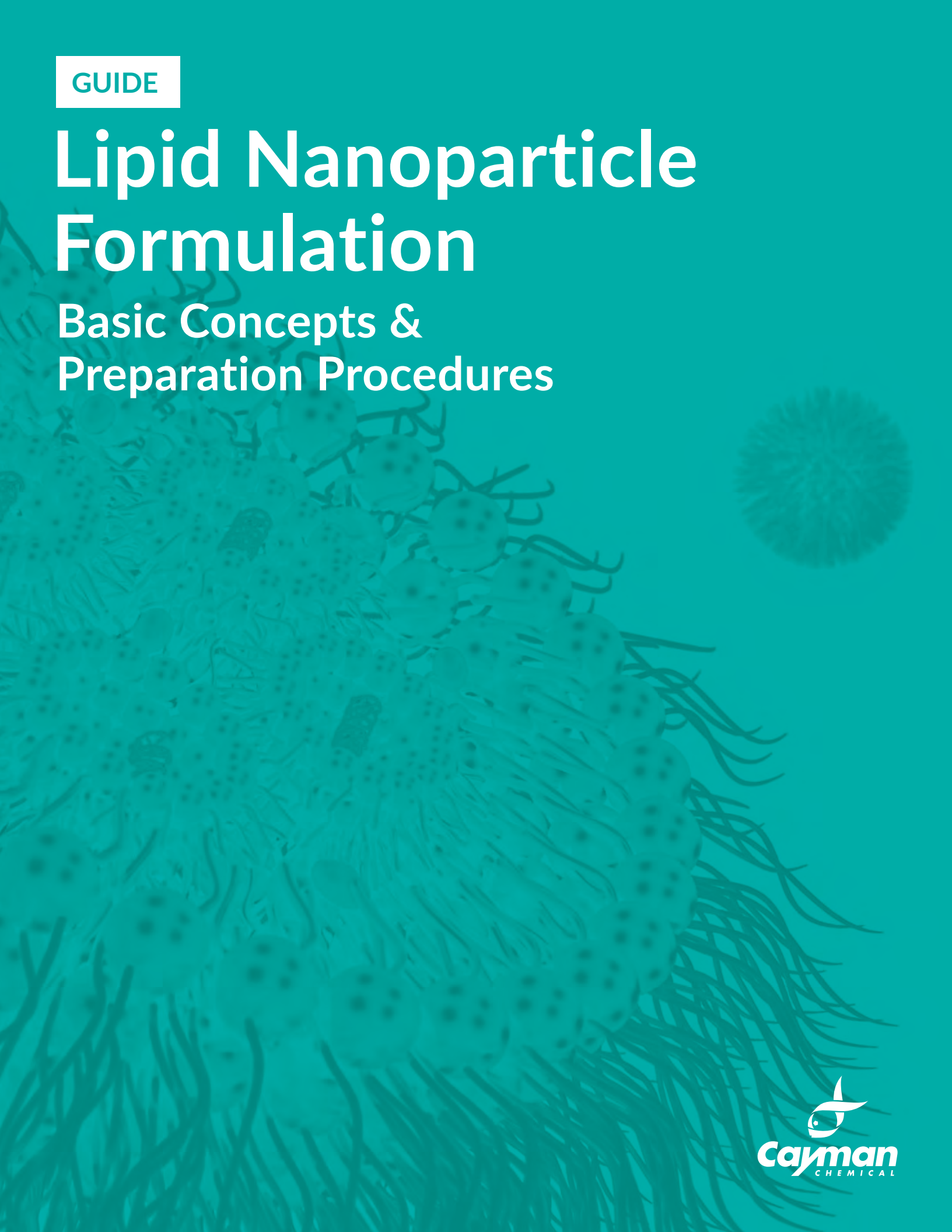
GUIDE

Lipid Nanoparticle Formulation

Basic Concepts & Preparation Procedures



Cayman CHEMICAL

The success of mRNA-based COVID-19 vaccines could not have been possible without decades of research on lipid-based drug delivery (LBDD). LBDD systems are highly versatile and have been used to deliver various bioactive molecules to targeted cells and tissues. LBDD has several advantages over conventional drug delivery methods, including increased drug stability, bioavailability, and distribution.

Lipid nanoparticles (LNPs) are a significant advancement for the delivery of nucleic acid-based therapeutics. Nucleic acids encapsulated within LNPs are protected from enzymatic degradation during the delivery process and are efficiently delivered to cells, where the therapeutic cargo is released.

Use this guide to learn about LBDD systems, the cargoes they deliver, and to explore basic concepts and procedures for the preparation of LNPs.

