Electronic Supplementary Information

Lung-selective nucleic acid vectors generated by in vivo lung-targeting-protein decoration of polyplexes

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Content

1. Materials and experimental....3
Fig. S1....7
Fig. S2....7
Fig. S3....8
Fig. S4....8
Fig. S5....9
Fig. S6....9
Fig. S7....10
Fig. S8....10
Fig. S9....11
Fig. S10....11
Fig. S11....12
Fig. S12....12
Fig. S13....13
Fig. S14....13
Fig. S15....14
Fig. S16....15
Fig. S17....15
Fig. S18....16

1. Materials and experimental

1.1. Materials

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE, 97%), 4-amino-1-butanol (ABOL, 98%), *N*,*N*′-bis(acryloyl)cystamine (BAC, 98%), triethylamine (99.5%), and glutathione (GSH, 98%) were purchased from Innochem Technology (Beijing, China). *N*,*N*-dimethylformamide (DMF), methanol (MeOH), and chloroform were purchased from Sinopharm Chemical Reagent (Shanghai, China). PEI 25K and Ribogreen RNA Reagent were purchased from Maokang Biological Technology (Shanghai, China). Cell Counting Kit-8 (CCK8) was purchased from Beyotime Biotechnology (Shanghai, China). Lipofectamine 2000 was purchased from ThermoFisher Scientific (Shanghai, China). JetPEI was purchased from Polyplus. ARCA EGFP mRNA (5-moUTP) and EZ CapTM Firefly luciferase mRNA were purchased from APExBIO Technology (Shanghai, China).

1.2. Experimental

Synthesis of poly(*N*,*N*′-bis(acryloyl)cystamine-*co*-4-amino-1-butanol) (pabol). The synthesis of pabol was based on previous reports.[1] Briefly, 4-amino-1-butanol (ABOL) (170 μL, 1.80 mmol), *N*,*N*′-bis(acryloyl)cystamine (BAC) (478 mg, 1.80 mmol) and triethylamine (25.0 μL, 0.180 mmol) were added into a reaction tube with 450 μL MeOH/H_2O (4:1, v/v), equipped with a stir bar. Polymerization was carried out in the dark at 50 °C under a static nitrogen atmosphere for about 7 days that the molecular weight could reach over 10.0 kDa. Then, to terminate the reaction, excess MeOH (50 mL) was added to the system. The solution was acidified with 1.0 M HCl to achieve

pH 4. Subsequently, the polymer was purified by dialysis (using a molecular weight cut off = 1000 Dalton) against acidic water (pH 5), with the water being refreshed 6 times over 3 days. Finally, the polymer was collected after freeze-drying.

Synthesis of poly((*N,N'*-bis(acryloyl)cystamine-*co*-4-amino-1-butanol)-*co*-1,2-distearoyl-sn-glycero-3-phosphoethanolamine) (polylipo). Polylipo was synthesized by aza-Michael polyaddition of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) and ABOL to BAC. In a typical experiment, BAC (717 mg, 2.70 mmol), ABOL (249 μL, 2.63 mmol), DSPE (52.1 mg, 0.0680 mmol), and triethylamine (37.6 μL, 0.270 mmol) were added into a mixed solvent, chloroform/DMF (1080 μL, v/v = 1/1) in a reaction tube charged with a stir bar. Polymerization was carried out in the dark at 50 °C under a static nitrogen atmosphere for a duration of 7 to 14 days, depending on the targeted molecular weight. Upon reaching the targeted molecular weight, the reaction mixture was diluted with DMF (30 mL), then acidified with 1.0 M HCl to achieve a pH of approximately 4. Subsequently, the polymer was purified by dialysis (using a molecular weight cut off = 1000 Dalton) against acidic water (pH 4), with the water being refreshed 6 times over 3 days. The resulting polymers in their HCl-salt form were collected after freeze-drying.

Preparation of polyamidoamines/nucleic acids polyplexes. In the preparation of polylipo/mRNA polyplexes, as an example, the polymer was dissolved in DNase/RNase-free water at a concentration of 50 mg/mL and stored at 4 °C in advance. In a typical procedure, mRNA stock solution (3 μL) was diluted to 240 μL HEPES buffer (20 mM HEPES, 5 wt% glucose in water, pH 7.0). Simultaneously, the

predetermined amount of polymer stock solution was diluted to 60 μL using the same buffer in a DNase/RNase free centrifuge tube, which was equipped with a magnetic stir bar. The tube was placed on a stir plate and then stirred at 1200 rmp. Then, the mRNA solution was added to the polymer solution at a rate of 160 μL/min using syringe pumps. The mixed solution was kept on the stir plate and stirred for 30 min to reach equilibrium. A series of polylipo/mRNA polyplexes were prepared at various weight ratios of polymer to mRNA ranging from 20:1 to 70:1. The pabol/mRNA polyplexes solution was prepared as previously described at the weight ratios of polymer to mRNA of 45:1. [1] PEI, jet PEI, and lipofectamine 2000 nanoparticles were prepared, according to the manufacturer's protocols at a weight ratio of vectors to mRNA of 3:1.

