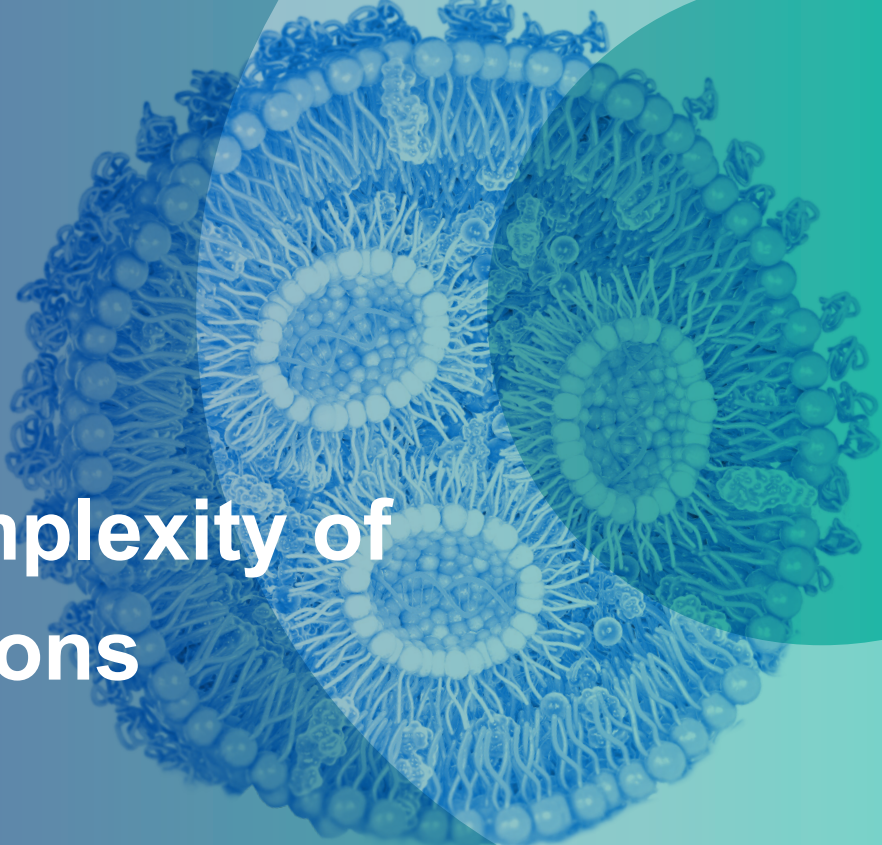


Inside the Complexity of LNP Formulations



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Understanding the mechanism of action is essential to developing effective LNP formulations.

Lipid nanoparticles (LNPs) are the leading non-viral delivery system for oligonucleotide (messenger RNA, antisense RNA, short-interfering RNA, plasmid and linearized DNA, etc.) therapeutics developed for the treatment of a wide range of diseases and genetic conditions. During the preclinical development phase, LNP formulations are optimized for potency and safety based on the target disease indication and route of administration. Understanding the mechanisms of action of LNPs is vital to developing formulations that effectively protect their cargos, deliver them to target tissues/organs, and release them when desired.

Why LNPs for Delivery of Oligonucleotides?

LNPs, as their name suggests, are small particles typically less than 100 nanometers in diameter. They represent effective delivery systems for nucleic acid therapeutics because they both protect sensitive DNA and RNA constructs from enzymatic degradation in the bloodstream and facilitate their crossing of cellular membranes and release in cellular interiors. Compared to other nanoparticle delivery technologies, LNPs often have longer half-lives, enhanced pharmacokinetics, and reduced toxicity. They also present reduced safety and immunogenicity concerns compared to viral vectors.

Multi-Component Lipid Compositions

LNPs comprise a mixture of several lipids surrounding a therapeutic payload (typically DNA

or RNA). Most LNPs are formulated using four different lipid types: a structural helper lipid, often a phospholipid; a lipid with a hydrophilic polyethylene glycol (PEG) chain (PEG lipid); cholesterol; and an ionizable lipid.

Phospholipid helper lipids provide rigidity and stability. They also can modify the surface charges of LNPs and the fusogenic properties of cell membranes. Although considered optional components, these helper lipids are often essential for achieving the desired activity *in vivo*. DSPC is one of the most established helper lipids for LNPs, with proven use in marketed mRNA vaccines such as Comirnaty and Spikevax.

PEG lipids are anchored in the lipid matrices of LNPs by their lipid tails through noncovalent interactions. Their hydrophilic groups reside on the surfaces of the LNPs where they block interaction of the lipid surface with serum proteins, conferring a degree of colloidal stability. Over time, the PEG-lipids are adsorbed on the vast pool of endogenous serum lipoproteins and the LNP core is exposed. This promotes absorption of serum proteins and immunoglobulins onto the LNP surface, which ultimately governs targeting and uptake into specific tissues.

