SUPPLEMENTARY INFORMATION:

RNA Lipid Nanoparticles Stabilized During Nebulization Through Excipient Selection

SUPPLEMENTARY FIGURES

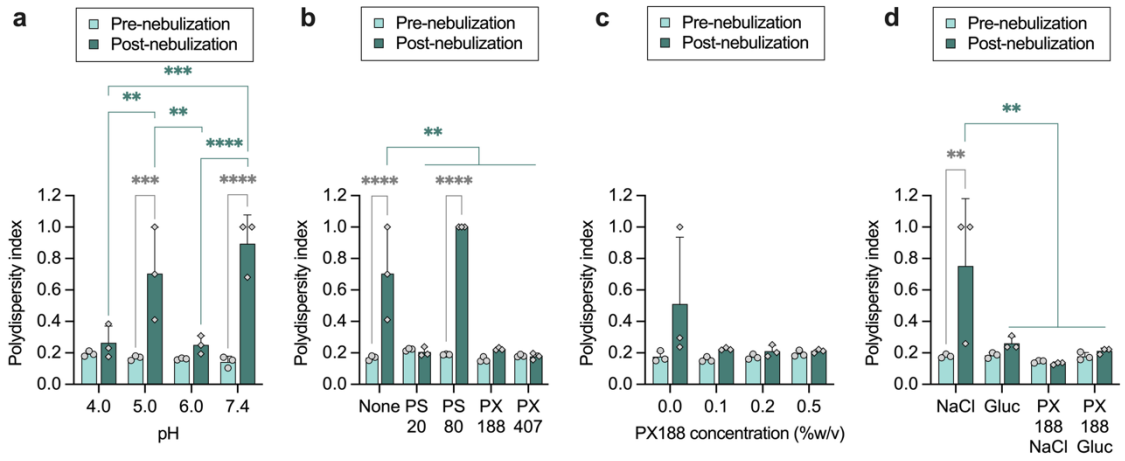

Figure S1. Polydispersity indices measured by dynamic light scattering for **a** LNP nebulization in 20 mM citrate (pH 4.0, 5.0, or 6.0) or 20 mM phosphate (pH 7.4) buffers, **b** LNP nebulization in 20 mM citrate buffer (pH 5) with varying surfactant polymers (0.1% w/v; values for "none" replotted from panel **a** for comparison), **c** LNP nebulization in 20 mM citrate buffer (pH 5) with varying concentrations of poloxamer 188 (PX188; values for 0.1% w/v replotted from panel **b**), and **d** LNP nebulization in 20 mM citrate buffer (pH 5.0) with varying iso-osmotic solutions (300 mOsm/L of sodium chloride (NaCl) or glucose (Gluc)) (n=3, mean ± standard deviation, ordinary two-way ANOVA, comparisons in gray made between pre-nebulization and post-nebulization conditions (Sidak's post hoc test), comparisons in teal made for post nebulization conditions between each buffer (**a,d** comparison between all groups, Tukey's post hoc test; **b,c** comparison between no excipients and each buffer, Dunnett's post hoc test), **p<0.01, ***p<0.001, ****p<0.0001, polydispersity indices measured as "multimodal" were assigned a value of 1.0).

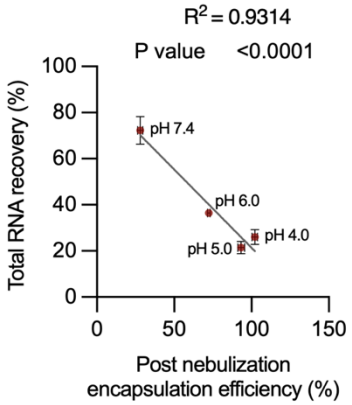

Figure S2. Increased total RNA recovery (%) correlates with reduced post-nebulization encapsulation efficiency (%) at different buffer pH (n=3, mean ± standard deviation, values reproduced from Figure 2**b**,**c**, simple linear regression with p value for test for non-zero slope).

