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Engineered ECM Models: Opportunities to Advance Understanding of Tumor Heterogeneity

Adrian A Shimpi[a], **Claudia Fischbach**[a,b]

[a]Nancy E. and Peter C. Meinig School of Biomedical Engineering, Cornell University, Ithaca, NY 14853, USA

[b]Kavli Institute at Cornell for Nanoscale Science, Cornell University, Ithaca, NY 14853, USA

Abstract

Intratumoral heterogeneity is a negative prognostic factor for cancer and commonly attributed to microenvironment-driven genetic mutations and/or the emergence of cancer stem cells (CSCs). How aberrant extracellular matrix (ECM) remodeling regulates the phenotypic diversity of tumor cells, however, remains poorly understood due in part to a lack of model systems that allow isolating the physicochemical heterogeneity of malignancy-associated ECM for mechanistic studies. Here, we review the compositional, microarchitectural, and mechanical hallmarks of cancer-associated ECM and highlight biomaterials and engineering approaches to recapitulate these properties for *in vitro* and *in vivo* studies. Subsequently, we describe how such engineered platforms may be explored to define the spatiotemporal dynamics through which cancer-associated ECM remodeling regulates intratumoral heterogeneity and the CSC phenotype. Finally, we highlight future opportunities and technological advances to further elucidate the relationship between tumor-associated ECM dynamics and intratumoral heterogeneity.

Introduction

Intratumoral heterogeneity is a hallmark of cancer and is characterized by the presence of different cancer cell subpopulations that severely limit patient outcomes due to their varied proliferative, invasive, and therapy resistance capabilities [1,2]. Historically, tumor cell heterogeneity has been attributed to oncogenic mutations that increase cell fitness in response to environmental pressures or chemotherapy [3]. However, phenotypic differences caused by epigenetic reprogramming as well as transient changes in gene expression, phosphoproteomics, and metabolic signaling are equally important [2,4,5]. Moreover, the

Corresponding Author: Claudia Fischbach (cf99@cornell.edu).

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self-renewal and therapy resistance of cancer stem-like cells (CSC) contributes to clonal diversity within tumors [6]. Indeed, an increase in CSCs due to transformation of tumor cells or environmental selection pressures promotes tumor development, metastasis, and treatment response [6]. Which role the tumor microenvironment (TME) plays in the emergence of CSCs and which effect this has on tumor heterogeneity is not well understood.

Within the TME, cancer cell phenotypes are regulated through crosstalk with tissue-resident stromal cells including cancer-associated fibroblasts (CAFs), adipocytes, endothelial cells, and infiltrating immune cells [7]. Much emphasis has been placed on how secretory functions of these cells control tumor heterogeneity and progression. Yet their impact on the physical properties of the TME may be similarly critical [8]. In particular, CAFs are widely studied for their role in changing the quantity, biochemical composition, and mechanical properties of extracellular matrix (ECM) in tumors [8], and these alterations regulate the geno- and phenotype of tumor cells as well as CSC quantity and functions [9]. Nevertheless, CAF-dependent ECM changes are not homogeneous, but are subject to spatial and temporal variations (Figure 1). How ECM heterogeneity is functionally linked to tumor heterogeneity remains unclear due in part to the lack of relevant model systems.

Both *in vivo* and *in vitro* studies have advanced our understanding of how tumor cell interactions with the ECM affect tumor progression and therapy response. However, the high cost and species-dependent differences between humans and mouse models, as well as shortcomings associated with 2-D cell culture make it challenging to isolate mechanistic links between ECM remodeling and tumor cell state. Engineered model systems can recapitulate and isolate TME-associated ECM changes to probe their effect on tumor heterogeneity as a function of CSC enrichment. Indeed, simply switching tumor cell culture from conventional 2-D to 3-D culture impacts several hallmarks of malignancy including cellular metabolism [10], invasion [11], and therapy resistance [12]. Furthermore, 3D culturing of cancer cells enriches for CSCs in part through activation of the epithelial to mesenchymal transition and altering cytokine secretion [13]. Here, we will summarize current knowledge of ECM changes in the TME, highlight model systems to mimic these changes for mechanistic studies, and outline strategies to further improve the impact engineered ECM models have on our understanding of tumor heterogeneity and the role of CSCs in this process (Figure 1).

