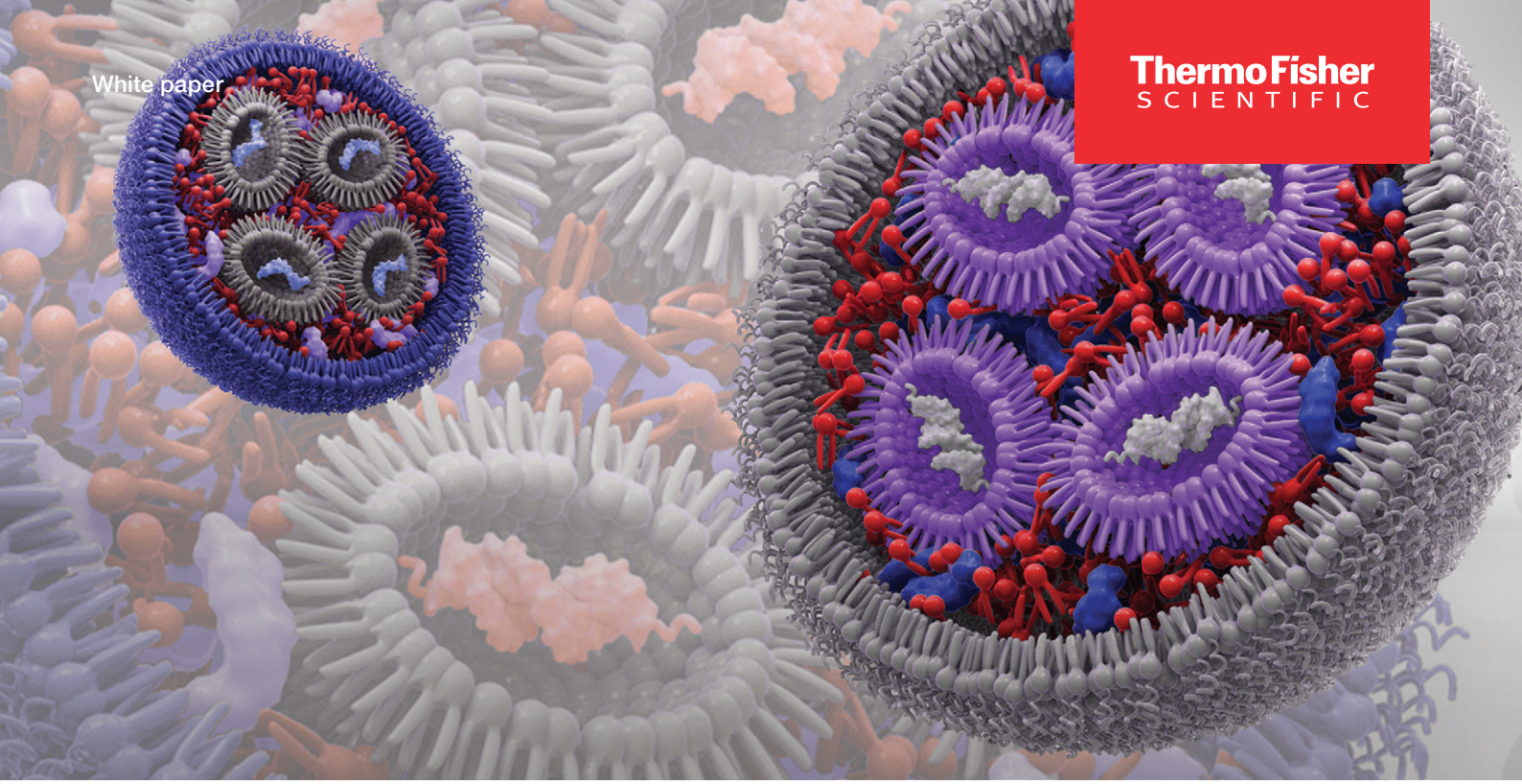

White paper

Advancing LNP-based vaccine and therapeutic delivery

Cryo-TEM and automated image analysis improve LNP characterization

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Keywords

Cryo-TEM, lipid nanoparticle, gene therapy, vaccine, LNP characterization, CQA, Amira Software

