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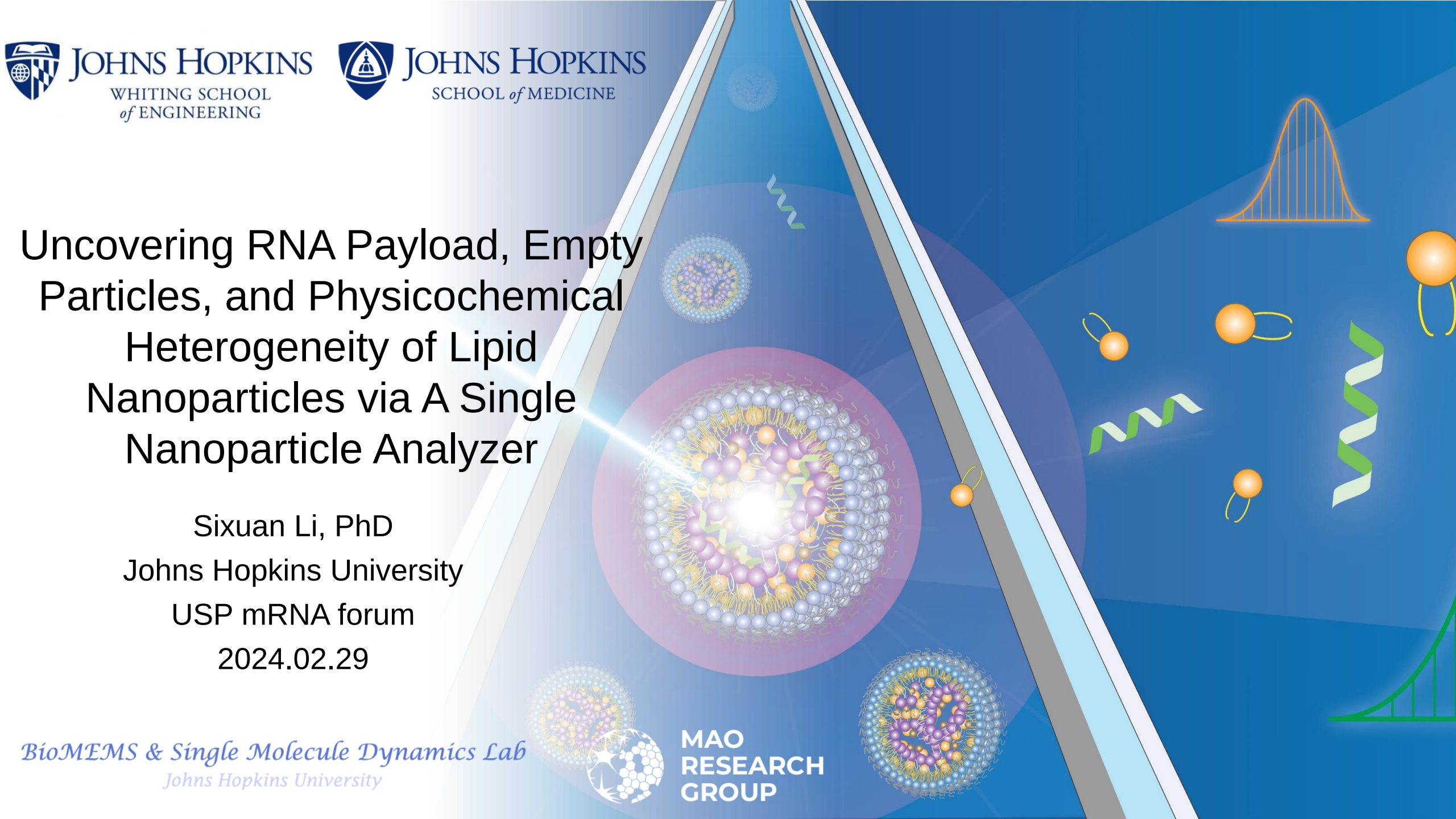
Uncovering RNA Payload, Empty Particles, and Physicochemical Heterogeneity of Lipid Nanoparticles via A Single Nanoparticle Analyzer

Sixuan Li, PhD
Johns Hopkins University

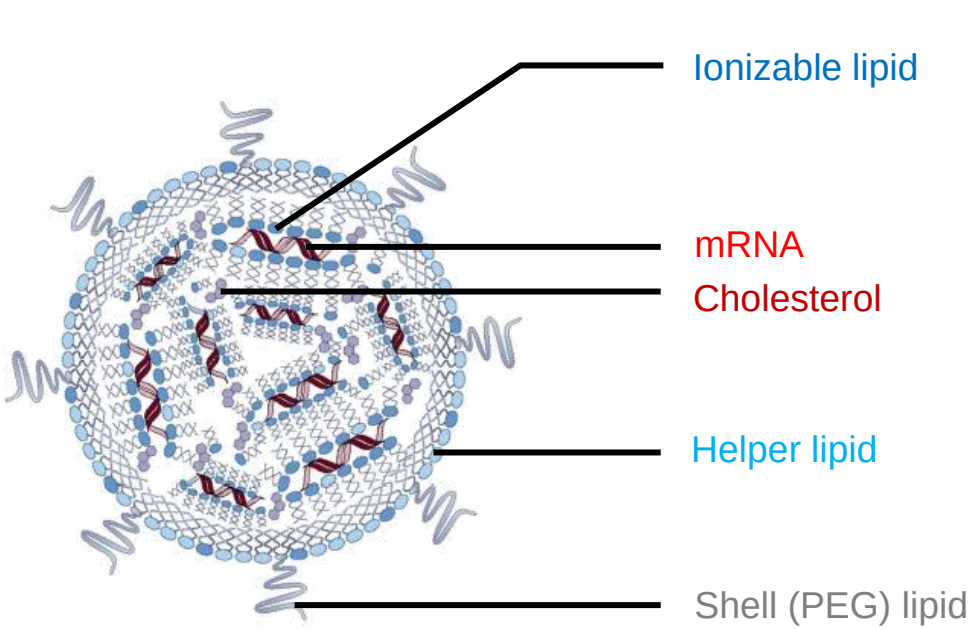
USP mRNA forum
2024.02.29

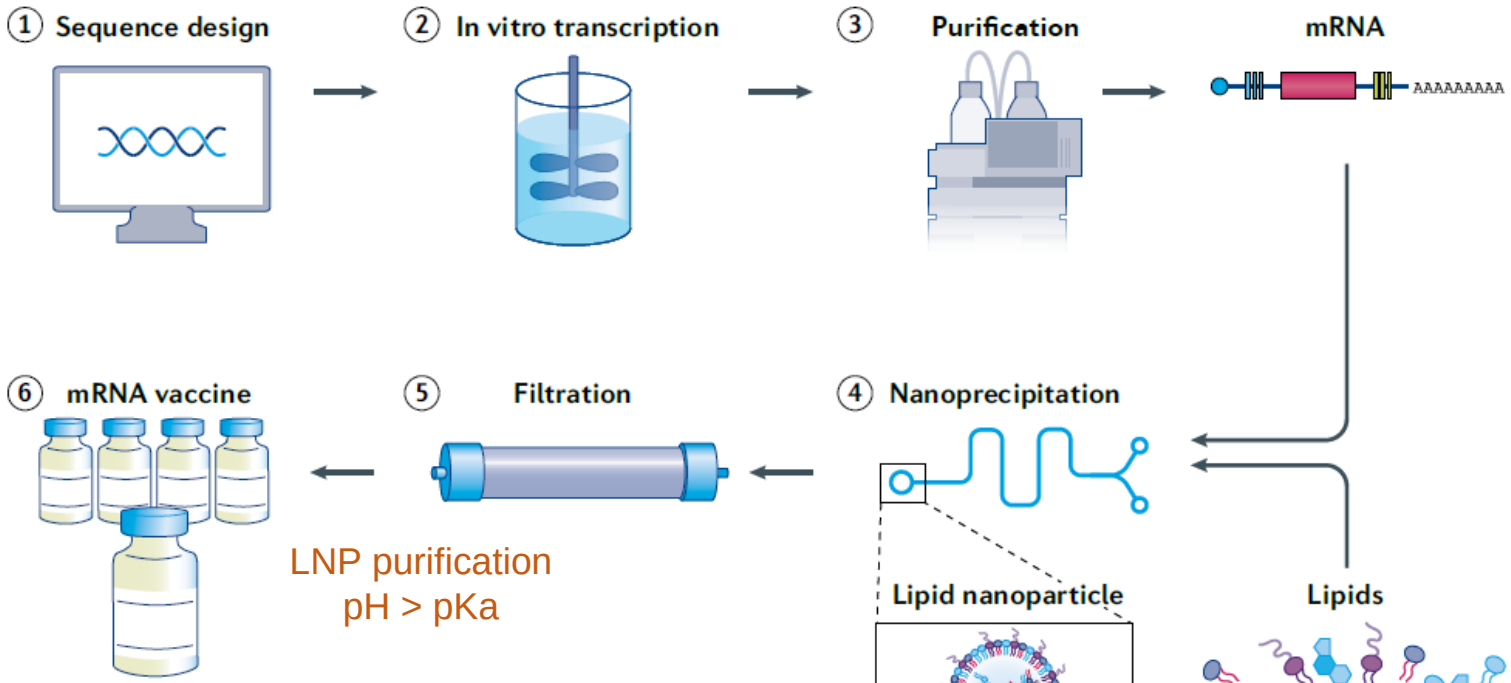


BioMEMS & Single Molecule Dynamics Lab
Johns Hopkins University

MAO RESEARCH GROUP

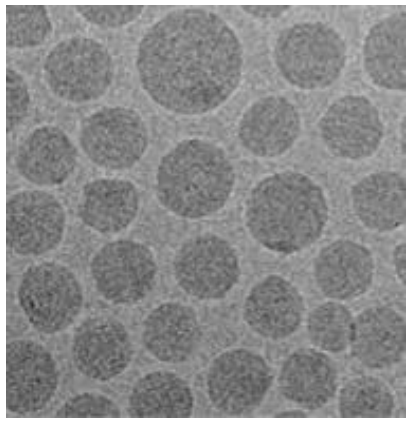
mRNA lipid nanoparticle (LNP) formulation process