Animal models. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Tongji University and approved by the Animal Ethics Committee of Shanghai Tenth People's Hospital affiliated to Tongji University [SHDSYY-2024-1231].

1.3. Characterization

^{1}H NMR

Polymers (3.0-5.0 mg) were dissolved in DMSO-d_6, and conducted on a Bruker AV 400 MHz at room temperature.

Size exclusive chromatography (SEC)

The molecular weights and molecular weight distribution were determined on Agilent 1260 Infinity Quaternary LC. HPLC grade DMF (containing 0.1 wt% LiBr) was used as the eluent. The flow rate was set at 1.0 mL/min and the column temperature was

fixed at 60 °C.

Dynamic light scattering (DLS)

Polyplexes formed between polyamidoamines and nucleic acids were examined on a Zetasizer Nano series instrument (Malvern Zetasizer NanoZS90) to determine the hydrodynamic diameter (D_h), polydispersity (PD) and zeta potential (ζ) of the polyplexes.

Transmission electron microscopy (TEM)

The samples were pipetted onto a carbon-coated copper grid and left to stand for 20 min. The liquid was then pipetted off, and this procedure was repeated three times. TEM images of BSA@polyplexes were imaged on JEM-2100 electron microscope.

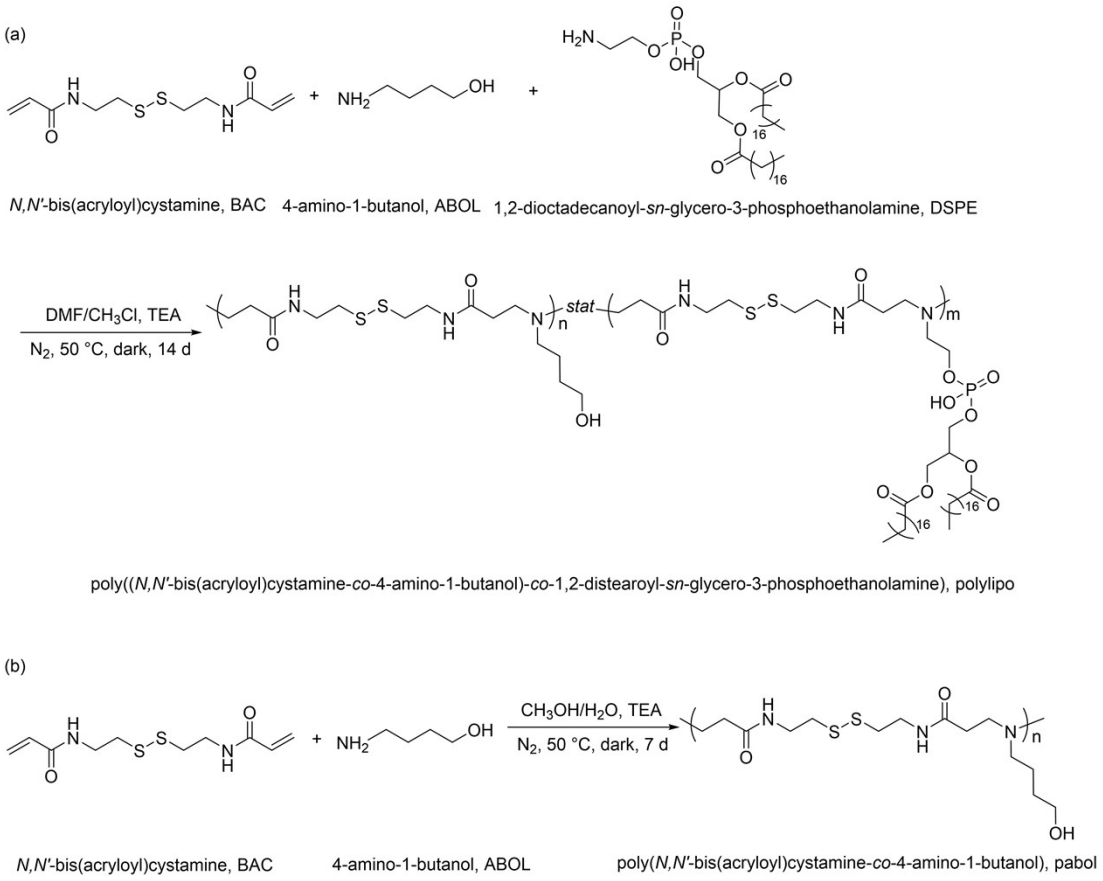

Fig. S1 Synthetic routes of polylipo and pabol.

