

1 *Supplementary Material*

2 **Aerosolizable Lipid Nanoparticles for Pulmonary**  
 3 **Delivery of mRNA through Design of Experiments**

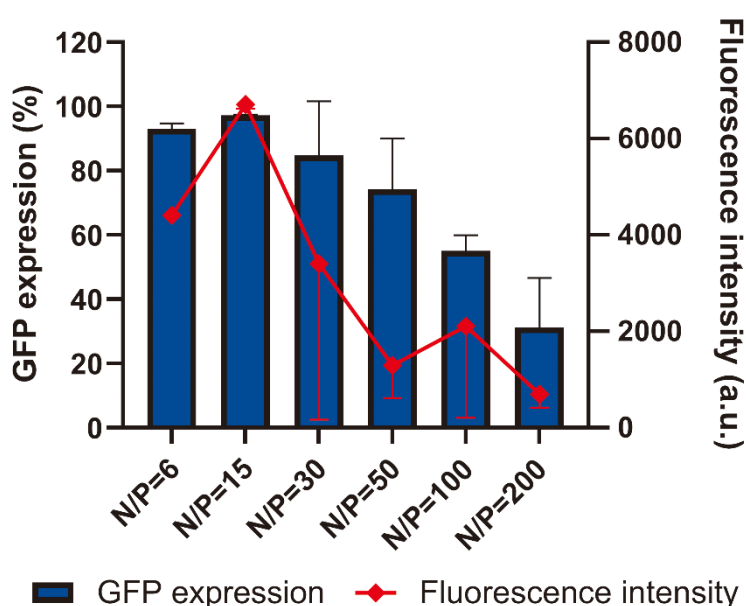
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11 **Figure S1:** Intracellular uptake of LNP formulations at different N/P ratios in HEK-293 cells measured  
 12 by percent GFP expression (left axis) and fluorescence intensity (right axis).

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14 **Table S1:** Limits of experimental design space

Component	Lower limit (molar ratio)	Upper limit (molar ratio)
Ionizable lipid	0.4	0.6
Phospholipid	0.1	0.2
PEG-lipid	0.01	0.05
Cholesterol	0.15	0.49

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17 **Table S2:** LNP compositions for 18 formulations.

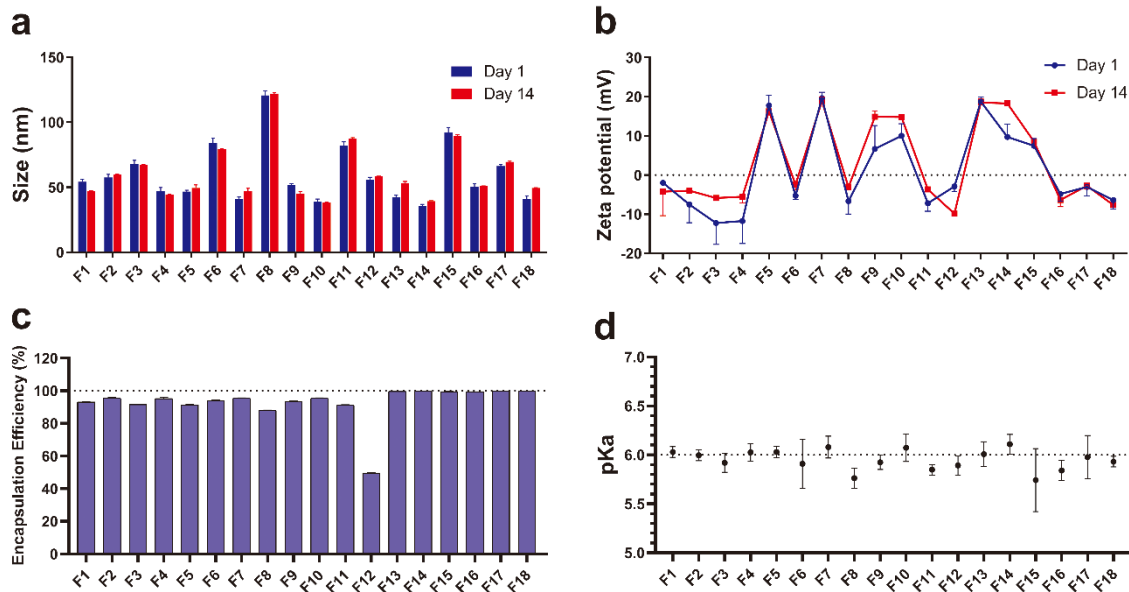
Formulation #	Phospholipid	PEG-lipid	Molar composition			
			Dlin-MC3-DMA	Phospholipid	PEG-lipid	Cholesterol
1	DOPE	DMG-PEG	0.6	0.2	0.05	0.15
2	DOPE	DMPE-PEG	0.4	0.2	0.01	0.39
3	DSPC	DMG-PEG	0.5	0.14	0.01	0.35
4	DOPE	DMPE-PEG	0.6	0.15	0.03	0.22
5	DSPC	DSPE-PEG	0.4	0.2	0.05	0.35
6	DPPC	DMG-PEG	0.4	0.1	0.01	0.49
7	DPPC	DSPE-PEG	0.4	0.1	0.05	0.45
8	DPPC	DMG-PEG	0.6	0.2	0.01	0.19
9	DOPE	DSPE-PEG	0.6	0.1	0.05	0.25
10	DOPE	DSPE-PEG	0.4	0.2	0.03	0.37
11	DPPC	DMPE-PEG	0.6	0.2	0.01	0.19
12	DSPC	DMG-PEG	0.6	0.2	0.05	0.15
13	DSPC	DSPE-PEG	0.5	0.1	0.05	0.35
14	DOPE	DSPE-PEG	0.4	0.15	0.05	0.4
15	DPPC	DSPE-PEG	0.6	0.1	0.01	0.29
16	DSPC	DMPE-PEG	0.5	0.2	0.03	0.27
17	DOPE	DMG-PEG	0.4	0.16	0.01	0.43
18	DPPC	DMPE-PEG	0.4	0.1	0.03	0.47

