

WHITE PAPER — CRYO-TEM

Seeing Through the Fog

Using Cryo-TEM to Characterize LNPs from Formulation Through Manufacturing

Lipid nanoparticle (LNP) performance is governed by structural attributes that bulk analytical methods cannot resolve. Dynamic light scattering (DLS) and RiboGreen assays report an average size and an average encapsulation efficiency; they do not report whether particles are unilamellar or multilamellar, whether they have atypical morphologies, whether an aqueous “bleb” compartment has formed, or whether payload is distributed evenly or concentrated in one compartment. Cryogenic transmission electron microscopy (cryo-TEM), combined with quantitative image analysis, resolves all of these attributes on a per-particle basis from a single dataset.

The work presented here follows a single mRNA LNP formulation through a few examples of changes it can encounter during development and manufacturing. Four mixing platforms were used to produce the same formulation; DLS and RiboGreen indicated the four batches were equivalent, yet cryo-TEM revealed a systematic size difference and a near two-fold difference in bleb frequency between platforms. Deliberate formulation and process changes (tangential flow filtration versus dialysis, buffer ionic strength, and payload length) shifted size, distribution width, and bleb percentage by amounts ranging from modest to dramatic: bleb percentage fell from 46% to 1% on a buffer change alone. The same analysis resolved changes that were never part of the design, an unregulated mixing flow rate and an over-concentrated batch carrying free mRNA, and quantified the morphological reorganization in a formulation held for ten weeks at 4 °C.

Together these datasets show that cryo-TEM is not a confirmatory imaging step at the end of development. It is a decision-making tool throughout formulation screening, process development, scale-up, batch-to-batch comparison, and storage assessment.

Introduction

Designing lipid nanoparticles for effective drug delivery requires simultaneous optimization of stability, encapsulation efficiency, and therapeutic efficacy. This is achieved through careful selection of core components, such as lipid composition, payload properties, and buffer conditions, and, increasingly, through addition of targeting moieties, synthetic lipid, or polymeric additives, and other modifications intended to enhance specific functionality.

Formulation is only half of the problem. The choice and optimization of the manufacturing process directly influences the physicochemical characteristics of the resulting particle: its size, the uniformity of the population, and the internal arrangement of lipid and payload. Mixing geometry, flow rate, buffer exchange strategy, concentration step, and storage all leave a structural signature on the product. A comprehensive understanding of the physicochemical–activity relationship is what ultimately produces stable, reproducible formulations and effective LNP-based vaccines and therapeutics.

This is where cryo-TEM contributes. Cryo-TEM provides information on size, uniformity, payload distribution, aggregation, shape, lamellarity, and bleb formation, all within a single dataset, on discrete particles preserved in a near-native, vitrified state, and on a per-particle rather than a bulk basis. Combined with automated image analysis, that visual information becomes quantitative and statistically meaningful.

What Cryo-TEM Measures That Bulk Methods Do Not

Before turning to the data, it is worth being specific about what cryo-TEM adds. Four attribute classes are routinely extracted from a single cryo-TEM dataset:

- **Size and size distribution.** Area equivalent diameter (AED) is measured directly from particle densities in the image, particle by particle. Because the full distribution is captured, D10/D50/D90 and SPAN are available, and small subpopulations of outsized particles are visible rather than averaged away.
- **Payload distribution.** Particles whose lipid or aqueous phase shows a dense, mottled appearance are consistent with nucleic acid loading. Depending on image contrast, the fraction of loaded particles is therefore countable, rather than inferred from a bulk fluorescence measurement.

- **Morphology and internal organization.** Uniformly dense particles, particles with a distinct aqueous bleb compartment, and multilamellar or multivesicular structures are directly distinguishable and can be counted as population percentages.
- **Bulk-phase content.** Material outside the particles, such as unencapsulated nucleic acid, aggregates, and precipitates, is visible in the background of the same image.

None of these attributes is exotic; all of them are difficult or impossible to obtain any other way. The practical consequence is that two batches can pass every routine analytical ensemble assay and still be structurally different products. The examples below show exactly that happening, more than once.

It is also worth noting what cryo-TEM is not. It is not a high-throughput bulk assay, and it does not replace DLS, NTA, or RiboGreen for routine monitoring. Sample-to-sample it is slower and more resource-intensive than a plate reader. Its value is complementary: it resolves the attributes those methods average over, and it is at its most powerful when a bulk method returns an answer that is either surprising or suspiciously unremarkable. Table 1 summarizes how the attribute coverage of the common techniques compares.

