

Development of a Robust LC–MS/MS Method for Quantifying SM-102 in Plasma and Tissues to Support LNP Biodistribution Studies

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Introduction

Lipid nanoparticles (LNPs) are widely used for the delivery of mRNA therapeutics, with ionizable lipids such as SM-102 playing a central role in intracellular delivery, pharmacokinetics (PK), and biodistribution of mRNA-LNPs. Despite their importance, sensitive and robust analytical methods for quantifying ionizable lipids across biological matrices remain limited. LC-MS/MS approaches are increasingly recognized as essential tools for understanding LNP biodistribution and clearance in preclinical models.

Methods

A high-resolution LC–MS/MS method was developed for the sensitive quantification of SM-102 in plasma and tissue matrices. The assay achieved a lower limit of quantification (LLOQ) of 1 pg/mL in plasma and was validated across liver, spleen, and muscle. The method was applied to biodistribution studies in C57BL/6J mice at 6 and 24 hours post-dose in plasma and tissues following intramuscular (IM) administration of SM-102-based LNPs containing mRNA doses of 50 and 500 µg/kg. Lipids were extracted from plasma and tissue samples and analyzed using a single LC–MS/MS workflow.

Results

The method demonstrated high sensitivity, selectivity, and robustness across all matrices. SM-102 was successfully quantified in plasma and tissues following IM administration at both dose levels; however, detection was dose- and time-dependent. At the higher mRNA dose (500 µg/kg), SM-102 was quantifiable across multiple tissues at all assessed time points. In contrast, at the lower mRNA dose (50 µg/kg), quantifiable levels were primarily observed at early time points, with concentrations falling below the limit of quantification at later time points. Tissue distribution profiles showed highest concentrations at the injection site and liver, with measurable levels in the spleen and plasma.

Conclusion

This study presents a sensitive and robust LC–MS/MS platform for quantifying the ionizable lipid SM-102 *in vivo* across multiple biological matrices. The method supports comprehensive biodistribution studies of LNP systems and provides a foundation for future work incorporating lipid metabolite characterization and biodegradation profiling. In combination with emerging mRNA-focused approaches such as long-read sequencing, this platform contributes to a more complete understanding of LNP pharmacokinetics to support the development of next-generation mRNA therapeutics.

Light-Activated Lipid Nanoparticles for Spatially Controlled Nucleic Acid Delivery

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Introduction

Lipid nanoparticles (LNPs) have emerged as leading non-viral vectors for nucleic acid delivery. However, their therapeutic potential remains constrained by inefficient endosomal escape and the limited ability to control intracellular cargo release with spatial precision. Here, we developed a light-activated LNP platform that enhances the intracellular delivery of large nucleic acids and enables localized transfection of selected cell populations.

Methods

Cationic LNPs encapsulating either mRNA or pDNA were assembled with citrate-coated gold nanoparticles (AuNPs) through electrostatic interactions to form light-activated LNP-AuNP hybrids. Following cellular uptake, the hybrids were activated using a 532 nm nanosecond pulsed laser. Transfection efficiency and cell viability were evaluated in HeLa cells and primary human mesenchymal stem cells (MSCs) by flow cytometry and CellTiter-Glo metabolic assay, respectively. Laser-triggered endosomal escape was investigated using calcein-release imaging and transmission electron microscopy. Spatially controlled delivery was performed by image-guided laser irradiation of predefined regions or selected cells and then visualized by confocal imaging.

Results

Laser activation substantially enhanced the delivery of both mRNA and pDNA. In HeLa cells, mRNA transfection increased to over 75% while maintaining approximately 90% cell viability. For pDNA delivery, laser irradiation approximately doubled the transfection efficiency compared with non-irradiated controls. Mechanistic studies confirmed efficient laser-triggered endosomal disruption, resulting in cytosolic cargo release. In hard-to-transfect primary MSCs, light-activated LNP-AuNP hybrids achieved nearly 40% mRNA transfection efficiency, whereas conventional LNP formulations yielded only approximately 2-4%. Importantly, image-guided laser activation enabled spatially

restricted transgene expression in predefined patterns and even in individually selected cells within heterogeneous co-cultures.

Conclusions

Light-activated LNP-AuNP hybrids provide a versatile strategy for the intracellular delivery of large nucleic acid cargos by externally triggering endosomal escape. Beyond markedly improving transfection efficiency, this platform enables spatially resolved gene delivery with single-cell precision, offering new opportunities for *ex vivo* cell engineering and next-generation RNA therapeutics.

Optimization and comparative evaluation of the production of targeted lipid nanoparticles using click chemistry and one-pot conjugation approaches

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Abstract

RNA therapeutics have demonstrated significant potential in the treatment of infectious diseases through delivery by lipid nanoparticles (LNPs). However, the pronounced hepatic accumulation of conventional LNPs limits their application in cancer immunotherapy, where selective targeting of tumour-draining lymph nodes (TDLNs) could provide a major therapeutic advantage. TDLNs are key immunological sites enriched in progenitor exhausted T cells, which play a central role in sustaining anti-tumour immunity. Because LNP biodistribution is strongly influenced by their physicochemical properties, extrahepatic delivery strategies increasingly rely on surface functionalization with ligands, such as antibodies, that recognize specific receptors on cell populations outside the liver. Here, we describe and compare two antibody-conjugation strategies for the generation of targeted LNPs (tLNPs): a modular click-chemistry approach and a one-pot formulation method. We optimized both strategies to achieve efficient and reproducible antibody grafting while preserving nanoparticle integrity. Click chemistry enabled modular incorporation of distinct antibody linkers and complementary functionalized PEG anchors on the LNP surface, whereas the one-pot method allowed direct antibody functionalization during nanoparticle formulation. Both approaches were systematically evaluated through formulation optimization, physicochemical characterization, quantification of grafted antibodies, and assessment of specific delivery *in vitro*. Antibody conjugation preserved nanoparticle stability, and the number of antibodies grafted per nanoparticle was quantified using the CBQCA assay. Functionally, tLNPs obtained with click chemistry enhanced specific delivery and transfection in HER2-positive cancer cells by 5-fold, with a 7-fold increase in protein expression. In PD-1-transduced immune cells, tLNPs increased transfection by 3- to 20-fold alongside an increase by 8- to 10-fold in protein expression depending on the type of LNP used, all showing minimal toxicity. These results support receptor-mediated uptake as the basis for improved and specific delivery and establish both conjugation strategies as robust platforms for the development of extrahepatically targeted LNPs for cancer immunotherapy, allowing for ongoing *in vivo* studies.

