Electronic Supplementary Material (ESI) for New Journal of Chemistry.

Supporting Information

Tuning charge density and crosslinking of precise amphiphilic oligo(ethanamino)amides for efficient and biocompatible gene delivery

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Chemical structure of synthesized oligomers

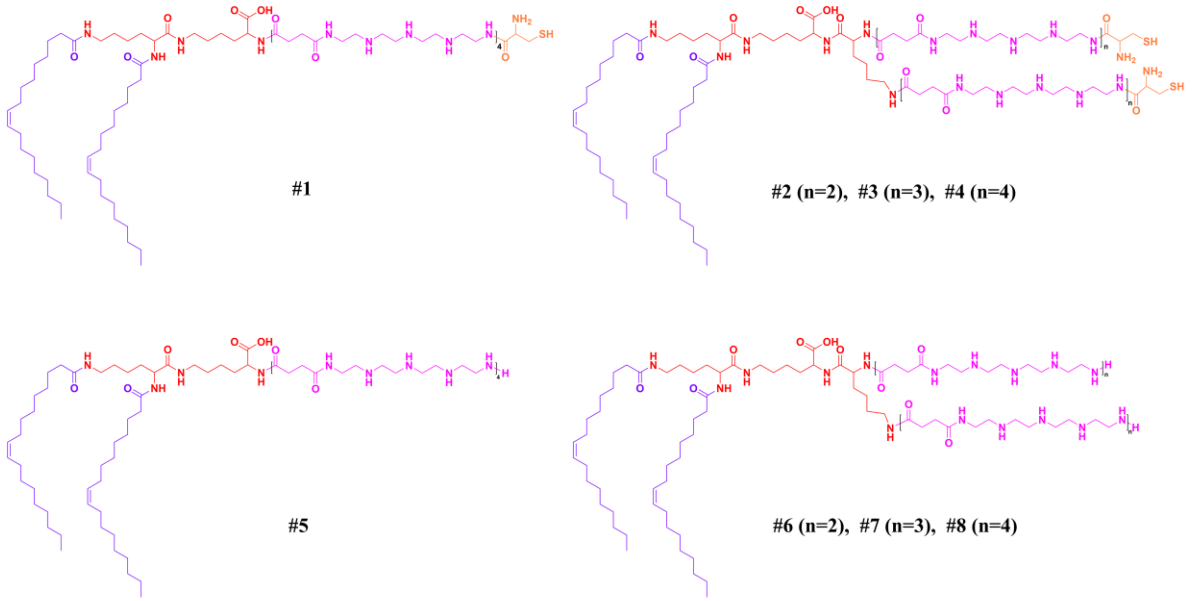

Figure S1. Chemical structure of synthesized oligomers.

Analytical data

Stp(Boc$_3$)-Fmoc:

^{1}H NMR (500 MHz, Chloroform-d) δ (ppm) = 1.46 (m, 27 H, -CH_3), 2.4-2.7 (m, 4 H, -CH_2- succinic acid), 3.30-3.50 (m, 16 H, -CH_2- tetraethylenepentamine), 4.17-4.48 (m, 3 H, -CH_2-, -CH- Fmoc), 7.3-7.8 (m, 8 H, Fmoc).

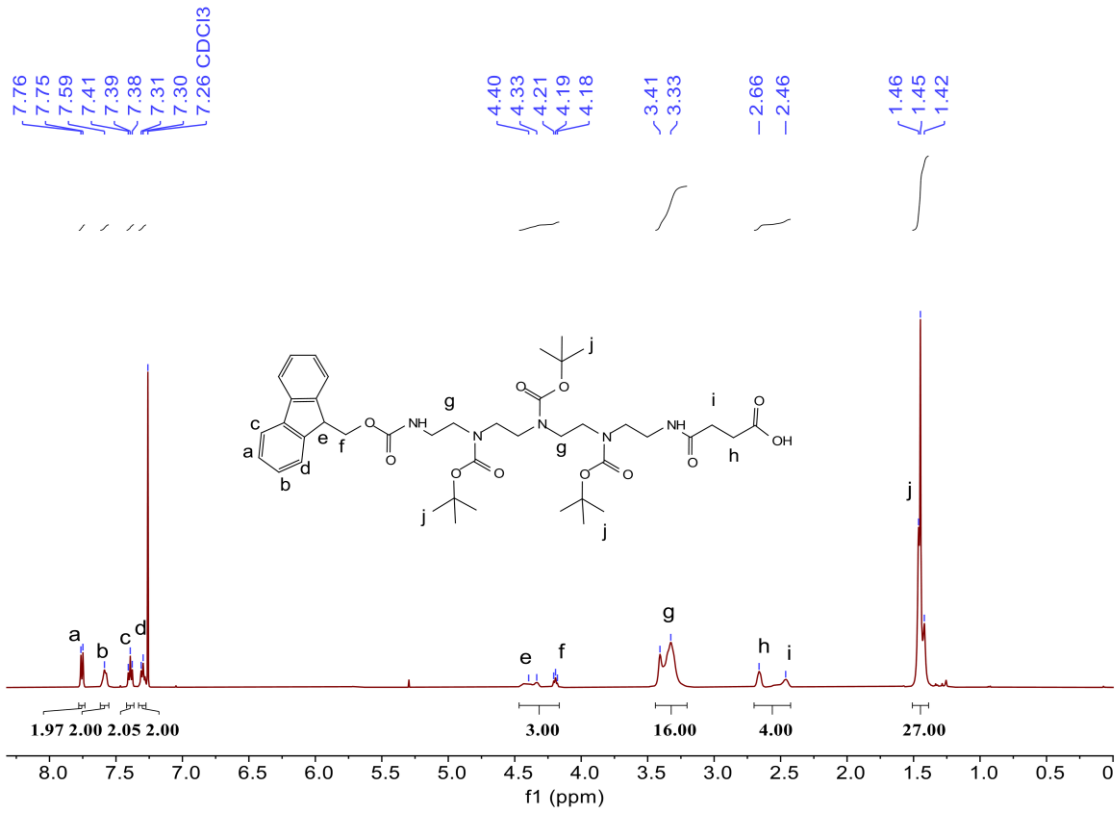

Figure S2. ^{1}H NMR spectrum of Stp(Boc$_3$)-Fmoc

Oligomer #1:

^{1}H NMR (300 MHz, D_2O) δ(ppm)= 0.7-0.94 (s, 6 H, -CH_3 Oleic acid), 1.2-1.63 (m, 70 H, -CH_2- Oleic acid, β-, γ-, δ-lysine, β-cysteine), 1.9-2.30 (m, 12 H, -CH_2- α-Oleic acid, ξ-Oleic acid), 2.5-2.7 (s, 16H, -CH_2- succinic acid), 3.12-3.25 (m, 6 H, -CH_2- ε-lysine, -SH- cysteine), 3.26-3.78 (m, 64H, -CH_2- tetraethylenepentamine), 4.22-4.35 (m, 3 H, -CH- α-lysine, α-cysteine), 5.08-5.46 (m, 4 H, -CH=CH- Oleic acid).

