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PERSPECTIVE

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Nucleic acid drug and delivery techniques for disease therapy: Present situation and future prospect

Tianjiao Wang[1,2] | **Youhong Tang**[3] | **Yuandong Tao**[4] | **Huixia Zhou**[4] | **Dan Ding**[1]

[1]State Key Laboratory of Medicinal Chemical Biology, Key Laboratory of Bioactive Materials, Frontiers Science Center for Cell Responses, Ministry of Education, and College of Life Sciences, Nankai University, Tianjin, China

[2]State Key Laboratory of Advanced Medical Materials and Devices, Tianjin Key Laboratory of Biomedical Materials, Key Laboratory of Biomaterials and Nanotechnology for Cancer Immunotherapy, Institute of Biomedical Engineering, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, PR China

[3]Australia–China Joint Centre for Personal Health Technologies, Medical Device Research Institute, Flinders University, Bedford Park, South Australia, Australia

[4]Department of Pediatric Urology, Senior Department of Pediatrics, The Seventh Medical Center of Chinese PLA General Hospital, National Engineering Laboratory for Birth Defects Prevention and Control of Key Technology, Beijing, China

Correspondence
Yuandong Tao, Huixia Zhou and Dan Ding.
Email: tao_yuandong@163.com, huixia99999@163.com and dingd@nankai.edu.cn

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Abstract

Over the two decades, RNA drugs have gradually made their way from bench to bed. Initially, RNA was not an ideal drug since RNA molecules degrade easily and have a relatively short half-life in the circulation system. Nevertheless, the chemical modification extended the half-life of RNA in recent years, which makes RNA drugs a new star in drug discovery industry. RNA molecules hold many properties that facilitate their application as therapeutic drugs. RNAs could fold to form complex conformations to bind to proteins, small molecules, or other nucleic acids, and some even form catalytic centers. Protein-encoding RNAs are the carriers of genetic information from DNA to ribosomes, and various types of non-coding RNAs cooperate in the transcription and translation of genetic information through various mechanisms. To date, three mainstream RNA therapies have drawn widespread attention: (1) messenger RNA that encodes therapeutic proteins or vaccine antigens; (2) small interfering RNA, microRNA (miRNA), antisense oligonucleotides that inhibit the activity of pathogenic RNAs; and (3) aptamers that regulate protein activity. Here, we summarized the current research and perspectives of RNA therapies, which may provide innovative highlights for cancer therapy.

KEYWORDS
mRNA, nucleic acid drugs, small RNA

1 | NUCLEIC ACID DRUGS DERIVATION

In 1990, scientists from the University of Wisconsin reported for the first time that messenger RNA (mRNA) directly injected into animals could achieve protein expression.[1] After that, a study by Scripps Research Institute demonstrated the potential of mRNA for disease treatment through injecting mRNA encoding Vasopressin into the brain to relieve diabetes insipidus in rats.[2] Noteworthy, direct injection of mRNA molecules could be absorbed by antigen-presenting cells (APCs) and induce an immune response.[3] The common production manner of mRNA vaccines is enzyme engineering, in which the immunogenicity, pharmacokinetics, and dose can be strictly controlled, and the stability and immunogenicity and vaccine stability can be maintained simultaneously via the optimization of the mRNA codon.[4]

The successful clinical application of RNA drugs faces several impediments: (1) the molecular weight and negative charge of nucleic acid restrict it pass through the biofilm; (2) RNA is easily degraded by RNase enzymes in the plasma and tissue environment, quickly cleared by liver and kidney and recognized by the immune system; (3) encapsulated in endocytosome after entering the cell obstructs its function. These problems make drug delivery the major technical obstacle facing the development of RNA drugs. At present, the modification of nucleic acid molecules to enhance stability and to avoid attack by the immune system and the use of drug delivery platforms, such as lipid nanoparticles (LNP) and *N*-acetylated galactosamine (GalNAc) coupling technology, are the two mainstream approaches.[5–7]

