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Advances in Bioactive Hydrogels to Probe and Direct Cell Fate

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Abstract

Advanced cell culture techniques are increasingly needed to better understand basic cell physiology, predict in vivo response, and engineer de novo functional tissue substitutes. Toward this concept, hydrogels have emerged as biomimetic in vitro culture systems that allow cells to be grown in or on user-defined microenvironments that recapitulate many critical aspects of native tissue. Hydrogel biofunctionality can be engineered predictably and precisely via the tailorability of the hydrogel's chemical and mechanical properties, each of which directly influences cell fate. In this review, we highlight state-of-the-art hydrogel platforms that have been used to assay and define cell behavior, placing an emphasis on recent directions in systems that offer dynamic control of material properties in time and space. We review current understanding of cell-material interactions in 2D and discuss recent and future efforts, as well as challenges, in extending this work to 3D. Ultimately, advances in hydrogel culture systems, synthetic approaches, and biological assays that can be performed in 3D are providing new opportunities to recapitulate fully the native cell niche.

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INTRODUCTION

Engraftment: incorporation of transplanted cells or tissue into the body of the host

Hydrogels: cross-linked networks that imbibe large quantities of water

4D: 3D spatial control in time

Spatiotemporal: controlled in time and space

Interest is growing in utilizing cell-based therapies in medicine. As far back as the fifteenth century, blood transfusions have been successful in reviving patients upon substantial fluid loss (1). In the modern day, bone marrow transplants are regularly used to treat patients affected by various types of cancers (e.g., leukemia, lymphoma) by restoring stem cells that were destroyed via radiation and/or chemotherapy (2). Additionally, the Edmunton protocol for islet transplantations has enabled patients who have severe problems in regulating their blood sugar with insulin to manage their diabetes through a cell-based therapy (3). From injection of stem cells into patient hearts after myocardial infarction to delivery of dopaminergic neurons to the brain to treat Parkinson's disease to use of human embryonic stem cell–derived oligodendrocytes to treat spinal cord injuries, >20,000 US Food and Drug Administration clinical trials are currently taking place that utilize cell-based therapies for patient treatment (**http://clinicaltrials.gov**).

Although the number of cell-based therapies continues to rise, many of these approaches are currently suboptimal, as only a few of the delivered cells survive the process, which yields engraftment rates of <10% and more typically 1–2% (4). As cells naturally reside in a 3D niche within the body, a major research effort lies in designing material carriers to increase cell survival upon implantation. Furthermore, as researchers seek to use different types of progenitor cell populations, new techniques must be developed to expand, grow, and direct them in culture, all of which necessitate new types of culture systems. Hydrogels have proven particularly beneficial in this regard, owing to their numerous similarities with the cells' native environment. Hydrogels have been successfully utilized as carriers during autologous chondrocyte implantation to repair and regenerate functional cartilage, to treat chronic wounds with tissue-engineered skin equivalents comprised of neonatal foreskin keratinocytes and dermal fibroblasts in collagen, and to deliver cardiac-derived stem cells to patients with severe refractory heart failure.

To further increase cell survival, integration, and engraftment as well as to accelerate and promote more functional tissue regeneration within these hydrogel carriers, recent efforts have focused on the development of bioactive scaffolds that better mimic the cell's in vivo surroundings and that actively promote user-defined cellular functionalities (e.g., proliferation, differentiation, matrix production) within these 3D constructs. Unfortunately, as researchers possess only a nascent understanding of the effects of cell-material interactions on physiology, a challenge lies in how best to design hydrogel carriers rationally for specific cell-based therapies and tissue engineering applications. By developing and utilizing programmable bioactive hydrogels that afford biophysical and biochemical tunability, cell response to individual user-defined cues can be fully elucidated. This knowledge can then be incorporated into next-generation biomaterials that will further increase the success of future cell-based therapies (**Figure 1**).

This contribution begins by reviewing the basic concepts in hydrogel structure-property relationships and presents the foundational and pioneering work utilizing hydrogels as 2D cell culture platforms. We then examine how knowledge ascertained from 2D studies has been translated into 3D culture systems, which cell functions may require a 3D environment to understand fully, and several challenges that are introduced in growing and assaying cells within a 3D environment. Finally, we highlight new directions in the development of tunable materials with 4D spatiotemporal control of the cell microenvironment and discuss how these techniques can provide insight into basic cell physiology as well as a better understanding of how cells receive and exchange information within their material niches. Ultimately, this knowledge provides feedback to researchers in the field for their quest to design better cell carrier systems for regenerative medicine and improved cell culture systems.

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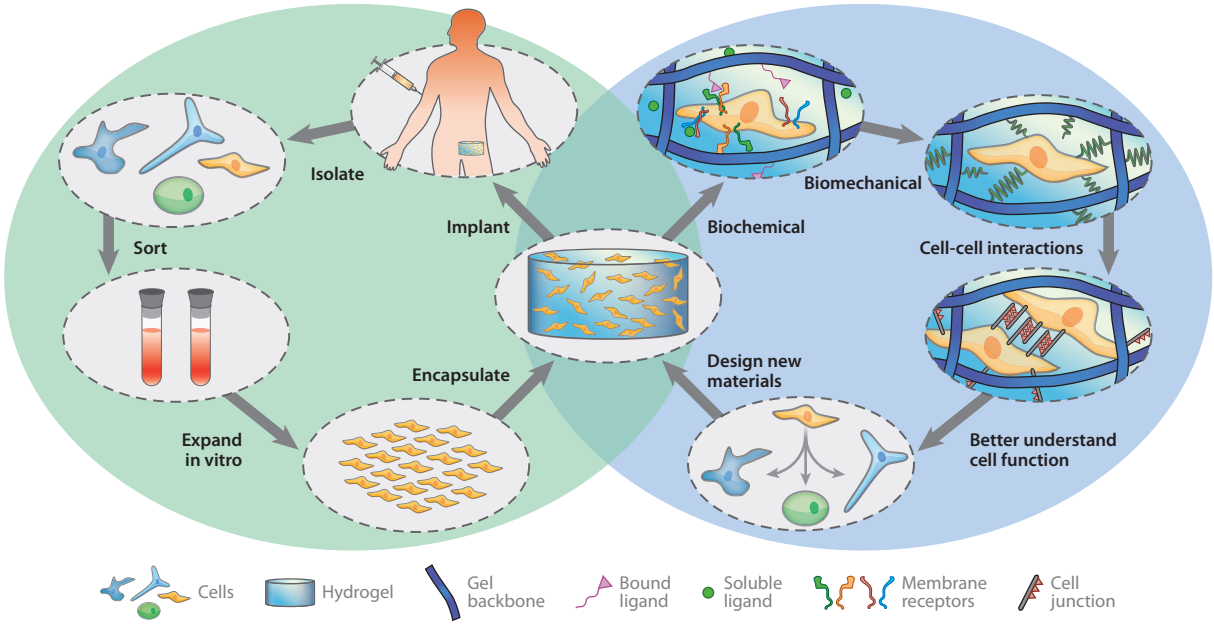

Figure 1

Bioactive programmable hydrogels, whose biochemical and biophysical properties can be tuned in time and space, offer user-defined control of the pericellular environment and are used to better understand cell response to specific cues within a native-like synthetic niche. Critical gel aspects derived from these 3D culture experiments are then incorporated into advanced biomaterial platforms to be used in conjunction with patient or donor-isolated cells to create autologous or allogeneic implantable tissue substitutes. Progenitor cells that are isolated from the patient also can be used for fundamental biological studies, thereby intertwining the methods to achieve basic cellular understanding and tissue engineering applications in a repeating cycle of rational biomaterial design.

