Supporting information for

Polyamino acid-based phosphatidyl polymer library for *in vivo* mRNA delivery with spleen targeting ability

Hanqin Zhao [a,b], Sheng Ma [a,c], Yibo Qi [a,b] , Yuxi Gao [a,d], Yuyan Zhang [a,b], Minhui Li [a,b], Jie Chen [a,c], Wantong Song [a,b,c*], Xuesi Chen [a,b,c*]

[a] Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, 130022, China

[b] School of Applied Chemistry and Engineering, University of Science and Technology of China, Hefei, 230026, China

[c] Jilin Biomedical Polymers Engineering Laboratory, Changchun, 130022, China

[d] State Key Laboratory of Supramolecular Structure and Materials, College of Chemistry, Jilin University, Changchun, 130012, China

Materials

5-benzyl L-glutamate N-carboxyanhydride (Chengdu Enlai biological technology Co.,Ltd., > 99.0%) were used as received. Glycidol, ethylene glycol chlorophosphite, Pluronic F-127, propylamine, n-butylamine, n-hexylamine, n-octylamine, N,N-diisopropylethylenediamine, 2-morpholinoethylamine, Di-n-hexylamine, diethylenetriamine, n-octanol, n-decyl alcohol, 1-dodecanol, 1-tetradecanol, 1-hexadecanol, 1-octadecanol, N,N'-diisopropylcarbodiimide (DIC) and 4-dimethylaminopyridine (DMAP) were purchased from Macklin Biochemical Technology (Shanghai). Organic solvents hexane, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), and tetrahydrofuran (THF) were purchased from Energy Chemical. Dulbecco's modified phosphate buffered saline (PBS), Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), and trypsin-EDTA (0.25%) were obtained from Meilunbio. EGFP and luciferase encoding mRNAs were purchased from Golden Transfer Science and Technology (Changchun, China).

TransDetect® Single-Luciferase（Firefly）Reporter Assay Kit were purchased from TransGen Biotech (Beijing, China).

Instruments

^{1}H NMR spectra were performed on a Bruker AV-300 or Bruker AV-500 NMR spectrometer. Polyplex sizes and ζ potentials were measured by Dynamic Light Scattering (DLS) using a Malvern Zetasizer Nano ZS (He-Ne laser, λ = 632 nm). Flow cytometry analysis was performed on BD FACSCelesta Flow Cytometer. Confocal laser scanning microscope images were obtained via ZEISS LSM 780 Laser Scanning Microscope. The fluorescent and bioluminescent images were obtained via PerkinElmer IVIS® Lumina LT Series III.

Synthesis of polyamino acid backbone PLG

Poly (L-glutamic acid) (PLG) was prepared via the ring-opening polymerization of 5-benzyl L-glutamate N-carboxyanhydride (Glu-NCA). The Glu-NCA (5.27g, 20.0 mmol) and anhydrous DMF (50 mL) were prepared in 250 mL dried flasks and stirred at room temperature until the Glu-NCA dissolved. The initiator n-hexylamine (102 mg,

1.01 mmol) was then added and the solution was stirred for 72 h at 30 °C. Acetic anhydride (510 mg, 5.00 mmol) was then added and the solution was reacted at 30 °C for 12 h for blocking residual amino groups. The precursor products protected by benzyl groups were obtained through ether precipitation and deprotected by acetic acid solution of hydrobromic acid. The crude products were dialyzed (MWCO 1000) with deionized water for 3 days and freeze-dried to obtain the product (yield 74%). The PLG had an average of 18 L-glutamic acid repeating units according to the ^{1}H-NMR spectra.

Synthesis of cationic polymers PLG-*g*-An

PLG (1.30 g, 10.0 mmol -COOH), DMAP (1.47 g, 12.0 mmol) and DIC (1.89 g, 15.0 mmol) was dissolved in anhydrous DMF (50 mL) in 250 mL dry flasks. Glycidol (889 mg, 12.0 mmol) was added and reacted for 24 h under a nitrogen atmosphere at 50 °C. The crude product was precipitated in ether three times and vacuum dried for 24 h to give PLG-g-epoxy polymer (yield 86%). The grafting rate of epoxy groups was calculated as 67.8% based on the ^{1}H-NMR spectra.

Then, PLG-*g*-epoxy polymer (200 mg) was reacted with excess amine A1-A8 (epoxy/amine molar ratio, 1/5) in DMF at 50 °C for 48 h to obtain PLG-*g*-An polymer. The product was precipitated in ether three times and vacuum dried for 24 h to give the PLG-*g*-An polymer (yield all above 85%).

Synthesis of alkylated dioxaphospholane oxide molecules (PLs)

Esterification of 2-chloro-2-oxo-1,3,2-dioxophosphane (COP) with corresponding alkylated alcohols were utilized to prepare alkylated dioxyphosphate oxide molecules (PLs). The alkylated alcohols (1.00 mmol) and triethylamine (106 mg, 1.05 mmol) were dissolved in anhydrous tetrahydrofuran (20 mL). Subsequently, dissolve COP (150 mg, 1.05 mmol) in THF (10 mL) and drop the solution into the mixture at under ice water bath. The reaction continued overnight at 25 °C. Triethylamine hydrochloride was removed via filter and the filtrate was evaporated to obtain PLs molecules. The yield of PLs molecules was above 90%.

