Supporting Information for

Engineering lipid nanoparticles for enhanced intracellular delivery of messenger RNA through inhalation

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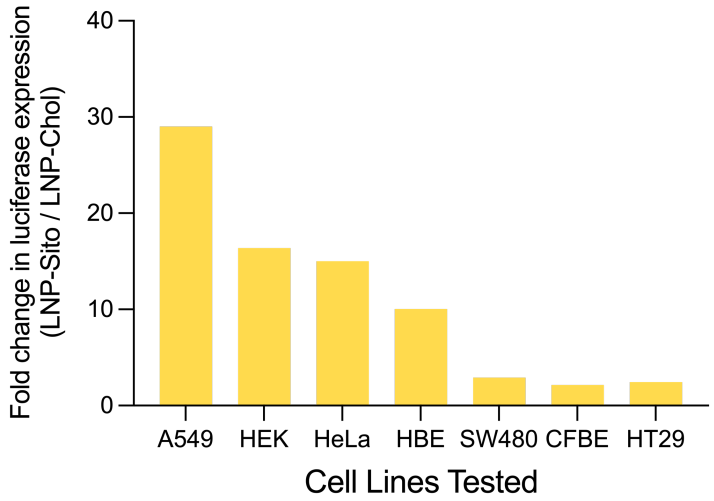
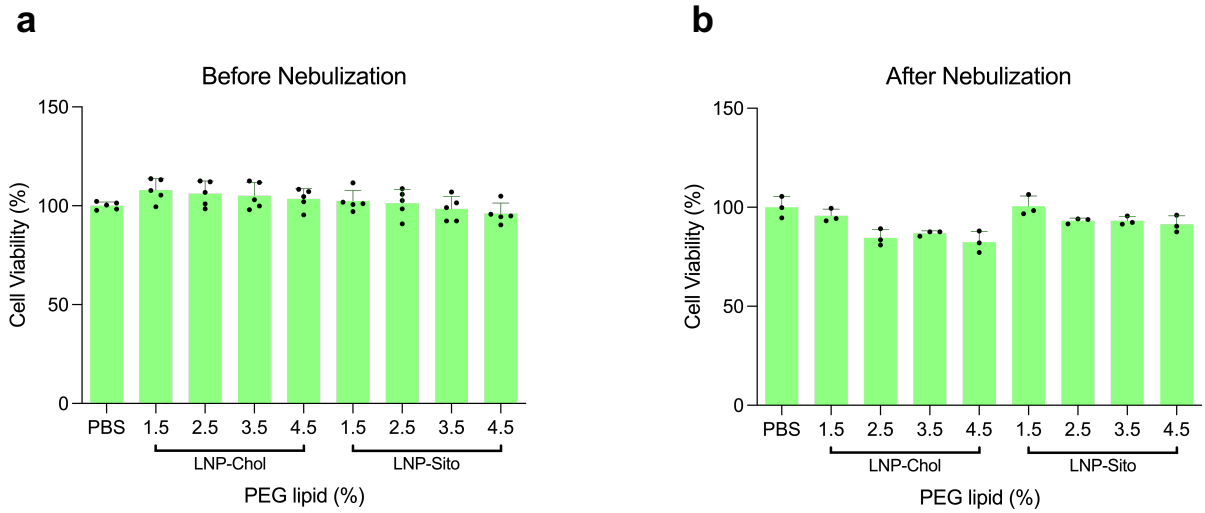
Figure S1. Comparison between LNP-Chol/1.5 and LNP-Sito/1.5 for delivering *Fluc* mRNA to various cell lines.



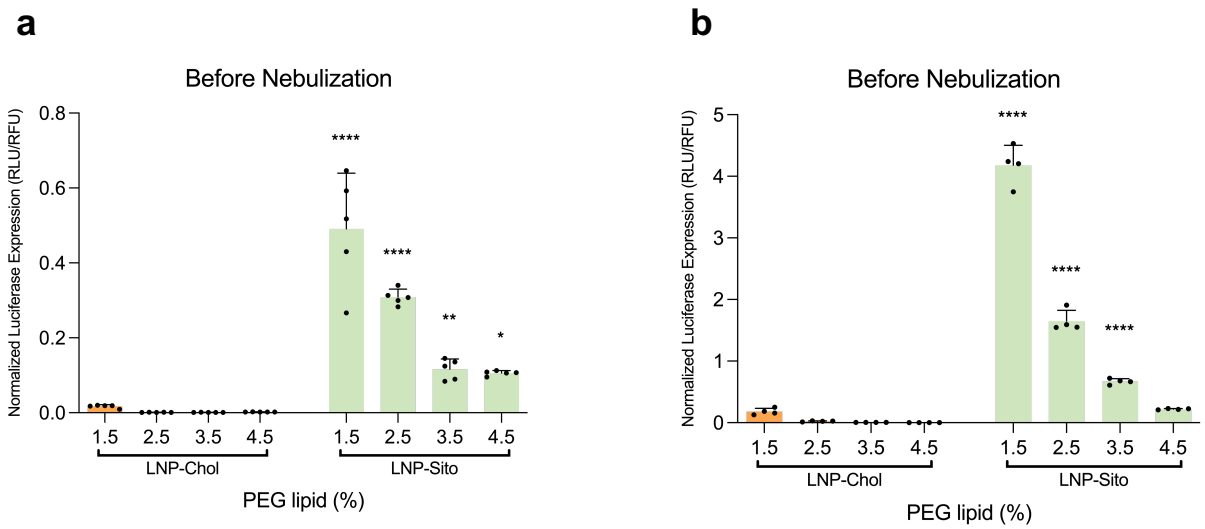
Data were present as the mean luciferase expression of LNP-Sito/1.5 divided by the mean luciferase expression of LNP-Chol/1.5. (n=4-6)

