Bio-inspired 3D microenvironments: a new dimension in tissue engineering



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# Bio-inspired 3D microenvironments: a new dimension in tissue engineering

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## Abstract

Biomaterial scaffolds have been a foundational element of the tissue engineering paradigm since the inception of the field. Over the years there has been a progressive move toward the rational design and fabrication of bio-inspired materials that mimic the composition as well as the architecture and 3D structure of tissues. In this review, we chronicle advances in the field that address key challenges in tissue engineering as well as some emerging applications. Specifically, a summary of the materials and chemistries used to engineer bio-inspired 3D matrices that mimic numerous aspects of the extracellular matrix is provided, along with an overview of bioprinting, an additive manufacturing approach, for the fabrication of engineered tissues with precisely controlled 3D structures and architectures. To emphasize the potential clinical impact of the bio-inspired paradigm in biomaterials engineering, some applications of bio-inspired matrices are discussed in the context of translational tissue engineering. However, focus is also given to recent advances in the use of engineered 3D cellular microenvironments for fundamental studies in cell biology, including photoresponsive systems that are shedding new light on how matrix properties influence cell phenotype and function. In an outlook for future work, the need for high-throughput methods both for screening and fabrication is highlighted. Finally, microscale organ-on-a-chip technologies are highlighted as a promising area for future investment in the application of bio-inspired microenvironments.

## Introduction

Tissue engineering is an interdisciplinary field in which technologies and principles from life science, engineering and medicine are synergistically combined to develop functional substitutes for damaged or diseased tissues or organs [1]. In this pursuit, it has become increasingly clear that it is crucial for biomaterial scaffolds created to facilitate tissue engineering be inspired by the natural three-dimensional (3D) structure of tissues [2, 3]. Biologic tissues are composite materials that exhibit characteristic and precisely oriented structures on size scales ranging from nanometers to centimeters [4]. A closer look at these structures reveals cells, which are on the order of tens to hundreds of micrometers, organized in distinct configurations within an extracellular matrix (ECM).

The natural ECM is a dynamic and hierarchically organized material that regulates essential cellular functions including adhesion, migration, proliferation, differentiation and morphogenesis [1]. This complex, heterogeneous network of soluble and insoluble proteins [5], growth factors [6] and polysaccharides provides the mechanical framework to facilitate cell anchorage, cell-cell interactions and tissue formation. Cell-secreted, insoluble matrix proteins such as collagens and elastins are arranged in an anisotropic fibrous architecture that provides both nanoscale (10–300 nm) and microscale (10–100 $\mu$m) topographic cues [5, 7].

Although this framework is critical for guiding cell shape and orientation to form specialized structures that are adapted to the particular function of the tissue [8], the matrix environment is not static. Instead the ECM is dynamically degraded, synthesized and remodeled by cells in both healthy and diseased tissue [9]. Much of our understanding of the mechanisms that control cellular functions such as migration, proliferation and differentiation has been learned from studying cells *ex vivo* on two-dimensional (2D) stiff glass or plastic surfaces [10, 11]. However, cellular organization in 3D is crucial to recapitulate tissue microarchitecture including cell spacing, density and access to diffusible, soluble signals and study the resulting biological function.

Pioneering research conducted by Bissell and colleagues nearly two decades ago demonstrated that blocking cell surface $\beta$1-integrins led to reversion of malignant breast cancer cells to a nonmalignant phenotype in 3D cell culture, a phenomena that had not been previously observed in 2D [12]. Since the publication of this groundbreaking work, cancer researchers have been using complex 3D models [13] to investigate cell-cell paracrine signaling [14], the impact of matrix stiffness on cancer cell phenotype [15, 16] and potential targets for therapeutics [17, 18]. This paradigm of bio-inspired 3D culture is now being applied broadly to the study of various diseases. However, the recognizance that cell biology in 3D culture systems more accurately mimics what occurs in tissues has also nucleated a new sub-discipline of tissue engineering, one aimed at using bio-inspired matrices for fundamental studies of cell biology. For example, 3D cell culture environments have recently been used to develop and validate a new model for cell migration that replaces the classical persistent random walk model previously described on 2D cell culture substrates [19, 20].

The importance of mimicking the dynamic nature of the ECM is also increasingly clear. For instance, Burdick *et al* developed a sequentially crosslinkable hyaluronic acid-based hydrogel system to spatiotemporally pattern different mechanical properties and degrees of biodegradability into a scaffold [21]. Initially this system was studied in 2D and revealed that stem cells exhibited high (low) degrees of spreading and underwent osteogenesis (adipogenesis) on stiffer (softer) regions [21]. Upon translation to 3D, different results emerged—permissive regions that allowed for cellular degradation of the surrounding material led to osteogenesis because of degradation-mediated traction forces, regardless of cell spreading or matrix stiffness [22]. These findings underscore the distinction between traditional tissue culture vessels and the complexities of the 3D physiological microenvironment, as well as the importance of translating fundamental cell culture studies and downstream applications into 3D. Variations in cell morphology in 3D versus 2D could be attributed to a number of key factors including spatial distribution of adhesions [23], scaffold topography, matrix mechanical properties and degradability [24].

This contribution will review recent advances in the design, fabrication and application of bio-inspired, 3D cellular microenvironments for both improving the current understanding of how cells interact with a changing microenvironment and translational applications in tissue engineering and regenerative medicine.

## Challenges in tissue engineering scaffold design and fabrication

To motivate the need for bio-inspired, 3D microenvironments in tissue engineering, it is important to consider the limitations of current design and fabrication approaches. For both translational and fundamental tissue engineering applications, two key challenges that must be addressed include (1) the development of dynamic, heterogeneous structures that include a vascular network and (2) the advancement of technologies for high-throughput manufacturing and screening.