PL1: ^{1}H NMR (CDCl$_3$, ppm): δ 0.89 (t, 3H, -CH$_2$CH$_2$CH$_2$CH$_3$), 1.27 (m, 10H, -CH$_2$(CH2)$_5$CH$_3$), 1.69 (m, 2H, -OCH$_2$CH$_2$CH$_2$CH$_2$-), 4.15 (m, 2H, -OCH$_2$CH$_2$CH$_2$CH$_2$-), 4.40 (m, 4H, OCH$_2$CH$_2$O-).

PL2: ^{1}H NMR (CDCl$_3$, ppm): δ 0.88 (t, 3H, -CH$_2$CH$_2$CH$_2$CH$_3$), 1.26 (m, 10H, -CH$_2$(CH2)$_5$CH$_3$), 1.69 (m, 2H, -OCH$_2$CH$_2$CH$_2$CH$_2$-), 4.15 (m, 2H, -OCH$_2$CH$_2$CH$_2$CH$_2$-), 4.40 (m, 4H, OCH$_2$CH$_2$O-).

PL3: ^{1}H NMR (CDCl$_3$, ppm): δ 0.88 (t, 3H, -CH$_2$CH$_2$CH$_2$CH$_3$), 1.26 (m, 10H, -CH$_2$(CH2)$_5$CH$_3$), 1.67 (m, 2H, -OCH$_2$CH$_2$CH$_2$CH$_2$-), 4.15 (m, 2H, -OCH$_2$CH$_2$CH$_2$CH$_2$-), 4.40 (m, 4H, OCH$_2$CH$_2$O-).

PL4: ^{1}H NMR (CDCl$_3$, ppm): δ 0.87 (t, 3H, -CH$_2$CH$_2$CH$_2$CH$_3$), 1.25 (m, 10H, -CH$_2$(CH2)$_5$CH$_3$), 1.66 (m, 2H, -OCH$_2$CH$_2$CH$_2$CH$_2$-), 4.13 (m, 2H, -OCH$_2$CH$_2$CH$_2$CH$_2$-), 4.38 (m, 4H, OCH$_2$CH$_2$O-).

PL5: ^{1}H NMR (CDCl$_3$, ppm): δ 0.87 (t, 3H, -CH$_2$CH$_2$CH$_2$CH$_3$), 1.25 (m, 10H, -CH$_2$(CH2)$_5$CH$_3$), 1.68 (m, 2H, -OCH$_2$CH$_2$CH$_2$CH$_2$-), 4.13 (m, 2H, -OCH$_2$CH$_2$CH$_2$CH$_2$-), 4.37 (m, 4H, OCH$_2$CH$_2$O-).

PL6: ^{1}H NMR (CDCl$_3$, ppm): δ 0.86 (t, 3H, -CH$_2$CH$_2$CH$_2$CH$_3$), 1.24 (m, 10H, -CH$_2$(CH2)$_5$CH$_3$), 1.66 (m, 2H, -OCH$_2$CH$_2$CH$_2$CH$_2$-), 4.13 (m, 2H, -OCH$_2$CH$_2$CH$_2$CH$_2$-), 4.39 (m, 4H, OCH$_2$CH$_2$O-).

Synthesis of phospholipidated polymer PLG-PPs and Cy5-PLG-PPs

Phospholipidated polymer PLG-PPs was prepared by orthogonal reaction using cationic polyamino acids (PLG-*g*-An) and PLs molecules as raw materials. The PLs modified on each PLG-*g*-An polymer were approximately 50% of the mole number of amine groups. The required amount of PLG-*g*-An polymer and PLs molecules were reacted in anhydrous DMSO (with an initial raw material concentration of 10 mg/mL) at 75 °C for 48 h. Finally, the DMSO solvent was removed through vacuum drying to obtain PLG-PPs polymer library. The subsets of the library were named PAn-PLm based on the combination of different An (n=1-8) and PLm (m=1-6). Crude PLG-PPs were used for initial mRNA transfer screening experiments. The polymers with good performance were redissolved by DMF and precipitated three times with ether. The precipitant was vacuum dried for 24 h and then used for *in vivo* evaluation.

For Cy5-PLG-PPs, it is obtained via the reaction between Cy5-NH$_2$ and the residual carboxyl groups on PLG-PPs. Specifically, PLG-PPs (20 mg) and anhydrous DMF (10 mL) were added in a 50 mL dry flask and stirred at room temperature until

PLG-PPs dissolve. Then 0.58 mg (3.0 μmol) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) and 0.29 mg (2.5 μmol) of N-hydroxy succinimide (NHS) were added to the solution and stirred at room temperature to activate the carboxyl groups. 30 min later, 1 mg (1.5 μmol) of Cy5-NH$_2$ were added and the solution was stirred at 50 °C in a nitrogen atmosphere for 24 hours. The crude product was dialyzed with deionized water under dark (MWCO 3500) for 3 days and finally be freeze-dried (yield 82%).

pK$_a$ measurement of individual polymers

Firstly, 0.5 mg PLG-PPs were dissolved in 5 mL NaCl saturated ethanol. Then, dilute 0.01 M HCl aqueous solution was added to the ethanol solution mentioned above for 10 μL each time and the resulting pH values were recorded. The pK$_a$ of PLG-PPs was calculated based on Henderson-Hasselbalch equation.

Polyplex preparation and characterization

PLG-PPs polymer was dissolved in ethanol and pre acidified with triple volume citric acid buffer (10 mM, pH 4.4). Mix mRNA diluted with citric acid buffer with the above solution using a pipette and let stand for 15 min. Fix the weight ratio of ZPP/mRNA at 50/1. Subsequently, 2.5 wt.% F127 was mixed in the aforementioned mixed solution and incubated for 15 min to obtain the PLG-PPs/mRNA polyplex. Dilute the polymer to the required concentration with 1× PBS buffer for subsequent experimental verification.

