Supporting Information

of

Synthesis and evaluation of cyclic cationic polymers for nucleic acid delivery

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1. **Experimental details**
2. **Figure S1-S7**

Materials

2-(Dimethylamino)ethyl methacrylate (DMAEMA, 99%, Aldrich) and oligo(ethylene glycol) monomethyl ether methacrylate (OEGMA, M_n= 300 g/mol and pendent EO units DP~4.5, Aldrich) were purified by passing through a column filled with basic alumina to remove the inhibitor prior to polymerization. Copper (I) chloride (CuCl) was washed with acetic acid and ethanol in turn to remove Cu^{2+}. Copper(I) bromide(CuBr, 99.999%), α-bromoisobutyryl bromide (98%),*N*,*N*,*N*′,*N*″,*N*″-pentamethyldiethylenetriamine(PMDETA, 99%), bipyridine (bpy), propargyl alcohol (99%), 2-propanol, *N,N*-dimethylformamide and all the other reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO) andused as received. ATRP initiator, propargyl 2-bromoisobutyrate, for the synthesis of linear precursor was prepared according to reported procedures.[1]Endotoxin-free plasmid pCMV-Luc2 (Photinuspyralis luciferase under control of the cytomegalovirus (CMV) enhancer/promoter) was produced with the Qiagen Plasmid Giga kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. YOYO-1 Iodide was purchased from Invitrogen (Carlsbad, CA).

Synthesis of linear polymer precursors (Linear p(DMAEMA)-N_3)

The linear precursor with Br terminus was prepared by ATRP in 2-propanol using propargyl 2-bromoisobutyrate as the initiator and bpy/CuCl as the catalyst. A reaction flask with a magnetic stirrer and a rubber septum was charged with solutions of (**a**)propargyl 2-bromoisobutyrate (0.10 g, 0.5 mmol), DMAEMA (1.97 g, 12.5 mmol), bpy (0.156 g, 1.0 mmol), and 2-propanol (3.125 mL), (**b**) propargyl 2-bromoisobutyrate (0.05 g, 0.25 mmol), DMAEMA (1.97 g, 12.5 mmol), bpy (0.078 g, 0.5 mmol), and 2-propanol (3.125 mL), or (**c**) propargyl 2-bromoisobutyrate (0.025 g, 0.125 mmol), DMAEMA (1.97 g, 12.5 mmol), bpy (0.039 g, 0.25 mmol), and 2-propanol (3.125 mL) to obtain [monomer (M)]/[initiator (I)] ratios of 25, 50 or 100, respectively. The flask was degassed by bubbling with Ar to remove any trace of oxygen in the system, and then CuCl (equivalent molar ratio to initiator) was

introduced into the flask under the protection of Ar flow. Finally, the reaction mixture was sealed followed by immersing the flask into an oil bath preheated at 40°C to start the polymerization. The polymerization was stopped by exposing the reaction mixture to air after 5 h. The monomer conversions in all reaction were determined to be ≥ 96 % by ^{1}H NMR analysis. The reaction mixture was diluted with 2 mL of DMF, and then directly subjected to dialysis against distilled water to remove the copper catalyst and unreacted monomers. The product was harvested by freeze-drying. Yield: 70~80%. Afterward, linear precursor with azide terminal waso btained as follows: p(DMAEMA)-Br (1.0 g) and NaN_3 in a 20-fold molar excess were dissolved in a 10 mL of water/DMF (1/4, v/v) mixed solvents in a round-bottom flask with a magnetic stirrer. The reactionmixture was stirred at 45 °C for 48 h. After purification by extensive dialysis to remove residual sodium salts, the linear precursors, linear-p(DMAEMA)-N_3homopolymer and were collected by freeze-drying. Yield: 90% (0.9 g)

