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Trends in Pharmacological Sciences

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Special Issue: Advances in Drug Delivery Systems

Review

Paving the Road for RNA Therapeutics

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Therapeutic RNA molecules possess high potential for treating medical conditions if they can successfully reach the target cell upon administration. However, unmodified RNA molecules are rapidly degraded and cleared from the circulation. In addition, their large size and negative charge complicates their passing through the cell membrane. The difficulty of RNA therapy, therefore, lies in the efficient intracellular delivery of intact RNA molecules to the tissue of interest without inducing adverse effects. Here, we outline the recent developments in therapeutic RNA delivery and discuss the wide potential in manipulating the function of cells with RNAs. The focus is not only on the variety of delivery strategies but also on the versatile nature of RNA and its wide applicability. This wide applicability is especially interesting when considering the modular nature of nucleic acids. An optimal delivery vehicle, therefore, can facilitate numerous clinical applications of RNA.

Highlights

RNA can manipulate targets that were previously undruggable by monoclonal antibodies (mAbs) or small molecules.

RNA therapy is a safer alternative to DNA therapy and is versatile as it can either increase or decrease gene expression in order to introduce new transcripts for protein replacement therapy and more.

Clinical translation of RNA therapeutics has accelerated in recent years.

Chemical manipulation of RNA molecules render them more stable and hence increase their potency and applicability.

Various carriers for the *in vivo* delivery of RNA molecules have been invented that include antibody–RNA conjugates, aptamer–RNA conjugates, lipid nanoparticles, and polymers.

Delivery to extrahepatic tissues might require targeting moieties.

Current Landscape of Biopharmaceutical Industry

For many years pharmaceutical companies have relied on small-molecule therapeutics to generate drugs. These small molecules bind to pockets of target proteins such as enzymes, receptors, or other proteins, mostly in an antagonistic way. By doing so they interfere with a certain biological process resulting in a therapeutic effect [1]. While small-molecule drugs possess certain favorable characteristics (such as ease of production, possibility of oral administration, favorable pharmacokinetics, and the ability to pass through the cell membrane), their potential is limited as they rely on the druggability of the target. Whether a biological target is druggable or not depends on a variety of factors such as the presence of suitable pockets in the protein structure in which small molecules can dock, a suitable size to accommodate binding, and the degree of polarity. Docking into a deep cavity is crucial in achieving sufficiently high binding affinities (generally with a K_D in the nanomolar range or better) [1]. It is estimated that out of the ~20 000 human proteins, only ~3000 are druggable [2]. As of 2017, approved drugs targeted only 667 human proteins [3]. This not only indicates that more drugs can be developed to reach the 3000 druggable targets, but also highlights that the vast majority of 20 000 human proteins remain undruggable.

To tap into this unexplored potential, we need to look beyond small-molecule drugs. Over the years, more complex, biological macromolecules such as monoclonal antibodies (mAbs) have entered the pharmaceutical arena. Major benefits of their use, compared with small-molecule drugs, include long half-life, ability to target a broader group of proteins (due to the vast mAb repertoire), ability to be engineered to widen their applicability and increase their specificity, and lower toxicity. Disadvantages of the more complex biological macromolecules include more complicated pharmacological profiles, higher cost of production, and limits in route of administration (mostly intravenous) [4].While small molecules still dominate the pharmaceutical market, biologics have started to gain a higher share in the last few years. In 2018, 17 of the 59 newly approved drugs were biologics [5,6]. Altogether, this shows that besides traditional small-molecule drugs, larger biomolecules are becoming increasingly important to treat diseases.

In line with the trend of developing more specific and efficacious medicines, a new therapeutic avenue is gaining momentum: that of nucleic-acid-based therapy. Examples of such therapeutic

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agents include the use of oligos [7], plasmid DNA [8], mRNA [9], **ribozymes** [10,11] (see Glossary) and RNAi-related nucleic acids such as miRNA [12], siRNA [13], and short hairpin RNA (shRNA) [14]. While the use of mAbs are limited to cell surface receptors or secreted proteins, nucleic acids can interfere with protein expression itself and therefore circumvent the druggability issue during drug development.

While clinical development of RNA therapeutics has faced decades of significant challenges in terms of potency and immunogenicity, in recent years, the field has gained some momentum with the recent approval of two siRNA-based drugs patisiran and givosiran [15,16] within a short period of time. This, combined with a well-filled clinical pipeline of mRNA therapeutics [17], shows the potential of clinical development of RNA therapeutics in the coming years. Because of the rapid developments in this field, this review focuses specifically on RNA therapeutics, its delivery, challenges, and finally, the outlook on clinical applications.

Types of RNA Therapeutics

RNA therapeutics span from antisense oligonucleotides (ASOs), siRNA, miRNA, mRNA, RNA aptamers, short activating RNA (saRNA), to single guide RNA (sgRNA) for CRISPR/Cas9 systems. Below, we discuss each of these classes of RNA therapeutic molecules.

ASOs

ASOs are short, single-stranded oligonucleotides that are complementary to a target mRNA to which they hybridize and thereby modulate protein expression. ASOs can be based on both DNA and RNA [18].While classical ASOs are DNA based and act by formation of DNA–RNA hybrids that serve as a substrate for RNase H, other ASO functionalities also exist and open the door for RNA-based ASOs. For example, ASOs can hybridize close to the start codon and sterically interfere with translation or hybridize to untranslated regions and sterically interfere with RNA-binding proteins (Figure 1). Besides inhibiting translation, ASOs can also increase translation efficiency of the target protein by sterically inhibiting the translation of an upstream open reading frame and can alter splicing by binding to splice sites or to exonic/intronic inclusion signals [19]. An example of an advanced stage RNA-ASO is a splice-modulating oligonucleotide, QR-110, that is currently being investigated in clinical trials for use against a severe type of inherited retinal dystrophy (Table 1) [20]. Furthermore, other types of RNA-ASOs are anti-miRs which bind directly to the mature strand of the target miRNA and block its functionality [21]. **Locked nucleic acids (LNAs)** enhance the functionality of anti-miRs by increasing their stability [22].

