

Mechanistic Insights into mRNA–LNP Interactions: Role of Ionizable Lipid Content in Regulating mRNA Intracellular Release and Translation

Hoang Quan Truong¹, Urmila Kafle^{1,2}, Cao Thuy Giang Nguyen¹, Yaohao Li², Fanfei Meng^{1,2 *}

¹ Department of Biomedical and Nutritional Sciences, University of Massachusetts Lowell, 3 Solomont Way, Lowell, MA 01854, USA

² Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, TX, USA

* Corresponding author:

Fanfei Meng, Ph.D.

Phone: 713-743-2629

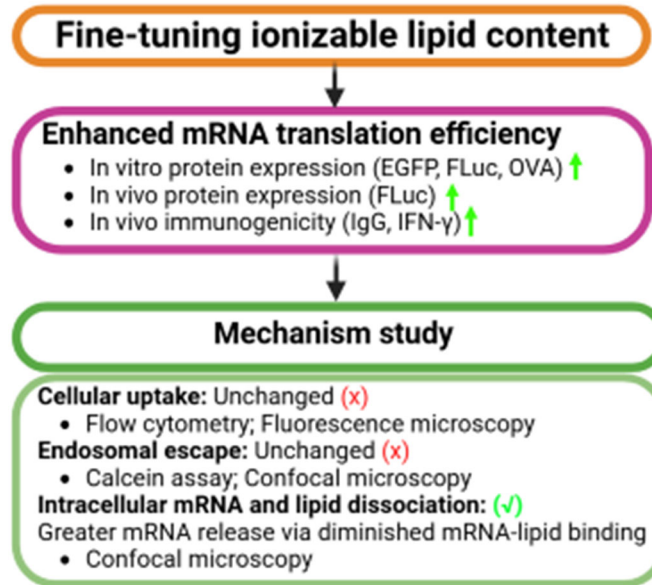
E-mail: fmeng6@central.uh.edu

Supporting Table 1. Molar ratios of lipid components in modified LNP formulations based on the clinically approved Moderna mRNA vaccine.

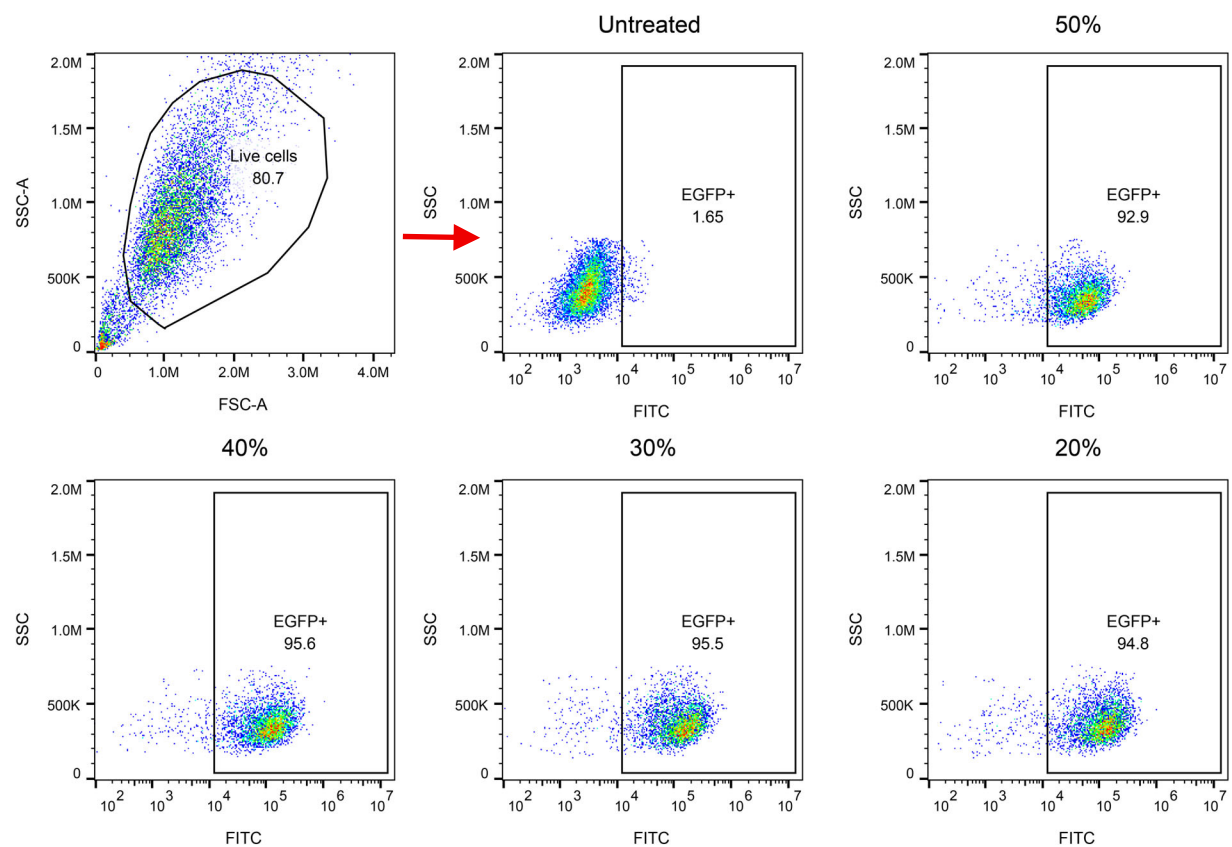
Name	SM-102	DSPC	Cholesterol	PEGylated lipid
50	50	10	38.5	1.5
45	45	10	38.5	1.5
40	40	10	38.5	1.5
30	30	10	38.5	1.5
20	20	10	38.5	1.5

