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Review

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Synthesis and High-Throughput Processing of Polymeric Hydrogels for 3D Cell Culture

Stuart B. Lowe,[†,‡] Vincent T. G. Tan,[†,‡] Alexander H. Soeriyadi,[†,‡] Thomas P. Davis,[∥,§,#] and J. Justin Gooding*[,†,‡,∥]

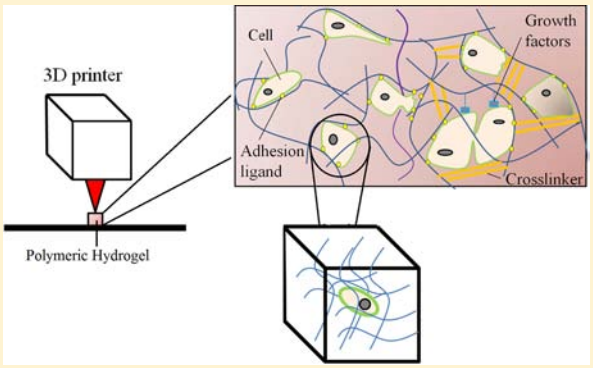
[†]School of Chemistry and [‡]Australian Centre for NanoMedicine, The University of New South Wales, Sydney, NSW 2052, Australia

[∥]ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, Parkville, VIC 3052, Australia

[§]Department of Chemistry, University of Warwick, Coventry, CV4 7AL, United Kingdom

[#]Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC 3052, Australia

ABSTRACT: 3D cell cultures have drawn a large amount of interest in the scientific community with their ability to closely mimic physiological conditions. Hydrogels have been used extensively in the development of extracellular matrix (ECM) mimics for 3D cell culture. Compounds such as collagen and fibrin are commonly used to synthesize natural ECM mimics; however they suffer from batch-to-batch variation. In this Review we explore the synthesis route of hydrogels; how they can be altered to give different chemical and physical properties; how different biomolecules such as arginylglycylaspartic acid (RGD) or vascular endothelial growth factor (VEGF) can be incorporated to give different biological cues; and how to create concentration gradients with UV light. There will also be emphasis on the types of techniques available in high-throughput processing such as nozzle and droplet-based biofabrication, photoenabled biofabrication, and microfluidics. The combination of these approaches and techniques allow the preparation of hydrogels which are capable of mimicking the ECM.



INTRODUCTION

It is well accepted that cells behave differently in 3 dimensions in contrast to their behavior on 2-dimensional substrates as commonly used in cell biology.[1] However, generating a standardized "3D tissue-culture plate" has proven challenging, with the requirements demanded by the myriad of different cell culture applications. Initially, research into 3D cell culture utilized naturally derived polymers, such as ECM proteins (for example, collagen, fibrin) and even commercially available basement membrane extract (Matrigel, BD Biosciences). The natural diversity of these biologically derived materials can result in batch to batch variability in the ECM mimicking materials.[2] Hence considerable research is motivated toward preparing synthetic ECM mimics. Section 1 of this review will focus on synthetic polymer hydrogels as mimics of the ECM. Synthetic polymers possess a number of advantages over natural polymers, including greater control over batch-to-batch variation; tailorable molecular weight; tailorable domain sizes; modular incorporation of biochemical functionality; and the potential to scale-up production. Hydrogels are a class of materials defined by IUPAC as polymer network gels, swollen with water.[3] Hence, hydrogels recapitulate many of the properties of native ECM, as described below. The lack of versatile hydrogel systems for 3D cell culture has been recognized as a key factor restricting progress in the field.[4] This versatility could be addressed through various bioconjugation and modular modification techniques which will be discussed in Section 1. Figure 1 highlights extensively the diverse range of conjugation reactions possible for both hydrogel formations and biomolecules attachment.

Traditional hydrogel casting methods with the random placement of cells within a premolded structure fail to capture the spatial organization and internal microstructure of native tissue.[5] Existing techniques for high-throughput processing of materials are now being adapted for use with biomaterials[6] and integrated with computer control for precise patterning in 3D[7] (see Section 2). These techniques are known by a variety of names with sometimes overlapping definitions, for example: layered manufacturing, rapid prototyping, rapid tooling, solid free-form fabrication, direct writing, additive manufacturing, biofabrication, 3D printing. Here, we will use the term 'high-throughput processing', and concentrate mainly on the techniques used to achieve high throughput spatial control over 3D hydrogels.

One of the key opportunities in the field of 3D cell culture is the convergence of synthetic polymer hydrogels with high-

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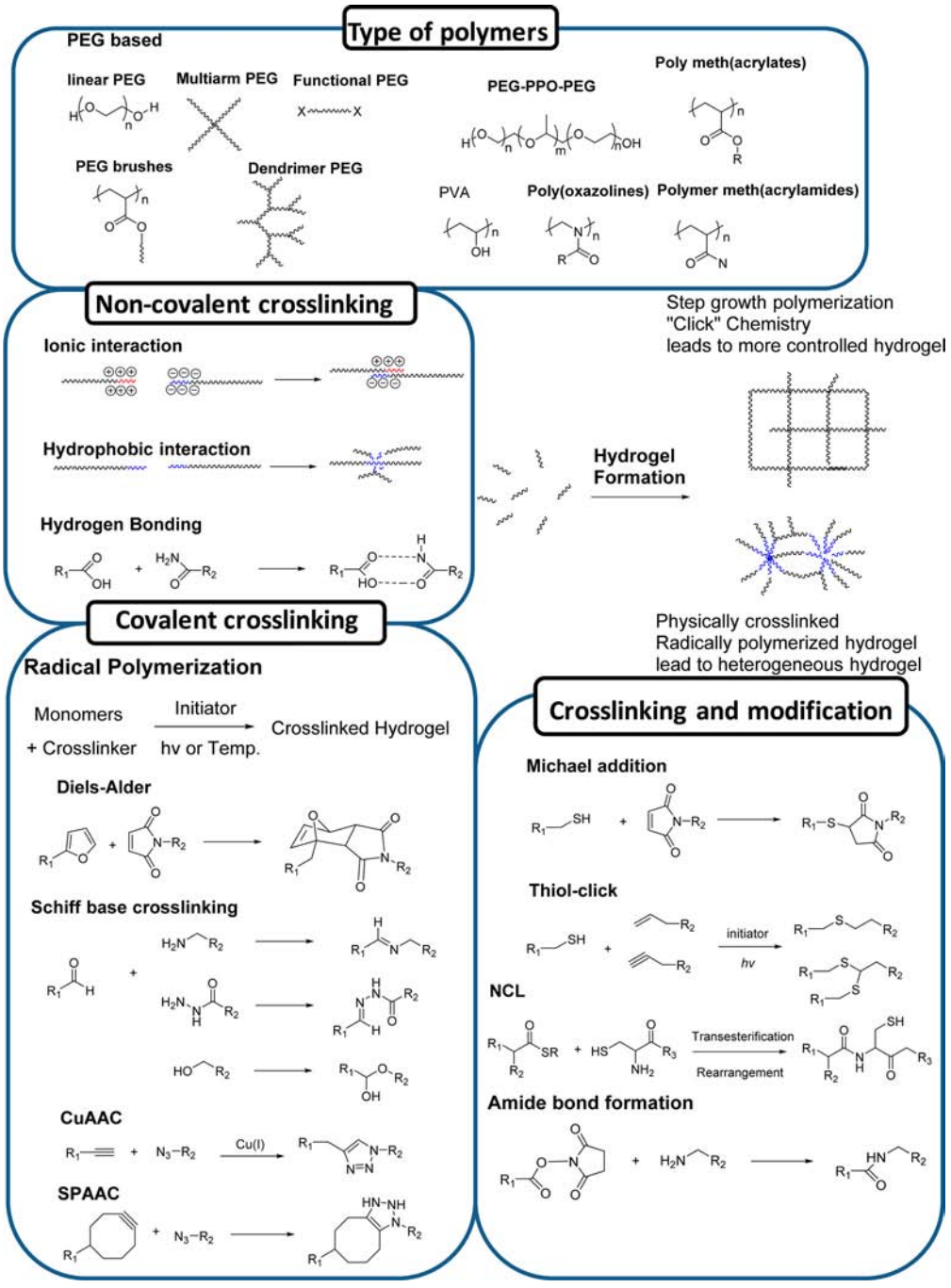

Figure 1. Overall schematic representation of hydrogel formation. There are a wide range of polymer types that have been used in hydrogel formation. Cross-linking mechanisms can be divided into two major mechanisms: noncovalent cross-linking or covalent cross-linking. There are also several chemistries that could be employed for both cross-linking and modification either at the same time or simultaneously.

throughput processing. Advanced polymer and high-throughput processing technology will allow exquisite control over both the chemical interactions and structural/mechanical interactions between synthetic polymeric hydrogels and cells.

■ CELL–ECM INTERACTIONS IN VIVO

Cells in biological tissues function in the presence of an extracellular matrix (ECM). The ECM's function is primarily structural and segregational, providing a hydrogel network within which cells can live, providing a barrier separating different tissues.[8] Collagen fibers make up the majority of the network fibers, with proteoglycan components promoting hydration, cellular binding, and network cross-linking via biorecognition interactions.

Cell organization within tissues is mediated by cell–ECM adhesions, and these adhesions are also responsible, in part, for cell sensing and behavior in response to external stimuli.[9] Binding to the ECM is mediated by integrins, cell membrane proteins that recognize and can bind to specific peptide motifs found within ECM components such as fibronectins, laminins, and proteoglycans.[8] In fact, adhesive peptide sequences have been discovered in many ECM protein;[10] for a summary of cell-adhesive peptides, see Zhu and Marchant's review on designed hydrogel scaffolds.[11] The most well-known cell adhesion

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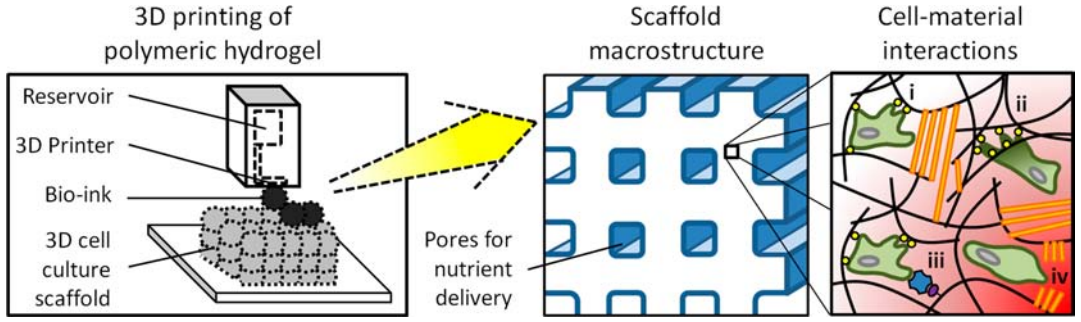
Scheme 1. Illustration of an Integrated Synthetic/High-Throughput Processing Strategy for 3D Cell Culture[a]



[a]Left to right: high-throughput processing of polymeric hydrogel (left-hand panel) involves loading of hydrogel precursor solution containing synthetic polymer and cells (bio-ink) into a 3D printer, and building up a 3D structure. Key material parameters at this stage are viscosity, surface tension, and nature of hydrogel cross-linking. Cross-link density will also affect the mechanical properties of the 3D scaffold. The scaffold macrostructure (central panel) could be built in a layer-by-layer or droplet-by-droplet fashion, allowing for spatial organization of cell types within a scaffold. For scaffolds larger than ~200 μm, channels/pores are necessary to transport nutrients to cells. At the cellular level, cell−material interactions dominate (right-hand panel), and a synthetic polymer (black fibers) should aim to recapitulate at least some of the functions of the native ECM. For example, (i) integrin binding sites (yellow) for cell adhesion allow cells to spread, move, and sense; (ii) cells sense and respond to changes in stiffness via mechanotransduction, mediated by integrin binding; (iii) sequester and release of growth factors (blue) is mediated by affinity binding sites (purple) incorporated into the polymer structure; (iv) proteolytically degradable cross-links (orange) allow cells to migrate through the hydrogel via mesenchymal migration, and cells may be stimulated to migrate in response to a biomolecular concentration gradient (red) in a process known as chemotaxis.

peptide is the arginylglycylaspartic acid (RGD) sequence, found naturally within fibronectin.[12] RGD plays several roles within the ECM, from facilitating cell adhesion to controlling cell morphology and motility, and has also been found to enhance cell viability.[13] Other examples of cell adhesion peptides include the YIGSR and IKVAV sequences derived from laminin.[14] Using chemical patterning of cell adhesion points to direct cells to specific regions in a hydrogel is described in Section 1.3.1−1.3.2.