Figure S2. *In vitro* cell viability of HeLa cells treated with LNP solution or nebulized LNP containing *Fluc* mRNA.



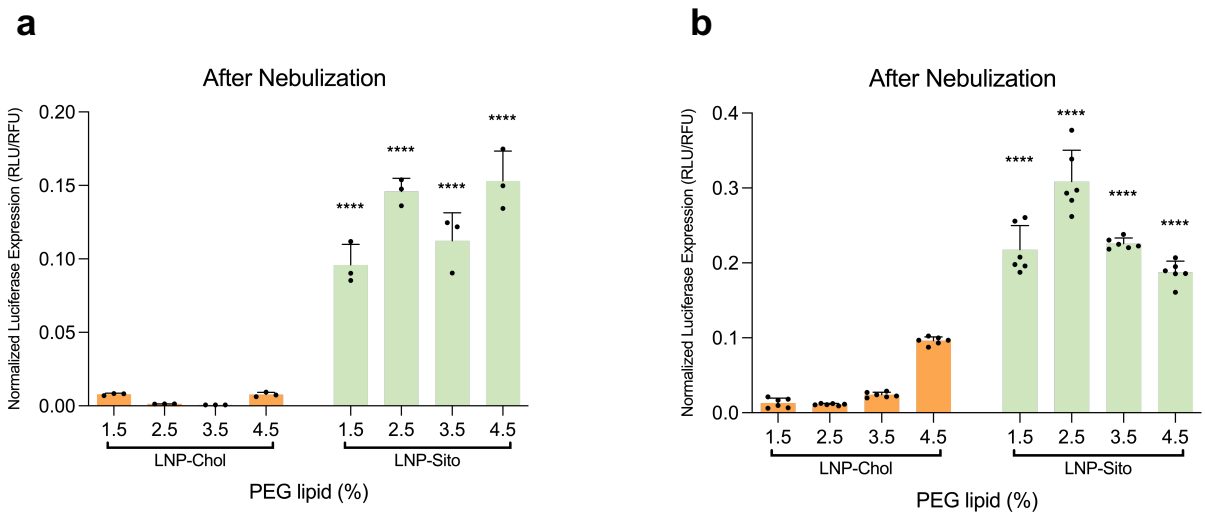
Cell viability results of HeLa cells treated with (**a**) LNP solution at a dose of 50 ng/well and (**b**) LNP aerosol at a dose of 1 μg *Fluc* mRNA per well for 24 h.

Figure S3. *In vitro* mRNA transfection assay after treating A549 and 16HBE14o- cells with LNP solution.



Luciferase expressions were measured in (**a**) A549 and (**b**) 16HBE14o- cells treated with LNP solution at a dose of 50 ng and 200 ng *Fluc* mRNA per well, respectively. Statistical analysis was performed using Two-way ANOVA with Sidak's multiple comparison tests. ****p<0.0001; **p<0.01; *p<0.05.

Figure S4. *In vitro* mRNA transfection assay after treating A549 and 16HBE14o- cells with nebulized LNP.



Luciferase expressions were measured in (**a**) A549 and (**b**) 16HBE14o- cells treated with LNP aerosol at a dose of 1000 ng and 500 ng *Fluc* mRNA per well, respectively. Statistical analysis was performed using Two-way ANOVA with Sidak's multiple comparison tests. ****p<0.0001.