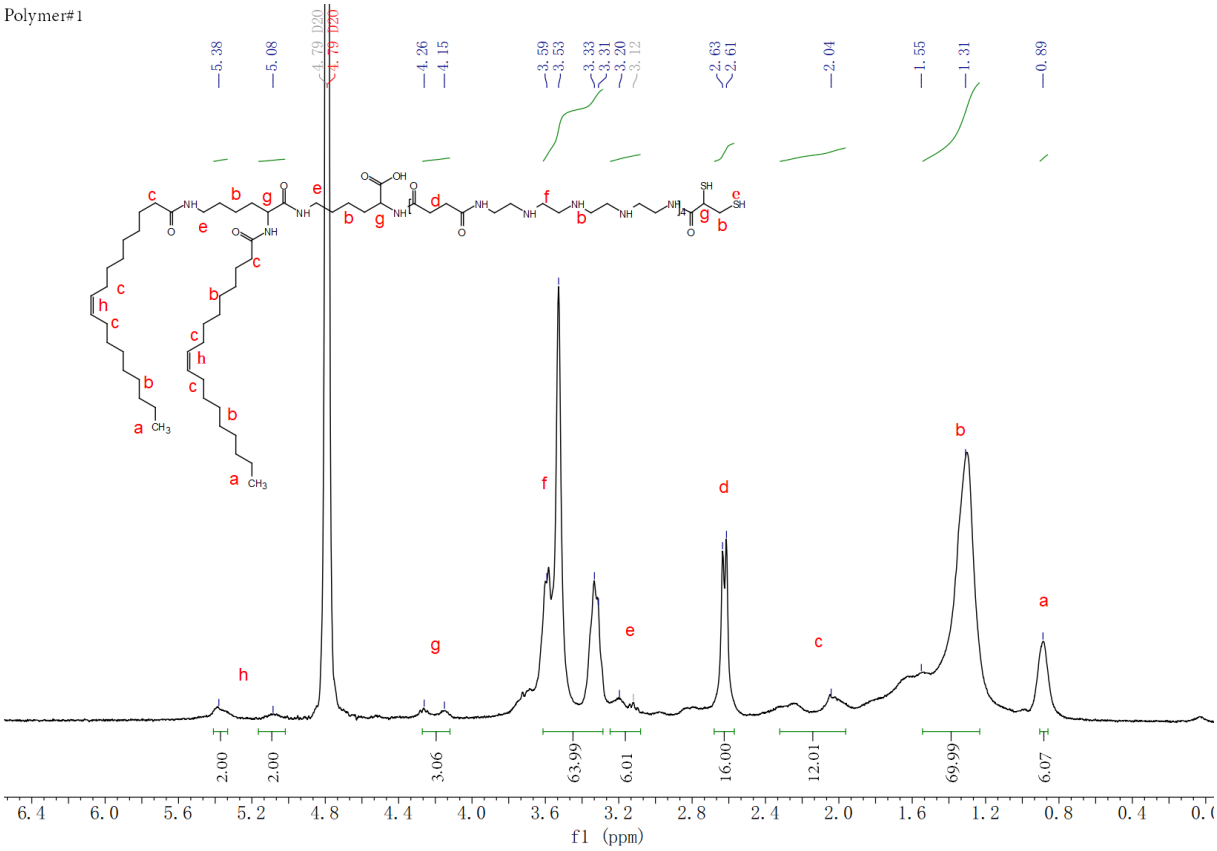

Figure S3. ^{1}H NMR spectrum of oligomer #1.

Oligomer #2:

^{1}H NMR (300 MHz, D_2O) δ(ppm)= 0.87-0.94 (s, 6 H, -CH_3 Oleic acid), 1.23-1.60 (m, 84 H, -CH_2- Oleic acid, β-, γ-, δ-lysine, β-cysteine), 2.02-2.30 (m, 12 H, -CH_2- α-Oleic acid, ξ-Oleic acid), 2.55-2.7 (s, 16 H, -CH_2- succinic acid), 3.14-3.25 (m, 8 H, -CH_2- ε-lysine, -SH- cysteine), 3.26-3.80 (m, 96 H, -CH_2- tetraethylenepentamine), 4.22-4.35 (m, 5 H, -CH- α-lysine, α-cysteine), 5.08-5.46 (m, 4 H, -CH=CH- Oleic acid).

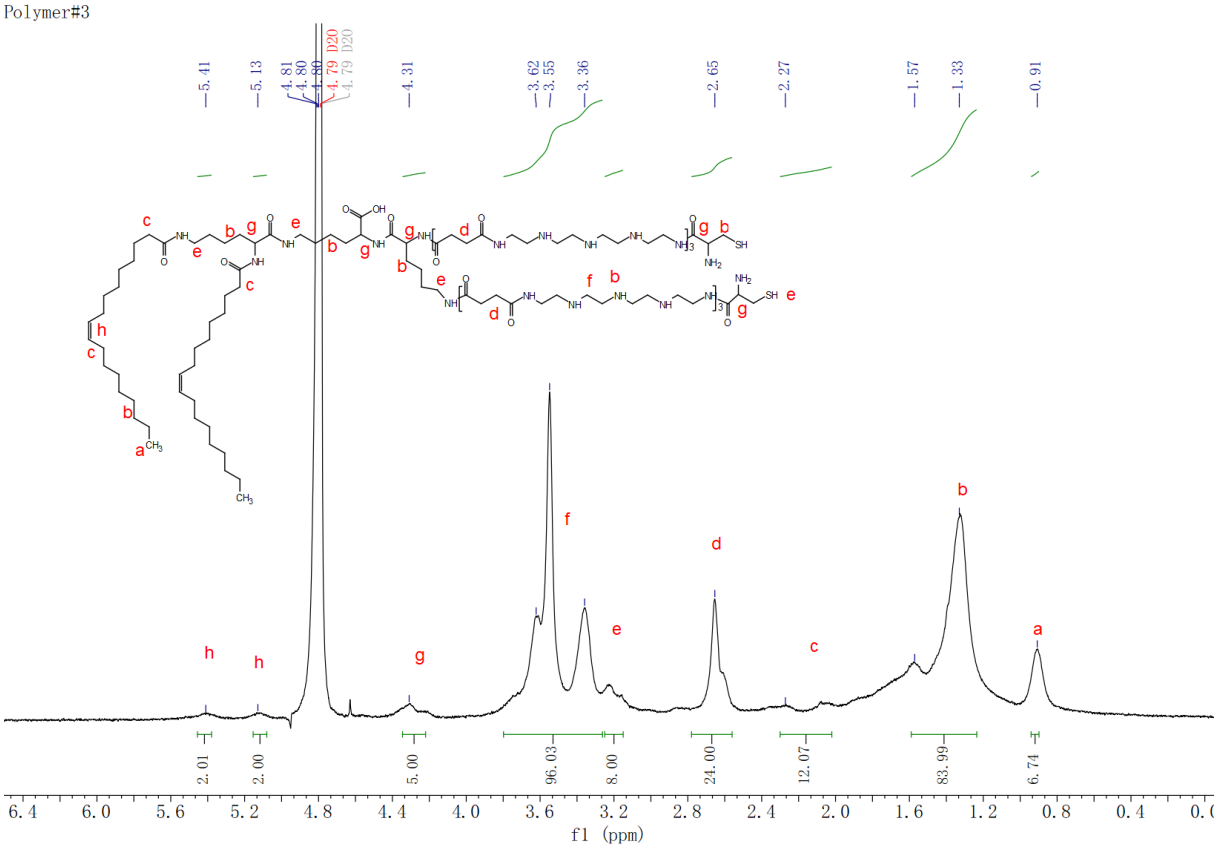

Figure S4. ^{1}H NMR spectrum of oligomer #2.

Oligomer #3:

^{1}H NMR (300 MHz, D_2O) δ(ppm)= 0.9-0.94 (s, 6 H, -CH_3 Oleic acid), 1.23-1.60 (m, 84 H, -CH_2- Oleic acid, β, γ, δ lysine, β cysteine), 2.02-2.30 (m,12 H, -CH_2- α Oleic acid, -ξ Oleic acid), 2.56-2.85 (s, 24 H, -CH_2- succinic acid), 3.14-3.25 (m, 8 H, -CH_2- ε lysine, -SH- cysteine), 3.26-3.80 (m, 96 H, -CH_2- tetraethylenepentamine), 4.22-4.35 (m, 5 H, -CH- α lysine, cysteine), 5.08-5.46 (d, 4 H, -CH=CH- Oleic acid).