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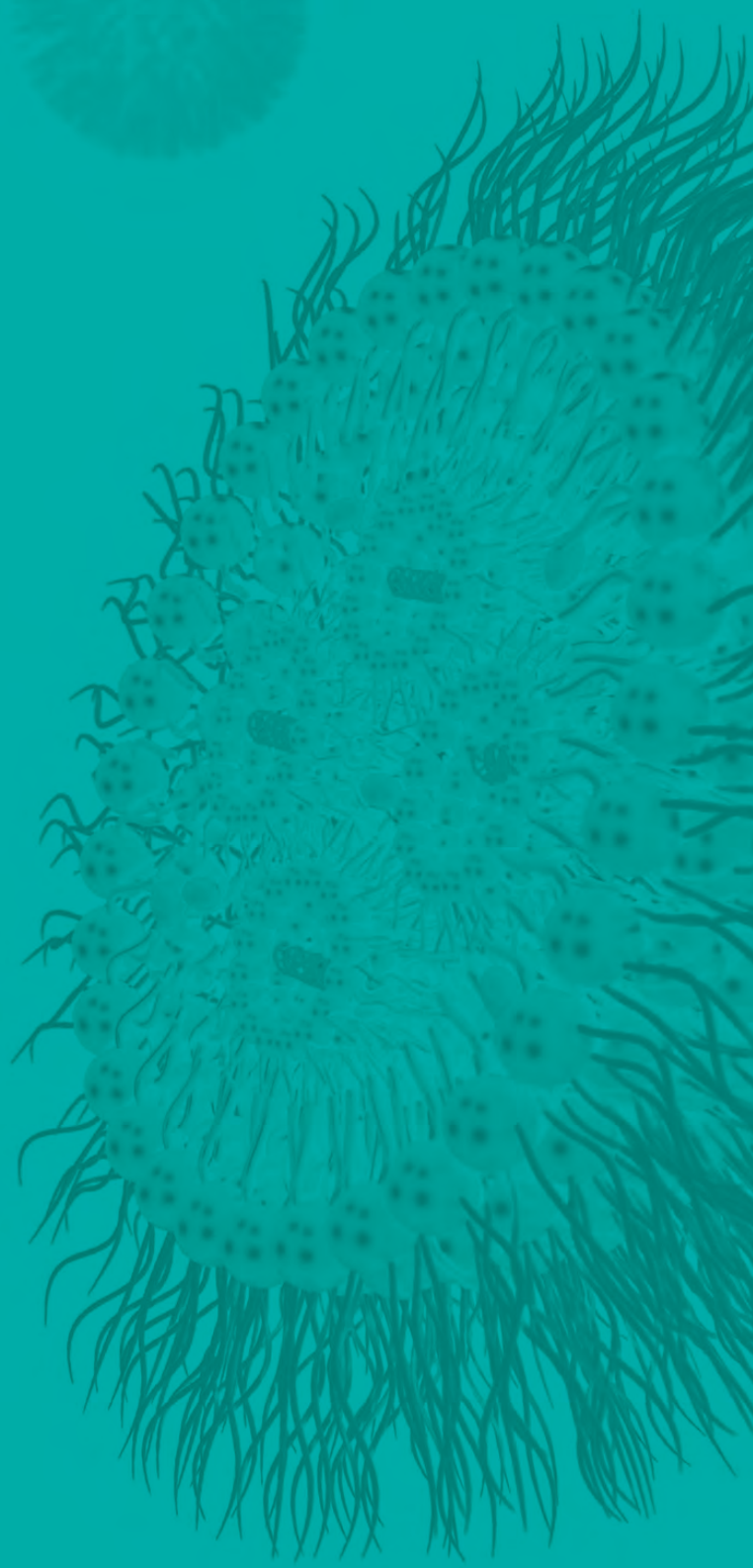


Types of LBDD Systems

Lipid nanoparticles (LNPs): a lipid shell surrounding an internal core composed of reverse micelles that encapsulate and deliver nucleic acids, like siRNA and mRNA, and plasmid DNA (pDNA).

Liposomes: contain one or more lipid bilayers and an aqueous core. They are further classified by lamellarity and size. Liposomes are used for hydrophobic and/or hydrophilic small molecules.

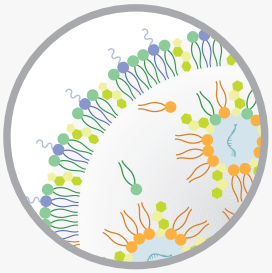
Solid lipid nanoparticles (SLNs): a surfactant shell surrounding a core matrix composed of solid lipids. They are used for hydrophobic and/or hydrophilic cargo.

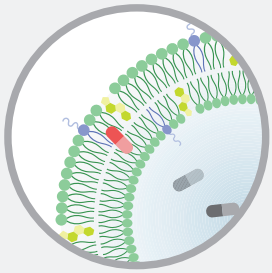
Nanostructured lipid carriers (NLCs): a surfactant shell surrounding a core matrix composed of solid and liquid lipids. They are used for hydrophobic and/or hydrophilic cargo.

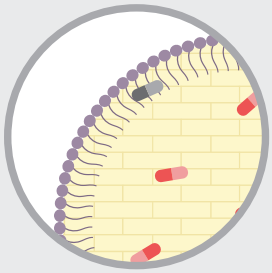
Micelles: self-assemblies of lipid monolayers in aqueous solutions. They have a hydrophobic core, where the phospholipid tails are oriented towards the interior, and can be used for small hydrophobic cargo.

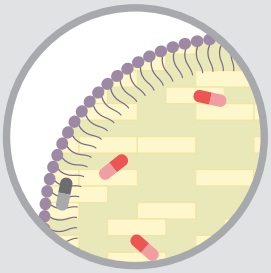
Reverse micelles: an inverted structure compared with traditional micelles. They form a hydrophilic core, with the phospholipid tails oriented towards the exterior, and can be used for small hydrophilic cargo, like nucleic acids in LNPs.

