

Supporting Information: Synthesis of Poly(meth)acrylates with Thioether and Tertiary Sulfonium Groups by ARGET ATRP and their use as siRNA Delivery Agents

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Experimental

Materials and Methods

Instrumentation. Monomer conversion was measured using ¹H NMR spectroscopy in DMSO-d₆ using a Bruker Avance 500 MHz spectrometer at 27 °C. Number and weight average molecular weights (M_n and M_w) and the dispersity index (M_w/M_n) were estimated by gel permeation chromatography (GPC). For pMTE(M)A, analysis was conducted using (Polymer Standards Services (PSS) columns (guard, 10⁵, 10³, and 10² Å) with THF eluent at 35° C, flow rate = 1.00 mL/min and differential refractive index (RI) detector (Waters 2414)). Linear PMMA was used for standards using WinGPC 7.0 software from PSS. For t-MTE(M)A, a Water 2695 Series with a data processor, equipped with three columns (Waters Ultrahydrogel Linier, 500 and 250), using 100 mM sodium phosphate buffer with 0.2 vol % trifluoroacetic acid (pH 2.5) as an eluent at a flow rate 0.5 mL/min, with detection by a refractive index (RI) detector. Polyethylene glycol standards were used for calibration.

Synthesis of 2-(methylthio)ethyl methacrylate (MTEMA). EDC-HCl, (12.04 g, 77.5 mmol) was dissolved in dry 50 mL DCM in a 100 mL RBF which was then submerged in an ice bath. 2-(Methylthio)ethanol (5 g, 4.71 mL, 54.3 mmol) was added to the flask with stirring. Then MMA (5.13 g, 5.06 mL, 60 mmol) [or AA (4.3 g, 4.1 mL, 60 mmol)] was added and solution became clear, pale yellow. Lastly, DMAP (0.073 g, 0.60 mmol)

was added and the flask was sealed and contents stirred for 48 hours. After the reaction was completed, the solution was poured into a separatory funnel and washed with saturated sodium bicarbonate solution three times, then water, and brine. The solution was dried and solvent was removed to form 2-(methylthio)ethyl methacrylate (MTEMA) in 63.0% yield. MTEMA: ^1H NMR (δ , ppm, in CDCl_3): 6.14 (s, 1H, =CH), 5.60 (s, 1H, =CH), 4.34 (t, 2H, CH_2O), 2.79 (t, 2H, CH_2S), 2.19 (s, 3H, CH_3S), and 1.97 (s, 3H, CH_3C). MTEA: ^1H NMR (δ , ppm, in CDCl_3): 6.41-6.47 (dd, 1H, =CH), 6.10-6.19 (dd, 1H, =CH), 5.84-5.88 (dd, 1H, =CH), 4.35 (t, 2H, CH_2O), 2.78 (t, 2H, CH_2S), and 2.18 (s, 3H, CH_3S). Figure S1,S2

General Procedure for ARGET ATRP of MTEMA and MTEA. A series of ARGET ATRP reactions were carried out under systematically varied conditions to determine optimal conditions for ARGET ATRP of MTEMA and MTEA.

(2-Methylthio)ethyl methacrylate (MTEMA) (0.5 g, 3.0 mmol), copper(II) chloride (0.4 mg, 3.0 μmol , TPMA (2.6 mg, 9 μmol), EBPA (11 mg, 7.8 μL , 45 μmol), and acetone (0.5 mL) were added to a 5 ml RBF and a Schlenk line adapter was added and the flask sealed. A solution of 55 mg/mL ascorbic acid in 1:1 acetone:DMF was prepared and then the flask was sealed. Both solutions were degassed *very* slowly for 10 minutes. Then 0.1 mL of ascorbic acid solution was added to the reaction flask and the flask was placed in a 35 $^\circ\text{C}$ oil bath. The polymerization was allowed to proceed for 24 hours.

(2-Methylthio)ethyl acrylate (MTEA) (0.5 g, 3.4 mmol), copper(II) bromide (0.76 mg, 3.0 μmol , TPMA (2.8 mg, 10.0 μmol), EBiB (9.5 mg, 7.2 μL , 49 μmol), and acetone (0.5 mL) were added to a 5 ml RBF and a Schlenk line adapter was inserted and then sealed. A solution of 60 mg/mL ascorbic acid in 1:1 acetone:DMF was prepared in a

separate flask and then the flask was sealed. Both solutions were degased *very* slowly for 10 minutes. Then 0.1 mL of the ascorbic acid solution was added to the reaction flask and the flask was placed in a 35 °C oil bath. The polymerization was allowed to proceed for 24 hours.

General Procedure for ARGET ATRP of Dimethyl Sulfonium Methacrylate (DMS⁺MA) and Dimethyl Sulfonium Acrylate (DMS⁺A). A series of ARGET ATRP reactions were carried out with systematically varied conditions to determine optimal conditions for ARGET ATRP of *t*-MTE(M)A.

t-MTEMA (1.0 g, 3.1 mmol), copper(II) chloride (0.37 mg, 2.8 μmol), TPMA (2.7 mg, 9.2 μmol), EBPA (7.5 mg, 5.4 μL, 31 μmol), DMF (0.1 mL), and methanol (0.5 mL) was put in a 5 mL RBF and sealed with a Schlenk adapter. A solution of 50 mg/mL ascorbic acid in methanol was prepared. Both solutions were degased very slowly for ten minutes. Then 0.1 mL of the ascorbic acid solution was added and the reaction flask was placed in a 50 °C oil bath.

t-MTEA (0.5 g, 1.6 mmol) copper(II) chloride (0.27 mg, 2.1 μmol), TPMA (2.0 mg, 7.0 μmol), EBiB (4.5 mg, 3.2 μL, 23.0 μmol), DMF (0.1 mL), and methanol (0.5 mL) were added to a 5 mL RBF and sealed with a Schlenk adapter. A solution of 36.5 mg/mL ascorbic acid in methanol was prepared. Both solutions were degased very slowly for ten minutes then 0.1 mL of the ascorbic acid solution was added and the reaction flask was placed in a 50 °C oil bath.

General Procedure for De-alkylation Studies of DMS⁺A via NMR. A series of experiments were performed via ¹H NMR analysis. 0.3 mL of a solution of 47.2 mM of *t*-MTEA in DMSO-*d*₆ was prepared. A separate 0.3 mL solution of 47.2 mM tetra-*n*-

butylammonium bromide in DMSO-d₆ was also prepared. The two solutions were mixed together and transferred to a NMR tube. Scans were recorded every 12 hours for 72 hours. This was repeated with the chloride salt. The final concentrations of both components were 23.6 mM (Figure S7-S10). To test the effects of an excess of salt, two 0.3 mL solutions of 236 mM were made of both salts and the same procedure was repeated. To test the effects of a more polar solvent the previous procedure was repeated in D₂O. Neutral monomer peaks started forming at 4.27 ppm, 2.76 ppm, and 2.11 ppm.

