Review

Mona Teng, Ziting Judy Xia, Nicholas Lo, Kashif Daud and Housheng Hansen He*

Assembling the RNA therapeutics toolbox

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Abstract: From the approval of COVID-19 mRNA vaccines to the 2023 Nobel Prize awarded for nucleoside base modifications, RNA therapeutics have entered the spotlight and are transforming drug development. While the term "RNA therapeutics" has been used in various contexts, this review focuses on treatments that utilize RNA as a component or target RNA for therapeutic effects. We summarize the latest advances in RNA-targeting tools and RNA-based technologies, including but not limited to mRNA, antisense oligos, siRNAs, small molecules and RNA editors. We focus on the mechanisms of current FDA-approved therapeutics but also provide a discussion on the upcoming workforces. The clinical utility of RNA-based therapeutics is enabled not only by the advances in RNA technologies but in conjunction with the significant improvements in chemical modifications and delivery platforms, which are also briefly discussed in the review. We summarize the latest RNA therapeutics based on their mechanisms and therapeutic effects, which include expressing proteins for vaccination and protein replacement therapies, degrading deleterious RNA, modulating transcription and translation efficiency, targeting noncoding RNAs, binding and modulating protein activity and editing RNA sequences and modifications. This review emphasizes the concept of an RNA therapeutic toolbox, pinpointing the readers to all the tools available for their desired research and clinical goals. As the field advances, the catalog of RNA therapeutic tools continues to grow, further allowing researchers to combine appropriate RNA technologies with suitable chemical modifications and delivery platforms to develop therapeutics tailored to their specific clinical challenges.

Keywords: RNA therapeutics; delivery of nucleic acids; chemical modifications

Introduction

Looking back at history, RNA therapeutics has been rapidly advancing since the initial key discoveries, for example, the discovery of mRNA in the 1960s [1]. In the following decades, additional milestones were made on mRNA, including the discovery of the 5′-cap, the first delivery to cells with liposomes, the development of *in vitro* mRNA synthesis platforms as well as the first cationic lipid-mediated mRNA delivery [2–6]. In the late 1990s, RNA interference (RNAi) was discovered and the first antisense oligo (ASO) drug was approved for cytomegalovirus (CMV) retinitis [7, 8]. During the 2000s, the importance of pseudouridine modification on mRNA was recognized and the first human trial of an mRNA vaccine against melanoma was initiated [9, 10]. In 2018, the first short interfering RNA (siRNA) drug patisiran was approved, showcasing the rapid therapeutic development in a short span of 20 years given that siRNA was initially discovered in 1998 [11]. In 2020, two COVID-19 mRNA vaccines were approved for emergency use and administered to millions of individuals worldwide, marking a historical milestone as the first widely-used RNA therapeutics [12, 13].

The development of RNA technologies stems from the basic understanding of how cellular RNA functions, for example, how endogenous mRNA is translated into proteins by cellular machinery and how siRNA and microRNA (miRNA) mediate gene silencing. In addition, understanding how endogenous RNAs are dysregulated in disease context can reveal novel therapeutic targets. This review will first introduce the types and functions of naturally occurring RNAs in cells and how they can be leveraged for therapeutic development. Following that, different types of RNA therapeutic agents will be summarized along with short briefings on the advances in medicinal chemistry and delivery strategies, although the latter is not the main focus of this review. Together, the RNA therapeutics toolbox is assembled and continues to expand for diverse cellular applications.

***Corresponding author: Housheng Hansen He**, Department of Medical Biophysics, Temerty Faculty of Medicine, University of Toronto, Toronto M2J 4A6, ON, Canada; and Princess Margaret Cancer Centre, University Health Network, Toronto M2J 4A6, ON, Canada, E-mail: hansenhe@uhnresearch.ca. https://orcid.org/0000-0003-2898-3363
Mona Teng, Department of Medical Biophysics, Temerty Faculty of Medicine, University of Toronto, Toronto, ON, Canada; and Princess Margaret Cancer Centre, University Health Network, Toronto, ON, Canada
Ziting Judy Xia, Nicholas Lo and Kashif Daud, Princess Margaret Cancer Centre, University Health Network, Toronto, ON, Canada

Types of naturally occurring RNAs

The repository of naturally occurring RNA in biological systems is highly versatile and covers a wide range of cellular functions. In general, cellular RNA can be grouped into two categories based on their coding potential: (1) messenger RNA (mRNA) that acts as templates for protein translation and (2) noncoding RNA (ncRNA) that does not associate with ribosomes or encode for proteins. While it is estimated that over 75 % of the human genome can be transcribed, only around 1 % of it encodes proteins and the majority are transcribed into ncRNA [14, 15]. ncRNA can be broadly categorized into three classes: (1) infrastructural or "housekeeping" RNA like ribosomal RNA (rRNA), transfer RNA (tRNA), spliceosomal RNA and small nucleolar RNA (snoRNA), (2) small regulatory RNA like siRNA, miRNA and small PIWI-interacting RNA (piRNA), and lastly (3) long noncoding RNAs (lncRNAs) that are typically longer than 200 nucleotides [16].

Coding RNA – mRNA

The biological significance of mRNA has been long recognized since the first discovery that they serve as templates for protein translation. Pre-mRNA is typically transcribed by RNA polymerase II [17]. The maturation of pre-mRNA can occur co- or post-transcriptionally, which typically includes intron excision, exon ligations, 7-methylguanosine (m7G) capping to the 5′ end, and formation of a 3′ end by cleavage and addition of a poly(A) tail [18]. Transcriptome-wide analyses revealed that more than 90 % of genes can undergo alternative splicing events and incorporate different combinations of exons or intron retention, leading to different coding sequences and downstream protein sequences [19–21]. Most mRNA undergoes the canonical translation initiation in the cytoplasm, which involves the binding of the ribosomal preinitiation complex to the 5′ cap [22]. The transcription, processing, and translation of mRNA are tightly regulated by multiple quality control mechanisms, which may be dysregulated in diseases, leading to mRNA abnormality that can occur at any stage of mRNA biogenesis.

Noncoding RNA – infrastructural RNA

Infrastructural RNAs like rRNA, tRNA, and spliceosomal RNA form machinery crucial for protein synthesis and life. rRNA represents the most abundant class of RNA, accounting for 80–90 % of total RNA by mass inside the cell [23, 24]. It is produced from ribosomal DNA clusters organized in the nucleolus and is transcribed by RNA polymerase I. tRNA makes up around 10–15 % of total RNA by mass and is transcribed by RNA polymerase III (Pol III) [24, 25]. It is an adapter molecule that links a specific codon in the mRNA with its corresponding amino acid. tRNA displays a highly conserved "cloverleaf" secondary structure and represents the most extensively modified RNA in cells, with about 10–20 % of residues being modified [26–28]. Other infrastructural "housekeeping" RNAs include spliceosomal small nuclear RNAs (snRNAs), which are about 150 nucleotides long on average. They are assembled with proteins to form the spliceosomes and allow for catalysis in the splicing reaction [29]. snoRNAs are widely present in the nucleoli of eukaryotic cells and are reported to play a role in rRNA, tRNA, and mRNA modifications [30].

