Supporting Information

Aza-Crown Ether Locked on Polyethyleneimine: Solve the Contradiction between Transfection Efficiency and Safety during *In Vivo* Gene Delivery

Shengran Li[a, b, †], Lin Lin[c, †], Wenliang Wang[d], Xinxin Yan[a, b], Binggang Chen[a, b], Sangni Jiang[a,b], Sanrong Liu*[,a], Xiaojing Ma*[, a], Huayu Tian[b,c], Xifei Yu*[, a, b]

a Laboratory of Polymer Composites Engineering, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, Jilin 130022, China. E-mail: liusanrong@ciac.ac.cn, xjma@ciac.ac.cn, xfyu@ciac.ac.cn.

b University of Science and Technology of China, Hefei, Anhui 230026 China.

c Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, Jilin 130022, China.

d Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

† The authors contributed equally to this work

Table of Contents

1 Materials and characterization

1.1 Materials

Tetraethylene glycol, diethanolamine, 5-bromovaleric acid and branched PEI_{25k} were purchased from Sigma-Aldrich (Shanghai, China), Tosyl chloride and 2-Bromoethanol were purchased from Aladdin (Shanghai, China), peptide GE11 was purchased from Yuanpeptide (Nanjing, China), and dialysis tube were purchased from Baijin Biotechnology (Changchun, China). pGL3 luciferase plasmid and luciferase assay system were purchased from Promega (Madison, WI). The plasmid DNA (pGFP) and DNA (caspase-3) were purchased from Invitrogen (Carlsbad, CA, USA). The plasmids were propagated in Escherichia coli DH5α and extracted using Endo-Free Plasmid Kit (Qiagen, Hilden, Germany). Nucleic Acid Labeling Kit, Cy5-DNA was purchased from Ribo Bio (Guangzhou, China). Fetal bovine serum (FBS), cell culture media (RPMI1640), trypsin, Celltiter-Blue, 4',6-diamidino-2-phenylindole (DAPI) were purchased from Powertek Biotechnology (Beijing, China). Male BALB/C nude mice (5–6 weeks old; weight, 18–22 g) were purchased from Vital River Company (Beijing, China) and kept at an SPF-level laboratory (Northeast Normal University).

1.2 Characterization

The ^{1}H and ^{19}F NMR spectra were recorded on the 400 NMR (AV-400 Bruker) and 500 NMR (AVANCEⅢ 500HD, Bruker). The molecular weight of materials was performed on Maldi-tof-MS. The charge and the size distribution of the delivery systems in aqueous medium was measured via DLS on Zetasizer Nano-ZS (Malvern). The morphology of these nanoparticles was measured by transmission electron microscopy (TEM) using JEOL JEM-1011 electron microscope (Bruker). The GFP expression and cellular transportation trace was monitored by confocal laser scanning microscopy (LSM 700 Carl Zeiss Microscopy) and flow cytometry (FACS, Guava easyCyte 6-2L from Milipore). The relative light units (RLU) of luciferase plasmid expression were measured by luminometer (Glomax). What's more, the synergy microplate reader (Synergy H1, from Bio Tek) was used to detect the call viability.

2 Synthetic procedures

2.1 The synthesis of 1[1]

Briefly, **1** was synthesized as follows. Tetraethylene glycol (8.06 g, 0.0415 mol) was dissolved in THF (50mL), then potassium hydroxide (9.3 g, 0.166 mol) was dissolved in water (24 mL) and added to the solution. After that, Tosyl chloride (17 g, 0.089 mol) in THF (47 mL) was added dropwise to the solution and react for 12 h. After reaction, ice water (43 mL) was added to the solution and stirred. Then, the product was extracted with CH_2Cl_2 for three times. The organic layer was concentrated by rotary evaporato to give colorless liquid (7.5 g, yield 93%). ^{1}H-NMR of product **1** was shown in **Figure S1**. ^{1}H NMR (500 MHz, $CDCl_3$): 7.78, 7.32 (d, CH_3***Ar***-), 4.18 (t, -SO_3C***H***$_2$-), 3.70 (t, -SO_3CH_2C***H***$_2$-), 3.52 (t, -OC***H***$_2$C***H***$_2$O-), 2.47 (s, C***H***$_3$Ar-).

2.2 The synthesis of 2[2]

A 250mL, three-neck round bottom flask was charged with a magnetic stir bar, 80 mL tBuOH, and KOtBu (1.47 g, 13.14 mmol). The mixture was warmed to 40°C stirred for 30 min to dissolve the KOtBu, and then **1** (2.2 g, 4.38 mmol, in 16mL dioxane) and diethanolamine (460 mg, 4.38 mmol, in 17 mL tBuOH) were added dropwise (simultaneously from two different dropping funnels) over the course of 12 h. Note: Slow additionof the solutions is crucial; the slower the addition, the higher the yield. After addition, the reaction mixture was allowed to cool. The reaction mixture was then filtered twice through a Büchner funnel and the solvent was removed on a rotary evaporator. Deionized water was added to the brown, sticky residue and the resulting solution was first extracted with hexane (hexane phase discarded), followed by CH_2Cl_2. The CH_2Cl_2 phases were collected, dried over $MgSO_4$, and solvent was removed on a rotary evaporator. The resulting dark brown residue was distilled through a bulb-to-bulb distillation under high static vacuum using a heat gun to yield the product as a colorless liquid (340 mg, 30 percent). The ^{1}H-NMR spectra was shown in **Figure S2**. ^{1}H NMR (500 MHz, D_2O): 3.65 (t, -OC***H***$_2$C***H***$_2$O-), 3.03 (t, -NHC***H***$_2$$CH_2$O-).

2.3 The synthesis of 3

The product of **2** (30 mg, 0.115 mmol) was dissolved in acetonitrile (6 mL), then potassium carbonate (62 mg, 0.45 mmol) and 5-bromovaleric acid (30 mg, 0.17 mmol) were added to the solution and stirred for 48h at 60°C. After reaction, the mixture was filtered through a funnel and added with HCl (8.4 mg, 0.23 mmol) and stirred for 2 h. The solution was centrifuged to get the supernatant, then rotary evaporated to give **3** as yellowish solid (34 mg, yield 82%). The ^{1}H-NMR result was shown in **Figure S3**. ^{1}H NMR (500 MHz, D_2O): 4.15 (t, -NH-C***H***$_2$$CH_2$-), 3.65 (t, -OC***H***$_2$C***H***$_2$O-), 3.26 (t, -NHC***H***$_2$$CH_2$O-), 2.45 (t, -$CH_2$C***H***$_2$COOH), 1.71 (m, -$CH_2$C***H***$_2$C***H***$_2$$CH_2$-).

2.4 The synthesis of 4

The product of **2** (22 mg, 0.084 mmol) was dissolved in acetonitrile (5 mL), then potassium carbonate (35 mg, 0.252 mmol) and 2-bromoethanol (15 mg, 0.12 mmol) were added to the solution and stirred for 48 h at 60°C . After reaction, the mixture was filtered through a funnel and purified by column chromatography to give **4** as yellowish liguid (23 mg, yield 90%). The ^{1}H-NMR result was shown in **Figure S4**. ^{1}H NMR (500 MHz, D_2O): 3.88 (t, -$CH_2C\boldsymbol{H}_2OH$), 3.67 (t, -$C\boldsymbol{H}_2CH_2OH$), 3.62 (t, -$OC\boldsymbol{H}_2C\boldsymbol{H}_2O$-), 3.10 (t, -$NHC\boldsymbol{H}_2CH_2O$-).

