Balance of chemistry, topography, and mechanics at the cell–biomaterial interface: Issues and challenges for assessing the role of substrate mechanics on cell response

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Abstract

Cells respond to three main categories of physicochemical cues: chemical, topographical, and mechanical. While surface chemistry and topography have been studied extensively, substrate mechanics has only recently been appreciated. Recent technologies of creating surfaces with well-defined chemistry and topography combined with sensitive surface characterization techniques have unquestionably deepened our understanding of surface chemical and topographical effects on cell behavior. In contrast, much less is known about substrate mechanics effects on cell behavior. This review summarizes the types of substrata and characterization methods that have been used to investigate substrate mechanics effects on cell behavior. We also speculate on the relationships between changes in substrate elasticity that occur naturally in vivo (e.g. wound healing) and cellular response. We present recent developments in creating substrata with well-defined mechanical properties in our own laboratory and conclude by discussing the major challenges and issues of determining whether substrate mechanics effects on cells are a material-independent phenomenon. This relatively new field would benefit significantly from contributions by surface scientists, and we hope that this review will stimulate the development of methods to create novel substrata with tunable mechanical properties and sensitive microscale techniques to probe their mechanical properties. Such model systems in which chemistry, topography, and mechanics can be independently controlled will facilitate the quest for design principles and material selection rules to control cell response.

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Keywords: Surface tension; Adhesion; Surface structure, morphology, roughness, and topography; Atomic force microscopy

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1. Introduction

1.1. Rationale and organization for this review

The ultimate goal of engineering the cell–material interface is to control cellular responses. Ideally, one would prefer to utilize predictive models [1–3] of how surface properties affect cell behavior rather than resorting to trial-and-error. Though we are still far from this goal, we can attack this problem by considering the factors that surface scientists can control to modulate cell–substrate interactions. These can essentially be classified into three categories of stimuli or cues to which a cell will respond: chemical, topographical, and mechanical. These three different types of cues can have very similar effects (Fig. 1; note that cell shape can be controlled by changing the surface chemistry, topography, or mechanics of the substrate). The surface scientist is already quite familiar with the numerous studies that have systematically investigated cellular response to surface chemistry [4] and topography (e.g. grooves and ridges) [5,6]. However, similar investigations regarding substrate mechanics have largely been overlooked.

In this review article, we will first discuss materials and methodologies that have been used to investigate how differences in elastic modulus can modulate cell response. Recently a pioneering study [7] stimulated a number of groups including ours to investigate the effects of substrate elasticity on cell behavior. At first glance, substrate mechanics may seem irrelevant to hard surfaces such as titanium and glass; however, soft tissue formation resulting from a wound healing response is inevitable whenever any surface (regardless of its stiffness) is implanted in the body.

Second, we will discuss the importance of the development of new tools for investigating biointerfacial cellular phenomena. Recent innovations in biomaterials science were critical for rapid

Fig. 1. Modulation of cell–substrate interactions can be achieved by either tuning either surface chemistry (A), topography (B), or substrate mechanics (C). (A) Fibroblasts on type I collagen-coated polyacrylamide substrata [13]. Top panel has lower surface concentration than bottom panel. (B) Vascular smooth muscle cells (VSMCs) on polydimethylsiloxane (PDMS) substrata with no topographical features (top panel) and with microgrooved topography (bottom) [63]. Both textured and nontextured PDMS were treated with fibronectin. (C) VSMCs on polyacrylamide substrata with different elastic modulus [52]. The lower panel is stiffer than the upper panel. Substrata were modified with collagen.
advancements in our understanding of cellular response to topographical and chemical cues. For example, it has been well known for over 50 years that cells respond to topographical cues [8]. However, systematic studies of this phenomenon were not possible until recent developments in microfabrication enabled the manufacture of surfaces with topographical features on the length-scale of cells [8]. Similarly, several technological advances have been critical for studying the effect of chemical cues [4]: (1) the ability to create surfaces with well-controlled chemistries, and (2) the development of sensitive surface characterization techniques to validate the surface chemistry. It would follow then that three critical issues must be considered in systematic studies of substrate elasticity on cell behavior. First, methodologies are needed to create substrata with well-defined mechanical properties at the microscale and mesoscale to match the range of length scales from individual cells up to whole tissues. Second, accurate measurement methods are required to assess the local microscale mechanical properties of the substrata. (Methods to measure bulk mechanical properties are already well established.) Third, experiments and predictive models are needed to quantitatively identify the range of mechanical properties over which a cell may respond, which may vary with cell type.

