A reversible and repeatable thiol-ene bioconjugation for dynamic patterning of signaling proteins in hydrogels

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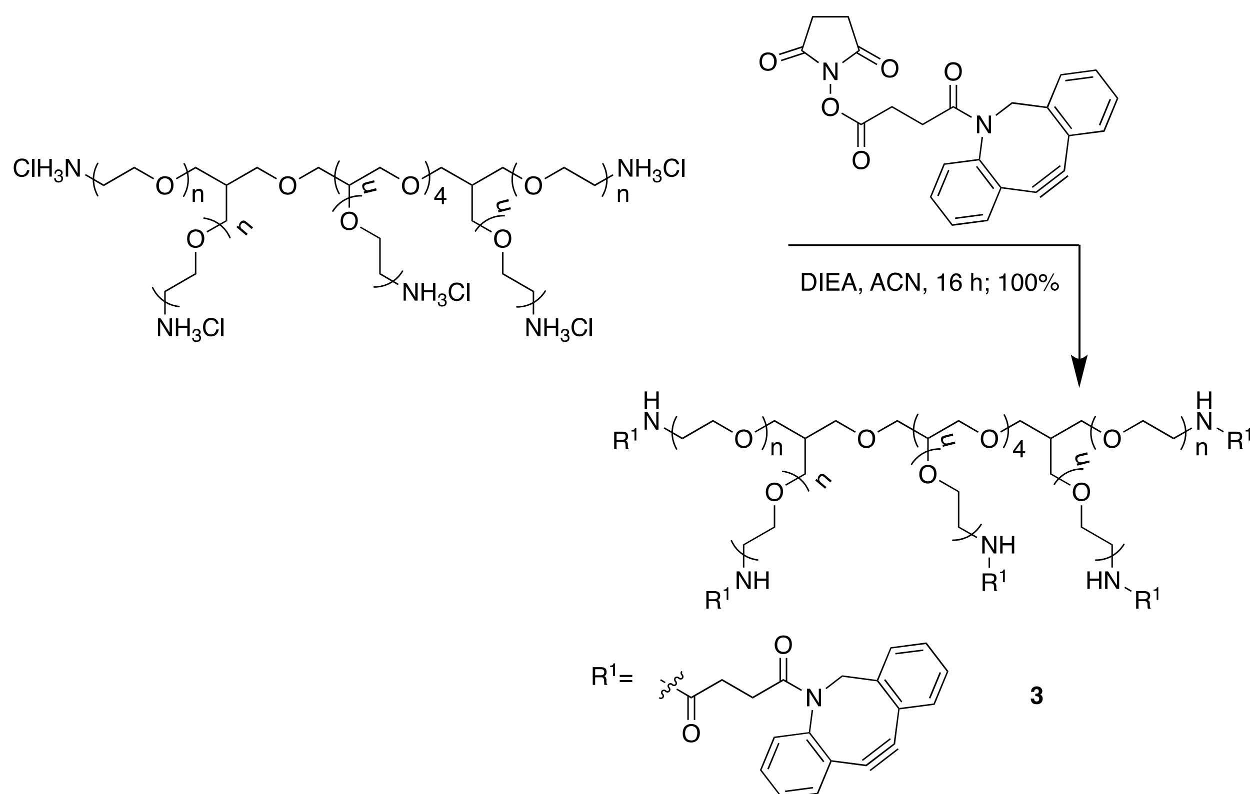
Index

S1. General synthetic methods 2
S2. Generation of azide-functionalized coverslips 7
S3. Hydrogel synthesis 7
S4. Thiolation and fluorescent labeling of proteins 7
S5. Characterization of transferrin swelling via fluorescence recovery after photobleaching 8
S6. Theoretical LAP consumption 9
S7. General procedure for photopatterning, protein release, and visualization of patterning by immunostaining 9
S8. Quantification of transferrin patterning via ELISA 11
S9. Generation of GFP-Smad3 cell line, cell culture, and *in situ* patterning and release of TGF-β1 12
S10. NMR Spectra 14
S11. References 18

S1. General synthetic methods

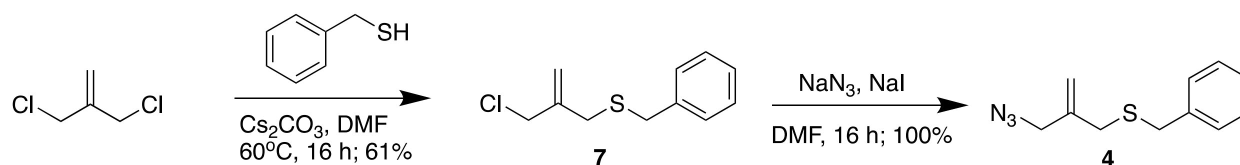
All moisture- and oxygen-sensitive reactions were carried out in flame-dried glassware under a nitrogen atmosphere. Unless otherwise noted, all reagents and solvents were the highest commercially available grades and used without further purification. All chemicals were purchased from Sigma Aldrich unless noted otherwise. *N*,*N*-dimethylformamide (DMF) and acetonitrile (ACN) were purified via a solvent purification system (Innovative Technologies) and water (H_2O) was purified with a MilliQ purification system (Millipore). Analytical thin layer chromatography (TLC) was used to monitor reactions and was performed on 0.25 mm pre-coated Silica Gel 60 F254 (Merck). Compounds were visualized with ultraviolet light (254 nm) and/or charring with *p*-anisaldehyde (15 g *p*-anisaldehyde, 5 mL H_2SO_4, 1 mL AcOH, 250 mL ethanol). Flash chromatography was performed on 230 – 400 mesh SiliaFlash® P60 silica gel (Silicycle).

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on Bruker AV-III 400 MHz spectrometer. Chemical shifts were reported relative to residual solvent peaks in parts per million ($CHCl_3$: ^{1}H δ 7.26, ^{13}C δ 77.0). Peak multiplicity is reported as singlet (s), doublet (d), multiplet (m), doublet of doublet (dd), etc. All spectra are reported in the supplemental information. High resolution electrospray ionization mass spectra (HRESI-MS) were obtained on a Thermo Finnigan LTQ Orbitrap (electrospray ionization, time-of-flight analyzer). Peptide analysis was performed with α-cyano-4-hydroxycinnamic acid (CHCA) matrix on a Voyager DE-STR MALDI-TOF (matrix-assisted laser desorption ionization, time-of-flight analyzer).



