

Rational design of rigid mRNA folding architecture to enhance intracellular processing and protein production

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Material and antibodies. 8-[(2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]amino]-octanoic acid, 1-octylnonyl ester (SM-102), DiIC18(7); 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR), chlorpromazine hydrochloride, cytochalasin-D, methyl- β -cyclodextrin, nocodazole, 2-deoxyglucose, wortmannin, dynasore and D-Luciferin (potassium salt) were purchased from MedChemExpress. 3,6-bis[4-[bis(2-hydroxydodecyl)amino]butyl]-2,5-piperazinedione (CKK-e12) was purchased from Echelon Biosciences. 4-(dimethylamino)-butanoic acid, (10Z,13Z)-1-(9Z,12Z)-9,12-octadecadien-1-yl-10,13-nonadecadien-1-yl ester (MC3) and 1,2-dioleoyl-3-dimethylammonium propane (DODAP) were purchased from BroadPharm. Distearoylphosphatidylcholine (DSPC) and 1,2-Dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000) were purchased from Avanti Polar Lipids. Cholesterol, magnesium chloride, zinc chloride, ferrous chloride tetrahydrate, copper(II) chloride, calcium chloride, aluminum chloride hexahydrate, manganese(II) chloride tetrahydrate, dithiothreitol (DTT), CH₃COOK, (CH₃COO)₂Mg, digitonin, cycloheximide, Triton X-100 and Sodium azide (NaN₃) were purchased from Sigma. All mRNA used in this study, including those encoding firefly luciferase (FLuc), enhanced green fluorescent protein (eGFP), mCherry, clustered regularly interspaced short palindromic repeats (CRISPR) associated protein 9 encoding (Cas9) mRNA and human erythropoietin encoding (hEPO) mRNA, and the self-amplifying RNA backbone, were purchased from TriLink BioTechnologies or ShanghaiCynbio. The primary mRNA platform investigated was (5moU)-modified and HPLC-purified to ensure high integrity and minimize immunogenicity. To evaluate the generality of the MARF strategy, we also tested unmodified mRNA and mRNA with m¹ Ψ modifications. Unless otherwise specified, all mRNA used in this study is 5moU modified. Cyanine5 (Cy5)-labeled FLuc mRNA (Cy5-mRNA) was purchased from APEXBio. Proprotein convertase subtilisin/kexin type 9 (Pcsk9) single guide RNA (Pcsk9-sgRNA), and enhanced green fluorescent (eGFP) protein single guide RNA (eGFP-sgRNA) were purchased from Integrated DNA Technologies. Pur-A-Lyzer Midi Dialysis Kits (molecular weight cutoff is 3,500) were purchased from Sigma. Antibodies used in this study include: Anti-mouse puromycin antibody (Developmental studies hybridoma bank, PMY-2A4), anti-mouse CK 18 antibody (Abcam, ab315096), anti-mouse F/480 antibody (Servicebio, GB113373), anti-mouse CD31 antibody (Servicebio, GB12063), anti-mouse PCSK9 antibody (Abcam, ab185194), anti-mouse HSP90 (Santa Cruz, sc-101494).

Nanoflow analysis. Nanoflow analysis was performed using the Nanoflow (NanoFCM Inc., China). Cy5-mRNA loaded LNPs and MARF-LNPs were prepared as described (DiO (0.5 wt% total lipids) was added into organic phase to label nanoparticles). LNP and MARF LNP were diluted with PBS to achieve an optimal range of 3000 to 20000 particles. Events were recorded for 1 min on two channels of 488 nm and 670 nm with a single photon counting avalanche photodiode detector. All channels were calibrated with 250 nm standard fluorescent silica beads ((S16M-Exo beads). Data were analyzed using the NanoFCM Professional Suite software (NanoFCM Inc., China).

Encapsulation number of mRNA. The encapsulation number of mRNA in each LNP was calculated as:

$$e = \frac{C_{all\ RNA} \times \zeta}{C_{all\ LNP} \times P_{Cy5^{+}Dio^{+}}}$$

where $C_{all\ RNA}$ represented the concentration of all mRNA involved in self-assembly with LNP. ζ represented mRNA encapsulation efficiency calculated using Quant-iT RiboGreen. $C_{all\ LNP}$ represented the concentration of all LNPs counted by NanoFCM. $P_{Cy5^{+}Dio^{+}}$ represented the proportion of LNPs (Dio⁺) encapsulating mRNA (Cy5⁺).

Encapsulation efficiency of metal ions. To quantify encapsulation efficiency of metal ions, we used Inductively Coupled Plasma Mass Spectrometry (ICP-MS). MARF-LNP were digested in freshly prepared aqua regia at room temperature for 24 hours. The solution was then diluted into deionized water ensuring the concentration of H⁺ < 2%. Samples were analyzed in triplicate, and encapsulation efficiency (EE) was calculated as:

$$EE = \frac{M_{In\ retention}}{M_{Total}}$$

where $M_{In\ retention}$ represented the amount of metal ions encapsulated in LNPs and M_{Total} represented total amount of metal ions involved in initial assembly with mRNA which in this case corresponds to 400-fold the Fluc mRNA concentration.

Western blot. The knockdown of *Pcsk9* gene was confirmed using Western blot. Hepa1-6 cells were cultured in 6-well plates (1×10^6 cells per well). After 24 hours of incubation, cells were treated with either MARF LNP or LNP (2.5 μ g RNA per well, 1:1, Cas9 mRNA/sgPcsk9, wt/wt). After 48 hours of incubation, the cells were lysed in RIPA buffer (Thermo Fisher) with a protease inhibitor cocktail (100 $\times$, Thermo Fisher) for 30 minutes at 4 °C. The cell lysates were centrifuged at 13,000 g for 15 minutes at 4°C and the supernatants were collected. Protein concentration was determined using a BCA protein assay kit (Thermo Fisher). Equal amounts of protein were separated by 10% SDS-PAGE and transferred onto PVDF membranes. Next, the membrane was blocked for 1 hours using 5% BSA in PBST (Thermo Fisher). After blocking, the membrane was incubated overnight at 4°C with primary antibodies against HSP90 (1:5000, Santa Cruz Biotechnology) and PCSK9 (1:1000, Abcam). Following PBST washes, the membrane was incubated with appropriate HRP-conjugated secondary antibodies (1:10000 dilution, Abcam) for 1 hours. After washing three times with PBST, the bands were visualized using ECL detection reagents (Bio-Rad).

Isolation of protein corona Adsorbed to LNP and MARF LNP. Isolation of protein corona was prepared according to previously described method with few modifications^{1,2}. LNP and MARF LNP were prepared and diluted to a final lipid concentration of 1 mg/mL with $1 \times$ PBS. LNP and MARF LNP were further incubated with mouse plasma at a 1:1 volume ratio and incubated under 37 °C for 2 hours. After incubation, the mixture was loaded onto a 0.7 M sucrose water solution and centrifuged at 15,300 g for 1 hour. The supernatant was removed and washed three times with PBS. After the final wash, the pellet was resuspended in 100 μ L 2% SDS solution and incubated under 95 °C for 10 minutes for the downstream analysis. 30 μ L SDS solution isolated from the surface of NPs was loaded on 10 % SDS-PAGE protein gel and separated at 120 V. Gel was

further visualized by Coomassie brilliant blue overnight. The gel was destained by deionized water and imaged. Further proteomics analysis of protein corona was conducted in the Quantitative Proteomics Core at the Cancer Science Institute of Singapore.

Endosomal escape. HEK-293T cells were cultured in Lab-Tek Chambered Coverglass (3×10^4 cells/well) overnight. Cells were treated with either Cy5-mRNA loaded LNP or MARF LNP (250 ng mRNA per well). After incubation for 24 hours under 37 °C, cells were washed three times with PBS, and LysoTracker Green was introduced in culture media for 1 hour to stain the endosomes. Subsequently, cells were washed again with PBS for three times, and Hoechst was added for 10 minutes to stain the nuclei for confocal laser microscopy observation.

For calcein leakage assay, flow cytometry and confocal microscopy were conducted. HEK-293T cells were seeded in 96 well plates (1×10^4 cells /well) or Lab-Tek Chambered Coverglass (3×10^4 cells/well) and cultured overnight. The next day, cells were treated with or without Bafilomycin A₁ (100 nM) and calcein (100 μM). LNPs and MARF LNPs were introduced to each well at a FLuc mRNA concentration of 250 ng/mL. After 4 hours incubation, cells were washed with PBS three times and then quantified using flow cytometry or observed by confocal microscopy.

FRET-based quenching assay. A 20-nt RNA oligonucleotide (GACCGAGAUGGACACGACCC), dual-labeled with BHQ2 and BHQ3 quenchers, was designed. 5 μg Cy5-mRNA and 0.25 nmol quenchers were first incubated for 2 hours at 4°C. RNAs solutions were later incubated with different ratios of Mn²⁺ for 12 hours at 4°C to form folded RNAs. After incubation, fluorescence spectra of folded RNA were measured by using an Edinburgh Instruments F-980 fluorescence spectrometer. Subsequently, 2.4 nmol EDTA were incubated with folded RNAs to recover structure of folded RNAs for 40 minutes and measured the fluorescence spectra. HEK-293T cells were seeded in Lab-Tek Chambered Coverglass (3×10^4 cells/well) overnight. The next day, LNP and MARF LNP encapsulating Cy5-mRNA and Quenchers with the same ratio described above were added into the cells at a Cy5-mRNA concentration of 250 ng/mL, followed by incubation at 37°C. After 1, 2, 4 and 6 hours, cells were washed with PBS for three times, and LysoTracker (green) dissolved in culture media was introduced and incubated for 1 hour. Subsequently, cells were washed with PBS for three times and Hoechst was added to cells for 10 minutes to stain the nucleus. Finally, live cells were observed via confocal laser microscopy.

Hemolysis. The hemolysis assay was conducted following a previously established protocol with modifications³. Blood sample was collected from mice and washed five times with 1× PBS. The red blood cells (RBCs) were then diluted in either citrate buffer saline (pH = 5.5, 20 mM citrate buffer, 130 mM NaCl) or 1 × PBS to prepare 4% vol/vol RBC solution. 200 μL RBC solution was added to 800 μL of LNP or MARF LNP (containing 1 μg mRNA), which had been pre-diluted in either 1× PBS or citrate buffer saline. The mixtures were incubated at 37°C for 1 hour. After incubation, the samples were centrifuged at 4°C at 1000 × g for 5 minutes. The supernatant was then transferred to a 96-well plate, and UV absorbance was measured at 540 nm using a plate reader (Thermo Fisher). As controls, 1× PBS and buffer served as negative control, while

water was used as a positive control. The percentage of hemolysis was calculated using the following formula:

$$\frac{\text{Sample absorbance} - \text{Negative control absorbance}}{\text{Positive control absorbance} - \text{Negative control absorbance}} \times 100\%$$

Stability of metal-assisted folded RNA. Fluc mRNA and Fold RNA (Mn) were dissolved separately in a nuclease-free water solution. And then, the solutions were incubated at 37°C for 12 hours. Subsequently, the solutions were analyzed using non-denaturing agarose gel electrophoresis. The mean gray values of different RNA brands were analyzed by ImageJ.

In vivo toxicity. BALB/c mice were intravenously or intramuscularly injected with FLuc mRNA loaded LNP or MARF LNP (2.5 mg/kg) and PBS were administered as control group. Serum was collected at 4, 24 and 48 hours to measure aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN), and creatinine (CREA), following the manufacturer's protocol (NanJing Jiancheng Bioengineering Institute). Major organs (heart, liver, spleen, lung, and kidney) were collected for H&E staining. Additionally, serum and lymph nodes proinflammatory cytokines, including IL-6, IFN- β , TNF- α and CXCL 10, were evaluated using ELISA kits (BioLegend).

Whole blood cells counting. BALB/c mice were administrated with mRNA (2.5 mg/kg FLuc mRNA) through i.v. injection. After 48 hours, whole blood was collected in EDTA anticoagulants for whole blood cells counting (BeiJing BowlinMan).

Puromycin labelling of nascent protein polypeptide chains. HEK-293T cells were cultured in 6-well plates (1×10^6 cells per well). After 24 h incubation, the medium was replaced with 1.5 mL of fresh medium containing eGFP or mCherry mRNA loaded MARF LNP or LNP (2.5 μ g mRNA per well) respectively. After 24 hours, puromycin was added to the culture medium to a final concentration of 36 μ M and incubated at 37 °C for 30 minutes to label newly translating polypeptide chains. Cells were washed with ice-cold PBS, and then lysed on the ice for 10 minutes using lysis buffer (100 mM CH₃COOK, 5 mM (CH₃COO)₂Mg, 1 mM dithiothreitol, 100 μ g/mL digitonin, 1 $\times$ EDTA-free protease inhibitor cocktail and 50 mM HEPES, pH = 7.4). Lysates were obtained by centrifugation at 20,000 $\times g$ for 5 min at 4°C and protein concentrations were measured using the BCA assay kit (Biobasic). 20 μ g of total protein samples were analyzed using 10% Tris-Tricine based gels and stained by Ponceau S staining solution (Sigma) or transferred to 0.2 mm nitrocellulose membrane (Biorad) for anti-puromycin western blot. Puromycin-labelled nascent proteins were detected by anti-puromycin antibody (PMY-2A4, DSHB).

Polysome profiling of HEK-293T cells. HEK-293T cells were cultured in 10 cm dishes until reaching 70-80% confluency and then treated with culture medium containing the indicated concentration of metal ions for 2 hours. 15 minutes prior to cell harvesting, cycloheximide was added to the medium at a final concentration of 100 μ g/mL to freeze translating ribosomes. Cells were washed with ice-cold 1 $\times$ PBS, and then lysed in ice for 10 minutes using 200 μ L lysis buffer (100 μ g/mL cycloheximide, 100 mM CH₃COOK, 5 mM (CH₃COO)₂Mg, 1

mM dithiothreitol, 1% Triton X-100, 1X EDTA-free protease inhibitor cocktail and 50mM HEPES, pH7.4). Lysates were obtained by centrifugation at $20,000 \times g$ for 5 min at 4°C. Lysate samples were loaded onto a 12 mL 10%–50% sucrose gradient and subjected to ultracentrifugation at 36,000 rpm for 2 h at 4°C in an SW41 rotor (Beckman). A piston gradient fractionator system (Biocomp) was used to monitor UV absorbance at 260 nm across the gradient.

Establishment of cell aging models. HEK-293T cells (both under 10 passages) were cultured in 6-well plates (5×10^5 cells per well). After 12 hours incubation, cells were treated with Doxorubicin at a final concentration of 250 nM. Cells were replaced with fresh medium after 24 hours treatment. After incubation for 24 hours, cells were ready for downstream analysis.

Mechanical property fitting of LNP and MARF LNP models. We constructed the mechanical model of LNP and MARF LNP by a cross-linking network of R beads. The number of cross-linking bonds for two models is fitted by comparing the simulated mechanical strength in dry state with the experimentally measured one. The more the cross-linking bonds there are, the stronger the mechanical strength becomes. Specifically, the LNP model is constructed by a chain of 3,000 beads that are randomly cross-linked by 3,000 bonds, and the MARF LNP model is constructed by a chain of 4,000 beads that are randomly cross-linked by 20,000 bonds. The bond interactions employ a harmonic spring with force constant $k_{bend}=1000.0 \text{ k}_B\text{T}$ and rest length $r_0=0.95 \text{ nm}$. The interactions between R beads and R beads employ the potential of $U_{LJ}(r)$ with $b=\sigma$.

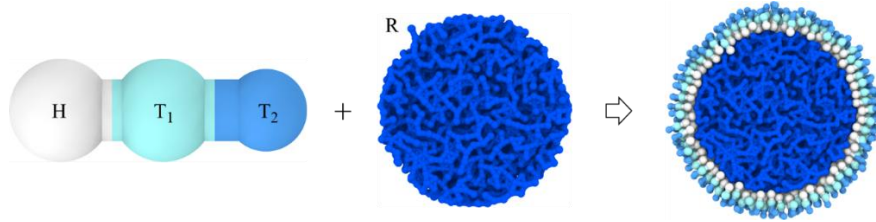
CG-MD simulations on endocytosis. The LNP and MARF LNP are wrapped by 2,500 and 1,300 lipids, respectively, to construct the wrapped LNP model and wrapped MARF LNP model. The initially random wrapped lipids become an ordered membrane in 100 μs MD simulation. The interactions between LNP and membrane are set as $U_{COS}(r)$ between H and R beads and $U_{WCA}(r)$ for other bead pairs, while the interactions between MARF LNP and membrane are set as $U_{COS}(r)$ between H and T_2 beads and $U_{WCA}(r)$ for other bead pairs. The cell membrane is represented by a vesicle model consisting of 30,000 lipids. The interactions between LNP/MARF LNP membrane and cell membrane are set as $0.6U_{LJ}(r)$ between H_{LNP} and H_{cell} beads and $U_{WCA}(r)$ for other bead pairs. The simulation of endocytosis on LNP/MARF LNP by cell in solution was conducted up to the time of 3,000 μs .

CG-MD simulations on translocation. The translocation process was simulated by driving the LNP and MARF LNP going through a hole on a plane. The plane is stacked by small beads at hexagonal arrangement with a bead-bead distance of 0.8 nm. The hole is dug on the plane with a radius of 20 nm. The translocation of wrapped LNP and MARF LNP through the hole is driven by a force of 0.015 per bead in reduced unit. The interactions between plane and the nanoparticles of wrapped LNP/MARF LNP employ the potential of $U_{WCA}(r)$.

All-atom MD simulations on the potential of mean force. All-atom MD simulations were conducted using the Gromacs 2023.4 simulation package⁵. All-atom force field parameters of the RNA dinucleotide were taken

from Amber99sb-ildn⁶ with parmbosc0⁷ and χ OL3⁸ corrections, and the TIP3P water model was used. The particle-mesh Ewald method was employed with a Fourier spacing of 0.12 nm, and a grid interpolation up to order 4 to manage long-range electrostatic forces and maintain the electroneutrality condition. The LINCS algorithm was used to constrain bonds involving hydrogen atoms, and a 2 fs time step was set. Coulomb real space interactions were truncated at 1.2 nm, and LJ interactions were similarly truncated at 1.2 nm. Long-range dispersion corrections for energy and pressure were applied to mitigate errors from truncated LJ interactions. Initially, the RNA was placed in a cubic box with dimensions of $4.0 \times 4.0 \times 4.0$ nm³, which was filled with TIP3P water molecules. Next, we carried out energy minimization, equilibration, and production run for each simulation setup. Energy minimization was achieved using the steepest descent algorithm. For pre-equilibration, we conducted NPT simulations using the Berendsen scheme. During simulation, the temperature was maintained at 300 K with the Berendsen thermostat, and the pressure was held at 1 bar using the Berendsen barostat⁹. Production runs were conducted in the NPT ensemble using the isotropic Parrinello-Rahman barostat, while the temperature was regulated with the velocity rescaling thermostat incorporating a stochastic term. The coupling constants for pressure and temperature were set to 5.0 and 0.1 ps, respectively. We used umbrella sampling simulations to calculate the potential of mean force (PMF) as a function of the distance of a single cation binding to RNA. For the backbone, we used the distance between the cation and the O2P atom. For metal ions in the system, a window spacing of 0.1 nm (roughly 20 windows) was used and 100 ns was performed for each umbrella simulation. The PMF was calculated using the weighted histogram analysis method (WHAM)¹⁰.

Coarse-grained molecular dynamics simulation method



The model of lipid, LNP/MARF LNP, and wrapped LNP/MARF LNP, where a lipid is represented by three beads named as H (head bead), T₁ (first tail bead 1), and T₂ (second tail bead), and LNP/MARF LNP is represented by a cross-linked network of R beads.

For large-scale molecular dynamics (MD) simulations of physical behaviors of lipid-wrapped LNP/MARF LNP, we employed the solvent-free coarse-grained (CG) model that was initially developed by Deserno et al¹¹ and refined in the application by Shi et al¹². The following potentials are used in the model to describe the interactions between beads:

$$U_{LJ}(r) = 4.0\varepsilon \left[\left(\frac{b}{r} \right)^{12} - \left(\frac{b}{r} \right)^6 \right] (0 < r < r_{cut} = 2^{1/6}b + w),$$

$$U_{WCA}(r) = 4.0\varepsilon \left[\left(\frac{b}{r} \right)^{12} - \left(\frac{b}{r} \right)^6 + \frac{1}{4} \right] (0 < r < r_{cut} = 2^{1/6}b),$$

$$U_{\text{COS}}(r) = \begin{cases} \varepsilon + U_{\text{WCA}}(r), & (r < r_{\text{cut}} = 2^{1/6}b) \\ -\varepsilon \cos \frac{\pi(r-r_{\text{cut}})}{2w}, & (r_{\text{cut}} < r < r_{\text{cut}} + w) \end{cases},$$

$$U_{\text{Harmonic}}(r) = \frac{1}{2}k_{\text{bend}}(r - r_0)^2.$$

In the potentials, ε and σ are used as the units of energy and length. The parameter ε can be deduced from the temperature, which is set as $1.1 k_B T$. The bead diameter, σ , is set as 1 nm , by comparing a typical membrane thickness of 5 nm . There are one head bead and two tail beads in one lipid, as typical DPPC lipid has 50 atoms which will be modeled as 3 beads. We choose $b_{\text{head,head}} = b_{\text{head,tail}} = 0.95\sigma$ and $b_{\text{tail,tail}} = \sigma$. The interactions between head beads and head beads, and between head beads and tail beads employ $U_{\text{WCA}}(r)$ potential, while the interactions between tail beads and tail beads employ $U_{\text{COS}}(r)$ potential with $w = 1.6\sigma$. The neighboring beads in a lipid are connected by a harmonic spring with force constant $k_{\text{bend}} = 1000.0 k_B T$ and rest length $r_0 = 0.95 \text{ nm}$. The head bead and the second tail bead are also connected by a harmonic spring with force constant $k_{\text{bend}} = 10.0 k_B T$ and rest length $r_0 = 4.0 \text{ nm}$. Mechanical property fitting simulation of LNP and MARF LNP in the dry state is conducted through conventional MD without solvents, while the wet-state simulations of endocytosis and translocation are achieved by introducing the implicit solvent model of lipid-wrapped LNP/MARF LNP along with Langevin molecular dynamics. All CG-MD simulations were performed on an in-house package¹³.

