1 Thiol-ene and photo-cleavage chemistry for controlled presentation of biomolecules in
2 hydrogels

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Abstract

Hydrogels have emerged as promising scaffolds in regenerative medicine for the delivery of biomolecules to promote healing. However, increasing evidence suggests that the context that biomolecules are presented to cells (e.g., as soluble versus tethered signals) can influence their bioactivity. A common approach to deliver biomolecules in hydrogels involves physically entrapping them within the network, such that they diffuse out over time to the surrounding tissues. While simple and versatile, the release profiles in such system are highly dependent on the molecular weight of the entrapped molecule relative to the network structure, and it can be difficult to control the release of two different signals at independent rates. In some cases, supraphysiologically high loadings are used to achieve therapeutic local concentrations, but uncontrolled release can then cause deleterious off-target side effects. *In vivo*, many growth factors and cytokines are stored in the extracellular matrix (ECM) and released on demand as needed during development, growth, and wound healing. Thus, emerging strategies in biomaterial chemistry have focused on ways to tether or sequester biological signals and engineer these bioactive scaffolds to signal to delivered cells or endogenous cells. While many strategies exist to achieve tethering of peptides, protein, and small molecules, this review focuses on photochemical methods, and their usefulness as a mild reaction that proceeds with fast kinetics in aqueous solutions and at physiological conditions. Photo-click and photo-caging methods are particularly useful because one can direct light to specific regions of the hydrogel to achieve spatial patterning. Recent methods have even demonstrated reversible introduction of biomolecules to mimic the dynamic changes of native ECM, enabling researchers to explore how the spatial and dynamic context of biomolecular signals influences important cell functions. This review will highlight how two photochemical methods have led to important advances in the tissue regeneration community, namely the thiol-ene photo-click reaction for bioconjugation and photocleavage reactions that allow for the removal of protecting groups. Specific examples will be highlighted where these methodologies have been used to engineer hydrogels that control and direct cell function with the aim of inspiring their use in regenerative medicine.

Keywords: thiol-ene; hydrogel; patterning; regenerative medicine; photo-cage; immobilization

1. Introduction

Scaffolding matrices have emerged as promising platforms for the delivery of therapeutics and cells *in vivo* [1]. Matrices loaded with releasable biomolecules can be implanted *in vivo* to promote tissue regeneration by signaling to endogenous cells or to cells delivered along with the matrix [2]. A common strategy for controlling the release of biomolecules involves their physical entrapment within scaffolding matrices [3-5], causing many high molecular weight molecules, such as cytokines and growth factors, to slowly diffuse from the matrix where they can direct cellular migration, differentiation and proliferation to promote tissue regeneration [6]. For example, Hubbell *et al.* demonstrated the promotion of bone regeneration *in vivo* through implantation of a poly(ethylene glycol) (PEG) hydrogel that was designed to elute bone morphogenetic protein 2 (BMP-2) to the surrounding tissues as the gel degraded [7]. Diffusion controlled release systems are versatile and can be used to deliver large doses without any chemical modification of the biological signal. However, there is limited control over the release profile, especially when loading multiple biomolecules, and maintaining a physiologically relevant concentration over extended time periods typically necessitates supraphysiological loading that can lead to deleterious off-target side-effects [8]. Indeed, the high levels of BMP-2 loaded in scaffolds used for regenerating bone tissue via diffusion controlled delivery systems led to severe ectopic bone growth [9]. With the rising use of regenerative medicine inspired therapeutics, there is a significant need for new biomaterials that allow versatile and precise control over the delivery of multiple biomolecules, from small molecules to proteins, in a manner that maintains their activity and ability to signal to targeted cells.

Figure 1. A cell in its microenvironment. ECM proteins (shown as yellow rods) sequester biomolecules, such as growth factors and cytokines (shown as orange spheres). Cells engage these biomolecules through cell surface receptors to elicit signaling. Signaling can lead to cell spreading and proliferation, and the production of enzymes that enable the cell to remodel and degrade the ECM (ECM degradation sequences shown as orange stripes). Figure adapted from Kyburz *et al* [10].

1
2 In designing biomaterial platforms to direct cellular function, it is necessary to
3 consider the role that the extracellular matrix (ECM) plays in influencing cells *in vivo*
4 (Figure 1). Tissues are composed of cells and their associated ECM—the complex,
5 tissue-specific, three-dimensional structure that provides cells with the requisite
6 mechanical and biological signals for healthy function [6]. In native tissues, the ECM
7 sequesters soluble biomolecules [11], a process that functions to shield them from
8 proteolytic degradation [12]. Moreover, the bioactivity of many proteins, such as
9 transforming growth factor- β (TGF- β) and fibroblast growth factor (FGF), is potentiated
10 by affinity binding to the ECM [11-13]. This suggests that cells can interact with
11 biomolecules through the ECM directly and that the context in which a cell interacts with
12 biomolecules can affect its function. Thus, an emerging approach to mimic biomolecule-
13 ECM interactions is to use affinity binding [14, 15] or covalent immobilization [16] of
14 biomolecules to matrices.

