

The Art of Engineering Biomimetic Cellular Microenvironments

Ross C. Bretherton and Cole A. DeForest*

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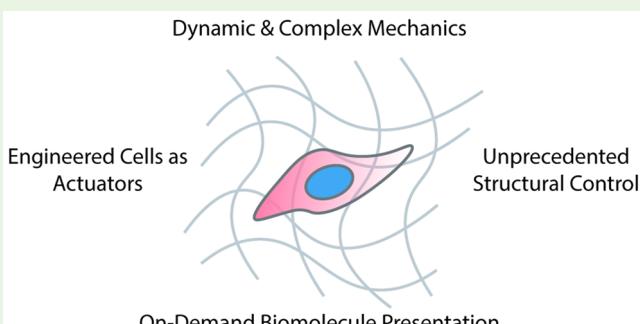
ABSTRACT: Cells and their surrounding microenvironment exist in dynamic reciprocity, where bidirectional feedback and feedforward crosstalk drives essential processes in development, homeostasis, and disease. With the ongoing explosion of customizable biomaterial innovation for dynamic cell culture, an ever-expanding suite of user-programmable scaffolds now exists to probe cell fate in response to spatiotemporally controlled biophysical and biochemical cues. Here, we highlight emerging trends in these efforts, emphasizing strategies that offer tunability over complex network mechanics, present biomolecular cues anisotropically, and harness cells as physiochemical actuators of the pericellular niche. Altogether, these material advances will lead to breakthroughs in our basic understanding of how cells interact with, integrate signals from, and influence their surrounding microenvironment.

KEYWORDS: *dynamic biomaterials, hydrogel, microenvironment, cell fate*

INTRODUCTION

It is now widely accepted that the extracellular matrix (ECM) evolves in space and time, harboring persistent recollections of past cellular states. These biological memories are most distinctly present as state-dependent cell-secreted proteins tethered to the ECM and anisotropic variations in matrix mechanics.^{1,2} Accompanying progression of many diseases, particularly those with a fibrotic element, the ECM binds an altered set of secreted growth factors and develops distinct mechanics from healthy tissue.^{3,4} With seminal studies highlighting the role of ECM-presented cues in driving significant changes in cell fate,⁵ researchers now appreciate why seeding healthy cells onto a diseased matrix is often sufficient to induce unhealthy cell phenotypes.⁶ Therefore, cells exist in dynamic reciprocity with their environment: extracellular cues alter cell behavior, and cells in turn shape their surroundings through secreted bioactive and structural proteins.⁷

Studies over the past many decades underscore the need to further decouple the ECM's role in guiding cell behavior throughout development, health, and disease. Engineered microenvironments can provide a user-defined platform in which to precisely tune individual aspects of the ECM to probe and direct encapsulated cell response, increasingly with four-dimensional (4D) and reversible control. To this end, the community has innovated and established a variety of modular hydrogel biomaterial designs that recapitulate critical complexities of the native cellular niche. Here, we highlight recent advances in the synthesis and manipulation of dynamic



biomaterials and discuss future strategies to mimic complex biological microenvironments *in vitro*.

ENGINEERING TISSUE MECHANICS BEYOND THE MODULUS

Tissue mechanics clearly play an important role in development and disease, and the varied mechanical properties of tissues cannot be fully captured by a single elastic modulus. Although most covalently cross-linked polymeric hydrogels are linearly elastic, whole tissues exhibit complex mechanical properties such as strain stiffening/softening and viscoelasticity.⁸ Though substrate stiffness is often seen as the classic mechanical parameter to characterize and manipulate in an engineered biomaterial, recent efforts have moved to decouple ECM elasticity, viscosity, and fiber thickness/architecture toward elucidating their individual roles on influencing cell function (Figure 1).

Underpinning the importance of complex mechanics when engineering cellular microenvironments is the understanding that cellular mechanosensation on and within soft materials is inherently dynamic.^{9,10} Cells adhere to the matrix through

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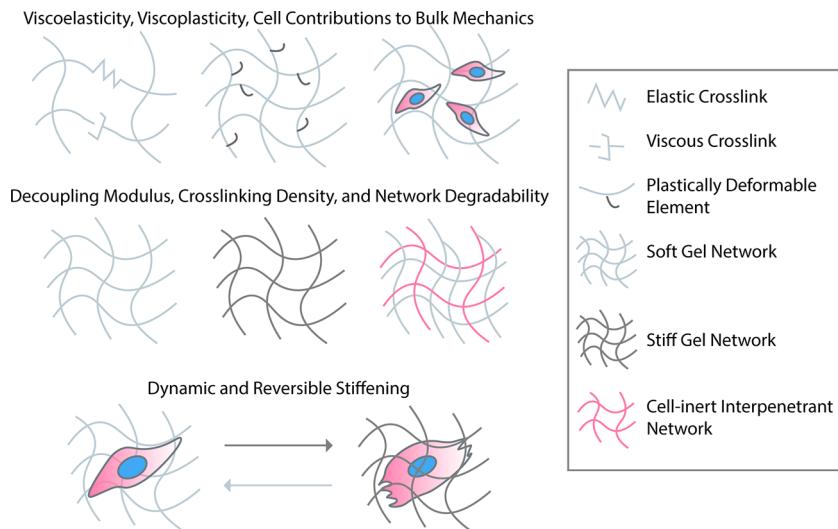


Figure 1. Mimicking complex mechanical aspects of microenvironmental signaling. With the use of modular and synthetic biomaterials, researchers have begun to elucidate the mechanical effects of ECM on cell function beyond its modulus. These properties include the time-dependent components of viscoelasticity and viscoplasticity, as well as dynamic changes to the modulus. Though material modulus and cross-link density are often used interchangeably in the biomaterials community, current efforts seek to decouple material cross-linking, stiffness, degradability, and fibral architecture so as to elucidate their independent effects on cell function.

membrane-bound integrins that are clustered into focal adhesions linking the actin cytoskeleton to the ECM. As cells exert spatiotemporally varied forces on their surroundings, time-dependent microenvironmental viscous behaviors complement substrate stiffness in establishing dynamic mechanical reciprocity between intracellular and extracellular tension.