Supporting Table 2. Molar ratios of lipid components in modified LNP formulations based on the clinically approved Pfizer/BioNTech mRNA vaccine.

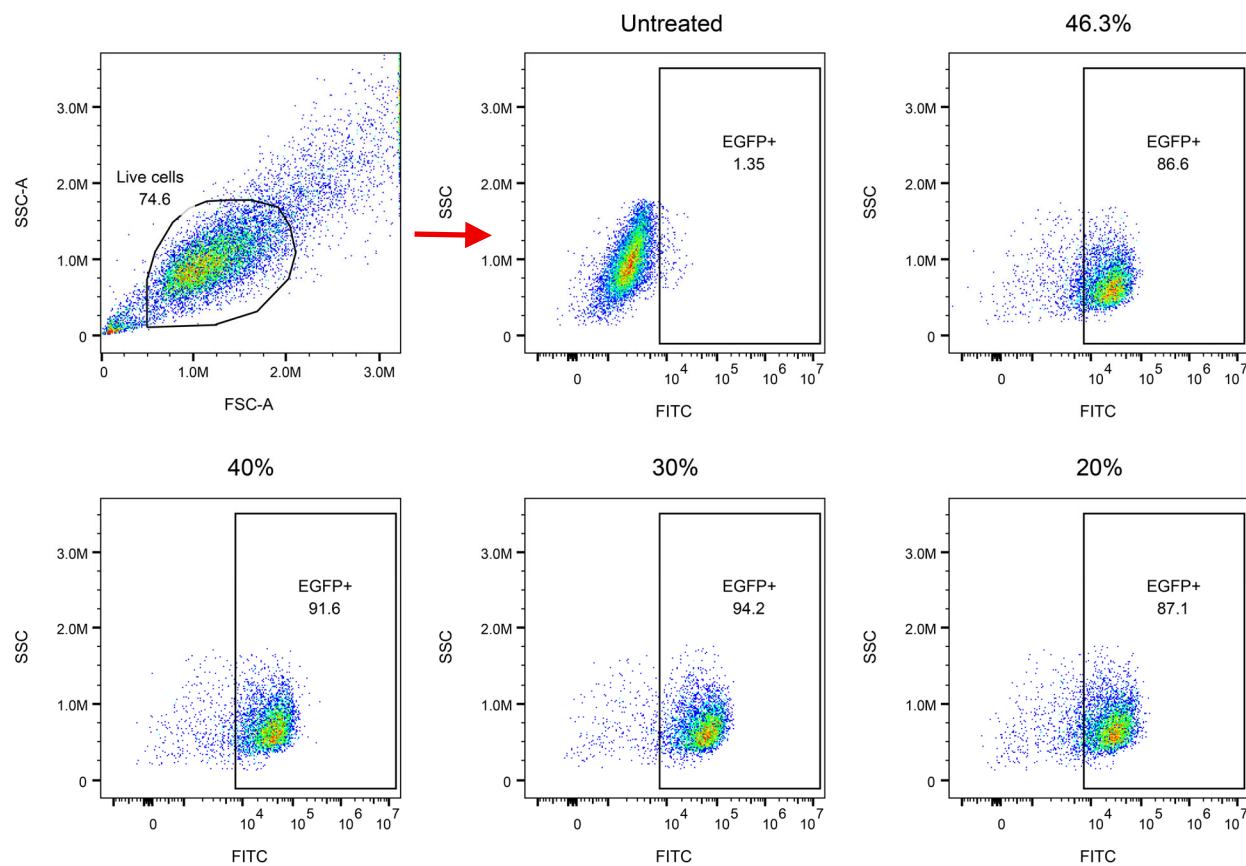
Name	ALC-3015	DSPC	Cholesterol	ALC-0159
46.3	46.3	9.4	42.7	1.6
40	40	9.4	42.7	1.6
30	30	9.4	42.7	1.6
20	20	9.4	42.7	1.6



