extracellular signal-regulated kinase (ERK) pathways, have both been shown to be strongly influenced by the use of 3D collagen and fibrin gel environments compared to 2D models.[136] Moreover, in the same study, by comparing 2D cell culture systems with collagen and fibrin matrices, dramatic morphological differences were reported when culturing human umbilical vein endothelial cells (HUVEC) in a 3D matrix compared to 2D culture plates. For example, in 3D, HUVEC vacuoles rapidly grow in size and adjacent cells coalesce to form tubelike structures after only 4 h.[136]

The onset of cancer cell dedifferentiation such as the transition from epithelial to mesenchymal phenotype is now considered an ensemble of complex processes in which extracellular cues play a major role.[137] The importance of signaling pathways leading to dedifferentiation has been studied widely in vitro. However, data from plastic 2D culture plates show important differences from results obtained with cells cultured on porous substrates or 3D collagen gels. The differences range from important morphological differences upon biochemical exposition[138] to variations in apoptosis levels.[139]

3D models have recently been used to study the mechanisms of apoptosis in adult tissues. It has been known for a long time that very important differences exist between the survival of cancer cells embedded in 3D spheroid bodies compared to tumors grown on flat surfaces when exposed to death stimuli.[140,141] Also, the presence of a large number of adhesion molecules for cancer cells in a 3D environment is suspected to be responsible for the drug resistance of certain types of cancers.[142] These phenomena have been an obstacle for the in vitro discovery of novel anticancer agents.

Aberrations in the apoptosis pathway in adult tissues are known to be an important factor in the pathogenesis of cancer, among other diseases.[143] Although thorough apoptosis studies have been carried out using traditional 2D cell cultures, studies in more relevant 3D culture models are now thought to lead to a more conclusive understanding of its mechanisms and, thus, of cancer.[144] The loss of important cell−ECM interactions has been associated with apoptosis for many tissue types. For example, the loss of specific integrin-mediated interactions has been shown to result in apoptosis for neural and mammary gland tissue, among others.[145,146] While 2D cultures have been used to reveal important adhesion mechanisms for apoptosis, experiments conducted using 3D systems have pointed toward the likelihood of more complex apoptosis signaling regulation. For example, lumen formation by cell apoptosis during mammary morphogenesis was shown to be due not simply to the loss of ECM interactions, as suggested by 2D studies, but to much more intricate phenomena.[145] Among others, the polarity induced by 3D epithelial tissue architecture is thought to be a critical aspect

of apoptosis regulation,[143] as are the differences in mitochondria homeostasis modulation in 3D compared to 2D cultures.[147]

Although the causes of the previous observations for different cell types in 3D compared with traditional 2D culture systems are not always understood, some general conclusions can be drawn as to the underlying mechanisms guiding these events. 3D environments have the capacity to convey a large spectrum of mechanical information, as well as to present a large array of textures and shapes to attached cells, when compared to flat surfaces. As such, both mechanical properties and textures are considered driving phenomena that help explain the large variations observed between flat surfaces and matrices along with two other key aspects of 3D environments: the chemical composition of scaffold material and its gas, nutrient, and waste permeability. These parameters can often have dramatic and overlooked effects in many experimental schemes of biomaterials science and have likely resulted in misleading observations. We will now attempt to outline the known effects of these phenomena and their mechanisms of action in cells.

4.4. Properties of Cell Culture Systems

4.4.1. Mechanical Properties. Mechanical properties of cell culture systems elicit and regulate, in an indirect manner, a large number of cell responses in both 2D and 3D systems and should be considered as important for cell physiology as biochemical properties. For example, such properties influence morphogenesis, bone formation, and neutrophil activation and are responsible for several stress-related pathologies.[148−150] They affect cellular migration, morphology, and adhesion.[151]

Mechanical loading is the imposition of stresses and/or strains on a body through a mechanical force. Should the force on the body be of appreciable magnitude and the body behaves in an elastic manner, deformation occurs. There are three ways to deform an elastic body via mechanical loading, through tensile extension, bulk compression, and shear stress. Quantitatively, the elasticity of a body is defined by the elastic modulus of the material: Young's modulus in the case of tensile loading, the bulk modulus in the case of bulk compression, and the shear modulus in the case of shear stress. Cells undergo each type of mechanical loading in both 2D and 3D cell culture systems.[152] Furthermore, many materials used in tissue engineering and cellular scaffolds possess viscoelastic behavior. Therefore, to describe these systems, it appears necessary to investigate viscoelastic material functions (e.g., stress relaxation, creep and recovery).

In an exclusively intuitive sense, a feedback phenomenon appears to be present between the matrix elasticity and the cell. Cells modify their intracellular signaling according to the elasticity of their local environment. Studies show that the elasticity of the 3D surrounding porous gel matrix of the cells has an impact on cell shape and cytoskeletal tensions, which in turn dramatically influences the fate of cells.[153] Likewise, cell plasma membrane elasticity equally influences cell behavior and fate, as discussed by Sackmann and Bruinsma.[154] Thus, 3D cell culture systems must be designed to provide mechanical loads with a magnitude, orientation, and type relevant to those of a physiological system.

The nature of the porous ECM scaffold, noticeably its elasticity, has a profound impact on the behavior of entrapped cells. Specifically, the nature and affinity of focal adhesions between cells and scaffold material is a key to force retroaction. Cells in hydrogels such as collagen and fibrin portray many focal adhesions, whereas cells in synthetic substrates, such as polyacrylamide gels, portray fewer focal adhesions.[151] Ligand-coated synthetic gels may improve, in some cases, cell−material focal adhesions.

Techniques such as AFM and micropipet aspiration can be used to study the effect of mechanical loading on cells. Typically, mechanical loads are applied on macroscopic scaffolds in which cells are entrapped. This can be accomplished on flat planes (e.g., tissue culture wells), yielding a 2D system.[152] Systems able to apply tensile loads on cell monolayers at known magnitudes are available (e.g., Bio-Flex plate, Flexcell Intl Corp.) and have been used to grow smooth muscle cells (SMCs) in strained collagen layers.[149] 2D surfaces are relevant to the study of endothelial cells lining blood vessels. However, important efforts are conducted to use 3D scaffolds, which are more representative of the in vivo reality for most tissue types. In such systems, scaffolds can be "loaded" through the attachment of the whole scaffold to vessel walls or "unloaded" as free-floating scaffolds.[127] Furthermore, complex bioreactor systems can also be used to control shear stresses around entrapped cells.[155]

Evaluation of the effects that mechanical loads have on cell behavior is challenging. In 2D systems, compression of silicone sheets or membranes is often used.[129,151] Silicone sheet elasticity can be varied for such systems, but must be quantified, which is a tedious procedure. Furthermore, while silicone sheets aptly illustrate unidirectional forces, multidirectional forces are unquantifiable in this system. Apparata that monitor gel deformation have been developed for 3D systems.[129]

Fibroblasts remain predominantly studied to correlate the mechanical loading to cell response within a system. Fibroblasts are typically seeded in mechanically loaded or unloaded type I collagen gels in which specific growth factors can be added, or cultured as a monolayer on a gel-coated well, with some form of force-sensing device. Fibroblasts were found to be highly sensitive to the mechanical loading of their environment, which is logical considering their role in tissue homeostasis, i.e., in synthesis of ECM components such as collagens and proteoglycans and in tissue remodeling and repair.[152] For example, fibroblast gene expression and growth factor responses in multiple mechanically defined environments, both 2D and 3D, show very important variations, as presented in a review by Wang et al.[152] Also, collagen fibril density was shown to modulate fibroblast proliferation and morphology.[128,156] Typically, fibroblasts are known to proliferate when placed in unloaded matrices or 2D culture systems compared to mechanically loaded systems. Finally, force monitoring has shown that, when growth factors are present in a 3D system, fibroblasts generate contractile forces.[129]

The influence of mechanical loading on the behavior of other types of cells including SMCs, endothelial cells, myoblasts, hepatocytes, osteoblasts, neurons, and neutrophils has been studied in the past. SMCs show increased proliferation and altered response to growth factors when subjected to mechanical strain in vitro.[157,158] SMC behavior has also been shown to be dramatically different in 2D collagen-coated surfaces compared to 3D collagen gels, noticeably in protein expression and contractile activity.[159] Myoblasts plated on collagen-coated polyacrylamide gels in vitro present normal myotube striation only at intermediate matrix elasticity that corresponds to in vivo healthy muscle tissue.[160] Hepatocytes maintain a differentiated phenotype and aggregate only in relatively elastic materials (such as Matrigel).[161] Neuron regeneration after injury in the central and peripheral nervous system is enhanced by the use of elastic

hydrogels that are hypothesized to lower the density of an otherwise impenetrable scar tissue at injury.[162] Alkaline phosphatase activity, a marker of osteoblast activity, is enhanced up to sevenfold when exposed to dynamic flow (i.e., elevated shear stresses).[163] When mechanically deformed in vitro in narrow channels to mimic in vivo deformation occurring in capillaries, neutrophils activate and extend pseudopods, thus increasing their migratory tendencies.[150]

It appears that the influence mechanical loading has on cell behavior in culture systems, whether it be 2D or 3D, is substantial. However, understanding is incomplete. The underlying difficulty is that the mechanical loading of a system is macroscopic in nature whereas cell response is molecular in nature. Drastic variations in materials (e.g., cell phenotype, scaffold material, etc.) between subsequent studies complicate conclusions. Nevertheless, analytically, one can draw some general conclusions, using fluid mechanics and the Buckingham-π theorem (presented in a later paragraph), relating how material mechanical properties influence cell response and behavior (i.e., cell spreading and morphology) to mechanical loading. Observations in the physics community suggest that, as Singer and Nicolson's Fluid-Mosaic model discussed earlier eludes,[32] a cell can be considered a viscoelastic body. As such, the cell simultaneously has the properties of a liquid (i.e., viscosity) and a solid (i.e., elasticity). Furthermore, materials composing gel matrix scaffolds (e.g., fibrin, collagen, etc.) portray the same properties.

During spreading (i.e., the process of adhesion) on a 2D surface, the cell behaves like a viscoelastic fluid body undergoing wetting and dewetting transitions.[154,164] Specifically, when a cell is interacting with a material, there is a disruption in its molecular architecture.[165] This disruption is a result of the generated force by the cell through specific short-range LR and nonspecific long-range molecular interactions (discussed earlier in section 3) between the cell and the material. For the relations of such forces to the elastic behavior of a cell in a molecular and theoretical context, the reader is referred to a thorough review by Sackmann and Goennenwein.[166] Likewise, in a 3D matrix scaffold, cell spreading and/or migration is somewhat parallel to the shape and/or movement of a drop of fluid in a surrounding fluid medium.

The Buckingham-π theorem provides a means of using dimensional analysis to identify dimensionless numbers that describe the physical attributes of a system.[167,168] It has been extensively used in fluid mechanics. If a cell has fluidlike properties, then some of the dimensionless numbers of fluid mechanics may be applicable to cell behaviors that resemble those of a fluid in contact with a solid, namely, spreading and subsequent morphology. Utilization of the Buckingham-π theorem can perhaps shed some insight into the nature of macroscopic cell behaviors, as cell adhesion is somewhat parallel to the wetting of a fluid on a surface. Perhaps dimensionless numbers can be developed in the future that yield a quantitative basis for cell behaviors much like the well-established Reynold's number gives a quantitative basis between turbulent and laminar flow regimes in fluid mechanics. Such analysis could presumably then be utilized to design systems and select materials that elicit and control a desired cell response and could be invaluable tools to the biomaterials and tissue engineering community.

The molecular nature of the viscoelastic cell behavior has been under intense investigation over the past decade within the physics community. Two progressive experimental techniques that have been developed are magnetic bead microrheometry[169] and reflection interference contrast microscopy.[170] Theoretical studies have also been performed by Bruinsma and Sackmann[164] and Nardi et al.[171] Experimentally, viscoelastic parameters (e.g., elastic moduli, relaxation time, etc.) of fibroblasts and HUVEC have been measured.[169,172] In another study, materials analogous to the cell membrane were fabricated and their viscoelastic properties were measured.[173] From a molecular perspective, it appears that the molecules actin and myosin (heavily involved in cellular adhesion, as discussed earlier in section 2.2) give cells their viscoelastic properties as addressed by Uhde and coworkers.[174−176] The viscoelastic properties of scaffold materials have also been studied experimentally, and parameters (e.g., Young's modulus, Poisson ratio, etc.) have been measured. In addition to more natural scaffold materials such as collagen[177] and soft hyaluronic acid gels,[178,179] synthetic polyelectrolyte multilayer films[180] have been studied. For a comprehensive review of progress in studies of this manner, the reader is referred to recent articles by Smith and Sackmann[181] and Trepat et al.[182]

The literature on the viscoelastic properties of cells and scaffolding materials is particularly relevant to the biomaterials community. Such studies give more analytical insight as to how mechanical properties of a system influence cell behavior. A particularly relevant study is that of Feneberg et al.[172] Here, a confluent monolayer of HUVEC was subjected to a mechanical load, and its viscoelastic properties were measured using magnetic bead microrheometry. Such an experiment is designed to be analogous to the shear load HUVEC experience in the lumen of blood vessels. Thus, this study is directly relevant to relentless in vitro efforts to develop perfused microvessels by the biomaterials and tissue engineering communities.