The binding of integrins to the ECM induces conformational changes and molecular rearrangements that result in anchoring of the intracellular cytoskeleton to the ECM. The integrin−ECM interaction allows cells to transduce mechanical forces (mechanotransduction)[9a] and also to perform signaling and migration, although the interactions here are poorly understood.[15] Since the cells are sensitive to mechanical forces, the stiffness of the ECM plays a role in cell behavior[16] and also influences stem cell differentiation, which is one of the ways in which tissue development is controlled, wherein different tissue types have a range of mechanical stiffnesses[11,17] (elasticity is also an important property in mechanically active tissues[18]). Hence, in order to target a specific tissue or niche, control over mechanical stiffness is required,[1d,5] as discussed in Section 1.1.

Access to nutrients is crucial for cell survival. The ECM permits diffusion of nutrients, although the diffusion limit of oxygen in biological tissue, for example, is only 100−200 μm.[19] Hence, in the body, most cells are located within 100 μm of a capillary and this represents a limit to the bulk size of ECM-mimicking structures. Consequently, for larger structures, the porosity of scaffolds at the 100-μm length scale becomes a crucial parameter in tissue engineering.[20] Porosity of the ECM at the 10-μm length scale also has an effect on the stimuli response of cells.[21] Interestingly, the pore size of the ECM has even been observed to influence the mechanism of cell migration, from ameboid, "squeezing" motion through narrow orifices; to mesenchymal migration, proteolytic involving degradation of impenetrable barriers.[22] Hence, control over the microscale structure of synthetic hydrogels is desirable, and steps toward achieving this goal are described in Section 2.

As described above, proteolysis is one of the major mechanisms through which cells can migrate, or, in the case of metastatic cancer cells, invade into surrounding tissue;[23] proteolysis also allows cells to dynamically remodel the ECM in order to repair tissue. There are many examples in the literature of hydrogels incorporating protease-degradable components, and these are discussed in Section 1.4. In chemotaxis, migration of cells toward an attractant molecule (such as nutrients), cells first sense gradients in molecular concentration via the G protein-linked signaling pathway, and then respond by migrating toward or away from the stimulus.[24] The directional sensing mechanism is also implicated in other cell behaviors such as stem cell differentiation, angiogenesis (blood vessel formation), and cancer cell invasion (the first step in the development of tumor metastases).[25] Human tissues often contain multiple cell types in a precise spatial organization.[4,5] *In vivo*, chemical gradients during development can direct cells to specific locations within a nascent organ. Using 3D bioprinting, it is possible to spatially pattern cells within 3D cell cultures using high-resolution control of "bio-ink" dispensing, which is discussed in Section 1.3. The formation of chemical gradients in hydrogels is discussed in Section 1.3.3.

The development of tissues is influenced by the presentation of growth factors during development. Growth factors are soluble proteins capable of triggering a multitude of cell responses, including migration, proliferation, and differentiation of specific subsets of cells.[26] Growth factors also interact with the ECM. The activity of growth factors is modulated by diffusion through and binding to the ECM, among other effects, and tissue-specific response can be elicited by the correct spatial and temporal presentation of growth factors. For example, the development of vascular tissue is mediated by a temporally controlled cocktail of growth factors including, among others, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).[27] Each growth factor has a specific role to play in the formation of blood vessels which could be harnessed in a synthetic tumor model to promote vascularization to allow nutrient delivery to the hydrogel core (*vide supra*). Thus, the presentation and controlled release of growth factors

would be a desirable characteristic for synthetic hydrogels, and fortunately nature provides inspiration toward achieving this goal. Growth factors can be stabilized and sequestered spatially via interactions with proteoglycans (e.g., heparin sulfate) that form a bridge between growth factors and the ECM. Such affinity binding interactions have been utilized to biofunctionalize synthetic hydrogels, as discussed in Section 1.3.2. Temporal control over ligand presentation is described in Section 1.3.1.

There are a plethora of cell behaviors that are influenced by the ECM. Synthesizing polymeric hydrogels for 3D cell culture allows one to take a reductionist approach into determining the effects of specific ECM characteristics on cell behavior.[28] To be an effective ECM mimic, a synthetic material must attempt to simulate at least some of the functions of the native ECM described above, such as cell adhesion, mechanical stiffness/elasticity, access to nutrients, plasticity, spatial organization, and spatial and temporal presentation of biochemical cues (see Scheme 1).

1. SYNTHESIS OF POLYMERIC HYDROGELS FOR 3D CELL CULTURE

In designing a polymeric hydrogel for 3D cell culture, the biocompatibility of both the base materials and of the hydrogel cross-linking method is of primary importance. Second, should the hydrogel be biofunctional (contain biomolecules that interact with cells to stimulate a biological response) or bioinert (non-interacting with cells, also known as a "blank slate" hydrogel)? Third, depending on the cross-linking used, the hydrogel may be static (maintaining its chemical and physical properties over time) or dynamic (changing its chemical or physical properties in response to an external stimulus). Finally, when designing polymers for high-throughput fabrication of 3D cell culture models (see Section 2), account must be taken of material processability, in particular, properties such as viscosity and surface tension.[4] In addition to processability, in some applications much more robust "tough" hydrogels may be required. Tough hydrogels can be generated if there is a strong affinity between polymer strands. For example, Bakerich et al.[29] generated a tough gel where entanglement of polymer chains involved both ionic and covalent interactions, improving the gel strength by 500%.

The most popular polymeric hydrogel components described in the literature are poly(ethylene glycol) (PEG) and its derivatives. PEG is nontoxic, antifouling, and nonimmunogenic, making it somewhat of a "blank slate" for investigating biological interactions[30] (although, hyperbranched poly(glycerol) has been shown to possess even lower cytotoxicity than PEG).[31] PEG is also easy to handle in both physical and chemical terms being soluble in water and organic solvents. Many variations of PEG polymers have been utilized for a range of biomedical applications,[32] with the most basic form of PEG being linear PEG homopolymer, which can be obtained commercially and is mainly synthesized via anionic or cationic polymerization.[30a] The BAB block copolymer of PEG with poly(propylene ethylene glycol) (PEG−PPG−PEG), known as Pluronic, is also widely used.[33] As well as being biocompatible and bioinert, Pluronic is also thermoresponsive, leading to widespread use in injectable systems. Telechelic PEG,[34] PEG stars,[35] and PEG dendrimers[36] have also been utilized, and the terminal hydroxyl of PEG can be easily modified to incorporate different functional groups, e.g., thiol, acrylate, or carboxyl.[37] The protein antifouling property of PEG means that it is relatively inert to the deposition of ECM secreted by cells. Hence "blank" PEG hydrogels must be biofunctionalized prior to cell interaction (see Sections 1.3 and 1.4).

Derivatives of poly((meth)acrylates) are heavily utilized in polymer hydrogels due to their aqueous stability. In particular, poly(2-hydroxyethyl methacrylate) (PHEMA) has been used in various biomedical applications.[30a,38] (Di)acrylated and (di)-methacrylated PEG polymers (PEGDA, PEGDMA, PEGA, PEGMA) are also common components in photopolymerized hydrogels[13,39] (see Sections 1.1 and 1.2 below). Certain poly((meth)acrylamide) polymers are of interest as they can imbue thermoresponsive properties[40] (see Section 1.2). However, one concern over the use of meth(acrylamide)s is the material's biocompatibility and biodegradability. As can be seen from the review by Malda et al.,[4] despite the fact that several acrylamide-based hydrogels have been fabricated, there are few reported biocompatibility studies. Besides the main types of polymer mentioned above, several other synthetic polymers have been used as hydrogels.[30a] As a final example, recently, poly(oxazoline) and its derivatives have been employed as attractive materials for hydrogel formation.[41] The breakthrough in the traditionally cumbersome preparation of poly(oxazoline) was made via the use of microwave reactors. For further general strategies on poly(oxazoline)-based hydrogels, readers are invited to consult a recent review by Kelly and Wiesbrock.[42]

As well as the intrinsic properties of the polymer, the choice of hydrogel cross-linking strategy can affect various aspects of the final hydrogel such as mechanical strength, porosity, functionality, and water content.[43] Biologically inert methods of cross-linking are preferable, as in high-throughput processing, cells and proteins would be mixed with gel precursor solution prior to cross-linking (see Section 1.1). The cross-linking strategy can also be static or dynamic, depending on the final requirements of the system.

Figure 1 summarizes the types of polymers utilized in polymeric hydrogels and the coupling chemistries applied to cross-link and biofunctionalize them. Cross-linked networks can be formed mainly through noncovalent cross-linking, covalent cross-linking, or a combination of both.[44] As the name suggests, noncovalent cross-linking generally refers to a noncovalent interaction between polymer chains, and is highly prevalent in biological systems.[45] Noncovalent cross-linking interactions can include H-bonding, hydrophobic interaction, ionic, stereocomplexation, or $\pi-\pi$ stacking.[4] To avoid confusion, we would like to clarify that while in much of the literature these interactions have been termed physical cross-linking, we classify these interaction as noncovalent as it is indeed still a chemical interaction. By contrast, covalent, commonly termed "chemical" cross-linking, relies on creating a polymeric network via the formation of covalent bonds, often requiring the addition of an initiator or cross-linker molecule.

A prominent example of noncovalent cross-linking is the hydrophobic interaction which dominates the behavior of several classes of stimuli responsive polymers. The stimulus in question is often pH or temperature whereby a polymer's interaction with water molecules encounters a phase transition, forming cross-linking bridges rather than remaining hydrated.[4] Another mechanism is the ionic interaction which is an electrostatic attraction between polyelectrolyte molecules with opposite charges. A class of hydrogel which has not yet found much application in 3D cell culture is the self-healing hydrogel made via H-bonding. For example, Cui and Del Campo[46]

reported a hydrogel comprising predominantly hydrophilic poly(2-(dimethylamino)-ethyl methacrylate) PDMAEMA co-polymerized with monomer containing 2-uerido-4-pyridone (UPy), resulting in a self-healing property. This property relied on multivalent H-bonding of the UPy units and could have future application in injectable materials.