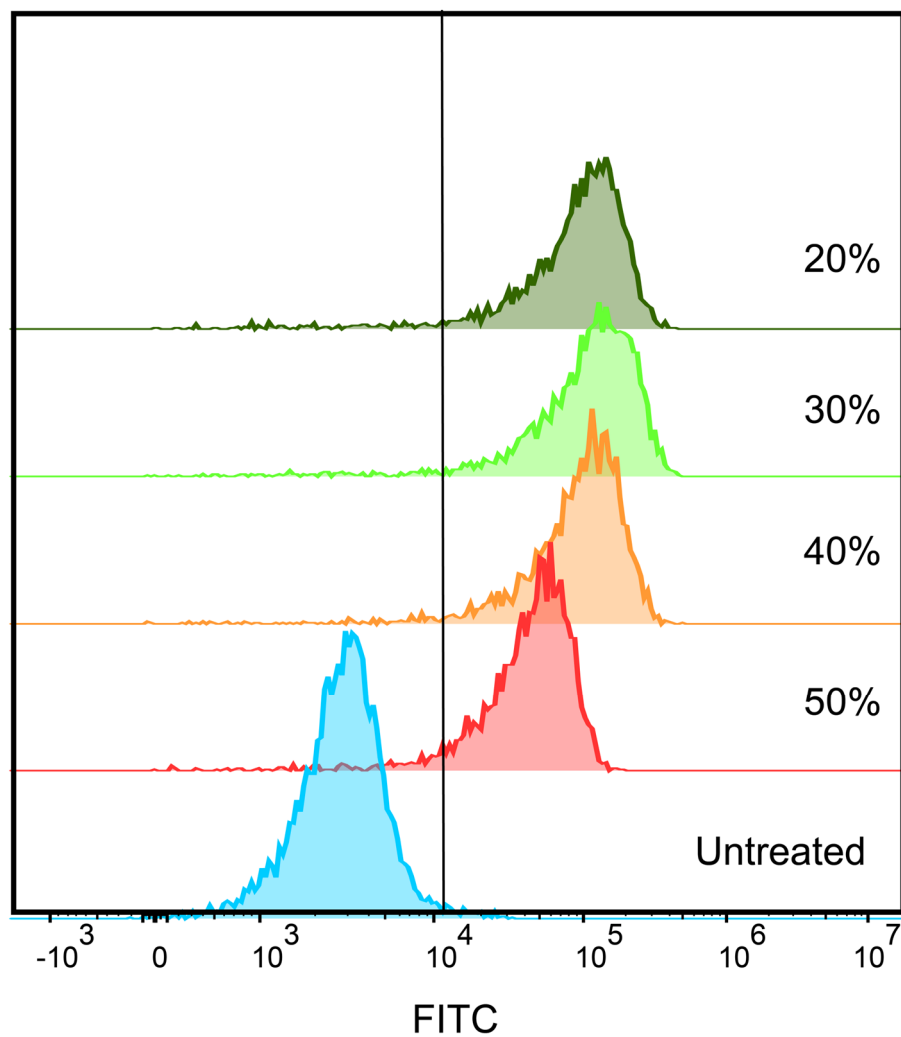
Supporting Fig. 1. Diagram illustrating the hypothesis-driven workflow for evaluating how fine-tuning ionizable lipid content improves mRNA-LNP performance.