2 | mRNA DELIVERY SYSTEM

Evaluation of the administration mode and special design of pharmaceutical formulations are crucial to ensure the metabolic stability and immune activity of mRNA vaccines. The delivery system should not only protect mRNA from degradation of nucleases but also cross the cell membrane with a phospholipid bilayer to promote mRNA uptake. A great number of mRNA delivery techniques have been proposed and many of them have entered the clinical trials, including nanoparticle liposomes, protamine carriers, polymer carriers, and LNP, etc.[8] In recent years, the reports on novel and modified delivery systems for mRNA have rapidly emerged.[9,10]

2.1 | Protamine carrier

Protamine is a natural cationic protein, which can be complex the mRNA molecules with negative charge into nano-sized particles to protect mRNA from RNase degradation in serum.[11] However, the tight junction of protamine to mRNA affects the efficacy of the mRNA vaccine and limits its application. In addition, the expression of antigen is greatly affected by the ratio of protamine to mRNA. The RNActive platform developed by CureVac company in Germany has successfully tackled this problem. In this protamine-mRNA complex, protamine acts as a TLR7/8 antagonist to induce Th1 cell response and the mRNA encoding target protein is presented to APCs to induce adaptive immune response.[12,13] CV9103 is such a self-adjuvanted mRNA vaccine that targets antigens of prostate cancer and has been proved immunogenic and tolerant in a phase I/II trial.[14] In a clinical trial for stage IV non-small-cell lung cancer patients, the CV9202 antigen-induced immunity was observed in 21 of 25 patients (84%), and 12 of 26 patients (46.2%) achieved stable disease.[15] A Phase I/II clinical trial of CV9202 vaccine in combination with the anti CTLA-4 tremelimumab and/or anti-PD-L1 durvalumab is performed, and corresponding outcomes are not published yet (Figure 1).

2.2 | LNP system

With two mRNA vaccines approved for vaccination against the COVID-19, the LNP system is becoming the most popular delivery technology at the moment. The LNP is commonly composed of four components including ionizable lipids, neutral helper phospholipids, cholesterol, and PEGylated lipids (Figure 2). In relative acidic environment, the ionizable cationic phospholipids carries a positive charge and realize electrostatic complexation with negatively charged mRNA molecules to form complex and improve mRNA stability.[17] Upon attachment, the cationic phospholipids fuse with negatively charged cell membranes, trigger membrane destabilization, and promote mRNA molecule delivery. After internalization into cells, the more acidic environment and hydrolases in lysosomes protonate and destroy the bilayer structure of LNP and release mRNA.[17] LNPs can also be expelled from the cell via opposite exocytosis, which is also a concern for mRNA administration via LNPs.

LNPs primarily accumulate in the liver tissue upon intravenous administration. Studies have shown that the apolipoprotein E (ApoE) in the blood can be easily adsorbed to the surface of LNPs, which consequently

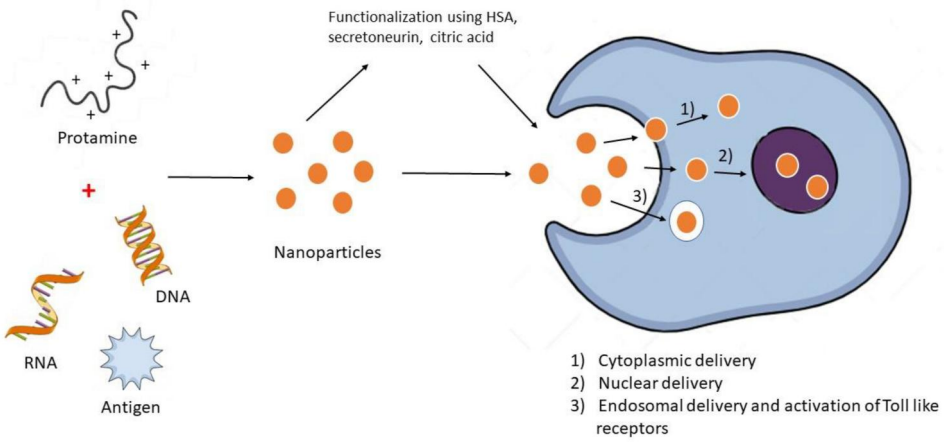

FIGURE 1 The structure of protamine carrier. Reproduced with permission.[11] Copyright 2021, MDPI.

