Supporting Information

Enhancing Spleen-Targeted mRNA Delivery with Branched Biodegradable Tails in Lipid Nanoparticles

Yupeng Ren,[a†] Ling Zeng,[b†] Yingsen Tang,[b] Jing Liao,[b] Meng Jiang,[b] Xinxiu Cao,[a*] Hui Fan,[a*] Jinjin Chen[b*]

a. Hunan Provincial Key Laboratory of Advanced Materials for New Energy Storage and Conversion, School of Materials Science and Engineering, Hunan University of Science and Technology, 2 Taoyuan Street, Xiangtan 411201, P. R. China.

b. Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Guangdong-Hong Kong Joint Laboratory for RNA Medicine, Medical Research Center, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, China.

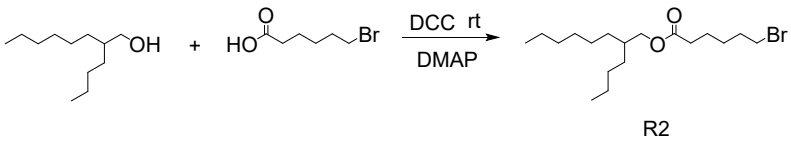
†These authors contributed equally.

*To whom correspondence may be addressed. Email: xxcao@hnust.edu.cn, huifan@hnust.edu.cn, chenjj365@mail.sysu.edu.cn.

Materials and methods

Chemicals were purchased from Guangzhou Lithium Court Technology Co. 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-dimyristoyl-rac-glycero-3-methoxy polyethylene glycol-2000 (DMG-PEG 2000) were purchased from Shanghai Bide Pharmaceuticals Technology Co. Cholesterol was purchased from Guangdong Lusheng Trading Co. The mRNA encoding firefly luciferase (Luc) was purchased from ApexBio. NMR spectra were recorded using a Bruker Advance II 400 MHz NMR spectrometer with deuterated chloroform ($CDCl_3$) as solvents.

Synthesis of tail

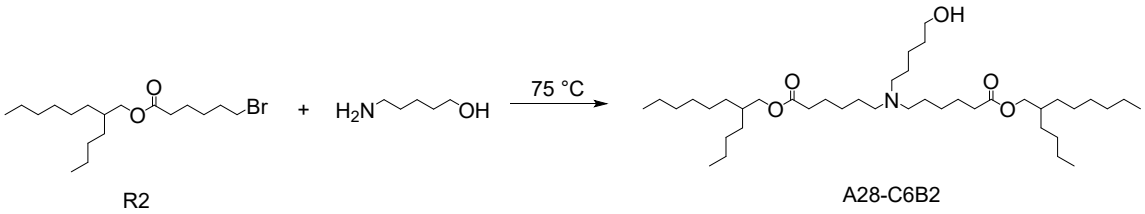


R2

Synthesis of 2-butyloctyl 6-bromohexanoate (R2)

3.33 g (0.018 mol, 1eq) of 2-butyloctan-1-ol and 3.49 g (0.018 mol, 1eq) of 6-bromohexanoic acid were added to a round-bottom flask. An appropriate amount of DCM was added, and stirring was initiated. Then 4.43 g (0.022 mol, 1.2eq) of DCC and 0.22g (0.002mol, 0.1eq) of DMAP were weighed and added to a round-bottomed flask and stirred until dissolved. The product was separated and purified using petroleum ether: ethyl acetate. The purified product was then subjected to NMR detection (Fig. S1). Other materials were synthesized using the same principle.

Synthesis of lipids



R2 A28-C6B2

Synthesis of bis(2-butyloctyl) 6,6'-((5-hydroxypentyl) azanediyl) dihexanoate (A28-C6B2)

2-butyloctyl 6-bromohexanoate (0.002mol, 2eq) was reacted with 5-aminopentan-1-ol (0.001mol, 1eq) in a molar ratio of 2:1 in a feeding reaction. The product was separated and purified using petroleum DCM: methanol. The purified product was then subjected to NMR detection (Fig. S4). Other materials were synthesized following the same principle.

Cell culture

HeLa cells were cultured in DMEM medium with 10% serum and 1% penicillin/streptomycin, 37 °C, and 5% CO_2 present.