Standardized Preparation of MARF LNP and Quality Control of Metal Incorporation

Materials and Reagents

1. mRNA: All 5moU-modified, m¹Ψ-modified, and unmodified mRNA used in this study, including FLuc, eGFP, mCherry, Cas9, hEPO, and the self-amplifying RNA, were purchased from TriLink BioTechnologies or Shanghai Cynbio. All mRNA were purchased at the concentration of 1 mg/mL . All mRNA passes agarose gel mobility-purified to ensure high integrity and minimize immunogenicity. And all mRNA was stored at -80°C in RNase-free tubes. Each lot was accompanied by a certificate of analysis (CoA).
2. Metal salts: magnesium chloride, zinc chloride, ferrous chloride tetrahydrate, copper(II) chloride, calcium chloride, aluminum chloride hexahydrate, manganese(II) chloride tetrahydrate were purchased from Sigma with analytical grade
3. Buffers: 10 mM citrate buffer ($\text{pH } 3.0$, RNase-free) for RNA folding; $1\times\text{PBS}$ ($\text{pH } 7.4$) for dialysis and storage. All buffers were prepared in metal-free consumables and filtered ($0.22 \mu\text{m}$).
4. Lipids: sm-102 were purchased from MedChemExpress. CKK-e12 was purchased from Echelon Biosciences. MC3 and DODAP were purchased from BroadPharm. DSPC and DMG-PEG2000 were purchased from Avanti Polar Lipids. All chemicals were analytical grade.
5. Consumables: Low-binding centrifuge tubes, ultrafiltration devices (MWCO $3\text{--}10 \text{ kDa}$), dialysis tubing (MWCO $6\text{--}8 \text{ kDa}$), pre-cleaned glassware.
6. Key instruments: ICP-MS, DLS/Zetasizer, Cryo-EM, AFM, microfluidic mixing device (NanoAssemblr or equivalent).

Step 1. Folded RNA Preparation.

1. Preparation of metal ions stock solution: Dissolve metal ions salt into RNase-free water at a concentration of 10 mM.
2. Incubation: Incubate mRNA (1 mg/mL) with metal ions solution (Metal : mRNA = 400 :1 mol/mol) under 4 °C for 12 hours to form folded RNA complex. Record temperature, volumes, and lot numbers.

Step 2 MARF LNP Formulation.

1. Organic phase: Dissolve ionizable lipid: DSPC: cholesterol: DMG-PEG2000 at 50:10:38.5:1.5 molar ratio in ethanol.
2. Aqueous phase: Use folded RNA in 10mM citrate buffer (pH 3.0).
3. Mixing: Rapid injection followed by vortexing at 3,000 rpm for 5 min.
4. Stabilization: Incubate at 4 degree for 4 h to allow nanoparticle maturation.

Step 3. Purification and Concentration

1. Dialysis: Transfer to dialysis tubing (MWCO 6–8 kDa) against excess 1×PBS (pH 7.4) for 12 h at 25 °C, replacing the outer buffer at least three times.
2. Concentration: Use ultrafiltration devices (MWCO 10 kDa) at 3,000 × g to achieve the desired volume.
3. QC sampling: Aliquot for (a) metal quantification, (b) nanoparticle physicochemical characterization, (c) potency assays, and (d) archival storage (–80 °C).

Step 4. Quantification of Metal Incorporation

1. Separation of Bound and Free Metal

Centrifugal ultrafiltration (MWCO 3 kDa) to separate LNP-bound metal (retentate) from free metal (filtrate).

2. ICP-MS Quantification

- 1) Sample preparation: Digest in freshly prepared aqua regia at room temperature for 24 hours. Dilute directly into deionized water ensuring the concentration of $H^+ < 2\%$.
- 2) Calibration: Matrix-matched standards (0.1–100 ppb), blanks, and certified reference material (CRM) included.
- 3) ICP-MS analysis: Monitor isotope of interest (e.g., ^{55}Mn).
- 4) Calculations: Encapsulation efficiency (%) = (metal in retentate / total metal added) × 100%

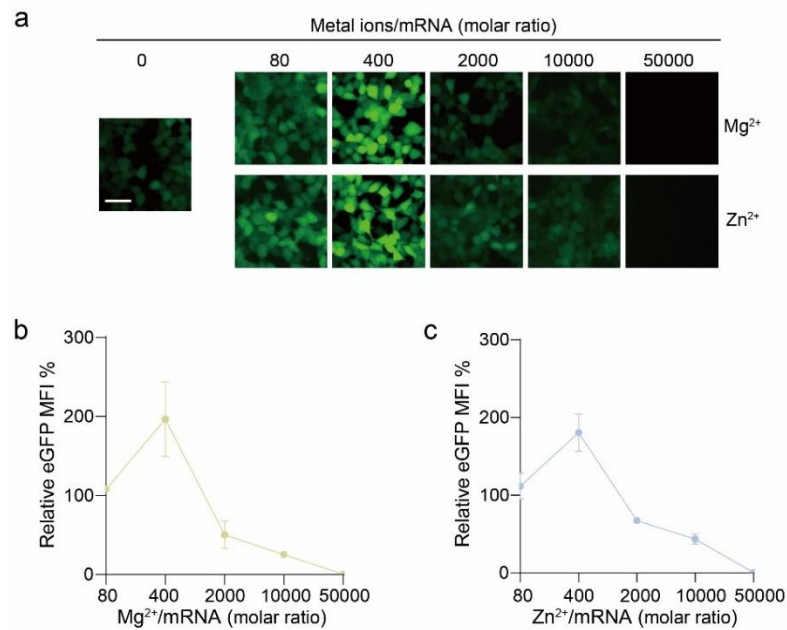
Step 5. Quality Control

1. Metal Encapsulation Assays: Inductively coupled plasma-mass spectrometry (ICP-MS) to evaluate total metal ions encapsulation efficiency (target: 30%), ensuring <10% deviation from target levels.
2. Nanoparticle Characterization: Dynamic lighting scattering (DLS) and cryogenic electron microscopy (cryo-EM) are applied to assess size distribution (target: hydrodynamic diameter <200 nm, zeta potential -5-5 mV, PDI< 0.3) and morphology. Atomic force microscopy (AFM) performed in liquid environments is employed to evaluate Young's modulus of nanoparticles. Ribogreen is used to evaluate mRNA encapsulation efficiency (target: >90%).
3. Functional Performance Tests: Evaluate transfection efficiency in vitro using luciferase or eGFP mRNA in HEK-293T cells, targeting > 2-fold enhancement in protein expression compared to control LNPs. for in vivo performance, assess liver expression after intravenous (I.V.) administration, aiming for >5-fold

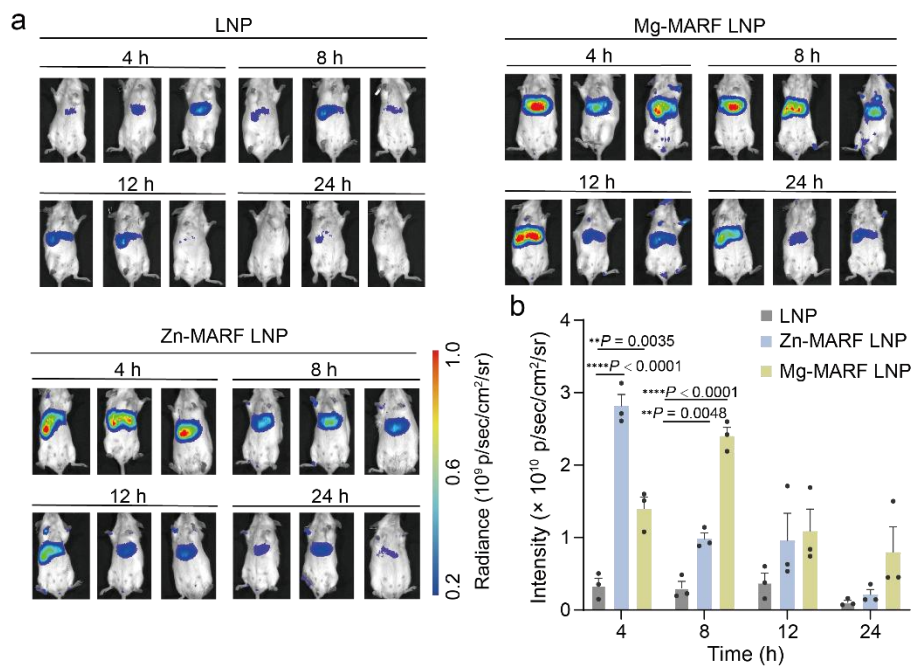
improvement at 4 hours post-injection, verified via IVIS.

4. Batch comparability: Evaluate successive batches through multiple key parameters, including particle size and polydispersity index, zeta potential (via DLS), mRNA encapsulation efficiency (via RiboGreen), and transfection efficiency (via in vitro luciferase assays).

Supplementary Figures



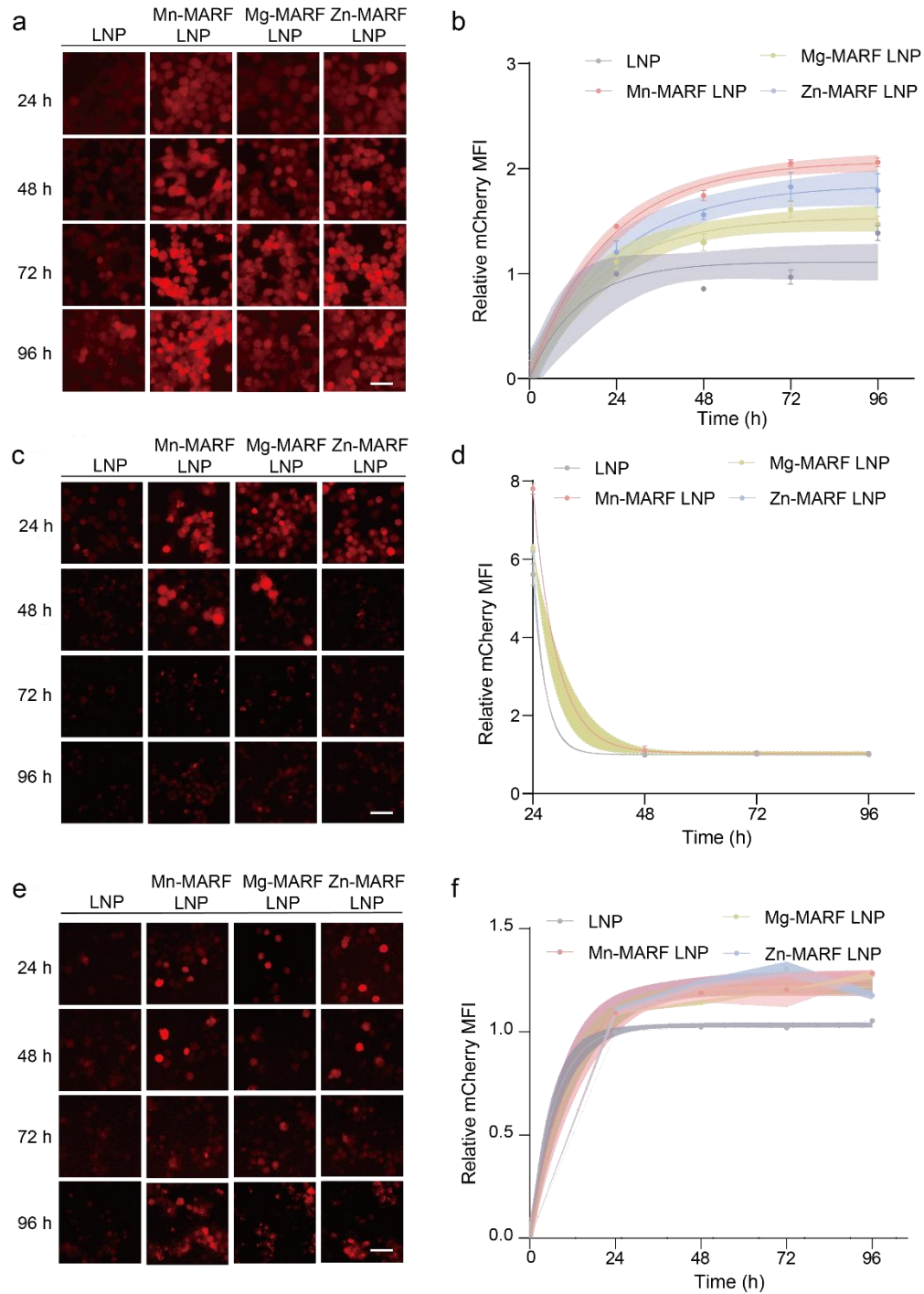
Supplementary Fig. 1 Transfection efficiency of eGFP mRNA incubated with different ratios of metal ions. (a) The fluorescence microscopy images of HEK293T cells after 24 h of incubation with eGFP-mRNA loaded Mg-MARF LNP or Zn-MARF LNP at different molar ratios of Mg²⁺ and Zn²⁺. **b,c** Relative intensity of eGFP in (a). The results were obtained from n=3 biologically independent samples. Data were presented as mean $\pm$ s.e.m. Scale bar: 50 μ m.



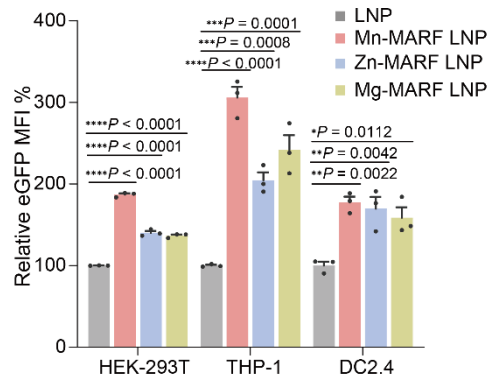
Supplementary Fig. 2 In vivo Fluc mRNA expression delivered by MARF LNP. (a) Bioluminescence images of mouse treated with FLuc-mRNA loaded LNP, Mg-MARF LNP or Zn-MARF LNP (mRNA:Mg²⁺/Zn²⁺=1:400) through i.v. injection at different time points. (b) Quantitative analysis of bioluminescence signal in (a). Data are presented as mean $\pm$ s.e.m. (n=3 biologically independent animals).

a					b		
Formulation(mol%)	Metal ions	Particle size (nm)	Zeta potential (mV)	PDI	Metal ions	pK _a	Encapsulation efficiency (%)
SM-102(50%) Cholesterol(38.5%) DSPC(10%) DMG-PEG20000(1.5%)	LNP	120.71	0.23	0.21	LNP	6.56	95.19
	Mn	120.36	0.20	0.15	Mn ²⁺	6.86	95.92
	Mg	106.58	0.03	0.18	Mg ²⁺	6.93	95.64
	Zn	119.74	0.07	0.16	Zn ²⁺	6.73	93.13
	Fe	116.49	0.07	0.17	Fe ²⁺	6.80	96.30
	Cu	129.29	0.10	0.21	Cu ²⁺	6.95	96.06
	Ca	116.00	0.03	0.13	Ca ²⁺	6.77	94.08
MC3(50%) Cholesterol(38.5%) DSPC(10%) DMG-PEG20000(1.5%)	LNP	140.43	0.07	0.22			
	Mn	151.65	0.10	0.22			
	Mg	133.89	0.07	0.23			
	Zn	142.92	0.33	0.23			
	Fe	137.98	0.00	0.19			
	Cu	223.43	0.13	0.19			
	Ca	144.83	0.03	0.22			
cKK-e12(50%) Cholesterol(38.5%) DSPC(10%) DMG-PEG20000(1.5%)	LNP	141.98	0.40	0.26			
	Mn	113.50	0.60	0.23			
	Mg	131.79	0.20	0.25			
	Zn	164.77	0.10	0.27			
	Fe	157.16	0.13	0.26			
	Cu	159.78	0.36	0.27			
	Ca	133.93	0.63	0.19			
DODAP(50%) Cholesterol(38.5%) DSPC(10%) DMG-PEG20000(1.5%)	LNP	110.58	-0.03	0.22			
	Mn	140.28	0.07	0.16			
	Mg	124.55	0.03	0.18			
	Zn	118.31	0.13	0.17			
	Fe	151.68	0.10	0.25			
	Cu	117.51	-0.03	0.19			
	Ca	142.90	0.07	0.22			

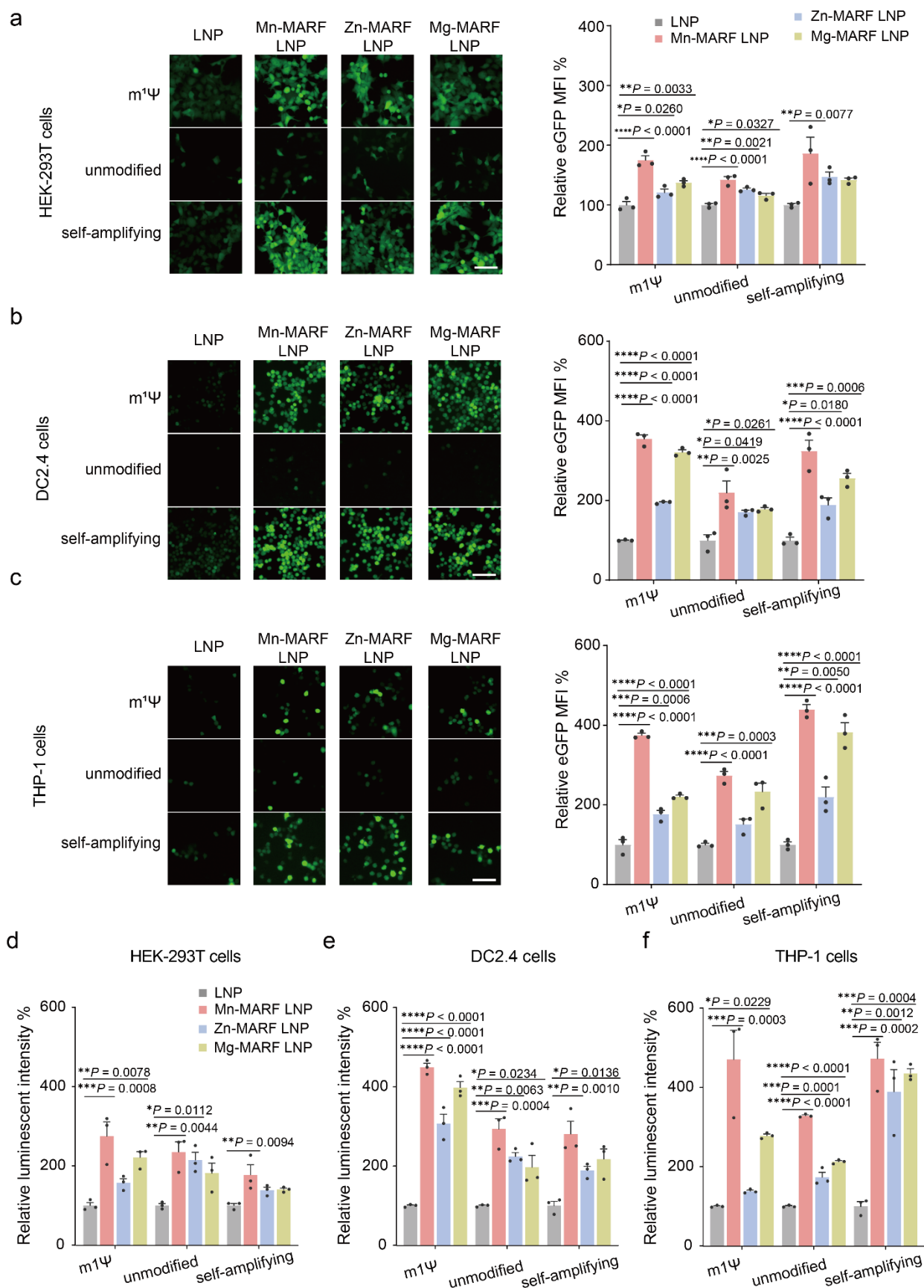
Supplementary Fig. 3 Characteristics of nanoparticles. (a) Zeta potential of various control LNP and MARF LNPs. (b) apparent pK_a and encapsulation efficiency of various MARF LNPs (SM-102 as ionizable lipid). The results were obtained from n=3 independent samples.