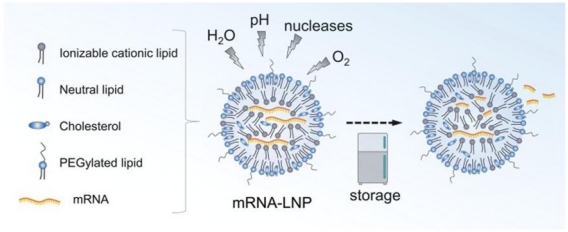

FIGURE 2 The structure of the mRNA-LNP vaccine. Reproduced with permission.[16] Copyright 2021, Elsevier. mRNA-LNP, messenger RNA-lipid nanoparticles.

facilitates the entrance into hepatocyte through the interaction between ApoE with low density lipoprotein receptor.[18] The selection of components and their ratios is critical for LNP-mediated mRNA delivery efficacy.[19] Accumulating evidence has suggested that organ-specific targeted delivery can be achieved through specific lipid structures. For example, lipids with imidazole groups are able to deliver mRNA to T lymphocytes. The lead structure 93-O17S has been reported to achieve 6.5% and 8.2% gene recombination in CD8- and CD4-positive mouse spleen T cells, respectively.[20] Moreover, Ma and colleagues developed an LNP based on neurotransmitter-derived lipids (NT lipids) inspired by the neurotransmitters such as tryptamine derivatives that could permeate the blood-brain barrier (BBB), which successfully delivered proteins, nucleic acids, and small molecule agents to neuronal cells through the BBB, achieving efficient brain delivery.[21] Cheng et al. report a selective ORgan targeting (SORT) approach that adds a fifth component to enable tissue-specific delivery of mRNAs. Adding 30% anionic lipids, 20% ionizing lipids, or 50% permanent cationic lipids to establish 5A2-SC8 LNPs that could achieve mRNA delivery in mouse spleen, liver, and lung, respectively. The SORT system provides a common strategy for organizational positioning that could be reproduced across different LNP delivery systems.[22] Dilliard et al. proposed a targeting mechanism for SORT LNPs. At least three processes were found to be required after injection, including PEG-based lipid desorption, interaction with specific serum proteins, and interaction with the corresponding receptor on the target cell. They next analyzed the protein corollaria of each SORT LNP and found that ApoE, β2-GPI, and Vtn, may contribute primarily to targeting the liver, spleen, and lung, and spleen, respectively. Deciphering these mechanisms is pivotal to guide the development of new LNP systems and elevate the efficacy of the screening process. Noteworthy, other than the lipid composition of LNP, the molar ratio of N/P (N refers to the amino group of ionizable cationic lipids, P refers to the phosphate group of mRNAs) also greatly affects the characteristics of LNP. Typically, the N/P ratio of 3:1–6:1 is adopted to encapsulate larger nucleic acid payloads, and as the N/P ratio decreases, each LNP can obtain a higher loading capacity of mRNA, while the mRNA encapsulation efficiency may decrease. In general, a higher N/P ratio could cause a higher encapsulation rate but may cause higher in vivo toxicity. This ratio needs to be fine-tuned to achieve better delivery efficiency.[23]

Cationic LNPs are stable complexes formed between synthetic cationic lipids and anionic nucleic acids and are currently the most widely used non-viral delivery system for nucleic acid drugs. The scalable production of mRNA vaccines or therapeutics relies on strong replicability, with the key factors being successful sequence modifications and assembly of the delivery system. However, the development of a scalable production chain is of paramount importance, as each link in the chain can potentially pose limitations. For example, it involves hundreds of raw

materials, including enzymes, nucleotides, lipids, among others, and materials with technical barriers such as enzymes and lipids may be supplied by only a few vendors. The complexity, structural requirements, in vivo delivery efficiency, and safety need to be considered for successful LNP application. Specifically, when choosing the lipid components for an LNP formulation, its worth considering the following aspects: biocompatibility, lipid bilayer fluidity, the size and charge of LNPs, encapsulation efficiency and stability.