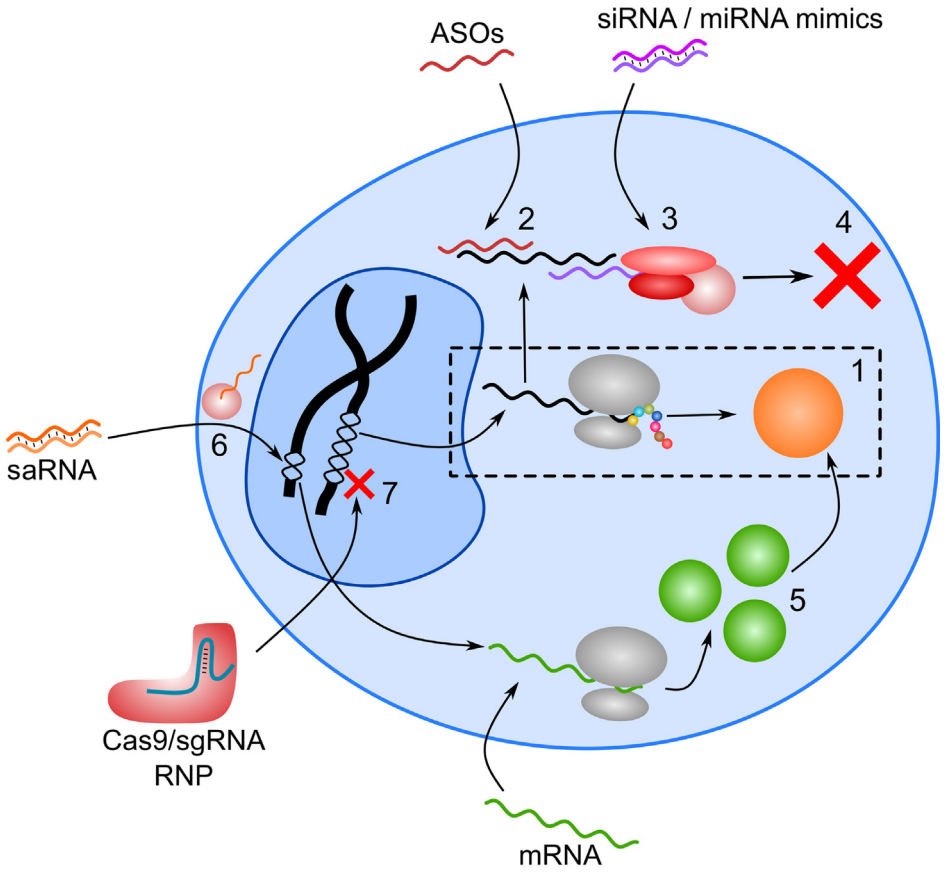
siRNA and miRNA

siRNAs are short (20–25 nucleotides), double-stranded RNA molecules that use the RNAi pathway to degrade a target mRNA in a sequence-specific manner. Upon delivery into the cytoplasm, argonaute (AGO)2 cleaves the passenger (sense) strand and the guide (antisense) strand of the siRNA is loaded into the RNA-induced silencing complex (RISC). The guide strand then guides the RISC to the target mRNA which is recognized and cleaved (Figure 1). The RISC and guide strand can be recycled and therefore one siRNA molecule can drive the cleavage of multiple mRNA molecules resulting in highly efficient gene silencing [23].

Another type of RNA that uses the RNAi pathway are miRNA mimics (Figure 1). miRNA mimics are synthetic, double-stranded RNAs that mimic a naturally occurring miRNA. These miRNA mimics can replenish altered miRNA functionality. For instance, in certain types of cancer, miRNAs that silence oncogenes are downregulated and miRNA mimics can give a therapeutic effect in such cases [22]. For examples of siRNA and miRNA therapeutics that are currently in advanced clinical trials for various disease indications, see Table 1.

Glossary

Asialoglycoprotein receptor (ASGR): a C-type lectin that is highly expressed on hepatocytes and removes desialylated glycoproteins from the circulation.
Endosomal escape: when a payload is internalized through endocytosis, it enters endocytic vesicles called endosomes. The payload needs to escape from the endosome in order to reach the cytosol, failure to escape results in transport to late endosomes and eventually lysosomes resulting in degradation of the payload.
Enhanced permeability and retention (EPR) effect: a theory by which high-molecular-weight nontargeted drugs accumulate in cancer tissues or tissues with inflammation due to hypervascularization and leaky vascular walls.
Locked nucleic acid (LNA): nucleic acid analog that possesses a 2'-O, 4'-C methylene bridge that reduces the flexibility of the pentose ring. LNAs have higher resistance against nucleases and altered hybridization properties.
Macropinocytosis: ingestion of extracellular liquid and dissolved molecules by cells.
Ribozymes: RNA enzymes; RNA molecules with catalytic activity.



LEGEND

RISC

AGO2

Target protein

Ribosome

Therapeutic protein

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mRNA

The concept of transfecting mRNA directly into cells instead of transfecting DNA has been around for a few decades [24,25]. mRNA is seen as a safer alternative to DNA [26] for therapeutic purposes such as protein replacement therapy, as it degrades quickly leading to no concerns about potential adverse effects from long-lasting expression or genomic integration. However, mRNA expression is more difficult to regulate than DNA expression, which remains a concern in mRNA therapy, as possible toxicity can arise from mRNA expression at off-target sites, leading to unwanted protein expression. For instance, unspecific expression of factor VIII, factor IX, and interleukin-12 has been shown to be dangerous [27]. Strategies that are commonly used for spatial control of DNA expression (such as use of transcription factors, inclusion of tissue specific promoters [28]) and for temporal control [such as the use of transcriptional on/off switches, e.g., tetracycline-responsive repressor (TetR) [29]] are not available for mRNA control. This, along with the unstable nature of mRNA, has delayed its potential for *in vivo* applications, and the field of mRNA therapeutics received a major boost only as advances in chemistry created more stable modified nucleotides (see later) and sophisticated regulation systems for mRNA expression were generated. mRNA therapeutics have found a role in protein replacement therapy [e.g., vascular endothelial growth factor (VEGF)-A delivery after myocardial infarction] [30], vaccines for infectious diseases (e.g., expression of viral antigens in dendritic cells) [31,32], or for *in vivo* production of mAbs [33]. For examples of mRNA therapeutics that are currently in advanced clinical trials for various disease indications, see Table 1.

RNA Aptamers

Aptamers are short single-stranded oligonucleotides that can consist of both DNA and RNA. Aptamers were first generated in 1990 using the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) selection method. Using SELEX, aptamers that selectively bind small molecular ligands or proteins with high affinity and high specificity are selected from a library [34,35]. To date, only one RNA aptamer has received FDA approval: pegabtanib, which is used for treatment of age-related macular degeneration (mechanism of action is the binding to the VEGF isoform 165) [36]. Several other aptamers are currently being investigated in clinical trials (Table 1). Besides the therapeutic potential of RNA aptamers, aptamers are also used solely as targeting moieties to aid delivery of other RNA payloads such as siRNA (see more discussion in the section 'Delivery of RNA Therapeutics').

saRNA

saRNAs are 21-nucleotide, double-stranded, noncoding RNA that possess two nucleotide overhangs on both ends [37] (Figure 1). saRNAs are initially loaded on the AGO2 protein where the passenger strand is cleaved. The saRNA–AGO2 complex then enters the nucleus and binds to promoter regions of genes to enhance transcription [38]. In a study by Zhao *et al.*, a combination of saRNA and siRNA was used to modulate the balance between the transcription factors CCAAT/enhancer-binding protein α and β (CEBPA, a tumor suppressor and CEBPB, an oncogene, respectively), where saRNA-driven activation of CEBPA decreased proliferation and migration in the differentiated hepatocellular carcinoma (HCC) cell lines [39]. saRNAs have recently

Figure 1. Overview of Different Mechanisms of Action of Different RNA Therapeutics. (1) Without therapeutic RNA molecules, the translation of a pathogenic protein proceeds without inhibition (shown in the broken line box). (2) ASOs hybridize to the target mRNA, while the (3) siRNA/miRNA mimics utilize the RISC in the RNAi pathway to (4) inhibit translation of target mRNA. (5) Overexpression of a therapeutic protein that counteracts the function of the pathogenic protein can be done by delivering the mRNA of the therapeutic protein. (6) saRNA can be delivered to the cell where it binds to AGO2, is imported to the nucleus, and in turn activates an endogenous gene. (7) A more permanent approach to remove the pathogenic protein is by gene knockout using Cas9 and sgRNA RNPs. Abbreviations: AGO2, argonaute 2; ASO, antisense oligonucleotide; RISC, RNA-induced silencing complex; RNP, ribonucleoprotein; saRNA, small activating RNA.

