Supplementary Information

Cyclic Poly(ß-amino ester)s with Enhanced Gene Transfection Activity Synthesized through Intra-molecular Cyclization

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Materials and Methods

Materials

4-amino-1-butanol (S4, Energy Chemical, 97%), 1,4-butanediol diacrylate (B4, Aladdin, >90% purity), 2,2'-Azobis(2-methylpropionitrile) (AIBN, Meryer, 99%), dimethyl sulfoxide (DMSO, Shanghai Macklin, 99.9%), dimethylformamide (DMF, J&K Scientific, 99.9%), diethyl ether (Sinopharm chemical reagent Co. Ltd, 99%), and deuterated chloroform ($CDCl_3$, Shanghai Macklin, 99.9%) were used as received. Picogreen dsDNA Quantitation Reagent (Yeasen Biotechnology), alamarBlue (Yeasen Biotechnology), Amplite Gaussia Luciferase cellular assay kit (AAT Bioquest) were used as per manufacturers' protocols. Plasmid encoding green fluorescence protein (pCMV-GFP) was purchased from Aldvron (USA), plasmid encoding cell secreted Gaussia luciferase (pCMV-Gluc) was obtained from New England Biolabs (UK).

LPAE synthesis and purification

LPAEs were synthesized *via* the "A2+B2" Michael addition strategy according to previous publications [1]. Firstly, B4 (0.792 g) and S4 (0.356 g) with the stoichiometric ratio of acrylate to amine at 1.0:1.0, were mixed to react at 90℃ under magnetic stirring. Gel permeation chromatography (GPC) was used to monitor the growth of weight average molecular weight (M_w), number average molecular weight (M_n) and polydispersity index ($Đ$). When the M_w was approaching to 50 kDa or 20 kDa, the reaction was stopped by cooling down to room temperature (RT) and then diluted with DMSO to a final concentration of 100 mg/mL. B4 (0.396 g) was further added to end-cap the polymer at RT for another 48 hours. To purify the crud LPAE, the reaction

mixture was added slowly to excessive diethyl ether under vigorous stirring over 15 min, and then kept undisturbed for 5 minutes. Afterwards, the supernatant was removed, the precipitation process was repeated three times. To end-cap the LPAE, excessive S4 was added into the LPAE solution and the mixture with a final concentration of 100 mg/ml was maintained to react at room temperature for 48 hours.

CPAE synthesis and purification

Taking the synthesis of CPAE-1 as an example, other CPAEs were synthesized in a similar manner. Briefly, LPAE-1 (3.800 g) and AIBN (0.008 g) were dissolved in butanone (50.0 ml). Oxygen was removed by bubbling argon through the mixture for 30 min at RT. The mixture was then reacted at 70℃ under magnetic stirring at 600 rmp. GPC was used to monitor the MW of polymer during the reaction process. To stop the reaction, the mixture was exposed to air and cooled down to RT. To purify the crud CPAE, the reaction mixture was added slowly to excessive diethyl ether under vigorous stirring over 15 min, and then kept undisturbed for 5 minutes. Afterwards, the supernatant was removed, the precipitation process was repeated three times.

GPC measurements

M_w, M_n and $Đ$ of polymers were determined by an Agilent 1260 infinity Ⅱ gel permeation chromatography (GPC) equipped with a refractive index (RI) detector. 10.0 mg of sample was dissolved in 1 mL of DMF containing 0.1% LiBr, and then filtered through a 0.22 μm filter. GPC columns (PolarGel-M Gard, 50 mm × 7.5 mm, and PolarGel-M, 300 mm × 7.5 mm, two in series) were eluted with DMF (plus 0.1% LiBr)

at a flow rate of 1 mL/min at 50 °C. GPC column were calibrated with linear poly(methyl methacrylate) (PMMA) standards.

NMR measurements

Chemical composition and structures of polymers were confirmed by nuclear magnetic resonance spectra (^{1}H NMR). Polymers were dissolved in $CDCl_3$ and NMR measurements were carried out on a Varian Inova 400 MHz Spectrometer (Bruker, Switzerland). The chemical shifts of sample were reported in parts per million (ppm) relative to the solvent $CDCl_3$ (7.26 ppm).