Attribute	DLS	NTA	RiboGreen	Cryo-TEM
Mean particle size	Yes (hydrodynamic, intensity-weighted)	Yes (hydrodynamic, number-weighted)	No	Yes (area equivalent diameter, per particle)
Full size distribution / SPAN	Limited	Yes	No	Yes
Detection of minor large-particle subpopulations	Biased — large particles dominate signal	Limited	No	Yes
Encapsulation efficiency	No	No	Yes (bulk average)	Indirect — loaded-particle fraction
Payload distribution across particles	No	No	No	Yes
Lamellarity (unilamellar / multilamellar / multivesicular)	No	No	No	Yes
Bleb formation	No	No	No	Yes
Particle shape and circularity	No	No	No	Yes
Unencapsulated nucleic acid in the bulk phase	No	No	Indirect	Yes (directly visualized)
Aggregation / fusion	Indirect	Indirect	No	Yes

TABLE 1 Attribute coverage across common LNP characterization methods. Cryo-TEM is unique in returning nearly all listed attributes from a single dataset on discrete particles.

Four Mixing Platforms, One Formulation

In collaboration with Phosphorex, a single mRNA LNP formulation, a standard four-component lipid system (MC3, cholesterol, DSPC, DMG-PEG-2000) carrying polyA, was produced on four different mixing platforms: a T-mixer, the Ignite+ (Precision NanoSystems), an impingement jet mixer, and the Sunshine (Unchained Labs). Each platform has a distinct design and operating principle. The mixing flow rate on each was independently optimized so that all four batches would be comparable by DLS and RiboGreen, and all four were dialyzed into the same final buffer (20 mM Tris-HCl, pH 7.4).

By the standard assays the exercise succeeded: the four preparations appeared similar in size and displayed similar mRNA encapsulation efficiency. The question this study set out to answer is whether they were, in fact, the same product.

SIZE DISTRIBUTION ANALYSIS

Cryo-TEM images were acquired at 73,000 $\times$ (0.197 nm/pixel) and 28,000 $\times$ (0.510 nm/pixel). At first inspection the overall size range across the four platforms does appear comparable (Figure 1). All four preparations contain particles with an entirely dense interior alongside particles that are only partially dense, with the dense region corresponding to the lipid or oily phase and the less dense region to an aqueous compartment commonly referred to as a bleb.

Quantifying the images tells a more precise story. Particles were automatically traced to determine the size distribution. LNPs from the Ignite+, the jet mixer, and the Sunshine are closely matched, with mean area equivalent diameters spanning 60–63 nm. Particles from the T-mixer are measurably smaller, averaging 52 nm, and the distribution carries some notably large outliers (Table 2; Figure 2, left).

