Supporting Information for

Design and Synthesis of a Polyguanidium Vector with Enhanced DNA Binding Ability for Effective Gene Delivery at Low N/P Ratio

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Experimental Procedures

Materials

All solvents and reagents were purchased from Shanghai Adamas Reagent, Ltd. or Shanghai Macklin Biochemical Co., Ltd. Water was purified by a Milli-Q water purification system. All other solvents were dried and stored over activated 4 Å molecular sieves. Triethylamine (TEA) and diisopropylethylamine (DIPEA) was dried over KOH pallets. All other reagents were used without further purification unless otherwise noted. YOYO-1 was purchased from Invitrogen (Carlsbad, CA, U.S.A.). Endotoxin-free plasmid purification kit was purchased from TIANGEN (Beijing, China). Luciferase assay kit was purchased from Promega (Madison, WI, USA). Plasmid DNA (pDNA) encoding luciferase, and GFP were obtained using the plasmid purification kit.

Instrumentation

Nuclear Magnetic Resonance (NMR). NMR spectra were recorded using a Bruker Avance II 400 MHz spectrometer in the NMR Laboratory, Institute of Chemical Biology and Nanomedicine, Hunan University. The data was processed in MestReNova 6.1 and aligned/annotated in Adobe Illustrator CC or Microsoft Paint of Windows 10.

Mass Spectrometry. High resolution mass spectral analyses were provided by the Mass Spectrometry Laboratory, School of Chemical Engineering, Dalian University of Technology, using an Agilent electron spray ionization (ESI) mass spectrometer instrument.

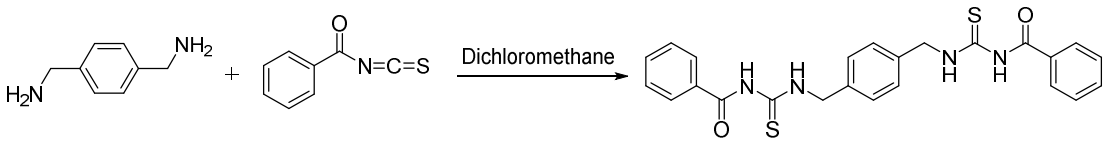
Analytical Gel Permeation Chromatography (GPC). GPC experiments were performed on a Waters system equipped with a Waters 1515 isocratic pump and a Waters 2414 refractive index detector. Separations were performed at 35 °C using Water $NaNO_3$ (0.2 M) and NaH_2PO_4 (0.01 M) as the mobile phase.

The molecular weight of polymers was determined based on a PEG-based standard curve. The obtained data points were imported into OriginPro (version 8.1), plotted and smoothed, and saved as vector image files (*.ai) for coloring and annotation in Adobe Illustrator CC.

Transmission Electron Microscopy (TEM). TEM experiments were conducted on a JEM-2100 Plus transmission electron microscope. Sample solutions were added on the copper grid, dried at room temperature overnight, and used directly for imaging.

Syntheses

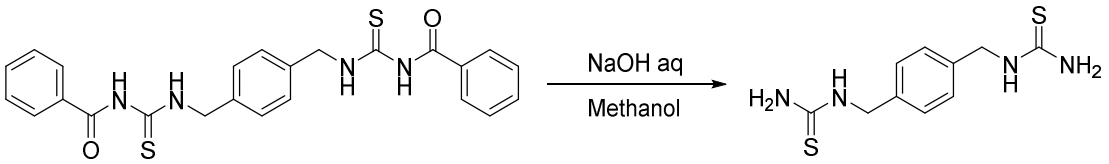
Synthesis of the dibenzoyl bisthiourea intermediate (G1)



1,4-(aminomethyl)benzene (2.6 g, 19.1 mmol) was suspended in DCM (170 mL) in a round-bottom flask placed in an ice-water bath and benzoyl isothiocyanate (6.54 g, 40.1 mmol) was added dropwise in the resulting suspension. The reaction was stirred at room temperature for 24 h. The resulting mixture was filtered through a Brinell funnel and the crude product was washed with dichloromethane several times. The resulting solid was dried to afford the product as a white solid (7.3 g, 82%). ^{1}H NMR: (400 MHz, DMSO-d_6): δ 11.37 (s, 2H), 11.23 (*t*, *J* = 5.5 Hz, 2H), 7.94 (*d*, *J* = 7.4 Hz, 4H), 7.63 (*t*, *J* = 7.4 Hz, 2H), 7.51 (*t*, *J* = 7.7 Hz, 4H), 7.40 (*s*, 4H), 4.87 (*d*, *J* = 5.5 Hz, 4H). ^{13}C NMR (101 MHz, DMSO-d_6): δ 181.0, 168.5, 136.9, 133.4, 132.7, 129.0, 128.8, 128.3, 48.4. ESI-MS: Calculated for $C_{24}H_{23}N_4O_2S_2^+$ ([M$^+$H]$^+$): 463.12; found: 463.69.

Synthesis of the bisthiourea intermediate (G2)

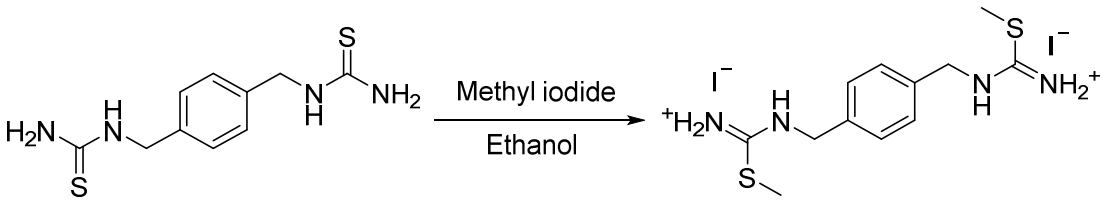
Compound **G1** from the previous step (6.98 g, 15.1 mmol) was suspended in methanol (150 mL) in a round-bottom flask and aqueous sodium hydroxide (5.0



G1 **G2**

M, 12.1 mL, 60.5 mmol) was added dropwise. The reaction was stirred at room temperature for 24 h. The resulting mixture was filtered through a funnel and the crude product was washed with methanol several times and the resulting solid was dried to afford **G2** as a white solid (3.6 g, 93%). This crude product was used in the next step without further purification. ^{1}H NMR: (400 MHz, DMSO-d_6): δ 7.97 (*s*, 2H), 7.26 (*s*, 4H), 7.06 (*s*, 4H), 4.61 (*s*, 4H). ^{13}C NMR (101 MHz, DMSO-d_6): δ 183.9, 138.3, 127.8, 47.7. ESI-MS: Calculated for $C_{10}H_{15}N_4S_2^+$ ($[M^+H]^+$): 255.07; found: 255.37.

