Supporting Information

Logic-Based Delivery of Site-Specifically Modified Proteins from Environmentally Responsive Hydrogel Biomaterials

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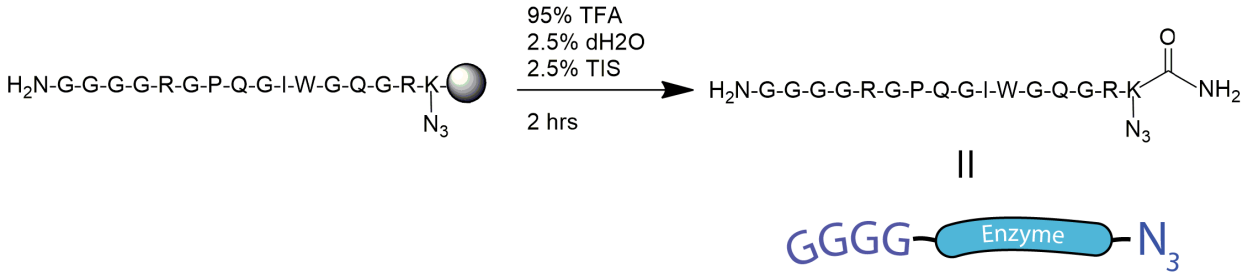
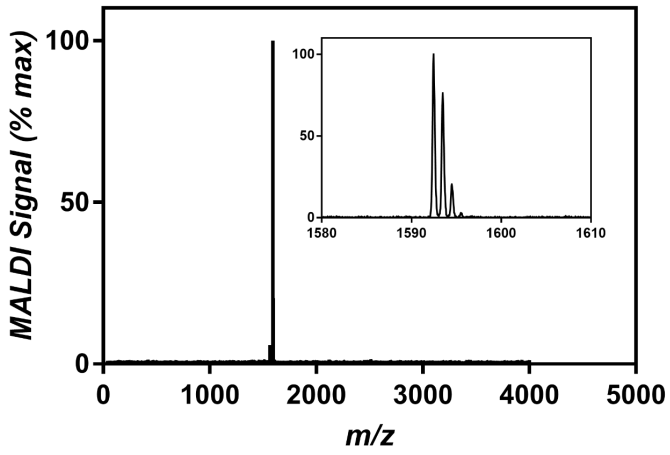
General Synthetic Information

Chemical reagents and solvents were purchased from either Sigma-Aldrich or Fisher Scientific and used without further purification. Distilled water (dH_2O) was obtained from a U.S. Filter Corporation Reverse Osmosis system equipped with a desalination membrane. All chemical reactions were performed under inert nitrogen atmosphere in flame-dried glassware and were stirred with Teflon-coated magnetic stir bars. Solvent was removed under reduced pressure with a Buchi Rotovap R-3 by using either V-700 vacuum pump or Welch 1400 high vacuum pump. All peptides were synthesized using Fmoc-based, microwave-assisted, solid-phase peptide synthesis methodology on a CEM Liberty 1. All peptides were purified by semi-preparative reverse-phase high pressure liquid chromatography (RP-HPLC) performed on Dionex Ultimate 3000 equipped with RS multiple variable wavelength detector, automated fraction collector, and C18 column. Peptide characterization was performed by using Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) on Bruker AutoFlex II. Lyophilization was performed on a Labconco FreeZone 2.5 Plus freeze-dryer equipped with Labconco rotary vane 117 vacuum pump. Lumen Dynamics OmniCure S1500 Spot UV curing system was used for photochemical cleavage reactions, where light intensity was determined using a Cole-Palmer radiometer (Series 9811-50, $\lambda = 365$ nm). All cell cultures were maintained in Thermo-Fisher Scientific MaxQ 4000 Benchtop Orbital Shaker-Incubator. Cells were lysed using Fisher Scientific Sonic Dismembrator (Model 505). Protein concentrations were measured on Thermo-Scientific NanoDrop 2000 Spectrophotometer. Protein mass characterization was performed on SYNAPT G2-Si Mass Spectrometer equipped with a liquid chromatography column (LC-MS). Fluorescence measurement data was acquired from SpectraMax M5 spectrometer. Fluorescence microscopy was performed on Leica SP8X confocal microscope. The synthesis of 2,5-dioxopyrrolidin-1-yl 4-azidobutanoate (N_3-OSu), 2,5-dioxopyrrolidin-1-yl 4-(4-(1-((4-azidobutanoyl)oxy)ethyl)-2-methoxy-5-nitrophenoxy) butanoate (N_3-*o*NB-OSu), PEG-tetraBCN, and N_3-PEG-N_3 were performed as reported in literature.[1] Fmoc-Lys(N_3)-OH was synthesized as previously reported.[2]

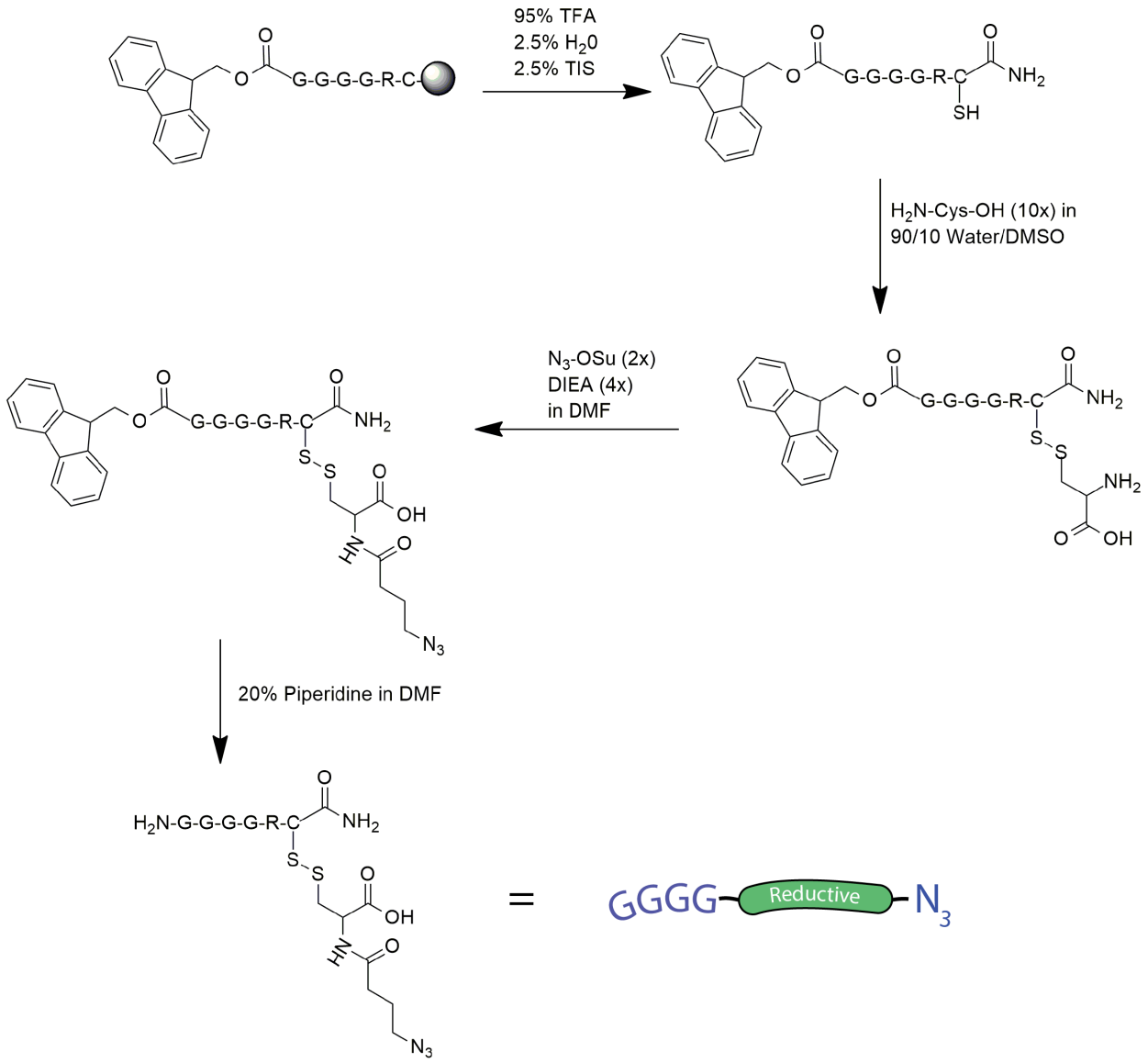
Method S1: Synthesis of Enzymatically-Degradable, Sortaggable Peptide (GGGG-E-N_3)



H-GGGGRGPQGIWGQGRK(N_3)-NH_2 was synthesized on rink amide resin (0.25 mmol scale) *via* standard Fmoc-based, microwave-assisted, solid-phase peptide synthesis methodologies; Fmoc-Lys(N_3)-OH was utilized to introduce azide functionality at the C-terminus. The peptide was deprotected and cleaved from resin by treatment with trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/dH_2O (95:2.5:2.5, 15 mL) for 2 hours. Following cleavage, the peptide was precipitated in and washed with ice-cold diethyl ether (2x), purified by RP-HPLC operating with 55 min gradient (5-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the final peptide (H-GGGGRGPQGIWGQGRK(N_3)-NH_2, denoted GGGG-E-N_3) as a white solid (97.6 mg, 0.0613 mmol, 24.5% yield). Peptide purity was confirmed by MALDI-TOF mass spectroscopy: calculated $[M+^1H]^+$, 1592.70, observed 1592.46.

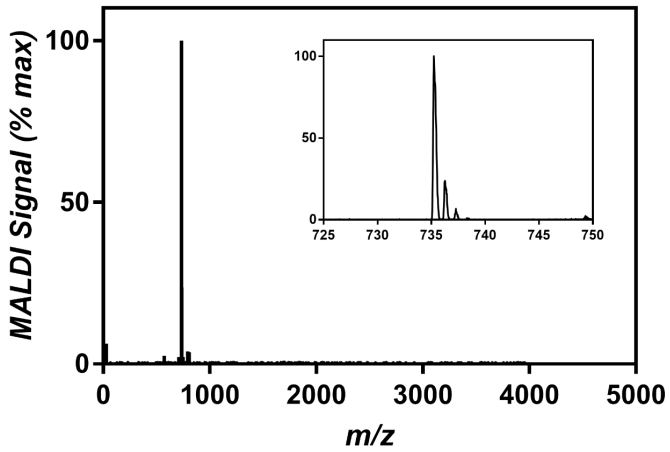


Method S2: Synthesis of Reduction-Degradable, Sortaggable Peptide (GGGG-R-N_3)