HYDROGELS AS PLATFORMS FOR CELL CULTURE AND TISSUE ENGINEERING

Hydrogels represent an emerging and highly attractive class of biomaterials whose unique set of properties has recently expanded their use into in vitro cell culture platforms (5–8). Their high water content, tissue-like elasticity, and facile transport of nutrients and waste render them ideal candidates for mimics of the cell's extracellular matrix (ECM), whereas their optical clarity enables microscopy-based assays of cell function to be performed and quantified readily. Furthermore, many hydrogels can be formed under mild, cytocompatible conditions that enable cell encapsulation and are modified easily to contain user-defined chemical functionalities, mechanical properties, and degradability. Hydrogels can be synthesized from a variety of starting materials, including both naturally and synthetically derived polymer systems.

Naturally derived components are commonly employed in the synthesis of hydrogels for cell culture owing to their inherent bioactivity, biocompatibility, and biodegradability. Gels have been created from a wide variety of sources, including collagen (9, 10), fibrin (11–13), hyaluronic acid (14–17), dextran (18, 19), and Matrigel™ (20), through a variety of cross-linking methods (e.g., physical, covalent, ionic interactions) with varying degrees of success. As these biomolecules inherently contain cell-signaling attributes, cells generally thrive in these materials. Nevertheless, the same endogenous epitopes and cues that enable high viability and proliferation rates within these naturally derived materials convolute basic cell biology studies and make it challenging to assess the isolated effects of single cues on cell function. In addition, the numerous interactions

Extracellular matrix (ECM): the material that surrounds the cell in vivo

between the cells and the biomolecules, coupled with the batch-to-batch variability of many of these products, result in hydrogels whose physical and chemical properties are difficult to predict and engineer. As such, synthetic polymer-based hydrogels, in which the exact composition and chemistry of the cell microenvironment are known explicitly, have evolved and are becoming increasingly attractive as culture platforms to gain information on the effects of specific biochemical and/or biophysical signals on cell function.

Poly(ethylene glycol) (PEG): the most commonly employed polymer in synthetic hydrogels

Cross-linking density (ρ_x): number of physical or chemical cross-links in a given volume; dictates many key hydrogel properties

Synthetic hydrogels in regenerative medicine applications are typically formed by reacting bioinert molecules, typically monomers and polymers, to form a cross-linked network in the presence of cells. As the chemical makeup of these gels is defined precisely by the selection of network components, the resulting hydrogels form with consistent and predictable properties and are readily tunable to create systems with user-desired functionality. For example, numerous synthetic hydrogels for 3D cell culture are formed from polymer building blocks based on poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), and poly(*N*-isopropylacrylamide) (PNIPAm). PEG is often considered the gold standard for synthetic scaffolds owing to its high hydrophilicity and bioinert structure that is highly resistant to protein adsorption. As PEG-based hydrogels lack functional sites to interact directly with cells in any way other than physical support, they are generally considered a blank slate that will permit, but not promote, basic cell function. In addition, the hydroxyl end groups of PEG are easily modified with other chemical functionalities (e.g., acrylates, methacrylates, maleimides, thiols, azides) that can react with each other to form a 3D network (21–23). PVA contains the same chemically modifiable hydroxyl functionalities, which are pendant to the polymer backbone, and is also water soluble and biocompatible. Its structure enables a high level of functionalization, as each chain can be decorated with many pendant reactive groups, which gives rise to hydrogels with unique and complex structures (24–28). Hydrogels have also been formed with PNIPAm, a polymer whose backbone undergoes conformational refolding above and below 32°C, which enables systems whose physical properties can be modulated with temperature (29–31). A variety of other synthetic polymer-based systems are also being explored (32).

In addition to the chemical composition and the reaction mechanism that forms the hydrogel, a variety of other parameters are important in gel properties (**Figure 2**). The cross-linking density (ρ_x) refers to the number of elastically active chains that are present in a given volume of the material and directly influences material properties such as (*a*) the water content of the hydrogel, as measured by the volumetric swelling ratio (*Q*, the ratio of water-swollen gel volume to dry volume); (*b*) the mesh size of the network (ζ), which dictates the diffusivity of molecules through the network (*D*); and (*c*) the gel mechanics, including the gel shear modulus (*G*). Based on rubber

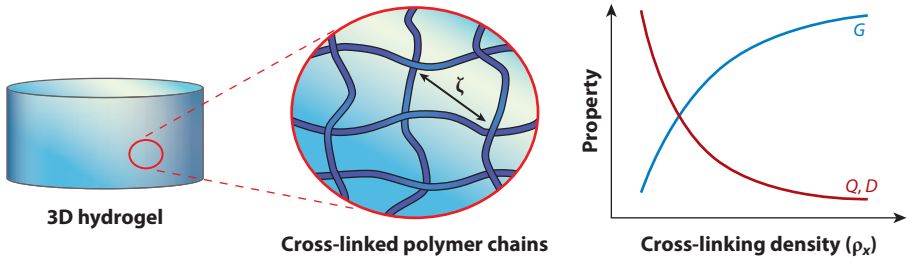

Figure 2

Many basic hydrogel properties, including gel mechanics and shear modulus (*G*), water content (*Q*), and diffusivity of molecules through the network (*D*), are directly related to the network mesh size (ζ) and cross-linking density (ρ_x).

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elasticity theory, for highly swollen networks these parameters can be related by (33):

$$G = RT\,\rho_x Q^{-\frac{1}{3}},$$

where R is the universal gas constant and T is the temperature. As G can be measured directly through network formation with rheometric techniques, the network cross-linking density can be calculated as the material evolves and subsequently degrades. Nevertheless, nearly every hydrogel property is related to the cross-linking density, so it is often difficult to create materials with desired mechanical properties defined a priori. From Flory-Rehner theory, for highly swollen materials, the swelling ratio scales as $Q \sim \rho_x^{-3/5}$, which implies that the shear modulus scales as $G \sim \rho_x^{6/5}$; Anseth et al. (34, 35) have confirmed this experimentally. Furthermore, Peppas et al. (36) described the relationship between molecule diffusivity within the hydrogel and network structure as:

$$D = D_0\left(1 - \frac{r_s}{\zeta}\right)e^{-Y\left(\frac{Q}{Q-1}\right)},$$

where D_0 is the diffusivity of the molecule in pure solvent, r_s is the hydrodynamic radius of the diffusing particle, and Y is the ratio of the critical volume required for translational movement of the encapsulated particle to the average free volume per molecule of solvent; it is commonly approximated as unity.