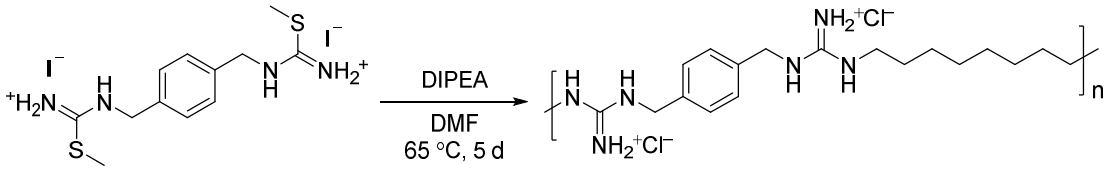
Synthesis of the bis-isothiourea monomer (G3)



G2 **G3**

The crude intermediate **G2** from the previous step (3.61 g, 14.2 mmol) was suspended in dry ethanol (150 mL) in a round-bottom flask and methyl iodide (4.82 g, 34.0 mmol) was added in the mixture. The reaction was stirred at 35 ^{o}C for 72 h. Solvent was evaporated *in vacuo* and the residue was washed with dry ethanol. The resulting solid was dried to afford pure monomer **G3** as a white solid (7.1 g, 93%). ^{1}H NMR: (400 MHz, DMSO-d_6): δ 9.89 (*s*, 2H), 9.18 (*s*, 4H), 7.36 (*s*, 4H), 4.58 (*s*, 4H), 2.65 (*s*, 6H).^{13}C NMR (101 MHz, DMSO-d_6): δ 168.4, 135.5, 128.3, 46.6, 14.3. High resolution ESI-MS: Calculated for $C_{12}H_{19}N_4S_2^+$ ($[M+H]^+$): 283.1045 ; obtained 283.1054.

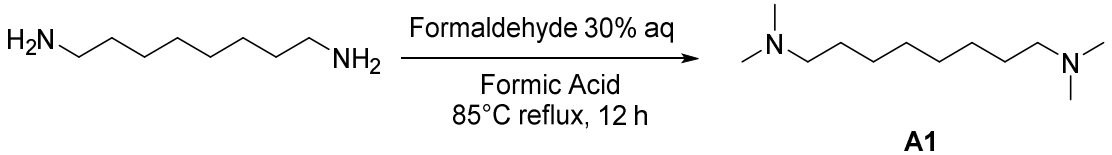
Synthesis of PPODG



G3 **PPODG**

The monomer 1,1'-(1,4-phenylenebis(methylene)) bis(2-methylisothiouronium) iodide **G3** (1.18 g, 2.20 mmol) and 1,8-octanediamine (0.31 g, 2.2 mmol) and *N,N*-Diisopropylethylamine (1.1 g, 8.8 mmol) were added into a 20-mL glass vial containing anhydrous DMF as solvent (10 mL). The reaction was stirred at 65 °C for 5 d. The mixture was added brine and dialyzed against water to remove smaller molecules and to replace I^- with Cl^- (MWCO for membrane = 1 kDa). The polymer was obtained through lyophilization as a white solid (0.44 g, 30%).

Synthesis of *N, N, N', N'*-tetramethyl-1,8-diaminooctane (A1)

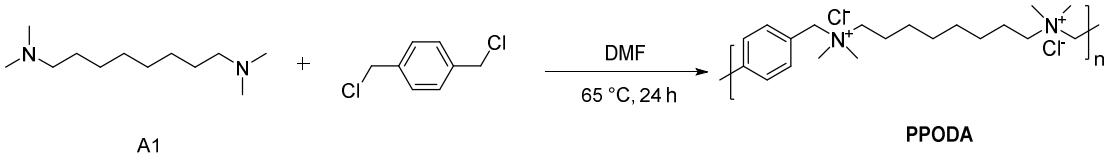


A1

The synthesis of this monomer was analogous to a literature procedure.[1] Formic acid (7.07 g, 15.4 mmol) was slowly added to 1,8-diaminooctane (3.69 g, 25.6 mmol) in a round-bottom flask under ice water bath. Aqueous formaldehyde solution (31.5 g, 385 mmol) was added in the mixture. And the reaction was stirred at 85 °C under reflux for 12 h. When the reaction cooling to room temperature, HCl solution (5.0 N, 50 mL) was added and the mixture was evaporated using a rotary evaporator. The residue was dissolved in 50 % aqueous NaOH solution (50 mL) and extracted with CH_2Cl_2 (80 mL × 3). The combined extracts were washed with brine (80 mL × 2). The organic layer was dried with anhydrous $MgSO_4$, filtered and evaporated to give the product as pale yellow oil (3.67 g, 72%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.26 – 2.17 (*m*, 16H), 1.45 (*dd*, *J* = 14.2, 7.0 Hz, 4H), 1.29 (*d*, *J* = 2.3 Hz, 8H). ^{13}C NMR (101

MHz, $CDCl_3$) δ 59.97, 45.54, 29.55, 27.78, 27.47. ESI-MS: Calculated for $C_{12}H_{29}N_2^+$ ($[M^+H]^+$): 201.23; found: 201.18.

Synthesis of PPODA



A1 PPODA

The monomer **A1** *N,N,N',N'*-tetramethyl-1,8-diaminooctane (0.12 g, 0.60 mmol) and 1,4-bis(chloromethyl)benzene (0.10 g, 0.60 mmol) were added in a 7-mL glass vial containing dry DMF (2 mL) as solvent. The reaction was stirred at 65 °C for 24 h. The product was purified by dialysis against water (membrane MWCO = 1 kDa). After lyophilization the polymer was obtained as a white solid (0.18 g, 81%).

