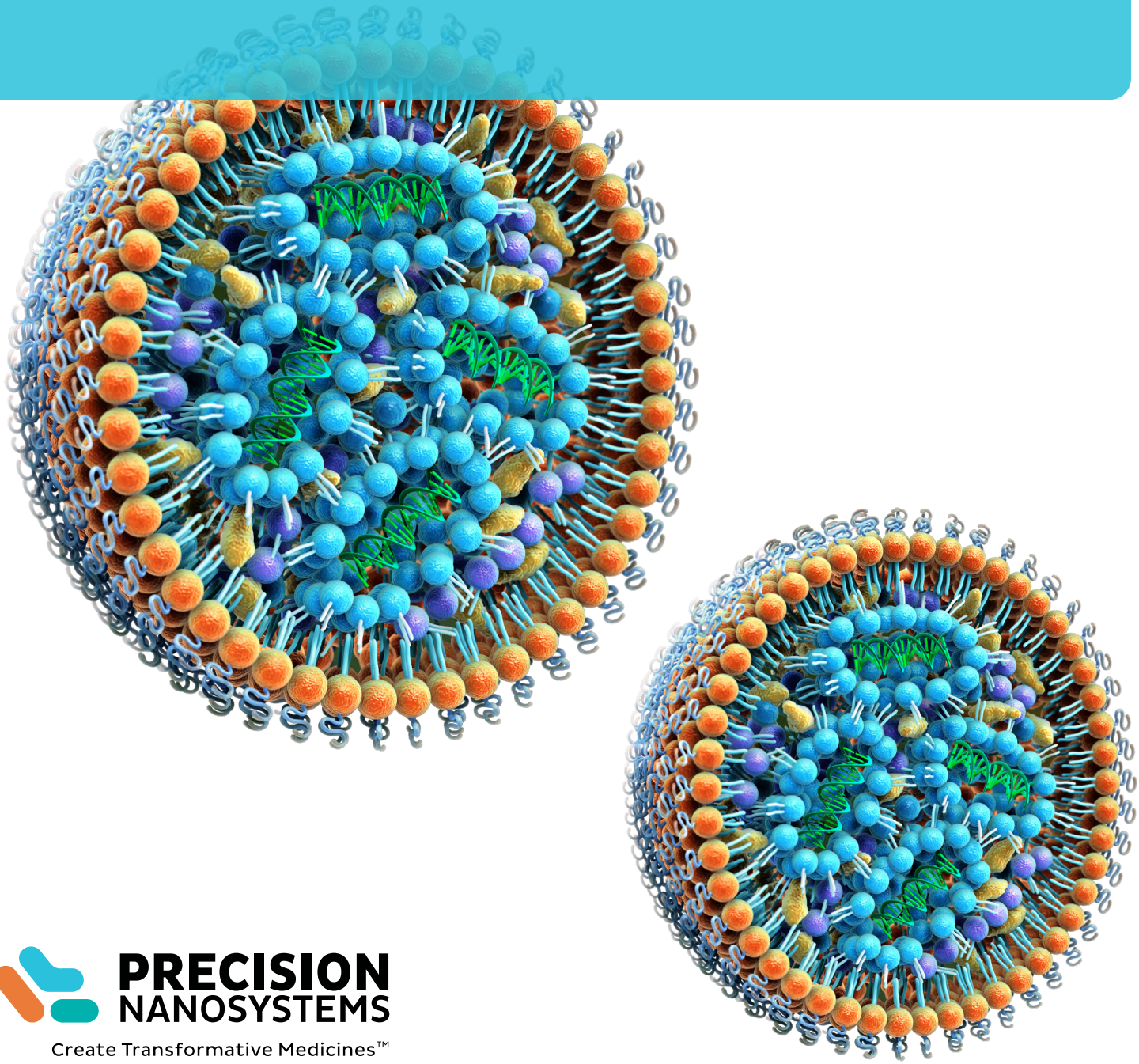
Lipid Nanoparticles (LNPs)

Gold Standard Delivery Technology for Small Molecules and Nucleic Acids



PRECISION NANOSYSTEMS

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Introduction and Biomedical Applications of Lipid Nanoparticles

In medicine, the biggest hurdle is getting the active pharmaceutical ingredient (API) into the target cells at the disease site, referred to as the drug delivery challenge. Nucleic acid drugs in particular face many hurdles in their journey from vial to the cytoplasm of target cells where they are active. Hydrolysis, ubiquitous nucleases, the host's immune system, trafficking within the body, cellular uptake and release into the cytoplasm all impact the safety and efficacy of nucleic acid drugs.

"LNPs are the industry leader in RNA delivery and play a critical role in every RNA drug delivery experiment."

- Anna Blakney, Ph.D., PEng | Assistant Professor, Michael Smith Laboratories & School of Biomedical Engineering

Lipid nanoparticles (LNPs) function as a drug delivery system (DDS) for nucleic acids as it protects the cargo from degradation and mediates a targeted, safe, and efficient intracellular delivery due to their:

- **Modularity:** Lipid excipients can be mixed, matched, and modified to deliver a variety of nucleic acid APIs to different targets in the body
- **Capacity:** Allow the delivery of larger mRNAs and the co-delivery of multiple RNAs, enabling new therapeutic mechanisms

