
Supplementary information

Optimization of lipid nanoparticles for the delivery of nebulized therapeutic mRNA to the lungs

In the format provided by the authors and unedited

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a

Group A

Components				Molar Ratio				Lipid:RNA Ratio	N:P Ratio	Included in Study	Diameters	PDI
Lipomer	Cholesterol	PEG	Helper	Lipomer	Cholesterol	PEG	Helper					
7C1	Cholesterol	C14PEG2K	DOPE	60	10	2	28	10	6.8	No	243.1	Multimodal
7C1	Cholesterol	C14PEG2K	DOPE	55	10	2	33	10	6.6	No	271.4	0.552
7C1	Cholesterol	C14PEG2K	DOPE	45	10	2	43	10	6.1	No	242.6	Multimodal
7C1	Cholesterol	C14PEG2K	DOPE	35	10	2	53	10	5.6	No	204.89	0.237
7C1	Cholesterol	C14PEG2K	DOPE	30	10	2	58	10	5.2	No	211.62	0.418
7C1	Cholesterol	C14PEG2K	DOPE	60	10	2	28	10	6.8	No	290.4	0.314
7C1	Cholesterol	C14PEG2K	DOPE	55	10	2	33	10	6.6	No	327.7	Multimodal
7C1	Cholesterol	C14PEG2K	DOPE	45	10	2	43	10	6.1	No	288.98	Multimodal
7C1	Cholesterol	C14PEG2K	DOPE	35	10	2	53	10	5.6	No	281.32	0.469
7C1	Cholesterol	C14PEG2K	DOPE	30	10	2	58	10	5.2	No	615.22	Multimodal

Group B

Components				Molar Ratio				Lipid:RNA Ratio	N:P Ratio	Included in Study	Diameters	PDI
Lipomer	Cholesterol	PEG	Helper	Lipomer	Cholesterol	PEG	Helper					
7C1	Cholesterol	C14PEG2K	DOPE	80	20	0	0	10	7.5	No	885.68	Multimodal
7C1	Cholesterol	C14PEG2K	DOPE	75	25	0	0	10	7.5	No	244.8	Multimodal
7C1	Cholesterol	C14PEG2K	DOPE	70	30	0	0	10	7.4	No	265.8	0.469
7C1	Cholesterol	C14PEG2K	DOPE	65	35	0	0	10	7.3	No	52	Multimodal
7C1	Cholesterol	C14PEG2K	DOPE	60	40	0	0	10	7.2	No	59.1	Multimodal
7C1	Cholesterol	C14PEG2K	DOPE	55	45	0	0	10	7.1	No	128.1	Multimodal
7C1	Cholesterol	C14PEG2K	DOPE	50	50	0	0	10	7.0	No	288.98	Multimodal
7C1	Cholesterol	C14PEG2K	DOPE	45	55	0	0	10	6.8	No	281.32	0.469

Group C

Components				Molar Ratio				Lipid:RNA Ratio	N:P Ratio	Included in Study	Diameters	PDI
Lipomer	Cholesterol	PEG	Helper	Lipomer	Cholesterol	PEG	Helper					
7C1	Cholesterol	C14PEG2K	DOPE	80	0	0	20	10	7.3	No	615.22	Multimodal
7C1	Cholesterol	C14PEG2K	DOPE	75	0	0	25	10	7.2	No	552.4	Multimodal
7C1	Cholesterol	C14PEG2K	DOPE	70	0	0	30	10	7.1	No	885.68	Multimodal
7C1	Cholesterol	C14PEG2K	DOPE	65	0	0	35	10	6.9	No	237.07	Multimodal
7C1	Cholesterol	C14PEG2K	DOPE	60	0	0	40	10	6.8	No	215.74	Multimodal
7C1	Cholesterol	C14PEG2K	DOPE	55	0	0	45	10	6.6	No	262.11	0.537
7C1	Cholesterol	C14PEG2K	DOPE	50	0	0	50	10	6.4	No	608.6	Multimodal
7C1	Cholesterol	C14PEG2K	DOPE	45	0	0	55	10	6.2	No	834.5	Multimodal

b

Group D

Components				Molar Ratio				Lipid:RNA Ratio	N:P Ratio	Included in Study	Diameters	PDI
Lipomer	Cholesterol	PEG	Helper	Lipomer	Cholesterol	PEG	Helper					
7C1	Cholesterol	C14PEG2K	DOTAP	60	10	25	5	10	5.7	No	123.3	Multimodal
7C1	Cholesterol	C14PEG2K	DOTAP	50	23.5	6.5	20	10	6.3	No	133.1	Multimodal
7C1	Cholesterol	C14PEG2K	DOTAP	35	18	2.5	44.5	10	5.7	Yes	161.2	0.408
7C1	Cholesterol	C14PEG2K	DOTAP	20	37.5	15	27.5	10	3.8	Yes	117.3	0.323
7C1	Cholesterol	C14PEG2K	DOTMA	60	10	25	5	10	5.7	Yes	149.4	0.453
7C1	Cholesterol	C14PEG2K	DOTMA	50	23.5	6.5	20	10	6.3	Yes	143.7	0.311
7C1	Cholesterol	C14PEG2K	DOTMA	35	18	2.5	44.5	10	5.7	No	107.3	0.526
7C1	Cholesterol	C14PEG2K	DOTMA	20	37.5	15	27.5	10	3.8	No	130.7	Multimodal
7C1	Cholesterol	C14PEG2K	DDAB	60	10	25	5	10	5.7	Yes	108.6	0.459
7C1	Cholesterol	C14PEG2K	DDAB	50	23.5	6.5	20	10	6.3	Yes	107.4	0.186
7C1	Cholesterol	C14PEG2K	DDAB	35	18	2.5	44.5	10	5.8	No	118.2	Multimodal
7C1	Cholesterol	C14PEG2K	DDAB	20	37.5	15	27.5	10	3.9	Yes	124.8	0.362