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19 **Table S3:** PDI of pre-nebulized LNP at Day 1 and Day 14, and nebulized LNP.

Formulation #	PDI		
	Pre-nebulized LNP - Day 1	Pre-nebulized LNP - Day 14	Nebulized LNP
1	0.261 ± 0.005	0.278 ± 0.022	0.245 ± 0.051
2	0.093 ± 0.009	0.128 ± 0.009	0.232 ± 0.024
3	0.080 ± 0.004	0.101 ± 0.004	0.268 ± 0.005
4	0.161 ± 0.006	0.172 ± 0.014	0.293 ± 0.008
5	0.205 ± 0.006	0.365 ± 0.151	0.591 ± 0.076
6	0.035 ± 0.029	0.042 ± 0.015	0.366 ± 0.058
7	0.241 ± 0.010	0.486 ± 0.029	0.425 ± 0.017
8	0.041 ± 0.002	0.045 ± 0.026	0.128 ± 0.009
9	0.199 ± 0.013	0.208 ± 0.033	0.402 ± 0.050
10	0.155 ± 0.017	0.197 ± 0.008	0.507 ± 0.062
11	0.058 ± 0.012	0.082 ± 0.010	0.235 ± 0.005
12	0.111 ± 0.015	0.188 ± 0.008	0.220 ± 0.008
13	0.149 ± 0.005	0.429 ± 0.025	0.668 ± 0.131
14	0.240 ± 0.006	0.343 ± 0.010	0.877 ± 0.114
15	0.057 ± 0.017	0.142 ± 0.005	0.575 ± 0.090
16	0.115 ± 0.010	0.157 ± 0.003	0.231 ± 0.013
17	0.121 ± 0.005	0.135 ± 0.009	0.213 ± 0.005
18	0.214 ± 0.007	0.366 ± 0.008	0.310 ± 0.025

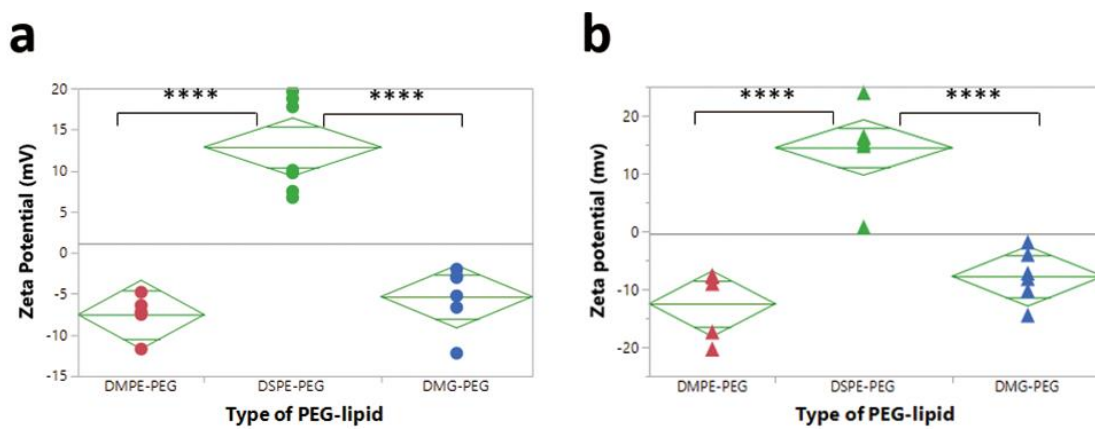
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22 **Figure S2:** Characterization of LNP formulations. (a) size, (b) zeta-potential, (c) encapsulation  
 23 efficiency, and (d) pKa. Stability of the lipid nanoparticles was evaluated by measuring size and  
 24 zeta-potential at day 1 and after 14 days from preparation and storage at 4 °C. (mean ± SD, n=3).