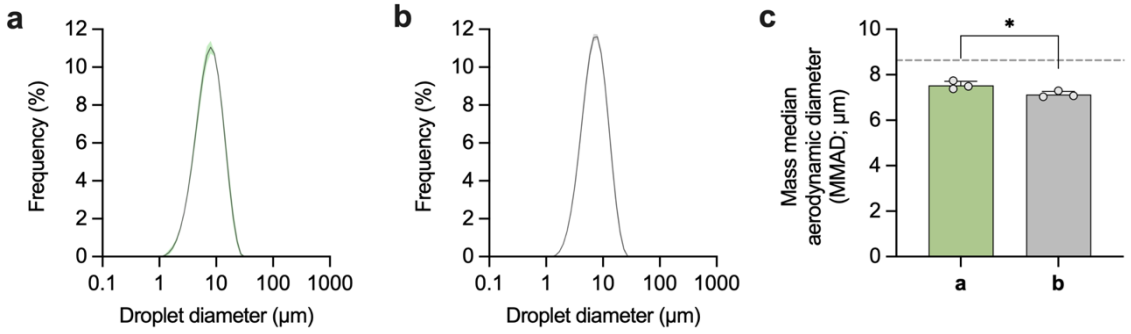

Figure S3. Droplet size measurement distributions for nebulized solutions of LNPs in 20 mM citrate (pH 5.0) with 5% (w/v) glucose and **a** without or **b** with 0.2% PX188 (n=3, mean with error envelope). **c** Calculated mass median aerodynamic diameters from distributions in **a** and **b** with grey dashed line indicating measurement of nebulized water (n=3, mean ± standard deviation, unpaired two-tailed t-test, *p<0.05).

