Development of Guanidinium-rich Protein Mimics for Efficient siRNA Delivery into Human T Cells

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I. MATERIALS AND INSTRUMENTATION

Materials: Maleic anhydride, furan, 4-dimethylaminopyridine (DMAP), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), 1,3-di-boc-2-(2-hydroxyethyl)guanidine, benzyl alcohol, ethyl vinyl ether, trifluoroacetic acid (TFA), methanol (MeOH), ethyl acetate (EtOAc), dimethyl sulfoxide (DMSO), pentane, 2,2,2-trifluoroethanol (TFE), and tetrahydrofuran (THF) were obtained as reagent grade from Aldrich, Fisher Scientific, Fluka, or Acros and used as received. Sterile, RNase-free phosphate buffered saline (Amresco) and heparin, from porcine intestinal mucosa, were purchased from VWR and bovine serum albumin (BSA) and DMEM/high with L-glutamine and sodium pyruvate were purchased from Sigma Aldrich and used as received. 3rd generation Grubbs catalyst (Dichloro-di(3-bromopyridino)-N,N'-Dimesitylenoimidazolino-Ru=CHPh; G3) was synthesized as described previously by Grubbs and coworkers.[1] Dichloromethane (CH_2Cl_2) (HPLC grade, Fisher Scientific) was distilled from CaH_2 under nitrogen. Biotech CE dialysis tubing membranes with a MWCO 100-500 g/mol were purchased from Spectrum Medical Industries. FITC-siRNA (sc-36869), siRNA to *hNOTCH1* (sc-36095), and RNase-free water were purchased from Santa Cruz Biotechnologies and R9, DeliverX Plus siRNA, Xfect, N-ter, RNAiMAX, and JetPEI were purchased from Peptide 2.0, Inc., Affymetrix, Clontech, Sigma Aldrich, Life Technologies, and Polyplus Transfection, respectively. Gibco RPMI 1640 glutaMAX and fetal bovine serum were purchased from Life Technologies and Penicillin/Streptomycin (10K/10K), MEM non-essential amino acids solution (10 mM, 100X), and sodium pyruvate solution (100 mM) were purchased from Lonza. Anti-human CD3 epsilon MAb (Clone UCHT1) and human CD28 MAb (Clone 37407) were purchased from R&D Systems, anti-human NOTCH1 PE, Annexin V PE apoptosis detection kit, 7-AAD viability stain, and Foxp3/transcription faction staining buffer set were purchased from eBiosciences. hPBMCs were purchased from from Stemcell Technologies, Inc. in $2.5x10^6$ cells/aliquot (Product # 70047.2). For experiments, cells from different donors were used. The company obtained these cells using institutional review board approved consent forms and protocols.

Instrumentation: ^1H and ^{13}C NMR spectra were recorded at 300 MHz and 75 MHz, respectively, using a Bruker DPX-300 NMR spectrometer. Chemical shifts (δ) are reported in

ppm and coupling constants (*J*) in Hz. The abbreviations used for splitting patterns are: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; tt, triplet of triplets; q, quartet; m, multiplet; comp, overlapping multiplets of magnetically non-equivalent protons; br, broad.

Mass spectral data were obtained at the University of Massachusetts, Mass Spectrometry Facility from a JEOL JMS 700 instrument (JEOL, Peabody, MA).

A CombiFlash® RF 200 automated chromatography system with a variable UV-Vis detector (λ= 200-780 nm, Teledyne ISCO, Lincoln, NE) was used for purification of monomer **13**. 120 g Redi*Sep* Rf Flash Columns were used for the separations. All other compounds were purified using standard silica gel chromatography methods.

Gel permeation chromatography (GPC) chromatograms for protected polymers were obtained using an Agilent 1260 series system equipped with a refractive index (RI) and ultraviolet (UV) detectors, a PL Gel 5 µm guard column, two 5 µm analytical Mixed-C columns, and one 5 µm analytical Mixed-D column (Agilent). All columns were connected in series and incubated at 40 °C. THF was used as the eluent with a flow rate of 1.0 mL/min. ~ 3 mg/mL samples were prepared using THF as the diluent and toluene as the flow marker. All samples were filtered through 0.45 µm PTFE syringe filters (Restek, Bellefonte, PA) Poly(methyl methacrylate) and polystyrene standards were used for the calibration.

GPC chromatograms for deprotected polymers were obtained using an Agilent 1260 series system equipped with a refractive index (RI) and ultraviolet (UV) detectors, an HFIPgel guard column (7 mm x 50 mm; Agilent) and three HFIPgel columns (7 mm x 300 mm; Agilent). All columns were connected in series and incubated at 40 °C. TFE with 20 mM NaTFA salt was used as the eluent with a flow rate of 1.0 mL/min. ~ 3 mg/mL samples were prepared using TFE with 20 mM NaTFA salt as the diluent and methanol as the flow marker. All samples were filtered through a 0.45 µm PTFE syringe filters (Restek, Bellefonte, PA). Poly(methyl methacrylate) standards were used for the calibration.

Flow cytometry (FCM) experiments were performed using a Becton Dickinson LSRII (BD Biosciences) with eight color analysis capabilities and two excitation lasers: 488 nm and 633 nm. Fluorescence signals were collected for 10,000 cells.

II. SYNTHETIC PROCEDURES AND CHARACTERIZATIONS

Monomer Synthesis:

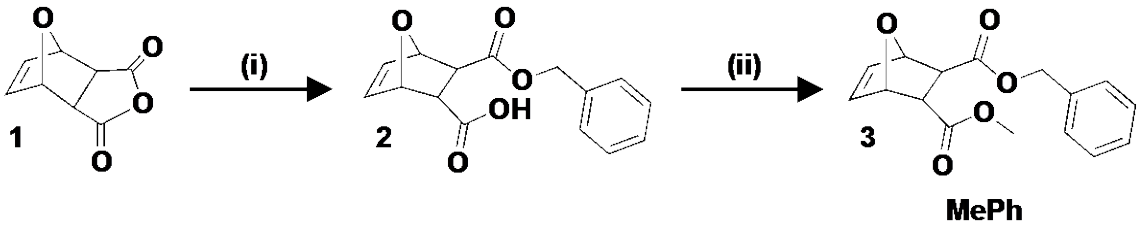

Figure S1. Synthesis of hydrophobic monomer **MePh**. i) Benzyl alcohol, DMAP, CH_2Cl_2,, RT, overnight; ii) MeOH, DMAP, EDC, CH_2Cl_2,, 0°C to RT, overnight;

Synthesis of 1: Maleic anhydride (100.0 g, 1.02 mol) was dissolved in 1 L toluene and 150 mL of furan (140.7 g, 2.05 mol) was added. The solution was stirred for 3 days at room temperature (RT) according to the literature. The product (**1**) was then filtered, washed with hexanes, and dried under vacuum overnight at RT to obtain a colorless powder. Spectroscopic data and yield were consistent with the literature.[2]

^{1}H NMR (300 MHz, DMSO-d_6): δ = 6.57 (s, 2H), 5.35 (s, 2H), 3.31 (s, 2H);

Synthesis of 2: The procedure reported by Lienkamp *et al*. was followed with minor modifications.[2] One equivalent of **1**, 1.25 equivalents of the corresponding substituted benzyl alcohols, and 10 mol% DMAP were dissolved in a minimal amount of freshly distilled CH_2Cl_2 and the reaction mixture was stirred under nitrogen at RT overnight. **2** precipitated from solution and was isolated using vacuum filtration and washed with cold CH_2Cl_2. **2** was then dried under vacuum at RT overnight.

2. Yield: 60 %, white powder

^{1}H NMR (300 MHz, DMSO-d_6): δ = 12.46 (br, 1H), 7.36 (comp, 5H), 6.45 (m, 2H), 5.12 (m, 2H), 5.01 (q, 2H), 2.77 (d, 2H);

^{13}C NMR (75 MHz, DMSO-d_6): δ = 172.74, 171.59, 136.79, 136.61, 136.07, 128.45, 128.05, 128.02, 80.18, 79.84, 65.99, 46.82, 46.10;

HR-MS (FAB) *m/z* $[M+H]^+$: 275.0914 (calc.), 275.0903 (found).

Synthesis of 3 (MePh): The procedure reported by Lienkamp *et al*. was followed with minor modifications.[2] One equivalent of **2**, one equivalent of MeOH, and 10 mol% DMAP were dissolved in CH_2Cl_2 and stirred at RT under nitrogen. The solution was then cooled down to 0°C in an ice bath and one equivalent of EDC was added. The solution was allowed to stir

overnight under nitrogen and gradually return to RT. The reaction mixture was then concentrated *via* rotary evaporation and purified by either column chromatography with EtOAc/CH_2Cl_2 (1/4, v/v) as the eluent or by using a CombiFlash purification system, with a 120 g silica cartridge and EtOAc/CH_2Cl_2 (1/4, v/v) as the eluent. Pure fractions were combined and then concentrated using rotary evaporation. The sample was dried under vacuum overnight at RT to obtain a white solid.

3: Yield 82 %, white powder.

^{1}H NMR (300 MHz, CD_3CN): δ = 7.38 (comp, 5H), 6.44 (comp, 2H), 5.14 (d, 2H), 5.06 (comp, 2H), 3.50 (s, 3H), 2.84 (q, 2H).

^{13}C NMR (75 MHz, CD_3CN): δ = 173.35, 172.84, 137.95, 137.91, 137.51, 129.84, 129.60, 129.50, 81.64, 81.63, 67.72, 52.75, 47.96, 47.89.

HR-MS (FAB) *m/z* $[M+H]^+$: 289.1076 (calc.), 289.1078 (found).

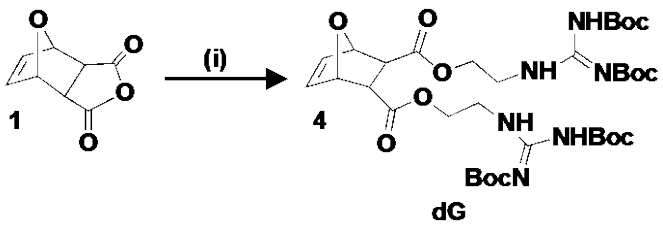

Figure S2. Synthesis of diguanidine monomer. i)1,3-di-boc-2-(2-hydroxyethyl)guanidine, DMAP, EDC, CH_2Cl_2, 0°C to RT, overnight;

Synthesis of 4 (dG): The procedure reported by Lienkamp *et.al*. was followed with minor modifications.[2] One equivalent of **1**, two equivalents of 1,3-Di-Boc-2-(2-hydroxyethyl)guanidine, and 10 mol% DMAP were dissolved in CH_2Cl_2 and the reaction mixture was stirred overnight at RT under nitrogen. After one day, the solution was cooled down to 0°C in ice bath and one equivalent of EDC was added. The solution was allowed to stir overnight under nitrogen and gradually return to RT. The reaction mixture was then concentrated using rotary evaporation and purified using a CombiFlash purification system, with a 120 g silica cartridge and EtOAc/CH_2Cl_2 (1/4, v/v) as the eluent. Pure fractions were combined and then concentrated using rotary evaporation. The sample was dried under vacuum overnight at RT to obtain a white solid.

Yield = 60 %, white solid

^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.49 (s, 2H), 8.24 (s, 2H), 6.47 (s, 2H), 5.16 (s, 2H), 4.14 (comp, 2H), 4.04 (comp, 2H), 3.53 (m, 4H), 2.82 (s, 2H), 1.47 (s, 18H), 1.39 (s, 18H);

^{13}C NMR (75 MHz, CD_3CN): δ = 172.43, 164.51, 157.35, 153.72, 137.56, 84.05, 81.41, 79.50, 63.72, 47.49, 40.21, 28.38, 28.09;

HR-MS (FAB) *m/z* $[M+H]^+$: 755.3827 (calc.), 755.3814 (found).

Polymers:

Figure S3. Synthesis of Boc-protected homopolymer PTDMs (5a-f). i) Dichloro-di(3-bromopyridino)-*N,N'*-Dimesitylenoimidazolino-Ru=CHPh (G3) catalyst, CH_2Cl_2, RT, 90 min; ii) Ethyl vinyl ether, RT, overnight; Polymers were synthesized with m = 5, 10, 20, 40, 60 and 80.

Homopolymer Synthesis of 5a-f: 5, 10, 20, 40, 60, or 80 equivalents of **4** and one equivalent of G3 catalyst were each dissolved in 1.5 mL of dry CH_2Cl_2 in separate schlenk flasks. The catalyst flask also contained a small stir bar. Three freeze-pump-thaw cycles were used to remove air. Following the third thaw step, the monomer flask was kept under nitrogen while the catalyst flask remained under vacuum. Monomer **4** was then cannulated into the vigorously stirring catalyst solution at RT. After 1.5 hr, the reaction was quenched with 3 mL of ethyl vinyl ether. The quenched polymer solutions were allowed to stir overnight at RT. The solutions were then transferred to 20 mL scintillation vials and concentrated using rotary evaporation. **5a-e** were then dissolved in a minimal amount of THF and added drop-wise to 100 mL of cold, stirring pentanes to precipitate the polymers. After 5-10 minutes of stirring, **5a-e** were isolated using vacuum filtration with fine sinter funnels. Polymers were dried under vacuum at RT overnight.

