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Breaking the final barrier: Evolution of cationic and ionizable lipid structure in lipid nanoparticles to escape the endosome

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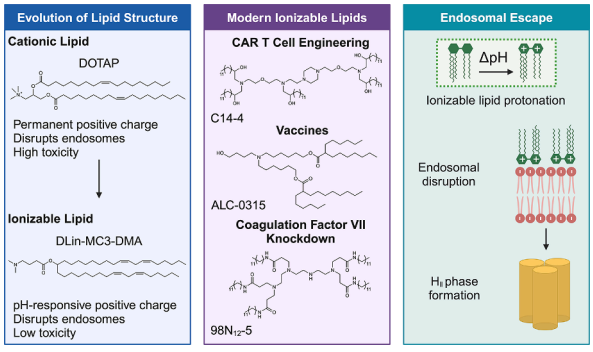
GRAPHICAL ABSTRACT



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ABSTRACT

In the past decade, nucleic acid therapies have seen a boon in development and clinical translation largely due to advances in nanotechnology that have enabled their safe and targeted delivery. Nanoparticles can protect nucleic acids from degradation by serum enzymes and can facilitate entry into cells. Still, achieving endosomal escape to allow nucleic acids to enter the cytoplasm has remained a significant barrier, where less than 5% of nanoparticles within the *endo*-lysosomal pathway are able to transfer their cargo to the cytosol. Lipid-based drug delivery vehicles, particularly lipid nanoparticles (LNPs), have been optimized to achieve potent endosomal escape, and thus have been the vector of choice in the clinic as demonstrated by their utilization in the COVID-19 mRNA vaccines. The success of LNPs is in large part due to the rational design of lipids that can specifically overcome endosomal barriers. In this review, we chart the evolution of lipid structure from cationic lipids to ionizable lipids, focusing on structure–function relationships, with a focus on how they relate to endosomal escape.

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Additionally, we examine recent advancements in ionizable lipid structure as well as discuss the future of lipid design.

1. Introduction

Since the late 1990s, nucleic acids have been employed in the clinic as therapeutics and vaccines for a range of diseases. The ability to introduce nucleic acids, including plasmid DNA, messenger RNA (mRNA), and small interfering RNA (siRNA), into the cellular environment presents an opportunity to modify the transcriptional and translational pathways responsible for diseases that have been difficult to effectively treat with earlier strategies [1–5]. However, a significant barrier to the delivery of these therapeutics is their large size, negative charge, and hydrophilicity, which prevents them from entering cells and leads to rapid nuclease-mediated degradation and immune recognition [6,7]. Lipid-based drug delivery systems, including nanoemulsions, liposomes, and lipid nanoparticles, have thus been used to overcome biological barriers to nucleic acid delivery since the 1980s [8–11]. Charged constituent lipids have been widely incorporated into lipid-based systems to neutralize the negatively charged nucleic acid backbone and promote encapsulation and cellular uptake [12]. Cationic lipids, which carry a permanently positive charge, have predominantly been employed in liposomal delivery systems [13]. Subsequent generations of lipid-based delivery systems demonstrated enhanced physical stability and encapsulation efficiency, with lipid nanoparticles (LNPs) emerging as the most clinically advanced delivery system for RNA therapeutics and vaccines [14]. Most notably, LNPs have served as the carrier for three FDA approved therapeutics and vaccines, Onpattro for the treatment of transthyretin amyloidosis, and the Moderna (Spikevax) and Pfizer/BioNTech (Comirnaty) mRNA COVID-19 vaccines [15,16]. The canonical LNP consists of four lipid components—phospholipid, cholesterol, PEGylated lipid, and ionizable lipid (IL)—with the IL component playing a critical role in facilitating RNA protection and endosomal escape [17–21]. Unlike earlier cationic lipids, ILs exhibit a neutral charge at physiological pH that minimizes toxicity, and a positive charge at acidic pH which promotes nucleic acid encapsulation and endosomal escape [22]. In this review, we provide a historical account of the transition from cationic to IL structures, the development of clinically relevant ILs, and structure–function investigations that guide the design of new IL structures. Furthermore, we discuss endosomal escape mechanisms that elucidate IL behavior and describe several characterization strategies to explore these mechanisms. Finally, we provide perspectives and directions for future investigation of IL structure–function relationships with the goal of arriving at a clear and representative list of design rules for future ILs.

2. Endosomal escape

Drug delivery vehicles can substantially increase the amount of therapeutic that enters cells, and often, the entry pathway inside the cell is through endocytosis. Many drugs, especially RNAs, cannot achieve their mechanism of action until they reach the cytosol, so escaping the endosomal-lysosomal maturation pathways is essential for therapeutic efficacy. Strikingly, despite the trivial distance, endosomal escape is one of the most significant barriers in drug delivery, with estimates that less than 5% of nucleic acid payloads in endosomes escape into the cytosol [23,24]. This barrier is a result of billions of years of cellular evolution to prevent foreign constructs from entering cells. Moreover, cells are equipped with sophisticated detection systems to hinder the entry of foreign nucleic acids—a result of the constant war between cells and viruses. For the latter, nucleotide chemical modifications have been pivotal in obfuscating the nucleic acid from immune recognition [25–27].

Endocytosis is a dynamic process that encompasses a vast area of entry mechanisms, often differentiated into clathrin-dependent and clathrin-independent pathways, depending on whether the endocytic vesicles contain the cytosolic protein clathrin (Fig. 1). This review will focus solely on the mechanisms relevant for LNPs and other lipid vehicles. For an in-depth analysis of the endosomal maturation process, we recommend the seminal review by Jatta Huotari and Ari Helenius [28]. Lipid-based drug delivery vehicles, including LNPs, often enter cells via clathrin-mediated endocytosis, likely due to the sheer volume of clathrin-coated pits on cells, and macropinocytosis, which, due to its ability to capture relatively large objects, is a natural sink for drug delivery vehicles [23,29–31]. After initial entrapment and budding of the plasma membrane, most nanoparticles are transferred to early endosomes that have a pH of 6.5. Early endosomes then are converted to late endosomes and then lysosomes, which have more acidic pH values of 5.5 and 4.5, respectively. This pathway represents a canonical description of endocytosis; however, macropinocytosis buds, or macropinosomes, can immediately be transferred to late endosomes, further complicating the pathway [32]. Moreover, early endosomes are often recycled back to the plasma membrane [24].

Most nanoparticles need to be equipped with mechanisms that allow them to escape before the endosomes mature into the lysosome, where the highly acidic environment could degrade the payloads, or before the endosomes are recycled outside of the cell. Most strategies to induce endosomal escape involve either fusing with the membrane, rupturing the membrane, or a combination of both methods [33,34]. With lipid-based nanoparticles, membrane fusion is feasible due to the cell-like architecture of the vehicles as well as the tendency to employ cationic or induced-cationic lipids and peptides that are attracted to the negatively-charged endosomal membrane. The use of positively-charged species can also be employed to destabilize endosomes by introducing groups that weaken the integrity of the membrane [35]. Another strategy to break open endosomes includes mechanical swelling, in which the nanoparticles, due to acidification, grow or elongate and induce enough strain to burst open the endosome; although, this route is more common for hydrogel- and polymer-like drug delivery vehicles [36,37]. Lastly, the proton sponge effect is another avenue hypothesized to induce endosomal escape, via increasing osmotic pressure [38,39]. Here, the nanoparticle contains a high buffering compacity that requires the cell to continuously pump protons, and thus chloride ions, into the endosome until osmotic pressure causes it to burst.