Preparation of LNPs

The LNPs were produced using the ethanol dilution method. Ionizable lipids, cholesterol, DSPC and DMG-PEG2000 were dissolved in ethanol at a molar ratio of 16:4:2:1. Luc-mRNA was dissolved in 25 mM sodium acetate buffer solution. The ethanol phase and the aqueous phase were vortexed in a volume of 1:3 and then the mixtures were dialyzed with ultrapure water for more than 12 hours at room temperature. The hydrodynamic diameter and polydispersity index (PDI) of the LNPs were measured at 25 °C using a Zetasizer Nano ZSP (Malvern Instruments). These LNPs loaded with mLuc were injected intravenously into 4-week-old ICR mice. mLuc

expression was assessed using the In Vivo Imaging System (IVIS) at six hours post-injection in major organs including the heart, liver, spleen, lung and kidney.

In vitro transfection of LNPs

To evaluate the delivery efficiency of Luc-mRNA in vitro, 1×10^4 Hela cells were inoculated into 96-well plates and incubated in a 37 °C incubator for 24 h. LNPs containing 200 ng of mRNA were added to each well and incubated at 37 °C for 24 h. The DMEM medium was drained and washed twice with PBS, and 100 μL of cell lysates were added to each well. After 5 minutes, 20 μL of the lysates were transferred to a white enzyme-labeled plate. Luciferase substrate was added and fluorescence was read using an enzyme marker.

Cellular uptake of LNPs

To study the cellular uptake of LNPs, LNPs encapsulating 2% DID were incubated with Hela cells at 37 °C for 1 h. Cells were digested with 0.25% trypsin, washed three times with PBS and analyzed using a CytoFLEX flow cytometer (Beckman Coulter).

pKa of LNPs

The pKa of LNP was determined using the 6-p-toluidino-2-naphthalenesulfonic acid (TNS) assay, following previously published methods. 20 μM of LNPs and 6 μM of TNS were dissolved in 100 μL mixed buffer of 20 mM sodium phosphate, 25 mM citrate, 20 mM ammonium acetate and 150 mM NaCl. Fluorescence was determined at each pH with settings of λ_{ex}=322 nm, λ_{em}=431 nm. By assuming that minimum fluorescence represents zero charge, and maximum fluorescence represents 100% charge, pKa was estimated by measuring the pH at the point exactly halfway between the values of minimum and maximum charge.

Membrane permeability of LNPs

Hemolysis of red blood cells (RBCs) was performed according to a previously published method. Briefly, mouse RBCs were isolated from mouse blood by centrifugation at 3000 rpm for 15 minutes. RBCs were washed three times with PBS and suspended in PBS at pH 5.5 or 7.4. 200 μL of RBC solution was added to each well of a 96-well plate. LNPs and 1% Triton-X100 were added to each well and incubated at 37 °C for 1 h. The plate was centrifuged at 3000 r for 5 min. 100 μL aliquots from each well were transferred to a transparent 96-well plate. The absorbance at 540 nm of each well was measured using a BioTek Synergy H1 multimode microplate reader (Agilent).

In vivo biodistribution

100 μg of LNPs incorporated with 2% DiD dye were injected into 6-week-old ICR mice via tail vein approach. Six hours after injection, the distribution of DiD in the spleen was assessed using IVIS. Mice were necropsied and spleens were collected. The cell suspension was ground and filtered through a 70-μm strainer. 2×10^6 cells were then incubated for 1 h in 100 μL of flow cytometry staining buffer (eBioscience) containing fluorophore-conjugated relevant antibodies listed in Table S1 at the recommended concentration of 4 °C. Data were collected by an LSR-II flow cytometer (BD Biosciences) and analyzed by FlowJo-v10. Gating information is shown in Fig. S7.

Determination of subcellular type of mRNA expression in spleen

Ai9 mice were injected with LNPs/mCre containing 15 μg Cre mRNA and 300 μg LNPs by I.V. injection. Forty-eight hours after the injection, the mice were killed and the spleen was collected. The cell suspensions were prepared by grinding and filtrating

through a 70-μmstrainer. Then 2×10^6 cells were incubated in 100 μL flow cytometry staining buffer (eBioscience) containing fluorophore-conjugated antibody of interest listed in Table S1 at the recommended concentration of 4 °C for 1 h. Then the cells were kept at 4 °C for analysis after washing twice with staining buffer. Data were collected by LSR-II flow cytometer (BD Biosciences) and analyzed by FlowJo-v10. Gating information is shown in Fig. S8.

