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Engineering lipid nanoparticles with improved endosomal escape for cytoplasmic delivery of RNA drugs

Ziqing Yan[1,2,†], Guangchao Qing[1,2,†] and Xing-Jie Liang[1,2,*]

[1] Laboratory of Controllable Nanopharmaceuticals, Chinese Academy of Sciences (CAS) Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, CAS Center for Excellence in Nanoscience, National Center for Nanoscience and Technology, Beijing, China
[2] University of Chinese Academy of Sciences, Beijing, China

† The authors contributed equally to this work.

* Corresponding author; Email: liangxj@nanoctr.cn.

Highlights:

- This review focuses on the engineering of lipid nanoparticles (LNPs), highlighting the potential alternatives to improve their endosomal escape for enhancing the delivery efficiency of RNA drugs.
- This review explores emerging and prospective techniques such as screening next-generation ionizable lipids and introducing functional molecular moieties to modify LNP components, to provide a roadmap for their advanced engineering.
- This review emphasizes the promise of artificial intelligence, particularly artificial neural networks, in lipid screening and structure optimization, presenting a time-efficient approach for LNP improvement.

Abstract: RNA therapy, with its capabilities in regulating physiological processes directly by affecting the central dogma, holds great potential in the prevention and treatment of various diseases. However, RNA-derived drugs typically require proper delivery vectors to function effectively and also face problems such as low delivery rate due to multiple biological barriers, especially in the intracellular endosome-lysosome pathway, where only a small fraction of RNA molecules can escape and enter the cytosol. A variety of RNA delivery vectors have been developed to address those challenges. Among them, lipid nanoparticles are one of the most promising candidates. Proper engineering of the constituents of LNP is a promising approach to facilitate endosomal escape of RNA. In this review, we will discuss the advantages of LNPs, along with the relationship between their molecular constituents and functions. Then, we will introduce several proposed mechanisms of endosomal escape and the properties of LNPs that may influence these mechanisms. Finally, the current efforts in promoting endosomal escape based on lipid molecule modification or the addition of other materials will be presented. Through the comprehensive review of the current status of efficient LNP-based endosomal escape, we hope this review will provide a valuable guideline for the development of promising RNA delivery vectors.

Keywords: RNA delivery; lipid nanoparticles; endosomal escape; biological barriers

1. Introduction

The rise of RNA drugs has drawn immense attention and is expected to come into play in various diseases [1,2]. Effective cellular delivery is a prerequisite for successful RNA therapies. However, the physiological barriers often impede the accumulation of foreign substances, including RNA drugs, at target sites, resulting in a prominent bottleneck in clinical practice [3–5]. In recent years, the emergence of versatile delivery vehicles, especially lipid nanoparticles (LNPs), has enabled the overcoming of constraints imposed by physiological barriers-imposed constraints to achieve efficient RNA delivery [4,6,7].

In this review, we will summarize the advantages and potential applications of RNA therapy, conclude the latest advances in delivery vectors for RNA therapy, as well as the progress made. Specifically, we will focus on the impact of endolysosomal pathways on RNA therapy and summarize the role and shortcomings of LNPs, in promoting endolysosomal escape and facilitating successful cytosolic delivery of RNA. The latest advances and potential strategies to improve the understanding of LNP-based RNA therapy have also been summarized.

1.1. Potential and application of RNA-based therapy

RNA therapy, referring to leveraging artificially designed RNA-based molecules to achieve biological pathway regulation, has shown its immense potential in disease prevention and treatment [2]. As we deepen our understanding of actively regulating gene expression to reach therapeutic aims, these RNA molecules present growing capabilities to regulate physiological functions or provide protection against viruses and tumours [8–10]. Due to the various subcategories of RNA and their proficient utilization of naturally existing cellular mechanisms, a wide variety of functions can be achieved. Typical cases include selective gene silencing mediated by the short interfering RNA (siRNA) and its associated RNA interference (RNAi) mechanism [11], direct cytoplasmic delivery of messenger RNA (mRNA) to produce proteins [12], modulation of protein functions through protein-specific aptamers binding [13], and even gene editing based on CRISPR systems [14]. Besides making flexible use of relatively well-understood regulation processes, RNA therapy possesses several other advantages including being capable of targeting undruggable targets [15–17], faster and more convenient production [2], reaching long-term effect [18], being useful for rare disease [19], and quicker development cycle [20], further enabling it to become an attractive goal of innovative research in medical and pharmaceutical science [2].

The practicability and efficacy of RNA-based therapies have been proved further through clinical trials of RNA-based drugs and the mass-scale commercialization of medicines open to the public [21,22]. A variety of RNA-derived drugs (or vaccines) that target distinct diseases ranging from virus infection, and cancer, to rare genetic diseases by using multiple types of RNA molecules have been released to the market [2,20]. Among them, recently developed COVID-19 vaccines based on mRNA therapy undoubtedly attract the most attention [23]. In a representative COVID-19 mRNA-based vaccine, involves using an LNP delivery system to deliver nucleic acids encoding the viral antigen into dendritic cells, a major type of antigen-presenting cell. This process induces an antigen-specific immune response, enabling a rapid defense upon pathogen invasion [24]. The advantageous characteristics such as ease of scale-up and flexibility to address virus mutations enabled RNA vaccines to manifest some of the

superiority in infection control [23,24]. The wide vaccination of representatives such as Spikevax [25] and Comirnaty [25] produced by Moderna and Pfizer–BioNTech, respectively, proves the promising prospects for clinical transformation of RNA therapy.

Apart from mRNA-based therapy applied in COVID-19 vaccines, other applications of RNA therapy that involve the usage of different RNA molecules to treat autoimmune disease, fibrosis, or rare diseases like pre-eclampsia also present promising results in frontier researches [26,27]. Other typical examples of already commercialized drugs include Onpattro, which employs LNPs to deliver small interfering RNA to reduce the accumulation of transthyretin proteins, thereby treating the hereditary variant transthyretin amyloidosis [28], and Pegaptanib, an antisense oligonucleotide therapy designed to block erroneous exon skipping [29]. Such newly developed medicines present the flexibility of RNA therapy to manipulate gene expression and protein function. Combining with the current success in RNA-derived drugs, RNA therapy has become a highly promising field that may promote pharmaceutical breakthroughs.

1.2. Bottleneck of RNA-delivery and current approaches

Despite RNA-based agents' potential to address multiple pharmaceutical challenges, certain unfavorable features of RNA molecules make it difficult for them to become promising pharmaceutical agents on their own. Several characteristics of RNA molecules, ranging from relatively large size of some mRNA, negative charge bearing in a physiological environment, slight immunogenicity to susceptibility to endo- and exo-nuclease [30], lead to the demand for properly engineered structure to enhance *in vivo* RNA delivery. Correspondingly, such modifications include, for instance, the addition of other functional molecules or the encapsulation of RNA drugs with delivery systems to boost their transfer across biological barriers [31,32].

Currently, several developed RNA carriers can prevent harmful effects caused by loaded RNA molecules, which otherwise be directly exposed to the physiological environment. Mature delivery system can be based on viral vectors, LNPs, cationic polymer-based polyplexes, exosomes, spherical nucleic acids, and DNA nanostructures[16]. Each kind of these systems is capable of transporting RNA molecules via covalent bonding, electrostatic-driven encapsulation, or the utilizations of naturally existing transportation systems [4]. Effective delivery systems facilitate the passage of RNA molecules across multiple barriers and their eventual transfect into the cytosol or attachment to specific molecules, thereby increasing the proportion of RNA that ultimately participates in the targeted physiological mechanism [31,33].

Among those carriers, LNPs, as used in the COVID-19 vaccines, stands out as one of the most widely adopted RNA delivery vehicles [34]. Unlike dominat recombinant viral vectors, LNPs are artificially synthesized through a process facilitated by electrostatic interactions between ionizable (or cationic) lipids and negatively charged RNA, spontaneously forming spherical structures around the loaded RNA molecules in microfluid systems [35]. Unlike liposomes, LNPs typically lack a strictly bounded phospholipid-bilayer structure, which is designed to promote endosomal escape [7]. Given the biophysical properties of lipids, LNPs offer benefits such as improved permeability, better-guaranteed safety and tolerability in the human body, and ease of design and mass production, enabling the refinement on RNA-based therapies [36,37]. Table 1 summarizes the ongoing clinical trials involving LNP-based RNA delivery systems, with data sourced from the ClinicalTrials.gov database.

In the previous section of this introduction, we have adequately discussed topics covering the potential and current application of RNA-based therapy, the necessity of an RNA delivery system, and

the validity of LNPs in implementing RNA transportation. However, based on previous studies on LNPs, there is still room for improvement for this delivery system. In this review, we will focus specifically on one physiological barrier, known as endolysosomal barrier, faced by the LNP delivery system, and discuss how molecular modifications to LNP structures may contribute to improved endosomal escape.

Table 1. LNP-based RNA delivery systems selected from phase I, II, III, and IV clinical trials.