Table 1. Overview of Clinical Trials of Various RNA Therapeutics Discussed in This Review[a]

Candidate	Type of RNA	Vehicle	Route of administration	Biological target	Condition or disease	Clinical stage	Year (first posted)	Clinical trial number
Antisense oligonucleotides								
QR-010	ASO	NA	Inhalation	Cystic fibrosis transmembrane conductance regulator (ΔF508 mutation)	Cystic fibrosis	Phase Ib	2015	NCT02532764
QR-421a	ASO	NA	Intravitreal injection	Usherin (exon 13 of USH2A)	Retinitis pigmentosa	Phase I/II	2018	NCT03780257
QR-110	ASO	NA	Intravitreal injection	Centrosomal protein 290 (p.Cys998X mutation)	Leber's Congenital amaurosis	Phase II/III	2019	NCT03913143
QR-1123	ASO	NA	Intravitreal injection	Mutant P23H of the rhodopsin gene	Retinitis pigmentosa	Phase I/II	2019	NCT04123626
Candidate	**Type of RNA**	**Vehicle**	**Route of administration**	**Biological target**	**Condition or disease**	**Clinical stage**	**Year (first posted)**	**CT identifier**
siRNA and miRNA								
TD101	siRNA	NA	Intralesional injection	Keratin 6A	Pachyonychia congenita	Phase I	2008	NCT00716014
PF-04523655	siRNA	NA	Intravitreal injection	RTP801	Age-related macular degeneration	Phase II	2008	NCT00713518
					Choroidal neovascularization Diabetic retinopathy Diabetic macular edema	Phase II	2011	NCT01445899
ALN-RSV01	siRNA	NA	Inhalation of nebulized solution	Nucleocapsid protein of respiratory syncytial virus	Lung transplant patients infected with respiratory syncytial virus	Phase IIb	2010	NCT01065935
ALN-VSP02	siRNA	siRNA-LNP[b] (2 siRNAs)	i.v. infusion	Kinesin spindle protein and vascular endothelial growth factor	Solid tumor	Phase I	2009/2010	NCT01158079 NCT00882180
TKM-080301	siRNA	Stable nucleic acid–lipid particles (SNALPs)	i.v. infusion	Polo-like kinase 1	Neuroendocrine tumors Adrenocortical carcinoma	Phase I/II	2010	NCT01262235
					Primary or secondary liver cancer	Phase I	2011	NCT01437007
					Advanced hepatocellular carcinoma	Phase I/II	2014	NCT02191878
QPI-1007	siRNA	NA	Intravitreal injection	Caspase 2	Nonarteritic anterior ischemic optic neuropathy	Phase II/III	2015	NCT02341560
					Acute primary angle closure glaucoma	Phase II	2013	NCT01965106
ALN-PCS02	siRNA	siRNA–LNP	i.v. infusion	Proprotein convertase subtilisin/kexin type 9	Elevated LDL-cholesterol	Phase I	2011	NCT01437059

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Table 1. (continued)

Candidate	Type of RNA	Vehicle	Route of administration	Biological target	Condition or disease	Clinical stage	Year (first posted)	CT identifier
siG12D-LODER	siRNA	Miniature biodegradable polymeric matrix	Intratumoral using endoscopic ultrasound biopsy needle	KRAS	Pancreatic cancer	Phase II	2012	NCT01676259
DOPC-encapsulated siRNA	siRNA	Neutral liposome	i.v. infusion	EphA2	Advanced malignant solid neoplasm	Phase I	2012	NCT01591356
Atu027	siRNA	Liposomal siRNA	i.v. infusion	Protein kinase N3	Pancreatic cancer	Phase Ib/IIa	2013	NCT01808638
SYL040012	siRNA	NA	Ophthalmic administration	β2-Adrenergic receptor	Open angle glaucoma Ocular hypertension	Phase II	2014	NCT02250612
ND-L02-s0201	siRNA	LNP	i.v. infusion	Heat shock protein 47	Hepatic fibrosis	Phase I	2014	NCT02227459
					Idiopathic pulmonary fibrosis	Phase II	2018	NCT03538301
QPI-1002	siRNA	NA	i.v. injection	P53	Delayed graft function	Phase III	2015	NCT02610296
					Risk for AKI following cardiac surgery	Phase III	2018	NCT03510897
ALN-PCSSC (Inclisiran)	siRNA	GalNAc-siRNA	s.c. injection	Proprotein convertase subtilisin/kexin type 9	Elevated LDL-cholesterol	Phase I	2014	NCT02314442
					Atherosclerotic cardiovascular disease (or risk equivalents)	Phase II	2015	NCT02597127
					Renal impairment	Phase I	2017	NCT03159416
					High cardiovascular risk and elevated LDL-cholesterol	Phase II	2017	NCT03060577
					Atherosclerotic cardiovascular disease Elevated LDL-cholesterol	Phase III	2018	NCT03399370 NCT03400800
					Homozygous Familial Hypercholesterolemia	Phase III	2019	NCT03851705
					Atherosclerotic cardiovascular disease (or risk equivalents) Hypercholesterolemia Elevated LDL cholesterol	Phase III	2019	NCT03814187
SYL1001	siRNA	NA	Ophthalmic administration	Transient receptor potential cation channel subfamily V Member 1	Dry eye disease	Phase III	2017	NCT03108664
ALN-GO1 (Lumasiran)	siRNA	GalNAc-siRNA	s.c. injection	hydroxyacid oxidase 1	Primary hyperoxaluria type 1	Phase III	2018	NCT04152200 NCT03905694 NCT03681184

ALN-AT3 (Fitusiran)	siRNA	GalNAc-siRNA	s.c. injection	Antithrombin	Hemophilia	Phase IIIα	2018	NCT03549871 NCT03974113 NCT03754790 NCT03417102 NCT03417245
ALN-AAT02	siRNA	GalNAc-siRNA	s.c. injection	Mutant of α-1 antitrypsin	ZZ Type α-1 Antitrypsin deficiency liver disease	Phase I/II	2018	NCT03767829
Patisiran (label expansion studies)	siRNA	LNP	i.v. infusion	transthyretin	hATTR with cardiomyopathy hATTR progression post liver transplant Polyneuropathy	Phase III	2019	NCT03997383 NCT03862807 NCT02510261 NCT04201418 (Phase IV)
ALN-CC5 (Cemdisiran)	siRNA	GalNAc-siRNA	s.c. injection	Complement component C5	IgA nephropathy Berger disease	Phase II	2019	NCT03841448
ALN-AGT01	siRNA	GalNAc-siRNA	s.c. injection	Angiotensinogen	Hypertension	Phase I	2019	NCT03934307
ALN-TTRsc02 (Vutrisiran)	siRNA	GalNAc-siRNA	s.c. injection	Transthyretin	Amyloidosis, hereditary transthyretin amyloidosis	Phase III	2018	NCT03759379
					Transthyretin amyloidosis (ATTR) with cardiomyopathy	Phase III	2019	NCT04153149
ARO-APOC3	siRNA	TRiM™ formulation	s.c. injection	Apolipoprotein C3	Hypertriglyceridemia Familial chylomicronemia	Phase I	2018	NCT03783377
ARO-ANG3	siRNA	TRiM™ formulation	s.c. injection	Angiopoietin like protein 3	Dyslipidemia	Phase I	2018	NCT03747224
RG6346 (DCR-HBVS)	siRNA	GalNAc-siRNA	s.c. injection	Hepatitis B surface antigen	Chronic hepatitis B viral infection	Phase I	2018	NCT03772249
KrasG12D siRNA (iExosomes)	siRNA	Mesenchymal Stromal cell derived Exosomes	i.v. infusion	KRAS	Pancreatic cancer patients with KrasG12D mutation	Phase I	2018	NCT03608631
OLX10010	siRNA	cp-asiRNA	i.d./s.c. injection	Connective tissue growth factor	Hypertrophic scar	Phase I	2018	NCT03569267
ARO-AAT	siRNA	TRiM™ formulation	s.c. injection	Mutant of α1-antitrypsin (Z-AAT)	α1-Antitrypsin deficiency	Phase II	2019	NCT03946449
ARO-HSD	siRNA	TRiM™ formulation	s.c. injection	Hydroxysteroid 17β-dehydrogenase 13	Nonalcoholic steatohepatitis	Phase I	2019	NCT04202354
ARO-HIF2	siRNA	TRiM™ formulation	i.v. infusion	Hypoxia-inducible factor 2α	Clear cell renal cell carcinoma	Phase I	2019	NCT04169711
DCR-PHXC	siRNA	GalXC-siRNA	s.c. injection	Lactate dehydrogenase A	Primary hyperoxaluria type 1 and type 2	Phase II	2019	NCT03847909 NCT04042402 (Phase 3)
JNJ-3989	siRNA	TRiM™ formulation	s.c. injection	Hepatitis B surface antigen	Chronic hepatitis B viral infection	Phase I	2019	NCT04208386
						Phase II	2020	NCT04439539