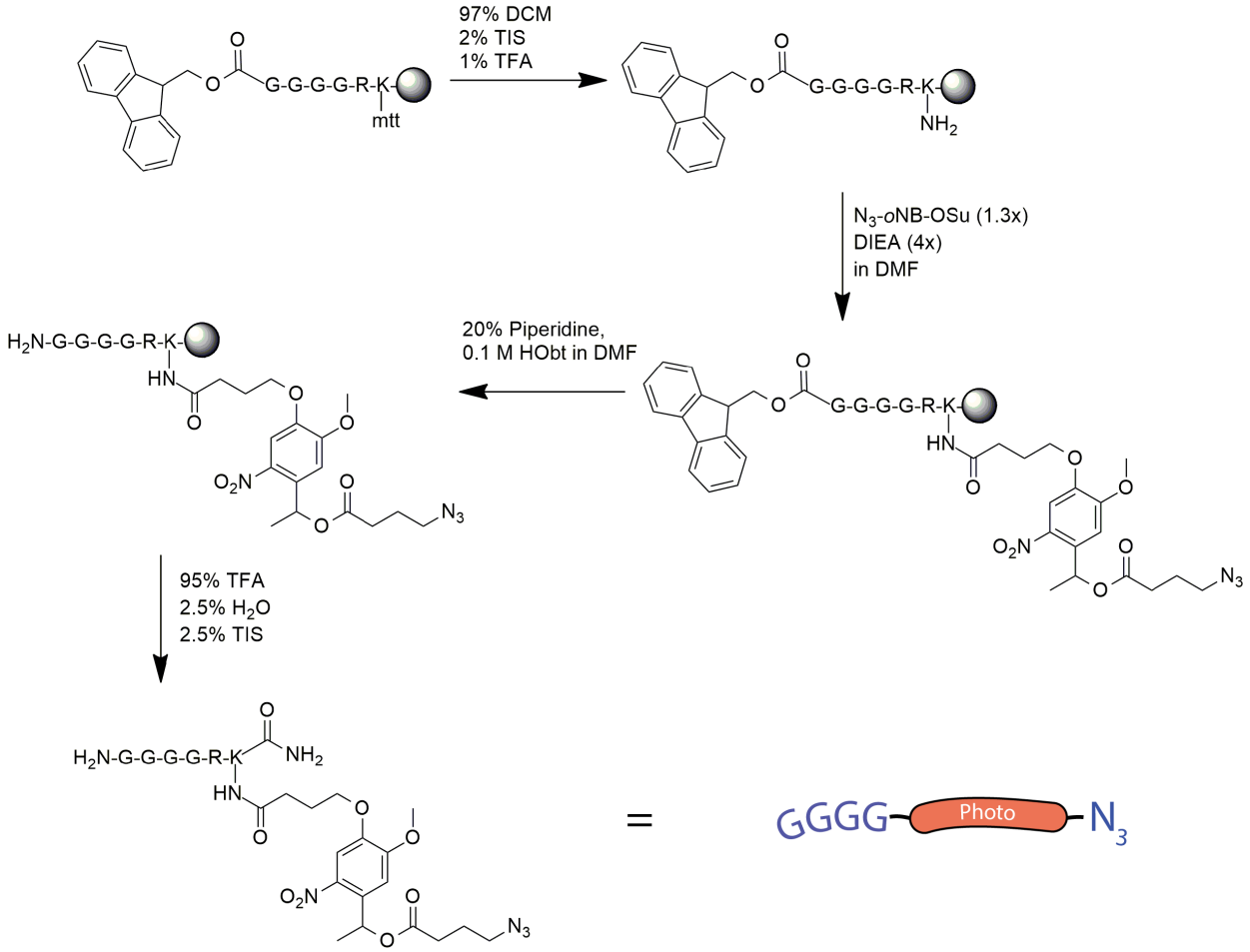


Fmoc-GGGGRC-NH_2 was synthesized on rink amide resin (0.25 mmol scale) *via* standard Fmoc-based, microwave-assisted, solid-phase peptide synthesis methodologies. The peptide was deprotected and cleaved from resin by treatment with TFA/TIS/dH_2O (95:2.5:2.5, 15 mL) for 2 hours. Following cleavage, the peptide was precipitated in and washed with ice-cold diethyl ether (2x), purified by RP-HPLC operating with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the intermediate peptide as a white solid. The intermediate peptide (50 mg, 0.069 mmol, 27.6% yield) and H-Cys-OH (84 mg, 0.69 mmol, 10x) were dissolved dH_2O/DMSO (9:1) and reacted at room temperature for 48 hrs. The solution was concentrated by rotary evaporation, dissolved in dH_2O, purified by RP-HPLC with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to give the intermediate peptide (Fmoc-GGGGRC(H-C-OH)-NH_2 with cysteines linked *via* disulfide bond)

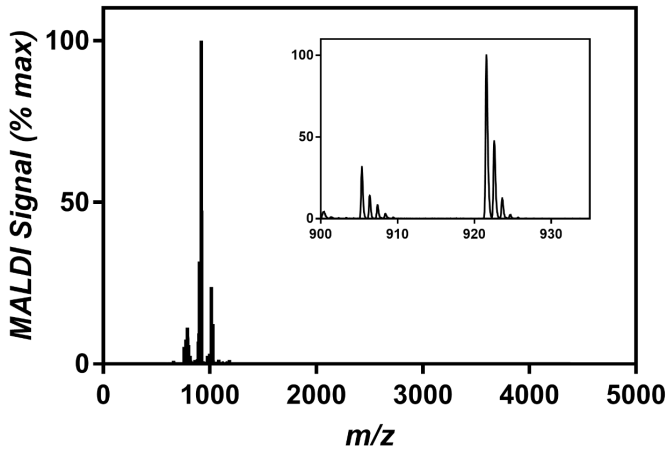
as a white solid. The intermediate peptide (8.2 mg, 0.0097 mmol, 14% yield) was reacted overnight with N_3-OSu (4.38 mg, 0.0194 mmol, 2x) and DIEA (5.01 mg, 0.039 mmol, 4x) in minimal DMF to introduce azide functionality onto the peptide. The reaction mixture was purified by RP-HPLC with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and the product fraction was lyophilized to obtain the intermediate peptide, Fmoc-GGGGRC(N_3-C-OH)-NH_2 with cysteines linked *via* disulfide bond (9 mg, 0.0094 mmol, 97% yield). The N-terminal Fmoc group was cleaved by incubating the peptide in piperidine (20%) in DMF (9 mL) for 10 mins. The deprotection reaction mixture was concentrated *via* rotary evaporation, purified by RP-HPLC operating with 55 min gradient (5-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the final product (H-GGGGRC(N_3-C-OH)-NH_2 with cysteines linked *via* disulfide bond, denoted GGGG-R-N_3) as a white solid (1.5 mg, 0.002 mmol, 21.3% yield). Peptide purity was confirmed by MALDI-TOF mass spectroscopy: calculated $[M+^1H]^+$, 734.97, observed 735.33.



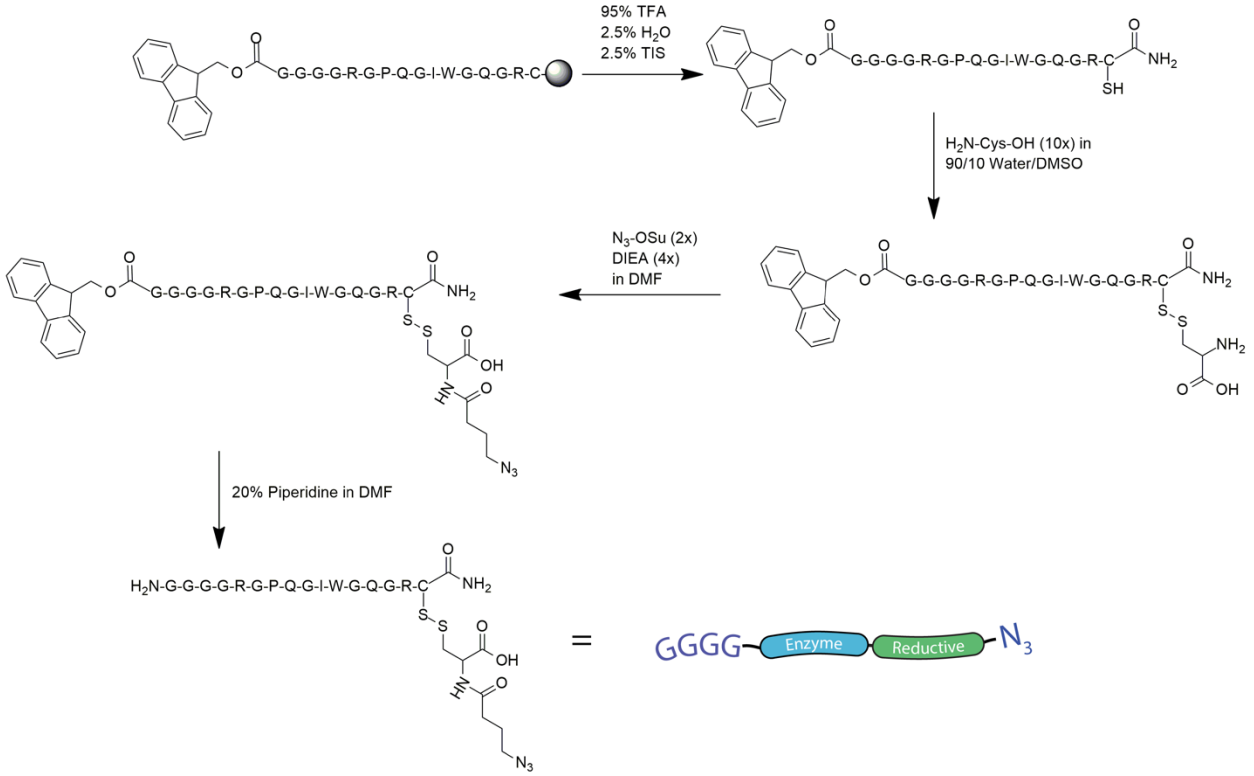
Method S3: Synthesis of Photo-Degradable, Sortaggable Peptide (GGGG-P-N_3)



Fmoc-GGGGRK(Mtt)-NH_2 was synthesized on rink amide resin (0.25 mmol scale) *via* standard Fmoc-based, microwave-assisted, solid-phase peptide synthesis methodologies. The acid-labile N-methyltrityl (Mtt) moiety protecting the ε-amino group of the lysine side chain was removed by treatment with DCM/TIS/TFA (97:2:1, 9x15 mL, 10 min each). The resin-bound peptide was reacted overnight with N_3-*o*NB-OSu (165 mg, 0.325 mmol, 1.3x) and DIEA (129.25 mg, 1 mmol, 4x) in minimal DMF to introduce *o*NB and azido functionality onto the ε-amino group of the lysine side chain. Fmoc deprotection was achieved on resin by treatment with piperidine (20%) and 1-hydroxybenzotriazole (HOBt, 0.1 M) in DMF (2x15 mL, 10 mins each). The peptide was deprotected and cleaved from resin by treatment with TFA/TIS/dH_2O (95:2.5:2.5, 15 mL) for 2 hours. Following cleavage, the peptide was precipitated in and washed with ice-cold diethyl ether (2x), purified by RP-HPLC operating with 55 min gradient (5-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the final product (H-GGGGRK(*o*NB-N_3)-NH_2, denoted GGGG-P-N_3) as a yellow solid (41.1 mg, 0.45 mmol, 17.83% yield). Peptide purity was confirmed by MALDI-TOF mass spectroscopy: calculated $[M+^1H]^+$, 921.96, observed 921.47.