General Procedure for De-alkylation Studies of poly(DMS⁺A) via NMR. The same procedure was repeated for the case of the polymer. Using a $M_n = 8200$, DP ~ 47 . A 0.3 mL solution of 84.7 mM (based on sulfonium) was prepared in DMSO-d₆. A solution of the same concentration of the salt was also prepared. The two solutions were mixed and transferred to a NMR tube. Scans were taken every 24 hours. This was repeated for chloride as well as with a 5 times excess of salt. The procedures were also repeated with D₂O as solvent. Figure S11-S14

Biocompatibility of PEO_{2k}BPA-*b*-poly(DSM⁺MA)_{nk} MC3T3-E1.4 cells were seeded at 25,000 cells/mL in 24-well plates, at 0.5 mL/well. Polymer treatments of 50 µg/mL and 100 µg/mL were administered after seeding. After 24 and 48 hours in culture, cells were washed 3x with 1X phosphate buffered saline (PBS) and co-stained with calcein AM (1:2000 dilution) and ethidium homodimer (EthD-1) (1:500 dilution) in PBS. Cultures were incubated at 37 °C for 30 minutes away from light, and visualized at 10x magnification.

MTS-based viability was performed to quantitate cell viability, and was performed using the CellTiter 96® AQueous One Solution kit (Promega, G380). MC3T3 cells were

seeded at 25,000 cells/mL in 96-well plates, at 0.2 mL/well. Polymer treatments were administered at 50, 100, 200 and 400 $\mu\text{g/mL}$. The MTS assay was executed 48 hours after treatments, as per the manufacturers directions. Briefly, existing culture medium was aspirated and replaced with 100 μL fresh complete medium. 20 μL MTS reagent was added to each well and incubated at 37 °C and 5% CO_2 for 1 hour. Sample absorbance was measured at 492 nm. Absorbance values from media-alone groups were subtracted from all other groups; cohorts receiving polymer doses were normalized to untreated cell cohorts to calculate percent cell viability.

Extended viability studies were conducted using the Quant-iT™ PicoGreen® dsDNA Assay kit (Life Technologies, P7589). Cells were seeded at 15,000 cells/mL in 24-well plates at 0.5 mL/well. Polymer treatments were delivered every 48 hours along with fresh α -MEM culture medium (10% fetal bovine serum, 1% penicillin/streptomycin). At experimental endpoints (days 4, 7 and 10), cells were lysed using Cell Signaling Lysis Buffer (Cell Signaling Technology, #9803). PicoGreen-based DNA content was evaluated after combination of 100 μL lysates with 100 μL PicoGreen dye (1:200 dilution in 1X TE buffer) for 3 minutes in 96-well assay plates. Sample fluorescence was analyzed at 492 nm (excitation) and 520 nm (emission) wavelengths.

PEO_{2k}BPA-*b*-poly(DSM⁺MA)_{nk}-siRNA Complexation Analysis siRNA complexation was determined by preparing weight:weight ratios of polymers with siRNA (Ambion, AM4611) in nuclease-free water (Ambion, AM9932). The total volume of each solution was 25 μL and siRNA quantities were held constant among samples at 500 ng. Samples were incubated at 4 °C for 1 hour, after which glycerol (Sigma-Aldrich, G5516) was added to a final volume of 10% as a loading buffer. Polymer:siRNA solutions were

loaded into 2% agarose (Sigma-Aldrich, A5304) gels in 1X TBE buffer (Bio-Rad Laboratories, #161-0770EDU). A 100V potential was applied across gels for 30 minutes, after which they were stained in 0.5 $\mu\text{g/mL}$ ethidium bromide (Sigma-Aldrich, E1510) in 1X TBE buffer for 1 hour. Gels were imaged under UV transillumination at 365 nm. For siRNA protection studies, samples were treated with 0.1U RNase A (Sigma-Aldrich, R4875) for 30 minutes prior to addition of glycerol loading buffer. For siRNA dissociation studies, polymer:siRNA solutions were incubated with heparan sulfate at the indicated heparan sulfate:siRNA ratio for 30 minutes prior to the addition of glycerol. ImageJ gel analysis was utilized for quantitation of siRNA band intensities.

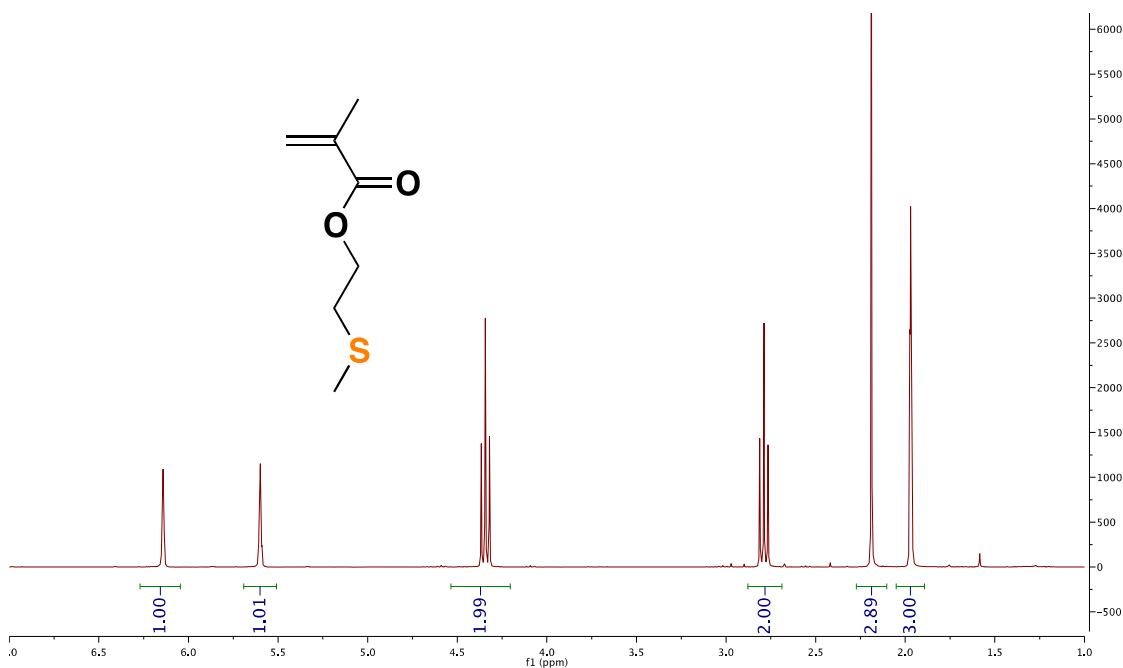


Figure S1. 500MHz ^1H NMR spectrum of MTEMA in CDCl_3 at room temperature.