The earliest and easiest method to create a covalently cross-linked hydrogel is through free radical polymerization. Free radical polymerization (FRP) provides a robust, fast method of producing covalently cross-linked hydrogels. FRP generally involves mixing initiator, monomer, and cross-linker. The subsequent chain reaction results in polymeric network formation with the kinetics and mechanical stiffness directly controlled by the ratio of cross-linker and type of monomer. FRP commonly employs off-the-shelf reagents such as hydrophilic (meth)acrylated monomers (e.g., 2-hydroxyethyl (meth)acrylate) and/or (meth)acrylated cross-linkers (e.g., ethylene di(meth)acrylate, PEGDA, PEGDMA).[30a] With regard to high-throughput processing, most notably, acrylated PEGs (for example, PEG-diacrylate, PEGDA) are used in photoenabled processing (see Section 2.3 below). The disadvantage of FRP is the heterogeneity within the polymer network (Figure 1) needs external stimulus (temperature or UV light) and residual double bonds that may potentially react with biological substances.[30a] For milder reaction conditions, hydrophilic polymers generally bear functional groups (such as hydroxyl, carboxylic acid, or amide) and thus there are a variety of reaction mechanisms available such as amide bond formation, Schiff base formation, or isocyanate reaction with hydroxyl or amide.[44] Chemical cross-linking can also be performed by utilizing an enzyme as a catalyst to avoid the addition of any chemicals and/or external stimulation.

An important class of reaction developed in the past decade is "click" reactions which are fast, high yield, and orthogonal, and require only mild reaction conditions.[47] "Click" chemistry has affected many materials science and engineering fields including hydrogel fabrication. The most prevalent click chemistry is the copper catalyzed azide−alkyne Huisgen cycloaddition (CuAAc). It was one of the first to be used in synthesizing well-defined PEG hydrogels.[48] In order to avoid the requirement of using copper catalysts which might be cytotoxic, DeForest and Anseth[49] have utilized a copper free strain-promoted azide−alkyne cycloaddition (SPAAC) first developed by Bertozzi[50] to design a PEG-based hydrogel. While there are not many applications of "clicked" synthetic hydrogels fabricated via high-throughput processing to date, we feel that nozzle based extrusion (see Section 2.1) would be a suitable candidate technique as it allows materials to be in a hydrogel state prior to deposition. Another alternative is to have a mixed droplet where each of the clickable units (in the case of SPAAC) is applied in two different channels, and once mixed, it will form hydrogel in minutes. Diels−Alder is another reaction that could be classified as a "click" reaction, which involves a reaction between diene and dienophile occurring in aqueous conditions without any catalyst or initiators.[51] For example, Kirchof et al. demonstrated a PEG based hydrogel made via Diels−Alder reaction with gelation time ranging from 14 to 171 min and storage modulus ranging from 2 to 40 kPa depending on the formulation.[52] Another example of the Diels−Alder reaction is the bio-orthogonal "click" chemistry of tetrazine-norbornene, which is fast, cyto-compatible, and orthogonal to photochemical patterning.[53]

"Thiol" click chemistry is another class of highly ulitized "click" chemistry. There are two types of thiol "click" mechanism which are the base catalyzed thiol-Michael addition and photoinitiated thiol−ene and thiol−yne reactions.[54] Alternatively, another interesting method of cross-linking is through native chemical ligation which is based on the reaction of a thiol with a thioester.[55] This mild reaction (neutral pH, at temp 25−37 °C, no catalyst) is attractive due to its regioselectivity and chemoselectivity.[55,56]

Many of the coupling chemistries applied for hydrogel cross-linking can also be used in biofunctionalization (see Sections 1.3 and 1.4). Many groups have reported the use of thiol−ene "click",[57] Michael additions,[58] succinimide groups,[39a,59] chelating monomers,[40a] azide−alkyne cycloaddition,[60] visible light,[61] and UV light[61] and even combinations such as light initiated thiol−ene Michael addition[13] as methods to attach biomolecules. A further advancement is the introduction of stimulus-responsive functional groups such as photocleavable *o*-nitrobenzyl ethers[39b] to introduce dynamic character into both bioinert and biofunctionalized polymeric hydrogels.

1.1. Static Bioinert Polymeric Hydrogels. As stated in the introduction, the mechanical properties of a hydrogel regulate various cellular behaviors such as proliferation, migration, and spreading.[16] In the body, different tissue types have a range of mechanical stiffness.[11,17] The brain has an elastic modulus of 240−490 Pa, as compared to fat tissue which has an elastic modulus of only 17 Pa.[17c] The vast range of elastic moduli (mechanical stiffness) within the body makes it imperative that the hydrogel is able to be designed to mimic the elastic modulus accordingly.[17c] In order to target a specific tissue or niche, control over mechanical stiffness is required. In practice, the number of cross-links is key to increasing hydrogel rigidity. This can be achieved by increasing concentration of the polymer, increasing the number of cross-linkable units per polymer, or, in covalently cross-linked hydrogels, by increasing cross-linker concentration. For photo-cross-linked hydrogels, increasing the irradiation time is also an option, although this runs the risk of causing DNA damage in cells. In the case of noncovalently cross-linked hydrogels, polymer design can influence the number and nature of hydrogel-forming domain interactions. This is where advances in polymer design have allowed greater tailorability in hydrogel properties. In the last 20 years or so, a new range of tools to create polymers with defined structures have been developed. Techniques such as living polymerization,[62] controlled radical polymerization,[63] "click" chemistry,[47,54] and self-assembly[64] have allowed much greater control over the hydrogel properties than before. It should be noted that synthetic peptide-based hydrogels are an important class of bioinspired materials that undergo non-covalent cross-linking through self-assembly.[65] The field of synthetic peptide hydrogels has been reviewed by Thordarson and co-workers,[65] and we invite interested readers to consult their article. Similarly, DNA-based responsive hydrogels have been recently reviewed by Xiong et al.[128]

Most noncovalent cross-linking techniques are associated with a stimulus response, which can be advantageous in processing, and is discussed in more detail in the Section 2. An example where noncovalent cross-linking was used to form static hydrogels was described by Vrana et al.[66] Using the cryogenic gelation of poly(vinyl alcohol) (PVA) to form a scaffold,[30a] bovine arterial smooth muscle cells could be stored frozen, encapsulated within a scaffold, before being thawed for study. After thawing, the cells had modest (50%) viability.

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However, hydrogels formed via noncovalent cross-linking tend to be mechanically weak (see below) and may even require a postprocessing covalent cross-linking step. An exception is shown in the electrostatic cross-linking of two families of ABA block copolymer possessing polyelectrolyte A blocks of opposing charge (and universal B block, PEG).[67] The maximum storage modulus obtained with this system was 4 kPa. However, in general, covalent cross-linking is stronger and more stable than noncovalent cross-linking, as illustrated in the sections below. That said, there are a few examples of cases where polymeric hydrogel precursors have been mixed with stiffer materials in order to simulate stiffer tissues such as bone.[58,68]

In mechanically active tissues, such as the bladder, lungs, and blood vessels, tissue elasticity is an important parameter. For the most part, polymeric hydrogels tend not to withstand high elastic strains; however, Greishaber et al.[60] reported an elastin-inspired cross-linking strategy capable of producing a material with a compression modulus of up to 120 kPa when hydrated. By using the ionic cross-linking of alginate through Ca^{2+} and the covalent chemical cross-linking of polyacrylamide, Sun et al.[69] created a highly stretchable and tough hydrogel which could be stretched over 20 times its initial length. The elastic modulus at rupture could be designed to be as high as 100 kPa. While such gels could be stretched significantly, the recovery rate of such hydrogels is slow and hence is currently a topic of further research. Nowak et al.[70] synthesize a hydrogel with rapid recovery rates whereby the hydrogel recovered 80% of its strength by 10 seconds. We expect more work will be done in the near future in this particular area as it could indeed be a significant parameter in 3D cell culturing hydrogels.

It has been shown that electrical stimulation of stem cells can direct differentiation to a neuronal lineage.[71] Controlled electrical stimulation of polymeric hydrogels could be achieved using conducting polymers. Mawad et al.[72] synthesized a covalently cross-linked hydrogel from poly(3-thiopheneacetic acid) and carbonyldiimidazole cross-linker. Although the hydrogel was initially synthesized in DMSO, it was thoroughly washed and proceeded to take up water, with a swelling ratio of up to 850% of its initial volume. The electrical conductivity of the hydrogel was on the order of 10^{-3} S/cm and the hydrogel was electroactive at physiological pH and temperature. Murine skeletal muscle myoblast C2C12 cells seeded on top of the hydrogels were found to maintain their viability after 72 h incubation.

Static bioinert polymeric hydrogels are of interest mainly due to the effect of mechanical properties on cell behavior. Preparation of substrates with step changes or continuous changes in mechanical properties may be useful in mimicking entire tissues; this will be covered in Section 1.2.

1.2. Dynamic Bioinert Polymeric Hydrogels. Dynamic materials are designed to alter their physical/chemical properties in response to external stimuli. When considering polymeric materials suitable for high-throughput processing and subsequent hydrogel formation, it may be desirable to incorporate stimulus-responsive behavior. Relevant stimuli may include temperature,[23,44] pH,[73] or light[74] (see also Section 1.3 for light related responses).

Thermoresponsive polymeric hydrogels are commonly applied as injectable materials for 3D cell culture and tissue repair. For example, Abdi et al.[75] mixed Pluronic F127 with alginate and hyaluronic acid to form an injectable delivery vehicle for rat skeletal muscle cells, with a gel transition temperature located between room temperature (22 °C) and body temperature (37 °C). Poly(*N*-isopropylacrylamide) (PNIPAAm) can also be utilized to induce a thermoresponsive sol−gel transition (with a cloud point of around 32 °C[76]), and a secondary covalent cross-linking interaction can be used to strengthen the resulting hydrogel. In work by Boere et al.,[77] a triblock ABA copolymer was synthesized (with the A blocks comprising a statistical copolymer of NIPAAm and *N*-(2-hydroxypropyl)methacrylamide-cysteine together, with a central linear PEG B block). This polymer formed a noncovalently cross-linked hydrogel at 37 °C, followed by native chemical ligation-mediated cross-linking via a PEG-thioester cross-linker. The polymer−cross-linker mixture was found to be liquid at room temperature, exhibiting a reversible sol−gel transition up to 30 min after noncovalent cross-linking at 37 °C. The native chemical ligation reaction was found to produce a nonreversible covalently cross-linked hydrogel with a storage modulus of 1 kPa after 3 h incubation. PNIPAAm-based hydrogels have also been used to encapsulate viable mesenchymal stem cells for up to 3 weeks.[78] An alternative methacrylamide compound, *N*-(2-hydroxypropyl)-methacrylamide (HPMA), was used to form dual-cross-linked networks with thermoresponsive properties. In a study by Censi et al.,[79] partially methacrylated HPMA-lactate was copolymerized with PEG to form a P(HPMA-lac)-PEG-P(HPMA-lac) ABA copolymer. The HPMA blocks endowed the hydrogel with a gel transition temperature of 21 °C and storage modulus of 300 Pa (noncovalent cross-linking) at 37 °C. UV irradiation induced covalent cross-linking via methacrylate polymerization, increasing the storage modulus to up to 100 kPa. Equine chondrocytes encapsulated in the hydrogels were found to have 85% viability after 3 days in culture.

One possible drawback of injectable materials for hydrogel formation is degradation of mechanical properties induced by hydrogel swelling under physiological conditions. Kamata et al.[80] synthesized a nonswellable hydrogel by incorporating hydrophilic ("swelling", hydrogel-forming) and thermoresponsive ("shrinking", substituent) components into the hydrogel network. The hydrophilic network-forming units were 4-armed PEG with either amine or *N*-hydroxysuccinimide (NHS) terminal groups, while the thermoresponsive substituents were 4-armed poly(ethyl glycidyl ether-*co*-methyl glycidyl ether) with amine terminal groups. Covalently cross-linked hydrogels were formed by amide bond formation (via NHS coupling), with the rate of cross-linking being controlled by phosphate and citrate buffer ionic strength, allowing the mixture to remain liquid for long enough (up to 1 h) to be injectable. Hydrogels possessing no thermoresponsive substituents were found to have swelled to 250% of their initial volume at 37 °C, whereas hydrogels composed of 40% molar ratio of thermoresponsive substituent were found to be nonswelling. The 40% substituted hydrogels could be stretched to 7-fold extension before failure, whereas the nonthermoresponsive hydrogels possessed only 2-fold extension before failure. This system is another example of a dual-cross-linking strategy improving the mechanical properties of a hydrogel.

