Supplementary Information for

Engineered modular biomaterial logic gates for environmentally triggered therapeutic delivery

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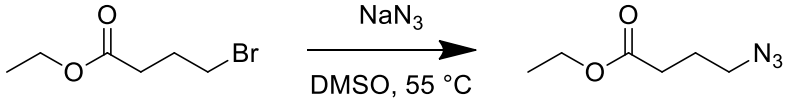
General synthetic information

Chemical reagents and solvents were purchased from either Sigma-Aldrich or Fisher Scientific and used as received. Peptide synthesis reagents were purchased from either ChemPep or Chem-Impex and used as received. Deionized water (dH_2O) was generated by a U.S. Filter Corporation Reverse Osmosis System with a Desal membrane. Synthetic chemical reactions were performed under a nitrogen atmosphere in oven-dried glassware and stirred with a Teflon-coated magnetic stir bar unless otherwise noted. Solvents were removed *in vacuo* with a Büchi Rotovapor R-3 equipped with a V-700 vacuum pump and V-855 vacuum controller and a Welch 1400 DuoSeal Belt-Drive high vacuum pump. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) data was collected at 298 K on Bruker instruments and chemical shifts are reported relative to tetramethylsilane (TMS, $\delta = 0$). Microwave-assisted peptide synthesis was performed on a CEM Liberty 1. Semi-preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) was performed on a Dionex Ultimate 3000 equipped with a variable multiple wavelength detector, automated fraction collector, and Thermo 5 µm Synchronis silica 250 x 21.2 mm C18 column. Lyophilization was performed on a LABCONCO FreeZone 2.5 Plus freeze-dryer equipped with a LABCONCO rotary vane 117 vacuum pump. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry was performed in reflectron positive ion mode on a Bruker AutoFlex II using a matrix of α-cyano-4-hydroxycinnamic acid:2,5-dihydroxy benzoic acid (2:1). High-resolution mass spectrometry (HRMS) was performed on a Thermo Linear Trap Quadrupole Orbitrap Xcalibur 2.0 DS. The light source for the photochemical cleavage was a Lumen Dynamics OmniCure S1500 Spot UV Curing system with an internal 365 nm filter and an external 360 nm cut-on longpass filter. Light intensity was measured using a Cole-Parmer Radiometer (Series 9811-50, $\lambda = 365$ nm). Fluorescence readings were acquired on a SpectraMax M5 spectrometer using Thermo Scientific Nunc black polypropylene 96-well plates. Rheological measurements were performed on an Anton Paar MCR301 equipped with a C-PTD200 Peltier plate and a CP25-1 cone and plate geometry. Fluorescent microscopy was performed on a Nikon Eclipse TE2000-U. Confocal microscopy was performed at the University of Washington Keck Microscopy Center on a Leica SP8X. Polymerase chain reaction (PCR) was performed in a Bioer LifeECO thermal cycler. Protein expression was performed in a Thermo Scientific MaxQ 4000 shaker incubator. Cells were lysed using a Fisher Scientific Model 505 Sonic Dismembrator. Mammalian cell culture was performed in a NuAire LabGard ES NU-437 Class II Type A2 Biosafety Cabinet. Cells were maintained in a Sanyo inCu saFe® MCO-17AC incubator at 37 °C and 5% CO_2. Flow cytometry was performed at the University of Washington Pathology Flow Cytometry Core Facility on a BD Biosciences LSR II Flow Cytometer.

Supplementary Method 1: Synthesis of 2,5-dioxopyrrolidin-1-yl 4-azidobutanoate (N_3-OSu)

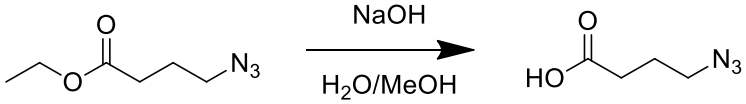
4-azidobutanoic acid (N_3-COOH) was synthesized following a published synthetic route[1], and used as a synthetic precursor for 2,5-dioxopyrrolidin-1-yl 4-azidobutanoate (N_3-OSu).

Synthesis of ethyl 4-azidobutanoate:



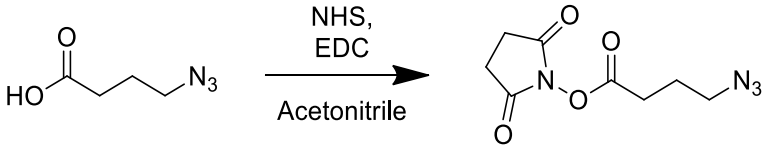
Ethyl-4-bromobutyrate (49.6 g, 254 mmol) and sodium azide (24.7 g, 378 mmol, 1.5x) were dissolved in dimethyl sulfoxide (DMSO, 375 mL) and reacted at 55 °C overnight under a nitrogen atmosphere. The reaction mixture was diluted with water (250 mL) and extracted into diethyl ether (3 x 250 mL). The organic layer was washed with water (250 mL) and brine (250 mL), dried over $MgSO_4$, filtered, and concentrated *in vacuo* to give the intermediate ethyl-4-azidobutanoate (37.7 g, 240 mmol, 94.5% yield) as a clear liquid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.17 (q, *J* = 7.1 Hz, 2H), 3.38 (t, *J* = 6.7 Hz, 2H), 2.43 (t, *J* = 7.2 Hz, 2H), 1.94 (p, *J* = 7.1 Hz, 2H), 1.29 (t, *J* = 7.1 Hz, 3H).

Synthesis of 4-azidobutanoic acid (N_3-COOH):



Ethyl-4-azidobutanoate (37.66 g, 240 mmol) was dissolved in aqueous sodium hydroxide (1 M, 250 mL). Methanol was added to homogenize the solution (175 mL) and the reaction mixture was stirred at room temperature for 3 hr. The methanol was removed *in vacuo* and aqueous hydrochloric acid was added dropwise to the reaction mixture until the pH became 1. The product was extracted into diethyl ether (3 x 250 mL), dried over magnesium sulfate ($MgSO_4$), filtered, and concentrated on a rotary evaporator to give 4-azidobutanoic acid (denoted N_3-COOH) as a faint yellow liquid (30.7 g, 237 mmol, 98.8% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.64 (br s, 1H), 3.41 (t, *J* = 6.7 Hz, 2H), 2.50 (t, *J* = 7.2 Hz, 2H), 2.01 – 1.88 (m, 2H). These spectral data matched those previously reported[1].

Synthesis of 2,5-dioxopyrrolidin-1-yl 4-azidobutanoate (N_3-OSu):



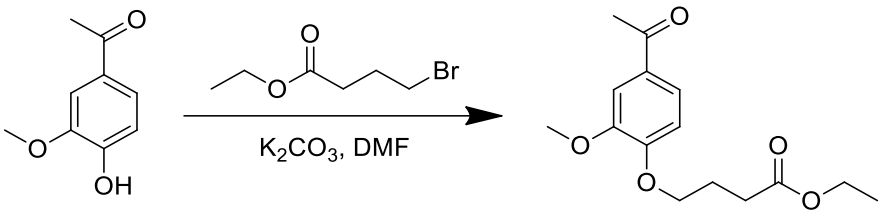
N_3-COOH (5.00 g, 38.7 mmol), N-hydroxysuccinimide (5.79 g, 50.3 mmol, 1.3x), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl 9.65 g, 50.3 mmol, 1.3x) were combined in a flame-dried flask and purged with nitrogen. Dry acetonitrile (50 mL) added *via* syringe to solubilize the reagents. The reaction mixture was stirred overnight at room temperature. The acetonitrile was removed *in vacuo* and the reaction products were dissolved in dichloromethane (DCM, 100 mL), washed with water (3 x 100 mL), dried over $MgSO_4$, filtered,

and concentrated *via* rotary evaporation to give the pure 2,5-dioxopyrrolidin-1-yl 4-azidobutanoate product (denoted N_3-OSu) as a white solid (7.98 g, 35.3 mmol, 91% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.45 (t, *J* = 6.6 Hz, 2H), 2.85 (s, 4H), 2.74 (t, *J* = 7.2 Hz, 2H), 2.09 – 1.96 (m, 2H).

Supplementary Method 2: Synthesis of 2,5-dioxopyrrolidin-1-yl 4-(4-(1-((4-azidobutanoyl)oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoate (N_3-*o*NB-OSu)

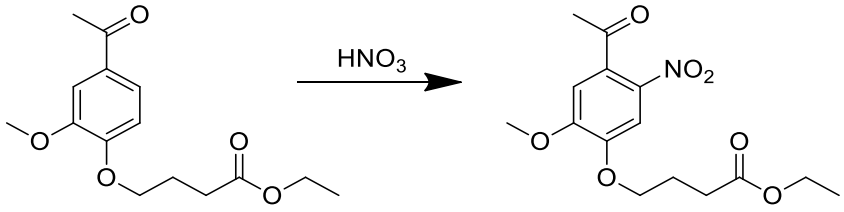
The synthesis of N_3-*o*NB-OSu was based on a published synthetic route[1] involving the reaction of 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoic acid[2] and N_3-COOH (Supplementary Method 1) with minor modifications.

Synthesis of ethyl 4-(4-acetyl-2-methoxyphenoxy)butanoate:



Acetovanillone (30.0 g, 180 mmol) and ethyl-4-bromobutyrate (42.3 g, 217 mmol, 1.2x) were dissolved in dimethylformamide (DMF, 150 mL) in a flame-dried flask. Potassium carbonate (37.4 g, 271 mmol, 1.5x) was added, and the reaction mixture was stirred overnight at room temperature under a nitrogen atmosphere. The reaction mixture was slowly poured into stirring water (1.5 L), and stirring was continued at room temperature for 2 hours. The mixture was stored overnight at 4 °C to precipitate the product. The product was vacuum filtered and lyophilized to give the intermediate, ethyl 4-(4-acetyl-2-methoxyphenoxy)butanoate, as a yellow solid (49.29 g, 175.8 mmol, 97.4% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.54 (m, 2H), 6.89 (d, *J* = 8.2 Hz, 1H), 4.20 – 4.09 (m, 4H), 3.91 (s, 3H), 2.60 – 2.48 (m, 5H), 2.18 (p, *J* = 6.8 Hz, 2H), 1.26 (t, *J* = 7.1 Hz, 3H).

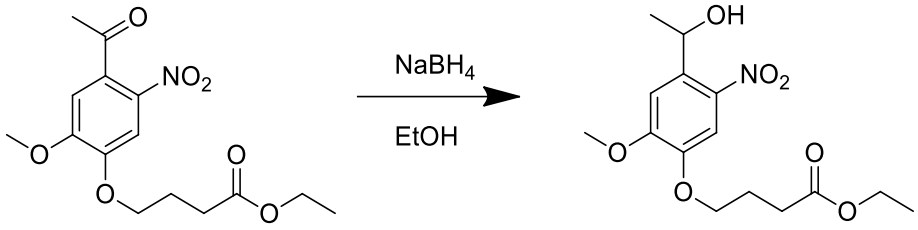
Synthesis of ethyl 4-(4-acetyl-2-methoxy-5-nitrophenoxy)butanoate:



Nitric acid (140 mL) was cooled in an ice bath in a round bottom flask (1 L). Ethyl 4-(4-acetyl-2-methoxyphenoxy)butanoate (48.65 g, 173.6 mmol) was added in small portions to the continually stirred solution, allowing for full dissolution before subsequent additions (approximately 40 minutes to dissolve the product). The reaction mixture was stirred while in the ice bath, and progress was monitored *via* thin-layer chromatography (TLC, hexanes/ethyl acetate/acetic acid, 50:50:1, product R_f ~ 0.5). Upon reaction completion (~40 minutes), the reaction mixture was added dropwise to continually stirred ice-cold water (1.5 L). This mixture was further agitated at room temperature for 1 hour, after which the crude product was precipitated overnight at 4 °C. The precipitate was filtered, rinsed with ice-cold water, and lyophilized to give a yellow-brown solid. The solid was dissolved in 65 °C ethanol (700 mL), cooled to room temperature, and recrystallized overnight at 4 °C. The product was vacuum filtered, rinsed with -20 °C ethanol, and dried to give the intermediate, ethyl 4-(4-acetyl-2-methoxy-5-nitrophenoxy)butanoate, as a yellow solid (33.90 g, 104.2 mmol, 60.0% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.60 (s, 1H), 6.74 (s, 1H), 4.20 – 4.11

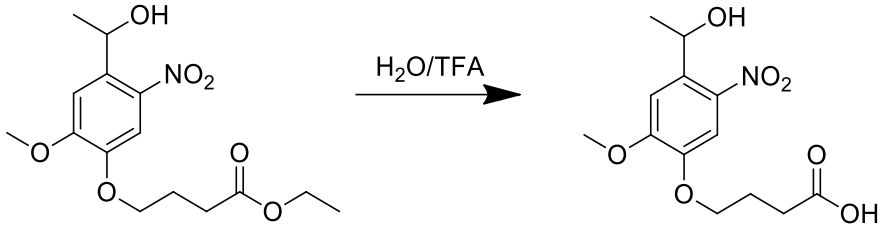
(m, 4H), 3.95 (s, 3H), 2.54 (t, *J* = 7.2 Hz, 2H), 2.49 (s, 3H), 2.19 (p, *J* = 6.7 Hz, 2H), 1.26 (t, *J* = 7.1 Hz, 3H).

Synthesis of ethyl 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoate:



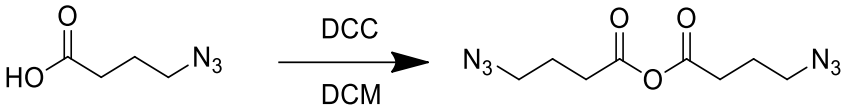
Ethyl 4-(4-acetyl-2-methoxy-5-nitrophenoxy)butanoate (47.12 g, 144 mmol) was dissolved in anhydrous ethanol (700 mL), purged with nitrogen, and heated to 38 °C. Sodium borohydride (5.33 g, 141 mmol, 0.97x) was added to the reaction mixture in equal portions every 5 minutes over a 40 minute period. The mixture was purged with nitrogen and stirred overnight at 38 °C. The reaction mixture was added to water (7 L), stirred 1 hour at room temperature, and the product was precipitated overnight at 4 °C. The suspension was filtered, rinsed with ice-cold water, and lyophilized to yield the intermediate, ethyl 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoate, as a dark yellow solid (37.84 g, 115.6 mmol, 80% yield). ^{1}H NMR (300 MHz, DMSO) δ 7.53 (s, 1H), 7.36 (s, 1H), 5.48 (d, *J* = 4.4 Hz, 1H), 5.33 – 5.19 (m, 1H), 4.17 – 3.99 (m, 4H), 3.91 (s, 3H), 2.48 (m, 2H), 1.99 (p, *J* = 6.8 Hz, 2H), 1.37 (d, *J* = 6.2 Hz, 3H), 1.19 (t, *J* = 7.1 Hz, 3H).

Synthesis of 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoic acid:

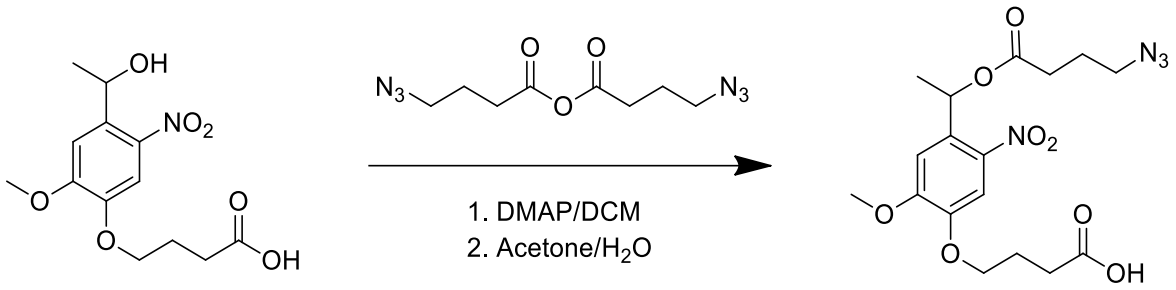


Ethyl 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoate (37.84 g, 115.6 mmol) was added to a solution of water (1 L) and trifluoroacetic acid (TFA, 96 mL) in a round-bottomed flask (2 L). The reaction mixture was stirred and heated to 90 °C. After 6 hours, additional TFA (48 mL) was added to the reaction mixture. After another 18 hours, additional TFA (48 mL) was added to the reaction mixture. After another 6 hours, the reaction mixture was filtered to remove the black solid. The filtrate was cooled to room temperature, upon which the precipitate was dissolved in minimal aqueous sodium hydroxide (1 M, 150 mL) and acidified to a pH of 1 *via* dropwise addition of hydrochloric acid. The precipitated product was vacuum filtered, washed, and lyophilized to give 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoic acid as a yellow solid (20.08 g, 93.6 mmol, 81.0% yield). ^{1}H NMR (300 MHz, DMSO) δ 12.18 (s, 1H), 7.55 (s, 1H), 7.37 (s, 1H), 5.50 (m, 1H), 5.27 (m, 1H), 4.07 (t, *J* = 6.5 Hz, 2H), 3.92 (s, 3H), 2.40 (t, *J* = 7.3 Hz, 2H), 1.96 (p, *J* = 6.8 Hz, 2H), 1.37 (d, *J* = 6.2 Hz, 3H). These spectral data matched those previously reported[2].

Synthesis of 4-(4-(1-((4-azidobutanoyl)oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoic acid (N_3-*o*NB-COOH):

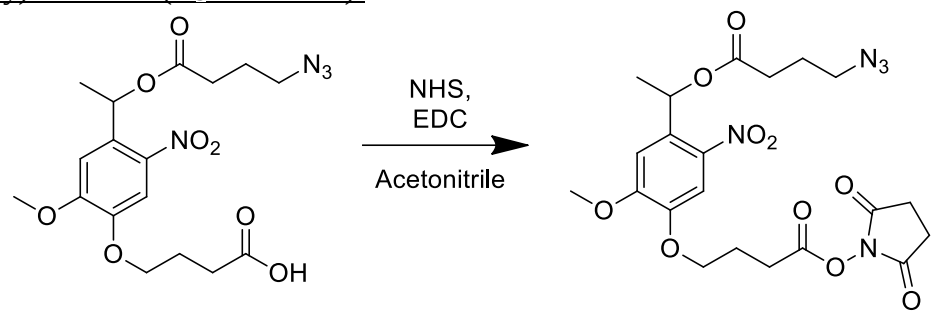


N_3-COOH (Supplementary Method 1, 64.6 g, 500 mmol) and N,N'-Dicyclohexylcarbodiimide (33.0 g, 160 mmol) were combined in a flame-dried flask, purged with nitrogen, dissolved in anhydrous DCM (400 mL), and reacted at room temperature for 60 minutes. The reaction mixture was filtered to remove the dicyclohexylurea byproduct, concentrated *in vacuo*, and filtered. The crude product was repeatedly redissolved in anhydrous DCM (~90 mL), concentrated, and filtered until urea formation ceased.



4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoic acid (10.0 g, 33.4 mmol), 4-dimethylaminopyridine (DMAP, 205 mg, 1.67 mmol) were added to the crude anhydride and dissolved in minimal DCM (250 mL). Pyridine (2.69 mL, 33.4 mmol) was added to the reaction and stirred at room temperature overnight under a nitrogen atmosphere. The reaction mixture was washed with saturated aqueous sodium carbonate (250 mL), 1 M hydrochloric acid (250 mL), and concentrated *in vacuo*. The intermediate was dissolved in water/acetone (50:50, 1400 mL) and stirred at room temperature overnight. The acetone was removed *in vacuo* and the product was extracted into DCM (3 x 300 mL). The organic layer was washed with 1 M hydrochloric acid, brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude mixture was purified on a silica flash column (20-40% Ethyl acetate in hexanes with 1% acetic acid) and concentrated to give 4-(4-(1-((4-azidobutanoyl)oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoic acid (denoted N_3-*o*NB-COOH) as a yellow solid (11.59 g, 28.24 mmol, 85% yield). ^{1}H NMR (500 MHz, DMSO) δ 12.08 (s, 1H), 7.57 (s, 1H), 7.10 (s, 1H), 6.21 (q, J = 6.4 Hz, 1H), 4.07 (t, J = 6.4 Hz, 2H), 3.93 (s, 3H), 3.32 (t, J = 6.8 Hz, 2H), 2.43 (t, J = 7.3 Hz, 2H), 2.38 (t, J = 7.3 Hz, 2H), 1.95 (p, J = 6.8 Hz, 2H), 1.76 (p, J = 7.1 Hz, 2H), 1.58 (d, J = 6.5 Hz, 3H). These spectral data matched those previously reported[1].

Synthesis of 2,5-dioxopyrrolidin-1-yl 4-(4-(1-((4-azidobutanoyl)oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoate (N_3-*o*NB-OSu):

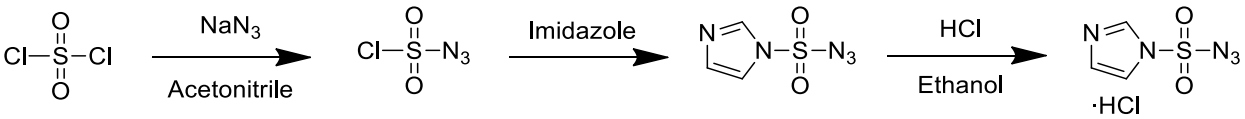


N_3-*o*NB-COOH (5.51 g, 13.4 mmol), N-hydroxysuccinimide (2.01 g, 17.5 mmol, 1.3x), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl 3.35 g, 17.5 mmol, 1.3x) were combined in a flame-dried flask and purged with nitrogen. Dry acetonitrile (50 mL) added *via* syringe to solubilize the reagents. The reaction mixture was stirred overnight at room temperature. The acetonitrile was removed *in vacuo* and the reaction mixture was dissolved in DCM (100 mL), washed with water (3 x 100 mL), dried over $MgSO_4$, filtered, and concentrated *in vacuo* to give the product (denoted N_3-*o*NB-OSu) as a yellow solid (6.69 g, 13.2 mmol, 98% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.62 (s, 1H), 7.03 (s, 1H), 6.52 (q, J = 6.4 Hz, 1H), 4.20 (t, J = 6.0 Hz, 2H), 4.00 (s, 3H), 3.42 – 3.31 (m, 2H), 2.91 (t, J = 7.3 Hz, 2H), 2.88 (s, 4H), 2.55 – 2.42 (m, 2H), 2.32 (p, J = 6.7 Hz, 2H), 1.93 (p, J = 6.9 Hz, 2H), 1.65 (d, J = 6.4 Hz, 3H).

Supplementary Method 3: Synthesis of (*R*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-azido-hexanoic acid (Fmoc-Lys(N_3)-OH)

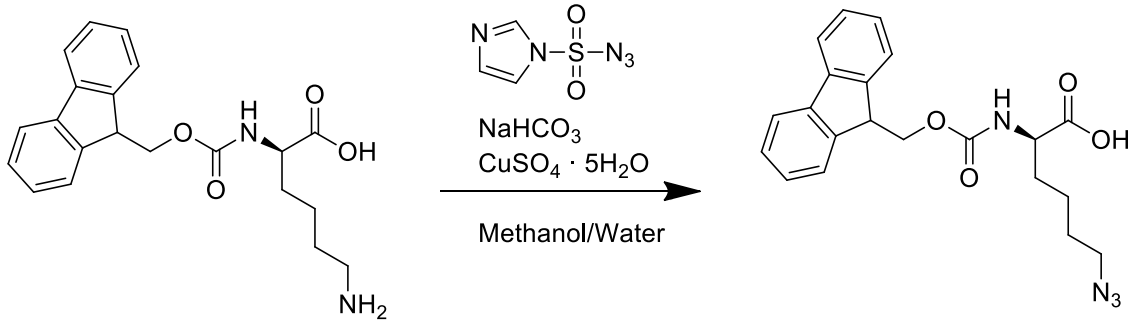
Imidazole-1-sulfonyl azide hydrochloride (Stick's reagent) and (*R*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-azidohexanoic acid (Fmoc-Lys(N_3)-OH) were synthesized following known synthetic routes[3,4] with minor modifications.

Synthesis of imidazole-1-sulfonyl azide hydrochloride:



Sulfuryl chloride (27.0 g, 16.2 mL, 200 mmol) was added dropwise to a suspension of sodium azide (13.0 g, 200 mmol) in acetonitrile (200 mL, 0 °C), and the reaction was stirred overnight at room temperature. The mixture was cooled in an ice bath, and imidazole (25.9 g, 380 mmol) was added in small portions to the stirred reaction mixture. The reaction mixture was brought to room temperature and stirred for 3 hours. The reaction mixture was diluted with ethyl acetate (400 mL), washed with water (2 x 400 mL), washed with saturated aqueous sodium carbonate (2 x 400 mL), dried over $MgSO_4$, and filtered. Hydrochloric acid in ethanol [formulated under a nitrogen atmosphere by dropwise addition of acetyl chloride (23.5 g, 300 mmol) into ice-cold anhydrous ethanol (75 mL)] was added dropwise to the stirring filtrate. The product was precipitated in an ice bath and filtered. The filter cake was washed with ice-cold ethyl acetate (3 x 100 mL) to yield the pure product, imidazole-1-sulfonyl azide hydrochloride [Stick's reagent[3]], as a white powder (25.86 g, 123.4 mmol, 61.7% yield). ^{1}H NMR (300 MHz, D_2O) δ 9.45 – 9.42 (m, 1H), 8.06 – 8.01 (m, 1H), 7.65 – 7.60 (m, 1H); ^{13}C-NMR (75 MHz, D_2O) δ = 137.7, 123.3, 120.1. These spectral data matched those previously reported[3].

Synthesis of (*R*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-azidohexanoic acid (Fmoc-Lys(N_3)-OH):



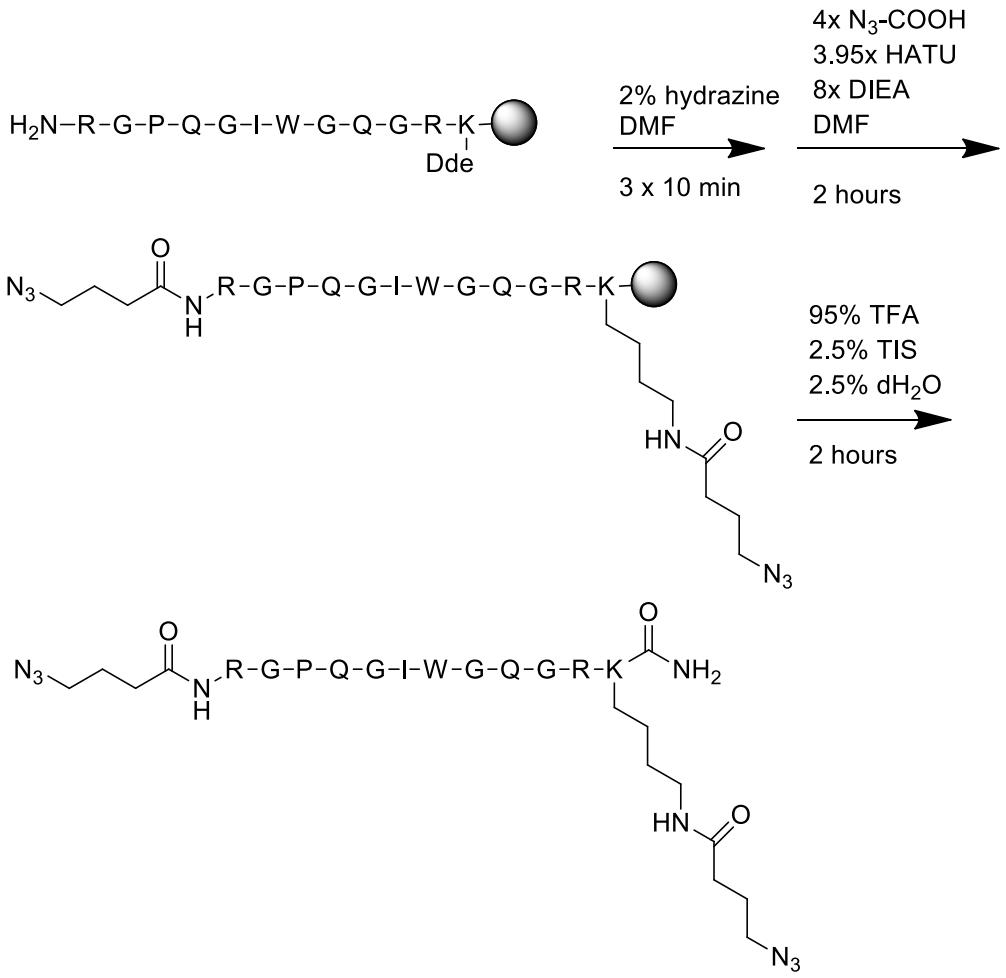
Fmoc-Lys-OH (11.00 g, 29.86 mmol) was dissolved in water (30 mL) with hydrochloric acid (aqueous 37%, 2.47 mL, 1x). A solution of water/methanol (1:2, 130 mL), imidazole-1-sulfonyl azide hydrochloride (7.50 g, 35.8 mmol, 1.2x), sodium bicarbonate (16.22 g, 193.1 mmol, 6.5 x), and aqueous $CuSO_4 \cdot 5H_2O$ (74.6 mg in 2 mL dH_2O, 0.299 mmol, 0.01x) were added, in order, to the reaction mixture and stirred for 17 hours at room temperature. The methanol was removed *in vacuo*, and the mixture was diluted with water (300 mL) and adjusted to a pH of 2 with hydrochloric acid. The crude product was extracted into ethyl acetate (3 x 150 mL), dried over

$MgSO_4$, filtered, and concentrated on a rotary evaporator. The crude product was purified on a silica column with a mobile phase of toluene/ethyl acetate/acetic acid (85:10:5) and concentrated to give the pure product [(*R*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-azidohexanoic acid, denoted Fmoc-Lys(N_3)-OH], as a light-yellow solid (10.82 g, 27.4 mmol, 92% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.93 (s, 1H), 7.79 (d, J = 7.5 Hz, 2H), 7.63 (d, J = 7.0 Hz, 2H), 7.39 (dt, J = 26.7, 7.2 Hz, 4H), 5.43 (d, J = 8.2 Hz, 1H), 4.65 – 4.34 (m, 3H), 4.25 (t, J = 6.8 Hz, 1H), 3.38 – 3.14 (m, 2H), 2.04 – 1.35 (m, 6H). These spectral data matched those previously reported[4].

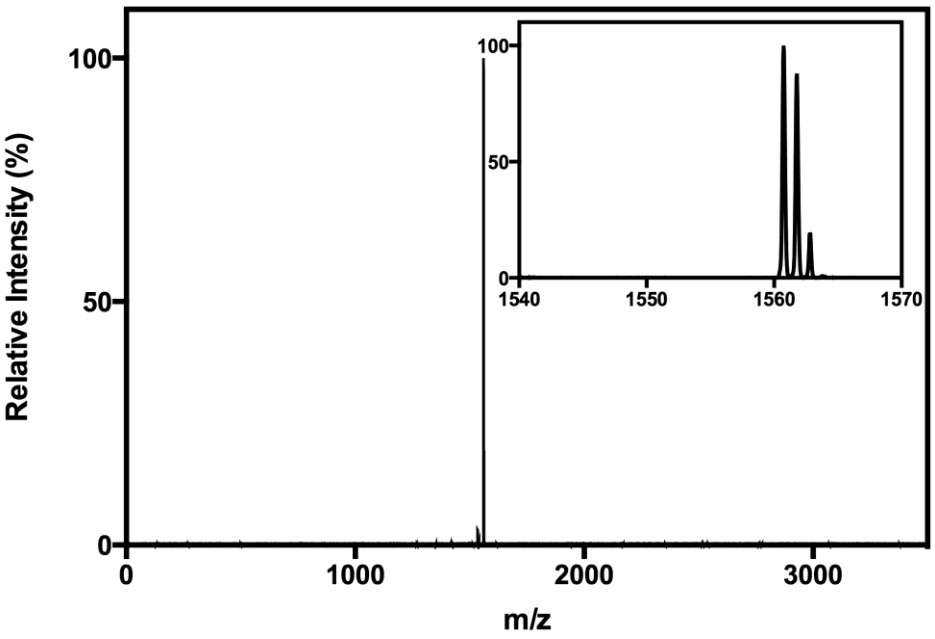
Supplementary Method 4: Fmoc solid-phase peptide synthesis

Automated microwave-assisted Fmoc solid-phase peptide synthesis was performed on a CEM Liberty 1 (0.5 mmol scale). Fmoc deprotections were performed in 20% piperidine (v/v) in DMF with 0.1 M 1-hydroxybenzotriazole (HOBt) at 90 ^{o}C for 90 seconds. Amino acids (except arginine and cysteine) were coupled to resin-bound peptides upon treatment (75 ^{o}C for 5 minutes) with Fmoc-protected amino acid (2 mmol, 4x), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 2 mmol, 4x), and *N*,*N*-diisopropylethylamine (DIEA, 2 mmol, 4x) in a mixture of DMF (9 mL) and *N*-Methyl-2-pyrrolidone (NMP, 2 mL). Cysteine couplings were performed using the same reagents as above but the reaction was performed at 50 ^{o}C for 30 minutes. Arginine couplings were performed using the same reagents as above but the reaction was performed at 25 ^{o}C for 45 minutes, drained, and repeated at 75 ^{o}C for 5 minutes.

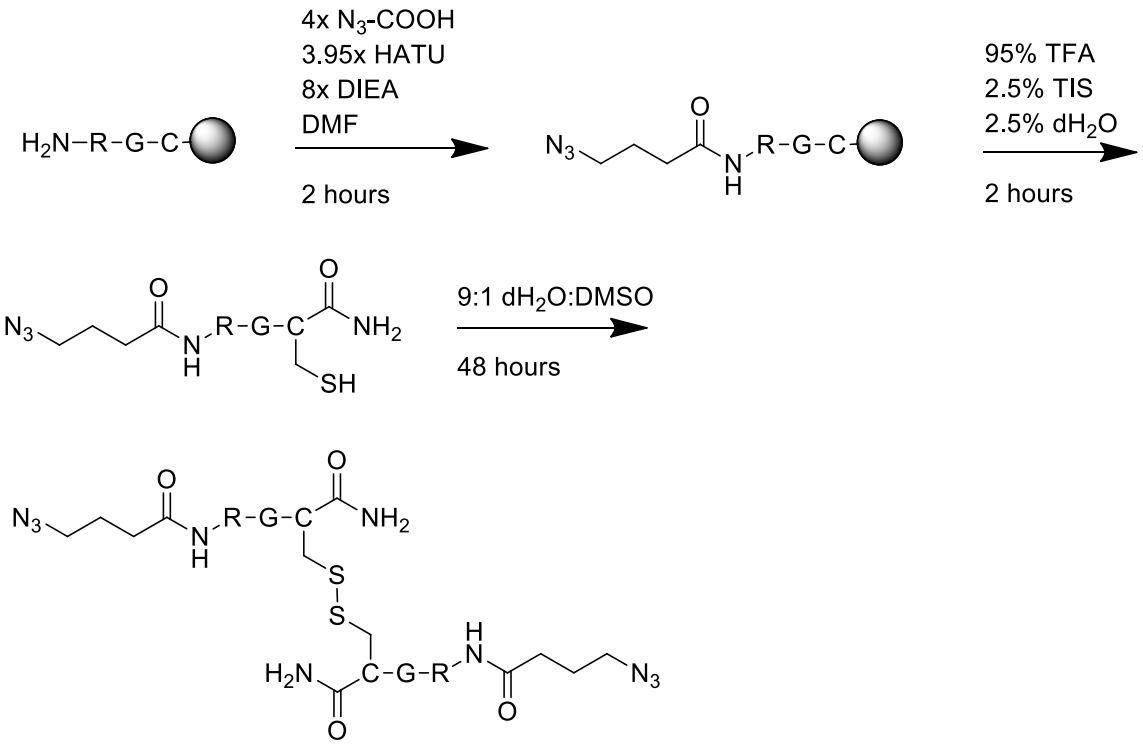
Supplementary Method 5: Enzymatic "YES" degradable crosslinker synthesis (E)



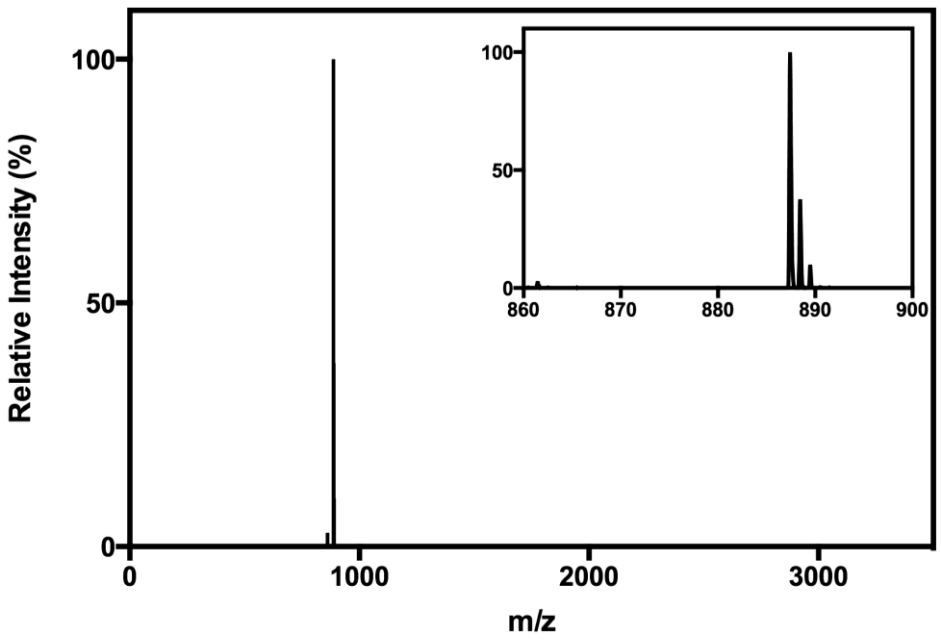
The resin-bound peptide H-RGPQGIWGQGRK(Dde)-NH_2 was synthesized by microwave-assisted Fmoc solid-phase peptide synthesis (SPPS, Supplementary Method 4) on Rink amide resin (0.5 mmol scale). The resin was treated with hydrazine monohydrate (2%) in DMF (3 x 10 min) to remove the N-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl) (Dde) protecting group. N_3-COOH (0.517 g, 4.0 mmol, 4x, Supplementary Method 1) was pre-activated upon reaction with 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU, 1.502 g, 3.95 mmol, 3.95x) and DIEA (1.034 g, 8.0 mmol, 8x) in minimal DMF for 5 minutes and then reacted with the resin for 90 minutes to functionalize the N-terminus and the ε-amino group of the lysine with an azide. The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/triisopropylsilane (TIS)/dH_2O (95:2.5:2.5, 30 mL) for 2 hours, and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the final product (N_3-RGPQGIWGQGRK(N_3)-NH_2, denoted E) as a yellow solid (222.0 mg, 0.142 mmol, 28.4% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{66}H_{106}N_{29}O_{16}^+$ [M + $^1H]^+$, 1560.8; observed 1560.9.


