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Review

Nanomaterials for Therapeutic RNA Delivery

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SUMMARY

Recent years have witnessed tremendous developments and breakthroughs in the field of RNA-based therapeutics. The distinct mechanisms of exogenous RNAs and analogs, including messenger RNAs, small interfering RNAs, microRNAs, and antisense oligonucleotides, have brought them unprecedented potential to treat a variety of pathological conditions. However, the widespread application of RNA therapeutics is hampered by their intrinsic features (e.g., instability, large size, and dense negative charge) and formidable host barriers. Development of safe and efficient vectors is key for successful delivery and translation of RNA therapeutics. In this review, we first present an overview of RNA therapeutics and their clinical translation. We then discuss their delivery challenges and highlight recent advances in nanomaterial-based RNA-delivery platforms. Finally, the potential concerns and future developments of RNA delivery systems are discussed.

INTRODUCTION

The delivery of RNA-based therapeutics with transient activity for genetic engineering has long been pursued for the treatment of pathological conditions ranging from infectious diseases, metabolic disorders, neurological diseases, cancers, and heritable disorders. Recently, this idea has grown from a scientific concept into a clinical reality.[1] RNA therapeutics show several advantages compared with traditional small molecules or protein-based therapeutics, including: (1) manipulation of any gene of interest without the limitation of undruggability; and (2) ease of design and manufacture by chemical synthesis methods or *in vitro* transcription systems.[2] During the last three decades, both academia and industry have spent great efforts in developing RNA molecules into therapeutic agents.

Despite the great promise of RNA-based therapeutics for disease management, their systemic applications have, until recently, been hampered by the instability and inefficient *in vivo* delivery of RNAs. Currently, several delivery technologies are being developed to facilitate RNA delivery, including mechanical forces (e.g., electroporation), viral vectors (e.g., lentivirus), and non-viral vectors (e.g., lipofection). Nevertheless, mechanical transfection is restricted by significant cytotoxicity and specialized equipment with limited practical use *in vivo*,[3] and viral vectors are concerned with potential carcinogenesis, high immunogenicity, broad tropism, confined gene-packaging capacity, and difficulty for large production.[4–6] Non-viral vectors hold the potential to overcome these shortcomings, especially with respect to safety and manufacture. For instance, polymers have been shown to have low toxicity and immunogenicity, and they can be easily synthesized in large quantities.[6,7] Additionally, the fast disclosure of genetic information enabled by next-generation sequencing platforms (e.g., Illumina sequencing technology) and the scalable production of RNA payloads provided by some biotechnology

Progress and Potential

RNA therapeutics hold great promise to treat many diseases including infectious diseases, cancers, and genetic disorders. However, their intrinsic properties (e.g., instability and large size) and formidable barriers have hindered their broad applications. There is a growing demand for developing safe and efficient vectors for RNA delivery. Nanomaterials (NMs), with the potential to overcome multiple physiological barriers, have emerged as promising non-viral vectors. With one marketed RNA drug, patisiran, and many drug candidates in clinical trials, NM-based delivery platforms are bridging the gap between RNA molecules and medicinal applications. Specifically, the unprecedented speed of mRNA vaccine development against the COVID-19 pandemic addresses the great value of NMs. In this review, we first give an overview of RNA therapeutics and their clinical translation. We then summarize recent advances in RNA-delivery NMs. Finally, we provide perspectives on future developments of NMs.

companies (e.g., TriLink) have made non-viral vectors more attractive as delivery systems, especially in the development of messenger RNA (mRNA) vaccines for virus outbreaks.[8] However, non-viral vectors have long suffered from low transfection efficiency compared with viral vectors, which is a result of their inferior capability to escort RNAs across multiple physiological barriers to target cells. This situation is rapidly evolving due to significant advances in materials science and nanotechnology, which have yielded many promising delivery candidates and have deepened the understanding of biological behaviors of RNA-delivery nanomaterials (NMs). Furthermore, recent developments in nucleotide modification chemistry have greatly propelled this field by increasing the stability and activity while decreasing the immunogenicity of RNAs.[9,10]

In this review, we provide an overview of RNA therapeutics and their clinically advanced delivery platforms. We also give a brief introduction of various biological barriers for *in vivo* delivery of RNA therapeutics and highlight recent advances in NM-based RNA-delivery platforms and their biomedical applications (Scheme 1). Finally, we discuss the potential concerns and future developments of RNA-based therapeutics.

RNA THERAPEUTICS AND THEIR CLINICAL TRANSLATION

Four major classes of RNA therapeutics have received the most attention: antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), microRNAs (miRNAs), and mRNAs. Their modes of action are briefly discussed in this section as well as their key advances and clinical developments.