Miscellaneous protocols

Gel Retardation Assay for DNA Condensation Evaluation. PPODG and plasmid DNA were dissolved in DI water at 0.2 mg/mL to prepare their respective stock solutions. The polymers were added to DNA solutions dropwise at N/P ratios of 1, 2.5, 5, 10 and 15, and the mixtures were incubated at room temperature for 20 min to allow the formation of the polymer/DNA complexes. The complexes with various N/P ratios were loaded on a 1% agarose gel at 100 ng DNA/well followed by electrophoresis at 110 V for 50 min. To quantitatively determine the level of DNA condensation, Ethidium bromide (EB) exclusion assay was performed. Plasmid DNA was stained with EB at the DNA/EB weight ratio of 10 at RT for 1 h. The polymers (0.2 mg/mL) were added into the DNA/EB solutions at the weight ratios of 1, 2.5, 5, 10 and 15. The mixtures were incubated at RT for another 30 min to allow DNA condensation before evaluation by fluorescence intensity (λ_{ex} = 510 nm, λ_{em} = 590 nm). The DNA condensation efficiency (%) was calculated according to the following

equation:

$$DNA\ condensation\ efficiency\ (\%) = (1-\frac{F-F_{EB}}{F_0-F_{EB}})\times 100$$

where F_{EB}, F, and F_0 denote the fluorescence intensity of pure EB solution, DNA/EB solution with polymer, and DNA/EB solution without any polymers, respectively.

DLS and Zeta-Potential Measurement. PPODG and plasmid DNA were dissolved in DI water at 0.2 mg/mL to prepare their respective stock solutions. The polymers were added to DNA solutions dropwise at N/P ratios of 1, 2.5, 5, 10 and 15, and the mixtures were incubated at room temperature for 20 min to allow the formation of the polymer/DNA complexes. Particle sizes and zeta potentials of polymer/DNA complexes at various N/P ratios were evaluated by dynamic laser scattering (DLS) on a Malvern Zetasizer instrument.

Confocal Microscopy. Confocal microscopic images were obtained on an Olympus Fluoview FV-1000 instrument. HeLa cells incubated in DMEM in cell culture dish (10^5 cells/well) were treated with polyplexes containing the YOYO-1 DNA at a concentration of 1 μg DNA/well and the N/P ratio of 2.5. Following incubation at 37 ^oC for 4 h, cells were washed with PBS for 3 times, fixed with paraformaldehyde (4%), stained with DAPI (2 μg/mL), and subjected to observation using a confocal microscope.

Isothermal Titration Calorimetry. The experiment parameters of ITC study was set as the following: total injections = 15 or 20, cell temperature = 25 ^oC, reference power = 10 μcal/s, initial delay = 60 s, syringe concentration (PPODG or PPODA) = 0.2 mg/mL, cell concentration (eGFP plasmid) = 0.01 mg/mL, initial cell volume = 15 μL, stirring speed = 400 RPM. For each injection, 1 μL of polymer solution was added into the cell over 10 s. The spacing between injections was set at 120 s.

***In vitro* Transfection.** HeLa or MCF-7 cells were seeded on 96-well plates at 10^4 cells/well and incubated overnight until the cells reached 70% confluence. The cell culture medium was replaced by serum-free DMEM (100 μL/well), into

which polyplexes at the N/P ratios of 1, 2.5, 5, 10 and 15 were added at 0.2 μg DNA/well. After incubation for 4 h, the medium was replaced by complete DMEM containing 10% FBS. Cells were further cultured for another 20 h before quantification of luciferase expression level using a Bright-Glo Luciferase assay kit and cellular protein level using a BCA kit. For the PEI/DNA polyplexes, the N/P ratio was fixed at 5.

Cell Uptake Evaluation. Plasmid DNA was firstly labeled with YOYO-1 at one dye molecule per 50 bp DNA on average. HeLa cells were seeded on 96-well plates at 1×10^4 cells/well and cultured overnight until the cells reached 70% confluence. The cell culture medium was changed to serum-free medium (100 μL/well) into which the complexes were added (0.2 μg DNA/well). After incubation at 37 °C for 4 h, cells were washed with cold PBS for three times to remove the surface coated complexes and subsequently lysed by the RIPA lysis buffer (100 μL/well) at RT for 20 min. The YOYO-1-DNA level was evaluated by spectrofluorimetry (λ_{ex} = 485 nm, λ_{em} = 530 nm) and the protein level was evaluated by the BCA kit. The cell uptake level was calculated as ng YOYO-1-DNA associated with 1 mg of cellular protein. To explore if the cell uptake of the polyplexes involves the energy-dependent endocytosis pathway, HeLa cells were cultured on 96-well plates and incubated with polyplexes for 4 h at 37 °C or 4 °C as described above. Results were expressed as percentage uptake of control cells that were treated with complexes at 37 °C for 4 h.

Cytotoxicity Evaluation. HeLa or MCF-7 cells were seeded on 96-well plates at 1×10^4 cells/well and cultured overnight until the cells reached 90% confluence. The medium was replaced by serum-free medium (100 μL/well) into which the free polymers were added at the final concentrations of 1, 2, 5 and 10 μg/well. After incubation at 37 °C for 4 h, the medium was refreshed with complete DMEM and further incubated for another 20 h before the assessment of cell viability using the MTT assay. Cells without polymer treatment served as the negative control. The results were presented as

percentage viability compared to control cells. The cytotoxicity of the DNA-polymer polyplexes were also evaluated using the same method at the transfection dosages at a DNA amount of 0.1 or 0.2 μg/well and a N/P ratio at 2.5 or 5.

Characterization

Polymerization conditions

Table S1. Solvent and temperature effect on the condensation polymerization affording PPODG. Reaction time = 5 d.

Solvent	DMF	DMF	DMF	DMF	DMSO	DMF + ACN (1:1)
Temperature (°C)	50	65	80	90	65	65
M_n (kDa)	1.6	1.7	1.6	1.7	1.6	1.6

Discussion: for most condensation polymerizations, the molecular weight of the final polymers is controlled by the reactivity of the monomers (end groups). Due to the low reactivity of the isothiourea used in this polymerization, only low molecular weight products could be obtained, even with long reaction time and high polarity solvents. Because the reactivity set a high limit (which is low, ~2.5 kDa) for the polymerization, and small oligomers were easily removed by dialysis (MWCO = 1 kDa), the final polymers all had pretty narrow PDI (< 1.1).

As low molecular weight is desired for lower cytotoxicity, we consider this a good inherent control over polymer property and homogeneity.

