

# A Reversible and Repeatable Thiol–Ene Bioconjugation for Dynamic Patterning of Signaling Proteins in Hydrogels

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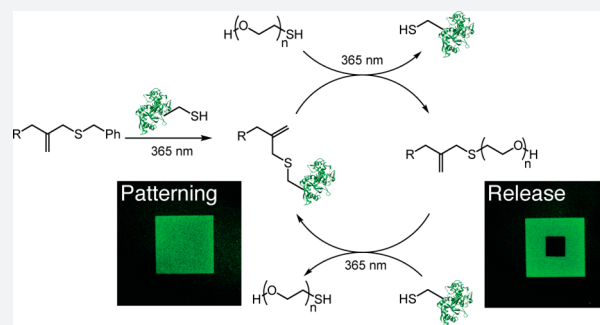
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## Supporting Information

**ABSTRACT:** Biomolecule-functionalized hydrogels have emerged as valuable cell culture platforms to recapitulate the mechanical and biochemical properties of the extracellular niche. The typical strategy to functionalize hydrogels with biomolecules involves directly tethering them to the hydrogel backbone resulting in a static material. Thus, this approach fails to capture the dynamic changes in biomolecule composition that occur during biological processes or that may be required for regenerative medicine applications. Moreover, it also limits the scope of biomolecules to simple peptides, as signaling proteins generally have poor stability under cell culture conditions and lose their bioactivity over time. To that end, we sought to develop a bioconjugation reaction that would enable reversible and repeatable tethering of signaling proteins to hydrogels, so that spent protein could be released on-demand and replaced with fresh protein as needed. Specifically, we designed an allyl sulfide chain-transfer agent that enables a reversible, photomediated, thiol–ene bioconjugation of signaling proteins to hydrogels. Upon addition of a thiolated protein to the allyl sulfide moiety, the previously tethered protein is released, and the “ene” functionality is regenerated. Using this approach, we demonstrate that protein patterning can be achieved in hydrogels through a thiol–ene reaction, and the patterned protein can then be released through a subsequent thiol–ene reaction of a PEG thiol. Importantly, this process is repeatable through multiple iterations and proceeds at physiologically relevant signaling protein concentrations. Finally, we demonstrate that whole signaling proteins can be patterned and released in the presence of cells, and that cells respond to their presentation with spatial fidelity. Combined, these data represent the first example of a methodology that enables fully reversible and repeatable patterning and release of signaling proteins from hydrogels.



## INTRODUCTION

Synthetic hydrogel materials seek to recapitulate critical aspects of the extracellular matrix for modeling cellular microenvironments.<sup>1</sup> Hydrogels synthesized from poly(ethylene glycol) (PEG) polymers act as a “blank slate,” enabling complete user control over the mechanical and biochemical signals presented to cells.<sup>2</sup> Manipulating cellular phenotypes *in vitro* often requires the presentation of biochemical signals to achieve desired alterations in cellular adhesion,<sup>3</sup> migration,<sup>4</sup> and differentiation.<sup>5,6</sup> To that end, biomolecules, including peptides and proteins, are grafted on to the scaffold via direct conjugation to the polymer backbone to facilitate their presentation to cells.<sup>7</sup>

While any bioconjugation reaction can be adapted to achieve biomolecule tethering,<sup>8,9</sup> photomediated bioconjugations,<sup>10,11</sup> including the thiol–ene photoclick reaction, are particularly attractive as they enable spatial presentation, or patterning, of

cues.<sup>10–19</sup> Biomolecule patterning via the thiol–ene reaction involves the addition of a thiolated biomolecule to alkene functionalities on the polymer backbone to afford a thioether adduct.<sup>20</sup> The reaction is radical mediated and initiated by light to enable spatial control over where in the hydrogel the reaction proceeds. Moreover, the process is cytocompatible and can be performed in the presence of cells.<sup>21</sup> Performing the thiol–ene reaction in conjunction with photolithographic methods enables the assembly of biomolecule gradients and complex three-dimensional patterns with high precision to design materials capable of mimicking the inherently heterogeneous nature of the cellular niche.<sup>22–24</sup> Because these approaches generally afford a covalent tether between the biomolecule and polymer backbone, the resulting materials

