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Enhancing RNA-lipid nanoparticle delivery: Organ- and cell-specificity and barcoding strategies

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ABSTRACT

Recent advancements in RNA therapeutics highlight the critical need for precision gene delivery systems that target specific organs and cells. Lipid nanoparticles (LNPs) have emerged as key vectors in delivering mRNA and siRNA, offering protection against enzymatic degradation, enabling targeted delivery and cellular uptake, and facilitating RNA cargo release into the cytosol. This review discusses the development and optimization of organ- and cell-specific LNPs, focusing on their design, mechanisms of action, and therapeutic applications. We explore innovations such as DNA/RNA barcoding, which facilitates high-throughput screening and precise adjustments in formulations. We address major challenges, including improving endosomal escape, minimizing off-target effects, and enhancing delivery efficiencies. Notable clinical trials and recent FDA approvals illustrate the practical applications and future potential of LNP-based RNA therapies. Our findings suggest that while considerable progress has been made, continued research is essential to resolve existing limitations and bridge the gap between preclinical and clinical evaluation of the safety and efficacy of RNA therapeutics. This review highlights the dynamic progress in LNP research. It outlines a roadmap for future advancements in RNA-based precision medicine.

1. The rise of siRNA and mRNA therapeutics and the need for precision delivery

1.1. Lipid nanoparticle-enabled mRNA therapeutics

Ribonucleic acid (RNA)-based therapeutics such as small interfering RNA (siRNA) and messenger RNA (mRNA) are on the rapid rise in clinical development for genetic medicines. The most well-known clinical applications are perhaps the two mRNA-based COVID-19 vaccines from Moderna (Elasomeran/Spikevax®) and Pfizer/BioNTech (Tozinameran/Comirnaty®), both using ionizable lipid nanoparticles (LNPs) as non-viral gene delivery systems for formulating the mRNAs [1,2]. LNPs play a critical role in shielding the RNAs from ribonuclease (RNase)-mediated degradation and, more significantly, facilitating the escape of RNA from endolysosomal degradation to enter the cytosol — a process known as endosomal escape — upon the LNPs' cellular uptake via endocytosis [3,4]. As depicted in Fig. 1 (left panel), LNP-formulated mRNAs are essential for the in vivo translation of both endogenous and exogenous peptides/proteins. This delivery method enables the direct production of biological therapeutics, such as vaccination antigens [2], therapeutic proteins, e.g., erythropoietin [5], antibody therapeutics [6,7], novel cellular receptors, e.g., the chimeric antigen-receptors (CARs) for cellular immunotherapies [8], and agents for genome editing, specifically those encoding CRISPR-Cas9 mRNA and single-guide RNA (sgRNA) [9–11]. The incorporation of a replicase sequence upstream of the therapeutic mRNA transforms it into self-amplifying RNA (saRNA), enhancing the translation of therapeutic proteins [12]. Beyond the clinically approved mRNA COVID-19 vaccines, both Moderna and BioNTech are developing over forty mRNA therapeutics for a range of applications, from Zika virus vaccines [13], personalized cancer vaccines [14], tolerogenic autoimmune/allergy vaccines [15,16], and various protein therapeutics, [17] e.g., Human erythropoietin (hEPO) [18]. The recent FDA approval of the first CRISPR-Cas9/sgRNA-based therapy (exagamglogene autotemcel/Casgevy™, delivered ex vivo via electroporation) further highlighted the potential of mRNA in encoding CRISPR-Cas for gene editing in various

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preclinical studies [19–22]. Significant milestones were achieved with two-phase I trials of lung targeting CRISPR-mRNA-LNPs dosed in 2024 for treating cystic fibrosis (RCT2100, NCT06237335) and primary ciliary dyskinesia (RCT1100, NCT05737485), highlighting the rapidly expanding use of the LNP-enabled gene editing [23].

1.2. siRNA therapeutics enabled by N-Acetylgalactosamine (GalNAc)-conjugates and lipid nanoparticle formulations

siRNA molecules, typically 20–25 nucleotides per strand and forming a double-stranded structure, play a crucial role in the RNA interference (RNAi) pathway. These double-stranded RNAs modulate gene expression by recruiting the RNA-induced Silencing Complex (RISC), which results in the targeted degradation of mRNA and the suppression of gene and protein expression (see Fig. 1, right panel) [24]. Initiation of this process involves the Argonaute protein (AGO) family, which facilitates the removal of the passenger (sense) strand of the siRNA [25], thus allowing the guide (antisense) strand to direct the RISC to the complementary mRNA sequence. This interaction leads to mRNA cleavage and degradation, silencing gene function [26,27]. Endogenously, siRNAs contribute to the regulation of gene expression and genomic stability. Exogenously, they have been synthesized for targeted gene knockdown in mammalian cells since 2001 [28]. The clinical trajectory of siRNA therapeutics began with the FDA approval of Patisiran (Onpattro®) in 2018 for the treatment of hereditary transthyretin amyloidosis (hATTR) [29]. Following this, four additional siRNA drugs have been authorized for hATTR (Vutrisiran/ Amvuttra®), acute hepatic porphyria (Givosiran/ Givlaari®), primary hyperoxaluria type 1 (Lumasiran/ Oxlumo®), and to lower low-density lipoprotein cholesterol (Inclisiran/ Leqvio®) [30–33].

1.3. The rising need for precision delivery of siRNA and mRNA therapeutics

siRNA therapeutics exhibit distinct dose-dependent pharmacodynamics and require periodic administration to sustain their efficacy [34], similar to small molecule drugs, except that siRNA therapeutics have significantly longer dosing intervals ranging from every 3 weeks to every 6 months [35]. The enduring effect of siRNA is unique and distinct from traditional sustained-release/long-acting dosage forms known for small molecular drugs. This necessitates a greater need for precision in RNA therapeutic delivery as the off-target effect will also become long-lasting [36,37]. In contrast, mRNA therapeutics often result in transient protein production [38]. Therefore, the most effective approach to harnessing their therapeutic potential is often through leveraging the immune responses, as seen with mRNA vaccines. A two-dose regimen can induce a robust and sustained immune response. To ensure strength and safety, careful design and precision delivery of mRNA therapeutics are essential. This maximizes efficacy and minimizes the risk of autoimmunity and other off-target effects [39]. In the context of CRISPR/ Cas9 gene editing, the consequences of the off-target effects could be significant [40,41]. Precision RNA delivery is critical when utilizing LNPs for delivering Cas9-encoding mRNA and sgRNA. Achieving organ-specific and cell-specific delivery for genome editing remains one of the

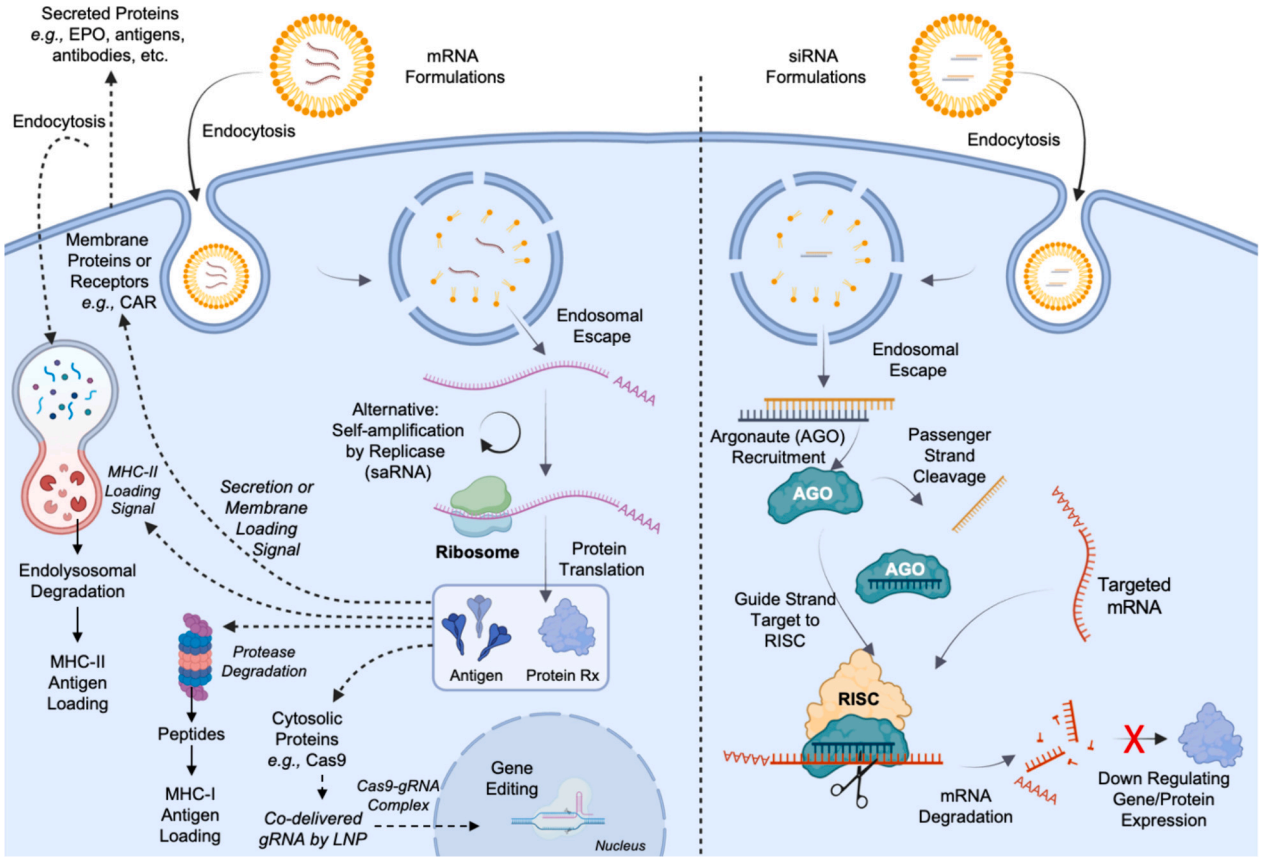

Fig. 1. Mechanism of Action of mRNA and siRNA therapeutics. mRNA and siRNA therapeutics both aim to alter protein functions by enabling protein translation or inhibiting protein translation. When formulated in lipid nanoparticles, both enter cells via endocytosis, which makes the "endosomal escape" of RNA-LNPs the primary biological barrier in their bioavailability. By carefully designing RNA sequences, mRNA serves as multifunctional platforms to produce intracellular proteins or antigens, membrane-bounded proteins or receptors, and secreted proteins that could also be recycled and endocytosed by the cell (left panel). On the contrary, siRNA enabled sequence-specific degradation of mRNA through the recruitment of RNA-induced Silencing Complex (RISC), leading to down-regulation of protein expression (right panel), which could serve as a stand-alone therapy or as emergency brakes for mRNA therapies to counteract any unintended consequences of mRNA therapy by silencing the same mRNA (This figure is created with BioRender.com).

biggest challenges for ensuring safety and efficacy [41,42]. As the field of RNA therapeutics burgeons, the success of these modalities hinges on the precision of delivery systems to minimize off-target effects [43]. While siRNA and mRNA therapies offer distinct pharmacological advantages and challenges, their full potential can only be capitalized with precision delivery.

Following the rapid development of both RNA therapeutics and lipid nanoparticles (LNPs), recent reviews have addressed various aspects of this multidisciplinary field, such as organ-specificity [44], non-liver targeting LNPs [45], spleen-targeted LNPs [46], mRNA-LNPs design [47,48], and clinical trials of siRNA therapeutics [49], etc. This review aims to serve as a one-stop shop by holistically examining the history and advancements in liposomal RNA delivery systems. We focus on state-of-the-art organ-specific and cell-specific RNA delivery strategies using LNPs and discuss the latest applications of DNA/RNA barcoding technologies for enhancing the targeting-specificity of LNPs. Additionally, we summarize current clinical trials of mRNA and siRNA therapeutics to highlight these innovative RNA therapies' translational potential and future directions.

2. A 60-year journey from liposomes to RNA-delivering ionizable lipid nanoparticles

Liposomal membraned formulations such as liposomes and LNPs are clinically approved therapeutic carriers capable of delivering small molecules and biologics such as proteins, peptides, and nucleic acids [50–52]. Liposomes and LNPs are self-assembled in aqueous buffers but are structurally different from each other. Liposomes typically consist of one to multiple lipid bilayers for entrapping large amounts of aqueous buffer inside the lipid bilayer membrane, often referred to as the aqueous core of the liposomes. LNPs' core predominately comprises lipids and hydrophilic small water pockets formed by ionizable lipids and nucleic acids in their inner core [53], shelled by a lipid mono-layer [54] or lipid bilayer membrane [55]. As demonstrated by the COVID-19 mRNA vaccine, LNPs have demonstrated their transformative potential for enabling RNA therapeutics. Finding the path for enabling this journey is a collective effort built over 60 years of research on lipid chemistry and formulations, as summarized in Fig. 2 (see **Table S1** for indexed references).

