Protein-Polymer Conjugation *via* Ligand Affinity and Photoactivation of Glutathione *S*-Transferase

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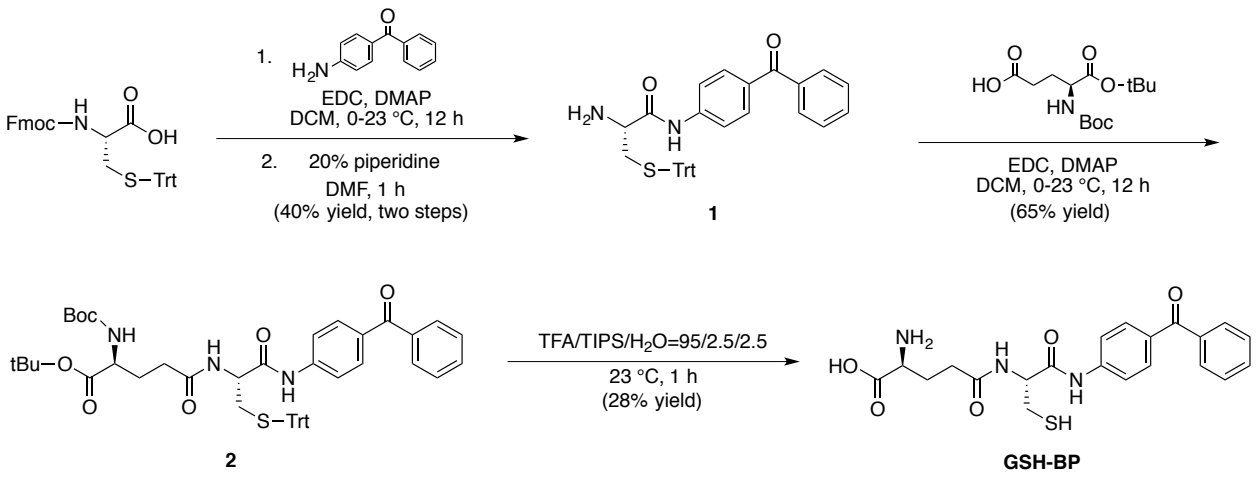
Supporting Information

Scheme S1. Synthesis of GSH-BP.



Synthesis of 1. In a round bottom flask over ice, Fmoc-Cys(Trt)-OH (1.63 g, 2.79 mmol) was dissolved in anhydrous DCM (5 mL) and stirred to dissolve. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, 0.97 g, 5.07 mmol) was then added to the reaction mixture. In a separate flask, 4-aminobenzophenone (4-ABP, 0.51 g, 2.58 mmol) was dissolved in anhydrous DCM (5 mL). After keeping in an ice bath for 20 min, the 4-ABP solution was added dropwise to the reaction flask. 4-Dimethylaminopyridine (DMAP, 61.4 mg, 0.5 mmol) was then added, and the reaction was stirred from 0 °C to 23 °C for 12 h. The completion of the reaction was confirmed by thin layer chromatography (TLC, EtOAc:Hexanes=1:2, stained with ninhydrin). The crude mixture was washed with saturated $NaHCO_3$ three times. The organic layer was then dried over $MgSO_4$ and concentrated *in vacuo*. 20% piperidine in DMF (11 mL) was added to the crude solid, and allowed to stir at 23 °C for 1 h. The solvent was removed *in vacuo* at 40 °C. The crude product was purified via silica gel flash chromatography with EtOAc : Hexane = 1 : 2, then changed to DCM : MeOH = 95 : 5 to obtain **1** with 40% yield containing impurities, and was brought forward to the next step. ^{1}H NMR (500

MHz in MeOD) δ: 7.81-7.14 (m, 24H), 3.44-3.41 (t, *J*= 6.15 Hz, 1H), 2.67-2.46 (m, 2H). ^{13}C NMR (500 MHz in MeOD) δ: 196.25, 172.80, 144.60, 142.37, 137.77, 132.63, 132.17, 131.10, 129.48, 129.35, 128.09, 127.61, 126.52, 118.84, 118.15, 116.98 66.44, 54.71, 36.85. DART-MS (± 1.0) observed (predicted): H^+ 543.2064 (543.2062).

Synthesis of Protected GSH-BP 2. In a round bottom flask over ice, Boc-Glu-OtBu (93.9 mg, 0.31 mmol) was dissolved in anhydrous DCM (2 mL) and stirred to dissolve. EDC·HCl (98.9 mg, 0.52 mmol) was then added to the reaction mixture. In a separate flask, **1** (140 mg, 0.26 mmol) was dissolved in anhydrous DCM (2 mL). After keeping over ice for 20 min, the solution of **1** was added dropwise to the reaction flask. DMAP (5.8 mg, 0.05 mmol) was then added, and the reaction was stirred from 0 °C to 23 °C for 24 h. The crude mixture was washed with saturated $NaHCO_3$ three times. The organic layer was then dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was purified using silica gel flash chromatography with EtOAc : Hexane = 1 : 2 to obtain 140 mg white solid with 65% yield containing slight amount of impurities. ^{1}H NMR (500 MHz in MeOD) δ: 7.81-7.16 (m, 24H), 4.49-4.46 (m, 1H), 4.03-3.84 (m, 1H), 2.71-2.49 (m, 2H), 2.41-2.26 (m, 2H), 2.16-1.77 (m, 2H), 1.52-1.36 (m, 18H). ^{13}C NMR (500 MHz in MeOD) δ: 197.51, 174.77, 173.14, 170.96, 158.09, 145.82, 143.80, 139.90, 134.29, 134.05, 133.53, 132.50, 132.39, 130.82, 130.68, 130.31, 129.47, 129.22, 129.03, 127.94, 120.47, 120.38, 114.07, 82.75, 80.54, 68.00, 55.34, 55.25, 54.64, 54.52, 34.89, 32.86, 28.73, 28.58, 28.24. IR: ν = 3292, 2972, 1703, 1652, 1595, 1522, 1445, 1366, 1310, 1278, 1251, 1149, 1047, 923, 845, 741. ESI-MS (± 1.0) observed (predicted): H^+ 828.3785 (828.3638).

Synthesis of GSH-BP. Protected GSH-BP **2** (41.5 mg, 0.05 mmol) was weighed in a glass vial. Under argon, trifluoroacetic acid (TFA, 1.33 mL), triisopropylsilane (TIPS, 35 μL, 0.17 mmol), and H_2O (35 μL) were added to the reaction with a final ratio of TFA/TIPS/H_2O = 95/2.5/2.5. Upon the addition of TFA, the solution immediately turned bright yellow. After the addition of TIPS, the color quickly turned pale yellow with the formation of a white precipitate. The reaction was allowed to stir for 1 h at 23 °C and then dried *in vacuo* to remove TFA. The solid was dissolved in 30% acetonitrile (ACN) and filtered for HPLC purification. HPLC chromatography was carried out with a gradient elution from 30% to 70% ACN in 30 min with a flow rate of 10 mL/min. The collected fractions were lyophilized to obtain 6.1 mg white solid with 28% yield. ^{1}H NMR (500 MHz in MeOD) δ: 7.79-7.51 (m, 9H), 4.67-4.58 (m, 1H), 3.70-3.58 (m, 1H), 3.04-2.83 (m, 2H), 2.56-2.53 (t, *J*= 7.13 Hz, 2H), 2.22-2.07 (m, 2H). ^{13}C NMR (500 MHz in MeOD) δ: 196.16, 173.88, 172.48, 169.69, 142.55, 137.73, 132.71, 132.17, 130.99, 129.41, 128.10, 119.06, 56.77, 54.20, 31.31, 26.36, 25.33. IR: ν = 3293, 2977, 1645, 1595, 1517, 1446, 1407, 1365, 1311, 1279, 1252, 1174, 1149, 1047, 938, 924, 851, 793, 741. ESI-MS (± 1.0) observed (predicted): H^+ 430.1450 (430.1392). UV-Vis (MeOH) λ_{max}= 295 nm.

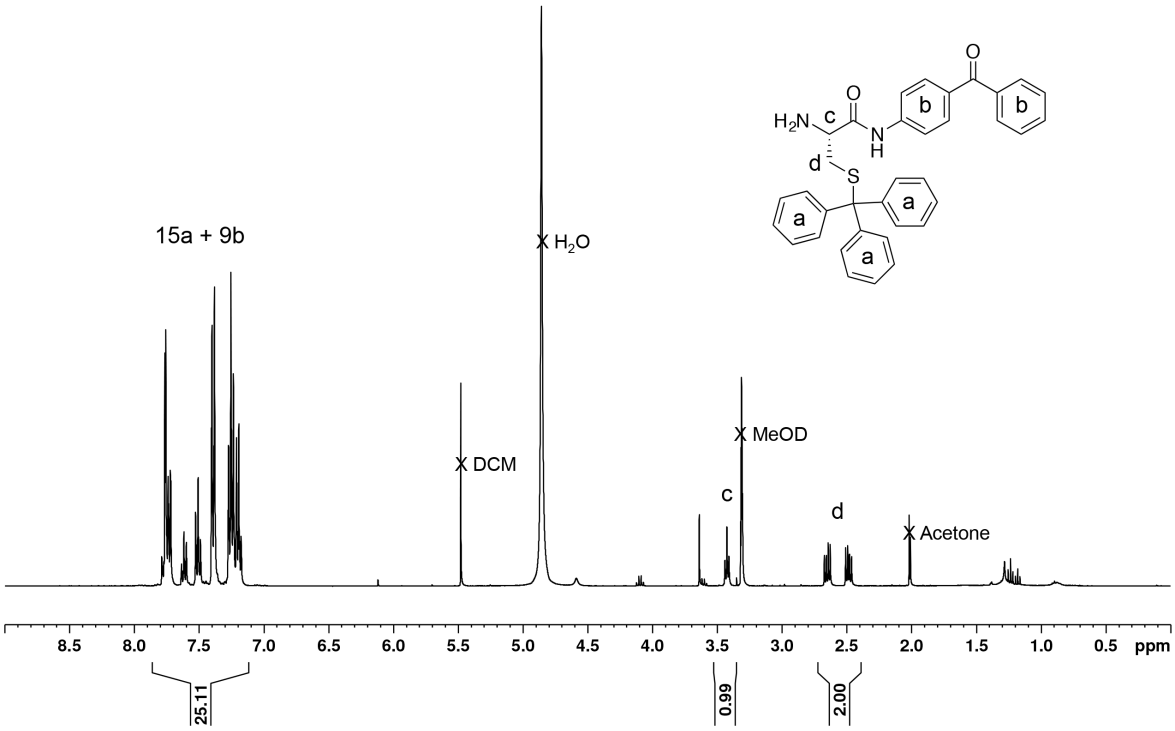


Figure S1. ^{1}H NMR spectrum of **1** (in MeOD).