2.3 | mRNA-based cancer therapy

Cancers are the secondary leading cause of death worldwide. Great efforts have been made to explore effective therapies including small molecular drugs and biological macromolecule drugs for chemical therapy, immune therapy, photochemical therapies.[24–26] The mRNA-based cancer immunotherapy holds immense potential, as mRNAs can be tailored to produce specific proteins, prompting the desired immune reactions.[27] A prevalent approach involves administering mRNA designed to encode tumor antigens, thus promoting immunization. Similarly, mRNA can be utilized to encode cytokines, immune checkpoint inhibitors, or other functional proteins, aiming to remodel the tumor microenvironment and reinstate immune fitness. In addition, the integration of mRNA with conventional therapeutic techniques presents an appealing avenue for enhancing cancer treatment efficacy.[28]

mRNA vaccines that express tumor-associated antigens (TAAs) currently play an important role in cancer treatment. The mRNA is first delivered to APCs where TAAs or neoantigens are produced. Major histocompatibility complex on the APC surface binds to T lymphocytes, activating CD8- and CD4-positive T cells and ultimately killing tumor cells (Figure 3).[29] These vaccines can be administered intravenously, intratumorally, intradermally, or intramuscularly. A great number of cases have entered clinical trials, among which a small number have advanced to Phase II (Table 1). For example, several ongoing clinical trials are evaluating the FixVac (cancer antigen) mRNA-LNP vaccine. BNT111 is the first in a series of mRNA-LNP vaccines that share FixVac (cancer antigen) fixed combinations to incorporate a shared TAA. BNT111 encodes at least one of four melanoid-associated antigens that show high prevalence and immunogenicity in melanoma. A Phase I dose-escalation trial (NCT02410733) evaluated its efficacy and safety and suggested that 39 of the 50 patients (75%) showed an immune response to one or more TAAs, during which BNT111 was able to induce CD4- and CD8-positive T cell responses. Of the 17 patients

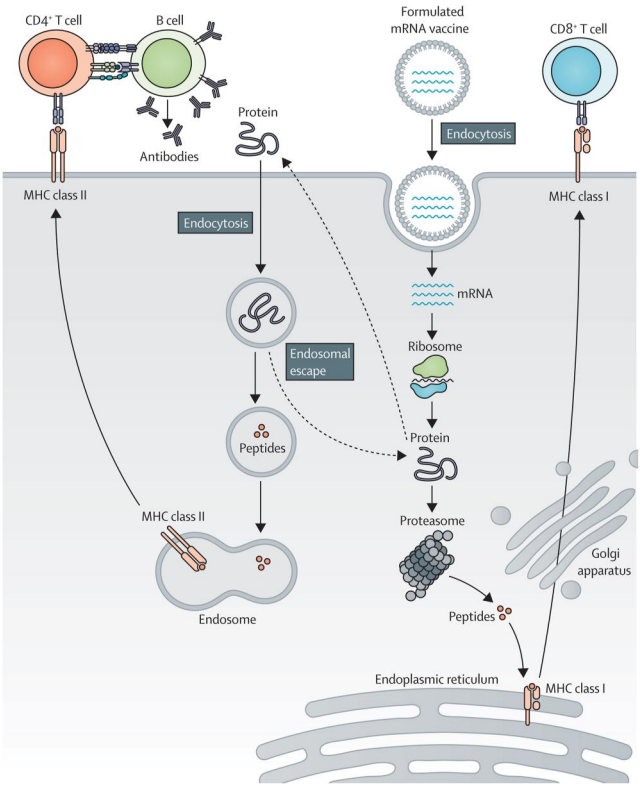

FIGURE 3 mRNA-LNP activates antigen-specific T cell activation. Reproduced with permission.[31] Copyright 2022, Elsevier. mRNA-LNP, messenger RNA-lipid nanoparticles.

who received combined therapy of BNT111 with anti-PD-1, 6 patients (35%) presented a partial response and 2 patients (12%) had stable disease. Of the 25 patients treated with BNT111 alone, 3 patients (12%) had partial remission and 7 patients (28%) had stable disease.[30] FixVac BNT111 is currently being tested in a randomized Phase II clinical trial (NCT04526899) that uses BNT111 alone or together with cemiplimab (an anti-PD-1 antibody) for the treatment of stage III and IV melanoma that is insensitive to anti-PD-1 antibodies or that is recurrent and unresectable. A Phase I/II clinical trial (NCT04382898) is currently underway to assess the effectiveness of the cancer vaccine BNT112, which encodes five TAAs, for the therapy of patients with metastatic prostate cancer, either alone or in combination with cemiplimab.[31] Apart from the FixVac vaccine, various studies are actively investigating the clinical effectiveness of an innovative mRNA-LNP vaccine platform known as personalized neoantigen-specific immunotherapy (iNeST), also referred to as BNT122. The iNeST approach involves utilizing mRNA-lipid complex vaccines that are tailored to encode the specific individual tumor mutations. This promising strategy is currently undergoing evaluation in multiple clinical trials. For example, for patients with triple-negative breast cancer, iNeST in combination with another lipid-formulated RNA that encodes TAAs

TABLE 1 mRNA-LNP vaccines in clinical trials.

Vaccine	Conditions	Stage/status	NCT number
mRNA-1345	Respiratory syncytial virus (RSV)	Phase I (recruiting)	NCT04528719
VAL-506440; mRNA-1440	Influenza A virus [H10N8]	Phase I (completed)	NCT03076385
VAL-339851; mRNA-1851	Influenza A virus [H7N9]	Phase I (completed)	NCT03345043
mRNA-1325	Zika virus	Phase I (completed)	NCT03014089
mRNA-1893		Phase I (completed)	NCT04064905
Rabipur®	Rabies virus	Phase I (active, not recruiting)	NCT03713086
mRNA-1647 and mRNA-1443	Cytomegalovirus (CMV)	Phase I (–)	NCT03382405
TAA mRNA	Melanoma	Phase I (recruiting)	NCT02410733
mRNA-2752	Solid tumors	Phase 1 (recruiting)	NCT03739931
TAA and neo-Ag mRNA	Breast cancer	Phase I (recruiting)	NCT02316457
ChulaCov19 mRNA vaccine	COVID-19	Phase I (not yet recruiting)	NCT04566276
SAM-LNP-spike	COVID-19	Phase I (recruiting)	NCT04776317
SAM-LNP-spike	COVID-19	Phase I (active, not recruiting)	NCT04758962
SARS-CoV-2 mRNA vaccine	COVID-19	Phase III (not yet recruiting)	NCT04847102

Abbreviations: mRNA-LNP, messenger RNA-lipid nanoparticle; TAA, tumor-associated antigen.

(BNT114) and p53 is receiving clinical trial (NCT02316457).[31]

RNA therapy has several important advantages, including high specificity to the target, modular development through sequence replacement, predictability in terms of pharmacokinetics and pharmacodynamics, and relative safety (most of them do not alter the genome). However, this therapy also faces some challenges: despite the modular "plug-and-play" design concept for RNA therapeutic drug development, their efficacy and safety still need to be determined through testing. Compared to other biological molecules, RNA molecules are relatively unstable. When exogenous RNA molecules are introduced into the human body, the levels of protein expression in cells are limited and often trigger immune responses in the body. These issues can be mitigated by optimizing RNA molecules through various methods, including chemical modifications.

3 | SMALL NUCLEIC ACID DRUG AND DELIVERY SYSTEM

Small nucleic acid drugs, that is, oligonucleotide drugs, including antisense oligonucleotides (ASO), microRNA (miRNA), small interfering RNA (siRNA), nucleic acid aptamer (RNA aptamer) and others. Among them, the ASO is discovered the earliest with drugs approved the most, and the development is more mature.[32] However, the ASO drug is not as effective as the siRNA drug.