The encapsulation efficiency of mRNA and mRNA integrity

The prepared formulations were sampled using the RiboGreen RNA Assay from Beijing Solarbio Science & Technology Co., Ltd. The reagents used in this experiment were provided in the kit. RNA-LNP was diluted a suitable number of times using 1×TE buffer to detect the concentration of free RNA outside LNP, and RNA-LNP was diluted a suitable number of times using Triton X-100 to detect the concentration of total RNA. Pipette 100 μL of diluted samples separately and add to an all-black 96-well plate. Dilute the RiboGreen reagent in the kit with 1X TE Buffer according to a certain ratio, and then add 100 μL of each reagent into the 96-well plate with samples, and incubate at 37 °C for 5min under the protection of light. The Fluorescence of each well was measured using a BioTek Synergy H1 Multi-Mode Microplate Reader (Agilent) at the λex=485 nm, λem=528 nm setting. Gating information is shown in Fig. S9.

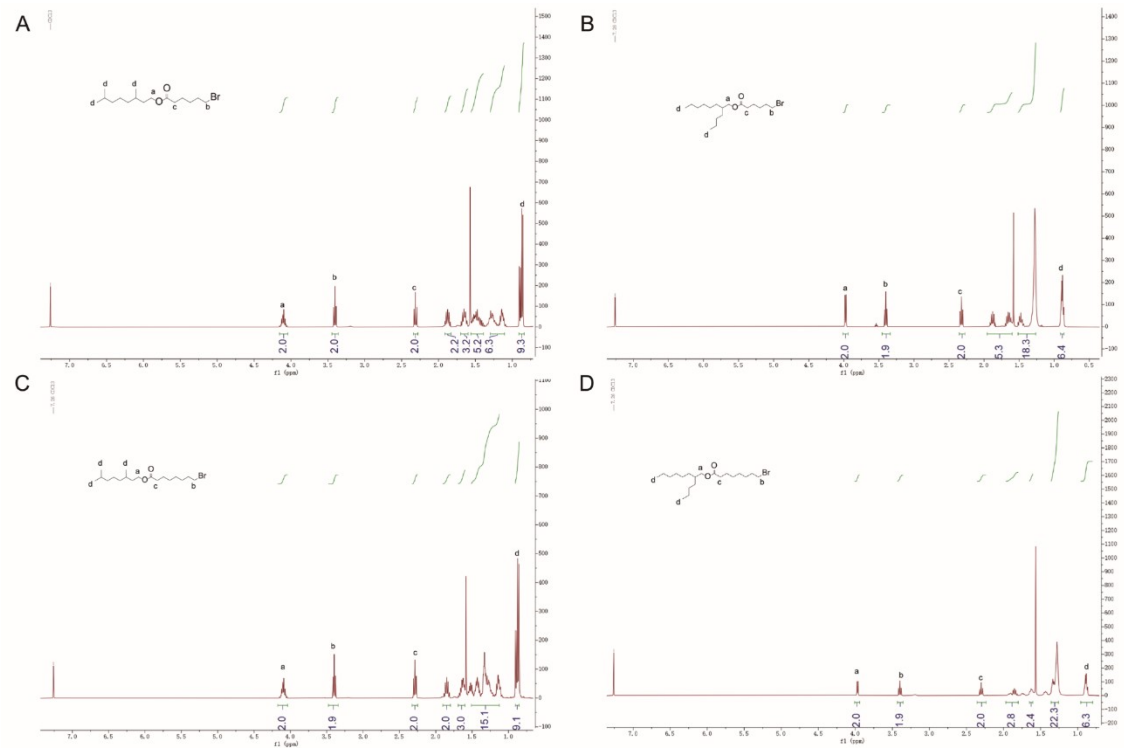