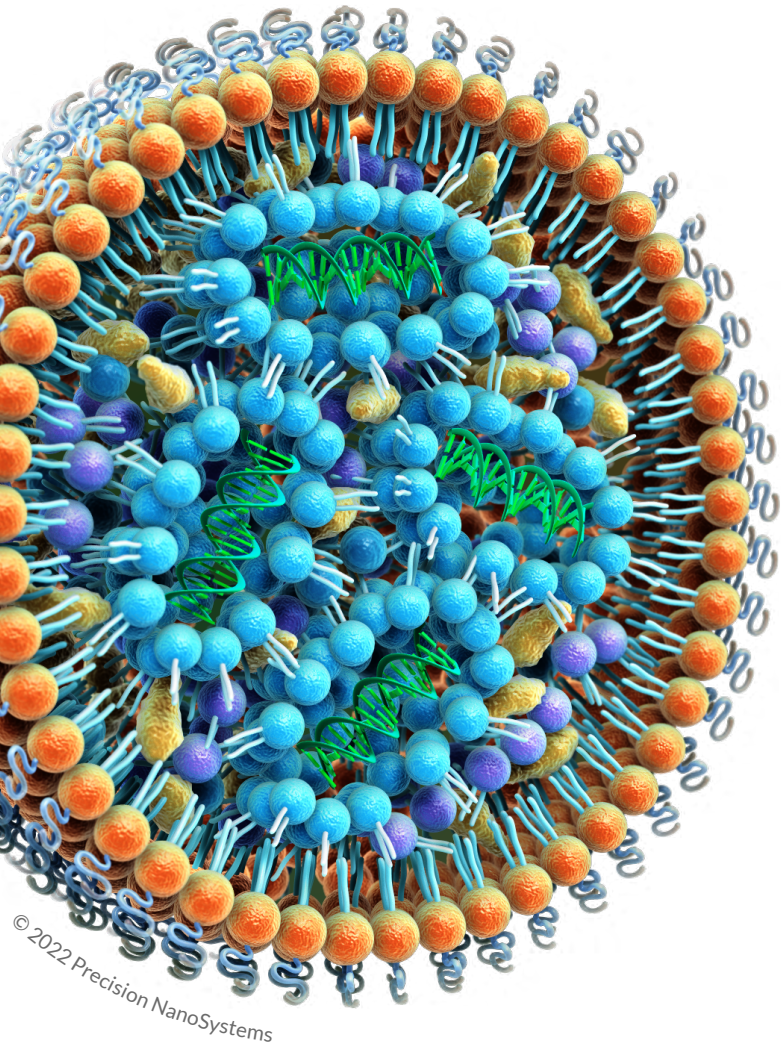
The LNPs have proven to diminish cytotoxicity and organ toxicity of the new and current treatments and improve the encapsulated drug's efficacy and pharmacokinetic profile.

LNPs are collectively safe, efficient, extremely valuable, and versatile tools for drug delivery applications.

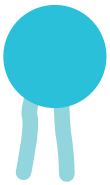
Lipid Nanoparticles	Active Ingredient	Example Application
	Nucleic Acid	siRNA knockdown
		Cell and Gene therapy
		mRNA therapeutics
		Plasmid transfection
		Nucleic acid vaccines
		CRISPR/Cas9 genomic editing

Table 1. Biomedical Applications of LNP

Anatomy of a LNP

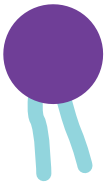


Structure of a Lipid Nanoparticle
Each LNP consists of four components that serve a purpose



1. Cationic Ionizable lipids: Binds nucleic acid and shifts charge with pH changes to limit cytotoxic effects

2. Cholesterol: Binds apolipoprotein E(ApoE) and mediates endocytosis via low-density lipoprotein (LDL) receptor



3. Helper Phospholipids: Aid in LNP stability, intracellular uptake, and endosomal escape

4. PEG-lipids: Hydrophilic lipids that create a barrier of water to protect LNPs from aggregating during assembly and increase bloodstream circulation lifetime

Figure 1. Schematic representation of a mRNA lipid nanoparticle

How LNP based nucleic acid (RNA/DNA) delivery works?