Cholesterol is included to boost the fluidity of LNPs (prevent solidification). It also provides some stabilization and promotes the fusion of LNPs to cell membranes.

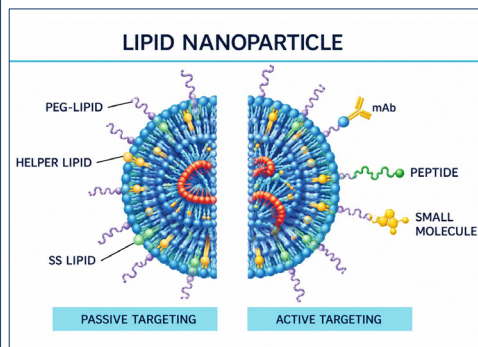
Ionizable lipids are the most important lipid component in LNP formulations, as they form complexes with nucleic acids, thereby enabling these therapeutic payloads to be embedded within LNP matrices. They are neutral at physiological pH (~7.4) and carry a positive charge under acidic conditions (pH < 6.5). At low pH, therefore, they interact with negatively charged oligonucleotides and cell membranes, providing both stability and increased cellular uptake. They must, however, be carefully designed to allow for initial complexation of DNA or RNA yet be sufficiently biodegradable to release the payload at the right time and place.

One of the primary functions of the ionizable lipid is complexation of the nucleic acid so that it can be effectively dispersed in the LNP matrix. Thus, the first step in forming an LNP is mixing the nucleic acid in an acidic buffer solution (~pH 4) with the ionizable lipid in ethanol. Under these conditions, the ionizable lipid becomes positively charged and complexes the nucleic acid. After mixing with the helper

lipid, PEG-lipid, and cholesterol, a nanoparticle forms comprising microdomains of nucleic acid and positively charged lipid dispersed within a hydrophobic lipid matrix with a PEG “coating” on the surface. The ionizable lipids on the surface are neutral at physiological pH, while the PEG groups form hydrogen bonds with water, creating a hydrophilic corona.

Examples of ionizable lipids used in approved LNP formulations include Dlin-MC3-DMA (ONPATRO®, Alnylam Pharmaceuticals), SM-102 (SpikeVax®, Moderna) and ALC-0315 (Comirnaty®, Pfizer/BioNTech). Cationic lipids with permanent positive charges have also been investigated in LNP formulations.

The choice of specific lipids depends on the properties of the therapeutic payload and the target tissues/organs, as well as any requirements for conjugation to targeting ligands (see below). In addition to selecting the specific lipids, formulation involves determining the optimal ratios of the four lipids among themselves and to the payload.



LNPs as Delivery Vehicles: Balancing Strong Membrane Fusion with Endosomal Release

LNPs, when they enter the bloodstream, are neutral and surrounded by the PEG-based hydrophilic corona. This PEG layer is eventually shed from the outer surface of the LNP, leaving the interior exposed, which promotes surface adsorption of serum proteins resulting in the formation of the protein corona. The specific proteins involved in the protein corona play a critical role in the subsequent LNP biodistribution and can interact with cell surface receptors to mediate entry of the LNP into cells.

LNPs can enter cells through two different mechanisms. LNPs with adsorbed serum proteins on their surfaces can undergo receptor mediated endocytosis in cells containing a complementary receptor on their surfaces.

LNPs with dense PEG coatings can undergo non-receptor-mediated uptake in the absence of a protein corona. Regardless of the mechanism, LNPs that successfully enter cells end up in their endosomal compartments, which, as they mature, become acidic, allowing the ionizable lipids in the LNPs to develop positive charges.

The positively charged ionizable lipids in the LNPs undergo charge-charge interactions with negatively charged, anionic lipids of the endosomal membrane. These interactions lead to fusion of the LNP with the membrane due to a shift from the standard bilayer structure to inverted non-lamellar or hexagonal phases. Indeed, the selection of lipids to modulate the hexagonal phase shift has been a useful strategy for improving endosomal escape. During the fusion process, the endosomal compartment is destabilized or ruptured, resulting in release of the nucleic acid cargo. In the final step, hydrolysis of lipids in the cytosolic compartment accelerates release of the charge-coupled nucleic acid cargo, and the *in vivo* lipid degradation kinetics are closely linked to enhanced RNA expression.

As such, LNP formulations must be designed to support a combination of stability in the bloodstream and efficient cellular uptake followed by robust membrane fusion, rapid endosomal escape, and effective degradation and dissipation of the lipid once it is released from the endosome. Furthermore, LNP formulations must have the appropriate size and surface properties to ensure interaction with the target organ/tissue type.

