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Microstructured Extracellular Matrices in Tissue Engineering and Development: An Update

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Abstract—Microstructured extracellular matrix (ECM), which contains heterogeneous features of the same size scale (5–100 μm) as tissue organoids, has become an important material for the engineering of functional tissues and for the study of tissue-level biology. This review describes methods to generate this class of ECM, and highlights recent advances in the application of microstructured ECM to problems in basic and applied biology. It also discusses computational techniques to analyze and optimize the microstructural patterns for a desired functional output.

Keywords—Biomimicry, Bio-inspired design, Micropatterning, Soft lithography, Multi-scale biomaterials.

INTRODUCTION

The ability to manipulate the extracellular matrix (ECM) has long played a critical role in the fields of tissue engineering and cell biology. Such manipulations include changes in ECM composition (e.g., adding basement membrane components to type I collagen), dimensionality (e.g., presenting collagen as a three-dimensional hydrogel instead of as a two-dimensional adsorbed layer), and mechanical state (e.g., pre-stressing a deformable matrix). Advances in these areas have been driven primarily by the appreciation that how mammalian cells behave is inextricably linked to the chemical and/or physical properties of the surrounding ECM.[27]

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Over the past decade, in conjunction with the emergence of methods to pattern ECM with micrometer resolution, it has become apparent that heterogeneous presentation of signals within ECM offers immense potential to enhance its functionality. Given that native tissues exhibit a texture at the length scale of organoids (i.e., 5–100 μm), we and others have suggested that mimicking such textures in a "microstructured" ECM may provide new types of substrata that can better replicate the complex signals observed *in vivo*.[19] Several years ago, we summarized some of the latest findings in this nascent approach, primarily in the areas of microvascular tissue engineering and epithelial branching morphogenesis.[29] At that point, the techniques for patterning three-dimensional ECM were still in development, and the structures that could be designed were rudimentary. Since then, many versatile new techniques have been described, and numerous studies have applied microstructured substrata to basic and applied biological problems in a wide variety of organ systems.

The purpose of this paper is to update our previous review and provide a starting point for future studies. We define microstructured ECM as one that possesses a microscale three-dimensional texture, in which the texture plays an essential role in shaping the biological response of cells to the ECM. We also include examples of patterned ECM-mimetic materials that are not naturally adhesive, but that can be derivatized with adhesive peptides (e.g., agarose, alginate, silk); many envisioned applications in tissue engineering favor these materials for their biocompatibility and mechanical strength. The underlying theme is that the microscale, rather than molecular-level, spatial organization of the ECM (or ECM mimic) modulates cell behavior.

Outside of the scope of this review are studies that use spatially uniform ECM. For example, we have omitted discussion of anisotropic, aligned collagen gels, or micropatterned gels in microfluidic devices in which the ECM acts primarily as a miniature version of a bulk gel. For these latter materials, we refer the reader to recent reviews.[37,54]