Supplementary Fig. 4 *In vitro* mCherry mRNA transfection in different cell lines. The mCherry-mRNA loaded LNP, Mn-MARF LNP, Mg-MARF LNP and Zn-MARF LNP (mRNA: $\text{Mn}^{2+}/\text{Mg}^{2+}/\text{Zn}^{2+}=1:400$) were transfected into different cell lines, including **(a, b)** HEK-293T cells, **(c, d)** DC2.4, and **(e, f)** THP-1 cells. The fluorescence microscopy images of cells after 24, 48, 72 and 96 hours of incubation with mCherry-mRNA loaded LNP, Mg-MARF LNP and Zn-MARF LNP **(a, c and e)**. Relative intensity of mCherry **(b, d and f)**. The results were obtained from $n=3$ biologically independent samples. Data were presented as mean $\pm$ s.e.m. Scale bar: 50 μ m.

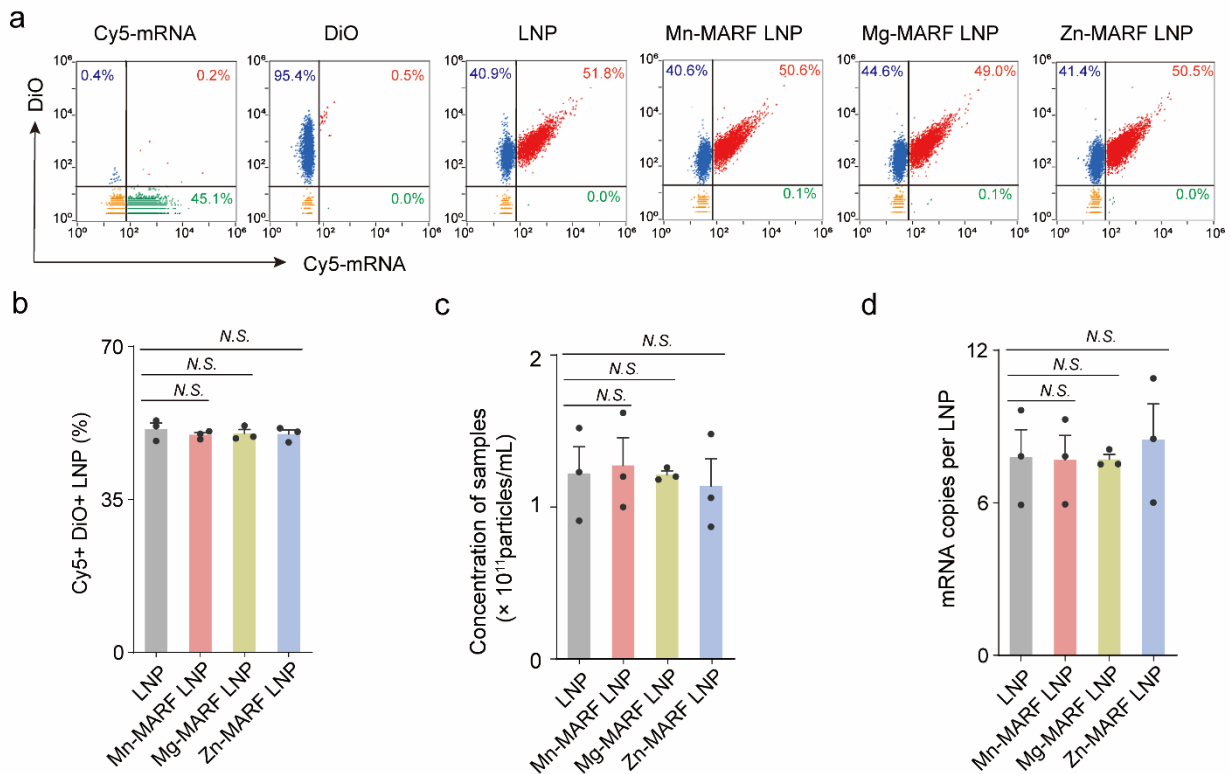


Supplementary Fig. 5 In vitro eGFP mRNA transfection in different cell lines. MARF LNP encapsulating eGFP mRNA were transfected into different cell lines, including HEK-293T cells, DC2.4, and THP-1 cells. The transfection efficiency of eGFP mRNA at 24 hours was quantified by ImageJ. The results were obtained from n=3 biologically independent samples. Data were presented as mean $\pm$ s.e.m.

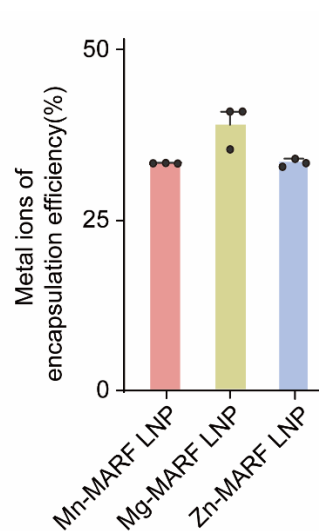


Supplementary Fig. 6. MARF strategy enhanced protein expression of delivered mRNA with

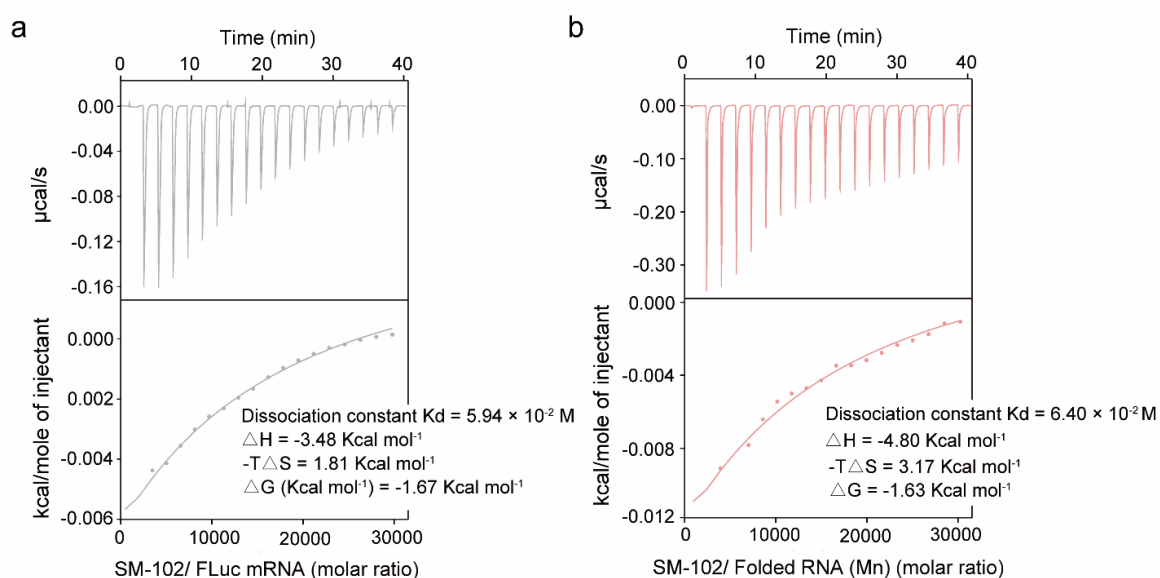
various chemical modifications *in vitro*. *In vitro* transfection of LNP, Mn-MARF LNP, Mg-MARF LNP, and Zn-MARF LNP encapsulating unmodified mRNA, m¹Ψ-modified mRNA, or self-amplifying mRNA encoding eGFP in (a) HEK293T cells, (b) DC2.4 cells, and (c) THP-1 cells. Fluorescence microscopy images (left) and relative eGFP intensities (right) were obtained after 24 h of incubation with LNP, Mg-MARF LNP, or Zn-MARF LNP. Relative luciferase expression was measured in (d) HEK293T, (e) DC2.4, and (f) THP-1 cells following treatment with control LNP or Mn-, Mg-, and Zn-MARF LNPs encapsulating FLuc mRNAs with different modifications. The results were obtained from n=3 biologically independent samples. Data were presented as mean ± s.e.m. Scale bar: 50 μm.



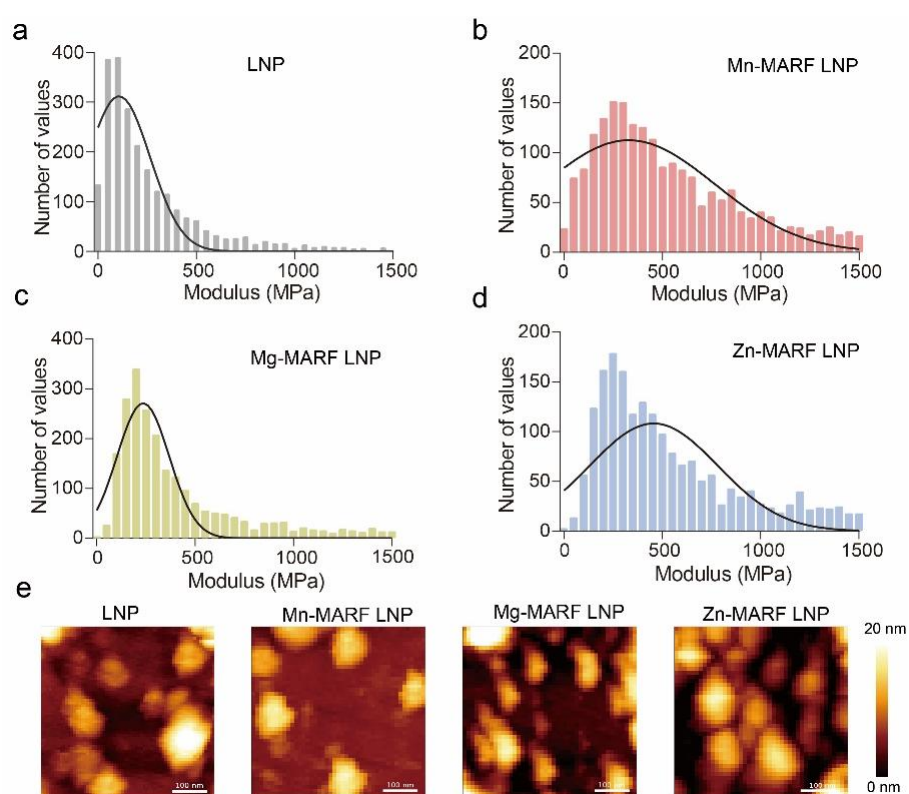
Supplementary Fig. 7. Quantification of mRNA encapsulation in LNP and MARF LNP by NanoFCM. a, Representative bivariate dot plots of Cy5-mRNA⁺ (x-axis) particles and DiO⁺ (y-axis) particles. b, Proportion of Cy5-mRNA/DiO double positive particles. c, Particle concentration of LNP, Mn-MARF LNP, Mg-MARF LNP and Zn-MARF LNP in the solution. d, Counts of mRNA encapsulated in each particle.



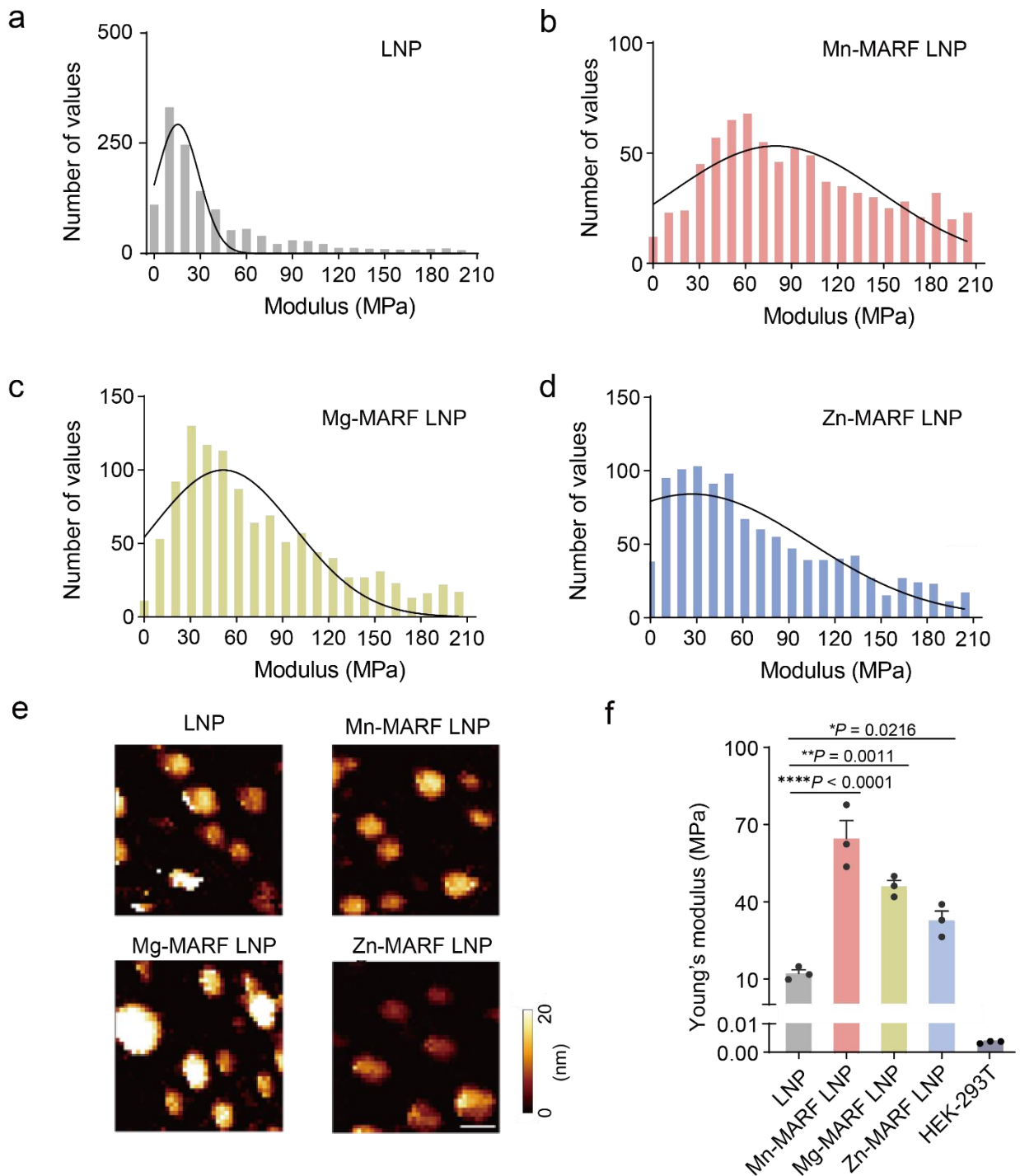
Supplementary Fig. 8. Quantitative determination of metal ion encapsulation efficiency in Mn-, Mg-, and Zn-MARF LNP by inductively coupled plasma mass spectrometry (ICP-MS).



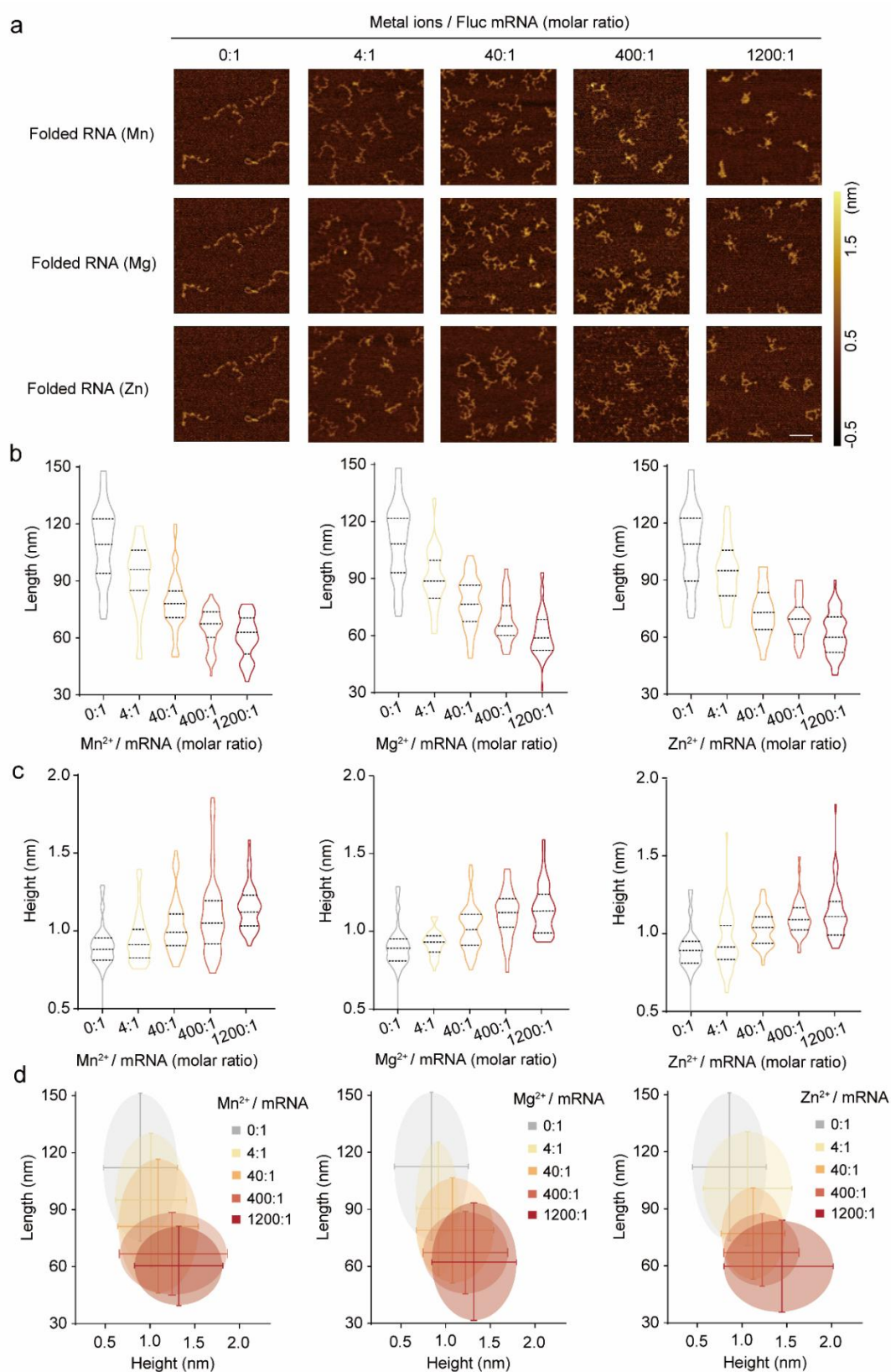
Supplementary Fig. 9. Thermodynamic characterization of mRNA-ionizable lipid. a,b Calorimetric titration curve (upper) and curve fitting (below) of SM-102 titrated to (a) FLuc mRNA or (b) folded FLuc RNA (Mn) in citrate buffer : ethanol mixture (3:1, v/v) at 298 K.



Supplementary Fig. 10. Young's modulus of MARF LNP in dry-state. Young's modulus histogram of (a) LNP, (b) Mn-MARF LNP, (c) Mg-MARF LNP, and (d) Zn-MARF LNP. The Young's modulus was calculated by assessing 2500 force-distance which were collected during the AFM imaging. (e) AFM images of different formulations. Scale bar: 100 nm.

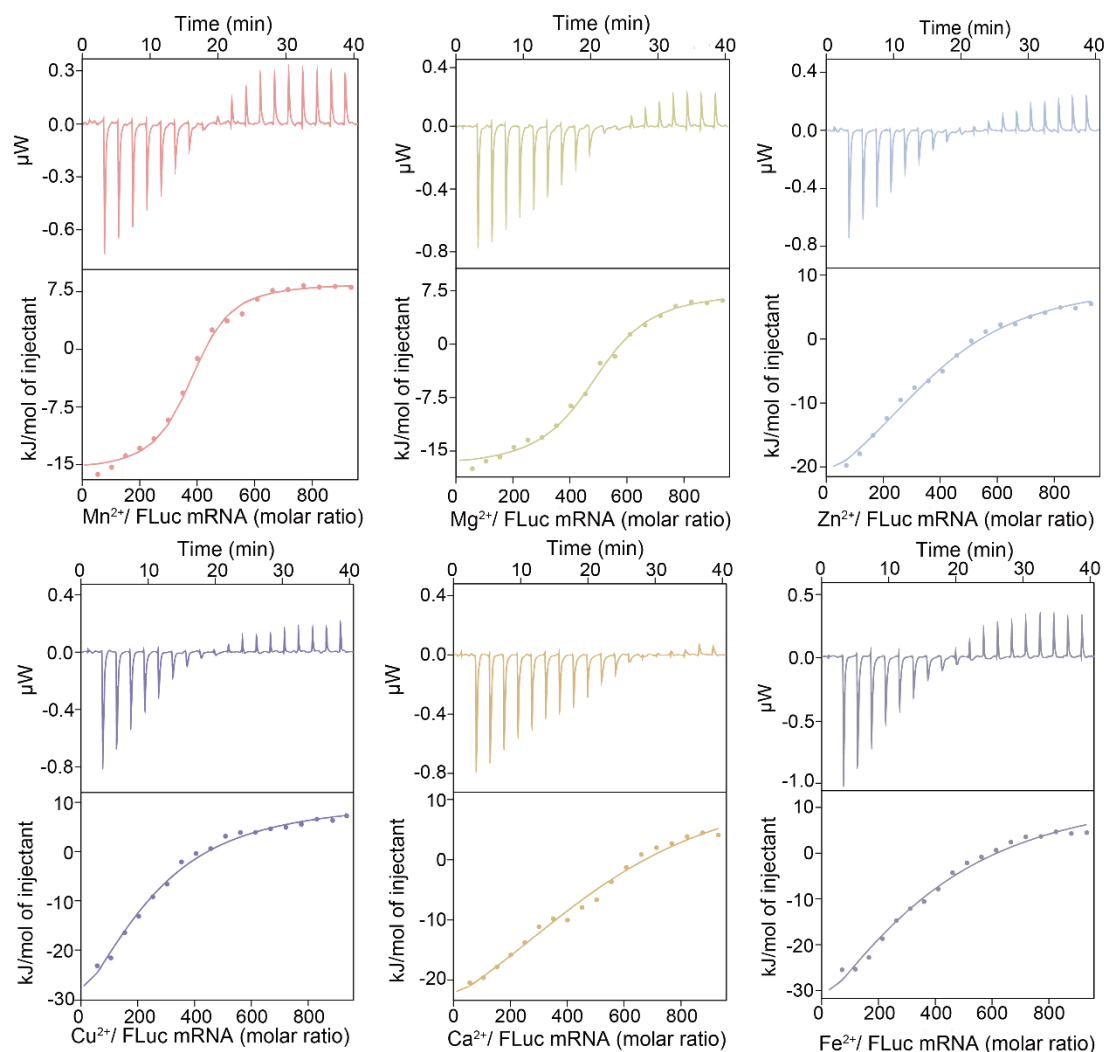


Supplementary Fig. 11. Young's modulus of MARF LNP in wet-state. Young's modulus histogram of (a) LNP, (b) Mn-MARF LNP, (c) Mg-MARF LNP, and (d) Zn-MARF LNP in physiological buffer. The Young's modulus was calculated by assessing 1500 force-distance profiles collected during the AFM imaging. (e) AFM images of different formulations. Scale bar: 100 nm. (f) Young's modulus of control LNP and Mn, Mg, Zn-MARF LNPs.