TWO-DIMENSIONAL HYDROGEL SUBSTRATES TO ASSAY CELL FUNCTION IN VITRO

As researchers seek to develop hydrogels that provide sufficient biochemical and biophysical cues to direct the function of encapsulated cells so as to engineer and recreate fully functional tissue, it is often arduous to know which specific biological cues are important in promoting a desired cellular response and therefore which cues should be included in vitro. This complexity stems, in part, from a fundamental difficulty in visualizing and quantifying the dynamically presented, spatially complex milieu of cues found in vivo. As such, researchers have turned to 2D cell culture platforms to better understand basic biological function on synthetic constructs whose microenvironmental properties can be tuned systematically and controlled explicitly. These flat substrates, most commonly rigid tissue culture polystyrene petri dishes but also soft hydrogel surfaces, are primarily responsible for our rudimentary understanding of cell behavior. These 2D substrates not only stimulate cell growth, which enables cultured cells to be kept alive and proliferating ex vivo for anywhere from days to decades, but also provide a simplified platform on which the effects of chemical functionality, ligand presentation, material stiffness, surface topography, and cell shape can be dictated precisely in a user-defined manner (**Figure 3**).

Effects of Chemical Ligand Composition on Two-Dimensional Cell Function

Much of the information that cells receive from their local microenvironment comes in the form of biochemical cues. Protein signals, both soluble and membrane-bound, bind to transmembrane receptors known as integrins that are located on the cell surface (37). When a cell first comes into contact with the ECM or another surface, physical and chemical associations between signaling ligands and surface receptors dictate the rate of cell attachment, spreading, migration, growth, and differentiation (38). Focal adhesions that serve as the mechanical linkage between the cell and the ECM are formed upon attachment and begin to promote the formation of cytoskeletal stress fibers and membrane protrusions including filopodia and lamellipodia (39, 40). Although focal

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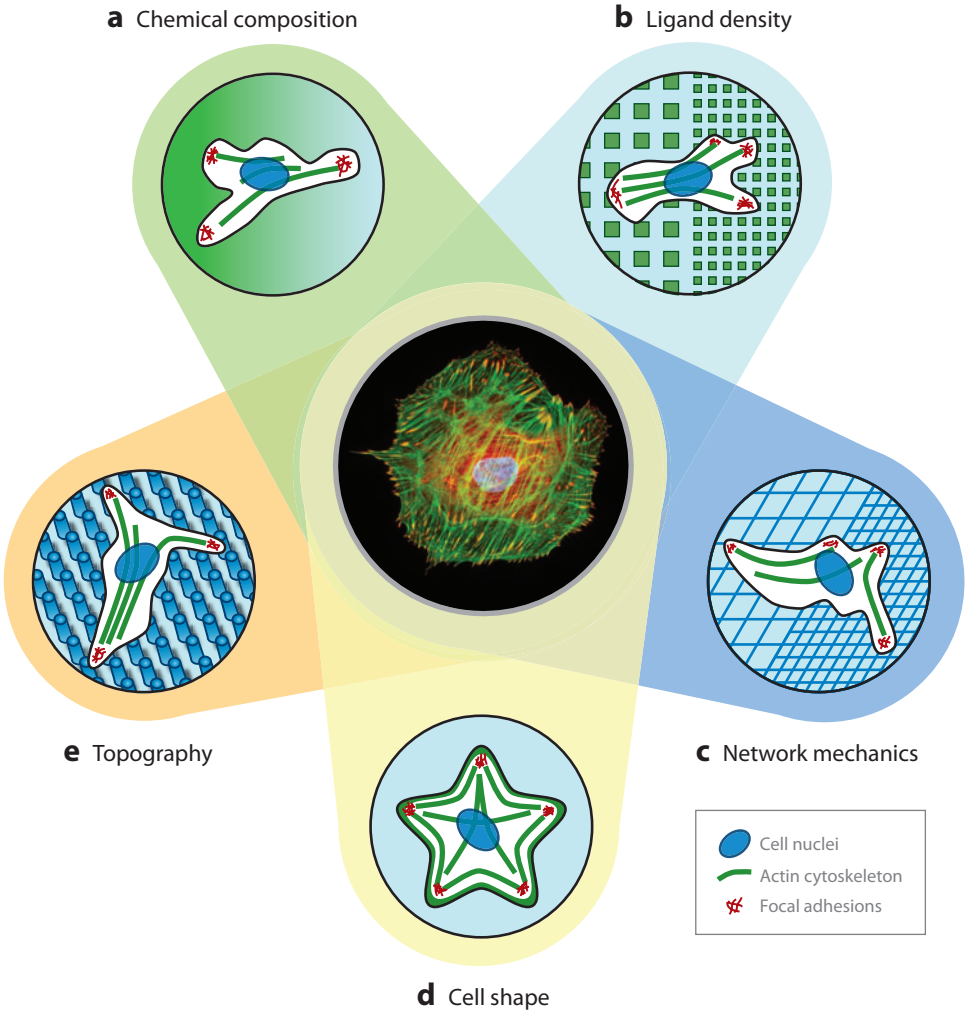

Figure 3

Cell function is dictated by changes in (*a*) chemical composition, (*b*) ligand density, (*c*) network mechanics, (*d*) shape imposed by the surrounding matrix, and (*e*) topography. Although techniques exist to control each of these aspects in 2D culture, an ongoing challenge lies in the development of material platforms that allow similar network tailorability in 3D.

adhesions can form between the cell and several different adhesive ligands, biomaterial scientists have routinely incorporated the arginine-glycine-aspartic acid (RGD) tripeptide into hydrogel materials to promote cell interactions. RGD is found within fibronectin, laminin, osteopontin, collagen, and many other adhesion proteins (41). RGD binds to a variety of heterodimeric integrin receptors, including $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (42, 43), and is commonly employed to impart cell adhesivity into otherwise inert biomaterial constructs (22, 44).

RGD: the cell-adhesive, protein-derived tripeptide sequence arginine-glycine-aspartic acid

To date, the major independent variable that researchers have investigated is the effect of the overall adhesion ligand concentration on basic cell functions (**Figure 3*a***). For example, cell morphology has been correlated with RGD concentration; cells tend to spread isotropically at higher surface ligand densities and randomly extend pseudopodia from the cell membrane when cultured

on surfaces with lower adhesivity (45). Additionally, although the strength of cell-stratum attachment increases linearly with surface chemical adhesivity, a biphasic relationship exists between the speed of cellular migration along a 2D substrate and the bulk RGD concentration (46, 47). Owing to their inability to attach initially, cells cultured on low RGD concentrations are unable to migrate along a synthetic surface, whereas those on highly adhesive constructs exhibit such strong interactions with the substrate that it is difficult for the cell to disrupt molecular bonds to support migration. Although they are cell-type dependent, maximal 2D migration rates typically occur at surface ligand densities of between 10^3 and 10^4 moieties μm^{-2}. In addition, the distance between integrin-binding sites and the total number of ligands clustered together in a fixed location govern migration and cytoskeletal organization. For example, when fibroblasts were seeded on PEG-based hydrogels containing 1, 5, or 9 RGD ligands per cluster, they exhibited increased adhesion, motility, and stress fiber formation at higher nanoscale RGD clustering (48, 49).