The efficacy of these methods, particularly the proton sponge effect, have been contested over the past decades, with conflicting results emerging [40]. This is due to both the complexity of the *endo*-lysosomal process as well as the lack of understanding of how internal and external structure and physicochemical parameters of drug delivery impact endosomal escape. Thus, it is important to consider the cell type, specific internalization mechanism, the pathway of the endosome, and how the size, shape, charge, and components of the vehicle influence these parameters. For example, Vermeulen *et al.* utilized mathematical modeling and quantitative confocal microscopy to discover that endosomal size and leakiness substantially impact the potency of the proton sponge effect, where cells with smaller and less leaky endosomes exhibit higher levels of endosomal escape compared to those with larger and more leaky endosomes [39]. On the nanoparticle side, researchers from the University of Copenhagen and AstraZeneca concluded via NMR and computation simulations that individual lipid shape and head group pK_a influences lipid shape and packing in LNPs, and that these parameters strongly affect endosomal escape [41]. Due to the complicated dynamics of endosomal escape, in the following sections, we discuss the evolution of lipid structure in lipid-based drug delivery vehicles from the perspective of efforts to design structures that more efficacious and

safely allow nucleic acid cargo to escape the endosome.

3. Cationic lipids

Cationic lipids were first used for non-viral gene delivery in the late 1980s. In 1987, Felgner and colleagues described the use of DOTMA, a synthetic cationic lipid, for plasmid DNA transfection of mammalian cells [42]. DOTMA has since been used for efficient RNA transfection into multiple cell types [43]. Several cationic lipid structures were developed in the following years, setting a new standard for non-viral gene transfer of plasmid DNA. This class of lipids consists of a permanently positively-charged head group. A review by Zabner provides additional information on commonly used cationic lipid structures [44]. While initial cationic lipid delivery systems were limited by inefficient plasmid transfection, addition of a neutral phospholipid was shown to increase efficiency [44]. Thus, cationic lipids were commonly used in cationic liposomes, or lipoplexes. The canonical cationic liposome contains two lipid species, a cationic lipid and a neutral phospholipid, sometimes referred to as a helper lipid.

Cationic lipids were necessary for nucleic acid encapsulation, and later were found to improve endosomal escape. This is due to their tendency to induce membrane phase changes upon electrostatic binding to the negatively charged phospholipids in endocytic membranes. The structure of cationic lipids determines its packing parameter, which can be modeled by $P = \frac{v}{al_c}$, where v represents the hydrocarbon volume, a is the head group area, and l_c is the lipid length[45]. Here, packing parameters of $P > 1$ correspond to nanostructures with negative curvature due to the relatively larger volume of the hydrocarbon chains. Such structures tend to form inverted hexagonal phases (H_{II}) after binding with anionic phospholipids in the endosomes, producing endosomes that are far less stable and more prone to membrane fusion and disruption. The helper lipid also plays a role in facilitating H_{II} phase formation, with the most common lipid being dioleoyl phosphatidylethanolamine (DOPE) [46,47]. This zwitterionic lipid, when mixed with cationic lipids initially forms lamellar phases; however, when placed in more hypertonic solution, such as in the endosome, the system transitions to a H_{II} phase that can intercalate into endosomal membranes, prompting destabilization.

Several additional cationic lipid structures have been evaluated for liposome-mediated nucleic acid transfection. DOTAP was investigated as a biodegradable derivative of DOTMA as it contains ester bonds in place of ether bonds to link the chains to the backbone for mRNA transfection [48]. Moreover, cationic cholesterol derivatives have been used for gene delivery [49–53]. Cholesterol derivatives with charged guanidinium, methyl imidazole, and pyridine headgroups exhibited particularly high DNA transfection activity [52,53]. For example, DC-Chol was synthesized in only one synthetic step, and when formulated into liposomes led to greater transfection and less toxicity than the DOTMA control formulation [51]. Using confocal microscopy, others have demonstrated using colocalization that DC-chol preferentially localizes in the early endosome, rather than the late endosome, suggesting that the cation has an increased fusogenetic nature at this stage[54]. Fang and colleagues modified the amine substituents to include hydroxyl groups in their BHEM-Chol cationic lipid structure [49]. When formulated into liposomes, BHEM-Chol was found to be the major driving force for membrane perturbation, enhancing fusion of the cationic liposome with the cell membrane.

The structures of cationic lipids derived from cholesterol and other lipids have been investigated in a systematic manner to examine structure–function relationships between cationic lipids and transfection activity. Farhood and colleagues identified a tertiary amine compound with a succinyl spacer-arm that has the highest DNA transfection activity compared to other structures varying in the degree of amine substitution and the presence of a spacer-arm [50]. Meanwhile, Felgner and colleagues investigated systematic structural changes to both cationic and neutral lipid structures in cationic liposomes. Cationic lipid derivates were designed from 2,3-dialkyloxypropyl quaternary ammonium compounds containing a hydroxyalkyl moiety on the quaternary amine and varied in hydroxyalkyl chain length on the quaternary amine and alkyl chain substitutions [55]. It was found that a hydroxyethyl moiety and

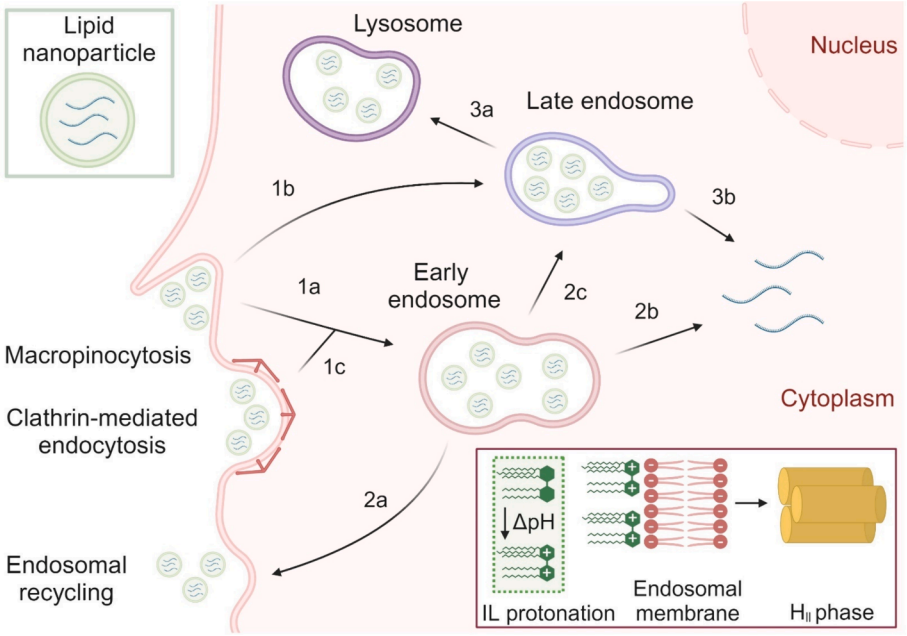

Fig. 1. Endosomal escape of lipid nanoparticles. Lipid nanoparticles (LNPs) enter cells through macropinocytosis, which will transfer the nanoparticles into early endosomes (1a) or late endosomes (1b). The LNPs can also enter early endosomes via clathrin-mediated endocytosis (1c). From the early endosomes, many LNPs are recycled outside of the cell (2a), whereas only a fraction of the nucleic acids can escape (2b). Some early endosomes will mature into late endosomes (2c) and then lysosomes (3a). LNPs can also undergo escape from late endosomes (3b). Endosomal escape of LNPs mainly proceeds via the protonation of ionizable lipids (ILs) in the acidic endosomes that bind to negatively charged endosomal membranes, causing a mesophase transition into a hexagonal (H_{II}) phase that releases the nucleic acid into the cytoplasm (bottom right).

dimyristyl alkyl chains achieved the highest transfection efficiency, and using chloroquine, which blocks early endosome progression into late endosomes, it was found that the myristyl chains induced less signal after chloroquine administration, signifying that these groups assist with cargo release during late endosomes. A meta-analysis of cationic lipid structures found that lipids with a 14-carbon chain length and mono-unsaturated in place of saturated chains provided optimal transfection [56]. This finding marked an early structural guideline for the development of new cationic lipids, suggesting that clear design rules could be identified and utilized to predict ideal structures for lipid-based nucleic acid delivery systems.

