

Polymer Hydrogels to Guide Organotypic and Organoid Cultures

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Human organotypic and organoid cultures provide increasingly life-like models of tissue/organ development and disease, enable more realistic drug screening, and may ultimately pave the way for new therapies. A broad variety of extracellular matrix-based or inspired materials is instrumental in these approaches. In this review article, the foundations of the related materials design are summarized with an emphasis on the advantages and limitations of decellularized and reconstituted biopolymeric matrices as well as biohybrid and fully synthetic polymer hydrogel systems applied to enable specific organotypic and organoid cultures. Recent progress in the fabrication of defined hydrogel systems offering thoroughly tunable biochemical and biophysical properties is highlighted. Potentialities of hydrogel-based approaches to address the persisting challenges of organoid technologies, namely scalability, connectivity/integration, reproducibility, parallelization, and in situ monitoring are discussed.

1. Introduction


Mammalian morphogenesis is governed by distinct cell–matrix interactions which are reciprocally modulated across scales in space and time to enable formation, homeostasis, and regeneration of complex tissues and organs. Although much of our current understanding of cell fate control results from the creative use of simplistic 2D culture systems, growing evidence suggests that this format often induces aberrant cellular states like flattened morphology, altered gene expression, or abnormal polarization, and can therefore be misleading.^[1] This was impressively shown by cultures of normal human breast

epithelial cells in 2D which lead to a tumorous cell behavior, while the recapitulation of bonafide cell–matrix interactions, i.e. 3D culture in native-like environments, reverted cell growth to normal.^[2] The relevance of physical microenvironmental characteristics for cell fate determination recognized by these early findings of Bissell was later impressively proven by Engler et al.,^[3] and motivated the search for alternative culture formats that better mimic the spatiotemporal signals acting on cells in vivo. Highly hydrated polymeric structures—hydrogels—derived from or designed with inspiration (and/or components taken) from extracellular matrices (ECMs), the biopolymeric structures that surround cells in tissues, are critical enablers in many of the related approaches.

In particular, the use of reconstituted type I collagen hydrogels derived from rat tails,^[4] the identification of fibronectin as an important cell adhesion receptor ligand,^[5] the generation of a basement membrane (BM) matrix-based hydrogel from chondrosarcoma murine cells^[6] widely known as Matrigel, and the characterization of laminins^[7] contributed much to the progress in establishing effective 3D culture protocols.^[8] Organotypic cultures, here defined as tissue- or organ-analogs obtained by tissue-like assemblies of terminally differentiated cells (primary cells or cell lines) or derived from isolated tissue fragments, have led to seminal insights into tissue development and homeostasis in healthy and diseased conditions. However, even these more sophisticated 3D cultures often cannot fully account for the complexity of living matter. With the discovery of pluripotent stem cells (PSCs) and their capabilities to self-organize into structures resembling early stages of developing tissues, 3D cultures entered the next stage of complexity. Such assemblies are commonly referred to as organoids. The term was already used in 1946 to define a tumor-derived mass isolated from human tissue^[9] and later applied in several studies on the dissociation, re-aggregation, and self-organization of tissue preparations resembling the organ of origin.^[10] To date, organoids are generally considered as 3D multicellular structures generated in vitro from pluripotent (embryonic or induced) or tissue-derived stem cells that not only histologically resemble native organs, but also exhibit a certain level of their basic functionality.^[11] Impressive 3D recapitulations of miniature organ-specific 3D structures exemplifying this approach include intestine,^[12] brain,^[13] optic cup,^[14,15] stomach,^[16] liver,^[17] and pancreas^[18] among others. These cultures provide unprecedented opportunities to study

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human organ development and disease, serve as increasingly valuable tools for drug screening, and could enable the production of replacement tissues for regenerative medicine in the future. However, so far these examples rely on a limited set of culture-supporting materials and, in a majority of the studies, on the use of Matrigel, a permissive ECM preparation derived from mouse sarcomas which is afflicted with fundamental limitations due to its ill-defined composition, lacking reproducibility and tunability.^[19] Its animal-derived origin also restricts the use of the generated tissues in therapeutic applications. In consequence, a variety of better defined natural and synthetic polymeric hydrogel materials has been explored as alternatives to Matrigel for directing survival, growth, assembly, and differentiation of cells.^[20] These materials include complex reconstituted decellularized ECMs (dECMs), which efficiently support cell growth and differentiation in many explored systems but remain of limited reproducibility and tunability.^[21] Aiming at reducing the complexity and increasing the reproducibility, assemblies of natural single biopolymer components such as type I collagen, laminin, gelatin, fibrin, hyaluronic acid (HA), or mixtures thereof have been investigated.^[22] These components are ECM-derived and bioactive, i.e., contain cell adhesion and remodeling sites, however, the resulting hydrogels are often limited in processability and of relatively poor mechanical properties. During the last decade, a range of biohybrid- and fully synthetic hydrogel materials^[22b,23,24] has proven equivalent or even superior results compared to biopolymer-based systems in organoid and organotypic cultures.^[25] While the related materials cannot yet recapitulate the complexity in composition, structure, and bioactivity of ECM *in vivo*, their precise and consistent fabrication together with a modular composition provide high reproducibility and enable effective customization. The most common polymer base component in biohybrid and fully synthetic hydrogels is poly(ethylene glycol) (PEG)^[25] which can be end-functionalized with reactive groups that allow for cross-linking under cytocompatible conditions (see Section 3.4 for further details).^[26] Synthetic polymer hydrogels are often blended or covalently modified with ECM proteins (e.g., laminin) or cell-adhesive peptides (e.g., RGD) at precisely controlled concentrations. Incorporating matrix metalloproteinase (MMP)-cleavable degradation sites is widely used to tailor the cell-driven remodeling of biohybrid and synthetic hydrogel materials.^[27] Several lead examples meanwhile demonstrate distinct advantages of synthetic hydrogel-supported cultures when compared to Matrigel-based protocols.^[25b,28] It can be anticipated that more complex synthetic culture environments will massively progress the holistic and hypothesis-driven modulation of exogenous cues for the benefit of cell biology, biotechnology, and regenerative medicine.^[25]

In this review, we aim at providing an overview on the use of hydrogel-based culture systems for organotypic and organoid cultures ranging from very complex ECM-derived preparations over more simple biopolymer-based matrices to biohybrid and fully synthetic engineered materials. To set the scene, we briefly summarize some key aspects of ECM and their roles in morphogenesis. In the main part of the article, potentialities of the different hydrogel materials used in organotypic and organoid cultures are surveyed referring to groundbreaking exemplary applications. Finally, current challenges of organotypic and organoid cultures



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are being discussed with regard to the capabilities of hydrogel-based technologies that may allow for better addressing them. In particular, we consider emerging activities to use hydrogels

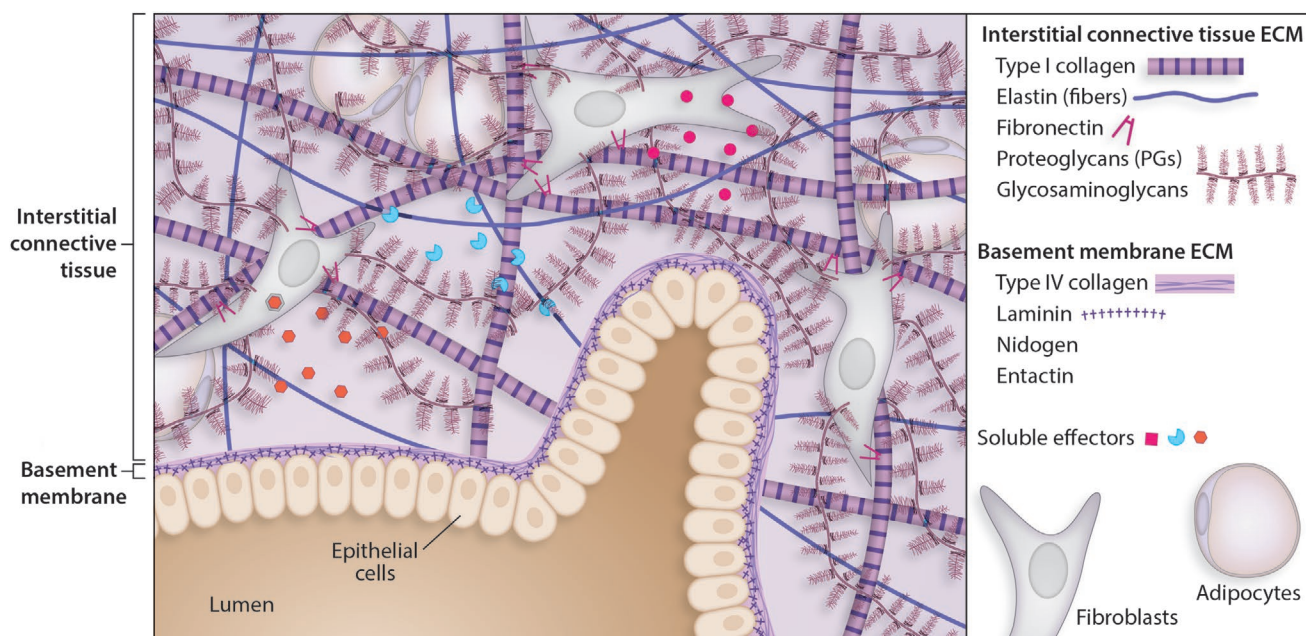


Figure 1. Schematic view of major classes of ECM components and main types of their supramolecular assemblies.

for introducing or facilitating scalability, connectivity/integration, reproducibility, parallelization, and in situ monitoring.

2. ECM in Morphogenesis

2.1. Emergence of ECM in Development

During embryonic development, totipotent stem cells proliferate and differentiate into tissues that give rise to complex organs affording a variety of specialized adaptive functions, thus creating the ever-stunning performance of living organisms. The involved developmental processes—as well as those governing homeostasis and regeneration—are inseparable from the dynamic and complex organization of ECM. First and foremost, ECM is being formed and (re-)organized by the cells upon proliferation, differentiation and assembly in characteristic patterns. In turn, matrix-based biochemical (specifically factor binding), structural (scaffolding), and mechanical (viscoelastic) cues act on cells in a spatiotemporal controlled manner and mediate adhesion, migration, proliferation, differentiation, as well as specific functional states of cells.

The development of a mammalian embryo from the fetus to a fully developed organism is a well-orchestrated, tightly controlled process accompanied and enabled by characteristic changes in the structure and composition of the tissue-associated ECM. Several loss-of function studies as well as work on ECM mutations have revealed the key role of ECM proteins such as laminin in development as their absence has led to birth defects or embryonic lethality.^[29] The first emergence of ECM molecules in a developing metazoan embryo was reported to occur at the eight cell stage with the expression of Laminin111,^[30] followed by type IV collagen deposition which

coincides with the assembly of a BM at the interface of the primitive endoderm and the inner cell mass, and underneath the parietal endoderm (Reichert's membrane), which extends over the trophoctoderm.^[30c,31] At this stage, the cells of the developing embryo lose their totipotency and develop into the pluripotent blastocyst. The herein assembled BM constitutes the earliest ECM that can be recognized as distinct structural entity. The emergence of characteristic ECM variants—as schematically depicted in **Figure 1**—comprises the formation of supramolecular assemblies of fibrillar proteins (e.g., collagens, elastin), glycoproteins (e.g., fibronectin, laminins), proteoglycans (e.g., perlecan, syndecan), glycosaminoglycans (e.g., heparan sulfate (HS), hyaluronan (HA)), which acquire a plethora of tissue- and organ-specific cross-scale structures out of combinations of two main elements, i.e. the interstitial/volume-filling, connective tissue-type ECM (IM) and the basal lamina/BM-type ECM.^[32] BMs are rich in laminins and type IV collagen, and form fine-structured, sheet-like assemblies containing nidogens as well as heparan sulfate proteoglycans (HSPGs).^[33] Their evolutionary emergence coincided with the origin of multicellularity in animals, suggesting that they are essential for the tissue formation.^[34] The IM is composed of fibronectin, elastic fibers, collagen I, vitronectin, glycosaminoglycans, and various matrix-cellular proteins such as thrombospondin,^[35] and undergoes alterations in the density and length of the contained collagen I fibrils during development but remains rather unchanged in postnatal growth.^[36] The fibrillar ECM proteins and glycoproteins provide cell adhesion binding sites governing cellular matrix anchorage and migration.^[37] Cell-secreted, soluble signaling molecules—morphogens/growth factors, cytokines—as well as proteases and protease inhibitors are reversibly associated with ECM structures and further tailor their functionalities. For example, the binding of the TGF- β dimer to fibrillin, vitronectin, and fibronectin results in an inactivation of the growth

factor which can be reversed upon proteolytic cleavage. This mechanism represents a major regulatory event in cardiac development,^[38] but has also been shown to be implicated in bone development.^[39] The binding of soluble factors is largely afforded by glycosaminoglycan (GAG) components of the ECM that electrostatically complex various morphogens, by that controlling their spatiotemporal distribution and availability, which is of fundamental importance in development.^[40] For instance, in the embryonic mouse midbrain the FGF8 gradient formation was shown to be regulated by the distribution of heparan- and chondroitin sulfate-GAG side chains of syndecans.^[41] Also, branching in the developing lung is controlled through GAG-mediated FGF signaling.^[42] Low O-sulfated HS facilitates diffusion and directs FGF10 and other distal signals from their source to target cells embedded in ECM containing GAGs of a higher sulfation degree.^[43] Furthermore, it has been shown that highly sulfated HS in BM reduces the rate of FGF2 movement through the matrix by several fold (100–1000) compared to when HS binding does not occur.^[43d]

It has been clearly demonstrated that a dysregulation in ECM deposition and function can have detrimental effects on the cells and their functions. For instance, abnormal BM dynamics can directly affect epithelial cell function, leading to pathological transformations.^[44] In particular, the role of ECM in cancer formation and metastasis is a subject of increasingly active research and covered by several excellent review articles.^[32e,44,45]

2.2. ECM Mechanics

The material properties of ECM manifest themselves in specific local and integral physical properties. In particular, given the physical coupling that results from cell–matrix adhesion, ECM creates and transmits mechanical signals including tensile, tractive and compressive forces as well as fluid shear forces which can activate intracellular signaling pathways and the cytoskeletal machinery^[46] acting on cells in development across scales.^[36a,47] Early in development, cell–cell contacts predominate, e.g., during the compaction phase at morula stage.^[47e] Forces derived intracellularly are transmitted via cell–cell contacts (e.g., cadherins) to neighboring cells or via traction to the ECM (e.g., integrins, focal adhesion (FA) complex)^[48] as it was holistically described by the tensegrity model.^[49] On the molecular level, conformational changes occur upon force application from a bent to an extended state of the integrin transmembrane adhesion receptors that lead to an increase of the affinity of the receptor for the ECM ligands.^[50] Forces are also transduced from the cells to various ECM ligands. For instance, ECM proteins such as fibronectin can unfold in response to mechanical stress to reveal cryptic binding sites that reinforce integrin adhesions and promote FA complex assembly.^[29c,51]

Assembly and remodeling during embryonic development lead to changes in ECM density, stiffness, viscoelasticity, as well as topology and fibrosity, thereby directing cell differentiation and the formation of functional tissue structures.^[32e,52] Mechanosensors and adhesion molecules located on the cell surface sense the changes of stiffness of the surrounding tissue and transduce this information into biochemical signals leading to changes of intracellular signaling including the Hippo

pathway.^[53] As recently explored in detail, ECM stiffness influences cellular processes including proliferation, migration, and stem cell fate specification.^[3a,54] The rigidity of the surrounding matrix was shown to alter the branching efficacy in endothelial sprouting as well as mammary branching by changing the actinomyosin contractibility.^[55] In mammary gland morphogenesis, branching is initiated at sites of highest mechanical stress and is stimulated by local activation of FA kinase. Induced by the contraction of the epithelium, matrix remodeling through localized fibronectin fibril assembly and deposition^[56] leads to the activation of a signaling cascade affecting Snail2 and E-Cadherin expression.^[55d,57] These changes induce a switch in cell behavior at the base of the progressing clefts associated with a more motile and less adhesive cell phenotype allowing the formation of transient intercellular gaps.^[57a] At the same time, metalloproteinase-mediated ECM cleavage and degradation at the edge of the terminal buds leads to thinner ECM sheets at the ends of the buds^[56b] than at the ducts allowing a better cell migration as well as an easier epithelial cell-budding process,^[29c,58] leading to a facilitated outgrowth of the tissue.

Shear stress, tension, or compression of cells are sensed by mechanosensitive ion channels, changes in receptor–ligand binding, deformation of the cytoskeleton and the primary cilium,^[36a] and are thought to direct the formation of organ systems. To which extent cells and tissues deform under forces depends on their intrinsic rheological properties.^[59] For instance, tensile forces, arising from stretching of bronchial epithelium during intrauterine breathing, support the development of the smooth muscle in the lung.^[60] Shear forces generated by maternal blood flow promote fetal hematopoiesis and the morphogenesis of the embryonic heart.^[61] Likewise, the formation of blood vessels,^[62] kidneys,^[63] muscle,^[64] joints,^[65] and bone^[66] has been demonstrated to critically depend on mechanical forces mediated by environmental ECM.

2.3. Transitory ECMs in Wound Healing and Regeneration

As another fundamentally important scenario, transitory ECM types control wound healing and regeneration. Upon injury, the fibrin clot formed through thrombin-mediated activation of fibrin(ogen) creates the initial, non-cellular matrix that itself undergoes an enzyme-triggered degradation process accompanied by the release of bioactive effectors, protein fragments and growth factors. Fibrin-based matrices trigger pro-inflammatory reactions and provide the first matrix for the cells involved in healing. This transitional matrix changes the biochemical and mechanical environment and instructs cell behavior during regeneration. Specific transitory ECM types have been shown to be instrumental for the powerful cardiac and skeletal muscle regeneration in newts.^[67] HA, tenascin-C and fibronectin are differentially expressed in the regenerating tissue,^[68] directly affecting cell behavior including proliferation, migration, myotube fragmentation and myoblast fusion.^[67b] Especially the expression of tenascin-C was shown to increase newt cardiomyocyte cell cycle reentry *in vitro*,^[69] highlighting opportunities to be investigated for future regenerative therapy approaches.

Our knowledge on the specific roles of particular ECM variants in stem cell regulation during development, homeostasis,

and regeneration creates the basis of materials design approaches that effectively template the balance of exogenous matrix signals and cellular ECM remodeling to direct organotypic and organoid cultures. The subsequent main part of the review (Section 3) is intended to provide a survey of the current status of the related efforts. While many protocols rely on the biochemical signature of tissue-specific ECM,^[70] epithelial and neural organoids were already successfully generated in animal-derived ECM that mimics a BM-like embryonic environment.^[6,34,71] Further sophistication in the rational design of emergence-inducing hydrogel matrices—together with other technological steps as outlined in Section 4—is anticipated to pave the way for more faithful *in vitro* models of tissues and organs. Importantly, not only the compositional (biochemical) but also physical (structural and mechanical) matrix features as well as their characteristic variations in space and time require consideration in the related approaches.

3. Engineering Cell- and Tissue-Instructive Hydrogel Materials

A vast array of biomaterials has been explored to support 3D cell cultures. The applied systems are mostly hydrogels, i.e., highly hydrated but insoluble polymer structures, and range from biopolymer-derived materials, including decellularized matrices and single biopolymeric preparations, to semi-synthetic or fully synthetic materials with thoroughly tunable mechanical and chemical properties. The above-described basic functional features of ECM environments generally serve as a design blueprint and are implemented in the engineered materials to different degrees—scaffolding, cell adhesion, complexation and redistribution of soluble signal molecules, tissue-like mechanical properties and remodeling, i.e., susceptibility to enzymatic cleavage, and assembly of cell-secreted matrix components are considered key criteria (compare **Table 1**).^[72] Many classical approaches to 3D culture made use of reconstituted type I collagen gels and BM/Matrigel preparations. However, to better and more precisely cope with the multifactorial regulation of cell fate control in organotypic and organoid cultures, a number of tunable hydrogel materials has been recently developed for tuning selected exogenous cell-instructive parameters to decipher their impact. In the first part of this section we summarize the related use of decellularized matrices and biopolymer-based hydrogels. Thereafter, we report on the application of biohybrid as well as fully synthetic polymeric hydrogel systems.

3.1. Multicomponent Biopolymer Hydrogels

3.1.1. Tissue- or Cell Culture-Derived Decellularized Matrices

At the beginning of the 20th century Loeb and Fleisher reported on the culture of animal-derived pieces of tissues *in vitro*.^[73] The approach has been refined and applied in studies on skeletal development^[74] and in investigations of kidney,^[75] lung,^[76] salivary gland,^[77] liver,^[78] pancreas,^[79] and mammary gland tissues.^[80] Considering the limited nutrient diffusion

across thicker tissues, organ slices, e.g., derived from the lung,^[81] small intestine,^[82] colon,^[82b,83] brain,^[84] and aorta^[85] have been cultured to characterize the histotypic relationships between various cell types within tissues and organs.^[86] A rather faithful recapitulation of tissue growth *in vitro* was achieved by using primary or pluripotent stem cell-derived cells in combination with decellularized ECMs.