Relative Intensity (%)
100
50
0
0
1000
2000
3000
m/z
100
50
0
1540
1550
1560
1570

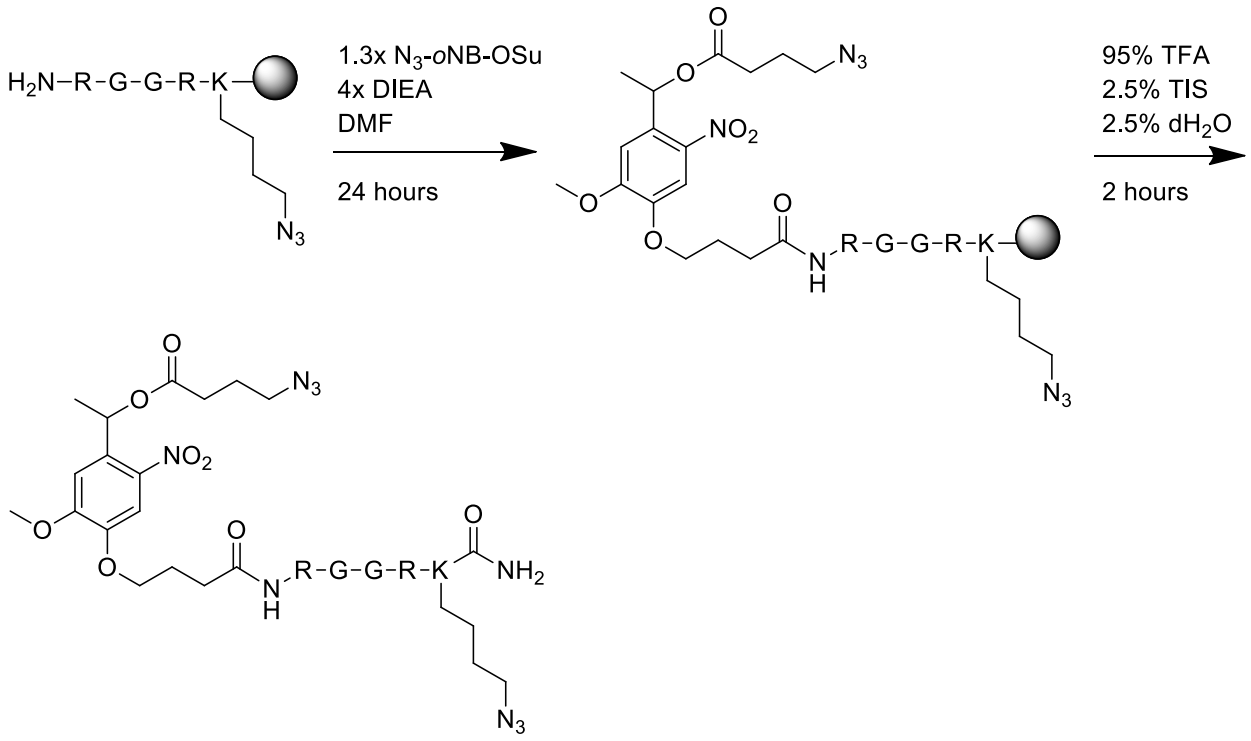
Supplementary Method 6: Reductive "YES" degradable crosslinker synthesis (R)



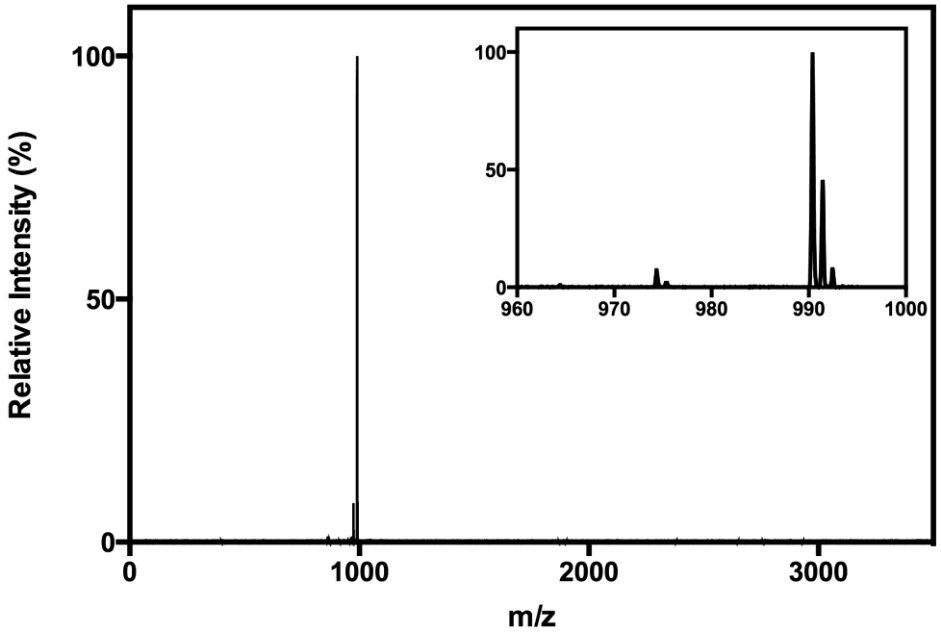
The resin-bound peptide H-RGC-NH_2 was synthesized by microwave-assisted Fmoc SPPS (Supplementary Method 4) on Rink amide resin (1.0 mmol scale). N_3-COOH (Supplementary Method 1, 0.517 g, 4.0 mmol, 4x) was pre-activated upon reaction with HATU (1.502 g, 3.95 mmol, 3.95x) and DIEA (1.034 g, 8.0 mmol, 8x) in minimal DMF for 5 minutes and then reacted with the resin for 90 minutes to functionalize the N-terminus with an azide. The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/TIS/dH_2O (95:2.5:2.5, 30 mL) for 2 hours, and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was dissolved in a dH_2O/DMSO (90:10) solution (100 mL) and reacted at room temperature for 48 hours. The peptide was concentrated and purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the final product (N_3-RG<u>C</u>(N_3-RG<u>C</u>-NH_2)-NH_2 dimerized *via* <u>disulfide bond</u>, denoted R) as a yellow solid (139.5 mg, 0.1572 mol, 31.5% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{30}H_{55}N_{20}O_8S_2^+$ [M + $^1H]^+$, 887.4; observed 887.4.


Relative Intensity (%)
100
50
0
0
1000
2000
3000
m/z
100
50
0
860
870
880
890
900

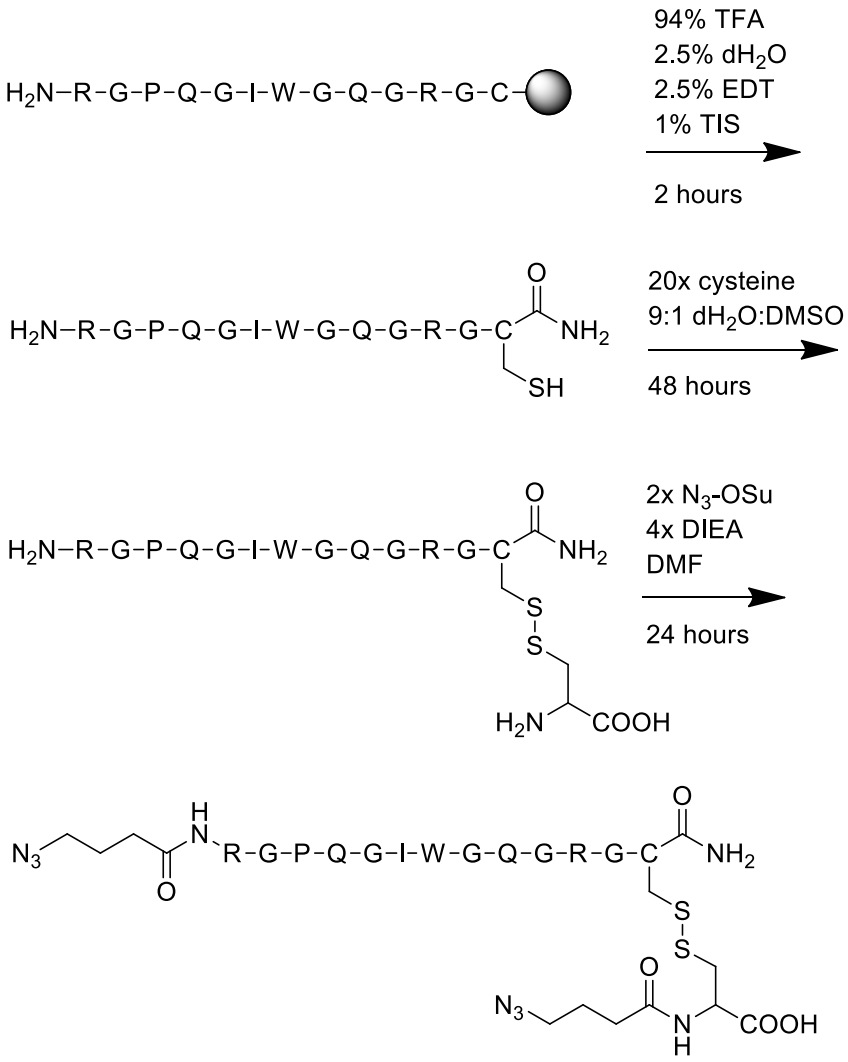
Supplementary Method 7: Photo "YES" degradable crosslinker synthesis (P)



The resin-bound peptide H-RGGRK(N_3)-NH_2 was synthesized by microwave-assisted Fmoc SPPS (Supplementary Method 4) on Rink amide resin (0.5 mmol scale) using a C-terminal Fmoc-Lys(N_3)-OH (Supplementary Method 3). Resin was subsequently treated with N_3-*o*NB-OSu (Supplementary Method 2, 0.65 mmol, 330 mg) and DIEA (2.0 mmol, 258 mg) in minimal DMF to introduce *o*NB functionality to the N-terminus. The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/TIS/dH_2O (95:2.5:2.5, 30 mL) for 2 hours, and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the final product (N_3-*o*NB-RGGRK(N_3)-NH_2, denoted P) as a yellow solid (147.4 mg, 0.1488 mol, 29.8% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{39}H_{64}N_{19}O_{12}^+$ $[M + {}^1H]^+$, 990.5; observed 990.4.

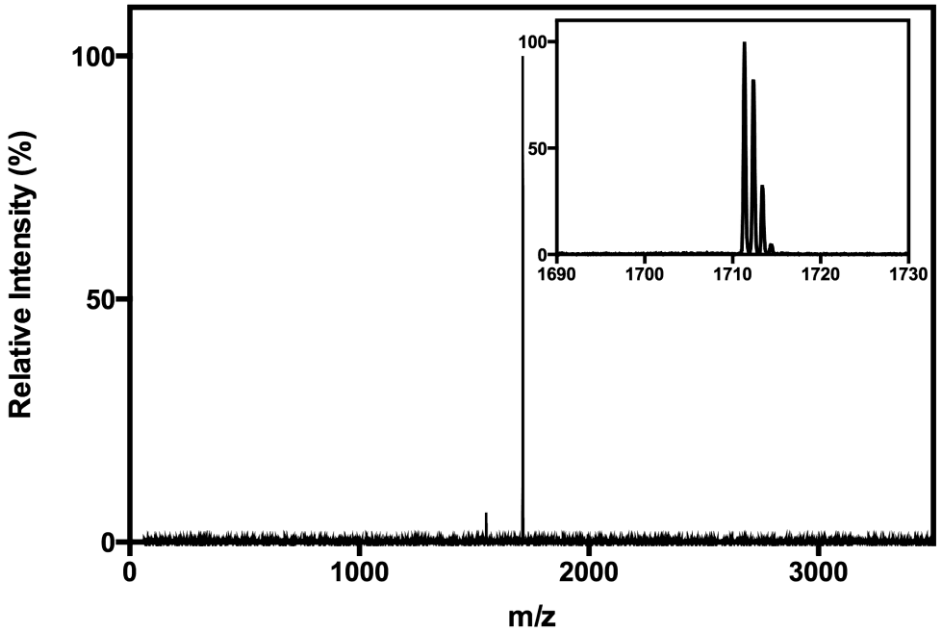

Relative Intensity (%)
100
50
0
0
1000
2000
3000
m/z
100
50
0
960
970
980
990
1000

Supplementary Method 8: Reductive "OR" Enzymatic degradable crosslinker synthesis (RVE)

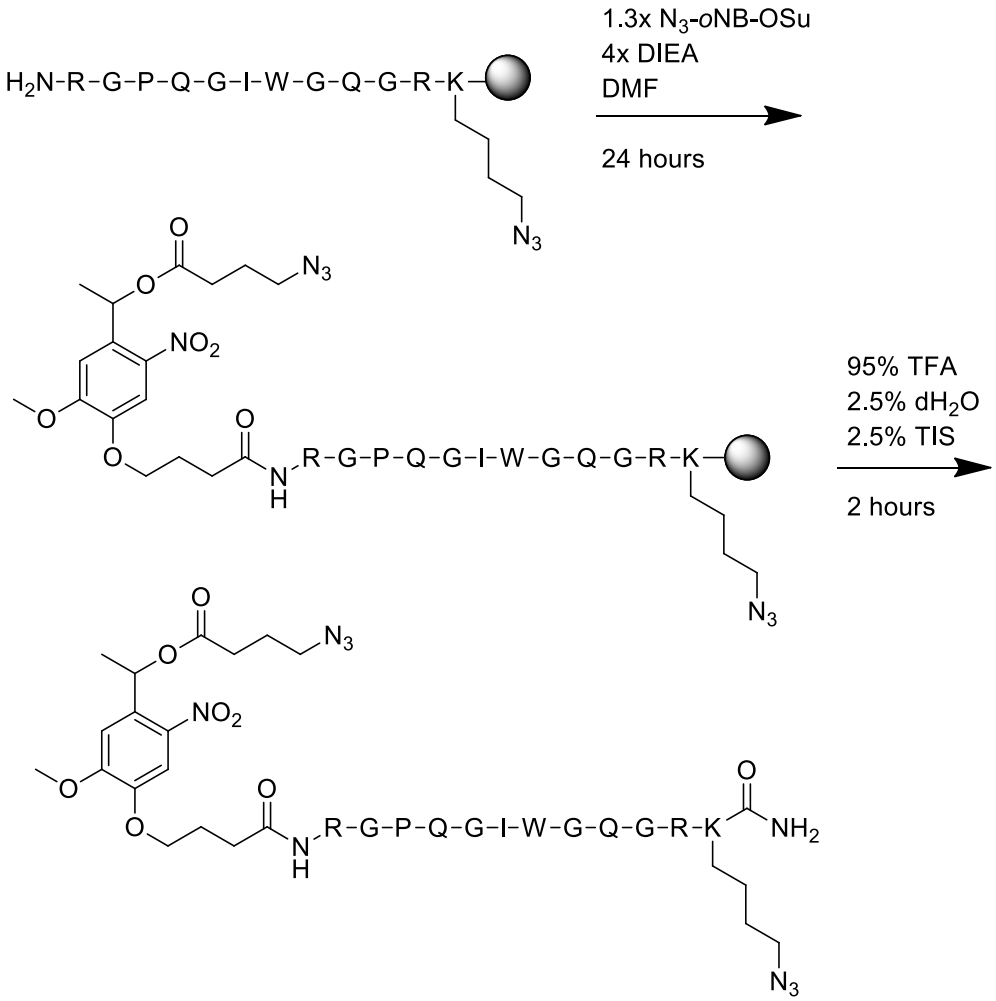


The resin-bound peptide H-RGPQGIWGQGRGC-NH_2 was synthesized by microwave-assisted Fmoc SPPS (Supplementary Method 4) on Rink amide resin (0.5 mmol scale). The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/dH_2O/1,2-ethanedithiol (EDT)/TIS (94:2.5:2.5:1, 30 mL) for 2 hours, and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (H-RGPQGIWGQGRGC-NH_2) as a white solid (417.4 mg, 0.305 mmol). The purified peptide and cysteine (0.74 g, 6.1 mmol, 20x) were dissolved in a dH_2O/DMSO (90:10) solution (50 mL) and stirred at room temperature for 48 hours. Product was filtered, concentrated *in vacuo*, and purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (H-RGPQGIWGQGRGC(H-C-OH)-NH_2 with cysteines linked *via* disulfide bond) as a white solid (241.4 mg, 0.162 mmol). To introduce azide functionalities requisite for hydrogel crosslinking, N_3-OSu (Supplementary Method 1, 146.6 mg, 0.648 mmol,

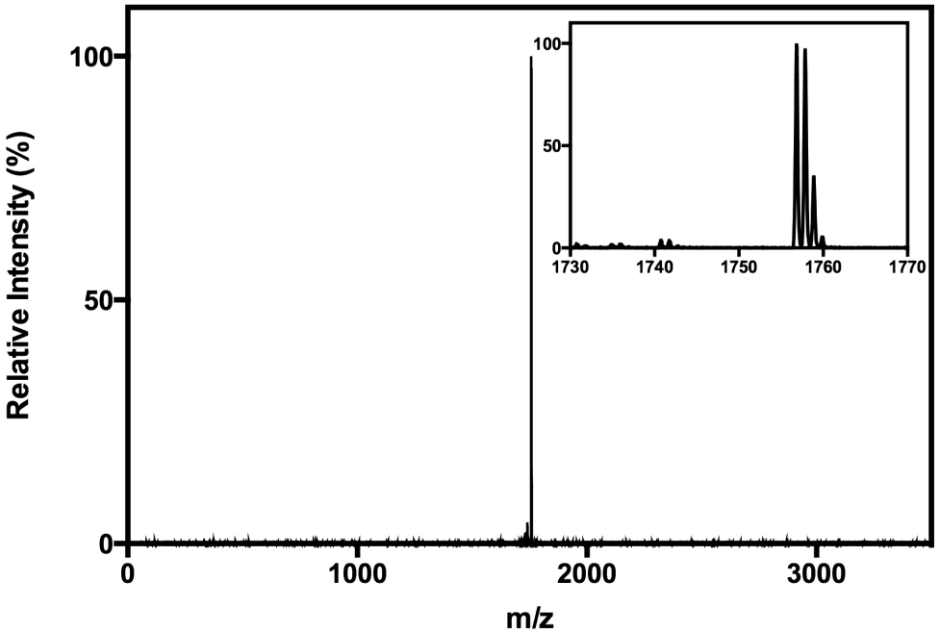
2x) was dissolved in minimal DMF with DIEA (167.5 mg, 1.30 mmol, 4x) and reacted with the peptide overnight at room temperature. The peptide was purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the final product (N_3-RGPQGIWGQGRG<u>C</u>(N_3-<u>C</u>-OH)-NH_2 with cysteines linked *via* <u>disulfide bridge</u>, denoted RVE) as a white solid (89.8 mg, 0.0525 mmol, 10.5% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{68}H_{107}N_{30}O_{19}S_2^+$ [M + $^1H]^+$, 1711.7; observed 1711.5.



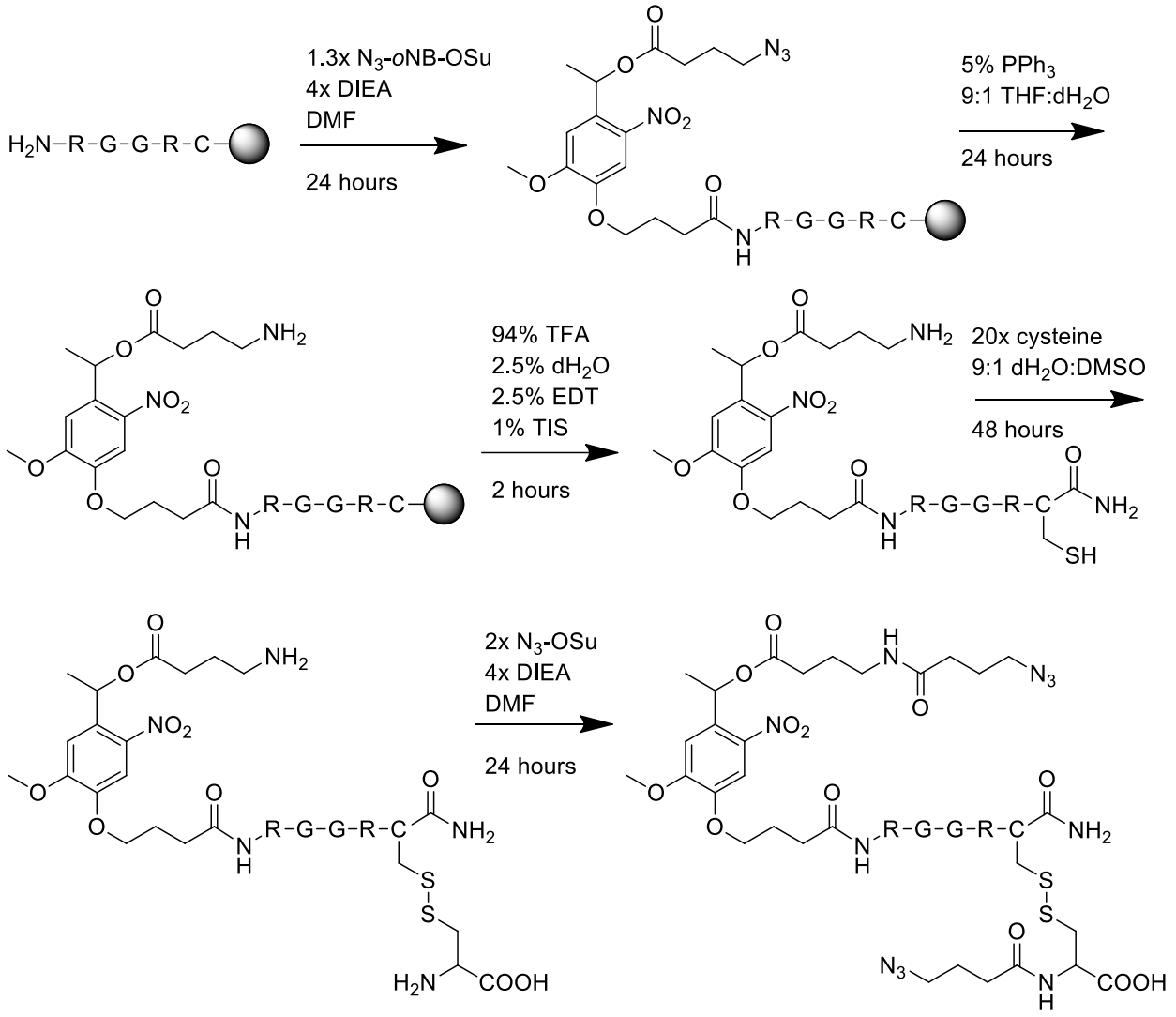
Supplementary Method 9: Enzymatic "OR" Photo degradable crosslinker synthesis (EVP)



The resin-bound peptide H-RGPQGIWGQGRK(N_3)-NH_2 was synthesized by microwave-assisted Fmoc SPPS (Supplementary Method 4) on Rink amide resin (0.5 mmol scale) using a C-terminal Fmoc-Lys(N_3)-OH (Supplementary Method 3). Resin was subsequently treated with N_3-*o*NB-OSu (Supplementary Method 2, 0.65 mmol, 330 mg) and DIEA (2.0 mmol, 258 mg) in minimal DMF to introduce *o*NB functionality to the N-terminus. The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/TIS/dH_2O (95:2.5:2.5, 30 mL) for 2 hours, and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the final product (N_3-*o*NB-RGPQGIWGQGRK(N_3)-NH_2, denoted EVP) as a yellow solid (159.9 mg, 0.0910 mmol, 18.2% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{75}H_{114}N_{29}O_{21}^+$ $[M + {}^1H]^+$, 1756.9; observed 1756.9.

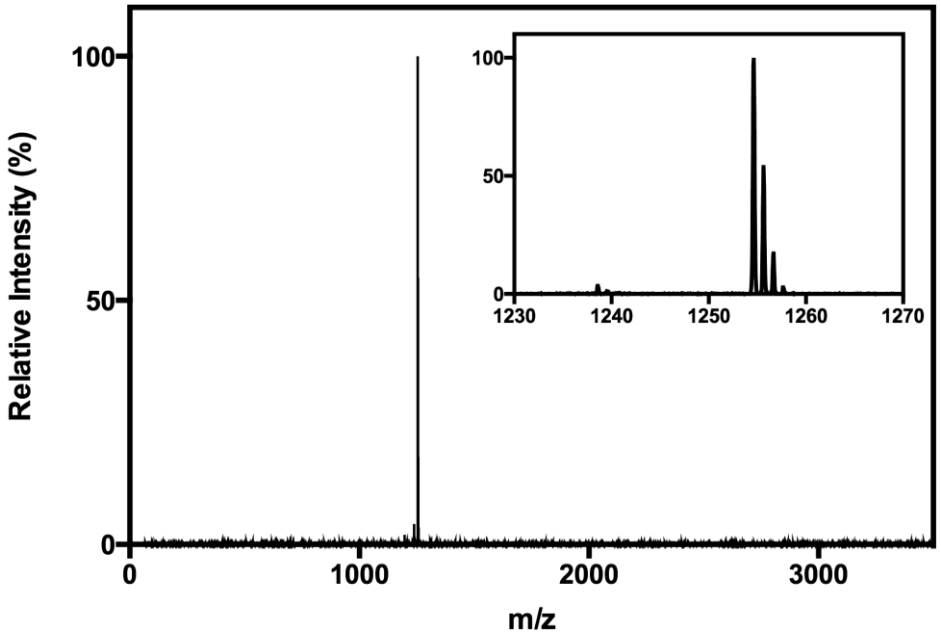

Relative Intensity (%)
100
50
0
0
1000
2000
3000
m/z
100
50
0
1730
1740
1750
1760
1770

Supplementary Method 10: Reductive "OR" Photo degradable crosslinker synthesis (RVP)

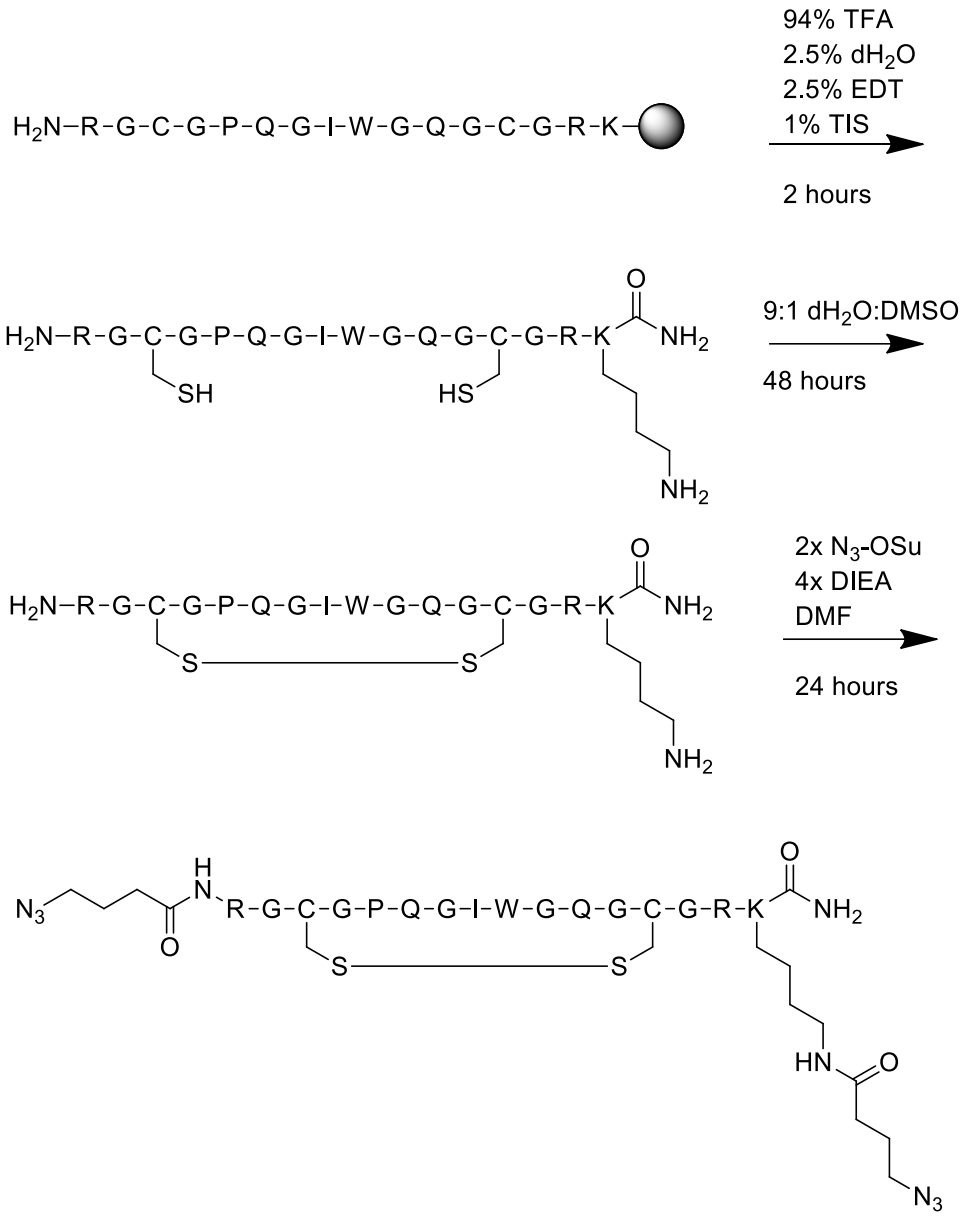


The resin-bound peptide H-RGGRC-NH_2 was synthesized by microwave-assisted Fmoc SPPS (Supplementary Method 4) on Rink amide resin (0.5 mmol scale). Resin was subsequently treated with N_3-*o*NB-OSu (Supplementary Method 2, 0.65 mmol, 330 mg) and DIEA (2.0 mmol, 258 mg) in minimal DMF to introduce *o*NB functionality to the N-terminus. The N-terminal azide was reduced to an amine by a Staudinger reduction; resin was washed with tetrahydrofuran (THF)/H_2O (90:10, 3 x 20 mL), followed by reaction with 5 wt% triphenylphosphine in THF/H_2O (90:10, 30 mL) for 18 hours. The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/dH_2O/EDT/TIS (94:2.5:2.5:1, 30 mL) for 2 hours, and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (H-*o*NB-RGGRC-NH_2) as a yellow solid (146.4 mg, 0.160 mmol). The purified peptide and cysteine (0.39 g, 3.2 mmol, 20x) were dissolved in a dH_2O/DMSO (90:10) solution (50 mL) and stirred at room temperature for 48 hours. The peptide was filtered, concentrated *in vacuo*, and purified using RP-HPLC operating

with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (H-*o*NB-RGGRC(H-C-OH)-NH_2 with cysteines linked *via* disulfide bond) as a yellow solid (120.8 mg, 0.117 mmol). N_3-OSu (Supplementary Method 1, 121 mg, 0.468 mmol, 2x) was dissolved in minimal DMF with DIEA (167.5 mg, 0.963 mmol, 4x) and reacted with the peptide overnight at room temperature. The peptide was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the final product (N_3-*o*NB-RGGRC(N_3-C-OH)-NH_2 with cysteines linked *via* disulfide bond, denoted RVP) as a yellow solid (32.2 mg, 0.0257 mmol, 5.1% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{47}H_{76}N_{21}O_{16}S_2^+$ $[M + {}^1H]^+$, 1254.5; observed 1254.6.

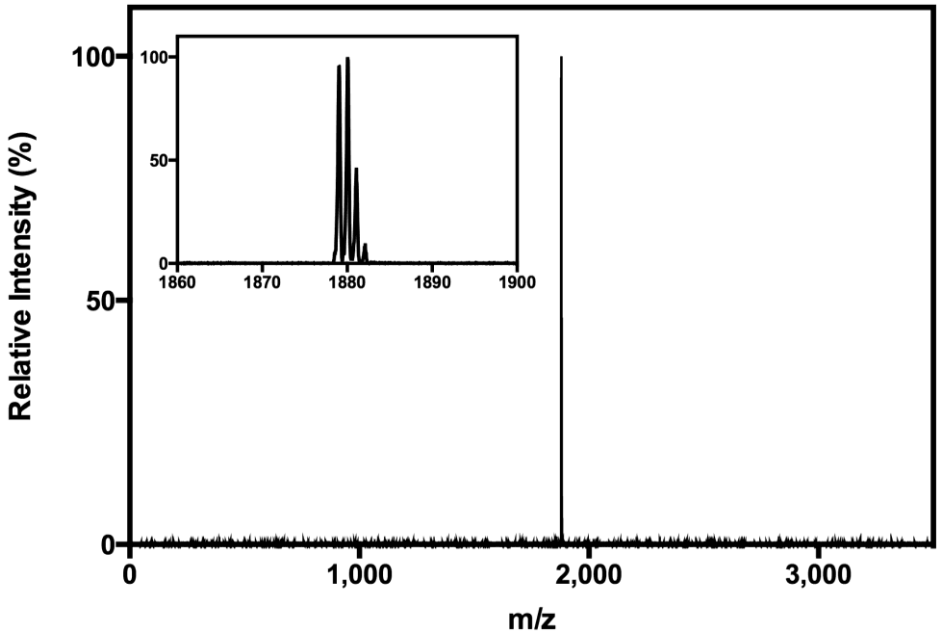


Supplementary Method 11: Reductive "AND" Enzymatic degradable crosslinker synthesis (R∧E)

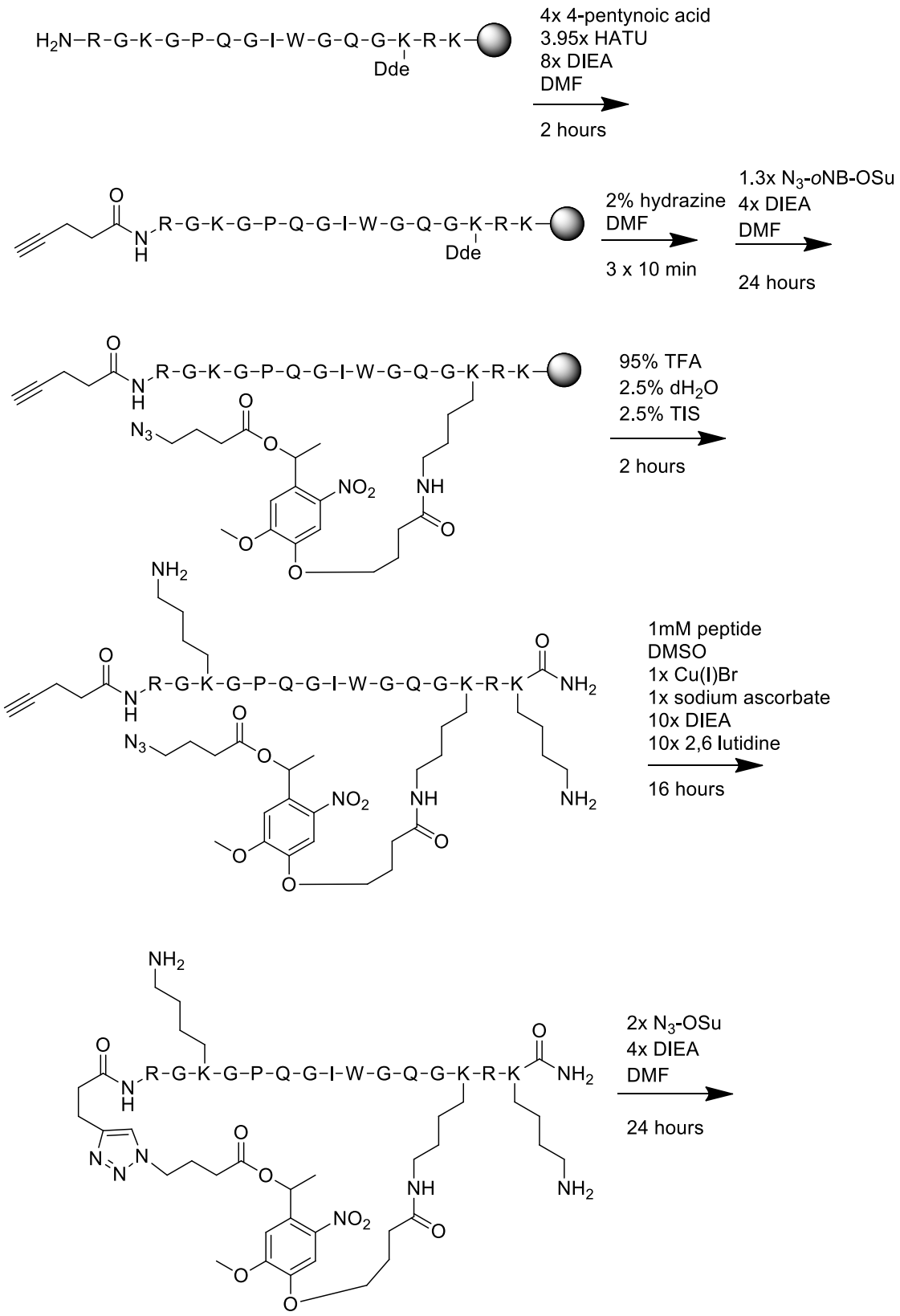


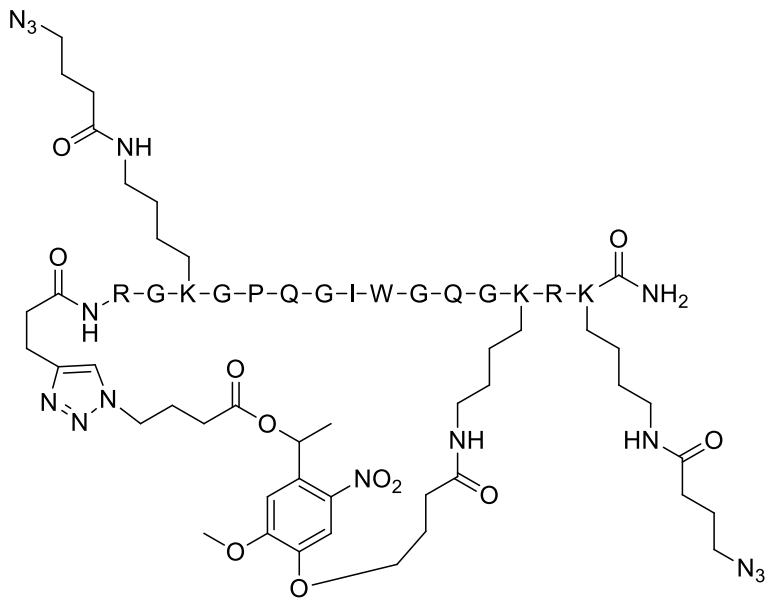
The resin-bound peptide H-RGCGPQGIWGQGCGRK-NH_2 was synthesized by microwave-assisted Fmoc SPPS (Supplementary Method 4) on Rink amide resin (1.0 mmol scale). The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/dH_2O/EDT/TIS (94:2.5:2.5:1) for 3 hours and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (H-RGCGPQGIWGQGCGRK-NH_2) as a white solid (531.0 mg, 0.320 mmol). To promote disulfide-mediated intramolecular stapling, the purified peptide was dissolved at 0.5 mM in a dH_2O/DMSO (90:10) solution (700 mL) and reacted at room temperature with no

agitation for 48 hours. Stapled product was concentrated *in vacuo* and purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (H-RGCGPQGIWGQGCGRK-NH_2 with intramolecular stapling *via* cysteine-cysteine disulfide bond) as a white solid (489 mg, 0.295 mmol). To introduce azide functionalities requisite for hydrogel crosslinking, N_3-OSu (Supplementary Method 1, 267 mg, 1.18 mmol, 2x) was dissolved in minimal DMF with DIEA (305 mg, 2.36 mmol, 4x) and reacted with the peptide overnight at room temperature. Stapled peptide was purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the final product (N_3-RGCGPQGIWGQGCGRK(N_3)-NH_2 with intramolecular stapling *via* cysteine-cysteine disulfide bond, denoted R∧E) as a white solid (197 mg, 0.105 mmol, 10.5% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{76}H_{120}N_{33}O_{20}S_2^+$ $[M + {}^1H]^+$, 1878.9; observed 1879.0.