Scheme S1. Synthesis of 8-armed PEG_{40K}-DBCO **3**.

8-armed poly(ethyleneglycol) dibenzylcycooctyne 3. To a flask containing 8-armed PEG_{40K}-NH_3Cl (0.25 g, 0.00625 mmol, JenKem Technologies) was added ACN (0.9 mL). *N*-hydroxysuccinimidyl dibenzylcycolooctyne (DBCO, Click Chemistry Tools) was added as a 40 mM stock in ACN (1.48 mL, 0.059 mmol). *N,N*-diisopropylethylamine (DIEA, 19 μL, 0.11 mmol) was added, and the solution was stirred for 16 h. The macromer was precipitated in ice cold diethyl ether and pelleted via centrifugation (20 min at 3.0 rcf). The pellet was air dried overnight and dialyzed (10K MWCO) against H_2O for 24 h. Freeze drying afforded **3** as a pure, white powder (0.25 g, 0.00625 mmol, 100%) with 86% DBCO functionalization as determined by ^{1}H NMR. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (d, *J* = 7.7 Hz, 6H), 7.57 – 7.51 (m, 7H), 7.45 – 7.29 (m, 40H), 6.15 (s, 8H), 5.18 (d, *J* = 13.9 Hz, 7H), 3.66 (s, 3632H), 2.84 (ddd, *J* = 16.8, 8.5, 6.5 Hz, 7H), 2.49 (ddd, *J* = 15.0, 8.5, 6.4 Hz, 7H), 2.19 (dt, *J* = 15.3, 6.2 Hz, 7H), 1.96 (dt, *J* = 16.7, 6.2 Hz, 7H), 1.76 (s, 95H).

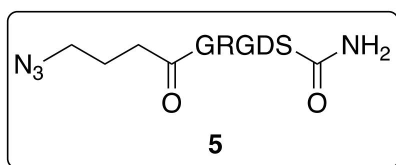


Scheme S2. Synthesis of allyl sulfide **4**.

2-chloromethyl-1-propene 3-benzyl sulfide 7. To a flask containing a suspension of Cs_2CO_3 (5.18 g, 23.0 mmol) in DMF (57 mL) was added 3-chloro-2-chloromethyl-1-propene (2.0 mL, 17.2 mmol) and benzyl mercaptan (2.2 mL 18.9 mmol). The suspension was heated to 60°C and stirred for 16 h. H_2O (70 mL) was added, and the aqueous layer was extracted with diethyl ether (70 mL). The combined organic layers were washed with H_2O (2 x 50 mL), dried with Mg_2SO_4, filtered and concentrated under reduced pressure. The crude oil was purified by column chromatography (0.5:99.5 → 1:99 EtOAc:hexanes) to afford **7** as a clear liquid (2.23 g, 10.45 mmol, 61%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 – 7.23 (m, 5H), 5.31 (q, J = 1.1 Hz, 1H), 5.11 (q, J = 1.1 Hz, 1H), 4.30 – 4.21 (m, 2H), 3.66 (s, 2H), 3.24 (d, J = 1.1 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 140.91, 137.88, 129.06, 128.54, 127.11, 117.49, 46.02, 35.39, 34.09. HRMS (EMM): calculated for $C_{11}H_{13}ClS$ $[M+Li]^+$: 219.0587, found 219.0596.

2-azidomethyl-1-propene 3-benzyl sulfide 4. To a solution of **7** (0.50 g, 2.35 mmol) in DMF (8 mL) was added NaN_3 (0.15 g, 2.35 mmol) and NaI (1.76 g, 11.75 mmol). The suspension was stirred for 16 h. EtOAc (10 mL) was added, and the organic layer was washed with H_2O (2 x 10 mL), brine (10 mL), dried with $MgSO_4$, filtered and concentrated under reduced pressure to afford **4** as a clear liquid (0.52 g, 2.35 mmol, 100%). ^{1}H NMR determined the product as sufficiently pure. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.20 (m, 5H), 5.23 (q, J = 1.2 Hz, 1H), 5.13 (tt, J = 1.0, 0.6 Hz, 1H), 4.00 – 3.90 (m, 2H), 3.67 (s, 2H), 3.14 (d, J = 1.1 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 139.22, 137.82, 129.05, 128.54, 127.12, 116.55, 53.92, 35.36, 34.74. HRMS (EMM): calculated for $C_{11}H_{13}N_3S$ $[M+Li]^+$: 226.0990, found 226.0996.

Azidobutyl-Gly-Arg-Gly-Asp-Ser-CONH$_2$ 5. Peptide synthesis was carried out on Rink Amide



MBHA resin (EMD Millipore) using standard HATU/Fmoc coupling chemistry conditions on a Protein Technologies Tribute Peptide Synthesizer. After synthesis of the Resin-Ser-Glu-Gly-Asp-Gly-NH$_2$ peptide, 4-azidobutanoic acid[1] was coupled to the *N*-terminus manually. To a vial containing 4-azidobutanoic acid (0.13 g, 1.0 mmol) was added HATU (0.38 g, 1.0 mmol) and DMF (4 mL). DIEA (0.35 mL, 2.0 mmol) was added and the yellow solution was transferred to a reaction vial with a fritted filter containing the resin (0.25 mmol). The solution was stirred for 4 h. The solvent was filtered, and the resin was washed with DMF (3x), and CH_2Cl_2 (3x). The resin tested negative for free amines via the Kaiser Ninhydrin test. To cleave the peptide from the resin, the resin was swelled for 30 min in CH_2Cl_2. A solution of 88:5:5:2 trifluoroacetic acid (TFA):phenol:H_2O:triisopropyl silane (4 mL) was added. The suspension was stirred for 1 h. The filtrate was collected, and the resin was washed with TFA (2x). The combined TFA washes were concentrated to ~1 mL by gently blowing air over the solution. The peptide was precipitated into ice cold diethyl ether (45 mL) and pelleted via centrifugation (15 mins at 3.0 rcf). The peptide was purified by high performance liquid chromatography (HPLC) on a Waters 2545 HPLC equipped with an XSELECT CSH Prep C18 column using a 20:80 ACN:H_2O $\rightarrow$ 95:5 ACN:H_2O gradient. MALDI-TOF analysis revealed peptide **5** as sufficiently pure (Fig. S1).