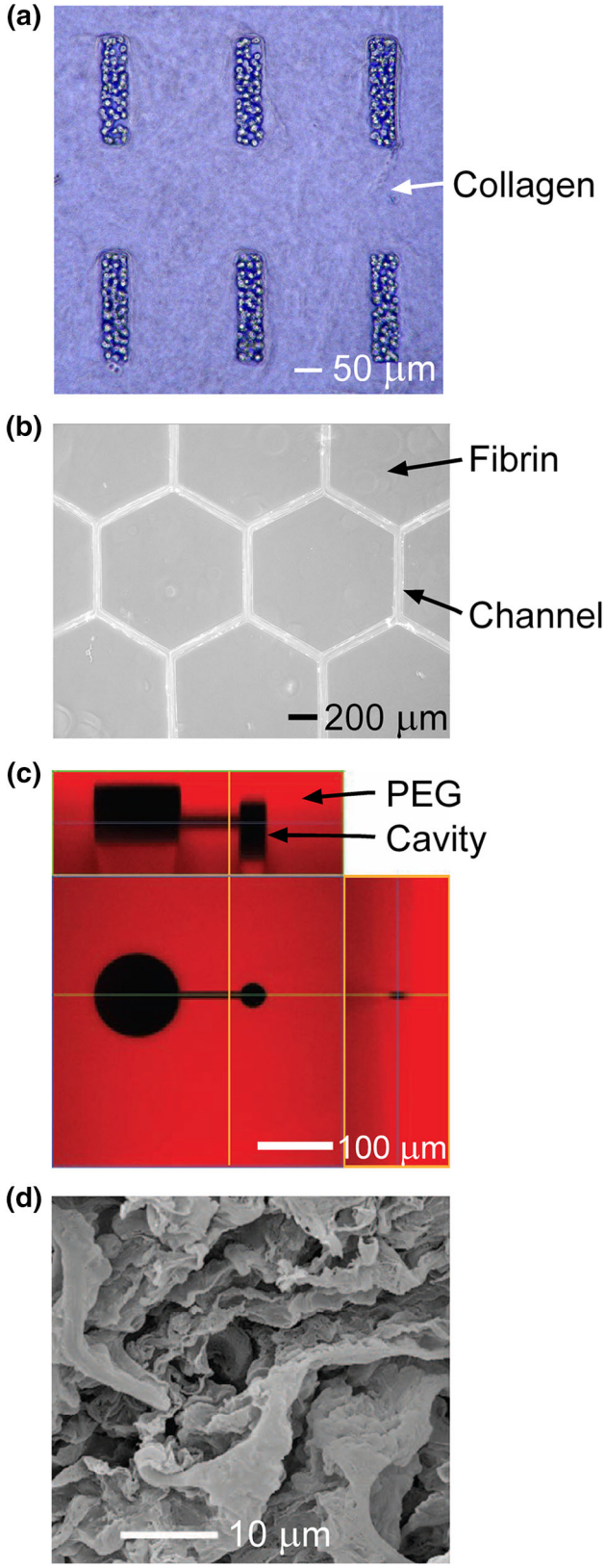
METHODS TO FORM MICROSTRUCTURED GELS

Compared to the soft lithographic techniques for patterning relatively sturdy polymers such as polydimethylsiloxane (PDMS), methods for patterning ECM with micrometer resolution are less well-developed. This disparity arises because ECM is typically much softer and more chemically fragile than well-crosslinked polymers, and because ECM is hydrated in its native state. Current techniques for forming microstructured ECM are largely adapted from standard soft lithography. More recently, photopatterning techniques have been developed for ECM mimics as well as for pure ECM. Another interesting approach, decellularization of intact organs, has yielded centimeter-scale gels that retain native ECM organization.

Micromolding

To create ECM gels with a surface texture, we showed a decade ago that passivating the surface of PDMS molds with serum albumin or other protein-resistant coatings would enable release of molded type I collagen, fibrin, Matrigel, or gelatin without distortion.[45] The surface features of these gels can be used to direct the microscale localization of cells; when indentations are molded onto the gel, seeded cells tend to settle and become trapped within them (Fig. 1a).[28,30,57] Manual assembly of a flat ECM gel over the patterned one yielded cell-filled cavities that were completely surrounded by ECM. Similar approaches used thin needles as templates to mold ECM, thereby forming open or blind-ended channels in one step.[7,47,51] The resolution of these micromolding techniques is on the order of 5 μm; for even finer structures, photoablation has been recently developed for collagen gels (see below).

For applications that involve large interstitial fluid pressures or cyclic mechanical strain, the passive adhesion that occurs when flat and patterned ECM gels are pressed together can be insufficient. In these cases, chemical or thermal bonding of the gels is often required. In early work, Stroock and colleagues[5] demonstrated that transient depolymerization (of alginate) was a viable strategy for bonding two passively adherent gels. Since then, we have extended their approach to



ECM gels using chaotropes and other perturbants (such as glycerol for type I collagen), and others have shown that silk and agarose gels are also amenable to

◀ **FIGURE 1. Examples of microstructured ECM. (a) Molded type I collagen gel, with mammary epithelial cells seeded within the resulting cavities.[28] (b) Patterned fibrin gel, after dissolution of a sacrificial gelatin mesh.[13] (c) PEG gel with a photo-generated cavity.[20] (d) Decellularized whole lung.[36] Reprinted with permission from the American Association for the Advancement of Science and the Royal Society of Chemistry.**

bonding.[4,23,38] By iteratively molding, stacking, and bonding thin layers of alginate, Zheng *et al.*[58] recently showed that three-dimensional interconnected microscale networks could be constructed within gels.

The additive molding techniques described above have been complemented by negative ones that use a molded material as a sacrificial template. Application of sacrificial approaches requires the use of a template that can be removed without disrupting the ECM that is formed around it. For type I collagen, appropriate sacrificial materials include Matrigel, gelatin, and alginate. Matrigel can be liquefied at ~4 °C or preferentially digested with dispase, gelatin can be liquefied at ~37 °C, and alginate can be liquefied with calcium ion chelators.[13,44,46] The advantages of the sacrificial approach are that the resulting patterned collagen gels are monolithic (i.e., one does not need to worry about adhesion between preformed gels) and that virtually no mechanical force is applied to the gel during the process. Interfacial diffusion of gels does not appear to be an issue, as the features remain sharp after dissolution of the sacrificial material (Fig. 1b). Given the popularity of soft lithography and the numerous additive and sacrificial techniques now available for generating microstructured collagen gels, it is not surprising that these methods have become the standard in terms of feature resolution and ease of use.