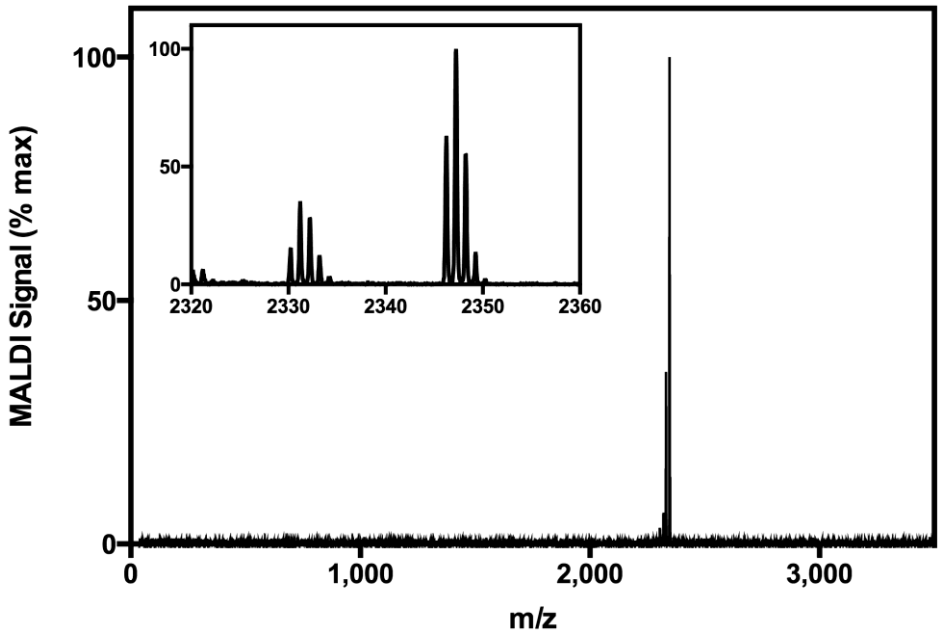
Supplementary Method 12: Enzymatic "AND" Photo degradable crosslinker synthesis (E∧P)



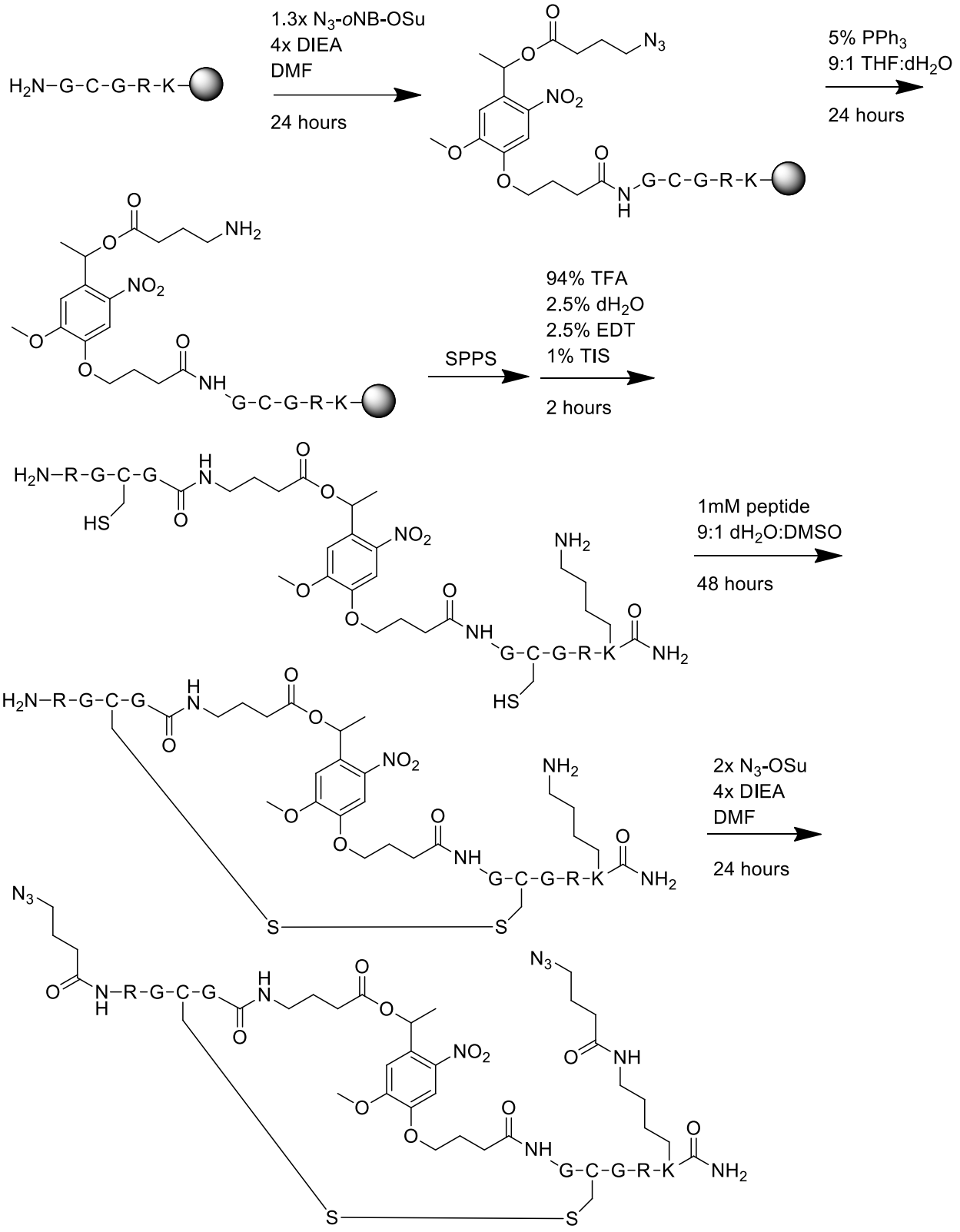


The resin-bound peptide H-RGKGPQGIWGQGK(Dde)RK-NH_2 was synthesized by microwave-assisted Fmoc SPPS (Supplementary Method 4) on Rink amide resin (0.5 mmol scale). 4-pentynoic acid (0.196 g, 2.0 mmol, 4x) was pre-activated upon reaction with HATU (0.751 g, 1.975 mmol, 3.95x) and DIEA (0.517 g, 4.0 mmol, 8x) in minimal DMF for 5 minutes and then reacted with the resin for 90 minutes to functionalize the N-terminus with an alkyne. The resin was treated with hydrazine monohydrate (2%) in DMF (3 x 10 min) to remove the Dde protecting group. Resin was subsequently treated with N_3-*o*NB-OSu (Supplementary Method 2, 0.65 mmol, 330 mg) and DIEA (2.0 mmol, 258 mg) in minimal DMF to introduce *o*NB functionality to the ε-amino group of the unprotected lysine. The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/TIS/dH_2O (95:2.5:2.5, 30 mL) for 2 hours, and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (yne-RGKGPQGIWGQGK(*o*NB-N_3)RK-NH_2) as a yellow solid (116 mg, 0.0546 mmol). To induce CuAAC-mediated intramolecular stapling (copper(I)-catalyzed azide-alkyne cycloaddition), pure linear peptide (1 mM) was dissolved in nitrogen-purged DMSO (55 mL) containing copper(I) bromide (7.8 mg, 0.055 mmol, 1x), sodium ascorbate (10.8 mg, 0.055 mmol, 1x) in water (550 μL), 2,6 lutidine (55.8 mg, 0.546 mmol, 10x), and DIEA (70.6 mg, 0.546 mmol, 10x). This mixture was allowed to react under nitrogen at room temperature for 16 hours. The mixture was concentrated *in vacuo*, redissolved in dH_2O, passed through an ion exchange column (Dowex M4195 resin, 5 grams), and lyophilized. Stapled product was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (yne-RGKGPQGIWGQGK(*o*NB-N_3)RK-NH_2 with an intramolecular staple *via* a triazole linkage between the alkyne and *o*NB-N_3 side chains) as a yellow solid (36 mg, 0.017 mmol). To introduce azide functionalities requisite for hydrogel crosslinking, N_3-OSu (Supplementary Method 1, 15.4 mg, 0.068 mmol, 2x) was dissolved in minimal DMF with DIEA (17.6 mg, 0.69 mmol, 4x) and reacted with the peptide overnight at room temperature. The stapled peptide was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded

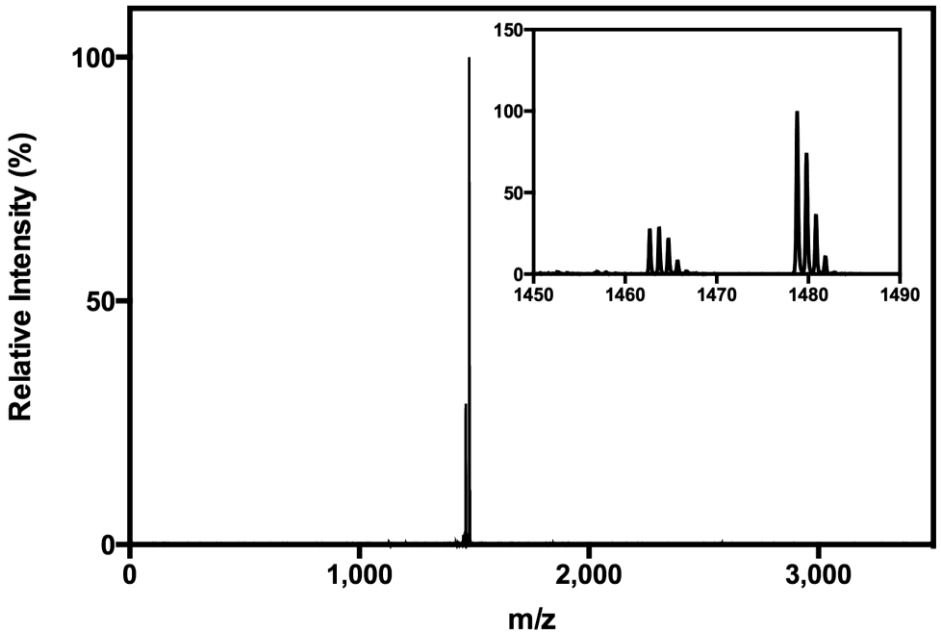
the final product (yne-RGK(N_3)GPQGIWGQGK(*o*NB-N_3)RK(N_3)-NH_2 with an intramolecular staple *via* a triazole linkage between the alkyne and *o*NB-N_3 side chains, denoted E∧P) as a yellow solid (11.2 mg, 0.00477 mmol, 0.95% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{108}H_{166}N_{41}O_{30}^+$ $[M + {}^1H]^+$, 2346.2; observed 2346.2.



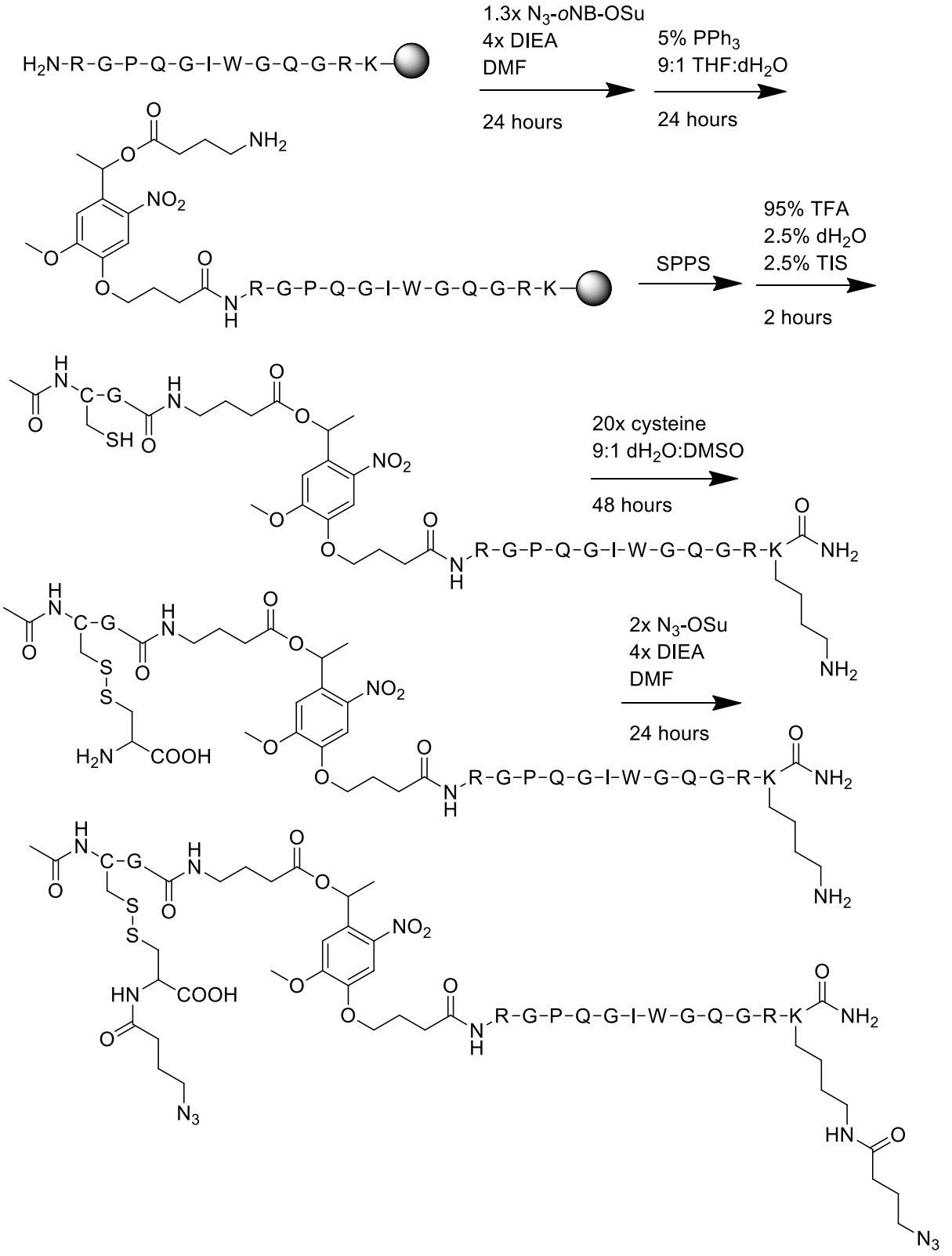
Supplementary Method 13: Reductive "AND" Photo degradable crosslinker synthesis (RΛP)



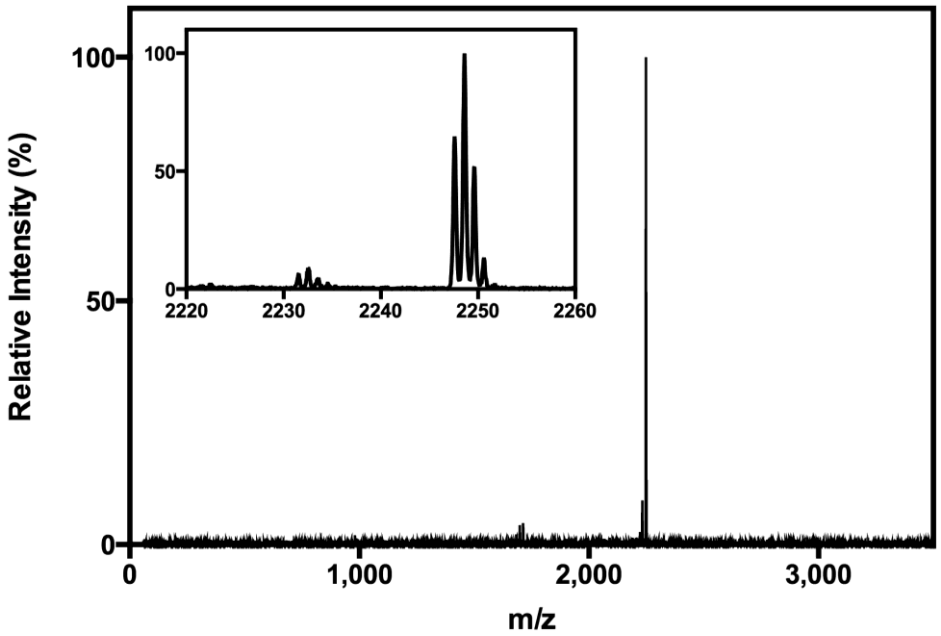
The resin-bound peptide H-GCGRK-NH_2 was synthesized by microwave-assisted Fmoc SPPS (Supplementary Method 4) on Rink amide resin (0.5 mmol scale). Resin was subsequently treated with N_3-*o*NB-OSu (Supplementary Method 2, 0.65 mmol, 330 mg) and DIEA (2.0 mmol, 258 mg) in minimal DMF to introduce *o*NB functionality to the N-terminus. The N-terminal azide was reduced to an amine by a Staudinger reduction; resin was washed with THF/H_2O (90:10, 3 x 20 mL), followed by reaction with 5 wt% triphenylphosphine in THF/H_2O (90:10, 30 mL) for 18 hours. The peptide sequence H-RGCG was appended to the N-terminus by standard microwave-assisted Fmoc SPPS (Supplementary Method 4). The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/dH_2O/EDT/TIS (94:2.5:2.5:1, 30 mL) for 2 hours, and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%) lyophilization yielded the pure intermediate (H-RGCG-*o*NB-GCGRK-NH_2) as a yellow solid (119 mg, 0.0946 mmol). To promote disulfide-mediated intramolecular stapling, the purified peptide was dissolved at 0.5 mM in a dH_2O/DMSO (90:10) solution (200 mL) and reacted at room temperature with no agitation for 48 hours. Stapled product was concentrated *in vacuo* and purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the intermediate (H-RGCG-*o*NB-GCGRK-NH_2 with intramolecular stapling *via* cysteine-cysteine disulfide bond) as a yellow solid (101 mg, 0.0807 mmol). To introduce azide functionalities requisite for hydrogel crosslinking, N_3-OSu (Supplementary Method 1, 73.0 mg, 0.323 mmol, 2x) was dissolved in minimal DMF with DIEA (83.4 mg, 0.646 mmol, 4x) and reacted with the peptide overnight at room temperature. The stapled peptide was purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the final product (N_3-RGCG-*o*NB-GCGRK(N_3)-NH_2 with intramolecular stapling *via* cysteine-cysteine disulfide bond, denoted RΛP) as a yellow solid (20.0 mg, 0.0135 mmol, 2.7% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{57}H_{92}N_{25}O_{18}S_2^+$ $[M + {}^1H]^+$, 1478.6; observed 1478.8.



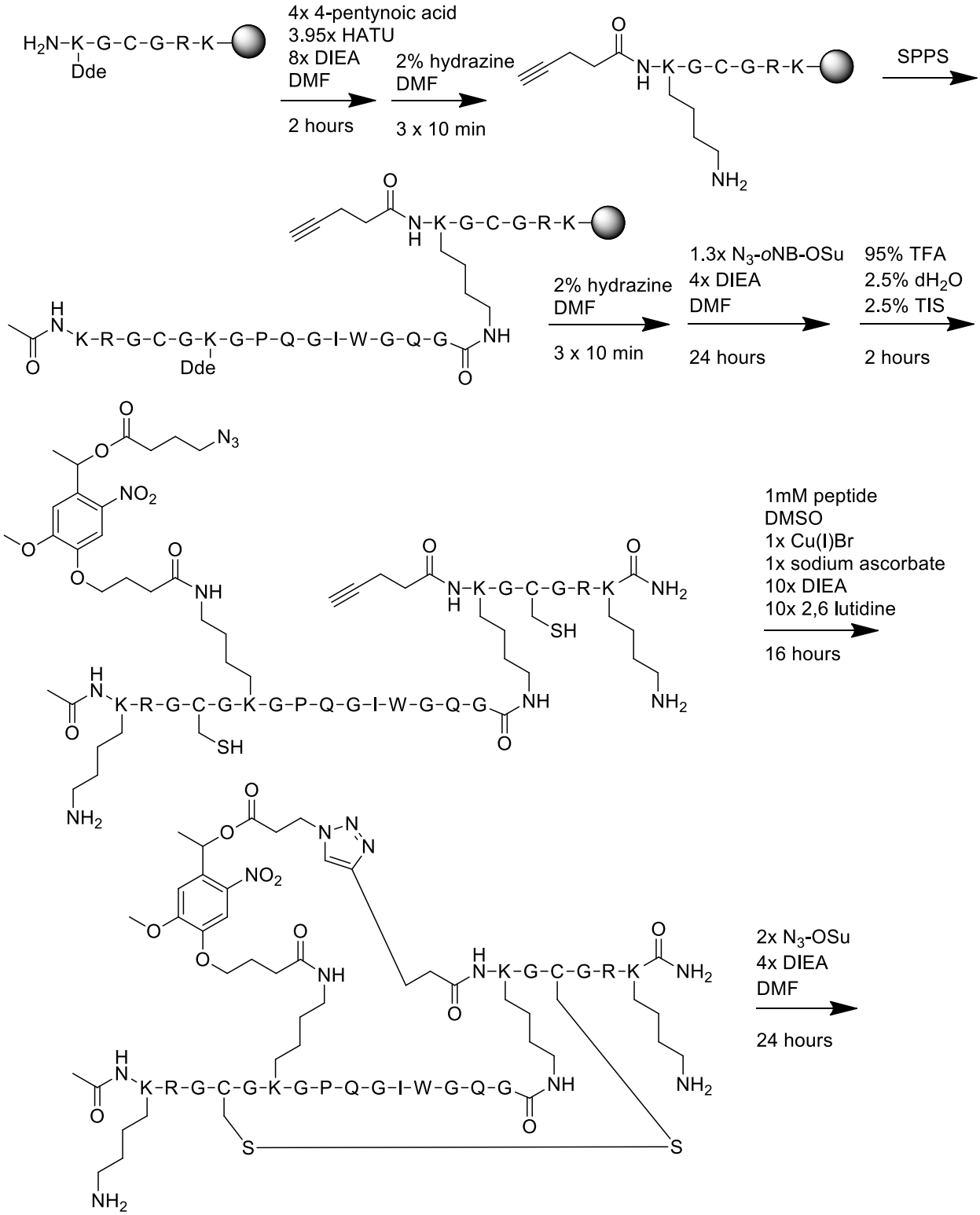
Supplementary Method 14: Enzymatic "OR" Reductive "OR" Photo degradable crosslinker synthesis [RVEVP]

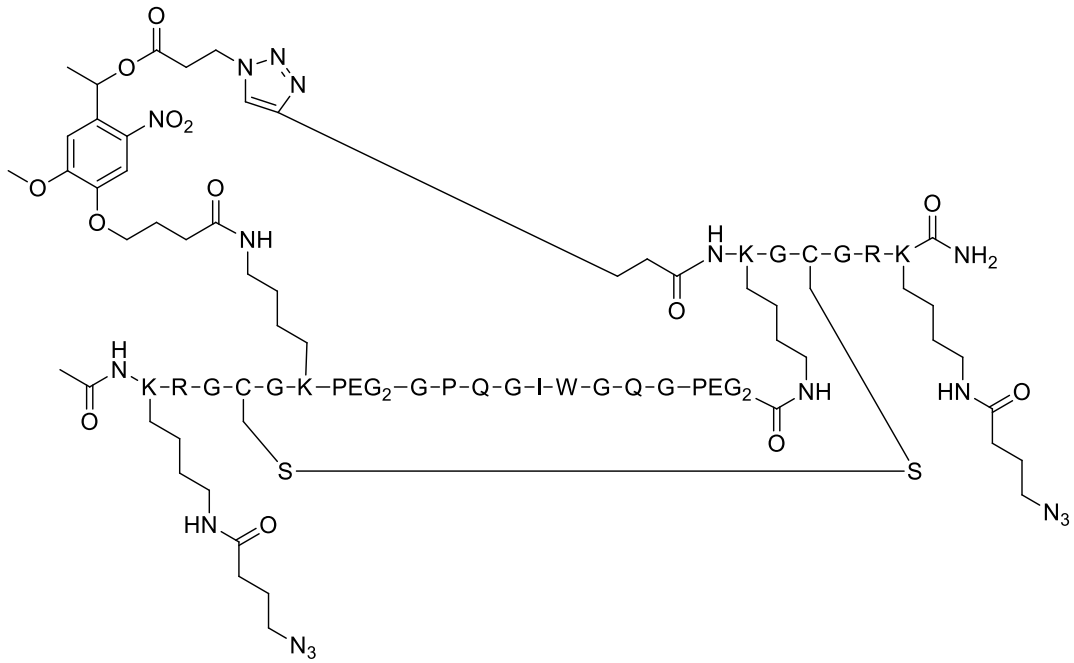


The resin-bound peptide H-RGPQGIWGQGRK-NH_2 was synthesized by microwave-assisted Fmoc SPPS (Supplementary Method 4) on Rink amide resin (0.5 mmol scale). Resin was subsequently treated with N_3-*o*NB-OSu (Supplementary Method 2, 0.65 mmol, 330 mg) and DIEA (2.0 mmol, 258 mg) in minimal DMF to introduce *o*NB functionality to the N-terminus. The N-terminal azide was reduced to an amine by a Staudinger reduction; resin was washed with THF/H_2O (90:10, 3 x 20 mL), followed by reaction with 5 wt% triphenylphosphine in THF/H_2O (90:10, 30 mL) for 18 hours. The peptide sequence Ac-CG was appended to the N-terminus by standard microwave-assisted Fmoc SPPS. The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/TIS/dH_2O (95:2.5:2.5, 30 mL) for 2 hours, and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (Ac-CG-*o*NB-RGPQGIWGQGRK-NH_2) as a yellow solid (81 mg, 0.0425 mmol). The purified peptide and cysteine (103 mg, 0.850 mmol, 20x) were dissolved in a dH_2O/DMSO (90:10) solution (100 mL) and stirred at room temperature for 48 hours. The peptide was filtered, concentrated *in vacuo*, and purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (Ac-C(H-C-OH)G-*o*NB-RGPQGIWGQGRK-NH_2 with cysteines linked *via* disulfide bond) as a yellow solid (30.9 mg, 0.0152 mmol). To introduce azide functionalities requisite for hydrogel crosslinking, N_3-OSu (Supplementary Method 1, 13.8 mg, 0.0608 mmol, 2x) was dissolved in minimal DMF with DIEA (15.7 mg, 0.122 mmol, 4x) and reacted with the peptide overnight at room temperature. The peptide was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the final product (Ac-C(N_3-C-OH)G-*o*NB-RGPQGIWGQGRK(N_3)-NH_2 with cysteines linked *via* disulfide bond, denoted R$\vee$[E$\vee$P]) as a yellow solid (32.2 mg, 0.0257 mmol, 5.1% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{93}H_{143}N_{34}O_{28}S_2^+$ $[M + {}^1H]^+$, 2248.0; observed 2248.6.



Supplementary Method 15: Enzymatic "AND" Reductive "AND" Photo degradable crosslinker synthesis [R∧E∧P]

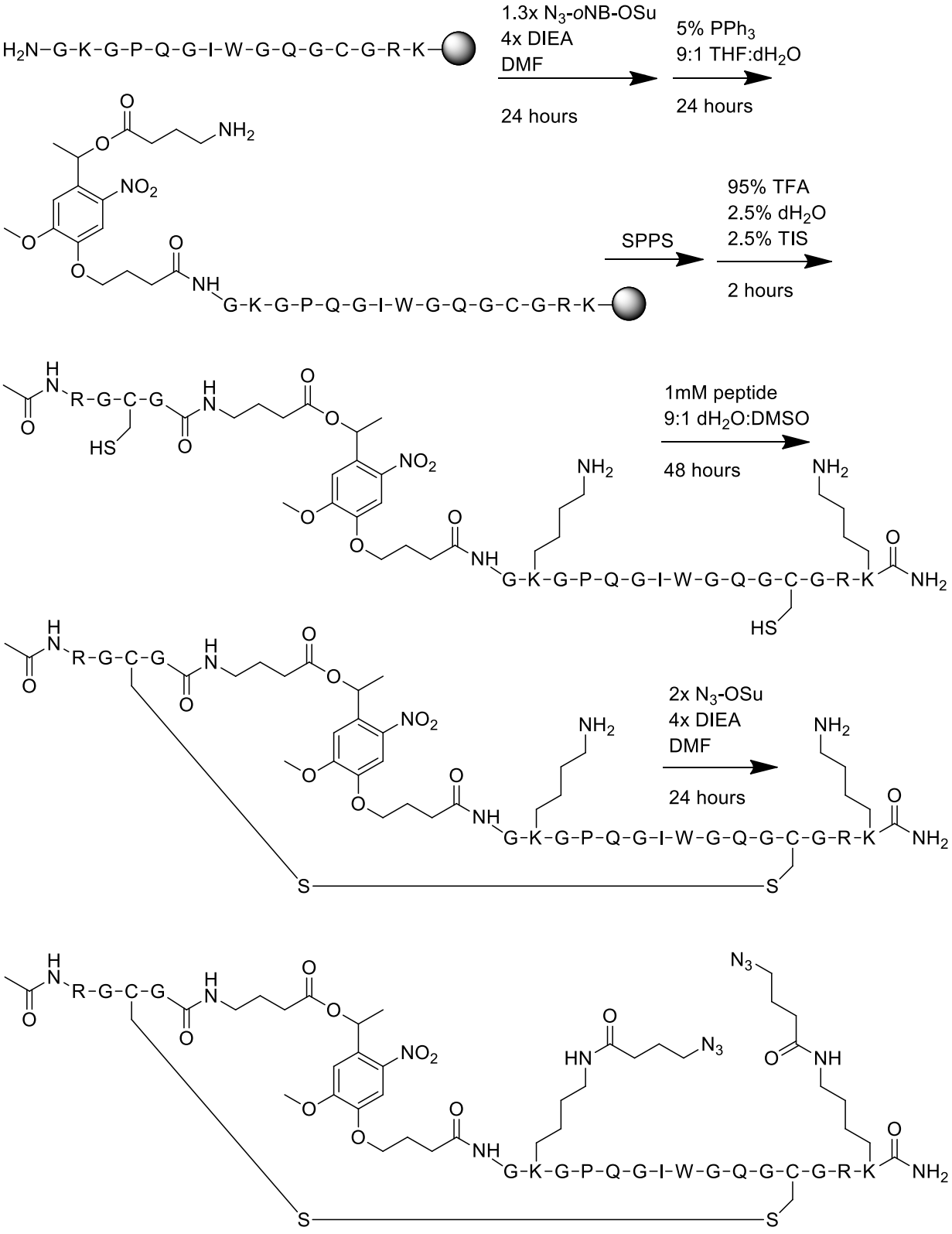




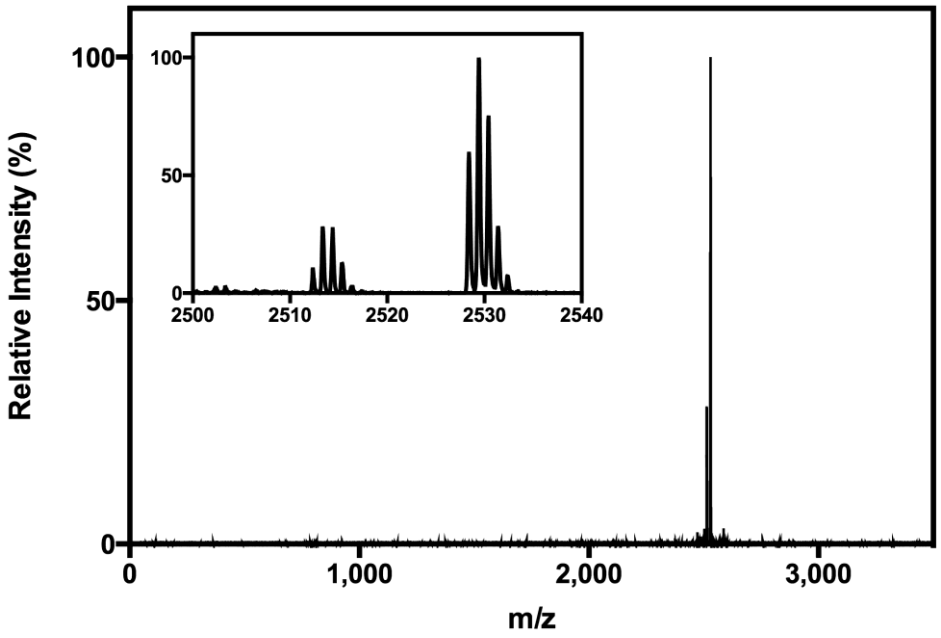
The resin-bound peptide H-K(Dde)GCGRK-NH_2 was synthesized by microwave-assisted Fmoc SPPS (Supplementary Method 4) on Rink amide resin (0.5 mmol scale). 4-pentynoic acid (0.196 g, 2.0 mmol, 4x) was pre-activated upon reaction with HATU (0.751 g, 1.975 mmol, 3.95x) and DIEA (0.517 g, 4.0 mmol, 8x) in minimal DMF for 5 minutes and then reacted with the resin for 90 minutes to functionalize the N-terminus with an alkyne. The resin was treated with hydrazine monohydrate (2%) in DMF (3 x 10 min) to remove the Dde protecting group. Fmoc-8-amino-3,6-dioxaoctanoic acid (residue denoted PEG_2) was incorporated into the peptide sequence Ac-KRGCGK(Dde)-PEG_2-GPQGIWGQG-PEG_2, which was appended to the ε-amino group of the unprotected lysine by standard microwave-assisted Fmoc SPPS. The resin was again treated with hydrazine monohydrate (2%) in DMF (3 x 10 min) to remove the second Dde protecting group. Resin was subsequently treated with N_3-*o*NB-OSu (Supplementary Method 2, 0.65 mmol, 330 mg) and DIEA (2.0 mmol, 258 mg) in minimal DMF to introduce *o*NB functionality to the ε-amino group of the unprotected lysine. The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/TIS/dH_2O (95:2.5:2.5, 30 mL) for 2 hours, and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (Ac-KRGCGK(*o*NB-N_3)-PEG_2-GPQGIWGQG-PEG_2-K(yne)GCGRK-NH_2) as a yellow solid (88 mg, 0.030 mmol). To induce CuAAC-mediated intramolecular stapling, pure linear peptide (1 mM) was dissolved in nitrogen-purged DMSO (30 mL) containing copper(I) bromide (4.3 mg, 0.030 mmol, 1x), sodium ascorbate (5.8 mg, 0.030 mmol, 1x) in water (300 μL), 2,6 lutidine (31.4 mg, 0.35 mmol, 10x), and DIEA (38.3 mg, 0.35 mmol, 10x). This mixture was allowed to react under nitrogen at room temperature for 16 hours. The mixture was concentrated *in vacuo*, redissolved in dH_2O, passed through an ion exchange column (Dowex M4195 resin, 5 grams), and lyophilized. Double-stapled product was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (Ac-KRG<u>C</u>GK(*o*NB-<u>N_3</u>)-PEG_2-GPQGIWGQG-PEG_2-K(<u>yne</u>)G<u>C</u>GRK-NH_2 with an intramolecular

staple *via* a triazole linkage between the alkyne and *o*NB-N_3 side chains and a second intramolecular staple *via* cysteine-cysteine disulfide bond) as a yellow solid (20.0 mg, 0.0068 mmol). To introduce azide functionalities requisite for hydrogel crosslinking, N_3-OSu (Supplementary Method 1, 6.2 mg, 0.027 mmol, 2x) was dissolved in minimal DMF with DIEA (7.1 mg, 0.54 mmol, 4x) and reacted with the peptide overnight at room temperature. The double-stapled product was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the final product [Ac-K(N_3)RGCGK(*o*NB-N_3)-PEG_2-GPQGIWGQG-PEG_2-K(yne)GCGRK(N_3)-NH_2 with an intramolecular staple *via* a triazole linkage between the alkyne and *o*NB-N_3 side chains and a second intramolecular staple *via* cysteine-cysteine disulfide bond, denoted R∧(E∧P)] as a yellow solid (4.2 mg, 0.0013 mmol, 0.26% overall yield). Peptide purity was confirmed using HRMS: calculated for $C_{134}H_{210}N_{47}O_{40}S_2^+$ $[M + 3\ ^1H]^{3+}$, 1061.181; observed 1061.185.

