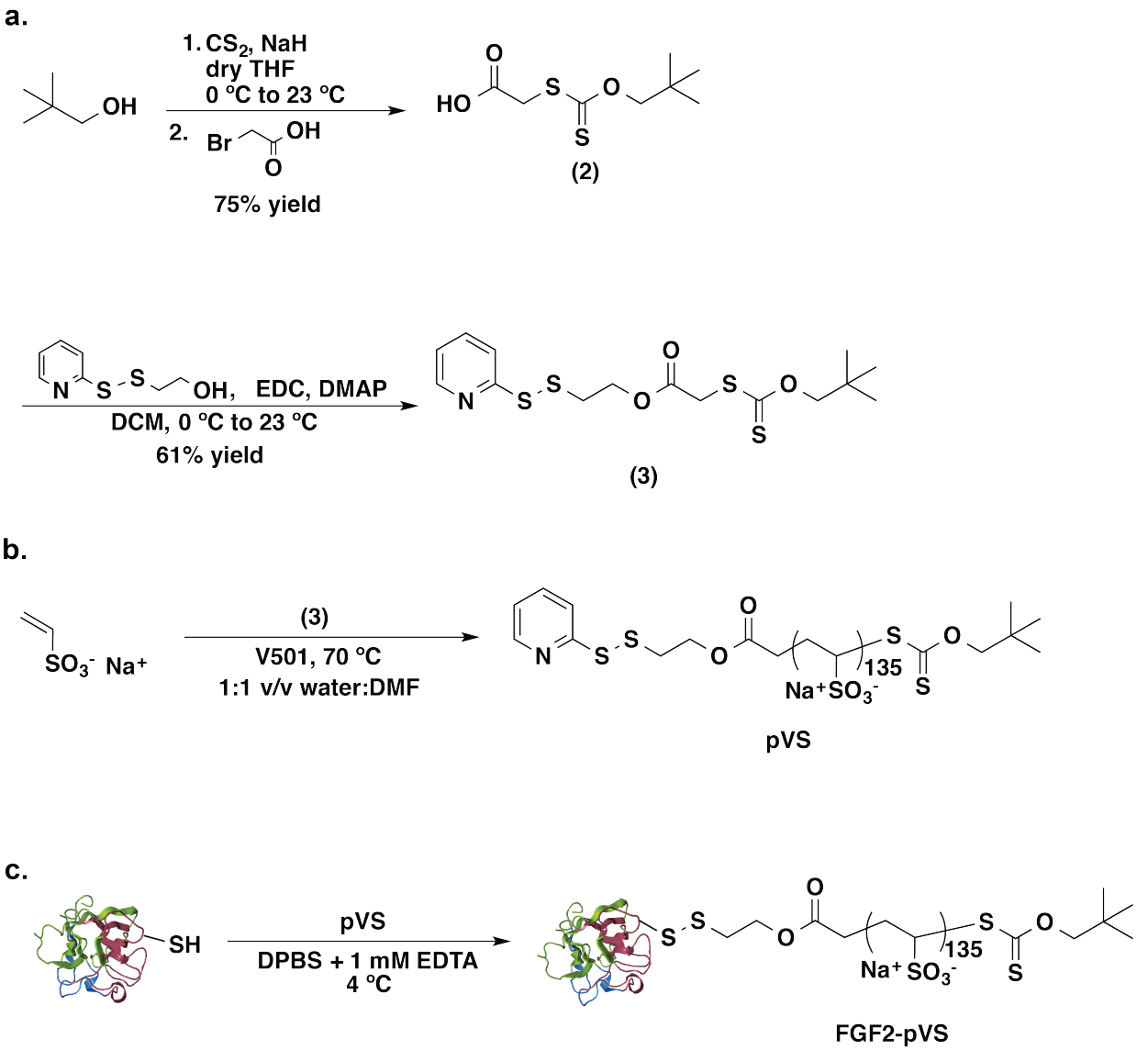
A Heparin-Mimicking Block Copolymer Both Stabilizes and Increases the Activity of Fibroblast Growth Factor 2 (FGF2)

Samantha J. Paluck, Thi H. Nguyen, Jonghan P. Lee, Heather D. Maynard*

Department of Chemistry and Biochemistry and California NanoSystems Institute, University of California, Los Angeles, 607 Charles E. Young Drive East, Los Angeles, California 90095-1569 United States

Supplemental Information



Scheme S1. a) Synthesis of xanthate CTA, b) RAFT polymerization of pVS polymer and c) protein polymer conjugation of pVS to FGF2 on a heparin column. Protein structure modified from PDB 1CVS using PyMOL software.

pVS Synthesis Overview. Reversible addition-fragmentation chain-transfer (RAFT) polymerization of vinyl sulfonate (VS) was performed with the xanthate CTA to yield PDS end functionalized pVS (Scheme S1b). VS monomer was added with a feed ratio of [VS]:[CTA1]:[V501] = 300:1:0.5 to yield a polymer with a degree of polymerization of 135 and molecular weight dispersity ($Đ$) of 1.16. Next, covalent conjugates were prepared through

disulfide exchange of the PDS-pVS with a surface exposed cysteine on FGF2 (Scheme S1c).

CTA Syntheses

*Synthesis of 2-(((neopentyloxy)carbonothioyl)thio)acetic acid, (**2**).* A 3-neck round bottom flask was purged with argon and dry THF (35 mL) was added. The round bottom was submerged in an ice bath and cooled to 0 °C. Sodium hydride, 60% in mineral oil (1.90 g, 47.5 mmol NaH) was added to the reaction flask and then neopentyl alcohol (3.13 mL, 28.8 mmol) was added drop-wise over 10 minutes. Following hydrogen evolution, the reaction was stirred for 10 minutes and then carbon disulfide was added (1.95 mL, 32.4 mmol); the reaction turned yellow. After 20 minutes, 2-bromoacetic acid (1.00 g, 7.20 mmol) was added and the reaction was slowly warmed to 23 °C over 5 hours. The reaction was quenched with 30 mL of methanol followed by 15 mL of water. THF was removed *in vacuo* and the crude mixture was washed twice with dichloromethane. The aqueous layer was acidified with 1 M HCl and then extracted three times with dichloromethane. The combined organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The product was further purified by recrystallization in warm water. The crystals were washed with cold water and lyophilized to dryness (1.2 g, 75% yield). δ ^{1}H NMR (400 MHz, $CDCl_3$): 9.95 (1H, br. s, COO**H**), 4.26 (2H, s), 3.97 (2H, s), 1.01 (9H, s). δ ^{13}C NMR (400 MHz, $CDCl_3$): 212.11, 174.24, 84.22, 37.44, 31.84, 26.50. FT- IR (cm^{-1}): 2955, 2868, 2701, 2594, 1717, 1467, 1419, 1398, 1357, 1303, 1273, 1228, 1173, 1066, 1028, 955, 933, 913, 880, 793, 665. HRMS-ESI (expected, observed): $[M-H]^-$ = (221.0305, 221.0306).

*Synthesis of 2-(pyridin-2-yldisulfanyl)ethyl 2 (((neopentyloxy)carbonothioyl)thio)acetate, (**3**).* A 3-neck round bottom flask was purged with argon and 2-pyridyl disulfide ethanol (0.40 g, 2.13 mmol) and (**1**) (0.31 g, 1.40 mmol) were added and dissolved in 14 mL of dry dichloromethane.

The reaction flask was submerged in an ice bath and cooled to 0 °C. EDC (0.27 g, 1.39 mmol) and DMAP (0.034 g, 0.28 mmol) were added to the reaction flask and the reaction allowed to warm to 23 °C over 2 hours. The reaction was washed twice with saturated sodium bicarbonate and then twice with water. The organic layer was dried over anhydrous magnesium sulfate and the solvent was removed in *vacuo*. The product was purified by silica gel chromatography in 2:1 hexane:ethyl acetate (Rf = 0.4) to give a yellow oil (329 mg, 61% yield). δ ^{1}H NMR (400 MHz, $CDCl_3$): 8.49-8.48 (1H, d, J = 4.68 Hz), 7.71-7.65 (2H, m), 7.14-7.11 (1H, m), 4.44-4.41 (2H, t, J = 6.44 Hz), 4.26 (2H, s), 3.93 (2H, s), 3.09-3.05 (2H, t, J = 6.44 Hz), 0.99 (9H, s). δ ^{13}C NMR (400 MHz, $CDCl_3$): 212.51, 167.62, 159.59, 149.75, 137.15, 120.97, 119.93, 84.04, 63.55, 37.57, 37.12, 31.85, 26.55. FT- IR (cm^{-1}): 2959, 2871, 1739, 1572, 1446, 1416, 1365, 1273, 1225, 1147, 1116, 1059, 1029, 986, 950, 931, 906, 880, 759, 716, 616, 555, 529, 508, 486, 479, 462. HRMS-ESI (expected, observed): $[M_{H+}]$ = (392.0482, 392.0439).