15 In addition to how biomolecules are presented to cells, the choice in the
16 biomaterial scaffold itself can influence cellular function. In fact, there is a growing
17 appreciation for the role of biophysical cues, and their potential synergies with
18 biochemical cues, on numerous cell processes [17-19]. One striking example comes from
19 the Blau group, where they demonstrated that muscle satellite stem cells cultured on
20 hydrogels with mechanical properties similar to skeletal muscle engrafted more
21 efficiently and led to significantly more muscle regeneration than those cultured on gels
22 in which the cells could sense the stiffness of the underlying tissue culture polystyrene
23 (TCPS) [20]. Further, the authors discovered that satellite stem cells from aged mice
24 require both the appropriate biophysical (substrate stiffness) and biochemical (mitogen
25 activated protein kinase inhibitor) signals to efficiently reprogram them to a proliferative
26 phenotype capable of muscle repair [21]. Taken together, these results demonstrate how
27 both the context in which biomolecules are presented to cells and the scaffold on which
28 they are presented can affect a biological response.

29 The emerging role of scaffold interactions with cells and biomolecules have led
30 the field of regenerative medicine to focus on the design of ECM mimics capable of
31 delivering viable cells and biomolecules to promote healing *in vivo* [22]. Hydrogels hold
32 promise to fulfill these needs because they allow for complete user control over their
33 material properties [23]. Hydrogels are synthetic scaffolds formed by the crosslinking of
34 hydrophilic synthetic polymers or biomacromolecules such as poly(ethylene glycol) [24],
35 poly(vinyl alcohol) [25], collagen [26], hyaluronan [27], and alginate [28] to afford
36 matrices. Many mild crosslinking strategies have been developed, so hydrogels can be
37 synthesized directly in the presence of tissues [29], cells [30], and biologics [7]. Physical
38 or chemical crosslinks render the hydrogels insoluble, but they typically imbibe large
39 amounts of water [31]. The high water content imparts mechanical properties that are
40 similar to many soft tissues and transport properties that are desirable for delivering
41 signals to cells [32]. Numerous material properties can be **adjusted** by changing the
42 crosslinking density of the polymer network to engineer gels with properties of interest
43 for selected applications [33].

44 Of the many materials from which a hydrogel can be assembled, there has been
45 significant interest in PEG hydrogels. From an application standpoint, PEG is widely
46 used in clinical medicine, and from a fundamental standpoint, PEG minimizes non-

specific adsorption of proteins found in culture media and *in vivo* [34]. When designing systems to signal to delivered exogenous cells or endogenous cells, these properties are particularly advantageous and afford the user better control over the biological signals that are presented to cells. PEG hydrogels also allow one to directly study the effects of biological signals on cells without confounding results from biologically active hydrogel matrices, such as collagen or hyaluronan.

Figure 2. Bioorthogonal click reactions include the thiol-Michael reaction, strain-promoted azide-alkyne cycloaddition (SPAAC), oxime formation, inverse electron demand Diels Alder (IEDDA) reaction, and the radical-mediated thiol-ene reaction. R_1 and R_2 can represent either a biomolecule or a hydrogel backbone.

Since PEG itself is bioinert, strategies to functionalize PEG hydrogels are increasingly important and are the focus of this review article. PEG macromolecules are easily amenable to a wide variety of chemistries that can be used to attach biomolecules and impart biological signals within the hydrogel. Specifically, ‘click’ chemistries have been widely adopted within the field of regenerative medicine because they are mild, selective, high yielding, and possess rapid reaction kinetics [35]. Importantly, a subset of click reactions are bioorthogonal (i.e., inert to functional groups found in cells and free of toxic reagents and byproducts), so they can be performed in the presence of cells [36, 37]. The most commonly employed bioorthogonal click reactions include thiol-Michael conjugate addition [38], strain-promoted azide-alkyne cycloaddition (SPAAC) [39], oxime formation [40], and inverse electron demand Diels-Alder (IEDDA) reactions [41] (Figure 2). These click chemistries all occur spontaneously and irreversibly upon addition of biomolecules, and are highly useful for the formation of PEG hydrogels or for post-polymerization modification of hydrogels with pendant biological functional groups. An advantage of bioorthogonal click reactions is the homogenous nature of the reaction throughout the gel with minimal side reactions, but in some experiments and applications, it may be desirable to control the spatial distribution of biomolecules, reversibly tether multiple biomolecules, or release biomolecules at a later time. These caveats are

important when considering the fact that the native ECM is highly dynamic and its composition is regularly changing, especially during events such as wound healing [11, 42, 43].

As a result, light-mediated chemistry has emerged as a powerful methodology to incorporate biomolecules within hydrogels with precise user control, and two photochemical methods have recently emerged as leaders in regenerative medicine: thiol-ene photo-click chemistry [44] (Figure 2) and photocleavage reactions [45]. The thiol-ene reaction is a bioorthogonal click reaction between a thiolated molecule and an alkene [31] and photocleavage reactions function to unmask a functionality or release a biomolecule. Upon irradiation, photocleavable cages degrade to expose a reactive handle for subsequent bioconjugation or to restore the bioactivity of a biomolecule. Alternatively, photocleavable linkers degrade to release an immobilized biomolecule. Importantly, since both thiol-ene and photocleavage reactions require light to proceed, only locations in the hydrogel that are exposed to light undergo the photochemical process. Moreover, many wavelengths, light intensities, and irradiation times are cytocompatible and can be used for these reactions to proceed [46]. Thus, using photochemistry, complex patterning of biomolecules can be achieved where traditional bioconjugation strategies fail.

