Supporting Information

Bottlebrush Copolymers for Gene Delivery: Influence of Architecture, Charge Density, and Backbone Length on Transfection Efficiency

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Materials

pHR' CMV GFP plasmid was provided by Dr John James (University of Wawick) and isolated using a Qiagen Maxi Prep kit, following the established protocol. Opti-MEM and Dulbecco's Modified Eagle Media (DMEM) were purchased from Thermo Fisher. Foetal bovine serum (FBS) was purchased from LabTech.com. Ethidium bromide (10 mg mL^{-1} solution), Heparin (sodium salt from porcine intestinal mucosa), and 2-Ethyl-2-Oxazoline were purchased from Sigma-Aldrich. Human embryonic kidney cells 293 (HEK293T, CRL-3216) were purchased from American type culture collection (ATCC). Methyl p-toluenesulfonate (MeTos) was purchased from VWR. Acetonitrile (99.9%, Extra Dry, AcroSeal™) was purchased from Fisher Scientific.

Characterisation

Proton Nuclear Magnetic Resonance (^{1}H-NMR)

^{1}H-NMR spectra were recorded using a Bruker DPX-300 or DPX-400 NMR spectrometer which operated at 300.13 and 400.05 MHz, respectively. The residual solvent peaks were used as internal references. Deuterated chloroform ($CDCl_3$) (δ_H = 7.26 ppm) and deuterated methanol (MeOD) (δ_H = 3.31 ppm) were used as the solvents for all measurements.

Size Exclusion Chromatography (SEC)

Aqueous SEC was measured on a System from PSS (Mainz, Germany) using set of NOVEMA Max columns (1 x 30 Å, 8 x 3000 mm; 2 x 1000 Å, 8 x 300 mm) including RI and UV (280 and 309 nm), as well as a 7-angle MALLS detector (SLD 7000). Water with 0.3 Vol% TFA or formic acid and 0.1 mol L^{-1} NaCl was used as the eluent and poly(vinyl pyridine) was used as calibration. For MALLS measurements, dn/dc was determined by performing different injections varying the injections volume and thus the injected sample amount. The area under the RI-curve was integrated and fitted against injected mass to determine the dn/dc (slope of the linear regression).

Transmission Electron Microscopy

In all cases the Formvar/carbon coated copper grids were subjected to glow discharge to render their surface hydrophilic. Then, 5 µl of sample was added to the grid and incubated for 1 min. After addition of the sample, excess solution was removed by blotting with tissue. 2 % uranyl acetate was added for a total of 3 min incubation (3 drops of 1 minute each). Again, excess liquid was removed by blotting and the grid was allowed to airdry. Imaging was done on a Jeol 2100Plus TEM fitted with a Gatan OneView IS camera and images analyzed by Fiji (www.fiji.sc).

Dynamic Light Scattering/Zeta-Potential Measurements

Size and zeta-potential measurements were carried out using an Anton Paar Litesizer 500 at a polymer concentration of 0.1 mg mL^{-1} and a temperature of 25 °C. The instrument is equipped with a semiconductor laser diode (40 mW, 658 nm). Size measurements were obtained at a scattering angle of 173 ° (back scatter). PDi was calculated using **Equation S1**. Zeta-potential measurements were carried out using Anton Paar omega cuvettes and modelled using the Smoluchowski theory.

$$PDi = \frac{\sigma^2}{d^2} \qquad \textbf{S1}$$

Statistical Analysis

To determine the statistical significance, analysis of variance (ANOVA) was performed using a Bonferroni test for means comparison. Statistically significant differences were indicated with * for $p < 0.001$. All statistical analyses were performed with data of n = 5 in OriginPro (Version 2019b).

Synthetic Procedures

Synthesis of Poly(2-Ethyl-2-Oxazoline)

To a dry microwave vial was added methyl tosylate (9 µL, 0.06 mmol), 2-ethyl-2-oxazoline (1.8 mL, 18 mmol) and acetonitrile (2.7 mL). The vial was sealed and placed into a microwave reactor before heating at 140 °C for 15 minutes. After polymerization, the vial was opened to air and the solvent removed using rotary evaporation. The polymer was dissolved in dichloromethane and washed with 3 x Na2CO3 and 3 x brine. Precipitation in diethyl ether and filtration yielded the purified polymer.

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 3.75 – 3.13 (m, 968 H, backbone), 3,10 - 2.92 (m, 3 H, Methyl group (α-end)), 2.54 - 2.13 (m, 484 H, CH_2 side chain), 1.23 – 0.98 (m, 726 H, CH_3 side chain);

SEC (DMF, PMMA calibration): M_n = 21,000 g mol^{-1}, $Đ$ = 1.49;

Synthesis of Poly[(2-Ethyl-2-Oxazoline)-co-(Ethylenimine)]

PEtOx containing polymer was dissolved in 1 M HCl (concentration of amide = 0.48 M) and added to a microwave reaction vial with a magnetic stirrer and sealed. The vial was placed in a Biotage Initiator+ Eight microwave reactor at 120 °C for a predetermined amount of time. After the reaction, the solution was made basic by addition of 4 M NaOH, before dialysis using an Amicon Ultra-15 centrifugal filter unit (MWCO = 3 kDa) to remove any salt formed. The solution was lyophilized to yield the product.

^{1}H NMR (300 MHz, D_2O) δ ppm: 3.75 – 3.13 (m, 184 H, backbone), 2.85 - 2.62 (m, 784 H, CH_2, EI), 2.54 - 2.13 (m, 92 H, CH_2 side chain), 1.23 – 0.98 (m, 138 H, CH_3 side chain);

SEC (H_2O, 0.3% formic acid, 1M NaCl, PVP calibration): M_n = 13,800 g mol^{-1}, $Đ$ = 1.49;

Experimental Procedures

Cell Culture

Human embryonic kidney cells 293 (HEK293T) were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10 % foetal bovine serum (FBS) and 1 % penicillin/streptomycin. Cells were grown as monolayers at 310 K under an atmosphere of 5 % CO2, and passaged once they have reached 70-80 % confluency (once every three days).

Polyplex Formation

Polyplex formation was carried out as follows. To an Eppendorf tube was added water and polymer followed by thorough mixing. pDNA ([pDNA]final = 35 µg mL^{-1}) was added to the Eppendorf tube to make a total volume of 100 µL and vortexed for 30 seconds before a 30 minute incubation at room temperature. The concentration of pDNA was fixed in all samples and the concentration of polymer varied to the make the targeted N/P ratio.

Agarose gels (0.8 % w/v) were prepared by adding 1.6 g agarose powder to 200 mL 1 X TAE buffer and heating in a microwave to dissolve the agarose. The solution was cooled before addition of ethidium bromide (EthBr) (20 µL of 10 mg mL^{-1} EthBr stock). The gel was poured into a casting tray and combs inserted to form the wells. To visualize polyplex formation, 20 µL of the polyplex prepared above was added to 5 µL SDS-free loading buffer and added to the wells of the agarose gel. The gel was run in 1 X TAE buffer at 100 V for 30 minutes before imaging with an Azure 600 imaging system.

Ethidium Bromide Displacement

Ethidium bromide/pDNA complexes were first prepared by incubating EthBr (1 μg mL^{-1}) and pDNA (15 μg mL^{-1}) in H_2O for 30 minutes at room temperature. 50 μL of polymer was aliquoted into a black 96-well plate at appropriate concentrations for the targeted N/P ratio. 50 μL of pDNA/EthBr was added to the well and mixed before incubation for 30 minutes at 37 °C. The fluorescence of each well was measured using a Cytation3 plate reader ($\lambda_{excitation}$ = 525 nm, $\lambda_{emission}$ = 605 nm). pDNA/EthBr in the absence of polymer was used as the control.

Polyplex Stability towards Heparin

Polyplexes were prepared as described above and 20 μL added to a black 96-well plate along with 1 μL EthBr (2.5 μg mL^{-1} in H_2O). Heparin solution (0.05 or 0.25 mg mL^{-1}) was added in 5 or 10 μL increments. After each addition the solution was incubated for 10 minutes at 37 °C before reading the fluorescence of each well using a Cytation3 plate reader ($\lambda_{excitation}$ = 525 nm, $\lambda_{emission}$ = 605 nm). A control of pDNA/EthBr complex was measured and diluted equally with H_2O throughout the experiment.