A Single-Dose mRNA-LNP Vaccine Encoding Membrane-Anchored Antigens Provides Robust Protection Against Pneumonic Plague

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The success of the mRNA-lipid nanoparticle (LNP) platforms has prompted significant interest in their application against bacterial pathogens. However, adapting these platforms requires optimizing the structural design of host-expressed bacterial antigens to ensure robust immunogenicity. While membrane anchoring is a promising strategy known to enhance the efficacy of viral mRNA vaccines, its application in bacterial mRNA platforms remains largely unexplored. Here, we applied this approach to develop a bivalent mRNA-LNP vaccine against *Yersinia pestis*, the causative agent of plague, utilizing the major antigens F1 and LcrV. We evaluated glycosylphosphatidylinositol (GPI)-anchored and transmembrane variants alongside secreted monomeric and hFc-conjugated formats. Our findings demonstrate that membrane-tethering of the F1 and LcrV antigens significantly improves overall immunogenicity. Whereas secreted monomeric and hFc-conjugated variants elicited predominantly cell-mediated and serological immune responses, respectively, the membrane-anchored bivalent mRNA vaccine generated a robust and balanced immune response that facilitated protection in a single-dose vaccination regimen. By meeting the single-dose biodefense benchmark prioritized by the World Health Organization Target Product Profile, this construct emerges as a highly translational candidate for a rapid-response plague vaccine. Ultimately, this study demonstrates that extending established antigen-tethering strategies to bacterial targets is a highly effective approach for generating the balanced immunity required to overcome complex bacterial infections.

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Title: Lipid nanoparticles with enhanced endosomal escape improve mRNA delivery to retinal cells

Purpose: Inherited retinal degenerations (IRDs) cause vision loss and blindness. IRDs are often monogenic disorders, making gene therapy a promising treatment strategy. Adeno-associated viruses (AAVs) are commonly used to deliver gene therapy products, however, they are limited by a 4.7kb packaging capacity and emerging safety concerns regarding their viral origin. Lipid nanoparticles (LNPs) can be used as alternative non-viral delivery vesicles for gene therapy. The major bottleneck of LNP-mediated nucleic acid delivery is inefficient endosomal escape. As a result, high doses are often required for effective retinal transfection. To address this, we compared a widely used gold standard LNP (inspired by the clinical Onpattro formulation; LNP1) to an LNP with enhanced endosomal escape (LNP2), aiming to improve delivery efficiency to retinal cells.

Methods: We created LNPs containing eGFP-encoding mRNA, with or without a fluorescent label (DiD) to track the cellular uptake of the particles. LNPs were screened in the ARPE-19 cell line and primary rat retinal pigment epithelium cells. Cellular uptake and transfection efficiency were quantified by fluorescence microscopy and flow cytometry analysis. We also subretinally injected Brown Norway rats with LNP (100, 200, and 300 ng of mRNA per eye) and terminated them 24 h post-injection. Eyes and major organs were collected for transfection and biodistribution analyses.

Results: LNP2 outperformed LNP1 in transfection efficiency, despite similar cellular uptake levels. Additionally, at high doses, LNP2 exhibited lower cytotoxicity than LNP1. Improved retinal transfection was also observed *in vivo* with LNP2 compared to LNP1, though further analysis is ongoing to determine cellular specificity and biodistribution.

Conclusion: LNP2 achieved high transfection efficiency in the retina at lower doses than LNP1. Future studies will focus on determining the long-term safety and efficacy of this formulation for gene-editing applications.

Design Rules for Endosomal Escape: How Peptide Sequence Shapes Suitability for Next-Generation Targeted RNA Delivery

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Background: Endosomal escape remains one of the most persistent barriers limiting the efficacy of RNA therapeutics.^[1,2] Although lipid nanoparticles dominate current clinical delivery, their escape mechanisms are heterogeneous, incompletely understood and challenging to quantify.^[3] Peptide-based systems offer a modular alternative with tuneable membrane activity and compatibility with targeted delivery modalities such as antibody-oligonucleotide conjugates (AOCs), peptide-oligonucleotide conjugates and emerging RNA formulations.^[4,5] However, the field lacks quantitative frameworks for comparing escape-relevant behaviour across peptide designs and linking sequence features to intracellular fate.

Methods: A panel of four rationally designed membrane-active peptides - three endosomal escape peptides (EEPs 1-3) and one hybrid EEP-CPP construct - was synthesised *via* solid-phase peptide synthesis. All peptides were purified and validated by analytical HPLC and mass spectrometry. Physicochemical characterisation included Fourier-transform infrared spectroscopy, circular dichroism, and small-angle X-ray scattering to evaluate secondary structure. Cytocompatibility was evaluated across 0-10 μ M (1hr incubation) using CellTiter-Glo[®] and Alamar Blue assays, and membrane integrity was assessed using LDH release and calcein red-orange leakage assays. Confocal microscopy was used to quantify the rate and extent of internalisation and subcellular localisation, with uptake kinetics derived from puncta-level intensity measurements, lysosomal association, and image-level metadata.