Tuning Viscoelasticity and Viscoplasticity. Nondegradable synthetic polymer hydrogels exhibit dominantly elastic mechanics. This is in stark contrast with the varying stress relaxation responses of soft tissues, which can be on the order of several minutes.⁸ In addition, the viscoelastic properties of tissue have been reported to change throughout the course of disease; patients with cardiomyopathies exhibit increased cardiac muscle viscosity that further contributes to progressive diastolic dysfunction throughout the disease course.¹¹ Conversely, cancerous tissues will also stiffen, but with a lower degree of stress relaxation.¹² The viscous behavior of biomaterials has been tuned primarily independent from the storage modulus through encapsulation of non-cross-linked entrapped polymer elements. For example, linear polyacrylamide can be incorporated within a cross-linked polyacrylamide gel to endow this popular and linearly elastic biomaterial platform with tunable viscosity.¹³ Increasing polyacrylamide viscosity attenuated seeded hepatic myofibroblasts spreading, restoring hallmarks of quiescent hepatic stellate cell phenotype. By incorporating poly(ethylene glycol) (PEG) spacers into an alginate hydrogel, stress relaxation rates can be increased with faster relaxation that drives cell spreading.¹⁴ In hyaluronan, the introduction of noncovalent guest–host cross-links has been used to independently increase the loss modulus of the hydrogel with the same viscosity-dependent effect on cell spreading.¹⁵

In addition to the reversible elastic deformation of substrates, cells can also sense the plasticity or irreversible deformation of a biomaterial.^{16,17} Many natural biomaterials that are noncovalently cross-linked (e.g., gels based on collagen, fibrin, reconstituted basement membrane, agarose, alginate) exhibit some degree of time-dependent plasticity–viscoplasticity.¹⁶ Cells encapsulated in these types of materials

can plastically remodel their surroundings over time in a manner dependent on integrin-based cellular force transmission and the strength of material cross-links within the gel. Plasticity can be modulated in a cell adhesion-independent manner through interpenetrating networks of varied molecular weight alginate and ionic cross-linking embedded in a reconstituted basement membrane.¹⁷ In this system, highly plastic networks promoted the spreading and invasive behavior of cancer cells independent of matrix modulus or enzymatic degradability. The effects of material plasticity on cell function have also been explored by incorporating PEG spacer side chains into an alginate hydrogel that are either covalently tethered, dynamically bound and able to rearrange, or free sliding within the network.¹⁸ Increased plasticity had profound effects on the transcriptome of mesenchymal stem cells (MSCs) seeded on the gel, especially with respect to pathways regulating focal adhesion remodeling and cell spreading. Gels with intermediate substrate plasticity promoted optimal spreading of MSCs, whereas cell spreading on highly plastic gels could be improved by attenuating cell contraction with the myosin inhibitor blebbistatin.

Whereas many tissues stiffen with compressive strain and soften with extension or shear, natural polymeric hydrogels such as collagen or fibrin do the opposite. These findings highlight the often-overlooked contribution of cells to the overall stiffness and mechanical behaviors of a tissue. Especially at lower strains, the passive stiffness of cells and their cytoskeleton plays a dominant role in dictating tissue stiffness.¹⁹ As determined rheologically through progressive decellularization of otherwise intact tissues, cells also contribute to the compressive strain stiffening behavior through both passive stiffness and active contraction.¹⁹ These findings offer some explanation as to why many of the most successful hydrogel systems for engineering functional and interconnected constructs are far softer than the tissues they aim to recapitulate.

Independent Control over Gel Mechanics and Network Properties. Another interesting development in the space of engineered tissue mechanics has been the decoupling

of stiffness, fiber architecture, and cross-link density in cell-compatible hydrogels. In collagen gels, stiffening the microenvironment by increasing the collagen weight percentage was shown to decrease angiogenic sprouting but stiffening the microenvironment without increasing fiber density through nonenzymatic glycation does the opposite.²⁰ A similar study using pulmonary fibroblasts found that although cells cultured on stiffer gels were more prone to myofibroblastic activation, increased cross-linking density diminished such phenotypic change when cultured in three-dimensional (3D) materials.²¹ The authors were then able to supplement the hydrogel system with electrospun polymeric fibers in a manner that did not impact bulk storage modulus, demonstrating that increased fiber density promoted fibroblast proliferation and primed for activation.²¹ Together, these studies reveal the distinct and sometimes opposing effects of fiber density, cross-link density, and substrate stiffness in an engineered biomaterial—three parameters that are often taken for granted as interchangeable in the field. Network cross-link concentration and cell-degradability have also been decoupled in a hydrogel system in which elastin-like polypeptides with varying rates of proteolytic degradation were cross-linked by copper-free click reaction with a suite of nondegradable PEG macromers ranging from 2 to 8 arms.²² Both a low cross-link density and rapid cleavage kinetics were necessary to form functional endothelial networks from encapsulated brain microvascular cells.²²