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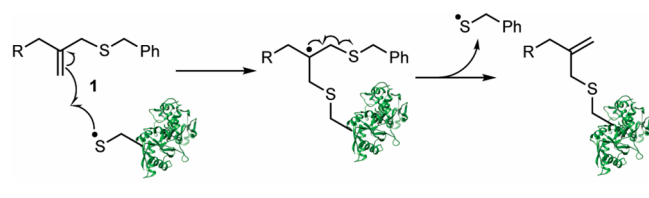
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are static and do not allow for user control over dynamic changes that occur during many biological processes. Indeed, biomolecules must be presented over time, as cells interpret signals from biomolecules on the order of seconds to days to change their phenotypes.<sup>25</sup> Introducing biochemical cues into a hydrogel network in a dynamic fashion would greatly improve extracellular matrix mimicry to enable specialized cell culture platforms.<sup>26</sup> For example, platforms for stem cell expansion and differentiation could be improved through the sequential presentation of multiple different biochemical cues over time.<sup>27</sup> Additionally, spatiotemporal patterning of multiple different cues could be used to engineer more accurate *in vitro* disease models where the composition of extracellular matrix proteins changes over the course of disease progression.<sup>28</sup>

To address the limitations associated with traditional covalent bioconjugation, new photomediated strategies have emerged that enable the patterning and subsequent release of biomolecules in hydrogels through the inclusion of a photolabile linkage.<sup>29,30</sup> Upon irradiation, the photolabile linker is cleaved, releasing the tethered biomolecule from the hydrogel. Because the bioconjugation handle is cleaved and thus consumed, patterning and release can only be performed once. From an experimental design standpoint, it may be necessary to sequentially pattern and release multiple different biomolecules of interest. Perhaps more importantly, the patterning of whole signaling proteins has remained a significant challenge due to their low stability under cell culture condition. For protein patterning to be practical, it is necessary that spent protein can be released from the hydrogel and replaced with fresh protein as needed.

Given these considerations, we sought to develop a methodology that would not only enable reversible patterning and release of proteins, but also be repeatable so that protein patterning and release can be performed through multiple iterations. We drew inspiration from the allyl sulfide chain-transfer agent which has been used extensively in the design of stress relaxing polymer systems.<sup>31–33</sup> We hypothesized that incorporating pendant allyl sulfide moieties onto the hydrogel backbone would enable full reversible and repeatable tethering of whole signaling proteins from a hydrogel through a photomediated thiol–ene click reaction (Scheme 1). Due to

**Scheme 1. Mechanism of the Thiol–ene Reaction between a Thiyl Radical-Containing Protein and the Allyl Sulfide Moiety 1 (R Represents the Hydrogel Backbone)**



the nature of the allyl sulfide as a chain-transfer agent, upon addition of protein to the allyl sulfide handle, any previously tethered protein would be released, and the alkene would be regenerated enabling subsequent thiol–ene reactions. We previously demonstrated that the allyl sulfide chain-transfer agent can be used to reversibly pattern peptides in hydrogels.<sup>34</sup> However, the patterning of whole signaling proteins poses a significant challenge over peptides—proteins are employed under dilute concentrations (picomolar to nanomolar)

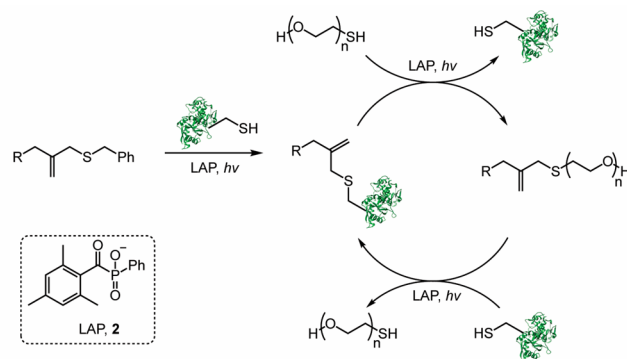
compared to peptides (millimolar) and are significantly bulkier, both inhibiting their reaction rates toward addition and release. Herein, we describe the development of a new allyl sulfide chemical handle that enables reversible and repeatable patterning of signaling proteins in hydrogels. Using this approach, we demonstrate that multiple different proteins can be patterned in and released from hydrogels through sequential thiol–ene reactions. We also demonstrate that this process is cytocompatible, and signaling proteins can be patterned and released in the presence of cells to control cell phenotype.

## RESULTS AND DISCUSSION

Drawing inspiration from the regenerative nature of chain-transfer agents employed in reversible addition–fragmentation chain-transfer (RAFT) polymerizations, we postulated the allyl sulfide chain-transfer agent **1** would enable reversible tethering and release of thiol-containing proteins (Scheme 1).<sup>31–34</sup> While we drew inspiration from RAFT polymerizations, which typically cannot be performed in the presence of oxygen, a key advantage of the thiol–ene reaction is it is not prone to oxygen inhibition and thus can be performed under cell culture conditions.<sup>35</sup> We hypothesized that photoinitiation will lead to the generation of thiyl radicals on the protein of interest. Attack of the thiyl radical at the allyl sulfide moiety results in the formation of an unstable intermediate that rapidly undergoes  $\beta$ -scission resulting in covalent tethering of the thiolated protein, release of benzyl mercaptan, and regeneration of the alkene. Since the radical intermediate is asymmetrical, the more stable radical will be favored for release upon  $\beta$ -scission. In our system, both the benzyl mercaptan radical and the protein thiyl radical have similar stabilities.<sup>36</sup> Thus, we hypothesized that initial product formation will favor the protein adduct, since protein concentrations is higher relative to released benzyl mercaptan.