As mentioned earlier, cells reside *in vivo* in a complex and ever-changing 3D microenvironment. In order to recapitulate these cell-matrix interactions, it is necessary to design and develop heterogeneous micro-architectures that facilitate dynamic delivery of signaling cues. Traditional biomaterial scaffold manufacturing processes, such as electrospinning, solution-casting, particulate leaching, freeze-drying and gas foaming, have been employed to create highly porous scaffolds, which facilitate transport of nutrients, waste and cell-secreted signaling molecules, cellular infiltration and presentation of physical architectural cues on the appropriate size scale [25]. However, these techniques are often limited by the inability to precisely control pore size, shape, arrangement, interconnectivity and hierarchical structure. Recent advances in computational topology design and additive manufacturing [26] have made it possible to specifically control and investigate the delicate balance between temporary mechanical function and porosity for biological mass transport required by regenerative medicine applications. For example, the supply of nutrients and oxygen is limited *in vivo* by diffusion to cells within 100–200 $\mu$m of a capillary [27]. Although scaffolds designed with pore sizes between 0.8 and 8 $\mu$m have been shown to permit complete infiltration of host cells and neovascularization regardless of material composition [28], their inability to provide sufficient blood supply immediately after implantation can lead to improper cellular integration or hypoxia-related cell death within a construct [29]. While many constructs will vascularize over time, this process is often too slow to provide adequate nutrition to the center of a large construct. Therefore, it is essential to develop new bio-inspired strategies for enhancing vascularization to ensure survival of large tissue-engineered constructs.

With regards to the need for dynamic materials, cells receive signals and morphogenic cues via precise

spatial and temporal delivery throughout our lifetimes. Beginning in embryonic development [30, 31], biochemical activity, cell-cell signaling and mechanical interactions individually and collectively vary both spatially and temporally to instruct cellular development and ultimately tissue formation. To harness the potential of cells to modulate endogenous healing [32–34] or restore damaged tissue, it is important to first understand how cells receive information *in vivo* and then to apply this information to the rational design of bio-inspired microenvironments [35]. Bio-inspired materials with tunable dynamic properties can facilitate this fundamental work. For instance, the Anseth research group recently used photoresponsive matrices to demonstrate that human mesenchymal stem cells (hMSCs) initially grown on substrates delivering supraphysiological doses of mechanical stiffness retained the tendency to differentiate down the osteogenic pathway, despite subsequent culture on dynamically softened materials [36]. These findings imply that hMSCs have a mechanical memory and that temporal control over delivery of mechanical cues is imperative to recapitulate the native microenvironment. Emerging strategies for spatiotemporal control of stem cell fate, specifically, have been reviewed by Kinney and McDevitt [37].

The resulting vast experimental space includes limitless spatiotemporally diverse combinations of soluble chemical cues, tethered adhesive ligands, substrate stiffnesses and micro- and nanotopographies. Designing and developing new bio-inspired, 3D microenvironments that exploit these characteristics therefore requires high-throughput screening and manufacturing capabilities for the development of biomaterials [38, 39] as well as the application of subsequent products [40]. Emerging approaches to address these key challenges will be highlighted throughout this contribution.

## Fabrication of bio-inspired 3D microenvironments

### Chemistries and materials

Because of their high water content and tunable mechanical properties, hydrogels are well suited for creating bio-inspired materials that mimic the ECM found in soft tissues (figure 1) [2, 41, 42]. In fact, the ECM can be considered as a prototypical hydrogel. However, to be useful as 3D microenvironments, the hydrogel matrix must be formed under cytocompatible conditions that permit cell encapsulation. This design goal has been achieved using a variety of chemical crosslinking strategies as well as non-covalent, self-assembling systems.

The conventional approach to chemical crosslinking involves the chain-growth polymerization of vinyl-functionalized polymers. For example, poly(ethylene glycol) (PEG) based macromers terminated with either acrylate or methacrylate end groups were used to develop some of the first bio-inspired synthetic hydrogels that remains widely used today [43–45]. An important advantage of PEG-based hydrogels is their non-fouling, biologically inert nature, which provides a unique opportunity to engineer bio-inspired 3D microenvironments from the bottom up [46]. Bio-instructive components that mimic aspects of the native ECM (e.g. synthetic peptides to enable integrin-mediated cell adhesion) can be readily incorporated into hydrogel networks. The mechanical properties of the matrix can also be tuned over a wide range by varying the PEG concentration and molecular weight. Notably, chain polymerization strategies are also widely used to create ECM-mimetic hydrogels from natural biopolymers, including alginate, hyaluronic acid (HA), and gelatin [47–51]. These biopolymers are rendered crosslinkable by functionalizing side-chain hydroxyl, carboxyl, and amine groups with acrylate or methacrylate groups. While alginate hydrogels require modification with bioactive components to function as ECM mimics, HA and gelatin (i.e. denatured collagen) are derived from natural ECM proteins and inherently contain bioactive sites [52]. However, it should be noted that synthetic peptides have been added to HA matrices to impart additional biological properties. Examples include functionalization with N-cadherin mimetic peptides [53], as well as matrix metalloproteinase (MMP)-sensitive crosslinks, integrin-binding motifs, and heparin for growth factor sequestering [54–55]. The mechanical properties of biopolymer hydrogels can be tuned by varying the degree of functionalization, the molecular weight of the macromolecular monomers, and their concentration during gel formation.

Several alternatives to chain-growth polymerization have been proposed for crosslinking both PEG-based and biopolymer-based hydrogels. These include thiol-X reactions (i.e. Michael-type addition, photoinitiated thiol-ene addition) [56, 57], Diels-Alder chemistry [58, 59], oxime and hydrazone ligation [60–62], copper-catalyzed azide-alkyne cycloadditions (CuAAC) [63, 64], strain-promoted azide-alkyne cycloadddditions (SPAAC) [65, 66], and tetrazine-based cycloadditions [67, 68]. All of these alternatives fall under the paradigm of click chemistry, which encompasses reactions that are fast, efficient, specific, proceed under mild conditions, and produce inoffensive byproducts [69, 70]. An important benefit of using click crosslinking chemistries is that their specificity can be exploited to create well-defined materials. In the case of thiol-X chemistries, the incorporation of bio-inspired peptides that mimic features of the native ECM is facile and can be readily achieved by inserting cysteine residues into the sequence [71]. This approach has been used in both PEG- and HA-based hydrogel matrices.