Biomaterials with Dynamic and Reversible Mechanical Control. Temporal evolution of stiffness has been another expanding locus of dynamic biomaterial development. As disease pathophysiology is progressive and chronic, simply lifting cells from a substrate mimicking a healthy mechanical environment and placing them in a diseased environment may not be sufficient to recapitulate the gradual compensation of cells to their changing microenvironment. To overcome this barrier, dynamic materials whose cross-linking density can be altered *in situ* have been the subject of great interest from the field. Many hydrogels that irreversibly stiffen have already been developed, including those based on release of calcium for alginate cross-linking,²³ Michael-type addition,²⁴ radical polymerization,²⁵ photoinitiated thiol–ene reaction,²⁶ enzymatic cross-linking,^{27,28} and anthracene dimerization.²⁹ Similarly, bioorthogonal softening or material degradation can be accomplished through inclusion of a photodegradable moiety (e.g., *ortho*-nitrobenzyl, allyl sulfide, ruthenium complexes) within a cross-link,^{30–33} passive cross-linker hydrolysis,³⁴ or even enzymatic transpeptidation *in situ*.³⁵

With unidirectional control over substrate moduli well established, the field has since turned toward materials capable of reversible stiffening and softening.³⁶ One reversible stiffening cycle can be readily programmed into materials through progressive cross-linking with a subsequently cleavable cross-link, resulting in a system where unidirectional stiffening and softening are controlled orthogonally to one another.³⁷ When true reversibility is desired, the conformational change of a photoresponsive chemical group such as an azobenzene can be exploited for repeat cycling of hydrogel stiffness.³⁸ Through site-specific modification of both the N- and C-termini, full-length proteins may be incorporated as functional cross-links within a biomaterial. Because the end-to-end translational movement lengths associated with stimulus-sensitive proteins is typically much larger than that obtained by small molecules, systems utilizing photoactivatable proteins

can enable cyclic control over stiffness spanning a significantly larger dynamic range.^{39,40} Using the conformational change of the optogenetic protein pair light, oxygen, and voltage sensing domain 2 (LOV2)-Jα, our group developed one such gel that softens in response to cytocompatible blue light and rapidly recovers its native stiffness in the dark.³⁹ Intriguingly, fibroblast activation was enhanced within these gels when subjected to pulsatile stiffening relative to both persistently soft and stiff controls; these results suggest that cells are not only sensitive toward the static substrate stiffness, but the temporal element of stiffening as well.

Cross-linking gel materials with photoresponsive proteins containing an engineered phytochrome that reversibly dimerize upon red/near-IR light exposure have also provided a route to dynamic mechanical control.⁴⁰ In this work, human MSCs were seeded onto the gel and subjected to 24 h of mechanical priming in the soft configuration, followed by an additional day of either static or dynamic photoswitching of the material. Transcriptomic analysis of cells on the static and dynamic substrates revealed that material stiffness for the first 24 h after seeding was more important than any subsequent dynamic alterations to the modulus. Notably, transforming growth factor-β1 (TGF-β1) and yes-associated protein (YAP) pathways were influenced by mechanical priming and less sensitive to more recent modulus switching, underscoring their known roles underpinning longer-term cellular mechanical memory. In contrast, mitogen-activated protein kinase (MAPK) signaling was a key pathway distinguishing slow (160 min) and fast (10 min) cycling of the substrate modulus, supporting its role as a more acute downstream effector of cell mechanotransduction.

With recent efforts to characterize the effects of more complex mechanical and physical properties (e.g., viscoelasticity, material plasticity, strain softening/stiffening, network architecture) on cell fate and function, we anticipate that the field's next step will be to innovate strategies to reversibly control these material properties as has been done for substrate stiffness. Dynamic modulation of substrate viscoelasticity poses an interesting challenge, as properties such as stress relaxation are already time-dependent and reliant on noncovalent interactions within the hydrogel network.

■ CONTROLLING DYNAMIC PRESENTATION OF BIOACTIVE LIGANDS

Biological tissues are dynamic, not just mechanically, but also biochemically (Figure 2). Cells are exposed to tightly regulated cues in the form of secreted proteins and factors from other cells and the extracellular environment, which in turn influence cell phenotype. Uniform decoration of engineered microenvironment with small molecules, peptides, and whole proteins has become fairly straightforward; several chemical strategies are now in existence to covalently functionalize hydrogels with bioactive elements.^{41–46} For nonspecific tethering of proteins to a scaffold, custom and commercially available small molecules (e.g., activated esters) can be used to stochastically install functional handles for biomaterial tethering (e.g., azides, alkynes, maleimides, (meth)acrylates) onto a protein under gentle, aqueous conditions. For controlled tethering to a scaffold and minimal impact on protein activity, site-specific modification techniques including through the use of sortase,⁴² N-myristoyltransferase (NMT),⁴³ and the SpyCatcher/Tag pair⁴⁴ have proven useful. Further flexibility of chemical group placement within a protein can be achieved