The overall strategy to achieve sequential protein tethering and release is outlined in Scheme 2. Photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphine **2** (LAP) was selected due to its rapid rate of initiation upon irradiation with 365 nm light and cytocompatibility.<sup>37</sup> After protein tethering to the hydrogel backbone, its release can be achieved through a

**Scheme 2. Proposed Strategy<sup>a</sup> To Reversibly Tether Signaling Proteins to Hydrogels through the Allyl Sulfide Moiety**

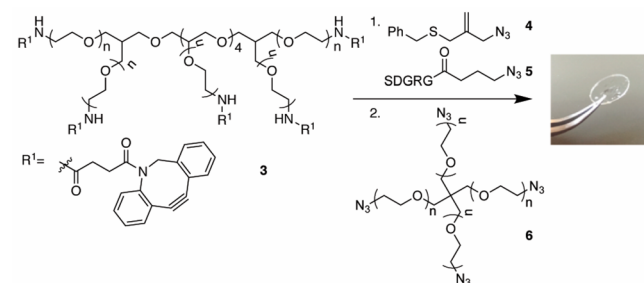


<sup>a</sup>Thiolated signaling proteins are tethered through a thiol–ene reaction. To release the protein, a subsequent thiol–ene reaction is performed with mPEG-SH. This process of protein tethering followed by release should be repeatable. R represents the hydrogel backbone.

subsequent thiol–ene reaction of a PEG thiol. This process results in tethering of PEG, regeneration of the allyl sulfide alkene, and release of the previously tethered protein. A third thiol–ene reaction can then be performed to tether a new protein of interest, while releasing the PEG thiol. Since the alkene is regenerated during each reaction, this process should be repeatable, thus enabling a methodology to reversibly pattern and release signaling proteins of interest with spatial fidelity within a hydrogel.

To synthesize allyl sulfide hydrogels (Scheme 3), we employed strain-promoted azide–alkyne cycloaddition

### Scheme 3. Synthesis of Allyl Sulfide SPAAC Hydrogels on an Azide-Functionalized Glass Coverslip

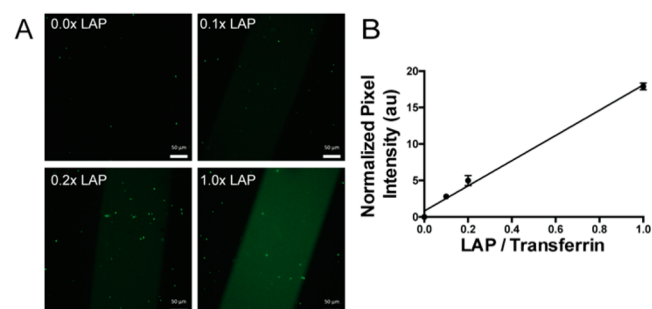


(SPAAC) polymerization.<sup>38</sup> Eight-armed PEG<sub>40K</sub>-dibenzylcyclooctyne (DBCO) **3** was functionalized with azido allyl sulfide **4** and the cell adhesive azido-RGDS peptide **5** to give a final gel concentration of 1 and 2 mM, respectively. Polymerization was performed between eight-armed PEG<sub>40K</sub>-DBCO and four-armed PEG<sub>20K</sub> azide **6** on an azide-functionalized coverslip to give a final gel composition of 10 wt % PEG. Gels were swelled in the presence of nPEG<sub>7</sub>-N<sub>3</sub> to quench any unreacted DBCO that could also participate in the thiol–ene bioconjugation during protein tethering. The inclusion of a large excess of allyl sulfide (1 mM) in the hydrogels relative to the concentration of signaling proteins that will be employed during tethering (<100 μM) ensures that any nonspecific addition of cysteine-containing serum proteins should not interfere with the overall tethering reaction efficiency.

With allyl sulfide gels in hand, we first sought to optimize protein tethering to the allyl sulfide hydrogels. We selected human transferrin, an 80 kDa iron transporting protein, as a model protein for kinetic studies.<sup>39</sup> Transferrin is larger than most signaling proteins, so its optimization of tethering and release should be translatable to other signaling proteins of interest. To ensure reactive thiols on transferrin are available to undergo the thiol–ene reaction, transferrin was treated with *N*-hydroxysuccinimidyl-PEG<sub>1K</sub> thiol to functionalize lysine residues nonspecifically.