Decellularization aims at isolating the ECM from its inhabiting cells while preserving posttranslational modifications of the molecular components as well as tissue-specific structural matrix features across scales. The approach was first reported in 1948^[87] and applied in 1964 for the preparation of dermal homografts.^[88] Later, decellularization was applied to the small intestinal submucosa for generating vascular graft constructs^[89] followed by various different tissue engineering applications. Several commercially available scaffolds for medical tissue repair such as AlloDerm, CuffPatch, MatriStem, Pelvicol, and Dura-Guard are based on the decellularization of dermis, small intestinal submucosa, urinary bladder, and pericardium of humans and animals.^[90] The rising number of patients with late- or end-stage failure of organs^[91] and the shortage of suitable donor organs resulted in a steadily increasing interest in the medical use of decellularized organs, including kidney,^[92] lung,^[93] heart,^[94] pancreas,^[95] and liver.^[90] An important advantage of these scaffolds is the readily re-establishing vascular networks at all hierarchical levels.^[96] Depending on the tissue of origin, the mechanical properties of these materials can however vary widely. A prominent translational experiment for the use of dECM scaffolds concerns the treatment of end-stage renal disease (**Figure 2**).^[92] Processed dECM scaffolds obtained by a specific detergent treatment (**Figure 2a**) were reseeded with suspended human umbilical venous endothelial cells (HUVECs) through the renal artery and suspended rat neonatal kidney cells through the ureter and cultured in an organ chamber (**Figure 2b**). Glomerular ECM structures were found to become predominantly repopulated with podocytes, whereas tubular structures were repopulated with tubular epithelial cells with re-established polarity, highlighting the value of native ECM for tissue regeneration. After several days in organ culture, regenerated kidney constructs produced urine *in vitro* (**Figure 2c**). In these regenerated kidneys, macromolecular sieving, glucose and electrolyte reabsorption were partially restored and creatinine clearance improved with increased renal arterial perfusion pressures, indicating successful engraftment and function of endothelial cells, podocytes, and tubular epithelial cells (**Figure 2d**).^[92]

In most of the reported examples, organ-derived dECM scaffolds were repopulated using HUVECs and tissue-specific progenitors. However, the alternative use of PSCs is considered attractive for scalable therapeutic applications.^[97] Accordingly, rat and human lung scaffolds have been repopulated with endothelial and perivascular cells derived from hiPSCs.^[98] Also, a decellularized human heart has been repopulated with hiPSC-derived cardiomyocytes^[94,99] leading to visible contractions with recordable repolarizations upon stimulation. For a comprehensive review on translational research toward clinical dECM applications the reader is referred to a related review.^[100]

Not only tissue can provide dECM, but also cells cultivated *in vitro*. However, the choice of the matrix-producing cell type

Table 1. Survey of polymer hydrogel systems for organotypic and organoid culture.

Origin	Basic materials characteristics			Cell-instructive properties			Applications in organotypic and organoid cultures	
	Composition	Physicochemical properties	Processing and handling	Scaffolding	Adhesiveness	Mechanical stimulation		Growth factor presentation
Decellularized matrices: dECM scaffolds								
From tissues/organs through decellularization-combination of physical (e.g., sonication), chemical (pH, surfactant, electrolyte), and enzymatic treatment (e.g., DNase)	Nearly complete set of tissue-specific ECM biopolymers Might contain growth factors	Large variation among tissues Generally softer than the tissue of origin Cross-scale heterogeneity of properties as in tissues/organs	Commonly used as macroscopic materials for re-seeding	Cross-scale structural templating Rapid generation of tissue- and organ-specific constructs, including intact vascular network Supports alignment of cells as in tissue of origin	Provides adhesive anchorage through ligand types and distribution as in tissue of origin	Varies locally and resembles target tissue closely	Effective redistribution (sequestration and release) of cytokines, chemokines, growth factors as in functional tissue Limited tunability	Kidney, ^[90] lung, ^[93,98] heart, ^[94,99] pancreas, ^[95] liver ^[90]
From cell cultures derived through decellularization-processed as above	Nearly complete set of tissue-specific ECM biopolymers Might contain growth factors Can be influenced by choice of cells, culture conditions, and decellularization protocols	A few hundred nm to 100 μm thick Tissue-analog ultrastructure Elasticity (AFM): 0.1–1 kPa ^[107] Optionally enhanced by MMC (deposition of fibrillar ECM proteins and GAGs) ^[110–112]	2D cell culture only	Local structural templating Rapid generation of tissue-specific constructs Supports alignment of cells with certain degrees of three-dimensionality	Provides adhesive anchorage through ligand types and distribution as deposited by cells in culture	Varies locally similar to tissues	Effectively redistributes cytokines, chemokines, growth factors as in functional tissue (sequestration and release) Limited tunability	hESC culture, ^[102g] osteogenic differentiation, ^[102b,104b,c] HSC niche ^[107]
Decellularized matrices: dECM hydrogels								
Decellularized organs (processed as above) are chopped into small pieces, ground into a powder and then digested into a liquid solution	Nearly complete set of tissue-specific ECM biopolymers Might contain growth factors Large compositional differences in Collagen I and GAG content ^[31]	Fibrillated ultrastructure with a differently interwoven network Stiffness range: 20 Pa (porcine testis) ^[31] to 550 Pa (porcine dermis) ^[31] Tuning of stiffness by chemical cross-linkers is possible Optionally modulation of architecture and mechanical properties by MMC ^[123]	Suitable for embedding cells ECM fiber self-assembly upon neutralization at 37 °C, working on ice avoids premature fibrillogenesis Can be used for extrusion-based bioprinting ^[133]	Locally template tissue-specific assembly of structures Inducing in vivo like modulation of cell metabolic activity, proliferation, morphology and differentiation	Provides localized adhesive anchorage through ligand types and distribution similar to the tissue of origin	Differs from tissue of origin (softer)	Affords complex-ation of cytokines, chemokines, growth factors similarly to functional tissue Redistributes growth factors effectively (sequestration and release) Limited tunability	Mouse kidney stem cells, ^[17b] skeletal injury model using C2C12 myoblasts, ^[31] rat and human islet cultures, ^[118] mammary, ^[132] testicular organoids, ^[131] cardiac progenitor culture, ^[119,120,122] differentiation of hESCs, ^[122] murine and human intestinal organoids, human gastric organoids, hepatocyte and liver duct organoids as well as pancreatic ducts ^[70]

Table 1. Continued.

Basic materials characteristics			Cell-instructive properties				Applications in organotypic and organoid cultures		
Origin	Composition	Physicochemical properties	Processing and handling	Scaffolding	Adhesiveness	Mechanical stimulation	Growth factor presentation	Susceptibility for remodeling	
Decellularized matrices: Matrigel, Cultrex BME, EHS matrix									
From murine Engelbreth-Holm-Swarm tumor-derived decellularization	Mixture of ECM proteins from BMs reconstituted at high protein concentrations (8–22 mg mL ⁻¹) Main components: laminin (60%), collagen IV (30%), entactin (8%); GAGs, growth factors (IGF1, FGF2, EGF, PDGF, NGF, VEGF, etc.), other proteins of unknown roles ^[134b] Also available as growth factor reduced (GFR) variant Varying composition influencing hydrogel properties among suppliers Exposure of cryptic laminin motifs to cells due to purification process ^[314]	Forms a nonporous, viscoelastic ^[126b] soft (450 Pa) hydrogel ^[127b] that contains regions of increased stiffness (1–2 kPa) ^[127b] Chemical crosslinking for elevated stiffness possible ^[315]	Suitable for embedding cells Processed at 4 °C, gels at 37 °C	Provides a BM-like local structural support to guide self-assembly and epithelialization Enables human embryonic stem cell maintenance	Provides cell-adhesive protein domains at sufficiently high densities to promote cell attachment ^[79]	Promotes growth of soft tissues, specifically supports epithelialization and tubulogenesis	Undergoes enzymatic degradation and matrix remodeling (IGF-1, TGF- β , EGF, PDGF, bFGF, NGF, VEGF) ^[124a]	Presents a range of growth supporting factors (IGF-1, TGF- β , EGF, PDGF, bFGF, NGF, VEGF) ^[124a]	HUVECs to form vascular tubular networks; ^[138,139b,c] MDCK-acini formation and branching; ^[319] acini formation from several organs; ^[140–143] branching morphogenesis from several organs; ^[316] organoids from: intestine; ^[12,157] colon; ^[138] gallbladder; ^[159] liver; ^[17,18a] pancreas; ^[18b] stomach; ^[16,170] kidney; ^[172] esophagus; ^[166a] fallopian tube; ^[163] mammary gland; ^[160] lung; ^[318] prostate; ^[162] salivary gland; ^[165b] taste buds; ^[161] brain; ^[13] midbrain-like organoids; ^[168] neuroepithelial cysts; ^[169] murine limb bud ^[173]
Decellularized matrices: Matrigel, Collagen I									
Combination of Matrigel and Collagen I at different ratios	Incorporation of Matrigel into collagen I increases pore size, stiffness, and heterogeneity of underlying mesh (213.53 Pa vs 30.22 Pa) ^[317]	Suitable for embedding cells Neutralization of solubilized collagen I on ice, mixing with Matrigel and cells, casting at 37 °C	Mimics disorganized BM found in invasive cancer conditions ^[50] Locally supports BM formation and epithelial polarization contrary to Collagen I hydrogels ^[320]	Similar to individual components	Supports cyst formation in soft gels Induces a transition from acinar to protrusive acinar and invasive morphology in epithelial cultures in stiffer gels	Allows increased MMP-mediated matrix degradation with increasing Matrigel concentration ^[317]	Similar to individual components, however less concentrated	Blood vessel organoid; ^[272] angiogenesis; ^[321] MDCK cyst; ^[315,320] mammary; ^[320] epithelial morphogenesis models ^[319]	

Table 1. Continued.

Basic materials characteristics			Cell-instructive properties				Applications in organotypic and organoid cultures	
Origin	Composition	Physicochemical properties	Processing and handling	Scaffolding	Adhesiveness	Mechanical stimulation	Growth factor presentation	Susceptibility for remodeling
Decellularized matrices: Matrigel, Alginate								
	Combination of Matrigel and Alginate	Stiffness adapted by varying the calcium concentration (1×10^{-3} M \rightarrow 21×10^{-3} M)	Suitable for embedding cells Casted using a double syringe filled with mixed alginate precursor, Matrigel and cells in one side and Ca-solution in the other side	Locally mimics healthy and diseased mammary tissue	Similar to individual components	Supports cyst formation in soft gels Induces invasive epithelial morphogenesis upon an increase in stiffness (100 Pa \rightarrow 2000 Pa)	Similar to individual components, however less concentrated	Similar to individual components Mammary breast cancer model ^[236,238]
Biopolymer-based hydrogels: Laminin								
Isolated from the murine Engelbreth-Holm-Swarm tumor or obtained by recombinant production	Laminin/entactin complex Individual laminin proteins or fragments to decorate hydrogels	Viscoelastic, soft polymer networks ^[9] The supramolecular structure resembles a quasi-regular mesh array of interconnecting struts (and somewhat flexible) Reconstituted laminin polymer struts measured 31 ± 8 nm ^[322]	Suitable for embedding cells High-concentration laminin/entactin is processed at 4 °C, gels at elevated temperatures via a cooperative heat-gelation requiring divalent cations (calcium)	Provides a BM-like local support to guide cell expansion, differentiation and morphogenesis Supports apical-basal polarity induction	Allows adhesion via β 1-mediated integrin signaling involving different α -subunits (skeletal muscle: α 7, hESC: α 6, endothelial cells: α 1, α 2, α 3, α 6) Gamma chain of Laminin 111 is involved in adhesion	Promotes growth of soft tissues, (e.g., neuronal cultures) and cyst formation	Presents several growth factors based on the presence of heparin-binding sites in the LG subunits (e.g., members of the VEGF/PDGF family, VEGF-A165, placental growth factor (PlGF)-2, PDGF-AA, PDGF-BB, PDGF-CC, FGF-2, FGF-7, FGF-10, FGF-18, BMP-2, BMP-3, NT-3, and BDNF) ^[326]	Allows for proteolytic degradation and association of further ECM components Neuroepithelial cysts ^[69a]
Biopolymer-based hydrogels: Fibrin								
Formed from fibrinogen, isolated from human plasma, upon addition of thrombin	Proteinaceous network of self-assembled fibrin monomers	Viscoelastic protein network Very soft (<100 Pa) Fibrillated ultrastructure ^[323] Pore size: ≈ 4 μ m ^[17] Varying the concentrations of fibrinogen and thrombin allows for tuning the mechanical properties Contains three putative β -integrin binding sites and a non-RGD dodecapeptide sequence in the gamma chain ^[324] Dynamically altered mechanical stimulation	Suitable for embedding cells Gel assembly by combining fibrinogen and thrombin using a dual-barrel syringe Enzymatic cleavage of fibrinogen by thrombin to induce fibrin formation	Provides local structural support for neurite or vessel outgrowth Supports polarized epithelial growth and tubulogenesis Promotes engraftment, vascularization, and function of transplanted islets	Anchors α v/ β 1 and α 5/ β 1, α 1b/ β 3 integrin on platelets, ICAM-1 on fibroblasts and VE-Cadherin on endothelial cells	Promotes neuronal outgrowth in soft hydrogels (\approx 50 Pa) Promotes sprouting of endothelial cells in soft, malleable hydrogels ^[325]	Serves as a depot for growth factors ^[78] FXIIIa crosslinking metallopeptinases can be used to incorporate engineered factors such as VEGF ^[79] Serum plasminogen in the medium also sustains degradation	Degrades upon activity of metalloproteinases and plasminogen from encapsulated cells Angiogenesis-like 3D capillary networks, ^[79] Pancreatic islets, ^[80] neurite growth, ^[327] angiogenesis, ^[328] epithelial growth and branching ^[58]

Table 1. Continued.

Origin	Basic materials characteristics			Cell-instructive properties			Applications in organotypic and organoid cultures		
	Composition	Physicochemical properties	Processing and handling	Scaffolding	Adhesiveness	Mechanical stimulation		Growth factor presentation	Susceptibility for remodeling
Biopolymer-based hydrogels: Fibrin, hyaluronic acid, laminin									
Fibrinogen and thrombin isolated from salmon ^[228]	Combination of a self-assembled fibrin network and an interpenetrating network of hyaluronic acid and laminin	Elastic modulus strongly depends on protein concentration (2.7 mg mL ⁻¹ -30 Pa; 8 mg mL ⁻¹ -800 Pa) Increased stiffness upon HA addition (119.23 ± 16.48 Pa → 202.3 ± 17.33 Pa) Fibrillated, interwoven network in which HA may surround fibrin fibers	Suitable for embedding cells Gel assembly by using a dual barrel syringe containing thrombin and a combination of salmon fibrinogen, thiolated HA and laminin	Provides local structural support for neurite outgrowth of rodent neurons and for mimicking the neurovascular niche Provides local guidance for vessel formation in laminin supplemented hybrid gels	Similar to individual components Binding and activation of integrins is affected due to a different location of the RGD sequence in salmon fibrin	Provides a stiffness similar to CNS Supports proliferation and differentiation of neural progenitors and neurite outgrowth	Similar to individual components	Degrades slower than human or bovine-derived fibrin gels thereby promoting a longer scaffolding	Neurovascular niche ^[176]
Biopolymer-based hydrogels: Fibrin, laminin/Matrigel									
Self-assembled fibrin network supplemented with laminin/entactin or GFR Matrigel	Sparse network of thick fibers of varying stiffness (1.5-7.7 mg mL ⁻¹ fibrin: 24 ± 10 Pa-492 ± 78 Pa) Integrated polymer network formation of laminin within the fibrin scaffold	Suitable for embedding cells Gel assembly using a dual barrel syringe containing thrombin and a combination of fibrinogen, fibronectin, nanocellulose, GFR Matrigel or laminin, and cells	Provides local support to guide cell growth and cyst formation Provides BM-like local structural support due to the incorporation of laminin to allow epithelialization, polarization and organoid differentiation	Provides sufficient adhesion sites to promote intestinal cyst formation Promotes organoid differentiation upon laminin incorporation	Supports cyst formation in soft gels (24 ± 10 Pa -270 ± 8 Pa, 1.5-6 mg mL ⁻¹ fibrin)	Elevated stiffness (492 ± 78 Pa; 7.5 mg mL ⁻¹ fibrin) leads to dense spheroid formation without differentiation into budding organoids	Similar to individual components	Degrades slowly upon the addition of laminin or Matrigel	Murine intestinal organoids, human pancreas organoids, human pancreatic ductal adenocarcinoma organoids, human small intestinal organoids, and human liver organoids ^[177]

Table 1. Continued.

Basic materials characteristics			Cell-instructive properties				Applications in organotypic and organoid cultures	
Origin	Composition	Physicochemical properties	Processing and handling	Scaffolding	Adhesiveness	Mechanical stimulation	Growth factor presentation	Susceptibility for remodeling
Biopolymer-based hydrogels: Collagen I								
Isolated from rat tail tendon via acid solubilization	Triple helical, fibrous protein	Reconstituted fibrillar structure	Suitable for embedding cells	Exhibits local structural and mechanical properties reminiscent of connective tissues	Provides several integrin-binding domains	Promotes epithelial and endothelial organotypic cultures and epithelial organoid formation in soft, tissue-like hydrogels	–	Contains target sequences of matrix metalloproteinases (MMP)
To harvest highly crosslinked collagens (e.g., from porcine skin, bovine skin) a combination of neutral salt solution with proteolytic extraction (pepsin digestion) is often used ^[22a]	Highly conserved among mammals (100 Pa - 2 kPa) adjustable by varying pH, gelation temperature, and collagen concentration ^[320]	Limited range of elasticity (100 Pa - 2 kPa) adjustable by varying pH, gelation temperature, and collagen concentration ^[320]	Self-assembling within 30 min upon neutralization at 37 °C	Supports polarized epithelial growth and tubulogenesis	GFOGER protein motif (where O is hydroxypoline) represents a high-affinity binding site for all collagen-binding integrins ^[321] including $\alpha2\beta1$, $\alpha1\beta1$, but also $\alpha1\beta1$, $\alpha10\beta1$, $\alpha\beta3$, $\alpha\beta5$	Changes in stiffness induce switch from acinar to invasive tumor morphology	–	Smooth muscle cells, ^[184] MSC differentiation, ^[185] adipocytes culture and differentiation, ^[186] epithelial growth and branching of several organs. ^[3-190,322]
	Increase of stiffness by cross-linking, ^[200] ultracentrifugation, ^[201] gel compression, ^[202] evaporating the solvent ^[203] or chemical modification ^[204]	Increase of stiffness by cross-linking, ^[200] ultracentrifugation, ^[201] gel compression, ^[202] evaporating the solvent ^[203] or chemical modification ^[204]	Working on ice avoids premature fibrillogenesis	Allows for axonal outgrowth without network formation				tumor modeling, ^[191-194] angiogenesis ^[196,197,323]
	Mild stiffness adjustment via transglutaminase-mediated crosslinking ^[198] and MMC ^[207c]	Mild stiffness adjustment via transglutaminase-mediated crosslinking ^[198] and MMC ^[207c]	Can be used for extrusion-based bioprinting					Human and murine intestinal organoid cultures ^[209,210]
Biopolymer-based hydrogels: Collagen I, laminin, fibronectin, hyaluronic acid								
Collagen I supplemented with HA (150 and 500 kDa), laminin, and fibronectin	Significantly higher swelling ratio than pure collagen gels (306.94 ± 6.29 vs 290.10 ± 0.81, $p < 0.01$)	Significantly increased elasticity relative to collagen gels (Young's modulus: 256.7 ± 20.0 Pa vs 559.2 ± 204.0 Pa)	Suitable for embedding cells	Local structural support mimicking mammary tissue	Promotes integrin-mediated cell adhesion similar to breast tissue	Supports polarized epithelial growth and maturation by softness	Similar to individual components	Allows for proteolytic degradation and association of further ECM components
	vs 290.10 ± 0.81, $p < 0.01$)	Significantly increased elasticity relative to collagen gels (Young's modulus: 256.7 ± 20.0 Pa vs 559.2 ± 204.0 Pa)	incubated with cells at 37 °C, partial assembly occurs within 5 min, full solidification within 1h	Provides a BM-like support due to the incorporation of laminin guiding epithelial tissues with ductal and lobular morphologies similar to human breast tissue				Mammary tissue growth ^[175]

Table 1. Continued.

Basic materials characteristics			Cell-instructive properties				Applications in organotypic and organoid cultures	
Origin	Composition	Physicochemical properties	Processing and handling	Scaffolding	Adhesiveness	Mechanical stimulation	Growth factor presentation	Susceptibility for remodeling
Biopolymer-based hydrogels: Gelatin								
Soluble derivative of collagen I generated via irreversible denaturation either through acidic hydrolysis (H ₂ SO ₄ or HCl) or alkaline hydrolysis (NaOH)	Polymer with the same amino acid sequence as Collagen I but lacking the triple helical character	Amorphous, thermo-responsive material containing refolded triple helix segments of 100–200 amino acids in length Highly variable physico-chemical properties with low intrinsic viscosity, better solubility and less antigenicity compared to collagen I Tuning of stiffness by chemical cross-linkers or blending with polysaccharides is possible Photocrosslinking after methacryloyl functionalization (GelMa) ^[334] provides increased mechanical tunability (2–30 kPa)	Suitable for embedding cells Gels spontaneously form when cooled below 37 °C GelMa requires a photo-initiated free-radical polymerization reaction GelMa was applied to neovascularization ^[223]	Provides local structural support for fetal rat ventricular cell growth leading to rhythmic contraction of embedded cells Stimulates vessel ingrowth in gelatin sponge after subcutaneous transplantation GelMa provides porous scaffold for fibroblasts and cardiomyocytes	Provides adhesion through presentation of peptides (RGD)	Spreading and networking of embedded fibroblasts and cardiomyocytes by adjusting the mechanical properties (4.5 ± 0.8 kPa) resulting in increased metabolic activity ^[339] Mimics the mechanical properties of neonatal hearts	Electrostatically complexes positively charged growth factors (e.g., bFGF, SDF-1, VEGF) ^[340]	Contains target metalloproteinases (MMP) rendering it degradable Cardiac grafts based on fetal rat ventricular muscle, ^[2,14] angiogenesis, ^[2,15a] colonoids, ^[223] cardiac fibrosis ^[339]
Biopolymer-based hydrogels: Hyaluronic acid								
From rooster combs, shark skin, bovine eyeballs, or human umbilical cords ^[335] Animal-free production of high MW HA (>1 MDa) via microbial fermentation (Streptococci)	Hydrophilic, high MW polysaccharide composed of a repeating disaccharide unit of glucuronate and N-acetylglucosamine The only non-sulfated glycosaminoglycan Not covalently associated with a core protein HA-batches vary in MW and MW distribution and can contain protein impurities ^[336]	Randomly interconnected chains lacking structural complexity Poor mechanical properties Low MW HA (50 kDa) viscous fluid (G' = 1–100 Pa); high MW HA (1200 kDa) network formation through chain entanglement (G' = 50–1000 Pa) ^[338] Hydrazide-, thiol-, and methacrylate-functionalization for tunable stiffness, pore size, degradation rates Thiol-modified HA crosslinks slowly by disulfide bridge formation (G' 13 Pa) ^[337]	Suitable for embedding cells Cell embedding by mixing SH-HA with crosslinker and cells Can be lyophilized to form sponges MeHA requires a photo-initiated free-radical polymerization reaction	Mimics natural microenvironment of hESC and of tumors Provides local microenvironment to support neurite outgrowth	Supports cell adhesion via CD44, CD168 or ICAM-1 Allows CD44-mediated differentiation toward bone	Provides resistance to compressive forces Supports endothelial cell proliferation in soft environments and inhibits angiogenesis in high MW, stiff gels	Binds IGFBP-3 and -5 Provides limited capacity to bind growth factors (e.g., VEGF, ^[341] BMP, ^[343] NGF, SDF-1 α) ^[342]	Allows for hyaluronidases-mediated remodeling ^[267] or for degradation by reactive oxygen species ^[346] Engineered MMP degradation sites allow further degradation Undifferentiated hESC growth, ^[344] bone and cartilage engineering, ^[345] neural differentiation, ^[266] tumor modeling ^[229–231]

Table 1. Continued.

