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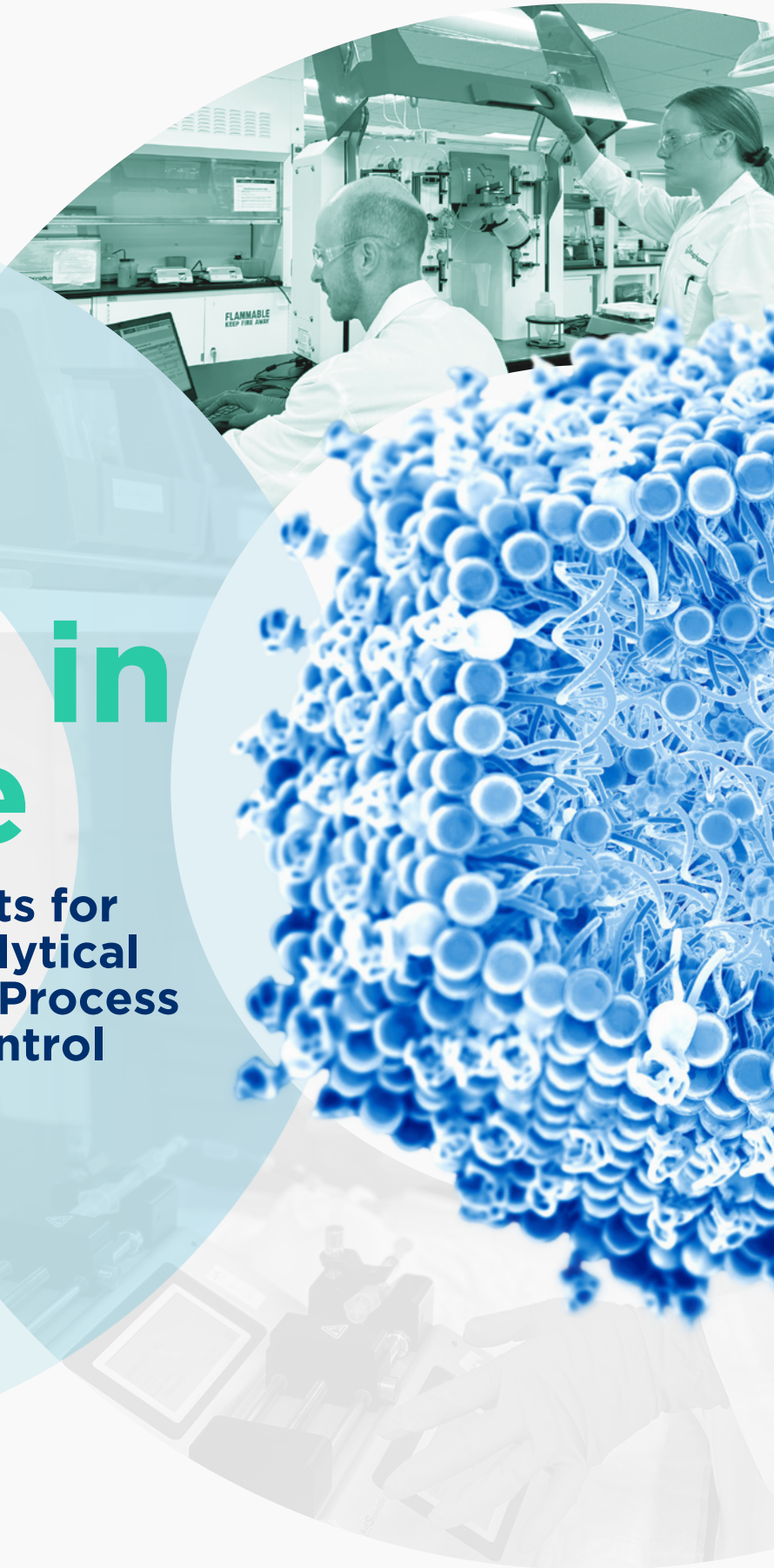
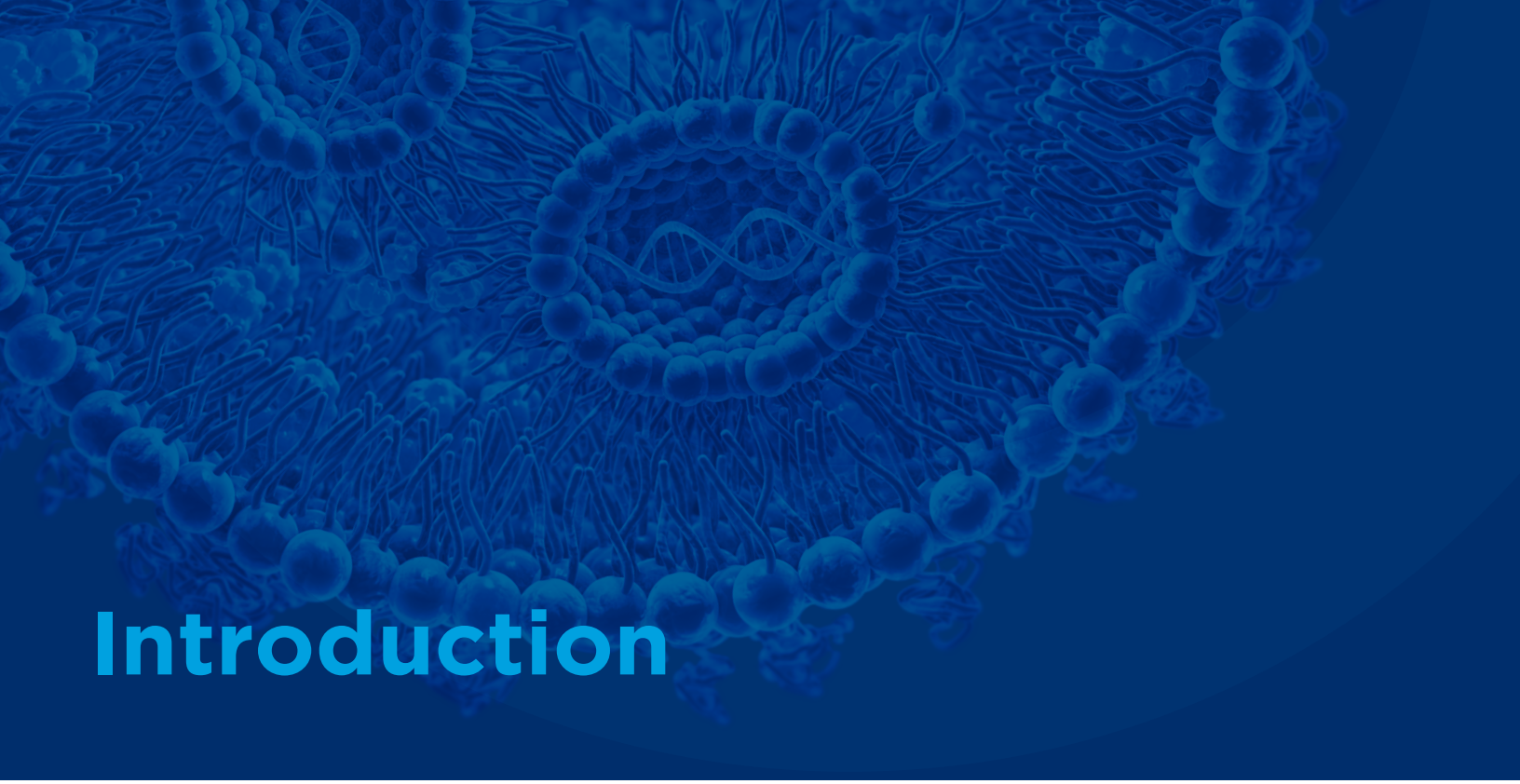