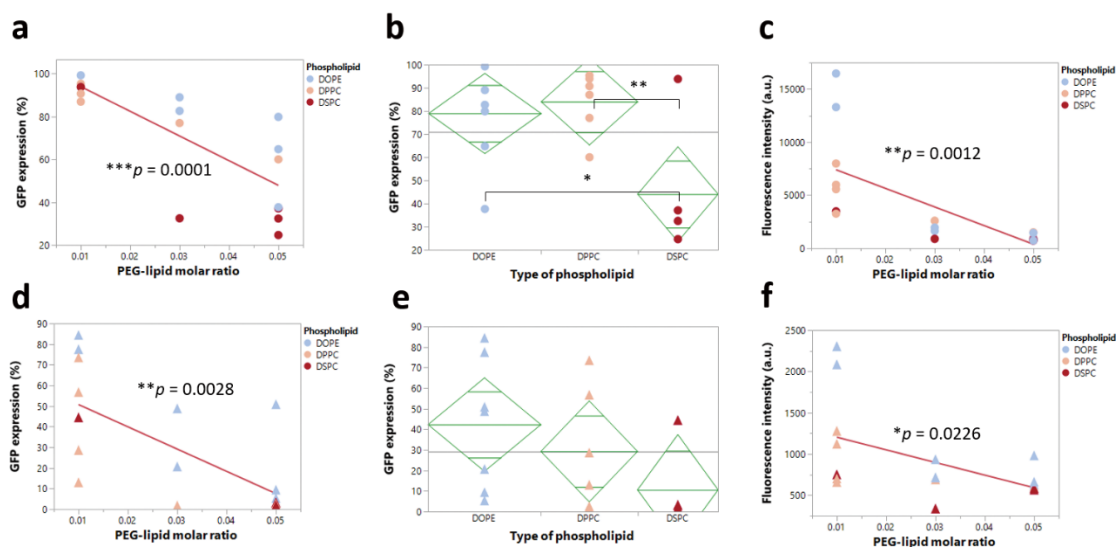
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27 **Figure S3:** Significant effects of type of PEG-lipid on zeta potential before (a) and after (b)  
 28 nebulization.

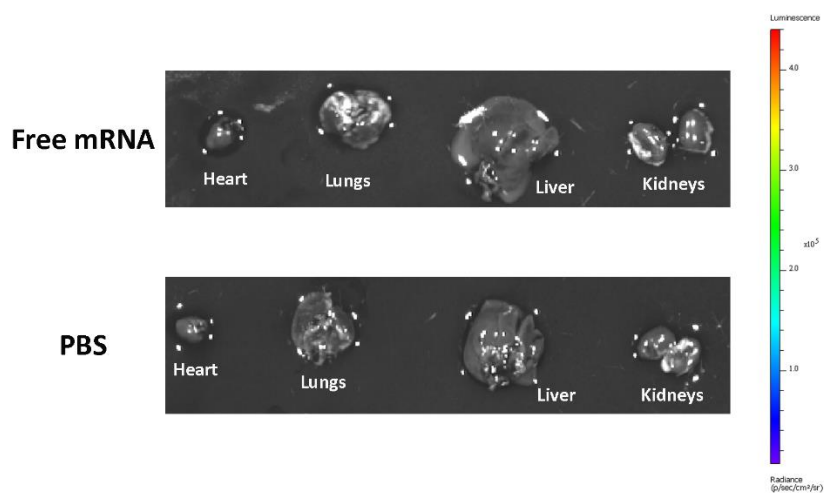
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31 **Figure S4:** Correlation analysis between intracellular uptake (percent GFP expression and  
 32 fluorescence intensity) and PEG-lipid molar ratio or type of phospholipid in NuLi-1 cells. Significant  
 33 effect of PEG-lipid molar ratio (a) and type of phospholipid (b) on the percent GFP expression before  
 34 nebulization. (c) Significant effect of PEG-lipid molar ratio on the fluorescence intensity before  
 35 nebulization. Significant effect of PEG-lipid molar ratio (d) and no significant effect of type of  
 36 phospholipid (e) on the percent GFP expression after nebulization. (f) Significant effect of PEG-lipid  
 37 molar ratio on fluorescence intensity after nebulization.

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40 **Figure S5:** Representative images of the luciferase expression in lungs, heart, liver, and kidneys in  
 41 negative control groups measured by IVIS imaging.