Photopatterning

The tremendous success of photolithography in generating micro- and nanoscale features has motivated many groups to consider similar light-based approaches for patterning ECM gels and mimics. Most of this work has relied on methacrylate- and acrylate-based photopolymerization, since the required light frequencies are generally acceptable for use with living cells. West, Anseth, and others have led the way in synthesizing (meth)acrylated derivatives of polymers such as polyethylene glycol (PEG) and patterning their polymerization using both single-layer and multilayered format. While early efforts generated patterns by UV exposure through a simple mask,[21] recent work has become far more elaborate. For instance, Hahn *et al.*[16] showed that two-photon activation can generate three-dimensional patterns of acrylated PEG with microscale resolution throughout the bulk of the gel. More recently, Kloxin *et al.* synthesized a photodegradable PEG acrylate, and showed that cavities could be ablated in a preformed gel (Fig. 1c). Although PEG itself is non-adhesive to cells, incorporation of adhesive groups is relatively straightforward (e.g., through co-polymerization of acrylated RGD peptide). Moreover, the (meth)acrylate photochemistry is versatile, and has been extended to create micropatterns in protein-based gels such as gelatin.[32]

Surprisingly, it is even possible to directly photopattern ECM gels, as recently shown by Friedl and co-workers.[18] This technique uses two-photon absorption to ablate collagen gels, although the completeness of the ablation and the identity of the resulting chemical byproducts remain unclear. Confocal reflectance microscopy showed that features as fine as ~3 μm could be generated with this approach.

Direct Writing

Direct writing of microscale features in ECM is still in early development, but holds much promise. The main approach adapts inkjet printing for use with viscous ECM, and can print open structures such as tubes and networks with a resolution on the order of 100 μm.[8] More recently, direct writing of a sacrificial sugar into robust, three-dimensional networks has enabled generation of centimeter-scale patterned ECM gels that contain interconnected channels for perfusion.[25] As with all direct writing techniques, these patterning methods are serial rather than parallel. As a result, simultaneously improving resolution and speed remains challenging; writing from multiple jets may eventually be needed for high-resolution applications.

Decellularization

With the demonstration by Ott *et al.*[34] that organs can be completely decellularized by perfusion with detergents, whole-organ decellularization has become a heavily investigated technique for generating microstructured ECM (Fig. 1d). The composition of the resulting ECM is largely preserved in its native state, and its geometric complexity far exceeds that of even the most elaborate features that can be currently molded or printed. By design, this technique routinely yields organ-scale ECM, and thus it holds tremendous promise for engineering functional tissues in clinically relevant volumes.

Other Methods

Microscale self-assembly based on surface tension has been used to create composites of patterned gels.[9] This process submerges free-standing, microscale gels

in a non-aqueous solvent, which promotes aggregation of the gels to minimize the interfacial area between aqueous and non-aqueous phases.

MICROSTRUCTURED ECM IN TISSUE ENGINEERING

The underlying motivation for using patterned ECM as scaffolds for engineering tissues is to provide the nascent construct with microscale spatial information that can potentially direct the growth and eventual functionality of the construct as it matures. Studies in this area have assumed that replicating tissue architecture is advantageous (although, as discussed below, the extent of replication required is still unclear). The studies summarized below consist of those that use patterned reconstituted ECM (chiefly, collagen-based gels) or decellularized organs as scaffolds.

Microstructured Reconstituted ECM as Scaffolds

The need for adequate tissue vascularization has driven much of the recent progress in applying microstructured ECM to engineered tissues. In contrast to growth factor- and progenitor cell-based approaches to vascularization, a microstructured approach strives to encode the geometry of the desired vascular networks explicitly within the scaffold. The scaffolds in these studies contain open channels and networks that enable immediate perfusion. We showed several years ago that single channels that were molded within type I collagen gels could act as templates to guide the formation of single "tubes" of endothelial cells (Fig. 2a).[7] These tubes, and others made by similar molding techniques, display many of the functions possessed by native microvessels, including a solute-selective barrier,[7,56] hemostasis,[57] reactivity to shear stress and inflammatory signals,[7,39,57] ability to undergo growth factor-induced angiogenesis,[31,57] and support of leukocyte adhesion.[7]

Besides providing channels to direct the formation of tubes that support flow, using microstructured ECM holds the advantage that the resulting vessels have defined size, shape, and location. This geometric consistency enables independent control over the different mechanical stresses applied to vessels. For instance, placing an empty blind-ended channel next to a vessel (to mimic lymphatic drainage) allowed control over interstitial fluid pressures and vascular stability.[55] This study correlated the observed spatial distribution of endothelial adhesion to the scaffold with calculated profiles of interstitial and vascular pressure. In this way, it concluded that a minimum level of vascular transmural pressure was required to maintain stable adhesion.

The channels within microstructured ECM can also be used directly for soft tissue regeneration (Fig. 2b).[58] Here, the well-controlled orientation of microscale pores accelerated vascular in-growth, compared to that observed in a commercially available dermal scaffold and in a homogeneous collagen gel, both of which had randomly oriented pores. In some of the patterned scaffolds, the channels were filled with collagen, at a lower density than in the surrounding gel. Surprisingly, whether the channels were filled or not was of lesser importance to the final result; the presence of microstructure within the scaffold was the dominant factor in enhanced vascularization.