EBOOK

Particle Science in Practice

Approaches and Insights for Lipid Nanoparticle Analytical Methods, Formulation, Process Design, and Quality Control



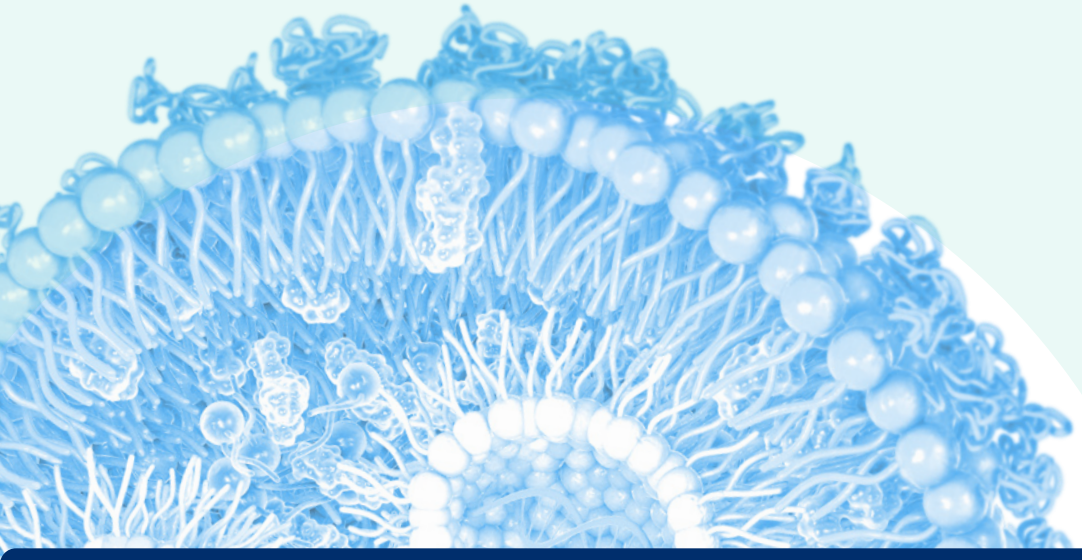
Introduction

Lipid nanoparticles (LNPs) have become a foundational technology for delivering nucleic acid medicines and other advanced therapeutics. Their use spans mRNA vaccines, siRNA therapeutics, and increasingly complex applications such as *in vivo* gene editing. However, navigating an LNP program from early feasibility to the clinic requires more than a promising formulation. It requires coordinated work across formulation science, process design, analytical method development, and CMC strategy at each stage of development.

This eBook assembles insights from the Phosphorex team and comprehensively examines the factors required for LNP program success. Also included in the discussion is the significant progress being made in engineering target-appropriate biodistribution, including particle architectures such as conjugated targeting ligands and corresponding process design.

Grounded in practical insights and experience, this eBook offers a picture of what it takes to move an LNP-based therapeutic from early feasibility to clinical readiness.

Contents



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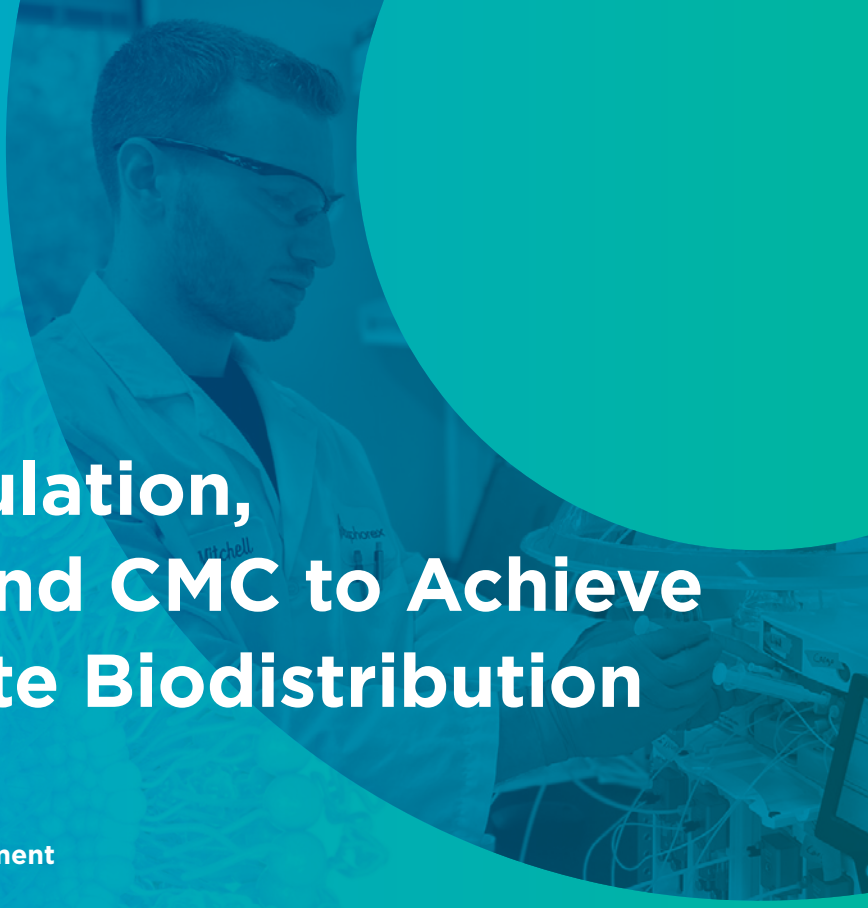

Optimizing Formulation, Process Design, and CMC to Achieve Target-Appropriate Biodistribution

An Interview with:
Evan Turek, Senior Director of Business Development
Theresa Logan, Head of Laboratory Operations

Lipid nanoparticles (LNPs) and polymer-based delivery systems have transformed the drug development landscape, enabling the development of new classes of nucleic acid medicines and advanced therapeutics. Yet despite promising early-phase data, many programs stall before reaching the clinic. Achieving target-appropriate biodistribution while ensuring manufacturability, scalability, and CMC robustness remains a central challenge.