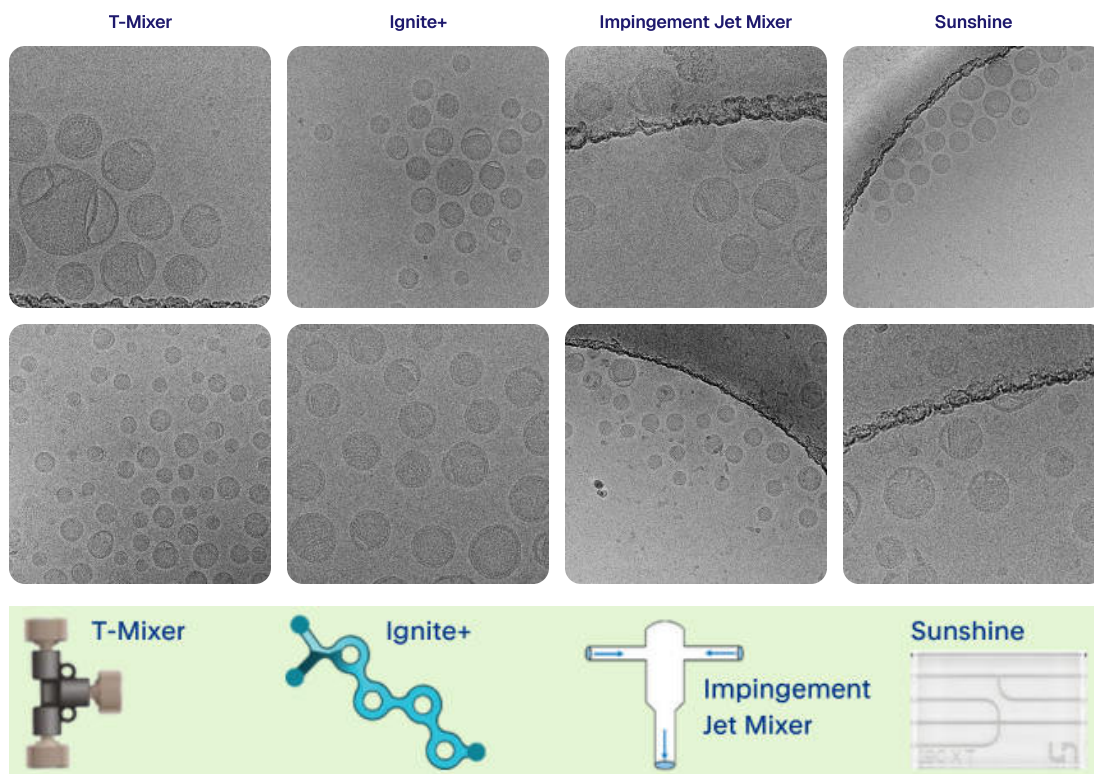


FIGURE 1 Cryo-TEM images of the same mRNA LNP formulation prepared on four mixing platforms. (Top row) 73,000 $\times$, scale bar 100 nm. (Middle row) 28,000 $\times$, scale bar 200 nm. (Bottom) The four mixing platforms: T-mixer, Ignite+, impingement jet mixer, and Sunshine.

	T-Mixer	Ignite+	Jet Mixer	Sunshine
SIZE				
Mean AED (nm)	52 ± 18	60 ± 14	63 ± 20	60 ± 15
Min / Max (nm)	20–257	23–161	27–193	29–221
Circularity	0.92	0.91	0.92	0.96
PAYLOAD				
Mottled	79%	89%	78%	84%
MORPHOLOGY				
Bleb features	27%	46%	45%	40%
No blebbing	72%	54%	54%	59%
Other	0.8%	0%	2%	1.1%

TABLE 2 Quantitative size and morphology analysis of LNPs generated on four mixing platforms. Mean area equivalent diameter (AED) ± standard deviation, size range, and circularity, with the percentage of particles showing a dense mottled appearance in the lipid or aqueous phase (indicative of payload), the percentage showing bleb formation, and the percentage showing other structures such as multivesicular or multilamellar character.

CRYO-TEM VERSUS DLS

Comparing the cryo-TEM measurements with the DLS data generated by Phosphorex shows the two techniques following a broadly similar trend, but with two informative differences (Figure 2, right). First, the absolute size measured by cryo-TEM is consistently smaller than that measured by DLS. This is expected: DLS reports a hydrodynamic diameter, whereas cryo-TEM measures directly from the particle densities in the image.

Second, and more consequential, DLS does not reproduce the finding that the T-mixer particles are smaller than the other three. The most likely explanation is the small population of much larger particles present in that sample. Larger particles scatter disproportionately, so a minor subpopulation can pull an intensity-weighted DLS average upward and mask a genuine shift in the bulk of the distribution.

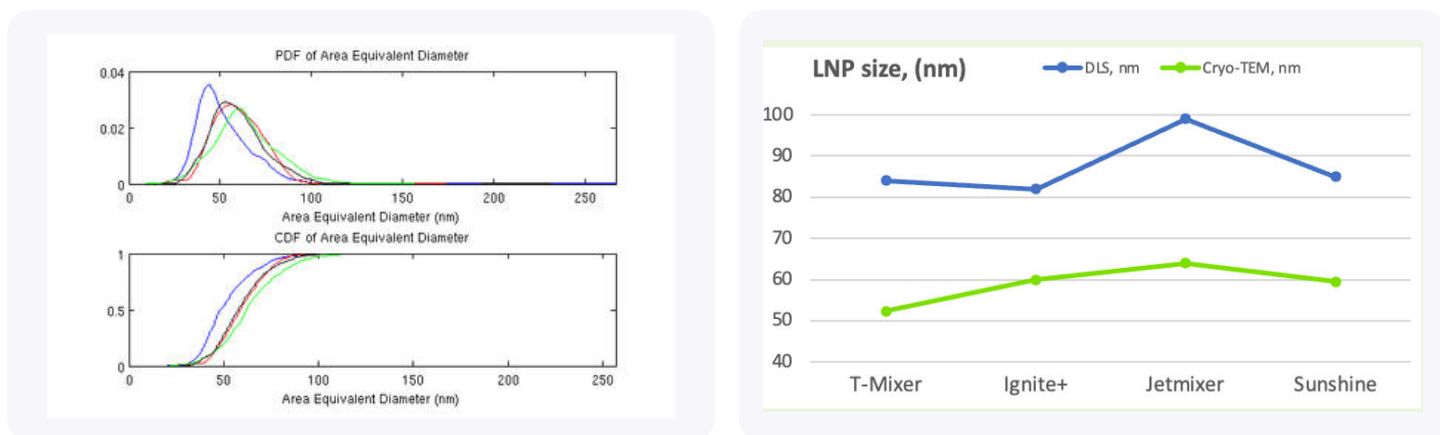


FIGURE 2 (Left) Probability and cumulative distribution functions of area equivalent diameter for the four mixing platforms, from image analysis of cryo-TEM images. (Right) Mean diameters calculated from cryo-TEM data compared with DLS measurements. The two techniques track a similar trend, but DLS does not reproduce the smaller mean size of the T-mixer preparation.

