Review

Xiao-Hua Qin[a], Aleksandr Ovsianikov*[,a], Jürgen Stampfl*[,a] and Robert Liska[a]

Additive manufacturing of photosensitive hydrogels for tissue engineering applications

Abstract: Hydrogels are extensively explored as scaffolding materials for 2D/3D cell culture and tissue engineering. Owing to the substantial complexity of tissues, it is increasingly important to develop 3D biomimetic hydrogels with user-defined architectures and controllable biological functions. To this end, one promising approach is to utilize photolithography-based additive manufacturing technologies (AMTs) in combination with photosensitive hydrogels. We here review recent advances in photolithography-based additive manufacturing of 3D hydrogels for tissue engineering applications. Given the importance of materials selection, we firstly give an overview of water-soluble photoinitiators for single- and two-photon polymerization, photopolymerizable hydrogel precursors and light-triggered chemistries for hydrogel formation. Through the text we discuss the design considerations of hydrogel precursors and synthetic approaches to polymerizable hydrogel precursors of synthetic and natural origins. Next, we shift to how photopolymerizable hydrogels could integrate with photolithography-based AMTs for creating well-defined hydrogel structures. We illustrate the working-principles of both single- and two-photon lithography and case studies of their applications in tissue engineering. In particular, two-photon lithography is highlighted as a powerful tool for 3D functionalization/construction of hydrogel constructs with μm-scale resolution. Within the text we also explain the chemical reactions involved in two-photon-induced biofunctionalization and polymerization. In the end, we summarize the limitations of available hydrogel systems and photolithography-based AMTs as well as a future outlook on potential optimizations.

[a]Additive Manufacturing Technologies group of TU Vienna: amt.tuwien.ac.at; Austrian Cluster for Tissue Regeneration.
***Corresponding authors: Aleksandr Ovsianikov and Prof. Jürgen Stampfl,** Institute of Materials Science and Technology, Vienna University of Technology, Favoritenstr. 9, 1040 Vienna, Austria, E-mail: aleksandr.ovsianikov@tuwien.ac.at; juergen.stampfl@tuwien.ac.at
Xiao-Hua Qin and Prof. Robert Liska: Institute of Applied Synthetic Chemistry, Vienna University of Technology, Getreidemarkt 9, 1060 Vienna, Austria

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Introduction

Breakthroughs in tissue engineering offer the potential to tackle many healthcare issues ranging from population aging to cancer [1, 2]. Tissue engineers are aiming to restore or replace the lost functions of damaged tissues or failed organs by integrating biomaterials and cells. Porous biomaterial scaffolds have gained much attention since they provide cells with not only mechanical support and nutrients, but a permissive microenvironment which ultimately facilitates cellular functions. Particularly, biodegradable polymers have been widely used as scaffolding materials since they could serve their functions only for a defined period. The time-scale of degradation process can be carefully designed to match a specific application [2, 3].

In vivo, tissues include three-dimensional (3D) macroscopic architectures of repeating subunits on the length scale of 100–1000 μm [4–6]. To mimic such structures, traditional additive manufacturing technologies (AMT) have been explored to create 3D scaffolds with physiologically-relevant features [7]. However, as the understanding of the matrix biology goes deeper, there is a growing need to engineer the local microenvironment at a subcellular scale (1–10 μm) with biochemical, physical and cellular stimuli [8–10]. To achieve this goal, multiphoton microfabrication shows the greatest promise to create extracellular matrix (ECM) mimetic hydrogels with μm-scale resolution [11].

In this review, we present an overview of recent advances in lithography-based additive manufacturing of bioactive hydrogels. Specifically, this article begins with an introduction to design and synthesis of

photopolymerizable hydrogels. The discussion then shifts to case studies where the integration of hydrogel chemistry and AMT has facilitated biologically-relevant studies in tissue engineering. We will highlight the use of multiphoton processing techniques in state of the art biomaterials research.

Photopolymerizable hydrogels

Hydrogels are attractive biomaterials because their structural and biochemical properties are highly similar to the extracellular matrix (ECM) of most tissues (Figure 1). In addition, they present superior biocompatibility and cause minimal cellular damage when used for cell culture [12]. Chemically, hydrogels are crosslinked networks formed by hydrophilic monomers or macromers. Because of the macromolecular pores in the gel network, hydrogels provide high permeability for nutrients, oxygen and cellular wastes [13]. In addition, physical properties of hydrogels can be adjusted to match the mechanical properties of soft tissues [14, 15].

Although hydrogels could be formed by conventional polymerization methods (heat, ionic interaction and redox), photopolymerization has drawn the most interests in the area of hydrogels [16, 17]. Certain types of hydrogels can be formed in situ upon exposure to light in the presence of light-sensitive compounds (photoinitiators). When UV or visible light interacts with photoinitiators, free radicals are generated to initiate polymerization and finally form crosslinked hydrogel networks.

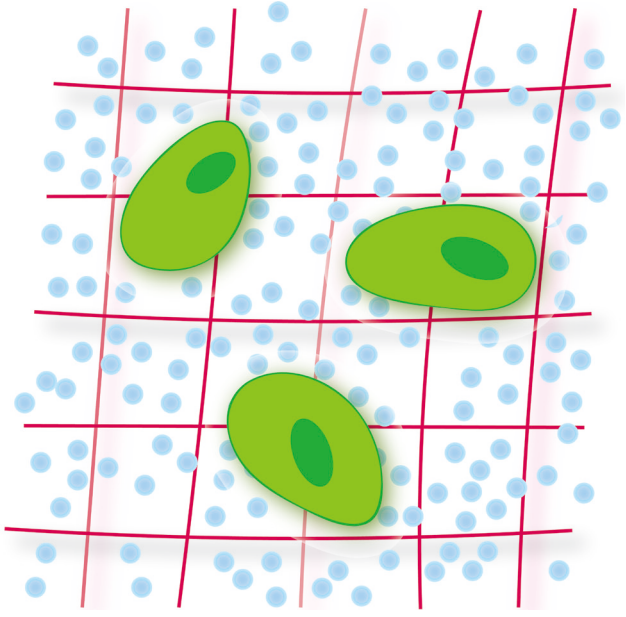


Figure 1 Hydrogel scaffolds for cell culture and tissue engineering.

Compared to conventional polymerization methods, photopolymerization presents numerous advantages, including fast reaction kinetics (below several minutes), spatiotemporal control over the polymerization process and processability at room temperature and physiological conditions [18]. All these attributes have enabled a wide range of photopatterning and stereolithography-based AMT techniques [19–21]. Integration of these AMT techniques with hydrogels have facilitated further tissue engineering applications, such as in situ cell encapsulation, spatiotemporal immobilization of bioactive ligands. In this section, we provide a materials selection guide to water-soluble photoinitiators and hydrogel precursors, which are essential components for photolithography-based additive manufacturing of 3D hydrogel constructs.

Water-soluble photoinitiators

When one designs a photopolymerization system, photoinitiator is the primary component to be considered since the selection of the photoinitiator is critical to the polymerization efficiency. First, the absorption spectrum of the selected photoinitiator should overlap to a large extent with the irradiation profile of the utilized light source. Second, the selected photoinitiator should have high efficiency in generating free radicals.

According to the radical generation mechanisms, radical photoinitiators can be divided into two categories: cleavable photoinitiators (Type-I) and bimolecular photoinitiating system (Type-II). Upon irradiation, Type-I photoinitiators such as Irgacure 2959 (I2959) undergo cleavage from the excited triplet state and generate two radicals for initiating polymerization (Figure 2A). In contrast, the initiating mechanism of Type-II systems (e.g., benzophenone/tertiary amine) is more complex. The exited benzophenone initiates a fast electron transfer from the lone pair of tertiary amine followed by a slow proton transfer process, providing the H-donor radical for initiating polymerization (Figure 2B). Notably, compared to Type-I photoinitiators, Type-II systems are much less efficient due to the bimolecular process, back electron transfer and especially the solvent cage effect in aqueous solutions [22].

Besides, it is noteworthy to mention the initiation mechanism of two-photon active initiators (Figure 2C). These initiators essentially have highly conjugated π-systems, good coplanarity and strong donor/acceptor groups. In the simplest case (i.e., radical polymerization), the initiation mechanism is currently accepted as: after intra- and

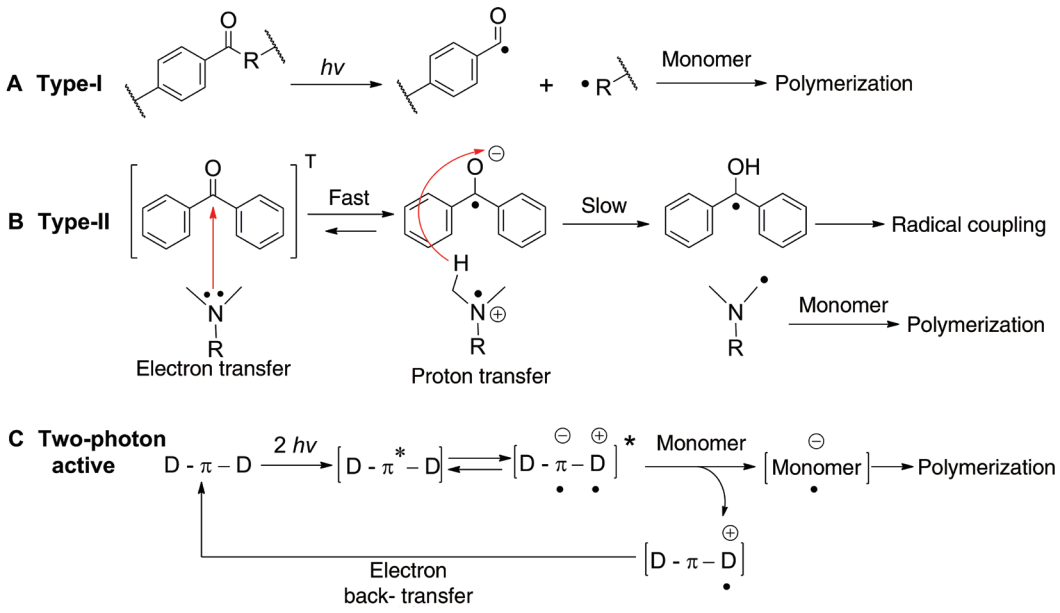


Figure 2 Photoinitiating mechanisms of Type-I (A), Type-II (B) and two-photon active (C) initiators.