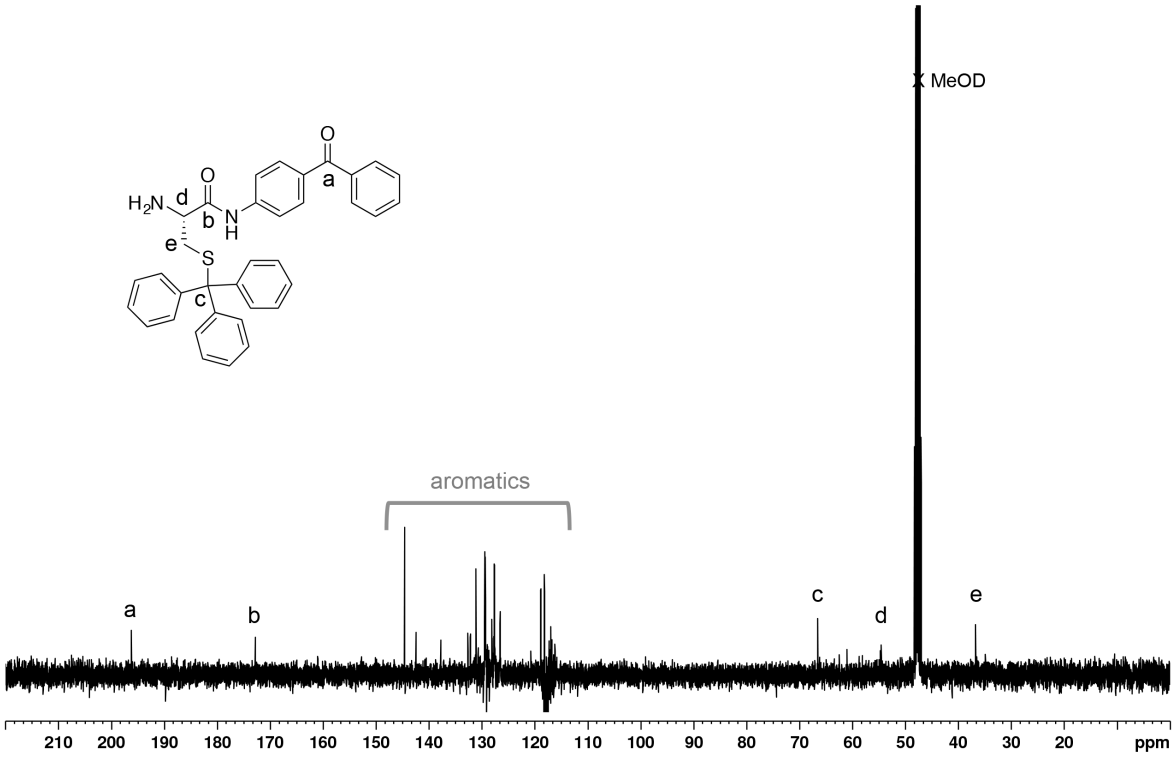


Figure S2. ^{13}C NMR spectrum of **1** (in MeOD).

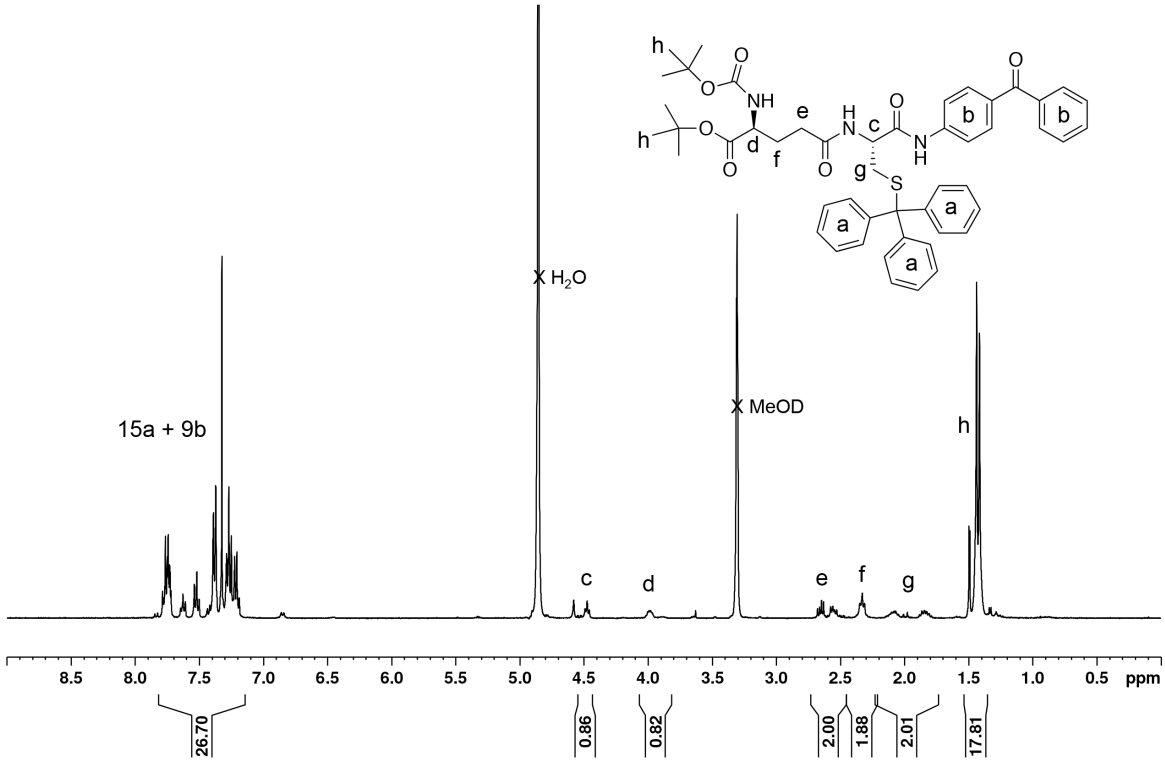


Figure S3. ^{1}H NMR spectrum of **2** (in MeOD).

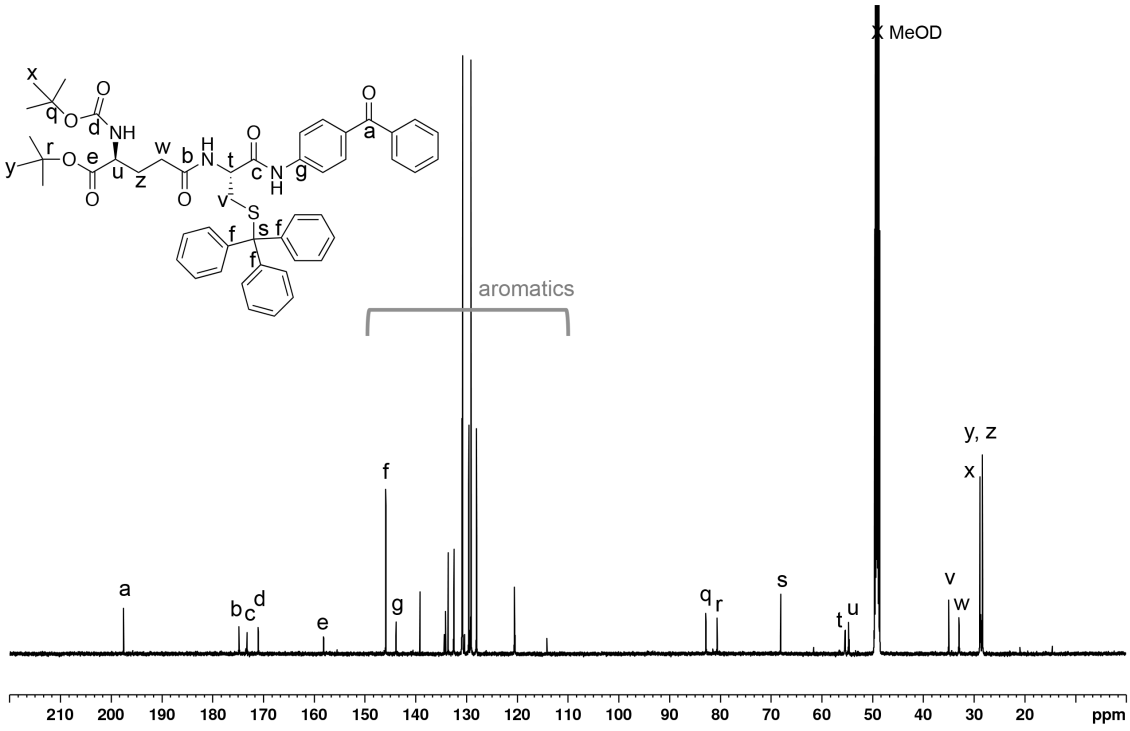


Figure S4. ^{13}C NMR spectrum of **2** (in MeOD).