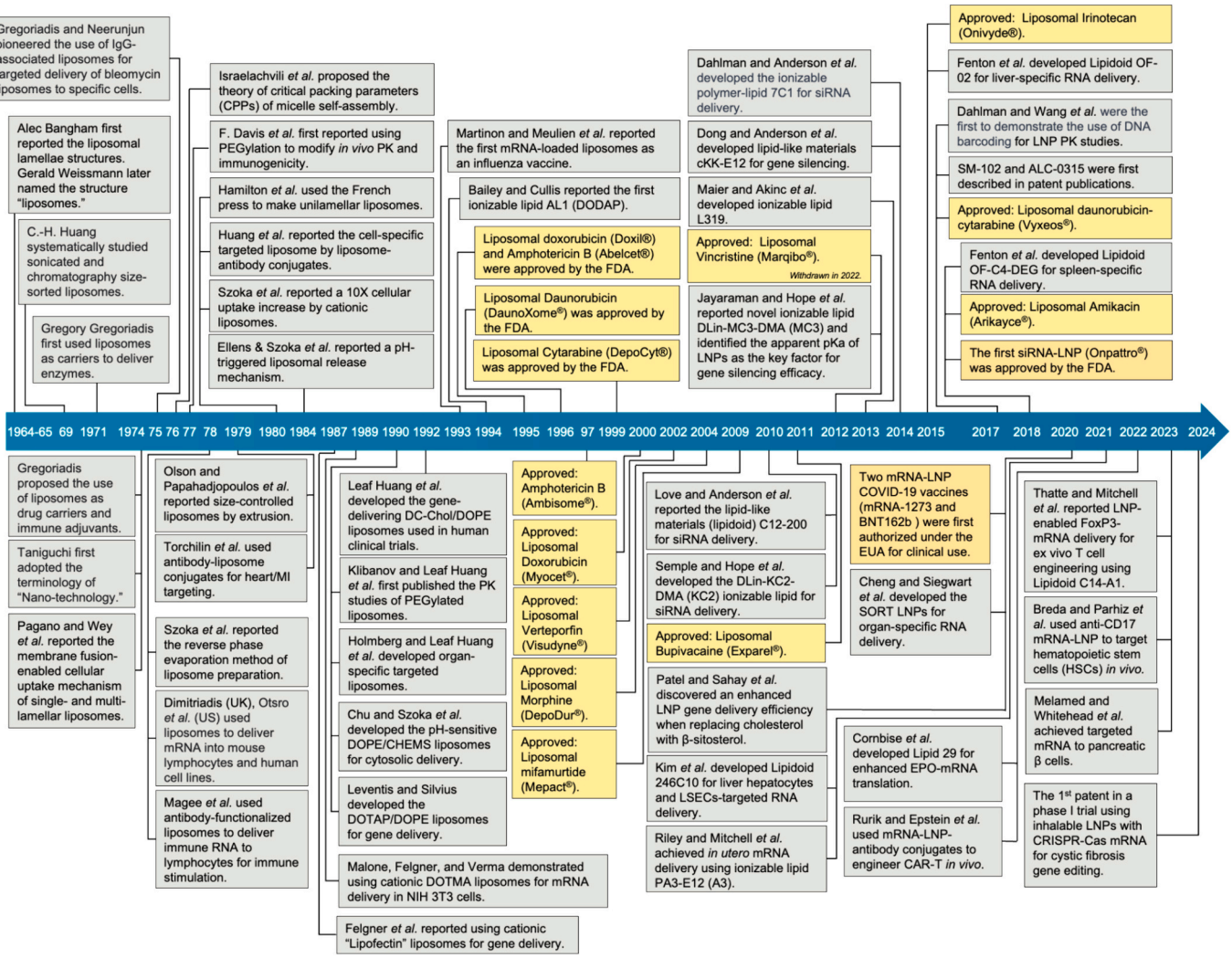

Fig. 2. A 60-year journey from liposomes to ionizable lipid nanoparticles for RNA delivery. This retrospective timeline summarizes the development of liposomes and lipid nanoparticle formulation, with selected milestones highlighting key discoveries for drug and gene delivery. Full itemized information is summarized in **Table S1**.

2.1. Early discoveries of liposomes for therapeutic delivery

The journey into the realm of the minuscule, which would eventually lead to the development of RNA-delivering liposomal structures, was inspired by Richard Feynman's visionary 1959 talk, "There's Plenty of Room at the Bottom." This lecture sparked a wave of research focused on the scientific exploration at the microscopic scales and the advancement of sophisticated microscopy techniques. Coinciding with this surge was Alec Bangham's seminal microscopy discovery of liposomal lamellar structures in 1964, initially described as "swollen phospholipids." By controlling the sizes and properties of the liposomal lamellar structures using sonication and chromatography [56], these phospholipid vesicles, resembling cellular membranes, have since evolved into highly biocompatible carriers capable of encapsulating diverse therapeutic agents as delivery systems, first proposed and demonstrated by Gregory Gregoriadis in the early 70s for in vivo enzyme delivery [57–59]. Coinciding with the burst of nanotechnology [60], liposomes soon evolved from basic biomembrane/vesicles to versatile therapeutic carriers due to their ability to encapsulate biological molecules [58,59,61]. In 1978, one year after Ostro et al. incorporated high molecular weight RNA into liposomes, Dimitriadis (UK) and Ostro et al. (US) independently reported their successful use of liposomal formulations for delivering mRNA for inducing protein translation in mouse lymphocytes and human cell lines [62,63]. This officially opened the era of developing gene-delivering liposomal formulations. During that time, liposomal formulations typically comprised egg- or soy-derived charge-neutral phosphatidylcholine (PC) lipids with a blend of varying amounts of cholesterol. Encapsulating hydrophilic RNAs into PC-based liposomes has one major challenge, which is the low encapsulation efficiency when using simple lipid film hydration methods (i.e., making liposomes by dissolving hydrophilic cargo such as RNAs in aqueous buffers then vortexed it with dried lipid film to foster self-assembly of multi-lamellar liposomes). In the same year, F. Szoka, Jr. and D. Papahadjopoulos reported the "*reverse phase evaporation method*" that largely improves the encapsulation efficiency of hydrophilic molecules. This is achieved by forming reverse-micelle emulsion from the lipids using an immiscible buffer and solvent pair, followed by solvent evaporation to form large unilamellar liposomes [64]. The lipid film hydration method and reverse phase evaporation have been widely used in studying RNA delivery since then.

2.2. Cationic liposomes for gene delivery

Building on early works that studied cellular interactions of positively charged liposomes, which showed higher cell association compared to negatively charged liposomes [65], cationic lipids, e.g., N-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA), were first reported for use in mRNA delivering liposomes in the late 80s [66,67]. Cationic liposomes complex nucleic acids via charge interactions, forming mixtures known as "lipoplexes." At that time, lipoplexes were used as a much-improved gene transfection method, compared to the cationic polymer-based transfection method using diethylaminoethyl-dextran (DEAE-Dextran) and the calcium phosphate co-precipitation methods developed in the 60s' and 70s' [68,69]. The advancement of cationic liposomes for gene delivery finally entered human clinical trials with leading formulation using cationic cholesterol derivatives with cationic lipid blend, i.e., DC-Chol/DOPE [70–73]. Parallel to cationic liposomes is the development of pH-sensitive/responsive liposomal formulations by Szoka et al. with compositions like pH-responsive cholesterylhemisuccinate (CHEMS) and cationic dioleoylphosphatidylethanolamine (DOPE), which significantly improved the cytosolic delivery of non-pH-sensitive formulation such as the mixture of CHEMS and neutrally charged dioleoylphosphatidylcholine (DOPC) [74]. This is achieved by incorporating pH-sensitive lipids capable of changing their self-assembly properties, thus destabilizing the lipid bilayer at lower pH values, resembling the acidifying process of the endosome to lysosome trafficking when liposomes are endocytosed [75]. Escaping endolysosomal trafficking is recognized as a critical biological barrier for the efficacy of liposome-encapsulated mRNA and siRNA.

2.3. The critical packing parameters theory of lipid self-assembly

Liposomes and LNPs are both made of self-assembled lipid structures, yet they are structurally different, driven by their functional need to encapsulate different therapeutic molecules. Despite the differences, liposomes and LNPs share nearly identical engineering principles, such as the critical packing parameters (CPPs) theory of lipids/micelles self-assembly. The concept of CPPs was first proposed by Israelachvili et al. in 1976, which provides a generalized self-assembly theory linking thermodynamics, molecular interactions free energies, and geometry [76]. The CPP is represented by this equation: $N_s = V_c / a_e * L_c$, where V_c = the volume of the hydrophobic chain, i.e., the volumetric space occupied by the hydrophobic part of the molecule, which is typically averse to water. The L_c = length of the hydrophobic chain, usually given in nanometers (nm). The a_e = the area per molecule at the hydrophilic-hydrophobic interface also called the effective headgroup area. This is the area occupied by the hydrophilic (water-attracting) part of the molecule at the interface where the molecule meets water. It's an important factor in determining how the molecules pack together. Amphiphilic surfactants and soap molecules typically have an $Ns \ll 1/3$, representing their smaller hydrophobic tails compared to the hydrophilic headgroups, leading to a conical-shaped molecule favoring the formation of spherical micelles. A $1/3 \leq N_s < 1/2$ typically forms cylindrical micelles. A flexible bilayer is typically formed when $1/2 \leq N_s < 1$, and the planer bilayer is formed when N_s values ≈ 1. When $N_s > 1$, the lipids will favor the formation of inverse micelles or other structures where the hydrophilic headgroups are internalized. Assembling lipid building blocks with different CPPs into liposomes or LNPs is like packing differently shaped objects (representing the lipid molecules) into boxes (representing the space in water). Depending on the shape of the objects, nature arranges them differently to fit (self-assemble) most efficiently. Similarly, the shape of the lipid molecules (influenced by their CPP) determines how they'll pack together in water, leading to different assembled structures. The ability of lipids to assemble into varying structures depending on the geometry of the lipid components is known as "lipid polymorphism."

2.4. The development of ionizable lipids and lipid nanoparticles for gene delivery

Observations on cationic liposomal formulations for gene delivery have led to extensive lipid polymorphism studies that ultimately give birth to the first ionizable cationic lipids 1,2-dioleoyl-3-dimethylammonium propane (DODAP, also known as AL1) in 1994 by Bailey and Cullis [77]. DODAP liposomes have an apparent pKa of 6.58, which forms cationic liposomes at pH 4 when co-formulated with helper supporting lipids such as phosphatidylcholine (PC), dioleoylphosphatidylethanolamine (DOPE), and cholesterol. However, fusion of lipid membranes was observed when pH raised from 4.0 to 7.5 due to the formation of one particular lipid polymorph, i.e., the H_2 Phase (Hexagonal Phase). The H_2 phase refers to a specific arrangement in which the lipid molecules organize themselves into cylindrical tubes that pack together in a hexagonal pattern, destabilizing lipid bilayers. This observations on pH-dependent lipid polymorph of ionizable lipids as the pH decreases, from L_2 (inverse micelle), H_2 (hexagonal), and Q2 (cubic) to lamellar structures, have led to the creative use of ionizable lipids such as D-Lin-MD3-DMA (MC3) or RNA delivery via forming LNPs [78]. This enabled the first FDA approval of LNP-formulated siRNA Patisiran (Onpattro; Alnylam) in 2018 [79–82]. Similar lipid molecular designs and pH-dependent self-assembly principles were adapted for ionizable lipids ALC-0315 (BioNTech/Pfizer) and SM-102 (Moderna) for delivering the