Fig. S1 (A) Mass spectrum and ^{1}H NMR of R1. 400 MHz, Chloroform-d: δ 4.15–4.04 (m, 2H), 3.40 (t, J=6.8 Hz, 2H), 2.31 (t, J=7.4 Hz, 2H), 1.89 (h, J=6.9, 6.3 Hz, 2H), 1.75–1.60 (m, 3H), 1.58–1.40 (m, 5H), 1.35–1.09 (m, 6H), 0.88 (dd, J=12.6, 6.6 Hz, 9H). (B) Mass spectrum and ^{1}H NMR of R2. 400 MHz, Chloroform-d: δ 3.97 (d, J=5.8 Hz, 2H), 3.40 (t, J=6.7 Hz, 2H), 2.32 (t, J=7.4 Hz, 2H), 1.88 (p, J=6.9 Hz, 3H), 1.68-1.60 (m, 2H), 1.54–1.25 (m, 18H), 0.89 (h, J=3.9 Hz, 6H). (C) Mass spectrum and ^{1}H NMR of R4. 400 MHz, Chloroform-d: δ 4.10 (tq, J=9.0, 4.1 Hz, 2H), 3.40 (t, J=6.8 Hz, 2H), 2.29 (t, J=7.5 Hz, 2H), 1.85 (p, J=7.0 Hz, 2H), 1.67–1.58 (m, 3H), 1.52–1.12 (m, 15H), 0.88 (dd, J=12.7, 6.6 Hz, 9H). (D) Mass spectrum and ^{1}H NMR of R5. 400 MHz, Chloroform-d: δ 3.97 (d, J=5.8 Hz, 2H), 3.40 (t, J=6.8 Hz, 2H), 2.30 (t, J=7.5 Hz, 2H), 1.94–1.79 (m, 3H), 1.65–1.60 (m, 2H), 1.31 (dt, J=21.0, 3.8 Hz, 22H), 0.89 (td, J=6.7, 3.9 Hz, 6H).

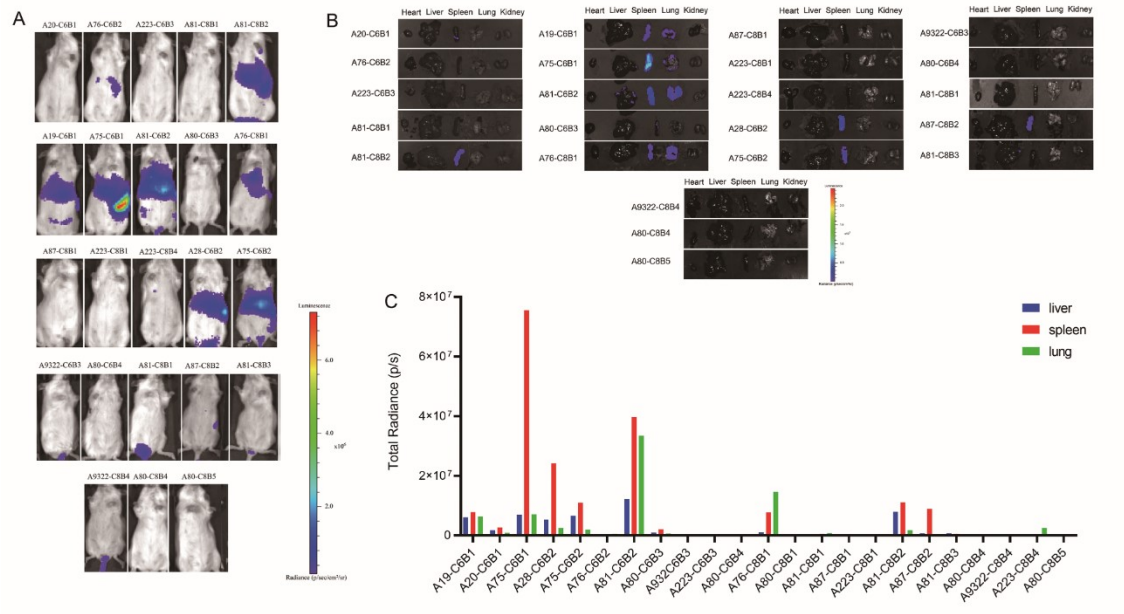


Fig. S2 (A) Representative IVIS images of mice at 6 h post I.V. Injection of twenty-six LNPs (2 μg of mLuc per mouse). (B) Ex vivo images of major organs from mice treated with LNPs/mLuc. (C) Quantification of mRNA expression by LNPs in various organs.