Aside from improving the mechanical properties of hydrogels, some of the cross-linking and coupling chemistries described thus far can be used for introducing biofunctionality into hydrogels, which will be discussed in the following sections.

1.3. Static Biofunctionalized Hydrogels. As described in Section 1, there are a variety of synthetic polymers that can be

Table 1. List of Various Biomolecules and Their Corresponding Functions and Conjugation Strategies

biomolecules	function	conjugation strategy	reference
RGD and RGD derivatives peptides	Cell Adhesion	Amide bond formation	Hern and Hubbell (39a) Man (63)
CRGD	Cell adhesion	Thiolene	Salinas (15) Farrugia (84)
Gradient RGD	Cell adhesion	Microfluidic and photopolymerization	Burdick (93) Delong (88)
Laminin derived recognition	Cell adhesion	Peptide acrylation and photopolymerization	Weber (85)
(MMPs)-cleavable peptide	Cell migration and remodeling	Michael type addition	Lee (86)
(MMPs)-cleavable peptides	Cell migration and remodeling	Conjugated-PEG and photopolymerization	West (105)
Gelatin		Acrylated gelatin	Duan (157)
Hyaluronic acid		Acrylated HA	Duan (157)
Fibronectin fragment	Cell adhesion	Enzyme cross-linking	Menzies (87)
VEGF	Growth factor	Michael type addition	Seliktar (88)
VEGF	Growth factor	VEGF-modified PEGDA	Phelps (90)
BMP-2	Growth factor	Affinity binding	Pratt (91)
bFGF	Growth factor	Amide bond formation	Delong (90)

exploited to form hydrogels. However, for biological applications, one of the most common polymer chains used is PEG.[30c,d] PEG is soluble in water and organic solvents, is nontoxic, is antifouling, and is nonimmunogenic; making it somewhat of a "blank slate" for investigating biological interactions.[30c,d] PEG's terminal hydroxyl can be easily modified to incorporate different functional groups, e.g., thiol, acrylate, or carboxyl.[37] Formation of the hydrogels can be done via cross-linking,[40a] initiators requiring either energy/heat[39a] or light.[39b] In the following section, various types of modified PEG and the coupling chemistries used to attach biomolecules are outlined (see also Table 1).

Before considering coupling chemistry, mention should be made of physical entrapment of ECM proteins into hydrogel networks (so-called ECM composites). Jung et al.[81] synthesized a PEG-based hydrogel network (cross-linked via native chemical ligation) supplemented with the ECM proteins collagen and laminin. The ECM proteins were present at low concentrations so as not to affect the mechanical properties and porosity of the base hydrogel. Human mesenchymal stem cells co-encapsulated in the hydrogels were found to have 93% viability over 21 days in culture as compared with the no ECM control, with 60% viability. In addition, human mesenchymal stem cells in hydrogels containing collagen only became elongated and aligned with the collagen fibers, as would be expected in the native ECM.

1.3.1. Short Peptide Sequences. As discussed in the introduction, cells interact with the ECM and sense their environment via transmembrane receptors called integrins.[9b,82] ECM proteins contain short peptide motifs that bind integrins and allow cells to adhere to the ECM, forming the basis for sensing and signaling. It is known that one of the ligands bound by integrin is the arginylglycylaspartic acid (RGD) peptide sequence found naturally within fibronectin.[12] RGD plays several roles within the ECM, from facilitating cell adhesion to controlling cell morphology and motility; they also enhance cell viability.[13] Therefore, one of the first steps toward producing biofunctional hydrogels was to incorporate of RGDs into polymer matrices. Herein, we describe methods for functionalizing polymers with RGD and other integrin binding sequences.

Hern and Hubbell[39a] found that human foreskin fibroblast cells cultured on PEGDA hydrogels did not fully spread and adhere. In order to improve cell adhesion, YRGDS peptide was conjugated to amine-reactive NHS ester-modified PEGA and subsequently inserted into the PEGDA hydrogel in a photopolymerization step. The hydrogel functionalization with RGD permitted up to 80% of the cells to fully spread on the hydrogel surface. The PEGylation of the YRGDS (providing a spacer between the bulk hydrogel and the peptide sequence) was found to promote specific interaction between cells and the hydrogel (no attachment to YRDGS peptide). Mann et al.[59] used the same method of RGD peptide bioconjugation, and as well as RGD cell adhesion units, protease-cleavable units were incorporated into the hydrogel allowing dynamic remodeling of the hydrogel by cells (see Section 1.4.1).

Salinas et al.[13] found that a 5 mM RGD peptide concentration could be used to sustain 77% human mesenchymal stem cell viability after 2 weeks in culture. In this work, thiol−ene "click" chemistry was used to incorporate homobifunctional cysteine-modified RGD peptides into a PEGDMA hydrogel network. In the presence of light and a photoinitiator, both the chain polymerization of PEGDMA and the thiol−ene "click" reactions proceeded simultaneously, yielding a cross-linked hydrogel network, and, depending on the thiol-methacrylate stoichiometry, pendant RGD groups providing cell attachment points. Farrugia et al.[83] synthesized a hydrogel that was highly resistant to cell attachment, composed of a copolymer of 2-(dec-9-enyl)-2-oxazoline and 2-ethyl-2-oxazoline, cross-linked via thiol−ene "click" with 2-mercaptoethanol. Similar hydrogels functionalized with cysteine-terminated RGD peptides were capable of encapsulating human dermal fibroblasts and maintaining cell viability for up to 8 days.

A range of adhesive peptide sequences, other than RGDs, have been identified.[10a] In order to investigate the effect of cell−matrix interactions on pancreatic β-cells with the goal of improving pancreatic cell transplant, Weber et al.[84] followed the procedure of Hern and Hubbell[39a] to create PEGA-conjugated analogues of the laminin adhesion sequences IKLLI, IKVAV, LRE, PDSGR, and YIGSR as well as the collagen type I sequence DGEA. The peptides were incorporated into the hydrogel in a photopolymerization step with PEGDMA. Two of the sequences, IKLLI and IKVAV, were found to promote β-cell viability and insulin secretion, and the sequences PDSGR and YISGR were found to have a synergistic effect on β-cell viability and insulin secretion. In another example, the short peptide sequences TTSWSQ and AEIDGIEL were found to promote stem cell self-renewal of mouse embryonic stems cells by integrin activation.[85] Menzies et al.[86] incorporated a recombinant fibronectin domain containing the RGD cell adhesion sequence and the synergistic PHSRN sequence into a

polymeric covalently cross-linked hydrogel. The hydrogel was composed of 8-arm PEG-hydroxyphenyl propionic acid cross-linked via horseradish peroxidase-mediated oxidative coupling of the phenolic hydroxyl groups. The fibronectin domain was incorporated into the hydrogel by dimerization of the hydroxyphenyl groups found on tyrosine residues with those on the hydroxyphenyl propionic acid. Human mesenchymal stem cells encapsulated in the hydrogels had 81% viability after 4 days in culture, and the cells cultured in control gels containing no fibronectin were more rounded in morphology, with poorly structured actin cytoskeleton, indicating poor attachment.

The bioconjugation of cell adhesion sequences into hydrogels improves cell adhesion and viability, and can be used to control stem cell fate in some situations. However, different proteins are required to further enhance the hydrogel function, for example, to promote stem cell differentiation or to allow for matrix remodelling. Biofunctionalization with proteins is discussed in the following subsection. In addition, random spacing of growth factors is not necessarily the most effective way to engineer the desired cellular response, as cells have been shown to be sensitive to ligand spacing.[87] The chemical patterning of cell adhesion points is described in Section 1.3.3.

1.3.2. Growth Factors. As stated in the Introduction, the development of tissues via differentiation of specific subsets of cells is influenced by the presentation of growth factors.[26] They are necessary additions to hydrogels if the 3D cell culture is to recapitulate some of the behaviors of native cell growth *in vivo*.

Seliktar et al.[88] reported that the covalent attachment of vascular endothelial growth factor (VEGF) into a PEG hydrogel caused latent MMP-2 to be up-regulated by human umbilical cord endothelial cells and human dermal fibroblasts cultured on the gels. *In vivo*, the upregulated MMP-2 liberates additional growth factors from the ECM, as part of the angiogenesis cascade.[27] Seliktar et al.[88] used PEG components consisting of 4 arms which were functionalized by vinyl sulfone (VS) or acrylate functionality at the OH-termini to form the hydrogel. VEGF could not be directly attached to the hydrogel; however, a recombinant VEGF mutant was engineered with an additional cysteine group at the C-terminus to allow covalent conjugation to the hydrogel. VEGF-modified PEGDA hydrogels were subsequently used by Phelps et al.[89] demonstrating enhanced reperfusion in a mouse model of hind-limb ischemia. It should be noted that as well as VEGF, both Seliktar et al. and Phelps et al. incorporated protease-cleavable units into their hydrogel allowing dynamic remodeling of the hydrogel by cells (see Section 1.4.1). Delong et al.[90] demonstrated that bFGF, which is another growth factor implicated in angiogenesis, induced a 41% increase in proliferation and 15% increase of migration of smooth muscle cells on PEG hydrogels. The bFGF was incorporated into the hydrogel via NHS-mediated amide bond formation.

An alternative growth factor binding strategy utilized by nature is affinity binding interactions. Pratt et al.[91] implemented an affinity-binding strategy to incorporate bone morphogenetic protein-2 (BMP-2) into a PEG hydrogel, demonstrating bone regenerating properties. The BMP-2 was incorporated via heparin bridging. First, *N*-acetyl-GCGK-*β*Ala-FAKLAARLYR-KA sequence, known for its ability to bind various morphogenic growth factors via a heparin bridge,[92] was covalently coupled via Michael addition to a PEG-VS hydrogel. Heparin and BMP-2 formed a bridging complex with the binding peptide to noncovalently present the growth factor within the hydrogel. Further work on affinity binding-and-release of soluble growth factors has been reported by Lin et al.[93]

Incorporating growth factors into hydrogels has been more challenging than simple short peptide sequences, since the chemical modification of proteins may alter their functionality. Heparin bridging and affinity binding may prove to be more successful strategies in the long term.

1.3.3. Static Biofunctionalized Hydrogels with Chemical Patterning. Cell behavior can be influenced by gradients in chemical/physical properties. For the analysis of cell migratory behaviors (e.g., chemotaxis,[24a,25] durotaxis[94]) differential concentrations of chemoattractants or cross-linker molecules would be required, necessitating a regiospecific control over the coupling chemistry. The main method of producing chemical gradients in hydrogels is via the use of mechanical mixers and photopolymerization, as discussed below.