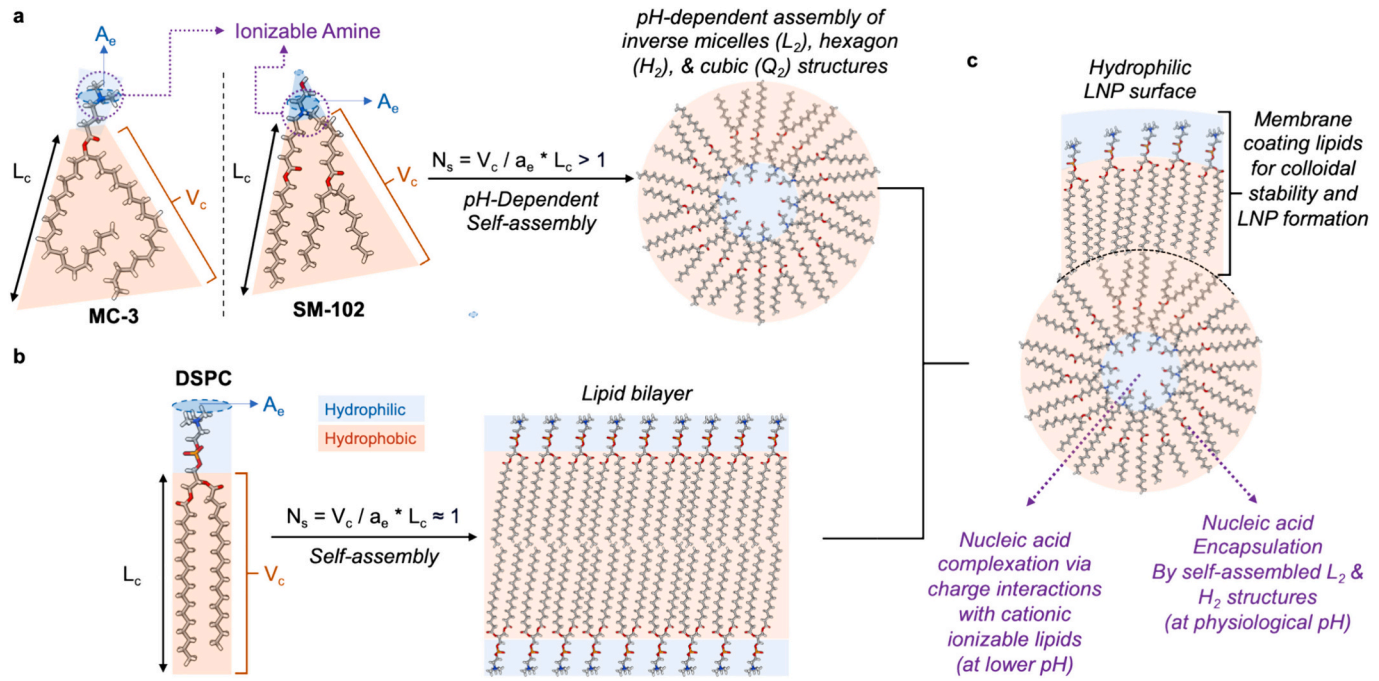

Fig. 3. Demonstration of the Critical Packing Parameter (CPP) and its application in designing ionizable lipids for effective mRNA delivery in lipid nanoparticles. CPP is a determinant of lipid molecular arrangement in aqueous environments, defined by the equation $N_s = V_c / a_e * L_c$, where V_c = the volume of the hydrophobic chain, L_c = length of the hydrophobic chain, a_e = the effective surface area of the hydrophilic head. The most likely self-assembled structures of lipid molecules in water may differ depending on their corresponding CPP. **(a)** Gene-delivering ionizable lipids such as MC-3 and SM-102 have a much larger V_c and a much smaller head group, yielding a CPP >1, conducive to forming pH-responsive, non-lamellar structures like inverse micelles, with internal ionizable heads and external hydrophobic tails. [78,268]. **(b)** In contrast, membrane lipids such as DSPC with a CPP approximately equal to 1 result in a bilayer structure due to its cylindrical shape, with hydrophilic heads at the water interface and hydrophobic tails tucked away. **(c)** At lower pH levels, the ionizable amines on the SM-102 headgroups become cationic, facilitating electrostatic complexation with anionic nucleic acids. This interaction ensures the nucleic acids are securely encapsulated within the interior, with the hydrophobic tails of SM-102 oriented outward. However, such a hydrophobic exterior is inherently unstable in aqueous environments. To overcome this, additional membrane-forming lipids are incorporated into the nanoparticle formulation. These lipids assemble into a stabilizing hydrophilic monolayer around the exterior, providing a compatible interface with the aqueous medium for enhanced colloidal stabilities, which is essential for the practical application of the lipid nanoparticles for mRNA delivery.

COVID-19 mRNA vaccine [78]. As shown in Fig. 3a, gene-delivering ionizable lipids such as MC-3 and SM-102 have a much larger Vc and a much smaller head group, yielding a CPP >1, conducive to forming pH-responsive, non-lamellar structures like inverse micelles, with internal ionizable heads and external hydrophobic tails. [68,240]. In contrast, membrane lipids such as DSPC with a CPP approximately equal to 1 result in a bilayer structure due to its cylindrical shape, with hydrophilic heads at the water interface and hydrophobic tails tucked away (Fig. 3b). At lower pH levels, the ionizable amines on the SM-102 headgroups become cationic, facilitating electrostatic complexation with anionic nucleic acids. This interaction ensures the nucleic acids are securely encapsulated within the interior of the ionizable inverse micelles, with the hydrophobic tails of SM-102 oriented outward. However, such a hydrophobic exterior is inherently unstable in aqueous environments. To overcome this, additional membrane-forming structural supporting lipids are required for the formation of stable LNPs. This external lipid membrane layer provides a hydrophilic interface with the aqueous medium and enhances colloidal stabilities, leading to the final LNP formulations used in the clinics (Fig. 3c).

3. Overviews of lipid components in liposomes and lipid nanoparticle formulations

The rapidly evolving field of LNPs for RNA delivery has seen an explosion of new cationic and ionizable lipids, making it challenging to remain abreast of developments without a foundational understanding of the lipids involved. Fig. 4 categorizes the lipids utilized in liposome and LNP formulations into seven primary groups: saturated and unsaturated fatty acids (Fig. 4a, section 3.1); phospholipids, e.g. phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphate (PA) (Fig. 4b, section 3.2); polyethylene glycol conjugated (PEGylated) lipids (Fig. 4c, section 3.3); cationic lipids and helper lipids (Fig. 4d, section 3.4); cholesterol and sterol-derivatives (Fig. 4e, section 3.5); ionizable inverse micellar lipids (Fig. 4f, section 3.6); and ionizable lipidoids (lipid-like multi-tail molecules, Fig. 4g, section 3.7). This simplified classification provides rough guidance in the vast chemical landscape of lipids used in lipid-formulated delivery systems. In the forthcoming sections, we aim to provide an account for these lipid categories, offering insights into the cutting-edge advancements in lipid-facilitated RNA delivery.

3.1. Saturated and unsaturated fatty acids

Fatty acids (Fig. 4a) are the basic building blocks of the hydrophobic lipid tails and major factors for determining critical packing parameters: the length of the lipid chain (Lc) and the volume of the hydrophobic lipid chain (Vc). To drive the critical packing parameter towards inverse micelles, unsaturated fatty acids with one to five unsaturated bonds are often used to increase the bulkiness of the lipid tails when synthesizing gene-delivering ionizable lipids.

3.2. Phospholipids

PCs such as DPPC (dipalmitoyl phosphatidylcholine) and DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) are fundamental components in assembling lipid bilayers (Fig. 4b). These bilayers, which are

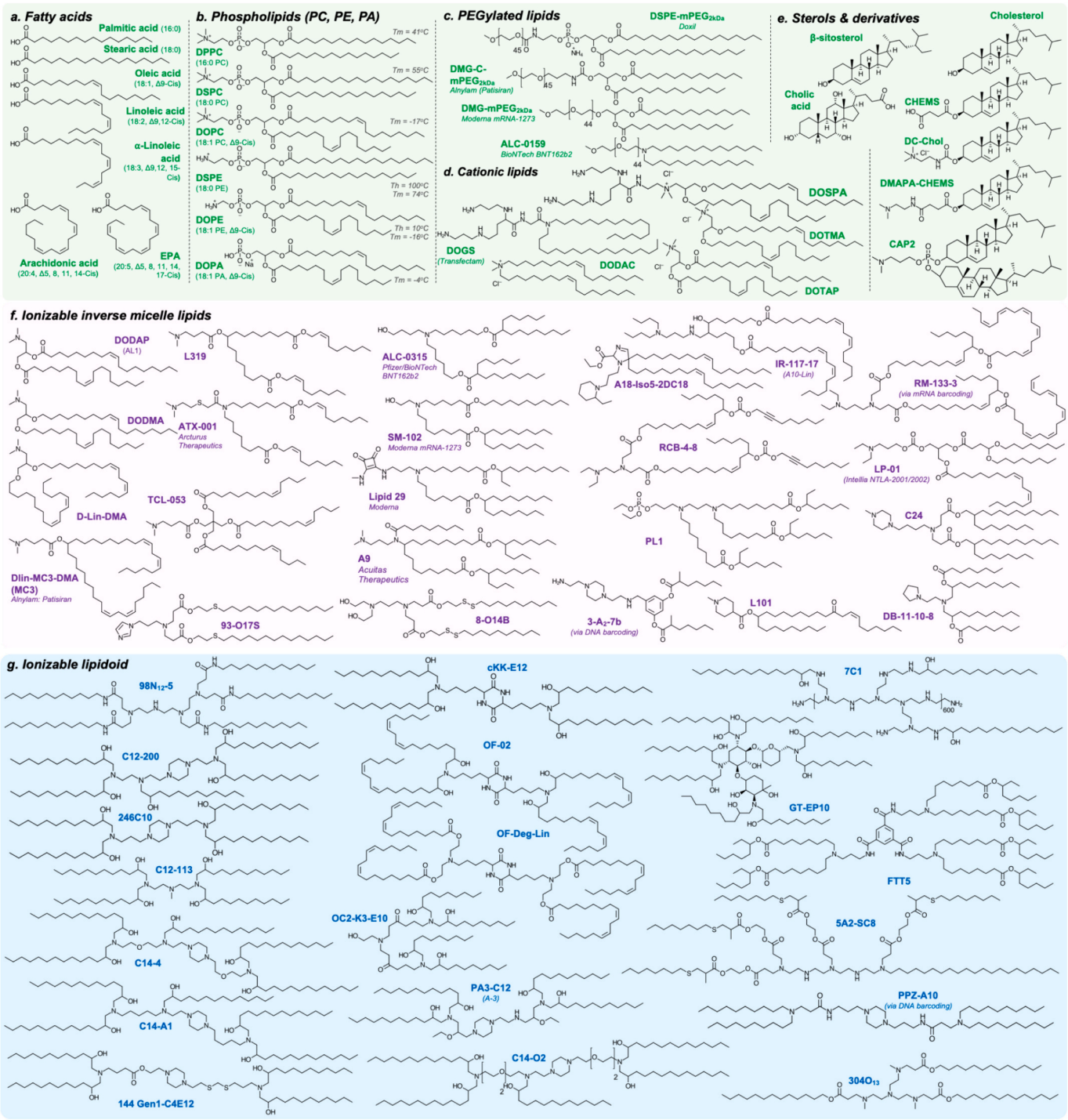

Fig. 4. Lipids for formulating RNA delivering ionizable lipid nanoparticles.

biomimetic mirrors of cell membranes and nuclear envelopes, excel in encapsulating nucleic acids and serving as selective barriers for molecular transport. The intrinsic properties of these bilayers underpin the design of liposomes and LNPs, providing the necessary encapsulation and protection for therapeutic delivery. The bilayer's behavior and functionality hinge on the physicochemical characteristics of the membrane structural lipids. The lipid tails' length and saturation level govern the bilayer's fluidity and phase/melt transition temperature (T_m). Lipid bilayers below T_m are packed in a more organized gel state with lower membrane mobility and permeability. Bilayer above the T_m has more lipid mobility, leading to less order packing and higher membrane permeability. Long saturated tails confer increased stability and a higher phase transition temperature, resulting in more rigid bilayers compared to short saturated lipid tails. Conversely, the incorporation of unsaturated fatty acids like oleic and linoleic acid disorganized the lipid bilayer, leading to significantly reduced Tm, enlarging the hydrophobic volume of the tails, altering the critical packing parameter, and decreasing the bilayer stability [83,84]. Lipid headgroups also influence the T_m, which generally follows the trend of PC < PS (phosphatidylserine) < PE (phosphoethanolamine) < PA (phosphate) [85]. In addition to the melt transition temperature, the hexagonal phase transition (T_h) of PE is used to describe the transition between the lamellar

bilayer and the inverse hexagonal phase, which destabilizes the bilayer (bad for liposome formation) and favors inverse hexagonal assembly (good for encapsulating RNAs in LNPs, to be stabilized by lipid monolayer, as shown in Fig. 3c). Similar to the T_m, the presence of unsaturated lipid tails also significantly reduces the T_h for PE [78,86,87].

3.3. PEGylated lipids

PEGylation, i.e., chemically conjugating polyethylene glycol on biomolecules, was first reported by Frank Davis in 1978 as a strategy to reduce immunogenicity while increasing blood circulation time [88]. The discovery of the enhanced permeation and retention (EPR) effect of macromolecular in solid tumors in 1986 [89] has led to great interest in developing PEGylated lipids (e.g., DSPE-PEG_{2kDa}, Fig. 4c), which have since been tested in liposomes and found to significantly increase blood circulation time, first reported in the literature by Leaf Huang et al. in 1990 [90], which ultimately led the first approval of liposomal drug, the PEGylated liposomal doxorubicin in 1995 [91]. Through prolonged blood circulation, PEGylated liposomes are able to take advantage of the EPR effect to enhance tumor drug accumulation, leading to a series of clinical approvals of liposomal chemotherapeutics, e.g., daunorubicin, cytarabine, irinotecan, etc. (Fig. 2 and **Table S1**), which opened the golden era of NanoMedicine and bio-nano interfacial studies [92–98,269,270]. Many more liposomal chemotherapeutics are still in development, especially for those capable of inducing cancer immunogenic cell death, e.g., mitoxantrone and oxaliplatin [50–52,99,100]. PEGylated lipids are also present in LNPs, e.g., DMC-C-PEG_{2kDa} (Patisiran/Alnylam), DMG-$mPEG_{2kDa}$ (Moderna), and ALC-0159 (Pfizer/BioNTech), due to their critical roles for enhancing formulation stabilities and altering LNP's pharmacokinetics upon systematic injections [101–103].

3.4. Cationic lipids and helper lipids

Cationic Lipids, e.g., DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), DOGS, DOSPA, DOTMA, etc. (Fig. 4d), are positively charged amino lipids that are essential for the formation of complexes with negatively charged phosphodiester backbone of RNA molecules to form the "lipoplex." Their primary function is to encapsulate the RNA and protect it from enzymatic breakdown. The cationic charges also promote cellular uptake by fostering electrostatic attractions with the cell membranes, which are generally negatively charged. Compared to DOTMA, the first cationic lipid used for RNA delivery, DOTAP changes the lipid tail conjugation from the ether (more stable) to ester (more degradable and cleavable), which is more biocompatible/biodegradable but less efficient in gene transfection in vivo [104]. DOPE (dioleoylphosphatidylethanolamine, Fig. 4b), a helper lipid, when combined with DOTMA or DOTAP, adds to the flexibility and fusogenic potential of the liposome or LNP membrane [105]. This fusion is crucial for the delivery system, as it aids in merging with the cell membrane and facilitating the release of RNA into the target cells. Liposomes and LNPs made of DOTAP/DOPE can be tailored to exhibit a range of zeta potentials, resulting in cell-type varying gene delivery efficiency in vitro [106].