Polyplex formulation

Polyplexes were prepared according to previous studies[2]. Briefly, polymers were first dissolved in DMSO to form a stock solution of 100 mg/mL. According to the polymer/DNA weight ratio (w/w), polymer stock solution and DNA were diluted to 10 μL with sodium acetate solution (0.025 M, pH = 5.2), respectively. And then, polymer solution was added to DNA solution and vortexed at a high speed for 25 ~ 30 seconds. The mixed solution was kept undisturbed for 25 min to allow for polyplex formation.

Size and zeta potential measurement of polyplexes

Typically, 0.5 μg of DNA was used for each sample preparation. Polyplexes were prepared as mentioned above and then diluted to 1.5 mL with deionized water or sodium acetate solution (0.025 M, pH=5.2). Size and zeta potential were measured with a Malvern Instruments Zetasizer (Nano ZSE) at a scattering angel of 90°. All experiments were repeated at least four times.

DNA binding affinity of CPAEs

1 μg of DNA was used for each sample preparation. Polyplexes with 3 μg of DNA were prepared as above and then equal volume of Picogreen solution (prepared by diluting 80 μl of Picogreen with 15.2 mL of sodium acetate, 0.025 M, pH = 5.2) was added and incubated for another 5 minutes. Afterwards, the mixture solution was aliquoted for three and diluted by 200 μL of serum-free Dulbecco's Modified Eagle Medium (DMEM) in a black 96-well plate. Fluorescence measurements were carried out using a plate reader (Synergy Hybrid H1, Biotek) with an excitation at 490 nm and an emission at 535 nm. All experiments were repeated at three times. Samples prepared with naked DNA were used as negative controls, samples prepared without DNA were used as blanks. DNA binding affinity of CPAEs was calculated as below:

DNA binding affinity (%) = $1-(F_{Sample}-F_{Blank})/ (F_{DNA}-F_{Blank})$ (1)

Where the F_{Sample}, F_{DNA} and F_{Blank} are the fluorescence intensity of sample, control and blank, respectively.

Cell culture

Human embryonic kidney 293T (293T) cells obtained from ATCC were cultured in DMEM containing 1% penicillin/streptomycin (P/S) and 10% FBS. All the cells were cultured in a humid incubator (37 °C, 5% CO_2) under standard culture conditions.

Gene transfection efficiency evaluation

293T cells were seeded in 96-well plates at a density of 2.5×10^4 cells/well and cultured at 37 °C overnight. GFP and Gluc plasmids were used as reporter genes to evaluate the gene transfection efficiency of polymers. 0.5 μg of DNA was used for each well, polyplexes were prepared at the w/w ratio of 50:1, 100:1, 150:1 and 200:1,

respectively. Afterwards, 80 μL of fresh culture medium was added to dilute the polyplexes solution to 100 μL. Cell supernatant was removed, and then 100 μL of polyplex-containing DMEM was added. Four hours later, the medium was replaced with fresh medium, and cells were cultured for another 44 hours. The expression of GFP was visualized with a fluorescence microscope (Olympus CKX53). To quantify the Gluc activity of cells after transfection, 60 μL of cell supernatant was harvested and transferred to the wells of a white plate, and then 80 μL of the pre-prepared Gluc Assay Work Solution was added and the intensity of luminescence, in terms of relative light unit (RLU), of the mixture solution was detected using a microplate reader (Synergy Hybrid H1, Biotek) immediately. All experiments were conducted in triplicates.

Cell viability evaluation

The viability of cells after transfection was measured with alamarBlue assay. Briefly, after transfection, cell supernatant was removed, cells were washed using phosphate-buffered saline solution (PBS, 0.1 M, pH = 7.4) twice, then 100 μL of alamarBlue solution (diluted to 10% with DMEM) was added. Cells were incubated for another 1 to 4 hours, and then fluorescence intensity of the supernatant was measured with a microplate reader (Synergy Hybrid H1, Biotek) with an excitation at 530 nm and emission at 590 nm. Fluorescence intensity of cells without any treatment is defined as 100% viability.

Statistical Analysis

Gluciferase activity and cell viability were analyzed by Student's *t*-test, results are

presented as average value ± standard deviation (SD). Average value and SD are calculated from at least three independent experimental results. p value < 0.05 is considered statistically significant.