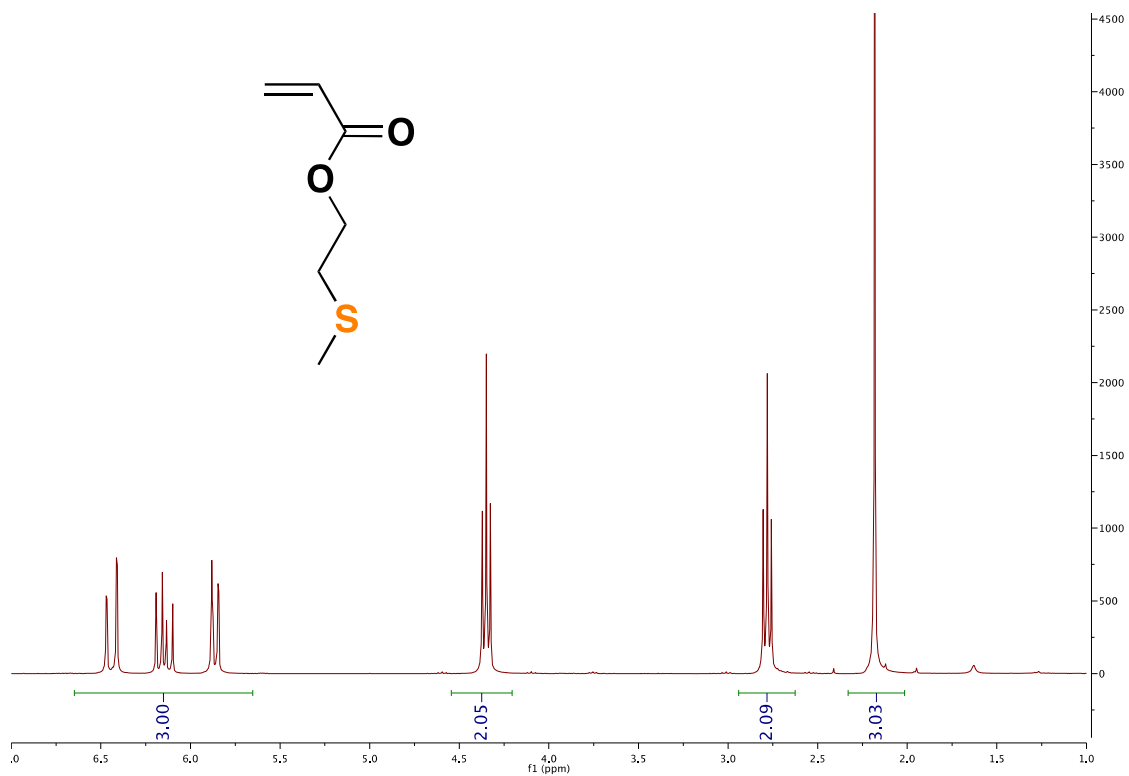


Figure S2. 500MHz ¹H NMR spectrum of MTEA in CDCl₃ at room temperature.

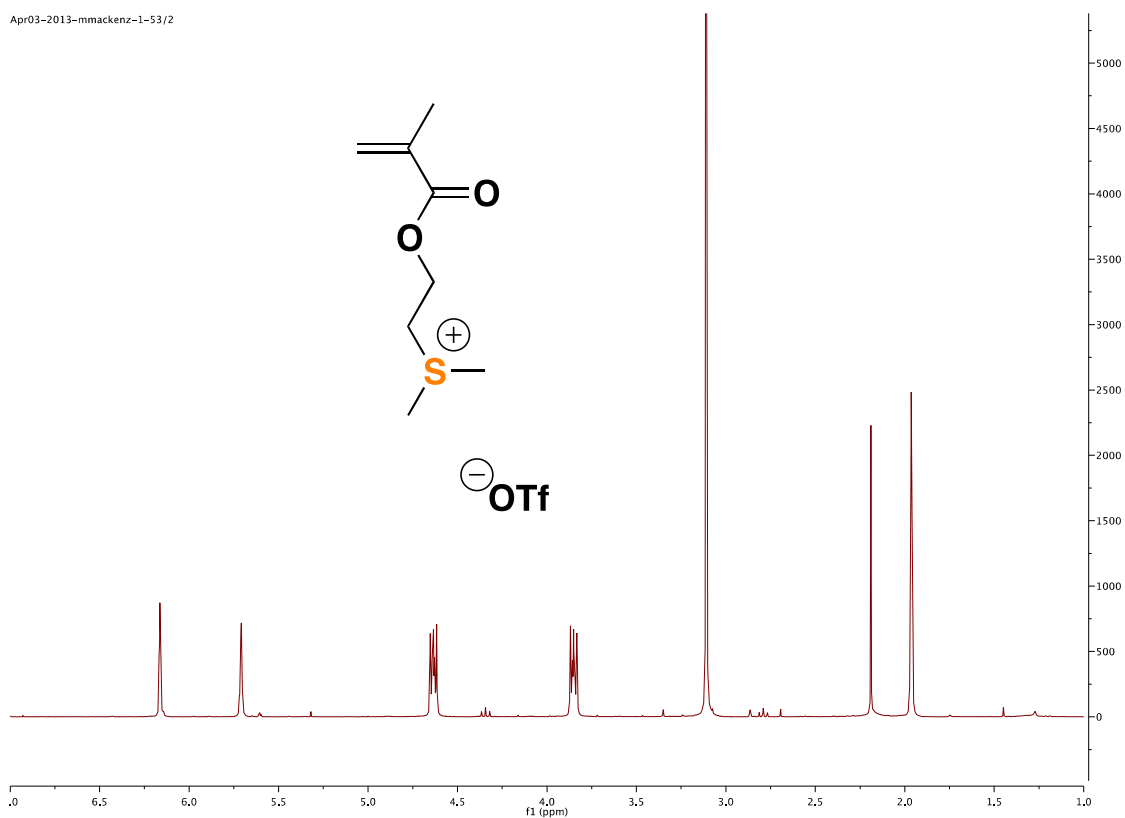


Figure S3. 500MHz ^1H NMR spectrum of DMS^+MA in d -DMSO at room temperature.