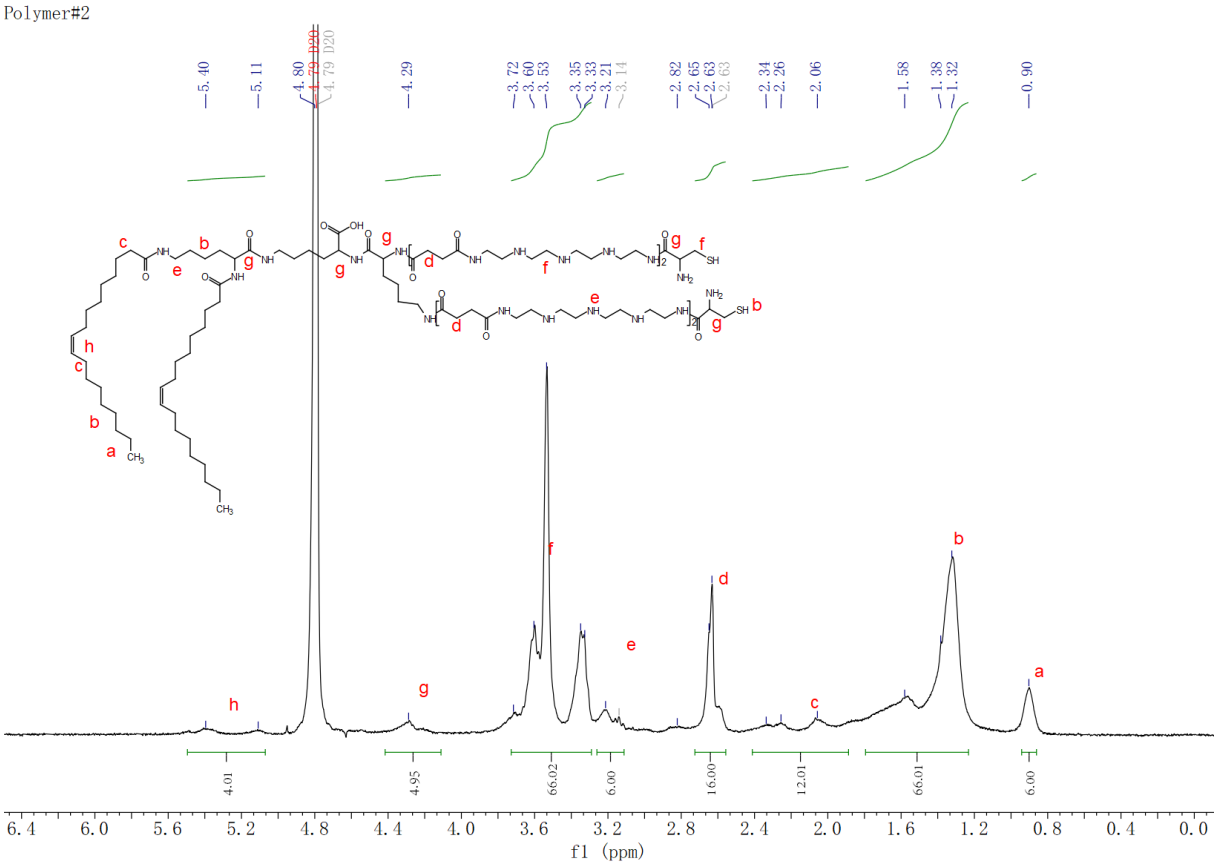

Figure S5. ^{1}H NMR spectrum of oligomer #3.

Oligomer #4:

^{1}H NMR (300 MHz, D_2O) δ(ppm) = 0.91-0.96 (s, 6 H, -CH_3 Oleic acid), 1.26-1.77(m, 90 H, -CH_2- Oleic acid, β, γ, δ lysine, β cysteine), 2.01-2.39 (m, 12 H, -CH_2- α Oleic acid, -ξ Oleic acid), 2.57-2.75 (s, 32 H, -CH_2- succinic acid), 3.14-3.26 (s, 8 H, -CH_2- ε lysine, -SH- cysteine), 3.32-3.74 (s, 128 H, -CH_2- tetraethylenepentamine), 4.22-4.37 (s, 5 H, -CH- α lysine, cysteine), 5.1-5.48 (d, 4 H, -CH=CH- Oleic acid).

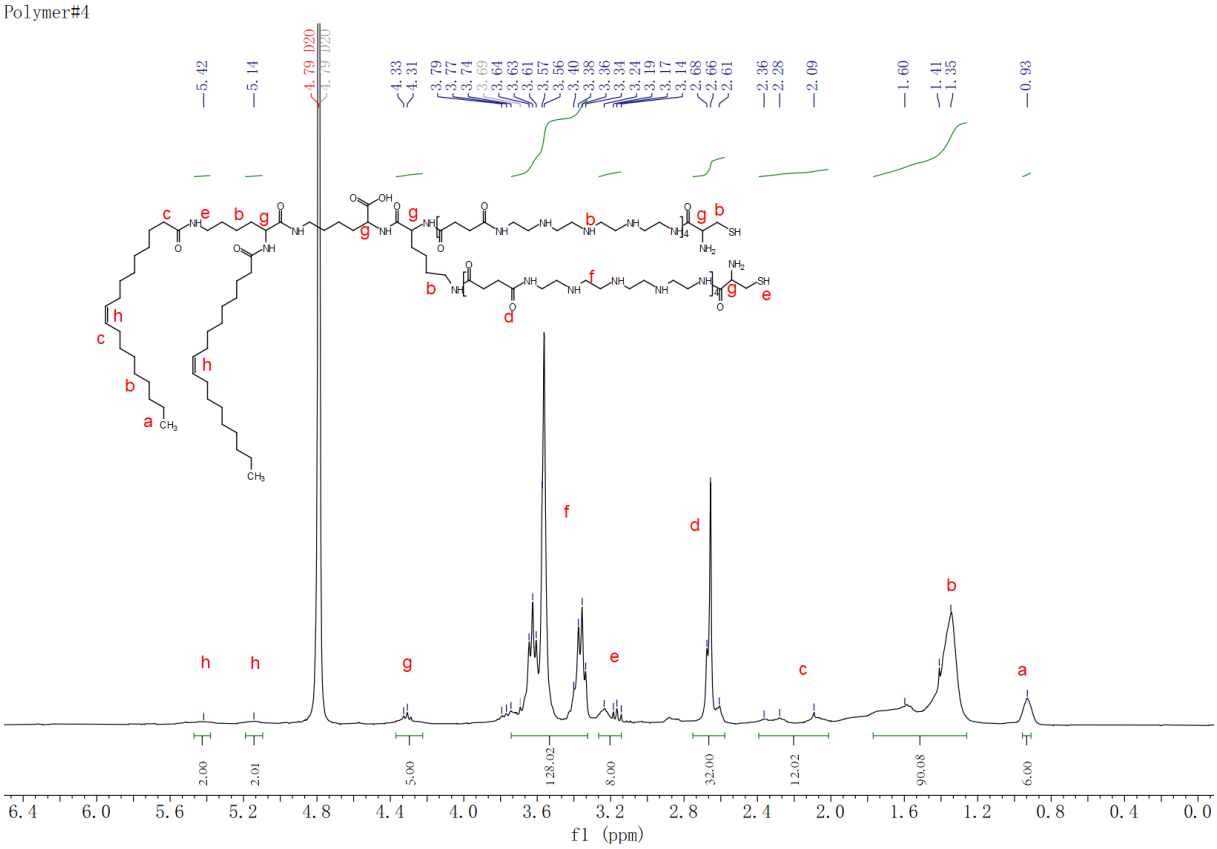

Figure S6. ^{1}H NMR spectrum of oligomer #4.