LNPs compared to other LBDD systems

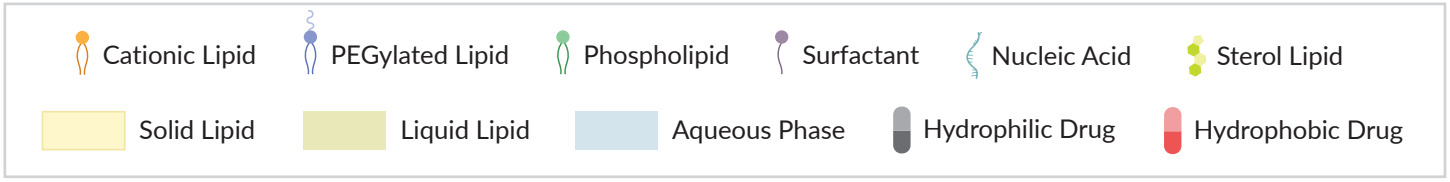








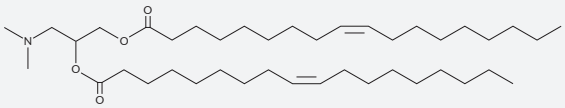
	Lipid Nanoparticle	Liposome	Solid Lipid Nanoparticle	Nanostructured Lipid Carrier
Lipid Shell	Monolayer	Bilayer	Surfactant	Surfactant
Internal Core	Reverse micelles	Aqueous	Solid lipids	Solid and liquid lipids
Cargo	Nucleic acids	Hydrophobic and/or hydrophilic small molecules	Hydrophobic and/or hydrophilic small molecules	Hydrophobic and/or hydrophilic small molecules
Size	~50-150 nm	~50-1,000 nm	~40-1,000 nm	~40-1,000 nm



Structural Components

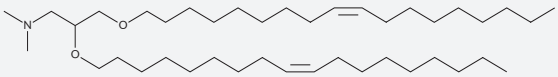
LNPs are composed of ionizable cationic lipids, helper lipids, which include glycerophospholipids, sterol lipids, and PEGylated lipids to protect nucleic acids, which are contained within an aqueous phase. Many of the same structural components used in LNPs are components of other LBDD particles. Lipids and molecules that contain them, like surfactants, can be used to tailor the behavior and properties of LBDD particles. Examples of lipid components are listed below for each class.

Ionizable cationic lipids circumvent the untoward cytotoxicity associated with **cationic lipids**. These lipids possess a transient cationic charge that is acquired at low pH (typically <7), forming reverse micelles that encapsulate nucleic acids in the LNP core. As these lipids have near-neutral charge at physiological pH, they deliver nucleic acid cargo without cytotoxicity.



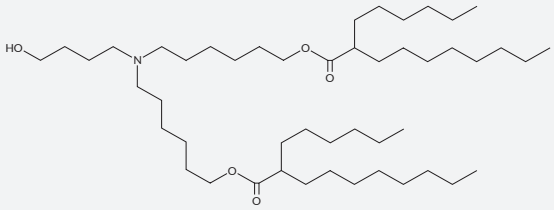
DODAP

pK_a Value of Tertiary Amine: 5.59



DODMA

pK_a Value of Tertiary Amine: 6.59



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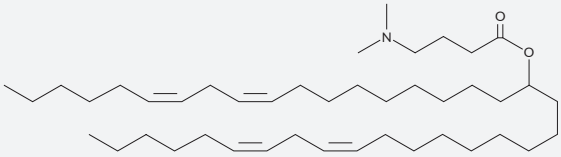
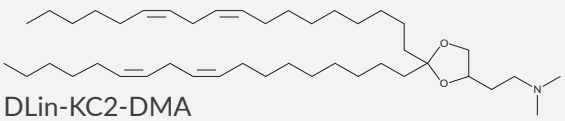
pK_a Value of Tertiary Amine: 6.09



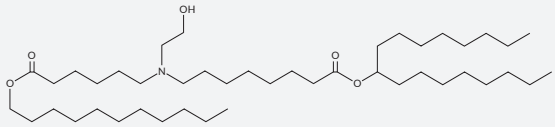
DLin-MC3-DMA

pK_a Value of Tertiary Amine: 6.44



DLin-KC2-DMA

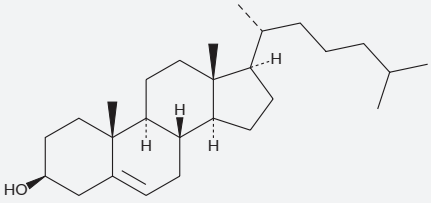
pK_a Value of Tertiary Amine: 6.70



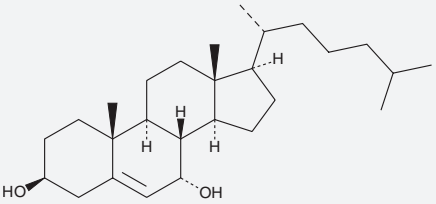
SM-102

pK_a Value of Tertiary Amine: 6.68

Sterol lipids, such as **cholesterol**, fill lipid membrane packing defects and provide structural integrity. They also aid in membrane fusion of the LNP and target cell, and some cholesterol derivatives, like **7α-hydroxy cholesterol**, have been used to improve the delivery of nucleic acid cargo.



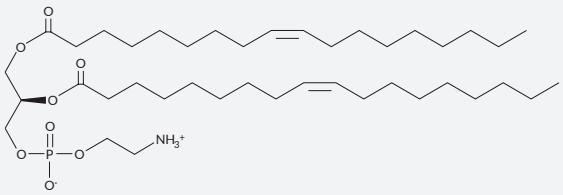
Cholesterol



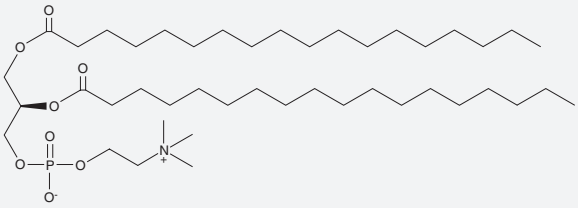
7α-hydroxy Cholesterol

Glycerophospholipids, colloquially known as helper lipids, are a class of phospholipid that contains a hydrophilic head group and two hydrophobic fatty acyl tails attached to a glycerol backbone. The hydrophilic head determines the surface charge of the LBDD particle, which can be neutral, anionic (negative), or cationic (positive).