A key advantage of the thiol–ene reaction is its high efficiency, whereby a single initiation event mediates hundreds or thousands of subsequent reactions.<sup>20</sup> Thus, under idealized conditions, the thiol–ene bioconjugation proceeds with catalytic photoinitiator with respect to the biomolecule. Signaling proteins, however, are often employed under dilute conditions (<100 μM). At these concentrations, we hypothesized that radical propagation may be inhibited, and thus the reaction would proceed with dependence upon LAP concentration. To that end, we first sought to determine the effect of photoinitiator stoichiometry on protein tethering efficiency. Allyl sulfide hydrogels were swollen with 100 μM

thiolated transferrin and a range of concentrations of LAP (0, 10, 20, or 100 μM). The hydrogels were irradiated with 5 mW/cm<sup>2</sup> 365 nm light for 180 s through a chrome photomask with a repeating 250 μm wide striped pattern. After washing away unreacted transferrin, protein tethering was visualized via immunostaining for transferrin. Normalized fluorescence intensity was measured as a readout of transferrin immobilization. After quantifying pixel intensity, a 1:1 stoichiometric ratio of LAP to transferrin yielded the most intense transferrin patterns (Figure 1A). Immobilization of transferrin scaled



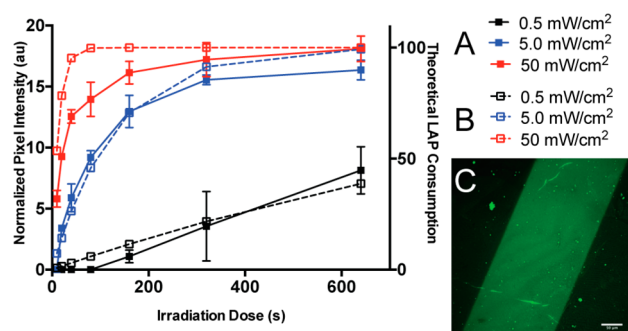
**Figure 1.** Protein patterning efficiency is a function of LAP concentration. Thiolated transferrin (100 μM) and LAP (0, 10, 20, 100 μM) were swelled into allyl sulfide hydrogels and irradiated through a chrome photomask with 5 mW/cm<sup>2</sup> 365 nm light for 180 s. Hydrogels were washed with PBS and immunostained for transferrin for visualization (A). Pixel intensity was averaged for the patterned region and subtracted from the average pixel intensity of the nonpatterned region to obtain normalized pixel intensity (B). Scale bars represent 50 μm.

linearly with increasing LAP concentration ( $R = 0.998$ ), suggesting radical propagation is indeed inhibited, and stoichiometric LAP relative to transferrin is required for efficient protein immobilization (Figure 1B).

Continuing with stoichiometric LAP, we next explored the effect of light intensity ( $I_0$ ) and light dose on protein patterning. Hydrogels were swelled with 100 μM thiolated transferrin and 100 μM LAP and irradiated through a chrome photomask with 365 nm light (0.5, 5, 50 mW/cm<sup>2</sup>) for a range of light doses (0–600 s). After immunostaining for transferrin, normalized pixel intensity was measured for each gel. Protein patterning increased with increasing light intensity and dose (Figure 2A). For both  $I_0 = 5$  and 50 mW/cm<sup>2</sup>, protein tethering plateaued at similar intensities, but the rate at which protein tethering plateaued increased with higher light intensity. Since we expected that propagation would be inhibited under these conditions, we hypothesized that this plateau in the protein signal may correlate with LAP consumption. Indeed, the theoretical rate of photolysis of LAP (eq S5) followed the same trend as protein patterning, further supporting our observations that protein patterning is dependent upon LAP initiation and that propagation is inhibited under these conditions (Figure 2B). With these data combined, we selected  $I_0 = 5$  mW/cm<sup>2</sup> for 180 s with stoichiometric LAP as the optimal conditions to achieve maximal protein tethering to allyl sulfide hydrogels (Figure 2C).

A key advantage of the allyl sulfide moiety is the regeneration of the alkene upon tethering of the protein of interest, thus enabling a subsequent thiol–ene reaction to release the bound protein. Importantly, this process should be