Oligomer #5:

^{1}H NMR (300 MHz, D_2O) δ(ppm)= 0.87-0.94 (s, 6 H, -CH_3 Oleic acid), 1.23-1.60 (m, 56 H, -CH_2- Oleic acid, β-, γ-, δ-lysine, β-cysteine), 2.02-2.30 (m, 12 H, -CH_2- α-Oleic acid, ξ-Oleic acid), 2.55-2.7 (s, 16 H, -CH_2- succinic acid), 3.14-3.25 (m, 4 H, -CH_2- ε-lysine), 3.26-3.80 (m, 64 H, -CH_2- tetraethylenepentamine), 4.22-4.35 (m, 2 H, -CH- α-lysine), 5.08-5.46 (m, 4 H, -CH=CH- Oleic acid).

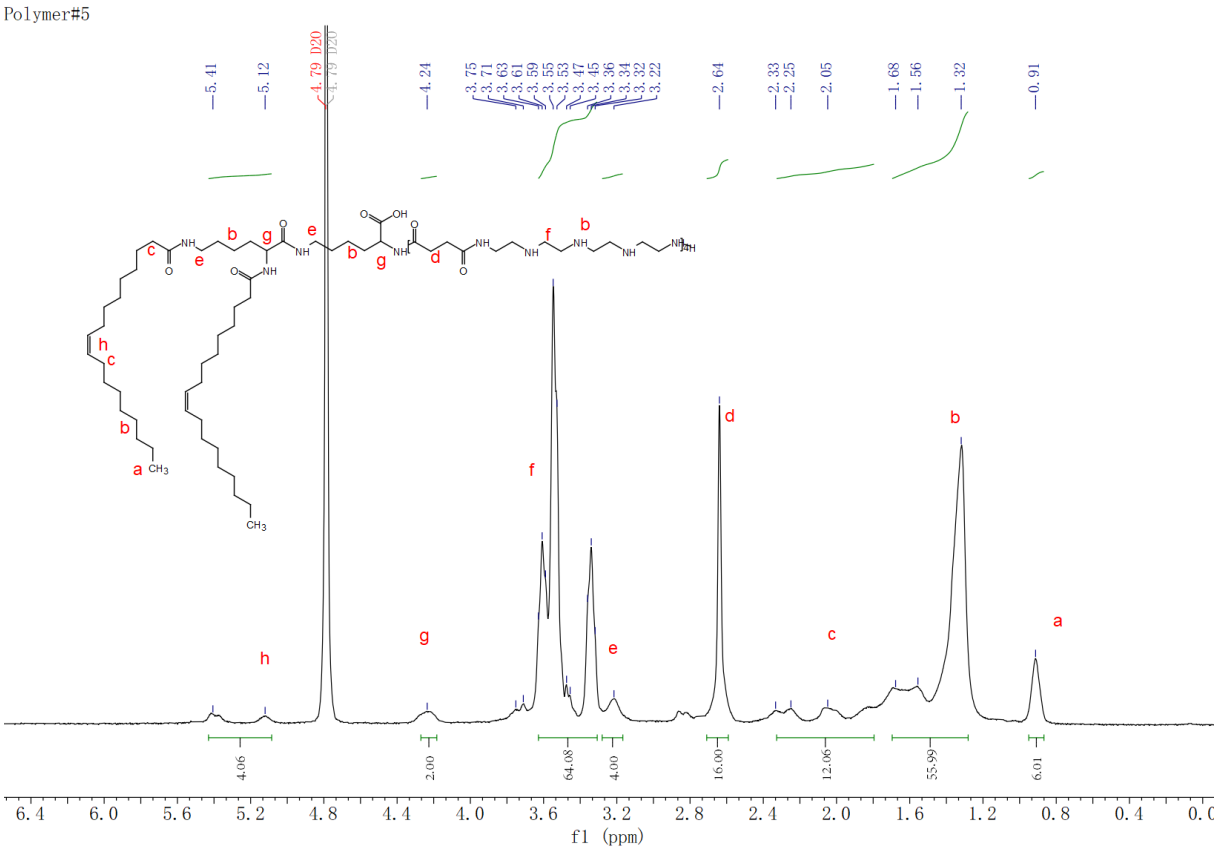

Figure S7. ^{1}H NMR spectrum of oligomer #5.

Oligomer #6:

^{1}H NMR (300 MHz, D_2O) δ(ppm)= 0.87-0.95 (s, 6 H, -CH_3 Oleic acid), 1.22-1.86 (m, 74 H, -CH_2- Oleic acid, β, γ, δ lysine), 1.89-2.48 (m, 12 H, -CH_2- α Oleic acid, -ξ Oleic acid), 2.56-2.72 (m, 16 H, -CH_2- succinic acid), 3.15-3.28 (s, 6 H, -CH_2- ε lysine), 3.30-3.65 (m, 96 H, -CH_2- tetraethylenepentamine), 4.24-4.36 (s, 3 H, -CH- α lysine), 5.08-5.43 (d, 4 H, -CH=CH- Oleic acid).

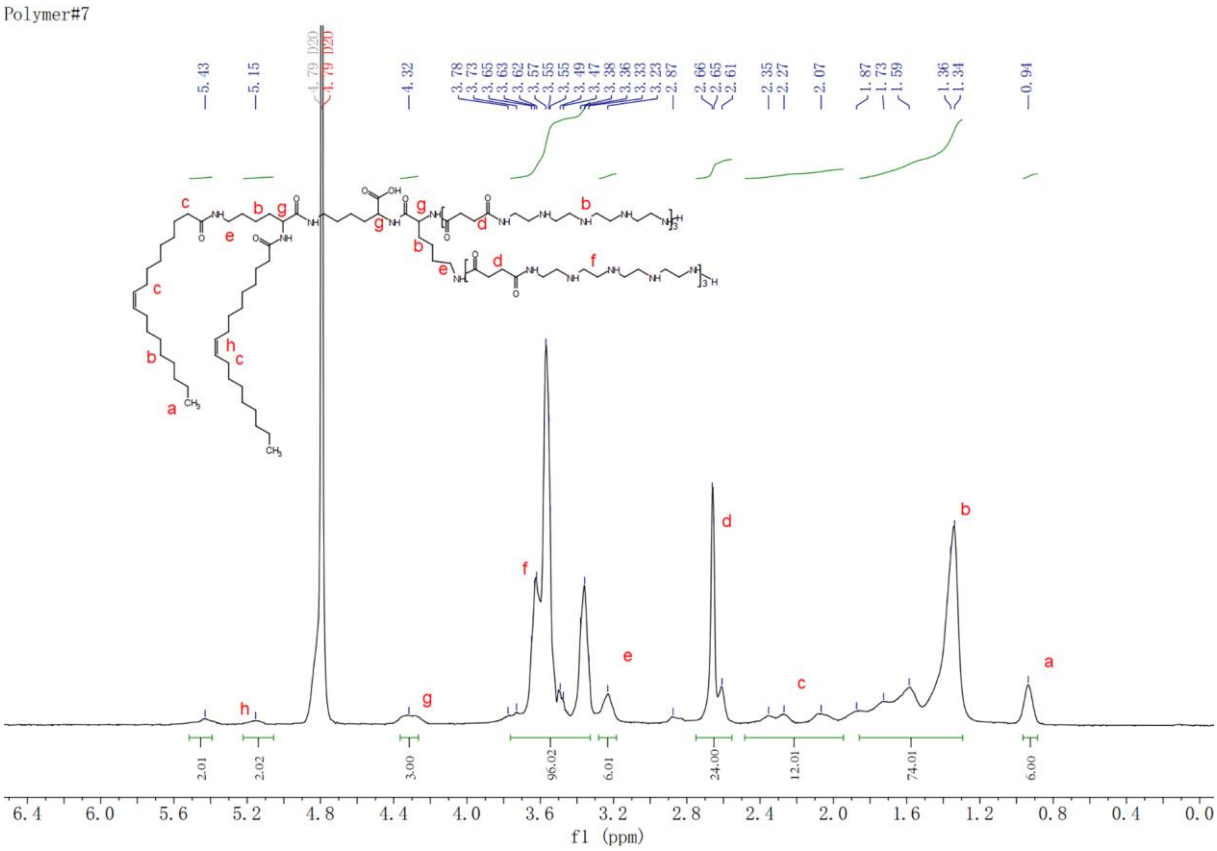

Figure S8. ^{1}H NMR spectrum of oligomer #6.