Basic materials characteristics			Cell-instructive properties				Applications in organotypic and organoid cultures		
Origin	Composition	Physicochemical properties	Processing and handling	Scaffolding	Adhesiveness	Mechanical stimulation	Growth factor presentation	Susceptibility for remodeling	
		Photo-crosslinking after methacryloyl functionalization (MeHA) provides increased mechanical tunability (150–2600 Pa) ^[347] Microbial fermented HA synthesis provides pure, well-defined batches ^[348]							
Biopolymer-based hydrogels: Alginate									
Extracted from brown algae (<i>Laminaria hyperborea</i> , <i>Laminaria digitata</i> , <i>Laminaria japonica</i> , <i>Ascophyllum nodosum</i> , and <i>Macrocystis pyrifera</i>) Alginate synthesized by bacteria (Azotobacter or Pseudomonas)	Anionic, linear unbranched polysaccharide composed of binary copolymers of (1-4)-linked α -L-guluronic (G-Block) and β -D-mannuronic acid (M-Block) residues of widely varying composition and sequence depending on its origin	Tunable physicochemical properties (e.g., 0.7% alginate: 200 Pa; 3% alginate: 12 kPa) Enhanced stiffness by increasing the length of G-block and MW (32 000–400 000 g mol ⁻¹) Azotobacter derived alginate contains higher fraction of G blocks, provides higher stiffness, ^[349] and provides more defined structures Ultrastructure consists of submicron size pores with sizes depending on G and M block content Higher stiffness leads to lower permeability ^[350] Photo cross-linking (methacrylate modified alginate) and chemical/thermal crosslinking protocols are available ^[233] limited long-term stability in physiological conditions due to release of divalent ions (exchange with monovalent cations)	Suitable for embedding cells Formed by non-covalent, electrostatic complexation via ionic cross-linking (Ca ²⁺ /Ba ²⁺) under mild gelation conditions ^[233] Gelation rate is critical for uniformity and strength Allows cell recovery by exchange of divalent (calcium) by monovalent cations (sodium) resulting in gel fragmentation	Polymer network embedding Provides structural guidance and tunable mechanical strength	Does not contain intrinsic adhesion sites for cellular adhesion receptors Promotes adhesion after chemical modification with adhesive ligands, e.g., RGD	Supports cell viability and differentiation of mesenchyme containing hindgut spheroids in 1% alginate gels of higher stiffness compared to Matrigel ^[232] Supports differentiation of intestinal organoid in the presence of ECM secreting mesenchyme and neurite outgrowth in soft, unmodified gels (0.6 kPa) ^[232,351]	–	Does not allow enzymatic remodeling as mammals lack alginase Partial oxidation by periodate make alginate gels degradable (rate depends on degree of oxidation, pH and temperature)	Intestinal organoids ^[232]

Table 1. Continued.

Basic materials characteristics			Cell-instructive properties				Applications in organotypic and organoid cultures		
Origin	Composition	Physicochemical properties	Processing and handling	Scaffolding	Adhesiveness	Mechanical stimulation	Growth factor presentation	Susceptibility for remodeling	
Biopolymer-based hydrogels: Silk fibroin									
From spiders, the larvae of <i>Bombyx mori</i> , other moth genera and numerous other insects	Insoluble fiber-forming protein consisting of layers of antiparallel β -sheets	Tight packing of sheets results in rigid structure and tensile strength Ultrastructure varies from leaf-like morphology to sponge-like structures with pore sizes of $5.0 \pm 4.2 \mu\text{m}$ depending on initial protein concentration and gelling temperature Tunable mechanical properties (1% silk protein: 4.8 kPa - 8% silk protein: 33.1 kPa) ^[352] adjustable up to 400 kPa ^[353]	Suitable for embedding cells Formation through physical crosslinking of large hydrophobic blocks of amino acids forming β -sheets when activated by environmental changes ^[341] Blending with other ECM materials is often applied	Polymer network embedding Provides structural guidance and tunable mechanical strength	Promotes RGD-mediated cell adhesion in fibroin <i>Antheraea pernyi</i> and <i>Antheraea mylitta</i> Does not promote cell adhesion when fibroin protein from other species is used	Mimics stiffness of murine (49.1 \pm 6.7 kPa) or rat (46.8 \pm 8.9 kPa) brain Promotes axonal outgrowth by adjusting the stiffness and collagen I supplementation	Does not promote intrinsic GF binding Cell-mediated GAG deposition can result in factor complexation of silk scaffolds	– Cartilage/bone cultures, ^[243,244] cortical brain ^[246] and brain tumors ^[247]	
Recombinant protein-based hydrogels									
Host system expression of proteins and polypeptides starting from designed amino acid sequences	Defined polypeptide containing a fibronectin-derived amino acid sequence and elastin-like structural domain	Modular design decoupled biochemical and mechanical properties Adhesive cues (RGD-containing fibronectin domain) and elastomeric structural cues (elastin-like domain) $E = 0.5\text{--}50 \text{ kPa}$ ^[232d]	Cell embedding through THPC-mediated covalent crosslinking Material processing: porous scaffolds, ^[354] fibrous scaffolds, ^[355] microfabrication strategies for controlled cross-scale cell alignment ^[26]	Provides local support to guide cell self-assembly and epithelialization	Enhances intestine differentiation through RGD adhesive domain	Induces murine intestine differentiation more efficiently in soft ($G' = 180 \text{ Pa}$) hydrogels	–	Allows for mechanical deformation in soft hydrogels Promotes MMP-mediated remodeling within the fibronectin-derived domain in stiffer matrices	Murine intestine ^[2,52e]
Biohybrid hydrogels									
PEG (polyethylene glycol); commonly obtained by ring-opening polymerization of ethylene oxide) + proteins (animal derived)	8 arm PEG and alkaline-treated gelatin	Tunable physicochemical properties Incorporation of non-modified ECM proteins Modulation of remodeling properties through variation of PEG:gelatin ratio $E = 2.6\text{--}6.8 \text{ kPa}$ ^[255d]	Suitable for embedding cells FXIIIa-mediated crosslinking (PEG-Gln + Lys residues on gelatin) Crosslinking of additional Lys-containing proteins (e.g., laminin-111, laminin-521) ^[255c–356]	Provides local support to guide cell differentiation and self-assembly Supports matrix mineralization through nucleation sites within the gelatin component Provides a BM-like local structural support through the incorporation of laminins	Mediates adhesion via β 1-mediated integrin signaling enhances vasculogenesis and osteogenesis through incorporation of laminin-111 Induces stronger liver differentiation in the presence of laminin-111 and laminin-521 compared to Matrigel	Promotes the differentiation of vascularized MSC-derived bone and liver when elastic moduli are adjusted to be similar to those of Matrigel (2.6 kPa)	Gelatin electrostatically complexes positively charged growth factors (e.g., bFGF, SDF-1, VEGF) ^[340]	Promotes degradation of the gelatin component and replacement by secreted ECM from encapsulated cells	Vascularized human MSC-derived bone and liver ^[255d]

Table 1. Continued.

Origin	Basic materials characteristics			Cell-instructive properties			Applications in organotypic and organoid cultures	
	Composition	Physicochemical properties	Processing and handling	Scaffolding	Adhesiveness	Mechanical stimulation		Growth factor presentation
8 arm PEG and ECM proteins	Tunable physicochemical properties Enables chemical modification for the incorporation of ECM proteins and degradable peptides Changing solid content enables modification of mechanical properties ($G' = 0.5\text{--}8\text{ kPa}$) ^[25h]	Suitable for embedding cells FXIIIa-mediated crosslinking (PEG-Gln + PEG-Lys) Lys-containing ECM proteins can be incorporated ^[25b]	Provides local support to guide cell expansion, differentiation, and morphogenesis Provides a BM-like local structural support based on laminin incorporation	Mediates adhesion via β 1-integrin signaling through the incorporation of laminin	Sustains murine pancreas colony formation and progenitor maintenance in soft ($G' = 250\text{ Pa}$) hydrogels Promotes apical-basal polarity and dorsal-ventral patterning of murine neural tube in hydrogels with intermediate stiffness ($G' = 2\text{--}4\text{ kPa}$) ^[26g]	Incorporation of laminin allows presentation of several growth factors based on the presence of heparin-binding sites in the LG subunits (e.g., members of the VEGF/PDGF family, VEGF-A165, placental growth factor (PlGF)-2, PDGF-AA, PDGF-BB, PDGF-CC, FGF-2, FGF-7, FGF-10, FGF-18, BMP-2, BMP-3, and BDNF) ^[26g]	Promotes cell-mediated degradation due to the incorporation of MMP-degradable peptides MMP-mediated degradation needed for patterning of murine neural tube	Murine pancreas, ^[18j] murine neural tube ^[25b]
8 arm PEG, RGD and laminin	Mechanically dynamic hydrogel tunable, hydrolysis-mediated softening profile (8 arm PEG-VS-Gln/8 arm PEG-VS-Lys, 8 arm PEG-Acr-Gln/8 arm PEG-Acr-Lys Different VS:Acr ratio for different softening profiles) G' from $\approx 1.3\text{ kPa}$ down to $\approx 200\text{ Pa}$ Adhesion sites: RGD, laminin-111 ^[25a]	Suitable for embedding cells FXIIIa-mediated crosslinking (PEG-Gln + PEG-Lys) Laminin-111 added in the hydrogel premix	Provides dynamic local support to guide cell proliferation, differentiation and cell self-assembly Provides a BM-like local structural support by incorporating laminin-111 driving murine intestinal organoid differentiation	Mediates cell adhesion via β 1-integrin signaling involving various α -integrin subunits (α_5 , α_6) based on RGD and laminin-111 supplementation	Softening profile sequentially supports stem cell expansion and morphogenesis through YAP-dependent mechanism	Incorporation of laminin allows presentation of several growth factors based on the presence of heparin-binding sites in the LG subunits (e.g., members of the VEGF/PDGF family, VEGF-A165, placental growth factor (PlGF)-2, PDGF-AA, PDGF-BB, PDGF-CC, FGF-2, FGF-7, FGF-10, FGF-18, BMP-2, BMP-3, and BDNF) ^[26g]	Localized viscoelastic remodeling allows self-organization and differentiation of the expanding organoid	Murine intestinal organoid ^[25a]

Table 1. Continued.

Origin	Basic materials characteristics				Cell-instructive properties				Applications in organotypic and organoid cultures
	Composition	Physicochemical properties	Processing and handling	Scaffolding	Adhesiveness	Mechanical stimulation	Growth factor presentation	Susceptibility for remodeling	
PEG (polyethylene glycol; commonly obtained by ring-opening polymerization of ethylene oxide) + proteins (recombinant)	8 arm PEG and recombinant jagged1	Tunable physicochemical properties $G' = 74\text{--}2157$ Pa Adhesive peptide: RGD Cell-mimicking motif: jagged1 Cell-degradable peptide: GPQ-W ^[268–356]	Suitable for embedding cells FXIIIa-mediated crosslinking ^[356]	Provides local, endothelial cell-mimicking support for the induction of perivascular commitment in human MSCs	Mediates cell adhesion via β 1-integrin signaling involving various α -integrin subunits (α_5, α_7) based on RGD-supplementation	Promotes capillary formation in soft ($G' = 500$ Pa) hydrogel variants	–	Provides cell-mediated degradation based on the incorporation of MMP-degradable peptides	Human perivascular niche ^[268]
PEG (polyethylene glycol; commonly obtained by ring-opening polymerization of ethylene oxide) + carbohydrates (animal derived)	4 arm PEG and heparin	Decoupled biochemical and mechanical properties $G' = 0.2\text{--}6$ kPa Adhesive peptides: RGD, IKVAV Cell-degradable peptides ^[256a] Variation of heparin sulfation pattern (local charge density) and heparin concentration (global charge density) ^[293]	Suitable for embedding cells in situ gelation via Michael type addition (thiol-maleimide) crosslinking reaction Material processing: porous scaffolds, ^[357] microgels, ^[280] bioprinting ^[358]	Provides local support to guide cell self-assembly Allows for material processing for controlled cross-scale cell alignment and organization ^[135–137]	Mediates cell adhesion via β 1-integrin signaling involving various α -integrin subunits (α_5, α_7) based on RGD-supplementation	Promotes a better vascular network formation, tumor vascularization and epithelial morphogenesis in soft ($G' = 200\text{--}500$ Pa) hydrogels	Promotes heparin-mediated binding and release of multiple growth factors	Promotes MMP-mediated remodeling necessary for migration, expansion and morphogenesis	Human endothelial cells, ^[256] vascularized human tumor spheroids, ^[238–262] human mammary epithelium, ^[251] human proximal tubules ^[25d]
8 arm PEG and HA	Elastic hydrogels with different mechanical properties ($G' = 284\text{--}3000$ Pa) Structural/adhesive component: HA Cell-degrading peptide: GPQ-W	Suitable for embedding cells FXIIIa-mediated crosslinking of HA-Gln and 8 arm PEG-Lys containing a MMP-sensitive peptide	Provides local structural support mimicking HA-rich tumor microenvironment	Promotes hyaladherin (e.g., CD44)-mediated adhesion	Elicits an invasive mammary epithelial phenotype both in stiff ($G' = 2000$ Pa) and soft ($G' = 284$ Pa) hydrogels	Provides a limited capacity of HA to bind growth factors (e.g., VEGF, BMP, NGF, SDF-1 α) ^[342]	MMP-mediated degradability induces a more invasive phenotype in mammary cancer epithelial colonies	Human mammary cancer ^[265]	
4 arm PEG and HA	Tunable physicochemical properties $G' = 134\text{--}282$ Pa Structural/adhesive component: HA Adhesive peptides: RGD, IKVAV, YIGSR (combinatorial presentation)	Suitable for embedding cells In situ gelation via Michael-type addition (thiol-maleimide)	Provides a local structural and mechanical support mimicking HA-rich neural stem/progenitor cell niches	Mediates cell adhesion via β 1-integrin signaling involving various α -integrin subunits (α_5, α_7) based on RGD-supplementation Supports an increased cell viability during growth and differentiation of Neural stem/progenitor cells due to adhesion to RGD	Provides stiffness mimicking mechanical properties of CNS	Provides a limited capacity of HA to bind growth factors (e.g., VEGF, BMP, NGF, SDF-1 α) ^[342]	Hyaluronidases-mediated remodeling ^[359]	Human neural stem/progenitor cells ^[360]	

Table 1. Continued.

Basic materials characteristics			Cell-instructive properties				Applications in organotypic and organoid cultures		
Origin	Composition	Physicochemical properties	Processing and handling	Scaffolding	Adhesiveness	Mechanical stimulation	Growth factor presentation	Susceptibility for remodeling	
Pam (polyacrylamide; commonly obtained by free radical polymerization of acrylamide) + proteins	Pam and vitronectin	Linearly elastic hydrogel not suitable for cell encapsulation $E = 0.2\text{--}40\text{ kPa}$ ^[360] Inert when unmodified, functionalized with ECM proteins to allow cell adhesion	2D cell culture only Acrylamide and bis-acrylamide mixed together with ammonium persulfate and TEMED Functionalization with proteins through chemical crosslinking (e.g., NHS chemistry)	Provides a local mechanical support to guide 2D cell differentiation	Mediates integrin based cell adhesion involving $\alpha_4\beta_5$ and $\alpha_5\beta_1$ integrins	Enhances 2D differentiation of hPSCs and allows more efficient morphogenesis in soft embryonic-like environments (CAM-like, $E = 1\text{ kPa}$)	Promotes the binding of several growth factors (e.g., IGF, EGF, TGF- β) due to vitronectin supplementation ^[361]	–	Human kidney organoid ^[283c]
Fully synthetic hydrogels									
PEG (ring-opening polymerization of ethylene oxide) + peptides (solid phase synthesis)	4 arm PEG, adhesive and degradable peptides	Independent control over biochemical and mechanical properties $E = 0.85\text{--}8.0\text{ kPa}$ Adhesive peptides: RGD, AG73, FOGER, IKVAV Cell-degradable peptides: GPQ-W, IPES	Suitable for embedding cells In situ gelation via Michael type addition (thiol-maleimide) crosslinking reaction	Local support to guide cell expansion, differentiation and morphogenesis Serves as injection vehicle for in vivo organoid delivery	Mediates integrin based cell adhesion Allows to define critical ranges of RGD density ($>250 \times 10^{-6}\text{ M}$) for morphogenesis by fine tuning of adhesive peptide concentration	Allows to define critical ranges of elasticity for morphogenesis by fine tuning of mechanical properties (canine epithelial cyst formation: $E = 4\text{ kPa}$; murine inner medullary collecting duct tubulogenesis: $G' = 200\text{ Pa}$; human intestinal organoid morphogenesis: $G' = 100\text{ Pa}$)	–	Allows MMP-specific (e.g., MT1-MMP or MMP1, 2 and 8) degradation to modulate cell expansion, morphogenesis and survival	Canine epithelial kidney cells, ^[269a] murine inner medullary collecting duct, ^[269b] human intestinal organoids ^[270]
PEG (ring-opening polymerization of ethylene oxide) + antibiotic	PEGDE and amikacin ("Amikagel")	Elastic, tunable physicochemical properties Unspecific adhesiveness modulated through variation of amikacin:PEGDE ratio High mechanical stiffness ($E = 37 \pm 5\text{ kPa}$ to $266 \pm 5\text{ kPa}$)	Amikacin hydrate and PEGDE mixed at different stoichiometric ratios, dissolved in water, vortexed and filtered gelation occurs at 40°C for 7.5 h	Supports the robust generation of beta-like spheroids and heterogeneous islet-like organoids without embedding	Unspecific adhesive properties of amikacin Low amikacin: PEGDE ratio results in lower adhesiveness and promotes spontaneous cell aggregate formation	Promotes the induction of uniform, easy to handle spheroids in stiff (E = 300 kPa) hydrogels	–	–	Human pancreas organoid ^[271a]

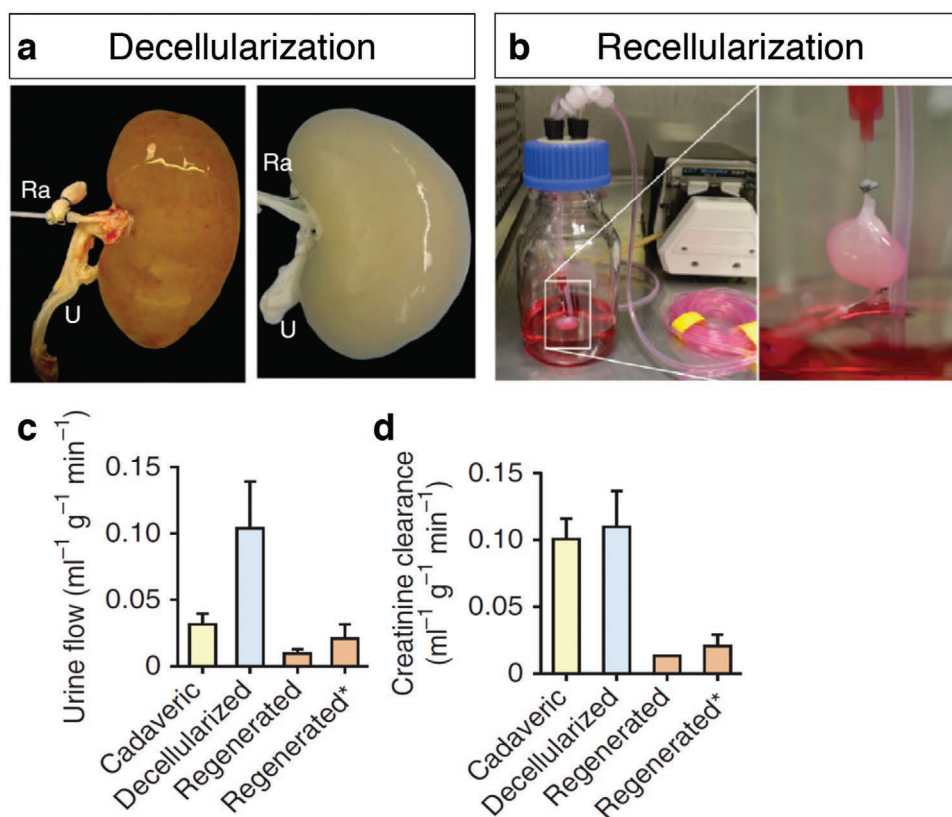


Figure 2. Generation of decellularized and re-cellularized kidneys. a) Perfusion decellularization of porcine kidneys. b) Cell-seeded decellularized rat kidney in whole-organ culture. c) Average urine flow rate (mL min^{-1}) for decellularized, cadaveric and regenerated kidneys perfused at 80 mm Hg and regenerated kidneys perfused at 120 mm Hg (regenerated*). d) Average creatinine clearance in cadaveric, decellularized, and regenerated kidneys perfused at 80 mm Hg and regenerated kidneys perfused at 120 mm Hg (regenerated*). Reproduced with permission.^[92] Copyright 2013, Springer Nature.