Dynamic light scattering (DLS) was used to analyze the size, polydispersity (PDI), and ζ potential of the polyplexes via Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The hydrodynamic diameter of nanoparticles was reported in percentage intensity mode and is the average of three independent measurements.

Cell culture

Human Embryonic Kidney cells (293T Cells) were cultured in Dulbecco's Modified Eagle's Medium (4500 mg L^{-1} glucose) with 10% FBS and 1% Penicillin/Streptomycin (P/S). Cells were cultured at 37 °C and 5% CO$_2$ in a humidified atmosphere.

In vitro mRNA delivery

For *in vitro* transfection, 293T cells (MeilunBio®) were cultivated in Dulbecco's Modified Eagle's Medium at 37 ℃ and 5% CO_2 conditions. On the day before the experiment, $1×10^5$ cells per well were inoculated onto a 24 well plate (Costa). Nanoparticles (NPs) containing 100 ng Luc mRNA were added to each well and incubated for 24 h. Then the cells were lysed and luciferase substrates were added. Luminescence quantification was performed using the GloMax® 20/20 Luminometer.

Endosomal escape and cellular uptake assay

Endosomal escape and cellular uptake of polyplexes were determined by confocal imaging. 293T cells were seeded at a 20,000 cells/well density into BIOFIL™ 20mm Confocal Dishes. 24 h later, the cell culture media were replaced by 180 μL fresh media (10% FBS) and cells were treated by 20 μL polyplexes with 2.5 μg Cy5-PLG-PPs and 50 ng mRNA per well. After 8 h incubation, cells were washed three times with PBS and stained by Lysotracker Green DND-26 (1/15000 dilution) and Hoechst 33342 (0.1 mg/mL) for 15 min at 37 ℃, then cells were imaged by confocal microscopy (LSM 780, Zeiss).

Animal declaration

All animal studies and procedures were conducted in accordance with the guidelines approved by the Animal Welfare and Ethics Committee of Changchun Institute of Applied Chemistry, Chinese Academy of Sciences. Female BALB/c mice aged 6-8 weeks were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd.

***In vivo* biodistribution and mRNA delivery**

For the *in vivo* biodistribution experiments, Cy5-PLG-PPs/mRNA polyplexes were prepared via the preparation mentioned above. Afterward, the polyplexes were intravenously (*i.v.*) administered to 6-8 weeks old BALB/c mice with the Luc mRNA doses of 0.25 mg/kg. 6 h later, mice were sacrificed. The organs (heart, lung, liver, spleen, kidney, and lymph nodes) were isolated and imaged on the PerkinElmer IVIS® Lumina LT Series III. These images were processed with the Living Image analysis software (Perkin Elmer).

For the *in vivo* mRNA delivery experiments, PLG-PPs/mRNA polyplexes were prepared via the preparation mentioned above. Afterward, the polyplexes were

intravenously (*i.v.*) administered to 6-8 weeks old BALB/c mice with the Luc mRNA doses of 0.25 mg/kg. 24 h later, 100 μL of D-Luciferin potassium salt solution (30 mg/mL) was intraperitoneally administered. 10 min later, mice were sacrificed. The organs (heart, lung, liver, spleen, kidney, and lymph nodes) were isolated and imaged on the PerkinElmer IVIS® Lumina LT Series III. These images were processed with the Living Image analysis software (Perkin Elmer). For positive control experiments, SM102/mRNA polyplexes were prepared via the reported methods[1,2]. Other procedures were performed the same as described above.

Cell isolation and staining for flow cytometry

Flow cytometry was used to detect EGFP positive cells in the spleen of different cell types. The single cell suspension was obtained by passing the spleens of BALB/c mice through 70-μm cell strainer. Then the red blood cells in suspension were lysed via red blood cell lysis buffer (1×). On the day before the experiment, $5×10^5$ cells per well were inoculated onto 6 well plates (Costa).Then single cells were incubated with PLG-PPs/mRNA polyplexes (EGFP mRNA, 1 μg per well). After 24 h incubation, the cells were rinsed with flow cytometry staining buffer. Then, the mixture was centrifuged (300 g, 5 min), and the precipitated cells obtained were resuspended in the flow cytometry staining buffer. The cells were mixed with antibodies and incubated in darkness at 4 °C for 20 min. Then, the cells were washed with flow cytometry staining buffer and resuspended in flow cytometry staining buffer (500 μL) for final analysis.

The antibodies used in this study were PE-Cy7 anti mouse CD45 (Biolegend, 103110), APC anti mouse CD19 (Biolegend, 115512), PE anti mouse CD49b (Biolegend, 108907), APC-Cy7 anti mouse CD11c (Biolegend, 117324), APC anti mouse CD8 (Biolegend, 100711), APC-Cy7 anti mouse CD4 (Biolegend, 100414) and PE anti mouse F4/80 (Biolegend, 123110).

***In vivo* biosafety assay**

PLG-PPs/mRNA polyplexes were *i.v.* administered to BALB/c mice at a mRNA dose of 0.5 mg/kg, which was higher than that required to induce high expression in spleen. PBS was *i.v.* administered as the negative control. 24 h later, the whole blood was collected into BD Microtainer tubes and serum was separated. The liver function

(ALT and AST) and renal function (UA, BUN and CRE) were evaluated via corresponding assay kit purchased from Nanjing Jiancheng Bioengineering Institution. Tissue sections (heart, liver, spleen, lung and kidney) and H&E staining were conducted at the Servicebio Technology (Wuhan, China).