NMR characterization

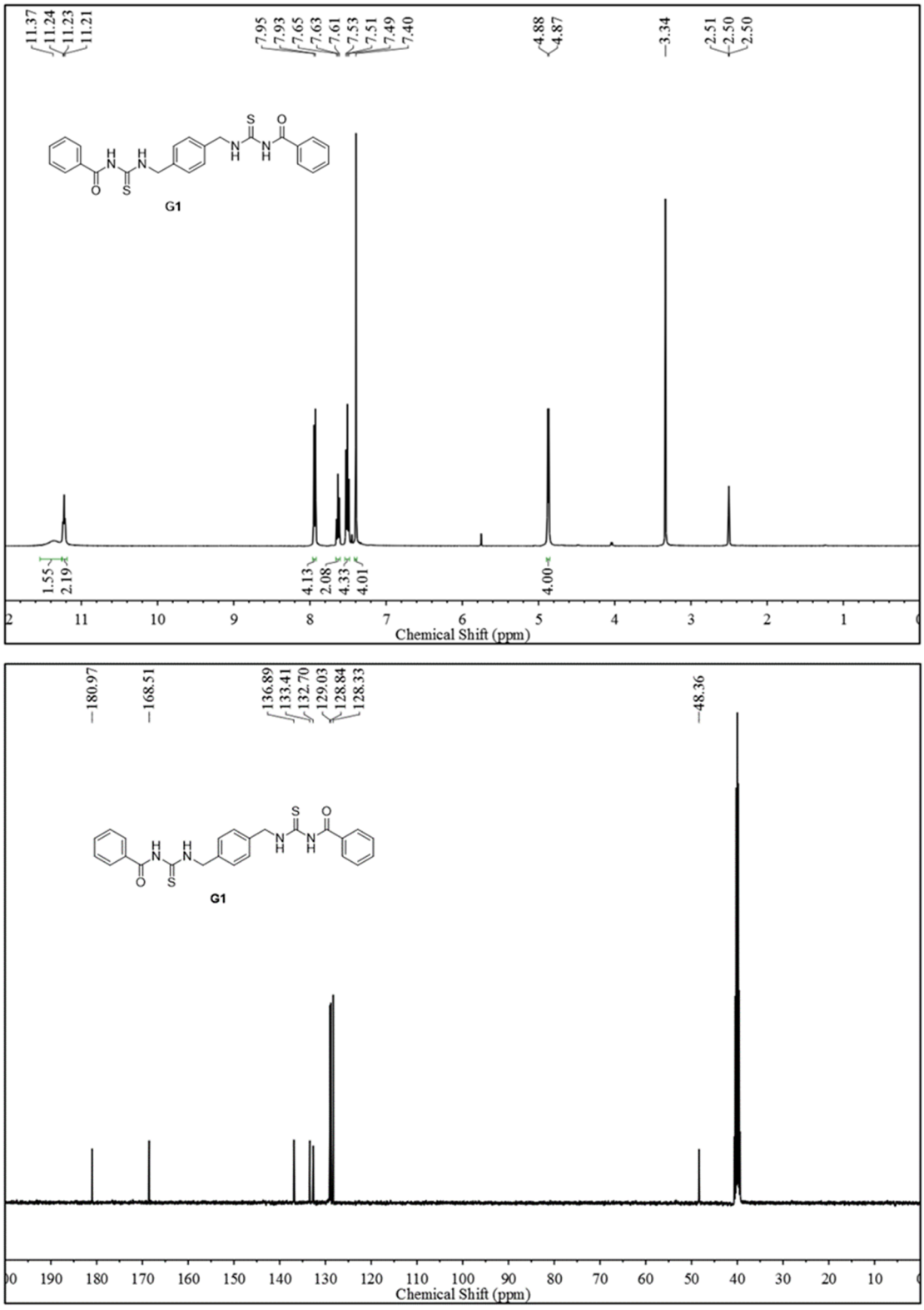


Figure S1. Structure, ^{1}H NMR and ^{13}C NMR spectra of the product **G1**.

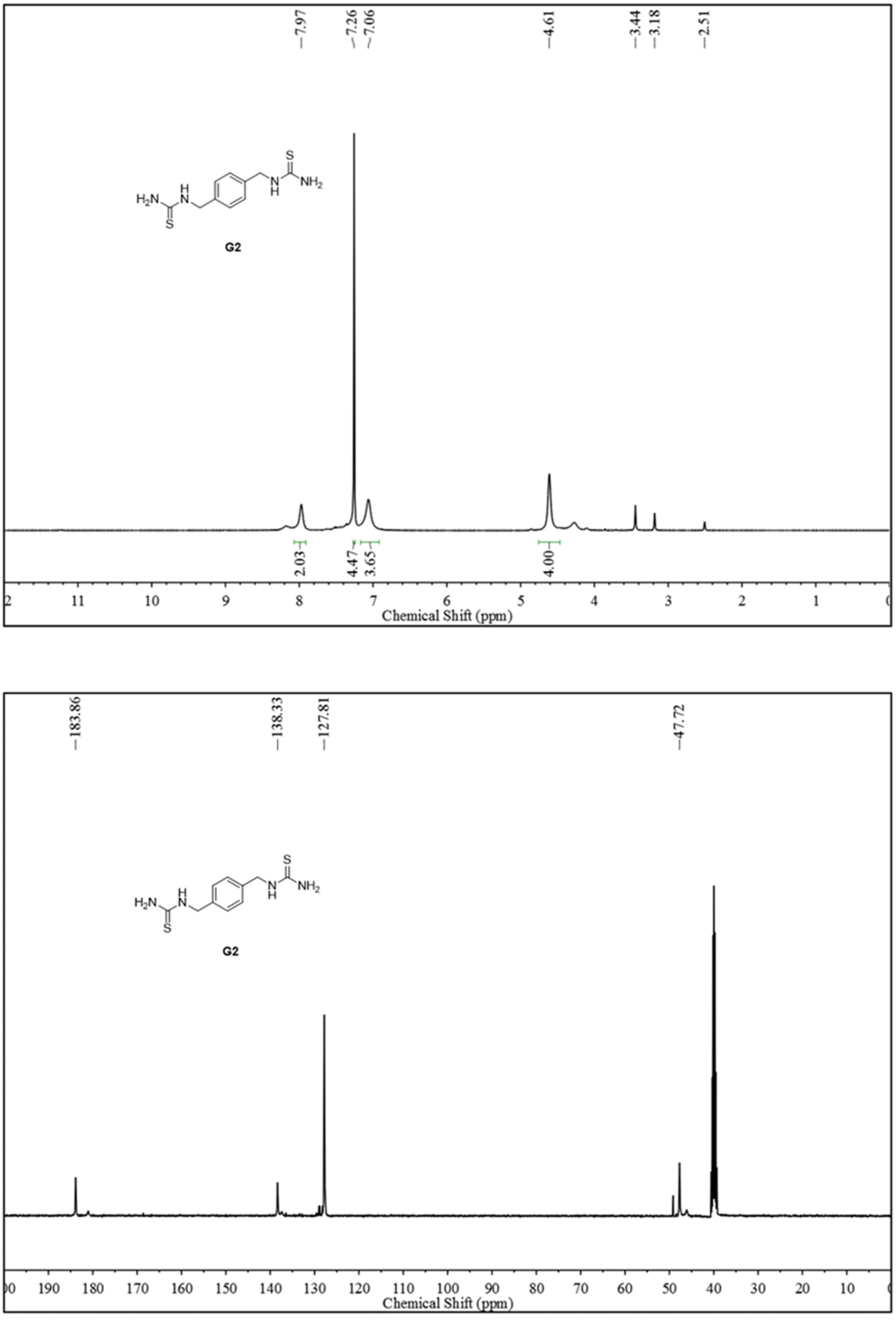


Figure S2. Structure, ^{1}H NMR and ^{13}C NMR spectra of the product **G2** (crude).