3.5. Cholesterol and sterol-derivatives

Sterols (Fig. 4e), such as cholesterol, are planar, rigid, hydrophobic molecules present in abundance for stabilizing natural cell membranes [107,108]. Increasing cholesterol concentration in the bilayer inhibits lipid movements, thus decreasing membrane fluidity, making the bilayer more rigid. When used in high concentrations, such as in clinically approved liposomal formulations (nearly 40% in molar ratio), the melting phase transition temperature of the liposomes could completely disappear, leaving highly stable liposomes suitable for drug encapsulation [109]. In the context of gene delivery, cholesterol serves as a helper lipid for cationic DOTAP formulations to stabilize the lipoplexes and improve gene transfer efficiency [110]. As successful gene delivery requires the use of membrane-destabilizing lipids, cholesterol is also routinely used in LNP formulations to stabilize the formulation. Substituting cholesterol with other sterol derivatives has been shown to alter LNP biodistribution and gene delivery efficiency [111,112]. Alternatively, acidic cholesterol derivatives such as cholesteryl hemisuccinate (CHEMS) are used to formulate pH-responsive gene delivery liposomes when co-formulated with non-bilayer lipid DOPE. CHEMS is negatively charged at natural pH, which stabilizes DOPE to form liposomes. When protonated at a lower pH and losing the negative charge, the CHEMS can no longer stabilize DOPE; thus, the bilayer is destabilized, favoring the gene delivery [113]. This ionizable property of CHEMS has also been used to stabilize bilayer-embedded cationic lipid drug conjugations for tumor targeting [52]. On the other hand, Cationic derivatives of cholesterol, such as DC-Chol, could also be co-formulated with DOPE as cationic liposomes as the first cationic liposomes being tested in clinical trials in 1992 [70,71]. More recently, ionizable lipid MC3-DMA-inspired DMAPA-CHEMS and dual-cholesterol cross-linked amino-phosphate (CAP2) were able to form ionizable LNPs for siRNA and mRNA delivery [114,115]. In addition to chemically synthesized cholesterol derivatives, natural sterols such as β-sitosterol were found to enhance the endosomal escape of LNPs, facilitating RNA delivery [111,116]. Replacing cholesterol with bile acid was found to alter the biodistribution of mRNA-LNPs and reduce liver distribution [112].

3.6. Ionizable inverse micellular lipids

The earliest developed ionizable lipid is the 1,2-dioleoyl-3-dimethylammonium-propane (DODAP, also known as AL1, Fig. 4f) [77]. DODAP is structurally similar to the cationic gene delivering lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) with just one difference in the head group. DODAP has a tertiary amine head (ionizable) compared to DOTAP's quaternary amine head group (always cationic). This ionizable head enables pH-dependent lipid polymorph, as mentioned in section 2.4, favoring hexagonal/inverse micellular assembly with a critical packing parameter >1. The ionizable head group of DODAP (ester tail) and DODMA (ether tail) results in less toxicity compared to cationic cousins DOTAP (ester tail) and DOTMA (ether tail) [117]. Increasing the numbers of cis-unsaturated bonds in the DOTMA tails generates D-Lin-DMA (di-linoleyloxy tails, two cis-double bonds/tail) and D-Len-DMA (di-linolenyloxy tails, three cis-double bonds/tail). D-Lin-DMA was found to be the most fusogenic and the most efficient one for siRNA delivery and gene knock-down, followed by D-Len-DMA, with DOTMA being the least efficient of the three lipids [118]. Continuously optimizing the head group chemistry landed a pKa value at the sweet spot of ~6.4, which led to the clinical use of DLin-MC3-DMA (MC3) [29,82,119]. Structured derivatized from MC3, lipid L319 introduced ester-conjugated unsaturated tails for enhanced biodegradability, followed by a head group-modified version ATX-001 [120]. Similarly, TCL053 kept the MC3 head group but replaced the linoleyloxy tails with three ester/degradable lipid tails, exhibiting low immunogenicity for repeated dosing in skeletal muscle [121]. Ionizable lipids with amino alcohol head groups, e.g., ALC-0315 and SM-102, showed enhanced endosomal escape with enhanced mRNA translation; both adopted cleavable ester linkages to reduce in vivo circulation half-life and live accumulation (compared to MC3) and thereby enhance biocompatibility [122,123]. Derivatizing from SM-102 to replace the hydroxyl headgroup with squaramide led to the Lipid 29 with significantly increased in vivo mRNA-protein translation for model protein hEPO [18]. Ester conjugated cleavable tail design has dominated the chemical space of ionizable inverse micellular lipids, e.g., A9 [124], IR-117-17 (A10-Lin) [22], RCB-4-8 [21], PL-1 [125], L101 [126], RM-133-3 [127], LP-01 [128,129], C24, 3-A_2-7b [20], and DB-11-10-8 [130], etc. Replacing ether/ester with thioether and bio-reducible disulfide bonds has also been attempted in 93-O17S [131], 8-O14B [132], and O12B/O16B/

N16B derivatives, providing alternative degradation mechanism in the ionizable lipid designs [133,134].

3.7. Ionizable lipidoids

Parallel to the development of the traditionally looking ionizable lipids, high-throughput polymer synthesis using ultra-large-scale automation-assisted combinatorial chemistry rapidly expanded the chemical space for gene delivery biomaterials, screening hundreds to thousands of structural derivatives of polycationic, pH-responsive, and biodegradable polymers in the early 2000s by Akinc, Anderson, Lynn, and Langer et al. [135–139]. This is achieved using amine and parallel synthesis compatible chemical reactions, e.g., Michael addition (reacting with alkyl-acrylate or alkyl-acrylamides), reductive amination [140], ring-opening reactions, etc. [141]. Using the parallel synthesis toolboxes validated in prior polymer works for synthesizing "lipid-like" molecules called "lipidoids." The first lipoids library contained a molecular library with amide linkages, two or more alkyl tails, 8–12 carbons in alkyl tails, and secondary amines. A total of 1200+ lipidoids were synthesized from alkyl-acrylate, alkyl-acrylamide, and amino molecules building blocks using Michael addition by Akinc et al. in 2008 for screening siRNA-delivering lipids with the Lipoids $98N_{12}$–5 as the top candidate (Fig. 4g) [142]. For in vivo siRNA silencing, lipidoid derivatives with amide conjugated tail exhibited significantly higher siRNA gene silencing efficiency compared to lipidoids with ester-conjugated tail. This is similar to the observation from cationic lipids between DOTMA (ether tail) and DOTAP (ester tail) mentioned previously [106]. When formulated into siRNA-LNPs, over 90% of the $98N_{12}$–5 LNPs were distributed to the liver [143].

On the other hand, Love et al. reported a second-generation lipidoid library of 126 lipidoids synthesized by nucleophilic epoxide ring opening reaction. They discovered the top candidate, C12–200, which is also a polycationic lipidoid stabilized by a piperazine ring with five nondegradable amino alcohol lipid tails. siRNAs delivered in vivo by C12–200 LNPs were found to be 100 times more efficient than $98N_{12}$–5 (LNP01), which has since become benchmarking lipidoids for siRNA delivery [144], derivatizing into many structurally similar lipidoids with 4–5 non-cleavable lipid tails via amine-mediated nucleophilic epoxide ring opening for varying applications, e.g., 246-C10 (4-tails) [145], C14–4 (ether-linked, 5 tails, for CAR-T engineering) [8], C14-A1 (stretched version, 5 tails), 144 Gen1-C4E12 (4 tails with a cleavable disulfide bond), PA3-C12 (A-3 for in utero mRNA delivery) [146], C12–113 (TLR7/8 agonist) [147], OC2-K3-E10 (muscle mRNA delivery) [148], PPZ-A10 (immune cell targeting) [149], and C14-O2 (CAR-macrophages/monocytes) [150], etc. In parallel, Whitehead et al. looked into developing lipidoids with cleavable ester-conjugated tails for siRNA delivery [141]. A total of 1400+ lipidoids with alkyl-acrylates ester builder blocks for generating easter-conjugated cleavable tails. siRNAs delivered in vivo by lead lipidoid $304O_{13}$ were found to be similarly potent compared to siRNAs C12–200, yet the cleavable $304O_{13}$ exhibited much lower liver toxicity compared to the non-cleavable C12–200 at higher siRNA/LNP doses [151].

Learning from pair-wise comparisons between cationic DOTMA and DOTAP, ionizable lipids DODAP and DODMA, and lipidoids 98 N12–5 amide vs. ester tail studies, ester-conjugated lipid tails often performed more poorly for in vivo RNA delivery due to rapid degradation although being more rapidly cleared from the body makes them more biocompatible [106,143]. Lipidoids with diketopiperazine (DKP) core structures were synthesized by Dong et al., led to the discovery of cKK-E12 lipidoid with an ApoE-facilitated hepatocyte uptake mechanism (similar to 246-C10 [145]), leading to more efficient in vivo siRNA-gene silencing compared to C12–200 [152]. Fenton et al. further derivatized cKK-E12 for mRNA delivery with lipidoid OF-02 (cKK core with 4 non-cleavable 1,2-amino alcohol linoleyl tails), which has a near identical liver-only biodistribution but significantly higher in vivo mRNA translation of hEPO compared to cKK-E12 [153]. Replacing the OF-02's non-cleavable tails with ester led to OF-Deg-Lin, which dramatically changed the in vivo mRNA delivery to specifically targeting the spleen [154]. Other functional lipidoid scaffolds include the phenyl core FTT5 with hyper-branched tails [155], dendrimer-like 5A2-SC8 [156], polymer-based 7C1 [157], aminoglycosides-based GT-EP10 [158], etc.

4. Organ-specific and cell-specific RNA delivery via targeted LNPs

4.1. Early development of organ-specific and cell-specific liposomal delivery systems

As the early generation of "organ-specific" delivery systems, liposomes are typically biodistributed to the liver and spleen, i.e., the primary organs associated with the reticuloendothelial system (RES)/mononuclear phagocyte system (MPS). The PEGylation of liposomes delayed the uptake by the RES/MPS and prolonged blood circulation [90]. Ultimately, PEGylated liposomes shared biodistribution similar to that of non-PEGylated liposomes, which accumulate in the liver and spleen after intravenous injection [52,159]. Unfortunately, compared to the EPR-driven liposomal PK, antibody-liposome conjugates failed to demonstrate additional PK benefits for tumor targeting [160]. Increasing the particle size of liposomes is known to increase spleen uptake [161]. It is well-known in the 80s that liposomal surface charges are known to impact its pharmacokinetics [162]; for example, increasing cationic lipids, e.g., DOTAP and DOTMA, in the liposomal formulations strongly enhance lung accumulation [163–165].

Further, sending liposomes to targeted cells has always been the goal at the birth of liposomal drug delivery in the 70s. Gregoriadis and Neerunjun explored the use of non-covalently functionalizing liposomes with IgG as a strategy for homing bleomycin-encapsulating liposomes to target specific cells in 1975 [166]. Magee et al. used IgG-functionalized liposomes for targeted delivery of "immune RNA" to lymphocytes for immune stimulation in 1978 [167]. Covalently functionalized antibody-liposome conjugates were explored for target delivery to myocardial infarction sites by Torchilin et al. in 1979 and for cell-type specific targeting by Huang et al. in 1980 [168,169]. Antibody-liposome conjugates were the major way to achieve site-specific/organ-specific therapeutic delivery, as demonstrated by Holmberg and Leaf Huang et al. in 1990 [170]. Reported in the same year by Klibanov and Leaf Huang et al., PEGylated liposomes significantly prolonged blood circulation [90], facilitating the tumor Enhanced Permeation and Retention (EPR) effect, which eventually took over the major focus for developing liposomal formulations for cancer delivery following the approval of Doxil® in 1995 [91]. On the other hand, antibody-drug conjugates are still being developed as the major targeted drug delivery platform for cancer to this date. Compared to liposomal delivery systems, antibody-drug conjugates are limited to very low drug loading capacity and cannot provide barrier protection for therapeutic RNAs from enzymatic degradation [171]. Thus, there is still a need to develop site-specific LNP systems for precision RNA therapeutics.

4.2. Liver/spleen-, liver-, spleen-, and lung-specific LNPs

At the front end of this development are the selective organ targeting (SORT) LNPs developed by Cheng and Siegwart et al. using ionizable lipids 5A2-SC8, MC2, and lipidoids C12–200. By adjusting the ratio of supporting lipids such as DOTAP or 18PA, the SORT LNPs could achieve exclusive organ-targeted mRNA delivery to the lung (with 50% DOTAP), liver (<10% DOTAP), and spleen (10–15% DOTAP with 10–40% 18PA). The ability of SORT LNPs to achieve organ-specific mRNA delivery is proposed for targeted CRISPR-Cas gene editing [172,173], which has led the phase 1 clinical trials of CT1100 (Lung SORT LNPs by ReCode Therapeutics) for Cystic Fibrosis in February 2024. LoPresti et al. reported a variation of the SORT LNPs using the ionizable lipid 304O13. Liver-favored distribution was observed when co-forming LNPs with the

neutral lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), sphingomyelin (SM), and ceramide (Cer). Spleen-favored distribution was achieved when co-formulating LNP formulations with zwitterionic lipid phosphatidylserine (PS) and anionic lipids phosphatidylglycerol (PG) and phosphatidic acid (PA). Cationic DOTAP and ethyl phosphatidylcholine (EPC) shifted the LNP distribution to the lungs [174]. When delivered via intratracheal (IT) administration, MC3 and DOTAP co-formulated LNPs have been shown to effectively deliver mRNA to alveolar epithelial cells (AECs) and fibroblasts in fibrotic lungs [175].