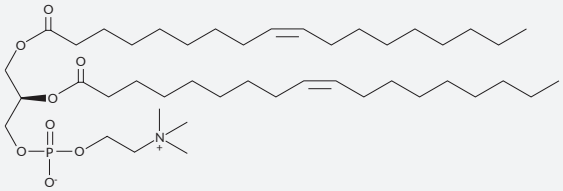
> **Neutral phospholipids** improve the efficacy of membrane fusion and can distribute or modify the net surface charge of the lipid particle. The phospholipid head groups with an overall neutral charge are **phosphatidylcholine (PC)** and **phosphatidylethanolamine (PE)**.



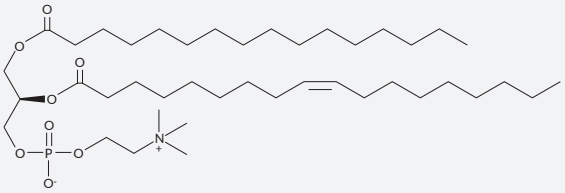
1,2-DOPE



1,2-DSPC

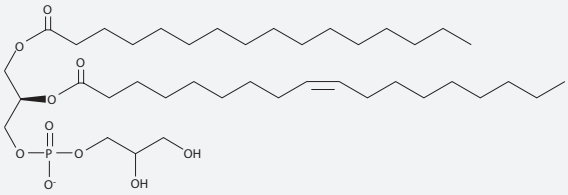


1,2-DOPC

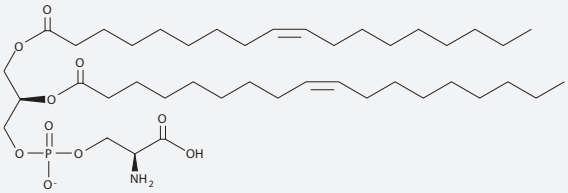


1,2-POPC

> **Anionic lipids** are typically used for the delivery of small molecules and are incorporated into neutral LBDD systems to prevent storage aggregation. They can modify the net surface charge of the lipid particle and influence cellular targeting. **Phosphatidylglycerol (PG)**, **phosphatidylinositol (PI)**, **phosphatidylserine (PS)**, and **phosphatidic acid (PA)** are phospholipids with anionic head groups.

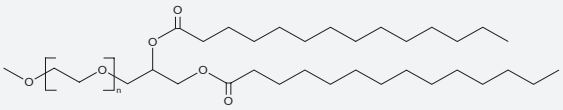


1,2-POPG

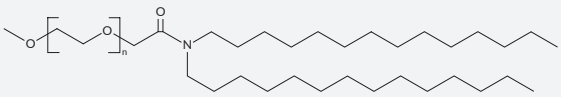


1,2-DOPS

PEGylated lipids prevent serum protein adsorption, inhibiting uptake by the mononuclear phagocyte system (MPS), a major obstacle in the delivery of LBDD systems. Some PEGylated lipids also contain terminal functional groups, such as **amine** or **maleimide**, which can be used to conjugate other molecules that improve cellular targeting and uptake.



DMG-PEG(2000)



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The **aqueous phase** solubilizes hydrophilic molecules, like nucleic acids in LNPs and hydrophilic drugs in liposomes, whereas hydrophobic cargo can be solubilized in the lipid phase.

Surfactants are a major component of the lipid shell of SLNs and NLCs. They reduce the interfacial tension between the lipophilic core and aqueous phase due to their amphipathic nature and improve stability during storage. Lecithin, which contains **phosphatidylcholines**, is one surfactant that has been used in SLNs and NLCs.

Solid lipids, colloquially known as fats, are solid at ambient temperature and used in the preparation of both SLNs and NLCs. Solid lipids used to prepare SLNs or NLCs are typically saturated and include **glycerolipids**, such as triacylglycerols, and fatty acids, including stearic acid, as well as fatty alcohols and fatty esters.

Liquid lipids, also referred to as oils, are fluid at ambient temperature. NLCs are formulated with a mixture of solid and liquid lipids, which increases drug loading capacity and prevents drug leakage. Liquid lipids used in NLCs are typically unsaturated and include **oleic acid**, **α-tocopherol**, and **squalene**.

Read our article *The Heads and Tails of Lipid-Based Drug Delivery* to dive deeper into the biophysical properties of lipids used in LBDD systems.

www.caymanchem.com/lipid-properties

Cargo

LNPs are superior for the encapsulation of nucleic acids, whereas other LBDD systems are preferable for the delivery of small molecule inhibitors or lipids. The requisite LBDD system for your application depends on the cargo, and the localization of the cargo within the LBDD particle depends on its physicochemical properties.

Nucleic Acids

mRNA, siRNA, and pDNA are common nucleic acid cargo. Nucleic acids are negatively charged and best encapsulated within LNPs using ionizable cationic lipids. mRNA-containing LNPs are the basis for most COVID-19 vaccines, and LNPs containing transthyretin-targeting siRNA are used to treat hereditary amyloidogenic transthyretin (ATTRv) amyloidosis.