Several cationic liposome formulations have been commercialized including Lipofectin, which consists of DOTMA and DOPE, and Mega-Fectin, which consists of DOTAP and either DOPE or cholesterol [43,48]. Other cationic lipids present in commercialized formulations include DDAB, a quaternary ammonium lipid, and DOSPA, a quaternary ammonium lipid with a spermine-containing head group [57]. TransfectAce consists of DDAB and DOPE, and Lipofectamine, which has been commonly used for mRNA delivery to a variety of cell types, consists of DOSPA and DOPE [58–60]. The structure of Lipofectamine was further optimized for delivery of siRNA and miRNA, and commercialized as Lipofectamine RNAiMAX [61]. While these commercial reagents have been effective as *in vitro* positive controls, their severe systemic toxicity makes them unsuitable for *in vivo* applications [62–64].

While cationic lipids have been successfully used for RNA delivery, several critical challenges limited their potential as clinically useful therapeutics. The association of their use with toxicity, including damage to cellular physiology, inflammation, and tissue damage, was of particular concern [65–69]. Additionally, their short circulation half-life and imprecise association with negatively charged intracellular and extracellular components presented additional barriers to their clinical use [22,70].

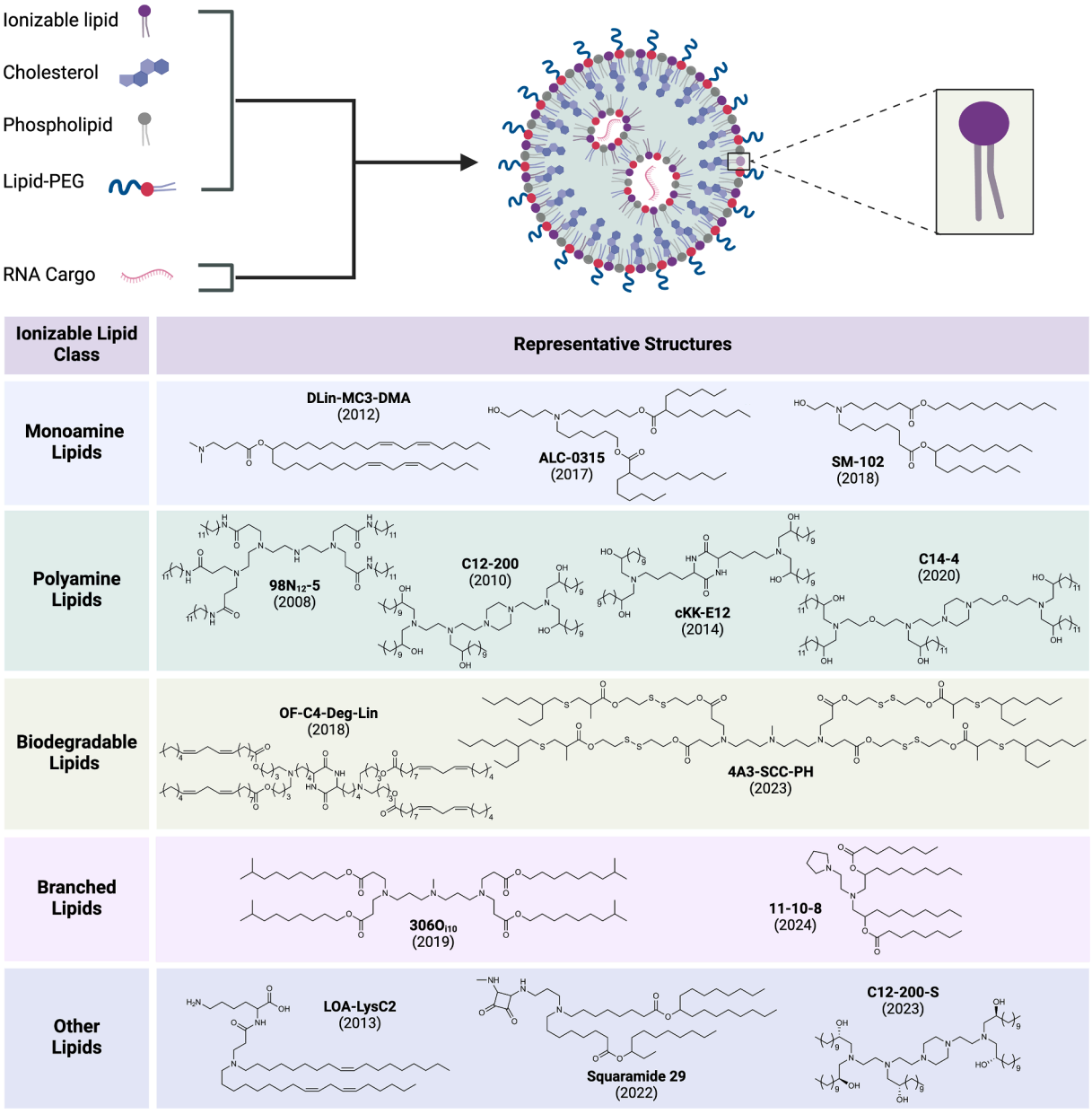

Fig. 2. Overview of major ionizable lipid structural classifications. Schematic of lipid nanoparticle (LNP) formulation with RNA cargo and four excipient lipids, including the ionizable lipid (IL). ILs can be broadly classified as monoamine (containing one amine), polyamine (containing multiple amines), biodegradable (containing degradable moieties, most commonly an ester), branched (containing branched tails), or other lipids based on structural features. Many ILs can be classified into multiple groups. Representative structures are shown for each group with the year the structure was first reported.

4. Ionizable lipids

Unlike cationic lipids, ILs exhibit a predominantly neutral charge under physiological conditions and thus are generally less toxic than cationic lipids[71]. Early studies that used ionizable lipids (ILs) for RNA delivery resulted in reduced *in vivo* toxicity, which increased the use of ILs for RNA delivery. DODAP (1,2-dioleoyl-3-dimethyaminopropane) was the first IL used for nucleic acid encapsulation [72]. The structure of DODAP consists of two acyl chains, each containing one double bond, and the IL exhibits a pK_a of 6.6–6.7 [73–75]. Early IL structure–function studies investigated the number of double bonds in each acyl chain. Heyes and colleagues found two double bonds per chain to be the optimal choice for IL design due to the balance achieved between enhanced silencing activity of encapsulated siRNA with a greater number of double bonds and sufficient encapsulation efficiency of siRNA with a lesser number of double bonds [72,76]. Thus, IL structures containing acyl chains with two double bonds were widely used and investigated in subsequent years for RNA delivery. While ILs have been used in a variety of delivery vehicles and nucleic acid types, here we focus on the structural development of ILs used for lipid nanoparticle (LNP) mediated RNA delivery (Fig. 2).

