NanoImaging Services

Characterization of LNP Payload Distribution and Loading Percentage

Cryo-TEM as an Orthogonal Technique for Lipid Nanoparticle Characterization

Introduction

Distribution of nucleic acid cargo among lipid nanoparticles (LNPs) has important implications for the dosage, efficacy, and safety of a vaccine or therapeutic. Uneven payload distribution may lead to higher than necessary dosage of components such as PEG-lipids, while also making it difficult to predict the amount of RNA or DNA that will reach the cytosol of target cells.

Optimization of an LNP formulation requires reliable methods that can assess key characteristics. While LNP size, zeta potential, and encapsulation efficiency can be measured with established techniques with known strengths and weaknesses, options for determining LNP payload distribution are fewer and less tested.

We sought to develop a cryogenic transmission electron microscopy (cryo-TEM) image analysis method that would provide an estimate of the percentage of loaded and empty LNPs. First, we imaged LNPs prepared with or without mRNA in order to visualize and identify cargo encapsulation. Particles in the mRNA-LNP formulation were then visually classified as loaded or non-loaded based on their internal appearance.

While this provides an estimate of loading percentage, visual classification by an analyst is a subjective process. To automate classification, we developed an analysis method that uses a metric of particle texture and a quantitative loading threshold to estimate the percentage of loaded LNPs. Next, we asked whether payload distribution varied with size or particle type (i.e. unilamellar vs blebbed particles). To test whether the textural analysis method provided internally consistent estimates, we used a second LNP formulation and mixed LNPs prepared with and without mRNA at known ratios.

Finally, we collaborated with Spectradyne to compare cryo-TEM results with payload distribution measurements made using an ARC™ particle analyzer. Before diving into these experiments, we'll first take a look at previous studies which measured LNP loading percentage, existing commercially available methods, and cryo-TEM studies which examined RNA encapsulation.

Studies Measuring LNP Payload Distribution Using Fluorescence

While other methods provide bulk encapsulation efficiency (Oude Blenke, 2018) or size-dependent payload distribution (Jia, 2021), only techniques using single particle fluorescence detection have been used to estimate the percentage of particles that contain cargo (Zhu, 2014, Li, 2022, Li, 2024, Geng, 2023, Münter, 2024).

Payload distribution and its impact on mRNA delivery has been studied using a common 4-lipid component LNP formulation encapsulating fluorescent mRNA (Li, 2022). In this study, the formulation was modified to also include a small percentage of fluorescently tagged helper lipid in order to detect individual particles regardless of loading status. Using a microcapillary flow system, multi-laser cylindrical illumination confocal spectroscopy (CICS), and single-molecule detection, the percentage of loaded particles and the number of mRNA copies per LNP was measured at different N:P ratios and PEGylated lipid molar ratios. After dialysis into a neutral buffer, empty particles made up a majority (upwards of 70%) of total LNPs in all conditions. Loaded LNPs most frequently contained two RNA copies, showing that payload was not evenly distributed amongst particles. The in vivo effect of uneven payload distribution has not been extensively studied, however, when mRNA dose was kept the same, but additional empty LNPs were injected, expression of luciferase mRNA in the liver, but not spleen, decreased (Li, 2022). Even if uneven payload distribution does not affect mRNA delivery in all cases, the excess ionizable and PEGylated lipid dosage associated with empty LNPs has significant safety implications (Wang, 2024). Further exploration of how payload distribution affects cargo delivery is needed, but before that can be done, methods for characterization of payload distribution need to be evaluated and made accessible.

Li and coauthors also used this technique (a microcapillary flow system and CICS) to measure the loading percentage of siRNA-LNPs formulated with different PEGylated lipid molar ratios (PEG mol%) (Li, 2024). Unlike the high number of empty particles found in mRNA-LNPs (Li, 2022), a majority of siRNA-LNPs contained cargo, except at the highest PEG mol% where slightly more than half of the particles were empty. The average number of siRNA copies ranged between ~10-125 copies per loaded LNP and was inversely related to PEG mol%.

Another type of single particle fluorescence analysis was used to compare payload distribution of differently sized cargo (Münter, 2024). LNPs containing fluorescent siRNA, mRNA, or plasmid DNA were immobilized on slides and imaged using confocal microscopy. Analysis showed that the majority of particles were loaded across all three cargo types. Empty particles were more prevalent in plasmid DNA-LNPs (~25% empty) and least prevalent in siRNA-LNPs (~6%), showing a potential relationship between nucleic acid size and payload distribution homogeneity. A lower number of empty particles was also found when mRNA-LNPs were prepared with different tangential-flow filtration (TFF) methods (Geng, 2023). A NanoAnalyzer (NanoFCM) was used to measure the percentage of empty particles after one or two-step TFF in three formulations. For each formulation, two-step TFF produced better payload homogeneity and empty particles accounted for only ~5-20% of all LNPs.