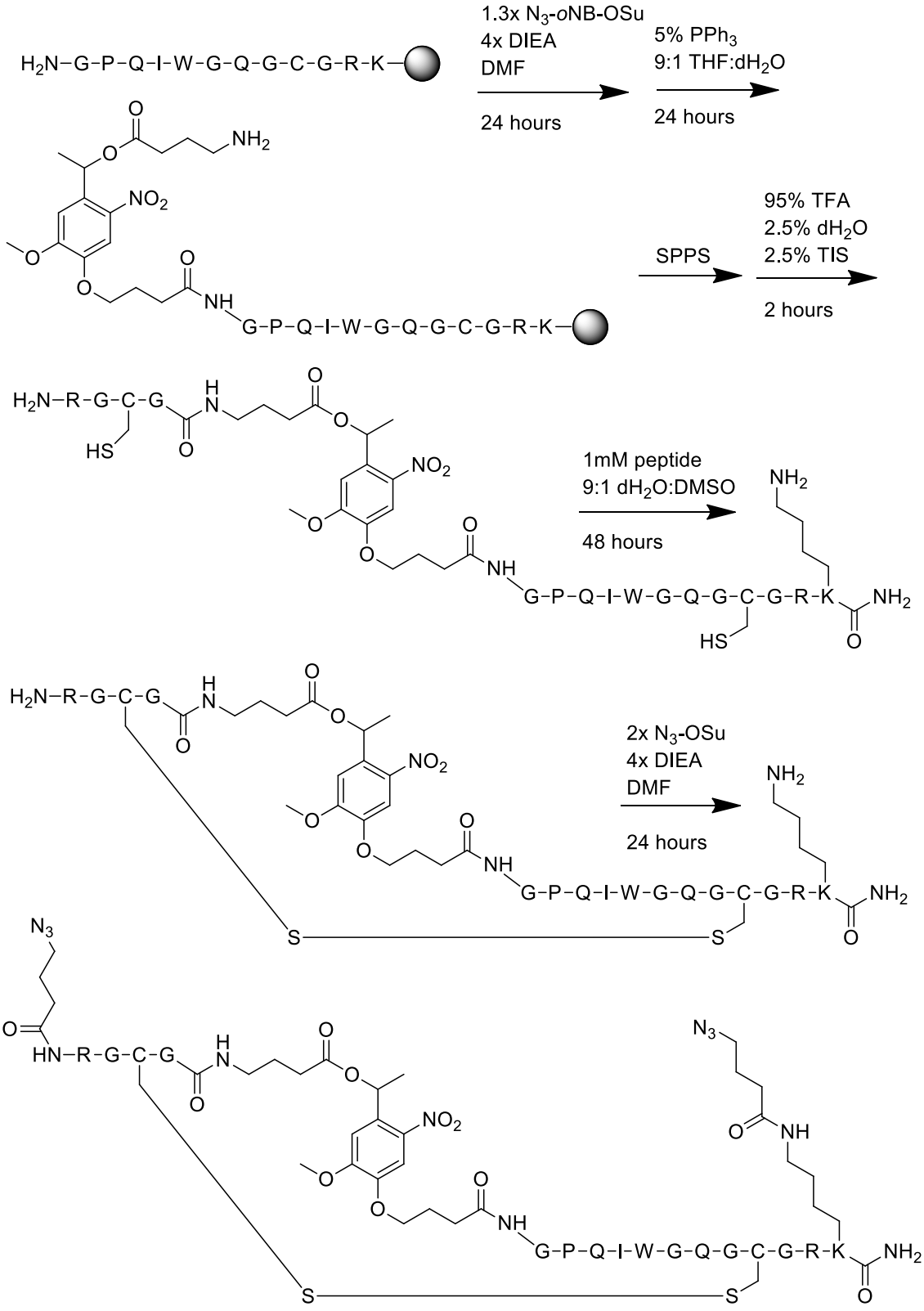
Supplementary Method 16: Enzymatic "AND" (Reductive "OR" Photo) degradable crosslinker synthesis [E∧(R∨P)]



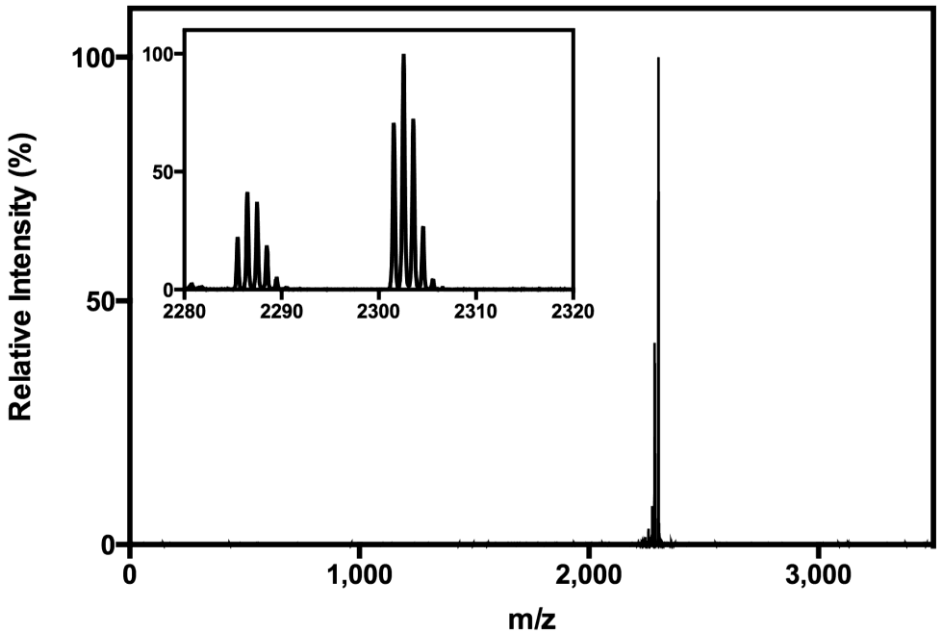
The resin-bound peptide H-GKGPQGIWGQGCGRK-NH_2 was synthesized by microwave-assisted Fmoc SPPS (Supplementary Method 4) on Rink amide resin (0.5 mmol scale). Resin was subsequently treated with N_3-*o*NB-OSu (Supplementary Method 2, 0.65 mmol, 330 mg) and DIEA (2.0 mmol, 258 mg) in minimal DMF to introduce *o*NB functionality to the N-terminus. The N-terminal azide was reduced to an amine by a Staudinger reduction; resin was washed with THF/H_2O (90:10, 3 x 20 mL), followed by reaction with 5 wt% triphenylphosphine in THF/H_2O (90:10, 30 mL) for 18 hours. The peptide sequence Ac-RGCG was appended to the N-terminus by standard microwave-assisted Fmoc SPPS. The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/TIS/dH_2O (95:2.5:2.5, 30 mL) for 2 hours, and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (Ac-RGCG-*o*NB-GKGPQGIWGQGCGRK-NH_2) as a yellow solid (57.9 mg, 0.0251 mmol). To promote disulfide-mediated intramolecular stapling, the purified peptide was dissolved at 0.5 mM in a dH_2O/DMSO (90:10) solution (50 mL) and reacted at room temperature with no agitation for 48 hours. Stapled product was concentrated *in vacuo* and purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (Ac-RG<u>C</u>G-*o*NB-GKGPQGIWGQG<u>C</u>GRK-NH_2 with intramolecular stapling *via* cysteine-cysteine <u>disulfide bond</u>) as a yellow solid (38.5 mg, 0.0167 mmol). To introduce azide functionalities requisite for hydrogel crosslinking, N_3-OSu (Supplementary Method 1, 15.1 mg, 0.0668 mmol, 2x) was dissolved in minimal DMF with DIEA (17.3 mg, 0.134 mmol, 4x) and reacted with the peptide overnight at room temperature. The stapled peptide was purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the final product [Ac-RG<u>C</u>G-*o*NB-GK(N_3)GPQGIWGQG<u>C</u>GRK(N_3)-NH_2 with intramolecular stapling *via* cysteine-cysteine <u>disulfide bond</u>, denoted E∧(R∨P)] as a yellow solid (14.0 mg, 0.0055 mmol, 1.1% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{105}H_{162}N_{39}O_{31}S_2^+$ [M + $^1H]^+$, 2529.2; observed 2529.4.



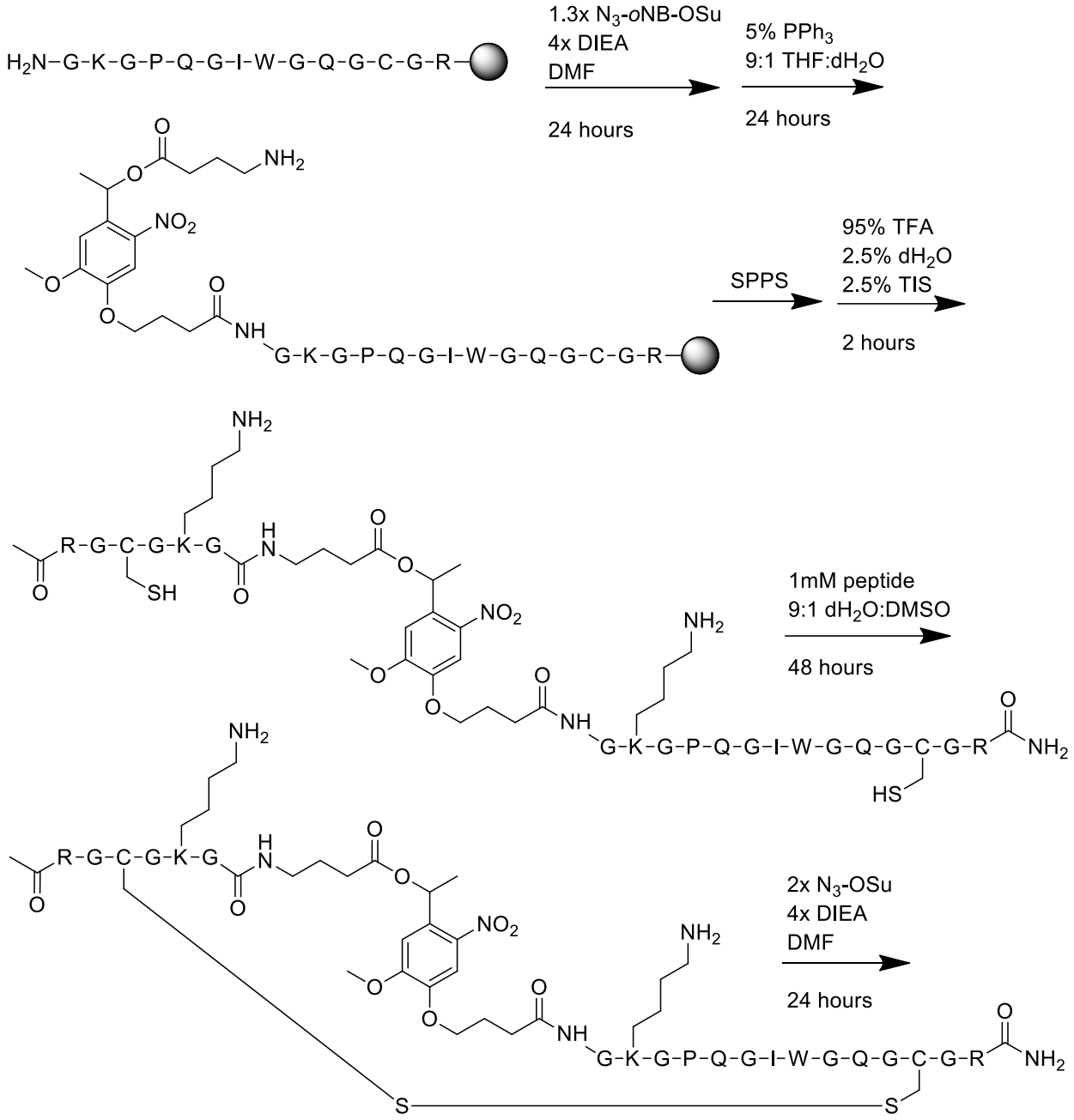
Supplementary Method 17: Reductive "AND" (Enzymatic "OR" Photo) degradable crosslinker synthesis [R∧(E∨P)]

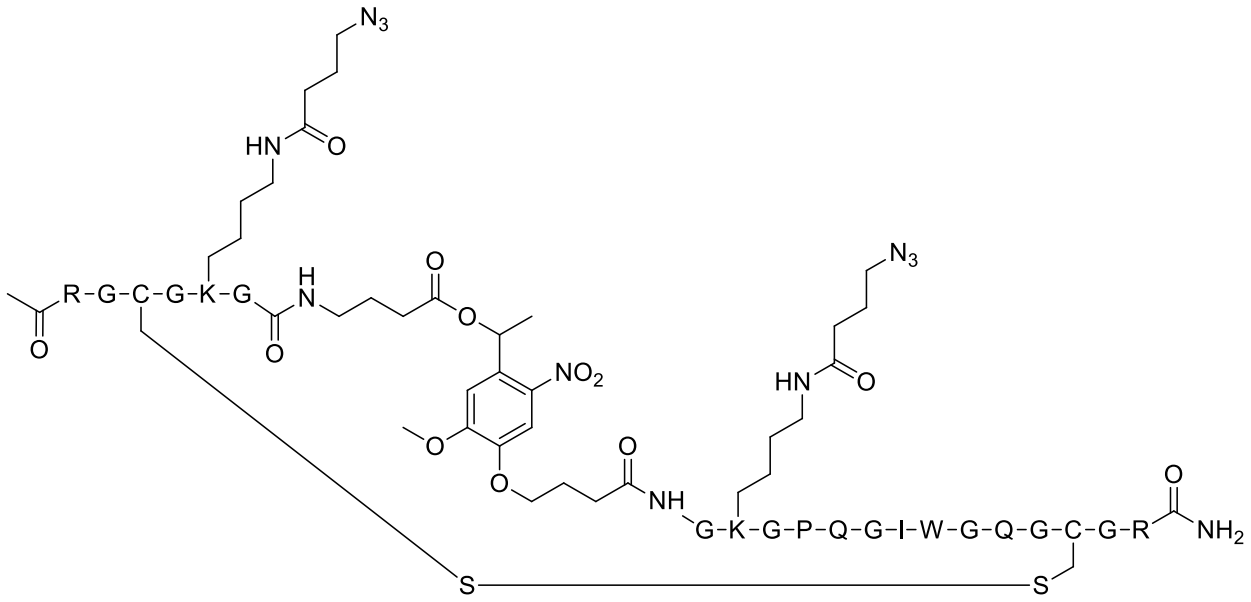


The resin-bound peptide H-GPQGIWGQGCGRK-NH_2 was synthesized by microwave-assisted Fmoc SPPS (Supplementary Method 4) on Rink amide resin (0.5 mmol scale). Resin was subsequently treated with N_3-*o*NB-OSu (Supplementary Method 2, 0.65 mmol, 330 mg) and DIEA (2.0 mmol, 258 mg) in minimal DMF to introduce *o*NB functionality to the N-terminus. The N-terminal azide was reduced to an amine by a Staudinger reduction; resin was washed with THF/H_2O (90:10, 3 x 20 mL), followed by reaction with 5 wt% triphenylphosphine in THF/H_2O (90:10, 30 mL) for 18 hours. The peptide sequence H-RGCG was appended to the N-terminus by standard microwave-assisted Fmoc SPPS. The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/TIS/dH_2O (95:2.5:2.5, 30 mL) for 2 hours, and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (H-RGCG-*o*NB-GPQGIWGQGCGRK-NH_2) as a yellow solid (58 mg, 0.028 mmol). To promote disulfide-mediated intramolecular stapling, the purified peptide was dissolved at 0.5 mM in a dH_2O/DMSO (90:10) solution (55 mL) and reacted at room temperature with no agitation for 48 hours. Stapled product was concentrated *in vacuo* and purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (H-RGCG-*o*NB-GPQGIWGQGCGRK-NH_2 with intramolecular stapling *via* cysteine-cysteine disulfide bond) as a yellow solid (23.5 mg, 0.0113 mmol). To introduce azide functionalities requisite for hydrogel crosslinking, N_3-OSu (Supplementary Method 1, 10.2 mg, 0.0452 mmol, 2x) was dissolved in minimal DMF with DIEA (11.7 mg, 0.0904 mmol, 4x) and reacted with the peptide overnight at room temperature. The stapled peptide was purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the final product [N_3-RGCG-*o*NB-GPQGIWGQGCGRK(N_3)-NH_2 with intramolecular stapling *via* cysteine-cysteine disulfide bond, denoted R∧(E∨P)] as a yellow solid (11.2 mg, 0.0049 mmol, 1.0% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{95}H_{145}N_{36}O_{28}S_2^+$ $[M + {}^1H]^+$, 2302.0; observed 2302.5.

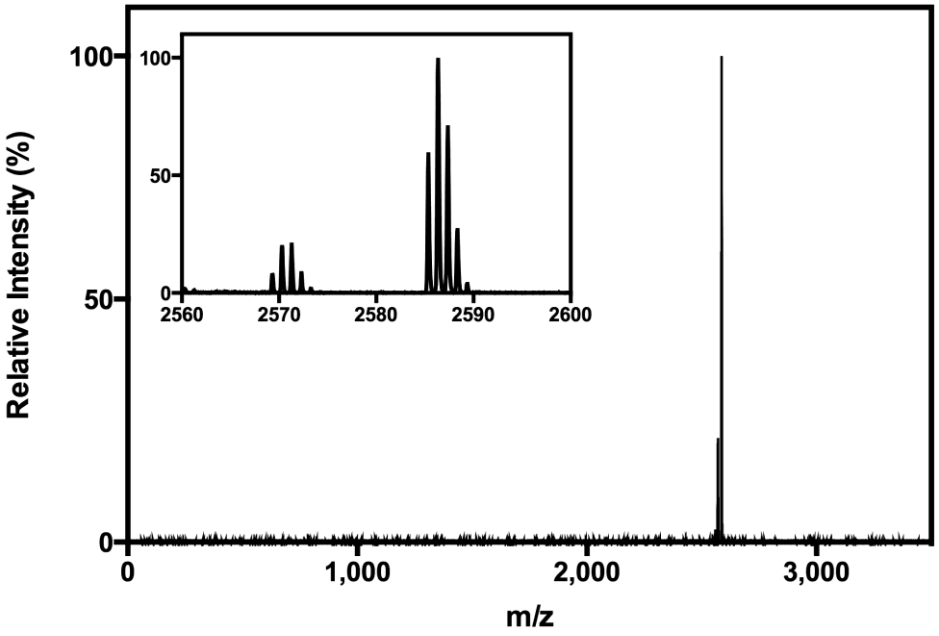


Supplementary Method 18: Photo "AND" (Reductive "OR" Enzymatic) degradable crosslinker synthesis [P∧(R∨E)]

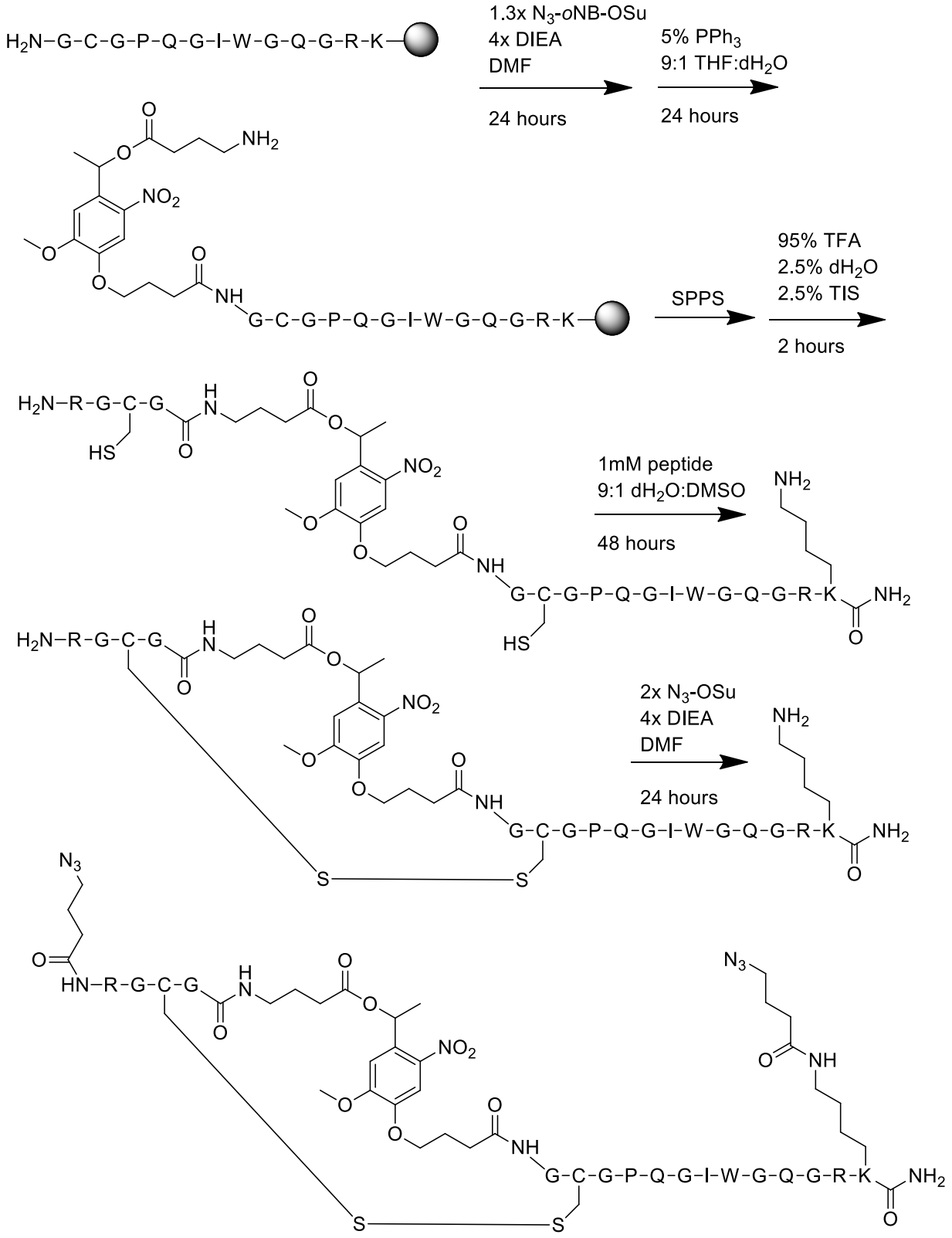




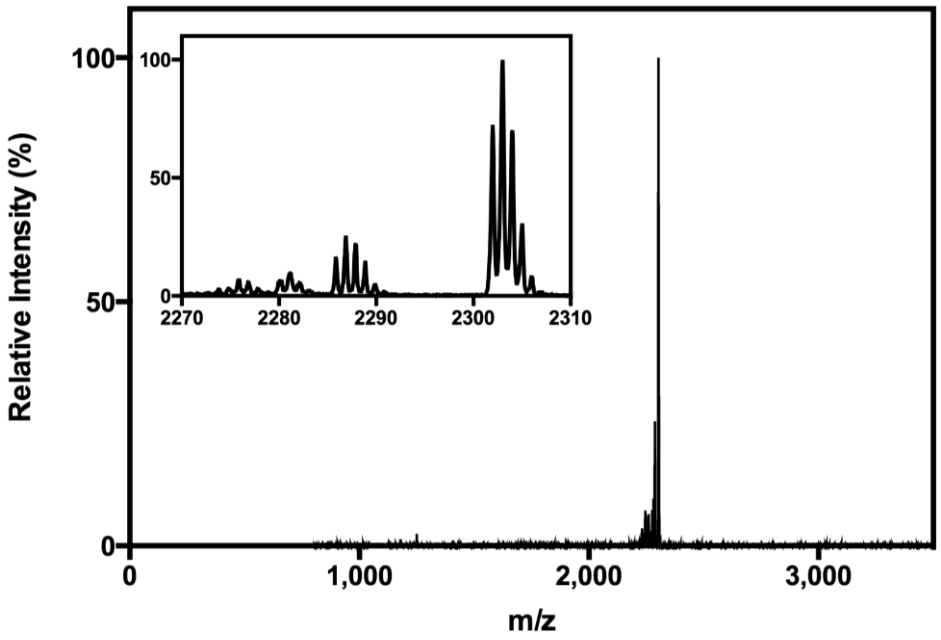
The resin-bound peptide H-GKGPQGIWGQGCGR-NH_2 was synthesized by microwave-assisted Fmoc SPPS (Supplementary Method 4) on Rink amide resin (0.5 mmol scale). Resin was subsequently treated with N_3-*o*NB-OSu (Supplementary Method 2, 0.65 mmol, 330 mg) and DIEA (2.0 mmol, 258 mg) in minimal DMF to introduce *o*NB functionality to the N-terminus. The N-terminal azide was reduced to an amine by a Staudinger reduction; resin was washed with THF/H_2O (90:10, 3 x 20 mL), followed by reaction with 5 wt% triphenylphosphine in THF/H_2O (90:10, 30 mL) for 18 hours. The peptide sequence Ac-RGCGKG was appended to the N-terminus by standard microwave-assisted Fmoc SPPS. The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/TIS/dH_2O (95:2.5:2.5, 30 mL) for 2 hours, and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (Ac-RGCGKG-*o*NB-GKGPQGIWGQGCGR-NH_2) as a yellow solid (93.1 mg, 0.0393 mmol). To promote disulfide-mediated intramolecular stapling, the purified peptide was dissolved at 0.5 mM in a dH_2O/DMSO (90:10) solution (80 mL) and reacted at room temperature with no agitation for 48 hours. Stapled product was concentrated *in vacuo* and purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (Ac-RG<u>C</u>GKG-*o*NB-GKGPQGIWGQG<u>C</u>GR-NH_2 with intramolecular stapling *via* cysteine-cysteine <u>disulfide bond</u>) as a yellow solid (55.3 mg, 0.0234 mmol). To introduce azide functionalities requisite for hydrogel crosslinking, N_3-OSu (Supplementary Method 1, 21.2 mg, 0.0936 mmol, 2x) was dissolved in minimal DMF with DIEA (24.2 mg, 0.187 mmol, 4x) and reacted with the peptide overnight at room temperature. The stapled peptide was purified using RP-HPLC operating with a 55 minute linear gradient (5–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the final product [Ac-RG<u>C</u>GK(N_3)G-*o*NB-GK(N_3)GPQGIWGQG<u>C</u>GR-NH_2 with intramolecular stapling *via* cysteine-cysteine <u>disulfide bond</u>, denoted P∧(R∨E)] as a yellow solid (11.0 mg, 0.0043 mmol, 0.9% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{107}H_{165}N_{40}O_{32}S_2^+$ [M + $^1H]^+$, 2586.2; observed 2586.4.


Relative Intensity (%)
100
50
0
0
1,000
2,000
3,000
m/z
100
50
0
2560
2570
2580
2590
2600

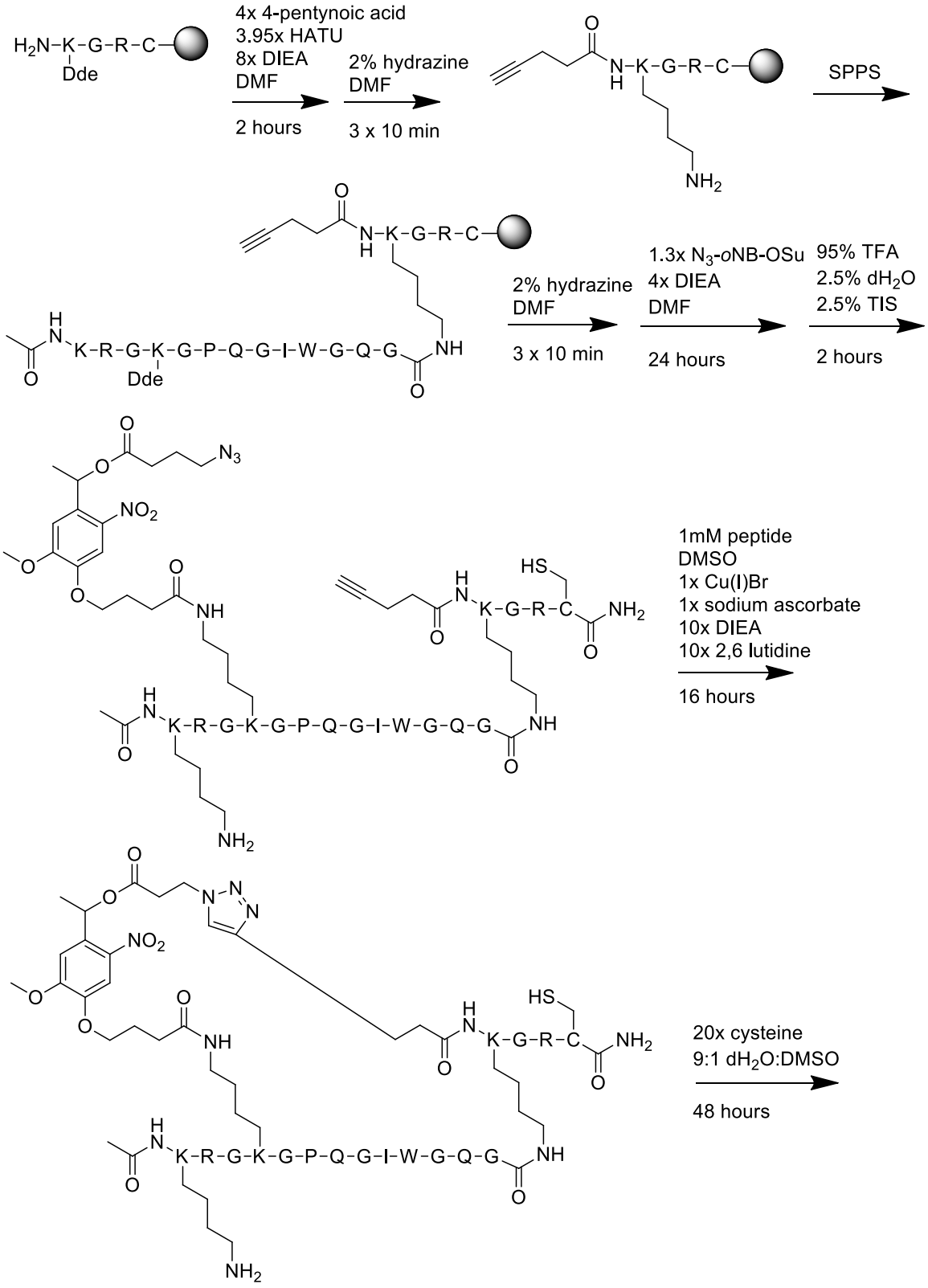
Supplementary Method 19: Enzymatic "OR" (Reductive "AND" Photo) degradable crosslinker synthesis [E∨(R∧P)]

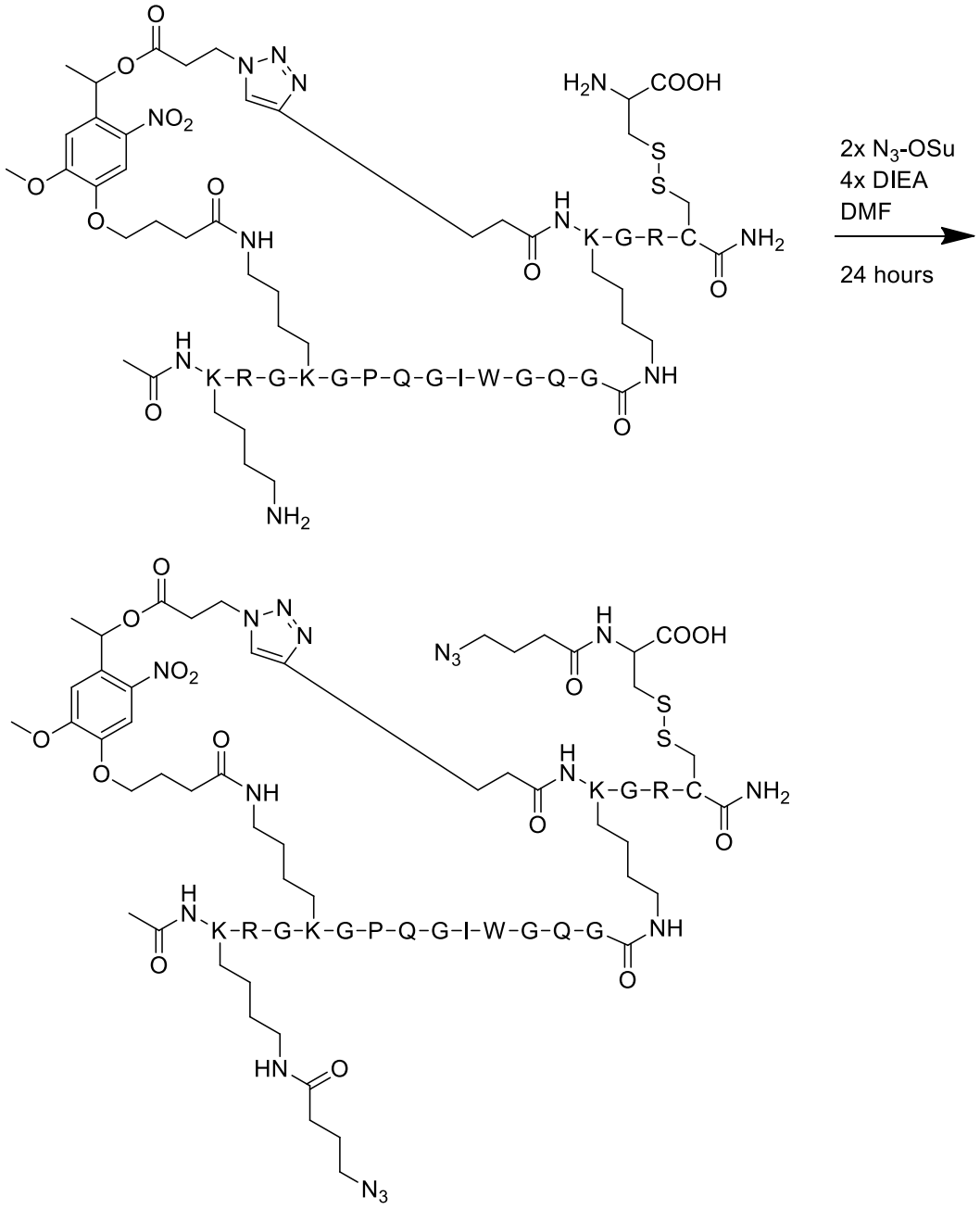


The resin-bound peptide H-GCGPQGIWGQGRK-NH_2 was synthesized by microwave-assisted Fmoc SPPS (Supplementary Method 4) on Rink amide resin (0.5 mmol scale). Resin was subsequently treated with N_3-*o*NB-OSu (Supplementary Method 2, 0.65 mmol, 330 mg) and DIEA (2.0 mmol, 258 mg) in minimal DMF to introduce *o*NB functionality to the N-terminus. The N-terminal azide was reduced to an amine by a Staudinger reduction; resin was washed with THF/H_2O (90:10, 3 x 20 mL), followed by reaction with 5 wt% triphenylphosphine in THF/H_2O (90:10, 30 mL) for 18 hours. The peptide sequence H-RGCG was appended to the N-terminus by standard microwave-assisted Fmoc SPPS. The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/TIS/dH_2O (95:2.5:2.5, 30 mL) for 2 hours, and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (H-RGCG-*o*NB-GCGPQGIWGQGRK-NH_2) as a yellow solid (85 mg, 0.0408 mmol). To promote disulfide-mediated intramolecular stapling, the purified peptide was dissolved at 0.5 mM in a dH_2O/DMSO (90:10) solution (80 mL) and reacted at room temperature with no agitation for 48 hours. Stapled product was concentrated *in vacuo* and purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (H-RGCG-*o*NB-GCGPQGIWGQGRK-NH_2 with intramolecular stapling *via* cysteine-cysteine disulfide bond) as a yellow solid (33.9 mg, 0.0163 mmol). To introduce azide functionalities requisite for hydrogel crosslinking, N_3-OSu (Supplementary Method 1, 14.7 mg, 0.0652 mmol, 2x) was dissolved in minimal DMF with DIEA (16.9 mg, 0.130 mmol, 4x) and reacted with the peptide overnight at room temperature. Stapled product was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the final product [N_3-RGCG-*o*NB-GCGPQGIWGQGRK(N_3)-NH_2 with intramolecular stapling *via* cysteine-cysteine disulfide bond, denoted E∨(R∧P)] as a yellow solid (14.9 mg, 0.0065 mmol, 1.3% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{95}H_{145}N_{36}O_{28}S_2^+$ $[M + {}^1H]^+$, 2302.0; observed 2303.0.