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software). Two tailed unpaired Student's t-test was used to compare the significance of two group comparisons. One-way ANOVA followed by Dunnett's multiple comparisons test was used to determine the significance of the indicated multiple replicate group comparisons. Data are expressed as mean ± s.d. ns = not significant, P-values < 0.05 (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****) were considered to be statistically significant.

Supplemental Figures
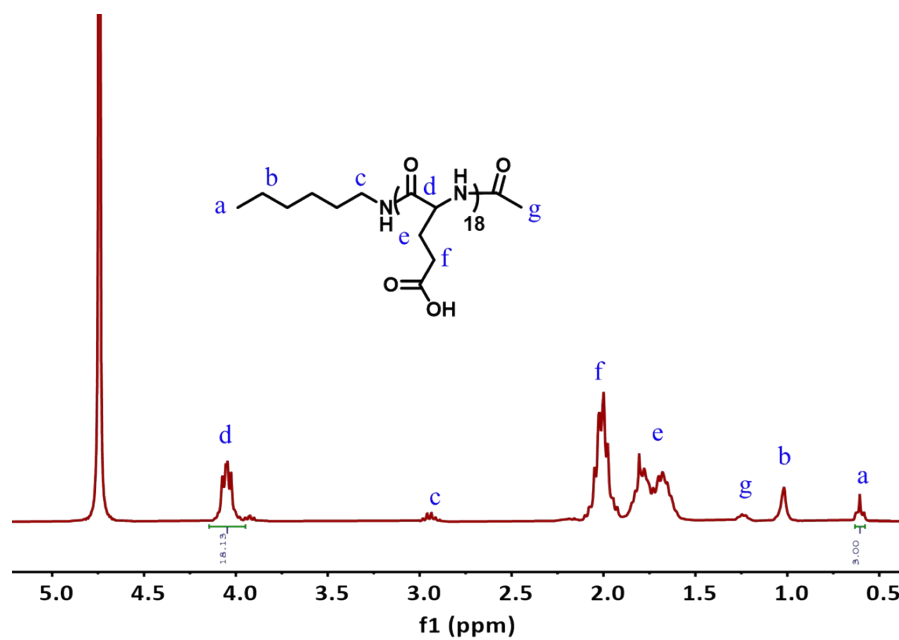
Figure S1. ¹H NMR spectrum of PLG polymer in D$_2$O with 5 wt.% DCl. The repeating unit was calculated to be about 18 using ¹H NMR spectrum.
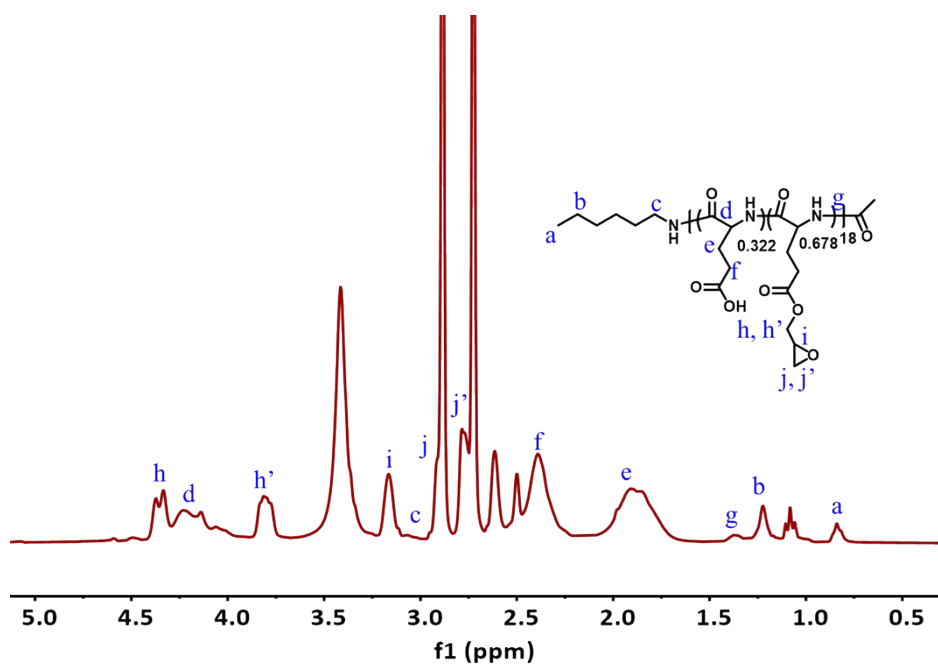
Figure S2. ¹H NMR spectrum of PLG-*g*-epoxy polymer in DMSO-d$_6$. The grafting rate of epoxy groups was calculated to be about 67.8% using ¹H NMR spectrum.
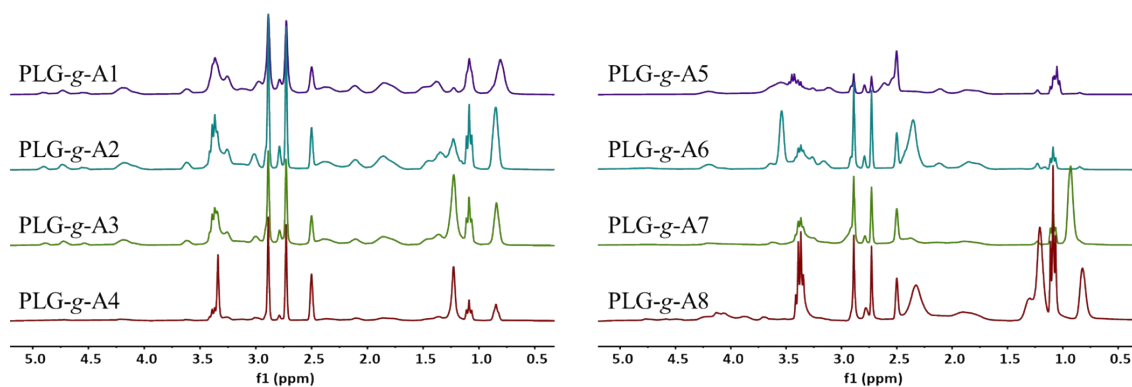
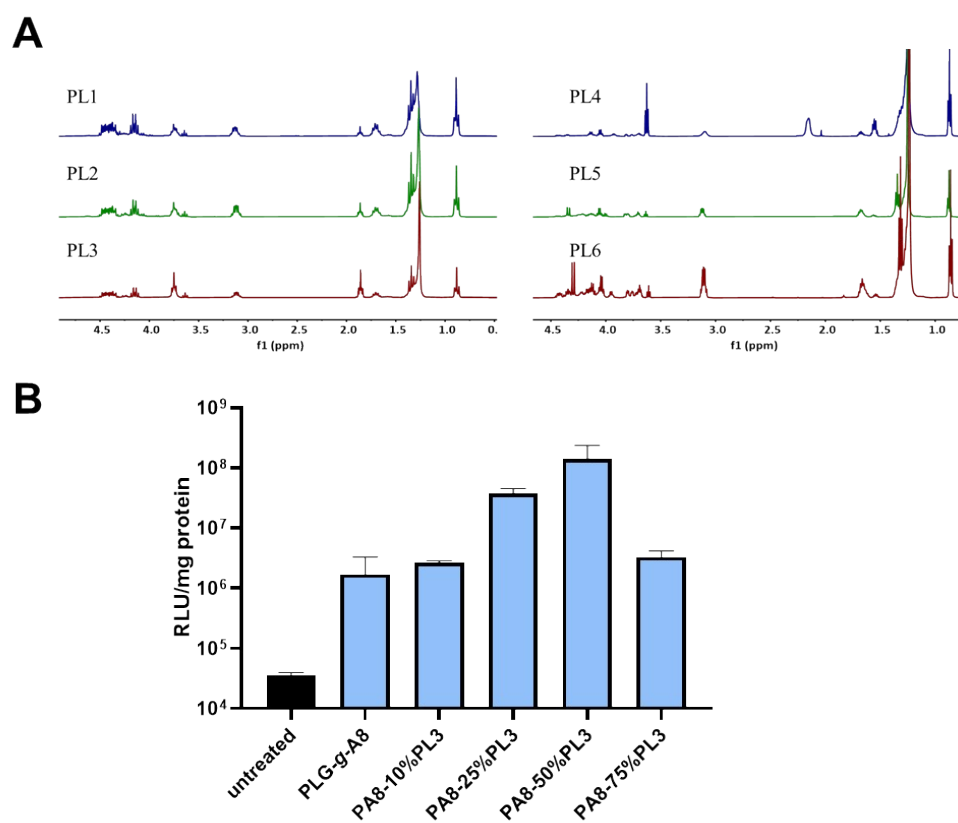**Figure S3.** ^{1}H NMR spectrum of PLG-*g*-An polymer in DMSO-d$_6$.