requires careful consideration as, for instance, a fibroblast-derived matrix was found to be unsuitable to sufficiently mimic the lung microenvironment in composition, structure and complexity.^[101] Various types of matrices deposited by different cells (including fibroblasts, mesenchymal stroma/stem cells (MSCs), HEK293 cells, chondrocytes) have been investigated for their capability to sustain cell growth, maintain stemness, or guide differentiation.^[102] Lanza and his team introduced the use of decellularized embryonic fibroblast cultures for the growth of human embryonic stem cells.^[102b] A majority of the work on decellularized cell cultures relied on MSCs, because these cells naturally secrete many structural ECM proteins and modifying enzymes.^[103] Reported critical parameters for the dECM quality are the passage number, the age of the donor, and dECM preparation method.^[104] Human and mouse MSCs-derived dECM was reported to contain collagen types I and III, fibronectin, small leucine rich proteoglycans, and major components of the BM, including perlecan and laminin,^[105] and showed to influence the differentiation potential of MSC in culture.^[104b,106] In a systematic extension of this approach, we used the culture of human MSCs under various conditions for dECM fabrication in combination with a particular polymer pre-coating technology effectively avoiding matrix delamination upon harvesting (Figure 3a). The obtained osteogenic-like (osteoECM) and collagen-rich (aaEMC) dECM preparations (Figure 3b) were thoroughly characterized for compositional and structural properties

and used to re-seed MSCs, which were shown to respond to the type and alignment of the particular dECM variants.^[107] These matrices were demonstrated to reconstitute microenvironmental cues of the bone marrow niche of human hematopoietic stem and progenitor stem cells to degrees that outperformed earlier attempts to maintain or proliferate this clinically relevant cell type by suspension culture and in cultures on Matrigel^[100] or on reconstituted ECM coatings (Figure 3c,d).^[108]

To increase the amount of dECM obtained with this approach of surface-immobilized matrix fabrication, macromolecular crowding (MMC), a method to enforce the precipitation of soluble ECM components during culture,^[109] was successfully applied. For instance, crowding through the addition of charged macromolecules stimulated primary embryonic fibroblasts to deposit significantly more collagen compared to standard culture conditions.^[110] MMC also influenced the adipogenic differentiation capacity of human bone marrow-derived MSCs by enhancing the pro-adipogenic microenvironment.^[111] We showed that MMC induces MSCs to form thicker fiber bundles and tighter ECM networks, thus influencing both the average topography and the elasticity of the decellularized substrate. Furthermore, we demonstrated that MMC can also impact GAG deposition, enhancing the retention of growth factors by the produced dECM.^[112]

Despite of the advantageous similarity of dECM to the native structure and composition of in vivo microenvironments^[102f] and the demonstrated translational potential of decellularized

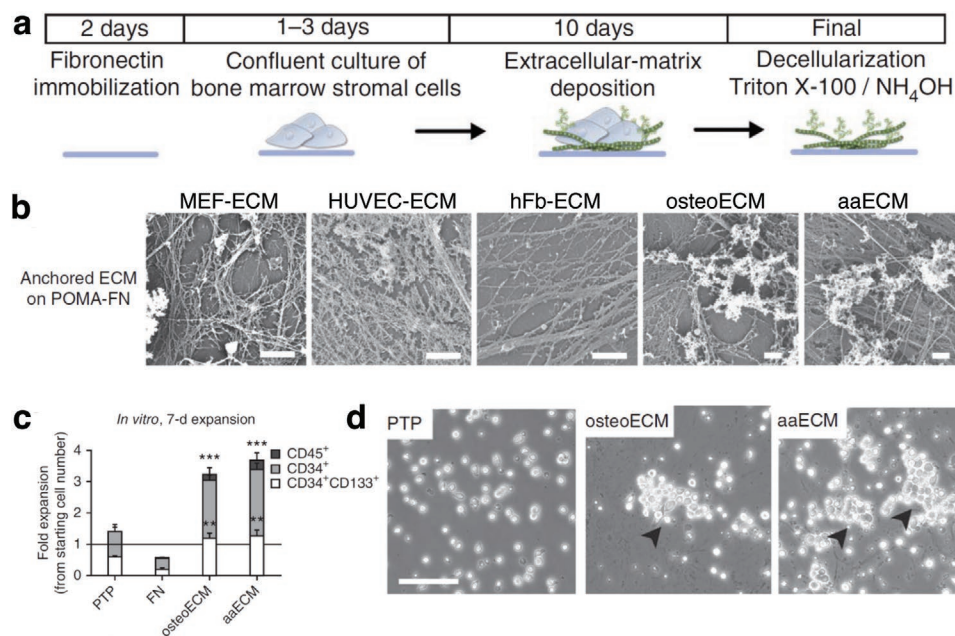


Figure 3. Surface-anchored MSC-derived dECM in vitro. a) Timeline for the generation of decellularized bone marrow MSC-derived ECMs. b) Scanning electron micrographs of the indicated cell-made matrices. c) In vitro expansion of human CD34+ HSPCs over 7d on plasma-treated tissue culture plastic (PTP), fibronectin, or MSC-derived ECM stimulated for osteogenic differentiation (osteoECM) or deposition of collagen-rich ECM (aaECM). d) Phase-contrast images showing cells on the indicated substrates at day 7. Arrowheads indicate cells in close contact with the underlying ECM. Scale bars, 2 μm b); 100 μm . d) Significance determined via one-way ANOVA; significance is in comparison to PTP. c) Reproduced with permission.^[107] Copyright 2013, Springer Nature.

organ scaffolds,^[113] the standardization of macroscopic dECM fabrication, the limited control over composition and compositional variations, and the persisting risk of immunogenicity demand further research to improve the usability of the method^[113] as well as research for better controllable alternative materials.

3.1.2. Solubilized dECM Preparations

With the discovery that dECM can be solubilized and re-precipitated, its utility in vitro and in vivo expanded significantly.^[114] To obtain solubilized dECM, decellularized materials are chopped into small pieces, grounded into a powder and then solubilized by enzymatic treatment to produce a solution which gels upon elevating the temperature to 37 °C.^[115] These material properties allow for the injection by minimally invasive delivery routines using a catheter or a syringe,^[116] and its growth-supportive characteristics for a wide range of cells explains its widespread use in cell culture applications, including organotypic and organoid cultures.^[117]

Despite the loss of spatial structural information in solubilized preparations, dECM hydrogels retain key biochemical cell-instructive characteristics of the tissue of origin which can greatly affect metabolic activity, proliferation, morphology, and differentiation of cells in vitro. For instance, when growing rodent and human islet cultures in bladder or pancreas-derived dECM hydrogels the cultured islets exhibited dynamic interactions with the dECM hydrogels, forming protrusions comprised of fibroblast-like sprouts that serve as conduits for

endothelial cell extensions. Long-term cultures of human and rat islets showed elevated insulin secretion upon glucose stimulation clearly exceeding the reference cultures.^[118] The use of dECM hydrogels was furthermore successfully demonstrated in cardiac regeneration.^[119] Culturing rat cardiac progenitor cells (CPCs) on a porcine ventricular dECM hydrogel showed enhanced cardiac progenitor function, increased cardiac marker expression and stronger serum-induced proliferation compared to type I collagen gels. The use of this dECM hydrogel was extended to human CPCs^[120] and applied for bioprinting applications^[121] in combination with GelMa, a gelatin-based matrix, demonstrating a 30-fold increase in cardiogenic gene expression when compared to pure GelMA reference patches. This result motivated the development of the dECM medical gel material VentiGel, which formed the basis for a promising first-in-man study in early and late myocardial infarction patients.^[122]

Another study highlighted that human embryonic stem cells (hESCs) embedded in hydrogels containing graduated amounts of porcine heart dECM gels show a dose-dependent increase in the expression of cardiac markers and contraction capacity.^[123] Obtained from naturally derived ECM, these preparations, however, lack a well-defined and reproducible composition,^[71c] vary in mechanical and biochemical properties and are very limited in tunability of their signaling characteristics.^[124] In a recent own study, we could modulate the cell-instructive properties of kidney derived dECM matrices by MMC (Figure 4),^[125] which was found to significantly affect the architecture and the elastic modulus of the matrices, with diameters and relative alignment of fibrils increasing depending on the concentrations of the

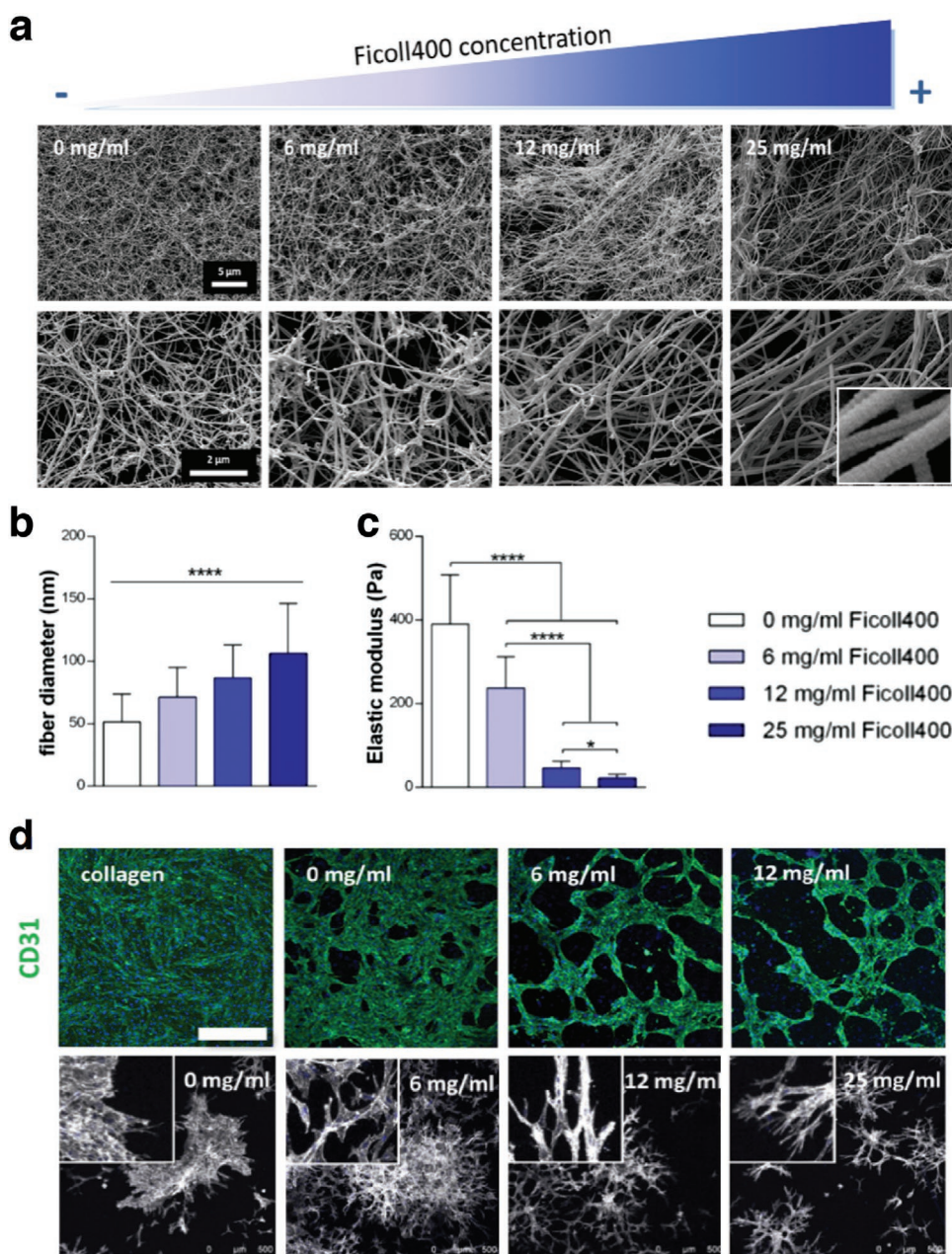


Figure 4. Kidney-derived dECM fibrillated hydrogels reconstituted in media containing different Ficoll400 concentrations. a) SEM micrographs of the matrices at 10 000 \times (top) and 40 000 \times (bottom) magnification. Inset: zoomed-in picture of ECM fibers assembled at 25 mg mL⁻¹ Ficoll400 showing the typical collagen D-bands. b) Fiber diameters calculated from SEM images. The average fiber diameter significantly increases with increasing Ficoll400 concentrations (**** p < 0.0001; one-way ANOVA; error bars, S.D.). c) Atomic force microscopy data of elastic moduli of the kidney-derived ECM scaffolds. MMC modulates the microscale mechanics of the reconstituted matrices, inducing a significant decrease in the elastic modulus with increase of the Ficoll400 concentration applied (* p < 0.05; **** p < 0.0001; one-way ANOVA; error bars, S.D.). d) Cellular response to reconstituted kidney matrices prepared with or without MMC. upper row: Formation of tube-like structures by HUVECs cultured on collagen gels and kidney-derived matrices visualized by confocal microscopy. Cells were stained for CD31 (green). Lower row: Confocal images of fluorescent tdTomato KSCs (gray) cultured on collagen gels and kidney-derived matrices. Insets: zoom-in. Scale bars, a) 5 μ m upper row, 2 μ m lower row, d) 500 μ m. Reproduced with permission.^[125] Copyright 2017.

crowding agent used (Figure 4a–c). The MMC-enhanced dECM preparations were clearly superior in supporting the morphogenesis of HUVECs and murine KSCs (Figure 4d).

While solubilized dECM preparations can provide a desirable performance, protocols relying on such materials remain

hard to standardize.^[126] Due to the preparation, materials obtained from solubilized dECM are in general soft and often much softer than the tissue from which they have been derived. For example, the integral elastic modulus of Matrigel was determined to be around 450 Pa by different methods,^[127] however,

on the microscale, it was found to be heterogeneous with distinctly stiffer regions (1–2 kPa).^[127b] Intentional stiffness modulation was achieved by incorporating solubilized dECM preparations in hybrid composite materials, i.e., by mixing with other biopolymers or with synthetic polymers,^[123,128] and through chemical or UV-mediated cross-linking.^[129]

The potential of the tissue-specific molecular fingerprint of dECM-derived hydrogels to direct cell differentiation has been clearly demonstrated for organoid and tumoroid cultures as well. Porcine testicular organoids obtained from testicular single cells^[130] embedded in tissue-specific dECM gels underwent seminiferous tubule organization comparable to the native organ.^[131] The culture of freshly isolated, pre-aggregated testicular cells in the very soft decellularized testicular dECM gel (storage modulus below 20 Pa) was clearly superior to the reference culture in collagen hydrogels (storage modulus 75–100 Pa). The dECM gel-grown testicular organoids were maintained for 45 days in culture and secreted stem cell factor and testosterone demonstrating functionality.

In a very recent study a porcine intestinal tissue-derived dECM gel was prepared, characterized and used to grow murine and human intestinal organoids, human gastric organoids, hepatocyte and liver duct organoids as well as pancreatic ducts.^[70] The organoids were successfully cultured for up to 8 passages over a time span of two months. Human fetal pancreatic organoids produced by means of this material were transplanted subcutaneously in immunodeficient mice where they preserved their tissue organization for several weeks. Small intestinal organoids obtained with this approach were likewise subcutaneously transplanted in mice and retrieved with matured properties after one month. The development of these hydrogels might open up the potential for human organoids to be used clinically.

Various tumoroid cultures in dECM have been recently reported, including lung cancer, breast cancer, hepatocellular carcinoma, colorectal cancer, and glioblastoma. Mollica et al. reported a self-gelling human mammary-derived dECM hydrogel culture system that induced significant changes in protein and gene expression profiles in embedded normal mammary and breast cancer cells (MCF/MDA-MB-468) when compared to standard Geltrex cultures.^[132] dECM in cancer research has been comprehensively covered by a recent review and will therefore not be addressed further.^[133]

3.1.3. Matrigel

Despite of the above-given examples of the successful use of tissue-specific dECM gel applications, most organoids of all three developmental lineages as well as organotypic cultures are currently grown in permissive murine sarcoma-derived dECM hydrogels available under the trade names Matrigel, Cultrex BME, or EHS matrix. Thus, the origin of the material is not a healthy, functional tissue. The solubilized BM preparations consist of a heterogeneous mixture of several extracellular matrix components, primarily laminin, collagen IV and nidogen, as well as proteoglycans and several growth factors,^[134] which are generally growth-supporting but variable and ill-defined.^[134b]

Tubulogenesis and branching morphogenesis are key developmental processes for endothelial as well as many epithelial cells. Accordingly, these processes are often recapitulated *in vitro* by modulating matrix signals acting on the cells.^[135] To induce branching, the cells have to loosen their cell–cell junctional contacts, activate proteases that degrade the surrounding BM and acquire an extensively invasive and motile behavior to initiate new branch formation/vessel sprouting.^[136] Vascular endothelial cell tubulogenesis was widely studied *in vitro* by embedding vascular endothelial cells in Matrigel, Collagen I, or fibrin.^[137] The high amounts of matrix-associated pro-angiogenic growth factors present in Matrigel (VEGF, TGF- β , PDGF, and FGF2) allow for tubular morphogenesis within a few hours after embedding.^[138] To avoid overstimulation, growth factor reduced Matrigel was used, however, the formation of lumenized endothelial capillaries remained controversial.^[139] Although these 3D cultures are very useful to screen for pro- and anti-angiogenic compounds, they lack shear forces from perfusion-based cultures involved in blood vessel formation *in vivo*.

Culturing epithelial cells derived from different organs including the mammary gland,^[140] lung,^[141] kidney,^[142] prostate,^[143] and others in BM-derived dECM hydrogels has led to the self-organized formation of spherical 3D structures surrounding a lumen similar to *in vivo* acini.^[144] This phenomenon was intensely studied using the Madin-Darbin Canine Kidney (MDCK) cells representing an early model of a generic epithelium that has greatly helped to understand the molecular machinery involved in epithelial cyst formation and branching. One key finding from the work in Matrigel reflects the necessity of early laminin signaling to establish the correct initial orientation of apico-basal polarity in growing cysts. In the presence of laminin, lumen formation occurs without an increase of apoptosis, however, in materials without laminin or when lumen formation is genetically delayed, apoptosis is an important pathway for lumen formation.^[142,145] The group of Mostov showed that during the onset of MDCK cell branching canonical markers of cell polarity are transiently lost resembling a partial transition from epithelial to mesenchymal cell phenotypes.^[146] The small GTPase Cdc42 acts as a master regulator of cell polarity in this system and has also been shown to be involved in tubulogenesis of endothelial^[147] as well as other epithelial tissues.^[148] As a result of this shift in polarity a reorientation of cell division along a newly growing branch of cells is observed without the loss of cell–cell contact.^[149] To identify growth factors necessary for tubulogenesis (e.g., hepatocyte growth factor (HGF) in the case of MDCK cells),^[150] less complex hydrogels like collagen type I were used. With respect to branching in other epithelial tissues, multiple growth factors have often been identified to regulate a single branching system. For instance, branching of the kidney is driven by glial cell line-derived neurotrophic factor (GDNF), epidermal growth factor (EGF), HGF, and FGFs,^[151] whereas EGF and HGF regulate branching of the mammary gland and lung.^[152]

However, the limited number of cell types (usually one or two) included in these organotypic models constrains the complexity of the tissue models, as well as their developmental repertoire.^[153] With the discovery that pluripotent embryonic

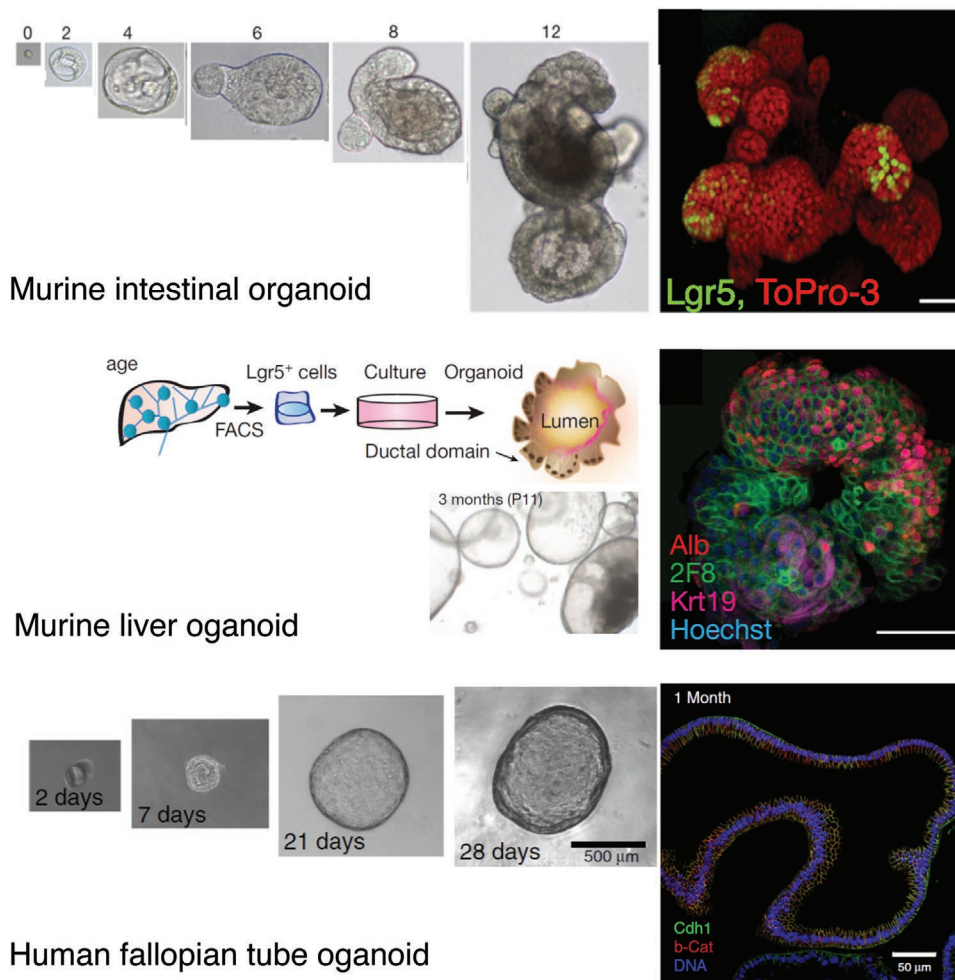


Figure 5. Depicted examples of organoid culture from single adult stem cells or tissues. a) Bright field pictures depicting single-cell-derived murine intestinal organoid growth. 3D reconstructed confocal image after 3 weeks in culture. Lgr5–GFP⁺ stem cells (green) are localized at the tip of crypt-like domains. Counterstain, ToPro-3 (red). Reproduced with permission.^[12] Copyright 2009, Springer Nature. b) Single-cell-derived hepatic organoids acquire hepatocyte fate and display hepatocyte functions in vitro. Scheme of experimental layout; representative brightfield image. Confocal image (z-stack projection) for hepatocyte-specific markers. Alb (red), Krt19 (magenta) and hepatocyte surface marker 2F8 (green). Nuclei were counterstained with Hoechst (blue). Reproduced with permission.^[17] Copyright 2013, Springer Nature. c) Time course of a monoclonal organoid generated from a single EpCAM⁺ cell sorted and embedded in Matrigel in a 96-well format. Confocal analysis of the organoids after 1 month in vitro reveals stability of the phenotype, with unchanged morphology and staining pattern of the adhesion markers. Reproduced with permission.^[163] Copyright 2015, Springer Nature. Scale bars, 50 µm.