5a (m=5): ^{1}H NMR (500 MHz, CD_3CN): δ = 11.53 (br, 2H), 8.35 (br, 2H), 5.88 (trans) and 5.62 (cis) (br, 2H total), 5.04 (cis) and 4.66 (trans) (br, 2H total), 4.17 (br, 4H), 3.56 (br, 4H), 3.17 (br, 2H), 1.47 (s, 18H), 1.42 (s, 18H).

5b (m=10): ^{1}H NMR (300 MHz, CD_3CN): δ = 11.52 (br, 2H), 8.35 (br, 2H), 5.86 (trans) and 5.59 (cis) (br, 2H total), 5.05 (cis) and 4.65 (trans) (br, 2H total), 4.17 (br, 4H), 3.55 (br, 4H), 3.15 (br, 2H), 1.46 (s, 18H), 1.41 (s, 18H).

5c (m=20): ^{1}H NMR (300 MHz, CD_3CN): δ = 11.54 (br, 2H), 8.35 (br, 2H), 5.86 (trans) and 5.59 (cis) (br, 2H total), 5.05 (cis) and 4.66 (trans) (br, 2H total), 4.17 (br, 4H), 3.55 (br, 4H), 3.14 (br, 2H), 1.46 (s, 18H), 1.41 (s, 18H).

5d (m=40): ^{1}H NMR (500 MHz, CD_3CN): δ = 11.54 (br, 2H), 8.35 (br, 2H), 5.86 (trans) and 5.61 (cis) (br, 2H total), 5.05 (cis) and 4.66 (trans) (br, 2H total), 4.16 (br, 4H), 3.55 (br, 4H), 3.14 (br, 2H), 1.46 (s, 18H), 1.41 (s, 18H).

5e (m=60): ^{1}H NMR (500 MHz, CD_3CN): δ = 11.54 (br, 2H), 8.35 (br, 2H), 5.86 (trans) and 5.61 (cis) (br, 2H total), 5.05 (cis) and 4.66 (trans) (br, 2H total), 4.17 (br, 4H), 3.56 (br, 4H), 3.17 (br, 2H), 1.46 (s, 18H), 1.41 (s, 18H).

5e (m=80): ^{1}H NMR (500 MHz, CD_3CN): δ = 11.54 (br, 2H), 8.35 (br, 2H), 5.86 (trans) and 5.61 (cis) (br, 2H total), 5.05 (cis) and 4.66 (trans) (br, 2H total), 4.16 (br, 4H), 3.55 (br, 4H), 3.15 (br, 2H), 1.46 (s, 18H), 1.41 (s, 18H).

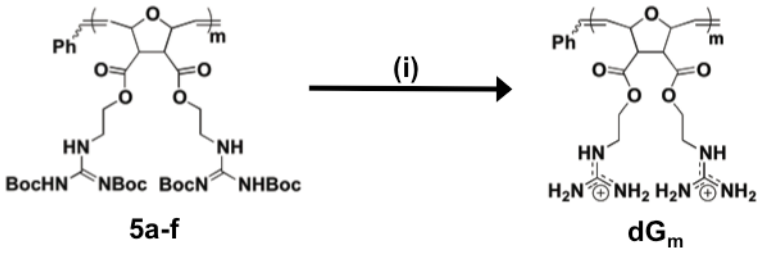

Figure S4. Deprotection of boc-protected homopolymers (5a-f) to yield the dG_m series of PTDMs. i) TFA/CH_2Cl_2 (1:1), RT, overnight. dG_m series further purified by dialysis with molecular weight cut-off : 100-500 g/mol.

Deprotection Procedure to Yield dG Polymer Series: 5a-e were dissolved in 2 mL of CH_2Cl_2 and allowed to stir. 2 mL of TFA was then added drop-wise to the solution and allowed to stir overnight at RT. Excess TFA was removed by azeotropic distillation with MeOH. During this process, 5-7 mL of MeOH was added and then the sample was concentrated using rotary evaporation. This process was repeated 7-9 times to ensure complete TFA removal.

Following this, samples were dissolved in a water/MeOH mixture, transferred to Biotech CE dialysis tubing membranes with a MWCO 100-500 g/mol and dialyzed against RO water until the conductivity of the water remained < 0.2 µS (2-3 days on dialysis). The dG series was then aqueous filtered and isolated from water by lyophilization.

dG$_5$: ^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.93 (br, 2H), 7.42 (br, 8H), 5.84 (trans) and 5.60 (cis) (br, 2H total), 4.96 (cis) and 4.63 (trans) (br, 2H total), 4.10 (br, 4H), 3.38 (br, 4H), 3.33 (br, 2H).

dG$_{10}$: ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.99 (br, 2H), 7.43 (br, 8H), 5.83 (trans) and 5.60 (cis) (br, 2H total), 4.96 (cis) and 4.62 (trans) (br, 2H total), 4.05 (br, 4H), 3.40 (br, 4H), 3.27 (br, 2H).

dG$_{20}$: ^{1}H NMR (300 MHz, DMSO-d_6): δ = 7.98 (br, 2H), 7.43 (br, 8H), 5.82 (trans) and 5.57 (cis) (br, 2H total), 4.96 (cis) and 4.57 (trans) (br, 2H total), 4.05 (br, 4H), 3.37 (br, 4H), 3.28 (br, 2H).

dG$_{40}$: ^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.98 (br, 2H), 7.44 (br, 8H), 5.82 (trans) and 5.58 (cis) (br, 2H total), 4.97 (cis) and 4.61 (trans) (br, 2H total), 4.03 (br, 4H), 3.40 (br, 4H), 3.27 (br, 2H).

dG$_{60}$: ^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.98 (br, 2H), 7.44 (br, 8H), 5.82 (trans) and 5.58 (cis) (br, 2H total), 4.97 (cis) and 4.57 (trans) (br, 2H total), 4.03 (br, 4H), 3.38 (br, 4H), 3.28 (br, 2H).

dG$_{80}$: ^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.98 (br, 2H), 7.43 (br, 8H), 5.82 (trans) and 5.58 (cis) (br, 2H total), 4.96 (cis) and 4.57 (trans) (br, 2H total), 4.03 (br, 4H), 3.37 (br, 4H), 3.32 (br, 2H).

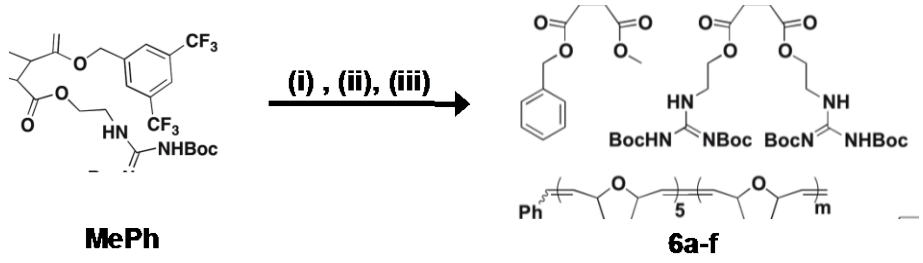

Figure S5. Synthesis of Boc-protected block copolymer PTDMs (6a-f). i) Dichloro-di(3-bromopyridino)-*N,N'*-Dimesitylenoimidazolino-Ru=CHPh (G3) catalyst, CH_2Cl_2, RT, 10 min; ii) dG, CH_2Cl_2, RT, 90 min; iii) Ethyl vinyl ether, RT, overnight; Polymers were synthesized with n=5 and m = 5, 10, 20, 40, 60 and 80.

Block Copolymer Synthesis of 6a-f: 5 equivalents of **3**, 5, 10, 20, 40, 60, or 80 equivalents of **4,** and one equivalent of G3 catalyst were each dissolved in 1.5 mL of dry CH_2Cl_2 in separate schlenk flasks. The catalyst flask also contained a small stir bar. Three freeze-pump-thaw cycles were used to remove air. Following the third thaw step, the flasks containing **3** and **4** were kept under nitrogen while the catalyst flask remained under vacuum. Monomer **3** was then cannulated into the vigorously stirring catalyst solution at RT. After 10 min, a small aliquot (0.05 mL) was removed from the solution for analysis and then monomer **4** was cannulated into the vigorously stirring polymerization solution at RT reaction. After 1.5 hr, the polymerizations were quenched with 3 mL of ethyl vinyl ether and allowed to stir overnight at RT. The solutions were then transferred to 20 mL scintillation vials and concentrated using rotary evaporation. **6a-f** were then dissolved in a minimal amount of THF and added drop-wise to 100 mL of cold, stirring pentanes to precipitate the polymers. After 5-10 minutes of stirring, **6a-f** were isolated using vacuum filtration with fine sinter funnels. Polymers were dried under vacuum at RT overnight.

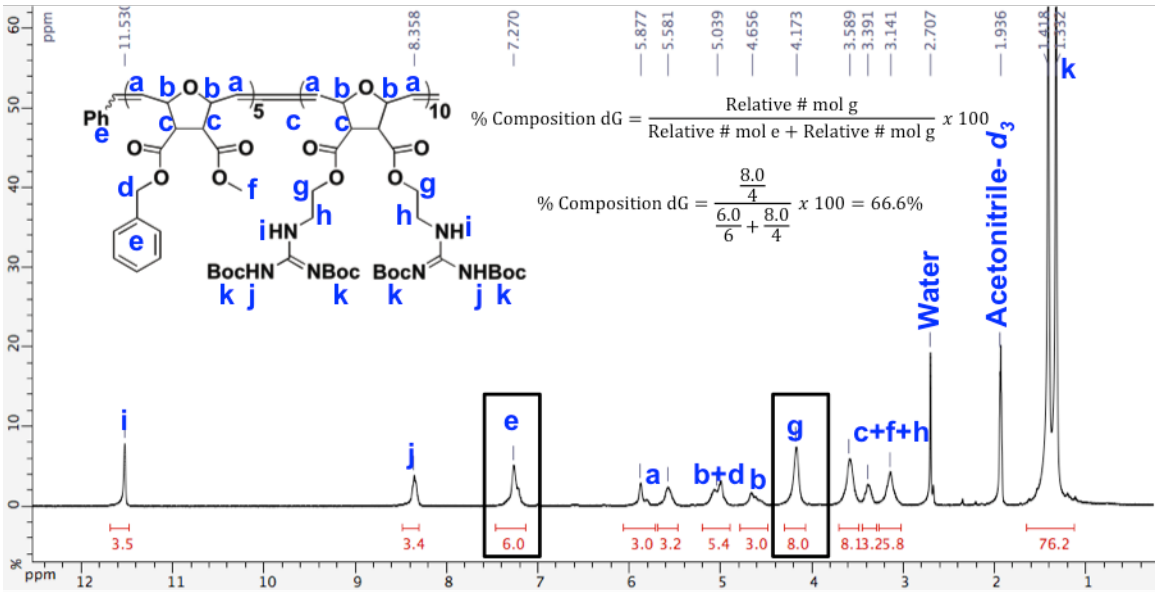

Figure S6. Representative ^{1}H NMR spectrum of **6b** to demonstrate the determination of block copolymer composition from unique peaks from each distinct repeat unit (dG and MePh). Data reflects true integrations for the polymer peaks and are not modified to display the number of hydrogen atoms expected to be found for each monomer, when analyzed independently.

Note: All ^{1}H NMR data reported below for **6a-f** reflects the numbers of hydrogen atoms expected to be found for each type of monomer repeat unit and do not necessarily reflect their relative abundance in the polymer samples.