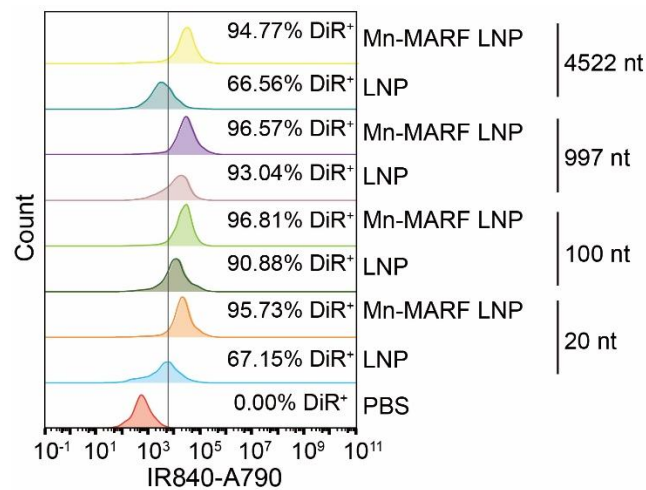


Supplementary Fig. 12. Controlling structure of folded RNA through changing ratio of metal ions. a, Morphology changes of naked FLuc-mRNA, Folded RNA (Mn), Folded RNA (Mg), and Folded RNA (Zn) driven by metal ions

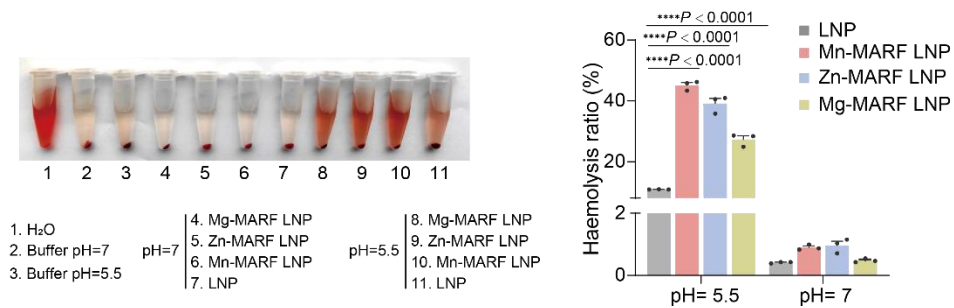
characterized by AFM. Scale bar: 50 nm. Length (b) and height (c) changes of naked FLuc-mRNA, Folded RNA (Mn), Folded RNA (Mg), and Folded RNA (Zn). d, Correlation between length and height of naked FLuc-mRNA, Folded RNA (Mn), Folded RNA (Mg), and Folded RNA (Zn).



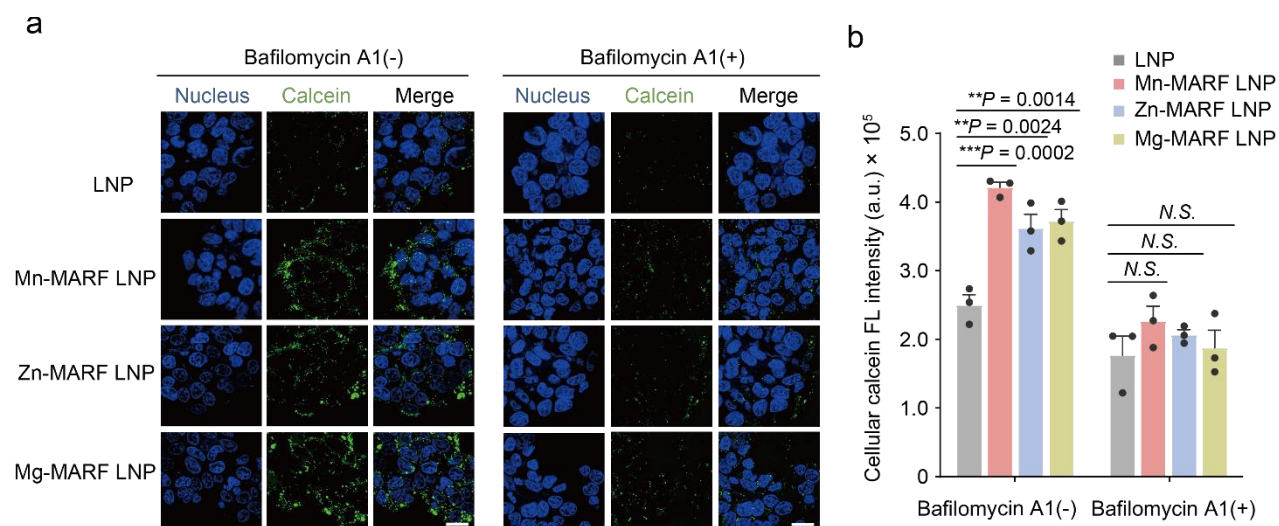
Supplementary Fig. 13. Binding thermogram and thermodynamic parameters for the titration of different metal ions into FLuc mRNA. Calorimetric titration curve (upper panel) and curve fitting (lower panel) of Mn^{2+} , Mg^{2+} , Zn^{2+} , Cu^{2+} , Ca^{2+} , and Fe^{2+} titrated to FLuc-mRNA at 298 K in enzyme-free water.



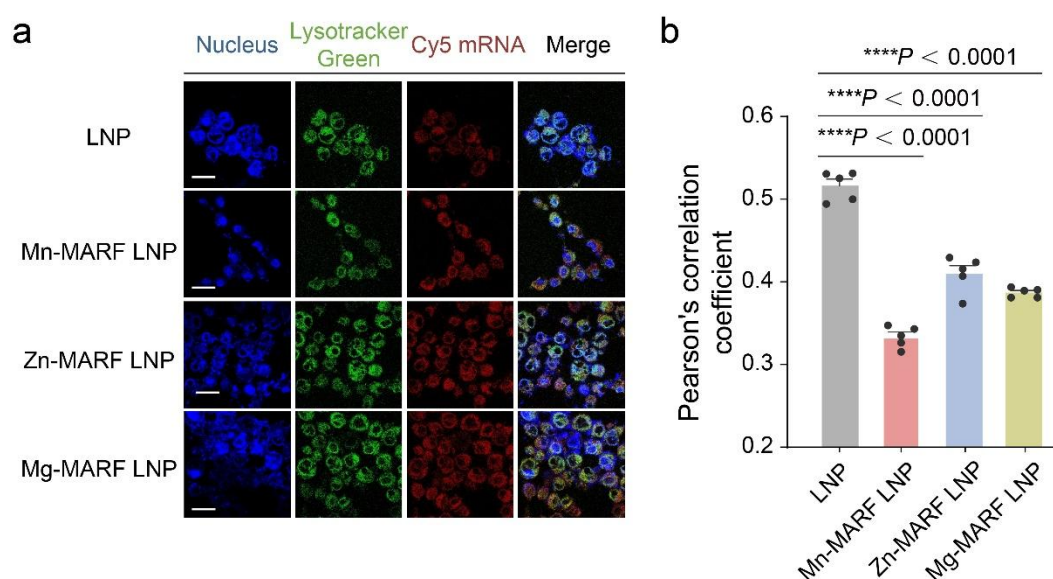
Supplementary Fig. 14. Intracellular uptake of MARF LNP with varying lengths of RNA. Flow cytometry plots of DiR-labeled LNPs encapsulated with RNAs with various lengths.



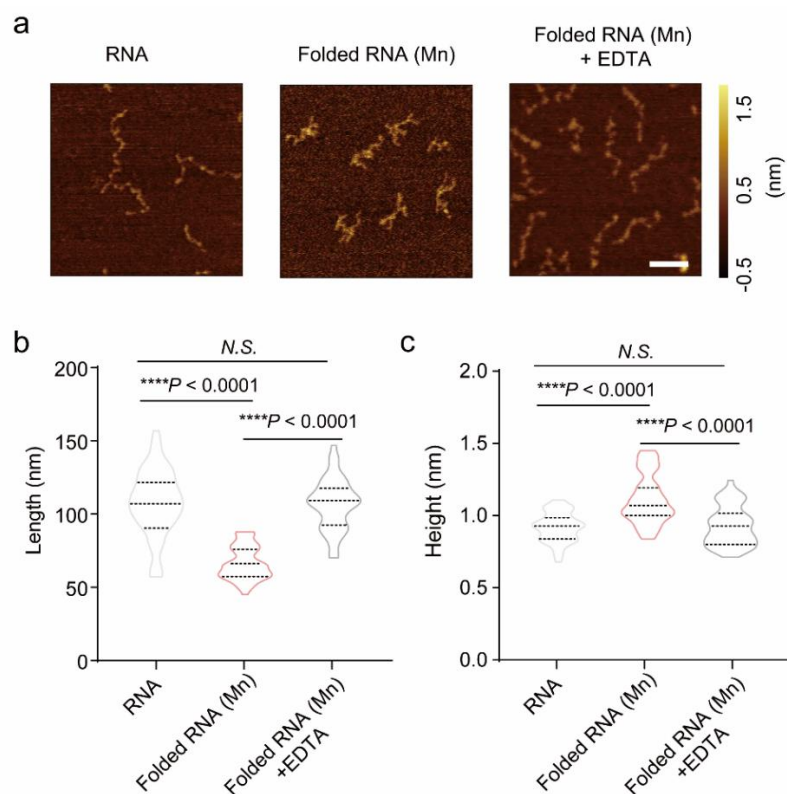
Supplementary Fig. 15. Haemolysis effect of MARF LNP. Haemolysis assay of FLuc-mRNA loaded LNP, Mg-MARF LNP, Zn-MARF LNP, and Mn-MARF LNP in acidic and physiology pH (left). RBCs were incubated with LNPs at 37 °C for 1 h before the supernatant was transferred into a clean 96-well plate to determine the absorption at 540 nm (right).



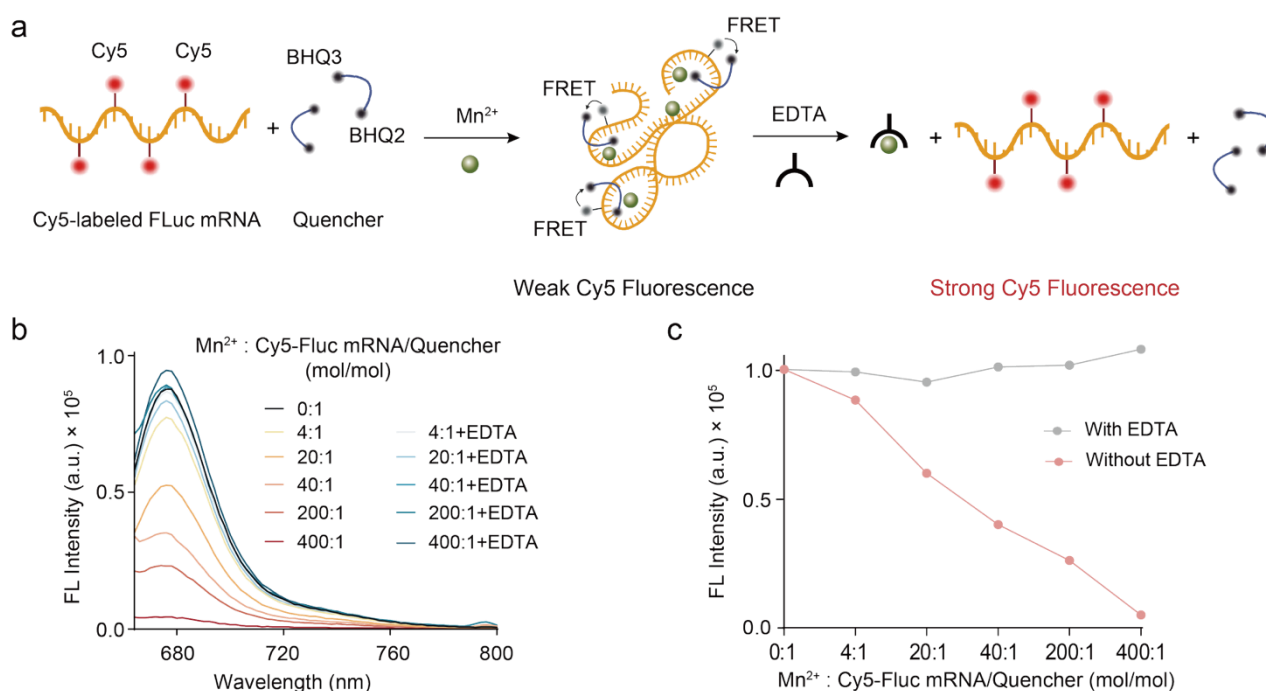
Supplementary Fig. 16. Calcein leakage assay of MARF LNP. a,b, Representative CLSM images of HEK-293T cells incubated with calcein as well as LNP or MARF-LNP with (a) or without (b) bafilomycin A1. Scale bars, 20 μ m. c, Quantification of calcein leakage by flow cytometry.



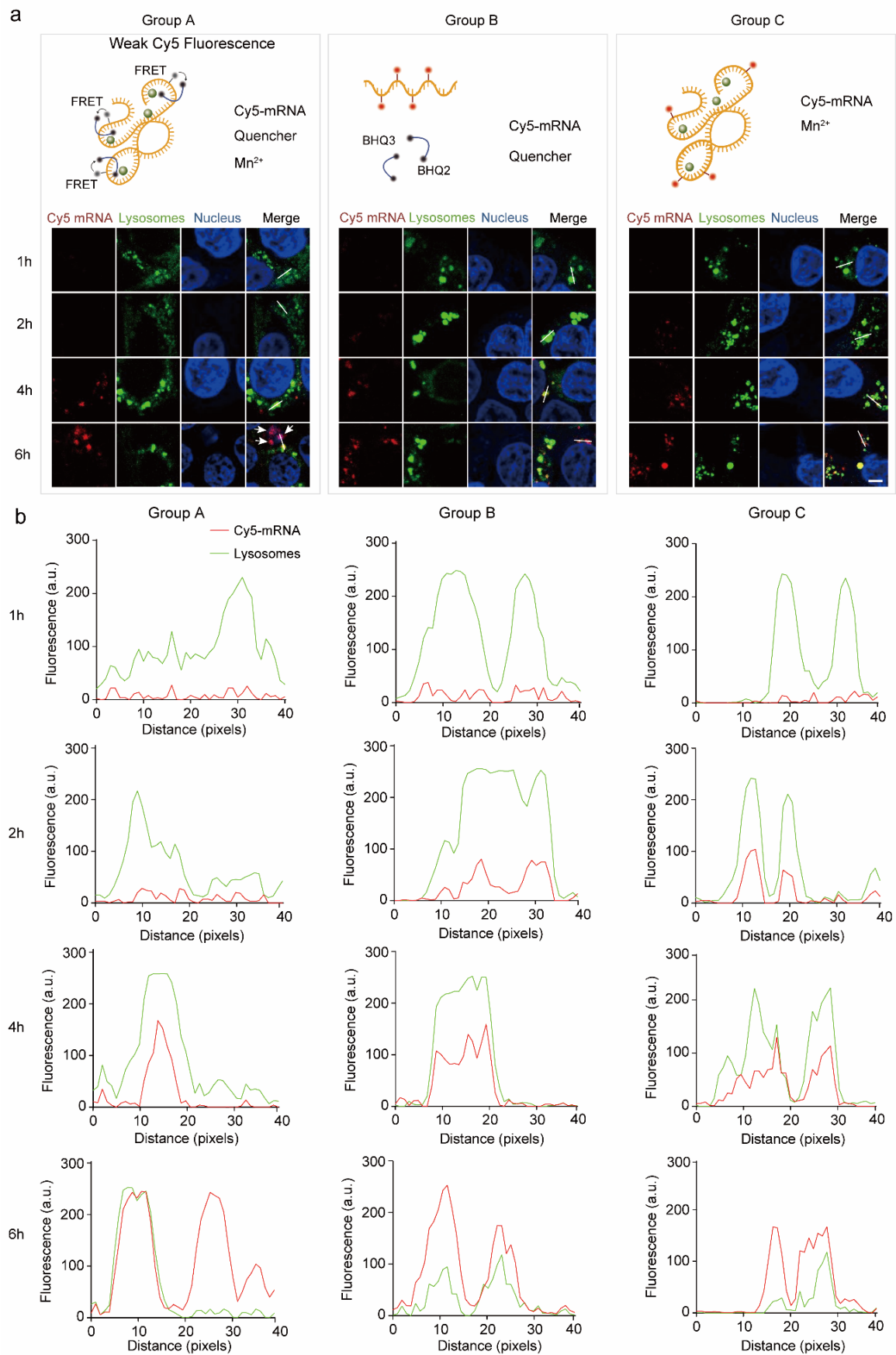
Supplementary Fig. 17. Co-localization of Cy5-mRNA with endo-lysosome. a, Representative confocal images of HEK-293T cells incubated with LNP, Mn-MARF LNP, Zn-MARF LNP or Mg-MARF LNP for 24 hours. Blue channel for nuclei of HEK293T cells with Hoechst 33342: λ_{ex} : 405 nm, λ_{em} : 419-476 nm; Green channel for endo-lysosomes labeled with Lysotracker green: λ_{ex} : 488, λ_{em} : 492-588 nm; Red channel for Cy5-labeled mRNA: λ_{ex} : 633, λ_{em} : 637-749 nm. Scale bars, 20 μ m. b, The Pearson's correlation coefficient of green and red signals from (a) was analyzed by imageJ (n=5).



Supplementary Fig. 18. Morphology of naked RNA and folded RNA with or without the presence of EDTA. a, Representative AFM images of RNA and folded RNA with or without the presence of EDTA. Scale bar: 50 nm. b, The length and height (c) of RNA, folded RNA (Mn) and folded RNA (Mn)+ EDTA were calculated by randomly selecting 50 RNA molecules.

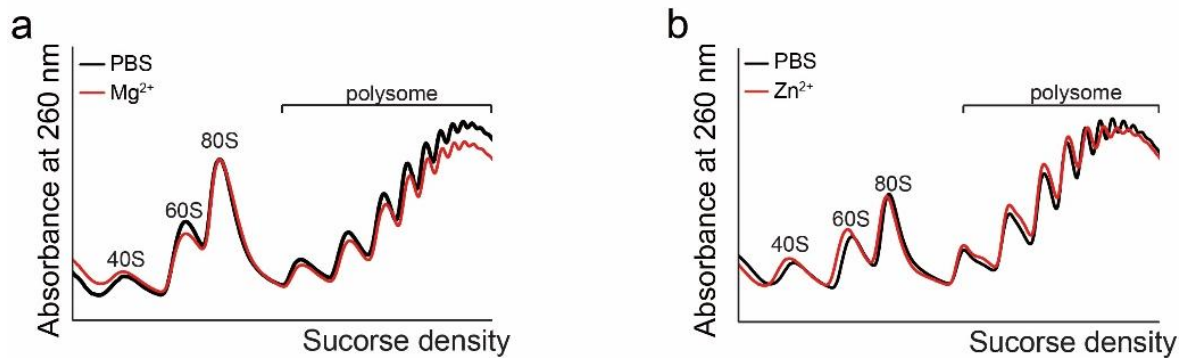


Supplementary Fig. 19. Fluorescence of Cy5-mRNA/quencher complexes in FRET assay. a, Schematic of FRET-based quenching assay. b, Fluorescence spectrum and (c) intensity quantification of Cy5-Fluc mRNA/quencher complexes under different metal ions conditions.