PAYLOAD DISTRIBUTION AND MORPHOLOGY

Cryo-TEM also reports on payload. The fraction of particles displaying the dense mottled appearance associated with mRNA loading is slightly higher for the Ignite+ (89%) and Sunshine (84%) than for the T-mixer (79%) and jet mixer (78%), a modest but consistent difference.

The most striking result is structural. Three of the four platforms produced populations in which 40–46% of particles carried bleb formations. The T-mixer produced markedly fewer: 27% (Table 2, Figure 3). This is close to a two-fold difference in a structural attribute that has been directly linked to transfection potency, arising purely from the choice of mixing platform, in batches that DLS and RiboGreen reported as equivalent.

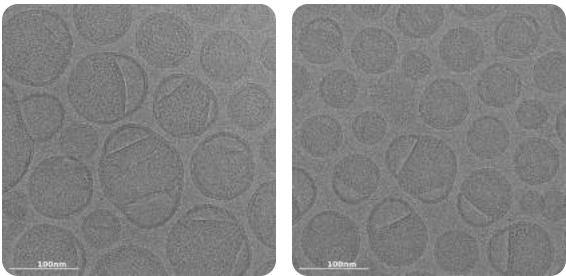


FIGURE 3 Cryo-TEM images of LNPs with bleb formations. The dense region corresponds to the lipid phase; the adjacent lower-density region is the aqueous bleb compartment. Scale bar 100 nm.

PART II

Intentional Formulation and Process Changes

Process changes introduced during LNP manufacturing are usually aimed at product quality, scalability, efficiency, or cost. Formulation changes are more often aimed at performance as a gene delivery vehicle (stability, encapsulation efficiency, payload delivery). In both cases the relevant question is the same: what did the change actually do to the particle?

Three deliberate changes were examined, each against the same reference condition (polyA payload, dialysis into 20 mM Tris-HCl, pH 7.4): substituting tangential flow filtration for dialysis, raising the ionic strength of the final buffer, and shortening the payload. Quantitative results for all four conditions are given in Table 3 and Table 4, with representative images in Figure 4.

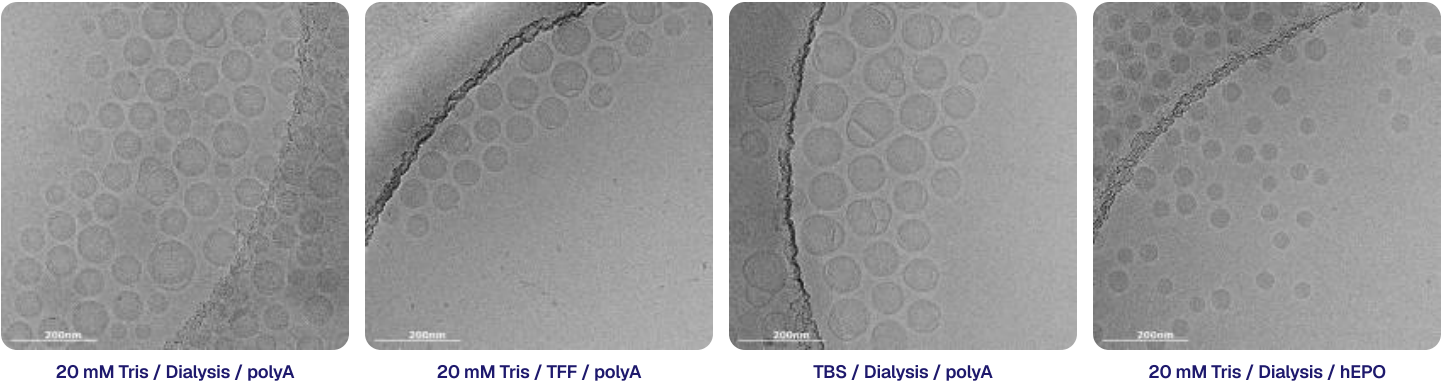


FIGURE 4 Representative cryo-TEM images for the four formulation and process conditions. Scale bar 200 nm.