This review will highlight recent examples where photochemistry has been employed to control the presentation of biomolecules within hydrogels to direct cell function. Specifically, we first explore the thiol-ene photo-click reaction and its advantages in allowing patterning of both peptides and proteins. Yet proteins are inherently challenging to tether to hydrogels, especially in radical-mediated processes such as thiol-ene, so we also present specific examples where photocleavage reactions are employed to reveal functionalities for subsequent protein patterning. Finally, we examine how both of these strategies have been modified to achieve reversible patterning of biomolecules within hydrogels. We aim to present a review that inspires the field of regenerative medicine to consider how contextual presentation of biomolecules can influence their function, and we review herein two powerful photochemical methods to control the context in which biomolecules are presented to cells.

2. Strategies in photochemical patterning of biological signals

The context in which a biomolecule is presented to a cell can affect its resultant biological signal. Whether a biomolecule is presented in soluble form or bound to the ECM can potentially affect its rate of internalization (and thus stability) or its ability to cluster cell surface receptors [47]—both of which can alter the observed signal output. Indeed, many growth factors require direct interactions with the ECM for signal transduction. For example, FGF-2 binds to heparan sulfate, a component of the ECM, for bioactivity [12]. Consequently, an emerging strategy in regenerative medicine involves covalently tethering biomolecules to the hydrogel matrix to mimic biomolecule-ECM interactions. In doing so, biomolecules are protected from degradation and delivered to cells in a sustained manner at physiologically relevant concentrations [48]. Significant work has been directed toward tethering peptides and proteins uniformly within hydrogels. In such an approach, proteins are functionalized with a reactive handle and covalently tethered to hydrogels during polymerization or post-polymerization via click chemistry (Figure 2) [28, 49-55]. Still, another way in which the context of biomolecule

1 presentation can be important is in its spatial arrangement within the ECM. Not all cells
2 within a tissue are exposed to the same biochemical signals at any given time [56]. In fact,
3 anisotropic signal presentation in the stem cell niche can direct asymmetric stem cell
4 division [57]. To that end, it is often desirable to control the spatial distribution of
5 biomolecules within hydrogels. Thus, one begins to consider methods to assemble
6 complex biomolecule patterns or concentration gradients within hydrogels to influence
7 specific cell populations without influencing neighboring cell populations. Photochemical
8 reactions have emerged as a means to achieve user-directed covalent immobilization of
9 biomolecules to direct cell function.

11 **2.1 Thiol-ene chemistry: a photo-click reaction**

13 The thiol-ene photo-click reaction was first identified in 1905 [58], but its full
14 utility as a bioorthogonal click reaction was not fully realized until recently. Thiol-ene
15 chemistry meets all the criteria of a click reaction [35], as it involves simple reactive
16 moieties and does not produce any byproducts. It is highly efficient, and can be employed
17 in reactions with a variety of substrates, ranging from small molecule derivatization [59,
18 60], to bioconjugation chemistry [61], to polymerization [62]. Importantly, the thiol-ene
19 reaction has been demonstrated as bioorthogonal—reactive alkenes are rarely found in
20 nature and native thiols primarily exist as unreactive disulfides or buried within proteins.
21 For these reasons, of all the bioorthogonal photoclick reactions, thiol-ene has been
22 explored most extensively in the biomaterials community. The thiol-ene reaction has
23 been reviewed comprehensively [44, 63-65], but we shall provide herein a brief overview
24 of the reaction with particular emphasis on its utility in regenerative medicine.

25 As its name implies, the thiol-ene reaction involves the addition of a thiol to an
26 alkene (“ene”) [63, 64]. It is a radical-mediated process that is initiated by light, heat or
27 redox processes; however light initiation is advantageous because it is cytocompatible.
28 For light-mediated thiol-ene chemistry, the reaction begins with radical initiation upon
29 irradiation to afford a thiyl radical (Scheme 1). Efficient initiation is typically achieved
30 with Norrish type I photoinitiators such as lithium phenyl-2,4,6-
31 trimethylbenzoylphosphinate (LAP) [66] and Irgacure 2959 (for ultraviolet initiation)
32 [28] or Irgacure 819 (for visible light initiation) [67]. Chain transfer involves addition of
33 the thiyl radical to the alkene followed by proton abstraction from a thiol-containing
34 molecule to generate a new thiyl radical. The chain transfer process is highly efficient
35 wherein a single radical causes up to tens of thousands of addition events to occur.
36 Termination occurs rapidly with a bimolecular reaction between any of the radical
37 species generated in the reaction. It is important to note that the efficiency of the thiol-ene
38 reaction relies heavily on the alkene moiety chosen. Electron rich and strained alkenes
39 like vinyl ethers and norbornenes, respectively, react most rapidly toward thiyl radicals
40 whilst electron poor alkenes such as acrylates and maleimides react slowly [68]. The
41 slower free-radical addition of thiyls to acrylates and maleimides should not, however, be
42 confused with the rapid base catalyzed Michael-addition of thiols to acrylates. Indeed,
43 mixed mode polymerizations can occur in photopolymerized thiol-acrylate systems [69].
44 Thus, for efficient thiol-ene mediated step-growth polymerization and bioconjugation,
45 norbornenes or vinyl ethers should be employed.

1 **Scheme 1.** Mechanism for the photo-click thiol-ene reaction.