4.4.2. Biochemical Properties. Cell−material adhesions are governed by short-range specific LR interactions and long-range nonspecific molecular interactions that are dependent on the chemical properties of both the cell and material (a 2D modified surface or a 3D gel matrix scaffold). Different phenotypes have different cell receptors, and different matrix materials have different ligands. Together, these are the biochemical parameters of a cell culture system. Examples of cell receptors include the integrins $\alpha5\beta1$ and $\alpha v\beta3$ (discussed earlier in section 2.2), whereas an example of an adhesive ligand is the RGD sequence found in fibrin and collagen gel scaffold materials, among others.

Adhesion receptors on the cell surfaces bind to various ligands of the ECM or other cells, regulating cell signaling cascades (e.g., through kinase- and phosphatase-dependent pathways) and yielding the final properties of living cells and tissues. Cell−material and cell−cell adhesion sites, whether specific or not, may be largely different in in vitro 2D systems compared to 3D systems. For example, focal and fibrillar adhesions in 3D systems differ from adhesions on 2D substrates in their integrin/paxillin content and localization, as well as in tyrosine phosphorylation of FAK, as illustrated by Figure 9.[183] Moreover, a number of adhesion molecules present in natural ECM materials are sometimes difficult to obtain and localize in in vitro systems. For example, Matrigel, a collagen derivative used to mimic the in vivo basal membrane, is known to possess a large number of trace molecules, including growth factors, which have the capacity to elicit substantial effects on seeded cell populations. Matrigel is widely used in 3D culture systems as a matrix scaffold and can lead to irreproducible results because the trace molecules are not all known. Novel polymeric materials can also create unexpected

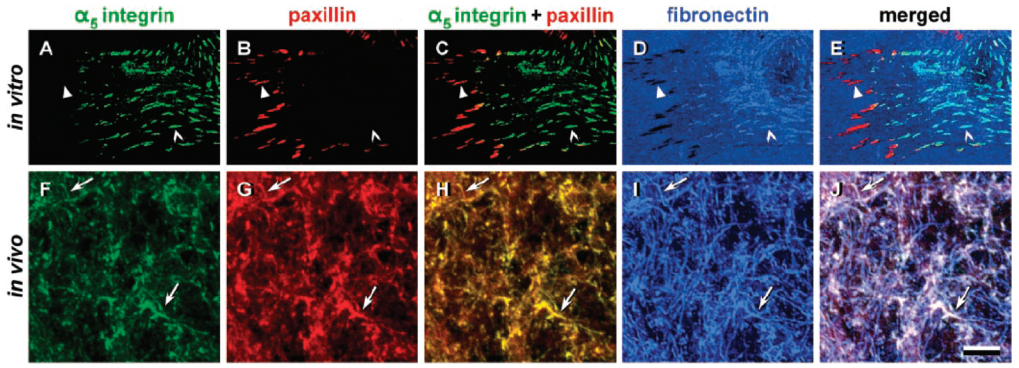

Figure 9. In vivo 3D matrix adhesions differ from focal or fibrillar adhesions on 2D substrates. (A−E) Confocal images of indirect immunofluorescence staining of NIH-3T3 mouse fibroblasts in vitro on a 2D fibronectin-coated coverslip; (F−J) transverse cryostat craniofacial mesenchyme sections of an E13.5 mouse embryo. α5 integrin [(A) and (F), green] and paxillin [(B) and (G), red] colocalize in a fibrillar organization in mesenchymal tissue [(H), yellow in merged image indicates overlap of red and green labels], but not on a 2D substrate in vitro (C). Fibronectin [(D) and (I), blue] localizes to fibrillar structures in vivo, and merged images indicate substantial overlap of all three molecules [(J), white compared to (E)]. Note that focal adhesions (filled arrowheads) and fibrillar adhesions (open arrowheads) show differential localization of the α5 integrin and paxillin markers only on traditional flat 2D substrates in vitro. The 3D matrix adhesions (arrows) identified by triple localization are present in 3D environments in vivo. Scale bar: 5 μm. Reprinted with permission from ref 183. Copyright 2001 AAAS.

responses from cells. Therefore, it is important to be cautious when comparing cell behavior on a plastic dish and in different scaffolds, whether natural or synthetic.

Cell adhesion from chemical niches varies widely depending on cell phenotype. Moreover, physiologic state, ligand distribution, concentration, and synergistic effects of numerous nonadhesive niches affect cell response. Chemical niches can consist of proteins (such as growth factors), ions, sugars, dissolved gases, or synthetic materials, among others.

Chemical niches are presented to embedded cells in 3D scaffolds through two means: (i) diffusion of molecules through the porous scaffold and (ii) ligands that constitute the composition of the scaffold. Although the first mechanism is dependent on transport properties (discussed in the next section), the second mechanism depends on the chemical composition of the scaffold. For example, collagen and fibrin gels contain the RGD sequences, and Matrigel (composed of both laminin and collagen) contains RGD sequences of collagen and the YIGSR (tyrosine-isoleucine-glycine-serine-arginine) sequence of laminin. Both the RGD and YIGSR sequences are responsible for initial cell adhesion to scaffold materials composed of such sequences. Natural polymers such as collagen will often contain trace molecules that are likely to interact with entrapped cells. On the other hand, synthetic materials can be devoid of such residues, although commercial plastics often contain plastic additives and those can affect cell responses.[184] In the case of mass transport, angiogenic growth factors such as VEGF and fibroblast growth factor (FGF), known to stimulate migration and proliferation, can be supplemented into culture media and delivered to the embedded cells via convection and/or diffusion. As an example, consider angiogenesis. A relevant scaffold material is fibrin gel, as it is well-established that it stimulates angiogenesis.[185,186] Suppose that the response of the endothelial cells in the system is inadequate; one could then supplement the culture media with VEGF or FGF to further simulate an angiogenic response.

Deciphering how chemical niches (i.e., parameters) influence cell response and behavior is not a trivial task from an analytical standpoint. Traditionally, techniques involve cell labeling using antibodies or other markers specific for a chemical niche. Nearly all labeling requires that the cells be fixed and/or permeabilized.

In addition to the microscopic techniques that were used to obtain the insights into cellular adhesion discussed in section 2.2, spectroscopic methods also exist to allow quantitative assessment of membrane protein dynamics in living cells. These include fluorescent-correlation spectroscopy (FCS), image-correlation spectroscopy (ICS), and fluorescence-recovery after photobleaching (FRAP).[187] Such techniques have yet to be exploited by the biomaterials and tissue engineering community. FCS tracks fluctuations in fluorescence intensity of labeled molecules in a very small volume (~1 fL) and correlates such fluctuations to the respective diffusion coefficient.[188] Two membrane proteins can be labeled simultaneously, and their interaction can be assessed.[189] This particular case is known as fluorescence cross-correlation spectroscopy (FCCS). Cell membrane proteins can be relatively immobile, making FCS and FCCS inapplicable. When such a condition arises, FRAP applies.[190] Fluorescence fluctuations of FCS and FCCS can be analyzed as a function of space, producing an image in the techniques of ICS and image cross-correlation spectroscopy (ICCS), respectively.[191,192]

Studies have been published that provide some further insight into how cells respond to materials and biochemical niches with respect to traditional cell labeling. ICCS has recently been used to quantitatively measure the fraction of actin that interacts with α-actinin at the boundary of a migrating Chinese hamster ovarian cell on fibronectin-coated glass.[193] In separate studies, ICS was used to track the dynamics of the α5 integrin and its association with α-actinin and paxillin.[194,195] An extension of ICS, known as spatiotemporal image correlation spectroscopy (STICS), allows one to quantify the direction and flow rate of membrane proteins during cellular processes, such as migration and proliferation.[196]

The full potential of these spectroscopic techniques is beginning to be unraveled. Such techniques would be very powerful in understanding the true influence of chemical niches and other material properties on cell adhesion and behavior.

4.4.3. Transport Properties. 2D and 3D cell culture systems are governed by drastically different mass transport phenomena. 3D matrix scaffolds drastically reduce gas and nutrient exchange rates between the cells and their surrounding medium. This has already been illustrated in Figure 7. In 3D systems, oxygen has first to transfer from air to the culture medium, and

then must diffuse through the scaffold to reach the cells. Although the first transfer step is present in 2D and 3D systems, the last step is of high importance in 3D systems, drastically effects cell behavior, and, in many cases, results in significant cell death. Furthermore, if introduction of a chemical niche into a 3D system via medium supplementation is desired, transport properties must be assessed to ensure that such a niche is delivered to the embedded cells in an adequate fashion.

A review of mass transfer phenomena and requirements for 3D cell culture systems is given by Martin and Vermette.[155] Very briefly, mass transport via convection (i.e., flow of nutrient medium) is required for gel scaffold thickness ranging from 100 μm to 1 mm, as diffusion alone is not sufficient to provide oxygen and nutrients. As such, for larger thickness and 3D systems, reactor configurations must provide flow through the scaffold, while minimizing possible damage or undesired shear stress to the cells. Engineering principles must be used to evaluate convective and diffusive effects and shear stresses imposed on cells, and then to design 3D cell culture systems that provide sufficient mass transfer in a physiologically adequate fashion. Hollow fiber reactors and hypothetical cell assemblages with functional "blood" vessels broadly constitute the most adequate reactor systems to achieve optimal mass transfer and minimize shear forces.

Oxygen is only sparsely soluble in typical culture media (e.g., human blood carries 50 times more oxygen due to hemoglobin). As such, oxygen carriers need to be developed to allow dense tissue growth in 3D culture systems. Most research on oxygen carriers is done as a potential for blood substitutes as opposed to increasing the oxygen content in culture media for cell culture systems. Oxygen content in culture media can be increased by employing, for example, perfluorocarbon (PFC)-based or hemoglobin-based oxygen carriers. A review on their preparation and progress is provided by Centis and Vermette.[197] Briefly, while they dramatically increase oxygen solubility, some oxygen carriers appear to be somewhat cytotoxic. PFC-based carriers have been shown to be highly toxic to fibroblasts and thus do not appear to be applicable in biomaterials and tissue engineering applications.[198] Furthermore, liposome-encapsulated hemoglobin shows significant toxicity to HUVEC monolayers in 2D with 50% cell death in the first 24 h.[199] On the other hand, when cells were embedded in a 3D fibrin gel and grown using a culture medium containing liposome-encapsulated hemoglobin, no cytotoxicity was observed and even cell proliferation increased compared to controls.[200] It appears that if one wishes to enhance the oxygen solubility of culture media, a fine balance is required to ensure an adequate increase in oxygen delivery and minimal toxic impact to cells.

4.5. Materials for Three-Dimensional Cell Culture Systems

4.5.1. Natural Materials. Ideally, in vitro systems should be fabricated from the same molecules present in in vivo systems, while adjusting the exact composition and localized growth factors to mimic specific cell responses. Because of difficulties in purifying ECM molecules for scaffolds with well-defined local mechanical and chemical properties, this requirement is somewhat unrealistic. However, ECM gels, such as fibrin, collagen, and Matrigel, can be used, only to some extent, to acquire desired matrix rigidity.[151] Furthermore, the exact ECM composition including trace chemical factors and local mechanical properties for most tissue types is not well-known.

The protein collagen is known to be present in living tissues in over a dozen types and in various fibrillar forms depending on the tissue. However, collagens can be difficult to purify while maintaining their architecture. Small amounts of impurities may cause unexpected cell responses in culture systems fabricated from extracted natural ECM. Accordingly, collagen gels have been noted to show important differences from in vivo systems relative to cell attachment, migration, and proliferation.[183] The 3D movement of lymphocytes in native lymph nodes appears random and significantly faster than what was observed in vitro in collagen gels.[201] Moreover, cell−cell interactions are seemingly prolonged in vivo compared to in vitro model systems.[202] Cells can be mixed with a collagen solution prior to polymerization to embed cells within the matrix. Importantly, the density of collagen fibers and their orientation has a large effect over cell migration and morphology.[203] Moreover, the mechanical loading of the material impacts cell fate. Mass transfer limitations for collagen-based systems have until now prevented any reported growth of large tissues. Collagen matrices may be blended with fibronectin or glycosaminoglycans such as hyaluronan to achieve in vivo-like matrices.[204,205] However, such blends are also difficult to reproduce and can yield different collagen structures as well as varying cell responses.[206] The supernatant of a Schwannoma, which predominantly contains laminin, collagen IV, and nidogen, known as Matrigel, is often used to mimic the basement membrane. However, as a large number of molecules can be found in Matrigel, it is often difficult to reproduce observations made using this material.[207] As such, care must be taken when interpreting results obtained from collagen-based systems as the fabrication method and exact source of the material is likely to have an important influence on the observations.

Fibrin is used mainly in applications where very low levels of trace chemicals are desired as it is possible to obtain relatively pure reactives (i.e., fibrinogen from blood plasma and recombinant thrombin). Fibrin can be covalently modified to incorporate growth factors or other biochemical niches.[208,209] Furthermore, it is possible, to some extent, to modulate the porosity and mechanical properties of fibrin by adding other compounds. For example, hyaluronic acid, a natural proteoglycan, increases fibrin gel porosity and hydration, thus promoting cell migration.[210] Aligned poly-L-lactic fibers can be added prior to fibrin gel formation to guide fibroblast and keratinocyte migration,[211] whereas biodegradable knitted elastomeric fabric can be added to improve the mechanical properties of tissue-derived cardiac constructs designed for in vivo implantation.[212] Magnetic beads coated with thrombin can be used to align fibrin fibers in 2D at the nanoscale level using magnetic fields.[213] The main drawback in using fibrin is that its mechanical properties change over time and degradation occurs. Aprotinin, a plasmin inhibitor, may be added to slow degradation.[214]

4.5.2. Synthetic Materials. Synthetic materials are being studied as replacements of certain natural materials. Such materials have the mandate to behave in a similar fashion to natural materials. As such, synthetic materials must allow specific localized interactions with certain types of cells, be nontoxic, and permit cell migration. In some cases, synthetic polymers are desirable as they allow the isolation of specific niches for cell study. For example, to some extent, using ligand-coated polyacrylamide gels for 3D cell culture allows simultaneous tuning of chemical and mechanical niches.[151] Because frequent novel materials are reported in the literature, a very large number of materials can be used. Degradation of the synthetic material is sometimes preferred to allow cell migration and tissue development.

