Electronic Supplementary Material (ESI) for Polymer Chemistry.


# Synthesis of Disulfide-Bridging Trehalose Polymers for Antibody and Fab Conjugation Using a Bis-Sulfone ATRP Initiator.

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*Supporting Information*

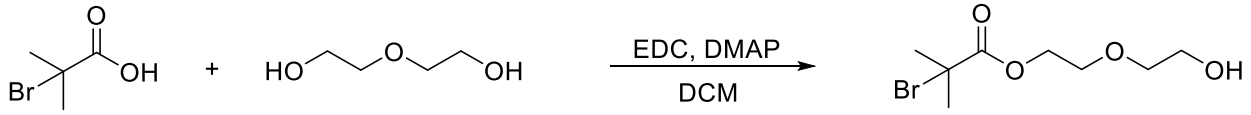
**Contents**

Experimental

All chemicals were purchased from Sigma-Aldrich and Fisher Scientific and were used without purification unless otherwise noted. For PEGMA, inhibitor was removed by passing through a plug of basic alumina. Herceptin® was purchased from the UCLA pharmacy. Fab specific (anti-Human IgG, Fab Specific−Peroxidase antibody produced in goat) and whole molecule (Anti-Human IgG (whole molecule)−Peroxidase antibody produced in rabbit) imaging antibodies were purchased from Sigma-Aldrich. MilliporeSigma™ Millex™ hydrophilic PTFE filters were used for stability studies. Trehalose was purchased from The Healthy Essential Management Corporation (Houston, TX) and was azeotropically dried with ethanol and kept under vacuum until use. Recombinant $Her_2$ antigen (Erb2 Fc Chimera) was purchased from R&D Solutions.

**Analytical Techniques**. Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker DRX 500 MHz, Bruker AV 500 MHz, and Bruker AV 600 MHz spectrometer. Proton NMR spectra were acquired with a relaxation delay of 2 s for small molecules and 10 s for polymers. Mass spectrometry for both proteins and small molecules was obtained on an Agilent Q-TOF 6530 LC/MS. For trehalose polymers, size exclusion chromatography (SEC) was conducted on a Malvern Viscotek GPCmax equipped with a TDA 305-040 Quadruple Detector Array (RI + Viscosity + LALS/RALS + UV) and 0.05 M sodium sulfate in water + 10 % methanol as eluent at a flow rate of 1.0 mL/min. For PEGMA polymerizations, SEC was conducted on a Shimadzu high performance liquid chromatography (HPLC) system with a refractive index detector (RID-10A), one Phenomenex Phenogel 10 µm guard column, and two Polymer Laboratories PLgel 5 µm mixed D columns in DMF eluent with LiBr (0.1 M) at 50 °C (flow rate: 0.80 mL/min). Trehalose monomer was purified by preparatory reverse phase high performance liquid chromatography (HPLC) on a Shimadzu system equipped with a UV detector using a Luna 5 µm C18 100A column (5 µm, 250 x 21.2 mm) with monitoring at $\lambda$ = 215 nm and 254 nm. Gradient solvent system (water:methanol = 90:10 to 40:60 over 20 min) was used as the mobile phase at a flow rate of 20 mL/min. For SDS PAGE, BioRad Any kD Mini-PROTEAN-TGX™ gels were used for the Fab and Fab conjugate while 4–15% Mini-PROTEAN® TGX™ gels were used for Herceptin and the Herceptin conjugate. SDS-PAGE protein standards were obtained from Bio-Rad (Precision Plus Protein Prestained Standards). Protein and conjugate purifications were conducted via FPLC on a Bio-Rad BioLogic DuoFlow chromatography system with UV monitoring at 280 nm (cation exchange column: 1mL GE Healthcare SPHP column, protein A: 1 mL MabSelect protein A GE Healthcare). Western blot was imaged with SuperSignal West Pico Chemiluminescent substrate paired with a CCD camera. Small molecule purification was done via flash chromatography and conducted on a Biotage Isolera One auto-column system.

**Ethylene glycol initiator synthesis.**




To an oven-dried scintillation vial with a stir bar, α-bromoisobutyric acetic acid (0.5 g, 2.99 mmol, 1 equivalent) and diethylene glycol (1.42 mL, 15 mmol, 5 equivalents) were added. The reagents were dissolved in 10 mL of dry DCM and cooled to 0 ºC. To the reaction mixture, *N*-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 0.69 g, 3.6 mmol, 1.2 equivalents) and 4-dimethylaminopyridine (DMAP, 0.037g, 0.299 mmol, 0.1 equivalents) was added. The reaction was allowed to warm to 22 ºC and proceed for 3 hrs. The product was purified via flash column chromatography with a 1:1 hexanes:ethyl acetate mobile phase ($R_f$ product ~ 0.3) to afford 492 mg (64.5 % yield) of product as a clear oil. $^1$H NMR (500 MHz, Chloroform-d) δ 4.37 – 4.32 (m, 2H), 3.78 – 3.70 (m, 4H), 3.64 – 3.59 (m, 2H), 1.94 (s, 6H). $^{13}$C NMR (126 MHz, Chloroform-*d*) δ 171.70, 72.36, 68.73, 64.96, 61.76, 55.68, 30.73. IR: $\nu$ = 3442, 2873, 1732, 1462 cm$^{-1}$. HRMS calculated for $C_8H_{15}BrO_4Na$ ([M+Na]$^+$) = 277.0051, observed = 276.9304.

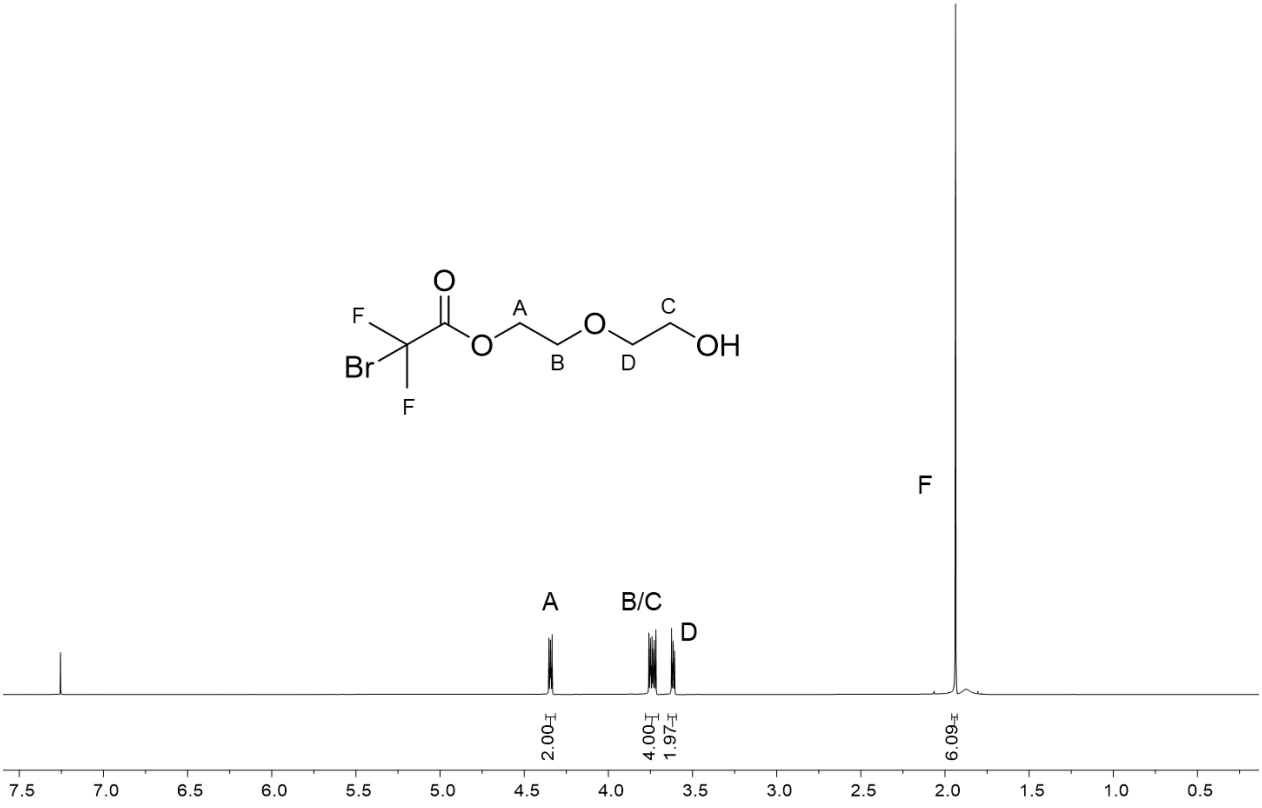



**Figure S1**. $^1$H NMR of ethylene glycol initiator ($CDCl_3$).

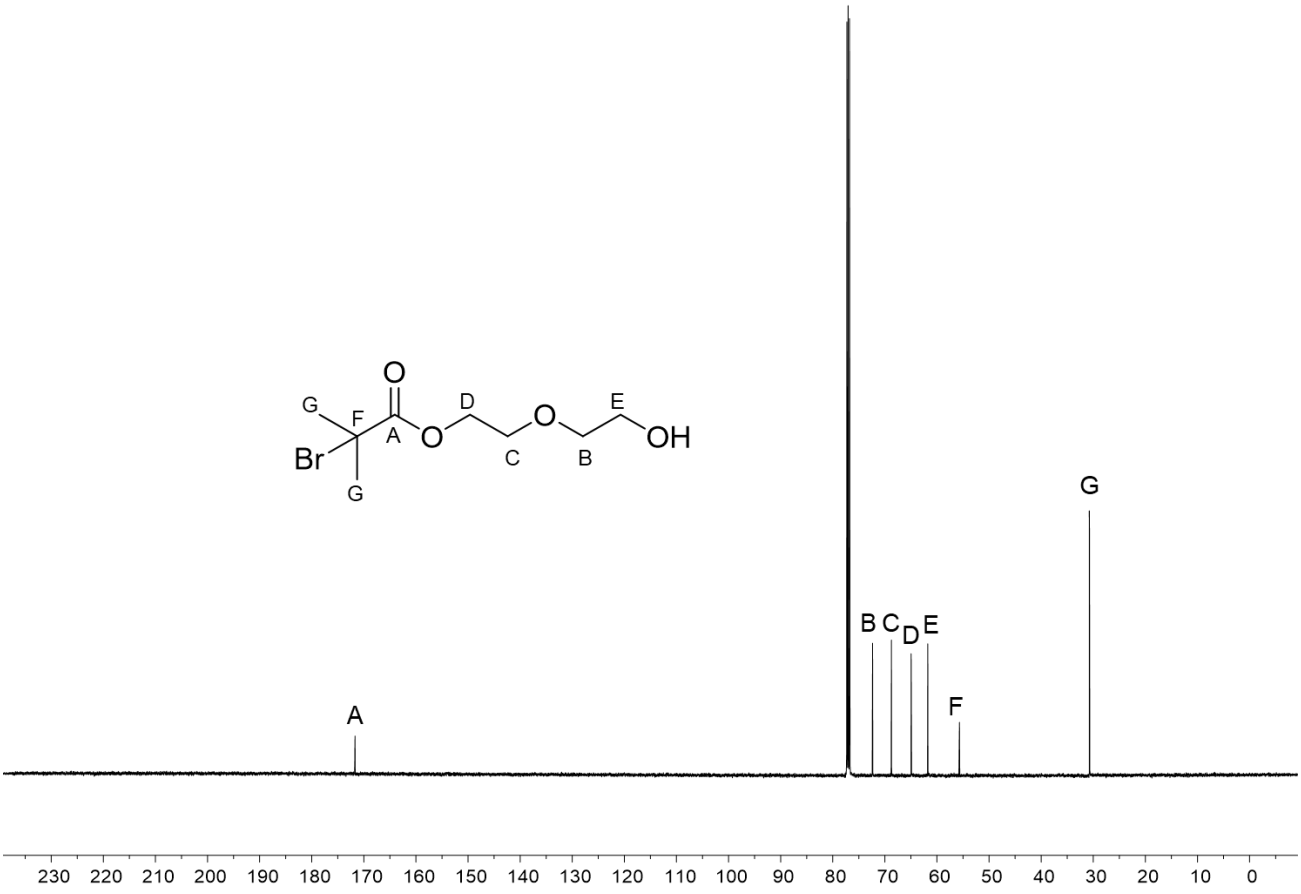
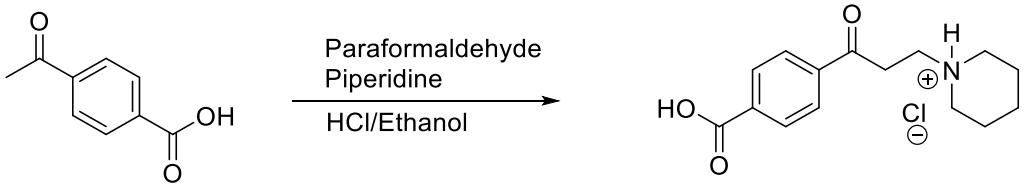