2.5 The synthesis of ACE-GE11 (5)

The product of **4** (11 mg, 0.028 mmol) was dissolved in DMF (2 mL), then added with N-hydroxysuccinimide (6.4 mg, 0.056 mmol) and N, N'-diisopropylcarbodiimide (8.6 μL, 0.056 mmol) and stirred for 24 h at room temperature. Then peptide GE11 (20 mg) dissolved in DMF (2 mL) and triethylamine (30 μL) were added to the solution and stired for another 24 h. After reaction, the mixture was precipitated by diethyl ether for 3 times. The residue was dissolved in water and freeze-drying to give **5** as white liquid (24 mg, yield 94%). The ^{1}H-NMR result was shown in **Figure S5**. ^{1}H NMR (500 MHz, D_2O): 3.70 (t, -$OC\boldsymbol{H}_2C\boldsymbol{H}_2O$-), 2.90 (t, -$NHC\boldsymbol{H}_2CH_2O$-), 2.75 (t, -$NH\text{-}C\boldsymbol{H}_2CH_2$-), 2.32 (t, -$CH_2C\boldsymbol{H}_2CO$-GE11), 2.00 (m, -$CH_2C\boldsymbol{H}_2C\boldsymbol{H}_2CH_2$-), other chemical shifts owed to GE11. The ^{13}C-NMR result was shown in **Figure S6**. The maldi-tof-MS result of **5** was shown in **Figure S7**.

3 Preparation of nanoparticles of ACE-GE11/(PEI/DNA)

Plasmid DNA was mixed with PEI_{25k} (2 mg/mL) in water to form nanopaticles at different N/P ratios. N/P ratio means total amines in PEI_{25k}/ total phosphates in DNA. Every 43 molecular weight per N for PEI, and every 330 molecular weight per P for DNA. The mass ratio of PEI/DNA is 0.13x when N/P=x. Then ACE-GE11 was caculated according to NMR result in Figure S9 and mixed with the complex of PEI/DNA. The solution of the nanopaticles of ACE-GE11/(PEI/DNA) were stored at 4 ℃. The size and zeta potential of these nanoparticles were detected by DLS (**Figure S8** and **S9**).

4 Agarose gel electrophoresis assay

PEI_{25k} and DNA were mixed at different N/P ratios and incubated for 20 min. The complexes were electrophoresed on a 0.8% agarose gel at 140 V for 20 min. DNA bands were visualized by staining with ethidium bromide (EB) and excitation by UV transillumination. The result was shown in **Figure S10**.

5 The lock and unlock ability of ACE on PEI

To study the binding stoichiometry and kinetics of ACE on PEI. We added enough ACE to PEI solutions (2 mg/mL in water). After stirred for different length of time (5, 10, 15, 20, 30, 40 ,60 min), the solutions were dialyzed immediately to remove the unbound ACE. After freeze-drying, the bound ACE was eluted by CH_2Cl_2. Then CH_2Cl_2 was removed under vacuum to get the ACE for weighing. The lock ratio was calculated by the molar ratio of ACE/(-NH_3^+ on PEI). The result was shown in **Figure 1b**. For NMR detection, ACE was mixed with PEI_{25k} for 30min in water, then dialysis with PBS to remove the ACE which not bind on the amino cations on PEI. The complexes were freeze-drying and dissolved in D_2O. The ^{1}H-NMR result was shown in **Figure S11**. The lock ratio of ACE on PEI was caculated by the integral in NMR result.

The binding affinity of ACE on PEI. Because ACE could not only bind to –NH_3^+, but also to Na^+, we put the ACE/PEI complex in different amount of NaCl solutions. The solutions were dialyzed immediately to remove the unbound ACE. After freeze-drying, the bound ACE was eluted by CH_2Cl_2. Then CH_2Cl_2 was removed under vacuum to get the ACE for weighing. The lock ratio was calculated by the molar ratio of ACE/(-NH_3^+ on PEI). The result was shown in Figure S12 .

ACE detached from PEI in acidic condition. The complexes of ACE/PEI were added to the acid solutions with different pH values and stirred for 2 h, then dialysis with PBS to remove the ACE that not bind on PEI, the solution was freeze-drying and dissolved in D_2O. The ^{1}H-NMR result was shown in **Figure S13**, chemical shifts at 2.6-3.0 belonged to PEI and 3.1-3.2, 3.6-3.8 belonged to ACE. The molar ratio of ACE and PEI was caculated according to the integral values, then the ratio of ACE detached from PEI was caculated based on the original molar ratio in Figure S9 minus the present molar ratio in Figure S10.The complexes of ACE/PEI were added to the water (pH=5.0) and stirred for 0.5, 1 and 2 h, then dialysis with PBS to remove the ACE that not bind on PEI, the solution was freeze-drying and dissolved in D_2O. The ^{1}H-NMR result was shown in **Figure S14**, and the ratio of ACE detached from PEI was caculated by the integral values in NMR results.

6 Gene transfection

For GFP gene transfection, A549 and NIH-3T3 cells were seeded in 24-well plates at a density of 60,000 cells per well in 600 μLof 10% FBS-containing RPMI 1640 medium and incubated for 24 h. Before transfection, the medium was replaced with fresh medium (500 μL) or with medium contains FBS. Solutions of nanoparticles of ACE-GE11/(PEI/DNA), ACE/(PEI/DNA) and PEI/DNA at different N/P ratios were added at a dose of 2 μg DNA (pGFP) per well and cultured for 4 h. The transfection medium was replaced with 600 μL of fresh RPMI-1640 medium supplemented with 10% FBS. The cells were incubated for an additional 48 h. The images were then acquired by using a confocal laser scanning microscope (CLSM).

For luciferase gene transfection, NIH-3T3 and A549 cells were seeded in 96-well plates at a density of 10,000 cells per well in 100 μL of 10% FBS containing RPMI 1640 medium and incubated for 16 h to reach 70–80%. The medium was replaced with fresh medium (90 μL) or with medium contains different amount of FBS. Solutions of nanoparticles of ACE-GE11/(PEI/DNA) and PEI/DNA at different N/P ratios were added at a dose of 0.5 μg DNA (pGL3) per well and cultured for 4 h. The transfection medium was then replaced with 100 μL of fresh RPMI-1640 medium that was supplemented with 10% FBS. The cells were incubated for an additional 48 h. The determination of luciferase plasmid expression was performed according to the standard protocol described in the manufacture's manual (Promega). Protein content of the lysis solution was determined by the Bradford protein assay kit. The luciferase activity was normalized with respect to the protein concentration (relative luciferase light units per milligram of protein). All data are presented with at least three independent measurements.

For the flow cytometry test, A549 and NIH-3T3 cells were plated in 24-well plates at 90,000 cells per dish in 600 μL of 10% FBS-containing RPMI-1640 and incubated for 12 h. The medium was replaced with fresh medium (500 μL) or with medium contains 10% FBS before use. Solutions of ACE-GE11/(PEI/DNA) and PEI/DNA complexes at N/P=10 were added at a dose of 2 μg DNA (pGFP) per dish and cultured for 4 h. Then, the transfection medium was replaced with fresh medium (600 μL) that was supplemented with 10% FBS. The cells were incubated for an additional 48 h. The medium was removed, and the cells were rinsed with PBS, detached by Trypsin, isolated, washed twice with PBS, and resuspended in PBS. The GFP-positive cells were measured by flow cytometry.

7 Intracellular transportation

DNA was labeled with Cy5 before use. NIH-3T3 and A549 cells were seeded in glass-bottomed petri dishes at 80,000 cells per dish in 10% FBS containing RPMI-1640 medium (1.8mL) and incubated for 12 h before use. The medium was replaced with RPMI-1640 (1.5 mL) without FBS. Solutions of ACE-GE11/(PEI/DNA) and PEI/DNA complexes at N/P=10 added at a dose of 2 μg Cy5-DNA per dish and cultured for 0.5 h, 1 h and 3 h. After timed incubation, the medium was removed, and the cells were washed with PBS three times. The cells were immersed by 4% paraformaldehyde for 30 min, which was subsequently removed. The cellular nuclei were stained with DAPI (blue) for 5 min. The cells were washed three times with PBS before observation by CLSM.