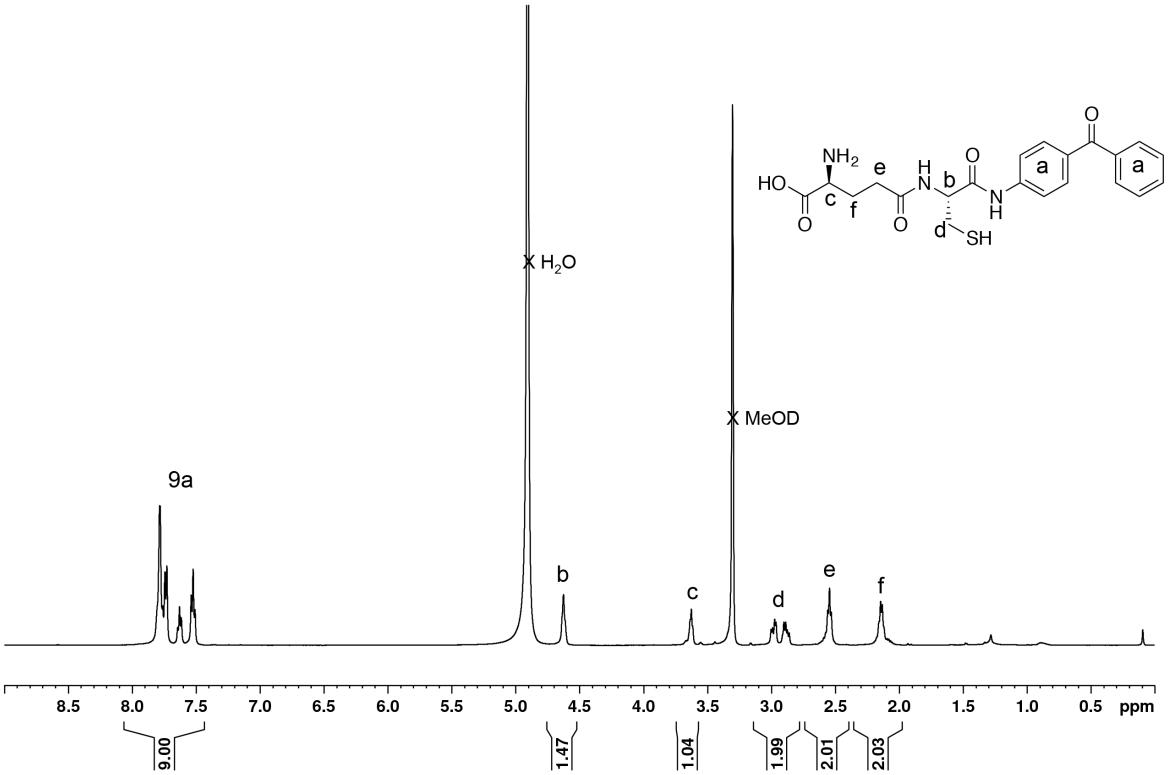


Figure S5. ^{1}H NMR spectrum of **3** (in MeOD).

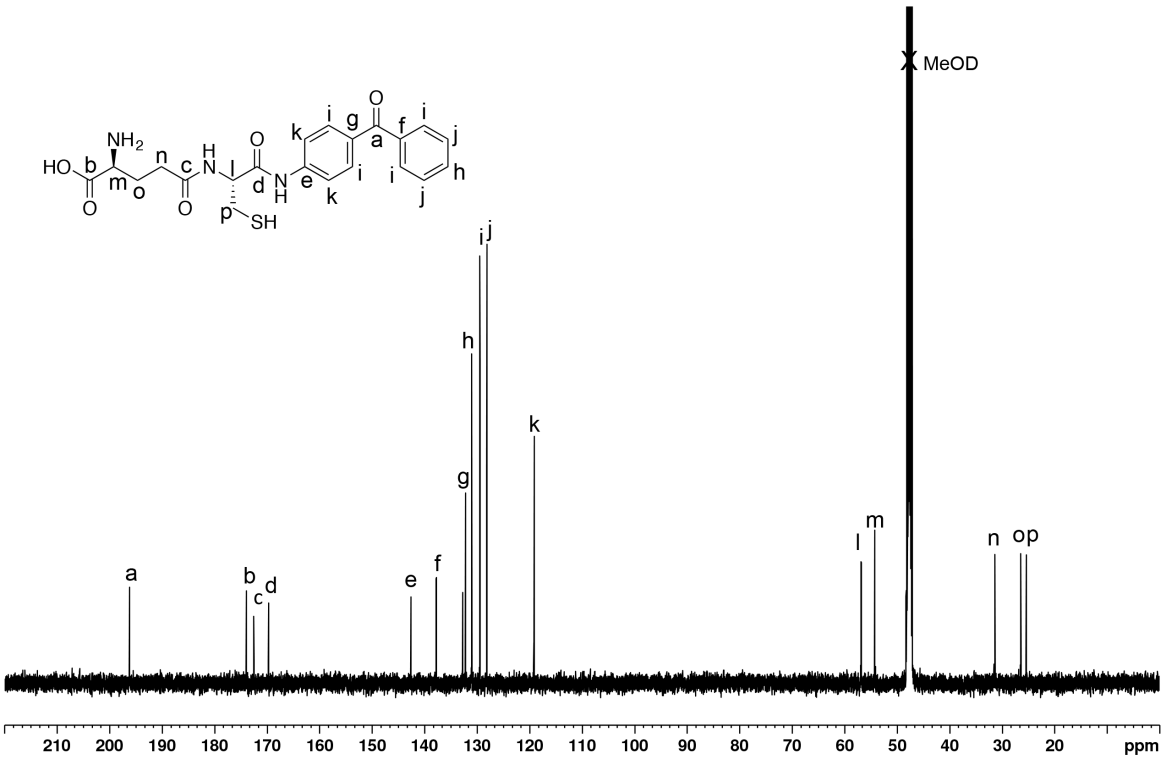
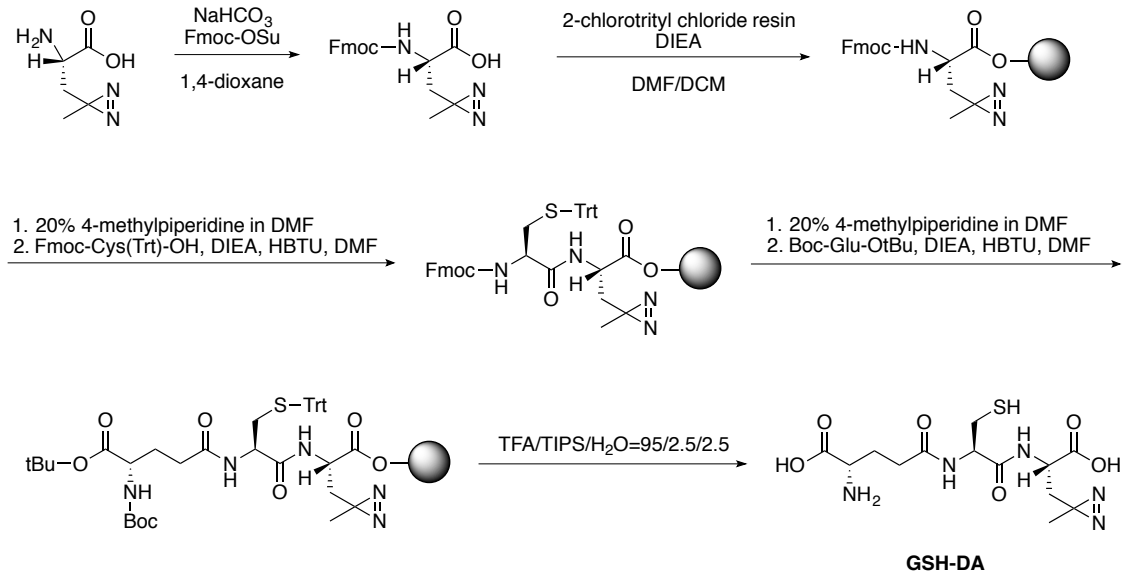


Figure S6. ^{13}C NMR spectrum of **3** (in MeOD).

Scheme S2. Synthesis of GSH-DA.



Synthesis of Fmoc-PhotoLeu. Fmoc-PhotoLeu was synthesized using a previously reported procedure.[1] Fmoc-OSu (118 mg, 0.35 mmol) was dissolved in 1,4-dioxane (2 mL). L-Photo-Leucine (50 mg, 0.35 mmol) and $NaHCO_3$ (47 mg, 0.56 mmol) were dissolved in H_2O (1.5 mL). 1,4-dioxane (2.5 mL) was added to this solution before adding Fmoc-OSu solution over 15 minutes. The reaction was then stirred for 26 hours at 23°C. HCl was added to the solution to acidify to pH 3 before extracting 3 times into EtOAc. Organic layers were combined and dried over $MgSO_4$ and concentrated *in vacuo*. Silica gel chromatography (5% MeOH in CH_2Cl_2 + 0.1% AcOH) yielded 107 mg (85%) as a beige solid. All steps were carried out in the dark. ^{1}H NMR (500 MHz in MeOD) δ: 7.81-7.63 (d, *J*= 7.5 Hz, 2H), 7.74-7.68 (dd, *J*= 4.6, 7.0 Hz, 2H), 7.41-7.34 (t, *J*= 7.5 Hz, 2H), 7.33-7.27 (dt, *J*= 1.05, 7.5 Hz, 2H), 4.42-4.30 (m, 2H), 4.28-4.22 (t, *J*= 7.15 Hz, 1H), 4.15-4.07 (dd, *J*= 4.3, 10.5 Hz, 1H), 2.03-1.95 (dd, *J*= 4.4, 14.95 Hz, 1H), 1.67-1.59 (dd, *J*= 10.6, 14.95 Hz, 1H), 1.06-1.00 and 0.93-0.89 (s, rotamer, 3H). ^{13}C NMR (500 MHz in MeOD) δ: 173.39, 157.04, 143.81, 141.18, 127.38, 126.76, 124.94, 119.50, 66.70, 50.10, 36.19, 23.60, 18.48.