Introduction

The rapid expansion of nucleic-acid-based therapeutics and vaccines has positioned lipid nanoparticles (LNPs) as one of the most critical delivery platforms in modern drug development. Since their pivotal role in the first mRNA COVID-19 vaccines, LNPs have become central to a wide range of therapeutic modalities, including mRNA vaccines, siRNA drugs, gene-editing systems, self-amplifying RNA, and DNA delivery.[1-5] The clinical and commercial success of LNP-based drugs, such as Onpattro, Comirnaty, and Spikevax, has further demonstrated the platform's versatility and therapeutic potential.[6-9] Between 2002 and 2024, ~189 clinical trials have been conducted, covering ~132 unique mRNA modalities across 18 disease areas.[10] This growing demand necessitates advanced characterization techniques that can enhance the performance, efficacy, and safety of development processes.

This white paper explores the use of a cryo-transmission electron microscopy (cryo-TEM) with an automated image analysis workflow for the comprehensive characterization of LNP formulations, aiming to enhance our understanding and optimization of these critical therapeutic delivery tools.

Significance of LNP formulation quality

LNPs are small, spherical structures, broadly 20 to 200 nanometers in size, which are generally composed of a combination of ionizable lipids, phospholipids, cholesterol, and PEG-lipids (Figure 1). They are most often used to encapsulate and protect nucleic acid therapeutics, such as mRNA and siRNA, whose efficacy and safety are intrinsically linked to the physical properties of the LNPs (i.e., size, shape, and internal structure). This is because these attributes directly impact biodistribution, cellular uptake, payload release kinetics, immunogenicity, etc.[11-14] Stability issues, influenced by various factors such as temperature, freeze-thaw cycles, ionic strength, adsorption, pH, and shear stress during manufacturing, often lead to product loss and reduced infectivity.[15,16]

Comprehensive characterization of these properties is a critical step in the development and manufacturing of LNP-based products. Traditional ensemble light-scattering techniques, such as dynamic light scattering (DLS) and related optical methods, measure only bulk hydrodynamic properties and cannot resolve the morphological heterogeneity or structural variations that can impact LNP performance. These approaches depend on idealized assumptions in their mathematical models and perform best with uniform, well-defined particle populations. As a result, their accuracy and interpretive power decline when applied to heterogeneous systems like LNPs, requiring the use of orthogonal technologies for detailed characterization.[17]

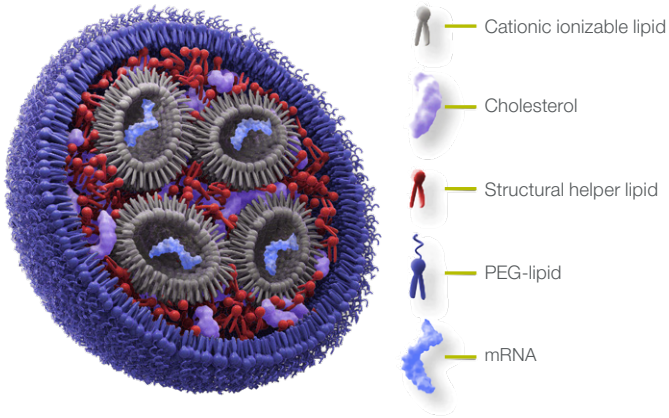


Figure 1. A simplified illustration of an LNP particle.

Regulatory guidelines for the quality of LNP-based formulations

Regulatory agencies across major regions (including the US FDA, the European Medicines Agency, the MHLW/PMDA of Japan, and China's NMPA) consistently emphasize the analysis of several key morphological features to confirm the efficacy and quality of nanoparticle-based therapeutics. This includes critical quality attributes (CQAs) such as particle size distribution, shape, morphology, lamellarity, as well as surface characteristics and internal structure. These properties are expected to be assessed using complementary and orthogonal analytical techniques. (A selection of these regulatory guidelines can be found in references 18–24.)

Advantages of cryo-TEM in LNP characterization

In the pharmaceutical R&D and process development landscape, precise characterization of delivery vehicles at the single particle level can be highly valuable. Cryo-TEM is a powerful technique that allows for the direct visualization of nanoparticles in their native, hydrated state. Unlike traditional negative-stain TEM, which requires chemical fixation or staining (that can distort particle morphology), cryo-TEM rapidly vitrifies the sample in a thin layer of amorphous ice. This flash-freezing process preserves the delicate structures and properties of LNPs in a near-native state.

