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Supporting Information

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Reactive and Bioactive Cationic α-Helical Polypeptide Template for Nonviral Gene Delivery**

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Supporting Information

***Materials*.**

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received unless otherwise specified. VB-Glu-NCA was prepared and polymerized according to published procedures.[1] Pierce BCA assay kits were purchased from ThermoFisher Scientific (Rockford, IL, USA). Luciferase assay reagent was purchased from Promega (Madison, WI, USA). Lipofectamine 2000 (LFA) and the fluorescent dye YOYO-1 were purchased from Invitrogen (Carlsbad, CA, USA).

Instrumentation.

NMR spectra were recorded on a Varian UI400 MHz, a UI500NB MHz or a VXR-500 MHz spectrometer. Tandem gel permeation chromatography (GPC) experiments were performed on a system equipped with an isocratic pump (Model 1100, Agilent Technology, Santa Clara, CA, USA), a DAWN HELEOS 18-angle laser light scattering detector (also known as multi-angle laser light scattering (MALLS) detector, Wyatt Technology, Santa Barbara, CA, USA) and an Optilab rEX refractive index detector (Wyatt Technology, Santa Barbara, CA, USA). The detection wavelength of HELEOS was set at 658 nm. Separations were performed using serially connected size exclusion columns (100 Å, 500 Å, 10^3Å and 10^4 Å Phenogel columns, 5 μm, 300 × 7.8 mm, Phenomenex, Torrance, CA) at 60°C using DMF containing 0.1 M LiBr as the mobile phase. The MALLS detector was calibrated using pure toluene with no need for external polymer standards and was used for the determination of the absolute molecular weights. The molecular weights (MWs) of all polymers were determined based on the dn/dc value of each sample

calculated offline by using the internal calibration system processed by the ASTRA V software (version 5.1.7.3, Wyatt Technology, Santa Barbara, CA, USA). Circular dichroism (CD) measurements were carried out on a JASCO 720 CD Spectrometer. Ozone was produced by an OZV-8S ozone generator manufactured by Ozone Solutions Inc (Hull, IA, USA). Lyophilization was performed on a FreeZone lyophilizer (Labconco, Kansas City, MO, USA).

Cells and Plasmids.

COS-7, HEK293, MDA-MB-231 and HeLa cells were obtained from ATCC. The cells were cultured according to their ATCC protocols at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM). The growth medium was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Federally registered hESC line H9 was purchased from WiCell Research Institute and routinely maintained under feeder conditions. The expression vectors pCMV-Luc coding for the luciferase gene and pEGFP-N1 coding for green fluorescent protein were obtained from Elim Biopharmaceuticals (Hayward, CA, USA).

Synthesis of PVBLG-X (X = 1-31).

Method 1: Poly(γ-(4-aldehydebenzyl-$_L$-glutamate) (20 mg), amine (3-5 molar equivalents relative to the Glu repeating unit) and the reducing agent $NaBH(OAc)_3$ (5-10 molar equivalents) were mixed in DMF (2 mL). The reaction mixtures were stirred at 50-60^{o}C for 24-48 h. The solution was then poured into 3 M HCl (3 mL), followed by dialysis against water and lyophilization (***X = 1-5***).

Method 2: Poly(γ-(4-aldehydebenzyl-$_L$-glutamate) (20 mg) and amine (3-5 molar equivalents relative to the Glu repeating unit) were stirred in DMF (2 mL) at 50-60^{o}C for 24h, followed by the addition of the reducing agent $NaBH_4$ (5-10 molar equivalents). The resulting solutions were stirred at room temperature for another 24 h. The mixture were then poured into 3 M HCl (3 mL), followed by dialysis against water and lyophilization (***X = 6-7***).

Method 3: Poly(γ-(4-aldehydebenzyl-$_L$-glutamate) (20 mg) and amine (3-5 molar equivalents relative to the Glu repeating unit) were stirred in DMF (2 mL) at 50-60^{o}C for 24h, followed by the addition of the reducing agent borane pyridine (5-10 molar equivalents). The resulting solutions were stirred at 50-60^{o}C for another 24 h. The mixtures were then poured into 3 M HCl (3 mL), followed by dialysis against water and lyophilization (***X = 8-31***).

The yields of the products PVBLG-X (X = 1-31) were around 50-75% after dialysis. Grafting efficiencies were analyzed by ^{1}H-NMR integration and all of the polymers have grafting efficient around 90% except for PVBLG-7 (70 % grafting density).

General procedure for the analysis of polymer by circular dichroism (CD).

Circular dichroism experiments were performed on a J-720 CD spectrometer. Polymer samples were prepared at concentrations of 0.05 mg/mL in DI water unless otherwise specified. The solution was placed in a quartz cell with a light path of 0.5 cm. The mean residue molar ellipticity of each polymer was calculated based on the measured apparent ellipticity by following equations reported in literature: Ellipticity ([θ] in deg·cm^2·dmol^{-1}) = (millidegrees × mean residue weight)/(pathlength in millimeters × concentration of polypeptide in mg ml^{-1}).[2, 3] Experiments were carried out at room temperature. For pH-dependency studies, polymers were dissolved in buffer solutions at specific pH values. The background of the buffer was subtracted from each spectrum. Sample CD spectra for the top-performing PVBLG$_n$-8 polymer as well as the random coil PVB-DL-G$_n$-8 can be seen in Figure 3c. The data indicates that PVBLG$_n$-8 helicity is maintained over pH values ranging from pH 2 to pH 7.4. Figure S1 indicates there was no detectable helicity change of the PVBLG$_n$-8 before and after the formation of the polyplex with DNA, as elicited by comparing the θ (mdeg) value of "pDNA + PVBDLG$_{150}$-8" and the "polyplex" at 222 nm. A more complete analysis of the pH and temperature stability of PVBLG$_n$-X helicity can be found elsewhere.[1]

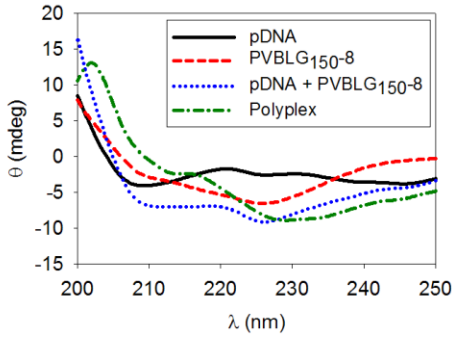

Figure S1. Circular dichroism spectra of free plasmid DNA (0.05 mg/mL), free $PVBLG_{150}$-8 (0.05 mg/mL), the numerical addition of the free DNA and $PVBLG_{150}$-8 curves as well as the actual polyplex formed by mixing plasmid DNA and $PVBLG_{150}$-8 at a 1:1 weight ratio (concentration of DNA and $PVBLG_{150}$-8 were 0.05 mg/mL and 0.05 mg/mL after mixing). For comparison and simplicity reasons, the raw data instead of normalized spectra are shown.

Gel Retardation Studies.

A solution of DNA (1 μg/10 μL) was prepared in doubled distilled water. Appropriate amounts of select $PVBLG_n$-X polymers (X = 1-9) dissolved in double distilled water were added to the DNA solution (10 μl) to achieve the desired $PVBLG_n$-X:DNA weight ratio. Complexes were incubated at room temperature for 15 min, after which loading dye was added and the solution (10 μl) was run on a 1% agarose gel (70 V, 70 min). DNA was stained with ethidium bromide and visualized on a Gel Doc imaging system (Biorad, Herclues, CA, USA).