One must have an appreciation for the multiple signals that affect cell behavior; therefore, it is critical to determine the role of substrate mechanical properties on cell response. Findings from these types of basic science studies may challenge current approaches and inspire more integrative methodologies of designing scaffolds for tissue engineering and other devices that interface with cells.

1.2. Scope

We focus our discussion on recent studies in this new research area and also discuss key experimental factors to consider in future investigations assessing the impact of substrate elasticity on cell behavior. These studies have primarily been carried out under static conditions: comprehensive reviews of effects of external mechanical forces such as cyclic strain or shear stress can be found elsewhere [9–11]. Moreover, under such static conditions, viscoelastic effects are minimal because strains generated from cellular traction stresses are well within the elastic range of the substrata [12,13].

In order to separate out effects due to differences in chemistry from mechanics, we focus our discussion on model material systems in which these properties can be independently controlled—unlike materials that naturally contain adhesive functionalities (e.g., fibrin, collagen). These properties can also be tuned independently in the synthetic degradable materials reviewed by Anseth et al. [14]; however, the mechanical properties of such degradable materials are time-dependent and therefore will not be included in this discussion. For a more biological perspective of the possible cellular mechanisms of sensing substrate compliance effects, see [15,16].

2. Review of recent literature

2.1. Engineering of substrata to evaluate effects of substrate mechanical properties on cell behavior

Though countless substrata have been used to investigate cell–substrate interactions, surprisingly few have been used to systematically study the effects of substrate elasticity on cell behavior. These include polyacrylamide (PAAM), polydimethylsiloxane (PDMS), alginate, and agarose (Table 1). For each of these materials, the degree of crosslinking is easily tuned to modulate the mechanical properties. Another common feature of these materials is that they require surface modification to promote cell adhesion. In most cases, covalent modification is needed (i.e. the surface is resistant to protein adsorption); thus, one can present specific adhesive ligands at the surface with minimal interference from other molecules in the solution.

By far, the majority of studies examining substrate mechanics effects on cell behavior have been carried out with PAAM substrata. The PAAM system is attractive because it is an extremely well-characterized system, and it is quite simple.
Table 1
Investigations of substrate stiffness effects on cell behavior

<table>
<thead>
<tr>
<th>Substrate/modification</th>
<th>Modulus/method</th>
<th>Cell type</th>
<th>Main finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAAM/collagen I (covalent)</td>
<td>$E$: 15–70 Pa$^2$/microindentation</td>
<td>Fibroblasts [7,44]</td>
<td>Reduced cell spreading and increased motility on softer substrata; increased growth and decreased apoptosis on stiffer substrata</td>
</tr>
<tr>
<td>PAAM (gradient)/collagen I (covalent)</td>
<td>$E$: 14 and 30 kPa/microindentation</td>
<td>Fibroblasts [42]</td>
<td>Preferential migration towards stiffer substrata</td>
</tr>
<tr>
<td>PAAM/polylysine (covalent) and Matrigel (physisorption)</td>
<td>$G^*$: 50–500 Pa/rheometry</td>
<td>Spinal cord neurons [37]</td>
<td>More neurite branching on softer substrata</td>
</tr>
<tr>
<td>PAAM/collagen I (covalent)</td>
<td>–</td>
<td>Endothelial cells [49]</td>
<td>Tubulogenesis increased for softer substrata</td>
</tr>
<tr>
<td>PAAM/collagen I (covalent or physisorption)</td>
<td>$E$: 2.5–40 kPa/uniaxial tension and atomic force microscopy</td>
<td>VSMCs [31]</td>
<td>Reduced cell spreading on softer substrata</td>
</tr>
<tr>
<td>PAAM (radial gradient)/collagen I (covalent)</td>
<td>$E$: 2.5–11 kPa/microindentation</td>
<td>VSMCs [48]</td>
<td>Preferential migration and accumulation of cells onto stiffer regions of substrata</td>
</tr>
<tr>
<td>Silicone rubber/no pretreatment</td>
<td>–/wrinkle method</td>
<td>Glioblastoma [64]</td>
<td>Reduced cell spreading and greater motility on softer substrata; Preferential migration and accumulation of cells onto stiffer regions of substrata</td>
</tr>
<tr>
<td>PDMS (mechanically patterned substrata)/fibronectin (physisorption)</td>
<td>$E$: 12 kPa–2.5 MPa/uniaxial tension</td>
<td>Fibroblasts and endothelial cells [51]</td>
<td>Preferential migration and accumulation of cells onto stiffer regions of substrata</td>
</tr>
<tr>
<td>Alginate/RGD (covalent)</td>
<td>$E_c$: ~12–127 kPa (Ca$^{2+}$); ~16–147 kPa (Ba$^{2+}$)/dynamic uniaxial confined compression</td>
<td>Chondrocytes [65]</td>
<td>Reduced cell spreading on softer substrata</td>
</tr>
<tr>
<td>Alginate/RGD (covalent)</td>
<td>$E$: 2–10 kPa$^{b}$</td>
<td>Skeletal myoblasts [66]</td>
<td>Rate of growth and differentiation higher on stiffer substrata; Rate of neurite extension greater on softer substrata</td>
</tr>
<tr>
<td>Agarose/no modification</td>
<td>$G^*$: 2–13 Pa/rheometry</td>
<td>Dorsal root ganglia neurons [3]</td>
<td></td>
</tr>
</tbody>
</table>


