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Endosomal escape and current obstacles in ionizable lipid nanoparticles mediated gene delivery: lessons from COVID-19 vaccines

Semmal Syed Meerasa [a], Aqeel Ahmad [b,*], Amer Ali Khan [b], Shafiul Haque [c,d], Imran Saleem [e,*]

[a] Department of Physiology, College of Medicine, Shaqra University, Shaqra, Saudi Arabia
[b] Department of Medical Biochemistry, College of Medicine, Shaqra University, Shaqra 11961, Saudi Arabia
[c] Department of Nursing, College of Nursing and Health Sciences, Jazan University, Jazan 82911, Saudi Arabia
[d] School of Medicine, Universidad Espiritu Santo, Samborondon 091952, Ecuador
[e] School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool L3 3AF, UK

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ABSTRACT

During last pandemic of COVID-19, two vaccines based on ionizable lipid nanoparticles (ILNP) were developed for COVID-19 prevention: Pfizer/BioNTech Vaccine (BNT162b2) and Moderna Vaccine (mRNA-1273). The observed efficacy of these two vaccine formulations catalyzed a global intensification of scientific inquiry into the therapeutic potential of these ionizable lipids, driving research efforts aimed at developing novel agents for a diverse range of pathologies. Successful ILNP-based delivery requires both selection of a suitable ionizable lipid and elucidation of its endosomal escape mechanism. This review focuses current knowledge on lipid diversity, emphasizing the structural and functional attributes of ionizable lipids essential for endosomal escape. A detailed analysis of COVID-19 vaccine lipid components, correlating their physicochemical properties with cellular and humoral immune responses, and exploring their implications for therapeutic innovation. Finally, we evaluate current challenges and future directions in ILNP-based therapy development.

1. Introduction

Significant advancements in nanoparticle (NP) technology have yielded a wide spectrum of delivery systems for genes and therapeutics. (Ahmad et al., 2019). Many peptide-based NPs have been created as a result of their various distinctive properties (Ahmad et al., 2021), including several pH sensitive endosomolytic peptides that demonstrated substantial toxicity at endosomal pH but low toxicity at physiological pH were discovered (Ahmad and Khan, 2022; Ahmad et al., 2015; Ahmad et al., 2021). Several toxic peptides have also been created to increase gene delivery, but their extreme toxicity prevented them from being widely used (Paray et al., 2021). To improve the endosomal escape of genes and drugs, lysosomotropic agents modifying NP, such as chloroquine, methylamine, and ammonium chloride, were created (Pei and Buyanova, 2018). Various polymer-based NP were also developed that improved endosomal escape and, as a result, improved gene delivery (Zhang et al., 2011a). Several of the polymers are naturally biocompatible and degradable (Samir et al., 2022).

Of the NPs mentioned above, lipid NPs were the most researched materials for DNA and RNA transport (Hou et al., 2021). Many varieties of lipid NP have been produced, such as cationic liposomes, which have numerous properties like as low toxicity, low immunogenicity, high condensation capacity, and cellular internalization. Nevertheless, limited stability, and poor endosomal escape are some of the

Abbreviations: ILNP, Ionizable lipid nanoparticles; NPs, nanoparticles; DOPE, dioleoylphosphatidylethanolamine; PEG, Poly(ethylene glycol); LNPs, lipid nanoparticles; WHO, World health organization; DODMA, 1,2-Dioleyloxy-3-dimethylaminopropane; DODAP, 1,2-dioleoyl-3-dimethylammonium-propane; NMR, Nuclear magnetic resonance; PEI, Polyethyleneimine; PLGA, Polylactic-co-glycolic acid; TTR, transthyretin; iPhos, ionizable phospholipids; PC, Phosphatidylcholine; PG, phosphatidylglycerol; PS, phosphatidylserine; PtdIns, phosphatidylinositol; PA, diacylglycerol, phosphatidic acid; PE, phosphatidylethanolamine; SAXS, synchrotron small-angle x-ray scattering; iLAND, thermostable ionizable lipid-like nanoparticle; hATTR, hereditary transthyretin amyloidosis; DSPC, Distearoylphosphatidylcholine; DLS, Dynamic light scattering.

* Corresponding authors.
E-mail addresses: semmalsyed@su.edu.sa (S.S. Meerasa), draqeel@su.edu.sa (A. Ahmad), ameralikhan@su.edu.sa (A.A. Khan), shhauqe@jazanu.edu.sa, shafiul.haque@hotmail.com (S. Haque), I.Saleem@ljmu.ac.uk (I. Saleem).

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characteristics that preclude their continuing usage (Zhang et al., 2011b). DOPE-containing lipid NPs with improved endosomal escape were further created (Zhang et al., 2011b). Although the PEG chain was added to this lipid NP to promote blood circulation, this alteration reduces endosomal escape and consequently gene expression (Zhang et al., 2011b).

Two COVID-19 vaccinations that were recently produced and authorized by the WHO for use in emergency situations to prevent COVID-19 were described in detail in later section (Verbeke et al., 2022). This result provides hope to other scientist to investigate further and create innovative medicines based on ionizable lipids for treating disorders. In the past, ILNP were created for commercial usage as Patisiran/Onpattro and were used to treat polyneuropathy (Urits et al., 2020). This review categorizes and discusses a range of ionizable lipids, classified based on their structural and functional attributes. We further examine commercially available ionizable lipid nanoparticle (ILNP)-based products employed in therapeutic applications. Finally, we provide a comprehensive analysis of the diverse endosomal escape mechanisms utilized by these ILNPs to facilitate cytosolic delivery.

2. Ionizable lipid types and their applications in RNA delivery

Ionizable lipids are crucial for RNA delivery, particularly in gene therapy and vaccine therapy. They can be classified functionally and structurally into five categories: unsaturated, multi-tailed, polymer-lipid, biodegradable, and branched-tailed ionizable lipids. They play a role in encapsulating RNA, ensuring stability, and being absorbed into cells while facilitating endosomal escape to bring about gene silencing or expression (Table 1) (Akinc et al., 2008; Dahlman et al., 2014; Dong et al., 2014; Fenton et al., 2017; Hajj et al., 2019; Han et al., 2020; Hassett et al., 2019; Karmacharya et al., 2022; Liu et al., 2021; Love et al., 2010; Maier et al., 2013; Meng and Grimm, 2021; Miao et al., 2020; Qiu et al., 2021; Sabnis et al., 2018; Sato et al., 2016; Sato et al., 2012; Semple et al., 2010; Suzuki and Ishihara, 2021; Whitehead et al., 2014; Xu et al., 2013; Yamamoto et al., 2016; Yonezawa et al., 2020; Zhang et al., 2020; Zhao and Huang, 2014). The following is a summary of their features and applications.

2.1. Unsaturated ionizable lipids

Unsaturated ionizable lipids contain one or more double bonds and are less stable but enhance membrane fusion and endosomal escape. When lipid unsaturation is increased, the phase structure transitions from bilayer to inverted hexagonal, facilitating RNA delivery (Fig. 1).

A number of unsaturated ionizable lipids such as DODMA, DLinDMA, and DLenDMA have shown strong gene silencing activities (Heyes et al., 2005). Tamura and Harashima searched for lipids producing such an endosomal escape and prepared pH-sensitive lipids such as YSK05 and YSK13-C3 to induce gene silencing in hepatocytes (Sato et al., 2012; Sato et al., 2021). Moreover, DLin-KC2-DMA and its variant DLin-KC3-DMA have been shown to result in potent liver gene silencing in vivo (Jayaraman et al., 2012; Liu and Huang, 2010; Semple et al., 2010). More recently, Δ9-linoleic acid-derived ionizable lipids, such as OF-02, have demonstrated efficient delivery of mRNA to the liver and liver targeting potential for therapeutics (Karmacharya et al., 2022). Also, A18-Iso5-2DC18, a heterocyclic ionizable lipid produced from alkylene-ketone that successfully delivered mRNA while also inducing interferon genes (STING) pathway (Miao et al., 2019).

Ge et al revealed that enhanced tail unsaturation in ionizable lipids increases mRNA encapsulation in LNPs. Moreover, it allows improved endosomal escape with higher transfection efficiency. Tail unsaturation also facilitates better in vivo delivery performance. Additionally, it offers modulation of LNP immunogenicity for safer therapeutic use (Ge et al., 2025). An unsaturated amino lipid, citronellol-derived (named 4A3-Cit), was very effective. It was discovered to be inducing mRNA expression in vivo many times higher than saturated lipids. The study concluded that unsaturation maximized the fusion of lipid nanoparticles with endosomal membranes for endosomal escape and effective mRNA delivery (Lee et al., 2021).