Condition	Mean AED (nm)	StdDev AED (nm)	D10 / D50 / D90 (nm)	SPAN
20 mM Tris / Dialysis / polyA	60.0	13.9	44.2 / 58.7 / 77.0	0.56
TBS / Dialysis / polyA	43.0	13.6	34.8 / 41.4 / 51.3	0.40
20 mM Tris / TFF / polyA	84.9	23.3	65.2 / 83.6 / 102.2	0.44
20 mM Tris / Dialysis / hEPO	64.8	18.0	45.9 / 62.9 / 83.2	0.59

TABLE 3 Size distribution analysis for the four formulation and process conditions. Mean area equivalent diameter (AED), standard deviation, D10/D50/D90, and SPAN.

Condition	Morphology			Internal appearance	
	Dense w/ blebbing	Uniform dense	ML / MV	Mottled	Evenly dense
20 mM Tris / Dialysis / polyA	46.0%	54%	0%	89%	11%
TBS / Dialysis / polyA	1.0%	98.6%	0.4%	83%	17%
20 mM Tris / TFF / polyA	60.1%	38.0%	1.9%	89%	11%
20 mM Tris / Dialysis / hEPO	11.8%	88.2%	0%	95%	5%

TABLE 4 Morphology and internal appearance for the four formulation and process conditions. ML/MV denotes multilamellar or multivesicular particles.

PURIFICATION METHOD: TANGENTIAL FLOW FILTRATION VERSUS DIALYSIS

When tangential flow filtration (TFF) was used instead of dialysis (into the same final 20 mM Tris buffer) the particles in the final solution were substantially larger. Machine learning-based image analysis measured 84.9 ± 23.3 nm for TFF against 60.0 ± 13.9 nm for dialysis, a roughly 40% increase in mean diameter.

TFF also produced a considerably higher proportion of particles with bleb formations: 60.1% versus 46.0%. The purification step, in other words, is not structurally neutral. A change made for scalability reasons materially altered both the size and the internal architecture of the product.

BUFFER IONIC STRENGTH

The second change concerned the ionic strength of the buffer into which particles are exchanged after initial mixing. Particles were generated in a low pH buffer and then dialyzed into one of two neutral pH buffers: 20 mM Tris, or Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris-HCl, pH 7.6).

The difference is visible before any analysis is run. LNPs in 20 mM Tris are generally larger and show blebs far more frequently than LNPs in TBS. Quantitatively, mean AED falls from 60.0 ± 13.9 nm in Tris to 43.0 ± 13.6 nm in TBS, and the distribution narrows appreciably (SPAN 0.40 versus 0.56).

Those size differences could plausibly be detected by NTA or DLS. What could not be detected by either is the morphological result: 46% of particles in the low ionic strength buffer carry bleb structures, against 1% in the high ionic strength buffer. A change to the dialysis buffer all but eliminated a structural feature associated with transfection potency.

PAYLOAD LENGTH

The third change held lipid formulation, buffer composition, and dialysis method constant and varied only the payload. A short mRNA (hEPO, 858 nucleotides) was substituted for the long polyA chain (approximately 6,000 nucleotides) used elsewhere in this work.

Particle size was comparable between the two: 64.8 ± 18.0 nm for hEPO against 60.0 ± 13.9 nm for polyA. Morphology was not. Bleb formation dropped from 46.0% with polyA to 11.8% with hEPO. The hEPO LNPs also showed a slightly higher proportion of particles with a mottled interior (95% versus 89%), consistent with a marginally greater fraction of particles carrying payload.

Unintentional Process Changes and Stability

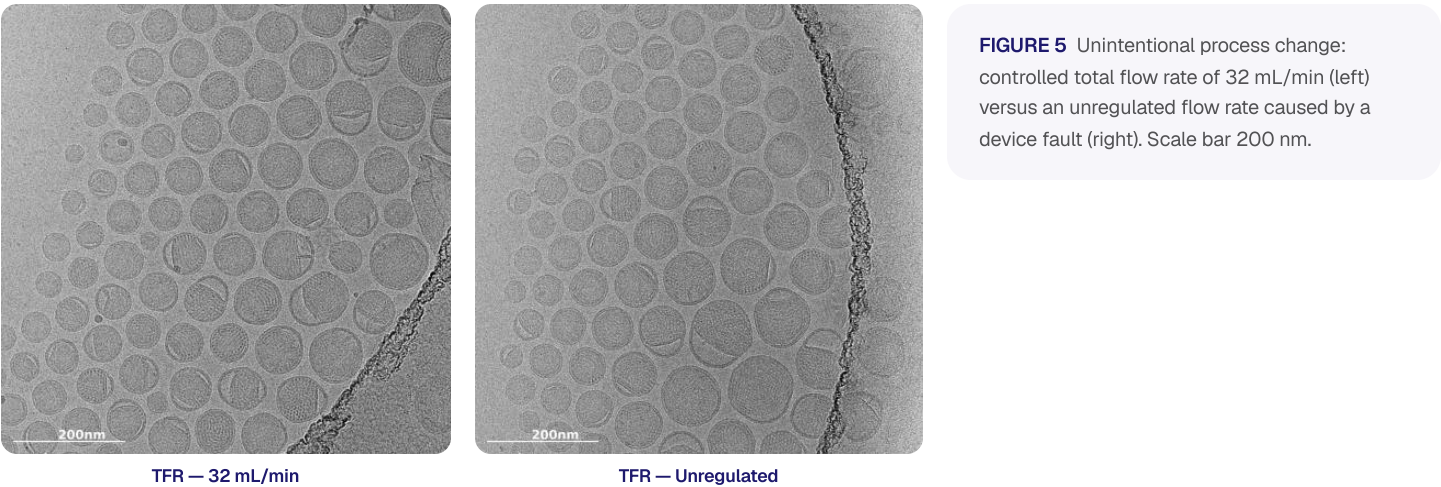
Characterizing anticipated changes is one use of cryo-TEM. Catching unanticipated ones is another, and arguably the more valuable of the two. NIS routinely performs batch analysis, and in most cases batches look comparable. Occasionally a batch returns entirely unremarkable DLS sizing results while showing morphology that is clearly deviant, a divergence that only direct imaging will surface.