Oligomer #7:

^{1}H NMR (300 MHz, D_2O) δ(ppm) = 0.89-0.97 (s, 6 H, -CH_3 Oleic acid), 1.29-1.86 (m, 74 H, -CH_2- Oleic acid, β, γ, δ lysine), 1.95-2.48 (m, 12 H, -CH_2- α Oleic acid, -ξ Oleic acid), 2.56-2.73 (m, 24 H, -CH_2- succinic acid), 3.18-3.28 (s, 6 H -CH_2- ε lysine), 3.33-3.76 (m, 96 H, -CH_2- tetraethylenepentamine), 4.21-4.39 (s, 3 H, -CH- α lysine), 5.09-5.48 (d, 4 H, -CH=CH- Oleic acid).

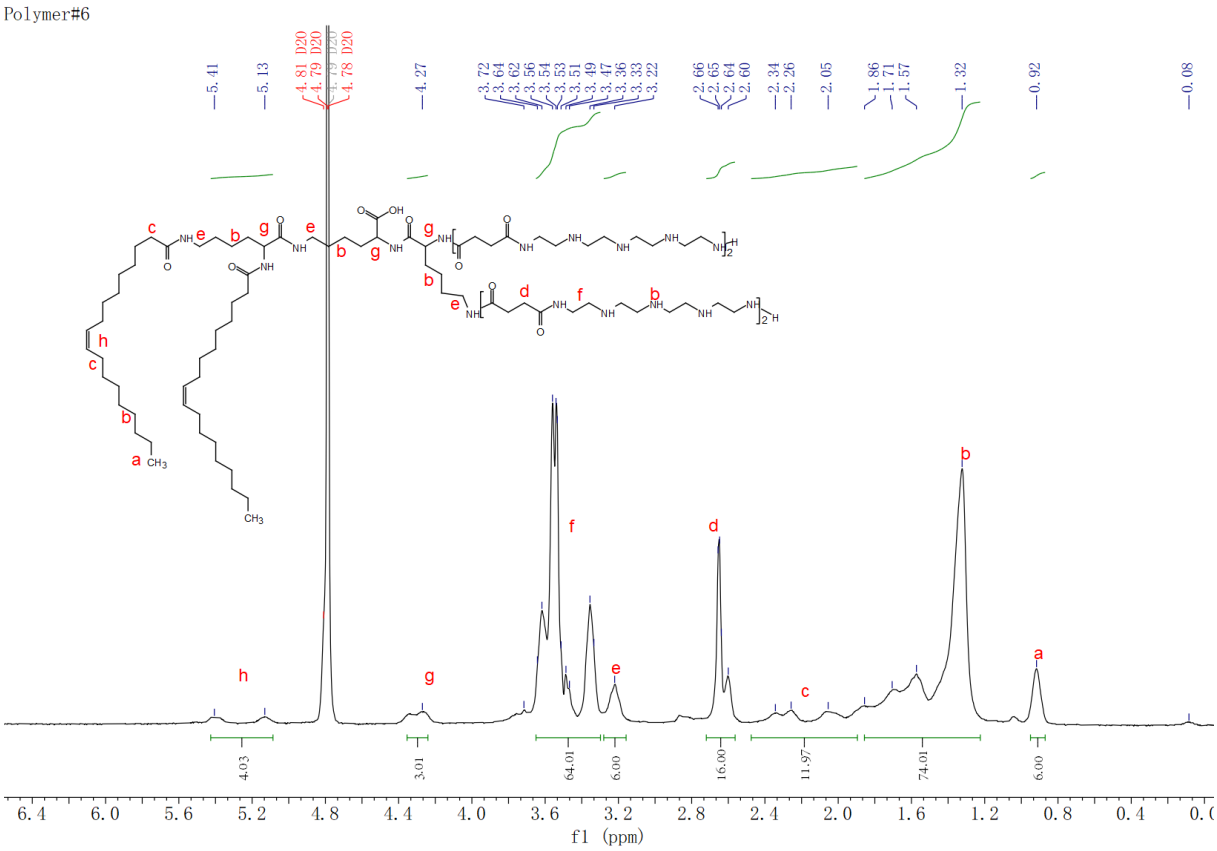

Figure S9. ^{1}H NMR spectrum of oligomer #7.

Oligomer #8:

^{1}H NMR (300 MHz, D_2O) δ(ppm) = 0.89-0.97 (s, 6 H, -CH_3 Oleic acid), 1.27-1.88 (m, 76 H, -CH_2- Oleic acid, β, γ, δ lysine), 1.94-2.49 (m, 12 H, -CH_2- α Oleic acid, -ξ Oleic acid), 2.55-2.72 (m, 32 H, -CH_2- succinic acid), 3.17-3.27 (s, 6 H -CH_2- ε lysine), 3.29-3.65 (m, 128 H, -CH_2- tetraethylenepentamine), 4.25-4.35 (s, 3 H, -CH- α lysine), 5.09-5.48 (d, 4 H, -CH=CH- Oleic acid).