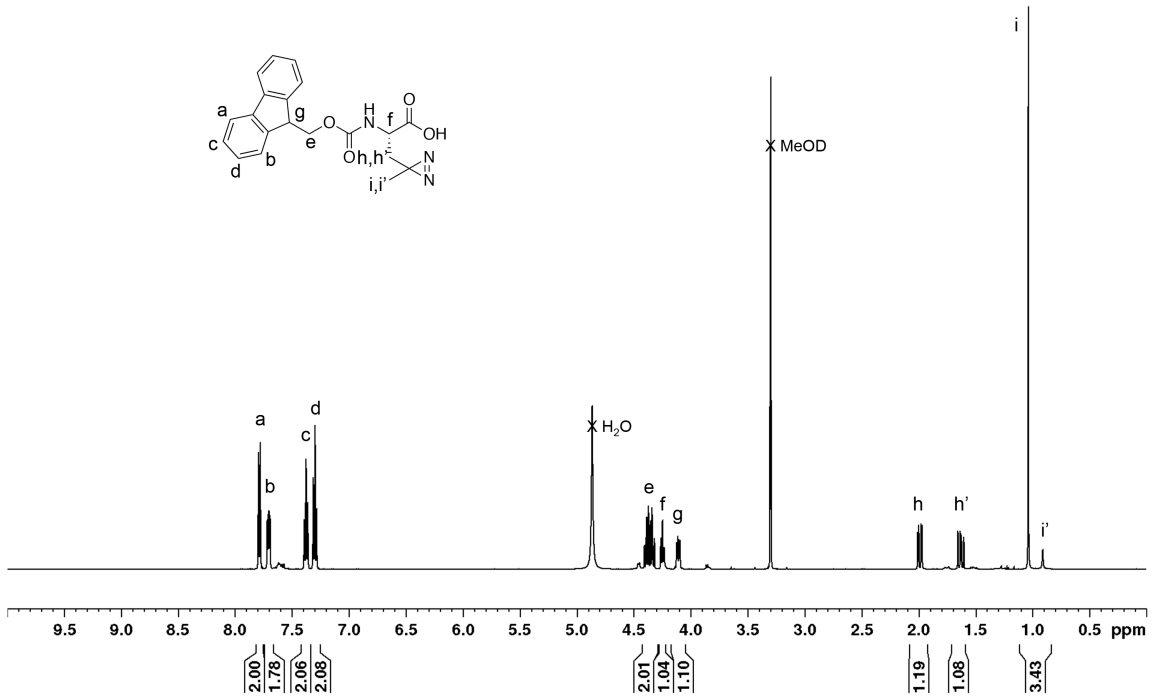


Figure S7. ^{1}H NMR spectrum of Fmoc-photo-Leucine (in MeOD).

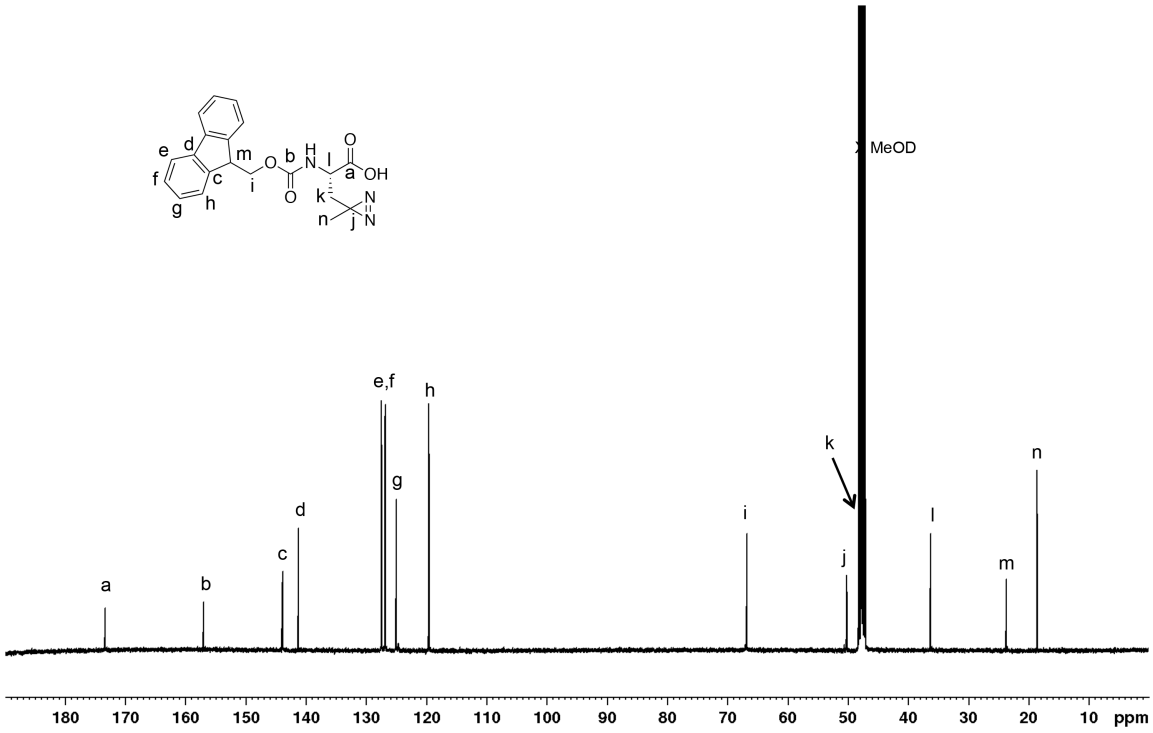


Figure S8. ^{13}C NMR spectrum of Fmoc-photo-Leucine (in MeOD). Note: It is anticipated that the carbon peak k is under the MeOD peak.

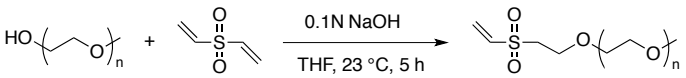
Synthesis of GSH-DA:

Resin Loading.[2] Fmoc-PhotoLeu (106 mg, 0.29 mmol) and DIEA (202 µL, 1.2 mmol) were dissolved in anhydrous DCM (2 mL). A small amount of DMF (200 µL) was added to dissolve the acid. The solution was added to the 2-chlorotrityl chloride resin (219 mg, .29 mmol) and mixed for 45 minutes in a peptide synthesis flask. The solution was drained and resin was rinsed with DCM/MeOH/DIEA (17:2:1), DCM, DMF, DCM, MeOH, DCM, DMF. The loaded resin was stored under argon until further use.

Amino Acid Coupling. 20% 4-methyl piperidine in DMF (10 mL) was added to resin in synthesis flask for 20 minutes to remove the Fmoc group. Solution was drained and resin was washed with DMF/DCM/MeOH/DCM/DMF. Fmoc-Cys(Trt)-OH (250 mg, 0.38 mmol) and HBTU (161 mg, 0.42 mmol) were dissolved in DMF (10 mL) and DIEA (148 μL, 1.5 mmol) was added before adding solution to resin and mixing for 3 hours at 23°C. Solution was drained and resin was washed again. The Fmoc group was removed as described previously. Boc-Glu-OtBu (129 mg, 0.41 mmol) and HBTU (161 mg, 0.42mmol) were dissolved in DMF (10 mL) before adding DIEA (148 μL, 1.5 mmol). This solution was added to resin and mixed at 23°C for 2 hours before draining solution and washing the resin.

Peptide Cleavage. The peptide was cleaved from the chlorotrityl resin using 95% TFA, 2.5% TIPS and 2.5% H_2O. The resin was mixed in cleavage cocktail for 45 minutes before draining the solution and rinsing the resin with DCM twice. The filtrate was concentrated *in vacuo* before precipitating in cold diethyl ether. The crude peptide was purified using HPLC with a C18 column with a gradient elution from 5% to 95% ACN in 30 min with a flow rate of 10 mL/min. The trityl group was subsequently deprotected using a solution of 95% TFA, 2.5% TIPS and 2.5% H_2O to yield 16.7 mg of pure GSH-DA as a white solid after precipitation in cold diethyl ether. ESI-MS (± 1.0) observed (predicted): H^+ 376.1289 (376.1291).

Scheme S3. Synthesis of VS-PEG.