AN UNREGULATED MIXING FLOW RATE

In the first example, a loose device cable was discovered after the lipid and mRNA phases had been mixed. The concern was that the flow rate had been unregulated during mixing, so cryo-TEM was used to determine whether LNP size or structure had been affected.

Mean particle size was essentially unchanged: 64.4 nm for the affected batch against 64.0 nm for the batch mixed at a controlled 32 mL/min total flow rate, with an identical SPAN of 0.72 (Figure 5; Table 5). The distribution, however, was wider: standard deviation 21.5 nm versus 17.2 nm — reflecting a minor fraction of particles up to 200 nm in size in the affected batch. A modest morphological shift accompanied it: bleb-containing particles fell from 55.7% in the controlled batch to 47.7% in the affected batch.

The practical conclusion was reassuring rather than alarming: the deviation did not materially damage the batch. But that conclusion was only available because the batch was imaged. Average size alone would have said nothing at all, and differences of this magnitude can matter when functional activity is later found to vary.



	Mean AED (nm)	StdDev AED (nm)	D50 (nm)	SPAN	Dense w/ blebbing	Uniform dense	ML / MV
TFR — 32 mL/min	64.0	17.2	62.0	0.72	55.7%	44.1%	0.2%
TFR — Unregulated	64.4	21.5	62.4	0.72	47.7%	52.1%	0.1%

TABLE 5 Controlled versus unregulated total flow rate (TFR). Mean size is unaffected; the unregulated batch shows a broader distribution and a shift in bleb frequency.

OVER-CONCENTRATION AND FREE MRNA

In the second example, an LNP formulation was inadvertently held on the concentration step too long, raising the possibility of over-concentration. The cryo-TEM images show a high density of punctate and linear material distributed throughout the background (Figure 6A). These densities are unencapsulated mRNA, released into the bulk phase and readily detected with the direct electron detectors used here.

Free nucleic acid in the bulk phase is a quality attribute with obvious implications for potency and for immunogenicity. It is also one that a bulk encapsulation assay may register only as a modest percentage shift, without indicating what has happened or where.

STORAGE AND SHELF-LIFE

The most conspicuous structural changes are typically seen in LNPs that have been stored too long, at too high a temperature, or without a stabilizing agent such as sucrose where the formulation requires a cryoprotectant.

Figure 6B shows a formulation stored for 10 weeks at 4 °C in 20 mM Tris buffer, pH 7.4. Particles expected to be approximately 60 nm now show a mean area equivalent diameter of 82 nm across a very wide size range spanning 866 nm. Many particles have developed more than two internal compartments, with abundant large aqueous bleb structures. The population has not simply drifted larger; it has reorganized.

This is what makes cryo-TEM useful for tracking how a formulation changes over time in storage. By imaging at defined time points post-manufacture, changes in payload distribution, size, and internal structure related to aggregation, fusion, or degradation can be tracked directly, rather than inferred from a drifting bulk average.