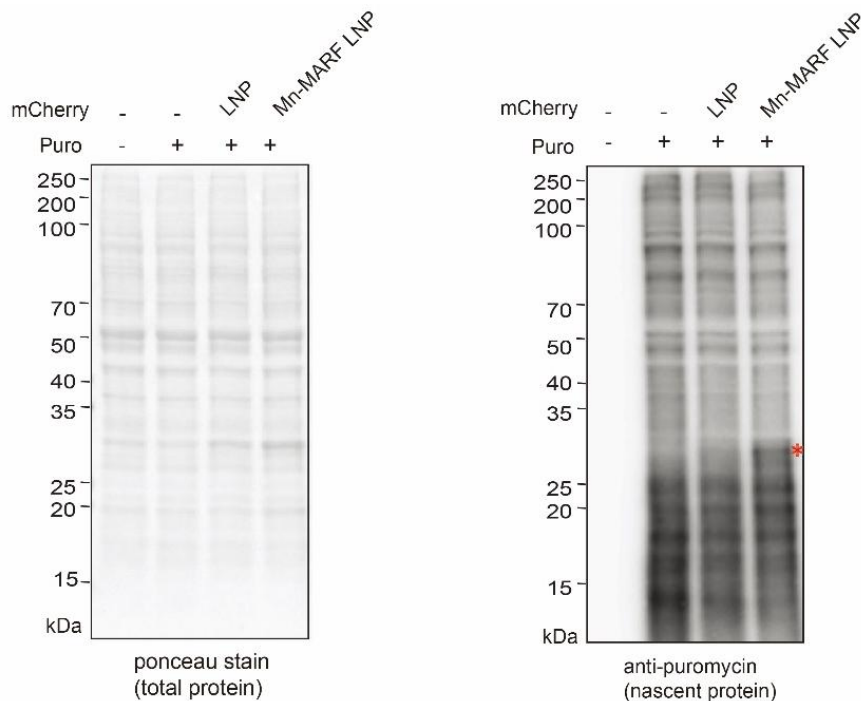


Supplementary Fig. 20. Reversibility and intracellular dynamics of the Mn²⁺-mRNA. a, Schematic of the FRET-based

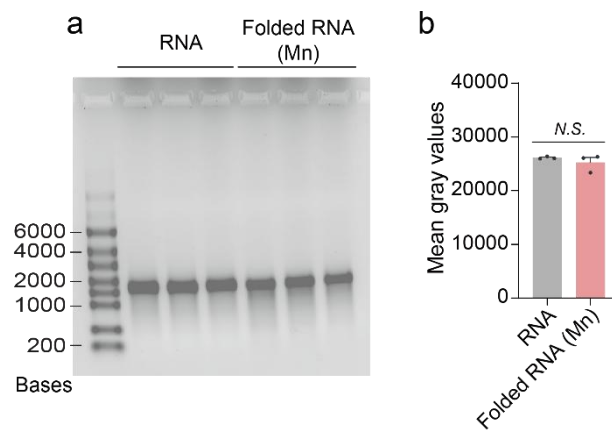
RNA folding reversibility assay (upper). Confocal microscopy images of HEK-293T cells treated with Cy5-labeled FLuc mRNA under three conditions: (Group A) pre-folded with Mn^{2+} and quencher strand, (Group B) with quencher strand only, and (Group C) with Mn^{2+} only (Bottom). Violet channel for Cy5-labeled mRNA: λ_{ex} : 633 nm, λ_{em} : 637–749 nm; Green channel for endosomes labeled with LysoTracker: λ_{ex} : 488 nm, λ_{em} : 492–588 nm; Blue channel for nuclei of HEK293T cells with Hoechst 33342: λ_{ex} : 405 nm, λ_{em} : 419–476 nm. Scale bars: 5 μ m. b, The fluorescence intensity profiles along the white line in (a).



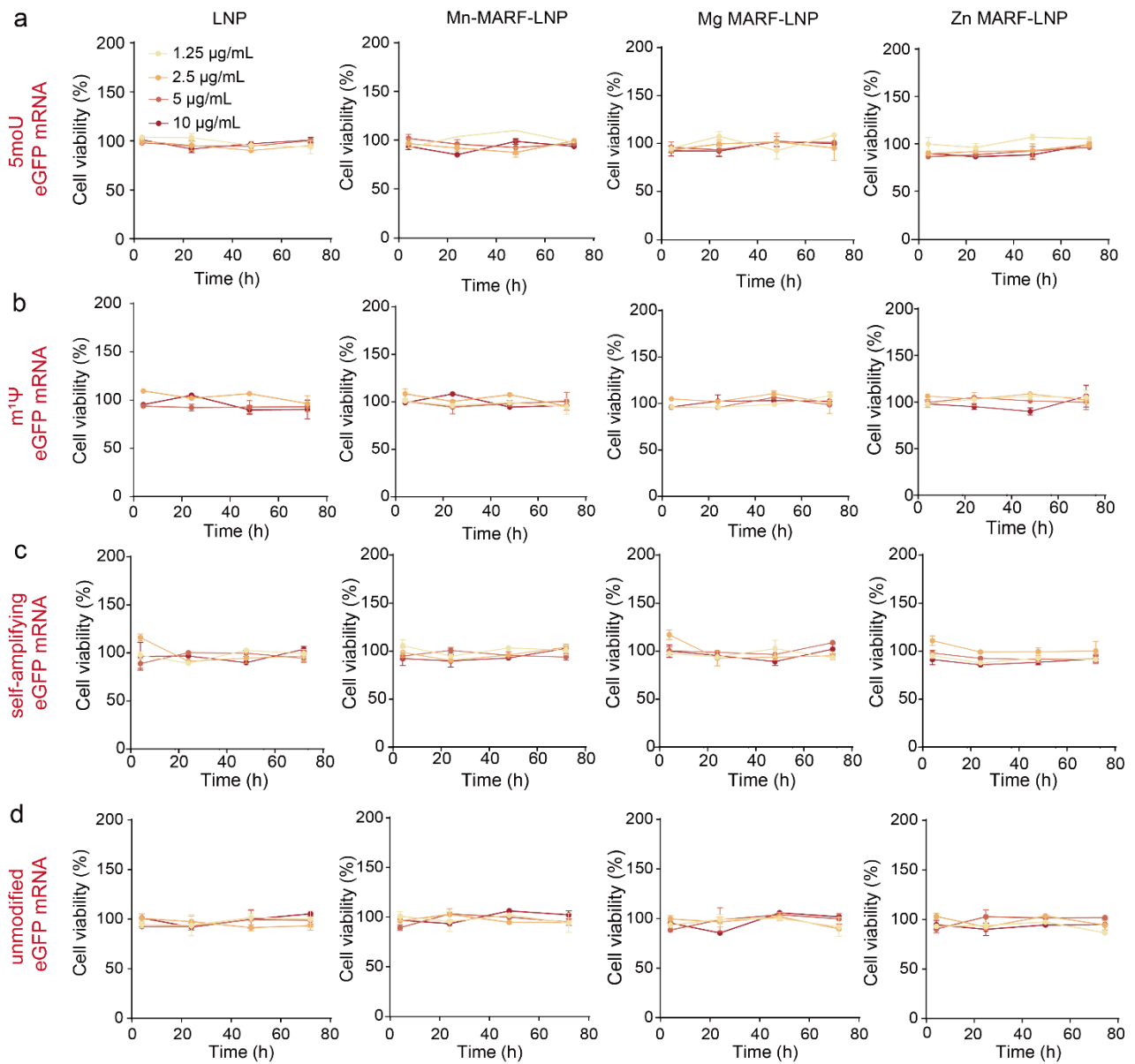
Supplementary Fig. 21. Polysome profiling of mRNA delivered via MARF LNP. Polysome profile analysis comparing HEK-293T cells treated with 3 μ M of (a) Mg^{2+} and (b) Zn^{2+} .



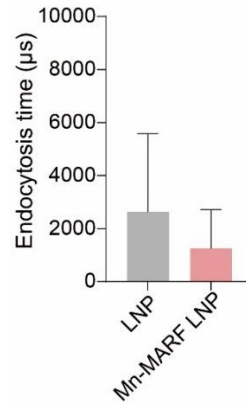
Supplementary Fig. 22. Puromycin blotting of whole lysates from HEK-293T cell treated with LNP and Mn-MARF LNP. Anti-puromycin antibody was applied to detect puromycylated peptides as an indicator of translation efficiency.



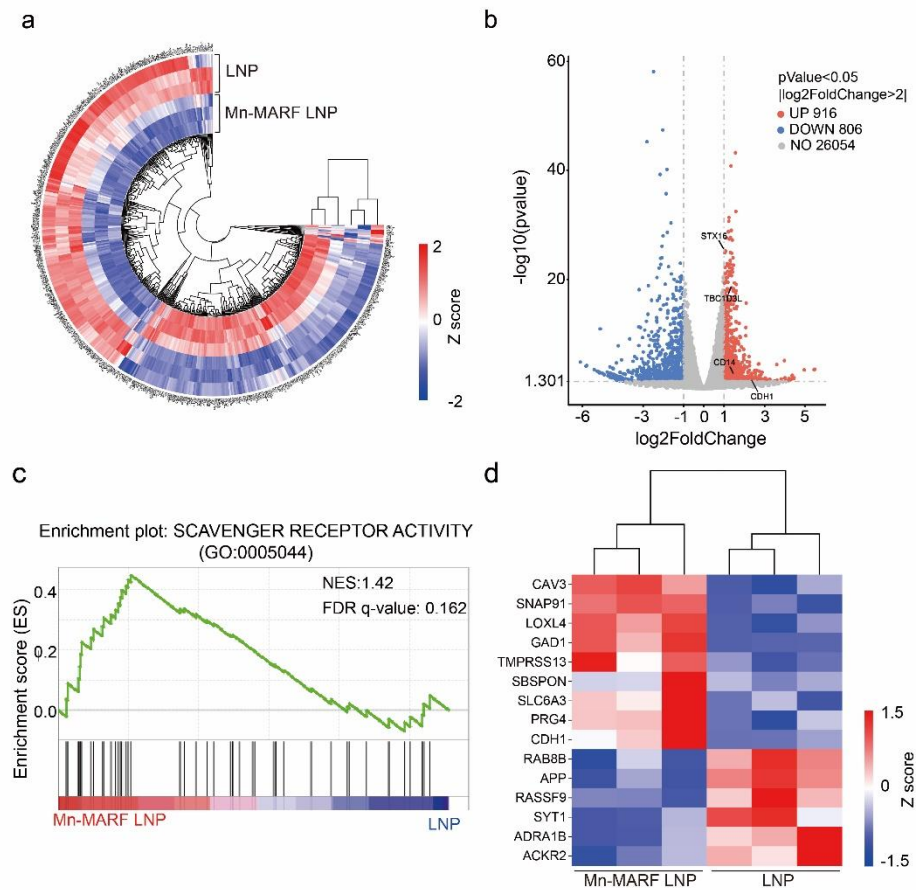
Supplementary Fig. 23. Storage stability of folded RNA. (a) Integrity and migration of mRNA and folded mRNA characterized by denaturing agarose gel after incubation at 37 °C for 12 hours. (b) The mean gray values of different RNA bands were analyzed by ImageJ (n=3). Data were presented as mean ± s.e.m.



Supplementary Fig. 24. Cytotoxicity of LNP, Mn-MARF LNP, Mg-MARF LNP, and Zn-MARF LNP encapsulating eGFP mRNAs with different chemical modifications, including mRNA (5moU), mRNA (m¹Ψ), self-amplifying RNA, and unmodified mRNA, was evaluated in HEK293T cells at varying concentrations over 4, 24, 48, and 72 h. The cell viability was determined by standard CCK8 method. The results are presented as mean $\pm$ standard deviation ($n = 3$).

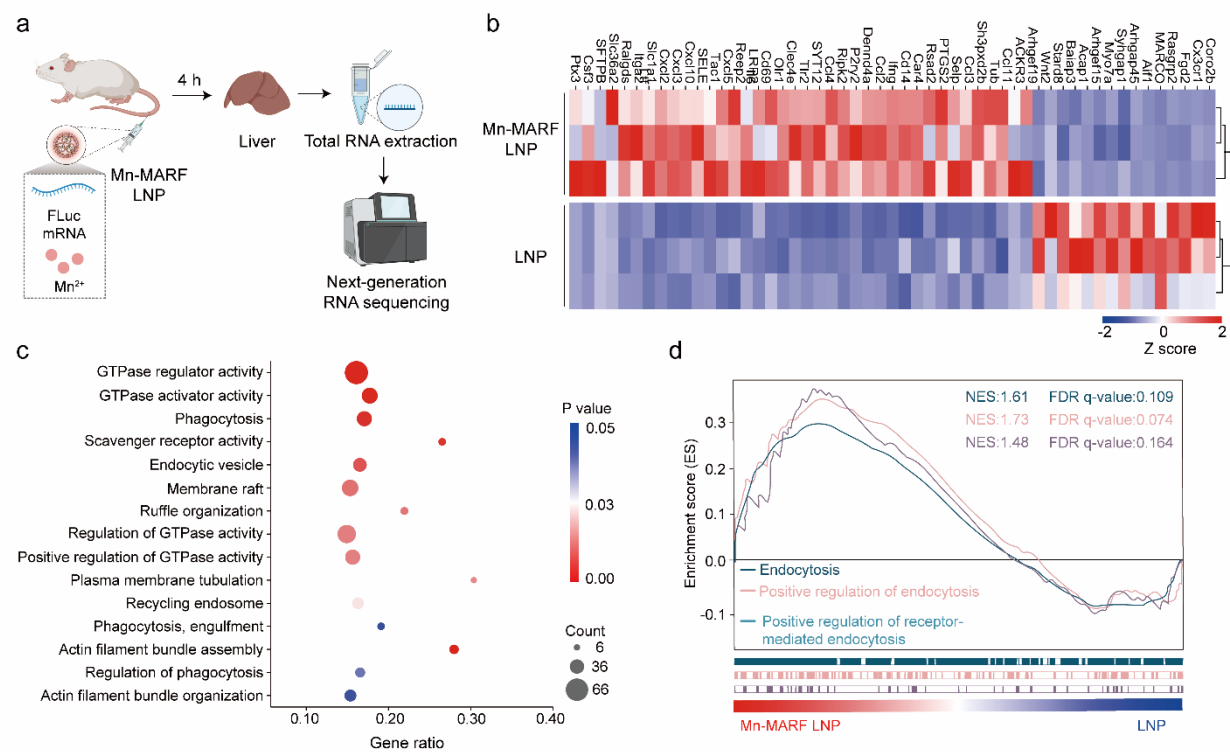


Supplementary Fig. 25 Endocytosis time of control LNP and Mn-MARF LNP based on MD simulation.

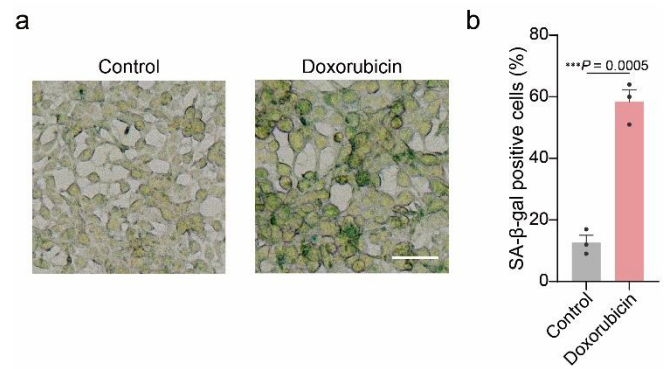


Supplementary Fig. 26 Transcriptomic analysis of HEK-293T cells 12 hours after treatment of Mn-MARF LNP and LNP. **a**, Circular heatmap showing all relative gene expression levels associated with endocytosis. **b**, Volcano plot of the differentially expressed genes (DEGs) in Mn-MARF LNP and LNP treated groups. Upregulated and downregulated genes are shown in red and blue dots. Some key genes related to endocytosis are labeled. **c**, Gene Set Enrichment Analysis (GSEA) enrichment plot of genes that modulate scavenger receptor activity pathway (MARF LNP vs. control LNP). **d**,

Heatmap indicates the expression of differentially expressed genes involved in clathrin- and caveolae-mediated endocytosis pathway in both Mn-MARF LNP and LNP-treated groups.

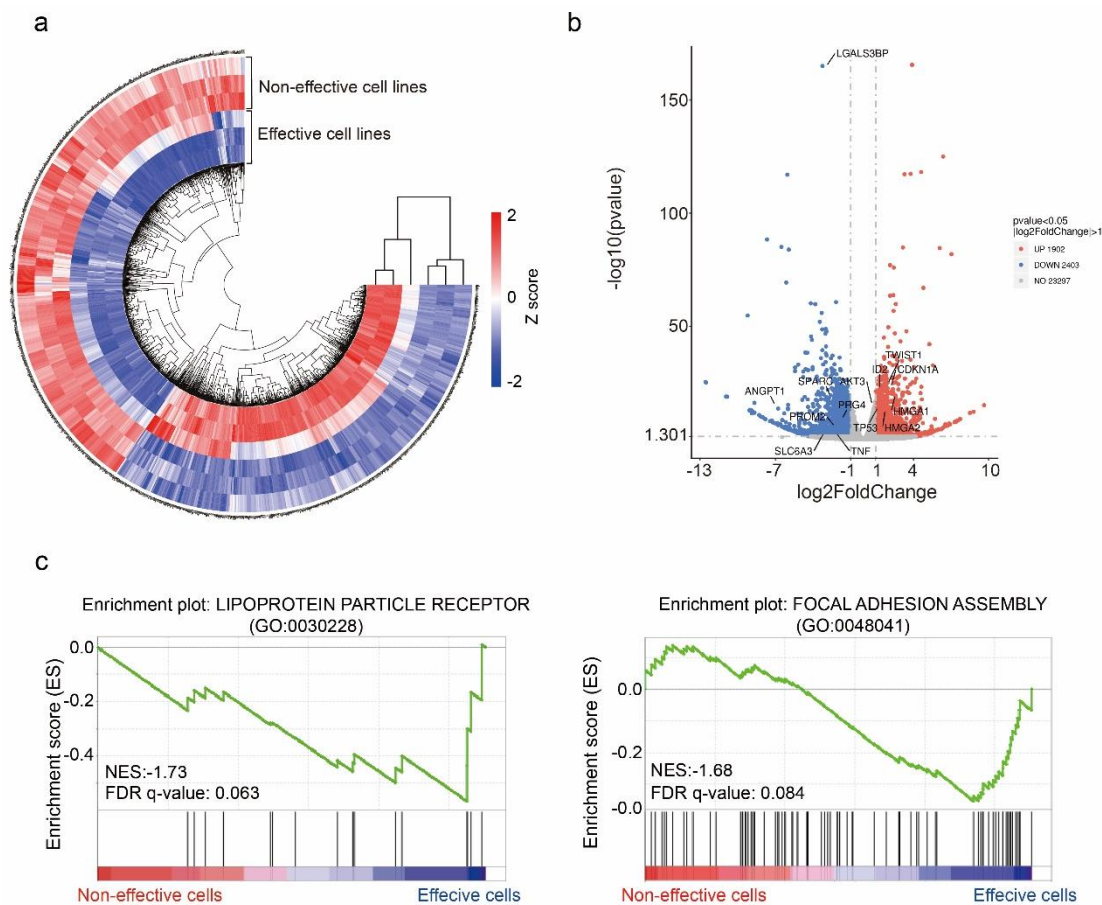


Supplementary Fig. 27. Transcriptomic analyses of liver tissues from mRNA-LNP injected mice. a, Schematic overview of the designed scRNA-seq experiment and workflow. Six-to eight-week-old BALB/c mice were injected with Mn-MARF LNPs or control LNPs (cLNPs). 4 hours post-injection, mice were sacrificed, and livers were harvested for downstream analysis. b, Heatmap of the relative expression of significantly regulated genes relevant to endocytosis in Mn-MARF LNP and LNP treated groups. (n=3, $p < 0.05$, $|\log_2 \text{FoldChange}| > 2.5$). c, Gene ontology (GO) enrichment analysis of the genes related to endocytosis from the comparison between the gene expression in Mn-MARF LNP and LNP treated groups. d, Gene set enrichment analysis (GSEA) for comparing Mn-MARF LNP and LNP treated groups.

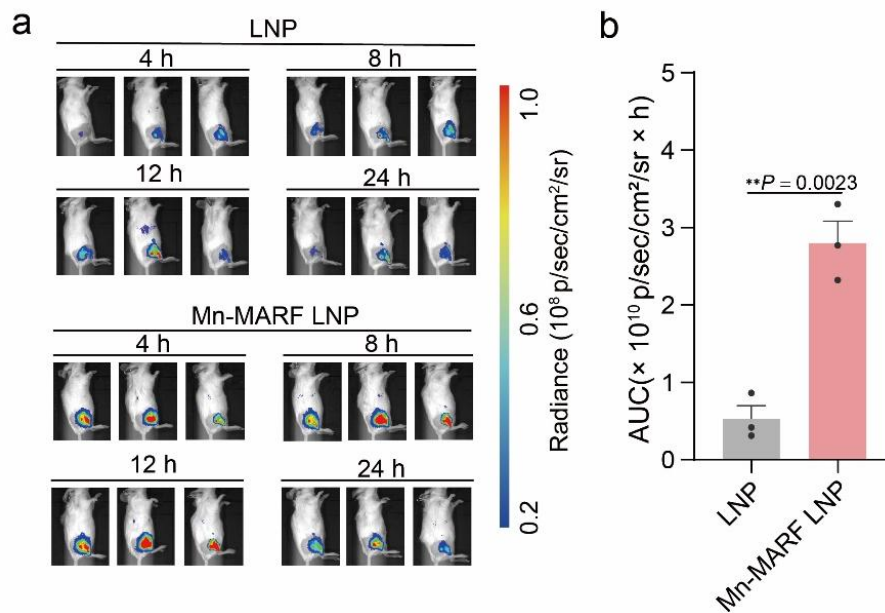


Supplementary Fig. 28. *In vitro* transfection of mRNA delivered by MARF LNP and LNP in aging cells. a, Representative SA-β-gal-stained images of young and aging HEK-293T cells induced by Doxorubicin. Scale bar: 50 μm.