2.2. Biodegradable ionizable lipids

Biodegradable cationic and ionizable lipids are designed with cleavable linkages (e.g., ester, amide) that can be hydrolyzed by endogenous enzymes into nontoxic metabolites, such as fatty acids, glycerol, or natural precursors, ensuring safety and sustainability. In contrast, non-biodegradable lipids often contain stable linkages (e.g., ether, carbamate) that resist enzymatic breakdown, leading to persistence in tissues and potential toxicity (Jörgensen et al., 2023). Thus, biodegradability enhances both therapeutic safety and environmental compatibility. To decrease buildup of nanoparticles and enhance biocompatibility, there are biodegradable lipids with ester or disulfide linkages (Tanaka et al., 2020; Wang et al., 2014). For example, L319, which was MC3 derivative, had more efficient RNAi delivery and more effective clearance (Maier et al., 2013). Lipid 5, the ester-modified tail-containing, had five times higher expression of genes over MC3 along with clearance within 24–48 h (Sabnis et al., 2018).

SM-102 and ALC-0315, the main lipids in COVID-19 mRNA vaccines, are effective in delivery and degrade quickly in vivo (Hassett et al., 2019). Among other biodegradable lipids, 304O13 and OF-Deg-Lin have demonstrated stronger gene silencing in immune cells, suggesting their potential for targeted immunotherapy (Fenton et al., 2017; Whitehead et al., 2014). In addition, bioreducible ionizable lipids like 306-O12B have significantly accelerated genome-editing applications through CRISPR-Cas9-mediated gene silencing (Qiu et al., 2021).

2.3. Ionizable polymer-lipids

Hybrid polymer–lipid nanoparticles combine the best of lipid- and polymer-based systems to promote RNA delivery. They can form stable nanoparticles and are generally effective at transfecting endothelial cells. Polyethyleneimine (PEI)-derived ionizable lipids (ILs), including 7C1, are commonly used for this purpose (Dahlman et al., 2014).

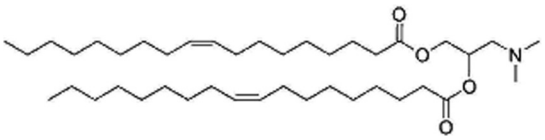
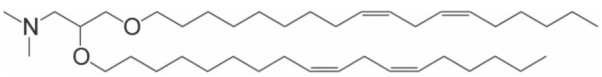
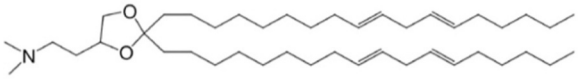
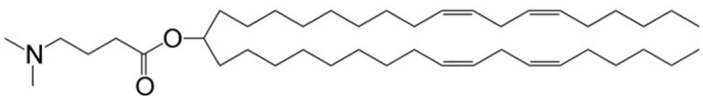
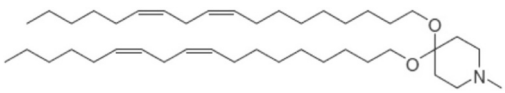
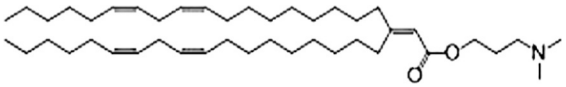
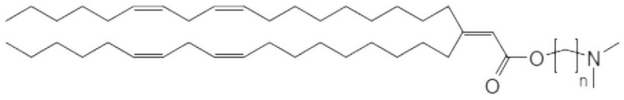
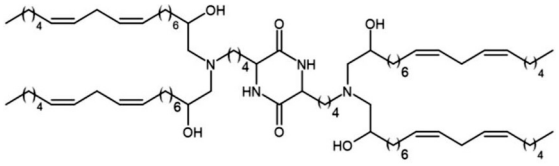
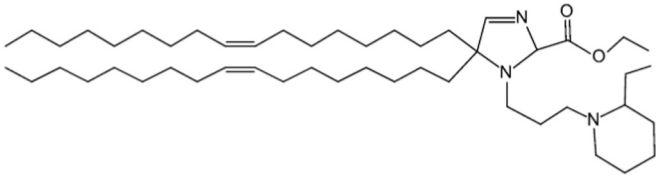
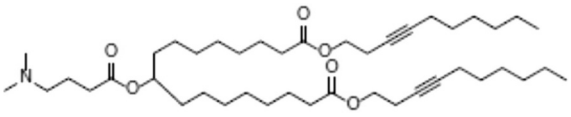
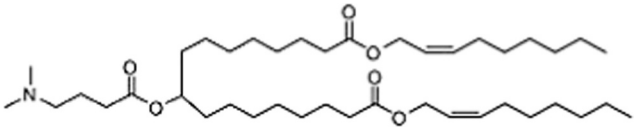
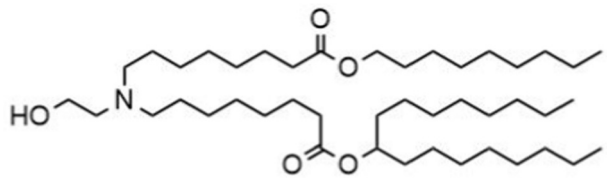
The term “dendrimer” is a modification of the Greek term dendron (tree), which indicates their tree-like structure. Buhleir and co-workers were the first to synthesize cascade-like molecules in 1978, which served as the starting point for dendritic polymers. Donald A. Tomalia and colleagues at Dow Laboratories further developed this field between 1979 and 1985 by preparing well-defined, branched macromolecules, which were termed dendrimers by Tomalia (Wang et al., 2022). Similarly, dendrimer ionizable lipids (e.g., PG1.C12 and PG1.C15) have delivered potent gene silencing in liver endothelial cells and hepatocytes (Khan et al., 2014). Another potential approach is conjugating dendrimers with PLGA-PEG to promote siRNA encapsulation and in vivo gene knockdown efficacy. The hybrid system with enhanced biocompatibility and higher transfection efficiency can thereby serve as a proficient candidate for clinical application (Wang et al., 2014).

2.4. Branched-tail ionizable lipids

With better endosomal escape and higher ionizable potential, branched-tailed ionizable lipids are better than linear analogs in RNA transfection. Research has shown that lipids 306Oi10 outperform linear analogs for mRNA delivery (Hajj et al., 2019). Functionalized branched-tail derivatives (FTT) have also displayed remarkable efficiency upon application in hemophilia A models, improving hFVIII protein expression and allowing for effective base editing (Zhang et al., 2020).

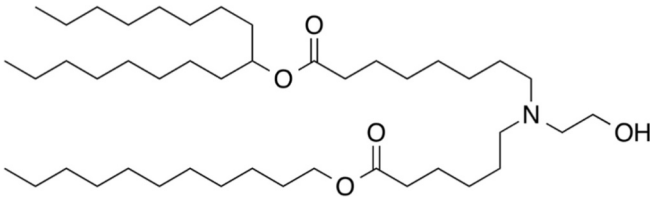
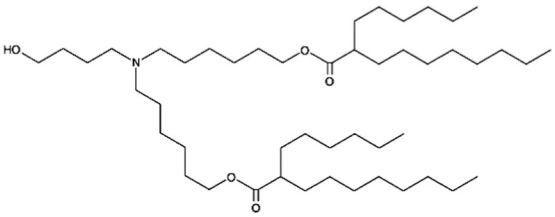
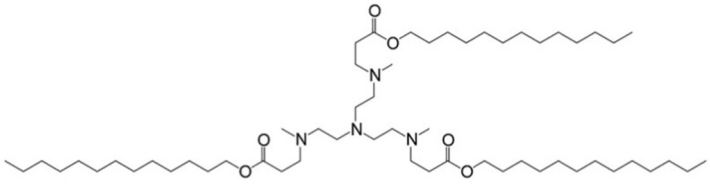
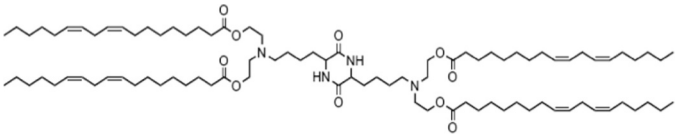
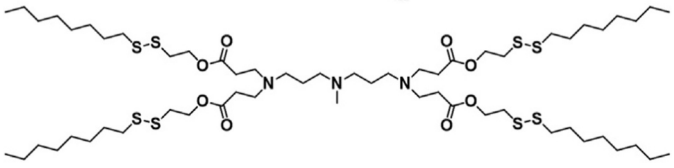
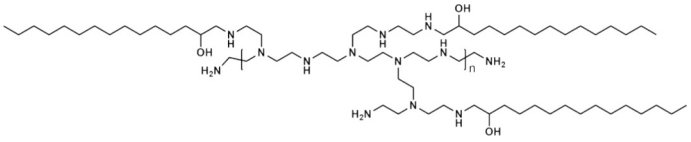
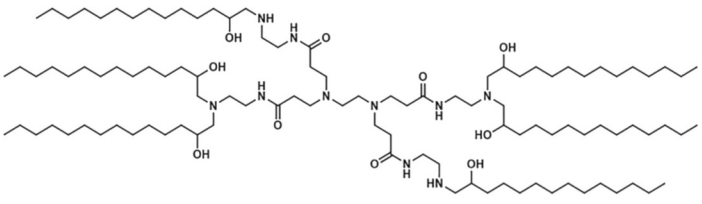
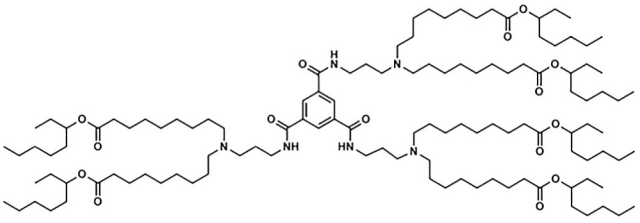
There are many recent articles based on branched-tail ionizable lipids despite these advances. When tested in vivo, Hashiba et al found that branched ionizable lipids had high stability and effectiveness and caused considerable genome editing in mice (Hashiba et al., 2022). Further studies are needed to improve their design, physiochemical