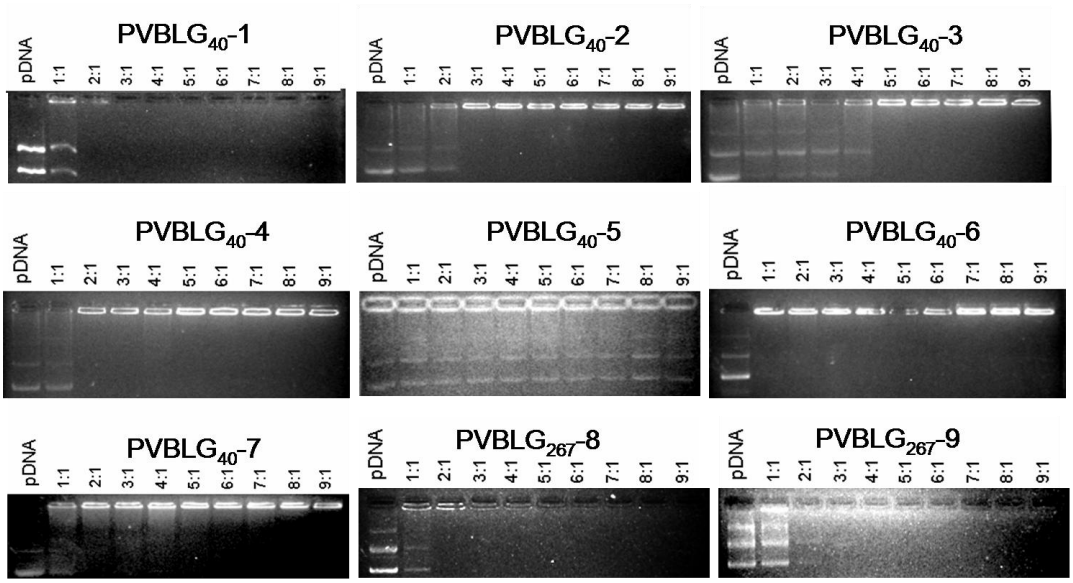

Figure S2. Gel retardation evaluating the ability of various $PVBLG_n$-X polymers to bind and condense DNA at various weight ratios. The numbers above the lanes correspond to the polypeptide:DNA weight ratio tested. Of the polymers tested, only $PVBLG_{40}$-5 was unable to bind and condense DNA at weight ratios up to 9:1

Complex Formation and Transfection.

DNA/polymer complexes were prepared at room temperature by dissolving DNA (0.35 μg) in 150 mM NaCl, 20 mM HEPES (175 μl). An equal volume of PLL, 25-kDa branched PEI or $PVBLG_n$-X in 150 mM NaCl, 20 mM HEPES was added to achieve the desired polypeptide:DNA weight ratio (typically ranging from 1:1 to 35:1). The final complexes were incubated at room temperature for 15 min before further use. Cells (COS-7, HEK293, MDA-MB-231 or HeLa) were cultured in DMEM supplemented with 10% horse serum and 1% penicillin-streptomycin according to ATCC protocols and plated in 96-well plates at 1×10^4 cells/well 24 h prior to transfection. Immediately before transfection, the growth medium was replaced with fresh serum-free DMEM and polyplex solution (50 μl) was added to each well (0.05 μg DNA/well). The transfection medium was replaced with fresh serum-supplemented growth medium 4 h post-transfection. For studies involving drug treatment (methyl-β-cyclodextrin at 10 mg/ml, chlorpromazine at 5 μg/ml, bafilomycin A1 at 5 nM, nocodazole at 20 μM), cells were incubated with serum-free DMEM containing the drug 30 min prior to transfection. Luciferase expression was quantified 24 h post-transfection using the Promega Bright-Glo luciferase assay system (Promega, Madison, WI, USA). Luciferase activity was measured in relative light units (RLU) using a PerkinElmer plate reader with luminescence capabilities (Waltham, MA, USA). Results were normalized to total cell protein using the Pierce BCA protein assay kit (Rockford, IL, USA). Transfections were performed in triplicate.

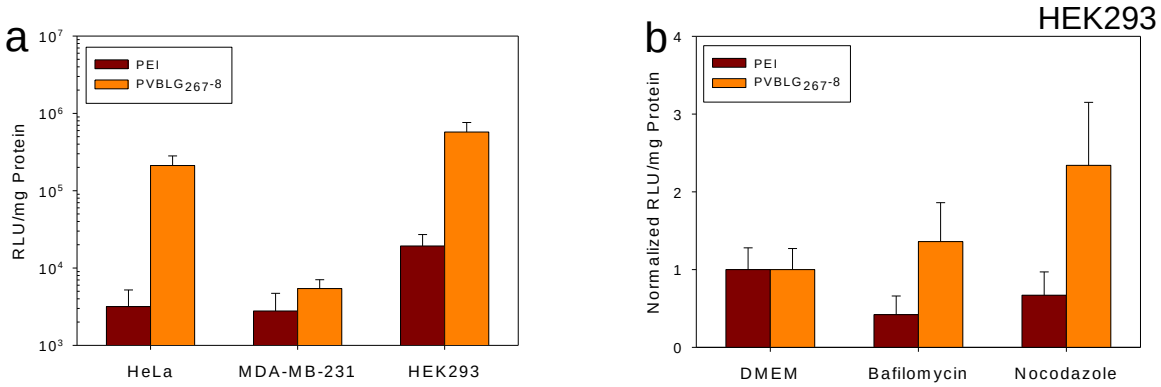

Figure S3. a) In vitro transfection of HeLa, MDA-MB-231 and HEK293 cells with 25-kDa branched PEI and the top-performing $PVBLG_{267}$-8 polymer. b) In vitro transfection of HEK293 cells transfected with complexes of 25-kDa PEI or $PVBLG_{267}$-8 in the presence of intracellular processing inhibitors. In both experiments, a $PVBLG_{267}$-8:DNA weight ratio of 30:1 was used, corresponding to a final $PVBLG_{267}$-8 concentration of 10 μg/ml.

Transfection in Serum.

DNA/polymer complexes were prepared at room temperature by dissolving DNA (0.35 μg) in 150 mM NaCl, 20 mM HEPES (175 μl). An equal volume of PVBLG$_{150}$-8 in 150 mM NaCl, 20 mM HEPES was added to a 25:1 polypeptide:DNA weight ratio. The final complexes were incubated at room temperature for 15 min before further use. Cells (COS-7) were cultured in DMEM supplemented with 10% horse serum and 1% penicillin-streptomycin according to ATCC protocols and plated in 96-well plates at 1×10^4 cells/well 24 h prior to transfection. Immediately before transfection, the growth medium was replaced with fresh serum-free DMEM or fresh DMEM containing 10% serum and polyplex solution (50 μl) was added to each well (0.05 μg DNA/well). The transfection medium was replaced with fresh serum-supplemented growth medium 4 h post-transfection. Luciferase expression was quantified 24 h post-transfection using the Promega Bright-Glo luciferase assay system (Promega, Madison, WI, USA). Luciferase activity was measured in relative light units (RLU) using a PerkinElmer plate reader with luminescence capabilities (Waltham, MA, USA). Results were normalized to total cell protein using the Pierce BCA protein assay kit (Rockford, IL, USA). Transfections were performed in triplicate.

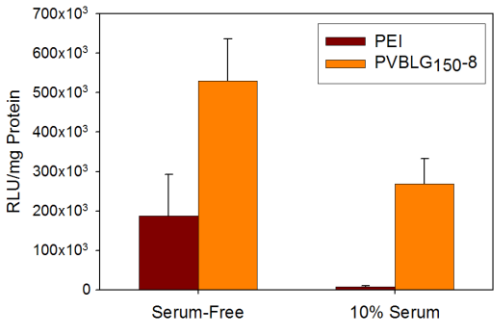

Figure S4. In vitro transfection of COS-7 cells with either PEI or PVBLG$_{150}$-8 polymer in the absence and presence of 10% fetal bovine serum. A PVBLG$_{150}$-8:DNA weight ratio of 25:1 was used, corresponding to a final PVBLG$_{150}$-8 concentration of 8.25 μg/ml.

Uptake.