Small noncoding RNA

miRNA and siRNA

miRNA and siRNA are around 20–35 nucleotides long and represent one of the most studied ncRNA classes due to their critical regulations on gene expression, mostly gene silencing. miRNAs are endogenous and expressed from an organism's genome, whereas siRNAs are typically thought to be primarily exogenous in origin, derived from viruses, transposons, or transgene triggers [31]. miRNAs are processed from stem-loop precursors with incomplete double-stranded regions, while siRNAs are excised from long, fully double-stranded RNAs [32]. Despite some differences, they both associate with Argonaute proteins, especially Argonaute 2 (AGO2), and form core components of the RNA-induced silencing complex (RISC) [31–33]. Once associated with RISC, the sense strand (or passenger strand) is cleaved by AGO2 while the antisense strand (or guide strand) remains in the complex to guide RNA target selection [34, 35]. The regulatory effect following target binding depends on the degree of sequence complementarity. siRNA typically directs RISC to perfectly complementary targets and induces target degradation [36]. miRNA often binds to the 3′ untranslated region (UTR) of mRNA with some mismatches and bulges. miRNA nucleotides 2–8 represent the seed region, which is the key region for target base pairing and recognition [31]. In general, a perfect complementarity between miRNA and target would induce target cleavage, whereas the presence of mismatches would promote repression of mRNA translation. Given their critical gene silencing functions, the potential of utilizing endogenous or engineered miRNA and siRNA to modify gene expression is

quickly recognized and they are heavily investigated as tools for RNA therapeutics. The landscape of miRNA and siRNA is frequently altered during disease progression, marking them as potential therapeutic targets.

piRNA

piRNA is an animal-specific class of small ncRNA with a length of 21–35 nucleotides that primarily silence transposable elements, especially in the germline, regulate gene expression, and fight against viral infection [37]. Over 90 % of the primary piRNAs are single-stranded and are transcribed by Pol II from piRNA-encoding genes located on specific genomic loci called "Piwi clusters" [38]. Modified and processed piRNAs are loaded into the PIWI-clade Argonautes (PIWI proteins), which then guide the complex to the nascent target transcript and induce target degradation or heterochromatin formation at the gene locus, thereby suppressing the target gene expression [39, 40].

Long noncoding RNA (lncRNA)

lncRNAs are typically longer than 200 nucleotides to differentiate from small ncRNAs like miRNA and tRNA. They are mainly transcribed by RNA pol II and can be spliced and polyadenylated similar to mRNA transcripts, although not required [16], display tissue-specific expression patterns and are dynamically regulated during development and disease progression. lncRNA has been suspected to be transcriptional noise due to their generally low expressions, low levels of sequence conservation compared to protein-coding genes, and low visibility in genetic screens. However, a couple of lncRNAs have been reported with conserved functions, such as H19 and XIST, essential for developmental processes [41–43]. Characterized examples of lncRNA have demonstrated that their functions include but are not limited to regulating genome organization, the assembly of membraneless organelles, and mRNA stability and translation, through general mechanisms of RNA-RNA, RNA-DNA, and RNA-protein interactions [16, 44–46]. lncRNA has been gaining interest as therapeutic targets given their gene expression regulations, functional versatility and dysregulation in diseases.

Circular RNA (circRNA)

circRNA is a class of lncRNA with a covalently enclosed loop structure, which confers higher stability than linear RNA and intrinsic resistance to exonuclease degradation [47–49]. They are produced by a non-canonical splicing event called backsplicing, in which a downstream splice site is linked to an upstream splice site [50]. High-throughput total RNA-seq has revealed that thousands of circRNAs exist in eukaryotic cells with tissue-specific expression patterns, including in fungi, plants, worms, fish, insects, and mammals [51–55]. circRNA can exert diverse regulations in biological processes through mechanisms of RNA-RNA, RNA-protein, and RNA-DNA interactions, similar to other lncRNAs. Although the majority of circRNAs are noncoding, a small fraction of them may encode for peptides through the presence of internal ribosomal entry site (IRES) and, in some cases, N6-methyladenosine (m^6A) dependent protein translation [56–59].

Enhancer RNA (eRNA)

eRNAs are transcribed from gene enhancers, which are *cis*-acting regulatory DNA elements that work in tandem with a given promoter to regulate the transcription of the downstream genes [60–63]. Global profiling and annotation estimated 40,000–65,000 eRNAs expressed in human cells [64–67]. Evidence suggests that eRNA can be functional and contribute to gene control by altering the chromatin environment and by interacting with transcriptional regulators [61].

RNA therapeutics toolbox

RNA therapeutics offer several advantages over other types of therapeutics, such as small molecules or antibodies. Small molecules are traditionally developed to bind structured regions of a protein, which may not be applicable to all proteins due to the presence of intrinsically "disordered" domains that lack definitive structure. In fact, it is estimated that only about 3,000 out of the 20,000 human proteins are "druggable" and approved drugs target only about 700 proteins so far [68, 69]. Antibodies are effective against cell-surface or circulating proteins but cannot steadily access cytoplasmic targets. In addition, many ncRNAs have been reported to be significantly altered in diseases and can drive disease progression. The existence of "undruggable" proteins and ncRNA targets emphasizes the need for alternative avenues and targeting RNA shows great promise for its diverse potential. In comparison to small molecules and antibodies, RNA-based therapeutic agents like ASO, siRNA and mRNA can be manufactured quickly at a large scale. The incredible speed of production is demonstrated by the COVID-19 mRNA vaccines, which were developed a few months after the start of the pandemic. Another advantage of RNA-based therapeutics is that they represent a

streamlined platform and can be quickly modified to target different RNAs or express different proteins. In addition, RNA tools are highly engineerable and can be designed to mediate different effects tailoring to various purposes. For example, ASOs can be engineered to induce target RNA cleavage as well as splicing modulation. In the following sections, RNA-targeting tools and RNA-based therapeutics will be discussed in detail based on their mechanisms of action and therapeutic effects, covering both FDA-approved therapeutics as well as upcoming technologies with clinical potentials.

Templates for protein expression

The feasibility of using *in vitro* transcribed (IVT) mRNA as templates for protein production was first demonstrated in 1990 by directly injecting naked mRNA encoding for chloramphenicol acetyl-transferase, luciferase and β-galactosidase in the mouse skeletal muscle [70]. Since the initial success, IVT mRNA quickly gained interest for various applications, especially for protein replacement for diseases caused by endogenous protein dysregulation and for expressing antigens for cancer and infectious disease vaccines. IVT mRNA typically mimics the endogenous mRNA with 5 sections: 5′ cap, 5′ UTR, an open reading frame that encodes the protein of interest, 3′ UTR and poly(A) tail. With advances in RNA synthesis and delivery technology, it becomes clear that mRNA has the potential to be engineered to encode for virtually any peptides or proteins in a time-efficient fashion.