Table 1
Selected examples of different classes of ionizable lipids.

Ionizable lipid	Structure	References
	Unsaturated Ionizable lipid	
DODAP		(Yonezawa et al., 2020)
DLin-DMA		(Yonezawa et al., 2020)
DLin-KC2-DMA		(Carrasco et al., 2021)
DLin-MC3-DMA		(Carrasco et al., 2021)
YSK05		(Sato et al., 2012)
YSK13-C3		(Sato et al., 2021)
YSK13		(Sato et al., 2016)
OF-02		(Karmacharya et al., 2022)
A18-Iso5-2DC18		(Han a et al., 2020)
A6		(Miao et al., 2020)
	Biodegradable ionizable lipids	
L319		(Maier et al., 2013)
Lipid 5		(Sabnis et al., 2018)

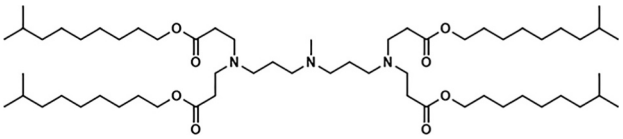
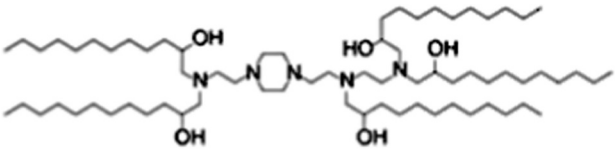
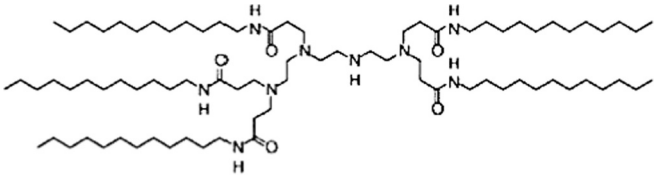
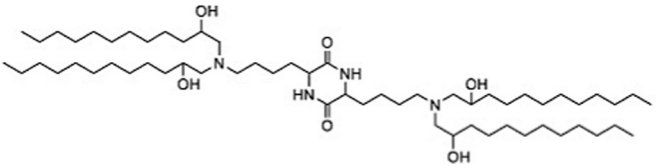
(*continued on next page*)

Table 1 (*continued*)

Ionizable lipid	Structure	References
SM-102		(Suzuki and Ishihara, 2021)
ALC-0315		(Suzuki and Ishihara, 2021)
$304O_{13}$		(Whitehead et al., 2014)
OF-Deg-Lin		(Fenton et al., 2017)
306-O12B		(Qiu et al., 2021)
Ionizable polymer-lipids		
7C1		(Dahlman et al., 2014)
G0-C14		(Xu et al., 2013)
Branched-Tail Ionizable Lipid		
FTT5		(Zhang et al., 2020)

(*continued on next page*)

Table 1 (*continued*)

Ionizable lipid	Structure	References
306Oi10		(Hajj et al., 2019)
Multi-tail ionizable lipids		
C12-200		(Love et al., 2010)
$98N_{12}$-5		(Akinc et al., 2008)
cKK-E12		(Dong et al., 2014)

properties, and gene delivery capabilities.

2.5. Multi-tail ionizable lipids

Lipids having many tails are known as multi-tail ionizable lipids. Many distinct forms of multi-tail ionizable lipids have been created, each with its own set of unique qualities such as high endosomal escape, allowing them to be widely used in gene delivery systems. Akinc et al. (2008) created a variety of ionizable lipids, including 98 N-12–5, which contained five tails and was effectively employed for in vivo hepatic gene silencing in the liver (Akinc et al., 2009; Akinc et al., 2008). The same group created a class of ionizable lipids generated from epoxide, C12-200, that are often non-biodegradable in nature (Love et al., 2010). The research demonstrated that a single intravenous injection of C12-200 having a concentration of 0.2 mg/kg per siRNA resulted in more than 65 % silencing of numerous genes in the liver of mice in vivo, with no apparent adverse effects (Love et al., 2010). Furthermore, a C12-200 modified formulation was utilized to silence a mutant transthyretin (TTR) gene, which might be employed to treat TTR amyloidosis (Sekijima et al., 2008). cKK-E12, an epoxide-derived multi-tail ionizable lipid composed of four amino alcohol-based lipid tails and dilysine-derived diketopiperazine core, was also produced (Dong et al., 2014). The DLS experiment and Cryo-TEM revealed that the cKK-E12 LNP was 70 nm in size and 35–85 nm in diameter (Dong et al., 2014). The data depicted that Pten was mostly silenced by cKK-E12 LNP in hepatocytes but not appreciably in endothelial cells or leukocytes in mouse liver tissue (Dong et al., 2014).

Several multi-tail ionizable phospholipids (iPhos) that can transport mRNA and single-guide RNA in vivo have been created by Siegwart and his team. Following that, they created an improved iPhos with one pH-sensitive zwitterionic component and three hydrophobic tails. To modify the gene, two key lipids, 9A1P9-5A2-SC8 and 9A1P9-DDAB, were utilized to transport mRNA and sgRNA (Liu et al., 2021). In the acidic environment of the endosome, these lipids create a perfect cone shape structure and change the membrane into a hexagonal form, disrupting the endosomal membrane and releasing the cargo into the cytoplasm (Meng and Grimm, 2021).

3. Structure of lipids and their role in endosomal escape

Based on their shapes, lipids are categorized into three groups: conical lipids, cylindrical shape lipids, and inverted conical lipids (Corin and Bowie, 2020; Janmey and Kinnunen, 2006; Vial et al., 2021; Zhukovsky et al., 2019) (Fig. 1). The concentration of small and big head-group kinds of lipids, as well as the amount of saturated and unsaturated lipids composition, have a substantial impact on lipid packing (Alberts et al., 2002; de Kroon et al., 2013; Jacquemyn et al., 2017). Lipids exist in two phases: solid phase (also known as gel phase) and liquid phase (Jouhet, 2013; Koynova et al., 2009; M'Baye et al., 2008).