Polymer Toxicity Against HEK293T Cells

The cytotoxicity of the polymers was determined in HEK293T using the XTT assay. Cells were seeded in a 96-well plate at 10,000 cells/well and left to incubate at 37 °C for 24 hr in DMEM. Polymers were dissolved in serum free DMEM at 1.1 mg mL^{-1} and filtered through a 0.22 μm filter before dilution. Foetal bovine serum (FBS) was added, and the concentration adjusted to 1 mg mL^{-1}. The media was replaced with polymer containing media, using serial dilutions to incubate the cells with polymers at different concentrations (1 mg mL^{-1} to 0.0156 mg mL^{-1}) and incubated for 18 hr at 37 °C. After drug exposure, the XTT assay was used to determine cell viability. Cell viability was determined in triplicate in three independent sets of experiments and their standard deviation calculated.

Transfection of HEK293T Cells

HEK293T cells were seeded in a 24-well plate at a density of 100,000 cells/well and incubated for 18 hours. After incubation, the media was removed and replaced with 300 μL of Opti-MEM Reduced Serum Media. Polyplexes were prepared as described above and incubated for 30 minutes, before dilution with 350 μL of Opti-MEM. After 1 hour the media was removed from the wells and replaced with polyplex containing media ([pDNA] = 10 μg mL^{-1}) and incubated at 37 °C for 5 hours. After incubation, the media was removed, and the cells washed once with warm DMEM. The media was replaced with fresh DMEM and incubated for 43 hours. The cells were then washed with 500 μL PBS and harvested with trypsin/EDTA (150 μL) before addition of 300 μL DMEM. The suspended cells were transferred to an Eppendorf tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was removed, and the cells washed with 500 μL PBS then centrifuged at 1000 rpm for 5 minutes. The supernatant was removed, and the cells resuspended in 100 μL PBS before addition of 100 μL of 8 % formaldehyde and 15 minutes incubation at room temperature to fix the cells. Once fixed the cells were centrifuged at 1000 rpm for 5 minutes and the pellet washed with 200 μL of PBS followed by re-centrifugation and resuspension in 200 μL of fresh PBS. The suspension was transferred into FACS tubes for analysis by flow cytometry. Samples were analyzed using a LSRII flow cytometer (488 nm laser with 530/30 filter and 561 nm laser with 585/15 filter).

Hemolysis Assay

Sheep red blood cells (RBCs) were prepared by washing with PBS *via* centrifugation until the supernatant was clear. Polymers were dissolved in PBS to a concentration of 1.024 mg mL^{-1} as the highest concentration and a serial dilution was performed. A solution of 1 % Triton X-100 was used as a positive control and a solution of PBS was used as a negative control. 100 μL of 6 % (v/v) of RBCs in PBS was added to each well of a 96 well plate. Then 100 μL of each polymer solution was added to

make up a total volume of 200 µL and was mixed before being incubated at 37°C for 2 hours. The 96-well plates were centrifuged at 600 x *g* for 10 minutes and 100 µL of the supernatant was transferred to a new plate. The absorbance at 540 nm was measured and normalized with the positive and negative control. Positive control was used as 100% cell lysis.

Figures

Nuclear Magnetic Resonance

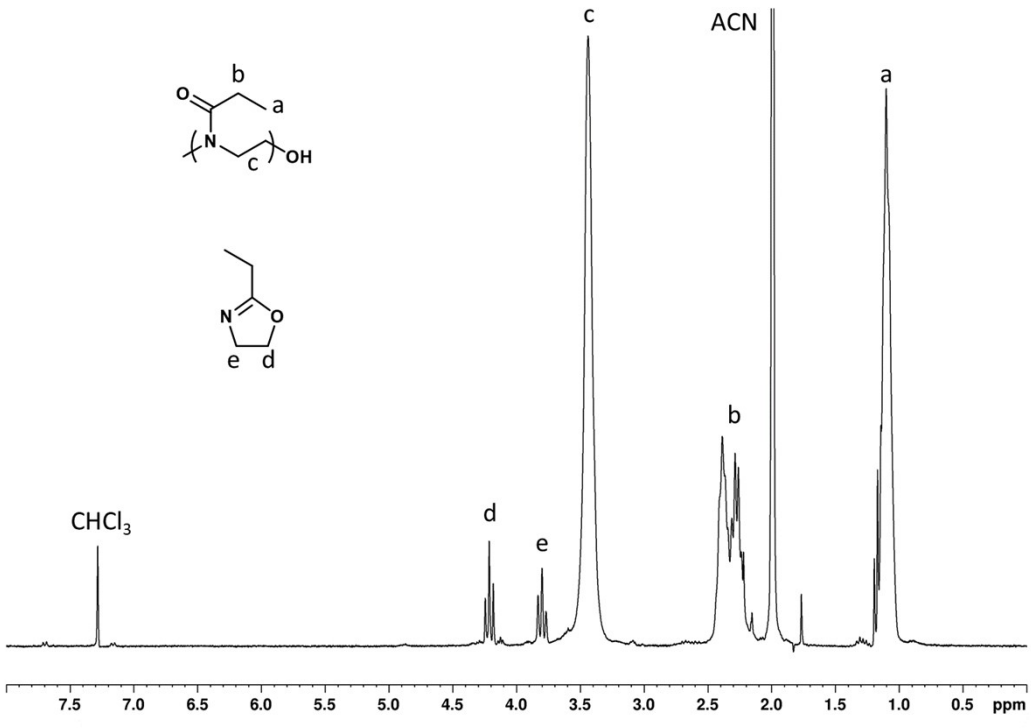

Figure S1 Assigned t = final ^{1}H NMR spectrum of **L-242** in $CDCl_3$.

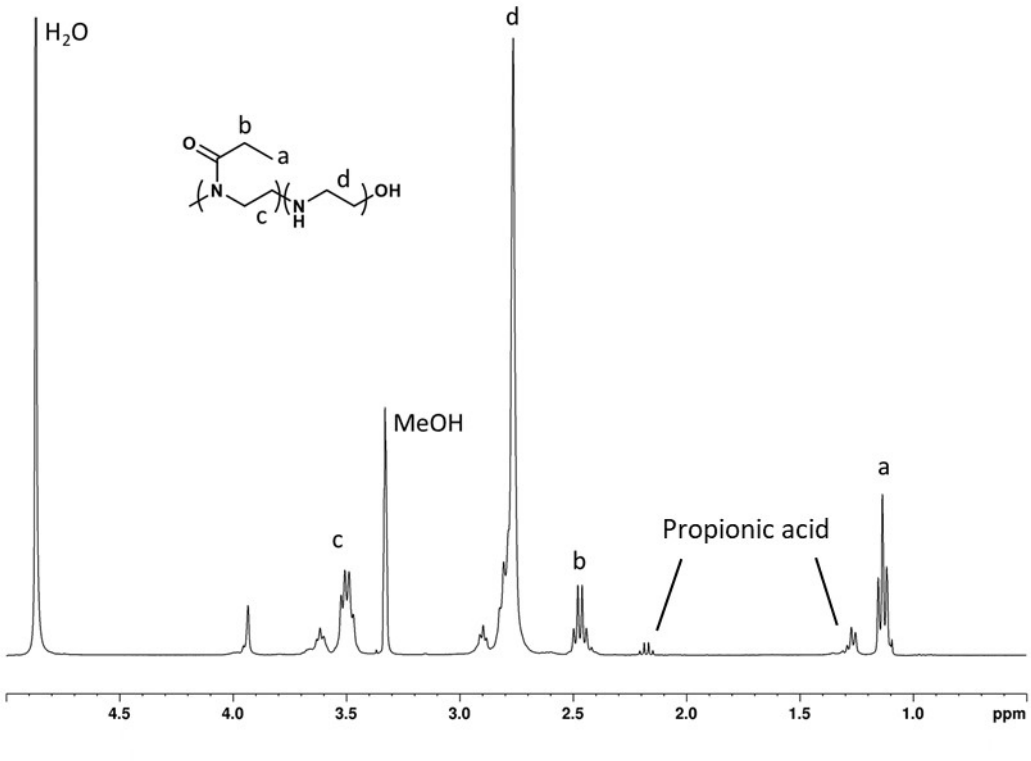

Figure S2 Assigned ^{1}H NMR spectrum of **L-242(81%)** in MeOD.