$^a$ Reported values are most likely underestimates of the actual values [32].

$^b$ Values inferred from [24] where a relationship between elastic modulus and guluronate content is reported for alginic acid.

to tune the mechanical properties by adjusting the crosslinker:monomer ratio. The crosslinker is typically bis-acrylamide, and the free-radical polymerization can be initiated chemically, thermally, or with a photoinitiator and UV light (Fig. 2A). Furthermore, PAAM is straightforward to modify with cell-adhesive molecules: a large number of strategies to chemically modify PAAM have been worked out in detail [17] due to its extensive use in chromatography. For example, PAAM can be functionalized through reactions with bifunctional linkers or copolymerization with acrylic monomers containing reactive groups such as N-hydroxysuccinimidyl ester (NHS-ester) that in turn react with the primary amine groups of cell-adhesive peptides and proteins [18].
Fig. 2. Schematics of materials used for studying substrate compliance effects on cell behavior. Polyacrylamide (A) and polydimethylsiloxane (PDMS; B) are formed by free-radical-initiated reactions between a crosslinker and a monomer or oligomer. Alginate (C) can be crosslinked either by electrostatic interactions (i.e. binding to Ca$^{2+}$, as shown) or also by covalent bonds formed through reactions at its hydroxyl or carboxyl functional groups. Agarose gels (D) form through chain entanglement, a thermoreversible process. Abbreviations: APS: ammonium persulfate; TEMED: $N,N',N''$-tetramethylethlenediamine; AIBN: 2,2'-azo-bis-isobutylnitrile.
One can also easily tune the mechanical properties of PDMS by varying the ratio of oligomer and crosslinker (Fig. 2B). However, the difficulty of chemical modification of PDMS, compared to PAAM, has been attributed to three main factors: chemical inertness, hydrophobicity, and high chain mobility (migration of chains from bulk to surface). Chain mobility is probably the key limiting factor in modifying PDMS; various surface treatments are used to create oxidized layers on hydrophobic materials such as polystyrene and polyactic acid, but surface reconstruction in PDMS leads to unstable and short-lived oxidized layers on PDMS. Thus, the physical adsorption of cell-adhesive molecules onto PDMS is relatively inefficient even after surface activation [19]. Recently, two promising methods have been used to treat the surface of PDMS: (1) layer-by-layer self-assembly of alternately positively and negatively-charged polymers [20], and (2) a clever method of stretching the substrate during surface treatment (exposure to UV-ozone followed by silane vapor) and then releasing the tension to enable the formation of a tightly-packed monolayer of functionalized silanes [21]. It is interesting to note that exposure to UV has also been used to deliberately lower the elastic modulus of silicone rubber (in this case, polymethylphenylsiloxane) films [22].

For the above two examples (PAAM and PDMS), modulation of the degree of crosslinking is typically achieved by covalent bonding. In the next example, alginate, the process of gelation can be achieved either via electrostatic interactions (i.e. binding to divalent cations such as Ca$^{2+}$, Mg$^{2+}$, Ba$^{2+}$, and Sr$^{2+}$) in which case the gelation is reversible (Fig. 2C), or via irreversible covalent bonds (as in the case for both PAAM and PDMS). The alginates are a family of seaweed-derived copolymers that vary in the fraction of its two monomers, $\beta$-D-mannuronate and $\alpha$-L-guluronate. Alginates with higher guluronate content or longer blocks of repeated guluronate residues are able to bind more divalent cations; consequently, alginates rich in guluronate are also associated with greater mechanical rigidity [23,24]. The impact of these two different crosslinking methods on the resulting mechanical properties is apparent from a recent study in which the ionic interactions between alginate and Ca$^{2+}$ were shown to impart higher toughness whereas covalent crosslinking alginate with adipic dihydrazide led to more brittle hydrogels [25]. Like PAAM, alginate is hydrophilic and is non-adhesive in terms of protein adsorption and cell adhesion. Therefore, alginate has also been covalently modified with cell-adhesive peptides (e.g. RGD) using NHS-ester-based strategies [26] similar to those applied to PAAM. In this case, a reactive alginate-NHS-ester intermediate was created using N-hydroxysulfosuccinimide (sulfo-NHS) and 1-ethyl-(dimethylaminoproyl) succinimide (also referred to as EDC or EDAC). The NHS-ester intermediate then readily reacted with a primary amine of a protein or peptide [18].