Hydrogels are typically assembled using thiol-ene chemistry through the polymerization of multi-armed macromers containing alkene functionalities (typically the highly reactive norbornene [70]) with a dithiol crosslinker (such as a proteolytically degradable peptide to facilitate cell spreading and migration) [27, 71]. Thiol-ene polymerization has a number of advantages over traditional chain growth polymerization reactions. First, thiol-ene polymerization proceeds via step-growth polymerization allowing for the evolution of a more homogenous network structure [62]. It is also not inhibited by the presence of oxygen, which typically leads to longer polymerization times and requires higher radical dosing in traditional radical polymerizations [68]. Furthermore, the thiol-ene reaction also has a significantly lower concentration of free radicals during propagation compared to traditional free radical polymerizations [72]. These combined properties of thiol-ene polymerization act to minimize damage to the encapsulants, and thus polymerization can be performed in the presence of cells, tissues, proteins and other signaling molecules. Perhaps the most significant benefit of the thiol-ene reaction is the ability to utilize photoinitiation in a step-growth polymerization, which allows for control over both the time when and location where polymerization occurs.

The use of photoinitiation can also allow for spatially patterning of thiolated biomolecules. If the gel is polymerized off-stoichiometry (i.e., with excess alkene macromers), unreacted alkenes will be present within the resulting hydrogel. These alkenes can be used for post-polymerization functionalization with thiol-containing biomolecules through a subsequent thiol-ene reaction. Consequently, biomolecules are covalently incorporated within the hydrogel only at locations where light is directed. In doing so, one can assemble complex patterns and concentration gradients of biomolecules within hydrogels. While thiols can be cumbersome to work with due to their propensity to oxidize and form disulfides, these concerns are minimized because disulfides are still reactive in the thiol-ene reaction [73].

Nevertheless, there are some considerations to keep in mind when employing thiol-ene chemistry, especially in the presence of biological systems. First, biomolecules may need to be functionalized with a free thiol to facilitate their attachment to the hydrogel. Modification of biomolecules (especially proteins) can alter or diminish their

function. Additionally, the thiol-ene reaction involves irradiation with light and the generation of free-radical species that can potentially lead to cytotoxicity issues [46, 74] and protein damage [72]. If care is taken when selecting the reaction conditions, most deleterious effects can be minimized, rendering the thiol-ene photo-click chemistry a powerful technique to both tether and pattern biomolecules within hydrogels.

2.1.1 Biomolecule patterning with thiol-ene chemistry

The discovery that peptides can mimic certain biological functions of the native ECM proteins from which they were derived has made their use commonplace within regenerative medicine [75]. Modification of hydrogels with these short peptide sequences (typically 3 – 10 amino acids) that are readily synthesized affords the ability to introduce biological function into otherwise biologically inert hydrogels. One has precise control over the biological signals incorporated within the hydrogel because peptides are significantly less complex, and generally more stable [76], than their native protein analogues, which often contain multiple signaling domains [77]. Many biologically active peptides have been developed for use in regenerative medicine [78]. Most commonly employed is the fibronectin-derived RGD peptide sequence. Fibronectin is a ubiquitous protein in the ECM that binds to various integrin receptors and promotes cell adhesion, spreading, and proliferation [79]. Thus, incorporation of RGD into hydrogels allows integrin-mediated cell attachment and improves the survival of anchorage dependent cells in hydrogel environments [80]. Laminin is another protein found extensively in native ECM that plays a role in facilitating cell adhesion, migration and differentiation [81]. The peptide sequences YIGSR [82] and IKVAV [83] have been found to bind cell surface receptors, similarly to fibronectin. These sequences have been widely explored in applications related to neuronal cultures and promoting neurite outgrowth, respectively [84]. Glycosaminoglycan (GAG)-binding peptides have also been identified. For example, Kiessling *et al.* identified the vitronectin-derived peptide sequence GKKQRFRRNRKG that binds to GAGs on human embryonic stem cells to influence their differentiation state [85-87]. Peptides can also be employed to sequester signaling molecules within the hydrogel through affinity binding. For example, the peptide WKNFQTI was used to limit the release of its binding partner, monocyte chemotactic protein, from PEG hydrogels [88]. Thus, peptides can be incorporated within hydrogels to facilitate cell attachment, direct cell fate, or control the release of signaling molecules. Numerous ECM-derived peptide epitopes have been identified and excellent reviews on this topic can be found [89, 90].

In 2008, the Anseth lab demonstrated the first example of peptide patterning in hydrogels via the thiol-ene photo-click chemistry [62]. PEG hydrogels were synthesized via an azide-alkyne cycloaddition polymerization to afford hydrogels bearing pendant alkene functionalities. The authors hypothesized that peptides containing cysteine residues can be efficiently patterned through a thiol-ene reaction between the thiol on cysteine and the pendant alkene functionality on the hydrogel. To that end, they synthesized a fluorescently labeled RGDSC peptide, and swelled the hydrogel with a solution of the peptide and a photoinitiator. Mask-based photolithography was employed to perform the thiol-ene reaction at specific locations. Fluorescent patterns were observed suggesting that the thiol-ene reaction was confined to regions that were exposed to light.

These findings demonstrated that the thiol-ene reaction could indeed be employed to spatially pattern peptide sequences within hydrogels with micron scale resolution and on time scales requiring only seconds to minutes. Moreover, it validated that incorporating cysteine within peptide sequences is a practical method to generate thiol-containing peptides for patterning.

Figure 3. 3D hydrogels were generated via SPAAC that contained pendant alkene functionalities for subsequent thiol-ene reactions post polymerization (A). Cell-mediated cleavage of the peptide cross-linker occurs at the residues highlighted in red. Three different fluorescently labeled cysteine-containing peptides were patterned sequentially and imaged via confocal microscopy (B). Three different gels were synthesized containing no RGD (C), uniform RGD (D) or patterned RGD (E) and NIH 3T3 cells expressing GFP were allowed to spread in the gels.