Commercially Available Methods for Loading Percentage Measurement

Three methods which use single particle fluorescence to measure the percentage of loaded particles are commercially available. Two instruments, Spectradyne's ARC particle analyzer and NanoFCM's Flow NanoAnalyzer, use a flow-based system to measure LNPs as they individually pass a fluorescence detector; while ONI's Nanoanalyzer uses super resolution microscopy to precisely locate individual LNPs on a slide. All three methods use a fluorescent nucleic acid stain to detect cargo.

- **Spectradyne's ARC particle analyzer** measures fluorescence when a particle is detected with resistive pulse sensing, which also provides size and concentration information.
- **NanoFCM's NanoAnalyzer** uses light scattering to estimate particle size and concentration. Scattering events and fluorescence peaks are correlated to detect empty and loaded LNPs as well as the presence of free nucleic acid.
- **ONI's Nanoanalyzer** uses dSTORM imaging of fluorescently labelled LNPs to pinpoint the locations of individual particles. Although a dSTORM compatible nucleic acid stain is not available, the less precise fluorescence signal of the cargo can be computationally matched with the dSTORM resolved LNPs.

Cryo-TEM Studies Examining LNP Cargo

Cryogenic Transmission Electron Microscopy (cryo-TEM) is an orthogonal method which can be used to simultaneously evaluate several LNP characteristics. It has been used extensively to characterize LNP size, morphology, and lamellarity. Cryo-TEM has also been used to investigate the presence of RNA cargo in LNPs.

Internal details of LNPs, such as a dense mottled or grainy appearance, are often evident in cryo-TEM images. Association between a dense mottled interior appearance and nucleic acid loading was supported with cryo-TEM imaging of LNPs labeled with thionine, a cationic dye that binds RNA (Brader, 2021).

The presence of RNA has also been inferred from the spacing of repeated structures seen in LNPs specifically designed to form lamellar or hexagonal superstructures (Pattipeiluhu, 2024). Cryo-TEM images of LNPs formulated with and without siRNA were analyzed using fast Fourier transforms to determine lattice spacing. One siRNA-LNP formulation showed two populations with different lattice spacing of structures - one population had spacing similar to that of the formulation without RNA, while the other population showed greater lattice spacing. Because the difference in spacing between populations matched the calculated width of a siRNA molecule, the two particle populations were interpreted as empty and siRNA-filled particles. While this type of analysis is useful for LNP formulations that have regularly repeating structures, it is not relevant to many LNP formulations which have particles with an amorphous lipid phase.

To test whether cryo-TEM could feasibly be used to estimate loading percentage, we analyzed images of LNPs using both a visual semi-quantitative and fully quantitative approach. In collaboration with Spectradyne, the same samples were measured with an ARC particle analyzer to understand how cryo-TEM image analysis compares with a fluorescence-based technique.

Visualizing RNA in LNPs

To visualize RNA in LNPs, cryo-TEM images were collected of two LNPs samples with the same lipid formulation, but produced in either the absence (-mRNA) or presence (+mRNA) of ~860 nucleotide (nt) mRNA. The samples were diluted 2-fold with PBS and applied to a holey carbon TEM grid. Then excess volume was blotted away, creating a very thin layer of sample. Immediately after blotting, the grids were vitrified in liquid ethane, instantly preserving the particles in a near-native, hydrated state.

Particles with different morphologies and appearances were seen in the cryo-TEM images including unilamellar particles with dense interiors associated with a lipid phase; and biphasic particles with a dense, lipid phase and a much smaller and less dense aqueous phase (**Figure 6, pg 8**). Many multi-compartmental particles with blebs were also observed - these particles are recognized by the presence of one or more aqueous compartments (blebs) which protrude from a lipid phase compartment. A bilayer is often visible delineating the outer border of aqueous blebs.

In addition to the overall interior density level that is associated with either an aqueous or lipid phase, a grainy appearance across some or all of a particle's interior can be observed in some LNPs produced in the presence of nucleic acid. This appearance has been correlated with RNA loading in one formulation (Brader, 2021). An assumption of cryo-TEM LNP payload analysis is that this grainy appearance is generalizable to different nucleic acid cargos and lipid formulations. The amount, density, and distribution of the pattern varies among particle interiors even within the same sample (**Figure 1B** and **Figure 3**). In comparison, LNPs made without RNA have particle interiors with a relatively even appearance (**Figure 1A** and **Figure 3**). Some variation in interior density in non-loaded LNPs may be associated with the arrangement of lipids, while the greater levels of variation observed in RNA-LNPs are likely attributable to the added electron density conferred by the presence of nucleic acid.