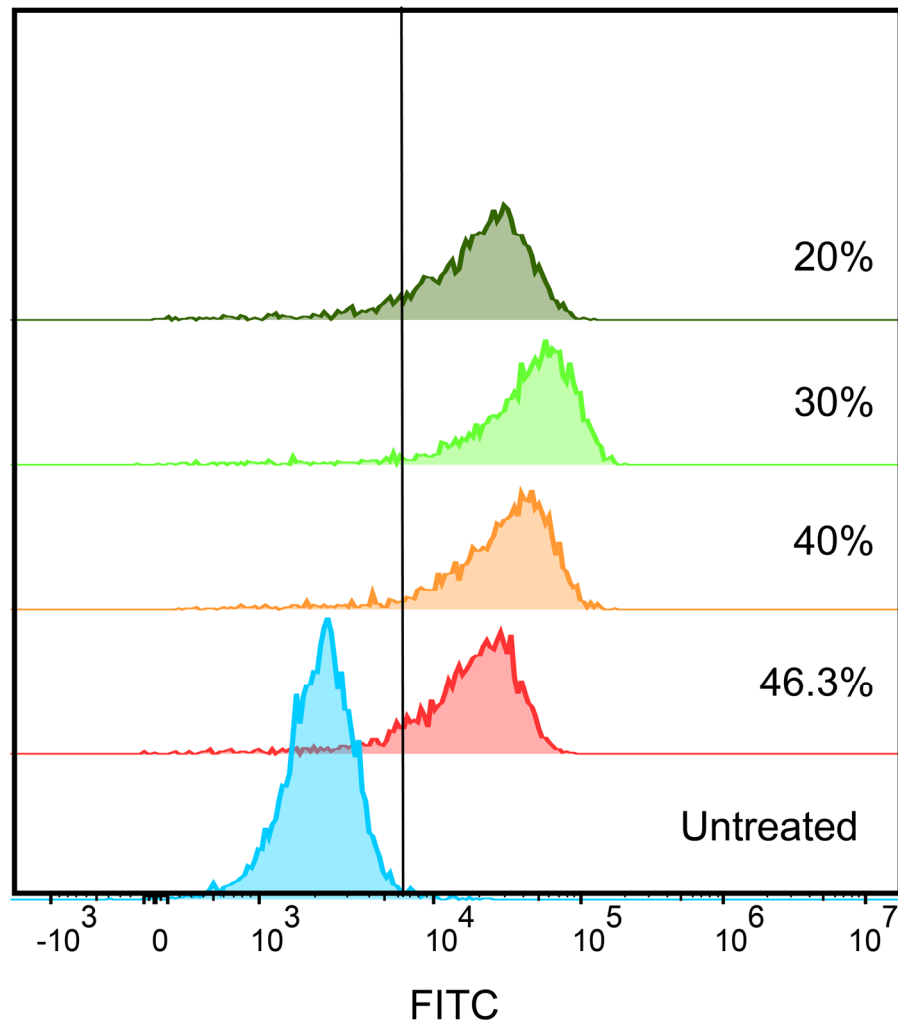
Supporting Fig. 2. Gating strategy and percentage of EGFP⁺ HEK293 cells after treatment with mRNA-LNPs containing varying amounts of ionizable lipid (Moderna version).



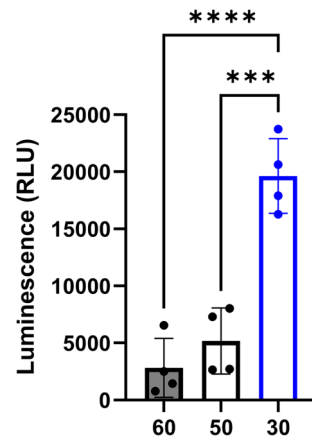
Supporting Fig. 3. Gating strategy and percentage of EGFP⁺ HEK293 cells after treatment with mRNA-LNPs containing varying amounts of ionizable lipid (Pfizer/BioNTech version).



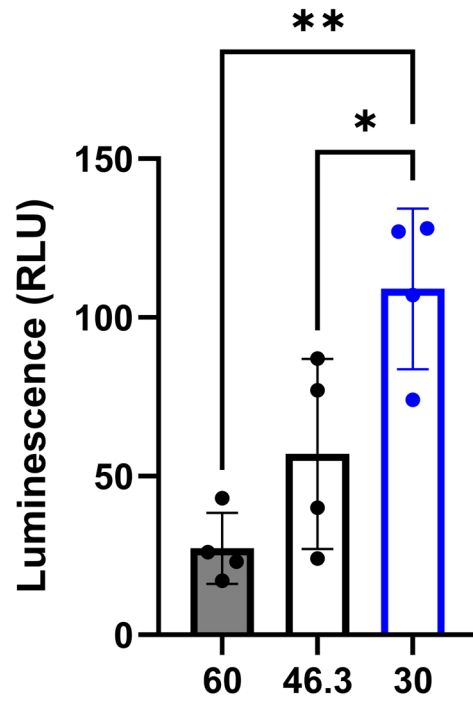
Supporting Fig. 4. Histogram showing EGFP⁺ HEK293 cells after treatment with mRNA-LNPs containing varying amounts of ionizable lipid (Moderna version).



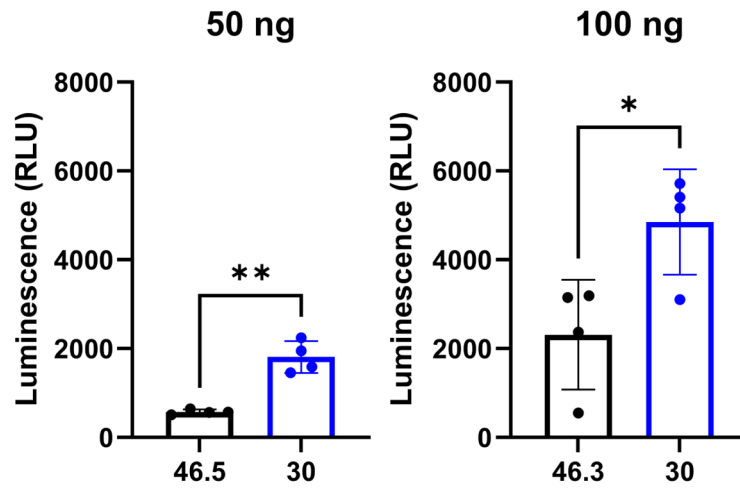
Supporting Fig. 5. Histogram showing EGFP⁺ HEK293 cells after treatment with mRNA-LNPs containing varying amounts of ionizable lipid (Pfizer//BioNTech version).