and tissue-specific stem cells can self-organize into complex organ-like structures, a reductionist cell model of in vivo biology became available which might close the gap between single cell type culture systems, 3D organotypic cultures and the complexity of in vivo tissues.^[154] During the last years a variety of 3D organoids has been derived from pluripotent and tissue-resident stem cells generating “miniature organ” structures of the three developmental lineages endoderm, mesoderm and ectoderm.^[11b,21,155] The vast majority of these cultures relied on the use of Matrigel which thereby became the gold standard material for organoid cultures—despite all its well-known limitations.^[21,156]

Embedding adult epithelial tissue-derived stem cells in Matrigel under growth supporting conditions leads to the self-organization of small, yet complex structures resembling their

organ of origin. Using single Lgr5-positive cells isolated from the murine intestine as starting population, Hans Clevers’ lab was the first to successfully grow in Matrigel 3D intestinal organoids characterized by a central lumen surrounded by crypt-like buddings that, within 2 weeks, developed into complex structures composed of stem, progenitor and mature intestinal cell types within a spatial organization as seen in vivo (Figure 5).^[12,157] Based on Lgr5 expression, stem cell populations from colon,^[158] stomach,^[16] liver,^[17] pancreas,^[18] gallbladder,^[159] mammary gland,^[160] and the taste buds^[161] were identified and successfully cultured in Matrigel as tissue-specific organoids. All of these cultures depend to a varying extent on a potent source of Wnt, a potent activator of tyrosine kinase receptor signaling like EGF, inhibition of BMP/TGF- β signaling and matrix environments as presented by Matrigel.^[11a] Likewise,

other stem cell populations were isolated from different epithelial organs (prostate,^[162] fallopian tube,^[163] lung,^[164] and salivary gland^[165]) and differentiated in Matrigel into tissue-specific organoids. The growth conditions were similar, yet their Wnt dependence seems to vary. While salivary gland organoids can be cultured long-term upon robust Wnt pathway activation,^[165a] for the prostate stimulation with R-spondin was not essential but strongly induced luminal cell differentiation, leading to a prostate-like pseudostratified structure. All mentioned examples represent simple or two-layered epithelia. Only the keratinizing stratified epithelium of the esophagus consists of several cell layers: the small basal-like cells are in contact with the extracellular matrix, large flat suprabasal-like cells reside in the interior, and hardened keratinized material can be found in the center.^[166]

In parallel to the generation of tissue-derived organoids, the first studies on PSC-derived organoids of the neuroectoderm were published, which, however, did not rely on matrix-guidance but on inhibition of members of the transforming growth factor beta (TGF β)-superfamily and the wingless-related integration site (Wnt) family. Such organoids fully depended on the initial formation of embryoid bodies of PSCs and therefore could not be derived clonally.^[167] When generating the optic cup organoid from mouse and human PSCs, the Sasai group also started to add Matrigel to the culture medium to stimulate epithelialization and ectoderm differentiation of the cells, however, the cells were at no time point fully embedded in the hydrogel.^[14,15] Lancaster et al. achieved a broader brain regional identity by using a minimal medium to prevent the expansion of non-neuroectodermal cells without inhibiting members of the TGF β and Wnt families (**Figure 6a**).^[13] Also, embedding in Matrigel allowed a dramatic reorganization and expansion without the need for replating the aggregates. With this approach, a variety of brain regions was generated within single organoids, including hindbrain, cortical or retinal regions. Likewise, less complex, regionally restricted brain organoids were generated from PSCs in Matrigel.^[168] The reconstitution of the neural tube to recapitulate spinal cord patterning in 3D was investigated by one of the authors (Meinhardt). Neuroepithelial cysts were clonally derived from murine embryonic stem cells within a Matrigel droplet^[169] and specified into a 3D patterned cervical neural tube structure upon the generic addition of RA to the culture medium (**Figure 6b**).

Besides neuroectodermal differentiation also other epithelial tissues were reconstituted from PSCs in vitro using Matrigel as supporting matrix, including stomach (**Figure 6c**),^[170] lung,^[171] and kidney.^[172] McCracken et al. observed that PSCs differentiated along the endodermal lineage were able to recapitulate in vivo stomach organogenesis upon embedding in Matrigel. The gastric organoids underwent folding and formed immature pits and glands in vitro similar to in vivo (**Figure 6c**).^[170]

Recently, even the formation of a murine limb bud was recapitulated in a 3D setting in vitro.^[173] The differentiation was mainly guided by the temporally restricted addition of growth factors but also through the addition of 4% Matrigel to the culture medium to allow for ectodermal differentiation. When transplanting the obtained structures, myoblasts and neuronal fibers from the host embryo invaded the grafts. Furthermore, the engineered limb bud structures were incorporated into

developing limb buds in vivo and were shown to differentiate into multiple cell types of limb progenitors. The technology might thus enable the generation of other complex mesenchymal/epithelial tissues from PSCs.

In sum, dECM materials have provided very powerful means for supporting cell growth and differentiation and, in combination with stem cells, enabled the creation of true-to-life tissue and organ in vitro models. However, the complexity and compositional variations of dECMs limit the standardization of the protocols and complicate targeted intervention as well as mechanistic biological studies. Given these constraints, the multifaceted, successful application of Matrigel in the formation of a plethora of different tissue models closely resembling their in vivo counterparts is even more impressive. Animal-derived extracts restrict the application of the explored technologies in regenerative and personalized therapies as well as in drug discovery and screening. Thus, although the dECM-based work has been fundamental, there is an urgent need for alternative materials which are less immunogenic and allow for the liberal modification of cell-instructive characteristics.

3.2. Hydrogel Preparations Based on Isolated Biopolymers

The use of dECM materials for organotypic and organoid cultures has impressively shown the importance of ECMs in cellular fate control and enabled a large variety of culture systems. However, dECM represents the least controllable hydrogel material. Therefore, materials to decipher the relevance of individual ECM components as well as integral matrix properties such as stiffness were explored as alternatives. Hydrogels composed of isolated ECM biopolymers and combinations thereof including HA, gelatin, collagen I, high concentration laminin, and chondroitin sulfate^[174] have been used to generate culture systems of less complexity. The application of these materials has enriched our knowledge on biological mechanisms in development, disease, and regeneration significantly. However, biopolymer-based hydrogel preparations also share some of the limitations of dECM gels, including batch-to-batch compositional variations.

3.2.1. Hydrogels Based on Combinations of Isolated Biopolymers

To reconstruct in vivo microenvironments in a more simplistic but defined way, different engineered biopolymer composite systems were created out of several isolated ECM proteins as well as carbohydrate components. For instance, Sokol et al. engineered a hydrogel scaffold that aimed at mimicking the human breast tissue environment by assembling its main components collagen I, laminin, and fibronectin as well as hyaluronan (**Figure 7a**).^[175] The proteins mainly provide adhesion sites and resistance to tensile forces while the non-sulfated, highly hydrated GAG provides resistance to compressive forces. The multicomponent ECM hydrogels exhibited indeed a significantly higher swelling ratio and increased elasticity relative to plain collagen I-based reference gels due to the inclusion of hyaluronans (**Figure 7b**). While previous attempts of the in

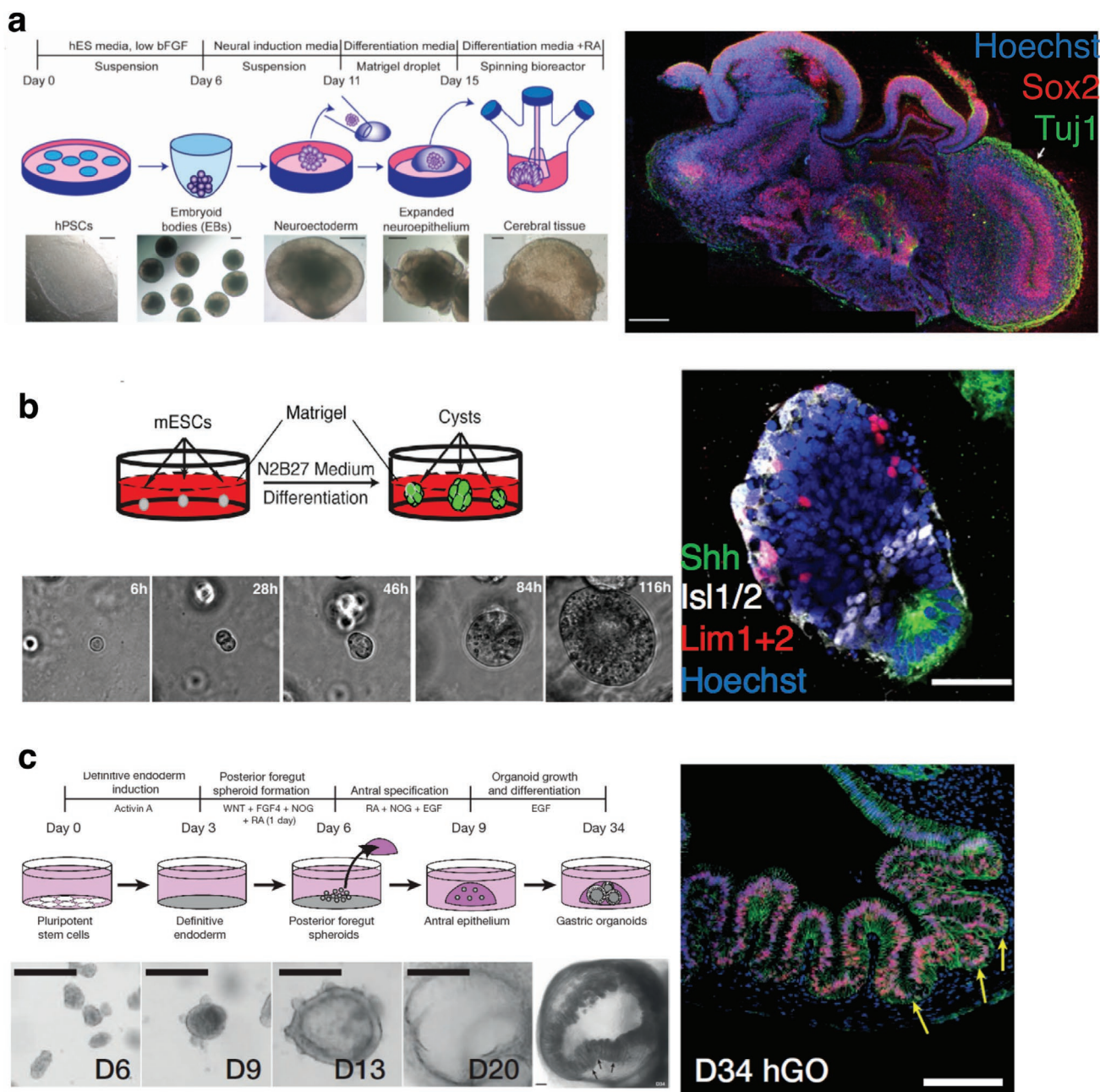


Figure 6. Depicted examples of organoid culture from pluripotent stem cells. a) Cerebral organoid (“mini-brain”) formation using a spinning bioreactor. Sectioning and immunohistochemistry revealed complex morphology with heterogeneous regions containing neural progenitors (Sox2, red) and neurons (Tuj1, green) (arrow). Reproduced with permission.^[13] Copyright 2013, Springer Nature. b) Neuroepithelial cyst formation from single murine embryonic stem cells embedded in Matrigel. Immunostaining for sonic hedgehog (Shh -green), Isl1/2 (white), Lim1+2 (red) indicate floor plate formation (green) and regionally restricted expression of both neuronal markers. Reproduced with permission.^[169a] Copyright 2014, Elsevier. c) Schematic representation of the *in vitro* culture system used to direct the differentiation of pluripotent stem cells into 3D gastric organoids. Morphological changes during growth of gastric organoids. By 4 weeks, the epithelium of hGOs exhibited a complex folded and glandular architecture and expressed PDX1 throughout the epithelium. Reproduced with permission.^[170] Copyright 2014, Springer Nature.

in vitro reconstitution of human mammary tissue in 3D collagen I hydrogels or Matrigel failed to induce a significant ductal growth, the ECM composite gels enabled clusters of patient-derived breast cells to self-organize, expand, and differentiate to complex structures with ductal and lobular morphologies that closely resembled the epithelial structures present in the

human breast (Figure 7c). Immunostaining of day 11 organoids revealed that outgrowths have matured and contained distinct luminal and basal layers (Figure 7e). Terminal ductal lobular units were enriched in mammary stem cells as indicated by co-expression of SLUG and SOX9 (Figure 7f) which were identified as the leader cells to direct ductal elongation.

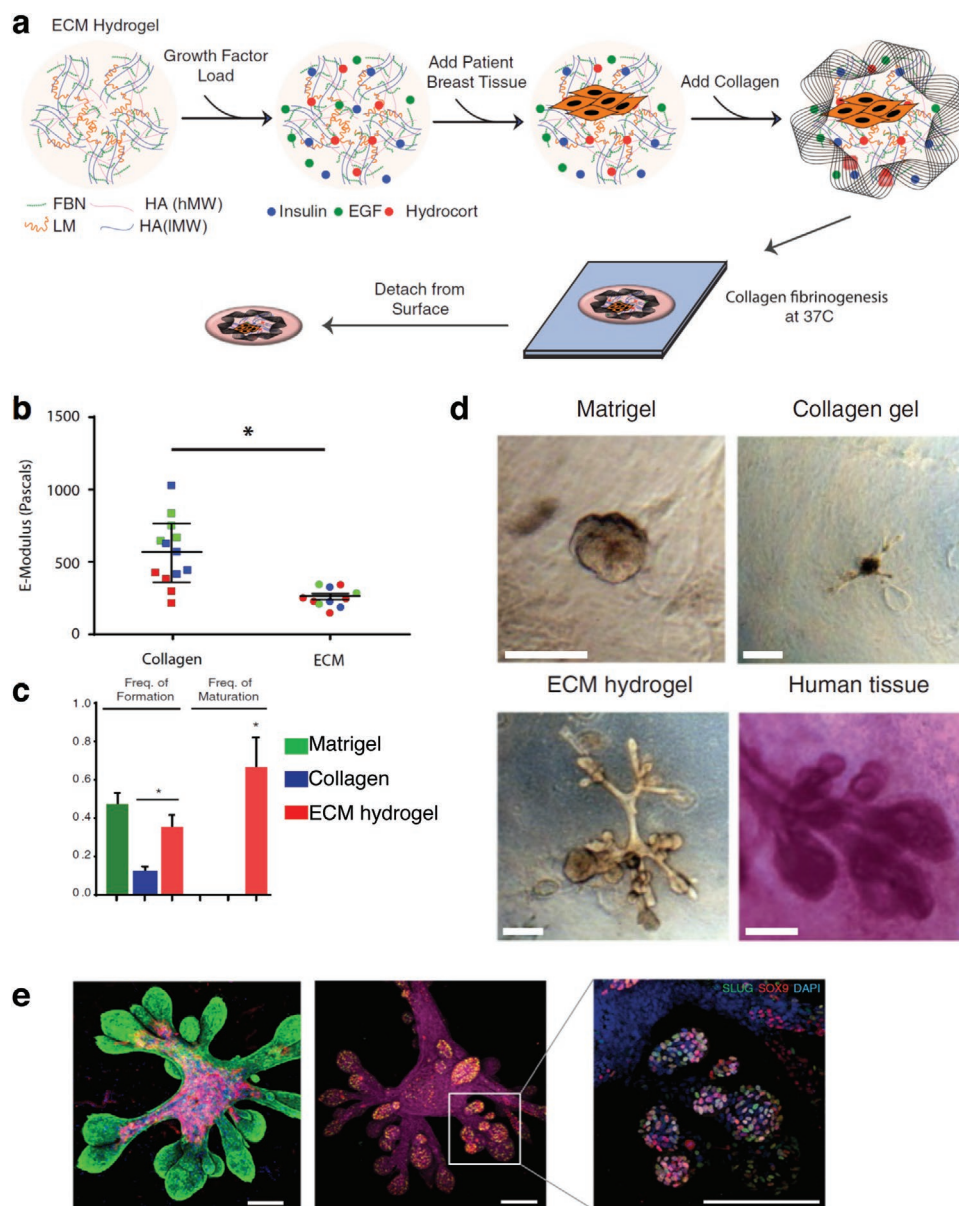


Figure 7. Growth of human breast tissues from patient cells in hydrogel scaffolds. a) Schematic representation of hydrogel scaffold assembly. b) Measurement of Young's modulus of at least three independent replicates for collagen gels and ECM hydrogels. Plotted are the mean and standard deviation for the replicates. c) Quantification of outgrowths in Matrigel, type I collagen, or ECM hydrogels next to representative bright-field whole-mount images of carmine-stained human breast tissue. d) Comparative morphology of human breast tissue in vivo and ex vivo. Representative bright-field images of ex vivo organoid growth after 10 days in Matrigel, polymerized type I collagen, or ECM hydrogels next to representative bright-field whole-mount images of carmine-stained human breast tissue. e) Immunofluorescence of luminal CK8/18 (red) and myoepithelial CK14 (green) marker expression at 11 days after seeding revealed that outgrowths have matured and the CK8/18⁺ cells fully lined the luminal layer, while the CK14⁺ cells were basally localized. f) Mammary stem cells are enriched and localized to the leading edge of elongating outgrowths and side branches as indicated by immunofluorescence stainings of day 10 organoids for SLUG (green), SOX9 (red) and actin (purple). Scale bars, d,e,f) 200 μ m. Reproduced under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>).^[175] Copyright 2016, Springer Nature.

A similar approach was used to engineer scaffolds resembling the neurovascular niche of the human brain to support human neural stem cell proliferation and differentiation as well as vessel formation to potentially use the obtained constructs therapeutically in the treatment of the damaged central nervous system (CNS).^[176] The used scaffold contained fibrin, HA, and laminin. While fibrin hydrogels are quickly degraded when transplanted

in vivo, the addition of HA and laminin rendered the gels more resistant to degradation and kept the overall stiffness comparable to brain tissue (202.3 \pm 17.33 Pa). Co-cultures of fetal-derived neural stem/progenitor cells and human endothelial colony-forming cell-derived endothelial cells showed that this composite hydrogel allows for significant vessel formation while promoting neural stem cell proliferation and differentiation.

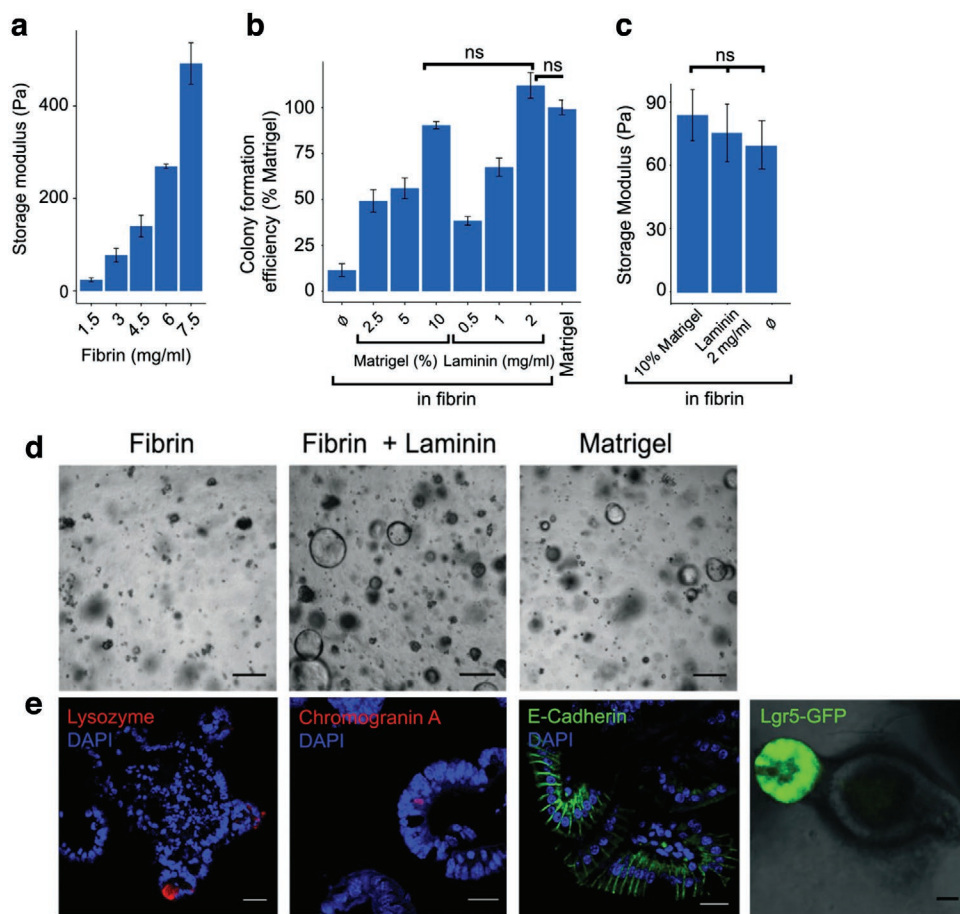


Figure 8. Fibrin/laminin hydrogels support murine small intestinal organoid growth. a) Storage modulus of fibrin hydrogels with different fibrin concentrations. Error bars: SD, $n = 3$. b) Matrigel and Laminin supplementation support colony formation efficiency of mouse small intestinal stem cells seeded in fibrin gels. Error bars: SD, $n = 6$. c) Quantification of the changes in mechanical properties associated with the addition of Matrigel or laminin to fibrin hydrogels (3 mg mL^{-1}), as measured by rheometry. Error bars: SD, $n = 3$. d) Mouse small intestinal stem cells four days after seeding in fibrin based hydrogels supplemented with laminin, or in pure Matrigel. Morphology in brightfield microscopy. e) The organoids were stained for Paneth cells (lysozyme), enteroendocrine cells (Chromogranin A), epithelial cells (E-Cadherin), and stem cells (Lgr5-GFP). Lgr5-positive and lysozyme positive cells were mostly observed in crypt-like domains. Scale bars, d) $200 \mu\text{m}$, e) $20 \mu\text{m}$. Reproduced under the terms of the Creative Commons Attribution Non-Commercial License CC BY-NC.^[177] Copyright 2018, Advanced Materials, published by Wiley-VCH.