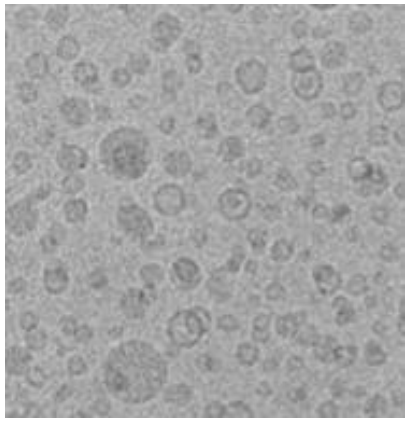


2021, *Nature Reviews Drug Discovery*, 20, 817-838

After filtration, pH 7.4

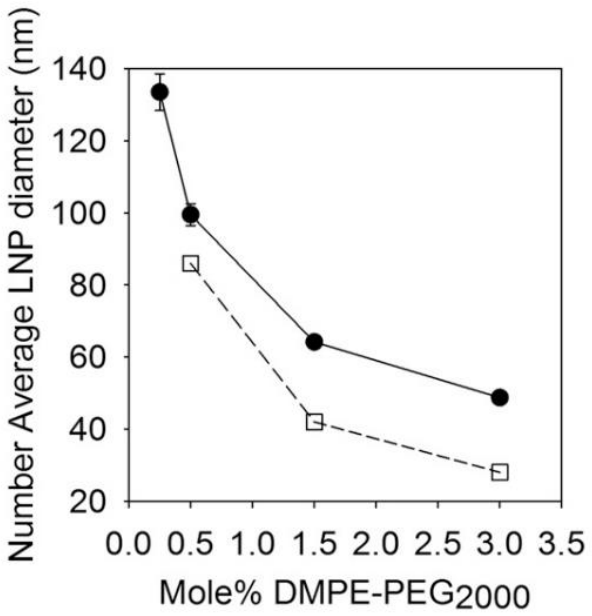


Before filtration, pH < pKa

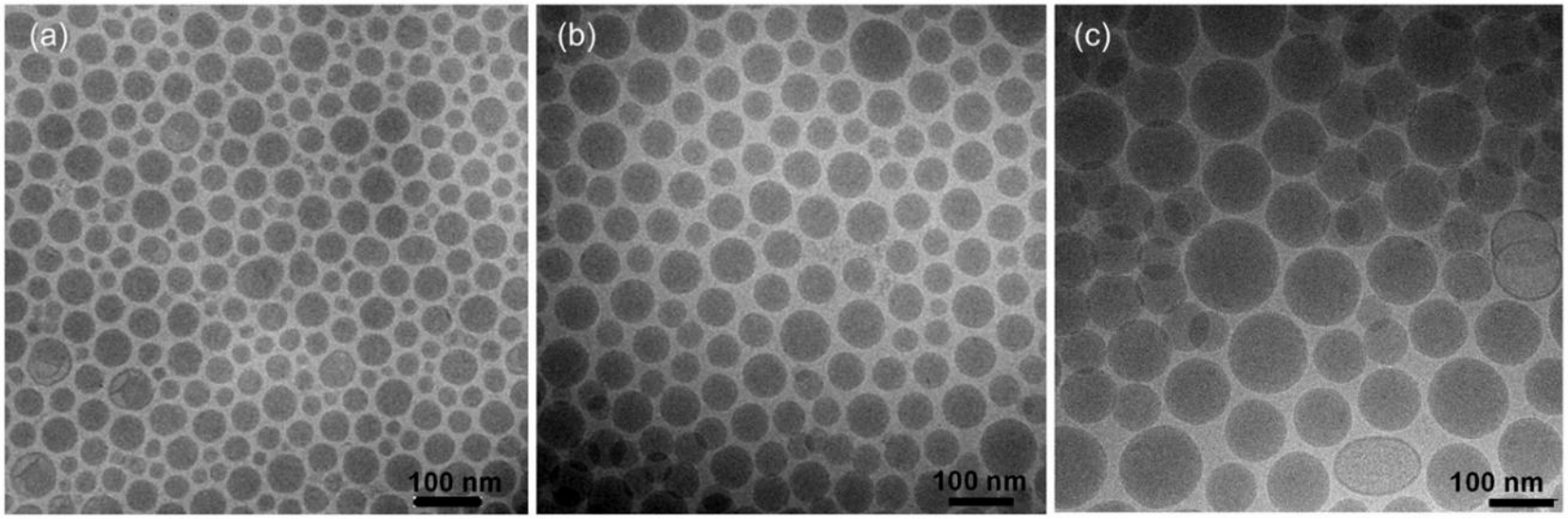


2018, *ACS Nano*, 12, 4787-4795

mRNA LNP properties to be characterized



Decreasing PEG lipid dosage



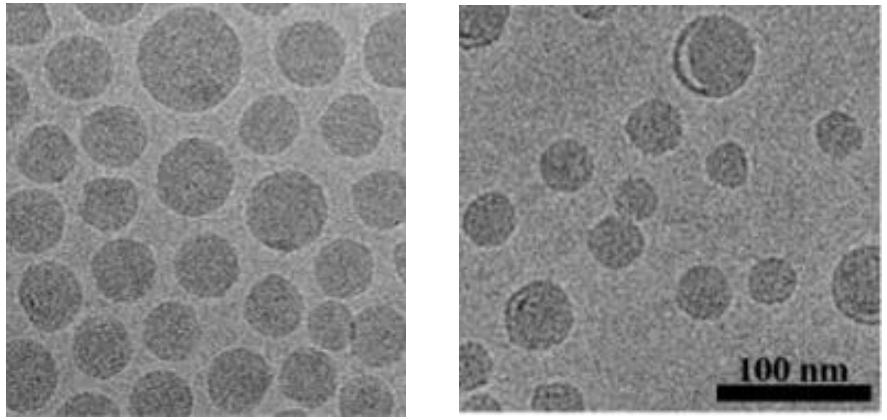
2018, *PNAS*, 115, E3351-E3360

Payload distribution and capacity of mRNA LNPs

- How does LNP size correlate to the mRNA payload?
- How are mRNAs distributed in LNP sample?
- How many mRNAs can be loaded into single LNP?
- Is every LNP loaded with mRNA? Empty LNP exist?
- What effect does empty LNP have on delivery and transfection?

After filtration, pH 7.4

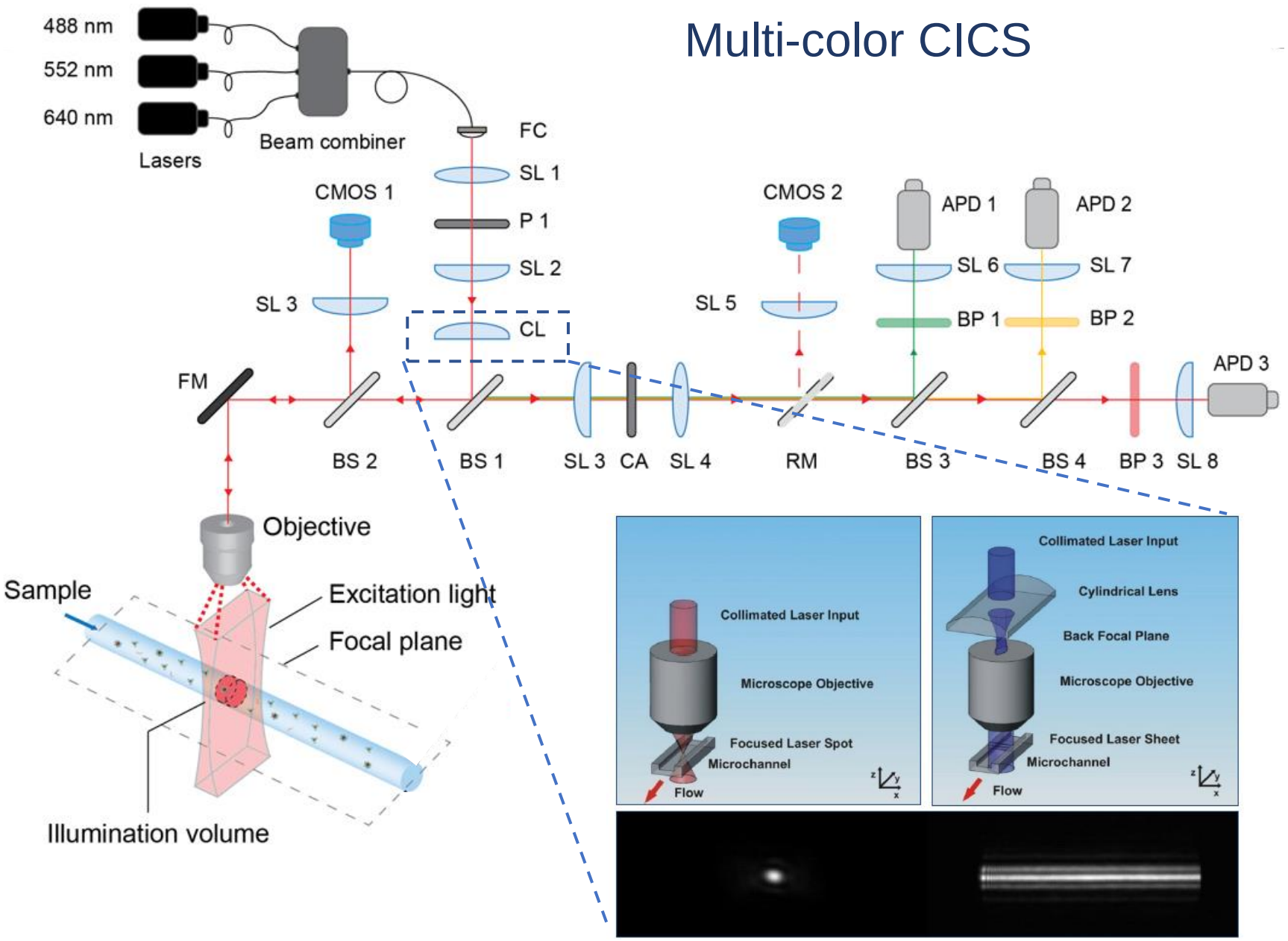
Formulated without nucleic acid, pH 7.4



2018, *ACS Nano*, 12, 4787-4795

Cylindrical illumination confocal spectroscopy (CICS): a single molecule detection platform

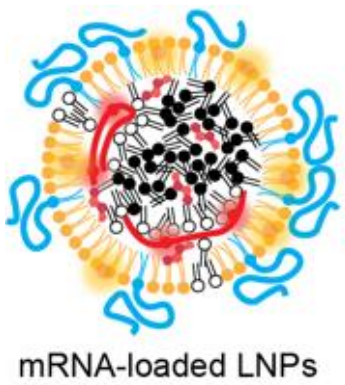
Multi-color CICS



CICS single nanoparticle detection platform

- Single molecule sensitivity
- Light sheet enables high mass detection efficiency and fluorescence uniformity
- High throughput (up to 6000 particles/min)
- Tiny sample consumption (<1 uL per sample)

mRNA LNP subpopulation identification by 3-color fluorescence coincidence analysis



mRNA-loaded LNPs

Empty LNPs

Free mRNAs

YOYO-1

Fluorescent helper lipids TMR-PC

Cy5

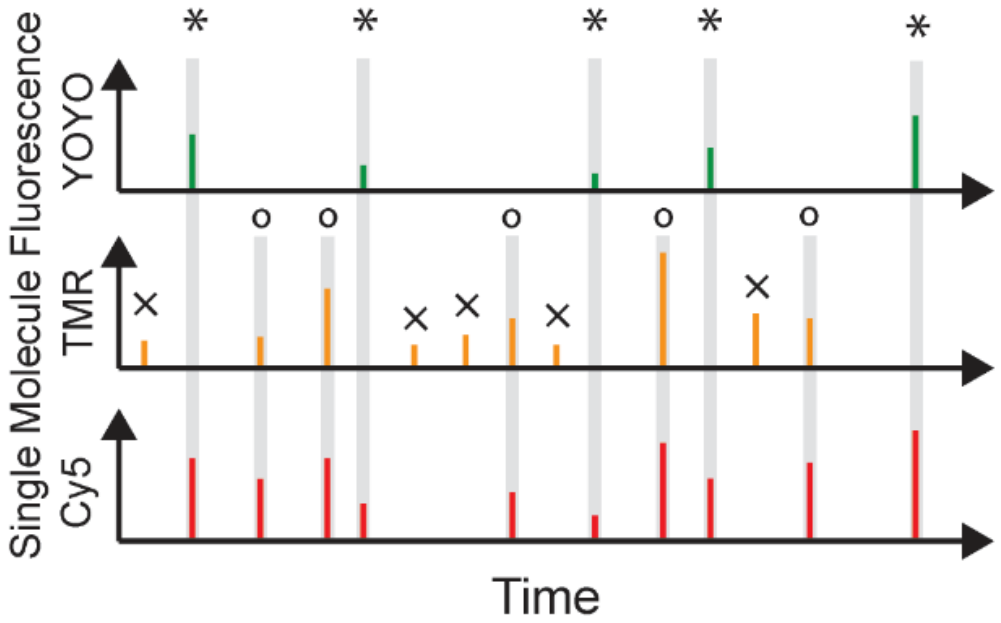
Charged ionizable lipids

Deprotonated ionizable lipids

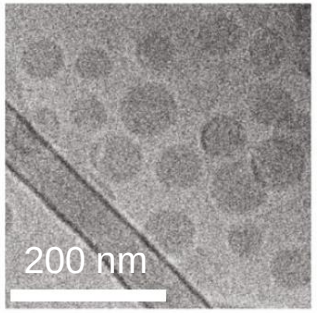
Helper lipids (DSPC)