Results: All peptides met expected design metrics, demonstrating $\geq 95\%$ purity, sequence identity, cytocompatibility, and minimal membrane disruption. Structural analyses revealed mixed α -helical/coil conformations, with fluorophore labelling modulating helicity. Lysosomal localisation was consistently high across constructs (Manders ~ 0.97 - 0.99), indicating lysosomal trapping as a shared fate. Uptake kinetics, however, differentiated therapeutic potential. The hybrid EEP-CPP construct showed strong, sustained internalisation, aligning with RNA delivery formats that benefit from efficient cell entry. In contrast, the “true” EEPs displayed tightly regulated or minimal uptake - EEP1 showed low internalisation consistent with pH-dependent activation, whilst EEP2 internalised rapidly but accumulated weakly - properties desirable for AOCs, where the antibody should drive uptake and the peptide should mediate selective endosomal escape. EEP3 exhibited heterogeneous, non-saturating uptake but maintained high lysosomal association. Together, these findings establish a clear design principle: peptide sequence determines therapeutic suitability, with CPP-like hybrids favouring RNA-focused applications and low-uptake, pH-responsive EEPs offering the greatest potential for AOC-mediated intracellular delivery.

Future Directions and Conclusions: This work provides a quantitative framework linking peptide sequence, structure, and intracellular behaviour with single-cell precision, offering actionable design rules for next-generation delivery systems. The next phase will verify endosomal escape mechanisms using peptide-oligonucleotide conjugates, AOCs, LNPs, and surface-functionalised delivery systems as orthogonal contexts to test sequence-dependent performance. These studies will be supported by vesicle-based disruption assays and Gal8-GFP live-cell reporters to quantify escape under controlled biophysical conditions. Together, these integrated approaches will define sequence-to-function rules for safe enhancement of endosomal escape and support the rational development of RNA delivery systems that extend beyond classical LNPs.

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Harnessing Memory T Cells Through Dendritic Cells *Via* Functionalised Lipid Nanoparticles

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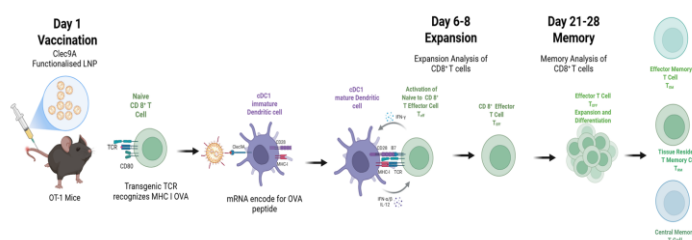
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The success of mRNA lipid nanoparticles (LNPs) as vaccines and now Immunotherapeutics have gained increasing attention due to their high tunability ranging from lipid molar ratio formulations, new generations of ionizable lipids,^[1] to cell-specific targeting features using monoclonal antibodies (mAB).^[2] Issues arise with only acute memory responses observed and no real long-term immunity like we observe with measles, yellow fever and small-pox vaccines. Dendritic cells (DCs) an antigen presenting cell (APC) are the main orchestrators between the adaptive and innate and are crucial in the formation and maintenance of memory T cell response.^[3] DCs contain a vast number of endocytic receptors that maybe the key enhancing memory cell formation, as they identify different pathogens from viruses, bacteria, fungus to necrotic cells Clec9A expressed on conventional DC1 (cDC1) detects necrotic cells and viral antigens and presenting towards CD8⁺ t cells^[4] which could improve long term memory cells. The use of ligand targeting peptides to functionalise LNPs is showing promising alternative than mAB conjugates^[5] over would more cost effective and scalable option for the future.



This project investigates a Clec9A targeting peptide^[6] containing a spacer group with DBCO and is functionalised to Pfizer-like LNP which shows comparable *in vivo* results to Moderna LNPs. Functionalisation will be completed through of non-commercialised Pfizer Pegylated Azide (ALC-0519 Azide) via Strain-Promoted Azide-Alkyne Cycloaddition (SPAAC). A high-density vs a low-density peptide functionalised LNP will be further investigated to observe if ligand density influences uptake. Functionalised LNPs encapsulate with ovalbumin (OVA) mRNA injected to the mice with APCs taken them up and presenting to T cells that will express the OT-1 t cell receptors. Time points of 7-8 days for expansion T cell analysis and 21-28 days for T cell memory analysis of spleen and liver will be determined by specific surface proteins expressed of on Tissue resident memory T cells, effector memory T cells and central memory T cells.

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Investigating the Impact of Biological Interactions on Lipid Nanoparticle Function and RNA Delivery Efficiency

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Background: Lipid nanoparticles (LNPs) are a leading platform for the delivery of RNA therapeutics, however, their in vivo behaviour following parenteral administration remains poorly understood. Upon exposure to biological fluids, endogenous proteins and biomolecules, including apolipoproteins, absorb onto the surface of LNPs, forming a protein corona that significantly alters their critical quality attributes (CQAs)^[1]. These changes have implications for LNP biodistribution, cellular uptake and therapeutic efficiency^[2], such as apolipoproteins promoting hepatic uptake, resulting in LNP accumulation in the liver^[3]. Understanding how biological fluid affects LNP structure and function is therefore essential for the design and optimisation of future RNA delivery systems targeting beyond the liver.

Methods: SM-102 LNPs encapsulating eGFP mRNA (N/P 10) were fabricated by microfluidic mixing at a lipid molar ratio of 50:10:1.5:38.5 (SM-102:DSPC:DMG-PEG2000:Cholesterol), with 3:1 aqueous: organic flow rate ratio and total flow rate of 15 mL/min. LNPs were characterised using dynamic light scattering, electrophoretic light scattering, nanoparticle tracking analysis and Ribogreen assays to assess particle size, polydispersity index, zeta potential and encapsulation efficiency. Frit-inlet asymmetric flow field-flow fractionation (AF4) was used to evaluate changes in LNP size and particle characteristics following incubation with high-density lipoprotein (HDL) and serum^[4,5]. Transfection studies were conducted in A549 and HepG2 cells in serum-containing and serum-free media, with and without HDL pretreatment. eGFP expression was assessed by fluorescence microscopy after 24 hours, and cell viability was evaluated using Alamar blue under the same conditions.

Results: SM-102 LNPs successfully transfected HepG2 cells in both serum-containing and serum-free media. In contrast, transfection in A549 cells was only observed in the presence of serum, suggesting a potential role for other serum proteins in enhancing cellular uptake in this cell line. Pretreatment of LNPs with HDL showed no transfection of these LNPs in both cell lines. This was due to HDL-induced alteration in LNP CQAs, including aggregation, changes in particle geometry and increased particle size as indicated by AF4 analysis.

