## Supplementary Information Appendix

# Optimization of phospholipid chemistry for improved lipid nanoparticle (LNP) delivery of messenger RNA (mRNA)

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## Supporting Information Figures

**A**

### RNA Encapsulation

**B**

### Zeta Potential (mV)

**C**

### Z-Average (nm)

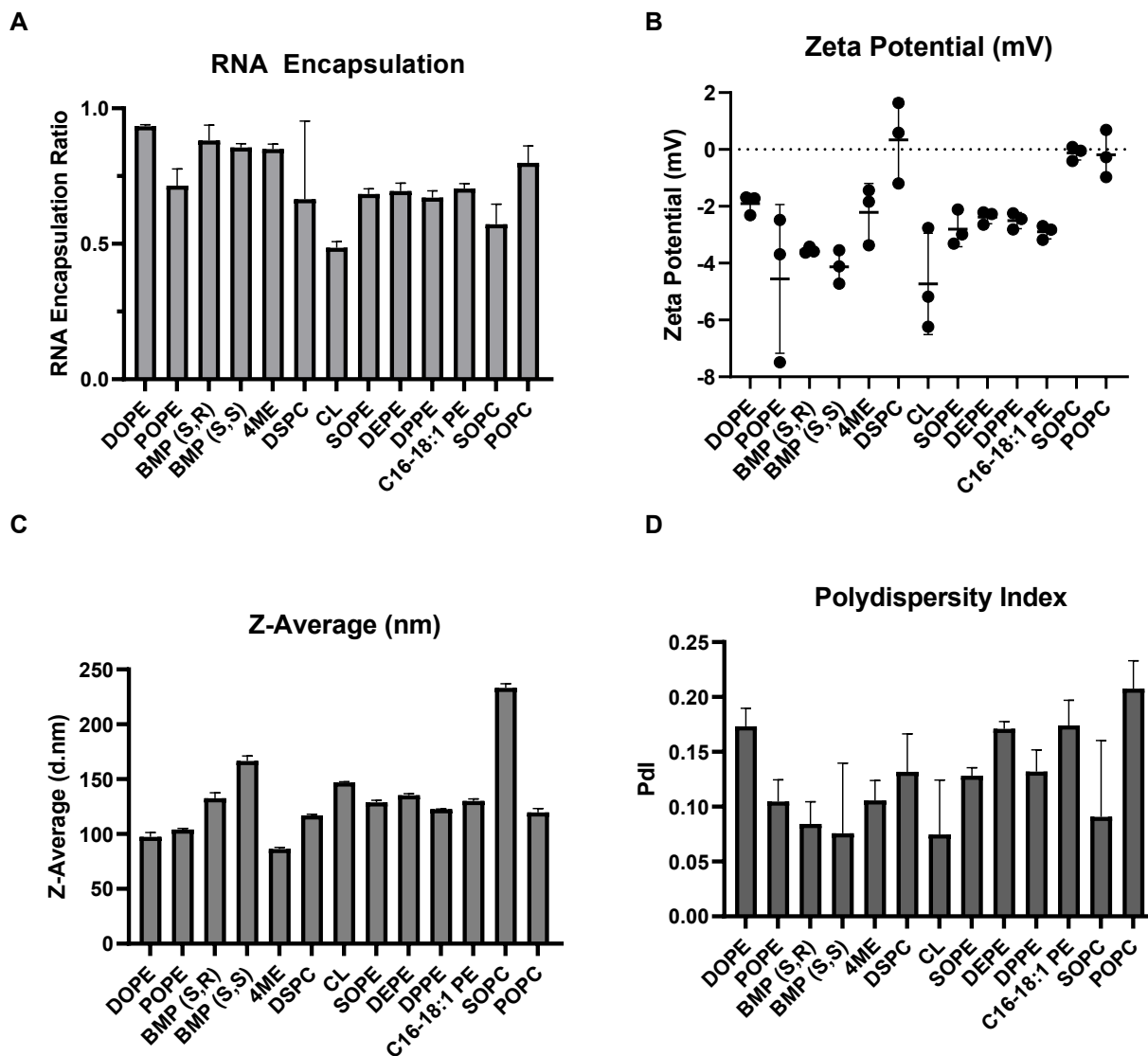**D**

### Polydispersity Index

**Figure S1. Characterization of four-component LNPs.** (A) RNA encapsulation results for 4A3-SC8:cholesterol:phospholipid (see x-axis):DMG-PEG2000 (molar ratio = 38.5:30:30:1.5) LNPs (n=4). (B) Surface charge, (C) hydrodynamic diameter, and (D) polydispersity index was also measured for the series of LNPs (n=3).
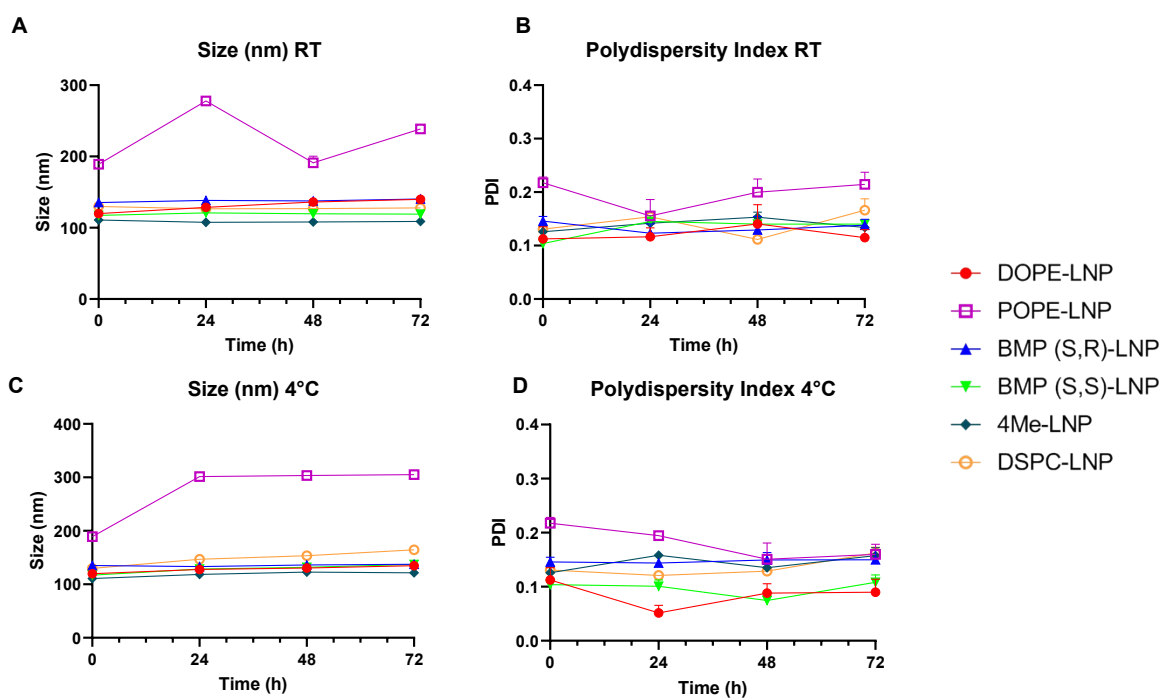
**Figure S2. LNPs formulated with different phospholipids are stable at room temperature and 4 ˚C.** 4A3-SC8 LNPs were formulated with different phospholipids using the molar ratios 4A3-SC8:Chol:DMG-PEG:PL 38.5:30:3:30 (%mol/mol) and a molar ratio of lipid to mRNA of 10000:1 (weight ratio 23:1). The size and PDI were monitored for 72h at room temperature (A-B) and 4˚C (C-D).
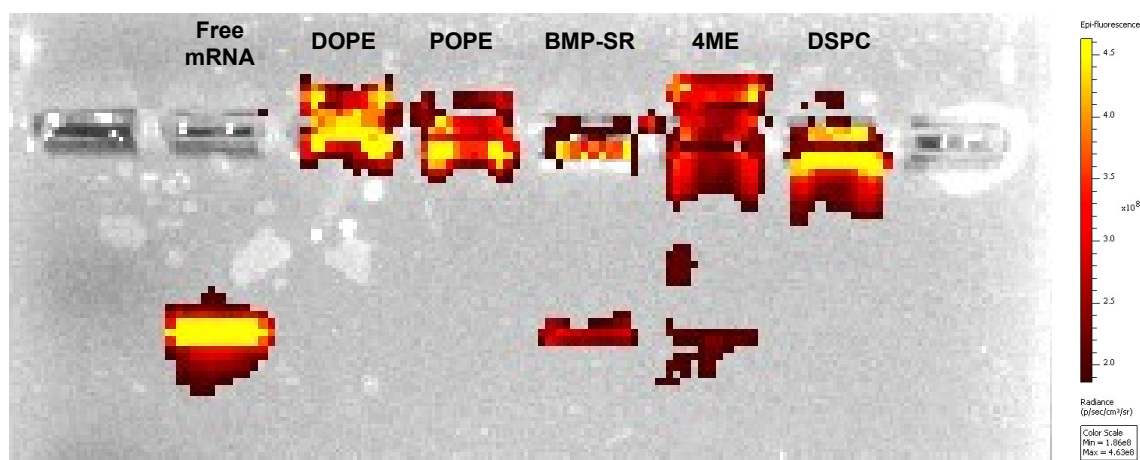