4.1. Monoamine lipids

The use of ILs in RNA LNP therapeutics and vaccines can be traced back to the development of DLinDMA (1,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane), an IL containing two double bonds on each of its two acyl chains. DLinDMA represents the earliest generational iteration in a group of structures that led to the IL employed in the first clinically approved RNA LNP therapy (Fig. 3). DLinDMA was first examined in a structure–function study within a library of structures that differed in the number of double bonds per acyl chain. DLinDMA contained two double bonds per acyl chain, whereas the other ILs contained between zero and three [76]. Semple and colleagues used the structure of DLinDMA to guide the design of similar IL structures with increased delivery activity based on the proposed mechanism of action *in vivo*. They synthesized DLin-KC2-DMA (2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane)), which varies from DLinDMA by the addition of a ketal ring linker and additional methylene groups between the DMA headgroup and ketal ring linker [77]. DLin-KC2-DMA demonstrated a 10-fold increase in potency for silencing of hepatic Factor VII (FVII) compared to DLinDMA. Jayaraman and colleagues then conducted a screening of 53 novel lipids, the structures of which were derived through modifications to the head group of DLin-KC2-DMA. DLin-MC3-DMA (dilinoleylmethyl-4-dimethylaminobutyrate) was identified as one of the most active ILs in the screening library, and structurally varied from DLin-KC2-DMA via replacement of the ketal ring linker with an ester group [78]. Additionally, they reported a strong correlation between IL pK_a value and *in vivo* FVII silencing activity and thus concluded an optimal IL pKa value of 6.2–6.5 for hepatic-gene silencing applications of LNPs. LNPs formulated with DLin-MC3-DMA, also referred to as MC3, demonstrated a pK_a of 6.44 within the optimal range and were 1000-fold more potent in silencing FVII activity compared to LNPs formulated with DLinDMA. Thus, MC3 was positioned as a lead IL for use in clinical applications of RNA LNPs due to its high activity. An LNP formulated with MC3 underwent preclinical investigation for transthyretin (TTR) gene silencing, and was then FDA approved in 2018 as Onpattro (patisiran) for treatment of hereditary transthyretin (hATTR) amyloidosis in humans following a two-stage clinical trial [79]. The optimization of MC3 resulted in clinical translation of the first approved RNA LNP therapeutic, further validating the success of the structural optimization process and demonstrating the value of structure–function studies for guiding design of new IL structures.

One of the foundational studies on MC3-based LNPs employed a combination of quantitative fluorescence imaging and electron microscopy to measure in real-time, the endosomal escape of Alexa647-conjugated siRNA[23]. The researchers found significant differences in uptake in *in vitro* cultures compared to intravenous administration *in vivo*, where accumulation in the liver can be faster than in certain cell cultures due to PEG shedding. Moreover, using siRNAs conjugated to 6 nm gold nanoparticles as a model, it was found that only 1–2 % of siRNAs are released from the endosome, further demonstrating the difficulty in overcoming endosomal escape. Compared to other conjugation moieties, gold nanoparticles offered high contrast for electron microscopy imaging, enabling more quantitative analysis of LNP and siRNA uptake and endosomal escape. Although the release levels are low, often, only a few thousand copies of RNAs are needed to induce their therapeutic effect[80]. The essential role of the IL was further demonstrated in a study that compared EGFP accumulation and translation in mouse livers using LNPs containing DLinDMA, DLin-KC2-DMA, or MC3 [81]. Despite all three LNPs having similar accumulation in the liver, MC3 DLin-KC2-DMA LNPs achieved higher EGFP signal compared to DLinDMA. Through a combination of small angle X-ray scattering (SAXS) and molecular dynamic (MD) simulations, it was determined that MC3 and DLin-KC-DMA lipids can mediate greater endosomal fusion via transitioning more quickly from an inverse micellar phase to a H_{II} phase at pH 6.5. Further SAXS analysis of MC3 mRNA nanoparticles compared to DOTMA, DODMA, DOTAP, and DODAP lipoplex mRNA nanoparticles revealed a correlation between pK_a and mesophase reconstruction, highlighting the role of protonation in reordering LNP internal structure during endosomal escape [82].

The endosomal escape properties of MC3 can result in simultaneous cross-cellular transfection via extracellular vesicles (EVs). During the endocytic maturation sequence, intraluminal vesicles of late endosomes

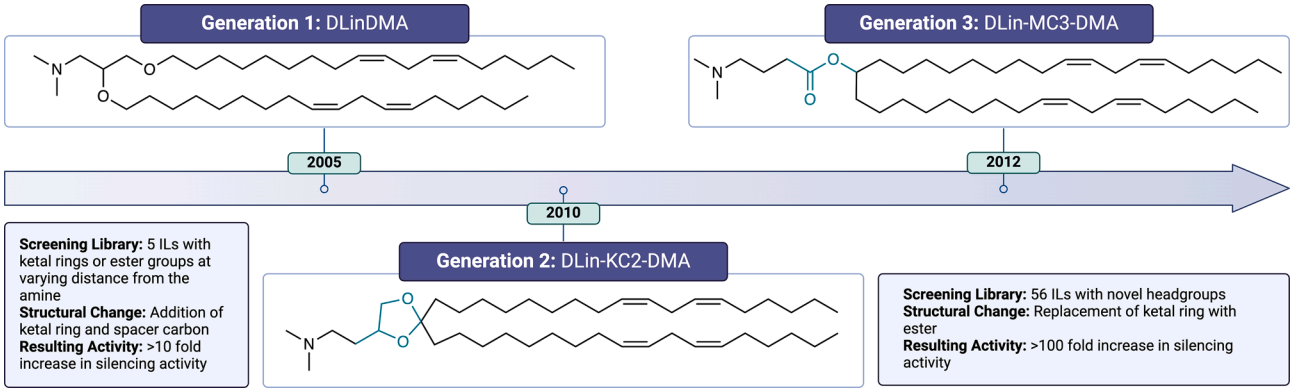

Fig. 3. Structural evolution of DLin-MC3-DMA ionizable lipid. Lead IL structures from each systematic investigation to optimize the original DLinDMA structure. An overview of the screening library from which each lead was identified, the structural changes that distinguish each structure from the prior lead, and the resulting activity improvement over the prior lead are provided.

can be secreted extracellularly, forming EVs that then can transfect nearby cells [83]. This can result in unintentional transfection of non-targeted cells and organs or can be exploited as a secondary delivery mechanism to transfer mRNA to nearby sites of interest, as has been showcased for pancreatic delivery [84]. This feature has been utilized to enhance the delivery of VEGF-A, a protein involved in angiogenesis, to the heart [85]. Here, it was found that VEGF-A LNP administration created EVs containing VEGF-A mRNA that were secreted mainly by cardiac progenitor cells to enhance angiogenetic function in cardiac tissue. Moreover, EV-based transfection lowered the production of inflammatory cytokines, pointing to a potential method to utilize LNPs to induce EV transfection in a less immunostimulatory manner.