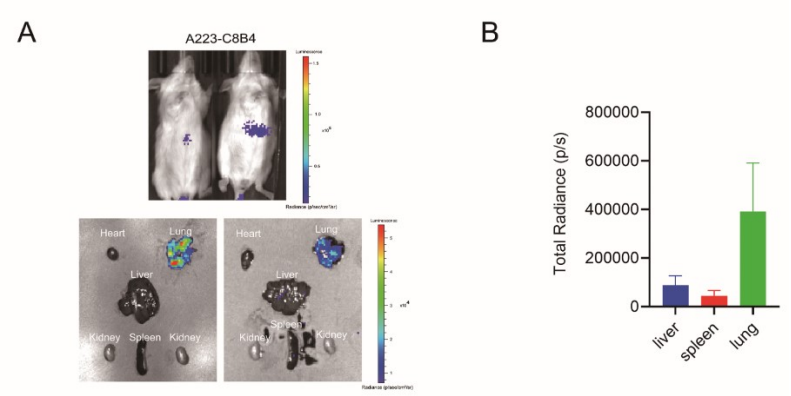


Fig. S3 (A) Representative IVIS images of mice at 6 h post intravenous injection of A223-C8B4. (B) Quantification of mRNA expression by A223-C8B4 in various organs.

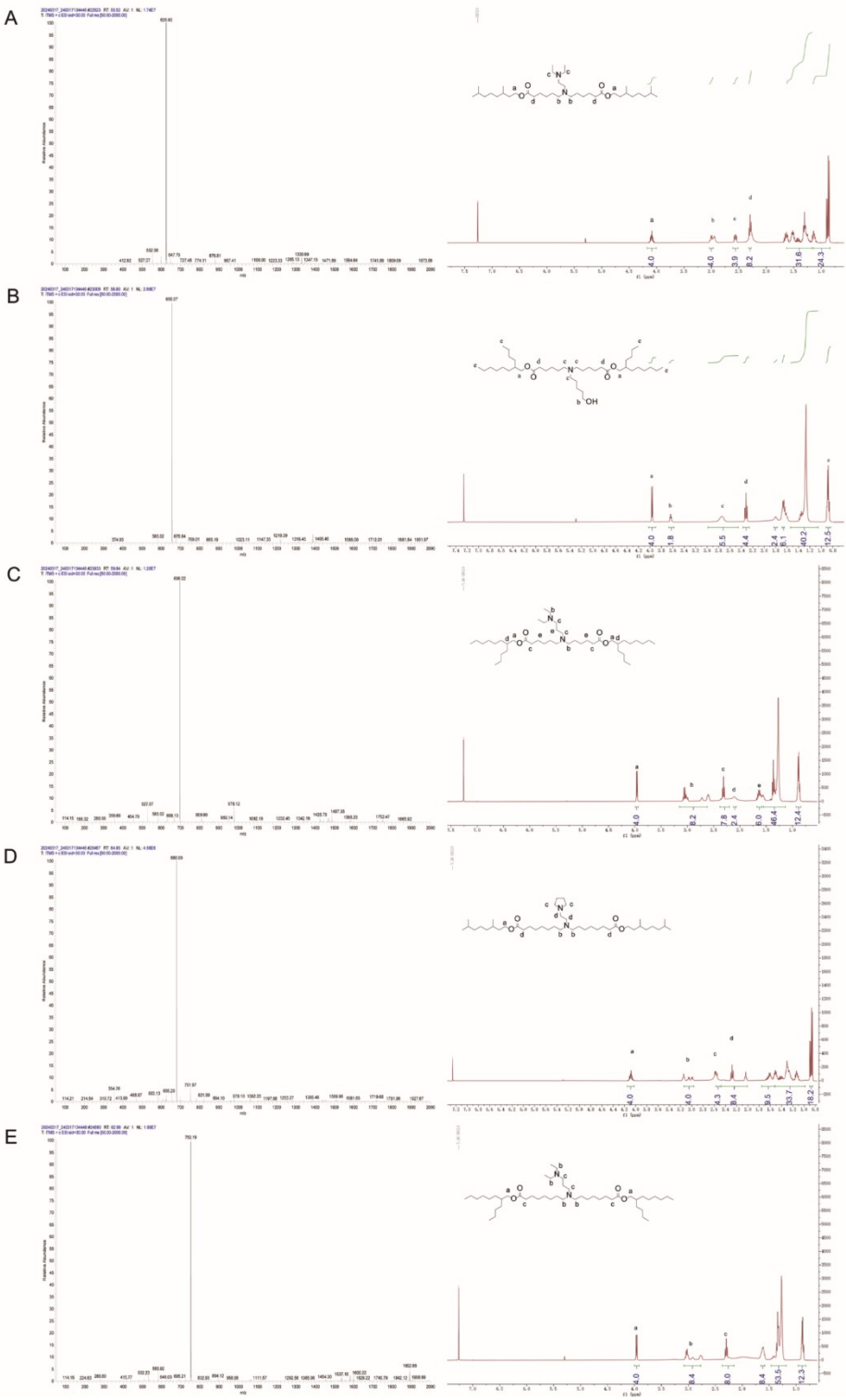


Fig. S4 (A) Mass spectrum and ^{1}H NMR of A75-C6B1. 400 MHz, Chloroform-d: δ 4.09 (td, J=7.4, 4.4 Hz, 4H), 3.00 (q, J=9.3, 8.3 Hz, 4H), 2.61–2.52 (m, 4H), 2.31 (d, J=7.4 Hz, 8H), 1.62–1.17 (m, 32H), 0.88 (dd, J=12.6, 6.6 Hz, 24H). (B) Mass spectrum and ^{1}H NMR of A28-C6B2. 400 MHz, Chloroform-d: δ 3.97 (d, J=5.8 Hz, 4H), 3.64 (t, J=5.3 Hz, 2H), 2.74 (s, 4H), 2.32 (t, J=7.4 Hz, 6H), 1.83–1.78 (m, 2H), 1.69–1.65 (m, 6H), 1.44–1.15 (m, 40H), 0.88 (td, J=6.6, 4.0 Hz, 12H). (C) Mass spectrum and ^{1}H NMR of A81-C6B2. 