Similar channel-based approaches have also been used to form other tubular structures. Ying and co-workers showed that channels in type I collagen and/or Matrigel could support the growth of tubes of MDCK renal epithelial cells.[41] We have also shown that Caco-2 intestinal epithelial cells and primary human renal epithelial cells will form mature tubes with thick epithelium in such scaffolds (unpublished results). Finally, it has long been appreciated that arrays of parallel channels can help guide the regeneration of neurons by mimicking endoneurial tubes.[3]

To direct the growth of non-tubular tissues, several studies have used channel-free microstructured ECM. For instance, molding of collagen against a surface that contained high-aspect-ratio pits yielded ~100-μm-wide "pillars" of collagen that mimicked the undulating shape of intestinal villi.[44] The fragile geometry necessitated the use of a sacrificial technique with alginate in the molding process. When seeded with Caco-2 cells, these scaffolds generated epithelial structures that looked remarkably similar to native villi in size and spacing. Other studies have used microstructured scaffolds to drive the formation of mechanically anisotropic muscular tissue.[10] It is unclear whether the microscale texture of the substratum is critical, or whether scaffold anisotropy at any length scale would yield a similar result.

Decellularized Organs as Scaffolds

Recent studies have shown that many whole organs, including heart,[34] lung,[33,36] liver,[50] and kidney,[43] can be decellularized and recellularized *via* a primary artery and vein (Fig. 2c). As discussed above, decellularized organs present a level of ECM patterning that is simply not achievable by state-of-the-art molding or printing techniques. One must keep in mind, however, that native ECM architecture may not necessarily be the optimal choice when engineering tissues. Intact basement membrane may inhibit cell migration, and mass transport in the absence of an intact endothelium may differ from that in native organs due to fluid shunting.

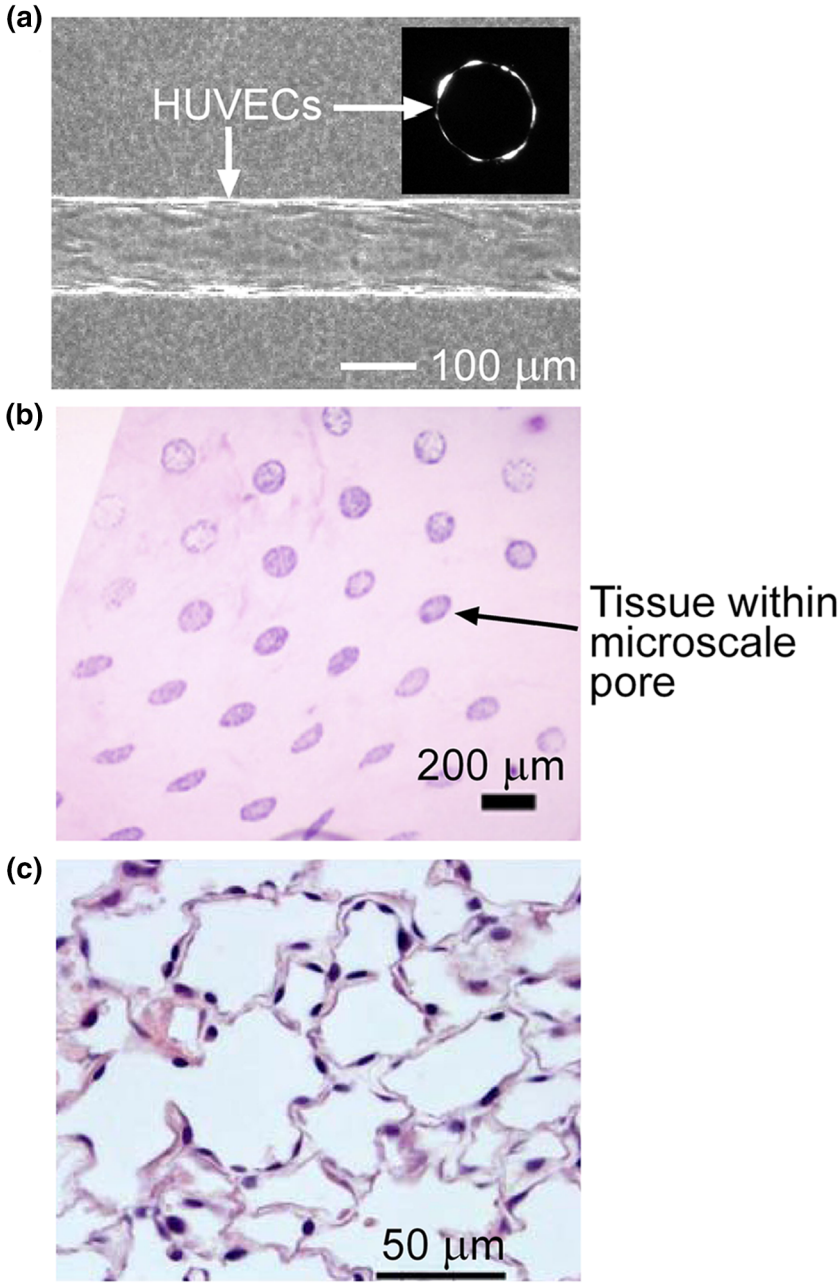


FIGURE 2. Engineered tissues that used microstructured ECM as scaffolds. (a) Perfused endothelial (HUVEC) tubes in a microfluidic collagen gel.[7] (b) Soft tissue that formed within a patterned collagen implant.[58] (c) Recellularized lung.[36] Reprinted with permission from Elsevier and the American Association for the Advancement of Science.

