Supporting Information

One-pot synthesis of dynamically crosslinked polymers for serum-resistant nucleic acid delivery

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Experimental

Materials, cells, and animals

PEI 25k, Wortmannin (WTM), methyl-β-cyclodextrin (mβCD), ellagic acid (EA), and YOYO-1 were purchased from Aladdin (Shanghai, China). jetPEI was purchased from Polyplus-transfection SA (Illkirch, France). PEI 1.8k was purchased from Alfa Aesar (Waltham, MA). Epigallocatechin gallate (EGCG) was purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). Rosemary acid (RA) was purchased from RHAWN (Shanghai, China). Nordihydroguaiaretic acid (NDGA) was purchased from Shanghai Xianding Biotechnology Co., Ltd. (Shanghai, China). Chlorpromazine (CPZ) and genistein (GNT) were obtained from TCI (Shanghai, China). 2-Acetylphenylboronic acid (2-APBA) and 4-Acetylphenylboronic acid (4-APBA) were purchased from Bide Pharmatech Ltd. (Shanghai, China). 3-Acetylphenylboronic acid (3-APBA) was purchased from Meryer (Shanghai, China). RT was obtained from Macklin, Inc (Shanghai, China). Bright-Glo™ Luciferase Assay System was purchased from Promega (Madison, WI). D-Luciferin was purchased from Glpbio (California, USA). Hoechst 33342, Lysotracker Deep Red, and 3-(4,5-dimethylthiahiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Beyotime, Co., Ltd. (Shanghai, China). Ethidium bromide (EB) was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Heparin was obtained from Solarbio (Beijing, China). sgRNA was purchased from Fenghui Biotechnology (Changsha, China). Cas9-mRNA and Cy5-mRNA were purchased from Absin Biotechnology (Shanghai, China). NHS-Cy5 and Lipofectamine™ MessengerMAX™ (Lipo-MAX) transfection reagent were purchased from Thermo Fisher Scientific (Germany). All the chemicals were used as received without further purification. All the chemicals were used as received without further purification. pLuc and pEGFP were gifted by Prof. Rui Peng's group (Soochow University).

B16F10 (murine melanoma), MDA-MB-231 (human breast cancer), HepG-2 (human hepatoellular carcinoma), HeLa (human cervical adenocarcinoma), NIH-3T3 (mouse

embryonic fibroblasts) and MSC (mouse mesenchymal stem) cells were purchased from the American Type Culture Collection (Rockville, MD, USA). HeLa-GFP cells were purchased from Fenghui Biotechnology (Changsha, China) established by infecting HeLa cells with lentivirus harboring a GFP-expressing cassette. Cells were cultured in DMEM (Gibco, Grand Island, NY) containing 10% FBS at 37 °C with 5% CO_2.

C57BL/6 mice (6-8 weeks old, 18-20 g) were purchased from Slaccas Experimental Animal Co., Ltd. (Shanghai, China) and housed in a specific pathogen-free (SPF) animal lab. Animal experiments were conducted following protocols approved by Soochow University Laboratory Animal Center, China. The approval number for the laboratory is SYXK(Su)2017-0043.

Synthesis and characterization of polymers

PEI 1.8k, 2-APBA, 3-APBA, 4-APBA, EGCG, and RA were separately dissolved in ultrapure water at 1 mg/mL. RT and NDGA were separately dissolved in 10% ethanol solution at 1 mg/mL, and EA was dissolved in DMSO at 10 mg/mL. PEI 1.8k, polyphenols, and APBA were added into ultrapure water (1 mL) at the molar ratio of 1/1/4, and the solution was stirred at room temperature for 1 h to form the ternary polymers. Without purification, the ternary polymer solution was directly used to complex with DNA to form NCs as described below.

PEI 1.8k (1 mg) and NHS-Cy5 (0.36 mg) were added into deionized water (1 mL) at the molar ratio of 1/1, and the solution was stirred at room temperature for 24 h following by dialysis against deionized water to prepare PEI $1.8k^{Cy5}$. Then, Cy5-labeled 2-PEI-RT^{Cy5} was prepared according to the same method as described for 2-PEI-RT.

To confirm the formation of imine between PEI 1.8k and APBA, the chemical structure of PEI 1.8k/2-APBA was characterized by ^1H nuclear magnetic resonance (^1H NMR) spectroscopy (Bruker AVANCE NEO 400 MHz). To confirm the formation of boronate ester between APBA and polyphenol, the fluorescence titration assay[1] was performed, wherein EGCG (0, 1.25, 2.5, 5, and 10 mM in deionized water) was added

at incremental concentrations into the mixture of PEI 1.8k/2-APBA (concentration of boronic acid at 1×10^{-3} mol/L) and ARS (1×10^{-4} mol/L) in buffer (52.1 wt% methanol in water containing 10 mmol/L KCl, 2.7 mmol/L KH_2PO_4, 2.7 mmol/L Na_2HPO_4, pH = 8.21). The fluorescence emission spectra of the ARS/PEI 1.8k/2-APBA/EGCG mixtures were recorded within the range of 535-675 nm (λ_{ex} = 495 nm). The FT-IR spectra and absorption spectra of the final ternary polymers were recorded on a Fourier transform infrared spectrometer (Vertex70 & Hyperion 1000) and a UV/Vis spectrophotometer (GENESYS 150, Thermo scientific), respectively.

Preparation and characterization of polymer/DNA NCs

DNA was dissolved in ultrapure water at 0.5 mg/mL and was mixed with ternary polymer solution at various polymer/DNA weight ratios. The mixture was vortexed for 5 s and incubated at room temperature for 30 min to allow formation of the polymer/DNA NCs. DNA condensation was qualitatively evaluated *via* electrophoresis of freshly prepared NCs on 1% agarose gel at 100 V for 40 min. To further quantify the DNA condensation level, EB exclusion assay was adopted.[2] EB was mixed with NCs at the DNA/EB weight ratio of 10/1, and the mixture was incubated at room temperature for 1 h. The fluorescence intensity was measured by spectrofluorimetry (λ_{ex} = 510 nm, λ_{em} = 590 nm). The pure EB solution and the DNA/EB solution without any polymer were used as negative and positive controls, respectively. The DNA condensation efficiency (%) was defined as follows:

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

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

The size and zeta potential of NCs were determined using a Malvern Zetasizer (Nano ZS90). The morphology of NCs was observed by transmission electron microscopy (TEM, FEI Tecnai F20, acceleration voltage = 200 kV).

Acid-triggered polymer degradation and DNA release

2-PEI-RT, 3-PEI-RT, and 4-PEI-RT were treated with HCl (pH 6.0) at room temperature for 1 h, and the absorbance at 250-500 nm before and after acid treatment was monitored using a UV/Vis spectrophotometer. To monitor the acid-triggered DNA release, freshly prepared NCs were treated with HCl (pH 6.0, 1 h) before being subjected to the heparin replacement assay as described before.[3]

In vitro gene transfection

B16F10 cells were seeded on 96-well plates at 1.5×10^4 cells per well and cultured in DMEM containing 10% FBS for 24 h. The medium was replaced with fresh DMEM containing 10%, 20%, 30%, or 50% FBS, and polymer/pLuc NCs at various polymer/pLuc weight ratios were added at 0.1 μg pLuc per well. After incubation at 37 °C for 6 h, NCs were removed and cells were further cultured in fresh medium for 18 h. Luciferase expression was quantified using the Bright-Glo™ luciferase kit, and the cellular protein level was determined using the BCA kit (Pierce). The transfection efficiency was expressed as relative luminescence unit (RLU) associated with 1 mg of cellular protein (RLU/mg protein). The transfection efficiencies of polymer/pLuc NCs were also determined in HepG-2, MDA-MB-231, NIH-3T3, and MSC cells using the same method as described for B16F10 cells.

To further determine the percentage of transfected cells, B16F10 cells were seeded on a 24-well plate at 5×10^4 cells per well and cultured for 24 h. The medium was replaced by fresh DMEM containing 10% FBS, and polymer/pEGFP NCs (w/w = 10, 0.5 μg pEGFP per well) were added. After incubation for 6 h, NCs were removed and cells were further cultured for 42 h. Cells were then observed using fluorescence microscopy.

Intracellular kinetics

YOYO-1-labeled pLuc (YOYO-1-DNA, one dye molecule per 50 bp DNA) was used to evaluate the cellular uptake level as well as intracellular distribution of NCs. Briefly, YOYO-1-DNA was allowed to form NCs with polymers as described above. B16F10 cells were seeded on 96-well plates at 1.5×10^4 cells per well and cultured

for 24 h. The medium was replaced by fresh 10% FBS-containing DMEM, into which polymer/YOYO-1-DNA NCs were added at 0.1 μg YOYO-1-DNA per well. After incubation at 37 °C for 6 h, cells were washed three times with cold PBS containing heparin (20 U/mL), and lysed with the RIPA lysis buffer (100 μL per well). YOYO-1-DNA content in the lysate was quantified by spectrofluorimetry (λ_{ex} = 485 nm, λ_{em} = 530 nm), and the protein content was determined using the BCA kit. The cell uptake level was determined as μg YOYO-1-DNA/mg protein.