Agarose, a repeated disaccharide composed of 3,6-anhydro-$\alpha$-L-galactose and $\beta$-D-galactose, is also a seaweed-derived copolymer with reversible gelation chemistry; however, agarose gelation is thermoreversible (Fig. 2D). In other words, at higher temperatures (>65 °C), agarose chains adopt a random coil configuration and become fluid. Gelation occurs as the temperature is decreased: chain mobility decreases, leading to aggregation and physical entanglement of chains. The gelation temperature ranges from 17 to 40 °C, depending on the degree of hydroxyethyl substituents on its side chains [3]. One can increase the hydrogel stiffness by increasing the concentration of agarose. Similar to PAAM and alginate, agarose has low interactions with cell surface receptors. In order to promote cell adhesion, agarose has been covalently modified with cell-adhesive peptides, such as RGD and the laminin-derived motif YIGSR, by using the activating agent $N,N$-carbonyldiimidazole (CDI) [27,28]. The chemistry of CDI is similar to the sulfo-NHS/EDC method used to modify alginate in that it creates a reactive intermediate (in this case an imidazole carbamate), which then readily reacts with a primary amine of a protein or peptide [18].

From these different methodologies of substrate preparation, several questions can be raised that relate to the assessment of substrate mechanics effects on cell behavior. The main issues are the choice of material and the extrapolation of findings from one cell/material system to another. In other words, are the general relationships between
substrate compliance and cell behavior independent of the material and cell type? The answer is particularly important if one aims to elucidate ‘design principles’ and ‘material selection criteria’ for specific biomedical applications. For example, it would be unsafe to implant PAAM gels in vivo because of the known toxicity of the acrylamide monomer. However, for reasons outlined above, there have been far more in vitro studies using PAAM than any other material to investigate substrate mechanics effects. Ideally one could translate findings from model systems such as PAAM to more biocompatible scaffolding materials.

2.2. Methods to characterize mechanical properties of substrata

In order to assess the role of substrate mechanical properties in modulating cellular response, it is critical to have accurate methods of measuring the mechanical properties of the substrate. Studies to date (Table 1) have largely focused on determining the elastic Young’s or shear modulus at low strain. For all of the substrata, there are various measurement techniques one can use to determine the elastic modulus. These include rheometry, microindentation, dynamic uniaxial compression, uniaxial tension, and atomic force microscopy (AFM). In principle, one could compare the results from such studies irrespective of the specific method used to determine modulus. Nevertheless, in practice, there are two important considerations: (1) bulk versus local mechanical properties, and (2) the validity of comparisons made between different techniques. Regarding the first issue, it is important to note that the majority of the techniques listed in Table 1 (and Table 2) measure the bulk elastic moduli of the materials. However, bulk techniques are not sensitive to local variations in the mechanical properties that may be present: for example, it is well known that gels often contain heterogeneities on the micron scale, i.e. on the length-scale of cells [29]. In contrast, microindentation and AFM [30] have been used to measure the local elastic moduli of materials. To address the second problem, direct comparison of the mechanical properties of gels from multiple measurement techniques is required. While there have been few such studies, there appears to be some agreement between different measurement techniques: recently it has been shown that the elastic moduli of PAAM gels obtained by AFM match values found by uniaxial tension [31,32] and rheometry [33]. However, two different measurement techniques do not always give similar values for the elastic modulus: for example, the tensile modulus of gels can be 1.6 times higher than the compressive modulus [34,35].

To determine whether substrate stiffness effects are material-independent, one should conduct studies using different materials with similar elastic moduli, keeping the cell type and adhesive ligand type and density constant. Note that there is limited overlap between the elastic moduli for the different materials studied to date (Table 1): agarose ~ 10–40 Pa; alginate ~ 12–147 kPa; PAAM ~ 150 Pa–40 kPa; and PDMS ~ 0.1–2 MPa. However, these values do not represent the full range of achievable moduli for these materials: PAAM gels with an elastic modulus of up to 100 kPa have been reported [36], and a recent study reported values of

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Type</th>
<th>Elastic modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vein</td>
<td>Saphenous</td>
<td>0.027</td>
</tr>
<tr>
<td>Artery</td>
<td>Thoracic aorta</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Abdominal aorta</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Carotid artery</td>
<td>1.0–3.0</td>
</tr>
<tr>
<td></td>
<td>Iliac artery</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Anterior cerebral artery</td>
<td>5.5</td>
</tr>
<tr>
<td>Nerve</td>
<td>Sciatic</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>Bovine spinal cord</td>
<td>(Shear): 50 Pa</td>
</tr>
<tr>
<td></td>
<td>Human gray matter</td>
<td>(Shear): 200 Pa</td>
</tr>
<tr>
<td>Tendon</td>
<td>Patellar</td>
<td>300–350</td>
</tr>
<tr>
<td></td>
<td>Flexor digitorum profundus</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>Extensor digitorum longus</td>
<td>1600</td>
</tr>
<tr>
<td>Cartilage</td>
<td>Bovine articular</td>
<td>(Compressive): 0.5–1</td>
</tr>
</tbody>
</table>

*Table adapted from data presented by Abe et al. [67] and references therein.

b Measured at 1 kPa.
c Measured at 13.3 kPa.
d Mouse tissue.
e From [37].
f From [68,69].
the tensile modulus up to 3.7 MPa for high molecular weight agarose [35] (although the gelation temperature is much higher than the softer agarose gels, thereby limiting its practical use, e.g. cell entrapment during gelation). Nevertheless, a comparison of the cell behavior on these stiffer agarose gels and PDMS would provide insight into the validity of cell responses to mechanical properties, independent of substrate type.

The design of materials with physiologically relevant elastic moduli should use the mechanical properties of tissues and remodeled ECM as a rough guide. One can immediately see that the elastic moduli for tissues (Table 2), with the exception of bovine spinal cord and human gray matter [37], are largely much higher than the materials listed in Table 1. The high values reported for tendon are not surprising, given that the main component is type I collagen; values of up to several GPa have been reported for the elastic modulus of aligned collagen fibrils [38]. While it is clear that the elastic modulus can reach very high values in vivo an important point is that there can also be local regions in the tissue that exhibit much lower values for the elastic moduli. These local regions would not be detected using the bulk measurement techniques that largely have been used to generate the values reported in Table 2. Such local differences in the mechanical properties can occur during ECM remodeling. Examples of local remodeling of the ECM include the deposition and breakdown of type I collagen during wound healing. Furthermore, Jacot et al. [39] have found that the elastic modulus of peripheral bypass grafts can double in the six months following surgery. The degree of this change, combined with the degree of increase in wall thickness, strongly suggests a role of increasing collagen content in the graft wall. The elastic modulus of type I collagen can vary over an enormous range based on its microstructure: on the order of GPa for single molecules of collagen and highly aligned collagen fibers [40] but only 5–25 kPa for randomly oriented fibers [41]. Thus, there is a wide range of mechanical properties characteristic of tissues and ECM; however, most investigations of substrate stiffness effects on cell behavior have only focused on the lower end of this range (Table 1).

2.3. Effects of substrate stiffness on cell behavior

In 1997, Wang’s group was the first to demonstrate that substrate stiffness could be used to modulate cell behavior [7]. The authors observed reduced cell spreading and greater motility of fibroblasts on soft PAAM substrata compared to relatively stiffer PAAM. In further studies employing gradient compliant substrata (i.e. materials characterized by a spatial gradient in elastic modulus), they were able to show that fibroblasts preferred to migrate toward stiffer regions of the substrate, a phenomena they called ‘durotaxis’ [42]. This relationship between cellular behavior (spread area, motility, and directional migration) and substrate stiffness does not appear to be unique to fibroblasts and PAAM, but also applies to a number of different cell types and materials (Table 1). One would expect similar behaviors for fibroblasts, myofibroblasts, and vascular smooth muscle cells (VSMCs) because they have similar function, but it is striking that the same dependence on substrate stiffness has also been found for neurite extension in neurons (i.e. increased rate of extension on softer substrata) [3]. Perhaps this should not be surprising as neurite extension can be considered to be analogous to migratory behavior in other cell types such as fibroblasts [43]. It is also interesting to note that these trends are independent of the adhesive ligand (Table 1): e.g. polylysine, fibronectin, collagen, and RGD. Lastly, in addition to cell morphology and migration, higher proliferation rates have been noted on higher-modulus (stiffer) substrata for both fibroblasts [44] and skeletal myoblasts [26]. While more studies are clearly required, a large number of cellular phenomena appear to have a common dependence on substrate stiffness regardless of cell type. Therefore, it is reasonable to state that substrate mechanics, like chemistry and topography, is a key regulator of cell behavior.

One could question whether the observed behavior of cells on substrata with different stiffness (Table 1) has any physiological relevance. For example, there are numerous examples of cell accumulation in vivo, but are these a result of changes in substrate stiffness or changes in the bio-
chemical environment? Atherosclerosis is one example where ECM remodeling leads to the undesirable accumulation of VSMCs. Interestingly, it has been reported that during the early stage of atherosclerosis, the initial lesion becomes softer than normal tissue [45,46]. As the disease progresses, a fibrous and calcified cap forms and the stiffness of these advanced lesions becomes much higher than that of the normal tissue [46,47]. Although it is not clear the extent to which changes in matrix stiffness are responsible for VSMC accumulation in atherosclerosis, studies on PAAM substrata have shown that VSMCs accumulate on stiffer regions of substrata in vitro [48]. It is interesting to note that large changes in tissue stiffness are not unique to the progression of vascular disease but are also characteristic of arthritis and osteoporosis. Thus, it is not surprising that substrate stiffness affects the behavior of a wide variety of cell types (Table 1).

In general, the projected cell area increases with substrate stiffness (Table 1), but this observation is based mainly on studies of single cells. However, one particular example of endothelial cell tubulogenesis (i.e. tube formation, a key step in angiogenesis) has strikingly demonstrated the impact of elastic modulus in a multi-cellular process [49]. Specifically, soft substrates were found to be more effective than stiff substrates in promoting tubulogenesis. This finding is consistent with the observations on soft substrata at the single-cell level: for substrates with lower stiffness, the spread area of a cell is lower and cell–substrate interactions are weakened, thereby promoting cell–cell interactions that lead to the formation of more organized cellular aggregates [50].

Another implication of this significant difference between cell behavior on soft and stiff substrata is that one can deliberately modulate the spatial mechanical properties of a substrate or matrix to trigger local changes in cell behavior. One well-known in vivo example is angiogenesis, where locally released matrix metalloproteinases break down the surrounding ECM, resulting in highly localized capillary sprouting. Angiogenesis is a complex physiological process, and it is highly likely that both biochemical and biomechanical events are involved. However, the finding that a critical step in angiogenesis known as tubulogenesis can be triggered solely by changes in substrate elasticity has important implications for designing material scaffolds for tissue engineering. In other words, the use of substrata with spatial gradients in their mechanical properties to locally control morphogenesis could be an alternative approach to current methods that incorporate biochemical signals such as growth factors.

The majority of the studies mentioned (Table 1) were carried out with substrata that have uniform mechanical properties. However, as we have just discussed, the local modulation of mechanical properties can have significant effects on cellular behavior. Therefore, an entirely new class of designed biomaterials with specifically designed microscale mechanical properties could be founded upon these phenomena. Recent work from our laboratory in this area will be discussed in the next section.

3. Recent developments in substrate design from our laboratory

3.1. Development of substrata with controllable elastic modulus at the microscale

As discussed above, substrata with uniform properties are critical for determining the impact of substrate elasticity on cell behavior; however, substrata with local differences in elastic modulus can actually reveal phenomena that would otherwise be undetected from studies on uniform substrata. For example, a landmark study by Lo et al. [42] used a gradient-compliant gel to demonstrate that cells prefer to migrate towards stiffer regions of a substrate. For example, a landmark study by Lo et al. [42] used a gradient-compliant gel to demonstrate that cells prefer to migrate towards stiffer regions of a substrate. However, they did not observe long-term accumulation of cells on the stiffer region of the substrate. This is in contrast to two recent studies [48,51] where cell accumulation was observed on stiffer regions of the substrate. A possible reason for this discrepancy may be due to differences in the level of control over the gradient patterns. Moreover, the studies that observed cell accumulation in stiffer regions of the substrate used micropatterning techniques to precisely control the local elastic modulus at
the micron scale, whereas the Lo et al. method involved simply positioning two drops of different polymer solutions side-by-side.

Recently, we have developed two different methods to fabricate substrata with microscale control over the mechanical properties (Fig. 3) [48,52,53]. We validated each technique by quantifying the local mechanical properties with either microindentation [48] or atomic force microscopy [52] (Fig. 3). Both methods use photopolymerization in combination with either micropatterning or microfluidics to obtain microgradient compliant gels. The simpler of the two fabrication methods (Fig. 3A) involves photopolymerization of the substrate in the presence of a mask with varying opacity [48]. The mask imparts spatial control of the light intensity, thereby controlling the degree of crosslinking and elastic modulus [54]. This micropatterning technique is extremely versatile and the types of patterns in mechanical compliance that one can generate are bounded only by one’s imagination, with the obvious limit that feature sizes are dictated by the resolution of the mask. The second method (Fig. 3B) uses a microfluidics technique developed recently to generate solution gradients using soft lithography [55]. By controlling the solution composition (e.g. bis-acrylamide concentration) at the inlets, one can obtain a specific gradient profile of crosslinker concentration which can then be converted into a microgradient compliant gel via photopolymerization [52,53]. Note that photopolymerization is not required for the microfluidics method (Fig. 3B) because the gradient in mechanical compliance is obtained by controlling the bis-acrylamide concentration, not the light intensity. While our two fabrication methods used PAAM as the basis for creating microgradient gels, these techniques should be readily adaptable to other polymer systems.

Using the simple micropatterning method (Fig. 3A) with a radial gradient pattern, we have shown that directional migration of VSMCs can be controlled by tuning the mechanics of the substrate (Fig. 4): VSMCs migrate preferentially from softer to stiffer regions of PAAM substrata [48]. Note the contrast of the ‘random walk’ of VSMCs migrating on a constant mechanically compliant gel (Fig. 4, inset). Importantly, with the radial gradient gel, cells accumulated on the stiffer region of the substrate. On the gradient gels formed using microfluidics, we find a similar dependence of the degree of cell spreading on substrate compliance [52] to what was observed on uniform compliant gels. Therefore, an attractive feature of these microgradient
gels is their potential as high throughput cell-based assays on a single substrate that varies over a wide range of elastic moduli. The motivation for such a device is based on the observations that cell response depends on substrate elasticity (Table 1); this in turn implies that cellular response to biochemical factors (e.g. small molecule drugs and other therapeutic agents) could also be modulated by substrate elasticity. Specifically, local heterogeneities in tissue properties may result in differential cellular response to drugs. Thus, substrate elasticity could be used as an additional variable in in vitro cell-based assays to reveal effects from drugs that would otherwise be undetected.

3.2. Surface modification issues with PDMS

As discussed earlier, one can create PDMS substrata with high elastic modulus values that are comparable to the values found for tissues (Table 2). But surprisingly, there have been few studies that have used PDMS to directly investigate substrate stiffness effects on cell behavior (Table 1). Moreover, these studies have typically used noncovalent methods (i.e. physisorption) to treat the PDMS surface with adhesive molecules. However, recent studies have reported poor cell adhesion to PDMS coated with fibronectin and attributed this finding to low adsorption efficiency of FN untreated PDMS [19]. In order to enhance cell adhesion to PDMS substrata, we used the layer-by-layer (LbL) method [20,56,57] (Fig. 5C). Briefly, alternating layers of the polycation polyethyleneimine (PEI) and the polyanion polystyrene sulfate (PSS) are assembled onto the PDMS (Fig. 5A). LbL-treatment of PDMS appears to be stable because cells do not form aggregates and remain adherent to the substrate even after one week (Fig. 5C). Though it is encouraging that this LbL method can be used to enhance cell adhesion onto PDMS, it is not clear whether this treatment changes the effective elastic modulus of the substrata seen by the cells, i.e. does surface chemistry alter substrate mechanics or vice versa? Clearly, a better understanding of the surface mechanics of coatings such as those obtained by LbL-treatment is needed. Furthermore, such studies would reveal whether one could use coatings to tune the surface elasticity of substrates to provide appropriate mechanical signals to cells—similar to how chemical coatings are currently used to present specific biochemical cues to cells.

4. Issues and considerations

Striking the right balance between substrate mechanics, chemistry, and topography in order to achieve specific and well-controlled cellular responses is a difficult challenge. Controlling
substrate properties is further complicated by their complex interdependent nature and the fact that these properties are changing spatially and temporally during cellular remodeling. Therefore, in order to generate meaningful design principles for advanced biomaterial surfaces, it is critical to elucidate the manner in which each of these factors act independently, synergistically, or antagonistically to impact cellular behavior. As we will see, many of these issues are not yet resolved, mainly due to the lack of appropriate experimental techniques; therefore, these challenges provide opportunities for surface scientists to uniquely contribute innovative techniques and design methods to this field.

4.1. Effects of crosslinking density on chemistry

Several chemistry-related issues arise when one modulates the substrate stiffness by altering the crosslink density. One could argue that the crosslink density alters the number of sites available for the covalent attachment of adhesive molecules. Furthermore, the number of free chain ends can vary with crosslink density; these free chains could act to ‘mask’ or ‘repel’ interactions with cells. Consequently, how can one be confident that the effects observed on less crosslinked substrata (i.e. softer) are not in fact due to ligand density variations or free chain ends that are acting as ‘repellers’? In fact, the situation can be more complex: instead of acting as a ‘repeller’, the chain end can act as an ‘attractor’ and promote cell adhesion if it is functionalized with a cell-adhesive ligand. Consider the case of increasing crosslink density where there is a mixed population of unliganded and liganded free chain ends: reduction of chain mobility would lower the number of repellers but at the same time limit access to attractors. Thus, there may be an optimal crosslink density to promote cell adhesion that can be modulated by the incorporation of adhesion sites.