In fact, a recent comparison of decellularized dermis and a collagen-based dermal scaffold concluded that the latter led to faster vascularization.[40] Thus, whether using native or engineered ECM, one must be judicious in choosing which microstructural features are retained, discarded, or ignored. Nevertheless, for organs with complex epithelial architectures such as the kidney, it is difficult to imagine how anything other than a decellularized organ could contain epithelial organoids (and their interconnections) at the densities required for effective organ replacement.

MICROSTRUCTURED ECM IN STUDIES OF DEVELOPMENT

Some of the earliest uses for ECM as a laboratory reagent were for studies of tissue morphogenesis and

development. ECM proteins are present within the embryo as early as gastrulation in vertebrates, and show a clear distribution pattern even at these early stages.[17] Replicating the microstructure of the ECM around and within developing tissues provides chemical and mechanical signals that are largely absent from homogeneous ECM; these signals may prove to be absolutely essential for normal development. One illustrative example is in the study of the formation of the branched networks of epithelial tubes that line most major organs. Kidneys, lungs, mammary glands, salivary glands, and prostate epithelia all form into ramified architectures during development *via* a recursive process known as branching morphogenesis.[1] These morphogenetic movements can be replicated in the culture setting by embedding clusters of epithelial cells within native ECM gels of either type I collagen or Matrigel. In these cluster-based assays, new multicellular branches emerge randomly from the surface of the organoids and penetrate into the surrounding ECM *via* active remodeling.[42] However, when epithelial organoids are formed within micromolded cavities of ECM, such that they take on reproducible architectures (Fig. 3a), new branches only arise from stereotyped locations defined by the initial tissue geometry.[30] This geometric signal is transmitted to the tissue in the form of concentration gradients of morphogens[30,35] as well as gradients of mechanical stress[11,12] which induce patterns of gene expression within the tissue.[22] It will be interesting to determine whether the signals induced by microstructured ECM are shared by the development of all branching organs.

In addition to its multitude of roles in regulating normal development, ECM architecture also appears to play a major role in the development of pathological tissues, including tumors. Microstructured ECM has proven especially useful for mimicking and distinguishing the physical effects of the microenvironment around tumor cells during tumor progression. Using a similar needle-based micromolding strategy as described above for endothelial tubes, we recently showed that breast tumor cell aggregates cultured within a blind-ended channel of collagen gel could be subjected to precise and varying amounts of interstitial fluid pressure (Fig. 3b).[47] Importantly, this strategy enabled isolation of the chemical and mechanical effects on the tumor cells resulting from interstitial fluid pressure, and revealed that high pressures within tumor aggregates inhibit cell invasion.

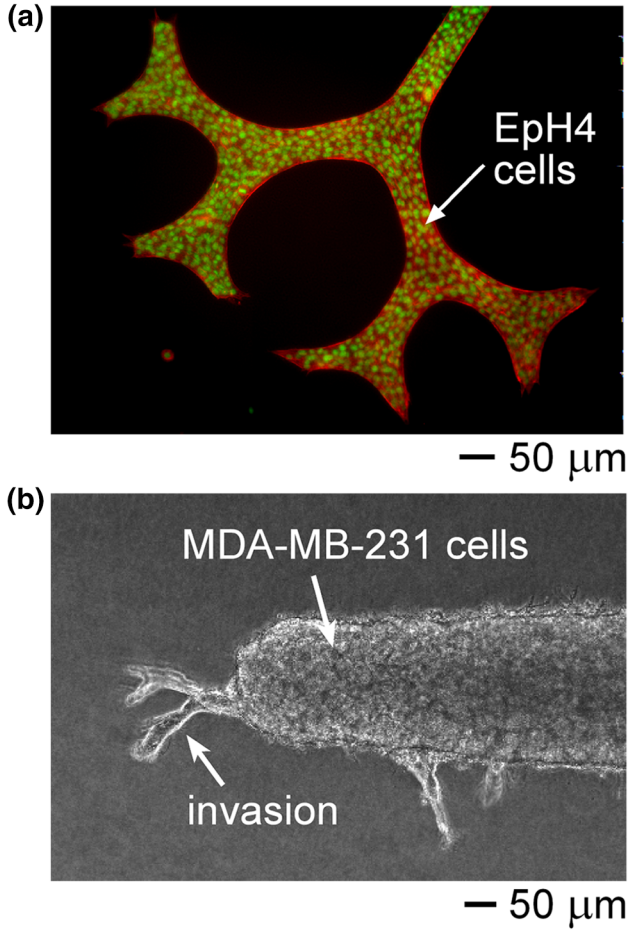


FIGURE 3. Development of microscale tissues within microstructured ECM. (a) Branching tubules of EpH4 mammary epithelial cells in a patterned collagen gel.[30] (b) Invasive aggregates of MDA-MB-231 tumor epithelial cells in a blind-ended channel in collagen.[47] Reprinted with permission from the American Association for the Advancement of Science and the Public Library of Science.