8 Cytotoxicity assay

NIH-3T3 and A549 cells were transferred to 96-well flat-bottomed plates with a density of 9,000 cells per well and incubated for 24 h. Afterwards, the medium was removed and the fresh serum-free RPMI-1640, including ACE-GE11/PEI_{25k} and PEI_{25k} at different concentrations were added. After incubation for 24 h, 10 μL of Celltiter-Blue reagent was added to each well, and the cells were incubated for another 3 h. The cell viability was detected by using a microplate reader (lex=560 nm, lem=590 nm). The cell viability was calculated by using Equation:

cell viability (%)= fluorescent intensity (sample) / fluorescent intensity (control) X 100%

For hemolytic test, fresh mouse blood was collected from heart, and the red blood cells (RBCs) were washed three times with PBS. After that, the RBCs were diluted and suspended with 10 mL PBS. First, 0.3 mL RBCs suspension was mixed with 1.2 mL PBS as negative control group, and with 1.2 mL water as positive control group. Various PEI and ACE-GE11/PEI dissolved in 1.2 mL PBS with different concentrations were added to the RBCs suspension (0.3 mL), and then incubated for 2 h at 37℃. Finally, the samples were centrifuged with 12000 r/min for 10 min, the supernatant was collected and measured the absorbance intensity at 541 nm with microplate spectrophotometer. The percent hemolysis was calculated according to the following equation:

percent hymolysis (%)= absorbance intensity of samples / absorbance intensity of positive control X 100%

9 Animals and tumor model

Male Balb/c mice, 4-6 weeks old, and nude mice (21 ± 2 g) from Vital River Company (Beijing, china) were used. All of the animal procedures were in accordance with the Animal Care and Use Committee of Northeast Normal University. A549 tumor was established by subcutaneous injection of 1×10^7 A549 cells into the desirable position and the tumor volume was determined via the following equation: volume=$0.5\times a\times b^2$, where a and b were represented for length and width, respectively. The length and width of the tumor were measured via Vernier caliper.

10 In Vivo Imaging Detection

For real-time dynamic gauging of the accumulation of cy5-dna at the tumor site, in vivo imaging was conducted via Maestro In Vivo Imaging System (Cambridge Research & Instrumentation, Inc.). The complexes of PEI/cy5-dna and ACE-GE11/(PEI/cy5-dna) were injected into the A549 tumor bearing nude mice via tail vein at cy5-dna dose of 1 mg/kg body weight when the tumor volume reached 100-300mm^3. The florescence imaging was conducted at 3, 6, 12, and 24 h after injection. After 24 h, the mice was sacrificed and the tumor and various organs (heart, liver, spleen, lung, and kidney) were excised and prepared for fluorescence imaging.

11 Pharmacokinetic experiments

6-week-old Balb/c mice were obtained to detect the pharmacokinetic of ACE-GE11/(PEI/DNA) and PEI/DNA. The complexes of cy5-DNA and samples were injected intravenously into mice tail vein with the concentration of cy5-DNA was 1 mg/kg of mouse body weight (n=3 per group). At fixed time (1/6, 0.5, 1, 2, 4, 6, 8, 12, 24 h), the blood samples (10 μL) were collected into heparin. Then, the plasma was extracted and the fluorescence intensity of cy5 was measured by microplate reader (λ ex=640 nm, λ em=680 nm). The concentration of cy5-dna was calculated via standard curve.

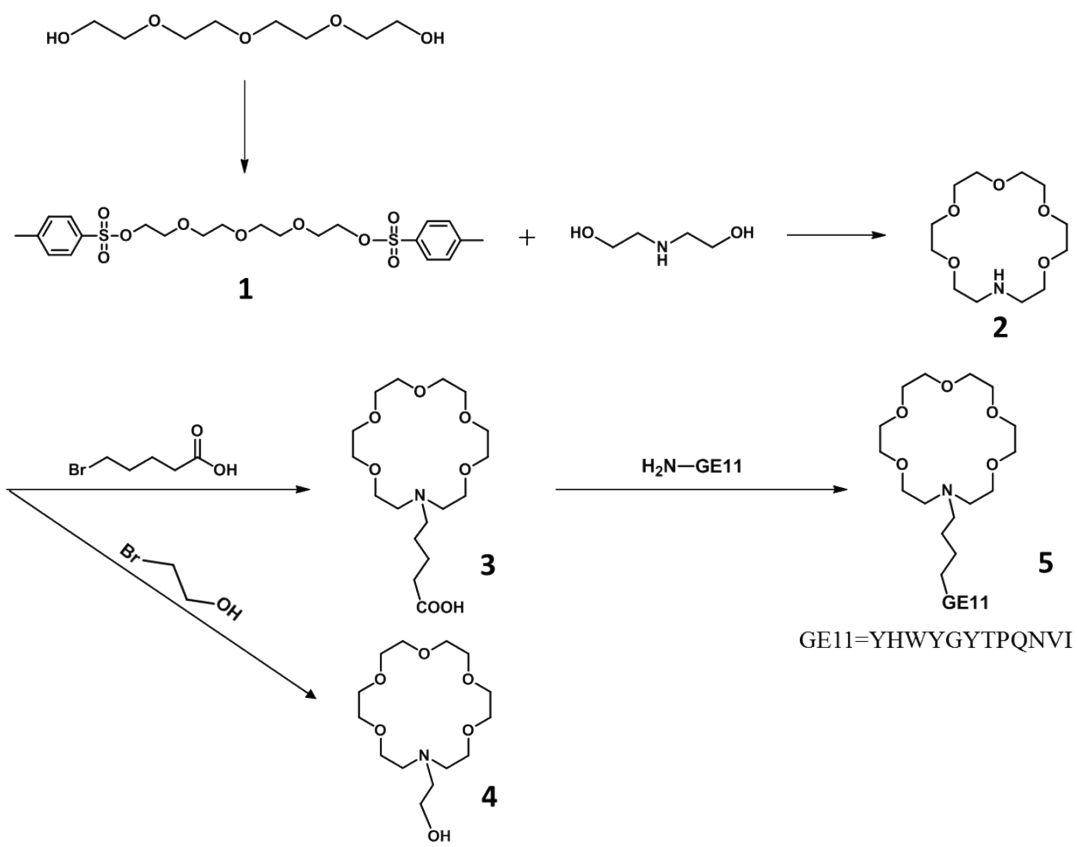
12 In vivo antitumor efficacy

The A549 tumor-bearing mice were randomly divided into four groups and treated with different formulations: (1) saline; (2) PEI_{25k}/DNA (DNA: caspase-3 1 mg/kg, N/P=10); (3) ACE-GE11/PEI_{25k} (PEI: 3mg/kg); (4) ACE-GE11/(PEI_{25k}/DNA) (DNA: caspase-3 1 mg/kg, N/P=10). The body weight and tumor volume were measured every other day. The treatment efficacy was evaluated by monitoring the relative changes of tumor volume and body weight. After 18 days, all of the mice were sacrificed, and the tumors and major organs (heart, liver, spleen, lung, and kidney) were excised to fix in 10% neutral buffered formalin and stained via hematoxylin and eosin (H&E) for pathological analysis. The tumor suppression ratio was calculated by (PBS's tumor volume – treatments' tumor volume) / PBS's tumor volume x 100%.

13 Statistics

All of the measurements presented are expressed as mean ± standard deviation (S.D.). Student's t-test was used to compare the statistical significance (*P < 0.05, **P < 0.01, ***P < 0.001).

Figures



Scheme S1. The detailed synthetic procedure of ACE-GE11.