DNA complexes were formed at their respective optimum transfection weight ratios as described above. The intercalating dye YOYO-1 was added at the ratio 15 nl YOYO-1 per 1 μg of DNA. Cells (COS-7 or HEK293) were cultured in DMEM supplemented with 10% horse serum and

1% penicillin-streptomycin according to ATCC protocols and plated in 24-well plates at 5×10^4 cells/well 24 h prior to transfection. Immediately before transfection, the growth medium was replaced with fresh serum-free medium and polyplex solution (50 μl) was added to each well (0.25 μg DNA/well). For studies involving drug treatment (methyl-β-cyclodextrin at 10 mg/ml, chlorpromazine at 5 μg/ml, bafilomycin A1 at 5 nM or nocodazole at 20 μM), cells were incubated with DMEM containing the drug 30 min prior to transfection. Four hours post-transfection, the cells were rinsed with PBS (0.5 mL × 2) to remove surface-bound complexes. Next, trypsin in PBS (0.05%, 100 μl) was added to each well. The cells and trypsin were incubated for approximately ten minutes before formaldehyde (4%, 400 μl) was added to each well. The cells were then collected and FACS analyses were performed on a BD Biosciences LSR II flow cytometer (Franklin Lakes, NJ, USA). Data were analyzed using the FCS Express software package (De Novo Software, Los Angeles, CA, USA). Transfections and uptake measurements were performed in triplicate.

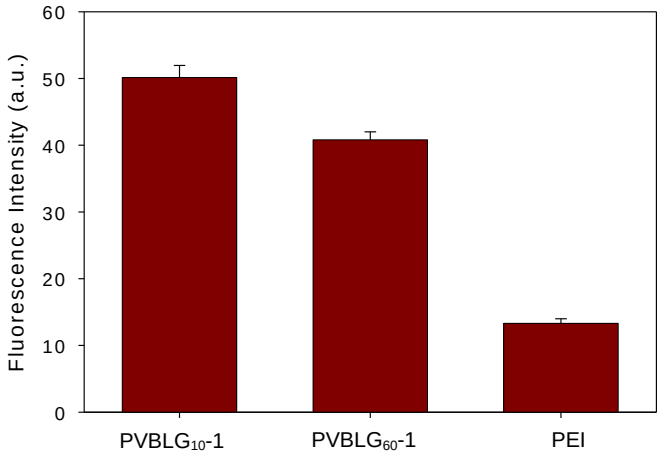

Figure S5. COS-7 cell uptake of YOYO-1 labeled complexes formed with either 25-kDa PEI or $PVBLG_{10}$-1 or $PVBLG_{60}$-1. The observation that both $PVBLG_{10}$-1 and $PVBLG_{60}$-1 show enhanced uptake compared to PEI while only $PVBLG_{60}$-1 has comparable transfection efficiency compared to PEI (see Figure 2a) suggests that the increased performance of the $PVBLG_n$-X polymers is likely not due solely to enhanced uptake.

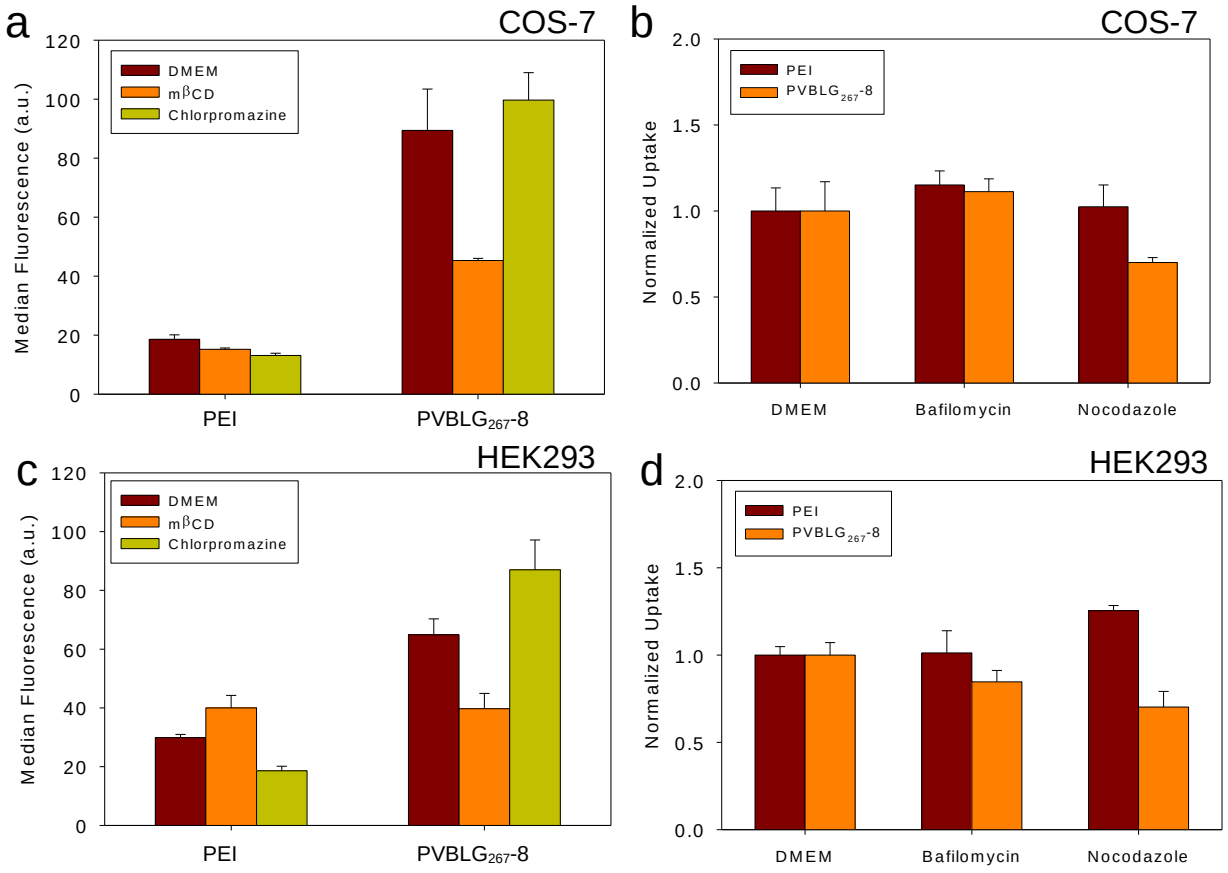

Figure S6. a) COS-7 cell uptake of YOYO-1 labeled complexes formed with either 25-kDa PEI or $PVBLG_{267}$-8 in the presence of endocytic inhibitors (mβCD = caveolae-mediated uptake inhibition, chlorpromazine = clathrin-mediated uptake inhibition). b) Normalized COS-7 cell uptake of YOYO-1 labeled complexes formed with either 25-kDa PEI or $PVBLG_{267}$-8 in the presence of intracellular processing inhibitors (bafilomycin A1 = endosome acidification inhibitor, nocodazole = microtubule depolymerizer). c) HEK293 cell uptake of YOYO-1 labeled complexes formed with either 25-kDa PEI or $PVBLG_{267}$-8 in the presence of endocytic inhibitors. c) Normalized HEK293 cell uptake of YOYO-1 labeled complexes formed with either 25-kDa PEI or $PVBLG_{267}$-8 in the presence of intracellular processing inhibitors. In all experiments, a $PVBLG_{267}$-8:DNA weight ratio of 30:1 was used, corresponding to a final $PVBLG_{267}$-8 concentration of 15 μg/ml.

Dynamic Light Scattering.

DNA complexes were formed in double distilled water at various $PVBLG_{267}$-8 to DNA weight ratios as indicated above. Following incubation at room temperature for 15 minutes, the complexes were diluted in water or PBS (1.8 mL) and subjected to size measurement on a Brookhaven Instruments Corporation 90 Plus Particle Size Analyzer Holtsville, NY). Five sets of measurements were performed for each sample.