Table S1. Feed ratio of monomers for the synthesis of LPAE-1

	Feed Ratio (mole)	**Stoichiometric Ratio**	**Mass Ratio**
S4	4.0	4.0	0.356 g
B4	4.0	4.0	0.792 g
DMSO (for end-capping)			10.3 mL
B4	2.0	2.0	0.396 g

Table S2. MW and *Đ* of LPAE-1 during polymerization

Time (h)	M_n **(kDa)**	M_w **(kDa)**	***Đ***
48	4.4	14.2	3.2
72	4.8	17.3	3.6
80	5.1	23.9	4.7
96	5.2	37.8	7.3
100	5.5	48.0	8.7
After purification	7.7	51.4	6.7

Table S3. Feed ratio of monomers for the synthesis of CPAE-1

	Feed Ratio (mole)	**Mass Ratio**
LPAE-1	1	5.140g
AIBN	0.5	0.008 g
Butanone		50 mL

Table S4. MW and *Đ* of CPAE-1 during intra-molecular cyclization

Time (h)	M_n **(kDa)**	M_w **(kDa)**	***Đ***
0	7.7	51.4	6.7
2	8.1	51.2	6.3
5	8.1	51.0	6.3
24	8.4	50.8	6.1
48	8.0	51.1	6.3

Table S5. Feed ratio of monomers for the synthesis of CPAE-2

	Feed Ratio(mole)	**Mass Ratio**
LPAE	1	5.140g
AIBN	1	0.016 g
Butanone		50 mL

Table S6. MW and *Đ* of CPAE-2 during intra-molecular cyclization

Time (h)	M_n **(kDa)**	M_w **(kDa)**	***Đ***
0	7.7	51.4	6.7
48	12.6	33.6	2.7

Table S7. Feed ratio of monomers for the synthesis of CPAE-3

	Feed Ratio (mole)	**Mass Ratio**
LPAE	1	5.140 g
AIBN	4	0.066 g
Butanone		50 mL

Table S8. MW and *Đ* of LPAE-2 during polymerization

Time (h)	M_w **(kDa)**	M_w **(kDa)**	***Đ***
5	4.6	8.1	1.8
10	6.9	17.0	2.5
After purification	8.6	18.7	2.2

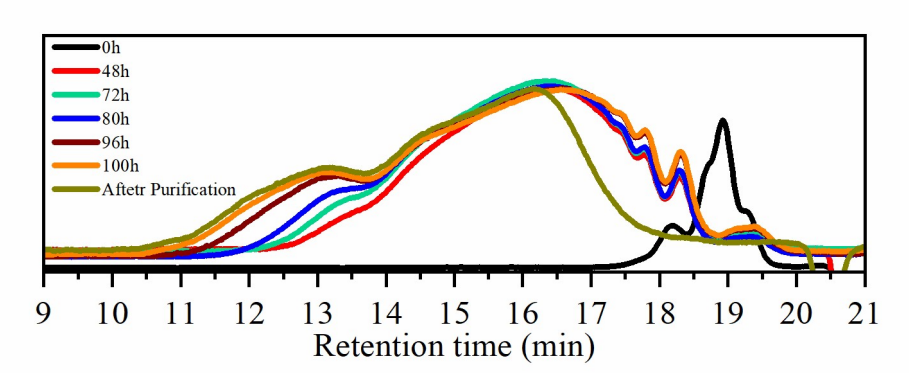

Figure S1. MW evolution of LPAE-1 during the polymerization process.