Extracellular Matrix Changes in the Tumor Microenvironment

Compositional Changes

Most prior research studying the role of ECM remodeling in cancer focused on the composition of the ECM and ECM-associated proteins (collectively referred to as the matrisome [14]). Changes in the matrisome relative to healthy tissue are characteristic of aggressive cancers including breast [15] and pancreatic cancer [16]. For example, fibronectin is often increased during tumorigenesis, regulates all stages of the metastatic cascade through integrin-dependent signaling, and impacts CSC marker expression [17–19]. Additionally, fibronectin provides the initial scaffolding for collagen deposition [20] and could, therefore, contribute to the elevated concentration of different collagen types in the

TME (in particular, type I and VI) that ultimately promote tumor invasion and metastasis due in part to altering tumor cell stemness [9,21–23].

Stromal accumulation of glycans such as hyaluronic acid (HA) and heparan sulfate can also predict worse clinical prognosis and contributes to CSC properties [24,25]. An increase of free glycans such as heparan sulfate, due to upregulation of glycosidases, promotes tumor progression [26] possibly by perturbing the nuanced interactions between protein- and glycan-based ECM components. Indeed, disrupted biosynthesis of glycans such as HA affects ECM bulk properties by stimulating collagen and fibronectin deposition [27]. Similarly, binding of heparan sulfate to the $FnIII_{12–14}$ domain of soluble fibronectin initiates deposition of fibrillar fibronectin within the ECM [28]. While these changes can indirectly promote tumor cell stemness, upregulation of glycans also increases engagement of cell surface receptors including CD44, which, in turn, activates expression of transcription factors linked to stemness [29,30]. Finally, most glycans carry a high negative charge density impacting the sequestration and bioactivity of growth factors and cytokines associated with stemness [6,31].

Physical Changes

Varied composition simultaneously alters the physical properties of the ECM (Figure 2), which independently regulate malignancy [32]. Greater ECM rigidity due to increased collagen deposition, cross-linking, and linearization is the most widely appreciated of these changes [33,34]. In fact, increased tissue density and stiffness are well known biomarkers of malignant tissue [35] and widely studied at the bulk tissue level. However, the biophysical properties of tumors vary in space and time with functional consequences on tumor heterogeneity [15,16,34]. For example, the invasive front of tumors exhibits increased ECM fiber alignment and stiffness relative to more benign tumors [34,36,37], while these features are reduced in more central regions of tumors due to a comparatively reduced ECM content [15,34]. Increased ECM fiber alignment, in turn, locally activates mechanotransduction and invasion via positive mechanical feedback and strain-dependent biochemical changes of the ECM [20,38,39]. For example, partial unfolding of fibronectin fibers due to tumor-induced stromal cell contractility increases ECM deposition and stiffness [40–42], exposes cryptic binding sites to soluble factors [43], and mechanically activates latent growth factors and cytokines stored in the ECM [44]. Such changes could increase tumor cell stemness and thus, tumor heterogeneity by modifying the signaling networks between tumor cells and the TME [45].

Glycan-protein interactions also contribute to microarchitectural changes of the ECM by either cross-linking individual protein fibers or physically interweaving with them to increase fiber diameter [27,43,46]. Importantly, the high negative charge density associated with increased glycan content increases tumor osmotic pressure, which can promote tumor heterogeneity by altering interstitial fluid flow to direct migration [47] or by impairing drug delivery [25,31,32]. As compositional and physical ECM changes are closely interconnected in the TME, engineered model systems are necessary to deconvolve how their individual and combined effects promote heterogeneity through altering CSCs.

Model Systems of the Extracellular Matrix

Decellularized scaffolds generated from tissue, patient samples, or deposited by cells in culture (cell-derived matrices, CDMs) mimic the native biochemical and physical properties of the ECM (Figure 3a) [23,48–50]. In particular, CAF-derived CDMs are often used to recapitulate tumor-associated ECM and promote the malignant potential of both tumor and stromal cells by activating mechanosignaling [49,50]. Despite their obvious benefits, the complexity of decellularized scaffolds and inability to selectively control substrate mechanics and architecture make it challenging to delineate mechanistic details of how ECM compositional and physical parameters impact tumor heterogeneity.