Small Molecules

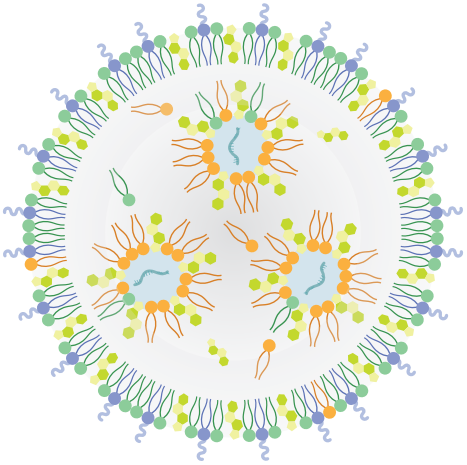
Hydrophobic and/or hydrophilic small molecules can be solubilized in the lipophilic or aqueous compartments, respectively, of liposomes, SLNs, and NLCs.

Hydrophobic drugs are dispersed in the lipophilic compartments of LBDD systems. **Amphotericin B**, an antifungal agent, and **verteporfin**, a photosensitizing agent, are examples of FDA-approved hydrophobic drugs that have been formulated in liposomes.

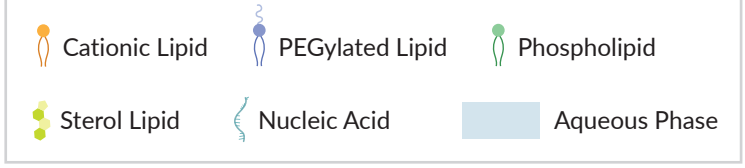
Hydrophilic drugs are solubilized in the aqueous compartments of LBDD systems. **Doxorubicin**, an antitumor antibiotic, is a hydrophilic drug. Doxil® is a form of doxorubicin encapsulated in liposomes and was the first LBDD formulation to be approved by the FDA.

Design

Several factors should be considered when selecting lipids and how they are formulated into LNPs. Below, we outline these factors and how each of them influences the physiological behavior of LNP particles.



Lipid Nanoparticle



The **lipid molar ratio** determines the lipid composition of the particles and influences their size, polydispersity, and efficacy. We recommend starting with a literature review to identify lipid molar ratios that have been previously developed for similar applications.

Lipid molar ratios for LNPs in FDA-approved agents

	Patisiran	BNT162b2	mRNA-1273
Cargo	siRNA	mRNA	mRNA
Ionizable Cationic Lipid	DLin-MC3-DMA	ALC-0315	SM-102
Neutral Phospholipid	1,2-DSPC	1,2-DSPC	1,2-DSPC
Sterol Lipid	Cholesterol	Cholesterol	Cholesterol
PEGylated Lipid	C-DMG-PEG(2000)	ALC-0159	DMG-PEG(2000)
Lipid Molar Ratio[a]	50:10:38.5:1.5	46.3:9.4:42.7:1.6	50:10:38.5:1.5

[a]*Ionizable cationic lipid:neutral phospholipid:cholesterol:PEGylated lipid*

The **lipid:nucleic acid weight ratio** influences the encapsulation efficiency. Most LNPs are formulated with a lipid:nucleic acid weight ratio of 10-30:1.

The ionizable lipid **nitrogen:nucleic acid phosphate (N:P) molar ratio** represents the charge balance between the cationic tertiary amine of the ionizable cationic lipid and the anionic phosphate group in the nucleic acid backbone. This property is the basis for the complexation of ionizable cationic lipids with nucleic acids. LNPs commonly have an N:P ratio around six.

The **lipid acid dissociation constant (lipid pK_a)** is the pH at which the ionized and nonionized forms of a lipid exist in equal concentrations. Lipid pK_a impacts the LNP encapsulation efficiency, efficacy, delivery, and toxicity. For RNA delivery, the lipid pK_a generally ranges from 6-7.

Three important parameters for an **aqueous buffer** are its composition, ionic strength, and pH. Buffers stabilize nucleic acids in solution, and ionizable cationic lipids become protonated and positively charged in the acidic aqueous buffer upon mixing. Commonly used buffers in LNP preparations are 25-50 mM sodium acetate or sodium citrate, pH 4-5. After preparation, LNPs are dialyzed into a neutral buffer, such as PBS, pH 7.4, for storage and use.

The **particle size** alters the pharmacokinetics of the administered particle. Smaller particles typically have longer circulation half-lives, as they evade elimination by the MPS. Particles less than 100 nm can easily pass through fenestrated endothelium to penetrate target tissues. The particle size is dependent on the preparation method. Depending on the LNP preparation method, extrusion can be used to achieve smaller, more uniform particle sizes.

The two most commonly used **routes of administration** for LNPs are intravenous and intramuscular injection. Of note, formulations optimized for a given route of administration are generally not applicable for other routes of administration.

> **Intravenously administered** LNPs with net positive, neutral, and negative charges can be targeted to the lungs, liver, and spleen, respectively. The inclusion of **cholesterol** or **PEGylated lipids** in the formulation, as well as increasing the LNP size, increases distribution to the spleen.

> **Intramuscular administration** is commonly used for vaccines, as it facilitates lymph node targeting and activation of the immune response. When a vaccine is administered, antigen-presenting cells (APCs), like macrophages and dendritic cells, are recruited to the delivery site, where they can encounter vaccine antigens. They then migrate to lymph nodes where they stimulate adaptive immune responses.

The **preparation method** determines the properties of LNPs, including size, homogeneity, and encapsulation efficiency. When selecting a preparation method, cost, scalability, reproducibility, and time commitment should also be considered.