Synthesis of cyclic polymers, Cyclic p(DMAEMA), by intra-chain click cyclization

In a typical procedure, 150 mL of DMF was placed in a three-neck flask and degassed by bubbling Ar for 1 h. 50-fold molar equivalents of CuBr and PMDETA were then charged into the flask under the protection of argon flow. A solution of HC≡C-p(DMAEMA)-N_3linear precursor (0.1 g) in degassed DMF (10 mL) was added to the catalyst solution via a syringe pump at the rate of 0.4 mL/h. The reaction was carried out at 100 °C in an Ar atmosphere for 25 h. At the end of the polymer solution addition, the mixture was allowed to proceed for another period of 23 h. After the mixture was cooled to room temperature, DMF was removed under reduced pressure and the concentrated residue was transferred directly to a dialysis tube (MWCO: 3.5 kDa, Fisher Scientific) and, dialyzed against distilled water to remove the cooper catalyst. The resulting cyclic polymer, cyclic p(DMAEMA), was obtained by freeze-drying. Yield: 70% (0.070 g)

Characterization of polymers.

^1H NMR spectra were recorded on a Bruker AV 500 (Bruker Corporation, Billerica, MA) nuclear magnetic resonance (NMR) instrument using $CDCl_3$ as a solvent. Fourier transform infrared (FTIR) spectra were recorded on a Bruker Vector 33 FTIR spectrometer. Samples were pressed into potassium bromide (KBr) pellets for measurements. Gel permeation chromatography (GPC) was used to determine molecular weight and polydispersity (M_w/M_n, PDI) of polymer samples prepared. SEC Tosoh TSK-GEL R-3000 and R-4000 columns (Tosoh Bioscience, Montgomeryville, PA) were connected in series to a Agilent 1200 series (Agilent Technologies, Santa Clara, CA), refractometer Optilab-rEX and triple-angle static laser light scattering detector miniDAWN TREOS (Wyatt Technology, Santa Barbara, CA). HPLC-grade DMF containing 0.1 wt% LiBr at 60 ^oC was used as the mobile phase at a flow rate of 1 mL/min.

Preparation and characterization of DNA polyplexes

The pCMV-Luc2 plasmid was diluted in double-distilled H_2O to a concentration of 0.1 mg/mL and mixed with an equal volume of polymer (also diluted in double-distilledH_2O) at different N/P (polymer protonatable nitrogens to DNA phosphates) ratios, as previously described.[2]After mixing, the polyplexes were allowed to incubate for 10 min at room temperature. Each polyplex solution (containing 2μg DNA) was mixed with 40 μL of distilled water, and then used to determine the particle size of the polyplexes by dynamic light scattering (DLS) performed on a Brookhaven Instruments Corp ZetaPALS instrument (Holtsville, NY) at a wavelength of 659.0 nm and a detection angle of 90°. The measurements were performed in triplicate. YOYO-1 quenching assay was performed as described previously.[4]

Gel retardation assay

The DNA binding ability of the polymers was investigated by agarose gel retardation assay. The polymer/DNA complexes prepared at varying N/P ratios from 0.5/1 to

6.0/1 were electrophoresed through a 1% (w/v) agarose gel containing ethidium bromide at 100 V in TAE buffer solution (40 mMTris-HCl,1 v/v % acetic acid, and 1 mM EDTA).

Acid−base titration

The buffering capacity of various linear and cyclic polymers was determined by acid–base titration over a pH range from 2.0 to 11.0. Briefly, polymer was dissolved in 0.15M NaCl aqueous solution (0.2 mg/mL). The solution was brought to a starting pH of 10.0 with 0.1 M NaOH and then was titrated with 0.1 M HCl using a pH meter. The buffering capacity was determined as μmol of H^+ per mg of polymer required to decrease the pH of 0.2 mg/mL polymer solution from 7.4 to 5.0.

HeLa cell culture

HeLa cells, human cervical carcinoma cells (ATCC CCL-2), were maintained in minimum essential media (MEM) containing L-glutamine (Gibco), 1% antibiotic-antimycotic (Gibco), and 10% fetal bovine serum (FBS, Invitrogen) at 37 °C and 5% CO_2.