COMPUTATIONAL DESIGN OF THE MICROSTRUCTURE

With the notable exception of decellularized organs, microstructured ECM has consisted of reconstituted or synthetic hydrogels whose basic physical properties (elastic moduli, hydraulic permeability) are readily measured. In principle, these measurements enable computational prediction and optimization of the forces and flows within such scaffolds. The most common approach has been to treat the patterned ECM as linearly elastic and locally isotropic, although empirical corrections (e.g., ones that result from cell-induced changes in ECM concentration) have been proposed.[12] The properties of the ECM are generally assumed to depend only on the local network structure. In the hydrated state, the patterned ECM is treated as a poroelastic material with incompressible fluid and solid constituents, and its deformation is described by biphasic mixture theory.[26,53] Viscous effects that are independent of bulk fluid flow are usually assumed negligible over the time-scales of most experiments. Below, we list the standard governing

equations that have been used in computational analysis of microstructured ECM, and highlight recent studies that apply these equations to questions in tissue engineering and development.

Fluid and solute transport are described by the modified Darcy's Law and the reaction–diffusion-convection equation, respectively:

$$\nabla P = -\frac{\mu}{\kappa}\mathbf{v} + \mu\nabla^2\mathbf{v} \tag{1}$$

$$\frac{\partial C}{\partial t} + \mathbf{v}\cdot\nabla C = D\nabla^2 C + R \tag{2}$$

where P is the interstitial fluid pressure, μ is the viscosity of the interstitial fluid, κ is the Darcy permeability of the ECM, $\mathbf{v}$ is the interstitial fluid velocity, C is the solute concentration, D is the solute diffusion coefficient in the ECM, and R is the solute production rate (negative for solute consumption). Since the average pore size in the ECM is orders of magnitude smaller than the patterned feature size in most studies (0.1–1 μm vs. 10–100 μm), the Brinkmann modification of Darcy's Law [the last term in Eq. (1)] is typically omitted.

When the ECM contains microscale channels, fluid transport in them is governed by the Navier–Stokes equations:

$$\rho\left[\frac{\partial \mathbf{v}}{\partial t} + (\mathbf{v}\cdot\nabla)\mathbf{v}\right] = -\nabla P + \mu\nabla^2\mathbf{v} \tag{3}$$

where ρ is the density of the (Newtonian) fluid within the channels. In almost all studies, the Reynolds number in the channel is small enough that inertial and entrance effects are negligible. One must keep in mind that no-slip boundary conditions do not necessarily apply when the channel wall is a porous ECM; the degree of slip will depend on the interstitial flows tangential or normal to the ECM channel wall. Solute transport within channels is described by Eq. (2) without the reaction term R.

Deformation of the ECM is usually treated using the constitutive relation of isotropic, linear poroelasticity with incompressible interstitial fluid and ECM fibers:

$$\sigma_{ij} = 2G\varepsilon_{ij} + \left(2G\frac{\nu}{1-2\nu}\varepsilon_{kk} - P\right)\delta_{ij} \tag{4}$$