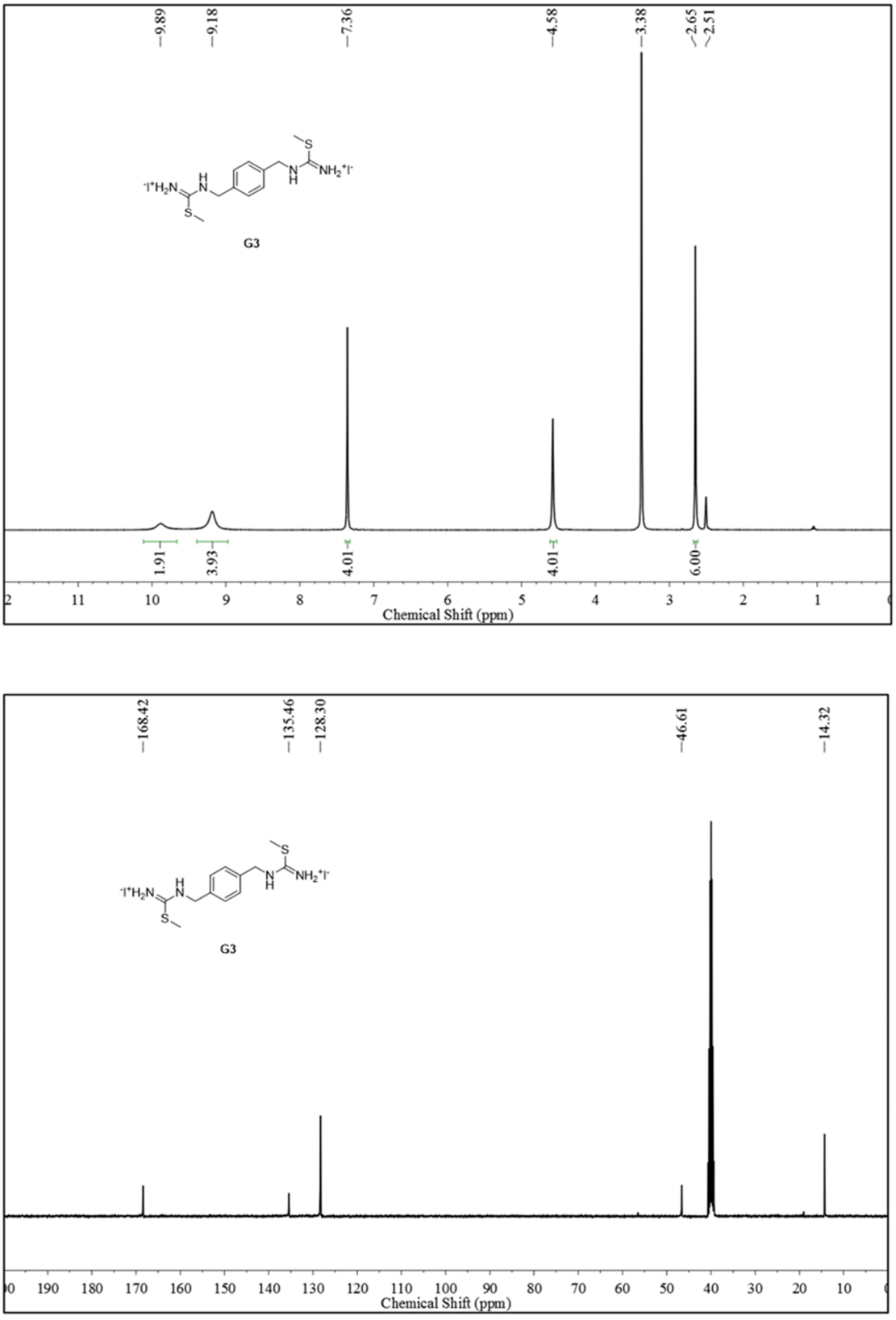


Figure S3. Structure, ^{1}H NMR and ^{13}C NMR spectra of the product **G3**.