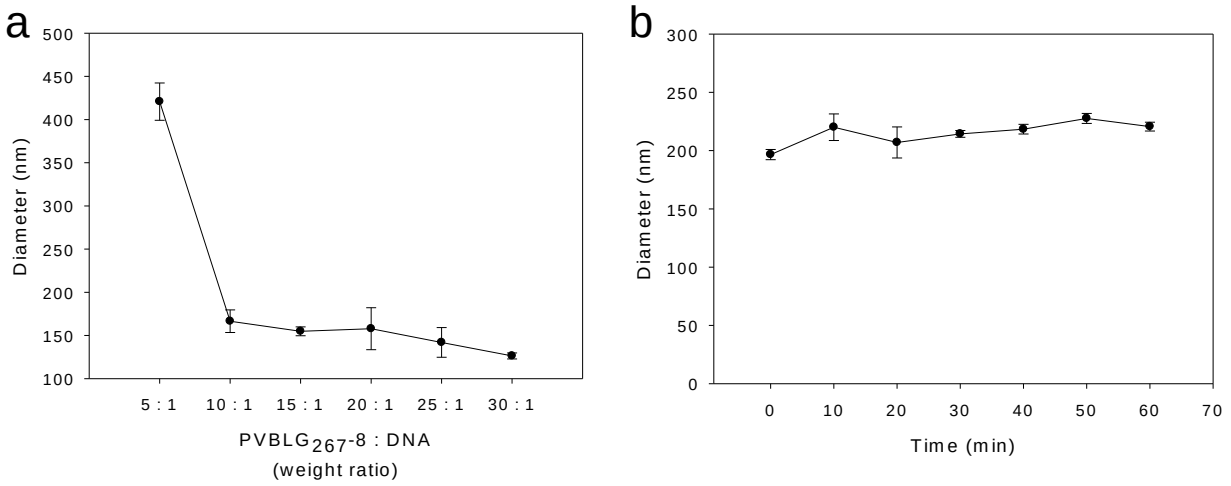

Figure S7. a) Measured diameter of $PVBLG_{267}$-8/DNA complexes at various polypeptide:DNA weight ratios. b) Diameter of $PVBLG_{267}$-8/DNA complexes (30:1 polypeptide:DNA ratio) over time in PBS.

Cytotoxicity Measurements.

The cytotoxicity of the PVBLG-X polymers was characterized using the MTT cell viability assay (Sigma-Aldrich, St. Louis, MO). Cells (COS-7) were seeded in 96-wells plates at 1×10^4 cells/well and grown overnight at 37 °C, 5% CO2 in medium containing 10% horse serum and 1% penicillin-streptomycin. Approximately 24 h after seeding the medium was replaced with serum-supplemented DMEM and the uncomplexed material was added to the cells at final concentrations between 0 and 50 μg/ml. After four hours of incubation, the medium was replaced with serum-containing medium and grown for another 20 h, after which reconstituted 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, 10 μl) was added. The plates were then incubated for another four hours and MTT solubilization solution (100 μl, Sigma-Aldrich, St. Louis, MO, USA) was added and the absorbance at 570 nm was read using a PerkinElmer plate reader (Waltham, MA, USA). The background absorbance of cells killed with ethanol was subtracted from the viable cell absorbance and normalized to cells grown in DMEM. Each experiment was repeated four times at each concentration.

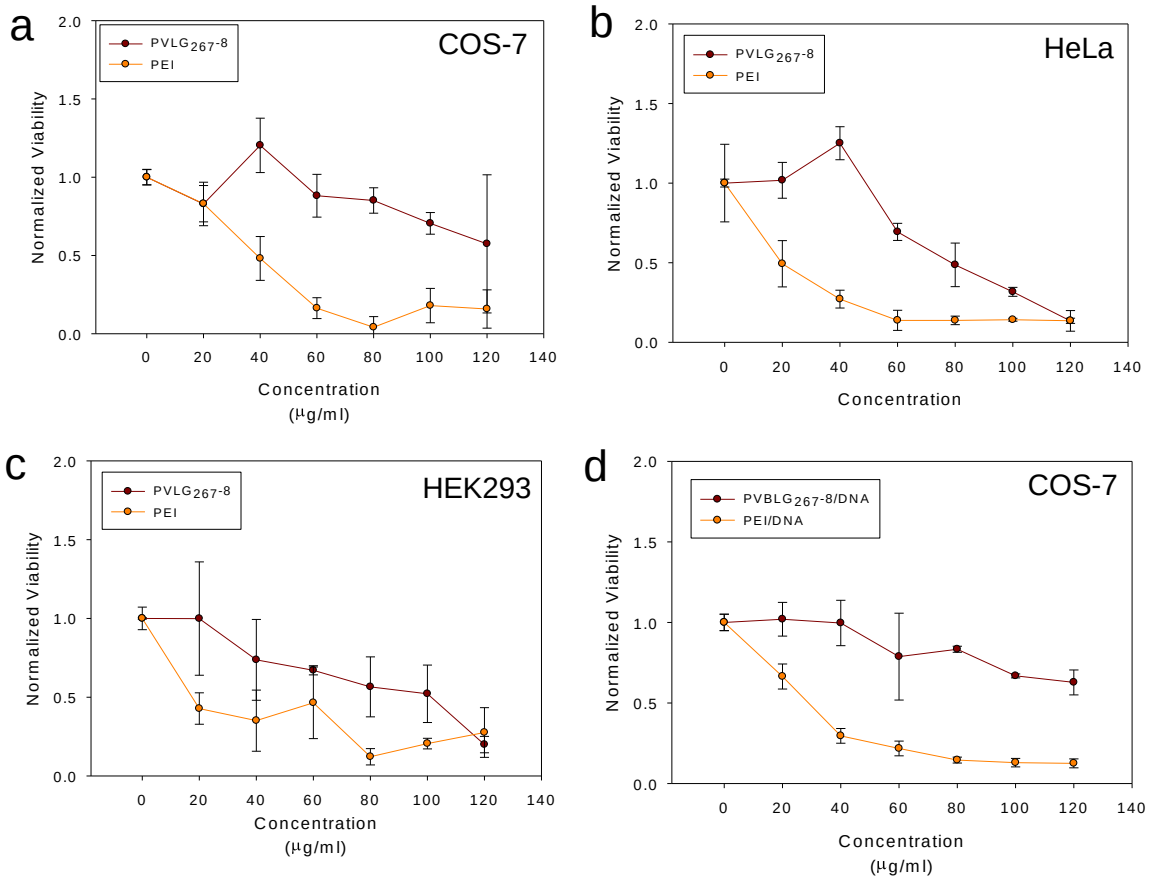

Figure S8. Toxicity of PEI and the top-performing PVBLG$_{267}$-8 polymers in (a) COS-7, (b) HeLa and (c) HEK293 cells as well as toxicity of PEI/DNA and PVBLG$_{267}$-8/DNA complexes in (d) COS-7 cells.

Fluorescence Microscopy.

DNA complexes were formed at the previously determined optimum transfection weight ratio as described above. COS-7 and HEK293 cells were cultured in DMEM supplemented with 10% horse serum and 1% penicillin-streptomycin according to ATCC protocols and plated in 6-well plates containing coverslips at 20 × 10^4 cells/well 24 h prior to transfection. Immediately before transfection, the growth medium was replaced with fresh serum-free medium containing 250 nM calcein. PVBLG$_{267}$-8 was added to cells at 0, 15 or 50 μg/ml. Sixty minutes post-transfection, the cells were rinsed with PBS (2 mL × 2) to remove surface-bound complexes and formaldehyde (4%, 1 mL) was added to each well. Following a 10-min incubation, the cells were rinsed with PBS (2 mL × 2) and mounted on glass slides. Cells were visualized with a Zeiss Axiovert 40 CFL fluorescence microscope equipped with a 40x objective.