The specificity of click crosslinking can also enable the utilization of sequential reactions, which has been key for engineering phototunable matrices that can mimic the dynamic properties of the native ECM. For example, in HA hydrogels primary crosslinking by thiol-acrylate Michael addition has been combined with secondary crosslinking via acrylate chain

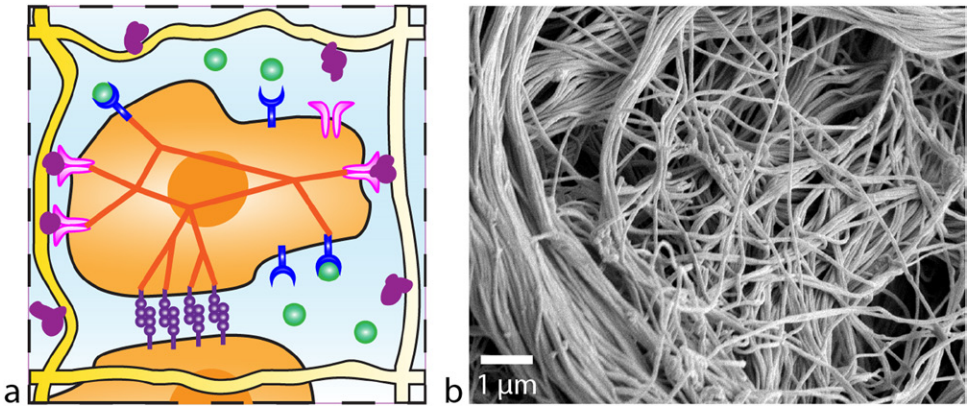


**Figure 1.** (a) Schematic of an engineered hydrogel microenvironment. Hydrogels are a class of highly hydrated materials that can be tuned to represent a vast experimental space including limitless spatiotemporally diverse combinations of soluble chemical cues (green), tethered adhesive ligands (purple), substrate stiffnesses and micro- and nanotopographies. These parameters can be exploited to design and develop new bio-inspired, 3D microenvironments that mimic the complexity of (b) the natural ECM depicted in a scanning electron micrograph of a porcine urinary bladder. Reprinted with permission from Kirschner *et al* [42]. Copyright WILEY.

polymerization to stiffen matrices [72] and interrogate the effects of cell shape and cell-mediated degradation on stem cell differentiation as well as vascular network formation in 3D cultures [54, 22]. Dynamic changes in biochemical composition have been achieved in PEG-based matrices by coupling CuAAC [64], SPAAC [65, 73], tetrazine [67], and oxime [61] click crosslinking with thiol-ene photoaddition reactions. Photocleavage reactions are also a powerful tool for biochemical patterning. Uncaging nitrobenzyl-protected reactive moieties like thiols [74–76], amines [77], and aminooxy [66] groups to enable secondary reactions (e.g. crosslinking for stiffening, conjugation of proteins and peptides) is another important and useful strategy that has been combined with click crosslinking. Several studies have also used click crosslinking in combination with nitrobenzyl and coumarin photocleavage reactions to photodegrade hydrogels as well as to release bioactive molecules such as growth factors [69, 78–80]. However, it should be noted that photodegradable chemistries have also been effectively used in non-click hydrogel systems [81–84].

While many molecularly crosslinked hydrogels are amorphous and uniform on the size scale of a cell, supramolecular assembly of peptide-based amphiphiles allows the formation of nanofibrillar hydrogel matrices that mimic the hierachical structure of ECM proteins and is another important and active area of research. In these materials, self-assembly occurs via non-covalent interactions between peptide subunits [85, 86]. Thus, the molecular structure and chemistry of the peptides are important. Peptide amphiphiles comprising a hydrophobic tail, a $\beta$-sheet sequence, and charged residues that promote water solubility and self-assembly into nanofibrillar matrices have been extensively investigated for tissue engineering and regenerative medicine [87–93]. A number of naturally occurring motifs that promote self-assembly are also known and have been used to develop ECM-like hydrogels for tissue engineering, including $\beta$-sheet, $\beta$-helix, and triple-helical collagen mimetic peptides [94–96]. An important advantage of peptide-based nanofibrillar matrices is their modularity. Bioactive sequences such as integrin-binding motifs can be introduced and displayed from the nanofibers [97], as can crosslinkable moieties to increase the matrix stiffness [98]. Enzymatically cleavable regions and releasable drug conjugates can also be incorporated [99]. Recently, a generalizable strategy for incorporating gradated combinations of multiple proteins in $\beta$-sheet fibrillizing matrices was reported [100]. Supramolecular chemistry has also been used to engineer shear thinning HA hydrogels for bioprinting [101], although the materials produced were not nanofibrillar.

## Bioprinting

Many of the materials and chemistries summarized above are amenable to processing via conventional material fabrication techniques like solution casting and photopolymerization. However, additive manufacturing is a particularly powerful and emerging fabrication technique that warrants special consideration. Advances in additive manufacturing technologies have already significantly improved the field's ability to make implantable devices that are customized to specific patients. In fact, the U.S. food and drug administration (FDA) has emphasized the promising future of additive manufacturing for advancing personalized medicine. During an American Association for Cancer Research meeting FDA Commissioner Margaret Hamburg said, '3D printing is transforming our concept of personalized medicine and medical intervention opportunities'. Using 3D computer models created from bioimaging data (e.g. a computed tomography (CT) scan), additive manufacturing processes, which fabricate 3D objects in a layer-by-layer fashion, can be used to fabricate tissues that replicate patient anatomy [102].