Passive vs. Active Targeting

One of the major challenges facing LNP delivery of oligonucleotide therapies is the tendency of ionizable lipid-based formulations to accumulate in the liver rather than the intended organ/tissue, significantly limiting their effectiveness against diseases affecting other parts of the body. For the passive targeting approach, the physicochemical properties (size, shape, surface charge, etc.) of the LNPs are adjusted through lipid selection, molar ratios, etc. in order to drive tropism towards the intended organ/cell type. Such formulation compositional changes ultimately influence the types of serum proteins bound to the LNP surface and thus alter the *in vivo* fate of the LNP.

Alternatively, the active targeting approach involves modifying the LNP surfaces with ligands

that bind to specific receptors on cells in target organs/tissues. For instance, modified PEG lipids containing functional groups at the ends of the PEG chains can be used. Antibodies, antibody fragments, peptides, aptamers, or other targeting ligands can then be conjugated to the functional groups via covalent chemistries. The ligands can be attached to the PEG-lipids prior to LNP formulation or to the LNP surface following therapeutic payload encapsulation using post-insertion and, most commonly, post-formulation conjugation methodologies, respectively.

Many Trade-Offs in Controlling LNP Drug Product Behavior

Understanding the mechanism of LNP delivery of nucleic acids and the roles different lipids play helps guide formulation choices necessary to achieve the desired pharmacological behavior. In addition to ligand choice for active targeting, particle size and zeta potential influence tissue targeting. Particle size also impacts the rate of biodistribution, and the PEG structure determines the circulating half-life in the body.

The choice of lipids and their ratios, and overall, the ratio to the payload, affect LNP stability, with aggregation and scalability among the major challenges to LNP development. In general, the total lipid content must be in excess of the nucleic acid/oligonucleotide content to form sta-

ble LNPs. Changes in particle size, propensity for aggregation, and low encapsulation efficiency indicate an incorrect component balance.

Tolerability is also influenced by the choice of lipids and their ratios, as well as the dosage form. Whether the route of administration is systemic or local (e.g., intraocular, intranasal, etc.) must also be taken into consideration. Modulation of the regulatory sequences of the nucleic acid/oligonucleotide will, meanwhile, impact the expression performance.

These numerous factors must be investigated when formulating LNP products to simultaneously optimize tissue targeting, kinetics, stability, and tolerability. Fortunately, there are well established ratios of the various lipid components and lipid-to-cargo ratios that serve as good benchmarks against which novel compositions can be compared. Due to the enormous design space in terms of potential ratios of cholesterol, PEG, helper, and ionizable lipids in LNP formulations, the best approach is to work with an experienced LNP formulation developer with knowledge of optimal ratios for different payloads and therapeutic applications.

The Need to Prioritize Development Tasks

With so many variables to consider during formulation development, it is important to establish a staged development strategy. The first step is to characterize and bench-

mark any novel ionizable lipids to be considered for the formulation via an *in vitro* / *in vivo* potency (transfection) and tolerability assessment, such as a rodent maximum tolerated dose study combined with cell-based assays for *in vitro* cytotoxicity and/or macrophage activation that indicate undesirable inflammatory properties.

Once the list of ionizable lipids has been narrowed, a design-of-experiments approach is used to investigate the effects of different helper lipids, PEG lipid content, cholesterol ratios, and other factors on LNP critical quality attributes and assess the feasibility of fabricating LNPs of high quality. A standard ethanol dilution process is used, and formulation feasibility is assessed by measuring particle size, polydispersity index (PDI), and encapsulation efficiency, followed by stability after one freeze-thaw cycle. Mapping the formulation design space in this way enables the determination of workable LNP compositions that retain favorable quality attributes in terms of size, PDI, and cargo encapsulation.

Formulations determined to provide stable LNPs can then be subjected to *in vivo* screening. In specific cases, *in vitro* activity screening can be considered where there is an established *in vitro* and *in vivo* correlation. Ultimately, biodistribution and tissue/cell targeting are evaluated *in vivo* using rodents or non-human primates.

Partnering for LNP Formulation Development Success

LNP formulation development is complex and requires knowledge and understanding of lipid chemistry and bioactivity, as well as the lipid intellectual property space. Factors impacting LNP stability and bioactivity, tissue biodistribution, manufacturing, and tolerability should all be optimized in a stage-appropriate manner throughout the product development cycle. Given the complexity of LNP drug products, analytical development should be initiated early, particularly with emphasis on assay- and purity-indicating methods.

Working with an outsourcing partner experienced in LNP formulation development and manufacturing can dramatically accelerate timelines. Partners with access to proprietary ionizable lipids, advanced screening technologies, and established formulation development strategies can further streamline projects while also significantly reducing program risk.