To understand the cell-instructive properties of individual biopolymers and to reduce variations based on poorly defined culture conditions, the number of xeno-derived products within hydrogels has to be reduced. Multiple approaches aimed at restricting the number of biopolymer components in hydrogels for 3D cultures to just two or one. Referring to the fact that the EHS tumor-derived matrix is rich in laminin, a fibrin matrix containing purified laminin was developed as a potential alternative. The material was shown to support the long-term expansion of epithelial organoids (Figure 8).^[177] The stiffness of the fibrin hydrogel was adjusted via its concentrations in the gels (1.5 mg mL^{-1} fibrin: $24 \pm 10 \text{ Pa}$; 6 mg mL^{-1} fibrin: $270 \pm 8 \text{ Pa}$, Figure 8a) and allowed efficient formation of round cysts (Figure 8b). By supplementing the hydrogels with 10% of Matrigel or 2 mg mL^{-1} laminin (Figure 8c) epithelial organoids formed with the same yield as in pure Matrigel cultures (Figure 8d) and contained the main cell types of the organoids including Goblet cells (not shown), Paneth cells (lysozyme), enteroendocrine cells (Chromogranin A), epithelial cells (E-Cadherin), and stem cells

(Lgr5-GFP) (Figure 8e). Lgr5-positive and lysozyme-positive cells were mostly observed in crypt-like domains. The engineered system was also used to successfully grow human pancreas organoids, human pancreatic ductal adenocarcinoma organoids, human small intestinal organoids, and human liver organoids. While the reported data show that Matrigel can be replaced by a less complex hydrogel system that is tunable in stiffness and adhesiveness, the used laminin was derived from the EHS tumor which hampers its use for regenerative therapies due to its animal-derived origin. Furthermore, the stereotypical patterning of intestinal stem cells seen in Matrigel could not yet be fully reproduced in the modified fibrin hydrogels.

3.2.2. Fibrin

Fibrin is readily generated from soluble fibrinogen in the presence of thrombin, i.e., from blood upon activation of the blood coagulation cascade, and serves in vivo as a provisional matrix

with key regulatory functions in wound healing.^[178] Engineered fibrin matrices containing covalently linked VEGF-constructs were successfully used for directing vascular endothelial cells toward angiogenesis-like 3D capillary networks.^[179] Accordingly, the use of fibrin-based materials is clinically well established, and several more recent applications demonstrate its tissue-instructive potential as, for example, in pancreatic islet transplantation where fibrin matrices functionalized with VEGF-A165, PDGF-BB and an integrin-binding fibronectin domain rendered the transplant pro-angiogenic.^[180] Fibrin mediated signaling also plays an important role for tumor growth,^[181] as shown in a study where tumorigenic cells were selected by culturing single cancer cells in fibrin matrices of ≈ 100 Pa in stiffness.^[182]

3.2.3. Collagen I

Collagen I is a highly abundant structural protein dominating the interstitial connective tissue-type ECM of skin, bone, cartilage, tendon, and ligament. Being one of the earliest 3D methods used to study the response of cancer cells,^[183] it is now widely used in its well-established solubilized and re-precipitated fibrillar gel format as a minimalistic 3D culture system for a vast range of studies ranging from culturing smooth muscles,^[184] MSC differentiation,^[185] adipocyte culture and differentiation,^[186] recapitulating epithelial growth and branching of the mammary,^[187,188] salivary,^[188] kidney,^[189] and pancreatic ductal epithelium,^[190] tumor modeling (pancreas,^[191] breast,^[192] colorectal,^[193] and skin,^[194,195]), as well as angiogenesis.^[196] It has been shown that a change in collagen I gel-stiffness alters endothelial cell spreading, angiogenic sprouting and glycation products.^[197] Collagen I is a substrate of transglutaminase and can thus be cross-linked through ϵ -(γ -glutamyl) lysine,^[198] which resulted in enhanced fibroblast attachment, spreading and proliferation.^[199] Collagen I gels can also be tuned in stiffness by cross-linking with glutaraldehyde,^[200] ultracentrifugation,^[201] gel compression,^[202] evaporating the solvent,^[203] or further chemical modification methods.^[204] Covalently linked HA-collagen I hydrogels^[205] and non-covalently intercalated heparin-collagen I-systems^[109b,206] have been developed to modulate the fibrillar structure and the mechanical properties of collagen-I-based matrices. Recently, the properties of collagen I hydrogels were also modulated by MMC,^[207] which allowed the tuning of fibrillogenesis kinetics and fiber architecture.^[207c,d] As a cell-compatible method, MMC was recently implemented for the bioprinting of hierarchical porous collagen-based structures.^[208]

Type I collagen hydrogels have been applied in intestinal organoid cultures using murine^[209] or human^[210] small intestinal crypts, and were shown to support the long-term in vitro maintenance and expansion of fully elaborated intestinal epithelial organoids similar to EHS matrix-based cultures. In yet another example, collagen I hydrogels were used to cultivate hPSC-derived polycystic kidney disease (PKD) organoids, and were instrumental in revealing the role of polycystin-1 (which is mutated in PKD) in the remodeling of the ECM micro-environment by kidney organoid epithelia.^[211] Other examples of the successful use of collagen I gels include the culture of murine stomach tissue, murine and human colon tissue.^[209d]

Recently, Matrigel/collagen I hydrogels have been used for the development of human blood vessel organoids through the directed differentiation of human embryonic as well as induced PSC lines in a 96-microwell format.^[212] Transplanting these structures into immunocompromised mice allowed the formation of a stable, perfused vascular tree, including arteries, arterioles and venules. Culturing these organoids under hyperglycemic conditions simulating a diabetic environment has led to a significant increase of type IV collagen, fibronectin, laminin, and perlecan within the BM of the vessels, demonstrating that the cells can actively remodel their microenvironment depending on the stimulus received. This study impressively showed that human blood vessel organoids can serve as a tissue surrogate suitable for modelling and identifying the regulators of diabetic vasculopathy.

3.2.4. Gelatin

Gelatin, a soluble derivative of collagen obtained by breaking the triple-helix structure into single-stranded molecules, easily forms gels,^[213] and commercially available gelatin-based foams (e.g., Gelfoam, water-insoluble gelatin sponge) have been, for example, seeded with cells derived from fetal rat ventricular muscle to form functional cardiac grafts.^[214] Gelatin promotes angiogenesis with a lower performance compared to collagen I, however, methacrylated gelatin materials were reported to support vascularization similarly or better as compared to collagen I materials.^[215] Gelatin methacryloyl (GelMA) is a gelatin derivative containing a majority of methacrylamide groups and a minority of methacrylate groups, which undergoes photoinitiated radical polymerization.^[216] By varying the degree of methacryloyl substitution, GelMA with finely tuned mechanical properties can be produced,^[217] allowing its application for the formation of organized, stable vascular networks,^[218] to engineer a load-bearing material through the addition of components such as carbon nanotubes, graphene oxide, inorganic nanoparticles, or other bio- and synthetic polymers to mimic tissues such as skeletal muscle, bone and cartilage,^[219] to engineer skin,^[220] and to investigate ovarian cancer.^[221] GelMa has also been applied to bioprinting of tissues analogs,^[222] including organoids. For instance, bovine colon organoids pre-differentiated in Matrigel were removed from the hydrogel, embedded in 7.5% GelMA (w/v) supplemented with 0.1 mg mL⁻¹ Matrigel and applied to bioprinting into 96 well culture plates.^[223] The colonoids remained viable and proliferative for 48 h post-print.

3.2.5. Hyaluronic Acid

HA^[224] is the only non-sulfated glycosaminoglycan abundant in CNS tissue and it is enriched during embryonic development and in adult neural stem/progenitor cell niches. Due to its inherent high degradability and low stiffness, several approaches were investigated to generate more controllable hydrogels with respect to its stiffness, degradability, and functionality.^[225] Modifications include hydrazide- or thiol-functionalization or methacrylation of HA. In a study by Zhu et al. acrylated HA was used to generate a hydrogel via Michael-type addition using a

bis-cysteine-containing MMP-degradable cross-linker to adjust the stiffness of the gel and a small RGD peptide to promote adhesion.^[225] Embedded vascular MSCs proliferated in the gel matrices and formed 3D multicellular networks with branches and sub-branches within 7 days. Through the addition of Noggin to the same gel-based culture system the cells formed a different type of network with a thicker network geometry, whereas the addition of BMP-2 led to 3D spheroid formation. The combined application of both morphogens led to complex multicellular morphologies in which miscellaneous morphological features coexisted, characteristic that was speculated to mimic the morphogenesis of trabecular bone and bone nodule formation. The observed differences were attributed to a Turing instability-controlled reaction-diffusion process of the morphogens.

Lindborg et al. reported a HA-chitosan hydrogel for the induction of cerebral organoid growth^[226] and cultured iPSCs within this material using E8 medium without the addition of neural induction reagents. The gels had a Young's modulus of about 10 kPa and were thus significantly stiffer than native CNS tissue (0.5–1 kPa^[227]) or materials used in earlier published applications.^[3a,228] However, within 2 weeks of culture organoid structures formed by self-organization, suggesting that the particular matrix composition was effectively supportive.

Since HA is a major component of the tumorous ECM, it is often used to recapitulate the tumor microenvironment in vitro. Several cancer models were set up as a basis for drug screening and disease modeling. For example, the group of Soker grew breast cancer, colon, liver and colorectal cancer spheroids but also intestinal organoids in HA-based microcarrier beads which allowed their usage in drug screens, cancer metastasis and drug resistance assessment.^[229] Using hydrazide-functionalized HA together with aldehyde functionalized HA allowed the establishment of a prostate cancer model that was used for testing the efficacy of cancer drugs.^[230] Combining thiolated-HA with thiolated gelatin or methacrylated collagen to provide cell adhesion motifs allowed the development of a set of patient-specific tumor-derived organoids from a large variety of tumor biopsies (lung, mesothelioma, melanoma, colorectal, appendical, sarcoma tumors) which were used in drug screens revealing patient-specific selective responses to drugs.^[231]

3.2.6. Alginate

Another widely applied single-biopolymer gel system is represented by the polysaccharide alginate, which is not a component of the mammalian ECM but originates from algae or bacteria, and, was successfully used to grow intestinal organoids.^[232] Soluble alginate forms a non-covalent, electrostatically complexed hydrogel upon calcium chloride addition, i.e., under mild gelation conditions;^[233] the obtained gel materials can be controlled with respect to physical and biochemical properties,^[234] and show a tissue-mimetic viscoelastic behavior.^[235] Capeling et al. hypothesized that human intestinal organoids (HIOs) may create their own niche and thus may be amenable to growth in substrates lacking inherent cell recognition.^[232] Alginate gels were adjusted to a storage and loss modulus similar to Matrigel, as well as to a stiffness comparable to a previously established PEG hydrogel culture.^[25c] The latter alginate gel type yielded

the most human intestinal organoids, but the total amount was significantly lower than in Matrigel (**Figure 9a–c**), probably due to the inability of the cells to remodel the hydrogel and a lack of serum proteins or growth factors. Alginate-grown HIOs could be passaged and maintained for at least 90 days in vitro without significant decreases in expression of key markers, further demonstrating the utility of alginate as a replacement to Matrigel in the presence of ECM producing cells. However, when embedding MSC-free, primary human enteroids into alginate hydrogels, the alginate did not support organoid growth, while enteroids expanded robustly in Matrigel (**Figure 9d**).^[232]

Further underpinning this conclusion, the group of Scaglione reported that a non-invasive breast cancer model can be established using alginate gels without the addition of cell adhesive proteins,^[236] whereas an invasive breast cancer model requires a more permissive environment established through the addition of Matrigel to the alginate scaffold in a 1:1 ratio.^[237] The Matrigel-blended alginate hydrogel helped to understand the epigenomic changes that underlie the tumorigenic impact of extracellular matrix mechanics.^[238]

3.2.7. Silk Fibroin

Silk fibroin, a fiber-forming protein that is not a component of the mammalian ECM either, is increasingly used for tissue engineering as well.^[239] For details of silk-based engineered biomaterials the reader is referred to the excellent related literature.^[239,240] Of particular interest, silk hydrogels can be formed through physical cross-linking of large hydrophobic blocks of amino acids forming β -sheets when activated by environmental changes such as shear stress (i.e., sonication or vortexing), lower pH, higher temperature, and changes in osmolarity.^[241] Silk hydrogels support encapsulation of cells by appropriate transport/permeation characteristics, however, they lack mammalian ECM motifs. Chitosan-silk hydrogel scaffolds have been used to grow keratinocytes with the aim to generate a cornea replacement.^[242] Silk scaffolds have also been applied to study cartilage regeneration using human bone marrow-derived mesenchymal stem cells.^[243] The seeded cells proliferated more quickly and deposited three times more GAGs on the silk fibroin scaffold than on collagen matrices. Bone constructs were created by differentiating hMSCs into osteoblasts on porous silk fibroin scaffolds.^[244] To develop a minimal invasive therapy for brain applications, self-assembling silk hydrogels were used as a support for mesenchymal cells.^[245] Through the combination of silk with type I collagen fibrillated hydrogel the group of D.L. Kaplan was able to recapitulate the compartmentalized structure of cortical brain tissue by segregating cell bodies and axons.^[246] This in vitro tissue construct was reported to show biochemical and electrophysiological responsiveness resembling brain homeostasis and mechanical injury responses. The same approach was used to study primary pediatric and adult brain tumors, yet, the used hydrogel composition was changed to type I collagen gel or HA hydrogels supplemented with native porcine-derived decellularized brain ECM to mimic the tumor environment more closely.^[247] Silk scaffolds have also been used in a variety of tissue bioengineering approaches including the generation of a functional human intestinal

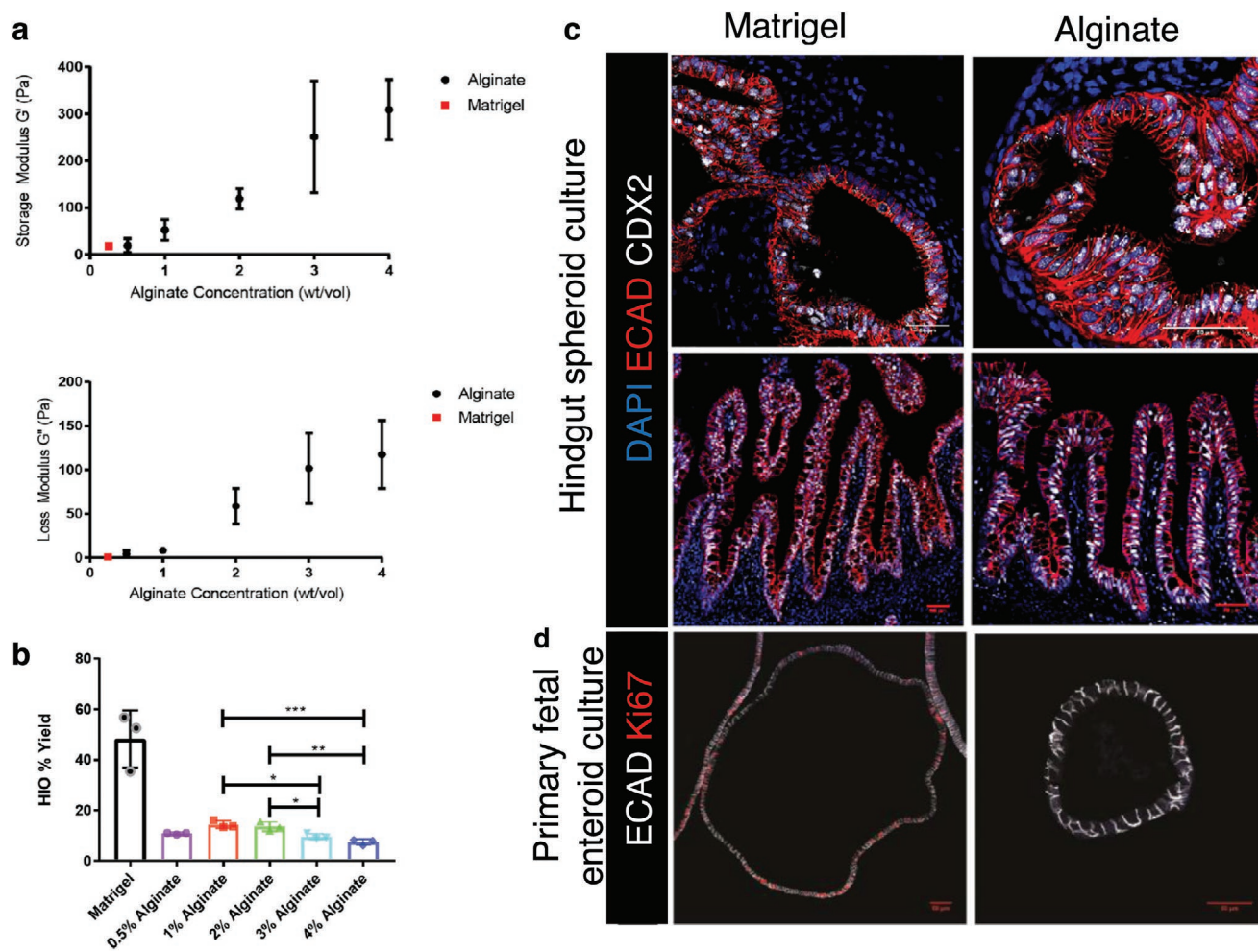


Figure 9. Alginate supports human intestinal organoid (HIO) survival in vitro. a) Rheological characterization (Storage modulus and loss modulus) of alginate hydrogels. Data shown are the mean \pm SD from $n \geq 3$ gels per condition. b) Quantification of HIO yield after 28 days in culture. Data shown are the average yields from three independent experiments with $n > 100$ spheroids per condition. Each point depicts overall yield from one experiment, while bars depict mean and SE. Significance was calculated with a one-way ANOVA and Tukey's multiple comparisons test. c) Representative fluorescent images of general epithelial marker staining in HIOs cultured in 1% alginate and Matrigel for 28 days (upper row) and transplanted HIOs from alginate and Matrigel (lower row). Markers shown are ECAD (epithelial marker) and CDX2 (intestinal epithelium marker). d) Representative images of intestinal enteroids embedded in alginate and Matrigel without mesenchyme after 7 days stained for ECAD and Ki67. Scale bars, 50 μ m. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. Reproduced with permission.^[232] Copyright 2018, Elsevier.

epithelium,^[248] skin reconstructs,^[249] bone reconstructs,^[250] or scaffolds for nerve regeneration.^[251]

3.3. Recombinant Protein-Based Hydrogels

Recombinant protein engineering offers an alternative strategy for the production of cell-instructive hydrogels that combine the advantages of both natural biopolymers and synthetic polymers.^[252] In this technology, the amino acid sequence of a target protein is encoded into a DNA plasmid which is then transfected into a host system (i.e., *Escherichia coli*) that translates the genetic information and expresses the engineered protein. Using the native cellular machineries, this technology allows the generation of tunable, defined, and reproducible materials composed of modular ECM peptide domains that carry specific structural or functional properties. Engineered ECM (eECM)

containing an elastin-like structural domain functionalized with RGD was generated and applied for the culture of an intestinal tissue explant from adult mice.^[252e] Both mechanical and adhesive properties of the eECM were shown to influence tissue differentiation, in vitro as the highest efficiency of successful organotypic culture was obtained with compliant matrices (≈ 180 Pa) with high concentrations ($\approx 3.2 \times 10^{-3}$ M) of cell-adhesive binding domains. The differentiation of the intestinal tissue cultivated within the eECM was demonstrated to be as efficient as the one obtained in softer (≈ 12 Pa) collagen-based cultures, thus enabling easier physical manipulation. The same hydrogel platform has been employed to study the influence of both matrix stiffness and degradability on the maintenance of stemness (self-renewal and differentiation capacity) of adult murine neural progenitor cells (NPCs).^[252d] Stemness was proved to be independent from the hydrogel mechanical properties over a wide range of neural-relevant stiffness

(≈ 0.5 – 50 kPa). Critical for self-renewal and differentiation was matrix remodeling, which was found to be independent from ECM-adhesion ligand clustering and cytoskeletal tension, but facilitated cadherin-mediated cell–cell contact and promoted β -catenin signaling.

3.4. Biohybrid and Fully Synthetic Polymer Hydrogels

As discussed in the previous subsections, biopolymer-based materials such as Matrigel and collagen type I gels are being extensively used in organotypic cultures to recapitulate epithelial morphogenesis, and so far are the most common materials for the generation of organoids of different tissue origins. Despite their often stunning efficacy, these matrices are characterized by an inherent lot-to-lot compositional variability that hampers the experimental reproducibility of many culture protocols. Furthermore, the complexity of such materials does not allow the decoupling of their biochemical and biophysical properties. For instance, when aiming at changing the mechanical properties of Matrigel or collagen type I-gels, the variation in polymer density similarly alters the distribution and organization of the adhesive sites within the matrix. In consequence, deciphering the contribution of specific ECM features to the regulation of tissue morphogenesis is hardly possible in biopolymer-based materials. Biohybrid or fully synthetic ECM-mimetics offer great opportunities to address this challenge, as the modularity of such biomaterials can allow for an independent control over their cell-instructive characteristics.