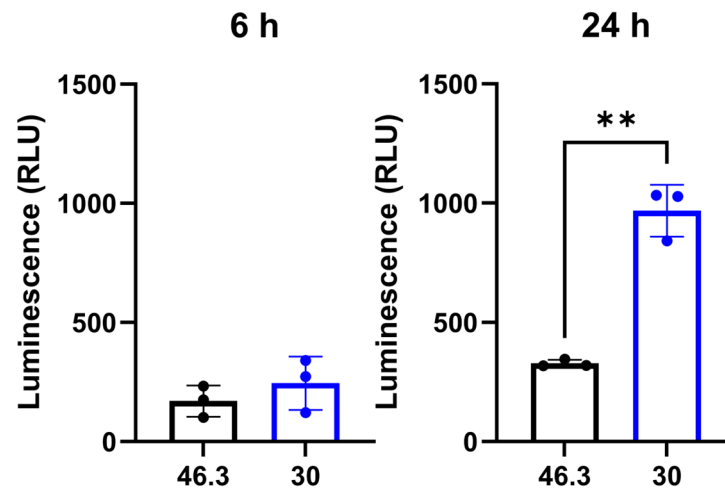
Supporting Fig. 6. Transfection efficiency of mRNA-LNPs (Moderna version). Luciferase expression at 24 h following transfection with Fluc-mRNA-LNPs with 50 ng mRNA per well in a 96-well plate on HEK-293 cells.



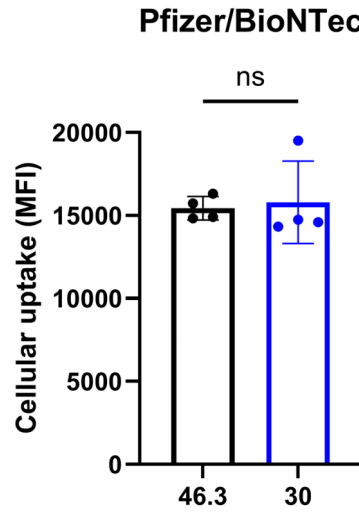
Supporting Fig. 7. Transfection efficiency of mRNA-LNPs (Pfizer/BioNTech version). Luciferase expression at 6 h following transfection with Fluc-mRNA-LNPs with 50 ng mRNA per well in a 96-well plate on HEK-293 cells.



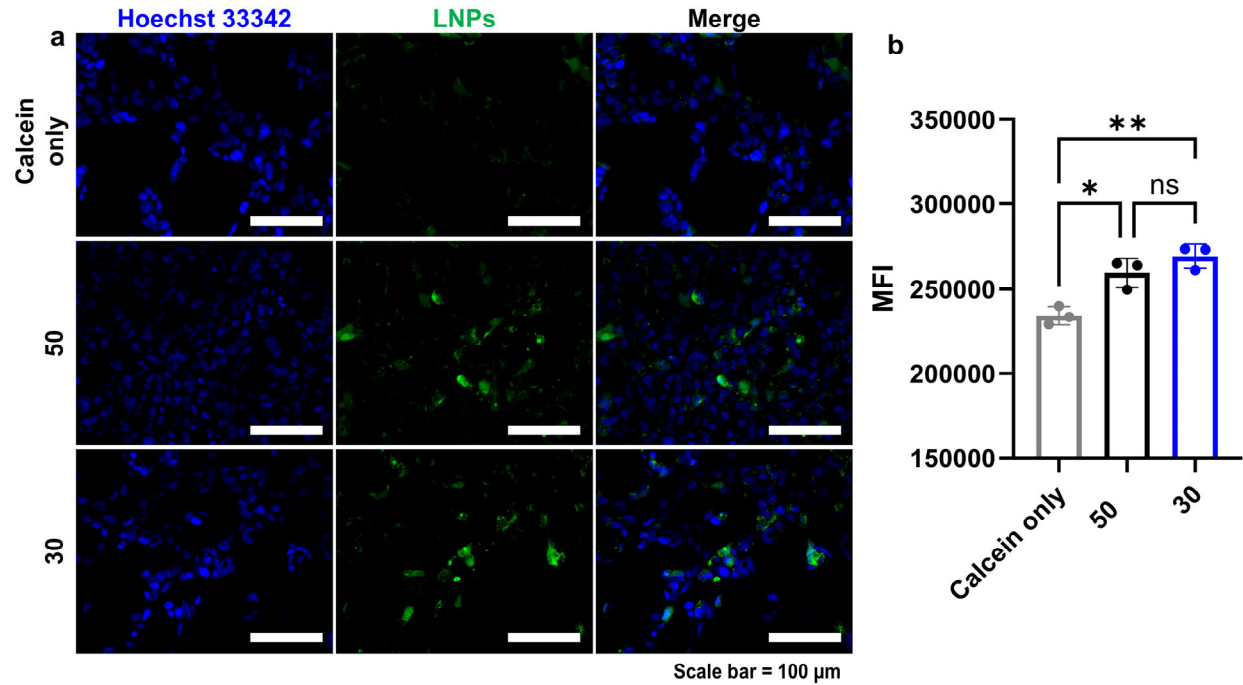
Supporting Fig. 8. Transfection efficiency of mRNA-LNPs (Pfizer version). Luciferase expression at 24 h following treatment with Fluc-mRNA-LNPs on HEK-293 cells with 50 ng or 100 ng mRNA per well in a 96-well plate.