**Figure S3. LNPs formulated with different PLs encapsulated mRNA.** LNPs were formulated with different phospholipids to encapsulate Cy5-labeled mRNA. The gel was imaged using an IVIS Lumina to obtain Cy5 fluorescence emission**.**
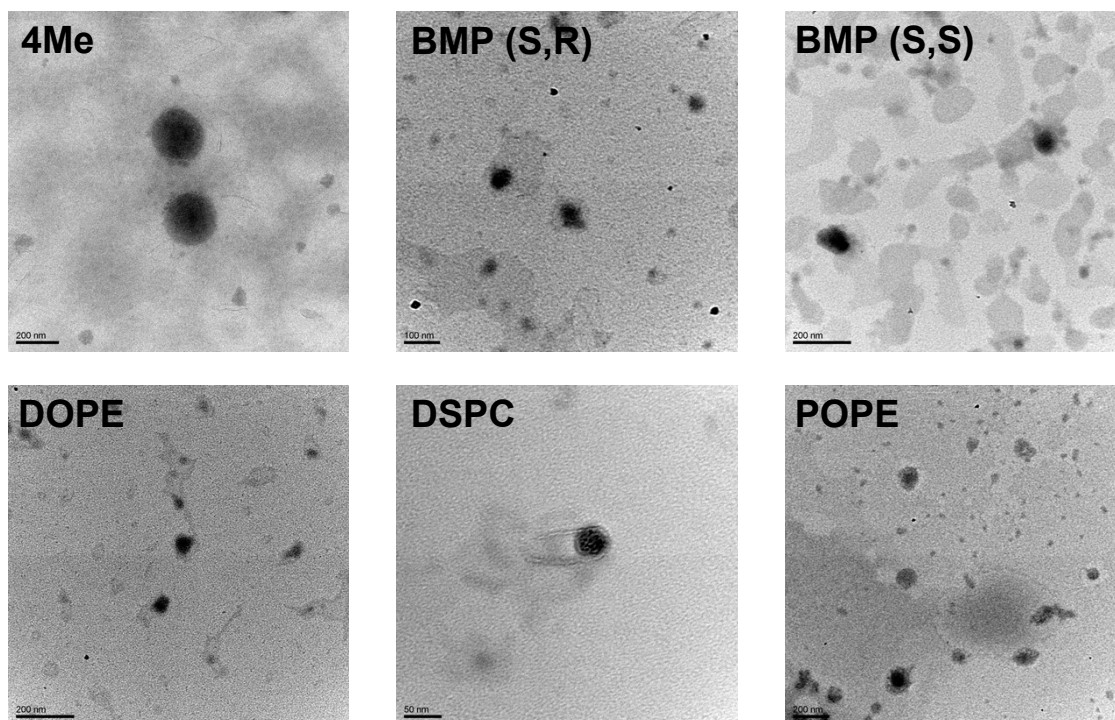
**Figure S4. Transmission Electron Microscopy (TEM) images of LNPs with different phospholipids.** LNPs were formulated with different phospholipids and imaged using TEM. The molar ratio used for the formulations was 4A3-SC8:Chol:Phospholipid:DMG-PEG = 38.5:30:30:1.5 (mol/mol); the mol ratio of 4A3-SC8 to mRNA was 10000:1 and the weight ratio of 4A3-SC8 to mRNA was 23:1.
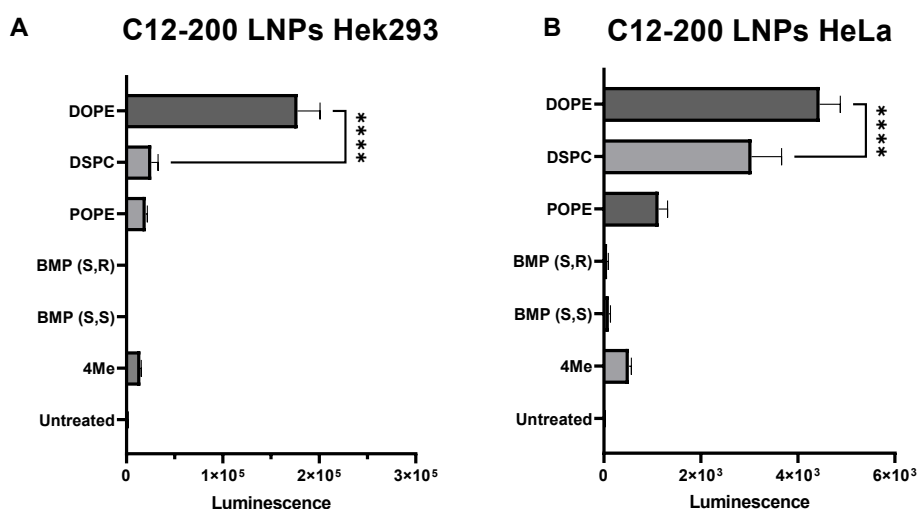