Figure S4. The characterization of PLm. (A)^{1}H NMR spectrum of alkylated dioxapholane oxide molecules (PL1-PL6) in CDCl$_3$. (B) The relationship between the modification rates of PL3 on PLG-*g*-A8 and the transfection efficacy of the PLG-PPs.
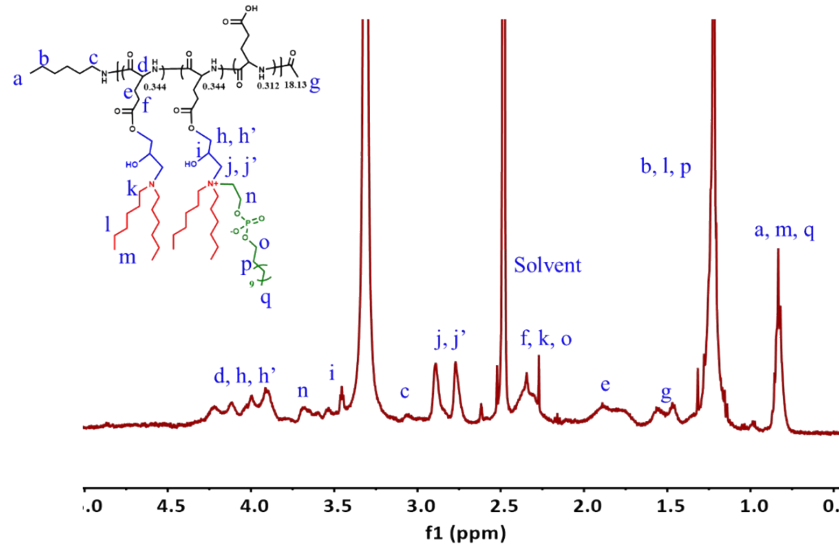
Figure S5. ^{1}H NMR spectrum of typical PLG-PPs(DMSO-d$_6$). PA8-PL3 was used as template. The sharp peak near 4.5 ppm (methylene group peak of PL3 disappeared post reaction, indicating that PL3 molecules reacted completely with PLG-*g*-A8 polymers).
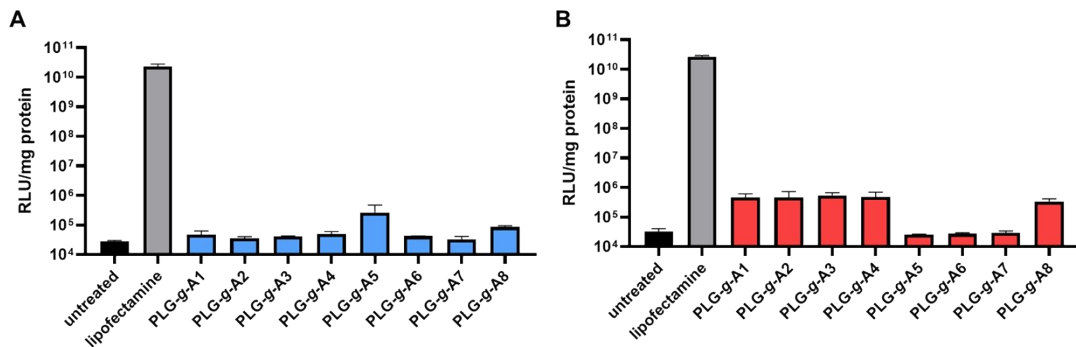
Figure S6. *In vitro* mRNA delivery mediated by PLG-*g*-An. Polymer/mRNA weight ratio was fixed at (A) 20/1 and (B) 50/1 (100 ng Luc mRNA per well). The data are presented as mean ± s.d. (*n* = 3).
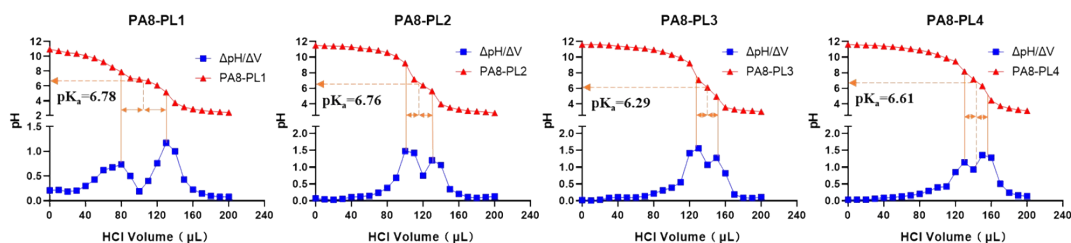
Figure S7. pK$_a$ of individual best performed PLG-PPs were measured by pH titration. The pK$_a$ values were calculated according to Henderson-Hasselbalch equation.
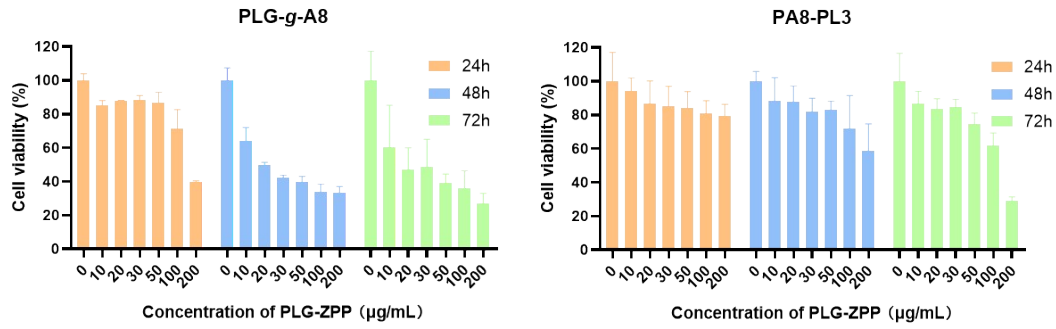
Figure S8. *In vitro* cytotoxicity of the preferred PLG-PPs using the standard CCK8 assay at a dose of 100 ng Luc mRNA. The data are presented as mean ± s.d. (*n* = 3).
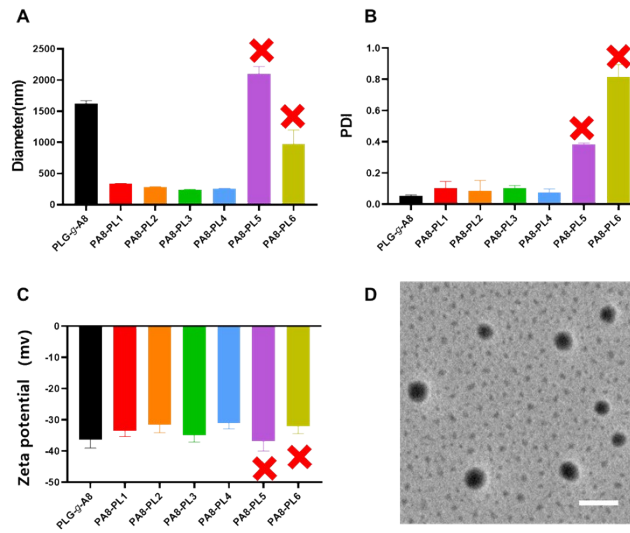