In this section, we will look into how the varied lipid structures affect transfection effectiveness. Data from the literature revealed that cationic lipid nanoparticles increased gene expression (Safinya, 2001). The findings demonstrated that cationic lipid NPs with DOPC as a helper lipid developed a multilamellar structure (Safinya, 2001). Koltover et al investigations demonstrated that replacing DOPC with DOPE causes a shift from the lamellar to inverted hexagonal phase, as detected by synchrotron small-angle x-ray scattering (SAXS) and optical microscopy (Koltover et al., 1998). DOPE destabilizes the development of lipid bi-layers, according to the findings (Litzinger and Huang, 1992). Huang and his colleagues discovered that cationic lipid NPs containing DOPE had strong transfection activity in A431 human cells (Farhood et al., 1995). Another study found that lipid NPs containing DOPE escape from endosomes by disrupting the endosomal membrane, resulting in considerable gene expression in L929 cells (Litzinger and Huang, 1992; Zhou and Huang, 1994). DOPC-containing lipid NPs, on the other hand, displayed low gene expression as compared to DOPE-containing lipid NPs (Litzinger and Huang, 1992). DOPE containing lipid NPs promotes fusogenic activity and inverted hexagonal lipid structures, whereas DOPC containing lipid NPs promotes bilayer structure. DOPE-containing lipid NPs demonstrated greater gene expression *in vitro* in 16HBE14o- cells and in vivo in mice than DOPC-containing lipid NPs (Du et al., 2014). After 6 or 22 h of incubation in 16HBE14o- cells, lipopolyplex

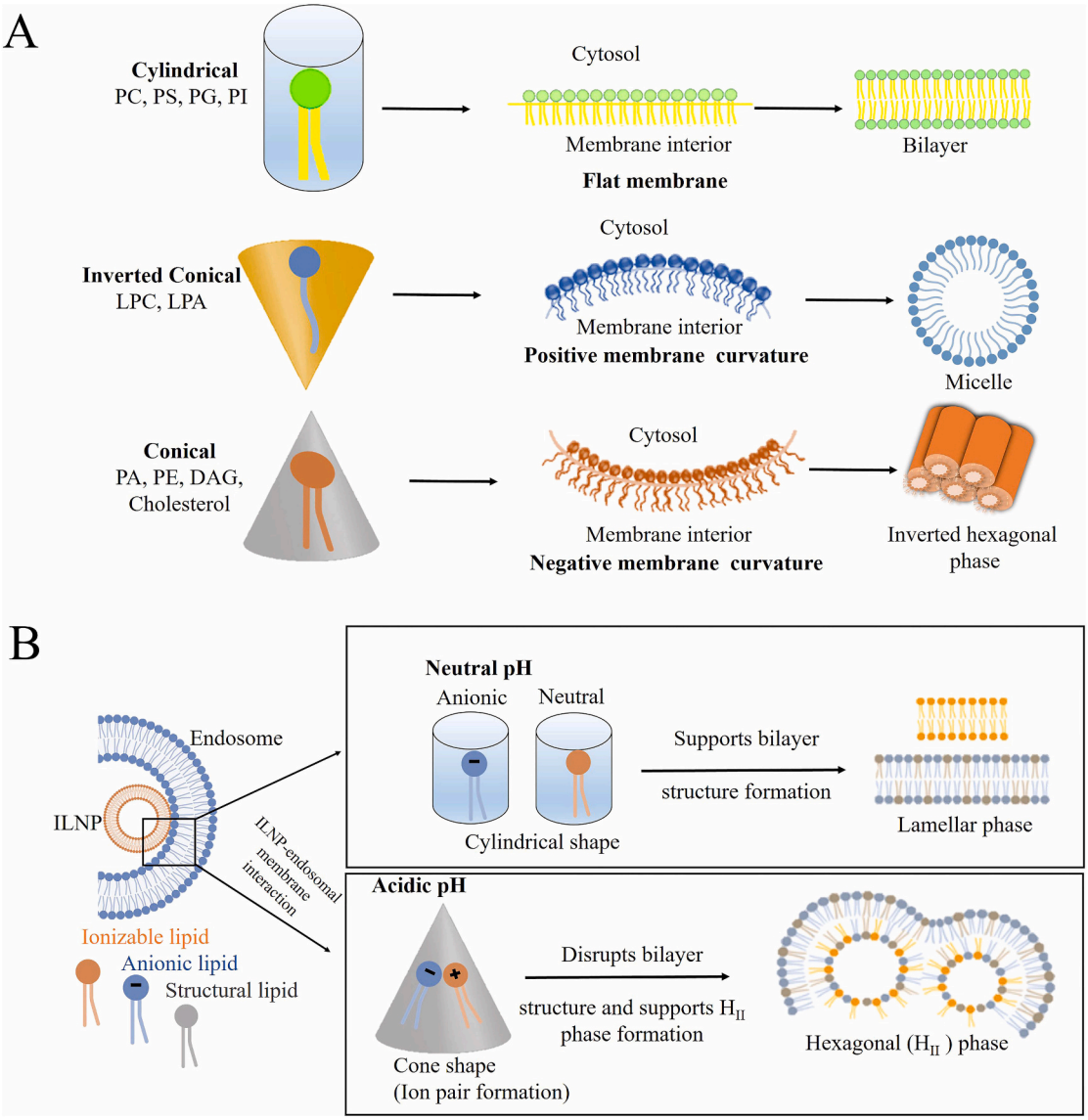


Fig. 1. Various forms of lipid structure and ionizable lipid characteristics. Panel A of the picture depicted cylindrical lipids such as PC, PG, PS, and PI, inverted cone lipids such as LPC and LPA, and cone lipids such as PA, DAG, PE, ionizable lipid and Cholesterol. Figure modified with permission from Zhukovsky et al 2019. (https://doi.org/10.1002/1873-3468.13563). Panel B demonstrated that at neutral or physiological pH, ionizable lipid favors the formation of bilayer structure, whereas at acidic pH, ionisable lipid becomes positively charged, interacting with anionic endosomal membrane and adopting cone shape structure, which favors the formation of hexagonal structure. Figure modified with permission from Schlich et al 2021 (https://doi.org/10.1002/btm2.10213). Note: Phosphatidylcholine (PC), Phosphatidylglycerol (PG), Phosphatidylserine (PS), Phosphatidylinositol (PI), Lysophosphatidylcholine (LPC), Lysophosphatidic acid (LPA), Phosphatidic acid (PA), Diacylglycerol (DAG), Phosphatidylethanolamine (PE).

formulation including DOPE lipids rapidly escape from endosome and the majority of DNA is located in cytoplasm and nucleus (Zuhorn et al., 2005). According to Zuhorn et al., the transfection effectiveness of the cationic lipid SAINT-2 (N-methyl-4(dioleyl)-Methylpyridinium-chloride)/DOPE and SAINT-2/DPPE lipoplexes in COS-7 cells was 70 % and 25 %, respectively (Mochizuki et al., 2013). According to the SAXS tests, SAINT-2/DPPE exhibits a lamellar phase that transforms into a mixed lamellar-hexagonal phase when it interacts with an anionic vesicle (Smisterová et al., 2001; Zuhorn et al., 2005). Another study, conducted by Mochizuki et al, found that adding DOPE to cationic liposomes increased gene expression in HepG2 cells (Mochizuki et al., 2013). Maslov et al demonstrated that a DOPE-modified cationic liposome improved transfection effectiveness in HEK293 cells (Maslov et al., 2012). Furthermore, the DOPE-modified cationic liposome demonstrated substantial gene silencing in BHK IR-780 cells (Maslov et al., 2012). Some in vivo tests were also performed on animals to determine the efficiency of transfection. DOPE-containing cationic liposomes, for example, demonstrated considerable gene expression in the lung, kidney, lymph node, heart, and liver of mice (Zhu et al., 1993). Additionally, mice's liver, spleen, and lung exhibited effective gene expression when DOPE-containing cationic liposomes were used (Thierry et al., 1995). Papahadjopoulos and his team conducted a significant investigation to examine the impact of cationic liposomes containing cholesterol and DOPE on their ability to carry genes both in vivo and *in vitro* (Hong et al., 1997). In comparison to cationic liposomes containing cholesterol, SKBR-3 (human breast cancer cells) cells expressed more genes when DOPE was added to them. Intriguingly, cationic liposomes containing DOPE revealed less gene expression in mice than those carrying cholesterol (Hong et al., 1997).

Lipids may interact with the anionic membrane of endosomes and have the capacity to flee from endosomes following acidification, Kim et al. have synthesized several novel ionizable molecules with pKa

values between 5.5 and 6.75 (Kim et al., 2021). Buschmann and his team carried out a thorough investigation of well-studied ionizable lipids including DLin-KC2-DMA (KC2), DLin-MC3-DMA (MC3), DLin-DMA (DLin), DODMA (DMA), and DODAP (DAP), and examined their physicochemical characteristics and transfection *in vitro* and in vivo (Carrasco et al., 2021).

In aqueous solution, the ionizable lipid exhibited pH-dependent behavior (Kulkarni et al., 2018; Zhang et al., 2022). The ionizable lipid has a positive charge at physiological pH but a neutral charge at acidic pH. In order for the siRNA to form a compound with negatively charged RNA or DNA, ionizable lipids are mixed with it at an acidic pH. Negatively charged endosomal membranes are electrostatically interacted with and destabilized by this positive charge ionizable lipid (Fig. 2). Due to the aforementioned characteristics, lipid formulations including ionizable lipids successfully release their payload into the cytosol and escape from the endosome (Zhang et al., 2022).