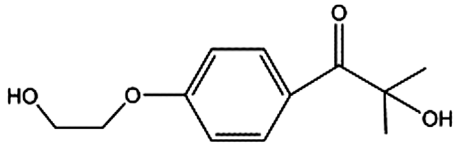
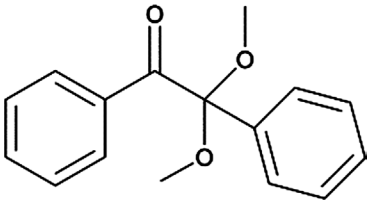
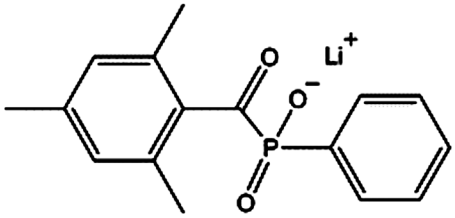
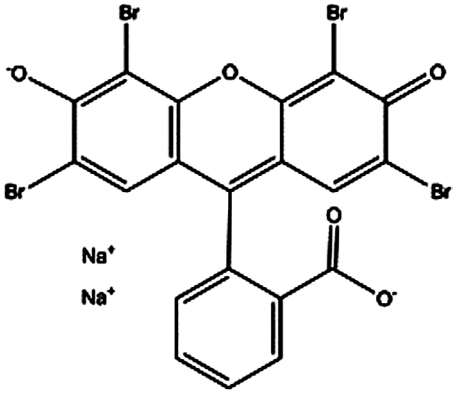
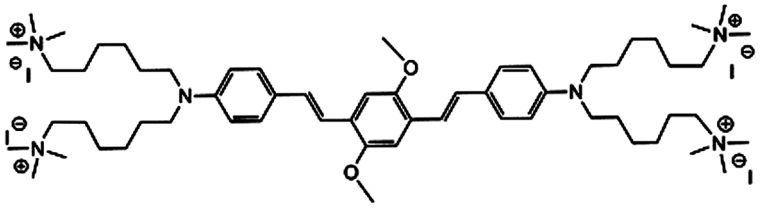
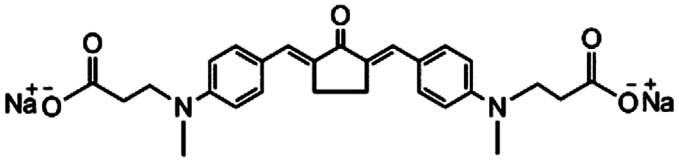
inter- molecular charge transfer interactions between the two-photon excited initiator and monomer, radicals are formed by electron transfer to initiate polymerization [23]. However, two-photon initiating mechanism for more complex systems (e.g., thiol-ene polymerization) remains largely unexplored.

For preparing hydrogels, more factors should be considered, particularly the water-solubility and cytocompatibility of photoinitiators. For in situ cell encapsulation, photoinitiators are used to polymerize gel precursor solutions containing cell suspension. After polymerization, ideally viable cells are encapsulated in hydrogel matrices. Table 1 presents an overview of state-of-the-art photoinitiators that have been used in preparing hydrogels. From the illustrated examples, it is important to note that each photoinitiator has different characteristics in terms of absorption profile, cytocompatibility, water-solubility and initiation mechanism.

For UV photopolymerization, classical Type-I photoinitiators with low cytotoxicity such as I2959 have been used for in situ cell-encapsulation studies [24, 29]. However I2959 suffers from low efficiency for cell-encapsulation purposes due to its limited absorption in the UV-A spectral range. The important n-π* transition of I2959 at ~330 nm has very low extinction coefficient. Combined with low initiator concentration due to limited solubility, its contribution to the initiation process is rather limited. While alternative cleavable initiators such as I651 possess much higher initiating efficiency at UV-A range, poor water-solubility often necessitates the use of organic solvents. Eosin Y has been used as a visible light initiator in combination with triethanolamine (TEA) [30]. While Eosin Y has strong absorption in the visible light range, it is less efficient than cleavable initiators due to intrinsic limitations of Type-II systems. Previous work by West and co-workers has explored the non-cytotoxic conditions of this three-component system [26]. It was found that the 'optimum' condition (~88% cell viability) was achieved using 0.01 mM Eosin Y and 0.1% TEA beyond which significant toxicity effects were observed. More recently, a Type-I visible light photoinitiator (lithium phenyl-2,4,6-trimethylbenzoylphosphinates, LAP, λ_{max}~375 nm) has proven to be much more efficient and less cytotoxic than I2959, thus making it a better photoinitiator for in situ cell encapsulation studies [31].

Very recently, Liska and co-workers have developed a series of water-soluble two-photon initiators for 3D microfabrication of hydrogels [28, 32]. For instance, a highly efficient initiator WSPI has been exploited to fabricate PEG hydrogels in the presence of living organisms [32]. Given that the synthesis of WSPI is sophisticated (>5 steps), a novel synthetic approach was established by Li et al. for the facile synthesis (2 steps) of carboxylate sodium salts of cyclic benzylidene ketone-based photoinitiators (e.g., P2CK) [28]. Importantly, it was demonstrated that cytotoxicity of P2CK solution on MG63 cells was as low as that of the well-proven biocompatible photoinitiator I2959.

Table 1 Water-soluble photoinitiators (PIs) for photopolymerization of hydrogels.

	PIs	Chemical structures	Water soluble	λ_{abs} (nm)	Reference
Type-I	I2959		Yes	276[a] 330[b]	[24]
	I651*		No	335[b]	[24]
	LAP		Yes	375[b]	[25]
Type-II	Eosin Y		Yes	514[a]	[26]
Two-photon active	WSPI		Yes	423[a]	[27]
	P2CK		Yes	470[a]	[28]

[a]π-π* absorption, [b]n-π* absorption; *ethanol has been used to increase the solubility of I651.

Photopolymerizable hydrogel precursors

Although the access to efficient photoinitiators is a prerequisite for additive manufacturing of hydrogels, design and synthesis of photopolymerizable monomers/macromers as hydrogel precursors are even more important. The up-to-date hydrogel platforms can be broadly divided into two classes associated with their origin: 1) synthetic

polymeric hydrogels that allow flexible tuning of chemical and mechanical properties; and 2) naturally-derived hydrogels that generally have superior biocompatibility and similar biological functions as their biomacromolecule origins.

Design considerations

To design appropriate hydrogel precursors for potential AMT applications in tissue engineering, several considerations should be addressed.

First, from a chemical point of view, hydrogel precursors should possess sufficient water solubility. To achieve this goal, polyethylene glycol (PEG) based synthetic polymers as well as certain naturally-derived polymers (e.g., gelatin, hyaluronan or HA) have drawn much attention due to their superior hydrophilicity. On the other hand, the photo reactivity of hydrogel precursors plays an important role in the feasibility to AMT. To fabricate an object with size scales that are clinically relevant (mm to cm scale), the photoreactivity of monomers/macromers directly determines both the writing speed and time costs of the manufacturing process.

Second, from a biological point of view, hydrogel precursors must meet further requirements.

- Cytocompatibility: For in situ cell encapsulation, cells are directly exposed to a series of materials at varying stages: initially hydrogel precursors, then radical-mediated polymerization process, subsequent crosslinked networks, photoinduced byproducts and finally degradation products. Therefore, all of these involved chemicals and related polymer chemistry must be cytocompatible or at least of very low cytotoxicity.
- Bioactivity: Apart from cytocompatibility, hydrogel scaffolds produced by AMTs should provide cells with a physiologically-relevant environment and thus support all cell functions that are occurring in vivo (adhesion, migration, proliferation, differentiation). In a natural environment of cells, cell attachment is regulated by the crosstalk between integrin receptors on cell membranes and integrin-binding motifs (e.g., RGD motif peptides) distributed on ECM components. To this end, researchers have utilized either synthetic materials in combination with RGD motifs or naturally-derived proteins containing RGD motifs (e.g., gelatin, fibrin, fibrinogen) to create biomimetic hydrogels for cell culture [8].
- Biodegradability: Since the ultimate goal of implantable hydrogels is to replace or repair damaged tissues, the degradation kinetics of printed hydrogels should be tunable to match the growth rate of new tissues.

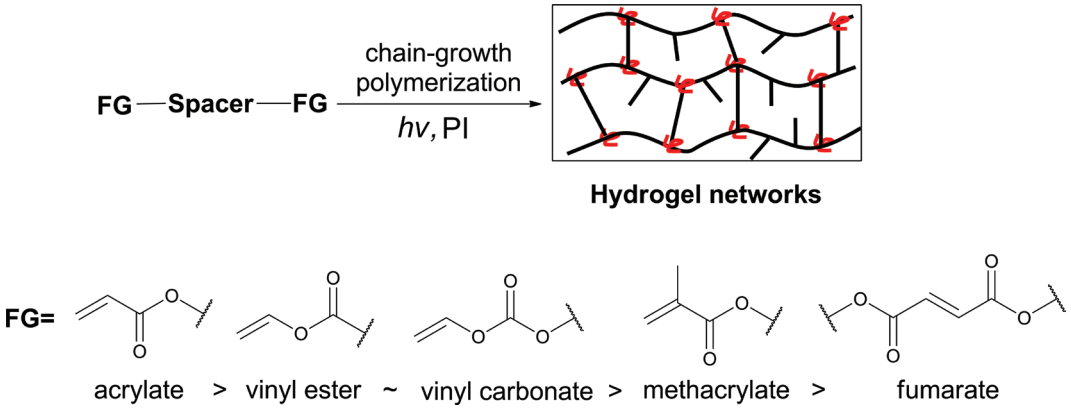
In the next sections, we discuss photopolymerizable hydrogel precursors that have been used in tissue engineering, proceeding from the chemistry of hydrogel formation, to synthetic hydrogels and naturally-derived hydrogels while discussing structure-property relationships and their applications in AMT.

Chemistry of hydrogel formation

The formation of hydrogel networks is based on the presence of multifunctional crosslinker during network formation. Thus, synthetic approaches to light induced network formation are not only limited to classical free-radical chain polymerization, but also alternative approaches based on click chemistry are currently investigated.

Chain polymerization: Free-radical chain polymerization is a facile approach to create hydrogels where a chain reaction propagates through vinyl-group containing monomers. The reactivity of vinyl monomers towards chain polymerization is dependent on the electronic environment of vinyl groups. Generally, it is accepted that the reactivity of vinyl monomers towards free-radical chain polymerization follows this sequence (Scheme 1): acrylate > vinyl ester ~ vinyl carbonate > methacrylate > fumarate [33].