With cryo-TEM, you can:

- **Estimation of multiple CQAs.** Quantify multiple critical quality attributes, such as shape, size, morphology, and lamellarity, from a single dataset.
- **Accurately analyze size and shape.** Capture detailed, high-resolution images of individual LNPs, which shows the presence of different shapes (e.g., spherical, elongated, or irregular), and enables you to precisely measure particle sizes.
- **Obtain internal, nano-structural insights.** Resolve the internal structure of LNPs, obtaining critical information on lipid packing and lamellarity. This shows how formulation conditions and composition affect internal architecture and, consequently, encapsulation efficiency and cargo release.
- **Assess homogeneity and consistency.** With quantitative assessment of sample homogeneity, you can see batch-to-batch variability, aiding in the optimization of manufacturing processes through improved reproducibility and product quality.
- **Visualize cargo encapsulation.** Observing direct visual evidence of cargo loading and distribution within the LNPs gives critical insights into the efficiency of the encapsulation process.
- **Optimize processes.** Refine manufacturing techniques, such as microfluidic mixing and extrusion, to produce more uniform and effective LNP formulations.

The cryo-TEM workflow

To begin, the LNP sample is placed in an appropriate buffer solution, which is then applied onto a specialized grid. The sample is then vitrified by rapidly plunging the grid into a cryogenic liquid (typically liquid ethane), converting the aqueous solution into amorphous ice, which preserves the LNPs in their native structural state. The grid is then loaded into a cryo-TEM microscope, where high-resolution images of the LNP particles are collected. Note that the vitrified grid is maintained at cryogenic temperatures throughout handling and imaging to avoid structural changes.

Figure 2. Example workflow combining LNP sample preparation, cryo-TEM imaging, and analysis.

Integrating cryo-TEM imaging with automated particle characterization

One of the major advantages of cryo-TEM imaging is its ability to deliver both high-resolution qualitative visualization and quantitative analysis of LNPs, revealing critical morphological features. Quantitative image analysis enables:

- **Insightful optimization** of LNP formulation and manufacturing parameters
- **Stability assessment** through monitoring of morphological changes over time
- **Classification and quantification** of structural features that influence delivery efficiency, safety, and potency

To further streamline this workflow, Thermo Fisher Scientific has developed a downstream pipeline for automated, AI-driven analysis of cryo-TEM images, leveraging Thermo Scientific™ Amira™ 3D Software. Once trained on curated, annotated cryo-TEM images, algorithms in Amira Software can process large datasets without manual intervention. By automating the extraction of morphological parameters, the workflow eliminates manual bottlenecks, reduces the time from imaging to statistical analysis, and enhances reproducibility. This integrated approach provides a scalable, high-throughput solution that strengthens development, quality control, and optimization of LNP-based therapeutics.

Materials and methods

Sample preparation

The cryo-TEM workflow typically begins with sample preparation of LNPs (Figure 2). Frozen LNP formulations were rapidly thawed at 37°C in a water bath, briefly held at room temperature during vitrification, and stored at 4°C for longer term use (i.e., days to weeks). LNPs are sticky and readily attach to the carbon film on cryo-TEM grids, so only a small number of particles reach the open holes where images are taken. To improve particle distribution in these imaging areas, two grid-modification approaches were evaluated prior to sample application.

A. Grid preparation with poly-L-lysine

Quantifoil R 1.2/1.3 TEM grids (300 mesh Cu) were glow discharged for 30 seconds at 20 mA. The coating solution consisted of poly-L-lysine dissolved in nuclease-free water to a final concentration of 10 mg/mL. 5 µL of the solution was applied to the carbon foil side of each grid and incubated for 30 seconds. Excess liquid was then blotted away with filter paper. 3 µL of LNP sample was subsequently applied to the foil side of the grid, incubated for 30 seconds, and blotted for 4 seconds (0 blot force, 100% humidity, 22°C). The grid was then vitrified in liquid ethane using a Thermo Scientific™ Vitrobot™ Mark IV System.