As a bioorthogonal click reaction, the thiol-ene photoclick-chemistry allows for the patterning of biomolecules in the presence of encapsulated cells [91-93]. For example, 4-arm PEG-azide **1** was polymerized via SPAAC with a proteolytically degradable bis-alkyne peptide **2** crosslinker that contained an alkene for subsequent thiol-ene functionalization (Figure 3) [94]. The authors demonstrated that three different fluorescent peptides could be patterned into the gel via thiol-ene chemistry (Figure 3B). Additionally, 3D peptide patterns were generated using multi-photon photolithography. By performing the polymerization in the presence of NIH 3T3 fibroblasts, the cells were encapsulated directly within the hydrogel during polymerization while maintaining high cell viability. Subsequent patterning of RGDSC peptides via thiol-ene chemistry allowed for user-directed cell spreading in specific regions of the gel (Figure 3C-E). Thus, thiol-ene chemistry can be used to pattern multiple peptide patterns within a gel, and importantly, peptide patterns can be generated in the presence of cells to direct their behavior.

Figure 4. NorHA **3** was employed by Burdick *et al.* to generate fibrous hydrogels via electrospinning (A). RGD (red) was photopatterned either parallel (B) or antiparallel to the fibers and NIH 3T3 cells were allowed to adhere to the gels. Cell localization within RGD patterns was quantified (D, E).

Thiol-ene photopatterning has also been explored in naturally derived hydrogels. Hyaluronic acid (HA) is a polysaccharide often employed in the synthesis of matrices for tissue engineering applications. In one example, norbornene functionalized HA **3** (NorHA) was polymerized via thiol-ene chemistry with dithiol crosslinkers to create hydrogels (Figure 4). Thiolated peptides were patterned into the resulting HA gels through thiol-ene reactions with unreacted, pendant norbornene functional groups [95]. Burdick *et al.* employed thiol-ene peptide patterning to explore the role of cell alignment in fibrous hydrogels. Covalently crosslinked hydrogel materials are typically amorphous with a molecular size mesh, but native ECM is composed of nanofibrous proteins that can provide structural cues to cells or allow more facile cell spreading and motility [96]. To combine the features of fibrous structure with the mechanical properties of a covalently crosslinked gel, the authors used electrospinning [97]. The resulting gels were photopatterned with 100 μm wide lanes of thiolated RGD using a mask either parallel or antiparallel to the fibers. On hydrogels patterned with RGD parallel to HA fibers, NIH 3T3 fibroblasts elongated in the direction of the hydrogel fibers as expected. Interestingly, gels with RGD patterned antiparallel to HA fibers still showed elongation relative to fiber alignment and not RGD pattern alignment. These data point to the importance of including structural features with spatially localized biochemical features in designing materials for regenerative medicine.

While early work focused on the patterning of peptides, thiol-ene chemistry is also beginning to see utility for patterning proteins within hydrogels. In order to ligate proteins to a hydrogel using thiol-ene chemistry, the hydrogel and protein must be functionalized with the appropriate reaction partners. In one scheme, a thiolated protein is

1 reacted with hydrogel functionalized with alkenes via an off-stoichiometric thiol-ene
2 polymerization. If the protein does not natively contain a free sulfhydryl, it can be
3 thiolated using Traut's reagent (2-iminothiolane), which converts native primary amines
4 to thiols under mild conditions [98]. Alternatively, alkenes can be coupled to proteins
5 through their primary amines so they can react with pendant thiols in hydrogels.
6 Regardless of the approach, the labeling reaction must be optimized to ensure minimal
7 reactive groups are appended to the protein to minimize deleterious effects on bioactivity
8 of labeling.

9 Alge *et al.* recently employed thiol-ene chemistry to achieve protein patterning in
10 hydrogels [99]. Hydrogels were synthesized via IEDDA polymerization of 4-arm PEG
11 tetrazene with bis-norbornene functionalized peptide crosslinker. Next, the authors
12 incorporated norbornene-labeled GGKGGC peptide uniformly to install pendant thiols
13 throughout the hydrogel. Two different fluorescently labeled bovine serum albumin
14 (BSA) proteins that had been functionalized with norbornene were patterned within the
15 hydrogel using mask-based photolithography. This work was instrumental in
16 demonstrating that thiol-ene chemistry can be employed to control spatial presentation of
17 proteins within hydrogels. While BSA is not biologically relevant, thiol-ene chemistry
18 has been used to tether growth factors, such as TGF- β , within hydrogels, but without
19 spatial control [50]. Still, these studies suggest that thiol-ene chemistry will be amenable
20 for patterning biologically active proteins within hydrogels.