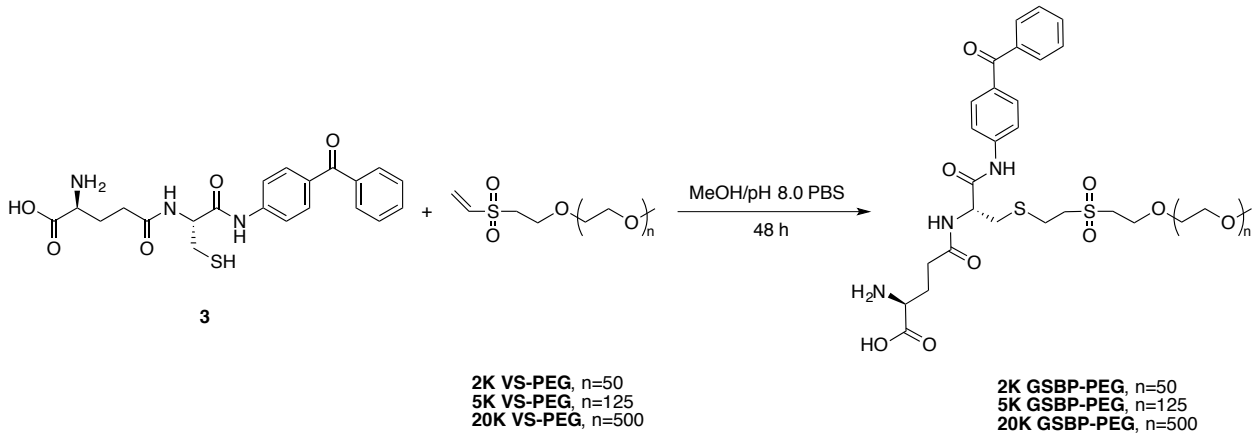


2K VS-PEG, n=50
5K VS-PEG, n=125
20K VS-PEG, n=500

Synthesis of VS-PEG. Here, we describe the synthesis of 2K VS-PEG. 5K and 20K VS-PEG were prepared according to this same method modified from a previous report.[3] To poly(ethylene glycol) methyl ether of average M_n ~2,000 (1.32 g, 0.66 mmol), THF (35 mL) was added and warmed to 30 °C. After the polymer had dissolved, the solution was cooled down to room temperature. Divinyl sulfone (0.2 mL, 1.98 mmol) was added, followed by addition of 0.1 N aqueous NaOH solution (2.4 mL). The reaction mixture was stirred at 23 °C for 5 h, then neutralized with 0.1 N HCl solution (2.4 mL) and filtered. The filtrate was concentrated *in vacuo* to remove most of the THF. The residual solution was diluted in approximately 10 mL of H_2O, washed with diethyl ether (x3 times), and extracted with chloroform (x3 times). The organic layer was then washed with H_2O, dried over $MgSO_4$, and concentrated *in vacuo*. The white solid was then dissolved in minimum amount of DCM, and precipitated into cold ether (50 mL x3). The precipitant was dried *in vacuo* to obtain 1.1 g of white powder with 79% yield. ^{1}H NMR of 2K VS-PEG (500 MHz in $CDCl_3$) (conversion: 89%) δ: 6.86-6.79 (dd, *J*= 9.95, 16.60 Hz, 1H), 6.42-6.36 (d, *J*= 16.65 Hz, 1H), 6.11-6.06 (d, *J*= 9.90 Hz, 1H), 4.00-3.22 (m, 200H), 3.40-3.37 (s, 3H). ^{1}H NMR of 5K VS-PEG (500 MHz in $CDCl_3$) (conversion: 85%) δ: 6.84-6.76 (dd, *J*= 9.90, 16.65 Hz, 1H), 6.40-6.34 (d, *J*= 16.65 Hz, 1H), 6.09-6.04 (d, *J*= 9.90 Hz, 1H), 3.96-3.20 (m, 500H), 3.37-3.34 (s, 3H). ^{1}H NMR of 20K VS-PEG (500 MHz in $CDCl_3$) (conversion: 90%) δ: 6.83-6.75 (dd, *J*= 10.00, 16.65 Hz, 1H), 6.38-

6.32 (d, *J*= 16.75 Hz, 1H), 6.07-6.03 (d, *J*= 10.00 Hz, 1H), 3.95-3.19 (m, 2000H), 3.36-3.33 (s, 3H).

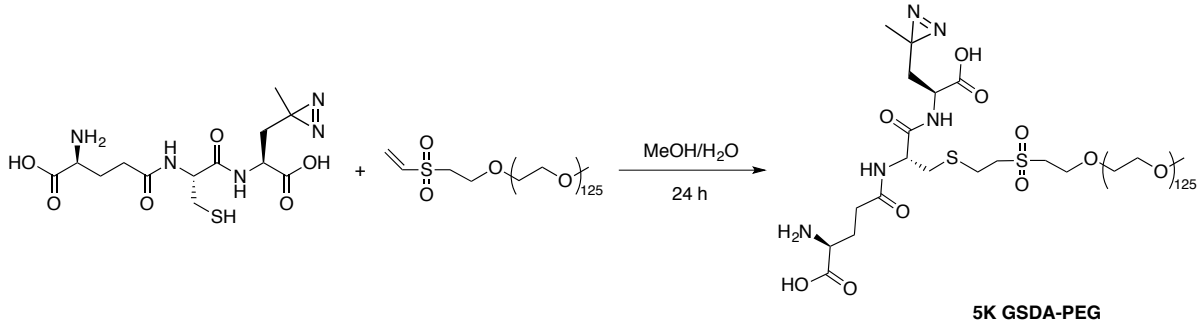
Scheme S4. Synthesis of GSBP-PEG.



Synthesis of GSBP-PEG. Here, we describe the synthesis of 2K GSBP-PEG. 5K and 20K GSBP-PEG were prepared according to the same method. Compound **3** (5.6 mg, 0.013 mmol) was dissolved in MeOH (0.2 mL). In a separate vial, VS-PEG (2K, 8.7 mg, 4.35×10^{-3} mmol) was dissolved in pH 8.0 phosphate buffer saline (0.2 mL). The two solutions were combined, and stirred under argon at 23 °C for 48 h. A small aliquot was taken for crude ^{1}H NMR in D_2O to confirm the disappearance of the vinyl peaks. The crude mixture was purified by HPLC with elution gradient of 60%-90% MeOH over 30 min. Alternatively, the crude mixture was purified by dialysis (MWCO 1,000) against H_2O for two days, followed by filtration to remove insoluble solids. The purified solution was then lyophilized to obtain 5.6 mg of white powder with 64% yield. ^{1}H NMR of 2K GSBP-PEG (500 MHz in D_2O) (conversion: 94%) δ: 7.88-7.49 (m, 9H), 4.76-4.69 (dd,

J= 6.20, 8.00 Hz, 1H), 3.96-3.37 (m, 200H), 3.36-3.34 (s, 3H), 3.20-3.15 (dd, *J*= 6.20, 13.75 Hz, 1H), 3.05-2.98 (m, 2H), 2.96-2.90 (dd, *J*= 8.20, 13.75 Hz, 1H), 2.58-2.52 (m, 2H), 2.18-2.10 (m, 2H). ^{1}H NMR of 5K GSBP-PEG (500 MHz in D_2O) (conversion: 100%) δ: 7.86-7.48 (m, 9H), 4.75-4.69 (dd, *J*= 6.20, 8.05 Hz, 1H), 3.96-3.40 (m, 500H), 3.36-3.34 (s, 3H), 3.21-3.14 (dd, *J*= 6.10, 13.70 Hz, 1H), 3.05-2.98 (m, 2H), 2.96-2.90 (dd, *J*= 8.20, 13.75 Hz, 1H), 2.58-2.52 (m, 2H), 2.17-2.09 (m, 2H). ^{1}H NMR of 20K GSBP-PEG (500 MHz in D_2O) (conversion: 95%) δ: 7.80-7.37 (m, 9H), 4.60-4.56 (m, 1H), 3.98-3.29 (m, 2000H), 3.27-3.25 (s, 3H), 3.08-2.86 (m, 4H), 2.48-2.33 (m, 2H), 2.08-2.96 (m, 2H).

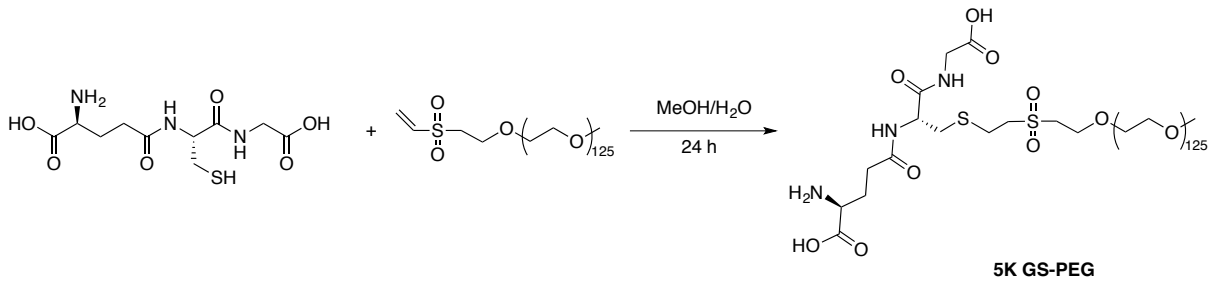
Scheme S5. Synthesis of 5K GSDA-PEG.



Synthesis of GSDA-PEG. GSH-DA (16.6 mg, 4.4×10^{-2} mmol) was dissolved in pH 8.0 phosphate buffer (1 mL). The solution was then added to VS-PEG (5K, 20.4 mg, 4.1×10^{-3} mmol), and the reaction mixture was stirred under Argon at 23 °C for 14 h. A small aliquot for crude ^{1}H NMR was taken in D_2O to confirm the disappearance of the vinyl peaks. The crude solution was concentrated via centrifugal filtration (0.5 mL, MWCO 3,000) and washed with H_2O for 5 times. The solution was then collected and

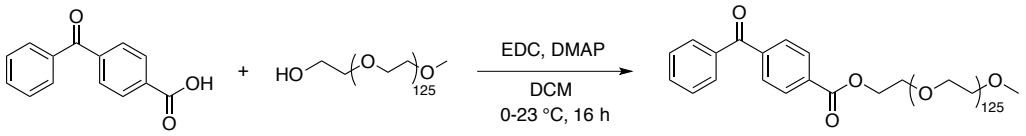
lyophilized. ^{1}H NMR (500 MHz in D_2O) (conversion: 75%) δ: 4.55-4.50 (dd, *J*= 5.05, 9.20 Hz, 1H), 4.08-4.02 (dd, *J*= 4.20, 9.50 Hz, 1H), 3.96-3.36 (m, 500H), 3.28-3.25 (s, 3H), 3.09-3.02 (dd, *J*= 5.00, 14.05 Hz, 1H), 2.97-2.89 (m, 2H), 2.88-2.81 (dd, *J*= 9.25, 14.05 Hz, 1H), 2.47-2.41 (m, 2H), 2.09-2.01 (m, 2H), 1.98-1.91 (dd, *J*= 4.20, 15.15 Hz, 1H), 1.54-1.46 (dd, *J*= 9.55, 15.15 Hz, 1H), 0.95-0.90 (s, 3H).

Scheme S6. Synthesis of 5K GS-PEG.



Synthesis of GS-PEG. Glutathione (153.7 mg, 0.5 mmol) was dissolved in pH 8.0, D-PBS (2 mL). The pH, which was around 3.0, was adjusted to 8.0 with 1 N NaOH and the total volume was diluted to 2 mL with additional phosphate buffer. The glutathione solution was added to VS-PEG (5K, 250 mg, 0.05 mmol), and the reaction mixture was stirred under argon at 23 °C for 24 h. A small aliquot for crude ^{1}H NMR was taken in D_2O to confirm the disappearance of the vinyl peaks. The crude was purified by dialysis (MWCO 1,000) against H_2O for two days, and then lyophilized. ^{1}H NMR of 5K GS-PEG (500 MHz in D_2O) (conversion: 86%) δ: 4.52-4.47 (dd, *J*= 5.10, 8.50 Hz, 1H), 3.90-3.39 (m, 500H), 3.27-3.23 (s, 3H), 3.05-2.97 (dd, *J*= 4.95, 14.10 Hz, 1H), 2.93-2.86 (m, 2H), 2.86-2.78 (dd, *J*= 8.65, 14.10 Hz, 1H), 2.47-2.35 (m, 2H), 2.08-1.99 (m, 2H).