To date, acrylated monomers are the state-of-the-art materials for AMT applications due to their high reactivity. A general method to synthesize acrylated macromers is based on esterification reaction between acryloyl chloride and hydroxyl groups of several synthetic building blocks [PEG, polyvinyl alcohol (PVA), etc.]. Although acrylates have superior reactivity, irritancy and potential cytotoxicity of unreacted acrylate groups is posing a challenge for their potential clinical-relevant applications. In contrast, methacrylates are much less cytotoxic as evidenced by their wide use as dental filling materials [34]. But the moderate photoreactivity of methacrylates raises many practical problems in most photolithography-based AMT. To address this challenge, we recently developed alternative photopolymers (vinyl esters) that represent a good trade-off between high reactivity and low toxicity [35]. Interestingly, there are very few monofunctional vinyl ester derivatives and only one hydrophobic difunctional vinyl esters (divinyl adipate) commercially available and a very limited number of photopolymerization studies related to vinyl esters have been reported so far [36]. Furthermore, hydrogels formed by vinyl ester chemistry remain largely



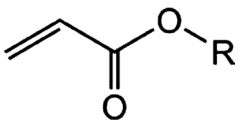
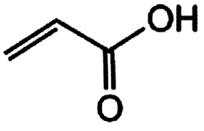
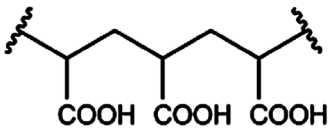
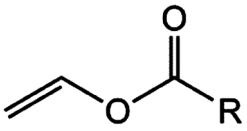
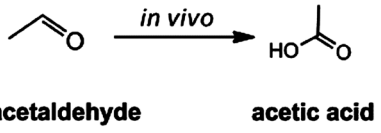
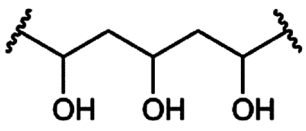
Scheme 1 Schematic showing the formation of hydrogel networks via free-radical chain-growth polymerization (FG: functional group).

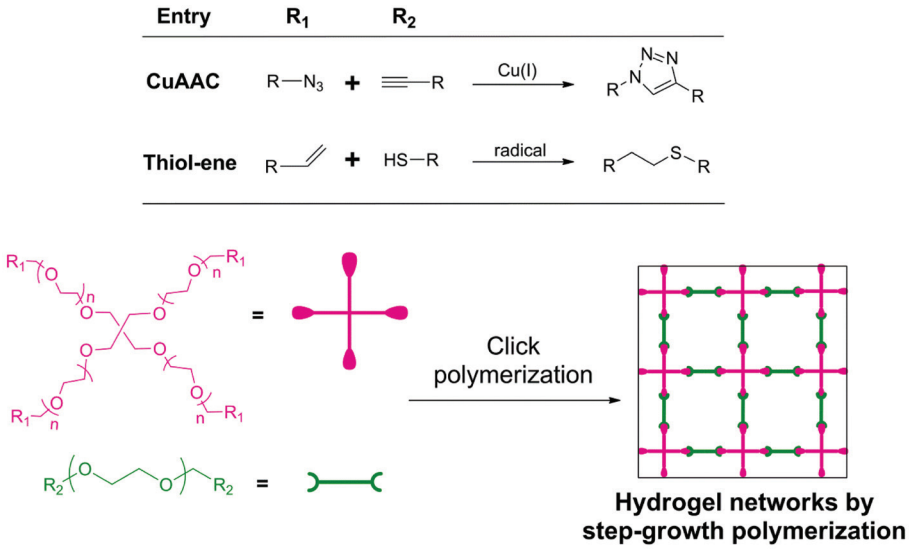
unexplored. We therefore synthesized PEG divinyl esters (PEGDVE) through a transesterification reaction between PEG dicarboxylic acids and vinyl acetate under the catalysis of palladium (II) acetate [37].

Table 2 presents a comparative analysis on the biocompatibility of acrylates and vinyl esters. Importantly, upon hydrolytic degradation, the major degradation products out of poly(vinyl esters) are FDA-approved poly(vinyl alcohol) (PVA) while polyacrylates result in high molecular weight poly (acrylic acids) which have proven very difficult to be excreted from the human body [35]. On the other hand, since it is unlikely to reach complete conversion of vinyl groups in radical photopolymerization, careful attention should be paid to the remaining unpolymerized vinyl groups (at least 10–20%). It is noteworthy that the remaining acrylate groups would form acrylic acids as degradation products that are highly cytotoxic and irritant while the residual vinyl ester groups can be easily hydrolyzed into acetaldehyde that can be easily metabolized into acetic acid in vivo under the catalysis of acetaldehyde dehydrogenase. Although vinyl esters are generally not as reactive as their acrylate analogs, our recent work demonstrated that the thiol-ene photo-click chemistry could greatly improve the reactivity of vinyl esters even to the level of acrylates [37]. Moreover, it is important to mention that the poor storage stability related to thiol-ene systems have recently been solved by using a combination of radical and acidic stabilizing system [38]. All these aspects suggest that water-soluble vinyl esters are promising hydrogel precursors for photolithography-based AMTs and tissue engineering applications.

Click-chemistry based polymerization: Besides chain polymerization, click chemistry is an emerging methodology to synthesize chemically-crosslinked hydrogels (Scheme 2). According to the initial concept proposed

Table 2 Comparative analysis of biocompatibility: polyacrylates vs. polyvinyl esters.

Monomers	Major degradation products	Hydrolyzed product of unpolymerized group
Acrylates		 Highly irritant
	 high M_w poly(acrylic acid)	acrylic acid
Vinyl esters		 acetaldehyde — acetic acid
	 low M_w poly(vinyl alcohol)	



Scheme 2 Schematic showing the formation of hydrogel networks via step-growth click-polymerization.

by Sharpless, click chemistry refers to unique chemical reactions between orthogonal groups that react with high selectivity under mild conditions [39]. For instance, copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) is highly specific and occurs in the presence of competing functional groups (Scheme 2). Researchers have successfully used CuAAC chemistry in combination with a series of hydrophilic macromers (PEG, PVA, HA) to prepare hydrogels [40–42]. However, spatiotemporal control is not readily achieved in these hydrogel systems. Interestingly, a collaborative study from Anseth and Bowman groups explored photoinitiated CuAAC reaction to create hydrogels with spatiotemporal control [43]. In this work, the CuAAC reaction was catalyzed by the photoreduction of Cu(II) to Cu(I) whereby the reduction was initiated by free radicals from activated photoinitiators. This method demonstrated comprehensive spatiotemporal control over the process of hydrogel formation, thus showing great promise for photopatterning or fabrication of hydrogels. But utilization of this method in AMT remains unexplored. Although hydrogels could also be formed via other click approaches like tetrazine-norbornene Diels-Alder reaction and oxime ligation [44, 45], these approaches generally lack of spatiotemporal control and thus are not suitable for direct use in AMT applications.

Radical-mediated thiol-ene photopolymerizations are alternative attractive click-approaches to create chemically-crosslinked hydrogels with spatiotemporal control. Such reactions are chemo-selective, robust and applicable to a wide range of macromers containing multiple ene or thiol groups. For instance, the pioneering work of Anseth and co-workers demonstrated that thiol-norbornene step-growth photopolymerizations are extremely robust systems for preparing hydrogels [46]. In their work, 4-arm PEG norbornenes could efficiently 'click' with a dithiol (i.e., a peptide with two cysteines), forming ideal hydrogel networks in <1 min [46].

The underlying mechanism of step-growth thiol-ene photopolymerizations is proposed below (Scheme 3A). Upon irradiation, free radicals are generated from photoinitiators followed by the H-abstraction reaction from thiols. The resulting thiyl radicals propagate across the ene functionalities, forming the carbon-centered radicals. Ideally, chain-transfer reactions are much more competitive than further chain-propagation reactions. Thus, the thiyl radicals are generated again upon highly efficient chain-transfer reactions.

Within the scope of ene groups, the conjugation rate with thiol groups differ greatly from one to another according to the electron density of the 'ene' groups [47]. For a specific thiol, electron-rich enes polymerize much faster than electron-poor enes. It is accepted that reactivity of ene groups towards radical-mediated thiol-ene polymerization follows this sequence (Scheme 3B): norbornene > vinyl ether > vinyl ester > allyl ether > vinyl carbonate > acrylate > N-substituted maleimide > methacrylate. It is important to note that some of these groups (e.g., norbornene, maleimide) undergo ideal step-growth polymerization while other functional groups (e.g., vinyl ester, vinyl carbonate, acrylate, methacrylate) undergo a mixed-mode (step-growth and chain-growth) polymerization mechanism. More

information related to thiol-ene photo-click chemistry could be found in comprehensive reviews of Hoyle and Bowman [47, 48].

Synthetic hydrogel precursors

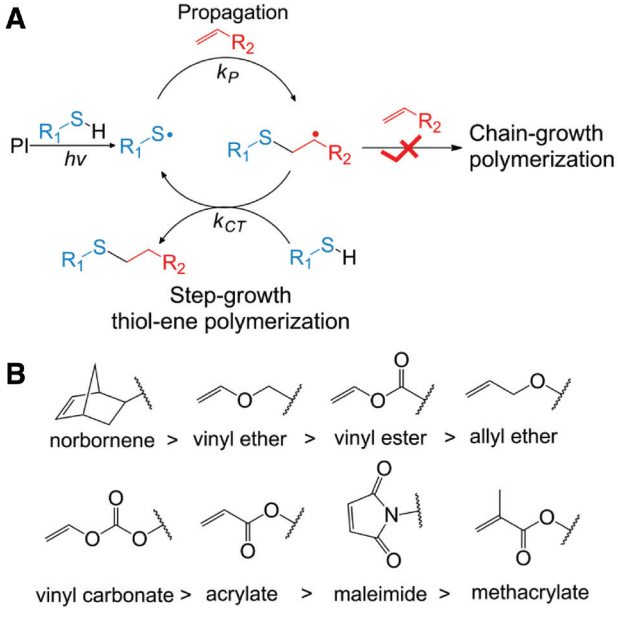
Synthetic hydrogels are increasingly explored for tissue engineering applications due to numerous advantages, such as easy access, cost efficacy, biologically blank state, and so forth. It is feasible to molecularly tailor the mechanical properties and degradation profiles of synthetic hydrogels and to match requirements for a specific application. Precursors of synthetic hydrogels include PEG, PVA, and poly(2-hydroxyethyl methacrylate) (PHEMA) (Figure 3). Considering that very few examples of PHEMA could be found in AMT applications, this section focuses on precursors based on PEG and PVA only.