22 **2.2 Photocleavage reactions**

24 While thiol-ene chemistry is a simple, but powerful, methodology to achieve
25 biomolecule tethering within hydrogels, patterning of proteins introduces additional
26 challenges. Proteins require much longer timescales for diffusion into the hydrogel
27 compared to low molecular weight peptides due to their larger size relative to the mesh of
28 the gel [100]. Thus, sequentially patterning multiple proteins can lead to impractical
29 timescales for creating regenerative biomaterials. Additionally, since thiol-ene
30 bioconjugation proceeds via a radical mechanism, protein damage can be a concern,
31 necessitating an alternative patterning approach. To that end, photocleavage reactions
32 offer a complementary strategy to achieve simultaneous patterning. These reactions
33 involve photocleavable or photocaging groups that undergo degradation upon irradiation
34 with light causing the release of a leaving group, which can be a reactive handle or a
35 biomolecule (Figure 5A) [45]. While they were originally developed for use in organic
36 synthesis, photocleavage reactions have emerged as important tools to achieve
37 biomolecule patterning within hydrogels [101]. In one approach, the photocleavable
38 group acts as a protecting group for a reactive moiety (Figure 5B). For example, they can
39 be used to mask thiol functionalities within hydrogels, which upon irradiation, cleave to
40 expose free thiols that can undergo thiol-Michael click reactions with maleimide-labeled
41 biomolecules [102]. Since this exposure of thiols is a photo-mediated process, the thiol-
42 Michael click reactions occur only in regions that are exposed to light. Alternatively,
43 photocleavable groups can be appended to the biomolecule itself as covalent inhibitors of
44 its function (Figure 5C). While the biomolecule is uniformly presented in the hydrogel,
45 its bioactivity is only restored in irradiated regions where photocleavage occurs [103].
46 Finally, biomolecules can also be attached to hydrogels using a photocleavable linker that

allows for spatially-controlled release of the biomolecule upon irradiation (Figure 5D) [104].

Figure 5. Methods to achieve patterning using photocleavable groups. A) Generic structures of ONB and coumarin photocleavable groups and their degradation products. B) The photocleavable group functions to mask a reactive handle (in this example, a thiol). Photolysis releases the thiol where it can undergo bioconjugation with a maleimide-labeled biomolecule. C) Photocleavable groups are attached to tethered biomolecules which inhibits their bioactivity. Patterning is achieved by releasing the photocleavable group to restore bioactivity in defined locations within the hydrogels. D) Biomolecules are tethered to hydrogels via a photocleavable group linker. Photolysis releases the biomolecule in defined regions.

Two of the commonly employed photocleavable groups in biomaterials are based on *o*-nitrobenzyl (ONB) and coumarin moieties. In its simplest form, ONB consists of a 1-nitrobenzyl moiety with a leaving group ortho to the nitro functionality (Figure 5A) [101]. ONBs are highly versatile and have been designed to mask and release carboxylic acids, thiols, alcohols, amines and phosphates for subsequent biomolecule conjugation [45, 105]. Coumarin derivatives contain a leaving group at the 3-position, and generally benefit from electron donating groups located at 6- or 7-position for efficient photolysis [101]. With both photocleavable groups, their absorption maximums can be tuned by altering the electronics of the molecules, and much effort has been directed toward identifying compositions that lead to photocleavage at a variety of wavelengths from near-IR to UV [101].

2.2.1 Patterning using photocleavage reactions

1
2 Photocleavage reactions have been used extensively to pattern peptides within
3 hydrogels [102, 103, 106-110]; however, their true utility is in patterning proteins. As
4 previously mentioned, one common approach is to use a photocleavable protecting group
5 to mask a reactive functionality [111, 112]. Upon photocleavage, the reactive group is
6 exposed allowing it to spontaneously react with biomolecules in regions that are
7 irradiated. For example, the Shoichet lab has used this methodology to achieve gradients
8 of proteins within hydrogels [113]. Coumarin-caged thiols within agarose hydrogels were
9 unmasked via photolithography and allowed to react with maleimide-labeled vascular
10 endothelial growth factor 165 (VEGF165). VEGF165 gradients were achieved by
11 controlling the laser power in one direction, and the authors explored whether endothelial
12 cells (ECs) could be directed along the concentration gradient. Interestingly, ECs cultured
13 on VEGF concentration gradients formed tubule-like structures in the direction of the
14 gradient, and the tubules appeared similar to those found *in vivo*. In a follow-up study, the
15 authors explored how ECs influence retinal stem and progenitor cell (RSPC) proliferation
16 and differentiation [114]. ECs and RSPCs were co-cultured on gradients of VEGF165
17 where they again formed tubule-like structures. Interestingly, differentiation of RSPCs
18 was inhibited in the presence of ECs, although direct contact was not required, suggesting
19 soluble EC-derived factors influence RSPC fate. These were the first findings that
20 suggest ECs influence RSPC quiescence and could motivate therapeutic strategies for
21 treating retinal degenerative disease.

22 This methodology has also been employed to achieve multi-protein patterning
23 within hydrogels [115]. Coumarin-caged thiols were unmasked in regions such that
24 subsequent thiol-Michael conjugate additions can be performed. Specifically, the authors
25 used two-photon photolithography to unmask thiols in specific regions of the gel, which
26 were then reacted with a maleimide-labeled barnase. After removal of the excess
27 maleimide-labeled barnase, additional thiols were unmasked at separate locations in the
28 gel. A maleimide-streptavidin conjugate was then added and allowed to react at this
29 second location. After washing, complementary barnstar-labeled sonic hedgehog
30 (barnstar-SHH) and biotin-labeled ciliary neurotrophic factor (biotin-CNTF) conjugates
31 were swollen into the gel; barnstar-SHH bound to patterned barnase, while biotin-CNTF
32 bound to patterned streptavidin. Importantly, the proteins were both introduced
33 simultaneously after the patterning and washing steps, greatly reducing the time required
34 for patterning and avoiding protein inactivation and confounding interactions that can
35 result from sequential patterning approaches. Using this multi-step, but highly controlled
36 approach, the authors were able to assemble complex, multi-protein three-dimensional
37 patterns within the hydrogel (Figure 6A, B). The authors verified that the proteins
38 retained their bioactivity upon tethering to the hydrogels using retinal precursor cells
39 (RPCs). For example, the authors assessed the ability of tethered SHH to mediate
40 transcription of the transcription factor Gli2. RPCs were plated on the agarose hydrogels
41 and *gli2* mRNA was quantified by RT-PCR (Figure 6C). Similar levels of *gli2* mRNA
42 were observed for cells exposed to tethered SHH compared to cells treated with soluble
43 SHH. Similarly, the authors monitored phosphorylated STAT3 by fluorescence
44 microscopy as a readout of CNTF function (Figure 6D). Only cells treated with tethered
45 CNTF or soluble CNTF exhibited staining for phospho-STAT3 suggesting CNTF is