Figure S5. A schematic of *in vivo* mRNA transfection assay after LNP inhalation

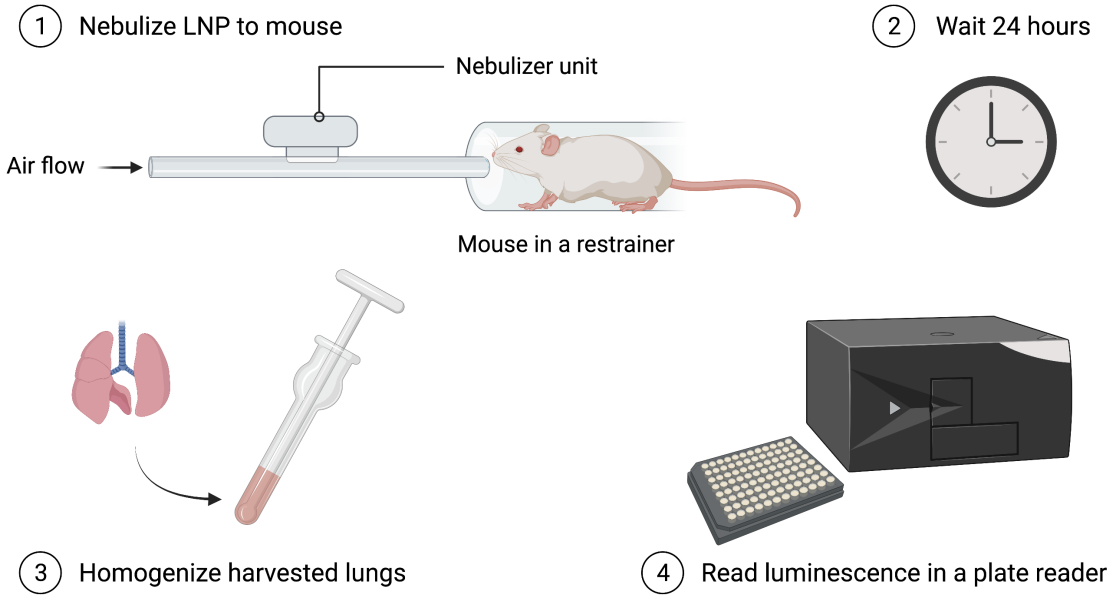
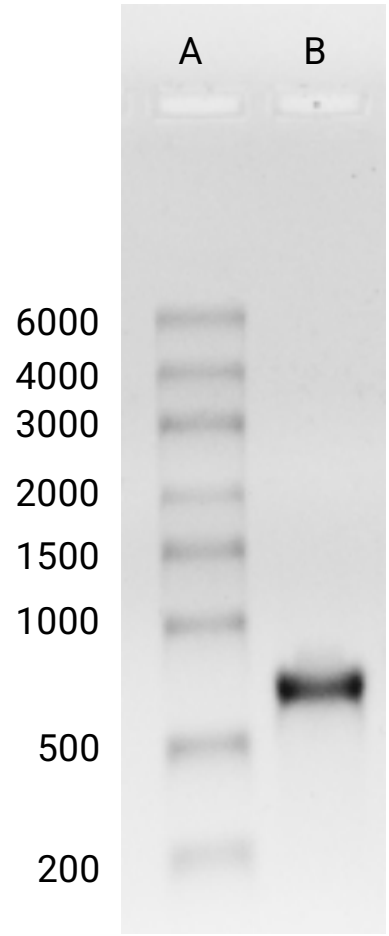
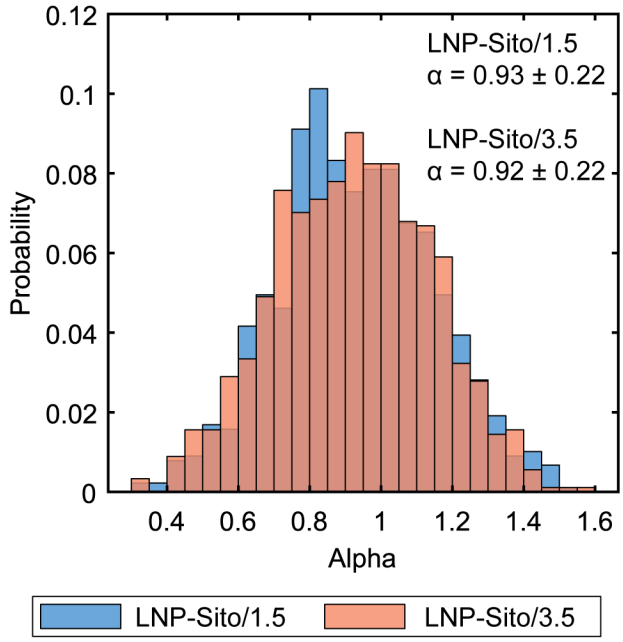