**Figure S2**. $^{13}C$ NMR of ethylene glycol initiator ($CDCl_3$)

**Mannich salt synthesis**.




Product was synthesized from modified procedure by Brocchini *et al.*[1] To a 250 mL round bottom flask with a stirbar absolute ethanol (25 mL), 4-acetyl benzoic acid (2 g, 12.2 mmol, 1 equivalent), piperidine (1.2 mL, 12.2 mmol, 1 equivalent), and paraformaldehyde (1.1 g, 36.5 mmol, 3 equivalents) were added. To this solution, concentrated HCl (1.13 mL of 37%, 13.7 mmol, 1.1 equivalents) was added. The reaction mixture was refluxed at 105 ºC for 12 h and then allowed to cool to 22 ºC. After cooling, 20 mL of acetone was added and the product was isolated via vacuum filtration to yield 4.47 g (61.6% yield) of a white powder. $^1H$ NMR (500 MHz, Deuterium Oxide) δ 8.03 – 7.95 (m, 4H), 3.57 (t, $J$ = 6.8 Hz, 2H), 3.52 – 3.39 (m, 4H), 2.91 (td, $J$ = 12.5, 3.1 Hz, 2H), 1.85 (dt, $J$ = 15.3, 3.6 Hz, 2H), 1.76 – 1.55 (m, 3H), 1.39 (qt, $J$ = 11.9, 3.7 Hz, 1H). $^{13}C$ NMR

(126 MHz, Deuterium Oxide) δ 199.12, 170.43, 138.60, 135.75, 129.77, 128.20, 53.55, 51.60, 33.10, 22.67, 20.96. HRMS calculated for $C_{15}H_{19}NO_3$ ([M+H]$^+$) = 262.1443, observed =262.1348.

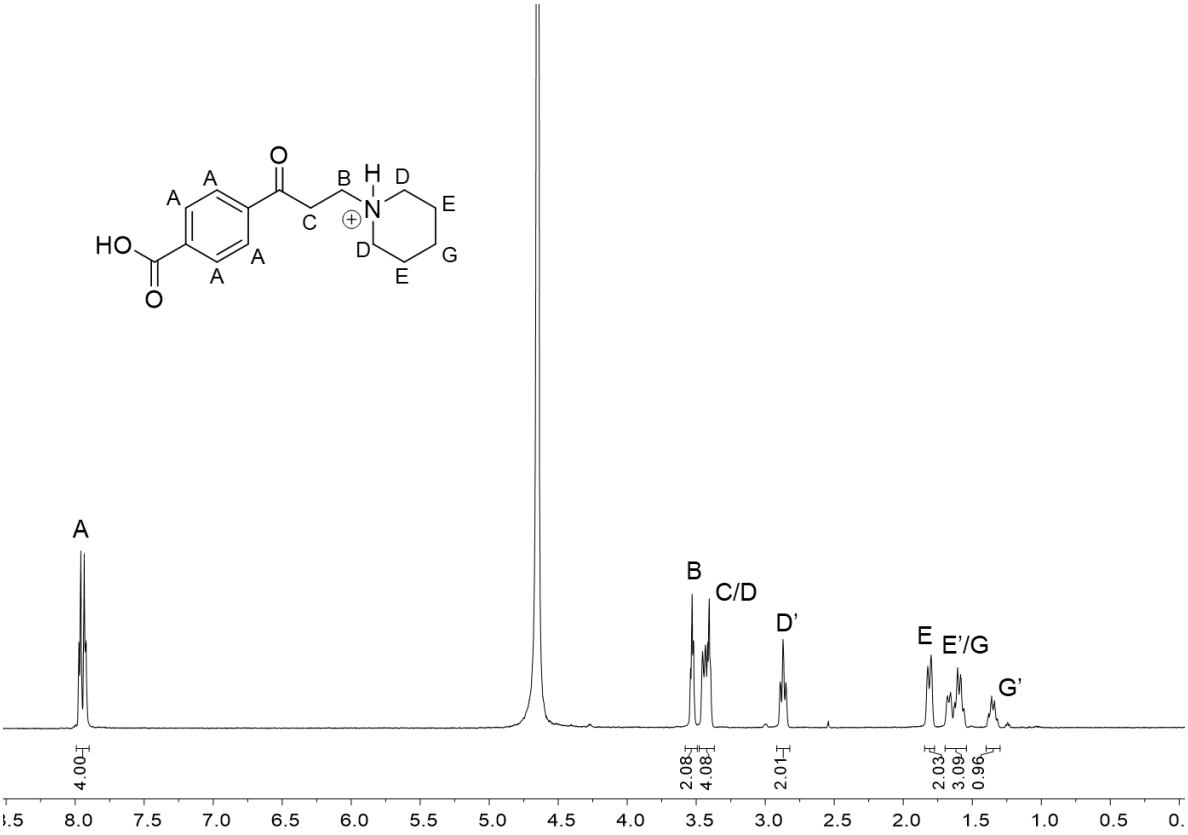



Figure S3. $^1$H NMR of mannich salt (D2O)

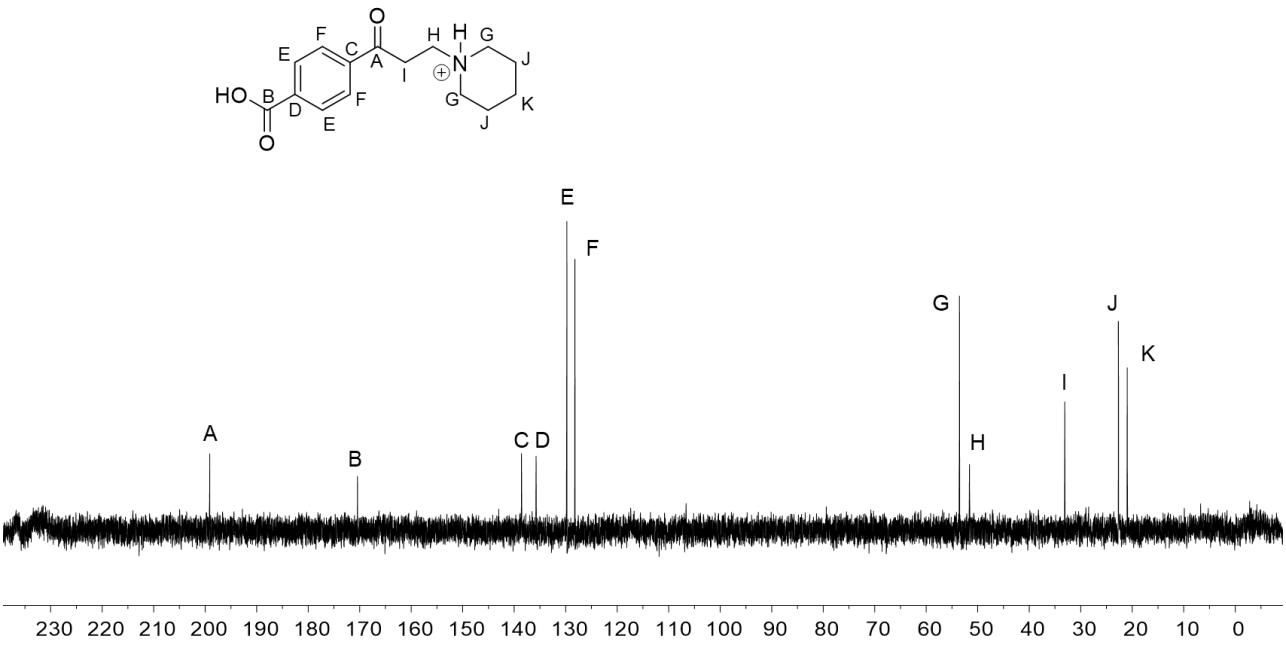
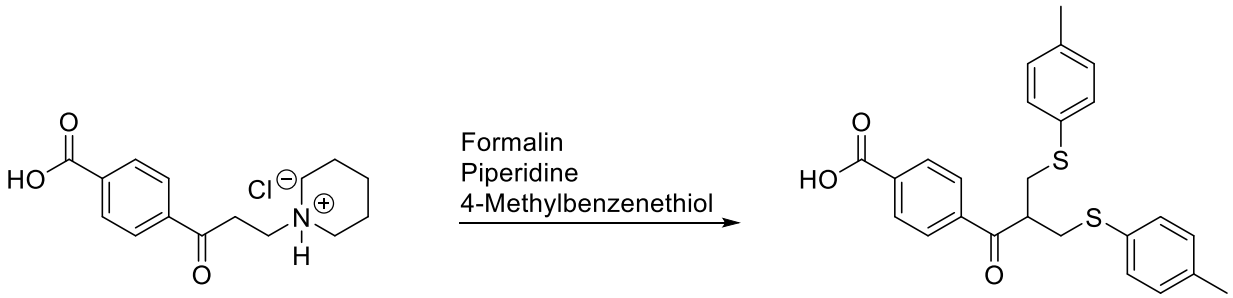