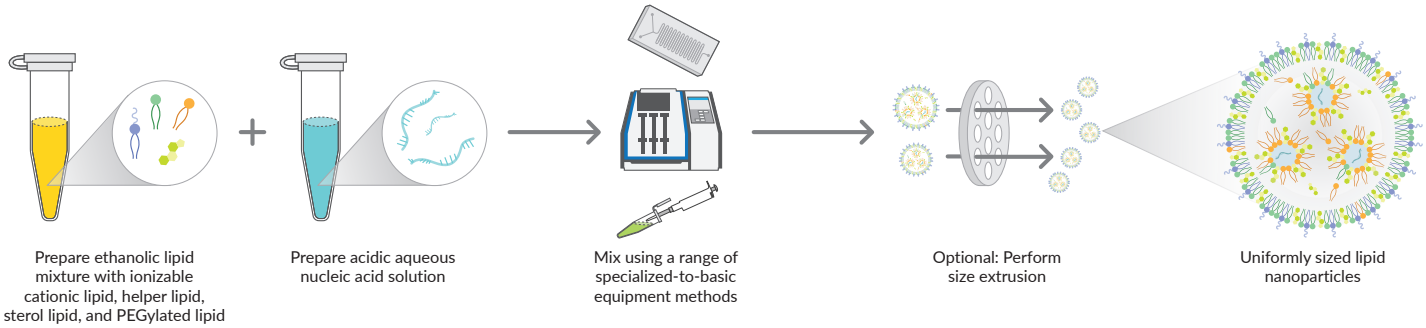
Procedures

In the next sections, we give a series of simple procedures to follow for producing LNPs. The entire range of the LNP life cycle is covered, starting with LNP preparation at the bench and ending with how to use and what to expect when testing LNPs in your *in vitro* or *in vivo* experiments.

LNP Preparation

Before beginning, ensure that all supplies, reagents, and working environments are RNase-free. siRNA and mRNA are chemically labile to RNases, which are enzymes that degrade RNA-based nucleic acids. The steps in LNP formulation are summarized below.

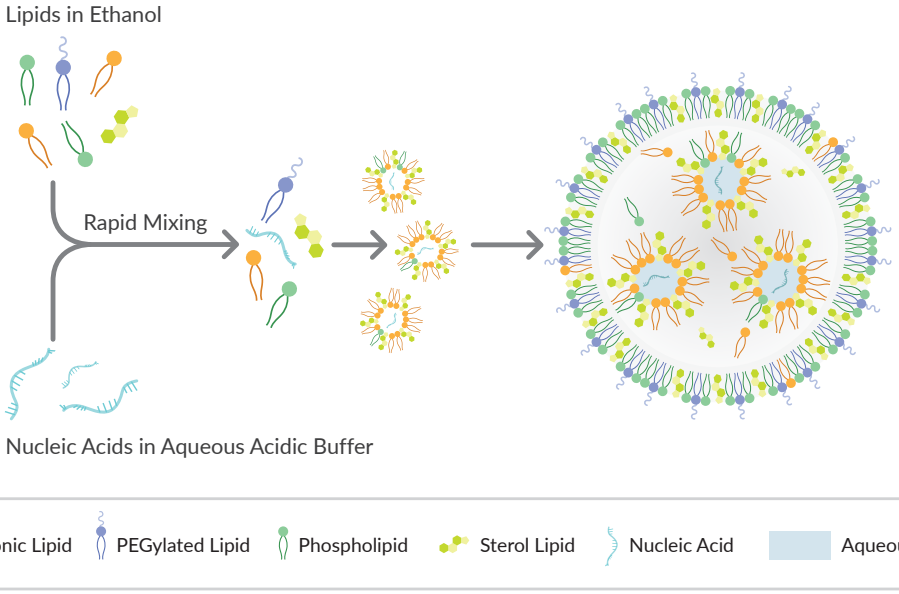
LNP preparation workflow



Mixing

LNPs are prepared by mixing an ethanolic lipid mixture with an acidic aqueous solution containing nucleic acids. A 1:3 ratio of ethanolic lipid mixture to aqueous buffer is generally used. Several methods are suitable for laboratory-scale, small-volume LNP production. Four of these methods, applicable for a range of specialized-to-basic equipment, are compared briefly on page 10.

Schematic of nucleic acid-containing LNP formation



Microfluidic Mixing Devices: Automated microfluidic devices or microfluidic chips are fast and efficient methods to prepare LNPs. These devices enable rapid mixing in a highly controllable, reproducible manner that achieves homogeneous LNPs and high encapsulation efficiency. In these devices, individual streams of the ethanolic lipid mixture and aqueous nucleic acid solution are rapidly combined. LNPs form as the two streams mix and are collected into a single collection tube. Parameters such as the flow rate ratio (FRR) and total flow rate (TFR) can be altered to fine-tune LNPs.

Hand-mixing: This is a simpler alternative method to ethanol-injection. Transfer the ethanolic lipid mixture into the aqueous acidic nucleic acid solution and mix for 15 seconds by rapid pipetting. Leave the mixture undisturbed for 10 minutes. As with the ethanol-injection method, hand-mixing of LNPs results in heterogeneous LNPs with low encapsulation efficiency and can yield variable results.

Feature comparison of LNP preparation methods

	Cost	Scalability	Encapsulation Efficiency	Reproducibility	Polydispersity Index
 Microfluidic Mixing Devices	High	High	High	High	Low
 Hand Mixing	Low	Low	Low	Low	High

Final Preparation

The final preparation of LNPs is performed after they have been formed during the mixing step. These steps help ensure that the LNPs are homogeneous, stable during storage and use, and free of any residual chemical or biological contaminants.

Extrusion: Extrusion reduces and unifies particle size. This step is generally performed with preparation methods that yield LNPs with variable particle sizes.

Dialysis: Dialyze the LNPs in storage buffer using appropriate molecular weight cut-off (MWCO) tubing. This step removes unencapsulated cargo, excess lipids, and ethanol from the final preparation. Dialysis also adjusts the pH of the LNPs from the acidic preparation buffer to the neutral storage solution. For larger volumes, tangential flow filtration is the optimal method for neutralizing pH and removing ethanol.