To explore how formulation, process design, and CMC must evolve to meet this challenge, we spoke with Theresa Logan, Head of Laboratory Operations, and Evan Turek, Sr. Director of Business Development at Phosphorex.

Why does biodistribution remain such a persistent hurdle for advanced drug delivery systems?

Theresa Logan (TL): For advanced delivery technologies, specifically LNPs, biodistribution has been a long-standing challenge, as most formulations naturally accumulate in the liver and spleen due to the protein coronas they accumulate. This has worked well for vaccines and hepatic targets, but as we work toward addressing broader therapeutic applications, this default distribution pattern is limiting. It restricts selective targeting of alternative tissue types, constraining therapeutic flexibility and increasing the risk of off-target exposure, immune activation, and toxicity.

However, significant progress is being made in engineering target-appropriate biodistribution through formulation composition, lipid or polymer chemistry, particle architecture, such as the use of conjugated targeting ligands, and corresponding process design.

Significant progress is being made in engineering target-appropriate biodistribution through formulation composition, lipid or polymer chemistry, particle architecture, such as the use of conjugated targeting ligands, and corresponding process design.

How do formulation and process design work together to influence biodistribution?

Evan Turek (ET): Among some in the industry, there's a tendency to segment formulation, process design, and CMC into considerations that are more discrete than they should be. Specifically, many think of a formulation's structural and compositional activity relationship as a nearly exclusive determinant of clinical potential. This approach neglects the importance of a composition's inherent relationship with clinically relevant process design and CMC, leaving manufacturability, scalability, and regulatory readiness at risk. In reality, these disciplines are deeply interdependent.

A formulation that shows promising biodistribution in the lab may behave very differently when scalable mixing methods, purification and concentration techniques, and filtration steps are introduced. Without trained expertise in the design and optimization of scalable processes and their impact on the control of critical quality attributes (CQA), a formulation's efficacy can be significantly disrupted as development advances.

Many candidates show promise in preclinical models but falter when transitioned to clinically relevant manufacturing conditions. But a specialized partner can identify these scalability risks before a lead candidate is even selected. By guiding innovators through small-scale feasibility and re-optimization, these partners ensure a balance of both scalable and efficacious critical quality attributes (CQAs). This harmony is essential for reproducibility *in vivo* performance and successful scale-up, ultimately preventing the costly waste of time and materials associated with process failure.

How do biodistribution, patient safety, and process design challenges differ between LNPs and polymeric delivery systems?

TL: During early feasibility, LNP-based systems often appear relatively simple to produce, allowing rapid test article production and dosing for expression in preclinical models. However, early-phase simplicity quickly becomes more complex as programs advance and innovators better understand the delicate and dynamic relationships among CQAs such as particle size and polydispersity index (PDI), process scalability challenges, and accompanying *in vivo* translation difficulties. For instance, the shear sensitivity of an LNP in a scalable downstream process may lead to particle aggregation, and stem from the specific properties of ionizable and helper lipid(s) selected at a small scale.

In the case of polymeric nanoparticles, the vehicle may exhibit superior inherent shear stability; however, it may also display a high propensity for premature cargo release during the extended process hold times required at larger manufacturing scales for clinical production. An experienced service provider like Phosphorex has the expertise to preserve the biodistribution and tolerability-dependent CQAs through process development for scale-up.

ET: Polymeric delivery systems are inherently complex from the outset. Varying payload concentration requirements often need robust formulation optimization. The connections between polymer chemistry, vehicle physical properties, and *in vivo* half-life warrant more process development diligence than a self-assembling lipid composition. These demands make early development less prescribed, but in some applications can ultimately yield a fabrication process with fewer risk exposures than an LNP. Importantly, polymeric systems can offer greater functionality for tuning biodistribution profiles, particularly for applications where liver accumulation is undesirable.

As a result, polymeric nanoparticles can enable alternative administration route strategies that LNPs may struggle to support, provided there is sufficient formulation, process development, and CMC expertise to manage and scale these intricate systems.

How do early-phase formulation decisions shape downstream manufacturability and clinical viability?

ET: We're still seeing too many cases of innovators nominating a small-scale formulation as the lead development candidate based solely on small-animal readouts. These lab-scale formulations too often have good data, but insufficient process feasibility and/or stability assessments, exposing the advanced drug delivery system innovator or adopter to significant risk. When the formulation candidate focuses only on chemistry, components, and molar ratios, teams miss the opportunity to design a delivery system that can realistically progress to clinical production. For "right first time" success, innovators or adopters should proactively conduct feasibility evaluations for GMP production at both clinical and commercial scales.

TL: Many programs fail not because the underlying structural activity relationship is flawed, but because early formulation decisions cannot support larger-scale development, much less clinical release for

Many programs fail not because the underlying structural activity relationship is flawed, but because early formulation decisions cannot support larger-scale development, much less clinical release for human use.

From a CMC perspective, downstream processing is a key step where reproducibility is preserved or deviations manifest.

human use. Even in the earliest phases, formulation decisions should be made with the GMP operation in mind to avoid limitations and challenges that can waste time and precious material.

What role does downstream processing play in achieving target-appropriate biodistribution?

TL: Downstream processing is all too often underestimated, particularly for lipid nanoparticles, yet it plays a critical role in determining whether on-target activity is preserved as a program advances. The selected unit operations and process conditions, such as tangential flow filtration (TFF), can each significantly influence particle size distribution, lipid composition maintenance, and RNA-lipid adduct formation, directly affecting *in vivo* performance and potentially compromising tolerability.

If these operations are not optimized with the same rigor as formulation composition, variability can be introduced, obscuring or even altering the desirable biological outcomes that qualified the candidate selection. From a CMC perspective, downstream processing is a key step where reproducibility is preserved or deviations manifest. We encourage early innovators to consider clinically relevant downstream processes to avoid significantly delaying the development timeline.

Why are well-constructed CMC programs so crucial for positioning a therapeutic for successful advancement to the clinic?

ET: An early program may demonstrate compelling expression, knockdown, or editing in the target tissues or cell types of small *in vivo* studies, but if innovators cannot convey a scalable process development plan that demonstrates CQA control, regulatory confidence erodes, and IND timelines can be significantly delayed or derailed altogether.

A strong CMC strategy links formulation intent to manufacturing reality. It addresses whether a delivery system can be reproduced consistently, scaled while preserving efficacy, and comprehensively characterized to ensure reliable safety.

Where do you see the most significant opportunities for advanced drug delivery systems in today's market?

ET: The industry buzz around non-viral delivery technologies for *in vivo* CAR-T is particularly energizing for us, and we see significant opportunity for innovators to further advance the viability of these treatments. This specialized field is attractive because *in vivo* CAR-T can deliver therapeutic payloads that effectively treat a wide range of cancers and autoimmune diseases, while limiting off-target adverse effects. Meanwhile, compared with *ex vivo* cell therapy, *in vivo* CAR-T significantly reduces the number of hospital visits required, thereby reducing patient burden.