***In vitro* transfection study**

Transfection studies in cultured cells were conducted as previously described.[1,2] HeLa cells were seeded at a density of 15,000 cells/well in MEM medium supplemented with 10% FBS and 1% antibiotic/antimycoticin 24-well plates. Cells were allowed to attach for 24 h at 37 °C, 5% CO_2. Polyplexes were formed at different N/P ratios using 1 μg of pCMV-Luc2 plasmid DNA in 20 μL total volume. Each sample was diluted to 200 μL with OptiMEM medium. The cells were washed once with PBS and the transfection solutions were added. After incubation at 37 °C, 5% CO_2 for 4 h, the cells were washed twice with PBS and the polyplex solution was replaced with complete cell culture media. After an additional 44 h at 37 °C, 5% CO_2, luciferase expression was quantified with a luciferase assay kit (Promega Corp.) according to the manufacturer's instructions, except that a freeze-thaw cycle at −80 °C was

included after the addition of the lysis buffer to ensure complete cell lysis. Luminescence intensity was measured on a Tecan Safire^2 plate reader (Tecan, Männedorf, Switzerland) with integration for 1 s. The total protein content in each well was measured by a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions so that the luciferase activity was normalized to the total protein content in each well. Each sample was tested with a sample size (n) = 3.

The effects of free polymer on transfection efficiency were evaluated through a modified polymer dosing transfection study. HeLa cells were plated at 15,000 cells per well in 24-well plates and incubated for 24 h at 37 ºC. Polyplexes were formed at N/P = 2 using 1 μg of pCMV-Luc2 plasmid DNA and poly-L-lysine (15-30 kDa, Sigma) in 20 μL total volume. Each sample was diluted to 200 μL final volume with OptiMEM medium. Cells were washed once with PBS and then polyplex solutions were added. Cells were incubated for 2 h at 37 ºC. After 2 h, free DMAEMA polymer was added to the wells to give a pDMAEMA concentration equivalent to N/P = 3 and cells incubated at 37 ºC for another 2 h. Cells were washed once with PBS, had fresh media added, and were incubated at 37 ºC for 44 h. After 44 h, cells were analyzed for luciferase activity and protein concentration as previously described.

Polyplex uptakeassay

Cellular uptake of polyplexes was evaluated via flow cytometry using fluorescently-labeled DNA. HeLa cells were plated at 30,000 cells per well in 12-well plates and incubated for 24 h at 37 ºC.pCMV-Luc2 plasmid was mixed with the bis-intercalating dye YOYO-1 iodide at a dye/base pair ratio of 1:100 and incubated at room temperature for 1 h. Polyplexes were formed at N/P = 5 by complexing YOYO-1 labeled DNA with pDMAEMA polymers. Cells were washed once with PBSand 2 μg of labeled polyplexes in OptiMEM were added to cells for 30 min at 37 ºC. Cells were washed twice with PBS, detached by treatment with trypsin, pelleted, and then resuspended in 1 mL of 2 μg/mL propidium iodide (PI) in PBS. Cells were incubated at 4 ºC in the dark for 10 minutes before being pelleted, washed once with

PBS, and analyzed by flow cytometry using the MACSQuant Analyzer (Miltenyi Biotec, Cologne, Germany), gating for PI- (live) cells.

Cytotoxicity study

The cytotoxicity of polymers was evaluated *in vitro* using the MTS assay. HeLa cells were plated overnight in96-well plates at a density of 2,500 cells per well per 0.1 mL. Polymers were prepared in serial dilutions in water and then diluted 10-fold in OptiMEM medium (Invitrogen). The cells were rinsed once with PBS and incubated with 40 μL of the polymer solution for 4 h at 37 °C, 5%CO_2. Cells were rinsed with PBS and the medium was replaced with100 μL complete growth medium. At 48 h, 20 μL of3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium(MTS) (Promega) were added to each well. Cells were then incubated at 37 °C, 5% CO_2 for 3 h. The absorbance of each well was measured at 490 nm using a plate reader.

Propidium Iodide Nanohole Assay.

Polymer-induced membrane disruption was evaluated through a nanohole assay adapted from Hong *et al*.[5] HeLa cells were plated at 30,000 cells per well in 12-well plates and incubated for 24 h at 37 ºC. Polymers were prepared in ddH_2O and then diluted 10-fold in OptiMEM medium. The cells were rinsed once with PBS and 400 μL of polymer and 100 μL of 1 mg/mL PI were added to each well. Cells were incubated at 37 ºC for 30 min. Cells were rinsed twice with PBS, trypsinized, pelleted, washed once with PBS, and then analyzed for PI staining by flow cytometry.