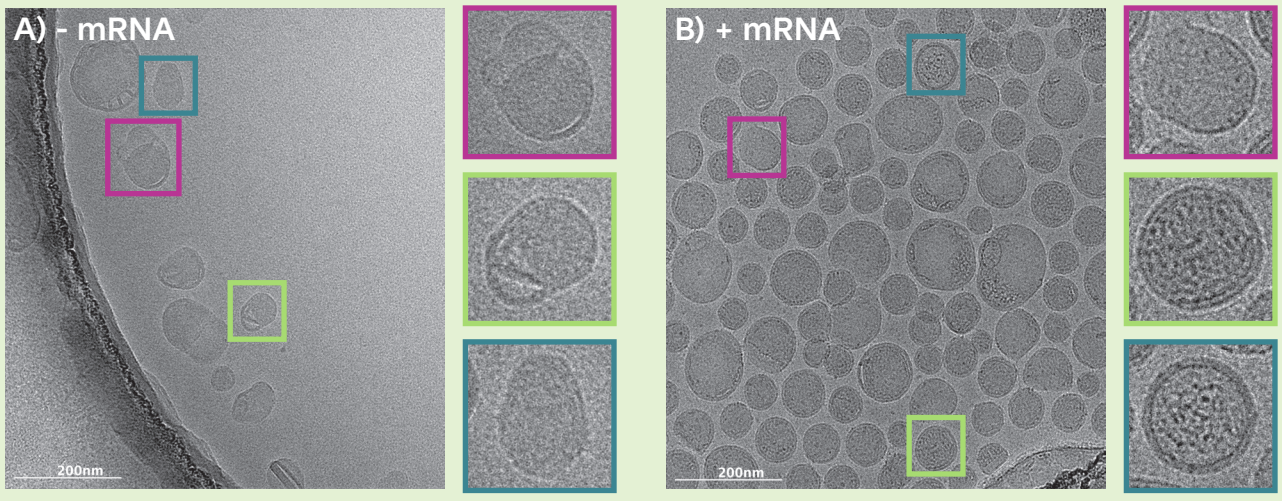


Figure 1 Cryo-TEM images of LNPs produced in the absence (A) or presence (B) of mRNA. Side panels show enlarged boxed regions.

Visual Evaluation of LNP Loading Status in Cryo-TEM Images

Individual particles within a cryo-TEM image can be assessed and classified for visual signs of nucleic acid loading, providing an estimate of the percentage of loaded particles. Particles in the +mRNA LNP images were classified as either Loaded or Non-Loaded by a trained analyst, while the -mRNA LNP images were used as a visual reference for non-loaded particles. 74% of particles in the +mRNA samples had visual signs of RNA loading (**Figure 2**).