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Table 1. (continued)

Candidate	Type of RNA	Vehicle	Route of administration	Biological target	Condition or disease	Clinical stage	Year (first posted)	CT identifier
BMT101	siRNA	cp-asiRNA	i.d. injection	Connective tissue growth factor	Hypertrophic scar	Phase II	2019	NCT04012099
DCR-A1AT	siRNA	GalXC-siRNA	s.c. injection	α1- Antitrypsin	α1-Antitrypsin deficiency	Phase I/II	2019	NCT04174118
ARO-ENaC	siRNA	NA	Inhalation of nebulized solution	Epithelial sodium channel α subunit	Cystic fibrosis	Phase I/IIa	2020	NCT04375514
AMG 890	siRNA	GalNAc-siRNA	s.c. injection	Lipoprotein(a)	Elevated plasma lipoprotein(a)	Phase II	2020	NCT04270760
STP705	siRNA	Histidine–lysine copolymer peptide	Intralesional injection	TGF-β, Cox-2	Cutaneous squamous cell carcinoma *in situ*	Phase I/II	2020	NCT04293679
Miravirsen	LNA-modified anti-miR	NA	s.c. injection	miR-122	Hepatitis C	Phase II	2010/2015	NCT01200420 NCT02508090 NCT02452814
TargomiRs	miR-mimic	EDV (nonliving bacterial minicells)	i.v. infusion	miR-16	Malignant pleural mesothelioma Non-small cell lung cancer	Phase I	2015	NCT02369198
RG-125 (AZD4076)	Anti-miR	GalNac	s.c. injection	miR-103/107	Nonalcoholic steatohepatitis	Phase I	2015	NCT02612662
SAR339375	Anti-miR	NA	s.c. injection	miR-21	Alport's syndrome	Phase II	2016	NCT02855268
MRG-106 (Cobomarsen)	LNA-modified anti-miR	NA	i.v. infusion	miR-155	Cutaneous T cell lymphoma Mycosis fungoides	Phase II	2018	NCT03713320
			s.c. and intratumoral injection i.v. infusion	miR-155	Lymphoma/leukemia	Phase I	2015	NCT02580552
MRG-110	Anti-miR	NA	i.d. injection	miR-92a	Wound healing	Phase I	2018	NCT03603431
MRG-201	miR-mimic	Cholesterol	i.d. injection	miR-29	Keloid	Phase II	2018	NCT03601052
mRNA								
Melanoma mRNA vaccine	mRNA	NA	i.d. injection	Melan-A Mage-A1 Mage-A3 Survivin GP100 Tyrosinase	Melanoma	Phase I/II	2005	NCT00204516
Melanoma mRNA vaccine	mRNA	Protamine-complexed mRNA	i.d. injection	Melan-A Mage-A1 Mage-A3 Survivin GP100 Tyrosinase	Melanoma	Phase I/II	2005	NCT00204607

CV9201	mRNA	RNActive® technology	i.d. injection	Tumor-associated antigens	Non-small cell lung cancer	Phase I/II	2009	NCT00923312
RBL001/RBL002	mRNA	NA	i.nod. injection	Melanoma antigens	Melanoma	Phase I	2012	NCT01684241
BNT114	mRNA	Proprietary size- and charge-based RNA-LPX	i.v. infusion	Shared TAA for TNBC and neoantigens identified by NGS	TNBC	Phase I	2014	NCT02316457
CV7201	mRNA	RNActive® technology	i.m. injection i.d. injection	Rabies virus glycoprotein	Rabies	Phase I	2014	NCT02241135
Lipo-MERIT	mRNA	Liposome-complexed mRNA	i.v. infusion	RBL001.1 RBL002.2 RBL003.1 RBL004.1	Melanoma	Phase I	2015	NCT02410733
mRNA-4157	mRNA	Lipid encapsulated mRNA	i.m. injection	Tumor-associated antigens (personalized)	Solid tumors	Phase I	2017	NCT03313778
					Melanoma	Phase II	2019	NCT03897881
BI 1361849	mRNA	RNActive® technology	i.d. injection	Tumor-associated antigens	Non-small cell lung cancer	Phase I/II	2017	NCT03164772
mRNA-1647 mRNA-1443	mRNA	Undisclosed	i.m. injection	Viral antigens	Cytomegalovirus infection	Phase I	2017	NCT03382405
mRNA1325	mRNA	LNP	i.d. injection	Viral antigen	Zika virus infection	Phase I	2017	NCT03014089
VAL506440	mRNA	LNP	i.d. injection	H10N8 antigen influenza	Influenza	Phase I	2017	NCT03076385
VAL339851	mRNA	LNP	i.d. injection	H7N9 Antigen influenza	Influenza	Phase I	2017	NCT03345043
mRNA-2416	mRNA	LNP	Intratumoral injection	OX40 ligand	Advanced malignancies	Phase I/II	2017	NCT03323398
CV9202 (BI 1361849)	mRNA	RNActive® technology	i.d. injection	Six non-small cell lung cancer antigens (NY-ESO-1, MAGE-C1, MAGE-C2, survivin, 5T4, and MUC-1)	Non-small cell lung cancer	Phase I/II	2017	NCT03164772
Neo-antigen mRNA	mRNA	NA	s.c. injection	Tumor antigen	Digestive tract adenocarcinomas	NA	2018	NCT03468244
CV7202	mRNA	LNP	i.m. injection	Rabies virus glycoprotein	Rabies	Phase I	2018	NCT03713086
mRNA1653	mRNA	LNP	i.d. injection	Viral antigen	Human metapneumovirus and human parainfluenza infection	Phase I	2018	NCT03392389
mRNA-2752	mRNA	LNP	Intratumoral injection	OX40L IL-23 IL-36γ	Advanced malignancies	Phase I	2018	NCT03739931
mRNA-1944	mRNA	LNP	i.v. infusion	Anti-Chikungunya virus mAb	Chikungunya virus infection	Phase I	2019	NCT03829384

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Table 1. (continued)

Candidate	Type of RNA	Vehicle	Route of administration	Biological target	Condition or disease	Clinical stage	Year (first posted)	CT identifier
BNT131 (SAR441000)	mRNA	Proprietary size- and charge-based RNA-LPX	Intratumoral injection	IL-12sc IL-15sushi IFNα GM-CSF	Metastatic neoplasm	Phase I	2019	NCT03871348
mRNA-1893	mRNA	LNP	i.m. injection	Undisclosed	Zika virus	Phase I	2019	NCT04064905
mRNA-3704	mRNA	LNP	i.v. infusion	Methylmalonyl-coenzyme A mutase	Methylmalonic acidemia	Phase I/II	2019	NCT03810690
mRNA-1273	mRNA	LNP	i.m. injection	SARS-CoV-2 glycoproteins	COVID-19	Phase III	2020	NCT04470427
BNT162	mRNA	LNP	i.m. injection	Undisclosed SARS-CoV-2 proteins	COVID-19	Phase I/II Phase II/III	2020	NCT04380701 NCT04368728
BNT122 (RO7198457)	mRNA	Proprietary size- and charge-based RNA-LPX	i.v. infusion	Tumor-associated antigens	Colorectal cancer stage II/III Advanced melanoma	Phase II	2020	NCT04486378 NCT03815058
					Locally advanced or metastatic solid tumors	Phase I	2017	NCT03289962
CVnCoV	mRNA	LNP	i.m. injection	SARS-CoV-2 spike protein	COVID-19	Phase I	2020	NCT04449276
RNA Aptamers								
NOX-E36	Aptamer	L-RNA, PEGylated	i.v. injection s.c. injection	Chemokine (cysteine–cysteine motif) ligand 2	Chronic inflammatory diseases Type 2 diabetes mellitus Systemic lupus erythematosus	Phase I	2009	NCT00976729
					Renal impairment	Phase I	2011	NCT01372124
					Type 2 diabetes mellitus albuminuria	Phase II	2012	NCT01547897