Conclusions and Future Work: These findings demonstrate that the LNP protein corona in biological fluid has a critical impact on RNA delivery efficiency and transfection efficiency, with distinct effects observed across cell types. Future work will focus on correlating transfection efficiency with internalisation and subcellular localisation and expanding these studies to additional cell lines and ionizable lipids.

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Developing Clinically Relevant Pulmonary Delivery Platforms for Antiviral RNA Therapeutics

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Respiratory viral infections remain a leading cause of global illness and mortality, and increasing global connectivity continues to heighten the risk of rapidly spreading airborne diseases. There is a critical unmet need for targeted and adaptable therapeutic platforms for the treatment of existing and emerging respiratory viral infections. RNA therapeutics delivered in lipid nanoparticles (LNPs) are well-established tools for treating respiratory viral infections. A versatile, inhaled platform for localised RNA delivery at the site of disease, adaptable for targeting different viral pathogens, could minimise systemic exposure and maximise therapeutic efficacy.

Dry-powder inhalation supports commercial translation by offering enhanced drug stability, dose consistency and compatibility with scalable manufacturing processes. By optimising LNP and spray-drying formulations, this project aims to engineer novel dry-powder formulations for the safe and effective delivery of antiviral RNA to the respiratory tract.

By establishing a flexible and targeted inhaled RNA delivery platform, this project will support the development of safer and more effective antiviral treatments while strengthening preparedness for future outbreaks and pandemics.

RNA Delivery Conference

Prato Italy

Abstract (flash talk/poster)

Towards real-time quality monitoring of RNA–lipid nanoparticle assembly using soft sensors

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Lipid nanoparticles (LNPs) remain the dominant delivery system for therapeutic RNA, yet small variations during nanoparticle assembly can lead to large differences in RNA encapsulation and particle stability, which ultimately impact critical quality attributes (CQAs) such as *in vivo* potency and immunogenicity. As RNA therapeutics expand beyond conventional messenger RNA (mRNA) vaccines to include self-amplifying RNA (saRNA) and circular RNA (circRNA), ensuring consistent nanoparticle formation during scale-up has become a major bottleneck for translational development. A CQA of RNA–LNPs is encapsulation efficiency (EE%), which influences RNA stability, cellular uptake, and innate immune sensing. However, current fluorescence-based assays used to quantify EE% are destructive, time-consuming, and poorly suited for continuous manufacturing processes. This limits the ability to monitor LNP formation in real time and gives insight into batch-to-batch variability that may affect RNA biodistribution and therapeutic performance.

Here, we present a non-destructive soft sensing strategy that estimates RNA encapsulation efficiency during LNP formation using real-time conductivity measurements. By monitoring conductivity changes between inlet and outlet streams during nanoparticle assembly, the approach captures the dynamic complexation between RNA and ionizable lipids. A mechanistic model based on a modified Langmuir isotherm links conductivity changes to the effective RNA–lipid complexation rate, enabling rapid estimation of EE% and mixing efficiency within seconds. Experimental results demonstrate that conductivity variations provide a robust proxy for RNA–lipid complex formation across different RNA species.

This in-line conductivity-based sensing Process Analytical Technology (PAT) framework enables real-time monitoring of RNA–LNP formation and offers a scalable solution for integrating PAT into RNA manufacturing. By enabling tighter control over nanoparticle assembly, this approach may help reduce variability in RNA delivery systems and support the translation of both mRNA, saRNA and circRNA therapeutics from laboratory discovery to optimal continuous manufacturing.

Next-generation lipid nanoparticles with mucolytic lipids for mRNA nasal delivery: Toward mucosal vaccination

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Mucosal vaccination represents a paradigm shift in immunization, enabling protective immunity directly at the sites where pathogens commonly initiate infection—something that traditional intramuscular vaccines often fail to fully achieve. The nasal mucosa offers a particularly attractive route, providing access to a dense and responsive immune network through a minimally invasive approach. However, the development of effective mucosal vaccines remains challenging due to enzymatic degradation, physical barriers, and rapid clearance, all of which limit antigen stability and uptake.

Messenger RNA (mRNA) vaccines, successfully validated during the COVID-19 pandemic, constitute a versatile and powerful platform for both infectious disease prevention and cancer immunotherapy. Their adaptation to mucosal delivery requires advanced carriers capable of overcoming local physiological barriers. Here, we report lipid nanoparticle (LNP) formulations derived from a novel ionizable lipid library, incorporating mucolytic lipids to enhance mucus penetration. LNPs were produced via microfluidic mixing, yielding highly reproducible batches with ~99% encapsulation efficiency and uniform particle sizes of approximately 100 nm.

These LNPs demonstrated robust transfection efficiency *in vitro* in CALU-3 airway epithelial cells and DC2.4 dendritic cells, enabling strong protein expression without detectable cytotoxicity. In addition, evaluation of the innate immune response *in vitro* revealed no significant induction of inflammatory markers, supporting a favorable safety profile. Following intranasal administration in BALB/c mice, the LNPs achieved efficient mRNA delivery, with expression predominantly localized in the lungs, as confirmed by *ex vivo* bioluminescence imaging. Among the tested formulations, LNP-ART2 exhibited superior stability and prolonged expression, maintaining detectable protein levels for up to three days. This performance was further reinforced by an optimized lyophilization process that preserved nanoparticle activity during long-term storage. Importantly, we demonstrate that an optimized version of these LNPs can be successfully nebulized without compromising their physicochemical properties or biological activity. Nebulized LNPs retained transfection efficiency comparable to their non-nebulized counterparts, supporting their suitability for inhalation-based delivery.

Ongoing work is focused on implementing a vaccination protocol in murine models, comparing conventional (intramuscular) and mucosal (intranasal) routes of administration to assess the immunogenic potential of these LNP vaccines.

Collectively, these findings establish a versatile and scalable LNP platform with mucolytic functionality, capable of overcoming key barriers in mucosal vaccination and advancing the development of next-generation mRNA vaccines for both respiratory and systemic protection.