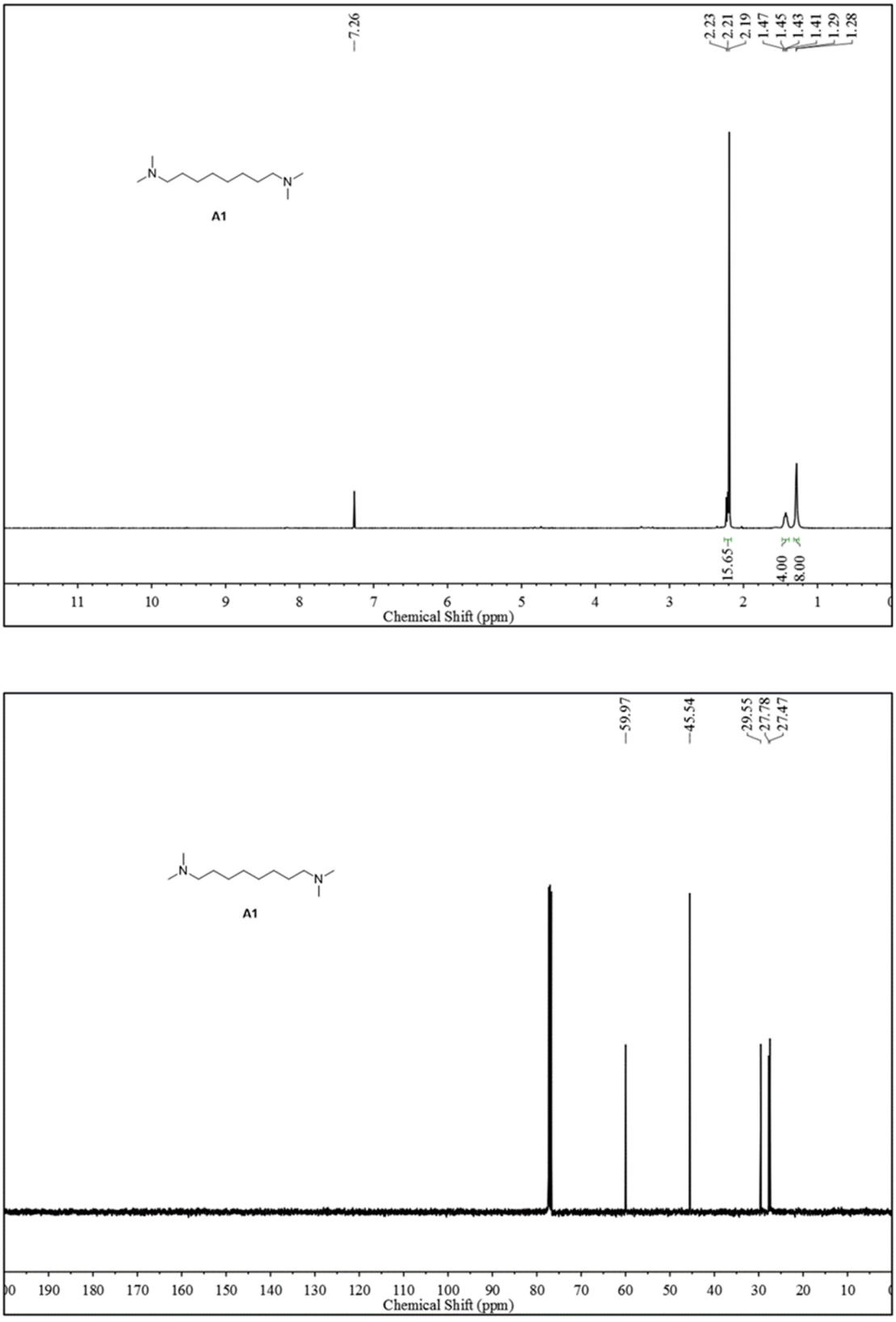


Figure S4. Structure, ^{1}H NMR and ^{13}C NMR spectra of the product **A1**.

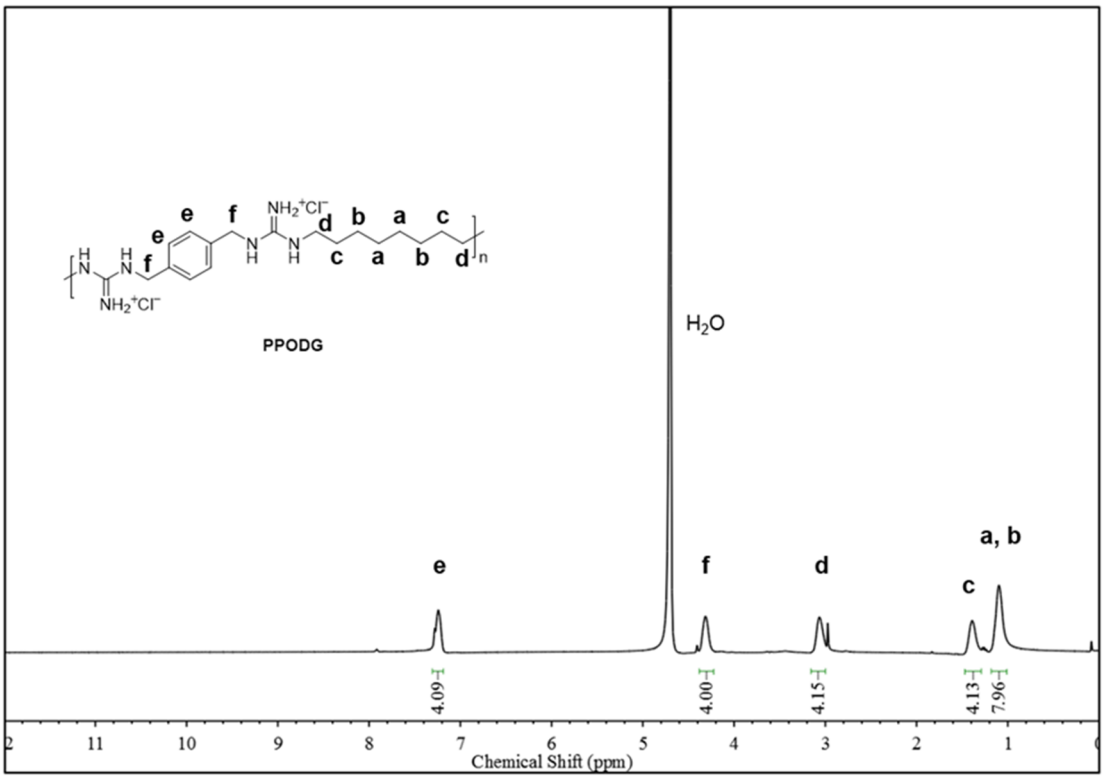


Figure S5. Structure and ^{1}H NMR spectrum of the polymer **PPODG**.