NOX-A12	Aptamer	L-RNA, PEGylated	i.v. injection	Chemokine (C-X-C motif) ligand 12 Stromal-derived factor 1	Autologous stem cell transplantation	Phase I	2009	NCT00976378
					Chronic lymphocytic leukemia	Phase II	2011	NCT01486797
					Multiple myeloma	Phase II	2012	NCT01521533
					Colorectal cancer Pancreatic cancer	Phase I/II	2017	NCT03168139
					Glioblastoma	Phase I/II	2019	NCT04121455
NOX-H94	Aptamer	L-RNA, PEGylated	Undisclosed	Hepcidin	Anemia of chronic disease	Phase II	2012	NCT01691040
					Anemia End-stage renal disease	Phase I/II	2014	NCT02079896
Zimura (ARC1905)	Aptamer	NA	Intravitreal injection	Complement component 5	Age-related macular degeneration	Phase II	2017	NCT03362190
					Stargardt disease 1	Phase II	2017	NCT03364153
saRNA								
MTL-CEBPA	saRNA	SMARTICLES® liposomal nanoparticle	i.v. infusion	CCAAT enhancer binding protein	Hepatocellular carcinoma Liver cancer	Phase I	2016	NCT02716012
Other								
CV8102	Noncoding ssRNA	NA	Intratumoral injection	Activation of TLR7/8/RIG-1	Melanoma Squamous cell carcinoma of the skin or head/neck Adenoid cystic carcinoma	Phase I	2017	NCT03291002

[a]Only the most advanced clinical stage for each RNA candidate is mentioned. Table was compiled using data from [I–IX], [25], and [81].

[b]Abbreviations: AKI, acute kidney injury; cp-asiRNA, proprietary cell-penetrating asymmetric interfering RNA by OliX Pharmaceuticals; GalXCL, proprietary siRNA formulation by Dicerna Pharmaceuticals; GM-CSF, granulocyte-macrophage colony-stimulating factor; i.d., intradermal; IFN, interferon; Il, interleukin; i.m., intramuscular; i.nod., intranodal; i.v., intravenous; LNA, locked nucleic acid; LNP, lipid nanoparticle; NA, not applicable; NGS, next-generation sequencing; s.c., subcutaneous; TRiM™, targeted RNAi molecule, platform for ligand-mediated targeted RNA delivery by Arrowhead Pharmaceuticals; TAA, tumor-associated antigen; TNBC, triple-negative breast cancer.

progressed to the clinical setting as well, and the first saRNA-based clinical trial is currently ongoing (clinical trial number[i]: NCT02716012; Table 1). This clinical trial uses liposomal nanoparticles, termed SMARTICLES, encapsulated with saRNA that activates the CEBPA gene for treatment of patients with HCC. CEBPA is considered a master regulator in normal liver function and its expression is decreased in HCC. Lowered CEBPA expression is seen in many liver abnormalities and increasing CEBPA expression in HCC might result in improved therapeutic outcome [40].

gRNA for CRISPR/Cas9-Directed Knockout

The development of CRISPR-Cas9 gene editing technology (Box 1) has further pushed the development of RNA therapeutics to the forefront. The codelivery of Cas9 mRNA and sgRNA against a certain genomic target has promising applications for gene knock out strategies (Figure 1). While in a laboratory setting, many options are available for the introduction of the Cas9 protein and sgRNA to cells, including plasmid DNA, viral transfection, or electroporation, but this does not easily translate to an *in vivo* clinical setting. [43]. Therefore, the most realistic method right now involves *ex vivo* manipulation of cells with re-introduction of edited cells into the body [44].

Chemical Modifications to Increase RNA Stability and Decrease Immunogenicity

While the field has seen significant progress, some of the major obstacles in RNA therapeutics are the unstable nature (due to the high stability and activity of RNases) and high immunogenicity of the RNA molecules [45]. Both single-stranded and double-stranded RNA molecules induce the production of type I interferons and various other proinflammatory cytokines through multiple signaling pathways, involving Toll-like receptor (TLR) 3, 7, or 8, or retinoic-acid inducible gene (RIG)/melanoma differentiation-associated (MDA)5 [46,47]. The high immunogenicity combined with low RNA stability necessitates chemical modifications of the RNA molecule to make advancement to the clinic more realistic. Such modifications can involve alterations of the ribose group, the phosphate backbone, the RNA termini, or modification of the nucleobases themselves [45]. For example, modifying the ribose on the 2'-O position dramatically increased the *in vivo* potency of siRNA. At least 13 ribose modifications have been reported previously and especially 2'-OMe, 2'-F, and 2′-O-methoxyethyl modifications turned out to be highly successful for increasing siRNA serum stability [45]. Effective phosphate modifications include phosphorothioates (PSs) and borine-modified phosphorus (boranophosphate). Such modifications replace nonbridging phosphate oxygen atoms with either sulfur or borane, leading to nuclease- resistant nucleotide linkages [45,48]. Another popular modification is the phosphorodithioate linkage (PS2) that replaces both nonbridging phosphate oxygens with sulfur [48]. Furthermore, as the RNA termini are vulnerable to exonucleases, protecting them is equally important. This has been done by including inverted thymidine residues at the 3' end [45]. Other strategies towards stabilizing RNA termini have included addition of palmitic acid [49] and the covalent attachment of aromatic compounds (such as phenyl, hydroxylphenyl, pyrenyl, and naphthyl derivatives) to the 5' sense strand of siRNAs [50].

Box 1. The CRISPR/Cas System

The CRISPR/Cas system, a form of acquired immunity in bacteria and archaea, has been harnessed as a genome-editing tool and has also revolutionized the field of RNA therapeutics. The CRISPR system consists of two distinct classes (1 and 2). Class 2 is the most frequently utilized for genome editing applications, in particular, CRISPR/Cas9. CRISPR/Cas9 requires the CRISPR-associated nuclease Cas9 along with a gRNA. The gRNA consists of two RNA molecules: the CRISPR/RNA (crRNA) and the transactivating RNA (tracrRNA). To simplify the tool, these two RNAs are combined on a single guide RNA chimera (sgRNA) [41]. While the gRNA guides the Cas9 nuclease to a specific genomic location, the Cas9 cuts the DNA, resulting in a double-strand break, which in eukaryotes can be repaired by two mechanisms: nonhomologous end joining (NHEJ) and homology-directed repair (HDR). The more prominent of the two DNA repair pathways, NHEJ, is prone to introducing indel errors during the repair causing frameshift mutations resulting in premature termination of translation, generating a knockout of the gene of interest. When an HDR (donor) template is introduced, HDR-directed repair can be utilized, which enables correction of mutated genes, insertion of genes, or replacement of genes [42].