Following the arrow of time: single-molecule to single-cell microscopy enables in-depth characterization and optimization of nanomedicines

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Lipid nanoparticles (LNPs) delivering mRNA vaccines and therapeutics are effective but remain inefficient, in part due to their substantial heterogeneity in size, shape, composition, and mRNA loading. Understanding how these biophysical properties in the micro world relate to therapeutic properties in the macro world requires characterization of individual nanoparticles in solution and connecting measurements made across multiple scales [1-6]. Here, we present a single-molecule to single-cell platform for quantitative imaging of nano medicines in out-of-equilibrium environments which makes use of CLiC (Convex Lens-induced confinement) microscopy [2-6], label-free iSCAT (interferometric scattering) and multi-channel fluorescence detection [1,3]. We are curious to measure, for the same suite of formulations, the correlated distributions of nanoparticle size and cargo [1], mass [3], mRNA payload and structure [2], interaction kinetics [5], and multi-step dynamics in living cells [1], and interpret how and why they work at a granular fundamental level. Excitingly, we investigate how formulation parameters influence LNP structure and heterogeneity, with back-to-back cryo-electron microscopy of the same nanoparticle formulations [2-6]. We explore how our approach and results can bridge the gap between nanomedicine behaviour in the micro (biophysical) world to the macro (therapeutic) world, and ultimately fuel data-driven optimization of nanomedicines for patients.

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Simultaneous confocal fluorescence and label-free imaging for accessible and quantitative characterization of lipid nanoparticles and structures inside live cells

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Optical microscopy has played a central role in establishing what we know about lipid nanoparticles (LNPs), cells, and subcellular structures, where different imaging methods have complementary strengths and limitations. Currently, there are numerous microscopy methods that can provide detailed fluorescence and label-free scattering information of LNPs in controlled environments, such as in solution [1] and when bound to glass [2]. However, due to the complex background and environment inside cells, it has remained a challenge to perform quantitative simultaneous label-free and fluorescence measurements of nanoparticles inside cells, where enabling such measurement would open a unique window into the dynamics of LNPs during their journey inside cells.

Here, we introduce confocal interferometric scattering (C-SCAT) microscopy which enables quantitative label-free characterization of nanoparticles throughout cells, where the measurements can be coupled with simultaneous fluorescence measurements for detailed multiparametric characterization [2]. Using a commercially available confocal microscope, we first validate C-SCAT using nanoparticles in controlled environments, spanning standard buffers to cell-like conditions, where the C-SCAT signal follows the expected particle polarizability. Moreover, we validate that the C-SCAT signal estimate remains consistent in live cells for a wide range of background amplitudes. We further demonstrate that C-SCAT relates scattering from subcellular structures such as actin filaments, endoplasmic reticulum, and mitochondria, to physical diameters, and can capture spatially resolved dynamics as exemplified by measuring intra-nuclear dynamics. Thus, C-SCAT can provide wide-ranging information about the cell dynamics while also being able to provide detailed single-particle information.

Together, C-SCAT is an accessible microscopy method that is well suited for detailed characterization of LNP-cell interactions and native intracellular dynamics. The method is ideally suited for live cell studies of cells that face labeling challenges, such as primary cells, and for measuring particle and cellular dynamics beyond the limitations of photobleaching. Our ongoing work focuses on detailed measurements of single-particle trajectories in live cells during LNP uptake and functional cargo release and developing a deeper and more actionable understanding of these dynamic processes.

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*Equal contribution

Insight into Lipid Nanoparticle Structure and Function from the Perspective of Molecular Dynamics Simulations

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Lipid nanoparticles (LNPs) have enabled the rapid development of RNA-based medicine in vaccination, cancer treatment and the therapy of rare diseases. These systems encapsulate RNA within assemblies of ionizable lipids (ILs), helper lipids, and a PEG shell, achieving high loading efficiency through electrostatic interactions of ILs with RNA, while maintaining low cytotoxicity due to the neutral charge of ILs under physiological conditions. Despite intense efforts in ILs design aiming to improve the RNA delivery efficiency, progress is hindered by a limited molecular-level understanding of LNP structure, its pH induced changes, and the mechanisms underlying endosomal escape.

Here, we employ molecular dynamics (MD) simulations to resolve the structure and functional dynamics of LNPs at atomic resolution. We show that lipid phase behavior within LNPs is strongly modulated by IL molecular shape^{1,2} and environmental pH.³ Furthermore, MD simulations provide a fully controlled environment to examine direct atomic-level characterization of RNA–lipid interactions⁴ and, with current computational capabilities, allow modeling of LNPs at near-realistic scales,⁵ including their interactions with endosomal membranes.¹ Notably, we capture the early stages of LNP–endosome fusion, revealing molecular events that initiate endosomal destabilization (Figure 1).

These findings provide composition-dependent mechanistic insight into LNP structure and function and establish MD simulations as a powerful platform for the rational, in silico design of next-generation RNA delivery systems.

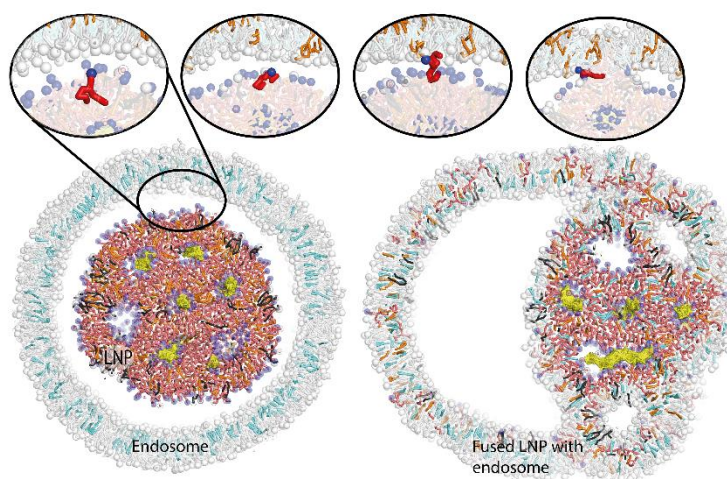


Figure 1: MD simulation insight into the fusion process of LNP (orange) with endosome (grey). The fusion of the LNP (left) is initiated by a single lipid handshake (upper insets) and results in deformed endosome (right).