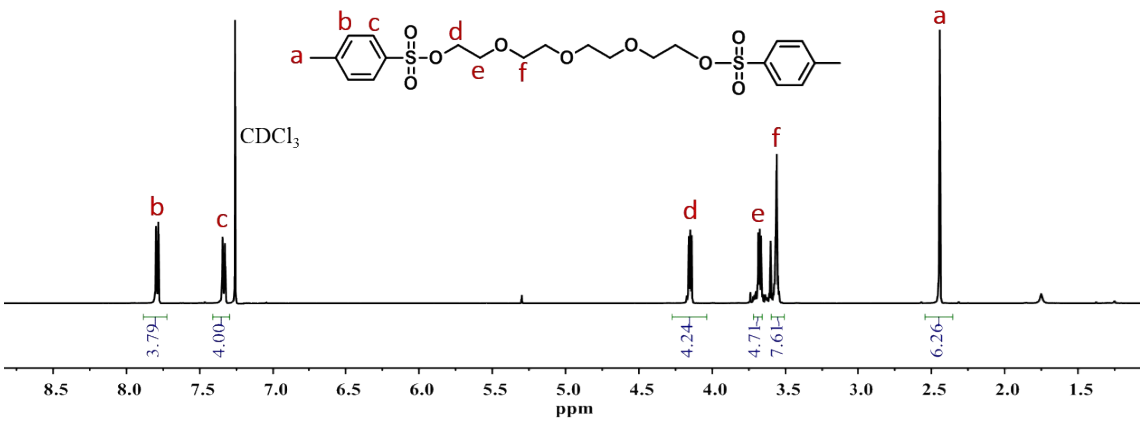


Figure S1. The ^{1}H-NMR spectrum of **1** in $CDCl_3$.

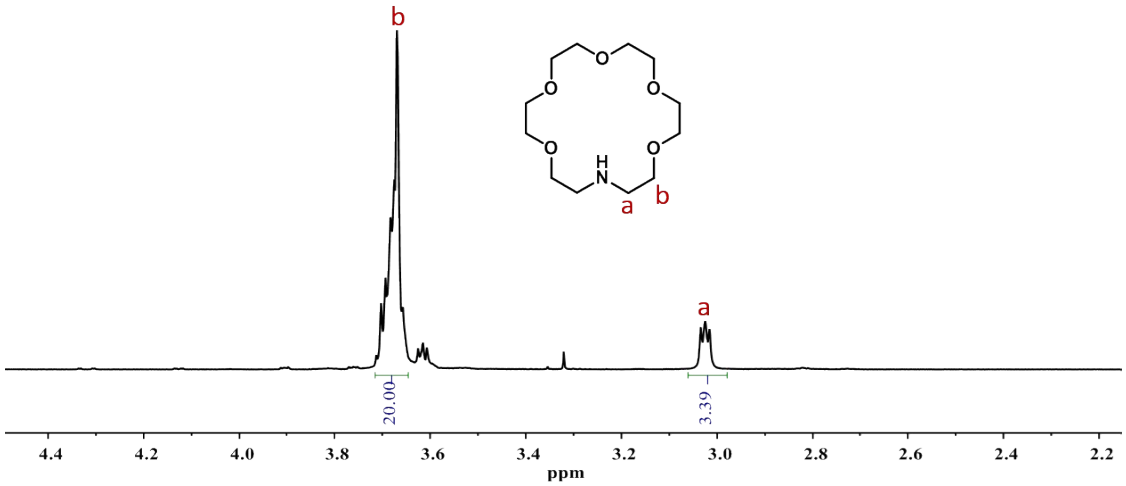


Figure S2. The ^{1}H-NMR spectrum of **2** in D_2O.

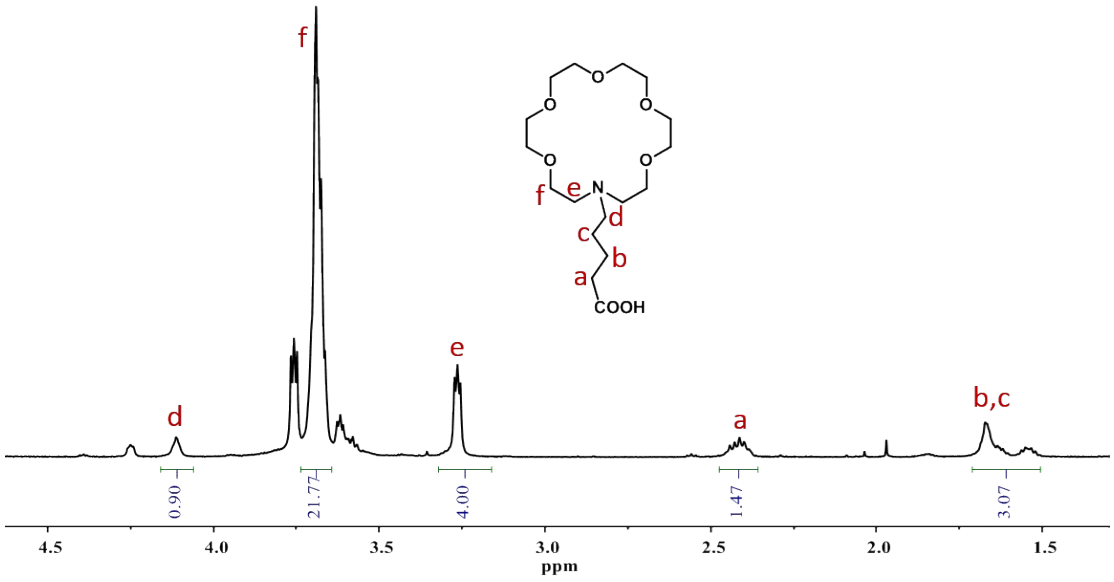


Figure S3. The ^{1}H-NMR spectrum of **3** in D_2O.

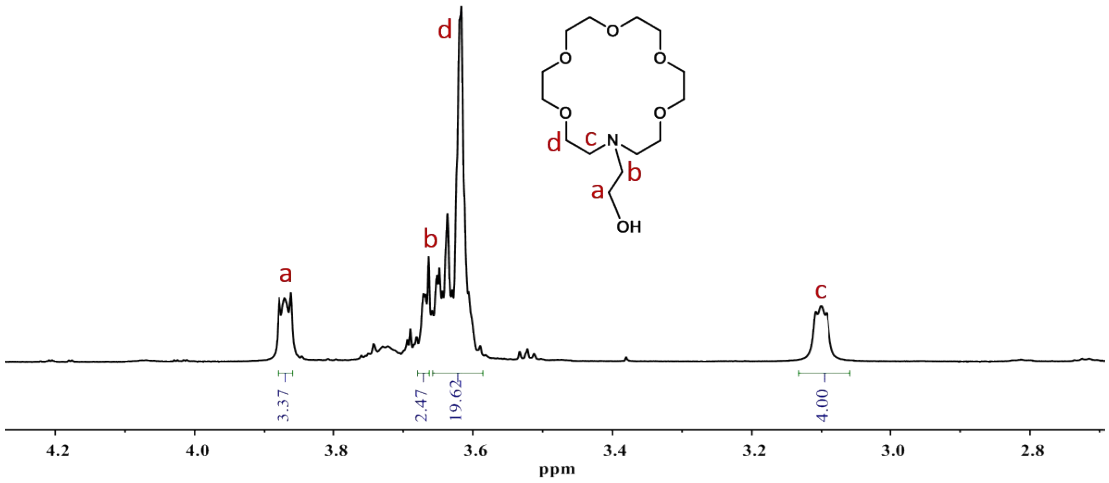


Figure S4. The ^{1}H-NMR spectrum of **4** in D_2O.