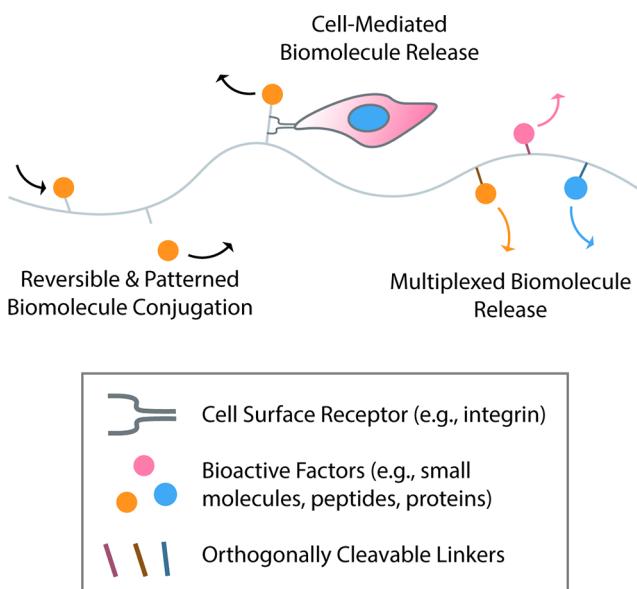


Figure 2. Recapitulating complex biochemical aspects of micro-environmental signaling. Exploiting bioorthogonal chemistries to immobilize or release biomolecules (e.g., small molecules, peptides, proteins) from biomaterials, reversible patterning of synthetic matrices may be achieved. New technologies such as traction-activated payloads (TrAPs) facilitate cell-mediated release of biomolecules from materials. With an ever-increasing suite of triggers for biomolecule release, the field is progressing toward systems capable of highly multiplexed triggers for on-demand and independent biomolecule release.

with noncanonical amino acid tagging and genetic code expansion.⁴⁵

User-Controlled Presentation of Bioactive Ligands. Heterogenous presentation of biochemical cues within hydrogels has most frequently been achieved using photopatterning, whereby directed light exposure is used to dictate when and where biomolecules are presented. Photomediated ligations, including those based on acrylates,^{47,48} thiol–ene,⁴⁹ oxime,⁵⁰ and enzymatic chemistries,⁵¹ have proven particularly useful for immobilizing small molecules, peptides, and even proteins into hydrogels. Photodegradation reactions, primarily based off of *ortho*-nitrobenzyl ester,⁵⁰ coumarin,⁵² or photocleavable proteins,⁵³ have found benefit for stimulating biomolecule release. These unidirectional material patterning approaches have enabled spatiotemporal control over proliferation, outgrowth, differentiation, and other complex cell fates within 3D gels.

Reversible biochemical control uniquely enables researchers to probe feedback loops between cells and their environment, which may be informative to identify tipping points in disease pathophysiology.³⁶ The first route to reversible payload tethering and release from biomaterial is simply combining an additive chemistry with an orthogonal subtractive one; this strategy has been successfully used to sequentially tether and release whole proteins to create complex and temporally evolving patterns capable of directing cell fate.^{50,54,55} However, this approach only allows one cycle of reversion, and current efforts seek to identify fully reversibly chemistries. One of the most promising approaches to date employs an allyl sulfide chain transfer, in which active radicals can help to trade one network-bound thiolated biomolecule for another.⁵⁶ While this reversible chemistry offers some repeatability, nonspecific

reactions associated with free-radicals limit cyclability and may be undesirable in the presence of cells. The reversible association of protein binding pairs has also been exploited through the optogenetic LOV2 Trap And Release of Protein (LOVTRAP) system in hydrogels, which also enabled repetitive cycles of protein patterning and release.⁵⁷ Although fully reversible, this strategy relies on noncovalent protein association with the gel that is comparatively unstable. Ongoing efforts seek to identify truly reversible and covalent strategies for biomolecule patterning.

Cell-Dictated Release of Bioactive Factors. Beyond user-directed ligand presentation, an emergent line of materials development focuses on systems that present biochemical cues in a cell-directed manner. The extracellular matrix acts not just as a structural scaffold but also as a reservoir for sequestered growth factors that become available to the cell upon matrix strain or remodeling.^{58,59} One well-characterized example is the sequestration of TGF- β 1 in the form of a large latent complex in the ECM, which when activated by strain or ECM degradation, causes fibroblast activation in the initiation of tissue repair.⁶⁰ Disrupting this sequestration capacity leads to dysregulated TGF- β 1 signaling, in turn causing developmental defects, cardiac disease, and cancers.⁶¹ The context of growth factor presentation is also incredibly important; when cells release sequestered growth factors from the ECM, receptor clustering with integrins can alter the nature of downstream intracellular signaling cascades. Therefore, mimicking cell-mediated release of bioactive factors from the ECM has been of great interest both for researchers wishing to deliver these factors therapeutically, as well as for those studying growth factor signaling through disease.

Growth factor sequestration within an engineered matrix for subsequent cell-mediated release can be accomplished through the inclusion of natural ECM components with growth factor affinity (e.g., fibronectin, heparin) or engineered components (e.g., antibodies, binding peptides).⁵⁸ More recently, synthetic aptamers have been designed to bind growth factors with high affinity and tether them to the matrix.⁶² By conjugating a cell-adhesive peptide to the free end of the aptamer, growth factors may be released through cell-mediated traction forces. The aptamer design of these Traction-Activated Payloads (TrAPs) could theoretically be modified to accommodate nearly any protein payload, while cell-selective release can also be achieved by modifying the cell-adhesive ligand through which the TrAP unravels. TrAP delivery of platelet-derived growth factor-BB (PDGF-BB) promoted denser cell growth in serum-free conditions that persisted for 2 weeks, suggesting that the growth factor could be stabilized by the aptamer in a manner similar to that of native ECM sequestration.

One of the most important aspects in designing a dynamic microenvironment for cells is the selection of an exogenous or cell-mediated trigger to induce material dynamics.⁶³ The number of triggers available to a researcher is ever-increasing and now includes bioorthogonal mechanisms including remote fields (e.g., light, ultrasound, magnetism)⁶⁴ and engineered enzymes.³⁵ Utilization of many of these exogenous triggers frequently requires specialty chemistries that are synthetically inaccessible and are limited in their capacity for multiplexing. Technologies for gene editing, specifically clustered regularly interspaced short palindromic repeats (CRISPR)/Cas, are now easily adaptable to target nearly any DNA sequence of interest, allowing researchers to quickly customize the DNA sequence that Cas targets by changing the sequence of the guide RNA.