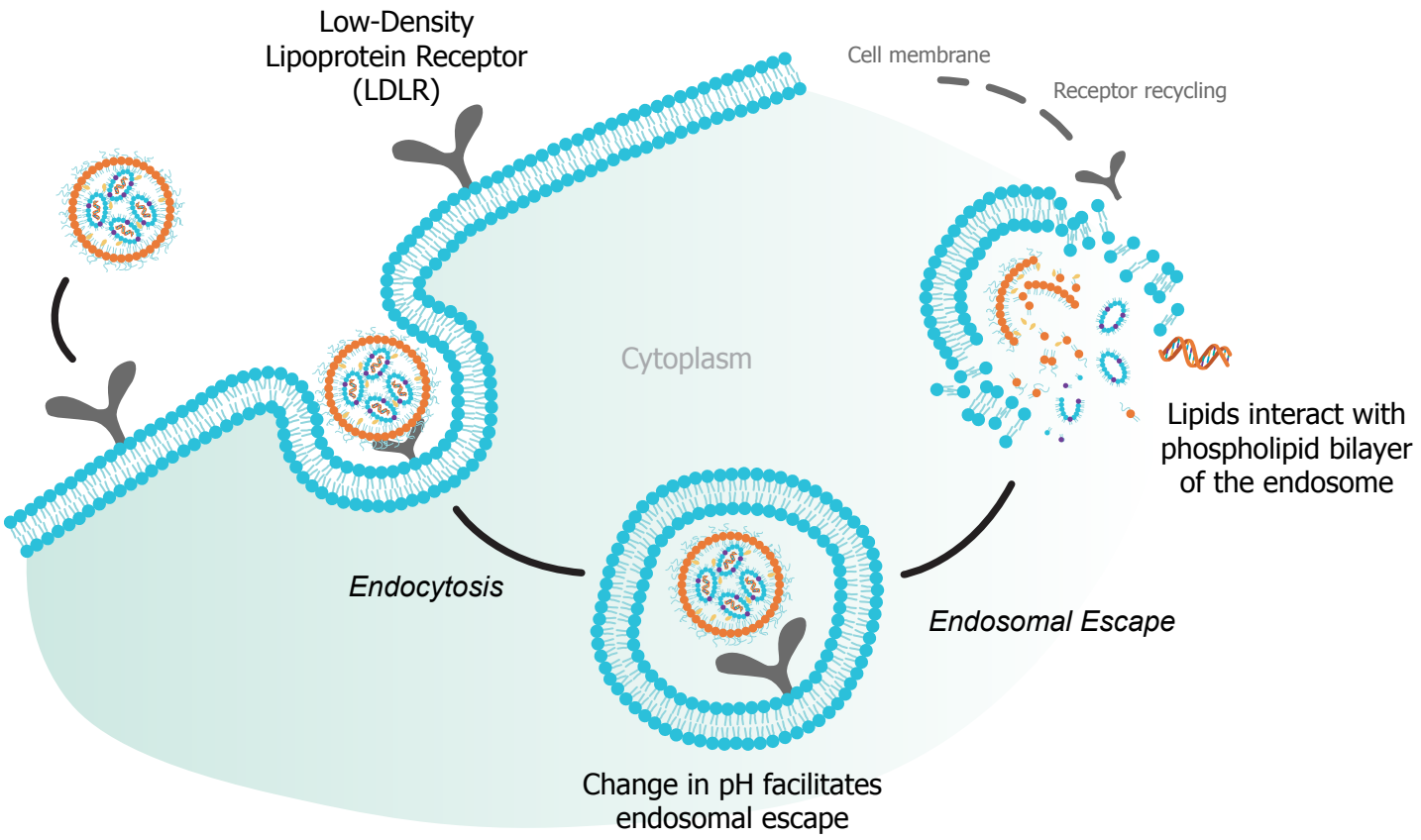


Figure 2. LNP delivery

The mRNA in orange color encapsulated in an LNP carrier is taken into the cell and then released. The delivery vehicle mediates uptake by endocytosis, and disruption of the endosome releasing the mRNA into the cytoplasm where it can interact with ribosomes which allow the creation of a new protein.

Boost Vaccine/Drug Potency by Altering Each of the Four LNP Components

A slight alteration in the chemical structure of the ionizable lipid, choice of PEGylated lipid, cholesterol, helper lipid, and the ratio of the four components can alter the property and LNP delivery efficiency.

1. Cationic Ionizable Lipid – Its uniqueness is its pH sensitivity. It holds a neutral charge under physiological pH to minimize toxicity and shifts charge with pH changes. During formulation at a lower pH, this quality enables electrostatic interaction with the negatively charged nucleic acid to ensure high efficiency. After cellular uptake by endocytosis, acidification of the endosome triggers lipid ionization that promotes interaction with oppositely charged lipids in the endosomal membrane, forming an ion pair (Figure. 3), that disrupts the endosome and eventually releases its cargo into the cell's cytoplasm.

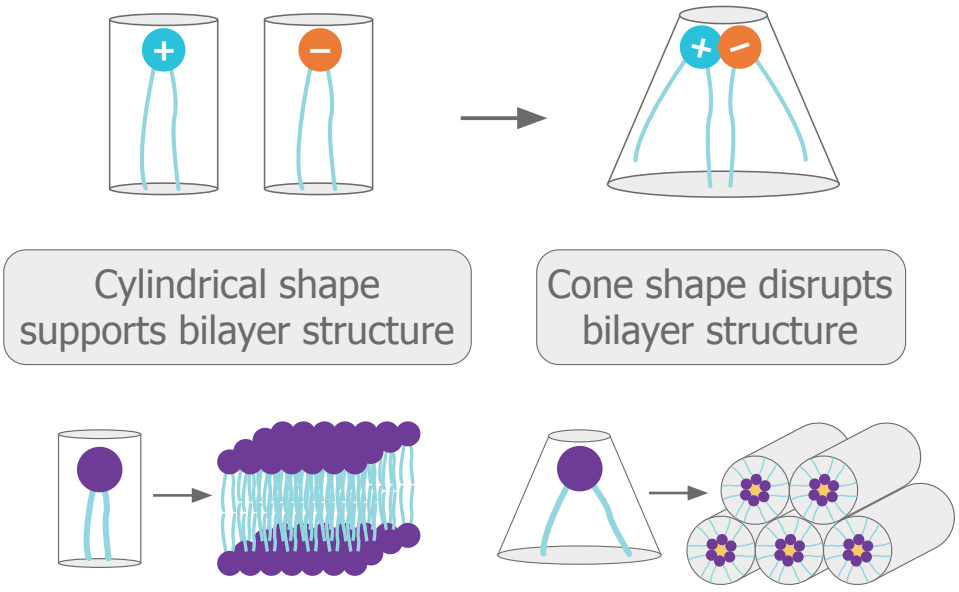


Figure 3. In the endosome, the lower pH environment ionizes the cationic lipids, which interact electrostatically with anionic lipids in the endosomal membrane. These ion pairs cause a phase transition into a porous hexagonal phase (HII) that disrupts the endosome and facilitates the release of the RNA into the cytoplasm.

The pKa (indication of acidic strength) is one of the significant factors known to determine ionizable lipids delivery efficacy of the LNP containing it. A study, "Mixing lipids to manipulate the ionization status of lipid nanoparticles for specific tissue targeting"[2], publicized a method that can predict the LNP pKa value. The researchers experimented with improving the targeting efficiency of an LNP by optimizing its dissociation constant (pKa) value, reflecting its ionization property or surface charge. In the study, pKa optimization with mixing different lipids into one formulation successfully targeted specific tissue (LSECs, liver sinusoidal endothelial cells).

In summary:

- The pKa value of the LNP is not dependent on the quantity of lipid used
- Optimizing the pKa value of the LNP by mixing lipids improves its targeting efficiency
- Gene silencing activity in the liver is dependent on the pKa value of LNP mix which can be optimized for specific uptake by LSECs.

As understood from the observations, testing and selecting appropriate formulations to manipulate the ionization status of lipid nanoparticles ensures effective therapeutics across applications.

It is worth noting that lipids that are effective at delivering nanoparticles to cells in culture can fail in animal studies. Furthermore, the laborious synthesis process makes it difficult to prepare rationally designed ionizable lipid candidates. Clinical ionizable lipids are all synthesized in multiple steps, posing scalability challenges. Aside from safety and potency, optimizing ionizable lipids for structural properties and additional functionalities such as targeting and immunomodulation is difficult. To address this issue, access to a proprietary lipid library for custom formulations is essential.