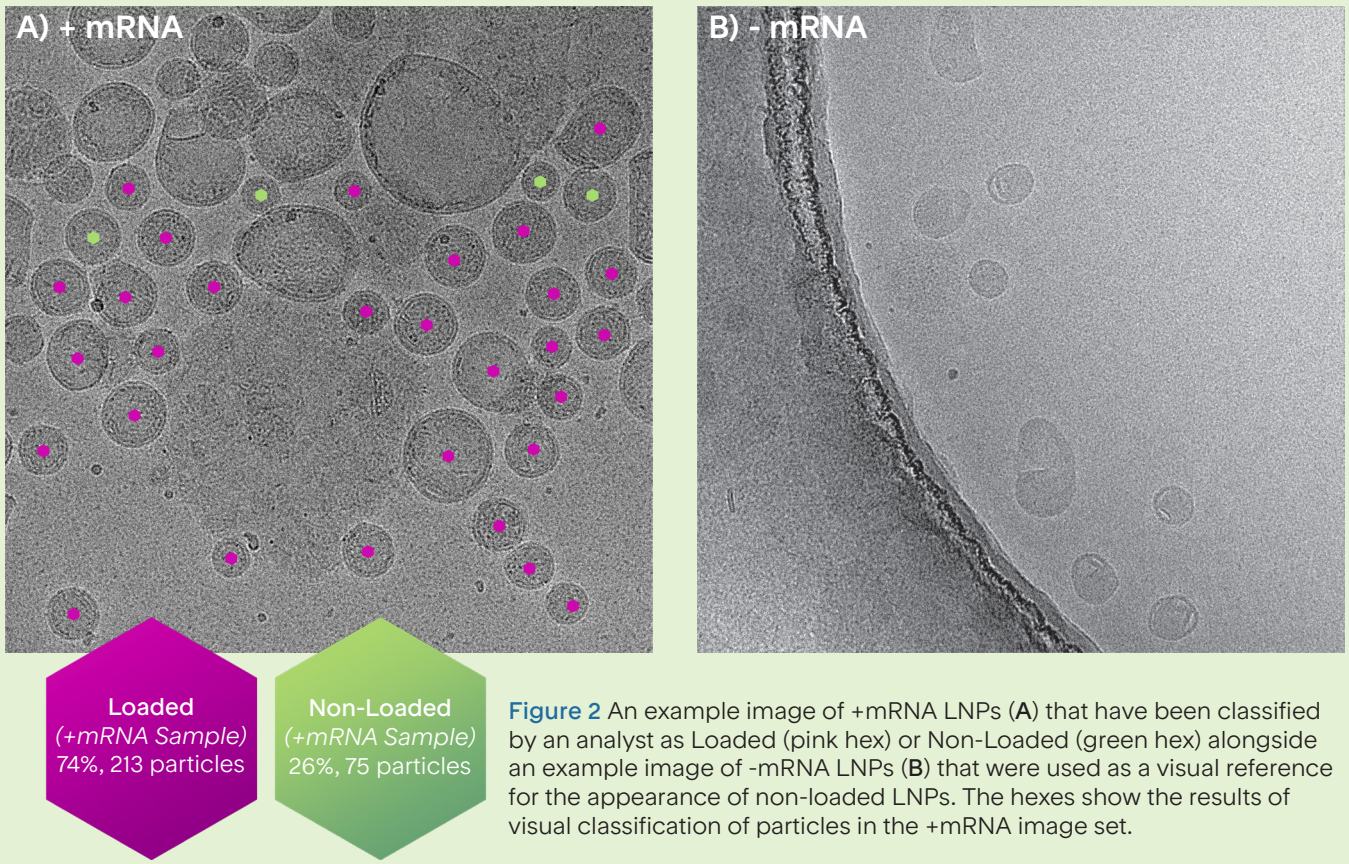


Figure 2 An example image of +mRNA LNPs (**A**) that have been classified by an analyst as Loaded (pink hex) or Non-Loaded (green hex) alongside an example image of -mRNA LNPs (**B**) that were used as a visual reference for the appearance of non-loaded LNPs. The hexes show the results of visual classification of particles in the +mRNA image set.

The classification of a particle based on visual signs of loading is often not straightforward. The densities seen within particle interiors vary in intensity, number, and location (**Figure 3**). All of these aspects of the visual pattern exist along a spectrum, making delineation into two categories challenging. While cryo-TEM images are information-rich and can provide a simultaneous overview of LNP size, lamellarity, morphology, and internal cargo, the subjective nature of visual analysis of loading status brings concerns of repeatability and analyst-to-analyst variation.

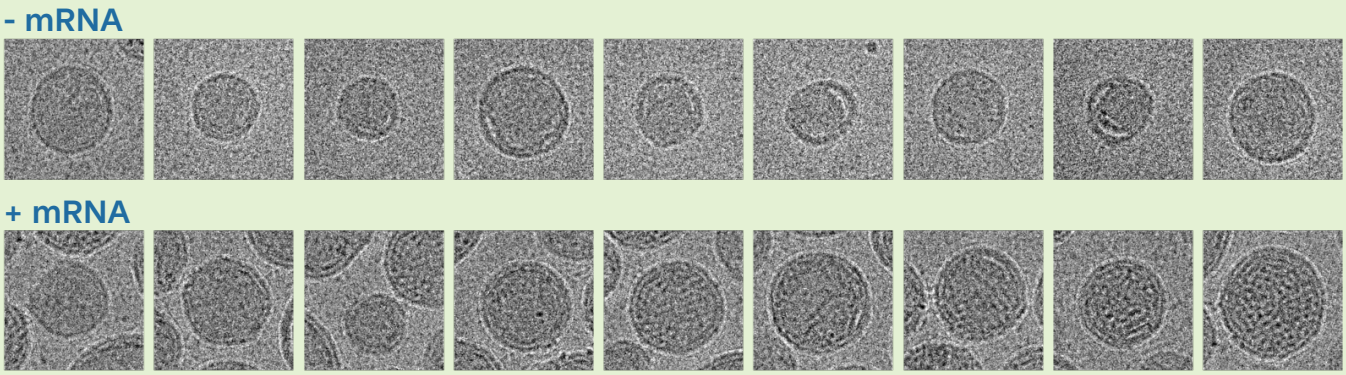


Figure 3 LNPs in the -mRNA sample (top row) have a fairly even interior appearance. In particles with multiple compartments, the appearance in both the lipid and aqueous phases is even regardless of the compartments' overall density levels. LNPs in the +mRNA sample (bottom row) show a spectrum of interior graininess, ranging from a fairly even appearance (bottom, far left) similar to particles in the -mRNA sample, to distinctly grainy (bottom, far right).

Quantitative Estimation of Loading Percentage from Cryo-TEM Images

The grainy pattern observed within RNA-loaded LNPs gives the particle interior a textured appearance relative to the more even appearance of non-loaded LNPs. Quantitative analysis of texture has been used in different biological contexts and imaging modalities because it captures variations in tissue or cellular characteristics that are otherwise difficult to quantify, such as to distinguish between healthy and cancerous mucosa in histology sections (Di Cataldo, 2016; Esgiar, 1998). To improve upon the visual classification of particles, we used a quantitative readout of texture to classify loaded and non-loaded particles.