We're excited that more innovations in this space are on the way, such as new target-site-binding ligands that enable more favorable CQA control through surface conjugation and purification steps of the conjugated LNP manufacturing process, without compromising binding affinity or resulting efficacy.

Additionally, facilitating new routes of administration for non-viral therapies is poised for significant research and will benefit patients with central nervous system (CNS), kidney, respiratory, and many other conditions. Given our experience with lipid, polymeric, and ligand-conjugated nanoparticles, these efforts are of significant interest to the Phosphorex team, and we are well-positioned to serve as a key resource for the industry as these programs advance.

Target-Appropriate Biodistribution Success Requires Comprehensive Strategies Throughout the Development Lifecycle

Achieving target-appropriate biodistribution is not just a formulation challenge; it spans formulation science, process design, and CMC. As delivery technologies mature, therapeutics become increasingly complex, and regulatory stringency rises. IND filing success will increasingly depend on the careful balance and integration of these disciplines from discovery through filing.

Phosphorex helps biopharma and biotech companies engineer targeted delivery vehicles by integrating formulation development, process design, and CMC strategy into a streamlined, clinically viable approach. By designing delivery systems with scalability, reproducibility, and regulatory expectations in mind from the outset, we enable partners to maintain delivery performance *in vivo* and de-risk the development path from candidate selection to IND filing.

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Derisk Therapeutic Nanoparticle and Microsphere Programs with Stage-Appropriate Analytical Method Development

Theresa Logan, Head of Laboratory Operations, Phosphorex

Formulation, process development, and analytics are interconnected. Advancing analytical method development in a stage-appropriate manner is critical to ensure the success of therapeutic nanoparticle and microsphere programs from feasibility to clinical manufacture. Whether programs involve lipid nanoparticles, polymeric nanoparticles, or polymeric microspheres encapsulating nucleic acids, small molecules, peptides, or biologics, including proteins, developing appropriate methods during the preclinical, early, and late clinical phases can accelerate development while reducing risk.

General Analytical Considerations for Therapeutic Nanoparticles and Microspheres

Therapeutic nanoparticles encapsulate active pharmaceutical ingredients (APIs), providing cargo protection, improved pharmacokinetics and bioavailability, and enabling targeted delivery, enhanced tissue penetration, and intracellular delivery. Therapeutic microspheres also encapsulate APIs, improving drug stability and bioavailability while enabling controlled and sustained release, which can reduce side effects and toxicity and improve patient compliance.

Lipidic and polymeric materials are the most commonly used compositions, including both synthetic and biomimetic lipids and polymers. Nanoparticles can encapsulate small molecules, proteins, peptides, and nucleic acid cargos, and their small size enables nonviral intracellular delivery. In contrast, microspheres are generally too large for intracellular delivery and therefore are unsuitable for nucleic acid

delivery. However, their larger size and depot-like properties extend the effective duration of drug exposure, increasing the apparent half-life and dosing interval and making microspheres well-suited for sustained-release drug delivery.

During development including in-process testing and clinical product release, analytical methods are required to assess both the loaded particles and their payloads. The physical and chemical properties of the particles must be characterized, along with endotoxin and bioburden levels. For loaded particles, attributes such as particle size, encapsulation efficiency, cargo content and integrity, and release kinetics must also be evaluated. However, a key challenge in characterizing cargos encapsulated within particulate-based drug delivery systems is extracting the cargo from the particles without degrading or compromising their integrity. This challenge is particularly significant for sensitive cargos such as proteins and nucleic acids.

In addition to gathering data to support regulatory submissions and meet regulatory requirements at each development phase, analytical methods should be developed from the outset to ensure that physicochemical properties, stability, and biological performance are well understood. This approach helps streamline program advancement and minimizes the need for rework. A risk-based approach to method development ensures that appropriate methods are implemented at each stage, progressively increasing understanding of physicochemical properties, stability, and biological performance as the program advances from discovery to the clinic. It also ensures that methods remain fit for purpose, with method qualification and validation activities expanding as programs progress toward late-stage clinical development and commercialization.

Benefits of Stage-Appropriate Analytical Method Development

Cost- and time-effective drug development requires selecting and implementing the right set of analytical methods at each stage. Developing stage-appropriate methods minimizes analytical costs while generating the data needed to support informed, data-driven decisions throughout formulation and process development. This approach ultimately enables preparation of an investigational new drug (IND) application and the collection of first-in-human proof-of-concept data.

Working with an experienced contract development and manufacturing organization (CDMO) can be critical to making informed method development decisions, particularly when formulating specialized products such as therapeutic nanoparticles and microspheres. A CDMO can leverage prior experience from similar projects to recommend which methods are needed early in formulation and/or process development to support the assessment of the formulation's critical quality attributes (CQAs) (e.g., particle size), and which can be delayed until development progresses towards the clinic.

Understanding Physicochemical Properties Paramount at the Proof-of-Concept Stage

Several attributes of therapeutic particles must be thoroughly understood at the proof-of-concept stage. First, the physicochemical properties of the particles include particle size distribution, surface charge, cargo content, encapsulation efficiency, integrity, and release kinetics.

Particle size strongly influences particle behavior, particularly biodistribution and cellular uptake. As such, it plays an important role in determining the organs and tissues in which the particles will accumulate. Particle size also dictates the sterilization strategy that must be employed. Nanoparticles can typically be filtered through 0.2-micron sterilizing filters, while microspheres, which are generally formulated for subcutaneous (SC) or intramuscular (IM) delivery, are typically manufactured via fully aseptic processes in a controlled, sterile environment or terminally sterilized via gamma irradiation or e-beam.

Surface charge affects protein adsorption, cellular interactions, and uptake, which are particularly important in the development of therapeutic lipid nanoparticles (LNPs).

Cargo content and stability are other important properties that must be well understood early in development. For instance, it is important to confirm that protein cargos are not denatured during the encapsulation process, and that sensitive nucleic acids remain stable once encapsulated and released from the delivery system. Significant project delays can result if instability is noted during process development and scale-up rather than early in the program.

In addition to gathering data to support regulatory submissions and/or meet regulatory requirements at each development phase, analytical methods should be developed from the outset to ensure that physicochemical properties, stability, and biological performance are well understood.

Additional Expectations for Polymeric Microspheres

Polymeric microspheres require analysis using several additional, non-standard methods. Understanding the release rate is critical for extended-release formulations, as it directly impacts product safety. These delivery systems are designed to release their payloads gradually over time, and an initial burst or rapid release of a large portion of the cargo can lead to toxicity.