The advent of bioprinting—3D printing of biocompatible materials including resorbable polymers,

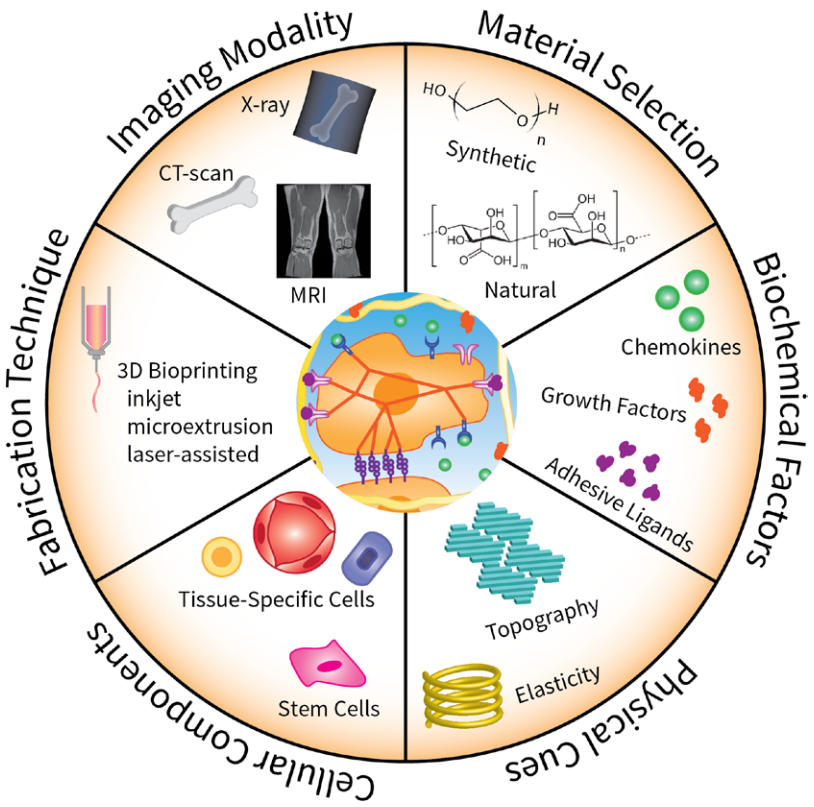


**Figure 2.** The physiological cellular microenvironment is both dynamic and complex. The tools available to create 3D bio-inspired microenvironments, which recapitulate the natural ECM include various imaging modalities, materials, chemical and physical cues, cellular components and fabrication techniques.

biologics, biomatrix, biofactors and cells—has given researchers unprecedented ability to recapitulate the native microenvironment by enhancing control over scaffold architecture on the macro- to micro- to nano-scale (figure 2) [103, 104]. Pioneers in this field created the first bioprinters by modifying commercial inkjet printers originally designed to print ink on paper [105, 106]. The ink cartridges were filled with bioink solutions [105, 106] comprising cells in a crosslinkable hydrogel matrix, and the paper was replaced with an electronically controlled platform to control vertical movement along the *z*-axis [105, 107] in addition to the x-y positioning provided by the printer. In recent years, the field has witnessed a revolution in grassroots innovation and creativity due to the development and availability of low-cost, open-source hardware and software [108] for fabrication [109]. For example, the Arduino electronic prototyping platform has been exploited to create a 3D bioprinter for less than $150 [110]. The biomaterials research community has embraced this open-source concept by collaborating to modify software/hardware and then contributing these improvements back to the community through both traditional publication of procedures in scientific journals [111] as well as the use of other nontraditional online forums such as Instructables [112]. Inkjet printers are now specifically designed and developed for bioprinting, and the fundamental technology is still the most widely used today. Inkjet bioprinting is now more inexpensive, flexible and accessible than ever before [106].

Inkjet bioprinting is a noncontact printing method that reproduces digital pattern information by using thermal or acoustic forces to precisely deposit 1 to 300 pl [113, 114] droplets of liquid at rates of 1 to 10 000 droplets per second [115] resulting in a resolution of ~50 $\mu$m [104]. Early research established that the inkjet printing process does not have a substantial impact on cell viability with reported survival rates of ~90% [105, 107]. The Wake Forest Institute for Regenerative Medicine, specifically Atala and colleagues, have embraced bioprinting as an approach to overcome the current challenges in tissue engineering scaffold fabrication. Seminal work by Atala *et al* in the field of bioprinting used inkjet technology to fabricate microparticles containing insulin-producing cells by coprinting the cells suspended in a sodium alginate solution directly into a calcium chloride solution. This method produced microparticles in the range of 30 to 60 $\mu$m and demonstrated high levels of cell viability [107]. Within the same year the Atala group reported producing tissue constructs using layer-by-layer printing of alginate/collagen inks containing cells that vascularized *in vivo,* as demonstrated by magnetic resonance imaging [116]. Recently a similar method was employed to print complex heterogeneous structures composed of two materials and three distinct cell types delivered to specific areas of the construct. Individual cell types retained both viability and appropriate phenotypic expression within the constructs *in vitro* and *in vivo*. Importantly, the bioprinted constructs induced