In both -mRNA and +mRNA image sets, particle interiors were first manually segmented to produce regions of interest (ROI). In particles with blebs, each compartment was segmented individually. Particles that overlapped with other particles or with ice contaminants were not included in analysis. Next, the texture of each ROI was measured using custom automated software. For blebbed particles, the compartment with the highest texture was retained in the dataset and used to classify the entire particle. The quantitative readout of texture was normalized across the full dataset, including particles from the -mRNA (89 particles) and +mRNA (374 particles) samples, and plotted as a histogram (Figure 4A). The -mRNA data was normally distributed and the 95% confidence interval (CI) was calculated. The upper limit of the -mRNA 95% CI, was then used as the threshold, above which particles in the +mRNA dataset were classified as Loaded. 69.4% of particles in the +mRNA sample were Loaded and had texture greater than the -mRNA 95% CI (**Figure 4B**).

These results show that particles imaged with cryo-TEM can be analyzed to provide a quantitative metric of texture and that the texture of most LNPs produced with mRNA exceeded the texture measured in LNPs made without cargo. The loading percentage estimated with the quantitative method is consistent with the visual classification, but has the added benefits of repeatability and a well-defined, objective, threshold for loading.

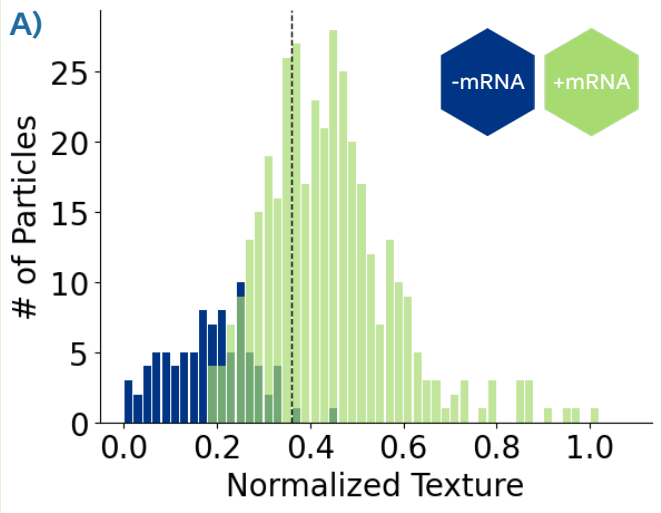


Figure 4 **A)** Histogram of normalized texture in - mRNA and + mRNA samples. The upper limit of the 95% CI (dashed line) of the - mRNA data was used as the threshold for loading classification in the +mRNA sample. **B)** Percentage of particles in the +mRNA sample that were classified as "Loaded" and "Non-Loaded".

Cargo Loading by Particle Size

To see whether loading status classification was related to particle size, the area equivalent diameter (AED) of the ROI(s) for each particle was plotted vs its normalized texture (**Figure 5A**). The size range of particle interiors was similar between particles classified as Loaded and Non-loaded. To visualize the size distribution of the loading classification results, particle internal AED was grouped into 10 nm bins and the percentage of Loaded particles was calculated for each bin (**Figure 5B**). Excluding bins that contained only a few particles, loading percentage ranged between 58-78%. Compared to the overall loading percentage of 69%, it appeared that smaller and larger particles were classified as loaded with similar frequency.

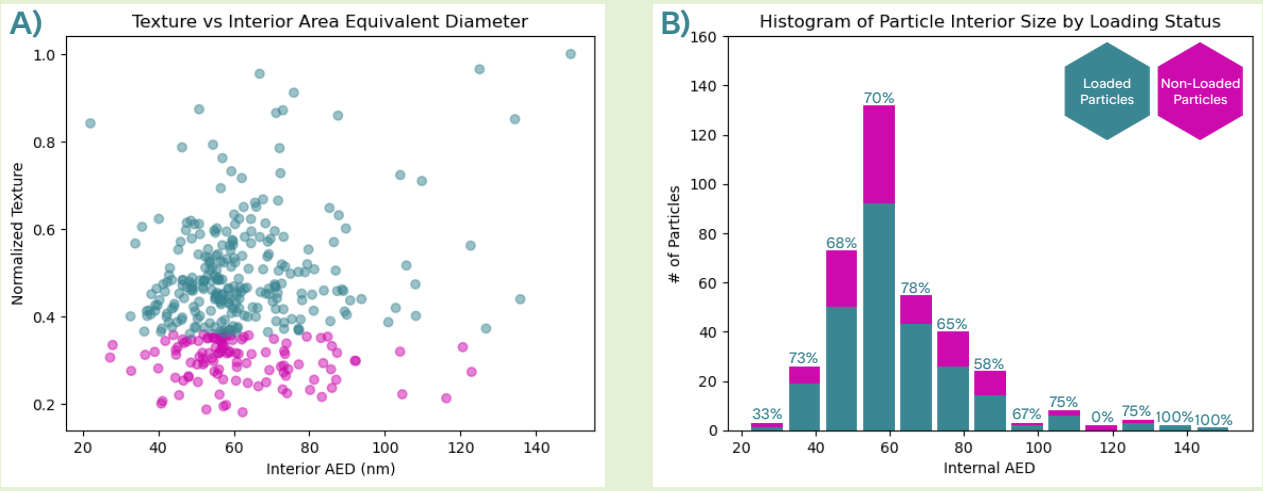


Figure 5 **A)** Interior Area Equivalent Diameter (AED) vs the normalized textural statistic for each particle analyzed in the +mRNA sample. For multicompartment particles, the AED of the constituent ROIs was summed and the ROI with the highest texture was plotted. **B)** A histogram of the data shown in 5A. Values above each bar show the percentage of particles that were classified as loaded for each 10 nm bin.