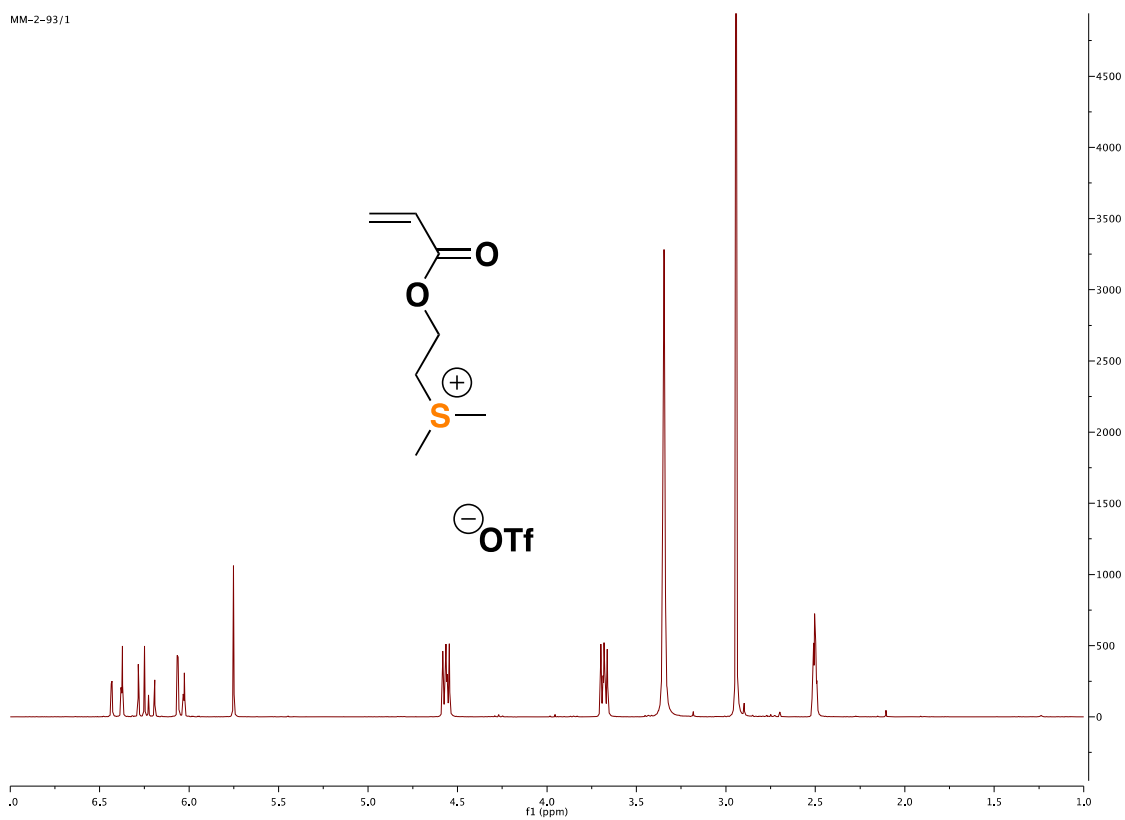


Figure S4. 500MHz ^1H NMR spectrum of DMS⁺A in *d*-DMSO at room temperature.

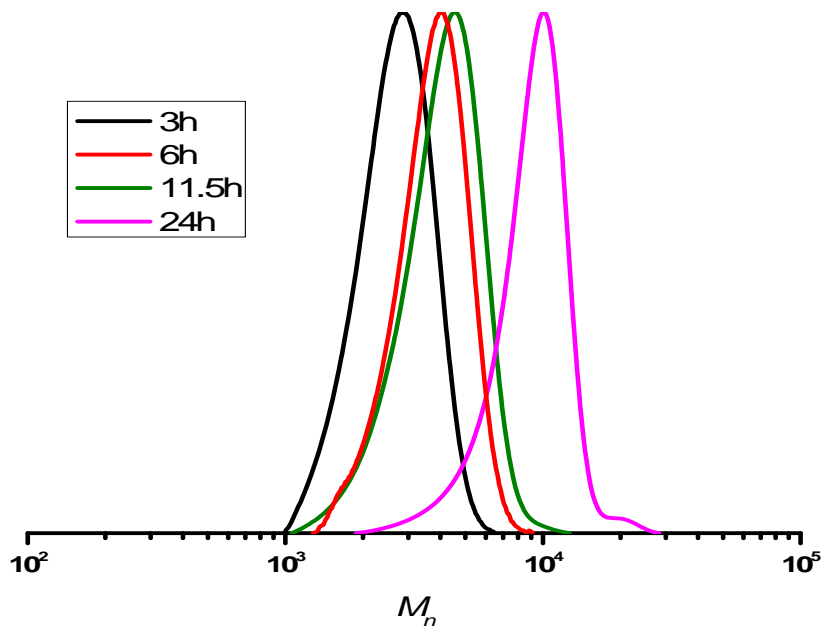


Figure S5. GPC traces of pMTEMA, Table 1, Entry 4. [MTEMA]:[EBPA]:[CuCl₂]:[TPMA]:[Ascorbic Acid]= 70:1:0.07:0.2:0.7, 40% [M] (v/v) at 35 °C.

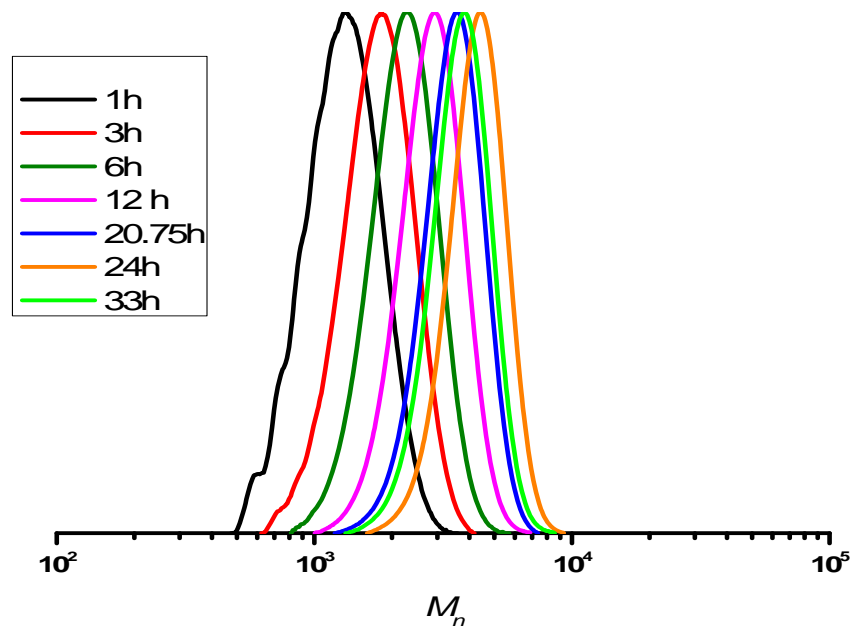


Figure S6. GPC traces of pMTEA, Table 1 Entry 8. [MTEA]:[EBiB]:[CuBr₂]:[TPMA]:[Ascorbic Acid]=100:1:0.09:0.3:0.9, 40% [M] (v/v) at 35 °C.