**Table 1.** Examples of 3D bio-inspired microenvironments in tissue engineering applications.

| Materials | Fabrication method | Cell source | Application | References |
|---|---|---|---|---|
| PCL Fibers/Alginate | Bioprinting | Chondrocytes | Cartilage tissue | Schuurman *et al* [125, 126] |
| Pluronic F127/GelMA | Bioprinting | HUVECs, HNDFs, 10T1/2s | Vascularized engineered tissue | Kolesky *et al* [128] |
| HA/N-Cadherin Mimetic Peptides | Chain-growth polymerization | hMSCs | Cartilage tissue | Bian *et al* [53] |
| MaHA/MMP-degradable crosslinker/BMP-2 | Thiol-ene polymerization | N/A | Bone tissue | Holloway *et al* [132] |
| Fibrin/PDGF-BB/BMP-2 | Supramolecular assembly | hAMSCs | Bone tissue<br>Skin regeneration | Vila *et al* [133, 134] |
| PCL | Bioprinting | N/A | Airway splint | Zopf *et al* [146] |
| dECM/PCL | Bioprinting | hASCs<br>hTMSCs | Heart, cartilage, and Adipose tissue | Pati *et al* [147] |
| Fibrin/Collagen | Bioprinting | AFSC | Skin regeneration | Skardal *et al* [148] |
| PEGDMA | Bioprinting | Chondrocytes | Cartilage tissue | Cui *et al* [149] |
| Alginate/Gelatin | Bioprinting | VICs, SMCs | Aortic valve conduits | Duan *et al* [150] |
| PEGDA/Collagen | Bioprinting | VICs | Aortic valve conduits | Hockaday *et al* [151] |
| PEG/MMP-degradable crosslinker/TGF$\beta$1 | Thiol-ene polymerization | Chondrocytes, hMSCs | Cartilage tissue | Sridhar *et al* [141] |
| PEG/MMP-degradable crosslinker/Vitronectin | SPAAC polymerization | hMSCs | Bone tissue | DeForest *et al* [67] |
| PCL/fibrin/collagen | Bioprinting | Chondrocytes | Cartilage | Xu *et al* [124] |
| PEG/MMP-degradable crosslinker/rhBMP2 | Thiol-ene polymerization | N/A | Bone tissue | Mariner *et al* [135] |
| PU/PCL/HA/gelatin/ Fibrin | Bioprinting | C2C12, NIH3T3 | Muscle-tendon | Merceron *et al* [131] |

adequate vascularization and therefore survived to form mature, functional tissues upon implantation *in vivo* [117]. The linear chains of alginate and/or collagen within these bioink suspensions form interchain associations in the presence of divalent cations [118] available in the solutions into which these materials have been printed to form natural hydrogels. Although natural hydrogels are inherently biocompatible and can be easily recognized and remodeled by cells [119], these natural networks are typically not mechanically robust and may contain endogenous signals that are difficult to isolate and control [120].

To overcome these limitations engineers and scientists are developing strategies to add mechanical stability to natural bioink components [121] or add biofunctionality to synthetic ones [122, 123]. Hybrid printing has emerged as an advanced fabrication technique for adding mechanical stability to bioprinted constructs. For instance, Tao *et al* combined inkjet printing with an electrospinning system to fabricate hybrid constructs consisting of alternating layers of electrospun polycaprolactone (PCL) for mechanical strength and rabbit elastic chondrocytes suspended in a fibrin-collagen hydrogel to create engineered cartilage tissue [124]. The resulting Young's modulus of the printed hybrid constructs (1.76 MPa) was four times higher than that of hydrogel constructs (0.41 MPa) and demonstrated the capability to produce cartilage specific ECM both *in vitro* and *in vivo* [124]. Similar results have also been achieved through a hybrid bioprinting technique that combined 3D deposition of PCL fibers with inkjet printing of cell-laden alginate hydrogel [125, 126].

Recent advances in bioink technology represent a promising strategy for researchers to address one of the key challenges in tissue engineering scaffold fabrication: the creation of heterogeneous 3D structures that include a vascular network. Chemical and material strategies similar to the ones outlined in the previous section have been translated into the creation of new bioink formulations [127]. Recently, Kolesky *et al* reported the development of a printing process that facilitates deposition of two dissimilar cell-laden inks and a fugitive ink to create space for vasculature simultaneously [128]. First a thermally reversible Pluronic F127-based ink was deposited to mimic natural 3D microvasculature. Next, gelatin methacrylate (GelMA) was added as both the bulk scaffold material as well as the cell-carrier. GelMA is comprised of gelatin, i.e. denatured collagen, modified with methacrylate moieties, which impart the ability for the scaffold to be covalently crosslinked by ultraviolet (UV) light in the presence of a photoinitiator. After covalent crosslinking, the construct was cooled below 4 °C subsequently liquefying the fugitive ink to facilitate removal and create space for a complex vascular network. This work demonstrated that the injection of human umbilical vein endothelial cells (HUVECs) led to the formation perfusable neovasculature [128]. A similar approach was employed by Miller *et al* using a slow-dissolving