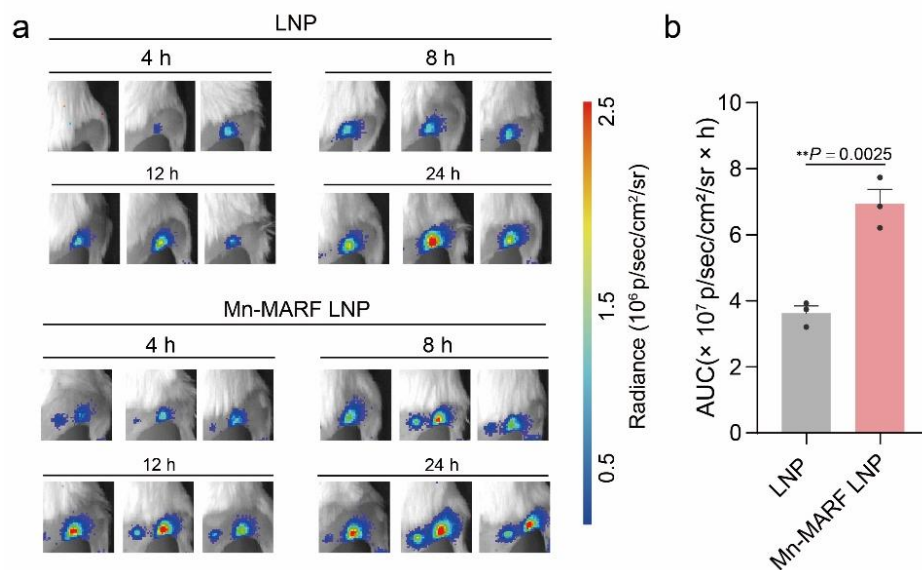
b, The average percentage of SA- β -gal positive cells obtained by analyzing of at least 100 cells (n = 3).



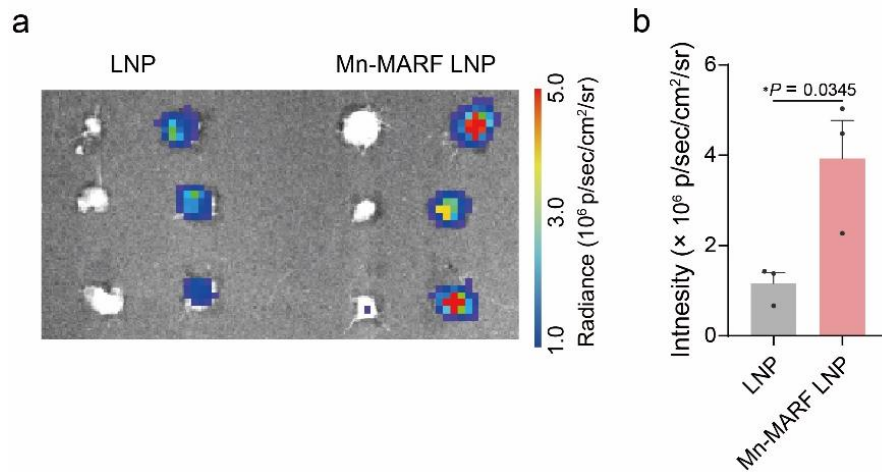
Supplementary Fig. 29. Transcriptomic analysis of young and aging HEK-293T cells lines. **a**, Circular heatmap showing all relative gene expression levels associated with cell aging. **b**, Volcano plot of the differentially expressed genes (DEGs) in effective and non-effective HEK-293T cell lines. Upregulated and downregulated genes are shown in red and blue dots. Some key genes related to endocytosis and cell aging are labeled. **c**, Gene Set Enrichment Analysis (GSEA) enrichment plot of genes that modulate lipoprotein particle receptor and focal adhesion assembly (MARF LNP vs. control LNP).



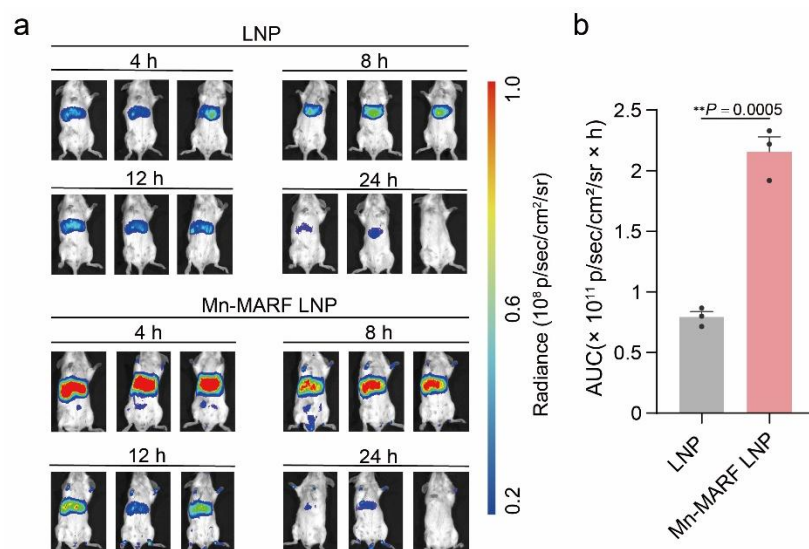
Supplementary Fig. 30 *In vivo* bioluminescence imaging of mice treated with control LNP and MARF LNP *via* i.m. administration. Whole-body bioluminescence (a) imaging and area under curve (AUC) (b) obtained from each mouse. The results were obtained from $n = 3$ biologically independent samples. Data were presented as mean $\pm$ s.e.m.



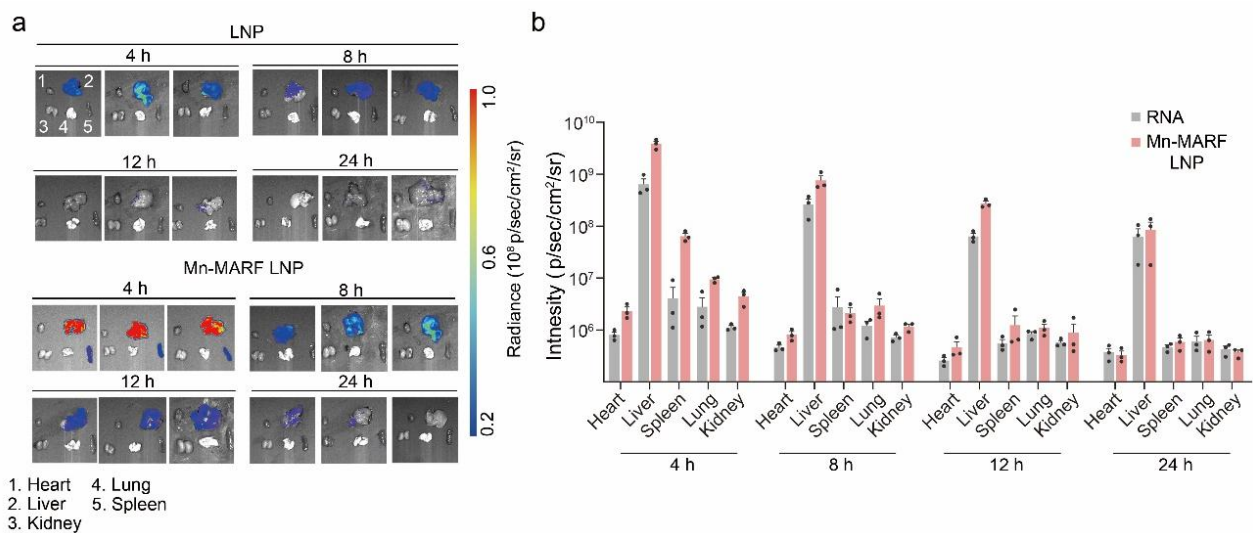
Supplementary Fig. 31 Lymph node bioluminescence imaging of mice treated with Mn-MARF LNP *via* footpad administration. Representative bioluminescence (a) imaging and area under curve (AUC) (b) obtained from each mouse. The results were obtained from $n = 3$ biologically independent samples. Data were presented as mean $\pm$ s.e.m.



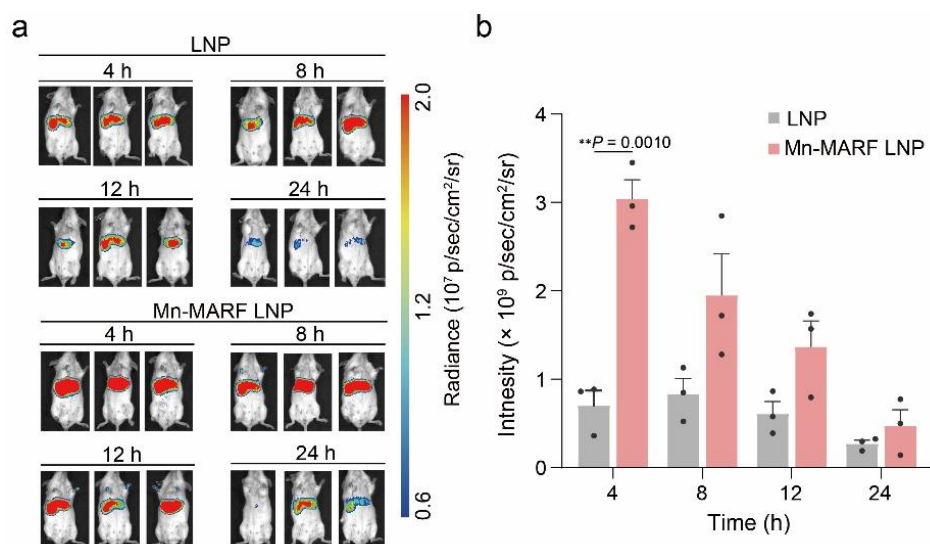
Supplementary Fig. 32 *Ex vivo* images of LNs after footpad injection. *Ex vivo* (a) imaging of LNs and (b) quantitative flux of luminescence at 24 hours post-administration. The results were obtained from $n = 3$ biologically independent samples. Data were presented as mean $\pm$ s.e.m.



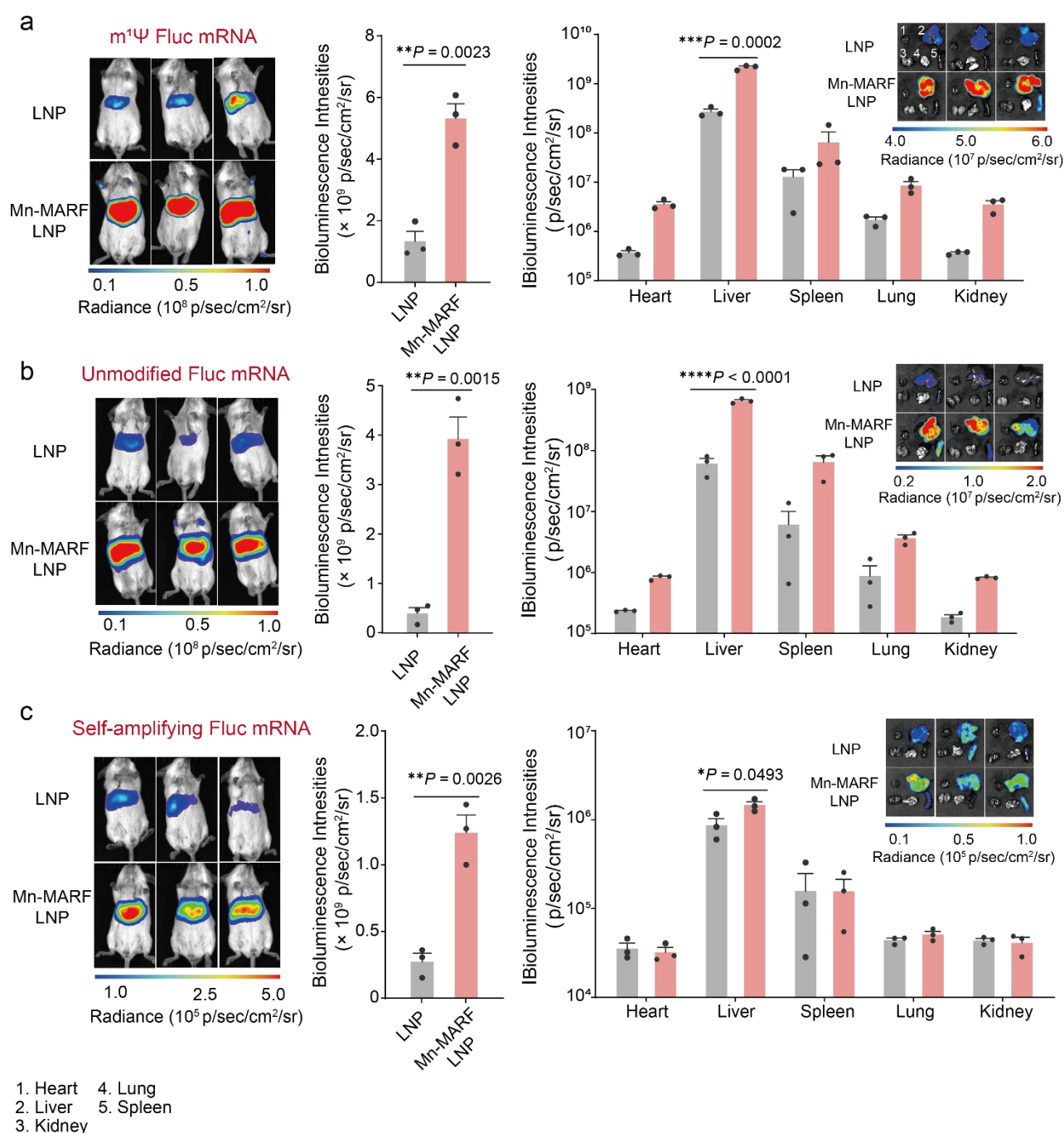
Supplementary Fig. 33 *In vivo* mRNA expression delivered by MARF LNP after i.v. administration (LNP is composed of SM-102 as ionizable lipid). Whole-body bioluminescence imaging (a) and area under curve (AUC) (b) obtained from each mouse. The results were obtained from $n = 3$ biologically independent samples. Data were presented as mean $\pm$ s.e.m.



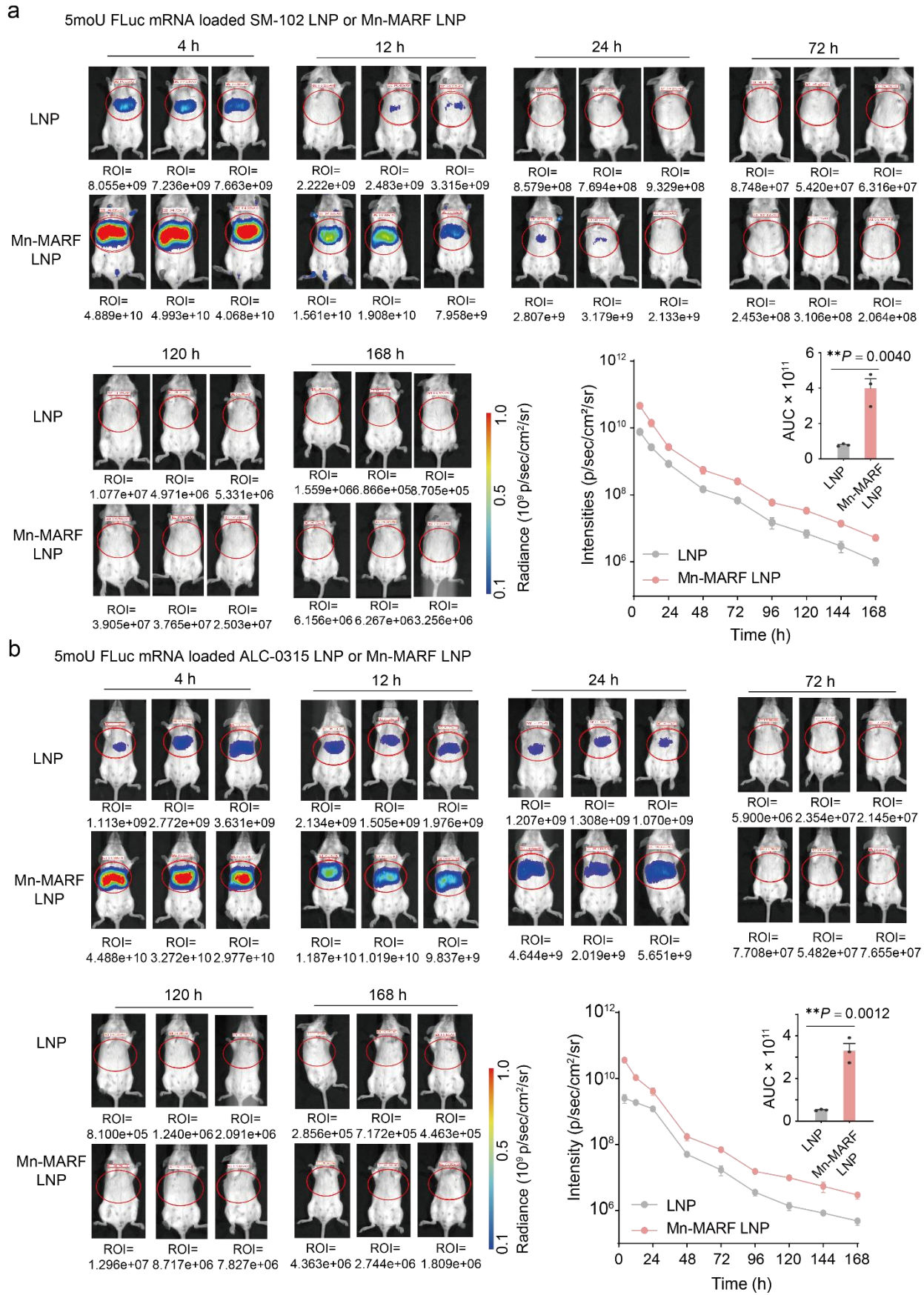
Supplementary Fig. 34 Biodistribution analysis of Mn-MARF LNP and LNP after i.v. administration. Bioluminescence imaging (**a**) and quantitative measurement (**b**) of major organs from BALB/c mice (6-8 weeks) at different time points after a single i.v. administration of either FLuc mRNA loaded LNP or Mn-MARF LNP (0.3 mg/kg). The results were obtained from $n = 3$ biologically independent samples. Data were presented as mean $\pm$ s.e.m.



Supplementary Fig. 35 *In vivo* mRNA expression delivered by MARF LNP after i.v. administration (LNP is composed of MC3 as ionizable lipid). Whole-body bioluminescence (**a**) imaging and (**b**) quantitative measurement of luciferase signals in targeted organs in BALB/c mice (6-8 weeks) at different time points after a single i.v. administration of either FLuc mRNA loaded LNP or Mn-MARF LNP (0.3 mg/kg). The results were obtained from $n = 3$ biologically independent samples. Data were presented as mean $\pm$ s.e.m.

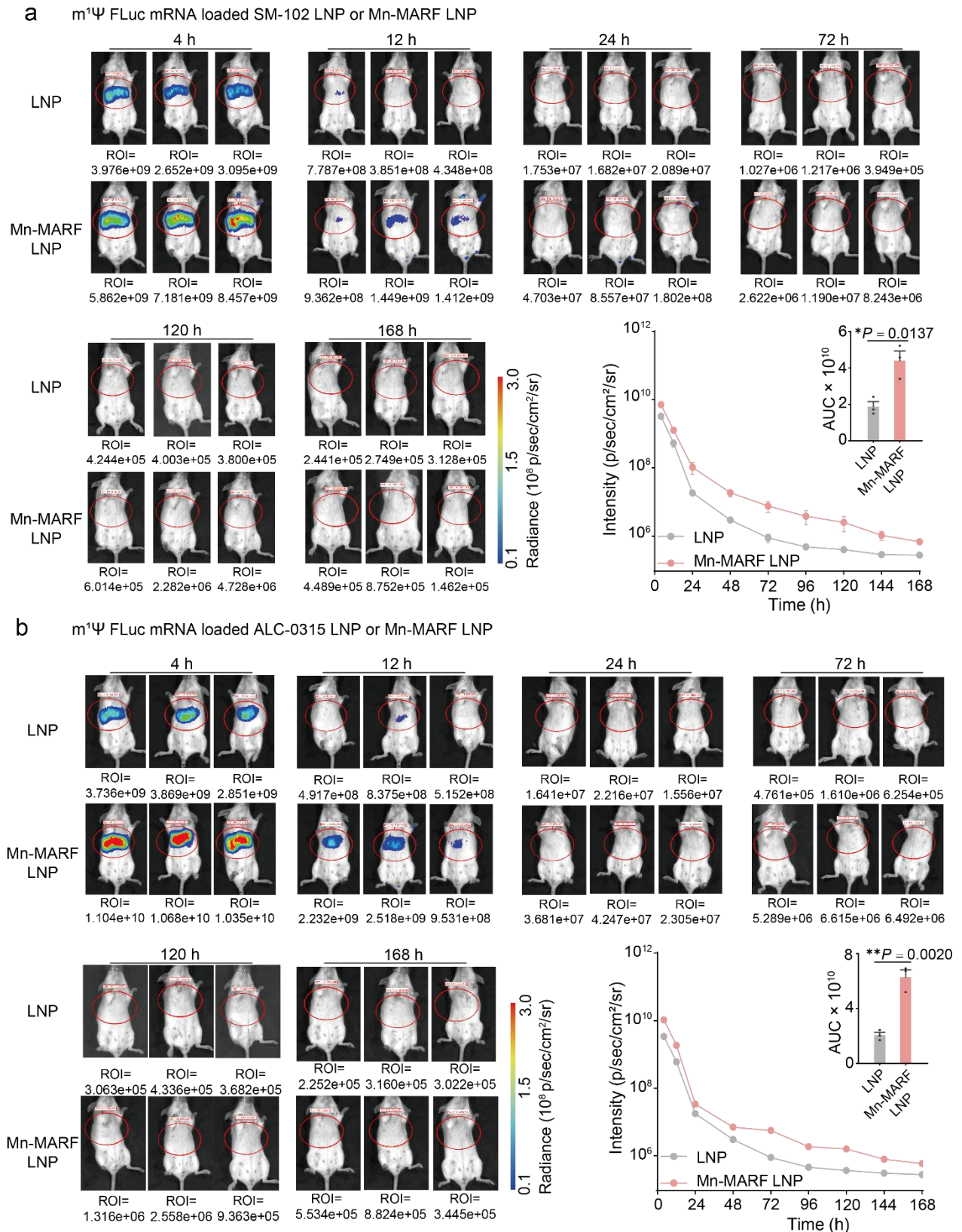


Supplementary Fig. 36. MARF strategy enhanced protein expression of delivered mRNA with various chemical modifications in vivo. Whole-body bioluminescence imaging (left) and corresponding signal intensities (middle) of female BALB/c mice (6–8 weeks) at 4 h post i.v. administration of control LNP, Mg-MARF LNP, or Zn-MARF LNP encapsulating FLuc mRNA with N1-methylpseudouridine (m¹Ψ) modification (a), unmodified FLuc mRNA (b), or self-amplifying FLuc mRNA (c). Bioluminescence imaging of major organs (inset, right) and quantitative analysis (right) were performed at 4 h following a single i.v. administration of differently modified FLuc mRNAs. Dosage: 0.3 mg/kg FLuc mRNA (n = 3/group).