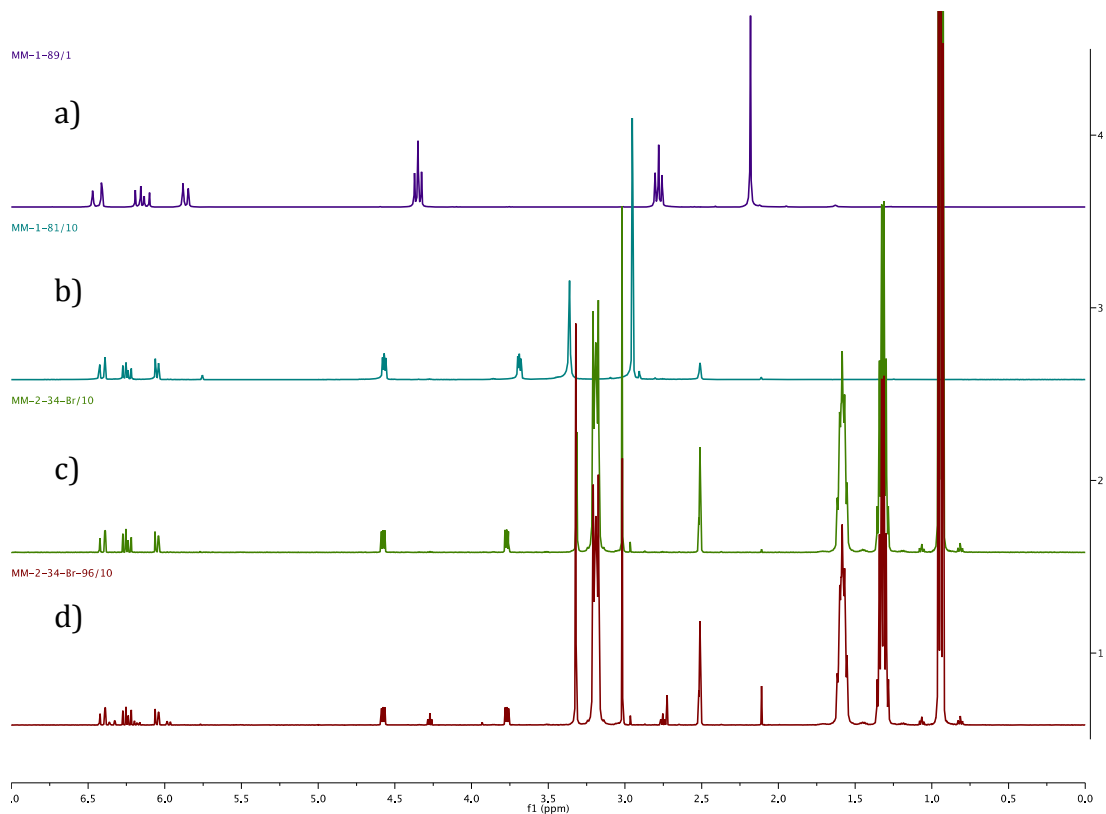


Figure S7. ^1H NMR spectra overlaid during monomer de-alkylation. $\text{DMS}^+\text{A}:\text{Br}^-$ 1:5 in d -DMSO at room temperature. (a) is a spectrum of MTEA. (b) is a spectrum of DMS^+A . (c) is at time = 0h. (d) is at time = 96h. $[\text{DMS}^+\text{A}]_0 = 0.0236$ M, $[\text{Br}^-]_0 = 0.118$ M.

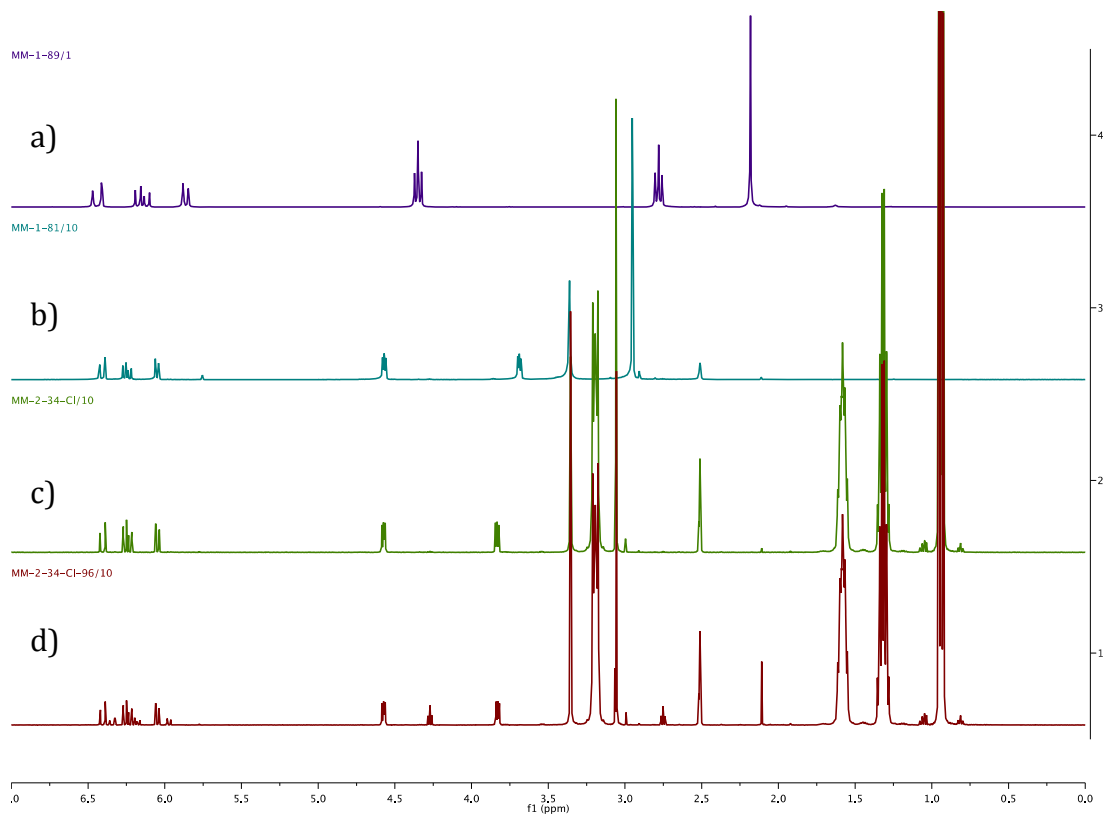


Figure S8. ^1H NMR spectra overlaid during monomer de-alkylation. $\text{DMS}^+\text{A}:\text{Cl}^-$ 1:5 in d -DMSO at room temperature. (a) is a spectrum of MTEA. (b) is a spectrum of DMS^+A . (c) is at time = 0h. (d) is at time = 96h. $[\text{DMS}^+\text{A}]_0 = 0.0236 \text{ M}$, $[\text{Br}^-]_0 = 0.118 \text{ M}$.