2. Cholesterol – The function of this component is to improve LNP stability and uptake by binding to Apolpoprotein E (ApoE) and mediating endocytosis via the low-density lipoprotein receptor (LDLR). Researchers from the study, "Naturally-occurring cholesterol analogues in lipid nanoparticles induce polymorphic shape and enhance intracellular delivery of mRNA,"[3] made similar observations by replacing cholesterol with various C-24 alkyl derivatives that, when incorporated inside an enhanced LNP (eLNP), can result in a substantial increase in gene delivery. The eLNPs relative to LNPs showed enhanced gene transfection and higher cellular uptake and retention, potentially leading to a steady release from the endosomes over time (Figure. 4).

In summary, replacement of cholesterol with sitosterol leads to:

- Higher cellular retention and improved gene expression
- Pathways with better sites for endosomal escape

Researchers suggest these enhancements could be due to the structural or surface lipid composition differences in eLNPs. The changes facilitate intracellular delivery, endosomal escape, or differential interactions of sitosterol with signaling components.

These findings show the importance of cholesterol in the subcellular transport of LNPs carrying mRNA and emphasize the need for designing customized composition nanoparticles to improve endosomal escape.

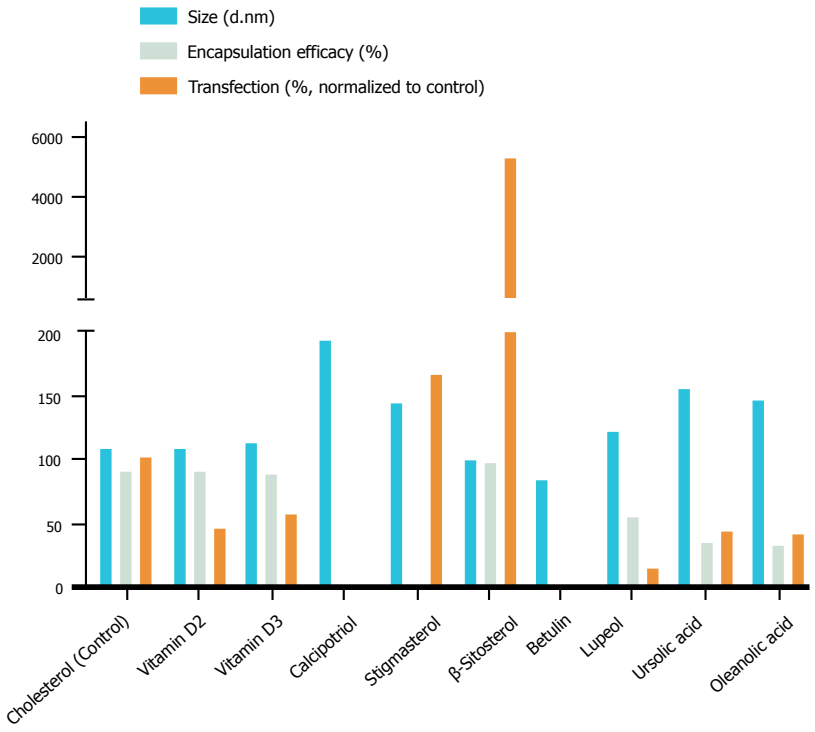


Figure 4.C-24 alkyl derivatives with variations in the tail and body were screened for their effect on the size, mRNA encapsulation efficiency (EE), and transfection efficiency of LNPs

(Adapted from Nat Commun 11, 983 (2020) Patel, S., Ashwanikumar, N., Robinson, E. et al. https://doi.org/10.1038/s41467-020-14527-2)

3. Helper Phospholipid – They aid in LNP stability, intracellular uptake, and endosomal escape. In a study, "Helper lipid structure influences protein adsorption and delivery of lipid nanoparticles to spleen and liver"[4], an analysis of different helper lipids was performed to specify the importance of analyzing and selecting helper lipids for various applications. Understanding the role of the helper lipid as well as its alterations in LNP formulation parameters, in LNP accumulation, ApoE adsorption, and biodistribution to different organs will help in the future design of LNPs for nucleic acid therapeutics.

Accumulation – Understanding the role of helper lipids in LNP accumulation differences in the liver and spleen enables the development of potential nucleic acid therapeutics. In the study, six hours after intravenous administration of LNPs in the mice, liver and spleen tissues were isolated and DNA from these tissues was extracted. Amplification of the b-DNA (a unique DNA barcode of each LNP formulated by pipette mixing) from the DNA samples was performed by polymerase chain reaction (PCR) followed by deep sequencing to determine the relative accumulation of LNPs in different tissues. The researchers used quantitative polymerase chain reaction (qPCR) to measure the total amount of b-DNAs in five nanograms of extracted DNA. The sequencing counts were normalized by multiplying the existing ratio of counts across liver and spleen tissues for a specific LNP formulation by the ratio of total b-DNAs as determined by qPCR. It was observed that a large majority of LNPs accumulated in either the liver or spleen. Next, the differences in accumulation in the liver and spleen of the LNPs formulated with two different helper lipids were analyzed. All LNPs formulated with dioleoylphosphatidylethanolamine (DOPE) were then compared to all LNPS formulated with distearoylphosphatidlycholine (DSPC), using a paired t-test.

It was found that overall, LNPs formulated with DSPC preferentially accumulated in the spleen while LNPs formulated with DOPE preferentially accumulated in the liver (Figure 5).