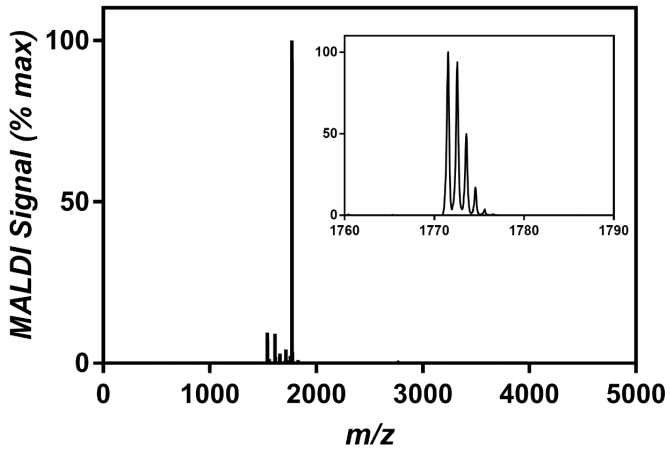

MALDI Signal (% max)
100
50
0
0
1000
2000
3000
4000
5000
m/z
100
50
0
900
910
920
930

Method S4: Synthesis of Enzyme-OR-Reductive-Degradable, Sortaggable Peptide (GGGG-EVR-N_3)

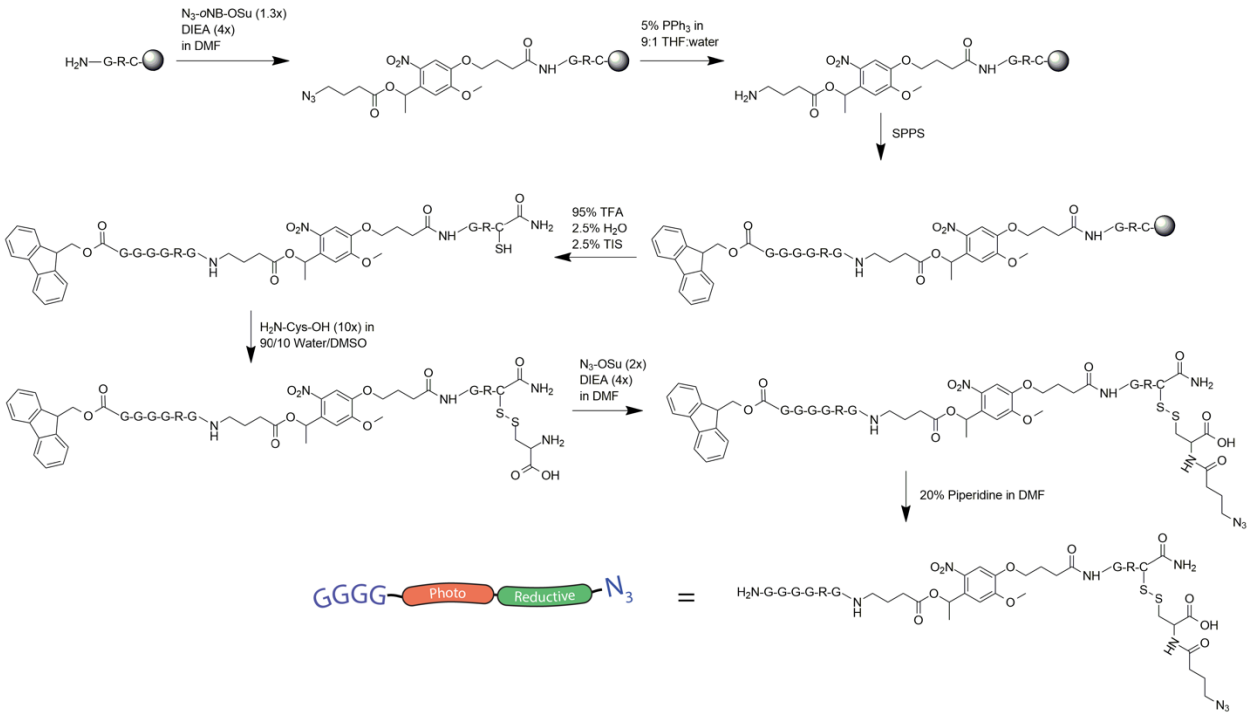


Fmoc-GGGGRGPQGIWGQGRC-NH_2 was synthesized on rink amide resin (0.25 mmol scale) *via* standard Fmoc-based, microwave-assisted, solid-phase peptide synthesis methodologies. The peptide was deprotected and cleaved from resin by treatment with TFA/TIS/dH_2O (95:2.5:2.5, 15 mL) for 2 hours. Following cleavage, the peptide was precipitated in and washed with ice-cold diethyl ether (2x), purified by RP-HPLC operating with 55 min gradient (5-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the intermediate peptide as a white solid (52 mg, 0.0295 mmol, 11.8% yield). The intermediate peptide and H-Cys-OH (35.71 mg, 0.295 mmol,10x) were dissolved in dH_2O/DMSO (9:1, 10 mL) and reacted at room temperature for 48 hrs. The solution was concentrated by rotary evaporation, dissolved in dH_2O, purified by RP-HPLC with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to give the intermediate peptide (Fmoc-GGGGRGPQGIWGQGRC(NH_2-C-OH)-NH_2 with cysteines linked *via* disulfide bond) as a white solid (38 mg, 0.0202 mmol, 68.5% yield). The intermediate peptide was reacted overnight with N_3-OSu (9.1294 mg, 0.0403 mmol, 2x) and DIEA (10.42 mg, 0.087 mmol, 4x) in minimal DMF to introduce azide functionality onto the peptide. The reaction mixture was purified by RP-HPLC with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and the product fraction was lyophilized to obtain the intermediate peptide (Fmoc-GGGGRGPQGIWGQGRC(N_3-C-OH)-NH_2 with cysteines linked *via* disulfide bond) as a white solid (25 mg, 0.0125 mmol, 61.8% yield). The N-terminal Fmoc group was cleaved by incubating the peptide in piperidine (20%) in DMF (25 mL) for 10 mins. The deprotection reaction mixture was concentrated *via* rotary evaporation, purified by RP-HPLC operating with 55 min gradient (5-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the final product (H-GGGGRGPQGIWGQGRC(N_3-C-OH)-NH_2 with

cysteines linked *via* disulfide bond, denoted GGGG-EVR-N_3) as a white solid (14.5 mg, 0.0082 mmol, 65% yield). Peptide purity was confirmed by MALDI-TOF mass spectroscopy: calculated $[M+^1H]^+$, 1771.8; observed 1771.6.

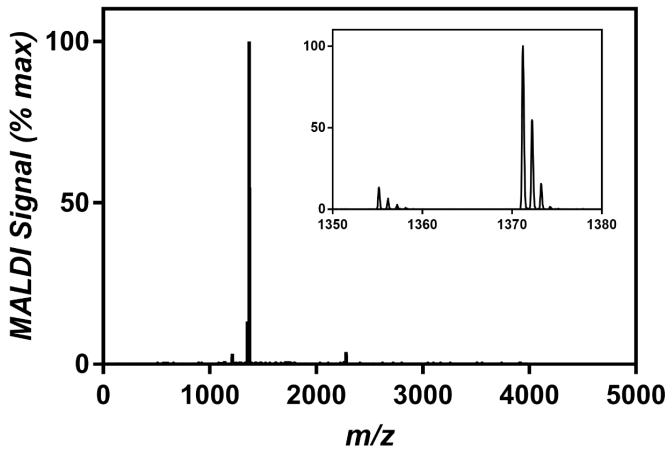


Method S5: Synthesis of Reductive-OR-Photo-Degradable, Sortaggable Peptide (GGGG-PVR-N_3)

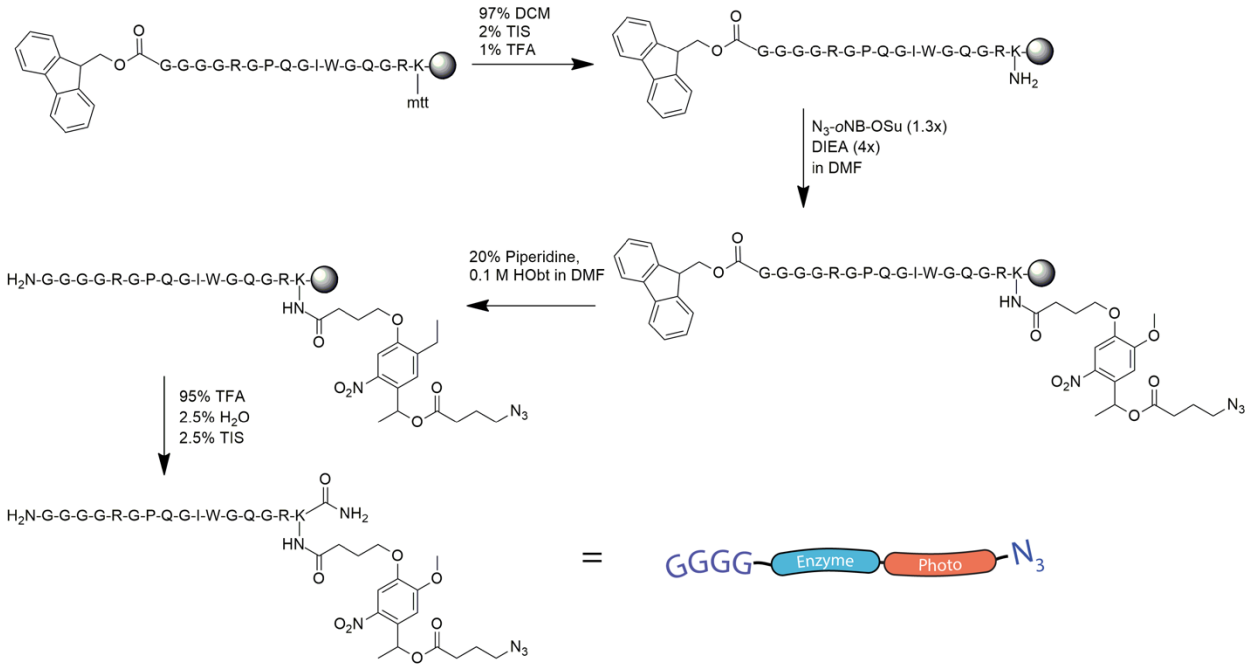


H-GRC-NH_2 was synthesized on rink amide resin (0.25 mmol scale) *via* standard Fmoc-based, microwave-assisted, solid-phase peptide synthesis methodologies. The resin-bound peptide was reacted overnight with N_3-*o*NB-OSu (165 mg, 0.325 mmol, 1.3x) and DIEA (129.25 mg, 1 mmol, 4x) in minimal DMF to introduce *o*NB and azido functionality onto the peptide N-terminus. The N-terminal azide was reduced to an amine by Staudinger reduction; the resin-bound peptide was washed THF/dH_2O (90:10, 3x20 mL) and reacted overnight with 5 wt% triphenylphosphine in THF/dH_2O (90/10, 30 mL). The peptide Fmoc-GGGGRG was appended to the N-terminus *via* standard microwave-assisted solid-phase peptide synthesis methodology. The peptide was deprotected and cleaved from resin by treatment with TFA/TIS/dH_2O (95:2.5:2.5, 15 mL) for 2 hours. Following cleavage, the peptide was precipitated in and washed with ice-cold diethyl ether (2x), purified by RP-HPLC operating with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the intermediate peptide (Fmoc-GGGGRG-*o*NB-GRC-NH_2) as a yellow solid (75 mg, 0.055 mmol, 22% yield). The intermediate peptide and H-Cys-OH (66.64 mg, 0.55 mmol, 10x) were dissolved in dH_2O/DMSO (9:1, 10 mL) and reacted at room temperature for 48 hrs. The solution was concentrated by rotary evaporation, dissolved in dH_2O, purified by RP-HPLC with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to give the intermediate peptide (Fmoc-GGGGRG-*o*NB-GRC(H-C-OH)-NH_2 with cysteines linked *via* disulfide bond) as a yellow solid (43.33 mg, 0.0292 mmol, 53.1% yield). The intermediate peptide was reacted overnight with N_3-OSu (13.22 mg, 0.584 mmol, 2x) and DIEA (15.13 mg, 0.117 mmol, 4x) in minimal DMF to introduce azide functionality onto the peptide. The reaction mixture was purified by RP-HPLC with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and the product fraction was lyophilized to obtain the intermediate peptide (Fmoc-GGGGRG-*o*NB-GRC(N_3-C-OH)-NH_2 with cysteines

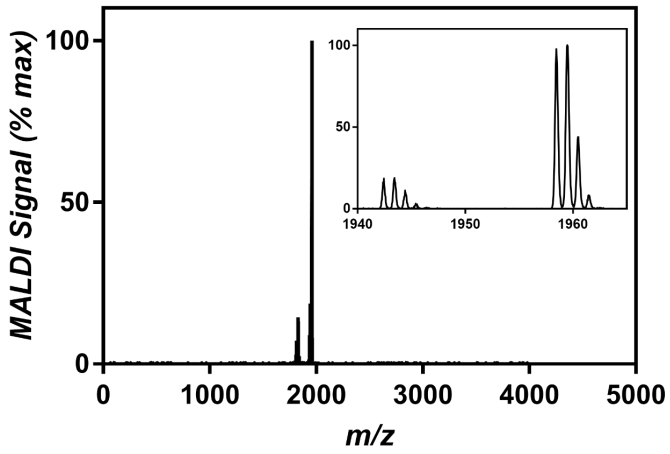
linked *via* disulfide bond) as a yellow solid (38.6 mg, 0.0242 mmol, 82.9% yield). The N-terminal Fmoc group was cleaved by incubating the peptide in piperidine (20%) in DMF (40 mL) for 10 mins. The deprotection reaction mixture was concentrated *via* rotary evaporation, purified by RP-HPLC operating with 55 min gradient (5-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the final product (H-GGGGRG-*o*NB-GRC(N_3-C-OH)-NH_2 with cysteines linked *via* disulfide bond, denoted GGGG-PvR-N_3) as a yellow solid (7.86 mg, 0.0057 mmol, 20% yield). Peptide purity was confirmed by MALDI-TOF mass spectroscopy: calculated $[M+^1H]^+$, 1371.65; observed 1371.52.