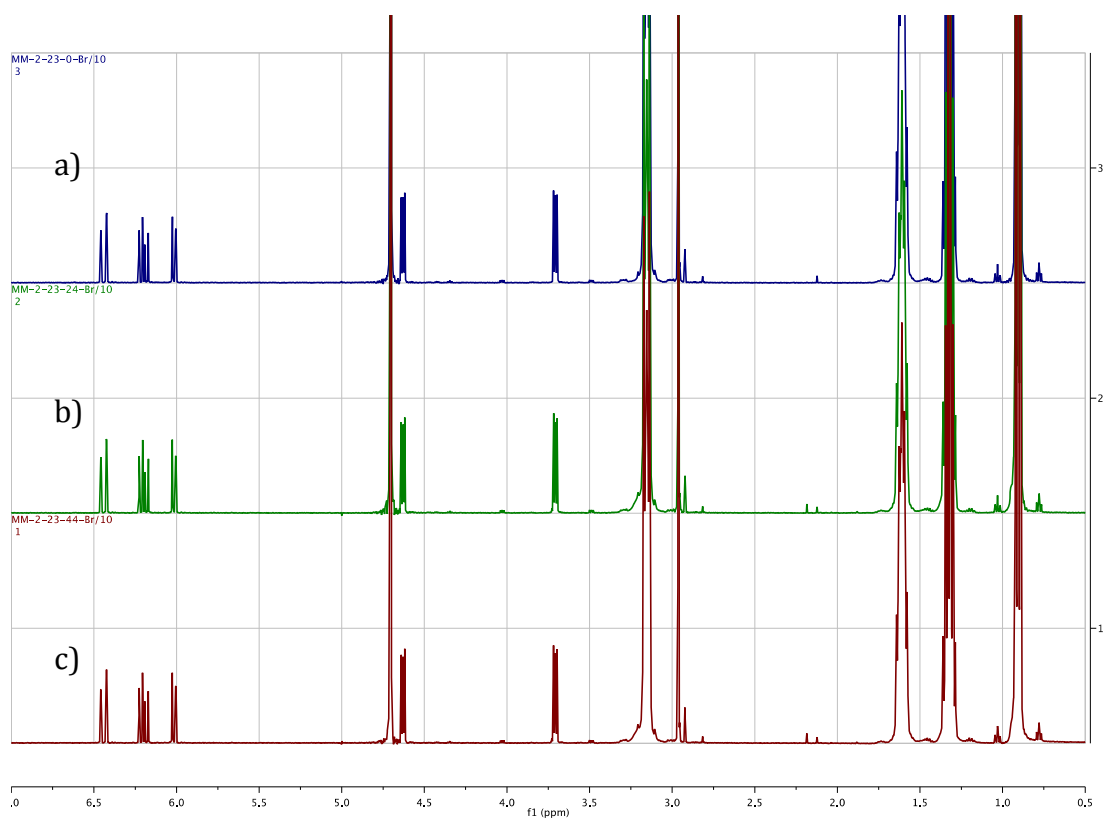


Figure S9. ^1H NMR spectra overlaid during monomer de-alkylation. $\text{DMS}^+\text{A}:\text{Br}^-$ 1:5 in D_2O at room temperature. (a) is at time = 0, (b) is at time = 24h, (c) is at time = 44h. $[\text{DMS}^+\text{A}]_0 = 0.0236 \text{ M}$, $[\text{Br}^-]_0 = 0.118 \text{ M}$.

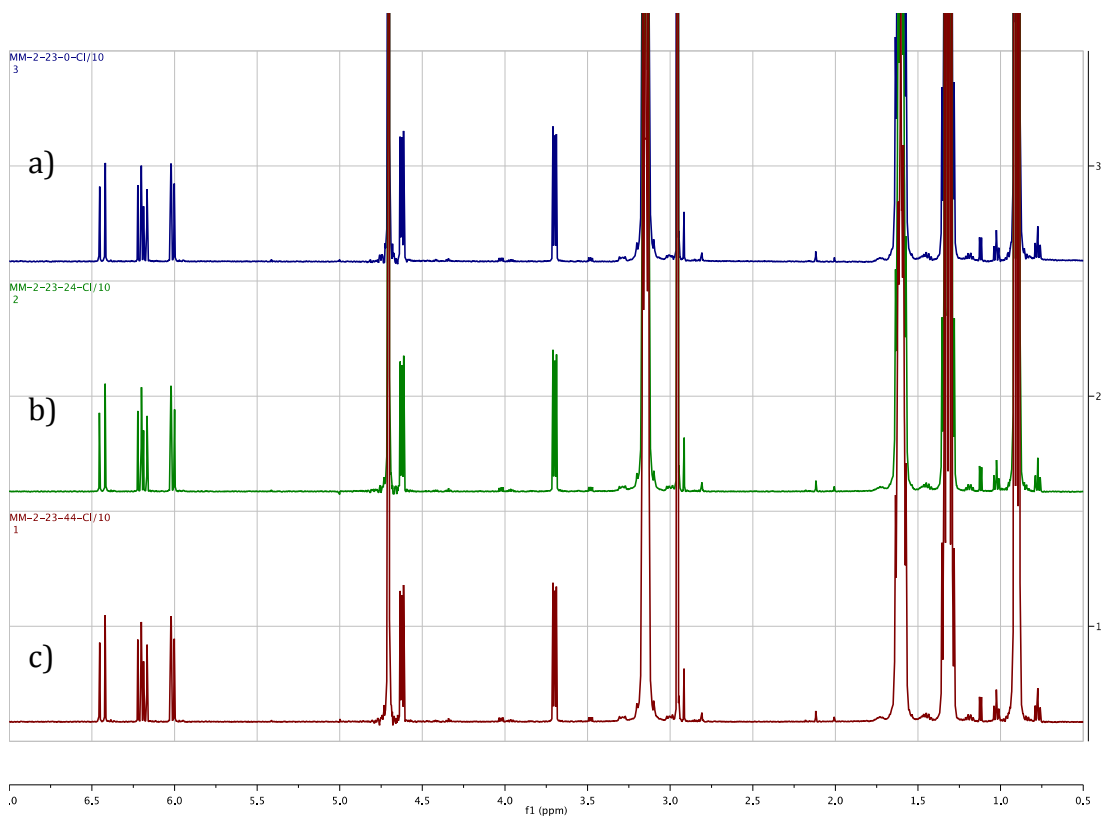


Figure S10. ^1H NMR spectra overlaid during monomer de-alkylation. $\text{DMS}^+\text{A}:\text{Cl}^-$ 1:5 in D_2O at room temperature. (a) is at time = 0, (b) is at time = 24h, (c) is at time = 44h. $[\text{DMS}^+\text{A}]_0 = 0.0236$, $[\text{Cl}^-]_0 = 0.118$ M.