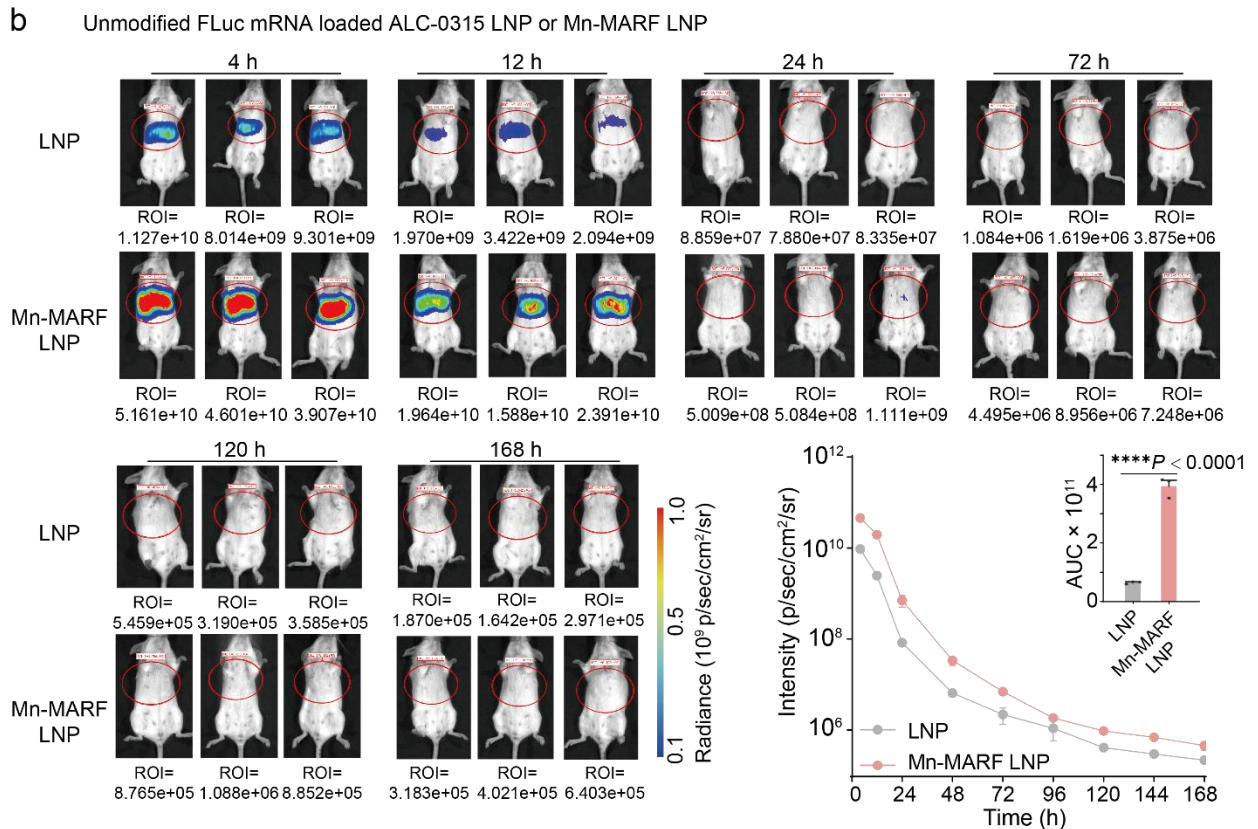
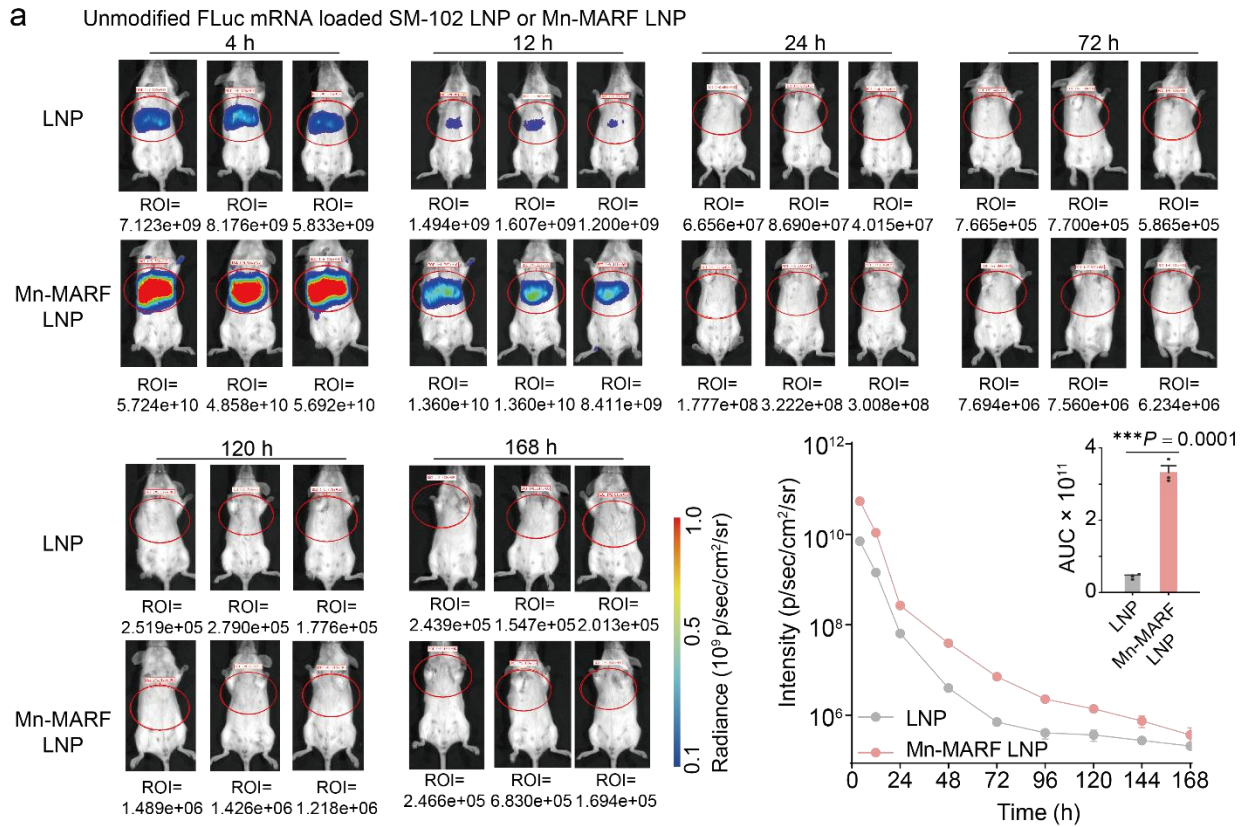
Supplementary Fig. 37. *In vivo* durability of protein expression of MARF LNP formulated with different ionizable

lipids (a) Whole-body bioluminescence imaging, quantitative signal analysis, and AUC in mice treated with FLuc mRNA (5moU) loaded control LNPs or Mn-MARF LNPs (SM-102). (b) Whole-body bioluminescence imaging, quantitative signal analysis, and AUC in mice treated with FLuc mRNA loaded control LNPs or Mn-MARF LNPs (ALC-0315). The results were obtained from $n = 3$ biologically independent samples. Data were presented as mean $\pm$ s.e.m.



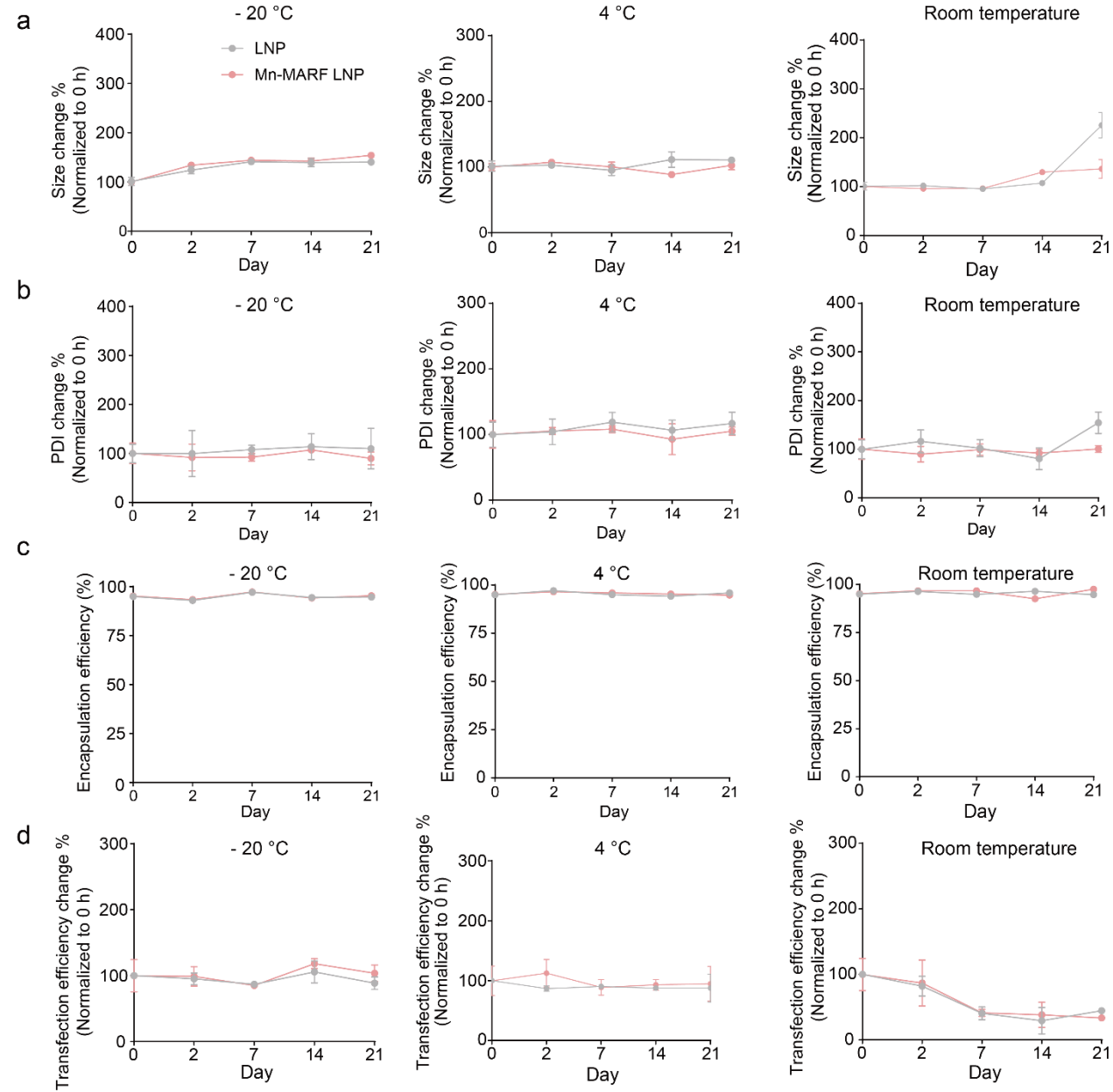
Supplementary Fig. 38. *In vivo* durability of protein expression of m¹Ψ modified mRNA delivered by MARF LNP formulated with different ionizable lipids. (a) Whole-body bioluminescence imaging, quantitative signal analysis, and AUC in mice treated with FLuc mRNA (m¹Ψ) loaded control LNPs or Mn-MARF LNPs (SM-102). (b) Whole-body

bioluminescence imaging, quantitative signal analysis, and AUC in mice treated with FLuc mRNA loaded control LNPs or Mn-MARF LNPs (ALC-0315). The results were obtained from $n = 3$ biologically independent samples. Data were presented as mean $\pm$ s.e.m.



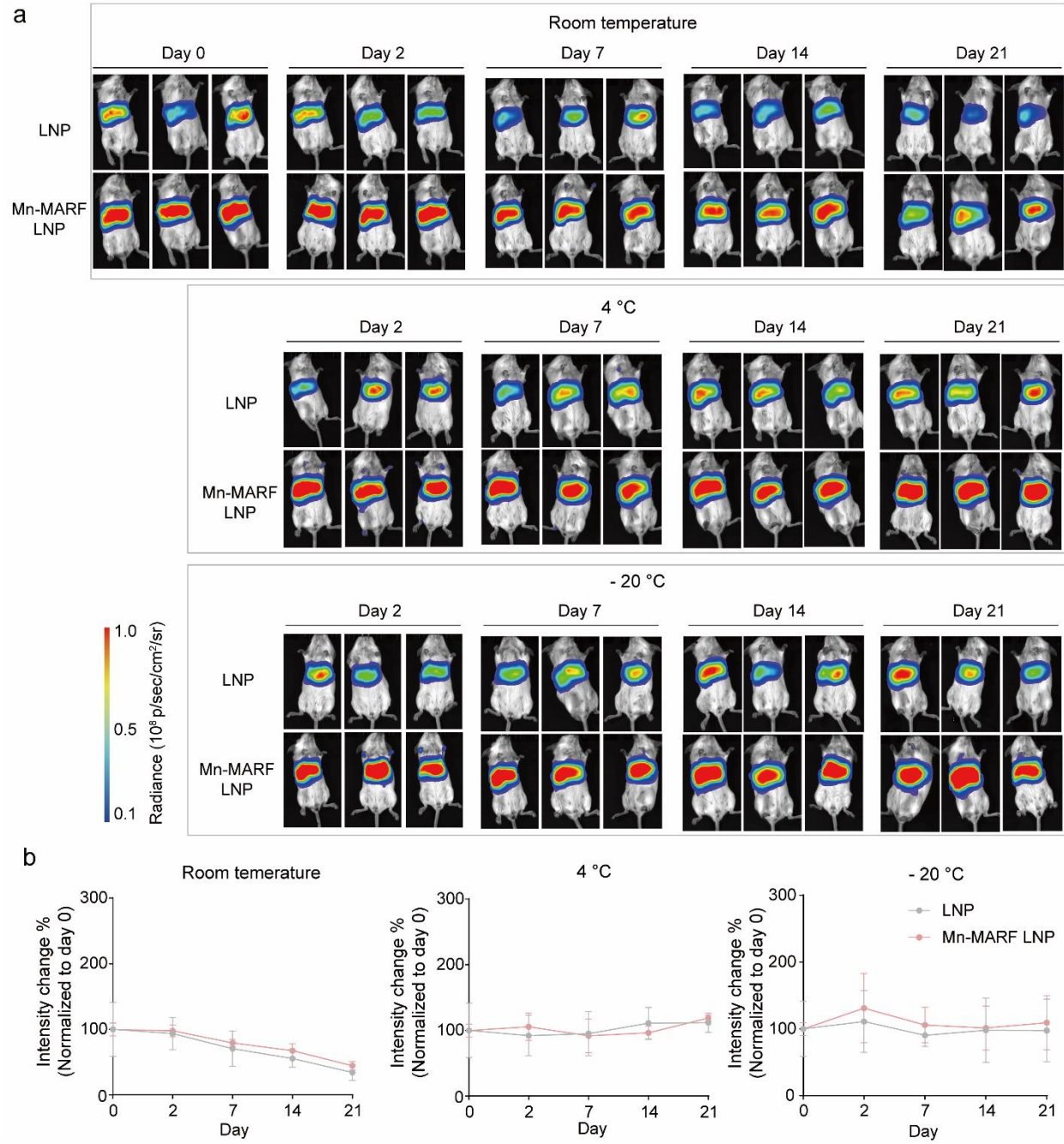
Supplementary Fig. 39. In vivo durability of protein expression mediated by unmodified mRNA following a single i.v. injection of MARF LNPs formulated with different ionizable lipids. (a) Whole-body bioluminescence imaging,

quantitative signal analysis, and area under the curve (AUC) in mice treated with unmodified FLuc mRNA loaded control LNPs or Mn-MARF LNPs containing SM-102 as the ionizable lipid. (b) Whole-body bioluminescence imaging, quantitative signal analysis, and AUC in mice treated with unmodified FLuc mRNA loaded control LNPs or Mn-MARF LNPs containing ALC-0315 as the ionizable lipid. The results were obtained from $n = 3$ biologically independent samples. Data were presented as mean $\pm$ s.e.m.

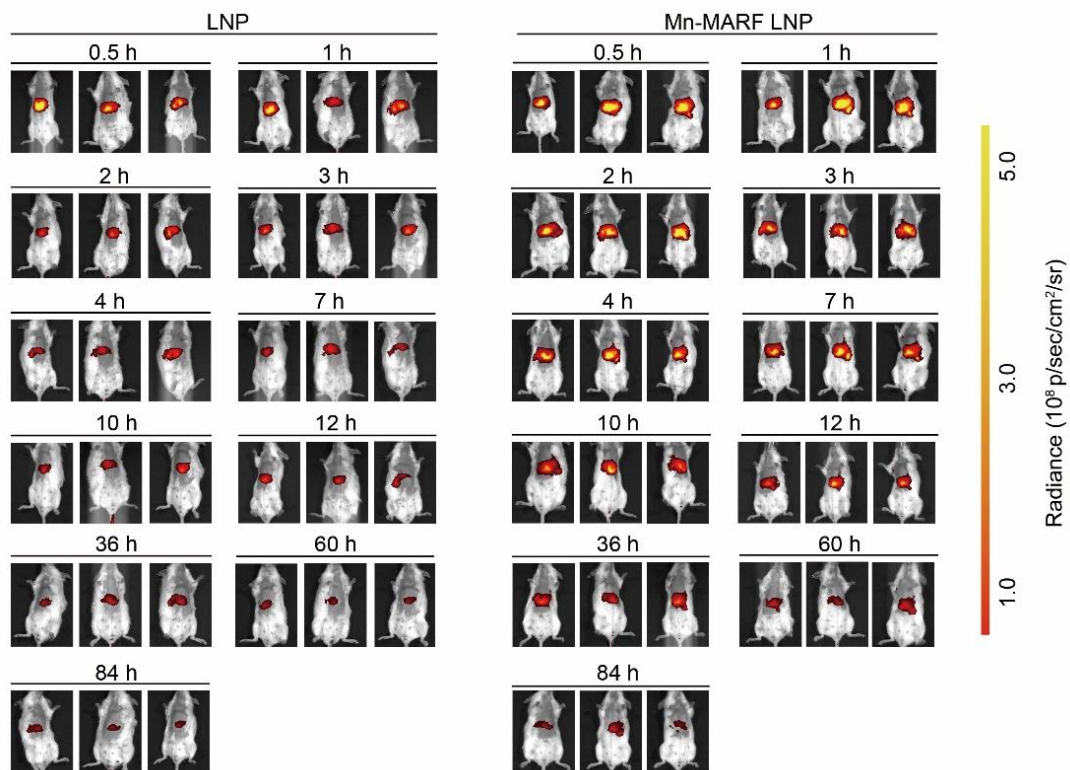


Supplementary Fig. 40. Stability of Mn-MARF LNP and control LNP over time at different storage temperatures *in vitro*. LNP and Mn-MARF (SM102) encapsulating FLuc-mRNA (5-moU) were stored in PBS at -20 °C, 4 °C, or 25 °C (room temperature) after different periods of time. The particle size (a), PDI (b) and mRNA encapsulation (c) of control LNP and Mn-MARF LNPs. (d) Relative protein expression in HEK-293T treated with control LNP, and Mn-MARF LNP encapsulated

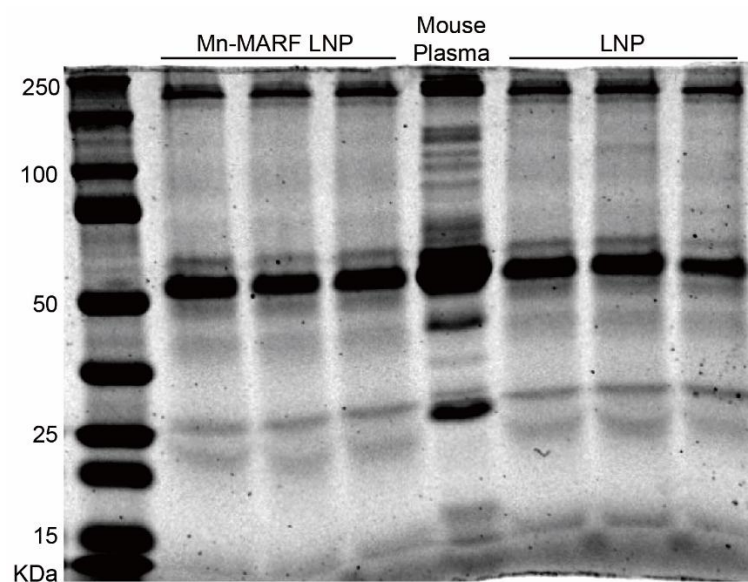
with eGFP-mRNA. The results were obtained from n=3 biologically independent samples. Data were presented as mean $\pm$ s.e.m.