To probe the internalization mechanism of the NCs, the cellular uptake study was performed at 4 °C or in the presence of various endocytic inhibitors including CPZ, GNT, mβCD, and WTM. Briefly, cells were pre-incubated with CPZ (10 μg/mL), GNT (100 μg/mL), mβCD (5 mM), or WTM (10 μg/mL) for 30 min at 37 °C followed by co-incubation with NCs and inhibitors for 6 h at 37 °C. In addition, cells were incubated with NCs at 4 °C for 6 h. The results were expressed as percentage uptake level of NCs at 37 °C for 6 h in the absence of inhibitors.

The internalization and intracellular distribution of NCs were also observed by confocal laser scanning microscopy (CLSM, ZEISS LSM 800). Briefly, B16F10, HepG-2, and MDA-MB-231 cells were seeded on coverslips in 24-well plates at 5 × 10^4 cells per well and cultured for 24 h before treatment with polymer/YOYO-1-DNA NCs (0.5 μg YOYO-1-DNA per well) for 6 h in DMEM containing 10% FBS. Cells were washed three times with cold PBS containing heparin (20 U/mL), stained with Lysotracker Deep Red (200 nM) for 40 min and Hoechst 33342 (5 μg/mL) for 20 min, and observed by CLSM.

Cytotoxicity

B16F10, HepG-2, MDA-MB-231, NIH-3T3, and MSC cells were seeded on 96-well plates (1.5 × 10^4 cells per well) and cultured for 24 h. Cells were then treated with polymer/pLuc NCs (0.1 μg DNA per well) at various polymer/DNA weight ratios in DMEM containing 10% FBS for 6 h as described above, and were further incubated for 18 h in fresh FBS-containing DMEM. Cells without NCs treatment served as the control. Cell viability was determined by the MTT assay, and the results were

presented as the percentage viability of control cells.

Preparation and characterization of 2-PEI-RT/mRNA NCs

mRNA was dissolved in ultrapure water at 0.5 mg/mL and was mixed with ternary polymer solution at various 2-PEI-RT/mRNA weight ratios. The mixture was vortexed for 5 s and incubated at room temperature for 30 min to allow formation of the 2-PEI-RT/mRNA NCs. mRNA condensation was qualitatively evaluated *via* electrophoresis of freshly prepared NCs on 1% agarose gel at 100 V for 30 min. The size and zeta potential of NCs were determined using the Malvern Zetasizer.

***In vitro* cell uptake of 2-PEI-RT/mRNA NCs**

HeLa cells were seeded on coverslips in 24-well plates at 5×10^4 cells per well and cultured for 24 h before treatment with free Cy5-mRNA, PEI 25k/Cy5-mRNA NCs, or 2-PEI-RT/Cy5-mRNA NCs (0.5 μg Cy5-mRNA per well) for 6 h in DMEM containing 10% FBS. Cells were washed three times with cold PBS containing heparin (20 U/mL), stained with Hoechst 33342 (5 μg/mL) for 20 min, and observed by CLSM. To quantify the cellular uptake level, cells were seeded on 24-well plates at 5×10^4 per well and cultured for 24 h. Cells were then incubated with free Cy5-mRNA, PEI 25k/Cy5-mRNA NCs, or 2-PEI-RT/Cy5-mRNA NCs (0.5 μg Cy5-mRNA per well) for 6 h. After being washed with PBS containing heparin (20 U/mL) for three times, cells were subjected to flow cytometric analysis (BD Biosciences C6-Plus). The cellular uptake level was represented by mean fluorescence intensity per cell.

***In vitro* gene editing efficiency**

2-PEI-RT, Cas9-mRNA, and GFP-targeting sgRNA (Table S1) were mixed at the weight ratio of 45/1/0.5, and incubated at 37 °C for 30 min to allow the formation of 2-PEI-RT/Cas9-mRNA/$sgRNA_{(GFP)}$ NCs. HeLa-GFP cells were seeded on glass-bottomed cell culture dish (d = 15 mm) at 1×10^5 cells per well and cultured for 24 h. Cells were then incubated with 2-PEI-RT/Cas9-mRNA/$sgRNA_{(GFP)}$ NCs for 24

h in fresh DMEM containing 10% FBS at 1 μg mRNA and 0.5 μg $sgRNA_{(GFP)}$ per well. Then, cells were incubated in fresh DMEM containing 10% FBS for another 48 h, stained with Hoechst 33342 (5 μg/mL) for 20 min, and observed by confocal laser scanning microscopy (CLSM). The fluorescence intensity was quantified using the Image J software. The mixture of Cas9-mRNA and $sgRNA_{(GFP)}$ was used as the negative control, and the commercial reagent Lipo-MAX was used as the positive control.

2-PEI-RT/Cas9-mRNA/$sgRNA_{(PLK1)}$ NCs were prepared from Cas9-mRNA and PLK1-targeting sgRNA ($sgRNA_{(PLK1)}$, Table S1) using the same method as described above. HeLa cells were seeded on 6-well plates (1×10^5 cells per well) and incubated for 24 h. Cells were incubated with 2-PEI-RT/Cas9-mRNA/$sgRNA_{(PLK1)}$ NCs (2 μg mRNA and 1 μg $sgRNA_{(PLK1)}$ per well) for 24 h at 37 ºC in fresh DMEM containing 10% FBS followed by incubation in fresh 10% FBS-containing DMEM for another 48 h. The genomic DNA was then isolated using the Cell/Tissue Genomic DNA Extraction Kit (GENERAY BIOTECH, China), and the gene editing efficiency of 2-PEI-RT/Cas9-mRNA/$sgRNA_{(PLK1)}$ NCs was determined using the next-generation sequencing (NGS) method performed by Tsingke Biotechnology Co., Ltd. (Beijing, China). In a parallel study, total RNA was isolated from HeLa cells using the Trizol reagent (Biosharp). The PLK1 mRNA level was determined by real-time PCR with specific primers (Table S2). Also, the PLK1 protein level was determined by Western blot using the anti-PLK1 rabbit monoclonal primary antibody (1:1000 dilution) and HRP-conjugated goat anti-rabbit IgG secondary antibody (1:10000 dilution). For the cell viability analysis, HeLa cells were seeded on 96-well plates at 1×10^4 cells per well and cultured for 24 h. After replacement with 10% FBS-containing DMEM (90 μL), 2-PEI-RT/Cas9-mRNA/$sgRNA_{(PLK1)}$ NCs or free Cas9-mRNA/$sgRNA_{(PLK1)}$ (10 μL, final concentrations of 0.2, 0.5, 0.8, 1, 2 μg Cas9-mRNA/mL) was added to each well and incubated with cells for 24 h. Cells were washed three times with cold PBS and incubated in 10% FBS-containing DMEM for another 48 h. Then, cell viability was determined by the MTT assay.

Evaluation of mitochondrial membrane potential

The mitochondrial membrane potential (ΔΨm) was determined using the mitochondrial dye JC-1 (Beyotime, Shanghai, China). B16F10 cells were seeded on coverslips in 24-well plate at 5×10^4 cells per well and cultured for 24 h. 2-PEI-RT/pLuc NCs, 3-PEI-RT/pLuc NCs, or 4-PEI-RT/pLuc NCs were added at 0.5 μg DNA per well and incubated with cells for 6 h followed by incubation in fresh medium for additional 18 h. Cells were then stained with JC-1 (500 μL) for 30 min and Hoechst 33342 (5 μg/mL) for 20 min, and observed by CLSM.

Hemolysis assay

Hemolysis ratios of NCs were deterined at the polymer concentration of 200 μg/mL. Briefly, fresh heparinized mouse blood was washed three times with PBS and then diluted to 10% (v/v) with PBS. Then, 2-PEI-RT/pLuc NCs, 3-PEI-RT/pLuc NCs, or 4-PEI-RT/pLuc NCs in PBS (20 μL) were added to diluted blood (180 μL). PBS (20 μL) was added as the negative control, and PBS containing 1% Triton X-100 (20 μL) was added as the positive control. After incubation at 37 ºC for 1 h, the mixture was centrifuged at 5000 rpm for 5 min. An aliquot of 100 μL supernatant was transferred to a 96-well plate and the absorbance at 350 nm was measured by the microplate reader. The hemolysis ratio was calculated as $[A_{sample}\text{-}A_{negative})/(A_{positive}\text{-}A_{negative})] \times 100\%$, where A_{sample}, $A_{negative}$, and $A_{positive}$ are the OD values of the supernatants from NCs, negative control, and positive control, respectively.