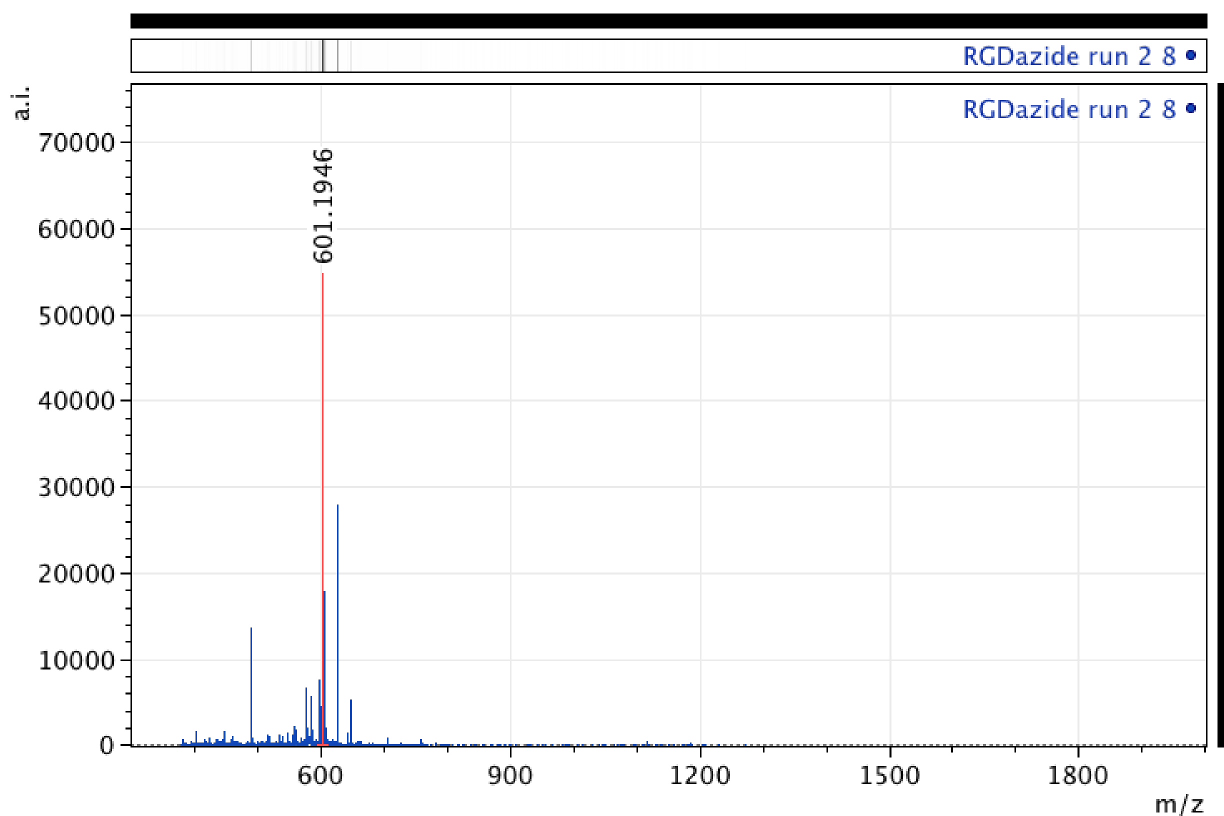
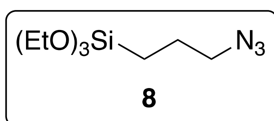


Figure S1. MALDI-TOF spectrum of purified peptide **5**.

3-azidopropyl triethoxysilane 8. To a flask containing NaN_3 (1.95 g, 30.0 mmol) and



tetrabutylammonium bromide (1.39 g, 4.0 mmol) was added ACN (75 mL). 3-chloropropyl triethoxysilane (4.82 mL, 20.0 mmol) was added. The solution was heated to 80 ^{o}C and stirred for 48 h. After cooling, the suspension was diluted with diethyl ether (25 mL). The suspension was filtered, and the solids were rinsed with diethyl ether (2 x 10 mL). The combined washes were concentrated under reduced pressure to afford **8** as clear liquid (4.93 g, 19.9 mmol, 99%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.82 (q, *J* = 7.0 Hz, 7H), 3.27 (t, *J* = 7.0 Hz, 2H), 1.79 – 1.64 (m, 2H), 1.23 (t, *J* = 7.0 Hz, 10H), 0.73 – 0.62 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 58.43, 53.80, 22.65, 18.26, 7.58. HRMS (EMM): calculated for $C_9H_{21}N_3O_3Si$ $[M+Li]^+$: 254.3110, found 254.1194.

S2. Generation of azide-functionalized coverslips

12 mm circular glass coverslips (Fisherbrand) were O_2 plasma treated. The coverslips were submerged in a solution of 20:3:1 toluene:3-azidopropyl trithoxysilane **8**:propylamine and allowed to react for 1 h. The coverslips were rinsed with toluene (3x) and dried in an 80 ^{o}C oven.

S3. Hydrogel synthesis

The following is a general procedure to synthesize three 15 µL hydrogels. To an epitube containing 10 wt% 8-armed PEG_{40K} DBCO **3** (36.75 µL, 14 mM final DBCO concentration) was added PBS (3.375 µL). A 35 mM stock of allyl sulfide **4** (1.5 µL, 1 mM final allyl sulfide concentration) and a 35 mM stock of azido RGDS **5** (3 µL, 2 mM final RGDS concentration) were added. The solution was immediately vortexed and allowed to react for 5 min. 20 wt% 4-armed PEG_{20K}-N_3 **6** (7.875 µL, 6 mM final N_3 concentration) was added[2], vortexed, and three 15 µL droplets were immediately pipetted onto a Sigmacote glass slide. Azide functionalized coverslips were placed on top of the droplets, and the gel was allowed to polymerize for 5 min. The gels were swelled in 10 mM $mPEG_7$-N_3 in PBS overnight prior to use in experiments. Gels have a Young's modulus (E) of 6 kPa as characterized by rheology (Fig. S2).