400 MHz, Chloroform-d: δ 3.96 (d, J=5.8 Hz, 4H), 3.03-2.60 (dq, J=14.9, 7.6 Hz, 8H), 2.31 (t, J=7.4 Hz, 8H), 2.11 (s, 2H), 1.67–1.59 (m, 6H), 1.55–1.13 (m, 46H), 0.88 (td, J=6.7, 4.0 Hz, 12H). (D) Mass spectrum and ^{1}H NMR of A76-C8B1. 400 MHz, Chloroform-d: δ 4.15–4.03 (m, 4H), 3.13–2.96 (m, 4H), 2.56 (d, J=7.5 Hz, 4H), 2.47–2.01 (m, 8H), 1.77–1.54 (m, 10H), 1.53–0.98 (m, 34H), 0.87 (dd, J=12.6, 6.6 Hz, 18H). (E) Mass spectrum and ^{1}H NMR of A81-C8B2. ^{1}H NMR (400 MHz, Chloroform-d) δ 3.96 (d, J=5.8 Hz, 4H), 3.09–2.77 (m, 8H), 2.30 (t, J=7.4 Hz, 8H), 1.62 (q, J=7.7,

6.9 Hz, 8H), 1.47–1.19 (m, 54H), 0.88 (td, J=6.7, 3.9 Hz, 12H).

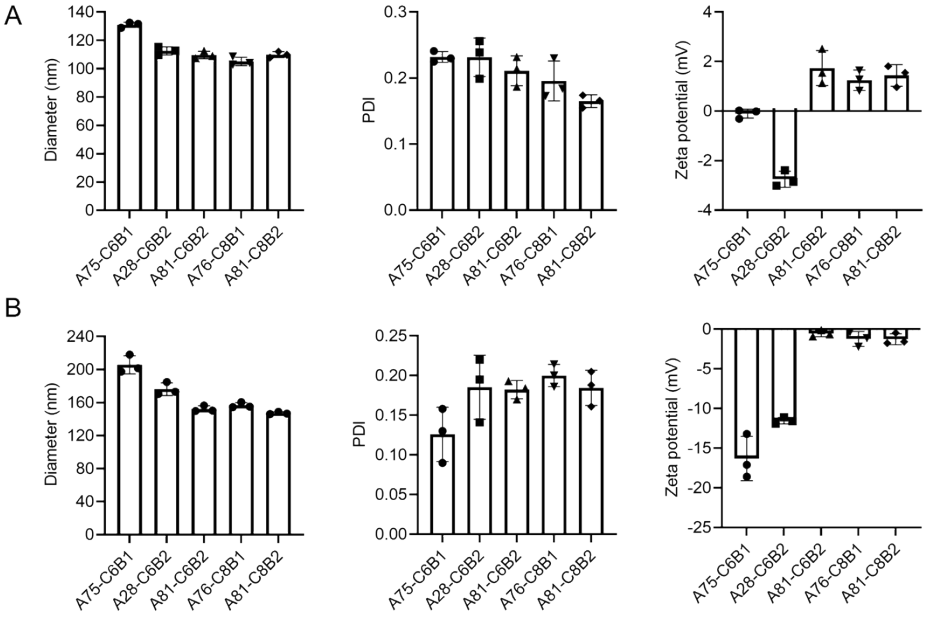


Fig. S5 The hydrodynamic diameter, polydispersity index (PDI), and zeta potential of LNPs (A) before and (B) after encapsulation of mRNA. Data were presented as mean ± s.d., n=3.