Process and formulation parameters can significantly influence the physicochemical properties of microspheres. Scaling up production from lab-scale equipment, such as probe-type sonicators or rotor-stator homogenizers, to clinically relevant unit operations, such as an inline homogenizer, can, for example, produce particles with altered API distributions and morphologies, particularly in porosity. The choice of solvent evaporation technique and the evaporation rate can also markedly affect API distribution, particle morphology, and release kinetics. Because these structural differences directly impact release behavior, scanning electron microscopy (SEM) is frequently used for detailed structural analysis, while Raman spectroscopy provides

information on the spatial distribution of the API within the microspheres.

Residual solvent content is another critical attribute, as it can affect the performance of polymeric microspheres. Monitoring solvent levels from the outset of process development, rather than waiting until clinical operations, is essential. Developing stage-appropriate analytical methods ensures a thorough understanding of solvent content at each process step and confirms consistency as the process is scaled.

Syringability and injectability are other important considerations for polymeric microspheres. Because these systems are used for sustained-release applications, the total amount of cargo that must be delivered over the intended release period should be evaluated early in development. This assessment informs key parameters, including required drug loading, injection volume, needle gauge selection, and helps determine whether the target dose can be delivered within the desired injection volume and needle specifications. Injectability and syringability can be studied cost-effectively using an Instron device, providing a preliminary understanding of these parameters before *in vivo* studies.

Phosphorex has the necessary experience and expertise to develop phase-appropriate analytics that assess the right attributes at the right time, with the sensitivity and robustness needed to support informed decision-making, meet or exceed regulatory expectations across all program phases, and enable rapid program advancement.

Confirming PK and Biodistribution is Crucial when Advancing to the Lead Formulation Stage

As programs advance toward lead formulation selection, understanding the pharmacokinetics (PK) and biodistribution of therapeutic nanoparticles becomes essential. Effective methods are needed to measure circulation half-life, cellular uptake, and organ accumulation, particularly when the nanoparticles are designed to target specific biological sites. Early characterization of these attributes informs formulation optimization and guides decision-making as the program progresses towards the clinic.

While *in vitro* studies provide valuable mechanistic information, it is critical to evaluate PK and biodistribution in relevant *in vivo* models as early as possible, starting with rodents and progressing to nonhuman primates (NHPs). For lipid nanoparticle (LNP) formulations, a major challenge is the limited translatability of results from *in vitro* systems to animal studies, including those in rats and NHPs. Strategies to improve this translatability are essential to increase the proportion of formulations that successfully reach clinical evaluation.

Targeted LNPs (tLNPs) present additional translational challenges because the cell-surface receptors used for targeting specific human cell- and/or tissue-types are often absent, expressed at different levels, or structurally different in small animal models. Humanized mice engrafted with human immune cells provide more predictive data than standard mice for immunogenicity, PK, efficacy, and toxicity, though the degree of humanization can significantly influence data interpretation. NHPs offer further human-relevant insights, particularly for systemic PK and immune responses. Despite these models, translatability limitations remain, making complementary data from *in vitro* studies, rodents, and humanized mice, combined with careful study design, critical for robust clinical translation.

Increasing Robustness and Confirmation of Identity, Safety, and Tolerability Required as Programs Move toward GMP

While at the preclinical stage, the focus is on determining whether a therapeutic nanoparticle or microsphere formulation solves a real biological problem and if that behavior translates into *in vitro* and then *in vivo* performance. Once that is successfully demonstrated, it is necessary to show that the biological effect is robust and reproducible. As programs get closer to the clinic, confirming the safety and tolerability of the formulation and the translatability of animal data into humans becomes paramount.

Assays in general remain similar as programs move from the proof-of-concept stage to early clinical studies. During the earliest development phases, ideal methods provide quick readouts to support evaluation of multiple formulations in parallel, yet are sufficiently robust and reproducible to ensure confidence in the ability to rank formulations. As programs advance into early clinical development and beyond, the robustness of the method becomes increasingly important and must reach a level that supports validation.

For all types of therapeutic nanoparticles and microspheres, identity determination also becomes increasingly important as programs move toward clinical manufacturing. It is essential to have robust methods to confirm the identity of both the cargo and the components of the particle delivery system (lipids, polymers, etc.). Typically, fluorescence-based assays or liquid chromatography (LC) methods are used to determine the cargo composition and encapsulation efficiency. The integrity of small-molecule, peptides, and protein cargos is typically assessed by LC, whereas a combination of capillary electrophoresis (CE) and LC is used for nucleic acid payloads.

For LNPs, it is also important to accurately establish lipid identity and determine the lipid composition. Because ionizable lipids may pose toxicity risks, regulators require confirmation of their safety. For polymeric systems such as poly(lactic-co-glycolic acid) (PLGA), despite being GRAS (generally regarded as safe) and widely used as pharmaceutical excipients, it is important to have appropriate polymer identity and content methods in place during early-phase clinical trials to ensure quality control, product performance, and batch-to-batch consistency.

Other assays that gain importance as programs advance towards the clinic include purity methods, which are typically based

Putting the Strategy into Practice at Phosphorex

Phase-appropriate method development at Phosphorex is achieved using several different approaches. Two good examples include a modified RiboGreen assay for analyzing messenger RNA (mRNA) payloads in LNPs and an *in vitro* release method for polymeric microspheres.

The RiboGreen assay is a sensitive, fluorescence-based method for quantification of mRNA in solution and encapsulation efficiency in LNPs. It is attractive for mRNA-LNP analysis because it is essentially a plug-and-play method that can be used across all LNP formulations. It is, in fact, the gold standard method used at the proof-of-concept/preclinical stage.

Because the RiboGreen method is a plate-based assay, it suffers from variability, which can reach 10%—a level not typically acceptable for API cargo methods. Phosphorex has overcome this variability issue by automating the conventional RiboGreen assay using robotic liquid handlers. This solution eliminates the variability associated with human operations, such as pipetting. It also enables the parallel screening of multiple formulations at higher throughput.

For analysis of polymeric microspheres, which are typically used in sustained-release formulations, the gold standard method for *in vitro* release assessment is the USP Apparatus 4 (Flow-Through Cell) method. This method is relatively complex in terms of development and parameter definition, and very limited in the number of samples it can test at once.

To simplify and accelerate analysis of the *in vitro* release properties of polymeric microspheres, Phosphorex adapted the USP Apparatus 2-like protocol for pharmaceutical dissolution testing. The sample is shaken in the *in vitro* release media within a vessel placed in a water bath, and the resulting data provide relative release rates (fast, medium, slow). This method is more straightforward and higher-throughput and thus provides sufficient information more quickly to appropriately rank formulations and identify the top candidates.

on size-exclusion chromatography (SEC) or ion-pair reverse-phase high-performance LC (RP-HPLC). The development of robust methods for detecting endotoxins and bacterial contaminants (e.g., sterility), residual solvents, and particulates also become essential as programs move toward the clinic.

Finally, as a program moves towards late-stage clinical studies and commercialization, scalability must be demonstrated. Appropriate analytics are needed to clearly demonstrate that any changes in process conditions that occur upon scaling do not impact the particle properties and *in vivo* performance of the therapeutic.