In this section, we provide an overview of synthetic materials used for organotypic and organoid cultures. We first give examples of modular biohybrid materials, where synthetic polymers are conjugated with biopolymers (protein- and carbohydrate-based) inherently retaining biological functions, and continue with fully synthetic materials, where the ECM is reduced to its fundamental components and defined bioactive motifs are integrated into tunable and highly reproducible cell-instructive polymeric networks.

3.4.1. Biohybrid Systems

While many biopolymer-based materials used for cell culture applications already possess cell adhesive sites and can be naturally degraded by cellular proteases, synthetic biomaterials, sometimes referred to as “blank slate” materials, are often inert, therefore requiring further functionalization for proper recapitulation of ECM-mimicking environments. Common synthetic polymers used for culture applications include PEG, poly(lactic-co-glycolic acid) (PLGA), polylactic acid (PLA), polyacrylamide (PAm), and several copolymers thereof.^[20,253]

PEG is the base component of numerous synthetic hydrogels for cell culture applications due to its hydrophilicity and chain flexibility resulting in generally low non-specific interactions with nearly all components of living matter. Furthermore, PEG is produced in a broad range of molecular weights and structures, and can easily be functionalized with reactive end groups

such as acrylates, maleimides, thiols, NHS esters, vinyl sulfones, and norbornenes, that allow the incorporation of bioactive molecules and polymerization under cyto-compatible conditions through several reactions (i.e., photopolymerization, chain polymerization, Michael addition, thiolene, and strain-promoted azide alkyne cycloaddition).^[26,254] Molecular weight and concentration, branching structure and cross-linking of the polymer chains can be adjusted to control the network structure of PEG-based hydrogels, and by that their physical properties including mesh size (molecular permeability) and mechanics (elasticity).

Several variants of PEG-based biohybrid hydrogels have been developed for the *in vitro* study of morphogenesis. As a common scheme in the formation of PEG-based biohybrid materials, PEG is conjugated with proteins^[25b,255] or carbohydrates^[25d,f,256] that can be naturally recognized by cellular integrin receptors and easily remodeled by cellular proteases, thus allowing a certain level of biological complexity within a finely tunable matrix. A recent study by Klotz et al.^[255c] presented the application of biosynthetic hydrogels based on 8 arm PEG and gelatine (gelPEG) for the culture of vascularized MSC-derived bone and liver tissue analogs. The gelPEG hydrogels outperformed Matrigel by better supporting the differentiation of both embedded cells. In particular, opposite to Matrigel-based cultures, bone-like tissue produced within gelPEG materials displayed mineralization due to nucleation/calcium deposition sites of the gelatin component. The authors further illustrated the tunability of the system, showing the covalent incorporation of additional, lysine-containing proteins into the hydrogel network. In another study, pancreas progenitor aggregates embedded into soft PEG-based hydrogels could be maintained and expanded when laminin 1 was covalently immobilized in the polymeric network.^[18b] Integrin recruiting was shown to be indispensable for progenitor maintenance, as non-functionalized PEG-hydrogels induced the progressive loss of pancreatic and epithelial phenotype and stopped the expansion of the cell aggregates. Not being as supportive as Matrigel in the generation of pancreatic organoids, this PEG-based matrix still illustrates the potential of modular, biohybrid hydrogels to functionally replace undefined multi-component materials.

As mentioned in Section 2, cells can sense the mechanical properties of the surrounding ECM and respond with different migration, proliferation, and differentiation programs. Pioneering studies by Engler et al.^[3a] have shown how 2D collagen-modified PAm hydrogels produced with a range of elastic moduli can specifically direct the differentiation of MSCs, inducing neurogenic, myogenic, or osteogenic lineage commitment on respectively soft (0.1–1 kPa), intermediate (8–17 kPa), and stiff (25–40 kPa) culture substrates. The concept of stiffness-dependent differentiation has been later confirmed and expanded for the culture of other cell types. hPSCs, for example, were shown to preferentially expand on stiff hydrogels following yes-associated protein 1 (YAP) nuclear translocation, and to differentiate on compliant substrates driven by cytoplasmic YAP sequestration.^[257] Importantly, additional studies have also demonstrated a strong interplay between matrix elasticity and biochemical signals in dictating cell differentiation.^[3b,258] The modular nature of biohybrid hydrogels facilitates high-throughput combinatorial studies of cell-instructive 3D microenvironments,

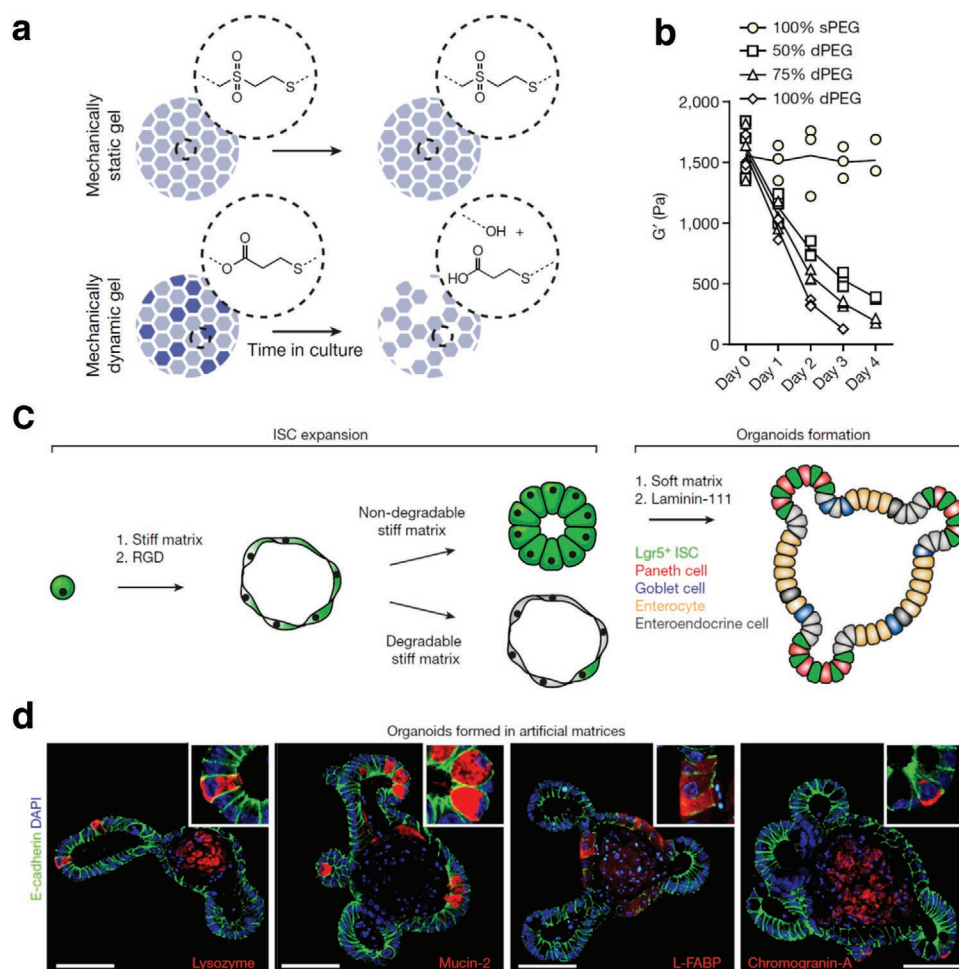


Figure 10. Mechanically dynamic hydrogels for intestinal stem cells (ISC) expansion and organoid formation. a) Schematic showing the principle for the formation of sPEG-dPEG mechanically dynamic hydrogels. b) Softening profile of the hydrogel as a function of dPEG. c) Schematic illustrating the mechanical regulation of different stages of intestinal organoid formation. d) Immunostaining of intestinal organoids generated within mechanically dynamic hydrogels. Scale bars, 50 μm . Reproduced with permission.^[259] Copyright 2016, Springer Nature.

where stiffness and biochemical properties can be tuned independently. For instance, an in-depth screening of mechanical properties, degradability, ECM components, and soluble factors was performed to identify the optimal conditions for neural tube development by mouse ESCs embedded in PEG-based hydrogels.^[25b] Apical-basal polarity and dorsal-ventral patterning were better promoted by non-degradable hydrogels with intermediate stiffness ($\approx 2\text{--}4$ kPa). Among laminin-111 (pure or entactin-rich), entactin, collagen IV, perlecan, fibronectin, and collagen I, the first was the best in inducing proliferation, differentiation, and apical-basal polarity. Minor or no significant impact was identified for bFGF. The authors of this study also compared the development of neuroepithelial cysts grown in the biohybrid hydrogel to those produced in the gold standard Matrigel. Notably, the optimized PEG-based system promoted the formation of a more homogeneous population of polarized neuroepithelial colonies, both in terms of size and morphology. The authors postulated that the heterogeneities found in the Matrigel-based cultures can be attributed to multiple non-directed or conflicting signals presented to the differentiating cells.

Tissue morphogenesis and organ development are complex, multi-step processes where cells go through sequential stages of proliferation, differentiation, and architectural/functional organization in response to exogenous signals that change over time. Biomaterial engineering offers the possibility of creating dynamic environments to guide *in vitro* morphogenesis. Lutolf and team^[28a,259] recently introduced a well-defined biohybrid hydrogel system whose dynamic mechanical properties can sequentially support the expansion of murine intestinal stem cells and their differentiation into organoids (**Figure 10**). In this hydrogel, a PEG backbone is functionalized with RGD and laminin-111 as minimal adhesive signals. The application of both a mechanically static PEG-vinylsulfone (sPEG) and a dynamic, hydrolytically degradable PEG-acrylate (dPEG) generates a polymeric network that undergoes progressive softening, so that initial high stiffness (≈ 1.3 kPa) can guide ISC expansion, while subsequent soft matrix characteristics (≈ 200 Pa) promote organoid morphogenesis. The softening profile of the hydrogel can be finely modulated over a wide range by adjusting the ratio between sPEG and dPEG, thus allowing for a systematic investigation of the organoid mechanical regulation. In this

regard, a YAP-dependent mechanism was identified as crucial for maintaining stemness and expansion of ISC. Following the unifying concept introduced by Brusatin et al.,^[260] the dynamic sPEG-dPEG hydrogel represents an example of material with “conforming properties,” which allows the self-organization of the expanding organoid thanks to localized viscoelastic remodeling. In fact, the hydrogel offers “conforming properties” at the intestinal crypt bottom, where cells experiencing high mechanical stress (activated YAP) retain stemness, while the rest of the organoid undergoes differentiation following YAP inactivation.

Although this development has been fundamental, the hydrogel being used in this study cannot account for the spatiotemporal distribution of morphogens required for more complex developmental processes. In vivo, morphogenesis is in fact regulated by molecular signals that are secreted and sensed by cells in specific spatiotemporal patterns mainly mediated by GAGs and proteoglycans. Aiming at recapitulating this ECM feature, we have developed a modular biohybrid hydrogel composed of 4 arm PEG (starPEG) and highly sulfated GAG such as heparin (serving as a model of the heparan sulfate component of proteoglycans).^[256a,261] Being strongly negatively charged, heparin anchors a plethora of small signaling proteins through electrostatic complexation with their positively charged surface domains, thus providing both protection from degradation and sustained release of growth factors. The cross-linking degree of the matrix can be modulated through the variation of the starPEG content, however, keeping the heparin concentration of the swollen gel constant. In this way, a complete decoupling of the physical and biomolecular properties of the hydrogel can be achieved. The material platform has been used for the systematic in vitro study of heterocellular angiogenesis, where matrix mechanics, bioactive components, and supporting mural cells were independently investigated.^[25e] Lumenized vascular networks formed when endothelial cells were embedded in soft (≈ 200 Pa), degradable hydrogels equipped with adhesive (RGD) peptides and the reversibly bound pro-angiogenic factors VEGF165, bFGF, and SDF-1 α . In particular, multifactorial delivery combined with mural cells allowed for the long-term culture of capillary networks of vascular endothelial cells from various sources. Since vascularization is a key process involved in tumor progression and metastasis, the starPEG-heparin hydrogel system was used to study the interaction between cancerous spheroids and endothelial cells.^[25e,262] Compared to Matrigel cultures, a more ordered and abundant angiogenic process occurred in starPEG-heparin hydrogels,^[262] where connections between the tumor spheroids and the endothelial cells were observed. Furthermore, the reconstituted tumor angiogenesis micro-environment was shown to be a more representative chemotherapeutic testing platform compared to standard 2D cultures. As discussed later in the manuscript (see Section 4), reaching proper in vitro vascularization is currently one of the biggest challenges toward the development of more physiologically relevant organoid cultures. Thoroughly tunable engineered materials, such as the starPEG-heparin hydrogels, can doubtlessly help to bridge the gap between the functionality of state-of-the-art organoids and their respective native organs.

It has been further demonstrated that starPEG-heparin hydrogels can serve as a tunable platform for the systematic and

independent investigation of the role of ECM cues in epithelial morphogenesis of mammary and kidney cells (Figure 11).^[25d,f] Nowak et al.^[25f] showed that human mammary epithelial cells embedded in compliant (200–350 Pa), MMP-degradable starPEG-heparin hydrogels formed polarized mammary acini without the need of additional adhesion ligands (Figure 11a). Conversely, stiff matrices (1.600 Pa) induced the development of unorganized colonies with an invasive phenotype. Both degradability and heparin incorporation were found to be necessary for the development of properly differentiated acini. In particular, a direct involvement of GAGs in the regulation of cell-ECM interaction was hypothesized, as only cells grown in heparin-containing matrices presented a continuous deposition of laminin-332 at the basal colony surface. Similar matrix properties were shown to induce the tubulogenesis of single human proximal tubule cells (Figure 11b).^[25d] The polarized tubular structures formed in soft MMP-cleavable starPEG-heparin hydrogels responded to cisplatin levels in ways comparable to the recorded human clinical response, demonstrating the suitability of this hydrogel system for nephrotoxicity testing.

Another major polysaccharide used for the generation of biohybrid hydrogels is HA, the only non-sulfated member of the GAG family. HA-based biohybrid hydrogels have been used to support organotypic cultures and tissue engineering constructs.^[256b–d] Being overexpressed in a number of tumors, HA is particularly useful for the realization of biohybrid materials that support the growth of in vitro 3D cancer models.^[230,263] Patient-derived prostate cancer cells cultivated in thiolated HA (HA-SH) and PEG-DA hydrogels maintain high viability compared to standard cultures, furthermore retaining their native epithelial phenotype and showing localization of the androgen receptor coherent with in vivo models.^[264] Mammary cancer epithelial cells—known to express the HA receptor CD44—embedded into modular HA-PEG hydrogels were shown to proliferate and form more invasive colonies compared to pure PEG matrices, independent of the stiffness of the matrix,^[265] highlighting the suitability of the system for in vitro studies on malignant and highly invasive cancers.

Abundant in the CNS and particularly in the adult neural stem cell/progenitor cell (NS/PC) niches, HA was combined with 4 arm PEG to produce hydrogels with mechanical properties approximating native CNS tissues (average complex shear modulus of 188 ± 42 Pa^[266]) and degradability mediated by cell-produced hyaluronidases.^[267] A study on the combinatorial presentation of different adhesion peptides to NS/PC within this biohybrid matrix revealed that RGD peptides increase viability and differentiation toward neurons and oligodendrocytes, both alone or in combination with YIGSR and IKVAV adhesion peptides. The 4 arm PEG-HA hydrogels were shown to support 3D cultures of human NS/PCs for at least 70 days.^[266] This study elegantly shows the potential of modular biohybrid hydrogels of recreating the biochemical and physical properties of specific physiological environments with high fidelity, allowing the precise presentation of cell-instructive cues including stiffness, degradability and adhesive sites.

Of note, the toolbox of cell-instructive biohybrid hydrogels is not limited to the recapitulation of adhesive and remodeling

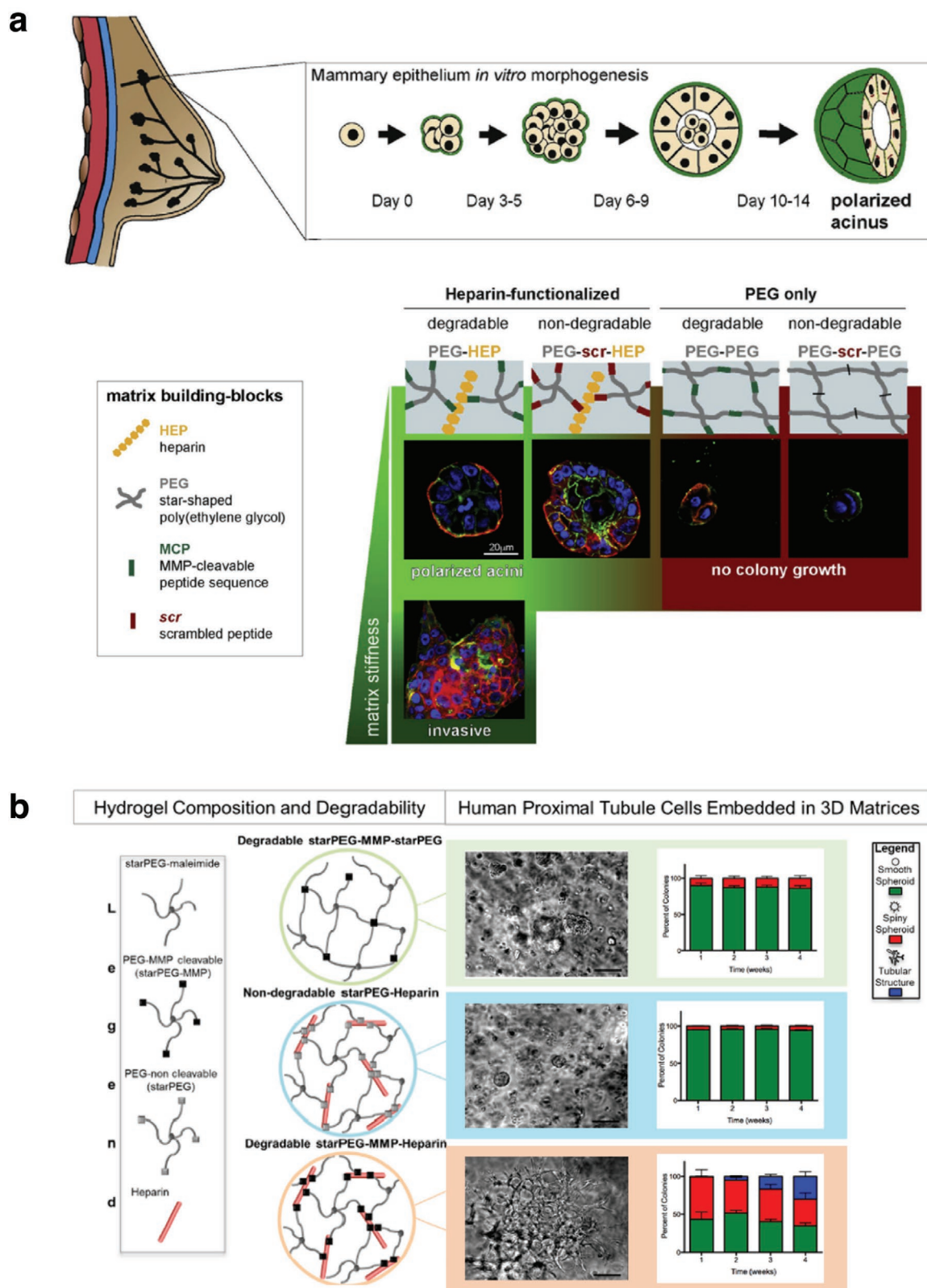


Figure 11. Application of modular starPEG-heparin hydrogels for the study of *in vitro* epithelial morphogenesis. a) Human mammary epithelial cell morphogenesis. Optimal formation of polarized acini occurs within soft, degradable PEG-heparin hydrogels. Scale bar, 20 μm . b) Human proximal tubule cell morphogenesis. Optimal tubulogenesis occurs within degradable starPEG-heparin hydrogels. Scale bar, 75 μm . Reproduced with permission.^[25d,f] Copyright 2017, Elsevier.

properties of the ECM. Synthetic polymers can be further engineered to mimic specific cell–cell interactions relevant for proper differentiation and morphogenetic processes. Blache et al., for example, have shown that PEG hydrogels can be functionalized with the recombinant Notch-activator Jagged1 to establish a fully defined perivascular niche model.^[268] The authors showed that MSCs co-cultivated with HUVECs form microcapillary networks when embedded within defined soft (74 Pa) degradable hydrogels. Furthermore, they have proved that MSCs undergo a typical phenotypic switch in the production of BM ECM demonstrating a perivascular commitment. Interestingly, the same phenotypic switch could be achieved in MSC monocultures when the PEG hydrogels were functionalized with recombinant Jagged1, which can mimic MSC–HUVEC interactions.

3.4.2. Fully Synthetic Systems

Biohybrid hydrogel materials constitute a big step toward more systematic and more reproducible scaffold-based cell culture studies, offering controlled matrix customization through the independent tuning of various biophysical and biochemical cell-instructive ECM features. Nevertheless, the use of animal-derived polymers in the realization of hydrogel networks still limits the precision in the materials design and constitutes a potential hurdle for the implementation of these materials in regenerative medicine applications. The identification of essential biofunctional subunits of ECM proteins and carbohydrates provides a base to create fully synthetic cell- and tissue-instructive hydrogel materials. For instance, synthetic polymers such as PEG can be functionalized with peptides containing integrin binding units and sequences that can be degraded by MMPs, in order to enable adhesion and cell-mediated matrix remodeling, respectively.^[27,254,24]

Following this approach, fully synthetic PEG-based hydrogels have been successfully tailored and applied to organotypic and organoid cultures of different origin, such as kidney,^[269] intestine,^[270] lung,^[270] pancreas,^[271] nervous,^[25b,169a,272] and immune system.^[273]

García and colleagues have shown that the epithelial morphogenesis of kidney cells is strongly influenced by mechanical properties, degradability, and availability of adhesive ligands of 4 arm maleimide-terminated PEG (PEG-4MAL) hydrogels functionalized with RGD peptides and a MMP-sensitive sequence (GPQ-W).^[269] In particular, their work demonstrates that MDCK cells undergo normal cyst formation, polarization, and lumen formation only in a narrow range of matrix elasticity conditions.^[269a] Furthermore, the authors showed that critical levels of RGD density and MMP-mediated degradability regulate the correct cyst differentiation. The same modular material platform has been recently used to investigate the independent contributions of physical and biochemical properties of the ECM to the tubulogenesis of the inner medullary collecting duct (IMCD) kidney cells.^[269b] Using PEG macromers of different size and at different density, the authors could finely modulate the storage modulus of the hydrogels, identifying 200 Pa as the optimal condition for tubule formation. RGD peptides were found to better stimulate proliferation and tubulogenesis of IMCD cells compared to laminin or collagen-derived

peptides, and furthermore a MT1-MMP (MMP14)-dependent degradation of the matrix was shown to be critical for the growth of luminal tubules.