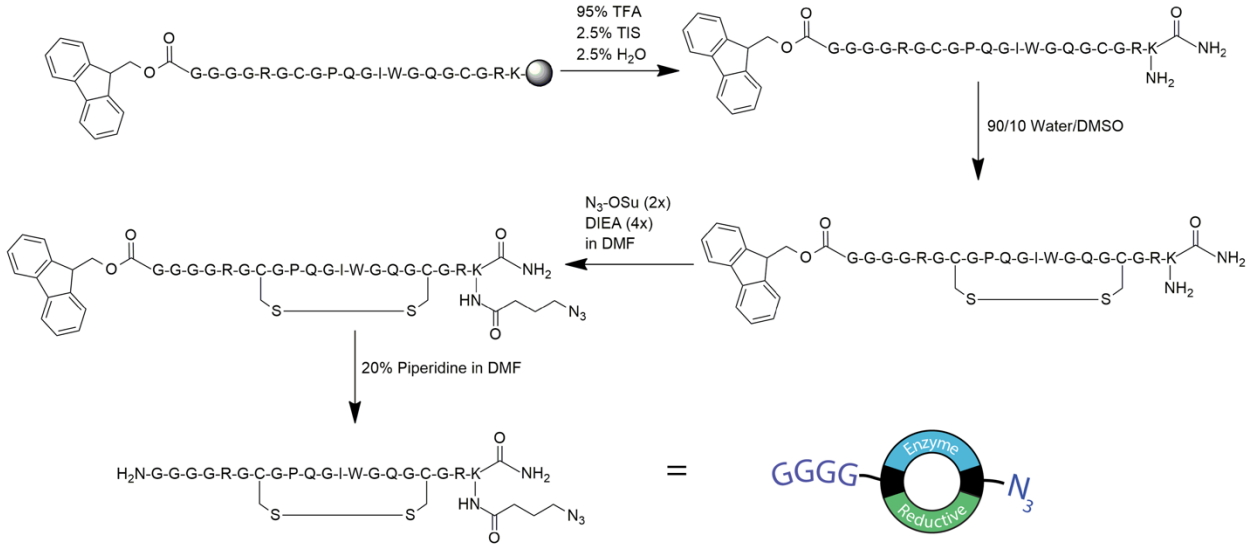
Method S6: Synthesis of Enzyme-OR-Photo-Degradable, Sortaggable Peptide (GGGG-EVP-N_3)



Fmoc-GGGGRGPQGIWGQGRK(Mtt)-NH_2 was synthesized on rink amide resin (0.25 mmol scale) *via* standard Fmoc-based, microwave-assisted, solid-phase peptide synthesis methodologies. The highly acid labile N-methyltrityl (Mtt) protection group on ε-amino group of the Lysine side chain was removed *via* treatment with Dichloromethane/ triisoproylsilane/ trifluoroacetic acid (97:2:1, 9x15 mL, 10 min each). The resin-bound peptide was reacted overnight with N_3-*o*NB-OSu (165 mg, 0.325 mmol, 1.3x) and DIEA (129.25 mg, 1 mmol, 4x) in minimal DMF to introduce *o*NB and azido functionality onto the ε-amino group of the Lysine side chain. The Fmoc deprotection was achieved on resin by treatment with a solution of piperidine (20%) and HOBt (0.1 M) in DMF (2x15 mL) for 10 mins. The peptide was deprotected and cleaved from resin by treatment with TFA/TIS/dH_2O (95:2.5:2.5, 15 mL) for 2 hours. Following cleavage, the peptide was precipitated in and washed with ice-cold diethyl ether (2x), purified by RP-HPLC operating with 55 min gradient (5-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the final product (H-GGGGRGPQGIWGQGRK(*o*NB-N_3)-NH_2, denoted GGGG-EVP-N_3) as a yellow solid (31.5 mg, 0.016 mmol, 6.4% yield). Peptide purity was confirmed by MALDI-TOF mass spectroscopy: calculated $[M+^1H]^+$, 1958.2, observed 1958.5.

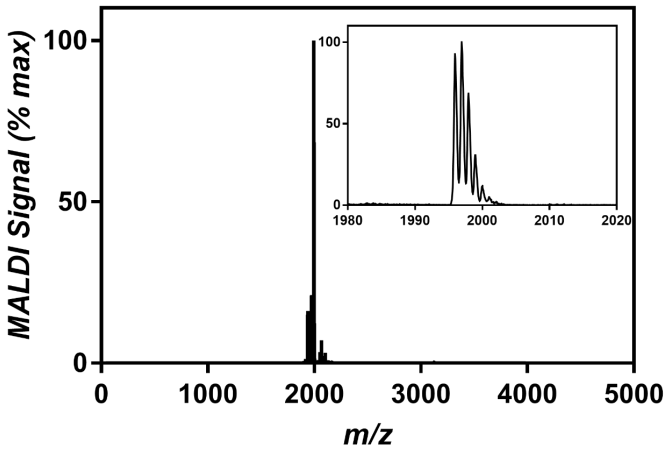

MALDI Signal (% max)
100
50
0
0
1000
2000
3000
4000
5000
m/z
100
50
0
1940
1950
1960

Method S7: Synthesis of Enzyme-AND-Reductive-Degradable, Sortaggable Peptide (GGGG-E∧R-N_3)

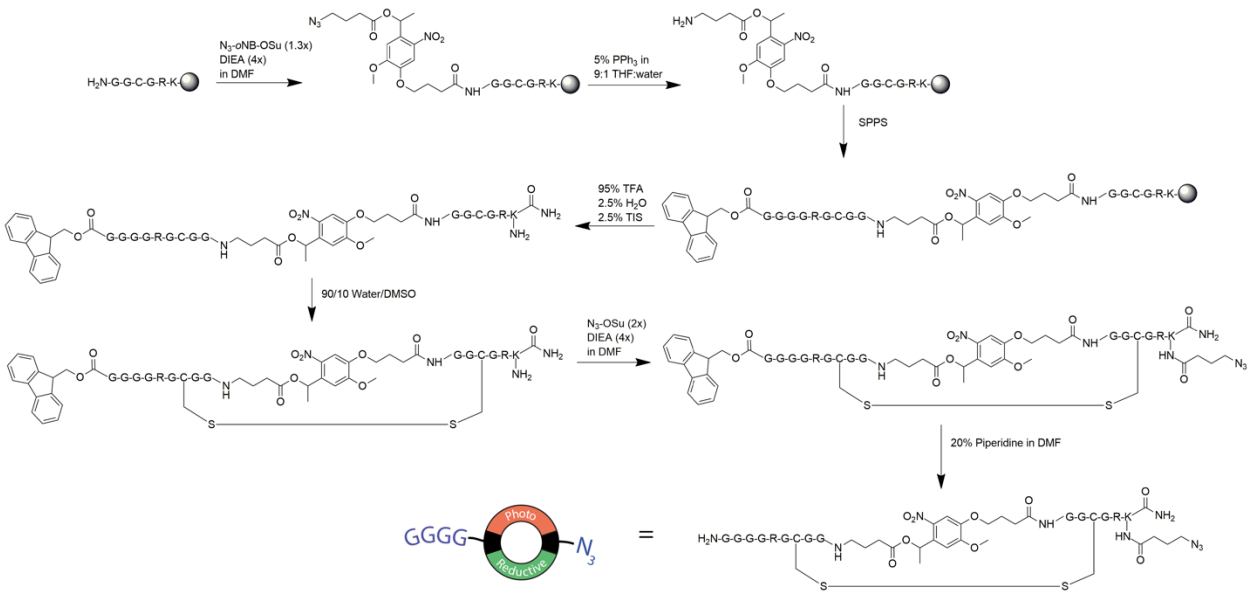


Fmoc-GGGGRGCGPQGIWGQGQGCGRK-NH_2 was synthesized on rink amide resin (0.25 mmol scale) *via* standard Fmoc-based, microwave-assisted, solid-phase peptide synthesis methodologies. The peptide was deprotected and cleaved from resin by treatment with TFA/TIS/dH_2O (95:2.5:2.5, 15 mL) for 2 hours. Following cleavage, the peptide was precipitated in and washed with ice-cold diethyl ether (2x), purified by RP-HPLC operating with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the intermediate peptide (Fmoc-GGGGRGCGPQGIWGQGQGCGRK-NH_2) as a white solid (126.5 mg, 0.059 mmol, 23.9% yield). The peptide was stapled *via* formation of an intramolecular disulfide bridge between the cysteine residues of the peptide; the intermediate peptide (1mM) was dissolved in a dH_2O/DMSO (90:10, 63 mL) solution and reacted at room temperature with no agitation for 48 hours. The stapled peptide was concentrated by rotary evaporation, dissolved in dH_2O, purified by RP-HPLC with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to give the intermediate peptide (Fmoc-GGGGRGCGPQGIWGQGQGCGRK-NH_2, stapled intramolecularly *via* cysteine-cysteine disulfide bond) as a white solid (47.6 mg, 0.0226 mmol, 37.7% yield). The intermediate peptide was reacted overnight with N_3-OSu (10.11 mg, 0.044 mmol, 2x) and DIEA (11.55 mg, 0.089 mmol, 4x) in minimal DMF to introduce azide functionality onto the peptide. The reaction mixture was purified by RP-HPLC operating with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the intermediate peptide (Fmoc-GGGGRGCGPQGIWGQGQGCGRK(N_3)-NH_2, stapled intramolecularly *via* cysteine-cysteine disulfide bond) as a white solid (34.4 mg, 0.0155 mmol, 68.6%). The N-terminal Fmoc group was cleaved by incubating the peptide in piperidine (20%) in DMF (30 mL) for 10 mins. The deprotection reaction mixture was concentrated *via* rotary evaporation, purified by RP-HPLC operating with 55 min gradient (5-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the final product (H-GGGGRGCGPQGIWGQGQGCGRK(N_3)-NH_2, stapled intramolecularly *via* cysteine-cysteine disulfide bond, denoted GGGG-E∧R-N_3) as white a solid

(8.3 mg, 0.0042 mmol, 27% yield). Peptide purity was confirmed by MALDI-TOF mass spectroscopy: calculated $[M+^{1}H]^{+}$, 1997.13; observed 1996.9.