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Intracellular structural evolution and fate of mRNA-LNPs as observed by Cryo-Electron Microscopy

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RNA delivery has emerged as a powerful therapeutic strategy, unlocking new possibilities for the treatment of previously undruggable diseases. The delivery of such therapeutics – particularly mRNA – relies on lipid nanoparticles (LNPs), yet significant challenges on their endosomal escape remain, making the delivery efficiency very low. Currently, our limited understanding of the mechanisms underpinning mRNA expression and intracellular fate of LNPs following endocytosis is a major bottleneck in advancing this technology. Addressing this knowledge gap requires advanced imaging methods, capable of capturing the structural evolution of LNPs and detecting RNA cargo in conditions that preserve biological integrity.

Cryo-electron microscopy (cryo-EM) is a powerful imaging technique for analyzing LNPs in a near-native state, offering a unique opportunity to observe their internal structure. *In vitro*, time-resolved cryo-EM imaging of LNPs during dynamic processes that mimic endocytosis, could provide insights into structural transitions that may influence delivery efficiency. Furthermore, *in cellulo* cryo-EM could provide the opportunity to study LNP behavior directly within intracellular compartments and during cellular trafficking.

In this poster we present a newly established state-of-the-art cryo-EM facility in Athens, Greece – the first of its kind in Southeast Europe – that can effectively support cutting-edge research in nanomedicine and RNA delivery. The facility workflows are optimized for high-resolution imaging of LNP structures, time-resolved studies, and *in situ* visualization of intracellular pathways involving LNPs and mRNA cargo. We showcase recent results generated by advanced imaging of mRNA-LNPs, dissecting their structural evolution and fate within biologically relevant environments. By utilizing the capabilities cryo-EM has to offer, this integrative technique will advance our understanding of LNP structure and dynamics, contributing to overcoming current limitations in RNA delivery.



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Exploring formation and clearance dynamics of clinical and novel mRNA-LNPs

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mRNA therapeutics have revolutionised vaccine platforms through the development and widespread use of Moderna and BioNTech/Pfizer's lipid nanoparticle (LNP)-formulated SARS-CoV-2 mRNA vaccines during the COVID-19 pandemic. Expansion of the technology beyond infectious disease vaccines, however, necessitates further fundamental understanding of these platforms under conditions encountered both in their development and biological application, so as to inform smart design and delivery.

We conducted two studies focusing on the LNP platform utilised for mRNA delivery. First, we interrogated the morphology and structural changes LNPs undergo during formulation, through fluorescence resonance energy transfer (FRET), small angle X-ray scattering (SAXS) and cryo-electron microscopy (cryoEM). We observed that particle structure changes significantly in the transition from an acidic environment to physiological pH and demonstrated that ionisable lipid pKa can determine the pH at which these changes occur.

Second, we investigated biological clearance of LNPs after repeated administration. Polyethylene glycol (PEG), part of the polymer-lipid component in LNPs required for particle stabilisation and its protein anti-fouling properties, has been known to trigger an immune response against it after repeated dosing. We developed a mouse model to investigate clearance rates of LNPs formulated with PEG-lipid or alternative hydrophilic polymer-lipids after two weekly doses. Our results demonstrated that PEG-LNPs were cleared rapidly from the circulation after a second weekly dose, while several polymer-LNPs, in particular positively charged POEGMA (POEGMA-2-(dimethylamino)ethyl methacrylate), exhibited minimal clearance after a repeated dose. LNP utilising PDMA (poly(N,N-dimethylacrylamide)) also displayed significantly lower clearance in the presence of anti-PEG antibodies.

These studies demonstrate the importance of lipid selection to harness the desired traits of LNP-based mRNA delivery systems and control their biological fate for diverse applications in nanomedicine.

Sustained Analgesia by Modulation of Synaptic Vesicle Endocytosis in Nociceptors Using a dCas9 Repression Strategy

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Abstract

Chronic pain is a common condition estimated to affect around 20% of people worldwide. Current treatments are limited by a lack of long-term efficacy, potentially dangerous side-effects, a significant risk of overdose and the requirement of regular administrations. Chronic pain is sustained by persistent synaptic transmission within nociceptive circuits. Synaptic vesicle endocytosis replenishes vesicle pools required for neurotransmission, but its nociceptor-specific contribution to pain and long-lasting therapeutic potential are unclear. We have identified SV endocytosis in nociceptors as a critical driver of ongoing pain and developed a mRNA-based strategy to target this mechanism.

A lipid nanoparticle-encapsulated CRISPR/dCas9-repressor system (dCas9-R) was employed to achieve sustained and reversible transcriptional and epigenetic repression of synaptic vesicle endocytic mediators following intrathecal delivery. dCas9-R mRNA was packaged into LNPs with sgRNA targeting the promoters of adaptor-associated kinase 1 (AAK1) and dynamin 1 (Dnm1). Multiple preclinical mouse models of acute and chronic pain were utilised to assess the analgesic potential of this strategy, including models of postoperative, inflammatory, neuropathic and osteoarthritic pain.

Endogenous expression of AAK1 and Dnm1 in lumbar dorsal root ganglia (DRG) was reduced by around 50% 48 hours after intrathecal administration of the dCas9-R construct. Administration of dCas9-R targeting AAK1 and Dnm1 eliminated both mechanical allodynia and thermal hyperalgesia throughout the course of a model of postoperative pain (7 days), and induced a significant level of analgesia in an inflammatory pain model (10 days). In longer-term models of neuropathic and osteoarthritic pain, administration of dCas9-R targeting either AAK1 or Dnm1 provided significant pain relief over a period of 28 days, suggesting the potential for this approach to induce chronic analgesia. Importantly, a single administration prevented or reversed pain hypersensitivity for extended durations without impairing normal nociception or locomotor activity.