Biodistribution and *in vivo* transfection

For the *in vivo* biodistribution study, C57BL/6 mice were subcutaneously injected with B16F10 cells (5×10^6) in the right flank. When the tumor volume reached 200 mm^3, 2-PEI-RT^{Cy5}/pLuc NCs (10 mg 2-PEI-RT^{Cy5}/kg, 1 mg pLuc/kg) were *i.v.* injected. Mice were sacrificed at 6 h post injection. The major organs (heart, liver, spleen, lung, and kidney) and tumor tissues were harvested and imaged (λ_{ex} = 633 nm, λ_{em} = 678 nm). For the *in vivo* transfection study, B16F10 tumor-bearing C57BL/6 mice (~100 mm^3) were *i.v.* injected with PBS, jetPEI/pLuc NCs, or 2-PEI-RT/pLuc

NCs (10 mg 2-PEI-RT/kg, 1 mg pLuc/kg). At 48 h post injection, mice were anesthetized and intraperitoneally injected with D-luciferin solution (100 μL, 15 mg/mL). After 10 min, bioluminescence imaging was conducted on a Maestro *in vivo* optical imaging system (Cambridge Research and Instrumentation, Inc.). In a parallel study, mice were sacrificed at 48 h post *i.v.* injection. The tumor tissues and major organs including heart, liver, spleen, lung, and kidney were harvested, washed three times with PBS, and homogenized with the passive lysis buffer containing protease inhibitor cocktail. The mixture was frozen in liquid nitrogen and thawed in 37 °C water bath for three cycles. The mixture was then centrifuged at 4 °C and 12000 rpm for 10 min. The supernatant was subjected to quantify the luciferase expression level using the Bright-Glo^{TM} luciferase kit and protein level using the BCA kit, respectively. Transfection efficiencies were represented as RLU/mg protein. On day 7, mice were sacrificed, and major organs were harvested, fixed with formalin, embedded in paraffin, sectioned, and stained with haematoxylin & eosin (H&E) before histological examination under an optical microscope.

Biocompatibility study

PBS or 2-PEI-RT/pLuc NCs were *i.v.* injected to C57BL/6 mice at 10 mg 2-PEI-RT/kg. At 24 h post injection, blood was collected. Hematological assessment was performed on a Cobas501 automatic hematology analyzer (Roche, USA) including white blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), platelet count (PLT), plateletocrit (PCT), mean platelet volume (MPV), platelet distribution width (PDW), and basophil ratio (BASOP).

Statistical analysis

Data were presented as mean ± standard deviation. Statistical analysis was performed using Student's *t*-test. Differences were considered to be significant at $*p < 0.05$ and very significant at $**p < 0.01$, $***p < 0.001$.

Reference

1. Z. Y. Zhao, X. Liu, M. Y. Hou, R. X. Zhou, F. Wu, J. Yan, W. Li, Y. J. Zheng, Q. M. Zhong, Y. B. Chen and L. C. Yin, *Adv. Mater.*, 2022, **34**, 2110560.
2. C. L. Ge, J. D. Yang, S. Z. Duan, Y. Liu, F. H. Meng and L. C. Yin, *Nano Lett.*, 2020, **20**, 1738-1746.
3. L. P. Zhu, J. M. Simpson, X. Xu, H. He, D. H. Zhang and L. C. Yin, *ACS Appl. Mater. Interfaces*, 2017, **9**, 23476-23486.

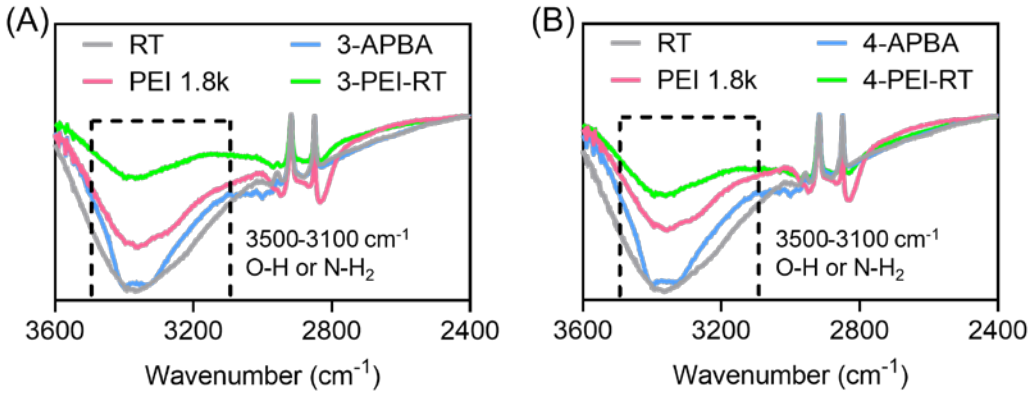

Fig. S1. (A) FT-IR spectra of RT, 3-APBA, PEI 1.8k, and 3-PEI-RT. (B) FT-IR spectra of RT, 4-APBA, PEI 1.8k, and 4-PEI-RT.

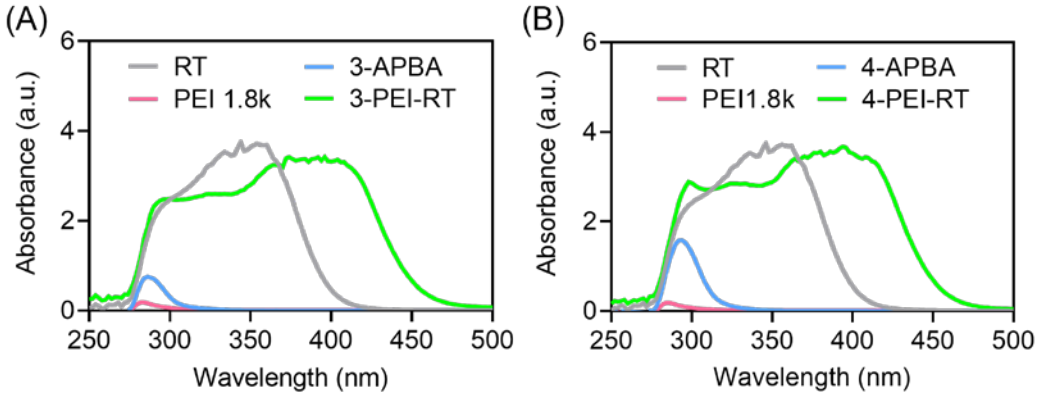

Fig. S2. (A) UV/Vis spectra of RT, 3-APBA, PEI 1.8k, and 3-PEI-RT. (B) UV/Vis spectra of RT, 4-APBA, PEI 1.8k, and 4-PEI-RT.