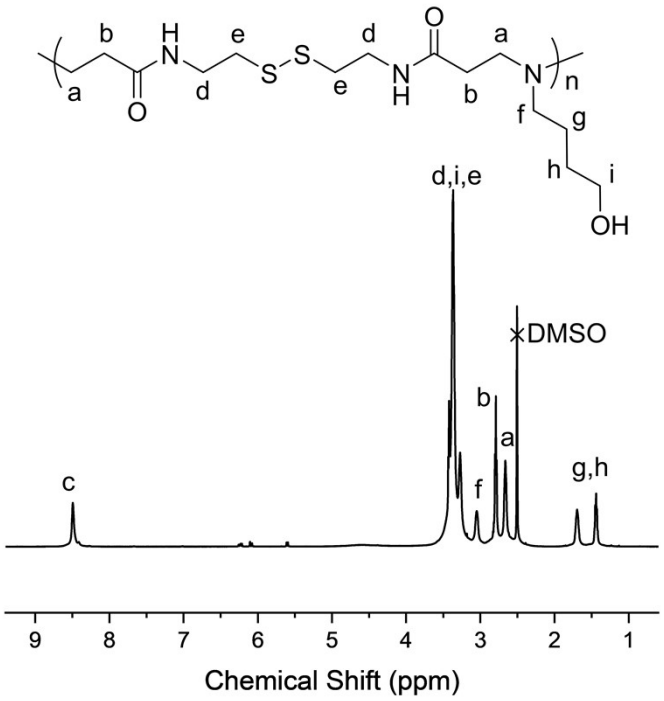

Fig. S2 Typical ^{1}H NMR spectra of pabol in DMSO-d_6.

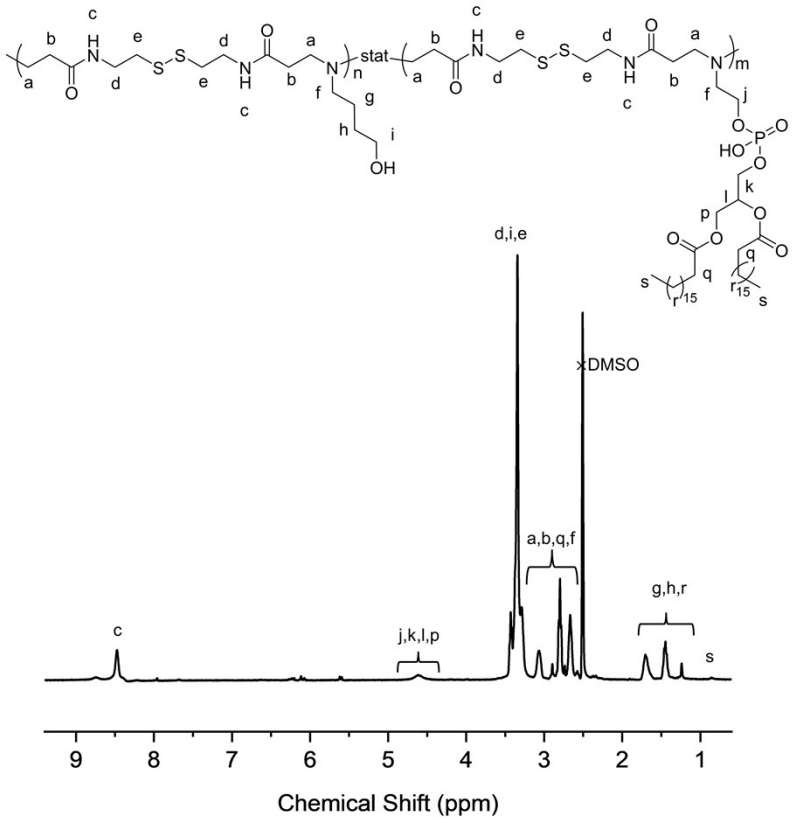

Fig. S3 Typical ^{1}H NMR spectra of polylipo in DMSO-d_6.

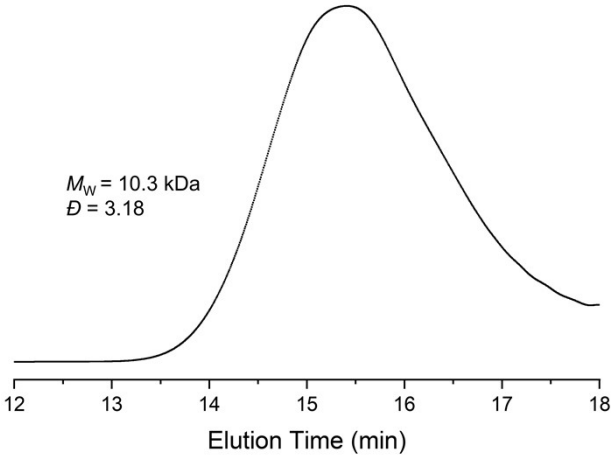

Fig. S4 SEC trace of pabol in DMF after 14 days of polymerization and 3 days of dialysis.