Poly(ethylene glycol): PEG and its derivatives have proved extremely versatile for tissue engineering applications, such as non-fouling coatings that are resistant to protein adsorption [49]. In recent years researchers have witnessed the wide use of PEG in the field of hydrogels due to its superior hydrophilicity, biocompatibility, and negligible immunogenicity [50]. PEG-based hydrogel precursors could be synthesized by introducing multifunctional vinyl or clickable groups to the pendant hydroxyl groups (Figure 4). For example, high M_w (3–20 kDa) (meth) acrylated PEG (PEGDA/PEGDMA) can be synthesized in one step by reacting (meth)acryloyl chloride with pendant hydroxyl groups [51] while lower M_w ones (0.3–1 kDa) are commercially available.

Researchers have also developed bioerodible PEG hydrogels using photopolymerizable macromers that contain a PEG central core but terminate with oligomers of alpha-hydroxy acids such as PLA [13, 52–54]. Specifically, the PLA-*b*-PEG-*b*-PLA triblock copolymers were firstly synthesized via classical ring-opening polymerization. An excess of (meth)acryloyl chloride was then reacted with PLA-b-PEG-b-PLA. The degradation rate of formed hydrogels can be tailored from <1 day to up to 4 months by controlling the crosslinking density of the gel or by selecting the type and amount of α-hydroxy esters. It is noteworthy to mention the reactivity differences between acrylated PEG and methacrylated PEG. For instance, 2 min exposure time was required for the former while 10 min was needed for the latter [52, 53].

As mentioned before, although PEGDA are the most widely used hydrogel precursor in the biomaterials community, their irritancy and potential cytotoxicity of unreacted acrylate groups might preclude them from further clinical-relevant applications [35]. To address this challenge, we recently developed PEG-based vinyl ester (PEGDVE) [37]. It was demonstrated that cytotoxicity of PEGDVE was more than one magnitude lower than its PEGDA/PEGDMA analogues. Furthermore, it is important to note that degradation products of PEGDVE hydrogels should be based on non-cytotoxic PVA and PEG.

Although the photoreactivity of PEGDVE are not comparable to PEGDA due to the presence of abstractable hydrogens, we recently circumvented this problem by using the robust thiol-ene chemistry [37]. Based on systematic photo-differential-scanning-calorimetry (Photo-DSC) studies, we suppose that vinyl esters are less prone to chain-growth homopolymerization when compared to acrylates, presumably because the formed radicals lack of resonance stabilization. In contrary, the low resonance stabilization effect might explain why these radicals



Scheme 3 (A) Proposed mechanism of radical-mediated thiol-ene photopolymerization and (B) reactivity sequence of variable "ene" groups towards thiol-ene conjugation.

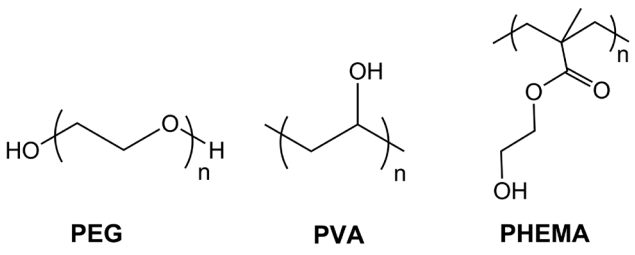


Figure 3 Examples of common synthetic hydrogels.

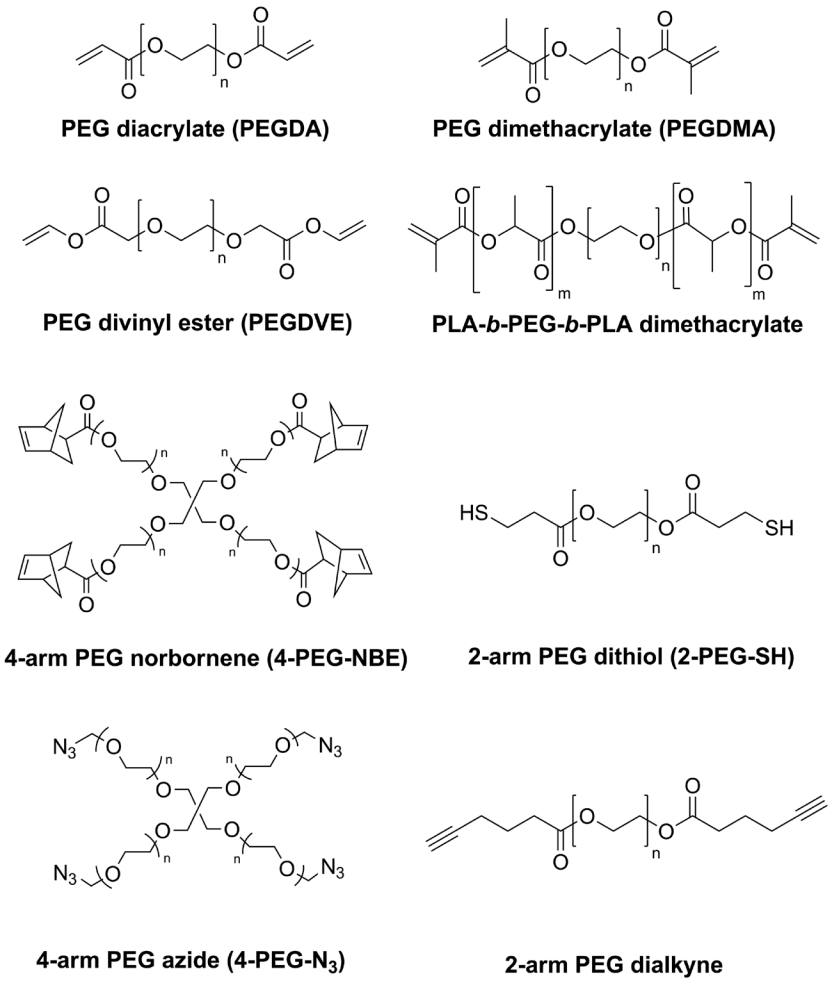


Figure 4 Chemical structures of PEG-based hydrogel precursors

show high reactivity towards chain transfer reactions with thiols. High reactivity and high conversion could be achieved in thiol-vinyl ester photopolymerizations.

Alternatively, PEG norbornenes are ideal hydrogel precursors in terms of high reactivity for thiol-ene step-growth photopolymerization. Fairbanks and Anseth demonstrated that thiol-norbornene photopolymerizations are suitable models to assemble hydrogels with CRGDS peptide in cell photo-encapsulation studies [46]. The robustness of thiol-norbornene click reactions even minimized the dose of photoinitiators required for cell-encapsulation and thus optimized the precursor's cytocompatibility. A recent study by McCall et al. proved that thiol-ene photopolymerizations are promising methods to form hydrogels for protein encapsulation while maintaining most bioactivities of proteins [55].

Poly(vinyl alcohol): PVA are hydrophilic polymers originated from partial hydrolysis of poly (vinyl acetate). PVA-based hydrogels have been widely used in space filling and drug delivery systems because of their superior biocompatibility (FDA-approved). In addition, PVA hydrogels are uniquely stronger than most other synthetic hydrogels [16]. Although aqueous PVA solutions can be physically crosslinked by crystallites formed by repeated freeze-thawing [56], the resultant hydrogels lack of stability at high temperature because of the melting crystallites. To get better stability, chemically crosslinked PVA hydrogels have been synthesized by using glutaraldehyde [57] or succinyl chloride [58]. However, these crosslinking agents are highly toxic and exclude the feasibility for in situ cell encapsulation applications.

In the last decade, researchers have extensively explored PVA-based hydrogels formed by photopolymerization in a minimally invasive fashion. Anseth and co-workers have developed photopolymerizable PVA macromers with varying degree of degradable ester linkages and reactive acrylate groups [59]. In this work, an acrylate terminated ester-anhydride was prepared and

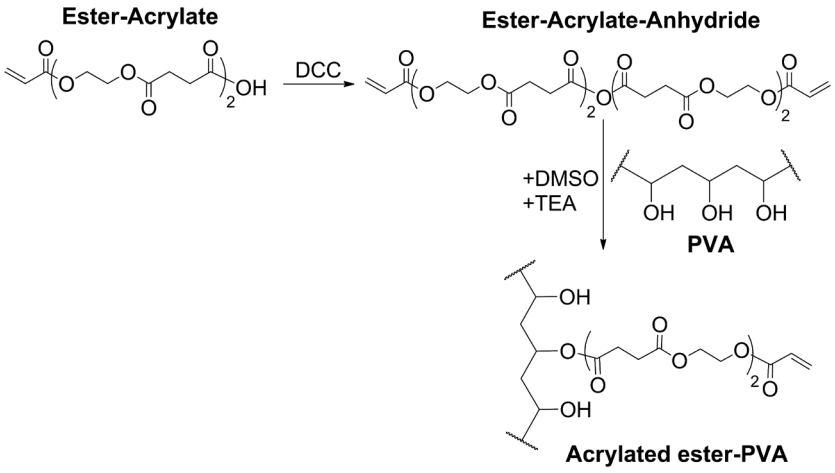


Figure 5 Synthetic scheme of acrylated ester-PVA.

subsequently reacted with pendant hydroxyl groups of PVA to form the desired acrylated ester-PVA (Figure 5). Synthetic hydrogels with tunable degradation profiles are important for most biodegradable hydrogel scaffolds in tissue engineering. To this end, Martens et al. have copolymerized methacrylated PEG and acrylated PVA macromers that are hydrolytically degradable to produce hydrogels [60]. In this work, it was found that degradation rates of copolymerized PEG-PVA gel were faster than the rates of PEG gel, but slower than the rates of PVA gel.

Although PVA hydrogels are not cell-adhesive like most other neutral polymers, they can be rendered cell-adhesive by covalently conjugating biological molecules (e.g., RGD motif, fibronectin) to the pendant hydroxyl groups of PVA (Figure 6). For instance, Schmedlen et al. have prepared bioactive PVA hydrogels that were not only non-cytotoxic but also supported the attachment and spreading of fibroblasts [61].

In this study, PVA was firstly modified with methacrylamide (for photocrosslinking) and amino groups (for RGD conjugation) via reaction of methacrylamidoacetaldehyde dimethyl acetal and aminobutyraldehyde diethyl acetal. Acetylated RGDS peptide was then immobilized on the modified PVA through an aminolysis reaction. It was found that the extent to which these hydrogels can support cell attachment and spreading was dose-dependent on the immobilized RGDS. In another work, Nuttelman et al. have covalently attached fibronectin on the surface of preformed PVA hydrogels through carbonyl diimidazol (CDI) chemistry [57]. The biofunctionalized PVA hydrogels drastically increased the rate of NIH3T3-fibroblast attachment and proliferation, and promoted 2D cell migration.