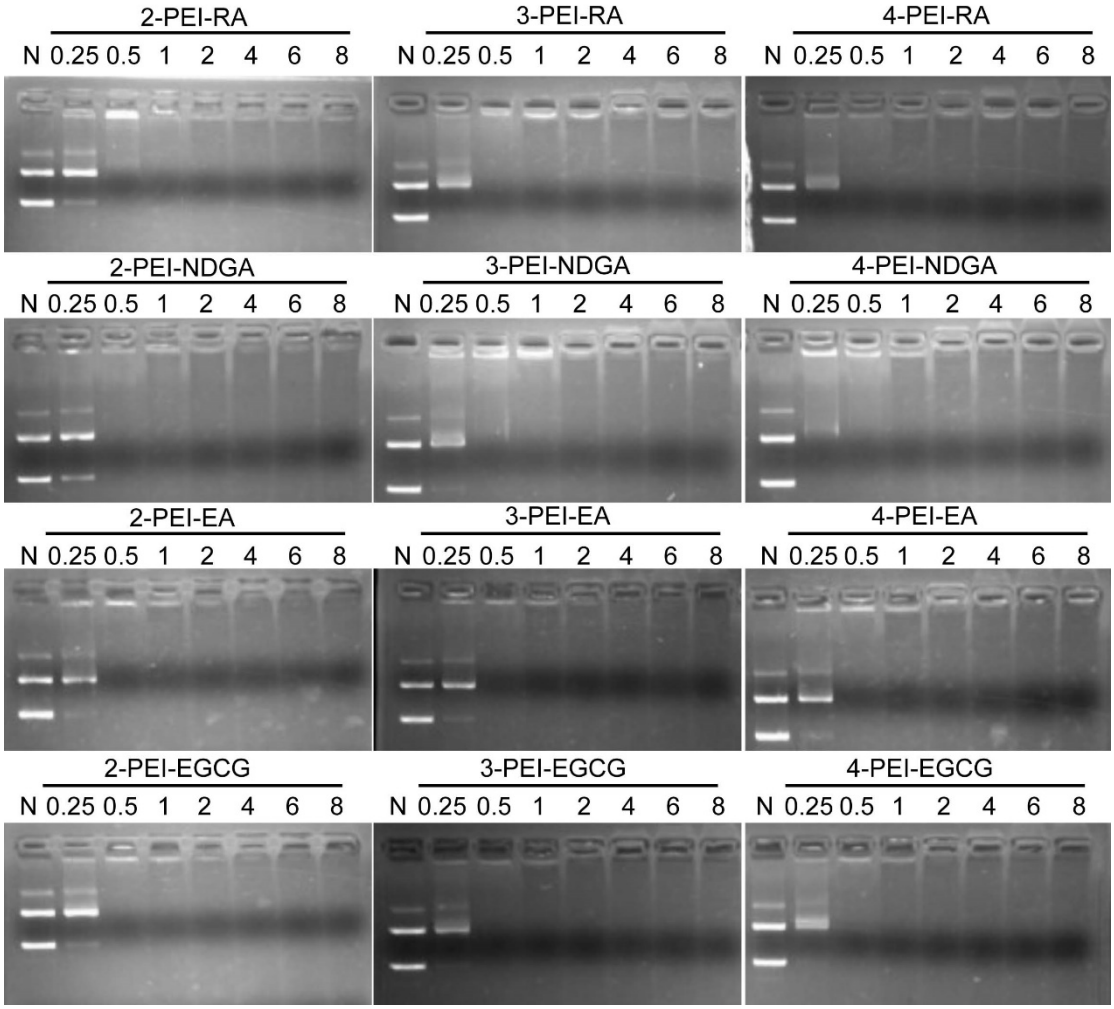

Fig. S3. DNA condensation by different polymers at various polymer/DNA weight ratios as evaluated by the gel retardation assay. N represents naked DNA.

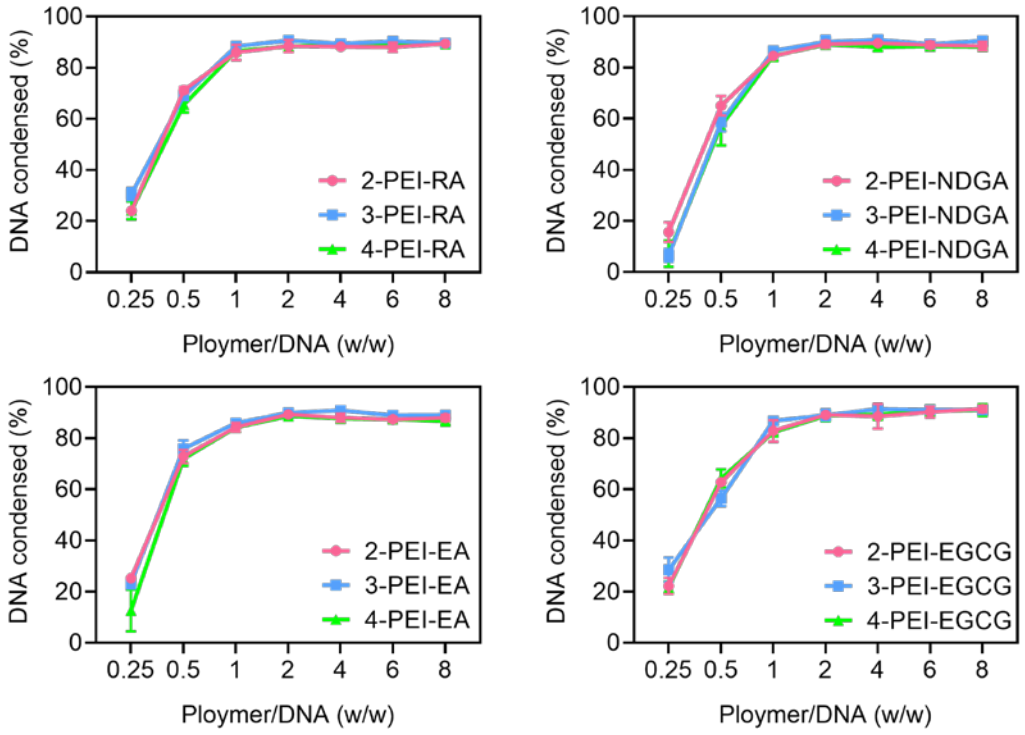

Fig. S4. DNA condensation by different polymers at various polymer/DNA weight ratios as evaluated by the EB exclusion assay (n = 3).

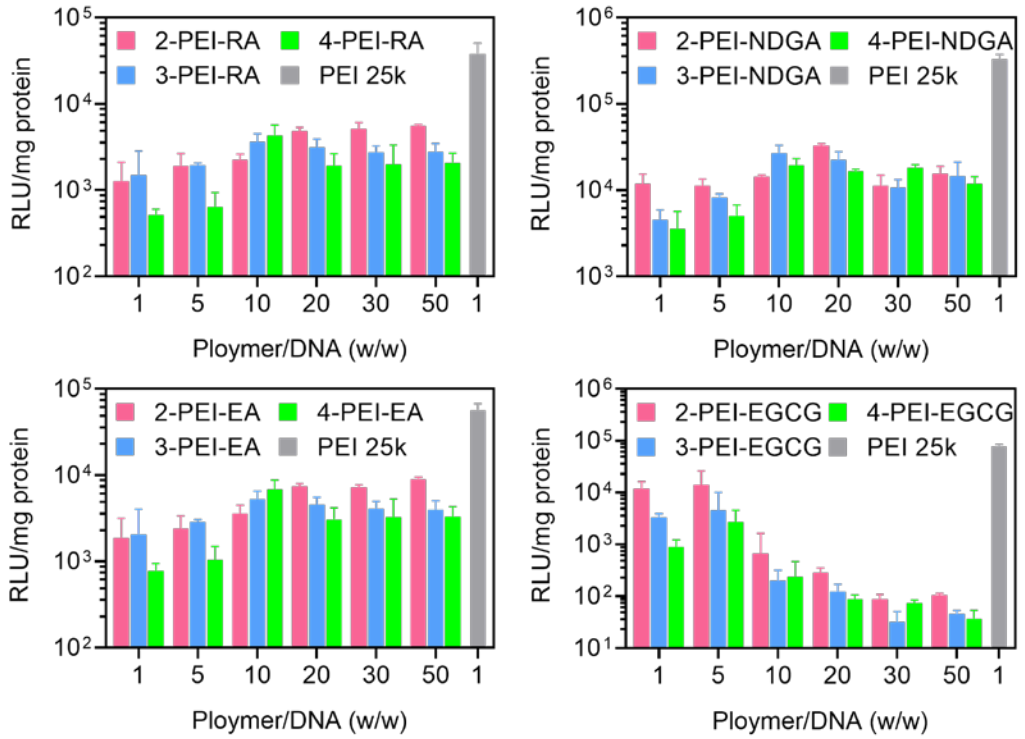

Fig. S5. *In vitro* DNA transfection efficiencies of different polymers in B16F10 cells at various polymer/pLuc weight ratios in DMEM containing 10% FBS (n = 3).

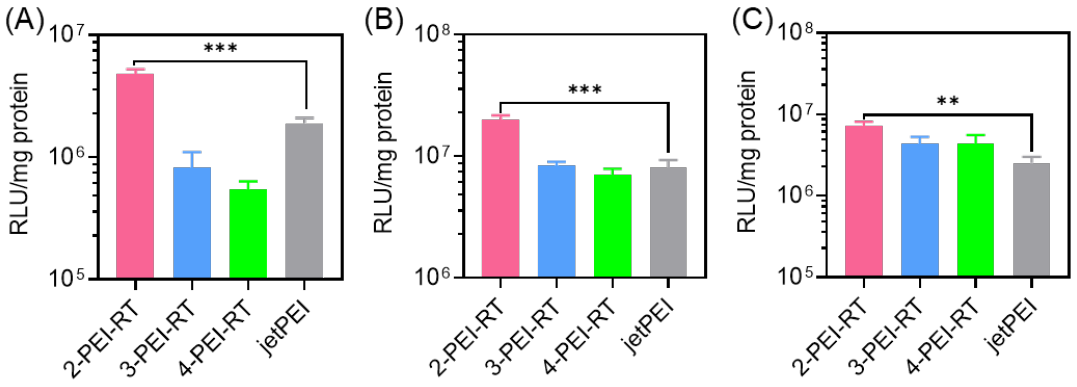

Fig. S6. *In vitro* transfection efficiencies of polymers/pLuc NCs in B16F10 (A, polymer/pLuc = 10, w/w), HepG-2 (B, polymer/pLuc = 20, w/w), and MDA-MB-231 (C, polymer/pLuc = 20, w/w) cells in DMEM containing 10% FBS (n = 3). jetPEI/pLuc NCs were used as the positive control according to the manufacture's protocol.