Synthesis of pVS. RAFT polymerizations were performed with a feed ratio of [VS]:[CTA1]:[V501] = 300:1:0.5 with a monomer concentration of 2.5 M. Vinyl sulfonate (VS) monomer (996 mg, 7.66 mmol) and V501 (3.6 mg, 0.013 mmol) were added to a Schlenk tube and dissolved in 1.5 mL of Milli-Q water (degassed prior to use). Next, **(2)** (10 mg, 0.026 mmol) was dissolved in 1.5 mL of DMF (degassed prior to use) and transferred to the Schlenk tube. The Schlenk tube was sealed and subjected to four freeze-pump-thaw cycles before immersion in an oil bath set to 70 °C. After 17 hours the polymerization was stopped by bubbling air through the Schlenk tube. The polymer was purified by dialysis in 1000 MWCO tubing against 1:1 v/v water:acetonitrile followed by 100% Milli-Q water. The resulting contents of the dialysis were lyophilized to dryness. δ ^{1}H NMR 500 MHz (D_2O): 8.82-8.81 (1H, d, *J* = 5.67 Hz), 8.50-8.46 (1H, td, *J* = 8.04, 1.67), 8.36-8.34 (1H, d, *J* = 8.36), 7.90-7.88 (1H, ddd, *J* = 7.06, 5.71, 0.95),

4.52 (2H, s), 4.19 (2H, s), 4.2-2.9 ($CH_2C\mathbf{H}SO_3Na$ polymer backbone), 3.41-3.39 (2H, t, J = 5.83), 2.9-1.4 ($C\mathbf{H_2}CHSO_3Na$ polymer backbone), 1.20 (9H, s). IR (cm^{-1}): 3419, 2957, 2878, 1725, 1642, 1448, 1366, 1148, 1027, 710, 610, 508. The NMR spectrum of the polymer was calibrated to the peak at 8.82-8.81 ppm, and the M_n of the polymer were calculated using the formula: [(polymer integrals 4.2-2.9 ppm)-4] * MW monomer * MW CTA. The polymer was synthesized with M_n by ^{1}H NMR of 17.9 kDa and a degree of polymerization of 135. The GPC trace in water showed M_n of 6.2 kDa with $Đ$ of 1.16.

Synthesis of p(SS-co-PEGMA): RAFT polymerization was performed with initial feed ratios of [SS]:[PEGMA]:[CTA]:[AIBN] = 35:10:1:0.2 and a monomer concentration of 1.0 M. Styrene sulfonate (382 mg, 1.85 mmol) was dissolved in 1.2 mL of degassed DMF in a Schlenk tube. CTA 3 (20 mg, 0.053 mmol) was dissolved in 1.2 mL of degassed water along with PEGMA M_n 300 (159 mg, 0.53 mmol) and AIBN (1.74 mg, 0.011 mmol) and then transferred to the Schlenk tube. The Schlenk tube was sealed and subjected to four freeze-pump-thaw cycles before immersion in an oil bath set to 70 °C. After 4 hours the polymerization was stopped by bubbling air through the Schlenk tube. The polymer was purified by dialysis in 1000 MWCO tubing against 1:1 v/v water:MeOH followed by 100% Milli-Q water. The resulting contents of the dialysis tubing were lyophilized to dryness. ^{1}H NMR 500 MHz (D_2O) δ: 8.40 (1H, s), 8.2-6.2 ($NaSO_3C_6\mathbf{H_4}$ side chains), 4.2-2.8 (PEGMA side chains), 2.8-0.0 (polymer backbone). The ^{1}H NMR spectrum of the resulting polymer was calibrated to the peak at 8.40 ppm, and the M_n of the polymer was calculated using the formula: M_n = [(integral of 8.2 – 6.2 ppm /4)*MW SS monomer]+[(integral of 4.2-2.8 ppm /20.4)*MW PEGMA monomer]+MW CTA. The M_n by NMR was 17.2 kDa. GPC was performed in DMF and M_n was calculated to be 19.8 kDa with $Đ$ of 1.21.

Cell Viability/Cytotoxicity: HUVECs or HDFs were trypsinized and resuspended in growth medium. The cells were plated at a concentration of 2,000 cells/well/100 μL and were incubated at 37 °C, 5% CO_2 over 16 hours to allow cells to adhere. After incubation the medium was replaced with 100 μL of polymer solutions in growth medium and incubated for another 24 hours at 37 °C, 5% CO_2. Extent of cell viability was assessed using a LIVE/DEAD® viability/cytotoxicity assay. The cells were washed with D-PBS then incubated with 1 μM calcein AM and 4 μM ethidium homodimer-1 in D-PBS at 37 °C, 5% CO_2. Fluorescent images were captured using red and green channels. NIH ImageJ Software was utilized to count the number of live and dead cells. Each sample assay was performed with 6 repeats.

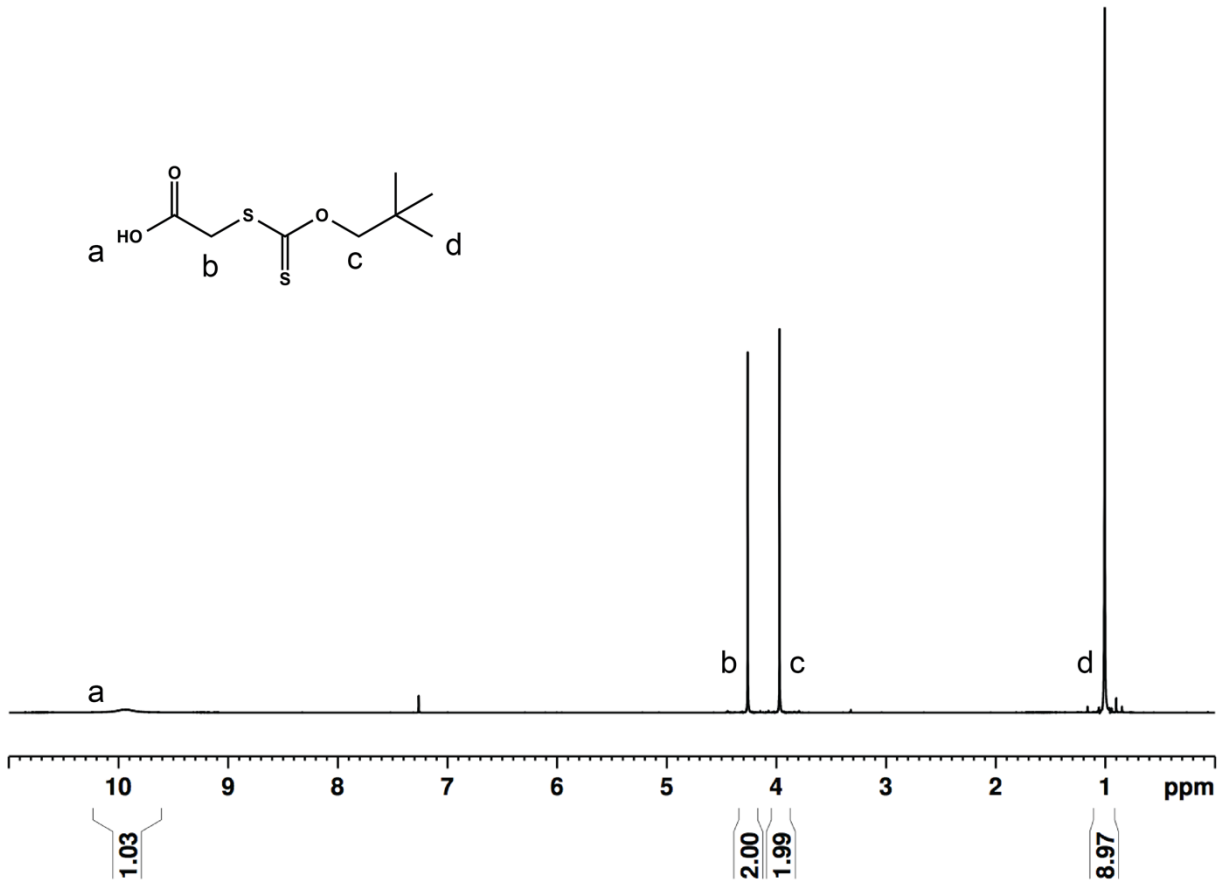


Figure S1. ^{1}H NMR (400 MHz, $CDCl_3$) of (**2**): 2-(((neopentyloxy)carbonothioyl)thio)acetic acid