Antigen expression for vaccination against infectious disease and cancer

In 2020, the Food and Drug Administration (FDA) approved the very first mRNA vaccines delivered by lipid nanoparticle (LNP) for providing immunity against the SARS-CoV-2 virus that initiated the global COVID-19 pandemic, BNT162b2 mRNA produced by Pfizer and BioNTech, and mRNA-1273 produced by Moderna (Table 1) (Figure 1) [12, 13]. Both mRNAs encode the SARS-CoV-2 spike protein, which mediates receptor binding and fusion of the viral and cellular membranes, and mutations are introduced to the coding sequence to stabilize the protein in the prefusion conformation [12, 13, 71]. mRNA vaccines have several advantages over traditional vaccines using live and attenuated pathogens, protein subunits or DNA: (1) mRNA does not integrate with the host DNA and is non-infectious, (2) the production of mRNA can be rapid, scalable and cost-effective and (3) mRNA can be engineered to encode for multiple antigens or variants of antigens to strengthen immune response [72, 73]. Since 2020, the formulations for the COVID-19 vaccines were quickly updated to protect against variants of the virus such as BA.4/5 and XBB, highlighting the feasibility of mRNA vaccines to be modified to closely follow virus evolution [74, 75]. To note, the versatility of mRNA vaccines was further demonstrated through the release of bivalent COVID-19 vaccines containing mRNAs encoding ancestral SARS-CoV-2 and variant spike proteins [76, 77]. Following the success of COVID-19 mRNA vaccines, mRNA vaccines against other infectious viruses, including ZIKA, cytomegalovirus (CMV), influenza, human metapneumovirus (hMPV), respiratory syncytial virus (RSV), Epstein-Barr, chikungunya, Nipah and rabies, are undergoing clinical trials.

In addition to infectious diseases, mRNA vaccines are being developed against cancer with the goal of activating endogenous immune response to eradicate cancer cells. The tumor antigen selection is a key step to effective vaccine design. Tumor antigens can be categorized into tumor-associated self antigens (TAAs) which are overexpressed in tumor cells but can also be present in normal tissues, and tumor- specific antigens (TSAs) which are neoantigens derived from mutations in tumor cells [78–80]. The delivery strategy for mRNA cancer vaccines can be cellular-based, such as through engineered dendritic cells, or through synthetic vectors such as LNP [78]. For example, dendritic cells transfected with mRNA encoding for antigens WT1, PRAME, and CMVpp65 are undergoing clinical trials for eliminating residual acute myeloid leukemia cells post-remission, and treatments showed good toleration by the patients (NCT02405338) [81, 82]. mRNA-4157 is an example of an mRNA cancer vaccine delivered by LNP for treating high-risk melanoma patients who have undergone surgery in combination with the anti-PD-1 drug pembrolizumab (NCT03897881). It is personalized to encode up to 34 different patient-specific neoantigens, demonstrating the feasibility of utilizing mRNA for personalized medicine, which may be technically challenging for traditional therapies [83]. In addition to expressing antigens, other immune activation strategies include delivering mRNA encoding inflammatory cytokines as well as antibodies [84–86].

Protein replacement therapies

The main goal of protein replacement therapy is to substitute or replenish specific protein deficiencies that result either from the protein being absent or non-functional due to mutations in affected patients. Gene or protein supplementation could be achieved in different ways including delivering DNA, editing the defective DNA sequence, delivering mRNA as well as directly delivering proteins. mRNA arises as an attractive candidate over proteins given the

Table 1: Current FDA-approved RNA therapeutics.

Therapeutics	Approval year	Disease (cellular target)	Type	Mechanism of action	Key modifications	Delivery strategy
Formivirsen	1998	CMV retinitis	ASO	RNA degradation	PS	Intravitreal
Pegaptanib	2004	Macular degeneration (VEGF)	Aptamer	VEGF antagonist	2′-OMe, 2′-F	Intravitreal, PEG conjugated
Mipomersen	2013	Familial hypercholesterolemia (ApoB)	ASO	RNA degradation	2′-MOE, PS	Subcutaneous
Nusinersen	2016	Spinal muscular atrophy (SMN2)	ASO	Splice switching	2′-MOE, PS	Intrathecal
Eteplirsen	2016	DMD (dystrophin exon 51)	ASO	Splice switching	PMO	Intravenous infusion
Patisiran	2018	hATTR amyloidosis (TTR)	siRNA	RNA degradation	2′-OMe, dT	Intravenous, LNP packaged
Inotersen	2018	hATTR amyloidosis (TTR)	ASO	RNA degradation	2′-MOE	Subcutaneous
Milasen	2018	Ceroid lipofuscinosis 7 (MFSD8)	ASO	Splice switching	PS, 2′-OMe	Intrathecal
Givosiran	2019	Acute hepatic porphyria (amino-levulinate synthase)	siRNA	RNA degradation	2′-OMe, 2′-deoxy-2′-F, PS	Subcutaneous, GalNAc conjugated
Golodirsen	2019	DMD (dystrophin exon 53)	ASO	Splice switching	PMO	Intravenous infusion
Lumasiran	2020	Primary hyperoxaluria type 1 (HAO1)	siRNA	RNA degradation	2′-OMe, 2′-deoxy-2′-F, PS	Subcutaneous, GalNAc conjugated
Inclisiran	2020	Hypercholesterolemia (PCSK9)	siRNA	RNA degradation	2′-OMe, 2′-deoxy-2′-F, PS	Subcutaneous, GalNAc conjugated
Viltolarsen	2020	DMD (dystrophin exon 53)	ASO	Splice switching	PMO	Intravenous infusion
Risdiplam	2020	Spinal muscular atrophy (SMN2)	Small molecule	Splice switching	NA	Oral
BNT162b2	2020	COVID	mRNA	Antigen expression	$m^1\Psi$	Intramuscular, LNP packaged
mRNA-1273	2020	COVID	mRNA	Antigen expression	$m^1\Psi$	Intramuscular, LNP packaged
Casimersen	2021	DMD (dystrophin exon 45)	ASO	Splice switching	PMO	Intravenous infusion
Vutrisiran	2022	hATTR amyloidosis (TTR)	siRNA	RNA degradation	2′-OMe, 2′-deoxy-2′-F, PS	Subcutaneous, GalNAc conjugated
Avacincaptad pegol	2023	Geographic atrophy (C5)	Aptamer	C5 antagonist	2′-F, 2′-OMe	Intravitreal, PEG conjugated
Tofersen	2023	Amyotrophic lateral sclerosis (SOD1)	ASO	RNA degradation	2′-MOE, PS	Intrathecal
Nedosiran	2023	Primary hyperoxaluria type 1 (LDH)	siRNA	RNA degradation	2′-Deoxy-2′-F, 2′-OMe, PS	Subcutaneous, GalNAc conjugated
Eplontersen	2023	hATTR amyloidosis (TTR)	ASO	RNA degradation	2′-MOE, PS	Subcutaneous, GalNAc conjugated

Note: CMV, cytomegalovirus; PEG, polyethylene glycol; VEGF, vascular endothelial growth factor; 2′-OMe, 2′-O-methyl; 2′-F, 2′-fluoro; 2′-MOE, 2′-methoxyethyl; PS, phosphorothioate; hATTR, hereditary transthyretin-mediated; LNP, lipid nanoparticle; PMO, phosphorodiamidate morpholino oligonucleotide; dT, 2′-deoxythymidine; DMD, Duchenne muscular dystrophy; $m^1\Psi$, 1-methyl-3′-pseudouridylyl; LDH, lactate dehydrogenase; GalNAc, N-acetylgalactosamine; ApoB, apolipoprotein B; SMN2, survival motor neuron 2; MFSD8, major facilitator superfamily domain containing 8; HAO1, hydroxyacid oxidase 1; PCSK9, proprotein convertase subtilisin/kexin type 9; LDH, lactate dehydrogenase; SOD1, superoxide dismutase 1; NA, not applicable.

short half-life of administered proteins and the ineligibility to substitute intracellular proteins such as transcription factors [87]. Compared to DNA delivery or gene editing, mRNA is generally considered safer due to its non-integrative nature and does not induce changes to the genome. mRNA-based replacement therapy has been extensively explored in various fields, including hematologic diseases, metabolic diseases, cancer, cardiac diseases, lung diseases, orthopedic diseases, and many more [88–92]. For example, AZD8601 is an mRNA encoding vascular endothelial growth factor A (VEGF-A) formulated in biocompatible citrate-buffered saline and is undergoing clinical trials to improve angiogenesis in patients undergoing coronary artery bypass grafting (NCT03370887) [93, 94].