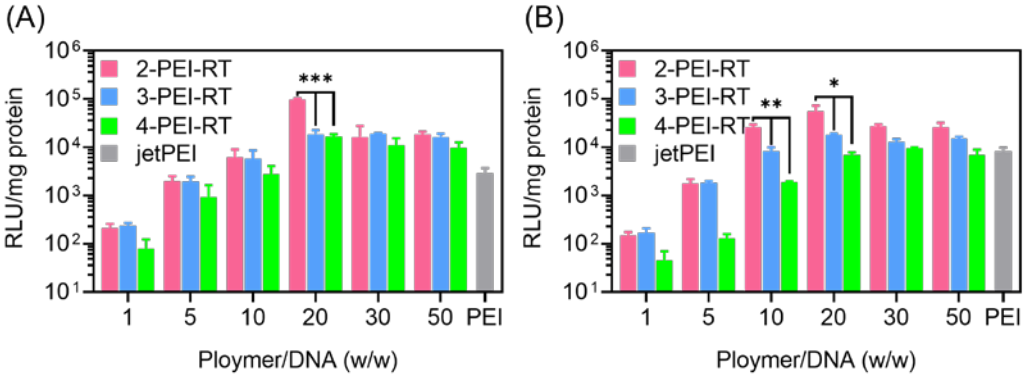

Fig. S7. Transfection efficiencies of polymers/pLuc NCs (1 μg pLuc/mL) at various polymer/pLuc weight ratios in NIH-3T3 (A) and MSC (B) cells in DMEM containing 10% FBS (n = 3). jetPEI/pLuc NCs were used as the positive control according to the manufacture's protocol.

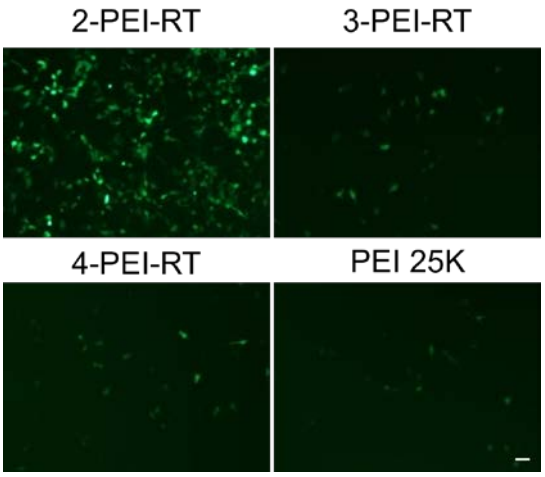

Fig. S8. *In vitro* transfection efficiencies of polymers/pEGFP NCs (polymer/pEGFP = 10, w/w) in B16F10 cells in DMEM containing 10% FBS (scale bar = 50 μm).

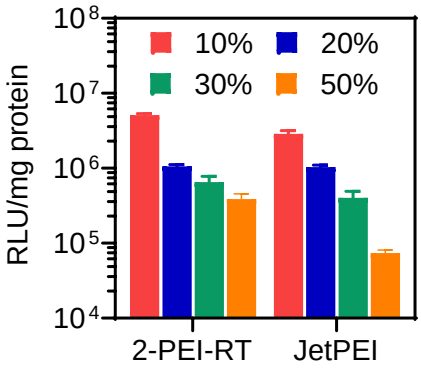

Fig. S9. Transfection efficiencies of 2-PEI-RT/pLuc NCs in B16F10 cells at the polymer/pLuc weight ratios of 10 in DMEM containing 10%, 20%, 30%, or 50% FBS (n = 3). jetPEI/pLuc NCs were used as the positive control.

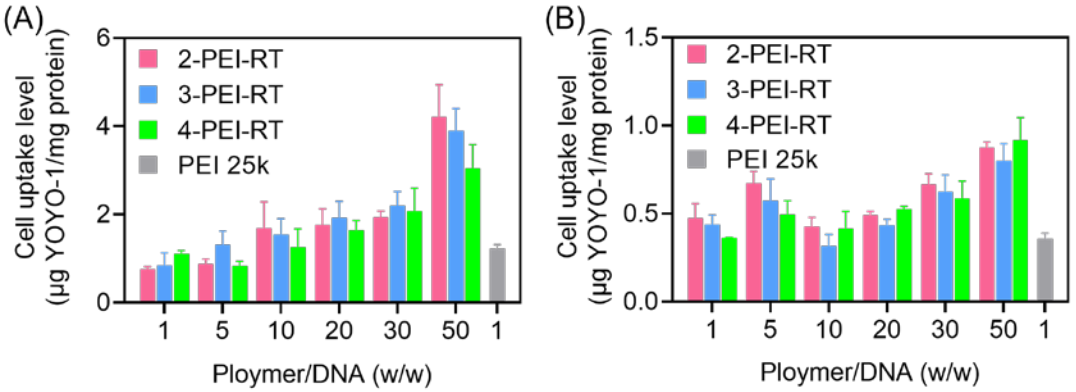

Fig. S10. Uptake levels of 2-PEI-RT/YOYO-1-DNA, 3-PEI-RT/YOYO-1-DNA, and 4-PEI-RT/YOYO-1-DNA NCs (1 μg DNA/mL) at various polymer/YOYO-1-DNA weight ratios in HepG-2 (A) and MDA-MB-231 (B) cells (n = 3).

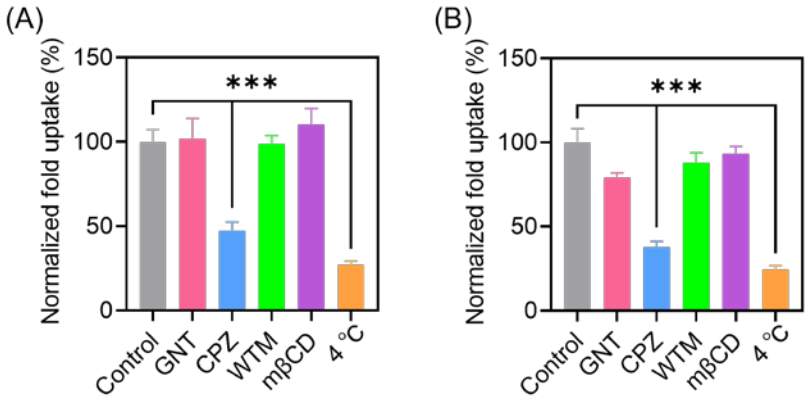

Fig. S11. Relative cellular uptake levels of 3-PEI-RT/YOYO-1-DNA NCs (A) and 4-PEI-RT/YOYO-1-DNA NCs (B) (1 μg DNA/mL, w/w = 10/1) in B16F10 cells at 4 °C or in the presence of various endocytic inhibitors (n = 3).