Synthetic polyacrylamide (PAA) gels are widely used to achieve tunable control of substrate stiffness. These gels can be functionalized with different adhesion ligands or full-length proteins (e.g. fibronectin, laminin) to promote cell adhesion and determine the effect of ECM mechanics in the context of varied adhesion receptor engagement (Figure 3b). Furthermore, PAA-based systems are easy to implement and can be tailored to mimic the spatial heterogeneity of ECM stiffness in tumors. Investigations of cancer cell migration on PAA gels with stiffness gradients, for example, have revealed that durotaxis of cancer cells requires an optimal stiffness [51,52]. However, time-dependent mechanical properties or viscoelasticity (e.g. strain-stiffening) are critical features of the native ECM that linearly elastic (i.e. time-independent) PAA gels cannot recapitulate [53].

Collagen type I-based hydrogels capture the viscoelastic and fibrillar nature of ECM in the TME (Figure 3c) [53]. Manipulation of collagen fibrillogenesis (e.g. by adjusting gelation temperature, collagen cross-linking agents, or the presence of a macromolecular crowding agent) enables selective control over collagen fiber structure and thus, scaffold microarchitecture and mechanics [39,54,55]. For example, lower casting temperature increases fiber diameter, scaffold pore size, and shear modulus independent of collagen concentration [39,55]. These differences have phenotypic consequences as stromal cells seeded into cold- versus warm-cast collagen hydrogels assume CAF-like characteristics enabled by localized strain-stiffening of the surrounding matrix [39,55]. The physical properties of collagen gels can be further adjusted by incorporating additional ECM components. For example, combining collagen and a dynamically crosslinked-HA hydrogel as a viscoelastic interpenetrating network (IPN) increases the compositional complexity and enables precise control over stress relaxation [56]. This toolbox can be additionally expanded by tuning HA crosslinker affinities, molecular weight, and concentration to generate faster stress relaxing gels that increase cell-mediated collagen fiber alignment, focal adhesion formation [56] and may affect cell cycle progression [57].

While collagen-based hydrogels permit a certain level of control over scaffold microarchitecture, collagen fibers *in vitro* differ significantly from those *in vivo*. Furthermore, isolating effects mediated by individual fiber properties (e.g. rigidity, thickness, and length) from bulk properties (e.g. scaffold porosity) is challenging, but can be accomplished with composite fibrous gel systems (Figure 3d). For example, electrospun methacrylated dextran (or dextran vinyl sulfone) fibers enable precise control over both bulk and individual fiber architecture and mechanics, while limiting fiber biochemical

activity such as collagen degradation to influence cell phenotypes [54,58,59]. Softer fibers in these systems are more readily recruited by cells to promote focal adhesion formation and cell proliferation, a phenotype typically seen on stiffer hydrogels [58]. Encapsulating electrospun fibers in a hydrogel furthermore revealed that fiber density can regulate fibroblast mechanosignaling independently of bulk stiffness [59]. Together, these results suggest that the physical properties of ECM fibers can influence cell phenotypes independent of ECM bulk and biochemical properties.

Biomaterials Systems to Elucidate the Interplay between Tumor and ECM Heterogeneity