Calcein is unable to cross intact membranes. As such, in the absence of an agent capable of pore formation, calcein is taken up by HEK293 cells in a pinocytic fashion, resulting in the appearance of small punctate intracellular fluorescent spots (Figure S9, 0 μg/ml). However, as the amount of $PVBLG_{267}$-8 in the extracellular medium is increased, the intracellular fluorescent signal becomes more diffuse, indicating membrane permeation and non-endocytic calcein uptake (Figure S9, 50 μg/ml). Although $PVBLG_{267}$-8 can function as an effective CPP when present in the medium at 50 μg/ml, such a high polypeptide concentration does not correspond with the optimum transfection formulation. At an intermediate concentration of $PVBLG_{267}$-8 which corresponds to the concentration of $PVBLG_{267}$-8 used in the optimum transfection formulation (i.e. 15 μg/ml), punctate fluorescent spots are observed. Taken as a whole, the data indicate that $PVBLG_{267}$-8 is able to form membrane pores in HEK293 cells when present at high concentration, although at lower concentrations—such as those that are relevant to in vitro gene delivery—pore formation does not occur and extracellular material must enter cells via an endocytic process. Endocytic uptake of transfection complexes formed with $PVBLG_{267}$-8 in HEK293 cells is also supported by the drug inhibition data of Figure S6c.

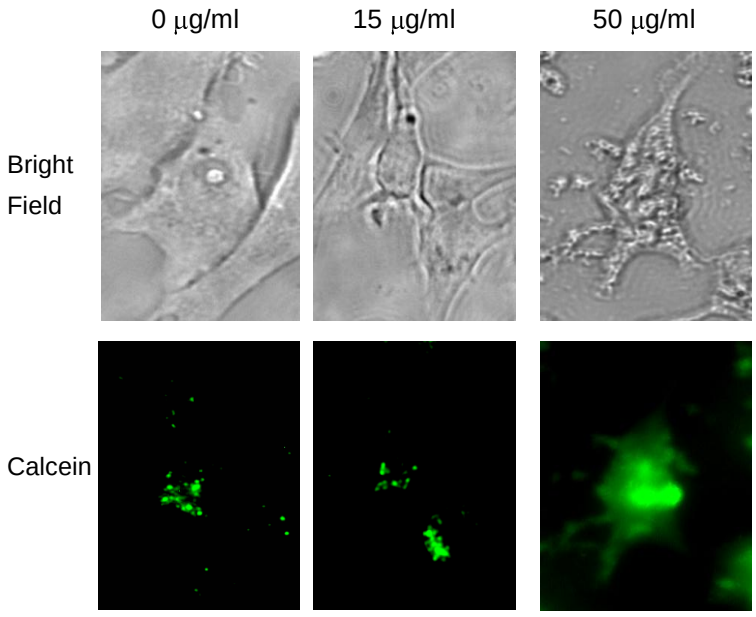

Figure S9. Clacein uptake in HEK293 cells treated with various concentrations of $PVBLG_{267}$-8. Punctate fluorescence observed in cells incubated with 0 and 15 μg/ml $PVBLG_{267}$-8 indicates pinocytic uptake. Diffuse fluorescence observed in cells treated with 50 μg/ml $PVBLG_{267}$-8 indicates cell membrane permeation and transmembrane uptake.

Transfection and Analysis of H9 hESCs.

DNA complexes were formed at $PVBLG_{267}$-8:DNA weight ratios between 15:1 and 30:1 as described above save for the use of pEGFP-N1 instead of pCMV-Luc. DNA complexes with Lipofectamine 2000 were prepared according to manufacturer recommendations. H9 hESCs were cultured in DMEM/F12 with 20% knockout serum replacement (KSR), 1 mM glutamine, 1% non-essential amino acid, 0.1 mM_mercaptoethanol, and 4 ng/ml bFGF and plated in 24-well plates at 5×10^4 cells/well 24 h prior to transfection. Immediately before transfection, the growth medium was replaced with fresh serum-free medium and polyplex solution (50 μl) was added to each well (0.25 μg DNA/well). For studies involving nocodazole treatment (10 μM), drug was added immediately prior to transfection. Four hours post-transfection, the cells were rinsed with PBS (0.5 mL) to remove surface-bound complexes and the media was replaced with growth media and incubated for 48 hours. After 48 hours, the cells were imaged under a fluorescent microscope to evaluate cell phenotype. The cells were then subjected to flow cytometry. First, trypsin in PBS (0.05%, 100 μl) was added to each well. Then cells and trypsin were incubated

for approximately ten minutes before formaldehyde (4%, 400 µl) was added to each well. The cells were then collected and FACS analyses were performed on a BD Biosciences LSR II flow cytometer (Franklin Lakes, NJ, USA). Data were analyzed using the FCS Express software package (De Novo Software, Los Angeles, CA, USA). Transfections and uptake measurements were performed in triplicate.

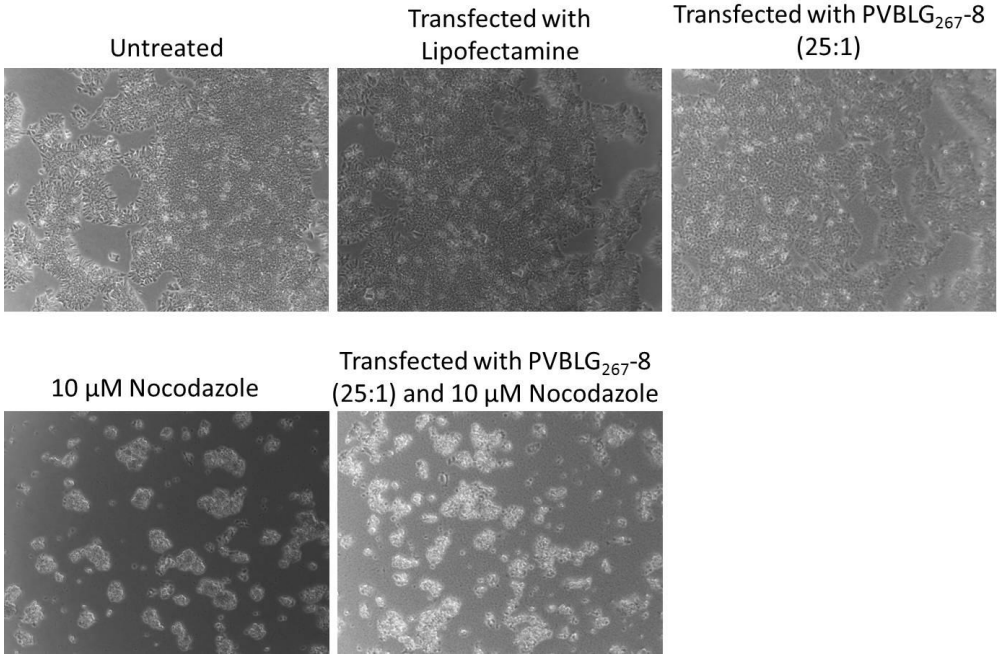

Figure S10. Phenotype of transfected and drug-treated H9 cells 48 hours post-transfection.

Confocal Microscopy.

DNA complexes were formed at the previously determined optimum transfection weight ratio as described above. The intercalating dye YOYO-1 was added at the ratio 15 nl YOYO-1 per 1 µg of DNA. COS-7 were cultured in DMEM supplemented with 10% horse serum and 1% penicillin-streptomycin according to ATCC protocols and plated in 6-well plates containing coverslips at 20×10^4 cells/well 24 h prior to transfection. Approximately 30 minutes before transfection, the growth medium was replaced with fresh serum-free medium containing 20 µM nocodazole. At transfection time, $PVBLG_{267}$-8/DNA complexes were added to the cells. Sixty minutes post-transfection, the cells were rinsed with PBS (2 mL × 2) to remove surface-bound complexes and formaldehyde (4%, 1 mL) was added to each well. Following a 10-min incubation, the cells were rinsed with PBS (2 mL × 2) and mounted on glass slides. Cells were

visualized with an Olympus BX60 confocal microscope equipped with a 100x oil immersion lens with an Argon laser for visualizing YOYO-1 (λ_{ex}=488).

The images obtained below in Figure S11 reveal that cells transfected in the presence of nocodazole appear to show larger fluorescent aggregates as compared with cells transfected in the absence of nocodazole. We believe this observation supports our assertion that nocodazole treatment causes material to accumulate in endocytic vesicles.