Group E

Components				Molar Ratio				Lipid:RNA Ratio	N:P Ratio	Included in Study	Diameters	PDI
Lipomer	Cholesterol	PEG	Helper	Lipomer	Cholesterol	PEG	Helper					
7C1	Cholesterol	C14PEG2K	DOPE	60	5	30	5	10	5.5	Yes	73.6	0.203
7C1	Cholesterol	C14PEG2K	DOPE	55	5	35	5	10	5.1	Yes	123	0.42
7C1	Cholesterol	C14PEG2K	DOPE	45	5	45	5	10	4.3	Yes	166	0.189
7C1	Cholesterol	C14PEG2K	DOPE	35	5	55	5	10	3.5	Yes	91.5	0.428
7C1	Cholesterol	C14PEG2K	DOPE	30	5	60	5	10	3.0	Yes	135.1	0.162
7C1	Cholesterol	C18PEG2K	DOPE	60	5	30	5	10	5.4	Yes	148.1	0.146
7C1	Cholesterol	C18PEG2K	DOPE	55	5	35	5	10	5.1	Yes	62.4	0.226
7C1	Cholesterol	C18PEG2K	DOPE	45	5	45	5	10	4.3	No	278.7	0.457
7C1	Cholesterol	C18PEG2K	DOPE	35	5	55	5	10	3.4	No	224.6	Multimodal
7C1	Cholesterol	C18PEG2K	DOPE	30	5	60	5	10	3.0	No	270.2	Multimodal

Group F

Components				Molar Ratio				Lipid:RNA Ratio	N:P Ratio	Included in Study	Diameters	PDI
Lipomer	Cholesterol	PEG	Helper	Lipomer	Cholesterol	PEG	Helper					
7C1	Cholesterol	C14PEG2K	DOPE	80	0	20	0	10	6.5	Yes	86.9	0.299
7C1	Cholesterol	C14PEG2K	DOPE	75	0	25	0	10	6.2	Yes	107.1	0.411
7C1	Cholesterol	C14PEG2K	DOPE	70	0	30	0	10	5.8	Yes	52	0.319
7C1	Cholesterol	C14PEG2K	DOPE	65	0	35	0	10	5.5	Yes	64.6	0.35
7C1	Cholesterol	C14PEG2K	DOPE	60	0	40	0	10	5.1	Yes	128.7	0.382
7C1	Cholesterol	C14PEG2K	DOPE	55	0	45	0	10	4.8	Yes	105.8	0.448
7C1	Cholesterol	C14PEG2K	DOPE	50	0	50	0	10	4.4	Yes	87.1	0.323
7C1	Cholesterol	C14PEG2K	DOPE	45	0	55	0	10	4.0	No	62.4	0.226

Supplementary Figure 1. (a) Detailed outline of nanoparticles included from groups that failed the quality control analysis (A, B, and C). **(b)** Detailed outline of nanoparticles included from groups that passed the quality control analysis (D, E, and F).

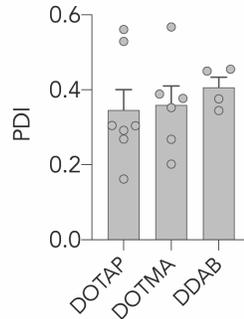
a

Mole Ratio	1	2	3	4	5	6	7	8	9
7C1	60	60	55	45	35	30	35	50	20
PEG	25	30	35	45	55	60	2.5	6.5	15
Cholesterol	10	5	5	5	5	5	18	23.5	37.5
Helper Lipid	5	5	5	5	5	5	44.5	20	27.5