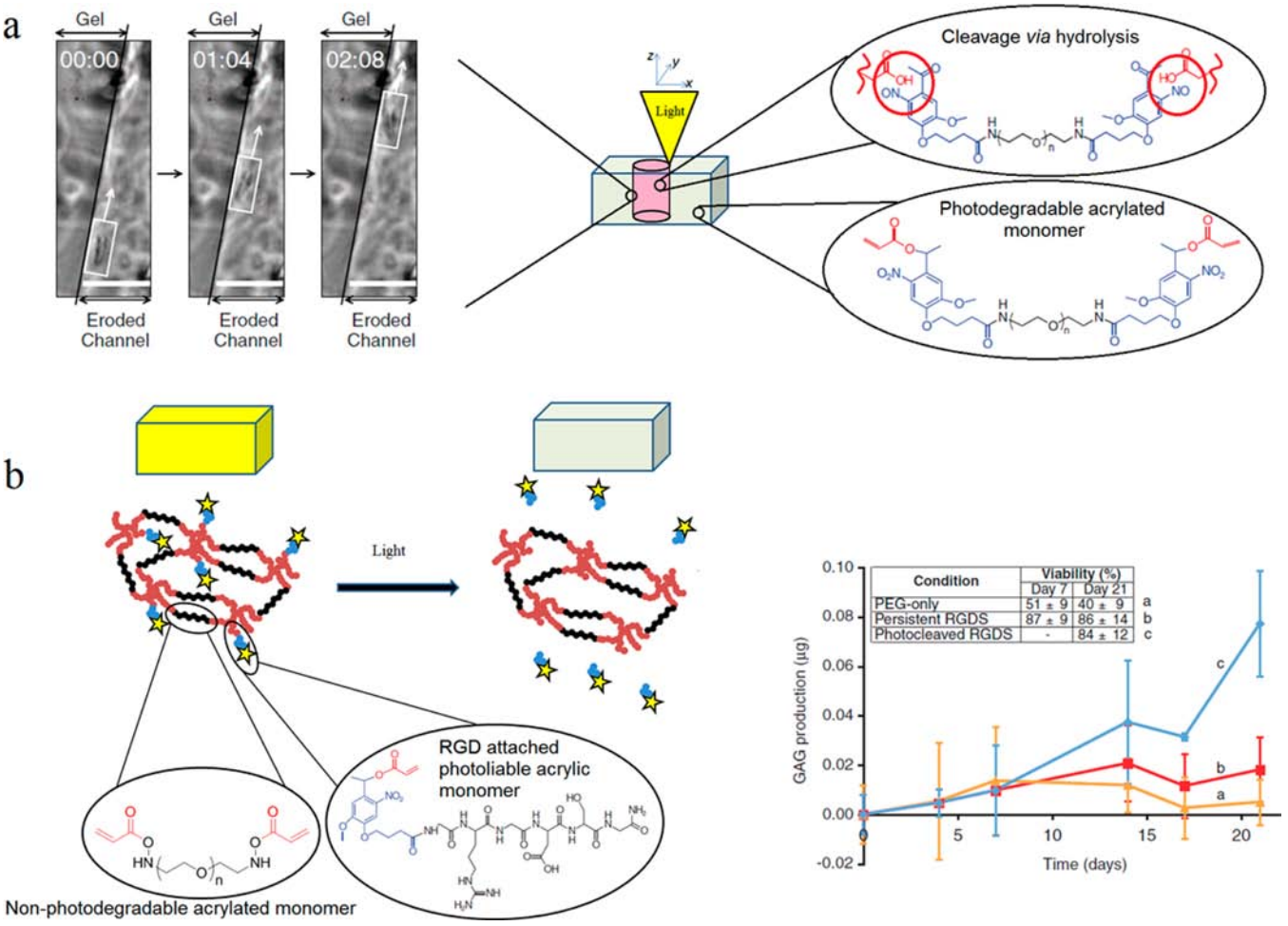
Cell responses to gradients in cell-adhesive RGD peptide concentration were observed by Burdick et al.[95] and DeLong et al.[90,96] Burdick et al. used a dual-inlet microfluidic system to prepare prepolymer solutions of PEGDA and PEGA-conjugated RGDs peptide in a concentration gradient. Photopolymerization subsequently cross-linked the RGDS in a static gradient patterned hydrogel (from 5 mM at one end and 0 mM at the other end of a 0.9 mm gel) . Between the two ends of the sample, a 5-fold decrease in cell attachment number was observed. Subsequently, DeLong et al. observed enhanced cell migration and preferential cell alignment with gradients of RGD peptide and basic fibroblast growth factor (generated in a mixing chamber and deposited into a mold using a peristaltic pump).

By contrast with gradients in peptide concentration, gradients in mechanical stiffness have also been prepared for the objective of allowing high-throughput screening of candidate matrices for stem cell differentiation, and also for the study of cell migration (specifically, durotaxis). Nemir et al.[97] used a temporally varying mixture of high- and low-MW PEGDA solutions (mixed in a similar chamber to that used by DeLong et al.[90,96]) cured via UV illumination to produce a spatial gradient of hydrogel stiffness. The compressive modulus ranged from ∼2 kPa to 100 kPa, and it was found that RAW 264.7 macrophages seeded on the hydrogels preferentially adhered in stiffer regions. Chatterjee et al.[98] used a similar mixer to create a temporally varying concentration of PEGDMA solution. The compressive modulus of these hydrogels ranged from 10 kPa to 300 kPa, and it was found that increased modulus (>225 kPa) led to osteogenic differentiation of MC3T3-E1 murine osteoblasts, in the absence of biomolecules or growth factors. Sunyer et al.[99] used a combination of acrylamide and bis acrylamide to synthesize a hydrogel. Photopolymerization and an opaque mask was used to gradually expose the hydrogel to light, causing the formation of a stiffness gradient within the hydrogel (with compressive modulus of 1 to 240 kPa, 115 kPa/mm). Murine 3T3 fibroblast cells were seeded on the hydrogel and it was shown that these cells preferred to adhere to a stiffer environment.

Gradient hydrogels have been shown to direct cell behavior, but for exquisite spatial control, materials processing (or biofabrication) tools would be required. Biofabrication techniques will be discussed in Section 2.

1.4. Dynamic Biofunctionalized Hydrogels. Establishing gradients or regions of different biomolecular concentrations will allow directed cell migration to specific niches within a hydrogel. However, for understanding the onset of many

Scheme 2[a]



[a]Outlining a photodegradable hydrogel with tunable biomolecular properties. (a) The base photodegradable acylated monomer (compound **1**) was used to synthesize the hydrogel, consisting of PEG (black), ethyl 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoic acid (blue), and acrylate terminal groups (red). Hydrogel cross-linking was carried out via redox-initiated free-radical polymerization in the presence of ammonium persulfate and tetramethylethylene diamine. When irradiated with UV light, the photolabile moiety (blue) was cleaved via hydrolysis, etching a channel. The eroded channel highlights cell movement through the channel from the image presented. (b) A non-photolabile acrylated monomer (compound **2**) is used to synthesize the hydrogel. An RGD-conjugated photolabile acrylic monomer (compound **3**) was attached to the hydrogel for hMSC studies. Upon irradiation, the RGD component was cleaved via hydrolysis and removed from the hydrogel. The graph outlines the RGD effect on hMSC where (a) consist of only PEG, (b) has RGD throughout the experiment, and (c) has RGD cleaved at day 7. (c) showed higher glycosaminoglycan production than (a) or (b). Reprinted with permission from AAAS.[39b]

cellular processes, a "starting gun" or temporal control over chemical functionality is required.[100] Temporal signaling is also a prerequisite for the differentiation of some stem cells.[26] In addition, stimuli-responsive gelation may be required for some materials processing methods to generating high-throughput 3D cell culture models (which will be described in Section 2). Temporally degradable systems have been recently reviewed by Kharkar et al.,[45] and here we describe a number of examples of temporally responsive hydrogels, including degradable hydrogels. We do not cover responsive hydrogels for drug delivery, which are well-known and have been reviewed elsewhere.[101]

Change in temperature can be used to induce a sol−gel transition, as described in Section 1, but temperature can be used as a trigger in more rigid structures, to promote cell migration. A photo-cross-linked hydrogel PEGDA containing a proportion of hydrolytically labile DTT bridges was synthesized by Hudalla et al.[102] At 37 °C, the DTT undergoes hydrolysis, which allowed for the migration and increased viability of human mesenchymal stem cells. Similarly, Matsusaki et al.[103] and Yoshida et al.[104] reported the use of bioreductants (cysteine) to degrade disulfide cross-linkers.[103,104] As the cross-linkers are degraded, the hydrogel 3D scaffold is compromised, and leads to the alteration of the hydrogel structures.

Photopolymerizable hydrogels were described in Section 1. Bulk modification and patterning of hydrogels was described in Section 1.3.3 above. As an extension to the standard stereolithographic techniques, and to allow cellular-level resolution, two-photon polymerization (2PP) was developed. 2PP utilizes radiating photons with energies exactly half as large as the energy required for normal stereolithography. When focused to a narrow spot, these photons can combine to induce photochemical reactions requiring the energy of two photons (hence the term two-photon) at an appreciable rate. The advantage of this procedure is that the radiation is not strongly

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absorbed by the surrounding medium. This means that exceptional resolution, as low as 0.1 μm, can be achieved.[105]

Kloxin et al.[39b] developed photoresponsive 3D cell culture systems activated by (i) bulk illumination to induce chondrogenesis and (ii) 2PP to induce cell migration, as a model for developing more relevant niches for stem cell culture in tissue regeneration. The photoresponsive hydrogel was composed of a PEGDA network (see Scheme 2) with photocleavable *o*-nitrobenzyl ether groups either (i) tethering RGDS cell adhesive peptide to the network or (ii) flanking the PEG group within the diacrylated polymer, thereby forming a photodegradable hydrogel network. Using the RGD-functionalized hydrogel in (i), Kloxin et al. observed elevated levels of chondrogenesis in hydrogels where RGD molecules were photocleaved by bulk illumination after 10 days in culture compared with persistent RGD presentation on control hydrogels. Using the photodegradable hydrogels in (ii), 2PP was employed to degrade channels of decreased mechanical stiffness in 3D patterns within the hydrogel. Fibrosarcoma cells were observed to migrate along photodegraded channels of reduced stiffness, demonstrating cell response to the hydrogel physical properties. By utilizing the photocleavable *o*-nitrobenzyl ether linkage, Kloxin et al. were able to demonstrate a generalized strategy for temporal control over hydrogel chemical and physical properties.

The same group[49] also developed an orthogonal coupling system for patterning RGD with visible light using thiol−ene coupling, and selectively degrading a hydrogel using UV light with the nitrobenzyl moiety described above. The hydrogel itself was cross-linked via strain-promoted azide−alkyne cycloaddition (SPAAC), with a storage modulus before degradation of 5 kPa. Murine NIH 3T3 fibroblasts were presented with dynamically degraded and/or RGD-patterned channels, and it was the combination of physical space as well as RGD patterning that was found to be necessary for cell migration.

The RGD tethering strategies described above have not been extended to more complex proteins, and in general, bioconjugation chemistries can alter protein structure and function. Recent work by Mosiewicz et al.[106] has allowed more fragile proteins to be attached to peptide-functionalized PEG hydrogels via the enzymatic activity of transglutaminase factor XIII. The peptide substrate of transglutaminase factor XIII was caged by 6-nitroveratryl chloroformate, a photolabile group. Upon photoactivation, the peptide substrate was exposed and available for enzymatic coupling to glutamine-terminated recombinant VEGF. In a soluble analogue of the system, the activity of VEGF was found to be maintained after transglutaminase factor XIII activity.

In the examples above, an external stimulus was required to change the properties of the hydrogel, but would it be possible to develop materials that were responsive to cellular activity (e.g., enzymatic degradation and remodeling)?

1.4.1. Dynamic, Enzyme-Responsive Hydrogels. It has been found that in order to migrate through a nonpermissive hydrogel region, cells need to be able to remodel their environment.[11,32] Typically this is achieved by proteolysis mediated by cell-secreted enzymes. Enzyme-responsive polymeric materials have been reviewed elsewhere,[107] but we include several pertinent examples below.