Figure S6. Agarose gel electrophoresis of *Nluc* mRNA



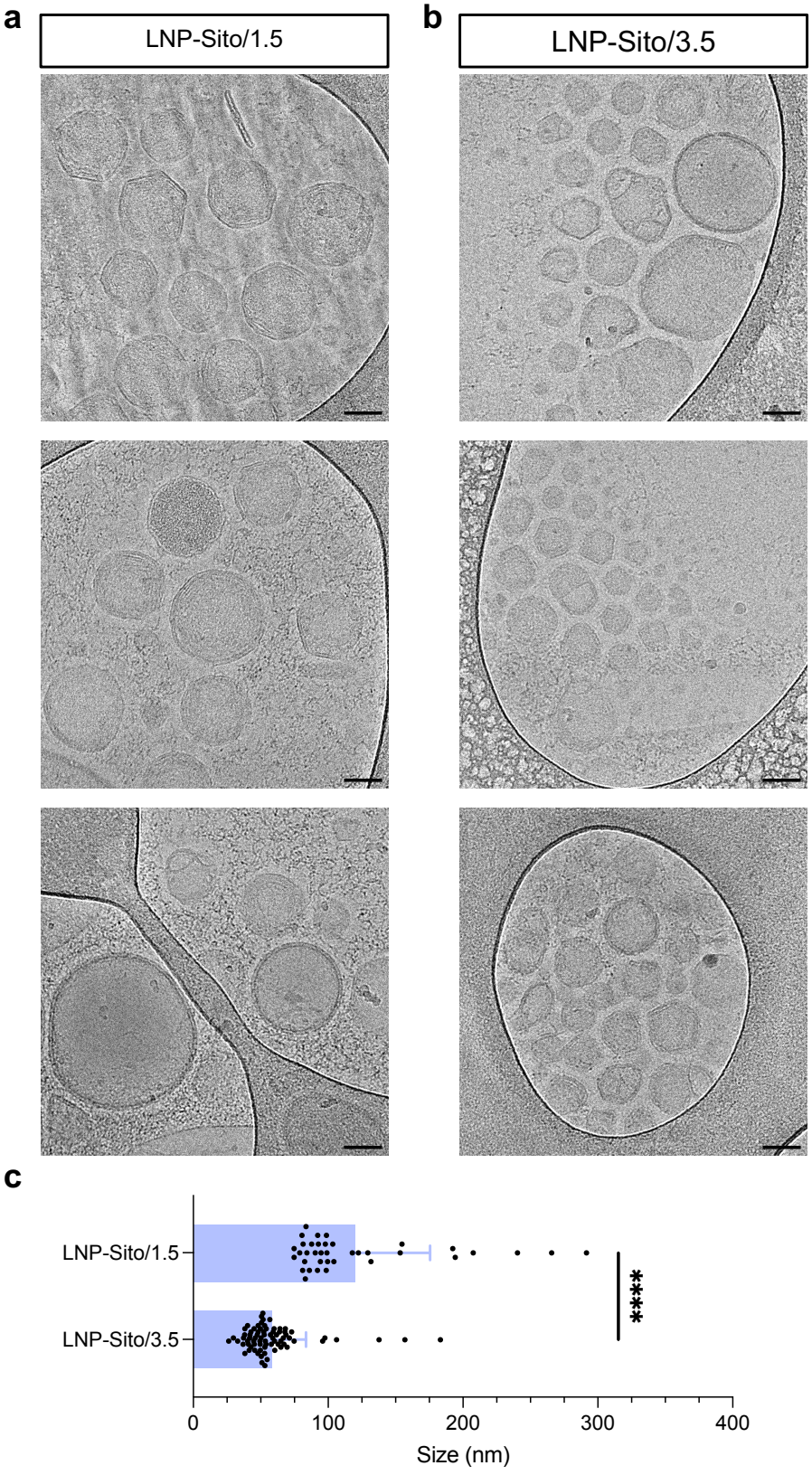
Nluc mRNA was analyzed on 1.5% formaldehyde-agarose gel electrophoresis. (**a**) RNA ladders and (**b**) *Nluc* mRNA. The predicted size of *Nluc* mRNA is approximately 800 bases.

Figure S7. Alpha comparison of LNP-Sito/1.5 and LNP-Sito/3.5.