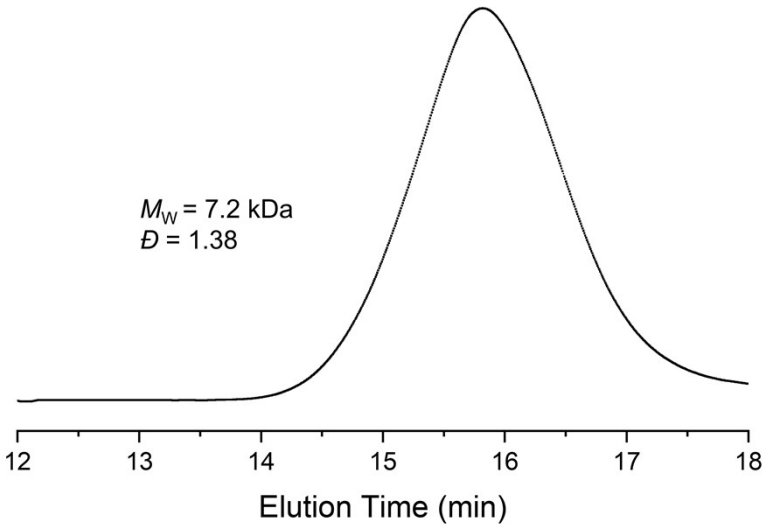

Fig. S5 SEC trace of polylipo in DMF after 14 days of polymerization and 3 days of dialysis.

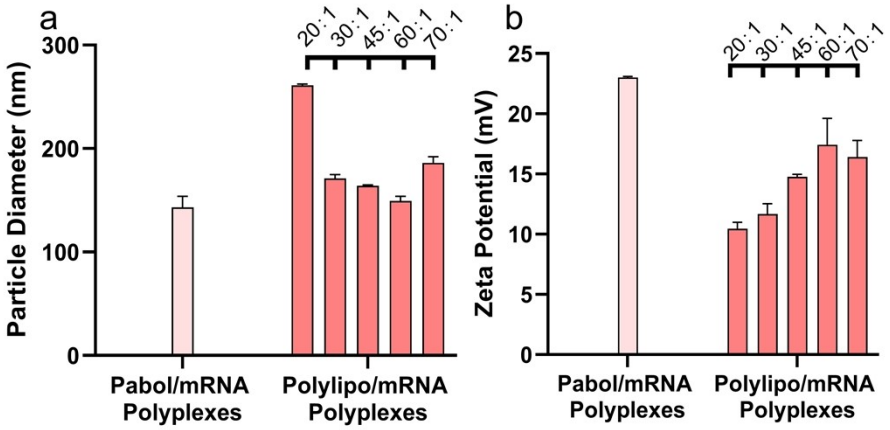

Fig. S6 (a) and (b) Hydrodynamic diameter and zeta potential values of polyamidoamines/mRNA polyplexes at pH 7.0. Polyplexes with various weight ratios of polymer to mRNA, ranging from 20:1 to 70:1, were prepared.

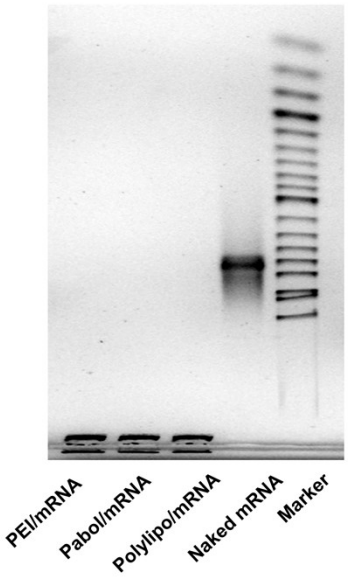

Fig. S7 Agarose gel electrophoresis image of PEI/mRNA, pabol/mRNA, polylipo/mRNA polyplexes, Naked mRNA, marker, respectively.

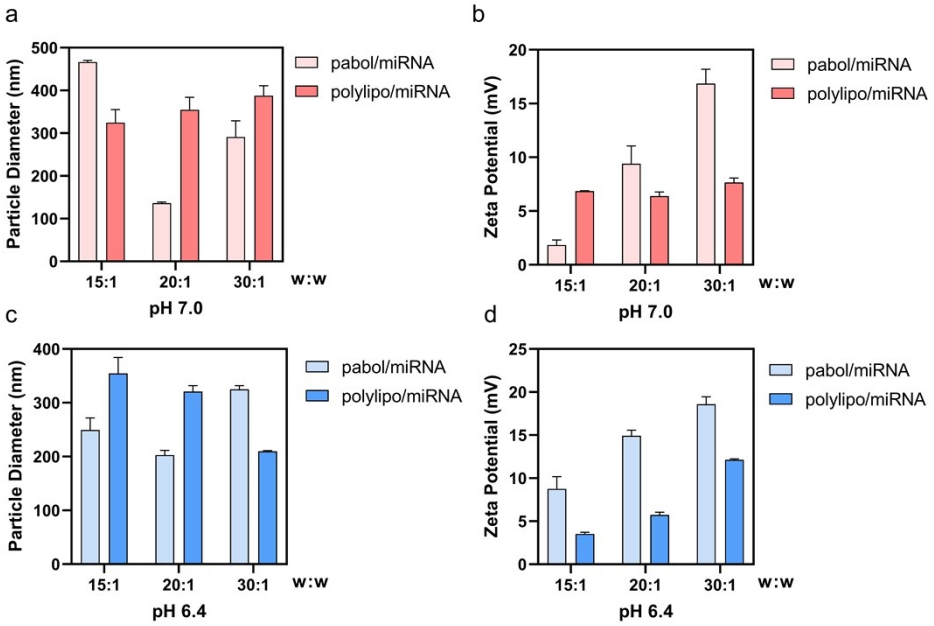

Fig. S8 (a) and (b) Hydrodynamic diameter and zeta potential values of polyamidoamines/miRNA polyplexes at pH 7.0, (c) and (d) Hydrodynamic diameter and zeta potential values of polyamidoamines/miRNA polyplexes at pH 6.4.