Scheme S7. Synthesis of 5K BP-PEG.



5KBP-PEG

Synthesis of BP-PEG. In a round bottom flask over ice, 4-benzoylbenzoic acid (181 mg, 0.8 mmol) was dissolved in DCM (4 mL). EDC·HCl (230.1 mg, 1.2 mmol), poly(ethylene glycol) methyl ether of average M_n ~5,000 (200 mg, 0.04 mmol), and DMAP (24.4 mg, 0.2 mmol) were then added to the reaction mixture. The reaction mixture was allowed to stir from 0 °C to 23 °C for 23 h. The crude mixture was washed once with H_2O, twice with saturated $NaHCO_3$, and twice with brine. The organic layer was then dried over $MgSO_4$ and concentrated *in vacuo*. The solid was purified by dialysis (MWCO 1,000) against H_2O for two days, followed by 0.22 μm PTFE syringe filtration to remove insoluble solids. The purified solution was then lyophilized. ^{1}H NMR (500 MHz in D_2O) (conversion: 100%) δ: 8.12-7.46 (m, 9H), 4.47-4.42 (m, 2H), 3.85-3.40 (m, 500H), 3.29-3.24 (s, 3H).

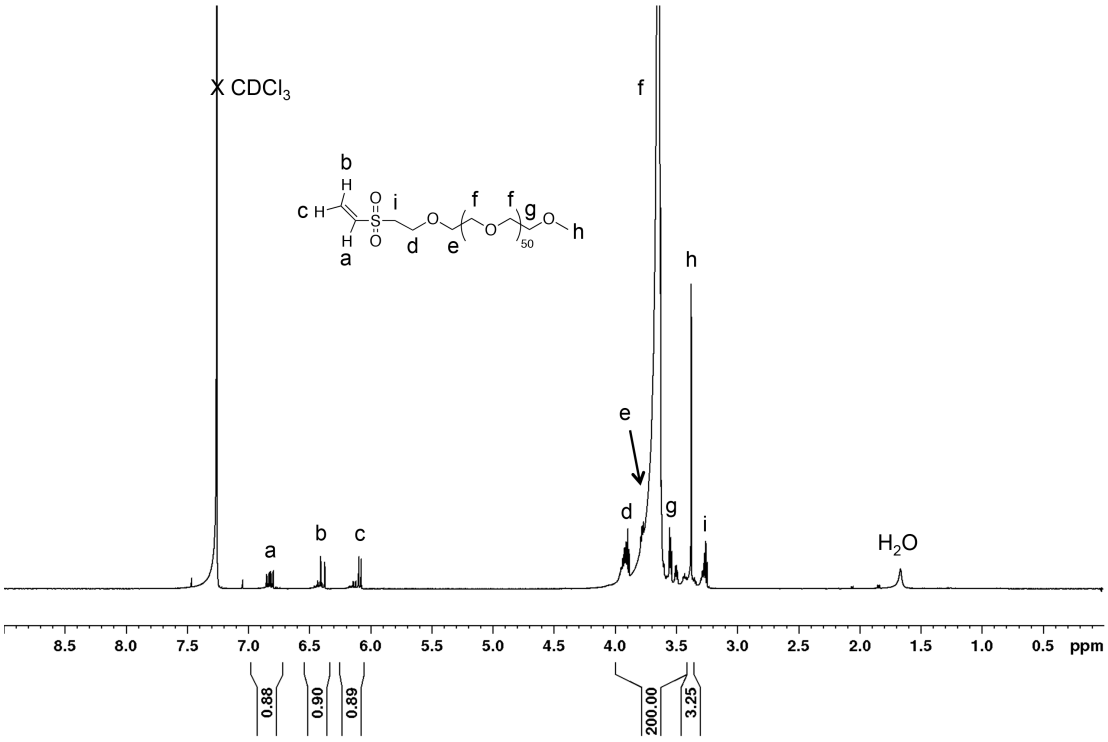


Figure S9. ^{1}H NMR spectrum of 2K VS-PEG (in $CDCl_3$).

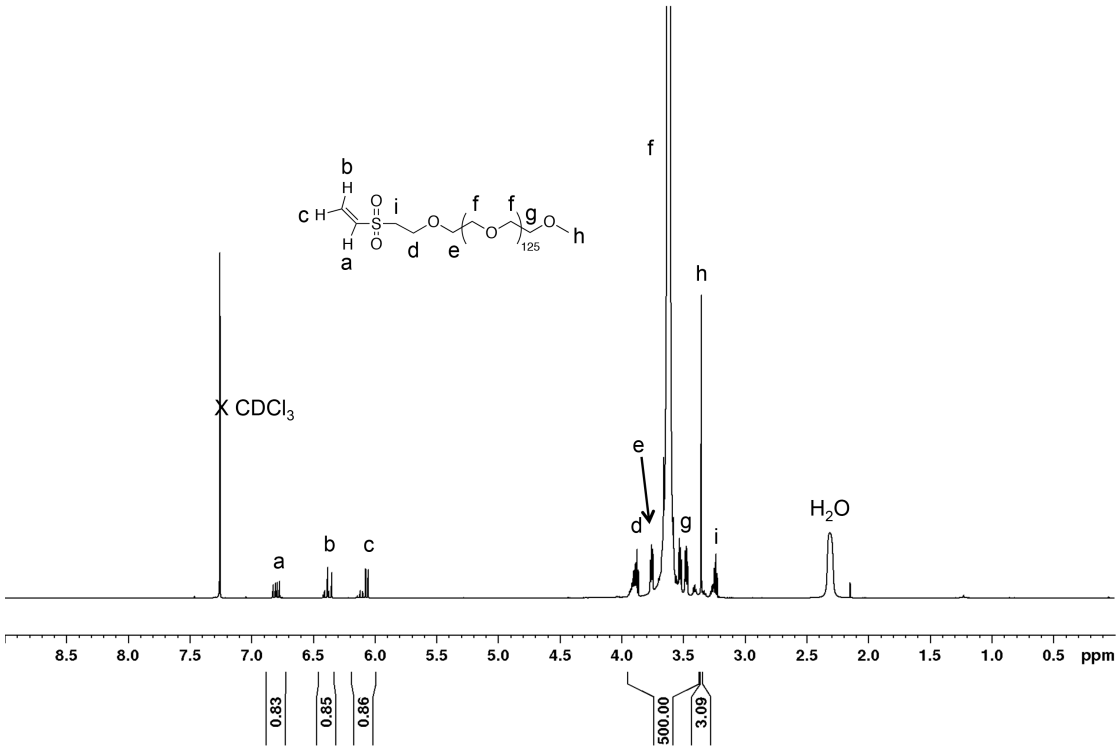


Figure S10. ^{1}H NMR spectrum of 5K VS-PEG (in $CDCl_3$).

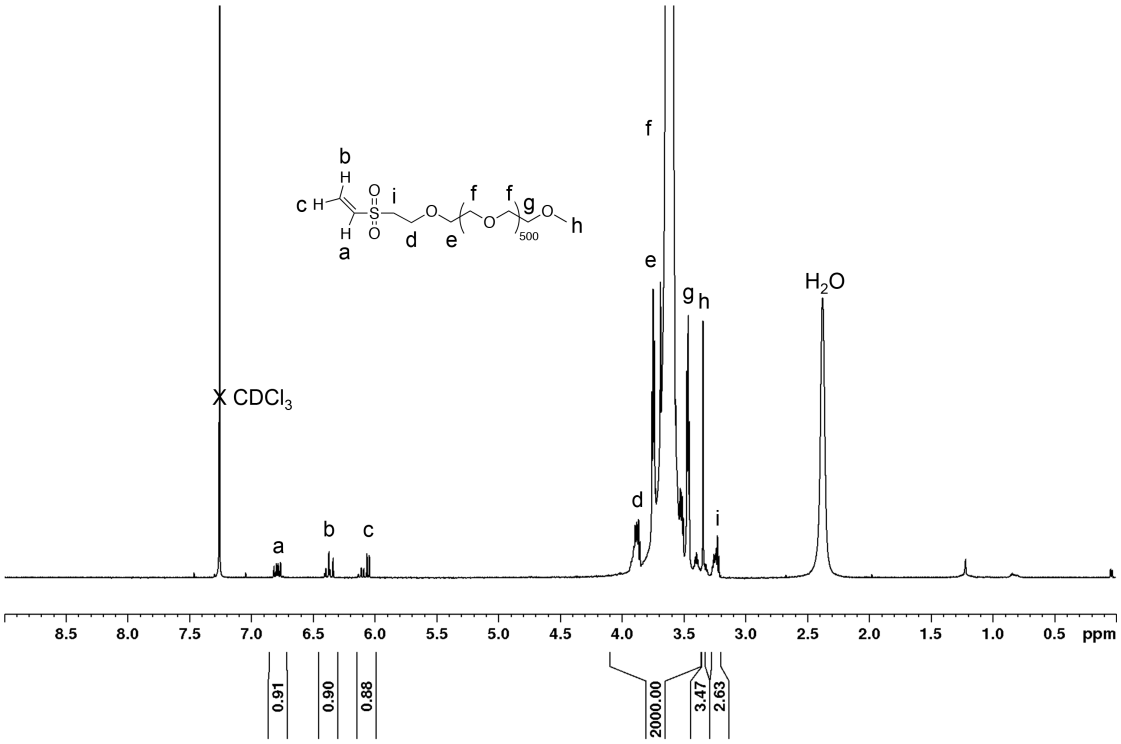


Figure S11. ^{1}H NMR spectrum of 20K VS-PEG (in $CDCl_3$).