Identifier	Phase	Sponsor	Intervention	Study title	Reference
NCT05057182 (Active, not recruiting, 2021)	IV	The University of Hong Kong	BNT162b2 mRNA vaccine (Cominarty®, BioNTech/Fosun Pharma), one dose (0.3mL after dilution) contains 30 micrograms of COVID-19 mRNA Vaccine embedded in lipid nanoparticles.	mRNA Vaccination to Boost Antibodies Against SARS-CoV-2 in Recipients of Inactivated Vaccines (the "mBoost" Study)	[38,39]
NCT06634420 (Active, not recruiting, 2025)	III	Intellia Therapeutics	(1) NTLA-2002 (2) Normal Saline IV Administration *NTLA-2002: CRISPR/Cas9 gene editing system delivered by lipid nanoparticle (LNP) for intravenous (IV) administration	HAELO: A Phase 3, Multinational, Randomized, Double-Blind, Placebo-Controlled Study to Evaluate the Efficacy and Safety of NTLA-2002 in Participants With Hereditary Angioedema (HAE)	[40]
NCT05658523 (Active, not recruiting, 2023)	III	Murdoch Childrens Research Institute	(1) Bivalent Moderna (2) Novavax *Bivalent Moderna: A single standard dose of the bivalent Moderna (mRNA-1273.214) COVID-19 vaccine containing equal amounts of mRNAs (25μg of each mRNA sequence) that encode the prefusion stabilized spike glycoproteins of the ancestral SARS-CoV-2 (Wuhan-Hu-1) and the Omicron variant (B.1.1.529 [BA.1]) with mRNAs encapsulated in lipid nanoparticles.	A Randomised Controlled Trial to Assess the Immunogenicity, Safety and Reactogenicity of a Bivalent mRNA Moderna COVID-19 Vaccine or a Protein-based Novavax COVID-19 Vaccine Given as a Fourth Dose in Healthy Adults in Australia	[41]
NCT05534035 (Uknown status, 2023)	III	Everest Medicines (Singapore) Pte. Ltd.	(1) PTX-COVID19-B (2) Vaxzevria® *PTX-COVID19-B is a preservative-free, sterile mRNA-lipid nanoparticle (mRNA-LNP) dispersion in an aqueous cryoprotectant buffer intended for IM injection.	A Phase III, Randomized, Observer-Blind Study to Evaluate the Safety and Superiority in Immunogenicity of PTX-COVID19-B Administered as Booster Vaccination Compared to Vaxzevria® in Adults Aged 18 Years and Older Who Were Previously Vaccinated With Vaxzevria®	[42,43]
NCT05534048 (Unknown status, 2023)	III	Everest Medicines (Singapore) Pte. Ltd.	(1) PTX-COVID19-B (2) Comirnaty® *PTX-COVID19-B is a preservative-free, sterile mRNA-lipid nanoparticle (mRNA-LNP) dispersion in an aqueous cryoprotectant buffer intended for IM injection.	A Phase III Study to Evaluate the Safety and Immunogenicity of PTX-COVID19-B Administered as Booster Vaccination in Previously Vaccinated Adults Aged 18 Years and Older	[42,43]
NCT04847102 (Unknown status, 2021)	III	Walvax Biotechnology Co., Ltd.	(1) SARS-CoV-2 mRNA Vaccine (2) Placebo *SARS-CoV-2 mRNA Vaccine is formulated by encapsulating the mRNA, which encodes the receptor-binding domain (RBD) of spike glycoprotein (S protein) of SARS-CoV-2 and is transcribed *in vitro* by the corresponding DNA template, in lipid nanoparticles (LNPs).	A Global, Multi-center, Randomized, Double-Blind, Placebo-controlled, Phase III Clinical Study to Evaluate the Protective Efficacy, Safety and Immunogenicity of SARS-CoV-2 Messenger Ribonucleic Acid (mRNA) Vaccine in Population Aged 18 Years and Older	[44,45]
NCT05468736 (Active, not recruiting, 2022)	II/III	Novavax	(1) SARS-CoV-2 rS/Matrix-M1 Adjuvant (Initial Vaccination Period) (2) Placebo	A Phase 2/3 Age De-escalating Study to Evaluate the Safety and Immunogenicity of a SARS-CoV-2 Recombinant Spike Protein Vaccine (SARS-CoV-2 rS) With Matrix-M™ Adjuvant in Children 6 Months to < 12 Years of Age	[46]
NCT06747858 (Recruiting, 2024)	II	Arcturus Therapeutics, Inc.	ARCT-032: CFTR mRNA formulated in lipid nanoparticles	A Phase 2, Open-label, Multiple Ascending-Dose Study to Evaluate the Safety, Tolerability and Efficacy of ARCT-032 in People With Cystic Fibrosis	[47,48]
NCT06488313 (Recruiting, 2024)	II	Arcturus Therapeutics, Inc.	ARCT-810: an investigational medicinal product comprising Ornithine Transcarbamylase (OTC) messenger RNA (mRNA) formulated in a lipid nanoparticle (LNP).	A Phase 2a, Open-label, Multiple Ascending Dose Study to Evaluate the Pharmacodynamics and Safety of ARCT-810 in Adolescent and Adult Participants With Ornithine Transcarbamylase Deficiency	[49]
NCT06309485 (Not yet recruiting, 2024)	II	Zhejiang Haichang Biotech Co., Ltd.	(1) WGI-0301 at MTD/RP2D dose IV infusion, QW (2) WGI-0301 at MTD/RP2D −1 dose IV infusion, QW (3) Sorafenib 400 mg PO, BID continuously (4) Sorafenib 400 mg PO, BID *WGI-0301 at MTD/RP2D: a lipid nanoparticle preparation of Archexin®, a 20-mer oligonucleotide that is complementary to Akt-1 mRNA, formulated for the treatment of advanced HCC.	An Open-Label Phase 2 Study of WGI-0301 Plus Sorafenib in Patients With Advanced Hepatocellular Carcinoma as Second Line Therapy	[50]
NCT05542693 (Unknown status, 2023)	II	Azidus Brasil	(1) RNA MCTI CIMATEC HDT 5μg (2) RNA MCTI CIMATEC HDT 10μg (3) Covishield®-AstraZeneca (4) Comirnaty®-Pfizer	Phase IIb, Randomized, Double-blind, Non-inferior, Multicenter Study to Evaluate the Safety and Immunogenicity of the Self-replicating Nanoparticle Carrier Replicon RNA Carrier (repRNA) Vaccine in Adults 18 to 65 Years of Age	[51]
NCT06714253 (Recruiting, 2025)	I/II	RiboX Therapeutics Ltd.	(1) RXRG001: circular ribonucleic acid encoding human aquaporin 1 encapsulated in a lipid nanoparticle (2) Placebo (saline)	A Phase I/IIa, Dose Escalation Study Evaluating the Safety, Tolerability, and Preliminary Efficacy of Intraductal Administration of RXRG001 to Parotid Gland(s) in Adults With Radiation-Induced Xerostomia and Hyposalivation	Not available
NCT06677307 (Recruiting, 2025)	I/II	Korro Bio, Inc.	KRRO-110: An RNA editing oligonucleotide encapsulated in a lipid nanoparticle (LNP) administered by intravenous (IV) infusion as a single dose in Part A (SAD), multi-dose in Part B (MAD).	A Phase 1/2a, Single- and Multiple-dose Escalation Study to Evaluate the Safety, Tolerability, Pharmacodynamics, and Pharmacokinetics of KRRO 110 in Healthy Adult Volunteers and in Adult Participants With Alpha-1 Antitrypsin Deficiency (AATD) (REWRITE)	[52]
NCT06735755 (Recruiting, 2024)	I/II	Beam Therapeutics Inc.	Single dose of BEAM-301 administered by IV BEAM-301 is designed to correct the G6PC1 c.247C>T allele via an A:T-to-G:C base-pair substitution, resulting in restoration of G6Pase-α catalytic activity.	A Phase 1/2, Dose-Exploration Study to Evaluate the Safety and Efficacy of BEAM-301 in Patients With Glycogen Storage Disease Type Ia (GSDIa) Homozygous or Compound Heterozygous for the G6PC1 c.247C>T (p.R83C) Variant	[53]
NCT06389877 (Recruiting, 2024)	I/II	Beam Therapeutics Inc.	BEAM-302: a lipid nanoparticle (LNP)-based therapy for the treatment of patients with AATD	A Phase 1/2 Dose-exploration and Dose-expansion Study to Evaluate the Safety and Efficacy of BEAM-302 in Adult Patients With Alpha-1 Antitrypsin Deficiency (AATD)-Associated Lung Disease and/or Liver Disease	[54]

Table 1. *Cont.*

Identifier	Phase	Sponsor	Intervention	Study title	Reference
NCT06686654 (Active, not recruiting, 2024)	I/II	Sanofi Pasteur, a Sanofi Company	(1) Investigational hMPV/RSV vaccine (2) Investigational RSV vaccine (monovalent) (3) Investigational hMPV vaccine (monovalent) (4) Licensed RSV Vaccine (5) Placebo	A Phase 1/2, Randomized, Observer-blind, Placebo-controlled Multi-arm Study to Evaluate the Safety and Immunogenicity of an hMPV/RSV mRNA Vaccine Candidate in Adult Participants Aged 60 Years and Older	[55]
NCT06917742 (Recruiting, 2025)	I	Capstan Therapeutics	(1) Single Escalating Dose of CPTX2309 (2) Multiple Escalating Dose of CPTX2309 *CPTX2309 is a targeted lipid nanoparticle designed to deliver an anti-CD19 CAR mRNA preferentially to $CD8^+$ T cells.	A First-in-Human, Phase 1, Single-Center, Open-Label, Single and Multiple Ascending Dose Study to Evaluate the Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of CPTX2309 by Intravenous Administration in Healthy Volunteers	[56]
NCT07019883 (Recruiting, 2025)	I	National Institute of Allergy and Infectious Diseases (NIAID)	(1) H5 AC-Anhui RNA Vaccine (2) H5 Astrakhan RNA Vaccine (3) Sodium Chloride, 0.9% *H5 AC-Anhui RNA Vaccine is a nucleoside-modified mRNA vaccine encoding a stabilized, antigenically central hemagglutinin (HA) from influenza A(H5), based on A/Anhui/1/2005 (clade 2.3.4) with substitutions 222QL, 224GS, 156TA, and 134TA. The 2119-nt mRNA is encapsulated in lipid nanoparticles (LNPs) for intramuscular delivery.	A Phase 1 Study to Evaluate the Safety and Immunogenicity of Two Doses of a Novel H5 Antigenically Central (AC)-Anhui mRNA-LNP Vaccine in Healthy Adults	[57]
NCT06694753 (Not yet recruiting, 2025)	I	HIV Vaccine Trials Network	(1) mRNA-1645-eODGT8 (2) mRNA-1645-CoreG28v2 (3) mRNA-1645-N332GT5 (4) Placebo *eOD-GT8 60mer is a self-assembling nanoparticle composed of 60 subunits of the engineered HIV-1 gp120 outer domain germline targeting version 8 (eOD-GT8) fused to an engineered form of a bacterial enzyme, Lumazine Synthase, through a 15-amino acid Glycine-Serine linker. eOD-GT8 60mer will be delivered using an mRNA lipid nanoparticle (LNP) platform. *core-g28v2 60mer is a nanoparticle composed of 60 protein subunits of an engineered core-gp120 fused to an engineered form of a bacterial enzyme, Lumazine Synthase, through a 21-amino acid Glycine-Serine linker. Core-g28v2 60mer will be delivered using an mRNA-LNP platform. *N332-GT5 gp151 is an HIV envelope glycoprotein gp151 trimer based on BG505 SOSIP MD39 (clade A) trimer with "germline-targeting" mutations added that confer the ability to bind germline precursors of BG18 class B cells. N332-GT5 gp151 will be delivered using an mRNA-LNP platform.	A Phase 1, Placebo-controlled, Blinded, Dose-escalation Study to Evaluate the Safety and Immunogenicity of mRNAs Encoding HIV Immunogens (eOD-GT8 60mer, Core-g28v2 60mer, N332-GT5 gp151) in Adult Participants Without HIV and in Overall Good Health in South Africa	[58]
NCT06954818 (Not yet recruiting, 2025)	I	CSPC Megalith Biopharmaceutical Co., Ltd.	(1) A zoster mRNA vaccine SYS6017 (2) Placebo *A zoster mRNA vaccine SYS6017 is an investigational lipid nanoparticle (LNP)-coated mRNA vaccine encoding the glycoprotein E (gE) of VZV, and indicated for active immunization for the prevention of zoster caused by the reactivation of latent VZV.	A Randomized, Observer-blind, Placebo-controlled, Adaptive Phase 1 Clinical Trial to Evaluate the Safety and Immunogenicity of SYS6017(a Zoster mRNA Vaccine) in Healthy Participants Aged 40 Years or More	[59]
NCT06689540 (Recruiting, 2024)	I	Shen Lin	MTS105: a combination of mRNA, which encodes a therapeutic protein, and its delivery vehicle, a lipid nanoparticle (LNP).	First-in-human Clinical Study of Mts105 for Advanced Hepatocellular Carcinoma	[60]
NCT05903339 (Active, not recruiting, 2024)	I	National Institute of Allergy and Infectious Diseases (NIAID)	(1) V3G CH848 Pr-NP1 60 mcg (2) 3M-052-AF 5 mcg (3) Alum 500 mcg (4) V3G CH848 mRNA-Tr2 50 mcg (5) ACU-026-001-1 2.0 mg (6) V3G CH848 Pr-NP1 100 mcg	A Phase 1 Clinical Trial to Evaluate the Safety and Immunogenicity of Ferritin Nanoparticles Expressing Native-like HIV-1 Envelope Trimers Followed by Boost With mRNA Lipid Nanoparticles Encoding a Native-like HIV-1 Envelope Trimer in Adults Without HIV	[61]
NCT05755620 (Active, not recruiting, 2023)	I	National Institute of Allergy and Infectious Diseases (NIAID)	(1) Influenza Virus Quadrivalent Inactivated Vaccine (2) Sodium Chloride, 0.9% (3) VRC-FLUNPF099-00-VP (H1ssF_3928) *VRC-FLUNPF099-00-VP (H1ssF_3928): The vaccine consists of modified nucleoside messenger RNA (mRNA) encapsulated in lipid nanoparticles (LNP), comprised of four lipid components: (ALC-307 (ionizable lipid), DSPC, cholesterol, and ALC-0159 [PEG lipid]).	A Phase 1, Open-Label, Comparator-Controlled, Dose-Escalation Study to Evaluate the Safety and Immunogenicity of a Single Dose of VRC H1ssF-3928 mRNA-LNP in Healthy Adults	[62]
NCT05945485 (Active, not recruiting, 2023)	I	National Institute of Allergy and Infectious Diseases (NIAID)	(1) DCVC H1 HA mRNA vaccine (2) Quadrivalent Recombinant Seasonal Influenza Vaccine (3) Sodium Chloride, 0.9% *DCVC H1 HA mRNA vaccine is a modified nucleoside messenger RNA (mRNA) vaccine encoding the full length HA of influenza A/California/07/2009 (H1N1) encapsulated in lipid nanoparticle (LNP) composed of ionizable lipid, DSPC, cholesterol, and PEG lipid for delivery	A Phase 1, Comparator-Controlled, Dosage-Escalation Study to Evaluate the Safety and Immunogenicity of Two Doses of DCVC H1 HA mRNA-LNP in Healthy Adults	Not available
NCT04601051 (Active, not recruiting, 2020)	I	Intellia Therapeutics	NTLA-2001: A clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene editing system delivered by lipid nanoparticles (LNPs) for intravenous (IV) administration	Phase 1 Two-Part (Open-label, Single Ascending Dose (Part 1) and Open-label, Single Dose Expansion (Part 2)) Study to Evaluate Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of NTLA-2001 in Patients With Hereditary Transthyretin Amyloidosis With Polyneuropathy (ATTRv-PN) and Patients With Transthyretin Amyloidosis-Related Cardiomyopathy (ATTR-CM)	[63]

*The introduction of the LNP-based RNA medicine. Data were sourced from the ClinicalTrials.gov database.

2. LNP and its intracellular RNA delivery mechanism

2.1. Structure and composition of lipid nanoparticles

Multifunctional requirements for RNA delivery vehicles include effectively encapsulating RNA molecules, avoiding immune system activation, crossing multiple biological barriers, and achieving accurate cell-selective (or organ-selective) and intracellular release. To fulfill these critical needs, an LNP used for RNA delivery must be a sophisticated entity composed of multiple functional components [34].

A typical LNP formulation consists of four key components: a cationic or ionizable lipid for mRNA encapsulation and endosomal release; a helper phospholipid that supports bilayer structure; a sterol to enhance membrane integrity and stability; and a poly (ethylene glycol)-ceramides (PEGylated) lipid that modulate nanoparticle size and circulation longevity (Figure 1). The representative examples of each component are summarized in Table 2.

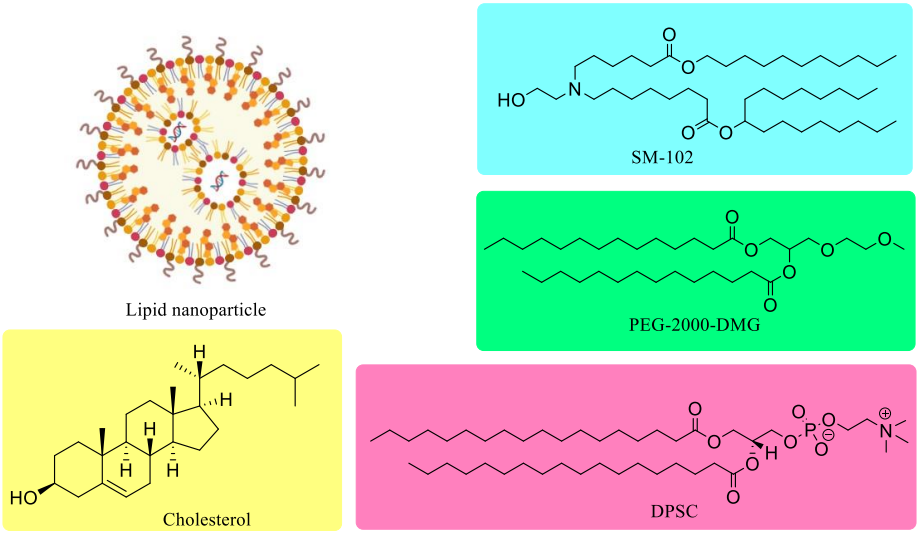


Figure 1. Schematic representation of the structure of LNP.

Cationic or ionizable lipids serve as the main constituent of LNP. The discovery of cationic lipids, artificially synthesized lipids such as N-[1-(2,3,-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), first laid the foundation of lipid-based RNA delivery systems [64]. The incorporation of polyamine group, quaternary ammonium salt, pyridinium, or imidazolium enables the synthesized lipids to carry positive charges under a neutral environment [65]. Coupled with helper lipids like dioleoyl-PC (DOPC) and dioleoyl-phosphatidylethanolamine (DOPE), those cationic lipids are capable of spontaneously forming particles that encapsulate mRNA molecules in the aqueous environment through electrostatic attractions [64]. Further experiments showed that lipid-based particles with this composition could achieve high mRNA transfection rates without additional molecules [37].

The innovative design of ionizable lipids, also known as pH-sensitive amino lipids, propelled the development of lipid-based nanoparticles after cationic lipids. Ionizable lipids become protonated on their ionizable nitrogen-containing headgroup in acidic conditions, such as during nanoparticle synthesis (to encapsulate negatively charged RNA molecules) and within acidic endosomal environment [66]. By contrast, they remain neutral under physiological condition. This feature not only significantly reduces the immunogenicity and potential risks of using LNPs but also enhances the disruption of the endosomal membrane to promote the intracellular delivery of RNA molecules [64].

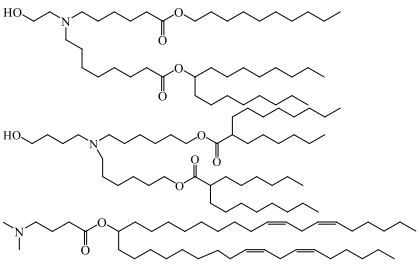
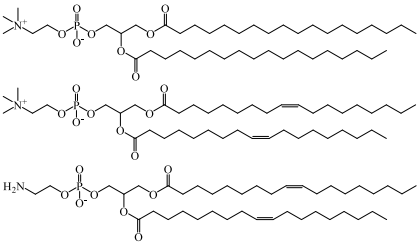
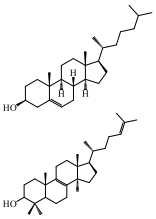
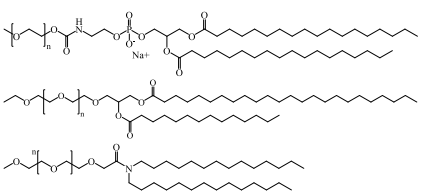
Various kinds of phospholipids and steroids, collectively referred to as helper lipids that mimic biological membranous structures, also contribute to the stability and delivery efficiency of LNPs. The geometric shape of a phospholipid molecule is determined by the relative size of its hydrophilic and hydrophobic regions, and also the existence of double bonds in alkyl groups [67]. These cylindrical or conical shapes influence the properties of LNPs, especially their phase transitions during endosomal

escape [34,68]. While sustaining particle integrity by preventing leakage of the RNA load and regulating fluidity in a biological environment, cholesterol also influences the RNA delivery process by reducing surface-bound proteins and promoting membrane fusion [68,69].

The inclusion of PEGylated lipids is also crucial for crossing multiple biological barriers. When participating in the microfluidic formulation of LNPs, PEGylated lipids tend to locate on the outer surface of the particle, due to the hydrophilicity and bulkiness of their chains [70]. PEGylated lipids dictate the surface properties of LNPs, manifesting as control over particle size and endocytosis, while attenuating immune cell activation and reticuloendothelial system-mediated clearance [68,71]. However, PEGylated lipids may hinder membrane destabilization and initiate an anti-PEG immune response [72].

Apart from the four primary lipids, additional molecules may be incorporated to further improve the functions of LNPs. For example, the addition of selective organ-targeting (SORT) molecules as the fifth constituent in LNPs allows tissue or organ selective RNA delivery despite the system's tendency to accumulate in the liver [73]. Together, these substances form the structural basis of RNA drugs within an LNP delivery system, with the specific proportions varying depending on the properties of the loaded RNA molecules.

Table 2. Composition of lipid nanoparticles.

Component	Role	Representation	
Ionizable lipid	Encapsulates mRNA/siRNA and enables endosomal escape for cytoplasmic release	SM-102	
		ALC-0315	
		DLin-MC3-DMA	
Phospholipid	Forms the foundational lipid bilayer of LNP	DSPC	
		DOPC	
		DOPE	
Sterol	Enhances membrane integrity and stability, aids in fusion	Cholesterol	
		Lanosterol	
PEGylated lipid	Controls particle size, reduces aggregation, and prolongs circulation time	DSPE-PEG	
		DMG-PEG	
		ALC-0159	

2.2. Intracellular delivery of lipid nanoparticle

2.2.1. Endolysosomal pathway as a limitation in RNA-drug delivery

After administration, LNPs undergo complicated biological pathways to finally realize their functions. Subsequent to circulation in the blood, LNP vehicles rely on multiple endocytosis pathways including but not restricted to clathrin- and caveolae-dependent and independent uptake to be internalized [74]. However, due to the presence of the endolysosomal pathway, cellular uptake of LNPs does not directly correlate with RNA drug efficiency [75]. In some cases, less than 2% of siRNA molecules are able to escape the endosome and fulfill their function, marking the endolysosomal pathway a crucial barrier in RNA delivery [76].

After endocytosis, together with other cell surface proteins and lipids, LNPs are incorporated into the peripheral endocytic carrier and form early endosomes through homotypic fusion [77]. Maturation of endosomes gradually occurs, through acidification mediated by vacuolar H^+-adenosine triphosphatase (V-ATPase)-dependent proton pumps and lipid and protein remodeling. Eventually, late endosomes fuse with lysosomes, where hydrolytic enzymes degrade various biomolecules at low pH [78]. It is known that LNPs and their RNA cargo will decompose and lose their function through this pathway (Figure 2) [79]. Therefore, finding specific strategies to escape endosomal entrapment is crucial for RNA therapy and LNP design. Next, we focus on several main mechanisms by which LNPs escape endosomes and summarize them in Table 3.

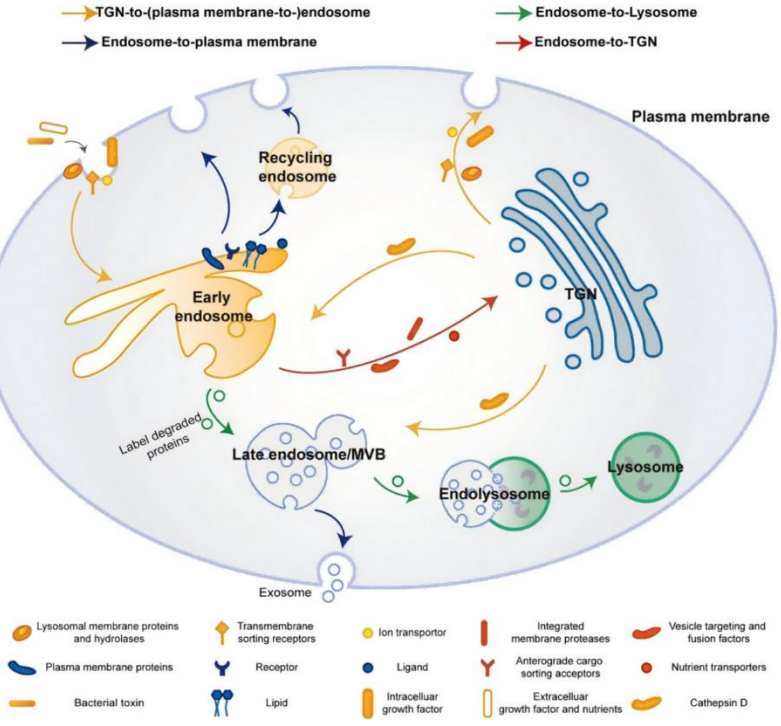


Figure 2. The schematic overview of the endolysosomal pathway. After uptake, endocytic vesicles fuse with the early endosome, which facilitates sorting of materials that have been taken up. At this stage, ingested materials can be recycled back to the plasma membrane, sent to the trans-Golgi Network (TGN), or degraded through endosomal acidification. Each compartment in the pathway is marked by characteristic proteins [79].

2.2.2. Proposed mechanisms of LNP endosomal escape

(1) Cone-shaped lipids/phase transition

One of the prevailing methods to overcome the endolysosomal barrier is to utilize the tailored properties of ionizable lipids, which become protonated under acidic environments but remain neutral under physiological circumstances [80]. Since an acidic environment is maintained within late endosomes (pH = 5.5), ionizable lipids that constitute the bilayer of nanoparticles can capture protons and therefore acquire positive charges. Those positively charged molecules can then interact with anionic phospholipids such as phosphatidylserine and lysobisphosphatidic acid in the bilayer structure of the endosome membrane [81]. The pH-dependent electrostatic attraction between the charged head groups of ionizable and anionic lipids disturb the lamellar geometry structure and induce a transition to an inverted cone geometry, leading to organization into a hexagonal phase [82] (Figure 3). This change in geometry can lead to the fusion of endosomal membrane and LNP, resulting in the exposure and release of the RNA payload into the cytosol [83]. This process was verified by techniques such as NMR spectrum with respect to phosphorous element, small-angle X-ray scattering, colorimetric analysis, and live-cell imaging [84].

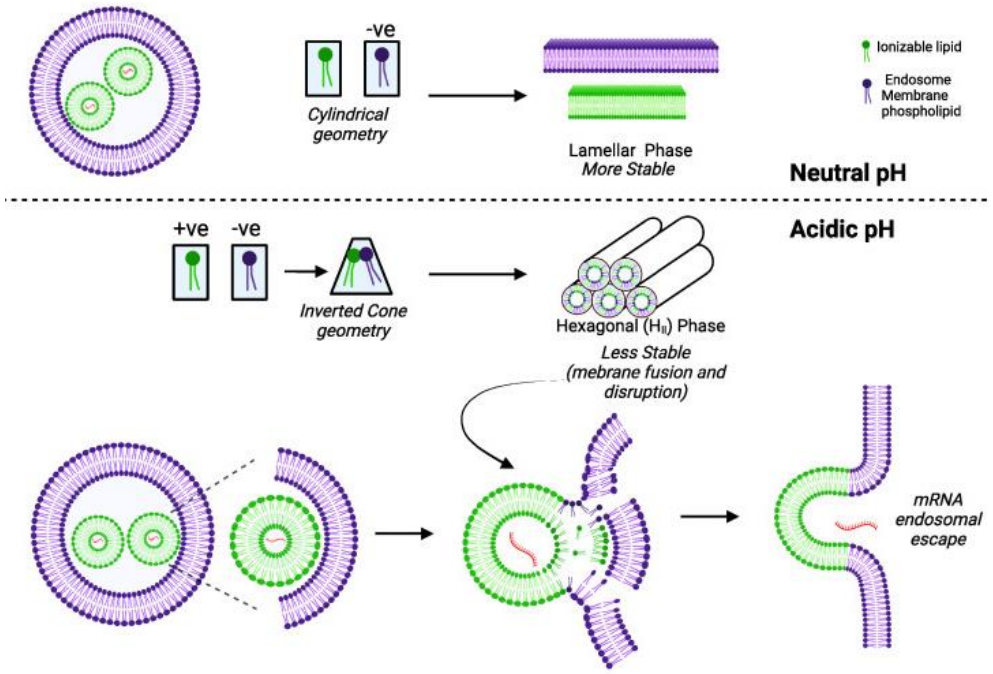


Figure 3. Schematic representation of the bilayer to hexagonal phase transition of LNP. Under the acidic pH of endolysosomal compartment, ionizable lipids are positively charged and interact with the anionic lipids present on the inner leaflet of the endosomal membrane. This interaction leads to the transition from bilayer to hexagonal phase transition resulting in endosomal membrane damage and cargo release [80].

The endosomal escape mechanism based on electrostatic attractions is highly pH-dependent. Moreover, other structural properties of the ionizable lipid and the specific cell type to be targeted also contribute to endosomal escape significantly. Relevant characteristics of the ionizable lipid molecule, including its precise shape, size (both for lipid tail and ionizable head group), pK_a value (the pH value

when half of the lipid molecules capture protons), and the temperature required for geometric change, together with the presence of other helper lipids, collectively determine the efficiency of endosomal escape [35,36]. Such findings lay the foundation for proper engineering refinements of LNP.

(2) Proton sponge effect

An alternate hypothesis about the mechanism of endosomal escape is the "proton sponge effect", which suggests that endosome vesicles can burst due to imbalanced osmotic pressure [85]. During the maturation of endosomes, V-ATPase continuously maintains an optimal pH environment for various enzymes inside the endosome membrane [86]. Along with this active transport, chloride ions enter the endosome through the membrane to maintain electrical potential. However, entering LNPs have buffering capacity, that is, they are able to bind hydrogen ions inside the organelle, due to their ability to become protonated under acidic conditions. This buffering or protonating effect induces electrostatic repulsion between lipid molecules, which destabilizes the membrane structure and also leads to increased influx of hydrogen ions and chloride ions (which enter as counterions) [87]. The incoming ions and water molecules that balance the osmotic pressure, together with the electrostatic repulsion of LNPs, cause swelling, bursting, and lysis of the endosome vesicle, thereby releasing the encapsulated payloads into the cytosol (Figure 4) [80,88].

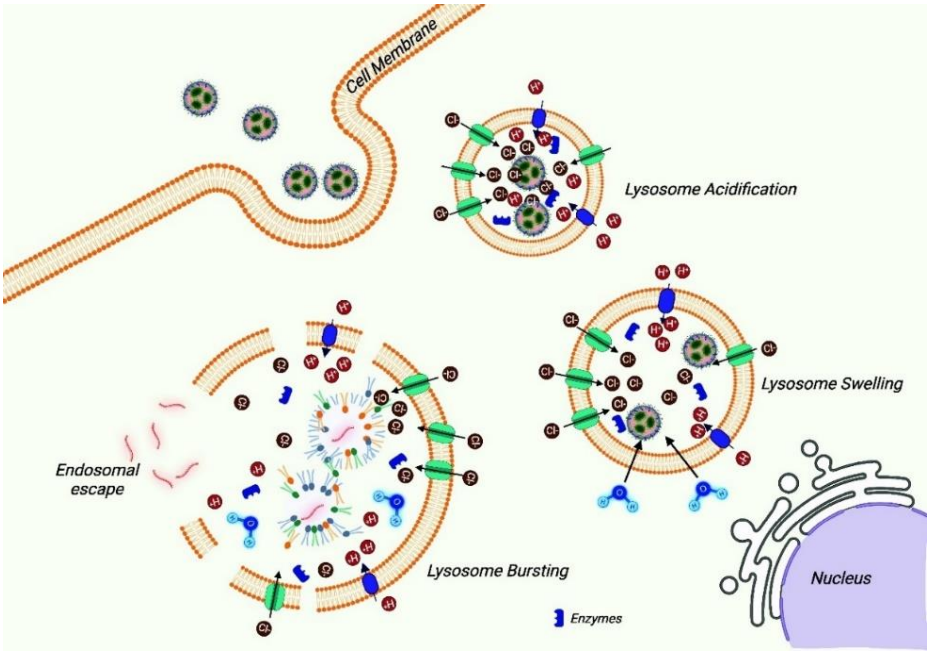


Figure 4. Schematic representation of the Proton Sponge Effect. Due to the buffering ability of ionizable lipids, there is a huge influx of protons by activation of the proton pump in endolysosomal compartments. To neutralize the membrane potential, an inflow of chloride ion is triggered creating an osmotic imbalance which is followed by water intake. This leads to endolysosomal compartment swelling and eventually burst, which releases the cargo [80].

To test this hypothesis, researchers have used the V-ATPase inhibitor bafilomycin A1 and ion-sensitive materials delivery systems to examine the role of the pH gradient in endosomal escape [88]. The results showed that bafilomycin A1 prominently reduced endosomal escape efficiency, whereas ion-sensitive materials improved it [89,90]. It's worth noting that since the pK_a value reflects the extent to which lipid

molecules become protonated at different pH levels, it remains a key factor in the proton sponge effect scenario. These results provide convincing evidence for this hypothesis [88,90]. However, the theory remains controversial, as the osmotic pressure generated by the pH gradient may be insufficient to cause membrane rupture [89]. In addition, complete endosomal lysis following escape is rarely observed [91,92].

(3) Photochemical internalization

Widely used to boost the trafficking of other drugs, photochemical reactions are also commonly adopted to trigger endosomal escaping events [93]. Photochemical internalization (PCI) technology relies on amphiphilic photosensitizers, a class of chemical compounds that can localize and accumulate in endocytic vesicles prior to endocytosis. Upon activation by external light, these photosensitizers generate membranous-destructive reactive oxygen species (ROS), which subsequently damage cellular components [94]. In addition, the short life-time of ROS may confine the damaging effect to their production site, minimizing side effects beyond endosomal escape [95]. The endocytic vesicles are thereby disrupted, resulting in the release of therapeutic particles into the cytoplasm [93].

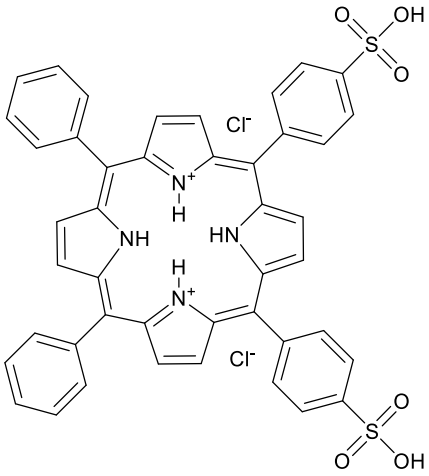


Figure 5. The chemical formula of meso-tetraphenylporphine disulphonic acid dihydrochloride.

Successful attempts leveraging PCI photosensitizers to promote intracellular delivery of RNA molecules have been made. For instance, it has been reported that the typical photosensitizer molecule meso-tetraphenylporphine disulphonic acid dihydrochloride (Figure 5), a meso-tetraphenylporphine derivative with two sulfonate groups on adjacent phenyl rings, can be activated by the light in the wavelength range of 375–450 nm, effectively facilitates the escape and release of siRNA molecules in nanoparticles [93]. Important factors related to PCI-induced endosomal escape include the concentration of the photosensitizers used and the duration of light exposure. It is also noteworthy that the rational design of photosensitizer molecules focuses on achieving longer retention time in the endocytic vesicles, which is contrary to LNP design principles [88,93,95,96]. Furthermore, the regulation of pK_a value by introducing specific functional groups such as sulfonic acid groups in PCI is also involved [95].

Table 3. Endosomal escape strategies of LNP.

Mechanism	Sub-Category	Core Principle	Representative examples	Advantages	Limitations
pH-response	Ionizable lipids	Proton sponge effect	SM-102, MC3, and ALC-0315	• Foundation of clinically approved LNPs • Good balance between efficacy and reduced toxicity *vs.* cationic lipids • Contributes to proton sponge effect	✧ Escape efficiency can be variable and suboptimal ✧ The proton sponge effect's dominance is debated ✧ Requires careful pK_a tuning (~6.2–6.5)
	pH-sensitive PEG-lipids	PEG corona attached via an acid-labile bond that cleaves in the endosome	Hydrazone-based PEG lipids	• Promotes "de-PEGylation" at the target site • Enhances cellular uptake and membrane fusion • Unveiling the hidden targeting ligands	✧ Adds complexity to formulation ✧ Cleavage kinetics must be optimized to match endosomal trafficking
Membrane fusion and disruption	pH-activated peptides	Peptides undergo conformational change in acid pH, forming membrane-disrupting α-helices	EEP13 and EEP14	• Extremely high escape efficiency • Bio-inspired and highly tunable • Low cytotoxicity at designed doses	✧ Can be immunogenic ✧ Adds significant complexity and cost to manufacturing ✧ Peptide stability can be an issue
	Cone-shaped lipids/phase transition	Conical lipids promote transition to non-bilayer phase, destabilizing the membrane	Dlin-MC3-DMA	• Enable highly efficient, direct membrane disruption and escape	✧ High association with cellular toxicity due to membrane destabilization ✧ Difficult to finely control the disruptive effect
	Chloroquine-mimicking lipids	Mimicking chloroquine's endosomolytic function	Endosomolytic chloroquine-like optimized lipid nanoparticles (ecoLNPs)	• Designed for higher efficiency and lower systemic toxicity than free chloroquine	✧ Still an emerging technology, and long-term toxicity profile not fully established
Lysosomal function modulation	Lysosome "replenishment"	LNPs deliver missing components to restore function in dysfunctional lysosomes	Acidic liposomes (LPPs)	• "Reverse" strategy is ideal for treating lysosomal storage diseases • Addresses the root cause of certain diseases	✧ Application is highly specific to certain disease pathologies
Photochemical internalization	Photosensitizer molecule	Generate membranous destructive ROS under light stimulation	Meso-Tetraphenylporphine disulphonic acid dihydrochloride	• Spatially localized effect • High specificity • Reduced off-target side effects	✧ Complex dual-parameter optimization ✧ Potential for operational variability and risk ✧ Design logic contrary to efficient delivery principles ✧ Dependence on external activation

3. Modification of LNP to enhance the endosomal escape

The optimization and functionalization of LNP have emerged as pivotal approaches for overcoming endosomal barriers in RNA therapeutic delivery. Such optimizations can be achieved through multiple strategies, including screening next-generation ionizable lipids, optimizing the formulation, introducing functional molecular moieties, and implementing precision modifications to existing architectures [76,78]. While substantial progress in ionizable lipid discovery and formulation optimization has been well-documented in recent literature [97–99], the subsequent sections will focus on functionalization of components. Specifically, we highlight cutting-edge molecular engineering strategies and their demonstrated efficacy in augmenting endosomal escape capabilities, with particular emphasis on innovative chemical modifications and bio-inspired design paradigms.

3.1. Cationic and ionizable lipid

Considering their unique alternating charge-carrying capability in neutral and acidic environments, ionizable (or cationic) lipid molecules serve as the most important components in LNPs—both for RNA molecule encapsulation and intracellular delivery. Therefore, engineering ionizable lipids to endow them with favorable properties is a crucial area of LNP refinement studies.

An ionizable lipid molecule typically consists of one headgroup (usually containing one nitrogen atom that can capture hydrogen ions), a linker region (usually composed of ether linkages), and several hydrophobic alkyl chains (Figure 6). Various approaches to optimize LNP design have been made by introducing distinct modifications to each part of an ionizable lipid molecule [100]. This previous research has yielded valuable insights into the structure-function relationship on a molecular basis and provides rationales for the rational optimization of LNPs for endosomal escape. For the alkyl chains, the degree of unsaturation and their length both affect the intracellular trafficking of LNPs [101]. Changes in the degrees of unsaturation influence several lipid properties, such as fluidity, phase transition temperature, hydrophobicity, packing parameter, and oxidation stability [101]. These alterations consequently shape the physicochemical properties, mRNA delivery efficiency, and immunogenicity of LNPs [101].

Differences in inductive effects and intermolecular interactions lead to varying pK_a value, and the degree of unsaturation also affects the phase transition temperature, which defines the temperature required for a geometry change to occur [102]. Both pK_a and phase transition temperature are negatively correlated with fusogenicity [97], the ability to trigger endosomal escape via the electrostatic interaction model. Accordingly, researchers conclude that alkyl chains with two unsaturated cis double bonds are most effective for the delivery of RNA molecules, and further increasing the double bonds may hinder cellular uptake prior to intracellular delivery [83,97]. This optimization approach is exemplified in DLin-MC3-DMA, which contains exactly two double bonds in each alkyl group and was the first FDA-approved ionizable lipid used to deliver siRNA molecules, as adopted in the drug Onpattro [103].

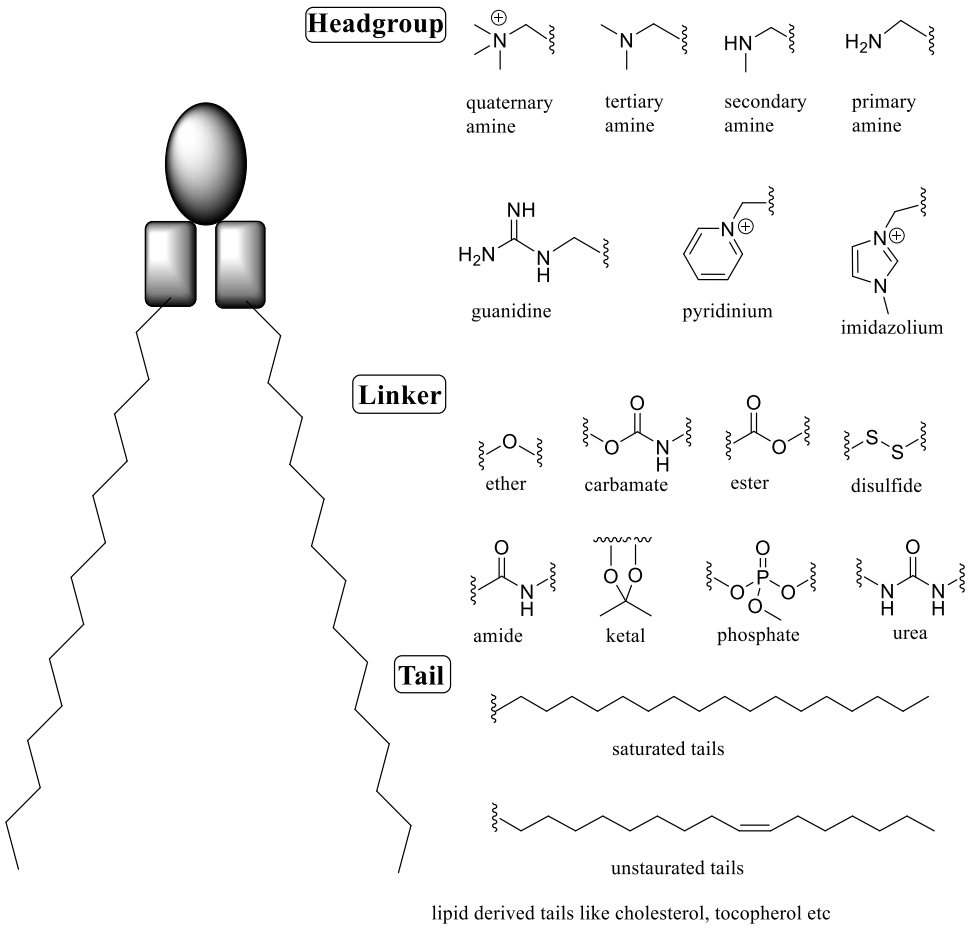


Figure 6. Schematic representation of the structure of ionizable molecules [34]. Reprinted with permission. Copyright 2022 American Chemical Society.

As for the linker part between the protonatable amine head group and the alkyl chain, ketal ring linkers appear to be the most effective in promoting endosomal escape, followed by alkoxy linkers, and then ester, carbamate, or thioether linkages [100]. Linker groups that resist hydrolysis in the endosomal environment should be used, which explains the relatively low efficiency of LNPs composed of ionizable lipids with ester linkages [104]. Therefore, future refinements and modifications of current LNPs are suggested, especially since most commercialized lipids still rely on ester groups. The optimal length of the linker group is also under discussion. Lipids with a longer alkyl group between the linkage and the nitrogen-containing group tend to exhibit a higher pK_a value and a lower phase transition temperature [83,97].

A wide variety of nitrogen-containing functional groups show potential for achieving ionizable properties. Choosing amine (tertiary or quaternary), imidazole, or piperazine (with varying numbers of methyl group substitutions) leads to different pK_a values due to inductive and conjugation effects [98,105]. Accordingly, modifying the nitrogen-containing groups can result in different likelihoods of endosomal escape. These design principles for ionizable lipids are applied in practice. Current approved RNA-LNP products, such as Onpattro, Comirnaty, and Spikevax, which used DLin-MC3-DMA, ALC-0315, and SM-102, respectively, as ionizable lipids all follow the principles mentioned above [98] (Table 2).

However, despite the extensive discussion on tuning the properties of ionizable lipid molecules, it should be noted that the efficacy of LNPs depends on multiple factors and does not increase monotonically with any single one. For example, studies indicate that the optimal pK_a for ionizable lipids

is approximately 6.4. Any deviation from this value, whether higher or lower, reduces RNA delivery efficiency, since the lipid must be protonated in acidic compartments while remaining neutral at physiological pH, imposing strict design restrictions [105]. It is also important to note that altering the functional groups or charge-carrying properties of ionizable lipids can affect the extracellular trafficking of LNPs [106]. This is because the surface charge influences the adsorption of proteins from biological fluids onto LNPs, and the resulting protein corona becomes the primary interface encountered by cells and tissues. Therefore, modulating the protein corona affects which cells and organs the LNP interacts with by determining the specific receptors engaged. For example, apolipoprotein E (ApoE) preferentially binds to neutral surfaces, enabling surface-charge-dependent binding between ApoE and LNPs [105]. This interaction subsequently promotes receptor-mediated uptake by liver cells. Moreover, LNPs with an electropositive surface that adsorb RGD motif-rich proteins such as vitronectin tends to accumulate in the lungs [107,108], whereas the enrichment of opsonins facilitates their rapid clearance by immune cells [109]. Another example involves ionizable lipids with piperazine groups, which can lead to accumulation in the spleen and liver [98]. These findings further manifest that engineering LNP structures is a sophisticated process involving multiple interrelated factors.

The identification of optimal ionizable lipids for facilitating endolysosomal escape presents a multifaceted challenge due to the large number of interdependent variables involved. Therefore, leveraging the power of machine learning algorithms to address this challenge is particularly promising. By using artificial neural networks, computational methods, and existing data on ionizable lipid properties related to endosomal escape, researchers may succeed in predicting pK_a or even overall LNP efficiency. Since machine learning algorithms are already being applied in the rational design of LNPs, this represents a cutting-edge approach with promising prospects [99].

3.2. Phospholipid

The rationale for optimizing phospholipid molecules incorporated into LNPs is similar to that for ionizable lipids, as both conform to the phase transition mechanism driven by electrostatic interactions. Integrating features of ionizable lipids into naturally derived phospholipids is a preferred method for the rational design of phospholipid [110]. Studies have shown that phosphatidylethanolamine (PE), particularly 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), which features a protonatable primary amine, often exhibits superior performance compared to phosphatidylcholine (PC) representatives such as 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), with its permanently charged quaternary amine, as well as phosphatidylserine (PS) molecules in the transfection efficiency of RNA payloads due to low colocalization with lysosomes [110] (Figure 7). This can be explained by the fact that PE lipids are commonly found in cellular membranes where they introduce membrane curvatures and increase tension, explaining their fusogenic properties, while PC and PS lipids help stabilize the bilayer [110]. Lipids with PC headgroups tend to adopt a cylinder shape that prevent their transition to a hexagonal conformation, whereas lipids with PE headgroups adopt a cone shape that facilitates membrane fusion at endosomal pH [67]. Extending the discussion on promoting hexagonal phase transition, it is established that a small charge-carrying group with large tail regions, often with three hydrophobic alkyl tails, is more likely to adopt cone shapes and promote endosomal escape [110].

Rational design of phospholipid in LNPs, especially through the inclusion of amine groups similar to those in ionizable lipids, can lead to multi-tailed ionizable phospholipid LNPs, where the engineered

phospholipid completely substitutes for ionizable lipids [110]. Since the use of phospholipid in LNPs is closely related to tissue or organ selectivity, further exploration of similar structures is highly promising [67].

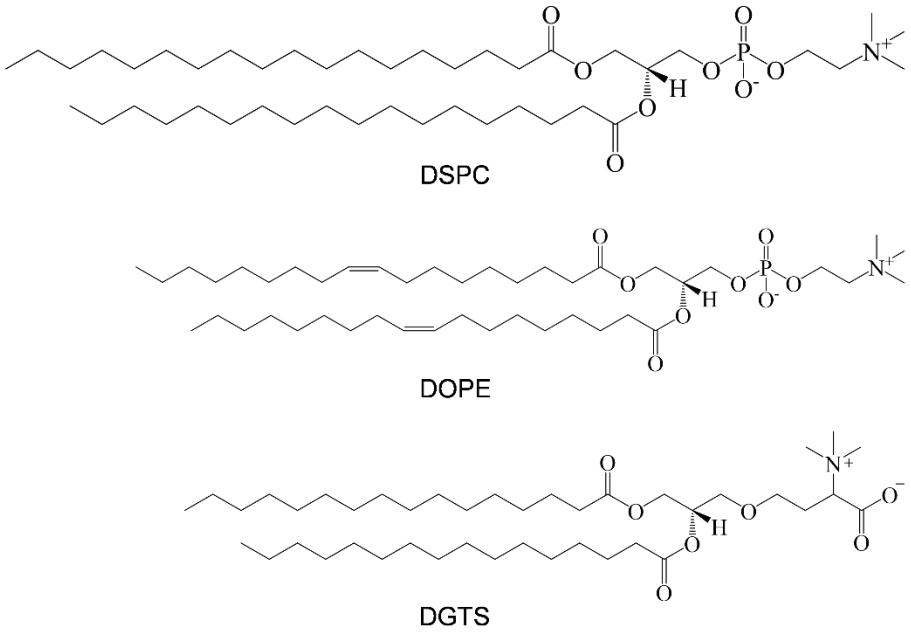


Figure 7. Structure of common phospholipids.

3.3. Steroid

Cholesterol or related steroid molecules play several crucial roles in the formation of LNPs. While cholesterol primarily modulates membrane fluidity and permeability to enhance lipid packing, it also plays a critical role in intracellular RNA delivery [111,112]. Several research groups have demonstrated that cholesterol enhances endosomal escape by influencing LNP morphology, prolonging the retention time in the endolysosomal pathway, and improving stability during phase transitions [111,113].

Multiple characteristics of cholesterol analogues impact their ability to facilitate endosomal escape. For example, phytosterols, C-24 alkyl derivatives of cholesterol, lack membrane-ordering ability (Figure 8). This is hypothesized to increase structural imperfections in nanoparticles composed of these sterols, due to their stereochemistry. These defects may contribute to variations in morphology and enhance fusogenic properties [112]. As part of our dietary intake, phytosterols may have a longer retention time in the endolysosomal pathway before undergoing further degradation or recycling process [112]. This is believed to be an advantage, since it provides more opportunities for membrane disruption and escape.

Accordingly, substituting cholesterol with sterol derivatives may increase the frequency of successful endosomal escape by several-fold. Researchers have employed visualization techniques to monitor endosomal escape and found that replacing cholesterol with certain C-24 sterol derivatives such as beta-sitosterol, fucosterol, campesterol, or stigmasterol notably alters the likelihood of successful RNA escape [112,114]. Thus, LNPs incorporating beta-sitosterol are the most promising candidate for facilitating endosomal escape. The varying morphological features and retention times of different LNPs have been confirmed [74].