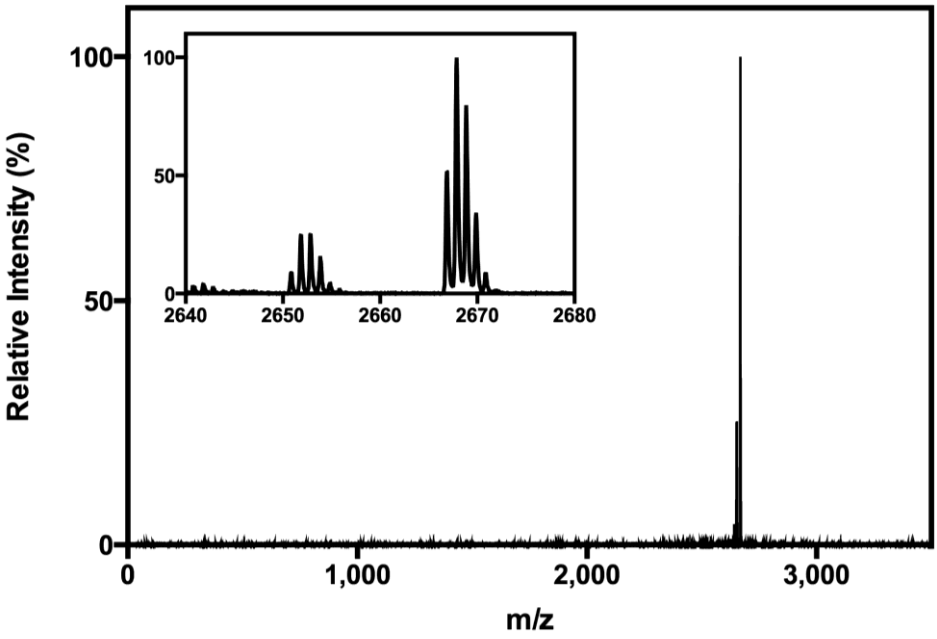
Supplementary Method 20: Reductive "OR" (Enzymatic "AND" Photo) degradable crosslinker synthesis [R∨(E∧P)]



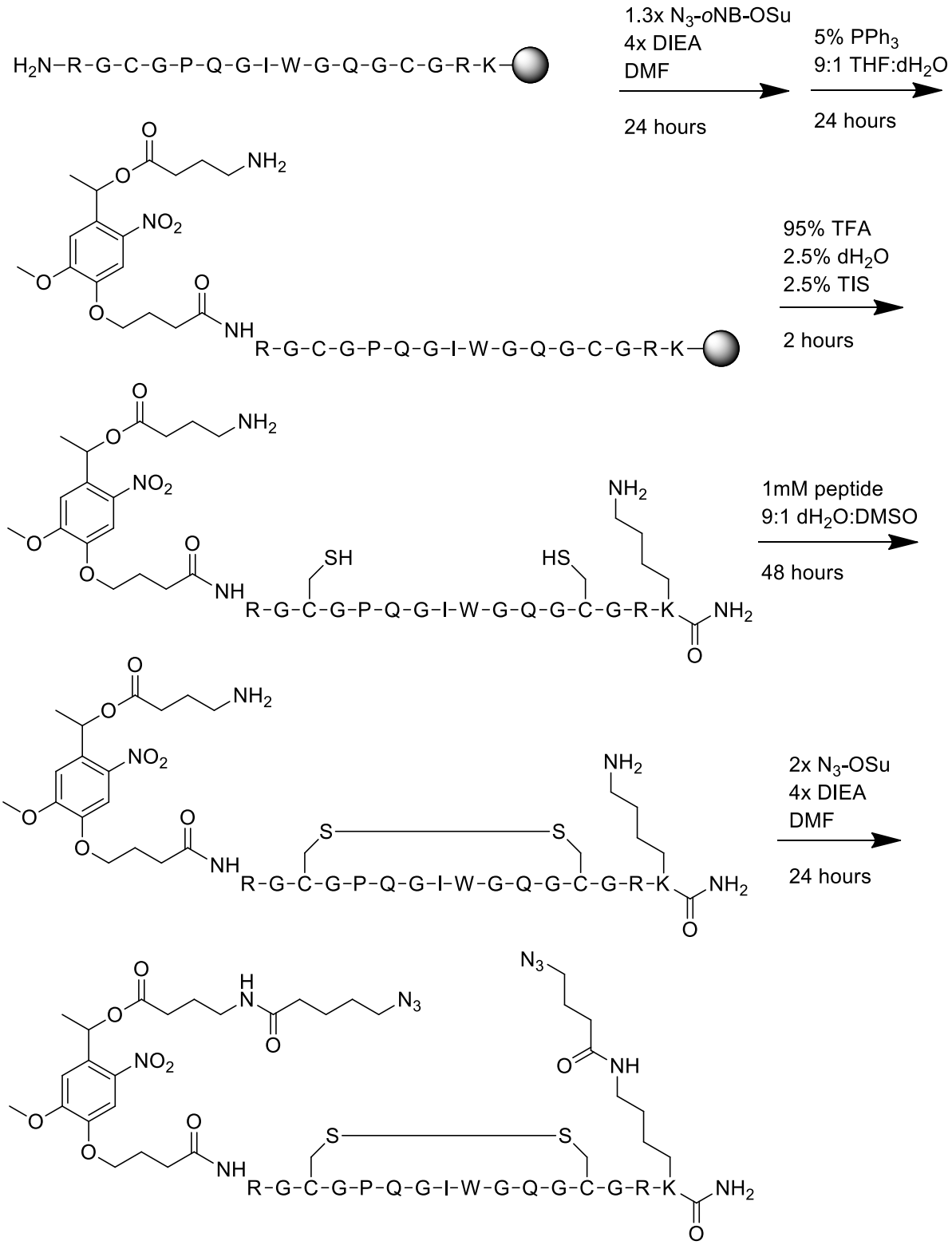


The resin-bound peptide H-K(Dde)GRC-NH_2 was synthesized by microwave-assisted Fmoc SPPS (Supplementary Method 4) on Rink amide resin (0.5 mmol scale). 4-pentynoic acid (0.196 g, 2.0 mmol, 4x) was pre-activated upon reaction with HATU (0.751 g, 1.975 mmol, 3.95x) and DIEA (0.517 g, 4.0 mmol, 8x) in minimal DMF for 5 minutes and then reacted with the resin for 90 minutes to functionalize the N-terminus with an alkyne. The resin was treated with hydrazine monohydrate (2%) in DMF (3 x 10 min) to remove the Dde protecting group. The peptide sequence Ac-KRGK(Dde)GPQGIWGQG was appended to the ε-amino group of the unprotected lysine by standard microwave-assisted Fmoc. The resin was again treated with hydrazine monohydrate (2%) in DMF (3 x 10 min) to remove the second Dde protecting group. Resin was subsequently treated with N_3-*o*NB-OSu (Supplementary Method 2, 0.65 mmol, 330 mg) and DIEA (2.0 mmol, 258 mg)

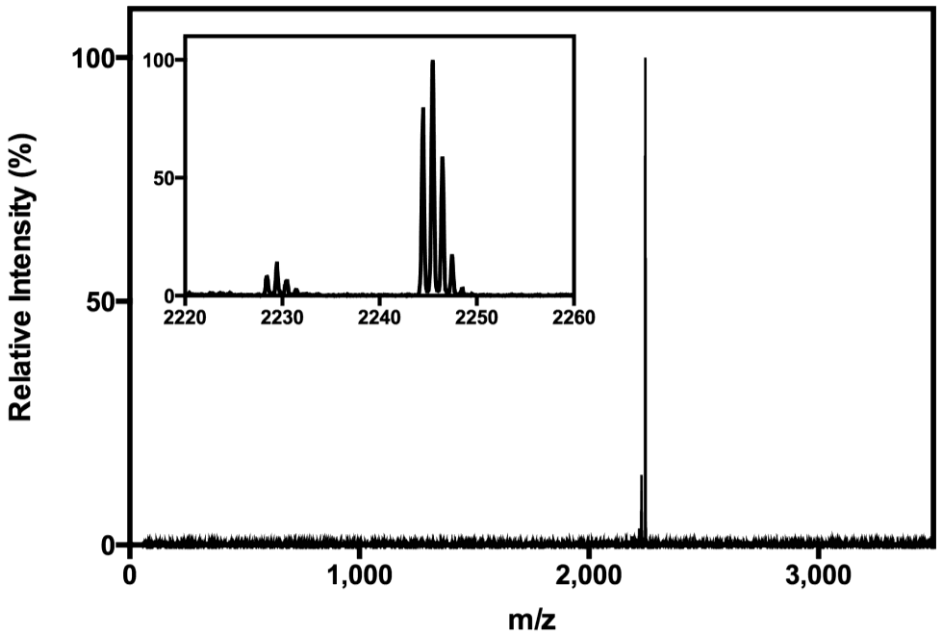
in minimal DMF to introduce *o*NB functionality to the ε-amino group of the unprotected lysine. The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/TIS/dH_2O (95:2.5:2.5, 30 mL) for 2 hours, and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (Ac-KRGK(*o*NB-N_3)GPQGIWGQGK(yne)GRC-NH_2) as a yellow solid (115 mg, 0.0494 mmol). To induce CuAAC-mediated intramolecular stapling, pure linear peptide (1 mM) was dissolved in nitrogen-purged DMSO (50 mL) containing copper(I) bromide (7.1 mg, 0.049 mmol, 1x), sodium ascorbate (9.8 mg, 0.049 mmol, 1x) in water (480 μL), 2,6 lutidine (52.9 mg, 0.494 mmol, 10x), and DIEA (63.8 mg, 0.494 mmol, 10x). This mixture was allowed to react under nitrogen at room temperature for 16 hours. The mixture was concentrated *in vacuo*, redissolved in dH_2O, passed through an ion exchange column (Dowex M4195 resin, 5 grams), and lyophilized. Stapled product was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (Ac-KRGK(*o*NB-$\underline{N_3}$)GPQGIWGQGK(<u>yne</u>)GRC-NH_2 with an intramolecular staple *via* a <u>triazole linkage</u> between the alkyne and *o*NB-N_3 side chains) as a yellow solid (55 mg, 0.0236 mmol). The purified peptide and cysteine (57 mg, 0.47 mmol, 20x) were dissolved in a dH_2O/DMSO (90:10) solution (20 mL) and stirred at room temperature for 48 hours. The stapled peptide was filtered, concentrated *in vacuo*, and purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (Ac-KRGK(*o*NB-$\underline{N_3}$)GPQGIWGQGK(<u>yne</u>)GR<u>C</u>(H-<u>C</u>-OH)-NH_2 with an intramolecular staple *via* a <u>triazole linkage</u> between the alkyne and *o*NB-N_3 side chains and cysteines linked *via* <u>disulfide bond</u>) as a yellow solid (30.0 mg, 0.0123 mmol). To introduce azide functionalities requisite for hydrogel crosslinking, N_3-OSu (Supplementary Method 1, 11.1 mg, 0.049 mmol, 2x) was dissolved in minimal DMF with DIEA (12.7 mg, 0.098 mmol, 4x) and reacted with the peptide overnight at room temperature. The stapled peptide was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the final product [Ac-K(N_3)RGK(*o*NB-$\underline{N_3}$)GPQGIWGQGK(<u>yne</u>)GR<u>C</u>(N_3-<u>C</u>-OH)-NH_2 with an intramolecular staple *via* a <u>triazole linkage</u> between the alkyne and *o*NB-N_3 side chains and cysteines linked *via* <u>disulfide bond</u>, denoted R∨(E∧P)] as a yellow solid (27.1 mg, 0.0102 mmol, 2.0% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{112}H_{172}N_{41}O_{32}S_2^+$ $[M + {}^1H]^+$, 2667.3; observed 2667.9.


Relative Intensity (%)
100
50
0
0
1,000
2,000
3,000
m/z
100
50
0
2640
2650
2660
2670
2680

Supplementary Method 21: Photo "OR" (Reductive "AND" Enzymatic) degradable crosslinker synthesis [P∨(R∧E)]



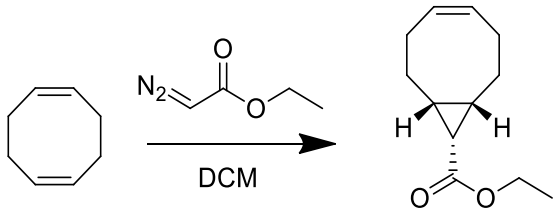
The resin-bound peptide H-RGCGPQGIWGQGCGRK-NH_2 was synthesized by microwave-assisted Fmoc SPPS (Supplementary Method 4) on Rink amide resin (0.5 mmol scale). Resin was subsequently treated with N_3-*o*NB-OSu (Supplementary Method 2, 0.65 mmol, 330 mg) and DIEA (2.0 mmol, 258 mg) in minimal DMF to introduce *o*NB functionality to the N-terminus. The N-terminal azide was reduced to an amine by a Staudinger reduction; resin was washed with THF/H_2O (90:10, 3 x 20 mL), followed by reaction with 5 wt% triphenylphosphine in THF/H_2O (90:10, 30 mL) for 18 hours. The peptide was simultaneously deprotected and cleaved from resin upon treatment with TFA/TIS/dH_2O (95:2.5:2.5, 30 mL) for 2 hours, and the crude peptide was precipitated in and washed with ice-cold diethyl ether (2x). The crude peptide was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (H_2N-*o*NB-RGCGPQGIWGQGCGRK-NH_2) as a yellow solid (99 mg, 0.0489 mmol). To promote disulfide-mediated intramolecular stapling, the purified peptide was dissolved at 0.5 mM in a dH_2O/DMSO (90:10) solution (100 mL) and reacted at room temperature with no agitation for 48 hours. Stapled product was concentrated *in vacuo* and purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the pure intermediate (H_2N-*o*NB-RGCGPQGIWGQGCGRK-NH_2 with intramolecular stapling *via* cysteine-cysteine disulfide bond) as a yellow solid (33.7 mg, 0.0167 mmol). To introduce azide functionalities requisite for hydrogel crosslinking, N_3-OSu (Supplementary Method 1, 15.1 mg, 0.0668 mmol, 2x) was dissolved in minimal DMF with DIEA (17.2 mg, 0.133 mmol, 4x) and reacted with the peptide overnight at room temperature. The stapled peptide was purified using RP-HPLC operating with a 43.4 minute linear gradient (20–100%) of acetonitrile in water containing TFA (0.1%); lyophilization yielded the final product [N_3-*o*NB-RGCGPQGIWGQGCGRK(N_3)-NH_2 with intramolecular stapling *via* cysteine-cysteine disulfide bond, denoted P∨(R∧E)] as a yellow solid (10.0 mg, 0.0045 mmol, 0.9% overall yield). Peptide purity was confirmed using MALDI-TOF: calculated for $C_{93}H_{142}N_{35}O_{27}S_2^+$ $[M + {}^1H]^+$, 2245.0; observed 2245.5.



Supplementary Method 22: Synthesis of 4-arm-PEG$_{20kDa}$-tetrabicyclononyne (PEG-tetraBCN) and fluorescent variants (PEG-tetraBCN-AF568, PEG-tetraBCN-FAM, PEG-tetraBCN-Cyanine5)

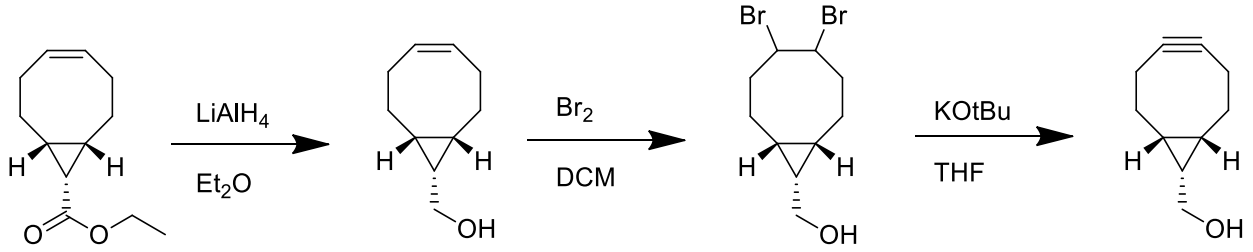
(1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl (2,5-dioxopyrrolidin-1-yl) carbonate (BCN-OSu) was synthesized from *(1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethanol* (BCN-OH)[5] following a published synthetic route[6].

Synthesis of (1*R*,8*S*,9*s*,*Z*)-ethyl bicyclo[6.1.0]non-4-ene-9-carboxylate (endo):



Ethyl diazoacetate (7.56 g, 7.0 mL, 66 mmol) in DCM (35 mL) was added dropwise to a 0°C solution of 1,5-cyclooctadiene (44.1 g, 50 mL, 408 mmol), rhodium(II) acetate dimer catalyst (1.0 g), and DCM (30 mL). The mixture was reacted at room temperature for 72 hours, filtered to remove the catalyst, and concentrated *in vacuo*. The product was purified on a silica column (0-1% ethyl acetate in hexanes) and concentrated to give both the exo (5.62 g, 28.9 mmol, 43.8% yield) and desired endo product (3.70 g, 19.0 mmol, 28.9% yield). Endo: ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.64 (m, 2H), 4.14 (q, J=7.1 Hz, 2H), 2.53 (m, 2H), 2.23 (m, 2H), 2.08 (m, 2H), 1.86 (m, 2H), 1.73 (t, J=8.8 Hz, 1H), 1.42 (m, 2H), 1.29 (t, J=7.1 Hz, 3H).

Synthesis of (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethanol:

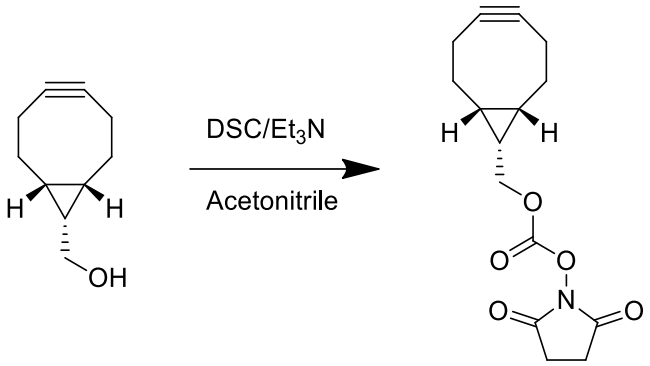


The endo intermediate, (1*R*,8*S*,9*s*,*Z*)-ethyl bicyclo[6.1.0]non-4-ene-9-carboxylate (3.70 g, 19.0 mmol), was dissolved in 0°C anhydrous ether (65 mL). Lithium aluminum hydride (750 mg, 19.8 mmol, 1.04x) in 0°C anhydrous ether (130 mL) was added dropwise to the ester over 15 minutes and the reaction was stirred at room temperature for 20 minutes. Minimal water was added to quench the solution and induced the formation of a grey precipitate. The solution was dried over $MgSO_4$, filtered, and concentrated *in vacuo* to yield the crude alcohol intermediate (1*R*,8*S*,9*s*,*Z*)-bicyclo[6.1.0]non-4-en-9-ylmethanol (2.91 g, 19.1 mmol, quantitative yield).

A solution of bromine (3.1g, 1.0 mL, 19 mmol) in DCM (13 mL) and added dropwise to a 0°C solution of the crude hydroxyl intermediate dissolved in anhydrous DCM (140 mL) until a yellow color persisted. The reaction was quenched with aqueous sodium thiosulfate (10 wt %, 50 mL). The product was extracted into DCM (2 x 70 mL), dried over $MgSO_4$, filtered, and concentrated *in vacuo* to yield the crude dibromide intermediate ((1*R*,8*S*,9*s*)-4,5-dibromobicyclo[6.1.0]nonan-9-yl)methanol (5.8 g, 19 mmol, quantitative yield).

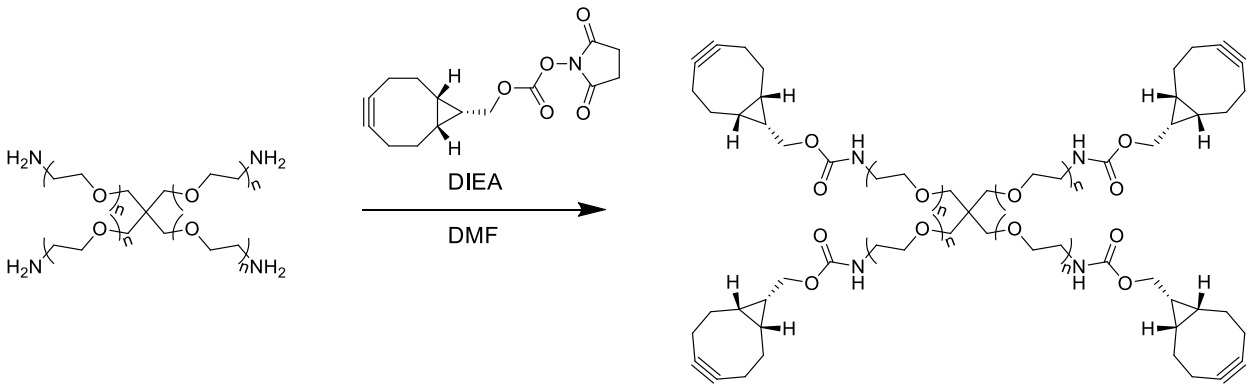
The dibromide intermediate was dissolved in 0°C anhydrous THF (120 mL) to which a solution of potassium tert-butoxide (1 M in THF, 50 mL) was added dropwise. The reaction mixture was stirred while refluxing at 75°C for 2.5 hours. The solution was cooled to room temperature and quenched with saturated aqueous ammonium chloride (150 mL). THF was removed *in vacuo* and the product was extracted into DCM (3 x 70 mL), dried over $MgSO_4$, filtered, and concentrated. The product was purified on a silica column (0-20% ethyl acetate in hexanes) and concentrated to yield pure (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethanol as a yellow oil (1.48 g, 9.85 mmol, 52% yield over 3 steps). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.62 (d, J=7.7 Hz, 2H), 2.59 (br s, 1H), 2.16 (m, 6H), 1.50 (m, 2H), 1.22 (m, 1H), 0.84 (m, 1H).

Synthesis of [(1*R*,8*S*,9*S*)-bicyclo[6.1.0]non-4-yn-9-yl]methyl 2,5-dioxopyrrolidin-1-yl carbonate (BCN-OSu):



(1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethanol (1.48 g, 9.9 mmol) and *N,N'*-Disuccinimidyl carbonate (5.1 g, 19.9 mmol, 2x) were dissolved in acetonitrile (75 mL) under a nitrogen atmosphere. Triethylamine (3.3g, 4.5 mL, 32 mmol) was added and the reaction was stirred overnight at room temperature. The reaction was diluted with 1:1 ethyl acetate:ether (200 mL) and washed with water (6 x 100 mL) and brine (2 x 50 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The product was purified on a silica flash column (3:1 hexanes:ethyl acetate) to yield the product [(1R,8S,9S)-bicyclo[6.1.0]non-4-yn-9-yl]methyl 2,5-dioxopyrrolidin-1-yl carbonate (denoted BCN-OSu) as a white solid (1.72 g, 5.9 mmol, 60% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.48 (d, J=8.4 Hz, 2H), 2.87 (s, 4H), 2.30 (m, 6H), 1.57 (m, 3H), 1.09 (m, 2H). These spectral data matched those previously reported[6].

Synthesis of 4-arm-PEG$_{20kDa}$-tetrabicyclononyne (PEG-tetraBCN):
4-arm-PEG$_{20kDa}$-tetrabicyclononyne was synthesized following a known synthetic route[6].



Four-arm poly(ethylene glycol) (PEG) tetraamine (M_n ~ 20 kDa, n ~ 113, 1.00 g, 0.050 mmol PEG, 0.200 mmol NH_2) and BCN-OSu (87.5 mg, 0.300 mmol, 1.5x) were dissolved in minimal DMF (10 mL) with DIEA (103.4 mg, 0.80 mmol, 4x) and stirred at room temperature overnight. The reaction mixture was diluted with water, dialyzed [molecular weight cut-off (MWCO) ~ 1 kDa, SpectraPor® 7], and lyophilized to yield the product (denoted PEG-tetraBCN) as a white powder (0.964 g, 0.0466 mmol, 93% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.23 (s, 4H), 4.13 (d, J = 8.0 Hz, 8H), 3.76 – 3.71 (m, 3.3 Hz, 8H), 3.63 – 3.61 (m, 1818H), 2.34 – 2.14 (m, 24H), 1.65 – 1.48 (m, 8H), 1.40 – 1.27 (m, 4H), 0.99 – 0.87 (m, 8H). Integral values of the ^{1}H NMR peaks characteristic of BCN (δ 2.24, 1.57, 1.34, 0.93) were compared to the PEG backbone (δ 3.62) to confirm that functionalization of amines with BCN exceeded 95%.

Synthesis of Alexa Fluor® 568 functionalized PEG-tetraBCN (PEG-tetraBCN-AF568):
Alexa Fluor® 568 cadavarine (0.963 mg, 1.19 μmol, 1x) and N_3-OSu (Supplementary Method 1, 0.536 mg, 2.38 μmol, 2x) were dissolved in minimal DMF (1 mL) with DIEA (0.615 mg, 4.76 μmol, 4x) and stirred at room temperature overnight. The reaction mixture was added directly to a solution of 4-arm-PEG$_{20kDa}$-tetraBCN (0.964 g, 0.0466 mmol, 40x) in phosphate-buffered saline (PBS, 30 mL) and stirred at room temperature for 3 hours. The reaction mixture was then dialyzed (MWCO ~ 10 kDa, SnakeSkin™) and lyophilized to give the product (denoted PEG-tetraBCN-AF568) as a pink powder (0.896 g, 0.0433 mmol, 93% yield).

Synthesis of FAM functionalized PEG-tetraBCN (PEG-tetraBCN-FAM):
5-FAM azide (AAT Bioquest, 20 μL of 100 mM in DMSO, 0.0020 mmol) and 4-arm-PEG$_{20kDa}$-tetraBCN (0.202 g, 0.0101 mmol) was dissolved in PBS (2 mL) and stirred for 2 hours at room temperature. The reaction mixture was diluted with water, dialyzed (MWCO 1kDa, SpectraPor® 7), and lyophilized to yield the product (denoted PEG-tetraBCN-FAM) as a yellow solid (0.204 g, 0.101 mmol, quantitative yield).

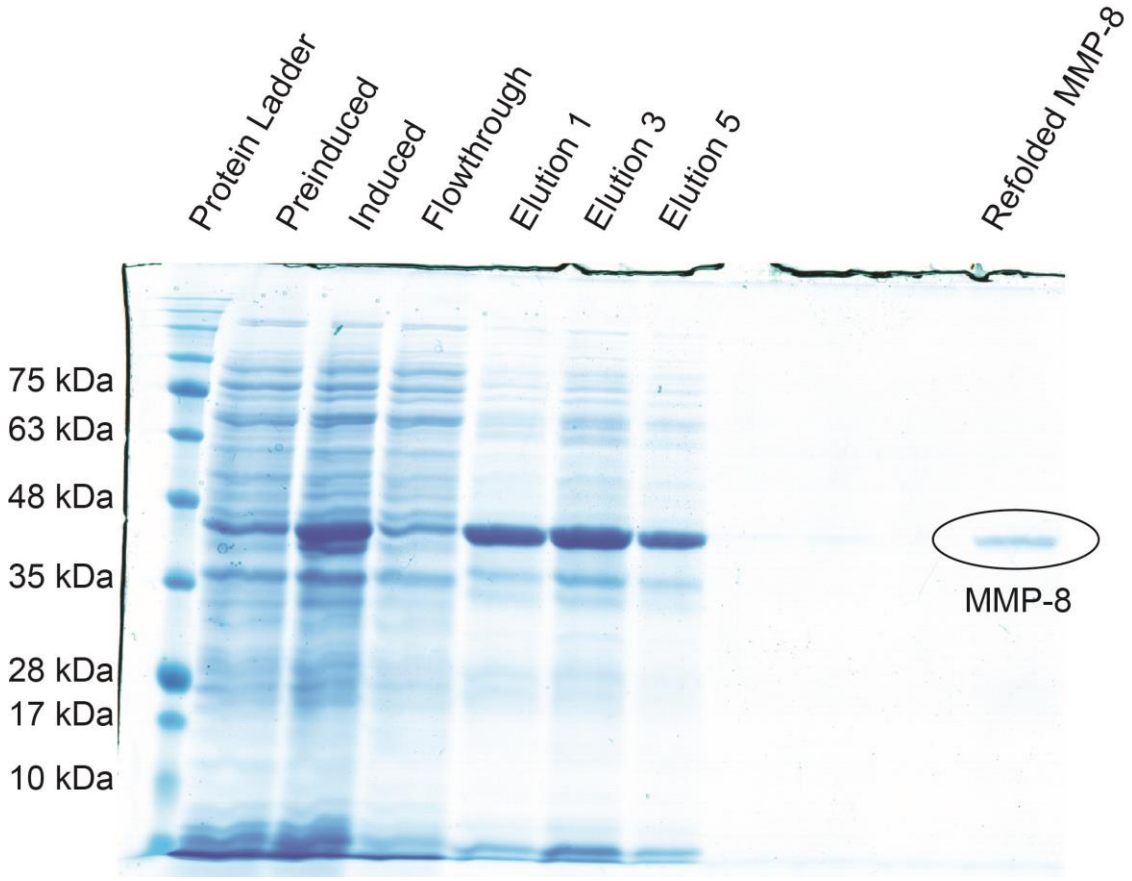
Synthesis of Cyanine5 functionalized PEG-tetraBCN (PEG-tetraBCN-Cyanine5):
Cyanine5 azide (Lumiprobe, 20 nmol) in DMSO (2 μL) was added to a solution of PEG-tetraBCN (2 μmol, 100x) in PBS (200 μL). The solution was mixed and reacted at room temperature for 2 hours. The blue product (denoted PEG-tetraBCN-Cyanine5) was used without further purification and kept as a 10 mM stock solution in PBS.

Supplementary Method 23: Recombinant expression and purification of matrix metalloproteinase-8 (MMP-8)

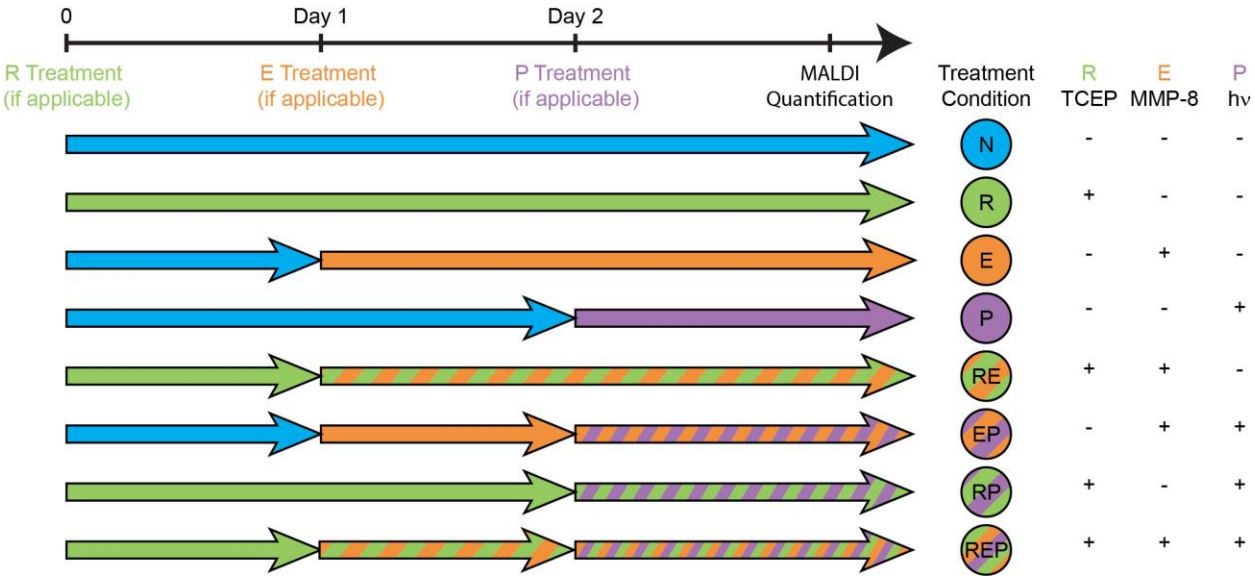
The MMP-8 gene was lifted from pCMV-Tag4A-MMP8 Wt (a gift from Yardena Samuels[7], Addgene plasmid # 29545), adding a C-terminal 6xHis purification tag and appropriate restriction enzyme cut sites (*Nde*I and *Xho*I) using PCR, placed into pET-28a (+) (EMD Biosciences), and expressed in BL21(DE3)pLysS (Promega Corporation). Cells were lysed *via* sonication, and the 6xHis-tagged protein was solubilized in 6 M urea prior to standard polyhistidine affinity purification using Ni-NTA (Fisher Pierce).

The purified protein was denatured and solubilized in MMP buffer (200 mM sodium chloride, 50 mM tris, 5 mM calcium chloride, 1 μM zinc chloride, pH adjusted to 7.5 with hydrochloric acid) containing 6 M urea and placed into dialysis tubing (MWCO ~3.5 kDa, SpectraPor® 7). The protein was dialyzed against MMP buffer containing progressively lower urea concentrations (4 M → 0 M) over the course of 48 hours at 4 °C, and then concentrated on a spin column (10 kDa MWCO) to yield the purified, refolded MMP-8.

Protein purity was assessed by SDS-PAGE analysis (12% Bis-Tris gel), and a single band corresponding to the expected molecular weight (43.2 kDa) was observed by Coumassie staining.



Supplementary Method 24: Assessing solution-based crosslinker degradation in response to external stimuli



To assess crosslinker fragmentation in response to different combinations of external stimuli, a series of solution-based studies were performed on nine of the synthesized peptides: E, R, P, R∨E, E∨P, R∨P, R∧E, E∧P, and R∧P. Peptide crosslinkers (40 nmol) were dissolved in MMP buffer (110 μL) and exposed to a unique input combination of enzyme, reductive, and light treatments (outlined in further detail below). In each study, degradation products for distinct crosslinker/treatment combinations were identified by MALDI mass spectrometry; observed cleavage products were compared with those expected to assess degradation modalities of the engineered crosslinker species.

Samples receiving the reductive input ($_R$) were treated with tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl, 2 μL, 100 mM in MMP buffer) and incubated overnight at 37 °C. To quench any unreached TCEP, these samples were further treated with hydroxyethyl disulfide (HEDS, 5 μL, 100 mM in MMP buffer) prior to incubation (4 hr, 37 °C). Samples not receiving reductive input were maintained at 37 °C.

Samples receiving the enzyme input ($_E$) were subsequently treated with MMP-8 (Supplementary Method 23, 5 μL, 0.2 mg mL^{-1} in MMP buffer) and all samples were incubated (20 hr, 37 °C).

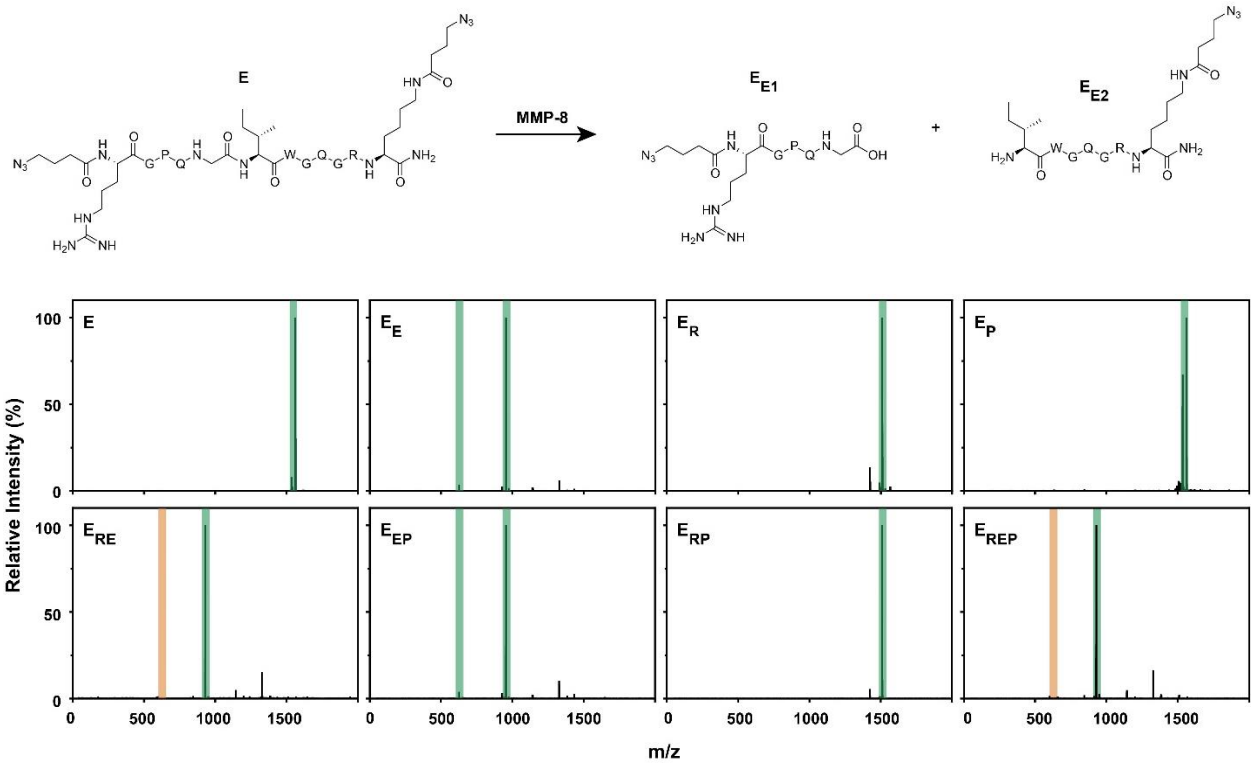
Samples receiving the light input ($_P$) were subsequently exposed to UV light (λ = 365 nm, 10 mW cm^{-2} incident light, 60 minute exposure).

All samples were diluted with acetonitrile/water (80:20, 100 μL) containing 0.1% TFA and characterized by MALDI-TOF as shown in Supplementary Figures 1-9. In these figures, green bars represent expected products that were observed while yellow bars represent expected products that were not observed. Treatment conditions are referenced using the crosslinker logical notation with a subscript indicating the inputs. For example, P_E corresponds to the "photo YES" crosslinker treated with enzyme; $(R∧E)_{REP}$ corresponds to the "reductive AND enzymatic" crosslinker (R∧E)

treated with every cue ($_{REP}$). Peptide fragments are referenced using the crosslinker logical notation with a subscript indicating the cues required to generate the product. If a crosslinker generates multiple fragments when treated with a set of cues, the products are differentiated by a numerical subscript. For example, $(R\wedge E)_{RE1}$ and $(R\wedge E)_{RE2}$ represent the two distinct products generated when the "reductive AND enzymatic" crosslinker (R∧E) is exposed to both reductive and enzymatic treatment.

There are inherent limitations in using MALDI to identify low-mass molecules due to interferance of the matrix molecules[8]; only molecules larger than 600 Da are within the MALDI range of detection (ROD). Additionally, molecules analyzed by MALDI that contain an *o*NB moiety generate the parent molecular ion as well as -16, -30, and -32 Da peaks corresponding to the laser-induced photodecomposition of the nitro (NO_2) group into nitroso (NO), amino (NH_2), and triplet nitrene (N:) groups, respectively[9,10]. When peptides were treated with reducing conditions, mass shifts of -26 and -52 Da were observed, correspond to a single and double reduction of an azide group (N_3) to an amino group (NH_2), respectively[11]. Free thiols (-SH) oxidized in the presence of reduced HEDS generated mass shifts of +76 Da, corresponding to the disulfide-containing species ($-SSCH_2CH_2OH$).

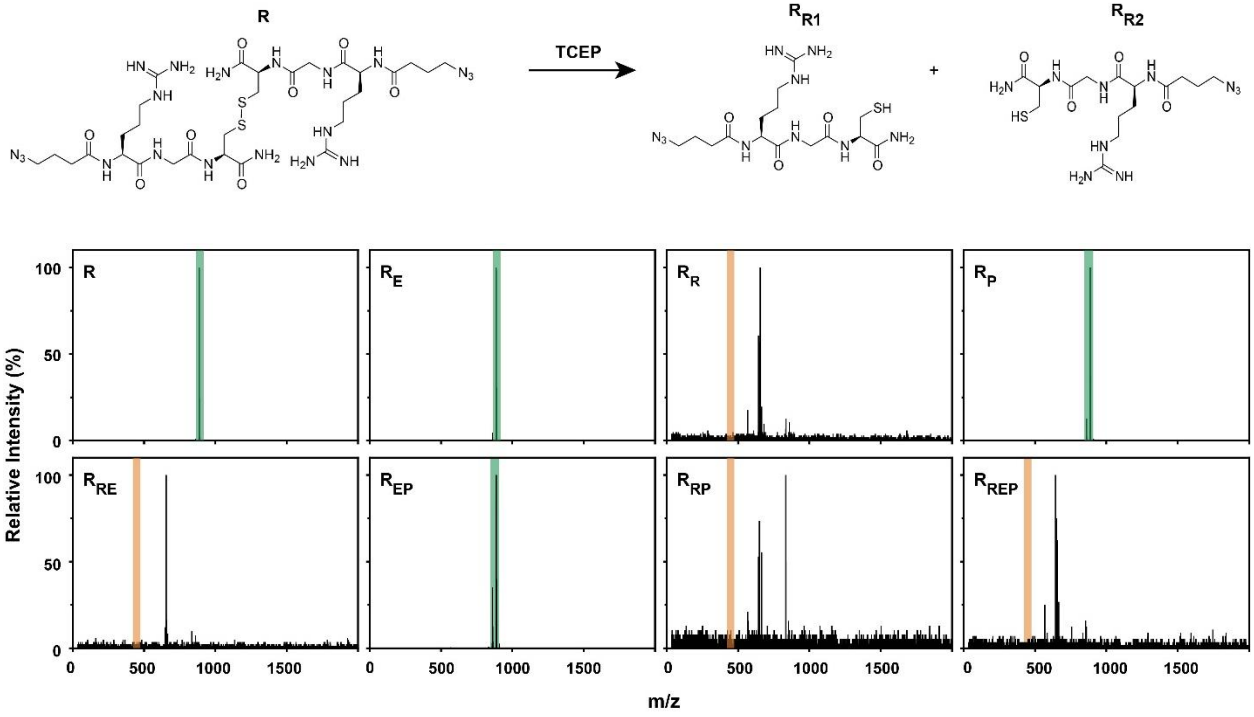
Supplementary Figure 1: Characterization of E crosslinker degradation in solution



Treatment	Expected Peptides	m/z (Da)	
		Calculated	Measured
E	E	$[M+H]^+$ = 1560.8; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 1534.8	1560.8; 1534.8
E_E	E_{E1}	$[M+H]^+$ = 625.3	625.5
	E_{E2}	$[M+H]^+$ = 954.5	954.6
E_R	E	$[M+H]^+$ = 1560.8; $[M+H: 2\ -N_3 \rightarrow 2\ -NH_2]^+$ = 1508.8	1509.0
E_P	E	$[M+H]^+$ = 1560.8; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 1534.8	1560.8; 1533.7
E_{RE}	E_{E1}	$[M+H]^+$ = 625.3; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 599.3	Below ROD
	E_{E2}	$[M+H]^+$ = 954.5; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 928.5	928.7
E_{EP}	E_{E1}	$[M+H]^+$ = 625.3	625.4
	E_{E2}	$[M+H]^+$ = 954.5	954.6
E_{RP}	E	$[M+H]^+$ = 1560.8; $[M+H: 2\ -N_3 \rightarrow 2\ -NH_2]^+$ = 1508.8	1508.9
E_{REP}	E_{E1}	$[M+H]^+$ = 625.3; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 599.3	Below ROD
	E_{E2}	$[M+H]^+$ = 954.5; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 928.5	928.6

Expected products were identified in all treatment conditions except product E_{E1}, presumably due to being below the MALDI range of detection (m/z > 600). Products were of good purity in all cases.