B. Grid preparation with a continuous carbon support film

Quantifoil TEM grids R 1.2/1.3 with a 3-nm continuous carbon layer (300-mesh Cu) were evaluated. Grids were glow-discharged under gentle conditions for 30 seconds at 7 mA to avoid damage to the carbon films. 3 µL of LNP sample was applied to the foil side, incubated for 30 seconds, and blotted for 2 seconds (0 blot force, 100% humidity, 22°C). It is worth noting that thicker continuous carbon can increase background and reduce contrast, so thinner carbon films (e.g., 2 nm) may be used when appropriate, though they are more fragile.

Note: LNP formulations exhibit sample-dependent behavior, so certain parameters, such as sample concentration (typically screened between 1–10 mg/mL) or blotting conditions (e.g., blot times of 2–6 seconds), may require individual optimization. The values above are provided only as initial guidance, and should be empirically optimized for each new sample.

Cryo-TEM image acquisition

Images of vitrified samples were collected on a Thermo Scientific™ Glacios™ Cryo-TEM at an accelerating voltage of 200 kV with a Thermo Scientific™ Falcon™ 4i Direct Electron Detector operated in counting mode. A 100-nm objective aperture was used to improve contrast. Each image was captured with an exposure time corresponding to a total electron dose of approximately 15–30 e/A^2, with a calibrated pixel size of ~0.20 nm. Grid mapping and image acquisition were performed with Thermo Scientific EPU Software.

TEM grid square selection on the image atlas

Even with optimized grid preparation, LNP samples rarely distribute uniformly across the grid, making careful grid-square selection essential during cryo-TEM imaging. An ice thickness of roughly 100–200 nm is often appropriate, but depends on the size of the LNP.

For the poly-L-lysine–treated grids, early screening included squares with varying apparent grid square sizes and ice thicknesses in order to determine which regions provide the best imaging conditions (Figure 3).

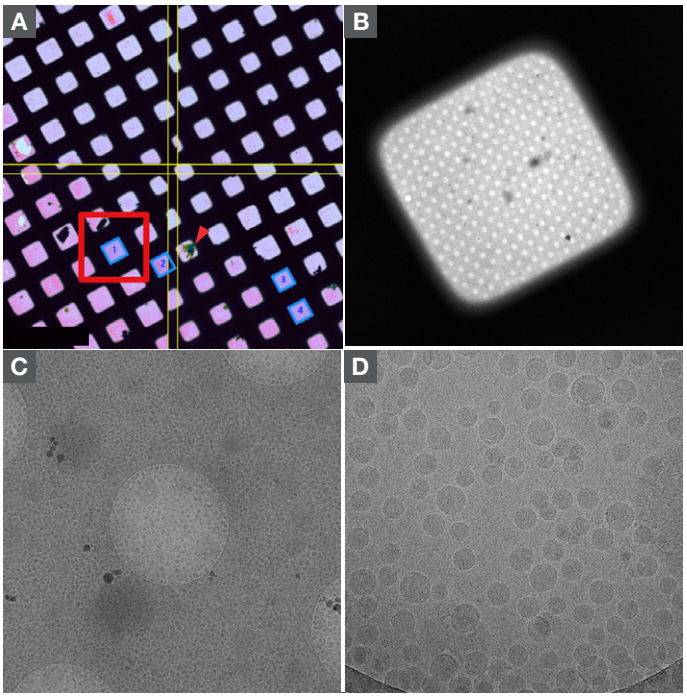


Figure 3. Screening of poly-L-lysine treated grids with EPU Software. A) The color gradient function is used to select grid squares with a range of ice thicknesses, while excluding regions where ice is too thin (uniform light pink) or too thick (green patch, red arrow). Representative images are shown at (B) grid-square, (C) foil-hole, and (D) data-acquisition magnifications.

Continuous carbon grids, meanwhile, generally have thinner ice compared to the poly-L-lysine–treated grids (Figure 4). As a result, grid squares with patches of slightly thicker ice could be selected, as these regions can yield higher particle density. These areas can be easily identified using the color gradient function in EPU Software (Figure 4A).