**Figure S4**. $^{13}C$ NMR of mannich salt ($D_2O$).

**Bis-sulfide acid synthesis**.




Product was synthesized from modified procedure by Brocchini *et al.*[1] To a 15 mL round bottom flask, Mannich salt (0.7 g, 2.35 mmol, 1 equivalent), 4-methylbenzene thiol (0.58g, 4.7 mmol, 2 equivalents), piperidine (0.1 mL, 1.01 mmol, 0.43 equivalents), formaldehyde (37% solution, 1 mL), ethanol (2 mL), and methanol (1 mL) were added. The solution was refluxed at 105 °C for 1 hr. After 1 h, the reaction was allowed to cool to 22 °C before adding formaldehyde (37% solution, 1 mL) again. The reaction was then refluxed for 3 more hours at 105 °C. The solution was then cooled to 22 °C and the solvent was removed *in vacuo*. To the crude, ~2 mL of methanol was added and heated to dissolve the suspension. The product was allowed to crystalize for 12 h at -20 °C and the product was isolated 400 mg (52.1% yield) via vacuum filtration as white crystals. $^1H$ NMR (500 MHz, Chloroform-*d*) δ 8.05 – 7.99 (m, 2H), 7.63 – 7.57 (m, 2H), 7.16 – 7.10 (m, 4H), 7.09 – 6.98 (m, 4H), 3.80 (p, $J$ = 8.4 Hz, 1H), 3.24 (dd, $J$ = 13.6, 7.4 Hz, 2H), 3.15 (dd, $J$ = 13.6, 6.1 Hz, 2H), 2.35 (s, 6H). $^{13}C$ NMR (126 MHz, Chloroform-*d*) δ 200.45, 168.80, 140.44, 137.29,

132.75, 131.58, 131.06, 130.27, 129.87, 128.34, 45.85, 36.39, 21.12. IR: $\nu$ =3015, 2919, 2650,1678 $cm^{-1}$. HRMS calculated for $C_{25}H_{24}O_3S_2$ ($[M+K]^+$) = 475.0804, observed = 474.9805.

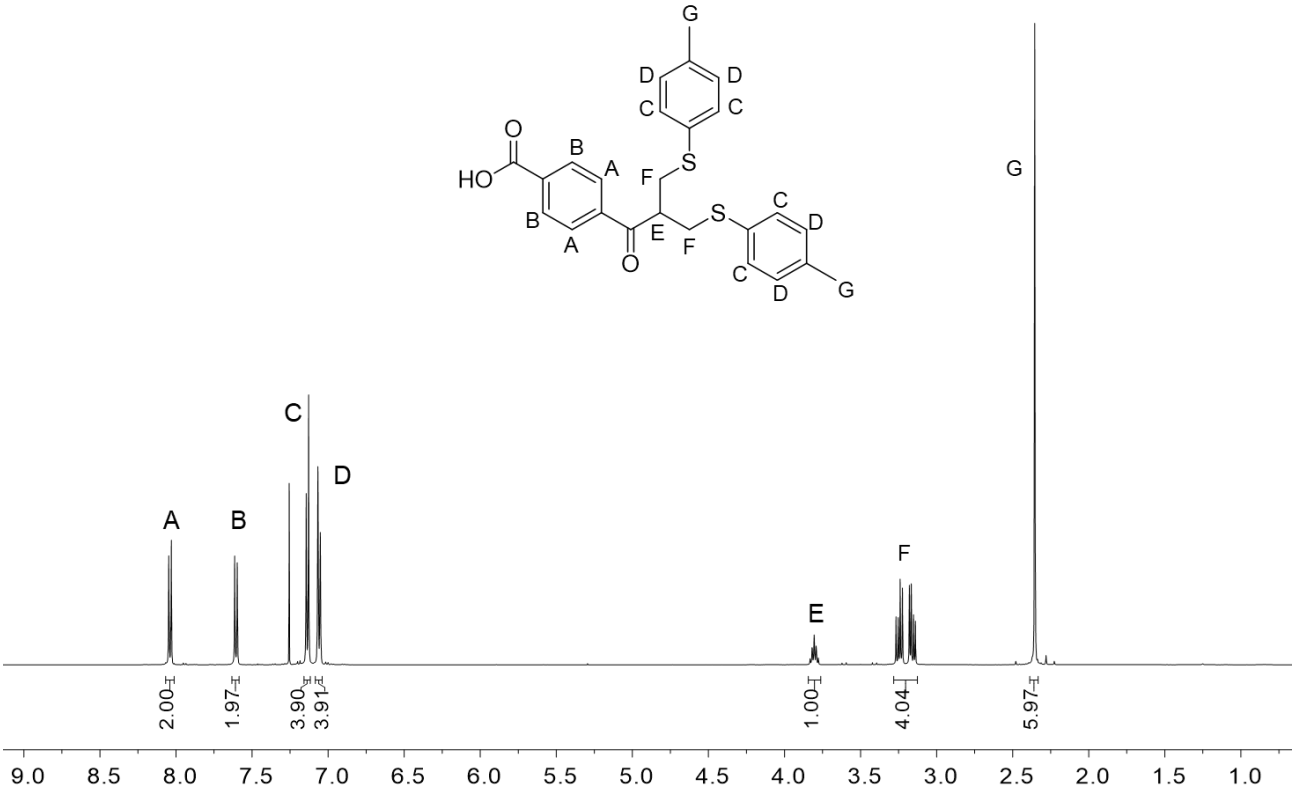



Figure S5. $^1H$ NMR of bis-sulfide acid (CDCl3)

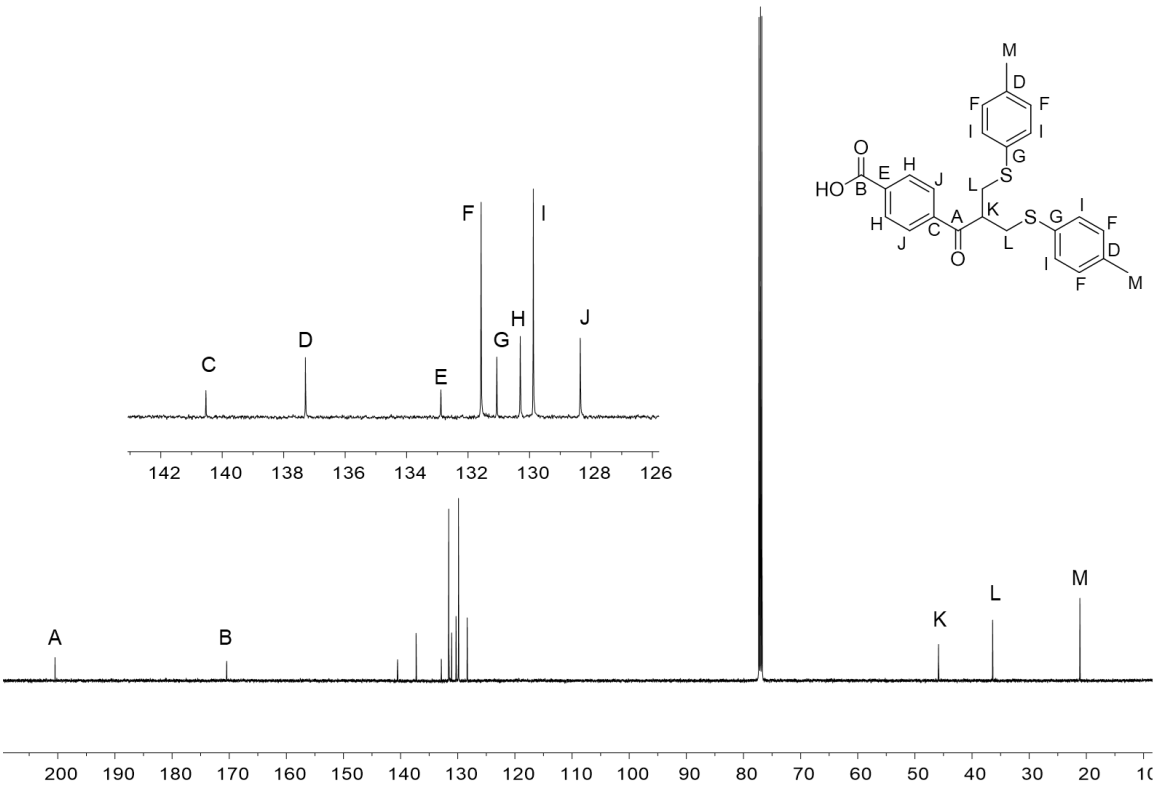
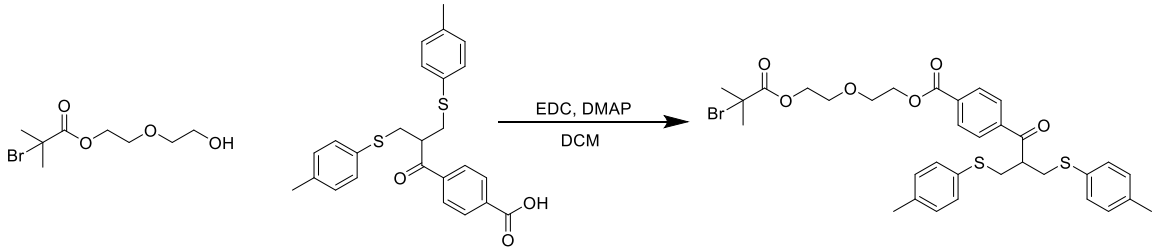



**Figure S6**. $^{13}C$ NMR of bis-sulfide acid ($CDCl_3$).

**Bis-sulfide initiator synthesis.**




To a 20 mL scintillation with a stir bar, diethylene glycol initiator (140 mg, 0.55 mmol, 1.2 equivalent), bis-sulfide acid (200 mg, 0.46 mmol, 1 equivalent), EDC (176 mg, 0.916 mmol, 2 equivalents), and DMAP (11.2 mg, 0.0916, 0.2 equivalents) were added and dissolved in 5 mL of dry DCM. Coupling was allowed to proceed for 12 h followed by concentration via rotovap. Product was purified with via flash chromatography with a gradient of Hex:EtOAc from 0% to 45% over 18 column volumes to yield 155 mg (56%) of a clear oil. $^1H$ NMR (400 MHz, Chloroform-*d*) δ 8.03 – 7.95 (dt, *J* = 8.7, 1.7 Hz, 2H), 7.59 (dt, *J* = 8.5, 1.7 Hz, 2H), 7.17 – 7.09 (dt, *J* = 8.2, 2.3 Hz, 4H), 7.09 – 7.01 (d, *J* = 7.9 Hz, 4H), 4.53 – 4.47 (m, 2H), 4.39 – 4.30 (m, 2H), 3.92 – 3.72 (m, 5H), 3.24 (dd, *J* = 13.6, 7.3 Hz, 2H), 3.15 (dd, *J* = 13.6, 6.2 Hz, 2H), 2.35 (s, 6H), 1.91 (s, 6H). $^{13}C$ NMR (126 MHz, Chloroform-*d*) δ 200.49, 137.23, 133.84, 131.52, 131.12, 129.88, 129.83, 128.26, 69.15, 68.81, 64.99, 64.45, 45.79, 36.40, 30.72, 21.13. IR: $\nu$ = 2921, 1723,