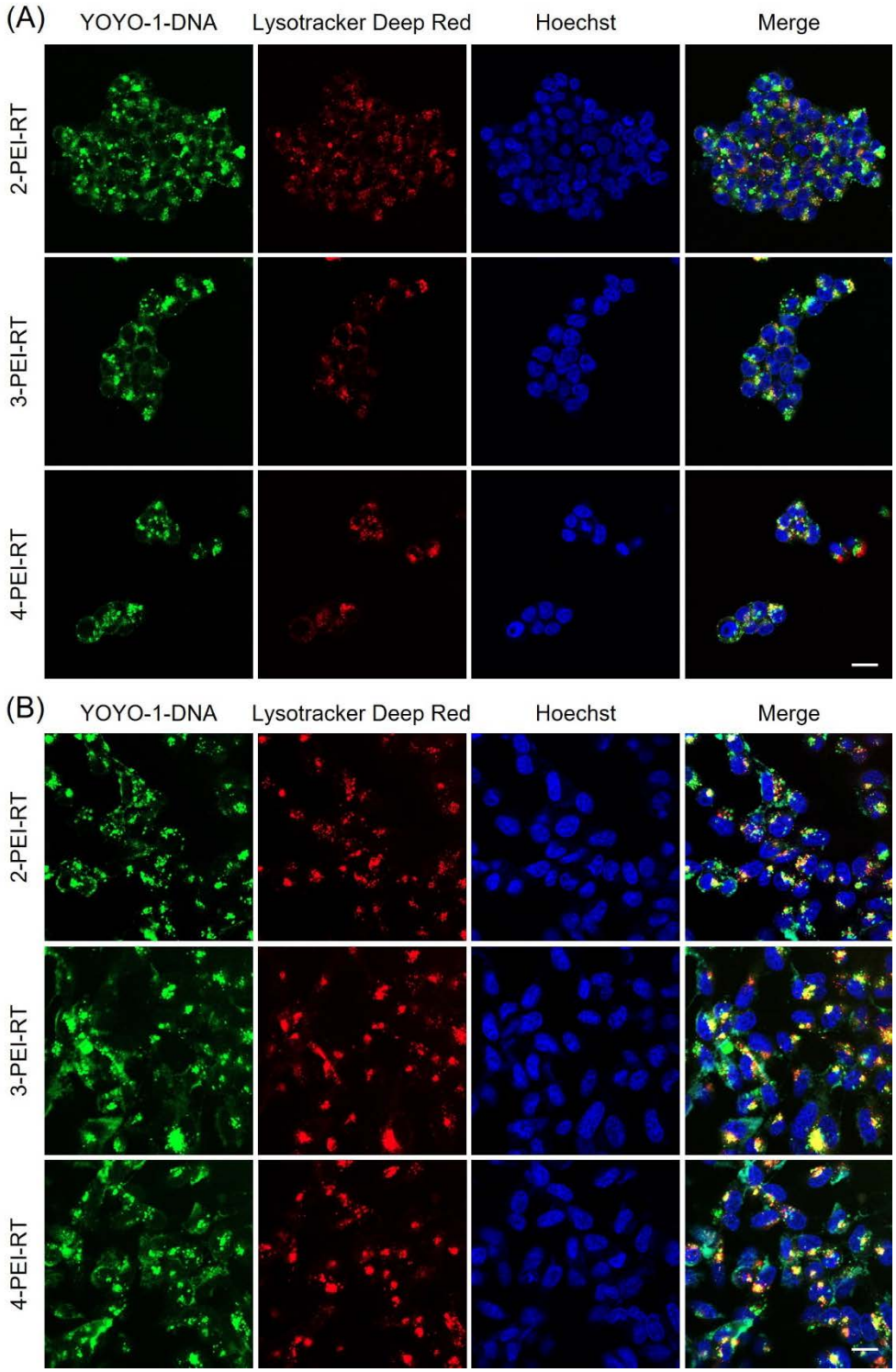

Fig. S12. CLSM images of HepG-2 (A) and MDA-MB-231 (B) cells following incubation with 2-PEI-RT/YOYO-1-DNA, 3-PEI-RT/YOYO-1-DNA, and 4-PEI-RT/YOYO-1-DNA NCs (1 μg DNA/mL, w/w = 10/1) at 37 °C for 6 h (scale bar = 20 μm). Nuclei were stained with Hoechst 33342 and endolysosomes were stained with Lysotracker Deep Red.

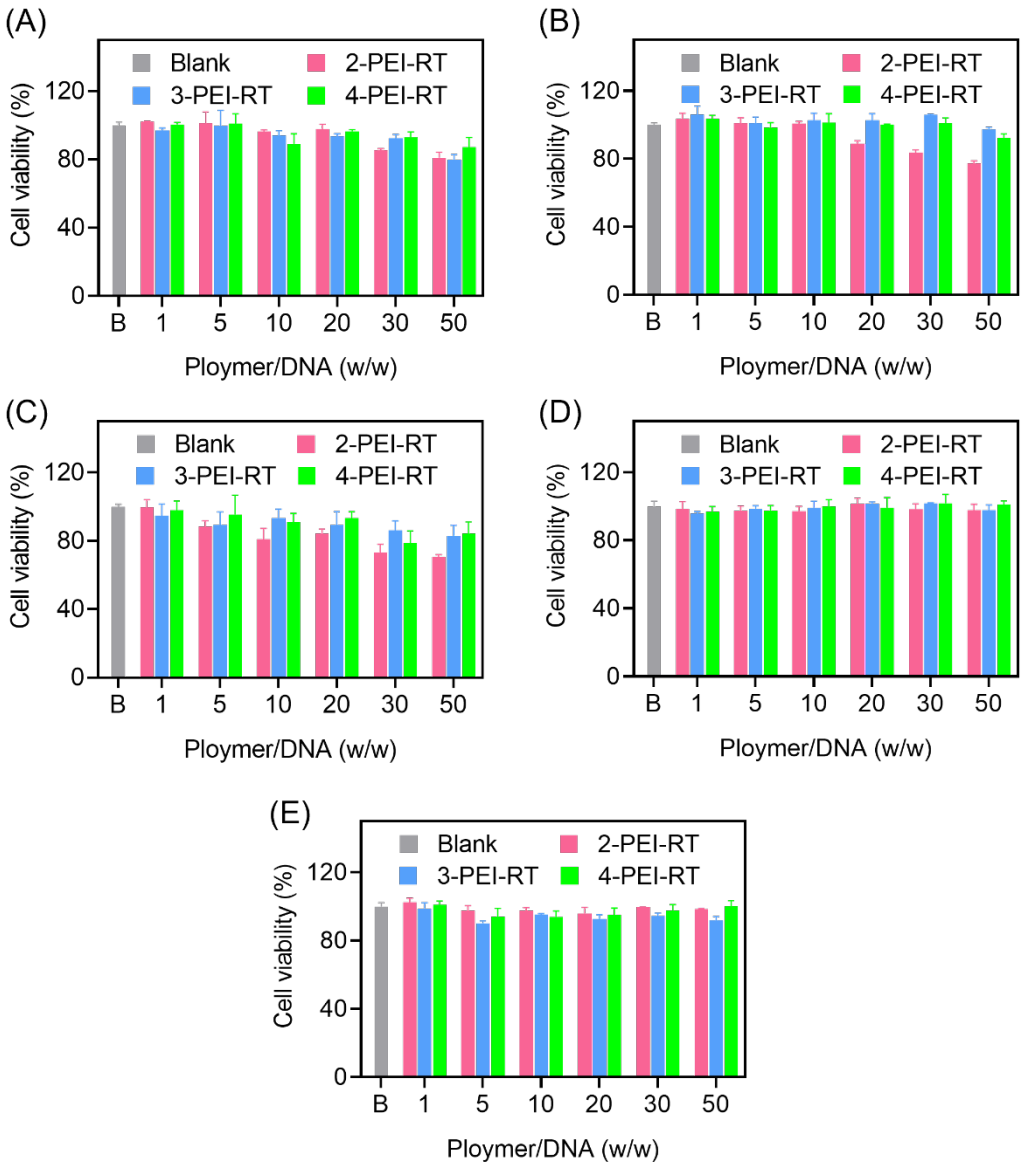

Fig. S13. Cytotoxicity of 2-PEI-RT/DNA, 3-PEI-RT/DNA, and 4-PEI-RT/DNA NCs (1 μg DNA/mL) at various polymer/DNA weight ratios in HepG-2 (A), B16F10 (B), MDA-MB-231 (C), NIH-3T3 (D), and MSC (E) cells following 24-h incubation as determined by the MTT assay (n = 3).

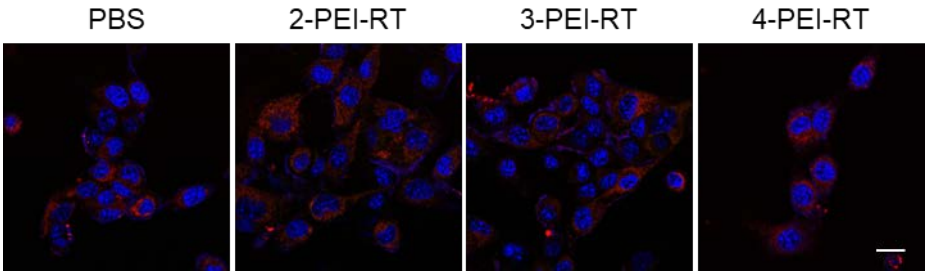

Fig. S14. Mitochondrial membrane potential (ΔΨm) of B16F10 cells stained with JC-1 after treatment with 2-PEI-RT/pLuc NCs, 3-PEI-RT/pLuc NCs, or 4-PEI-RT/pLuc NCs (w/w = 10/1, scale bar = 20 μm) for 24 h. Cell nuclei were stained with Hoechst 33342.