While MC3 has shown remarkable success for intravenous (IV) administration of RNA LNP therapeutics, it is not ideal for all administration routes. For example, vaccines administered using an intramuscular (IM) route necessitate minimal reactivity and high tolerability at the injection site, particularly when repeat dosing at therapeutically relevant levels is required [86]. A study using MC3 as the IL in an mRNA LNP vaccine demonstrated moderate local and system adverse events following IM administration, which is consistent with the known half-life of the IL in tissues [87,88]. These results indicated that the ideal IL structure for IV administration is different than that for IM administration. Early findings on the success of mRNA as a vaccine platform motivated the optimization of IL structures for vaccine-specific administration routes [89,90]. This work culminated in the development of two IL structures currently in use in FDA approved mRNA LNP vaccines, both of which are administered intramuscularly.

The development of IL structures utilized in the COVID-19 vaccines was the result of meticulous structure–function studies. Sabnis and colleagues investigated IL structures for IM mRNA administration using a structural evolution approach [91]. They began by investigating clearance of LNPs delivered through an IV route. Their initial screen identified ethanolamine as an ideal head group for optimal encapsulation and physiochemical properties but found that several early structures containing an ethanolamine head group and two dilinoleic tails demonstrated similar clearance rates to MC3. Thus, they introduced ester linkages into the structures to promote *in vivo* metabolism by esterases [92]. Structures with differing placement and substitution of esters demonstrated varying mRNA delivery efficiency and tissue clearance rates. A structure that contained two tails, one containing a secondary ester and the other containing a primary ester, demonstrated 3-fold greater mRNA expression than MC3 and optimal tissue clearance as no lipid was detected at 24 h post-administration. Further investigation into the relationship between the structure and activity of the lead IL focused on the position of the primary ester. It was found that clearance at 24 h decreased as the primary ester was moved closer to the amine group. Two lead structures were investigated in non-human primates, and one demonstrated improved delivery efficiency and rapid lipid clearance compared to MC3, representing a critical step to clinical use. This study highlights the importance of examining multiple IL structural motifs, as small changes to the structures resulted in significant changes to both mRNA delivery and lipid clearance. The endosomal escape properties of the lead IL and MC3 were determined using single-molecule fluorescence *in situ* hybridization (smFish) to compare the levels of mRNA detected in organelles to those in the cytosol. Moreover, using a fluorescently-tagged DOPE, the number of LNPs in each cell was also quantified. Interestingly, while MC3 LNPs accumulated 5-fold higher in cells than the lead lipid, the opposite trend was observed for cytosolic mRNA amounts, where MC3 LNPs had lower cytosolic mRNA than the leading lipid. This finding runs counter to the principle that greater accumulation will lead to enhanced delivery; instead, the kinetic and thermodynamic parameters of lipid phase transitions and endosomal disruption are likely more significant.

Hassett and colleagues then investigated analogous structures for IM administration of mRNA LNPs using a similar methodology [86]. Through evaluating several structures with slight structural modifications from the top-performing IL in the previous study, they determined that the lead structure from the IV administration screen was also the top performer for IM administration. The lipid demonstrated the greatest tolerability and highest mRNA expression following IM administration, significantly outperforming MC3 in both categories. This result is understandable considering that the structure was optimized in the previous study for rapid tissue clearance, which is an important determinant of immune reaction following vaccination. This IL structure was later named SM-102.

The structure and synthesis of the ALC-0315 IL were first disclosed in a patent application by Acuitas Therapeutics in 2017 [93,94]. While the structure of ALC-0315 resembles that of SM-102, it contains a butanolamine rather than ethanolamine head group. Additionally, two alkyl chains, one shorter in length than the other, are attached to each ester and the esters are connected to the rest of the molecule in a different orientation than in the SM-102 structure. The biodegradable nature of both ILs due to the inclusion of ester groups in the lipid tails allows for *in vivo* hydrolysis, and thus faster clearance and improved tolerability compared to MC3. This property makes both structures particularly successful for applications necessitating IM administration, including vaccines in which minimal tissue accumulation and adverse immune events are desirable. Indeed, mRNA LNP therapeutics formulated with both ILs have demonstrated clinical success as COVID-19 vaccines. The Moderna (Spikevax) formulation is formulated with SM-102 and the Pfizer/BioNTech (Comirnaty) formulation contains ALC-0315 [95–97]. Both formulations were granted Emergency Use Authorizations (EUAs) in 2020, with full FDA approval granted in 2021 for the Pfizer/BioNTech formulation and in 2022 for the Moderna formulation [98].

The endosomal escape properties of ALC-0315 and SM-102 have been investigated heavily. In a study by Yu and colleagues, it was determined that both ILs undergo a remarkably complex series of mesophase transitions within the *endo*-lysosomal pathway [99]. Using *in situ* time-resolved SAXS coupled with rapid flow mixing, the researchers determined that as the pH decreases, the LNPs, due to the ILs, undergo a transition from inverse micellar to a H_{II} phase, followed by transitions to inverse bicontinuous cubic phases and finally a lamellar phase. Additional studies have demonstrated that SM-102 may be better able to form a cubic structure at a lower pH compared to ALC-0315, which could explain why the SM-102 LNPs induce greater mRNA translation than ALC-0315 LNPs when applied to other applications, such as lung macrophages [100].

The success of both ILs for IM administration of LNP vaccines has motivated further studies to investigate new IL structures through a structural evolution methodology. Tilstra and colleagues used an iterative design methodology to optimize new ILs for IM administration and found that ionizable lipids consisting of an ethanolamine core and LNPs with a pK_a between 6.6 and 6.9 maximized mRNA delivery [101]. The varying structures of ILs approved for clinical use in different applications demonstrates the importance of optimizing IL structures to have desired functionalities.

4.2. Polyamine lipids

Unlike the monoamine ILs like those used in the FDA approved LNP formulations, polyamine ILs contain more than two tails. While monoamine structures are still being investigated and employed in the development of new LNP therapeutics, polyamine ILs have emerged as popular alternatives. The canonical polyamine IL consists of a polyamine core attached to alkyl tails, with the number of tails corresponding to the number of amine attachment sites [102]. As a result, polyamine ILs can be synthesized using a combinatorial approach. The simple synthesis scheme enables high-throughput screening of polyamine structures, which has led to the elucidation of many structure–function relationships [17]. Numerous core structures have been investigated, and alkyl epoxides, alkyl acrylates, and alkyl acrylamides are commonly used as electrophilic lipid tails.

Several lead polyamine cores have been identified through high-throughput screening of combinatorial libraries. The first such study screened a library of 700 polyamine ILs with acrylate tails in 2008, and revealed the 98 core, a linear structure consisting of two primary and two secondary amine groups, to be a lead performer for siRNA delivery [103]. Further, enhanced delivery performance was characterized by cores containing at least two amine groups. Love and colleagues used a similar approach to screen a library of 196 ILs that were formulated by reacting epoxides of different alkyl lengths with 14 polyamine cores [102]. The 200 core, which consists of an internal piperazine ring with a total of five amine groups and five amine attachment sites, was identified as a top performer. LNPs containing the C12-200 IL, formulated by reacting the 200 core with 12-carbon alkyl epoxide, achieved highly efficient *in vivo* gene silencing. C12-200 has since been considered a gold-standard IL for LNP-mediated delivery of siRNA and mRNA to the liver [104–110]. Dong and colleagues synthesized a combinatorial library of 103 ILs with lipoamino acid, lipopeptide, and lipopolypeptide cores [111]. A diketopiperazine core cKK was identified as a lead structure for selective gene silencing in hepatocytes, and cKK-E12, an IL with four 12-carbon epoxide tails, showed success in rodents and nonhuman primates. Our group screened a combinatorial library of eight polyamine cores and three epoxide tails of varying length to optimize mRNA delivery to primary human T cells [112]. C14-4, which consisted of five 14-carbon tails and a core consisting of a piperazine ring, five amine attachment sites, and ether moieties, was identified as a top performer. Interestingly, three of the top-performing cores contain piperazine rings, suggesting ring containing cores should be further investigated when designing new polyamine ILs [113].