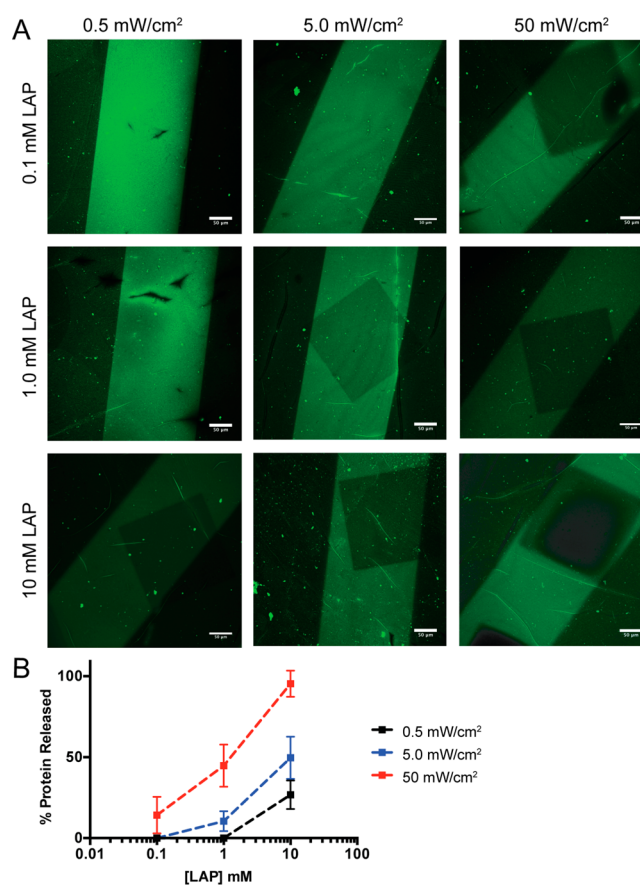




**Figure 2.** Protein patterning increases with light intensity and dose. Hydrogels were swelled with 100  $\mu\text{M}$  thiolated transferrin and 100  $\mu\text{M}$  LAP and irradiated through a chrome photomask with 365 nm light at specified intensities and doses. Gels were immunostained for transferrin, and normalized pixel intensity was measured (A, solid line). The rate of consumption of LAP was calculated with respect to  $I_0$  and dose (B, dashed line). Maximal protein patterning was achieved with 5  $\text{mW}/\text{cm}^2$  365 nm light for 180 s (C).

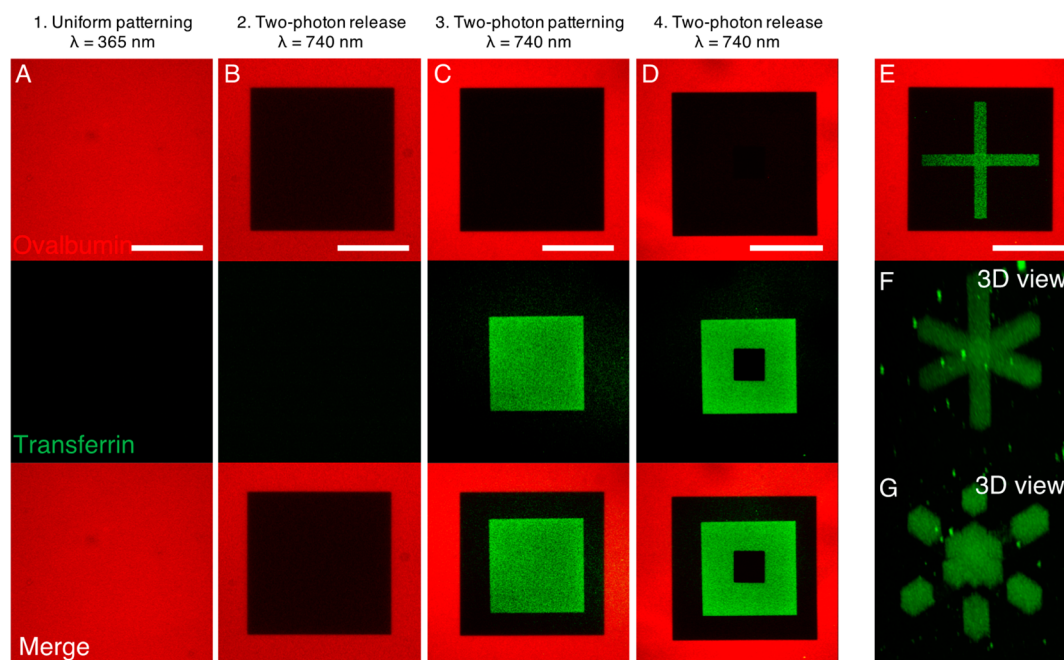
repeatable to afford a methodology to immobilize and release signaling proteins of interest in demand. We chose to release previously patterned protein from the hydrogels through a thiol–ene reaction of PEG thiol resulting in tethering of an inert PEG moiety to the hydrogel. To optimize the release conditions, we explored the effect of LAP concentration and  $I_0$  on the reaction. Hydrogels patterned with transferrin in 250  $\mu\text{m}$  stripes were swelled with a release solution consisting of 50  $\mu\text{M}$  PEG<sub>1K</sub> thiol and a range of catalytic LAP concentrations (0.1, 1.0, and 10 mM). By employing a large excess of PEG thiol relative to allyl sulfide concentration in the hydrogel (1 mM), reactivity with all allyl sulfide moieties should occur to ensure release of all tethered protein. Hydrogels were placed on a chrome photomask with a repeating 250  $\times$  250  $\mu\text{m}$  square pattern and irradiated with 365 nm light of varying intensity (0.5, 5.0, and 50  $\text{mW}/\text{cm}^2$ ) for 180 s. Thus, the release reaction occurs in 250  $\times$  250  $\mu\text{m}$  regions within the striped transferrin patterns. The hydrogels were then washed and immunostained, and normalized pixel intensity was measured (Figure 3A). Transferrin release increased with increasing LAP concentration and light intensity, as indicated by a decrease in fluorescence within the irradiated regions. Remarkably, when hydrogels were irradiated with  $I_0 = 50 \text{ mW}/\text{cm}^2$  365 nm light with 10 mM LAP, a >97% decrease in fluorescence signal intensity was observed relative to the original pattern signal intensity (Figure 3B). These results suggest near-complete release of protein can be achieved via this methodology. Furthermore, LAP concentration and light intensity may be tuned to achieve protein patterns of varied concentrations within the patterned regions.