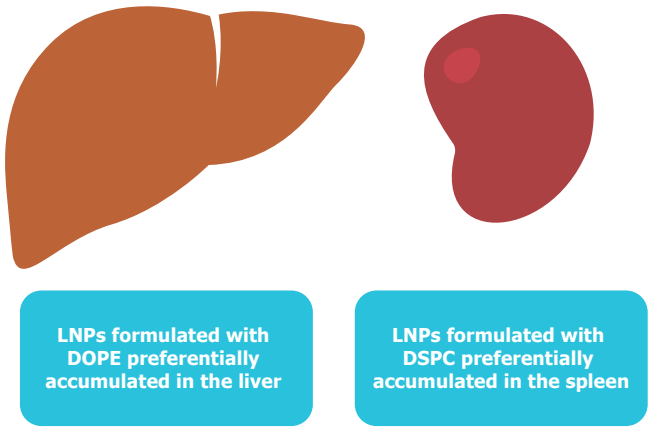


Figure 5.LNPs differential accumulation in Liver and Spleen

To further explore individual differences, delivery to the liver and spleen of individual LNP pairs that had different helper lipids were compared. LNP pairings were identical in all parameters except for the type of the helper lipid (DOPE or DSPC) incorporated into the formulation. In the spleen, LNPs formulated with DSPC accumulated to a larger degree than their DOPE-containing counterparts and in the liver, many LNPs formulated with DOPE accumulated to a larger degree than their DSPC-containing counterparts

In summary:

- LNP formulated with DOPE showed higher accumulation in liver
- LNP formulated with DSPC showed better accumulation in spleen

The data highlights preferential accumulation based on helper lipid considering the accumulation differences between DSPC-containing LNPs and DOPE-containing LNPs. This indicates that optimizing LNPs for targeted delivery with modified helper lipid chemistry is achievable while maintaining other parameters of formulation.

Adsorption – The study demonstrates how helper lipids determine LNPs adsorption preferences. Given the differences in liver and spleen accumulation, the researchers assessed the two helper lipids for ApoE adsorption to LNPs with Quartz Crystal Microbalance, QCM-D experiments probing for LNP-ApoE interactions. The QCM-D technique can monitor changes in the interfacial viscoelastic properties and mass-uptake. The high peak rate of LNPs on the ApoE-coated sensor results from specific interactions between ApoE and the LNP formulated with DOPE. It was observed that DOPE-containing LNP indicated irreversible and more significant adsorption (Figure 6). PBS rinses indicate that the particles irreversibly adsorbed to the ApoE layer.

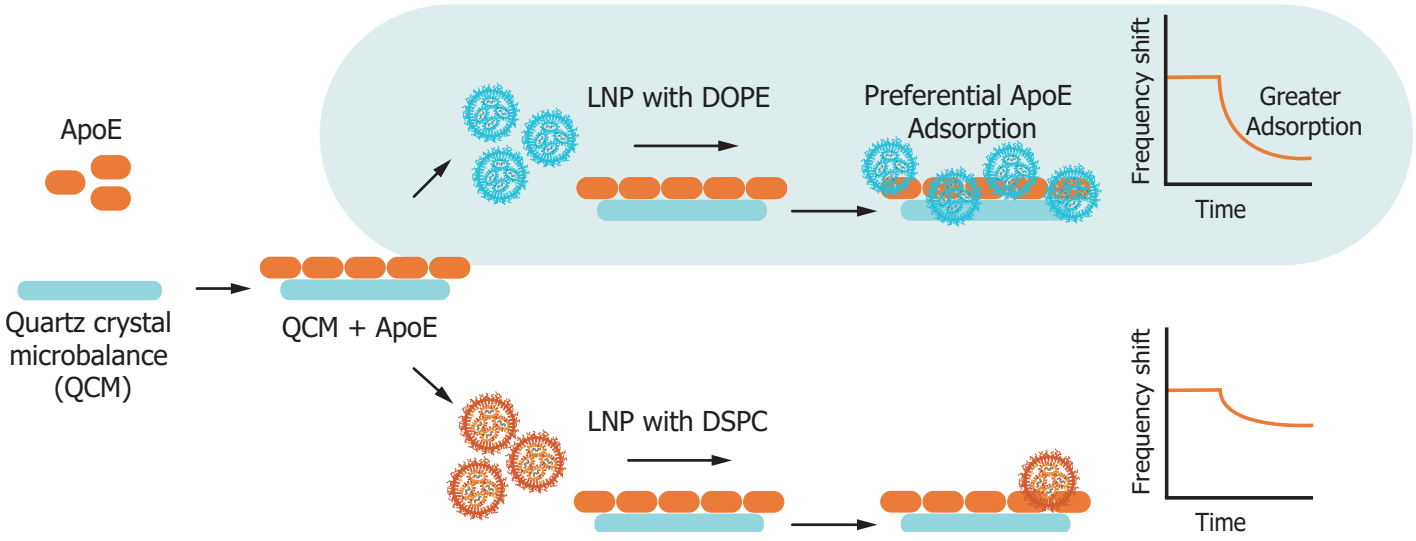


Figure 6. ApoE layers were irreversibly adsorbed on Au-coated quartz crystals, followed by the target LNP solution. Adsorption of particles results in a negative frequency shift of the quartz crystal. A larger decrease in frequency of DOPE-containing LNP than the DSPC-containing LNP, indicating greater adsorption onto the ApoE layer.

(Adapted from Biomater. Sci., 2021,9, 1449-1463)

In summary:

- DOPE-containing LNP has greater adsorption onto the ApoE layer
- DOPE-containing LNP adsorption is irreversible

The data suggest that LNPs formulated with DOPE interact preferentially with ApoE. Because the helper lipid DOPE is unsaturated, it increases the membrane fluidity of LNPs compared to the saturated DSPC, which could account for the observed stronger interactions with ApoE. In addition, the findings indicate that switching the helper phospholipid from DSPC to DOPE influences protein adsorption to LNPs.

Biodistribution – It is critical to consider biodistribution differences across organs when developing LNP formulations. LNPs formulated with mRNA encoding for firefly luciferase provide insight into the relationship between biodistribution and efficacy. The research also suggested that switching the helper phospholipids affected LNP delivery and mRNA transfection. LNPs were intravenously administered to two separate groups of C57BL/6 mice via tail vein injection. The researchers then quantified total luminescent flux six hours post-injection using an IVIS Spectrum imaging system. Reverse transcriptase qPCR (RT-qPCR) was performed to quantify the amounts of luciferase mRNA present in each group. It was observed that in the liver, mice injected with DOPE-containing LNP formulation had approximately two times the amount of mRNA as mice injected with and DSPC-containing LNP formulation. In the spleen, mice injected with DSPC-containing LNP formulation had approximately five times the amount of mRNA as mice injected with DOPE-containing LNP formulation. The observed data shows the biodistribution differences between DOPE-containing LNP formulation and DSPC-containing LNP formulation.