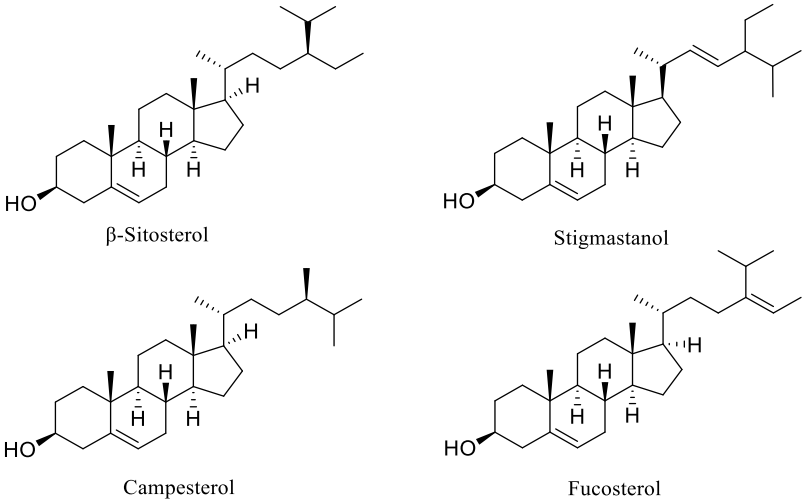


Figure 8. Structure of common steroids.

It has also been proposed that cholesterol and its derivatives contribute to the stabilization of ionizable lipid molecules that adopt a hexagonal phase during electrostatic interactions [115]. The organizing function of cholesterol molecules promotes the formation of a specific ordered structure in LNPs, where helper lipids including specific phospholipid and cholesterol, co-locate and orient toward interstitial, and ionizable lipids populate the interaxial directions. At specific ratios of the four constituent lipid molecules, this specific morphological distribution favors the conversion from the lamellar phase to the hexagonal phase, which effectively promotes endosomal escape effectively [115]. However, the transition becomes less favorable at higher proportions of cholesterol molecules, imposing additional constraints on the rational design of LNPs [115]. Moreover, the detailed structure-activity relationship between sterol molecules and the intracellular delivery of LNPs requires further research.

3.4. Inorganic ions

Integrating inorganic ions such as calcium, manganese, magnesium, carbonate, and phosphate ions into nanocarriers may enhance the potential for endosomal escape even after lysosome formation [80,116]. The incorporation of inorganic ions such as calcium and manganese ions into LNPs has been shown to enhance the endosomal escape efficiency of mRNA [117]. Mechanistically, both calcium and manganese ions facilitate escape by elevating the osmotic pressure, yet the underlying processes exhibit distinct characteristics. In the case of calcium-incorporated LNP, surface disruption triggers the release of calcium ions from LNPs from the liposomal core, as evidenced by an observed increase in zeta potential. As the endocytosed calcium ions exit endosomal compartments via calcium channels, they facilitate the influx of protons, accompanied by an influx of chloride ions and water driven by the electrochemical potential [80,118]. In contrast, the addition of manganese to LNPs enhances the lysosomal escape of mRNA by increasing the pH buffering capacity of the nanoparticles, forcing continued proton influx along with chloride entry [117]. This induces high osmotic pressure and swelling or even rupture of the endosome membrane [119]. Based on the understanding of membrane biophysics, other inorganic ions may also serve as lysosomal escape enhancers. Future research will likely focus more on how to rationally integrate these inorganic ions into the optimized design of LNPs.

3.5. Other potential compositions

Beyond the scope of conventional LNPs and their constituents, other molecules or vectors naturally possess the ability to disrupt endosomal membrane structures. Research on innovative hybrid systems combining LNP with viral vectors or other naturally occurring materials represents a highly promising approach to enhance the endosomal escape efficiency of non-viral delivery platforms [88].

Virus-inspired nanoparticles offer advantages such as high affinity toward phosphate-rich cell membranes and the ability to destabilize endosomes [120]. The inclusion of zinc coordinative ligands (zinc-dipicolylamine, Zn-DPA), which mimic the glycoprotein spikes of viruses, on the surface of nanoparticles effectively facilitates cell binding and endosomal escape, although in existing studies the ligands are typically incorporated into polymeric or cationic nanoparticles rather than LNPs [120]. Strong interactions between the spike-like zinc ligand and the phosphate groups of membrane lipids induce favorable membrane-disruptive events [120]. Another strategy involves incorporating DNA aptamer-based lysosome-targeting chimeras into LNPs. Consisting of a DNA tetrahedral scaffold and two aptamers specifically targeting proteins of interest and mannose 6-phosphate receptor, the chimera can be conjugated onto LNPs via click chemistry [121]. The resulting LNPs can mimic the spike protein-host receptor interaction-mediated viral fusion mechanism to achieve efficient intracellular nucleic acid delivery.

The introduction of polyamine polymers, including polyethylenimine, polyamidoamine, poly(β-amino esters), and poly(2-dimethylaminoethyl methacrylate), into nanoparticles enables proton buffering and uptake at endosomal pH levels, causing osmotic swelling and rupture of the endosomal membrane [122]. This represents a promising strategy to enhance the endosomal escape capability of LNPs. Polyamidoamine dendrimers exhibit favorable biocompatibility and biodegradability [123]. Incorporating a polyamine polymer derived from polyamidoamine dendrimers, which contain exclusively tertiary amines, into LNPs can enhance the endosomal escape efficiency from 20% to 80% [124]. It should be noted that polyamine polymers typically contain a mixture of primary, secondary, and tertiary amines, which may engage in nonspecific interactions with biomolecules and membranes, thereby potentially inducing toxicity [124]. Toxicity mitigation poses a critical challenge for their implementation in LNPs.

Some other naturally occurring materials classified as endosomolytic agents may also assist in endosomal escape. However, their cytotoxicity restricts their direct application *in vivo* and necessitates further modifications to improve their utility. Melittin, a naturally occurring antimicrobial peptide, is one such molecule. Derived from bee sting venom, this 26-amino acid cationic peptide exhibits membrane-lytic activity and can be conjugated to nanoparticles [125]. It is believed that the positively charged segment, a Lys-Arg-Lys-Arg sequence near the C-terminus of melittin, is able to perturb membrane structure and boost endosomal escape [126]. Several derivatives of melittin, such as maleic anhydride derivatives, have been synthesized offering improved intracellular delivery, reduced cytotoxicity, and modulatable charge-carrying properties [83,126].

Modification with complementary coiled-coil lipopeptides has been demonstrated to enhance the mRNA delivery efficiency of LNPs through fusion-mediated delivery [127]. One successful example is the heterodimeric coiled-coil pair CPE4 and CPK4, which feature PEG-based spacers and cholesterol anchors [128]. During the delivery process, CPE4 and CPK4 perform different tasks: CPK4 anchors to the cell membrane and promotes the formation of cellular protrusions by spontaneous membrane insertion, while CPE4 acts as a complementary linker to enable the bringing together of opposing LNP.

The interaction between the lipopeptides brings LNPs into close proximity with the cell membrane, ultimately leading to fusion [127]. This complementary helical-helical lipopeptide system substantially enhances mRNA delivery by LNPs to difficult-to-transfect induced pluripotent stem-cell-derived cardiomyocytes [129]. However, the requirement for pre-modifying target cells with CPK4, combined with a heavy reliance on specific CPK4-CPE4 recognition, may pose challenges for the further translation of this system. Furthermore, the amphipathic α-helical structure of such lipopeptides possesses intrinsic membrane activity. After LNPs are internalized into acidic endosomes, these helical structures may undergo conformational changes or directly interact with the endosomal membrane, thereby disrupting its stability to facilitate the cytosolic release of mRNA [127].

4. Conclusion and perspective

Up until now, the endolysosomal pathway remains a major barrier to effective LNP-based RNA drug delivery [66,82]. In this review, we have thoroughly discussed several current theories and hypotheses about the mechanism of endosomal escape, which enables RNA to reach the cytosol and function properly. Based on the proposed mechanisms, researchers have pursued diverse strategies to advance the rational design of LNPs. These efforts focus on elucidating structure-property relationship of various components, incorporating both natural and synthetic alternative ingredients, and integrating these insights to develop functional RNA drug carriers [130,131]. Considering the broader context of RNA therapeutics for disease prevention and treatment, further enhancing the endosomal escape capability of LNP carriers remains a crucial objective in pharmaceutical science. After weaving through current academic achievements, several methodologies and approaches seem particularly intriguing in further advancing the construction of next-generation LNP vehicles.

Firstly, utilizing naturally derived materials in more tailored and appropriate ways in nanocarriers, or drawing design principles and inspiration from naturally occurring structures may promote progress in LNP rational design. As illustrated in the preceding comparison of phospholipids such as DOPE and DSPC, their functional effects on LNPs can be attributed to their inherent biological roles within the human body [67]. In addition, incorporating virus-mimicking structures and naturally occurring membrane-disruptive molecules may offer promising avenues for nanoparticle innovations [120,126].

Furthermore, introducing artificial intelligence methods into medical science is another emerging field. High throughput screening conducted through machine learning algorithms, especially artificial neural networks, holds the potential for yielding valuable information for LNP design at the molecular level. Optimal lipid compositions and ratios can be accurately identified or predicted through a much less time-consuming process, substantially accelerating advancement in this particular field [78].

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Authors' contribution

Conceptualization, Xing-Jie Liang; investigation, resources, data curation, writing—original draft preparation, Ziqing Yan; writing—review and editing, Guangchao Qing and Xing-Jie Liang; visualization, supervision, project administration, funding acquisition, Xing-Jie Liang and Guangchao Qing. All authors have read and agreed to the published version of the manuscript.

Conflicts of interests

The authors declare no conflict of interests.

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