Three vital requirements for the materials are nontoxicity, ease of sterilization, and the promotion of cellular adhesion. However, while some of these materials can achieve well-defined porosities and other mechanical properties, many of these materials lack adequate chemical properties to promote cellular adhesion and thus require some type of additional chemical modification.

Transformed natural materials, such as cross-linked polysaccharides (e.g., agarose and alginate) may be used to encapsulate cells. Simple 3D constructs made of encapsulated cells in alginate or agarose beads are often encountered in biomaterials and tissue engineering literature, noticeably for the culture of chondrocytes, pancreatic islets, and hepatic cells. Encapsulation is reported to increase cell viability, tolerance to shear stress, and proliferation as well as inducing in vivo-like behavior.[161] Systems made from these polymers show flexibility in their mechanical properties as their elastic modulus is dependent on the molecular mass of the polymer.[151] They have been widely used in 3D systems where high shear stresses are present.[155]

In cancer research, tumor cells are frequently studied to assess the fundamental differences between 2D and 3D cultures. The cells are usually studied in the form of spherical assemblies termed spheroids.[215] These assemblies can be obtained by spontaneous aggregation from the 2D cultures of certain cell lines (e.g., breast cancer). Cancer cells can also be grown on agar, agarose, or recombinant basement membrane to help induce the formation of spheroids. Both techniques have yielded useful data, although inadequate mass transport limits spheroid size and viability of inner cells. Moreover, the interaction of cancer cells with stromal cells cannot be modeled with these systems. However, novel scaffolds are being developed to allow such cocultures.[215]

Bone cells necessitate rigid (i.e., inelastic) scaffolds to mimic the in vivo ECM. As such, composite systems made of polymers and ceramics are actively being developed to combine the mechanical properties of ceramics with the advantages of polymers (e.g., biodegradability, flexibility, and available chemical sites).[216,217] A review of materials developed to culture bone cells in 3D is presented by Rezwan et al.[218]

In most studies, cells are cultured in synthetic organic polymer scaffolds of various shapes, adhesion characteristics, mechanical properties, and porosities. An example is hydrogels that combine receptor binding sites to exert cell traction and protease-sensitive degradation sites to allow cell migration.[219] These hydrogels are fabricated from multiarm vinyl sulfone functionalized poly(ethylene glycol) backbones with either monocysteine adhesion peptides or biscysteine protease-sensitive peptides to allow localized degradation. This scheme allows a complex interaction between cells and synthetic materials somewhat akin to the interactions present in native tissues. An extensive list of polymers currently used is reviewed by Hutmacher.[220] Large efforts are also devoted to scaffold creation with predetermined architecture using computational topology design and solid free-form fabrication.[221]

4.6. Micropatterned Cell Cultures

The majority of in vitro cell culture systems, whether 2D or 3D, involve dispersing cells in a homogeneous fluid within a largely homogeneous construct such as a Petri dish or well plate. Unfortunately, cells in in vivo systems are surrounded by a complexity of physical and chemical microenvironments.[222] As such, large efforts have been made in the patterning of cells to surfaces at micrometer resolution in a technique known as micropatterning.

Micropatterning has a variety of potential applications. In fundamental biology, micropatterning can be used to position biochemical parameters to understand how the quantity and geometry of localized ligand distributions effects cell response and any subsequent behavior.[223,224] Biomaterials scientists and tissue engineers exploit any understanding gained to control cell response, growth, and behavior.[225,226]

Numerous techniques can be used to produce micropatterned surfaces. The most widely used is that of soft lithography.[227,228] Within soft lithography are the techniques of microcontact printing and microfluidic patterning. Another common technique is that of photolithography, where ultraviolet illumination is used to pattern a substrate. For a concise review of the engineering approaches to produce micropatterned surfaces, the reader is referred to an article by Falconnet et al.[229]

Few techniques exist to spatially localize biochemical niches in synthetic materials to genuinely mimic in vivo tissue architecture. This currently constitutes a limitation for the design of 3D systems to produce viable human tissue substitutes. Lithography-based deposition methods have been developed to produce well-defined 2D surfaces for cell study.[230] However, these techniques are not transferable to 3D systems. Layer-by-layer printing techniques have been reported to enable 3D positioning of matrix material. For example, 3D printing of porous hydroxyapatite scaffolds for bone engineering achieves well-defined dimensions, although lacking localized biochemical parameters.[231,232] Polylactic acid 3D printing from computer-aided design (CAD) models was also reported, again lacking localized biochemical parameters.[233]

Mircropatterning has also found use in further understanding how cells respond to surface topography.[234] However, quantitatively defining surface topography is challenging.[235] Early reports indicated that cell adhesion is enhanced when the roughness of a surface is increased.[236] Later, numerous studies would show that adsorbed protein conformation is sensitive to nanoscale topography features of the surface.[237−239] The nanoscale is a similar length scale to that of the proteins themselves. As mentioned earlier in section 2, protein adsorption directly impacts cell adhesion and subsequent response. Topographical features at the microscale can be too large to directly impact protein adsorption[240] and, thus, likely would have no direct impact on cell adhesion and response.

Cell migration has been shown to be somewhat controlled via the use of micropatterning. On flat surfaces, cell migration is essentially random in the absence of biochemical stimuli.[241] Later, micropatterning proteins showed that one can in actual fact guide migratory cells.[242−245] As suggested by Curtis and Wilkinson,[246] the guidance of migratory cells along micropatterned grooves and ridges can be explained by the fact that the actin in the cell cytoskeleton is organized in a way that requires minimal work. Thus, the cells migrate along the path of the grooves and ridges. However, controlling the direction of migration has yet to be achieved. While guiding the migration of cells is a step forward from that of adhesion only, far more control of cell behavior is needed to apply such micropatterned systems for tissue engineering applications. Patterning cell migration in a 3D environment can be achieved by fibers disposed in a given pattern within a gel, as was reported by us.[134,135]

When micropatterning techniques for modulating cell response first surfaced, it apprears they were touted as potential revolutionary tools for biology, biomaterials, and tissue engineering research. In biology, it appears micropatterning has substantiated

the fact that ligand composition, localization, and concentration dictates cell adhesion and subsequent behaviors such as morphology and survival. However, over a decade since its introduction in biomaterials and tissue engineering research, micropatterning appears to still remain a "potential" tool as opposed to a "valuable" tool. Perhaps the reason for this is that micropatterned systems do not directly alter cell−cell interactions or transport properties, both being major contributors to tissue growth and cell response to biomaterials surfaces. As such, the majority of culture systems based on micropatterned surfaces are more an extension of 2D systems rather than a truly 3D approach.

4.7. Current State and Future Direction of Three-Dimensional Cell Culture Systems

Cells cultured in 3D systems clearly demonstrate large differences, for example, relative to morphology and protein expression compared to those grown in 2D environments. This should not be surprising considering the extent to which biochemical, mechanical, and nutrient and waste transfer properties differ for the two types of systems. However, large efforts still need to be deployed to achieve the fabrication of 3D environments in vitro that can successfully reproduce the in vivo reality with low variability and relative ease of use to allow everyday use. 3D culture systems that have been reported in the literature often present limitations in nutrient transfer, give inadequate or unmeasurable biochemical cues to cells, or fail to give important mechanical signals. Progress is made every year to improve nutrient transfer through bioreactor design and attempts to induce channels in lumps of cells (e.g., by angiogenesis).

Petri dishes and multiwell plates currently constitute the simplest and most widely used "bioreactors" for 3D cultures. Cells are embedded within a polymerized porous scaffold material in a multiwell plate or Petri dish, and nutrient-rich supernatant is supplemented onto the matrix and changed during culture. Mass transfer in such systems is strongly limited because of inadequate diffusion of nutrients through the porous scaffold. As such, only thin sections of embedded cells can be used. Cells located closer to the supernatant beneficiate from enhanced oxygen and nutrient access. Mass transfer in such systems may be improved by the use of well inserts that suspend the embedded cells on a highly porous membrane that allows diffusion through the top and bottom of the scaffold containing embedded cells. Without convective mass transport, however, only thin sections of embedded cells can be used. More complex bioreactor systems are needed to enhance mass transfer.[155]

Novel materials are constantly reported to allow cell growth in vitro. The next few years should witness the emergence of integrated systems. However, some aspects of the in vivo environment should rapidly be addressed. For example, spatial localization of key biochemical niches is very likely to be important to reproduce the highly nonuniform in vivo tissues. Although developments are generated on flat surfaces to achieve this goal, very few 3D systems address this critical issue.

5. MODIFICATION, PROPERTIES, AND ANALYSIS OF SURFACES

5.1. Modified Surfaces in Biomaterials Science

An approach to understand and subsequently control the cell−surface interaction is the modification of surfaces with physical and chemical properties that result in protein adsorption with conformation/orientation favorable for a particular cell response, for example, the design of a surface using adsorbed VEGF and Fn to promote the migration and proliferation of endothelial cells.[82] Such surfaces would be beneficial to better understand the complex process of angiogenesis and revascularization of new and/or damaged tissues.[247]

Surface modification can employ plasma polymerization and/or SAMs, two approaches that will be further discussed below.

Table 3. Surface Modification Substrates and Chemicals

surface modification	substrate	modification compounds
self-assembled monolayers	noble metals	organothiol
	gold, Au(111)	organothiols (R−SH)
	copper	organosulfides (R−S)
	silver	organodisulfides (R−S−S−R)
	semiconductors	
	indium phosphide	
	gallium arsenic	
	silicon-based materials	organosilane
	glass	OTS (octadecyltrichlorosilane)
	quartz	APTMS [(3-aminopropyl)trimethoxysilane]
	mica	alkyltrichlorosilane
	silicon oxide	tridecafluoro-1,1,2,2-tetrahydroochyl-1-dimethylchlorosilane
	metallic compounds	
	aluminum oxide	
	zinc selenide	
plasma polymerization		amine functional monomers
	glass	*n*-heptylamine
	mica	ethylene diamine
	FEP	propylamine
	PTFE	allylamine
	Ti alloys	other functionalities
		acetaldehyde (CHO)
plasma treatment		
	glass	Ar
	mica	N_2
	FEP	O_2
	PTFE	He
	metals	H_2
low-fouling		phospholipids
	glass	PC (phosphatidylcholine)
	mica	phosphatidylserine
	FEP	synthetic polymers
	PTFE	PEG (poly(ethylene glycol))
		PEO (poly(ethylene oxide))
		dextran derivatives
		CMD (carboxymethyl dextran)

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Table 4. Properties of Cell Culture Surfaces

surface modification	functionality	properties: charge	properties: wettability
self-assembled monolayers[a]	CH_3	neutral	hydrophobic
	OH	neutral	hydrophilic
	NH_2	+	hydrophilic
	COOH	−	hydrophilic
	CF_3	neutral	hydrophobic
commercial substrates			
untreated polystyrene	n/a	neutral	hydrophobic
tissue culture grade polystyrene	n/a	−	hydrophilic
Primaria	NH_2/COOH	±	hydrophilic
polylysine-coated plastic	n/a	+	n/a
polymer grafted			
PEG-grafted	NH_2	all low-fouling[b]	
	COOH	all low-fouling[b]	
dextran derivatives	COOH	all low-fouling[b]	
	(CHO)	all low-fouling[b]	

[a] Note: For the row pertaining to SAMs, the functionality corresponds to the tail group. [b] The property "low-fouling" is somewhat interpretive as discussed in section 5.7. For low-fouling behavior, one must define the tolerance of acceptable adsorption for their particular application and then use the appropriate experimental method and conditions to produce a low-fouling surface.

Another approach is the design of low-fouling surfaces.[248] The term low-fouling relates to the resistance of a surface toward protein adsorption, which subsequently results in little to no cellular adhesion to the surface. Common low-fouling modifications involve the attachment of PEG or the equivalent poly(ethylene oxide) (PEO),[248−251] dextran derivatives,[17−19,252−254] or phospholipids[255−259] to surfaces.

In this section, we discuss the modification of substrates using SAMs and plasma polymerization. Different types and the advantages, disadvantages, and complexities of each are discussed. The surface properties that result due to such modifications and methods of analysis are outlined. Particular emphasis is placed on the low-fouling properties of modified surfaces and the limitations of surface analysis using contact angle measurements.

5.2. Surface Fabrication

As shown earlier in Figure 1A, prior to the introduction of proteins and/or cells, substrates can undergo modification for the purpose of enhancing their chemical and biological activity. Such modifications typically alter the chemistry and topography of the substrate.[240] As shown in Tables 3 and 4, there are a wide variety of materials used for surface modification in biomaterials science.[260] Materials that are typically used as substrates are composed of alloys or metals such as titanium or gold. Silicon-based materials such as glass and mica are also very common. In addition, the use of polymers such as polytetrafluoroethylene (PTFE) and fluorinated ethylene propylene (FEP) have been used as substrates.

Two available chemical surface modifications of substrates are the use of plasma polymerization[87,261] or SAMs.[21,44] Following the modification of a substrate using plasma polymerization or SAMs, the modified surface can be conjugated with a protein, chemical spacer (e.g., PEG, dextran derivatives), or any compound that will affect the surface activity.