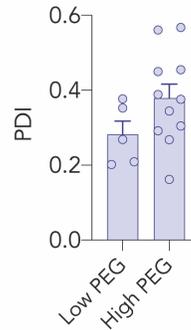
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Components				Molar Ratio				Lipid:RNA Ratio	N:P Ratio	Included in Study	Diameters	PDI
Lipomer	Cholesterol	PEG	Helper	Lipomer	Cholesterol	PEG	Helper					
7C1	Cholesterol	C14PEG2K	DOTAP	60	5	30	5	10	5.5	No	179.6	0.341
7C1	Cholesterol	C14PEG2K	DOTAP	55	5	35	5	10	5.1	Yes	68.5	0.292
7C1	Cholesterol	C14PEG2K	DOTAP	45	5	45	5	10	4.3	Yes	95.2	0.561
7C1	Cholesterol	C14PEG2K	DOTAP	35	5	55	5	10	3.5	Yes	65.5	0.305
7C1	Cholesterol	C14PEG2K	DOTAP	30	5	60	5	10	3.0	No	193.8	Multimodal
7C1	Cholesterol	C14PEG2K	DOTAP	60	10	25	5	10	5.7	Yes	68.8	0.162
7C1	Cholesterol	C14PEG2K	DOTAP	50	23.5	6.5	20	10	6.3	No	173.1	0.304
7C1	Cholesterol	C14PEG2K	DOTAP	35	18	2.5	44.5	10	5.7	Yes	145.5	0.269
7C1	Cholesterol	C14PEG2K	DOTAP	20	37.5	15	27.5	10	3.8	No	149.1	0.529
7C1	Cholesterol	C14PEG2K	DOTMA	60	5	30	5	10	5.5	No	106.9	Multimodal
7C1	Cholesterol	C14PEG2K	DOTMA	55	5	35	5	10	5.1	Yes	141.1	0.568
7C1	Cholesterol	C14PEG2K	DOTMA	45	5	45	5	10	4.3	Yes	103.4	0.389
7C1	Cholesterol	C14PEG2K	DOTMA	35	5	55	5	10	3.5	Yes	69.4	0.268
7C1	Cholesterol	C14PEG2K	DOTMA	30	5	60	5	10	3.0	No	96.2	Multimodal
7C1	Cholesterol	C14PEG2K	DOTMA	60	10	25	5	10	5.7	No	355.7	Multimodal
7C1	Cholesterol	C14PEG2K	DOTMA	50	23.5	6.5	20	10	6.3	Yes	163.2	0.353
7C1	Cholesterol	C14PEG2K	DOTMA	35	18	2.5	44.5	10	5.7	Yes	152.6	0.202
7C1	Cholesterol	C14PEG2K	DOTMA	20	37.5	15	27.5	10	3.8	Yes	81.9	0.377
7C1	Cholesterol	C14PEG2K	DDAB	60	5	30	5	10	5.5	No	60.2	0.412
7C1	Cholesterol	C14PEG2K	DDAB	55	5	35	5	10	5.1	Yes	89.8	0.455
7C1	Cholesterol	C14PEG2K	DDAB	45	5	45	5	10	4.3	Yes	79.5	0.376
7C1	Cholesterol	C14PEG2K	DDAB	35	5	55	5	10	3.5	Yes	72.2	0.45
7C1	Cholesterol	C14PEG2K	DDAB	30	5	60	5	10	3.0	No	81.5	Multimodal
7C1	Cholesterol	C14PEG2K	DDAB	60	10	25	5	10	5.7	Yes	65.4	0.345
7C1	Cholesterol	C14PEG2K	DDAB	50	23.5	6.5	20	10	6.3	No	206	Multimodal
7C1	Cholesterol	C14PEG2K	DDAB	35	18	2.5	44.5	10	5.8	Yes	130	0.209
7C1	Cholesterol	C14PEG2K	DDAB	20	37.5	15	27.5	10	3.9	No	125.5	Multimodal

c



d

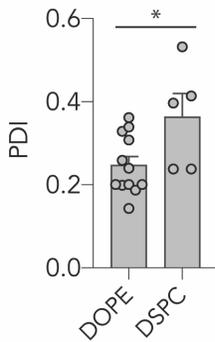