Size Exclusion Chromatography

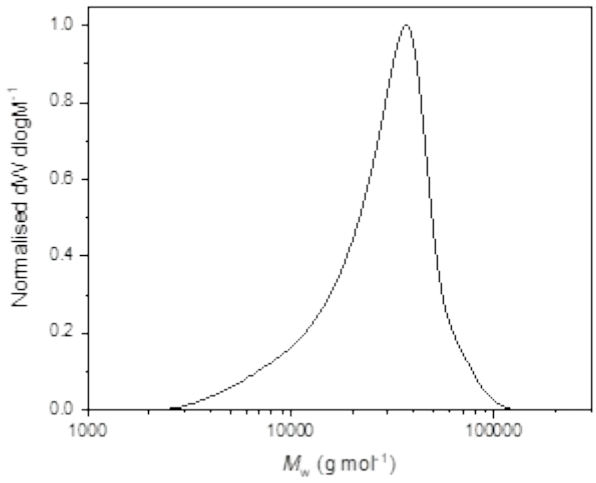

Figure S3 SEC curve for **L-242**, measured in DMF with a PMMA calibration.

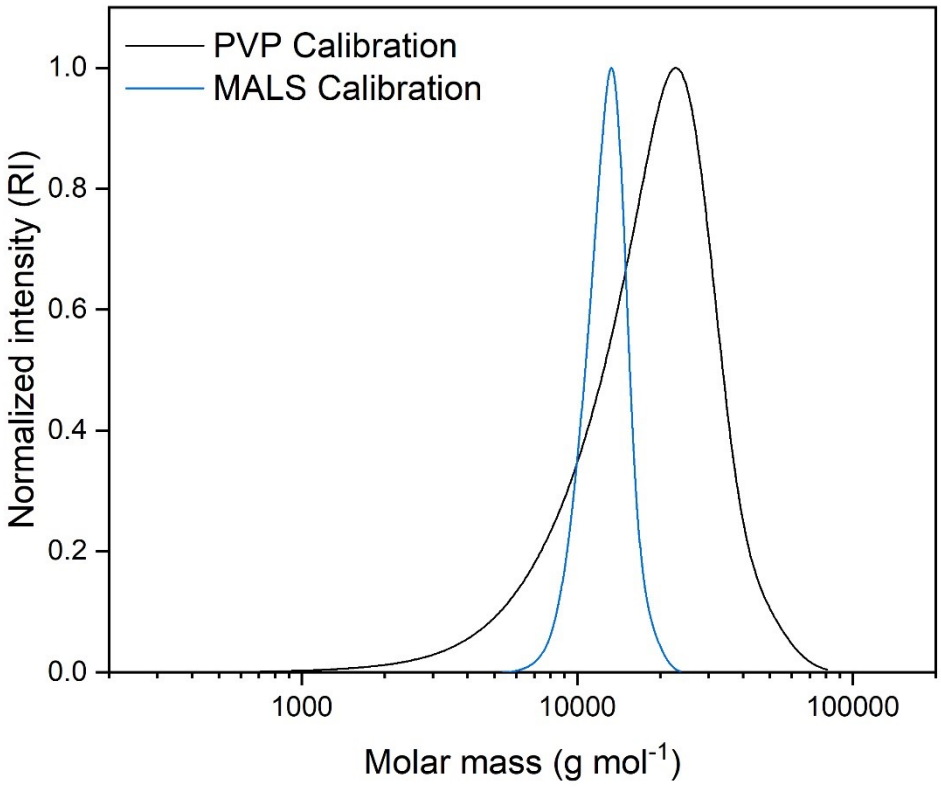

Figure S4 SEC trace of linear hydrolyzed polymer **L-242(81%)** in water (0.3% formic acid, 0.1 M NaCl), calibrated by a PVP standard or by MALS.

Agarose Gel Electrophoresis

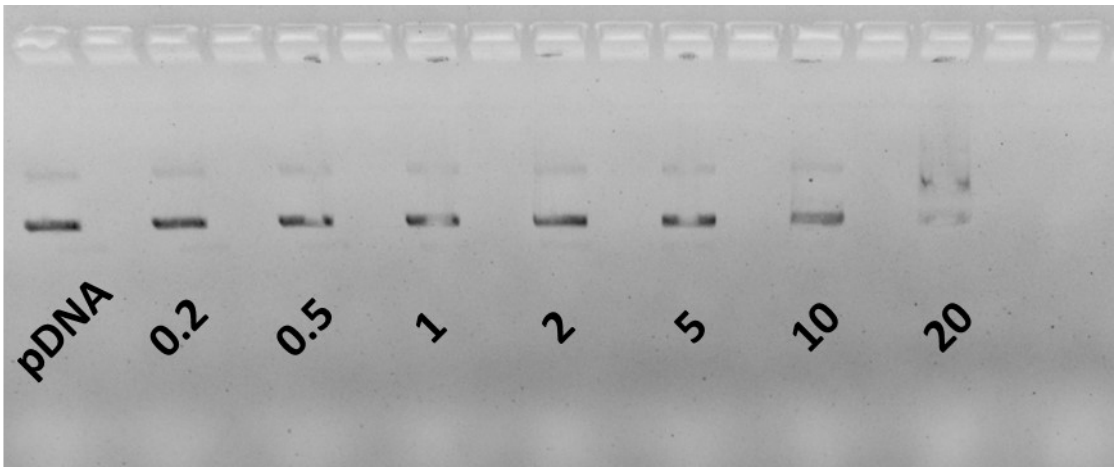

Figure S5 Polyplex formation of **B-10(32%)** at different N/P ratios.

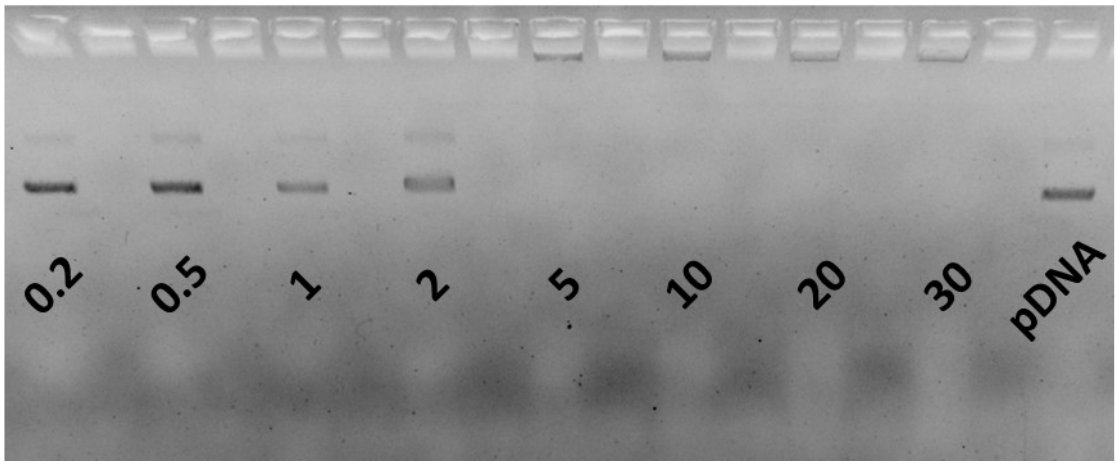

Figure S6 Polyplex formation of **B-10(46%)** at different N/P ratios.

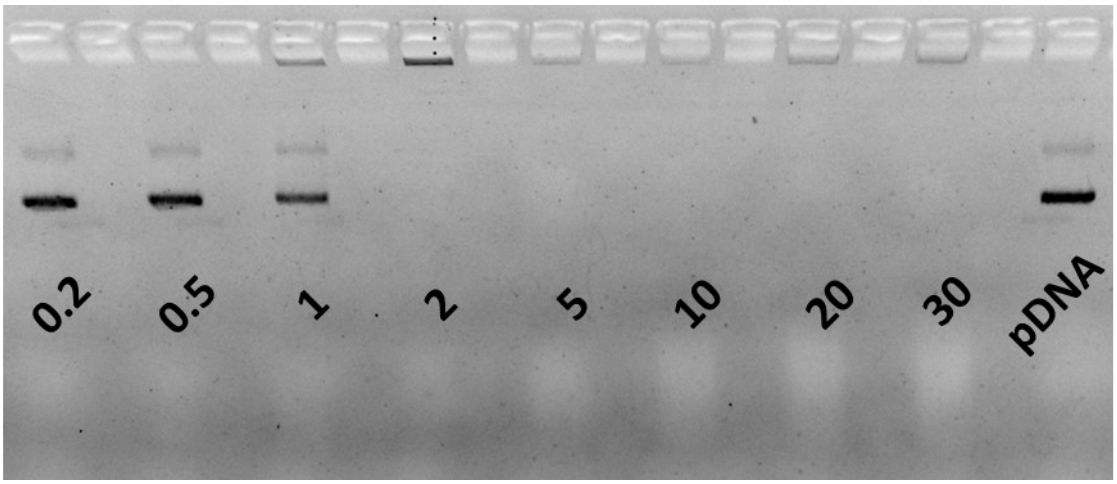

Figure S7 Polyplex formation of **B-10(69%)** at different N/P ratios.