References

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5. Hong, S.; Bielinska, A. U.; Mecke, A.; Keszler, B.; Beals, J. L.; Shi,X.; Balogh, L.; Orr, B. G.; Baker, J. R.; Banaszak Holl, M. M. *Bioconjugate Chem.* **2004**, *15*, 774-782.

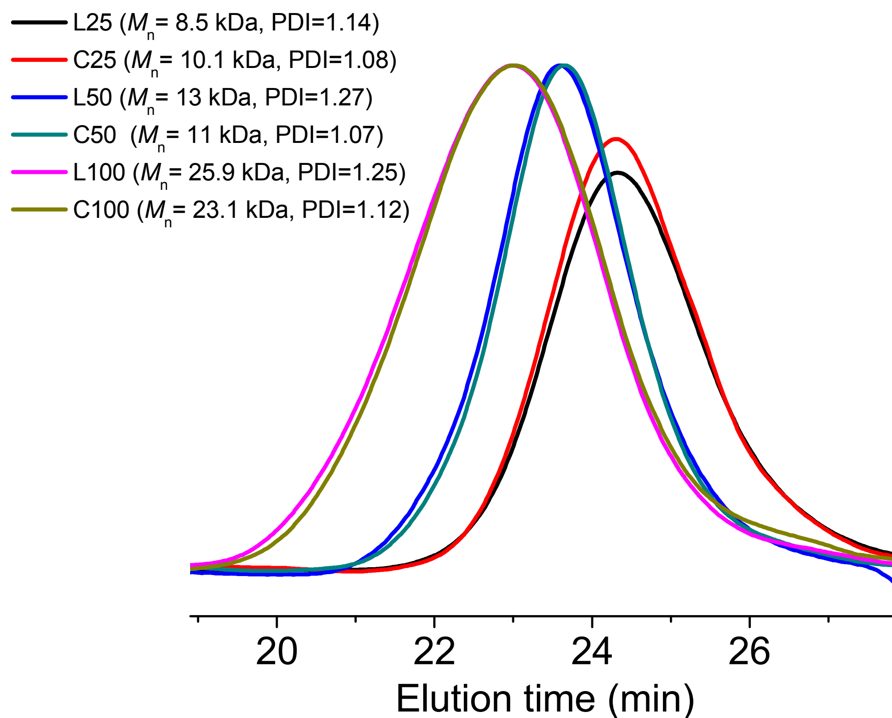

Figure S1. GPC traces of linear and cyclic pDMAEMA with various degree of polymerizations (DPs).

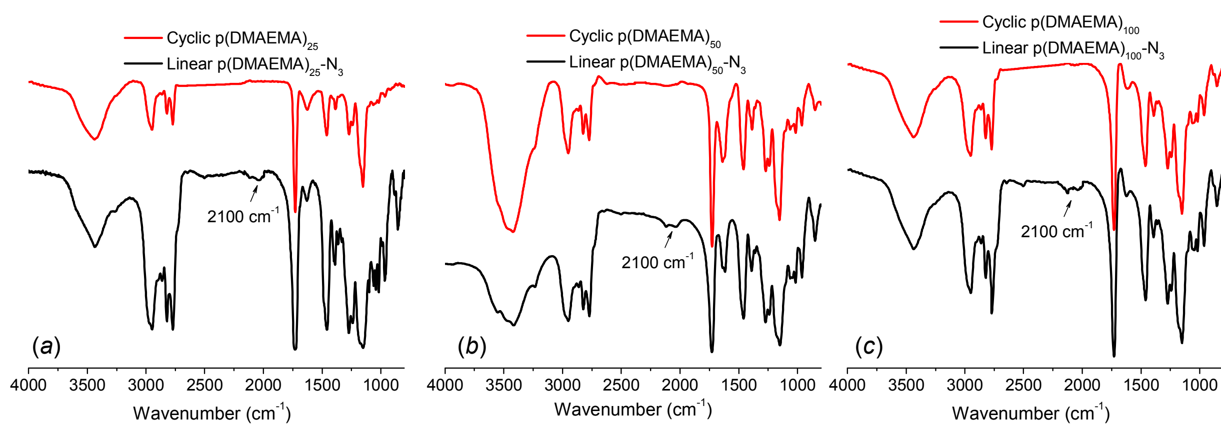

Figure S2. FT-IR spectra of linear p(DMAEMA)-N_3 and cyclic p(DMAEMA) polymers.