5.3. Self-Assembled Monolayers (SAMs)

Perhaps the most popular surface modification in cell−materials research is the use of SAMs.[262−265] A SAM consists of molecules each containing a head and tail group. The head group is adsorbed to a substrate while the tail group is available for further interaction. This is illustrated in Figure 10. Proteins can then be physisorbed or chemisorbed onto the SAM-modified surface, followed by the seeding of cells.

The popularity of SAMs in cell−materials research stems from the commercial availability of SAM molecules with specific head−tail group combinations. The availability of different tail groups allows researchers to alter the chemistry (see Table 4 for specific examples) of surfaces rapidly. Unfortunately, the popularity of SAMs is also due to a perceived notion that preparation can be performed with ease and simplicity. Such a notion should be taken with caution, as there is still much research currently being performed to understand the mechanisms of self-assembly and to identify the experimental conditions necessary to produce well-ordered homogeneous SAMs in a reproducible fashion. As shown in Table 3, two popular SAM systems include the use of organosilanes on hydroxylated glass, silicon oxides, or aluminum oxides and organosulfurs on noble metals.

The most popular and studied organosulfur SAM system is the organothiol (R−SH) on Miller oriented Au(111) surfaces. Here R represents the tail group and the remaining chemical functionality represents the head group. Other organosulfur−noble metal systems include organosulfides (R−S) and organodisulfides (R−S−S−R). Other noble metal substrates include silver and copper. The popularity of organothiol SAM on Au(111) is a result of the strong affinity of the S−Au bond and the impeccable, well-defined structure of the S adsorbed to the Au(111) surface.[266]

The formation of an organosulfur SAM is a complex and not completely understood phenomenon. It has generally been proven that the formation of organothiol SAM on Au(111) follows a two-step process. The first step occurs rather quickly (i.e., in a few minutes), which involves rapid chemisorption of the head SH group to the Au(111) surface in a diffusive fashion. Upon completion of this initial step, the monolayer has reached 80−90% of its final thickness. The second step is less rapid, taking hours. In this step, intermolecular Coulomb and VDW interactions occur between both the adsorbate−substrate and adsorbate−adsorbate, resulting in the formation of an organized 2D crystal surface structure.[267−269]

There is still much controversy regarding the influence of intermolecular electrostatic interactions to the degree of disorder.[270] Furthermore, even when assuming an ideal organomonolayer, one must still proceed with caution. It has been shown that, upon completion of an organosulfur SAM, assumed homogeneous, the strength of H-bonding between COOH or NH_2 tail ends of the chemisorbed SAM and organosulfur molecule tail ends that remain in suspension results in a partial multilayer.[271] This results in a surface of significant heterogeneity and roughness. Care must also be taken if one is preparing one's own gold surfaces. Proper annealing must be performed to ensure a high-quality Au(111) surface with large, flat, virtually defect-free terraces. Together, this indicates that organosulfur SAM preparation and fabrication must be pursued with caution as surface homogeneity is not guaranteed.

Organosilanes on modified glass surfaces are another popular SAM system in cell−materials research. They sometimes appear to be favorable over organothiols. The primary reason is that

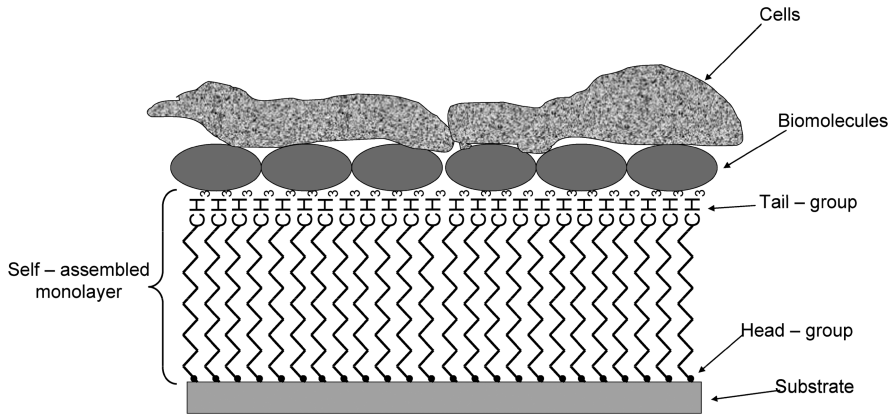

Figure 10. Self-assembled monolayer (SAM) exposed to proteins and cells. Figure not drawn to scale. The head group is chemisorbed to the substrate. The tail group (in this example, a hydrophobic methyl, CH_3 functionality) is available for interaction. In this particular example, the SAM is exposed to biomolecules (chemisorption or physisorption) prior to cell seeding. Alternatively, cells can be seeded directly to the SAM. When this is the case, the cell will synthesize and secrete ECM proteins to aid in adhesion.

glass can be used as the underlying substrate. For organothiols, vapor deposition of gold or another noble metal onto a silicon-based material such as mica or glass is required. Gold-coated substrates can also be purchased commercially. However, their high cost and partial transparency makes them unattractive. Shipping time can be lengthy as the gold-coated substrates are usually produced upon order to provide customers with oxide-free gold substrates. On the other hand, organosilane monolayers require bare glass surfaces and various chemical reagents, both of which are readily available commercially, further enhancing their convenience.

Organosilanes contain a reactive silane group on one end (i.e., head group) and a functional group (i.e., tail-group) on the other, which depends on the application or surface desired. The formation of organosilane SAMs has been extensively studied.[272] Many different silinization chemical reactions exist. Most simplistically, silinization is made on hydroxylated or amine surfaces. The head group of the silane SAM molecule contains a Si residue that interacts with the hydroxylated/amine surface to form a Si−O bond and subsequently release an intermediate. Different types of silanes are classified by the intermediate.

Although the transparency of glass, the availability of materials and reagents, and the perceived simplicity of silanization make organosilane SAMs attractive, the preparation and reproducibility of such surfaces are extremely sensitive to experimental conditions. For example, alkyltrichlorosilanes are sensitive to moisture conditions. The absence of water results in incomplete monolayer formation, whereas an excess of water results in solution polymerization.[273−277] Nonideal moisture conditions result in heterogeneous surfaces. To provide an example of the consequences of an incomplete monolayer, consider that COOH groups adsorb on metal oxide surfaces (e.g., aluminum oxides).[266] Thus, an incomplete monolayer could result in nonspecific adsorption of the carboxylic acid groups of a protein with the exposed aluminum oxide surface resulting in a modified surface with a significant degree of heterogeneity, nonspecific adsorption, and variable roughness, leading to a possible misinterpretation of the cell−surface interaction. The stability of some widely used alkyl-terminated organosilanes for biomedical applications such as cell culture and medical implantation has also been questioned by researchers.[278,279]

Regardless of whether or not one is using organothiols or organosilanes to study cell behavior, careful consideration must be taken regarding the formation, homogeneity, and stability of SAMs. In general, the belief that SAMs consisting of longer chains between the head−tail lead more rapidly to stable self-assembly is consistent, regardless of the SAM system under study. This is likely because of a larger quantity of electrostatic and VDW adsorbate−adsorbate interactions. The general difficulty among the development of SAMs is reproducibility, stemming from an incomplete understanding of the dynamics behind their formation. The aim of theoretical work on SAMs today is to better understand the surface and intermolecular interactions necessary for the formation of uniform, reproducible SAMs. Experimental characterization of SAMs to ensure reproducibility also still remains a difficult task.[280]

Self-assembly today, in general, remains a widely studied field.[265,281] The complexity and dynamics of SAM formation has attracted much interest and research in areas of experimental and theoretical physics, chemistry, and nanotechnology.[282] However, observed cell responses and functions sometimes may be due to unintentional surface heterogeneity, nonspecific adsorption, and roughness.

5.4. Plasma Polymerization

Plasma polymerization is a widely used technique to chemically modify inert substrates. Plasma polymerization can be used to introduce a thin film of amine functionalities. Subsequently, additional compounds such as PEG spacers,[87,248,251] polysaccharides such as CMD,[17−19] or any heterobifunctional cross-linking compounds[73,83] can be conjugated to the amine-functionalized substrate. This is usually followed by the covalent immobilization of ECM proteins and peptides to further enhance surface activity to biological species. The addition of spacers such as CMD and PEG with low-fouling properties is advantageous as many biomolecules (i.e., ECM proteins) undergo a significant reduction in activity upon adsorption directly to a substrate.[283] The spacer acts as a means of simultaneously retaining the activity of the biomolecule and preventing nonspecific adsorption of proteins and biomolecules.

Numerous methods of plasma surface modification exist, such as plasma treatment. However, in plasma treatment, the gas comprising the plasma consists of a nonpolymerizing gas. Thus, plasma polymerization should not be confused with plasma treatment. In plasma polymerization the plasma state is driven

by electric glow discharge, usually via radio frequency (RF) power generators. Because the plasma state is not generated via heating, plasma generated by electric glow discharge is often referred to as "low-temperature plasma". In general, plasma polymerization is often referred to as "glow discharge plasma polymerization", where organic monomer vapors are polymerized. Furthermore, it should be pointed out that glow discharge plasma polymerization should not be confused with "plasma graft polymerization" and "plasma radiation polymerization". The distinct features of the many plasma modifications are outlined in a nice review by Yasuda.[284] A comprehensive review on surface modification using plasma methods for biomolecule and cell immobilization can be found in an excellent review by Siow et al.[285]

For the production of reliable, high-quality plasma polymerization-modified substrates, tedious optimization of the plasma parameters is required. The quality and quantity of the thin film is highly dependent on experimental parameters such as monomer pressure, input power, deposition time, and nature of the monomer itself. Unfortunately, there are no universal quantitative values for one specific monomer or application. All parameters are dependent on the geometry and spatial dimensions of the chamber, all of which vary among laboratories. As a result, it is recommended that users perform optimization experiments. A very effective simple method is the use of a factorial experimental design for the determination of optimal plasma polymerization parameters.[286]

There are disadvantages when performing plasma polymerization on a surface.[260] Because of the complex composition of the plasma and a variety of reaction possibilities, when an organic monomer is introduced to a surface via plasma polymerization, there in actual fact exist many unknown functionalities on the surface.[287,288] Thus, for an amine monomer such as *n*-heptylamine, although the modified surface is primarily composed of amine functionalities, there in actual fact exist other nitrogen-containing nonamine (e.g., NH) functionalities. Postexperimental plasma polymerization conditions also can affect the properties of a modified surface. Immediately after plasma polymerization, the substrate can be placed in a liquid solution containing spacers (e.g., PEG, dextran derivatives, etc.) or heterobifunctional cross-linking molecules (e.g., sulfo-SMCC, etc.). Unfortunately, plasma polymerized thin films can be unstable in solution.[289] When some plasma polymerized thin films are exposed to a liquid solution, they can undergo swelling. This can lead to the breaking of adhesive bonds, resulting in partial delamination of the thin film.[87,283] Extreme cases of plasma polymerized thin film swelling have also resulted in significant reduction in thin film density and in the portrayal of porosity. As a result, molecules exposed to the thin film can interact with amine functionalities buried several layers in the thin film, resulting in surface roughness.[290] Another problem is that many surface-deposited plasma polymers undergo rapid oxidation, reducing their reactivity.[291] Swelling and oxidation problems can be somewhat reduced by using a rapid covalent mechanism immediately after plasma polymerization. Carbodiimide chemistry is often used for such a purpose. Some plasmas, particularly *n*-heptylamine in a study by Martin et al., have been shown not to swell when immersed in aqueous solutions.[286] Conversely, Vasilev et al. performed a detailed study on *n*-heptylamine plasma and found swelling that was dependent on the radio frequency power of the plasma process.[292] The reason for these somewhat contradictory results is perhaps due to the choice of underlying substrate. Martin et al. used FEP substrates, whereas Vasilev et al. used silicon wafers. In our personal experience, we found plasma polymer layers of *n*-heptylamine on silicon wafers to undergo significant delamination upon exposure to aqueous solution. An interesting study would be investigating the dependence of *n*-heptylamine plasma layer characteristics on various substrate compositions. A debate also exists on whether microwave frequency is superior to generate plasma as opposed to the commonly used radio frequency.[87,293]

The disadvantages of plasma polymerization manifest from the fact that technical exploitation of the process far outweighs any scientific research of the physics and chemical reactions that result in the modified surface. Any research into such mechanisms is largely based on speculation to explain empirical observations. Unfortunately, the physics of plasmas has largely been studied by physicists using a nonpolymerizing gas as plasma. Little theoretical and analytical work has been done on polymerizing plasmas. Also, few analytical instruments are commercially available to diagnose in situ plasma polymerization processes, probably owing to the often very corrosive nature of the generated byproducts. As a result, a complete picture of the process of glow discharge plasma polymerization does not exist.

Despite difficulties in the optimization, instability of the modified substrates in solution, and a sparse theoretical picture, plasma polymerization modified substrates remain of interest. The primary benefit of plasma polymerization modified surfaces is that their production is significantly quicker when compared to SAM-modified surfaces. The lengthy time required for the formation of SAMs can lead to problems if an inadequate time is allotted for their formation; the result is a heterogeneous surface. Of course, the choice of modification is equally dependent on the application and aim of the experiment being performed. If well fabricated and characterized, a SAM-modified surface, particularly an organosulfur, is superior to plasma polymerization modified surfaces because of the absence of unknown functionalities. With SAMs, the only surface functionality should be that of the tail group.