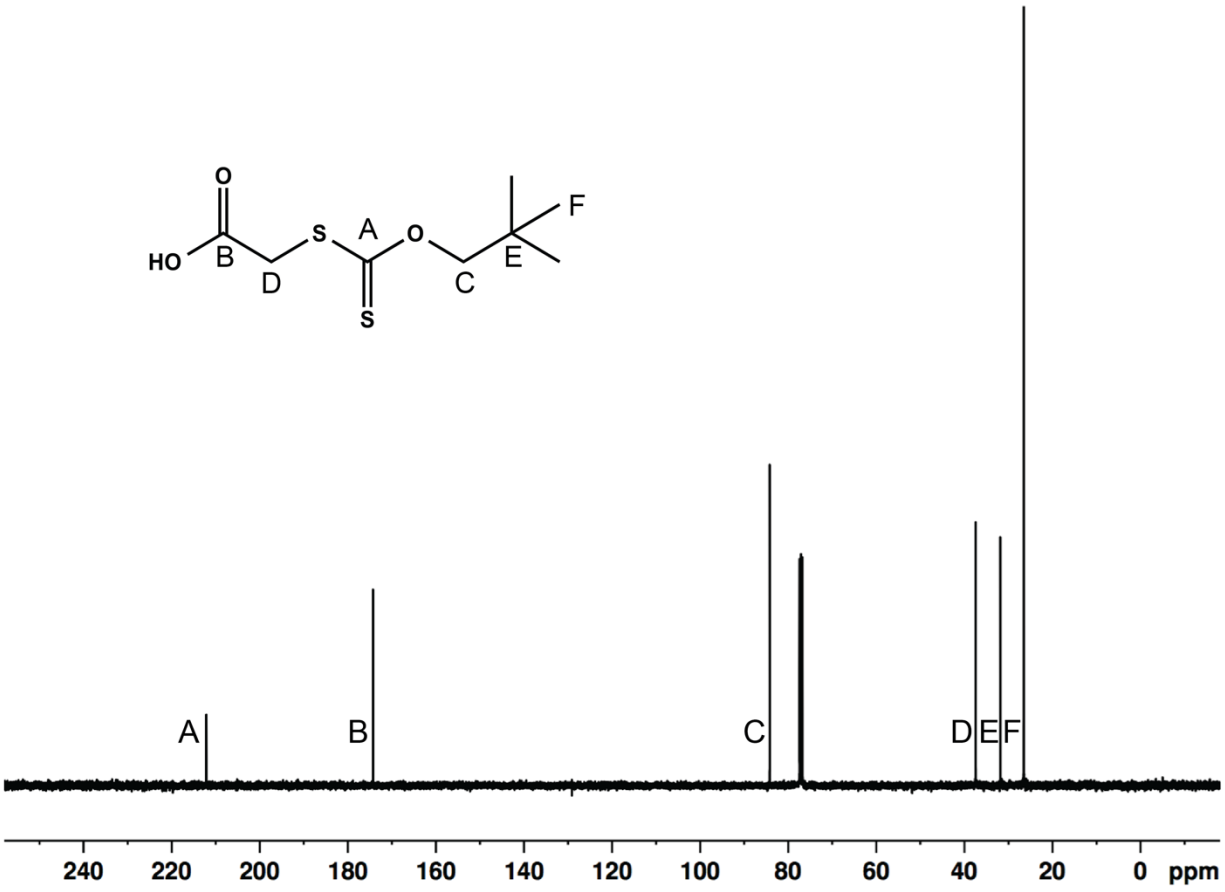


Figure S2. ^{13}C NMR (400 MHz, $CDCl_3$) of (**2**): 2-(((neopentyloxy)carbonothioyl)thio)acetic acid.

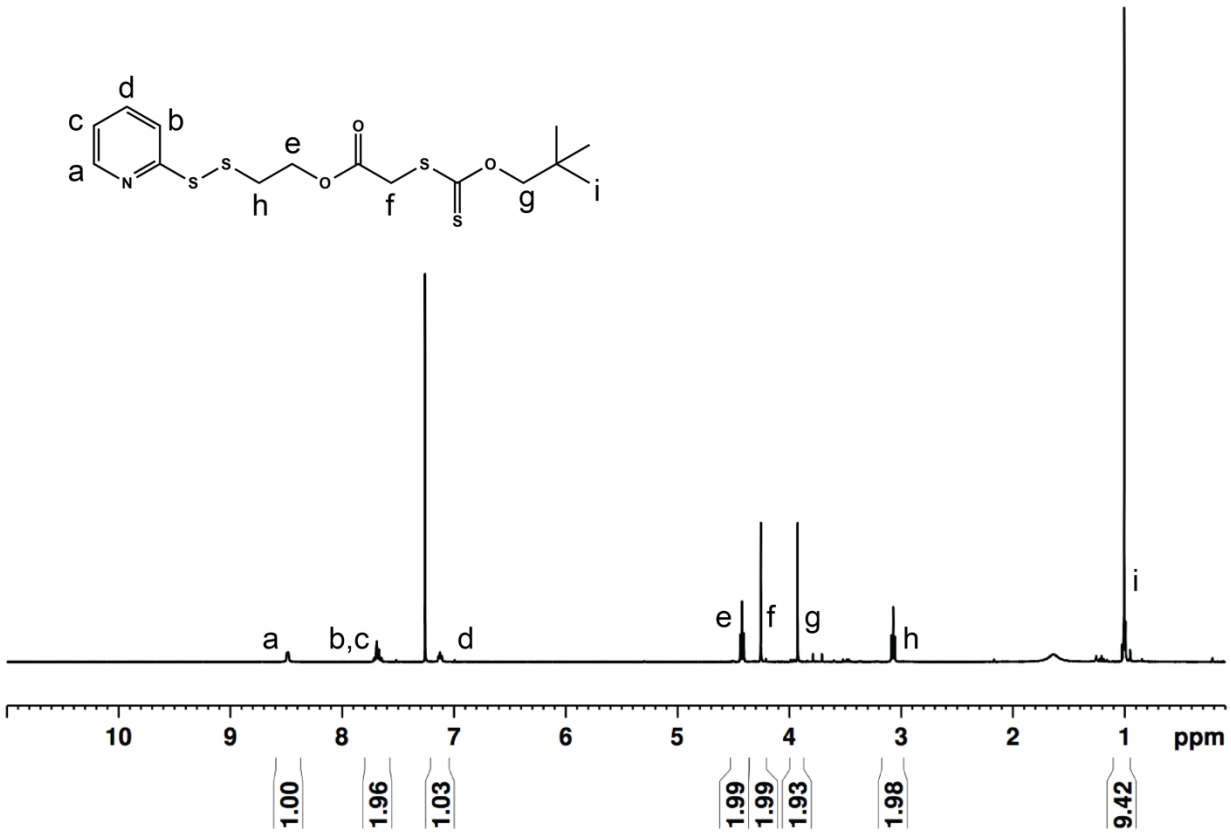


Figure S3. ^{1}H NMR (400 MHz, $CDCl_3$) of (**3**): 2-(pyridin-2-yldisulfanyl)ethyl 2-(((neopentyloxy)carbonothioyl)thio)acetate.