Filter-sterilize: Filtration is the recommended method for LNP sterilization. Filter-sterilize LNPs with a 0.22 µm filter before storage.

Stability & Storage

After LNPs have been prepared, they may either be used immediately or stored for later use. Below, we discuss factors that can compromise LNP integrity during storage and provide tips to limit storage instability.

Physical stability describes the structural integrity of LNPs during storage. Particle fusion or aggregation and leakage of encapsulated cargo are examples of physical instability.

- Ensure size distribution remains small and homogeneous:
  - Use anionic or PEGylated lipids to prevent particle fusion/aggregation
- Prevent cargo leakage:
  - Include cholesterol
- Follow storage requirements:
  - Adjust temperature, buffers, and pH
  - Avoid freeze-thaw cycles

Chemical stability defines the resistance of LNP lipid and cargo components to modifications in their molecular structure. Hydrolysis, oxidation, and transesterification can lead to nucleic acid and lipid degradation or the formation of lipid-nucleic acid adducts and loss of efficacy.

- Limit cargo degradation:
  - Use RNase-free reagents and supplies
  - Consider nucleic acid cargo with backbone modifications
- Prevent lipid oxidation:
  - Include **antioxidants** or cryoprotectants during storage
- Follow storage requirements:
  - Adjust temperature, buffers, and pH
  - Avoid freeze-thaw cycles

Biological stability relates to the capacity of LNPs to avoid early degradation. Factors that contribute to biological stability include lipid composition, particle size, and surface charge.

- Reduce serum protein opsonization:
  - Include PEGylated lipids
  - Decrease particle size
  - Achieve near-neutral zeta potential
  - Increase LNP hydrophilicity
- Prevent cargo leakage:
  - Follow storage requirements
  - Increase LNP stability with cholesterol or helper lipids

Storage is a critical parameter in the stability of LNP formulations. Generally, LNPs may be stored at 4°C for up to one week or, for long-term storage, lyophilized and held at -80°C. Storage temperature, buffers, and pH may need to be optimized. The inclusion of cryoprotectants is recommended when freezing with or without lyophilization.

Characterization

Characterization of LNP attributes prior to *in vitro* or *in vivo* use is critical for reproducibility.

The **LNP size** describes the average diameter of LNPs and influences their biodistribution and cellular uptake. The **polydispersity index (PDI)** is a measure of the LNP size distribution. Homogeneous, uniformly sized samples have small PDIs, and samples with heterogeneous size distributions have large PDIs. The LNP size and PDI of an LNP preparation can be reduced by optimizing lipid components, increasing the mixing rate, selecting a different preparation method, or by adding an extrusion step. These attributes can be measured by dynamic light scattering (DLS) using a specialized instrument.

The **zeta potential** is the electrostatic potential surrounding the LNP. In general, a near-neutral zeta potential is desirable. Anionic LNPs may be electrostatically repelled from negatively charged plasma membranes, and cationic LNPs can be cytotoxic. The zeta potential can be adjusted by altering the N:P ratio. Zeta potential is measured by a specialized instrument.

Encapsulation efficiency is the final amount of nucleic acid contained within the LNP compared to nucleic acid not encapsulated within the LNP. Microfluidic mixing yields the highest encapsulation efficiencies. Encapsulation efficiency can be measured using nucleic acid-binding fluorescent dyes in the presence and absence of detergent, such as 1% Triton X-100. This technique can also serve to report the encapsulated nucleic acid concentration.

Lipid and cargo integrity are essential for the efficacy and stability of LNPs. Refer to the stability and storage section on page 12 for more information. Our **Chemical Synthesis team** offers lipid characterization services.

The **apparent/global pK_a** of formulated LNPs determines the effect of the ionizable lipid on the apparent pK_a of LNPs. This parameter influences the LNP ionization and surface charge, stability, potency, and toxicity.

LipidLaunch™ LNP Apparent pK_a Assay Kit (TNS Method)

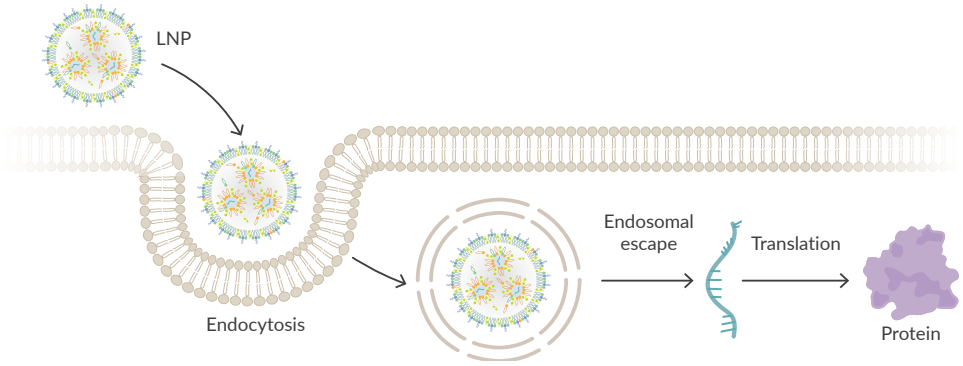
Item No. 702680

- Reagents to determine ionizable lipid effect on the apparent pK_a of LNPs

Learn more at **www.caymanchem.com/pka-assay**

Assessment

LNPs are internalized by target cells *via* endocytosis. Endosomal escape is the process by which the LNP cargo is delivered to the cytosol. Ionizable cationic lipids become positively charged in the acidic environment of the endosomal lumen, which disrupts the negatively charged endosomal membrane and promotes release of the encapsulated nucleic acid cargo into the cytosol, where translation occurs.