Cargo Loading by Particle Lamellarity

Of the 373 particles analyzed in the +mRNA sample, 32 had one or more aqueous blebs in addition to a lipid phase. The majority of particles were unilamellar and lipid filled, while a few particles were split between a predominantly lipid phase and a very small visible aqueous phase. Of the blebbed particles, 81.2% were Loaded, and 34.4% of them had two or more compartments (either lipid or aqueous) that were loaded.

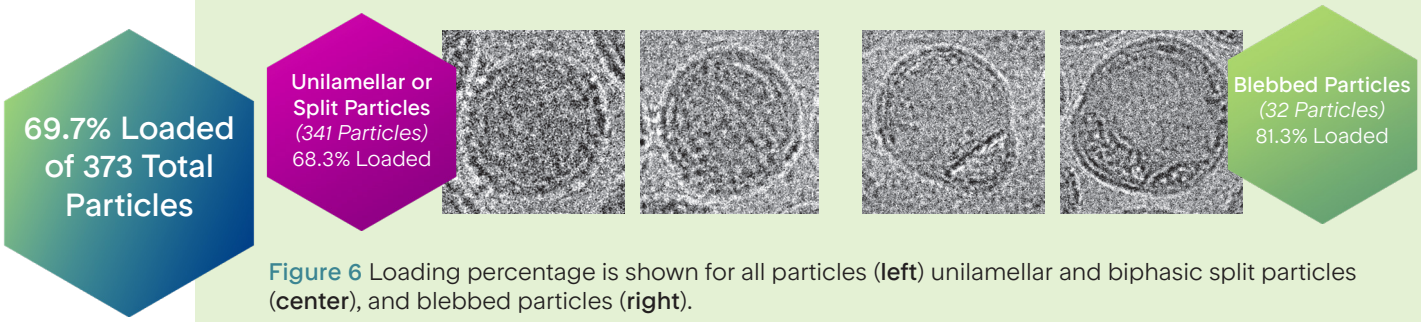
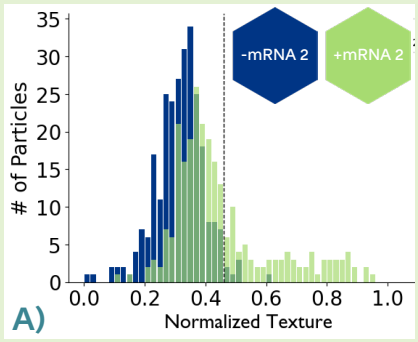


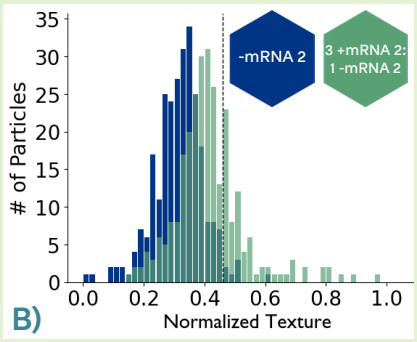
Figure 6 Loading percentage is shown for all particles (**left**) unilamellar and biphasic split particles (**center**), and blebbed particles (**right**).

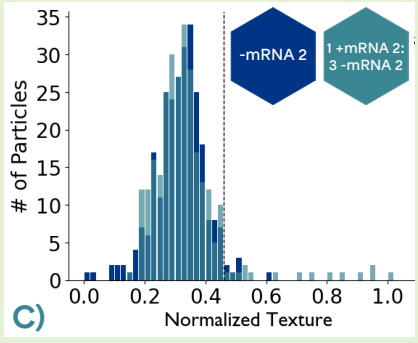
Cryo-TEM Textural Analysis of a Second Formulation

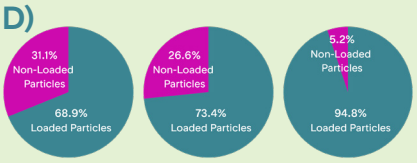
We used the same textural analysis to estimate the percentage of loaded particles in a second lipid formulation containing mRNA cargo ~4100 nt in length (+mRNA 2). A non-loaded control (-mRNA 2) of the same lipid formulation was also analyzed to determine the loading threshold. Additionally, +mRNA 2 and -mRNA 2 were mixed at ratios of 3:1 and 1:3 to test if the loading percentage measured in the mixtures would match the expected percentage based on the loading percentage measured in +mRNA2 and the mixing ratio.