3.1 | Small nucleic acid

ASO are single-stranded antisense oligonucleotide molecules between 12 and 30 bp in length, and usually are heterozygous molecules of DNA and RNA.[33] Initially, ASO are used to block the translation and transcription of DNA or RNA by base pairing, and are later found to recruit RNase, specifically degrade targeted mRNAs, interfere with pre-RNA splicing, and indirectly enhance the expression of certain proteins by regulating translation, which can be utilized to design ASO drugs for diverse targets.[34,35] To date, more than 50 ASO drugs are being evaluated in the clinical stage, covering multiple areas including the central nervous system, anti-infection, cardiovascular and cancers.[36]

The siRNA drug function through a post-transcriptional gene silencing mechanism, a highly conserved RNA interference process that has been found in many eukaryotes.[37] SiRNAs are artificially synthesized double-stranded RNA molecules between 15 and 30 bp in length, with siRNAs shorter than 21 bp bind with TRBP (TAR-RNA binding protein) and further bind to AGO 2 (Argonaute 2), forming the precursor of the silencing complex: pre-RISC (RNA-induced gene silencing

complex). After that, the guide strand in siRNA is selected for loading into pre-RISC, and positive-strand RNA is degraded to form mature RISC, which interacts with targeted mRNA protein, then AGO 2 protein in RISC exhibits activity of exonuclease to cleave mRNA and silence gene expression.[38] Accumulating evidence has introduced the therapeutic effects of siRNAs in multiple diseases. For example, siRNA targeting Fas for the first time proved to protect mice from fulminant hepatitis in an in vivo model.[39] Even though the siRNA drug still faces a certain bottleneck in the delivery of tissue cells, in August 2018, the Food and Drug Administration (FDA) approved the first-ever RNA interference drug, Alnylam's Onpattro (Patisiran), for the treatment of nerve damage caused by the rare disease hereditary transthyretin-mediated amyloidosis (hATTR) in adults. This approval came after the promising results observed in the Phase 3 clinical trial known as "Apollo" (NCT01960348).[40] This siRNA drug has been effectively delivered to organs and tissues, and it is widely recognized that with further breakthroughs in drug delivery systems, siRNA drugs will gradually replace ASO drugs and become the mainstream RNA interfering drugs.[41] Different from the siRNA that functions through pairing of 21 nucleotides, miRNA only requires 2- to 8-nucleotide pairing and usually acts on a bunch of targets but not only one signaling pathway. Relevant technologies need to be further broken through.

Aptamers are short single-stranded oligonucleotides that fold into unique three-dimensional structures and bind to a wide range of targets, including small molecules, proteins, metal ions, bacteria, viruses and whole cells, and its high specificity and binding affinity could reach the antibody level.[42,43] Compared with antibodies, the advantages of aptamers are outstanding, such as fast in vitro screening, cell-free chemical synthesis, small size, low immunogenicity and strong tissue penetration.[44] Nucleic acid aptamers were initially created through artificial screening in 1990, and the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) technology, which matches enriched ligands, provided a crucial research tool for nucleic acid aptamers. SELEX enables the selection of nucleic acid aptamers with high specificity and affinity for target substances from a random single-stranded nucleic acid sequence library. Although the SELEX technology has significantly advanced the research and development of nucleic acid aptamers, limitations such as patent barriers, the lack of research on their biological activity, and binding sites have restricted the clinical use and market availability of nucleic acid aptamers as therapeutic drugs.[45]

Currently, nucleic acid aptamer drugs used directly for therapy do not have clear commercial and therapeutic success stories, and this may be partly due to factors like patent protection and in vivo conditions. In 2004, Pfizer/Eyetech obtained FDA approval for the nucleic acid aptamer drug Pegaptanib (Macugen) for the treatment of AMD (age-related macular degeneration). It was the only nucleic acid aptamer drug that made it to the market. Pegaptanib is a 28-base RNA aptamer that specifically binds to the VEGF isoform VEGF165, thereby inhibiting endothelial cell proliferation, tissue-level vascular leakage, and angiogenesis.[46] However, due to the emergence of the more effective Lucentis, an antibody fragment for treating AMD, Pegaptanib has been phased out. E10030 (ARC127) is based on the PDGF-BB nucleic acid aptamer ARC126, with a 40 kDa branched polyethylene glycol added at the 5′ end to impede renal clearance, thereby extending its in vivo half-life.[47] However, due to its inability to demonstrate greater efficacy in improving vision when used in combination with ranibizumab than when ranibizumab is used alone in Phase III clinical trials, the Phase III clinical trial of E10030 has been declared a failure. Nevertheless, with increasing investments in various aspects of nucleic acid aptamer technology, the iterative development of novel nucleic acid aptamers is gradually opening new therapeutic possibilities.