As previously outlined, plasmas composed of nonpolymerizing gases can also be used for modification in a process known as plasma treatment. In biomaterials, plasma treatment can be used for surface cleaning (usually metallic materials) and modification.[294] Plasma treatments for cleaning are typically composed of N_2, O_2, Ar, He, or H_2. Unlike plasma polymerization where the surface acts as mechanical support for the thin film,[295] plasma treatment can etch atoms from the surfaces and replace them with new functionalities.[285] The most common plasma treatment is that using ammonia plasma to functionalize the surface with amines. Plasma-treated surfaces can significantly alter the physical morphology of the substrate. Thus, one's observations may be due to such morphological changes as opposed to chemical modification. Users of plasma treatment often do not address the resulting heterogeneity and morphological changes of the surface as being the contributors to varying cell response and/or biomolecule interaction. It is almost always assumed that the cell response and/or biomolecule interactions are a result of an assumed chemical substrate modification. However, this assumption is not always true as roughness and heterogeneity simultaneously contribute to such responses. Furthermore, plasma-treated films can undergo delamination exposing cells and/or biomolecules to the underlying substrate and not to the chemical modification.

Table 5. Static Water Contact Angle and Cell/Protein Responses

cells/proteins	materials	θstat	cell response	ref
stromal	various materials	16°−118°	no clear relationship between θ and cell number	310
chondrocyte	polystyrene-modified with plasma polymerization	39°−107°	cell number seemed to decrease as θ increased	381
chondrocyte, fibroblast	films of poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactide-co-glycolide) (PLGA) treated with chloric acid	37°−85°	cell number increased as θ decreased	313
fibronectin, MG63 cells	modified titanium−aluminum−vanadium alloy	4°−43°	no clear relationship between θ and binding of fibronectin or cell attachment	315
osteoblast	CO_2 laser-treated 316 LS stainless steel	78°−89°	cell proliferation increased as θ decreased	309
endothelial	plasma-treated polystyrene	27°−80°	different adhesion between nontreated and plasma-treated polystyrene but no clear relationship between θ and cell adhesion for the treated samples	319
osteoblast	titanium alloy substrates (rod and sheet of Ti-6Al-4 V)	75° (rod) 85° (sheet)	cell density seemed to decrease as θ decreased	307
fibroblast	SAMs of different alkyl chain lengths	58°−107°	cell coverage and detachment rate decreased as θ increased	305
fibroblast	silanes with SH, NH_2, and CH_3	0°−92°	no clear relationship between θ and cell attachment	29
osteoblast	titanium surface oxides	48°−83°	no clear relationship between θ and cell attachment or proliferation	322
smooth muscle	plasma-polymerized poly(ethylene terephthalate) (PET) and PTFE surfaces	42°−103°	no clear relationship between θ and cell density or growth	314
yeast	glass, polypropylene, polystyrene, stainless steel	33°−103°	yeasts seemed to be more firmly attached to hydrophobic supports	308
yeast	membranes made of polyacrylonitrile, polysulfone, modified polysulfone, polypropylene, and Teflon	35°−58°	no clear relationship between θ and initial cell adhesion rate	311

5.5. Properties and Analysis of Modified Surfaces in Cell Science

Cell behavior to a surface is dependent on the surface properties. Two of the most common surface properties considered by the researchers are the surface polarity (i.e., charge) and wettability. Wettability can be thought of as the tendency of a liquid drop to spread (i.e., wet) a surface. Table 4 summarizes the properties of common surfaces and their modifications. Included are common functional tail ends (i.e., surface chemistry) of SAMs, spacers used post-plasma polymerization, and commercially available surfaces unmodified by the user, along with their properties. Unmodified substrates are usually immediately used for standard cell culture practice. Plasma polymerization modified substrates with grafted CMD or PEG are further modified via the chemisorption of ECM proteins or other biomolecules with amide bonds[18] or Schiff base linkages.[296,297] For SAM-modified surfaces, the ECM proteins are introduced to the surface without the use of a catalyst to entice chemisorption relying solely on physisorption mechanisms.[44,298] However, chemisorption is possible for the SAM-modified surfaces should that be applicable.[82]

A great deal of emphasis has been placed on surface wettability and charge, as shown in Table 4. It is generally believed that hydrophilic surfaces are superior to hydrophobic for cell adhesion, with the reason being that proteins adsorb to hydrophilic surface with less affinity, allowing greater freedom of conformation and orientation. Subsequently, a greater number of cells adhere to a hydrophilic substrate relative to a hydrophobic surface. Proteins and other organic molecules portray a net charge that is dependent on their isoelectric point. A protein or organic molecule can interact with a surface of opposite charge through Coulomb interactions.

In addition to AFM and SFA discussed earlier, surface characterization typically involves combinations of X-ray photoelectron spectroscopy (XPS), contact angle measurements, immunochemistry (IC) and immunofluorescence (IF), secondary ion mass spectrometry (SIMS), and matrix-assisted laser desorption/ionization (MALDI). XPS is employed to ensure the addition of a thin film whether it be plasma polymerization, SAMs, or a protein film, as the spectrum is dependent on the atomic composition of the surface.[299] XPS has also been used to determine surface coverage of adsorbed proteins,[300] although the use of XPS to characterize surface coverage can have serious limitations. Contact angle measurement is used to determine the wettability of the substrate. While contact angle measurements are very common in biomaterials surface science, the information they provide is ill-defined. Furthermore, the contact angle of a surface is highly influenced by surface heterogeneity and roughness. The applicability of contact angle to biomaterials surface science is very questionable. We address this matter in detail in the next section. IC/IF employs the use of antibodies for the specific recognition of proteins or fragments thereof as a semiquantitative measurement of the surface protein density. Two other common methods of measuring the surface density of a protein are radio labeling and fluorescent conjugation using, for example, fluorescein or rhodamine. SIMS and MALDI can be used to determine the chemical composition of a surface.[301,302] Each of these characterization techniques are not without limitations. One must determine which techniques are most applicable to their research.

Mechanical properties of modified surfaces are often overlooked. One possible reason for this is that mechanical properties are generally difficult to explicitly quantitatively define.[303] For

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Table 6. Advancing, Receding, And Dynamic Water Contact Angle and Cell/Protein Responses

cells/proteins	material	θ	cell response	ref
endothelial	individual and mixed heparin/albumin layer on poly(acrylic acid)	θ_{ADV} = 25°−90°	no clear relationship between θ and cell attachment	316
albumin, fibrinogen	semi-interpenetrating polymer networks with PEG-diol	θ_{DYN} = 25°−58°[a]	protein adsorption decreased as θ decreased	306
hepatoblastoma	membranes made from polyetherimide, polyacrylonitrile-*N*-vinylpyrollidone copolymer, polyacrylonitrile, and polyvinylidendifluoride	θ_{ADV} = 53°−80°	no clear relationship between θ and cell adhesion	312
10% FBS in DMEM, chondrocyte	poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) blends	θ_{REC} = 46°−62°	no clear relationship between θ and protein adsorption or cell density	321
fibroblast	various materials	θ_{ADV} = 60°−110°	no clear relationship between θ and cell number	317
fibronectin	surface-modified poly(hydroxybutyrate) films	θ_{ADV} = 60°−81°	fibronectin displacement of preadsorbed fibronectin was not a function of θ	318
neutrophils	phosphorylcholine-containing polyurethanes	θ_{ADV} = 75°−92°	neutrophil attachment rate constants decreased as θ increased; however, very high hysteresis was observed (up to 92°)	320
FBS, fibroblast	CH_3, Br, $CH{=}CH_2$, NH_2, COOH, PEG, and OH terminated alkyl silane SAMs	θ_{ADV} = 15°−95°	no clear relationship between θ and FBS proteins desorbed from the surfaces, cell attachment (%), or cell spread areas	21

[a] For θ_{DYN}, both θ_{AVD} and θ_{REC} were measured and were within the range as shown.

example, although modification of surface roughness has been studied thoroughly in the past, it is difficult to quantitatively define "roughness". Many studies aim to chemically modify a surface while not considering any resultant roughness. Such roughness is unintentional,[246] and this may lead to studies in which one might believe they are observing cell responses to chemical modification, while they could be observing responses to both roughness and the chemical modification. Furthermore, the flexibility and the elasticity of a modified surface also have been shown to effect cell response, particularly migration and the formation of focal adhesion contacts.[304] In conclusion, one must consider that cell response is dependent on the chemistry, topography, and elasticity of the modified surface, not to mention the elasticity of the cell itself. We believe that disregarding any of these individual contributions toward cell response has resulted in confusion.

5.6. Contact Angle in Correlating Cell Responses Toward Biomaterial Surfaces

It is difficult to extract any relationship between cell responses and biomaterial contact angle measurements by water. Tables 5 and 6 summarize some results of establishing cell and protein interaction as a function of biomaterial water contact angle, θ.[21,29,305−322] Some studies tend to indicate that cell responses can be related to the water contact angle found on specific biomaterials, but overall, no clear links can be found. Comparison of results from different studies is complicated by the fact that experimental parameters often vary from one study to another. For example, cell phenotype, growth conditions, and cell densities are often different. Furthermore, ranges of water contact angle values between surfaces are often too narrow, making it difficult to establish meaningful correlation between water contact angle and cell response. More importantly, the analysis of Tables 5 and 6 points out that far more important material characterization should be considered when trying to correlate biomaterial properties to cell responses. These can include surface chemistry, surface molecular architecture approximated by surface force analyses, and surface roughness, only to name a few.

The usefulness of measuring water contact angles on solid biomaterials surfaces is therefore opened to question. Can water contact angle on a biomaterial surface predict protein adsorption and/or cell responses? This section briefly addresses such a question.

5.6.1. Hydrophobic Interactions. When investigating water contact angle results, in part, we look at the wettability of a solid surface. The wettability is categorized as either hydrophobic or hydrophilic. As mentioned earlier in section 3.2, the origin of hydrophobic interactions is poorly understood.

In an excellent review paper, Meyer et al.[99] reported the most recent progress in understanding hydrophobic interactions. The hydrophobic effect refers to the very low solubility of nonpolar solutes in an aqueous phase. For example, fluorocarbons are considered as being one of the most hydrophobic molecules. Many self-assembly processes are influenced by hydrophobic forces including micelle, vesicle, and bilayer formation, and protein (un)folding.[78]

Molecules or surfaces can interact with water by having either Lewis acid and/or Lewis base properties. H-bonds can also be referred to as Lewis acid (electron-acceptor) and Lewis base (electron-donor) interactions.[323,324] Some molecules can behave as either a Lewis acid or base, and water is an example. Surfaces bearing chemical moieties having Lewis acid and/or base characteristics should be more hydrophilic, if other surface properties are ignored.

As mentioned by Meyer et al.,[99] no deep quantitative understanding of the exact origin and nature of hydrophobic forces across water exist. Nonpolar groups are barely soluble in water. The strength of hydrophobic interactions is larger than that

described by the Lifshitz theory of VDW forces.[99,325,326] It is very difficult to estimate forces between hydrophobic surfaces because of (i) the possible presence of gas bubbles that can result in a density-depleted water layer around these surfaces, which can potentially bridge the surfaces and affect force measurement profiles;[327] (ii) the use of different experimental techniques and protocols to produce hydrophobic surfaces and/or to measure the resulting surface forces; and (iii) surface heterogeneity, surface roughness, and surface molecular structure and composition, which can result in different surface molecular rearrangements when hydrophobic surfaces are immersed in water.

An example involving hydrophobic forces in protein interactions is the use of hydrophobic interaction chromatography. In fact, protein separation can be carried out by hydrophobic interaction chromatography, a technique using hydrophobic interactions between nonpolar regions on the surface of proteins and hydrophobic ligands immobilized on the adsorbant.[328] It is reported that adsorption increases with mobile phases having high ionic strength. Elution is done by decreasing subsequently the eluent salt concentration.[328] The type of salt is known to affect hydrophobic interactions.[328] The main properties influencing protein retention during hydrophobic interaction chromatography experiments are salt concentration and type as well as the surface density of the hydrophobic ligands.[329] However, the concepts behind hydrophobic interaction chromatography have not been applied to study proteins−biomaterial interactions.

5.6.2. Contact Angle Measurements. Wettability involves the spreading of a liquid onto a solid substrate. In a sense, wettability is related to the water structure adopted over a solid surface. Wetting or spreading can be categorized into nonreactive or reactive. Considering Zisman's rule,[330,331] wetting surfaces are associated to high surface energy and nonwetting ones are referred to as low surface energy. The level of wetting is represented by the contact angle formed at the interface between a solid and a liquid.[332]

In reactive systems, such as those involved in cell−material interactions, the process of wetting also involves mass transport of molecules at the solid/liquid interface. A biochemical reaction and/or adsorption can occur between the surface and the molecules dispersed/dissolved in the contacting medium, and the produced surface species can also influence wetting.[332]

The contact angle between a liquid and a solid is the angle formed between the liquid and the solid surface. Contact angles can be measured by different methods including the static sessile drop method, the dynamic sessile drop, and the dynamic Wilhelmy, to name only a few.[332] A contact angle of 180° indicates that there is little to no wetting between the liquid and the solid surface. It is generally accepted that a liquid is said to wet a solid surface when the contact angle is <90°,[332] although no exact consensus is applied in the biomaterials literature. With a contact angle of zero, the liquid is considered to completely wet the solid. Superhydrophobic surfaces (contact angle > 150°) have been developed. These surfaces find applications for their potential antisticking, anticontamination, and self-cleaning properties.[331,333,334] Micro- and nanostructures appear to play a crucial role in achieving superhydrophobic surfaces, again pointing to the importance of surface structure on wettability.

