# RNA delivery biomaterials for the treatment of genetic and rare diseases

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## Abstract

Genetic and rare diseases (GARDs) affect more than 350 million patients worldwide and remain a significant challenge in the clinic. Hence, continuous efforts have been made to bridge the significant gap between the supply and demand of effective treatments for GARDs. Recent decades have witnessed the impressive progress in the fight against GARDs, with an improved understanding of the genetic origins of rare diseases and the rapid development in gene therapy providing a new avenue for GARD therapy. RNA-based therapeutics, such as RNA interference (RNAi), messenger RNA (mRNA) and RNA-involved genome editing technologies, demonstrate great potential as a therapy tool for treating genetic associated rare diseases. In the meantime, a variety of RNA delivery vehicles were established for boosting the widespread applications of RNA therapeutics. Among all the RNA delivery platforms which enable the systemic applications

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of RNAs, non-viral RNA delivery biomaterials display superior properties and a few biomaterials have been successfully exploited for achieving the RNA-based gene therapies on GARDs. In this review article, we focus on recent advances in the development of novel biomaterials for delivery of RNA-based therapeutics and highlight their applications to treat GARDs.

## Keywords

RNA delivery biomaterials; genetic and rare diseases (GARDs); RNA therapeutics; RNAi; mRNA; CRISPR

## 1. Introduction

Genetic and rare diseases (GARDs) represent a large category of disorders which share the similarity of each affecting a small portion of the population.[1, 2] While a single rare disease may affect only a small group of people, with the definition as fewer than 200,000 patients in the United States or less than 1 in 2,000 people in the European Union, the collective impact of rare diseases can be enormous.[1–3] Approximately seven thousand rare diseases affect around 30 million individuals in the US and over 350 million patients worldwide.[3–5] Despite the great efforts that have been devoted to supporting the development of orphan drugs, many rare diseases still lack effective cures and call for new therapeutics.[3] Since a majority of GARDs stem from genetic disorders, recent progresses in gene therapies could offer more options for treating GARDs.[3, 5]

In the past several decades, RNA-based therapeutics have been explored by researchers for diverse disease applications, ranging from cancers, infections, and autoimmune diseases to genetic disorders.[6–11] RNA vaccines, for example, have exhibited encouraging results in both infection therapy and cancer immunotherapy.[6, 7, 11–15] Another worthwhile application of RNA technology is to apply RNAs as a gene therapy tool to treat GARDs, which currently lack access to effective therapeutics. To date, a variety of RNA therapeutics have been developed for GARD therapies based on the versatile family of RNA molecules, including microRNA (miRNA), small interfering RNA (siRNA), messenger RNA (mRNA), long non-coding RNA (lncRNA), and genome editing systems containing RNA components. [6, 9, 16] These different RNA-based therapeutics can be used to treat genetic diseases, primarily at the RNA level via distinct mechanisms of action. For instance, RNA interference (RNAi) technology enables the downregulation of disease related genes and protein expression; mRNA mediates the expression and supplementation of therapeutic proteins; the CRISPR gene editing system facilitates gene engineering *in vivo* or through cell engineering *ex vivo*.[6, 9, 11, 17–20] Significant research has demonstrated the feasibility of RNA technologies to treat or cure GARDs (Figure 1).[17, 21]

In terms of RNA therapeutics, RNA molecules alone have difficulty penetrating target cells, are vulnerable to degradations in biological fluids, and are incapable of accumulating in target organs following systemic administration.[18] Although some naked RNAs and chemically modified RNAs were applied for therapies based on local administration or *ex vivo* applications, appropriate delivery methods are still essential for both enabling systemic applications of RNA therapeutics and further maximizing their therapeutic window.[22]

Several commonly used systemic RNA delivery methods include viral vectors, DNA/RNA nanotechnology and non-viral biomaterials.[20, 23, 24] Viral vectors are a type of efficient tool for gene transfection and account for many gene-therapy clinical trials.[20, 25, 26] Several viral vector mediated cellular and gene-therapy products, such as Kymriah for CAR-T immunotherapy on lymphoblastic leukemia, have already gained FDA approval.[27] Also, many viral vectors have been employed for treating GARDs, such as hemophilia, cystic fibrosis, or rare inherited retinal diseases, etc.[23, 28] In addition to viral vectors, DNA and RNA nanotechnologies have also been applied to deliver RNA therapeutics such as siRNA and miRNA, which are mainly used for cancer therapy.[24, 29–33] Non-viral RNA delivery biomaterials, such as ligand-RNA conjugates, lipid or polymer-based nanoparticles, and cell derived vehicles (exosomes), constitute another large category of RNA delivery vehicles. Different non-viral materials have been designed and optimized for accommodating efficient delivery of diverse RNA molecules as well as multi-component genome editing systems.[22, 34–36] Moreover, many of these biomaterials exhibit organ targeting properties, which make it possible for RNA therapeutics to achieve targeted effects at diseased sites.[18] In recent years, a number of non-viral delivery systems have been exploited for enabling RNA-based gene therapies on GARDs (Figure 2). Given the promising results from clinical trials and preclinical studies, biomaterials will continue to contribute to RNA therapeutics on GARDs.

In this review, we summarize the non-viral biomaterials developed for the RNA therapeutic delivery, including siRNA, mRNA, genome editing systems with RNA, miRNA, and lncRNA. We categorize these RNA delivery systems based on the different types of RNA payloads they afford and discuss in detail their different targeted organs, such as the liver, lungs, brain, muscle etc. In addition, we highlight clinical trials and therapeutic applications of these RNA delivery biomaterials in the field of GARD therapies. Finally, we briefly conclude with the advantages and limitations of different delivery vehicles and provide our future perspectives.

## 2. Small interfering RNA (siRNA)

siRNA, based on the RNA interference (RNAi) technology,[50] enables efficient downregulation of specific gene expression through complementary-based mRNA cleavage. [51] A number of genetic and rare diseases (GARDs) have shown their close link to specific genetic abnormalities, where mutant or misfolded proteins are expressed as a result and lead to disease symptoms.[1] Therefore, siRNA therapy can be applied for treating GARDs by targeted silencing of the disease-causing genes.[50, 52]

siRNA is typically double-stranded RNA oligonucleotides composed of 19–25 base pairs. [22] Their gene silencing mechanism involves the interaction of siRNA with an RNA-induced silencing complex (RISC), where siRNA is separated into sense and antisense strands. The sense strand of siRNA is subsequently discarded while the antisense strand recognizes the complementary mRNA and, together with the endonuclease Argonaute 2 (AGO2), guides the cleavage of targeted sequence.[9] Through these processes, siRNA could achieve targeted gene silencing and thus mediate the downregulation of gene activity. [51]

In recent years, siRNA for treating GARDs has aroused prominent interest in both pharmaceutical industries and academia - emphasized by the US FDA approval of the first siRNA drug, Patisiran, in 2018.[53] This is also marked by a growing number of siRNA-based therapies, which are entering clinical trials.[6, 51, 54] Early applications of siRNA in treating GARDs, such as TD101 siRNA (NCT00716014) for treating an inherited skin disorder, pachyonychia congenital, emerged with therapies based on local administration, since naked and unmodified siRNA are suspected to degrade in blood circulation.[55] However, many siRNA delivery biomaterials have been successively exploited to enable the long circulation and organ targeting effects of siRNA for more systemic applications.[52] To date, various delivery biomaterials have been developed for siRNA-based therapies and some of them have demonstrated potentials in translational applications. In this section, we summarize the non-viral siRNA delivery systems that have been applied for GARDs based on their different target organs, particularly the liver, lungs, and brain (Table 1). We also review several typical delivery systems, such as lipid and lipid derived nanoparticles (LNPs) and ligand-siRNA conjugates, which enable the siRNA-based therapeutics on GARDs.

## 2.1 Liver delivery of siRNA

**Lipid and lipid derived nanoparticles (LNPs)—**In 2018, Patisiran (ONPATTRO™) gained its approval by the US FDA and became the first marketed RNAi-based therapy. Patisiran is a siRNA drug encapsulated within LNPs that was developed by Alnylam® Pharmaceuticals for hereditary transthyretin amyloidosis (hATTR) therapy. hATTR is an inherited genetic disease characterized by the aggregation of mutant transthyretin (TTR) proteins, which leads to disease symptoms such as heart and nerve function impairment. Patisiran, by adopting the RNAi technique, can be used to target and inhibit the TTR mRNA from producing mutant TTR proteins. Meanwhile, the use of LNPs as a well-established delivery vehicle enables the efficient delivery and accumulation of siRNA into hepatocytes, where TTR proteins are primarily produced. The combined functionality of Patisiran's RNAi technique coupled with the use of LNPs as a delivery vehicle enables a robust reduction in the expression of the hATTR disease-linked gene, ultimately providing a therapeutic treatment for the genetic disease.[56, 57] The approval of Patisiran for hATTR therapy not only marks a significant milestone for the gene therapy based treatment of GARDs but also represents the fruit of research in the field of RNAi therapy.[53, 58]

In addition to Patisiran, a growing number of research studies reported siRNA delivery using LNPs. Many of these siRNA LNPs showed efficient delivery of siRNA to the liver. Except from the favorable physiological properties of the liver for LNPs accumulation, one potential mechanism of hepatocyte targeting effect by LNPs involves the interaction of ionizable LNPs with apolipoprotein E (apoE), a protein that facilitates the transport of molecules such as lipids and cholesterol.[59, 60] Binding on the surface of LNPs, apoE mediated the hepatocellular uptake of LNPs through multiple receptors, such as the low-density lipoprotein receptor (LDLR).[59, 60] The exceptional properties of LNPs for RNA delivery include, but are not limited to, the encapsulation and protection of RNAs from degradation during circulation, as well as the delivery of RNA payloads to the target organ following systemic administration.[18, 54, 61] Among the reported non-viral siRNA delivery platforms, LNPs represent a major intravenously (*i.v.*) administered siRNA delivery system

for clinical applications.[22, 38] The most commonly reported siRNA LNP formulation contains five key components, which are the lipid or lipid derivatives, phospholipid, cholesterol, polyethylene glycol (PEG), and the payload siRNA.[59] In addition to physiochemical properties of the main lipid material, the combination as well as the relative ratios of all the key components also have profound effects on the delivery efficiency of LNP formulations.[59, 62] To date, many different lipid and lipid derived materials have been exploited for establishing siRNA delivery LNPs.[59, 62]

Early exploration of systemic siRNA delivery LNPs mainly focused on lipid materials such as 1,2-dilinoleyloxy-*N,N*-dimethyl-3-aminopropane (DLin-DMA).[52] Further modification of the DLin-DMA lipid based on rational design identified DLin-KC2-DMA with an enhanced gene silencing activity.[63] Instead of generating a large library of compounds for screening, Semple and coworkers adopted a rational design strategy for the modification of the key structures, such as the head group and the linker region, within the ionizable lipid DLin-DMA.[63] Their rational design was guided by the knowledge of the relationship between the chemical structures of key groups within the lipid materials and the physicochemical properties as well as the *in vivo* activity of nanoparticles.[63] Semple et al. reported that DLin-KC2-DMA containing stable nucleic acid lipid particles (SNALPs) exhibited *in vivo* activity at siRNA doses of 0.01 mg/kg in the mouse model and was capable of silencing the *TTR* gene in nonhuman primates with $ED_{50}$ around 0.3 mg/kg.[63] A number of lipid materials were further developed by rational design of a large library of compounds followed by *in vitro* and *in vivo* screenings, such as the DLin-MC3-DMA (MC3) used in the Patisiran LNPs formulation, C12–200, and $304O_{13}$.[38, 64, 65] For example, MC3 lipid was identified through further modulating and screening on the head group structure of DLin-KC2-DMA.[66] All these lipid materials showed improved delivery efficiency as compared with DLin-KC2-DMA LNPs.[38, 64, 65] Some other LNPs were reported with organ specificity, such as cKK-E12 lipopeptide nanoparticles, wherein the lipid tails were conjugated to peptide derivatives and achieved liver targeting siRNA delivery.[67]

In 2013, Schmidt et al. reported siRNA LNPs targeting transmembrane serine protease 6 (Tmprss6) for β-thalassemia therapy.[68] β-Thalassemia is an inherited blood genetic disorder caused by the deficient or absent synthesis of β-globin (HBB). *TMPRSS6* gene encodes the matriptase-2 protein, which is expressed in hepatocytes and negatively modulates the production of hepcidin. Mutations of TMPRSS6 were found to elevate hepcidin expression and cause iron-refractory iron deficiency anemia (IRIDA).[69] By targeting and suppressing TMPRSS6 using the Tmprss6 siRNA LNPs, Schmidt et al. achieved decreased iron overload and ameliorated anemia in the mouse model of β-thalassemia intermedia.[68] In 2015, they employed the combination therapy of the Tmprss6 siRNA LNPs with Deferiprone, an oral iron chelator, for further mitigating anemia and secondary iron overload in murine β-thalassemia intermedia.[70]

In 2016, Dutta et al. explored the use of siRNA LNPs for treating primary hyperoxaluria type 1 (PH-1). PH-1 is a rare metabolic disorder characterized by the defect of a liver peroxisomal enzyme, alanine glyoxylate aminotransferase (AGT).[71] The deficiency of AGT activity could increase the production of oxalate in the liver, which leads to the

formation and accumulation of calcium oxalate crystals in kidneys.[72] Current treatment for the prevention of primary manifestations of PH-1 is limited to vitamin B6 therapy, however, patients who are not pyridoxine responsive may require a combined liver/kidney transplant.[71] Dutta et al. adopted siRNA LNPs to target and downregulate *hydroxyacid oxidase 1* (*HAO1*). *HAO1* gene encodes glycolate oxidase (GO), which can transform glycolate into glyoxylate, and thus can be used as a gene silencing target for reducing oxalate levels in the liver.[73] The identified lead HAO1 siRNA-LNP showed potent suppression of GO expression in both mice and nonhuman primates. Moreover, the reduction of GO protein in the liver also resulted in reduced urine oxalate levels and calcium oxalate accumulation in the PH-1 mouse model.[71]

Although a variety of LNPs have been developed and many studies have revealed their feasibility of siRNA-based therapies, few clinical applications of siRNA LNPs for GARDs are ongoing. Many preclinical studies adopted LNPs as a liver targeting delivery system to test gene silencing activity, whereas siRNA was further developed into *N*-acetylgalactosamine (GalNAc)-siRNA conjugates for clinical trials.[70, 71, 74]

**N-acetylgalactosamine (GalNAc) conjugates**—With the development of chemical modifications on RNA molecules, modified siRNA are reported to remain intact for long durations under circulation, which provides the possibility of delivering siRNA without the shielding of nanoparticles.[19, 75, 76] Hence, siRNA delivery through ligand conjugation emerged with efficient and targeted delivery of siRNA therapeutics.

One of the well-established ligand-siRNA conjugates is that of siRNA to GalNAc, which is known as a potent ligand to the asialoglycoprotein receptor (ASGPR). The mechanism of action for the ligands interacting with ASGPR has been thoroughly investigated since the 1960s.[77] The ASGPR is highly expressed on hepatocytes and GalNAc facilitates the uptake of its payload into hepatocytes through ASGPR via clathrin-mediated endocytosis. [77] Based on this mechanism, researchers developed multivalent GalNAc-siRNA conjugates for liver targeting siRNA delivery.[77] Results showed that by conjugating the engineered ASGPR ligand to the chemically modified siRNA molecule, GalNAc-siRNA conjugates could achieve systemic stability against nucleases, which would in turn improve pharmacokinetics. Most importantly, single or multiple low-volume subcutaneous injections of these conjugates resulted in effective suppression of the target gene in the liver.[51, 77, 78] As compared with many other siRNA delivery systems which are based on repeated high-dose *i.v.* administrations, this subcutaneously delivered GalNAc-siRNA conjugate is thus more desirable for some therapeutic purposes. Due to these favorable properties of GalNAc-siRNA conjugates, many linker chemistries and RNA modification methods have been subsequently explored by researchers for maximizing the therapeutic window. A series of GalNAc-siRNA conjugates were established in recent years to treat a variety of GARDs with the liver as the target organ (Table 1).[64, 68, 79, 80]

Moreover, Alnylam® Pharmaceuticals developed the Enhanced Stabilization Chemistry (ESC)-GalNAc-siRNA conjugate platform, aiming to further enhance the stability, potency, and duration of siRNA molecules in the liver following subcutaneous administration.[81] As compared with their first generation 'Standard Template Chemistry' (STC)-based GalNAc-

siRNA, in which RNA molecules were 2'-OMe and 2'-F modified and contained two terminal phosphorothioate (PS) linkages, the newly developed ESC platform features higher 2'-OMe content and four additional terminal PS linkages.[81] Many of these ESC-based GalNAc-siRNA conjugates have entered clinical trials, among which some have already reached Phase 3 clinical trials (Table 1). For example, acute hepatic porphyrias (AHP) is a rare genetic disease caused by an inherited defect in one of the enzymes in the heme biosynthesis pathway.[74, 82, 83] The elevated activity of the 5-aminolevulinic acid synthase 1 (ALAS1) enzyme, which regulates the heme pathway, can lead to the buildup of neurotoxic heme intermediates in the liver and result in disease symptoms.[82] ALN-AS1 (Givosiran), a GalNAc-conjugated RNAi therapeutic, could effectively prevent and treat induced attacks of AHP by targeting and silencing ALAS1.[74] Similarly, another GalNAc-siRNA conjugate (ALN-AT3, Fitusiran) targeting antithrombin (AT) was developed as a therapy for hemophilia A and B, inherited bleeding disorders, based on the reports that co-inheritance of prothrombotic mutations in patients results in the ameliorated clinical phenotype in hemophilia.[84] Additionally, ALN-PCSsc (Inclisiran) is a synthetic GalNAc-siRNA conjugate designed against the proprotein convertase subtilisinkexin type 9 (PCSK9). PCSK9 is known as a crucial regulator of the low-density lipoprotein receptor (LDLR), since it interacts with the LDLR and mediates the lysosomal degradation of the receptors. Therefore, the inhibition of PCSK9 offers a strategy for lowering the plasma LDL-cholesterol (LDL-C) level in patients. Inclisiran was reported to be able to significantly decrease the production of PCSK9 in liver and thus reduce the LDL-C level, which enables it to be used for the management of autosomal dominant hypercholesterolemia (ADH).[85, 86]

In addition to the GalNAc-siRNA conjugates developed based on the ESC delivery platform designed by Alnylam® Pharmaceuticals, Dicerna™ Pharmaceuticals recently initiated Phase 2 clinical trials (NCT03847909) for DCR-PHXC in treating primary hyperoxalurias (PH), and Silence Therapeutics, in year 2019, filed CTA for SLN124 for β-thalassemia and myelodysplasia syndrome.[87, 88]

**Other ligand conjugates—**Arrowhead Pharmaceuticals' Targeted RNAi Molecule platform (TRiM™) is another example of using ligand conjugation strategy to mediate targeted siRNA delivery.[89] The TRiM™ platform utilizes ligand-mediated delivery and stringent bioinformatics to design tissue-specific targeting conjugates.[89] Although detailed information about TRiM™ platform is not disclosed, it is known that the platform comprises a highly potent RNA trigger as well as optimized high affinity targeting ligand and a linker for each drug candidate based on their specific applications.[89] The TRiM™ platform provides advantages such as enabling the delivery of RNAi to tissues beyond the liver and through multiple routes of administration.[89]

ARO-AAT, one of the candidates built on the TRiM™ platform, was developed as a therapy for alpha-1 antitrypsin deficiency (AATD) and has completed a Phase 1 clinical study (NCT03362242). AATD is a rare genetic liver disease caused by the production of excessive alpha-1 antitrypsin (AAT) mutant Z (Z-AAT) in the hepatocytes.[90] Accumulation of misfolded Z-AAT in the liver can lead to highly variable liver diseases with a variety of clinical presentations, such as hepatic injury and hepatocellular carcinoma (HCC).[90, 91]

ARO-AAT is a liver targeting siRNA conjugate developed to suppress the production of Z-AAT protein by gene silencing with the aim to prevent accumulation of disease-related proteins in the liver, allow clearance of accumulated proteins, prevent repeated cycles of cellular damage, and reverse fibrosis associated with prior damage.[90]

Additionally, siRNA conjugations with cholesterol, aptamer, peptide and other moieties were also reported to facilitate the distribution and gene silencing effects of siRNA molecules in different organs, liver included.[92–94] These siRNA conjugates are under extensive exploration in preclinical studies.

## 2.2 Lung delivery of siRNA

Cystic fibrosis (CF) is a type of inherited rare disease characterized by a genetic mutation that causes mucus buildup in the lungs and pancreas. Patients with CF show symptoms such as having difficulty breathing or experiencing recurrent and persistent lung infections. To date, several publications have applied lung siRNA delivery systems for the treatment of CF. [95–98] Their common therapeutic strategy is based on the downregulation of epithelial sodium channel (ENaC). ENaC was found to be a target for CF therapy, since the upregulation of ENaC in the lungs of CF patients leads to the increased absorption of sodium and fluids from the airway, and eventually results in impaired mucociliary clearance.

In 2017, Manunta et al. reported the development of an ENaCsiRNA nanocomplex that induced effective *in vitro* and *in vivo* silencing of airway ENaC.[95, 96] This self-assembling ENaCsiRNA nanocomplex formulation was composed of a liposome (DOTMA/DOPE; L), an epithelial targeting peptide (P) and an siRNA (R).[95] Results showed that the established LPR nanocomplexes facilitated the translocation of siRNA through mucus, mediated efficient ENaCsiRNA transfections in primary CF epithelial cells, and reduced 30% of the αENaC and βENaC mRNA. These ENaCsiRNA nanocomplexes also modulated the activity of ENaC and showed effective delivery and silencing *in vivo*.[95, 96]

Based on ENaC inhibition mediated by siRNA, Arrowhead Pharmaceuticals developed the ARO-ENaC siRNA conjugates, by using the TRiM™ platform as the delivery technique. [98] ARO-ENaC is designed to target and suppress the production of the epithelial sodium channel alpha subunit (αENaC) in the airways of the CF lungs.[98] Preclinical studies showed that by adopting an integrin αvβ6 receptor ligand (EpL) as a targeting ligand, the ARO-ENaC siRNA conjugate can achieve the functional delivery of siRNA to the airway epithelium. ARO-ENaC thus mediated targeted silencing of αENaC mRNA in the lungs of rats, and dose-dependently improved mucociliary clearance in sheep after inhaled aerosol exposure.[98]

## 2.3 Brain delivery of siRNA

Although a variety of diseases require therapeutics to be delivered to the brain, brain targeted delivery has always been a formidable issue, despite the continuous efforts that have been made to help overcome the barrier. While RNAi holds great promise for treating genetic associated neurological disorders, efficient delivery of siRNA into the brain is still a remarkable obstacle to the clinical applications.[99–101] Strategies that have been applied for achieving functional siRNA delivery into the brain mainly rely on employing potent

protein or peptide ligands to target the receptors, such as transferrin, insulin and LDL receptors, which are highly expressed on the blood brain barrier (BBB).[102] In recent years, some brain targeting siRNA delivery systems, including spherical nucleic acid (SNA), stable nucleic acid lipid particles (SNALPs) and targeted exosomes, have demonstrated potentials for treating neurological genetic disorders.[63, 103] Two representative studies using brain targeting siRNA delivery systems to treat rare disease are summarized as follows.

Machado-Joseph disease (MJD) is a rare neurodegenerative disease, which lacks available treatment.[104] As an autosomal dominant disorder, MJD is caused by the abnormal repetition of CAG in the *ATXN3/MJD1* gene, which results in mutations in the ataxin-3 protein. In 2015, Conceiç~ao et al. successfully developed siRNA-SNALPs for the treatment of MJD, which incorporated a rabies virus glycoprotein (RVG) derived short peptide RVG-9r and encapsulated mutant ataxin-3 targeting siRNA.[37] The afforded formulation exhibited favorable features for systemic administration and *in vitro* efficacy to silence the mutant ataxin-3 in neuronal cells. *In vivo* studies further showed that RVG-9r targeted siRNA-SNALPs mediated silencing of MJD causing gene, ameliorated disease symptoms in the MJD mouse models following *i.v.* administration. Additionally, a safety profile of the repeated *i.v.* administrations of RVG-9r targeted mutant ataxin-3 targeting siRNA-SNALPs was further reported in a follow-up study.[105]

Another example of brain targeting siRNA delivery system adopted exosome vehicles for the treatment of a fatal neurological genetic disorder, Huntington's disease (HD).[106] The cause of HD is related to the heightened CAG repeats in the *huntingtin* (*HTT*) gene.[101, 106] Many studies have demonstrated that siRNA could mediate the reduction of HTT expression in the brain.[101, 107] In 2016, Didiot et al. efficiently loaded modified small interfering RNA (hsiRNAs targeting HTT mRNA) into exosomes, which are endogenous vesicles applied as siRNA delivery biomaterials. Results showed that the hsiRNA-loaded exosomes could be uptake into mouse primary cortical neurons and, therefore, facilitate the suppression of HTT mRNA and protein. Moreover, administration of hsiRNA-loaded exosomes through unilateral infusion into mouse striatum further showed *in vivo* efficacy of these established exosomes, where 35% of HTT mRNA were silenced in the brain of mice. [106]

## 3. Messenger RNA (mRNA)

mRNA functions as the bridge between DNA and proteins in eukaryotic cells.[8, 12, 119] Endogenous mRNA carries genetic information from DNA and is translated into functional proteins.[8, 12, 119] Alternatively, by using the *in vitro* transcription techniques (IVTs), mRNA can be synthesized and, therefore, have become a potential new drug class for diverse therapeutic applications.[34, 39, 120, 121] When introduced into cytosol via mRNA delivery systems, the IVT mRNAs can be translated to produce desired functional proteins. [8, 12, 119]

Many genetic and rare diseases (GARDs) are characterized by protein deficiencies or malfunctions that require protein replacement therapies (PRTs).[122, 123] Hence,

supplementation of exogenous proteins is a straightforward therapeutic approach.[8, 119, 120] Compared with protein drugs, mRNA can supplement both cytoplasmic and large transmembranic proteins.[8, 119, 120] Moreover, mRNA is more effective because one mRNA molecule can generate multiple copies of a protein.[8, 119, 120] Relative to plasmid DNA (pDNA), mRNA possesses temporary bioactivity and functions outside the nucleus, leading to a more controllable protein expression and avoiding the potential insertion of the genome.[8, 119, 120] Although mRNA has become a potential class of therapeutic medicines, several aspects of mRNA, including the translatability, stability, immunogenicity, and intracellular delivery have to be improved in order to achieve clinical applications.[8, 119, 120] Increased translatability and stability can be achieved though modifying and optimizing the cap structure, 5' and 3' untranslated regions (UTRs), coding sequences, and other structural elements.[8, 119, 120] Incorporating modified nucleosides, such as pseudouridine and 2−thiouridine into mRNA can reduce its immune-stimulatory effects.[8, 119, 120] Ultimately, intracellular delivery remains the major challenge for widespread applications of mRNA therapeutics.

Recently, many biomaterials have been developed for mRNA delivery, including lipids and lipid derived materials, polymers, and many others. [8, 39, 120, 124] Among them, lipids and lipid derived materials are the backbone for mRNA delivery systems.[8, 39, 124, 125] Cationic lipids, such as DOTMA (1,2-di-*O*-octadecenyl-3-trimethylammonium-propane), DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), zwitterionic DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine), and lipofectamine have been widely studied in mRNA delivery.[39, 126] Meanwhile, ionizable lipid and lipid derived materials, which acquire positive charges at low pH and become neutral at physiological pH, have been developed for decreasing the toxicity and immunogenicity while retaining the delivery efficiency.[8] 1,2-dilinoleyloxy-3 dimethylaminopropane (DLin-DMA) and its derivatives, DLin-KC2-DMA and DLin-MC3-DMA (MC3) are representative ionizable lipid materials.[63, 127–132] In 2018, the nucleic acid drug Patisiran (ONPATTRO™) based on MC3 lipid have been approved by the US FDA.[124] Another ionizable lipid material named cKK-E12 has been investigated to deliver mRNA for multiple applications.[67, 133–135] Moreover, its derivative with unsaturated fatty chains, OF-02, and its biodegradable derivative, OF-Deg-Lin, showed efficient mRNA delivery *in vivo*.[136–138] The efficacy of ionizable lipid and lipid derived materials such as *N,N,N*-tris(2-aminoethyl) benzene-1,3,5-tricarboxamide (TT3)[40, 139], C12–200[133, 140–142], and $306O_{110}$[143] have also been proved for *in vivo* mRNA delivery. In addition, other ionizable lipids developed by Moderna Therapeutics[47, 144], Intellia Therapeutics[48], and Arcturus Therapeutics[145, 146] have been tested in preclinical studies.

Cationic polymers, such as polyamines, polypeptides, and block polymers, are another category of biomaterials for mRNA delivery. Polyethylenimine (PEI) is one of the most developed cationic polymers [39], but its toxicity has limited the therapeutic applications.[8, 124] In addition to PEI, chitosan[147–149], poly(β-amino esters) (PBAEs)[41, 150, 151], poly(glycoamidoamine) brushes (TarN3C10)[152], and *N*-substituted polyaspartamides (PAsp)[44, 153–157] have been used for mRNA delivery. Charge-altering releasable transporters (CARTs)[158–160], polyamidoamine (PAMAM) or polypropylenimine-based dendrimers[161, 162], and poly(lysine)-based polypeptides[163], have also been developed

for *in vivo* mRNA delivery. Other biomaterials, including peptide complexes and inorganic materials for mRNA delivery, which mainly focus on cancer and other diseases, have been reviewed in the literature.[10, 34, 39, 121, 124]

In this section, we describe several representative mRNA delivery systems aiming at GARD therapy (Table 2), mainly based on liver and lung delivery biomaterials. Additionally, we highlight several mRNA delivery systems for other organs which may be further developed for GARD treatment.

### 3.1   Liver delivery of mRNA

For many GARDs, the liver is a preferred organ for mRNA-based PRTs because systemically administrated mRNA vehicles are predominantly trapped by the reticuloendothelial system (RES) in the liver. In addition, the interaction between apolipoprotein E (apoE) and biomaterials such as lipid and lipid derived materials facilitates the accumulation in the liver.[8, 18, 34, 39, 59, 120, 121, 124, 164, 165]

Hemophilia B is a blood genetic disease due to the deficiency of the blood coagulation factor IX (FIX).[166] Patients with hemophilia B are characterized by bleeding tendency, leading to recurrent bleeding episodes following trauma or spontaneous hemorrhages.[166] Repeated bleeding, especially into the joints, muscles, and soft tissues, causes chronic lesions including pain, deformities, and loss of movement.[166] Although the FIX PRT improves the outcome of hemophilia B, its short half-life, frequent intravenous (*i.v.*) administration method, and cost still limit the broader utilization.[167] Several lipid derived materials have been synthesized for mRNA-based PRT of Hemophilia B. For example, TT3 was developed for human FIX (hFIX) mRNA delivery.[40] After 6 hours of *i.v.* injection, the plasma concentration of FIX was 1740 ng/mL and the activity was 791 mIU/mL, which were well within the normal physiological values.[40] In another study, C12–200 was used to deliver hFIX mRNA to the liver of hemophilia B mice.[168] A single administration of lipid derived nanoparticles (LNPs) containing 0.5 mg/kg hFIX mRNA yielded 1800 ng/mL of FIX in the plasma.[168] The LNP formulation based on the ionizable ATX lipid developed by Arcturus Therapeutics was also prepared for delivering hFIX mRNA.[145] The therapeutic level of FIX in the plasma was maintained up to 6 days following a single *i.v.* administration of LNPs complexed with 2.0 mg/kg hFIX mRNA.[145]

Thrombotic thrombocytopenic purpura (TTP) is another blood genetic disease characterized by the clinical symptoms including haemolytic anaemia, thrombocytopenia, renal failure, and neurologic dysfunction.[169] One of the revealed pathogeneses of TTP is the inherited mutation of the zinc metalloproteinase gene (*ADAMTS13*).[169] The ATX-based mRNA delivery system provided an approach for TTP treatment.[170] One-time tail vein injection of formulated human ADAMTS13 (hADAMTS13) mRNA induced a therapeutic level of hADAMTS13 (140 ng/mL in the circulation at 24 h, and 90 ng/mL at 48 h) in ADAMTS13-deficient mice. Furthermore, therapeutic level was maintained for up to five days, which was significantly longer than the half-life (24 hours) of the recombinant human ADAMTS13 (rhADAMTS13) protein.[170]

Methylmalonic academia (MMA) is an amino acid metabolism disorder caused by the dysfunctional methylmalonyl-CoA mutase (MUT), leading to an excessive accumulation of methylmalonic acid.[171] Depending on the mutated genes, the metabolic disorder results in different clinical symptoms, including vomiting, acidosis, hyperammonemia, lethargy hypertonia, and failure to thrive.[171, 172] Although diagnosis and management have been improved, effective therapies remain elusive and patients still suffer from MMA associated complications and lifelong mortality.[171, 172] Systemic injection of human MUT (hMUT) mRNA, formulated with MC3 lipid, demonstrated a rapid (as early as 2 h after administration) and effective (maximum 90% at 24 h) reduction of plasma methylmalonic acid.[47] In addition, repeated administrations improved the growth and survival of MMA mice without obvious liver damage and inflammation.[47] Based on its mRNA platform, Moderna Therapeutics initiated a clinical trial (NCT03810690) to evaluate the mRNA-3704 in patients with MMA (Table 2).

Another amino acid metabolism disorder is hepatorenal tyrosinemia type 1 (HT-1) characterized by the mutated fumarylacetoacetate hydrolase (FAH), an important enzyme for tyrosine catabolism.[173–175] In HT-1 patients, the accumulation of tyrosine metabolites generally causes hepatic cirrhosis, renal dysfunction, neurologic crises, and cancer.[173–175] Clinical treatment of HT-1 combines a tyrosinerestricted diet and nitisinone, an inhibitor for preventing the formation of tyrosine metabolites.[175] However, long-term use of nitisinone increases the risks of developing hepatic neoplasms and liver failure.[162] In a recent report, the 5A2-SC8 dendrimer-based lipid nanoparticle (DLNP) was screened out to deliver human FAH (hFAH) mRNA in *Fah* knockout mice.[162] The mice were *i.v.* administrated with hFAH mRNA loaded DLNPs every 3 days for a month, during which hFAH expression was elevated in the liver.[162]

Ornithine transcarbamylase deficiency (OTCD) is also an amino acid metabolism disorder which is caused by the loss of ornithine transcarbamylase (OTC), a key enzyme in the urea cycle.[176] The deficiency of OTC results in hyperammonemia, giving rise to neurological injury, coma, and even death.[176] mRNA-based PRT is a promising alternative for liver transplant which is currently the only option for treating OTCD.[176] The Hybrid mRNA Technology (HMT) was developed for targeting expression of OTC in hepatocytes.[177] HMT contained two types of nanoparticles: the *N*-acetylgalactosamine (GalNAc) polymer micelle which allowed hepatic targeting and facilitated endosomal evasion, and the DOTAP LNP which protected mRNA from enzymatic degradation. After *i.v.* injection of OTC mRNA with HMT, a prolonged survival was observed in OTCD mice. Meanwhile, no obvious changes of cytokines or significant pathological lesions were detected in the blood and liver, respectively.[177] Another study for treating OTCD is MRT5201 developed by Translate Bio based on their proprietary mRNA therapeutic platform (MRT™).[178] MRT5201 aims to restore the OTC level in patients with OTCD using mRNA LNPs. Recently, a phase 1/2 clinical trial (NCT03767270) on MRT5201 has been launched (Table 2).

Fabry disease is a genetic disease due to the deficiency of lysosomal alpha galactosidase A (α-GLA).[179] The deficient α-GLA contributes to a progressive accumulation of glycolipids in many tissues and cell types, giving rise to acroparesthesia, angiokeratomas,

myocardial infarction, renal failure, and other clinical symptoms.[179] Two recent studies explored the therapeutic potential of mRNA LNPs in mice with Fabry disease. DeRosa et al. encapsulated human GLA (hGLA) mRNA in C12–200-based LNPs and *i.v.* dosed each mouse with 1.0 mg/kg mRNA LNPs or recombinant hunam GLA (rhGLA) proteins.[142] One week after administration, hGLA proteins in the heart and kidneys of the LNP treated group was 10-fold more than the rhGLA protein treated group. Meanwhile, Fabry disease associated biomarkers, globotriaosylceramide (Gb3) and globotriaosylsphingosine (lyso-Gb3), decreased by 92% and 88% in the LNP treated group. In contrast, these biomarkers decreased by only 67% and 61% in the rhGLA protein treated group. Similar effects were observed from the study using MC3-based LNPs to deliver hGLA mRNA.[129, 144] A single *i.v.* administration restored the α-GLA activity with concomitant clearance of Gb3 and lyso-Gb3 in plasma and different tissues in mice with Fabry disease. Moreover, the therapeutic effects were maintained for up to six weeks.[129]

Acute intermittent porphyria (AIP), a rare metabolic disease, is correlated with hepatic deficiency of porphobilinogen deaminase (PBGD), leading to over-accumulated porphyrin precursors.[180] Patients with AIP typically experience abdominal pain, psychosis, hypertension, and tachycardia.[180] A recent preclinical study demonstrated that *i.v.* injection of human PBGD (hPBGD) mRNA encapsulated in MC3-based LNPs restored hepatic PBGD expression followed by a normalization of porphyrin precursor levels.[130] In AIP mice, a single dose alleviated mitochondrial dysfunction, hypertension, and pain. Importantly, the PBGD expression in the liver lasted for at least 10 days, which provided a full protection throughout the diseased period (5 to 7 days).[130]

Additionally, there are three other GARDs (Crigler-Najjar syndrome type 1, alpha-1 antitrypsin deficiency, and glycogen storage disease type 1) which are currently treated by mRNA LNPs in preclinical studies (Table 2).[181–183]

### 3.2   Lung delivery of mRNA

In addition to liver delivery, several lung delivery systems were recently reported in the literature.[97, 126] For delivery of mRNA to the lungs, *i.v.* and intratracheal *(i.t.)* administration were mainly investigated in the past.[126] In general, lung-targeted mRNA delivery via *i.v.* administration is challenging because a substantial amount of mRNA nanoparticles will be trapped in the liver and spleen.[126] Furthermore, pulmonary capillaries are non-fenestrated, hindering free exchanges of large molecules from the blood to the lung tissues.[126] However, several strategies have been developed for lung targeting mRNA delivery after systemic administration. For example, coupling pulmonary affinity ligands to nanoparticles can significantly increase mRNA delivery to the lungs.[42] Some biomaterials, interactions with serum proteins or surface receptors of pulmonary cells, may contribute to the lung specific accumulation.[150] The pathologic characteristics of certain lung diseases, such as hyper-vascularization, may also be utilized to facilitate nanoparticles reaching these areas. In contrast to *i.v.* administration, *i.t.* administration via suspension nebulization or dry powder inhalation can directly deliver mRNA to the airways of the lungs. [184] Moreover, using *i.t.* administration, mRNA can be delivered to a variety of cell types, such as alveolar cells, pulmonary endothelium, and immune cells.[126, 184]

Cystic fibrosis (CF) is associated with the defective cystic fibrosis transmembrane conductance regulator (CFTR), functioning as the cAMP mediated chloride channel on epithelial cells.[185, 186] Patients with CF usually experience repeated airway infections, chronic pulmonary inflammation, and respiratory distress.[187] Some small molecules that aim to restore the chloride transport function of the mutant CFTR have shown promising clinical results. However, their narrow application range, poor improvements in lung function, and side effects limit their utilization.[188–190] Recently, MC3-based LNPs were prepared to intranasally deliver human CFTR (hCFTR) mRNA to *Cftr* knockout mice.[131] On day 3 post-administration, chloride secretion reached 55% of that in healthy mice and the secretion maintained more than 15 days.[131] In a separate study, Haque et al. complexed hCFTR mRNA with chitosan-coated PLGA nanoparticles to relieve CF symptoms. The restored lung function was determined by increasing CFTR expression and improved forced expiratory volume (FEV).[149] Another clinical candidate, MRT5005, designed by Translate Bio to address CF through delivery of CFTR mRNA, has been studied in a clinical trial (NCT03375047, Table 2). MRT5005 will be administered by nebulization to the respiratory tract of adult subjects with CF and its safety will be evaluated in the clinical trial.[178] LUNAR-CF, developed by Arcturus Therapeutics, is another mRNA-based therapeutic for treating CF.[191] Preclinical studies indicated LUNAR nanoparticles effectively delivered mRNA into pulmonary epithelial cells and were resistant to the degradation in CF sputum during nebulization.[191]

Several mRNA delivery systems were inclined to accumulate in the lungs, and thus are propitious for treatment of pulmonary GARDs. Schrom et al. prepared lung-targeted nanoparticles by mixing mRNA with preassembled C12-(2–3-2) oligoalkylamines lipid micelles.[192, 193] Compared with other organs, the stronger luciferase signal in the lungs indicated the pulmonary specificity. Based on these results, they further *i.v.* delivered angiotensin-converting enzyme 2 (ACE2) mRNA, a therapeutic agent for lung fibrosis, by the lung-targeted nanoparticles. After 6 hours, obvious accumulation of ACE2 mRNA and proteins were observed in the lungs.[192, 193] Kaczmarek et al. developed a hybrid polymer–lipid nanoparticle consisting of PBAEs and lipid-PEG for lung mRNA delivery. [150] Through optimization of the polymer construction and nanoparticle formulation, they obtained a nanoparticle which showed comparable lung-specificity but two orders-of-magnitude more effective in mRNA delivery than the base nanoparticle.[194] Recently, the same group synthesized hyperbranched poly(beta amino esters) (hPBAEs) to deliver mRNA within aerosol formulation to the lungs.[151] The hPBAE nanoparticles were stable without lipid-PEG and withstood the shearing forces from the vibrating mesh nebulizer. After 24 h of nebulization, luciferase signal was observed in the lungs but not in the spleen and liver. [151] Yan et al. identified the top material named PE4K-A17–0.33C12 from a poly-(trimethylolpropane allyl ether-co-suberoyl chloride) library[195]. The PE4K-A17–0.33C12 nanoparticles formulated with 5% pluronic F127 resulted in obvious luciferase expression in the lungs after systematic administration in mice.[195] An alternative strategy for selectively delivering mRNA to the lungs is coupling nanoparticles with affinity ligands. Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a typical target of endothelial cells of the lungs. Parhiz et al. developed PECAM-1 antibody modified LNPs for lung targeting delivery of mRNA.[42] Relative to non-targeted LNPs, systemic administration of

PECAM-1 coupled mRNA LNPs exhibited approximately 200-fold inhibition and 25-fold elevation of luciferase expression in the liver and the lungs, respectively.[42]

### 3.3   Other organ delivery of mRNA

Additionally, many biomaterials are developed and applied for delivering mRNA to other organs, such as skin, eye, nasal cavity, and heart. For these organs, topical delivery is the main administration route.

Williams-Beuren syndrome (WBS) is caused by spontaneous microdeletion of about 27 genes from chromosome 7q11.23.[196, 197] Patients with WBS suffer from approximately 50 % of elastin loss which is associated with complications such as cardiovascular diseases, nervous disorders, and developmental problems.[196, 197] Lescan et al. utilized tropoelastin (TE) mRNA/lipofectamine 2000 complexes to restore the elastin level in mesenchymal stem cells isolated from a WBS patient. After 24 h of treatment, about 13-fold higher amounts of elastin was observed compared to untreated cells. [198] Furthermore, in porcine skin, over 1.2-fold increased expression of elastin was observed after the subcutaneous delivery of the complexes.[198]

Inherited retinal degeneration (IRD) is caused by progressive death of retinal photoreceptors. [199] Patients typically experience visual disturbances in childhood and gradually lose their central vision.[199] Recently, Patel et al. studied the kinetics and localization of photoreceptor expression mediated by MC3-based mRNA LNPs in the eyes of albino BALB/c mice.[132] After a subretinal injection, the photoreceptor expression persisted for 96 h (began within 4 h and reached peak expression at 24 h), and mainly distributed in the retinal pigmented epithelium (RPE).[132]

There are also some mRNA delivery systems that may be applied to GARDs in other organs. Polyplex nanomicelles composed of PEG and poly (*N*-(*N*-(2-aminoethyl)-2-aminoethyl) aspartamide) (PAsp (DET)) were designed for the delivery of the mRNA encoding neutrophilic factor into the nasal cavity of the mice with olfactory dysfunction.[156] Intranasal administration of the nanomicelles promoted the recovery of their olfactory function. Magadum et al. recently reviewed mRNA delivery systems in cardiac therapy.[200] For example, an intramyocardial injection of mRNA expressing human vascular endothelial growth factor A (VEGF-A) with RNAiMAX decreased the morbidity of mice with myocardial infarction following the repair of heart function.[201]

## 4.   Genome editing systems containing RNA components

The advent of genome editing tools, including meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR-Cas9), CRISPR from *Prevotella* and *Francisella* 1 (CRISPR-Cpf1, CRISPR-Cas12a), and many others have enabled gene engineering to potentially correct disease-causing mutations.[202–206] Among them, CRISPR-Cas9 is one of the most widely used genome editing systems for *in vivo* applications. The CRISPR–Cas9 system composes of the Cas9 nuclease, a single guide RNA (sgRNA) and if needed, a donor DNA template for homology-directed repair (HDR).[202,

205, 207] The Cas9 nuclease utilizes a sgRNA to recognize the target sequence and subsequently cleaves the genome at the specific site. The system can be designed to edit a large number of sequences in the genome with different sgRNAs and donor DNA templates, thus making it a powerful genome editing tool.[202, 205, 207] However, there are some challenges for *in vivo* applications of therapeutic genome editing tools.[205–208] Firstly, genome editing components must reach the cell nucleus to achieve therapeutic effects. Thus, extracellular and intracellular barriers need to be overcome, including avoiding enzymatic degradation in the blood, circumventing clearance by the reticuloendothelial system (RES), penetrating cell membranes, escaping endosome entrapment, and infiltrating nuclear membranes. Secondly, the complexity and large molecular weight of these components are significant challenges for delivery materials. For example, the molecular weight of Cas9 protein is about 160 kDa, and the phosphate backbones of sgRNA and DNA are highly negatively charged. These features hinder the efficiency of delivery materials. Thirdly, genome editing components should be delivered to target tissues and edit specific sequences in a controllable manner to prevent off-target events and immune responses. Therefore, an effective delivery system for genome editing tools needs to overcome these challenges in order to achieve clinical applications *in vivo*.

Genome editing delivery systems are generally classified into categories of physical delivery, viral delivery, and non-viral delivery. Physical delivery such as electroporation and microinjection have been widely used, which was discussed by a few review articles.[207, 209, 210] Viral delivery, such as lentivirus (LV) and adeno-associated virus (AAV), is also an effective method to deliver genome editing components.[202, 210] In addition, researchers investigated numerous non-viral materials for delivery of genome editing tools. These materials are similar to those used for mRNA delivery.[202, 205, 207–211] Targeted delivery is crucial for achieving genome editing specificity and reducing off-target effects. Although many targeting strategies have been developed for drug delivery, they have not be thoroughly applied to genome editing delivery systems. Currently, tissue targeting genome editing delivery has been limited to topical administration except the liver. Therefore, targeted delivery of genome editing tools need be addressed in order to facilitate future clinical applications.

In this section, we summarize representative non-viral materials applied to deliver genome editing components containing mRNA or/and sgRNA for the treatment of genetic and rare diseases (GARDs). (Table 3). We also review some typical non-viral materials which may be developed for delivering genome editing components to treat GARDs in the future.

### 4.1 Liver delivery of genome editing components

Transthyretin (TTR), secreted by the liver and cerebral choroid plexus, is a transport protein for retinol and thyroxine.[212, 213] The aggregation of mutated transthyretins is associated with hereditary transthyretin amyloidosis (hATTR).[212, 213] In a recent study, LP01-based lipid derived nanoparticles (LNPs) were developed for delivering Cas9 mRNA and sgRNA to edit hepatic *Ttr* gene in mice and rats.[48] Following a single intravenous (*i.v.*) injection, over 90% reduction of serum TTR was observed and the knockdown effect persisted for at least 12 months. Moreover, multiple administrations achieved cumulative editing efficacy

without obvious cytokine stimulation and body weight loss.[48] The combined delivery of Cas9 mRNA and sgRNA by C12–200-based LNPs and DNA donor by AAV was used to treat hepatorenal tyrosinemia type 1 (HT-1) by correcting the *fumarylacetoacetate hydrolase* (*Fah*) gene on *Fah* knockout mice.[141] After a single treatment, the FAH expression in more than 6% of hepatocytes was repaired and the symptoms of hepatic damage were prevented in the following 30 days. Another material named zwitterionic amino lipid (ZAL) was synthesized in order to deliver genome editing components.[214] With *i.v.* injection of ZAL nanoparticles containing Cas9 mRNA and sgRNA, the stop gene sequences in Rosa26^tdTomato mice were edited, leading to strong tdTO fluorescent signal in the liver, lungs and kidneys.[214]

Autosomal dominant hypercholesterolemia (ADH) is associated with mutations in the proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene.[215] Patients with ADH usually suffer from coronary heart disease because of the high level of low-density lipoprotein cholesterol.[215] TT3 LNPs were used to deliver Cas9 mRNA and sgRNA to edit *Pcsk9* gene *in vivo*.[139] After *i.v.* injection of Cas9 mRNA TT3 LNPs following the injection of sgRNA TT3 LNPs, the PCSK9 protein level was remarkably decreased in C57BL/6 mice.[139] In another study, Anderson et al. developed enhanced sgRNA (e-sgRNA) by incorporating chemically modified nucleotides into guide RNA sequences.[135] They formulated Cas9 mRNA and e-sgRNA targeting *Pcsk9* with cKK-E12 LNPs.[134, 216] Following a single *i.v.* injection, hepatic PCSK9 protein was reduced by 80% and plasma cholesterol levels were reduced by about 40%.[135]

## 4.2   Lung delivery of genome editing components

Surfactant protein B (SP-B) deficiency is a lung genetic disease caused by mutated *SP-B* gene.[217] SP-B is essential for maintaining alveolar stability by interacting with lipid molecules to relieve surface tension.[217] Newborn infants with SP-B deficiency typically develop fatal respiratory failure within the first year.[217, 218] Currently, no effective treatment for SP-B deficiency is available.[218] Mahiny et al. utilized chitosan-PLGA nanoparticles to deliver ZFN mRNA and TALEN mRNA.[219] According to the efficiency of HDR, they finally chose ZFN mRNA loaded chitosan-PLGA nanoparticles to treat SP-B deficiency in mice.[219] Following intratracheal (*i.t.*) co-administration of nanoparticles encapsulating ZFN mRNA and AAV containing a DNA donor, SP-B expression was maintained for as long as 20 days in the lungs, which prevented the deterioration of lung function and resulted in longer lifetimes compared with negative controls.[219]

The peptide–poloxamine nanoparticle containing the *Sleeping Beauty (SB)* transposon system, which consists of *SB* transposon (pDNA) and transposase-encoding mRNA, was investigated to knock in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene in lung airway epithelium of cystic fibrosis (CF) mice. With the hydrophobic moiety of the peptide, the nanoparticle can overcome both the extracellular and intracellular barriers to achieve effective gene delivery *in vivo*.[45] After three-time *i.t.* administration, CFTR expression lasted for 18 weeks in CF mice and the level was similar to that of the wild type group. Meanwhile, lung pathohistological sections indicated no visible signs of

inflammatory infiltrates and blood sample analysis showed a normal level of white blood cells.[45]

## 4.3 Brain delivery of genome editing components

Fragile X syndrome (FXS) is a familial mental disease caused by abnormal trinucleotide sequence *CGG* in the *Fragile X mental retardation 1* (*FMR1*) gene.[220] The lack of effective therapeutic options causes patients with FXS to develop intellectual disabilities and autism spectrum disorders (ASDs).[220] Recently, CRISPR-Gold consisting of DNA functionalized gold nanoparticles and the PAsp (DET) polymer was developed for delivering Cas9 ribonucleoproteins (RNPs) to knock out the *mGluR5* gene. The overexpressed signaling of *mGluR5* gene is associated with both FXS and ASDs.[44] Through intracranial injection of 2 μL of CRISPR-Gold, both the mRNA and protein levels of mGluR5 decreased 40–50% in FXS mice. Furthermore, the treated FXS mice significantly resumed exaggerated repetitive behaviors after three weeks.[44]

The bioreducible LNP's encapsulation of negatively charged Cre recombinase or anionic Cas9 RNPs by electrostatic assembly may lead to an approach for correcting mental disorders.[221] The disulfide bonds in the bioreducible lipids were degradable in response to the intracellular environment, which promoted intracellular delivery efficiency and reduced lipid toxicity. After six days of intracranial injection of Cre recombinase complexed with bioreducible lipid 8-O14B, visible tdTomato signal in the brain of Ai9 mice indicated an effective DNA recombination.[221]

## 4.4 Muscle delivery of genome editing components

Duchenne muscular dystrophy (DMD) is an inherited muscle degeneration due to the dysfunctional dystrophin protein which connects the muscle cytoskeleton to surrounding extracellular matrix to maintain muscle integrity and strength.[222, 223] Mutations result in progressive muscle degeneration and loss of mobility.[222, 223] Although life expectancy has been extended due to advanced management, effective therapies for DMD are still urgently needed.[222, 223] Lee et al. used CRISPR-Gold to correct the mutated gene in DMD mice.[224] After two weeks of intramuscular injection of CRISPR-Gold with cardiotoxin in DMD mice, 5.4% of the dystrophin gene was repaired and obvious increase of dystrophin protein was observed in the treated muscle tissues. Restored muscle function was also demonstrated by increasing limb tension.[224]

PAsp(DET) polymer was designed to *in vivo* deliver Cpf1 and 5′ extended crRNA.[157] The 5′ extension of crRNA improved the serum stability and enhanced the nanoparticle formulation with PAsp(DET) polymer, resulting in a higher *in vivo* delivery efficiency than wild type Cpf1 RNPs. Two weeks after intramuscular injection of PAsp(DET) polymer encapsulating 200 pmol Cpf1 and 200 pmol 5′ extended crRNA on Rosa$^{26tdTomato}$ mice, the stop sequences of tdTomato were effectively deleted, and robust tdTomato signal was observed.[157]

### 4.5 Ear delivery of genome editing components

TMC1 (transmembrane channel-like protein 1) is an essential element for auditory transduction in hair cells.[225] Mutations in *TMC1* result in inherited hearing loss. [225] Most recently, Gao et al. applied lipofectamine 2000 to deliver Cas9 RNPs to disrupt the mutant *Tmc1* for the treatment of genetic deafness.[226] The intraaural injection of Cas9 RNPs/lipofectamine 2000 complexes into the scala media of neonatal $Tmc1^{Bth/+}$ mice significantly increased the survival of hair cells. Additionally, lower auditory response thresholds and improved acoustic responses were observed in injected mice.[226]

### 4.6 Other biomaterials for genome editing delivery

Many other distinctive delivery materials have been reported for delivering genome editing components and can possibly be developed for GARDs in the future. Ramakrishna et al. demonstrated that conjugating Cas9 and sgRNA with cell-penetrating peptides (CPPs) enhanced intracellular delivery efficiency because of the membrane-disturbed properties of CPPs.[227] Similarly, Wang et al. synthesized a membrane-penetrating cationic α-helical polypeptide which enhanced cellular internalization of Cas9 plasmid and sgRNA.[228] In addition to cellular uptake, endosomal trap is another barrier for genome editing delivery systems. Some materials, such as zeolitic imidazole frameworks (ZIFs) [229] and graphene oxide (GO)-PEG-polyethylenimine (PEI) [230] have been investigated to facilitate endosomal evasion. Furthermore, Mout et al. prepared cationic arginine gold nanoparticles (ArgNPs) to directly deliver Cas9 RNPs into the cellular cytoplasm without endocytosis. [231] For non-viral vectors, good biocompatibility enables the reduction of side effects and improves tolerability. Biodegradable lipid derived materials designed by Zhang et al. achieved efficient delivery of Cas9 mRNA *in vivo* without significant organic damage.[232] Timin et al. used hybrid microcarriers consisting of biocompatible $SiO_2$ capsules and degradable-polymer coatings for CRISPR-Cas9 delivery.[233] Other methods based on nucleic acid materials, including DNA nanoclews [234] and RNA aptamer-streptavidin complexes [235], were also explored for delivering gene editing components.

## 5. MicroRNA (miRNA) and long non coding RNA (LncRNA)

miRNA is small, non-coding RNA which regulates gene expression in biological systems primarily by silencing the target mRNA.[236] miRNA shares many similarities with siRNA. Specifically, both miRNA and siRNA could mediate gene silencing post-transcriptionally by targeting the mRNA. However, miRNA may regulate multiple mRNA targets whereas siRNA inhibits one specific mRNA target.[237] Studies have identified that the dysregulation of miRNA is closely associated with various disease statuses.[237, 238] Therefore, molecules that mimic the function of endogenous miRNA (miRNA mimics) as well as molecules that target endogenous miRNA (antimiRs) were widely investigated in preclinical studies.[237, 238] While many miRNA mimics and antimiRs have been focused on cancer therapies due to their biological roles and properties, several miRNA related therapies were reported for genetic and rare diseases.[237, 239] For example, MRG-201, developed by miRagen Therapeutics, is a cholesterol-conjugated miRNA duplex used for the treatment of Scleroderma and Keloids.[240] MRG-201 is a synthetic miRNA mimic of miR-29 designed to reduce the expression of collagen and other scar formation related

proteins.[238, 241] MRG-201 is now in Phase 2 clinical trials (NCT03601052). Recently, brain targeted nanoparticles for miRNA delivery have been reported. In 2011, Hwang et al. reported that the rabies virus glycoprotein (RVG)-labeled non-toxic SSPEI (RVG-SSPEI) nanomaterials were able to facilitate the *in vivo* delivery of miR-124a to neuron cells.[242] Combining RVG-SSPEI nanoparticles with mannitol solution could further improve the delivery of miR-124a across the BBB to the brain.[242]

Although extensive preclinical studies on miRNA therapeutics have been carried out, only a small fraction of them have reached phases of clinical development.[238] Besides, only a small portion of miRNA therapeutics targets diseases other than cancer.[237] It is still of great interest to develop more functional miRNA delivery biomaterials to enable both the better understanding the roles of miRNA *in vivo* and miRNA-based gene therapy on GARDs.

LncRNA is another important type of non-coding RNA molecule, which is typically more than 200 bases in length.[243] While only a small proportion of lncRNA transcripts have been functionally characterized to date, lncRNA is found to be involved in many different biological processes.[244] Therefore, it is realized that mutations affecting lncRNA expression could lead to diseases, as do protein coding genes. Numerous studies in the past several years showed that altered lncRNA transcriptional levels were associated with a variety of genetic disorders ranging from monogenic to complex disorders.[245] LncRNA may thus become a therapeutic target for treating GARDs.[246]

## 6. Conclusions and future perspectives

In summary, we provide an overview of delivery biomaterials for RNA-based therapies, with an emphasis on genetic and rare diseases (GARDs). Recently, an increasing number of RNA molecules are emerging as promising breakthrough therapies for many diseases.[3] A variety of delivery systems have been explored and developed to enable the RNA therapeutics for treating different GARDs (Figure 3).

Among various delivery materials, lipid and lipid derived nanoparticles (LNPs) exhibit compelling delivery efficiency towards many different types of RNAs, ranging from small RNA molecules such as siRNA to much larger mRNA molecules or more complicated genome editing systems containing RNA components. By exploring diverse chemical scaffolds of lipids, researchers can develop LNPs with optimized physiochemical and biological properties to match a specific application. However, LNPs still have some limitations. For example, a majority of LNPs accumulate in the liver which, to some extent, limits the use of LNPs to treatment of liver-oriented diseases. Additional efforts are needed in order to further develop new LNPs and expand their applications in different organs. This may be promoted by high-throughput technologies, optimizing screening methodologies, and modulating cellular signal pathways to identify tissue specific and highly efficient delivery systems.[133, 247–249]

Ligand conjugations have achieved prominent success in delivering small RNA molecules with the development of RNA chemical modification technology. Liver targeting *N*-

acetylgalactosamine (GalNAc) conjugation represents the lead conjugation strategy and has a growing number of RNA therapeutics in clinical trials. One of the most significant advantages of GalNAc-siRNA conjugations is that they can be administered subcutaneously, which improves the compliance of treatment. However, new ligands, especially for targeting organs other than the liver, need to be discovered. Linker chemistry can be further optimized to maximize the therapeutic window of the conjugates. Meanwhile, chemical modification of large RNA molecules is also an important area that is worth further investigation.

In addition, many other biomaterials, such as cell derived vehicles, gold nanoparticles, polymeric nanoparticles, and DNA/RNA nanotechnology, were also used as delivery systems for *in vivo* RNA therapeutics. Their applications in treating GARDs have not been widely explored yet. More categories of delivery systems await application for RNA-based GARD therapies.

Although many RNA delivery biomaterials have been explored for treating GARDs, a large number of rare diseases are still awaiting effective treatments. In addition to the need for deep understandings of the rare diseases and identifications of new therapeutic targets, more efforts are needed to expand the chemistry of biomaterials and enable the delivery of RNA therapeutics to different target organs and cell populations in GARD applications.

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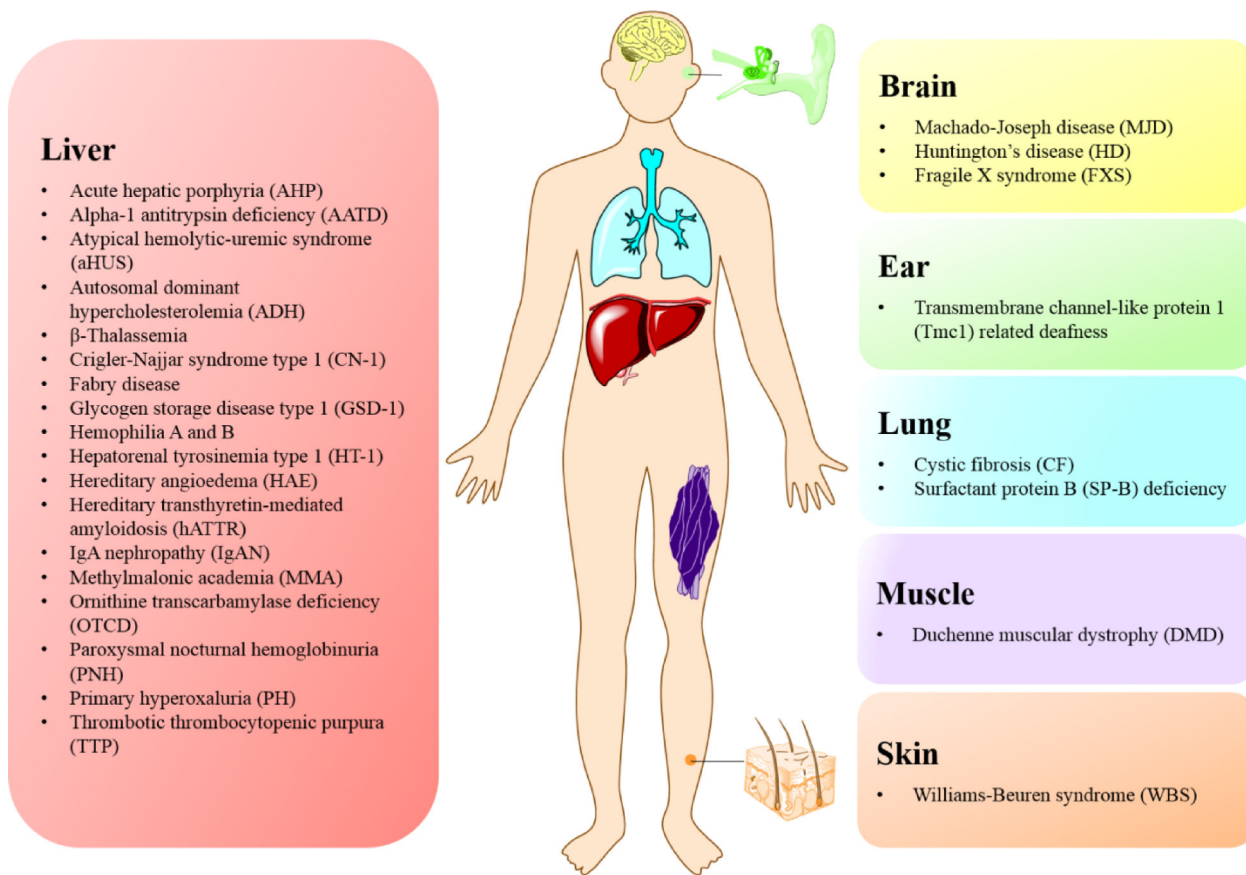
**Figure 1.**
A representation of genetic and rare diseases (GARDs) that have been explored using non-viral biomaterials based RNA therapeutics.
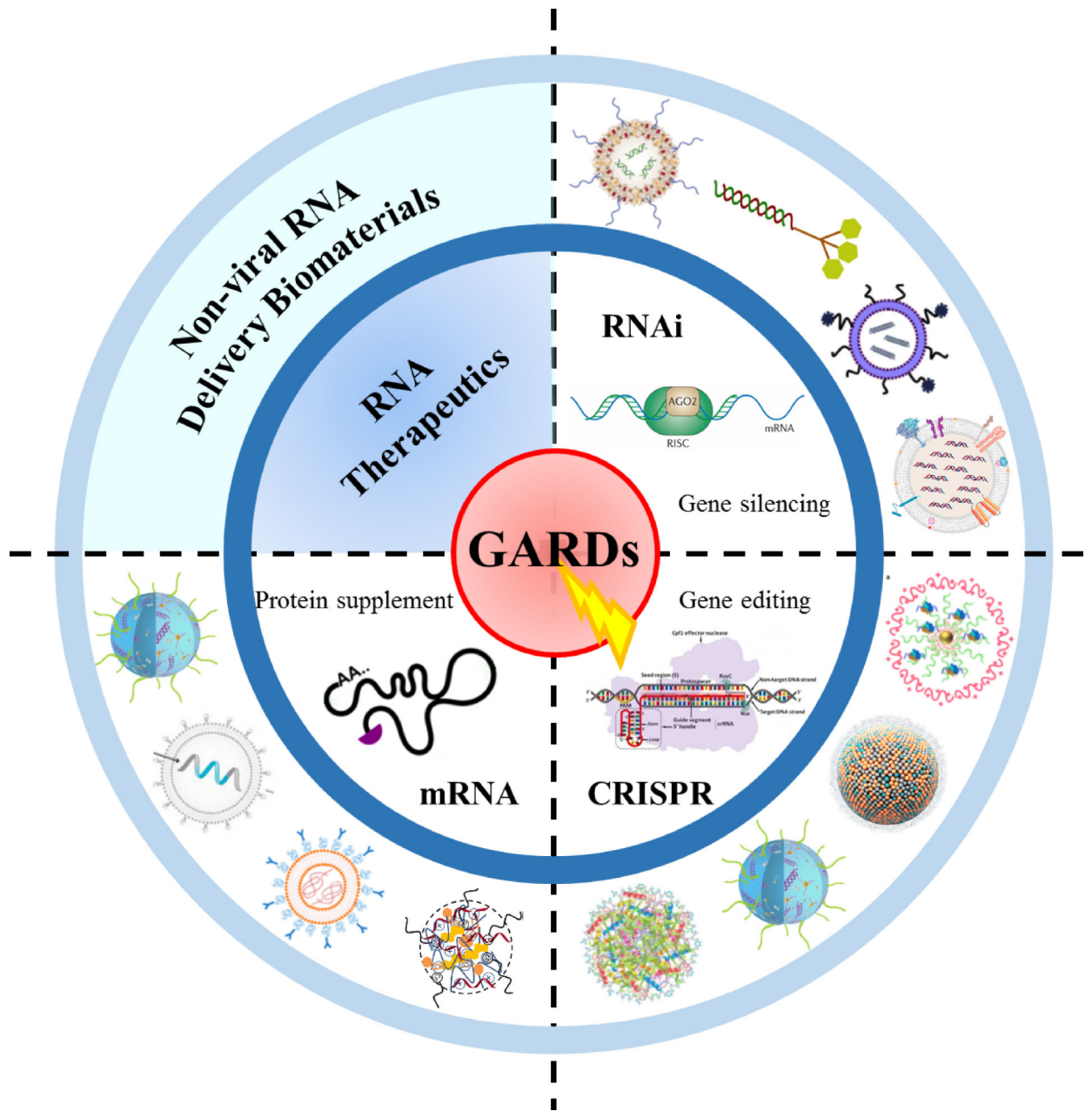
**Figure 2.**
An illustration of representative non-viral biomaterials (outer circle) together with versatile RNA therapeutics (inner circle) that have been exploited for treating genetic and rare diseases (GARDs). [9, 37–49]
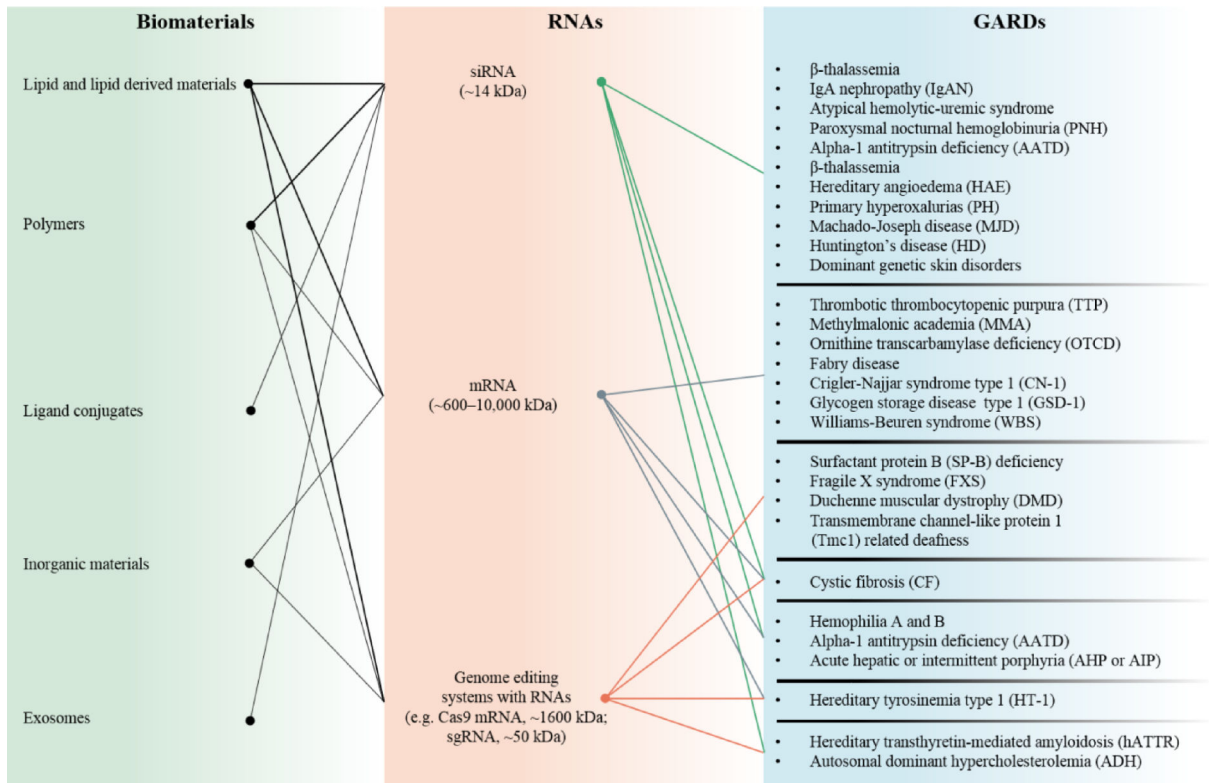
**Figure 3.**
A list of biomaterials and RNAs that have been explored for the treatment of different GARDs.

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**Table 1.**

List of siRNA delivery systems for the treatment of genetic and rare disease (GARD)

| Name of siRNA Therapeutics or siRNA | Delivery Material | Administration Route | Indication/Disease model | Target Organ | Clinical Trial | Reference |
|---|---|---|---|---|---|---|
| Patisiran (ALN-TTR02) | LNP (MC3) | *i.v.* | Hereditary transthyretinmediated amyloidosis (hATTR) | liver | FDA approved (ONPATTRO™) | [56, 58, 64] |
| DCR-PH1 | LNP | *i.v.* | Primary hyperoxaluria type 1 (PH-1) | liver | Pre-clinical | [71] |
| Tmprss6 siRNA | Ionizable LNP | *i.v.* | β-Thalassemia | liver | Pre-clinical | [68, 70] |
| Givosiran (ALN-AS1) | (ESC)-GalNAc delivery platform | subcutaneous | Acute hepatic porphyria (AHP) | liver | Phase 3 (NCT03338816) | [74] |
| Fitusiran (ALN-AT3) | (ESC)-GalNAc delivery platform | subcutaneous | Hemophilia A and B | liver | Phase 3 (NCT03754790) | [84] |
| Lumasiran (ALN-GO1) | (ESC)-GalNAc delivery platform | subcutaneous | Primary hyperoxaluria type 1 (PH-1) | liver | Phase 3 (NCT03681184) | [108, 109] |
| Vutrisiran (ALNTTRsc02) | (ESC)-GalNAc delivery platform | subcutaneous | Hereditary transthyretinmediated amyloidosis (hATTR) | liver | Phase 3 (NCT03759379) |  |
| Inclisiran (ALNPCSsc) | (ESC)-GalNAc delivery platform | subcutaneous | Autosomal dominant hypercholesterolemia (ADH) | liver | Phase 3 (NCT02963311) | [85] |
| Cemdisiran (ALNCC5) | (ESC)-GalNAc delivery platform | subcutaneous | IgA nephropathy (IgAN), atypical hemolytic-uremic syndrome (aHUS), paroxysmal nocturnal hemoglobinuria (PNH) | liver | Phase 2 (NCT03841448) Phase 1/2 (NCT02352493) | [110, 111] |
| ALN-AAT02 | (ESC)-GalNAc delivery platform | subcutaneous | Alpha-1 antitrypsin deficiency (AATD) | liver | Phase 2 (NCT03767829) | [112] |
| ALN-TMP | (ESC)-GalNAc delivery platform | subcutaneous | β-Thalassemia | liver | Pre-clinical | [80] |
| ALN-F12 | (ESC)-GalNAc delivery platform | subcutaneous | Hereditary angioedema (HAE) | liver | Pre-clinical | [113] |
| Revusiran (ALNTTRsc) | (STC)-GalNAc delivery platform | subcutaneous | Hereditary transthyretinmediated amyloidosis (hATTR) | liver | Phase 2 (NCT02292186) |  |
| DCR-PHXC | GalXC™ | subcutaneous | Primary hyperoxalurias (PH) | liver | Phase 2 (NCT03847909) | [87] |
| SLN124 | GalNAc-siRNA conjugation | subcutaneous | β-Thalassemia | liver | Pre-clinical | [88] |
| ARO-AAT | TRiM™ | subcutaneous | Alpha-1 antitrypsin deficiency (AATD) | liver | Phase 1 (NCT03362242) | [114] |
| ARC-AAT | DPCiv™ (Ex1) | *i.v.* | Alpha-1 antitrypsin deficiency (AATD) | liver | Phase 2 (NCT02900183) withdrawn | [90] |
| Lac-α-CDE (G3)/siRNA complex | Lac-α-CDE (G3) conjugate | *i.v.* | Familial amyloid polyneuropathy (FAP) | liver | Pre-clinical | [115, 116] |

| Name of siRNA Therapeutics or siRNA | Delivery Material | Administration Route | Indication/Disease model | Target Organ | Clinical Trial | Reference |
|---|---|---|---|---|---|---|
| GUG-β-CDE (G2, DS 1.8)/siTTR complex | GUG-β-CDE (G2) conjugate | *i.v.* | Familial amyloid polyneuropathy (FAP) | liver | Pre-clinical | [117] |
| ENaCsiRNA | nanocomplexes | oropharyngeal | Cystic fibrosis (CF) | lung | Pre-clinical | [95, 96] |
| ARO-ENaC | TRiM™ | intratracheal/ oropharyngeal/ nebulized | Cystic fibrosis (CF) | lung | Pre-clinical | [98] |
| siMutAtax3 siRNA | RVG-9r-targeted SNALPs | *i.v.* | Machado-Joseph disease (MJD) | brain | Pre-clinical | [37, 105] |
| hsiRNA-loaded exosomes | exosomes | striatum | Huntington's disease (HD) | brain | Pre-clinical | [106] |
| siRNA SNA-NCs | SNA-NCs | topical | Dominant genetic skin disorders | skin | Pre-clinical | [118] |

*i.v.*, intravenous; LNP, lipid and lipid derived nanoparticle; MC3, DLin-MC3-DMA; ESC, Enhanced Stabilization Chemistry; STC, Standard Template Chemistry; TRiM™, Targeted RNAi Molecule Platform; DPCiv™, Dynamic Polyconjugate (DPC) *i.v.* drug delivery vehicle; SNALPs, Stable Nucleic Acid Lipid Particles; SNA-NCs, Spherical Nucleic Acid Nanoparticle Conjugates.

List of mRNA delivery systems for the treatment of genetic and rare disease (GARD)

| Name of mRNA Therapeutics or mRNA | Delivery Material | Administration Route | Indication/Disease model | Target organ | Clinical Trial | Reference |
|---|---|---|---|---|---|---|
| hFIX mRNA | LNP (TT3) | i.v. | Hemophilia B | liver | Pre-clinical | [40] |
| hFIX mRNA | LNP (C12–200) | i.v. | Hemophilia B | liver | Pre-clinical | [168] |
| hFIX mRNA | LNP (ATX) | i.v. | Hemophilia B | liver | Pre-clinical | [145] |
| hADAMTS13 mRNA | LNP (ATX) | i.v. | Thrombotic thrombocytopenic purpura (TTP) | liver | Pre-clinical | [170] |
| hMUT mRNA | Ionizable LNP | i.v. | Methylmalonic academia (MMA) | liver | Phase 1/2 (NCT03810690) | [47] |
| FAH mRNA | Dendrimer LNP (5A2-SC8) | i.v. | Hereditary tyrosinemia type 1 (HT-1) | liver | Pre-clinical | [162] |
| hOTC mRNA | HMT (GalNAc and DOTAP) | i.v. | Ornithine transcarbamylase deficiency (OTCD) | liver | Pre-clinical | [177] |
| MRT5201 | MRT™ | i.v. | Ornithine transcarbamylase deficiency (OTCD) | liver | Phase 1/2 (NCT03767270) | [178] |
| hGLA mRNA | LNP (C12–200) | i.v. | Fabry disease | liver | Pre-clinical | [142] |
| hGLA mRNA | LNP (MC3) | i.v. | Fabry disease | liver | Pre-clinical | [129] |
| hPBGD mRNA | LNP (MC3) | i.v. | Acute intermittent porphyria (AIP) | liver | Pre-clinical | [130] |
| hUGT1A1 mRNA | Ionizable LNP | i.v. | Crigler-Najjar syndrome type 1 (CN-1) | liver | Pre-clinical | [181] |
| hSERPINA1 mRNA | Ionizable LNP | i.v. | Alpha-1 antitrypsin deficiency (AATD) | liver | Pre-clinical | [182] |
| hG6PC mRNA | Ionizable LNP | i.v. | Glycogen storage disease type 1 (GSD-1) | liver | Pre-clinical | [183] |
| hCFTR mRNA | LNP (MC3) | intranasal | Cystic fibrosis (CF) | lung | Pre-clinical | [131] |
| hCFTR mRNA | NP (chitosancoated PLGA) | i.t. or i.v. | Cystic fibrosis (CF) | lung | Pre-clinical | [149] |
| MRT5005 | MRT™ | nebulized | Cystic fibrosis (CF) | lung | Phase 1/2 (NCT03375047) | [178] |
| hCFTR mRNA | LUNAR LNP | nebulized | Cystic fibrosis (CF) | lung | Pre-clinical | [191] |
| hTE mRNA | lipofectamine 2000 | subcutaneous | Williams-Beuren syndrome (WBS) | skin | Pre-clinical | [198] |

i.v., intravenous; i.t., intratracheal; LNP, lipid and lipid derived nanoparticle; NP, nanoparticle; LUNAR, Lipid-enabled and Unlocked Nucleomonomer Agent-modified RNA; MRT™, mRNA Therapeutic Platform; HMT, Hybrid mRNA Technology; MC3, DLin-MC3-DMA.

**Table 3.**

List of CRISPR and other genome editing delivery systems for the treatment of genetic and rare disease (GARD)

| Name of the genome editing system | Delivery Material | Administration Route | Indication/Disease model | Target organ | Clinical Trial | Reference |
|---|---|---|---|---|---|---|
| Cas9 mRNA and sgRNA | LNP (LP01) | *i.v.* | Hereditary transthyretinmediated amyloidosis (hATTR) | liver | Pre-clinical | [48] |
| Cas9 mRNA, gRNA, and DNA template | LNP (C12–200) and AAV vector | *i.v.* | Hereditary tyrosinemia type 1 (HT-1) | liver | Pre-clinical | [141] |
| Cas9 mRNA and sgRNA | LNP (ZALs) | *i.v.* | Ai9 mouse | liver, lung, and spleen | Pre-clinical | [214] |
| Cas9 mRNA and sgRNA | LNP (TT3) | *i.v.* | Autosomal dominant hypercholesterolemia (ADH) | liver | Pre-clinical | [139] |
| Cas9 mRNA and enhanced sgRNA | LNP (cKK-E12) | *i.v.* | Autosomal dominant hypercholesterolemia (ADH) | liver | Pre-clinical | [135] |
| ZFN mRNA and DNA template | NP (chitosan-coated PLGA) and AAV vector | *i.t.* | Surfactant protein B (SP-B) deficiency | lung | Pre-clinical | [219] |
| *SB*100X-mRNA and pDNA | NP (peptide-poloxamine) | *i.t.* | Cystic fibrosis (CF) | lung | Pre-clinical | [45] |
| Cas9 and sgRNA | CRISPR-Gold | intracranial | Fragile X syndrome (FXS) | brain | Pre-clinical | [44] |
| Cas9 and sgRNA | LNP (8-O14B) | intracranial | Ai9 mouse | brain | Pre-clinical | [221] |
| Cas9, gRNA, and DNA template | CRISPR-Gold | intramuscular | Duchenne muscular dystrophy (DMD) | muscle | Pre-clinical | [224] |
| Cpf1 and 5' extended crRNA | NP (PAsp(DET)) | intramuscular | Ai9 mouse | muscle | Pre-clinical | [157] |
| Cas9 and sgRNA | lipofectamine 2000 | intraaural | Transmembrane channellike protein 1(Tmc1) related deafness | ear | Pre-clinical | [226] |

*i.v.*, intravenous; *i.t.*, intratracheal; LNP, lipid and lipid derived nanoparticle; NP, nanoparticle.