3.2 | Small nucleic acid delivery system

Research and development in the field of small nucleic acid drugs mainly focus on two directions, one the one hand, the development of gene sequencing technology and the reduction of the cost make it possible for the industrialization of small nucleic acid drugs,[48] on the other hand, the development of RNA modification technology[49] and breakthrough on drug delivery system increases the RNA stability in blood and efficacy and safety.[50,51]

The indications for small nucleic acid drugs cover a wide range.[52] Among them, cancers and rare diseases are the most widely applied fields of small nucleic acid drugs.[53,54] To date, more than 50 small nucleic acid drugs in the world are in the clinical research stage, covering nerve, cardiovascular, infection and tumor fields. Nine small nucleic acid drugs have been approved on the market, five of which are orphan drugs and are the first drugs in the field of the disease; to some extent, they meet the needs of rare patients who have not been treated before.[55] Among them, the world's first drug for the treatment of spinal muscular atrophy, Nusinersen, currently has the highest sales of small nucleic acid drugs with a global sales of $1.951 billion in 2021. Nevertheless, certain drawbacks retard the development and application of siRNA drugs. The first siRNA drug to be tested in

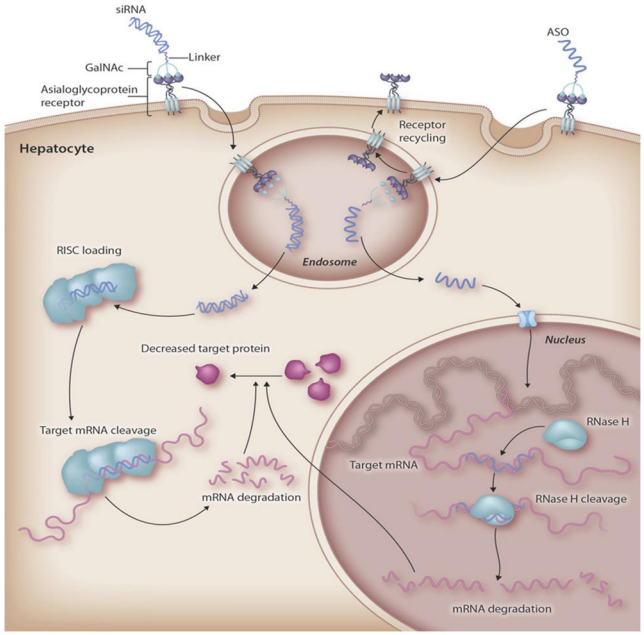

FIGURE 4 The delivery of siRNA and ASO by the GalNAc delivery system. Reproduced with permission.[60] Copyright, 2020, Elsevier. ASO, antisense oligonucleotides; GalNAc, *N*-acetylated galactosamine; siRNA, small interfering RNA.

a clinical trial, Bevasiranib, was discontinued in a Phase III trial due to poor results in 2009.

The off-target effect, poor targeting and stability are the most important factors affecting the efficacy of siRNA drugs: (1) poor targeting: siRNA is a negatively charged bioactive macromolecule, hence it is hard to target or penetrate the negatively charged cell membrane; (2) the antisense chain of siRNA could cause the suppression of some non-homologous genes through miRNA pathway, and sense chain could mediate the silencing of its homologous genes, leading to an off-target effect; (3) unmodified double-stranded RNA can also cause the activation of certain innate immune mechanisms, therefore inducing side effects in clinical use; (4) RNA is easy to be degraded by blood nuclease in circulation system, leading to its poor stability.

Similar to mRNA, the development of small nucleic acid drug delivery platform is the focus of the small nucleic acid pharmaceutical field. At present, in order to solve this problem, the research and development of drug delivery system technology is growing vigorously and mainly focus on LNP, GalNAc coupling technique, and the recent high profile exosomes.[56,57] Several small RNA drugs delivered by LNP and GalNAc have acquired new drug approvals. Patisiran is an RNA interference therapeutic agent delivered by the LNP system, which was proved to specifically inhibit the hepatic synthesis of transthyretin and approved for clinical use in 2018.[58] Inclisiran is a long-acting lipid-lowering drug that is delivered by the GalNAc system and requires only two injections a year; hence, it opens a new chapter in the use of small nucleic acids for common chronic diseases.