Numerous research have been done that provide clear explanations of the endosomal escape process (Suzuki and Ishihara, 2021). A thermostable, ionizable, lipid-like nanoparticle (iLAND) was created by Huang and his colleagues, and their endosomal escape mechanism was investigated (Hu et al., 2022) (Fig. 3). The most fluorescence was seen after three hours, suggesting that they were present in endosomes, and after that it started to decrease, indicating that ILNP had escaped from endosomes and was now in the cytosol (Fig. 3C) (Hu et al., 2022).

As we know, bafilomycin A decreased the gene silencing activity whereas the addition of chloroquine increased it (Heath et al., 2019; Wang et al., 2010). According to Pearson's correlations in cells, which were increased and decreased in the presence of chloroquine and bafilomycin A, respectively, in comparison to untreated cells, the Fig. 3D and E showed that endosomal escape of the cargo is increased by the addition of chloroquine while it is reduced in the presence of bafilomycin A. Fig. 3F demonstrates that these two chemicals had no impact on cellular internalization. Endosomal rupture was the primary mechanism responsible for this ILNP's endosomal escape. At physiological pH, the ionizable lipids, as seen in Fig. 3G, were neutral with columnar shapes that changed to hexagonal or conical shapes when the pH was decreased because the lipids' protonated amine groups make them cationic at this low pH. Because bafilomycin A reduces the cargo's endosomal escape, these findings further showed that the proton sponge mechanism is implicated in this endosomal escape mechanism (Fig. 3D and F) (Hu et al., 2022).

Physicochemical characteristics of lipid nanoparticles (LNP), i.e., lipid saturation and phase transition behavior, significantly impact their interaction with and regulation of the immune system. These factors

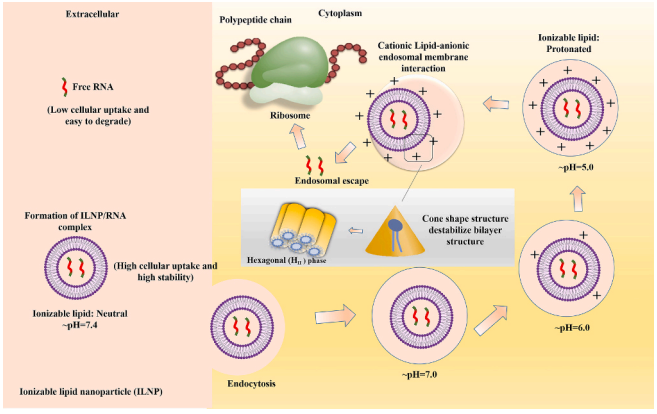


Fig. 2. The general endosomal escape mechanism of ILNP's endosomal escape. Ionizable lipids are neutral at physiological pH and positively charged at acidic pH. Naked RNA has limited permeability and is easily degraded by nucleases. Ionizable lipids create hexagonal structures at endosomal pH, breach the endosomal membrane, and discharge RNA into the cytoplasm.

control the in-vivo stability of LNP, biodistribution, cell uptake, and endosomal escape, which directly impact cellular as well as humoral immunity (Catenacci et al., 2024). Several studies, for example, found that proliferation of T cells is very dependent on LNP composition, and there are encouraging LNP-mRNA vaccine formulations. LNPs are most effective at activating T cells when their membrane Tm is set to near physiological or experimental temperature (37 °C) (Fedosejevs et al., 2025).

4. Clinical implications of ionizable lipid based nanocarriers

For the treatment of various disorders, a number of ionizable lipid-based particles were created; some are now undergoing clinical trials, while others are already available on the market (Table 2) (Adams et al., 2018; Baden et al., 2021; Buschmann et al., 2021; De Alwis et al., 2021; Gillmore et al., 2021; Kalnin et al., 2021; McKay et al., 2020; Polack et al., 2020; Rauch et al., 2021). We are aware that a mutation in the transthyretin (TTR) gene is the primary cause of hereditary transthyretin amyloidosis, a kind of autosomal dominant fartal illness (Adams et al., 2018). Patisiran, often referred to as Onpattro, is used to treat people with polyneuropathy brought on by hATTR amyloidosis in hepatocytes (Hoy, 2018) (Fig. 4). In vivo gene-editing treatment with this formulation (NTLA-2001) results in an 87 % decrease in blood TTR in individuals with hATTR and just moderate adverse effects (ClinicalTrials.gov number, NCT04601051) (Gillmore et al., 2021).

In conjunction with the University of Pennsylvania, Chulalongkorn University created the Ionizable lipid-containing vaccine ChulaCov19 for COVID-19, which entered the phase-1 and 2 clinical study (ClinicalTrials.gov Identifier: NCT04566276). It was tolerable, safe, and elicited dose-dependent immune responses with the greatest immunogenicity being observed in the 50 μg dose. Though stable for (low- and middle-income countries) LMIC distribution, its lack of prefusion stabilization of the spike and variant LNP preparation might have undermined breadth and durability of immunity. The ChulaCov19 vaccine did not quite fail in the strict sense of the word, being safe and immunogenic during clinical trials. It was not, though, widely implemented or licensed as a prominent COVID-19 vaccine due to numerous market- and situation-related factors (Puthanakit et al., 2024). Unfortunately, the vaccine has not yet received approval. Genevant ionizable lipid CL1 with nucleoside-modified mRNA is present in this formulation (Buschmann et al., 2021). The Arcturus Company has made additional attempts to produce the COVID-19 vaccine (LUNAR COVID-19), which entered phase 1 and phase 2 clinical trials but did not enter phase 3 clinical trials, and the data available at this time indicated that it is not approved for use (De Alwis et al., 2021). This formulation, which was administered intramuscularly (i.m.), comprises self-amplifying, full-length, unmodified mRNA together with the ionizable lipid Arcturus Lipid 2,2 (8,8) 4C CH3 (Buschmann et al., 2021). While, Preclinical and first-phase clinical trials of LUNAR vaccine produced robust and persistent immune responses. It is unknown but could be possible that subsequent trials could not achieve the very high efficacy seen in Pfizer-BioNTech and Moderna mRNA vaccines (De Alwis et al., 2021). The COVID-19 vaccine formulation LNP-nCoV saRNA, created by Imperial College, UK, entered Phase 1, but failed to enter Phase 2 as evidenced by the available data. It is constructed of Acuitas A9 ionizable lipid with self-amplifying spike mRNA (Buschmann et al., 2021). CureVac created the CVnCoV COVID-19 vaccine, which is made of ALC-0315, an ionizable lipid. This vaccine has entered Phase 1 but has yet to get clinical approval (Rauch et al., 2021). Rauch et al demonstrated that CVnCoV vaccination with a suboptimal dosage protects hamsters from SARS-CoV-2 (Rauch et al., 2021). At a dosage of 12 g, many volunteers experienced headache, weariness, myalgia, chills, and even fever in phase 1 clinical studies (Kremsner et al., 2021). Some volunteers reportedly experienced local soreness at the injection site (Kremsner et al., 2021). Another Ionizable lipid-based vaccine (MRT5500) was developed by Sanofi Pasteur translate Bio, which has C12-200 or an ICE