Biomaterials models have expanded our understanding of how biophysical alterations of the ECM influence tumor heterogeneity and stemness. For example, studies with PAA and fibrillar collagen gels suggest that ECM stiffness and microarchitecture synergize to increase CSC numbers and tumor metastatic burden [60]. Tumors cells cultured on stiff versus soft PAA gels increase stem cell marker expression and invasiveness, and hypoxia, an independent inducer of tumor cell stemness, further elevates these differences ultimately promoting tumor invasion and metastasis [61]. Comparing tumor cell migration in isotropic and anisotropic collagen gels additionally revealed that CSCs respond to collagen alignment with increased motility relative to their differentiated counterparts due to greater morphological plasticity and protrusion frequency [62]. Consistent with these findings, more weakly adherent cells migrate faster due in part to more labile focal adhesions, and these changes are predictive of metastatic potential [63]. While altered focal adhesion dynamics can be intrinsic to a specific cell phenotype, they are further regulated by collagen fiber structure. Adjusting collagen microarchitecture by altering gelation temperatures, for example, suggested that adhesion lifetimes depend on the balance between fiber mechanical properties and cell contractility [64]. While these connections have been elucidated with fibroblasts, fiber mechanical properties may also affect tumor cell stemness as focal adhesion-dependent cell signaling varies between CSCs and differentiated tumor cells [64,65].

Biomaterials systems have also increased understanding of how ECM-dependent changes of metabolism and DNA damage regulate tumor heterogeneity. Metabolic flexibility is a hallmark of cancer enabling tumor cells to produce energy and building blocks for growth. Furthermore, it maintains redox homeostasis by balancing reactive oxygen species (ROS) that influence tumor cell responses to DNA damage and thus, genomic instability as well as stem-like characteristics [66,67]. Interestingly, all of these mechanisms depend on the specific ECM environment in which tumor cells are located. Exposure of tumor cells to confining collagen microarchitectures (i.e. small pore sizes and short fiber length), for example, increases oxidative stress and ROS-responsive gene expression [54], which has been shown independently to promote the transition of quiescent CSCs to more proliferative CSCs [67]. In addition to affecting tumor cell phenotype through ROS-dependent changes in DNA damage, ECM-mediated cell confinement can alter DNA damage mechanisms directly. Indeed, tumor cell migration in confining ECM microarchitectures mechanically induces DNA damage due to nuclear deformation or rupture [68,69]. As CSCs are softer

and have lower ROS levels as well as increased antioxidant defenses relative to more differentiated tumor cells, they may be less susceptible to nuclear rupture-induced DNA damage under confining ECM conditions [67,70,71]. Collectively, these results imply that the ECM microenvironment impacts tumor heterogeneity by altering their metabolism and DNA damage response with functional consequences on tumor pheno- and genotype.

Conclusion and Future Perspectives

Studies with engineered ECM models suggest that CSCs interpret biophysical changes in the ECM differently than their differentiated counterparts. These differences may contribute to the pheno- and genotypic heterogeneity of tumors by inducing the transformation (e.g. through altered mechanotransduction) and selection (e.g. by affecting DNA-damage mechanisms) of tumor cells with stem-like properties. While these examples highlight how ECM changes alter cell behavior, ECM and cellular heterogeneity are reciprocally linked and it is the interplay between both that drives the evolution of the TME.

Although biomaterials approaches have advanced knowledge of how ECM biophysical properties regulate tumor cell heterogeneity, the opposite is much less clear; i.e., how specific tumor cell subpopulations affect ECM heterogeneity (Figure 1). Cells under nutrient-rich conditions, for example, increase ECM deposition and stiffness, while nutrient deprivation causes ECM degradation possibly explaining varied abundancy of ECM at the invasive front versus central regions of a tumor [72]. Furthermore, cells exposed to a specific ECM or biomaterial deposit new ECM, which, in turn, influences cell adhesion and proliferation independent of the initial materials properties [55,73]. Such changes can, for example, affect mechanosignaling-dependent, long-range interactions between distant cells [39,74]. Model systems that incorporate both cellular and ECM heterogeneity will be essential to better understand how the reciprocal links between both parameters affect tumor progression, therapy resistance, and metastasis (Figure 1).

Engineered tumor models conventionally use cell lines, which are intrinsically homogeneous and do not accurately reflect properties of the original cell source making it difficult to assign a specific result to a particular cancer [75]. Patient-derived organoids (PDOs) can mimic the intratumoral heterogeneity of a specific patient [76,77], but are challenging to expand and thus, have not been widely adapted in engineered model systems yet. Furthermore, their formation typically relies on the use of Matrigel, which is problematic given the poorly defined nature and batch-to-batch variability of Matrigel. Microengineered cell culture arrays can facilitate the large-scale formation of organoids at significantly reduced Matrigel concentrations, which has the potential to provide scalable methods to generate PDOs for mechanistic studies of ECM-dependent tumor heterogeneity [77].