1684, 1269, 1103, 805 $cm^{-1}$. ESI-MS calculated for $C_{33}H_{37}BrO_6S_2$ ($[M+Na]^+$) = 695.1112, observed = 695.1142

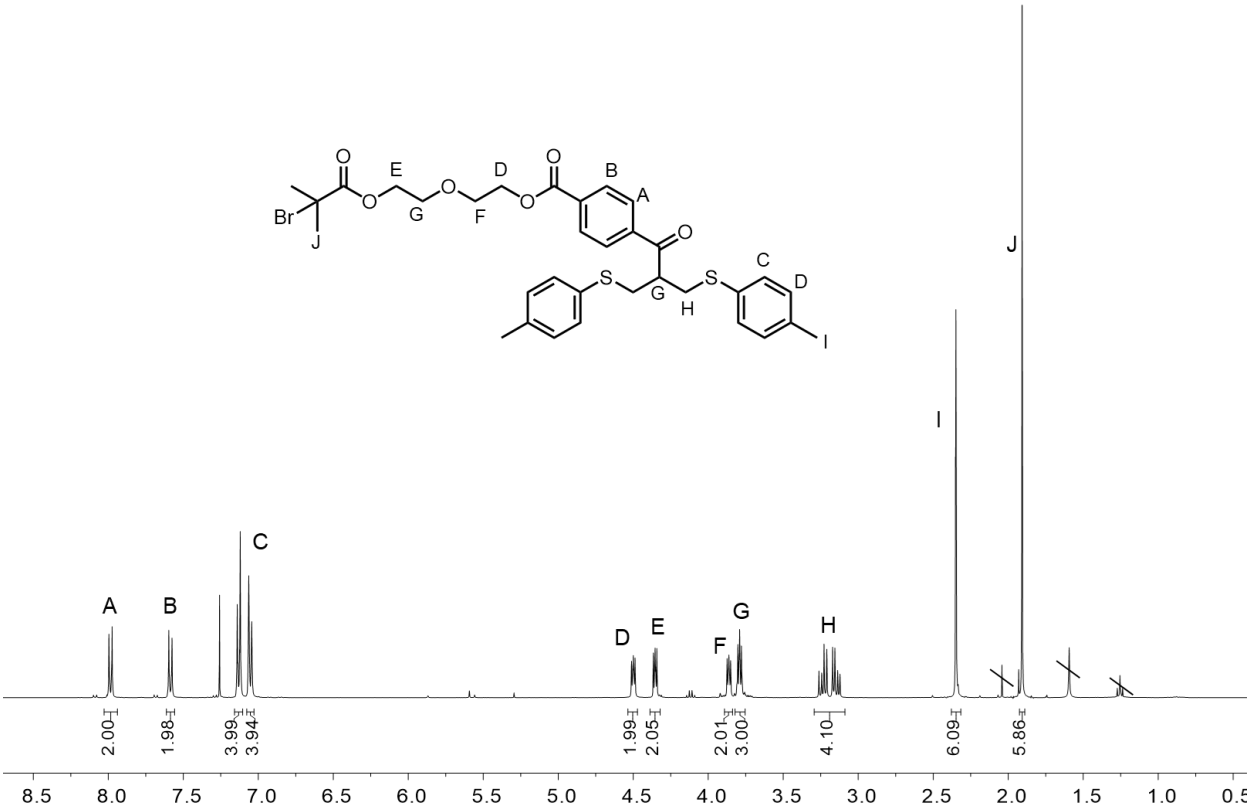



**Figure S7**. $^1H$ NMR of bis-sulfide initiator ($CDCl_3$).

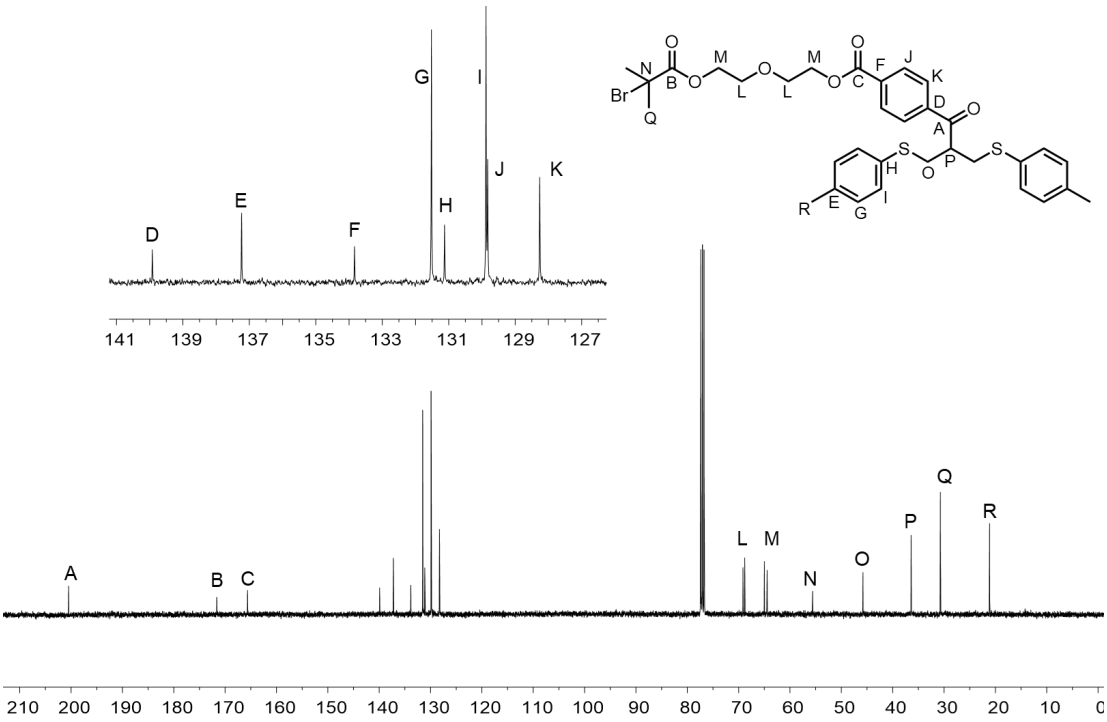
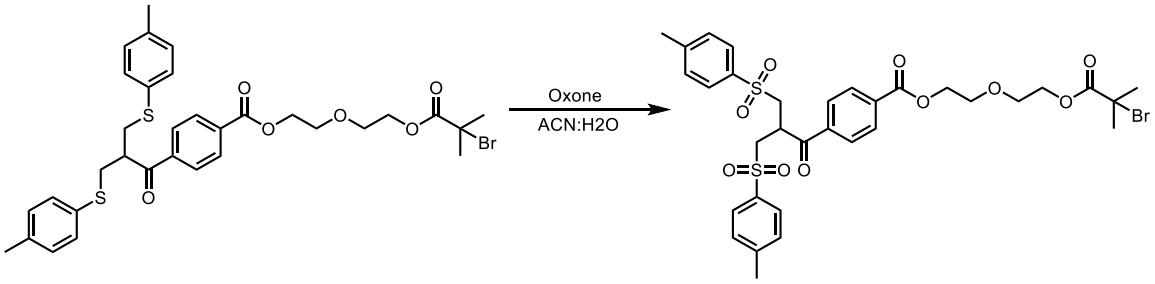



**Figure S8**. $^{13}C$ NMR of bis-sulfide initiator ($CDCl_3$).

**Bis-sulfone initiator synthesis.**




Bis sulfide (149 mg, 0.221 mmol, 1 equivalent) and oxone monopersulfate (1.63 g, 5.3 mmol, 24 equivalents) were dissolved in 10 mL of 1:1 acetonitrile:$H_2O$ in a scintillation vial with stir bar. Reaction was stirred vigorously for 4 h, after which complete conversion was observed via LCMS. Acetonitrile was removed via rotovap and DCM and water were added to the vial. The reaction was transferred to a separatory funnel and the organic layer was collected. The aqueous layer was extracted three times with DCM and organic layers were pooled before drying with $MgSO_4$. Solvent was removed via rotary evaporation and the resulting oil was re-dissolved in acetonitrile. The product was then precipitated into 15 mL of water, centrifuged, and the supernatant was discarded. The resulting residue was lyophilized to yield 137 mg (84%) of a white foam. It was found that without the final precipitation step, the product failed to initiate polymerization. We attribute this to excess oxone that likely interferes with the reduction of $CuBr_2$ during the AGET

ATRP process. $^{1}$H NMR (500 MHz, Chloroform-*d*) δ 8.04 (dt, *J* = 8.5, 1.7, 2H), 7.69 (m, 6H), 7.36 (d, *J* = 7.9 Hz, 4H), 4.51 (m, 2H), 4. (m, 3H), 3.87 (m, 2H), 3.80 (m, 2H), 3.61 (dd, *J* = 14.3, 6.6 Hz, 2H), 3.48 (dd, *J* = 14.3, 5.9 Hz, 2H), 2.48 (s, 6H), 1.92 (s, 6H). $^{13}$C NMR (126 MHz, Chloroform-*d*) δ 195.31, 171.62, 165.36, 145.57, 137.53, 135.33, 134.60, 130.22, 130.14, 128.48, 128.34, 69.11, 68.83, 64.98, 64.58, 55.65, 55.59, 35.70, 30.72, 21.75. IR: $\nu$ = 2926, 1723, 1694, 1271, 1141, 1106, 1085, 742 cm$^{-1}$. ESI-MS calculated for $C_{33}H_{37}BrO_{10}S_2$ ($[M+NH_4]^+$) = 754.1355, observed = 754.0900.