Naturally-derived hydrogel precursors

When it comes to biocompatibility issues, naturally-derived hydrogels are generally thought to be advantageous over synthetic hydrogels since natural gels may offer better biochemical and biological cues to surrounding cells. Most naturally-derived hydrogels are either components of natural ECM or provide similar properties that mimic the natural situations. Researchers have used a variety of naturally-derived materials, including collagen, gelatin, hyaluronic acid (HA), chitosan, alginate, and so forth [62–65]. Importantly, collagen is the major component of the

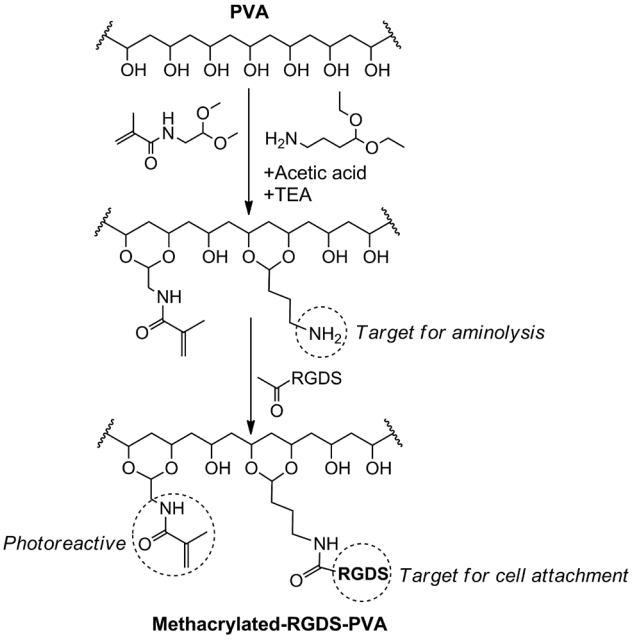


Figure 6 Synthesis of methacrylated-RGDS-PVA.

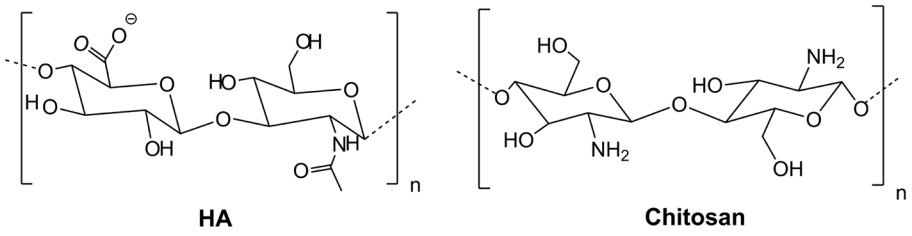


Figure 7 Structures of common substrates for naturally-derived hydrogels: hyaluronic acid (HA) and chitosan.

proteins in the ECM of human tissues and takes up approx. 25% of the total protein mass. Furthermore, HA is another important ECM component and widely distributed throughout connective, epithelial, and neural tissues. Chemically, HA is a non-sulfated, anionic glycosaminoglycan consisting of repeating disaccharide units. Similar to HA, both chitosan and alginate are linear, hydrophilic polysaccharides with superior biocompatibility (Figure 7). They have been shown to present favorable in vivo characteristics and thus they have been widely accepted as excellent hydrogel precursors for various tissue engineering applications [66–68].

Collagen: Collagen is one type of fibrous proteins and the most abundant protein in mammals. Collagen has a triple helical structure formed by polypeptide helices made of Gly-X-Y repeating units [69]. As a major structural protein of most ECM, Type-I collagen plays important passive roles in stress-bearing by providing skin, bone, tendon, and ligament with their characteristic tensile strengths. Since Type-I collagen covers a wide range of domains that could crosstalk with integrin receptors on the cell surfaces and other matrix proteins, collagen-based hydrogels are cell adhesive and favourable for cell migration [70]. Hydrogels based on collagen can be formed by either physically crosslinking treatments (i.e., irradiation, freeze-drying) [71, 72], or chemically crosslinking agents (i.e., glutaraldehyde, carbodiimide) [72, 73]. Yet, there are few examples of polymerizable collagen derivatives, due to the difficulty of collagen dissolution.

Gelatin: Compared to native collagen, gelatin is more cost-effective and has easier access. Gelatin products with different isoelectronic points can be prepared via acidic or alkaline denaturation [74]. Gelatin is derived from collagen Type I via heat denaturation and has gained increasing popularity in the biomaterials community. For this point gelatin hydrogels have been widely used as drug carriers in controlled delivery systems [75].

A general problem related to gelatin-based hydrogels is their poor mechanical properties which can be improved by physical or chemical crosslinking methods. Among them, photopolymerizable gelatin derivatives have received the most attention due to their great advantage for in situ cell photo-encapsulation. To render gelatin polymerizable, target functional groups could be introduced by modifying lysine units or glutamic/aspartic acid units (Figure 8). For instance, Van den Bulcke et al. have reported a derivative of gelatin modified with methacrylamide groups (Gel-MA) [76]. In this work Gel-MA was prepared through an amidation reaction between ε-amino groups of lysine units of gelatin and methacrylic anhydride. Gel-MA hydrogels have enabled in situ cell encapsulation by providing favorable cell viability and cell adhesion [77]. Klein, Malda, and co-workers exploited Gel-MA in combination with other photocrosslinkable derivatives of HA, alginate, chondroinite sulfate for cartilage tissue engineering applications [78, 79]. Notably, Ovsianikov et al. explored the two-photon polymerization of Gel-MA by using I2959 as photoinitiator [80]. Although proof-of-concept structuring succeeded, it is important to note that the photoreactivity of methacrylated macromers are generally too low for most photolithography-based AMT applications.

To tackle the reactivity issue, acrylamide derivative of gelatin (Gel-AC) was developed in the Liska group (unpublished results). Although photo-rheometry studies proved that Gel-AC indeed possessed high photoreactivity, cell-encapsulation attempts revealed that Gel-AC induced a drastic decrease of MG63 cell viability. It is presumably due

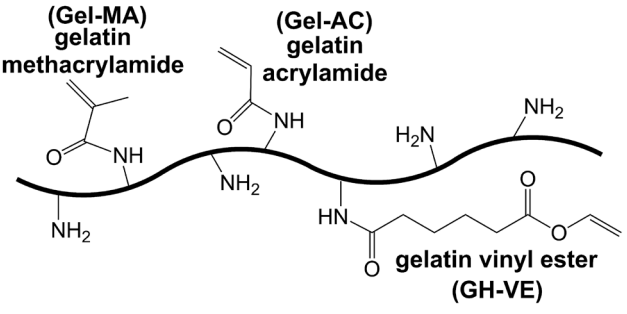


Figure 8 Structures of photopolymerizable gelatin derivatives: gelatin methacrylamide (Gel-MA), gelatin acrylamide (Gel-AC) and gelatin vinyl ester (GH-VE) for naturally-derived hydrogels.

to the Michael-addition side reactions between acrylamide moieties and amine groups of cell-surface proteins. To achieve a good trade-off between high reactivity and low toxicity, Qin et al. recently reported the first hydrogel precursor with vinyl ester groups, i.e., gelatin vinyl esters (GH-VE), which was proved to be low cytotoxic and enzymatically degradable [81]. Specifically, low MW gelatin (GH) was functionalized with VE groups through an aminolysis reaction between ε-amino groups of lysine units and an excessive amount of divinyl adipate (DVA). MTT assay revealed that GH-VE macromer presented negligible cytotoxicity on MG63 cells, which suggests that the modification did not negatively influence the cytocompatibility of the substrate. Although ^{1}H-NMR and MALDI-TOF analysis showed that there are more than two VE groups per macromer, the photoreactivity of GH-VE towards homopolymerization was quite limited. To overcome this limitation, the more efficient thiol-ene click chemistry was adopted in this study [81].

Since water-soluble multifunctional macrothiols are not commercially available, the authors selected reduced BSA (BSA-SH) as a model macrothiol to donate multiple free cysteine units for subsequent photo-click reactions with vinyl ester groups of GH-VE (Figure 9). The extent of cysteine units was controlled by changing the stoichiometry between disulfide bridges and reducing agent such as tris(2-carboxyethyl)phosphine (TCEP). It was found that both photoreactivity and crosslinking density of these hydrogels were dependent on the thiol/ene ratio, as proved by photo-rheometry and swelling studies [81]. Importantly, it was shown that the extent of cell attachment on these protein-based hydrogels could be regulated by the relative ratio between GH-VE (adhesive) and BSA-SH (repellent). By using a highly efficient initiator (i.e., WSPI) and two-photon thiol-ene lithography, it was feasible to produce complex hydrogel constructs in 3D at a writing speed as high as 50 mm/s.

Hyaluronic acid: HA or hyaluronan is a linear polysaccharide consisting of D-glucuronic acid and D-N-acetylglucosamine as repeating units. As the major component of ECM, HA plays an important role in wound healing, cell signaling and tissue morphogenesis. Due to its unique biological functions, HA has been widely used in biomedical applications such as for treatment of osteoarthritis and regeneration of vocal cord [82, 83]. Nevertheless, the native HA suffers from poor mechanical properties and short half-lives in vivo which makes it inappropriate for certain clinical applications such as articular cartilage regeneration. As such, many researchers have explored a variety of methods to chemically modify HA with polymerizable groups in order to obtain covalently crosslinked hydrogel networks [84–86]. These methods could effectively improve the mechanical strength and prolong the half-lives of HA hydrogels in vivo.

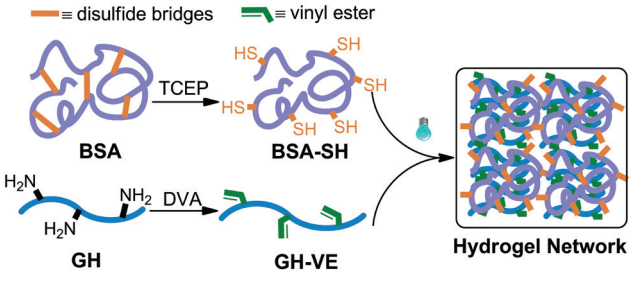


Figure 9 Schematic showing the hydrogel formation via thiol-ene photopolymerization using GH-VE and BSA-SH as precursors.

The principle targets for chemical modification in HA are primary and secondary hydroxyl groups and the carboxyl groups (Figure 10). For instance, photopolymerizable methacrylated HA (HA-MA) were synthesized by reacting either methacrylic anhydride (or glycidyl methacrylate) with the primary hydroxyl groups in HA [87, 88]. Khademhosseini et al. have explored the micromolding-based photopatterning of HA-MA hydrogels for cell encapsulation applications [89]. In this work, the irradiation conditions were harsh (300 mW/cm^2 UV light, 3 min), presumably as a result of the limited reactivity of HA-MA. Recently, Khetan and Burdick developed acrylated HA (HA-AC) [90]. Specifically, 2-hydroxy ethyl acrylate was firstly conjugated with succinate followed by a further esterification reaction with HA. However, the use of HA-AC in AMT has not been reported yet.