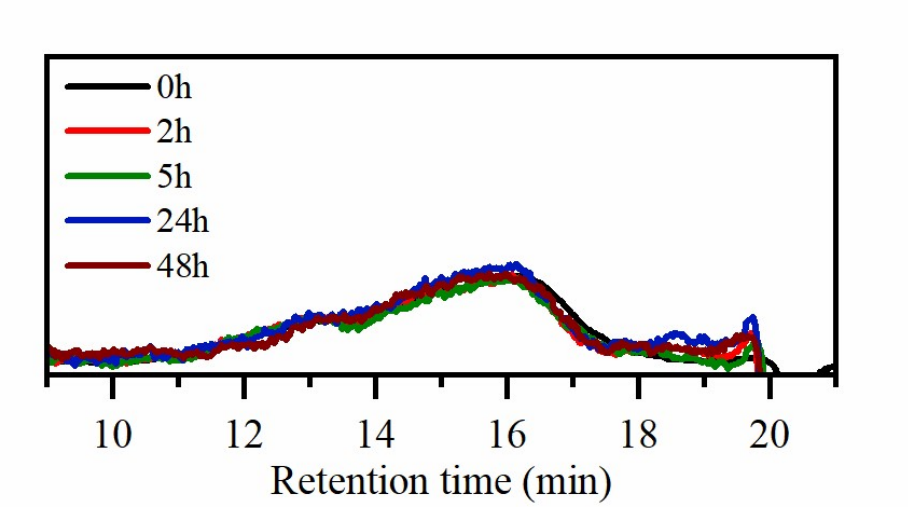

Figure S2. MW evolution of CPAE-1 during intra-molecular cyclization process.

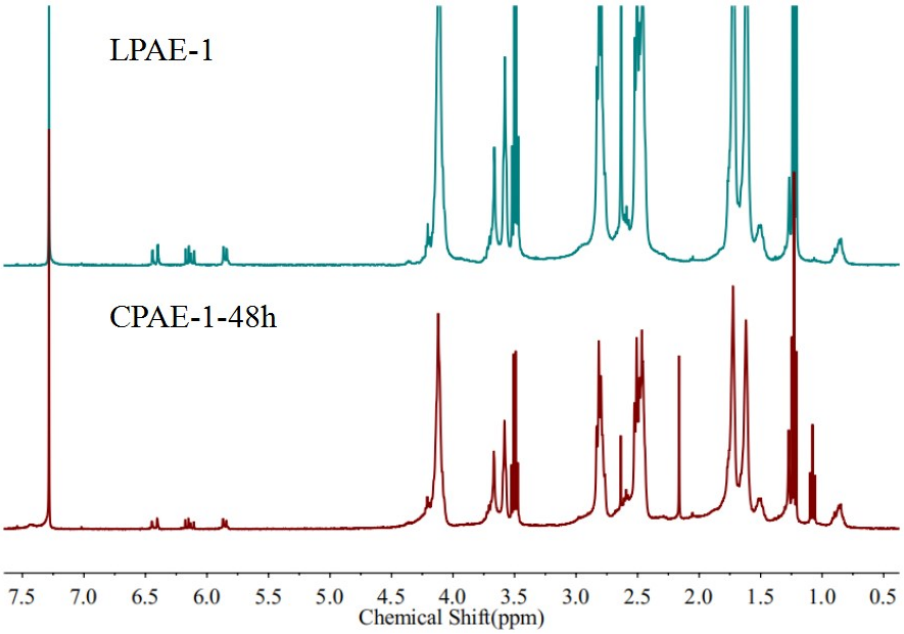

Figure S3. ^{1}HNMR spectra of LPAE-1 and CPAE-1.

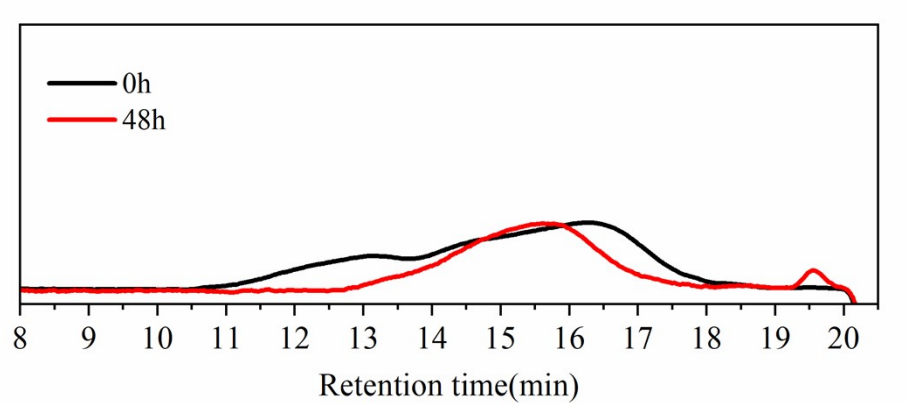

Figure S4. MW evolution of CPAE-2 during intra-molecular cyclization process.