Hubbell and co-workers pioneered the approach of cross-linking PEG hydrogels with MMP-degradable peptide cross-linkers.[108] This class of hydrogels are important to study cell migration, protease inhibition study, and drug efficacy studies. Lutolf et al.[109] synthesized a PEG hydrogel network chemically cross-linked with protease (specifically matrix metalloproteinase, MMP) degradable substrate to follow the invasion of primary human fibroblasts. This system was subsequently implemented in a rat model of bone regeneration therapy.[110] Taking the biomimetic approach further, the same group designed a PEG hydrogel network that is biomimetic in both its synthesis (through transglutaminase enzyme factor XIII) and degradation (through MMPs).[111] Similarly, Anseth and co-workers utilized specific peptide sequences to covalently cross-link PEG hydrogel to create collagenase[112] and elastase[113] responsive hydrogels.

Enzyme-degradable subunits have been utilized in many of the cell-adhesive or chemically patterned hydrogels described in previous sections. A growth factor encapsulating hydrogel capable of upregulating MMP-2 expression of human umbilical cord endothelial cells and human dermal fibroblasts was described in Section 1.3.2. In this hydrogel, Seliktar et al.[88] incorporated MMP-2 degradable cross-links, and the stimulation of latent to active MMP-2 by transforming growth factor β1 is an example of a temporally controlled enzyme-mediated degradation.

Kyburz et al.[114] investigated the effect on human mesenchymal stem cell migration of cross-linking network density and RGD peptide concentration in 4-arm PEG-norborene hydrogels bridged by MMP-cleavable peptide sequence KCGPQG↓IWGQCK (↓ indicates site of proteolysis). The PEG polymer end groups were functionalized with norbornene and the formation of the hydrogel was facilitated via a cysteine capped peptide sequence. Photoinitiators were used to activate the cysteine on both the cross-linking and RGD peptide sequence and allow attachment. Migration speeds were found to be highest in hydrogels of low cross-linking density and high RGD concentration. In contrast to the work of Kyburz et al., the norborene-tetrazine Diels−Alder reaction was used by Alge et al.[53] to cross-link proteolytically degradable hydrogels.

Singh et al.[115] used a patterned hydrogel to demonstrate durotaxis of human HT 1080 fibrosarcoma cells. Singh et al. used a norborene-terminated 8-arm PEG that was partially cross-linked with MMP-sensitive cysteine-terminated peptides, under bulk UV illumination. A lithographic mask was used to further cross-link specific regions that were 200 μm wide. The process used by Singh et al. resulted in a patterned hydrogel with regions of higher and lower stiffness.[115] Human HT 1080 fibrosarcoma cells encapsulated in the gels were found to migrate preferentially from the stiffer regions (elastic modulus 360 Pa) to the compliant regions (elastic modulus 100 Pa).

The group of West used 2PP (see above) to pattern hydrogels in 3D using photoactivatable biofunctional molecules to study cell migration and stem cell differentiation.[116] Building on this work, the West group were able to direct the migration of human dermal fibroblasts along specified tracks within a degradable PEG hydrogel.[117] The hydrogel backbone was created via UV photopolymerization of MMP-degradable (↓ indicates site of proteolysis) acrylate-PEG-(GGGL↓GPAGGK-PEG)$_n$-acrylate in the presence of initiator, a process that leaves a number of unreacted acrylates. After soaking in acrylate-RGDS peptide solution, 2PP was used to pattern cell adhesion points at high resolution within the hydrogel. Lee et al. found that when human dermal fibroblasts were encapsulated within the MMP-sensitive hydrogels, the cells underwent guided 3D

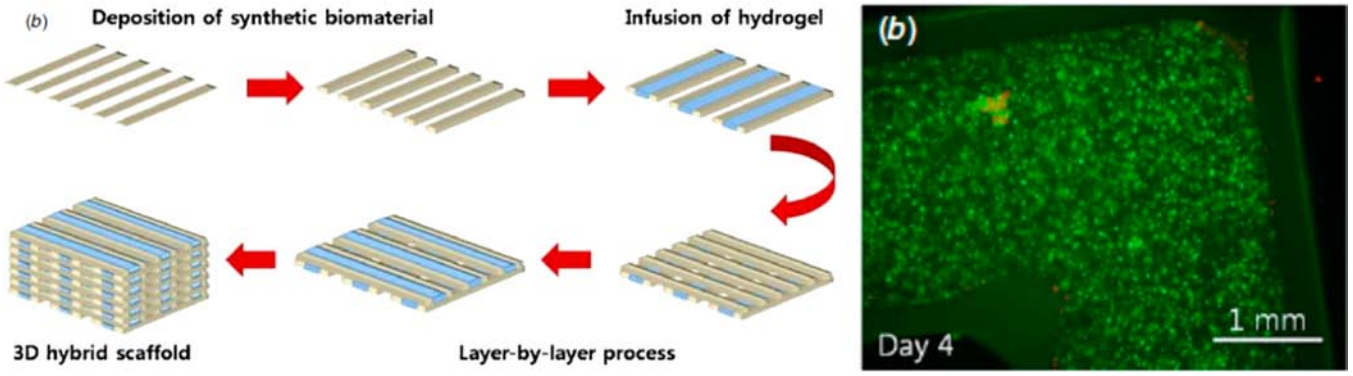

Figure 2. Use of a high-rigidity scaffold to form porous hydrogel architectures. Left: Layer-by-layer deposition was used to deposit poly(caprolactone)/poly(lactic-*co*-glycolic acid) (beige) and MC3T3-E1 osteoblasts in atelocollagen (blue). The entire porous structure also include empty pores to allow for the penetration of nutrients throughout the 3D structure. Right: After 4 days in culture, a fluorescent cell viability stain was applied, staining live cells green and dead cells red. Using fluorescence microscopy, the resulting viability images indicated a 98% cell viability. From Shim, J. H., Kim, J. Y., Park, M., Park, J., and Cho, D. W. (2011) Development of a hybrid scaffold with synthetic biomaterials and hydrogel using solid freeform fabrication technology, *Biofabrication* 3 (3), 034102. Reproduced by permission from IOP Publishing. All rights reserved.

migration only into the RGDS-patterned regions of the hydrogels.

This brief overview hopefully captures some of the extensive work that has been carried out using enzyme-degradable hydrogels. This modification is especially important when cells are encapsulated within matrices, such as during hydrogel formation. Methods for controlling spatial positioning of cell types and hydrogel structure are described in Section 2.

2. HIGH-THROUGHPUT PROCESSING TECHNIQUES FOR 3D CELL CULTURE

The role of biomolecular cues in influencing cell behavior and cell viability was explained in the previous section. Cells are also known to respond to the relative positioning of biomolecules, other cells, and the nanoscale topography of the extracellular environment.[5] Existing techniques for processing of materials are now being adapted for use with biomaterials[6] and integrated with computer control for precise patterning in 3D.[7] Material properties relevant for the fabrication of 3D cell cultures include viscosity and surface tension,[118] depending on the processing technique utilized; for some techniques, shear-thinning behavior may be beneficial.[4] In order to form a spatially patterned structure, the material must form a hydrogel to prevent redissolution and loss of spatial control;[118] Calvert[119] identified fidelity and reproducibility as key parameters to quantify in order to assess different techniques. Processing of single-cell droplets has been demonstrated by Nakamura et al.,[120] although with any single-particle technique, Poisson statistics will play a role.

Based on a review by Billiet on natural and synthetic hydrogel processing,[121] high-throughput processing techniques can be grouped into four categories: (i) nozzle-based; (ii) droplet-based; (iii) photoenabled; (iv) microfluidic. For each category, we will review the innovations allowing for elucidation of desired cellular response.

2.1. Nozzle-Based Biofabrication. Using a nozzle to cast a gel precursor solution or extrude a pregelled material is a key feature in the following systems. The nozzle-based systems allow for continuous, rapid deposition of materials, but compromises on resolution, with feature sizes on the order of 200 μm.[4] Multiple-nozzle systems have also been developed, which allow for the deposition of different materials[122] positioned in predetermined patterns[79,123] and which can interact chemically[124] or ionically.[125]

One of the motivations behind nozzle-based biofabrication is to gain more control over the positioning of cells within a hydrogel scaffold, where previously most cell-containing hydrogels contained a random distribution of cells (achieved by mixing cells with the gel precursor solution prior to bulk gelation). In an early demonstration of the concept, Smith et al.[126] designed a multinozzle extrusion device for deposition of synthetic/biomaterials and cells with a nozzle size of 90−350 μm and *xyz* positioning reproducibility of 5 μm. Using a cooled nozzle at 2 °C, the thermoresponsive polymer, Pluronic F127, was deposited in a layer-by-layer fashion on a glass slide heated to 25 °C in a predefined 3D pattern. In a separate experiment, human primary fibroblasts were mixed with Pluronic F127 before extrusion, with a resulting cell viability of 60%. In the case of hydrogel materials requiring a cross-linking agent to facilitate gelation, the rapid prototyping robot dispensing (RPBOD) system[124] allows for the simultaneous dual deposition of gel precursor (chitosan) and cross-linker (sodium hydroxide). By contrast, Cohen et al.[127] found a high printing fidelity (low macroscopic surface roughness and high reproducibility) when gel precursor (alginate) and cross-linker (calcium chloride) solutions were mixed thoroughly prior to extrusion. Using an appropriate mixing system (mixing gelatin and alginate) opens the way toward producing constructs with molecular concentration/mechanical properties gradients.[128]

3D bioplotting is a nozzle-based method compatible with 3D structure formation from low-viscosity precursors. In 3D bioplotting, the low-viscosity precursor solutions is deposited directly into a bath of material with similar rheological properties.[129] This material supports the 3D structure while it is being cast and can also promote hydrogel cross-linking (covalent[130] or noncovalent).[131] Maher et al.[130] created a 3D porous scaffold (interconnected pore size of 200−400 μm) of alginate, and ridges (separation of 200 μm) of cell-adhesive PEGDA-RGD gels. Fedorovich et al.[131a] used a 3D bioplotter to combine two differentially stained populations of goat bone marrow stromal cells within the same scaffold. To achieve the co-encapsulation, cells were mixed into ice-cold Pluronic F127 and plotted into culture medium at room temperature. Different syringes loaded with the two cell populations were interchanged between the printing of different layers. However,

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the cell viability in these constructs was low, with only 4% survival after 3 days. The authors pointed out that the use of membrane-stabilizing agents, as tested by Khattak et al.,[132] may go some way toward improving cell viability.

Apart from 3D bioplotting, other methods of maintaining hydrogel morphology during the fabrication process have been developed. Li et al.[128] and Zhao et al.[125] implemented a temperature-controlled deposition and manufacturing process based on the low-temperature gelation of gelatin. During fabrication, the hydrogel construct was held at low temperature (6−8 °C). However, for cell encapsulation and cell culture, it was necessary to introduce either glutaraldehyde or genipin (chemical cross-linkers), or a secondary hydrogel component and cross-linking mechanism (alginate/Ca^{2+} ions; fibrinogen/thrombin). For room-temperature gelation of Matrigel, Snyder et al.[133] used a cooled nozzle, in an inverse implementation to that shown by Li.