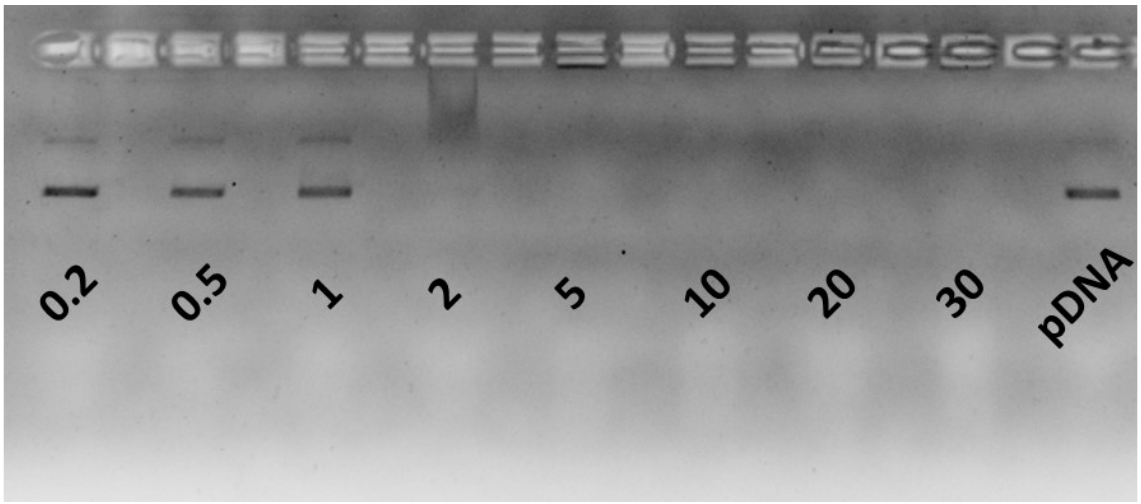

Figure S8 Polyplex formation of **B-10(79%)** at different N/P ratios.

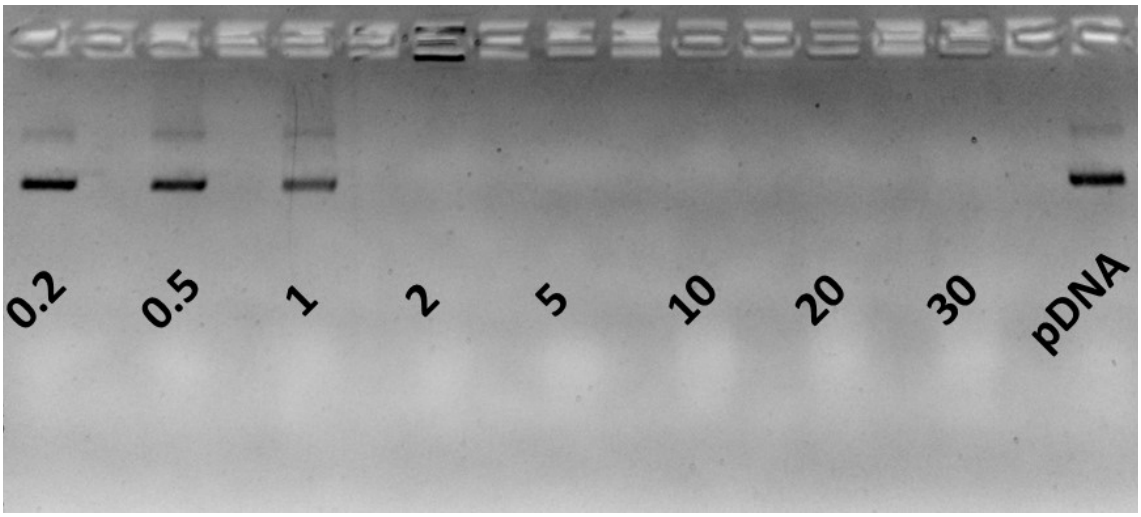

Figure S9 Polyplex formation of **B-25(67%)** at different N/P ratios.

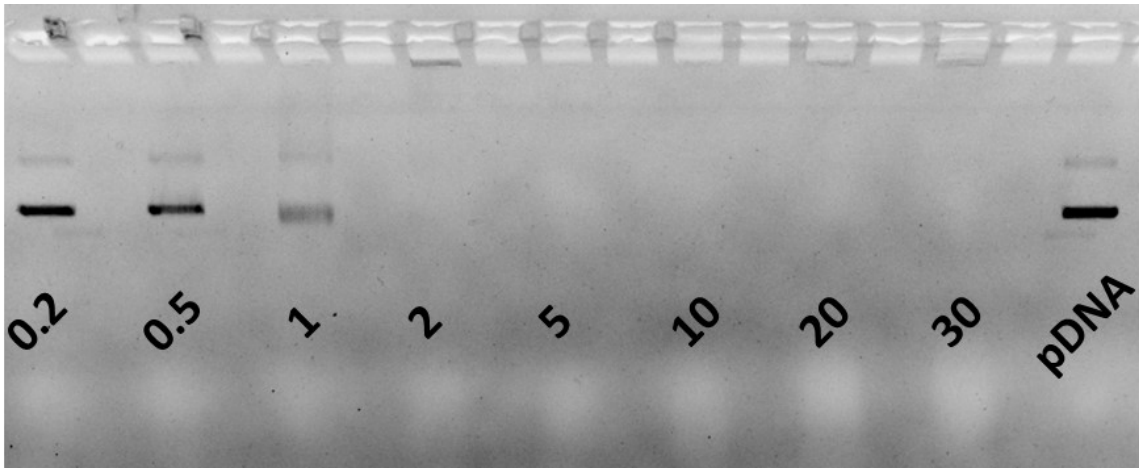

Figure S10 Polyplex formation of **B-50(67%)** at different N/P ratios.

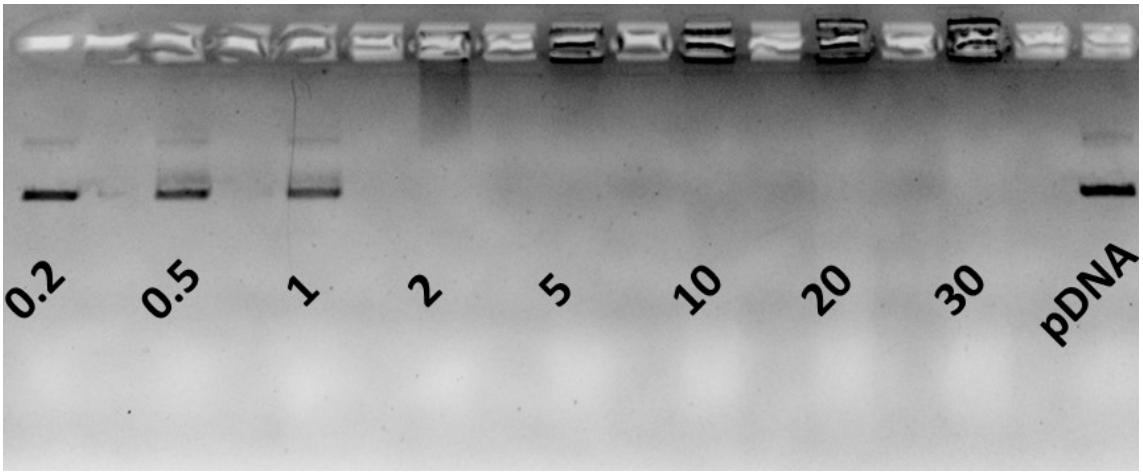

Figure S11 Polyplex formation of **L-242(81%)** at different N/P ratios.