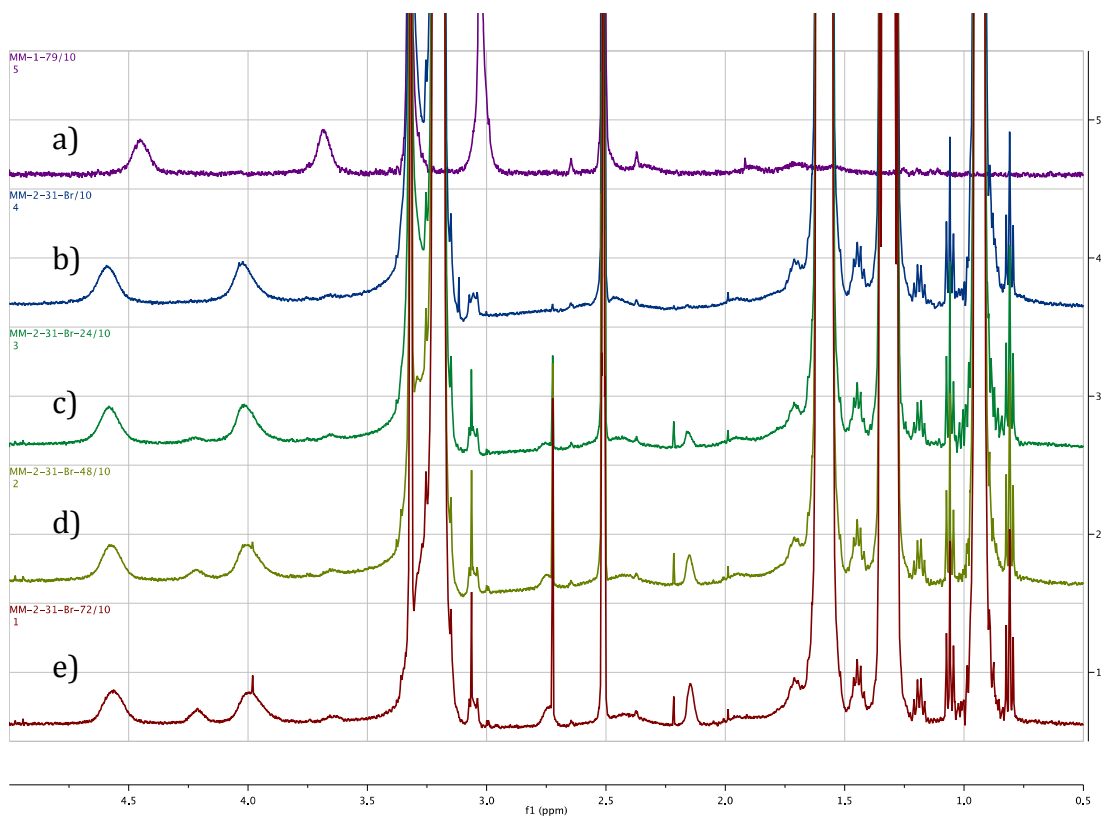


Figure S11. 500MHz ^1H NMR spectra overlaid during de-alkylation of poly(DMS^+A) with 5 eqv of Br^- in DMSO. $M_n = 8200$. (a) is poly(MTEMA), (b) is at time = 0h, (c) is at time = 24h, (d) is at time = 48h, (e) is at time = 72h. . $[\text{poly}(\text{DMS}^+\text{MA})]_0 = 0.0236 \text{ M}$, $[\text{Br}^-]_0 = 0.118 \text{ M}$.

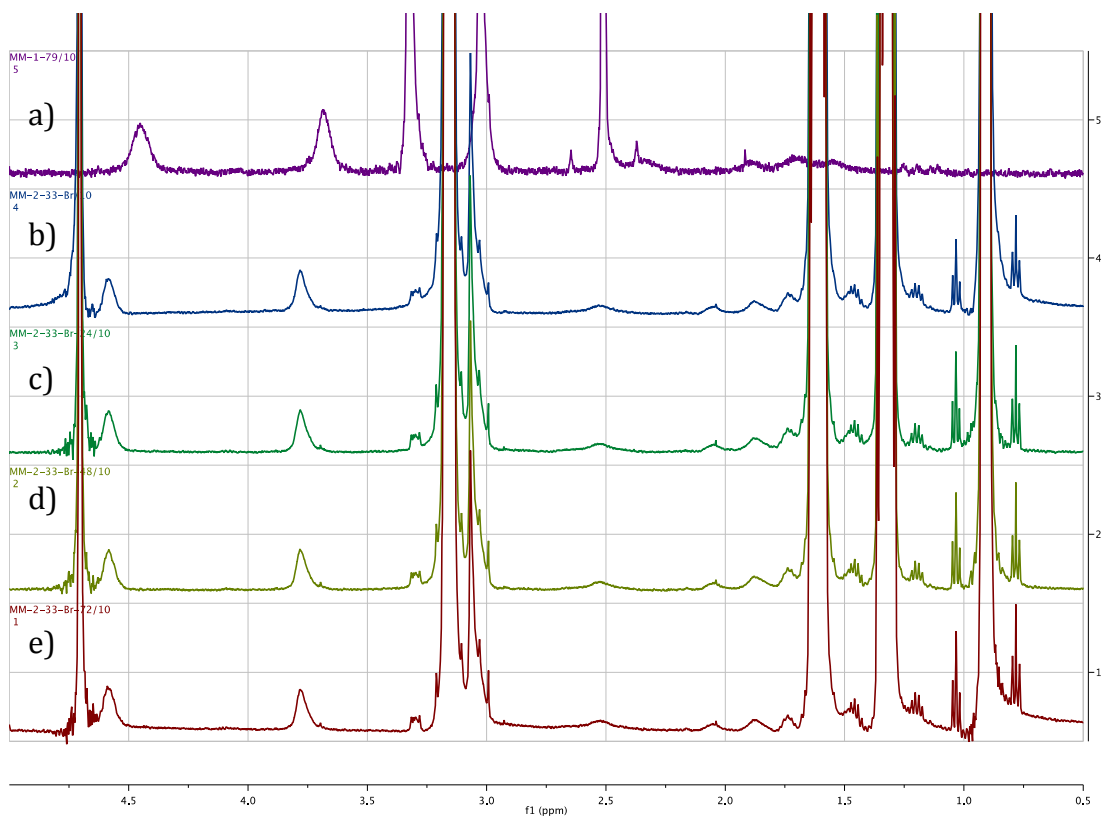


Figure S12. 500MHz ^1H NMR spectra overlaid during de-alkylation of poly(DMS $^+$ A) with 5 eqv of Br^- in D_2O . $M_n = 8200$. (a) is poly(MTEMA), (b) is at time = 0h, (c) is at time = 24h, (d) is at time = 48h, (e) is at time = 72h. $[\text{poly}(\text{DMS}^+\text{MA})]_0 = 0.0236 \text{ M}$, $[\text{Br}^-]_0 = 0.118 \text{ M}$.