In addition to bulk concentration, the local spacing between individual chemical ligands is important in regulating cell function (**Figure 3*b***) (50, 51). When cells are cultured on materials containing regularly spaced RGD ligands, cell spreading is observed only when the distance between moieties is <70 nm, likely owing to the restriction of integrin clustering (52). For example, when culturing MC3T3-E1 cells on an alginate-based hydrogel, Mooney et al. (53) observed upregulation of osteoblast proliferation and differentiation when RGD ligand spacing was decreased from 78 to 36 nm while maintaining the same bulk ligand concentration. Additionally, cells have been cultured on regularly patterned arrays of ECM protein dots of defined size that are separated by regions of nonadhesivity generated using microcontact printing techniques (54, 55). Although cells on these substrata adhere to and spread on surfaces containing adhesive regions that are <5 μm apart, an island spacing of 5–25 μm induces the cell to adapt to the shape of the ECM pattern and to form stress fibers that align with the underlying patterned adhesion sites (56). When these ECM spots are spaced at a distance of >25 μm, cells are unable to spread beyond the boundaries of the individual ECM spot upon which they were seeded. Furthermore, cells will proactively spread and move in a highly predictable direction when cultured on surfaces comprised of linear islands, as opposed to those that are circular or square, which can be used to actively direct cell migration (57).

Mechanotransduction: conversion of mechanical signaling cues into chemical cellular activity

Human mesenchymal stem cells (hMSCs): can undergo adipogenesis, osteogenesis, or chondrogenesis to become fat, bone, or cartilage, respectively

Effects of Matrix Elasticity on Two-Dimensional Cell Function

In addition to chemical cues, mechanical signals are known to dictate local cell behavior, and appreciation is growing for the role of mechanotransduction during cell expansion and differentiation (**Figure 3*c***) (58). Almost universally, cells will adhere more strongly to stiffer materials compared with soft substrates (59). Once attached, they spread out along the surface, forming many actin-myosin stress fibers, and extend processes further when cultured on compliant materials (60–62). Additionally, cells migrate significantly faster on uniform stiff surfaces and undergo biased cell movement toward increasing stiffness when cultured on a gradient of rigidity (63). Lo et al. (63) showed that NIH 3T3 fibroblasts that were cultured on flexible collagen-coated polyacrylamide sheets previously exposed to a gradient of bis-acrylamide cross-linker to create a stiffness gradient exhibited a migratory preference for stiff substrates in a process called durotaxis.

In addition to dictating cell morphology and locomotion, matrix elasticity is sufficient to induce lineage-specific differentiation of progenitor cells. For example, in seminal studies by Discher and colleagues (64, 65), the differentiation of human mesenchymal stem cells (hMSCs) was found to be dependent on the 2D matrix elasticity of the collagen-coated polyacrylamide substrate on which they were cultured. hMSCs grown on moderately firm substrates ($E \sim 10$ kPa, similar to that of muscle) exhibited upregulated myogenic markers, whereas hMSCs proceeded down an osteogenic

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pathway when cultured on a stiffer substrate ($E \sim 35$ kPa, similar to that of bone). Interestingly, after hMSCs are committed down a specific pathway on the basis of matrix programming, they are less responsive to soluble induction factors that typically correspond to a different lineage. Additionally, Healy and colleagues (66) found that soft PEG-peptide-based materials ($E \sim 0.5$ kPa, near the physiological stiffness of brain tissue) promote neural differentiation of neuronal stem cells in serum-free media, whereas stiff gels ($E \sim 1$–10 kPa) promoted differentiation to glial cells; Leipzig & Shoichet (67) have reported similar findings. Moreover, skeletal muscle stem cells (MuSCs) exhibit self-renewal in vitro when cultured on soft PEG hydrogel substrates that mimic muscle elasticity and are able to regenerate functional muscle tissue upon implantation, abilities that are lost when MuSCs are cultured on rigid substrates such as tissue-cultured polystyrene (68). Finally, in a study by Anseth and colleagues (69), the dynamic alteration of culture platform elasticity from a stiff to a soft material via a photodegradable PEG hydrogel enabled activation and subsequent deactivation of porcine valvular interstitial cells into their myofibroblastic phenotype; this further demonstrates that the mechanical properties of a substrate are capable of directing cell fate but also illustrates for the first time that this can occur in a dynamic, reversible manner.

Effects of Two-Dimensional Cell Shape on Function

In addition to the biochemical and biophysical material effects on 2D biological function, the importance of cell shape on fate has been recognized and studied over the past decade (**Figure 3*d***) (70–72). In groundbreaking work by Ingber and colleagues (54), individual cells underwent switchable genetic programming in response to shape-dependent cues. Capillary endothelial cells were seeded onto micropatterned gold substrates containing islands of ECM-derived adhesive proteins and spread to mirror the shape of the underlying pattern. By varying the size and shape of the islands, cells became apoptotic when cultured on small islands (<10-μm $\times$ 10-μm squares) that prevented cell extension but proliferated on larger islands (>25-μm $\times$ 25-μm squares) that permitted spreading, regardless of the type of patterned adhesive ligand employed (fibronectin, collagen type I, vitronectin). Cells were able to survive and grow on regions comprised of many smaller adhesive islands separated by areas of nonadhesivity even though they contained the same total area of ECM contact that resulted in apoptosis for a single small island. In addition, micropatterned islands have been used to study the process by which cells bind to and acquire shape on a substrate (73) as well as cell polarity (74–77).

Commitment of stem cells to different lineages also can be regulated by confining their particular geometry. Chen and colleagues (78) recently demonstrated that hMSCs become osteogenic when allowed to spread on a large pattern (10,000-μm^2 squares) but become adipogenic when confined to small islands (1,024-μm^2 squares) where they were unable to spread. Increased hMSC spreading resulted in higher cytoskeletal tension and greater expression of RhoA and its downstream effector Rho-associated protein kinase (ROCK), both of which are involved in regulating cytoskeletal organization (79). Although island size can regulate stem cell fate, the actual island shape of a given size can as well. Mrksich and colleagues (80) demonstrated that hMSCs display different osteogenic and adipogenic profiles when cultured on different patterns (rectangles with increasing aspect ratio or rounded pentagons with different subcellular curvature) of the same area and in media containing competing soluble differentiation cues; increased island curvature was correlated with additional cytoskeletal contractility and an osteogenic fate. Cell shape also has been found to regulate the commitment of hMSCs to chondrocytes and myocytes (81), epidermal homeostasis (82), and epithelial-mesenchymal transition (83), and it continues to be an important factor in guiding stem cell fate.