active upon tethering to hydrogels. These data demonstrate that photocleavable groups can be used to achieve patterning of bioactive proteins.

Figure 6. Complex 3D structures of SHH and CNTF were formed using two-photon photolithography in hydrogels viewed from top (A) and side (B). SHH was labeled with a green fluorophore and CNTF was labeled with a red fluorophore. (Scale bar represents 100 μ m). C) RPCs upregulate a key SHH signaling mediator, *Gli2*, in response to immobilized and soluble SHH as analyzed by RT-PCR. D) RPCs stained positive for phospho-STAT3 (red) in response to immobilized and soluble CNTF.

Similarly, photocleavable groups can also be used to mask substrates for an enzyme that mediates ligation of a protein to achieve protein patterning [116]. For example, Lutolf *et al.* generated PEG hydrogels containing pendant peptide **4** that contained a lysine residue masked by an ONB derivative (Figure 7A) [76]. Photolysis of ONB frees the lysine affording a peptide **5** that is the substrate for transglutaminase factor XIII (FXIIIa), which catalyzes the formation of an isopeptide bond between the N-terminal glutamine of the amino acid sequence NQEQVSPL and a lysine. Thus, the authors postulated that proteins could be linked to the hydrogel via FXIIIa upon photo-uncaging of the lysine on a peptide **4**. Indeed, using this methodology, VEGF and Fc-chimera IgG antibody were incorporated within hydrogels with spatial control. To verify that proteins maintained their bioactivity upon patterning via this method, the authors examined the ability of VEGF to mediate cell attachment. Recombinant VEGF fragments were patterned in defined shapes and MSCs were cultured on the gels (Figure 7B-C). Only regions that had been patterned with the VEGF fragments facilitated attachment of the MSCs. These combined studies demonstrate that photocleavage reactions are

powerful methodologies to achieve protein patterning within hydrogels with minimal effects on their bioactivities.

Figure 7. A) Photocaging strategy to unmask the peptide substrate for FXIIIa to perform protein ligations. VEGF fragments (21 kDa) were patterned into hydrogels and MSCs were cultured on top of the hydrogels for 3 h (A – C). (Scale bars represent 200 μ m)

3. Reversible control over biomolecule patterning

In the previous sections, we have outlined synthetic methods to achieve contextual presentation of biomolecules with respect to their interactions with the ECM, as well as their spatial distribution within matrices. Still, the ECM is highly dynamic and biomolecule expression in the niche varies in response to various external stimuli, which can affect cellular function. For example, a temporally regulated transition from Notch signaling to Wnt signaling is necessary for myogenic differentiation in muscle satellite cells to regenerate muscle tissue [117]. Thus, it is logical to consider the context of reversibility in the presentation of biomolecules. Many of the aforementioned approaches to mediate biomolecule tethering allow for conjugation, and in some cases release, of biological signals, but they are irreversible—in other words, it is not possible to introduce and remove signals multiple times using the same functionalities. Hence, the ability to dynamically control the presentation of biomolecules within hydrogels has been a contemporary topic in the regenerative medicine community [118]. Both thiol-ene chemistry and photocleavage reactions have been adapted to allow for patterning that is both spatial and temporal in nature.

3.1 Thiol-ene mediated reversible biomolecule patterning

To achieve reversibility in a thiol-ene reaction, the Anseth lab drew inspiration from the chain transfer agents used in reversible addition fragment (RAFT) polymerization [119]. Specifically, the authors postulated that allyl sulfide functional groups would allow for reversible thiol-ene chemistry—attack of a thiyl radical to the allyl sulfide would lead to regeneration of the alkene and release of the previous thiolated

molecule (Figure 8A). Thus, allyl sulfide functional groups should theoretically allow for infinite sequential thiol-ene reactions to proceed. To test this hypothesis, PEG hydrogels were synthesized via azide-alkyne cycloaddition polymerization of a 4-arm PEG azide and an allyl sulfide crosslinker. Using multiphoton photolithography, the authors patterned a square of fluorescent RGDS (Figure 8B). RGDS labeled with a different fluorophore was then incorporated and patterned in the arbitrary shape of a buffalo within the previously patterned square, thus exchanging the peptide within these regions (Figure 8C). Finally, a non-fluorescent RGDS peptide was patterned in the shape of 'CU' within the buffalo, performing a third exchange of biomolecule (Figure 8D). These data support that the allyl sulfide functionality can be employed to reversibly pattern biomolecules via thiol-ene chemistry within hydrogels. It is likely that this approach can be expanded to achieve reversible peptide and protein patterning to directly exchange the immobilized biochemical signals that cells receive. Allyl sulfide chemistry affords a plethora of opportunities to explore the effects of dynamic biochemical signaling in a more biophysically relevant 3D milieu, where the cell is not polarized as it is on 2D substrates. These studies may lead to a better understanding of the stem cell niche and also influence the regenerative properties of these materials upon transplantation due to the contextual presentation of biophysical and biochemical signals, similar to the findings of the Blau lab [20].