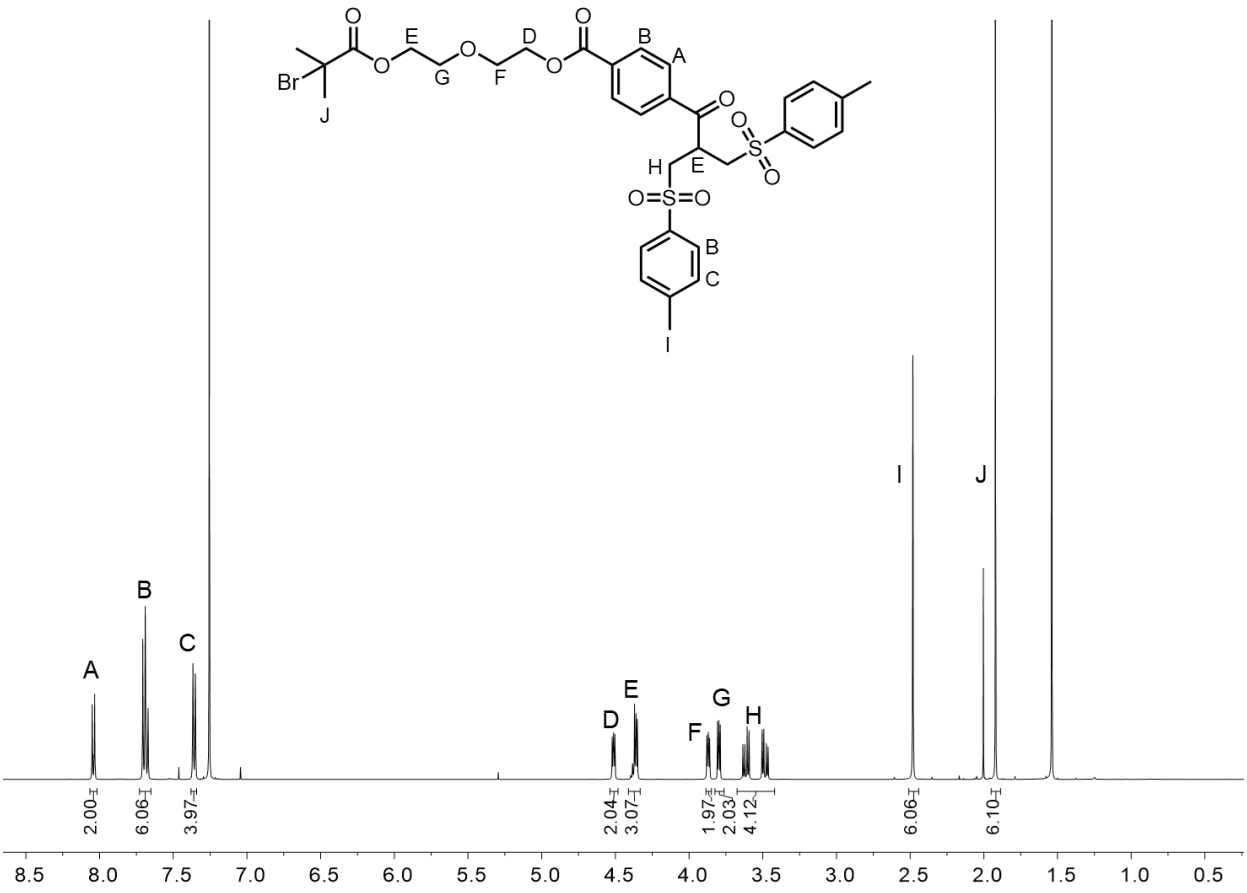



**Figure S9**. $^{1}$H NMR of bis-sulfone initiator ($CDCl_3$).

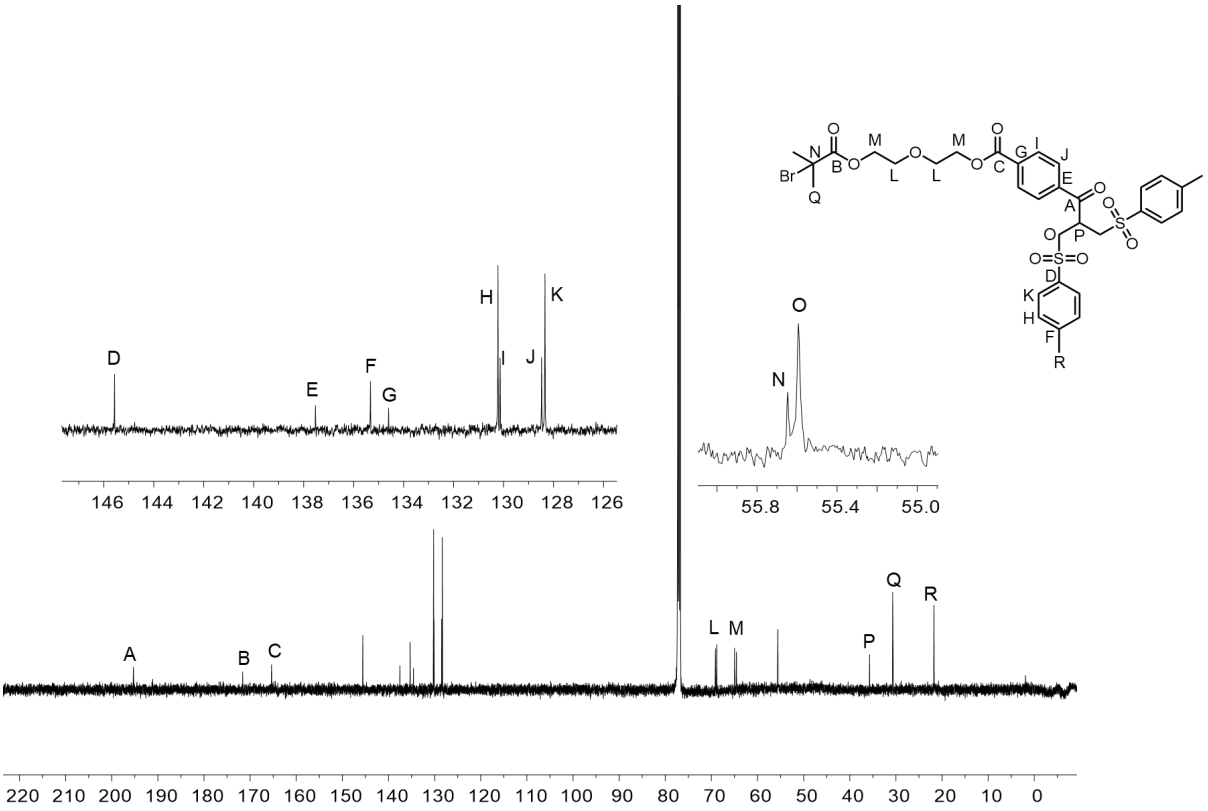
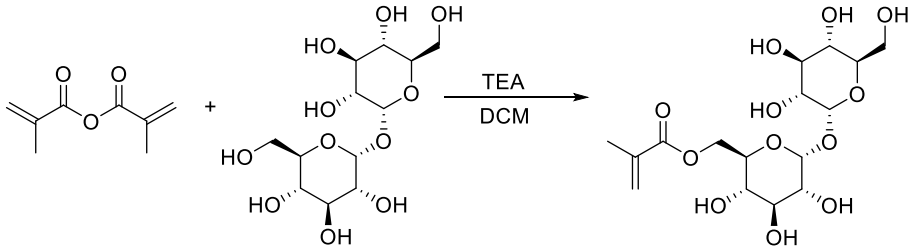



**Figure S10**. $^{13}C$ NMR of bis-sulfone initiator ($CDCl_3$).

**Trehalose methacrylate synthesis.**




Trehalose monomer was synthesized as previously reported.[2] To an oven-dried 250 mL flask, azeotropically dried trehalose (6.92 g, 20.2 mmol, 5 equivalents) was added and dissolved in dry DMSO. Dry TEA (8.41 mL, 60.7 mmol, 15 equivalents) and methacrylic anhydride (0.6 mL, 4.05 mmol, 1 equivalent) were added sequentially and the reaction was allowed to proceed for 16 h under argon. The crude was then precipitated into a chilled flask of 1800 mL of 8:2 Hex/DCM. The antisolvent was poured off and the remaining oil was dissolved in 70 mL of water. The sample was the purified via preparatory high-performance liquid chromatograph (10-60% gradient over 20 minutes). Fractions containing product were combined and 150 ppm of 4-methoxy phenol was added to prevent auto-polymerization. Methanol was removed via rotary evaporation in a 2-neck round bottom and a septum with a needle bubbling into the solution to provide oxygen and prevent autopolymerization. The remaining water was then lyophilized and 698 mg (41.4 %) of product was recovered via lyophilization as a fluffy solid. $^1H$ NMR (600 MHz, Deuterium Oxide) δ 6.01 (s, 1H), 5.60 (s, 1H), 5.04-5.01 (dd, J = 3.9 Hz, 2H), 4.35 (d, J = 12.3, 2.2 Hz, 1H), 4.22 (dd, J =

12.3, 5.2 Hz, 1H), 3.94 (m, J = 10.1, 5.3, 2.1 Hz, 1H), 3.75 – 3.64 (m, 4H), 3.61 (dd, J = 12.1, 5.2 Hz, 1H), 3.53 (dd, J = 9.9, 3.9 Hz, 1H), 3.48 (dd, J = 10.0, 3.9 Hz, 1H), 3.40 (t, J = 9.6 Hz, 1H), 3.29 (t, J = 9.5 Hz, 1H), 1.79 (s, 3H). ESI-MS calculated for $C_{16}H_{26}O_{12}$ ($[M+Na]^+$) = 433.1322, observed = 433.1458.

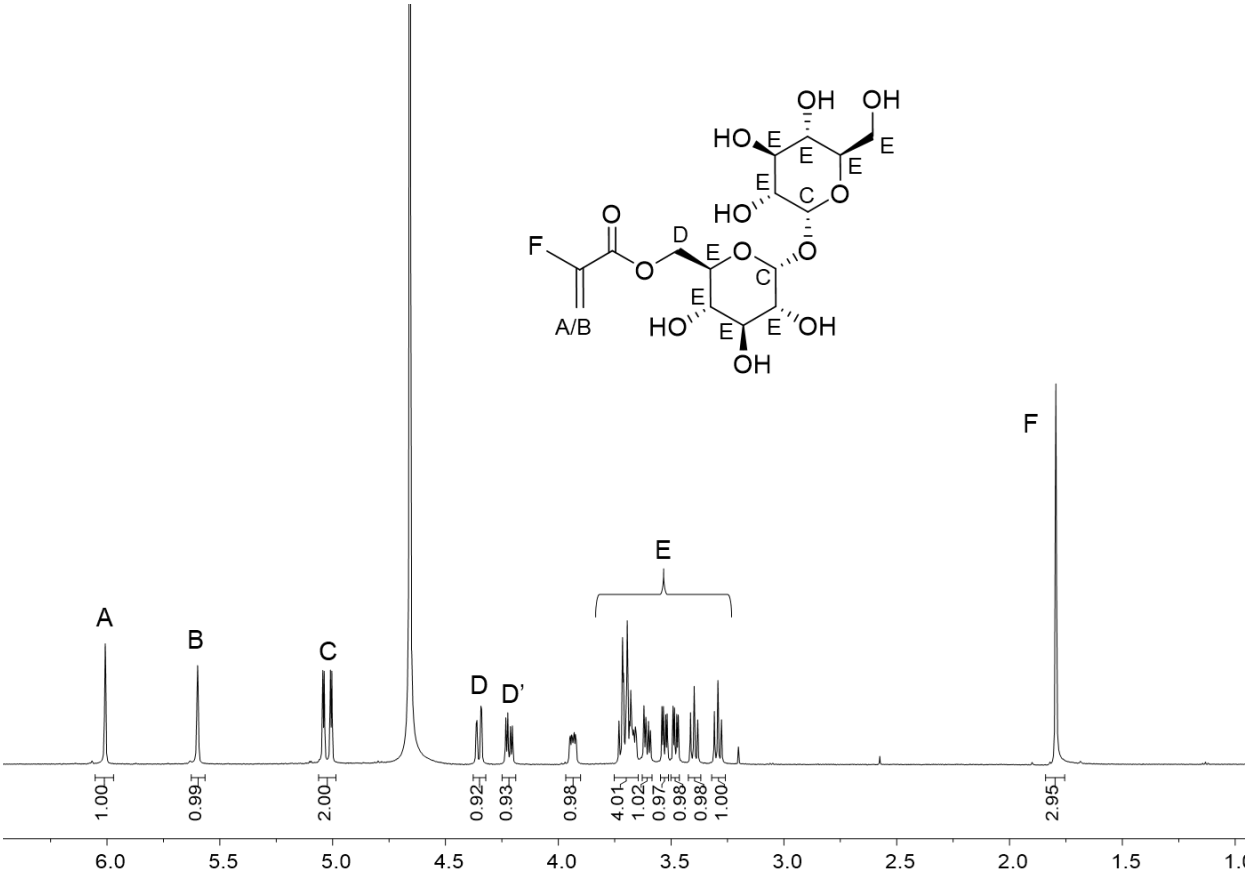
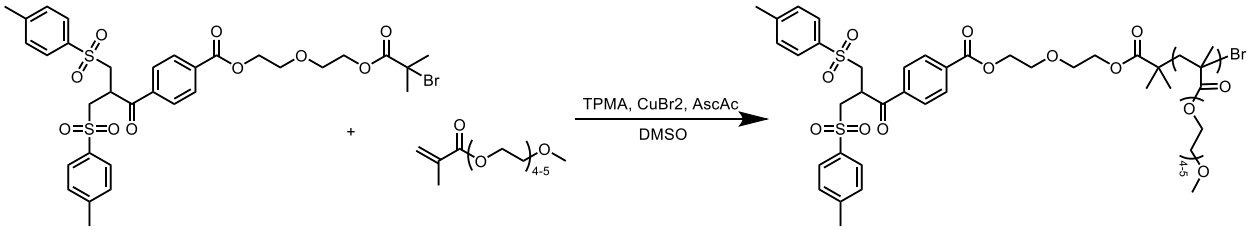