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Effects of Substrate Topography on Two-Dimensional Cell Function

Another important biophysical aspect of the cellular microenvironment that is responsible for guiding cell function lies in the topography of the underlying substrate (**Figure 3*e***). As the native matrix is not flat over the size scale that a cell experiences, owing to the fibrillar structure of and void space within various ECM components, researchers have attempted to understand the biological effects of nano- and microscopic variations in surface structure that are overlooked by standard 2D culture (84, 85). By culturing cells on grates, pits, and posts, the effects of topographic geometries on cell attachment, alignment, migration, and differentiation have been characterized for a variety of cell types and feature sizes (86, 87). Nanopatterned structures give rise to anisotropic stresses within the cell, which in turn influence cytoskeletal organization, cell morphology, and function (88).

One of the most common types of synthetic surface topographies for regulating cell function are grates, in which parallel lines of defined nano and microscopic width are depressed from the otherwise flat surface by a given depth. When cells are seeded on these materials, they display increased adhesion rates and, once attached, will polarize and elongate with a cytoskeleton that is parallel to the grating axis as well as undergo biased migration along the features (89, 90). Additionally, a neuronal precursor cell line (PC12) underwent neurite extension parallel to groove topography (91). As cell morphology is altered, genetic expression is also changed, as fibroblast seeding on nanograte induces an increase in fibronectin mRNA transcription and stability (92) that can then give rise to cellular differentiation. hMSCs that were seeded on 350-nm-wide nanograte underwent neuronal transdifferentiation with and without retinoic acid, as marked by significant upregulation of microtubule-associated protein 2 (MAP2), that was unobserved on unpatterned and micropatterned substrates (93). The size threshold below which grate topography appears to have little influence on cell function is on the order of 100-nm wide and 100-nm deep (94).

Nanopitted materials, otherwise flat surfaces that contain small nonconnected depressions of defined width and depth, have also been extensively employed to influence cell function. Cells typically experience a reduction in cellular adhesion and a decrease in spreading but an increase in filopodia formation when cultured on substrates containing pits of diameters >35 nm (95, 96). Nanopits have also been used to control the differentiation of hMSCs to osteoblasts. In the seminal work by Dalby et al. (97), hMSCs were seeded on surfaces with 100-nm-deep, 120-nm-diameter pits with 300-nm center-center spacing arranged hexagonally, square, randomly, and in a square pattern with ± 50 nm random variation in both the x and y dimensions. Osteogenic differentiation was observed solely on the square and random square patterns, although a higher frequency of osteocalcin-positive cells was found on the slightly irregular substrates compared with the completely ordered and completely random materials. This work further demonstrated that cell function depends not only on the size and shape of topographical features but also on their arrangement, as <50-nm alterations in feature placement drastically influenced cell fate and function. In a separate study, the noninducing square patterns were used to prevent hMSC differentiation, which enabled long-term maintenance of stem cell phenotype, growth, and multipotency up to at least 8 weeks of culture (98).

Cells have also been cultured on nano- and microposts, needle-like structures that extend outward from the surface of the material. In general, when cells are seeded on posts, adhesion, proliferation, and cell size are all decreased, partially owing to the decreased substrate surface area that is accessible. These phenomena are more pronounced as structure height increases from 50 to 600 nm (89). Beyond directing fate, microposts have been developed and extensively used by Chen and colleagues (99) to study the traction forces that cells exert on their substrates. When cells are cultured on elastic cylindrical beams, the deflection that underlying posts exhibit upon

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cell contraction can be quantified readily and, using these measurements, traction forces can be calculated directly from a force-displacement relationship for pure bending (100). By varying the height of micromolded elastomeric microposts, substrates of different stiffnesses can be readily generated, with shorter posts giving rise to increased material compliance, and the effects of culture stiffness on hMSC differentiation assayed (101). This unique approach enables the effects of material rigidity on cell function to be assayed explicitly while holding other surface properties constant and without changing base material chemical composition or cross-linking. Additionally, magnetic platforms with cobalt nanowires trapped within the microposts enable the study of cellular reaction to applied forces (102). Step forces applied to the culture led to increased local focal adhesion size that was confined only to the site of application; cells on nearby nonmagnetic posts showed no change.

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HYDROGELS AS PLATFORMS FOR THREE-DIMENSIONAL CELL CULTURE AND TISSUE ENGINEERING

Although 2D cell culture has provided the field with many useful insights and valuable knowledge about how cells function in response to highly defined cues from their local surroundings, these culture platforms introduce an asymmetry that may be unlike the cells' in vivo environment. This inadvertent cell polarization can cause changes in biological function, which were first noted by Bissell and colleagues (103) in the form of human breast epithelial cells that behaved normally when grown in 3D but exhibited tumor-like characteristics in 2D culture. Furthermore, one might expect dramatic variations in cell motility in 2D versus 3D environments, as the latter confine the cell to a dense matrix that it must degrade or deform. This disparity in cell function based simply on the dimension of culture has led to a growing interest in designing and utilizing 3D constructs to better understand how cells sense and receive information from their 3D niche. Unfortunately, biological techniques originally developed for characterizing cells in 2D culture, including commonplace methods such as immunostaining and isolating proteins and DNA, are much more difficult to perform in 3D and often require reoptimization and longer timescales when translated into platforms in which cells are encapsulated. Furthermore, many of the fabrication methods used to create highly defined cell substrata are not readily translated into 3D, so unique approaches must be developed to tune the biochemical and biophysical properties of hydrogels in 3D.

Initial efforts focused on developing basic hydrogel material platforms that permit cell viability and provide basic structural support, which enabled researchers to culture cells in 3D for the first time. However, more recent endeavors have concentrated on the synthesis of advanced, functionalized gels that are capable of promoting specific cell functions of interest in a manner that allows complex spatiotemporal presentation of those promoting signals. In vivo, cells are suspended in a 3D matrix of signaling cues that are dictated by the constantly remodeling surrounding ECM. The structural integrity of the ECM, which is comprised of fibrillar proteins including collagen, fibronectin, and laminin, imparts mechanical cues to cell function as well as chemical signals in the form of binding epitopes and cleavage sites (104). By mimicking critical biochemical and biophysical aspects of the native ECM, bioactive matrices have been created that impart user-defined cues to encapsulated cells that can be used to study cell fate in response to physiologically relevant cell-material interactions and to engineer tissue-like structures.

Biochemical Tunability of Bulk Hydrogel Properties

As cells receive a variety of complex chemical signals from their local microenvironment, interest is growing in the development and utilization of biochemically functionalized polymer-based

hydrogels for 3D cell culture applications that promote specific cell functions (6, 7, 105). Perhaps the simplest method to impart this desired biofunctionality within hydrogels lies in the encapsulation of full proteins. When the hydrogel mesh size is smaller than the hydrodynamic radius of the protein, it will remain trapped within the 3D network and available to provide cues to encapsulated cells. Although full proteins can be trapped within hydrogels to elicit a specific interaction, peptides have emerged as an alternative synthetic route that allows facile introduction of biofunctionality, as covalently bound ligands, into hydrogels. Peptides are short chains of amino acid residues linked together by amide bonds. Each amino acid consists of an amine and a carboxylic acid as well as an α-carbon with an R group that can contain a variety of functionalities (e.g., amines, thiols, alcohols, acids). As peptides contain the same amino acid building blocks as proteins, they can evoke biological functions similar to those evoked by the full proteins whose structure they mimic. They are easily synthesized and purified, and peptides can be systematically engineered to include a variety of nonnatural amino acids, which ultimately enables a greater level of synthetic biological control than is possible with their complex biomacromolecular counterparts (106). Peptides have already enabled the incorporation of integrin-binding epitopes (e.g., the fibronectin-derived RGD sequence) (22, 27, 41, 107), enzymatically degradable sequences (e.g., the collagenase-sensitive sequence GPQG↓ILGQ) (108–110), growth-factor sequestering properties (e.g., the basic fibroblast growth factor–binding KRTGQYKL sequence) (111–113), as well as additional bioactivity (114–117) into otherwise bioinert hydrogel formulations, which has permitted cells to interact directly with their surrounding material.