The group of García has also demonstrated the versatility of this fully synthetic tunable hydrogel in guiding the epithelial morphogenesis of tissues of different origin, such as intestine and lung.^[270] Optimal conditions were identified for the differentiation of HIOs (**Figure 12**). Hydrogels with a storage modulus of ≈ 100 Pa, 2×10^{-3} M RGD and GPQ-W degradable peptides supported the generation of HIOs from ESCs and iPSCs. This hydrogel represents the first fully synthetic matrix system guiding the development of HIOs in vitro, offering a viable alternative to previously published protocols that required animal-derived components for proper differentiation.^[28a,259] In view of potential future applications in regenerative medicine, the hydrogel was tested as a vehicle for the delivery of HIOs into murine intestinal mucosal wounds. The in situ polymerizing material was demonstrated to assist the engraftment and differentiation of HIOs. Furthermore, compared to non-embedded HIOs, hydrogel-embedded HIOs accelerated wound repair. As an additional advantage, the PEG-4MAL system is more than two times less expensive compared to Matrigel.^[270b]

In parallel to the generation of ECM-mimetic peptides, synthetic GAGs, analogs have been produced for the realization of materials capable of binding and stabilizing growth factors, thus protecting them from degradation and regulating their bioavailability.^[273b,274] For instance, the synthetic heparin mimic poly(sodium-4-styrenesulfonate) (PSS) was shown to have strong bFGF binding affinity and to regulate bFGF-mediated cellular processes.^[275] Knowing the involvement of bFGF in the in vitro self-renewal of hPSCs, Chang et al. developed a synthetic hydrogel consisting of PSS-functionalized PAM and tested it for the maintenance of hPSCs.^[274] This heparin-mimicking material supported the expansion of multiple hPSC lines over 20 passages, without affecting their pluripotency and karyotypic stability. Although not yet applied for the 3D culture of embedded single cells or tissue analogs, we envision that hydrogels containing fully synthetic GAG analogs will allow for unprecedented levels of control over in vitro tissue morphogenesis and organoid development.

4. Perspectives

The introduction of better defined and tunable hydrogel matrices for organotypic and organoid cultures has further raised our awareness of the key role of exogenous signals in the morphogenetic processes driving tissue and organ formation. For a long time, Matrigel has been applied as a universal and mainly permissive matrix in the culture of organoids of different origin, not considering the potentialities of tuning tissue and organ-specific exogenous cues. Extrapolating current trends, we envision the rational design of engineered matrices to soon allow for generating highly orchestrated sets of cross-scale environmental signals that encode the constraints of various dynamic tissue-specific milieus. Moreover, due to their xeno-free, chemically defined, and modular nature, engineered synthetic hydrogels guarantee higher levels of experimental accuracy and reproducibility, and can

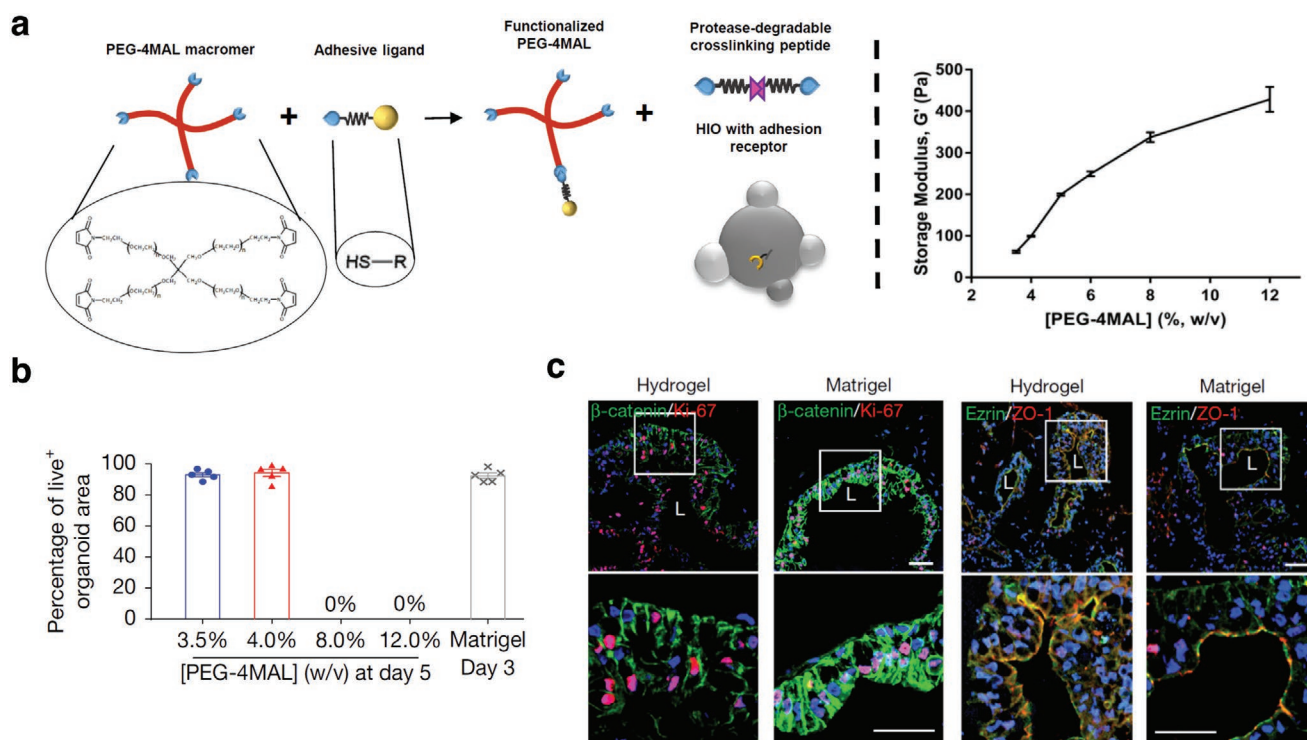


Figure 12. Fully synthetic PEG-4MAL hydrogels for the generation of human intestinal organoids. a) Schematic showing the principles of hydrogel formation and human intestinal organoid (HIO)/spheroid embedding. The storage modulus of the hydrogel can be tuned by varying the PEG-4MAL density (wt%). b) Hydrogels with 3.5–4.0% PEG-4MAL density (≈ 100 Pa) allow the formation of intestinal organoids with efficiency comparable to Matrigel. c) Immunostaining of intestinal organoids produced within 4.0% PEG-4MAL-RGD hydrogels or Matrigel. Scale bars, 100 μm . Reproduced with permission.^[25d] Copyright 2017, Springer Nature.

help to bridge the gap between in vitro studies and regenerative medicine applications. Even anticipating the advent of these developments and despite of the impressive progress in mechanistically deciphering tissue morphogenesis and the development of more and more robust differentiation protocols, state-of-the-art organoid technologies are still facing several fundamental limitations. Subsequently, we discuss how these main challenges of organoid cultures (Figure 13) can be addressed—at least in part—with the help of hydrogel-based materials to aim at more physiologically relevant tissue analogs.

4.1. Scalability and Connectivity/Integration

Organoids generated by in vitro cellular self-assembly generally only mature to the level of fetal tissues and grow up to a few millimeters in size.^[276] Engineering approaches to account for the high cell density and cross-scale hierarchical complexity of in vivo tissues will be indispensable for the development of organoids with more realistic size and architectural organization. This, in turn, will increase the maturation and the functionality of the organoids, and may expand their translational potential to enable whole-organ transplantation approaches. Biofabrication technologies and bottom-up assembling strategies can be instrumental to meet this challenge. Organoids can

be used as dynamic single building blocks or bio-inks, combined with engineered hydrogel units and matrices, for the precise realization of specific tissue geometries.^[277] For example, complex cell–cell interactions could be modelled combining region-specific organoids with other cell types, other organoids, or engineered signaling centers to generate, respectively, multilineage, multiregion, or polarized assembloids.^[278] Multiphasic hydrogel materials will become of utmost relevance in defining the regional specificity of the assembling units.^[279] Microgels with different biochemical and physical identity, for instance, could be used to pre-differentiate and support the individual building blocks which, when assembled, would generate organized functional tissues with controlled compartmentalization. Microgel-in-gel approaches would add an even higher level of spatially resolved tissue complexity. For instance, organoids-containing microgels could be embedded within a bulk hydrogel matrix for the incorporation of supportive cell populations (i.e., stromal and/or vascular compartment).^[280]

An important aspect hampering the maturation and growth of state-of-the-art organoids is related both to diffusion limitations and short lifespan. Bioreactors have been already applied in tissue engineering to ensure gas exchange, as well as transport of nutrients and waste products,^[281] and they were also shown to enhance lifespan, maturation, reproducibility and scaled-up production of organoids.^[282] The integration of functional and connectable vascular networks can further

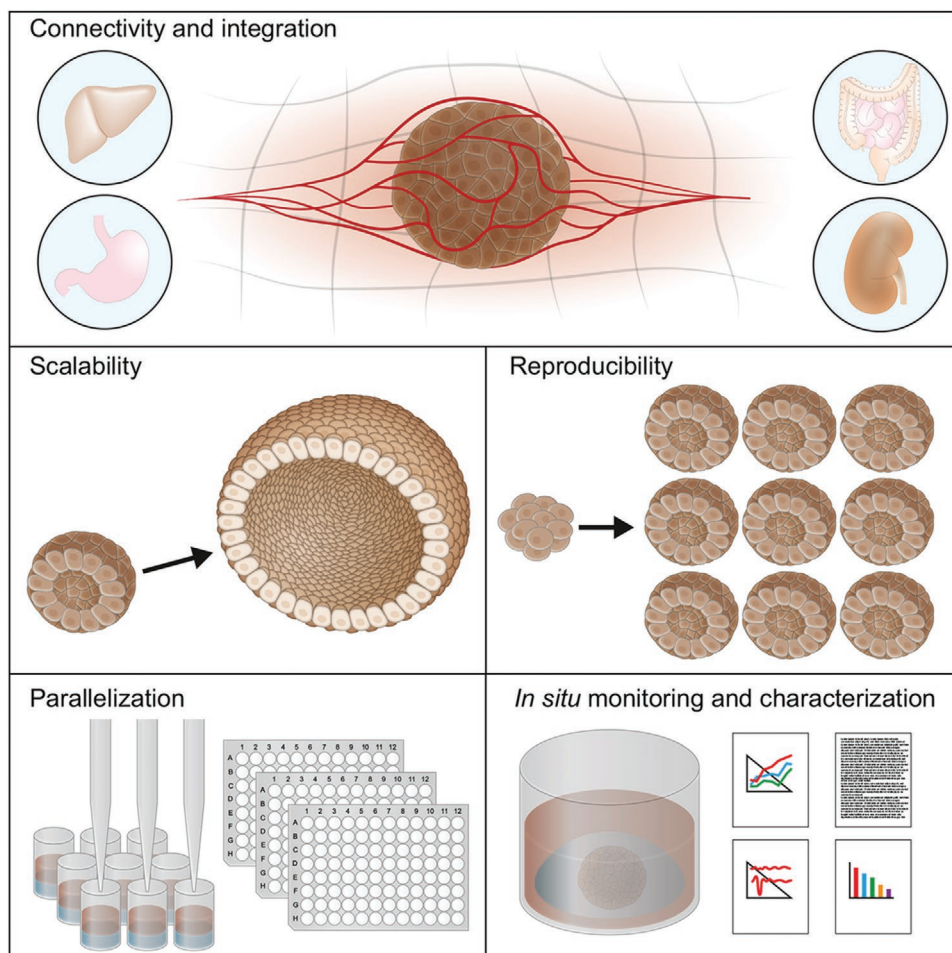


Figure 13. Current challenges of organoids cultures. a) Allow for connectivity and integration to increase realism and complexity. b) Produce size-relevant tissue-analogs with higher levels of structural organization and maturity. c) Balance self-organization and cell-guidance for robust and reproducible generation of organoids. d) Enable parallelization for high throughput screening and combinatorial approaches. e) Implement advanced in situ monitoring and in-depth characterization approaches.

promote the maturation of organoids, as it has been already established through in vivo and in ovo transplantation experiments.^[283] Furthermore, facilitating the mass transfer of oxygen and nutrients, it would allow for more efficient morphogenesis of scaled-up, size-relevant tissues.

Different engineering approaches can be applied to implement the connectivity of vascular structures to developing organoids. Among them, bioprinting combined with sacrificial molding has shown great potential.^[284] Particularly, recent work by Skylar-Scott et al. impressively demonstrated the vascularization of highly dense tissue constructs composed of organ building blocks (OBBs) through a new technique called SWIFT (sacrificial writing into functional tissue).^[284d] Following this biomanufacturing method, patient-specific iPSC-derived organoids were generated, resuspended into a cold collagen-Matrigel solution, and assembled at high density via centrifugation to yield a living matrix containing about 200 million cells mL⁻¹. Thanks to the viscosity and self-healing properties of the cell-ECM slurry, a sacrificial gelatin ink was printed within the tissue without generating defects or altering cell viability. Bringing the construct to 37 °C induced gelation of the

collagen-Matrigel solution, while allowing the gelatin ink to melt and evacuate, leaving behind a channel that was then used to form a branching vascular network. Perfused, patient-derived cardiac tissues showed high viability compared to control tissues with no embedded channel, where a necrotic core was observed within 12 h. Most importantly, the perfused cardiac OBBs fused together to form a solid tissue that could beat synchronously over a 7-day period. Using a similar strategy, Miller et al. generated a vascularized, monolithic cellularized tissue by casting a cell-containing ECM prepolymer solution onto a sacrificial carbohydrate glass network.^[284a] The authors demonstrated the high versatility of the system, as a wide range of natural and synthetic materials was compatible with the casting technique. A different approach to produce vascularized organoids would consist of co-culturing endothelial cells with the developing tissue. In this context, engineered hydrogels can define cell-instructive niches that guide vascular network formation and support their maintenance.^[25e,271b,285]

Considering connectivity and integration, yet another level of complexity could be reached by the realization of organoids-on-a-chip.^[286] Although on a miniaturized scale, organoids of

different origin could be connected together and integrated in microfabricated devices for the precise control over mechanical forces and biochemical factors. Engineered hydrogel materials would allow for organ-specific compartmentalization and spatiotemporally defined niche factors. These complex microphysiological systems, recreating the *in vivo* cross-talk between different organs, would offer extremely powerful models of human (patho)physiology for drug screening, studying disease mechanisms, and tailoring individualized therapies.^[287]

4.2. Reproducibility

While organoids mimic the microarchitecture of their respective tissues of origin, *in vitro* self-organization processes can only in part recapitulate the heterogeneous and hierarchical structure of *in vivo* organs. Intuitively, this is particularly true for more complex organoids, such as the kidney, where the emergence of functional structures with coherent architectural organization can strongly impede the experimental reproducibility. Furthermore, variability of organoid cultures can arise already from the first steps of differentiation, and be expressed as variations in cell type proportions and the presence of undesired cell populations.^[288] As pointed out by Lütolf and co-workers,^[153,289] a balance between self-organization and guided development is needed for robust and physiologically relevant morphogenesis. Several bioengineering strategies can be applied to control each of the critical stages of organoid development, taking into account the spatiotemporal heterogeneity of the ECM cues driving cell proliferation, migration, sorting, and architectural arrangement. In standard organotypic and organoid culture systems most of the cell-instructive cues are presented to the cells through a predominantly uniform fashion, both spatially and temporally. Hydrogel-based materials could be engineered in order to locally provide specific biochemical and mechanical signals able to trigger lineage commitment, spatial cell patterning, and organization. For instance, gradients of signaling molecules have been reproduced *in vitro* using microfluidic approaches^[290] or morphogen-loaded microparticles.^[291] Manfrin et al. have recently shown the importance of asymmetric signaling environments through the development of engineered signaling centers.^[290] Using a microfluidic-based approach, they generated spatiotemporally controlled counteracting gradients of BMP4 and NOGGIN that were able to break the radial symmetry of confined hPSC colonies to mimic the axial germ layer patterning naturally occurring during *in vivo* embryonic development. Biomaterials can be an alternative or complementary approach to microfluidics for the generation of controlled morphogen gradients,^[292] and hydrogel-based systems have currently reached high levels of tunability in this respect. Highly charged GAG-based biohybrid hydrogels whose integral space charge density and local charge density can be tuned independently are now available for unprecedented control over the binding and release of multiple signaling molecules.^[293] Microgel-in-gel systems based on these GAG-containing materials would allow for the culture of organoids in presence of microgels acting as engineered signaling centers precisely positioned in the supportive bulk matrix.

Likewise, the controlled presentation of adhesive ligands is critical for robust cell sorting and migration. Patterning of bioligands in specific shapes and sizes, for instance, was shown to enable controlled morphogenesis of neural rosettes.^[294] Engineered hydrogels can also allow for the dynamic positioning of adhesive ligands. PEG hydrogels can be functionalized with biomolecule-binding sites protected by photosensitive caging group for the precise control of the spatial and temporal distribution of bioligands.^[295] Upon irradiation at a defined time and on a specific region, adhesive peptides can be incorporated and used to spatially orient cell migration within the matrix.

Another critically important aspect to consider for controlled and more realistic *in vitro* morphogenesis is the dynamic physical nature of *in vivo* ECM. While in their tissue of origin cells interact with mechanically dynamic and dissipative environments, standard hydrogels used for organoid culture mainly behave as linearly elastic materials, where compressive forces build up upon colony growth and cannot be dissipated, thus hampering *in vitro* morphogenesis.^[259,296] Different strategies can be used to induce the progressive softening of covalently cross-linked hydrogels which include enzymatic degradation,^[27,24] photo-induced degradation,^[297] or passive hydrolysis.^[28a,259] Nevertheless, such approaches might be incompatible for long-term culture of organoids. Hydrogels with stress-relaxation characteristics generated through reversible cross-linking schemes^[296,298] not only improve organoid development, but may also allow for more controlled scalability of the tissue analogs.

4.3. Parallelization

Along with the development of strategies for the generation of size-scalable organoids, technologies for automated, parallelized, and miniaturized production are required. High throughput development of organoids through sophisticated liquid handling systems empowers combinatorial approaches for the rational design of both differentiation protocols and cell-instructive matrices.^[25b,299] Although modular hydrogel materials are particularly suitable for the high throughput and systematic investigations of cell-instructive matrix features, well-chosen combinations of functional polymers and gelation characteristics are required for effective and consistent network formation and homogeneous cell distribution.^[300] Furthermore, the concomitant development of new analytical and computational tools will be needed to further unravel the synergistic communication between cells and ECM. Ultimately, the parallelized and high throughput production of realistic organoids will be fundamental for the generation and the routine application of human disease models, paving the way for faster compound screening for large-scale pharmaceutical applications and individualized therapies.^[299a]

4.4. Monitoring and Characterization

While progress in culture and engineering protocols is allowing for the realization of more and more realistic tissue analogs,

the parallel development of accurate analytical methods and sophisticated in situ monitoring strategies is required for in-depth molecular and structural characterization of organoids. Single cell sequencing^[288,301] and RNA tomography^[302] can give valuable information on the transcriptional signature of the developing organoids. In particular, RNA tomography can be complementary with microscopy-based approaches and reveal spatially resolved gene expression within complex tissue analogs.

The analysis of self-organization during organoid formation requires scale-crossing technologies that allow both spatial (from micrometers to centimeters) and temporal (from milliseconds to days-weeks) resolution. Imaging technologies need to bridge cellular and subcellular information with tissue organization and functionality. Confocal,^[303] multiphoton laser scanning,^[303] and light sheet fluorescence microscopy^[304] are non-invasive optical sectioning techniques that allow for a fine resolution of whole organoid architectures. Furthermore, clearing methods have been developed to overcome light scattering. While some methodologies increase the transparency for whole organ imaging,^[305] others were optimized for the handling of fragile organoid structures.^[306] Other important advances concern the use of multiple fluorophores for multiplexed fluorescence microscopy. For instance, Gut et al.^[307] impressively showed the multiplexed and high throughput analysis of 40 different proteins through iterative indirect immunofluorescence imaging (4i) of fixed cell populations across length scales, simultaneously capturing properties at populational, cellular, and subcellular levels.

Organoids are a powerful tool to decipher key mechanisms of tissue development and live-cell imaging is particularly useful for the observation of the related dynamic processes. Light sheet microscopy allows for fast and detailed scanning of biological samples with minimized light exposure and phototoxicity. Furthermore, monitoring of in vivo transplanted organoids is today complemented with advanced lineage-tracing methods with multi-colored reporters.^[308] With a rapidly increasing number of sophisticated 3D imaging strategies becoming available, new image analysis tools are needed to extract the collected information. Addressing this need, Coutu et al.^[304–309] have recently introduced an open access software for the analysis of big imaging data sets with single-cell resolution.^[309]

Beyond the molecular and structural characterization, novel monitoring concepts are emerging in the context of in vitro tissue morphogenesis. As mechanical signaling is one of the key factors driving cellular behaviors, different technologies were developed for the quantification of the mechanical stress exerted and experienced by cells during tissue development. Biomaterial engineering currently offers valuable tools for the realization of stress-sensors. For instance, highly defined, cell-like microgels were recently designed^[310] for the quantification of isotropic and anisotropic stress in developing zebrafish^[311] and the measurement of compressive forces of PEG-heparin hydrogels on embedded breast cancer tumor spheroids.^[312] The incorporation of such mechanical probes in developing organoids would give new insights into the spatiotemporal dynamics of in vitro morphogenetic processes, and would allow for more accurate design of cell-instructive microenvironments.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

biomaterials, extracellular matrix, hydrogels, organoids, organotypic cultures

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