S4. Thiolation and fluorescent labeling of proteins

Human transferrin was obtained from Sigma Aldrich, human transforming growth factor-β1 (TGF-β1) was obtained from R&D Systems, and Alexa Fluor 555-labeled ovalbumin was obtained from ThermoFisher. All thiolations were performed via treatment with 10 eq. NHS-$PEG_{3.4K}$ thiol (Nanocs) in PBS pH 8.0 with 2 mM EDTA for 1 h. Unreacted PEG thiol could be removed via centrifugation on Zeba™ Spin Desalting Columns, 7K MWCO (ThermoFisher). The extent of thiolation was determined via Ellman's assay (1.3 thiols/transferrin). Generally, thiolated proteins were employed without purification. Fluorescently-labeled thiolated transferrin was generated by the inclusion of 10 eq. Alexa Fluor 488 5-SPD Ester (Thermo Fisher) during the thiolation step.

S5. Characterization of transferrin swelling via fluorescence recovery after photobleaching

The diffusion coefficient for proteins within the allyl sulfide hydrogels was estimated by using fluorescence recovery after photobleaching (FRAP).[3] The fluorescently-labelled protein (fluorescein-transferrin) was diffused into the hydrogel overnight. Photobleaching was performed on a Nikon A1R laser scanning microscope. Bleaching was initiated using a 1 s pulse of 405 nm light in a circular region (50 μM diameter), and performed in triplicate. A low numerical aperture objective (0.4) was used to approximate a columnar bleached volume in the vicinity of the focal plane, and diffusion was assumed to occur in the radial dimension only. The average fluorescence intensity was monitored over the recovery period and fit to the equation:

$$f(t) = e^{-\frac{\tau_D}{2t}}\left[I_0\left(\frac{\tau_D}{2t}\right) + I_1\left(\frac{\tau_D}{2t}\right)\right](1-m) \qquad \text{Eq. S1}$$

where $\tau_D \equiv \frac{w^2}{D_f}$

where I_0 and I_1 are modified Bessel functions of the first kind, w is the radius of the bleached volume (25 μm), and *(1-m)* is included to account for the steady-state offset, to yield the diffusion coefficient D_f = 11.9 ± 1.1 μm^2 s^{-1}. An estimate of the time needed for the protein to diffuse into the gel (~130 μm) can be provided by the characteristic diffusion time

$$t = \frac{L^2}{D_f} \approx 1400\ s \qquad \text{Eq. S2}$$

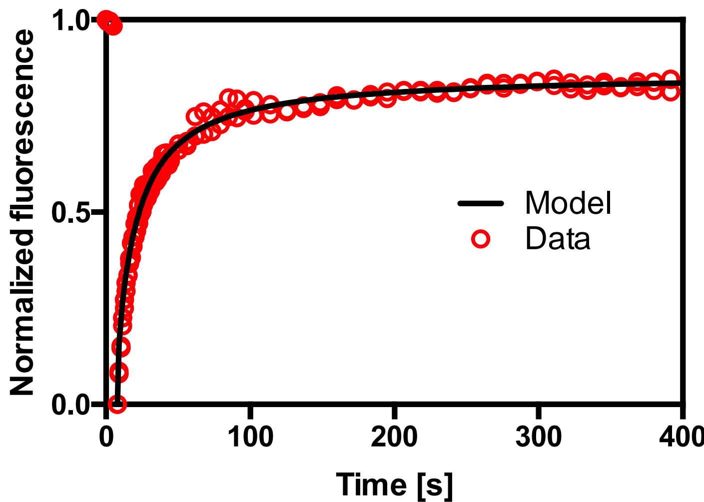


Figure S2: Fitting the fluorescence recovery data to **Eq. S1** provides the diffusion coefficient of the fluorescently-labelled transferrin within the hydrogel.

S6. Theoretical LAP consumption

Theoretical photoinitiator consumption was calculated assuming first-order kinetics of the photolysis reaction:

$$\frac{d[LAP]}{dt} = -k[LAP] \qquad \text{Eq. S3}$$

where the rate constant, *k*, is defined by:

$$k = \frac{\phi I \varepsilon\, ln(10)}{N_A h\nu} \qquad \text{Eq. S4}$$

where ϕ is the quantum yield of the initiator (assumed unity), *I* is the incident light intensity (10 mW cm^{-2}), and ε is the molar extinction coefficient at the wavelength of interest (ε_{365} 218 M^{-1} cm^{-1}). The frequency of light (ν), Avogadro's number (N_A), the Planck constant (*h*), and *ln(10)* are included for unit conversion. Solving, this gives k = 0.0153 s^{-1}. And the LAP concentration can then be calculated from the integrated rate equation:

$$[LAP] = [LAP]_0 e^{-0.0153t} \qquad \text{Eq. S5}$$

S7. General procedure for photopatterning, protein release, and visualization of patterning by immunostaining

Hydrogels were swelled with desired concentrations of thiolated transferrin and LAP for 4 h. Gels were placed on a chrome photomask of a desired pattern and irradiated through the mask with 365 nm light of desired light intensity and dose from an Omnicure Series 1000 light source. Gels were washed with PBS (3 x 1 h) to remove unreacted transferrin.

To release proteins, patterned hydrogels were swelled with PEG_{1K} thiol (Nanocs) and LAP of desired concentration for 1 h. Gels were irradiated through a chrome photomask with 365 nm light using an Omnicure Series 1000 light source. Gels were washed with PBS (3 x 1 h) to remove released transferrin.