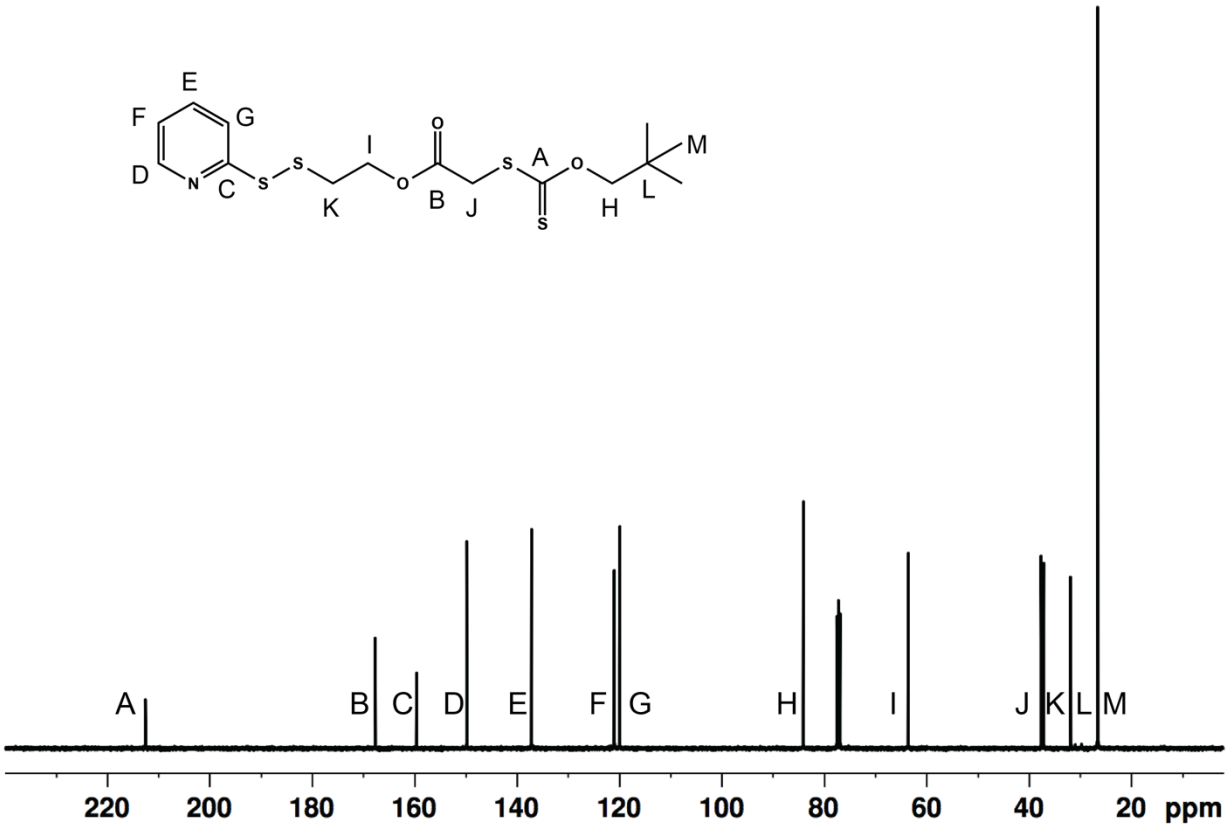


Figure S4. ^{13}C NMR (400 MHz, $CDCl_3$) of (**3**): 2-(pyridin-2-yldisulfanyl)ethyl 2-(((neopentyloxy)carbonothioyl)thio)acetate.