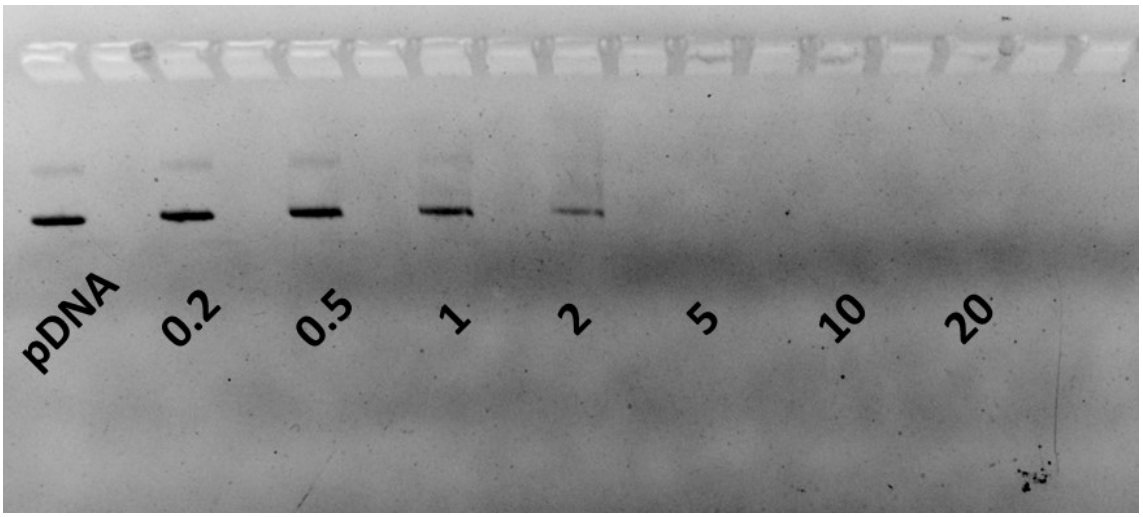

Figure S12 Polyplex formation of **lPEI** at different N/P ratios.

TEM microscopy images

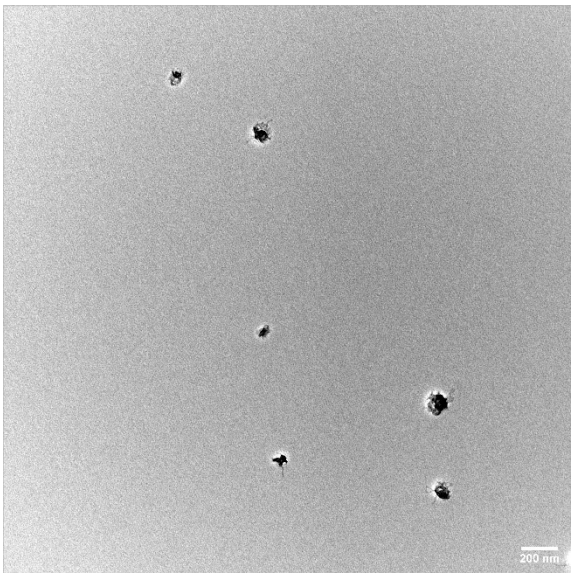

Figure S13 Transmission Electron Microscopy (TEM) images of polyplexes formed with **B-10(45%).**

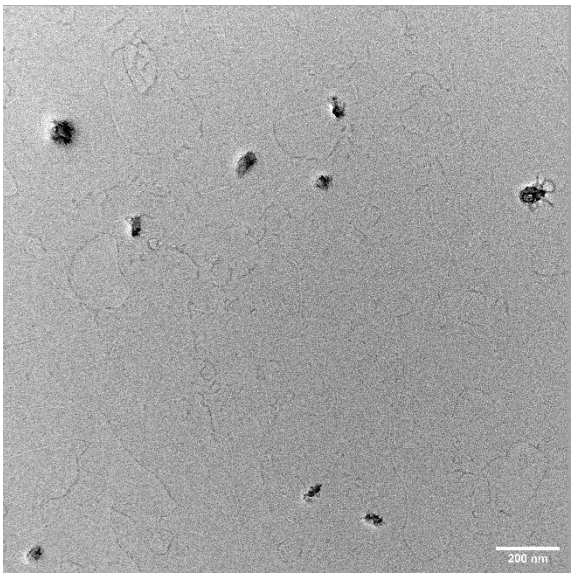

Figure S14 Transmission Electron Microscopy (TEM) images of polyplexes formed with **B-10(79%)**

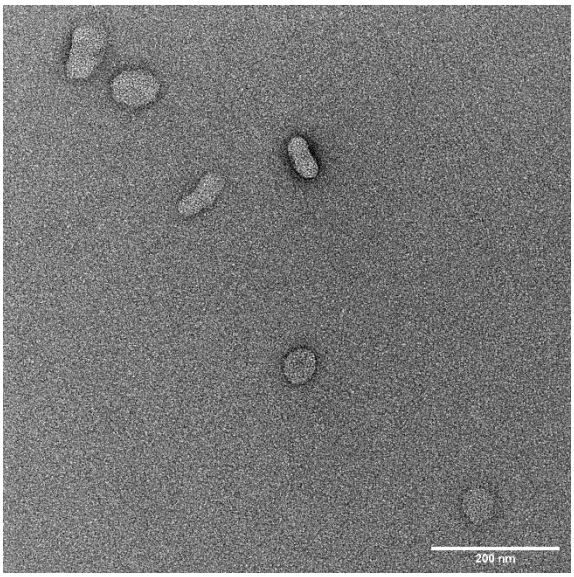

Figure S15 Transmission Electron Microscopy (TEM) images of polyplexes formed with **B-25(67%)**

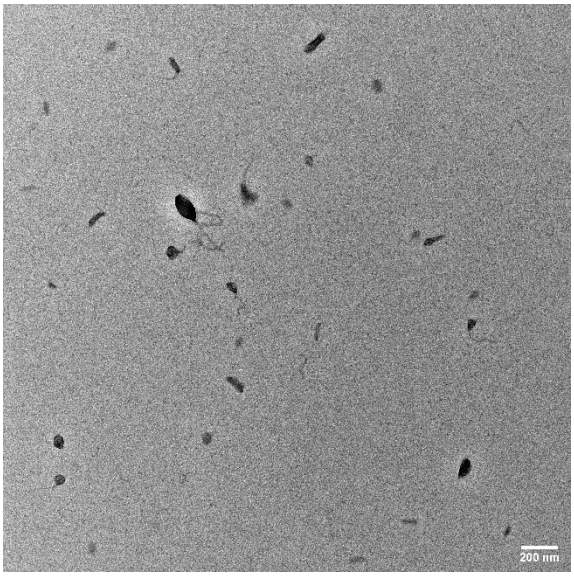

Figure S16 Transmission Electron Microscopy (TEM) images of polyplexes formed with **B-50(67%)**

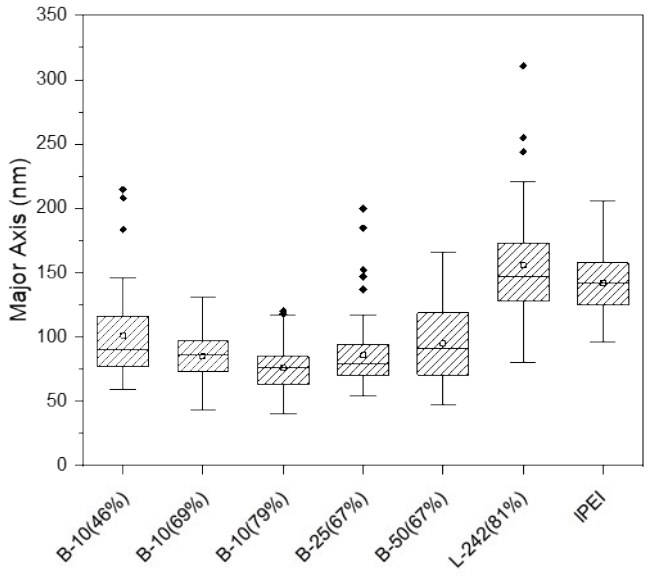

Figure S17: Major axis lengths determined from TEM images. n = 42 for **B-10(46%)**,n = 55 for **B-10(69%)**, n = 79 for **B-10(79%)**, n = 90 for **B-25(67%)**, n = 80 for **B-50(67%)**, n = 54 for **L-242(81%)**, n = 43 for **lPEI**. (□) = mean, (◆) = outliers.