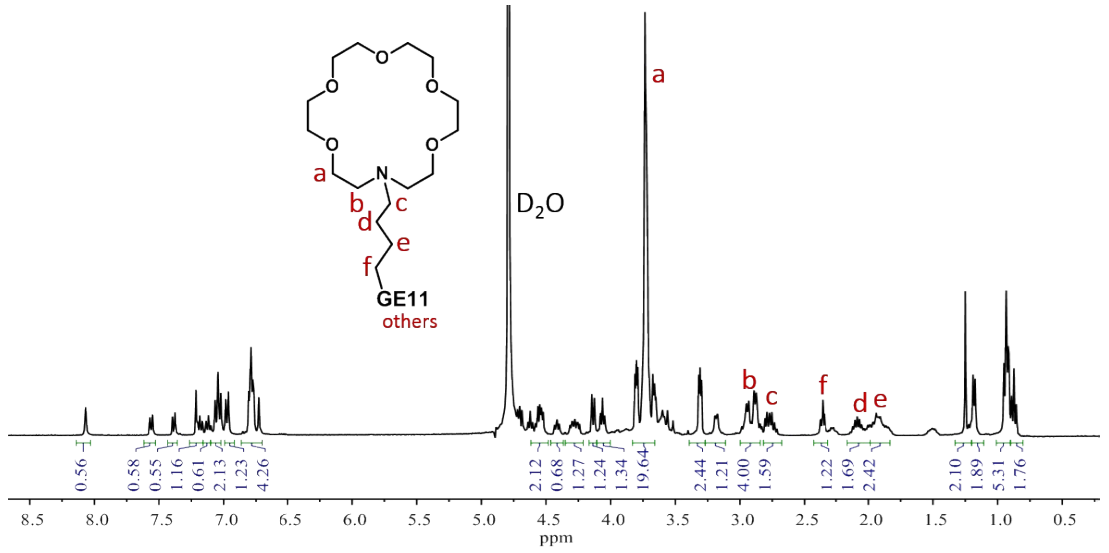


Figure S5. The ^{1}H-NMR spectrum of **5** in D_2O.

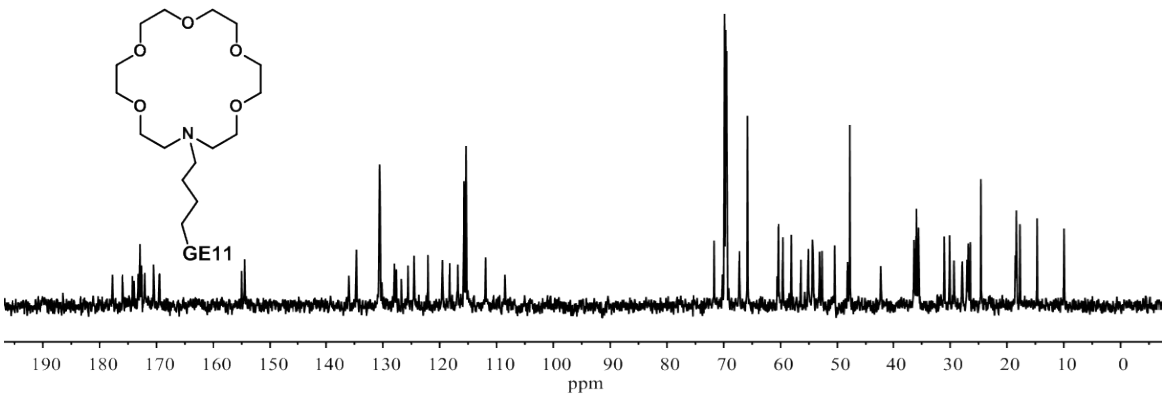


Figure S6. The ^{13}C-NMR spectrum of **5** in D_2O.

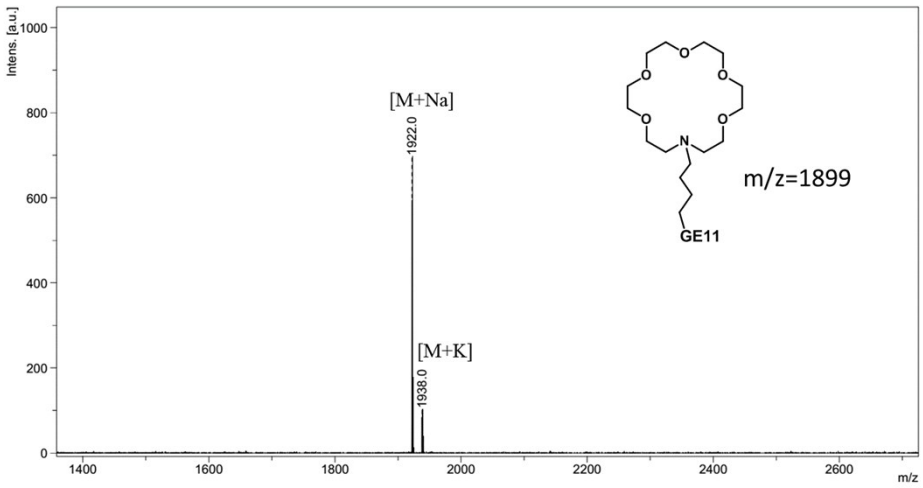


Figure S7. The maldi-tof MS of ACE-GE11.

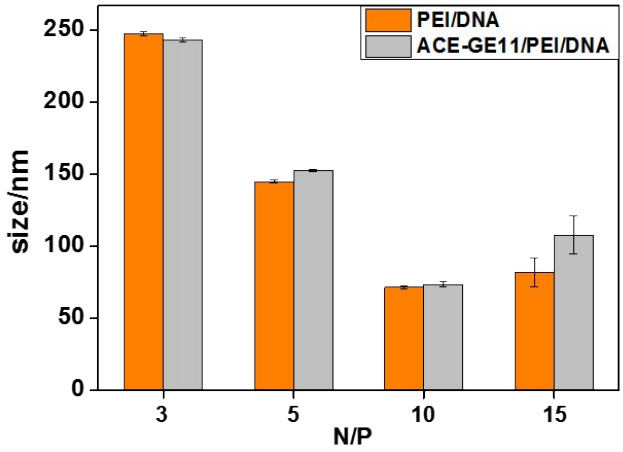


Figure S8. The size of the complexes of our materials and DNA at different N/P ratios detected by DLS.

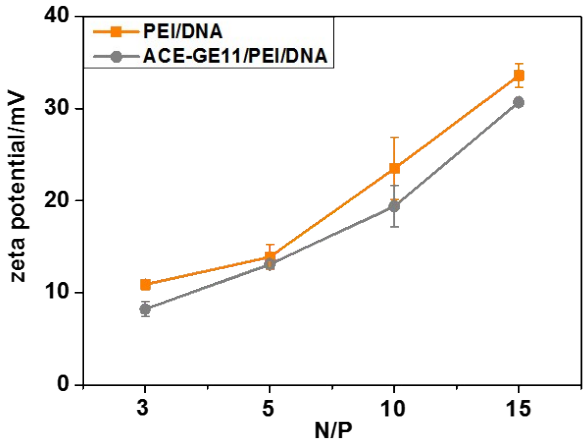


Figure S9. Zeta potential of the complexes of our materials and DNA at different N/P ratios detected by DLS.

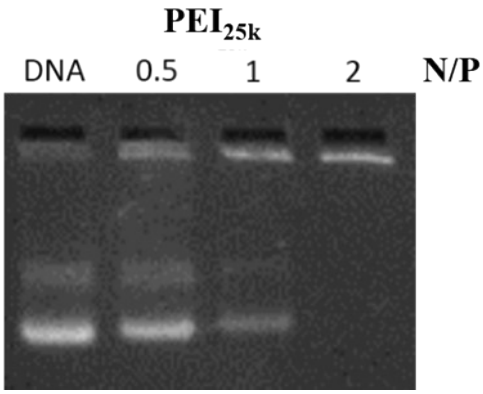


Figure S10. Agarose gel retardation assay of PEI_{25k}/DNA at different N/P ratios.

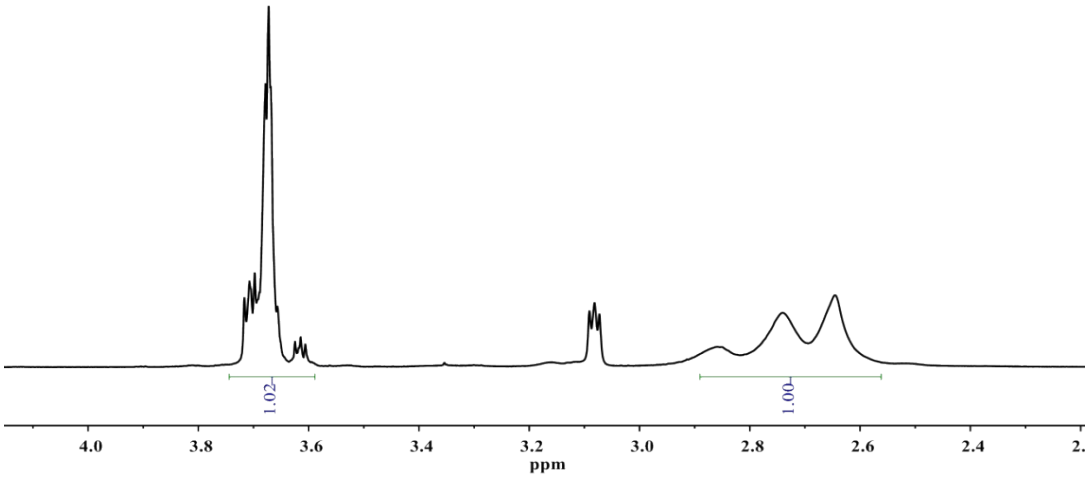


Figure S11. The ^{1}H-NMR spectrum of the complexes of ACE/PEI_{25k} in D_2O.