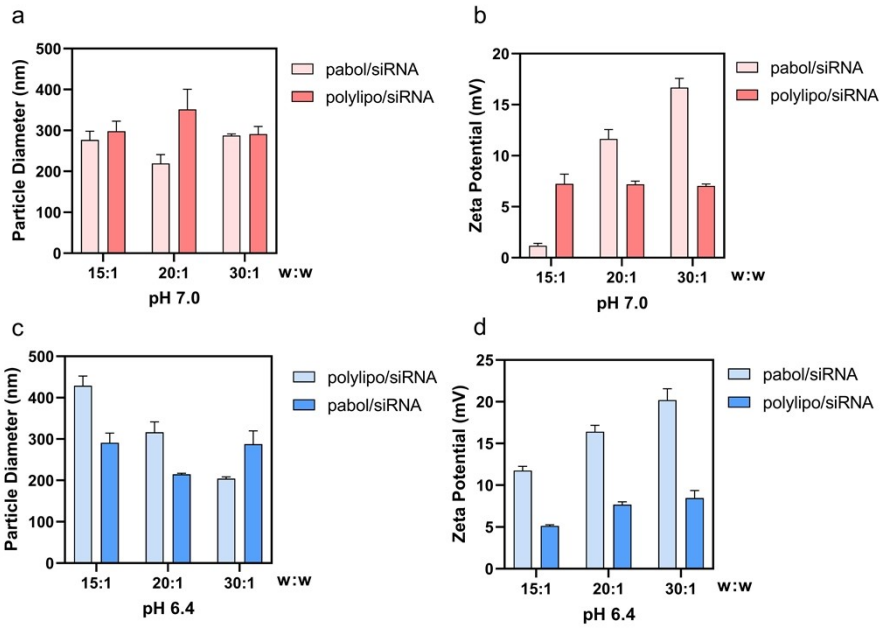

Fig. S9 (a) and (b) Hydrodynamic diameter and zeta potential values of polyamidoamine/siRNA polyplexes at pH 7.0, (c) and (d) Hydrodynamic diameter and zeta potential values of polyamidoamine/siRNA polyplexes at pH 6.4.

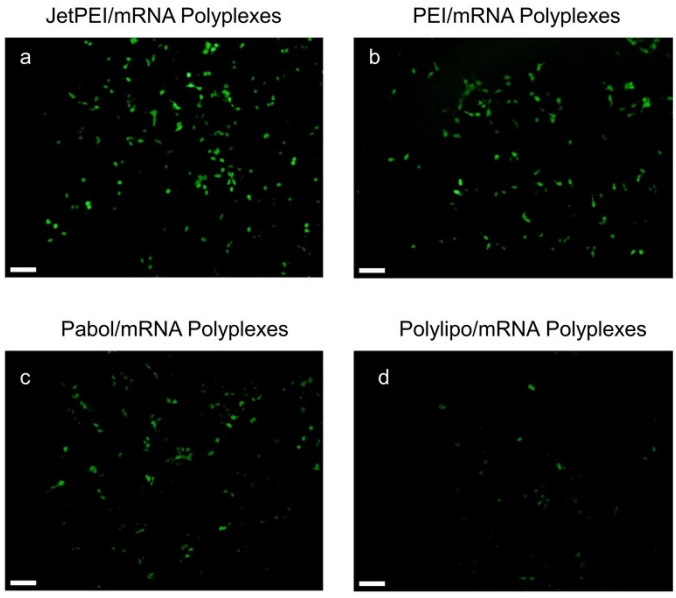

Fig. S10 In vitro transfection images to HEK cells of vectors including (a) jetPEI/mRNA, (b) PEI/mRNA, (c) pabol/mRNA and (d) polylipo/mRNA polyplexes 24 h post-transfection. Scale bars = 100 μm.

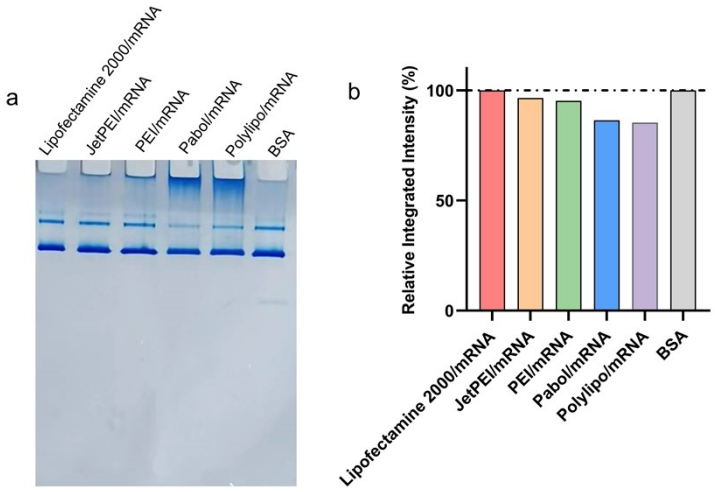

Fig. S11 (a) The interaction between BSA and lipofectamine 2000, jetPEI/mRNA, PEI/mRNA, pabol/mRNA and polylipo/mRNA polyplexes shown by polyacrylamide gel electrophoresis. (b) Quantified using ImageJ software.