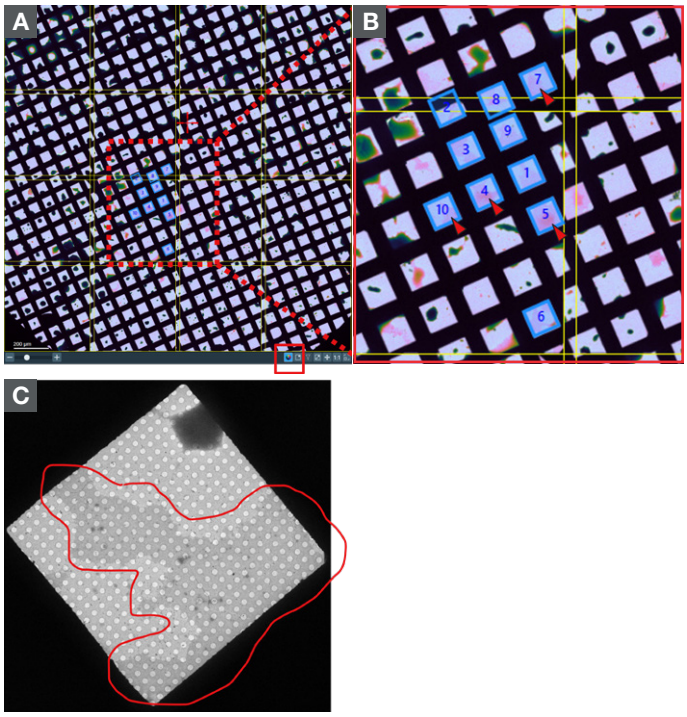


Figure 4. Screening of continuous carbon grids with EPU Software. A) Using the color gradient function (highlighted with a solid red box), grid squares with patches of slightly thicker ice can be easily identified. B) Close-up where these ice patches (pink) are indicated by red arrowheads, clearly differentiated from thinner (light pink squares) and very thick ice (green patches). C) Typical appearance of the desired ice patches (marked with a red outline) at grid-square magnification. The hole selection ice filter should be adjusted to only collect micrographs in this area.

All Thermo Scientific cryo-TEM systems are fully compatible with automated image analysis workflows enabled by Amira Software, as described in the following section. The Glacios Cryo-TEM also features an Autoloader capable of holding up to 12 vitrified grids simultaneously, enabling efficient screening and high-throughput data collection without repeated manual grid exchanges. Combined, this automated workflow reduces operator intervention, minimizes sample exposure to environmental conditions, and greatly accelerates multi-grid imaging campaigns.

Note that instruments such as the Thermo Scientific™ Talos™ 12 TEM and Thermo Scientific™ Tundra™ Cryo-TEM can also be used for LNP imaging. However, they only support single-grid loading and require manual grid insertion and removal for each dataset.

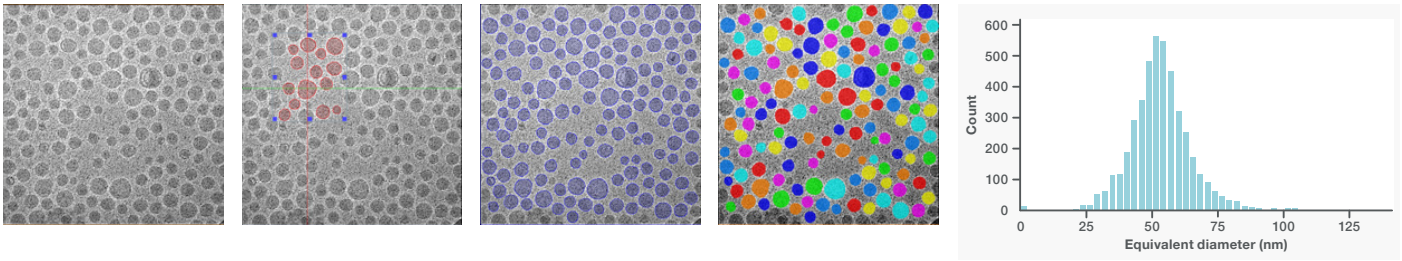


Figure 5. The LNP particle-picking and analysis workflow in Amira 3D Software.

Automated cryo-TEM image analysis with Amira 3D Software

Image analysis was performed with Amira 3D Software. A recipe was used to automate the workflow; this provides a predefined sequence of processing steps/operations and includes all the necessary parameters and instructions for tasks such as image enhancement, segmentation, and analysis (Figure 5).