To visualize gels via immunostaining, gels were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h. Gels were incubated with 5 µg/mL rabbit anti-human transferrin (Abcam ab82411) in 1% BSA in PBS overnight at 4 ^{o}C. Gels were washed with PBS with 0.1% Tween-20 (PBST, 3 x 1 h). Gels were incubated with goat anti-rabbit Alexa Fluor 488 (1:300) in 1% BSA in PBS for 1 h. After washing with PBST (3 x 1 h), gels were images on a Zeiss Examiner Z1 confocal microscope. For quantitative image analysis, all gels were visualized 10 µm from the surface of the gel with the same laser power and gain for each analysis.

Three-dimensional photopatterning was achieved *via* two-photon photoinitiation using a femtosecond pulsed laser (Coherent Chameleon) at 740 nm with a maximum laser output (measured at the objective) of 260 mW. Two-photon photopatterning was performed on a laser scanning microscope (Zeiss LSM 710) using a Fluar 10x 0.5 NA objective. A pixel size of 420 nm and pixel dwell time of 0.5 µsec were used, and regions were scanned in the z-dimension (Δz=1 µm) to produce three-dimensional patterns. Laser intensity and averaging were varied to determine the optimal laser settings for photopatterning and photorelease. Based on these results, 100% laser power and a single scan were used for both conjugation and release experiments.

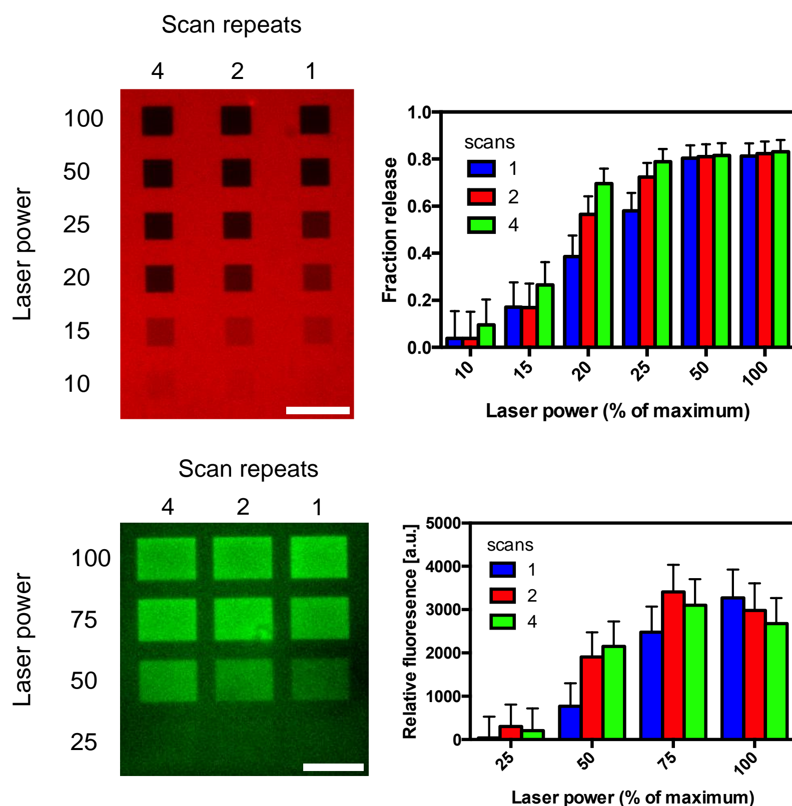


Figure S3. Characterization of two-photon protein release (ovalbumin-AF555) and photopatterning (transferrin-AF488) within allyl sulfide-functionalized hydrogels. Scale bars = 50 µm.

S8. Quantification of transferrin patterning via ELISA

Gels were swelled with known concentrations of thiolated transferrin (24, 240, 24,00, 24,000, or 240,000 pg/mL) and 100 µM LAP. The entire gel was irradiated with 5 mW/cm^2 365 nm light for 180 s using an Omnicure Series 1000 light source. Gels were washed with PBST (3 x 1 h), and then swelled with 50 mM PEG$_{1K}$ thiol and 10 mM LAP for 1 h. The entire gel was irradiated with 50 mW/cm^2 365 nm light for 180 s. Gels were placed in 300 µL PBS for 24 h. The concentration of transferrin was measured in the PBS solution using the Human Transferrin ELISA Kit (ThermoFisher). Actual concentration of transferrin tethered to the hydrogel was calculated using the known volume of the hydrogel (15 µL).

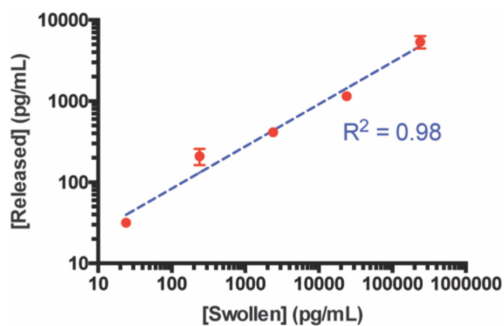


Figure S4. Hydrogels were swelled with known concentrations of thiolated transferrin and 100 μM LAP for 4 h. The gel was irradiated with 5 mW/cm^2 365 nm light for 180 s. Unbound protein was washed away, and gels were swollen with 50 mM PEG$_{1K}$-SH and 10 mM LAP and irradiated with 50 mW/cm^2 365 nm light for 180 s. Gels were placed in PBS and allowed to deswell overnight. Transferrin concentration of the PBS solution was determined via ELISA and plotted against initial transferrin concentrations (red). Power law analysis revealed a linear relationship between amount of transferrin swelled and amount detected after patterning and release (blue).

We observed a power law relationship between the amount of transferrin swelled into the hydrogel for patterning and the actual amount measured upon release (Fig. 5). Specifically, the amount of transferrin tethered to the hydrogel can be predicted by the following relationship: $[\text{actual}] \propto [\text{swelled}]^{1/2}$.

S9. Generation of GFP-Smad3 cell line, cell culture, and *in situ* patterning and release of TGF-β1

Mouse embryonic fibroblasts (MEFs) were maintained in DMEM High glucose media supplemented with 10% fetal bovine serum, penicillin/streptomyocin, and fungizone at 37 °C in 5% CO_2. Transgenic MEF cells stably expressing GFP-Smad3 were constructed using retroviral mediated gene transfer as previously described.[4] Cells stably expressing GFP-Smad3 were purified from the virus exposed population of MEFS using fluorescence activated cell sorting (FACS).