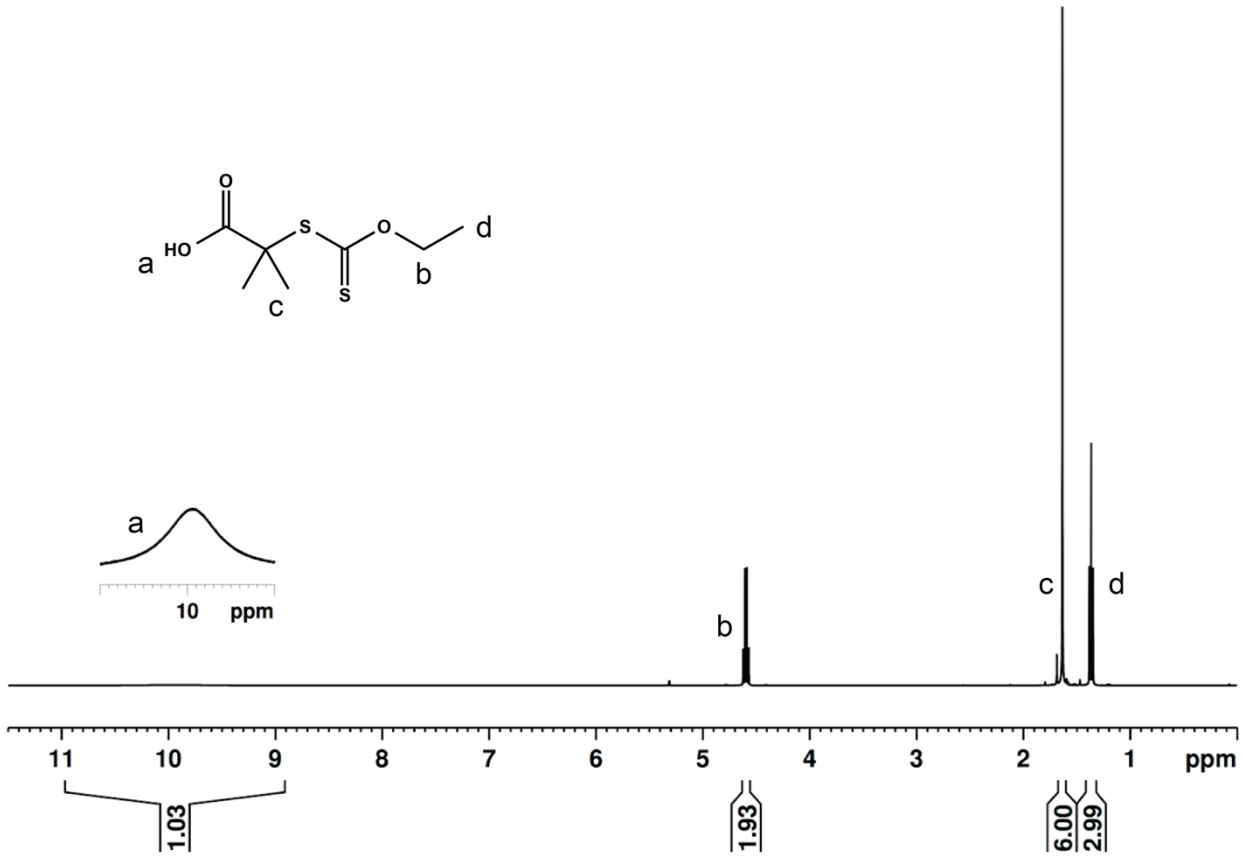


Figure S5. ^{1}H NMR (400 MHz, $CDCl_3$) of **(1).**

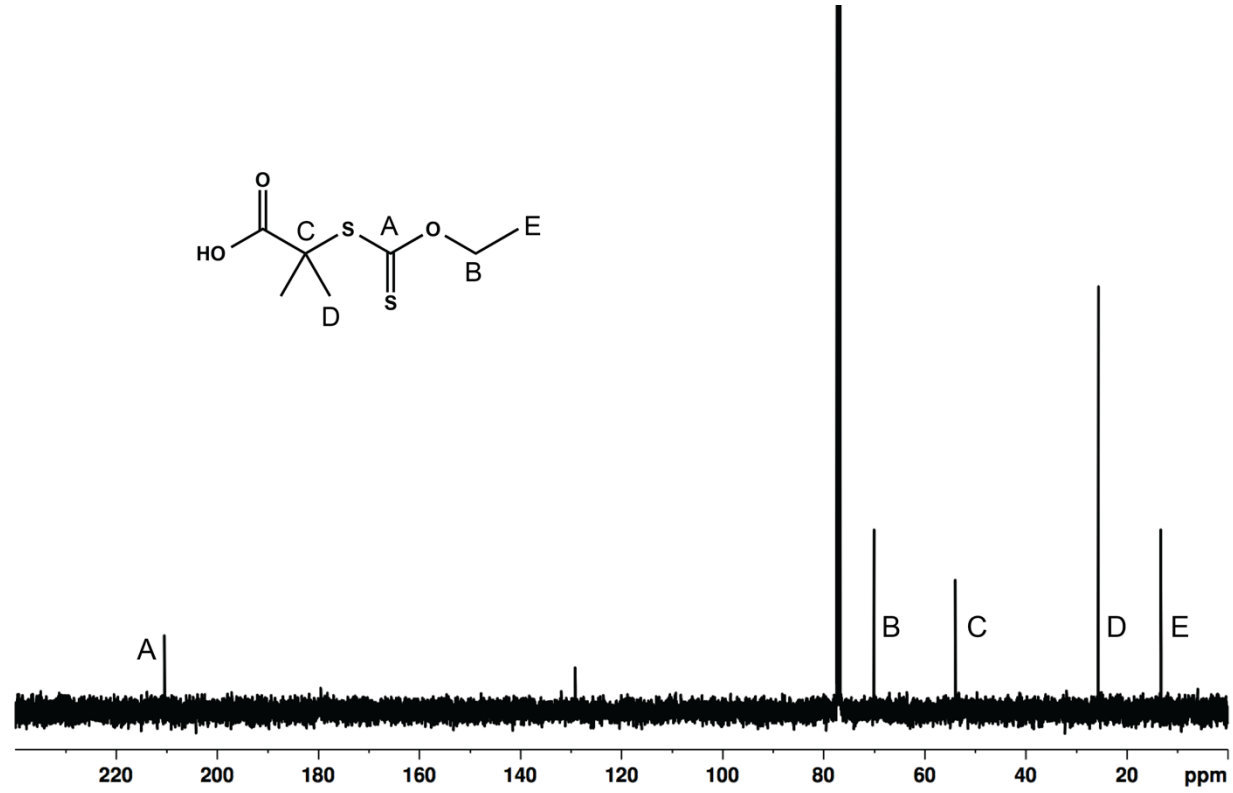


Figure S6. ^{13}C NMR (400 MHz, $CDCl_3$) of **(1).**

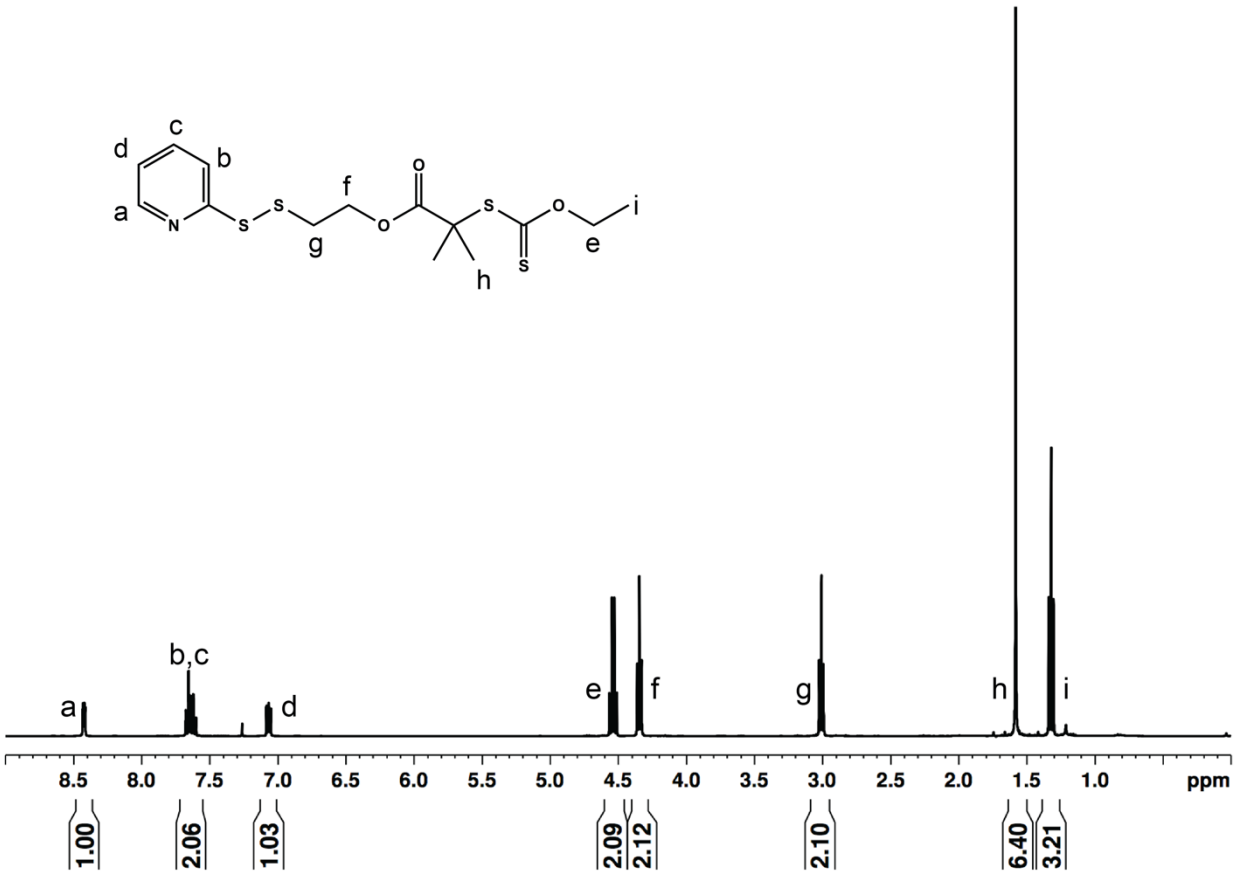


Figure S7. ^{1}H NMR (400 MHz, $CDCl_3$) of **CTA**: 2-(pyridin-2-yldisulfanyl)ethyl 2-((ethoxycarbonothioyl)thio)-2-methylpropanoate.

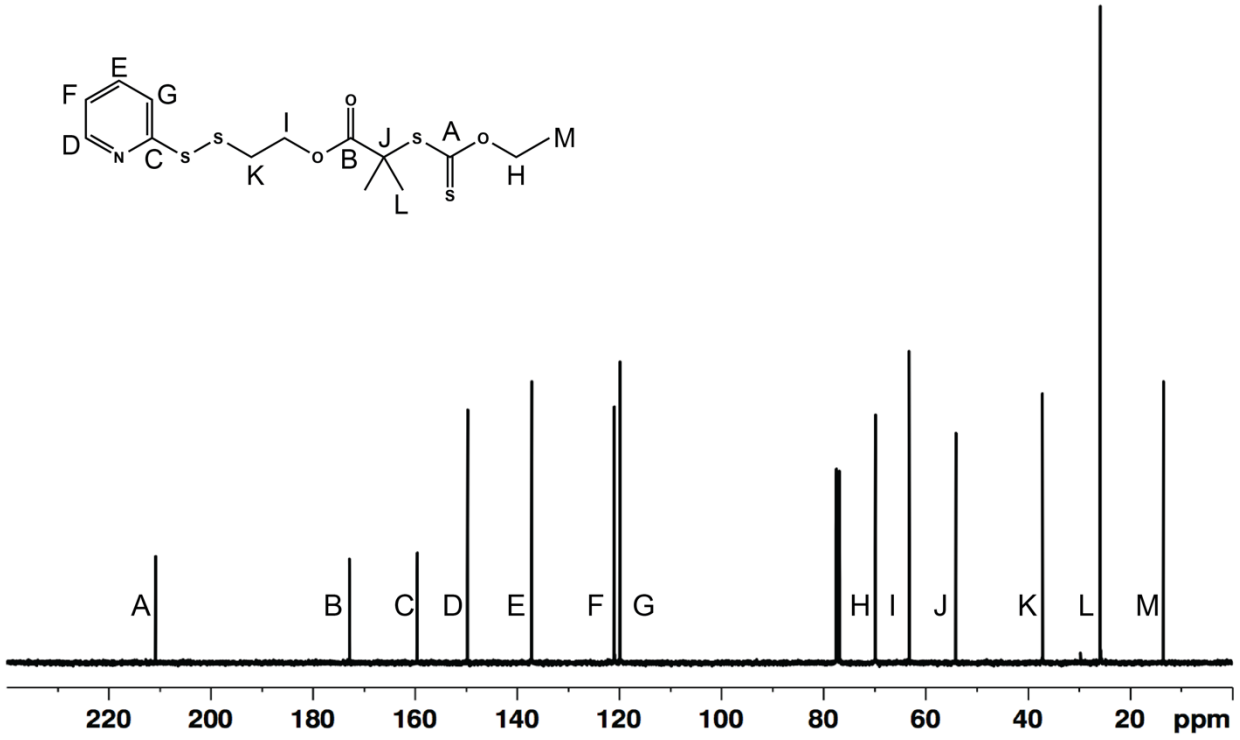


Figure S8. ^{13}C NMR (400 MHz, $CDCl_3$) of **CTA**: 2-(pyridin-2-yldisulfanyl)ethyl 2-((ethoxycarbonothioyl)thio)-2-methylpropanoate.