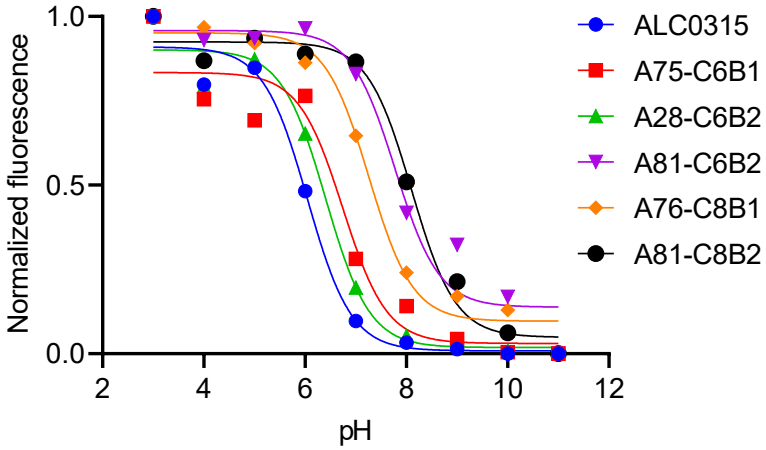


Fig. S6 Determination of pKa of LNPs using the TNS fluorescence assay.

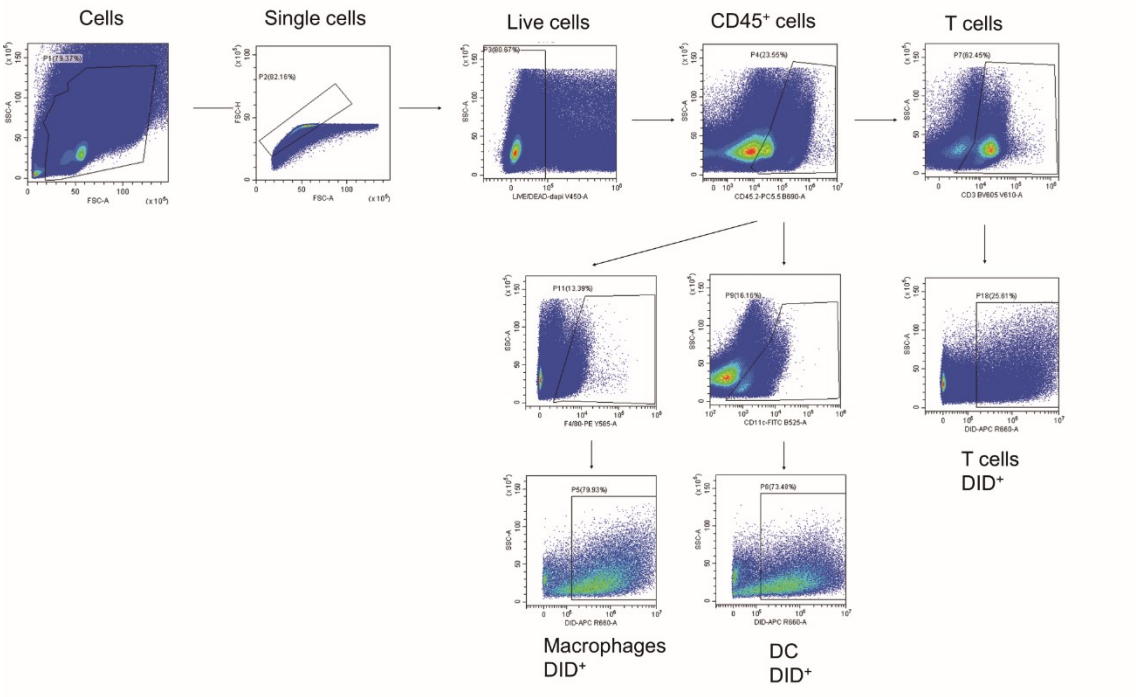


Fig. S7 The FACS gating strategy for analysis of DiD in splenocytes.

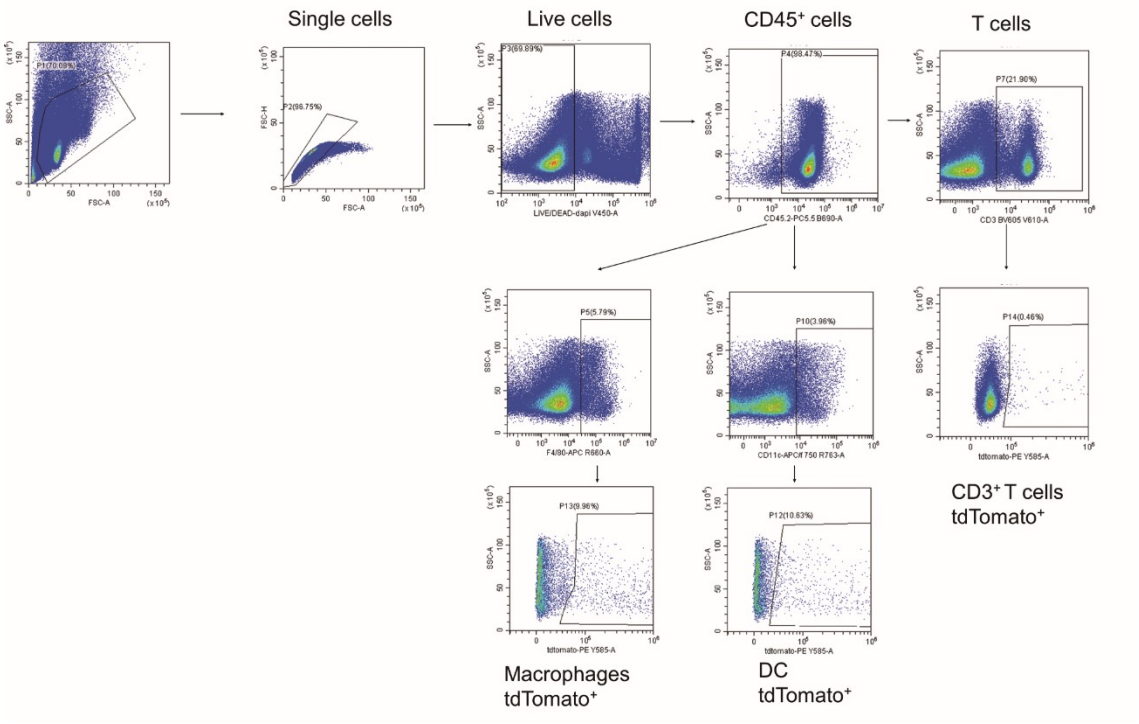


Fig. S8 The FACS gating strategy for analysis of tdTomato expression in splenocytes.

Table S1. Antibodies used for flow cytometry.

Target	Lable	Provider	Catalog
CD45	PerCp-Cy5.5	Biolegend	552950
F4/80	PE	Biolegend	157304
CD11c	FITC	Biolegend	103108
CD3	BV605	Biolegend	100237
CD4	PE/CY7	Biolegend	100528
CD8	BC650	Biolegend	344730
F4/80	APC	Biolegend	123165
CD11c	APC/Fire750	Biolegend	117352

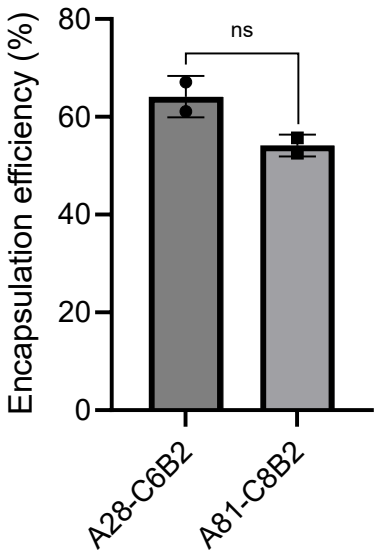


Fig.S9 mRNA encapsulation efficiency. Data were presented as mean ± s.d., n=2. The statistical significance was calculated by t tests: *p < 0.05, **p < 0.01, ****p < 0.001, ns represents not statistically significant.