While (meth)acrylated HA has been developed as photocrosslinkable hydrogel precursors, several optimizations could be addressed. Like most methacylate-based monomers, the methacrylated HA lack of photoreactivity which poses a challenge for potential uses in AMT applications. Additionally, it is well known that the efficient thiol-ene click chemistry could not improve the reactivity of methacrylates [47, 48, 91]. Although acrylated HA exhibits higher photoreactivity, the irritancy and potential cytotoxicity of acrylates are very likely problematic for FDA-approval. Very recently, Qin et al. reported a HA alternative with pendant vinyl ester groups (HA vinyl esters, HA-VE) to tackle these limitations [92]. A series of HA-VE with tunable degree of substitution were synthesized via lipase-catalyzed transesterification. The development of HA-VE was based on previous work: 1) vinyl esters are much less cytotoxic than their acrylates references [35], and 2) the moderate photoreactivity of vinyl esters could be greatly improved by using the robust thiol-ene chemistry [37]. Owing to the unique molecular design, degradation products of HA-VE hydrogels through hydrolysis are PVA and adipic acid (both FDA-approved). Importantly,

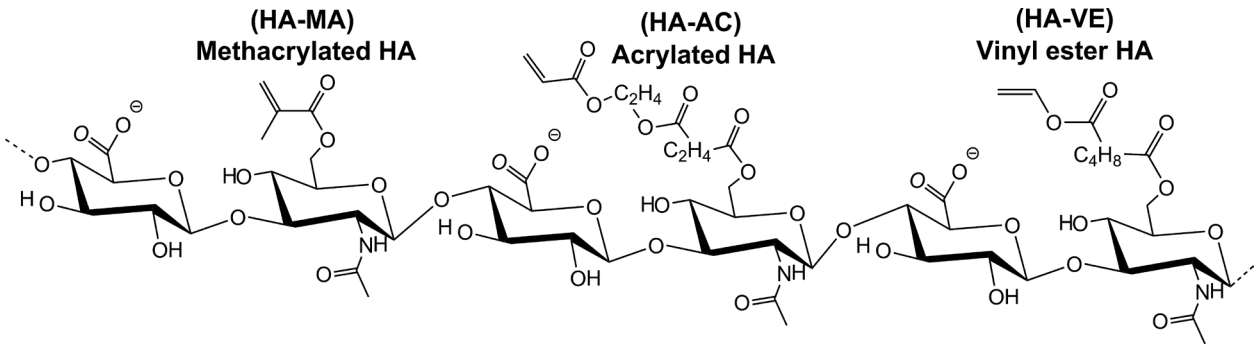


Figure 10 Chemical structures of hyaluronic acid (HA) derivatives: HA-MA, HA-AC and HA-VE.

the cytotoxicity of HA-VE on L929 fibroblasts was found to be significantly lower than that of HA-AC ($p<0.01$) and HA-MA ($p<0.05$) [92]. Although the reactivity of HA-VE towards homopolymerization was insufficient for 2PP, it was proved that thiol-ene chemistry could substantially improve its reactivity. This optimization enabled 2PP microfabrication of a complex hydrogel construct of HA-VE with μm-scale accuracy and high writing speed (50 mm/s).

Additive manufacturing of 3D hydrogels

As discussed before, hydrogels are superior scaffolding materials that mimic various aspects of the native ECM, including the natural architecture of tissues. From the engineering point of view, hydrogel scaffolds should be designed with "solute transport" properties, i.e., free transport of nutrients and wastes. Although small molecules such as glucose and oxygen can freely diffuse into and out of the gel networks, the transport of most biomacromolecules may largely depend on various parameters, including molecular weight and hydrodynamic radius of the solute, mesh size of the networks, and pore size of hydrogel scaffolds. The network mesh size is dictated by macromer size, macromer composition and crosslinking density of the gel network [13]; while the macroscopic porosity is dependant on the scaffold design and requirements for a specific application.

Importantly, researchers have proven that scaffolds with tuning porosity are critical for the integration of implanted scaffolds with host tissue. A milestone work of Brauker et al. has shown that engineered scaffolds with pore sizes between 0.8 and 8 μm always permitted the complete penetration of the host cells and neovascularization, regardless of the chemical nature of scaffolding material [93]. As one type of scaffolds, hydrogels follow the same principle. For example, hydrogels with porosity at the same range have enabled the infiltration of host cells as evidenced in the long-term success of PHEMA hydrogels for cornea replacement [94].

To fabricate porous hydrogel scaffolds, researchers have explored a variety of techniques, including particulate leaching, gas forming and so forth. Although such traditional techniques have shown the feasibility to generate porous structures, precise control over the pore geometry, size or interconnectivity are challenging to achieve, not to mention complex structures. In recent years, computer-aided-design (CAD)-based additive manufacturing technologies (AMT) have gained increasing popularity in the tissue engineering field. Importantly, the integration of clinical imaging data with AMT may allow the creation of a patient-specific construct for a specific tissue defect. Among these AMT techniques, photolithography-based AMT techniques have shown the greatest promise because of the use of light. Through various AMT devices light could be selectively delivered to targeted regions and induce local phase changes, thus providing full spatiotemporal control. For instance, digital light processing (DLP) technique could fabricate hydrogel structures that are uniform in z-direction but vary in structure in the x-/y-directions [95].

In recent years, cell biologists have gained substantial evidence on the significance of the third dimension in cell culture. Compared to 2D hydrogels, culturing conditions in 3D hydrogels represent the cell morphology and physiology that are mimicking the in vivo conditions much better [96]. In addition, the milestone work of Chen and Whitesides elegantly proved that the geometrical control of cell shape is effective to control cell proliferation and apoptosis [97].

Together, realization of 3D hydrogels with reproducible macroscale (porosity) and microscale features (local

geometry) is increasingly important for current tissue engineering research. To this end, two-photon polymerization (2PP) approach appears to be an ideal solution for creating 3D hydrogels with controlled porosity and user-defined microscale geometry. Here an overview on recent advances in photolithography-based additive manufacturing of 3D hydrogels is provided with an emphasis on the 2PP technique.

Single-photon lithography

According to the working principle, lithography-based AMT can be further divided into single-photon (SP) and multiphoton techniques. Mask-based SP photopatterning techniques offer several benefits, mainly because they are fairly easy to use. Bhatia and co-workers have developed a method to create 3D hydrogel constructs via iterative 2D photopatterning (Figure 11A) [99]. In this study, PEGDA-based hydrogel microstructures (Figure 11A) composed of overlaid layers were produced with a spatial resolution on the order of hundreds of microns. In a later study, the same group has successfully translated this approach into the fabrication of a functional 3D hepatic construct (Figure 11B) with complex internal architectures [98].

Since the presence of mechanical gradients within hydrogels plays an important role in regulating cell migration and other functions, researchers have explored photolithographical approaches to create 3D hydrogels with patterned stiffness [100–104]. West and co-workers have shown that mask-based UV patterning provided PEGDA hydrogels with spatially patterned and tunable stiffness [101]. In this work the stiffness gradient was realized by variation of crosslinking density of the hydrogel, which was further dictated by PEGDA macromers of varying molecular weight and controlled mixing and photopatterning procedure.

Another single-photon AMT technique is DLP where a pattern of UV or visible light is projected onto a layer of photopolymers positioned on top of a programmable stage. 3D hydrogel structures can be accordingly stacked up in a layer-by-layer manner. A typical DLP device (Figure 12A) comprises four main parts: 1 – light source, 2 – digital mirror device, 3 – optics, and 4 – building platform.

Chen, Roy and co-workers have created PEGDA-based hydrogel constructs with open structures and viable stromal cells (Figure 12B) for stem-cell-differentiation studies [105]. It has to be noted that there is a trade-off between build size and resolution when using DLP, since the number of pixels on the dynamic masks (a.k.a. digital mirror device) is typically around 2000×1000 pixels. When choosing a typical pixel size of 30 μm, the corresponding build size is then around 60×30 mm^2. Improving the resolution therefore leads to a reduction in build size.

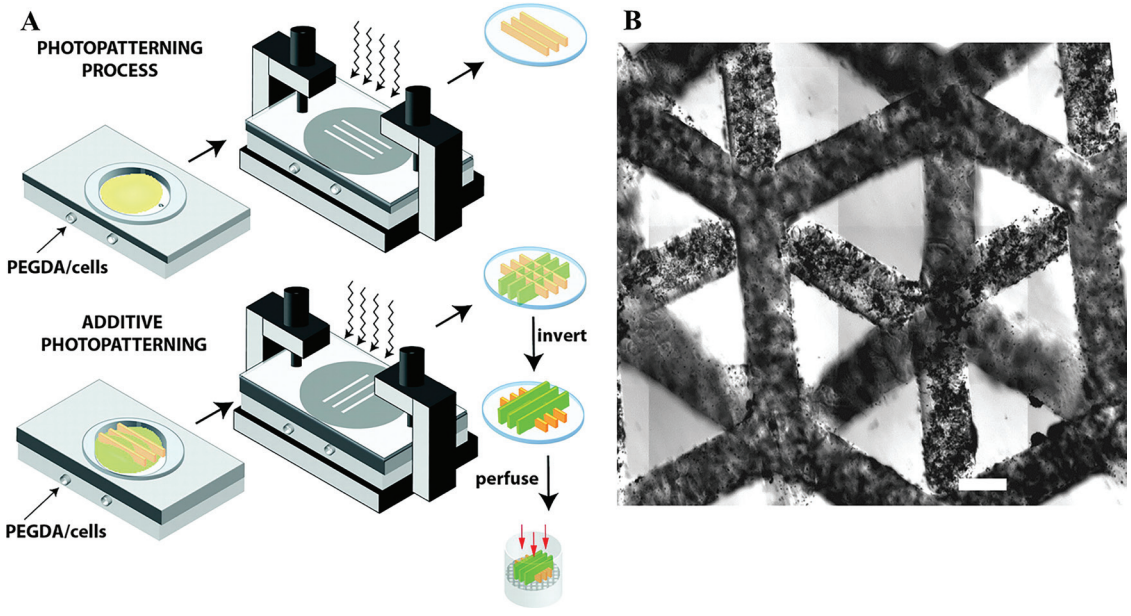


Figure 11 (A) Schematics of mask-based single-photon additive photopatterning and (B) high-magnification photomicrograph of a 3D hydrogel construct with interconnected porosity for mimicking a hepatic construct. (Scale bar: 500 μm, Reprinted with permission, Copyright FASEB Journal [98].)