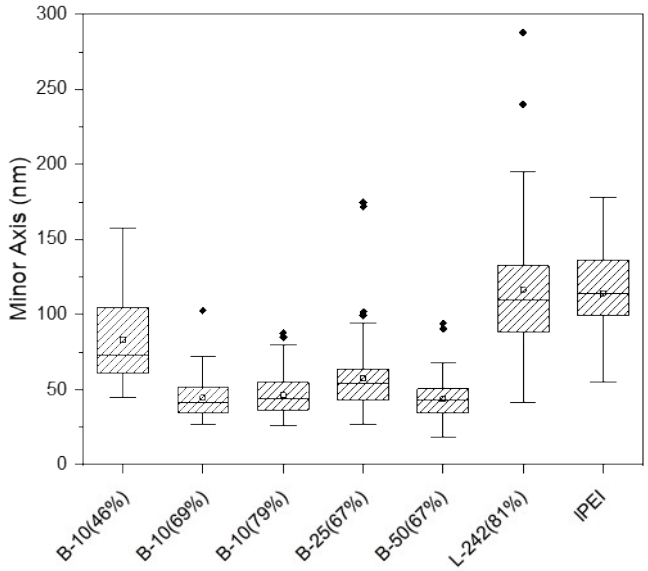

Figure S18: Minor axis lengths determined from TEM images. n = 42 for **B-10(46%)**,n = 55 for **B-10(69%)**, n = 79 for **B-10(79%)**, n = 90 for **B-25(67%)**, n = 80 for **B-50(67%)**, n = 54 for **L-242(81%)**, n = 43 for **lPEI**. (□) = mean, (◆) = outliers.

Flow Cytometry

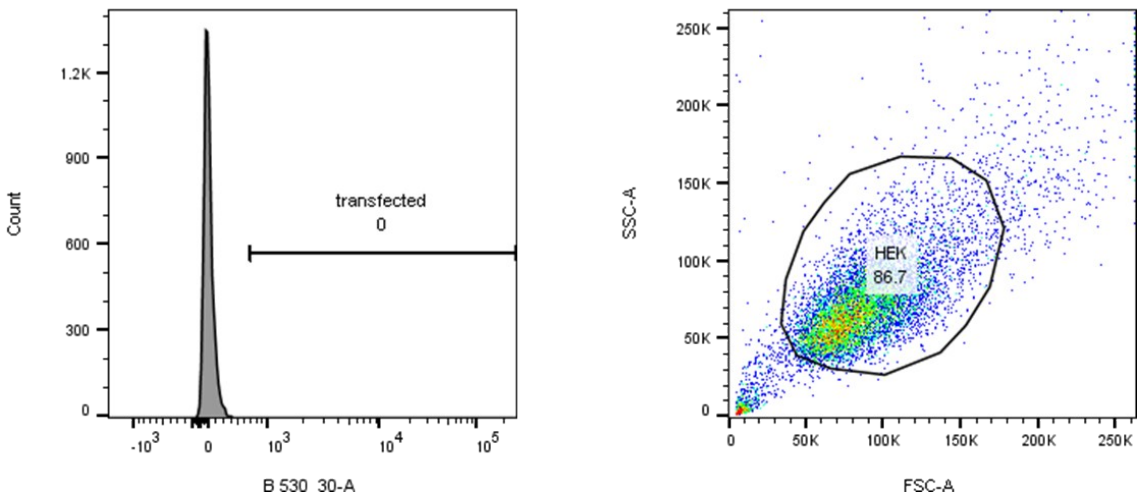

Figure S19 Flow cytometry results for transfection of HEK293T cells - blank (no transfection).

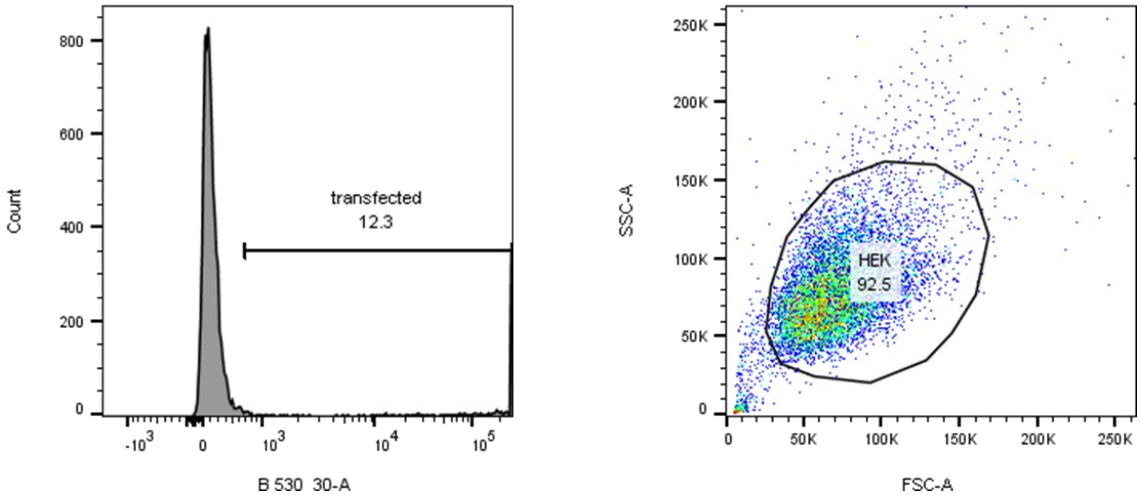

Figure S20 Flow cytometry results for transfection of HEK293T cells – **B-10(45%)**.

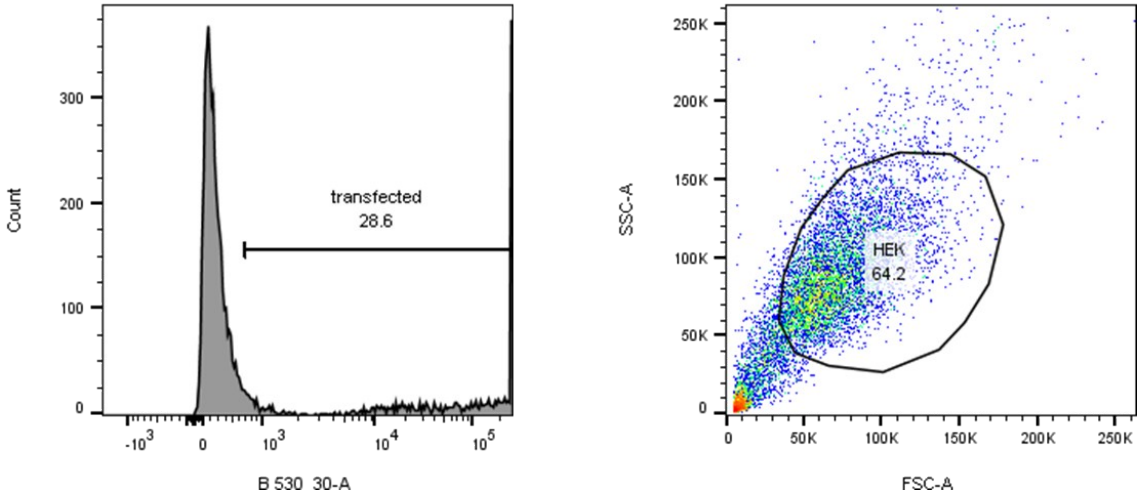

Figure S21 Flow cytometry results for transfection of HEK293T cells – **B-10(69%)**.