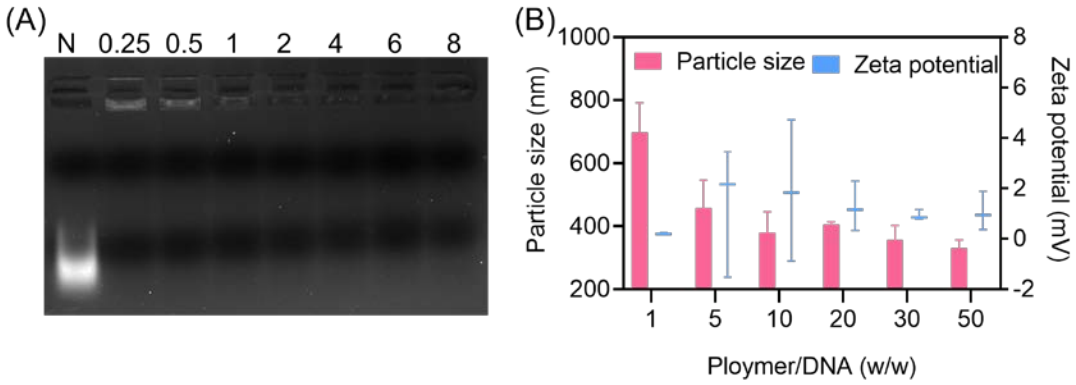

Fig. S15. (A) mRNA condensation by 2-PEI-RT at various 2-PEI-RT/Cas9-mRNA weight ratios as evaluated by the gel retardation assay. N represents naked Cas9-mRNA. (B) Size and zeta potential of 2-PEI-RT/Cas9-mRNA NCs at various 2-PEI-RT/RNA weight ratios (n = 3).

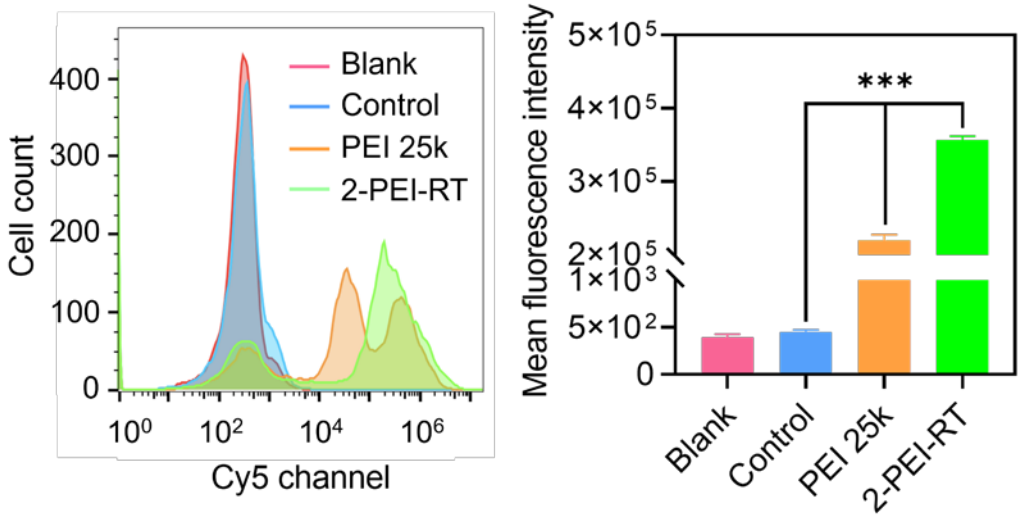

Fig. S16. Flow cytometry analysis of HeLa cells following incubation with 2-PEI-RT/Cy5-mRNA NCs (1 μg Cy5-mRNA/mL, w/w = 30/1) at 37 °C for 6 h (n = 3). PEI 25k/Cy5-mRNA NCs (w/w = 2/1) were used as the positive control.

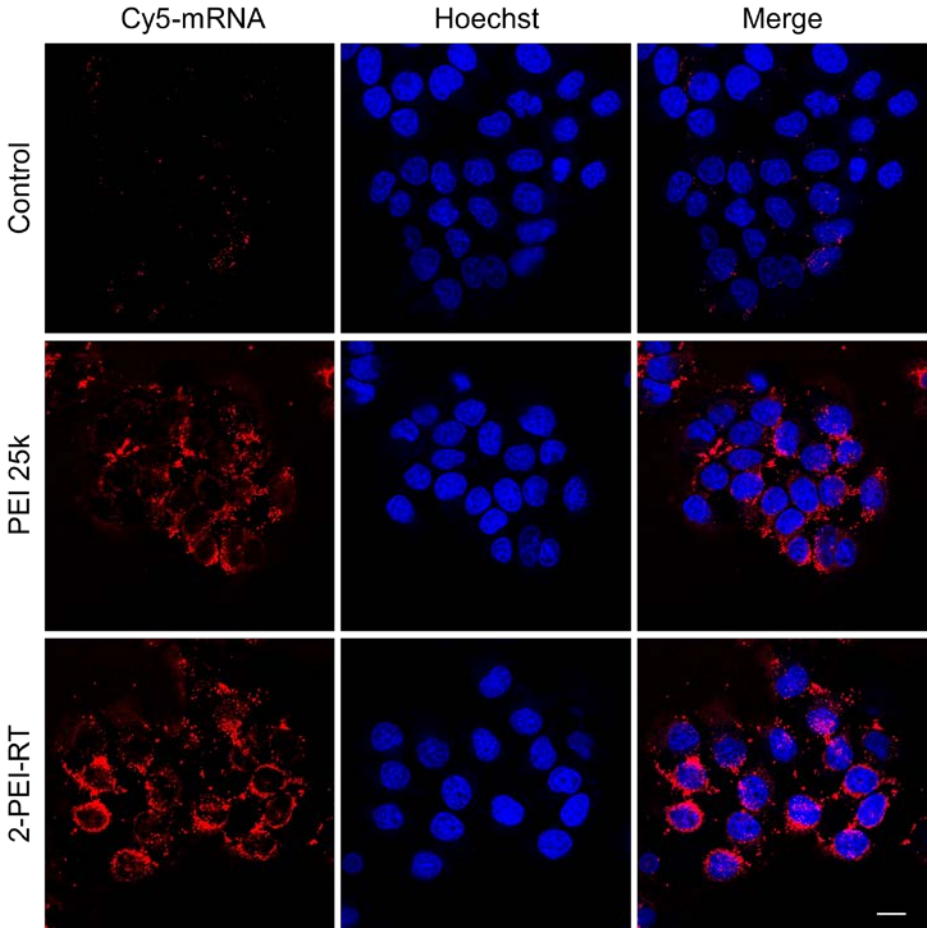

Fig. S17. CLSM image of HeLa cells following incubation with 2-PEI-RT/Cy5-mRNA NCs (1 μg Cy5-mRNA/mL, w/w = 30/1) at 37 °C for 6 h (scale bar = 20 μm). PEI 25k/Cy5-mRNA NCs (w/w = 2/1) were used as the positive control. Nuclei were stained with Hoechst 33342.

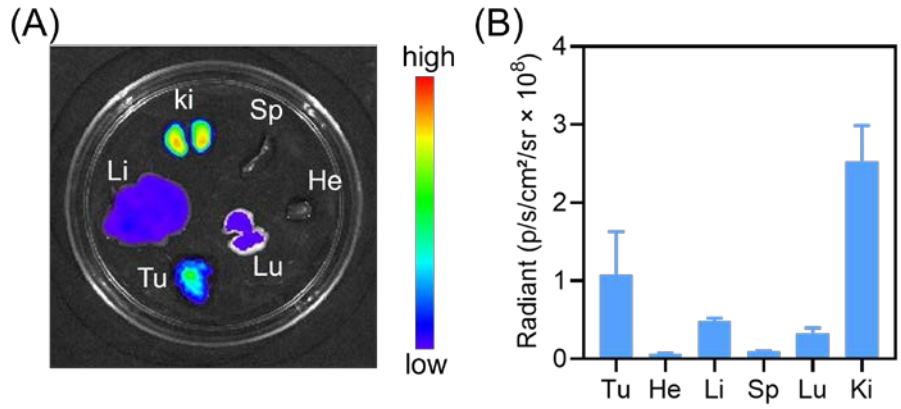

Fig. S18. Biodistribution of 2-PEI-RT^{Cy5}/pLuc NCs following *i.v.* injection. (A) *Ex vivo* fluorescence imaging of major organs and tumors isolated from mice at 6 h post *i.v.* injection (Tu, tumor; He, heart; Li, liver; Sp, spleen; Lu, lung; Ki, kidney). (B) Semi-quantitative fluorescence intensities of different organs and tumors in (A) (n = 4).