**Figure S11**. $^1$H NMR of trehalose methacrylate ($D_2O$).

**Representative PEGMA Polymerization.**




Stock solutions of $CuBr_2$ (16 mg/mL), tris (2-pyridylmethyl) amine (TPMA, 43 mg/mL), and bis-sulfone initiator (54 mg/mL) were prepared in DMSO in individual dram vials. To a scintillation vial containing PEGMA monomer (0.4 mL, 1.5 mmol, 200 equivalents), 100 µL of the $CuBr_2$ (1.6 mg, 7.4 µmol, 1 equivalent), 100 µL of the TPMA stock (4.3 mg, 15 µmol, 2 equivalents), and

100 µL of initiator (5.4 mg, 7.4 µmol, 1 equivalent) were added along with 2.8 mL of DMSO (0.45 M with respect to monomer). For percent conversion analysis, 40 µL of tetralin was added as an internal standard. The mixture was then transferred to a Schlenk tube and three freeze-pump-thaw cycles were conducted. Meanwhile, a stock solution (7.8 mg/mL) of ascorbic acid was sparged with argon. After the final freeze-pump-thaw cycle, the polymerization was initiated by adding 100 µL of the sparged ascorbic acid solution (0.78 mg, 4.43 µmol, 0.6 equivalents) to the Schlenk flask.

For kinetics, timepoints were collected through an argon-purged syringe and frozen immediately in liquid nitrogen to quench. After thawing, the timepoint was diluted in ~0.1 mL of DMF. A portion of the sample was used for GPC analysis while a separate aliquot was diluted 5-fold in acetonitrile for HPLC analysis. Percent conversion was determined via analytical HPLC by comparing the PEGMA monomer integration against the tetralin internal standard.

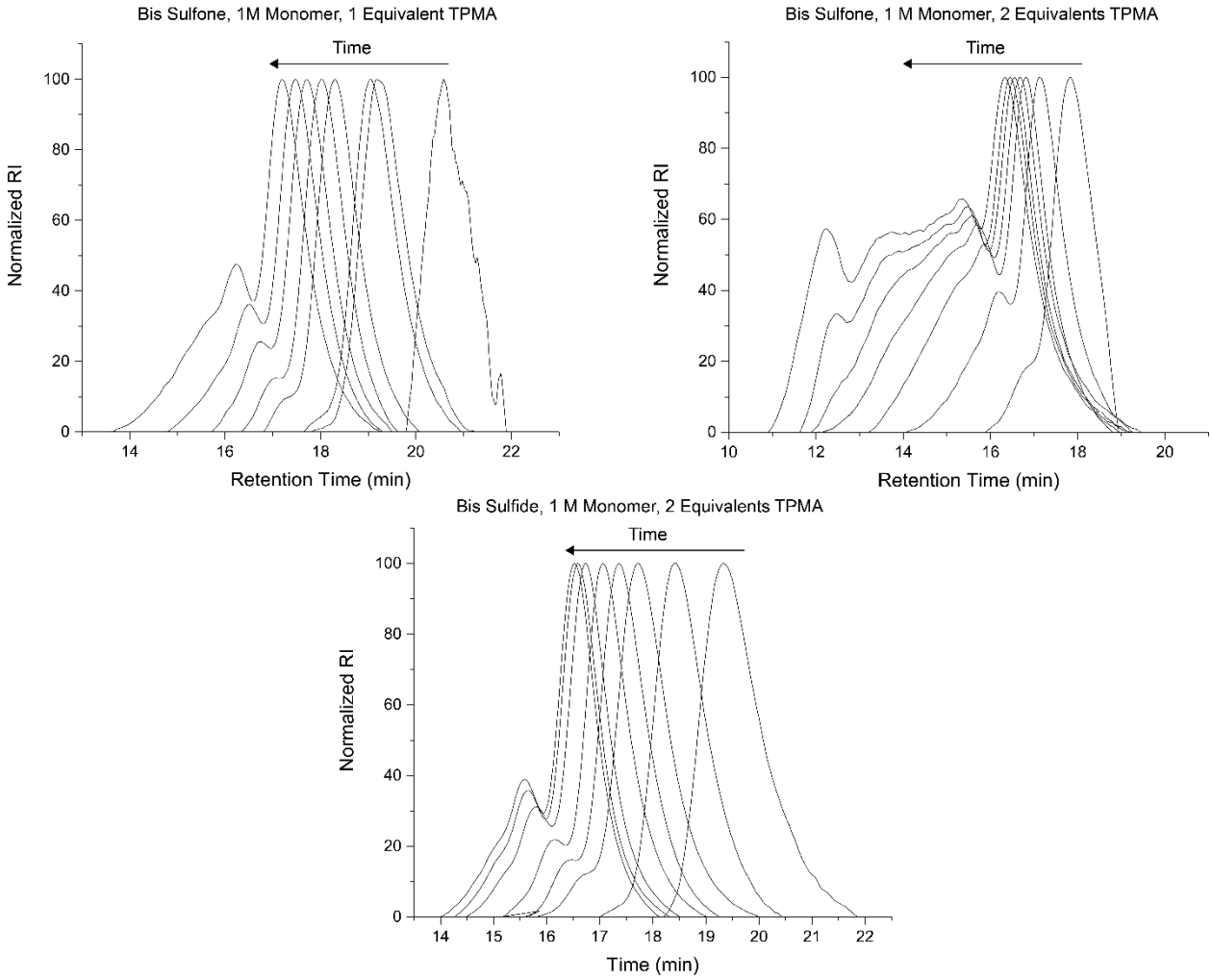
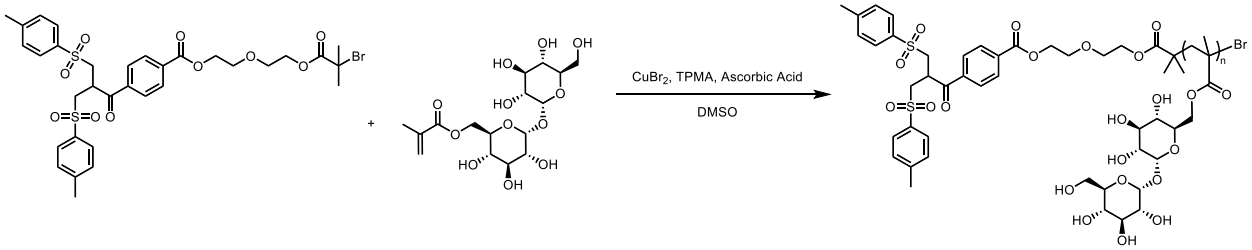



**Figure S12**. Overlaid GPC traces of tested conditions for polymerization optimization.

**Polymerization of trehalose monomer.**




Stock solutions of $CuBr_2$ (21.8 mg/mL), TPMA (56.6 mg/mL), and sulfone initiator (72.0 mg/mL) were prepared in dry DMSO in individual dram vials. To a scintillation vial containing trehalose monomer (100 mg, 0.24 mmol, 25 equivalents), 100 µL of the $CuBr_2$ stock (2.18 mg, 9.7 µmol, 1 equivalent), 100 µL of the TPMA stock (5.7 mg, 19.4 µmol, 2 equivalents), and 100 µL of the sulfone initiator (7.2 mg, 9.7 µmol, 1 equivalent) were added and the resulting solution transferred to a Schlenk tube. An additional 50 µL of DMSO was used to dissolve remaining reagents and also transferred to the Schlenk flask. Meanwhile, an 18.4 mg/mL solution of ascorbic acid was sparged with argon in dram vial with a septum for ~15 min. The solution in the Schlenk flask was degassed via 3 freeze-pump-thaw cycles and 56 µL of the sparged ascorbic acid solution (1.03 mg, 5.8 µmol, 0.6 equivalents) was added to initiate polymerization (final reaction volume = 406 µL, 0.6 M with respect to monomer). The polymerization was allowed to proceed for 16 h before quenching with liquid $N_2$. The polymer was purified via dialysis (MWCO = 3.5 kDa) against water for 2 days and recovered via lyophilization. $^1$H NMR (500 MHz, Deuterium Oxide) δ 5.10, 5.05, 4.23, 4.01, 3.93, 3.75, 3.66, 3.53, 3.35, 1.81, 0.97, 0.79.