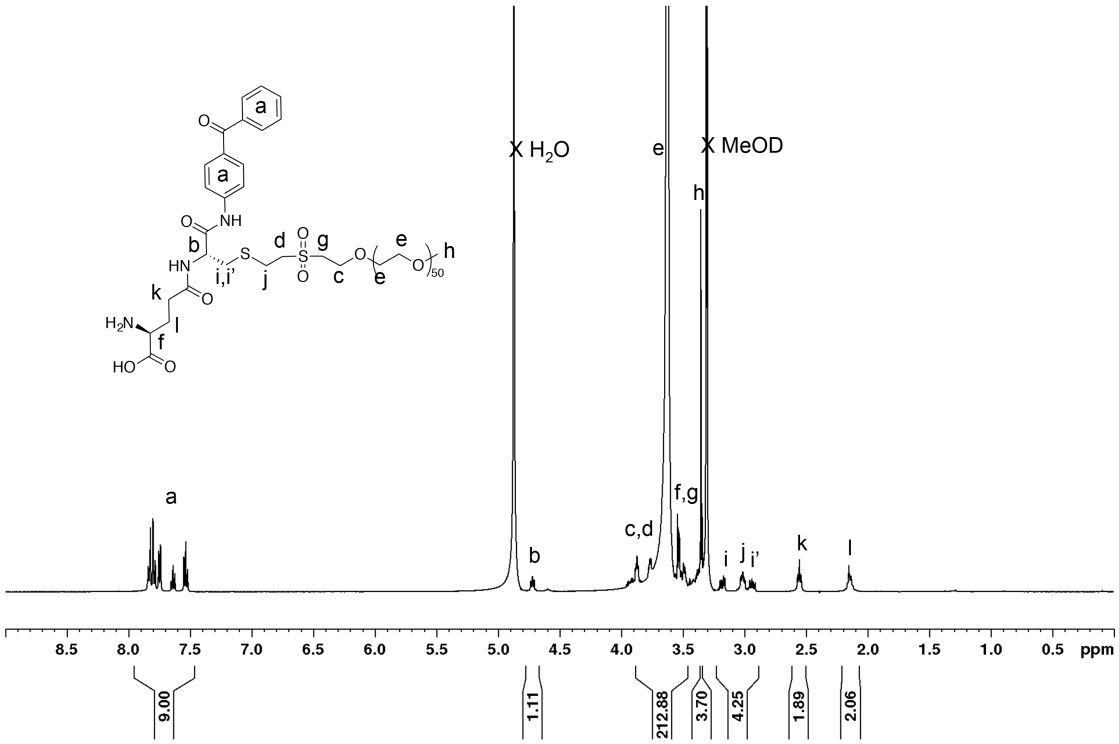


Figure S12. ^{1}H NMR spectrum of 2K GSBP-PEG (in MeOD).

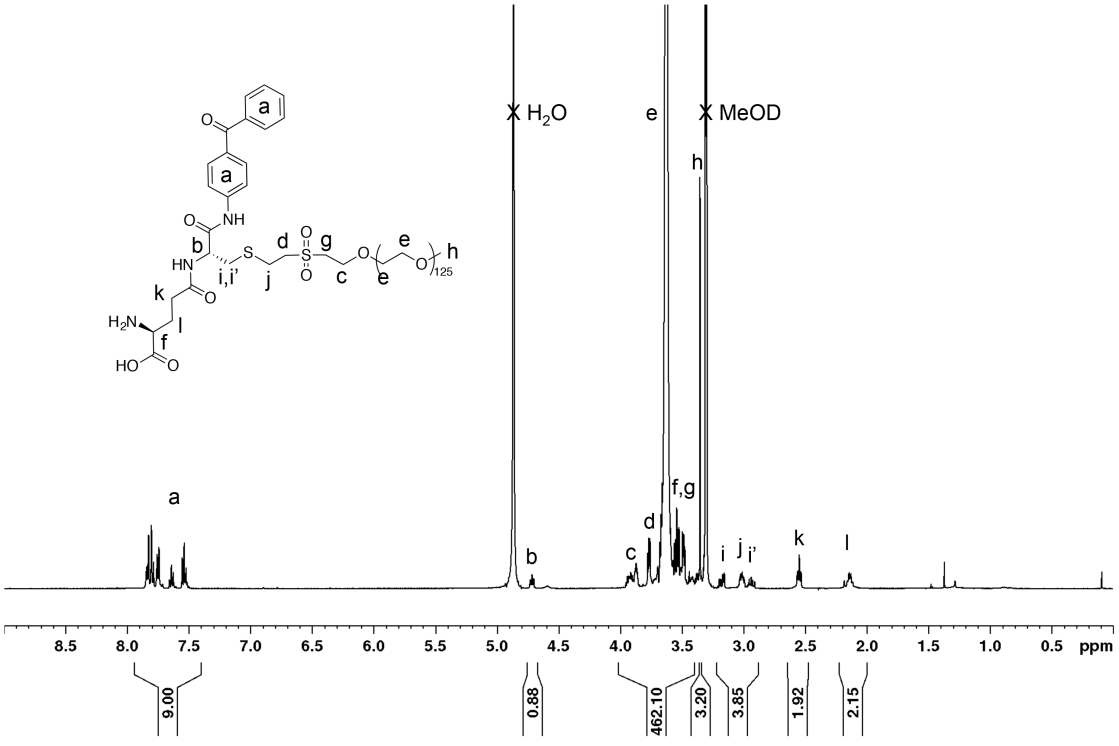


Figure S13. ^{1}H NMR spectrum of 5K GSBP-PEG (in $CDCl_3$).

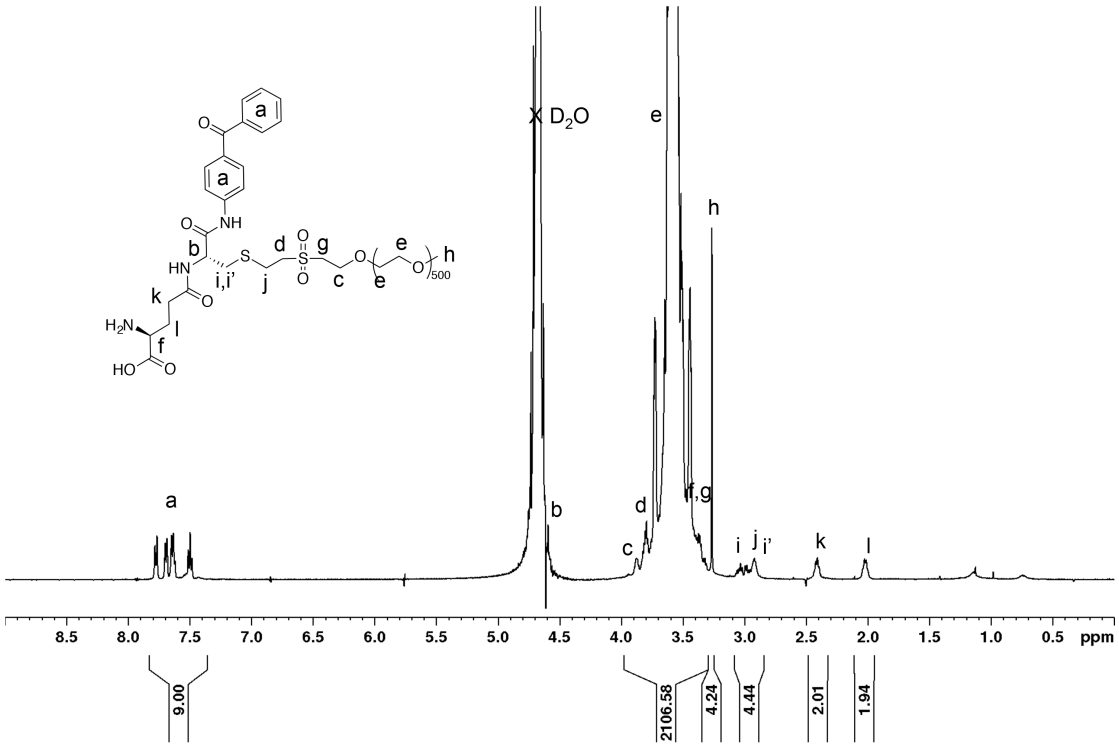


Figure S14. ^{1}H NMR spectrum of 20K GSBP-PEG (in MeOD).

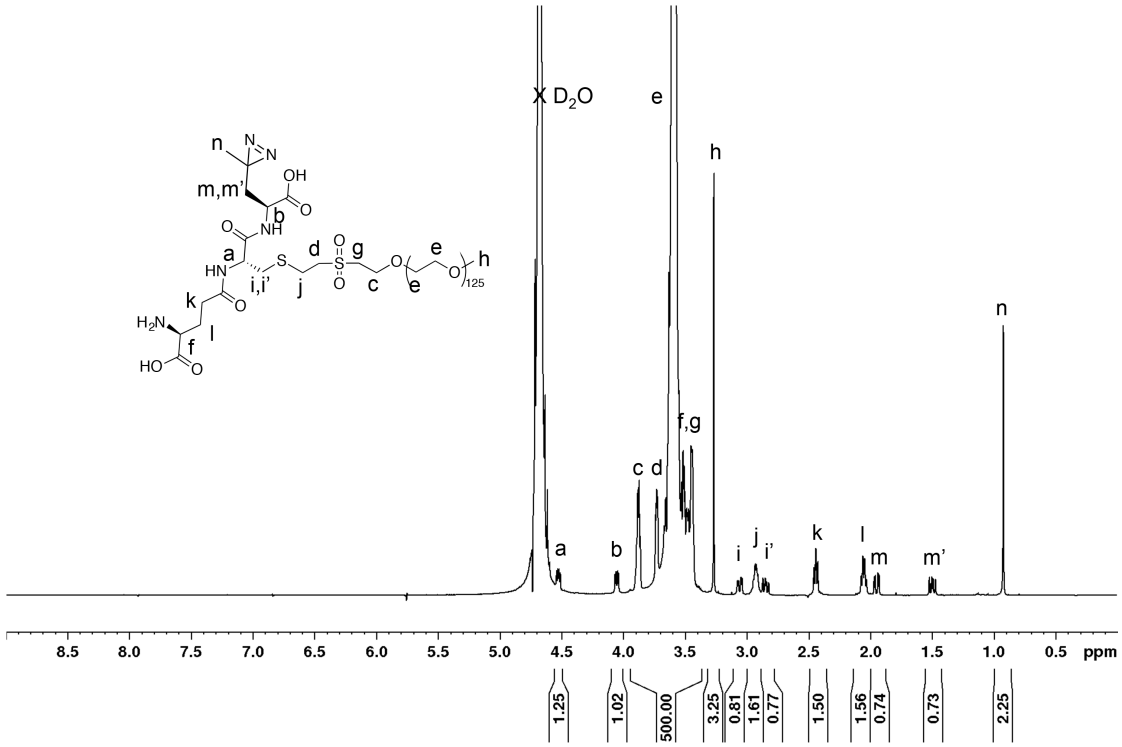


Figure S15. ^{1}H NMR spectrum of 5K GSDA-PEG (in D_2O).