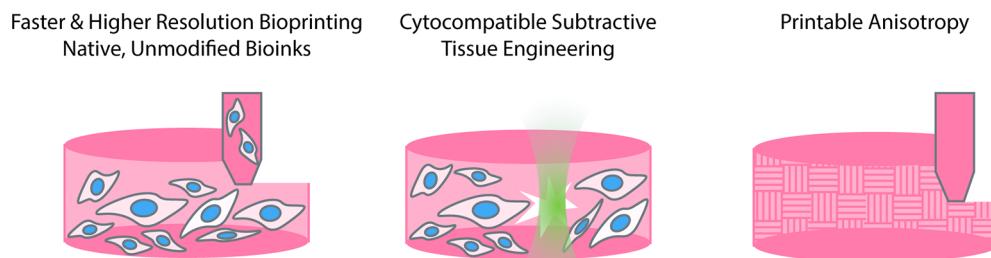


Figure 3. Advances in engineering complex tissue structures. Recent efforts in bioprinting have boosted resolution and speed, yielding faster, parallelizable, and more generalizable techniques for additive tissue construction. Photolabile hydrogel cross-links now permit creation of intricate voids and microvascular structures through subtractive engineering. Bioprinting dynamic materials with switchable anisotropy has also opened the door to local control over material microstructure.

This complete customizability is highly desired for programming the stimulus response of a biomaterial, and for this reason the CRISPR/Cas12a system has been recently adapted to the materials space.⁶⁵ Cas12a specifically recognizes a double-stranded DNA (dsDNA) trigger but upon sequence recognition collaterally cleaves nearby single-stranded DNA (ssDNA) nonspecifically.⁶⁵ By incorporating a payload tethered to a hydrogel backbone by ssDNA or incorporating ssDNA into the material cross-link, stimulus-responsive biomolecule release or bulk material degradation was induced upon introduction of a specific dsDNA trigger. By customizing the guide RNA and dsDNA trigger sequences, this strategy is easily adapted to any oligonucleotide trigger; because the mechanism for degradation is the nonspecific collateral cleavage of ssDNA downstream to dsDNA recognition, payload release is not entirely specific. Exploring material dynamics reliant only on specific dsDNA cleavage could open the door to near limitless multiplexing potential based on distinct target sequences and gRNAs.

FABRICATING COMPLEX TISSUE STRUCTURES

The heterogeneous and hierarchical structures of biological tissues provide both an inspiration and a challenge to engineers looking to recapitulate structurally facilitated tissue functions. The human body is full of branching structures scaling many orders of magnitude in size for gas, liquid, and nutrient transport, anisotropic tissues optimized for the generation of force, and gradients of stiffness or biochemical factors. Appropriate structures are often necessary for the function of bioengineered tissues; as one example, engineered heart muscles must align to mature and generate appreciable force, and large tissues are limited by nutrient diffusion and require perfusable vasculature. Recent advances in spatially controlling material structural properties have opened the door to tissues with greater functionality (Figure 3).

Additive Manufacturing of Engineered Tissues. Additive manufacturing of complex architectures with native and specialty biomaterials has hit maturity, and several advances to the field over the past few years now enable facile printing of exquisitely complex geometries. Specialty bioinks are no longer required to print high-resolution structures—with the development of freeform reversible embedding of suspended hydrogels (FRESH), native collagen can be sculpted down to a resolution of 20 μm through extrusion into a buffered gelatin slurry that is washed away upon incubation at 37 °C.⁶⁶ This technique has enabled perfusable vessels, a contractile ventricular model, and an at-scale heart to be printed using unmodified collagen.^{66,67} Though extrusion is

perhaps the simplest method for bioprinting, stereolithographic photopolymerization of materials enables highly parallelized fabrication of structures. Although this approach is much faster and offers fewer architectural constraints, cytotoxic photoinitiators/photoabsorbers have limited its use for bioprinting.⁶⁸ Recently, the cytocompatible food dye tartrazine was identified as an alternative photoabsorber for stereolithography, leading to the development of a technique termed stereolithography apparatus for tissue engineering (SLATE). By minimizing unwanted out-of-plane photopolymerization, SLATE has proven useful for the fabrication of complex and interlocking void spaces within hydrogels to recapitulate vascular geometries, and can be used to rapidly construct engineered tissues containing a wide variety of cell types, including primary human stem cells.⁶⁹

While stereolithographic polymerization of tissues enables precise and parallelized deposition of voxels of a single material by layer, it does not readily enable voxel-by-voxel control of material composition. To this end, multimaterial multinozzle arrays have been reported, capable of switching between up to eight materials at a frequency of up to 50 Hz at the nozzle head level.⁷⁰ Applied to bioprinting, this approach could provide unprecedented control over the composition of a biomaterial in 3D, with the benefits of parallelized material deposition. Open-microfluidic well-plate inserts can be exploited to sculpt gel precursors by capillary action, facilitating controlled deposition of 3D structures with nearly any hydrogel chemistry to further expand the palette of bioinks that can be 3D printed, eliminating requirements of photopolymerization and material extrudability.⁷¹