Textural analysis estimated 31.1% of particles were Loaded in the +mRNA 2 sample (**Figure 7**). This percentage was used to calculate the expected percentage of loaded particles in the mixture samples. The results were within ~3% of expected values for both mixtures (**Figure 7E**).









E)

	Expected	Measured
3:1 Mixture	23%	26.6%
1:3 Mixture	8%	5.2%

Figure 7 Histogram of normalized texture in -mRNA 2 alongside **A)** + mRNA 2 **B)** a 3:1 mixture and **C)** a 1:3 mixture of +mRNA 2: -mRNA 2. **D)** The % loaded is shown for all samples along with **E)** the expected and measured % loaded percentage for the two mixture samples calculated from the +mRNA 2 loading percentage and the mixing ratio.

These results show that textural analysis of cryo-TEM images can be applied to multiple lipid formulations and cargo lengths. The overall accuracy of the method is hard to assess in the absence of LNP standard materials, but importantly the results of the mixture experiment show that the method is internally consistent and provides measurements in line with expectations.

Measurement of LNP Loading with Spectradyne ARC Particle Analyzer

Using multiple orthogonal techniques is helpful when attempting to understand LNP loading percentage and payload distribution, especially as a "gold standard" technique has not yet been established. We compared the cryo-TEM results to measurement with Spectradyne's ARC particle analyzer, which uses a microfluidic chamber and fluorescence detector to quickly determine the percentage of loaded particles using a small sample volume. The instrument uses microfluidic resistive pulse sensing (Fraikin, 2011) to determine the size distribution and concentration of a sample, while simultaneously measuring fluorescence signal from individual particles. With a known fluorescence limit of detection as the threshold, the brightness of each particle is used to classify it as Full or Empty.

The - mRNA and + mRNA samples were both analyzed with the ARC instrument by staining with the intercalating nucleic acid stain SYTO 9, incubating for 45 minutes, diluting 100-fold, and then loading onto a microfluidic cartridge with a ~65-400 nm size range. Alongside the stained samples, unstained samples were also measured to test for any non-specific fluorescence signal.

Both stained and unstained -mRNA analysis showed a very small percentage of particles that had fluorescence above the detection threshold (**Figure 8A**). Similarly, the unstained +mRNA sample had only 1.7% of particles above threshold. In contrast, 47.6% of particles in the stained +mRNA sample were SYTO9-positive and classified as Full (**Figure 8B**). Loading analysis was restricted to particles larger than 85 nm measured diameter: Below 85 nm the loaded particles were not sufficiently bright for the full population of loaded particles to be resolved from the noise and for an accurate measurement of loading to be obtained.

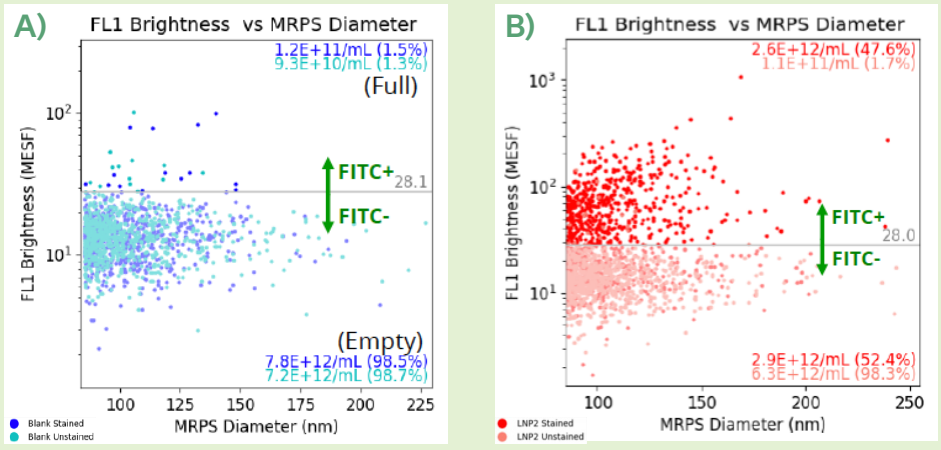


Figure 7 Diameter and fluorescence particle measurements of -mRNA (**A**) and +mRNA (**B**) samples that have been analyzed with or without SYTO9 staining.

Comparison of LNP Payload Distribution Estimates

Payload distribution of an LNP formulation is an important characteristic but is challenging to assess. An ideal method would accurately and specifically measure different types and lengths of payload, be sensitive enough to detect one copy of nucleic acid, quantify the number of copies encapsulated in each particle, measure payload distribution as a function of particle size and lamellarity, and work across different LNP formulations. Currently, no method has demonstrated all of these capabilities, but development and testing are ongoing.

ARC analysis measured 47.6% of particles were loaded, while cryo-TEM textural analysis estimated 69.4% were loaded. Comparison of more samples with different payload types and lengths would be helpful to understand the differences between the two methods. Like any method, payload distribution measurements using ARC and cryo-TEM textural analysis have strengths and weaknesses which should be kept in mind when interpreting results.