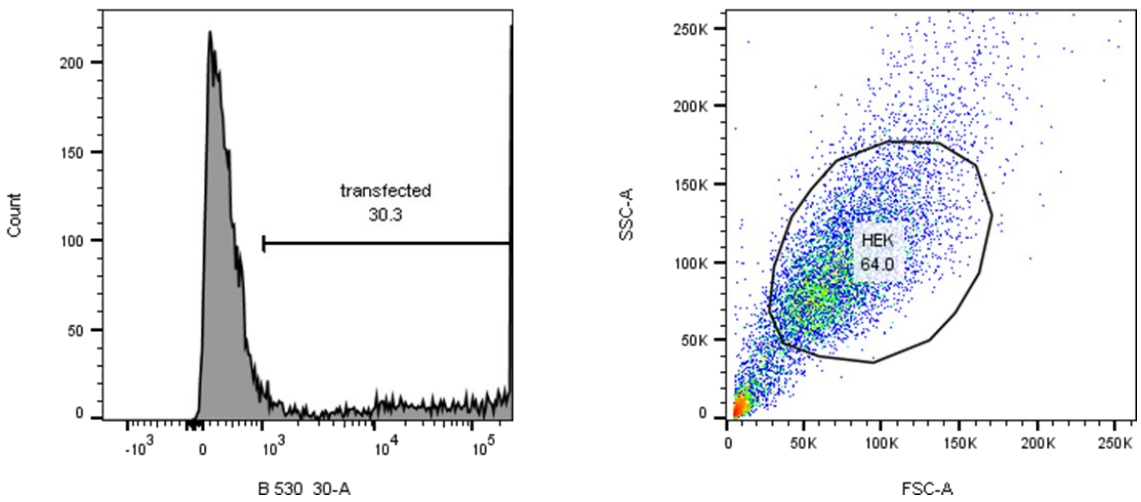

Figure S22 Flow cytometry results for transfection of HEK293T cells – **B-10(78%)**.

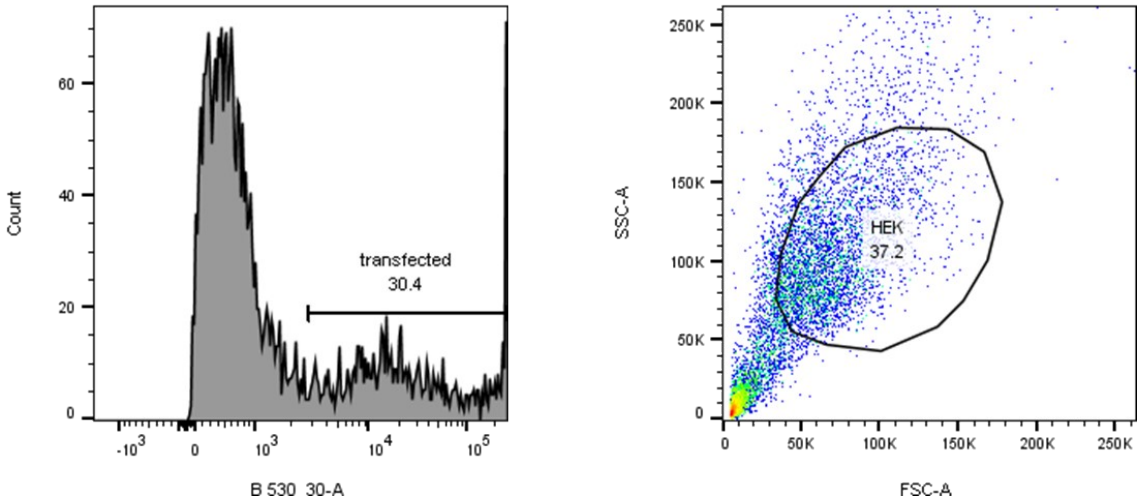

Figure S23 Flow cytometry results for transfection of HEK293T cells – **B-25(67%)**.

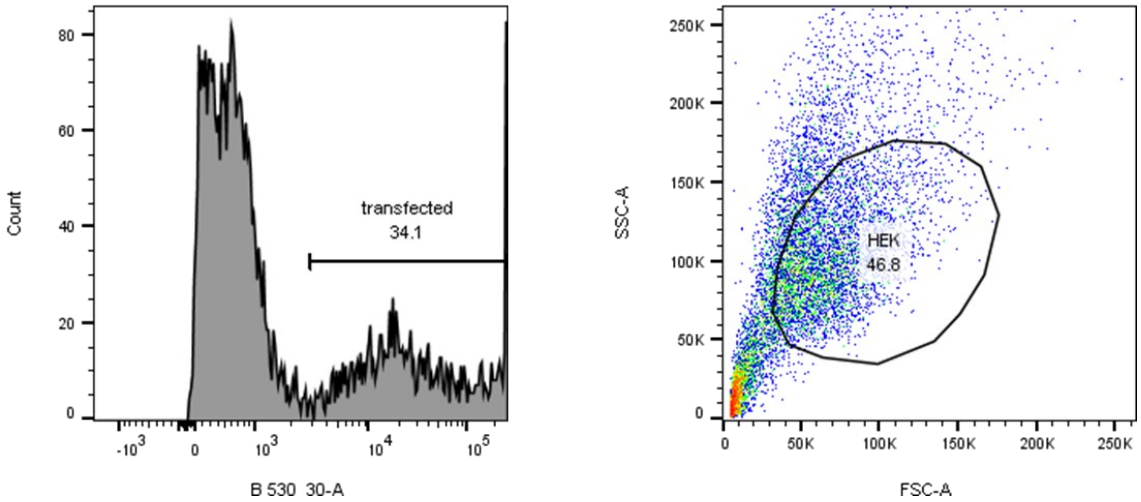

Figure S24 Flow cytometry results for transfection of HEK293T cells – **B-50(67%)**.

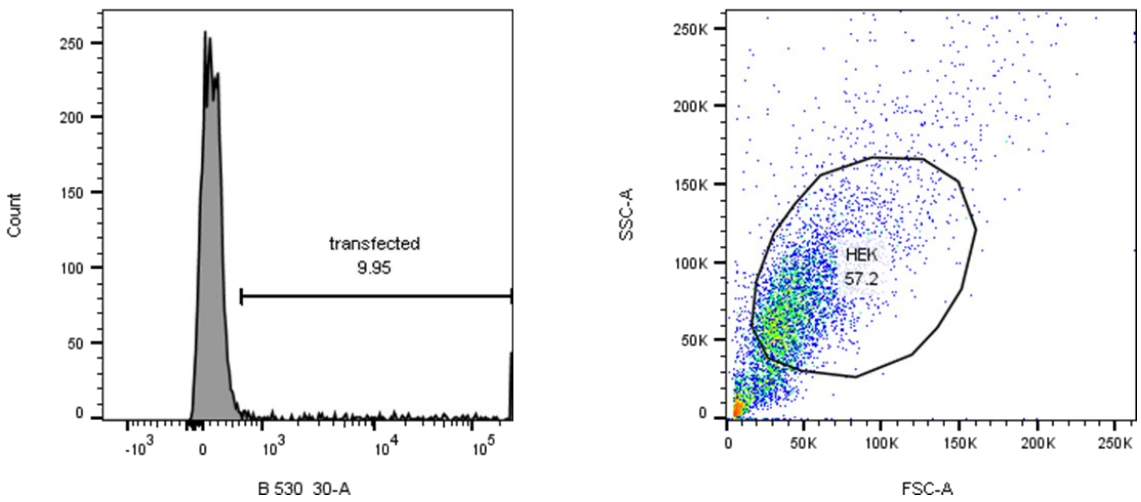

Figure S25 Flow cytometry results for transfection of HEK293T cells – **L-242(81%)**.

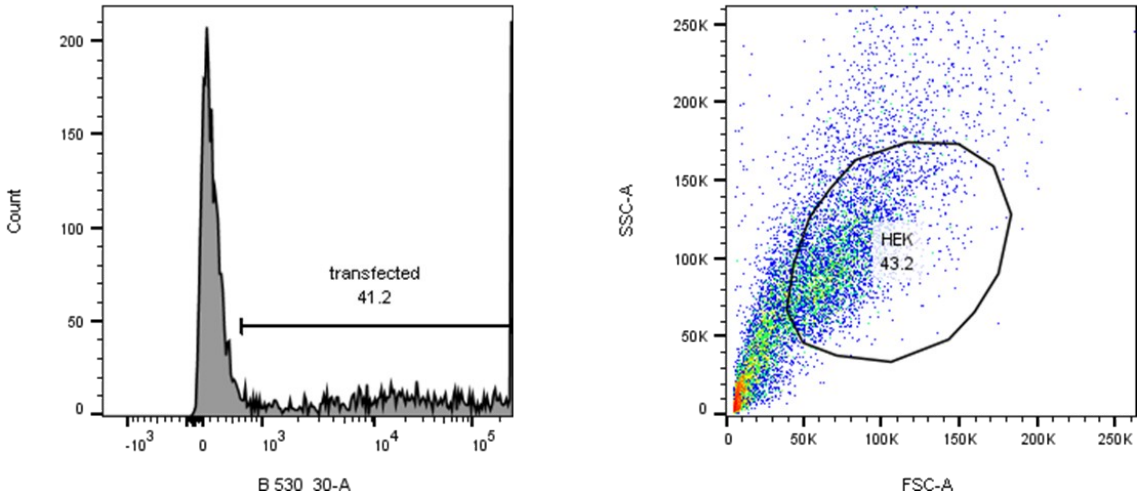

Figure S26 Flow cytometry results for transfection of HEK293T cells – **lPEI**.