Monitoring cellular responses to ECM heterogeneity in a spatiotemporally controlled manner is equally critical to delineate the functional and mechanistic relationships between cells and ECM in engineered tumor models. Highly, multiplexed imaging [78] as well as high-throughput image acquisition and analysis pipelines [77] permit maximizing the amount of information that can be extracted from precious samples. In addition, advances in spatial transcriptomics provide molecular information at near cellular resolution [79].

When combined with computational analysis tools of single-cell sequencing these methods yield spatially resolved information to better understand the mechanisms of ECM-dependent tumor heterogeneity [79].

In conclusion, compositional and physical changes of the ECM and their spatiotemporal variations synergistically contribute to tumor heterogeneity by guiding cell fate decisions in the TME. Engineered model systems that can recapitulate both cellular and ECM heterogeneity are critical to elucidate the mechanisms through which ECM characteristics and different cellular states are linked. When combined with enabling technologies to spatiotemporally profile cell states as a function of the ECM new insights into tumor progression, metastasis, and therapeutic resistance will emerge that have the potential to inform more efficacious anti-cancer therapies.

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When combined with a robotic system and automated image analysis, this platform enabled drug testing in precision medicine settings.

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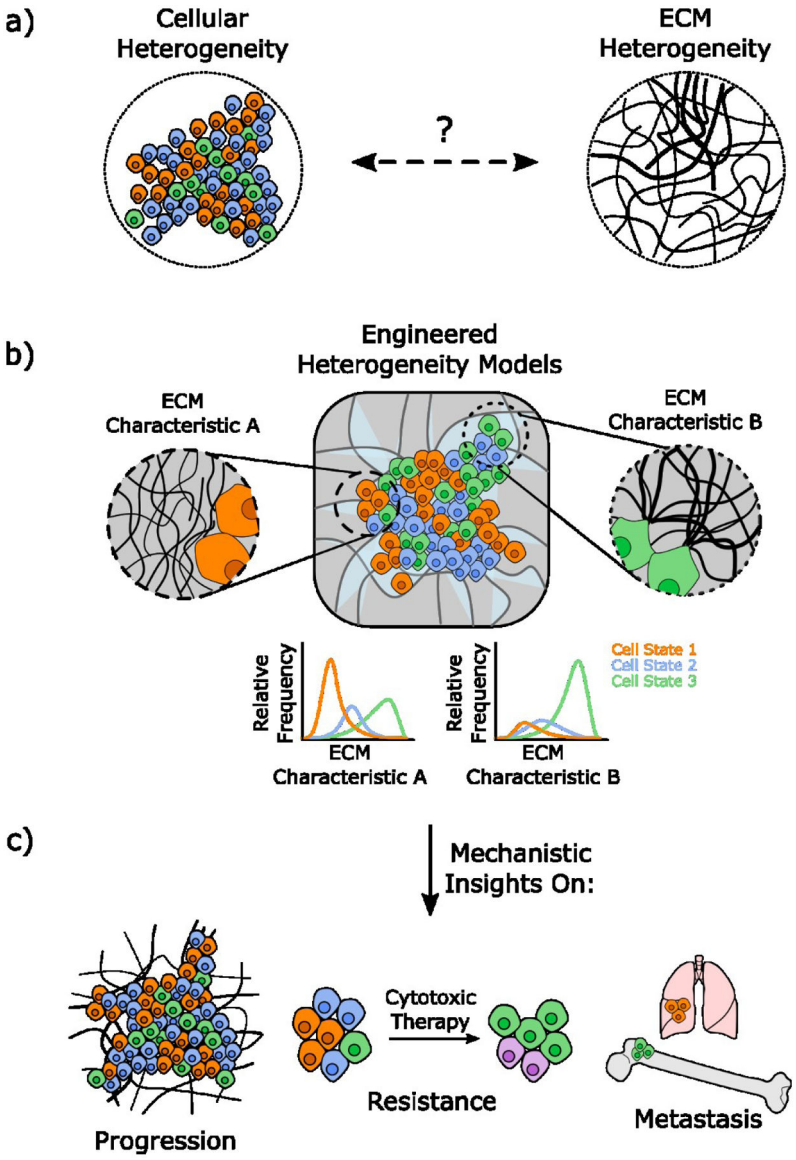