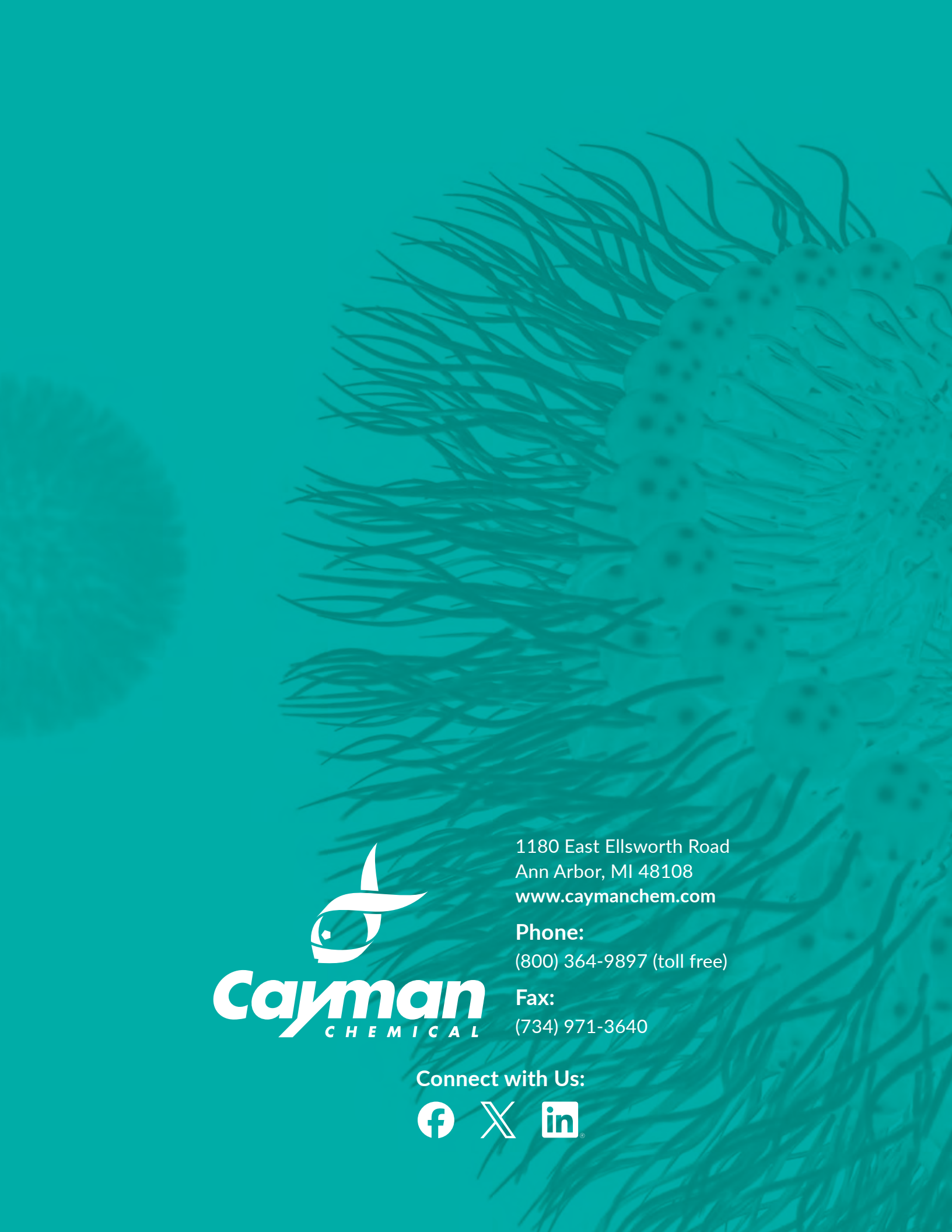
Cellular models and simple molecular biology techniques can be used to test the efficacy of LNPs *in vitro*. Measurement of knockdown or expression of the gene or protein of interest can be accomplished *via* qPCR or **Western blot**, respectively. Cell-based reporter or luciferase assays are also used to determine LNP efficacy.

Animal models provide valuable insight into the efficacy of LNP-based therapeutics *in vivo*. Protein expression of nucleic acid products *in vivo* follows a target-dependent time course. Nucleic acids encoding functional proteins produce changes in protein concentrations within hours, whereas those designed to elicit an antibody response can occur between several days to a couple of weeks. It is often necessary to use repeated dosing regimens to achieve sustained protein expression. **ELISAs** and multiplexed assays can be used to measure target protein responses, and flow cytometry can determine changes in cellular phenotype.

The **fate of LNPs** after administration depends on the lipid composition, LNP design, and the route of administration. Lipids used in LNPs are detectable in the tissues that LNPs are distributed to after administration. These lipids are biocompatible and rapidly degraded, and they are generally eliminated within 24 to 48 hours after administration. To determine lipid tissue concentrations, mass spectrometry-based approaches can be used. Our **Analytical Chemistry** team can assist you in the detection of lipids in various tissues and sample matrices.

Technical Support at any step of your research

Our technical support and product development scientists are here to help answer your questions about products, applications, and protocols, and to help you find additional resources. Contact our **Technical Support specialists.**



Cayman CHEMICAL

1180 East Ellsworth Road
Ann Arbor, MI 48108
www.caymanchem.com

Phone:
(800) 364-9897 (toll free)

Fax:
(734) 971-3640

Connect with Us: