Minireview

Capturing extracellular matrix properties in vitro: Microengineering materials to decipher cell and tissue level processes

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Abstract

Rapid advances in biology have led to the establishment of new fields with tremendous translational potential including regenerative medicine and immunoengineering. One commonality to these fields is the need to extract cells for manipulation in vitro; however, results obtained in laboratory cell culture will often differ widely from observations made in vivo. To more closely emulate native cell biology in the laboratory, designer engineered environments have proved a successful methodology to decipher the properties of the extracellular matrix that govern cellular decision making. Here, we present an overview of matrix properties that affect cell behavior, strategies for recapitulating important parameters in vitro, and examples of how these properties can affect cell and tissue level processes, with emphasis on leveraging these tools for immunoengineering.

Keywords: Biomaterials, extracellular matrix, micropatterning

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Introduction

Since the advent of in vitro cell culture in the early 20th century, epitomized by Harrison's development of the hanging drop technique to observe nerve fiber growth in 1907, it has provided a convenient, cost-effective method to study specific cell lines in minimal simplified growth conditions, free of many of the outside influences seen in vivo. This allows for isolation of single cell lines to investigate their properties, testing the effects of various pharmacological agents on specific cell types and a multitude of other applications under well-controlled conditions. However, these advantages come at a price; due to the differences between in vitro and in vivo cell culture conditions, cell characteristics change with long term in vitro culture. Cells adapt to the different culture conditions by changing their behavior and activities.[1]

With the accumulating evidence of the role that physical and mechanical factors such as forces,[2] shape,[3] and architecture[4] play in regulating cell behavior, the divide between in vitro cell culture and in vivo environments presents an obstacle to studying and manipulating cells in the laboratory. There have been several advances in materials and fabrication techniques that have allowed for modulation of the extracellular matrix (ECM) available to cells during in vitro culture. In fact, cells reside in very complex and dynamic extracellular matrices,[5–8] with very specific compositions, ligand presentations, mechanical properties, and organization that vary between different tissues.[9] Extracellular factors strongly influence many facets of cell behavior such as homeostasis,[10,11] morphogenesis,[12,13] self-renewal and differentiation of stem cells,[14] development,[6,15] and disease.[15,16] It thus becomes clear that, in order to be able to more fully study cell behavior in vitro, cell culture platforms in which these factors can be recapitulated and/or manipulated must be developed.[17]

Although methods to confine cells to specific shapes have been demonstrated since 1967,[18] the more recent spread of lithographic,[19] microfluidic,[20] and other patterning techniques have made micropatterning of cells much more convenient and accessible. The increasing use of both natural and synthetic soft materials[21–23] have allowed for manipulation of the form and mechanical properties of the ECM as well as ligand presentation. ECM proteins and synthetic peptides enable more precise study of specific cell–ECM interactions.[5] Degradable[24] and dynamically tunable[25] platforms elucidate how cells react to changes in their microenvironments. Techniques such as 3D printing[26] and nanopatterning[27] allow for investigating processes on tissue and subcellular scales, respectively. These advances, along with others, have enabled engineered in vitro environments to be much more accurate model systems for in vivo processes, yielding considerable insights on cellular behavior.[16,28]

In this minireview, we explore engineered environments to study and control the effects of ECM properties on cell activity. For both single cell and multiple cell systems, we consider relevant ECM properties with examples of in vitro model systems that capture these properties, highlighting some insights gleaned from such systems. We then highlight some applications of microengineered materials for the emerging field of immuno-engineering.

Engineered environments for single cell culture

Single cells experience a myriad of different signals from their ECM (Figure 1). Cells transduce and integrate these different factors into biochemical signals altering their behavior.[29] There are a variety of cellular apparatus used to detect extracellular signals such as growth factors and cytokine receptors, ion channels, cell–matrix, and cell–cell adhesion molecules.[30] Particularly, forces exerted by and on the cells through transmembrane receptors such as integrins play an important role through ''mechanotransduction'' via the cellular cytoskeleton.[31–34] Stem cells, with their plasticity, ability to differentiate down different lineages, and importance for regenerative medicine, are particularly sensitive to extracellular cues and thus are the focus of several of these studies.[35–37]

Matrix composition

Biochemical factors present in the extracellular space are numerous and present a multitude of signals to cells, allowing for functional complexity in cell behavior.[44] A wide variety of glycosaminoglycans (GAGs), proteoglycans, and different glycoproteins such as collagens, fibronectins (FNs), and laminins, combine together to provide a very rich signaling environment, which varies widely between different tissues. In fact, loss of function mutations in several of these proteins are embryonic lethal or post-natal lethal within four weeks,[44] highlighting their importance. However, due to the high complexity and organization, it is significantly challenging to recapitulate aspects of such an environment in vitro. A common strategy is adsorption[45] or chemical conjugation[46] of proteins onto synthetic tissue culture substrates. This method is more facile for studying the effects of single components of the ECM or simple combinations and is useful for deconstructing the roles of

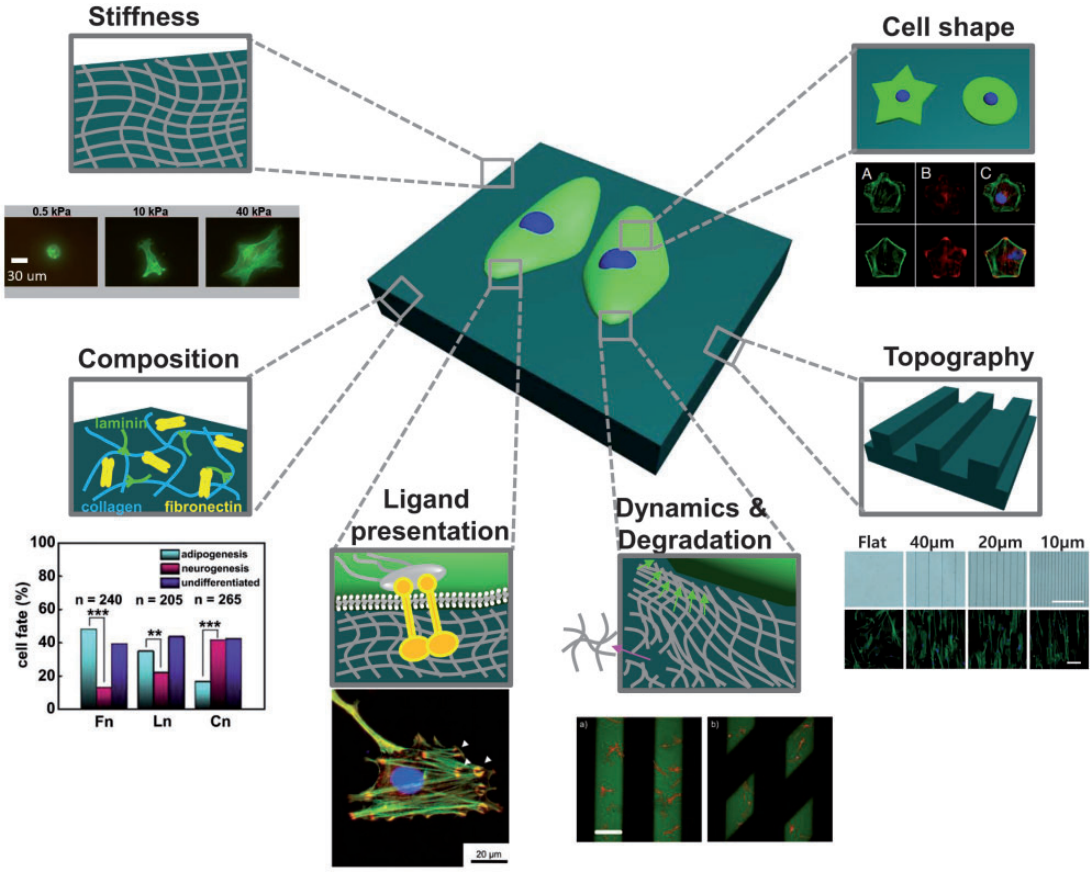

Figure 1 Matrix properties affect cell behavior in vitro: *elasticity*—MSC morphology (and cytokine secretions) is dependent on matrix stiffness.[38] *Composition*—MSC differentiation is highly dependent on the matrix protein conjugated to the surface (reprinted by Lee et al.,[39] Copyright (2013), with permission from Elsevier). *Ligand presentation*—fibroblast focal adhesions only form on 5 μm RGD functionalized gold islands with stress fibers running between adhesions (reprinted with permission by Aydin et al.,[40] copyright (2010) American chemical society). *Dynamics and degradation*—cell adhesion can be switched on and off by switching the conjugation of ligands at the surface (reprinted by DeForest and Anseth,[41] Copyright (2012), with permission from Wiley). *Topography*—substrate topography controls alignment and epigenetic reprogramming of cells (reprinted by permission from Macmillan Publishers Ltd: Nature Materials by Downing et al.,[42] copyright (2013)). *Cell Shape*—modifying cell shape can affect MSC cytoskeleton, focal adhesion formation and differentiation.[43] (A color version of this figure is available in the online journal.)

different ECM components and their interactions. Both adsorption and chemical conjugation, however, may alter protein conformation, potentially changing protein bioactivity.[47] Other strategies include the use of natural ECM components, such as GAG or collagen gels, to fabricate tissue culture environments[48] or using decellularized matrices.[49] These strategies recapture several aspects of the in vivo environment but relinquish some control over the precise environment presented to cells. Matrix composition has been found to influence diverse aspects of cell behavior such as extracellular signal-regulated kinases (ERK) activation by mechanical strain in smooth muscle cells,[50] endothelial cells network formation and their response to transforming growth factor-β,[51] secretome,[38] cancer progression,[52] and stem cell fate.[53] We and other groups have shown previously that for mesenchymal stem cells (MSCs), matrix composition can direct cell differentiation and mediate how cells respond to other cues.[39,54] Two current areas of active research are the use of cell-derived matrices to reconstitute in vitro environments[55] and synthesis of matrices that can better interact with growth factors via sequestration and other interactions.[56]

Ligand presentation

Cells will behave very differently depending on how the ligand presents to the cell. This mainly has to do with how cells interact with the proteins via focal adhesions, clusters of intracellular proteins, and transmembrane integrins.[57,58] These interactions physically transfer forces between the ECM and cells, facilitating mechanotransduction and cellular remodeling of the ECM.[29,59] Cell–matrix interactions are sensitive to ligand density, ligand spacing, receptor clustering, and ligand availability,[60] in addition to composition. Furthermore, the pliability of proteins to cell generated forces tunes the availability of cryptic signaling sites.[30] Several innovative methods have been developed to control these different aspects. The use of recombinant protein fragments or peptide sequences allows for tailoring of specific cell–matrix interactions since integrin pairs react with specific peptide sequences[31] with different affinities and outcomes. For example, using different FN III9–10 fragments with variable specificities to $\alpha_5\beta_1$ integrins allows control of $\alpha_5\beta_1$-mediated MSC osteogenesis.[61] Self-assembled monolayers of alkanethiolates on gold substrates can be used to present a more uniform interface to cells and control ligand density and affinity.[62,63] Block copolymer micelle nanolithography,[64] a technique by which very uniform arrangements of gold nanodots can be made, has been used to study effects of ligand spacing and density variations and, when combined with micropatterning, the effects of ligand clustering. The use of such methods has revealed the different binding affinities of integrins depending on peptide sequences[65] (even depending on cyclic vs. linear variants of Arginylglycylaspartic acid (RGD),[63] a commonly used peptide sequence from FN) or adhesion clustering.[66] Moreover, Spatz et al.[67] have demonstrated a threshold of ~60 nm of ligand separation for activation of integrin function and more recently have reported a more dominant role for local ligand density as opposed to global.[68] Finally, density of protein tethering alters the deformations exacted on proteins by cells, altering cell signaling, and MSC fate.[69]

Cell shape

One of the challenges of in vitro cell culture is cell heterogeneity and poor replicability of results. Micropatterning of cell shape diminishes much of the heterogeneity inherent in cell culture substrates and controls for several aspects of cellular structure such as spread area and spatial distribution of adhesions,[3] allowing for better control over experiments. Furthermore, control over cell shape facilitates geometric manipulation of the structure of the cytoskeleton.[3,70] There are multiple methods of micropatterning cells including lithography,[19] photo-patterning,[41] microfluidics,[71] and microcontact printing.[72] Micropatterning does not have to be with integrin ligands but can utilize other cellular components such as lipid bilayers.[73] Cell shape can determine the structure of the cytoskeleteon,[70] focal adhesions,[74] intermediate filaments,[75] internal cell organization,[76] nuclear forces,[77] and histone modifications.[78,79] Consequently, cell shape and size also influence cell viability,[80] stem cell multipotency,[81] and fate decisions.[39,82] Increasing the degree of cytoskeletal tension nudges MSCs toward an osteogenic, rather than adipogenic fate[43] and modulates integrin-mediated matrix interaction.[83]

Elasticity

With the elasticity of various tissues spanning orders of magnitude,[84] ECM elasticity is one of the most studied physical factors influencing cell behavior. Mechanics have also been implicated in a wide array of pathologies.[85,86] Cells respond to changes in ECM elasticity,[87] often by changing their own properties as evidenced by fibroblasts matching stiffness to their substrates.[88] Biological materials are usually heterogeneous in mechanical properties and often display nonlinear elastic behavior.[89] Synthetic materials such as polymeric hydrogels and natural materials are routinely fabricated with tunable stiffness, and materials with variable rigidities such as micropost arrays[90] have been used to probe stiffness response as well. Various cytoskeletal components and signaling pathways have been implicated in these processes including focal adhesion kinase, Rho/Rock,[35] and Yes-associated protein (YAP)/transcriptional co-activator with PDZ-binding motif (TAZ)[91] as well as nuclear elements such as lamin-A[92] and Linker of Nucleoskeleton and Cytoskeleton (LINC) complexes.[93] Early studies showed that cell motion and focal adhesions are regulated by substrate elasticity.[21] Engler et al.[94,95] demonstrated that MSC fate depends on substrate compliance, with optimal differentiation marker expression occurring on elasticities matching in vivo elasticity. Since then, the influence of substrate elasticity on modulating several aspects of cell behavior has been well documented.[96] It has further been reported that the effects on MSCs depend on how long they are exposed to a substrate and that MSC behavior is affected by their mechanical history.[97,98] The mechanism, or what exactly the cells are responding to, is variable, since changing material stiffness typically entails changing material porosity, matrix tethering, and other mechanical properties. Response to mechanical

properties has been attributed to matrix elasticity,[99,100] density of protein tethering,[69] viscoelastic creep,[101] traction forces,[102] and stress relaxation.[103]

Topography

As opposed to flat culture substrates, basement membranes, and ECM components such as collagen, which form submicron-sized fibrils, have a very hierarchical structure and are often textured, providing topographic signaling cues.[104] These cues, depending on their size, can interact with integrins up to whole cells. Advances in nanofabrication have allowed the formation of nanoscale gratings, posts, pits, aligned fibers, and other structures that can be made isotropic, anisotropic, or in gradient form.[105,106] Nanotopography can affect cell morphology, adhesion, migration, proliferation, and differentiation, generally through generation of anisotropic stresses in cells.[106] MSC differentiation has been reported to be guided by nanotopography, for example, to the neurogenic[107] or osteogenic[108] lineages. Recently, Downing et al.[42] have shown that microgrooves can modify the epigenetics and significantly improve the reprogramming of fibroblasts, demonstrating the large potential of topographic cues.

Dynamic and degradable environments

The constantly changing nature of in vivo ECM is well known.[52,109] As stated above, cells react to changes in ECM properties but are affected by previous environments. For example, there have been recent reports that MSCs ''remember'' their previous substrates[97,98] for at least 10 days with regard to nuclear localization of RUNX2, YAP, and osteogenic differentiation, although other properties such as cell area remain plastic or relatively unaffected by previous states. This is a new field of study, however, and more work is required to understand the mechanisms through which cells maintain this memory and its effect on cell behavior for longer term. Dynamic materials are hence desirable to construe the effects of changing microenvironments on cells. Switchable surfaces,[110] stimuli responsive materials,[111] and photoresponsive materials[41] have been used to modulate matrix properties such as ligand presentation, composition, stiffness, and cell shape during cell culture. Furthermore, substrate degradability may be desirable for both probing cell behavior and for in vivo use of engineered substrates.[24,112,113] A significant challenge remains engineering reversibility into these kinds of systems as opposed to one-directional changes.[114]

Other factors such as dimensionality,[23,115] mechanical load, and shear flow are also potent regulators of cell behavior. Cell behavior is typically very different between 2D and 3D environments as evidenced by several studies.[102,116,117] Although it is extremely challenging to control for multiple aspects of ECM structure in the same experiment, it is important to evaluate data in context of all the appropriate properties of the system and how they relate to the relevant in vivo environments. Different components such as hydrogels and nanopatterning or micropatterning can be combined to study the effects of multiple factors concurrently.[39,40] In fact, studies combining multiple cues often reveal crosstalk and interplay among different factors.[59] For example, MSC response to stiffness is dependent on matrix composition in terms of adhesion,[83] differentiation,[39,54] and therapeutic potential.[38] For this reason, it is imperative to take the whole biophysical system into consideration before making conclusions about the effects of certain parameters.