Similar to liposomes, LNPs are PEGylated and typically exhibit liver/spleen distribution following systematic administration. Leveraging liver biodistribution, Onpattro® LNPs (ionizable lipid = MC3 with non-cleavable lipid tails) are designed to treat liver hATTR amyloidosis [123,176]. The high liver accumulation of MC3 LNPs sparks the development of ionizable lipids with cleavable ester tails. Ester lipid tails have been used in clinically approved SM-102, ALC-0315 ionizable lipids, and many other structural derivatives sharing liver biodistributions for improving the biodegradability of ionizable lipids in liver [177]. On the lipidoid side, the classic lipidoid 5-tail piperazine-based C12–200 is known to target both liver and spleen when given intravenously [177]. The 4-tail piperazine-based 246C10, however, showed liver-only distribution in a CRISPR/Cas gene editing study targeting antithrombin (mAT) for treating hemophilia [11]. With 2 extra carbons in the lipid chain in 246C10, LNPs based on piperazine-based IC8 (246C12) were shown to distribute to the liver and spleen when delivering mRNA encoding B7H3 × CD3 bispecific T-cell engagers (BiTEs) antibodies. The liver was targeted as an in vivo antibody factory for BiTEs expression and secretion to treat hematologic malignancies and melanoma [178]. LNPs based on modified diketopiperazine (DKP)-based lipidoids, e.g., cKK-E12 (non-cleavable saturated tails) and OF-02 (non-cleavable unsaturated tails), showed a highly liver-focused distribution with higher protein translation efficiency by OF-02 compared to cKK-E12 [153]. Interestingly enough, by modifying DKP-lipidoids with cleavable ester and unsaturated tails, e.g., OF-Deg-Lin, although still distributed to the liver more than the spleen, the spleen accounted for over 85% of the mRNA expression [154]. Incorporating oleic acid (OA) in LNP formulations significantly enhanced liver distribution [164], whereas replacing cholesterol with cholesteric acid enables LNPs to exhibit a splenic tropism [112]. Additionally, incorporating different helper lipids in LNPs is known to alter their interaction with serum apolipoprotein E (ApoE). Having DOPE in the LNP formulation leads to higher ApoE binding and enhances liver delivery. Replacing DOPE with DSPC decreases ApoE binding, reducing liver uptake and increasing spleen distribution [179].

4.3. Placenta and fetus distribution of liver/spleen targeted LNP during pregnancy

Neither the liver-targeting LNPs formulated by DKP-lipidoids, e.g., cKK-E12 nor OF-02, nor the classic liver-and-spleen targeting lipidoids C12–200 distributed to the uterus/ovaries after systematic distribution [154]. By modifying the lipid tails of C12–200 with ether linkages, ionizable lipids A-4 showed an over 20× distribution to the spleen over the liver in non-pregnant mice. More interestingly, when given systematically in pregnant mice, LNPs A-4 are highly efficient in biodistribution to the placenta but not the fetus. A similar effect was observed on a similar ionizable lipidoid derivative B-5, which also has an ether structure [177]. This phenomenon, where highly spleen-targeted LNPs breaching into the placenta during pregnancy, is proposed for delivering mRNA-VEGFR to increase placenta blood flow for placental insufficiency. Whether this pregnancy-dependent biodistribution applies to other spleen-targeted LNPs, such as OF-Deg-Lin, remains to be investigated, and this unique observation/demonstration also highlighted the importance of developing precision RNA delivery to minimize undesirable off-target effects. Finally, in-utero delivery of LNP A-4 (PA4) was able to deliver mRNA more efficiently to the liver, lung, and intestine of the fetus with minimal fetal immunotoxicity or liver damage compared to mRNA delivered by MC3-LNP or jetPEI cationic polymer [146]. Meanwhile, the MC3 LNPs were shown to deliver mRNA to the heart, diaphragm, and skeletal muscle, in addition to the liver of the fetus via in-utero delivery [180].

4.4. Pancreatic distribution of liver-and-spleen targeted LNP via intraperitoneal injection

The administrative route is known to significantly impact the pharmacokinetic and protein translation half-live of mRNA-delivering LNPs [181]. When given intravenously, liver-targeting lipidoids 306Oi10 (ester lipid tails, 98.6% liver), 200Oi10 (a C12-C200 derivative with ester tails, 97.7% liver), and liver-and-spleen-targeting $514O_{6,10}$ (branched ester lipid tail, 68.3% liver and 24.6% liver) showed typical LNP biodistribution. However, given via intraperitoneal injections, significantly increased pancreas distribution was observed for 306Oi10 (14.7%), 200Oi10 (46.4%), and $514O_{6,10}$ (52.3%). This pancreatic distribution could be further enhanced by incorporating cationic lipid DOTAP in the LNP formulation, which universally increases pancreas mRNA expression for all three lipidoids tested [182].

4.5. LNPs for bone microenvironment RNA delivery

Bisphosphonate (BP) ligands, such as alendronate and pamidronate, have long been studied as drug conjugates for targeting metabolic bone diseases [183]. BP-lipid conjugates have been used for bone-targeting liposomal delivery of doxorubicin [184]. Similarly, BP-conjugated lipidoids 490BP-C14, while majorly accumulating in the liver and spleen after IV injections, were able to distribute to the bone microenvironment in mice and demonstrate mRNA-EGFP expression in the femur [185]. For genome editing therapy targeting haematopoietic stem cells (HSCs), incorporating covalent crosslinker/lipid crosslinkers (e.g., AA11, a 3-sulfo-N-hydroxysuccinimide modified fatty ester) in the LNP formulations has also been demonstrated to alter the serum protein coating on the LNP (also known as the protein corona), which led to bone marrow homing at 20% molar ratio in 5A2-SC8-based LNPs [186].

4.6. Eye-specific and ear-specific RNA delivery via localized LNP administrations

Represented by the FDA-approved adeno-associated virus (AAV)-based gene therapy, Luxturna™, eye-specific gene delivery via subretinal delivery has been widely explored for treating inherited retinal degeneration/blindness and other retinal degeneration diseases [187]. Using MC3/KC2 formulated LNPs, mRNA given via subretinal delivery was able to demonstrate gene expression in the eyes for 5 days, paving new ways for repeatable non-immunogenic gene therapy in the eyes [188].

On the other hand, while no FDA-approved gene therapies exist, AAV-gene therapy is being studied in clinical trials to restore hearing in autosomal recessive deafness [189,190]. Cationic lipid-mediated in vivo delivery of Cas9–guide RNA complexes via microinjection to the inner ear has shown great promise as a non-viral genome editing therapy in preclinical studies [191].

4.7. Immune cell-targeting LNPs for immunoengineering

Immunoengineering is a dynamic and growing field that leverages the immune system's capabilities to combat diseases, notably cancer and autoimmune disorders. LNPs have revolutionized this field by offering precise delivery of immunomodulatory molecules, transforming immune cells into precise, targeted therapeutic agents. An essential area of focus is the creation of tolerogenic vaccines that promote immune tolerance, potentially transforming treatments for autoimmune diseases such as multiple sclerosis [16], preventing anaphylaxis for peanut

allergies [15], and allergic airway disease [192,193]. These vaccines function by inducing antigen-specific regulatory T cells (iTregs). LNPs serve as delivery vessels, carrying mRNA that encodes the target antigens or allergens to specific antigen-presenting cells, such as liver sinusoidal endothelial cells (LSECs) or tolerogenic dendritic cells [194]. By employing mannose ligands that bind to the mannose receptor CD206 on LSECs and optimizing the LNP composition with PEGylated lipids, this mannose-mediated approach efficiently homes in on the target cells. This strategy, utilizing liver-targeting ionizable lipids like 246C10 and MC3 for LSEC targeting, has been validated in several studies [15,145,195]. Inflammatory diseases have also been addressed using LNPs. For instance, Veiga et al. attached anti-Ly6c antibodies to LNPs to home in on and treat Ly6c + inflammatory leukocytes involved in inflammatory bowel disease [196]. In cardiology, Rurik et al. utilized

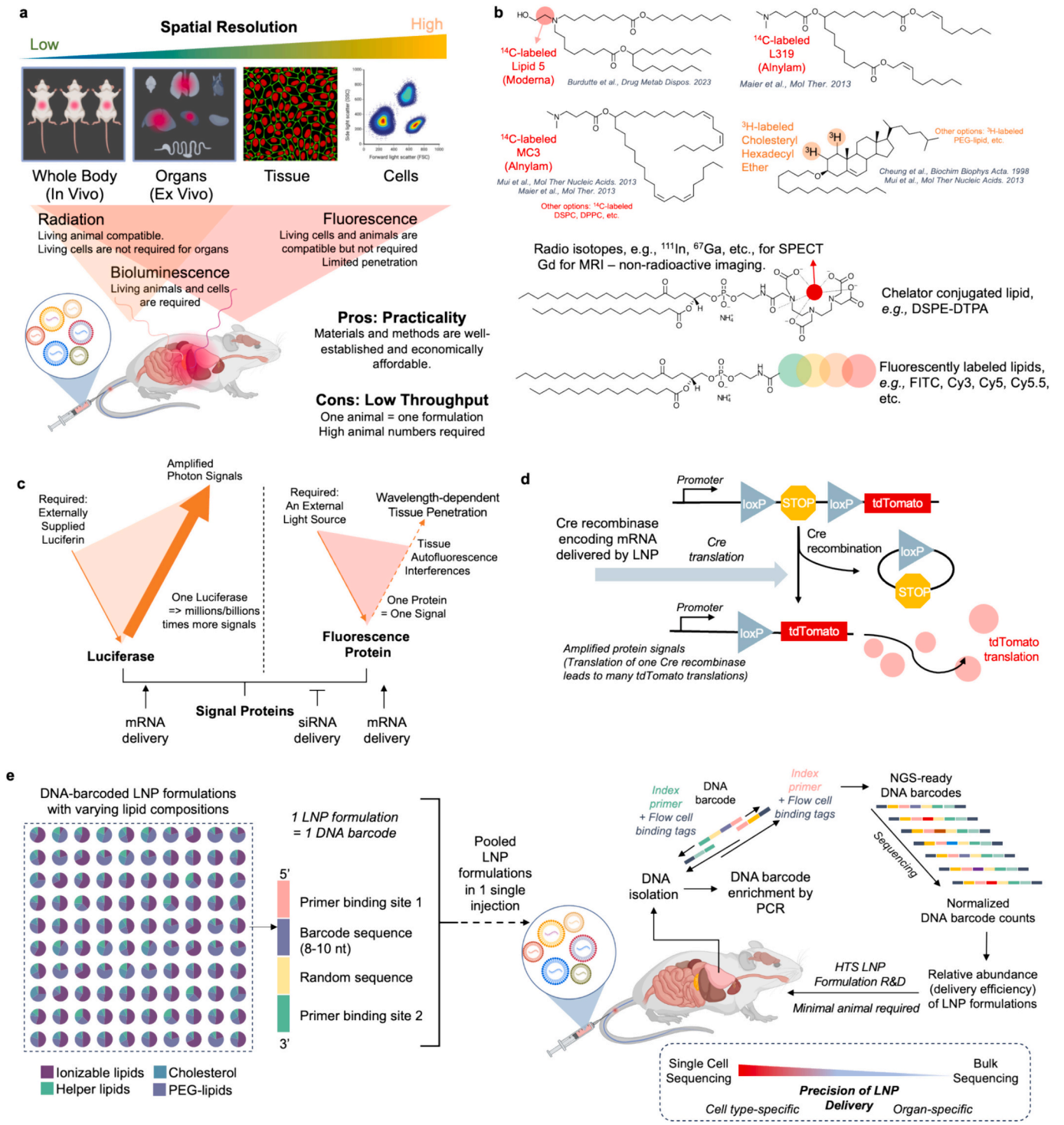

Fig. 5. Biodistribution tracers for developing organ-specific and cell-specific LNPs. a. Spatial resolution of commonly used molecular tracers for PK/PD studies. **b.** Molecular labeling techniques for tracking lipids in the LNPs. **c.** Comparing bioluminescent-based and fluorescent-based imaging techniques for tracking RNA delivery by LNPs. **d.** Illustration of using Cre-Lox system for detecting mRNA delivery. **e.** The DNA-barcoding workflow for HTS LNP formulation screening in vivo. (panels and d are partially created with BioRender.com).

LNPs outfitted with anti-CD5 antibodies to target T cells in the spleen. These modified T cells are then reprogrammed into CAR-T cells that target the fibroblast activation protein (FAP), offering a novel strategy to treat cardiac injury [197].

5. Barcoding technologies for high-throughput LNP formulation discovery

As mentioned earlier, gene editing by CRISPR-Cas-encoding mRNA is a burgeoning application of RNA therapeutics, driving the ever-increasing demand for cell-specific precision RNA delivery. This also means a significantly increased need to work with genetically modified transgenic animal models when developing such LNP formulations. Barcoded LNP formulations are becoming essential for developing next-generation gene editing therapeutics to facilitate LNP discovery with reasonable efficiency and feasibility [198].

5.1. DNA-encoded library (DEL) for high-throughput screening (HTS)

DNA-encoded library (DEL) is commonly used in fragment-based drug discovery, e.g., to discover proteolysis targeting chimeras (PROTACs) by screening for the protein binding of billions of compounds in one microcentrifuge tube. The DEL-enabled high-throughput screening (HTS) could be done in a few days instead of years when using the traditional drug screening method in microtiter plates. This is achieved by attaching every drug fragment (experiment variables) to a unique DNA sequence, i.e., the barcode [199]. The biggest advantage of the DEL/DNA-barcoding approach is that it allows sample pooling. In the context of PROTACs discovery, 1E4-1E12 DNA-tagged molecules could be pooled in one microtube for protein binding studies [200]. Non-binding molecules are washed away along with their DNA barcodes, and the remaining DNA barcodes of the "hit" molecules that bound to the target protein can then be amplified and tagged for DNA sequencing using standard polymer chain reactions (PCR) workflow. The identity of the drug candidates could then be "decoded" by next-generation DNA sequencing and ranked by their abundance to identify the top candidates/DNA barcode. Compared to traditional HTS drug screening, the DEL/DNA-barcoding approach could significantly reduce the time from years to days and resources required, allowing HTS screening in vitro and in vivo via sequencing.