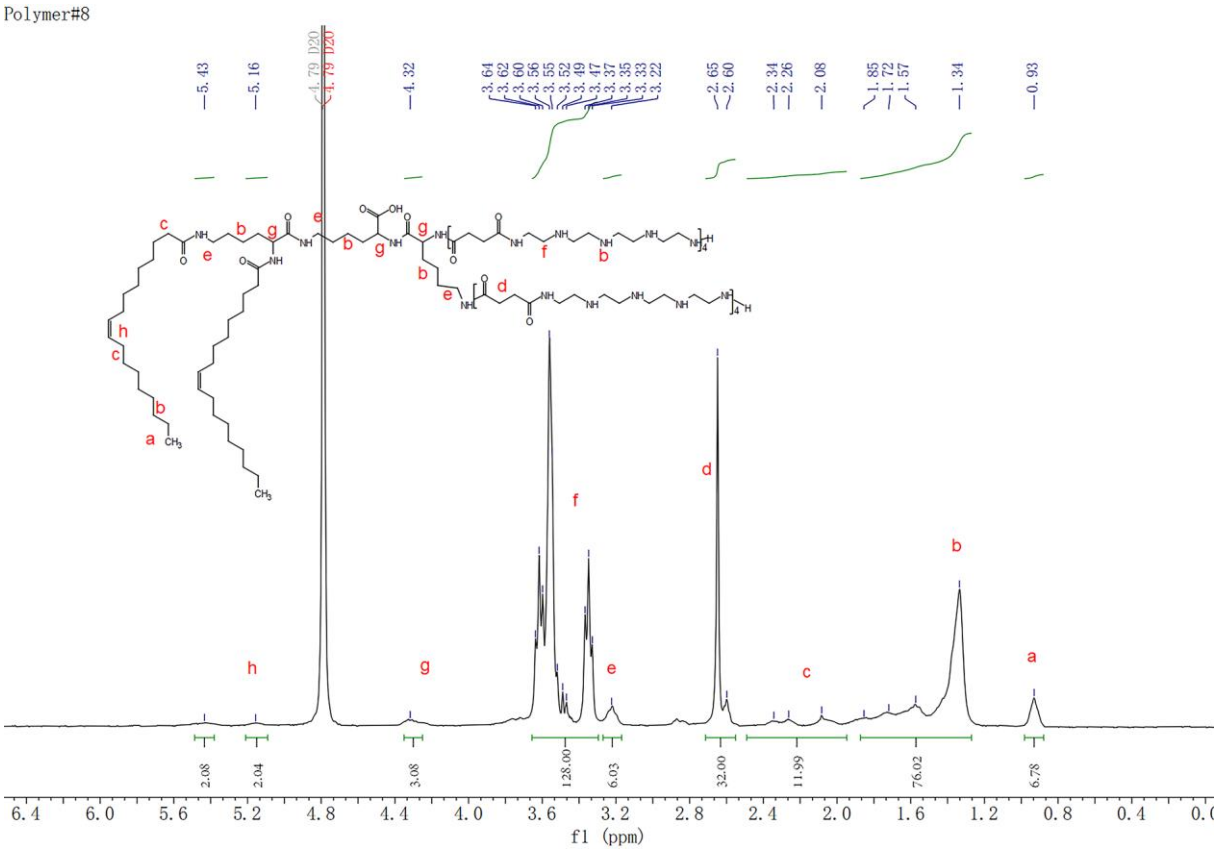

Figure S10. ^{1}H NMR spectrum of oligomer #8.

Buffer capacity of oligomers

The buffer capacity of oligomers was determined by alkalimetric titrations. The oligomer sample, containing 10 µmol protonable amines, was diluted in a total volume of 3 mL NaCl solution (50 mM) and was adjusted to pH 2 by addition of hydrochloric acid. Afterwards, a back titration with 0.05 M NaOH was performed until a pH of 11 was reached. To distinguish oligomer and solvent effects, a control titration of 50 mM NaCl solution without oligomer was performed. Volume differences (ΔV) between defined pH values were determined. Total endolysosomal buffer capacity C in the pH range between 5 and 7.4 was calculated according to the following formula[29]:

$$C_{pH5-pH7.4} = \frac{\left[\Delta V(sample)_{pH5.0-pH7.4} - \Delta V(NaCl)_{PH5.0-pH7.4}\right] \cdot 50mM}{10\mu moles} \cdot 100\%$$

Table S1. Total buffer capacity of oligomers between pH 5.0 to 7.4 measured by acidification to pH 2 and back titration with NaOH.

Oligomer number	Buffer capacity
#1	12.5%
#2	18.5%
#3	14.0%
#4	22.0%
#5	18.0%
#6	20.0%
#7	20.5%
#8	21.5%
25 KDa PEI	23.1%

Particle size and zeta potential of polyplexes

Table S2. Particle size (Z-average) and zeta potential of pDNA polyplexes (N/P 12) and siRNA polyplexes (N/P 24) in HBG buffer measured by DLS. Polyplexes were diluted 1:20 with HEPES buffer before measurement. Data were presented as mean ± SD (n=3)

Number	pDNA polyplexes			siRNA polyplexes		
	Z-average (nm)	PDI	Zeta potential (mV)	Z-average (nm)	PDI	Zeta potential (mV)
#1	117.7 ± 1.8	0.255 ± 0.019	11.9 ± 0.9	164.1 ± 1.1	0.224 ± 0.010	30.6 ± 1.0
#2	104.9 ± 1.2	0.257 ± 0.031	29.0 ± 0.3	138.0 ± 1.3	0.256 ± 0.006	31.3 ± 3.5
#3	104.2 ± 2.1	0.256 ± 0.027	25.0 ± 1.7	173.5 ± 3.0	0.323 ± 0.056	29.9 ± 1.0
#4	118.0 ± 1.4	0.246 ± 0.003	27.4 ± 0.6	171.2 ± 1.3	0.344 ± 0.091	18.7 ± 0.7
#5	108.5 ± 1.6	0.233 ± 0.024	17.8 ± 2.6	173.0 ± 2.8	0.350 ± 0.033	27.0 ± 3.1
#6	115.2 ± 0.4	0.244 ± 0.017	25.8 ± 3.4	182.8 ± 2.3	0.375 ± 0.072	33.7 ± 3.3
#7	166.6 ± 2.3	0.247 ± 0.012	33.0 ± 3.0	183.3 ± 2.7	0.341 ± 0.019	20.4 ± 1.3
#8	158.1 ± 3.5	0.270 ± 0.015	26.8 ± 3.0	175.2 ± 2.5	0.311 ± 0.008	29.2 ± 2.1

Nuclease and serum stability by gel shift assay

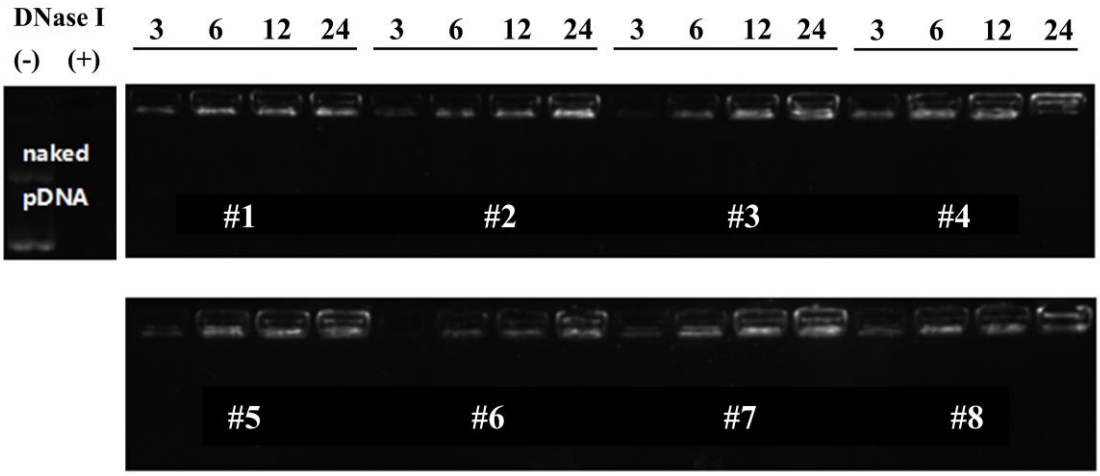

Figure S11. Gel retardation assays of oligomers/pDNA polyplexes formed at N/P 3, 6, 12, and 24 were incubated with DNase I. Naked pDNA was incubated with DNase I as the control (+), the untreated naked pDNA was the control (-). The polyplexes were incubated with DNase I (1 U/μL) and 10 μL $MgCl_2$ buffer (10×) at 37℃ for 15 min followed by reaction with 1 μL 50 mM EDTA at 65 °C for 10 min to terminate DNase I activity.