Templates for CRISPR-Cas9 effector

CRISPR/Cas9-mediated genome editing has become a heavily-investigated approach to treat genetic diseases as it can correct DNA sequences and provide long-lasting therapeutic benefits after a single treatment. Since genome editing occurs at the DNA level, it is out of the scope of this review. However, it is worth noting that delivering mRNA

encoding Cas9 effector has been extensively studied as an alternative to delivering Cas9 protein or DNA plasmids [95–98]. For example, NTLA-2001 is an *in vivo* gene editing agent composed of LNP encapsulating Cas9 mRNA and sgRNA targeting transthyretin (TTR) for the treatment of transthyretin amyloidosis [99]. It is undergoing phase I clinical trials, and TTR protein reduction was observed in patients (NCT04601051). Very recently, in November and December of 2023, the first CRISPR-based gene editing was approved for treating sickle cell disease in the UK and USA, marking a milestone [100]. As the technology continues to be approved and expanded, delivering Cas9 mRNA and guide RNA would be promising alternatives to the current Cas9 protein delivery approach.

Upcoming templates for protein expression

Prolonged RNA half-life and protein translation are often desired to increase vaccine immunogenicity and the duration of therapeutic effect while lowering the RNA amount required per dose. Several modified forms of translation templates are under investigation, including **self-amplifying RNA** (saRNA), **circRNA** and **trans-amplifying RNA** (taRNA). saRNA originates from alphavirus structures and is engineered by replacing the gene sequence coding for virus structural proteins with the gene of interest [101–103]. RNA from alphaviruses contains sequences coding for nonstructural proteins (nsP1-4) that function as replicases through RNA-dependent RNA synthesis [101–103]. Using the COVID-19 vaccine as an example, saRNA encoding the spike protein showed elicited a high immune response and may induce a higher neutralizing antibody titer than traditional mRNA [104–106]. However, one limitation is that saRNA is generally longer than traditional mRNA, which challenges its production and delivery. To overcome this challenge, trans-amplifying RNA (taRNA) has been developed, which involves a second mRNA encoding the alphaviral replicase. Preclinical studies of taRNA vaccines encoding virus antigens showed high immunogenicity and good antibody titer, demonstrating potential clinical utility [107–109]. circRNA as protein templates is also gaining interest due to their longer half-life than linear mRNA. Despite the lack of a 5′ cap, circRNA can be engineered to efficiently express proteins through IRES-dependent translation mechanisms [110–112]. In preclinical models, circRNA vaccines can be effectively translated into antigens and can elicit immune responses more potent and long-lasting than linear mRNA [113, 114]. These newer forms of translation templates hold great promise for bringing prolonged therapeutic effects for antigen expression and protein replacements.

Splicing regulators

RNA mis-splicing underlies numerous human diseases and may result from mutations or dysregulation in DNA sequences, RNA *cis*-regulatory elements, and spliceosomal factors [115]. **ASOs** are single-stranded synthetic nucleic acids (typically DNA) that are 8–50 nucleotides long and are designed to selectively base-pair to RNA of interest [116, 117]. For splicing modulation, ASOs (also referred to as splice-switching oligonucleotides [SSOs]) can be designed to promote both exon exclusion and inclusion by binding to intron-exon junctions or sequences involved in splicing (Figure 1) [116, 117]. Several ASOs are approved by the FDA to modulate RNA splicing in diseases like spinal muscular atrophy (SMA), Duchenne muscular dystrophy (DMD), and Batten disease (Table 1) [118–122]. For example, nusinersen is the first splicing-correcting ASO approved for SMA treatment, which is a neuromuscular disorder caused by loss of survival motor neuron (SMN) proteins. It promotes exon 7 inclusion of SMN2 mRNA and increases the expression of SMN protein by binding to the intronic splicing inhibitor in intron 7 (Figure 1) [118]. Recent studies demonstrated that ASOs can be personalized and designed to specifically correct splicing based on the patient's unique mutational profile, exemplified by Milasen, highlighting its potential in precision medicine [121].

Given that RNAs can adopt stable secondary and tertiary structures, **small molecules** targeting RNA have been investigated and they offer several advantages over RNA-based therapeutics including well-established delivery systems. The development of RNA-targeting small molecules has been challenging since only a few RNA structures have been solved and experimentally validated [123]. In recent years, several high throughput screening methods have been developed and identified thousands of compounds that can bind RNA [124]. Machine learning approaches are also being implemented to increase the efficiency of small molecule designs by predicting RNA structures as well as the binding affinity to RNAs [125, 126]. Risdiplam is the first FDA-approved small molecule that modifies *SMN2* mRNA splicing for the treatment of SMA, similar to nusinersen's mode of action as discussed above (Table 1) [127]. Some advantages of risdiplam over ASOs are the effective brain penetration and oral bioavailability. With advances in chemical synthesis, screening methods, and RNA structure availability, increasing small molecule candidates are being reported in preclinical studies for various disease models [123].