Other studies have systematically investigated the tail structure of polyamine ILs. Several studies have shown that ILs with mid-length alkyl tails are optimal, with short-tail ILs demonstrating low delivery efficacy and long-tail ILs having low solubility which complicates nanoparticle formation [102,114,115]. Further, our group demonstrated that the optimal tail length for mRNA delivery varies with mRNA size [114]. Other studies have optimized tail length for specific applications. Optimization of the piperazine ring containing 4 core discussed previously found that 14-carbon tails achieved the highest delivery efficacy for T cells, and the C14-4 IL has since been used in CAR T cell applications [112,116–118]. The same core was optimized for placental delivery, and it was found that the A4 IL, which consisted of 12-carbon tails, achieved *in vivo* delivery to the placenta [119]. Using a dendrimer core and epoxide tails, alkyl tail length was found to influence tissue tropism within the liver [120]. Tail length has also been investigated with acrylamide tails, which led to the finding that either two long amide tails or a greater number of shorter amide tails led to optimal delivery using the 98 core [103,121]. IL structure was further optimized by exploring the number of amide-containing 12-carbon tails, and it was found that 5 such tails were ideal for siRNA delivery. This optimal structure contains one less tail than reaction site, suggesting that polyamine structures with reduced tail number should be further explored. The role of double bonds in polyamine tails has also been investigated, with Fenton and colleagues investigating derivatives of cKK-E12 with varying numbers of double bonds and finding that two double bonds per tail led to increased delivery. Interestingly, these studies highlight the importance of several structural features of IL tails, as small structural changes are found to substantially impact LNP behavior. IL tail length in particular stands out for its ability to modulate organ targeting, delivery efficiency, and tissue selectivity. In the case of C14-4 and A4, a difference in tail length of two carbons distinguishes T cell delivery from placental delivery. In other cases, minor changes to tail length result in large differences in delivery efficiency and tissue targeting within the same organ.

Aside from identifying the functional implications of IL structural characteristics, systematic investigations have also revealed mechanistic changes that arise from small structural modifications. Paunovska and colleagues modified the tail length of ILs containing the diketopiperazine cKK core and found that tail length changes could drive the targeting of new cell types. While absorption of apolipoprotein E (ApoE) onto LNPs facilitates hepatocyte entry via the low-density lipoprotein receptor (LDLR), several ILs with modified tail lengths were shown to enter other cell types via ApoE and LDLR-independent pathways [122]. Other studies have demonstrated the role of ILs in protein corona formation, targeting of specific cell types, and other uptake pathways, further suggesting that investigating the mechanistic implications of small IL structural changes is critical to achieve a greater understanding of LNP behavior [14,123–129]. One area that has remained understudied is the location of the IL within the LNP. Structural changes that modulate IL lipophilicity could result in the placement of the IL on the nanoparticle surface or within the lipid core, which could impact interactions between LNPs and their environment, particularly due to changes in protein corona formation. While polyamine IL structures have demonstrated highly efficient delivery and gained popularity for their simple synthesis, their biocompatibility is an important factor. The majority of polyamine structures contain stable backbones and many contain no degradable moieties, particularly those with epoxide tails [17,130]. As a result, it is important to consider the biotoxicity and immune interactions of these structures when choosing ILs for therapeutic applications.

4.3. Biodegradable lipids

Biodegradable IL structures demonstrated success in both mRNA vaccines and Onpattro, which are all formulated with ester containing ILs. Ester linkers have been used for many applications due to their *in vivo* efficacy and enhanced biodegradation rate [17,77,87,91,131–135]. Other biodegradable IL structures contain degradable groups in place of the ester. Disulfide linkages have been investigated for mRNA delivery and specifically employed for *in vivo* genome editing [136–139]. Chen and colleagues investigated a library of 96 IL structures with degradable linkers, and found that the top performing structures contain four disulfide bond-bridged ester linkers [136]. These structures outperformed the corresponding structures that lacked disulfide bonds and contained disulfide bonds but no ester linker, and an enhanced endosomal escape and mRNA release mechanism was identified to support the increased efficiency of the disulfide bond-bridged structures. ILs containing degradable amide bonds have also been used for genome editing [140]. Systematic structure–function studies have also been used to investigate and characterize structural features of biodegradable ILs. Tail length, tail geometry, and linker spacing were systematically investigated using the biodegradable diketopiperazine cKK core discussed earlier [141]. Interestingly, changes to IL structure had a greater influence on mRNA delivery than siRNA delivery, consistent with other findings of varying IL efficacy with different cargo types [109,142]. Investigation of an IL library consisting of HEPES Good-derived IL structures that contained a piperazine core, asymmetric tails, and degradable ester and disulfide moieties identified key structure–function relationships and a lipid bilayer packing mechanism that mediated *in vivo* protein production [143]. Changes to lipid tail length, carbon linker length, and molecular weight were shown to influence physical properties and *in vivo* potency of the corresponding LNPs, and the inclusion of additional ester groups in IL tails was shown to reduce protein expression. Acrylate tails have also been employed in biodegradable IL structures, predominantly in combination with polyamine cores [107,144]. Whitehead and colleagues synthesized a library of 1400 degradable acrylate-tail ILs and identified four criteria capable of robustly predicting *in vivo* efficacy of the corresponding LNPs [107]. These criteria were the presence of a tertiary amine core, tails consisting of a 13-carbon chain linked to the acrylate, greater than two tails, and pK_a greater than 5.4, with pKa being the most influential factor. This study both illustrates the value of structure–function investigations of large IL libraries and presents clear design rules for new acrylate-containing ILs. Further, the coexistence of design rules for multiple structural features supports the idea that IL

structure influences several factors that determine LNP behavior. The mechanistic implications of minor IL structural variations could therefore change based on the nature of the variation and influence different facets of LNP behavior, including protein corona formation, endosomal escape, and cargo encapsulation. Thus, the sheer complexity of IL structure and its implications necessitates a deeper understanding of the role of each structural component to achieve an enhanced IL design process.