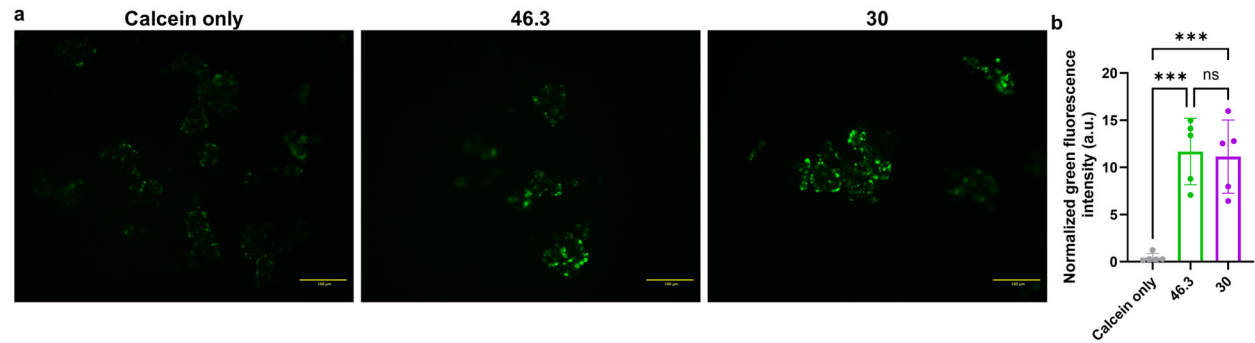
Supporting Fig. 9. Transfection efficiency of mRNA-LNPs (Pfizer/BioNTech version). Luciferase expression at 6 h and 24 h following treatment with Fluc-mRNA-LNPs on B16F10 melanoma cells with 50 ng mRNA per well in a 96-well plate.



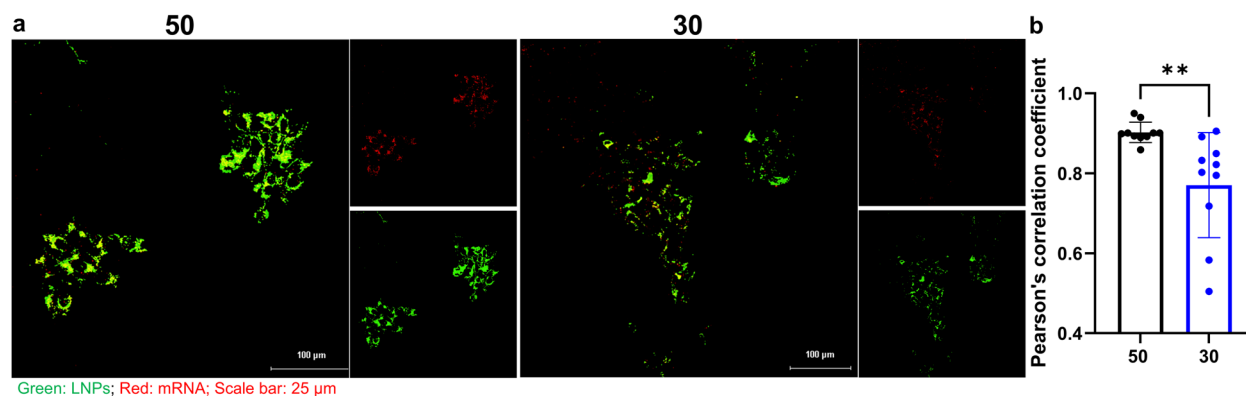
Supporting Fig. 10. Cellular uptake of mRNA-LNPs (Pfizer/BioNTec version). Quantification of uptake in HEK293 cells assessed by flow cytometry.