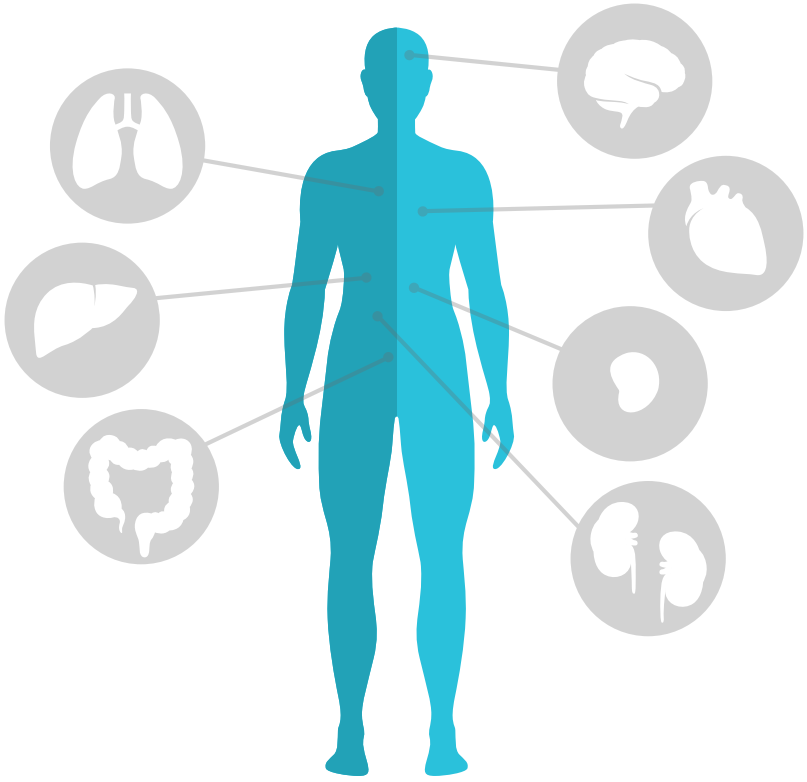


Figure 7. Biodistribution is fundamental to identifying target organs and anticipating safety and efficacy

In summary:

- In the liver, DOPE-containing LNP had approximately two times the amount of mRNA
- In the spleen, DOPE-containing LNP had around five times the amount of mRNA

The data indicate that the helper lipid identity is one of the factors accountable for the observed biodistribution differences between the two LNP formulations. These findings suggest that it is vital to experiment with various LNP formulations to optimize potential nucleic acid therapeutics.

4. PEG-Lipid – PEG lipids, when incorporated into LNPs, confer "stealth" properties. PEGylation creates a hydrophilic protective layer around the nanoparticles that can repel the absorption of opsonin proteins by steric repulsion, thereby blocking and delaying the first step in the opsonization process. As a result, it reduces LNP recognition by the immune system and increases systemic circulation. In addition, stabilization of LNPs with PEG-lipids increases the half-life of the LNPs in the blood, resulting in greater exposure of cells to the LNPs. Subsequently, the PEG shedding process is necessary to allow adsorption of ApoE which facilitates uptake in cells with LDLR.

Choice of PEG-lipid manipulates PEG shedding rate and anti-PEG antibody production – To effectively deliver LNP payloads, shedding some or all of the PEG-lipid coating from LNPs is required before cellular uptake. It is critical in the development of RNA interference-based therapeutics using LNP technology. The timing of this PEG shedding is an essential feature of an effective LNP. Shedding too quickly can limit biodistribution primarily to the liver, while shedding too slowly, the distribution of the LNP to extrahepatic tissues becomes possible with some hindrance to intracellular internalization of the LNP.

PEG-lipid functions:

- Electrical neutrality
- Significant spatial repulsion
- High hydrophilicity
- Drug release