Specificity for Payload

Measurement with SYTO9 and the ARC particle analyzer has been shown to be very specific for nucleic acid cargo. SYTO9 specificity for nucleic acid has been well established, and the current experiment shows only a small percentage of false positives detected in the stained -mRNA sample (Figure 6A). On the other hand, cryo-TEM analysis relies upon textural differences that lack nucleic acid specificity. While the presence of nucleic acids in particles adds density which contributes to the particle texture, texture may also be influenced by other factors, such as lipid arrangement or presence of background contaminants or material.

Payload Detection and Sensitivity

The lower limits of payload detection for the cryo-TEM and ARC methods are difficult to judge. In the current experiments, it is unknown how many copies of mRNA must be present in a particle before it is sufficiently different in texture or fluorescence to be classified as loaded. This detection limit may be different depending on the size or type of payload. Longer payload and/or more copies would be expected to bind more SYTO9 dye, making it easier to detect. Similarly, longer payloads occupy more space within the particle which could make textural detection easier.

In the ARC dataset, particles below 85 nm were excluded from the determination of loading percentage because it was suspected that detection in smaller particles, which may have fewer copies encapsulated, was not reliable. On the other hand, the cryo-TEM analysis showed smaller and larger particles were classified as loaded with similar frequency.

Cryo-TEM analysis is dependent on the difference in contrast between nucleic acids, lipids, and the buffer. Image contrast is affected by both the thickness of the ice and the presence of cryoprotectants. Complications of cryoprotectants can be avoided by diluting them to less than ~5% or exchanging into a buffer without cryoprotectants just prior to grid preparation. Care must be taken to control for conditions affecting contrast in order to produce an image set that is suitable for loading analysis.

Suitability Across Payloads

SYTO9 is suitable to measure different types of nucleic acid payload and is thought to bind well regardless of nucleotide sequence. In addition, the binding capacity of the payload should scale with the total mass of encapsulated RNA per particle.

A desired metric for LNP loading is mass of nucleic acid per particle. This unit is agnostic to the details of the payload and is therefore a generally reportable analytical metric that does not divulge sensitive proprietary information such as copy length.

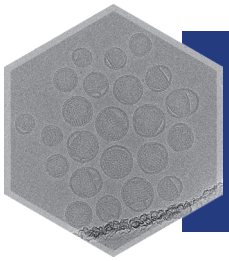
A calibration of fluorescence intensity to mass of RNA per particle is under development at Spectradyne that would permit measurement of this property for LNPs. With this calibration, the limit of detection (always an important parameter for any measurement) will be defined in terms of mass of nucleic acid, rather than units of fluorescence intensity (e.g., FITC-ERF).

Further tests are needed to understand how well cryo-TEM analysis can detect payloads below ~850 nt. If one copy of a shorter RNA often goes undetected, while a single copy of a longer RNA is easily detected, this analysis may not be useful for comparison of loading across payload sizes.

Suitability Across Lipid Formulations

SYTO9 is widely used as nucleic acid dye due to its ability to permeate prokaryotic and eukaryotic cells. Differences in SYTO9 permeability of bacterial species has been shown and is thought to be due to the structural differences of cell envelopes in gram negative and gram positive bacteria (McGoverin, 2020). In comparison to bacteria, permeating LNPs is a simpler proposition. It does not seem likely that differences in types or relative amounts of the lipid components of an LNP formulation would affect the ability of SYTO9 to permeate and interact with the cargo, although this has never been formally tested. It is likely that ARC analysis is suitable for measurement of payload distribution across lipid formulations.

Cryo-TEM textural analysis may be suitable for use in some but not all lipid formulations. Many LNP formulations, including the ones shown here, have particles with an amorphous lipid core with or without an additional aqueous phase. In an amorphous lipid or aqueous phase the densities associated with the nucleic acid provide the texture and pattern we rely on in the visual and quantitative methods presented. However, LNPs can be designed to produce different internal lipid structures (Pattipeiluhu, 2024), which may reduce the sensitivity of loading classification based on appearance or texture. If repeated structures are present in LNPs, analysis of lattice spacing in the cryo-TEM images may be a more appropriate method.



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Conclusion

Current and emerging methods for measuring payload distribution and loading percentage in LNP formulations require further testing. While investigation of these methods is ongoing, single particle fluorescence instruments, such as Spectradyne's ARC particle analyzer, represent the only type of methodology available to measure the percentage of particles which contain cargo. Using textural analysis of cryo-TEM images as a quantitative readout for loading, may represent another method to estimate the percentage of loaded particles.

Because there is no gold standard technique for loading percentage and no standard LNP samples with defined loading percentages to test new techniques, the use of multiple complimentary orthogonal techniques is useful in understanding payload distribution. Further testing will help to determine how different methods and instruments compare and the best use cases for each. Cryo-TEM loading analysis is likely to be suitable for identifying loading differences between batches of the same formulation and can provide added value when used alongside other cryo-TEM analysis methods which measure particle size, lamellarity, and morphology.

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