NMR used calculate molecular weight in addition to GPC. Proton A was set to an integration of 2 and used to determine the integration of peak D. The values were then plugged into the following formula: $\frac{\text{Integration of D}}{2} * 410.37$

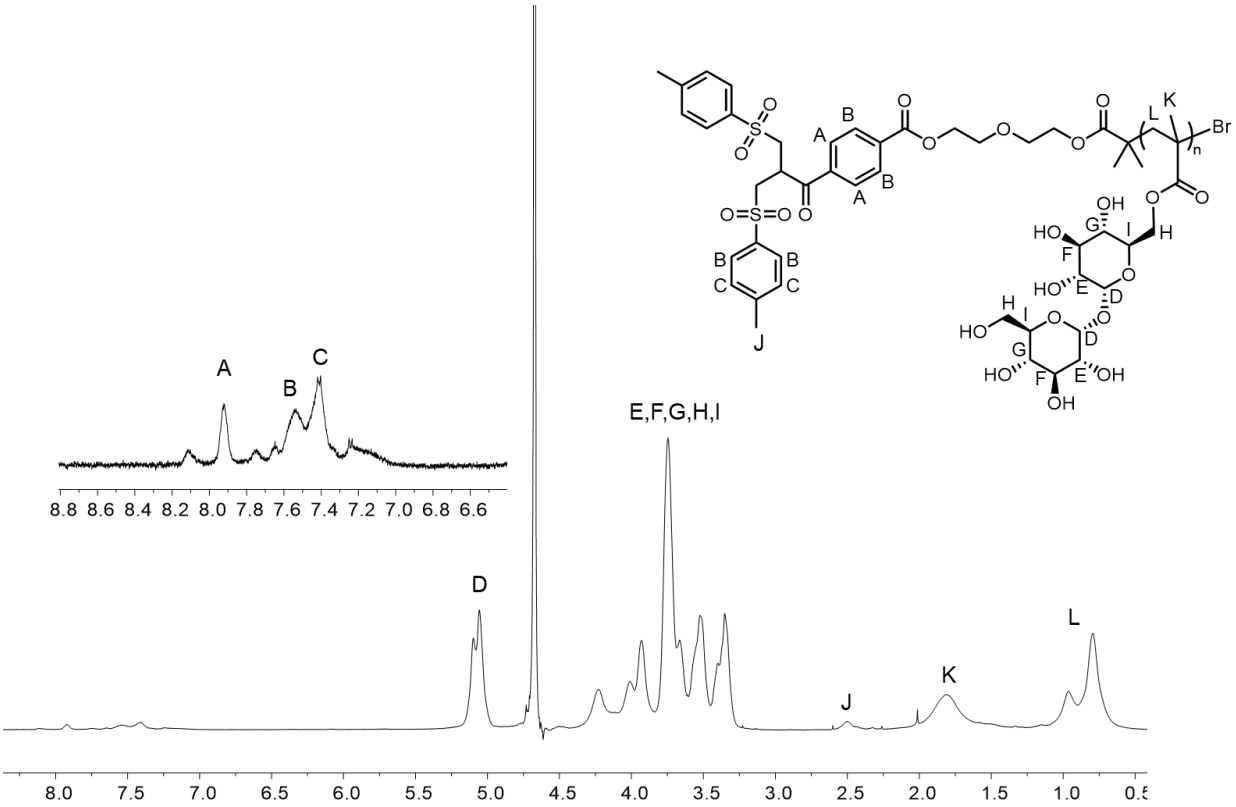
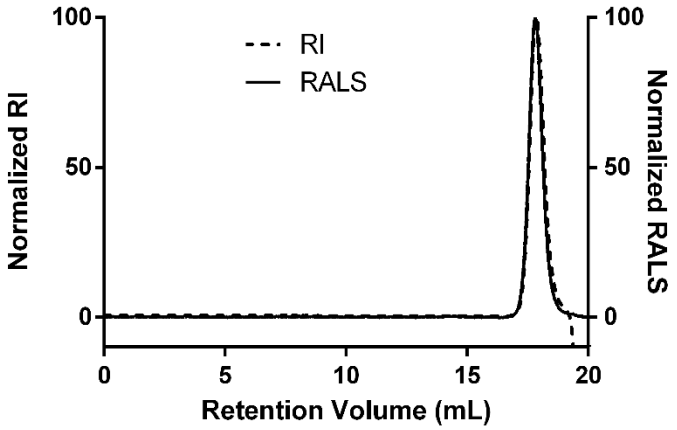



**Figure S13.** $^{1}$H NMR of bis-sulfone trehalose polymer (D2O).




| Standard Type | $M_n$ |
|---|---|
| Dextran Light Scattering | 39 kDa |
| PEG Conventional | 4.9 kDa |
| $^{1}$H NMR | 32 kDa |

**Figure S14**. Representative GPC trace of trehalose polymer along with molecular weight calculated via different methods.

**Fab preparation**. The FAB preparation was adapted from a reported literature reports.[3,4] First, 224 mg of cysteine was added to 12 mL of digestion buffer (20 mM $NaH_2PO_4$, pH 7.4, 10 mM EDTA) immediately before use. Then, 1.6 mL of immobilized papain resin was added to 2.4 mL of digestion buffer and the resulting suspension was centrifuged (1000xg, 2 min). The supernatant was discarded and the resin was resuspended in 2.4 mL of digestion buffer. This procedure was repeated a total of 3 times. After resuspending the final time, the slurry was added to a 15 mL conical tube. To the suspension, 4 milligrams of Herceptin (previously dissolved in 200 µL of digestion buffer) was added. The sample was purged with argon and incubated in thermoshaker (300 rpm, 37 °C) for 20 hrs. The crude reaction mixture was then centrifuged (1000xg, 2 min) and the supernatant was collected. Resin was resuspended in 2 mL of digestion buffer and the previous process of centrifugation followed by supernatant collection was repeated twice. The pooled supernatants were then purified via fast-protein liquid chromatography as outlined below.

A 1 mL Mabselect SuRe® was equilibrated with phosphate buffered saline (pH 7.4) at a flowrate of 0.7 mL/min. Sample was loaded and Fab was eluted from column with 12 column volumes (CV) of isocratic PBS. The $F_c$ was eluted with a linear gradient of 0-100% 50 mM glycine buffer (pH 2.7) over 20 column volumes. Fractions containing Fab were pooled, dialyzed into PBS buffer, concentrated via ultracentrifugation, and stored at 4 °C. Deconvoluted ESI-mass spectrometry of the reduced Fab displayed 3 masses corresponding to light chain of Herceptin (calculated = 24204.14, observed = 24203.64) and two different heavy chain papain cleavage sites differing by the tripeptide KTH (calculated without KTH peptide = 23443.10, observed = 23441.26 and calculated with KTH= 23837.72, observed = 23836.71).

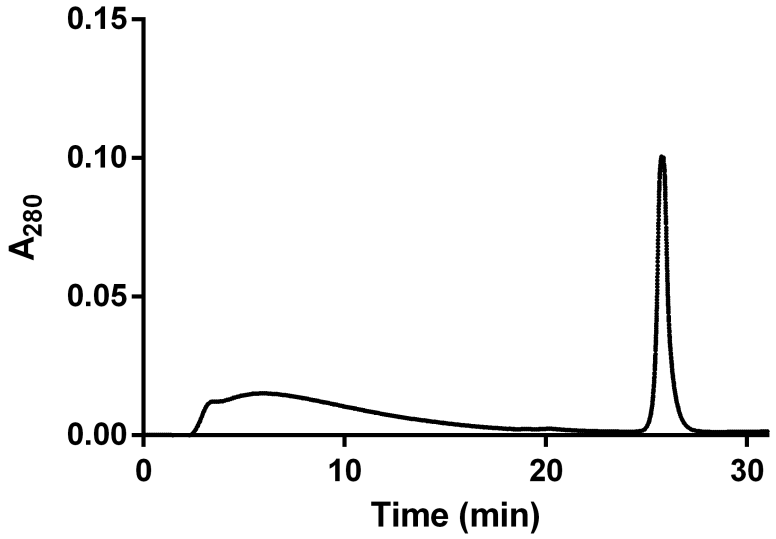



**Figure S15**. FPLC of Herceptin papain digest

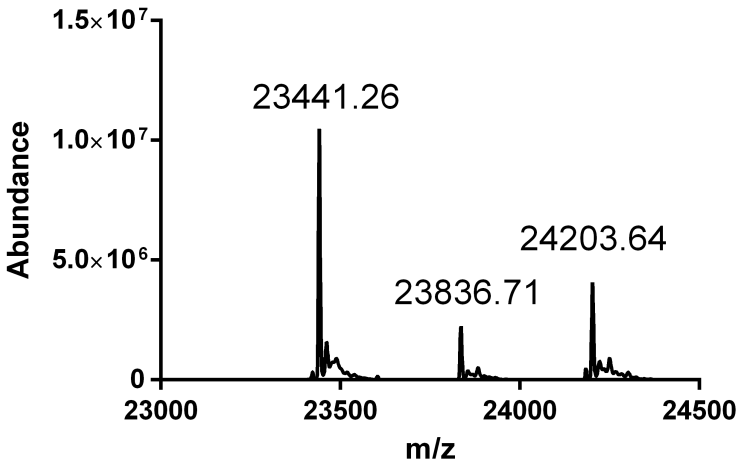



**Figure S16**. ESI-MS of purified, reduced Fab

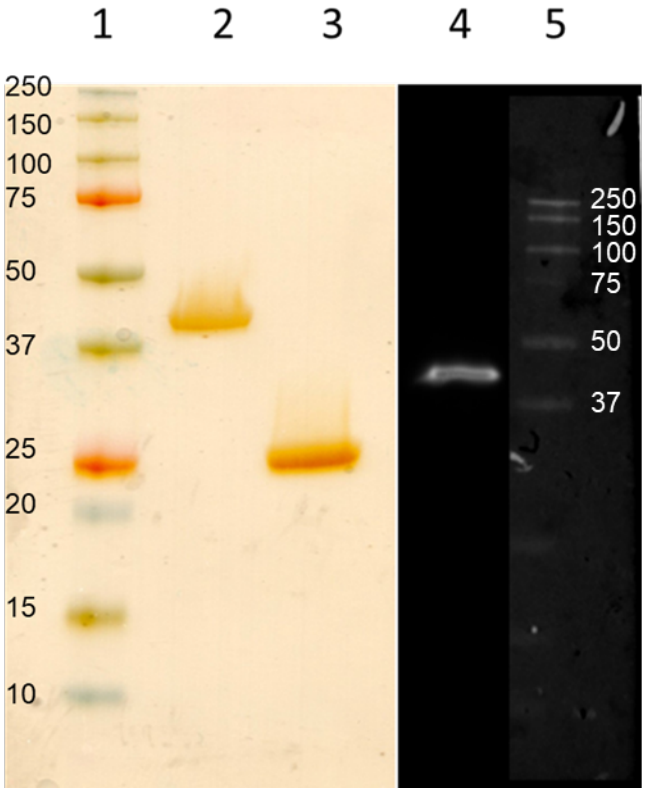



**Figure S17**. SDS PAGE visualized via silver staining (lane 1: ladder, lane 2: Fab, lane 3: Fab reducing) and western blot (lane 4: Fab, lane 5: protein ladder) of purified Fab

**Representative conjugation to Fab**. 1 mL of Fab stock was concentrated via Amicon 30 kDa ultra centrifugal filtration to a concentration of 9.1 mg/mL (91 µL volume, 832 ug protein, 1.7 x $10^{-8}$ mol). To this sample, 1 weight equivalent of DTT was added and reduction was allowed to

proceed at 22 °C for 1 hr. The sample was then buffer exchanged via 0.5 mL ZEBA desalting column into PBS+10 mM EDTA. A 100 mg/mL stock of 23 kDa trehalose polymer dissolved in MilliQ water was then added (4.16 mg, 1.72 x $10^{-7}$ mol, 10 equivalents). Conjugation was allowed to proceed at 22 °C for 12 h. Conjugate was purified from excess polymer via cation exchange chromatography using 1 mL GE Healthcare SPHP columns. Free polymer was eluted with isocratic 20 mM MES, pH 5.7 over 12 CV's. Conjugate was eluted with a linear gradient of 20 mM MES, pH 5.7, 300 mM NaCl over 15 CVs.