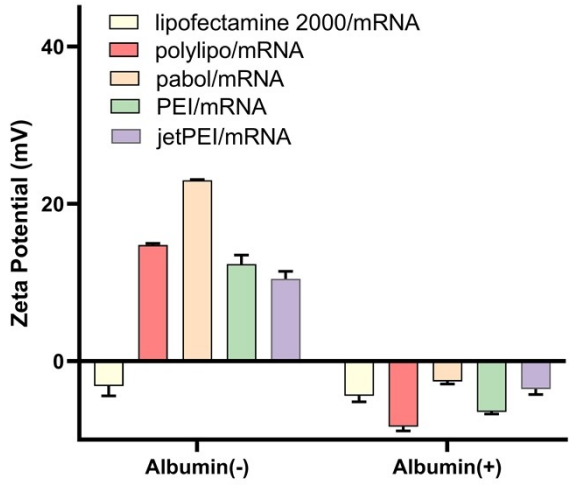

Fig. S12 Zeta potential change of nanoparticles after incubation with BSA.

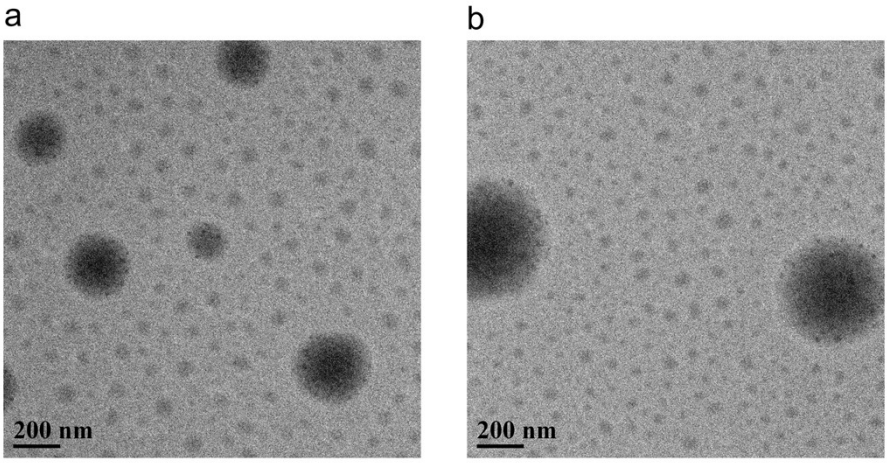

Fig. S13 TEM (scale bars = 200 nm) of (a) BSA@pabol and (b) BSA@polylipo polyplexes.

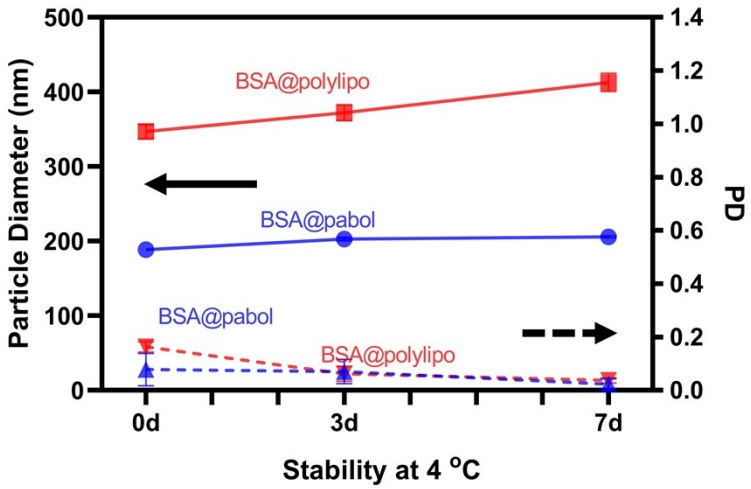

Fig. S14 The results of hydrodynamic diameter and PD changes in BSA@pabol and BSA@polylipo polyplexes stored at 4 °C.