The GalNAc delivery platform is based on the covalently linking of GalNAc to small nucleic acids, which can specifically bind to the asialoglycoprotein receptor on the surface of parenchymal hepatocytes, enabling rapid cellular endocytosis. This technique has high specificity of liver targeting and high enrichment in liver tissue with very low entrance into other tissues.[59] Compared to LNP, the GalNAc delivery system utilizes its high liver selectivity to achieve precise delivery. Alnylam is the first company to introduce GalNAc delivery technology, and several GalNAc-siRNA drugs, including Givlaari for acute hepatic porphyria and Lumasiran for hyperoxaluria, have been approved by the FDA (Figure 4).[60]

4 | CONCLUSION

In this perspective, we summarized representative nucleic acid drugs, delivery systems, and clinical applications. Current nucleic acid drugs mainly include mRNA, small RNAs and aptamers, which functions by encoding therapeutic proteins, inhibiting RNA expression, and regulating protein activity. The RNA delivery techniques have been widely studied for better delivery and efficacy and entered the clinical trials, especially the protamine carrier, LNP, and GalNAc. The RNA field is of great therapeutic potential. However, in addition to the hard issue in RNA drug design period such as immunogenicity, the core challenges, including drug delivery, off-target side effects, and general toxicity, are difficult to avoid in subsequent clinical trials. At present, although the delivery system represented by the non-viral nanoparticle platform is continuously developing, the problems of low efficiency and lack of targeting mechanism still need a breakthrough.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

ORCID

Dan Ding https://orcid.org/0000-0003-1873-6510

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AUTHOR BIOGRAPHIES

Tianjiao Wang received her Ph.D. degree from the Collage of Life Science in Nankai University in 2019. After her postdoctoral training in State Laboratory of Medicinal Chemistry Biology of Nankai University, where she mainly studied the application of supramolecular assembling peptides for targeted protein degradation in tumor therapy, she is now an assistant research fellow of Institute of Biomedical Engineering, Chinese Academy of Medical Sciences.

Youhong Tang is a Matthew Flinders Professor and was an Australian Research Council-Discovery Early Career Researcher in Flinders University, Australia. He was the Deputy Director of International Laboratory for Health Technologies and ACSRF-Joint Research Centre on Personal Health Technologies and is a Research Leader in Institute for NanoScale Science and Technology and a Management Committee in Medical Device Research Institute in Flinders University. He was elected as a Fellow of Royal Society of Chemistry (FRSC), UK in 2018 and a Fellow of Royal Australia Chemical Institute (FRACI), Australia in 2021. His research interests mainly focused on Chemo/ biosensors and their devices with aggregation-induced emission features.

Dr. Yuandong Tao received B.A. from Medical College of Xi'an Jiaotong University and Ph.D. from Academy of Military Medical Sciences. He is now a pediatric urologist at the Seventh Medical Center of Chinese PLA General Hospital. He is interested in understanding the pathologic mechanisms of acute and chronic kidney injury.

Dr. Huixia Zhou received her Ph.D. degree from Tongji Medical College of Huazhong University of Science and Technology and is now a pediatric urologist at the Seventh Medical Center of Chinese PLA General Hospital. She is renowned for her contribution to the field of pediatric urological minimal-invasive surgery and is one of the most experienced laparoscopic and robotic surgeons in China. She had established a standardized minimally-invasive surgery technique system in pediatric urology in the nation. She is also the Chairman of the Pediatric Urological Group of Chinese Urological Association (CUA).

Dan Ding received his Ph.D. degree from the Department of Polymer Science and Engineering in Nanjing University in 2010. After postdoctoral training in National University of Singapore, he joined Nankai University, where he is currently a professor in State Key Laboratory of Medicinal Chemical Biology, Key Laboratory of Bioactive Materials, Ministry of Education, and College of Life Sciences. He also conducted his work in The Hong Kong University of Science and Technology as a visiting scholar. His current research focuses on the design and synthesis of smart/functional molecular imaging probes and exploration of their biomedical applications.

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