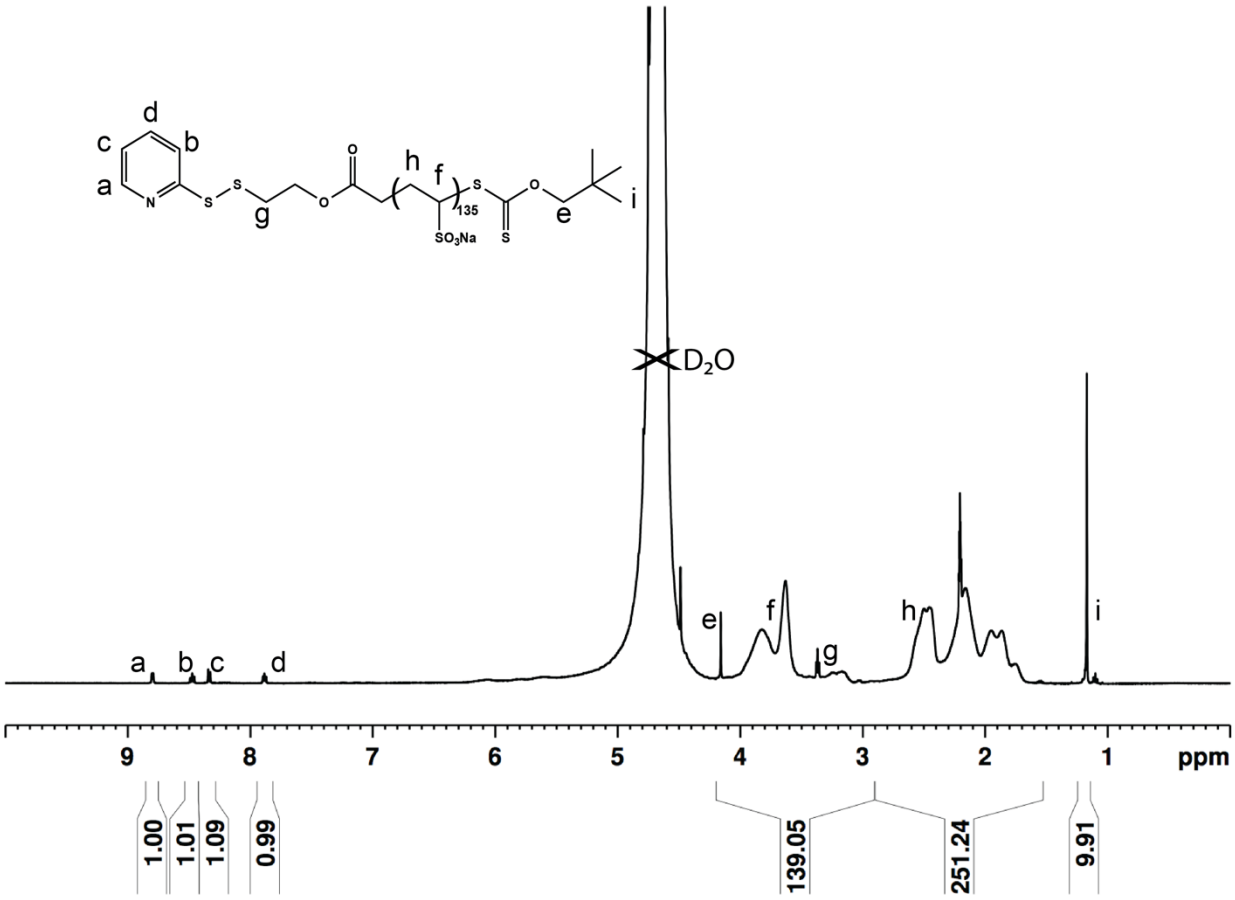


Figure S9. ^{1}H NMR (400 MHz, D_2O/CD_3CN) of pVS.

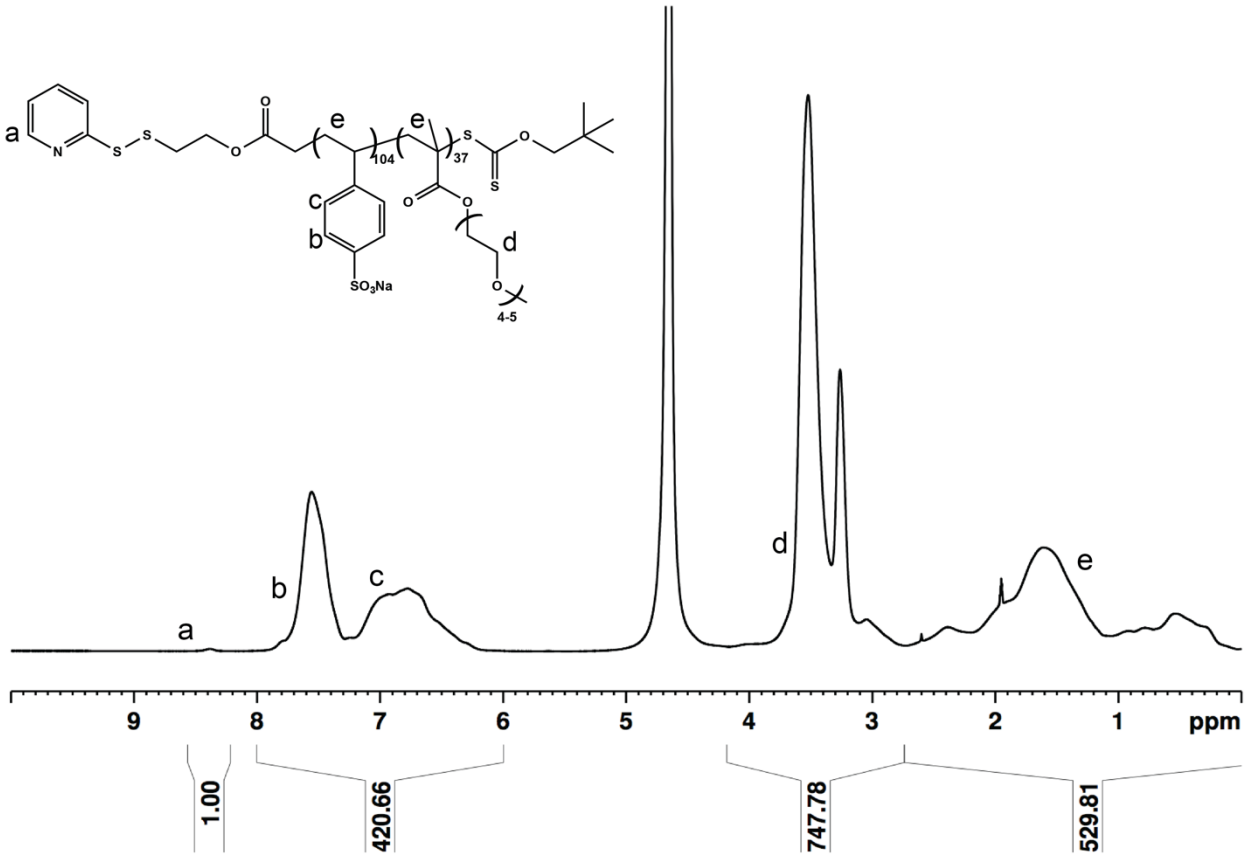


Figure S10. ^{1}H NMR (400 MHz, D_2O) of p(SS-*co*-PEGMA) macroCTA.