Supplementary Figure 2. (a) Molar ratios of individual LNPs nebulized. **(b)** The first iterations of individually tested LNPs were made of 7C1, cholesterol, C₁₄PEG₂₀₀₀, and positive helper lipids at 9 different molar ratios.

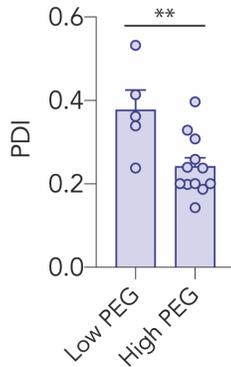
a

Components				Molar Ratio				Lipid:RNA Ratio	N:P Ratio	Included in Study	Diameters	PDI
Lipomer	Cholesterol	PEG	Helper	Lipomer	Cholesterol	PEG	Helper					
7C1	Cholesterol	C14PEG2K	DOPE	60	5	30	5	10	5.5	Yes	45.4	0.199
7C1	Cholesterol	C14PEG2K	DOPE	55	5	35	5	10	5.1	Yes	56.6	0.201
7C1	Cholesterol	C14PEG2K	DOPE	45	5	45	5	10	4.3	Yes	54.2	0.187
7C1	Cholesterol	C14PEG2K	DOPE	35	5	55	5	10	3.5	Yes	50.7	0.308
7C1	Cholesterol	C14PEG2K	DOPE	30	5	60	5	10	3.0	Yes	40.8	0.143
7C1	Cholesterol	C18PEG2K	DOPE	60	5	30	5	10	5.4	No	165.5	Multimodal
7C1	Cholesterol	C18PEG2K	DOPE	55	5	35	5	10	5.1	Yes	45.6	0.201
7C1	Cholesterol	C18PEG2K	DOPE	45	5	45	5	10	4.3	Yes	197.1	0.201
7C1	Cholesterol	C18PEG2K	DOPE	35	5	55	5	10	3.4	Yes	41.7	0.24
7C1	Cholesterol	C18PEG2K	DOPE	30	5	60	5	10	3.0	Yes	53.1	0.259
7C1	Cholesterol	C14PEG2K	DOPE	60	10	25	5	10	5.7	Yes	94.9	0.329
7C1	Cholesterol	C14PEG2K	DOPE	50	23.5	6.5	20	10	6.3	Yes	96.9	0.362
7C1	Cholesterol	C14PEG2K	DOPE	35	18	2.5	44.5	10	5.6	No	209.4	0.527
7C1	Cholesterol	C14PEG2K	DOPE	20	37.5	15	27.5	10	3.8	No	50.6	0.18
7C1	Cholesterol	C18PEG2K	DOPE	60	10	25	5	10	5.7	No	64.9	0.218
7C1	Cholesterol	C18PEG2K	DOPE	50	23.5	6.5	20	10	6.2	No	475.9	0.241
7C1	Cholesterol	C18PEG2K	DOPE	35	18	2.5	44.5	10	5.6	No	103.2	Multimodal
7C1	Cholesterol	C18PEG2K	DOPE	20	37.5	15	27.5	10	3.7	Yes	72.5	0.339
7C1	Cholesterol	C14PEG2K	DSPC	60	10	25	5	10	5.7	Yes	52.2	0.397
7C1	Cholesterol	C14PEG2K	DSPC	50	23.5	6.5	20	10	6.2	No	254.8	0.466
7C1	Cholesterol	C14PEG2K	DSPC	35	18	2.5	44.5	10	5.6	No	442.2	Multimodal
7C1	Cholesterol	C14PEG2K	DSPC	20	37.5	15	27.5	10	3.7	Yes	93.3	0.414
7C1	Cholesterol	C18PEG2K	DSPC	60	10	25	5	10	5.7	Yes	52.4	0.238
7C1	Cholesterol	C18PEG2K	DSPC	50	23.5	6.5	20	10	6.2	Yes	110.6	0.532
7C1	Cholesterol	C18PEG2K	DSPC	35	18	2.5	44.5	10	5.5	No	614.6	Multimodal
7C1	Cholesterol	C18PEG2K	DSPC	20	37.5	15	27.5	10	3.7	Yes	77.4	0.238