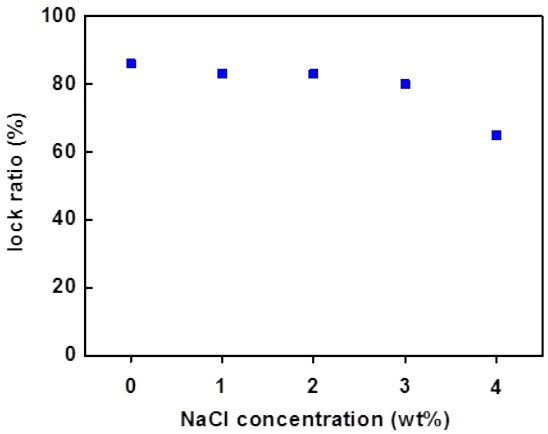


Figure S12. The lock ratio of ACE on PEI in different concentrations of NaCl solution.

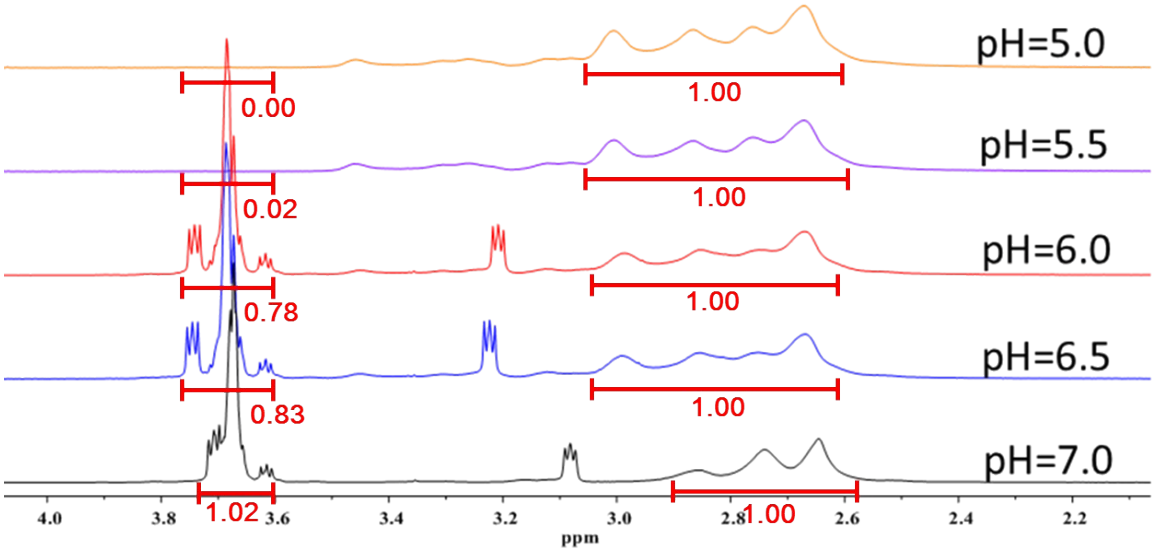


Figure S13. The ^{1}H-NMR spectrum of the complexes of ACE/PEI_{25k} at different pH after dialysis to remove the detached ACE. The integrals of PEI were unified at 1.00.

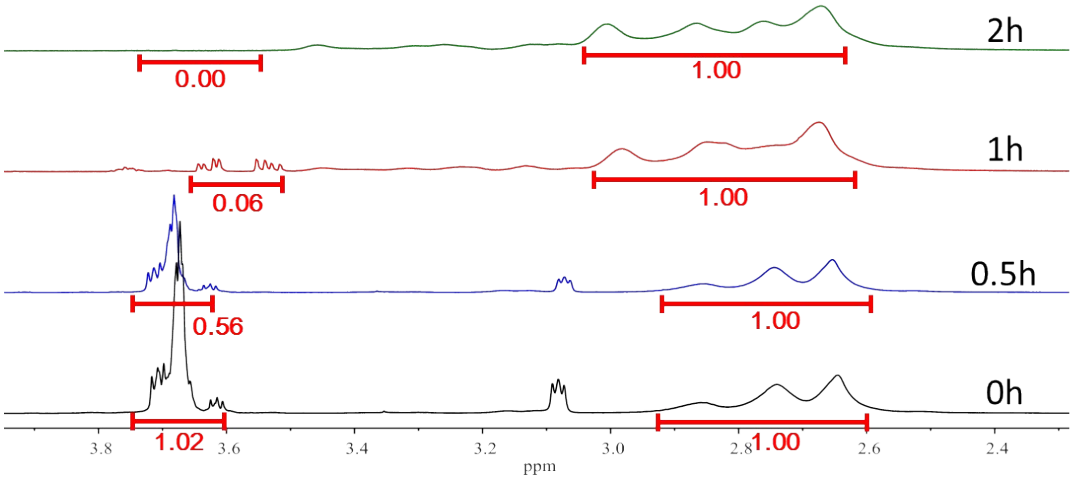


Figure S14. The ^{1}H-NMR spectrum of the complexes of ACE/PEI_{25k} at different times at pH=5.0 after dialysis to remove the detached ACE. The integrals of PEI were unified at 1.00.

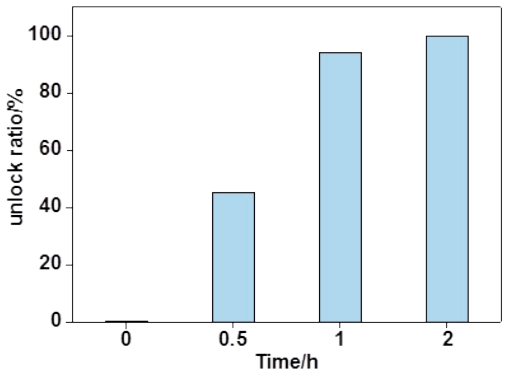


Figure S15. The ratio of ACE unlocked from PEI_{25k} at different times at pH=5.0.

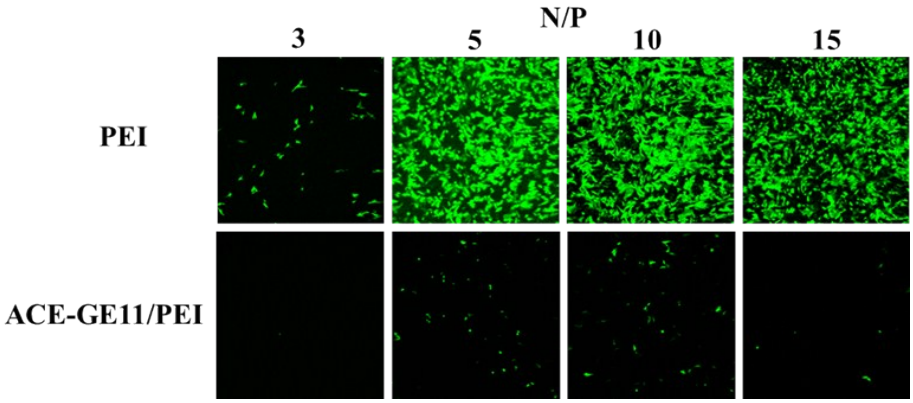


Figure S16. GFP gene transfection of our materials and DNA at different N/P ratios without FBS in NIH-3T3 cells.