5.2. Tracking LNPs in vivo with molecular tracers and DNA barcodes

In drug delivery system development, molecular tracers are instrumental in conducting pharmacokinetic (PK) and pharmacodynamic (PD) studies. As shown in Fig. 5a-d, these tracers allow for the tracking of LNPs at resolutions that range from whole-body to single-cell levels, each requiring specific probes for accurate localization [201]. Radioisotopes provide the best tissue penetration length and exceptional signal-to-noise ratios, which are directly translated from preclinical animal models to human studies. Through ^{14}C and ^{3}H labeling, LNPs can be used to radiolabeled without modifying chemical structures [101,120,202,203]. Alternatively, chelator-conjugated lipids, e.g., DSPE-DTPA, could also be incorporated into LNP formulations for PK studies. However, radiation-based tracers do not provide sufficient spatial resolution down to tissue and cellular levels, which are centric to gene delivery studies. Fluorescent imaging with lipid-conjugated dyes—such as DiI, DiO, DiD, DiA, and DiR—is advantageous for higher spatial resolution at the organ, tissue, and cellular levels. These dyes allow researchers to investigate the spatial distribution and cellular uptake of LNPs in small animal models, although their shallow tissue penetration limits their use [52,204]. However, tracking LNPs alone does not confirm the successful delivery and function of their cargo—mRNA or siRNA—within the target cells. To assess this directly, fluorescent reporter proteins like GFP, RFP, and mCherry are common tools for evaluating protein synthesis following mRNA delivery or for monitoring siRNA silencing efficiency by tracking the loss of fluorescence in genetically modified reporter cells. For a more dynamic and amplified signal, mRNA encodes bioluminescent proteins, such as firefly luciferase (fLuc) is commonly used. The enzyme fLuc catalyzes the emission of light in the presence of its chemical substrate, luciferin, enabling a single mRNA translation event to produce a substantially heightened luminescent output, offering a stark contrast to the one-to-one signal ratio seen with fluorescent proteins from mRNA translation. However, the bioluminescent method is restricted to living cells, as the enzymatic reaction requires a live cell environment (Fig. 5c). To bypass this limitation and to boost the visibility of successful mRNA delivery, especially at low delivery efficiencies, transgenic animals are engineered with a genetic control system for reporter protein expression. For example, transgenic mouse models such as Ai9$^{LSL\text{-}tdTomato}$ and Ai14$^{LSL\text{-}tdTomato}$ carry a tdTomato (a red fluorescent protein) reporter gene preceded by a loxP-flanked STOP cassette, i.e., the reporter gene is initially silenced. The administration of LNPs containing Cre recombinase mRNA to these mice triggers the expression of Cre in target cells. Cre recombinase then excises the STOP cassette, thus activating reporter tdTomato gene expression. The resulting bright red fluorescence serves as a robust and precise marker of successful Cre mRNA delivery and expression. This sophisticated strategy allows for the signal amplification necessary for analysis, even in fixed tissues or cells, as illustrated in Fig. 5d [205].

When DNAs are used as the molecular tracer, DNA *"barcodes"* could be formulated with the ionizable LNPs, where each varying formulation composition is designated with a unique DNA sequence (unnatural sequence) that is sandwiched with PCR primer binding sites (Fig. 5e). Similar to the DEL HTS screening, hundreds to thousands of DNA-barcoded LNPs could then be pooled into one single injection for evaluating the pharmacokinetics of LNP in one single animal [206]. After the experiment, the organ-specific or cell-specific biodistribution of LNP-delivered DNA barcodes could be extracted and amplified by PCR. By fusing PCR primers with next-generation sequencing (NGS)-enabling tags, the amplified DNA barcodes could then be sequenced and ranked by abundance at tissue-, organ-, and cellular levels, representing the biodistribution ranking of the barcoded LNP formulations. Using DNA barcoding with just 8 nucleotides, 4E8 combinations could be generated, equating to 65,536 sequence-unique DNA barcodes. Massive LNP formulations could be screened with minimal animal numbers, thus enabling HTS LNP formulation discovery in transgenic models that are not practically feasible using other molecular tracers.

5.3. Standard-, enhanced-DNA-barcoding and mRNA/peptide-barcoding strategies for LNPs

Dahlman et al. demonstrated the use of DNA-barcoded LNPs in 2017 by screening 30 siRNA-delivering LNP formulations per one mouse to identify the optimal PEG-lipid tail length, PEG molecular weights, and PEG % for delivery to the brain, heart, kidney, liver, lung, skeletal muscle, uterus, and pancreas, using LNPs based on lipidoids 7C1 and C12–200 [207]. Huayamares et al. screened 94 DNA-barcoded mRNA-LNP (7C1) formulations in one injection per animal to develop liver "de-targeted" formulation for enhancing mRNA delivery to a solid tumor model of neck squamous cell carcinoma (FaDu xenograft/HNSCC) [208]. While DNA-barcoding is extremely powerful for in vivo HTS of LNP formulations, detecting DNA barcodes reflects only the distribution of the LNP, i.e., the pharmacokinetics of the LNPs. For RNA therapeutics, delivering to the targeted organ or cells, however, does not equate to mRNA translation or siRNA gene silencing due to the endosomal escape barriers mentioned above. In other words, using DNA barcoding itself is not enough to evaluate the efficacy and pharmacodynamics of RNA therapeutics. Therefore, co-delivery of reporter RNA molecules is often advised to establish the PK/PD profile of RNA-delivering LNP formulations. For example, Sanchez et al. utilized 128 LNP formulations co-encapsulating DNA-barcode/Cre-mRNA using stereo-pure R- or S-C12–200 and racemic C12–200. By pooling 55 formulations/injection

per Ai9/Ai14$^{LSL\text{-}tdTomato}$ mouse, successful mRNA delivery and translation can be identified by isolating tdTomato-positive cells following NGS sequencing to decode the LNP formulation. The S-configured C12–200 was found to significantly enhance mRNA translation compared to the R-configured C12–200 [209]. Similar strategies have been demonstrated by Lokugamage et al. screening 82 cKK-E12-based LNP formulations co-encapsulating DNA-barcodes and Cre-mRNA, per Ai14$^{LSL\text{-}tdTomato}$ mouse, for identifying LNPs targeting liver Kupffer cells, endothelial cells, and hepatocyte [210]. Ni et al. investigated 128 structurally different ionizable lipidoids sharing a piperazine core and using Cre-mRNA/DNA barcoding to pool-inject 65 LNP candidates per Ai14$^{LSL\text{-}tdTomato}$ mouse, which led to the discovery of PPZ-A10 (Pi-A10) as a novel mRNA-delivering lipidoid targeting hepatic and splenic immune cells (Fig. 4g) [149].

In addition to NGS-based barcoding strategies, HTS proteomic profiling via LC-MS/MS provides an alternative avenue for evaluating mRNA-delivering LNPs in vivo. As demonstrated by Rhym et al., this is achieved by fusing reporter mRNA with a peptide-barcoding sequence, that both the reporter gene and the peptide-barcodes are detectable when mRNA delivery and translation are both successful [211]. Using mRNA barcoding, Rhym et al. evaluated 384 structurally different ionizable lipids in only 9 mice, identifying RM133–3 (Fig. 4f) as a novel ionizable lipid for mRNA delivery [127]. All considered, the combined use of fluorescent/bioluminescent molecule probes, standard-, enhanced-DNA-barcoding, and mRNA/peptide-barcoding strategies provide HTS LNP screening with precision and resolution down to single cell levels, paving new ways of fast-tracking organ-specific and cell-specific LNP for prevision RNA delivery, as summarized in Table 1.

6. Milestones and clinical landscape in the development of mRNA and siRNA therapeutics

6.1. A six-decade-long journey from discovery to clinical approvals of mRNA and siRNA therapeutics

Parallel to the discovery of liposomes, Sydney Brenner, François Jacob, and Matt Meselson discovered the role of mRNA in 1961 [212–214]. As an intermediate between DNA and protein translation, the discovery of mRNA and ways to synthesize stabilized mRNA (inclusion of PolyA tails and RNA capping technologies for enhanced stabilities and translation efficiency) for in vitro/in vivo protein expression alongside the gene delivery endeavors via liposomal formulations collectively embarked on a six-decade-long journey in developing mRNA therapeutics (**Fig. 6** and **Table S2**) [215–219]. Therapeutic applications of mRNA were explored in various directions in the '90s when Wolff et al. first demonstrated in 1990 using mRNA for direct in vivo gene transfer and protein expression in mouse skeletal muscle [219]. The successful use of mRNA for in vivo protein translation led to the exploration of mRNA-mediated diabetes treatments by Jirikowski in 1992 [220], anti-influenza cytotoxic T-cell induction using mRNA-liposomes by Martinon et al. in 1993 [221], mRNA cancer vaccines by Conry et al. in 1995 [222], mRNA-enabled dendritic cell vaccine by Boczkowski et al. in 1996 [223], mRNA-mediated overexpression of receptor in mammalian cells (conceptionally leading to modern-day CAR-T engineering via mRNA-LNP) by Karikó et al. in 1999 [224], and mRNA melanoma vaccine for cytotoxic T cell induction by Zhou et al. in 1999 [225] among many other mRNA-enabled vaccination studies [222,223]. More importantly, Katalin Karikó and Drew Weissman discovered that modified nucleoside significantly reduced the innate immune response to exogenous mRNA, enhancing its stability and efficacy [226]. Such advancements proved instrumental fifteen years later when mRNA technology was rapidly applied to develop mRNA-based COVID-19 vaccines. The discovery was later awarded the Nobel Prize in Physiology in 2023 [227]. On the other hand, siRNA was discovered for posttranscriptional gene silencing in 1999 by Hamilton and Baulcombe et al., nearly four decades after mRNA was discovered [228]. As shown previously in Fig. 1, siRNA functions as a targeted mRNA degrader, leading to targeted gene silencing. The unique mechanism of siRNA led to their first approval for clinical use in 2018 as Patisiran, an MC-3 lipid-formulated siRNA that silences the amount of wild-type and mutant transthyretin mRNA for treating hereditary transthyretin-mediated amyloidosis [29,229]. This culmination of decades of RNA research and innovation in RNA-LNP delivery systems collectively set the stage for the diverse clinical landscape of mRNA and siRNA therapeutics we see today.

6.2. The clinical landscape of mRNA and siRNA therapeutics

The burgeoning field of mRNA and siRNA therapeutics has transitioned from foundational research to clinical applications with unprecedented speed. The clinical landscape today is diverse, encompassing vaccines, gene editing, and gene silencing approaches that tackle a broad range of diseases. In the vanguard of mRNA therapeutics are the COVID-19 vaccines, Tozinameran and Elasomeran, known commercially as BNT162b2 and mRNA-1273. These vaccines utilize LNPs as delivery systems, successfully stimulating protective immune responses against SARS-CoV-2. Their development highlights the agility of mRNA platforms in adapting to pathogenic mutations, a contrast to traditional vaccine development methods. This flexibility was demonstrated when Moderna's mRNA-1273 entered clinical trials within two months of the viral genome's publication [230].

Beyond its pivotal role in combating COVID-19, mRNA therapeutics are expanding their reach to target a range of formidable infectious diseases [231]. Clinical trials are currently underway for next-generation vaccines aimed at preventing influenza, HIV/AIDS, and rabies [232–236]. Meanwhile, CRISPR-Cas9 gene editing has also advanced through the use of mRNA delivery. Targeted gene editing is achieved by encapsulating Cas9 mRNA and sgRNA within LNPs [9]. This innovative approach is now being tested in clinical settings. A phase I trial for gene editing in patients with Primary Ciliary Dyskinesia (RCT1100, NCT05737485, targeting the DNAI1 gene) and another for Cystic Fibrosis (RCT2100, NCT06237335, targeting the CFTR gene) are noteworthy. Both trials have dosed the first patient in 2024 via inhaled LNPs for lung delivery.

The realm of siRNA therapeutics is equally diverse and has been applied to a variety of cancers, including lung cancer, pancreatic cancer, breast cancer, prostate cancer, ovarian cancer, and liver cancer, among others [237–242]. Tailored siRNA molecules enable the targeting of specific cancer gene sequences, offering personalized treatment options. The versatility of siRNA is not limited to cancer; it has emerged as a powerful tool against genetic and metabolic diseases (**Table S3**) [29,49].