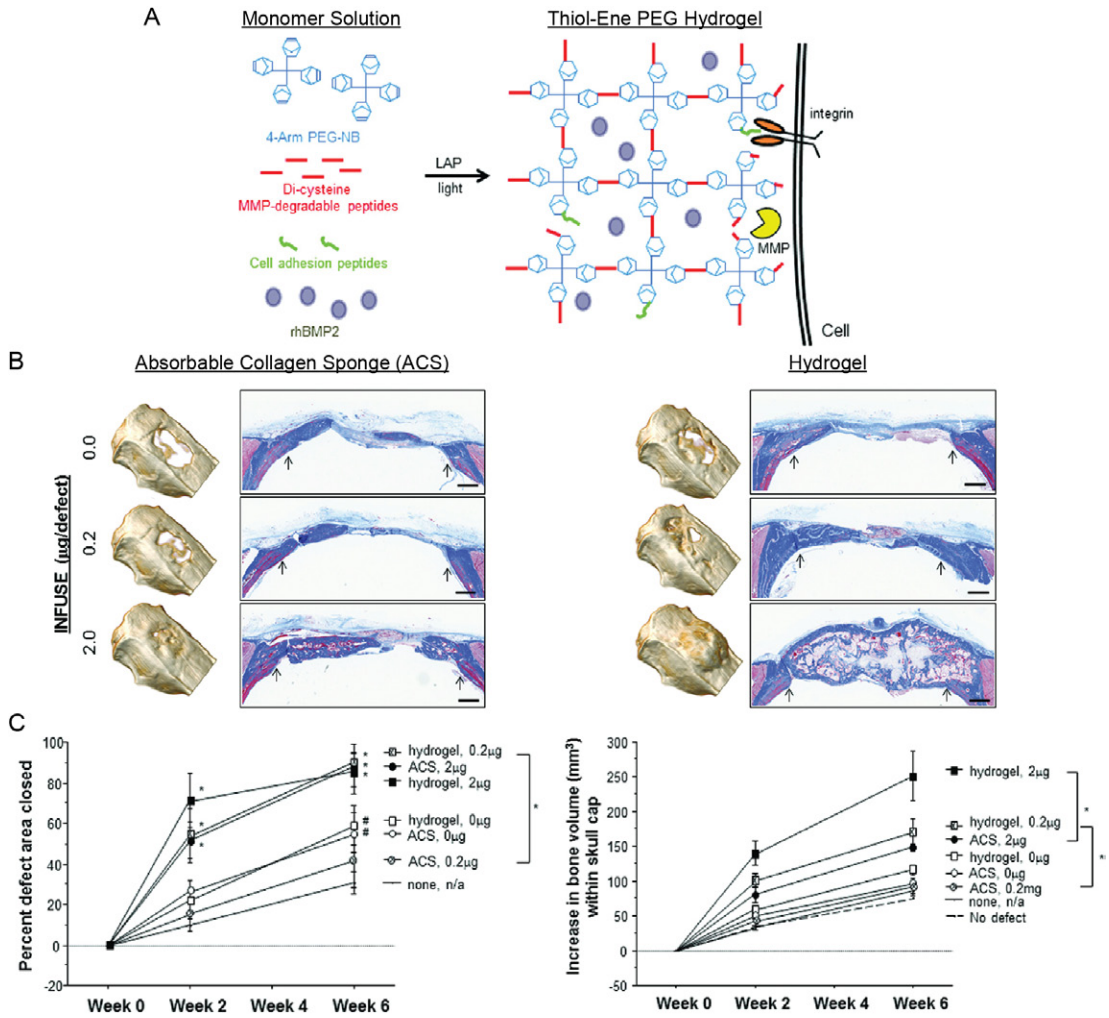


**Figure 3.** Bone tissue engineering using bio-inspired PEG-peptide hydrogels. (a) PEG hydrogels containing ECM mimetic MMP-degradable crosslinks and cell adhesive peptides were photopolymerized to encapsulate and deliver rhBMP2 (INFUSE). Qualitative (b) and quantitative (c) analyses of histological and $\mu$CT data after implantation into rat calvarial defects showed that both defect closure and bone volume were significantly enhanced using the hydrogel delivery system, even with a 10-fold lower dose of rhBMP2. Comparisons were made to an absorbable collagen sponge, which is the standard delivery material for INFUSE. Reproduced with permission from Mariner *et al* [135]. Copyright WILEY.

carbohydrate glass as a sacrificial material to fabricate perfusable vascular structures in cell-laden PEG hydrogels [129]. In another example, Highley *et al* exploited the structure and function of supramolecular hydrogels based on modified hyaluronic acid (HA) to develop a hydrogel-based 3D printing system where a shear-thinning hydrogel bioink was printed directly into a self-healing support hydrogels [101].

## Applications of 3D microenvironments in tissue engineering

The advances in chemistries, materials and manufacturing methods described above not only address key challenges in the development of dynamic, heterogeneous structures that more precisely mimic native tissue, but also move these technologies closer to application in the clinic (table 1) where the severe shortage of tissue and organs for transplantation is worsening every year [130]. Tissue engineering applications of bioprinting in particular are seemingly endless, and in fact, this technique has been used to create several tissue-mimics [104] including cartilage [124] and muscle-tendon complexes [131]

Many of the 3D ECM-mimicking materials created from natural and synthetic polymers have been utilized in preclinical tissue engineering studies. For example, methacrylated HA hydrogels modified with N-cadherin mimetic peptides and loaded with hMSCs have shown neocartilage formation after subcutaneous implantation in a murine model [53]. Similarly, natural, biodegradable maleimide-functionalized HA (MaHA) [132] and fibrin-based [133] hydrogels loaded with bone morphogenetic protein-2 (BMP-2) have been shown to enhance cellular ingrowth and bone regeneration in murine critical-sized calvarial defect models. Growth factors such as BMP-2 and platelet-derived growth factor-BB (PDGF-BB) have also been modified with the placenta growth factor-2 (PIGF-$2_{123–144}$) to enhance their ECM-binding affinity. When incorporated into fibrin hydrogels these super-affinity growth factors induced repair of both chronic