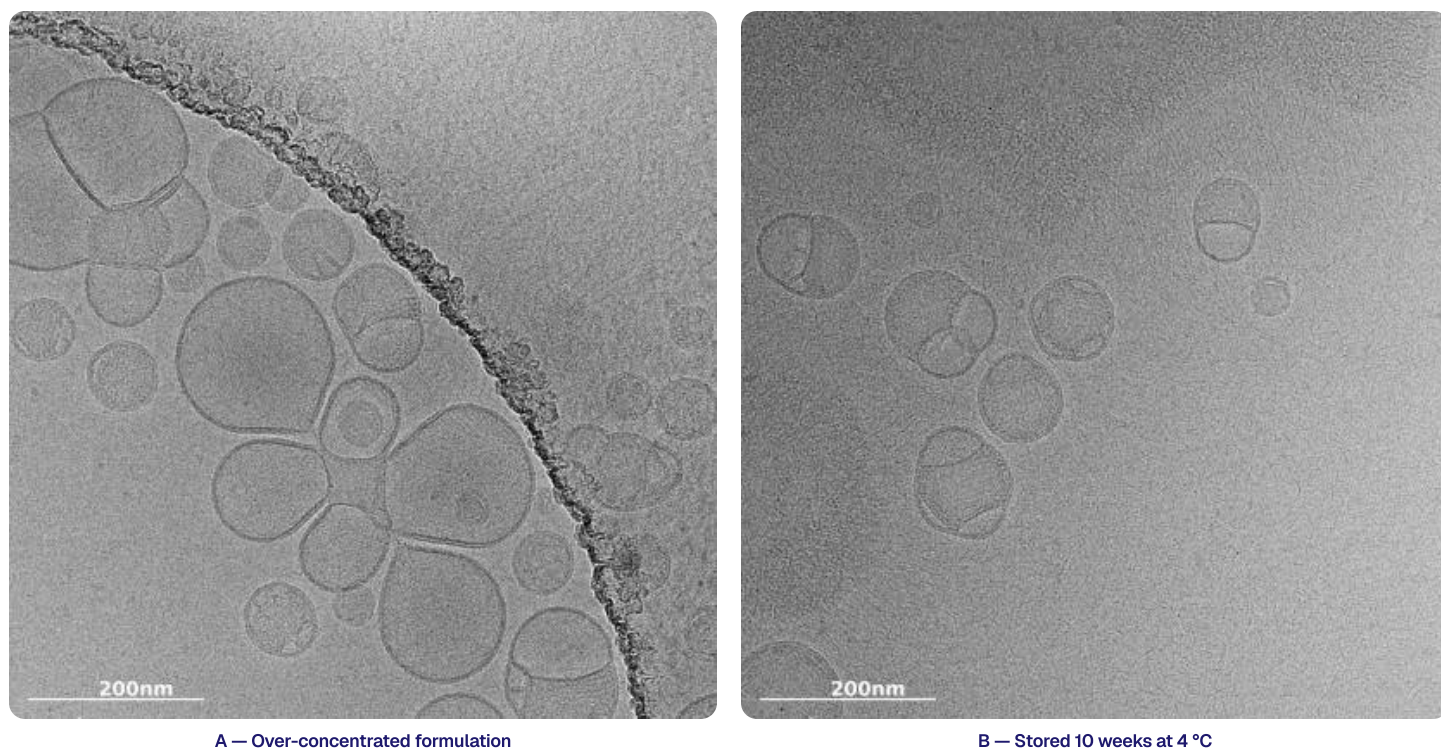


FIGURE 6 (A) An LNP formulation over-concentrated after mixing. Punctate and string-like material throughout the background is indicative of free mRNA. (B) An LNP formulation stored at 4 °C for 10 weeks in 20 mM Tris buffer, showing atypical morphology. Scale bar 200 nm.

Discussion

Across all three parts of this work, one pattern recurs: quality attributes that appear stable by bulk measurement are not necessarily stable at the level of the individual particle. Four mixing platforms tuned to equivalence by DLS and RiboGreen produced populations differing nearly two-fold in bleb frequency. A change of dialysis buffer moved bleb frequency from 46% to 1%. A change of purification method moved mean diameter by 40% and bleb frequency by 14 percentage points. In each case the structural consequence was invisible to the assays that were being used to declare the batches comparable.

Bleb formation itself remains an active topic in the field. Cheng and colleagues showed that bleb structure formation in LNPs is buffer dependent and that bleb formation leads to increased transfection efficiency². Pratsinis and colleagues

showed that LNP morphology relates to lipid selection and composition as well as to the manufacturing method³, consistent with what we observed here across four mixing platforms and three deliberate process changes.

The practical implication is that structural characterization belongs earlier in the workflow than it is often placed. During initial development, per-particle structural readouts let screening studies discriminate between candidate formulations and processes that bulk methods cannot separate, which both accelerates screening and reduces the risk of carrying a structurally inferior candidate forward. During scale-up and scale-out, the same readouts explain variations in activity or potency that would otherwise be unattributable. And when a batch doesn't look right, direct imaging turns an open question into a clear answer.