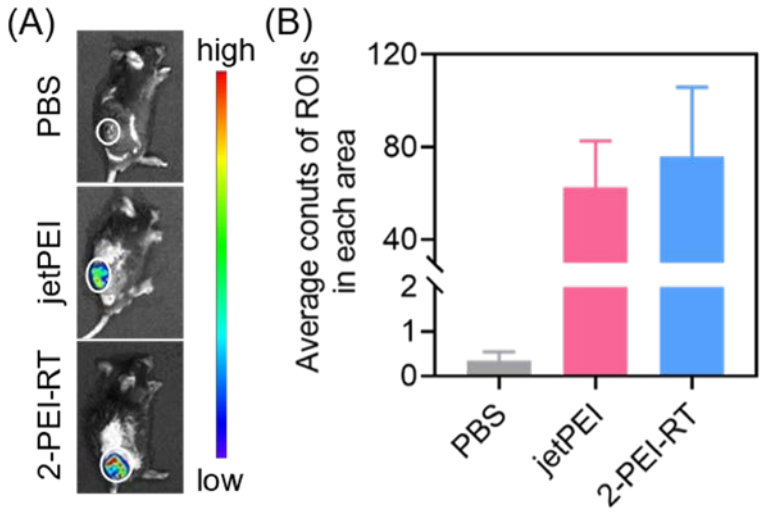

Fig. S19 *In vivo* transfection efficiencies of 2-PEI-RT/pLuc NCs in tumors. (A) Bioluminescence images of B16F10 tumor-bearing mice at 48 h post *i.v.* injection of PBS, jet PEI/pLuc NCs, or 2-PEI-RT/pLuc NCs (B) Semi-quantitative bioluminescence intensities of tumors in (A) (n = 3). D-luciferin was *i.p.* injected 10 min before imaging.

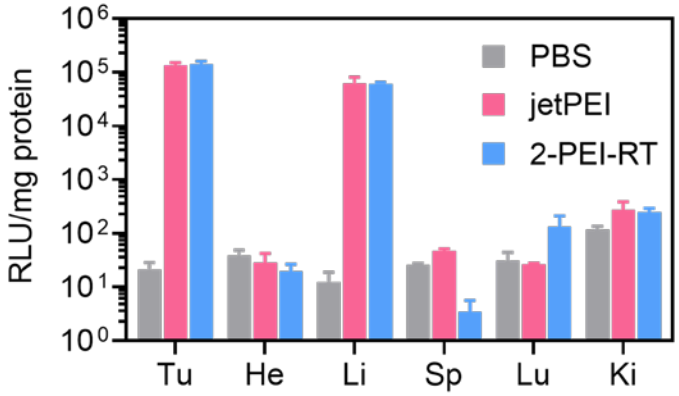

Fig. S20. Transfection efficiencies of jetPEI/pLuc NCs and 2-PEI-RT/pLuc NCs in major organs and tumors isolated from mice at 48 h post *i.v.* injection (Tu, tumor; He, heart; Li, liver; Sp, spleen; Lu, lung; Ki, kidney; n = 3).

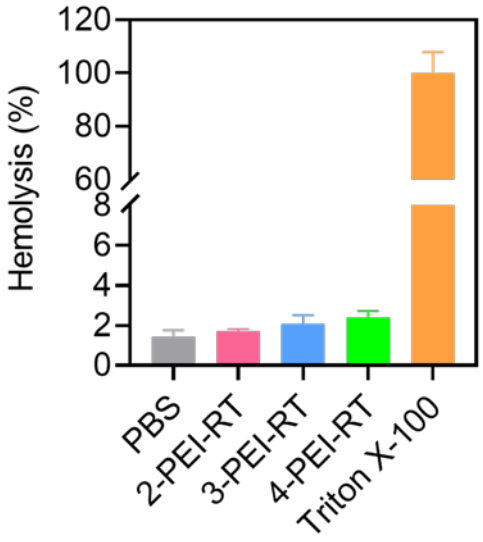

Fig. S21. Hemolysis activity of 2-PEI-RT/pLuc NCs, 3-PEI-RT/pLuc NCs, and 4-PEI-RT/pLuc NCs (200 μg polymer/mL, w/w = 10/1, n = 3). Triton X-100 (1%)-treated red blood cells served as 100%.

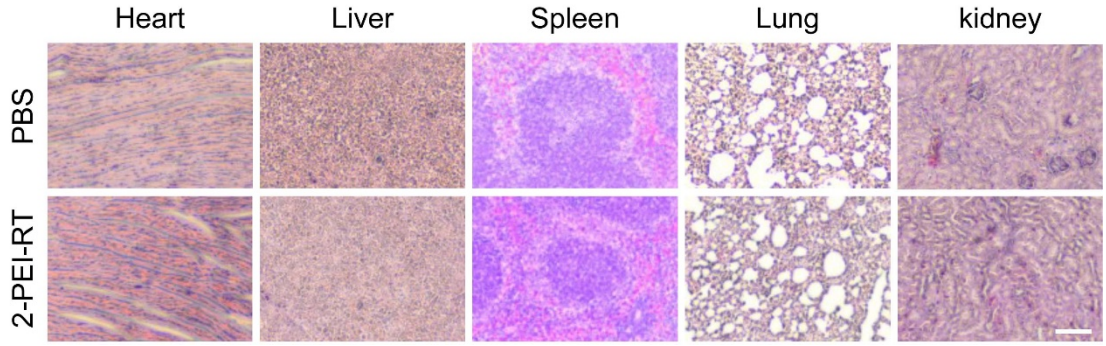

Fig. S22. H&E staining sections of major organs including heart, liver, spleen, lung, and kidney harvested from B16F10 tumor-bearing mice on day 7 after *i.v.* injection of 2-PEI-RT/pLuc NCs (scar bar = 100 μm).

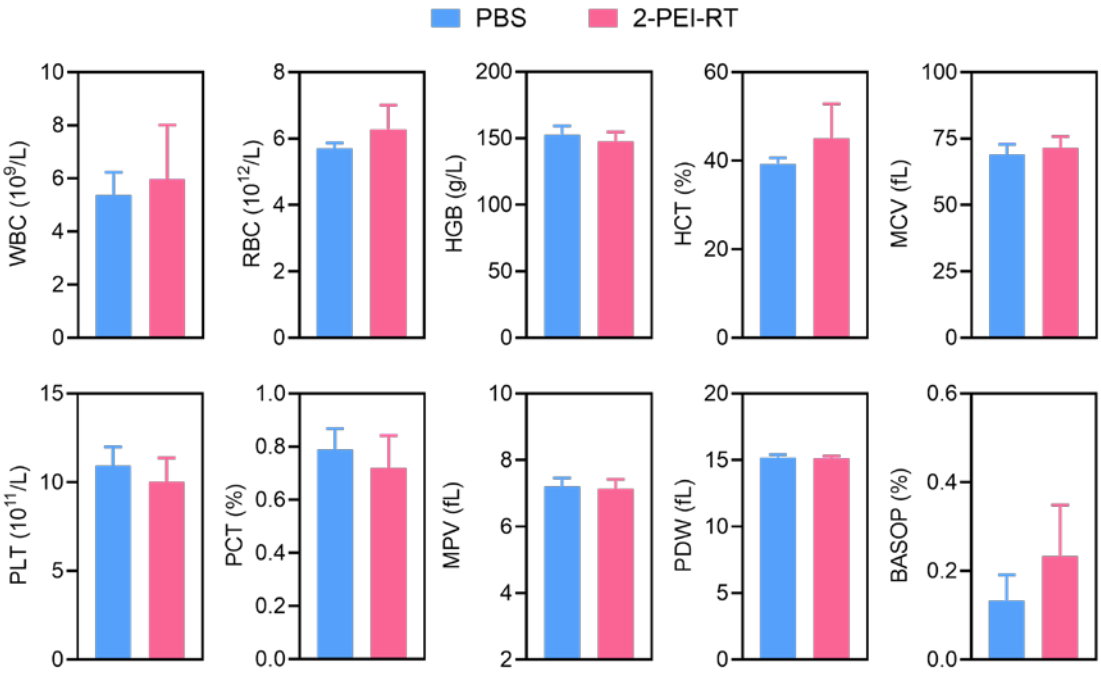

Fig. S23. Hematological parameters of mice at 24 h post *i.v.* injection of PBS or 2-PEI-RT/pLuc NCs (10 mg 2-PEI-RT/kg, w/w = 10/1) to healthy mice (n = 3).

Table S1. Sequences of sgRNA and the targeting DNA used in this study.

Name	Sequence (5'-3')	Targeting sequence
$sgRNA_{(GFP)}$ (targeting GFP)	GACAAGAUGUCCUCGGCGAAGUU UUAGAGCUAGAAAUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAA CUUGAAAAAGUGGCACCGAGUCG GUGCUUUU	GACAAGATGTCCTC GGCGAA
$sgRNA_{(PLK1)}$ (targeting PLK1)	CGGAGGCUCUGCUCGGAUCGGUU UUAGAGCUAGAAAUAGCAAGUUA AAAUAAGGCUAGUCCGUUAUCAA CUUGAAAAAGUGGCACCGAGUCG GUGCUUUU	CGGAGGCTCTGCTC GGATCG

Table S2. Primer sequences for real-time PCR assay.

Name	Sequence (5'-3')
PLK1-F	GGCAACCTTTTCCTGAATGA
PLK1-R	AATGGACCACACATCCACCT
β-actin-F	TCTGGCACCACACCTTCTACAATG
β-actin-R	GGATAGCACAGCCTGGATAGCAA