Scaffolds of higher rigidity can also maintain the cell-laden hydrogel morphology. Shim et al.[123] used a multinozzle system to engineer a hybrid scaffold made from alternating strips of poly(caprolactone)/poly(lactic-*co*-glycolic acid) and hyaluronic acid/gelatin/atelocollagen seeded with MC3T3-E1 osteoblasts/rat primary hepatocytes (see Figure 2). As can be seen from the micrograph, the channel containing hydrogel and cells was confined by the more rigid synthetic scaffold, and the synthetic scaffold was inert to cell attachment. The atelocollagen hydrogen supported MC3T3 osteoblasts, with up to 98% cell viability after 4 days in culture. Recently, Snyder et al.[68] reported the combination of two processes: multinozzle deposition and freeze casting; in the same structure, pore sizes ranging from submicrometer lamellae (freeze casting) to 100 μm pores (nozzle deposition) were observed. Cell viability was higher for cells seeded on the combined process scaffolds than on scaffolds produced by either process alone. Larger pores and channels within hydrogels have been fabricated using a sacrificial template, as described by Bertassoni et al.[134] In the method of Bertassoni et al., 100-μm to 1-mm-thick fibers of noncovalently cross-linked agarose were deposited on a methacrylated gel precursor. The methacrylates were subjected to photopolymerization, which did not affect the agarose. The agarose was subsequently manually removed from the hydrogel. Murine calvarial preosteoblast MT3T3 cells encapsulated in gelatin-methacrylate hydrogels exhibited viabilities of 60% after 7 days as compared with gelatin-methacrylate hydrogels possessing microchannels, which exhibited cell viability of 95% after 7 days.

Nozzle-based biofabrication is extremely versatile, as can be seen be the large number of examples and experimental setups described above. It is also compatible with the printing of mixtures of hydrogel precursor solutions and cells. The resolution of the structures printed via nozzle-based methods, however, is generally low and is limited by the gauge of the nozzle used, the diameter of which is restricted by considerations of viscous flow and extrusion.

2.2. Droplet-Based Biofabrication. By contrast with continuous deposition using a nozzle, the droplet-based systems employ a variety of methods with the aim of producing droplets with diameters ranging from 10 to 60 μm in diameter (or 1−100 pL in volume).[4] It has been demonstrated that droplets of so-called "bioink" can be positioned with lateral spacing on the order of magnitude of the droplet size (tens of μm),[120] and with layer heights of as small as 100 nm.[119] This method of biofabrication commonly makes use of off-the-shelf printer cartridges/print heads; however, these components may not be well-suited for delivery of biomaterials.[135] Parameters influencing the effectiveness of droplet-based biofabrication include surface tension and viscosity of solutions to be delivered, and clogging of orifices by cells and protein aggregates[4] (necessitating the use of low cell concentrations in bioinks).[136] It should be noted that inkjet printing is only compatible with materials with viscosities lower than 0.1 Pa·s.[118] There is ongoing effort to discover biocompatible materials and compatible processing techniques suitable for large-scale, reproducible biofabrication via droplet-based methods. Herein, we describe two types of inkjet droplet-based biofabrication, thermal inkjet printing and piezoelectric inkjet printing; and briefly cover advances in pneumatic and acoustic droplet generation and laser-induced forward transfer.

In thermal inkjet printing, ink from a reservoir enters a narrow channel leading to an orifice some tens of micrometers wide. A thin film resistor, making up part of the channel wall, undergoes resistive heating, vaporizing the ink in a layer a few micrometers thick. The expanding vapor forces ink through the orifice, forming a droplet. Despite the high temperatures close to the resistor, the bulk of the ink does not undergo substantial heating, and indeed, viable mammalian cells have been printed using the thermal inkjet method, first demonstrated by the Boland group.[137] It has been found, however, that the thermal inkjet process can induce transient pore formation in cells; however, this was used in a combined delivery-and-transfection system developed by the Yoo group.[138] The Yoo group also designed a single-cell alginate hydrogel droplet generator[139] with pancreatic β-cell viability of 89% observed.

In piezoelectric inkjet printing, ink enters a cavity in the printhead and is forced through a nozzle by the deflection of a piezoelectric pressure plate.[120] By forcing cells through a small orifice, some shear stress on the cells would be expected. However, Saunders et al. found that deposition of human HT 1080 fibroblast cells via piezoelectric inkjet did not have a detrimental effect on cell viability,[140] although some cell settling and orifice clogging was observed after a period of 20 min after bioink loading. Parsa et al.[141] used Pluronic F68 as a biocompatible surfactant to reduce the incidence of cell settling. Although the introduction of surfactant improved droplet resolution (highly reproducible droplet size), the variability in number of cells deposited in a specified volume suggests there was still some cell aggregation within the solution.

Similar to the work of Li et al. using nozzle-based technology (see Section 2.1 above), dual-cross-linking methods are also compatible with piezoelectric inkjet printing. Di Biase et al.[142] used thermoresponsive Pluronic F127 as a basis for physical gelation and stabilization of droplets produced via piezoelectric inkjet printing. The Pluronic F127 was chemically modified with terminal acrylates and mixed with PEGDA in solution at 5 °C. The printed structures were photocured with visible light of wavelength 460 nm. Following structure formation, human HT 1080 fibroblasts were seeded on top of the patterns using the same piezoelectric setup.

Pneumatic droplet generation employs controlling gas pressure in a syringe filled with bioink fluid. Applying a pressure pulse generates a droplet. The use of the pneumatic system has been shown to overcome the surface tension of high viscosity liquids (for example, collagen solutions with viscosities of up to 200 Pa·s).[143] Interestingly, pneumatic droplet generation is commonly combined with layer-by-layer assembly

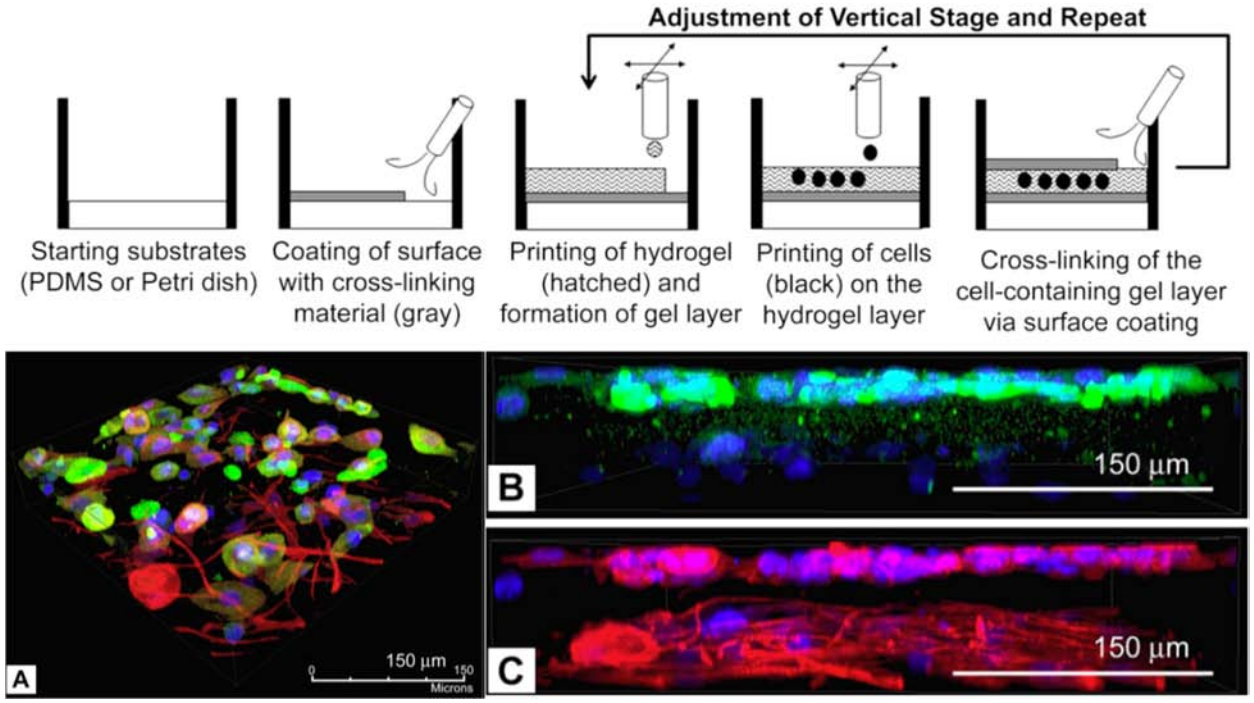

Figure 3. Layer-by-layer assembly of a human skin tissue model. Top: layer-by-layer deposition procedure. Cells printed on top of a collagen layer were encapsulated via cross-linking of collagen with sodium bicarbonate. Bottom: (A) 3D rendering of immunofluorescent staining captured using confocal microscopy. Keratin-rich areas are labeled in green, while β-tubulin-rich areas are labeled in red. Cell nuclei are labeled in blue. (B) Projection of keratin-stained area indicating the presence of keratinocytes (rich in keratin) in the top layer. (C) β-tubulin projection indicating the presence of keratinocytes in the top layer and fibroblasts in the bottom layer (both cell types contain β-tubulin). The spacing between the cell layers was 75 μm. Reprinted with permission from Elsevier.[143b]

by sequential deposition of gel precursor, followed by a nebulized cross-linking solution. The layer-by-layer assembly can be used to confine cells to a defined plane within the 3D structure (see Figure 3). In addition, the use of a nebulized cross-linker has the potential to improve the resolution of an engineered hydrogel microstructure as it avoids the coalescence of equally sized droplets containing precursor (for example, collagen) and cross-linker (for example, sodium bicarbonate) solutions, and the associated uncertainty in final droplet position.[143b] The compatibility of this method with high-viscosity bioinks seems to have alleviated the issue of clogging and cell settling.

An alternative means of avoiding clogging is to generate droplets using large orifices or via orifice-free techniques. Demirci et al.[144] utilized a piezoelectric acoustic-wave generator to form droplets of agarose by focusing of acoustic waves at the center of a circular geometry. This method was used to reliably encapsulate as few as 1−3 HL-1 cardiomyocyte cells per droplet. A laser vaporization method has been used to deposit rat Schwann and astroglial cells and pig lens epithelial cells on top of gelatin hydrogels, with the cells exhibiting high viability.[145] The technique is known as laser-assisted bioprinting, based on the process of laser-induced forward transfer (LIFT), and has been reviewed extensively by Guillemot et al.[146] and Koch et al.[147]

Droplet-based biofabrication promises to bring the required micrometer-level resolution to the production of 3D cell cultures. However, the issues of orifice clogging by cell aggregates and viscosity-matching of bioinks are issues that are still actively being investigated by the research community. Among the most promising droplet-based techniques are the more pneumatic, acoustic, and LIFT-based droplet generation methods. These provide greater tailorability compared with the inkjet methods that employ hardware that was not originally designed for bioprinting. In many cases, droplet formation is stabilized by means of a photocuring step. The use of photochemistry will be described in the following section and holds much promise in chemically and mechanically patterning bulk hydrogels.

2.3. Photoenabled Biofabrication. Photochemistry offers an alternative to physical deposition of biomaterials by allowing the chemistry of hydrogels to be altered *in situ*. The spatial resolution of photoenabled biofabrication is limited only by the theoretical diffraction limit and practically can achieve micrometer-level precision.[121] Lithographic techniques and focused laser targeting can be used individually or in tandem to mold desired structures (including pores, where the required pore size for tissue engineering is dependent on the cell type)[148] or introduce chemical patterning.[39c] The thickness and transparency of hydrogels to be fabricated via photoenabled techniques must be considered, due to the attenuation of light intensity in optically dense substrates.

One of the earliest techniques commercialized for non-biological rapid prototyping was stereolithography. In this process, UV light focused into a spot of diameter ~100 μm is used to selectively cross-link a photoactive solution. A movable stage submerges each completed layer to begin the cross-linking of the successive layer, building up a structure in a layer-by-layer fashion.[149] In early work on stereolithography for biofabrication, fibroblasts,[149,150] hepatocytes,[151] Chinese hamster ovary cells,[152] and stromal cells[153] were encapsulated in hydrogels with high viability observed.