First, the image is filtered to homogenize background intensities. This is followed by particle segmentation using an AI deep-learning based approach. Here, a small subpopulation of the data is semi-manually annotated for lipid nanoparticles using the AI-assisted selection tool in Amira Software. A convolutional neural network (i.e., a UNet with a VGG19 backbone[25,26]) is then trained on these annotations to recognize LNPs. The trained model is subsequently used to automatically detect LNPs across the entire dataset. After segmentation, the detected particles are separated and uniquely identified, enabling automatic, per-particle measurement of relevant CQAs. Finally, these measured CQAs can be statistically analyzed, and corresponding graphs generated in Amira Software.

Results

Grid modifications and their effect on LNP imaging

To overcome the strong affinity of the LNPs for the carbon support of the grids, two complementary strategies were tested in this white paper. First, the lipid concentration was increased to approximately 1–10 mg/mL to improve the likelihood that sufficient particles partition into the vitrified ice. Second, the grid surface chemistry was optimized to promote particle distribution into the holes. Two approaches were evaluated:

Chemical passivation

The success of passivating agents relies heavily on the surface charge of the LNPs. Treating grids with positively charged poly-L-lysine enhanced particle partitioning of the LNPs due to their positive surface charge (Figure 6B). Negatively charged poly-L-glutamic acid, meanwhile, led to sample aggregation (Figure 6D).

Ultrathin continuous carbon films

Ultrathin continuous carbon films (2–5 nm) provide additional adsorption sites while still being able to support vitrification that is suitable for imaging. The use of these films can increase overall particle density compared to the standard grids.

These grid modifications highlight the importance of matching the surface properties of the grids to the physicochemical characteristics of the LNP formulations, helping to achieve optimal particle distribution and image quality.

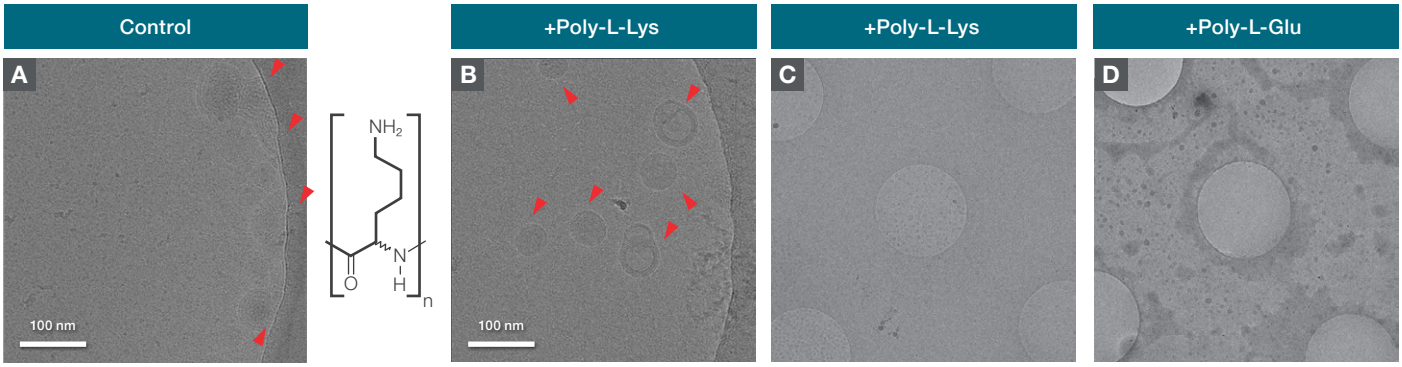


Figure 6. A) On untreated grids, LNPs (red arrowheads) preferentially adhere to the carbon foil. Pre-treatment with poly-L-lysine improves particle partitioning into grid holes and prevents aggregation (B and C). Conversely, passivation with negatively charged poly-L-glutamic acid induces LNP aggregation, with particles largely excluded from the holes and instead accumulating on the carbon surface (D).