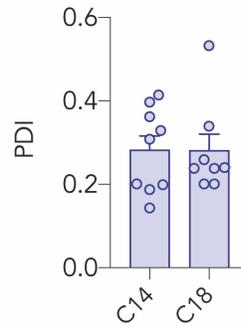
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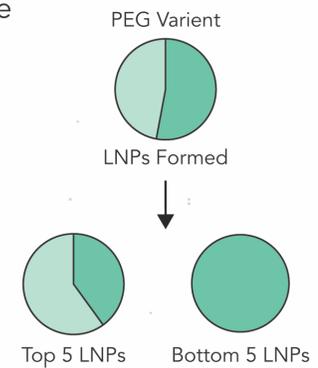
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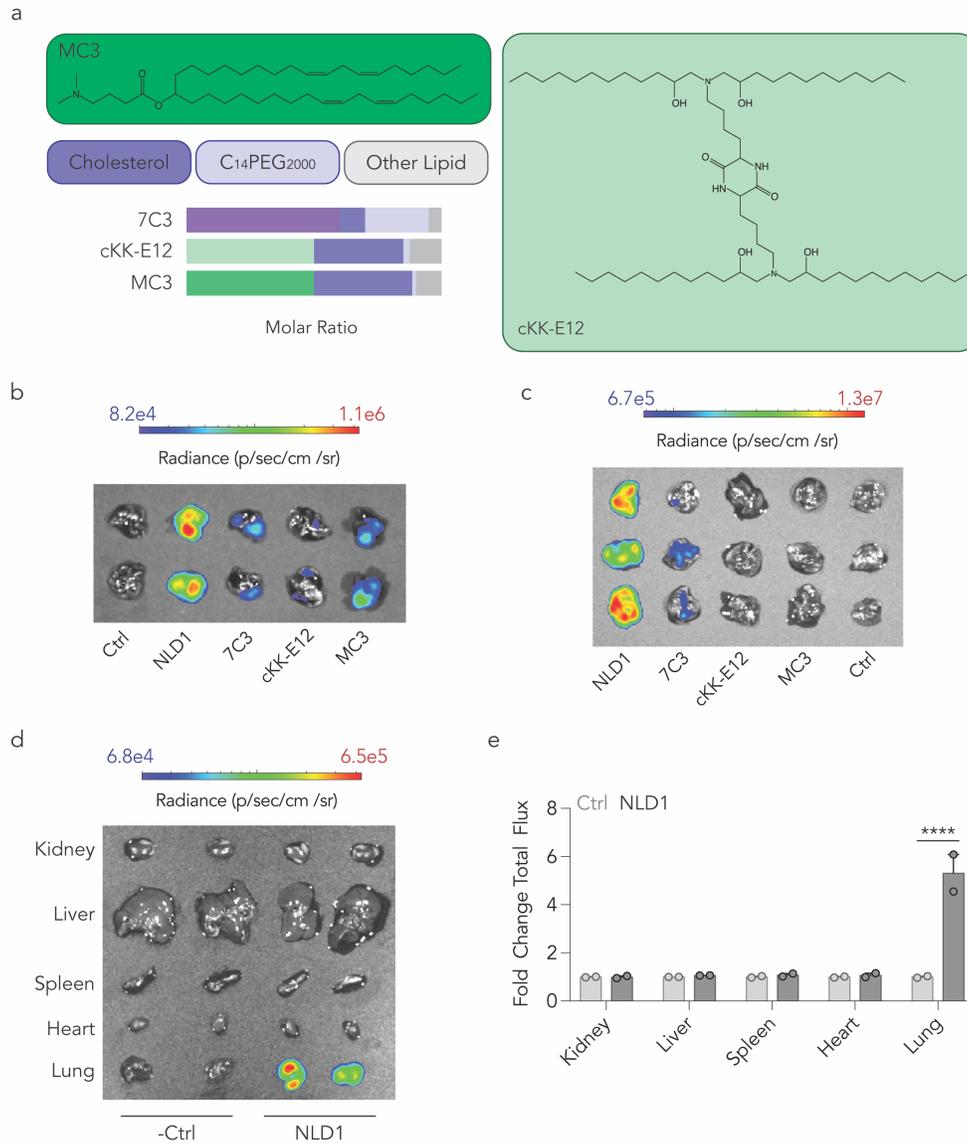
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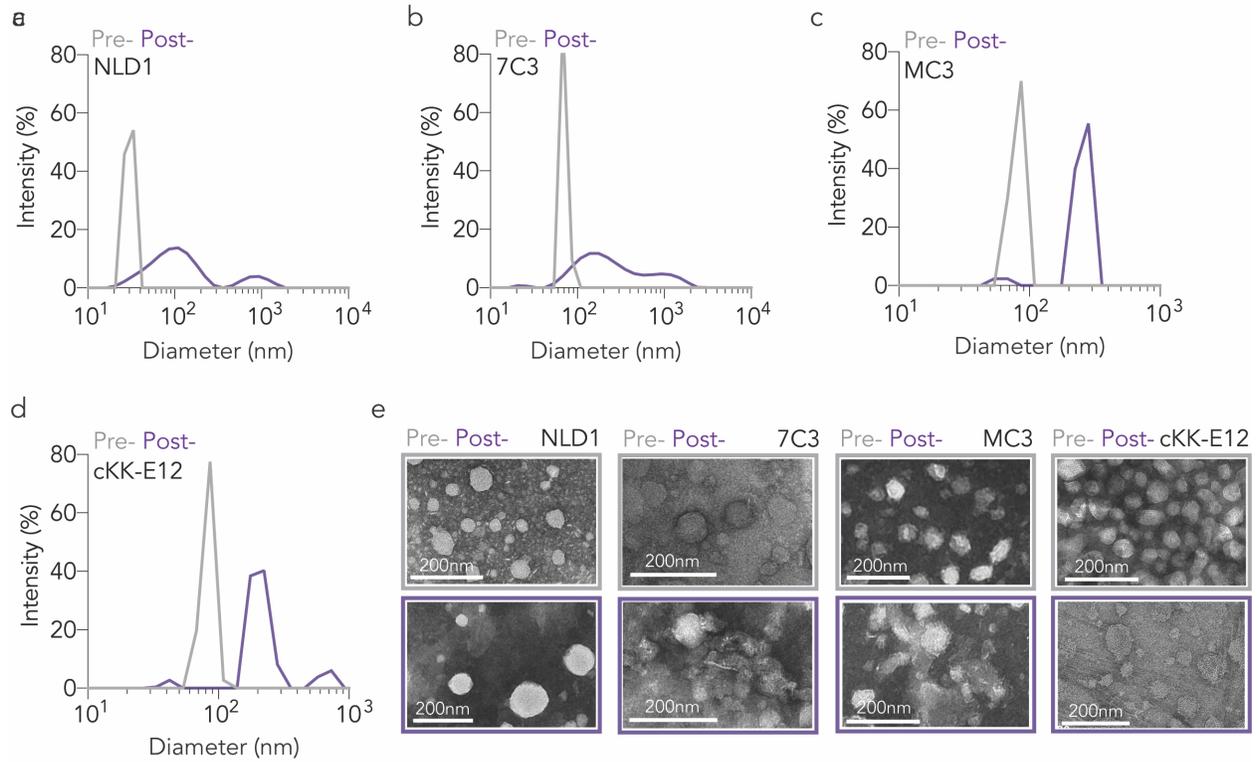
e