Delivery of RNA Therapeutics

The major challenge in RNA therapeutics is delivery and difficulties in intracellular uptake of RNA molecules due to the large molecular weight and negatively charged phosphate backbone that hinders internalization [51]. Previous studies on systemic administration of naked RNA has revealed that their pharmacokinetic profile is generally poor [52–54]. While chemical modifications of the RNA molecules dramatically increase the stability and decrease the immunogenicity, intravenous injection of naked RNA remains challenging where multiple barriers hamper RNA uptake. Cellular barriers include difficulties in both passing of the cell membrane as well as the subsequent **endosomal escape**. While other RNA molecules might be hampered with these cellular barriers, naked mRNA delivery somewhat circumvents these barriers as dendritic cells actively internalize naked mRNA by **micropinocytosis** [55,56]. Another barrier is the mononuclear phagocyte system (MPS), which effectively clears administered particulates through phagocytosis [57]. Although the smaller RNA molecules such as siRNA are not taken up by the MPS, a disadvantage of the small size is the increased clearance rate by the kidneys as the glomerular filtration barrier limit is about 8 nm [57]. Kidney clearance is therefore the major obstacle for intravenous administration of naked RNA. Fortunately, including PS modifications decreases the rate of kidney clearance of RNA molecules by increasing binding to plasma proteins, thereby increasing the serum half-life of the administered RNA [58].

When naked siRNA is injected intravenously, generally high doses are required. A study from 2012 reported that when ≤200 mg/kg siRNA was administered, the plasma concentration 30 min after injection was ~90% lower than 5 min after injection. At 2 h after injection, there was virtually no siRNA left in the plasma (<2%) due to rapid clearance by the kidneys. Even in partially nephrectomized rats, the clearance of siRNA was rapid. Higher dosages like 800 and 1200 mg/kg, however, yielded better results, which was mirrored in monkeys [52]. The fact that the clearance rate is slower with the higher doses indicates that the high dose, at least partially, saturates the kidney clearance system. However, the clearance is still fast and such high doses are clinically irrelevant both from a financial and safety point of view. Furthermore, the transient nature of siRNA will require multiple doses and dosing multiple times at such high concentrations seems unrealistic.

Local administration seems to be the more viable way of delivering naked RNA. Naked siRNA has been successfully delivered to eye, brain, and tumor tissues when injected locally [59]. Likewise, naked mRNA has also been successfully delivered locally to the heart. In 2018, Carlsson and colleagues reported efficient uptake of naked mRNA encoding VEGF by cardiomyocytes when injected directly into the heart. This resulted in an improved cardiac function in an animal model of myocardial infarction [60]. Especially for vaccination purposes, naked mRNA delivery is an option as dendritic cells actively internalize naked mRNA by micropinocytosis [55,56]. Other strategies that have been successful for naked mRNA delivery include intradermal injection of mRNA using hollow microneedles for expression in the skin [61], and injection of self-amplifying mRNA resulting in prolonged protein expression [62]. Below, we discuss several delivery strategies that have been developed to enable safe and efficient delivery of RNA therapeutics.

RNA Conjugated with a Targeting Moiety

The attachment of an active targeting moiety such as an antibody to the RNA can aid in both tissue/cell targeting and in internalization into target cells. Although it does not directly protect the RNA against degradation, it facilitates accumulation into the cells of interest [63]. Important characteristics of the targeting moiety are presence of active groups for conjugation purposes, good binding affinity, and reduced immunogenicity [64]. A recent notable success story of RNA conjugated to a targeting moiety is givosiran, which has been recently approved by the FDA.

Givosiran is composed of siRNA conjugated to three N-acetylgalactosamine (GalNAc) molecules. GalNAc binds the **asialoglycoprotein receptor (ASGR)** with high affinity and internalizes siRNA into the hepatocytes rapidly, although endosomal escape remains an issue, like in other delivery strategies [65]. This liver-specific uptake makes GalNAc suitable for siRNA delivery to the liver for hepatic diseases.

Antibodies are a popular choice as targeting moieties due to their specificity, high affinity, well-defined structure, and long *in* vivo circulation time [66]. Multiple studies have used antibody–siRNA conjugates (ARCs) with different methods of linking the antibody to the siRNA molecule. These conjugation methods can include either a covalent attachment (e.g., Sugo *et al.* used maleimide/thiol chemistry [67]) or a noncovalent attachment (e.g., Ibtehaj *et al.* used protamine as a linker using its electrostatic interactions with siRNA [68]) (Figure 2A). Moreover, linkers that allow for attachment between the siRNA and antibody can be made cleavable. A feature of cleavable linkers is that once the conjugate enters the target cell, the siRNA and antibody separate, which can alter the intracellular tracking including endosomal release and RISC loading [69–71]. pH sensitivity and photosensitivity are examples of strategies used in cleavable linker design [63].

An alternative to the use of full-length mAbs is the use of smaller antibody derivatives such as single-chain variable fragment (scFv) [72] (Figure 2B). Examples of such applications include the delivery of CXC chemokine receptor (CXCR)4–siRNA for $HER2^+$ breast cancer therapy [73] and CD44–siRNA targeted by a scFv against epidermal growth factor receptor (EGFR) in triple-negative breast cancer [74].

Aptamers

Aptamers have been used to direct therapeutic RNA molecules to target sites [75] (Figure 2C). In terms of specificity and affinity, they are highly comparable to antibodies but are smaller, have a higher stability, and are easier to generate [76]. In 2018, Zhou *et al.* used an RNA aptamer against gp120 (the exterior envelope glycoprotein in HIV that drives its entry into the host $CD4^+$ T cells) to deliver anti-HIV siRNA to infected T cells. The siRNA induces transcriptional gene silencing by targeting specific sites within the viral promoter, resulting in suppression of HIV infection and protection of $CD4^+$ T cells in mice [77]. Another study used an RNA aptamer against prostate-specific membrane antigen (PSMA), a cell-surface receptor that is highly upregulated in certain prostate cancers, to target prostate cancer cells in a mouse xenograft model engrafted with $PSMA^+$ human cancer cells. The RNA therapeutic delivered in this study was the CRISPR/Cas9 system against polo-like kinase (PLK)1. The results demonstrated high cell-type-specific delivery that translated into antitumor activity *in vivo* [78].

Lipid Nanoparticles

Initially developed as carriers for *in vivo* siRNA delivery [79], lipid nanoparticles (LNPs) are complex and large structures (~100 nm) that also have been used to deliver large RNA molecules such as mRNA *in vivo* [80]. LNPs can encapsulate large amounts of RNA [81] and protect them against RNase degradation and renal clearance (Figure 2D) [82]. Addition of polyethylene glycol (PEG) lipids has been proven to enhance LNP circulation time due to the steric barrier it creates around the LNP surface that protects the LNPs from interactions with plasma proteins, typically opsonins, that would target the LNPs for degradation by the MPS [83]. In fact, the use of PEG-lipids has become the standard since novel microfluidic mixing systems require the steric barrier provided by PEG to facilitate self-assembly. However, the use of PEG-lipids is a double-edged sword as the downside is that the same steric barrier inhibits interactions with the cell membrane and subsequent endosomal escape of therapeutic RNA [84]. Therefore, fine tuning of the amount of PEG-lipid and the length of the PEG chain is pivotal.

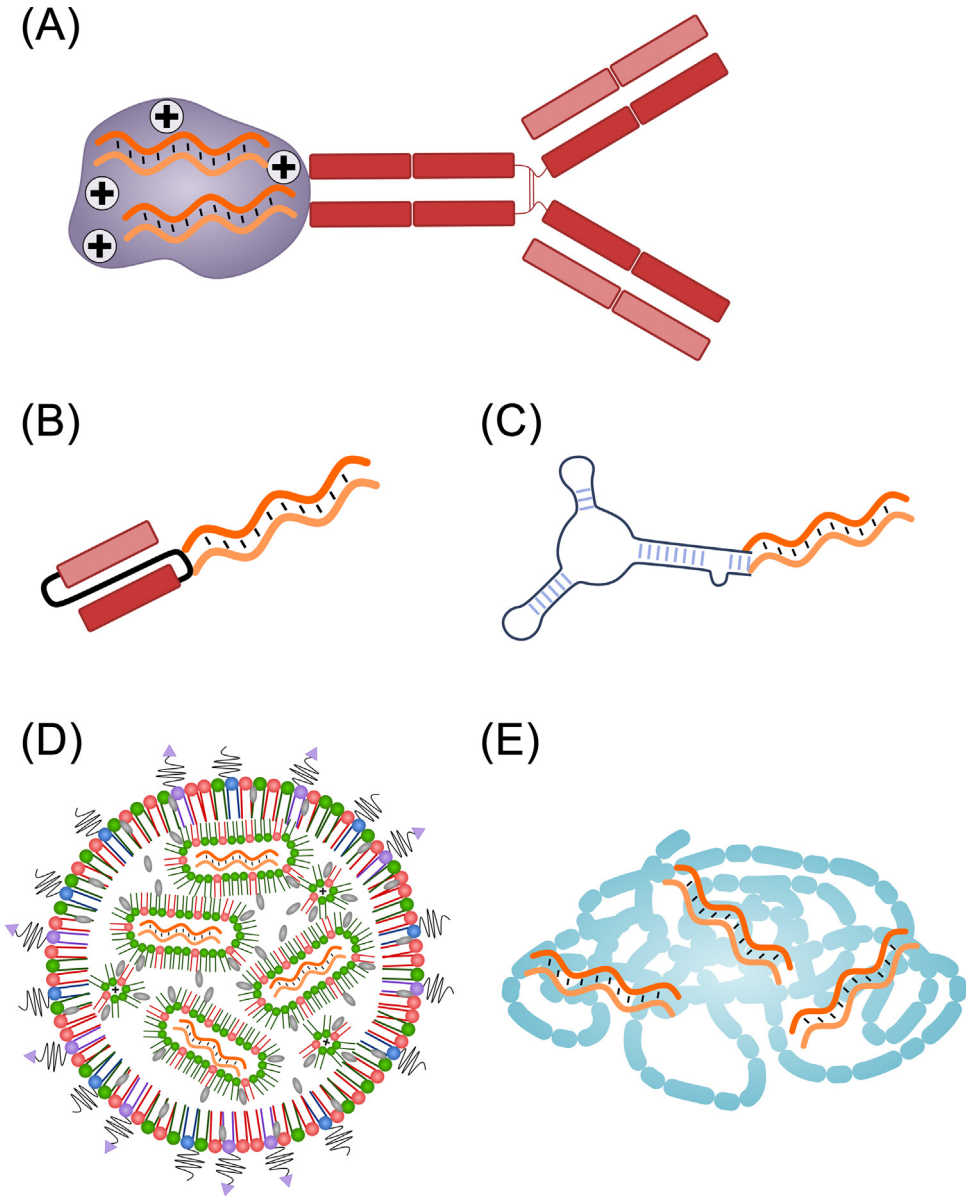

Figure 2. Examples of Delivery Vehicles for Different RNA Payloads. (A) Antibody conjugated to RNA molecules which can be mediated by using for instance positively charged protamine (shown as plus signs). (B) Conjugate of RNA with a single-chain variable fragment (scFv). (C) RNA–aptamer conjugates. (D) RNA encapsulated in lipid nanoparticles (LNPs). Cationic or ionizable lipids (shown in green) aid in encapsulating the RNA payload through electrostatic interactions. This way, the RNA is encapsulated in inverted micelles. Cholesterol (shown in grey) provides stability to the LNPs. The surface of the LNPs are generally coated with PEG (black lines). Reactive groups such as maleimide (purple triangles) can be linked to the PEG and are used to functionalize the LNPs with targeting moieties (chemical conjugation of targeting moieties). (E) Cationic polymers can encapsulate RNA therapeutics by electrostatic interactions.

The interaction of PEG-lipid with the plasma proteins upon injection results in release of PEG from the LNP, which further complicates optimization of the LNP formulation. An important study in 2013 reported on the desorption rate of PEG-lipids from the LNPs into the aqueous phase.

This desorption involves a transition state that requires a large amount of free energy. The length of the hydrophobic chain influences the amount of energy required to enter this activation state; the longer the hydrophobic chain, the more energy is required and hence the lower the *in vivo* PEG-lipid desorption rate. The results demonstrated that PEG-C18 desorption is low (0.2%/h), while initial desorption rates of PEG-C14 is ~2%/min [85]. Due to the positive and negative effects of PEG-lipids on *in vivo* intracellular RNA delivery, an optimal balance is required and, in most cases, including ~1.5% PEG-lipid (as percentage of the total lipid composition) is ideal. An increased concentration would result in a decrease of RNA delivery and can only be compensated by including shorter chain PEG-lipids (e.g. PEG-C14) that will enhance RNA desorption rates [85].

Polymers

Cationic polymers are well suited for the delivery of RNA due to their positive charge, chemical diversity, and compatibility with various targeting moieties (Figure 2E). Polymers can be linear or branched polymers, or consist of many branched repeats such as in the case of dendrimers [86]. Examples of the use of polymers for RNA delivery include polyethyleneimine (PEI) for the delivery of siRNA [87] and self-immolative polycarbonate-block-poly(α-amino)esters (dynamic materials that function as polycations that can lose their cationic charge to enhance endosomal escape) for mRNA delivery [9,88]. Dendrimers are popular for RNA delivery due to their tunable structure and monodispersity (all dendrimer molecules are as similar as possible and of a well-defined size upon synthesis). These hyperbranched macromolecules can be functionalized with many functional groups for use in, for instance, ligand attachment when targeted delivery is required [89,90]. Dong *et al.* described an example of targeted dendrimers for RNA delivery in 2018 where dendrimers encapsulated with siRNA were targeted with the RDGK peptide, which can target both the tumor endothelium (through $\alpha_v\beta_3$ integrin) and the tumor cells themselves (through neuropilin-1 receptor targeting). Using that system, the researchers silenced the cancer survival gene Hsp27 *in vivo* and achieved significant anticancer activity [91].

Moving to the Clinic

Since the FDA approval of the RNA therapeutic, pegaptanib (Macugen) in 2004 [92], the field of RNA has not progressed far. Besides the general difficulties in intracellular delivery of oligonucleotides to the cell/tissue of interest, RNA molecules are notoriously unstable due to the presence of ribonucleases. As discussed here, advances in nucleic acid chemistry combined with suitable delivery vehicles boosted the field, and these efforts eventually crystalized into the approval of the first siRNA-based drug, patisiran in 2018 [15]. Since then, the field of RNA therapeutics has received a boost, and this is evident when looking at the clinical pipeline that contains a wide range of RNA therapeutics at all stages of clinical development for a variety of medical conditions [34,93] (Table 1).

During the clinical development of patisiran, the LNP delivery vehicle seemed by far the most advanced and suitable carrier for siRNA delivery *in vivo*. The reason LNPs were so successful in hepatic gene silencing was due to the ability of ionizable LNPs to adsorb apoliporotein E (ApoE) in the circulation, giving rise to a natural targeting ligand that binds with high affinity to the low-density lipoprotein (LDL) receptor that is widely expressed on hepatocytes [94]. In late 2019, a second siRNA drug, givosiran, was approved [16]. Instead of LNPs, givosiran was delivered as a GalNAc-conjugated siRNA. Both currently approved siRNA-based drugs thus have a strong preferential uptake in the liver but use distinct mechanisms (ApoE vs GalNAc) [16,94].