Contact angles measured on solid surfaces can vary depending on how the material has been processed or cleaned. For example, the advancing contact angle of water on Teflon can vary from 98° to 130°, as summarized by Yekta-Fard and Ponter.[335] Although sometimes subtle, the environment can influence the contact angles on solid surfaces. Furthermore, the drop size can affect the contact angles.[335]

From an experimental side, incorrect experimental methods can be found in the literature dealing with contact angle measurements.[335] For example, solid surfaces are not always prepared and/or processed adequately (i.e., cleaning, fabrication, characterization, etc.). Often, many papers do not report the measurements of both the advancing and receding contact angles. Surface roughness also influences the contact angle,[332,336] although the effect of surface roughness remains unclear.

5.6.3. Contact Angle Hysteresis. Contact angle hysteresis is almost always observed when measuring contact angles on solid surfaces. The three main sources of hysteresis are surface roughness, chemical contamination, and solutes in the liquid.[337] The difference between advancing and receding angles (i.e., hysteresis) can be around tens of degrees.[338] Hysteresis of 1° or 2° can be considered within the uncertainty associated with contact angle measurements.[323] However, it is possible to encounter hysteresis up to 50°.[323] With these errors, it becomes difficult (nearly impossible) to draw any conclusions, even qualitative, on thermodynamic parameters that one may try to deduce from contact angle measurements. This situation might explain, in part, why no exact correlations have been found between contact angle experiments and protein adsorption or cell response.

As outlined above, surface roughness and heterogeneity are two main factors influencing contact angle hysteresis.[323,332,335] For example, commercial plastics can be heterogeneous and contain many additives used to facilitate their shaping and processing.[184] Even plastic made of only one polymer can have surface heterogeneity because of the mosaic formed by crystalline and amorphous regions.[335] Another cause of hysteresis with polar polymers is the possible reorientation of molecules or groups at the polymer surface under the influence of the liquid exposure. Hydroxyl groups on polymer chains can be buried within the bulk of the material when exposed to a nonpolar environment. But when contacting water, these molecules can move to the surface due to interaction with the polar aqueous environment. This process of molecular reorientation can be time-dependent.[339] It is thus likely that the speed at which a liquid wets a solid would also affect this process.

Many researchers in the biomaterials literature do not measure advancing and receding contact angles. It can be argued that it would be necessary to measure both advancing and receding angles if someone wishes to extract some information from these measurements, if any information is in fact available. However, analysis of the results listed in Tables 5 and 6 reveals that this information does not even provide more insight to establish a relationship between cell responses and water contact angle measurements on biomaterials.

5.6.4. Meaning and Relevance of Contact Angle Measurements. As stated by Vogler, "Even the coarsest categorization of biomaterials into fully-water-wettable and not fully-water-wettable fails to bring the protein adsorption picture into focus".[340,341] This statement can be extended to cell−material interactions, as revealed by results listed in Tables 5 and 6.

The above discussion reveals a clear advantage that justifies the use of surface force measurements by SFA and/or AFM combined with chemical analyses over contact angle measurements. By measuring contact angle, we see the wetting of a solid surface by water in the presence of a gaseous phase. Unfortunately, this situation is rarely encountered in cell culture experiments.

As mentioned by Yaminsky and Ninham,[338] only surface force experiments can reveal how interactions vary over separation distance between solid surfaces. Different forces can exist between surfaces, and these include VDW and Coulomb (treated together via DLVO theory), hydrophobic, and oscillatory, to name only a few. Knowing or approximating, just partly, these interactions is far more instructive for designing biomaterials surfaces aiming to modulate cell responses. The development of the SFA[115] and the AFM[119] has paved the way to the characterization of surface properties at the molecular level for hydrated systems that are not accessible by any other instruments. Unfortunately, SFA and AFM in force measurements mode do not appear sufficiently exploited by biomaterial surface scientists, as many still prefer the use of contact angle measurements to characterize and design biomaterials surfaces.

Many researchers apply the Gibbs function (or Gibbs energy) to explain contact angle or surface tension measurements. Even more audacious is attempting to disentangle or to predict the interactions between complex solid surfaces and even more complex biological fluids (e.g., blood) and living cells. The Gibbs energy behind the interaction between a solid surface and a contacting medium includes the chemical composition of the solid surface, the molecular architecture of the interface, the roughness and homogeneity of the solid surface, and the composition and properties of the contacting medium.

When a solid surface is exposed to or immersed into an aqueous solution, the molecular architecture of the solid−liquid interface is dictated by how the molecules attached on the solid surface and those dissolved or dispersed in the contacting media are disposed within the interface. It is necessary to treat this molecular architecture within the interface between a solid surface bearing hydrated layers and an aqueous medium, but this level of information is limited by the instruments available to probe such a dynamic interface. The molecular architecture of these interfaces can be depicted by molecules attached to a solid surface stretching more or less into the contacting aqueous phase and by some gradients of ions, water molecules, and biomolecules (e.g., amino acids, peptides, proteins, lipids, etc.) depending on the aqueous solution involved. In fact, the molecules on the solid surface can extend into the contacting medium, forming a hydrated network that can be penetrated by some dissolved (or dispersed) molecules contained in the contacting medium, depending on the sizes of both the molecules attached on the surface and those in solution (or in dispersion). It is also important to consider that molecules in solution or in dispersion can form complex concentration gradients within the interface between the solid material and the bulk contacting medium. For example, we agree with Vogler, who suggested that one should appreciate the difference between proteins in direct contact with a surface and proteins associated or entrapped in a bound hydration layer.[341] In fact, we believe that both should be assessed to correlate cell−material interactions.

One cannot assume continuity of the macroscopic physical properties at interfaces. Gradients of density and other thermodynamic properties in the normal direction can be significant.[338] The ion environment and solvation can affect the water structure at solid surfaces. The Hofmeister series has been suggested to classify ions in order of their ability to affect water structure. Some salts can modify the solubility of proteins and their structure stability. Again, the use of water contact angle values does not reveal the relative impact of these parameters on cell−material interactions. The interface thickness and solute concentrations within such an interface appear to be elegant parameters to use as one of the criteria to design low-fouling surfaces. However, very few techniques allow such characterization in complex biological fluids containing proteins, peptides, ions, and other molecules. Rather than contact angle measurements, perhaps quartz crystal microbalance (QCM) with dissipation monitoring, some spectroscopic methods, and AFM force measurements can be used to approximate, to some extent, these properties. To make the problem even more complex, often the exact composition of many biological fluids is unknown. Fetal bovine serum (FBS) is a good example where the exact composition is unknown along with some possible batch-to-batch variations. Consequently, it is very difficult, almost impossible, to rigorously apply thermodynamic concepts to the design of biomaterials surfaces with the aim to modulate cell−material interactions using these often-called "thermodynamic" analyses.

Perhaps water contact angle has been interpreted with overenthusiasm because of the fact that, by mass, human cells primarily consist of water? Thus, one may believe water contact angle measurements to be applicable contrary to our personal belief. However, as we have discussed, it is obvious that, in general, the interaction between water and matter remains poorly understood. An alternative explanation for the overuse of contact angle is the influence of the polywater theory. In fact, polywater was a hypothetical polymerized form of water.[342] This concept created scientific controversy and has been invalidated.

As proposed by Fowkes,[343−345] the thermodynamic free energy, which can also be referred to as the exergy of the system (i.e., the maximal amount of useful work that can be extracted from a system as it is brought to equilibrium with the surrounding environment) should be separated into several components corresponding to forces dictating the possible work of adhesion. These can include VDW forces, H-bonds, acid−base forces, Coulomb forces, and steric forces, to name only a few. Knowing or approximating these forces is far more instructive than trying to speculate the optimization of the surface free energy from contact angle measurements with the aim to modulate very complex cell−material interactions. No reliable method for the direct measurements of surface free energy of solids exists for such complex systems encountered in biomaterials.[346] Nevertheless, many still continue to extract these values from contact angle measurements obtained from various liquids on solids. Perhaps, it would be interesting to investigate the application of the Buckingham-π theorem[167,168] to develop dimensionless analyses of the forces involved in cell−material interactions.

Thorough thermodynamic concepts and contact angle measurements can hardly be applied when analyzing cell−material interactions, particularly if someone wishes to apply Gibbs energy to the design of biomaterial surfaces. Reaction kinetics and path selections should be considered using perhaps a constrained optimization strategy to direct a particular reaction while minimizing or delaying other unwanted reactions.

5.7. Low-Fouling Surfaces

Low-fouling surfaces reject nonspecific adsorption. As mentioned earlier, such surfaces are of particular interest in the development of materials suitable for medical implants as protein adsorption is believed to be the premier event in cell adhesion. Three particular low-fouling materials that have received much attention are phospholipids, particularly phosphatidylcholine (PC) and its derivatives, synthetic polymers such as PEG, and

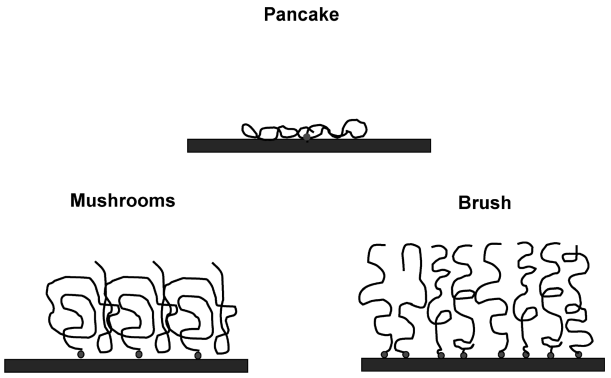


Figure 11. Surface-immobilized polymer conformations. Figure not drawn to scale. Adapted with permission from ref 248. Copyright 2003 Elsevier. The pancake, mushroom, and brush conformations were originally coined by de Gennes.[361−365]

finally polysaccharides, such as dextran derivatives. Other types of "low-fouling" materials for surface modification exist. For such materials, the reader is referred to a brief report by Hoffman.[347]

PC is a zwitterionic phospholipid making up a major portion of the outer cellular surface.[36] Thus, PC-treated surfaces have attracted much attention to mimic cell membranes.[255−259,348−354] However, many of these studies only report a reduction in protein adsorption and thus should not be considered low-fouling.[248] Furthermore, studies by Malmsten, in which the comparison of low-fouling characteristics of PC and PEG were made, revealed that PEG was found to be superior in terms of low-fouling characteristics.[355,356] Also, in two separate studies, Ruiz et al.[357] reported an 80% reduction in fibrinogen adsorption using PC whereas for the same protein Huang et al.[358] reported a 96−98% reduction in adsorption using PEG. Although experimental conditions will vary between different studies, PEG seems to be superior in terms of protein rejection over PC.

The polymer PEG has been widely studied in biomedical applications.[248] The attractiveness of PEG is manifested by its ability to conjugate covalently to proteins while decreasing immunogenic response and serum lifetime, as first reported by Abuchowski and co-workers.[359,360] PEG is the most studied low-fouling polymer. Although many advances have been made in understanding the physical and chemical mechanisms that result in low-fouling PEG surfaces, a clear picture remains elusive.

It is generally believed that PEG layers have steric repulsive forces of greater magnitude than the attractive VDW and DL forces. Such dominance in repulsive magnitude is dependent on the molecular architecture of the PEG layer. The factors governing protein attraction or repulsion are discussed in more detail later in this section. The most important parameter in preventing protein adsorption appears to be PEG surface density. As shown pictorially in Figure 11, high-density "brush" and "mushroom" PEG confirmations have superior low-fouling capabilities in comparison to low-density "pancake" conformations. Conformation is dependent on the radius of gyration of the polymer (R_g) and the distance between adsorption points on the surface (D). Steric repulsion of high magnitude results for the case when $R_g > D$ due to the "brush" conformation. At $R_g \approx D$, "mushroom" conformations form. Finally, for $R_g < D$, the resultant conformation is the "pancake".[361−365] Although this model of de Gennes and Alexander remains widely accepted, it is believed that the model used to describe this observation is not completely realistic.[366] Furthermore, there is also experimental evidence that in some cases tight "brush" conformations enhance protein adsorption.[250]

The production of PEG layers in a dense "brush" conformation is somewhat of a difficult task. This is due to steric hindrance of PEG chains as they come in contact with chains that are already adsorbed onto the surface. Producing PEG coatings under "cloud point" conditions seems an effective way of eluding steric hindrance to produce high-density PEG coatings, thus preventing protein adsorption.[249] PEG surfaces produced under "cloud point" conditions can also possess chemically available amine groups for specific biomolecule immobilization, making them ideal for biomaterial applications.[251] Such a feature is very beneficial because an ideal implant should simultaneously promote maximal cell adhesion for integration of the implant into the host while minimizing undesired host responses. For example, a PEG surface with chemically available amine groups allows the specific covalent grafting of biomolecules (e.g., Fn, RGD, etc.) for enhancing cellular adhesion (i.e., integrating the implant into the host) while minimizing adsorption favorable for undesired and/or prolonged inflammatory responses.

The abundance in literature of different PEG surface preparations and their interactions with various biological systems is vast. For a comprehensive review on the subject, the reader is referred to a detailed review by Vermette and Meagher.[248] Generally, one must perform surface characterization using techniques such as AFM, QCM, and surface plasmon resonance (SPR). Such a practice is not always followed. There are many different methods to graft PEG molecules on surfaces. Covalent immobilization of PEG chains is recommended because physisorbed chains have been shown to be unstable.[367] However, although silanated (a chemisorption of the polymer) PEG surfaces effectively reduced protein adsorption,[368] one should be cautious because silanated surface stability is questionable, as previously discussed in section 5.3 of this article.