Having optimized protein tethering and release from allyl sulfide hydrogels, we next sought to demonstrate that the process of protein patterning and release can also be controlled in three-dimensions and is repeatable through multiple iterations with multiple different proteins (Figure 4). To control spatial patterning in three-dimensions, multiphoton photolithography can be employed. Multiphoton patterning techniques rely on near-simultaneous absorption of two or more photons and therefore restrict initiation to the focal volume, imparting precise three-dimensional control over the protein tethering reaction.<sup>40</sup> Further, the nonlinear absorption increases the penetration depth for multiphoton strategies. We first patterned the thiolated and fluorescently labeled



**Figure 3.** Protein release depends on LAP concentration and light intensity. Hydrogels with patterned transferrin were swelled with 50 mM mPEG<sub>1K</sub>-SH and LAP (0.1, 1.0, 10 mM), placed over a chrome photomask with a repeating 250  $\mu\text{m}$   $\times$  250  $\mu\text{m}$  square pattern, and irradiated with 365 nm light at specific  $I_0$  (0.5, 5.0, 50  $\text{mW}/\text{cm}^2$ ). Immunostaining for transferrin was used to visualize patterning (A). Percent transferrin release was calculated by measuring the pixel intensity of the released region and pixel intensity of the original patterned region, normalized to the pixel intensity of unpatterned regions (B). Scale bar represents 50  $\mu\text{m}$ .

immunogenic protein ovalbumin. Ovalbumin was tethered uniformly to the hydrogel using the optimized exposure conditions (365 nm, 5  $\text{mW}/\text{cm}^2$ , 5 min; Figure 4A). Two-photon photolithography ( $\lambda = 740 \text{ nm}$ ) was then employed to release a 200  $\mu\text{m}$   $\times$  200  $\mu\text{m}$  square within the original transferrin pattern through a subsequent thiol–ene reaction of PEG thiol (Figure 4B). To demonstrate protein patterning could be performed a subsequent time, thiolated and fluorescently labeled transferrin was patterned through two-photon photolithography in a 133  $\mu\text{m}$   $\times$  133  $\mu\text{m}$  square within the released region (Figure 4C). Finally, a 44  $\mu\text{m}$   $\times$  44  $\mu\text{m}$  square pattern of transferrin was released through another thiol–ene reaction of PEG thiol (Figure 4D). Additionally, to leverage the 3D capabilities provided by two-photon patterning, a “coordinate axes” shape was patterned in the released area (Figure 4E,F). Selected 3D regions of this shape were then replaced with PEG<sub>1K</sub>-thiol (Figure 4G). These data demonstrate not only that the regenerative nature of the allyl sulfide handle as the same region of a hydrogel can be patterned multiple times, but also that multiple different proteins can be patterned sequentially.