Deep Expertise in Particulate-Based Delivery Solutions

The inherent complexity of lipid and polymeric nanoparticles often creates significant translational gaps between preclinical and clinical development, particularly in achieving cost-effective, scalable, clinically relevant production operations. Access to expertise in therapeutic nanoparticle and microsphere technologies and the ability to develop phase-appropriate analytical methods are essential to addressing those gaps.

Phosphorex has focused solely on particulate-based drug delivery systems since its founding. Technologies covered include polymeric nanoparticles and microspheres, lipid nanoparticles and liposomes containing nucleic acids (mRNA, small interfering RNA, plasmid DNA, and other oligonucleotides), small-molecule, peptide, and protein payloads.

Over more than two decades of applying the standard methods required by regulatory agencies for particulate-based systems, the company has established a deep toolbox of techniques applicable across all three platforms. Standard and specialized methods are applied that ensure thorough characterization and accurate quantitation of both particles and their cargos. That includes the use of technologies that enable careful extraction of cargos from particles without impacting their critical quality attributes.

Similarly, for targeted LNPs and polymeric particles, Phosphorex has extensive expertise in the conjugation and characterization of targeting ligands presented on the particle surface, enabling accurate characterization and quantitation of their targeting ability.

As importantly, Phosphorex has the necessary experience and expertise to develop phase-appropriate analytics that assess the right attributes at the right time, with the sensitivity and robustness needed to support informed decision-making, meet or exceed regulatory expectations across all program phases, and enable rapid program advancement. Contact us to discuss how we can help to advance your program.

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TECHNICAL BULLETIN

Effect of Downstream Buffer Type on LNP Size

Introduction

After self-assembly of lipid nanoparticles (LNPs) through mixing of lipid and cargo solutions, further dilution or buffer exchange is required to reduce/remove ethanol and stabilize the particles. During this process, the pH is raised resulting in de-protonation of the ionizable lipids which subsequently fuse to form the hydrophobic, amorphous core of the LNPs [1]. Small LNPs may also gradually fuse to form larger particles that are more energetically favorable through a process known as Ostwald ripening [2].

Particle size is one of the most significant parameters that affects LNP biodistribution [3]. In many cases, LNPs with a certain size range are required to achieve better tissue, organ, or intracellular targeting. The LNP size can be tuned via adjusting the PEG-lipid content, microfluidic mixing conditions, and formulation buffer composition [4]. However, the fusion and ripening phenomenon of LNPs during downstream processing often results in a particle size increase which increases the risk of LNP size exceeding the target range. In this study, we aim to compare the effect of different downstream buffer types on the LNP particle fusion, morphology and final size.

Methods

To fabricate the LNPs, DLin-MC3-DMA (MC3), Cholesterol, DSPC, and DMG-PEG2K at molar ratio of 50:38.5:10:1.5 in ethanol were mixed with polyA (model cargo with ~6,000 nucleotides) in 10 mM citrate buffer (pH 3) at an N/P ratio of 6 using Unchained Labs' Sunshine system and 190 µm Sunny XT cross-junction chip or Cytiva's NanoAssemblr™ Ignite+™ system and NxGen™ chip. The flow rate ratio of aqueous solution and lipid solution was 3:1, and total flow rate was 12 mL/min. To remove ethanol, the post mixing LNP solutions were dialyzed against different buffers including PBS (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.5 mM KH_2PO_4; pH 7.4), TBS (150 mM NaCl; 50 mM Tris/Tris-HCl; pH 7.6) and TRIS (20 mM Tris/Tris-HCl; pH 7.4) using a Thermo Fischer Scientific's Pierce™ Slide-A-Lyzer® dialysis cassette (MWCO: 20 kDa) overnight at 4˚C. Alternatively, ethanol can be diluted and sample reconcentrated via three rounds of bulk dilution (using PBS, TBS or TRIS) followed by re-concentration using EMD Millipore's Amicon® filters (MWCO: 100 kDa). After the buffer exchange, LNPs were further concentrated to ~200 µg/mL (polyA content) and then filtered by 0.2 µm syringe filter. LNPs for morphology examination using Cryogenic Transmission Electron Microscopy (cryo-TEM) were concentrated to ~500 µg/mL (polyA). The mean particle diameter and polydispersity index (PDI) were determined via dynamic light scattering (DLS).

Results

As shown in Fig. 1, LNPs fabricated via microfluidic mixing on Unchained Labs' Sunshine system were ~80 nm. The dialysis method was used to remove ethanol (~25%v/v) present in the LNP solution post mixing. After dialysis in TRIS buffer, the LNP size increased to ~95 nm whereas dialysis against PBS and TBS maintained the size near 80 nm. Particle size was well maintained during the subsequent concentration step using EMD Millipore's Amicon® filter, and slightly decreased post 0.2 µm filtration.

While PBS, TBS, and TRIS are commonly used buffer systems for LNP downstream processing, both PBS and TBS have significantly higher salt content (ionic strength) as compared to TRIS buffer alone. What's interesting is that the effect of salt concentration in LNP formulation buffer and downstream buffer on size are quite different. LNPs fabricated in formulation buffers with higher salt concentration often result in larger particles due to the increased London-van der Waals attraction [5].

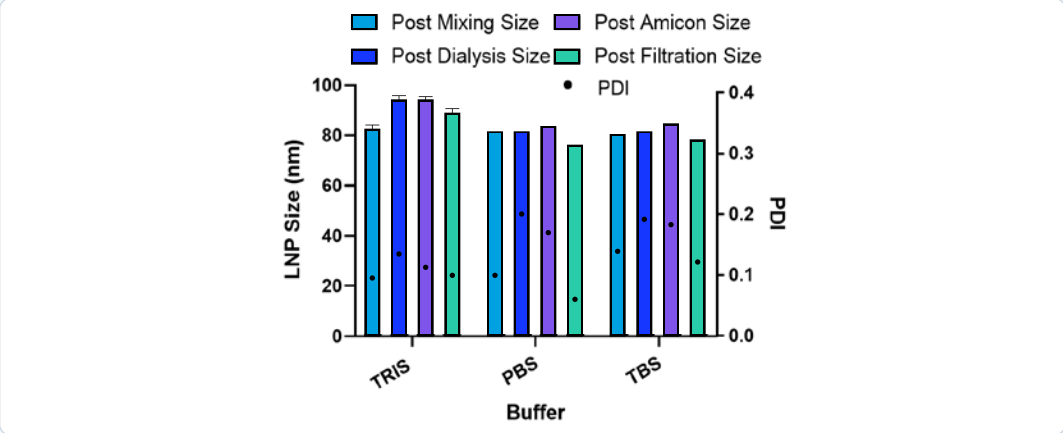

Figure 1: Effect of different downstream buffers on LNP size and PDI. LNPs were fabricated using Unchained Labs' Sunshine system and 190 µm Sunny XT cross-junction chip. The downstream processing steps include dialysis, concentration using Amicon® filters and 0.2 µm filtration.