Dynamic materials: constructs that offer nonstatic chemical and/or physical properties

Peptides are most commonly incorporated into hydrogels during the formation of the network itself. In this approach, peptides are synthesized and functionalized to include the same reactive substituents that are employed for network formation and are simply mixed at the desired concentration into the polymer precursor solution. Upon gelation, the peptide is homogenously distributed throughout the network and is able to signal encapsulated cells. Hern & Hubbell (107) exploited this technique to incorporate varying amounts of acryl-PEG-RGD, an acrylated version of the adhesive ligand RGD created by the coupling of monoacrylated PEG-*N*-hydroxysuccinimide to the N-terminal α-amino group of the peptide, into a gel formed by the radical chain photopolymerization of PEG diacrylate (PEGDA), an approach that many other researchers subsequently adopted (118–120). When cultured on these materials, osteoblasts exhibited a higher degree of spreading and cytoskeletal organization on gels as well as increased mineralization within gels containing additional RGD (22). Alternatively, a mixed-mode photopolymerization of acrylates in the presence of thiols enables cysteine-containing peptides to be incorporated directly into materials with no postsynthetic modification (121). In this system, the chain polymerization of acrylates is truncated with a step addition to a thiol, which gives rise to networks with unique structure. Finally, cysteine-containing peptides are also directly incorporated into hydrogels formed via the base-catalyzed Michael-type addition between electron-deficient alkenes (e.g., acrylates, vinyl sulfones) and thiols (122–124), or by the radical-mediated thiol-ene reaction (125). Although these approaches are fairly straightforward, the level of peptide incorporation directly influences the network structure and resulting gel mechanics, and the approach offers no spatiotemporal control over the gel's functionalization.

Dynamic Biochemical Tunability Within Hydrogels

Although the initial biochemical properties of synthetic hydrogels are readily controlled by the approaches outlined thus far, platforms that allow subsequent tunability of the presentation of these bioepitopes are particularly beneficial, as these dynamic materials enable cell function to be modulated in space and time as the experimenter sees fit. For example, the West lab (126–128)

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has exploited a classic photopolymerization technique that enables this level of dynamic control. Here, hydrogels were formed via the photoinitiated radical chain polymerization of PEGDA. Although the bulk of the acrylate functionalities are consumed during material formation, a small percentage of free reactive groups are present for subsequent conjugation to acrylate-containing monomers. Postgelation, acryl-PEG-RGD was swollen into the network with a small amount of photoinitiator, and subvolumes of the gel were selectively exposed to pulsed laser light, which locally induced chain polymerization and thus material functionalization. This technique enables multiple cues to be introduced post-gel formation and was used to guide human dermal fibroblast cell spreading within an enzymatically degradable gel.

Shoichet and colleagues (129, 130) have developed an alternative patterning approach that enables chemical immobilization in a more defined manner that does not modify the local gel mechanics. Here, an agarose gel was synthesized that contained photocaged thiols. In the presence of focused laser light, free thiols were liberated that spontaneously reacted with maleimide-functionalized RGD that was swollen into the gel. The unreacted peptides were swollen out of the gel to yield patterned channels downward from the surface of the gel. Dorsal root ganglia cells were seeded on top of these networks and were found to extend processes only into the adhesive biochemical domains of the gel. More recently, this approach was extended to pattern multiple full proteins within the same gel, which enabled migration of cells seeded on the surface into a prepatterned gel network (131). Although this approach does enable gel patterning without altering network mechanics, the cytotoxicity of maleimides ultimately precludes the use of this patterning scheme in the presence of cells.

Taking the lead from the growing philosophy of click chemistry, in which reactions proceed quickly and orthogonally to many common biological functionalities, Anseth and colleagues (132) synthesized idealized, step-growth networks on the basis of the strain-promoted azide-alkyne cycloaddition (SPAAC), the catalyst-free analog of the traditional copper-free variant. A photoreactive allyl ester pendant to the polymer backbone and uniformly distributed throughout the network was used as an anchor point to which proteins, peptides, and small molecules were patterned within the network with full spatiotemporal control via the photoinitiated thiol-ene photoconjugation reaction. Discrete patterns of multiple peptides deep within the gel and complex gradients along the material surface were generated with multiphoton-based and photolithographic techniques, respectively (132, 133) (see Photopatterning Within Hydrogel Networks sidebar). This patterning approach was fully cytocompatible, and localized patterning of RGD leads to volumetrically confined cell spreading within the gel. Additionally, by including a photodegradable moiety within the patterning peptide and initiating the thiol-ene reaction with visible light, introduced cues were subsequently removed upon UV light exposure, which demonstrated the potential for dynamic photoreversible biochemical patterning within hydrogel networks (134).

Dynamic Biophysical Control of Hydrogel Structure

In addition to chemical cues, mechanical signals are known to dictate local cell behavior, and appreciation is growing for the role of mechanotransduction during cell expansion and differentiation. As discussed previously, hMSCs undergo lineage-specific differentiation on the basis of the elasticity of the substrate on which they were cultured (64). Similar studies have been performed in 3D in which the matrix stiffness was found to induce adipogenesis and osteogenesis of hMSCs within compliant ($E \sim 2.5$ kPa) and stiff ($E \sim 110$ kPa) agarose gels, respectively (135). Although there is significant interest in understanding these mechanobiological phenomena and in

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PHOTOPATTERNING WITHIN HYDROGEL NETWORKS

As researchers seek to create biomaterial platforms that mimic the dynamic and heterogeneous nature of native ECM, light-based reactions are gaining popularity in the development of nonuniform, patterned culture systems. By utilizing photoinitiated reactions in the synthesis of and/or subsequent patterning of these constructs, the reaction can be controlled in time simply on the basis of when the light is turned on. Additionally, through a variety of techniques light can be delivered selectively to specific portions of the material to induce local changes, which enables full spatiotemporal reaction control. By projecting collimated masked light directly onto a sample, photolithographic techniques enable photoreactions to be confined to specific 3D volumes defined by a 2D mask. Alternatively, focused laser light provides full 3D control over in which hydrogel subvolumes a reaction of interest takes place. Although 3D patterning can be achieved using single-photon laser light, multiphoton-based approaches provide significantly better z-resolution than their single-photon counterparts and have ultimately enabled single-micrometer-scale patterning resolution in all three spatial dimensions. Though the effects of scattering and attenuation are nontrivial and can impose limits on light penetration depth and sample thickness, photochemical reactions continue to shine as a powerful tool in inhomogeneous biomaterial generation.