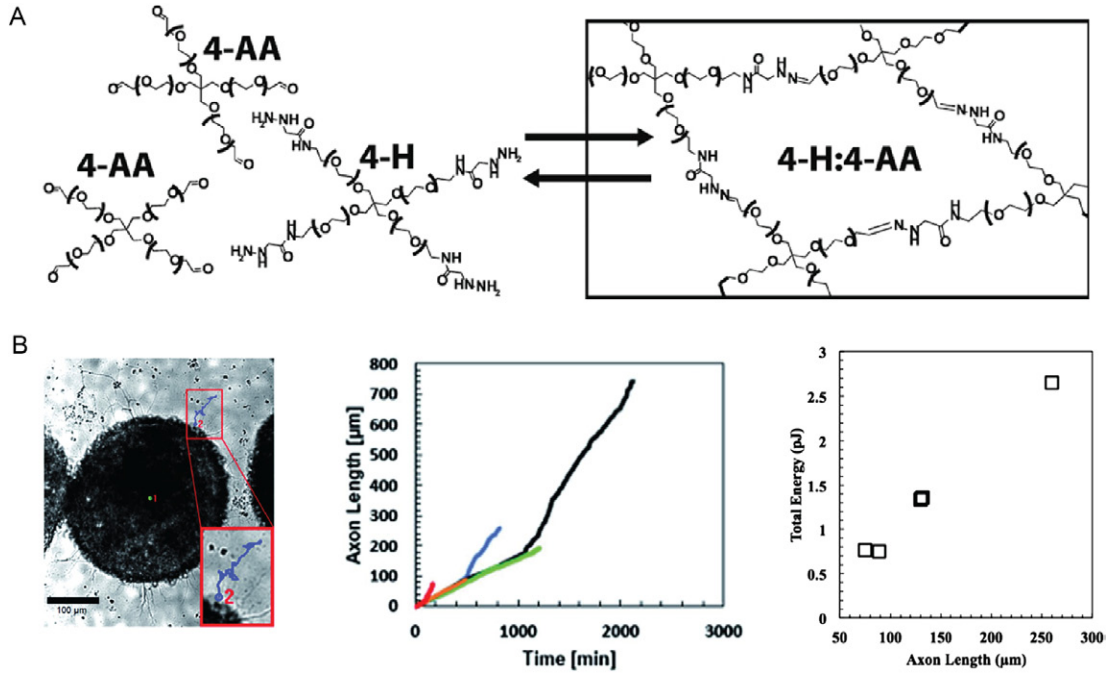


**Figure 4.** Bio-inspired hydrogels to study neurite extension. (a) Covalently adaptable PEG hydrogels with tunable stress relaxation characteristics were prepared by hydrazone crosslinking. (b) Embryoid bodies were differentiated into motor neurons and encapsulated within these hydrogels to study the biophysics of neurite extension. Axon outgrowth was tracked in real time, and the forces and energies involved in neurite extension were calculated based on the viscoelastic properties of the network. Reproduced with permission from McKinnon *et al* [145, 63]. Copyright WILEY.

wounds and bone defects in murine models [134]. Exciting results have also been obtained using biomimetic PEG hydrogels. For example, enzymatically degradable PEG hydrogels crosslinked using photoinitiated thiol-ene chemistry have been effectively used to deliver BMP-2 and regenerate rat calvarial defects, with Mariner *et al* showing superior results for defect closure and total bone volume compared to a clinically used absorbable collagen sponge (figure 3) [135]. The thiol-ene photo-click chemistry in particular has emerged as a versatile tool for synthesizing bio-inspired matrices, as it has been demonstrated to improve both protein bioactivity [136] and cell viability [137, 138] after encapsulation. It is also compatible with the covalent immobilization of signals such as the growth factor TGF$\beta$1 [139] to promote directed stem cell differentiation [140]. Results from recent work show this platform increases both viability of encapsulated chondrocytes and production of cartilage matrix over just 14 d of culture *in vitro* [141].

Bio-inspired 3D matrices have also been translated to clinical use. One of the most notable examples is the use of fibrin gels in matrix-assisted chondrocyte transplantation [142]. While the tunability of synthetic systems is an important advantage for this application as well as other similar cell-based tissue engineering therapies, the translation of these materials to the clinic has been slower. In an exciting study, Sharma *et al* used photopolymerizable PEG hydrogels supplemented with hyaluronic acid to augment microfracture surgery in 15 patients [143]. These results showed that patients had less pain and improved knee function after 6 months, and no adverse events were reported. Similar PEG-based hydrogels have also been FDA approved for use as tissue sealants, for example to close corneal incisions after intraocular lens implantation [144].

Because cell phenotype in 3D culture more closely represents that found in tissues, *in vitro* tissue engineering strategies using bio-inspired matrices are also having a significant impact in fundamental studies of cell biology. For example, covalently adaptable hydrogels with tunable stress-relaxation properties were recently used to study the biophysics of neurite extension from differentiated mouse embryoid bodies (figure 4) [145, 63]. Tunable PEG hydrogels have also been used to study drug responsiveness in melanoma cells in both 2D and 3D culture, with 3D analysis showing that metastatic melanoma cells increase their MMP activity and migration in response to BRAF kinase inhibition (figure 5) [145, 63]. The photoresponsive systems that allow for spatiotemporal control over the dynamic presentation of biomolecular signaling are also playing an important role in fundamental work, because they can recreate the heterogeneous biochemical milieu of the ECM and also mimic its dynamics. DeForest *et al* recently described a new bio-inspired 3D platform that exploits three bio-orthogonal chemistries to enable reversible, spatially controlled presentation of full-length proteins [67]. First, a polymeric hydrogel network was formed using a SPAAC reaction [79], then a photodeprotection-oxime-ligation sequence was introduced for protein attachment and finally an ortho-nitrobenzyl ester photoscission was employed for protein removal. This platform was subsequently used