6a (n=5; m=5): ^{1}H NMR (300 MHz, CD_3CN): δ = 11.53 (br, 2H), 8.35 (br, 2H), 7.34 (br, 6H), 5.86 (trans) and 5.60 (cis) (comp, 4H total), 5.07 (comp, 2H), 5.07 (cis) and 4.65 (trans) (comp, 4H), 4.17 (br, 4H), 3.51 (comp, 7H), 3.15 (br, 4H), 1.47 (s, 18H), 1.42 (s, 18H).
n:m = 5 : 5

6b (n=5; m=10): ^{1}H NMR (500 MHz, CD_3CN): δ = 11.54 (br, 2H), 8.36 (br, 2H), 7.33 (br, 6H), 5.86 (trans) and 5.59 (cis) (comp, 4H total), 5.05 (comp, 2H), 5.05 (cis) and 4.64 (trans) (comp, 4H total), 4.17 (br, 4H), 3.55 (comp, 7H), 3.14 (br, 4H), 1.46 (s, 18H), 1.41 (s, 18H).
n:m = 5 : 10

6c (n=5; m=20): ^{1}H NMR (500 MHz, CD_3CN): δ = 11.54 (br, 2H), 8.35 (br, 2H), 7.34 (br, 6H), 5.86 (trans) and 5.60 (cis) (comp, 4H) total, 5.05 (comp, 2H) 5.05 (cis) and 4.65 (trans) (comp, 4H total), 4.16 (br, 4H), 3.55 (comp, 7H), 3.15 (br, 4H), 1.46 (s, 18H), 1.41 (s, 18H).

n:m = 5 : 20

6d (n=5; m=40): ^{1}H NMR (500 MHz, CD_3CN): δ = 11.54 (br, 2H), 8.35 (br, 2H), 7.34 (br, 6H), 5.86 (trans) and 5.61 (cis) (comp, 4H) total, 5.05 (comp, 2H) 5.05 (cis) and 4.66 (trans) (comp, 4H total), 4.17 (br, 4H), 3.55 (comp, 7H), 3.14 (br, 4H), 1.46 (s, 18H), 1.41 (s, 18H).
n:m = 5 : 40

6e (n=5; m=60): ^{1}H NMR (500 MHz, CD_3CN): δ = 11.54 (br, 2H), 8.35 (br, 2H), 7.34 (br, 6H), 5.86 (trans) and 5.60 (cis) (comp, 4H) total, 5.05 (comp, 2H) 5.05 (cis) and 4.66 (trans) (comp, 4H total), 4.16 (br, 4H), 3.55 (comp, 7H), 3.14 (br, 4H), 1.46 (s, 18H), 1.41 (s, 18H).
n:m = 5 : 60

6f (n=5; m=80): ^{1}H NMR (500 MHz, CD_3CN): δ = 11.54 (br, 2H), 8.35 (br, 2H), 7.34 (br, 6H), 5.86 (trans) and 5.61 (cis) (comp, 4H) total, 5.05 (comp, 2H) 5.05 (cis) and 4.66 (trans) (comp, 4H total), 4.17 (br, 4H), 3.55 (comp, 7H), 3.14 (br, 4H), 1.46 (s, 18H), 1.41 (s, 18H).
n:m = 5 : 80

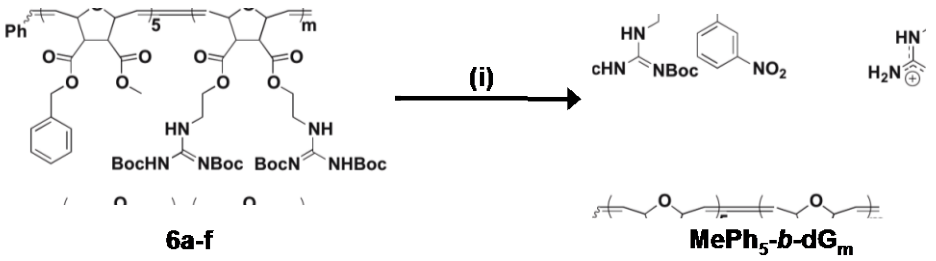

Figure S7. Deprotection of boc-protected block copolymers (6a-f) to yield the $MePh_5$-*b*-dG_m series of PTDMs. i) TFA/CH_2Cl_2 (1:1), RT, overnight. dG_m series further purified by dialysis with molecular weight cut-off : 100-500 g/mol.

Deprotection Procedure to Yield $MePh_5$-*b*-dG_m Polymer Series: 6a-f were dissolved in 2 mL of CH_2Cl_2 and allowed to stir. 2 mL of TFA was then added drop-wise to the solution and allowed to stir overnight at RT. Excess TFA was removed by azeotropic distillation with MeOH. During this process, 5-7 mL of MeOH was added and then the sample was concentrated using rotary evaporation. This process was repeated 7-9 times to ensure complete TFA removal. Following this, samples were dissolved in a water/MeOH mixture,

transferred to Biotech CE dialysis tubing membranes with a MWCO 100-500 g/mol and dialyzed against RO water until the conductivity of the water remained < 0.2 µS (2-3 days on dialysis). The $MePh_5$-*b*-dG_m series was then aqueous filtered and isolated from water by lyophilization.

$MePh_5$-*b*-dG_5: ^{1}H NMR (300 MHz, CD_3CN-d_6): δ = 7.88 (br, 2H), 7.35 (comp, 6H), 7.07 (comp, 8H), 5.87 (trans) and 5.62 (cis) (br, 4H total), 5.07 (comp, 2H), 5.07 (cis) and 4.68 (trans) (comp, 4H total), 4.15 (br, 4H), 3.42 (comp, 7H), 3.20 (br, 4H).

$MePh_5$-*b*-dG_{10}: ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.96 (br, 2H), 7.39 (comp, 14H), 5.83 (trans) and 5.59 (cis) (comp, 4H total), 5.01 (comp, 2H), 5.01 (cis) and 4.58 (trans) (comp, 4H total), 4.07 (4H, br), 3.38 (7H, br), 3.27 (4H, br);

$MePh_5$-*b*-dG_{20}: ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.98 (br, 2H), 7.40 (comp, 14H), 5.83 (trans) and 5.59 (cis) (comp, 4H total), 5.03 (comp, 2H), 5.03 (cis) and 4.61 (trans) (comp, 4H total), 4.04 (4H, br), 3.40 (7H, br), 3.28 (4H, br);

$MePh_5$-*b*-dG_{40}: ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.98 (br, 2H), 7.44 (comp, 14H), 5.82 (trans) and 5.59 (cis) (comp, 4H total), 4.97 (comp, 2H), 4.97 (cis) and 4.58 (trans) (comp, 4H total), 4.10 (4H, br), 3.40 (7H, br), 3.28 (4H, br);

$MePh_5$-*b*-dG_{60}: ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.99 (br, 2H), 7.44 (comp, 14H), 5.82 (trans) and 5.58 (cis) (comp, 4H total), 4.97 (comp, 2H), 4.97 (cis) and 4.61 (trans) (comp, 4H total), 4.04 (4H, br), 3.40 (7H, br), 3.28 (4H, br);

$MePh_5$-*b*-dG_{80}: ^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.99 (br, 2H), 7.33 (comp, 14H), 5.84 (trans) and 5.57 (cis) (comp, 4H total), 4.97 (comp, 2H), 4.97 (cis) and 4.60 (trans) (comp, 4H total), 4.04 (4H, br), 3.40 (7H, br), 3.31 (4H, br);

Molecular Weight Characterization

GPC Data

Table S1. Molecular weight characterization of Boc-protected homopolymer and block copolymer PTDMs.

Polymer	M_n[a] (Da)	M_w[a] (Da)	M_p[a] (Da)	Đ[a] (M_w/M_n)
5a	4,300	4,600	4,500	1.06
5b	6,400	6,800	6,700	1.07
5c	12,700	13,400	13,500	1.06
5d	23,600	27,200	30,500	1.15
5e	40,500	45,400	48,700	1.12
5f	50,200	59,200	71,000	1.18
6a	5,400	5,900	5,900	1.08
6b	8,300	9,000	9,300	1.08
6c	16,000	17,100	17,500	1.07
6d	27,600	31,500	36,800	1.14
6e	36,800	41,300	46,700	1.12
6f	45,000	57,100	71,100	1.27

[a]Number average molecular weight (M_n), weight average molecular weight (M_w), molecular weight at the peak maximum (M_p), and dispersity indices (Đ=M_w/M_n) determined by GPC using poly(methyl methacrylate) (PMMA) standards, tetrahydrofuran (THF) as the eluent, and toluene as the flow marker.

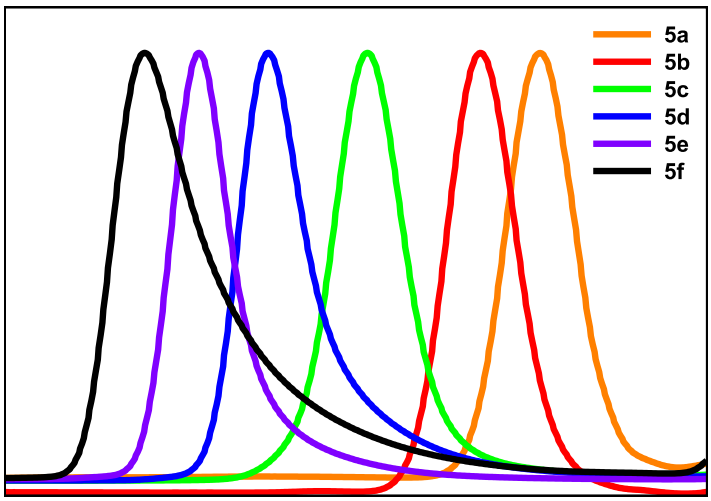

Figure S8. THF GPC chromatograms for boc-protected homopolymers PTDMs **5a-f**. A summary of molecular weight data can be found in **Table S1**.

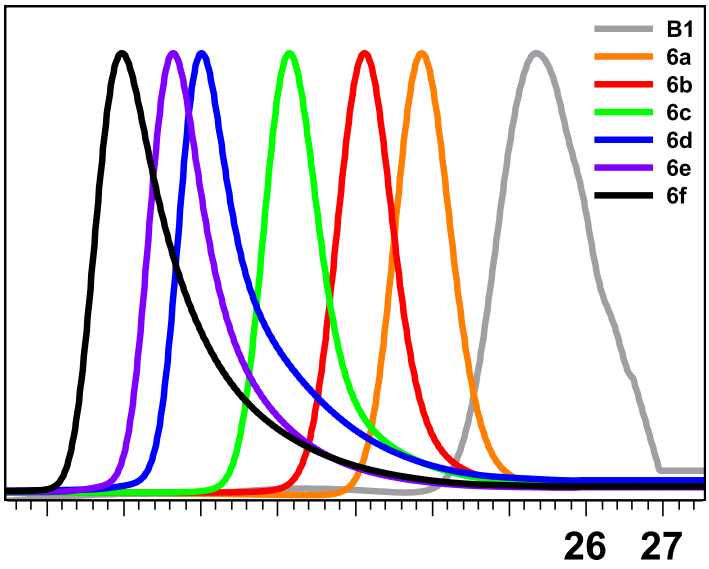

Figure S9. THF GPC chromatograms for boc-protected block copolymer PTDMs **6a-f**. **B1** (grey) is a representative chromatogram for the first block of the BCP PTDMs. A summary of molecular weight data can be found in **Table S1**.

GPC Data

Table S2. Molecular Weight Characterization of deprotected homopolymer and block copolymer PTDMs.

Polymer	M_n[a] (Da)	M_w[a] (Da)	M_p[a] (Da)	Đ[a] (M_w/M_n)
dG_5	8,800	9,300	9,000	1.05
dG_{10}	11,400	12,000	11,700	1.05
dG_{20}	18,300	19,400	19,400	1.06
dG_{40}	31,500	33,900	36,000	1.08
dG_{60}	45,600	49,100	50,800	1.08
dG_{80}	57,100	80,400	67,500	1.41
$MePh_5$-*b*-dG_5	10,200	10,900	10,100	1.07
$MePh_5$-*b*-dG_{10}	14,900	15,800	15,200	1.06
$MePh_5$-*b*-dG_{20}	21,800	23,500	23,500	1.08
$MePh_5$-*b*-dG_{40}	33,300	36,600	39,500	1.10
$MePh_5$-*b*-dG_{60}	39,700	43,700	45,600	1.10
$MePh_5$-*b*-dG_{80}	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]

[a]Number average molecular weight (M_n), weight average molecular weight (M_w), molecular weight at the peak maximum (M_p), and dispersity indices (Đ=M_w/M_n) determined by GPC

using poly(methyl methacrylate) (PMMA) standards, 2,2,2-trifluoroethanol (TFE) with 20 mM NaTFA salt as the eluent, and methanol as the flow marker. [b]not determined (n.d.) due to insolubility in TFE eluent.

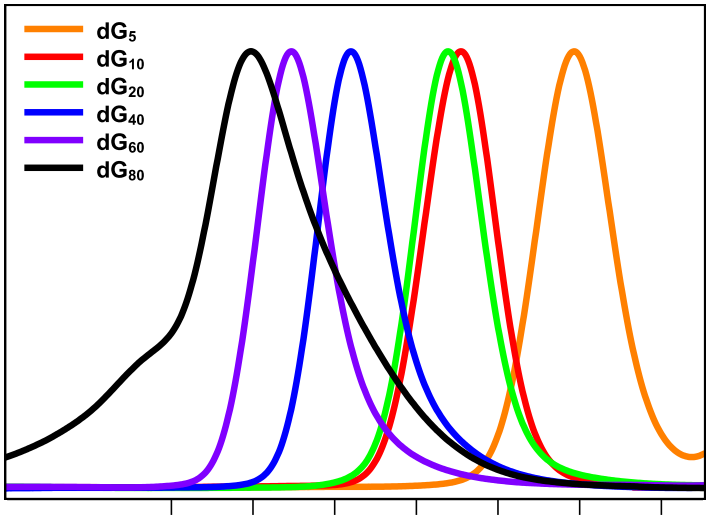

Figure S10. TFE GPC chromatograms for deprotected homopolymer PTDMs. A summary of molecular weight data can be found in **Table S2**.