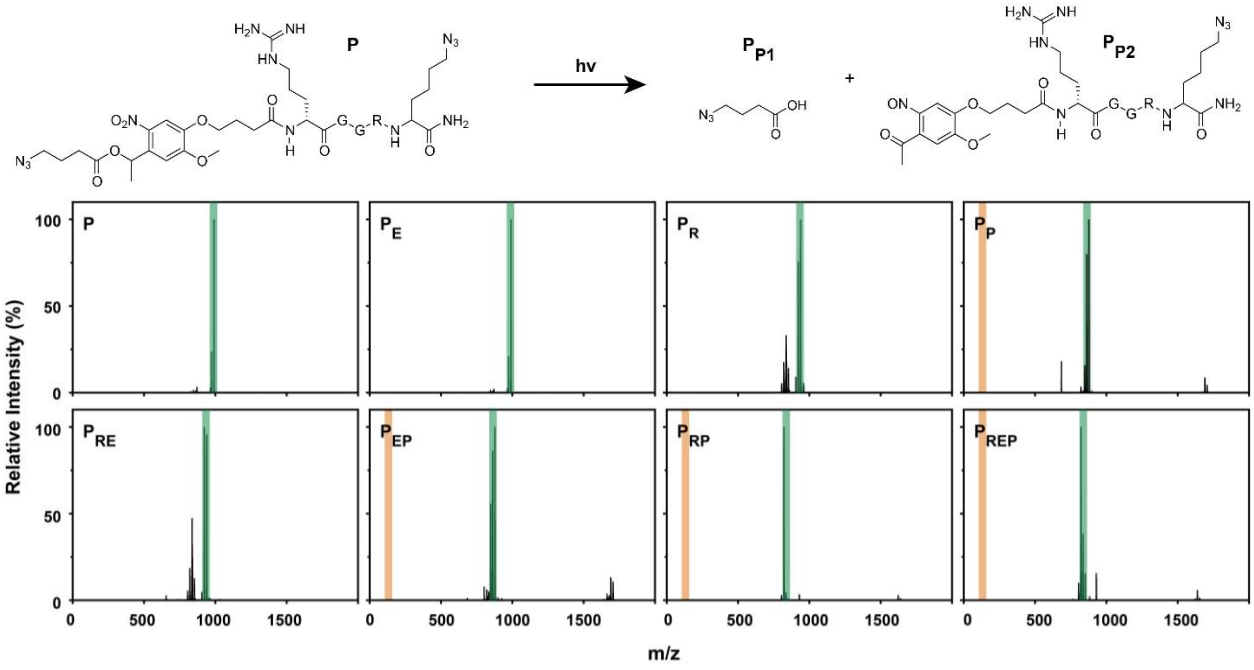
Supplementary Figure 2: Characterization of R crosslinker degradation in solution



Treatment	Expected Peptides	m/z (Da)	
		Calculated	Measured
R	R	$[M+H]^+$ = 887.4	887.4
R_E	R	$[M+H]^+$ = 887.4	887.4
R_R	R_{R1}	$[M+H]^+$ = 445.2	Below ROD
	R_{R2}	$[M+H]^+$ = 445.2	Below ROD
R_P	R	$[M+H]^+$ = 887.4; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 861.4	887.6; 861.6
R_{RE}	R_{R1}	$[M+H]^+$ = 445.2	Below ROD
	R_{R2}	$[M+H]^+$ = 445.2	Below ROD
R_{EP}	R	$[M+H]^+$ = 887.4; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 861.4	887.5; 860.4
R_{RP}	R_{R1}	$[M+H]^+$ = 445.2	Below ROD
	R_{R2}	$[M+H]^+$ = 445.2	Below ROD
R_{REP}	R_{R1}	$[M+H]^+$ = 445.2	Below ROD
	R_{R2}	$[M+H]^+$ = 445.2	Below ROD

Expected products were identified in all treatment conditions except R_{R1} and R_{R2}, presumably due to the products being below the MALDI range of detection (m/z > 600). In treatments R_R, R_{RE}, R_{RP}, and R_{REP}, the noisy background signal is likely because no expected products were in MALDI ROD.

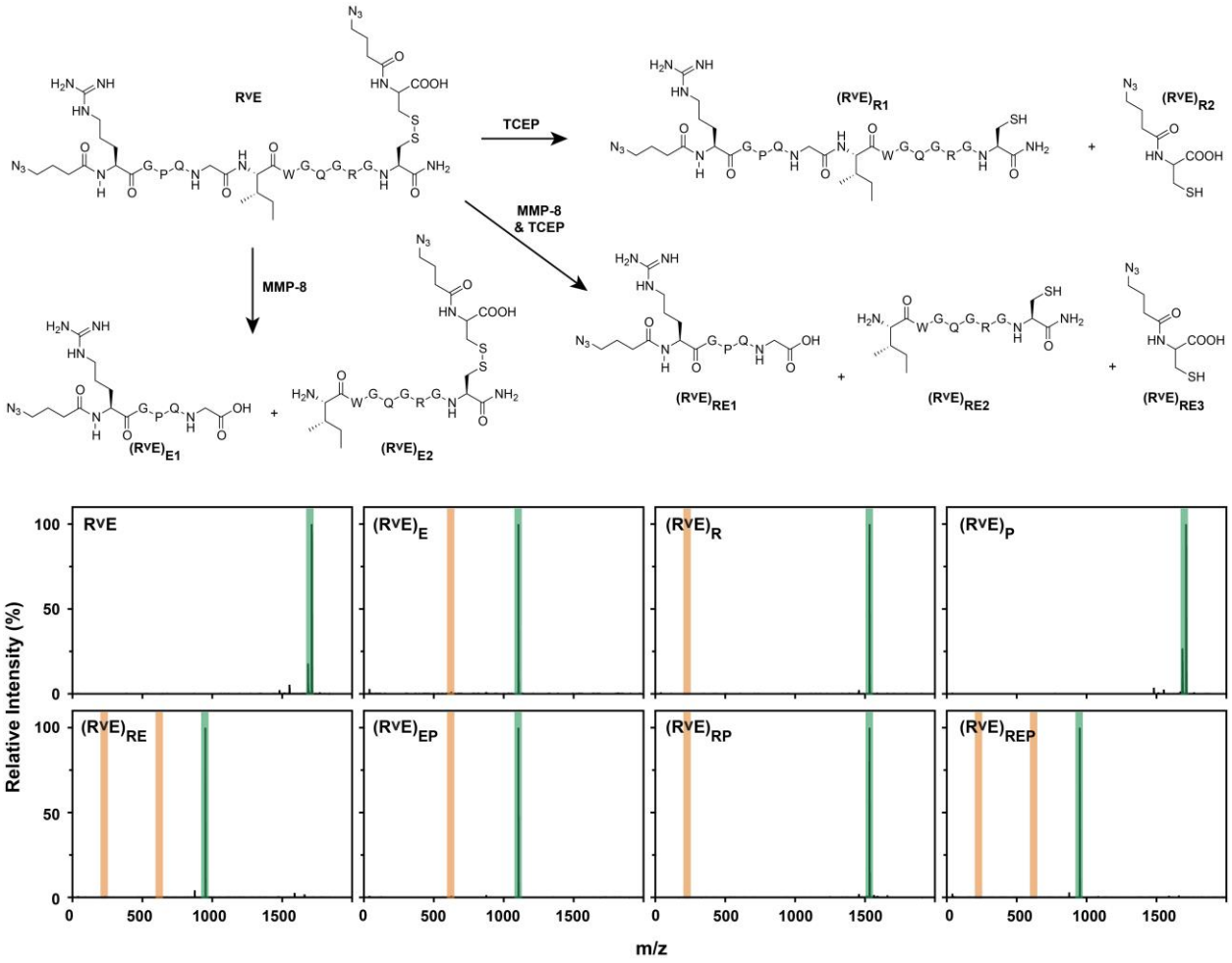
Supplementary Figure 3: Characterization of P crosslinker degradation in solution



Treatment	Expected Peptides	m/z (Da)	
		Calculated	Measured
P	P	$[M+H]^+$ = 990.5; [M+H: $-NO_2 \rightarrow -NO]^+$ = 974.5	990.6; 974.6
P_E	P	$[M+H]^+$ = 990.5; [M+H: $-NO_2 \rightarrow -NO]^+$ = 974.5	990.6; 974.6
P_R	P	$[M+H]^+$ = 990.5; [M+H: 2 $-N_3 \rightarrow$ 2 $-NH_2]^+$ = 938.5; [M+H: 2 $-N_3 \rightarrow$ 2 $-NH_2$ & $-NO_2 \rightarrow -NO]^+$ = 922.5; [M+H: 2 $-N_3 \rightarrow$ 2 $-NH_2$ & $-NO_2 \rightarrow -N]^+$ = 906.5	938.6; 922.6; 906.5
P_P	P_{P1}	$[M+H]^+$ = 130.1	Below ROD
	P_{P2}	$[M+H]^+$ = 861.4; [M+H: $-NO_2 \rightarrow -NH_2]^+$ = 847.4	861.6; 847.6
P_{RE}	P	$[M+H]^+$ = 990.5; [M+H: 2 $-N_3 \rightarrow$ 2 $-NH_2]^+$ = 938.5; [M+H: 2 $-N_3 \rightarrow$ 2 $-NH_2$ & $-NO_2 \rightarrow -NO]^+$ = 922.5; [M+H: 2 $-N_3 \rightarrow$ 2 $-NH_2$ & $-NO_2 \rightarrow -N]^+$ = 906.5	938.6; 922.5; 906.5
P_{EP}	P_{P1}	$[M+H]^+$ = 130.1	Below ROD
	P_{P2}	$[M+H]^+$ = 861.4; [M+H: $-NO_2 \rightarrow -NH_2]^+$ = 847.4	861.6; 847.5
P_{RP}	P_{P1}	$[M+H]^+$ = 130.1	Below ROD
	P_{P2}	$[M+H]^+$ = 861.4; [M+H: $-N_3 \rightarrow -NH_2]^+$ = 835.4; [M+H: -N3 → -NH2 & -NO → -NH2]+ = 821.5	835.5; 821.5
P_{REP}	P_{P1}	$[M+H]^+$ = 130.1	Below ROD
	P_{P2}	$[M+H]^+$ = 861.4; [M+H: $-N_3 \rightarrow -NH_2]^+$ = 835.4; [M+H: $-N_3 \rightarrow -NH_2$ & -NO → $-NH_2]^+$ = 821.5	835.6; 821.5

Expected products were identified in all treatment conditions except P_{P1}, presumably due to being below the MALDI range of detection (m/z > 600). Products were of good purity in all cases except for treatments P_R and P_{RE}.

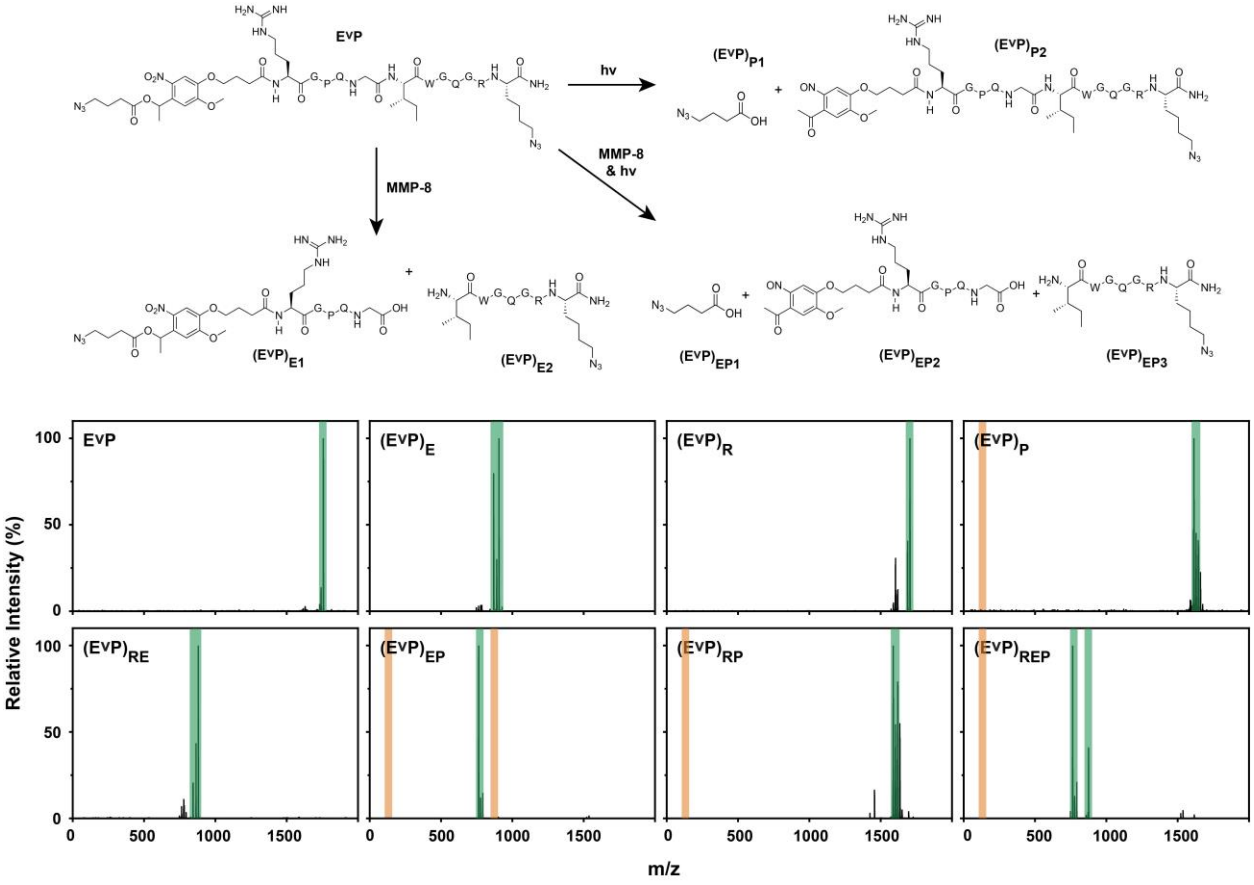
Supplementary Figure 4: Characterization of RVE crosslinker degradation in solution



Treatment	Expected Peptides	m/z (Da)	
		Calculated	Measured
RVE	RVE	$[M+H]^+$ = 1711.8; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 1685.8	1711.5; 1685.4
$(RVE)_E$	$(RVE)_{E1}$	$[M+H]^+$ = 625.3; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 599.3	Below ROD
	$(RVE)_{E2}$	$[M+H]^+$ = 1105.5	1105.4
$(RVE)_R$	$(RVE)_{R1}$	$[M+H]^+$ = 1481.7; $[M+H: -N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH]^+$ = 1531.8	1531.6
	$(RVE)_{R2}$	$[M+H]^+$ = 233.1; $[M+H: -N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH]^+$ = 283.2	Below ROD
$(RVE)_P$	RVE	$[M+H]^+$ = 1711.8; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 1685.8	1711.6; 1685.6
$(RVE)_{RE}$	$(RVE)_{RE1}$	$[M+H]^+$ = 625.3; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 599.3	Below ROD
	$(RVE)_{RE2}$	$[M+H]^+$ = 875.4; $[M+H: -N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH]^+$ = 950.4	951.4
	$(RVE)_{RE3}$	$[M+H]^+$ = 233.1; $[M+H: -N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH]^+$ = 283.2	Below ROD
$(RVE)_{EP}$	$(RVE)_{E1}$	$[M+H]^+$ = 625.3; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 599.3	Below ROD
	$(RVE)_{E2}$	$[M+H]^+$ = 1105.5	1105.4
$(RVE)_{RP}$	$(RVE)_{R1}$	$[M+H]^+$ = 1481.7; $[M+H: -N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH]^+$ = 1531.8	1531.7
	$(RVE)_{R2}$	$[M+H]^+$ = 233.1	Below ROD
$(RVE)_{REP}$	$(RVE)_{RE1}$	$[M+H]^+$ = 625.3; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 599.3	Below ROD
	$(RVE)_{RE2}$	$[M+H]^+$ = 875.4; $[M+H: -N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH]^+$ = 950.4	951.4
	$(RVE)_{RE3}$	$[M+H]^+$ = 233.1; $[M+H: -N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH]^+$ = 283.2	Below ROD

Expected products were identified in all treatment conditions except products $(RVE)_{E1}$, $(RVE)_{R2}$, $(RVE)_{RE1}$, and $(RVE)_{RE3}$, presumably due to being below the MALDI range of detection (m/z > 600). Products were of good purity in all cases.

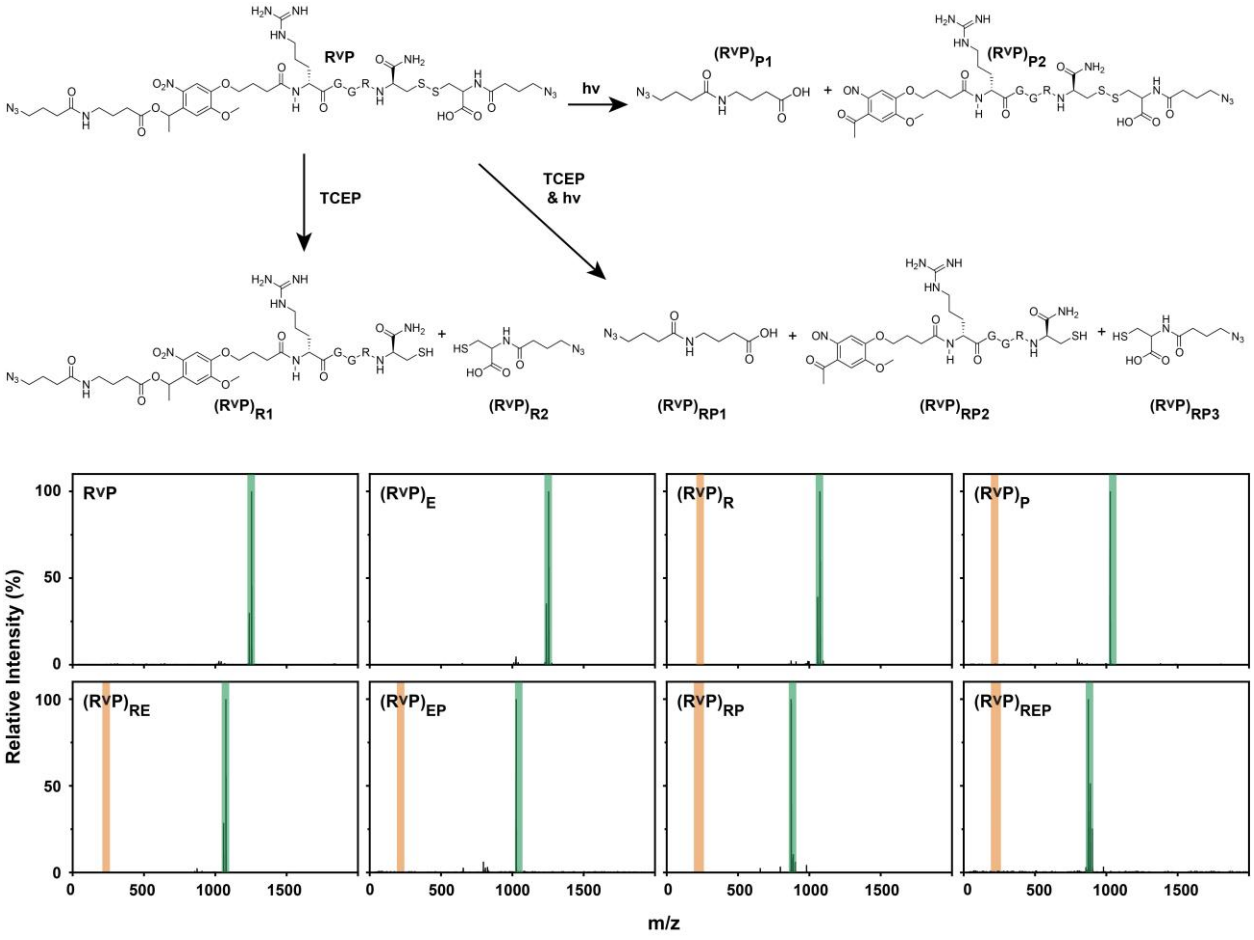
Supplementary Figure 5: Characterization of EVP crosslinker degradation in solution



Treatment	Expected Peptides	m/z (Da)	
		Calculated	Measured
EVP	EVP	$[M+H]^+$ = 1756.9; $[M+H: -NO_2 \rightarrow -NO]^+$ = 1740.9	1756.8; 1740.8
$(EVP)_E$	$(EVP)_{E1}$	$[M+H]^+$ = 906.4; $[M+H: -NO_2 \rightarrow -NO]^+$ = 890.4	906.5; 860.5
	$(EVP)_{E2}$	$[M+H]^+$ = 869.5	869.5
$(EVP)_R$	EVP	$[M+H]^+$ = 1756.9; $[M+H: 2\ -N_3 \rightarrow 2\ -NH_2]^+$ = 1704.9; $[M+H: 2\ -N_3 \rightarrow 2\ -NH_2\ \&\ -NO_2 \rightarrow -NO]^+$ = 1688.9	1704.8; 1688.8
$(EVP)_P$	$(EVP)_{P1}$	$[M+H]^+$ = 130.1	Below ROD
	$(EVP)_{P2}$	$[M+H]^+$ = 1627.8; $[M+H: -NO \rightarrow -NH_2]^+$ = 1613.8	1627.6; 1613.5
$(EVP)_{RE}$	$(EVP)_{E1}$	$[M+H]^+$ = 906.4; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 880.4; $[M+H: -N_3 \rightarrow -NH_2\ \&\ -NO_2 \rightarrow -NO]^+$ = 864.4	880.6; 864.6
	$(EVP)_{E2}$	$[M+H]^+$ = 869.5; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 843.5	843.7
$(EVP)_{EP}$	$(EVP)_{EP1}$	$[M+H]^+$ = 130.1	Below ROD
	$(EVP)_{EP2}$	$[M+H]^+$ = 777.4; $[M+H: -NO \rightarrow -NH_2]^+$ = 763.4	777.5; 763.4
	$(EVP)_{EP3}$	$[M+H]^+$ = 869.5; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 843.5	Not observed
$(EVP)_{RP}$	$(EVP)_{P1}$	$[M+H]^+$ = 130.1	Below ROD
	$(EVP)_{P2}$	$[M+H]^+$ = 1627.8; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 1601.8; $[M+H: -N_3 \rightarrow -NH_2\ \&\ -NO \rightarrow -NH_2]^+$ = 1587.8	1601.7; 1587.7
$(EVP)_{REP}$	$(EVP)_{EP1}$	$[M+H]^+$ = 130.1	Below ROD
	$(EVP)_{EP2}$	$[M+H]^+$ = 777.4; $[M+H: -NO \rightarrow -NH_2]^+$ = 763.4	777.4; 763.4
	$(EVP)_{EP3}$	$[M+H]^+$ = 869.5; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 843.5	Not observed

Expected products were identified in all treatment conditions except products $(EVP)_{P1}$, $(EVP)_{EP1}$, $(EVP)_{EP1}$, and $(EVP)_{EP3}$, presumably due to being below the MALDI range of detection (m/z > 600) or relative differences in ionization propensity with other species present. Products were of good purity in all cases except for treatment $(EVP)_R$.

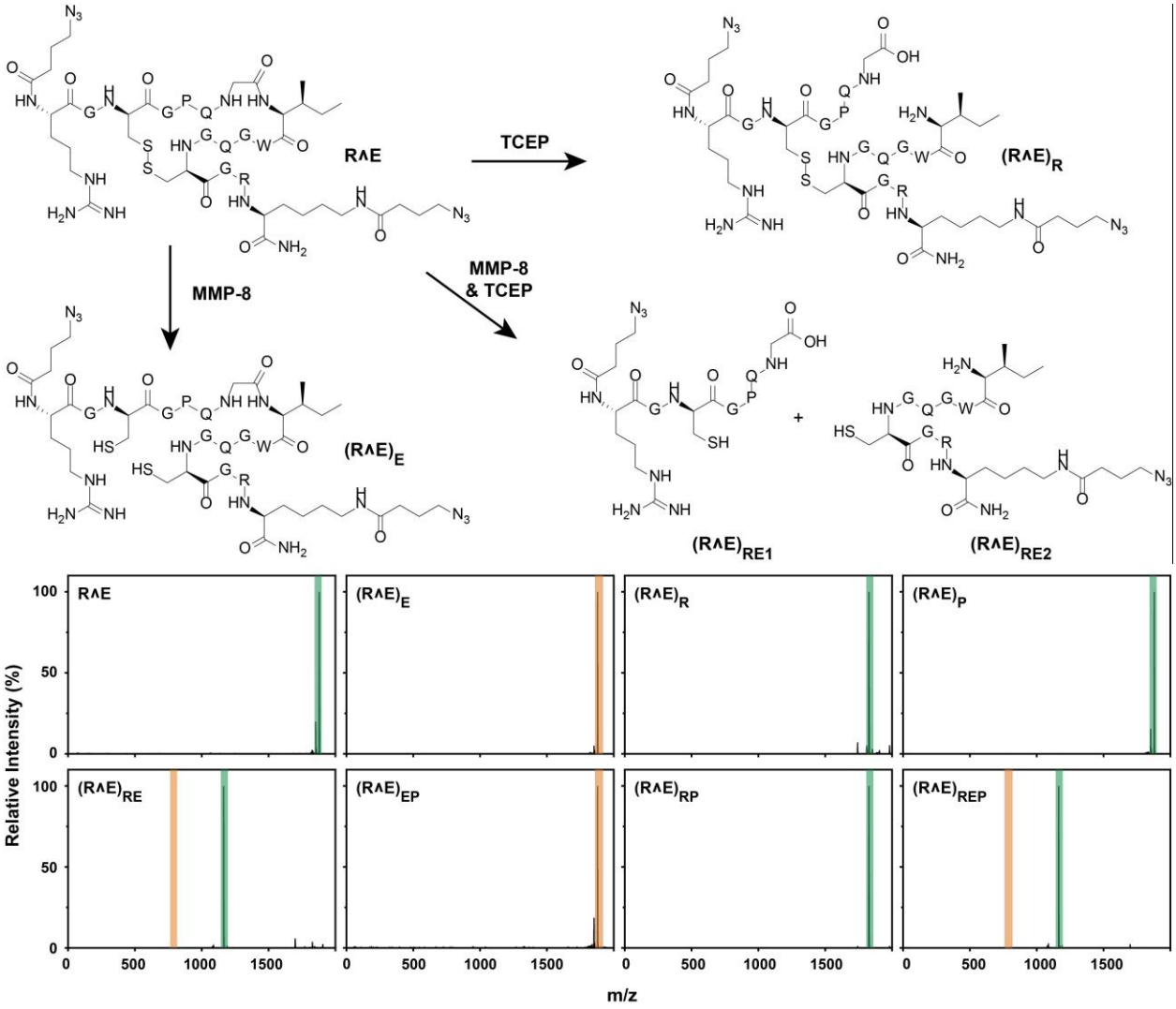
Supplementary Figure 6: Characterization of RVP crosslinker degradation in solution



Treatment	Expected Peptides	m/z (Da)	
		Calculated	Measured
RVP	RVP	$[M+H]^+$ = 1254.5; [M+H: $-NO_2 \rightarrow -NO]^+$ = 1238.5	1254.6; 1238.6
$(RVP)_E$	RVP	$[M+H]^+$ = 1254.5; [M+H: $-NO_2 \rightarrow -NO]^+$ = 1238.5	1254.6; 1238.5
$(RVP)_R$	$(RVP)_{R1}$	$[M+H]^+$ = 1024.5; [M+H: $-N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH]^+$ = 1074.6; [M+H: $-N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH$ & $-NO_2 \rightarrow -NO]^+$ = 1058.6	1074.5; 1058.5
	$(RVP)_{R2}$	$[M+H]^+$ = 233.1	Below ROD
$(RVP)_P$	$(RVP)_{P1}$	$[M+H]^+$ = 215.1	Below ROD
	$(RVP)_{P2}$	$[M+H]^+$ = 1040.4; [M+H: $-NO_2 \rightarrow -NH_2]^+$ = 1026.4	1026.5
$(RVP)_{RE}$	$(RVP)_{R1}$	$[M+H]^+$ = 1024.5; [M+H: $-N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH]^+$ = 1074.6; [M+H: $-N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH$ & $-NO_2 \rightarrow -NO]^+$ = 1058.6	1074.6; 1058.5
	$(RVP)_{R2}$	$[M+H]^+$ = 233.1	Below ROD
$(RVP)_{EP}$	$(RVP)_{P1}$	$[M+H]^+$ = 215.1	Below ROD
	$(RVP)_{P2}$	$[M+H]^+$ = 1040.4; [M+H: $-NO_2 \rightarrow -NH_2]^+$ = 1026.4	1026.5
$(RVP)_{RP}$	$(RVP)_{RP1}$	$[M+H]^+$ = 215.1	Below ROD
	$(RVP)_{RP2}$	$[M+H]^+$ = 810.4; [M+H: $-SH \rightarrow -SSCH_2CH_2OH]^+$ = 886.5; [M+H: $-SH \rightarrow -SSCH_2CH_2OH$ & $-NO_2 \rightarrow -NH_2]^+$ = 872.5	886.5; 872.5
	$(RVP)_{RP3}$	$[M+H]^+$ = 233.1	Below ROD
$(RVP)_{REP}$	$(RVP)_{RP1}$	$[M+H]^+$ = 215.1	Below ROD
	$(RVP)_{RP2}$	$[M+H]^+$ = 810.4; [M+H: $-SH \rightarrow -SSCH_2CH_2OH]^+$ = 886.5; [M+H: $-SH \rightarrow -SSCH_2CH_2OH$ & $-NO_2 \rightarrow -NH_2]^+$ = 872.5	886.5; 872.5
	$(RVP)_{RP3}$	$[M+H]^+$ = 233.1	Below ROD

Expected products were identified in all treatment conditions except products $(RVP)_{R2}$, $(RVP)_{P1}$, $(RVP)_{RP1}$, and $(RVP)_{RP3}$, presumably due to being below the MALDI range of detection (m/z > 600). Products were of good purity in all cases.

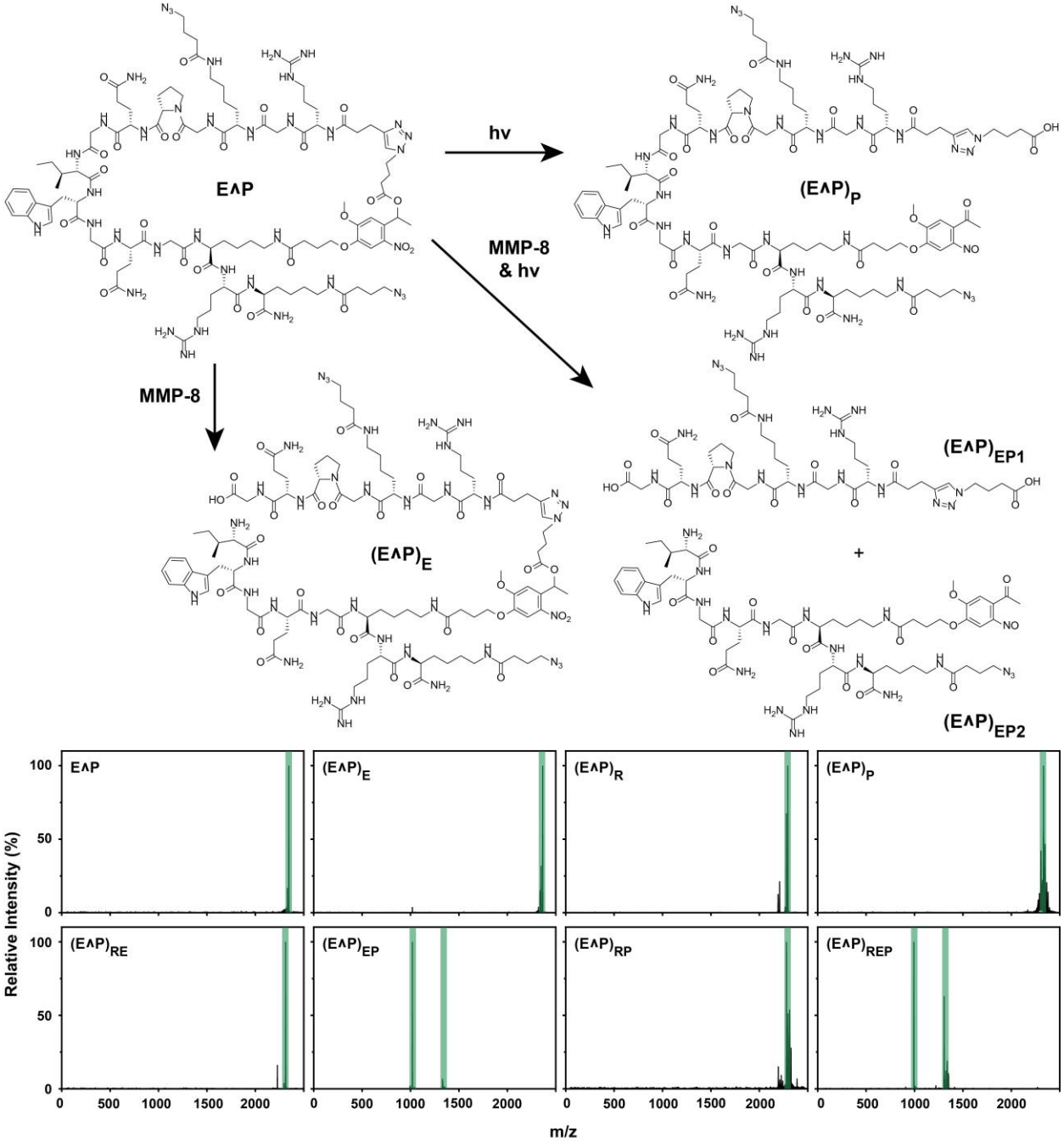
Supplementary Figure 7: Characterization of RΛE crosslinker degradation in solution



Treatment	Expected Peptides	m/z (Da)	
		Calculated	Measured
RΛE	RΛE	$[M+H]^+$ = 1878.9; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 1852.9	1878.6; 1852.6
$(RΛE)_E$	$(RΛE)_E$	$[M+H]^+$ = 1896.9; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 1870.9	Not observed
$(RΛE)_R$	$(RΛE)_R$	$[M+H]^+$ = 1880.9; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 1854.9; $[M+H: 2\ -N_3 \rightarrow 2\ -NH_2]^+$ = 1828.9	1826.6
$(RΛE)_P$	RΛE	$[M+H]^+$ = 1878.9; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 1852.9	1878.9; 1852.8
$(RΛE)_{RE}$	$(RΛE)_{RE1}$	$[M+H]^+$ = 785.3; $[M+H: -N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH]^+$ = 835.4	Not observed
	$(RΛE)_{RE2}$	$[M+H]^+$ = 1114.6; $[M+H: -N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH]^+$ = 1164.7	1164.4
$(RΛE)_{EP}$	$(RΛE)_E$	$[M+H]^+$ = 1896.9; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 1870.9	Not observed
$(RΛE)_{RP}$	$(RΛE)_R$	$[M+H]^+$ = 1880.9; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 1854.9; $[M+H: 2\ -N_3 \rightarrow 2\ -NH_2]^+$ = 1828.9	1826.8
$(RΛE)_{REP}$	$(RΛE)_{RE1}$	$[M+H]^+$ = 785.3; $[M+H: -N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH]^+$ = 835.4	Not observed
	$(RΛE)_{RE2}$	$[M+H]^+$ = 1114.6; $[M+H: -N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH]^+$ = 1164.7	1164.5

Expected products were identified in all treatment conditions except product $(RΛE)_{RE1}$, presumably due relative differences in ionization propensity with other species present. In conditions expected to produce $(RΛE)_E$, only the starting material was observed. Products were of good purity in all cases.

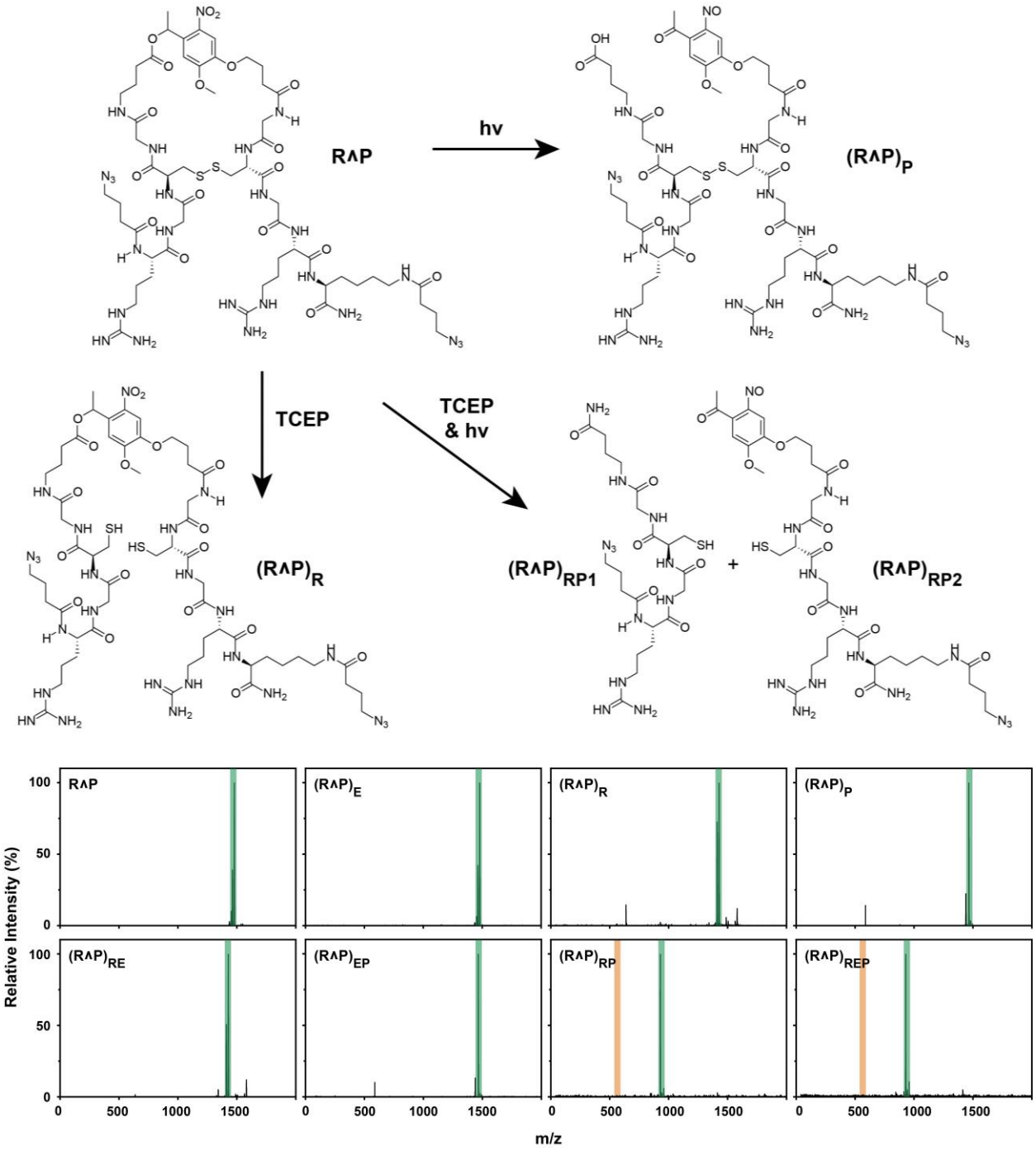
Supplementary Figure 8: Characterization of EΛP crosslinker degradation in solution



Treatment	Expected Peptides	m/z (Da)	
		Calculated	Measured
EΛP	EΛP	$[M+H]^+$ = 2346.2; [M+H: $-NO_2 \rightarrow -NO]^+$ = 2330.2	2346.0; 2330.0
$(E\Lambda P)_E$	$(E\Lambda P)_E$	$[M+H]^+$ = 2364.2; [M+H: $-NO_2 \rightarrow -NO]^+$ = 2348.2	2364; 2348.1
$(E\Lambda P)_R$	EΛP	$[M+H]^+$ = 2346.2; [M+H: 2 $-N_3 \rightarrow 2 -NH_2]^+$ = 2294.2; [M+H: 2 -N3 → 2 -NH2 & -NO2 → -NO]+ = 2278.2	2294.2; 2278.1
$(E\Lambda P)_P$	$(E\Lambda P)_P$	$[M+H]^+$ = 2346.2; [M+H: -NO→ -N or $-NH_2]^+$ = 2330.2/2332.2; [M+H: $-N_3 \rightarrow -NH_2]^+$ = 2320.2; [M+H: $-N_3 \rightarrow -NH_2$ & -NO → -N or $-NH_2]^+$ = 2304.6/2306.2	2346.2; 2332.1; 2330.2; 2320.1; 2306.1; 2304.1
$(E\Lambda P)_{RE}$	$(E\Lambda P)_E$	$[M+H]^+$ = 2364.2; [M+H: 2 $-N_3 \rightarrow 2 -NH_2]^+$ = 2312.2; [M+H: 2 $-N_3 \rightarrow 2 -NH_2$ & $-NO_2 \rightarrow -NO]^+$ = 2296.2	2312.2; 2296.2
$(E\Lambda P)_{EP}$	$(E\Lambda P)_{EP1}$	$[M+H]^+$ = 1345.7; [M+H: -NO→ -N]+ = 1329.7	1329.8
	$(E\Lambda P)_{EP2}$	$[M+H]^+$ = 1019.5; [M+H: $-N_3 \rightarrow -NH_2]^+$ = 993.5	1019.6
$(E\Lambda P)_{RP}$	$(E\Lambda P)_P$	$[M+H]^+$ = 2346.2; [M+H: 2 $-N_3 \rightarrow 2 -NH_2]^+$ = 2294.2; [M+H: 2 $-N_3 \rightarrow 2 -NH_2$ & -NO → $-NH_2]^+$ = 2280.2	2294.2; 2280.3
$(E\Lambda P)_{REP}$	$(E\Lambda P)_{EP1}$	$[M+H]^+$ = 1019.5; [M+H: $-N_3 \rightarrow -NH_2]^+$ = 993.5	993.6
	$(E\Lambda P)_{EP2}$	$[M+H]^+$ = 1345.7; [M+H: $-N_3 \rightarrow -NH_2]^+$ = 1319.7; [M+H: $-N_3 \rightarrow -NH_2$ & -NO → $-NH_2]^+$ = 1305.7	1319.7; 1305.8

Expected products were identified in all treatment. Products were of good purity in all cases.