For small-scale batches, bulk dilution and Amicon® concentration is another commonly used buffer exchange method to reduce the ethanol content and adjust pH in the post mixing LNP solution. In good agreement with our results obtained using dialysis method, LNPs diluted (10x) with TRIS buffer and re-concentrated using Amicon® filter three times had larger final particle size (~100 nm) compared to those processed with either TBS or PBS (~88 nm) (Fig. 2). It was also observed that the dilution/concentration (3x) method generated slightly larger particles than the dialysis method even when the same downstream buffer was used possibly due to different kinetics of ethanol removal which has been reported to impact the fusion of LNPs (Fig. 1 & Fig. 2) [2, 6]. Alternatively, multiple rounds of LNP concentration using Amicon® filter may result in LNP fusion and aggregate formation through interaction with the filter membrane.

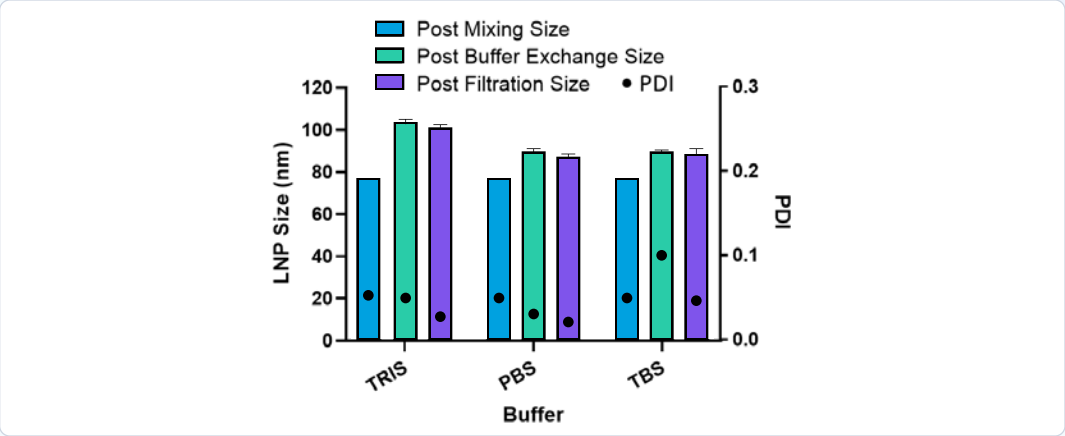

Figure 2: Effect of different downstream buffers on LNP size and PDI. LNPs were fabricated using Unchained Labs' Sunshine system and 190 µm Sunny XT cross-junction chip (Unchained Labs). The downstream processing steps included bulk dilution and Amicon® concentration three times followed by 0.2 µm filtration.

In Fig. 3, LNPs fabricated via Cytiva's NanoAssemblr™ Ignite+™ system were ~64 nm which is smaller than those fabricated via Unchained Labs' Sunshine system. After dialysis in TRIS buffer, the LNP size gradually increased during downstream processing and reached a final particle size of ~80 nm. By comparison, post mixing LNP size was well maintained during dialysis in TBS buffer and the following downstream steps (Fig. 3).

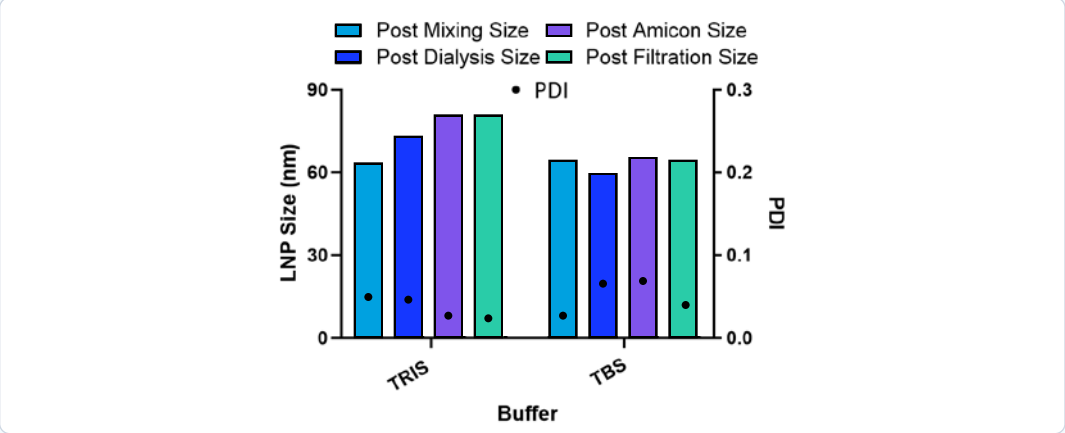

Figure 3: Effect of different downstream buffers on LNP size and PDI. LNPs were fabricated using Cytiva's NanoAssemblr™ Ignite+™ system and NxGen chip. The downstream processing steps include dialysis, Amicon® concentration and 0.2 µm filtration.

To determine whether downstream buffer type has additional impacts beyond particle size, we investigated LNP morphology via cryo-TEM. As shown in Fig. 4, LNPs dialyzed in TBS buffer showed normal solid core morphology (panel B), whereas LNPs dialyzed in TRIS buffer displayed distinct bleb structures (panel A). Other parameters have been reported to influence bleb structure formation including ionizable lipid type and formulation buffer composition [7]. LNP formulations prepared in presence of high concentrations of sodium citrate resulted in bleb structure formation and improved transfection efficiencies both *in vitro* and *in vivo*, possibly due to improved integrity of the mRNA cargo [7].

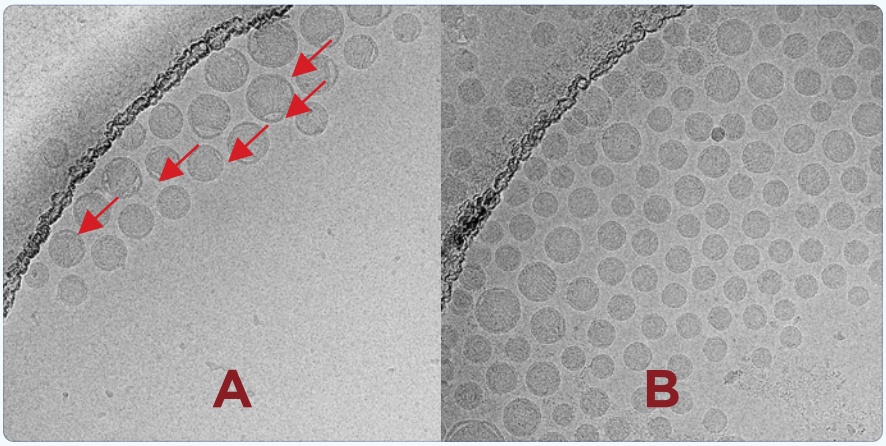

Figure 4: Cryo-TEM images of LNPs using TRIS (Panel A) or TBS (Panel B) buffer for downstream processing. Red arrows indicate the bleb structure of LNPs. LNPs were fabricated using Cytiva's NanoAssemblr™ Ignite+™ system and NxGen chip. The downstream processing steps include dialysis, Amicon® concentration and 0.2 µm filtration.