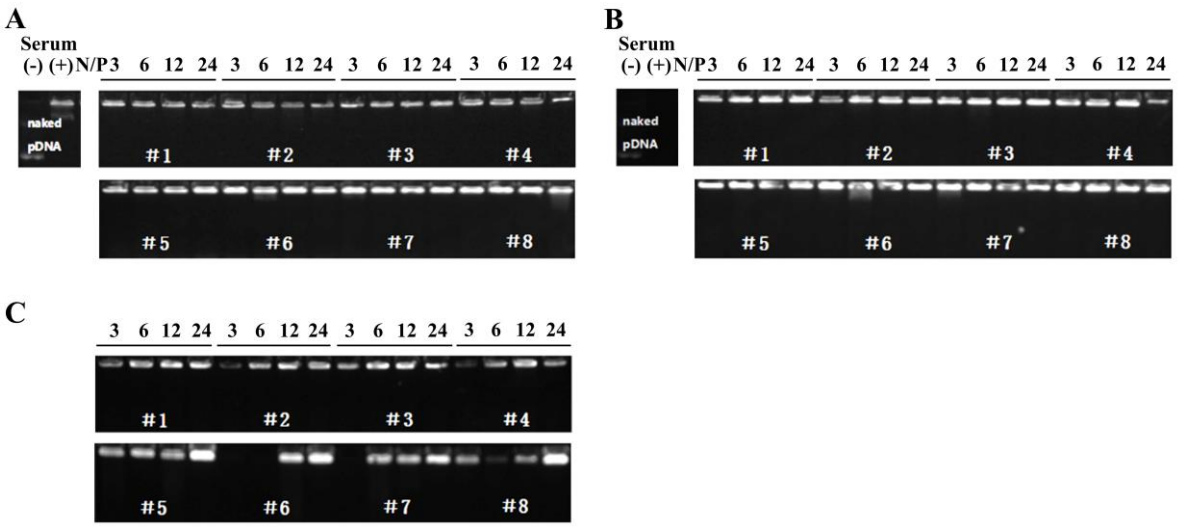

Figure S12. Gel retardation assays of stability of polyplexes in serum. Oligomers/pDNA polyplexes formed at N/P 3, 6, 12, and 24 were incubated with 50% FBS at 37℃ for certain time: (A) 0 h; (B) 12 h; (C) 24 h. Naked pDNA incubated with serum was the positive control (+), the untreated naked pDNA was the negative control (-).

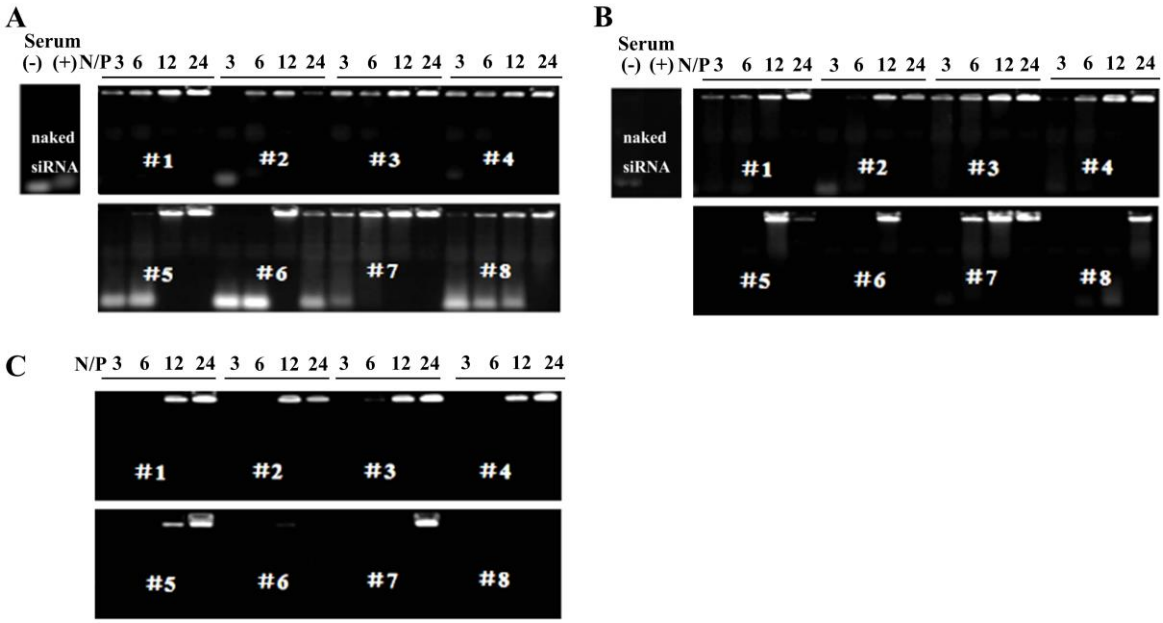

Figure S13. Gel retardation assays of stability of siRNA polyplexes in serum. Oligomers/siRNA polyplexes formed at N/P 3, 6, 12, and 24 were incubated with 50% FBS at 37°C for certain times, followed by (A) 0 h; (B) 12 h; (C) 24 h. Naked siRNA was incubated with serum as positive control (+) and untreated naked siRNA as negative control (-).

Size distribution of pDNA polyplexes after incubation with GSH

pDNA polyplexes formed by oligomer #1 or #5 at N/P 12 were assembled as described in section **2.3**. Equal volume of 20 mM glutathione solution was subjected to these polyplexes and samples was incubated at 37 °C for 2 h and analyzed by size distribution as described in section **2.5**.

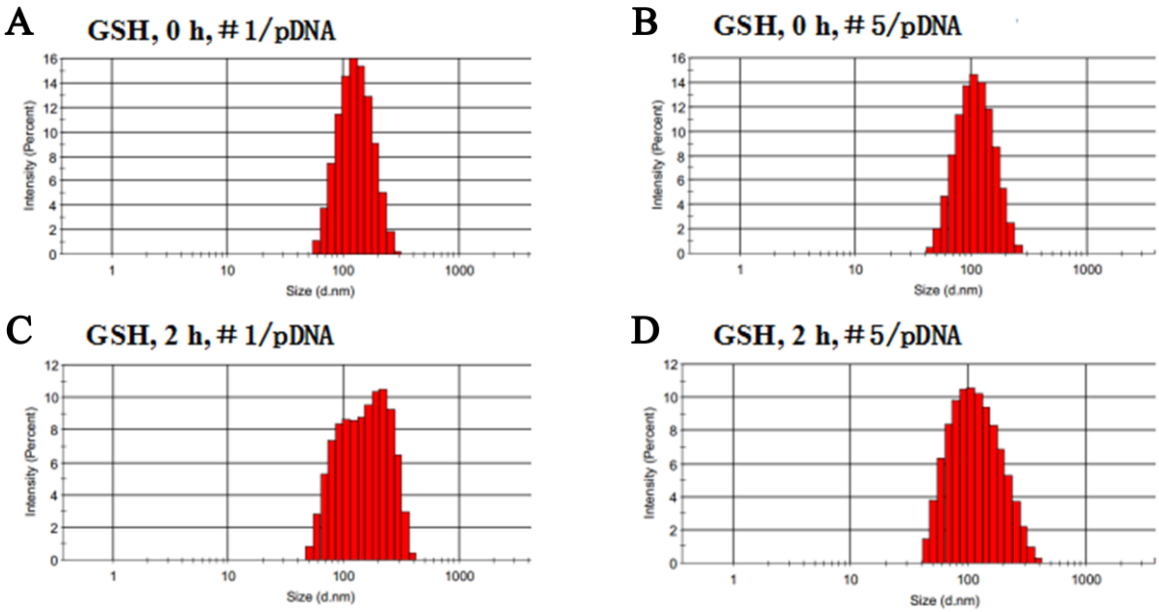

Figure S14. Size distribution of the #1/pDNA and #5/pDNA polyplexes (N/P 12) determined using DLS, after incubation with GSH (10 mM). (A) GSH, 0 h, #1/pDNA; (B) GSH, 0 h, #5/pDNA; (C) GSH, 2 h, #1/pDNA; (D) GSH, 2 h, #5/pDNA.