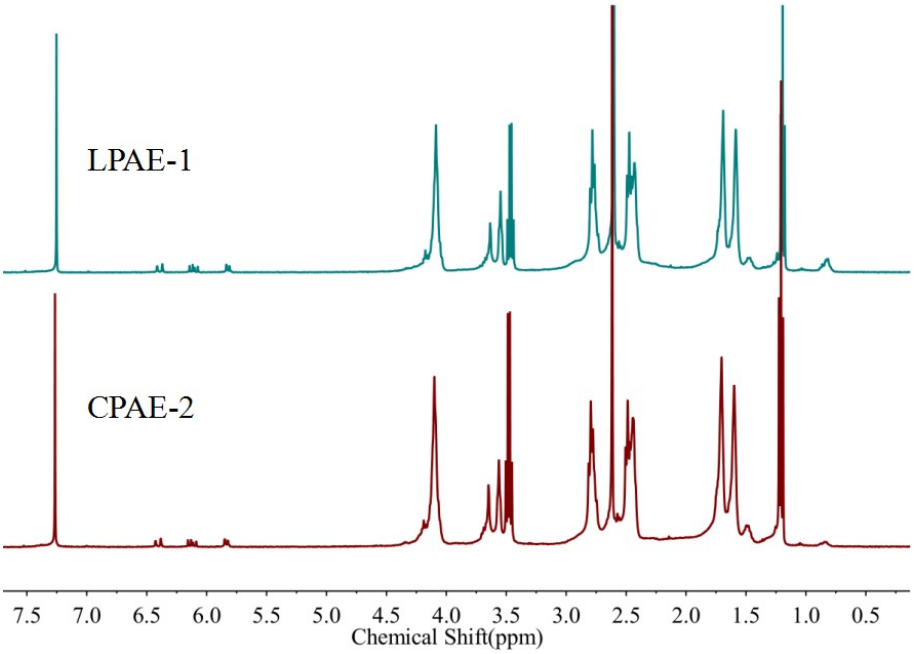

Figure S5. ^{1}HNMR spectra of LPAE-1 and CPAE-2.