Figure S9. The characterization of polyplexes prepared via PLG-*g*-PA8 and its derivative including the (A) diameter, (B) PDI and (C) ζ potential. PA8-PL5 and PA8-PL6 marked with red crosses could not be assembled into nanoparticles. (D) The typical TEM image of PLG-PPs/mRNA complexes (PA8-PL3 was chosen as the template). Scale bar = 500 nm. The data are presented as mean ± s.d. (*n* = 3).
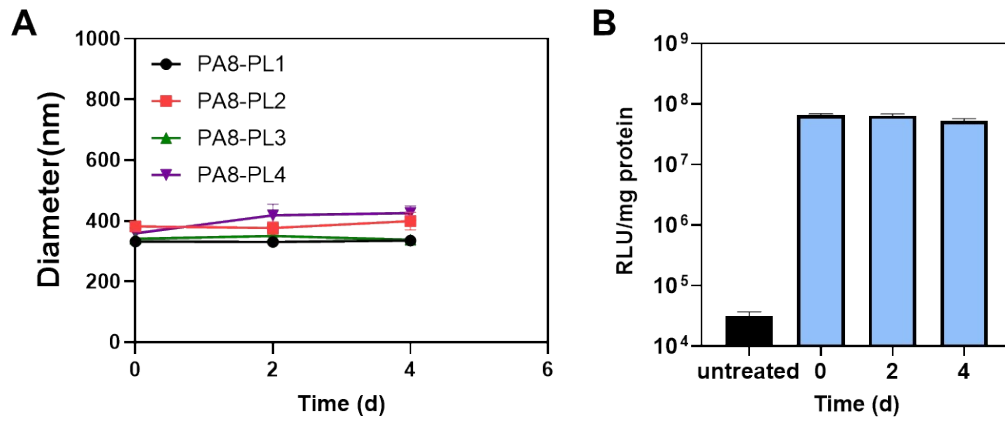
Figure S10. Long term stability of PLG-PPs/mRNA polyplexes. (A) The particle size of PA8-PLm (m = 1-4) evaluated at Day 0, 2, and 4. (B) The transfection efficiency of PA8-PL3 evaluated after 0, 2, and 4 days storage. The data are presented as mean ± s.d. (n = 3).
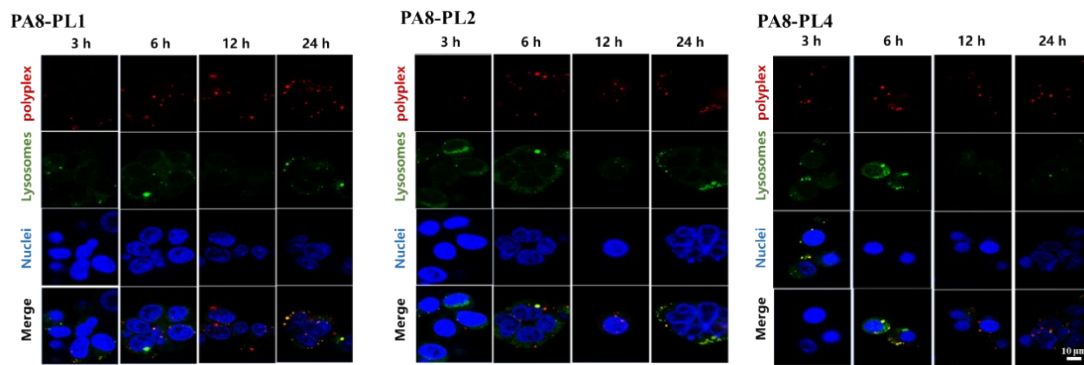