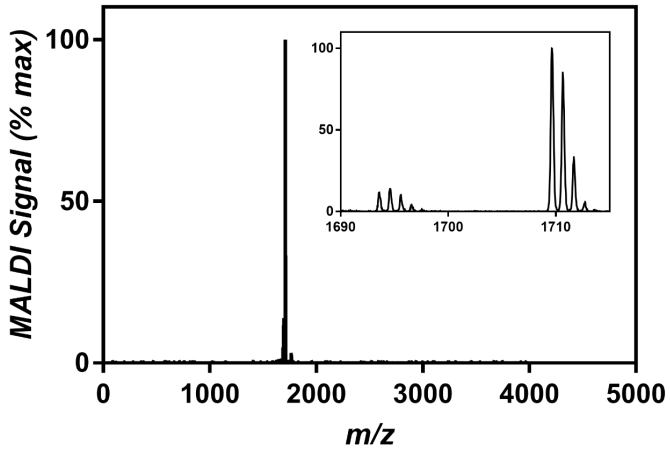


Method S8: Synthesis of Reductive-AND-Photo-Degradable, Sortaggable Peptide (GGGG-P∧R-N_3)

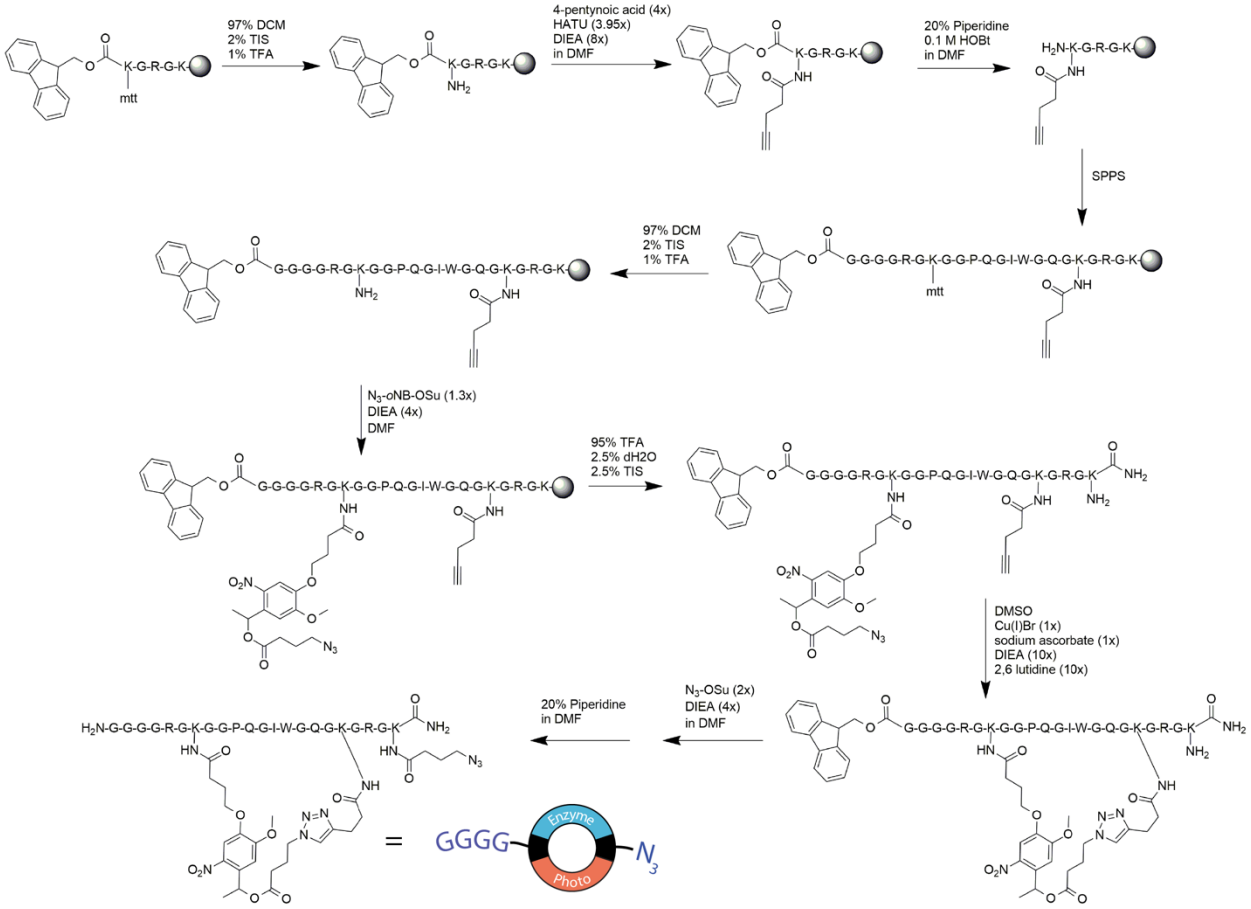


H-GCGRK-NH_2 was synthesized on rink amide resin (0.25 mmol scale) *via* standard Fmoc-based, microwave-assisted, solid-phase peptide synthesis methodologies. The resin-bound peptide was reacted overnight with N_3-*o*NB-OSu (165 mg, 0.325 mmol, 1.3x) and DIEA (129.25 mg, 1 mmol, 4x) in minimal DMF to introduce *o*NB and azido functionality onto the N-terminus. The N-terminal azide was reduced to an amine by Staudinger reduction; the resin-bound peptide was washed THF/dH_2O (90:10, 3x20 mL) and reacted overnight with 5 wt% triphenylphosphine in THF/dH_2O (90/10, 30 mL). Fmoc-GGGGRGCG-OH was appended to the N-terminus *via* standard microwave-assisted solid-phase peptide synthesis methodology. The peptide was deprotected and cleaved from resin by treatment with TFA/TIS/dH_2O (95:2.5:2.5, 15 mL) for 2 hours. Following cleavage, the peptide was precipitated in and washed with ice-cold diethyl ether (2x), purified by RP-HPLC operating with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the intermediate peptide (Fmoc-GGGGRGCG-*o*NB-GCGRK-NH_2) as a yellow solid (100 mg, 0.055 mmol, 22% yield). The peptide was stapled *via* formation of an intramolecular disulfide bridge between the cysteine residues of the peptide; the intermediate peptide (1 mM) was dissolved in a dH_2O/DMSO (90:10, 55 mL) solution and reacted at room temperature with no agitation for 48 hours. The stapled peptide was concentrated by rotary evaporation, dissolved in dH_2O, purified by RP-HPLC with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to give the intermediate peptide (Fmoc-GGGGRGCG-*o*NB-G<u>C</u>GRK-NH_2, stapled intramolecularly *via* cysteine-cysteine <u>disulfide bond</u>) as a yellow solid (60 mg, 0.0329 mmol, 59.8 % yield). The intermediate peptide was reacted overnight with N_3-OSu (10.5 mg, 0.464 mmol, 2x) and DIEA (11.98 mg, 0.093 mmol, 4x) in minimal DMF to introduce azide functionality onto the peptide. The reaction mixture was purified by RP-HPLC operating with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the intermediate peptide (Fmoc-GGGGRG<u>C</u>G-*o*NB-G<u>C</u>GRK(N_3)-NH_2, stapled intramolecularly *via* cysteine-cysteine <u>disulfide bond</u>) as a yellow solid (44 mg, 0.0227 mmol, 50%). The N-terminal Fmoc group was cleaved by incubating the peptide in piperidine (20%) in DMF (10 mL) for 10 mins. The deprotection reaction mixture was

concentrated *via* rotary evaporation, purified by RP-HPLC operating with 55 min gradient (5-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the final product (H-GGGGRGCG-*o*NB-GCGRK(N_3)-NH_2, stapled intramolecularly *via* cysteine-cysteine disulfide bond, denoted GGGG-P∧R-N_3) as a yellow solid (6.3 mg, 0.0037 mmol, 16.3% yield). Peptide purity was confirmed by MALDI-TOF mass spectroscopy: calculated $[M+^1H]^+$, 1709.9; observed 1709.6

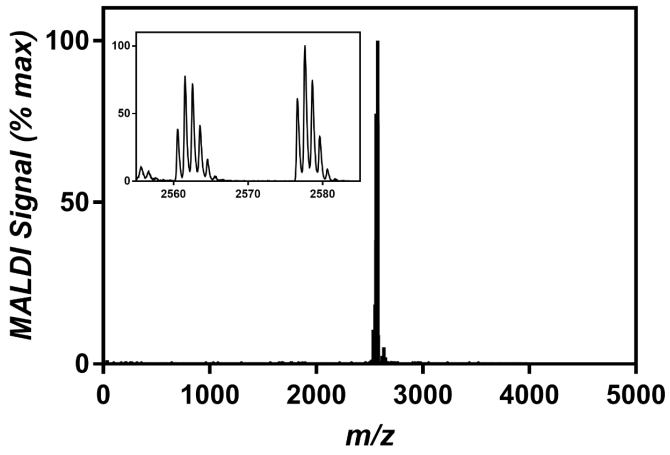


Method S9: Synthesis of Enzyme-AND-Photo-Degradable, Sortaggable Peptide (GGGG-EΛP-N_3)



Fmoc-K(Mtt)GRK-NH_2 was synthesized on rink amide resin (0.25 mmol scale) *via* standard Fmoc-based, microwave-assisted, solid-phase peptide synthesis methodologies. The highly acid labile N-methyltrityl (Mtt) protection group on ε-amino group of the Lysine side chain was removed *via* treatment with Dichloromethane/ triisoproylsilane/ trifluoroacetic acid (97:2:1, 9x15 mL, 10 min each). HATU coupling was used to functionalize ε-amino group of the Lysine side chain with an alkyne; 4-pentynoic acid (98.1 mg, 1 mmol, 4x) was pre-activated upon reaction with HATU (0.376 g, 0.99 mmol, 3.95x) and DIEA (260.7 mg, 2 mmol, 8x) in minimal DMF for 5 minutes and then reacted with the resin for 90 minutes. The Fmoc protecting group on the N-terminus of the peptide was cleaved on resin by treatment with a solution of piperidine (20%) and HOBt (0.1 M) in DMF (2x15 mL, 10 mins each). The peptide Fmoc-GGGGRGK(Mtt)GGPQGIWGQG was appended to the N-terminus *via* standard microwave-assisted solid-phase peptide synthesis methodology. The N-methyltrityl (Mtt) protection group on ε-amino group of the Lysine side chain was removed *via* treatment with Dichloromethane/ triisoproylsilane/ trifluoroacetic acid (97:2:1, 9x15 mL, 10 min each). The resin-bound peptide was reacted overnight with N_3-*o*NB-OSu (165 mg, 0.325 mmol, 1.3x) and DIEA (129.25 mg, 1 mmol, 4x) in minimal DMF to introduce *o*NB and azido functionality onto the ε-amino group of the Lysine side chain. The peptide was deprotected and cleaved from resin by treatment with TFA/TIS/dH_2O (95:2.5:2.5, 15 mL) for 2 hours. Following cleavage, the peptide was precipitated

in and washed with ice-cold diethyl ether (2x), purified by RP-HPLC operating with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the intermediate peptide (Fmoc-GGGGRGK(*o*NB-N_3)GGPQGIWGQGK(yne)GRK-NH_2) as a yellow solid (85 mg, 0.0316 mmol, 12.7% yield). The alkyne and azide functionalities present on the peptide side chains were stapled together *via* CuAAC (copper(I)-catalyzed azide-alkyne cycloaddition) click reaction; the linear peptide (1mM) was dissolved in nitrogen-purged DMSO (32 mL) containing cooper(I) bromide (4.53 mg, 0.032 mmol, 1eq), sodium ascorbate (6.19 mg, 0.032 mmol, 1eq) in water (316 µL), lutidine (32.23 mg, 0.316 mmol, 10 eq), and DIEA (40.84 mg, 0.316 mmol, 10eq); this mixture was allowed to react under nitrogen at room temperature overnight, concentrated *via* rotary evaporation, passed through ion exchange column (Dowex, M4195 resin, 5 g), and lyophilized. The stapled product was purified by RP-HPLC operating with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the intermediate peptide (Fmoc-GGGGRGK(*o*NB-N_3)GGPQGIWGQGK(yne)GRK-NH_2, stapled intramolecularly *via* triazole linkage between the alkyne and *o*NB-N_3 side chains) as a yellow solid (7.8 mg, 2.9 µmol, 9.18% yield). The intermediate peptide was reacted overnight with N_3-OSu (1.31 mg, 0.0058, 2x) and DIEA (1.498 mg, 0.0116 mmol, 4x) in minimal DMF to introduce azide functionality onto the peptide. The reaction mixture was purified by RP-HPLC operating with 43.4 min gradient (20-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the intermediate peptide (Fmoc-GGGGRGK(*o*NB-N_3)GGPQGIWGQGK(yne)GRK(N_3)-NH_2, stapled intramolecularly *via* triazole linkage between the alkyne and *o*NB-N_3 side chains) as a yellow solid (8 mg, 2.85 µmol, 98% yield). The N-terminal Fmoc group was cleaved by incubating the peptide in piperidine (20%) in DMF (8 mL) for 10 mins. The deprotection reaction mixture was concentrated *via* rotary evaporation, purified by RP-HPLC operating with 55 min gradient (5-100%) of acetonitrile in water containing TFA (0.1%), and lyophilized to obtain the final product (H-GGGGRGK(*o*NB-N_3)GGPQGIWGQGK(yne)GRK(N_3)-NH_2, stapled intramolecularly *via* triazole linkage between the alkyne and *o*NB-N_3 side chains, denoted GGGG-EΛP-N_3) as a solid (3.6 mg, 1.4 µmol, 49% yield). Peptide purity was confirmed by MALDI-TOF mass spectroscopy: calculated $[M+^1H]^+$, 2577.7; observed 2577.5.