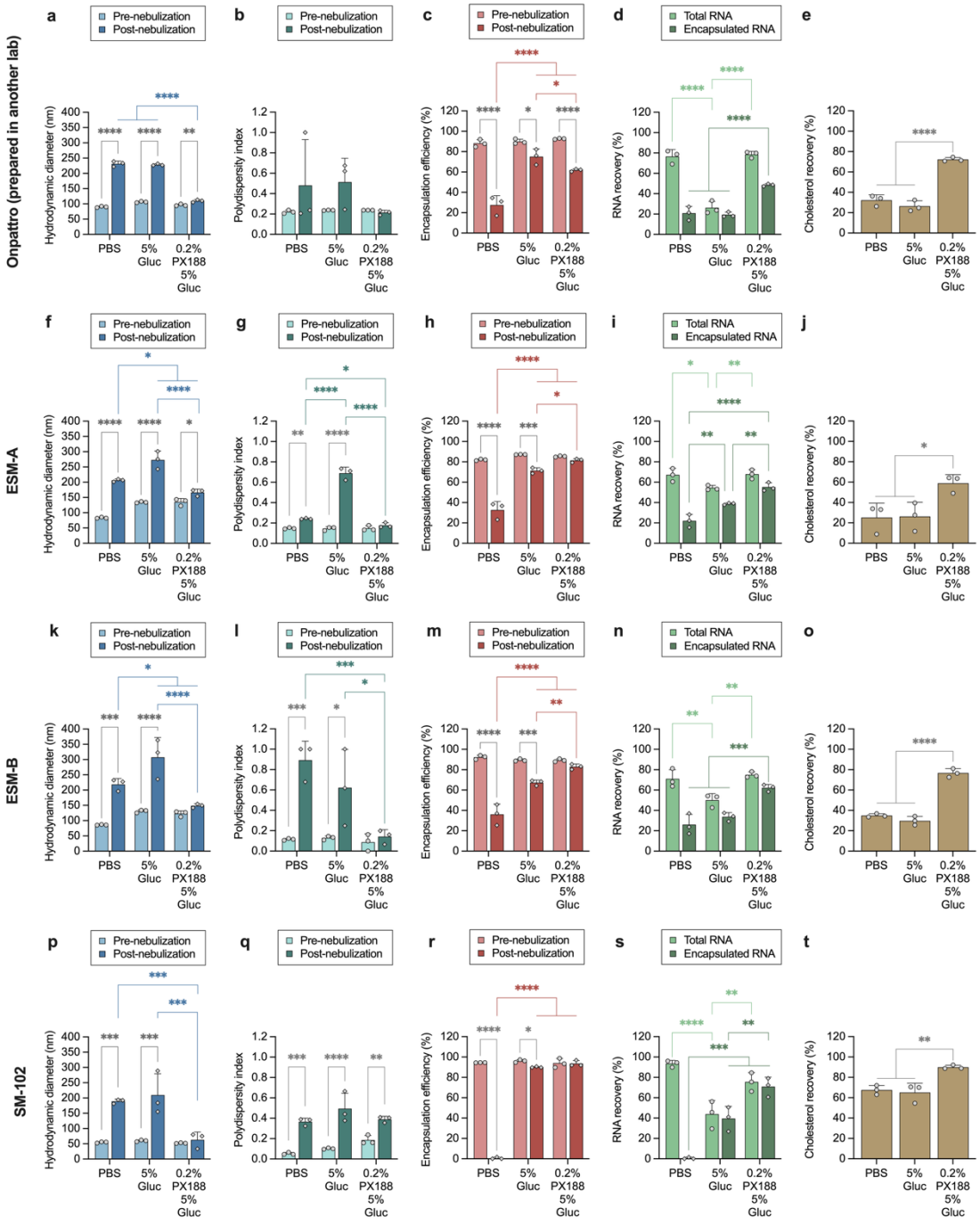

Figure S4. Optimal stabilization conditions are also effective for multiple types of LNPs encapsulating siFLuc. Measurement of **a** hydrodynamic diameter, **b** polydispersity index, **c** encapsulation efficiency, **d** total and encapsulated RNA recovery and **e** cholesterol recovery for Onpattro LNP (prepared in another laboratory) nebulization in various buffers. Measurement of **f** hydrodynamic diameter, **g** polydispersity index, **h** encapsulation efficiency, **i** total and encapsulated RNA recovery and **j** cholesterol recovery for ESM-A LNP nebulization in various buffers. Measurement of **k** hydrodynamic diameter, **l** polydispersity index, **m** encapsulation efficiency, **n** total and encapsulated RNA recovery and **o** cholesterol recovery for ESM-B LNP nebulization in various buffers. Measurement of **p** hydrodynamic diameter, **q** polydispersity index, **r** encapsulation efficiency, **s** total and encapsulated RNA recovery and **t** cholesterol recovery for SM-102 LNP nebulization in various buffers. (**a,b,c,d,f,g,h,i,k,l,m,n,p,q,r,s** – n=3, mean ± standard deviation, ordinary two-way ANOVA, comparisons in gray made between pre- and post-nebulization conditions (Sidak's post hoc test),

comparisons in blue, teal, or red made for post-nebulization conditions between each buffer (Tukey's post hoc test), comparisons in light green made between total RNA recovery measurements and comparisons in dark green made between encapsulated RNA recovery measurements (Tukey's post hoc test), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, polydispersity indices measured as "multimodal" were assigned a value of 1.0. **e,j,o,t** – n=3, mean ± standard deviation, ordinary one-way ANOVA with Tukey's post hoc test, comparison made between all groups and no excipients, *p<0.05, **p<0.01, ****p<0.0001).

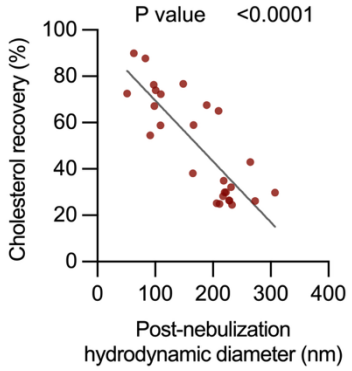

Figure S5. Increased post-nebulization LNP hydrodynamic diameter correlates with reduced cholesterol recovery (n=3, mean ± standard deviation, values reproduced from Figures 2–4 and Figure S4, simple linear regression with p value for test for non-zero slope).