These findings establish synaptic vesicle endocytic regulation as a mechanism underlying chronic pain and demonstrate the translational potential of LNP-delivered dCas9-R as a durable, non-opioid pain therapy.

Title: Respiratory delivery of self-amplifying RNA for vaccination against RSV

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Human respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract disease in newborns and young children, causing approximately 100,000 deaths annually in children under five years of age. To date, three RSV vaccines, Arexvy, Abrysvo, and mRESVIA, have been approved, but only for use in adults over 60 or in pregnant women. Among these, mRESVIA is an mRNA-based vaccine, while the other two are protein-based subunit vaccines. Self-amplifying RNA (saRNA) vaccines typically induce a stronger Th1-biased immune response compared to modified mRNA and may thus represent a promising alternative to decrease the risk of vaccine enhanced respiratory disease (VERD). Moreover, unlike the intramuscular route used by these three approved vaccines, which primarily elicits systemic immunity, respiratory delivery is expected to induce mucosal immunity that can neutralizing RSV upon entry. Respiratory vaccination could also improve pediatric RSV vaccine uptake by offering a more child-friendly alternative to injections. We therefore evaluated the efficacy of respiratory-delivered saRNA vaccines against RSV in a preclinical model. We designed two lipid nanoparticle-formulated self-amplifying RNA (saRNA) vaccines encoding a secreted version of either a wild-type (WT-F) or a prefusion stabilized (pre-F) RSV F protein. In an initial study, we investigated the systemic and local adaptive immune responses after intranasal, intratracheal, and intramuscular delivery of three doses of the saRNA^{WT-F} vaccine (0.25, 1 and 3.5 µg). Among the respiratory routes, intratracheal vaccination performed better than intranasal vaccination. Subsequently, we compared the humoral and cellular immune responses elicited by the pre-F and WT-F saRNA vaccines following both intratracheal and intramuscular administration using the most optimal doses per route, which was respectively 3.5 and 1.0 µg. Given its ability to induce stronger humoral and cellular immune responses, the saRNA^{pre-F} vaccine was selected for evaluation of protective efficacy against RSV infection in mice via both intratracheal and intramuscular routes. Notably, both routes provided complete protection against RSV challenge. In conclusion, our findings suggest that a saRNA vaccine encoding a secreted, prefusion-stabilized F protein could be effective against RSV when administered via the intratracheal route.

Protein Corona-Driven Design of Lipid Nanoparticles Enables Functional Gene Silencing in the Liver

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Abstract

Lipid nanoparticles (LNPs) are the most clinically advanced vehicles for nucleic acid delivery and exhibit intrinsic accumulation in the liver following systemic administration. However, hepatic tropism does not consistently translate into functional RNA interference due to limited intracellular processing and inefficient cytosolic release [1]. The protein corona (PC), formed upon exposure of nanoparticles to biological fluids, is increasingly recognized as a key determinant of nanoparticle biological identity, influencing biodistribution, cellular uptake, and intracellular trafficking [2-3]. In this study, we developed an LNP platform in which protein corona composition was modulated by varying lipid formulation parameters, to enhance functional siRNA delivery to the liver. Four LNP formulations with systematically varied cholesterol content were designed to influence PC composition and biological performance. Physicochemical characterization and proteomic analysis revealed that cholesterol-enriched formulations reshape the corona profile, influencing the adsorption of apolipoproteins, complement proteins, immunoglobulins, and coagulation factors, thereby affecting nanoparticle stability, immune recognition, and hepatic targeting [3]. Comparative screening identified a lead formulation with improved colloidal stability, high transfection efficiency, and enhanced liver accumulation *in vivo*. siRNA-loaded LNPs were efficiently internalized by Kupffer cells (KCs) and mediated robust gene silencing *in vitro*. In a primary biliary cholangitis (PBC) mouse model, systemic administration of siTIMP1-loaded LNPs selectively silenced TIMP-1 in KCs, leading to reactivation of extracellular matrix (ECM) degradation pathways, upregulation of MMP-9 and MMP-12, and suppression of pro-inflammatory chemokines (Ccl2, Cxcl9, Cxcl10). These effects were associated with reduced macrophage infiltration, alleviation of cholestasis, and attenuation of liver fibrosis. Collectively, these findings highlight how LNP formulation can modulate protein corona composition to improve functional hepatic siRNA delivery and support the development of LNP-based RNA therapeutics for fibrotic and inflammatory liver disease (**Figure 1**).

Cholesterol-Optimized LNPs Enable siTIMP1 Delivery to Liver Macrophages and Restore Hepatic Homeostasis