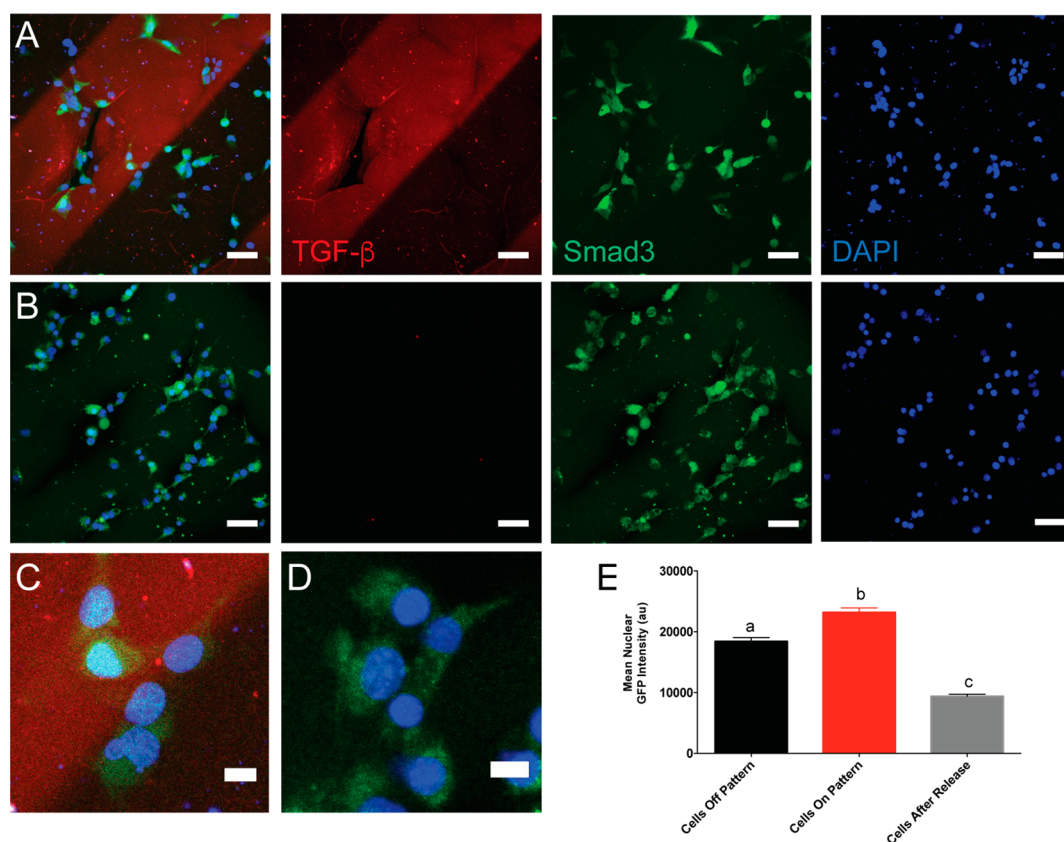
**Figure 4.** Sequential tethering and release of multiple proteins with 3D control. Thiolated ovalbumin (AlexaFluor 555 conjugate) was uniformly tethered to the hydrogel (A), then selectively released using PEG<sub>1K</sub>-thiol (B), followed by tethering (C) and release (D) of thiolated transferrin (AlexaFluor 488 conjugate). Two-photon photolithography is used to pattern proteins with 3D control. A “coordinate axes” shape of transferrin is patterned within the volume of released ovalbumin (E, middle plane; F, 3D view), and selected regions of the 3D shape can be removed (G). Scale bars 100  $\mu\text{m}$ .

Finally, we also characterized the concentration of transferrin tethered to the hydrogel through an enzyme-linked immunosorbent assay (ELISA) analysis (Figure S3). Transferrin patterning in the hydrogel proceeded through a broad range of physiologically relevant concentrations (10–10 000 pg/mL). Importantly, a linear relationship ( $R^2 = 0.98$ ) between the amount of transferrin swelled into the hydrogel and the amount patterned on the hydrogel was identified, suggesting the resultant protein concentration patterned in the hydrogel can easily be predicted *a priori*.

A key benefit of the thiol–ene reaction is that it is cytocompatible, so tethering of biomolecules can be performed in the presence of cells with minimal cytotoxicity. Moreover, since the thiol–ene reaction is light mediated, complex three-dimensional patterns of biomolecules can be assembled within the gel on-demand, vastly expanding the traditional experimental space for evaluating how cells respond to spatiotemporal protein cues. We sought to use this strategy of protein patterning followed by release with a signaling protein to verify that cells can respond to dynamic changes in protein patterning. We selected transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) for use as a signaling protein of interest. TGF- $\beta$ 1 is a growth factor principally implicated in many fibrotic diseases and is a target of interest for therapeutic intervention.<sup>41</sup> Moreover, TGF- $\beta$ 1 exists bound to the extracellular matrix *in vivo*, and cells interact with TGF- $\beta$ 1 through direct interactions with the matrix.<sup>42,43</sup> Thus, tethering TGF- $\beta$ 1 to hydrogels may function as a physiologically relevant presentation platform. When TGF- $\beta$ 1 binds to the TGF- $\beta$ 1 receptor on the surface of cells, a signaling cascade proceeds culminating in translocation of the transcription factor Smad3 from the cytosol to the nucleus. We postulated that the nuclear translocation of Smad3 could be used as a real-time readout of TGF- $\beta$ 1 signaling for our experiments. Thus,

we modified mouse embryonic fibroblasts (MEFs) via retroviral transfection with a GFP-Smad3 fusion, expressed at endogenous levels.<sup>44,45</sup> Importantly, Smad3 translocation is transient—cells treated with TGF- $\beta$ 1 exhibit rapid nuclear localization, and removal of TGF- $\beta$ 1 results in localization of Smad3 to the cytosol within minutes.<sup>44</sup>