Based on studies that led to givosiran, it seems that GalNAc-based siRNA delivery strategies have some important benefits over LNPs. In terms of toxicity, ease of production and the required dose frequency, GalNAc is superior [95]. Furthermore, GalNAc-based strategies can be

administered subcutaneously [96] as opposed to intravenously for LNPs. LNPs also require an extensive premedication regime to deal with infusion-related reactions [97]. Because of this, many siRNA candidates currently in the clinical pipeline are based on GalNAc or similar conjugates (see the siRNA section in Table 1). Alnylam currently has three candidate drugs in Phase III studies (fitusiran, lumasiran, and vutrisiran)[ii]. A fourth one, inclisiran, showed positive results in Phase III studies and is currently under review for approval [98]. Arrowhead Pharmaceuticals has two candidates in Phase II studies (ARO-AAT and JNJ-3989)[iii] and Dicerna is building a clinical pipeline with three programs (Phyox, RG6346, and Shine)[iv].

Multiple mRNA therapeutics are also being developed: Moderna has two candidate drugs in Phase II and an impressive early stage pipeline[v]; CureVac has several candidates in Phase I[vi]; and BioNtech has one candidate against melanoma in Phase II and many candidates in development[vii]. Moreover, the recent outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in accelerated development of several mRNA vaccines: mRNA-1273 (Phase III) by Moderna; BNT162 (Phase II/III) by BioNTech; and CVnCoV Vaccine (Phase I) by CureVac (Table 1).

While siRNA and mRNA candidates are the most abundant, dozens of other RNA therapeutic candidates have also entered clinical trials (Table 1). Indeed, four RNA-based ASOs are currently under clinical investigation: QR-010, QR-421a, QR-1123, and QR110, all developed by ProQR. Furthermore, anti-miR and miRNA mimics, including miravirsen, SAR339375, cobomarsen, and MRG-201 are all currently in Phase II clinical trials (Table 1).

While the first clinical trial with the *in vivo* delivery of CRISPR/Cas9 components was launched recently (clinical trial number: NCT03872479), the delivery is via viral vectors [35]. This might soon change as several studies have reported on the *in vivo* delivery of CRISPR/Cas9 components using RNA-LNP in animal models. Notable examples include: delivery of Cas9 mRNA with LNPs (although viral vectors were used to deliver the sgRNA and repair template) to correct a fumarylacetoacetate hydrolase splicing mutation in a mouse model of hereditary tyrosinemia [36], and delivery of both Cas9 mRNA and sgRNA with LNPs to knockout the mouse transthyretin gene, resulting in >97% reduction of serum proteins levels that lasted for at least 12 months [37].

Concluding Remarks and Future Perspectives

This review covered the recent advances in delivery of RNA therapeutics. Various RNA payloads were described with different roles, ranging from overexpression to silencing and knockout of the target gene. The versatility of RNA molecules makes them popular therapeutic agents for a wide range of medical conditions. However, issues with delivery have slowed down the field of RNA therapeutics for many years. Poor cellular uptake, fast clearance from the circulation, and the induction of immune responses have necessitated optimization of both the RNA molecules as well as the delivery vehicles. Chemical modifications of RNA molecules truly boosted the field of RNA therapeutics and facilitated the shift from completely encapsulated RNA nanoparticles to the use of less complex RNA conjugates (e.g., GalNAc). The ability to protect RNA molecules from degradation *in vivo* turned out to be crucial as early attempts with RNA-based therapy resulted in direct enzymatic degradation eliminating any therapeutic potential [95]. Additionally, the discovery of circular RNA (circRNA) provided the scientific community with even more possibilities to protect and deliver therapeutic RNA. As circRNA is stable against exonucleolytic decay, it might become an important agent for therapeutic applications for longer-lasting effects [99].

There is, however, still plenty of work to be done to advance the dream of RNA therapy. For example, most RNA-based therapeutics are chemically modified RNAs and it is important to

Outstanding Questions

What options can to be explored to enhance endosomal escape for RNA therapeutics?

As mRNA is expensive and difficult to deliver intracellularly, could saRNA replace mRNA to boost expression of endogenous genes, leaving mRNA dedicated only for the introduction of exogenous proteins (such as for vaccines or Cas9)?

How can we overcome the problem of saturation of certain intracellular components (such as AGO2) in situations where multiple RNAs are used to maximize efficacy for simultaneous silencing/knockout of one gene and overexpression of another gene?

pay attention to potential adverse effects of these modifications. Not only does modified RNA differ structurally from unmodified RNA but it can also affect the functionality. Therefore, some caution is necessary when focusing on an improved pharmacokinetic profile of synthetic RNA molecules with disregard to altered biological functionalities that might not be directly visible. To address this issue, a new class of RNAs has been explored. These are the bioengineered RNA agents (BERAs), which are made and folded in living cells and demonstrate favorable stability in human cells. They could represent a more natural alternative to the extensively chemical modified RNAs currently used [100].

Furthermore, in terms of delivery of RNA therapeutics, most strategies have focused on the 'low-hanging fruit' by delivering the RNA to the liver and most drug candidates have hence focused on liver- and kidney-related diseases (with some upcoming potential in the central nervous system as well) [101]. Future ambitions should focus on sites in the body that are harder to reach such as leukocytes that are dispersed over the body and intrinsically hard to transfect. Currently, it seems that this necessitates the inclusion of targeting moieties such as peptides, antibodies, other proteins, polysaccharides, and more. Besides cell targeting, such targeting moieties might also aid in enhancing internalization of the payload.

Internalization and endosomal escape is a real bottleneck in RNA delivery. While a small fraction of siRNA release in the cytosol is sufficient for knockdown, other RNA-based approaches still lack potency due to endosomal entrapment, and we wonder how delivery systems can enhance this (see Outstanding Questions). This is not trivial, as can be seen from the use of PEG, which on the one hand is a critical component in many delivery systems, while on the other hand, it inhibits endosomal escape.

While most delivery efforts for small RNAs such as siRNA are now invested in GalNAc-based or similar conjugates, larger delivery vehicles such as polymers or LNPs should not yet be disregarded. This is because other vehicles might aid in the improvement of extrahepatic delivery. We and others have demonstrated the feasibility of accurately targeting specific cell populations *in vivo* in animal models by functionalizing the surface of the delivery vehicle with targeting moieties [102–110]. Therefore, for leukocyte-implicated diseases such as chronic lymphocytic leukemia or autoimmunity, tumors with insufficient **enhanced permeability and retention (EPR) effect** or any other condition where specific targeting to the cell of interest is critical, a variety of delivery vehicles such as LNPs and dendrimers that can be functionalized with targeting moieties, are in high demand [111]. Perhaps, including targeting moieties can also bring down costs by maximizing accumulation of the therapeutic agents at the cell/tissue of interest. This is especially important for mRNA, which is expensive to prepare and might therefore be universally less accessible (see Outstanding Questions).

In conclusion, the field of RNA therapeutics has seen major developments at multiple levels (targeting, RNA modifications, delivery vehicles, etc.) and a wide variety of different RNA molecules are currently at different stages of (pre-)clinical development (Table 1). Owing to the invested efforts, RNA therapeutics moved from unrealistic dreams to genuine realities and many actors in the field are determined to drive the RNA revolution to the next level.

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Resources

[i]https://clinicaltrials.gov/

[ii]www.alnylam.com/alnylam-rnai-pipeline/

[iii]https://arrowheadpharma.com/pipeline

[iv]https://dicerna.com/pipeline

[v]www.modernatx.com/pipeline

[vi]www.curevac.com/our-pipeline

[vii]https://biontech.de/science/pipeline

[viii]www.cancer.gov/

[ix]www.olixpharma.com/

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