For patterning and release experiments *in situ*, hydrogels were swelled with 100 μM LAP, 10 μM Alexa Fluor 555-labeled thiolated transferrin, and 100 nM thiolated TGF-β1 for 5 min. Gels were irradiated through a chrome photomask for 180s with 5 mW/cm^2 365 nm light from an Omnicure Series 1000 light source. Gels were washed with media (3 x 5 min). 100,000 MEF GFP-Smad3 cells were seeded on the patterned hydrogels in media supplemented with 10 μM Y276322 (Stemcell Technologies), which was required for efficient cell spreading. After 3 h, cells were treated with 0.2

µg/mL Hoescht 33258 (ThermoFisher) for 5 min and imaged on a Zeiss Examiner Z1 confocal microscope. To release TGF-β1, gels were swollen with 50 mM PEG_{1K} thiol and 10 mM LAP for 1 h. Gels were irradiated with 50 mW/cm^2 365 nm light for 180 s and washed with media (3 x 30 min). Gels were imaged via confocal microscopy. Nuclear GFP intensity was measured in ImageJ via gating for Hoescht 33258 staining.

For cell viability studies, MEF GFP-Smad3 cells were seeded at 30,000 cells/well in a 24 well plate with and without 10 µM LAP. Cells were irradiated with 50 mW/cm^2 365 nm light for 180 s. After 1 h, cell viability was assed via Trypan blue staining on a Life Technologies Countess II FL cell counter (Fig. S4).

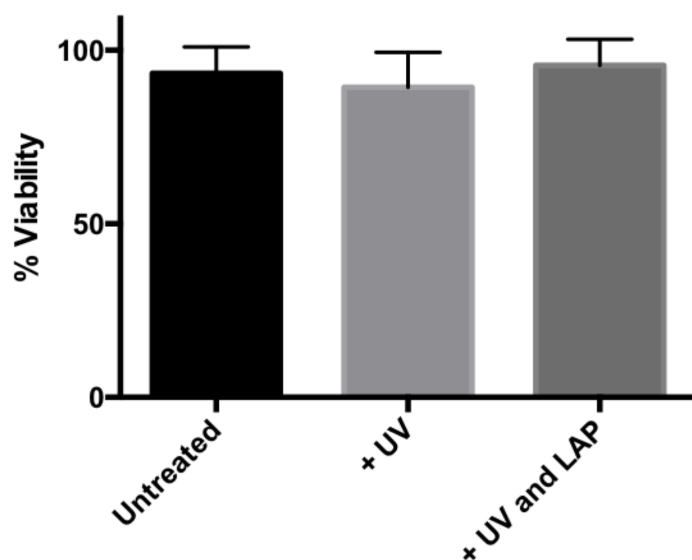
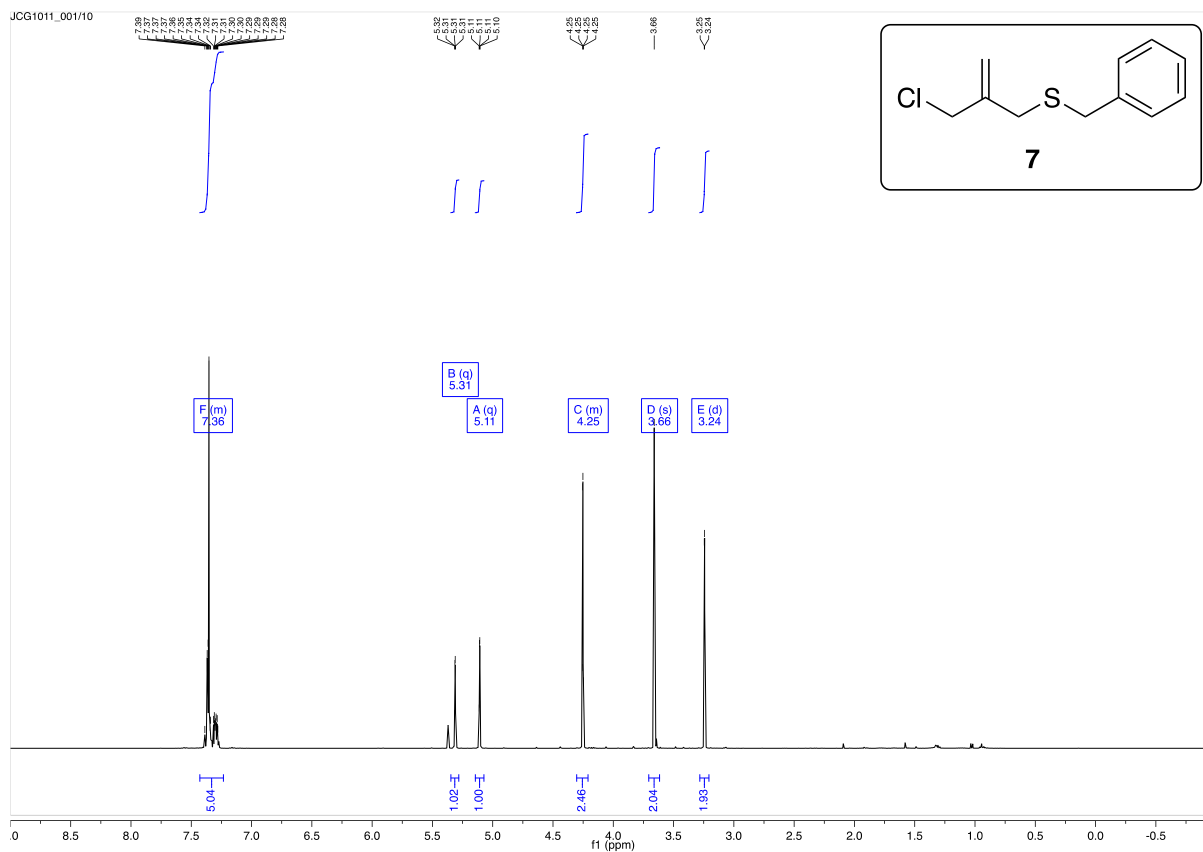
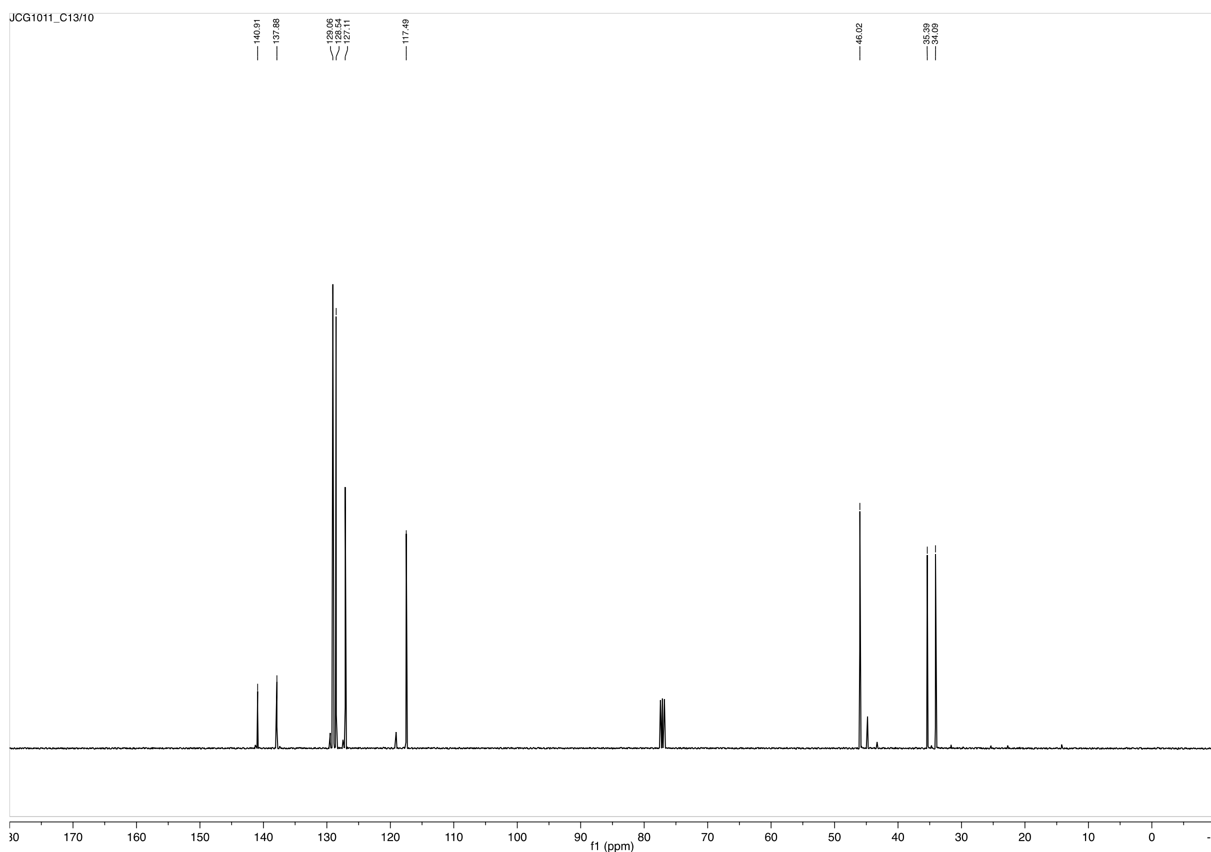