Although PEG has extraordinary abilities in protein repulsion subsequently resulting in the inability of cells to adhere to PEG, it has very limited capabilities for the dense grafting of peptides, proteins, etc. for engineering a specific reaction.[369] An example of a specific reaction could be the chemisorption of an ECM protein on the surface, thereby promoting cellular adhesion and, thus, implant integration. Such a limitation inspired Massia et al.[253] to develop surfaces with the polysaccharide dextran immobilized on a surface. The potentialy dense peptide grafting capabilities of dextrans result from their flexibility and the abundant presence of hydroxyl groups throughout the long polysaccharide chains.[252] Such hydroxyl groups can be modified into carboxyl[17] and aldehyde[296,297] functionalities.

The literature on dextran-derivative surfaces is not nearly as vast as that of PEG. It has been shown that the method of immobilization of CMD affects the low-fouling properties of the surface. CMD is a dextran upon which some of the abundant hydroxyl groups have been transformed to a chemically available carboxyl group. Carboxyl groups are more convenient for the chemisorption of biomolecules through the use of amide bonding, as shown in Figure 12B. Prior to CMD immobilization, surfaces premodified using acetaldehyde plasma polymerization and polyamine spacers had superior low-fouling capabilities to those treated with heptylamine plasma polymerization only.[254,370] The effect of immobilization conditions over CMD layer conformation and grafting density has also been investigated (Figure 12A). The electrolyte concentration upon initial adsorption affects the conformation with a high electrolyte

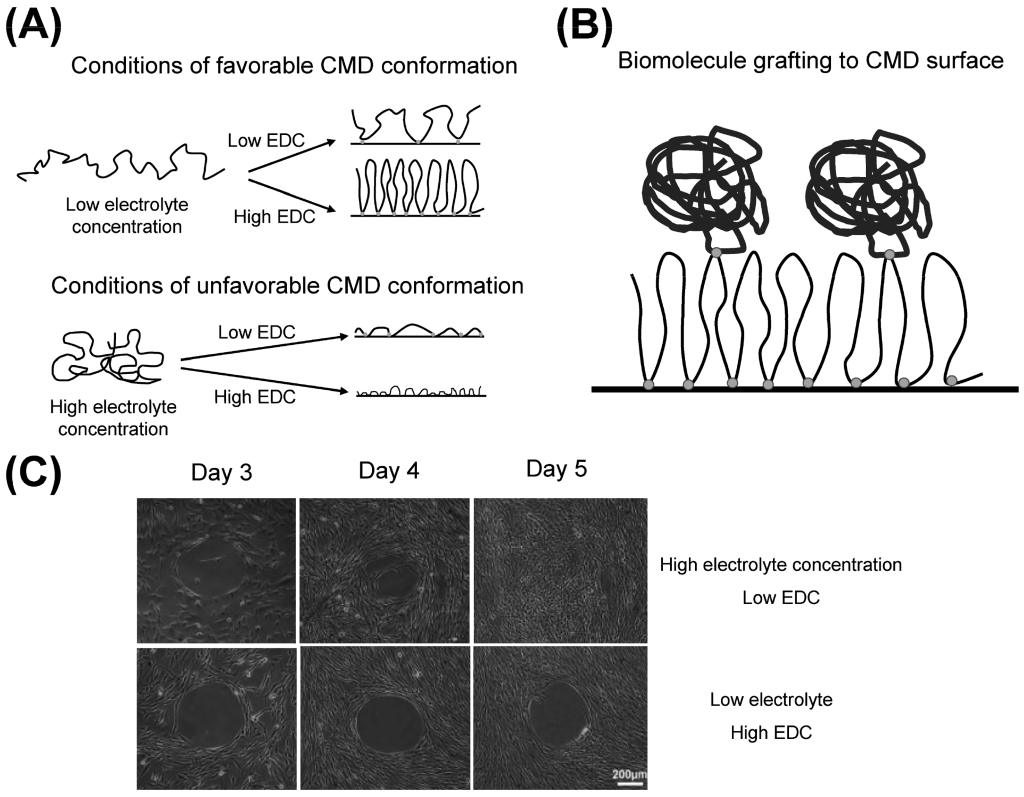


Figure 12. Dependence of CMD conformation on experimental conditions and its effect on cellular resistance. (A) Conformational dependence of surface-immobilized CMD. Adapted with permission from ref 17. Copyright 2007 American Chemical Society. (B) Biomolecule grafting to CMD surface. Figure not drawn to scale. (C) Resistance of fibroblast adhesion to spots of CMD. Adapted with permission from ref 19. Copyright 2007 John Wiley & Sons, Inc. Experimental details for both studies can be found within the references.

concentration, resulting in unfavorable CMD conformation.[17] The ratio of carbodiimide catalyst concentration to CMD carboxylation affects the final density. The optimized conditions were found to be a carboxylation degree of 70% and a carbodiimide catalyst molar ratio to carboxylation degree of 5 (i.e., EDC + NHS/COOH = 5). The resultant conformations are shown in Figure 12. Employing such conditions resulted in serum and cellular repellency similar to that of PEG (see Figure 12C)[17,19] while simultaneously allowing the grafting of biomolecules to study specific cell adhesion.[18] The dependence of cellular resistance to CMD conformation is shown in Figure 12. While dextran derivatives have some benefits over PEG, they have their limits depending on the application. For example, it has been shown that dextran derivatives activate the complement system in hematological studies.[371] PEG-grafted surfaces have proven to be more adequate for hematological studies.[372,373] For other types of polysaccharide surface compositions with low-fouling capability, the reader is referred to literature by Morra and Cassineli.[374]

It is worthwhile discussing in more detail the general physical mechanisms that govern protein repulsion. In general, for a low-fouling surface there exist two protein−surface separations at which adsorption can occur.[375] There is one very close and one further from the underlying surface, as shown in parts A and B of Figure 13. As shown in parts C and D of Figure 13, such is the result of the attractive VDW forces of the underlying surface and the repulsive steric force from the polymer/polysaccharide adlayer. We restrict the attractive interaction potential to that of the VDW for simplicity. However, other attractive forces (e.g., hydrophobic, electrostatic, etc.) can be present depending on the properties of the underlying substrate. The interaction potential for the VDW interaction is given by eq 2. As mentioned in section 3.2, there is no accepted interaction potential for the steric force. However, it has been shown previously that perhaps adsorbed polymer brushes yield a parabolic profile (as shown in Figure 13C) given by

$$w(r) \approx w_0\left(1 - \frac{r^2}{L_0^2}\right) \tag{5}$$

where $w_0 \approx (a^2/\sigma)^{2/3}$. Here, r is the separation distance, σ is the surface density of the surface immobilized polymer, a is the length of the monomer comprising the polymer chains, and L_0 is the thickness of the polymer adlayer in its free form (i.e., no compression from an adsorbate).[376] The first separation known as primary adsorption occurs very close to the surface (i.e., $\leq$1 nm). Secondary adsorption occurs at the boundary of the polymer/polysaccharide adlayer when the attractive VDW force overcomes the magnitude of the repulsive steric force. It should be pointed out that the boundary of the polymer/polysaccharide adlayer is not always a well-defined position in space.[78] Also, for this discussion we employ the VDW interaction potential between two small atoms or molecules. As mentioned earlier in section 3.2, the interaction potential of the VDW force is, in actual fact, dependent on the geometry and is different for macroscopic surfaces and macromolecules. Despite these technicalities, this model is still relevant for explaining as quantitatively and simply as possible primary and secondary adsorption of molecules onto "brush" polymer surfaces.

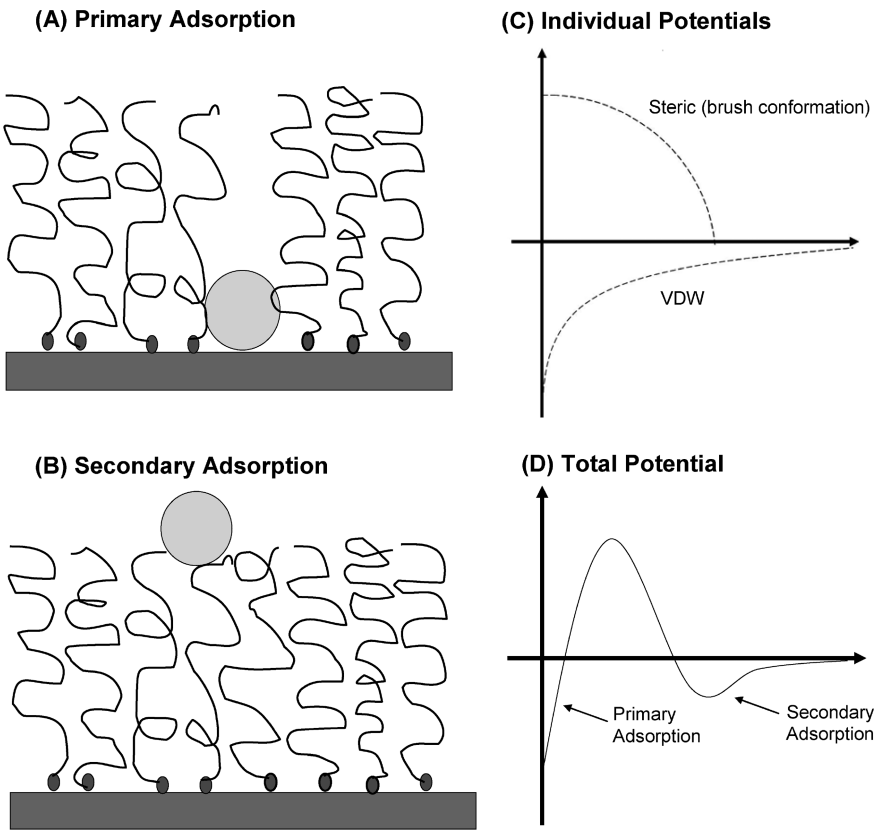


Figure 13. Primary and secondary adsorption of proteins on low-fouling surfaces. Figure not drawn to scale. (A) Primary adsorption of protein. (B) Secondary adsorption of protein. (C) Interaction potentials of individual attractive (e.g., VDW) and repulsive steric forces on a low-fouling surface. (D) Superposition of individual attractive and repulsive forces yielding the total interaction potential of a low-fouling surface.[375−377]

As discussed by Leckband et al.,[377] secondary adsorption is a direct result of the "brush" conformation outlined earlier. The ideal low-fouling surface has neither the primary nor secondary adsorption profiles in its interaction potential. Approaches to eliminating primary and secondary adsorption on a surface are discussed in excellent detail by Leckband et al.[377] Briefly, eliminating primary adsorption is generally very difficult as a result of the multiplicity of surface compositions of both proteins and the bare surface.[378] Thus, a realistic approach is to alter the interaction potential such that secondary adsorption at distances ≥ 1 nm is eliminated and the primary maximum is of such high magnitude that the protein never reaches the underlying surface in proximity of ≤ 1 nm.

Despite the advances in understanding the picture of low-fouling interactions between surfaces and proteins, an overall interaction picture remains elusive. As discussed in a review by Vermette and Meagher,[248] the surface grafting density of the adsorbed polymer is believed to be the most important parameter governing the repulsion of proteins. However, in many systems such a parameter cannot be properly characterized. The VDW interaction between proteins and PEG also requires more explicit attention. Furthermore, all PEG literature leaves absent the consideration of H-bonding in such a system whereas the solubility of PEG is based on H-bonding.

Interpretation of the term "fouling" also leads to confusion. It is advised that one draws caution when declaring their surface "nonfouling". The definition of "nonfouling" is zero adsorption. However, it can be argued that such a characteristic is impossible to theoretically verify as a result of a finite sensitivity of the technique used. For example, optical waveguide lightmode spectroscopy (OWLS) has a sensitivity of 1−2 ng/cm;[2] thus an adsorption density lower than the sensitivity would go undetected, leading to a false "nonfouling" categorization. Because of such controversy with the definition of "zero", the term "low-fouling" is deemed more appropriate.[248] Furthermore, a surface should also be tested with a wide range of proteins before concluding low-fouling behavior. Some surfaces may be resistant toward one protein but not to another. For example, in a study by Malmsten et al.,[379] it was shown that the smaller protein insulin had more adsorption relative to fibrinogen, a larger protein, on the same surface. Thus, one must test their surface with a wide array of proteins to ensure it is truly low-fouling.

The terms low-fouling and nonfouling also require consideration of the time frame of the experiment or application.[248] Some surfaces simply delay the adsorption of proteins rather than completely eliminate it. In one study, the surfaces were only exposed to proteins for 4 min,[380] which is an inadequate length if one is considering biomedical applications. For an application with short-term exposure to a physiological environment such as the case of a contact lens, a shorter time frame is adequate. However, the same surface would likely be inadequate for a long-term application. Thus, one must clearly define a tolerable threshold of adsorption when designing and deeming if the surface is adequate for their particular application.

6. CONCLUSIONS

The scientific discourse of cell−surface and cell−material interactions is vast, covering fundamental aspects of physics,

chemistry, and biology. There appears to be significant segregation between many researchers with varying scientific backgrounds studying, to some extent, systems related to cell−surface and cell−material interactions. We believe that such segregation is the primary contributing factor in the wide variety of results reported throughout this article and what we believe to be very limited understanding of cell behavior (i.e., adhesion, migration, proliferation, and differentiation) to modified surfaces and materials. Other contributing factors are limitations in the experimental and theoretical framework.