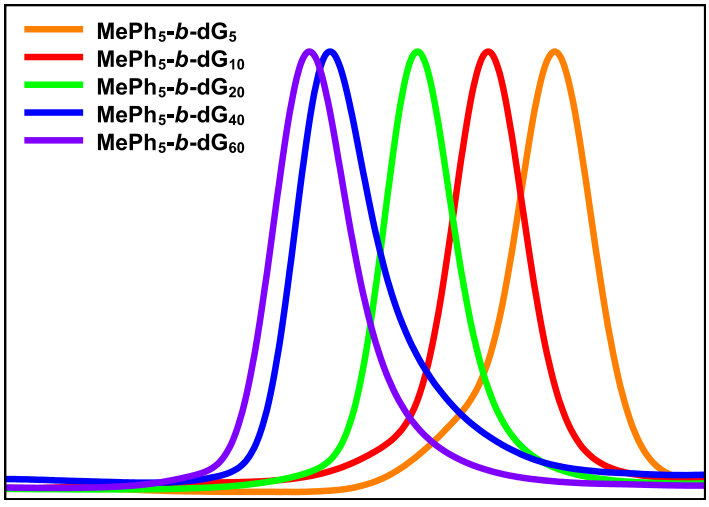

Figure S11. TFE GPC chromatograms for deprotected block copolymer PTDMs. A summary of molecular weight data can be found in **Table S2**.

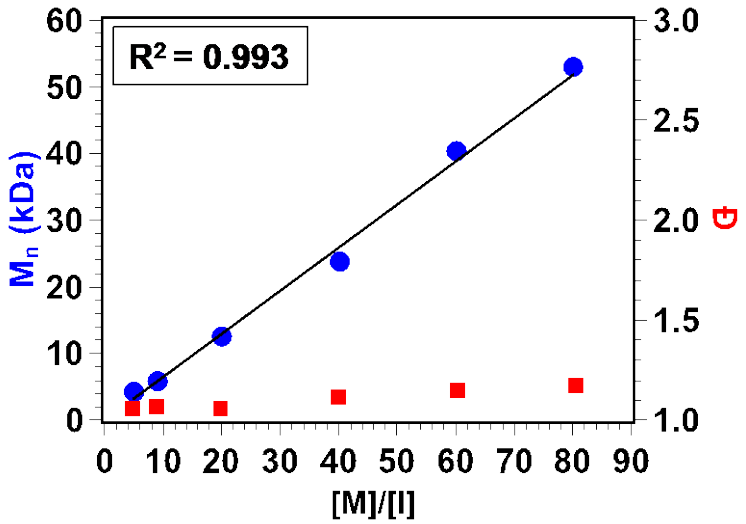

Figure S12. Plot of number average molecular weight (M_n) and dispersity index (Đ) with respect to monomer / initiator [M]/[I] ratio for the dG_m series. The linear relationship (R^2 = 0.993) reflects the controlled nature of the polymerization.

III. COMPLEXATION ASSESSMENT

Polymer/siRNA Complexation

PTDMs were complexed with siRNA at N:P ratios of 0.5:1, 1:1, 2:1, 4:1, 8:1, and 12:1 in microfuge tubes, with the siRNA amount held constant at 1 µg. Complexes were allowed to incubate at RT for 30 minutes prior to agarose gel electrophoresis with sodium boric acid conductive medium.

0.8% Agarose Gel Preparation and Processing

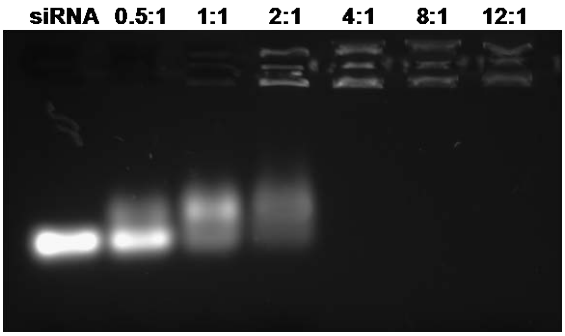

Figure S13. Gel retardation assay to assess PTDM / siRNA complex formation using dG_{10}. All samples were run on a 0.8% agarose gel and the N:P ratios tested ranged from 0.5:1 to 12:1, with 1 µg of siRNA per well.

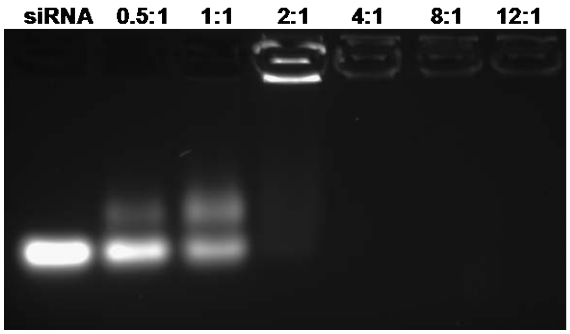

Figure S14. Gel retardation assay to assess PTDM / siRNA complex formation using dG_{20}. All samples were run on a 0.8% agarose gel and the N:P ratios tested ranged from 0.5:1 to 12:1, with 1 µg of siRNA per well.

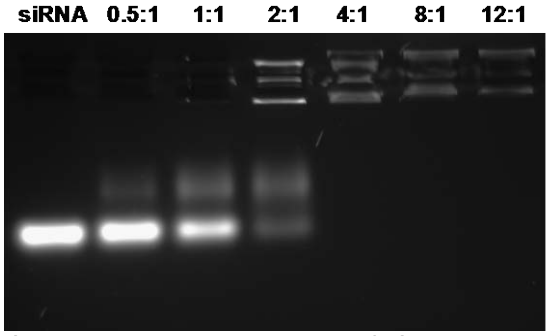

Figure S15. Gel retardation assay to assess PTDM / siRNA complex formation using dG_{40}. All samples were run on a 0.8% agarose gel and the N:P ratios tested ranged from 0.5:1 to 12:1, with 1 µg of siRNA per well.

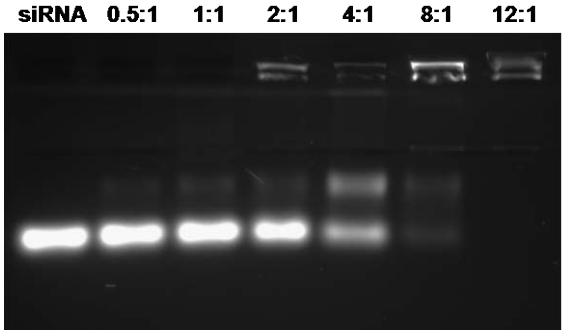

Figure S16. Gel retardation assay to assess PTDM / siRNA complex formation using dG_{60}. All samples were run on a 0.8% agarose gel and the N:P ratios tested ranged from 0.5:1 to 12:1, with 1 µg of siRNA per well.

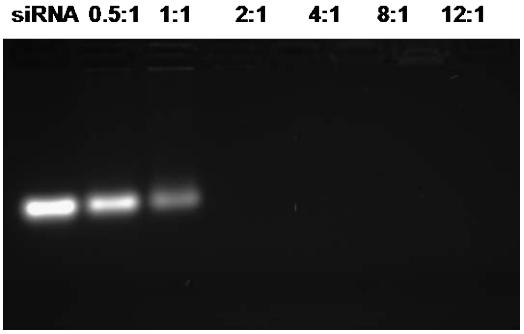

Figure S17. Gel retardation assay to assess PTDM / siRNA complex formation using $MePh_5$-*b*-dG_5. All samples were run on a 0.8% agarose gel and the N:P ratios tested ranged from 0.5:1 to 12:1, with 1 µg of siRNA per well.

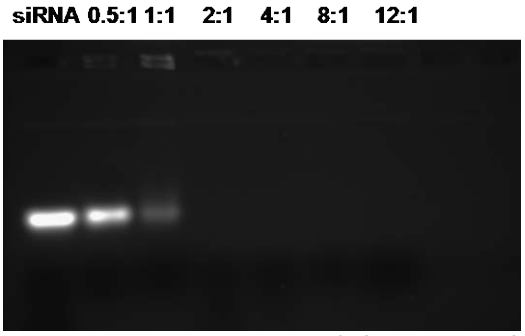

Figure S18. Gel retardation assay to assess PTDM / siRNA complex formation using $MePh_5$-*b*-dG_{10}. All samples were run on a 0.8% agarose gel and the N:P ratios tested ranged from 0.5:1 to 12:1, with 1 µg of siRNA per well.

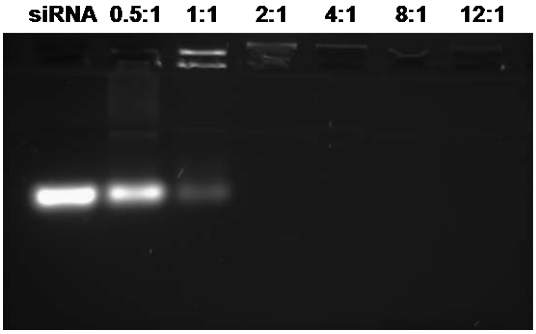

Figure S19. Gel retardation assay to assess PTDM / siRNA complex formation using $MePh_5$-*b*-dG_{20}. All samples were run on a 0.8% agarose gel and the N:P ratios tested ranged from 0.5:1 to 12:1, with 1 µg of siRNA per well.

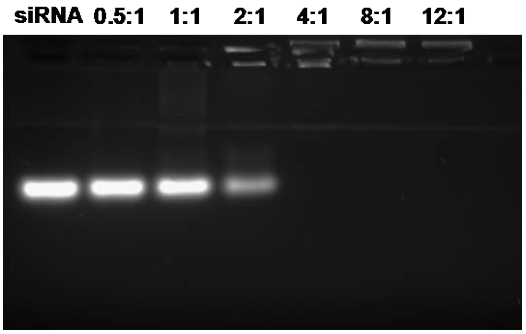

Figure S20. Gel retardation assay to assess PTDM / siRNA complex formation using $MePh_5$-*b*-dG_{40}. All samples were run on a 0.8% agarose gel and the N:P ratios tested ranged from 0.5:1 to 12:1, with 1 µg of siRNA per well.

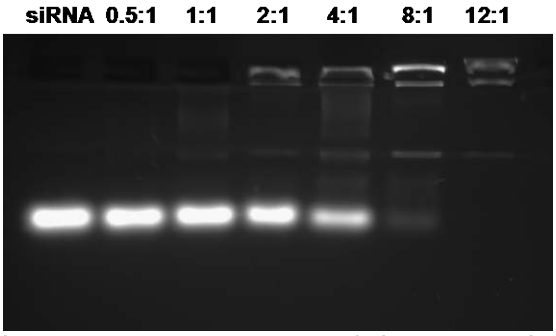

Figure S21. Gel retardation assay to assess PTDM / siRNA complex formation using $MePh_5$-*b*-dG_{60}. All samples were run on a 0.8% agarose gel and the N:P ratios tested ranged from 0.5:1 to 12:1, with 1 µg of siRNA per well.

IV. FITC-SIRNA DELIVERY AND CELL VIABILITY ASSAYS

FITC-siRNA Uptake in Jurkat T Cells

General Procedure: Polymers were dissolved in sterile DMSO to make 1 mM stock solutions. Polymers were stored at -20 °C in 50 µL aliquots. On the day of the experiment, Jurkat T cells were harvested, centrifuged, counted, and re-suspended in complete cell growth medium with 10% FBS to a density of $4x10^5$ cells/mL (1 mL final volume in a 12-well plate). Polymers with charge contents less than 40 were diluted to 0.1 mM with PBS and polymers with charge contents of 40 or larger were diluted to 0.005 mM with PBS (pH 7.4). Polymers were mixed with siRNA (10 µM stock solution, 50 nM in final well) at an N/P ratio, where N is the number of positively charged nitrogen groups in the polymer structures and P is the number of negatively charged phosphate groups in the FITC-siRNA duplexes, of 8/1 in PBS (100 µL total for each complex solution). This N/P ratio was previously optimized.[3] Complexes were incubated at RT for 30 minutes prior to adding them drop-wise to each well and gently pipetting the media in each well up and down to evenly mix in the complex solutions.. Cells were then

incubated at 37 °C in a 5% CO_2 atmosphere for 4 hr. After 4 hr, cells were harvested and washed 3 times with 500 µL of a 20 U/mL heparin solution. Cells were either re-suspended in 200 µL of FACS wash buffer after the final wash for analysis or prepared for viability staining. See procedure below. For flow cytometry analysis, the fluorescence signal was collected for 10,000 cells. The cell populations were gated in order to assess the percent of positive cells, which reflected the percentage of the cell population that received FITC-siRNA. The calculated median fluorescence intensity (MFI) represented the amount of cargo delivered to the cells. Results for percent positive cells and MFI can be found in the main text (**Figure 2**).