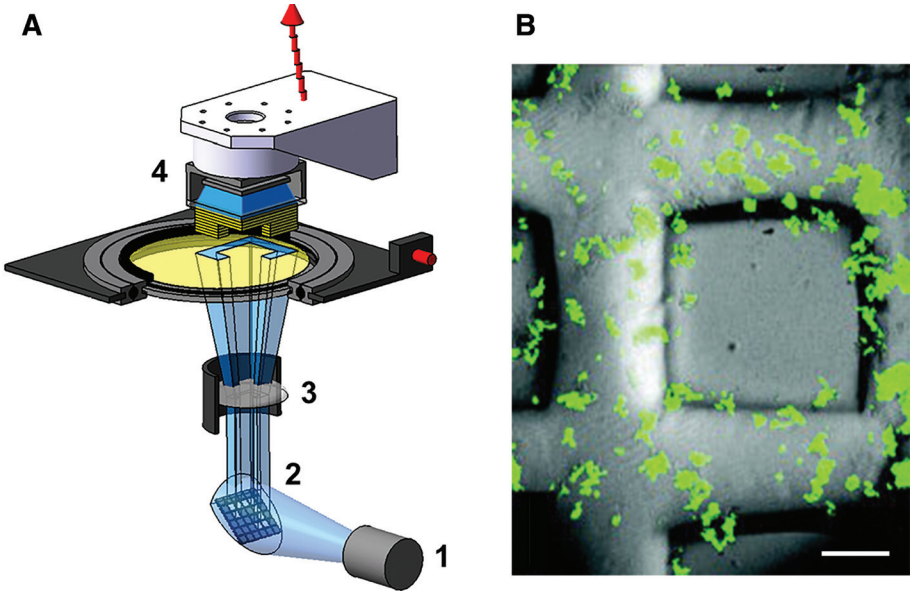


Figure 12 (A) Schematic showing a DLP system and its main functional parts: 1 – light source, 2 – digital mirror device, 3 – optics, 4 – building platform; and (B) overlaid micrograph of DLP-fabricated PEGDA scaffolds encapsulated with viable stromal cells (green) after 24 h incubation. (Scale bar: 200 μm, Reprinted with permission, Copyright Wiley [105].)

Multiphoton lithography

The success of hydrogels lies on their capability to establish the crosstalk between host cells and the gel matrix [10]. As the understanding of ECM biology goes deeper, rapid construction of 3D hydrogels with sub-cellular features becomes increasingly important. Early work of Hubbell and co-workers has comprehensively highlighted the importance of varying matrix effects on dictating cell fate [8, 106]. It is accepted that the length scale at which cells can sense and respond to their surrounding signals is on the scale of 1 μm or less. Therefore, from an engineering point of view, creating cellular microenvironment with spatial resolution on a (sub)micron-scale is essential for achieving biomimetic control over cell behaviors. As mentioned before, conventional lithographic techniques, relying on single-photon absorption, provide limited spatial resolution on the order of a few tens of microns at best. In contrast, multiphoton lithography (or multiphoton processing) has recently proven to be a very promising technological platform for overcoming this limitation [11, 80, 107–111].

Multiphoton processing techniques rely on the multiphoton absorption of laser radiation within the volume of a suitable photosensitive material [11, 109]. For simplicity, the following discussion is focused on two-photon absorption (TPA) and TPA-relevant reactions. TPA is a non-linear optical process where two photons of a given wavelength absorbed nearly simultaneously (~10^{-16} s) [109]. Since TPA-excited chemical events are highly localized within the focal volume of a laser beam, high spatial resolution can be achieved. By focusing a femtosecond near infrared laser within the precursor solution, hydrogels with user-dictated shapes can be produced with true geometrical control in 3D. When compared to SP lithography (mostly UV light), two-photon lithography (near infrared light) operates at higher penetration depth and is therefore able to produce 3D structures within the volume of the sample without the necessity to deposit material layer-by-layer. Another advantage of using near IR light is low probability of cell and tissue damage, making it promising for potential clinical-relevant applications [32].

In the last few years, researchers have sought to utilize the two-photon processing for creating customized 3D hydrogels in different ways, including two-photon-induced biofunctionalization of preformed 3D hydrogel networks and direct shaping of 3D hydrogel constructs via two-photon-induced polymerization (2PP).

Two-photon-induced biofunctionalization

In a natural environment, cells are embedded in the ECM, which is a complex 3D hydrogel network containing a variety of biochemical and biophysical signals. Within the ECM context, spatial and temporal access of

integrin ligands, morphogens and growth factors regulate a number of biological processes [4, 112–114]. For instance, formation of new blood vessels (neovascularization) is dictated by the highly delicate biochemical and biophysical environments of different cell types, and in particular the spatial and temporal presentation of a number of growth factors [115–117]. An excellent overview of channeled hydrogels for neovascularization goes to a very recent review by Redl and co-workers [118]. Apart from creating channeled hydrogel structures, spatiotemporal patterning of bioactive molecules in preformed 3D hydrogel constructs is an extremely useful tool to precisely control cell behavior. West and co-workers have shown that the two-photon patterning of RGD motifs in PEG-based hydrogels could effectively confine cell adhesion and cell migration in the area functionalized with RGD [119, 120]. In these studies hydrogel pellets were firstly formed by SP photopolymerization and then soaked in a solution of photoinitiator and acrylated RGD peptides.

Besides, researchers have also explored the two-photon uncaging concept for biofunctionalization of 3D hydrogels. In a recent study of Shoichet and co-workers, multiple growth factors were sequentially immobilized within agarose hydrogels (Figure 13) [121]. Specifically, agarose hydrogel matrices were firstly activated with carbonyldiimidazole (CDI) and then modified with 2-nitrobenzyl-protected cysteines [122]. Upon two-photon irradiation, cysteine pendant groups were liberated for thiol-ene conjugation with maleimide-containing barnase/streptavidin. Finally, growth factors terminated with orthogonal counterparts (barstar/biotin) were immobilized via affinity-binding within the 3D pattern.

A recent study by Ovsianikov et al. has established that bioorthogonal groups such as azides can be precisely immobilized within PEG matrices via 3D photografting [123]. Specifically, the grafting process was based on the decomposition of a commercially available aromatic diazide under three-photon excitation (3PA) and subsequent nitrene insertion, finally leaving pendant azide groups for further functionalization. However, it is noteworthy that three-photon absorption (3PA) is often less efficient and thus requires much higher laser intensity than TPA due to the lower probability of 3PA [124]. Li et al. recently developed a novel fluoroaryl azide compound (FAF-3) for efficient TPA grafting (Figure 14A). The rational molecular design provides FAF-3 with a large TPA cross section (178 GM at 800 nm) while the alkyne tail facilitates further bioorthogonal conjugation. Specifically, FAF-3 was precisely grafted within PEG matrices via TPA-induced photolysis and subsequent nitrene insertion. Since the grafting reactions are highly localized in a confined volume within the laser focal spot, arbitrary Yin-Yang pattern of alkyne groups (Figure 14B) was created in the PEG matrices with high spatial resolution (~3.6 μm) [125].

In addition, ultrafast scanning (~550 mm s^{-1}) was achieved due to the highly efficient grafting reactions. To realize 3D site-specific functionalization, a model compound (i.e., red fluorophore terminated with azide group) was further conjugated on the "clickable" Yin-Yang pattern through CuAAC reactions. After that the Yin-Yang pattern changed from green to red, indicating the success of site-specific functionalization. This work shows great potential for further biologically-relevant studies where matrix biofunctionalization is necessary. In theory, any

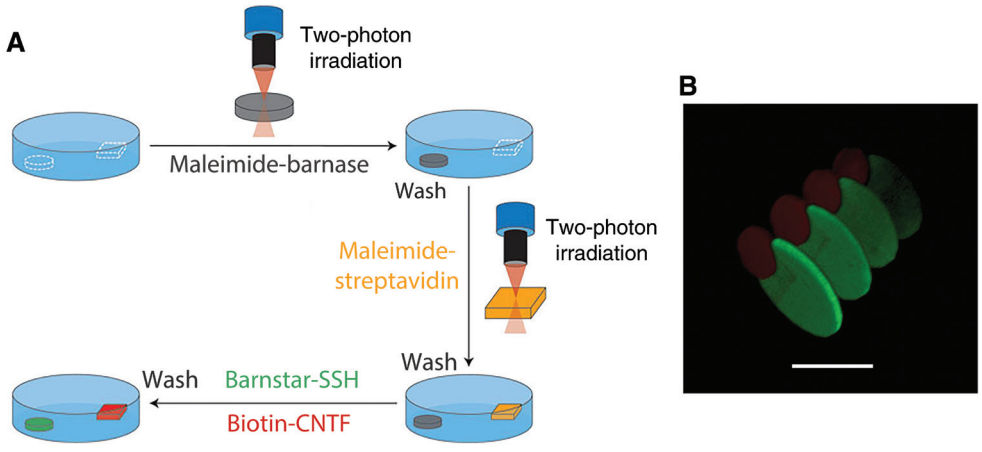


Figure 13 Two-photon biofunctionalization of alginate gels with various growth factors: (A) schematics of the protocol and (B) 3D projection of a confocal micrograph illustrating the patterned volumes and user-defined localization of two growth factors. Scale bar, 100 μm. (Reprinted with permission, Copyright Nature Publishing Group [121].)