The Molecular Shape Hypothesis

The molecular shape hypothesis is a strategy that can guide lipid selection and design.¹ This theory states that all lipids have a certain geometric shape that is determined by the size of the head group and the geometry of the lipid tail. For example, lipids with large head groups and very compact tails, when stacked together in a nanoparticle, promote a concave curvature, while lipids with two relatively saturated tails will form more of a planar, cylindrical shape with neutral curvature. LNPs comprising lipids with a mix of saturated and unsaturated tails and small head groups tend to have an inverse curvature, which can promote greater fusogenic membrane behavior.

However, there is currently no mathematical model to calculate the specific shapes and behaviors of different LNP formulations. This information can, however, be used as a guide to the potential fusion behavior of different helper lipids and thus help to narrow down the possible compatible options for a given therapeutic payload and formulation. Physical screening of different LNP formulations is still required, but the number of experiments can be dramatically reduced from 100 or more to a dozen or so.

Strategic Partnership to Advance Novel LNP Therapeutics

Since August 2024, NOF Corporation has been a strategic investor in Phosphorex. This collaboration combines NOF's high-purity phospholipids, diacylglycerol PEGs, and proprietary ionizable lipids with Phosphorex's nanoparticle manufacturing capabilities, tailored solutions, and enabling technologies to optimize the release rate, targeting ability, bioavailability, and deliverability of LNPs and other drug delivery systems.

Phosphorex and NOF experts are eager to help solve complex LNP problems and successfully develop novel therapies for patients in need. [Phosphorex](#) and [NOF](#) can be reached at their respective contact pages.

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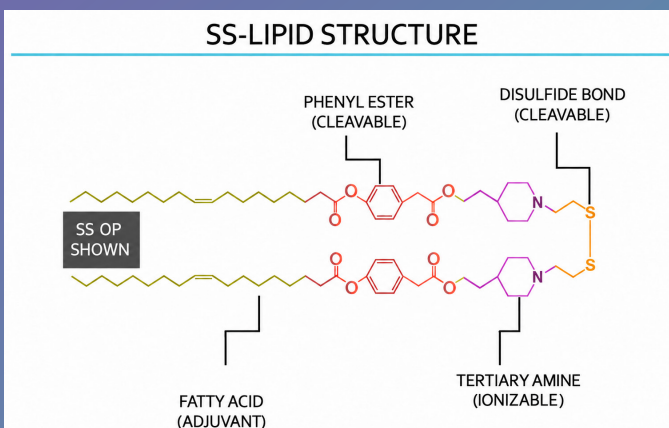


CASE STUDY

LNP Formulation Development at NOF

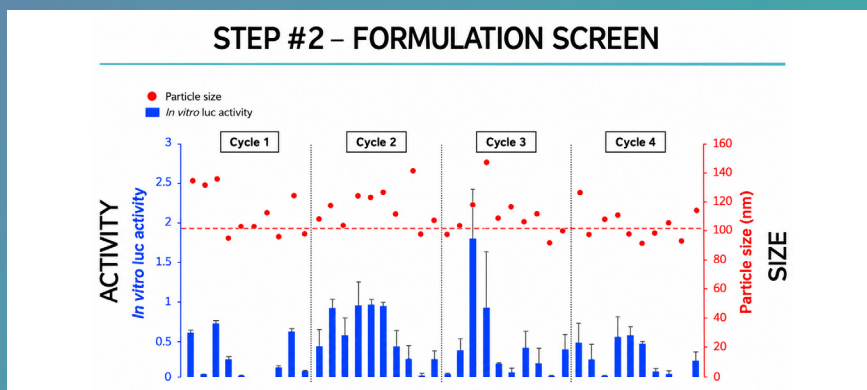
NOF Corporation has developed its own proprietary ionizable lipids (Figure 1). The COATSOME® SS Series contains two lipid tails joined together via disulfide bonds and that contain tertiary amine groups. The tertiary amine groups become protonated in the acidic environment of the endoplasm, while the disulfide bonds undergo cleavage in the cytoplasm. Cleavage of the disulfide bond then promotes lipid tail cleavage via ester bond breakage. As a result, these lipids are highly biodegradable and biocompatible.

Figure 1:
NOF Proprietary
Ionizable Lipid



These lipids exhibit good *in vivo* activity compared with commercial ionizable lipids when incorporated into standard formulations known to target the liver using material informatics, a machine learning-based approach that incorporates structural information and lipid control models, NOF explored the LNP formulation design spaces for the lead ionizable lipid candidates, evaluating 4,000 to 5,000 parameters and screening 20 to 40 LNPs at a time across multiple cycles. After several cycles looking at activity against the liver and target particle size, it was possible to identify the LNP formulations that afforded the most potent activity (Figure 2).

Figure 2: Formulation Screen using novel NOF ionizable lipids



As a result of the iterative screening efforts, COATSOME® SS Series proprietary ionizable LNPs against multiple targets are now available for the development of novel LNP therapeutics.