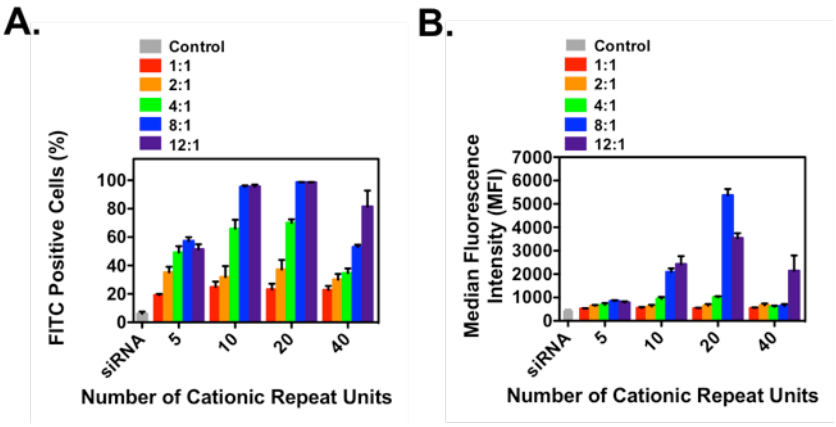

Figure S22. N/P ratio screening for FITC-siRNA delivery into Jurkat T cells using ROMP-based PTDMs. Jurkat T cells (cell density = $4x10^5$ cells/mL) were treated with polymer/FITC-siRNA complexes with an N/P ratio = 8/1 in complete media for four hours at 37°C and compared with untreated cells and cells only receiving FITC-siRNA. A) Percent positive cells. B) MFI of the cell population. Each data point represents the mean ± SEM of three independent experiments.

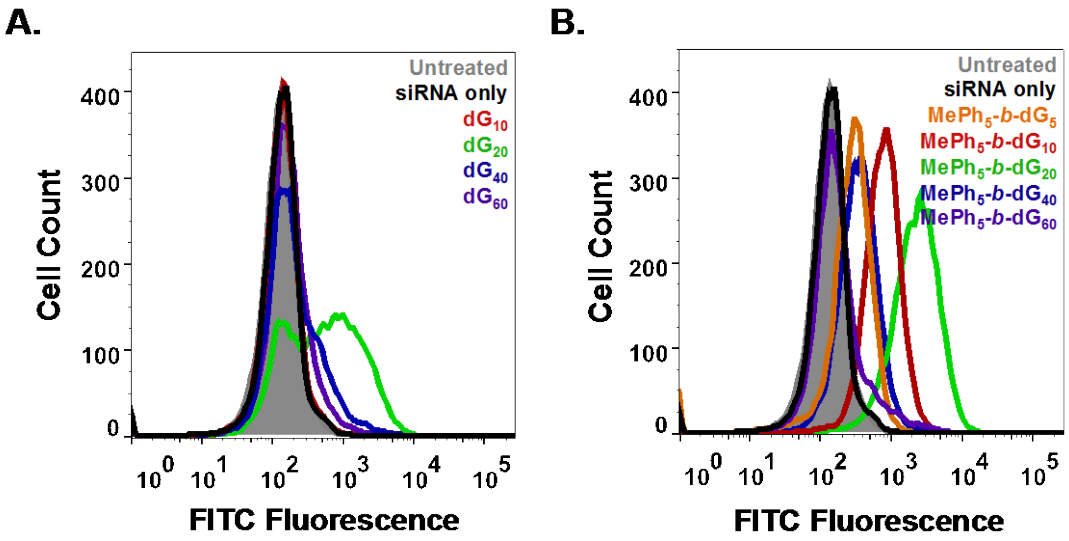

Figure S23. Representative histograms for FITC-siRNA delivery into Jurkat T cells using ROMP-based protein mimics. Jurkat T cells (cell density = $4x10^5$ cells/mL) were treated with polymer/FITC-siRNA complexes with an N/P ratio = 8/1 in complete media for four hours at 37°C and compared with untreated cells and cells only receiving FITC-siRNA. A) Overlay of representative histograms for cells treated with homopolymer/siRNA complexes. B) Overlay of representative histograms for cells treated with block copolymer/siRNA complexes.

Comparison to Commercially Available Reagents: For experiments in which polymers were compared to commercially available reagents, the setup was identical to the general procedure with the exception that all commercially available reagents were handled in accordance with the recommended procedures. Summaries of these conditions are documented below. The concentration of siRNA in the final experiment wells remained at 50 nM for consistency. For flow cytometry analysis, the fluorescence signal was collected for 10,000 cells. The cell populations were gated in order to assess the percent of positive cells, which reflected the percentage of the cell population that received FITC-siRNA. The calculated MFI values reflected the amount of cargo delivered to the cells. Results for percent positive cells and MFI can be found in the main text (**Figure 3**).

R9. This reagent was purchased from Peptide 2.0 and dissolved in PBS to make a 1 mM stock solution. The N:P ratio used for this experiment was 8:1 to be consistent with the ratios used for the polymeric reagents.

DeliverX. This reagent was purchased from Affymetrix. The recommended reagent quantities were used as documented in the user manual. No further optimization was performed. For

these experiments, DeliverX was first sonicated for five minutes to obtain a homogeneous solution. Following this, 5 µL of siRNA was dissolved in 45 µL of siRNA Buffer 1 and 7 µL of DeliverX was dissolved in 43 µL of siRNA Buffer 2. Both solutions were vortex mixed prior to mixing both solutions together. This solution was gently vortex mixed and then allowed to incubate at RT for 20 minutes prior to adding it to the cell suspension.

Xfect. This reagent was purchased from CloneTech. Optimization was not required as per the directions on the company's website and in the user manual. The amounts of siRNA and polymer delivery reagent to be used for each transfection were pre-determined by the company based on the well size and volume of media to be used. For these experiments 5 µL of siRNA was added to in 45 µL of Xfect reaction buffer. In a separate tube, 8 uL of Xfect polymer solution was added to 42 µL of Xfect reaction buffer. Both samples were vortex mixed prior to adding the polymer solution to the siRNA solution and vortex mixing. The complex was allowed to incubate at RT for 20 minutes prior to adding it to the cell suspension.

N-ter. This reagent was purchased from Sigma Aldrich. The ratio of siRNA to transfection peptide to be used was documented in the user manual, where it was also noted that this ratio did not require further optimization. For these experiments, 5 µL of siRNA was added to 45 µL of PBS and 7 µL of N-ter was added to 43 µL of RNase-free water. The solutions were gently vortexed before combining them together. The combined solution was then vortexed and allowed to incubate at RT for 20 minutes prior to adding to the cell suspension.

RNAiMAX. This reagent was purchased from Life Technologies. The ratio of siRNA to transfection reagent used was documented in the user manual. For these experiments, 5 µL of siRNA was added to 45 µL of serum free media and 15 µL of RNAiMAX was added to 35 µL of serum free media. These solutions were gently vortexed before combining the solutions and gently vortex mixing the combined solution. The complex was allowed to incubate at RT for 20 minutes prior to adding to the cell suspension.

JetPEI. This reagent was purchased from Polyplus Transfection. The ratio of siRNA to transfection reagent used was the same as documented for pDNA. For these experiments, 5 µL of siRNA was added to 45 µL of a 150 mM NaCl solution and 1.5 µL of JetPEI was added to 48.5 µL of a 150 mM NaCl solution. These solutions were gently vortexed before combining

the solutions and gently vortex mixing the combined solution. The complex was allowed to incubate at RT for 30 minutes prior to adding to the cell suspension.

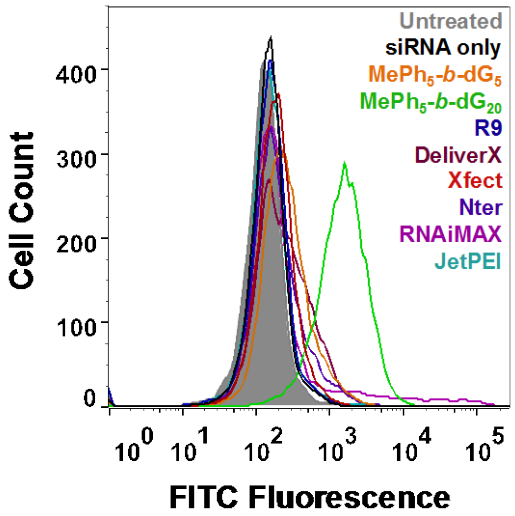

Figure S24. Representative histograms for FITC-siRNA delivery into Jurkat T cells using ROMP-based protein mimics and commercially available reagents. Jurkat T cells (cell density = $4x10^5$ cells/mL) were treated with polymer/FITC-siRNA complexes with an N/P ratio = 8/1 in complete media for four hours at 37°C and compared with untreated cells and cells only receiving FITC-siRNA.

Viability in Jurkat T Cells

General Annexin V / 7-AAD Viability Assay Procedure: This assay was used to assess apoptosis (Annexin V staining) as well as overall viability (7-AAD staining), in order to have a more complete understanding of how polymer/siRNA treatment affected the cell populations. Following wash steps documented above, cells were washed with 200 µL of 1X Annexin V binding buffer and subsequently spun down. Cells were re-suspended in 100 µL of an PE-Annexin V / binding buffer stock solution (1.2 mL of binding buffer + 60 µL PE-Annexin; Solution was scaled up or down as needed.) Cells were then incubated at RT for 15 minutes protected from light. After the incubation, cells were brought up to 200 µL with Annexin V binding buffer and spun down. Cells were re-suspended in 200 µL of a 7-AAD stock solution (2.4 mL of binding buffer + 60 µL of 7-AAD stain; Solution was scaled up or down as needed) and transferred to FCM tubes for analysis. For flow cytometry analysis, the fluorescence signal was collected for 10,000 cells. The cell populations were gated in order to assess the percent of positive cells, which reflected the percentage of dead cell in the population.

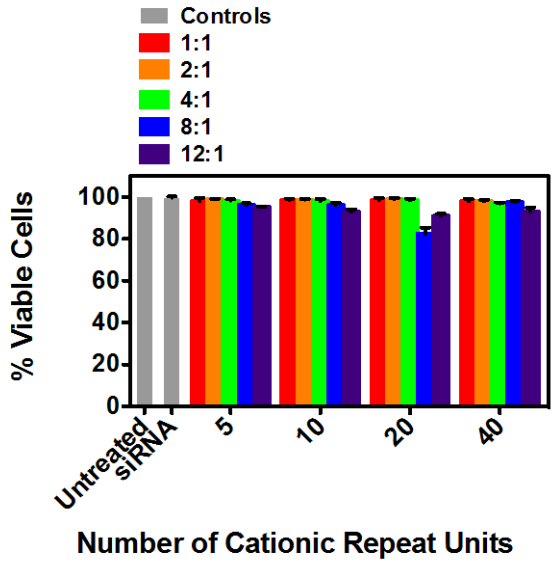

Figure S25. Percent viable cells using a 7-Amino-actinomycin (7-AAD) Jurkat T Cell viability assay. Jurkat T cells (cell density = $4x10^5$ cells/mL) were treated with PTDM/FITC-siRNA complexes with an N/P ratio = 8/1 in complete media for four hours at 37°C and compared with untreated cells and cells only receiving FITC-siRNA. Cells were stained at four hours. Each data point represents the mean ± SEM of three independent experiments.

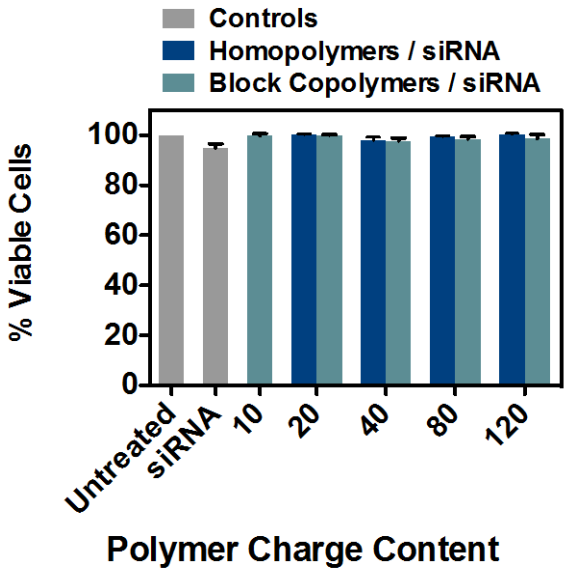

Figure S26. Percent viable cells using a 7-Amino-actinomycin (7-AAD) Jurkat T Cell viability assay. Jurkat T cells (cell density = $4x10^5$ cells/mL) were treated with PTDM/FITC-siRNA complexes with an N/P ratio = 8/1 in complete media for four hours at 37°C and compared with untreated cells and cells only receiving FITC-siRNA. Cells were stained at four hours. Each data point represents the mean ± SEM of three independent experiments.

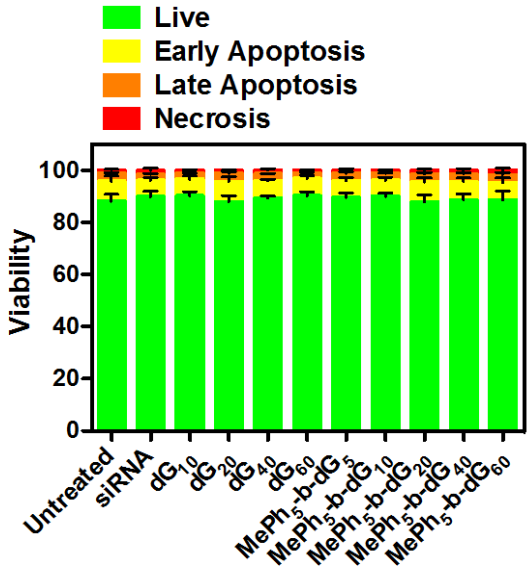

Figure S27. Percent viable cells using a 7-Amino-actinomycin (7-AAD) and Annexin-V Jurkat T cell viability assay. Jurkat T cells (cell density = $4x10^5$ cells/mL) were treated with polymer/FITC-siRNA complexes with an N/P ratio = 8/1 in complete media for four hours at 37°C and compared with untreated cells and cells only receiving FITC-siRNA. Cells were stained at four hours. Each data point represents the mean ± SEM of three independent experiments.