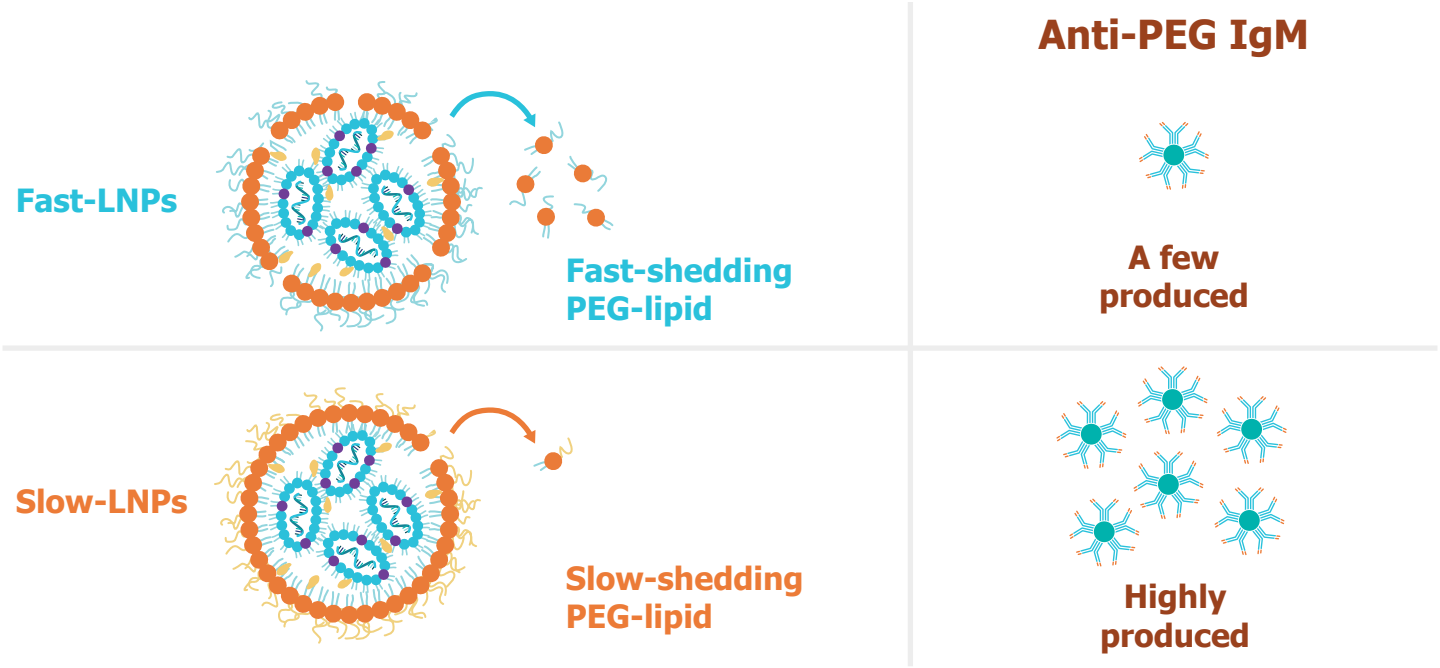


Figure 8. PEG shedding rate influences antibody production

(Adapted from International Journal of Pharmaceutics, Volume 588,2020,119792,ISSN 0378-5173, Takuya Suzuki et al https://doi.org/10.1016/j.ijpharm.2020.119792

In a study, "PEG shedding-rate-dependent blood clearance of PEGylated lipid nanoparticles in mice: Faster PEG shedding attenuates anti-PEG IgM production,"[5] researchers measured the PEG shedding profiles of DMG-LNP (LNPs conjugated to a fast-shedding PEG-lipid (short acyl chain)) and DSG-LNP (LNPs conjugated to a slow-shedding PEG-lipid (long acyl chain)). This experiment was carried out *in vitro* by incubating each LNP with mouse serum and measuring the amount of PEG-lipid still conjugated to the LNPs using nuclear magnetic resonance (NMR). When the experiment began, 80–87% of the PEG-lipid was bound to the LNPs and remained attached during incubation. In contrast, for the two DMG-LNPs, approximately 50% DMG-PEG shedding was completed within three hours and more than 80% within six hours.

The results indicate that DMG-PEG was shed from the LNPs faster than DSG-PEG, suggesting that shorter acyl chains are shed from LNPs faster than longer acyl chains. The anti-PEG IgM titers induced by PEG-DMG-LNP were approximately one-fifth to one-third of those induced by PEG-DSG-LNP. The data shows the effect of the PEG-DMG-LNP and PEG-DSG-LNP on the production of anti-PEG IgM (Figure 8).

Thus, the data indicate that the type of PEG-lipid, and therefore the rate of shedding, affects the degree of induction of anti-PEG IgM by LNPs.

The above findings suggests that the PEG shedding rate influences the immunogenicity of PEGylated LNPs. Manipulating the rate or degree of PEG shedding, by empirically selecting PEG lipid could be a potentially valuable means of controlling the cellular interaction and immunogenicity of LNPs.

LNP Safety and Optimization is Crucial – One does not fit all

Altering and optimizing the components of LNP affects its delivery efficiency, potency, and biodistribution, making it essential to test customized LNPs for each application. Another crucial consideration is the lipids' safety and toxicity. Nonhuman primates (NHPs) are the best predictor of human responses to LNP vaccines, administered Intramuscularly (IM). Precision NanoSystems' recent NHP study to evaluate immunogenicity of saRNA-LNP COVID vaccine candidates in non-naive rhesus primate models highlights the safety of one of its lipid formulations PNI-002 for RNA vaccine applications. In the study, WHO SARS-CoV-2 human IgG standard was used to establish a quantitative curve for relative quantification of anti-spike IgG in the serum specimens. It was observed that the non-naïve rhesus primate models demonstrated an immune response, high IgG levels, good safety, and did not appear to be genotoxic while testing the immunogenicity of the saRNA covid vaccine.

However, while lipid type, size, and surface charge all have an impact on lipid nanoparticle stability and safety, the role of payload in LNP stability and safety should not be overlooked. Several studies are currently underway to determine the stability of payloads like mRNA under various storage and other conditions.

