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REVIEW

Lipid-nanoparticle-enabled nucleic acid therapeutics for liver disorders

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Abstract Inherited genetic disorders of the liver pose a significant public health burden. Liver transplantation is often limited by the availability of donor livers and the exorbitant costs of immunosuppressive therapy. To overcome these limitations, nucleic acid therapy provides a hopeful alternative that enables gene repair, gene supplementation, and gene silencing with suitable vectors. Though viral vectors are the most efficient and preferred for gene therapy, pre-existing immunity debilitating immune responses limit their use. As a potential alternative, lipid nanoparticle-mediated vectors are being explored to deliver multiple nucleic acid forms, including pDNA, mRNA, siRNA, and proteins. Herein, we discuss the broader applications of lipid nanoparticles, from protein replacement therapy to restoring the disease mechanism through nucleic acid delivery and gene editing, as well as multiple preclinical and clinical studies as a potential alternative to liver transplantation.

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1. Introduction

Liver disorders, including alcoholic liver disease, fatty liver disease, hereditary diseases, liver carcinoma, viral hepatitis, and liver cirrhosis, pose a significant world health problem. Globally, the estimated prevalence of liver disease is almost 800 million, with a steady increase over the past two decades[1]. Although liver transplantation offers a potential cure for liver disorders, the availability of the organ donor and the necessity for immune-suppressing medication throughout the patient's rest of life makes the procedure challenging for a larger disease population[2,3]. More importantly, prolonged use of immune surprising agents makes patients vulnerable to infections and also induces systemic toxicity[4]. Towards local immune suppression, liver accumulating liposomal drug formulations are considered an additional or second-line treatment for liver diseases[5].

The liver is the largest abdominal organ and gland, accounting for more than 400 known physiological functions. The function of the liver includes the metabolism of lipids, carbohydrates, and amino acids, and the liver contributes significantly to the synthesis of different biochemicals[6].

The liver has different lobules comprising parenchymal (hepatocytes) and non-parenchymal cells. Hepatocytes occupy most of the space in the liver, whereas the significant non-parenchymal cells are Kupffer cells, sinusoidal endothelial cells, and hepatic stellate cells[7]. Of these, hepatocytes are responsible for major biochemical processes in the body. These processes include serum protein production to activate innate immunity against microorganisms in the liver; Kupffer cells are the body's primary phagocytotic center; and liver sinusoidal endothelial cells maintain immune homeostasis[8].

Defects in a single gene produce a corresponding malfunction protein, leading to monogenic liver disorders (IMDs) such as hemophilia, ornithine transcarbamylase deficiency (OTCD), and several more[9]. Correcting the defective gene either by supplementing the correct copy or restoring the defective pathway by regulating the expression of other genes is increasingly considered the first treatment line in these rare liver disorders[10]. Since most hereditary liver diseases are due to loss of function and reduced gene expression, gene therapy is evolving as a promising approach to alleviating these disease conditions. The primary goal of gene therapy is to supplement the correct copy of the malfunctioning gene, thereby correcting the deficient phenotype[11].

Viruses can transport their genomes to host cells and utilize cellular machinery to initiate genome expression. Several viruses are turning into gene-delivery vehicles by inserting a therapeutic gene into all or part of the viral coding areas. Despite this, viral vectors remain the most preferred for gene therapy, having been utilized in more than two-thirds of clinical trials to date[12]. Adenovirus, retrovirus, adeno-associated virus, lentivirus, herpes simplex virus, and poxvirus are the most often utilized viral vectors in clinical studies. Each of these viral vectors has distinct characteristics that provide distinct advantages for clinical gene transfer but are also associated with various inherent limitations, including pre-existing antibodies against the virus, the small packaging size of the transgene (5 kb), and neutralizing antibodies produced as a result of immunological reactions to the vector limit the use of viral vectors for liver disorders, reducing the effectiveness of re-dosing in AAV-mediated gene therapy[13] and lentivirus has random integration concerns.

US Food and Drug Administration (FDA) has approved multiple gene therapy products for monogenic liver disorders, including Adeno Associated Virus (AAV)-based gene therapies for Hemophilia A (*F8* gene in AAV5, ROCTAVIAN) and Hemophilia B (*F9* gene in AAV5 Hemgenix). Since clotting factor concentrates (CFC) have the risk of transmitting viral infection, approximately 75% of Hemophilia patients have been treated with a recombinant clotting factor. Frequent infusions of short half-life and immunogenic, recombinant-based factor or non-factor replacement therapies develop antibodies, making costly and inconvenient replacement therapy. Due to these limitations, nearly 70%−80% of hemophilic patients from developing countries need access to proper treatment[14].

Recently, clinical data suggested that AAV-based gene therapies for hemophilia A & B showed high F8 expression for at least one year in Hemophilia A patients, thus reducing the risk of spontaneous bleeding events and the treatment frequency compared to factor replacement therapy. Recently, the FDA approved an AAV5-based gene therapy, ROCTAVIANTM, for treating adults with severe hemophilia A. However, studies revealed that AAV-based gene therapy has shown inconsistent levels of F8 expression across patients and immune responses to viral vectors, preventing repeated treatment in hemophilia A patients. Due to the complex steps involved in vector manufacturing, AAV-based products have exorbitant costs of over $2 million. Hemgenix, with a charge tag of $3.5 million, is now the costliest drug on the planet[15]. Furthermore, AAV-based gene therapy could be inaccessible to a significant patient population, below 12-year-old patients, and over 80% due to pre-existing antibodies against the vector[16].

Recent advances in developing mRNA vaccines for coronavirus disease (COVID-19) using clinically translatable lipid nanoparticles have drawn attention to delivering the payloads efficiently to the target cells[17]. FDA emergency use authorization for Pfizer-BioNTech and Moderna mRNA-LNP vaccines in 2020[18]. Alnylam received FDA approval for the lipid-based siRNA drug Onpattro™ for hereditary transthyretin-mediated amyloidosis (hATTR amyloidosis, Table 1). Concurrently, emerging delivery vector systems based on polymeric and lipid nanoparticles currently targeting hepatocytes are explored[19]. Through the decades, lipid nanoparticles have allowed us to use non-viral vectors to efficiently deliver genes to liver cells. This review presents an overview of non-viral nucleic acid therapy for liver monogenic disorders involving gene expression and gene editing strategies using mRNA, siRNA, and anti-sense oligonucleotides as therapeutic molecules.

Apart from gene supplementing, researchers have developed alternative strategies, and one such ground-breaking discovery is gene editing technology. This technique provides efficient and precise modification by introducing a double-stranded break (DSB) in a particular genomic sequence followed by subsequent DNA break repair[20]. Gene-regulating approaches can treat a broader range of liver diseases, including inherited and acquired ones, with an *ex-vivo* or *in-vivo* approach.

There are two types of CRISPR-based technology grounded on the repair mechanism. The two main components of DSB repair pathways are those that use DNA templates for homology-directed repair (HDR) and those that carry out re-ligation (end-joining) of the broken DNA ends, typically with additional nucleotide deletions or insertions at the DSB site (HDR). The two main components of DSB repair pathways are those that use DNA templates for homology-directed repair and those that carry out re-ligation (end-joining) of the broken DNA ends, typically with additional nucleotide deletions or insertions at the DSB site (HDR).

Table 1 FDA-approved drugs for hepatic disorders.

Name of the drug	Gene of target	Indication	Company/product	Type of nucleic acid therapy	Route of administration	Trail status
Mipomersen	ApoB100 mRNA	hoFH	Genzyme (kynamro)	ASO	Subcutaneous	01/2013
Patisiran	TTR mRNA	hATTR	Alnylam Pharmaceuticals (onpattro)	siRNA	Intravenous	08/2018
Inotersen	TTR mRNA	hATTR	Ionis Pharmaceuticals (Tegsedi)	ASO	Subcutaneous	10/2018
Giosiran	ALAS1 mRNA	AHP	Alnylam Pharmaceuticals (Givlaari)	siRNA	Subcutaneous	11/2019
Lumasiran	HA01 mRNA	PH Type 1	Alnylam Pharmaceuticals (oxlumo)	siRNA	Subcutaneous	11/2020

(Advances in genome editing through control of DNA repair pathways)[21]. Base editors, which consist of a deaminase domain and a Cas9 variation (D10A nickase (nCas9) or catalytically deficient Cas9 (dCas9)), bypass several of the constraints of DSB-producing, nuclease-mediated genome editing. (Precision genome engineering through adenine and cytosine base editing)[22,23]. Prime editors are fusion proteins comprising a Cas9 nickase domain (inactivated HNH nuclease) and a reverse transcriptase domain designed by the researchers[24,25].

In this review, we summarize the advances with lipid nanoparticles for multiple nucleic acid therapeutic approaches that include gene silencing, editing, and supplementation, where the process can be done by siRNA/ASO, ZFN/TALEN/CRISPR/Base Editors, and plasmid/mRNA, respectively (Fig. 1).

2. Delivery vectors: Non-viral vectors

Recent decades have witnessed the transformation of nucleic acid therapeutics from bench to bedside, particularly for liver-targeted delivery. Even though viral vectors have been the preferred choice for targeting hepatocytes, their success is limited because of the previously mentioned drawbacks, such as pre-existing antibodies, insertional mutations, and packaging capacity[26]. On the other hand, an increasing number of non-viral vectors have been evolving as choices for delivering genetic material to the liver. It must meet specific criteria, including lipid-nanoparticle design, nucleic acid complexation, formulation, nucleic acid design, target cell characterization, administration mode for therapeutically relevant transfections, and acceptable safety profiles[27,28] (Fig. 2). This review discusses progress in the field of liver-directed nucleic acid therapy approaches.

Non-viral vectors come in many types, including polymers and solid lipid nanoparticles[29]. Lipid nanoparticles are multifunctional envelope-type nanodevices where pH-sensitive lipids deliver siRNA[30]. Other studies have used lipid-coated phosphate (LCP) nanoparticles for *in-vitro* transfection in mammalian cells and showed less toxicity[31]. Hepatocytes and hepatic stellate cells can receive nucleic acids through this LCP [32,33]. Lipid-coated calcium phosphate offers efficient pDNA delivery to the liver hepatocytes,

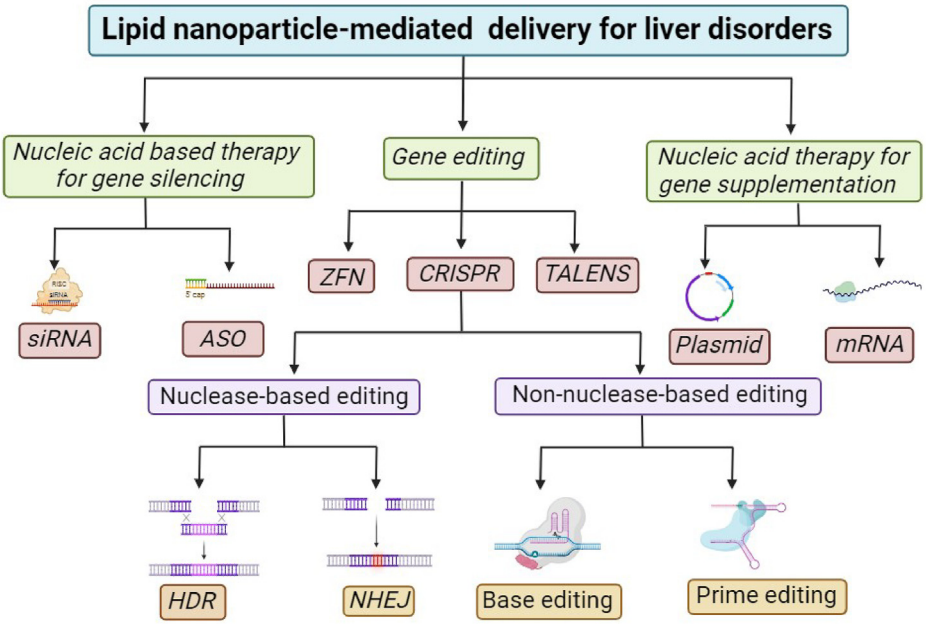

Figure 1 Lipid-mediated nucleic acid therapy is classified into three primary strategies. To facilitate the disease condition, gene expression is regulated by siRNA or ASO. Using nuclease-based and non-nuclease-based editing tools, gene editing corrects a specific mutant gene to return to the normal phenotype. Gene supplementation delivers functional nucleic acids in pDNA or mRNA form. siRNA, silencing RNA; ASO, anti-sense oligonucleotides; ZFN, zinc finger nucleases; CRISPR, clustered regularly interspaced palindromic repeats; TALENS, transcription activator like effector nucleases; HDR, homologous directed repeat; NHEJ, non-homologous end joining; BE, base editor; PE, prime editor.

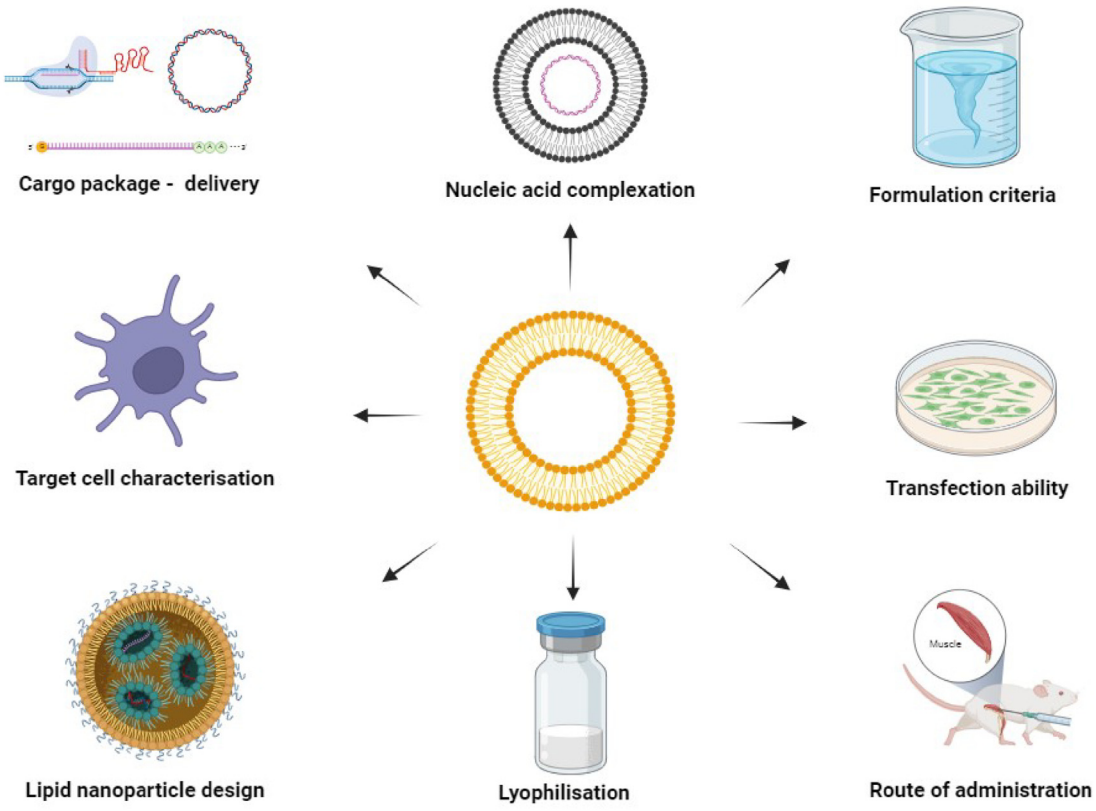

Figure 2 Criteria to attain efficient transfection. A liposome and any nucleic acid are combined to form a lipoplex. This lipoplex's efficiency in transfecting the cells depends on Lipid-nanoparticle design, nucleic acid complexation, formulation, DNA-vector design, characterization of the target cell, route of administration, lyophilization, and transfection ability.

not other organs. This formulated lipid nanoparticle system can reduce the amount of metastasis in the liver up to 10-fold[34]. Besides targeting hepatocytes, lipid-coated calcium phosphate is also used for hepatic stellate cells—furthermore, non-ionic surfactant-based vesicles, termed niosomes, target hepatocytes to treat liver fibrosis and malaria[35]. Both liver cancer treatment and liver cancer diagnosis employ nano-emulsions and nano-micelles[36,37]. Relaxin, which plays an imperative anti-fibrogenic role with nitric oxide-dependent pathways impeding fibroblast differentiation, proliferation, and activation, has been explored as a potential target against liver fibrogenesis[38].

Receptors can effectively target liver hepatocytes, including membrane-bound C-lectin receptors and asialoglycoprotein receptors (ASGPR). Hepatocytes have significant expression of it, with around 1.8 million receptors per cell[39]. Our previous findings reported that cationic lipids containing cyclic and open sugars efficiently delivered genes selectively to the mouse liver *via* the asialoglycoprotein receptor[40]. Folate receptors expressed as glycosylphosphatidylinositol on the membrane can also target hepatocytes[41]. The mannose receptor, Retinoid binding protein, platelet-derived growth factor receptor-beta, and collagen type VI are a few receptors studied for targeting hepatic stellate cells[42]. *In vitro*, the gene of interest is delivered by a process called transfection or lipofection. Liposomes and nucleic acids interact electrostatically to generate lipoplex. Endocytosis allows them to enter the target cell. Following early endosomal escape, nucleic acids are released into the cytoplasm and transported to the nucleus, transcription occurs, and functional protein is produced *via* cytoplasmic translation[43] (Fig. 3).

3. Lipid nanoparticle components and structural design for nucleic acid delivery

Lipid nanoparticles and nucleic acid begin to expand in the 1960s and finally join forces in the 1970s; the combined growth has been phenomenal to this day in a variety of fields (Fig. 4).

Lipid nanoparticles used for nucleic acid therapy consist of cationic lipids/ionizable lipids, helper lipids, phosphatidyl methylamines or phosphatidylcholine, and cholesterol. Polyethylene glycol (PEG) is also used to impart circulation stability and reduce particle aggregation. Cationic/Ionizable lipids form electrostatic complexes with negatively charged nucleic acids to facilitate intracellular delivery, protect nucleic acids from enzymatic degradation, and facilitate intracellular delivery (Fig. 5)[65]. The particle size and surface charge can be quantified using dynamic light scattering (DLS) and zeta potentials. The structures of nucleic acid-encapsulated lipid nanoparticles can be visualized with TEM or cryo-TEM (Transmission Electron Microscopy), and internal molecular arrangements of the lipids in the lipid nanoparticles can be analysed with small-angle X-ray scattering experiments (SAXS-D), differential scanning calorimetry (DSC) and fluorescence resonance energy transfer (FRET) experiments[66]. Physico-chemical properties of the lipid nanoparticles critically depend on the molecular architecture of the ionized/ionizable lipid, the nature of the helper lipids, and their composition, which determines the transfection efficiencies.

Concerning ionized/ionizable lipids, transfection efficiencies can be modulated by the type of hydrophilic head group, hydrophobic tail, and a linker group, such as an amide, ether, and ester,

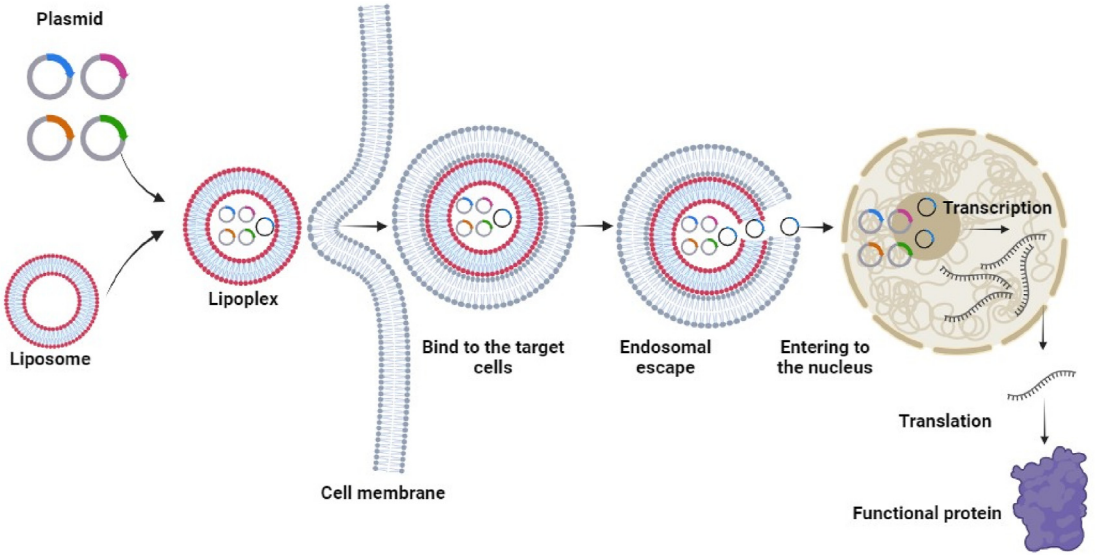

Figure 3 Outline of lipid-mediated gene delivery. Liposome and nucleic acid combine to form lipoplex by electrostatic interaction. They enter the target cell through endocytosis. After the early endosomal escape, nucleic acids are released into the cytoplasm, transported to the nucleus, undergo transcription, and are allowed by a translation in the cytoplasm to produce functional protein.

which usually holds the polar nitrogenous head group. Understanding the non-polar hydrophobic tail and these structural criteria is crucial in efficient transfection and gene delivery[66]. In ionizable lipids, head groups have either primary, secondary, or tertiary amines, which are protonated in an acidic pH environment and become uncharged at neutral pH. Efficient intracellular delivery of lipid nanoparticles critically depends on cellular uptake and early endosomal escape, which happen through either the proton sponge effect or membrane fusion in endosomes (Fig. 5). The balance between the hydrophilic head group and the hydrophobic tail is a critical parameter in designing the cationic lipids for developing stable lipid nanoparticle formulations[67]. Helper lipids like cholesterol impart rigidity to the liposomes, increasing the stability of the formulation[68]. We demonstrated that steroidal spirosolanes, analogous to cholesterol could be used as alternative methods to enhance transfections further[69]. Concurrently, the Sahay group demonstrated that using β-sitosterol, naturally-occurring cholesterol as a co-lipid in lipid nanoparticles, induced polyhedral shape and, in turn, enhanced intracellular delivery of mRNA. This study emphasized the influence of alternative steroidal co-lipids on the surface composition and structural properties of nanoparticles and their subcellular interactions to improve cytoplasmic delivery[70]. Recently, the Cullis group demonstrated that even buffers used to prepare LNPs were necessary for the transfections. In higher salt concentrations, 300 mM sodium citrate buffer at pH4, mRNA encapsulated LNP displayed distinctive mRNA-rich "bleb" structures with higher transfection properties[66].

As shown in Fig. 5, each functional nucleic acid molecule has a different mechanism of action. RNA molecules require cytoplasmic delivery, in which mRNA has to be translated to express functional protein. SiRNA and ASO cleave the target mRNA to restore the aberrant cascade. Plasmid DNA should be transported into the nucleus. Hence, specific strategies, such as adding a nuclear localization signal, are required to enhance the nuclear delivery of pDNA[71] (Fig. 5). Considering the nature and action of the nucleic acids, nanoparticle design strategies vary. As RNA is a linear molecule, it requires a higher lipid: nucleic acid ratio, whereas pDNA is circular and requires less lipid: nucleic acid ratio for better transfections. Hence, specific strategies are needed to develop the lipid nanoparticles for each type of nucleic acid molecule[72].

The general parameters considered for nanoparticles are long-term stability in storage after lyophilization, higher nucleic acid encapsulation efficiency, uniform particle size, surface charge ratio, low cytotoxicity, and scalable manufacturing. Overall, LNPs are a cost-effective choice for nucleic acid delivery due to their ease of manufacturing and reproducible scale-up procedures[73].

4. Liver-specific nucleic acid delivery

Either passive or active methods can typically achieve organ-specific nucleic acid delivery. Passive delivery can increase local nucleic acid concentrations and reduce non-specific transport to other organs, thus reducing adverse effects. This can be achieved by optimizing physicochemical properties, including the size, surface modification, or lipid composition[74]. Consequently, passive accumulation of nanoparticles requires either pathophysiological properties or specific tissue compatibility that complement the nanoparticle's properties. Recently, selective organ targeting (SORT) has been explored with lipid nanoparticles by optimizing the composition of ionizable and helper lipids[75].

Active targeting requires nanoparticles with covalently tethered receptor-homing ligands on their surfaces to deliver nucleic acids to specific cell types through receptor-mediated endocytosis[40]. A more targeted approach to relevant liver cell types reduces side effects and maximizes the therapeutic outcome. One of the most successful uses of non-viral transfections for organ-specific gene delivery is delivering genes to hepatocytes. Asialoglycoprotein receptors (ASGPR) are highly expressed in hepatocytes. Galactose is a potent ligand for ASGPRs. Hence, these galactosylated lipids can be the most promising implementation for delivering therapeutic genes to hepatocytes for liver-specific nucleic acid delivery. The targeting efficiency depends on the nature of the ligand and its distance to the ammonium ion center[40].

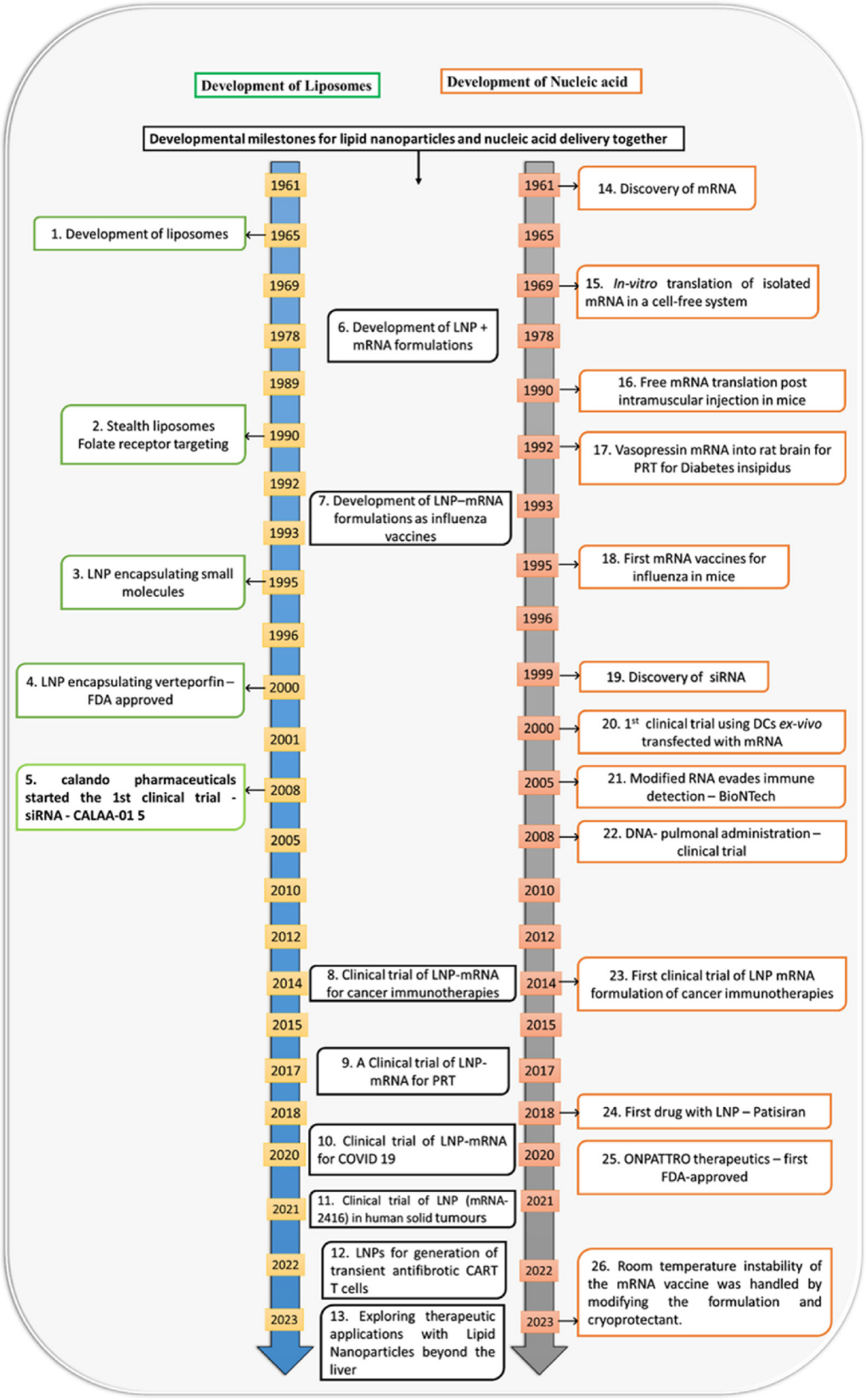

Figure 4 Timeline of nucleic acid and liposome development till 2023. Relevant references are represented as 1[44], 2[45], 3-4[46], 5[47], 6[48], 7[49], 8 (NCT02316457), 9 (NCT03375047), 10[50], 11[51], 12[52], 13[53], 14[54], 15[55], 16[56], 17[57], 18[58], 19[59], 20[60], 21[61], 22[60], 23 (NCT02316457), 24[62], 25[63], 26[64] within the figure.

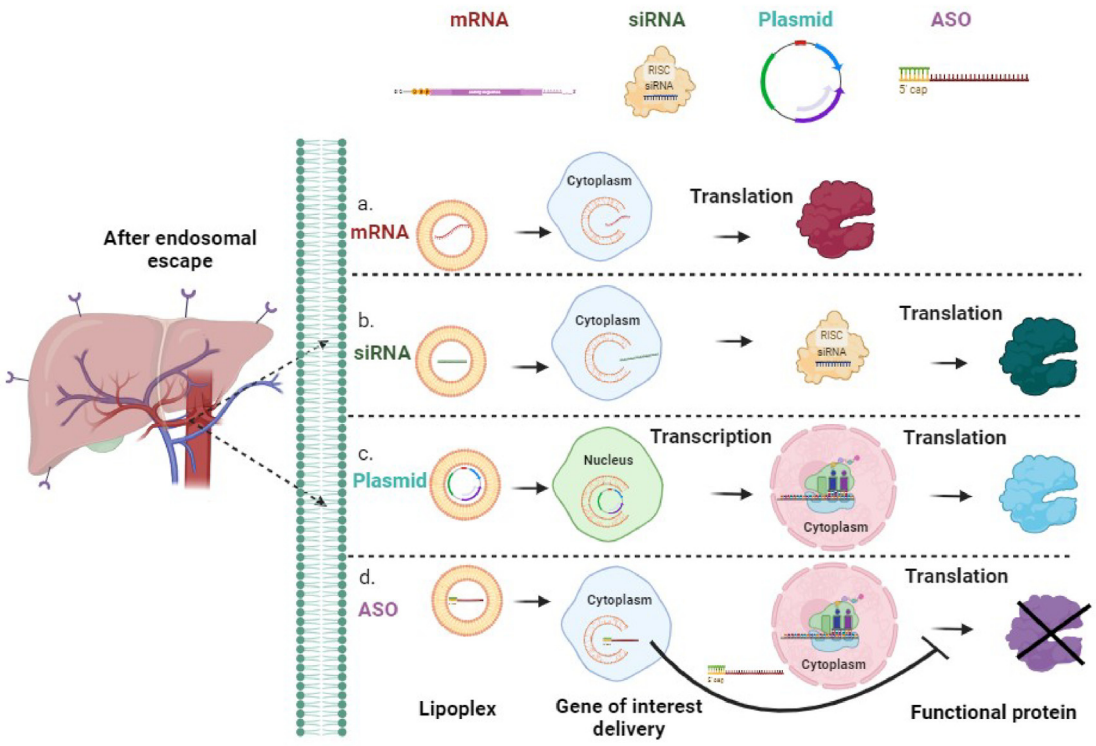

Figure 5 Representation of LNP-Based delivery of different nucleic acids to the liver to produce therapeutic protein. (a) Delivery of mRNA+LNP complex. Where mRNA is released into the cytoplasm, thereby making therapeutic protein. (b) siRNA encapsulated LNP performs sequence-specific gene-silencing (c) pDNA+LNP enters the nucleus, followed by transcription and translation in the cytoplasm. (d) Antisense Oligonucleotides are released in the cytoplasm, inhibiting specific gene expression by promoting mRNA degradation.

Exploring the combination of both strategies first to facilitate general (passive) liver uptake and, subsequently, ligand-mediated (active) cellular internalization may pave the way for efficient and precise liver-specific delivery.

Currently, multiple ongoing pre-clinical and clinical studies are in progress for treating liver disorders using liposome-mediated nucleic acid delivery (Tables 2 and 3)[76-90].

5. Nucleic acid therapy for gene silencing

When the mRNA is transported with the LNP complex, the mRNA is released into the cytoplasm, and therapeutic protein is produced. Regarding siRNA, the sequence-specific gene silencing by siRNA is encapsulated in LNP. Concerning pDNA with LNP, it penetrates the nucleus, followed by transcription and translation in the cytoplasm. In the cytoplasm, antisense oligonucleotides are produced, limiting particular gene expression by increasing mRNA breakdown (Fig. 5).

5.1. siRNA-based therapy

In the 2000s, the siRNA delivery concept was initiated for specific target gene knockdown, increasing the required protein or decreasing the redundant gene. Later, it became a therapeutic option with the help of the lipid nanoparticle-mediated delivery system. siRNA must be delivered in the cytoplasm to form the RNA-induced silencing complex (RISC complex). In 2006, the siRNA delivery commenced with apolipoprotein B specific siRNA encapsulated using stable nucleic acid lipid particles (SNALP) to silence diseases in non-human primates through intravenous administration[87]. When Onpattro®, a siRNA-based treatment, received FDA approval in 2020, it marked a significant advancement in treating the rare disease hereditary transthyretin amyloidosis (hATTR)[91]. A DLin-MC3-DMA lipid-encapsulated siRNA could achieve high levels of gene silencing *in vivo* without inducing systemic toxicity and is currently being used as a clinically approved LNP to deliver multiple siRNA products[54]. Many other potential products are being developed inspired by the clinical success of LNP-enabled siRNA-based drugs. They are currently at the pre-clinical stage for hereditary and acquired diseases. In addition, siRNA is also being explored in developing anti-viral therapeutics, including hepatitis B virus (HBV) and hepatitis B virus (HDV). Liver-specific GalNAc-PEG lipids were conjugated with modified siRNA and delivered to hepatocytes in chimeric mice, and a load of hepatitis B virus (HBV) was decreased[92]. ARB-1740 demonstrated potent inhibition of hepatitis delta virus with significantly reduced life cycle markers, including HBsAg, HBeAg, and HBcAg viral proteins in a dually-infected humanized mouse model[93]. Similarly, for the hepatitis C virus, systematically injected lipoid nanoparticles carrying PRK2 siRNA reduced HCV RNA in the blood[94]. Another siRNA drug, ARC-520, consists of two synthetic short interfering RNAs (siRNAs) conjugated to cholesterol, targeting mRNA transcripts of HBV polyadenylation sites[95]. It is the first RNAi-based antiviral therapeutic to enter the clinic and showed promising efficacy in phase-I clinical trials[96].

In addition, Fitusiran[97], Givosiran[98], Inclisiran[99], and Lumasiran[100] are the GalNac lipid conjugated siRNA therapeutics that are in clinical trials for treating hepatic diseases such as

Table 2 Current preclinical studies.

Nucleic acid	Gene of target	Cellular target	Target nucleic acid therapy	Animal model	Ref.
pDNA	Luciferase	Screening	Gene expression	ICR mice	76
mRNA	Glycogen storage disease type 1A	Hepatocytes	Gene expression	*G6pc*$^{-/-}$ mouse	77
mRNA	Ornithine transcarbamylase deficiency	Hepatocytes	Gene expression	*Otc*$^{spf\text{-}ash}$mice	78
mRNA	Alpha-1 antitrypsin (*AAT*) deficiency	Primary human hepatocytes	Gene expression	Slcw/SzJ, (NSG-PiZ) mice	79
mRNA	Crigler–Najar syndrome type 1	Hepatocytes	Gene expression	Gunn rats	80
mRNA	Haemophilia B	Hepatocytes	Gene expression	Haemophilic B mice	81
mRNA	Cre-recombinase luciferase	Screening	Gene expression	NMRI mice/reporter	82
mRNA	Arginase deficiency	Hepatocytes	Gene expression	*Arg1*$^{-/-}$ mice	
mRNA sgRNA	*TTR*	Hepatocytes/ATTRv amyloidosis	Gene editing	Sprague–Dawley	83
mRNA sgRNA	*PCSK9*	Hepatocytes/ hypercholesterolemia	Gene editing	C57BL/6	84
siRNA	*TTR*	Hepatocytes/ATTRv amyloidosis	Gene silencing	Cynomolgus monkeys	85
siRNA	*F7*	Hepatocytes/screening	Gene silencing	C57BI/6 mice	86
siRNA	*APOB*	Hepatocytes/ hypercholesterolemia	Gene silencing	Cynomolgus monkeys	87

Table 3 Current clinical studies.

Nucleic acid	Gene of target	Indication	Product	Type of nucleic acid therapy	Clinical stage	Ref.
sgRNA mRNA	*TTR* Cas9	ATTRv amyloidosis	NTLA-2001 (Intellia therapeutics/regeneron)	Gene editing	Phase 1 planned	88
mRNA	*CEBPα*	Advanced liver cancer	MTL-CEBPS (Mins Alpha)	Gene expression	Phase I (recruiting) NCT02716012	89
sgRNA mRNA	*TTR*		Intellia therapeutics	Gene editing	Phase I NCT04601051	
siRNA	*TTR*	ATTRv amyloidosis	Onpattro®, patisiran (Alnylam Pharmaceuticals)	Gene silencing	Approved (2018)	88
siRNA	*HBV*	Hepatitis	ARB-001467 (Arbutus Biopharma)	Gene silencing	Phase II (completed) NCT02631096	90

hemophilia B, acute hepatic porphyria, Low Density Lipoprotein, and primary hyperoxaluria type 1 respectively. A novel LNP drug containing ND-L02-s0201/BMS-986263 LNP with HSP47 siRNA was developed to treat liver and idiopathic pulmonary fibrosis and is in phase 1 clinical trials. The accumulation of insoluble collagen characterizes fibrosis because of the instigation of hepatic stellate cells (HSC) by HSP47. Systemic administration of BMS-986263 LNP loaded with siRNA HSP47 can reversibly inhibit HSP47 production[101].

LNP-siRNA-mediated gene transfer has been explored in Hepatic oncology. Multiple liver malignancies overexpress polo-like kinase. TKM-080301, a siRNA nanoparticle formulation, could reduce PLK1 levels and control cell cycle progression stages[102]. Recently, LNP has also delivered both siRNA and mRNA, which target the Factor VII gene and the luciferase protein, respectively[103]. The use of siRNA has also been investigated for controlling abnormally expressed adhesion molecules on hepatocytes, such as $\beta 1$ and αv integrin subunits, to treat Hepatocellular carcinoma[104].

Among the nucleic acid therapeutics, siRNA-based therapeutics were the most successful in the clinical trials for multiple monogenic liver disorders, owing to their ease of application and robust stability. These are aimed at balancing the disease cascade to alleviate the condition. The long-term consequences of repeated dosing are still elusive.

5.2. *Anti-sense oligonucleotides (ASOs)*

ASOs are synthetic, single-stranded oligonucleotides that bind to complementary RNA transcripts to form a hybrid ASO-RNA complex, resulting in cleavage by ribonuclease H. The Zamecnik group reported the first antisense oligonucleotides (ASO) as an antiviral drug targeting the Rous Sarcoma Virus and inhibiting its replication[105]. Bepirovirsen is an ASO antiviral to reduce the viral load of HBV. In a recent phase 2 clinical trial, HBsAg levels remained undetectable, and HBsAg DNA levels stayed below 20 IU per ml at 24 weeks after discontinuing the treatment in the infected patients[106]. Inotersen, a 2′-*O*-methoxyethyl-modified ASO to treat hereditary transthyretin amyloidosis, is an FDA-approved, clinically available therapeutic with an increased therapeutic outcome[107]. Targeting the proprotein convertase subtilisin/kexin type 9 (PCSK9) gene was investigated using bio-reducible LNP-based ASO delivery. Systemic ASO delivery could significantly silence the *PCSK9* gene in pre-clinical studies. The drug is found to be safe as there is no toxicity in the liver and kidney even after 5 mg/kg of LNP-ASO delivery[108].

Accumulation of toxic metabolites is one of the major concerns in many inherited metabolic liver disorders. To address this, ASO and siRNA have emerged as promising options for metabolic pathway reprogramming and substrate reduction therapy[109]. Chemically modified ASO for targeting apolipoprotein B (APOB)

to decrease circulating cholesterol levels is FDA-approved. Mipomersen, an ASO, hybridizes to the mRNA, cleaves the APOB mRNA, and reduces low-density lipoprotein cholesterol[110]. Non-alcoholic-steatohepatitis (NASH) is another metabolic disorder that leads to cirrhosis over the period. By targeting the expression of serine/threonine-protein kinase 25 (STK25), GalNAc-modified ASO is under investigation for treating NASH[111]. The application of ASO in controlling the expression of long non-coding RNAs (lncRNAs) in liver diseases such as cholangiocarcinoma and hepatocellular carcinoma is also being explored[112].

6. Nucleic acid therapy for gene supplementation

6.1. *Gene therapy (plasmid DNA-based delivery)*

Gene therapy means the use of genetic material for therapeutic purposes. Initially, gene therapy was considered solely for replacing the malfunctioning gene with its correct copy. However, attributing the advances in understanding disease mechanisms, gene therapy has expanded horizons to other approaches, from silencing pathogenic genes to precise modification at the genomic level[113]. Although, conceptually, it is simple, a safer and more efficient vector for delivering the genes systemically is still a significant challenge. Despite a few clinical successes with AAVs, their applications in monogenic liver disorders are limited due to pre-existing immunity to the virus, no possibility for vector re-administration, and treatment of 12-year-old children. More importantly, it highlights significant disease populations[114]. Non-viral vectors, such as lipid nanoparticles, polymers, and dendrimers, are the potential alternatives. Among these, lipid nanoparticles have been emerging as a promising alternative.

Multiple pre-clinical studies have used liposome-based plasmid DNA (pDNA) to treat monogenic liver disorders. In our structure-activity investigation studies, we evaluated efficiencies of galactose ligand binding both in cyclic pyranose and open chain form by varying spacer arm between ligand and quaternary ammonium center in the cationic lipids. We observed that cationic lipids in cyclic form with six carbon spacers and open form with two carbon spacers efficiently transfect luciferase reporter plasmids into mouse liver[40]. Hashida group proposed histidinylation of galactosylated cholesterol derivative (Gal-His-C4-Chol) to induce the "proton sponge effect" for superior gene delivery to hepatocytes[115]. Leong group developed chitosan-based nanoparticles encapsulating functional FVIII transgene incorporated in gelatin. In preclinical studies, 1%−4% of functional F8 protein expressions were detected in the plasma of hemophilia A mice. Although the levels are low, they are sufficient to control spontaneous bleeding[116]. To improve the efficacy of pDNA delivery and expression in the liver, the Akita group developed an advanced hepatic gene delivery system using a novel ssPalm derivative (an SS-cleavable and pH-activated lipid-like material: ssPalm) and an anti-inflammatory drug, Dexamethasone. The lipid nanoparticle system reduced the inflammatory responses induced by pDNA transfection and increased the transgene expression in mice[117].

Although multiple lipid nanoparticle systems exist for liver-specific delivery of pDNA, therapeutically relevant transgene expression with pDNA *in vivo* is still challenging.

7. mRNA-based therapy

mRNA as a therapeutic molecule offers multiple advantages, including its non-integrative nature, as it requires cytoplasmic delivery, which also contributes to its ability to transfect non-mitotic cells compared to plasmid DNA (pDNA)[118,119]. However, its therapeutic applications were limited due to the induction of immune responses to exogenous RNA, affecting translational efficiency[120]. These limitations were overcome by chemical modifications on mRNA such as pseudouridine (Ψ-UTP) and methyl pseudouridine (me1Ψ-UTP) modifications[121]. Concurrent to mRNA technology, advances in lipid nanoparticle manufacturing with microfluidic technology made it possible to produce uniform lipid nanoparticles with high encapsulation efficiencies of mRNA. Scientific advances on these fronts complemented each other to open a new therapeutic avenue[122]. It has emerged as versatile for treating several conditions, including gene editing[123], vaccine development[124], induced pluripotent stem cells (iPSCs) reprogramming[125], protein replacement therapy[126], antibody therapy[127], and chimeric antigenic receptors (CAR)[128]. As an alternative to protein replacement therapy, mRNA molecules are currently being researched for treating liver-associated viral illnesses and monogenic liver disorders[129,130].

Delivery of mRNA molecules for protein replacement therapy could address the challenges of delivering functional recombinant proteins, including repeated administration and antibodies against the recombinant protein[131]. Using chemically pseudo-modified, codon-optimized human methylmalonyl-CoA mutase mRNA encapsulated in biodegradable lipid nanoparticles, a functional enzyme was expressed, which in turn resulted in a reduction in plasma methylmalonic acid of 85% in a mouse model, namely Mut/; TgINS-MCK-Mutmice, Mut/; TgINS-CBA-G715V[13].

The fabrication of lipid nanoparticles enabled the precise and efficient delivery of mRNA simultaneously. The hybrid mRNA technology delivery system (HMT) was designed to treat ornithine transcarbamylase (OTC) deficiency. The OTC mRNA normalized the plasma ammonia and orotic acid levels in the urinary system in the OTCD mice model[78]. A novel series of amino lipids could efficiently deliver luciferase mRNA in rodents and nonhuman primates without adverse events[132].

LNP-mRNA delivery system has been utilized for the hepatic reticuloendothelial system (RES), where the systemically administered LNP-mRNA targets hepatocytes and paves the way for recovery from RES[133]. On the road to clinical studies, multiple administrations of these mRNA-lipid nanoparticles into nonhuman primates confirmed safety profiles and translatability. The technology is also being used in a proof-of-concept for systemic delivery of human porphobilinogen deaminase (hPBGD) encoding mRNA as a potential therapy for acute intermittent porphyria (AIP)[134].

Another example of mRNA delivery to restore a Phosphatidylcholine Transporter (hABCB4) is in the preclinical stage for treating lethal, progressive familial intrahepatic cholestasis type 3 (PFIC3), which does not have any effective treatment[135]. In preclinical evaluations, an array of RNAs (mRNA and dual mRNAs) encapsulated in LNP replace the enzyme in propionic acidemia/aciduria (PA) disorders. These RNAs encode the PCCA (alpha) and PCCB (beta) proteins, thereby expressing the desired protein to alleviate PA diseases in the liver[136].

Oral administration of mRNA encoding the cytoplasmic enzyme arginase 1 (ARG1) in biodegradable LNP restored 54% of the enzyme, thus overcoming the ARG1 deficiency at a clinically

relevant level in transgenic mice[137]. A loss-of-function mutation in *SLC25A13* causes a defective aspartate/glutamate transporter, leading to citrin deficiency. Intravenous administration of human codon-optimized citrin mRNA ameliorated disease conditions by reducing citrulline and blood ammonia levels[138].

In vivo, OF-02 LNP increases the production of EPO by two times. As a result, mRNA is efficiently delivered *in vivo* to express a specific protein[139]. Biodegradable lipids were utilized in Dlin-MC3-DMA by incorporating alkyne and ester groups (cKKE12 LNPs) to reduce toxicity and achieve earlier clearance of nanoparticles. The study identified endocytosis, ApoE-independent cellular uptake, and albumin-associated micropinocytosis. During mRNA release, the nanoparticles also promote membrane fusion, which enhances the delivery of mRNA[140]. In PKU animal models, intravenous delivery of a full-length mRNA encoding human PAH induced high levels of the human PAH enzyme and restored its metabolism[141].

Although mRNA delivery as a protein replacement therapy offers a safer and more effective alternative, these studies are still pre-clinical. UTR engineering, cell-specific codon optimization, and poly-A tail optimization would further enhance the efficiency and stability of mRNA to reduce repeated dosing.

8. Gene editing approaches

8.1. *Zinc finger nuclease (ZFN)*

ZFNs are the first generation of gene-editing tools. It has Cys2His2 fingers that recognize approximately three bps of DNA for editing[142]. *In vivo* gene editing using ZFN-encoded mRNA and LNP complexes knocked out more than 90% of the *TTR* gene. The mRNA used was ten times lower than previously reported. In addition, co-delivering therapeutic FIX transgene-loaded AAV with ZFN mRNA-LNP targeting intron 1 of the ALB locus showed effective *in vivo* integration and therapeutic protein expression. Thus, ZFN-mediated genome editing of hepatocytes *via* non-viral vectors exhibits sustained transgene expression and clinical safety[143].

8.2. *Transcription activator-like effector nucleases (TALENs)*

TALENs comprise a non-specific FokI nuclease domain fused to a customizable DNA-binding domain for target-specific gene editing[144]. The Criggler−Najjar syndrome type 1 is a rare inherited disease for which liver transplantation is the only cure—mutations in the *UGT1A1* gene cause this syndrome. To facilitate further research into this condition, using TALEN technology, mouse-derived liver cell lines were produced with *UGT1A1* gene deficiency[145].

8.3. *CRISPR/Cas9 gene editing*

CRISPR/Cas9 genome editing platforms are rapidly increasing in basic and clinical research. Delivering Cas9 protein and single-stranded guide RNA (sgRNA) is essential for the functional activity of the CRISPR/Cas9 system[146].

8.3.1. *Nuclease-based*

Julian D. Gillmore and his colleaguess[147] in collaboration with Intellia Therapeutics conducted phase 1 clinical trials to treat transthyretin amyloidosis using a gene editing drug, NTLA-2001 for reducing serum ATTR level. The drug consists of Cas9 protein encoding chemically modified mRNA and single guide RNAs (sgRNAs) targeting hATTR gene encapsulated in lipid nanoparticles (LNP)[147]. In another finding, a biodegradable lipid nanoparticle successfully delivered both the Cas9 mRNA and chemically modified sgRNA(s) to achieve robust delivery *in vivo*, in turn excision of the mouse transthyretin gene in the liver, and lowering serum protein levels by more than 97% for at least 12 months. LNPs were administered multiple times without compromising activity[83].

LNP-mediated delivery for acute lung inflammation, the delivered cas9mRNA and sgRNA reduced the frac intercellular adhesion molecule 2 (ICAM-2) fraction hepatocytes but not in the other major organs[148]. Cas9 protein and sgRNA targeting the transerytherin (*TTR*) gene were co-delivered to edit the TTR gene. The results revealed that the LNP system could knock down 97% of the TTR gene in a single administration in the murine model[149].

For the fumarylacetoacetate hydrolase gene, the combination of vectors has been explored. sgRNA and template were encapsulated in the viral vector, and Cas9-mRNA was packed in LNP to correct the mutation[150]. For *ANGPTI3* gene knockdown due to hypercholesterolemia, CRISPR Cas9 gene editing produced a therapeutic effect that lasted up to 100 days after a single liver administration.

Similarly, in HBV-infected mice, successive delivery of Cas9 mRNA and sgRNA targeting HBV DNA combined with TT3 could drastically reduce liver and serum HBsAg (HBV surface antigen), liver and serum HBeAg (HBV e antigen), and liver HBV RNA and DNA levels[151]. Several functionalized TT3 derivatives (FTT) with branched ester chains were created based on the chemical makeup of TT3. However, FTT5 demonstrated the highest potency for mRNA transport. Base editing efficiency in hepatocytes was 60% when ABE mRNA- and sgPcsk9-loaded FTT5 LLNs were administered intravenously to mice. This was more significant than the base editing efficiency of TT3 LLNs[152]. They were using cKK-E12 LNPs as a delivery vehicle to simultaneously encapsulate Cas9 mRNA and two chemically modified sgRNA targeting the Pcsk9 gene, one year after the coupled delivery of C12-200 LNPs and AAV2/8 together with components of the CRISPR system. *PCSK9* was gene-edited to a greater than 80% extent in the liver after a single intravenous injection of cKK-E12 LNPs, resulting in lower serum cholesterol and undetectable Pcsk9 levels[153,154]. Hemophilia can be recovered by inhibiting antithrombin (*AT*), an endogenous negative regulator of thrombin synthesis. Targeted gene editing of AT in mouse liver using 246C10 LNPs loaded with Cas9 and sgRNA improved thrombin production while inhibiting mouse AT (mAT) without causing off-target effects, hepatotoxicity, or significant anti-Cas9 immune responses[154].

More powerful LNPs have recently been described for transporting Cas9 mRNA and sgRNA to the liver. For instance, 306Oi10 LNPs, a branched-tail mRNA nanocarrier, increased the expression of proteins more than C12-200 and DLin-MC3-DMA LNPs did, allowing for powerful delivery of both CRISPR/Cas9 RNA components in the mouse liver, resulting in effective gene editing in mice[155].

CRISPR12/CR RNA RNA loaded in DNA Nanoclew (NC) was used to target and deliver the *PCSK9* gene of hepatocytes to lower cholesterol levels in serum. *In vivo*, approximately 48% and 45% of efficient *PCSK9* gene disruption and cholesterol reduction were observed[156]. SpCas9 mRNA was delivered within LNPs; an HDR donor DNA template and sgRNA expression cassette were

provided on an AAV8 vector that was co-administered along with the LNPs for HTI in mouse liver and corrected through HDR-based point mutation. The results demonstrated 0.8% correction of tyrosinemia and 24% indels in the liver, thereby inducing therapeutic genome editing[157].

8.3.2. Non-nuclease-based-base editors

Notably, CRISPR Base Editors, Adenine Base Editors (ABE), and Cytosine Base Editors (LipidCBE) are a recently developed gene editing tool that dispenses with the need to use template-based techniques like double-standard breaks to modify the target site precisely. Base editors work into the CRISPR/Cas system; sgRNAs recognize the binding site, and Cas9 is fused with deaminases, either cytosine (CBE: Base conversion from C to T) or adenosine (ABE: Base conversion from A to G)[158] (Fig. 6). The approach revolutionized gene editing in deactivating specific genes. It can be explored in basic and translational research, including developing disease-mimicking cellular models, animal models, and disease treatments.

Recently, ABE has been used to study the decline of blood low-density lipoprotein levels in mice and cynomolgus macaques. In this editing strategy, LNP has been used to encapsulate ABE mRNA and sgRNA targeting the *PCSK9* gene, showing a reduced level of LDL and PCSK9 (58% and 95%) in mice and 14% and 32% in macaques, respectively[159]. Another study shows that LNP-based base editor mRNA targeting the *PCSK9* gene reduced LDL (60%) and PCSK9 (90%) levels in cynomolgus monkeys[160]. It is evident, therefore, that LNP is an efficient way to deliver the base editor, which is a direct repair mechanism.

To generate fumarylacetoacetate hydrolase in the hepatocytes for tyrosinemia through hydrodynamic tail vein injection, the lipid nanoparticle used was encapsulated with a codon-optimized Adenine Base Editor mRNA and chemically modified sgRNA. To correct the disorder, point mutations in (G·C to T·A) genes were precisely edited by the ABE method[161].

9. Current challenges and limitations

Significant efforts have been made in recent years to develop non-viral gene therapies. Numerous lipid-based DNA delivery systems have been made, and successful siRNA clinical trials have produced the first RNAi therapy and received regulatory approvals. Nevertheless, limited transfection efficiency for specific tissues, imprecise targeting, and unintended immune system activation are barriers to using DNA vectors in clinical care. Therefore, it is necessary to continue researching effective and biocompatible nanomaterials for efficient transgenic expression and targeted DNA transport to target cells. Next-generation lipids' ongoing and successful development in RNA therapeutics illustrates how DNA therapies can be improved.

Regarding pDNA nucleic acid therapy, the payload size is another rate-limiting step in further developing viral and non-viral vectors, as many vectors are only capable of delivering transgenes less than 10 kb, but some plasmids are larger.

mRNA-based therapy may be a middle-ground strategy for treating these untreated diseases, improving patient survival and quality of life. However, mRNA-based treatment is still in its infancy, and many uncertainties must be resolved in clinical trials despite being an up-and-coming method. The absence of toxicity brought on by the build-up of LNPs will be crucial in determining the efficacy of mRNA-based therapy since minor variations in LNP tolerance may significantly impact long-term safety. Innovative LNPs with excellent pharmacokinetics and, more critically, a favorable toxicity profile assessed in non-human primates after repeated administrations are being developed because of active research in this area. The price of production and development will drop due to improvements in mRNA and LNP technology and the broadening of the indications, making this technology accessible for several rare and ultra-rare diseases.

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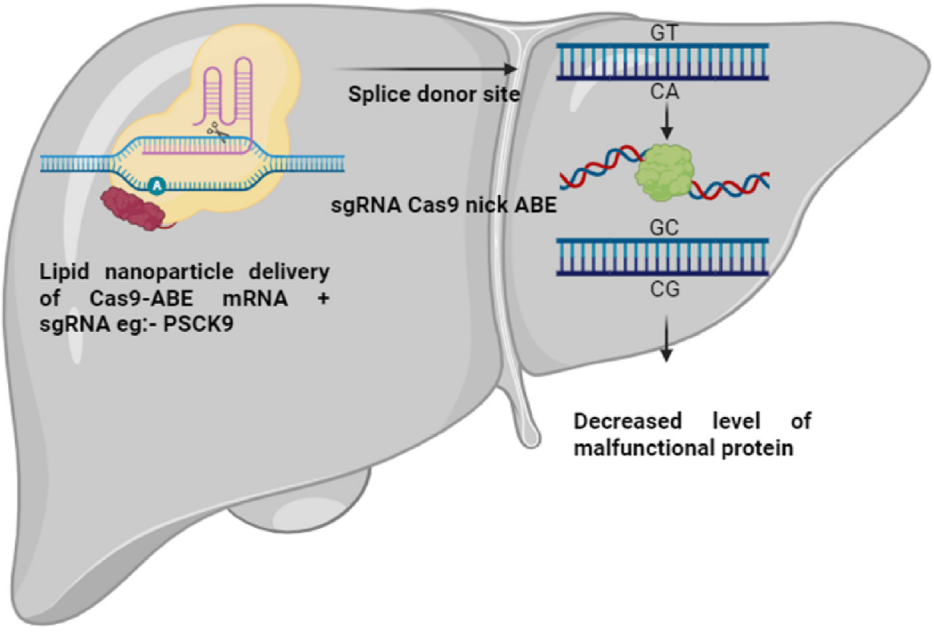

Figure 6 LNP-mediated base editing for liver disorders. LNP-encapsulated Cas9-ABE mRNA and sgRNA reach the liver, inducing point mutation without causing double-strand breaks, thereby reducing impaired protein levels.

rare and ultra-rare diseases. For those with monogenic disorders for whom liver transplantation is necessary, mRNA technology will be of significant interest due to the liver-targeting features of the LNPs created so far.

Because of the challenges associated with pharmacokinetics and the generally higher costs for development and manufacturing compared to small molecules, the main potential virtue is the anatomization of targets that are not available for small molecules. However, siRNAs currently only work in one direction: inhibition of gene translation. Consequently, development programs preferably focus on diseases unavailable to other treatment options and targets with validated overexpression and disease relevance.

10. Future perspective and conclusion remarks

The specificity of many techniques is closely tied to this; for instance, there is a considerable discussion regarding the specificity of CRISPR. Although base editing and prime editing offer a chance to improve specificity, no preclinical research utilizes these two methods. We hope that gene editing for treating liver disorders using lipid-mediated vectors will be investigated in the future.

The development of ionizable/cationic lipids and LNP technology has improved hepatocyte gene regulation from the bench to the bedside. LNPs are a versatile toolbox with numerous prospects for producing future gene treatments with more robust therapeutic benefits and improved toxicity profiles. This is because they are a multicomponent and modular platform. For instance, integrating lipophilic prodrugs in LNP systems has proven to be a desirable method for lowering the immunostimulatory effects of nucleic acid treatments. This is because it allows for the creation of combination therapies with additive therapeutic benefits. The development of more effective (ionizable cationic) lipids and advances in our understanding of nano-bio interactions *in vivo* are driving the ongoing refinement of LNP structures for the delivery of nucleic acid therapies.

Though, with the help of cutting-edge technology like CRISPR, we can edit the desirable gene, there are still some significant limitations, such as *in vivo* efficiency and off-target effects. Furthermore, the current *in vivo* gene editing strategy focuses on NHEJ-mediated gene knockouts but not HDR-based restoration of functional genes. For successful clinical trials, the future application of targeting components on the surface of LNP to increase its specificity will also be helpful. This is because delivery *via* LNP to different cell types needs to be clarified. Transient and efficient expression of LNP could provide a wide range of future therapeutic gene editing and gene delivery approaches.

In conclusion, developing regulatory frameworks to prompt the release of these products with enhanced safety and lower cost offers a substantial advantage to the patient population in developing and developed nations. The optimization of non-viral vectors to improve delivery specificity and mitigate cytotoxicity and the thorough characterization of vector safety profiles, followed by the conduct of broader clinical trials, are critical factors in the application of liver-targeted nucleic acid therapy to a variety of disorders originating in the liver.

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Author contributions

All the authors contributed to the writing and reviewing of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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