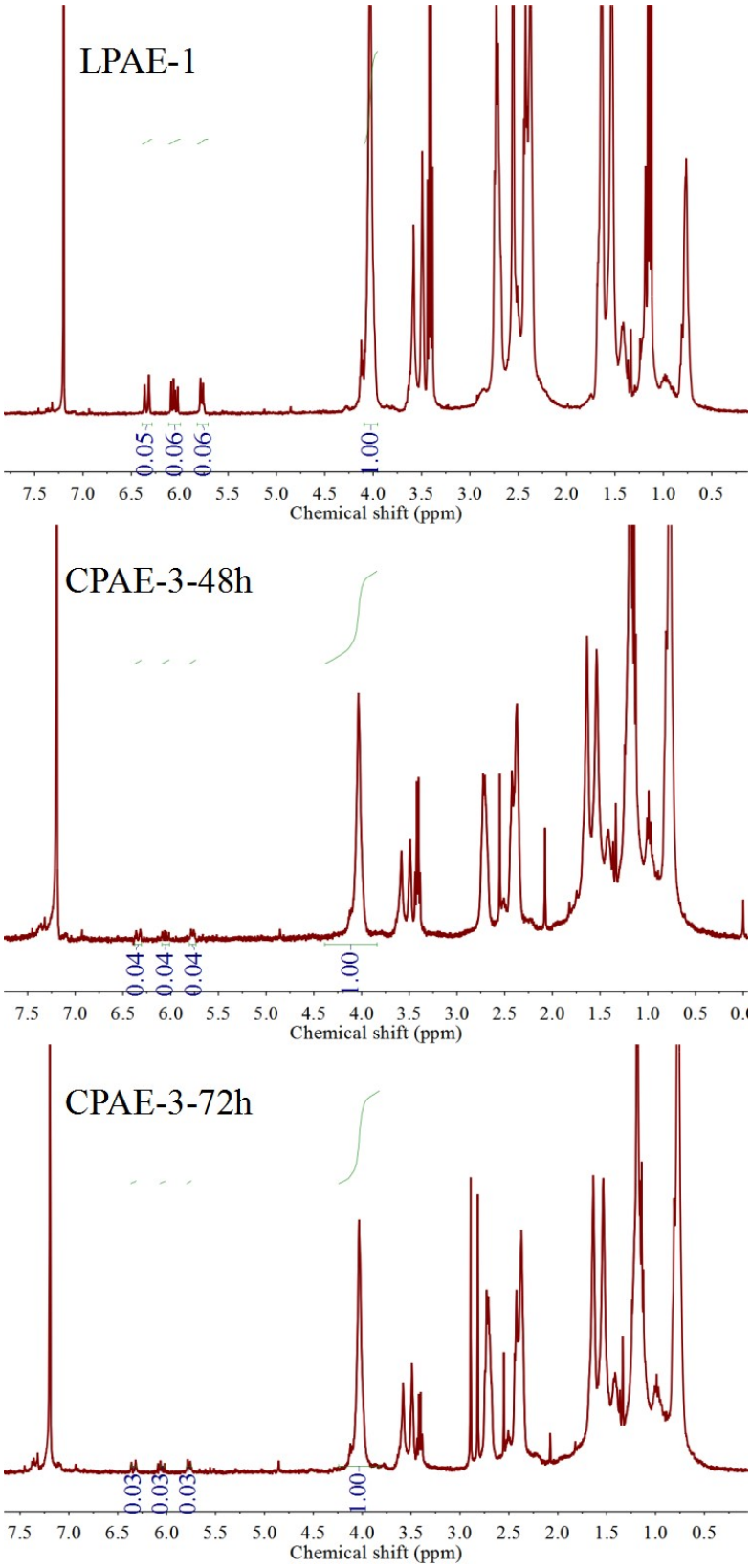

Figure S6. ^{1}HNMR spectra of LPAE-1, CPAE-3-48h, and CPAE-3-72h.

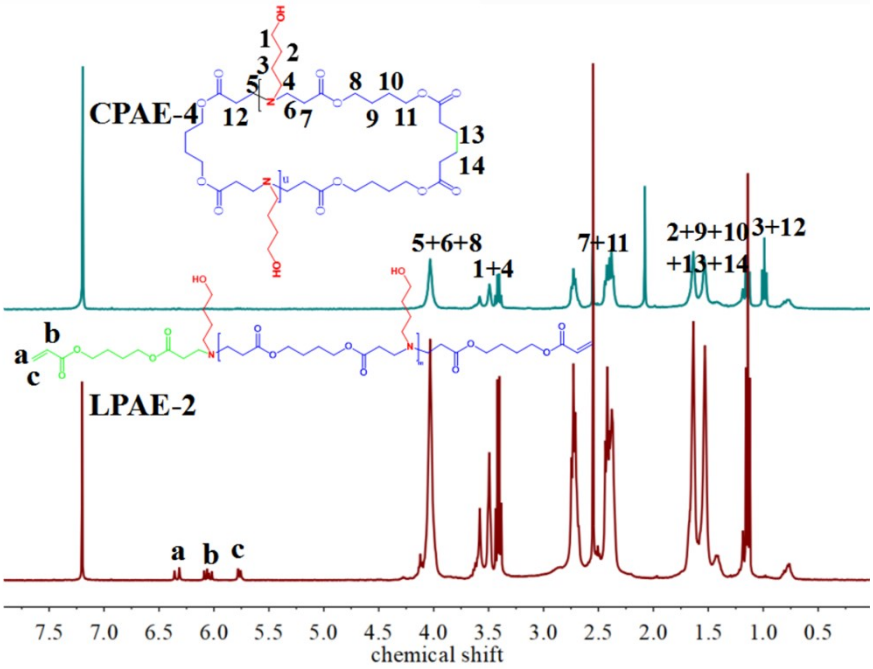

Figure S7. ^{1}HNMR spectra of LPAE-2 and CPAE-4.