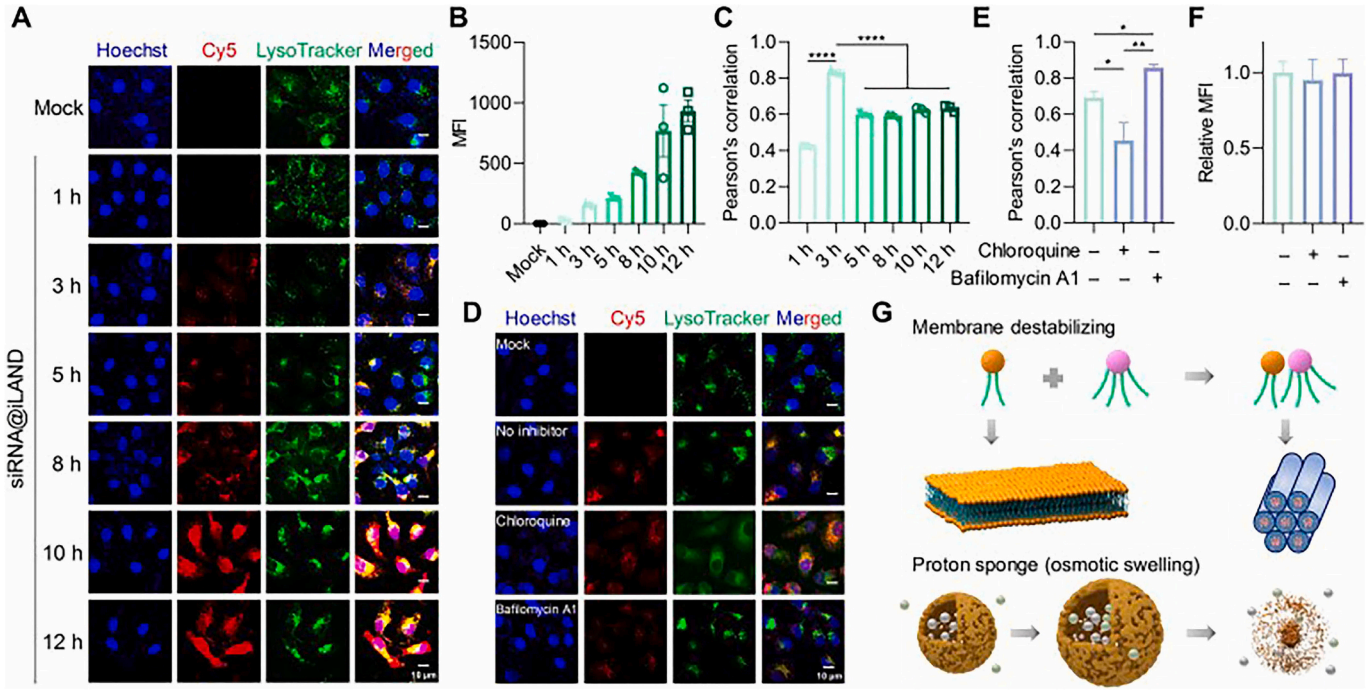


Fig. 3. Mechanism of siRNA-ILNP (siRNA@iLAND) cellular internalization and endosomal escape. The internalization of Cy5-siRNA-ILNP by HepG2 cells was demonstrated in panel A. Once siRNA-ILNP was transfected, the fluorescence intensity was displayed in panel B. Cy5-siRNA and endosome/lysosome co-localization research was displayed in panel C. In the presence of chloroquine and bafilomycin A1, panel D displayed ILNP's cellular uptake and endosomal escape. The co-localization analysis was shown in panel E, and the MFI of siRNA in the cells was shown in panel F. The endosomal escape mechanism was displayed in Panel G. Reprinted with permission from Hu et al, 2022 (https://doi.org/10.1126/sciadv.abm1418). Note: Ionizable lipid nanoparticles (ILNP), Mean fluorescence Intensity (MFI), Small interfering RNA (siRNA), Thermostable ionizable lipid-like nanoparticle (iLAND).

Table 2
Ionizable lipid-based drug/gene delivery system and their clinical implications.

Product Name	Ionizable lipid	Gene	Application	Route of administration	Clinical Trial (Phase)	Company	Ref. No.
Onpattro/ Patisiran	MC3	TTR siRNA	TTR knockdown	i.v.	Approved in 2018	Alnylam	(Urits et al., 2020)
NTLA −2001	LP01	Cas9 mRNA and TTR sgRNA	TTR knockdown	i.v.	NCT04601051	Intellia	(Gillmore et al., 2021)
ChulaCov19	CL1	nucleoside-modified mRNA	COVID-19 vaccine	i.m.	Not approved till now	Chulalong Korn University	(Buschmann et al., 2021)
LUNAR COVID-19	Lipid 2,2 (8,8) 4C CH_3	self-amplifying spike mRNA	COVID-19 vaccine	i.m.	Not approved till now	Arcturus	(Kremsner et al., 2021)
LNP-nCoV saRNA	Acuitas A9	self-amplifying spike mRNA	COVID-19 vaccine	i.m.	Not approved till now	Imperial College	(McKay et al., 2020)
CVnCoV	ALC-0315	unmodified mRNA	COVID-19 vaccine	i.m.	Not approved till now	CureVac	(Rauch et al., 2021)
MRT5500	C12-200 or from ICE- or cysteine based ionizable lipid families	unmodified mRNA	COVID-19 vaccine	i.m.	Not approved till now	Sanofi Pasteur	(Kalnin et al., 2021), Kremsner et al., 2021)
mRNA-1273	SM-102	Nucleoside modified spike mRNA	COVID-19 vaccine	i.m.	Approved	Moderna	(Baden et al., 2021)
BNT162b2	Acuitas ALC-0315	Nucleoside modified spike mRNA	COVID-19 vaccine	i.m.	Approved	BioNTech (Pfizer)	(Polack et al., 2020)

(imidazole cholesterol ester) or a cysteine-based Ionizable lipid family lipid component. This formulation elicited a substantial immunogenic response, yielding impressive neutralizing antibodies that may aid in the battle against COVID-19. The evidence available to date indicated that this vaccine entered phase 1/2 but was unable to obtain clinical approval (Kremsner et al., 2021). Recently, two ionizable lipid-based vaccines, Pfizer/BioNTech Vaccine (BNT162b2) and Moderna Vaccine (mRNA-1273), were authorized for the prevention of COVID-19, which are addressed in detail below(Baden et al., 2021; Corbett et al., 2020; Polack et al., 2020).

5. Lessons from COVID-19 vaccines

It is common knowledge that COVID-19 vaccines include essential ingredients like ionizable lipids. The COVID-19 vaccines (Moderna and Pfizer/BioNTech) are said to contain RNA as well as four other major components in their composition (Fig. 4). Lipids that are cationic or ionizable are one of their constituents. Moderna has the ionizable lipid SM-102, while Pfizer/BioNTech contains the ionizable lipid ALC-0315. The ionizable lipid facilitates the formation of RNA complexes, improves endosomal escape, and facilitates cellular absorption. PEG-lipids