Figure 8. A) Allyl sulfide strategy to reversibly perform thiol-ene reactions indefinitely to dynamically tether biomolecules. Red CRGDS was patterned via multiphoton patterning to afford a cube (B). Green CRGDS was patterned in the shape of a buffalo to exchange the two peptides (C). Finally, nonfluorescent RGDS was patterned in the shape of 'CU' to exchange peptides a second time (D).

3.2 Photocleavage mediated reversible biomolecule patterning

Reversible control over biomolecule patterning with proteins typically involves tethering biomolecules to the hydrogel scaffold via a photocleavable linker (Figure 3C) [104]. Thus, proteins can be patterned with spatial control, and then cleaved and released at a later time point. For example, the Tirrell lab recently exploited ONB functionality to achieve sequential protein patterning [120]. Specifically, the authors generated PEG hydrogels bearing pendant alkoxyamines **6** that are masked by an ONB protecting group (Figure 9A). Multiphoton photolithography was employed to unmask alkoxyamines to react with proteins such as **7** that contained an ONB linker tethered to an aldehyde. This

approach allows proteins to be patterned via unmasking of alkoxyamines on the hydrogel, and then released via photolysis of ONB group linking the protein to the hydrogel. Using this strategy, the authors were able to pattern BSA in complex patterns (Figure 9B) and then release BSA from the hydrogel at a later time point (Figure 9C). The authors then explored the role of spatial presentation of vitronectin (VTN) on the osteogenesis of human mesenchymal stem cells (hMSCs). It has previously been shown that hMSCs undergo osteogenesis, as determined by osteocalcin (OC) expression, in 3D environments that allow for cell spreading [27]. The authors postulated that by patterning the adhesive protein VTN in lines, osteogenesis could be induced solely in hMSCs exposed to VTN. Indeed, 3 days after VTN patterning, cells exposed to VTN expressed high levels of OC, while cells outside the VTN patterned regions did not (Figure 9E). VTN was then released at specific regions, and remarkably after 6 days, cells that had previously expressed OC lost expression once VTN was removed (Figure 9F). These data demonstrate that photocleavable groups can be used to not only pattern proteins, but also release them. Moreover, these studies demonstrate that user-directed addition and release of biomolecules can direct cell function.

Figure 9. SPAAC hydrogels were generated to bear masked alkoxyamine **6**. Upon photolysis of ONB, biomolecules can be appended through the gel by aldehyde **7** that has an ONB linker to cleave biomolecules after bioconjugation. Fluorescent BSA resembling structure **7** was patterned within the hydrogel using multiphoton photolithography (B). At a later time point, fluorescent BSA was released from the hydrogel in defined regions (C) (Scale bars represent 100 μ m). hMSCs were seeded within SPAAC hydrogels and treated with CellTracker red and stained for OC (green), a marker of osteogenesis. At 1 d, VTN was patterned in lines (shown as white dotted lines) and after 4 d, hMSCs stained for OC in patterned regions (E). VTN was released in regions after 4 d and hMSC OC expression was lost in those regions at 10 d (D).

4. Conclusions and Future Prospects

Hydrogels have emerged as versatile and tailorable scaffolds in regenerative medicine for the delivery of biomolecules to promote healing, and they have played a

pivotal role in understanding cellular processes in 3D environments. Indeed, numerous orthogonal chemistries have emerged to achieve complex spatial control over the presentation of biomolecules within hydrogels. Of these, thiol-ene and photocleavage reactions have been highly successful at controlling the 3D presentation of both peptides and proteins in hydrogels to direct cell function. With the ability to integrate biological signals, the field is increasingly focusing on developing chemistries that allow for the presentation of biomolecules with spatial and temporal control (termed ‘4D biology’). To that end, thiol-ene and photocleavage reactions have been adapted to allow for sequential and reversible tethering of biomolecules. Many biological processes are controlled by multiple signals acting in coordination, for example, opposing gradients of chemoattractants and chemorepellants, sequential changes in adhesive proteins and cytokine signals during differentiation of stem cells, and even dynamic changes in matrix stiffening in combination with inflammatory molecules that accelerate fibrotic diseases. To better understand which signals are important when trying to regenerate tissues or delivery drugs to target the treatment of disease, 4D hydrogel culture systems provide an important tool. However, the multitude of possible variables in the experimental space necessitates the integration of rationale material design with high throughput methods to rapidly create hydrogels with various temporal combinations of biophysical and biochemical cues. The advantage of creating such biomaterial arrays is that they can be easily integrated with existing biological and imaging methods to track and monitor key cellular processes in real time and in response to the matrix environment. While it is not possible, or perhaps even necessary, to recapitulate every aspect of native ECM-cell interactions *in vitro*, these approaches will be insightful and critical for identifying key biological signal combinations that have either positive or deleterious effects on tissue regeneration, wound healing, and disease progression. As such, findings from such studies should directly impact the design of future material-based therapeutics in regenerative medicine.

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