Figure S5. Cells were treated with or without LAP and irradiated with 50 mW/cm^2 365 nm light for 180 s. Cell viability was assessed after 1 h via Trypan blue staining.

S10. NMR Spectra


JCG1011_001/10
Cl
S
7
F (m)
7.36
B (q)
5.31
A (q)
5.11
C (m)
4.25
D (s)
3.66
E (d)
3.24
5.04
1.02
1.00
2.46
2.04
1.93
f1 (ppm)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46.02
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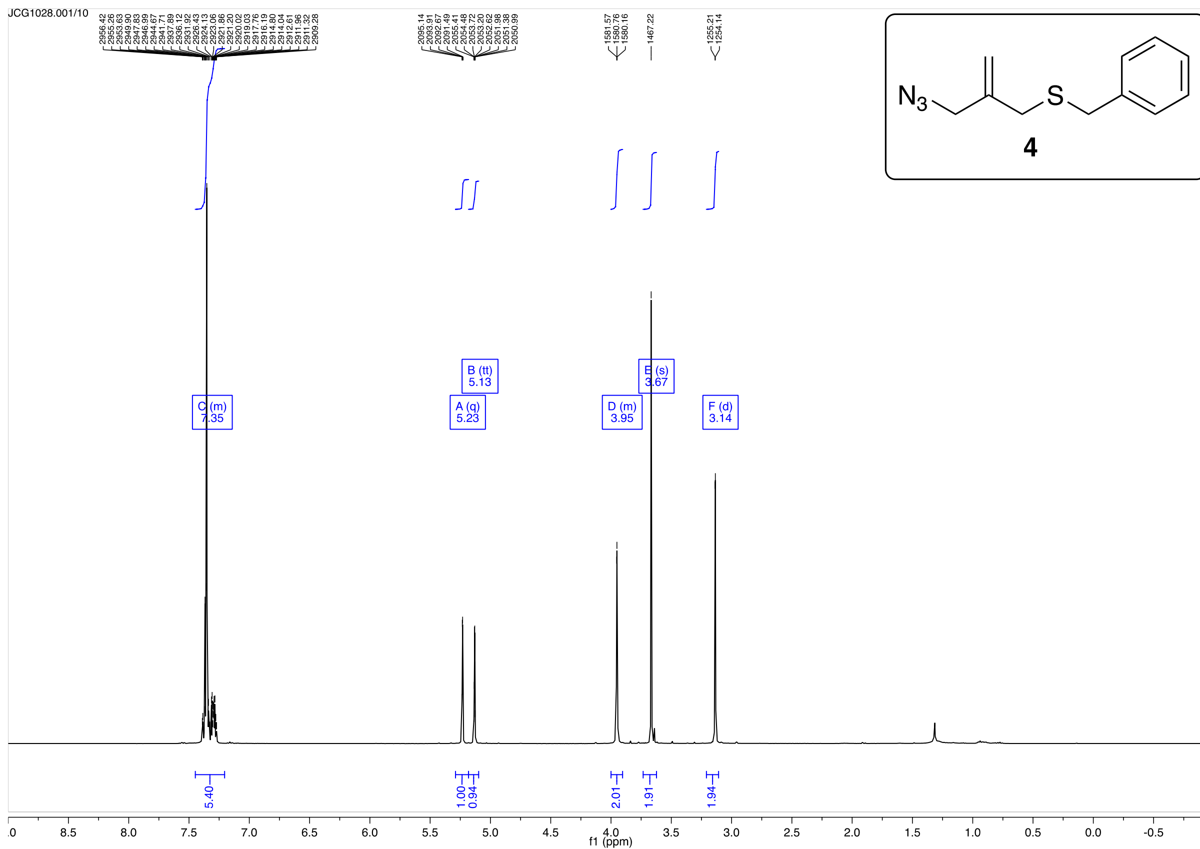
f1 (ppm)