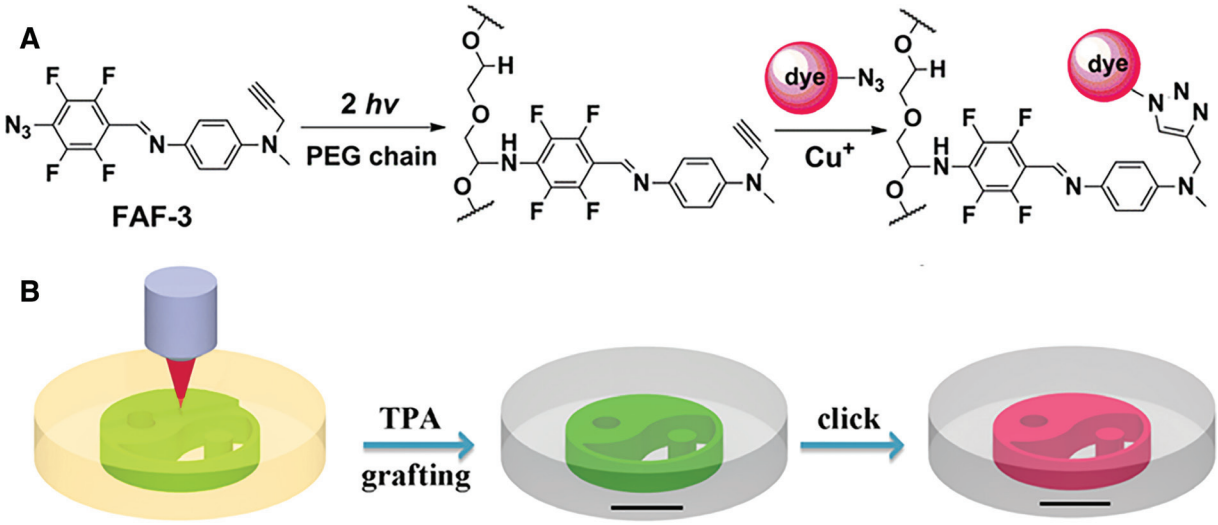


Figure 14 3D site-specific functionalization of PEG matrices via photografting: (A) grafting mechanism and subsequent CuAAC reaction; and (B) schematics of the grafting procedure. (Scale bar: 50 μm [125].)

bioactive molecule of interest (e.g., VEGF-2) with alkyne groups could be readily incorporated through CuAAC-based click reactions.

Two-photon-induced polymerization

Although arbitrary biochemical patterns can be created via two-photon-induced biofunctionalization, these methods are intrinsically limited by the preformation of hydrogel pellets and thus not suitable for fabrication of complex structures. In contrast, 2PP strategy presents great promise to fabricate 3D hydrogels with complex geometry. By scanning focused pulsed laser in a hydrogel precursor solution, 3D hydrogels with user-dictated structures can be fabricated with micrometer-scale resolution. Again, high resolution is accessible because of 2PP processes are localized in the focused volume. Although radical-mediated 2PP is fast, it is important to note that the resolution of 2PP is also influenced by: 1) optical parameters of the 2PP setup; 2) how far the free radicals can diffuse from the reaction center; and 3) the extent to which oxygen could quench the reactions.

Direct fabrication of 3D hydrogels via 2PP primarily relies on the access to cytocompatible hydrogel precursors with high photoreactivity. Synthetic hydrogel precursors that are suitable for 2PP fabrication are mostly based on acrylated macromers like PEGDA. Torgersen et al. reported the first example of 2PP fabrication of hydrogels in the presence of living organisms [32]. In this work, viable *Caenorhabditis elegans* were captured in a 3D woodpile hydrogel structure of PEGDA. Besides, Ober et al. reported the use of PEGDA in combination with 2-hydroxyethyl methacrylate (HEMA) for 2PP microfabrication [126]. Käpylä et al. recently reported the 2PP of (meth)acrylated poly(α-amino acid)s in comparison with commercial PEGDA (M_n=575, 10000 Da) [127]. These new precursors were applicable for 2PP microfabrication in a wide processing window. Stable and well-defined hydrogel microstructures were fabricated with 80% water content by using I2959 as the PI. However, cytocompatibility of these hydrogel systems were not reported in this study.

Alternatively, naturally-derived hydrogel precursors have also been used for 2PP fabrication of 3D hydrogels. For instance, Ovsianikov et al. reported the 2PP microfabrication of 3D hydrogel constructs with high resolution (10 μm) and defined porosity by using photopolymerizable methacrylated gelatin (Gel-MA) [80, 128]. It was found that Gel-MA enabled favourable cell-matrix interactions with mesenchymal stem cells, including proliferation, adhesion and osteogenic lineage (Figure 15) [128]. Very recently, Ovsianikov et al. reported the first example of 2PP processing of cell-containing hydrogel constructs [129]. In this study, Gel-MA and highly efficient two-photon PIs were exploited as the photosensitive precursor materials. After the 2PP encapsulation of MG63 cells in Gel-MA, cell damage was observed within the laser-exposed region though a small percentage of cells were found to be viable in the vicinity to the exposed region. Interestingly, control experiments (i.e., no PIs) revealed that the same laser radiation conditions for 2PP seemed not to induce cell damage. The authors thus propose that the localized cell damage during 2PP could be attributed to cytotoxic species such as initiating radicals and reactive oxygen species.

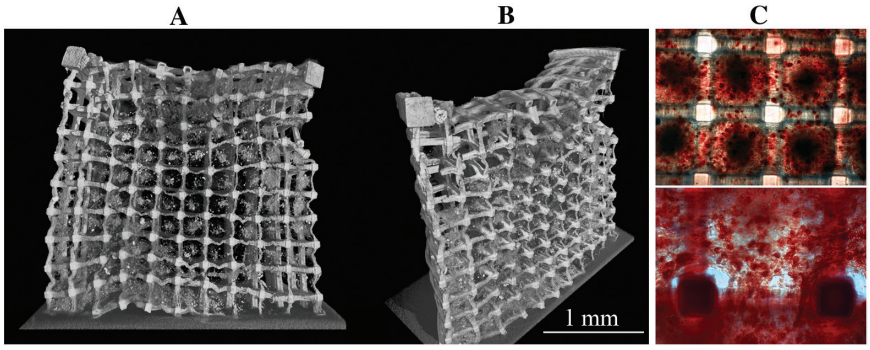


Figure 15 Osteogenic differentiation of mesenchymal stems cells (at day 20) on gelatin scaffolds produced by two-photon polymerization (2PP) technique: (A) and (B) Micro-CT images showing crystal-like mineral deposition within the pores; (C) alizarin red staining confirming calcific deposition by cells of an osteogenic lineage. (Reprinted with permission from Woodhead Publishing [128], scale bar: 1 mm.)

Besides chain-growth 2PP, Liska and Stampfl groups have recently explored the robust thiol-ene click chemistry for 2PP microfabrication of 3D hydrogels (Figure 16) [81, 92]. As depicted in Figure 9, vinyl ester derivative of gelatin (GH-VE) and reduced albumin (BSA-SH) were developed as the "ene" and "thiol" macromers. By integrating thiol-ene photo-click chemistry and two-photon lithography, it was feasible to simultaneously pattern two proteins in 3D at high writing speed (50 mm/s) and produce a 3D hydrogel construct with complex geometry with micrometer-scale resolution.

Summary and outlook

In summary, progressive integration of hydrogel chemistry, photopolymerization and varying AMT techniques has enabled the creation of 3D bioactive hydrogels for specific applications. It is critical to mention that tissue engineering of complex tissues or organs is still a big challenge. However, this challenge may be addressed from different aspects in the next stage of engineering evolution.

From the material point of view, water-soluble photoinitiators continue to be the key element of photolithography-based AMT. Besides water solubility, appropriate absorption wavelength (>350 nm) and high initiating efficiency will be a golden standard for the design of next generation of photoinitiators. Furthermore, cytotoxicity of photoinitiators and especially photoinduced cellular damage due to reactive oxygen species (ROS) poses a big challenge for AMT hydrogel fabrication in the presence of cells. This problem might be circumvented by incorporating ROS scavengers into the hydrogel formulation.

In addition, the selection of optimum gel precursor (natural or synthetic origin) remains the top concern of most researchers. Synthetic polymers are advantageous for most AMT techniques because of cost-efficacy and controllable property. But the use of synthetic polymers often necessitates the incorporation of bioactive motifs to foster favorable cell-material interactions. In comparison,

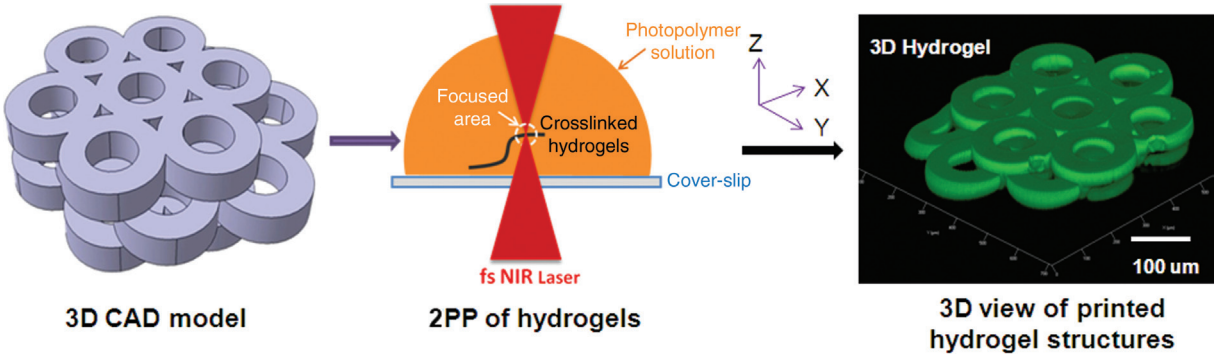


Figure 16 Illustration for 2PP microfabrication of 3D hydrogel scaffolds with well-defined architectures using protein-based hydrogel precursors [81].

the performance of naturally-derived polymers largely depends on the number of polymerizable groups which is further related to both the substrate chemistry and modification strategy. However, naturally-derived polymers often provide superior cytocompatibility. On the other hand, cytocompatibility of gel precursors and gel formation chemistry has gained increasing attention in recent years since whether cells can survive the hydrogel fabrication process is a critical parameter. We reason that the toxicity issue related to acrylate chemistry can finally be circumvented by using alternative vinyl ester chemistry since vinyl esters are much less irritant while providing PVA (FDA-approved) as the degradation products. Besides, gel formation chemistry has been extended to bioorthogonal reactions where light-triggered click reactions hold the most promise. In particular, radical-mediated thiol-ene reactions represent a number of advantages for potential integration with AMT hydrogel platforms, including spatiotemporal control, robust kinetics and mild reaction conditions.

It is well established that varying photolithography-based AMT techniques allow the fabrication of hydrogel scaffolds with appropriate macrostructure (porosity) and micro-scale patterns of biochemical, mechanical and gradient properties. But it is important to note that each of the reviewed AMT techniques may have pros and cons. For instance, DLP appears to be a promising technique for layer-by-layer assembly of a large 3D hydrogel construct whereas currently it is unable to achieve feature sizes below 30 μm. In contrast, 2PP seems to be the most promising technique to engineer hydrogels with (sub) cellular features. However, a major challenge related to 2PP is the long processing time required to fabricate a hydrogel construct with clinically-relevant sizes. The processing time can be shortened either by increasing the setup scanning speed or by improving the robustness of gel formation chemistry.

In the future, the fusion of rationally-designed hydrogel precursors, robust gel formation chemistry and optimized AMT platforms would help researchers to better understand cell-cell and cell-matrix interactions. The knowledge can be exploited for engineering complex tissues and designing therapeutic strategies for regenerative medicine.

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