Supplementary Figure 9: Characterization of RΛP crosslinker degradation in solution



Treatment	Expected Peptides	m/z (Da)	
		Calculated	Measured
RΛP	RΛP	$[M+H]^+$ = 1478.6; $[M+H: -NO_2 \rightarrow -NO]^+$ = 1462.6; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 1452.6	1478.7; 1462.5; 1452.5
$(RΛP)_E$	RΛP	$[M+H]^+$ = 1478.6; $[M+H: -NO_2 \rightarrow -NO]^+$ = 1462.6; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 1452.6	1478.7; 1462.6; 1452.6
$(RΛP)_R$	$(RΛP)_R$	$[M+H]^+$ = 1480.6; $[M+H: 2\ -N_3 \rightarrow 2\ -NH_2]^+$ = 1428.6; $[M+H: 2\ -N_3 \rightarrow 2\ -NH_2$ & $-NO_2 \rightarrow -NO]^+$ = 1412.6	1428.7; 1412.7
$(RΛP)_P$	$(RΛP)_P$	$[M+H]^+$ = 1478.6; $[M+H: -NO \rightarrow -NH_2]^+$ = 1464.6; $[M+H: -N_3 \rightarrow -NH_2$ & $-NO \rightarrow -NH_2]^+$ = 1438.6	1465.8; 1439.8
$(RΛP)_{RE}$	$(RΛP)_R$	$[M+H]^+$ = 1480.6; $[M+H: 2\ -N_3 \rightarrow 2\ -NH_2]^+$ = 1428.6; $[M+H: 2\ -N_3 \rightarrow 2\ -NH_2$ & $-NO_2 \rightarrow -NO]^+$ = 1412.6	1428.0; 1412.0
$(RΛP)_{EP}$	$(RΛP)_P$	$[M+H]^+$ = 1478.6; $[M+H: -NO \rightarrow -NH_2]^+$ = 1464.6; $[M+H: -N_3 \rightarrow -NH_2$ & $-NO \rightarrow -NH_2]^+$ = 1438.6	1465.5; 1439.5
$(RΛP)_{RP}$	$(RΛP)_{RP1}$	$[M+H]^+$ = 587.3; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 561.3	Below ROD
	$(RΛP)_{RP2}$	$[M+H]^+$ = 894.4; $[M+H: -N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH$ & $-NO \rightarrow -NH_2]^+$ = 944.5	930.6
$(RΛP)_{REP}$	$(RΛP)_{RP1}$	$[M+H]^+$ = 587.3; $[M+H: -N_3 \rightarrow -NH_2]^+$ = 561.3	Below ROD
	$(RΛP)_{RP2}$	$[M+H]^+$ = 894.4; $[M+H: -N_3 \rightarrow -NH_2$ & $-SH \rightarrow -SSCH_2CH_2OH$ & $-NO \rightarrow -NH_2]^+$ = 944.5	930.5

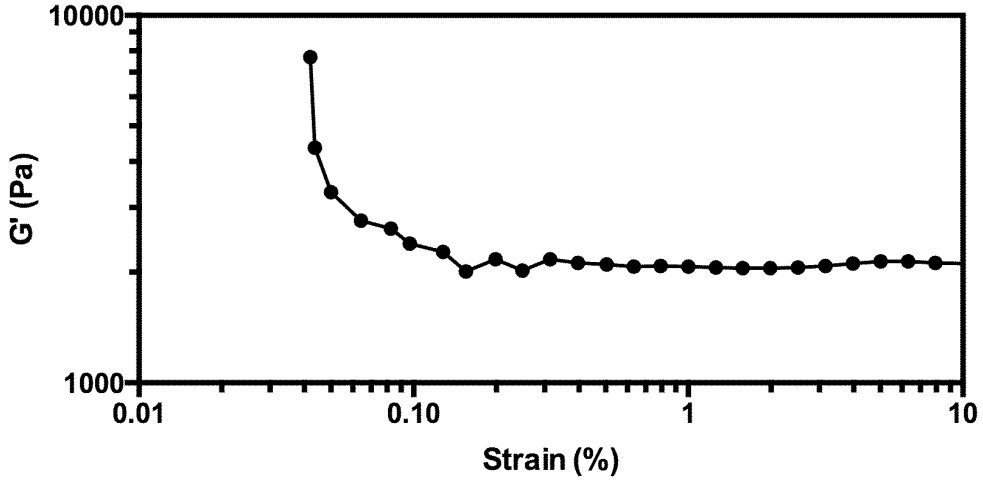
Expected products were identified in all treatment conditions except product $(RΛE)_{RP1}$, presumably due to being below the MALDI range of detection (m/z > 600). Products were of good purity in all cases.

Supplementary Method 25: *In situ* rheology of hydrogel formation

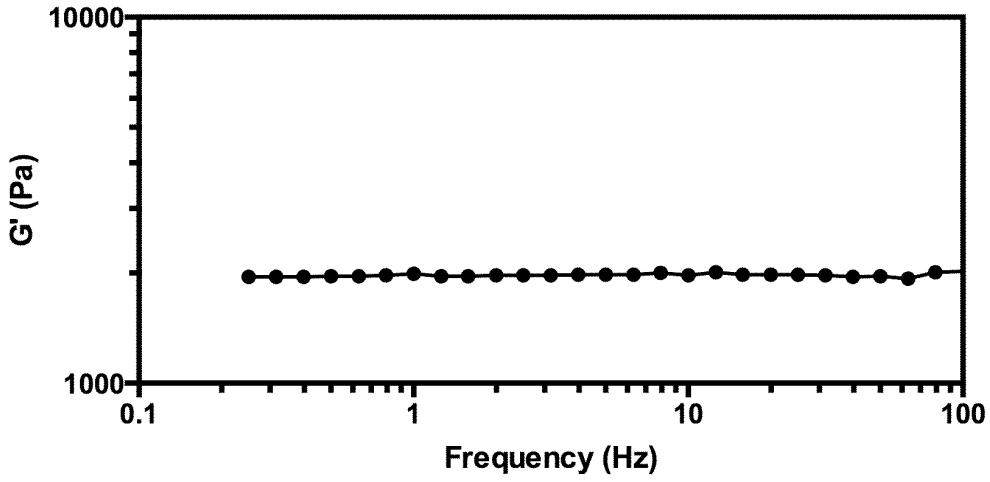
Strain and frequency sweeps:
Oscillatory rheology was performed on an Anton Paar MCR301 rheometer equipped with a cone and plate geometry (25 mm diameter, 1° cone) at 25 °C. Strain and frequency sweeps were used to determine the linear viscoelastic region (LVR), the set of imposed conditions from which the elastic modulus is independent[12].

A solution of PEG-tetraBCN (Supplementary Method 22, 2 mM) and the R crosslinker (Supplementary Method 6, 4 mM) in PBS was reacted *in situ* for 120 minutes to form a hydrogel network. For the strain sweep, the frequency was fixed at 25 Hz, and the storage (G') and loss (G") moduli were measured at various strains (0.01 – 10%). For the frequency sweep, the strain was fixed at 1%, and G' and G'' were measured at various frequencies (0.1 – 100 Hz). The sweeps demonstrate that at 25 Hz and 1% strain, the hydrogel networks are in the LVR because a perturbation to either the frequency or strain does not change the value of G'. These conditions were used for future rheological analyses.

Strain sweep:

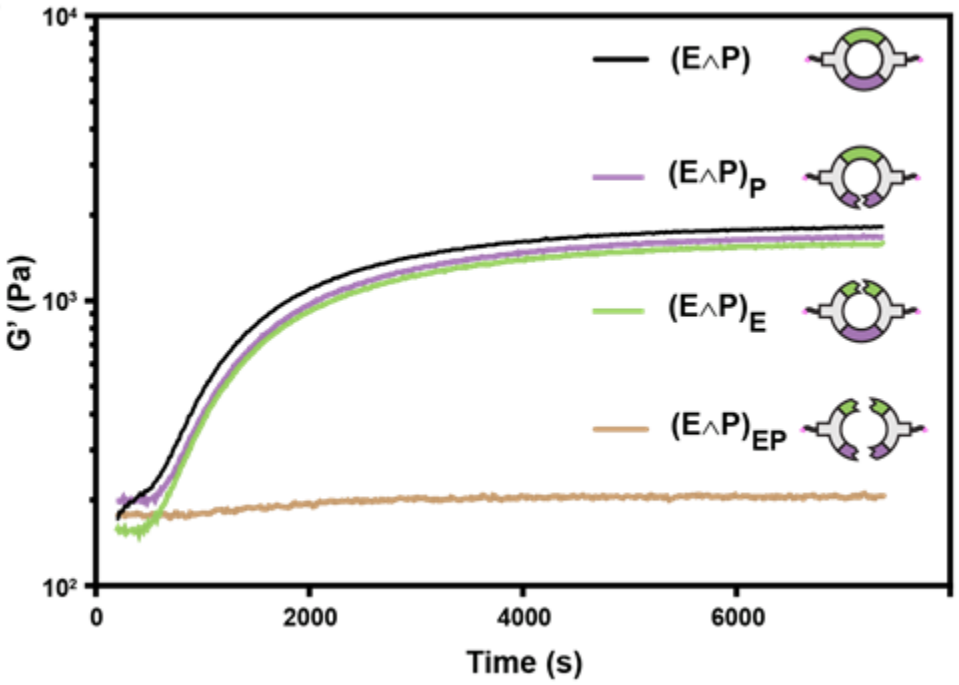


Frequency sweep:



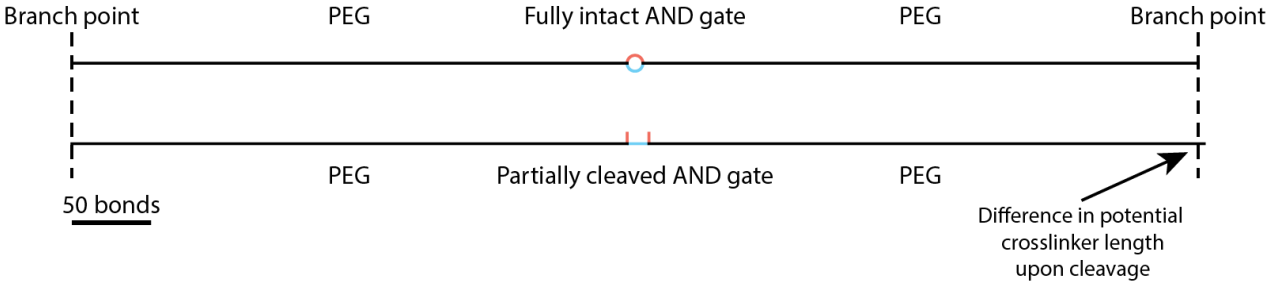
In situ hydrogel rheology:

Oscillatory rheology was performed on an Anton Paar MCR301 rheometer equipped with a cone and plate geometry (25 mm diameter, 1° cone) at 25 °C and 25 Hz with a 1% strain. A solution of PEG-tetraBCN (3 mM, Supplementary Method 22) and the E∧P crosslinker (6 mM, Supplementary Method 12) in MMP buffer was reacted *in situ*, while G' and G" were measured as a function of time over the course of two hours. Peptide crosslinkers were pre-treated with MMP and/or light as described in Supplementary Method 24 in experimental triplicate. Hydrogels with similar stiffnesses formed when the E∧P crosslinker was untreated (G' = 1660 ± 170 Pa), exposed to just light (G' = 1580 ± 130 Pa), or exposed to just enzyme (G' = 1540 ± 110 Pa). When the crosslinker was treated with both enzyme and light, a hydrogel did not form (G' = 200 ± 30 Pa). All samples had a final loss modulus (G") of ~ 50 Pa.



Supplementary Method 26: Assessing network relaxation following partial cleavage of AND-gate crosslinkers

To estimate the effect of partial AND-gate linker cleavage on bulk material properties, we compared the average number of elastically active covalent bonds between network branch points before and after a single degradation event. As these bonds contributed by the 4-arm PEG represent the overwhelming majority of those between crosslinks (>90%), minimal structural relaxation is predicted following the cleavage of a single stimuli-labile moiety (<3% for all AND-gated crosslinkers). This is consistent with rheology data, where AND-gate crosslinker treatment with a single input does not produce a significant change in final material stiffness (Supplementary Method 25).

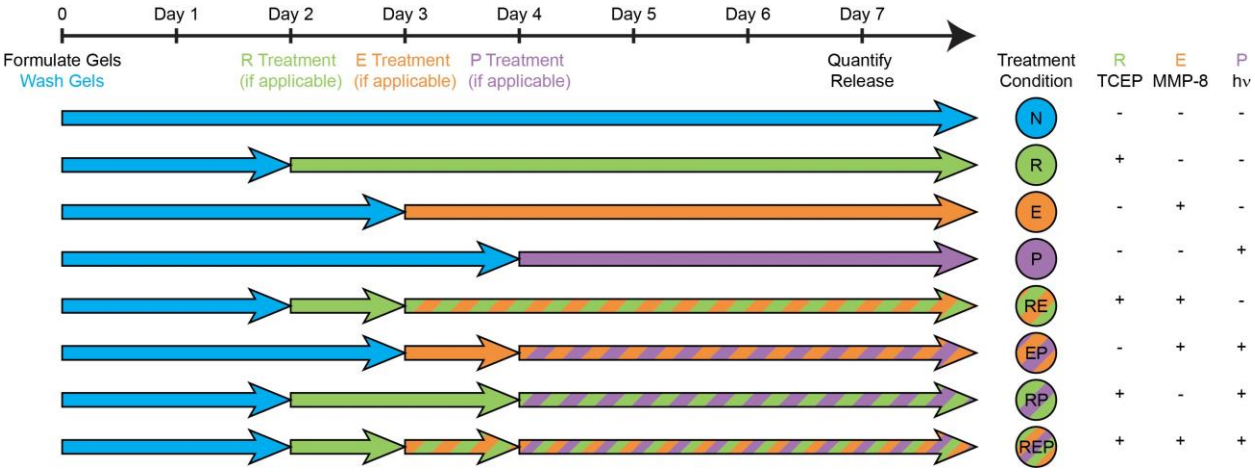


E∧P Crosslinker
Bonds from PEG macromer = 702
Bonds shared by both arms of peptide linker = 26
Bonds through E-arm = 30
Bonds through P-arm = 34
758 vs 762 indicates a **0.5% difference**

E∧R Crosslinker
Bonds from PEG macromer = 702
Bonds shared by both arms of peptide linker = 30
Bonds through E-arm = 30
Bonds through R-arm = 5
737 vs 762 is a **3.3% difference**

R∧P Crosslinker
Bonds from PEG macromer = 702
Bonds shared by both arms of peptide linker = 31
Bonds through R-arm = 5
Bonds through P-arm = 25
738 vs 758 is a **2.6% difference**

Supplementary Method 27: Logic-based hydrogel degradation in response to sequential stimuli



Fluorescent hydrogels were formulated from a precursor solution of PEG-tetraBCN-AF568 (2 mM) and a diazide peptide crosslinker (4 mM) in MMP buffer. Immediately upon addition of all components (65 μL total), the solution was vortexed and centrifuged. The precursor solution was transferred *via* a positive displacement pipette into microcentrifuge tubes (6 x 10 μL), centrifuged, and reacted at room temperature for 1 hour. To remove any fluorescent sol fraction, hydrogels were washed in MMP buffer (1 mL, 2 x 24 hours, 37 °C) prior to treatment. A total of 24 hydrogels were synthesized from each crosslinker (8 input combinations in experimental triplicate).

Samples receiving the reductive input ($_R$) were treated with tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl, 2 μL, 100 mM in MMP buffer) and incubated overnight (22 hr) at 37 °C. To quench any unreacted TCEP, these samples were further treated with hydroxyethyl disulfide (5 μL, 100 mM in MMP buffer) prior to incubation (4 hr, 37 °C). Samples not receiving reductive input were maintained at 37 °C.

Samples receiving the enzyme input ($_E$) were subsequently treated with MMP-8 (Supplementary Method 23, 5 μL, 0.2 mg mL^{-1} in MMP buffer) and all samples were incubated (20 hr, 37 °C).

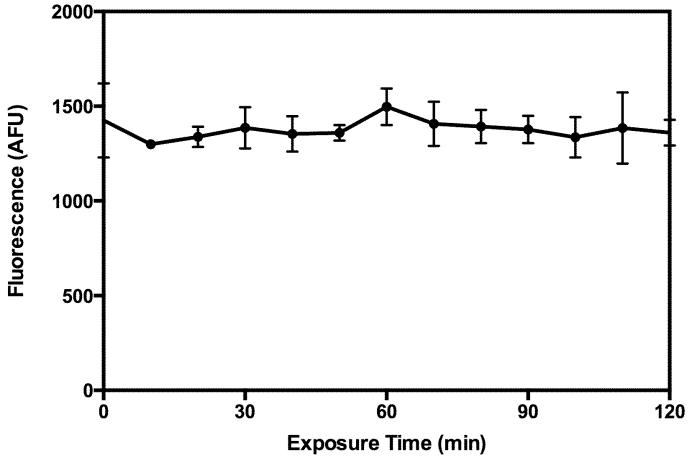
Samples receiving the light input ($_P$) were subsequently exposed to UV light (λ = 365 nm, 10 mW cm^{-2} incident light, 60 minute exposure) prior to incubation (72 hr, 37 °C).

After 8 total days of experimental treatment, the extent of gel degradation was assessed by quantifying supernatant fluorescence (excitation: 570 nm, emission: 610 nm, emission cut-off filter: 590 nm).

Supplementary Method 28: Characterization of PEG-tetraBCN-AF568 fluorescence

AF568 photobleaching study:

To assess the level of AF568 fluorophore photobleaching that occurs during light treatment conditions used in the hydrogel release studies (Supplementary Method 27), AF568 was dissolved in PBS (10 μM) and exposed to UV light (λ = 365 nm, 10 mW cm^{-2}) in experimental triplicate. Sample fluorescence (100 μL, 1 μM, excitation wavelength 570 nm, emission wavelength 610 nm) was measured following 10-minute intervals of light exposure. One-way analysis of variance (ANOVA) failed to reject the null hypothesis that fluorescence does not change upon light exposure (p-value = 0.82). This suggests that AF568 does not undergo significant photobleaching under light treatment conditions used in the hydrogel degradation studies.

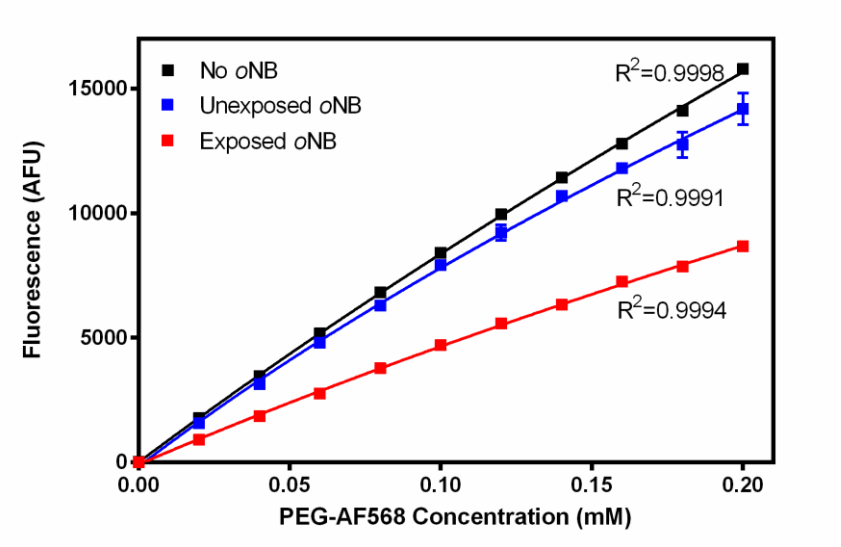


Quantification of PEG-tetraBCN-AF568 concentrations using fluorescence:

Fluorescence was used to determine the concentration of PEG-tetraBCN-AF568 in solution, and thus to quantify the extent of gel degradation in response to each combination of external stimuli. PEG-tetraBCN-AF568 fluorescence was significantly altered both in the presence of *o*NB, and in the presence of photocleaved *o*NB (*o*NB spectral properties change upon photodecomposition [1]). Therefore, three different calibration curves are required to correlate fluorescent signal with PEG-tetraBCN-AF568 concentration for crosslinkers that: 1) do not contain *o*NB; 2) contain *o*NB but have not been exposed to light; 3) contain photocleaved *o*NB.

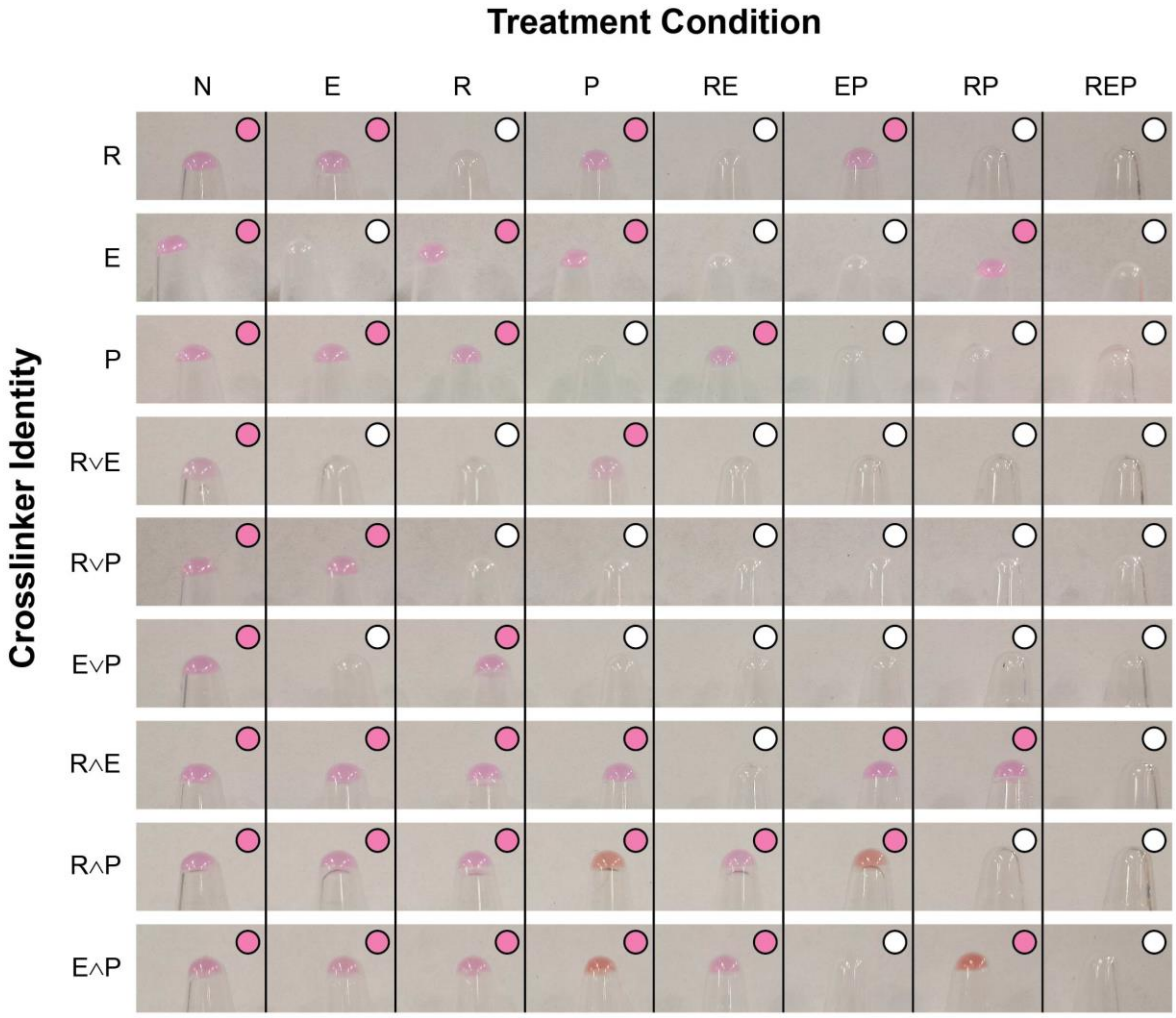
A calibration curve (shown below, black) was generated using pure PEG-tetraBCN-AF568 in MMP buffer, and was used for constructs that do not contain *o*NB. A second calibration curve (shown below, blue) was generated using PEG-tetraBCN-AF568 and P crosslinker (Supplementary Method 7, 1:2 molar ratio) in MMP buffer, and was used for systems that contain *o*NB but have not been exposed to light. The final calibration curve (shown below, red) was generated using PEG-tetraBCN-AF568 and P crosslinker (1:2 molar ratio) treated by the light condition used in the release studies (λ = 365 nm, 10 mW cm^{-2}, 60 minutes) in MMP buffer, and was used for systems that contain *o*NB and have been exposed to light.

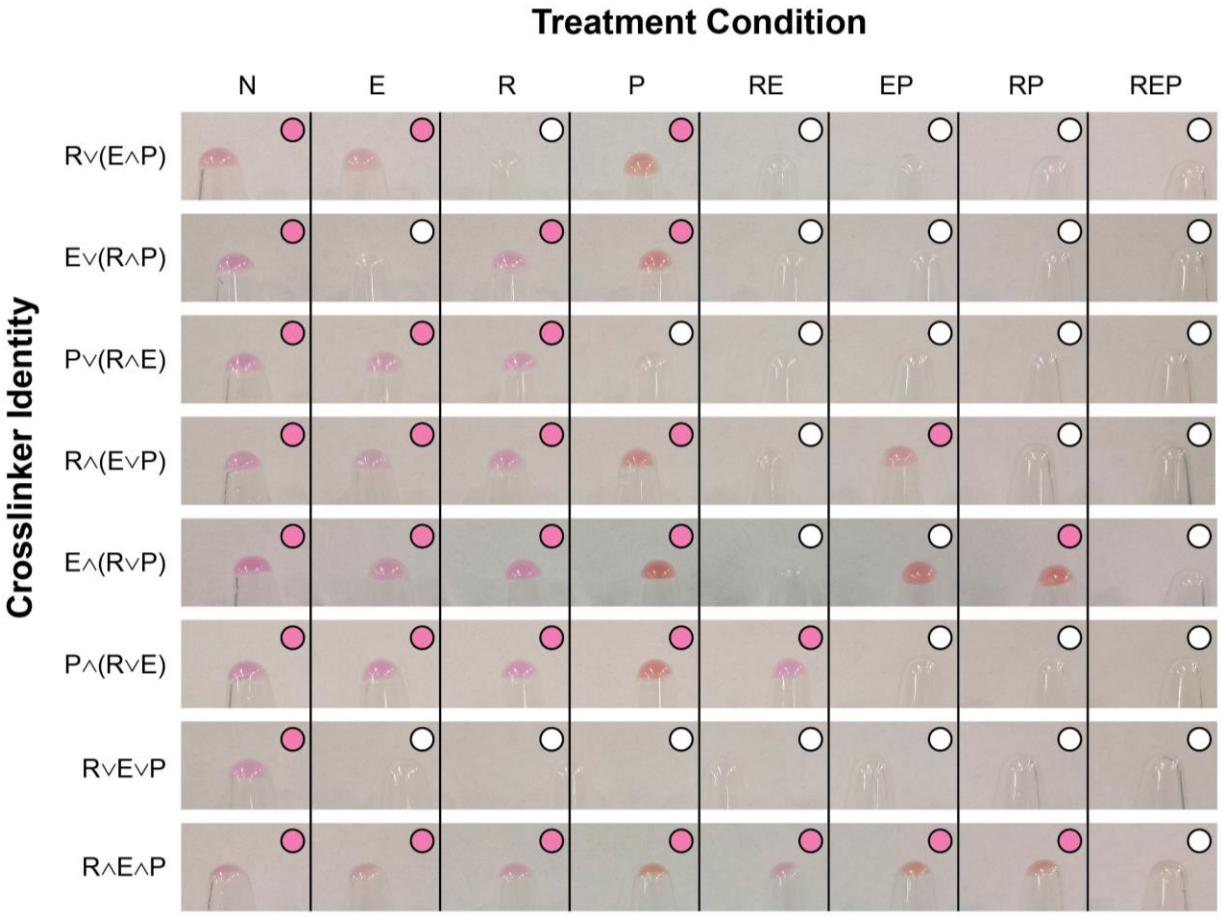
Sample fluorescence was found to be scale with PEG-AF568 concentration for all cases, enabling the extent of hydrogel degradation to be quantified for each crosslinker system in response to different input combinations.


No oNB
Unexposed oNB
Exposed oNB
R2=0.9998
R2=0.9991
R2=0.9994
Fluorescence (AFU)
15000
10000
5000
0
0.00
0.05
0.10
0.15
0.20
PEG-AF568 Concentration (mM)

Supplementary Figure 10: Gel photographs following logic-based degradation

Fluorescent hydrogels were prepared and treated as described in Supplementary Method 27. Following complete degradative treatment, samples were photographed with a digital camera. Treatments that did not result in material degradation retained a hydrogel (pink). The expected degradation behavior is indicated by a colored dot in each condition; a pink dot denotes conditions that are expected to keep the material intact while a white dot denotes those expected to yield gel degradation.

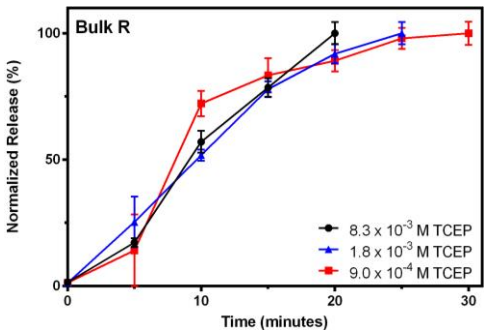


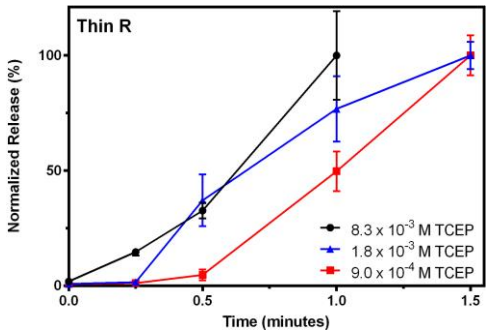

Treatment Condition
N
E
R
P
RE
EP
RP
REP
Crosslinker Identity
R∨(E∧P)
E∨(R∧P)
P∨(R∧E)
R∧(E∨P)
E∧(R∨P)
P∧(R∨E)
R∨E∨P
R∧E∧P

Supplementary Figure 11: Degradation kinetics of single-input hydrogels

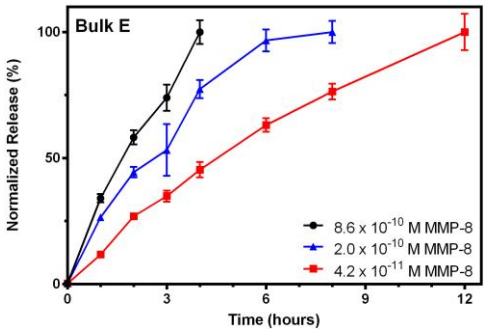
To assess the kinetics of hydrogel degradation, we examined the influence of input intensity and time on the response of single-input, YES-gated materials (R, E, and P). Fluorescent hydrogels were formulated as before (10 μL, Supplementary Method 27) in two different geometries: 1) as a bulk gel in a microcentrifuge tube, and 2) as a thin gel (~50 μm thickness) in a 24-well plate. Hydrogels were washed with MMP buffer (1 mL, 2 x 24 hours, 37 °C) prior to treatment. At each timepoint, the extent of gel degradation was assessed by quantifying supernatant fluorescence (excitation: 570 nm, emission: 610 nm, emission cut-off filter: 590 nm), in experimental triplicate.

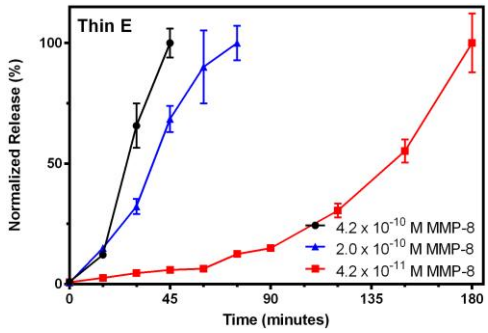
Reductively degradable hydrogels (R) were treated with TCEP·HCl (8.3 mM, 1.8 mM, or 0.90 mM in MMP buffer) at 37 °C.



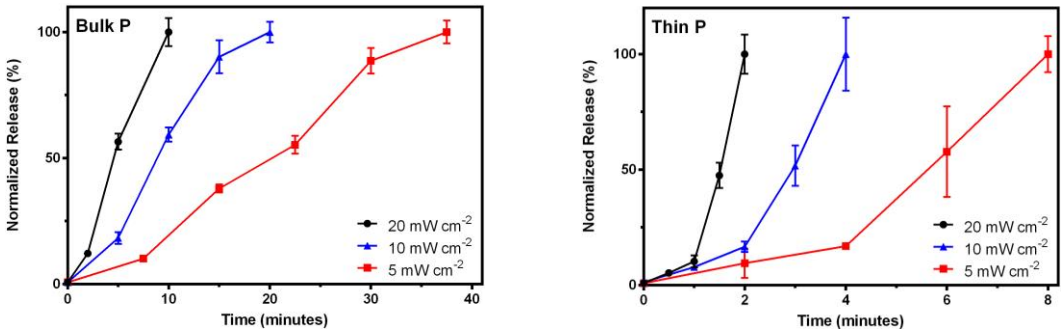


Enzymatically degradable hydrogels (E) were treated with MMP-8 (0.86 nM, 0.42 nM, 0.20 nM, or 0.042 nM in MMP buffer) at 37 °C.





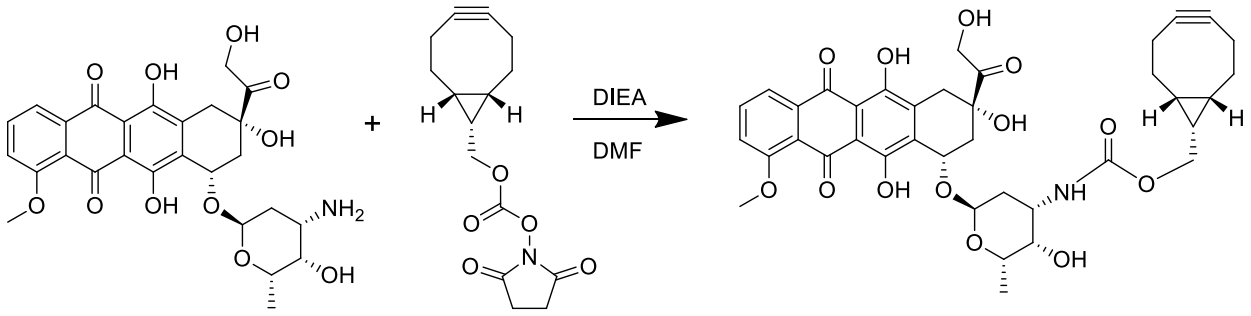
Photolytically degradable hydrogels (P), were treated with UV light (λ = 365 nm, 20 mW cm^{-2}, 10 mW cm^{-2}, or 5 mW cm^{-2}) in MMP buffer.



These experiments highlight that the timescale of logic-based material response is dictated, in part, by construct geometry. As each single-input gel/treatment combination yielded full degradation in <1.5 hours for thin gels or <12 hours for bulk gels, the experimental timeline outlined in Supplementary Method 27 was deemed appropriate to examine biocomputational responses of higher-ordered logical systems.