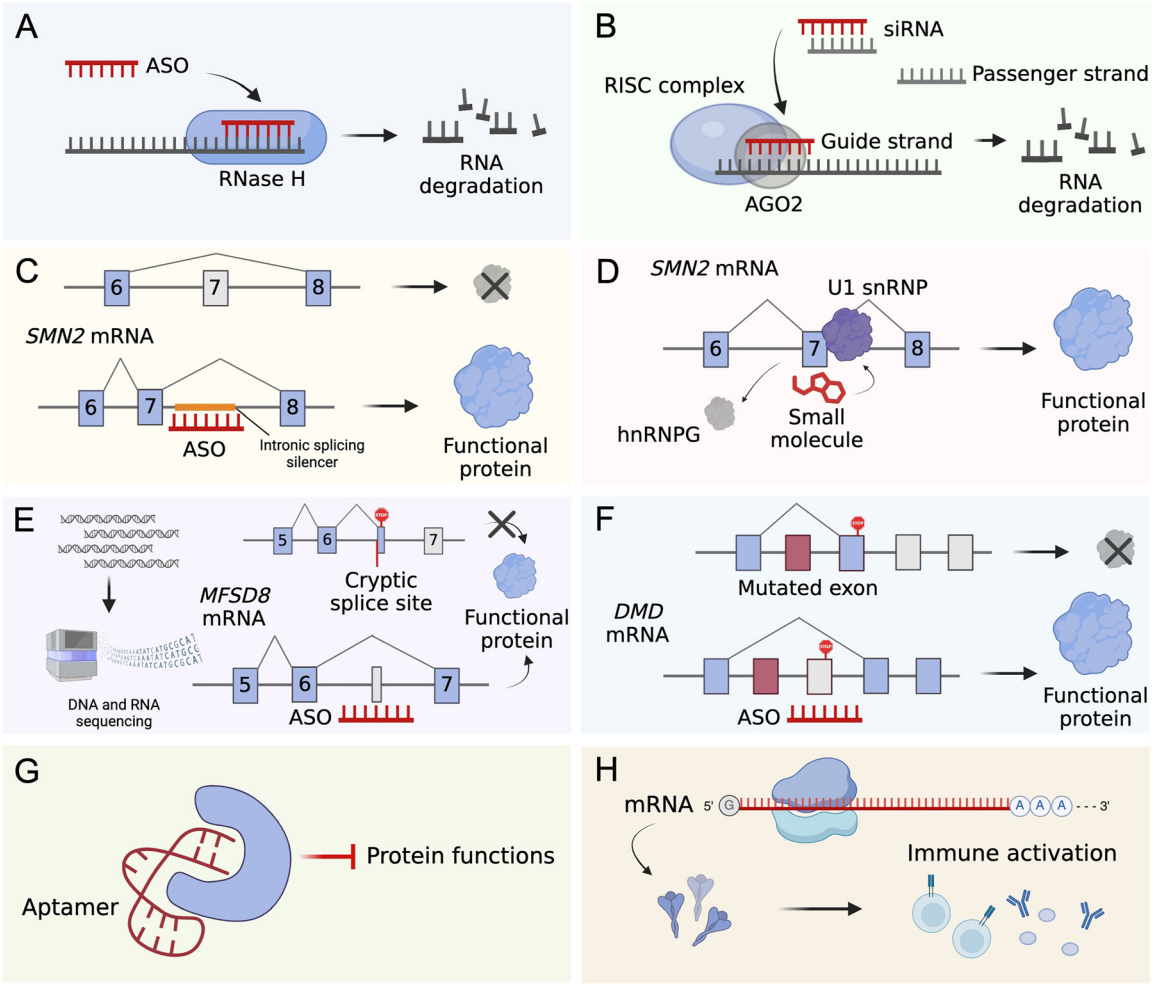

Figure 1: Mechanisms of action of FDA-approved RNA therapeutics. (A) ASO hybridizes to target RNA through sequence complementarity and recruits RNase H to mediate target degradation and sequential protein downregulation. Current FDA-approved degradation-inducing ASOs include formivirsen, mipomersen, inotersen, tofersen and eplontersen. (B) siRNA therapeutics are delivered as double-stranded molecules. The guide strand is loaded into the RISC complex and guides the complex to the RNA target for degradation. Current FDA-approved degradation-inducing siRNAs include patisiran, givosiran, lumasiran, inclisiran, vutrisiran and nedosiran. (C) Splice-switching ASO nusinersen binds to an intronic splicing silencer located in the intron of *SMN2* pre-mRNA and promotes exon 7 inclusion, which leads to functional protein production [230]. (D) Small molecule risdiplam binds to exon 7 of SMN2 pre-mRNA and mediates exon inclusion. The exact mechanism remains to be elucidated but it is proposed that risdiplam promotes the recruitment of U1 snRNP while displacing hnRNPG [231]. (E) Splice-switching ASO can be personalized based on a patient's specific mutational profiles, seen in the case of milasen. DNA and RNA sequencing of a patient revealed a cryptic splice site in the intron following exon 6 of *MFSD8* mRNA, leading to truncated transcript production. Milasen inhibits the splicing to the cryptic splice site and promotes functional exon 6-exon 7 splicing [121]. (F) Splice-switching ASO can bind to an exon of pre-mRNA and leads to the exclusion of that exon in the final mRNA, seen in the cases of eteplirsen, golodirsen, viltolarsen and casimersen. This promotes the generation of functional DMD proteins by skipping a mutated exon with premature stop codon or exon that induces frameshifts [119, 120, 122, 232]. (G) Aptamer folds into defined structures and suppresses protein functions, seen in the cases of pegaptanib and avacincaptad pegol. (H) *In vitro* transcribed mRNA encodes antigens and leads to downstream immune activation, seen in the cases of COVID-19 vaccines.

RNA degraders

Many human diseases are driven by the overexpression and the accumulation of unwanted proteins, such as oncogenes in cancer. Small molecules targeting proteins have been the mainstay of drug development; however, many proteins remain untargetable due to the lack of definitive structures. Degrading RNA is a promising alternative and can be applied to virtually any target in theory. ASOs and siRNAs are the main workforces currently approved by the FDA to degrade and downregulate RNA (Table 1) (Figure 1). The binding of **ASOs** to RNA forms an RNA-DNA hybrid that becomes a substrate for RNase H, especially RNase H1, which leads to RNA degradation [128, 129]. RNase H-recruiting ASOs can possess different chemical modifications compared to the splicing modulating ASOs, which will be discussed in detail

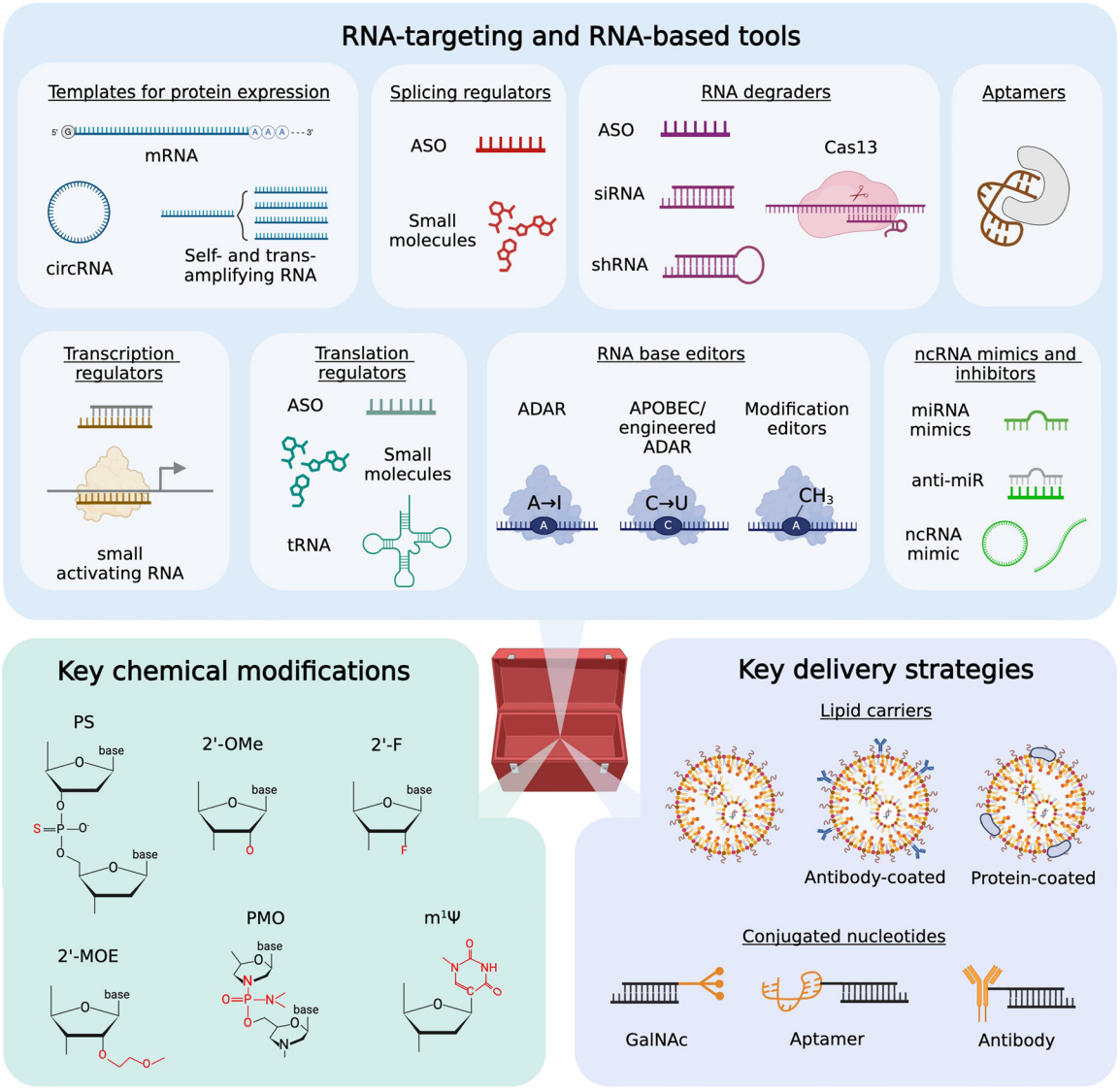

Figure 2: Overview of the RNA therapeutics toolbox. FDA-approved and upcoming tools are categorized based on their mechanisms of action and therapeutic effects. Examples of key chemical modifications and delivery strategies are also summarized, but these do not represent the full catalog of all existing ones.

in the chemical modifications section [117, 130, 131]. An example of an FDA-approved RNase H-recruiting ASO is mipomersen, which binds to mRNA encoding human apolipoprotein B (ApoB), leading to its degradation and thus a decrease in ApoB protein production (Table 1). The treatment preferentially reduces small low-density lipoprotein particle number in patients with hypercholesterolemia [132].

siRNA-mediated RNAi is another powerful tool to downregulate RNA and many are approved by the FDA. In contrast to single-stranded ASOs, siRNAs are double-stranded and trigger AGO2 and RISC-dependent RNA degradation. The selection of antisense or guide strand is essential to therapeutic efficacy and how to chemically engineer siRNAs to maximize the specific loading has been a subject of significant scientific inquiry, which will be discussed in later sections [131, 133]. The first siRNA therapeutics, patisiran, delivered by LNP, was approved by the FDA in 2018 to treat hereditary transthyretin-mediated (hATTR) amyloidosis by targeting the 3′ UTR of mRNA encoding both wild type and mutant transthyretin (TTR) [11, 134]. Other FDA-approved siRNAs, such as givosiran and inclisiran, are conjugated to N-acetylgalactosamine (GalNAc), which is a sugar molecule that can bind to the asialoglycoprotein receptor abundantly expressed on liver cells for liver-specific delivery [135, 136]. Due to the delivery limitations (discussed in sections below), the current FDA-approved

ASOs and siRNAs are targeting liver RNAs for liver-related diseases. However, numerous ASOs and siRNAs are under clinical trials for other diseases such as lymphoma, prostate cancer, pancreatic cancer, and many other cancers [137, 138]. As an example, siG12D-LODER is a biodegradable implant that releases siRNA against KRAS (G12D) for treating locally advanced pancreatic cancer in combination with chemotherapy [139]. KRAS is an example of oncogene with limited small molecule inhibitors, which highlights the need for RNAi therapeutics and its versatility to target the "undruggable" proteins.

Upcoming RNA degraders

Similar to the mechanisms of siRNA, short hairpin RNAs (**shRNAs**) can also be loaded into the RISC complex and undergo RNAi after being processed by Dicer. To further improve gene knockdown efficacy, bifunctional shRNAs have been developed that both degrade mRNA transcripts as well as inhibit mRNA translation in preclinical models [140]. Bifunctional shRNAs contain two stem-loop structures and get processed into siRNA-like (fully complementary to target) and miRNA-like (contains mismatches) effectors, which promote cleavage and translation inhibition, respectively [140]. pbi-shRNA™ EWS/FLI1 Type 1 LPX is an example of bifunctional shRNAs in clinical development for treating patients with advanced Ewing's sarcoma by targeting EWS-FLI1 fusion protein (NCT02736565) [141].

In addition to ASOs and RNAi-based degraders, **CRISPR-Cas13** systems have emerged as engineerable tools to target RNA for cleavage as well as for other purposes such as RNA editing. Several Cas13 variants have been reported, such as Cas13a, Cas13b, Cas13c, Cas13d, and Cas13X, which all involve an effector enzyme that cleaves RNA and a single-guide RNA (sgRNA) that guides the system to RNA of interest [142–144]. In cell line models, Cas13 systems have been reported with fewer off-targets compared to ASOs or RNAi, which could be advantageous [145]. Recent studies have demonstrated that Cas13 protein effector and sgRNA can be delivered in mice using adeno-associated viruses (AAV) as therapeutics to treat RNA viral infections and silence gene expressions in numerous disease models, indicating potential clinical utility [146–149].

Transcription and translation regulators

In some cases, modulating the transcription and translation efficiency of mRNA is desired without altering the copies of mRNA molecules itself. RNA oligonucleotides can be engineered to activate gene transcription and modulate translation efficiency, distinctive from the actions of the RNA degraders mentioned above. **Small activating RNAs** (also known as saRNAs, a separate identity from self-amplifying RNAs mentioned in previous sections) are about 20-nucleotide long double-stranded RNA that can target regions in the gene promoter and mediates RNA activation pathway (RNAa) to increase gene transcription through an AGO2-dependent mechanism [150–153]. Although no small activating RNAs have been approved by the FDA currently, many candidates are undergoing preclinical studies and early phases of clinical trials. For example, MTL-CEBPA is the first small activating RNA in clinical trials to upregulate the expression of CEBPA, a downregulated tumor suppressor, in patients with advanced liver cancer [154, 155].

RNA therapeutics can also be engineered to modulate translation efficiency to upregulate or downregulate protein expression. In preclinical studies, **ASOs** can inhibit protein translation through steric effects [156, 157]. On the other hand, ASOs can also increase protein production by blocking translation from undesired open reading frames and thus promoting production from the desired open reading frames [158–160]. **Small molecules** have also been identified in preclinical studies to modulate mRNA translation efficiency. For instance, synucleozid is a small molecule that binds to an iron-responsive element in the mRNA and inhibits the translation of α-synuclein that links to Parkinson's disease [161]. As the α-synuclein protein lacks a defined structure, this again highlights that targeting mRNA is a promising approach to address these "undruggable" proteins.

In recent years, synthetic **tRNAs** have gained interest for modulating translation. Nonsense mutations or DNA mutations that change a sense codon to a premature stop codon account for 11 % of human pathogenic mutations. Suppressor tRNAs have been engineered to recognize stop codons, leading to continued protein translation from transcripts with premature stop codons in cell models and preclinical animal models [162–164]. In theory, one suppressor tRNA could be applied to multiple diseases caused by nonsense mutations, signifying a wide range of potential applications.