**Figure S5. DOPE phospholipid increases C12-200 LNP mediated mRNA delivery.** Results for luciferase protein activity following delivery of firefly luciferase mRNA (A) Hek293T and (B) HeLa cells. N=4 ± stdev, one-way ANOVA **** $p<0.0001$, Turkey's test ** $p< 0.001$, * $p<0.01$.
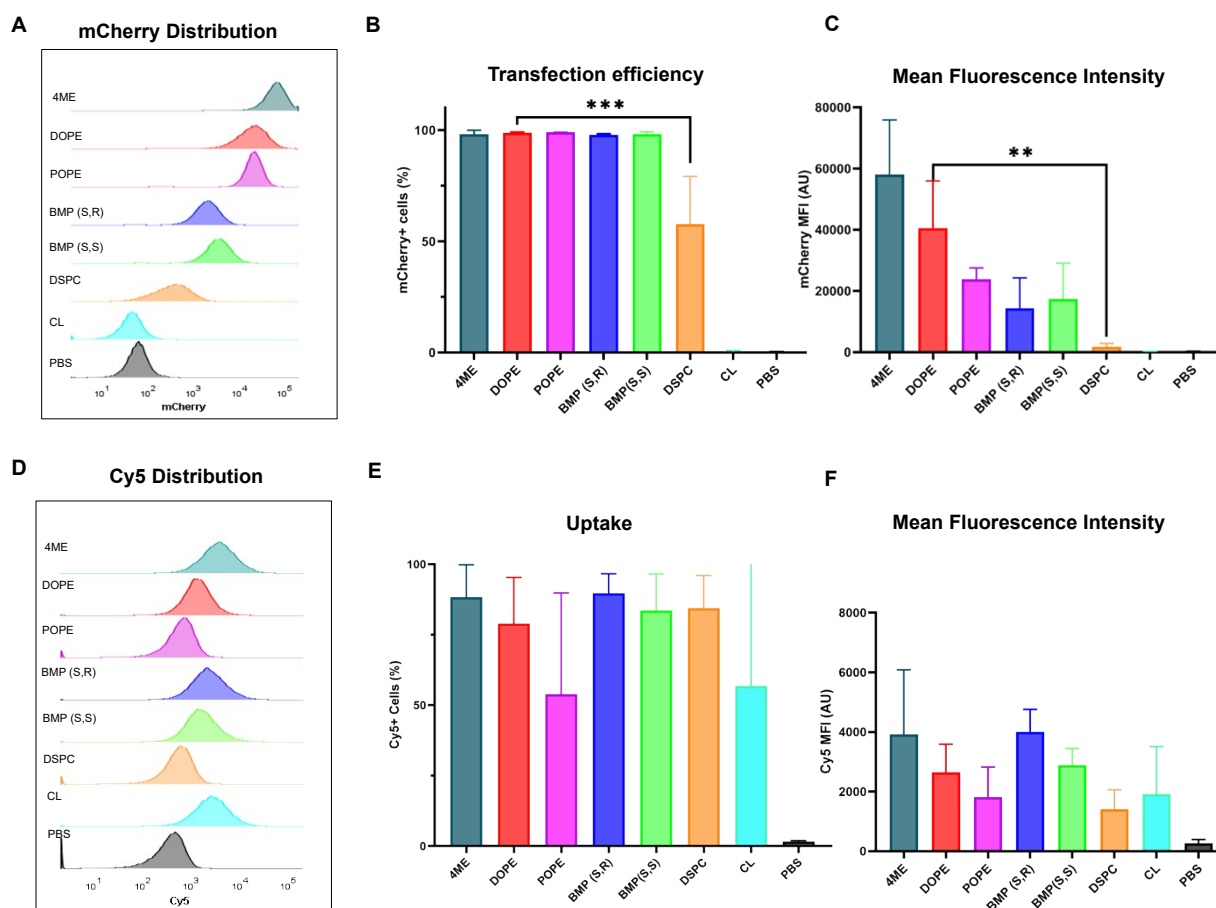
**Figure S6. Uptake of LNPs is not affected by phospholipid identity.** Distribution of mCherry fluorescence (A), percentage of mCherry positive cells (B), and mean fluorescence intensity of each LNP (C) obtained by flow cytometry after 48 h incubation of Hek293T cells with mCherry mRNA-LNPs (250 ng). Uptake of LNP was assessed after 48 h incubation of Hek293T cells with Cy5-labeled mRNA-LNP (250 ng) and flow cytometry was used to determine (D) distribution of Cy5 fluorescence, (E) percentage of Cy5 positive cells, and (F) mean fluorescence intensity. N=4 ± stdev, one-way ANOVA **** $p<0.0001$, Turkey's test ** $p< 0.001$, * $p<0.01$.