Cholesterol

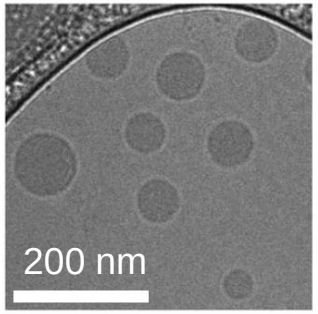
PEG lipid



LNPs with non-labeled mRNA and non-labeled DSPC



LNPs with Cy5-mRNA and TMR-PC helper lipid



Multi-color fluorescence coincidence analysis of mRNA LNP

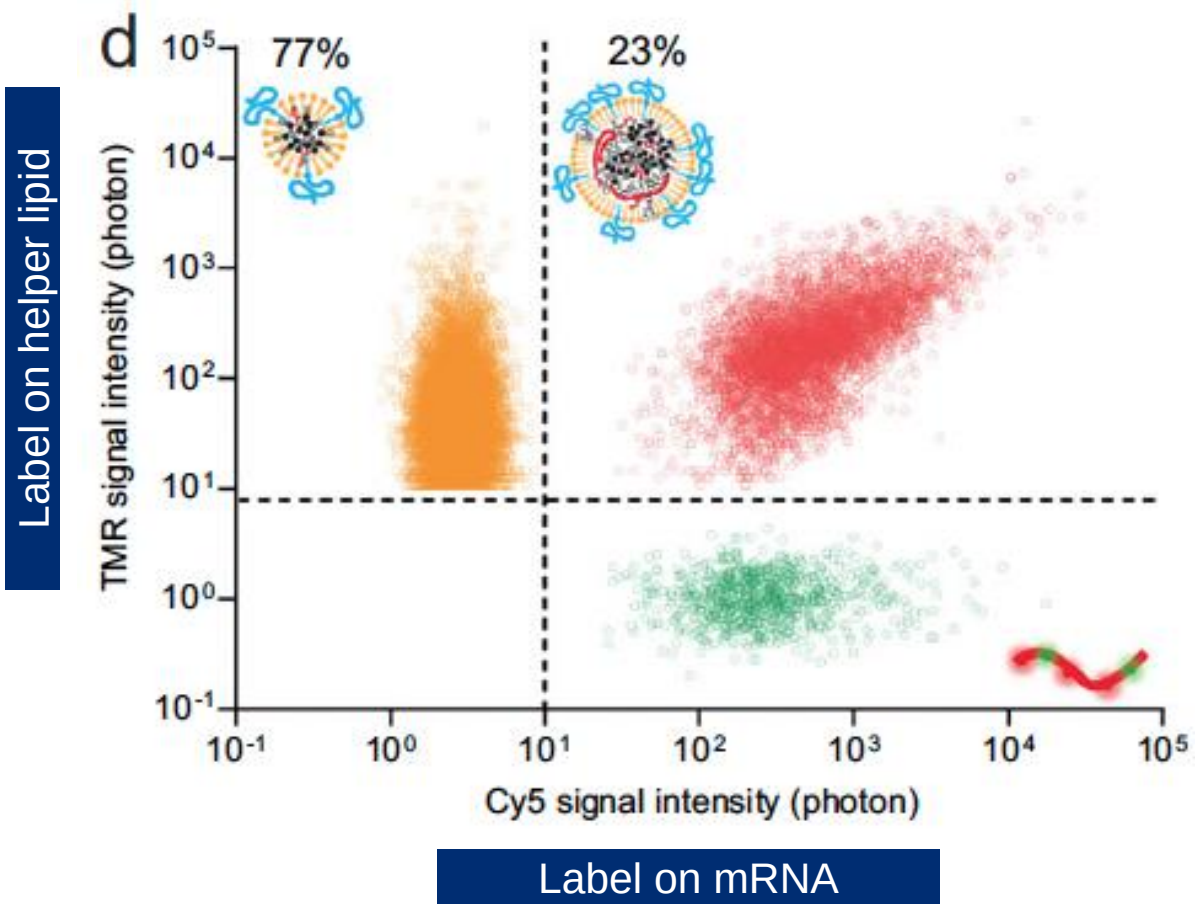
	○	×	∗
	mRNA loaded LNPs	Empty LNPs	Free mRNA
YOYO-1	-	-	+
TMR-PC	+	+	-
Cy5-mRNA	+	-	+

Characterization of a benchmark formulated mRNA LNP

Dlin-MC3-DMA : Cholesterol : DSPC : DMG-PEG2000 = 50 : 38.5 : 10 : 1.5*

- Same formulation as ONPATTRO® by Alnylam
- 20 µg/mL mRNA, N/P =6

Final formulation at pH 7.4



- Differentiate mRNA loaded LNP vs. empty LNP
- Quantify mRNA payload distribution in the formulation

For clarity, 10% of 195,090 signals are shown in the figure. The percentages labeled are relative to all TMR events.

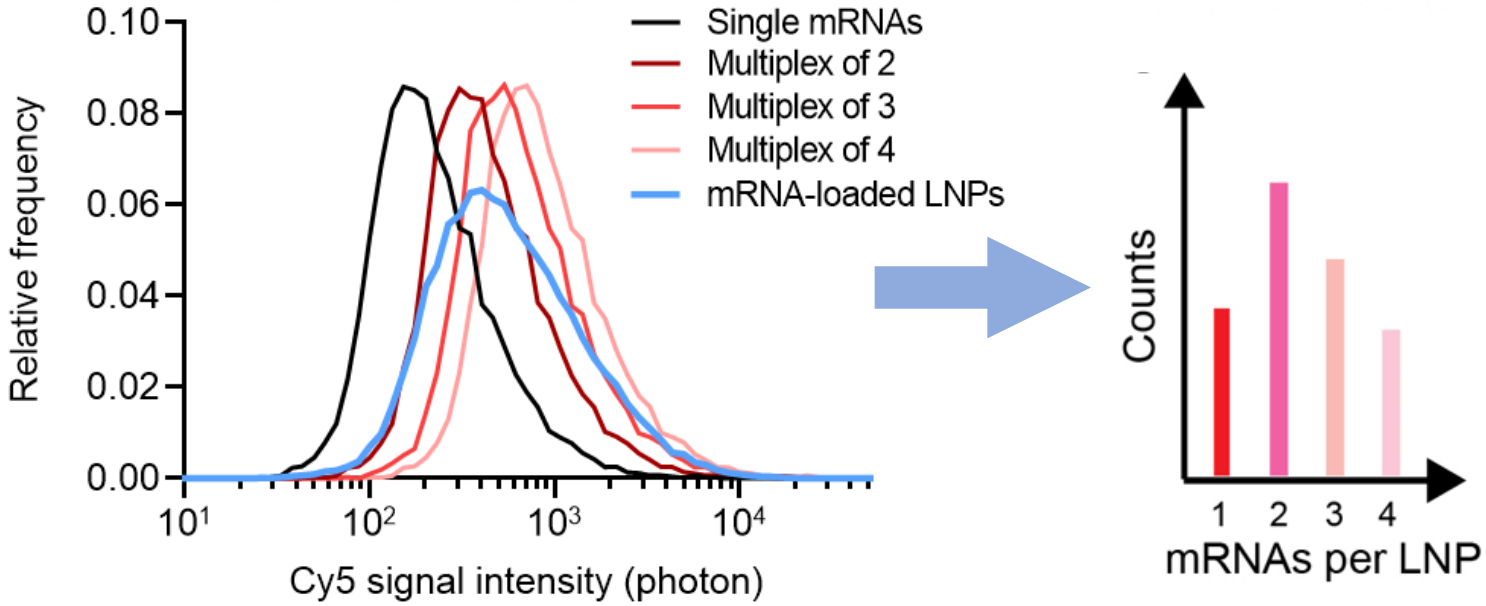
Quantification of mRNA payload and lipid content in single mRNA LNP

CICS platform+ Deconvolution algorithm -> accurate characterization of single LNP

- Deconvolution takes multiple variation in fluorescence detection into consideration
- Quantitative analysis at single mRNA LNP level:
  - mRNA payload and its distribution in the formulation
  - Relative helper lipid quantification and its distribution in the formulation

$$D^*_{LNP} = \sum_{n=1}^{N} w_n \times D_{RNA,n}$$

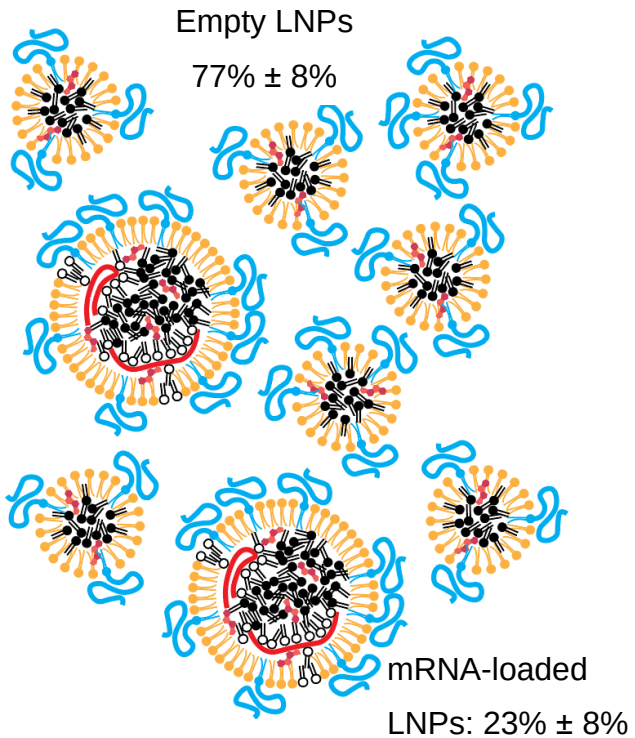
$$N = N^* = \sum_{n=1}^{N} w_n$$

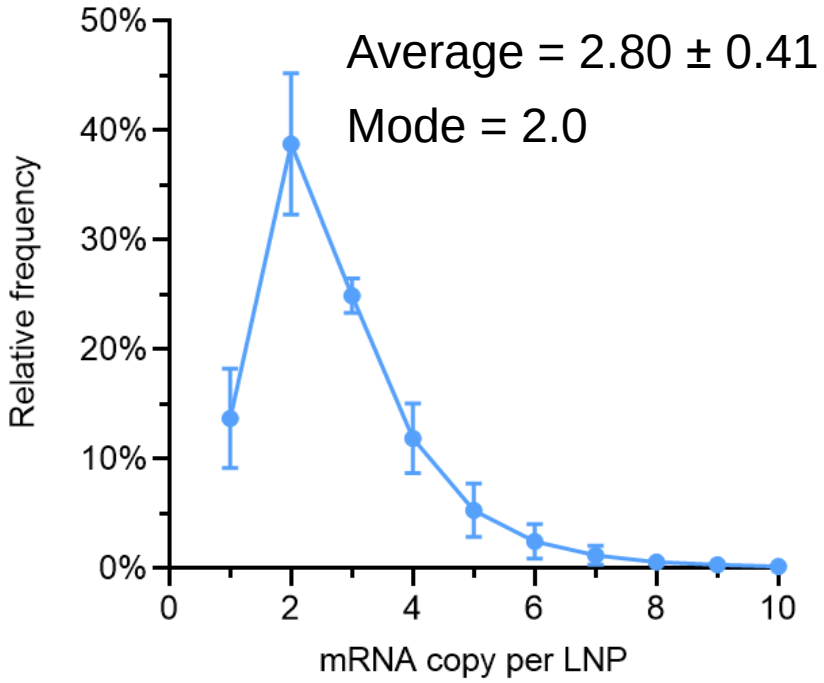


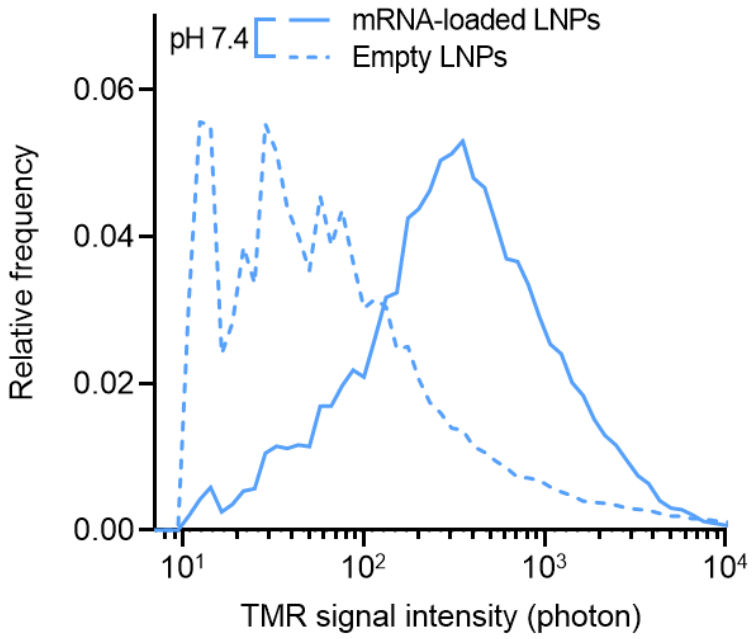
Single nanoparticle characterization of the mRNA LNP benchmark formulation

Dlin-MC3-DMA : Cholesterol : DSPC : DMG-PEG2000 = 50 : 38.5 : 10 : 1.5

Calculating mRNA payload distribution using deconvolution algorithm:







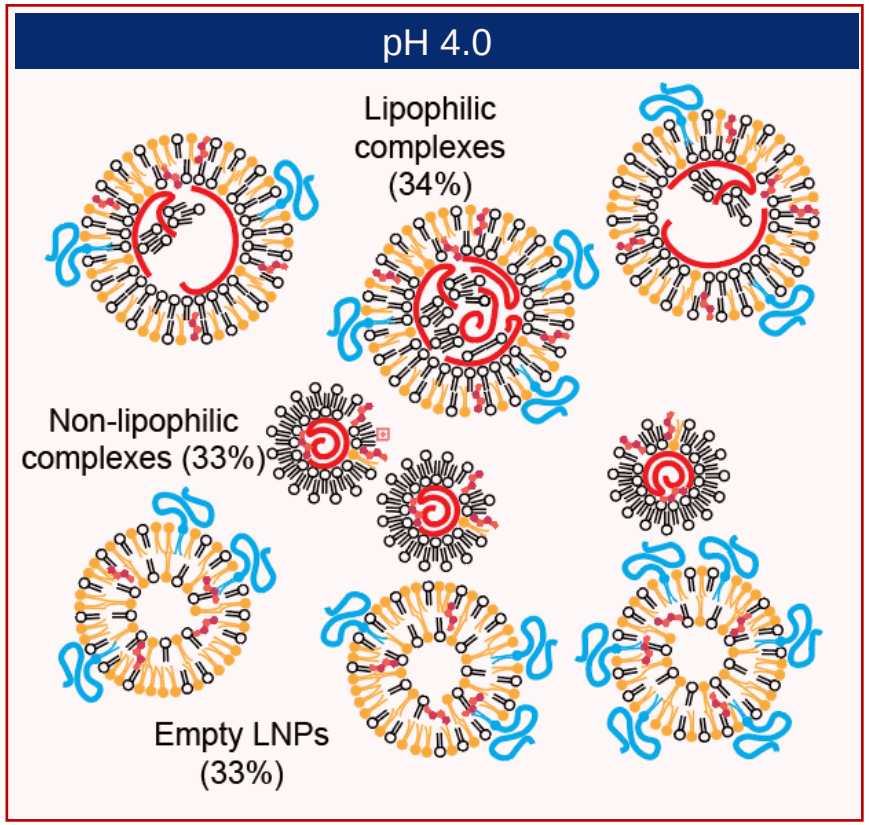
TMR-labeled helper lipid

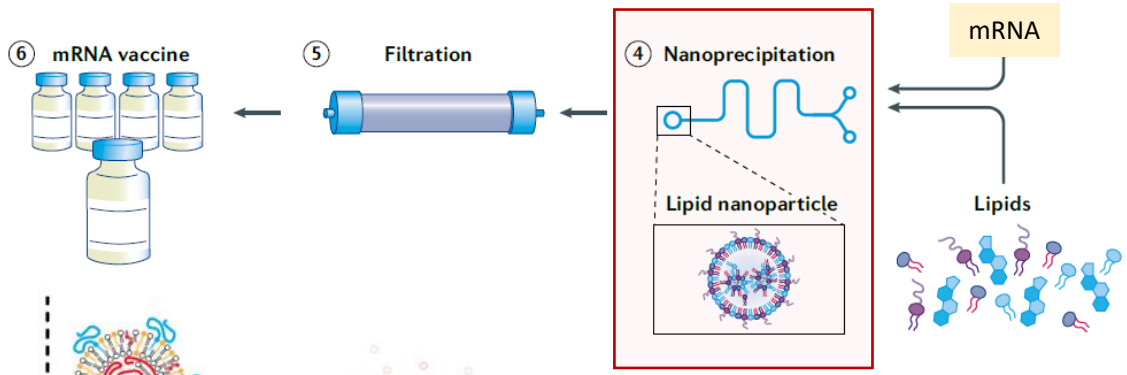
n = 6 independent formulation experiments;
Data are presented as mean values ± SD.

Charged ionizable lipids
Helper lipids
Deprotonated ionizable lipids
Cholesterol
PEG lipid
mRNA

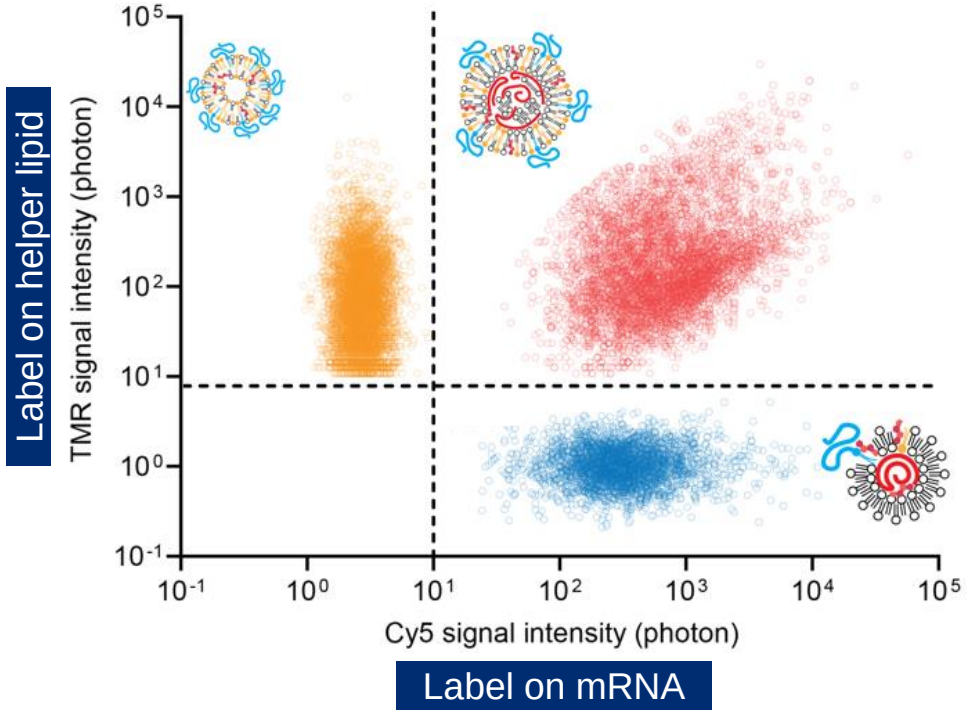
mRNA LNP composition change: Initial stage (pH 4.0)

Low PEG lipid level: DMG-PEG2000 < 1.5%





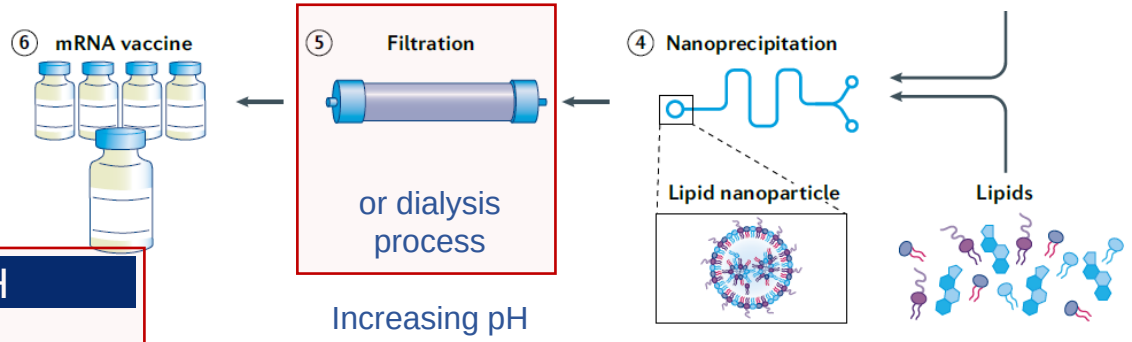
pH 4.0

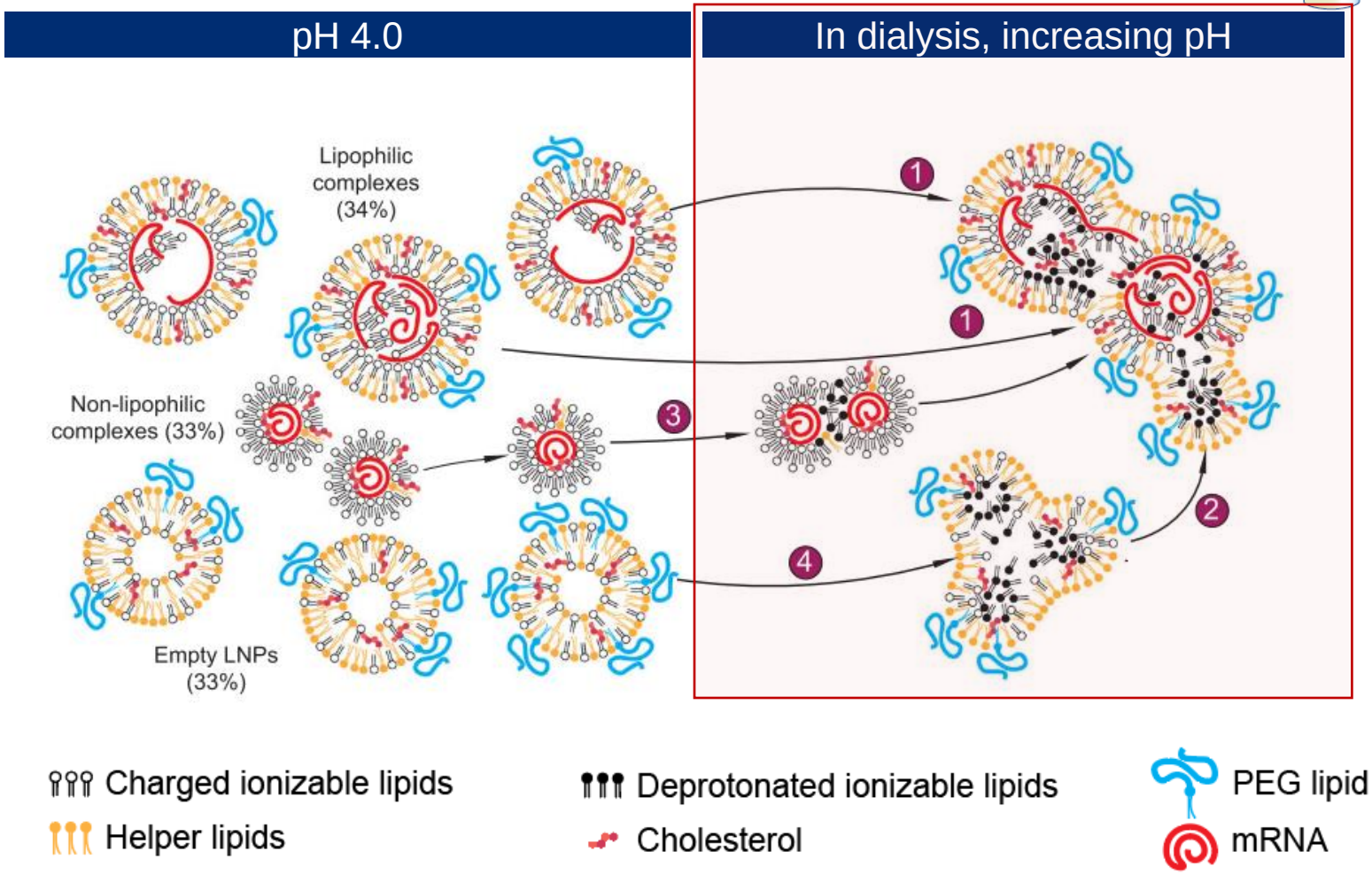


Charged ionizable lipids
Deprotonated ionizable lipids
PEG lipid
Helper lipids
Cholesterol
mRNA

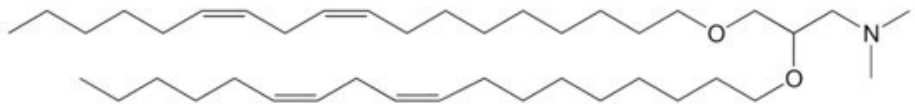
mRNA LNP composition change: During filtration process

Low PEG lipid level: DMG-PEG2000 < 1.5%





Dlin-MC3-DMA, pKa = 6.44



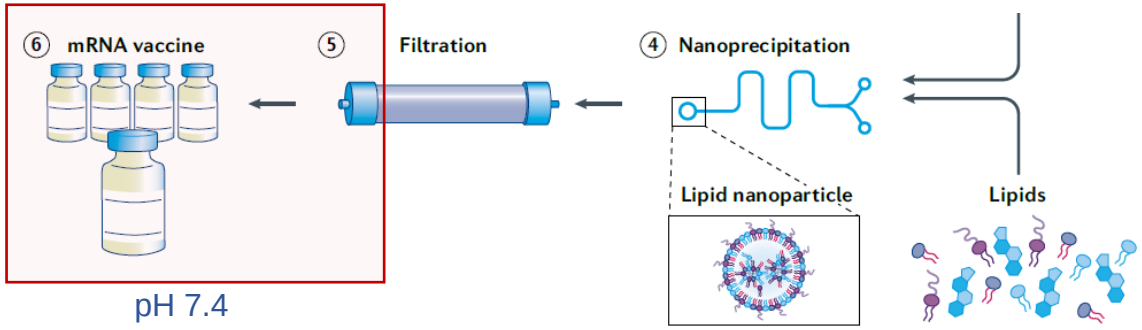
- Hydrophobic interactions of ionizable lipids
- Re-distribution of helper lipid
- Steric effects by PEG lipids