4.4. Branched lipids

ILs containing branched tail structures have been investigated for mRNA delivery and applied in clinical applications including the COVID-19 vaccines and CRISPR-Cas9 genome editing [91,110,140,145–149]. Hajj and colleagues identified an IL structure that consisted of the 306 polyamine core and acrylate tails. Introduction of a one-carbon branch into the tails increased delivery efficiency 10-fold over the straight tail version, which was attributed to strong surface ionization of the LNP containing the branched-tail structure at late endosomal pH [146]. It was then found that the branched-structure induced greater protein expression than C12-200 and MC3 and facilitated protein expression in all major liver cell types unlike many liver-targeted deliver systems [110]. While these studies demonstrated the high efficacy of branched-tail ILs, Hashiba and colleagues conducted the first systematic investigation of branched-tail ILs by synthesizing a library of 32 ILs with α-branched tails [147]. All structures consisted of an aminoalcohol head with two hydrophobic tails that varied in symmetry and total carbon number. *In vivo* screening of the library demonstrated that branched tails increased headgroup ionizability under acidic conditions and identified a top performer for gene editing applications. Another study used a combinatorial synthesis approach to compare branched-tail structures to their linear-tail equivalents across multiple polar headgroups and found that branched-tail structures led to greater mRNA delivery by inducing fewer surface charges and increased stability to mediate cellular uptake. Han and colleagues developed a combinatorial synthesis method to add branched tails to aminoalcohol heads using degradable linkers, enabling rapid and high-throughput synthesis of branched-tail structures using the one-pot, two-step, three-component reaction [149]. Screening of two libraries that varied in headgroup structure and tail length identified key structural criteria including total carbon number, symmetry, and headgroup to predict performance of new ILs in this class. Here, systematic structure–function studies explained the increased efficacy of branched-tail ILs and outlined clear design criteria for new structures.

4.5. Other lipids

Several other IL structural features have also been investigated. Mahon and colleagues systematically investigated the introduction of functional groups into IL tails and found that the success of introducing a functional group was dependent on the overall amine content and number of IL tails [150]. Tails that consisted of hydroxyl, ether, and carbamate functional groups predominantly increased delivery efficiency, demonstrating that the presence of functional groups can influence nanoparticle formation. Further, it was found that the most efficient ILs contained three or four amines, which is consistent with previous findings for optimal amine content [102,103]. A different study systematically investigated lysine-based ILs, which consisted of a zwitterionic lysine headgroup linked to a long-chain dialkylamine through an amide linkage, and found that tail saturation and headgroup composition affected siRNA knockdown by influencing IL protonation behavior and electrostatic membrane disruption [151]. Liu and colleagues further investigated zwitterionic lipid structures by investigating a combinatorial library of ionizable phospholipids, which contain a zwitterionic headgroup formed by an ionizable amine and a phosphate group, and three hydrophobic tails [152]. Systematic investigation of 572 structures identified a mechanism for increased endosomal escape that mediated *in vivo* efficacy and organ selectivity. The endosomal escape properties of these lipids were deduced by exploring the interactions of the corresponding LNPs with artificial endosomes. By mixing the two, the transition from a prototypical bilayer state to H_{II} phase was observed using ^{31}P NMR. Moreover, by incorporating a FRET reporter system within the artificial endosomes or the LNPs, the authors demonstrated that their novel lipids enhanced both lipid fusion and endosomal disruption. Although this approach deviates from the canonical four-component LNP delivery system since the phospholipid structure and IL pH-switching functionality are combined in the same structure, this class of structures is promising for selective organ targeting when employed in certain helper lipid combinations.

While these structural features increase LNP efficacy by promoting cellular entry and endosomal escape, the success of other structures is influenced by the specific interactions between IL chemical groups and mRNA. A study led by Moderna identified a novel aromatic squaramide IL structure by optimizing new structures for mRNA delivery [153]. Further investigation and advanced characterization of squaramide structures revealed that an optimal balance of intermolecular interactions including hydrogen bonding with sugars and pi-stacking with mRNA led to the success of the lead structure *in vivo*. This work demonstrates the value of considering non-traditional interactions between IL structural entities and mRNA beyond Coulombic attractions to develop LNPs with enhanced *in vivo* performance. Lastly, Da Silva Sanchez and colleagues investigated stereopure IL derivatives of C12-200 [154]. It was found that one stereopure enantiomer of C12-200 increased mRNA delivery 3-fold and 6-fold over the racemic IL and opposite enantiomer, respectively, due to higher *in vivo* tolerability. This result establishes the importance of considering the stereochemistry of LNP components when designing new structures, and is consistent with previous knowledge that stereochemistry is an important determinant of pharmacokinetics and safety of small-molecule drugs [155,156]. Interestingly, IL stereochemistry presents an alternative to the inclusion of esters or other biodegradable linkers in IL tails to improve LNP tolerability.

5. Future outlook and conclusions

LNPs have gained traction as delivery systems for RNA therapeutics. The clinical success of mRNA LNP vaccines during the COVID-19 pandemic demonstrated their effectiveness on a global scale, motivating the development of new LNP therapeutics for a wide range of diseases (Fig. 4). The collective body of literature on IL design has demonstrated that each segment of ILs can be optimized for a particular biological application, and that small molecular alterations can result in drastic changes in LNP performance. As ILs can broadly be segmented into the ionizable core, linker, and lipid, the structure of each component should be considered. The ionizable core, either monoamine or polyamine, largely determines the pK_a of the resulting LNP. This value can be modulated by adding nearby electron withdrawing or electron donating moieties. While ideal pK_a is often listed as ~ 6, it is important to adjust this value depending on the specific endosomal-lysosome pathway of the cell type of interest, as each cell type has slightly different biochemical pathways that could necessitate increasing or decreasing the pK_a. Moreover, a clear preference between monoamine and polyamine cores has yet to emerge, as each has shown promise in a variety of translational applications. The linker, though a less explored parameter, can enhance IL hydrophilicity and biodegradability. Linkers can be altered by adjusting the reacting electrophiles, which are commonly epoxides or acrylates. Underutilized electrophiles, such as isothiocyanates, aldehydes, and electron-poor aromatic groups, could provide unique structural templates. The lipid tail has proven to be an essential parameter in rational IL design. This is often examined by adjusting the lipid length; however, future IL designs should also test adding unsaturated groups to assist in mesophase transition during