Figure S11. Endosomal escape and cellular uptake fluorescence images of 293T cells treated with PA8-PLm (m=1, 2, 4) prepared polyplexes.
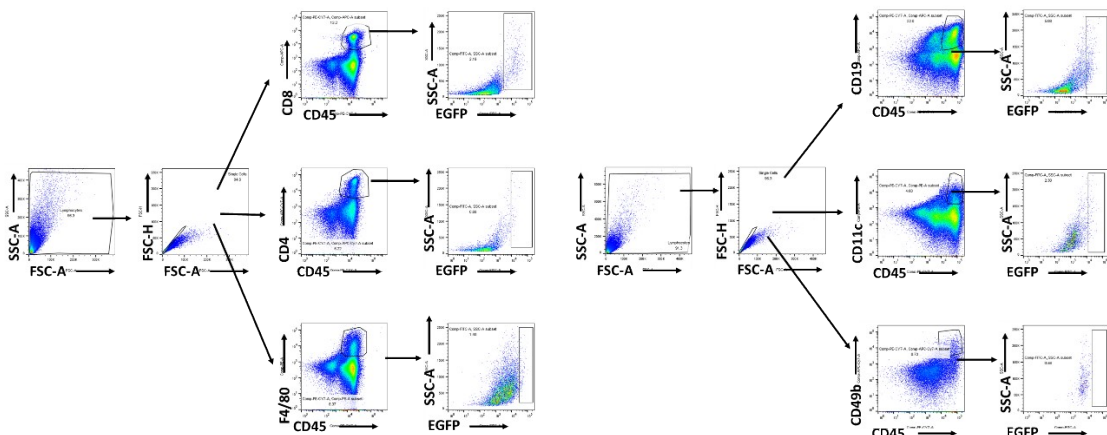

Figure S12. The FACS gating strategy for analysis of spleen cells treated by PLG-