RNA aptamers

RNA aptamers are single-stranded oligonucleotides (can also be DNA for DNA aptamers) with well-defined three dimensional structures that can directly bind to specific target molecules, such as proteins and biomolecules (Figure 1) [165, 166]. Given their abilities to bind targets with high affinity and specificity, RNA aptamers are also referred

to as nucleic acid antibodies. In comparison with traditional antibodies, RNA aptamers are advantageous in several aspects, including longer shelf life and ease of synthesis and modifications [167]. Pegaptanib is the first RNA aptamer approved by the FDA in 2004 for treating age-related macular degeneration (AMD) by blocking vascular endothelial growth factor (VEGF) binding to its receptor [168]. Recently in 2023, a second RNA aptamer, avacincaptad pegol, was approved by the FDA to inhibit complement C5 for treating geographic atrophy, an advanced form of AMD [169]. RNA aptamers are generated using systematic evolution of ligands by exponential enrichment (SELEX) to optimize for affinity and specificity [170]. In addition to the direct therapeutic effect, RNA aptamers can also serve as guides for site-specific drug delivery by directly conjugating to the drug molecules or by assembling onto the surface of nanocarriers [171].

Noncoding RNA mimics and inhibitors

miRNAs are key mediators of RNAi in cells and have the ability to regulate complex transcriptional networks. While there are no miRNA-based therapeutics approved by FDA currently, many mimics and inhibitors are in clinical development. miRNA mimics exploit the main advantage of endogenous miRNAs being able to target multiple mRNAs at once. TargomiR is an example of a miR-16 mimic in clinical development for treating patients with recurrent malignant pleural mesothelioma and non-small cell lung cancer (NCT02369198) [172]. To inhibit miRNA activity, single-stranded modified antisense nucleotides anti-miRs have been developed and numerous are undergoing clinical trials. RG-012 is an example of a complementary molecule that inhibits miR-21 from binding to other targets and was investigated for treating Alport syndrome (NCT03373786). Despite great therapeutic benefits in preclinical models, the development of RG-012 and many other miRNA-based therapeutics has been halted in clinics due to a lack of efficacy or adverse health effects, which will be discussed in later sections [173].

Several other noncoding RNA-based tools have been explored in the laboratory but have not yet been applied in the clinic. In addition to anti-miRs that specifically inhibit one miRNA, miRNA sponges have been engineered to prevent one or more miRNAs binding to other cellular targets, thus inhibiting miRNA activity [174, 175]. miRNA sponges are synthetic linear or circular transcripts with tandem binding sites to one or more miRNAs of interest and compete with other cellular mRNAs for miRNA binding [175]. In particular, circRNAs have the potential to be potent sponges, as demonstrated by ciRS-7, a natural circle containing more than 70 binding sites for miR-7 [176, 177]. lncRNAs and circRNAs have been reported to be extensively dysregulated in diseases compared to normal tissues and can drive pathogenesis. Although no therapeutics are currently in clinical trials to target or mimic lncRNAs, they can be novel targets given further understanding of their functions. lncRNAs have been explored as biomarkers and several are evaluated in clinical trials, for example, NCT03830619 for lung cancer and NCT04175691 for acute ischemic stroke [178, 179].

RNA base editors

RNA editing has emerged as a powerful tool to correct disease-causing mutations and to tackle situations where transient effect is desirable as opposed to permanent genome editing, such as acute pain, viral infection and inflammation. Although no editors have entered the clinical space, preclinical studies have demonstrated efficacy for various disease models mostly utilizing the endogenous effector enzyme adenosine deaminases (ADARs). ADAR represents a family of enzymes that deaminates double-stranded RNA and edits adenosine (A) to inosine (I), which is then functionally recognized as guanosine (G) by cellular machinery [180]. Notably, G-to-A mutations were estimated to account for 28 % of disease-causing mutations and, therefore, can be potentially corrected by ADAR-mediated RNA editing [181]. In addition to ADARs, APOBEC enzymes are another endogenous family of editors that mediate cytosine (C)-to-uracil (U) editing [182]. To recruit endogenous RNA editors to target RNA, a guide complementary to the target RNA must be delivered to the cells and its design is critical for target specificity and efficacy. The guide can be delivered as ASOs, gRNAs or DNA templates that are transcribed into gRNAs upon entry of the cell. In October 2023, Wave Life Sciences submitted the first RNA editing clinical trial application for WVE-006, which is a GalNAc-conjugated oligonucleotide that recruits endogenous ADAR to correct *SERPINA1* mutations for treating alpha-1 antitrypsin deficiency (AATD). RNA editors can also be exogenously delivered to cells which enable engineering of the proteins and the gRNA. Available engineered systems include: (1) covalently linking gRNA to the deamination effector, (2) fusing deaminase to an RNA-binding domain which binds to a corresponding RNA motif on the gRNA and (3) fusing deaminase to a catalytically inactive Cas13 protein [183]. ADAR deaminase domain has also been engineered to mediate C-to-U editing [184]. Besides sequence editing, tools are also available to edit RNA modifications, such as N6-methyladenosine (m6A), which contribute to the regulation of RNA stability, splicing, and translation [185].

Chemical modifications and delivery strategies

One of the biggest challenges to translate RNA therapeutics into the clinic is to effectively deliver the molecules to the target site. Oligonucleotides and mRNAs are typically large (mRNAs are about 300–1,500 kDa, double-stranded siRNAs are about 14 kDa, single-stranded ASOs are 4–10 kDa) and negatively charged, meaning that they do not readily pass through the plasma membrane [186, 187]. To achieve therapeutic effect, RNA therapeutics must resist nuclease degradation in the extracellular space, bypass renal clearance, evade sequestration by plasma proteins, traverse the plasma membrane, escape the endosomes, and avoid re-export by exocytosis [186, 188]. The therapeutics would need to overcome additional barriers such as the blood-brain barrier if the central nervous system is the desired target site.

One key approach to optimize the pharmacodynamics and pharmacokinetics of RNA therapeutics is to chemically modify the DNA and RNA molecules (Figure 2). While this review does not aim to cover the modifications in detail, several are worth mentioning given their incorporation in the FDA-approved therapeutics. The main goal of chemical modification is to increase the nuclease degradation resistance when delivered to the extracellular space while still retaining the affinity for complementary targets and preserving the mode of action. The exchange of phosphodiester (PO) to phosphorothioate (PS), the substitution of ribose 2′-O-position to 2′-O-methoxyethyl (MOE) and the development of phosphorodiamidate morpholino oligonucleotide (PMO) are examples of modifications incorporated into the FDA-approved therapeutics (Table 1) [131, 189]. It is worth noting that some modifications, such as MOE, at certain positions can affect RISC loading in the case of siRNAs and may not be suitable for RNA degradation-inducing therapeutics. Some common modifications adopted for siRNAs include 2′-O-methyl (OMe) and 2′-fluoro (F) nucleotides, which still enable RISC loading and target cleavage (Table 1) [131, 189]. On the other hand, target cleavage is not desired for splicing modulators like splice-switching ASOs. They commonly possess modifications such as MOE, PS, and PMO that confer high stability but do not trigger mRNA degradation [186, 190]. Similarly, aptamers are chemically modified to prevent rapid clearance *in vivo*. Spiegelmers are examples of modified aptamers composed of L-nucleotide chains as opposed to the natural D-nucleotides, which exhibit exceptional stability in serum [191]. While various chemical modifications are available, the optimal choice depends on the application and the desired mode of action, whether it is to trigger target degradation or act as steric blocks or aptamers. Therapeutic mRNAs face similar challenges, they are prone to nuclease degradation especially given their larger sizes and can lead to unwanted immunogenicity. One key solution is to incorporate pseudouridine (Ψ), which enhances RNA stability and decreases anti-RNA immune response given that it is an abundant modification found on naturally occurring cellular RNAs [192]. This finding was utilized in the COVID mRNA vaccine designs and awarded the Nobel Prize in 2023 given its significance [193–195]. A recent study indicates that pseudouridine in mRNA can cause +1 ribosomal frameshift in animals injected with BNT162b2 COVID mRNA vaccines, indicating potential off-target effects [196]. Although no adverse outcomes have been reported from mistranslation so far, the effect of chemical modifications requires further examination and optimization.