JCG1028.001/10
N3
S
4
C (m)
7.35
A (q)
5.23
B (tt)
5.13
D (m)
3.95
E (s)
3.67
F (d)
3.14
5.40
1.00
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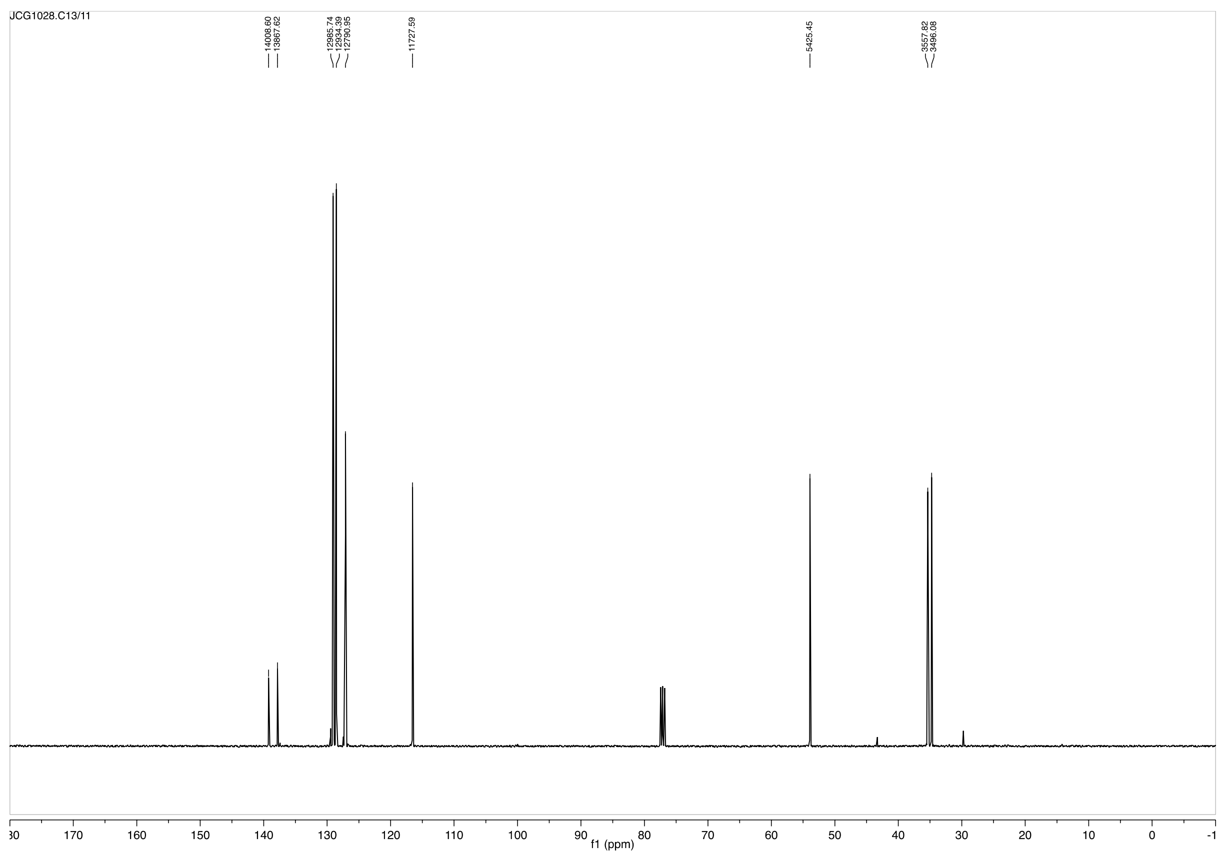
2.01
1.91
1.94
f1 (ppm)


JCG1028.C13/11
f1 (ppm)

JCG1071_001/10

$(EtO)_3Si$–$CH_2CH_2CH_2$–N_3

8

E (q) 3.82

C (t) 3.27

B (m) 1.72

D (t) 1.23

A (m) 0.68

6.73 2.31 2.26 10.04 2.00

f1 (ppm)

JCG1071_C13/10

58.43 53.80 22.65 18.26 7.58

f1 (ppm)

S11. References

[1] M. Huang, Z. Deng, J. Tian, T. Liu, Synthesis and biological evaluation of salinomycin triazole analogues as anticancer agents. *Eur. J. Med. Chem.* **2017**, *127*, 900-908.

[2] B. J. Adzima, Y. Tao, C. J. Kloxin, C. A. DeForest, K. S. Anseth, C. N. Bowman, Spatial and temporal control of the alkyne–azide cycloaddition by photoinitiated Cu(II) reduction. *Nature Chem.* **2011**, *3*, 256-259.

[3] E. A. Reits, J. J. Neefjes, From fixed to FRAP: measuring protein mobility and activity in living cells. *Nat. Cell Biol.* **2001**, *3*, E145-147.

[4] R. A. Erickson, X. Liu, Association of v-ErbA with Smad4 Disrupts TGF-β Signaling. *Mol. Biol. Cell* **2009**, *20*, 1509-1519.