Supplementary Figure 3. (a) The second iterations of individually tested LNPs were comprised of 7C1, cholesterol, C₁₄PEG₂₀₀₀, C₁₈PEG₂₀₀₀, and neutral helper lipids at 9 different molar ratios. **(b-d)** LNP PDI as a function of helper lipid, PEG amount, and PEG type, respectively. **(b)** **P* = 0.027, **(c)** ***P* = 0.0077, two-tailed, *t*-test. **(e)** PEG tail length found in the best and worst performing LNPs ranked by lung luminescence.

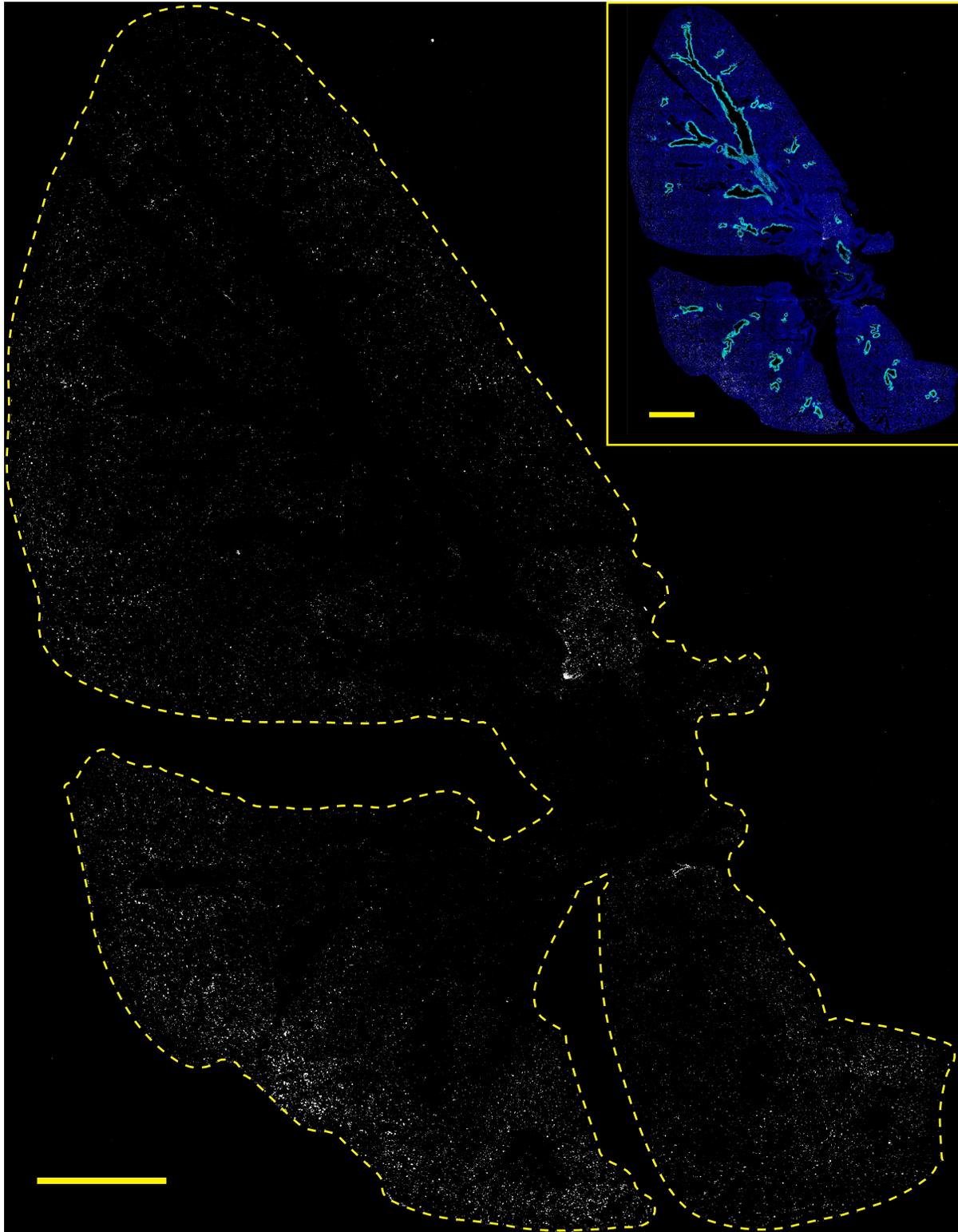


Supplementary Figure 4. (a) 7C3, cKK-E12, and MC3 were formulated by combining the ionizable material 7C3, cKK-E12, or MC3, C₁₄PEG₂₀₀₀, cholesterol, and a neutral phospholipid. LNPs were administered via nebulization to lungs and (b,c) luciferase luminescence was measured. (d,e) 48 hours post nebulization with NLD1, luciferase luminescence was measured in kidney, liver, spleen, heart, and lungs; we only found measurable luminescence in the lungs. **** $P < 0.0001$, One-way ANOVA, average \pm SEM, $n=2$ mice/group, biological replicates shown.