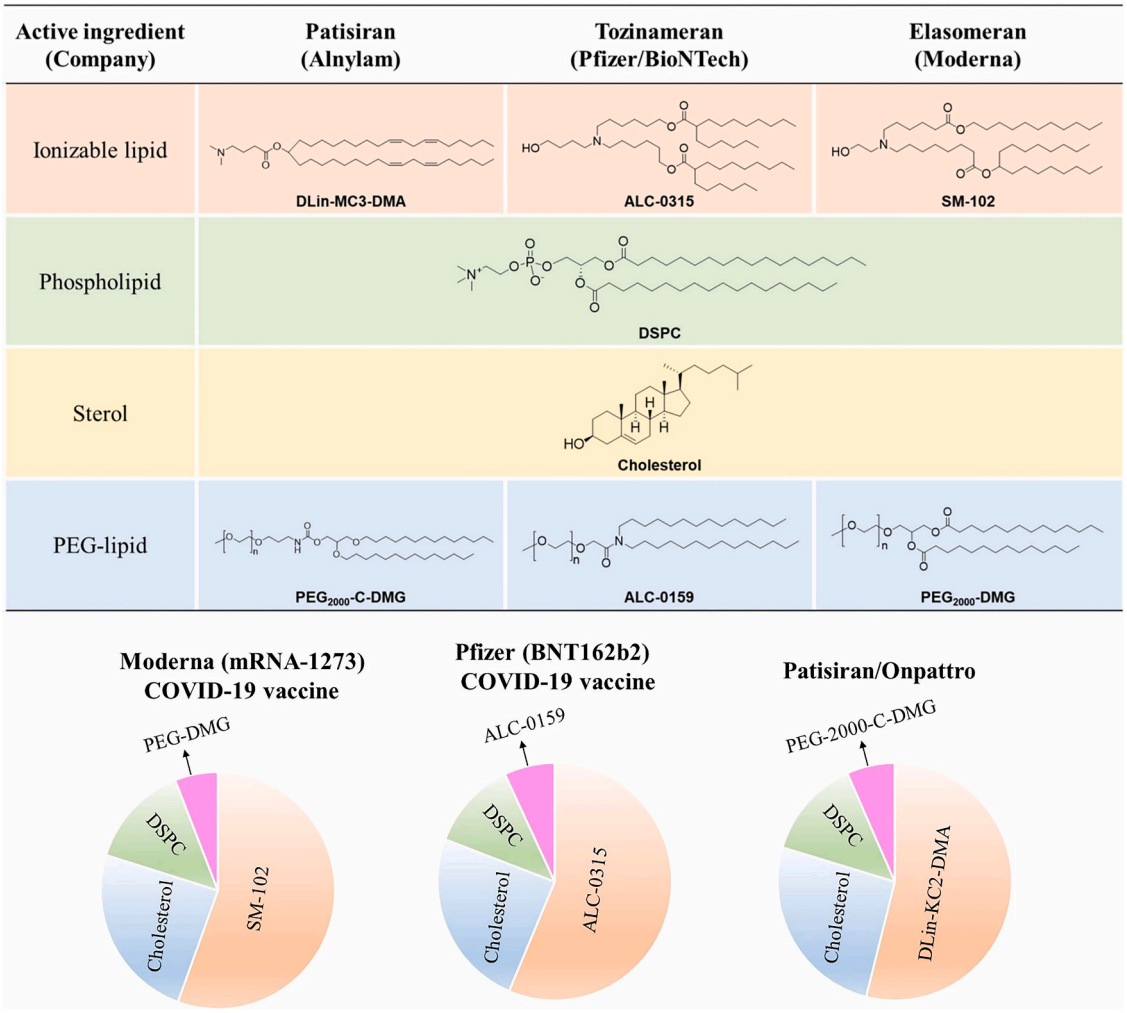


Fig. 4. Several ionizable lipids, helper lipids, and PEGs were employed in the formulation of COVID-19 vaccines and patisiran. Panel A showed the different types of lipids used in Patisiran and COVID-19 vaccines: Pfizer/BioNTech Vaccine (BNT162b2) and Moderna Vaccine (mRNA-1273) (Figure modified with permission from Suzuki and Ishihara, 2021, https://doi.org/10.1016/j.dmpk.2021.100424)). Panel B showed the molar ratio of different components used in Pfizer/BioNTech Vaccine, Moderna Vaccine and Patisiran formulations. Poly(ethylene glycol) (PEG).

are among the additional ingredients that have been discovered; Moderna has PEG-2000-DMG and Pfizer/BioNTech has ALC-0159. PEG-lipids assist in regulating the size and dispersion of NPs (Kauffman et al., 2015). PEG-lipids also stop the particles from aggregating, improving stability for storage (Leung et al., 2014). The PEGylation also prolongs blood circulation time and slows down the fast uptake of NPs by mononuclear phagocyte cells (Akita et al., 2015). The use of PEG-lipids with short acyl chains decreases immunogenicity while having no effect on gene silencing (Judge et al., 2006). The length, structure, and concentration of the PEG chain in the formulations are crucial characteristics that can impact DNA/RNA encapsulation, in vivo dispersal, and the effectiveness of NP transfection (Albertsen et al., 2022). Without PEG-lipids, LNP formulations are unstable, and particles are extremely polydisperse (Kulkarni et al., 2019). Dahlman and his colleagues' DLS studies revealed that LNP having little or no PEG-lipid had higher than 200 hydrodynamic diameters and were very unstable, demonstrating the necessity of PEG lipid in lipid-based drug delivery systems (Lokugamage et al., 2021). The same group reported that LNP containing 15 % PEG exhibited considerable luciferase expression, but LNP having greater concentrations of PEG exhibited decreased luciferase expression, showing the role of PEG in gene expression (Lokugamage et al., 2021).

Both Moderna and Pfizer/BioNTech COVID-19 vaccine formulations contain "helper" lipids such as cholesterol and phospholipids DSPC. By lengthening circulation half-lives and eliminating surface bound proteins, cholesterol maintains the NPs' integrity and stability (Semple et al., 1996). The cholesterol aids in the encapsulation of nucleic acid and minimizes the possibility of drug/gene leakage (Albertsen et al., 2022). According to the study, cholesterol has a single bilayer structure, and phospholipids offer stability to lipid nanoparticles while also increasing encapsulation and cellular transport of cargo (Kulkarni et al., 2017; Kulkarni et al., 2019). Phospholipids are amphipathic molecules that aid in the creation of bilayer structures, endosomal membrane fusion, and targeted specialized delivery. According to the existing data, saturated phospholipids, such as DSPC, are typically suited for short siRNA delivery, whereas unsaturated lipids, such as DOPE, are employed for prolonged mRNA delivery (Kauffman et al., 2015; Lokugamage et al., 2019). It should be noted that DSPC are present in both the Moderna and the Pfizer/BioNTech COVID-19 vaccines (Fang et al., 2022).

Hassett et al. discovered that ionizable lipid-containing LNP-mRNA with a pKa value of 6.6–6.9 elicited the most antibodies in mice when supplied through IM method (Hassett et al., 2019). As a result, one of the criteria for selecting ionizable lipids with pKa values in the aforementioned range is that they are ionizable. Moderna vaccine contains the ionizable lipid Lipid H' (SM-102) with a pKa value of 6.68. After intradermal injection of an ionizable lipid Lipid H carrying LNP-mRNA, mice produced substantial amounts of IgG antibodies (Hassett et al.,

2019) (Fig. 5). Furthermore, the lipid H demonstrated several physico-chemical features in Non-human Primates, including biodegradability, tolerability, gene expression, and immunogenicity (Albertsen et al., 2022). The pKa of the MC3 lipid utilized in Onpattro is around 6.35. The results revealed that MC3 modified LNP-mRNA had lower gene expression than lipid H modified LNP-mRNA (Hassett et al., 2019). In addition, antibody production in MC3 modified LNP-mRNA is three times lower than in H modified LNP-mRNA (Hassett et al., 2019). In addition, MC3 modified LNP had less mixed-cell inflammation and muscle fiber necrosis than lipid H modified LNP-mRNA (Hassett et al., 2019). It is worth noting that ILNP with a pKa of 6.2–6.6 is an excellent vector for delivering mRNA and siRNA through IV (Jayaraman et al., 2012) (3). The perfect pKa value of lipid is around 6.2–6.5 for LNP-mediated gene silencing in the liver, while the best pKa value for mRNA transport in the liver is around 6.2–6.8 (Hassett et al., 2019). The optimal pKa value for ILNP immunogenicity is between 6.6 and 6.8 (Hassett et al., 2019). Another COVID-19 vaccine with ionizable lipid (Pfizer-BioNTech COVID-19 vaccine) ALC-0315 has a pKa of around 6.09. (Zhang et al., 2022). According to the above facts and literature research, the optimum optimal pKa range for mRNA transport is around 6.0–6.8. The pH of the late endosome/lysosome is also about 6–4.5. When these ILNPs enter the endosome, the low pH causes protonation of their amine group, which changes the ILNP's overall neutral charge to cationic, permitting electrostatic contact with the negative charge of the endosomal membrane. The interaction of cationic ionizable lipid with negatively charged membrane results in the production of an ion pair that favors the formation of a conical shape, which induces the construction of a hexagonal shape structure, which allows the endosome to escape to the cytosol (Semple et al., 2010) (Fig. 1). The development of hexagonal structures promotes membrane bilayer breakdown via membrane fusion, resulting in endosomal cargo escape (Semple et al., 2010).

All three clinically authorized lipid formulations, patisiran, Pfizer, and moderna, have significant similarities and differences. As previously stated, they are mostly composed of four kinds of lipids. The ester bond in the lipid tail of both the ionizable lipids ALC-0315 and SM-102 makes them biodegradable (Suzuki and Ishihara, 2021). Because of this property, ALC-0315 and SM-102 lipid formulations cleared the circulation faster than LNP containing MC3 (Hassett et al., 2019). The pKa of the ionizable lipids found in these formulations ranges from 6 to 6.7. The ILNP prevents the RNA from being digested by nuclease enzymes. These ILNP also do not interact with serum proteins due to their neutral charge

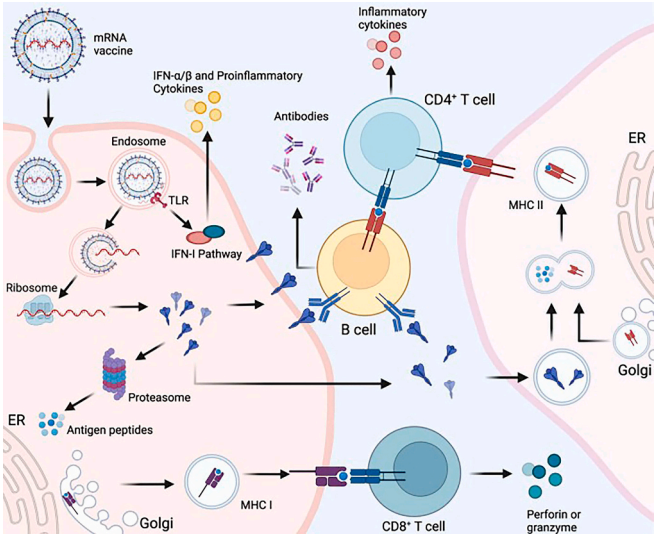


Fig. 5. Cellular, and humoral immune responses and self-adjuvant effects of mRNA-ILNP vaccines. Reprinted with permission from Fang et al.2022 (https://doi.org/10.1038/s41392-022-00950-y).

at physiological pH. The ILNPs enter cells via ApoE-dependent and/or ApoE-independent pathways, as demonstrated elsewhere. Following internalization into the endosome, ILNP causes upregulation or down-regulation of the targeted proteins via mRNA and siRNA, respectively (Suzuki and Ishihara, 2021) (Fig. 2). It should be mentioned that the makeup of all of these formulas varies. Sucrose is present in Pfizer and Moderna but not in Patisiran. Sucrose is known to work as a cryprotectant, preserving the physical characteristics of the vaccine during the freeze–thaw process (Ball et al., 2017). Stability is a critical issue with ILNP-based vaccinations or drug carriers. Patisiran (unopened vials) can be kept at 2–8C for 27 months, according to the data. This formation should not be frozen since it is made in PBS without sucrose and cannot withstand the freezing and thawing processes (Suzuki and Ishihara, 2021). The Pfizer vaccination may be stored at a temperature of −90 to −60 C for 6 months, whereas the Moderna vaccine requires a temperature of −25 to −15 C and can likewise be stored for 6 months (Suzuki and Ishihara, 2021).