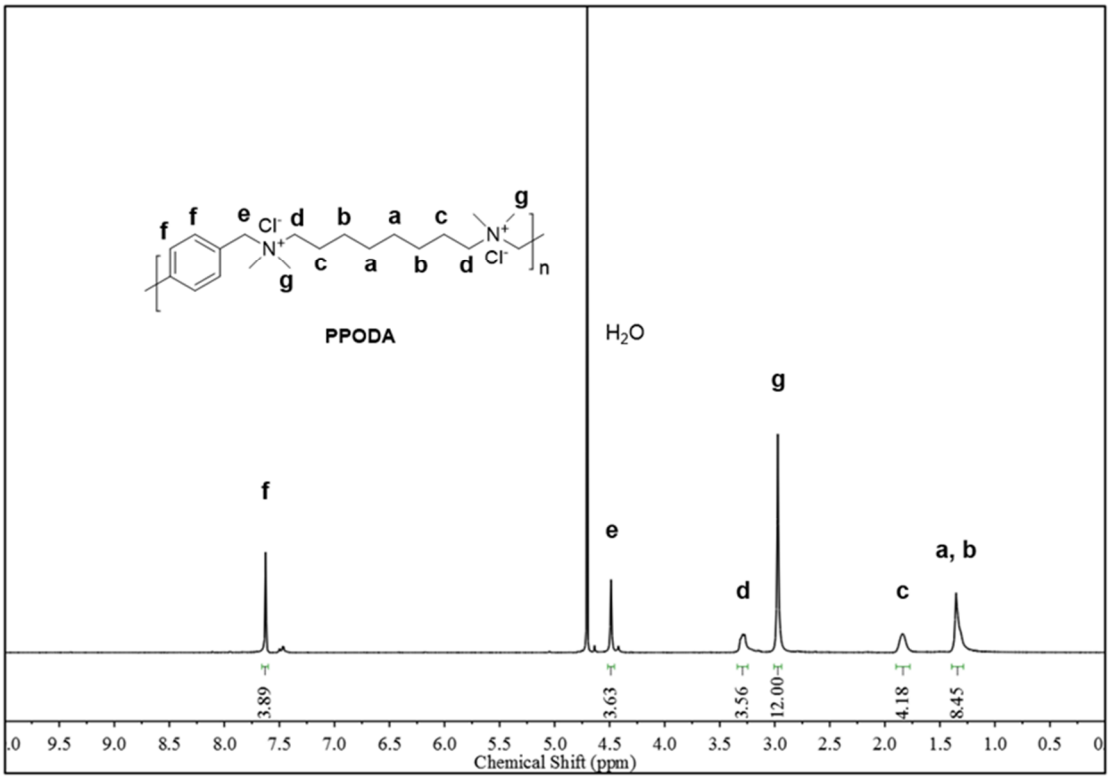


Figure S6. Structure and ^{1}H NMR spectrum of the polymer **PPODA**.

Additional TEM images of PPODG + GFP polyplexes

The solution of **PPODG** and GFP are mixed together in a N/P ratio of 5:1. After a few minutes, a droplet of the solution was added on the copper mesh. TEM characterization was performed after the sample was completely dried.

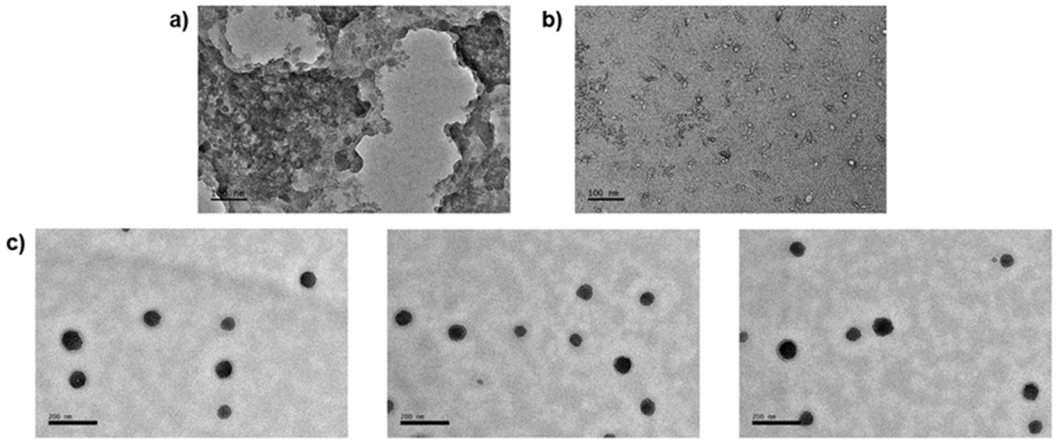
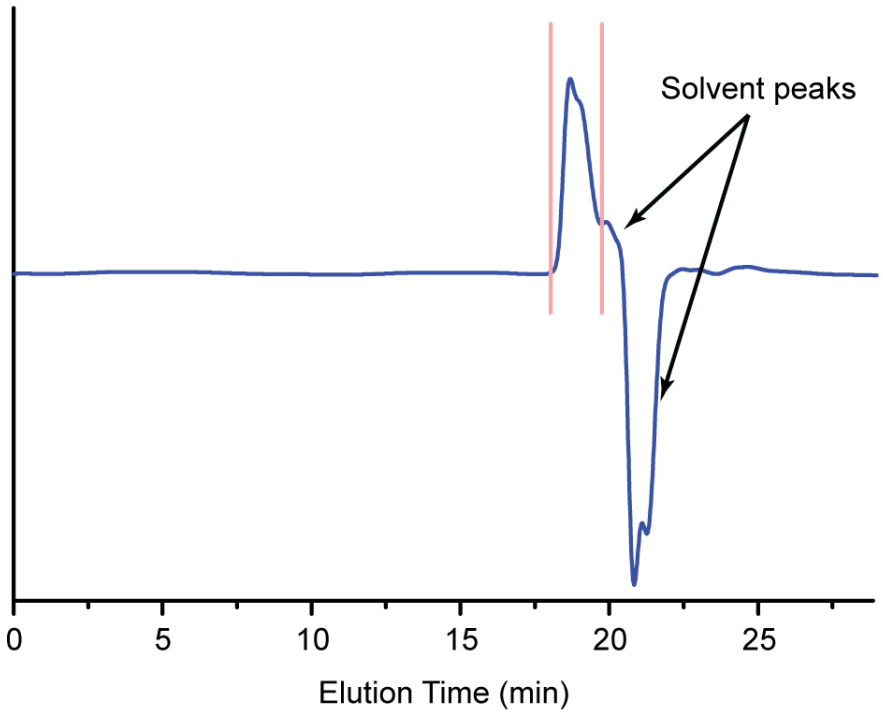


Figure S7. a) PPODG; b) GFP plasmid; c) Polyplexes formed from PPODG + GFP plasmid.