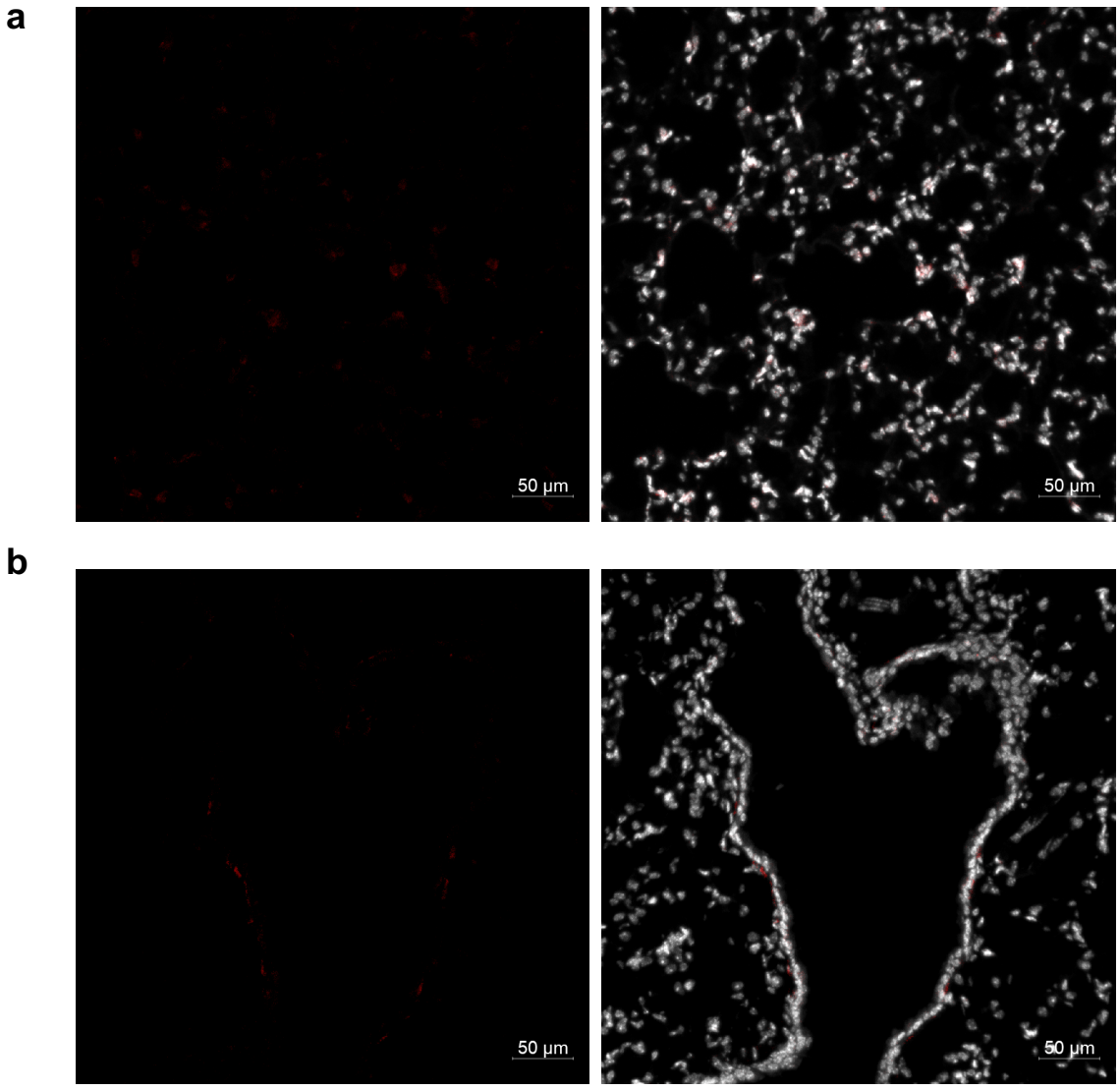
Analysis of trajectories for LNP-Sito/1.5 (blue, n=323) LNP-Sito/3.5 (orange, n=353), mean ± S.D. The two formulations do not show significant differences in alpha values (p=0.34)

Figure S8. Cryogenic transmission microscopy (cryoTEM) imaging of nebulized LNP-Sito/1.5 and LNP-Sito/3.5.