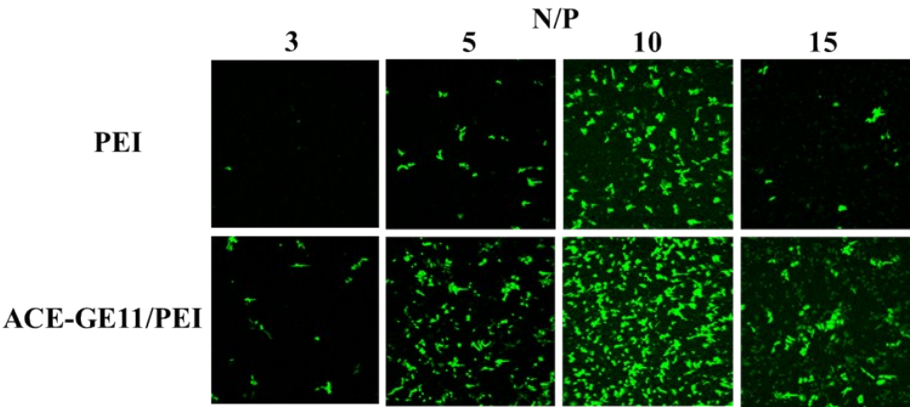


Figure S17. GFP gene transfection of our materials and DNA at different N/P ratios without FBS in A549 cells.

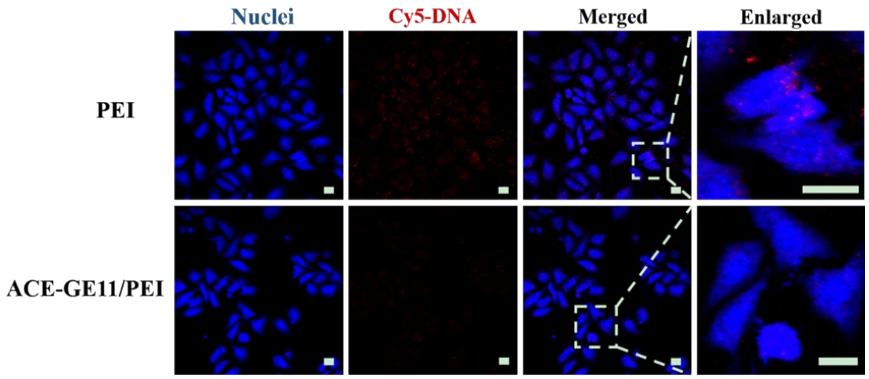


Figure S18. CLSM images of NIH-3T3 cells cultured with PEI/cy5-DNA and ACE-GE11/(PEI/cy5-DNA) complexes in serum-free medium for 1h; Cy5-DNA and cell nuclei stained with DAPI are shown in red and blue, respectively; scale bars: 20μm.

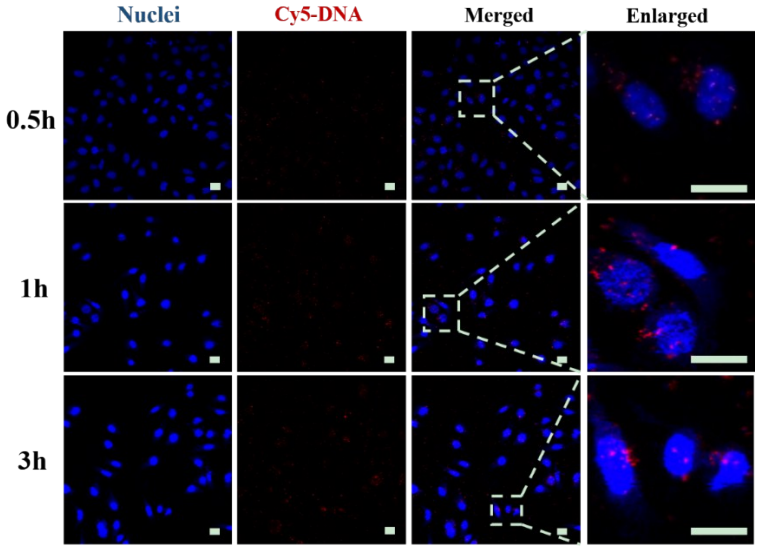


Figure S19. CLSM images of A549 cells cultured with PEI/cy5-DNA complexes in serum-free medium for 0.5h, 1h and 3h; Cy5-DNA and cell nuclei stained with DAPI are shown in red and blue, respectively; scale bars: 20μm.

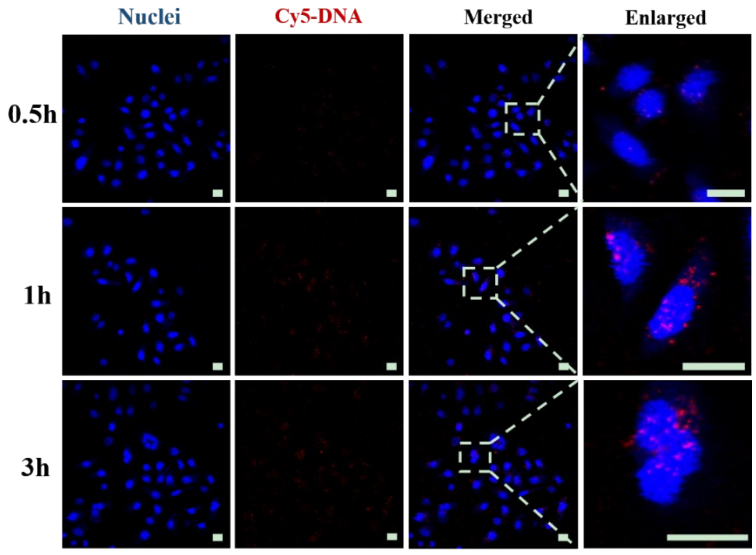


Figure S20. CLSM images of A549 cells cultured with ACE-GE11/(PEI/cy5-DNA) complexes in serum-free medium for 0.5h, 1h and 3h; Cy5-DNA and cell nuclei stained with DAPI are shown in red and blue, respectively; scale bars: 20μm.

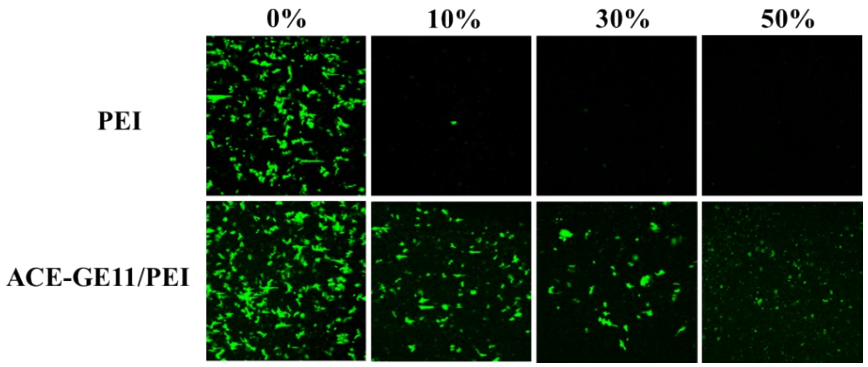


Figure S21. GFP gene transfection of our materials and DNA at N/P=10 with different amount of FBS in A549 cells.

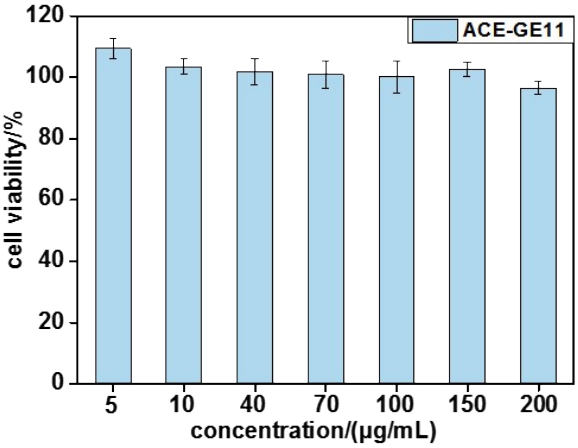


Figure S22. Cell viability of NIH-3T3 cells incubated with different concentrations of ACE-GE11 for 24h. The fluorescence intensity was measured by microplate reader after adding Celltiter-Blue reagent for 4h.