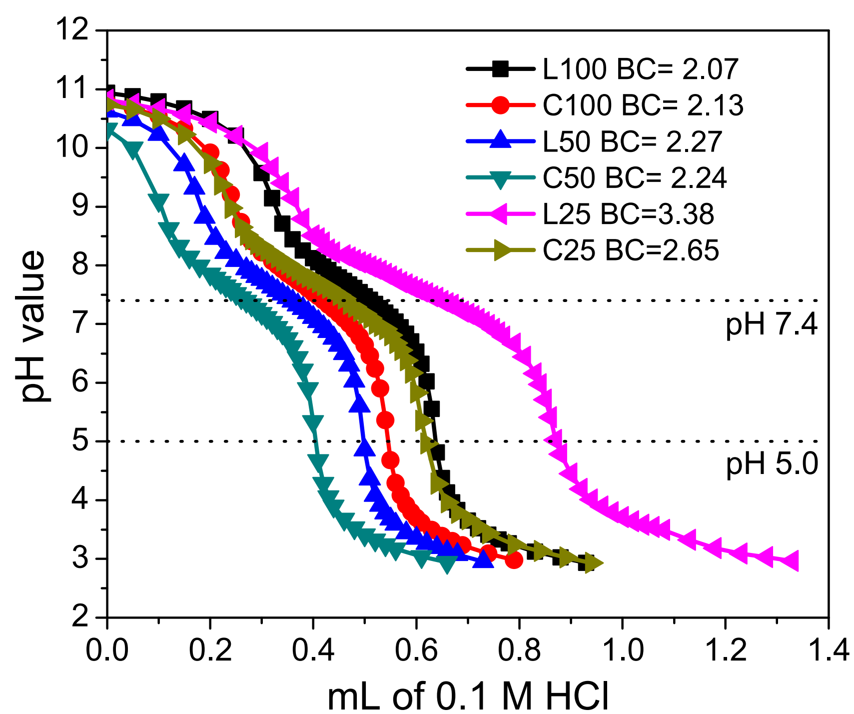

Figure S3. Acid-base titration of linear precursors and cyclic polymers.