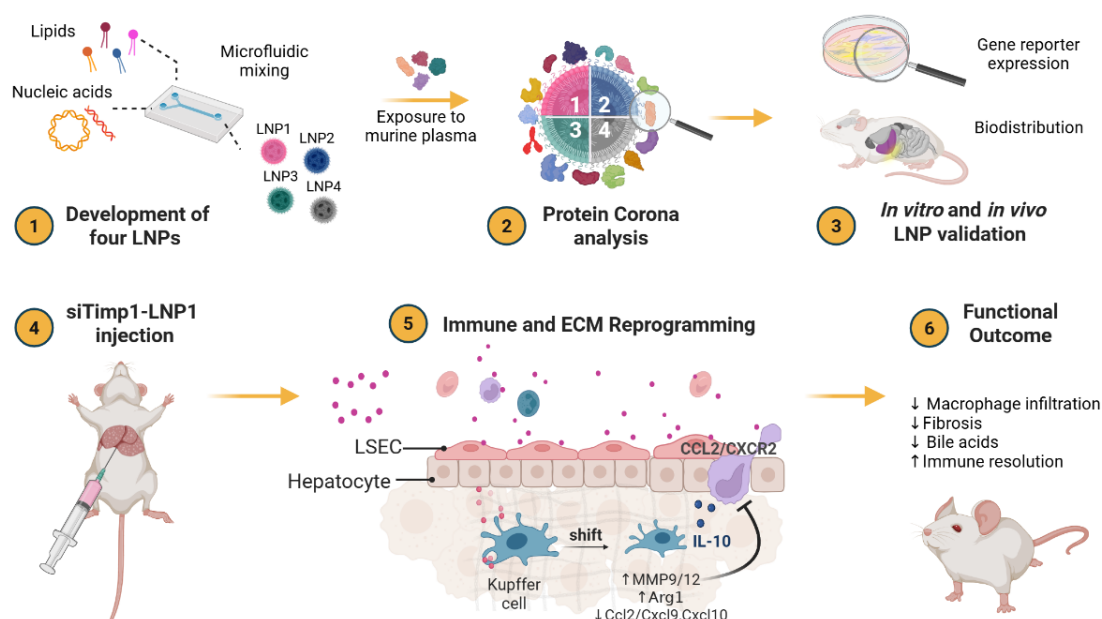


Figure 1. Overview of siTIMP1-LNP development and mechanism of action. Cholesterol-modulated LNPs are generated by microfluidics (1) and characterized before and after protein corona formation in plasma (2–3). The optimized siTIMP1-LNP is administered intraperitoneally (4), accumulates in liver Kupffer cells, and mediates RISC-dependent TimP1 silencing following endosomal escape (5). This induces macrophage reprogramming toward a resolving phenotype, reducing inflammation and fibrosis (6).

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The use of AAV and LNP to achieve highly efficient genome editing in the liver

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Introduction and Objective

Ornithine transcarbamylase (OTC) deficiency is a rare childhood hereditary metabolic disorder that is caused by a mutation in the enzyme OTC resulting in the partial or complete loss of the enzyme. The lack of this enzyme which is highly expressed in the liver causes a build-up of ammonia in the blood stream which can cause neurological damage. The current curative treatment is liver transplant. However it is suboptimal due to lack of donor organs, the accessibility to transplant and the requirement for life-long immunosuppression.

Other treatment options include adeno associated virus (AAV)-based gene therapy which has shown promising results and is currently in human clinical trials. Preclinical studies have demonstrated efficient gene editing in both mouse models and patient derived primary human hepatocytes with the use of dual AAV vectors. However, the use of this approach has safety concerns including immune related toxicity and non-specific targeting. In contrast, use of mRNA is safer because it avoids any possibility of integration of gene editing enzymes into the host genome, instead delivering gene editing machinery in a transient manner allowing the control of dosing and the related pharmacokinetics. Hence, we aim to investigate the use of the AAV technology with mRNA-LNP for gene editing. In order to optimise the delivery with LNPs, the initial studies were conducted with wild type mice with the objective of inserting reporter mu-GFP-nluc into a specific location in the mouse genome.

Materials and Methods

LNPs encapsulating *Staphylococcus aureus* Cas9 (SaCas9) or *Streptococcus pyogenes* Cas9 (SpCas9) mRNA either alone or paired with a single guide RNA (sgRNA) were formulated with similar lipid composition to the well-known Onpattro formulation. The AAV vectors encoded either the reporter gene (mu-GFP-nluc) or reporter gene (mu-GFP-nluc) with sgRNA. Mice were injected intravenously with the AAV followed by the LNP (2mg/kg) with a 7 day interval. Three weeks after the 2nd injection, tissues were harvested, weighed and homogenised using a gentleMACS dissociator. mRNA translation was characterised using a nanoluciferase based assay.

Results

Initial studies confirmed that the delivery of AAV encoding mu-GFP-nluc with sgRNA, followed by the delivery of LNP encapsulating SaCas9 resulted in more than 50% gene editing in the liver. Interestingly, similar results were also obtained with the delivery of AAV encoding mu-GFP-nluc, followed by the delivery of LNP encapsulating SpCas9 with sgRNA. These promising results have led us to further investigate the mRNA translation in other tissues to determine the selectivity of this approach. Further optimisations including time and dose of LNP and AAV are currently being investigated.

Conclusion

Overall, our results support that the dual AAV approach can be replaced with the combination of the AAV vector encoding the gene of interest and a LNP encapsulating SpCas9 and sgRNA. Data obtained from future studies will help further optimise the delivery and will form part of pre-clinical work to develop a treatment for (OTC) deficiency, by inserting of a working, healthy copy of the OTC cDNA.

Surface Effects on mcDNA-LNP Characterization: Implications for Analytical Reliability

Aleš Štrancar, BIA Separations Inc.

Lipid nanoparticles (LNPs) are established delivery systems for nucleic acids and other therapeutic compounds. Reliable product characterization is essential for process development, quality control, and formulation optimization. A frequently overlooked source of analytical variability is the interaction of the LNP and free payload with laboratory and manufacturing contact surfaces. These interactions can introduce systematic errors during sample handling, storage, and analysis.

This study investigates how surface interactions influence the analytical determination of encapsulation efficiency in minicircle (mc) DNA-LNP formulations. The impact of commonly used plastic and glass materials on mcDNA-LNP sample recovery and measured encapsulation efficiency was evaluated under controlled conditions. Quantification was performed using a **unique two-dimensional LNP chromatography approach that enables simultaneous analysis of free and encapsulated nucleic acids, quantification of different nucleic acid species, assessment of LNP heterogeneity, quantification of DNA/RNA-lipid adducts, evaluation of DNA/RNA fragmentation, monitoring of LNP–protein conjugation, and additional critical quality attributes-all within a single 2D HPLC workflow.**

Results demonstrated significant differences in the interaction of container materials with mcDNA-LNP formulations. **Plastic materials caused the greatest loss of measurable nucleic acid, while glass containers had a smaller impact.** Two-dimensional analysis revealed that this effect was primarily driven by depletion of the free nucleic acid fraction, whereas the encapsulated fraction remained largely unaffected (as protected by the lipids). **Consequently, selective adsorption of free nucleic acids produced artificially elevated encapsulation efficiencies.**

These findings demonstrate that both **appropriate sample preparation and handling, together with robust analytical methodologies, are required for accurate characterization of LNP products.** Such approaches improve confidence in analytical data, support reliable process evaluation, and enable more representative assessment of product quality throughout development and manufacturing.