Thiolated TGF- $\beta$ 1 (10 ng/mL) was patterned into 250  $\mu\text{m}$  stripes through a chrome photomask. To visualize TGF- $\beta$ 1 patterns during microscopy, gels were simultaneously patterned with 10  $\mu\text{M}$  fluorescently labeled thiolated transferrin. MEF GFP-Smad3 cells were seeded on top of the gels and allowed to spread for 3 h prior to imaging (Figure 5A). GFP signal was measured within the nucleus for cells both on and off the TGF- $\beta$ 1 patterns (Figure 5C), and we observed a  $\sim$ 1.2-fold increase in nuclear GFP signal for cells on top of the TGF- $\beta$ 1 patterns relative to those not on the pattern (Figure 5E). These data demonstrate that cells can signal through tethered TGF- $\beta$ 1 and that the TGF- $\beta$ 1 cellular response can be controlled through its spatial presentation. To remove the TGF- $\beta$ 1 signal from the hydrogels, the cell-laden gels were swelled with 50 mM PEG thiol and 10 mM LAP for 1 h. The gels were then flood irradiated with 50 mW/cm<sup>2</sup> 365 nm light for 180 s to release all patterned TGF- $\beta$ 1. After washing the gels with media for 90 min, cells remained attached and were subsequently imaged (Figure 5B). As expected, mean nuclear GFP intensity was  $\sim$ 2.0-fold lower after release of TGF- $\beta$ 1 (Figure 5D,E) compared to cells that were on the patterned regions initially. Finally, cell viability was also assessed before and after irradiation, and no significant decrease in viability was observed (Figure S4). Although we did not observe cells on patterns and off patterns influencing each other via paracrine signaling, open questions remain regarding how cells on patterns and off patterns communicate with each other and how patterning of signals may be used to influence entire cell



**Figure 5.** GFP-Smad3-expressing mouse embryonic fibroblasts respond to TGF- $\beta$ 1 patterned hydrogels. MEFs were seeded on hydrogels patterned with TGF- $\beta$ 1 (10 ng/mL) and cultured for 3 h (A). TGF- $\beta$ 1 was released *in situ* via a subsequent thiol–ene reaction with 50 mM PEG thiol, and cells were cultured for an additional 90 min (B). Inset of merged image shows cells within the pattern contain the Smad3 reporter in the nucleus (C, D). Mean nuclear GFP signal was measured for cells within the patterned region, outside the patterned region, and for cells after release of TGF- $\beta$ 1 (E). At least 260 cells were analyzed per condition. Letters a, b, and c denote groups that are statistically distinct ( $p < 0.001$ ) according to two-way ANOVA with Bonferroni testing for multiple comparisons. Data are shown as mean  $\pm$  standard error (SEM). TGF- $\beta$ 1 (red), DAPI (blue), Smad3 (green).

populations or define the length scales over which paracrine signaling occurs. Combined, these data demonstrate how the allyl sulfide functional handle can be used to pattern and release signaling proteins within hydrogel matrices in the presence of cells. Our patterning strategy also enables user control to manipulate cellular phenotype with spatial fidelity.

## CONCLUSIONS

In conclusion, we have designed an allyl sulfide handle that allows reversible thiol–ene protein patterning within cell-laden hydrogels. Due to the ability of the allyl sulfide to undergo chain transfer, the “ene” functionality is regenerated, and any tethered protein is released during each subsequent thiol–ene reaction. Moreover, this process of patterning and releasing proteins on-demand is repeatable, which enables facile replenishment of active proteins in hydrogel matrices. Importantly, we demonstrate that signaling proteins maintain their bioactivity and can be employed to control cell phenotype in a user-defined manner. TGF- $\beta$ 1 patterning induced localized cellular responses, and upon release of TGF- $\beta$ 1, cells returned to an unstimulated phenotype. We anticipate our protein patterning strategy will enable unique platforms to maintain and alter cell phenotype for precision medicine applications, where biomolecules can be tethered and released from a hydrogel to control cell phenotype for a wide array of applications, including immunoengineering and stem cell

expansion.<sup>46</sup> Combined, the allyl sulfide moiety is a powerful bioconjugation handle enabling real-time user control over the presentation of signaling proteins in hydrogels.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.8b00325.

Synthetic procedures and molecular characterization for all new compounds, hydrogel fabrication, protein thiolation and fluorescent labeling, protein swelling characterization by fluorescence recovery after photobleaching analysis, photokinetic calculations, procedures for single-photon and two-photon protein patterning and release, immunostaining protocol, ELISA protocol, and generation of GFP-Smad3 cell line and cell culture (PDF)

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## Notes

The authors declare no competing financial interest. No unexpected or unusually high safety hazards were encountered.

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