PPs/EGFP mRNA complexes *in vitro*. Gates for EGFP⁺ cells in major immune cell types were drawn based on PBS control group.
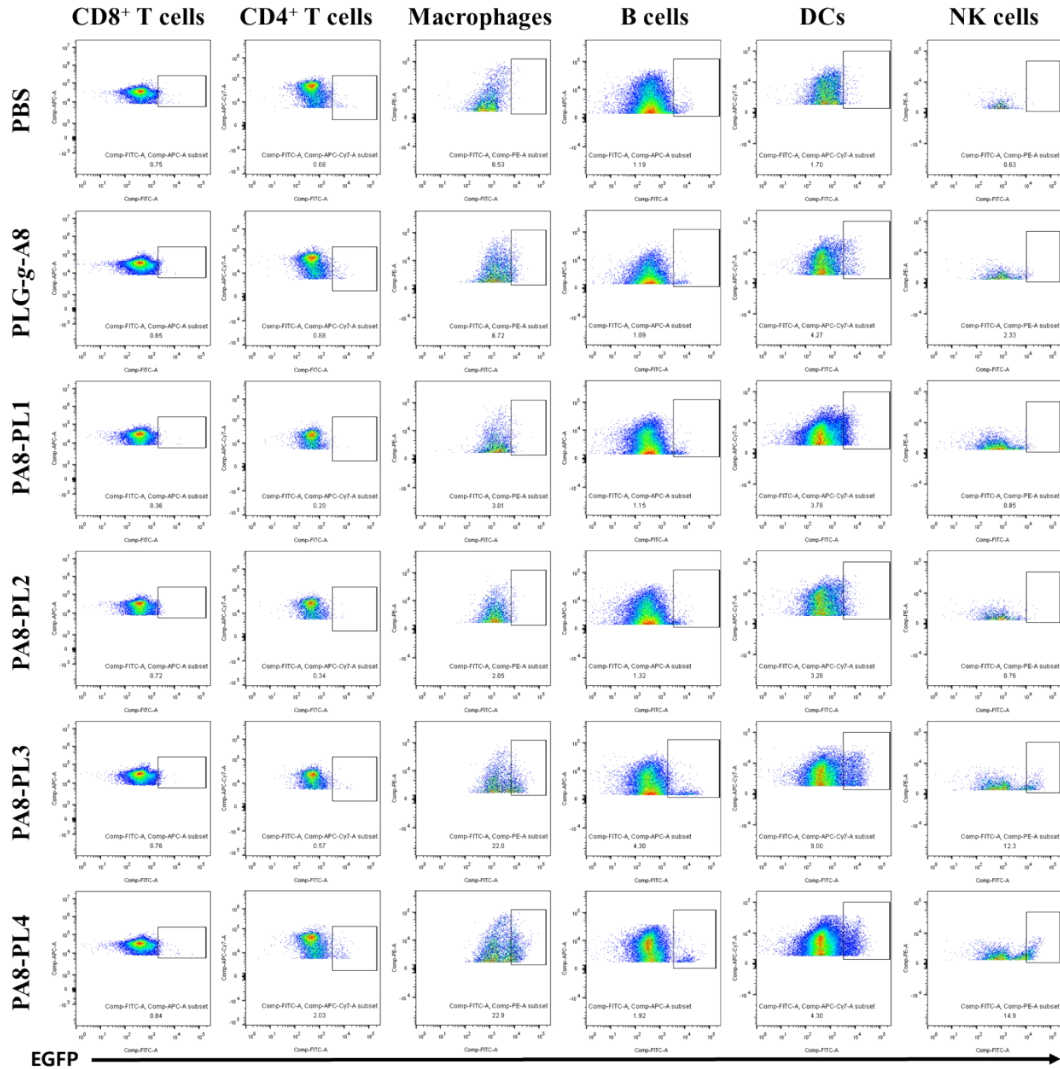

Figure S13. The cytometry graphs of EGFP expression in spleen cells treated via different PLG-PPs/EGFP mRNA complexes *in vitro*. Gates for EGFP⁺ cells in major immune cell types were drawn based on PBS control group.
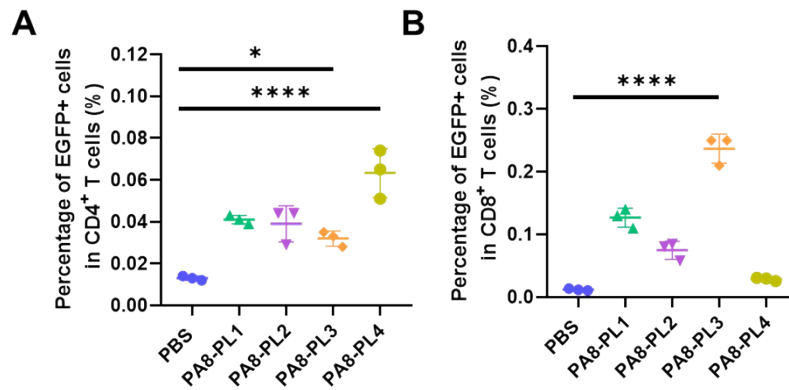
Figure S14. Flow cytometry analysis of harvested spleen cells after the intravenous injection of PLG-PPS/mRNA in mice. The histograms for EGFP^{+} cells in (A) CD4^{+} or (B) CD8^{+} T cells treated with different polymer groups. The data are presented as mean $\pm$ s.d. ($n = 3$). Statistical significance was analyzed by the two-tailed unpaired Student's t-test: *$p < 0.05$; ****$p < 0.0001$.

References

1. X. Wang, S. Liu, Y. Sun, X. Yu, S. M. Lee, Q. Cheng, T. Wei, J. Gong, J. Robinson, D. Zhang, X. Lian, P. Basak and D. J. Siegwart, *Nature Protocols*, 2023, **18**, 265-291.
2. R. Zhang, S. Shao, Y. Piao, J. Xiang, X. Wei, Z. Zhang, Z. Zhou, J. Tang, N. Qiu, X. Xu, Y. Liu and Y. Shen, *Advanced Materials*, 2023, **35**, 2303614.