Supplementary Method 29: Logic-based delivery of functional doxorubicin from hydrogels

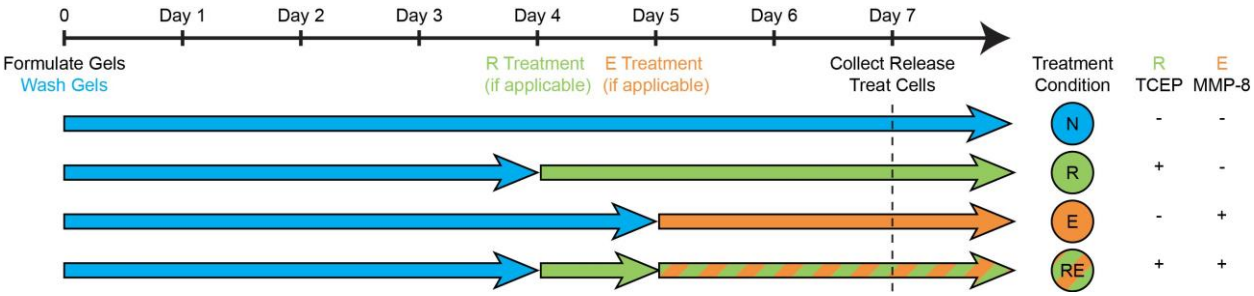
Synthesis of (1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethyl ((2S,3S,4S,6R)-3-hydroxy-2-methyl-6-(((1S,3S)-3,5,12-trihydroxy-3-(2-hydroxyacetyl)-10-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydrotetracen-1-yl)oxy)tetrahydro-2H-pyran-4-yl)carbamate (DOX-BCN):



Doxorubicin hydrochloride (DOX, 6.0 mg, 10 μmol), BCN-OSu (Supplementary Method 22, 6.0 mg, 21 μmol, 2x), and DIEA (5.3 mg, 41 μmol, 4x) were dissolved in minimal DMF and reacted overnight to functionalize doxorubicin with BCN. Complete functionalization of doxorubicin was confirmed *via* HPLC. The product (DOX-BCN) was used without any further purification.

Synthesis of RΛE-DOX crosslinker:
DOX-BCN (0.8 μmol) and RΛE crosslinker (4 μmol, 5x) were reacted in a mixture of DMF/PBS (300 μL) for 2 hours, whereby roughly 10% of RΛE-presented azides were modified with DOX. The product (RΛE-DOX) was used without any further purification.

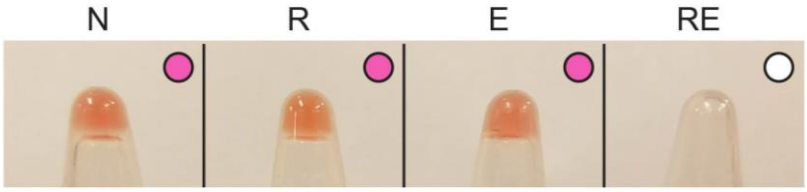
DOX-loaded hydrogel treatments:
DOX-loaded hydrogels were formulated from a precursor solution of PEG-tetraBCN (2 mM) and either RΛE (4 mM, control group) or RΛE-DOX linker (4 mM, experimental group) in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) buffer (5 mM HEPES, 3 mM $CaCl_2$, 5 μM $ZnCl_2$). Immediately upon addition of all components (95 μL total), the solution was vortexed and centrifuged. The precursor solution was transferred *via* a positive displacement pipette into microcentrifuge tubes (6 x 15 μL), centrifuged, and reacted at room temperature for 1 hour. To remove any unconjugated DOX, hydrogels were washed with HEPES buffer (1 mL, 4 x 24 hours, 37 °C) prior to treatment. A total of twelve hydrogels were synthesized (four input combinations in experimental triplicate).



Samples receiving the reductive input ($_R$) were treated with glutathione (GSH, 2 mM) and incubated overnight (24 hr) at 37 °C. Samples not receiving reductive input were maintained at 37 °C.

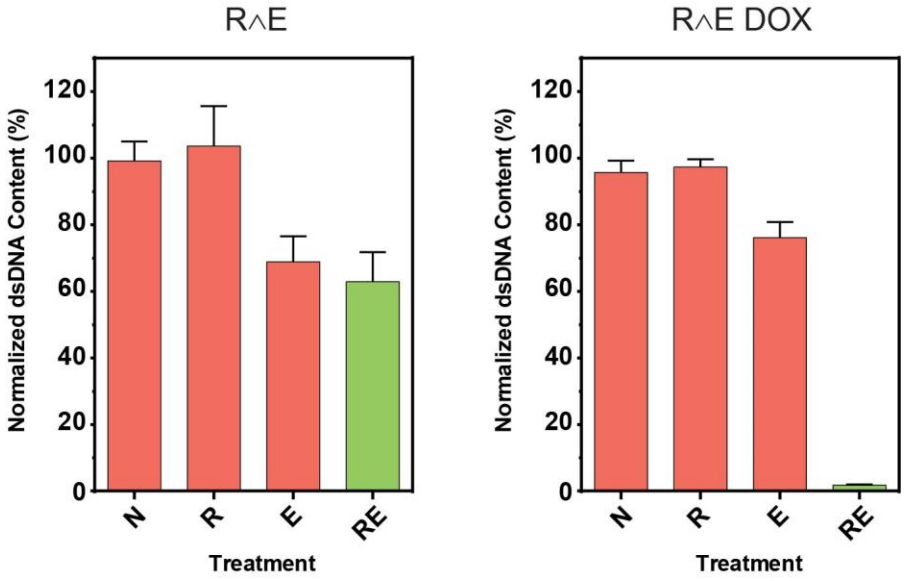
Samples receiving the enzyme input ($_E$) were subsequently treated with MMP-8 (Supplementary Method 23, 10 μL, 0.2 mg mL^{-1} in MMP buffer) and all samples were incubated (24 hr, 37 °C).

After experimental treatment, hydrogel supernatant (85 μL) was collected and combined with Dulbecco's Modified Eagle's Medium [DMEM, 85 μL, 2x containing 20% fetal bovine serum (FBS, Corning) and 2% Penicillin Streptomycin (PS, ThermoFisher)].



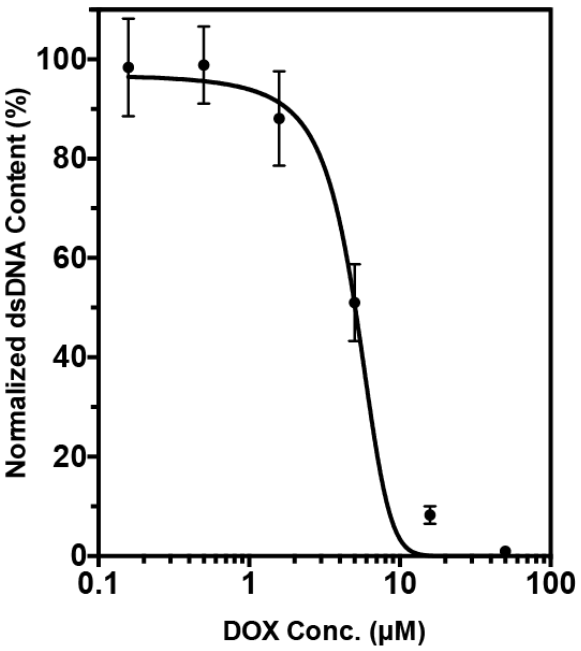
Biological response to DOX-hydrogel supernatant:

HeLa cells were seeded on a 96-well plate (2 x 10^3 cells per well) and cultured in DMEM supplemented with 10% FBS and 1% PS for 24 hours. The cells were then incubated in the DOX-hydrogel release solution (as described above), or in that from R∧E gels lacking DOX, for an additional 48 hours. A PicoGreen® dsDNA Assay (ThermoFisher) was performed to quantify the DNA concentration as a proxy for cell density.



Dose-response curve for doxorubicin construct:

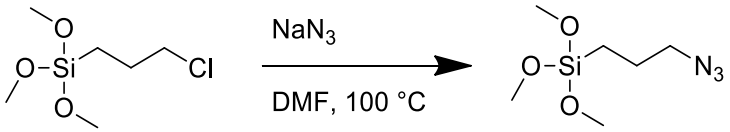
HeLa cells were seeded on a 96-well plate (2 x 10^3 cells per well) and cultured in DMEM supplemented with 10% FBS and 1% PS for 24 hours. The media was replaced with R∧E-DOX in a mixture of HEPES buffer and 2x media (1:1) and incubated for 48 hours prior to quantification of dsDNA by PicoGreen® analysis.


Normalized dsDNA Content (%)
100
80
60
40
20
0
0.1
1
10
100
DOX Conc. (μM)

Supplementary Method 30: Multi-logic hydrogel treatment and microscopy

Azide-functionalized glass slides were synthesized following a published route[6] based on a silanization procedure was derived from the work by Walba, *et al.*[13].

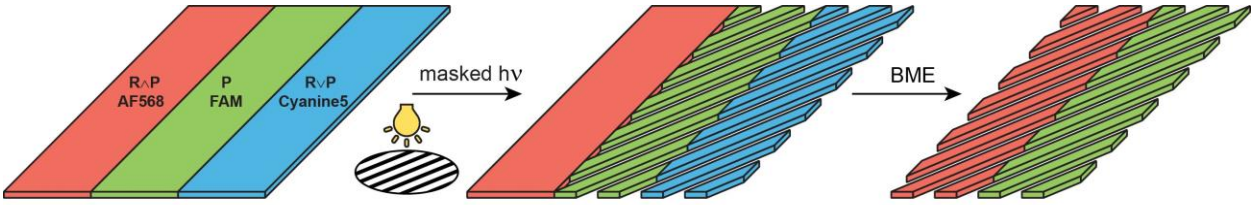
Synthesis of (3-azidopropyl)trimethoxysilane:



Anhydrous DMF (40 mL) was added to an oven-dried round bottom flask containing 3-chloropropyltrimethoxysilane (12 mL, 65.3 mmol, 1x) and sodium azide (6.38 g, 98.1 mmol, 1.5x) under a nitrogen atmosphere. The reaction was stirred overnight at 100 ºC, cooled to room temperature, and diluted with diethyl ether:dH_2O (1:1, 150 mL). The organic layer was washed with water (3x) and brine (1x), dried over $MgSO_4$, and concentrated *in vacuo* to yield a clear oil (12.74 g, 62.1 mmol, 95% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.57 (s, 9H), 3.26 (t, *J* = 6.9 Hz, 2H), 1.75 – 1.66 (m, 2H), 0.73 – 0.66 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 53.86, 50.72, 22.58, 6.46. These spectral data matched those previously reported[14].

Treatment of glass slides:
Plain glass slides were cleaned in piranha solution (50% sulfuric acid, 35% dH_2O, 15% hydrogen peroxide) for 30 minutes at room temperature. The slides were rinsed with water, acetone (3x) and then dried. The slides were incubated for 90 minutes in a solution of (3-azidopropyl)trimethoxysilane (70 mM) and n-butylamine (70 mM) in toluene. The slides were subsequently rinsed with toluene, wiped dry with a Kimwipe™, and baked overnight at 80 ºC. Functionalized slides were stable when stored at room temperature under ambient conditions for several weeks.

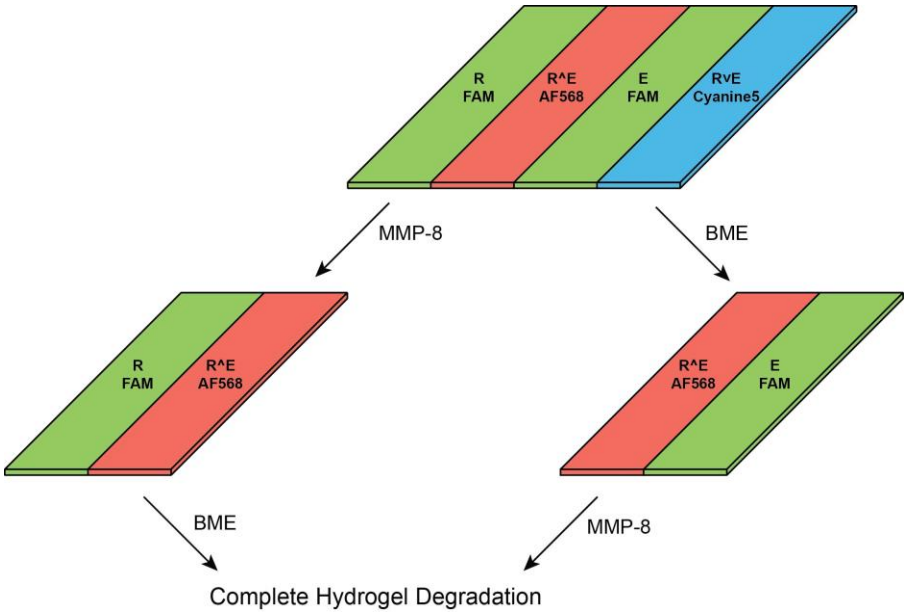
Preparation of three-region hydrogels differently sensitive to light and reductant:
Hydrogels were formed sandwiched between an azide-functional glass slide and a Rain-X®-treated glass slide separated by two 0.005" thick silicone spacers (McMaster-Carr). A precursor solution containing PEG-tetraBCN-FAM (Supplementary Method 22, 2 mM) and the P crosslinker (Supplementary Method 7, 4 mM) was injected between the silicone spacers (0.005 inches) and reacted for one hour. Subsequently, a precursor solution of PEG-tetraBCN-Cyanine5 (Supplementary Method 22, 2 mM) and the R∨P crosslinker (Supplementary Method 10, 4 mM) was injected between the FAM functionalized region and a silicone spacer and reacted for one hour. This was repeated on the other side of the FAM functionalized region with PEG-tetraBCN-AF568 (Supplementary Method 22, 2 mM) and R∧P crosslinker (Supplementary Method 13, 4 mM). Hydrogels were equilibrated and stored in PBS.



Treatment of three-region hydrogels sensitive to light and reductant:

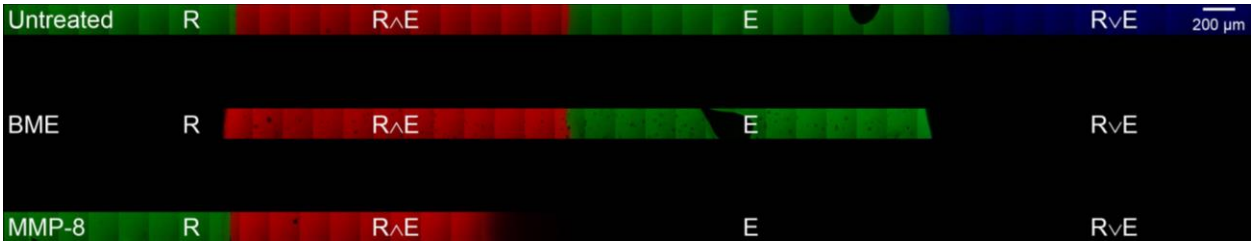
Preformed tri-color gels were exposed to UV light (λ = 365 nm, 10 mW cm^{-2} incident light, 10 minute exposure) through a slitted photomask (200 μm wide lines separated by 200 μm spaces), inducing degradation of the P- and R∨P-based gels in the UV-exposed regions. Following light treatment, gels were soaked in 2-mercaptoethanol (BME, 0.25 mM in 50 mL PBS) for 45 minutes at room temperature, yielding full degradation of the R∨P-based gel portion while leaving the P-based volume fully intact. The R∧P-based material degraded to reveal the original photopatterned exposure, demonstrating that both inputs are required for material degradation.

Preparation of four-region hydrogels differently sensitive to reductant and enzyme:
Hydrogels containing four regions, each with a distinct logical crosslinker and fluorophore, were prepared using the method described above. The regions from left to right were: 1) R crosslinker/FAM (Methods S6, S22), 2) R∧E crosslinker/AF568 (Methods S11, S22), 3) E crosslinker/FAM (Methods S5, S22), and 4) R∨E crosslinker/Cyanine5 (Methods S8, S22).



Treatment of four-region hydrogels sensitive to reductant and enzyme:
Preformed four-color gels were sequentially exposed to reducing conditions (0.25 mM BME, 45 minutes) and MMP-8 (0.20 nM, 2 hours, Supplementary Method 23), in either order. In response

to the initial treatment, two regions of the hydrogel degraded (R∨E and the relevant single-input region). Exposure to the second input induced full degradation on the remainder of the hydrogel.



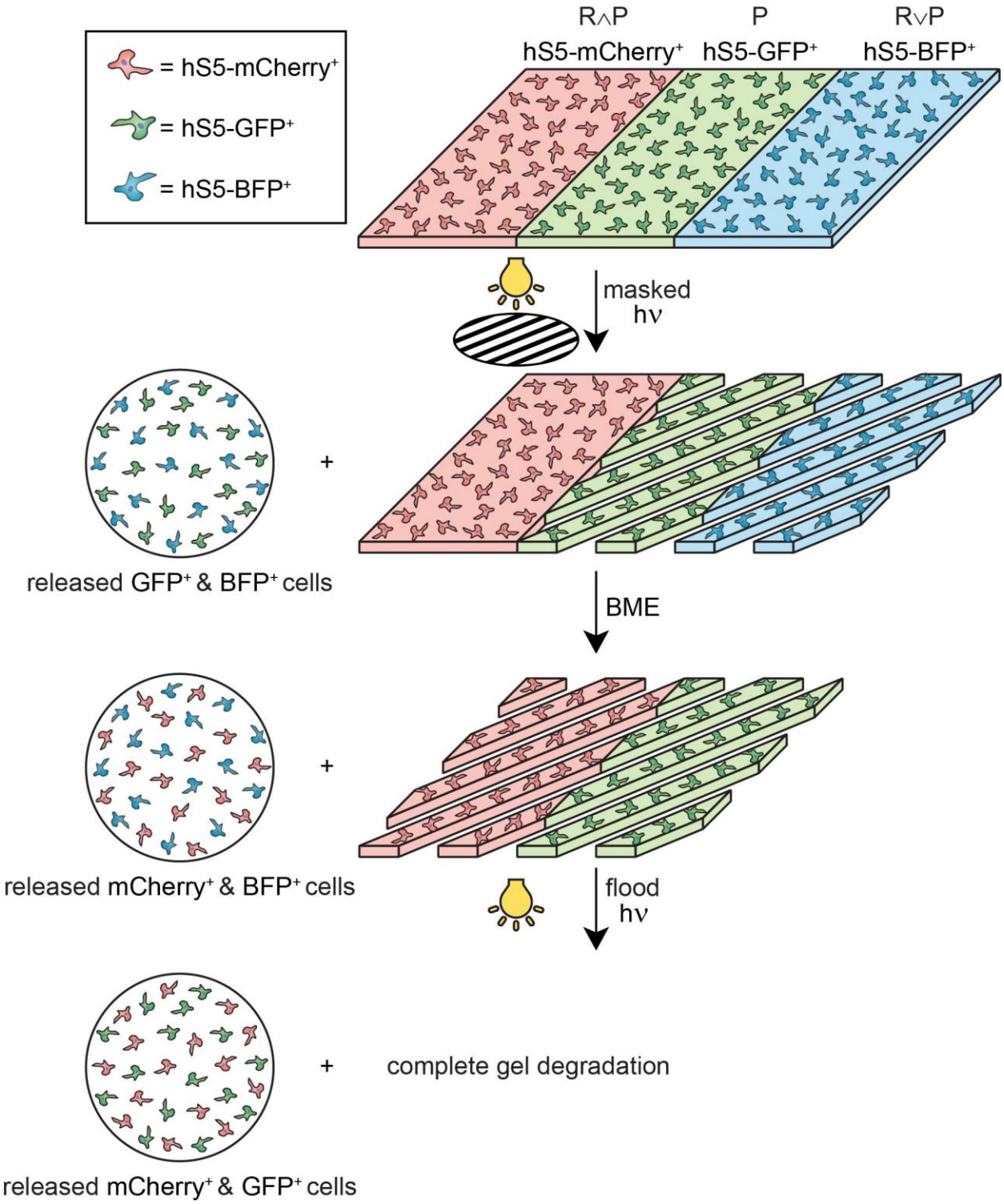
Hydrogel microscopy:

Hydrogels were imaged using fluorescent confocal microscopy. Three channels – one for each fluorophore – were simultaneously monitored. For visualization, fluorescence corresponding to AF568 (excitation 578 nm, emission 594-627 nm), FAM (excitation 515 nm, emission 520-569 nm), and Cyanine5 (excitation 646 nm, emission 663-675 nm) were false colored as red, green, and blue, respectively.

Supplementary Method 31: Hydrogel-encapsulated cell release studies

Four human bone marrow-derived stromal cell lines [hS5, a gift from the Ying Zheng and Beverly Torok-Storb labs at the University of Washington and the Fred Hutchinson Cancer Research Center, respectively[15]], including one non-fluorescent control and three each stably transfected to express a single fluorescent protein [mCherry, green fluorescent protein (GFP), or blue fluorescent protein (BFP)], were cultured at 37 °C in Roswell Park Memorial Institute medium 1640 (RPMI-1640, Corning) supplemented with 10% FBS and 1% PS.

Cell-laden multi-colored hydrogel preparation for microscopy:
Hydrogels were formed sandwiched between an azide-functional glass slide and a Rain-X®-treated glass slide separated by two 0.005" thick silicone spacers (McMaster-Carr). A precursor solution containing PEG-tetraBCN (Supplementary Method 22, 2 mM), the P crosslinker (Supplementary Method 7, 4 mM), and hS5-GFP$^+$ (40 x 10^6 cells mL^{-1}) was injected between the silicone spacers and reacted for one hour. Subsequently, a precursor solution of PEG-tetraBCN (2 mM), the R∨P crosslinker (Supplementary Method 10, 4 mM), and hS5-BFP$^+$ (40 x 10^6 cells mL^{-1}) was injected between the hS5-GFP$^+$ encapsulated region and a silicone spacer. Immediately afterwards, a precursor solution of PEG-tetraBCN (2 mM), R∧P crosslinker (Supplementary Method 13, 4 mM), and hS5-mCherry$^+$ (40 x 10^6 cells mL^{-1}) was injected between the other side of the hS5-GFP$^+$ encapsulated region and a silicone spacer. The hS5-mCherry$^+$ and hS5-BFP$^+$ encapsulated regions were simultaneously reacted for one hour. Hydrogels were stored in media overnight.


= hS5-mCherry+
= hS5-GFP+
= hS5-BFP+
R∧P
hS5-mCherry+
P
hS5-GFP+
R∨P
hS5-BFP+
masked
hν
+
released GFP+ & BFP+ cells
BME
+
released mCherry+ & BFP+ cells
flood
hν
+
complete gel degradation
released mCherry+ & GFP+ cells

Cell-laden hydrogel treatments for microscopy:

Preformed tri-color cell-laden gels were exposed to UV light (λ = 365 nm, 10 mW cm^{-2} incident light, 10 minute exposure) through a slitted photomask (200 μm wide lines separated by 200 μm spaces), inducing degradation of the P- and R∨P-based gels in the exposed regions and associated release of hS5-GFP^+ and hS5-BFP^+ cells. Following light treatment, gels were soaked in 2-mercaptoethanol (BME, 0.25 mM in 50 mL PBS) at 37 °C for 45 minutes, yielding full degradation of the R∨P-based gel portion (including hS5-BFP^+ release) while leaving the P-based gel fully intact. The R∧P-based gel degraded to match the original photopatterned exposure (causing release of hS5-$mCherry^+$ cells), demonstrating that both inputs are required for material degradation. Finally, gels were exposed to unmasked light to induce complete degradation of any remaining material and to release an equal number of hS5-GFP^+ and hS5-$mCherry^+$ cells.

Cell-laden hydrogel microscopy:

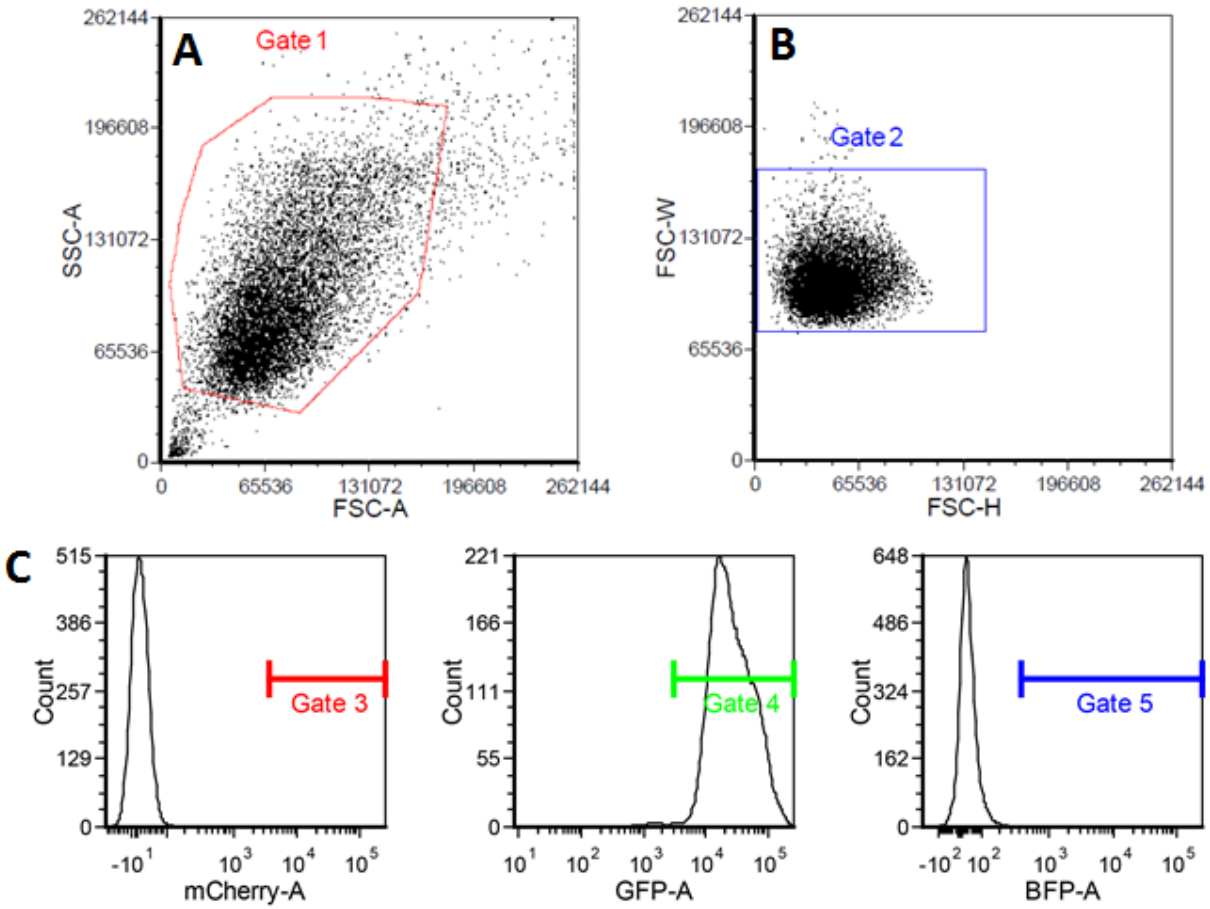
Following each degradative step, cells that remained encapsulated in the intact gel were imaged using fluorescent confocal microscopy. Three channels – one for each fluorescent protein – were simultaneously monitored. For visualization, fluorescence corresponding to mCherry (excitation 587 nm, emission 626-703 nm), GFP (excitation 489 nm, emission 499-570 nm), and BFP (excitation 405 nm, emission 413-494 nm) were false colored as red, green, and blue, respectively.

Quantifying cell release by flow cytometry:

Following each degradative step, released cells were harvested though successive gel rinsing with PBS and centrifugation. For each treatment condition, released cells were fixed using a solution of 4% formaldehyde in PBS (37 °C, 10 minutes), chilled on ice (1 minute), concentrated by centrifugation, and resuspended in PBS. Flow cytometry was performed on released cell populations using a BD Biosciences LSR II Flow Cytometer. Forward scattering (FS), side scattering (SS), and the fluorescence corresponding to BFP (405 nm and 100 mW laser line, 450/50 nm bandpass filter), GFP (488 nm and 100 mW laser line, 530/30 nm bandpass filter), and mCherry (561 nm and 150 mW laser line, 610/20 nm bandpass filter) were measured for each event. Flow cytometry was performed on standards of each cell line (non-fluorescent, mCherry, GFP, and BFP) and cells released from each treatment.

Analysis of flow cytometry data:

To account for spectral overlap of fluorescent proteins, compensation controls were calculated from the standards using automatic compensation in FACSDiva software. The gating tree was set as follows (shown below for GFP standard). *A:* FSC/SSC (the distribution of cell size and intracellular complexity, respectively, from light scatter) to *B:* FSC/pulse width (to isolate events corresponding to single cells) to *C:* histograms of each fluorescent channel. Gating for *A* and *B* was performed using non-fluorescent hS5 cells. Gating for *C* was performed for red, green, and blue fluorescence using hS5-$mCherry^+$, hS5-GFP^+, hS5-BFP^+ standards, respectively.

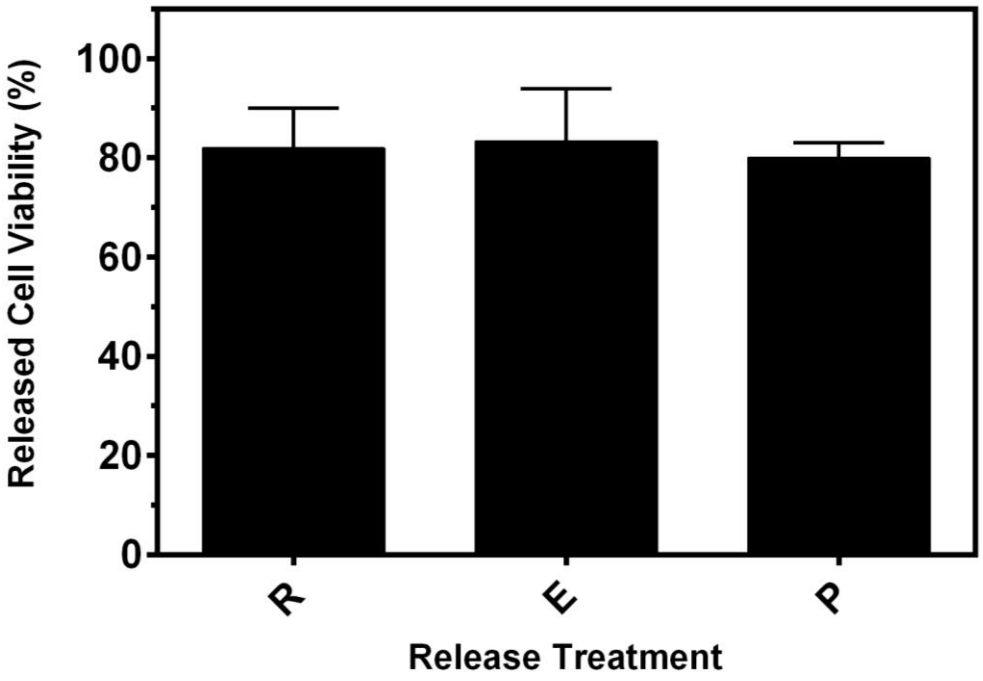

A
Gate 1
SSC-A
FSC-A
262144
196608
131072
65536
0
B
Gate 2
FSC-W
FSC-H
C
Gate 3
Count
mCherry-A
Gate 4
GFP-A
Gate 5
BFP-A

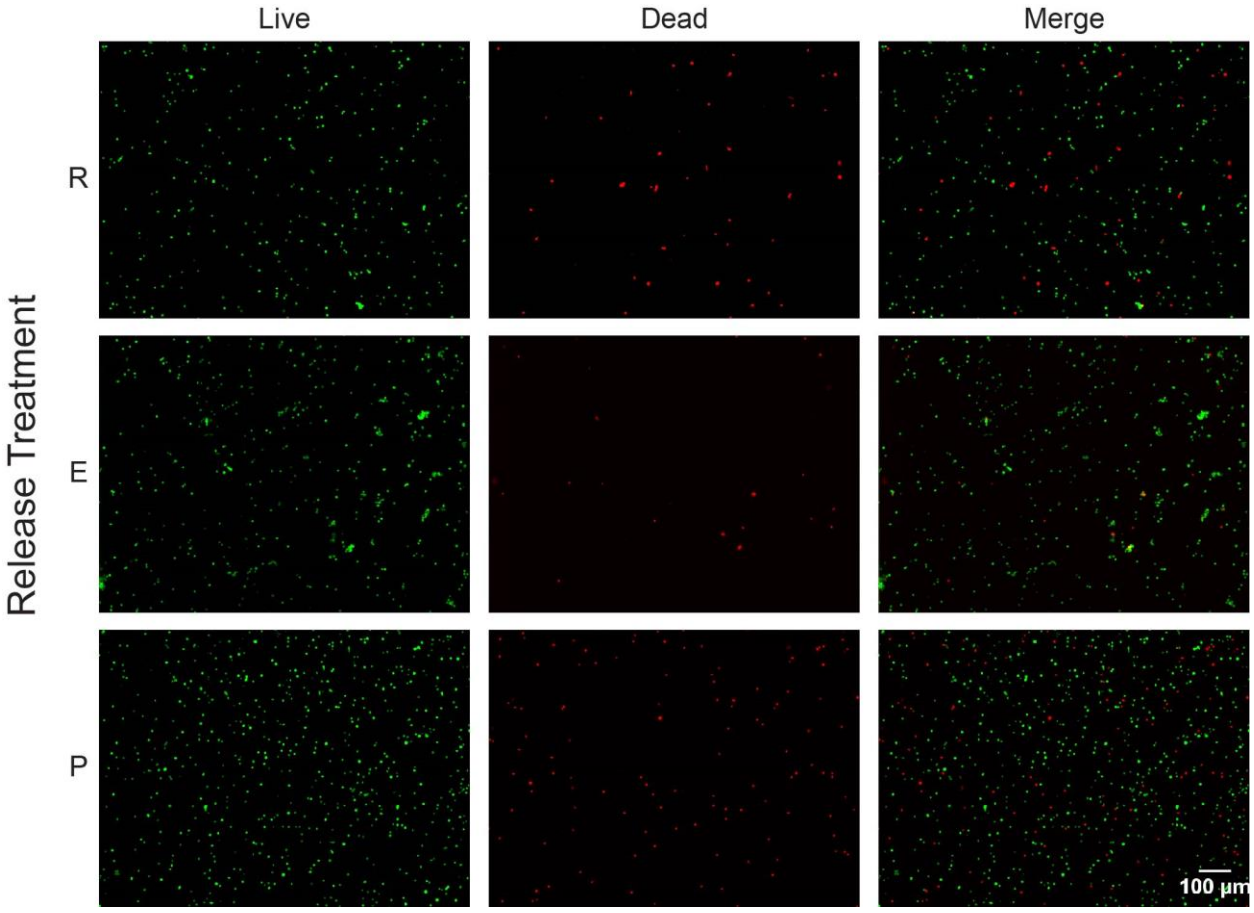
Supplementary Figure 12: Cell viability following hydrogel encapsulation and triggered release

Cell-laden hydrogel preparation for viability studies:
Single-input responsive hydrogels were formed sandwiched between an azide-functional glass slide and a Rain-X®-treated glass slide separated by two 0.005" thick silicone spacers (McMaster-Carr). A precursor solution containing PEG-tetraBCN (2 mM), a YES crosslinker (either R, E, or P; 4 mM), and hS5 (40 x 10^6 cells mL^{-1}) was injected between the silicone spacers and reacted for one hour. Hydrogels were stored in media for one hour prior to treatments. Each material was generated and assayed in experimental triplicate.

Cell-laden hydrogel degradation for viability studies:
Gels were degraded and the viability of the released cells was assessed. To induce gel degradation, R gels were treated with BME (0.25 mM in PBS) at 37 °C for 45 minutes; P gels were exposed to UV light (λ = 365 nm, 10 mW cm^{-2} incident light) at 25 °C for 10 minutes; E gels were treated with MMP-8 (0.20 nM in RPMI) at 37 °C for 60 minutes. All treatments yielded complete hydrogel degradation. Released cells were collected and isolated *via* centrifugation prior to Live/Dead® staining (Invitrogen).

Viability analysis:
Cells released from the gels and stained with the Live/Dead® assay were imaged on a Nikon Eclipse TE2000-U microscope using filter cubes corresponding to both live (excitation 480/40 nm filter, emission 535/50 nm filter) and dead (excitation 560/20 nm filter, emission 630/60 nm filter) staining. Viability was determined for each treatment by standard image analysis. Average cell viability and representative images for each treatment condition are presented below. For each release treatment, representative fluorescent images of live and dead cells are false colored green and red, respectively.




Live
Dead
Merge
Release Treatment
R
E
P
100 µm

References

1. DeForest, C. A. & Anseth, K. S. Cytocompatible click-based hydrogels with dynamically tunable properties through orthogonal photoconjugation and photocleavage reactions. *Nat. Chem.* **3,** 925–931 (2011).
2. Kloxin, A. M., Tibbitt, M. W. & Anseth, K. S. Synthesis of photodegradable hydrogels as dynamically tunable cell culture platforms. *Nat. Protoc.* **5,** 1867–1887 (2010).
3. Goddard-Borger, E. D. & Stick, R. V. An efficient, inexpensive, and shelf-stable diazotransfer reagent: Imidazole-1-sulfonyl azide hydrochloride. *Org. Lett.* **9,** 3797–3800 (2007).
4. Chapman, R., Jolliffe, K. A. & Perrier, S. Synthesis of self assembling cyclic peptide-polymer conjugates using click chemistry. *Aust. J. Chem.* **63,** 1169–1172 (2010).
5. Dommerholt, J. *et al.* Readily accessible bicyclononynes for bioorthogonal labeling and three-dimensional imaging of living cells. *Angew. Chemie - Int. Ed.* **49,** 9422–9425 (2010).
6. DeForest, C. A. & Tirrell, D. A. A photoreversible protein-patterning approach for guiding stem cell fate in three-dimensional gels. *Nat. Mater.* **14,** 523–531 (2015).
7. Palavalli, L. H. *et al.* Analysis of the matrix metalloproteinase family reveals that MMP8 is often mutated in melanoma. *Nat Genet* **41,** 518–520 (2009).
8. Zhang, S. *et al.* A novel strategy for MALDI-TOF MS analysis of small molecules. *J. Am. Soc. Mass Spectrom.* **21,** 154–160 (2010).
9. Sarver, A., Scheffler, N. K., Shetlar, M. D. & Gibson, B. W. Analysis of peptides and proteins containing nitrotyrosine by matrix-assisted laser desorption/ionization mass spectrometry. *J. Am. Soc. Mass Spectrom.* **12,** 439–448 (2001).
10. Strohalm, M., Kodíček, M. & Pechar, M. Tryptophan modification by 2-hydroxy-5-nitrobenzyl bromide studied by MALDI-TOF mass spectrometry. *Biochem. Biophys. Res. Commun.* **312,** 811–816 (2003).
11. Faucher, A.-M. & Grand-Maître, C. tris(2-carboxyethyl)phosphine (TCEP) for the reduction of sulfoxides, sulfonylchlorides, N-oxides and azides. *Synth. Commun.* **33,** 3503–3511 (2003).
12. Hiemenz, P. C. & Lodge, T. P. *Polymer Chemistry*. (CRC Press, 2007).
13. Walba, D. M. *et al.* Self-assembled monolayers for liquid crystal alignment: simple preparation on glass using alkyltrialkoxysilanes. *Liq. Cryst.* **31,** 481–489 (2004).
14. Godula, K., Rabuka, D., Nam, K. T. & Bertozzi, C. R. Synthesis and microcontact printing of dual end-functionalized mucin-like glycopolymers for microarray applications. *Angew. Chemie - Int. Ed.* **48,** 4973–4976 (2009).
15. Iwata, M., Torok-Storb, B., Wayner, E. A. & Carter, W. G. CDCP1 identifies a CD146 negative subset of marrow fibroblasts involved with cytokine production. *PLoS One* **9,** 1–11 (2014).