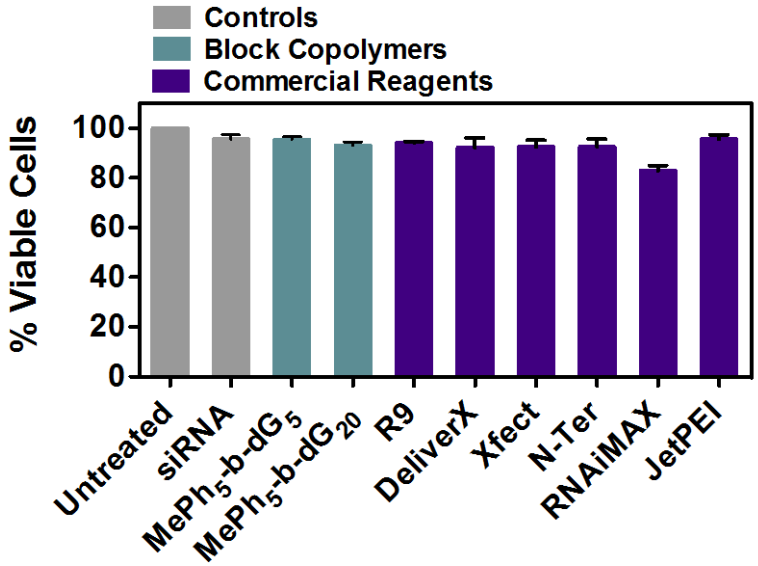

Figure S28. Percent viable cells using a 7-Amino-actinomycin (7-AAD) Jurkat T Cell viability assay. Jurkat T cells (cell density = $4x10^5$ cells/mL) were treated with PTDM/FITC-siRNA complexes with an N/P ratio = 8/1 or commercial reagents/FITC-siRNA complexes used as directed in complete media for four hours at 37°C and compared with untreated cells and cells

only receiving FITC-siRNA. Cells were stained at four hours. Each data point represents the mean ± SEM of three independent experiments.

Viability at 4 and 24 hr: To assess cell viability at 4 and 24 hr, $4x10^5$ cells/mL were treated as documented in the general FITC-siRNA uptake in Jurkat T cells procedure, with the exception that some cells were treated with just the polymer itself. After the 4 hr treatment, cells were split in half ($2x10^5$ cells/mL in each half) and washed three times with 500 µL of a 20 U/mL heparin solution. After the third wash, one half of the cells were viability stained as documented in the General Annexin V / 7-AAD viability assay procedure and analyzed by FCM and the other half were re-suspended in warm media and re-plated ($2x10^5$ cells/mL) for analysis at 24 hr. After 24 hr, the re-plated cells were harvested, washed three times with 500 µL of a 20 U/mL heparin solution, and prepared for viability staining and subsequent analysis. For flow cytometry analysis, the fluorescence signal was collected for 10,000 cells. The cell populations were gated in order to assess the percent of positive cells, which reflected the percentage of dead cell in the population. Note that the longer polymers with charge contents larger than 40 were not tested.

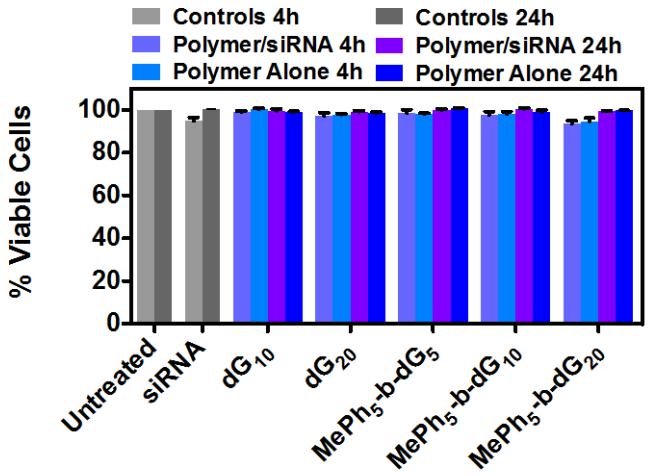

Figure S29. Percent viable cells using a 7-Amino-actinomycin (7-AAD) Jurkat T Cell viability assay. Jurkat T cells (cell density = $4x10^5$ cells/mL) were treated with PTDM/FITC-siRNA complexes with an N/P ratio = 8/1 or the same concentration of polymer with no siRNA in complete media for four hours at 37°C and compared with untreated cells and cells only receiving FITC-siRNA. Half of the cell populations ($2x10^5$ cells) were stained at four hours. The other half ($2x10^5$ cell) were re-plated in complete media and stained at 24 hours. Each data point represents the mean ± SEM of three independent experiments.

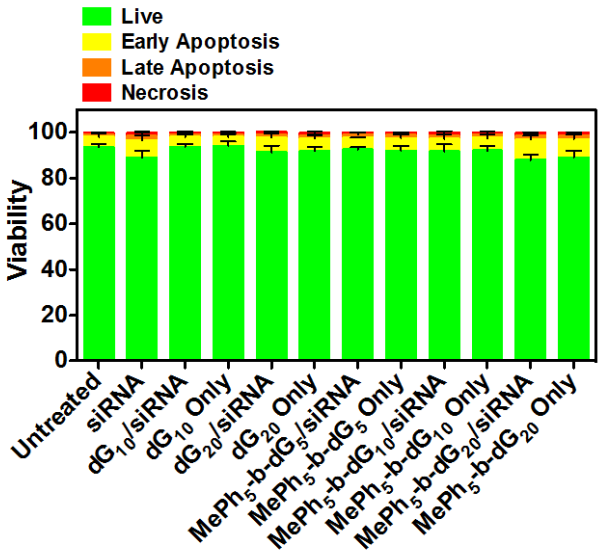

Figure S30. Percent viable cells using a 7-Amino-actinomycin (7-AAD) and Annexin-V Jurkat T cell viability assay at the four hour time point. Jurkat T cells (cell density = $4x10^5$ cells/mL) were treated with PTDM/FITC-siRNA complexes with an N/P ratio = 8/1 or the same concentration of PTDM with no siRNA in complete media for four hours at 37°C and compared with untreated cells and cells only receiving FITC-siRNA. Half the cell populations ($2x10^5$ cells) were stained at four hours and the other half ($2x10^5$ cells) were re-plated for a 24 hour time point. Each data point represents the mean ± SEM of three independent experiments.

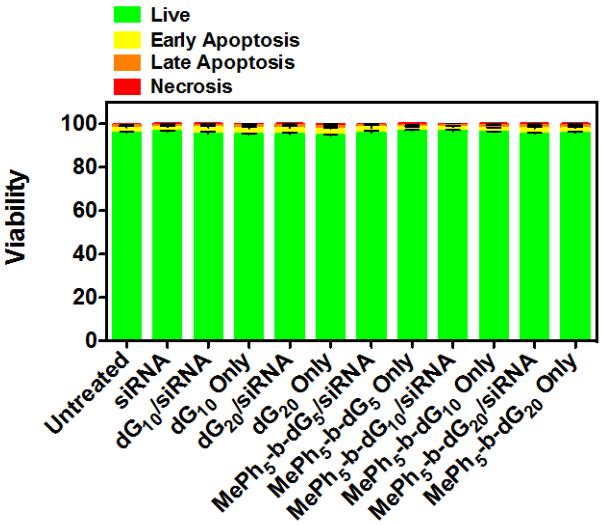

Figure S31. Percent viable cells using a 7-Amino-actinomycin (7-AAD) and Annexin-V Jurkat T cell viability assay at the 24 hour time point. Jurkat T cells (cell density = $4x10^5$ cells/mL) were treated with PTDM/FITC-siRNA complexes with an N/P ratio = 8/1 or the same concentration of PTDM with no siRNA in complete media for four hours at 37°C and compared with untreated cells and cells only receiving FITC-siRNA. Half the cell populations ($2x10^5$ cells) were stained at four hours and the other half ($2x10^5$ cells) were re-plated and stained at the

24 hour time point. Each data point represents the mean ± SEM of three independent experiments.

Cell Counts

In addition to viability staining at 4 and 24 hr, cells were counted at 24 hr to assess proliferation. 1/20 dilution of each experimental well in trypan blue was used for cell counts. Since Jurkat T cells have a doubling time of 24 hr, it was anticipated that healthy cell populations would double by the 24 hr time point. Cells were not counted at the 4 hr time point since they were initially counted at the start of the experiment.

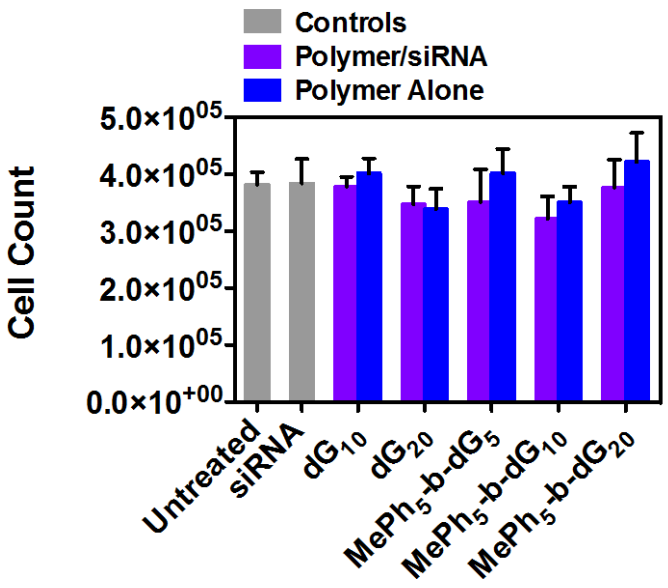

Figure S32. Cell counts 24 hours following PTDM/FITC-siRNA or polymer alone treatments. Jurkat T cells (cell density = $4x10^5$ cells/mL) were treated with PTDM/FITC-siRNA complexes with an N/P ratio = 8/1 in complete or the same concentration of PTDM with no siRNA media for four hours at 37°C and compared with untreated cells and cells only receiving FITC-siRNA. The entire cell population was counted 24 hours. Comparison wells receiving polymer and no siRNA were also used as controls. Each data point represents the mean ± SEM of three independent experiments.

FITC-siRNA Uptake in HeLa Cells

General Procedure: Polymers were dissolved in sterile DMSO to make 1 mM stock solutions. Polymers were stored at -20 °C in 50 µL aliquots. 48 hr prior to the experiment, HeLa T cells were trypsinized, harvested, centrifuged, counted, and re-suspended in complete cell growth medium with 10% FBS to a density of $5x10^4$ cells/mL (1 mL final volume in a 12-well plate). Cells were then incubated for approximately 48 hr at 37 °C in a 5% CO_2 atmosphere to allow

cells to become 70-90% confluent. On the day of the experiment, fresh media is added to the cells. Polymers with charge contents less than 40 were diluted to 0.1 mM with PBS and polymers with charge contents of 40 or larger were diluted to 0.005 mM with PBS (pH 7.4). Polymers were mixed with FITC-siRNA (10 μM stock solution, 50 nM in final well) at an N/P ratio of 4/1 in PBS (100 μL total for each complex solution). The N/P ratio optimization with the corresponding cell viability data can be found in Error! Reference source not found. and Error! Reference source not found.. Complexes were incubated at RT for 30 minutes prior to adding them drop-wise to each well. The 12-well plate was gently rocked back and forth to help evenly distribute the complex solutions. Cells were then incubated at 37 °C in a 5% CO_2 atmosphere for 4 hr. After 4 hr, cells were trypsinized, harvested, and washed 3 times with 500 μL a 20 U/mL heparin solution. Cells were either re-suspended in 200 μL of FACS wash buffer after the final wash for analysis or prepared for viability staining. See procedure below. For flow cytometry analysis, the fluorescence signal was collected for 10,000 cells. The cell populations were gated in order to assess the percentage of positive cells, which reflected the percentage of the cell population that received FITC-siRNA. The calculated MFI represented the amount of cargo delivered to the cells. Results for percent positive cells and MFI can be found in Figure S35.