Figure 1: Engineered model systems to study tumor heterogeneity.

a) Tumors are characterized by both cellular and ECM heterogeneity. To understand how the interplay between both parameters affects cell fate decisions in the TME b) engineered heterogeneity models are needed that recapitulate both. c) Mechanistic studies with these models will enable new insights into tumor progression, therapy resistance, and metastasis.

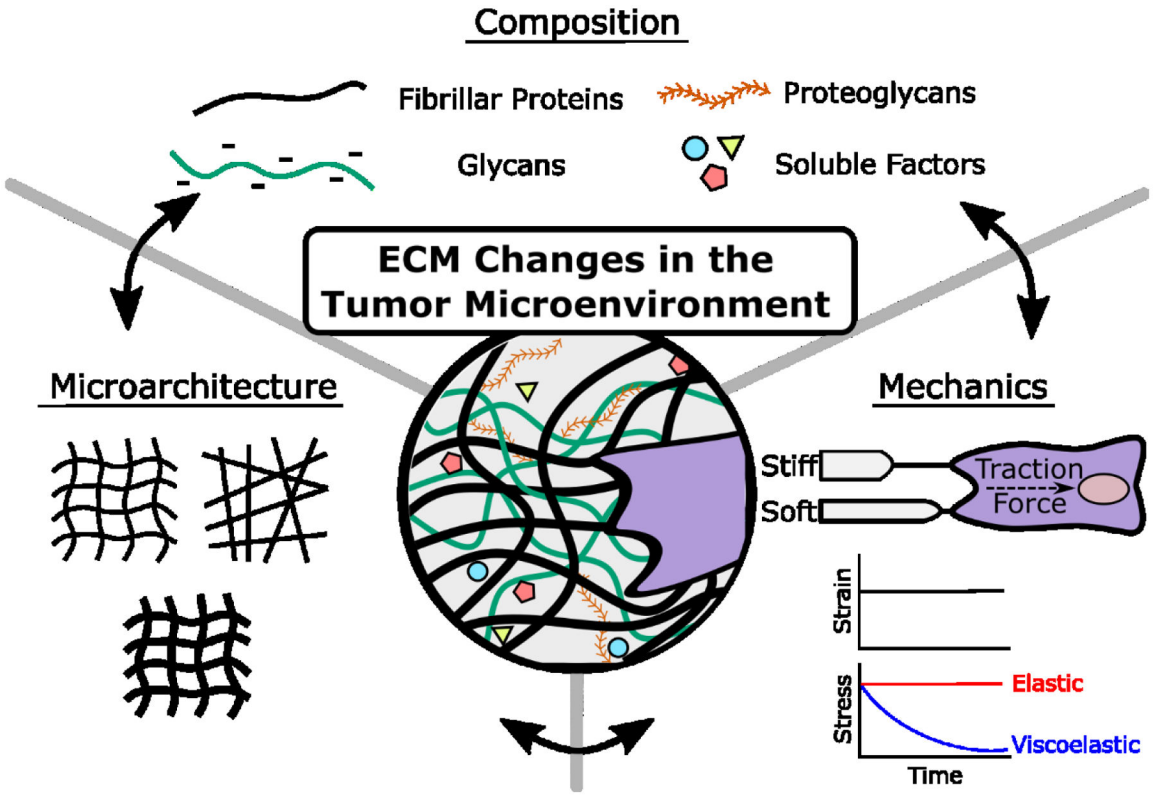

Figure 2: Extracellular matrix changes in the tumor microenvironment.