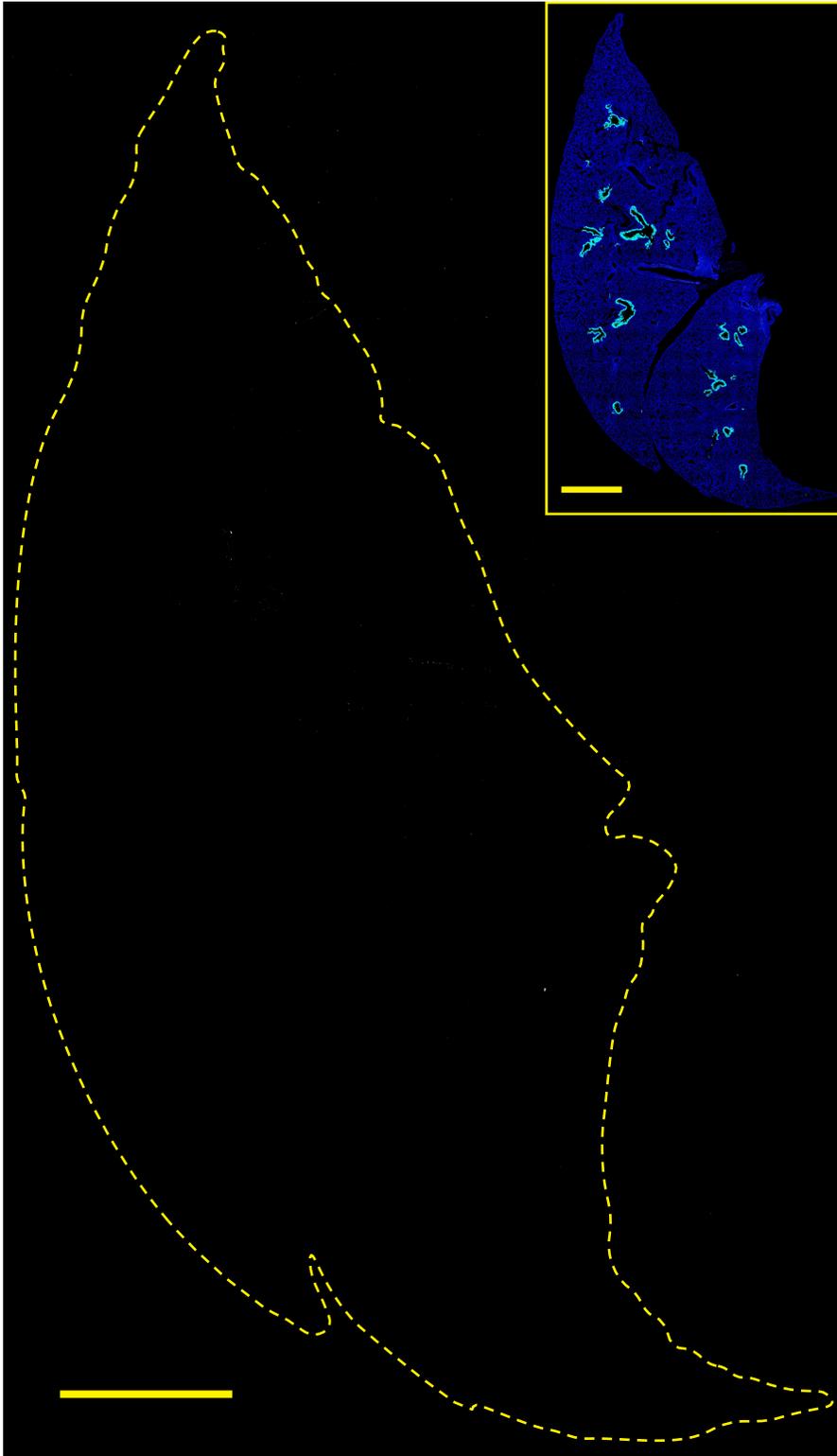


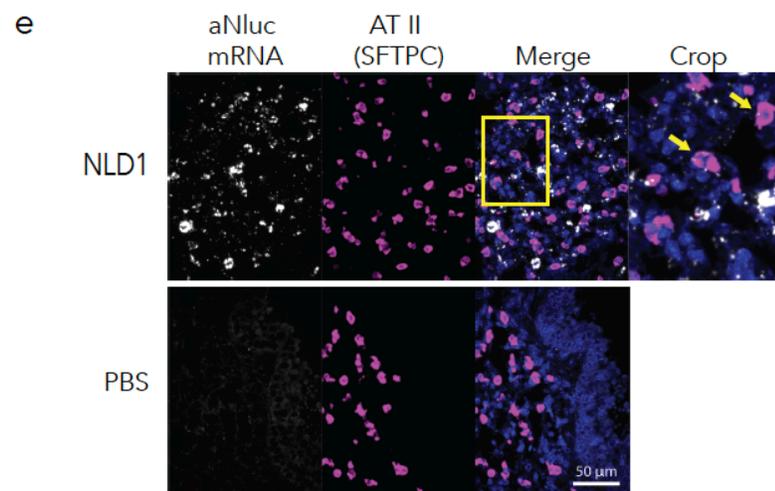
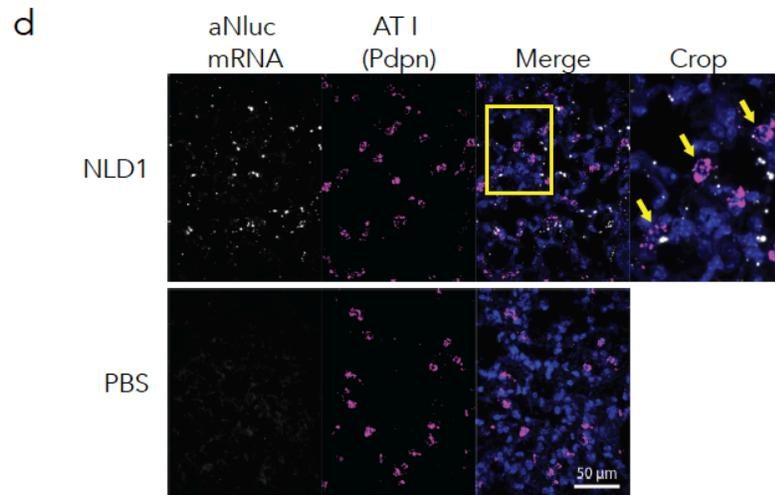
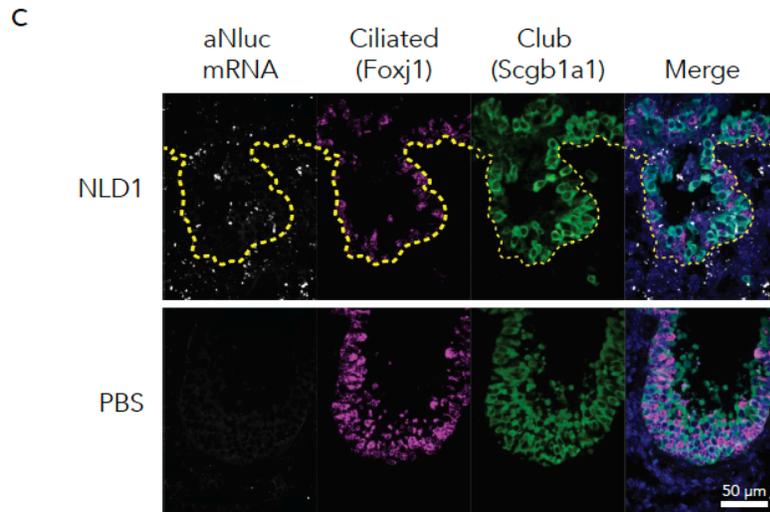
Supplementary Figure 5. (a) LNP diameter pre- and post-nebulization of NLD1, **(b)** 7C3, **(c)** MC3, and **(d)** cKK-E12. **(e)** TEM image of NLD1, 7C3, MC3, and cKK-E12 pre- and post-nebulization.