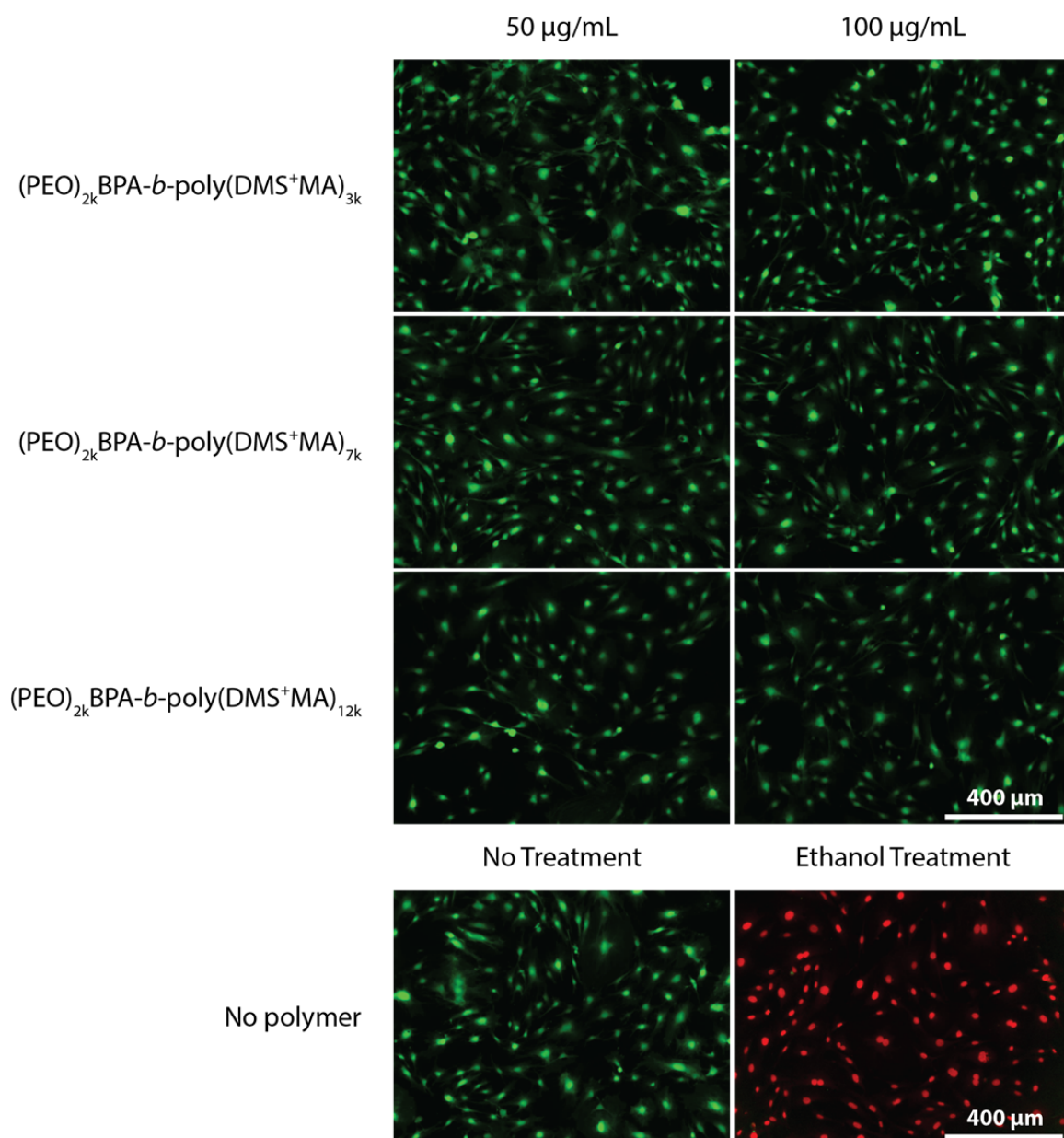


Figure S13. Live/dead staining of MC3T3 cells 24 hours following treatment by $\text{PEO}_{2k}\text{BPA-}b\text{-poly}(\text{DMS}^+\text{MA})_{3k/7k/12k}$ block copolymers. As determined by the predominant fluorescent calcein (green), major cytotoxicity is not a consequence of block copolymer delivery at 50 and 100 $\mu\text{g/mL}$. There were no discernable reductions in cell density caused by either polymer treatment.

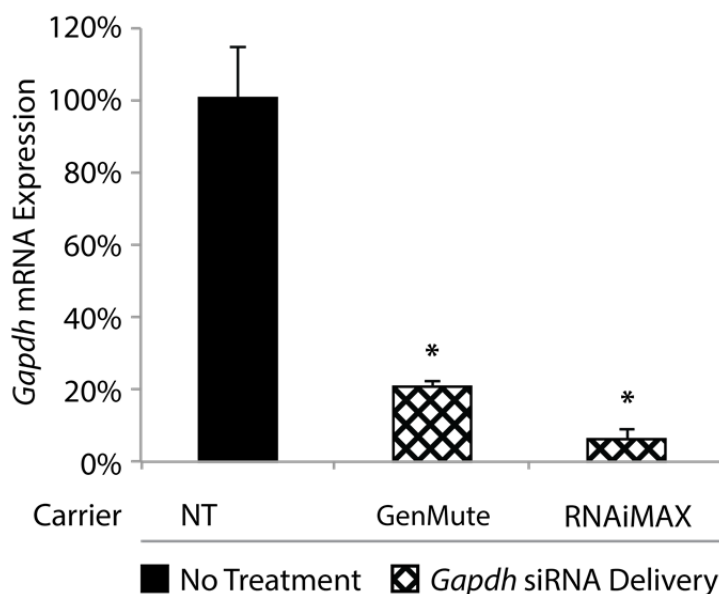


Figure S14. *Gapdh* mRNA expression by quantitative real-time PCR in MC3T3 cells 48 hours after RNAi treatment by GenMute and Lipofectamine RNAiMAX delivering *Gapdh* siRNAs. Both GenMute and RNAiMAX treatments were prepared according to the manufacturers protocol. *Gapdh* mRNA levels were reduced to 20.78±1.45% and 6.25±2.63% for GenMute and RNAiMAX treatments, respectively.

Table S1. Characterization of PEO_{2k}BPA-*b*-poly(DSM⁺MA) block copolymers synthesized by ARGET ATRP in 50% w/v acetone, 35°C.

Entry	[MTEMA] ₀ /[PEO _{2k} BPA] ₀ /[CuCl ₂] ₀ /[TPMA] ₀ /[Ascorbic Acid] ₀	Time, h	MTEMA Degree of polymerization ^[a]	$M_n^{NMR} \cdot 10^3$ ^[b]	M_w/M_n ^[c]
PEO _{2k} BPA- <i>b</i> -poly(DSM ⁺ MA) _{3k}	100/1/0.065/0.21/0.71	24	15	5.0	1.17
PEO _{2k} BPA- <i>b</i> -poly(DSM ⁺ MA) _{7k}	100/1/0.065/0.21/0.71	27	35	9.0	1.48
PEO _{2k} BPA- <i>b</i> -poly(DSM ⁺ MA) _{12k}	100/1/0.065/0.21/0.71	30	45	11.5	1.56

^[a] Degree of polymerization of MTEMA was determined using ¹H NMR comparing the ratio of the PEO_{2k}BPA protons to the methyl peaks of the poly(MTEMA). ^[b] M_n^{NMR} was calculated by adding the molecular weight of the macroinitiator to the corresponding molecular weight of the poly(MTEMA) block's degree of polymerization. ^[c] M_w/M_n was calculated using polyethylene oxide standards. The samples were analyzed on a aqueous GPC system using a 100mM sodium phosphate incl. 0.2 vol% Trifluoroacetic acid (~pH 2.5) running buffer with 3 Waters Ultrahydrogel columns in series (Linear, 250, 500) at a flow rate of 1 ml/min.