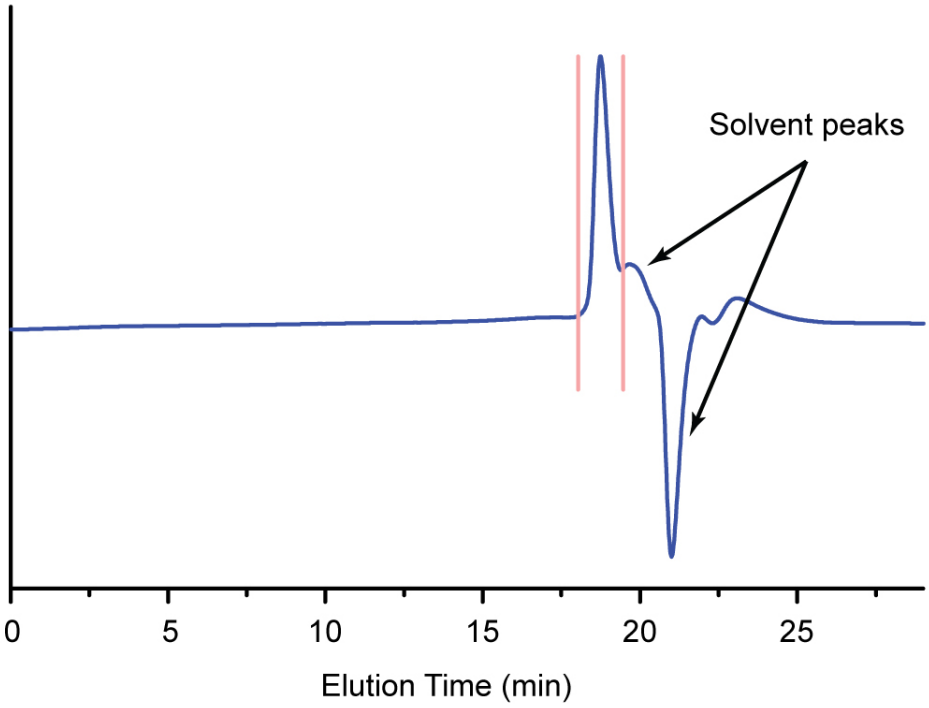
GPC characterization



Molecular weight result calculated using a PEG standard curve:

M_n = 1.7 kDa, PDI = 1.07

Figure S8. GPC curve and analysis result of **PPODG**.



Molecular weight result calculated using a PEG standard curve:

M_n = 1.9 kDa, PDI = 1.03

Figure S9. GPC curve and analysis result of **PPODA**.

Dynamic light scattering data

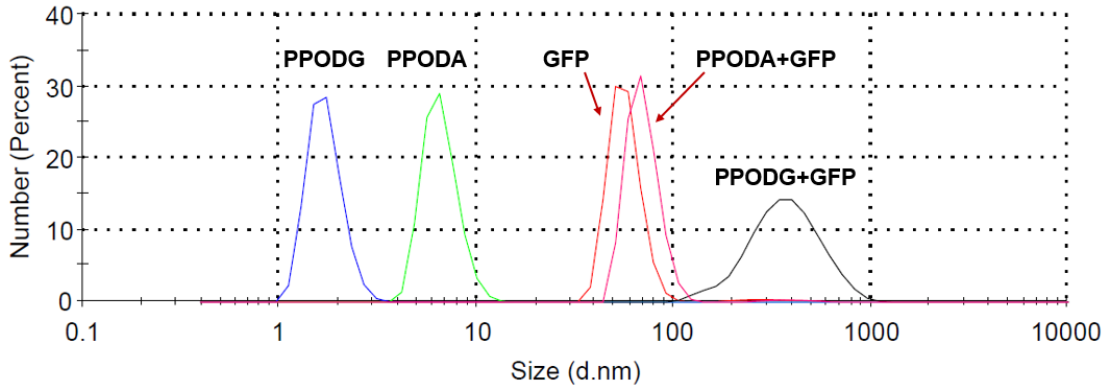


Figure S10. Particle size distribution of **PPODG** and **PPODA** mixing with plasmid GFP. Concentrations: 6 ng/μL for PPODG and PPODA and 1.2 ng/μL for GFP.

Discussion: From the result above, it can be clearly seen that at this concentration, PPODG has significantly higher affinity toward the plasmid and causes aggregation. PPODA mixed with GFP plasmid does not lead to aggregation, according to the data. Presumably, this is a result of the lack of guanidium groups on PPODA.

Isothermal titration calorimetry data

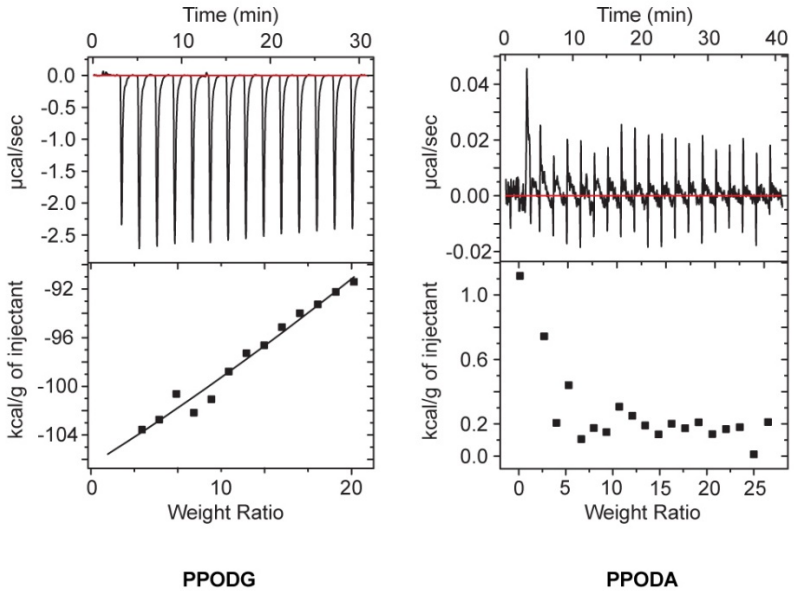


Figure S11. Results from the isothermal titration calorimetry experiments. Apparently, PPODG showed significant enthalpy change while PPODA showed negligible enthalpy change when they were mixed with eGFP plasmid.

Additional cellular uptake data

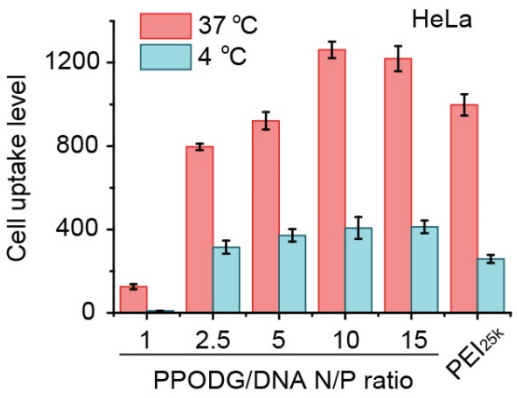


Figure S12. HeLa cell uptake levels of PPODG/DNA complexes at different N/P ratios at 37 °C and 4 °C. Uptake of PEI_{25k}/DNA complex at its optimized N/P ratio was used for comparison. Cell uptake level was quantified as ng YOYO-1 DNA per mg of protein.

References

1. Park, W.; Yu, D.; Na, K.; Jelfs, K. E.; Slater, B.; Sakamoto, Y.; Ryoo, R., *Chem. Mater.* **2011,** *23* (23), 5131-5137.