Peer pressure: The influence of multicellular interactions

In addition to all the factors influencing single cells during culture, there are multiple additional effects in play when multicellular constructs are considered together (Figure 2). In this situation, the position of a cell relative to other cells, cell–cell interactions, paracrine signaling, and interactions with different cell types act to instruct cellular outcomes and coordinated cell behavior. This is particularly apparent during development where the relative positions of cells can dictate their specification and differentiation.[6] Although scaffolds for studying these kinds of behaviors are typically on a larger scale than those for single cells, great care must be taken to optimize the experimental parameters and define the specific interactions being studied in order to deconstruct specific cues and determine their precise influence. Here, we present a brief overview of some of these factors.

In a typical in vivo niche, there are multiple cell types in contact in different ways. Cells in contact interact through cadherins; a family of cell adhesion molecules which mediate interactions. Cadherin based cell–cell contacts are involved in a plethora of biological processes such as development, differentiation, and disease.[118] Multiple platforms have been developed wherein homo- and heterotypic cell–cell contacts can be controlled from a single cell–cell contact up to large scale co-cultures.[119] Cells in contact have been shown to mechanically couple together,[120] allowing for large scale collective cell migration.[121] Tseng et al.[122] have shown that the organization of intercellular junctions are dependent on the ECM architecture. Studying interactions of heterotypic cells has shown interesting phenomena such as natural cell sorting due to adhesion effects[123] and self-assembly of multicellular structures.[124] Artificial boundaries between different cell types allow the investigation of interfacial interactions (in tumor-stroma, for example).[125]

Cohesive forces between cells stabilize them in contact. Differences in adhesion between homophilic and heterophilic cell–cell contacts may cause cell aggregation and sorting,[126] analogous to surface tension in fluids.[127] The shapes of individual cells within aggregates depend on their position within the aggregate, which specifies their cortical tension and degree of cell–cell adhesion.[128] However, several other factors change at the surfaces of patterned cell aggregates, thereby complicating the interpretation of behavior. Some of these factors are mechanical stresses due to traction forces,[129,130] cytokine gradients caused by uneven distribution of cells,[131] and differences in surface curvature. Often, these factors feed into each other, giving an extra layer of complexity which can, however, be elucidated by usage of more controlled patterning methods such as the use of microfluidics to precisely control cytokine gradients.[132]

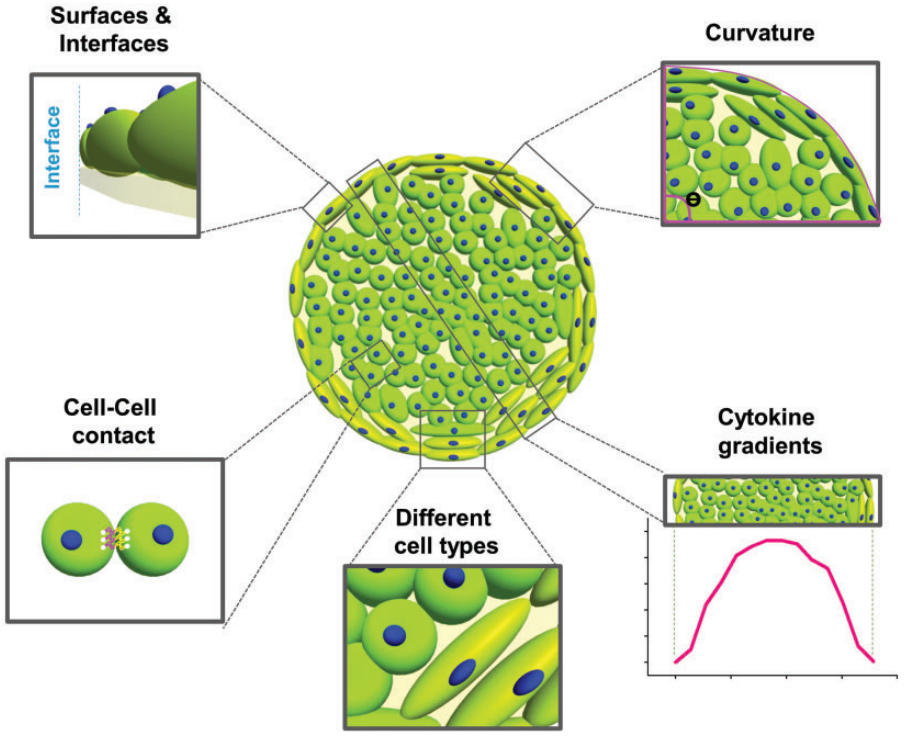

Figure 2 Interactions of multiple cells. Several factors are introduced when multiple cells are considered together including cell–cell contact, contact between different cell types, the introduction of interfaces and curvature, and cytokine gradients across the system. These factors control effects such as collective cell behavior and cell sorting, for example. (A color version of this figure is available in the online journal.)