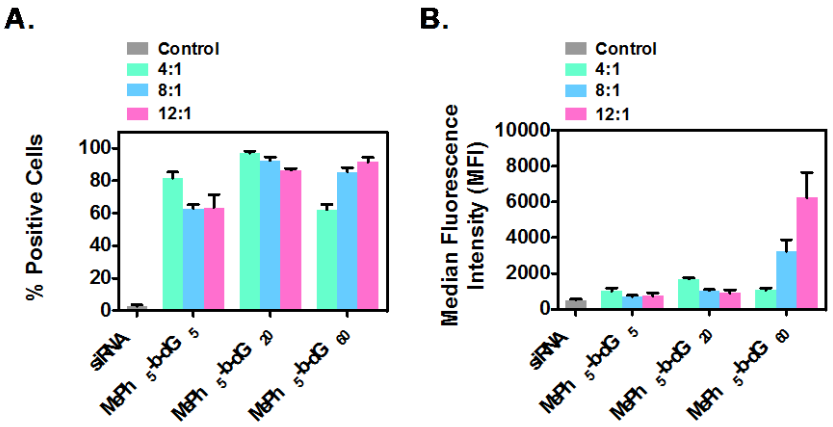

Figure S33. N/P ratio screening for FITC-siRNA delivery into HeLa cells using ROMP-based PTDMs. HeLa cells (cell density = $5x10^4$ cells/mL 48 hours prior to experiment; 70-90% confluent on the day of the experiment) treated with polymer/FITC-siRNA complexes with an N/P ratio of either 4/1, 8/1, or 12/1 in complete media for four hours at 37°C and compared cells only receiving FITC-siRNA. All data was compared to an untreated control. A) Percent positive cells. B) MFI of the cell population. Each data point represents the mean ± SEM of three independent experiments.

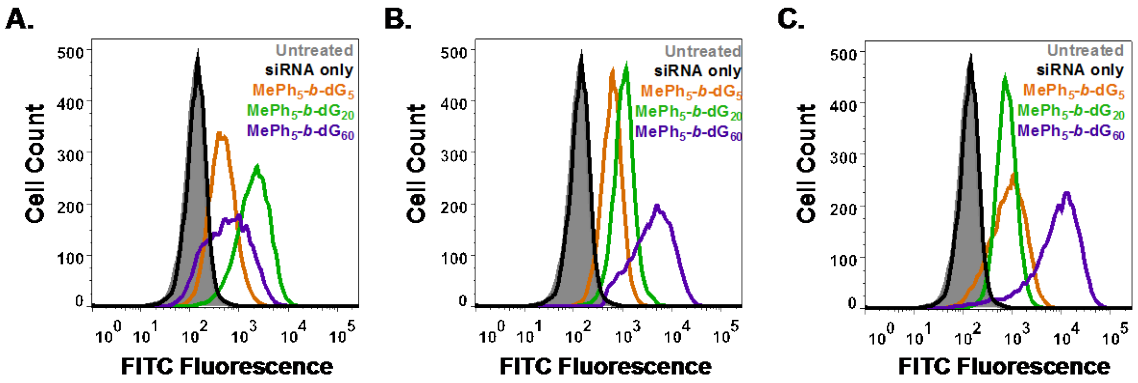

Figure S34. Representative histograms for FITC-siRNA delivery into HeLa cells using ROMP-based PTDMs. HeLa cells (cell density = $5x10^4$ cells/mL 48 hours prior to experiment; 70-90% confluent on the day of the experiment) treated with polymer/FITC-siRNA complexes with an N/P ratio = 8/1 in complete media for four hours at 37°C and compared cells only receiving FITC-siRNA and to an untreated control. A) Overlay of representative histograms for cells treated with PTDM/siRNA complexes with an N:P = 4:1. B) Overlay of representative histograms for cells treated with PTDM/siRNA complexes with an N:P = 8:1. C) Overlay of representative histograms for cells treated with PTDM/siRNA complexes with an N:P = 12:1.

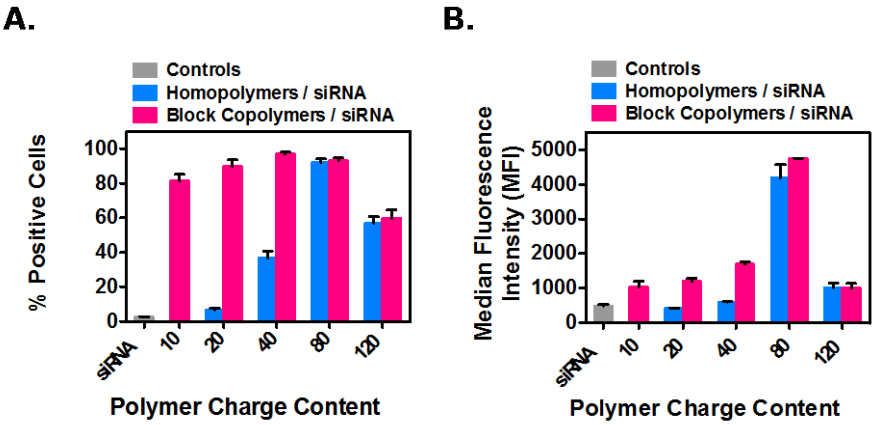

Figure S35. FITC-siRNA delivery into HeLa cells using ROMP-based protein mimics. HeLa cells (cell density = $5x10^4$ cells/mL 48 hours prior to experiment; 70-90% confluent on the day of the experiment) treated with polymer/FITC-siRNA complexes with an N/P ratio = 8/1 in complete media for four hours at 37°C and compared cells only receiving FITC-siRNA. All data was normalized to an untreated control. A) Percent positive cells. B) MFI) of the cell population. Each data point represents the mean ± SEM of three independent experiments.

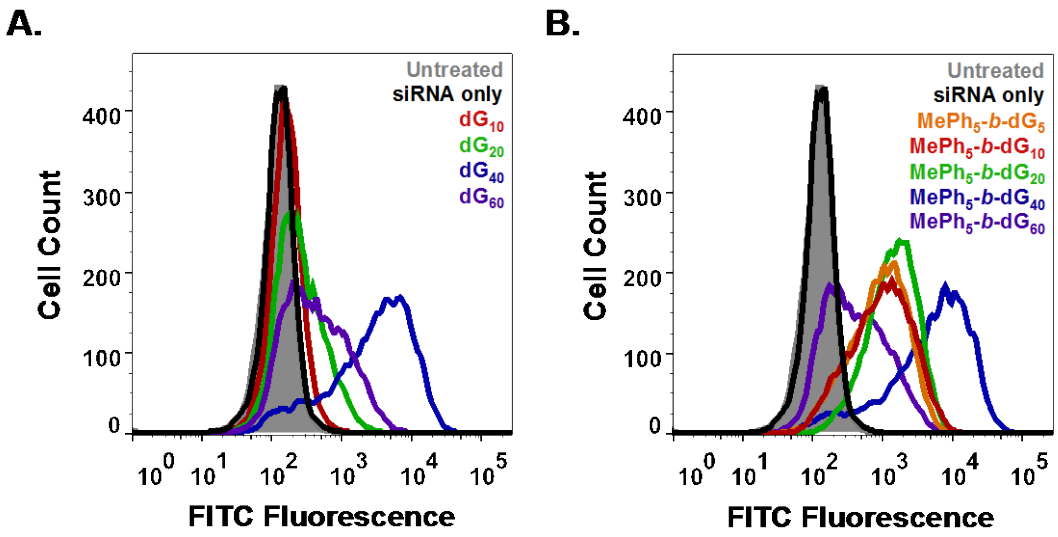

Figure S36. Representative histograms for FITC-siRNA delivery into HeLa cells using ROMP-based protein mimics. HeLa cells (cell density = $5x10^4$ cells/mL 48 hours prior to experiment; 70-90% confluent on the day of the experiment) treated with polymer/FITC-siRNA complexes with an N/P ratio = 8/1 in complete media for four hours at 37°C and compared cells only receiving FITC-siRNA and to an untreated control. A) Overlay of representative histograms for cells treated with homopolymer/siRNA complexes. B) Overlay of representative histograms for cells treated with block copolymer/siRNA complexes.

Viability in HeLa Cells

General 7-AAD Viability Assay Procedure: Following the wash steps documented above, cells were re-suspended in 200 µL of a 7-AAD stock solution (2.4 mL of FACS wash buffer + 60 µL of 7-AAD stain; Solution was scaled up or down as needed) and transferred to FCM tubes for analysis. For flow cytometry analysis, the fluorescence signal was collected for 10,000 cells. The cell populations were gated in order to assess the percent of positive cells, which reflected the percentage of dead cell in the population.

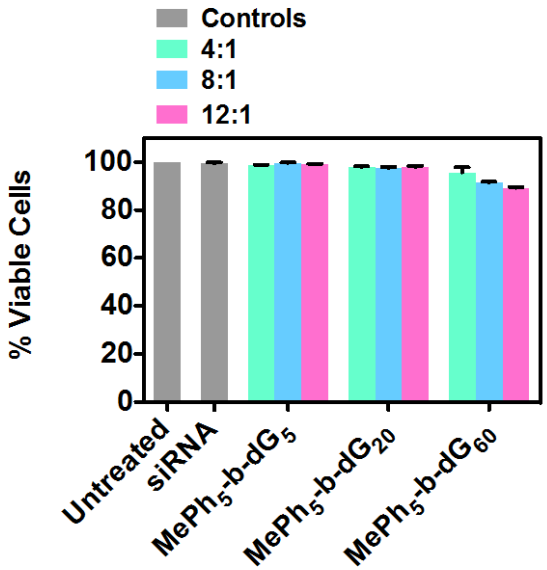

Figure S37. Percent viable cells using a 7-Amino-actinomycin (7-AAD) HeLa cell viability assay. HeLa cells (cell density = $5x10^4$ cells/mL 48 hours prior to experiment; 70-90% confluent on the day of the experiment) were treated with polymer/FITC-siRNA complexes with an N/P ratio of either 4/1, 8/1, or 12/1 in complete media for four hours at 37°C and compared with untreated cells and cells only receiving FITC-siRNA. Cells were stained at four hours. Each data point represents the mean ± SEM of three independent experiments.

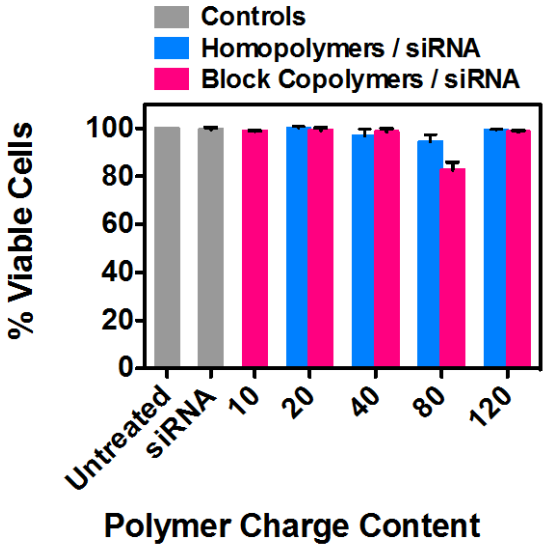

Figure S38. Percent viable cells using a 7-Amino-actinomycin (7-AAD) HeLa cell viability assay. HeLa cells (cell density = $5x10^4$ cells/mL 48 hours prior to experiment; 70-90% confluent on the day of the experiment) were treated with polymer/FITC-siRNA complexes with an N/P ratio = 8/1 in complete media for four hours at 37°C and compared with untreated cells and cells only receiving FITC-siRNA. Cells were stained at four hours. Each data point represents the mean ± SEM of three independent experiments.

V. *hNOTCH1 KNOCKDOWN IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS (*hPBMCs)

Cell enrichment: The night before the experiment, human peripheral blood mononuclear cells (hPBMCs) were thawed and enriched for the viable cell population, a majority of which were T cells, overnight. In this process, hPBMCs were thawed, added to 9 mL of warmed media (RPMI 1640 with 10% FBS), centrifuged, re-suspended in 2 mL of warmed media, and added to two wells of a 6-well plate. Each well was brought up to 3 mL with warmed media and cells were incubated at 37 °C in a 5% CO_2 atmosphere overnight.

Well-plate coating: The night before the experiment, a 24-well plate was antibody-coated with a solution contain anti-CD3 and anti-CD28. For a stock solution that can coat 12 wells, 3.940 mL of PBS was added to a 15 mL centrifuge tube along with 40 µL of a 0.5 mg/mL solution of anti-CD3 in PBS and 20 µL of a 0.5 mg/mL solution of anti-CD28. The solution was lightly vortexed and 300 µL of it was transferred to each of 12 wells. This stock solution preparation can be scaled up or down as needed.

hPBMC Treatment and Incubation: Polymers were dissolved in sterile DMSO to make 1 mM stock solutions. Polymers were stored at -20 °C in 50 µL aliquots. On the day of the experiment, hPBMCs were harvested, centrifuged, counted, and re-suspended in complete cell growth medium with 10% FBS to a density of $1x10^6$ cells/mL (1 mL final volume in a 12-well plate). Polymers with charge contents less than 40 were diluted to 0.1 mM with PBS and polymers with charge contents of 40 or larger were diluted to 0.005 mM with PBS (pH 7.4). Polymers were mixed with siRNA (10 µM stock solution, 100 nM in final well) at an N/P ratio of 8/1 in PBS (100 µL total for each complex solution). This N/P ratio was previously optimized.[3] Complexes were incubated at RT for 30 minutes prior to adding them drop-wise to each well and gently pipetting the media in each well up and down to evenly mix in the complex solutions. Cells were then incubated at 37 °C in a 5% CO_2 atmosphere for 4 hr. After 4 hr, the cells were harvested, centrifuged, re-suspended in complete cell growth medium with 10% FBS and transferred to the coated well plate for stimulation. At least one untreated sample was transferred to an uncoated well to serve as a stimulation control. Cells were incubated at 37 °C in a 5% CO_2 atmosphere for 48 hr.