Method S10: Protein Expression and Protein-Peptide Conjugation.

Protein Expression and Ni-NTA Immobilization

The pSTEPL-POI construct was transformed into BL21 (DE3) line.[3] A starter culture (20 mL) was grown overnight in LB containing ampicillin (0.1 mg/mL). The starter culture was used to inoculate LB (480 mL) containing ampicillin (0.1 mg/mL). The culture was grown to an OD600 of 0.4-0.6 before induction with Isopropyl β-D-1-thiogalactopyranoside (0.5 mM, IPTG). The culture was grown overnight at 25 °C. The cells were centrifuged (4000 x g, 10 mins) to obtain a pellet. The cells were re-suspended in STEPL Lysis Buffer (20 mM Tris-base, 50 mM NaCl, 10 mM imidazole, pH 7.5) containing phenylmethanesulfonyl fluoride (1 mM, PMSF) as a protease inhibitor. The cells were lysed using sonication; the cell suspension was kept on ice and subjected to short sonication burst cycles (1 sec pulse, 2 sec pause) with total sonication time 6 minutes. After sonication, the lysate was clarified by centrifugation (4000 x g, 10 minutes) and loaded onto Ni-NTA resin. The resin was washed with STEPL wash buffer (20 mM Tris-base, 50 mM NaCl, 20 mM imidazole, pH 7.5) to remove non-specifically bound proteins.

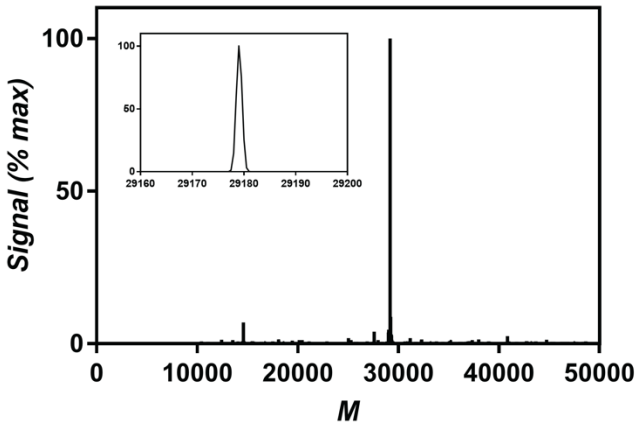
Note: pSTEPL constructs of EGFP was obtained as a gift from the Tsourkas lab.[3] Similar constructs for mCherry and mCerulean expression were prepared by standard molecular cloning techniques.[4]

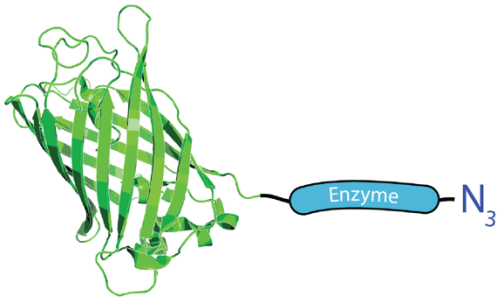
POI-Peptide Conjugation by STEPL Reaction

Ni-NTA column was loaded with EGFP as described. Sortaggable peptide was dissolved in STEPL buffer (5 mL) containing calcium chloride (0.1 mM, Ca^{2+}) and added to the column. To promote STEPL reaction, the column was reacted at 37 °C for 4 hours under gently agitation, resulting to the modification and subsequent displacement of POI with sortaggable peptide. After the STEPL reaction, the column flow through, containing the protein-peptide construct, was collected. Centrifugal membrane filter (Amicon Ultra-4, MW cutoff: 10 kDa) were used to simultaneously concentrate and further purify the protein-peptide construct.

Method S11: Synthesis of Enzyme-Responsive EGFP Pendant (EGFP-E-N_3)

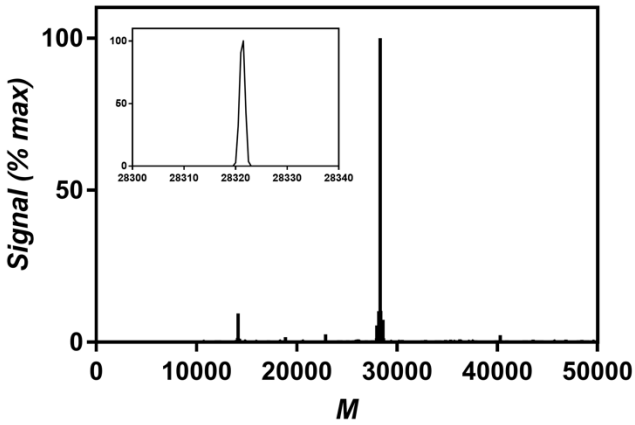
EGFP was expressed as a STEPL construct and loaded on the Ni-NTA column. Sortaggable peptide GGGG-E-N_3 (Method S1) was conjugated onto EGFP by STEPL (Method S10). Protein purity was confirmed by LC-MS: calculated [M], 29179.7; observed 29179.5.

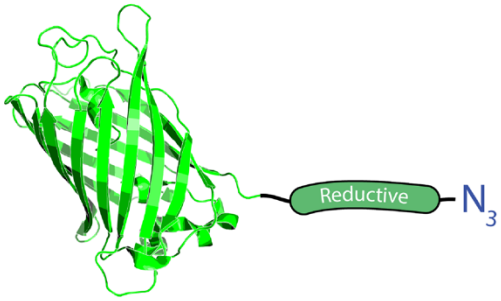




Method S12: Synthesis of Reductive-Responsive EGFP Pendant (EGFP-R-N_3)

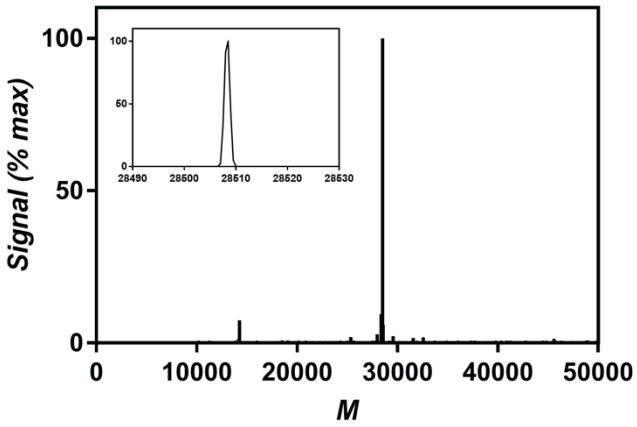
EGFP was expressed as a STEPL construct and loaded on the Ni-NTA column. Sortaggable peptide GGGG-R-N_3 (Method S2) was conjugated onto EGFP by STEPL (Method S10). Protein purity was confirmed by LC-MS: calculated [M], 28321.97; observed 28322.

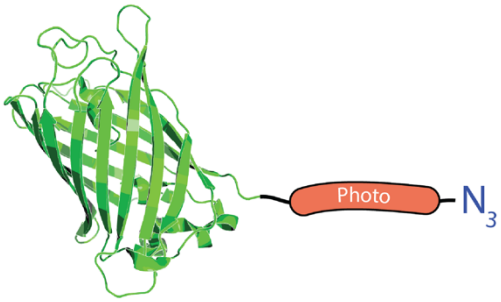




Method S13: Synthesis of Photo-Responsive EGFP Pendant (EGFP-P-N_3)

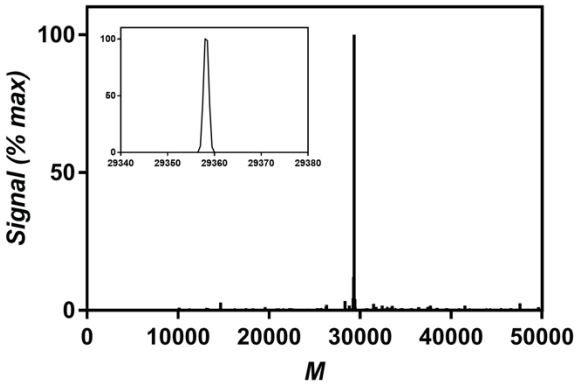
EGFP was expressed as a STEPL construct and loaded on the Ni-NTA column. Sortaggable peptide GGGG-P-N_3 (Method S3) was conjugated onto EGFP by STEPL (Method S10). Protein purity was confirmed by LC-MS: calculated [M], 28508.96; observed 28509.

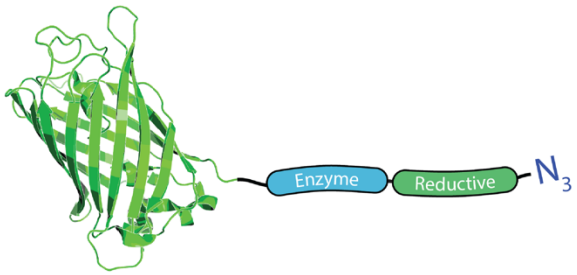




Method S14: Synthesis of Enzyme-OR-Reductive-Responsive EGFP Pendant (EGFP-EVR-N_3)

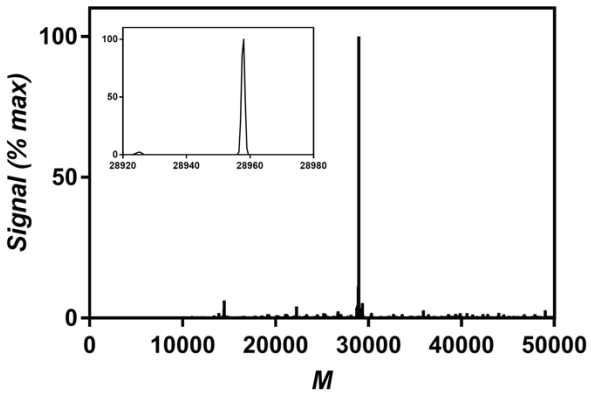
EGFP was expressed as a STEPL construct and loaded on the Ni-NTA column. Sortaggable peptide GGGG-EVR-N_3 (Method S4) was conjugated onto EGFP by STEPL (Method S10). Protein purity was confirmed by LC-MS: calculated [M], 29358.8; observed 29358.5.