A. Rapid qualitative assessment of LNPs with cryo-TEM imaging

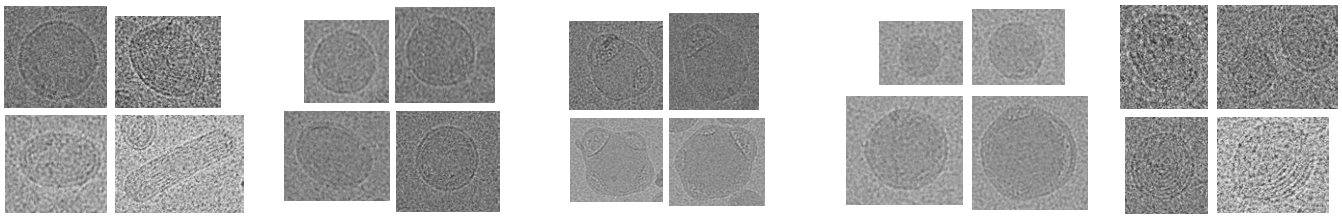
Cryo-TEM imaging enables rapid qualitative evaluation of key CQAs for LNPs. Initial visual inspection of the micrographs provides immediate insight into overall sample quality without requiring extensive downstream analysis (Table 1). Several qualitative features are readily identifiable, including overall particle shape and size distribution, along with morphological characteristics such as the presence of solid-core particles, blebbed or irregular structures, and multilamellar particles. These observations offer an early indication of formulation integrity, sample heterogeneity, and potential stability issues, guiding subsequent quantitative analysis and process optimization.

B. Precise quantification of LNP CQAs via automated image analysis

Quantitative analysis of cryo-TEM image data can provide more detailed and precise characterization. In this study, approximately 74 images were selected and analyzed using the automated workflow described in Figure 5. Through this process, around 4,305 particles were examined for CQAs such as shape, size, surface area, and particle elongation (Figure 7).

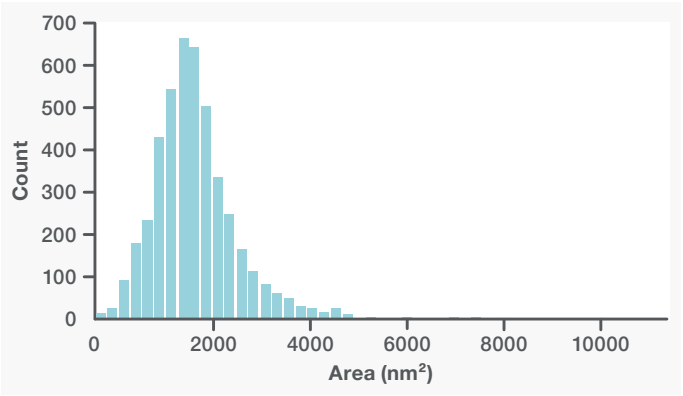
- **Equivalent diameter:** The LNPs exhibit a median diameter of 45.94 nm, with an average of 46.34 ± 10.21 nm, corresponding to a polydispersity index (PDI) of 0.049. This indicates a relatively uniform particle size distribution.
- **Particle area:** The median particle area is 1,658 nm^2, with an average area of 1,769 ± 815 nm^2.
- **Shape and morphology metrics:** Shape factor analysis reveals a median value of 1.02 and an average of 1.06 ± 0.14, indicating that most particles are near-spherical, with slight morphological variation. Elongation values are tightly clustered, with a median of 0.83 and an average of 0.80 ± 0.15, further confirming smooth, continuous particle outlines. Additionally, the elongation graph demonstrates that, although most particles were relatively round, there is a noticeable minority that deviates from this trend and appears more elongated.

Collectively, these quantitative metrics highlight the power of automated cryo-TEM workflows, accurately resolving subtle morphological and geometric variations across large LNP populations.

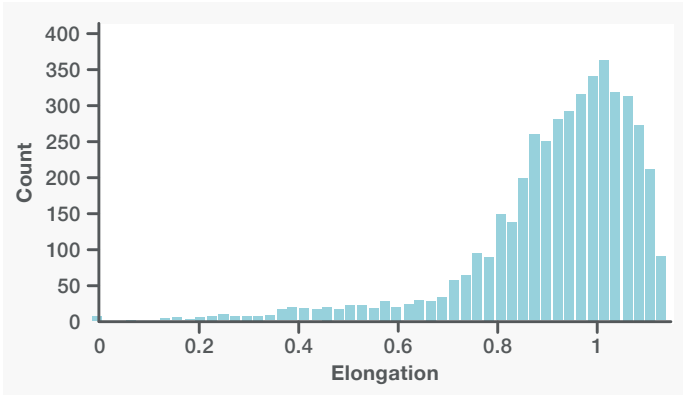


Shape analysis	Solid core	Blebbed particles	Size distribution	Lamellarity
Compare regular round particles to those with irregular shapes.	Evaluate subtle morphological differences, such as blebbed surfaces and solid-core structures, by directly visualizing variations in membrane continuity, surface topology, internal density, and overall core architecture.		Ascertain product heterogeneity by examining particle aggregation and size distribution. This attribute is important for the assessment of batch-to-batch variability, formulation development, as well as process development and validation.	Directly visualize single- and multi-lamellar structures, enabling precise characterization of membrane thickness, bilayer spacing, and contrast profiles. This provides quantitative insights into lamellarity distributions across entire LNP populations.