a

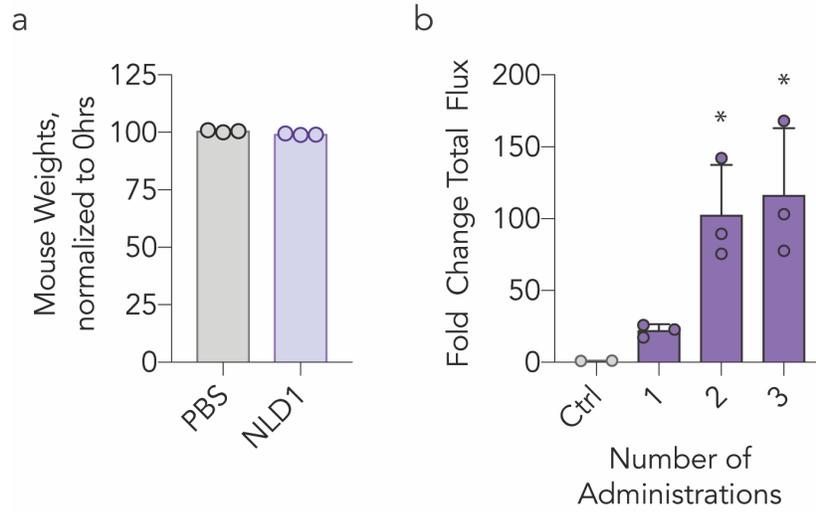


b



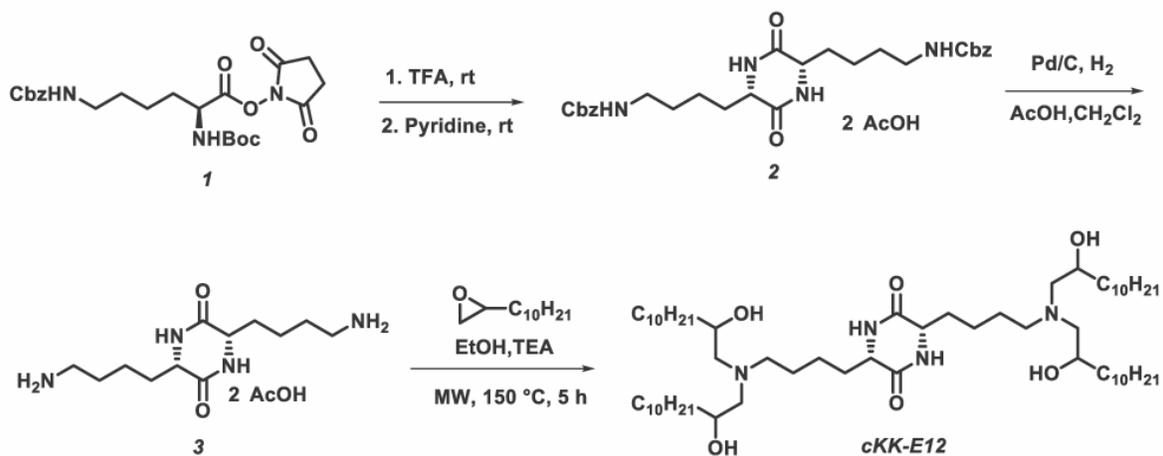


Supplementary Figure 6. (a) Biodistribution of AncNanoLuc mRNA throughout the lung of a mouse 4 hrs post nebulization with 100 µg mRNA. Small inset shows a lung section stained for Scgb1a1 mRNA to mark the bronchial airways (green), AncNanoLuc mRNA (white), and DAPI (blue). The expanded image shows only AncNanoLuc mRNA (white) with an outline of the lung section. The AncNanoLuc mRNA displays a punctate distribution with higher densities toward the periphery of the lung. (b) Negative control lung section treated with AncNanoLuc probes 4 hrs post nebulization with 1x PBS. Small inset shows a lung section stained for Scgb1a1 mRNA to mark the bronchial airways (green), AncNanoLuc mRNA (white), and DAPI (Blue). The expanded image shows only AncNanoLuc mRNA (white) with an outline of the lung section. No major background of the AncNanoLuc mRNA probes was observed in the PBS section. (c) FISH analysis for AncNanoLuc mRNA uptake in epithelial cell subtypes. Tissues were obtained 4 hours post nebulization with either 100 µg of AncNano-Luc mRNA in NLD-1 or 1x PBS and processed for FISH analysis. Probes against AncNanoLuc mRNA (white) show strong staining in treated tissues. Probes against Foxj1 mRNA marking ciliated bronchial epithelial cells (magenta) and Scgb1a1 mRNA marking bronchial club cells (green) were used to visualize the bronchiolar space. Uptake of AncNanoLuc mRNA was observed in these bronchial epithelial cells. (d,e) Probes against Pdpn mRNA and Sftpc mRNA were used to mark alveolar type I and II epithelial cells, respectively (magenta). Uptake of AncNanoLuc mRNA can be seen in both of these cell types as indicated by the yellow arrows. Scale bars represent 1mm.



Supplementary Figure 7. (a) Mice showed no significant weight loss after exposure to 50 μg of NLD1 to carry AncNanoLuc mRNA via nebulization. **(b)** NLD1 was readministered at a dose of 20 μg / mouse. * $P = 0.037$, * $P = 0.020$, One-way ANOVA, average \pm SEM, $n=3$ mice/group, biological replicates shown.

a



Supplementary Figure 8. (a) Chemical synthesis of cKK-E12.