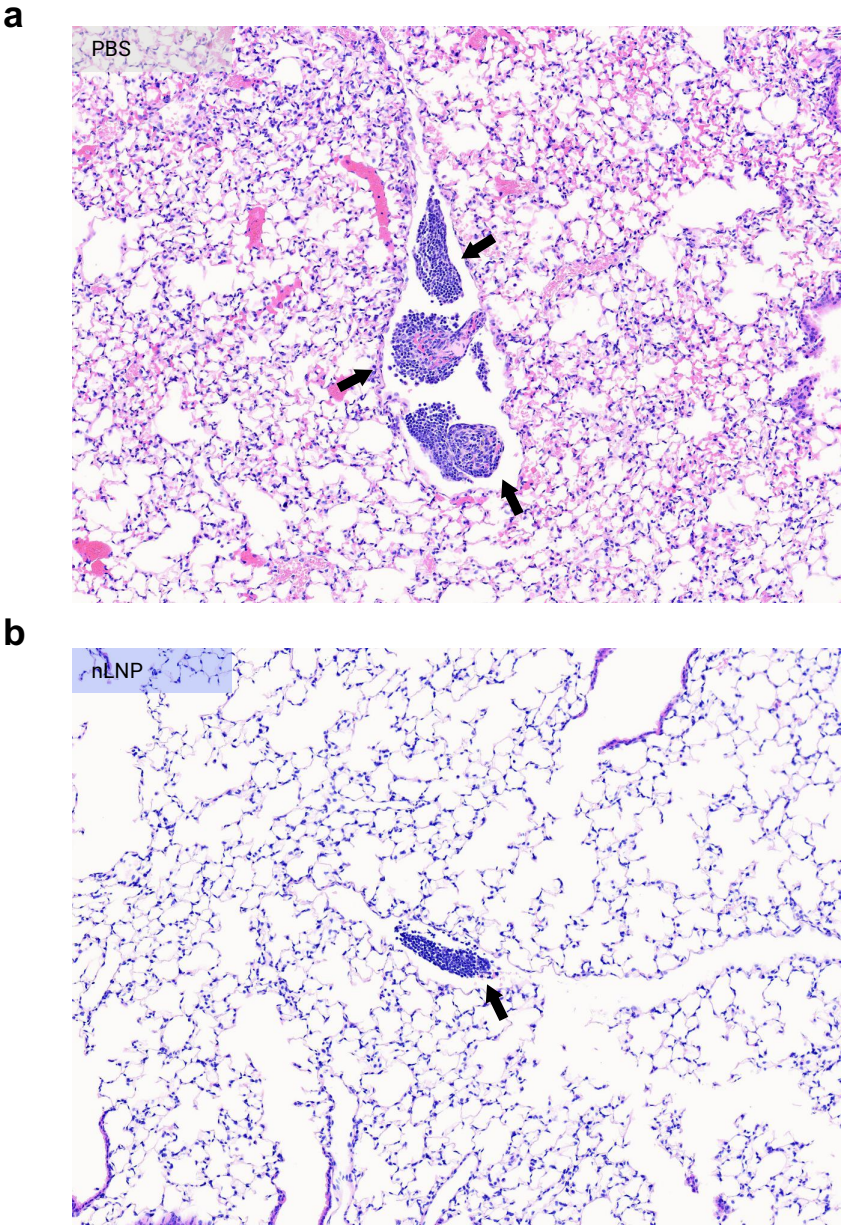
(**a,b**) Three representative cryogenic transmission electron microscopy (cryoTEM) imaging of (**a**) nebulized LNP-Sito/1.5 and (**b**) LNP-Sito/3.5. Scale bars indicate 50 nm. (**c**) Particle size measurement of LNP-Sito/1.5 (n=37) and LNP-Sito/3.5 (n=75) in cryoTEM data. Data were presented in mean ± standard deviation. ****p<0.0001; significant analysis by unpaired t-test.

Figure S9. Representative confocal images of immunohistochemistry images of untreated Ai9 mouse lungs.



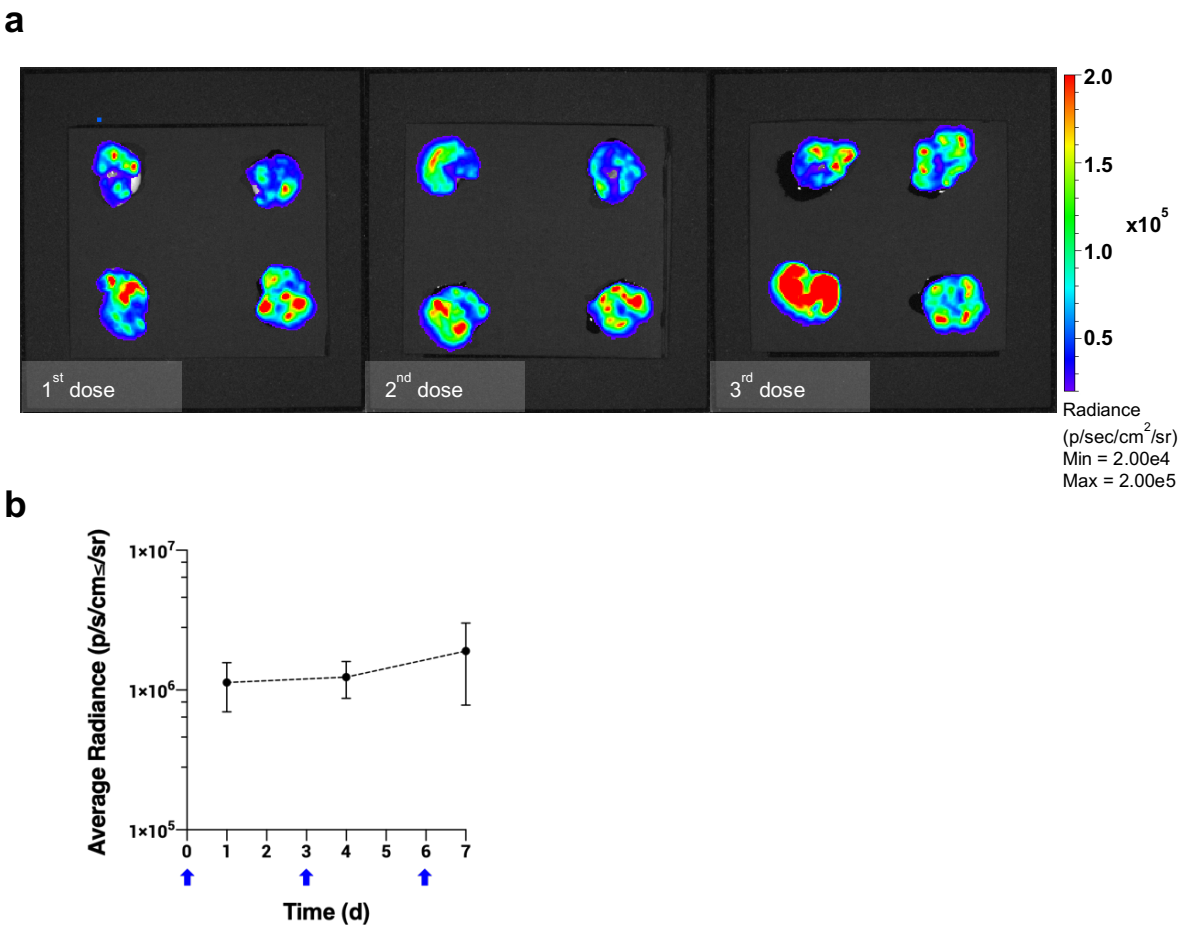
Representative immunohistochemistry of Ai9 mouse lung sections without nanoparticle inhalation. Alveolar spaces and airway bronchioles were presented with tdTomato (left) and the merged image (right). tdTomato and nuclei were pseudo-colored with red and white, respectively. 20x magnification. Scale bars refer to 50 μm.