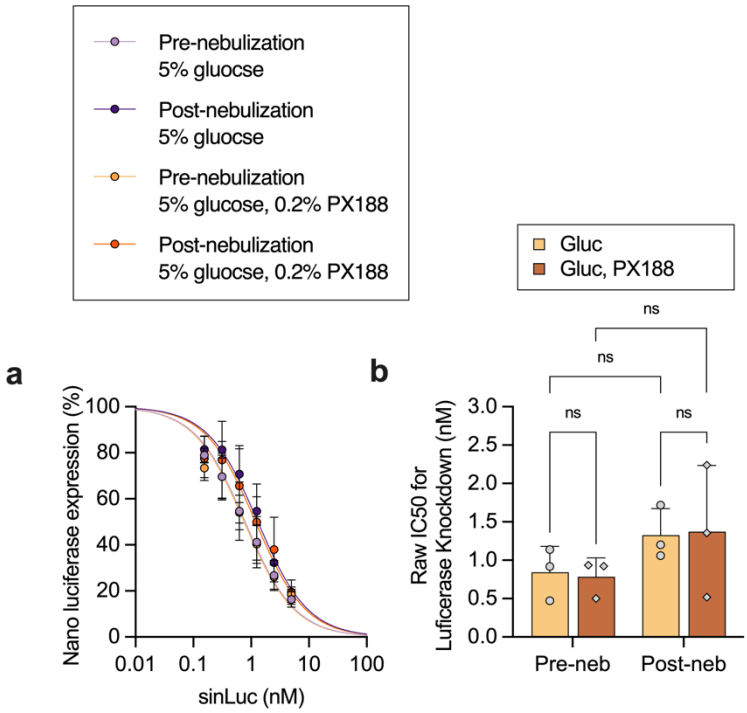

Figure S6. Dose-response curves to determine IC_{50} values of nano-luciferase knockdown. **a** Expression of nano-luciferase in Vero-nLucP cells after treatment with varying LNP concentrations in terms of encapsulated sinLuc. **b** Raw IC_{50} values for nLuc knockdown in Vero cells for LNPs nebulized in citrate buffer with 5% glucose (Gluc) with or without 0.2% poloxamer 188 (PX188; n=3, mean ± standard deviation, ordinary two-way ANOVA with Sidak's post hoc test, ns = not significant).