2PP is an extension to the standard stereolithographic techniques, and was described in Section 1.4 Koroleva et al.[154]

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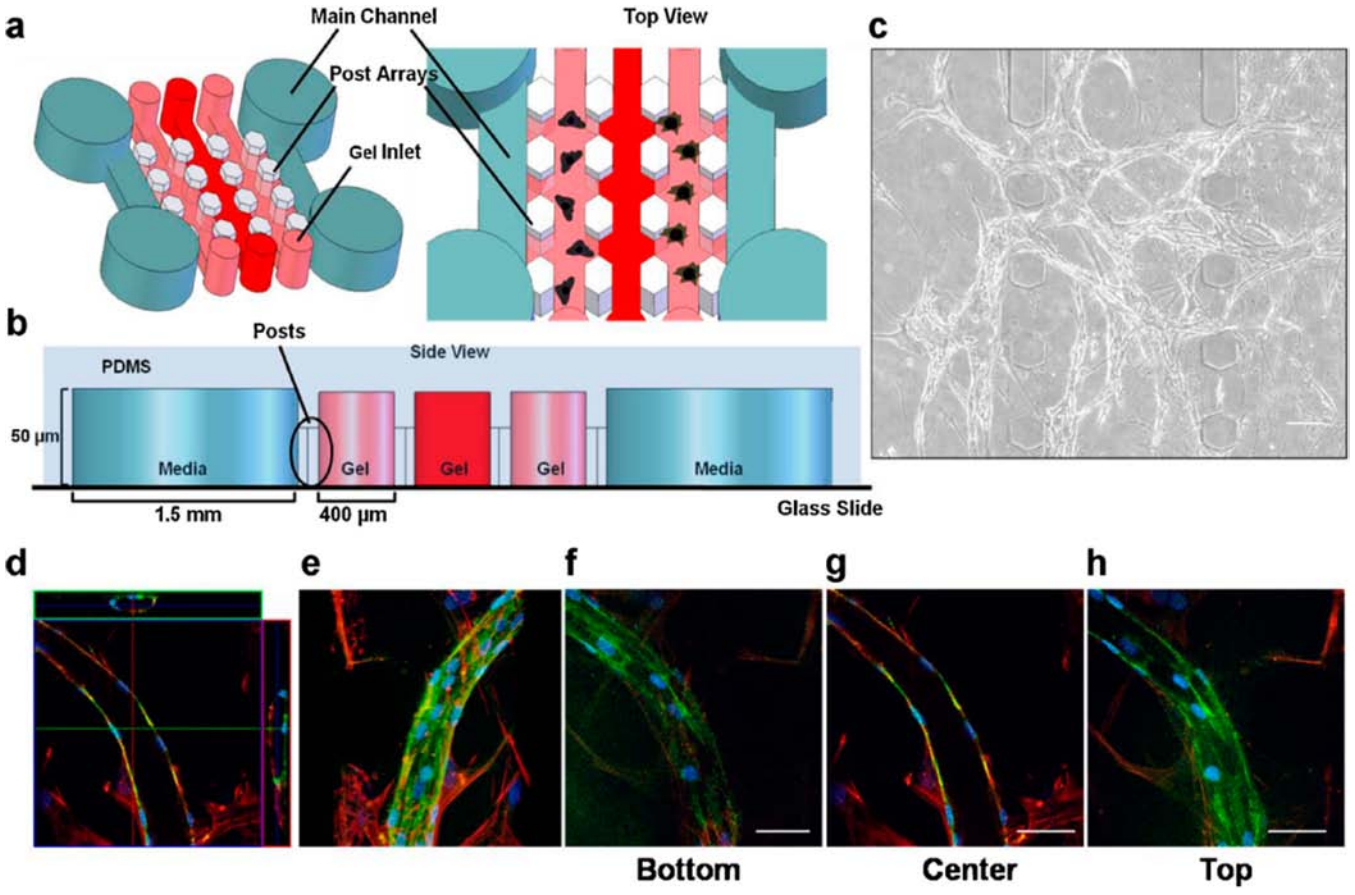

Figure 4. Microfluidic seeding of cell cocultures. (a,b) Schematic of microfluidic device used to coculture human umbilical vein endothelial cells (HUVECs, left-hand pink channel) and normal human lung fibroblasts (NHLFs, right-hand pink channel). The post array confines the flow of the gels to a strip 400 μm wide. (c) Phase-contrast image (day 14) of a primitive capillary plexus formed by HUVECs in the presence of NHLFs within fibrin gels in the microfluidic device. Scale bar: 100 μm. (d–h) Confocal microscope images from day 14 cocultures, with F-actin stained red, CD31 stained green, and cell nuclei stained blue. (d) Orthogonal image mode showing single *xy*, *xz*, and *yz* planes, indicating the presence of hollow lumens. (e) 3D projection of entire volume. (f–h) *xy* slices at different *z* heights clearly indicating hollow lumen. Scale bar: 50 μm. Copyright 2010 Wiley Periodicals, Inc.[164]

fabricated 3D scaffolds from four-arm poly(lactic acid) acrylate mixed with Igracure photoinitiator. As the 2PP technique is often highly time-consuming, the four-arm poly(lactic acid) scaffolds were subsequently used as masters for the creation of a PDMS mold for higher throughput of gel casting. This general method was also extended to the generation of high-resolution fibrin structures incorporating human pulmonary endothelial microvascular cells.[148]

Stereolithography is not the sole photoenabled biofabrication method capable of generating high-resolution structures. Barry et al.[155] combined direct ink writing with *in situ* photopolymerization to obtain hydrogel scaffolds with micrometer-sized features. By mixing high-molecular-weight poly(acrylamide) with monomeric acrylamide, *N*,*N*-methylene bis(acrylamide), diethoxyacetophenone, and deionized water, a shear-thinning and photopolymerizable solution was formed. By attaching a fiber-optic guide onto the printhead, it was possible to directly photopolymerize the solution as it was printed. Upon deposition and photocuring of the bioink, formation of hydrogel filaments with diameters as small as 5 μm and spacings of 20 μm (in regular patterns) was observed. This approaches the resolution achievable by 2PP. Barry et al. found that their hydrogels were not cytotoxic to cells seeded directly on top of the material.

Hockaday et al.[156] combined a multiple-nozzle deposition system[157] with a UV-LED for photo-cross-linking. Each nozzle contained a mixture of PEGDA of different molecular weights resulting in hydrogels of differing stiffness. Alginate was added to the PEGDA solutions to increase the solution viscosity and allow extrusion to occur. This setup was used to replicate a porcine aortic valve from a micro-CT scan, using stiff hydrogel (75 kPa elastic modulus) for the aortic wall and compliant hydrogel (13 kPa elastic modulus) for the leaflets. The replica geometric fidelity was assessed by surface deviation analysis using micro-CT scanning. For the largest models, up to 93% of the replica scan points lie within a 10% diameter threshold from the originals. Porcine aortic valve interstitial cells seeded on top of the printed scaffolds were found to be viable after up to 21 days in culture (last time point reported). The work of Hockaday et al. represents an attempt to incorporate biological tissue heterogeneity into *in vitro* models.

Combining the best of natural hydrogel (biocompatibility) and synthetic (tailorability), several examples have also shown methacrylated examples of gelatin and hyaluronic acid could lead to photo-cross-linkable polymer.[158] Recently, Duan et al.[159] showed an example where methacrylated derivatives of hyaluronic acid and gelatin were printed through extrusion with storage modulus ranging from 0.1 to 10 kPa to encapsulate

human valvular interstitial cells. The disadvantage of FRP is the heterogeneity within the polymer network and free radicals could react with biological substances if the technique is used for cell encapsulation and leads to cytotoxicity.

Photoenabled biofabrication has been used to create hydrogels with high-resolution patterning of physical and chemical properties, showing promise for biomedical applications.[160] There has also been demonstration of dynamic control of hydrogels using photocleavable subunits. This control has been used to direct cell migration within hydrogels. Perhaps combining nozzle- or droplet-based methods with photoenabled methods would allow for fine control over initial and final cell positioning within hydrogels.

2.4. Emerging Biofabrication Technology: Microfluidics. Microfluidic methods deal with picolitre volumes in micrometer-scale dimensions. They show great promise in a range of biomedical applications. Kobel et al.[161] and Velasco et al.[162] have reviewed the state of the art in using microfluidics to prepare cell-laden microgels with dimensions and patterning on the scale of tens to hundreds of micrometers. For example, Steinhilber et al.[163] used microfluidic emulsion formation to create droplets of 200 μm diameter, encapsulating yeast cells in a hydrogel composed of hyperbranched poly(glycerol) decaacrylate and PEGDA. The droplets were found to possess high stiffness (elastic modulus in the kPa range) and this may explain why the yeast cells were found to have low viability (~30% live cells after 12 h incubation).

Microfluidic technology could potentially be most effective in the preparation of cocultures of cells in precise spatial positions relative to each other. This would be particularly useful for studying interactions between cell populations in models of multicellular tissue types (e.g., blood vessels). Carrion et al.[164] used an array of narrowly spaced posts within a microfluidic device to produce 400-μm-wide, physically confined fibrin hydrogel strips, which were in physical contact to allow diffusion from one strip to another (Figure 4). Human umbilical vein endothelial cells (HUVECs) and normal human lung fibroblasts (NHLFs) were seeded in fibrin hydrogel strips on either side of a strip containing no cells. The microfluidic device was subject to flow of cell culture medium from adjacent channels allowing nutrient diffusion. After 14 days in culture, the HUVECs were found to have vascularized the "blank" hydrogel, with the NHLFs occupying a perivascular position, as would be expected in natural capillary formation.

Microfluidic systems have also been used to shape and manipulate hydrogels at the microscale (microgels).[165] Eydelnant et al.[165a] utilized electrostatic manipulation of discrete nano- and microliter droplets across open-electrode arrays containing hydrophilic patches to form hydrogels of reconstituted basement membrane, collagen, and agarose. Madin Darby canine kidney cells cultured inside the microsgels were found to retain up to 80% viability after 24 h in culture; the cells also began to form multicellular spheroids (as observed in native kidney) after 72 h in culture. Interestingly, this technique can be used to rapidly generate arrays of microgels for high-throughput testing.

CONCLUSION

The potential applications of biofabricated 3D cell culture models will be defined by the limitations of technology.[166] Advances in polymer synthesis have the potential to produce biocompatible materials with gentle cross-linking conditions, suitable for use with cells, while still retaining tailorable mechanical properties and biofunctionalization points. To date, most high-throughput processing approaches have used naturally derived materials as bioinks, but we envisage that this will begin to change in the near future as more synthetic polymeric hydrogels come on stream.

A putative strategy for realizing a fully functional, tailorable, and spatially defined 3D cell culture model (see Scheme 1) would involve a synthetic bioink with multiple domains including cross-linking domain(s) and biofunctionalized domain(s), mixed with cells in a dispensing mechanism. The transition from cell-gel precursor mixture to cell-encapsulated gel may be multistep involving physical cross-linking to form transitional structures such as droplets, followed by a chemical curing step to define a final hydrogel structure. Multiple materials could form part of the same structure, and dynamic control over materials properties, possibly by photocleavable chemical linkages, would allow for directed cell migration and/or stem cell differentiation. Such a platform would no doubt be indispensable for cell biologists in fields such as tissue engineering,[11,127,143b,156,167] stem cell research,[17a,168] morphogenesis,[169] tumor modeling,[170] disease modeling,[171] high-throughput single-cell analysis,[119,144,162] toxicology,[172] and drug discovery.[173]

AUTHOR INFORMATION

Corresponding Author

*E-mail: Justin.gooding@unsw.edu.au; Fax: +61 2 9385 6141; Tel: +61 2 9385 5384.

Notes

The authors declare no competing financial interest.

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