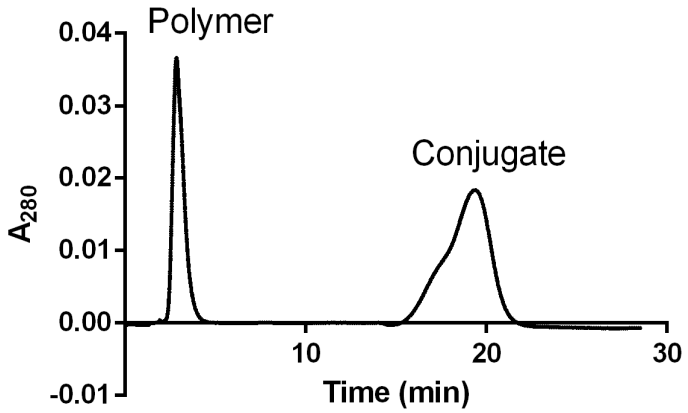



**Figure S18**. Representative FPLC trace of conjugates

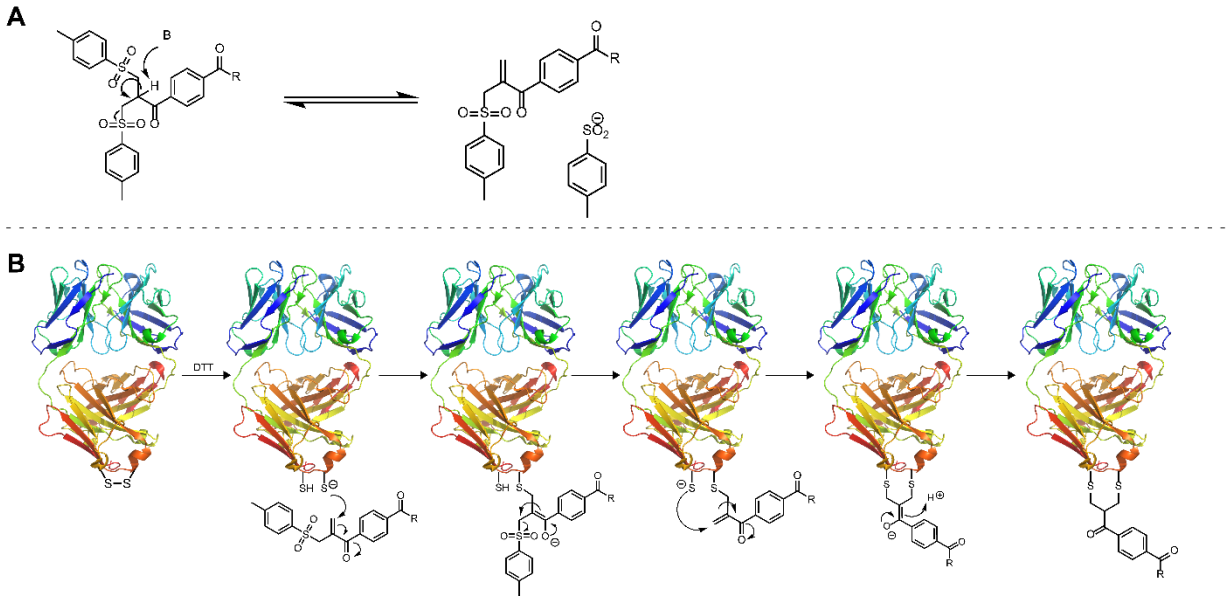



**Figure S19**. (A) Equilibrium of bis-sulfone and mono-sulfone at pH 7.4 and (B) mechanism of conjugation for the bis-sulfone.[4]

**Representative Herceptin Conjugation**. Herceptin (10 mg/mL, 100 µL) was dissolved in 20 mM sodium phosphate buffer with 20 mM EDTA. Next, 3.5 µL of a 1 mg/mL solution of TCEP was added and incubated for 2 h at 37 °C. TCEP was then removed via Amicon ultrafiltration (100

kDa cutoff) and 50 equivalents of 16 kDa bis-sulfone trehalose polymer (dissolved in water) was added. The conjugation was allowed to proceed for 16 h at 40 °C. The conjugate was purified via cation exchange chromatography with a HiTrap SPHP column. Free polymer was eluted with 8 column volumes of isocratic MES buffer (pH 5.6) followed by elution of the conjugate with a gradient of MES buffer (pH 5.6) + 300 mM NaCl over 12 column volumes.

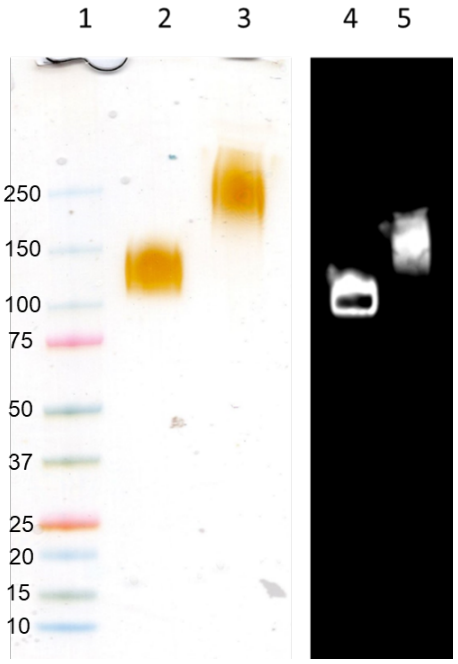



**Figure S20**. SDS PAGE visualized via silver staining (lane 1: ladder, lane 2: Herceptin, lane 3: Herceptin conjugate) and western blot (lane 4: Herceptin, lane 5: Herceptin conjugate) of Herceptin conjugate.

**General ELISA Protocol**. To the wells of a high-binding 96 well plate, 100 µL of a 1 µg/mL solution (diluted in 0.1 M carbonate buffer, pH 9.6) of recombinant $Her_2$ was added. The plate was covered with foil and incubated at 4 °C overnight for 12 h. The following day, the solution was aspirated and the plate was washed four times with ELISA wash buffer (PBS + 0.3% Tween 20). To the wells, 200 µL of blocking buffer (1% BSA in PBS, filtered with 0.22 µM filter) was added and incubated at 22 °C for 2 h. Again, 4 washes were performed with wash buffer before adding 100 µL of antibody and conjugate samples at appropriate dilution (dilution buffer = 1% BSA in PBS). The plate was incubated at 22 °C for 1 h before repeating aspiration and wash procedure. FAB-specific peroxidase labeled anti-human IgG (diluted 1:40,000 in dilution buffer) was then added (100 µL per well) and the plate was incubated at 37 °C for 45 min. After aspirating and washing the plate a final time, 100 µL of TMB ( 3,3',5,5'-Tetramethylbenzidine) substrate solution was added to the wells via a multichannel pipette and the plate was incubated in the dark for ~5 min. After sufficient development of blue color, 50 µL of 1 M sulfuric acid was added via multichannel pipette and absorbance of each well was measured at 450 nm.

**Stability Heat Ramp**. Fab protein was diluted to 0.1 mg/mL in PBS and 50 µL aliquots were divided among 24 separate 0.5 mL Lo-Bind eppendorf tubes. Three of the aliquots were stored at 4 °C to serve as a control. The remaining tubes were placed in a pre-heated water bath at 50 °C. At 30-minute intervals, 3 samples (triplicate samples) were removed and stored at 4 °C, and the temperature of the bath was increased by 5 °C. This procedure was repeated to a final temperature of 80 °C. Samples were then filtered through a 0.22 µM and remaining protein was analyzed via analytical HPLC (C3 stationary phase, 10-95% acetonitrile+0.1% TFA, 5% isocratic isopropanol + 0.1% TFA, 70 °C column temperature).

**Stability at 75 °C**. Fab and Fab conjugate were diluted to 0.1 mg/mL in PBS. For both sets of samples, 12 aliquots of 50 µL divided into separate lo-bind Eppendorf tubes. Three samples were stored at 4 °C for both the conjugate and Fab to serve as controls. The remaining samples were placed in a preheated 75 °C water bath. Three samples were removed from each group at 15 minutes, 30 minutes, and 60 minutes and stored at 4 °C until analysis. Samples were then filtered through a 0.22 µM and remaining protein was analyzed via analytical HPLC (C3 stationary phase,10-95% acetonitrile+0.1% TFA, 5% isocratic isopropanol + 0.1% TFA, 70 °C column temperature).

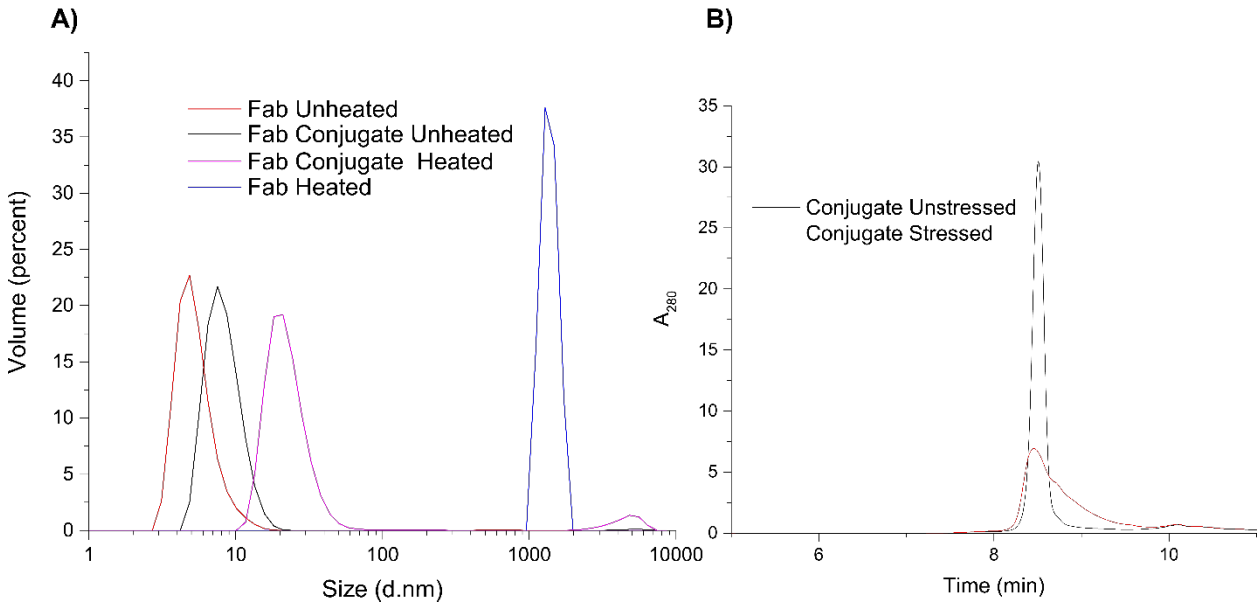



**Figure S21**. A) DLS of Fab and conjugate before (unheated) and after stressing (heated at 75 °C, 1 hr, 0.25 mg/mL). B) HPLC overlay of Fab conjugate before and after stressing at 75 °C. Oligomers observed via DLS do not appear to be resolved via reverse-phase HPLC, although there is a broadening of the peak.

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