Conclusion

Cryo-TEM is an exceptionally powerful tool for characterizing quality attributes that can be affected by any small change in formulation or process, whether that change is intentional or unexpected. Although size, payload distribution, and morphology can each be partially assessed by individual orthogonal techniques, cryo-TEM provides direct visualization of multiple quality attributes simultaneously (including attributes not visualizable by any other technique) from the same dataset, on discrete particles preserved in near-native condition.

The evidence presented here is that this is not a marginal advantage. Batches judged equivalent by DLS and RiboGreen differed nearly two-fold in bleb frequency depending on

mixing platform. A buffer change reduced bleb frequency from 46% to 1%. A purification change increased mean diameter by 40%. A process deviation that left mean size untouched broadened the distribution and shifted morphology. Ten weeks of refrigerated storage reorganized a population that a mean diameter would have described as merely somewhat larger.

When batches need to be compared, when material has been stored, or when something in the process has changed unexpectedly, that combination of attributes from a single dataset makes cryo-TEM a remarkably useful characterization technique — and one best deployed while formulation and process decisions are still being made.

Methods

Cryo-TEM grids were prepared undiluted using a Vitrobot (Thermo Fisher Scientific). Cryo electron microscopy was performed on a Thermo Fisher Scientific Glacios Cryo Transmission Electron Microscope operated at 200 kV and equipped with a Falcon 3 direct electron detector. After identifying suitable target areas at lower magnifications, high magnification images were acquired at nominal magnifications of 150,000 $\times$ (0.096 nm/pixel), 73,000 $\times$ (0.197 nm/pixel), and 28,000 $\times$ (0.510 nm/pixel) using Leginon software¹. Images were acquired at a nominal underfocus of $-5.5\text{ }\mu\text{m}$ to $-2.0\text{ }\mu\text{m}$ and electron doses of approximately $10\text{--}25\text{ e}^-/\text{\AA}^2$.

Image analyses were carried out both manually and using a custom machine learning-based segmentation algorithm, using a minimum of 750 particles per dataset, or all particles available.

The LNP formulation in all experiments described here was a standard four lipid component system (MC3, cholesterol, DSPC, DMG-PEG-2000) containing either polyA (approximately 6,000 nucleotides) or hEPO mRNA (858 nucleotides). LNPs were prepared by Phosphorex. For the mixing platform comparison (Part I), LNPs were prepared using a T-mixer, the Ignite+ (Precision NanoSystems), an impingement jet mixer, and the Sunshine (Unchained Labs), and dialyzed into a final buffer of 20 mM Tris-HCl, pH 7.4. For the formulation and process change studies (Parts II and III), LNPs were prepared using either the Ignite+ or an impingement jet mixer and processed into a final buffer of 20 mM Tris-HCl, pH 7.4 or Tris Buffered Saline (TBS; 150 mM NaCl, 50 mM Tris-HCl, pH 7.6) by dialysis or tangential flow filtration.

Notes on Designing a Cryo-TEM Study

A cryo-TEM study is only as good as the sampling behind it. A few practical points govern whether the resulting numbers will support a decision.

- **Particle counts.** The analyses reported here used a minimum of 750 particles per dataset, or all particles available. Population percentages such as bleb frequency are proportions, and their precision depends directly on the number of particles counted; small datasets will not reliably resolve the differences of a few percentage points that appear in Tables 2 and 4.
- **Magnification strategy.** Lower magnifications survey the grid and locate suitable areas; higher magnifications resolve internal structure. Both are needed. Size distributions and morphology classification were performed here from images at 73,000 $\times$ and 28,000 $\times$, with 150,000 $\times$ available for fine structural detail.
- **Sample preparation.** Grids in this work were prepared from undiluted, fresh material. Formulations containing up to 10% sucrose can oftentimes be vitrified as received; above that, the cryoprotectant degrades contrast enough that the sample must be diluted or buffer exchanged before imaging. Dilution and buffer exchange to remove sucrose both change the environment the particles sit in, and the buffer environment or the buffer exchange handling can alter morphology. Where a formulation can be imaged undiluted, it should be, because the alternative risks introducing the very artifact the study is trying to measure. Where it cannot, the handling step belongs in the interpretation of the result.
- **Consistent classification.** Morphology percentages are only comparable across samples if the classification criteria are held constant. Automated, algorithm-based segmentation and classification make this tractable at scale and remove much of the operator-to-operator variability that manual scoring introduces.
- **What to report.** Mean AED alone is insufficient. Reporting standard deviation, D10/D50/D90, SPAN, min/max, loaded-particle fraction, and morphology class percentages preserves the information needed to compare batches or track down what changed in an unexpected one.

LNP samples described in this work were prepared by Phosphorex. NIS gratefully acknowledges the collaboration.

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