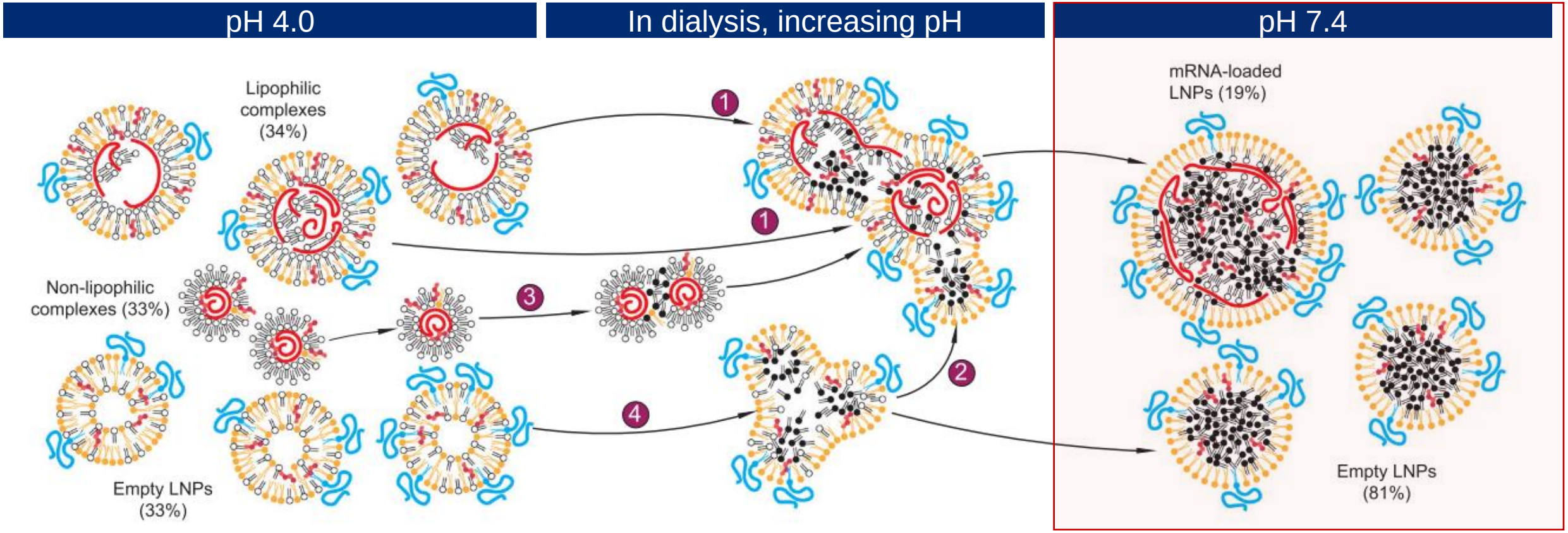
Possible composition change during dialysis

1. Merge between lipophilic complexes
2. Merge of empty LNPs & mRNA complexes
3. Merge of non-lipophilic complexes
4. Split of empty LNPs

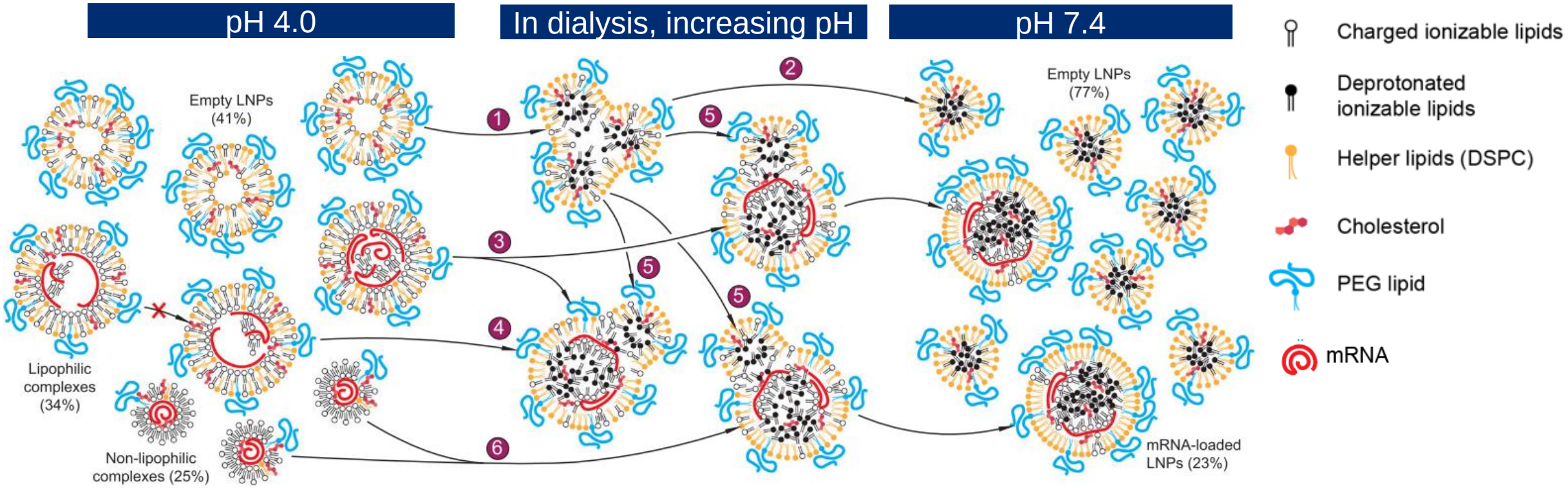
mRNA LNP composition change: Final stage (pH 7.4)

Low PEG lipid level: DMG-PEG2000 < 1.5%





mRNA LNP composition change during filtration: High PEG lipid concentration

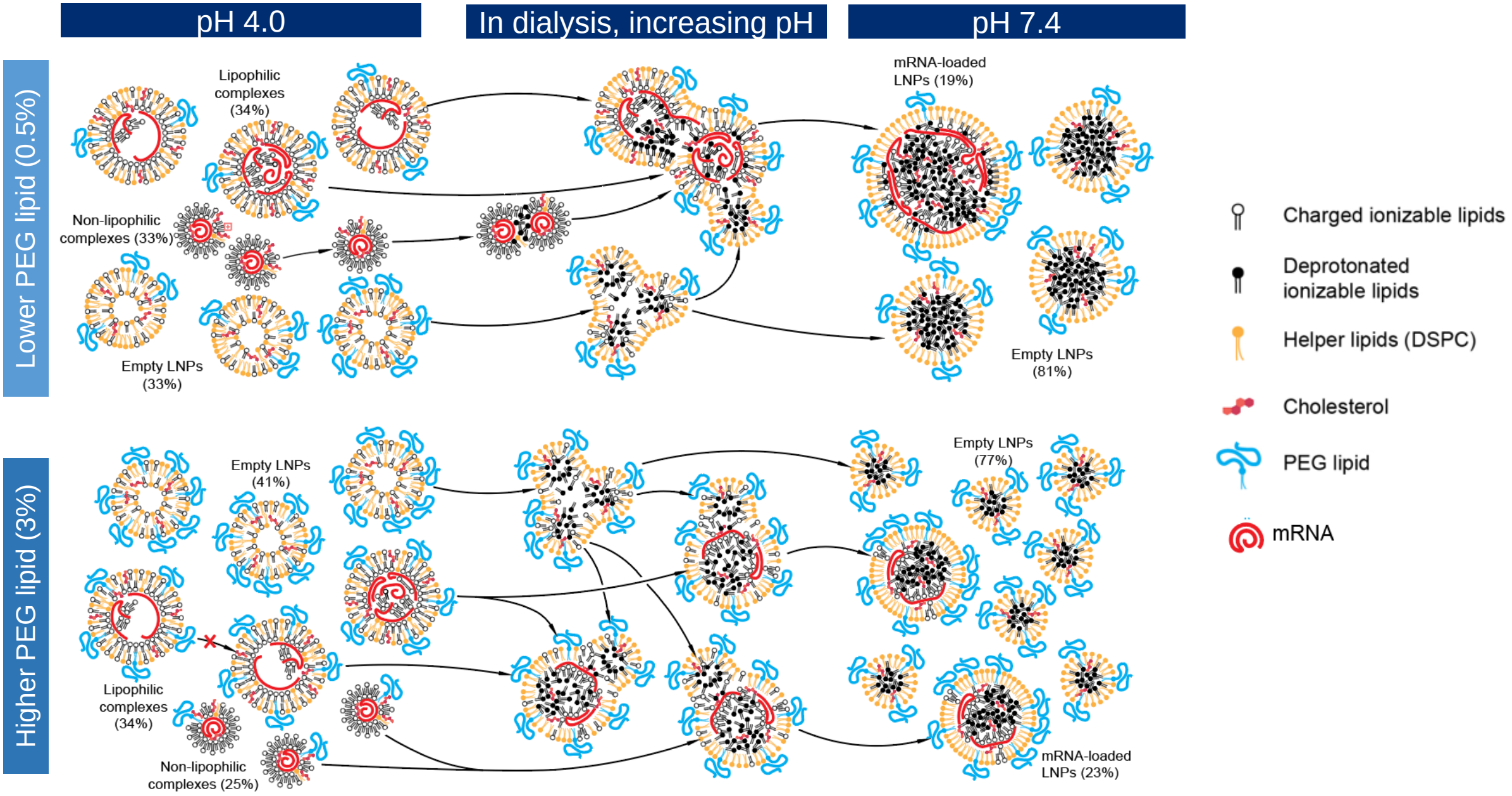


Possible composition change during dialysis:

1. Splitting of empty LNPs
2. Stabilization of empty LNPs
3. Splitting of lipophilic complexes with an initially high mRNA payload
4. Remaining a same mRNA payload for lipophilic complexes with an initially low or intermediate payload
5. Merge of empty LNPs with mRNA complexes
6. Merge of non-lipophilic complexes.

Note: The cross mark indicates the mRNA payload of lipophilic complexes does not increase without merge

mRNA LNP composition change during filtration: Effect of PEG lipid concentration



Composition and properties of the mRNA LNP benchmark formulation

Table 2 | Composition features of the benchmark LNP formulation at an mRNA concentration of 20 μg/mL and an N/P ratio of 6

	Before dialysis at pH 4.0 (i.e., the initial LNPs)	After dialysis at pH 7.4 (i.e., the final LNP product)
Number-average payload (mRNA copy per particle)	Lipophilic complexes: 3.43 ± 0.38 Non-lipophilic complexes: 1.34 ± 0.20 All nanoparticles: 2.51 ± 0.24	2.80 ± 0.41
Mode (most abundant) of mRNA payload	Lipophilic complexes: 2 Non-lipophilic complexes: 1	2
Populations	34% ± 8% lipophilic complexes 25% ± 4% non-lipophilic complexes 41% ± 10% empty LNPs	23% ± 8% mRNA-loaded LNPs 77% ± 8% empty LNPs
Particle number concentration*	Lipophilic complexes: $8.56 \times 10^{15} \pm 1.26 \times 10^{15}$ mL^{-1} Non-lipophilic complexes: $6.47 \times 10^{15} \pm 9.74 \times 10^{14}$ mL^{-1} } ~1.51×10^{16} mL^{-1} Empty LNPs: $1.11 \times 10^{16} \pm 5.04 \times 10^{15}$ mL^{-1}	mRNA-loaded LNPs: $1.29 \times 10^{16} \pm 2.22 \times 10^{15}$ mL^{-1} empty LNPs: $4.88 \times 10^{16} \pm 2.49 \times 10^{16}$ mL^{-1}
Encapsulation efficiency	N/A	94.2% ± 3.6% by RiboGreen** 85.6% ± 5.1% by CICS*
Average particle size***	106.3 ± 13.0 nm	120.5 ± 6.0 nm
Zeta-potential***	+45.1 ± 0.9 mV	−6.3 ± 1.3 mV

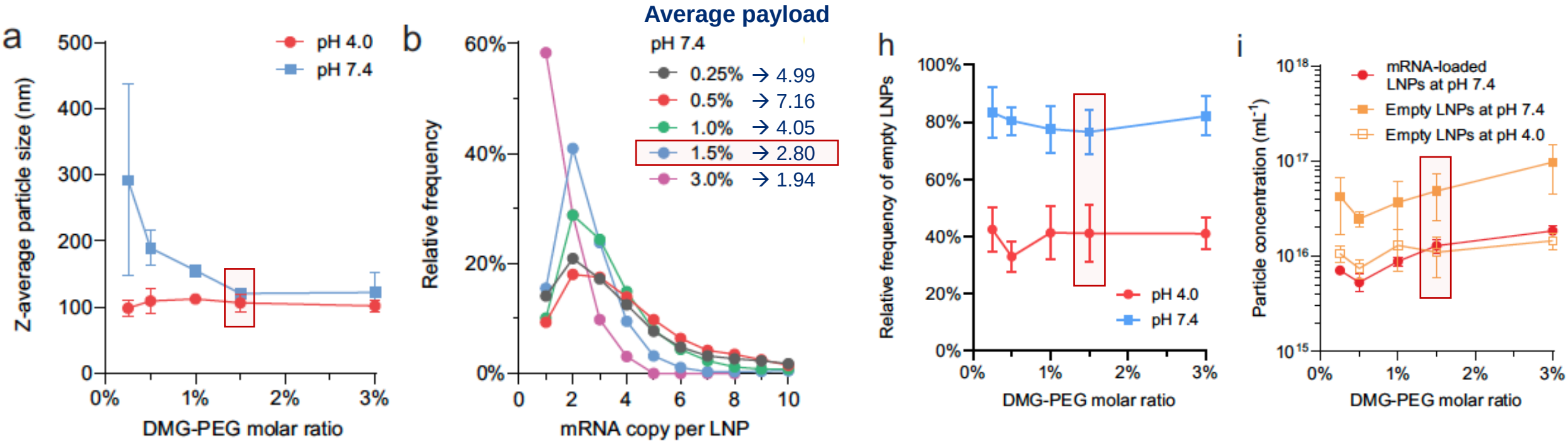
Lipid composition: DLin-MC3-DMA:cholesterol:DSPC:DMG-PEG2000 = 50:38.5:10:1.5.