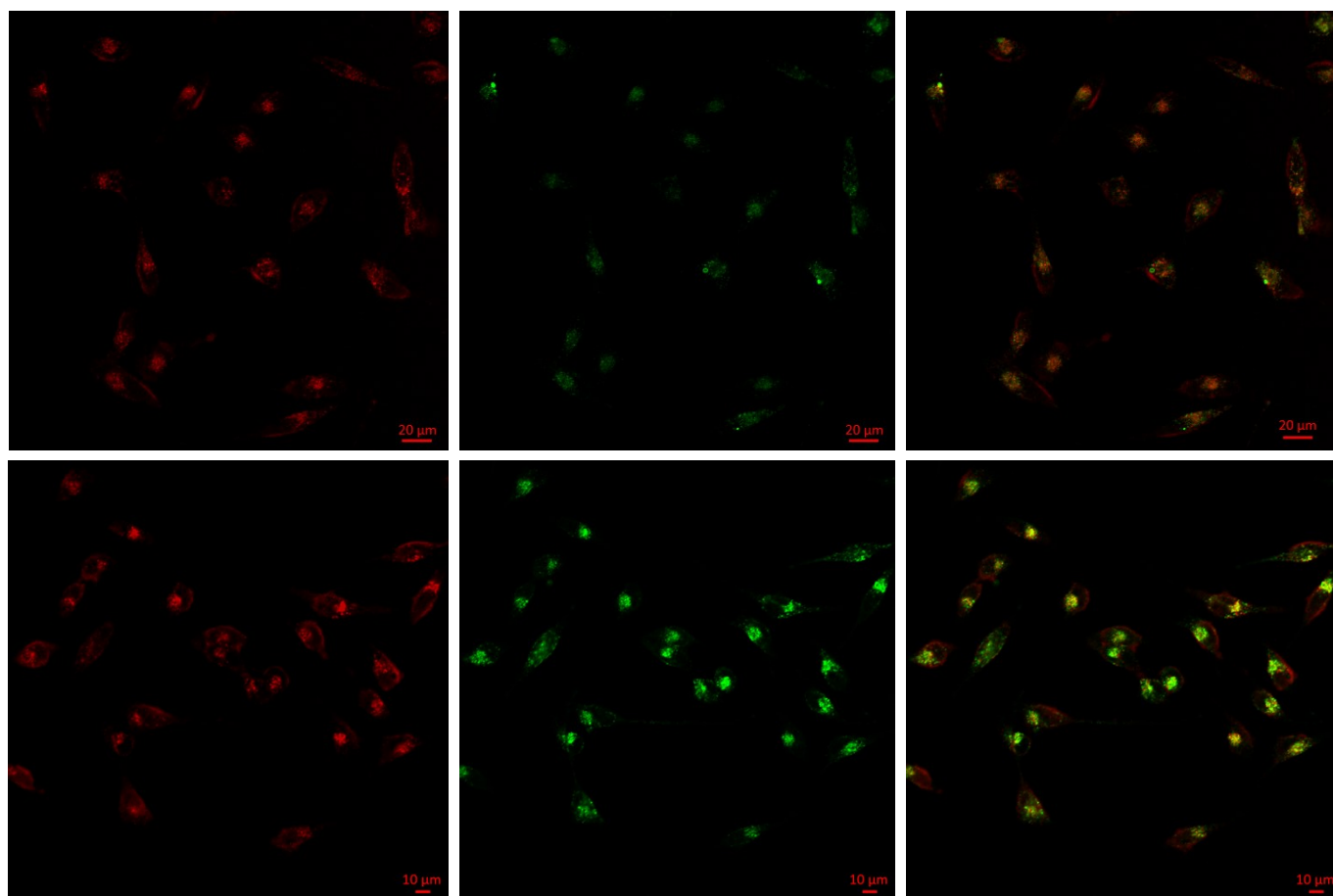**Figure S7. DOPE-LNP enhances endosomal escape.** Larger size confocal images from **Figure 3A** collected of Cy5-mRNA (290 ng) delivered by DOPE-LNP (top images) and DSPC-LNP (bottom images) after 24h incubation and staining with LysoTracker green.
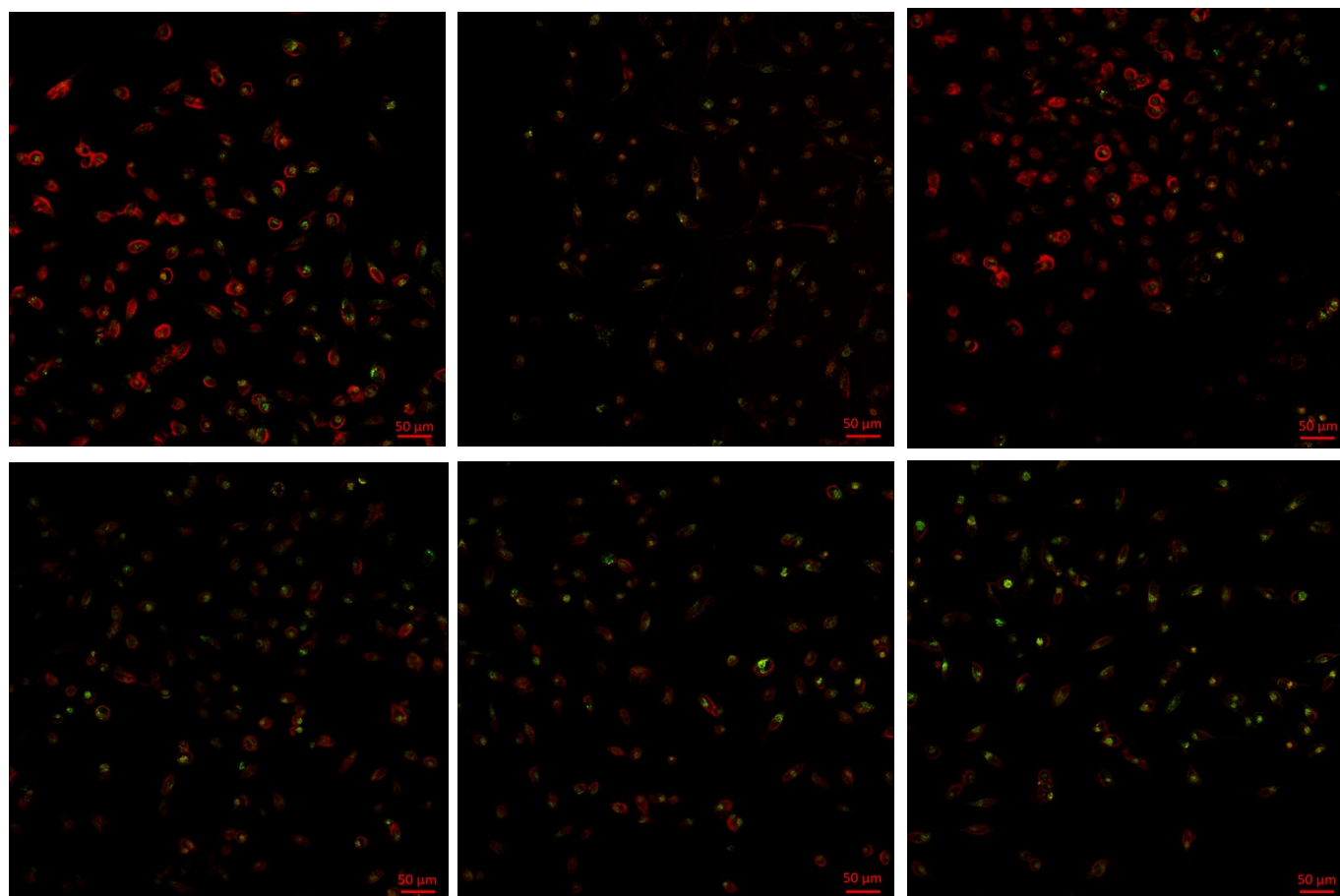
**Figure S8. Lysosome and Cy5-mRNA colocalization.** Additional confocal images collected of Cy5-mRNA (290 ng) delivered by DOPE-LNP (top images) and DSPC-LNP (bottom images) after 24h incubation and staining with LysoTracker green. These images were used to calculate the Pearson coefficient values.