Figure S10. Histological analysis of mononuclear clots in mouse lungs.



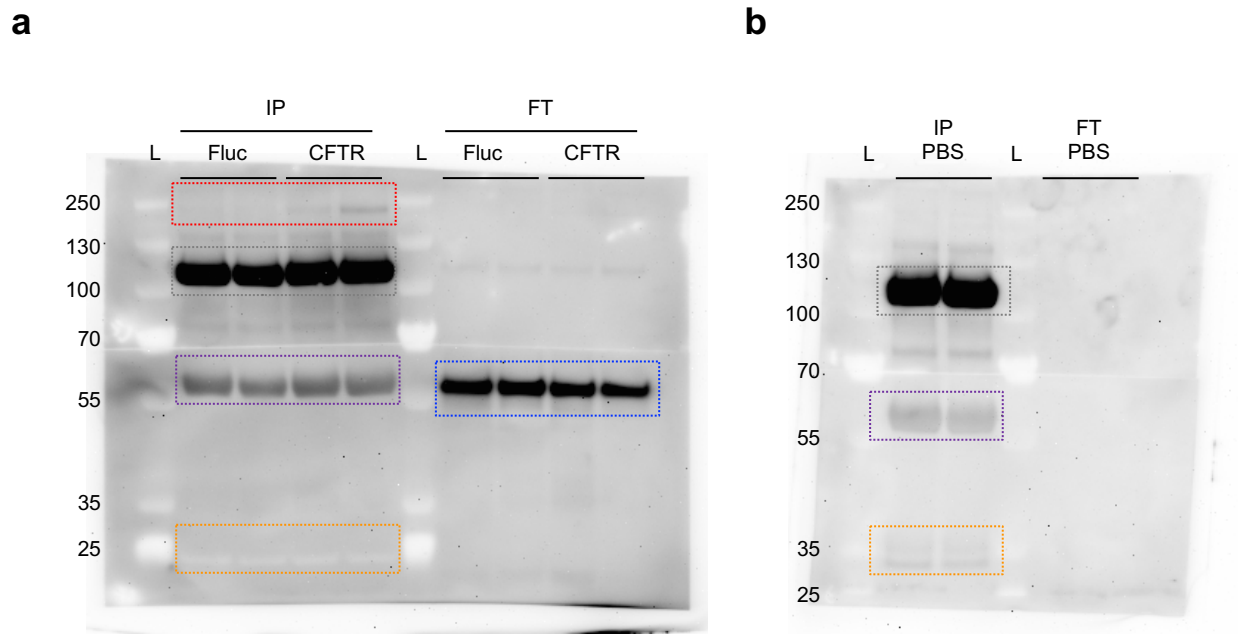
(**a**) PBS- and (**b**) nLNP-treated lungs had mononuclear clots (arrows). Focal, small clots of mononuclear cells, fibrin, and scattered erythrocytes are found in both vehicle and LNP treated lungs. Clots are found adjacent to pleura, within small vessels, or in alveolar ducts. The finding is considered to most likely be incidental and unrelated to treatment. Hematoxylin and eosin; 20x magnification.

Figure S11. ***Nluc*** **mRNA transfection in the mouse lungs after repeated inhalation.**



(**a, b**) After three inhalations of nLNP encapsulating *Nluc* mRNA, the mouse lungs were harvested, and the luminescence was imaged *ex vivo*. (**a**) *Ex vivo* images of the mouse lungs and (**b**) the average radiance of the luminescence in the images (n=4).