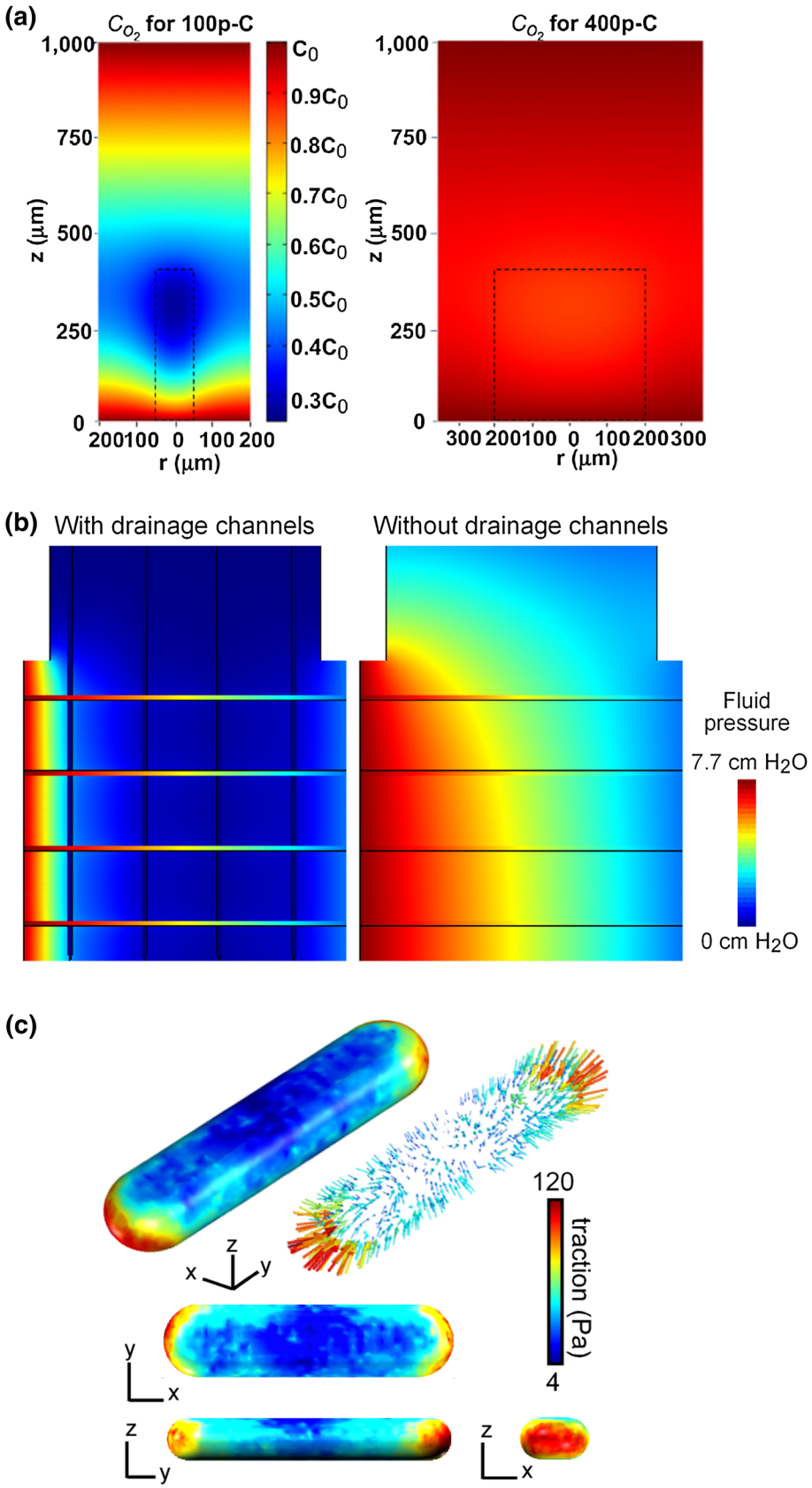
where σ_{ij} and ε_{ij} are the stress and strain tensors for the ECM, G is the shear modulus, and ν is the Poisson's ratio. The stress tensor $\sigma_{ij} + P\delta_{ij}$ refers to the stress borne by the ECM fibers. Immediately after application of external stress, the ECM acts as an incompressible material (i.e., $\nu = 0.5$). Thus, one must be careful when applying elastic moduli that are measured under relatively rapid deformations (e.g., in a rheometer) to slow, cell-induced deformation of ECM. Poisson's ratio, which is difficult to measure, is often assumed to be ~0.2; direct measurement of this value in a variety of reconstituted ECM would be helpful.[2] Viscous effects, such as a time-dependent shear modulus, can be incorporated by replacing the right-hand-side of equation (4) with an integral of shear rate over time.[24]

Optimization of Fluid and Solid Transport

Recent experimental and computational studies have established guidelines for the design of microstructured ECM to support the metabolism of engineered tissues (Fig. 4a).[6,49,58] It is well-known that the microvascular design in some native tissues (e.g., skeletal muscle) can be approximated by the Krogh model in which each vessel perfuses its surrounding volume. This design led Choi *et al.* and us to analyze the transport of solutes between parallel arrays of perfused channels and the surrounding ECM.[6,49] The patterned alginate gels in the Choi study were formed by micromolding of cell-containing precursors against a PDMS mold. Diffusion-limited solute distribution profiles under perfusion could be described well by solving Eq. (2) in the channels and ECM. Of note, the concept of a Krogh radius (the radius of the perfused volume that surrounds a channel) was found to apply, which enables straightforward estimation of the channel densities required to supply surrounding tissues with sufficient nutrients.[6]

We addressed the question of what channel designs are optimal by using the channel volume fraction as a minimization function.[49] Solving Eqs. (1)–(3), this study yielded analytical expressions for the channel spacing and diameter that would minimize the volume occupied by the channels while maintaining sufficient delivery of solutes to the surrounding tissue. Optimal dimensions were surprisingly large (on the order of 50–100 μm), a result that implies that true capillary-scale features may not be necessary in microstructured ECM to achieve adequate solute delivery.