Extensive limitations exist in both quantitatively modeling and measuring surface and intermolecular forces in cell−surface science. Such limitations stem from the complexities of biological systems. Biological systems involve many large molecules, nonlinear behavior, and somewhat chaotic thermodynamics, as such systems are never in or even approaching equilibrium. Together with limitations in computational and force measurement technology, it is somewhat unrealistic to attempt to quantify all aspects of a cell−surface or cell−material interaction in regards to surface and intermolecular forces. Furthermore, the origins of fundamental interactions such as hydrophobic have no quantitative basis and are very poorly understood. Nevertheless, we believe such forces require more attention in the future than they are currently attracting. Perhaps a good starting point would be to quantitatively determine to what extent Coulomb and DL interactions coerce with gravity during initial cell adhesion, subsequently either delaying or accelerating the LR interaction, the frontier between physical interactions such as Coulomb, VDW, double-layer, etc. and biological functions such as proliferation, apoptosis, etc.

As discussed in section 4, a traditional in vitro cell culture system is not representative of the in vivo physiological reality. Differences in observations between 2D and 3D cell culture systems manifest from alterations in the mechanical environment, cell signaling pathways (i.e., chemical niches), and transport properties between the two systems. The design of a system that adequately represents the mechanical and transport properties of the human body is extremely challenging. This is the reason for 2D systems being the principle vehicle used to study cell−material interactions. Furthermore, imaging cells in a 3D environment, at the same resolution as that in 2D systems, is extremely difficult.

Efforts in tissue engineering have advanced experimental simulation of the mechanical and transport properties of the human body in vitro through the use of gels composed of the ECM elements and bioreactors capable of dynamic mass transport, with both being more representative of the in vivo reality with respect to traditional 2D cell culture systems. In 3D cell culture systems, the mechanical environment is simulated using gels composed of ECM materials, namely, fibrin, collagen, and Matrigel, to name a few. We strongly believe that Matrigel should be avoided because its composition is complex and somewhat unknown. Furthermore, it is often difficult to reproduce observations using Matrigel. If possible, collagen should also perhaps be avoided because it is difficult to purify. The architecture of purified collagen varies based on the purification technique employed. Regardless of purification technique, collagen always contains impurities; this is likely the underlying reason for large variations in the results of previous literature that employs the use of collagens. Marine collagens are perhaps an alternative. Nevertheless, collagen is superior relative to Matrigel and finds use in biomaterials research. In our opinion, fibrin is the most reliable natural ECM gel material available today. Although problems with degradation have been reported, fibrin can be freer of impurities and can be modified with growth factors or other biochemical niches, allowing one to assess their role in 3D tissue development and function. The design of a bioreactor to simulate mass transport properties of the human body requires one to evaluate whether diffusive or convective transport is adequate for the delivery of nutrients and the removal of wastes while simultaneously minimizing shear stress onto the cells and tissues. Despite many advances in in vitro cell culture that is more representative of the in vivo reality, the limitations mentioned here are significant and one must factor such limitations into one's future research.

Although protein interactions are known to be the underlying mechanisms of cellular adhesion to materials, more detail into such mechanisms is necessary to better understand cell−surface and cell−material interactions. As discussed in section 2, cellular adhesion is a complex and dynamic process. Such dynamics are dependent on the particular availability of ligands on the modified surface. The availability of particular ligands is dependent on the physical and chemical properties from modification of the underlying substrate. Very little is known about how modifications to the substrate leading to the availability of ligands on the modified surface affect the dynamics of cellular adhesion. Such intercellular dynamics govern subsequent behaviors such as morphology, proliferation, and differentiation, to name a few. Cell labeling of adhesion proteins, which is fortuitous in itself, fixes the cells, resulting in an unprecedented loss of how cells behave to modified surfaces. Furthermore, it is performed after a random time (varying from hours to days) between different studies in the literature. Techniques capable of analyzing live cells are perhaps methods that can provide far more extensive insight into cell behavior to biomaterials than those of traditional fixation and labeling.

The specific influence of substrate modifications on the conformation of biomolecules adsorbed to the substrate is not well-known. Most literature restricts the resultant protein conformation to a substrate as either "extended" or "compact". Little research mentions or even suggests what the resultant secondary, tertiary, or quaternary structure of the adsorbed protein is. Furthermore, literature implies there is a conformational change of the protein from its "natural" conformation upon adsorption to a surface. However, the "natural" conformation is not explicitly defined, giving such statements no basis. Also, the proteins used in such studies are not ideal. Purification results in trace amounts of contaminants such as proteolytic fragments, to name one, and these contaminants effect cell adhesion and subsequent behavior, something that is not usually accounted for in the literature. In summary, attention to cell adhesion dynamics, adsorbed protein architecture, and considerations in the imperfections of purified proteins is required in future research.

Surface modifications also complicate research, as discussed in section 5. Methods such as SAMs and plasma polymerization for surface modification are not without disadvantages. For SAMs, organosilanes pose numerous problems due to their sensitivity to moisture conditions. Organosulfur SAMs pose fewer problems than organosilanes, but one still must be cautious of H-bonding between COOH and NH_2 tail groups of free and adsorbed organosulfur SAM molecules resulting in a partial multilayer. Otherwise, one might be deceived, believing that exposed cells and/or biomolecules are interacting with a flat COOH or NH_2 surface, when they are in actual fact interacting with a partial

multilayer composed of a combination of the tail and head groups of the SAM molecule with variable roughness. Fortunately, methods provided by Wang et al.[271] exist that remove such a partial multilayer. We believe that plasma treatment as a means of chemical substrate modification should not be employed because the resulting morphological alterations and significant delamination only weaken the merit of resultant cell response and/or biomolecule interaction being in fact due to the chemical modification of the substrate. The presence of unwanted chemical moieties (e.g., NH when ideally only NH_2) remains the premier problem when using plasma polymerization. Unfortunately, this is difficult, if not impossible, to control. We thus believe that the best substrate modification currently available for model studies is the use of organosulfur SAMs on Au(111)surfaces, whereas if someone wishes to study a coating that can be applied to a wide range of substrate chemistries, types, and geometries, plasma polymerization appears to be a good choice. However, we strongly urge adequate surface characterization prior to further modification using biomolecules or direct cell seeding.

More often than not, modified surfaces are not adequately characterized prior to the adsorption of biomolecules and/or the seeding of cells. Typically, the most common surface characterization technique used is XPS, which provides excellent information on surface composition but no explicit information on surface architecture. Thus, problems such as unintentional surface roughness and heterogeneity can go unnoticed with XPS. The second most common surface analysis technique used is water contact angle measurements. The misconceptions and complexities of water contact angle measurements are vast. The largest is their relevance to biological systems. When measuring contact angle, one is observing how a solid interface interacts with an aqueous liquid phase in the presence of air (a third phase). This third phase is rarely encountered in biology. Surface roughness, chemical contamination, and heterogeneity all cause contact angle hysteresis, something that is often overlooked by researchers in cell−material interactions, who in most cases use static water contact angle measurements. Furthermore, when a surface is wetted, H-bonding and Coulomb interactions can force molecules from the surface, creating a gradient of ions, thus influencing the contact angle. Finally, surface wettability, the property that is assessed via contact angle measurements, is a direct result of the hydrophobic properties of the surface; as discussed in sections 3 and 5, very little quantitative understanding of the origin and nature of hydrophobic interactions is known. With all these complexities, it is no wonder that no direct correlation can be made between contact angle and predicting the behavior of cells and/or biomolecule interactions on a surface as shown in Tables 5 and 6. Nevertheless, contact angle use remains rampant today in cell−surface and cell−material research. We strongly discourage the use of contact angle as a means of surface characterization. If one wishes to employ low-fouling surfaces in their research, the degree of acceptable adhesion must be established and considerations on the finite sensitivity of adsorption measurement techniques must be taken into account.

We recommend employing AFM along with XPS, thus acquiring both surface composition and architecture for biomaterial surface characterization. To access the physical properties of the surface, techniques such as AFM force measurements or SFA should be used. QCM with dissipation monitoring is one of the more advantageous means of assessing biomolecule adsorption to a surface. QCM with dissipation monitoring allows one to assess the quantity of biomolecules adsorbed to the surface while simultaneously giving information regarding the rigidity of the adsorbed biomolecules.

Cellular adhesion and behavior to modified surfaces is in fact governed by, for all intensive purposes, the superposition of surface and intermolecular interactions. We understand the difficulty, both experimentally and theoretically, of resolving the individual contributions of such interactions within the system. Nevertheless, such interactions at the molecular level described in section 3 govern macroscopic cell behavior and response. A fundamental understanding of this influence would provide invaluable tools for designing and utilizing in vitro systems and materials that elicit a desired and controllable cell response, thereby advancing not only fundamental cell biology, but also biomaterials science, tissue engineering, and the numerous other related subdisciplines.

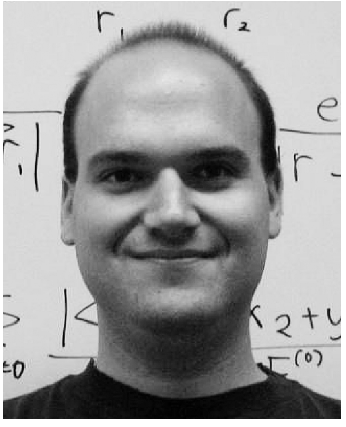
The purpose of this article was not to hunt down errors, but to establish the general concepts behind cell−material interactions. We do not pretend to better understand or to marginalize the work that has been presented throughout this article. Rather, we wished to present the reader a wide scientific spectrum of literature relevant to the subject of cell−material interactions as we believe many of the limitations and complexities of physics, chemistry, and biology are not known sufficiently across disciplines. We hope that this article directs future research considerations and interpretation toward general comprehension of cell behavior to surfaces and materials as opposed to expanding an overabundant mass of literature.

AUTHOR INFORMATION

Corresponding Author

*Corresponding author. Phone: +1 819-821-8000 ext. 62826. Fax: +1 819-821-7955. E-mail: Patrick.Vermette@USherbrooke.ca.

BIOGRAPHIES



Evan A. Dubiel holds a B.Sc. and a M.Sc. in Engineering Physics from the University of Saskatchewan (Canada). He is currently a Ph.D. candidate in Chemical and Biotechnological Engineering at the Université de Sherbrooke (Canada). His general research interests are in understanding how physical interactions in biological systems govern cell response and behavior and then utilizing such understanding for developing systems capable of growing

and maintaining suitable and sustainable human tissue substitutes.

Yves Martin (Ph.D.) has contributed to projects including bioreactor conception and development, drug delivery systems development, 3D cell signaling, stem cell research, and cell−material interactions during his graduate studies at the Université de Sherbrooke (Canada). Also, he has been working on the development of a medical device to evaluate platelet activation from whole blood samples at the Université de Sherbrooke. Dr. Martin is a managing editor of an issue of Frontiers in Bioscience, has published key scientific papers, is coinventor of patents, has participated in many scientific meetings, and has taught undergraduate chemical and biotechnological engineering students.

Patrick Vermette is a full professor and ingénieur in the Department of Chemical and Biotechnological Engineering (Université de Sherbrooke, Sherbrooke, Canada). He is also a researcher at the Research Centre on Aging (Sherbrooke, Canada). He received his Ph.D. from the University of New South Wales (Sydney, Australia), while working at CSIRO Molecular Science (Melbourne, Australia). He has built a state-of-the-art laboratory for biomaterials and tissue engineering. His research focus is in biomaterials, tissue engineering, and surface science and biotechnological engineering.

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LIST OF ABBREVIATIONS

ADV	advancing
Au(111)	Miller oriented (lmn) gold surface
AFM	atomic force microscopy
CAD	computer-aided design
CCBD	central cell binding domain
CMD	carboxymethyl dextran
DL	double-layer
DLVO	Derjaguin−Landau−Verway−Overbeek
DYN	dynamic
ECM	extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminoproplyl)carbodiimide
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FBS	fetal bovine serum
FCCS	fluorescent cross-correlation spectroscopy
FCS	fluorescent correlation spectroscopy
FEP	fluorinated ethylene propylene
FGF	fibroblast growth factor
Fn	fibronectin
FRAP	fluorescence recovery after photobleaching
GBD	gelatin binding domain
GRGDS	glycine-arginine-glycine-aspartic acid-serine
HBD	heparin-binding domain
H-bond	hydrogen bond
HGF	hepatocyte growth factor
HUVEC	human umbilical vein endothelial cell
IC	immunochemistry
ICCS	image cross-correlation spectroscopy
ICS	image correlation spectroscopy
LPA	lysophosphatidic acid
LR	ligand−receptor
MALDI	matrix-assisted laser desorption/ionization
MAP	mitogen-activated protein
NHS	*N*-hydroxysuccinimide
OWLS	optical waveguide lightmode spectroscopy
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEG	poly(ethylene glycol)
PEO	poly(ethylene oxide)
PET	poly(ethylene terephthalate
PFC	perfluorocarbon
PHSRN	proline-histidine-serine-arginine-asparagine
PGA	poly(glycolic acid)
PTFE	polytetrafluoroethylene
PLA	poly(lactic acid)
PLGA	poly(lactide-co-glycolide)
QCM	quartz crystal microbalance
REC	receding
RF	radio frequency
RGD	arginine-glycine-aspartic acid
SAM	self-assembled monolayer
SFA	surface force apparatus
SIMS	secondary ion mass spectrometry
SMC	smooth muscle cell
SPR	surface plasmon resonance

STICS	spatiotemporal image correlation spectroscopy
Sulfo-SMCC	sulfosuccinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate
VDW	van der Waals
VEGF	vascular endothelial growth factor
Vn	vitronectin
YIGSR	tyrosine-isoleucine-glycine-serine-arginine

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