Tumors are characterized by changes in ECM composition including increased levels of fibrillar proteins (e.g. collagen and fibronectin), glycans (e.g. hyaluronic acid, proteoglycans), and soluble factors (e.g. growth factors, cytokines). Compositional changes entail physical ECM changes including microarchitectural (e.g. ECM fiber linearization and thickness) and mechanical changes (e.g. ECM elasticity [time-independent] or viscoelasticity [time-dependent, e.g. stress relaxation]). Compositional and physical changes are interdependent and lead to changes in cell phenotype.

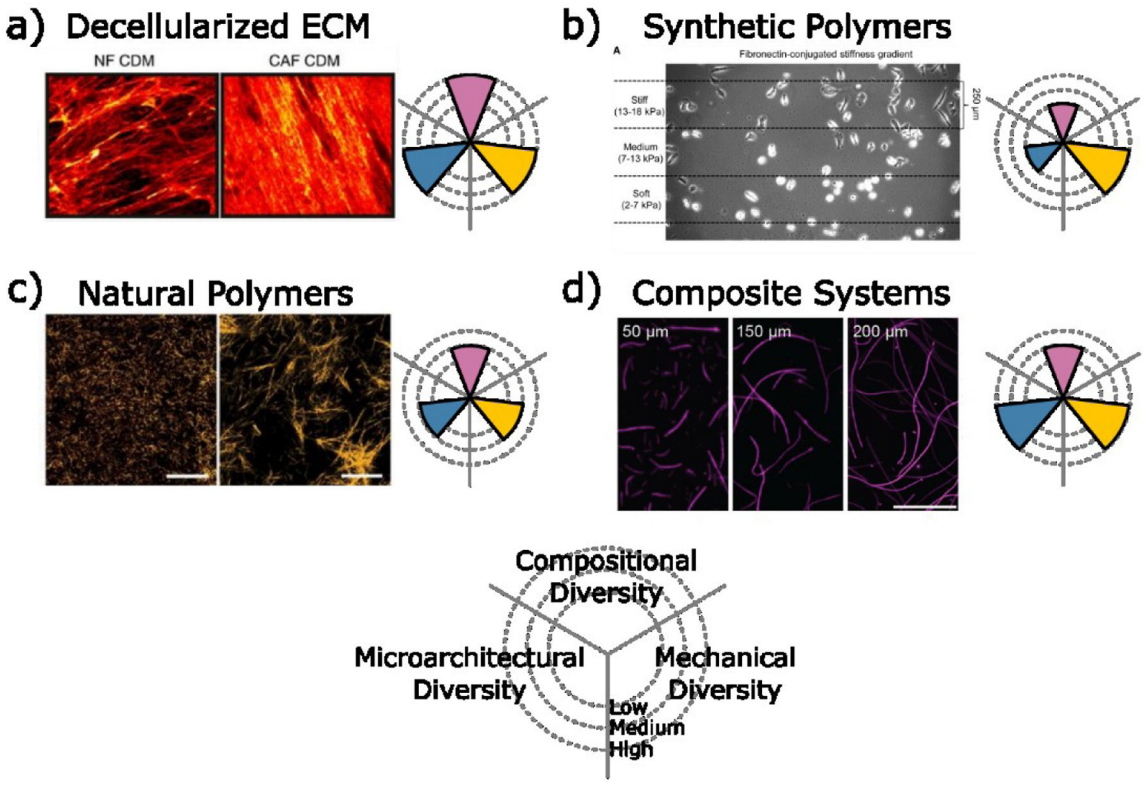

Figure 3: Examples of engineered model systems with controlled ECM properties.

a) Decellularized ECM from normal (NF) and cancer associated fibroblasts (CAF) visualized by collagen I staining [46]. Reproduced with permission of the Nature Publishing Group. b) Synthetic polymers such as polyacrylamide gels with stiffness gradients provide a range of mechanical diversity [47]. Reproduced with permission of Elsevier. c) Natural polymers such as collagen I provide control over ECM structure by adjusting the gelation parameters [51]. Reproduced with permission of the National Academy of Science. d) Composite systems of fibrous polymers encapsulated within hydrogels such as electrospun dextran vinyl sulfone fibers encapsulated in methacrylated gelatin enable microarchitectural and mechanical control [55]. Reproduced with permission of the American Chemical Society.