Another limitation of conventional stereolithography for additive manufacturing is its resolution (typically tens to hundreds of micrometers), which may not be small enough for applications exploring subcellular patterning of topographical and biological cues.⁷² An alternative means for additive manufacturing is multiphoton polymerization, a versatile strategy in which a femtosecond-pulsed laser can initiate polymerization on a voxel-by-voxel basis, enabling a much finer resolution (hundreds of nanometers).⁷³ Many of the conventional bioinks used for stereolithography also have multiphoton absorbance, and thus can be readily utilized with this technique. Though these methods offer unmatched spatial patterning resolution, one critical limitation lies in fabrication speed; as polymerization occurs one voxel at a time, these techniques are largely reserved for small-featured structures.⁷⁴

Just as the biomaterials field has successfully coopted multiphoton laser scanning from the photonics community for additive manufacturing, light sheet microscopy is now also

poised for adoption to rapidly generate relatively high-resolution structures. In light sheet microscopy, the sample is illuminated through one axis, whereas its fluorescence signal is detected through one perpendicular, enabling very fast scanning of large volumes that would be prohibitive to image by conventional laser scanning microscopy.⁷⁵ One early and very recent adaptation of this technique for additive manufacturing, xolography, polymerizes structures by illuminating the resin with a projected image and an intersecting light sheet at different wavelengths and along orthogonal axes.⁷⁶ Photopolymerization is achieved in a dual-color photoinitiator (DCPI) system, in which the activity of a benzophenone type II photoinitiator is optically regulated with an ultraviolet light-responsive spiropyran photoswitch. This approach improves upon the resolution of stereolithography by an order of magnitude, while generating large-scale objects at 4–5 orders of magnitude faster than multiphoton lithography. For biological applications, we anticipate that the first-generation DCPI will be limited by cytotoxicity and carcinogenicity, similar to its benzophenone precursor, but look forward to the development of biocompatible initiators for this uniquely enabling additive manufacturing technique.⁷⁷

Subtractive Manufacturing of Engineered Tissues.

Subtractive manufacturing, whereby patterned removal of a subset of bulk starting material, has also found utility for tissue fabrication; micrometer-scale resolution over complex void volumes have made these the strategy of choice for creating well-defined microvascular networks. Utilizing high-intensity femtosecond-pulsed lasers to induce nonspecific photoablation of hydrogel materials (e.g., PEG, collagen), early efforts demonstrated the feasibility of laser-based subtractive manufacturing as a technique to generate perfusable microvascular arrays within a biomaterial.^{78,79} Though nonspecific photoablation can be used to fabricate vessels down to the size of a human capillary,⁸⁰ the process is slow and requires high illumination intensities which are generally not cytocompatible. This process can be sped up dramatically through employment of photolabile moieties within the gel backbone, enabling material degradation and capillary-sized vessel patterning in the presence of living cells.^{30,81} Further improvements have been made by employing small molecule photosensitizers in conjunction with degradable gels.⁸² Despite these successes, there remains substantial room for improvements on the speed and throughput of subtractive tissue engineering, calling for the same ingenuity which catalyzed the additive manufacturing improvements reviewed above.

Engineered Control of Tissue Anisotropy. The microstructural alignment, or anisotropy, of an engineered biomaterial can impact a variety of tissue and cellular responses, including neurite outgrowth along aligned surfaces, fibroblast activation following myocardial infarction, and the coordinated contraction of muscle for force generation.^{83–85} Anisotropic biomaterials are traditionally fabricated through electrospinning or by directional freezing prior to lyophilization.^{86,87} Alternatively, as collagen in solution has negative diamagnetic anisotropy, aligned collagen hydrogels can be formed under a supermagnetic field.⁸³ Until recently, however, the magnetic alignment of collagen gels could be accomplished only in bulk and was limited by the specialized instrumentation required.⁸⁸

Even more recently, local spatial control of material anisotropy has been achieved on a bioprinter with a regular magnet by embedding streptavidin-conjugated iron nano-

particles into a collagen/agarose bioink such that magnetic field-induced particle movement during gelation promoted collagen fiber alignment.⁸⁹ By equipping the 3D bioprinter with a magnet, anisotropy could be induced upon ink deposition through pulsed magnetic fields.⁸⁹

In another powerful approach complementary to directed material alterations, tissue anisotropy can be introduced directly through acoustic patterning of cells.⁹⁰ In this technique, cells are forced into the pressure nodes of a standing ultrasound wave as a hydrogel is polymerized around them, thus generating repeating lines or points of cells within the gel as the basis for microstructural anisotropy. This approach has been successfully employed to pattern myoblasts into aligned engineered muscle.

Cell-Guided Construction of Engineered Tissues. All of the above fabrication techniques offer a high degree of user control over guiding biological structure. Yet, complex biological structures may also be fabricated by offering control over self-assembly to encapsulated cells and providing minimal cues to guide self-emergent tissue architecture. This approach to tissue construction comes from the philosophy of organoid biology, which harnesses stem-cell aggregation and self-guided differentiation into complex biological structures.⁹¹ The same cells or cellular aggregates used to form organoids can also be extruded through a syringe at high density and allowed to self-aggregate in a workflow termed bioprinting-assisted tissue emergence (BATE) to take organoid biology beyond the millimeter scale.⁹² This approach derives all complexity from organoid self-assembly, and as a result requires no specialized equipment for the syringe extrusion printing. The future of bioprinting holds larger and even more complex structures than ever, yet these advances may very well be delivered in simple and streamlined workflows.