Figure S12. Detection of hCFTR protein in the CFKO lungs after repeated inhalation of nLNP encapsulating *Fluc* or *hCFTR* mRNA.



(**a, b**) Results of Western blot following immunoprecipitation (IP) against hCFTR. (**a**) After inhalation of nLNP encapsulating *hCFTR* or *Fluc* mRNA, the CFKO mouse lungs were harvested, homogenized, and lysated for the IP and Western blot. (**b**) IP with PBS was performed as a control. hCFTR protein (red), α-Tubulin (blue), the hCFTR antibody (gray), and heavy chains (purple) and light chains (orange) of the antibody were marked. IP; immunoprecipitated samples, FT; flow-through samples, L; ladders

Table S1. Lipid molar compositions of the LNP formulations.

Formulation	**Sterol (%)**	**DSPC (%)**	**DMG-PEG$_{2K}$ (%)**	**DLin-MC3-DMA (%)**
LNP-Chol/1.5 (Onpattro®)	Cholesterol, 38.5	10	1.5	50
LNP-Chol/2.5	Cholesterol, 38.5	9	2.5	50
LNP-Chol/3.5	Cholesterol, 38.5	8	3.5	50
LNP-Chol/4.5	Cholesterol, 38.5	7	4.5	50
LNP-Chol/5.5	Cholesterol, 38.5	6	5.5	50
LNP-Chol/7.5	Cholesterol, 38.5	5	7.5	50
LNP-Sito/1.5	β-sitosterol, 38.5	10	1.5	50
LNP-Sito/2.5	β-sitosterol, 38.5	9	2.5	50
LNP-Sito/3.5	β-sitosterol, 38.5	8	3.5	50
LNP-Sito/4.5	β-sitosterol, 38.5	7	4.5	50

Data were presented as molar percentages of lipid contents of the LNP formulations.

Table S2. Size measurement of LNP-Chol and LNP-Sito containing various PEG-lipid content in DLS and CryoTEM.

	DLS		CryoTEM	
%DMG-PEG_{2K}	LNP-Chol	LNP-Sito	LNP-Chol	LNP-Sito
1.5%	82.79 ± 0.79 nm	94.10 ± 2.49 nm	50 ± 10 nm	70 ± 15 nm
2.5%	72.95 ± 2.00 nm	81.68 ± 1.90 nm	56 ± 15 nm	60 ± 8 nm
3.5%	65.20 ± 1.58 nm	68.46 ± 0.62 nm	41 ± 6 nm	43 ± 8 nm
4.5%	58.52 ± 1.37 nm	60.98 ± 1.05 nm	33 ± 8 nm	33 ± 11 nm

Data were presented as mean ± standard deviation. (DLS; *n*=3, CryoTEM; *n*=5-20)

Table S3. Size measurement of nebulized LNP-Sito/1.5 and 3.5 in CryoTEM.

	LNP-Sito/1.5 (n=37)	LNP-Sito/3.5 (n=75)
Size	120 ± 56 nm	58 ± 25 nm

Data were presented as mean ± standard deviation.

Table S4. Results of clinical chemistry of mouse sera collected after inhalation exposure.

	Results	
Parameter	PBS-treated	LNP-treated
ALP (U/L)	100.7 ± 7.2	107.3 ± 8.1
AST (U/L)	44.7 ± 5.5	62.3 ± 34.6
ALT (U/L)	19.3 ± 2.1	22.3 ± 5.9
GGT (U/L)	0.0 ± 0.0	0.0 ± 0.0
Albumin (g/dL)	2.7 ± 0.0	2.8 ± 0.1
Total Bilirubin (mg/dL)	0.2 ± 0.1	0.1 ± 0.1
Total Protein (g/dL)	4.2 ± 0.1	4.5 ± 0.1
Globulin (g/dL)	1.5 ± 0.1	1.7 ± 0.1
BUN (mg/dL)	22.0 ± 2.6	22.0 ± 2.6
Creatinine (mg/dL)	0.1 ± 0.1	0.1 ± 0.0
BUN/Creatinine Ratio	150.0 ± 132.3	220. ± 26.5

Data were presented as mean ± standard deviation. (n=3)

Table S5. Summary of Histopathological Findings.

	PBS-treated			**LNP-treated**		
Parameter	# Abnormal	Mean Group Score	Mean Lesion Score	# Abnormal	Mean Group Score	Mean Lesion Score
Congestion	0	0		1	0.7	2
Mononuclear clot /aggregate	1	0.3	1	3	1	1
Artifact	3	2	2	3	2	2
Sum-Scores:	1	0.3	1	4	1.7	3
No Significant Findings	2			0		

Microscopic changes were graded as to severity utilizing a standard grading system whereby 0 = no significant change, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe. International Harmonization of Nomenclature and Diagnostic (INHAND) Criteria standards are used as the basis of evaluation (https://www.toxpath.org/inhand.asp).