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elucidating the method by which physical forces in and on a cell contribute to its function, few 3D platforms enable these fundamental studies to be performed dynamically (136, 137).

As the biophysical parameters (e.g., stiffness, water content, molecule diffusivity) of a hydrogel are directly related to the cross-linking density of the network, efforts to control gel mechanical properties have centered on ways to alter the connectivity of the material dynamically. Some attempts have been made to synthesize 3D materials that stiffen with time. For example, by reacting thiolated hyaluronic acid with PEGDA via a slow Michael-type addition, the modulus of the material stiffened from $E \sim 1$ kPa to ~8 kPa over a period of 300 h; this process was designed to match the local stiffening of heart muscle cells during their development from mesoderm to adult myocardium, and it resulted in greater cardiomyocyte differentiation than in static culture (138). As an alternative to this preengineered strategy, West and colleagues (126) have used radical photopolymerized PEGDA hydrogels that have been formed with incomplete conversion and contain free acrylates for gel modification to obtain a user-defined increase in gel mechanics. A low-molecular-weight PEGDA and a small amount of photoinitiator were swollen into the network, and regions of the gel were selectively exposed to UV light to locally introduce additional cross-links within the material and alter the modulus from $E \sim 1$ kPa to ~3 kPa. The patterning also induced changes in the hydrogel transport properties, and fluorescently labeled dextran was imbibed only in the unpatterned regions of the gel. Similarly, materials containing a photosensitive amino acid, *p*-azidophenylalanine, were used to photocross-link artificial ECM proteins together; the resulting stiffness was directly related to total amount of light exposure (139). Additionally, hydrogels have been formed via a Michael addition between acrylate-functionalized hyaluronic acid and a reaction-limiting amount of bis(cysteine)-containing enzymatically degradable peptides (140, 141). Patterned regions of the remaining acrylates were cross-linked with masked UV light exposure to create a stiffer network. Although cells were able to attach to and spread in the base hydrogel, the increased density of nonenzymatically degradable cross-links in the patterned regions physically trapped the cells and prevented their migration through the material. This platform was used to pattern regions of spreading of encapsulated single cells and chick aortic arches as well as adipogenic/osteogenic hMSC fate by regulating cell shape within the materials.

Just as gels have been created that allow stiffening of the material in a preengineered or user-defined manner, a variety of systems have emerged that enable the gel to become more

compliant with or at a specific time. This has been achieved via the incorporation of degradable linkers within the polymer backbone that will fracture a cross-link when cleaved and thus alter the gel's mechanical properties. Hubbell and colleagues (21) introduced hydrolytically degradable gels in which macromers having a PEG central block were extended with oligo(DL-lactic acid) (PLA) or oligo(glycolic acid) (PGA) and terminated with acrylates. The PLA and PGA domains contained predictably hydrolyzable ester bonds, which liberate an acid and an alcohol, and the rate of network degradation was controlled between 1 day and 4 months on the basis of the number of PLA/PGA repeat units present. Statistical kinetic models of the degradation behavior of these materials have also been created (142–144), and similar methodologies have been applied to other networks (145). PLA-PEG-PLA gels have been used for the encapsulation of a variety of cell types [e.g., chondrocytes (146), osteoblasts (147), neural precursor cells (148)], with cell function correlated directly to degradation rates, and have demonstrated some of the benefits of nonstatic culture platforms. Regardless, the mass profiles of these platforms are fixed by their preengineered rates of degradation and cannot be altered post-gel fabrication.

By incorporating enzymatically degradable units into the network, the rate of material degradation is dictated locally by the amount of cell enzyme secretion and the susceptibility of the cross-linker sequence to cleavage (105, 109, 125, 149). Many peptide sequences have been incorporated into synthetic gels, each subject to different enzymatic activity and a wide range of degradation kinetics. The majority of these substrates have been susceptible to cleavage by collagenase (149), plasmin (119, 150), and/or matrix metalloproteinases (151, 152); a variety of sequences have been engineered to cleave upon treatment with a given enzyme. Unfortunately, most sequences have a degree of promiscuity and are not cleaved exclusively by a single enzyme (153). As cells are able to secrete the same enzymes responsible for network degradation, they can actively reform their surrounding microenvironment in a way that has enabled cell proliferation, migration, and differentiation over a timescale that is biologically dictated. In addition, enzyme has been delivered exogenously to these materials to degrade the network and liberate the cells (154). Although this approach does offer user-defined degradation, enzymes can have adverse effects on biological function and are often cost prohibitive for use in network degradation.

Light has been used to decrease the cross-linking density of the system to control network mechanical properties; it is advantageous, as degradation is triggered externally and mechanics are tuned in situ. Seliktar and colleagues (155) have used high-intensity pulsed laser light to photoablate physical channels in transparent hydrogels. With feature control on the micrometer scale, channels were created to guide neural outgrowth from a clot into a gel, which opens new avenues for the generation of guidance channels for treating nerve injuries. Although this technique can be applied to most biomaterials and patterned cell function by altering physical properties of the gel, the high light requirements are cytotoxic and prohibit patterning in the presence of cells. An alternative approach has focused on the synthesis of networks on the basis of the photoreversible dimerization of nitrocinnamate- or anthracene-functionalized polymers that are formed with 365-nm light and degraded with 254-nm light (156, 157). Unfortunately, full network degradation has not been demonstrated, and the cytotoxicity of 254-nm light limits its application to cell culture. More recently, attempts have been made to create photodegradable networks that undergo reverse gelation with cytocompatible conditions (158–163). For example, Anseth and colleagues (158) have designed linear PEG molecules that are flanked by photolabile acrylate moieties. This monomer was polymerized radically via a redox initiation scheme to create photodegradable hydrogels whose properties were tuned photochemically in the presence of cells. These gels have been used to influence migration (158), to create stiffness gradients throughout the material (164), and to study cytoskeletal reorganization of cells on 2D surfaces (165). Finally,

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disulfide cross-linked networks have been formed that degrade via a radical-mediated disulfide fragmentation reaction that can be controlled spatiotemporally (166).

Bioorthogonal: chemical reactions that occur in the presence of biological systems but without disrupting native biochemical processes

TECHNIQUES TO BETTER ELUCIDATE THREE-DIMENSIONAL CELL FUNCTION

Although a variety of synthetic platforms and patterning approaches have emerged that have enabled spatiotemporal control of either gel chemistry or gel degradation, independent control over both the biochemical and biophysical aspects of the material properties in 3D and in time has proved much more difficult and remains an active challenge within the field (**Figure 4**). This 4D control of hydrogel properties allows the dynamic tailorability of the cell's microenvironment and provides new tools to assay the synergistic effects of substrate rigidity and chemical makeup on cell function in a fully defined, synthetic niche. DeForest & Anseth (167) have made a preliminary effort in which three independent and bioorthogonal chemistries were used to synthesize and subsequently modify the chemical and mechanical aspects of a gel. By utilizing multiple wavelengths of light to initiate independently two photoreactions (i.e., the thiol-ene photocoupling and *o*-nitrobenzyl ether photodegradation), 4D control over the network functionality and architecture of a SPAAC-based hydrogel network was obtained. These reactions were used to create channels within the gel material and to functionalize portions of these channels selectively with RGD, thereby directing cell outgrowth within the gel through the combination of user-defined physical and chemical cues.