■ ENGINEERING CELLS AS MECHANICAL AND BIOCHEMICAL ACTUATORS OF THE MICROENVIRONMENT

Genetic engineering and synthetic biological approaches have become more accessible than ever, leading to several recent studies that use engineered cells themselves to pattern phenotype and the microenvironment (Figure 4). Both biochemical and mechanical actuation through synthetic gene circuits have been explored to spatially control tissue structure and function.

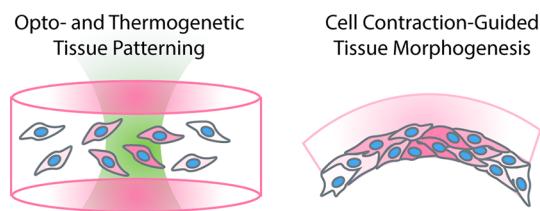


Figure 4. Employing cells as direct biochemical and mechanical actuators of the microenvironment. Borrowing tools from optogenetics, genetic engineering, and synthetic biology, an expanded toolbox exists to direct cell function within engineered materials. Opto- and thermogenetic cell patterning approaches provide a powerful route to directly modulate cell state within a material. Cells may also be used to guide macroscale tissue structure through controlled contraction.

The most extensive suite of remote triggers for cells may be borrowed from the field of optogenetics, which focuses on the use of light to precisely direct gene regulation and cell function. Optogenetic triggers, which come in the form of proteins that may dimerize, conformationally change, aggregate, or open/close a channel, have been developed to manipulate nearly every level of biological signaling.⁹³ Though we have already introduced studies which borrow optogenetic proteins to impart dynamic biomaterial properties, this rich toolkit can also afford control over cell adhesion to the matrix and neighboring cells,^{94,95} migration,⁹⁶ protein expression,^{97–99} and ion flux,¹⁰⁰ all of which could be used to control the extracellular microenvironment of an engineered tissue from the inside out. Paralleling development of orthogonal biomaterial triggers, wavelength-specific optogenetic triggers have also been developed and enable multiplexed control over cell function.^{101,102} Optogenetic control over cell migration can be combined with light-sensitive material chemistries.¹⁰³ For example, stem cells transfected with a photoactivatable Rac kinase were rendered susceptible to migration in the direction of a 458 nm light pulse, whereas hydrogel channels could be dynamically ablated through cleavage of an *ortho*-nitrobenzyl moiety as a PEG hydrogel cross-link.¹⁰³ Simultaneously controlling cell behavior from inside-out and outside-in offers unique opportunities to engineer the dynamic reciprocity between cell and environment that can be ubiquitously found throughout development and disease.

Using the human Heat Shock Protein Family A Member 7 (HSPA7) promoter, several past studies have successfully hijacked the heat stress response to induce transgene expression in response to mild heating.^{104,105} Recent work adopts this strategy for tissue engineering, employing perfusable channels through which hot fluid may be pumped, thus creating well-defined temperature profiles throughout the material that governs patterned gene expression of encapsulated cells.¹⁰⁶ This method was then used to induce spatial expression of liver enzymes through patterned expression of a Wnt signaling regulator.¹⁰⁶

Most biological tissues are not uniform slabs, and instead exhibit complex, curved geometries that spontaneously emerge throughout development and dictate tissue-specific functions. Just as bulk mechanical properties of a tissue can influence cell state, local topological and mechanical cues also dictate the behavior of tissues and the cells within them.^{107,108} Both as a means of replicating developmental emergence of tissue shape and as a strategy for creating appropriately shaped tissues for regenerative therapies, the actuation of cell contraction has been harnessed as a mechanism for generating curved structures from biomaterials.^{109,110} This can be accomplished by either patterning a contractile cell type or modulating that cell's ability to compact zones of a material. DNA-programmed assembly of cells has been used to localize seeding of mesenchymal cells onto collagen hydrogels and rationally induce folding of these gels into a user-specified shape by cellular contraction.¹⁰⁹ Through entirely synthetic cell patterning, this technique was used to recapitulate the incredibly complex tessellated curvature of embryonic chick gut lumen. During the dynamic folding process, actively contractile mesenchymal cells were found to be able to guide the migration of "passenger" vascular endothelial cells into the nascent folds. Alternatively, by spatially controlling the incorporation of a peptide that inhibits cell contraction in an evenly seeded gel, flat hydrogels can be made curved by

inducing regions of high contraction by cells in the material.¹¹⁰ These curved gels were fabricated to recreate the geometry of the human cornea and were able to guide the differentiation pattern of human epithelial stem cells into corneal epithelium. Though in this case contraction was indirectly induced by providing fetal bovine serum in the medium, logical "AND" gate cross-linkers susceptible to one exogenous stimulus and one cell-secreted factor could be harnessed for tighter control over cell remodeling and contraction of a material.¹¹¹

In the future, we envision that instead of producing a protein in bulk and biochemically patterning it within gels, proteins may instead be produced *in situ* by encapsulated cells under tight 4D transcriptional control by optogenetic or thermogenetic means. This concept has only recently been preliminarily explored, whereby bacteria transformed with an optogenetic protein plasmid (pDawn) and encapsulated within a gel expressed and secreted Red Fluorescent Protein in response to blue light.¹¹² With several strategies to genetically install chemical groups that facilitate gel-protein conjugation, secreted proteins could be sequestered by the hydrogel in a manner mimicking the role of the ECM.