Conclusion

In this study, the effect of downstream buffer type on LNP size was investigated. Use of buffers with higher ionic strength (e.g., TBS, PBS) resulted in better maintenance of LNP particle size during downstream processing. Downstream buffer choice may also influence LNP morphology including bleb structure formation as observed via cryo-TEM. In addition to optimization of the LNP size through formulation parameters and upstream mixing technique, the downstream processing parameters including buffer composition need to be carefully considered to achieve the target product profile. Phosphorex has extensive experience in upstream LNP formulation design and optimization, as well as great expertise in selecting the most appropriate downstream processing methods for each formulation. This enables fast formulation screening and seamless transfer to cGMP manufacturing of customized LNP products.

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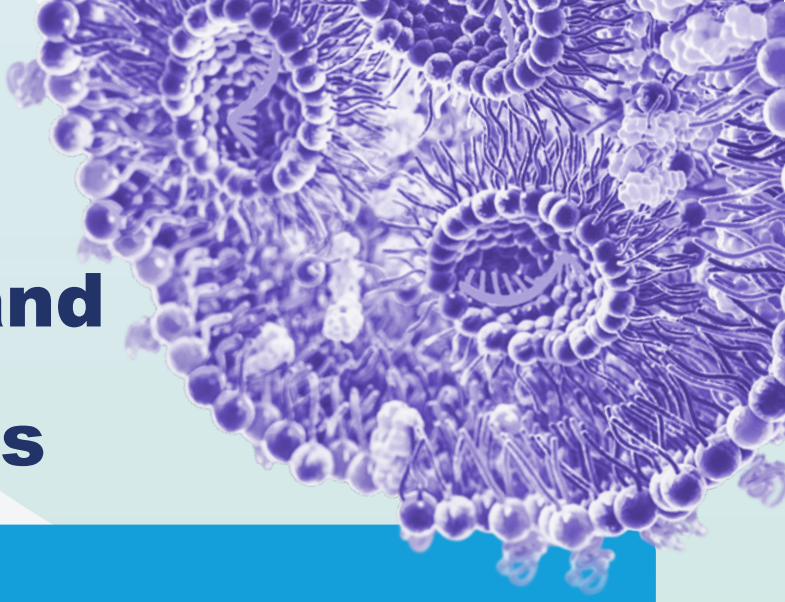
SUCCESS STORY

Process Quality and Control for Novel Gene Editing LNPs



⚠ Problem

A biopharma company developing a novel co-encapsulated gene-editing LNP system was dissatisfied with the batch quality produced by its incumbent supplier. Variability in particle attributes and cargo integrity raised concerns about suitability for preclinical development and clinical translation, prompting the search for a new delivery partner.

Solution

Phosphorex evaluated the co-encapsulated mRNA cargos in the quality LNPs it fabricated following clean process protocols, conducting integrity analysis by capillary electrophoresis to measure a weight ratio of 1.07:1.00 (wt:wt), respectively, for the intact cargo species versus the target theoretical 1:1 ratio (wt:wt)

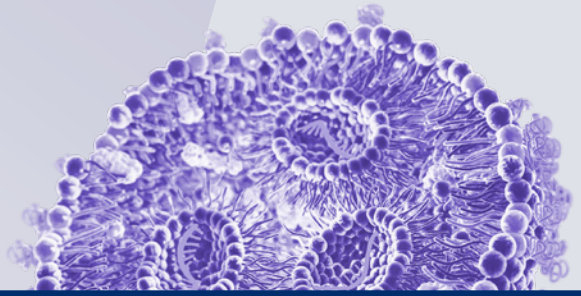
Outcome

Phosphorex delivered a high-quality LNP batch with a 77 nm mean particle size, PDI of 0.039, and 97% encapsulation efficiency following a freeze/thaw cycle at –80°C. mRNA cargo integrity closely matched the theoretical 1:1 ratio. The success led to repeated engagement to advance development.

At Phosphorex, we are committed to helping our partners navigate the complexities of precision drug delivery and advance their programs with confidence. We adapt to your modality, delivery target, and mission, bringing together comprehensive expertise through our Drug Delivery Engine to support every stage of development.

With payload-agnostic, non-viral delivery capabilities, deep experience, and an adaptive approach, we move at the pace of your program; iterating, refining, and aligning as needed. Through close collaboration and clear communication, we serve as a true extension of your team, focused on delivering meaningful progress.

Let's Solve Your Delivery Challenge ›



Behind the Solution

A biopharma client came to us after growing frustrated with inconsistent LNP batches from their previous CDMO service supplier. Their goal was straightforward: co-encapsulate two proprietary gene-editing cargos in a well-characterized LNP formulation suitable for preclinical studies. Variability in particle attributes and cargo integrity from prior batches had raised legitimate concerns about whether the material could support preclinical development, let alone clinical translation.

Our team approached the project leveraging phase-appropriate process protocols and rigorous analytical characterization at each stage. Cargo integrity was confirmed by capillary gel electrophoresis, which measured a weight ratio of 1.07:1.00 (wt:wt) for the intact cargo species against the theoretical 1:1 target, a result that gave the client confidence in both the encapsulation process and the analytical methodology supporting it.

The finished batch met demanding quality specifications across the board. Mean particle size was 77 nm with a PDI of 0.039, and encapsulation efficiency reached 97% after a freeze/thaw cycle at –80°C. Endotoxin levels held below 0.3 EU/ml. The high process yield produced enough frozen aliquots to fully support the client's preclinical testing needs without requiring additional manufacturing runs.

The numbers reflected consistent process control from formulation through fill-finish. Co-encapsulating two distinct mRNA cargoes at a defined ratio is technically demanding, and maintaining that ratio while meeting tight particle size and low PDI requirements requires both formulation expertise and disciplined execution. Our team rose to the occasion, which led to the client's decision to return for a follow-on optimization project. For gene-editing programs where cargo stoichiometry matters, innovators need this kind of process reliability to advance early feasibility work to material that can be effective in later phases of development.

Sarah Francis

Analytical Scientist

With a Bachelor of Science, Biomedical Engineering from Worcester Polytechnic Institute and a passion for particulate-based drug delivery, Sarah is rapidly climbing the ranks within Phosphorex's analytical team.

> Not many CDMOs have the expertise to co-encapsulate two distinct mRNA cargoes at a defined ratio and maintain that ratio while meeting tight particle size and low PDI requirements. I am proud to work alongside my colleagues, allowing Phosphorex to bring this much-needed expertise to the table.