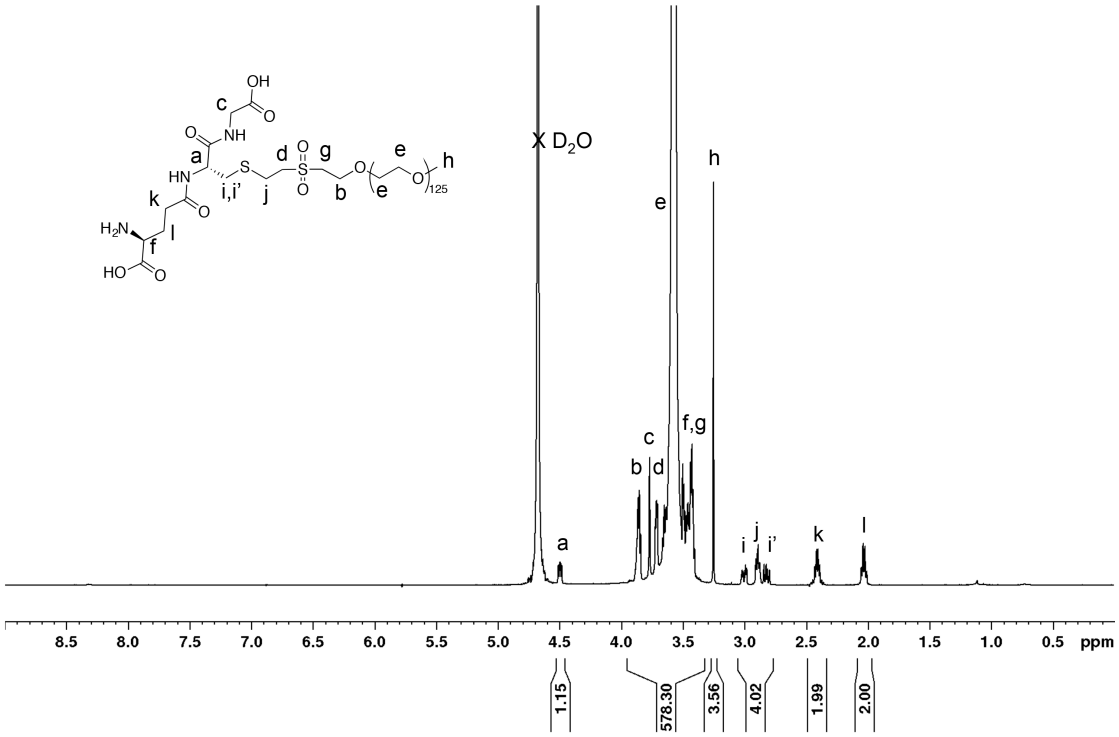


Figure S16. ^{1}H NMR spectrum of 5K GS-PEG (in D_2O).

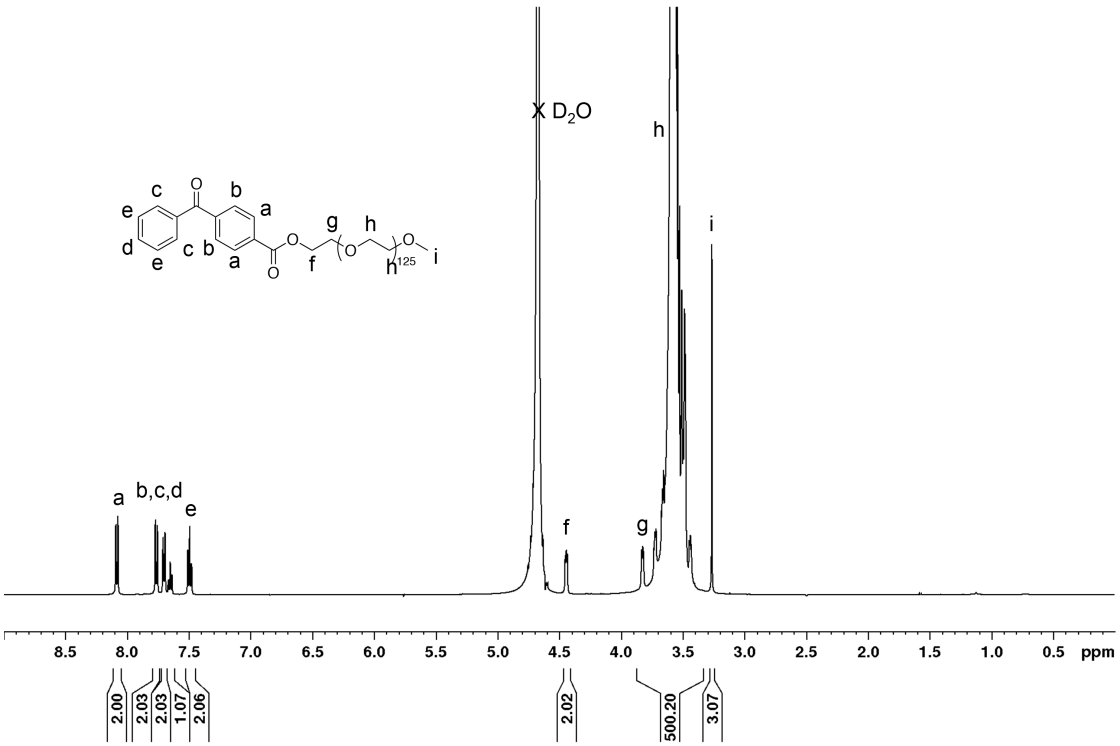


Figure S17. ^{1}H NMR spectrum of 5K BP-PEG (in D_2O).

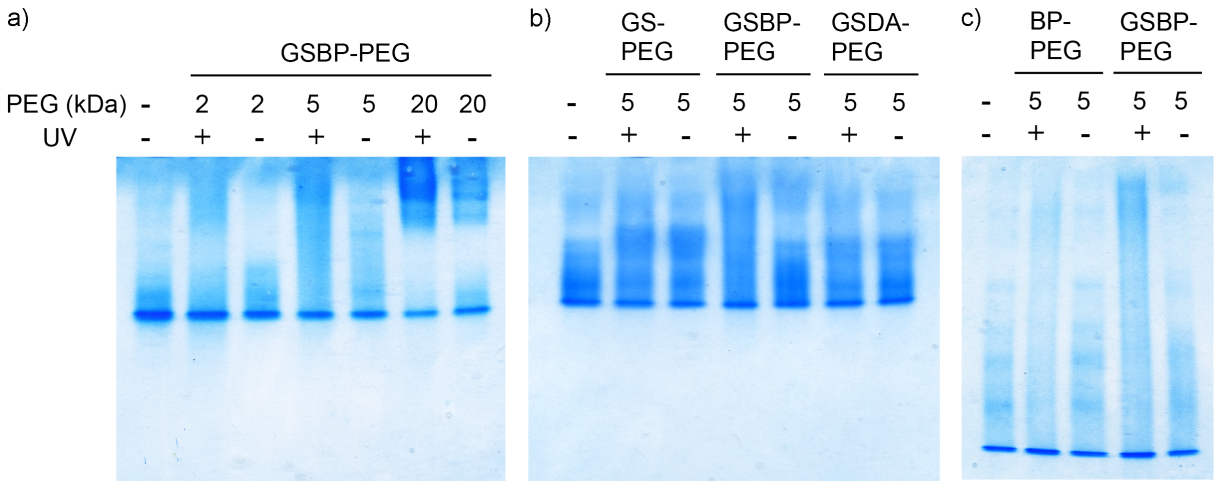


Figure S18. Native PAGE of a) GSBP-PEG conjugation to GST; b) comparison between GS-PEG, 5K GSBP-PEG, and GSDA-PEG; c) comparison between BP-PEG and 5K GSBP-PEG.

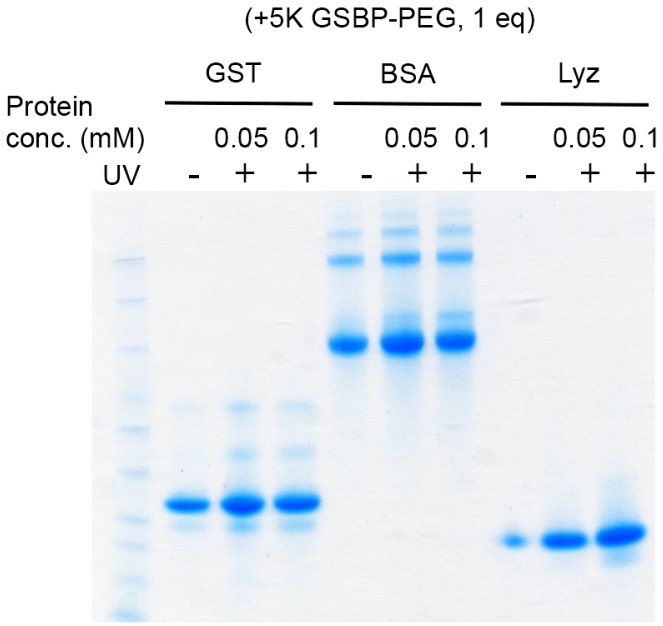


Figure S19. SDS-PAGE of the conjugation of 5K GSBP-PEG to GST, BSA, and Lyz at low concentrations. No conjugation is observed for Lyz.

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