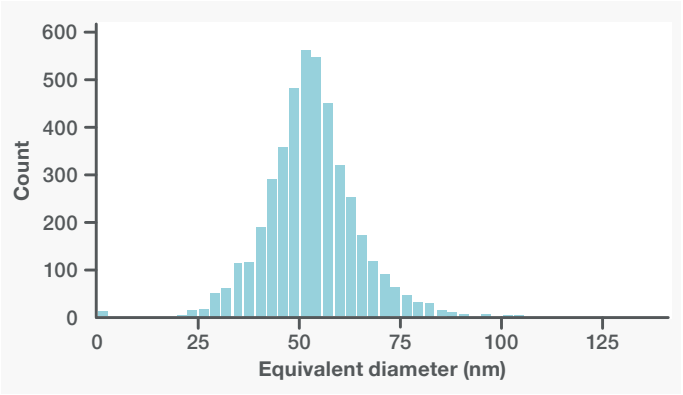
Table 1. Critical quality attributes revealed with cryo-TEM.



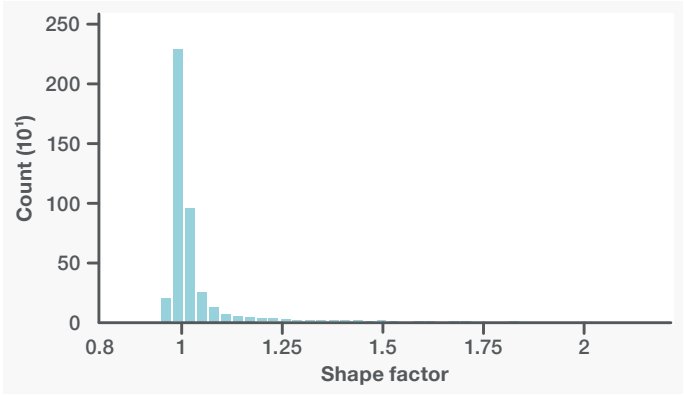
Note: Equivalent to the surface area of the particle.



Note: Values close to 1 indicate circularity, whereas values close to zero are more elongated.



Note: Defined as the diameter of a disk with the same area as the particle.



Note: Shape factor = 1 for a perfect circular shape. Higher values indicate objects that are less circular.

Figure 7. Quantitative characterization of select CQAs for the LNP samples.

Discussion

LNP formulations can be difficult to fully characterize due to their complex compositions, heterogeneous structures, and payload diversity. Traditional biochemical assays provide necessary information on particle titer and encapsulation efficiency, but lack the resolution to reveal morphological details, while bulk techniques such as DLS often obscure true size distributions in complex samples.

Cryo-TEM overcomes these limitations by directly visualizing LNPs in their native vitrified state, enabling high-resolution assessment of particle morphology, lamellarity, and shape, along with surface features such as blebbing. When combined with automated particle characterization in Amira Software, this approach provides scalable, unbiased quantification across tens of thousands of particles, greatly enhancing reproducibility and statistical confidence. This level of precise characterization is crucial for LNP process development, and for ensuring the quality of gene therapies and vaccines formulations. Furthermore, detailed morphological analysis of LNPs is increasingly necessary to adhere to stringent regulatory guidelines, which aim to ensure high standards of quality, efficacy, and safety.

Conclusions and perspectives

The integration of cryo-TEM imaging with automated image analysis will be instrumental in advancing the field of LNP-based nanomedicine. This powerful combination not only enhances the accuracy and detail of LNP characterization but also streamlines the workflow, making it more efficient and reliable. By leveraging these advanced technologies, researchers and manufacturers can gain a deeper understanding of LNP structure-function relationships, helping to ensure the development of high-quality, effective gene therapy and vaccine products that meet stringent regulatory standards.

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