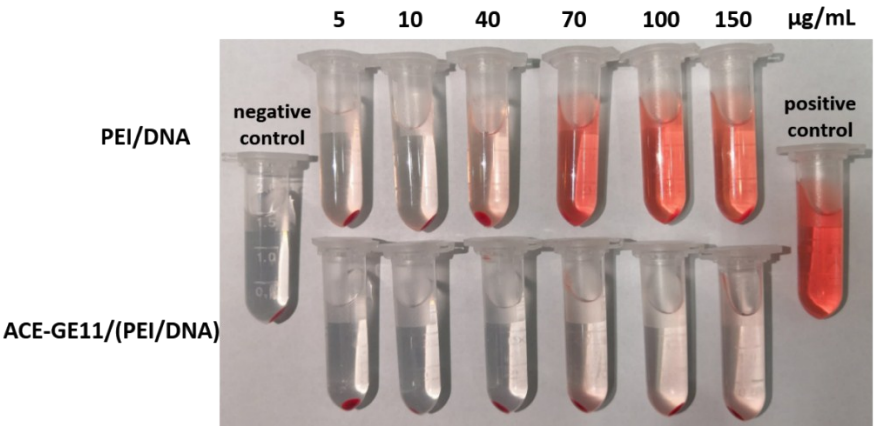


Figure S23. The hemolysis assay of PEI/DNA and ACE-GE11/(PEI/DNA).

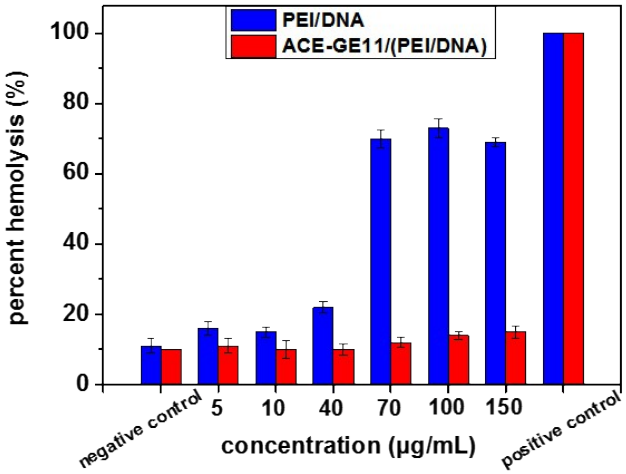


Figure S24. The quantified hemolysis test of PEI/DNA and ACE-GE11/(PEI/DNA).

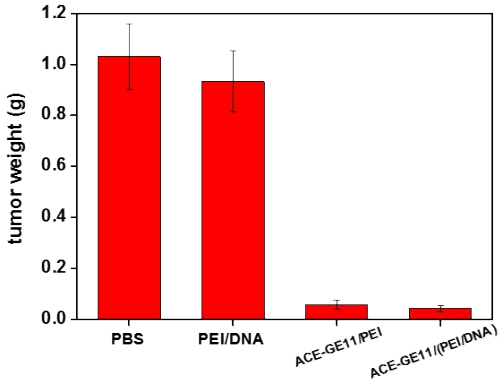


Figure S25. Tumor weights of the mice treated with different samples.

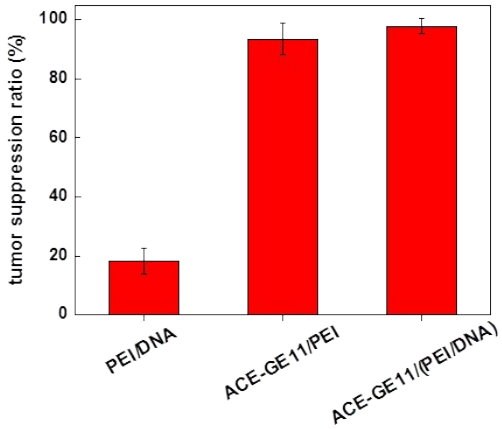


Figure S26. The tumor suppression ratio of different treatment groups.

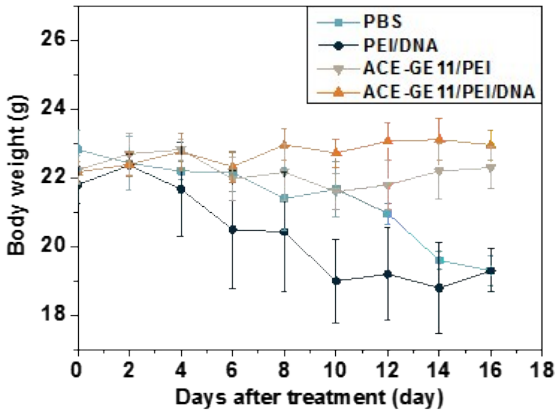


Figure S27. Body weight variation of the mice during the treatment.

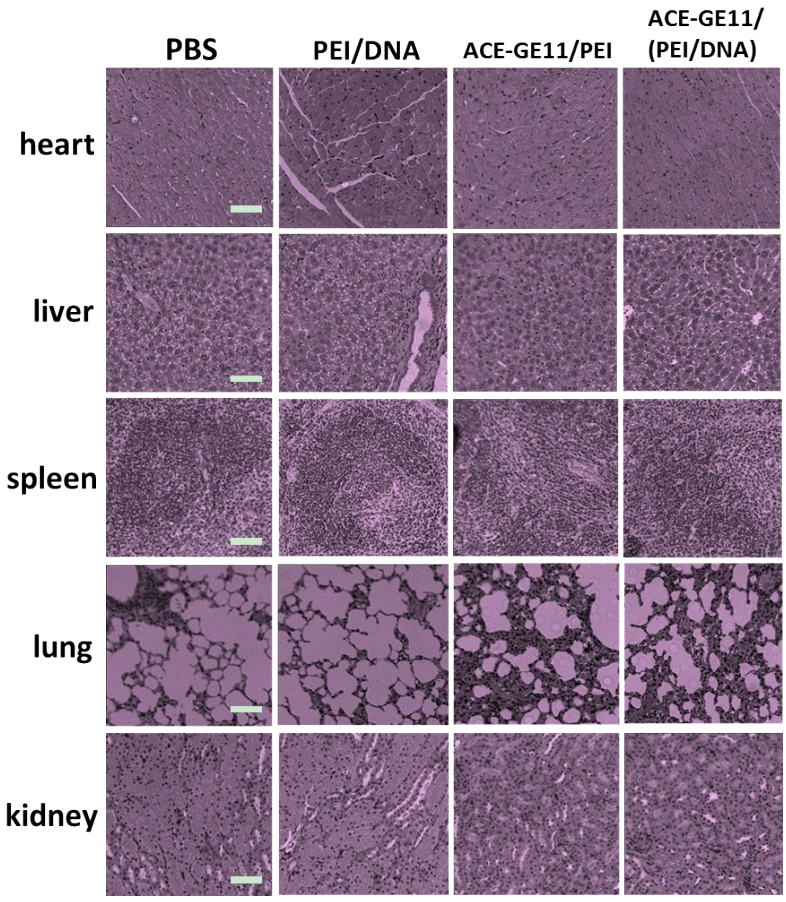


Figure S28. Histological analysis of normal organs treated via various formulations after 18 days. Scale bars: 50μm.

References

[1] H. Maeda, S. Furuyoshi, Y.Nakatsuji, M. Okahara, Bull. *Chem. Soc. Jpn.* **1983,** *56*, 212-218.
[2] L.G. Pap, N. Arulsamy, E. B. Hulley, *Polyhedron* **2018,** *141*, 385-392.