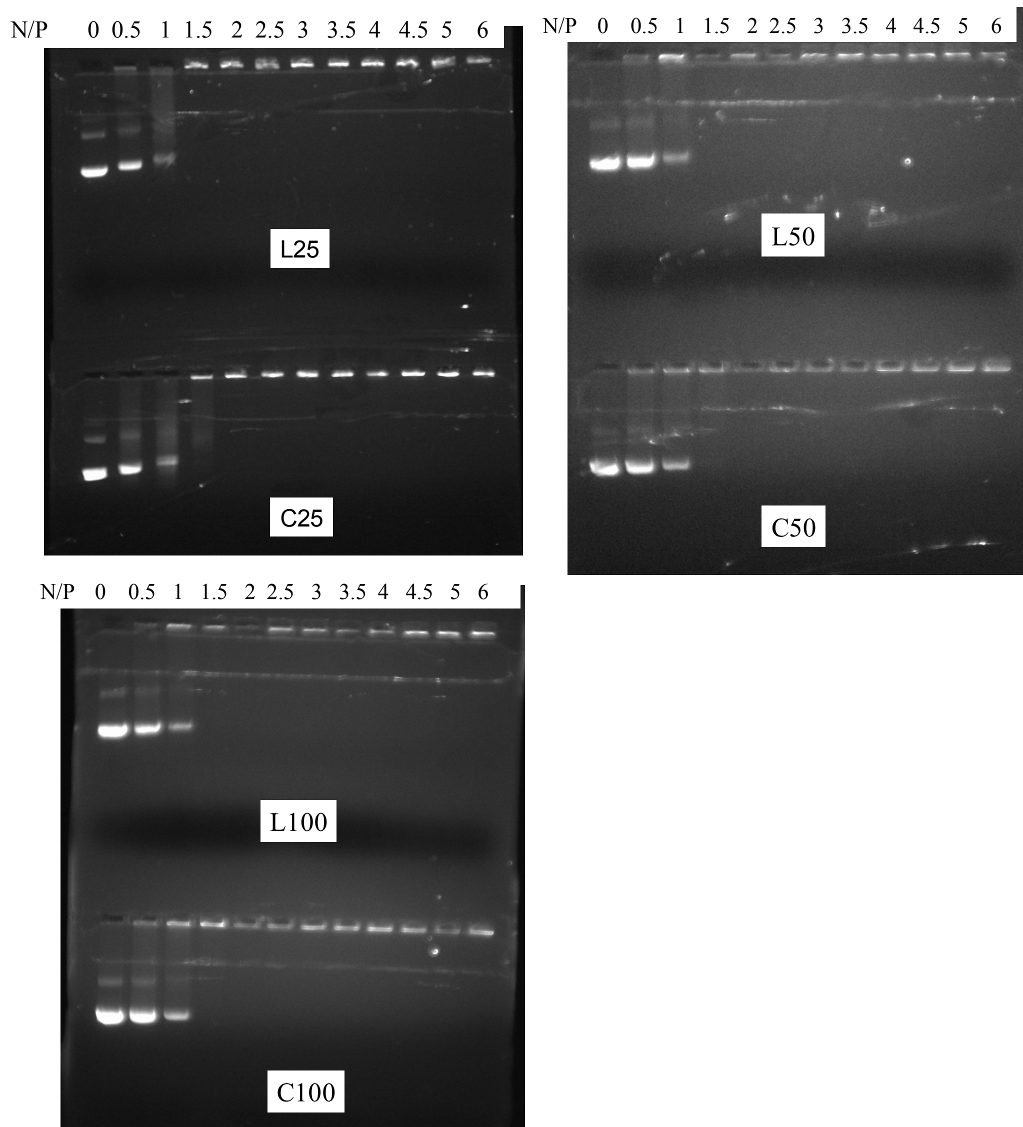

Figure S4. Agarose gel electrophoresis of various polyplexes formed by linear and cyclic polymers with plasmid DNA at N/P ratios ranging from 0/1 to 6/1.

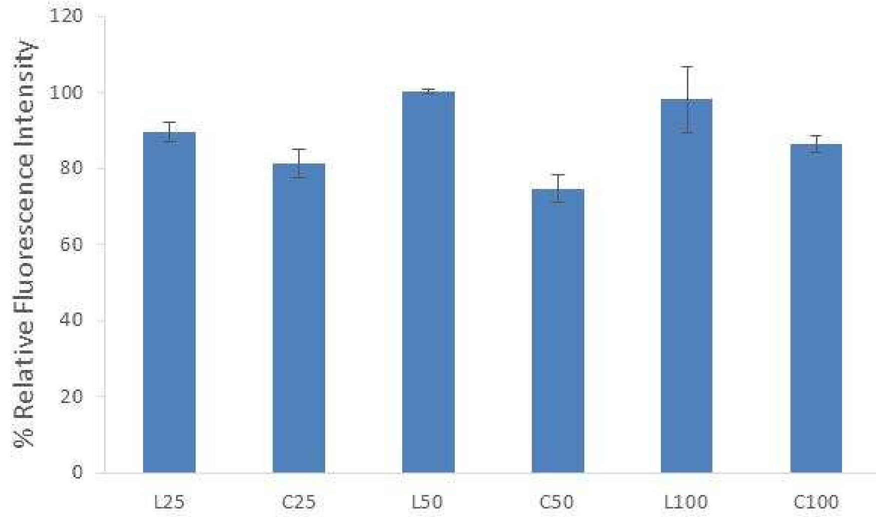

Figure S5. DNA condensation assessed by YOYO-1 fluorescence assay. Polyplexes containing YOYO-1-labeled plasmid were prepared in ddH_2O at N/P=5 with various polymers. YOYO-1 fluorescence in polyplex solution was normalized to unpackaged plasmid.