### Pearson's Coefficient

| DOPE-LNPs | DSPC-LNPs |
|-----------|-----------|
| 0.22 | 0.22 |
| 0.07 | 0.27 |
| 0.24 | 0.40 |
| 0.19 | 0.52 |

**Figure S9. Tabulated Pearson's Coefficients.** Colocalization coefficients obtained from the images in Figure 3 and Figure S4 using ImageJ's PSC colocalization plugin[1].

| | Molar Ratio | | | | | Molar Percentage | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Formulation | 4A3-SC8 | DOPE | Cholesterol | PEG-DMG | SORT PL | 4A3-SC8 | DOPE | Cholesterol | PEG-DMG | SORT PL |
| BMP-5 | 15 | 15 | 30 | 3 | 3.315 | 22.62 | 22.62 | 45.24 | 4.52 | 5.00 |
| BMP-10 | 15 | 15 | 30 | 3 | 7 | 21.43 | 21.43 | 42.86 | 4.29 | 10.00 |
| BMP-20 | 15 | 15 | 30 | 3 | 15.75 | 19.05 | 19.05 | 38.10 | 3.81 | 20.00 |
| BMP-30 | 15 | 15 | 30 | 3 | 27 | 16.67 | 16.67 | 33.33 | 3.33 | 30.00 |
| BMP-40 | 15 | 15 | 30 | 3 | 42 | 14.29 | 14.29 | 28.57 | 2.86 | 40.00 |
| POPE-20 | 15 | 15 | 30 | 3 | 15.75 | 19.05 | 19.05 | 38.10 | 3.81 | 20.00 |
| 4ME-20 | 15 | 15 | 30 | 3 | 15.75 | 19.05 | 19.05 | 38.10 | 3.81 | 20.00 |
| DSPC | 15 | 15 | 30 | 3 | 15.75 | 19.05 | 19.05 | 38.10 | 3.81 | 20.00 |
| CL | 15 | 15 | 30 | 3 | 15.75 | 19.05 | 19.05 | 38.10 | 3.81 | 20.00 |