Flow Cytometry Analysis:

Harvesting: After 48 hrs, cells were harvested, centrifuged, and washed with PBS. Cells were re-suspended in 200 µL of PBS and transferred to a 96-well plate. Cells were washed two additional times with FACS wash buffer. After the last wash, the cells were split in half, one part for intracellular staining for *hNOTCH1* protein and one part for viability staining.

Surface Staining: Following the wash steps documented above, cells were resuspended in 100 µL of FACS wash buffer and stained with 5 µL of FITC-labeled anti-CD8 and 5 µL of APC-labeled anti-CD4. Cells were incubated for 30 minutes on ice protected from light. After 30 minutes, the cells were brought up to 200 µL with FACS wash buffer and spun down. Cell were washed two additional times with 200 µL FACS wash buffer and then prepared for intracellular staining or viability staining. These stains were used to verify the CD4+ and CD8+ T cell populations present in the cell samples at 48 hours. Representative gating of cells is provided in Figure S[Bd1]. The cell populations were roughly 30% CD8+ T cells and 50% CD4+ T cells.

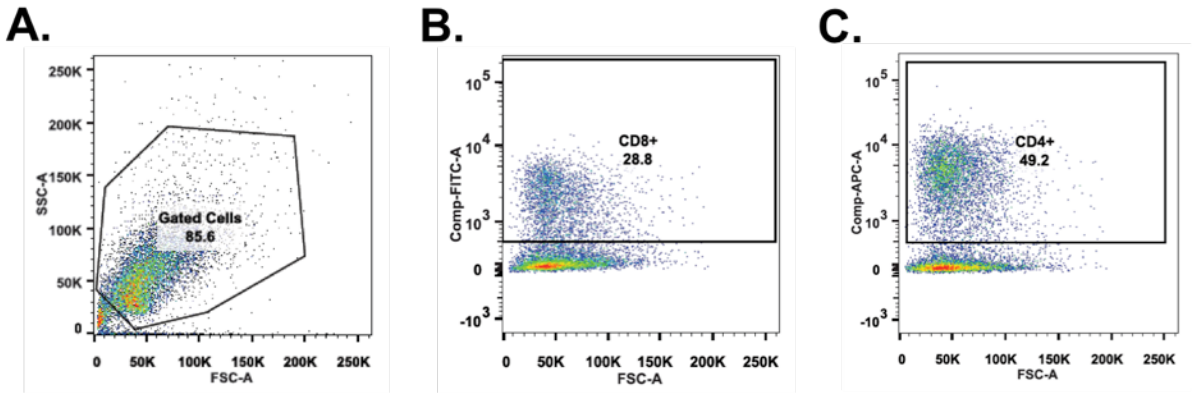

Figure S39. Representative flow cytometry cell gating to determine the fraction of the population that represent CD8+T cells and CD4+ T cells. A) Plot of forward vs. side scatter showing the gate on the cell population used for analysis. B) Plot of FITC fluorescence vs. forward scatter showing the gate on the FITC-positive cells (CD8+ T cells). C) Plot of APC fluorescence vs. forward scatter showing the gate on the APC-positive cells (CD4+ T cells).

Intracellular Staining: Following cell surface staining documented above, cells were re-suspended in 100 µL of the Foxp3 Fix/Perm Cocktail and incubated for 30 min on ice protected from light. After the 30 min incubation, the cells were brought up to 200 µL with the permeabilization wash buffer, which was prepared by diluting a 5 mL of a 10X stock solution

in 45 mL of deionized water. Cells were washed three times with the permeablization wash buffer. After the third wash step, cells were re-suspended in 50 µL of the permeablization wash buffer, stained with 2 µL of anti-human NOTCH1 PE, and incubated for 30 min on ice protected light. After the 30 min incubation, cells were washed three times with the permeablization wash buffer and then re-suspended in 200 µL of FACS wash buffer and transferred to FCM tubes for analysis. For flow cytometry analysis, the fluorescence signal was collected for 10,000 cells. The cell populations were gated in order to assess the percent of positive cells, which reflected the percentage of the cell population expressing *hNOTCH1* protein. The calculated MFI represented the amount of *hNOTCH1* protein present in the cells. The percent relative protein expression represents the percent positive cells multiplied by the MFI, normalized to the blank, and multiplied by 100%. Results for the percent relative protein expression can be found in the main text (**Figure 4**).

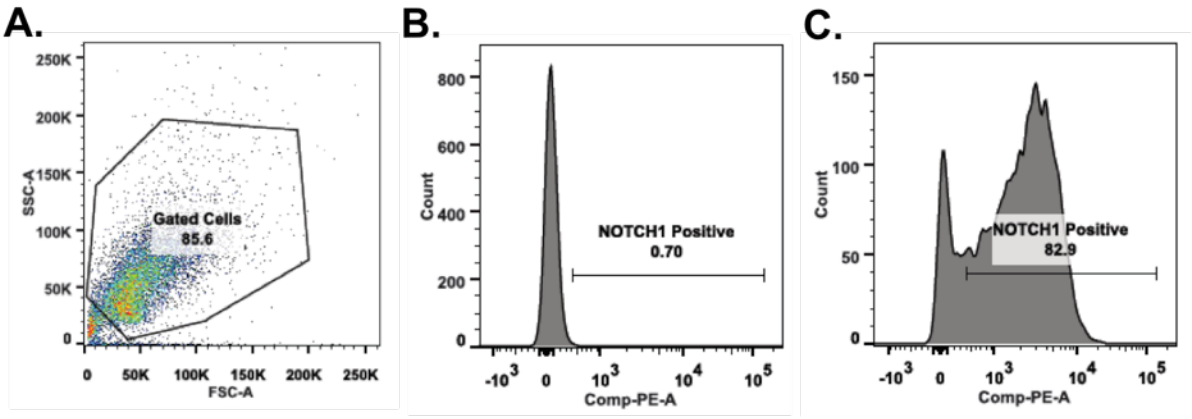

Figure S40. Representative flow cytometry cell gating to determine the NOTCH1 positive population. A) Plot of forward vs. side scatter showing the gate on the cell population used for analysis. B) Flow cytometry histogram showing the PE gate on the unstained samples. C) Flow cytometry histogram showing the PE-positive population in a treated sample.

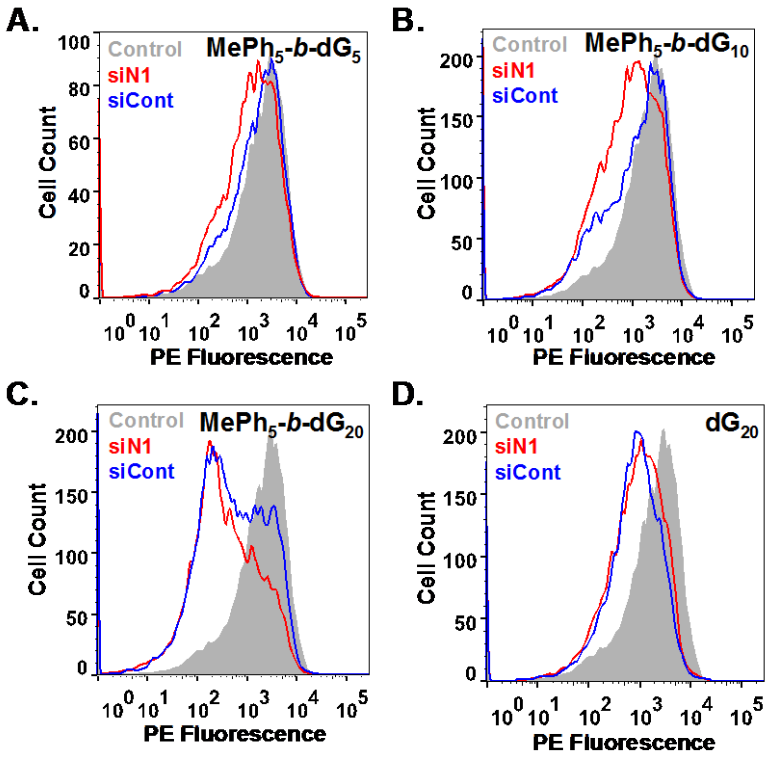

Figure S41. Representative histograms for *hNOTCH1* knockdown in PBMCs using ROMP-based PTDMs. PBMCs (cell density = $1x10^6$ cells/mL). Cells treated with PTDM/siN1 complexes or PTDM/siCont with an N/P ratio = 8/1 in complete media for four hours at 37°C. After treatment, cells were washed and then stimulated with plate-bound anti-CD3 and anti-CD28 for 48 hours. Untreated samples, samples receiving *hNOTCH1* siRNA (siN1), and samples receiving scrambled control siRNA (siCont) are represented in grey, red, and blue, respectively.

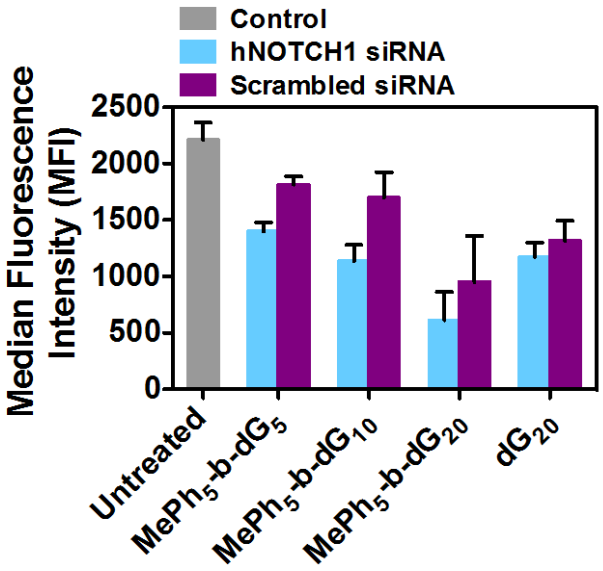

Figure S42. MFI for hNOTCH1 protein in hPBMCs (cell density = $1x10^6$ cells/mL). Cells treated with PTDM/hNOTCH1 siRNA complexes or PTDM/scrambled siRNA with an N/P ratio = 8/1 in complete media for four hours at 37°C. After treatment, cells were washed and then stimulated with plate-bound anti-CD3 and anti-CD28 for 48 hours. All data was normalized to an untreated control. Each data point represents the mean ± SEM of four independent experiments.

7-AAD Cell Viability Assay: Following the wash steps documented above, cells were re-suspended in 200 µL of a 7-AAD stock solution (2.4 mL of binding buffer + 60 µL of 7-AAD stain; Solution was scaled up or down as needed) and transferred to FCM tubes for analysis. For flow cytometry analysis, the fluorescence signal was collected for 10,000 cells. The cell populations were gated in order to assess the percent of positive cells, which reflected the percentage of dead cell in the population. Viability results can be found in the main text (**Figure 4**).

Western Blot

PBMCs, in the presence or absence of stimulation, were harvested at 48 hours post-treatment and whole cell lysates were prepared using RIPA buffer. Lysates were resolved on an 8% SDS-PAGE gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk in PBST and probed with Anti-cleaved Notch1 Val1744 (D3B8, Cell Signaling Technology) or Anti-Glyceraldehyde-3-Phosphate Dehydrogenase Antibody (6C5, Merck Millipore). Membranes were then washed and incubated with their corresponding horseradish peroxidase-conjugated secondary antibodies (GE Healthcare). Oxidation of the

chemiluminescent substrate (#34087, Thermo Fisher Scientific) was detected using a G:Box (Syngene).

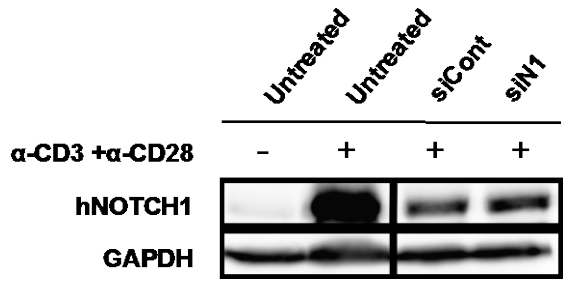

Figure S43. Western blot analysis showing knockdown of NOTCH1 by **$MePh_5$-*b*-dG_{20}**. Unstimulated, untreated cells (lane 1), untreated, stimulated cells (lane 2), treated with $MePh_5$-*b*-dG_{20}/scrambled siRNA complexes (siCont, lane 3) or $MePh_5$-*b*-dG_{20}/NOTCH1 siRNA complexes (siN1, lane 4). Total protein lysates were immunoblotted with antibodies that detect the active NOTCH1 intracellular domain and GAPDH. Untreated, unstimulated PBMCs (lane 1) were included as a negative control of the active NOTCH1 intracellular domain. siCont stands for scrambled siRNA and siN1 stands for siRNA to *NOTCH1*.

VI. REFERENCES

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(3) Tezgel, A. Ö. *et al. Mol. Ther.* **2013**, *21*, 201.

(4) Brody, J. R., Kern, S. E. *Biotechniques*. **2004,** *36*, 214.