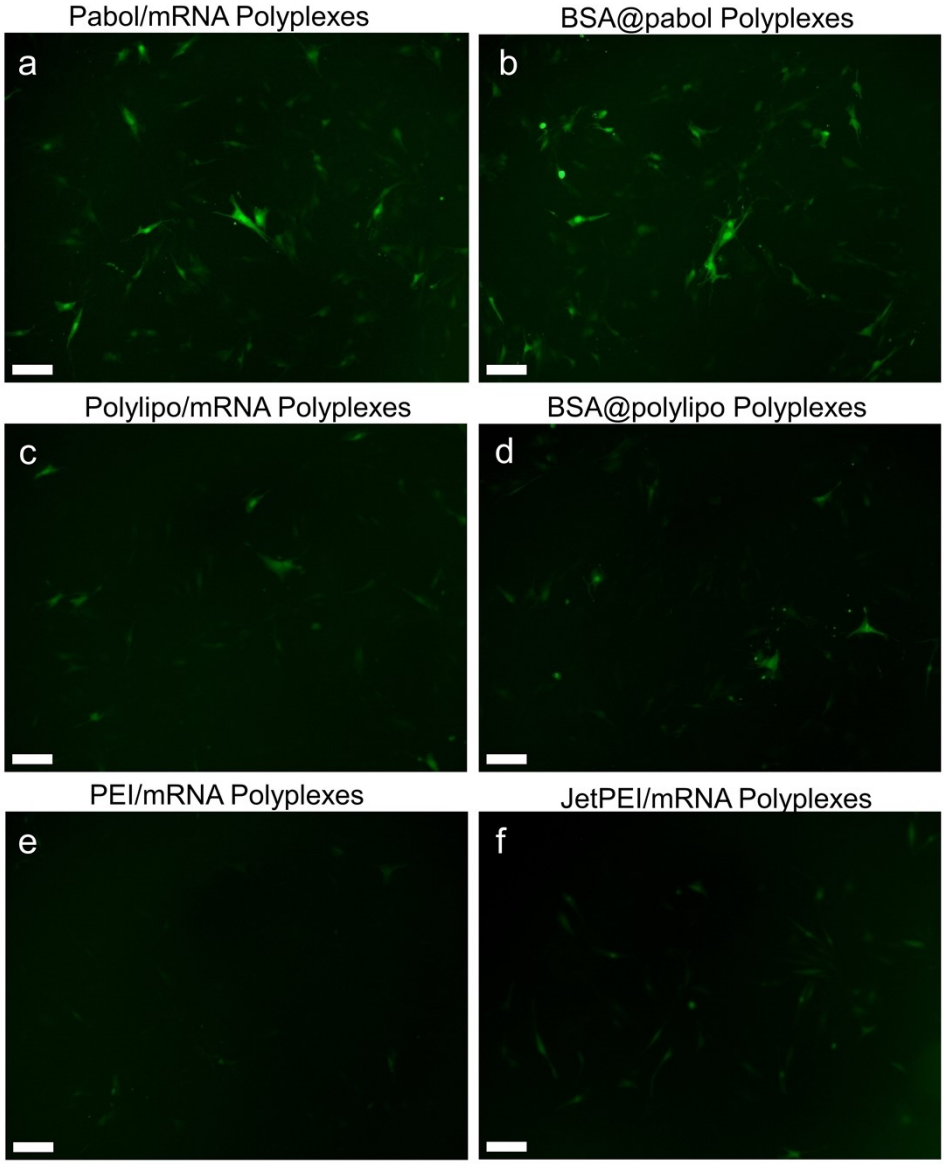

Fig. S15 In vitro transfection images to mouse lung primary microvascular endothelial cells of vectors including (a) pabol/mRNA, (b) BSA@pabol, (c) polylipo/mRNA, (d) BSA@polylipo, (e) PEI/mRNA and (f) jetPEI/mRNA polyplexes 24 h post-transfection. Scale bars = 100 μm.

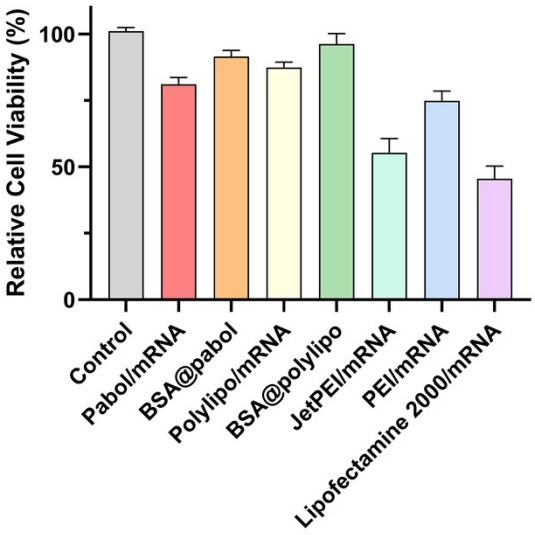

Fig. S16 Relative cell viability of mouse lung primary microvascular endothelial cells incubated with nanoparticles for 24 h (mean ± SD, n = 3).