*The calculations for these parameters from CICS data are detailed in Supplementary Discussion 2–5;

**The assay is described in Methods;

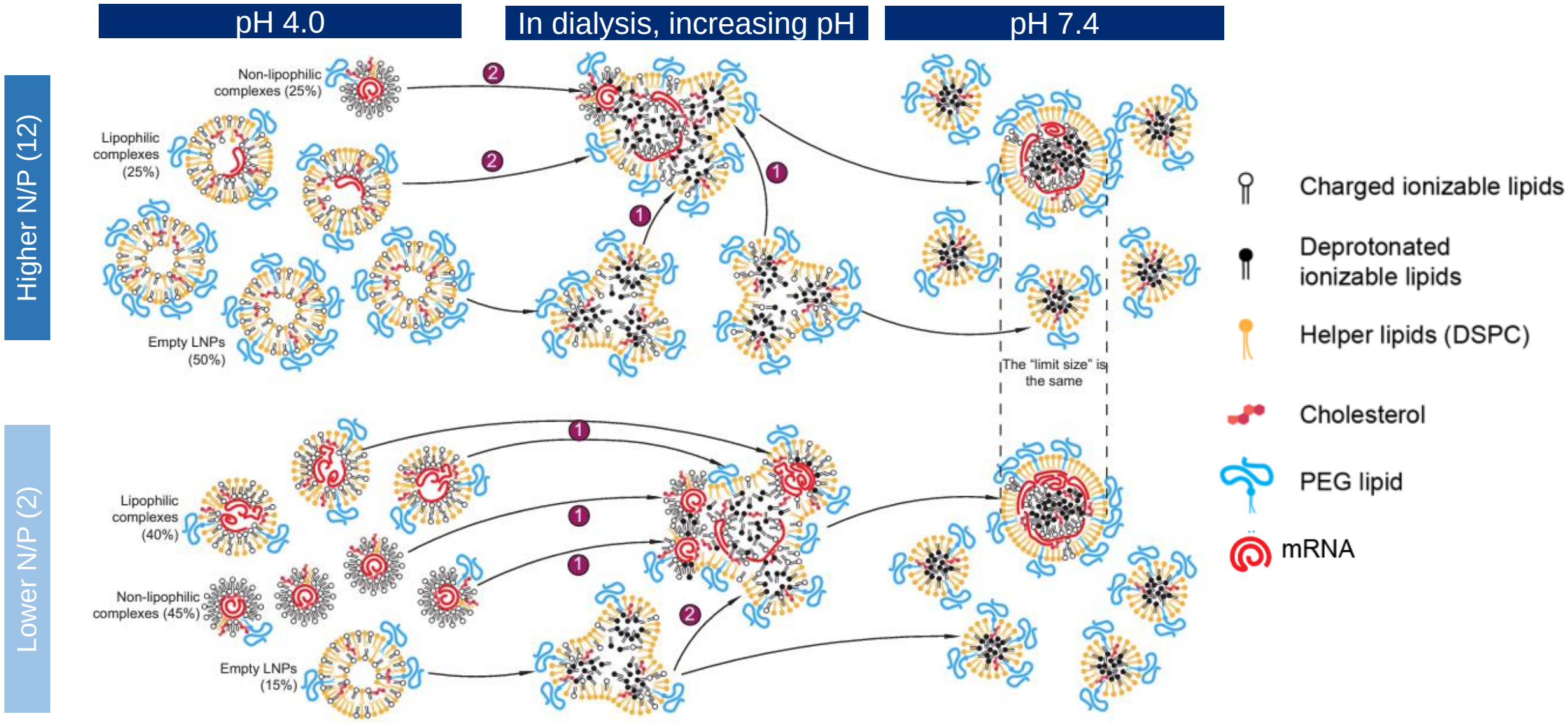
***The particle size is reported as z-average diameter assessed by dynamic light scattering (DLS), that counted all empty or mRNA-loaded LNPs. The zeta-potential was assessed by phase analysis light scattering (PALS).

Effect of PEG lipid concentration on mRNA LNP size and payload

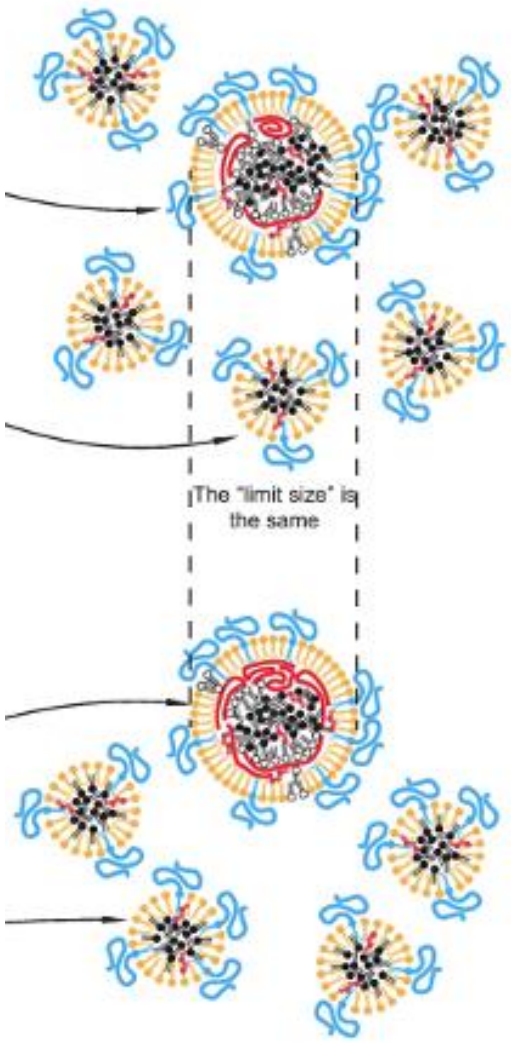


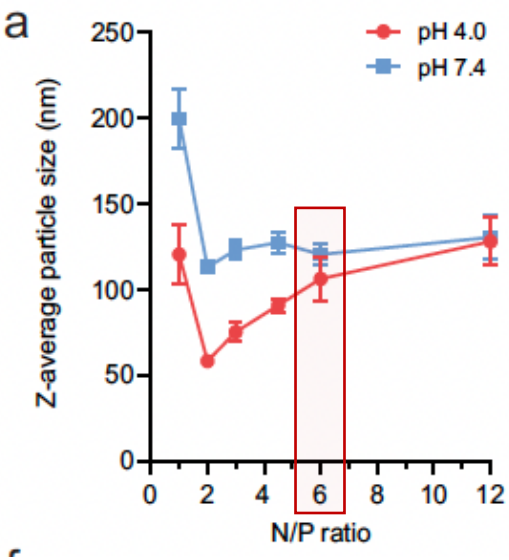
- LNP size decrease and became stable at higher PEG%
- mRNA payload capacity correlates with LNP size
- Relative constant empty LNP ratio at pH 7.4

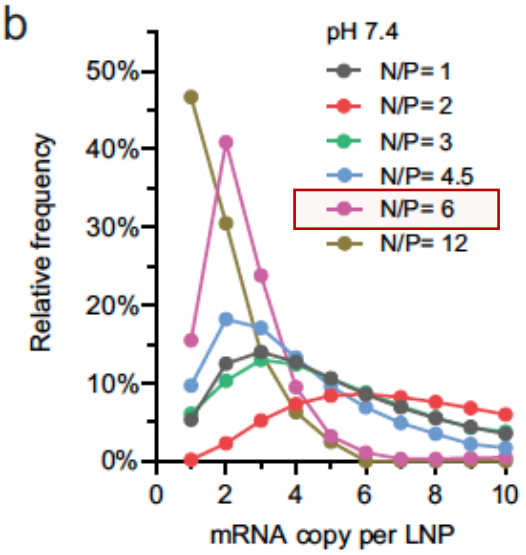
mRNA LNP composition change during filtration: Effect of N/P ratio

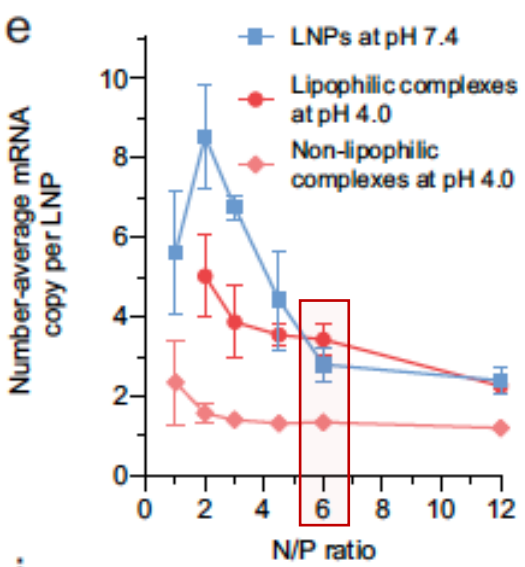


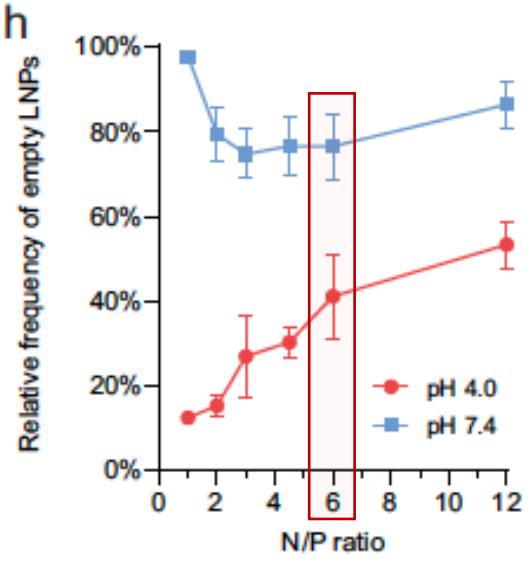
mRNA LNP composition change during filtration: Effect of N/P ratio (Cont.)

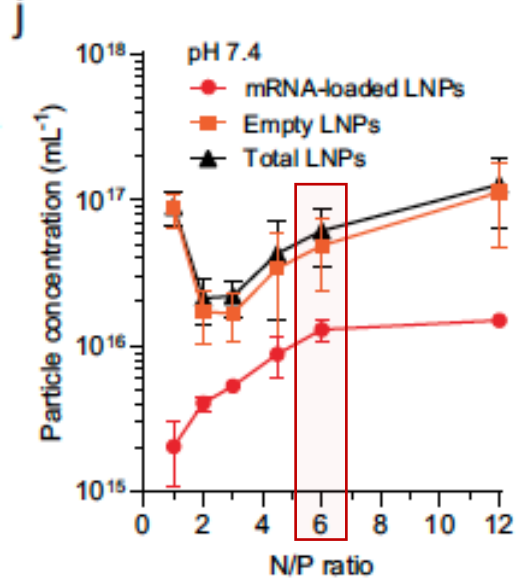












PEG lipid

mRNA

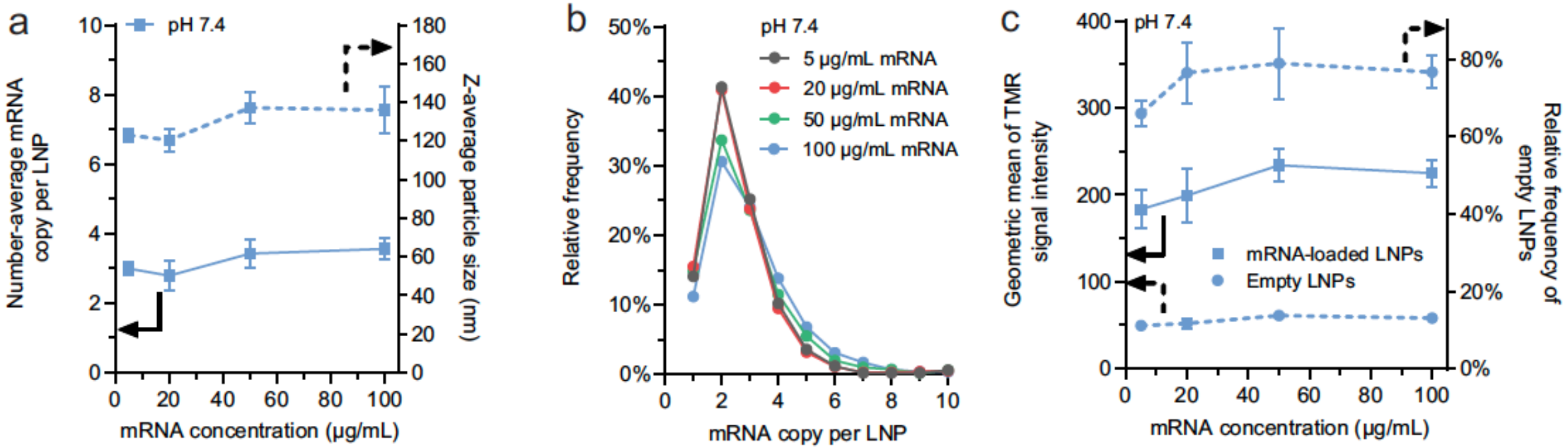
Charged ionizable lipids

Helper lipids

Deprotonated ionizable lipids

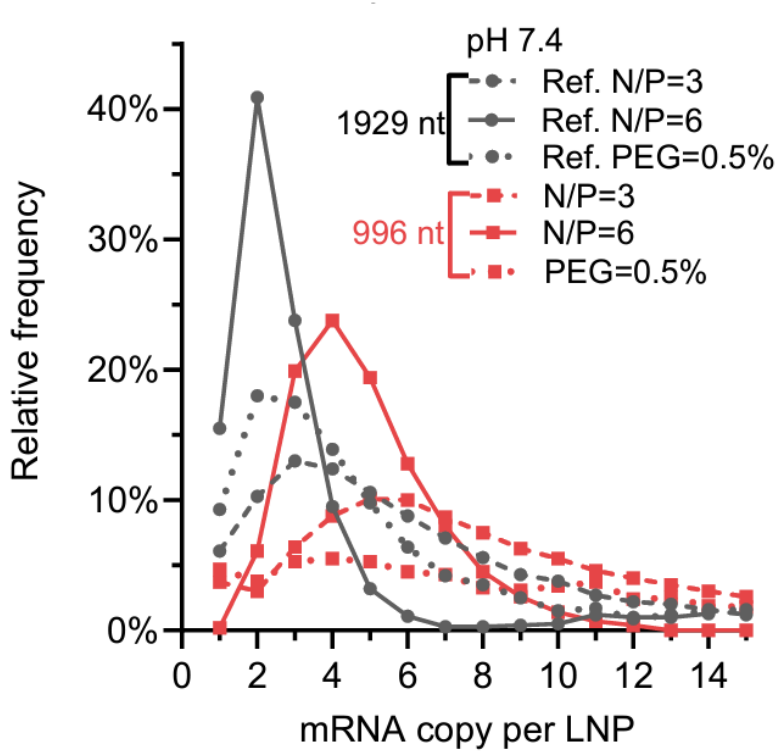
Cholesterol

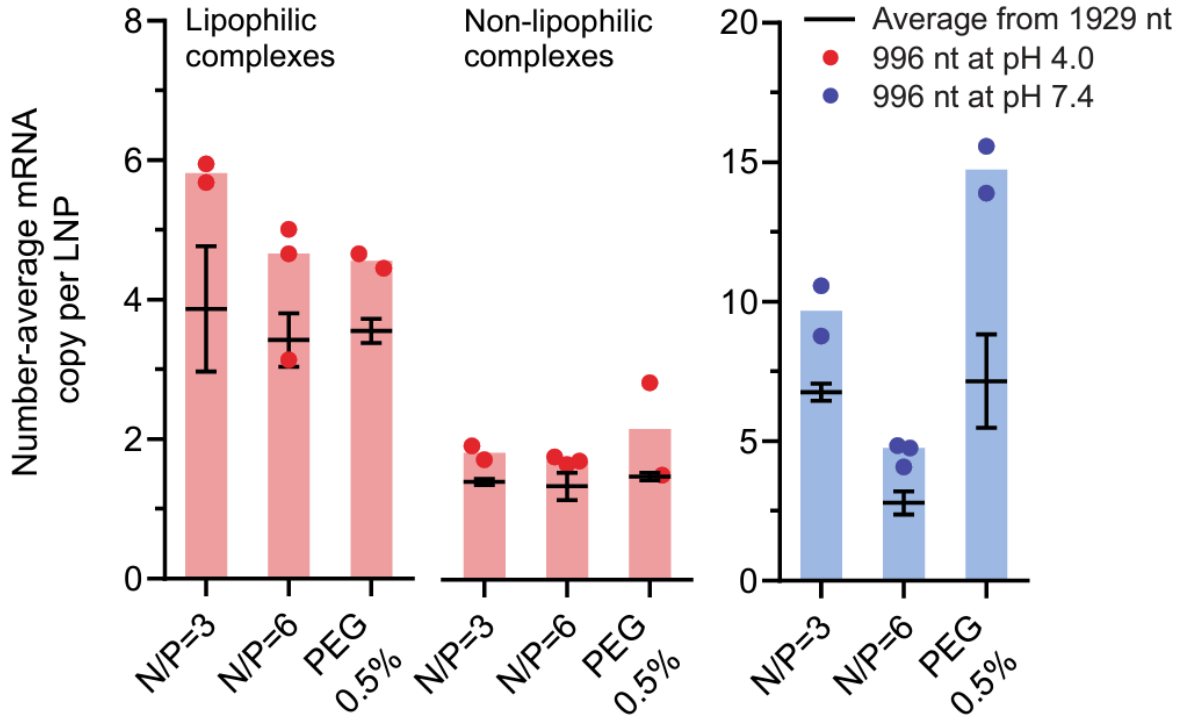
Characterization of payload in mRNA LNP: Effect of mRNA concentration



- Increase mRNA concentration while N/P=6
- mRNA concentration has no obvious effects on :
  - Payload level and distribution
  - Particle size
  - Empty particle percentage

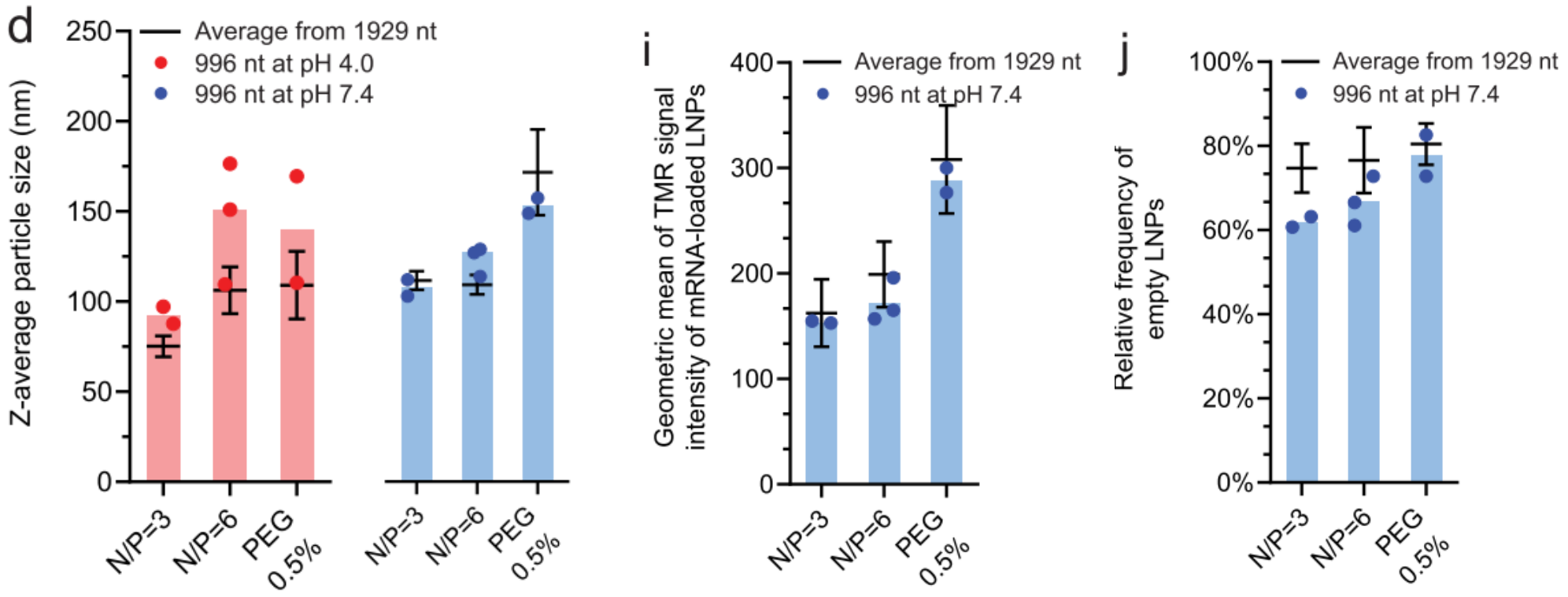
Characterization of payload in mRNA LNP: Effect of mRNA length





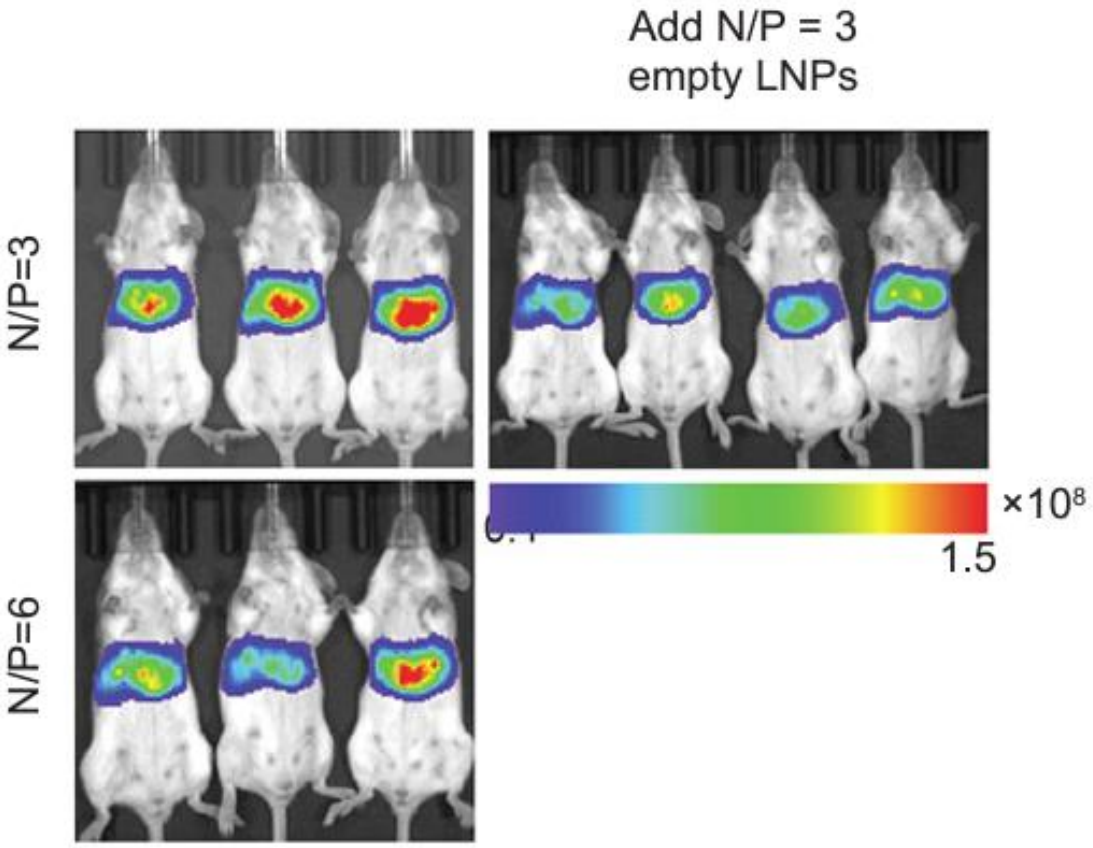
- mRNA length (1929 nt vs. 996 nt) has remarkable effects on payload and its distribution
  - Almost doubled payload for half mRNA length (mode of the payload distribution)