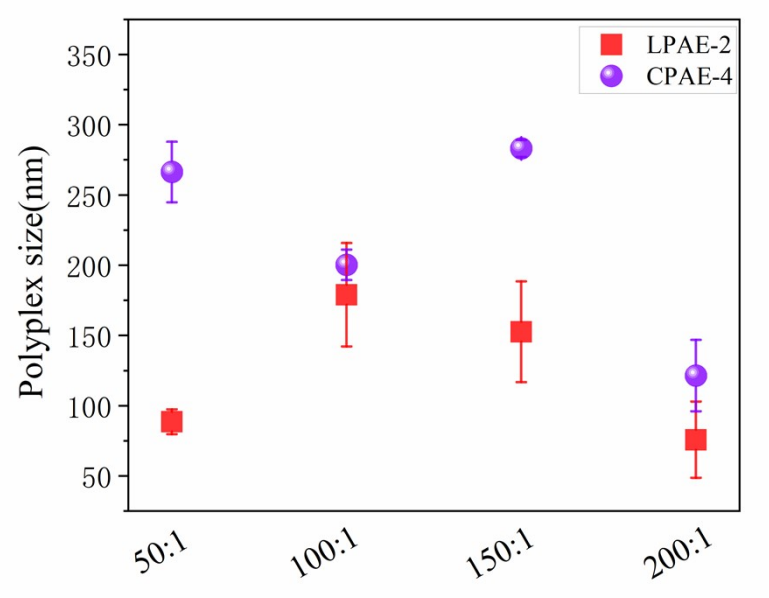

Figure S8. Size of LPAE-2/DNA and CPAE-4/DNA polyplexes in sodium acetate solution (0.025 M, pH = 5.2).

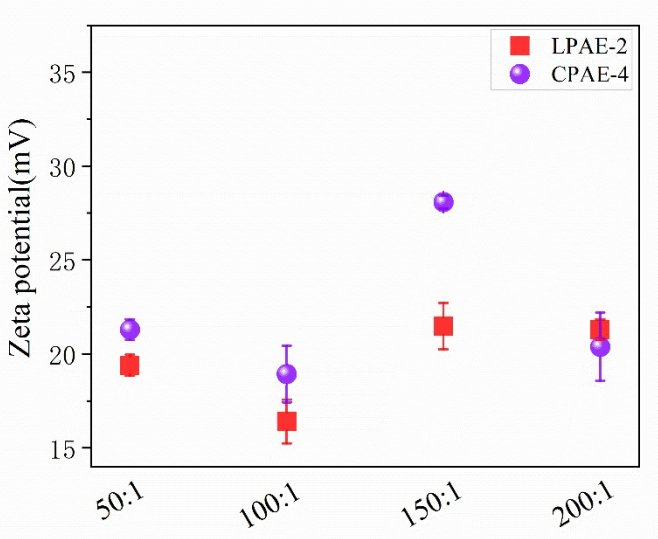

Figure S9. Zeta potential of LPAE-2/DNA and CPAE-4/DNA polyplexes in sodium acetate solution (0.025 M, pH = 5.2).

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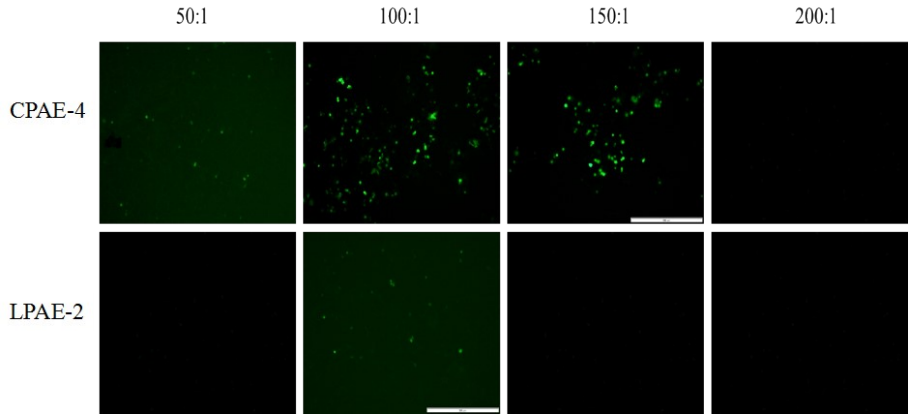

Figure S10. GFP images of 293T cells 48 hours post transfection with CPAE-4 and LPAE-2.