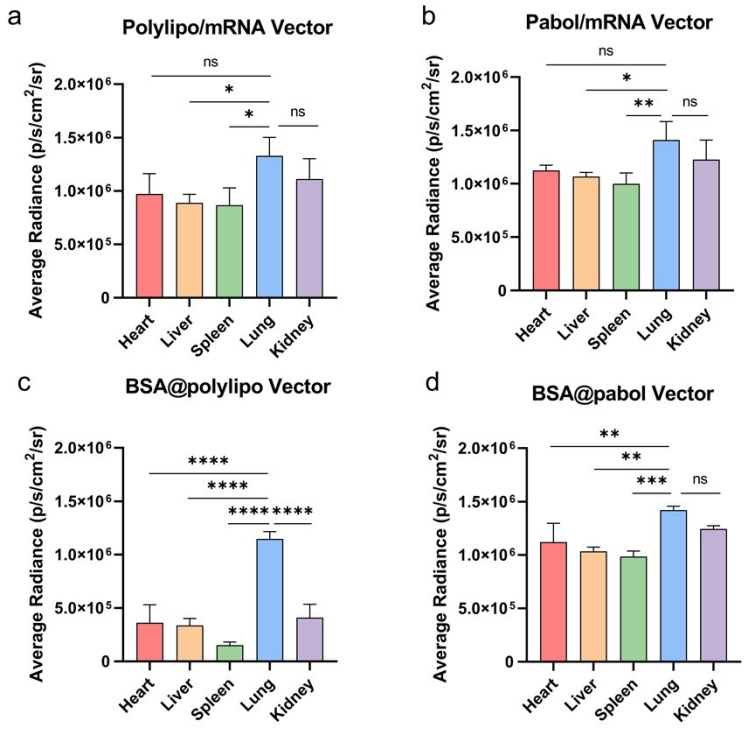

Fig. S17 Quantification of Fluc protein expression (mean ± SD, n = 3). Statistical significance was analyzed by one-way ANOVA. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, and ns: not significant.

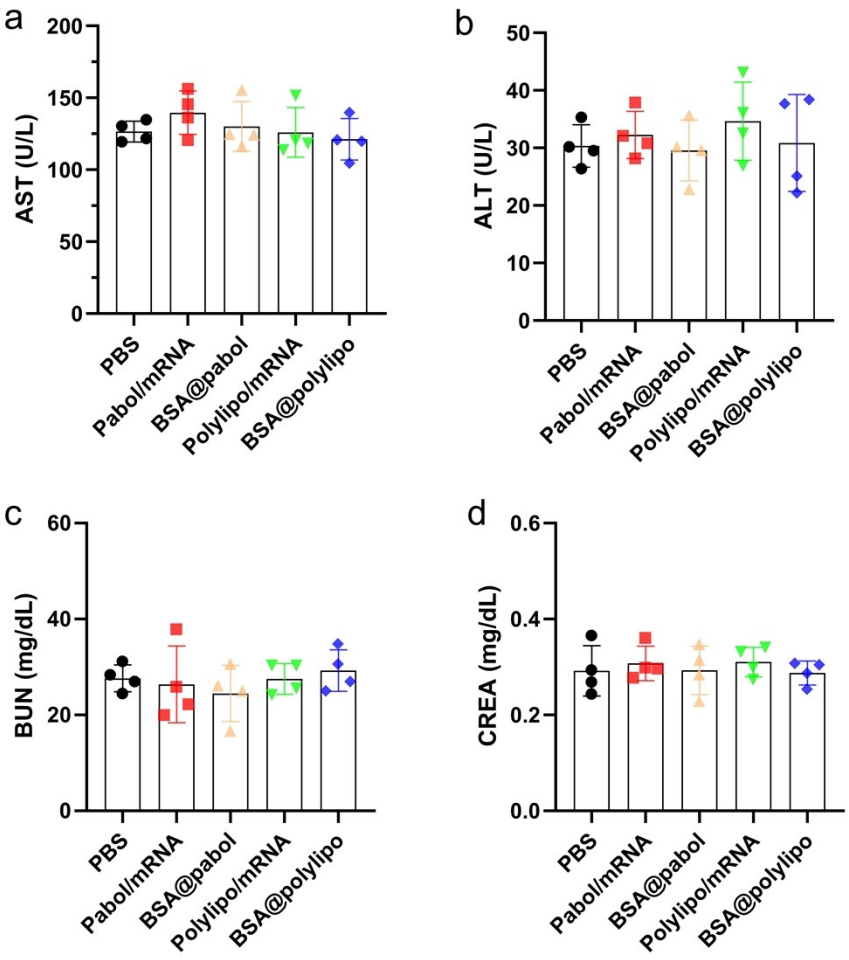

Fig. S18 Pabol/mRNA, BSA@pabol, polylipo/mRNA and BSA@polylipo polyplexes were well tolerated in vivo. Liver (AST and ALT) and kidney function (BUN and CREA) were evaluated 24 h after the subcutaneous injection of polyplexes (mean ± SD, n = 4). Statistical significance was analyzed by one-way ANOVA. ns: not significant.

Reference

1. A. K. Blakney, Y. Zhu, P. F. McKay, C. R. Bouton, J. Yeow, J. Tang, K. Hu, K. Samnuan, C. L. Grigsby, R. J. Shattock and M. M. Stevens, *ACS Nano*, 2020, **14**, 5711-5727.