The current main delivery strategies for RNA therapeutics include: (1) delivering nucleic acids without carriers, (2) conjugating nucleic acids to biomolecules such as lipids, aptamers, antibodies and sugars, and (3) packaging into cellular carriers like dendritic cells, or synthetic carriers like LNPs and polymeric nanoparticles (Figure 2). The “optimal” delivery platform depends greatly on the type of nucleic acid payload and the target tissue. While small RNA therapeutics such as ASOs and siRNAs can be delivered without carriers, mRNAs require a vehicle for entry into a cell due to their large sizes. The delivery of naked nucleic acids is typically through local injection. For example, splicing switch ASO nusinersen for treating spinal muscular atrophy is delivered through intrathecal injection or direct injection into the fluid surrounding the spinal cord [118]. However, these injections are invasive and methods to deliver the therapeutics across the blood-brain barrier are in need. LNP is a key class of drug delivery system approved by the FDA for liver siRNA (patisiran) delivery and for COVID mRNA vaccine delivery [11–13]. It is advantageous over other delivery platforms due to its good tissue penetration, weak immunogenicity, and lower toxicity compared to some other polymeric materials [188, 197]. These LNPs contain variations of four basic components: (1) ionizable lipids, (2) cholesterol, (3) helper lipids and (4) poly(ethylene glycol) (PEG)-lipid. One limitation of the current LNP formulations is that they mainly accumulate in the liver. While it is acceptable for treating liver diseases and producing antigens or proteins that are missing in inherited metabolic and hematological disorders, effective LNP delivery to other tissues remains challenging but has shown good improvements in preclinical studies by modifying the formulation and coating LNPs with tissue-targeting macromolecules [188, 198–201]. Using lung tissue as an example, numerous efforts are spent on developing inhalable or nebulized LNPs to maximize

therapeutic concentration in the lungs while limiting systemic exposure [202]. To withstand the shear stress during nebulization, LNP formulation is being optimized by modifying lipid components and composition as well as incorporating additional elements such as DNA hydrogel [203–205]. In addition to LNPs, other synthetic carriers include polymeric nanoparticles, micelles, dendrimers, and liposomes [188, 197]. Altogether, synthetic carriers are continuing to be optimized for composition as well as synthesis methods [206–209]. Other RNA therapeutic delivery strategies include directly conjugating nucleic acids to tissue-targeting macromolecules, for example, GalNAc for FDA-approved liver targeting. Asialoglycoprotein receptor that GalNAc binds to is an ideal receptor for active targeting since it is specifically expressed in liver cells and can lead to rapid endocytosis of nucleic acids upon GalNAc binding [188]. Ongoing research has reported that ligands, in the forms of sugars, proteins, aptamers, lipids, and more, can increase delivery to other organs, such as the kidney, lung, and heart [210–212]. This review offers an overview of some of the common chemical modifications and delivery strategies, however, ongoing research and upcoming technologies extend far beyond the material covered here and will likely transform the clinical application of RNA therapeutics in coming years.

Challenges and future outlooks

While the success and promise of RNA therapeutics are evident, several challenges remain to be tackled. As discussed in the above section, tissue-specific and extrahepatic deliveries remain an obstacle but multiple upcoming strategies have reported good improvement in preclinical studies. Other delivery challenges related to LNP include inefficient cellular uptake and poor endosomal escape to release cargo, which are under extensive investigation and optimization [188, 213].

A concern regarding therapeutics like ASOs and siRNAs is the target specificity and the undesired off-target effects. To improve target specificity and effectively select target sequences, machine learning algorithms have been developed to predict silencing efficacy as well as off-target effects, which can account for siRNA sequence features, RNA target sequence features and the presence of chemical modifications [214–216]. It is worth noting that machine learning algorithms are also being applied to improve LNP formulations, RNA aptamer, and small molecule designs, and will be versatile tools for various aspects of RNA therapeutic development [217–219]. Chemical modification is another method to improve siRNA target specificity. Although siRNAs are designed fully complementary to their targets, they can exhibit miRNA-like activities and bind to undefined sites through the miRNA-like seed region. To tackle that, modifications such as 2′-OMe can be introduced in the siRNA seed region and weaken the interaction with undesired transcripts [220]. Off-target effects can also be mediated by loading the passenger strand into RISC, which can be limited by several strategies including introducing different chemical modifications to the passenger and guide strands and trimming or fragmenting the passenger strand [221–224].

Another challenge faced by RNA therapeutics, or therapeutics in general, is the translation of experimental agents into clinical practice. Many potential RNA therapeutics have demonstrated promising results in preclinical studies, however, failed to translate into the clinics due to severe toxicity and limited efficacy in humans. For example, miR-21 inhibitor RG-012 and miR-34a mimic MRX34 both demonstrated good efficacy in preclinical models but clinical trials were halted due to severe adverse events [173]. The toxicity in some cases can be associated with off-target effects of the oligonucleotides and undesired on-target effects in tissues other than the intended target site due to systemic administration [179]. The dosing of RNA therapeutics is another factor that needs to be taken into consideration, which can impact the off-target rates and affect endogenous RNAi machinery [225, 226]. Another major cause of toxicity is the undesired immunogenicity. As a viral defense mechanism, multiple extra- and intracellular receptors exist to recognize both single-stranded and double-stranded RNA, leading to downstream immune activation. As an example, anti-VEGFR1 siRNA bevasiranib can induce anti-angiogenic effects through direct TLR3 stimulation, resulting in its termination of clinical development (NCT00499590) [227]. As introduced in previous sections, chemical modifications on oligonucleotides can significantly reduce immunogenicity by limiting their interaction with DNA or RNA sensors [228, 229]. Other upcoming strategies include designing smaller RNA therapeutics, given that TLR activation requires a length of at least 21 nucleotides, and combining RNA therapeutics with other therapies to reduce the dosing requirement [179].

The development of RNA therapeutics is an interdisciplinary effort and depends on advances in RNA-targeting and RNA-based technologies, molecular understanding of the mechanism of action, chemical modifications, delivery platforms and immunology. In this review, we summarize the latest tools available for the RNA therapeutic toolbox and highlight the diverse applications ranging from antigen expression for vaccines to mimicking and targeting ncRNA (Figur 2). As the toolbox continues to expand, researchers can assemble the “optimal” therapeutics by selecting the

appropriate tools for the desired mode of action and combine with chemical modifications and delivery strategies for a specific tissue of target, tailored for a clinical application. With more than dozens of RNA therapeutics currently undergoing clinical trials, there is no doubt that we will continue to witness growth of the field and application in clinics in the upcoming years, bringing benefits to patients, especially those with dire need for novel therapeutics.

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