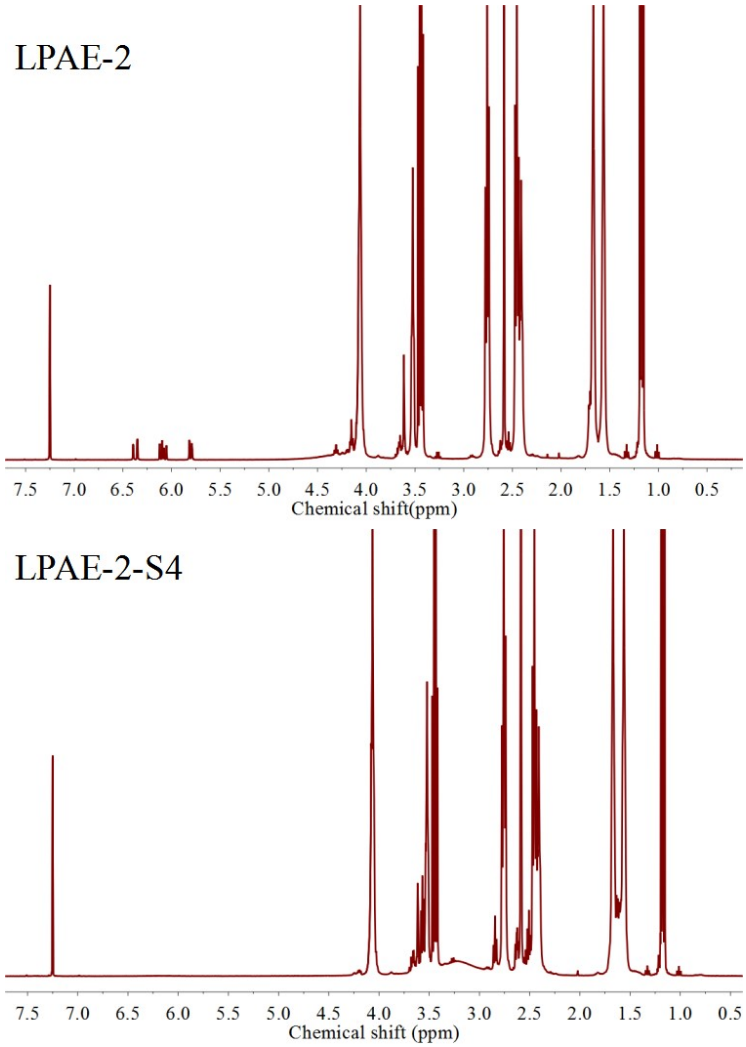

Figure S11. ^{1}HNMR spectra of LPAE-2 before and after end-capping (denoted LPAE-2-S4). It can be seen that after end-capping, all the vinyl groups have been consumed.

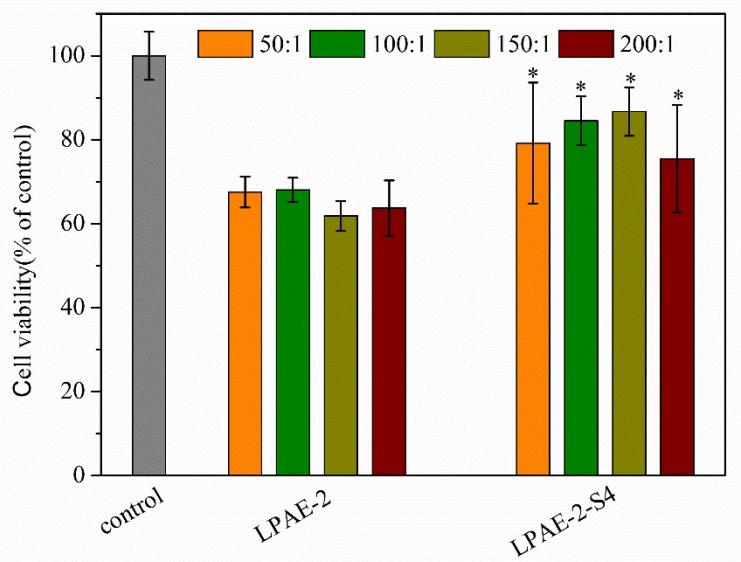

Figure S12. Viability of 293T cells after incubation with polyplexes formulated by LPAE-2 or LPAE-2-S4 with DNA for 48 hours.

References

1. S. Liu, Y. Gao, D. Zhou, M. Zeng, F. Alshehri, B. Newland, J. Lyu, J. O'Keeffe-Ahern, U. Greiser, T. Guo, F. Zhang and W. Wang, *Nat. Commun.*, 2019, **10**. 3307.

2. D. Zhou, L. Cutlar, Y. Gao, W. Wang, J. O'Keeffe-Ahern, S. McMahon, B. Duarte, F. Larcher,B. J. Rodriguez, U. Greiser and W. Wang, *Sci. Adv.*, 2016, **2**. e1600102.