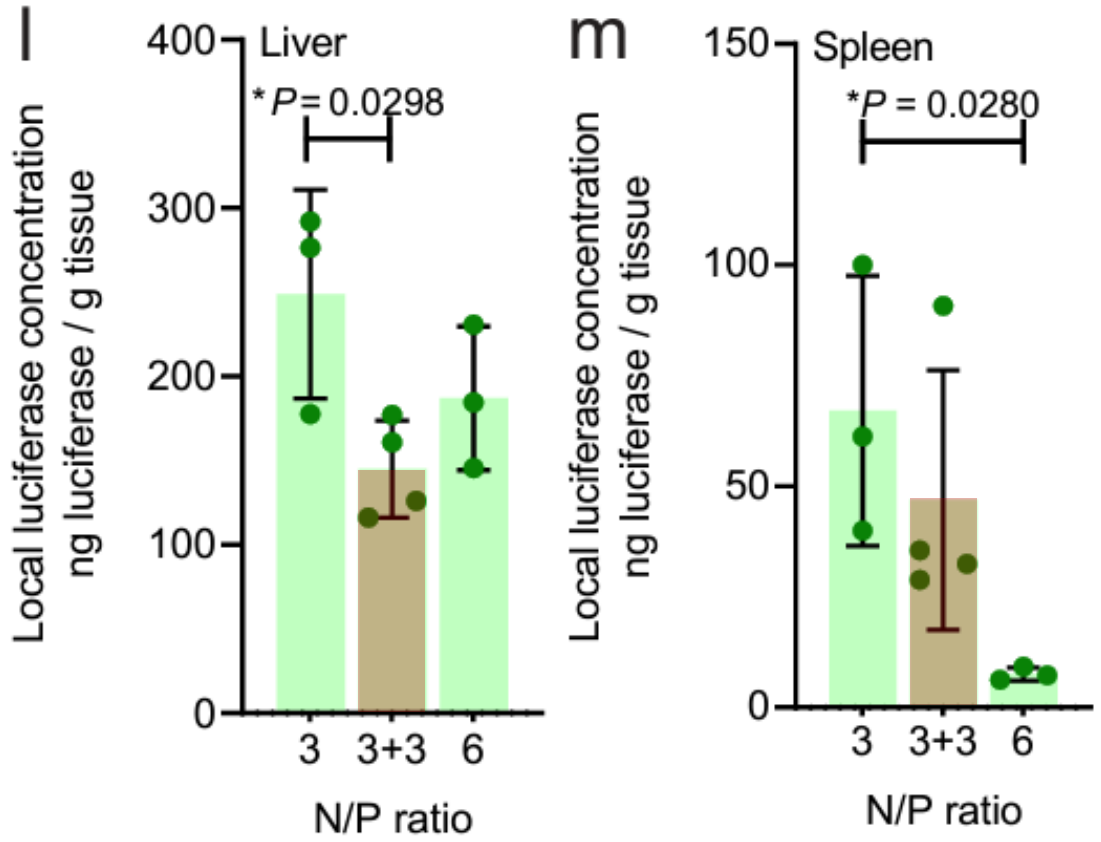
Characterization of payload in mRNA LNP: Effect of mRNA length (Cont.)



- mRNA length (1929 nt vs. 996 nt) has remarkable effects on payload and its distribution
  - No obvious change in LNP size @ pH 7.4
  - Helper lipid content of mRNA-loaded LNPs at pH 7.4 similar for two mRNA sizes
  - LNP assembly influenced by the lipid-to-mRNA mass (N/P) ratio, rather than mRNA concentrations.
  - Fraction of empty LNPs decreased for shorter mRNA

Characterization of payload in mRNA LNP: Effect of empty LNP





- N/P =3 & 6, have ~75% empty LNPs, N/P 3+3 with ~ 86% empty LNPs
- N/P = 6 contains ~ 50% mRNA payload compared to N/P = 3
- Fraction of empty LNPs and payload capacity may influence the transgene expression profile

Adjuvant effect of Empty LNP in mRNA vaccine

communications biology

ARTICLE

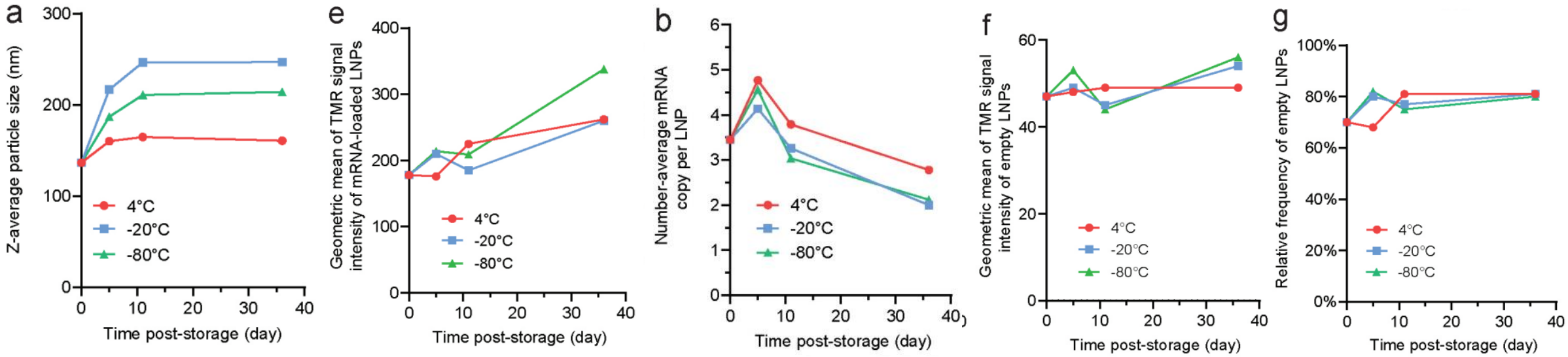
https://doi.org/10.1038/s42003-023-04555-1 OPEN

Lipid nanoparticles (LNP) induce activation and maturation of antigen presenting cells in young and aged individuals

Jennifer Connors[1,2], David Joyner[2,3,8], Nathan J. Mege[4,8], Gina M. Cusimano[1,2], Matthew R. Bell[1,2], Jennifer Marcy[3], Bhavani Taramangalam[1,2], Kenneth M. Kim[1], Paulo J. C. Lin[5], Ying K. Tam[5], Drew Weissman[6,7], Michele A. Kutzler[1,2], Mohamad-Gabriel Alameh[6,7]✉ & Elias K. Haddad[1,2]✉

- Drexel Univ., Acuitas Therapeutics and Upenn researchers discovered
  - Empty LNP (eLNP) could induce maturation of monocyte derived dendritic cells (MDDCs)
  - eLNP could lead to lower immune responses to SARS-CoV-2 mRNA-based vaccines

Monitoring stability of mRNA LNP at different storage conditions



- **Conditions**:
  - LNPs formulated with 5% sucrose as cryo-protectant
  - Thaw and test up to 36 days
- **Observations**:
  - mRNA LNP size increased over time (liposomal fusion): -20°C > -80°C > 4°C
  - Leakage of encapsulated payload: -20°C ≈ -80°C > 4°C
  - Empty LNP percentage and helper lipid content remains stable

Summary

- **Developed a multi-parametric analytical technique to characterize mRNA lipid nanoparticle**
  - Single nanoparticle analysis based on multi-color fluorescence
  - Multiple CQAs reported: mRNA payload distribution, EE%, empty NP%, lipid composition, stability, particle concentrations
  - High sensitivity, high throughput and low sample consumption for mRNA LNP formulation screening
- **Investigated the dynamic assembly mechanism of mRNA LNP during filtration and purification**
  - Provided mechanistic explanation of LNP composition change during the process
  - Analyzed the effect of formulation factors (N/P, PEG%, mRNA length etc.) on mRNA payload and its distribution

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Payload distribution and capacity of mRNA lipid nanoparticles

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Check for updates

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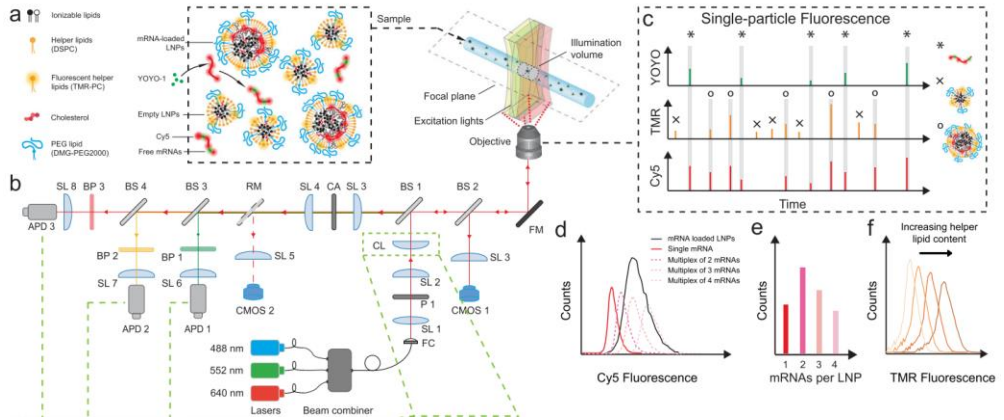

Outlook

- Improve the comprehensive understanding of structure-property-function relation of RNA LNP
  - **LNP size-payload-lipid content correlation**
  - **Multiple RNA co-packaging**
- Further optimize LNP formulation and provide insights to rational drug design and development
  - Methods to reduce empty LNP
  - Dialysis process
  - Antibody conjugation characterization
- Explore the pharmacological effect of payload characteristics of LNP

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Mechanical Engineering
Johns Hopkins University

Prof. *Hai-Quan Mao*, Ph.D.
Material Science & Engineering
Johns Hopkins University

Prof. *Efrosini Kokkoli*, Ph.D.
Chemical and Biomolecular Engineering
Johns Hopkins University

Andrew Li
PhD candidate

Dr. Kuangwen Hsieh
Assistant Research Professor

Fangchi Shao
PhD candidate

Dr. Yizong Hu
Currently Postdoc Fellow
MIT

Jinghan Lin
PhD student

Yining Zhu
PhD candidate

Zachary Schneiderman
PhD candidate

BioMEMS & Single Molecule Dynamics Lab
Johns Hopkins University

me.jhu.edu/thwang/

MAO RESEARCH GROUP

maogroup.jhu.edu

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Develop KNPs with precision control of composition, size, and payload capacity for macromolecular nanotherapeutics.

- Continuous and scalable production
- Payload uniformity control

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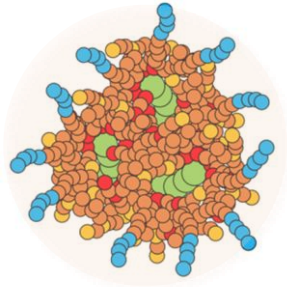
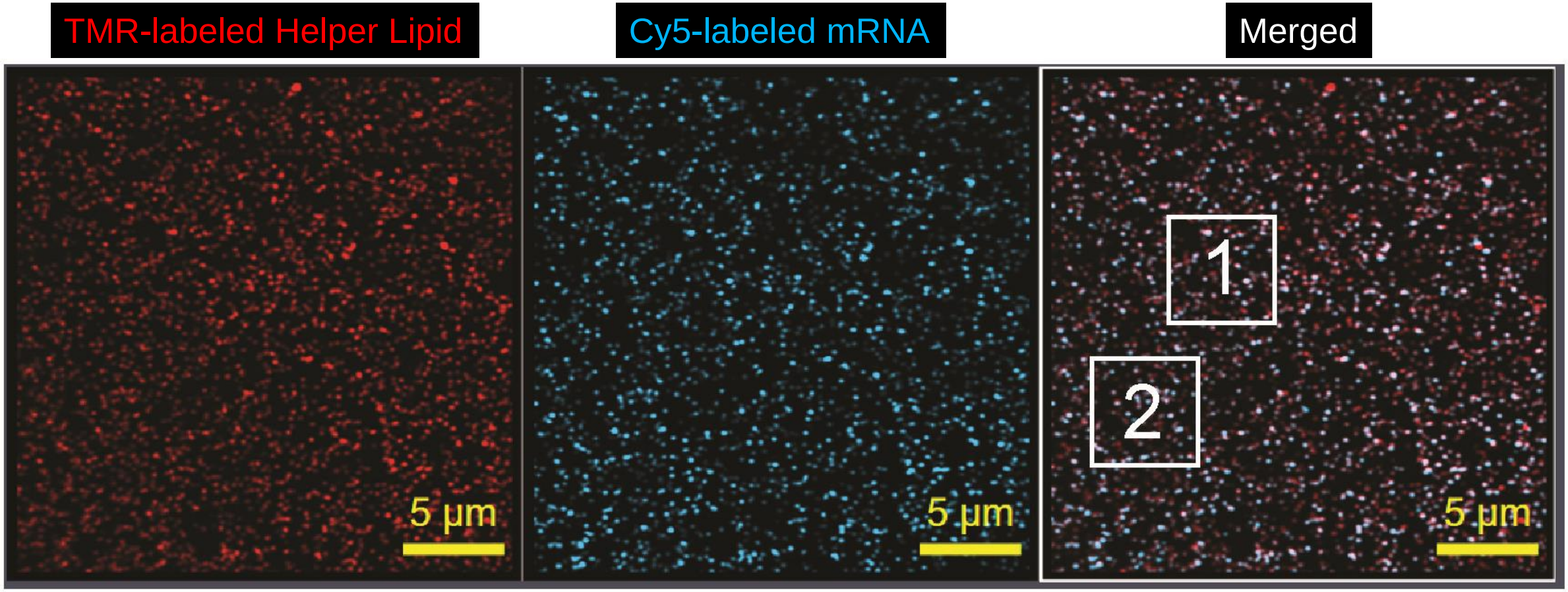
BioMEMS & Single Molecule Dynamics Lab

Johns Hopkins University

me.jhu.edu/thwang/



Airyscan super-resolution confocal microscope imaging of mRNA LNP



Resolution = 120 nm

Airyscan super-resolution confocal microscope imaging of mRNA LNP