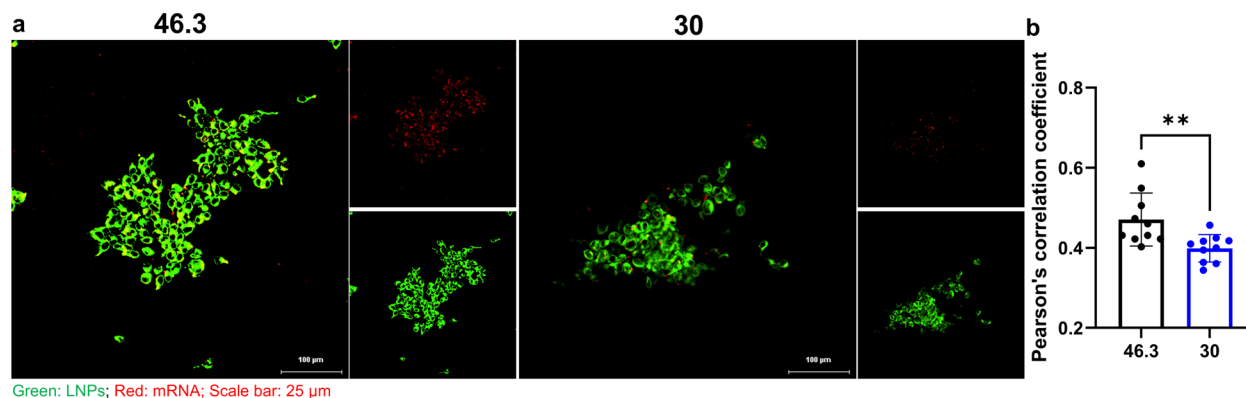
Supporting Fig. 11. Evaluation of endosomal escape mRNA-LNP (Moderna version) using the Calcein assay. (a) Representative fluorescence images of HEK293 cells. Cells were treated with mRNA-LNPs (50 ng mRNA per well) supplemented with 40 μ L of Calcein solution (40 μ g calcein per well) and incubated for 6 h. Post-incubation, cells were washed with PBS, stained with Hoechst 33342 (3 μ g/mL, 15 min), and fixed with 4% paraformaldehyde for 15 min. Calcein fluorescence was visualized using the iRiS™ Digital Cell Imaging System (Logos Biosystems; Anyang, South Korea) treated with LNPs. Nuclei were stained with Hoechst 33342 (blue). Scale bars, 100 μ m. (b) Quantification of fluorescence intensity from Calcein assay with flow cytometry. Data are presented as mean $\pm$ SD. *p < 0.05; **p < 0.01.



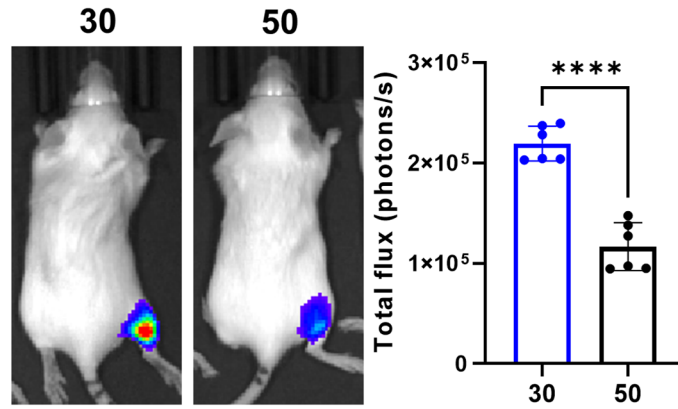
Supporting Fig. 12. Evaluation of endosomal escape of mRNA-LNP (Pfizer/BioNTech version) using the Calcein assay. (a) Representative fluorescence images of HEK293 cells. Cells were treated with mRNA-LNPs (50 ng mRNA per well) supplemented with 40 μ L of Calcein solution (40 μ g calcein per well) and incubated for 6 h. Post-incubation, cells were washed with PBS, stained with Hoechst 33342 (3 μ g/mL, 15 min), and then fixed with 4% paraformaldehyde for 15 min. Calcein fluorescence was visualized using the iRiS Digital Cell Imaging System (Logos Biosystems; Anyang, South Korea) after treatment with LNPs. Nuclei were stained with Hoechst 33342 (blue). Scale bars, 100 μ m. (b) Quantification of fluorescence intensity from Calcein assay with flow cytometry. Data are presented as mean $\pm$ SD. * p < 0.05; ** p < 0.01.