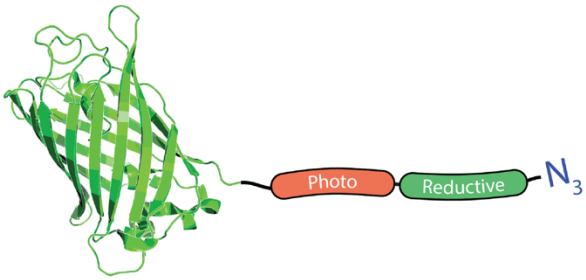




Method S15: Synthesis of Photo-OR-Reductive-Responsive EGFP Pendant (EGFP-PVR-N_3)

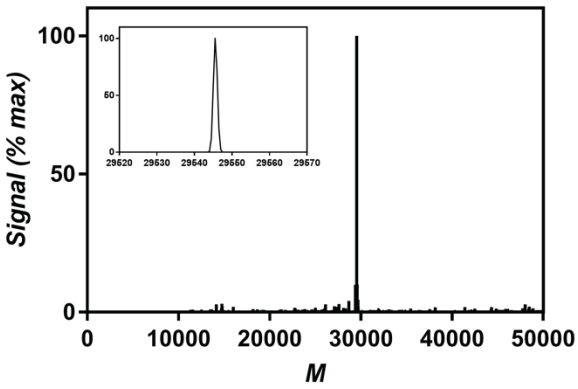
EGFP was expressed as a STEPL construct and loaded on the Ni-NTA column. Sortaggable peptide GGGG-PVR-N_3 (Method S5) was conjugated onto EGFP by STEPL (Method S10). Protein purity was confirmed by LC-MS: calculated [M], 28958.65; observed 28958.

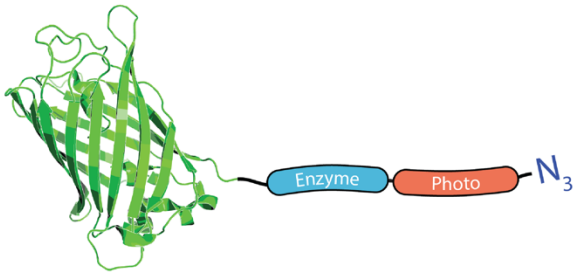




Method S16: Synthesis of Enzyme-OR-Photo-Responsive EGFP Pendant (EGFP-EVP-N_3)

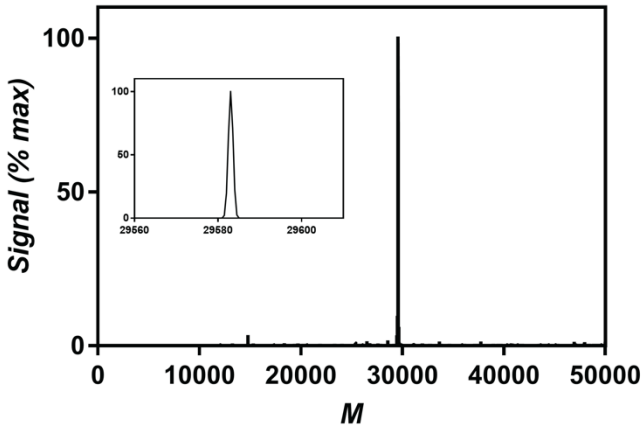
EGFP was expressed as a STEPL construct and loaded on the Ni-NTA column. Sortaggable peptide GGGG-EVP-N_3 (Method S6) was conjugated onto EGFP by STEPL (Method S10). Protein purity was confirmed by LC-MS: calculated [M], 29545.2; observed 29545.5.

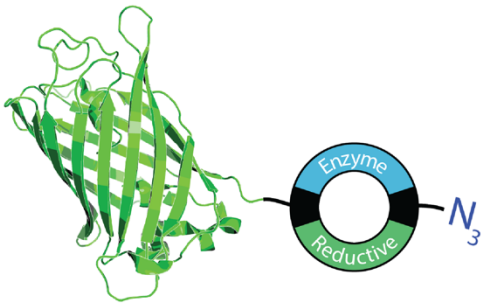




Method S17: Synthesis of Enzyme-AND-Reductive-Responsive-EGFP Pendant (EGFP-E∧R-N_3)

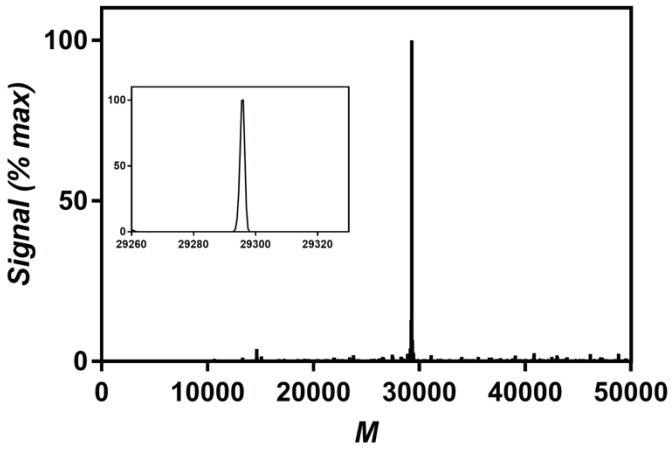
EGFP was expressed as a STEPL construct and loaded on the Ni-NTA column. Sortaggable peptide GGGG-E∧R-N_3 (Method S7) was conjugated onto EGFP by STEPL (Method S10). Protein purity was confirmed by LC-MS: calculated [M], 29584.13; observed 29583.5.

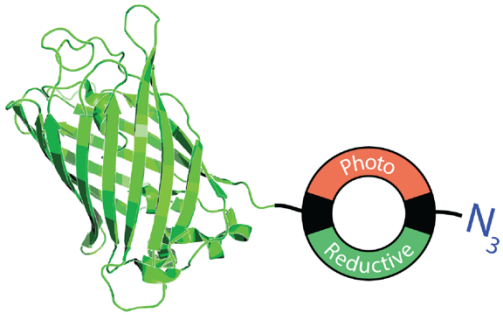




Method S18: Synthesis of Photo-AND-Reductive-Responsive-EGFP Pendant (EGFP-P∧R-N_3)

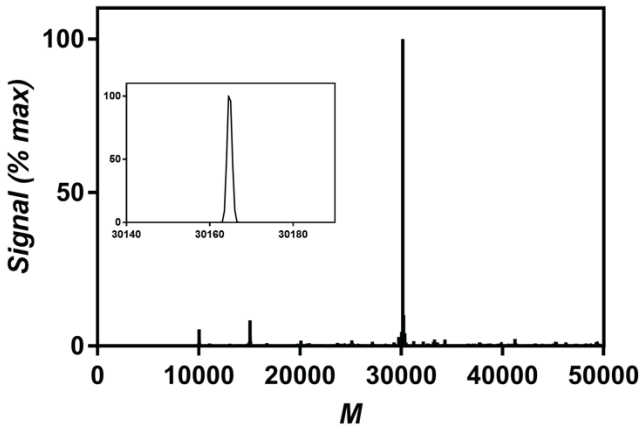
EGFP was expressed as a STEPL construct and loaded on the Ni-NTA column. Sortaggable peptide GGGG-P∧R-N_3 (Method S8) was conjugated onto EGFP by STEPL (Method S10). Protein purity was confirmed by LC-MS: calculated [M], 29296.9; observed 29296.5.

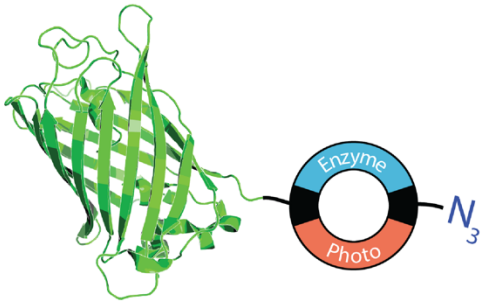




Method S19: Synthesis of Enzyme-AND-Photo-Responsive EGFP Pendant (EGFP-E∧P-N_3)

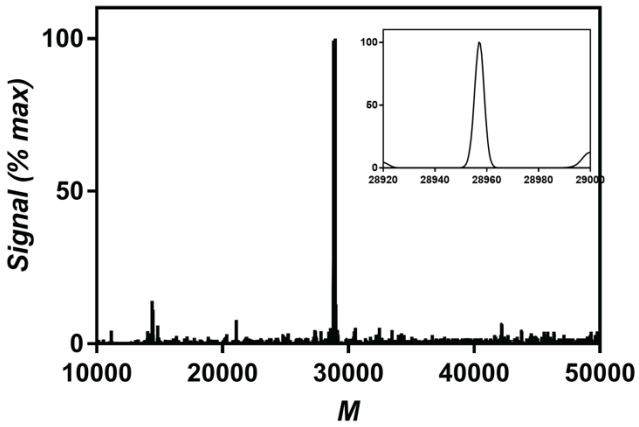
EGFP was expressed as a STEPL construct and loaded on the Ni-NTA column. Sortaggable peptide GGGG-E∧P-N_3 (Method S9) was conjugated onto EGFP by STEPL (Method S10). Protein purity was confirmed by LC-MS: calculated [M], 30165.7; observed 30165.

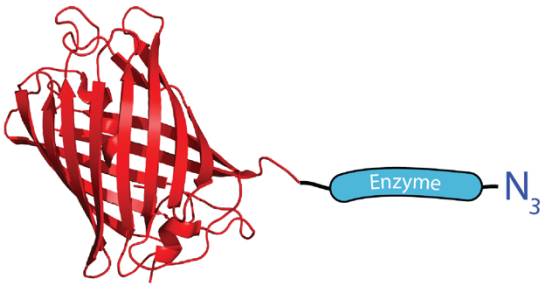




Method S20: Synthesis of Enzyme-Responsive mCherry Pendant (mCherry-E-N_3)

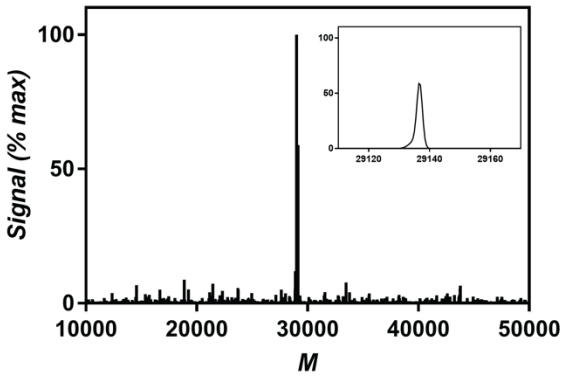
mCherry was expressed as a STEPL construct and loaded on the Ni-NTA column. Sortaggable peptide GGGG-E-N_3 (Method S1) was conjugated onto mCherry by STEPL (Method S10). Protein purity was confirmed by LC-MS: calculated [M], 28959.9; observed 28958.5.

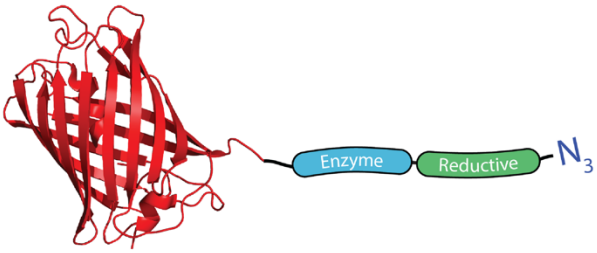




Method S21: Synthesis of Enzyme-OR-Reductive-Responsive-mCherry Pendant (mCherry- EVR-N_3)

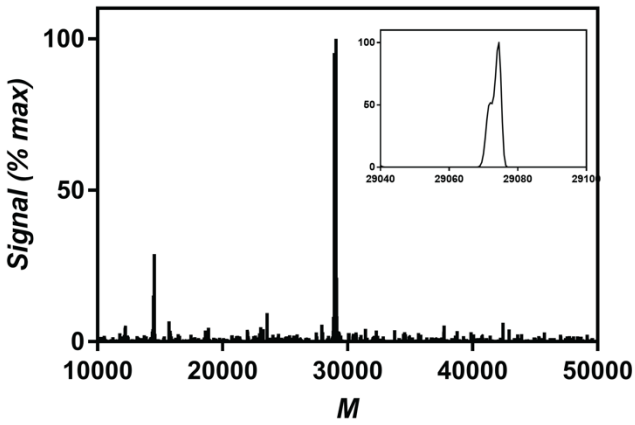
EGFP was expressed and loaded on the Ni-NTA column. Sortaggable peptide GGGG-EVR-N_3 (Method S4) was conjugated onto mCherry by STEPL (Method S10). Protein purity was confirmed by LC-MS: calculated [M], 29140.9; observed 29137.5.