An overview of the current clinical trials in mRNA and siRNA therapies is distilled from an extensive search of ClinicalTrials.gov (Fig. 7a). These trials are categorized into mRNA and siRNA therapeutics, with the mRNA trials further clustered as (i) mRNA therapeutics (which imparts therapeutic action) and (ii) mRNA-enabled cellular therapy (where mRNA-encoded proteins/receptors/ligands augments cell-based treatments) [243]. As shown in **Fig 7, b1**, mRNA therapeutics have reached clinical approval through the mRNA COVID-19 vaccines, with highly active pipelines across phase I-III. Diving into the indications of mRNA therapeutics, the majority of the trials are COVID-19 related, as expected, followed by the up-and-rising applications such as mRNA-vaccine for infectious diseases, oncology, genetic disorders, cardiovascular, etc. (**Fig. 7, b2**). Diving into the oncology-related indications, the top application is evidently the personalized mRNA cancer vaccines, followed by other indications in gastrointestinal, melanoma, liver cancer, pancreatic cancer, and others (**Fig. 7, b3**). As for infectious diseases, the leading applications are influenza vaccines (~45%), followed by Respiratory Syncytial Virus (RSV) vaccines, HIV vaccines, cytomegalovirus (CMV) vaccines, Rabies vaccines, and others (**Fig 7, b4**).

mRNA-enabled cellular therapy is on the rapid rise, with just a

Table 1
LNPs for organ-specific and cell-specific RNA delivery.

Targeted Organ(s) or Cell(s)	Barcoding (Y/N)	Ionizable Lipids (% molar ratio)	Supporting Lipids & Targeting Ligands	RNA Cargo	Animal	Proposed Applications	Ref.
Liver	No	MC3 50%	DSPC, lipid-PEG and β-sitosterol	mRNA	BALB/c mice	SARS-CoV-2	[257]
Liver		MC3	DOPE, lipid-PEG and Chol	mRNA	C57BL/6 J mice	Liver cancer	[258]
Liver		OF-02 35%	DOPE, lipid-PEG and Chol	mRNA	C57BL/6 mice	Protein/enzyme replacement therapy	[153]
Liver		G0-C14 5%	PDSA and lipid-PEG	mRNA	Nude, BALB/c, C57BL/6 mice	Liver cancer	[259]
Liver & Spleen		IC8 35%	DSPC, lipid-PEG and Chol	mRNA	NSG mice	hematologic malignancies and melanoma	[178]
Liver		246C10 26.5%	DOPE, lipid-PEG and Chol	mRNA sgRNA	C57BL/6 mice	Gene editing Hemophilia	[11]
Liver		5A2-SC8 24%	DOPE, lipid-PEG and Chol	mRNA sgRNA siRNA	C57BL/6 mice	Gene editing Liver cancer	[156]
Liver		GT-EP10 (A15) 20%	DOPE, Chol, DMG-PEG_{2kDa}	mRNA	C57BL/6 mice	Protein/enzyme replacement therapy	[158]
Liver Lung Spleen		5A2-SC8 39.5% 5A2-SC8 11.9% 5A2-SC8 16.67%	DODAP[a], DOPE, lipid-PEG and Chol DOTAP[b], DOPE, lipid-PEG and Chol 18PA[c], DOPE, lipid-PEG and Chol	mRNA sgRNA	C57BL/6 mice, $Ai14^{LSL\text{-}tdTomato}$ mice	Gene editing	[173]
Liver Lung Spleen		$304O_{13}$ 35%	DOPE, C14-PEG and Chol DOTAP, C14-PEG and Chol PC, C14-PEG and Chol	mRNA	C57BL/6NCrl mice	Multiple	[174]
Muscle		TCL053 60%	DPPC, lipid-PEG and Chol	mRNA sgRNA	hEx45KI-mdx44 mice	CRISPR-Cas gene editing *(Duchenne muscular dystrophy)*	[251]
Bone		490BP-C14 35%	DOPE, lipid-PEG and Chol	mRNA	C57BL/6 J mice	Skeletal diseases	[185]
Bone		5A2-SC8 19%	DOPE, Chol, DMG-PEG_{2kDa}, Crosslinker (20%)	mRNA	C57BL/6 & $Ai14^{LSL\text{-}tdTomato}$ mice	HSCs gene editing	[186]
Pancreases (via IP injection)		$306O_{i10}$ $200O_{i10}$ (2nd best) $514O_{i6,\ 10}$ (the best) 35%	Chol, C14-PEG_{2kDa} Helper: DOTAP (better) or DOPE or PS	mRNA	C57BL/6 mice	Diabetic, Pancreatic cancer	[182]
Lung alveolar epithelial cells (and fibroblasts (intratracheal)		MC3 10–25%	DOTAP (40%) DPPC, Chol, DSPE-PEG_{2kDa}	mRNA	C57BL/6 mice	Idiopathic pulmonary fibrosis	[175]
Lung (via inhalation)		IR-117-17 (A10-Lin) IR-19-Py 50.1%	DOTAP, Chol, C14-PEG_{2kDa}	mRNA	C57BL/6 J B6.Cg-Gt(ROSA) 26Sortm14(CAG-tdTomato)Hze/J C57BL/6 N Scnn1b-Tg	Lung diseases (e.g., cystic fibrosis, primary ciliary dyskinesia, α-1 antitrypsin deficiency, vaccination, and asthma	[22]
Fetal Liver (in utero)		PA3-C12/C14 (A-3/B-3) 35%	DOPE, Chol, C14-PEG_{2kDa}	mRNA	Balb/c	Protein/enzyme replacement therapy	[146]
Placenta (IV injection) Spleen		A-4 35%	DOPE, Chol, C14-PEG_{2kDa}	mRNA (VGEF-A) mRNA	Pregnant mice: gestational age E16 Non-pregnant mice	Placental insufficiency	[177]
Retinal		MC3, KC2 50%	DSPC, DMG-PEG_{2kDa}	mRNA	Balb/c	Retinal degeneration	[188]
Leukocyte		MC3 50%	DSPC, PEG-lipid and Chol Anti-Ly6c Abs	mRNA	C57BL/6 mice	Inflammatory Bowel Disease	[196]
T cells (Spleen)		L319 55%	DSPC, PEG-lipid and Chol Anti-CD5 Abs,	mRNA	C57BL/6 N, $Ai6^{LSL\text{-}ZsGreen1}$ mice	Cardiac Fibrosis	[197]
LSECs		MC3 50%	Mannose, DSPC, PEG-lipid and Chol + Mannose	mRNA	C3H/HeJ mice	Tolerogenic vaccine Peanut Allergy	[15]

(*continued on next page*)

Table 1 (*continued*)

Targeted Organ(s) or Cell(s)	Barcoding (Y/N)	Ionizable Lipids (% molar ratio)	Supporting Lipids & Targeting Ligands	RNA Cargo	Animal	Proposed Applications	Ref.
LSECs		246C10 26.5%	DOPE, PEG-lipid and Chol + Mannose	mRNA	C57BL/6, Ai14$^{LSL\text{-}tdTomato}$ mice	Tolerogenic vaccines	[145]
SECs/LSECs		MC3 50%	DSPC, PEG-lipid and Chol	mRNA	C57BL/6 mice	Tolerogenic vaccines	[195]
B cells		OF-Deg-Lin 35%	DOPE, PEG-lipid and Chol	mRNA	C57BL/6 mice	ImmunoEngineering	[154]
Liver		C12–200 50%	DSPC, lipid-PEG and Chol	siRNA	C57BL/6 mice	Multiple	[207]
Lung		7C1 70%	lipid-PEG and Chol				
Liver		Stereo-pure C12–200-based 40%	DOPE, lipid-PEG and 20α-OH	mRNA	Ai14$^{LSL\text{-}tdTomato}$ mice	Protein/enzyme replacement therapy	[209]
Tumor		7C1 50%	DSPC, lipid-PEG and Chol	mRNA	FaDu xenograft nude mice	Head and neck cancers	[208]
Lung		3-A_2-7b 35%	DOPE, Chol, C14-PEG_{2kDa}	mRNA-Cas9 sgRNA-VEGFR2	Ai14$^{LSL\text{-}tdTomato}$, C57BL/6 J mice	CRISPR-Cas gene editing (e.g., lung cancer)	[20]
Lung ECs		7C1 50%	18:1 Lyso PC, PEG-lipid and Chol	mRNA-SpCas9, sgRNA, siRNA	C57BL/6 J mice	CRISPR-Cas Gene editing for cystic fibrosis	[260]
Spleen ECs		7C1 60%	DOPE, PEG-lipid and Chol	mRNA	Ai14$^{LSL\text{-}tdTomato}$ mice	ImmunoEngineering	
Myeloid progenitors	Yes (DNA)	7C1 60%	PEG-lipid and Chol	N/A	C57BL/6 J mice	NA	[261]
Liver ECs		7C1 50%	18:1 Lyso PC, lipid-PEG and cholesteryl oleate	mRNA-SpCas9, sgRNA	C57BL/6 J mice (WT, LDLR$^{-/-}$, VLDLR$^{-/-}$)	CRISPR-Cas gene editing	[262]
BMECs, Lung and spleen		7C1-based 80%	PEG-lipid	siRNA and Bortezomib	C57BL/6 mice, MM.1S-xenograft NOD SCID mice	Multiple Myeloma	[263]
Liver ECs and KCs; Lung ECs		Stereo-pure 7C1-based 80%	PEG-lipid and Chol	siRNA	C57BL/6 J mice (WT, ApoE$^{-/-}$, LDLR$^{-/-}$, and VLDLR$^{-/-}$)	NA	[264]
KCs		cKK-E15 45%	DOPE, PEG-lipid and Chol	mRNA	Ai14$^{LSL\text{-}tdTomato}$ mice	ImmunoEngineering Vaccination	[210]
Lung ECs		cKK-E12 35%	18:0 DDAB, PEG-lipid and Chol	mRNA,	Ai14$^{LSL\text{-}tdTomato}$ mice, C57BL/6 J mice	Gene editing.	[265]
Liver KCs and ECs		cKK-E12 50%	DOPE, PEG-lipid and 20α-OH	mRNA	Ai14$^{LSL\text{-}tdTomato}$ mice	ImmunoEngineering Vaccination	[266]
Liver KCs Spleen macrophages		PPZ-A10 35%	DOPE, Chol, C18-PEG_{2kDa}	mRNA	Ai14$^{LSL\text{-}tdTomato}$ mice	ImmunoEngineering Vaccination	[149]
Lung ECs		RCB-4-8 30%	DOTAP, Chol, C14-PEG_{2kDa}	mRNA-SpCas9 sgRNA	C57BL/6, Ai9$^{LSL\text{-}tdTomato}$ mice	CRISPR-Cas gene editing for cystic fibrosis	[21]
Liver, spleen, lung	Yes (mRNA)	C12–200 35% (liver, spleen) 60% (lung)	DOPE, Chol, C14-PEG_{2kDa}	mRNA	C57BL/6	CRISPR-Cas9 gene editing, vaccines, immunoRx	[267]
Liver		RM-133-3 35%	DOPE, lipid-PEG and Chol	mRNA	C57BL/6 mice	Protein Rx (e.g., hEPO)	[127]

handful of trials breaching into phases II and II/III (**Fig. 7, c1**). The indications are overwhelming towards oncology (95%) with some application on infectious diseases (**Fig. 7, c2**). The leading application in oncology is mRNA-enabled antigen-loaded dendritic cell (DC) vaccines, followed by chimeric antigen receptor (CAR)-enabled T cell and NK cell therapies and mRNA-enabled antigen-loaded monocyte vaccines (**Fig. 7, c3**). Major targets in oncology are led by brain cancer (~30%), followed by melanoma, leukemia, prostate cancer, personalized cancer therapies, and others (**Fig. 7, c4**).

On the opposite spectrum, the development of siRNA therapies has higher clinical trial completion percentages compared to later-emerging mRNA trials, although the total volume of trials is fewer than mRNA trials (**Fig. 7, d1**). Clinical applications are led by genetic disorders (~40%), followed by oncology (~17%), eye, liver, and cardiovascular diseases, and others (**Fig. 7, d2**). Leading siRNA applications in genetic disorders include ATTR amyloidosis (~30%), primary hyperoxaluria, acute intermittent porphyria, hemophilia, myotonic dystrophy, and others (**Fig. 7, d3**). This is followed by cardiovascular diseases (CVD) and metabolic syndrome, with over 65% of the siRNA trials looking at hypercholesterolemia (exemplified by the approval of Inclisiran in 2021) and atherosclerotic diseases, followed by hypertension, hyperlipidemia, and thrombosis (**Fig. 7, d4**). As for oncology, siRNA is most studied for treating solid tumors, which came up on top ~33% of the trials, followed by liver, pancreatic, and skin cancer, and others (**Fig. 7, d5**). The siRNA-targeted oncogenes are summarized in **Table S3**. In sum, these pioneering clinical trials chart a transformative course for mRNA and siRNA therapeutics, revealing a horizon rich with potential for addressing some of the most pressing medical challenges of our time.