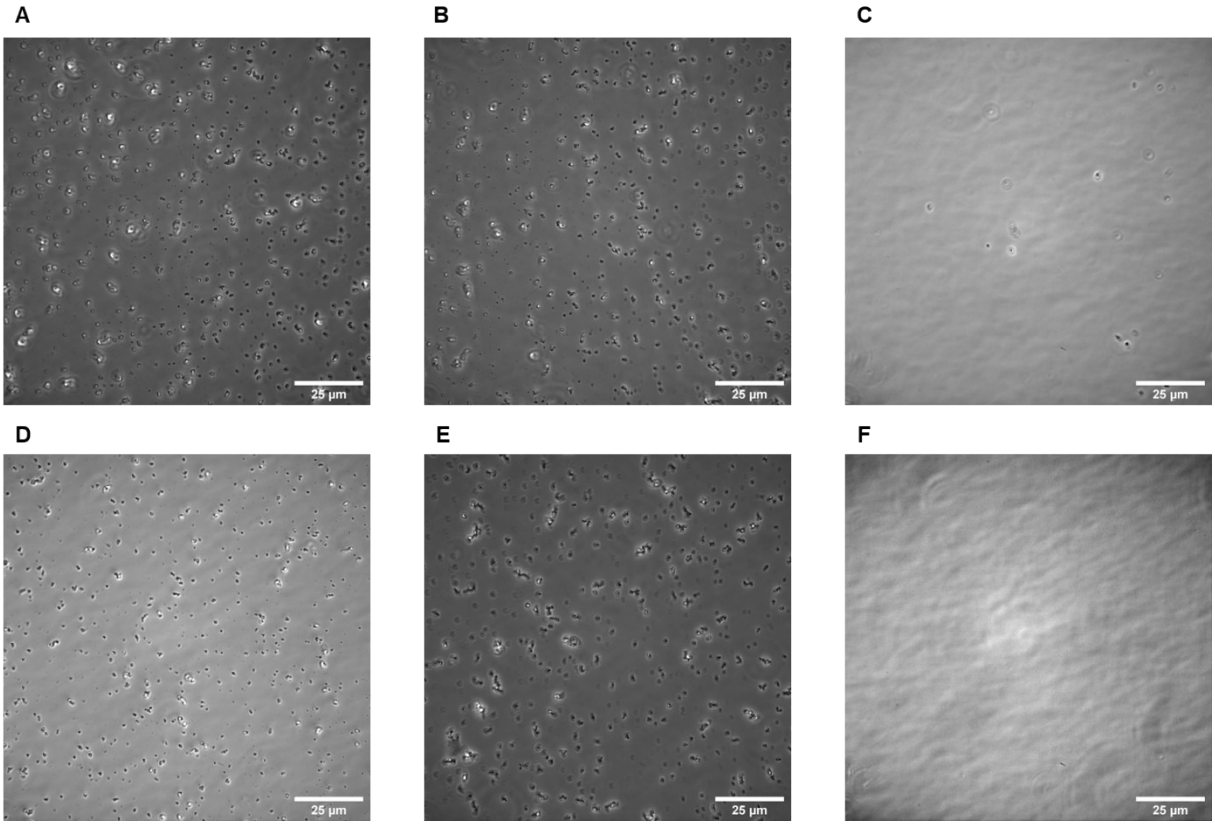

Figure S27 Incubation of **B-10(69%)** (A-C) or lPEI (D-F) polyplexes incubated with fresh Opti-MEM (A, D), cell supernatant Opti-MEM (B, E), or water (C, F). Scale bar = 25 µm. Images obtained using a Leica DMi8 microscope (100 x objective).

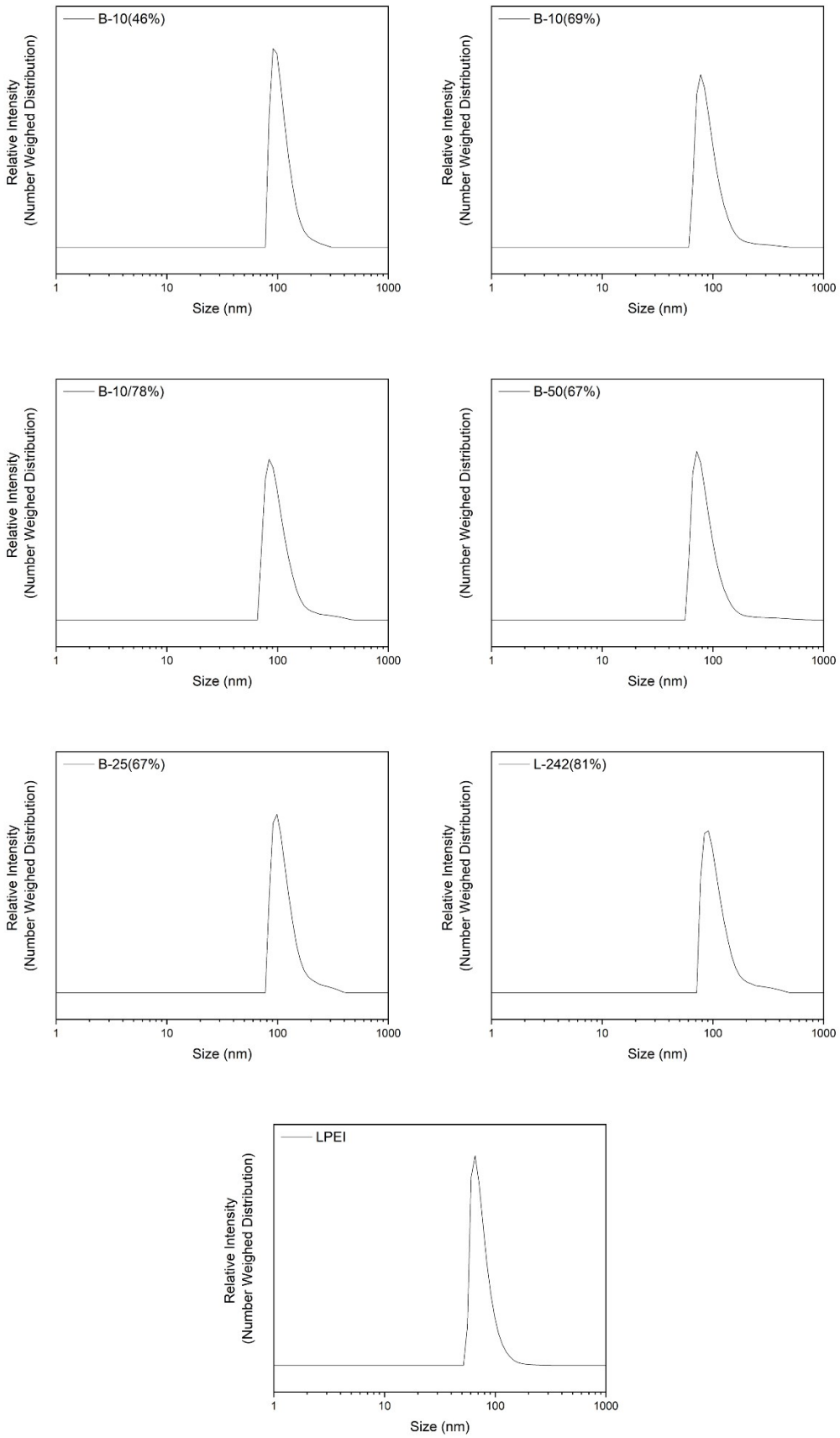

Figure S29 DLS plots (number weighed distribution) of polyplexes based on different polymers.

DLS plots