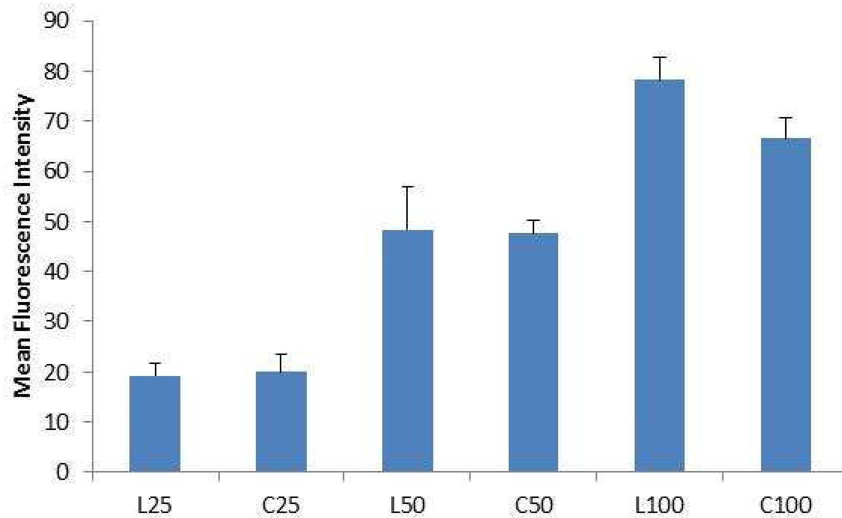

Figure S6. Polyplex uptake in HeLa cells. Plasmid DNA was labeled with YOYO-1 fluorophore and complexed with polymers at N/P=5. Polyplexes were exposed to HeLa cells for 30 min and then assessed for cell-associated fluorescence by flow cytometry.

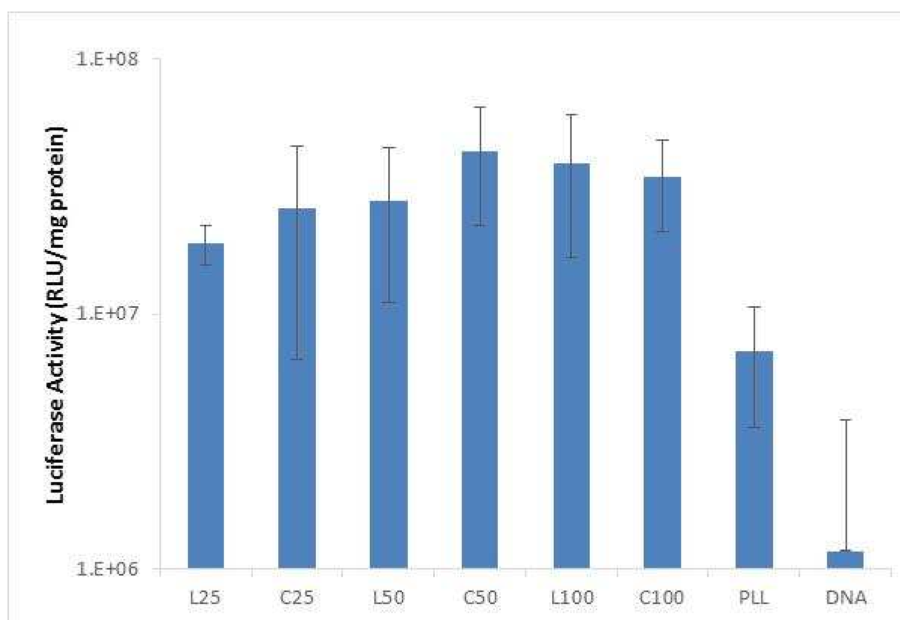

Figure S7. Effects of free cyclic and linear pDMAEMA polymers on transfection. Poly-(L)-lysine polyplexes complexed with luciferase plasmid was prepared at N/P=2 and exposed to cells. Two hours later, free pDMAEMA polymers were added to a total N/P=5. Luciferase expression was assessed 48 h after initial transfection.