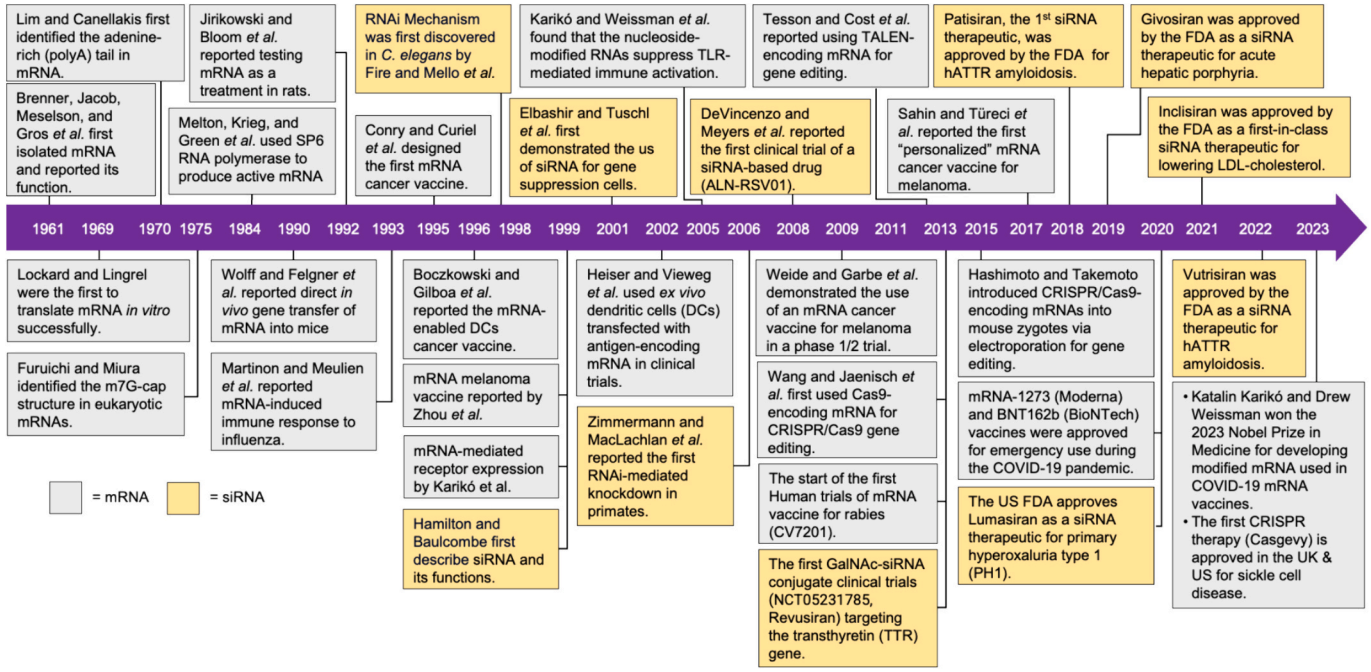

Fig. 6. A six-decade-long journey from discovery to clinical approvals of mRNA and siRNA therapeutics. This retrospective timeline summarizes the development of mRNA and siRNA therapeutics, with selected milestones highlighting key developments for enabling their clinical applications. Full itemized information is summarized in **Table S2**.

7. Challenges and future perspectives

Compared to traditional medicinal chemistry-driven drug discovery and development approaches for small molecular drugs, the development of RNA therapeutics, or genetic medicine in general, is often driven by gene sequencing results (e.g., mRNA vaccines or siRNA silencing), protein sequencing results (e.g., for protein replacement therapy), or function-driven protein engineering designs (e.g., CARs), followed by coding such therapeutic blueprints into the RNA sequences for in vivo expression (as mRNA) or silencing (as siRNA). These approaches led to a turnaround in the design and synthesis of RNA molecules for therapeutics compared to the development of new small molecule drugs through traditional chemistry due to the ability to rapidly synthesize RNA once the target sequence is known. More importantly, regardless of the RNA sequence, all could be delivered using LNP platforms to enable clinical translation. Still, significant challenges remained in improving the precision and efficiency of RNA delivery. As precision medicine continues to evolve, the FDA's validation of RNA-based therapeutics stands as a testament to the burgeoning potential of RNA-enabled treatment strategies. The requirement for precision RNA delivery has been significantly elevated from intramuscular mRNA vaccine injection to CRISPR-based gene editing, demanding the highest level of cell-specific RNA delivery. This precision delivery of RNA therapeutics is contingent on successfully navigating the complex in vivo environment. As both RNA molecules and lipids are inherently unstable and biodegradable under physiological conditions due to lipase or other enzymes, such as the ubiquitous presence of ribonucleases, delivering RNAs to biologically harsh environments, e.g., GI tract, remained highly challenging. Moreover, even when cell-specific RNA delivery is achieved, the efficiency with which these formulations can escape the endolysosomal trafficking once inside the cells is currently limited to approximately 2%, posing another significant barrier for delivering therapeutic RNA to their site of action. Additionally, failing to achieve precision delivery leads to off-target effects within the circulatory system and unintended toxicities. When delivering siRNAs, the off-target effect compounds over a prolonged period, furthering the problem [244,245].

For protein displacement mRNA therapies, e.g., human EPO, the current state of delivering hEPO-encoding mRNA via LNPs shows a duration of expression that has hardly reached beyond 24+ hours [246], a stark contrast to siRNA therapies, which can sustain effects for months. Chemical modification of lipid headgroups (e.g., Lipid 29) was shown to significantly increase the serum EPO concentration for ~3 days [18]. More recently, it was found that codon optimization for attenuating ribosome load plays a critical role in limiting translation-dependent mRNA decay, leading to an overall improved mRNA translation [247]. To address RNA's inherent instability, advances in chemical modifications have emerged as a pivotal strategy to bolster RNA stability and mitigate immune responses to exogenous mRNA [226]. Modifications like N6-methyladenosine (m6A) and pseudouridine (Ψ) not only fortify the RNA structure but also refine its interaction with cellular machinery, enhancing translation efficacy as seen in mRNA vaccines like Comirnaty® and Spikevax® [248–250]. The logistical demands of RNA therapies, particularly mRNA, which necessitate sub-zero storage conditions, underscore the need for innovation in formulation stability. This necessity was highlighted during the rapid deployment of COVID-19 vaccines and remains an area ripe for improvement. The most recent development by Moderna's next-generation mRNA-1283 COVID-19 vaccine can now store at 2–5 °C, highlighting another aspect of the importance of LNP formulation development for ensuring RNA stabilities.

The loading capacity of LNPs is another interesting aspect for future development. While most lipidoids were developed for siRNA delivery, delivering mRNA has been challenging due to the sharp increase in nucleotides embedded in mRNA. It is known that payload distribution and RNA loading capacity critically affect LNP formulation's physicochemical properties and in vivo PK/PD [103]. Developing LNP platforms capable of delivering large proteins, e.g., dystrophin for Duchenne muscular dystrophy, remains challenging. Although a CRISPR-based micro-dystrophin editing approach has been demonstrated for treating the Duchenne muscular dystrophy (DMD) mouse model, the editing effect remained limited by the transient expression of mRNA due to the limitations mentioned above [251].

Immunogenicity adds another layer of complexity to RNA therapy.

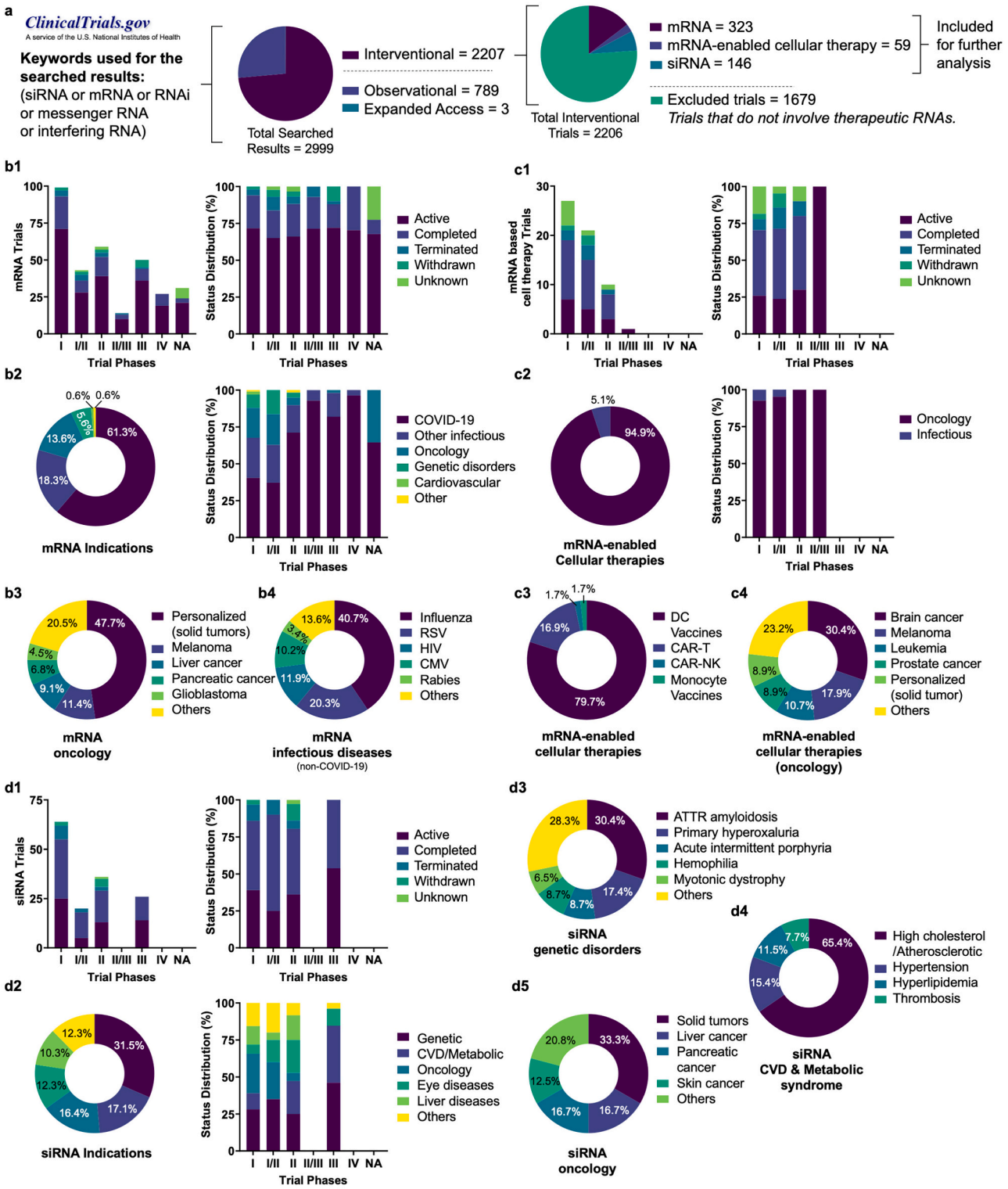

Fig. 7. Clinical Landscape of mRNA and siRNA Therapeutics. A. Search terms for clinicaltrial.gov using keywords included "siRNA," "mRNA," "RNAi," "messenger RNA," and "interfering RNA.". Exclusions were made for trials that did not involve mRNA or siRNA as therapeutic— remaining trials were included for further analysis (detailed in **Fig. S1**). B. mRNA therapies, C. mRNA/cell therapies, and D. siRNA therapies. E. Analyzing indications in mRNA therapy, the top 3 indications were classified by phase and marked in F. Conducting a similar analysis for mRNA/cell therapy and siRNA therapy, with mRNA/cell therapy labeled in G. H and siRNA labeled in I.J. Inclusion of trials: up to April 24, 2024. Detailed classification is available via the online supporting information.

For example, heterocyclic lipids are known to stimulate immune responses [252,253], which may be favorable when developing immunogenic mRNA vaccines for cancer or infectious diseases. However, when developing tolerogenic mRNA vaccines, the opposite is required, which has been demonstrated by formulating m1Ψ modified mRNA [16] and the incorporation of Dexamethasone lipid-conjugated prodrugs in LNP formulations [254]. More interestingly, the sensory mechanism of endosomal damage caused by the endosomal escape of LNPs is linked to

LNP-associated inflammation, leaving additional challenges for non-inflammatory RNA delivery [255].

Recent clinical developments reflect the complexity of translating preclinical successes into safe and efficacious human therapies. While liver RNA delivery via LNPs has been widely reported and is considered relatively mature in preclinical mouse models, Verve Therapeutics halted their phase I clinical trial (Heart-1) in April 2024 due to safety and tolerability concerns, which involves their liver targeting CRISPR-LNPs VERVE-101 targets *PCSK9* gene for lowering cholesterol [256]. Alternative LNP VERVE-102 is proposed to replace the original ionizable lipid in the VERVE-101 formulation. As the FDA mandates a 14-year follow-up period for patients who participated in base-editing therapies and trials, this represents one of the highest safety standards requirements for RNA therapeutics and echoes the need for developing organ- and cell-specific LNPs that not only perform and are compatible with preclinical models but, more importantly, to be safe for patient use. Yet, little is known, with more to be investigated to bridge the gap between preclinical and clinical studies of LNPs.

Looking ahead, DNA/RNA barcoding technologies emerge as vital tools in refining LNP formulation discovery. They enable the dissection of biodistribution at a cellular level, fostering efficient preclinical evaluations. The established efficacy of organ-specific LNPs in animal models paves the way for broader applications. Yet, extending cell-specific delivery to a wider array of cell types remains an uncharted and promising frontier for precision medicine. As many of the genome editing targets of LNPs are mostly different from the traditional liposomal biodistribution targets, discovering novel tropism-specific lipids and LNPs remains an up-and-rising challenge with the ever-increasing need for precision RNA therapeutics. In summary, the development of RNA therapeutics stands at a juncture of remarkable potential, where the depth of lipid and RNA engineering converges with the breadth of delivery platforms to chart the course for the next generation of precision RNA medicine.

Declaration of generative AI and AI-assisted technologies in the writing process

The author used Microsoft Bing and Grammarly to improve the manuscript's readability. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

CRediT authorship contribution statement

Pu-Sheng Wei: Writing – original draft, Data curation. **Nagasri Thota:** Writing – original draft, Data curation. **Greshma John:** Formal analysis, Data curation. **Evelyn Chang:** Formal analysis, Data curation. **Sunjae Lee:** Formal analysis, Data curation. **Yuanjun Wang:** Formal analysis, Data curation. **Zitao Ma:** Formal analysis, Data curation. **Yu-Hsuan Tsai:** Writing – review & editing, Formal analysis, Data curation. **Kuo-Ching Mei:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Data curation, 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2024.08.030.

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