■ ENGINEERING SIMPLICITY

The options available to a researcher wishing to engineer a biological microenvironment—material platforms, conjugation chemistries, mechanical and biochemical factors to consider—are nearly limitless. Contrary as it may sound, simplicity is also a key factor to consider when engineering complex microenvironments and is a crucial to the utility of any tool toward the study of disease. Many life-sciences laboratories are not equipped with the instrumentation or personnel for organic synthesis of the precursors and reagents used in many dynamic biomaterials. It is for this reason that Matrigel remains the one of the most widely used 3D matrices, despite limitations in batch variability and a lack of tunability.¹¹³ Conversely, biomaterials laboratories depend on expert collaborators to provide impactful applications for the uniquely enabling materials that they develop. In many ways, biomaterials development to study the microenvironment have outpaced the utilization of such materials for studying biology.

Many systems highlighted herein gain utility from simplicity. SLATE bioprinting uses U.S. Food and Drug Administration (FDA)-approved food additive dyes as photoabsorbers.⁶⁹ Recent studies pushing forward the culture of organoids in synthetic matrices have used hydrolysis as a trigger for the dynamic substrate softening required to support organoid formation.³⁴ BATE relies upon biology to provide emergent complexity in printed organoids, but only requires simple extrusion printing using a syringe and a manually controlled microscope stage.⁹² These types of systems are easy for a nonengineer to adopt and exploit to catalyze impactful and translatable findings with respect to any disease of interest.

Our group and others have made a turn toward genetically encoded approaches for hydrogel formation and modulation, circumventing many of the insurmountable barriers that synthetic organic chemistry have imposed on biology laboratories interested in using biomaterial tools.^{53,114–116} By coexpressing pre-existing enzymes for site-specific protein modification (e.g., NMT, sortase) alongside a protein of interest, bioorthogonal handles can be installed *in situ* for direct incorporation into a hydrogel network with no postsynthetic modification.⁵³ Spontaneous protein–protein binding (e.g., SpyCatcher/Tag ligation) can be exploited for

hydrogel cross-linking with no synthetic elements or catalysts, further distilling the field of synthetic hydrogel matrices into a format accessible to biologists.^{115,116}

FUTURE DIRECTIONS

As the field of dynamic biomaterials develops increasingly modular platforms and flexible bioconjugation chemistries, it is now possible to take many popular biomaterials systems off the shelf and simultaneously specify an expansive set of physiochemical properties in tandem. Future work will certainly continue to push the limits of stimulus responsiveness toward improved multiplexing, utilization of triggers with in vivo relevance, scaffolding elements that integrate biological signals and generate feedback, and full spatiotemporal regulation that matches all biological scales. With such expanded levels of customization, it will be increasingly important to seek out the simplest mechanisms of control required for any given experimental question.

We believe that the field still has much to borrow from the emergent and neighboring spheres of optogenetics and protein engineering. Current approaches typically use a naturally derived protein or peptide to impart a biomaterial with a biological function. Yet, with modern-day protein engineering tools, it is possible to optimize proteins computationally for sustained bioactivity and tunable release from a biomaterial scaffold.¹¹⁷ Furthermore, as *de novo* protein design continues to reach maturity, we envision a future in which protein elements may be rationally designed from grounds up as desired components of biomaterials.¹¹⁸ There is already a vast and rapidly expanding toolkit of structural components^{119–121} and protein logic gates^{122–124} that, in combination with site-specific modification strategies to incorporate these elements into a material, provide ripe grounds for exploration.

Lastly, although the philosophy of engineering complexity from the outside in has yielded unprecedented control over the biological microenvironment, approaches that harness self-emergent complexity such as BATE and the broader field of organoid biology have demonstrated that engineered tissues may also be constructed from naïve cell types; as complex 3D folds may be generated from relatively simple 2D cell patterns, these strategies promise to yield relatively mature engineered tissues with very few exogenous cues in a largely unsupervised manner. Such emergent strategies may prove more effective for some tissue engineering applications than traditional approaches involving patterned materials. Certainly, both philosophies should be considered by biologists, biomaterial scientists, and tissue engineers alike, and we turn toward many exciting future studies to identify which approach is best suited for a given biological application.

AUTHOR INFORMATION

Corresponding Author

Cole A. DeForest — Department of Bioengineering, Institute of Stem Cell & Regenerative Medicine, Department of Chemical Engineering, Department of Chemistry, and Molecular Engineering & Sciences Institute, University of Washington, Seattle, Washington 98105, United States;  orcid.org/0000-0003-0337-3577; Email: profcole@uw.edu

Author

Ross C. Bretherton — Department of Bioengineering and Institute of Stem Cell & Regenerative Medicine, University of

Washington, Seattle, Washington 98105, United States;

 orcid.org/0000-0002-9567-8556

Complete contact information is available at:

<https://pubs.acs.org/10.1021/acsbiomaterials.0c01549>

Author Contributions

R.C.B. and C.A.D. conceptualized, wrote, and edited the manuscript.

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Notes

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ABBREVIATIONS

3D, three-dimensional; 4D, four-dimensional; BATE, bioprinting-assisted tissue emergence; CRISPR, clustered regularly interspaced short palindromic repeats; DCPI, dual-color photoinitiator; dsDNA, double-stranded DNA; ECM, extracellular matrix; U.S. FDA, United States Food and Drug Administration; FRESH, freeform reversible embedding of suspended hydrogels; HSPA7, Heat Shock Protein Family A Member 7; LOV2, light, oxygen, and voltage sensing domain; LOVTRAP, LOV2 Trap And Release of Protein; MAPK, mitogen-activated protein kinase; MSC, mesenchymal stem cell; NMT, N-myristoyltransferase; PEG, poly(ethylene glycol); PDGF-BB, platelet-derived growth factor-BB; SLATE, stereolithographic apparatus for tissue engineering; ssDNA, single-stranded DNA; TrAP, traction-activated payload; YAP, yes-associated protein

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