Transfection of PEI pDNA polyplexes with and without chloroquine

For pDNA transfection with different amount of PEI, A549 cells were seeded into 96-well plates with 6000 cells per well 24 h before pDNA transfection. Before treatment, the cell culture medium was replaced with 80 μL fresh medium containing 10% FBS and with or without chloroquine (0.1 mM). pDNA polyplexes with 25KDa PEI containing 200 ng pDNA formed at N/P 5, 10, 15 and 20 in a total volume of 20 μL HBG were added to each well and incubated at 37 °C. After 4 h incubation at 37 °C, medium was changed again by 100 μL fresh medium containing 10% FBS, and cells were further cultured for 48 h after initial transfection. Then, the cells were washed with ice-cold phosphate buffered saline for three times, followed by adding firefly luciferase reporter gene assay cell lysis buffer with 100 μL per well. The plates were stand for 30 min for complete lysis at room temperature. Afterwards, 60 μL of the lysate were taken and reacted with 100 μL of luciferase substrate before measured immediately. The luminescence was quantified using a multi-mode microplate reader (Envision 2104 Multilabel counter, Perkin Elmer, USA).

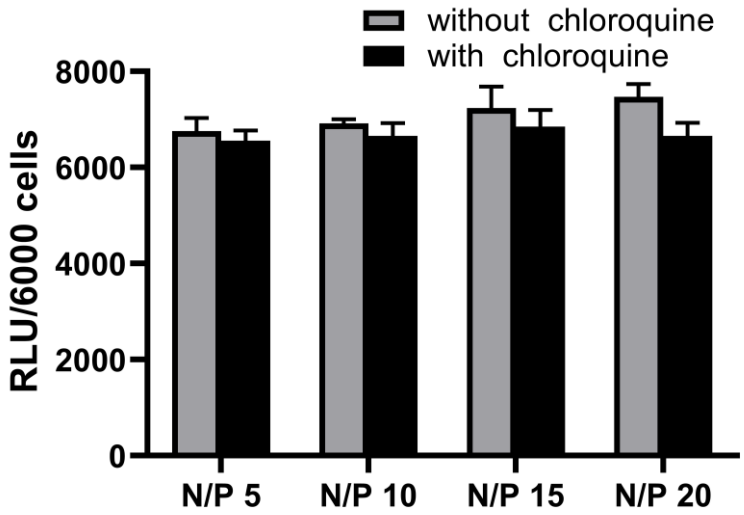

Figure S15. pDNA transfection with and without chloroquine in A549 cells with luciferase pDNA/25KDa PEI polyplexes formed at N/P 5, 10, 15 or 20 (200 ng pDNA per well). Luciferase activities at 48 h after transfection are presented in relative light units (RLU) as the mean ± SD (n = 3).

Cytotoxicity studies of pDNA polyplexes with chloroquine

The cytotoxicity of the formed pDNA polyplexes was assessed using MTT cell viability assay according to the provided protocols. A549 cells were seeded in 96-well plate at a density of 6×10^3 cells/well and incubated for additional 24 h, and then medium is changed. For pDNA polyplexes, the same procedure as the initial transfection has been applied and incubated for 4 h followed with medium change, then the cells were incubated for another 20 h. To test the cell viability, the medium was changed to 20 µL MTT solution (5 mg/mL dissolved in PBS) with 180 µL fresh medium, and cells were incubated for 4 h at 37°C. Unreacted MTT was then removed. DMSO (150 µL) was added to each well to dissolve the purple formazan product and the absorbance was measured at 570 nm.

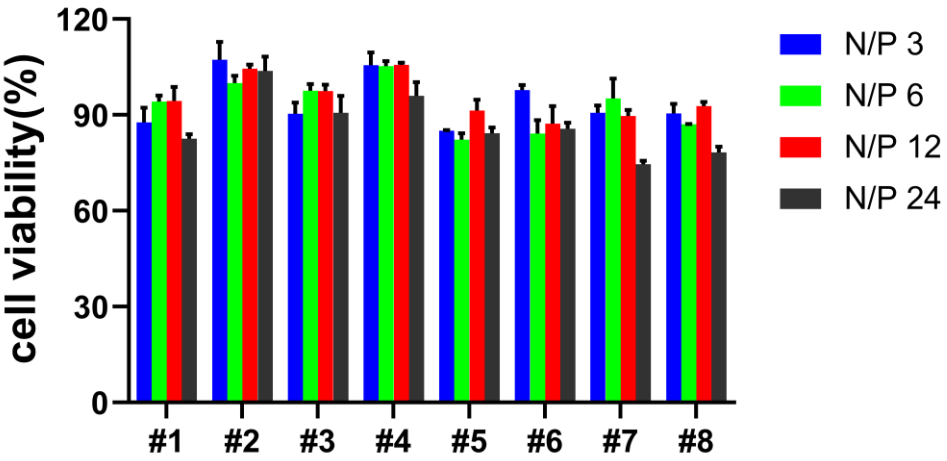

Figure S16. Cytotoxicity of oligomers/pDNA polyplexes formed at N/P 3, 6, 12, 24 in the presence of chloroquine in A549 cells. Cell viabilities are shown as mean ± SD (n = 4).

Hemolysis of oligomers

Cationic oligomers often occurred hemolysis in vivo, thus, hemolysis test was carried out to evaluated the cationic oligomers. A 2% SD rat blood cell suspension was prepared with normal saline. Blood sample were incubated with oligomer #1-8 at concentration of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5 μM for 3 h at 37°C. Normal saline blank negative control group and pure water positive control group were set. After incubation, the intact erythrocytes were separated by centrifugation at 1800 r/min for 12 min, the supernatants were transferred to 96-well plate, the hemolysis was calculated by comparing the absorbance at 570 nm of samples with negative and positive controls according to the following formula.

$$Hemolysis(\%) = \frac{A_{sample} - A_{negtive}}{A_{sample} - A_{positive}} \times 100\%$$

where A_{sample} is the absorbance of sample (mean value), $A_{negtive}$ is the absorbance of the negative control (mean value), and $A_{positive}$ is the absorbance of the positive control (mean value).

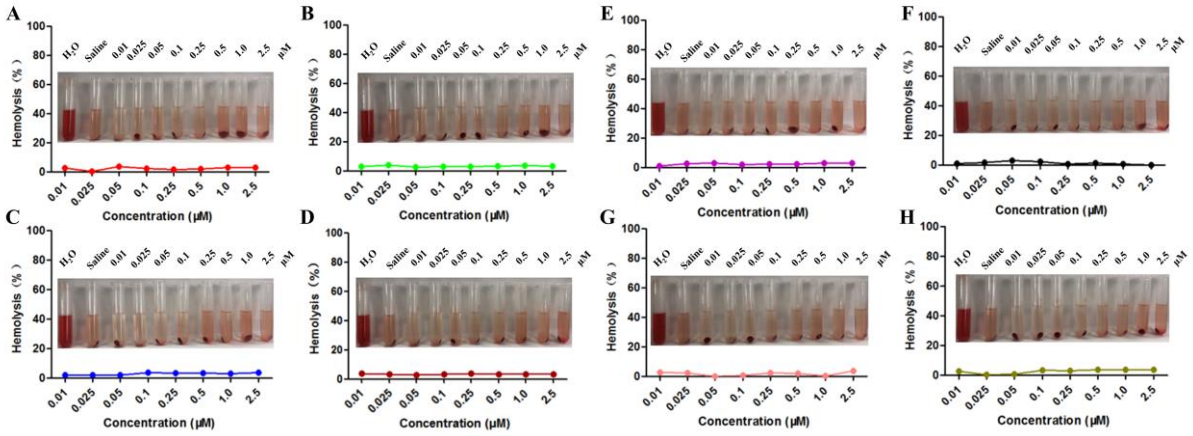

Figure S17. Hemolysis of oligomers of different concentration at pH 7.4. A, oligomer #1; B, oligomer #2; C, oligomer #3; D, oligomer #4; E, oligomer #5; F, oligomer #6; G, oligomer #7; H, oligomer #8.