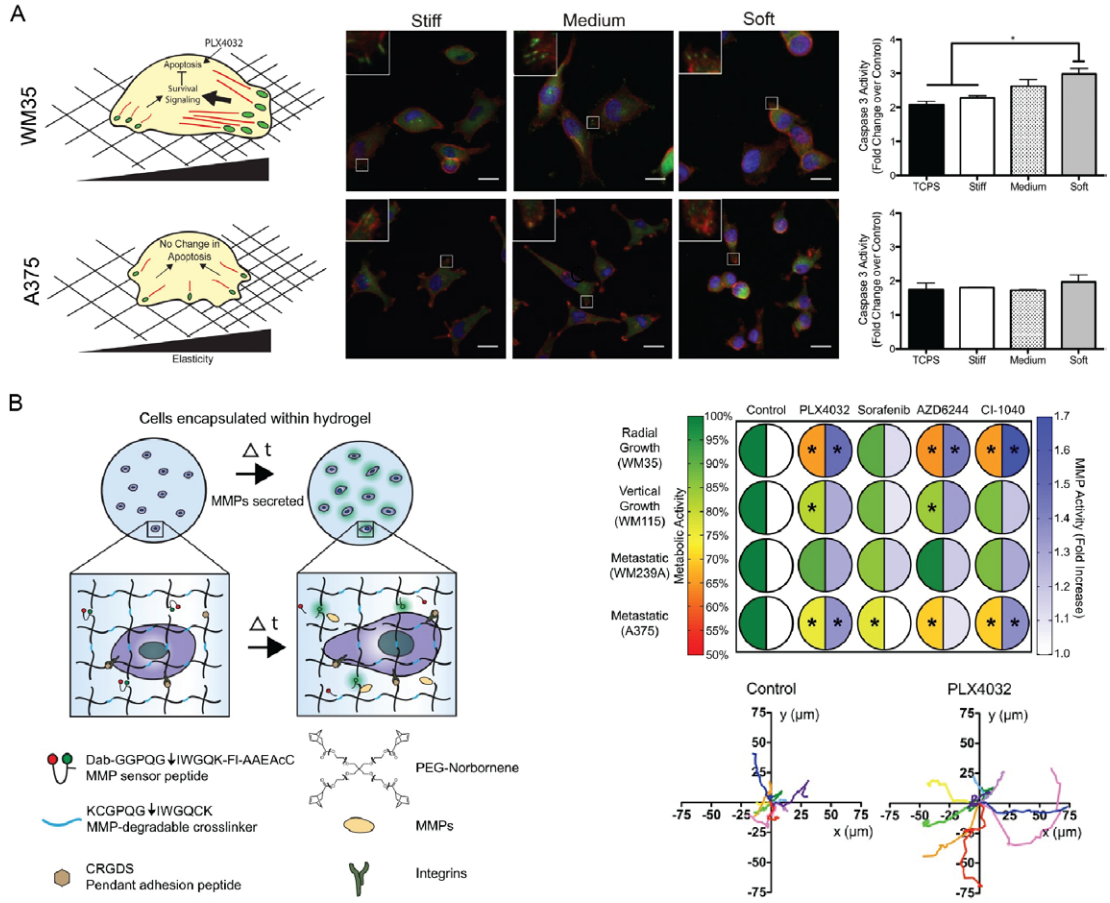


**Figure 5.** Bio-inspired hydrogels to study drug responsiveness of melanoma. (a) 2D culture of WM35 and A375 melanoma cells, which are from the radial growth and metastatic phases, respectively, on ECM mimetic PEG hydrogels with tunable stiffness revealed a stage-dependent response to BRAF kinase inhibition with PLX4032. This difference would have been masked if the cells had been cultured strictly on tissue culture plastic. (b) 3D culture of melanoma cells from varying stages of progression in ECM mimetic PEG hydrogels with a fluorogenic MMP sensor peptide revealed that A375 cells treated with PLX4032 exhibited increased MMP activity, which corresponded to increased cell elongation and single-cell migration, despite an overall decrease in metabolic activity. Reproduced from Tokuda *et al* [160], copyright 2014 with permission from Elsevier, and Leight *et al* [161].

to exert spatiotemporal control over encapsulated hMSC differentiation through the controlled presentation of the adhesion protein, vitronectin. During vitronectin presentation in precisely patterned areas, hMSCs increased output of osteogenic markers including alkaline phosphatase (ALP) and osteocalcin [67]. It should be appreciated that this and other photoresponsive platforms could also be broadly useful for interrogating the role of matrix-derived signals in development and disease. Moreover, knowledge gained regarding the influence of matrix properties on stem cell lineage commitment could also facilitate the future design of bio-instructive matrices for regenerative medicine.

## Future directions

A fundamental shift in design paradigms from 2D to 3D has begun to revolutionize the way bio-inspired microenvironments are created for evaluating cellular responses to material cues, engineering tissues, *in vitro* modeling of disease, and treating patients using precision medicine [152]. However, many technological challenges remain along the pathway to realizing precisely controlled microenvironments to present the matrix properties and signals that are necessary to promote healing, induce tissue regeneration, deliver therapeutics and monitor health in real-time. Addressing these complexities requires interdisciplinary collaboration among scientists, engineers, cell biologists, physicists and physicians. Here, we highlighted progress toward the ultimate goal of developing dynamic, heterogeneous structures that include a vascular network and advancement of technologies for high-throughput manufacturing and screening. As we begin to scale-up the innovations that overcome current limitations, new technical demands must be met. For example, manufacturing speeds and resolutions must simultaneously increase to create constructs of clinically relevant size.

One interesting approach to improving both resolution (increasing) and fabrication time (decreasing) is dynamic optical projection stereolithography, which uses a digital micromirror device to project patterns with theoretical resolution up to 10 $\mu$m[153] within seconds [154]. This continuous 3D printing approach has already been used to generate concave hydrogel microstructures that permit growth of cell clusters/

spheroids and their long-term maintenance of function, demonstrating the potential for patient-derived disease modeling [155]. Although, the limits of size, speed and precision have yet to be fully defined, another promising biofabrication technique for improved speed and resolution is granular printing [156]. At the point of injection, a granular gel that surrounds the part fluidizes and then rapidly solidifies trapping soft injected material in place. This physical approach to bioprinting overcomes many technical obstacles to producing complex, large aspect ratio 3D objects and has been shown to be compatible with the encapsulation of living cells [156].

While scale up and throughput will be important for the future success and translation of bioprinting, microscale organ-on-a-chip technologies that integrate bio-inspired microenvironments with fluid flow as well as other dynamic physiological processes are also a key area for future investment. Promising advancements have already been made in the area of liver- and heart-on-a-chip, and the two have even been integrated toward the development of microscale platforms that can potentially serve as functional representations of human physiology [157]. Tumor-on-a-chip models could significantly improve and enhance the development of new targeted therapeutics, especially when integrated with vascular and lymphatic vessels [158]. Recent studies have shown that bio-inspired 3D culture in biopolymer hydrogels can be used to produce an *in vitro* model of Alzheimer's disease [159], which could prove useful for the development of new therapeutics. These tools will become increasingly important and, when combined with advances in stem cell technology, could lead to exciting new possibilities in precision medicine.

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