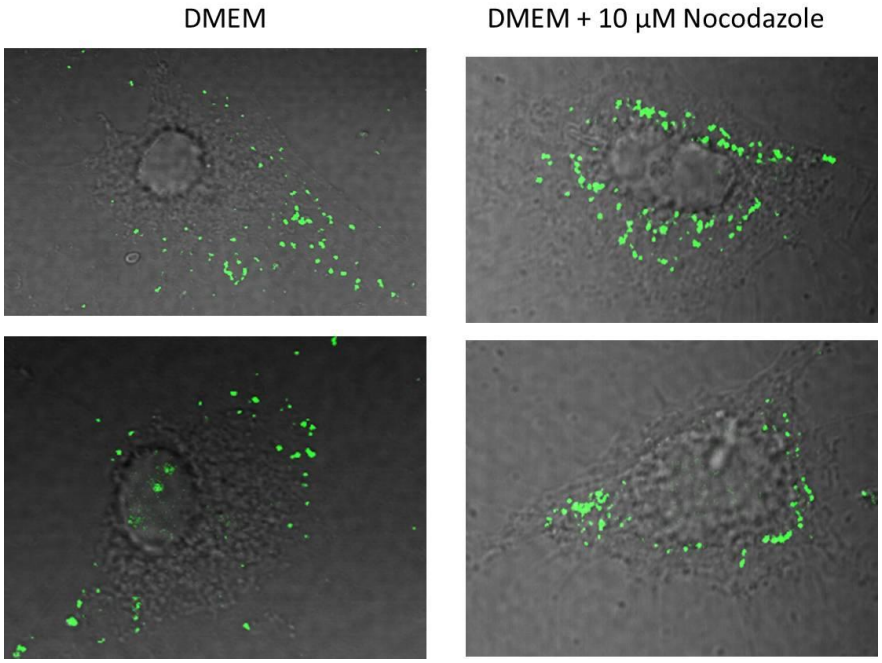

Figure S11. Confocal micrograph of COS-7 cells transfected with YOYO-1 labeled DNA/ $PVBLG_{267}$-8 complexes in the presence or absence of nocodazole. The appearance of smaller and more diffuse punctate fluorescence in the untreated cells (left column) as compared with the cells treated with nocodazole (right column) suggest that nocodazole is causing complexes to accumulate in endocytic vesicles.

NMR Spectra of Select $PVBLG_n$-X Polymers.

NMR spectra were recorded on a Varian UI400 MHz, a UI500NB MHz or a VXR-500 MHz spectrometer. The 1H-NMR spectra for $PVBLG_n$-1 through $PVBLG_n$-9 are shown below. The entire set of 31 side chains is not included for for the sake of brevity. However, this selection presented below does include the spectra for the top 3 side chains.

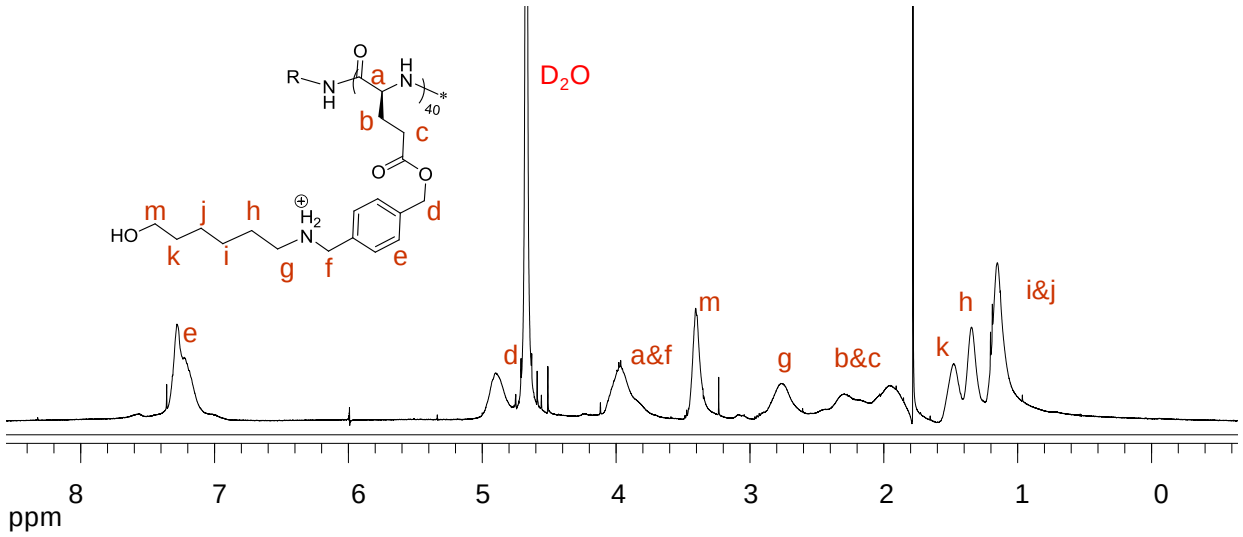

Figure S12. ^{1}H NMR of PVBLG$_{40}$-1 in D_2O.

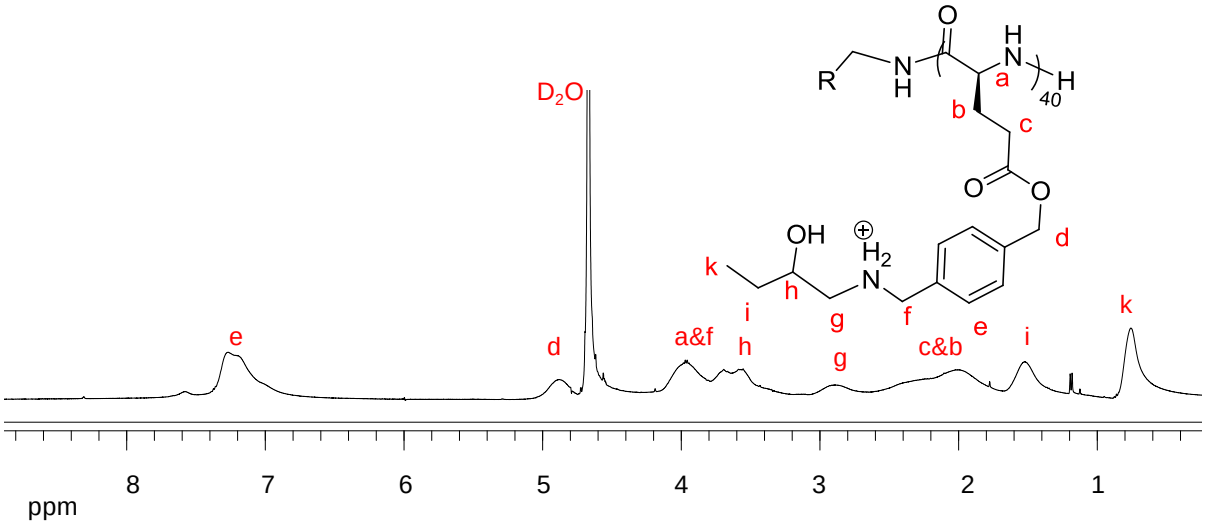

Figure S13. ^{1}H NMR of PVBLG$_{40}$-2 in D_2O.

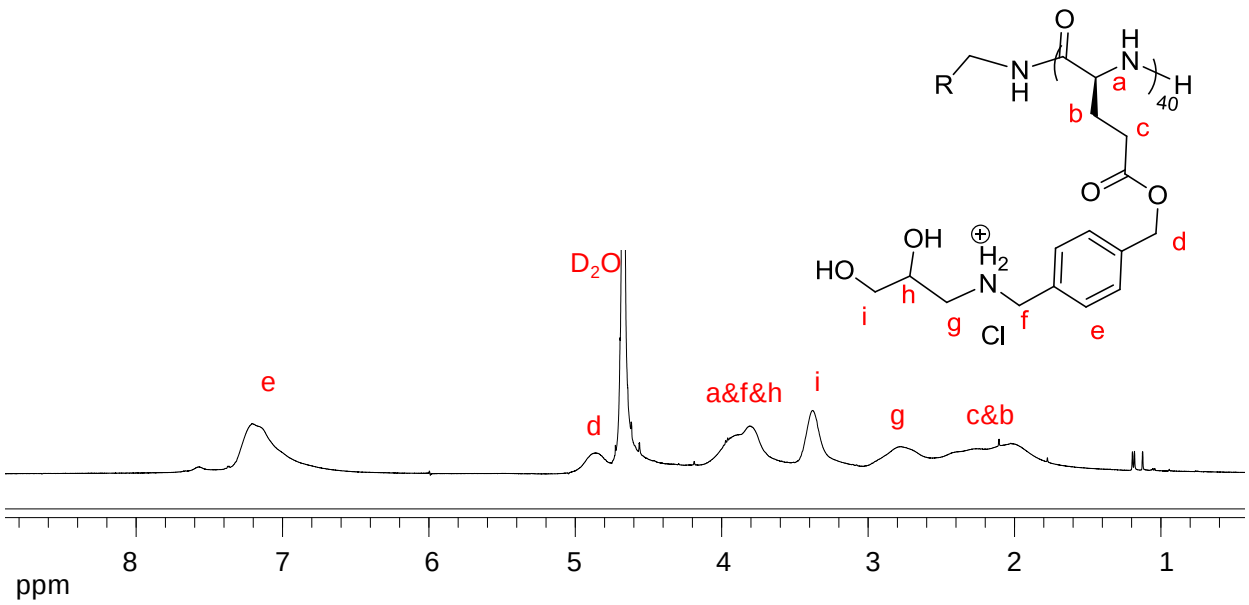

Figure S14. ^{1}H NMR of PVBLG$_{40}$-3 in D_2O.

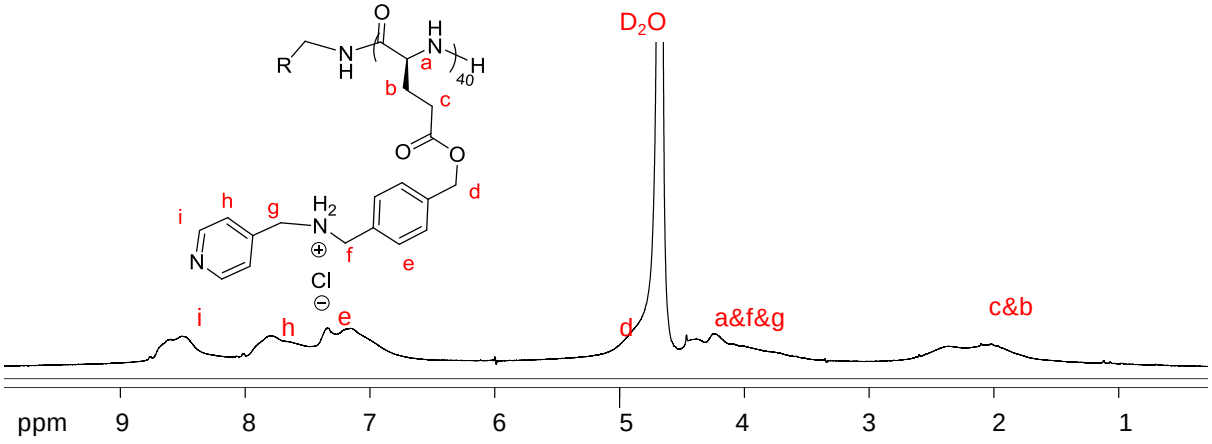

Figure S15. ^{1}H NMR of PVBLG$_{40}$-4 in D_2O.

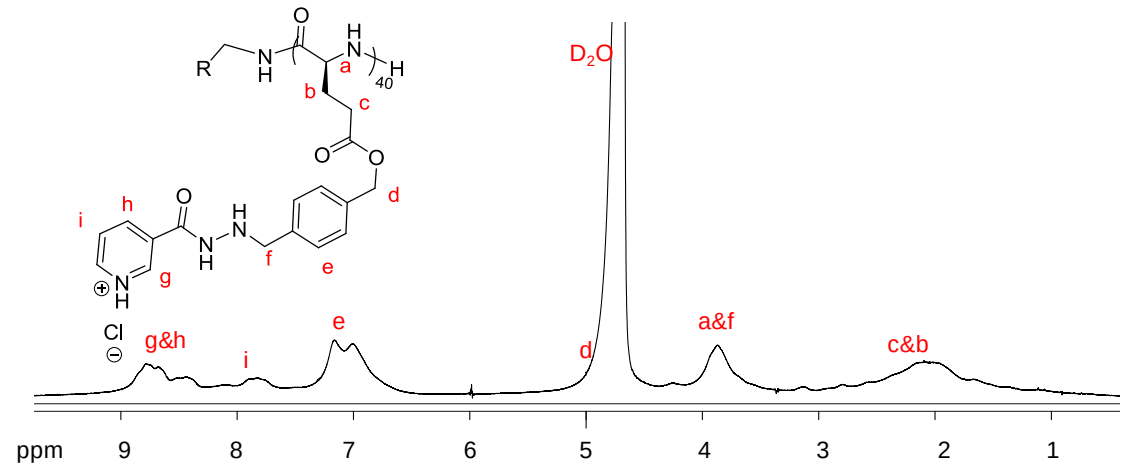

Figure S16. ^{1}H NMR of PVBLG$_{40}$-5 in D_2O.

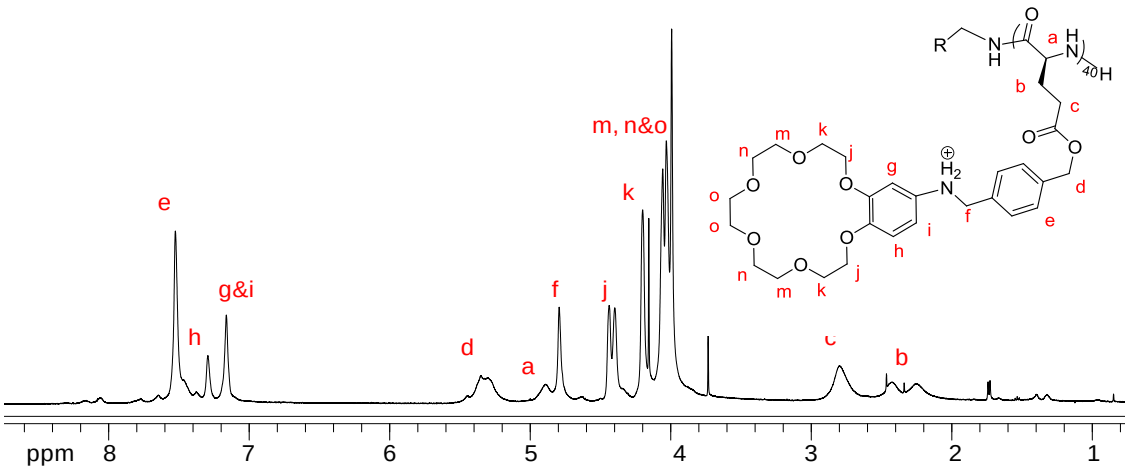

Figure S17. ^{1}H NMR of PVBLG$_{40}$-6 in D_2O.

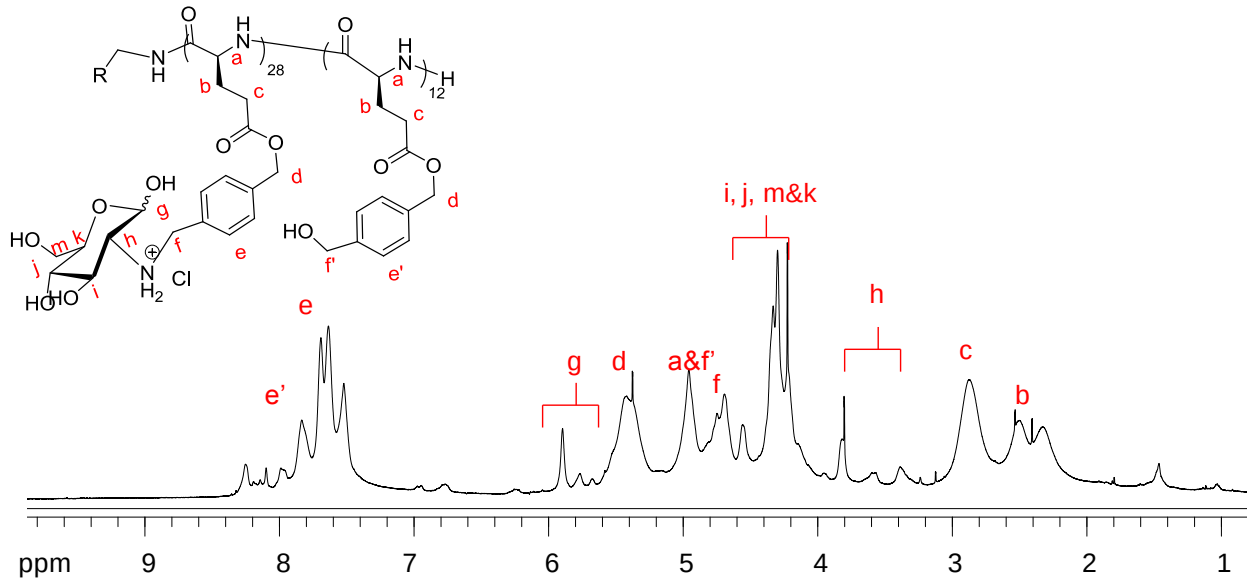

Figure S18. ^{1}H NMR of PVBLG$_{40}$-7 in D_2O.

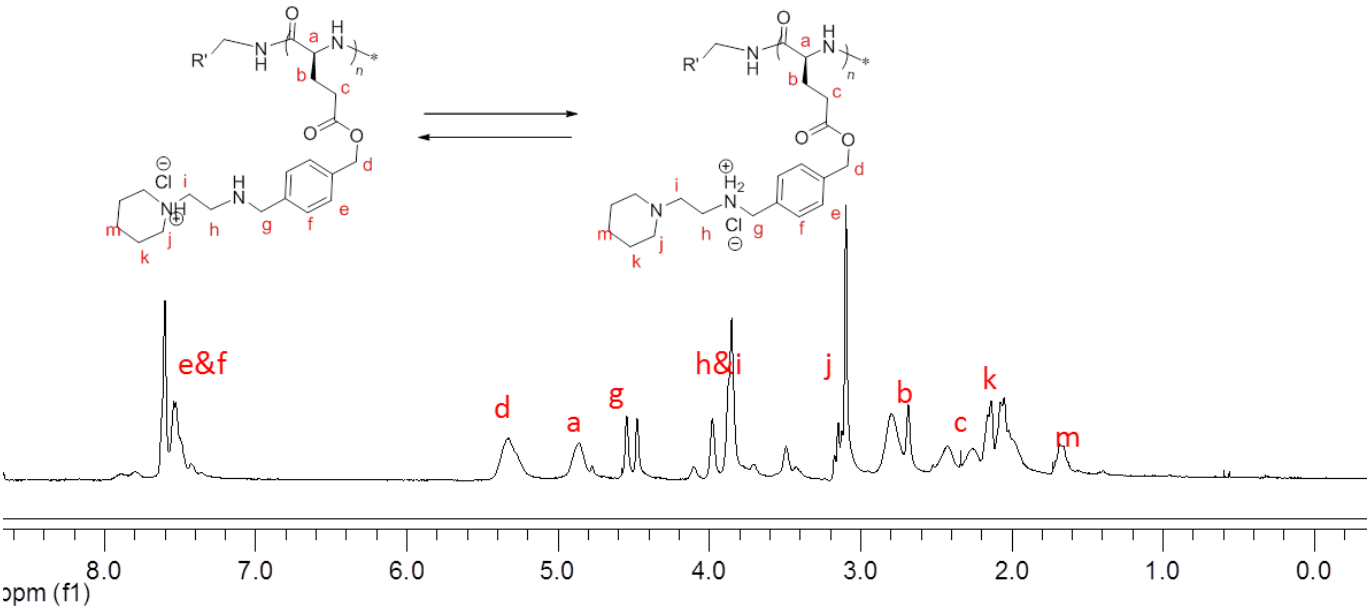

Figure S19 ^{1}H NMR of PVBLG$_{267}$-8 in TFA-d.

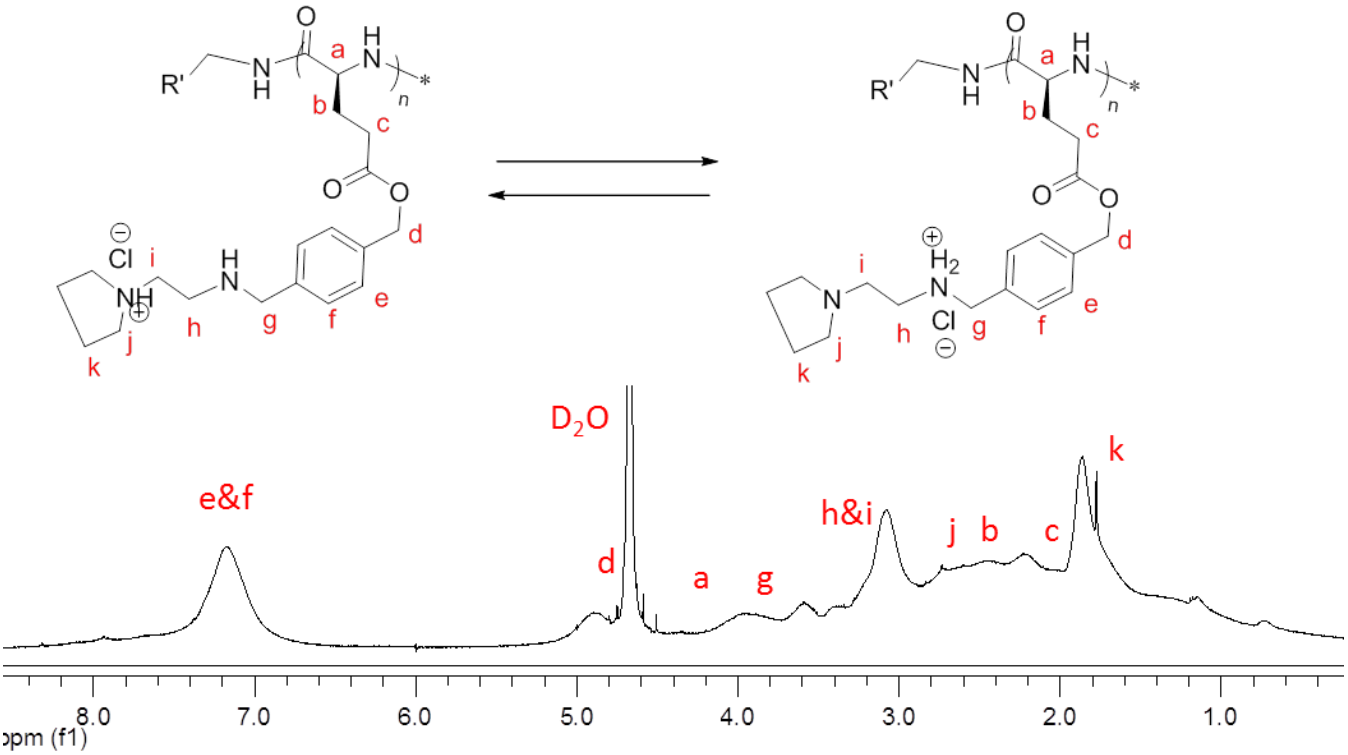

Figure S20. ^{1}H NMR of PVBLG$_{267}$-9 in D_2O.

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