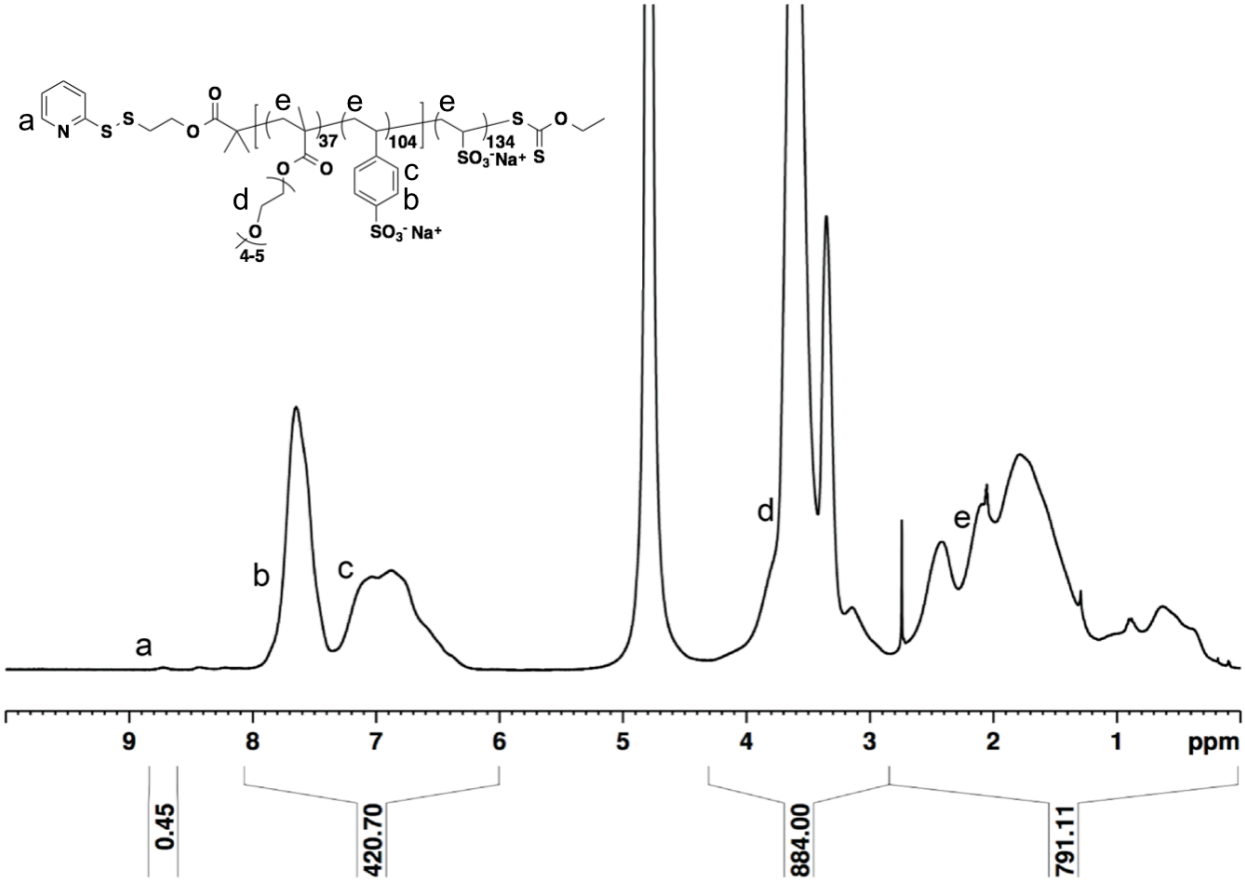


Figure S11. ^{1}H NMR (400 MHz, D_2O) of p(SS-*co*-PEGMA)-*b*-VS.

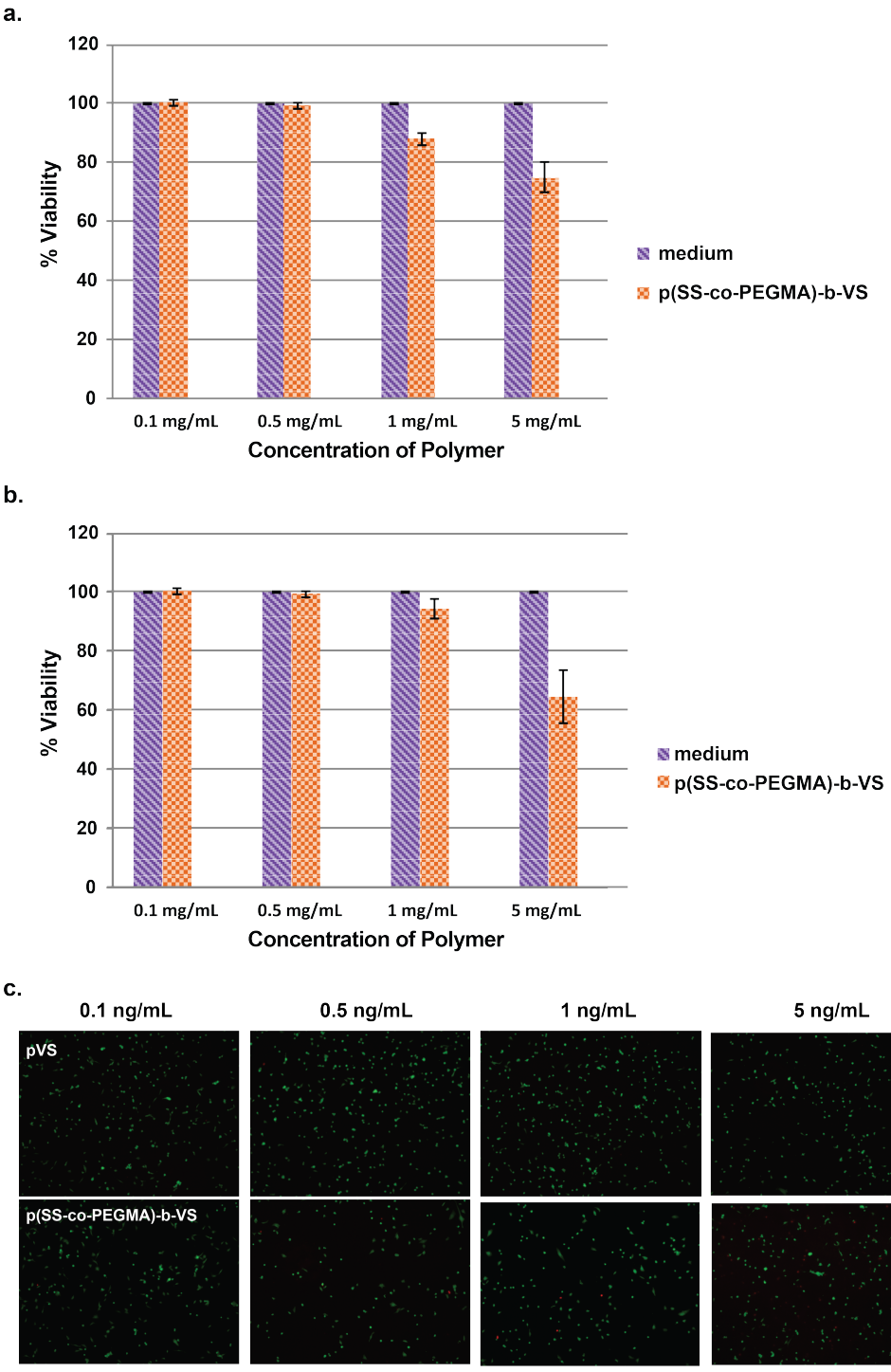


Figure S12. Polymer cytotoxicity in a) human umbilical vein endothelial cells and b) human dermal fibroblasts (100% represents value for blank media). c) representative images of cells with live cells stained green and dead cells stained red.