As researchers continue to develop novel cell-laden ECM mimetics that offer precise control over the cellular microenvironment, a growing challenge lies in the monitoring of encapsulated cell fate within these constructs. Successful techniques must be performed in aqueous medium that is dilute; at physiological temperatures, osmolarity, and atmosphere; and under sterile conditions. Owing to the optical clarity of hydrogels, microscopy-based techniques represent the most

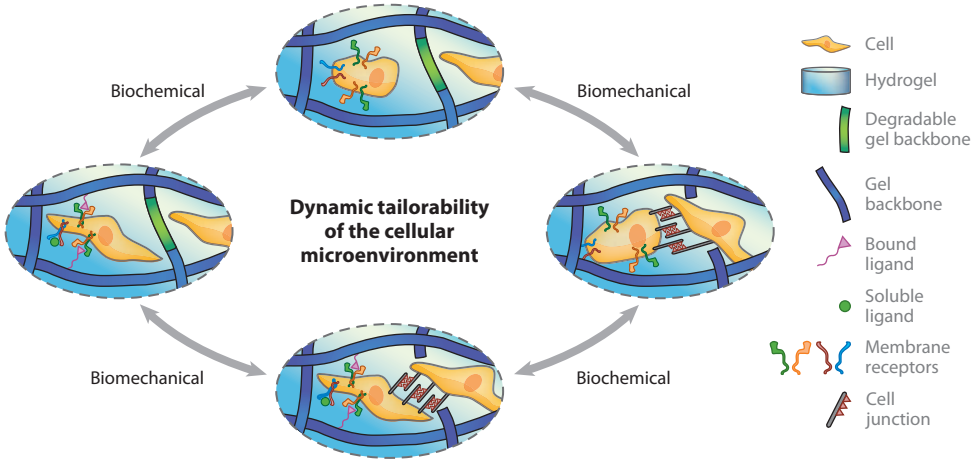

Figure 4

By utilizing multiple bioorthogonal chemistries, multifunctional synthetic biological hydrogels are created that offer independent control over the biochemical and biophysical aspects of material properties that dictate encapsulated cell function. An ongoing challenge lies in developing systems that enable reversible control of network structure and chemical presentation such that cues can be introduced systematically and subsequently removed in a manner that fully mimics the dynamic in vivo environment.

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common approach to monitoring cells within 3D constructs. Real-time tracking enables changes in cell morphology as well as migration speed and persistence to be quantified (68). Spinning disk confocal microscopy, which utilizes a series of moving pinholes to eliminate out-of-focus light, enables rapid performance of these tracking techniques in 3D and precise monitoring of cell position and shape within a material through time (168). One drawback of this technique is that cells must be fluorescently labeled, though this is readily achieved by immunolabeling (e.g., a primary antibody followed by amplification with a dyed secondary antibody), by genetic labeling (e.g., transfection), or with targeting proteins or fluorophores (169). The z-resolution and imaging depth for confocal-based techniques can be increased while minimizing light scattering with multiphoton-based approaches, in which absorbance of two or more photons of infrared light is required to excite and visualize a fluorophore (170). Although super-resolution techniques such as photoactivated localization microscopy and stochastic optical reconstruction microscopy continue to be developed and already provide resolution at 50 nm or better, most of these techniques have not been commonly applied to 3D systems (171, 172).

Photoactivated localization microscopy/stochastic optical reconstruction microscopy: super-resolution microscopy-based techniques that enable higher resolution that that defined by the diffraction limit of light

Beyond monitoring and tracking cellular functions in real time, additional techniques to quantify the effects of cells on their immediate surroundings and to determine how they are remodeling their extracellular environment are of growing interest. By entrapping fluorescent microparticles within a hydrogel material and monitoring their 3D position over time with confocal microscopy, movements in particle location can be used to calculate quantitatively the mechanical traction forces cells exert on their 3D surroundings (173, 174). Although these techniques provide unique insight into the distance over which a cell can feel its surroundings, they are extremely computationally intensive and cannot yet be performed in real time. Furthermore, the motion of encapsulated microparticles, owing to either Brownian movement or an external force (e.g., magnetism, optical tweezers), can be used to calculate the local gel cross-linking density and modulus via tracer particle microrheology (175). This technique provides a potential method to monitor quantitatively the network mechanics as encapsulated cells actively reform the material by simultaneously degrading the construct and replacing it with their own secreted matrix. Though qualitative, intramolecular fluorophore self-quenching techniques similar to Förster resonance energy transfer have been used to visualize collagenase activity and cell migration paths within hydrogels (117, 132). Although these techniques are each valuable in their own right, improved and complementary techniques are still needed to visualize and quantify cell-material interactions simultaneously and in real time.

CONCLUSIONS

Over the past few decades, research on the rational design of programmable and bioactive hydrogel systems has witnessed significant progress for 3D cell culture and tissue engineering applications. Through designing hydrogel systems that enable user-defined tailorability of the physical and chemical cues within the surrounding ECM, as highlighted here, researchers now have a better understanding of how cells function in response to temporal changes in the pericellular environment. Additionally, researchers are gaining a better understanding of the specific effects of material chemical composition, ligand density, network mechanics, surface topography, and cell shape on fundamental biological functions through newly developed techniques within these advanced systems. Such an increased understanding of fundamental cell response to the external environment may provide improved insight into the rational design of synthetic biomaterial scaffolds in applications ranging from the expansion and differentiation of stem cells to carriers for cell delivery and regenerative medicine.

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FUTURE ISSUES

1. Modular applications: As multiple cues are often necessary to elicit complex cellular functions, use of multiple chemistries in which each reaction is responsible for controlling a different aspect of the hydrogel environment will enable independent control of various biophysical and biochemical aspects in the presence of cells.
2. Bioorthogonal chemistries: The development of new and different synthetic reactions that enable precise control over when and where reactants are either joined together or fragmented apart will be crucial in altering material compositions.
3. Reversible functionalization: Systems that enable the introduction and subsequent removal of cell-directing cues will be imperative in recapitulating the dynamic in vivo environment and in directing tissue formation.
4. Effect of biomolecule structure: As patterning techniques further enable the introduction of various biochemical moieties, an important consideration will be their structure; cyclic, stapled, and multivalent peptides serve as crucial players in directing cell fate.
5. Chemical versus mechanical: Understanding the synergistic effects of chemical signaling moieties (soluble and immobilized) and network compliance will be vital in understanding and engineering biological function.
6. Full circle: By utilizing information gained from 2D and 3D culture studies to aid in the development of next-generation biomaterials that better mimic the native ECM, more efficient tissue-engineered products will benefit more patients with greater rates of success.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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5. Landmark review that helped to identify the scope of tissue engineering.

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