In addition to deconstructing the influences of different factors in the microenvironment, engineered matrices that can simultaneously control multiple cues may be used to optimize desired outcomes. For instance, 3D printing techniques have been developed that can control matrix composition, topography, elasticity, and spatial organization of different cell types which have been used to print vascularized, multiple cell-laden constructs.[133]

Micropatterning techniques for immune engineering

With the complexity and various roles of the immune system, immune cells have evolved very sophisticated machinery to respond to different situations with suitable behavior. For example, macrophages have both pro-inflammatory and pro-healing, anti-inflammatory phenotypes with significant plasticity between them. These phenotypes are regulated according to both secreted factors and the physical environment, with dysregulation occurring in cancer and obesity, for example.[134] Understanding interactions of immune cells with biomaterials is crucial for understanding and controlling foreign body reactions for implants and is a major field of study.[135] Macrophage adhesion, activation, and fusion, contributing to fibrogenic reactions to foreign bodies, are dependent on culture environment,[136,137] such as stiffness[138] and cell shape[139] and may cause macrophages to remodel their ECM.[140] Furthermore, ECM effects have been studied in inflammation, wound healing, immunomodulation, and immune response to cancer.[141,142]

With the rise of immunoengineering, and the potential for controlling immune behavior across a host of processes, it is important to study, and make use of, the modulation of immune cells via ECM. There have been a few reports of the use of patterning strategies to modulate immune cells. Micropatterning of cell–cell junctions has been used to study cell interactions such as immunological synapses (IS), the junction between T lymphocytes (T-cells) and antigen presenting cells. Doh and Irvine[143] have shown, using micropatterning of T-cell receptors (TCR) and intercellular adhesion molecules in different structures, that T-cell assembly of ISs was strongly dependent on the unique physical structure of the synapse with stable interactions on focal spots of TCR ligands. More recent work by Tabdanov et al.[144] using similar methodology showed the effects of structure of ISs on the cytoskeletal mechanics of T-cells. Mossman et al.[145] have used nanopatterning techniques to constrain IS formation, elucidating a correlation between radial TCR position and signaling.[145] Adhesive protein micropatterns have been shown to affect fibrogenic activation of macrophages, with relevance to foreign body response.[146] Moreover, patterning of other cell components, such as lipid bilayers, can be used to further probe these systems.[147]

Single cell micropatterning has also been used with macrophages, white blood cells that perform phagocytosis. Patterning of cell shape was used by McWhorter et al.[139] to modulate the phenotype of macrophages between pro-inflammatory (M1) and pro-healing (M2) states by controlling elongation of single macrophages. Increase in cell

aspect ratio led to enhancement of M2-related cytokines and polarization through an actomyosin contractility dependent mechanism. More recently, micropatterning of macrophages was used to study the cytoskeletal effects of edema toxin[148] where reproducible control over the actin cytoskeleton was used to normalize cell response to toxin.

Outlook

There are several reasons to implement physiologically relevant physical and chemical properties for in vitro scaffolds including the ability to study cells in a more complex ''natural'' environment, the development of more representative models to supplement or replace animal models, and the development of tissue engineering constructs which can be implanted and interfaced with existing tissue.[17] Studies using systems with tunable properties such as stiffness, composition, and cell shape will reveal dramatic changes in cell behavior compared to standard culture dishes, often with the recurring theme of large changes at physiologically relevant matrix properties. However, studies with multiple cues often reflect a coupling between these different factors, complicating the establishment of parameter–function relationships. Going forward, developing platforms that can capture the complexity of the native ECM while also having the ability to quantitatively, precisely, and specifically tune matrix properties to deconstruct and control the effects of various cues, are crucial for in vitro study of cells, development of model systems and development of scaffolds for tissue engineering and regenerative medicine applications.

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DECLARATION OF CONFLICTING INTERESTS

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