**Figure S10. Molar ratios and percentages for SORT LNPs.** SORT LNPs were prepared in 40:1 weight ratio of total lipids to mRNA and injected IV at 0.1 mg/kg[2].
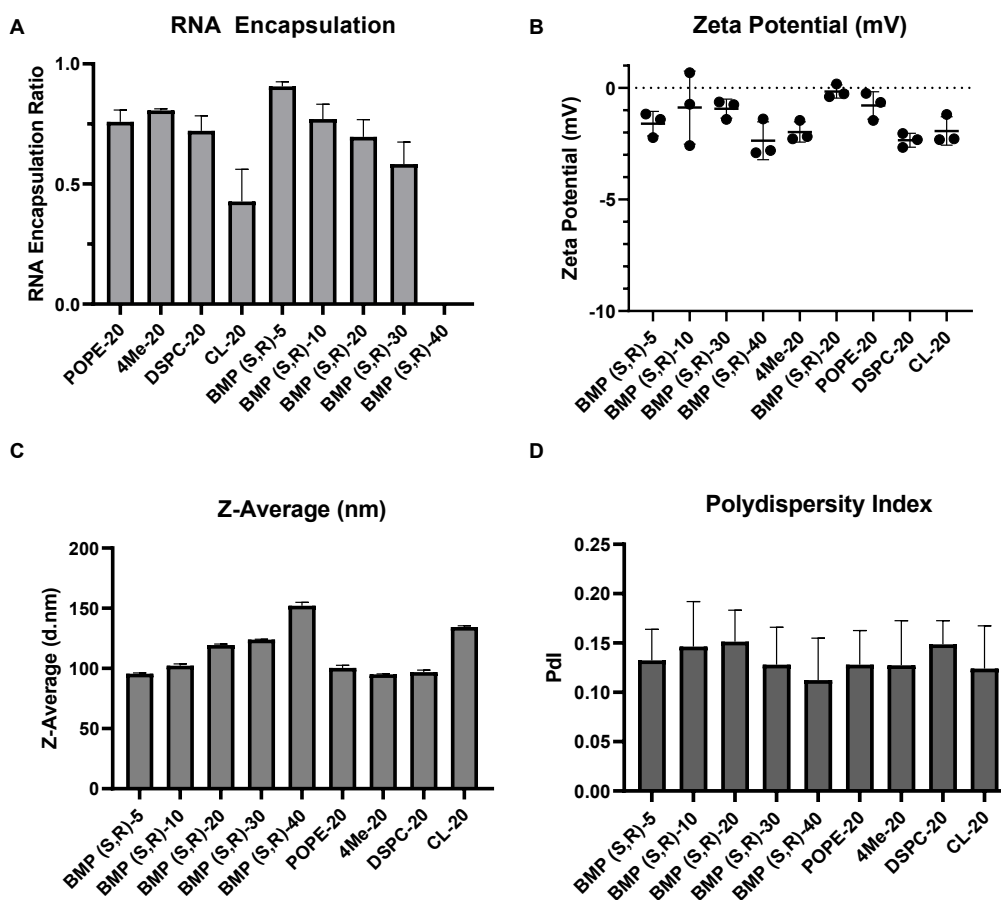


**Figure S11.** Characterization of five-component SORT LNPs. (A) RNA encapsulation results for 4A3-SC8:DOPE:DMG-PEG:phospholipid (see x-axis) (molar ratio = 15:15:30:30:3:x, where x was varied from 5% to 40% (x=3.315, 7, 15.75, 27, 42)) (n=4). (B) Surface charge, (C) hydrodynamic diameter, and (D) polydispersity index was also measured for the series of LNPs (n=3).

## Notes and references

1.      A. P. French, S. Mills, R. Swarup, M. J. Bennett and T. P. Pridmore, *Nat. Protoc.*, 2008, **3**, 619-628.
2.      Q. Cheng, T. Wei, L. Farbiak, L. T. Johnson, S. A. Dilliard and D. J. Siegwart, *Nat. Nanotechnol.*, 2020, **15**, 313-320.