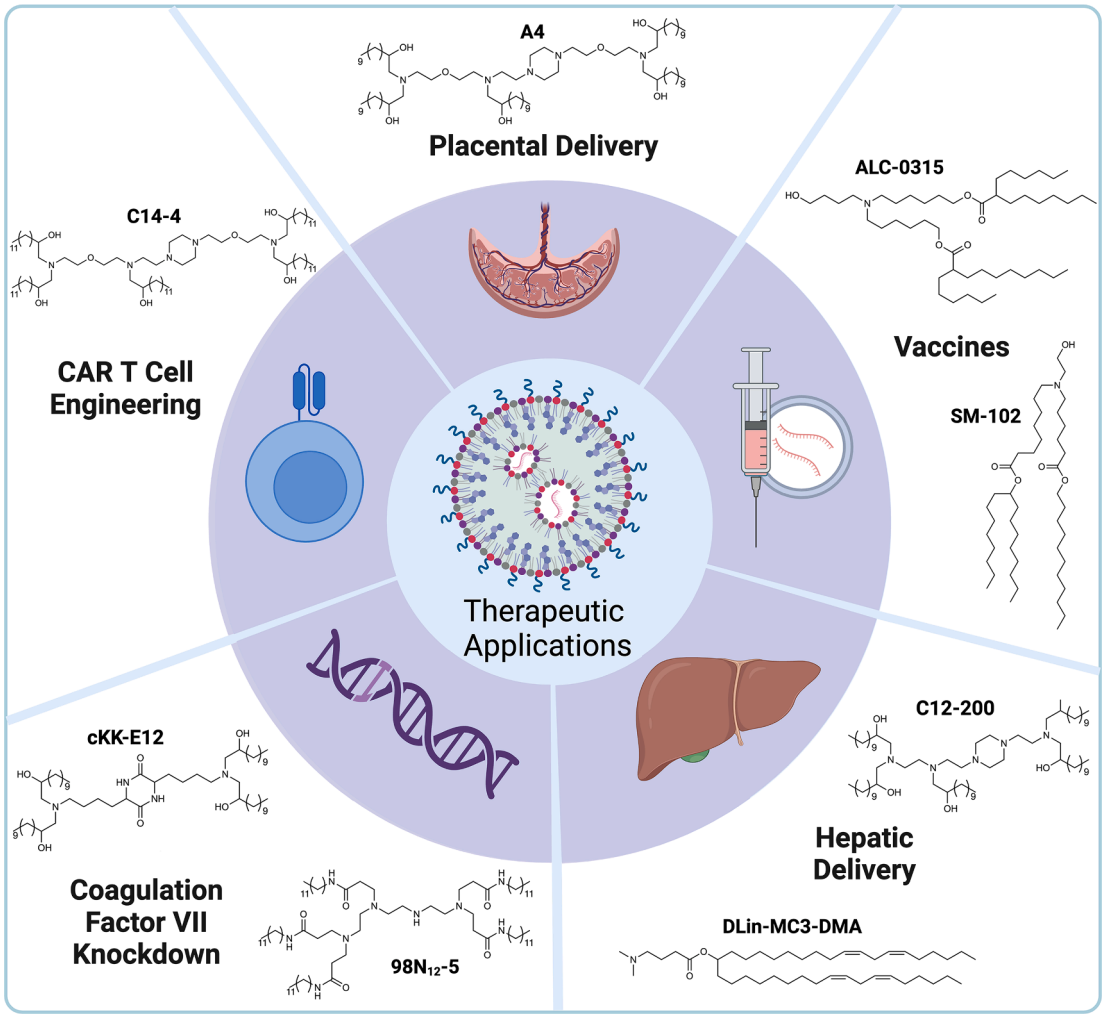

Fig. 4. Therapeutic applications of ionizable lipid structures. Overview highlighting IL structures (outer region) that correspond to specific therapeutic uses (inner ring). The structures of certain ILs have been optimized to enhance a specific therapeutic functionality of the corresponding LNP. While these structures have been investigated and reported for the highlighted purposes, this does not preclude their potential success in other applications.

endosomal escape. Branching also has emerged as an essential feature, where branching at the linker site, middle, and end of the lipid chain has enhanced LNP efficacy in different applications. Finally, as many ILs, particularly those similar to C12-200, exist as a combination of multiple stereoisomers, it will be important to begin identifying specific stereochemistries that are best suited for LNP-mediated transfection, especially as biological environments are inherently chiral. A summary of discussed IL structural parameters is found in Fig. 5.

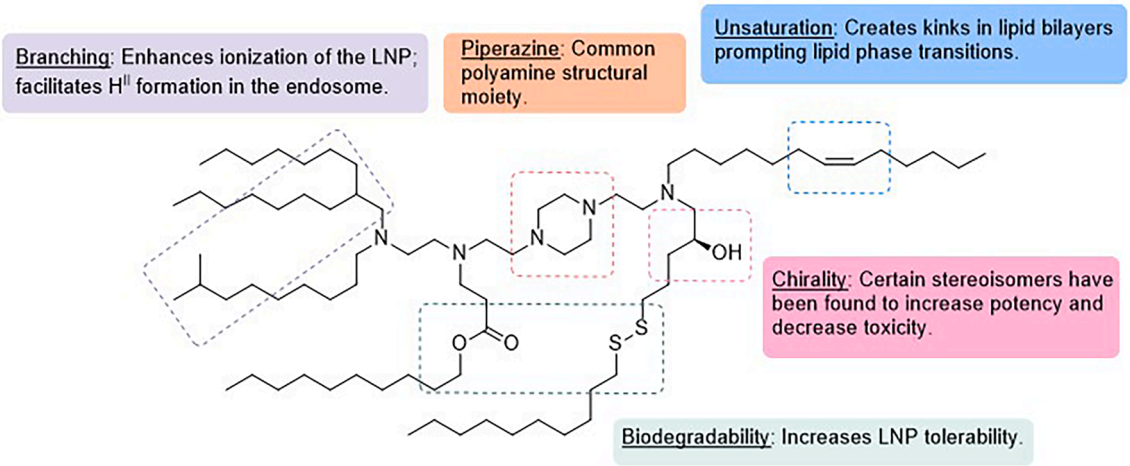

Fig. 5. Structural considerations when designing ionizable lipids. Each part of the IL can be optimized to enhance both efficacy and safety. This includes the mono- or polyamine core, lipid linker, and lipid tail.

While many LNPs have demonstrated success in ongoing clinical trials, other LNP therapeutics have failed to produce promising efficacy and safety data [157]. To better predict successful formulations when designing LNPs, it is crucial to first understand the differences between LNPs that succeed and fail in the clinic. Given the critical role of the IL in determining LNP efficacy, with minor IL structural changes often altering LNP efficacy, IL structure is a promising predictor of LNP success. To harness the predictive potential of IL structure, it is first necessary to develop a comprehensive understanding of how IL structure defines LNP function and the mechanisms underlying these relationships. There are several guiding questions that need further research to allow for rationally designed ILs, which include:

1. What is the relationship between IL pK_a and overall LNP pK_a?
2. How to achieve endosomal escape without permanently damaging the cell?
3. Does cell type impact the ideal pK_a range?
4. How does internal LNP packing during formulation impact overall delivery?
5. How does IL structure impact mesophase transitions in endocytic conditions?
6. What are the kinetic parameters for mRNA escape into the cytosol?

One major barrier hindering these answers is the lack of standardization in the LNP community. Factors such as lipid excipient purity, mRNA modifications, and formulation method all play a important role in determining the overall efficacy of an LNP formulation [158–160]. Such consensuses have been employed in other materials communities, including for perovskite photovoltaics, and have created rigorous testing protocols and universal qualifications standards that are paramount for external comparisons [161]. A second key barrier is the lack of methods to probe endosomal escape *in vivo*, as most methods either examine the LNPs themselves or within *in vitro* cultures. This is particularly essential for LNP research due to the growing number of studies that have observed poor correlation between *in vitro* and *in vivo* screening [18,149,162,163].

Robust IL structure–function studies have started to define these relationships, with some leading to early criteria for IL design [107]. Expanding these studies to examine a broader range of structures could lead to a comprehensive understanding of the implications of IL structure. Pairing these large studies with advanced characterization techniques could enable identification of the mechanisms responsible for these relationships. While this type of investigation could successfully enhance our understanding of IL structure, it is labor-intensive and time consuming to experimentally investigate a collection of IL structures large enough to define clear design rules. Artificial intelligence and machine learning strategies have shown success in drug discovery, leading to faster and more precise development processes [164,165]. Using similar techniques to conduct high-throughput investigations of new IL structures could greatly enhance our understanding of IL structure and its implications. From here, a clear IL design process could be defined for function-specific, cargo-specific, and organ-specific applications of LNPs. In practice, this could take the form of a short set of rules for IL design, similar to Lipinski's rules for small molecule drug design [166]. This would enable researchers to design effective and application-specific ILs by adhering to a clear and robust set of design rules. By empowering the identification of tailored IL structures that result in more effective LNPs for a wide range of diseases, this advancement would mark a new era of LNP development, allowing the delivery system to reach its fullest potential.

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.

Data availability

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

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