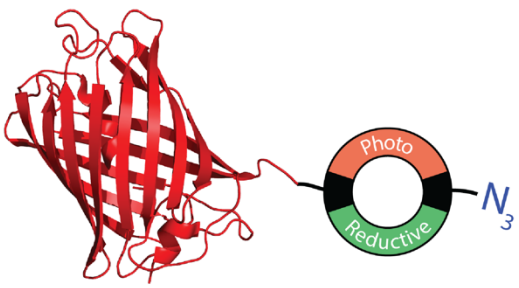




Method S22: Synthesis of Reductive-AND-Photo-Responsive-mCherry Pendant (mCherry-P∧R-N_3)

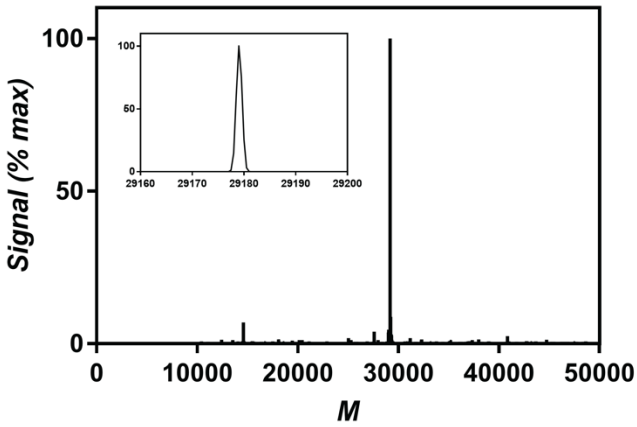
mCherry was expressed as a STEPL construct and loaded on the Ni-NTA column. Sortaggable peptide GGGG-P∧R-N_3 (Method S8) was conjugated onto mCherry by STEPL (Method S10). Protein purity was confirmed by LC-MS: calculated [M], 29079.0; observed 29075.5.

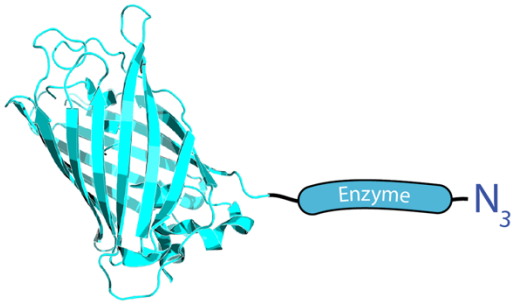




Method S23: Synthesis of Enzyme-Responsive mCerulean Pendant (mCerulean-E-N_3)

mCerulean was expressed as a STEPL construct and loaded on the Ni-NTA column. Sortaggable peptide GGGG-E-N_3 (Method S1) was conjugated onto mCerulean by STEPL (Method S10). Protein purity was confirmed by LC-MS: calculated [M], 29179.7; observed 29179.5.





Method S24: Relating Protein Concentration to Fluorescence

The EGFP calibration curve was obtained for peptide-protein conjugate (EGFP-E-N_3) dissolved in MMP buffer (50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, pH = 7.5). Fluorescence measurements ($\lambda_{excitation}$ = 475 nm, $\lambda_{emission}$ = 510 nm) were performed in triplicate.

The mCherry calibration curve was obtained for peptide-protein conjugate (mCherry-P-N_3) dissolved in MMP buffer. Fluorescence measurements ($\lambda_{excitation}$ = 575 nm, $\lambda_{emission}$ = 610 nm) were performed in triplicate.

The mCerulean calibration curve was obtained for peptide-protein conjugate (mCerulean-E-N_3) dissolved in MMP buffer. Fluorescence measurements ($\lambda_{excitation}$ = 433 nm, $\lambda_{emission}$ = 475 nm) were performed in triplicate.

In all cases, a linear relationship between concentration and fluorescence was observed over the measured range.

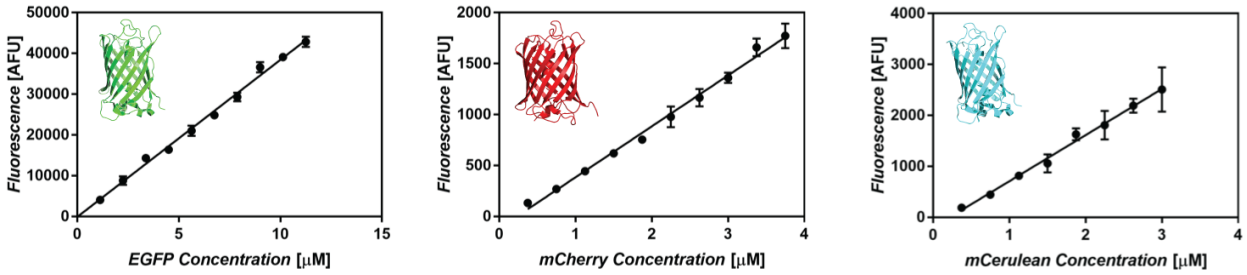
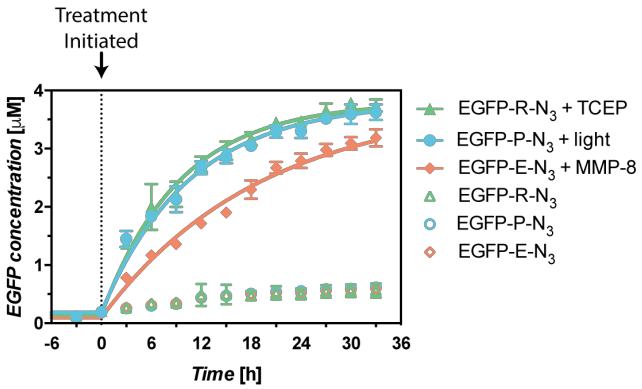


Figure S1: Time-Course Release of YES-gated EGFP Pendants to Single Input Treatments



Hydrogels (10 μL) were formulated from PEG-tetraBCN (M_n ~ 20,000 Da, 2 mM), N_3-PEG-N_3 (M_n ~ 3,500 Da, 4 mM), and either EGFP-R-N_3, EGFP-E-N_3, or EGFP-P-N_3 (50 μM each) in MMP buffer (50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, pH = 7.5). The protein and PEG-tetraBCN were pre-reacted for 4 hours prior to mixing with N_3-PEG-N_3. Immediately upon combination of all components in microcentrifuge tubes (0.6 mL), the solution was vortexed and centrifuged, resulting in cone-shaped gel solutions that were several mm tall. After one hour, formed hydrogels were washed for 12 hours in MMP buffer to remove unconjugated protein.

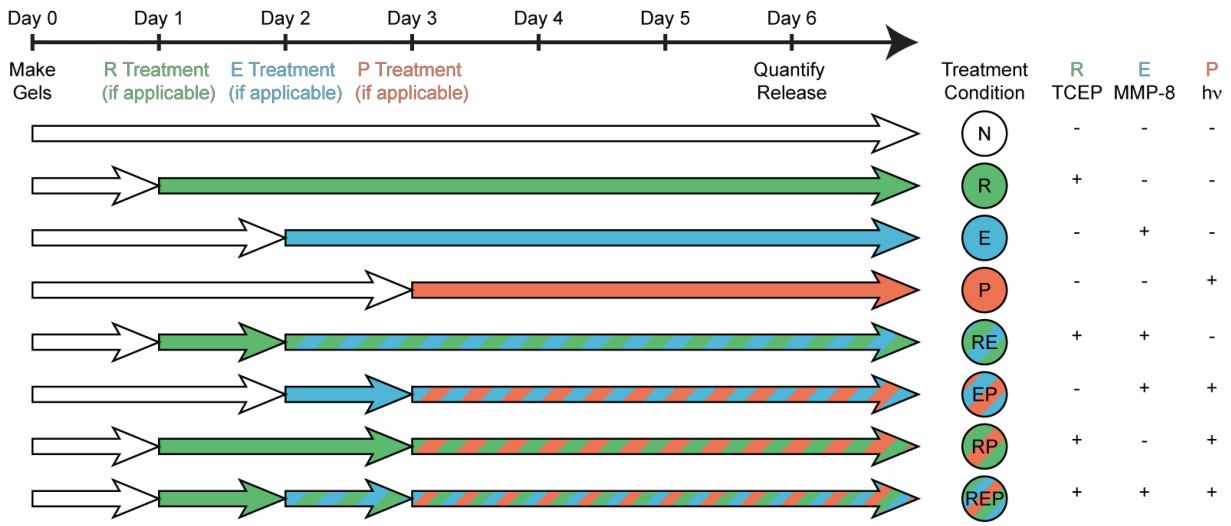
All treatments were performed at 4 °C in MMP buffer (100 μL). Supernatant fluorescence corresponding to released EGFP (λ_{ex} = 475 nm, λ_{em} = 510 nm) was measured every three hours for a total of 36 hours and used to calculate protein concentration (Method S24). Dashed line in plot indicates when treatments were initiated (time = 0).

Samples receiving the reductive input were treated with tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl, 2 μL, 100 mM in MMP buffer).

Samples receiving the enzyme input were treated with MMP-8 (2.5 μL, 0.4 mg mL^{-1} in MMP buffer).

Samples receiving the light input were exposed to UV light (λ = 365 nm, 20 mW cm^{-2} incident light, 10 minute exposure).

Method S25: Logic-based Protein Release in Response to Sequential Stimuli



Hydrogels (10 µL) were formulated from PEG-tetraBCN (M_n ~ 20,000 Da, 2 mM), N_3-PEG-N_3 (M_n ~ 3,500 Da, 4 mM), and sortagged azide-functionalized logical proteins (0.1 mM total, equal concentration of all proteins) in MMP buffer (50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, pH = 7.5). The protein and PEG-tetraBCN were pre-reacted for 4 hours prior to mixing with N_3-PEG-N_3. Immediately upon combination of all components in microcentrifuge tubes (0.6 mL), the solution was vortexed and centrifuged, resulting in cone-shaped gel solutions that were several mm tall. After one hour, formed hydrogels were washed for 24 hours in MMP buffer to remove unconjugated protein.

All treatments were performed at 4 °C in MMP buffer (100 µL).

Samples receiving the reductive input were treated with tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl, 2 µL, 100 mM in MMP buffer) and incubated overnight (22 hr). 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). Samples not receiving reductive input were maintained in MMP buffer.

Samples receiving the enzyme input were subsequently treated with MMP-8 (2.5 µL, 0.4 mg mL^{-1} in MMP buffer) and all samples were incubated (20 hr).

Samples receiving the light input were subsequently exposed to UV light (λ = 365 nm, 20 mW cm^{-2} incident light, 10 minute exposure) prior to incubation (72 hr).

After 6 total days of experimental treatment, protein release was quantified by measuring the fluorescence corresponding to EGFP (λ_{ex} = 475 nm, λ_{em} = 510 nm), mCherry (λ_{ex} = 575 nm, λ_{em} = 610 nm), and mCerulean (λ_{ex} = 433 nm, λ_{em} = 475 nm) in the supernatant.

Supplementary References

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