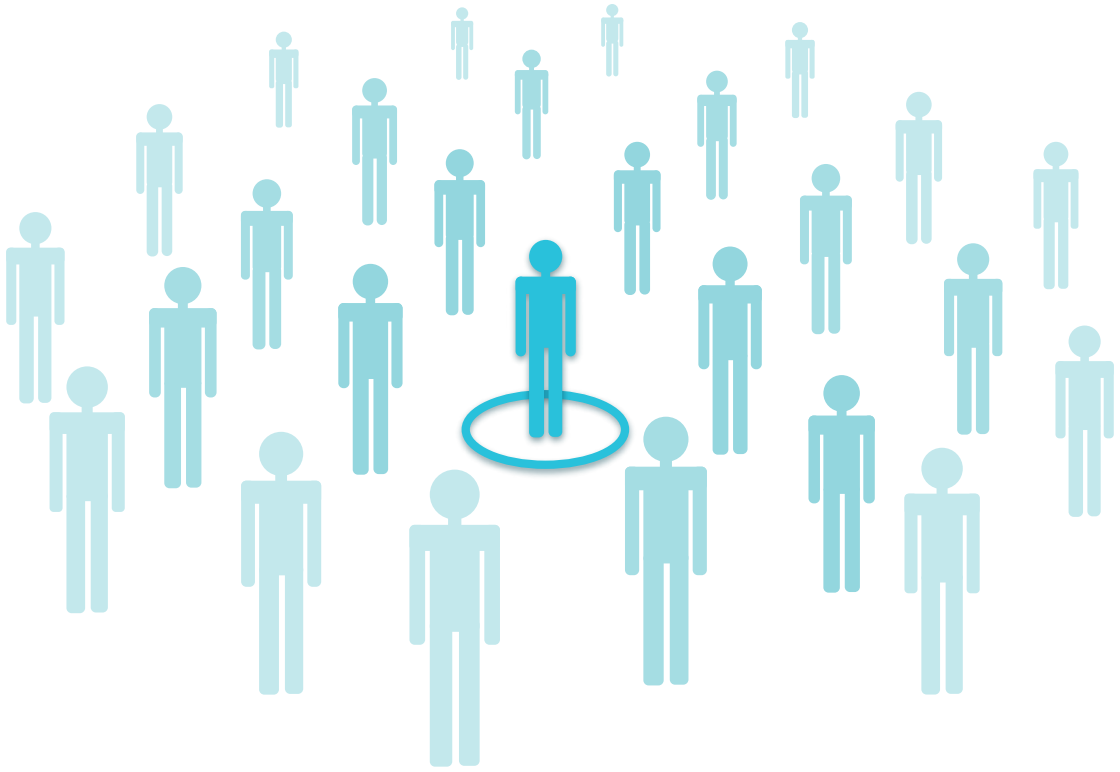


Figure 9. Optimize lipid nanoparticles to target therapeutics

Develop Optimal Formulations

Optimizing LNP formulations is complex, involving the selection of at least five different lipid components and optimization of a vast combination of compositional parameters. Precision NanoSystems provides a wide range of cGMP manufactured lipids to enable clients to tailor drug delivery systems to perfection. The lipids match the highest purity standards, ensuring efficiency, potency and biodistribution of active pharmaceutical ingredients, thereby increasing efficacy and safety. The GenVoy Delivery Platform comprises off-the-shelf RUO reagents such as GenVoy-ILM™ and a library of proprietary lipids available as custom formulations. Backed by extensive RNA-LNP drug formulation and development expertise, Precision NanoSystems helps scientists and drug developers select appropriate formulations to ensure effective therapeutics across applications. In addition, LNP manufacturing is scalable to advanced preclinical and clinical scales with NxGen™ NanoAssemblr® technology.

The era of mRNA medicines is on the horizon. It needs continuous commitment to innovate new tools and technology so that every scientist can become a genomic medicine developer, whether it is in established pharma, emerging biotechnology companies, or academic research labs. Precision NanoSystems can provide access to their exclusive proprietary lipid library and expertise to develop a bespoke lipid nanoparticle formulation for genomic medicine. And, with GenVoy Delivery Platform, it is easier to conceive modular manufacturing suites capable of standardizing RNA drug product manufacturing with highly scalable microfluidic production technology. Precision NanoSystems, now part of the Danaher Life Sciences Group, offers truly comprehensive end-to-end RNA drug development and manufacturing solutions.

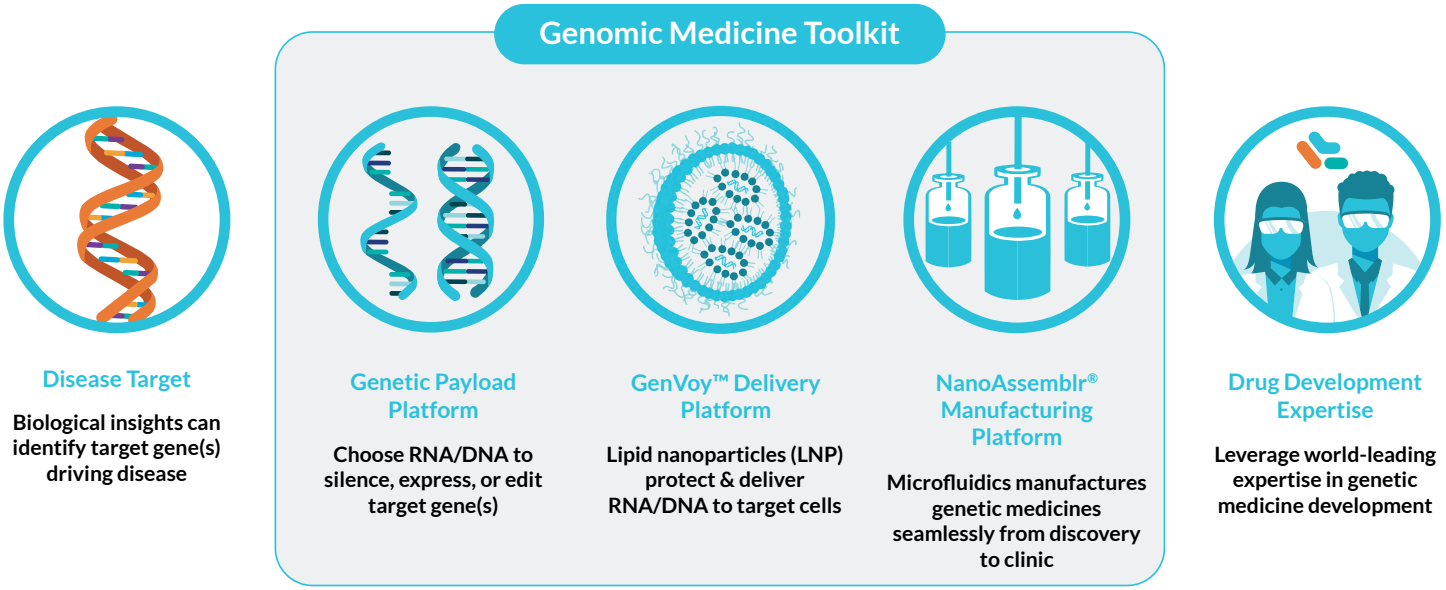


Figure 10. Genomic Medicine Toolkit to develop optimal formulations

If you need help with a lipid-based drug delivery problem, please contact Precision NanoSystems.

Precision NanoSystems offers:

- Access to Clinical Lipid Nanoparticle Library
- Proprietary lipid nanoparticle reagent kits for functional genomic studies
- GMP lipids for lipid nanoparticle formulation
- Formulation development and manufacturing
- Analytical services

Learn more about Precision NanoSystems Proprietary Lipid Library

Have questions about lipid formulations development? Learn more about formulation here.

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