By focusing solely on fluid flow with Eqs. (1) and (3), we also showed that similar computational models could be used to analyze the pressure distributions within scaffolds patterned with parallel vascular arrays (Fig. 4b).[48] The objective of this work was to determine the optimal placement of empty channels for draining the ECM, lowering interstitial fluid pressure, and increasing the mechanical stability of surrounding vessels. The models showed that each drainage channel was responsible for maintaining the stability of vessels located within a given distance, analogous to the Krogh radius for perfusion systems. Although the Krogh analogy is not exact (solute consumption and pressure loss are not governed by identical equations),


(a)
C_{O_2} for 100p-C
C_{O_2} for 400p-C
1,000
750
500
250
0
z (μm)
200100 0 100 200
300 200100 0 100 200300
r (μm)
C0
0.9C0
0.8C0
0.7C0
0.6C0
0.5C0
0.4C0
0.3C0
(b)
With drainage channels
Without drainage channels
Fluid pressure
7.7 cm H2O
0 cm H2O
(c)
120
traction (Pa)
4
z
x
y

◀ **FIGURE 4. Computational analysis of transport and mechanics in microstructured ECM. (a) Oxygen concentration within two collagen scaffolds of different microscale pore geometry, from solving Eq. (2) within the scaffold.[58] (b) Interstitial pressure within a vascularized fibrin gel with and without drainage channels, from solving Eqs. (1) and (3) within the channels, vessels, and gel.[55] (c) Cell-generated mechanical stress at the surface of a seeded collagen cavity, from solving Eq. (4).[12] Reprinted with permission from Elsevier and Wiley Periodicals.**

it serves as a convenient tool for assessing the drainage channel density required to stabilize vessels of a given density.

Control of Local Mechanical Stress

In addition to transmitting forces from fluid flow, ECM also transmits tensile forces from the contraction of cells embedded within it. This property has enabled investigation of the role of endogenous (tissue-generated) mechanical forces on cell behavior. By using micromolded cavities within collagen gels, we generated epithelial tissues of defined shape and size, and importantly, were able to predict the patterns of mechanical forces generated by each tissue, depending on its initial geometry (Fig. 4c).[12] Cells were more invasive when located in regions of the tissues that experienced higher relative levels of mechanical stress. Similarly, Guo and colleagues found, using clusters of cells patterned within collagen gels against an agarose support, that tension transmitted between tissues could direct their invasion.[14] Our understanding of these mechanical stresses and their effects on cells in three-dimensional tissues will likely benefit from the increasing resolution with which microscale structural features can be generated within ECM.

CONCLUSIONS AND FUTURE OPPORTUNITIES

The unique ability of microstructured ECM to approximate the three-dimensional geometry of tissues has enabled this class of materials to play an emerging role in studies of basic and applied biology. We believe that advances in ECM processing will allow several desirable structures to be attained in the near future, including (in order of increasing difficulty): ECM gels that are laminated by surface films of basement membrane, ECM that contains interconnections between microscale and millimeter-scale features, fractal trees that consist of open bifurcating ducts, and interwoven bifurcating networks that approximate the vascular and epithelial compartments of a branched tissue. Although organ decellularization can already produce many of these complex structures, there is still a need for the defined control afforded by lithographic techniques, which can theoretically produce ECM of nearly identical microscale size and shape.

For applications in tissue engineering, a major unresolved issue is to what extent native ECM microscale architecture is needed to obtain proper function.[52] Advantages of a microstructured ECM must be balanced against the added time and cost of manufacturing. For the vascular system, it is fairly clear that scaffolds that contain channels can support perfusion in ways that homogeneous scaffolds simply cannot. Even here, a hybrid approach that uses micropatterning techniques to generate most vessels and cellular self-organization to generate the finest structures (e.g., capillaries) may be more effective than methods based exclusively on one or the other vascularization strategy. We note that crude versions of this hybrid approach have already been implemented *in vivo* during ''vascular prefabrication,'' in which a large perfused vessel is used to generate capillary networks within a tissue or biomaterial.[15] Also, since current patterning techniques yield small (millimeter-scale) tissues, methods to pattern large (centimeter-scale) scaffolds without sacrificing resolution or speed would be very welcome.

For studies of cell and developmental biology, microstructured ECM offers the potential to ask direct questions about the roles of tissue mechanics, geometry, and organization in morphogenesis. While *ex vivo* morphogenesis can never supplant that of the embryo, we believe that this approach complements genetic strategies in the study of development. Our current understanding of tissue development is mainly genetic in nature, confined to the molecular signals that accompany morphogenesis. Microstructured ECM can help reveal how these molecular signals direct, or are directed by, the mechanical forces of tissue morphogenesis.

Advances in computational techniques will undoubtedly be important in this field as well. To date, most numerical models have examined artificially symmetric domains to limit the model geometry to a tractable size. The ability to solve relevant equations in the entire heterogeneous domain (e.g., to determine solute transport throughout a centimeter-sized decellularized scaffold) will require the development of efficient numerical schemes. Real-time, noninvasive imaging techniques to visualize functional behaviors throughout the thickness of the ECM will also be needed to provide data for correlation with numerical predictions.

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