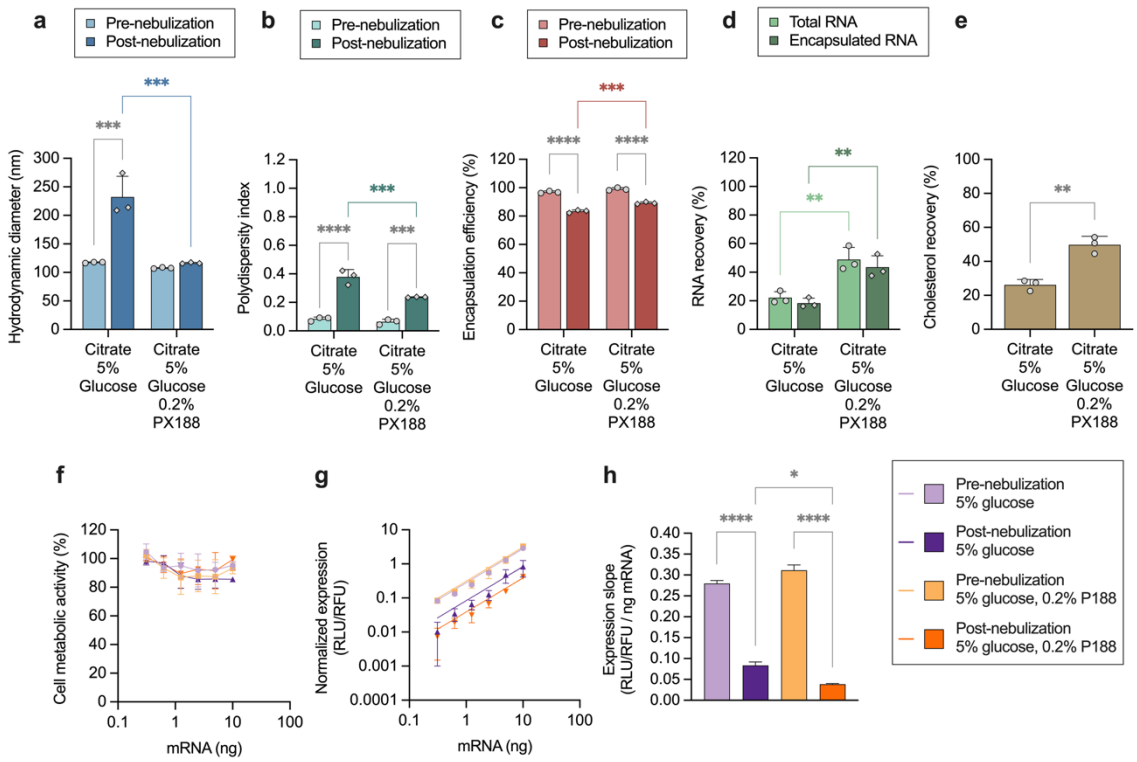

Figure S7. LNPs encapsulating mFLuc are stabilized during nebulization in optimal formulation conditions and result in FLuc expression in epithelial cells following treatment. Measurement of **a** hydrodynamic diameter, **b** polydispersity index, **c** encapsulation efficiency, **d** total and encapsulated RNA recovery and **e** cholesterol recovery for LNP nebulization in 20 mM citrate, pH 5.0 with 5% w/v glucose with or without 0.2% w/v poloxamer 188. **f** Cellular metabolic activity, **g** normalized FLuc expression and **h** slope of expression vs. mRNA dose plot from **g** for Vero-nLucP cells treated with LNPs before or after nebulization. (**a,b,c,d** – n=3, mean ± standard deviation, ordinary two-way ANOVA with Sidak's post hoc test, comparisons in grey between pre- and post-nebulization conditions, comparisons in blue, teal, and red made for post-nebulization conditions between buffers, comparisons in light green made between total RNA recovery measurements and comparisons in dark green made between encapsulated RNA recovery measurements, **p<0.01, ***p<0.001, ****p<0.0001, **e** – n=3, mean ± standard deviation, unpaired two-tailed t-test, **p<0.01, **h** – n=3, mean ± standard deviation, ordinary one-way ANOVA with Tukey's post hoc test, comparison between all groups, *p<0.05, ****p<0.0001).

SUPPLEMENTARY TABLES

Table S1. siRNA sequences used in LNP formulations.

Name	Target	Sequence
sinLuc	Nano luciferase	5'-GGAUUGUCCUGAGCGGUGAdTdT-3' 5'-UCACCGCUCAGGACAAUCCdTdT-3'
siFLuc	Firefly luciferase	5'-mGmGUmUCmCUGGAAmCAmAUmUGmCUUUUAmCdA-3' 5'-UGmUAAAAGmCAmAUmUGUUCCAGGAmACmCmAmG-3'

*where “d” denotes a DNA base and “m” indicates a 2’-O-methyl RNA base

Table S2. Characterization of LNP formulations used in nebulization experiments.

ID#	Name	**Lipid ratio** (Ionizable lipid: cholesterol: phospholipid: DMG-PEG)	Ionizable lipid	Phospholipid	RNA	N/P	Formulation buffer	Z-average diameter (nm)	PDI	Encapsulation efficiency (%)	Figures
1	Onpattro	50:38.5:10:1.5	MC3	DSPC	sinLuc	3	Acetate (25 mM)	71.8	0.129	93.9	2-5, S1-3,S5-6
2	Onpattro	50:38.5:10:1.5	MC3	DSPC	siFLuc	3	Acetate (25 mM)	81.3	0.224	88.2	S4
3	ESM-A	33:25.5:40:1.5	MC3	ESM	siFLuc	3	Acetate (25 mM)	76.4	0.113	82.0	S4
4	ESM-B	20:38.5:40:1.5	MC3	ESM	siFLuc	3	Acetate (25 mM)	80.0	0.098	92.3	S4
5	SM-102	50:38.5:10:1.5	SM-102	DSPC	siFLuc	6	Acetate (25 mM)	53.8	0.044	90.1	S4
6	SM-102	50:38.5:10:1.5	SM-102	DSPC	siFLuc	6	Acetate (25 mM)	77.3	0.093	94.4	S4
7	Onpattro	50:38.5:10:1.5	MC3	DSPC	mFLuc	6	Citrate (50 mM)	101.4	0.021	94.2	S7

Table S3. List of nebulization buffers tested.

ID#	Buffer	Concentration	pH	Additives	Figures
1	Citrate	20 mM	4.0	None	2,S1
2	Citrate	20 mM	5.0	None	2,3,S1
3	Citrate	20 mM	6.0	None	2,S1
4	Phosphate	20 mM	7.4	None	2,S1
5	Citrate	20 mM	5.0	0.1% w/v PS20	3,S1
6	Citrate	20 mM	5.0	0.1% w/v PS80	3,S1
7	Citrate	20 mM	5.0	0.1% w/v PX188	3,S1
8	Citrate	20 mM	5.0	0.1% w/v PX407	3,S1
9	Citrate	20 mM	5.0	0.2% w/v PX188	3,S1
10	Citrate	20 mM	5.0	0.5% w/v PX188	3,S1
11	Citrate	20 mM	5.0	300 mOsm/L NaCl	4,S1
12	Citrate	20 mM	5.0	300 mOsm/L Gluc	4,5,S1,S3-4,S6-7
13	Citrate	20 mM	5.0	300 mOsm/L NaCl, 0.2% w/v PX188	4,S1
14	Citrate	20 mM	5.0	300 mOsm/L Gluc, 0.2% w/v PX188	4,5,S1,S3-4,S6-7
15	Phosphate	11.8 mM	7.4	137 mM NaCl, 2.7 mM KCl	S4