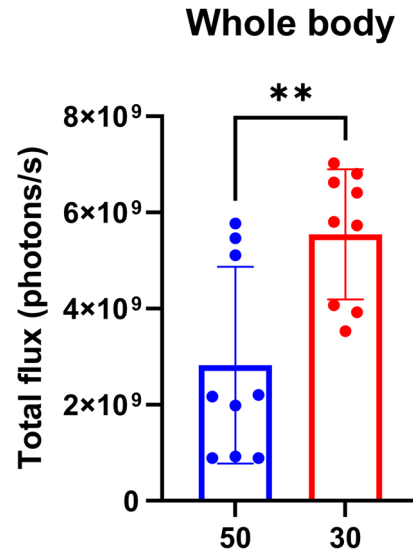
Supporting Fig. 13. Intracellular dissociation of mRNA from LNPs (Moderna version). (a) Representative confocal images of HEK293 cells after treatment with mRNA-LNP for 6 h: dual-labeled mRNA-LNPs: NBD-SM102 for LNPs (green) and Cy5 for mRNA (red). Scale bars, 100 μm . (b) Quantification of intracellular LNP and mRNA colocalization based on 10 images for each group, expressed as Pearson's correlation coefficient, and analyzed using ImageJ. Data are presented as mean $\pm$ SD. ** $p < 0.01$.



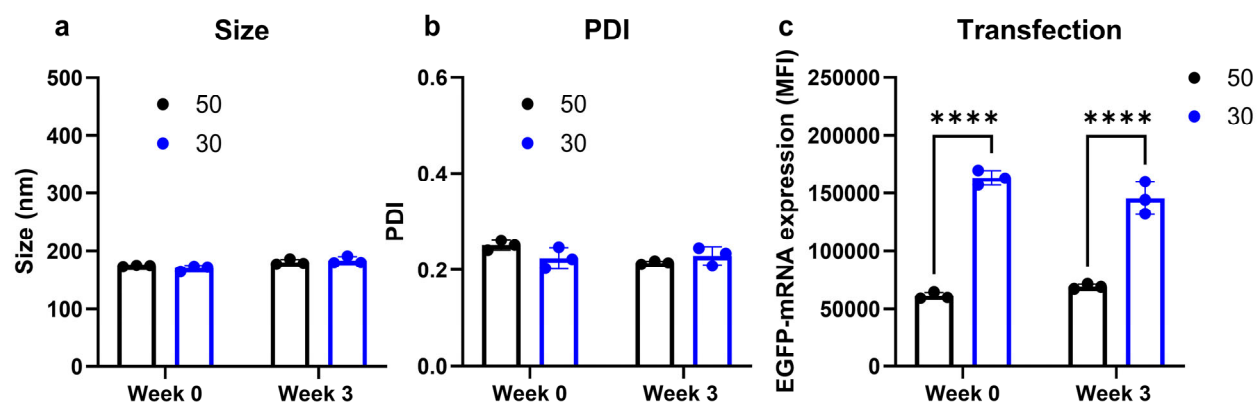
Supporting Fig. 14. Intracellular dissociation of mRNA from LNPs (Pfizer/BioNTech version). (a) Representative confocal images of HEK293 cells after treatment with mRNA-LNP for 6 h: dual-labeled mRNA-LNPs: NBD-Cholesterol for LNPs (green) and Cy5 for mRNA (red). Scale bars, 100 μm . (b) Quantification of intracellular LNP and mRNA colocalization based on 10 images for each group, expressed as Pearson's correlation coefficient, and analyzed using ImageJ. Data are presented as mean $\pm$ SD. ** $p < 0.01$.



Supporting Fig. 15. In vivo luciferase expression following administration of mRNA-LNPs. Representative bioluminescence images acquired 6 h after intramuscular (IM) injection of Fluc mRNA-LNPs (0.25 μ g Fluc mRNA per mouse) in BALB/c mice (left), with corresponding quantification of luminescence intensity (right). For each mouse, three images were acquired and quantified. Data are presented as mean $\pm$ SD. **** $p < 0.0001$.



Supporting Fig. 16. Quantification of whole-body bioluminescence at 6 h after IV injection of Fluc-mRNA-LNPs (2 μ g Fluc mRNA per mouse) in Balb/c mice. Bioluminescence images were acquired 10-20 minutes after intraperitoneal injection of luciferin (30 mg/mL, 100 μ L per mouse). For each mouse, three images were collected, resulting in a total of nine images analyzed per group. Representative images are shown in Fig. 7c. Data are shown as mean $\pm$ SD. ** $p < 0.01$, *** $p < 0.0001$.



Supporting Fig. 17. Stability and functional performance of mRNA-LNPs after storage. (a) Size, (b) PDI, and (c) transfection efficiency of freshly prepared and - 80 °C stored 50- and 30- groups. mRNA-LNPs were formulated and suspended in PBS containing 8% sucrose (w/v) solution, then stored at -80 °C. After three weeks of storage, samples were thawed on ice and analyzed for particle size, zeta potential, polydispersity index (PDI), and transfection efficiency. These parameters were compared with those of freshly prepared mRNA-LNPs to assess stability. Data demonstrates that both groups maintain physicochemical stability and transfection capability after frozen storage.