How these COVID-19 vaccines (ILNP-mRNA) internalize into cells and combat infections are significant topics, and we shall answer them here based on a literature review. Some review articles released by the facts concerning the cellular and humoral immune responses caused by COVID-19 vaccines such as mRNA vaccines (Fang et al., 2022; Hou et al., 2021) (Fig. 5). This COVID-19 vaccine, which is an mRNA vaccine, enters cells via the endocytic route. The ILNP-mRNA COVID-19 vaccines exit from the endosome to the cytosol in the manner described above. After being employed as an endogenous antigen and translated into proteins by the ribosome in the cytoplasm, mRNA is then shredded by the proteasome and exposed to CD8 + cytotoxic T cells via the MHC class I (Verbeke et al., 2019). Antigenic peptides are delivered to CD8 + cells via MHC class I via attaching to T cell receptors (TCR). This causes CD8 + T lymphocytes to release perforin and granzyme, which destroy the infected cells. This sort of infected cell destruction is known as activate cell-mediated immune responses (Fig. 5). Furthermore, proteins produced into the extracellular environment are picked up by antigen presentation cells (APCs), which breakdown them into smaller antigenic peptides and deliver them to CD4 + T cells via MHC class II. This procedure both activates cellular immune responses through cytokine release and humoral immune responses by stimulating B cells to generate antibodies (Cagigi and Loré, 2021). Furthermore, ILNP-mRNA COVID-19 vaccinations have a self-adjuvant effect by activating antiviral innate immunity (Verbeke et al., 2019). The ILNP-mRNA interacts to the Toll-like receptor (TLR) in the endosome, causing type I interferon (IFN-I) and inflammatory cytokines to be produced (De Beuckelaer et al., 2017).

6. Current obstacles and challenges

Many other types of delivery systems have been exhaustively researched, but only a handful of them have made it from the lab to the clinic. This is due to a number of factors. Most polymer-based NPs, for example, are very poisonous and non-biodegradable. Peptide-based NPs are unstable and destroyed in the body by proteases. The majority of inorganic NPs are not biodegradable or biocompatible. One of the major challenges is the NPs' toxicity. Many NP shown limited cellular uptake, and their mechanism is unknown. Many NPs have strong cellular uptake but become caught in endosomes and destroyed by hydrolytic enzymes.

The ILNP has a number of benefits over conventional delivery systems. In general, ILNP have a biodegradable nature and are biocompatible. The neutral charge of the ILNP at low pH also contributes to their low toxicity. They have reduced immunogenicity as well. They guard the RNA against being destroyed by nucleases. In comparison to naked RNA, they also boost the cellular uptake of RNA. Due to the simplicity of this composition, several varieties of sizeable ILNP with various applications may be created.

The key issue with the alternative delivery technique is that the majority of the research were conducted mostly *in vitro* on cell lines.

Information about in vivo is inadequate since there is a dearth of available in vivo data. Because it is well recognized that in vivo data is more realistic to reality, in vivo investigations are crucial in addition to *in vitro* studies. The advantage of ILNP is that the majority of their research have been performed in vivo, which moves them closer to the clinic from the lab. More research is needed to create biodegradable ionizable lipids since some ionizable lipids are inherently non-biodegradable, which can lead to immunogenicity and long-term toxicity. It has also been demonstrated that the majority of ionizable lipid accumulate in the liver, making the transfer of ILNP to non-hepatic tissue challenging. To overcome the barrier of extrahepatic delivery, scientists have come up with different novel techniques. Modulating the composition of the lipid nanoparticle (LNP) is one such salient method (Song et al., 2024). Through changing the proportions of ionizable lipids, phospholipids, cholesterol, and PEG-lipids, particle stability, biodistribution, and ultimately targeting of the nanoparticles to the outside of the liver can be regulated (Truong and Meng, 2025). Another widely used method is surface modification of LNPs. For instance, controlling the density of PEGylation can extend the systemic circulation time and improve tissue penetration and, as such, increase the probability of nanoparticles to access extrahepatic targets. Modifying the LNP surface with targeted ligands—e.g., antibodies, peptides, aptamers, proteins, or sugars—enables receptor-mediated internalization in targeted tissues and significantly improves delivery specificity (Cheng et al., 2025; Truong and Meng, 2025). At the same time, scientists are investigating many local delivery approaches for extrahepatic targeting (Truong and Meng, 2025). Due to the complexity and number of steps involved in the synthesis of the ionizable lipids, a significant amount of time and labor-intensive work is needed for this. Thus, new chemical techniques are urgently needed to simplify the synthesis of this substance. As a result, more study in this field is necessary, and more funding should be allocated in this direction.

One of the main obstacles to the development of ILNP-based therapies is instability. The majority of lipid-based NPs are unstable and readily broken down. Two ILNP-based mRNA COVID-19 vaccines have recently been produced and licensed for the treatment of COVID-19. For preservation, these vaccinations need to be frozen or ultra-frozen. They weren't used in low- and middle-income nations despite their highly effective immunization rates due to their highly unstable character. We must do research to create vaccinations that will remain stable at ambient temperature or in a typical refrigerator (4 °C). Furthermore, the shelf life of these COVID-19 vaccinations is only six months, when it should be at least a year. When compared to DNA, RNA has less stability. To boost the stability of the LNP, we may alternatively employ DNA instead of RNA. However, there is relatively little information known about DNA-ILNP-based gene delivery. The majority of the research has used RNA-LNP-based gene delivery. It is critical that we work on LNP-DNA-based delivery systems to understand their composition, cellular and endosomal escape mechanisms, physiochemical features, and pharmacokinetic parameters.

Traditional lipid nanoparticles (LNPs), while highly effective for nucleic acid delivery, are marred by their multi-component formulations, costly manufacturing process, and rigorous cold-chain storage requirements. Recent advances from the laboratories of Percec and Weissman have positioned ionizable amphiphilic Janus dendrimers (IAJDs) as a cost-effective and viable alternative (Arshad et al., 2024; Zhang et al., 2021). Unlike LNPs, which rely on four different lipid components, IAJDs are a single-component delivery system, thereby enabling simpler and cheaper large-scale synthesis. A 2024 paper from Weissman, and Percec group described the expedited, ten-gram-scale synthesis of sequence-defined IAJDs, which combine structural precision and multifunctionality (Arshad et al., 2024). Imperatively, these dendrimers exhibit unlimited stability at room temperature in air, eliminating the need for ultra-cold storage, and making them particularly attractive for global distribution of RNA vaccines and therapeutics (Arshad et al., 2024). This shift from multicomponent LNPs to single-component IAJDs is a significant step towards low-cost, stable, and scalable delivery technologies.

7. Conclusion

Ionizable lipids have emerged as a crucial component in drug and gene delivery systems, with enhanced biocompatibility, increased cellular uptake, and efficient endosomal release. Their pH sensitivity allows for more targeted release of cargo, which increases the stability and activity of nucleic acid-based therapeutics and small-molecule drugs. The addition of ionizable lipids to lipid nanoparticles (LNPs) has significantly enhanced mRNA vaccine and siRNA therapy's clinical efficacy and showcased their potential to revolutionize therapy. Their challenges such as immunogenicity, off-target, and large-scale production are currently under exploration.

CRediT authorship contribution statement

Semmal Syed Meerasa: Writing – review & editing, Writing – original draft, Visualization, Investigation, Conceptualization. **Aqeel Ahmad:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Investigation, Conceptualization. **Amer Ali Khan:** Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Shafiul Haque:** Writing – review & editing, Visualization, Project administration, Investigation, Conceptualization. **Imran Saleem:** Writing – review & editing, Visualization, Validation, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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