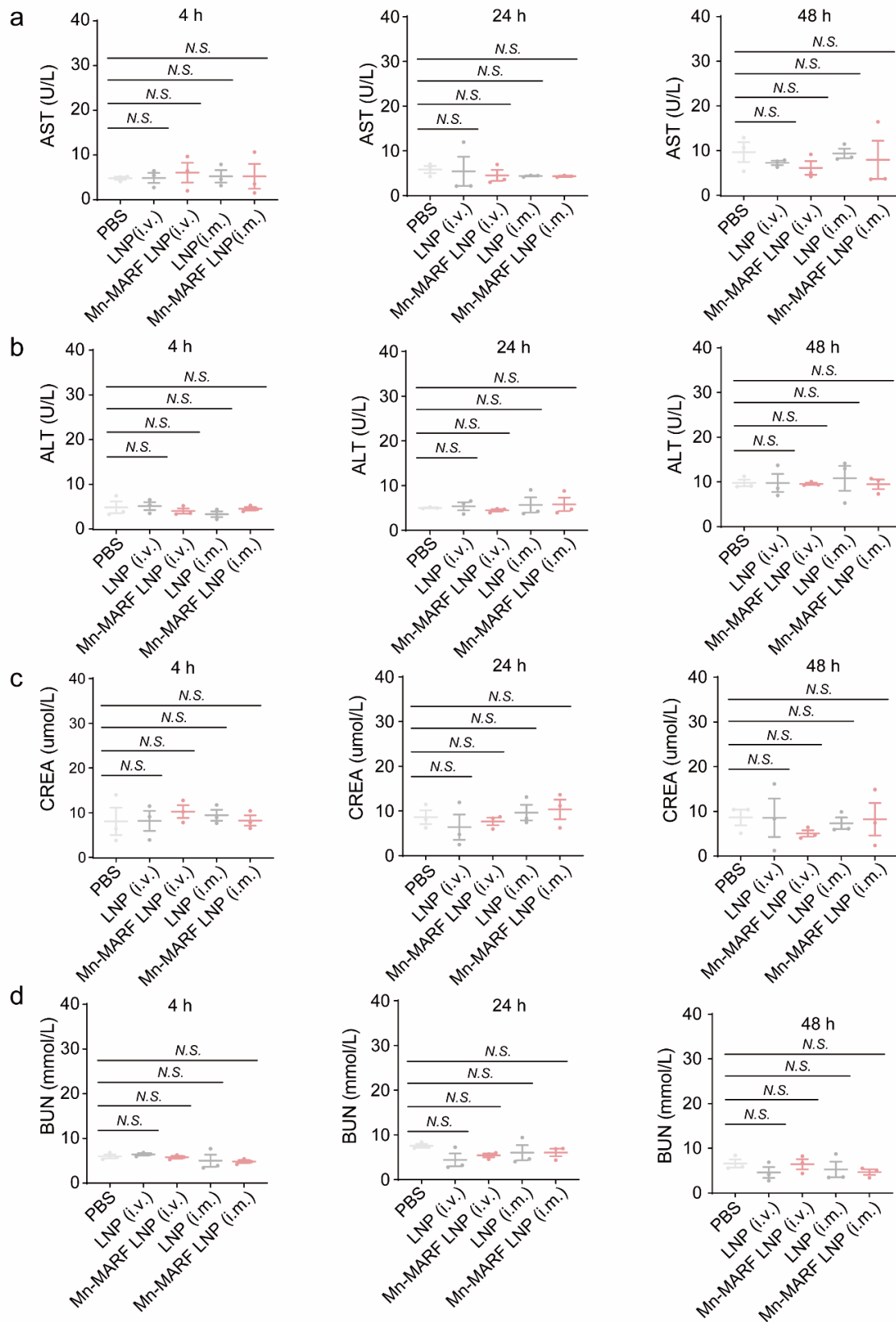
Supplementary Fig. 41. Stability of Mn-MARF LNP and control LNP over time at different storage temperatures *in vivo*. LNP and Mn-MARF (SM-102) encapsulating FLuc-mRNA (5-moU) were stored in PBS at -20 °C, 4 °C, or 25 °C (room temperature) after different periods of time. Whole-body bioluminescence imaging (a) and quantitative analysis (b) of bioluminescence signal from day 0 up to day 21 following i.v. administration of FLuc mRNA loaded control LNP and Mn-MARF LNP. Dosage: 0.3 mg/kg FLuc mRNA (n = 3/group).



Supplementary Fig. 42. *In vivo* fluorescence imaging of mice treated with DiR-labelled LNP and Mn-MARF LNP. Whole-body bioluminescence imaging of BALB/c mice (6-8 weeks) at different time points after a single i.v. administration of DiR labelled FLuc-mRNA loaded LNP or Mn-MARF LNP (0.3 mg/kg).

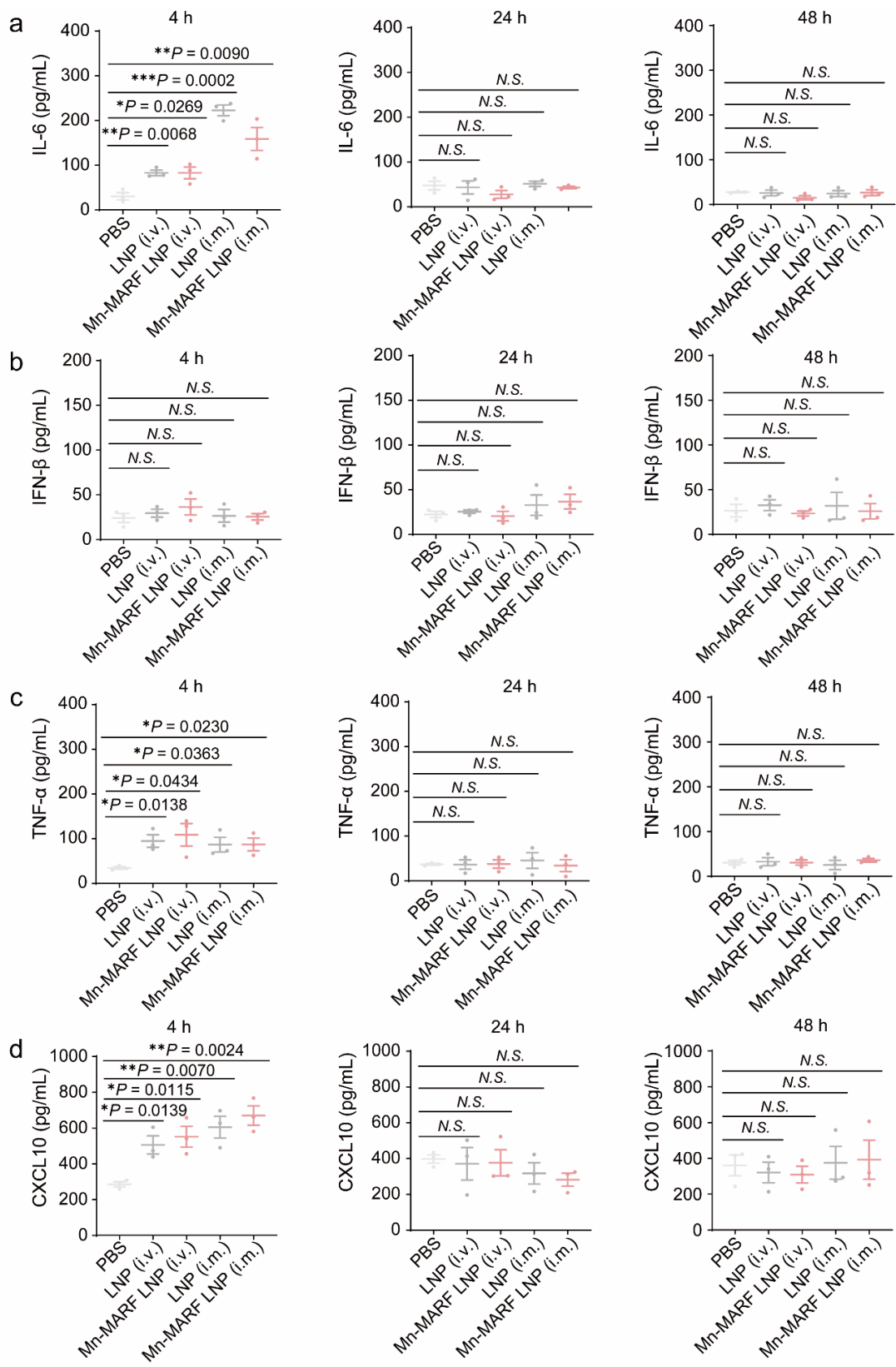


Supplementary Fig. 43. Images of SDS-PAGE gels showing protein corona absorbed on the LNP or Mn-MARF LNP.



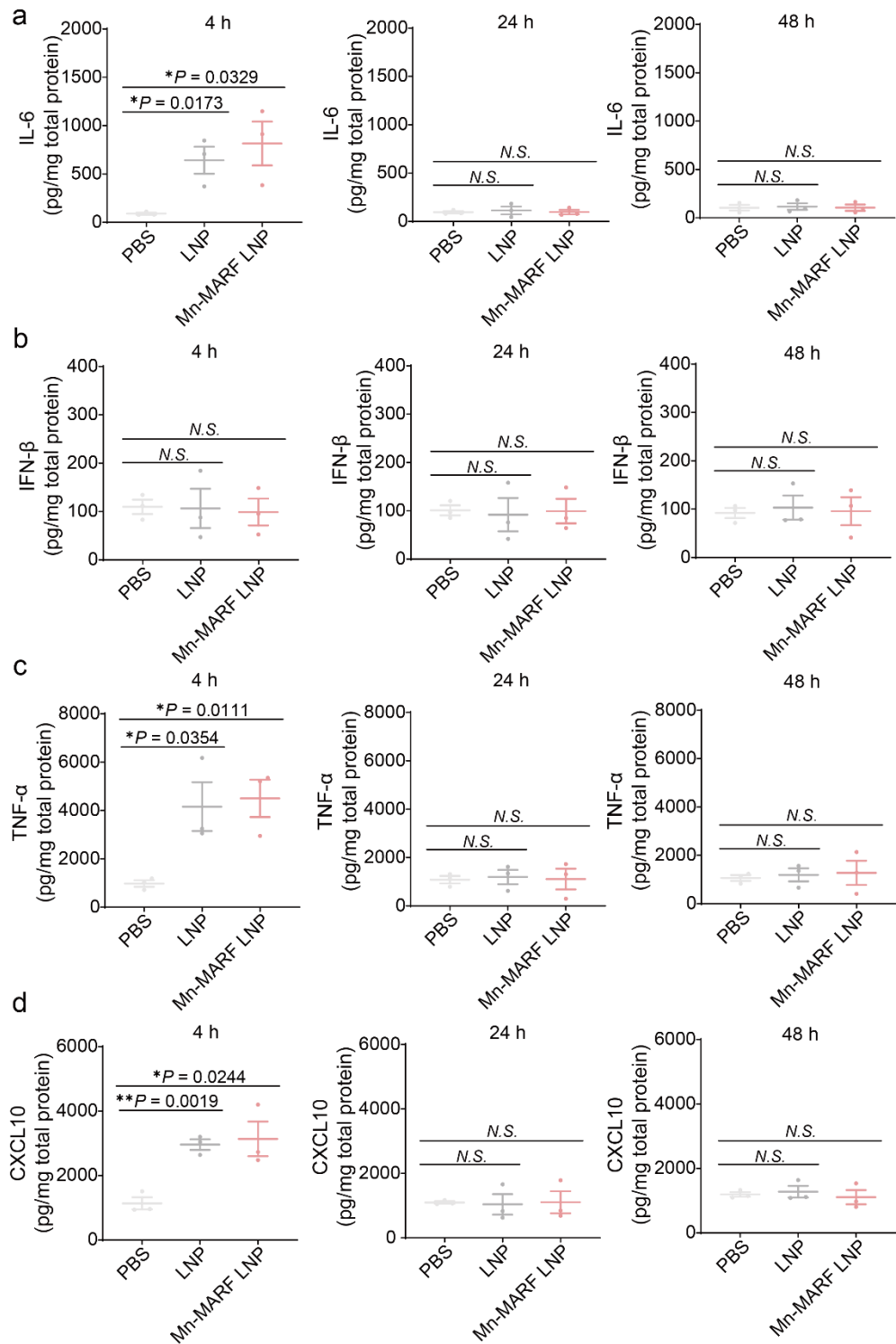
Supplementary Fig. 44. *In vivo* toxicity of Mn-MARF LNP and LNP following i.v. or i.m. administration. Liver and kidney damage biomarkers, including AST (a), ALT (b), CREA (c), and BUN (d), were measured in serum at 4, 24, and 48

h following treatment with MARF LNPs or control LNPs (2.5 mg/kg) administered via both i.v. and i.m. routes. The results were obtained from n = 3 biologically independent samples. Data were presented as mean $\pm$ s.e.m.



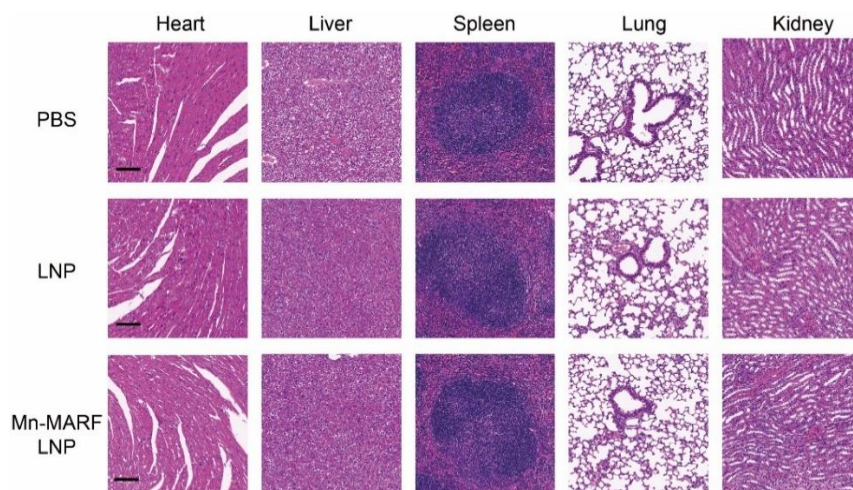
Supplementary Fig. 45. *In vivo* toxicity of Mn-MARF LNP and LNP following i.v. or i.m. administration.

Proinflammation markers, including IL-6 (a), IL- β (b), TNF- α (c), and CXCL10 (d), were measured in serum at 4, 24, and 48 h following treatment with MARF LNPs or control LNPs (2.5 mg/kg) administered via both i.v. and i.m. routes. The results were obtained from $n = 3$ biologically independent samples. Data were presented as mean $\pm$ s.e.m.

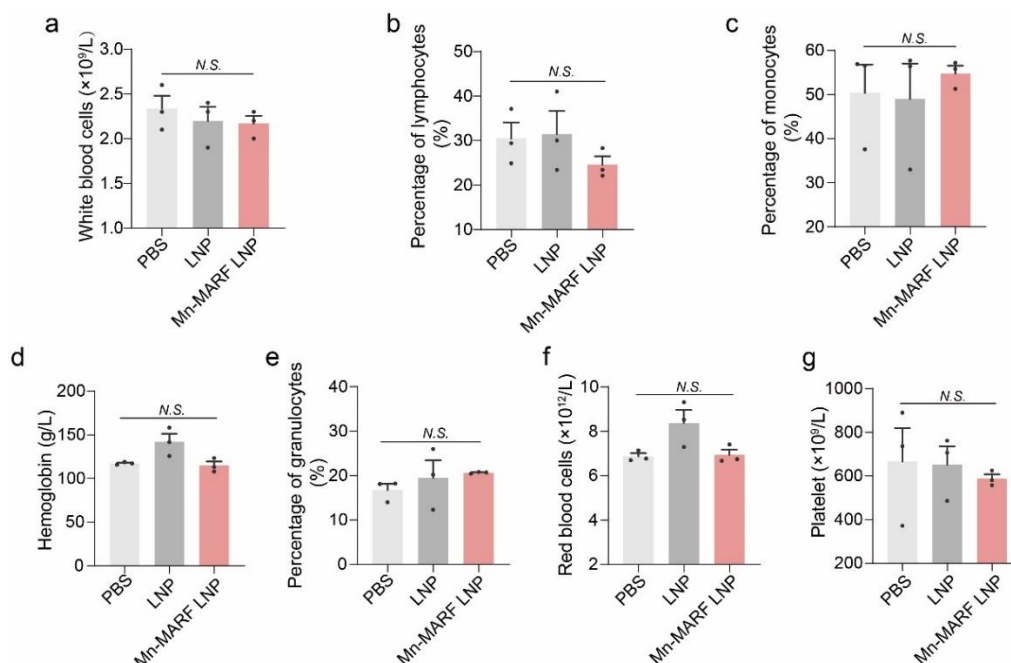


Supplementary Fig. 46. *In vivo* toxicity of Mn-MARF LNP and LNP following i.m. administration. Proinflammation

markers, including IL-6 (a), IL- β (b), TNF- α (c), and CXCL10 (d), were measured in draining lymph nodes at 4, and 24 h following treatment with MARF LNPs or control LNPs (2.5 mg/kg) administered via i.m. route. The results were obtained from n = 3 biologically independent samples. Data were presented as mean $\pm$ s.e.m.

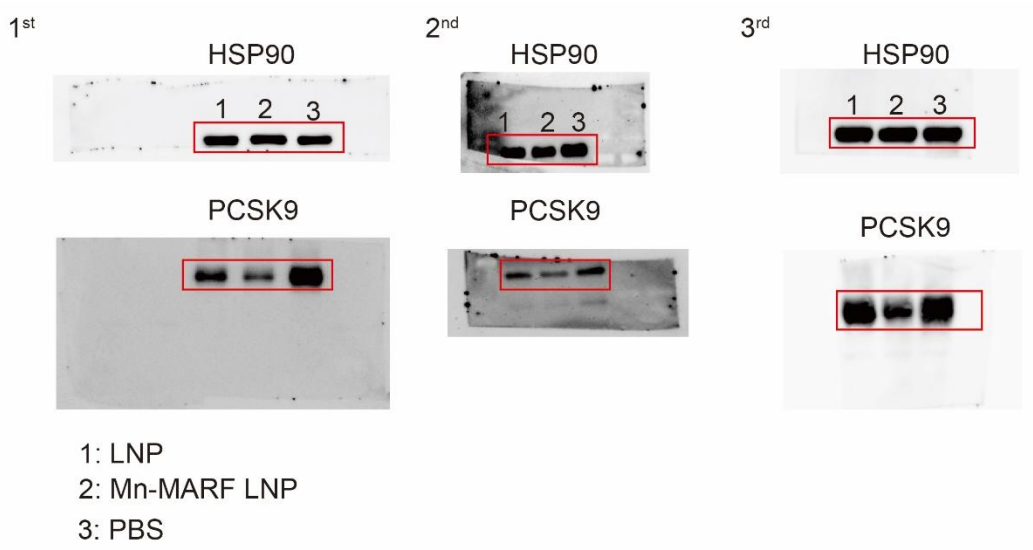


Supplementary Fig. 47 Histological analysis of major organs from mice treated with Mn-MARF LNP and LNP. Representative histology images of main organs (heart, liver, spleen, lung, kidney) after two doses treatment of Mn-MARF LNP and LNP through i.v. injection (1 mg/Kg). Scale bar= 50 μ m.



Supplementary Fig. 48 Biocompatibility analysis of Mn-MARF LNP and LNP. The whole blood was collected 48 hours after administration of one dose treatment of Mn-MARF LNP and LNP (2.5 mg/Kg) for hemocompatibility test, including (a) number of white blood cells, (b) percentage of lymphocytes, (c) percentage of monocytes, (d) concentration of hemoglobin,

(e) percentage of granulocyte, (f) number of red blood cells and (g) number of platelets. The results were obtained from n = 3 biologically independent samples. Data were presented as mean $\pm$ s.e.m.



Supplementary Fig. 49 Uncropped western blot images

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