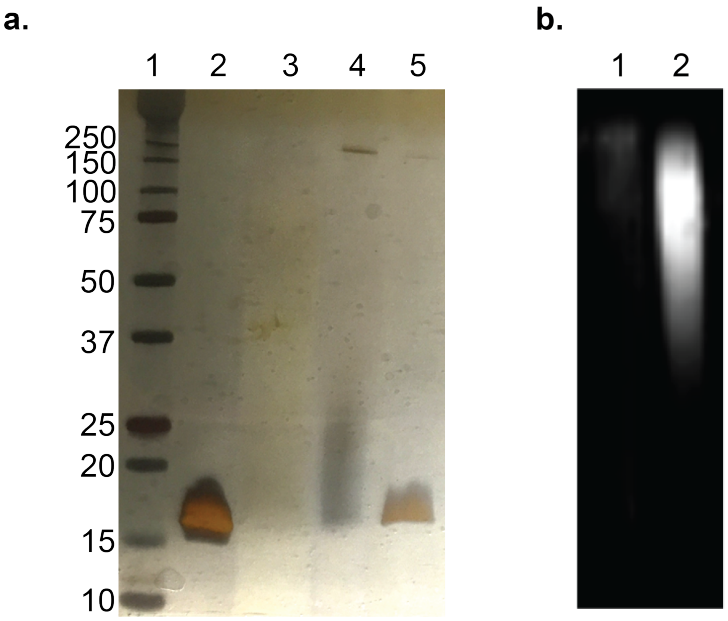
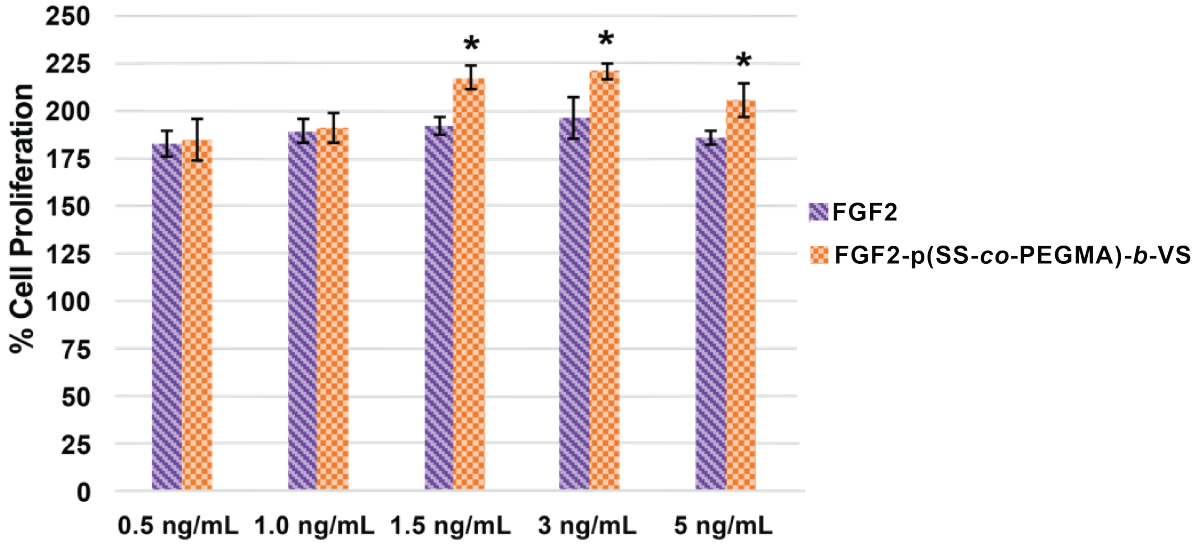


Figure S13. a) SDS-PAGE of crude conjugate (stained with silver nitrate). Lane 1: Protein ladder; Lane 2: FGF2; Lane 3: p(SS-*co*-PEGMA)-*b*-VS; Lane 4: FGF2-p(SS-*co*-PEGMA)-*b*-VS in non-reducing conditions; Lane 5: FGF2-p(SS-*co*-PEGMA)-*b*-VS in reducing conditions. b) Western blot of native-PAGE of purified conjugate. Lane 1: FGF2; Lane 2: FGF2-p(SS-*co*-PEGMA)-*b*-VS.

a.



b.

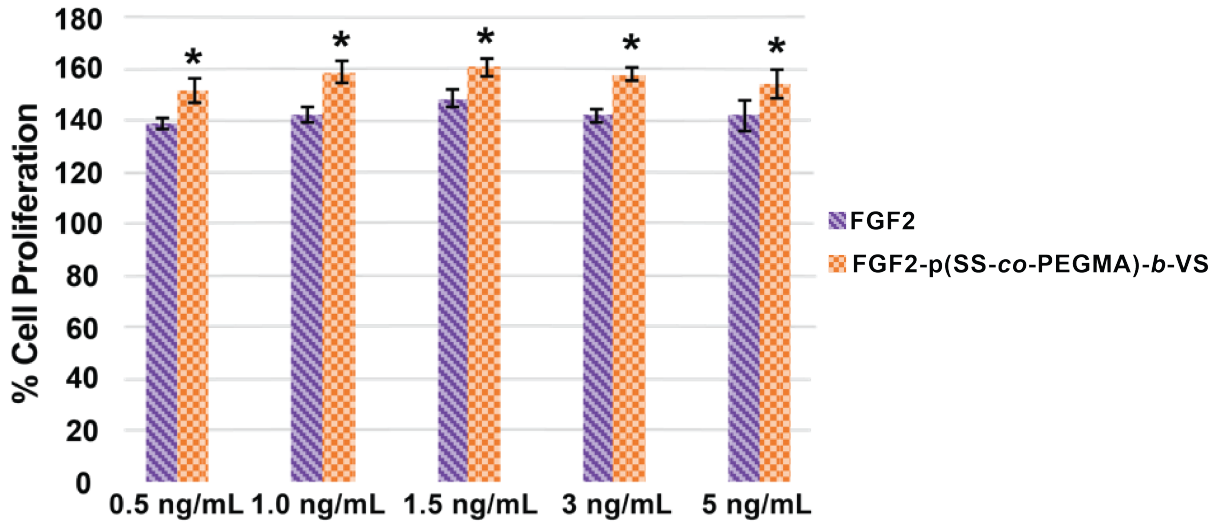


Figure S14. a) HDF cell growth when incubated with FGF2 or FGF2-p(SS-co-PEGMA)-b-VS. b) HUVEC cell growth when incubated with FGF2 or FGF2-p(SS-co-PEGMA)-b-VS. Data was normalized to cells incubated with medium alone (set to 100%). Six replicates per sample, error bars represent standard deviation. Statistical analysis was done using Student's t-test. * $p<0.01$ compared to FGF2.

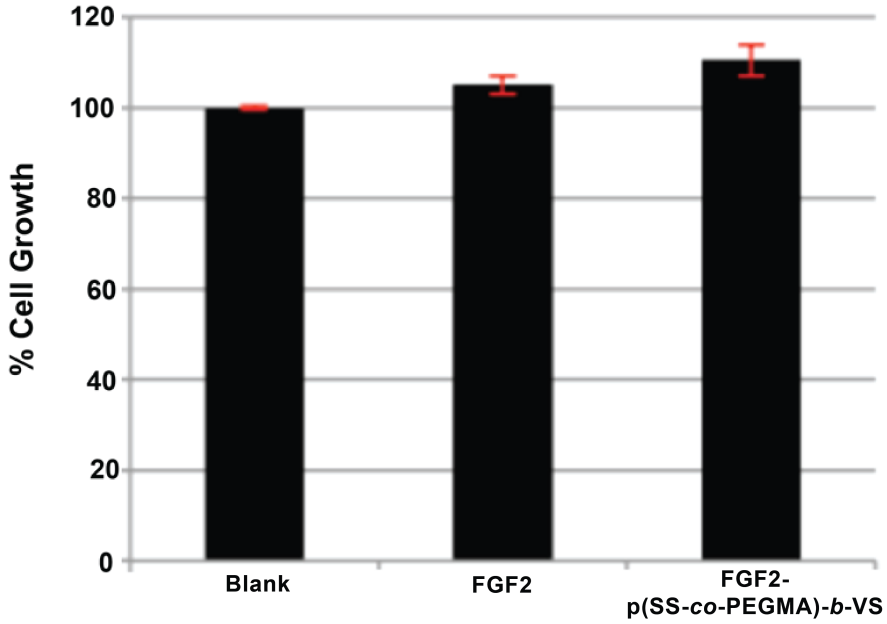


Figure S15. HUVEC growth measured after 18-hour incubation using CellTiter-Blue®. Data was normalized to blank medium (0%). Error bars represent SEM.