There is also the question of whether ligand density of a modified substrate changes with crosslink density. A number of groups including ours recently have quantified the density of adhesive molecules on substrata with different stiffnesses and found that ligand density does not vary significantly with crosslink density [31,42,48]. Moreover, in a recent study, Engler et al. independently varied both substrate elasticity and ligand concentration and demonstrated that increases in adhesive ligand density do not compensate for the low cell spreading on soft substrata [31]. Regarding the second issue, free chains can modulate surface interactions: a recent study reported a significant increase in friction between polymer layers in the presence of free chains [58]. In order to
minimize the number of free chains, but at the same time vary stiffness, one could use multifunctional end-linking agents to crosslink preformed functionally-terminated chains of varying molecular weight. While these types of studies would help clarify the issue of substrate elasticity versus surface chemistry, there is some evidence that differences in substrate elasticity are the driving force for the ‘durotaxis’ phenomenon (i.e. preferential migration towards stiffer substrata). Specifically, Lo et al. [42] used a microneedle to stretch a soft substrate and observed that ‘durotaxis’ could be simulated on this ‘prestressed’ soft substrate. They concluded that the microneedle acted to increase the apparent elastic modulus and was responsible for the preferential migration towards the microneedle. However, one could also argue that the cell responded to the externally applied force from the microneedle or that this force altered the alignment of the adhesive molecules, i.e. altered the topography of the surface.

4.2. Changes in chemistry and topography from active cell remodeling

Cells actively secrete their own ECM molecules and can also remodel their matrix in part by exerting tractional forces. A well-known example of matrix remodeling in vivo is fibroblast-mediated collagen compaction during wound healing [59,60]. This active remodeling can lead to reorganization and alignment of the matrix and can ultimately change substrate topography (e.g. alignment of ECM), chemistry (e.g. changes in ligand density), and even substrate mechanics (e.g. from anisotropy of aligned ECM due to cellular tractional forces). The degree to which remodeling can occur in an in vitro substrate system will depend on whether the adhesive molecules are physically adsorbed or covalently attached to the surface. Moreover, the nature of the covalent chemistry used to attach the adhesive molecules to a surface will impact protein conformation as well as its attachment strength (i.e. the number and type of bonds between the protein and the substrate). Furthermore, it is also likely that ligand presentation will affect cellular traction force generation: we have shown recently that cells exert much higher traction forces on FN- compared to RGD-modified PAAM [61]. Thus, it is critical to fully characterize the surfaces of the substrata in order to observe dynamic changes in structure and conformation. However, the surface characterization of soft materials is particularly challenging because many surface techniques require ultrahigh vacuum conditions (which significantly alters the structure of soft materials and tissues). Other techniques such as atomic force microscopy and environmental scanning electron microscopy (ESEM) allow the study of hydrated samples, but do not allow the resolution that one obtains by ultrahigh vacuum techniques. Freeze-fracture transmission electron microscopy (FFTEM) presents certain advantages, but one cannot view dynamic phenomena because the sample is frozen. Therefore, developments in fluorescence-based assays and other dynamic imaging methods would greatly impact the study of cell–biomaterial interactions on soft substrata.

5. Conclusions

The effect of substrate elasticity on cell behavior is still a relatively unexplored research field. Because this is such a new area, the number of materials that have been investigated is quite small. In fact, most studies have been conducted with PAAM, arguably a material that will never be implanted in vivo because of known toxicity of acrylamide monomer. Moreover, investigations have been narrowly focused on a small range of elastic moduli. In order to determine whether substrate elasticity effects on cell behavior are independent of material type and to explore the full range of the phenomena, we should evaluate other materials that exhibit a wider range of properties.

We propose an analogous approach that surface scientists have used previously to probe the role of substrate chemistry [4] and topography [62] on cell behavior: inventing novel techniques to create and characterize substrata with well-defined mechanical properties. Critical advances in surface science have enabled a deeper understanding of surface chemistry and topography at the cell–biomaterial interface. Therefore, we
anticipate that a similar approach will have great impact on our understanding of substrate mechanics. This integrative and systematic approach will allow us to test the balance of substrate chemistry, topography, and mechanics in engineering the cell–material interface to control cellular response.

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