Antisense Oligonucleotides

ASOs, discovered in 1978,[11] are the first clinically developed RNA-targeting therapeutics. ASOs are single-stranded, synthetically modified nucleic acid analogs of 15–30 nucleotides with a central DNA gapmer region and two RNA flanks.[12] ASOs bind to complementary mRNA sequences to form DNA-RNA duplexes and repress translation by RNase H-mediated mRNA cleavage and/or steric hindrance of ribosomes (Figure 1A). In 1998, formivirsen became the first RNase H-dependent ASO drug approved by the US Food and Drug Administration (FDA) for the treatment of cytomegalovirus retinitis in patients, which opened a new avenue for RNA therapeutics.[13] Interestingly, ASOs can also be designed to act on precursor mRNAs (pre-mRNAs) in the nucleus to mask alternative intron-exon junctions (splice sites) to generate a specific mRNA isoform that can restore stability or function to a mutated gene product (i.e., exon-skipping ASOs) (Figure 1B). Currently there are five ASO drugs on the market, three of which are exon-skipping ASO drugs.[14]

Small Interfering RNAs

siRNAs are 21- to 23-nucleotide-long RNA duplexes (~14 kDa) with an mRNA sequence (sense strand) and its complementary sequence (antisense active strand). After entering the cytosol, siRNA is loaded into the RNA-induced silencing complex (RISC), leading to the cleavage of the sense strand by endonuclease Argonaute 2 (AGO2). The activated RISC containing the antisense strand of siRNA selectively seeks out and degrades mRNA that is complementary to the antisense strand, thus blocking translation of mRNA to protein (Figure 1C). Since the discovery of RNA interference (RNAi) in 1998,[15] siRNAs have become a ubiquitous and powerful tool to silence the expression of virtually any gene in a highly efficient and specific manner, including those traditionally considered undruggable targets. After 20 years of continuous effort, the FDA approved the first siRNA drug, patisiran (Alnylam Pharmaceuticals), in August 2018 for the treatment of hereditary transthyretin

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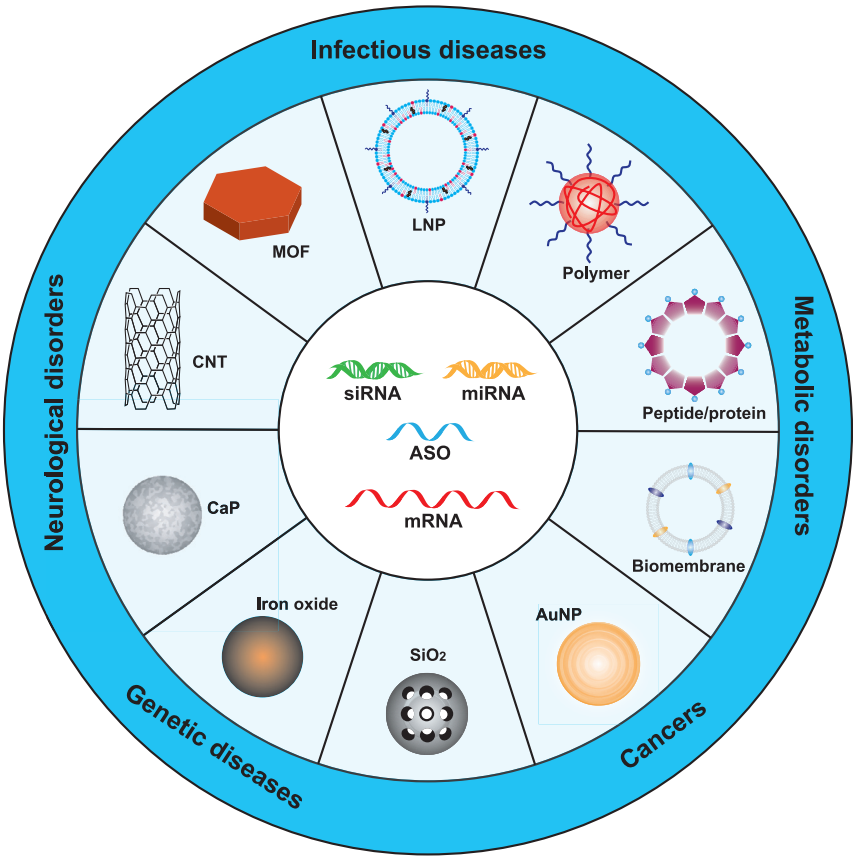

Scheme 1. An Overview of Nanomaterial-Based RNA Delivery Platforms and Their Biomedical Applications

Representative nanomaterials include lipid nanoparticle (LNP), polymer, peptide/protein, biomembrane, gold nanoparticle (AuNP), silica (SiO_2), iron oxide, calcium phosphate (CaP), carbon nanotube (CNT), and metal-organic framework (MOF).

amyloidosis (hATTR) with polyneuropathy, heralding a new era in RNAi therapy. Soon afterward, in 2019 Alnylam received the approval for their second siRNA drug, givosiran, for the treatment of acute hepatic porphyria.

MicroRNAs

miRNAs were first identified in 1993 as a class of endogenous non-coding RNAs of ~22 nucleotides involved in virtually every cellular process.[16] Since dysregulation of miRNAs is associated with numerous human diseases, regulation of miRNA expression has been proposed as an innovative therapeutic approach. Such a strategy is classified into two categories, namely mimicking or antagonizing (known as anti-miRs) the function of miRNAs. Synthetic double-stranded miRNA mimics, similar to siRNAs, can be loaded into the RISC (Figure 1D), leading to suppressed expression of mRNAs containing partially complementary sequences by repressing their translation or facilitating their degradation.[17] miRNAs are less specific and efficient than siRNAs, but one single miRNA can modulate multiple RNA transcripts and pathways.[12] In contrast, synthetic single-stranded anti-miRs, similar to ASOs, can sequence-specifically bind to mature miRNAs to interrupt their function on mRNAs (Figure 1E). With the advances in miRNA biology and delivery technology, miRNA-based therapeutics have been extensively investigated in preclinical studies, and some have reached clinical development.[18]

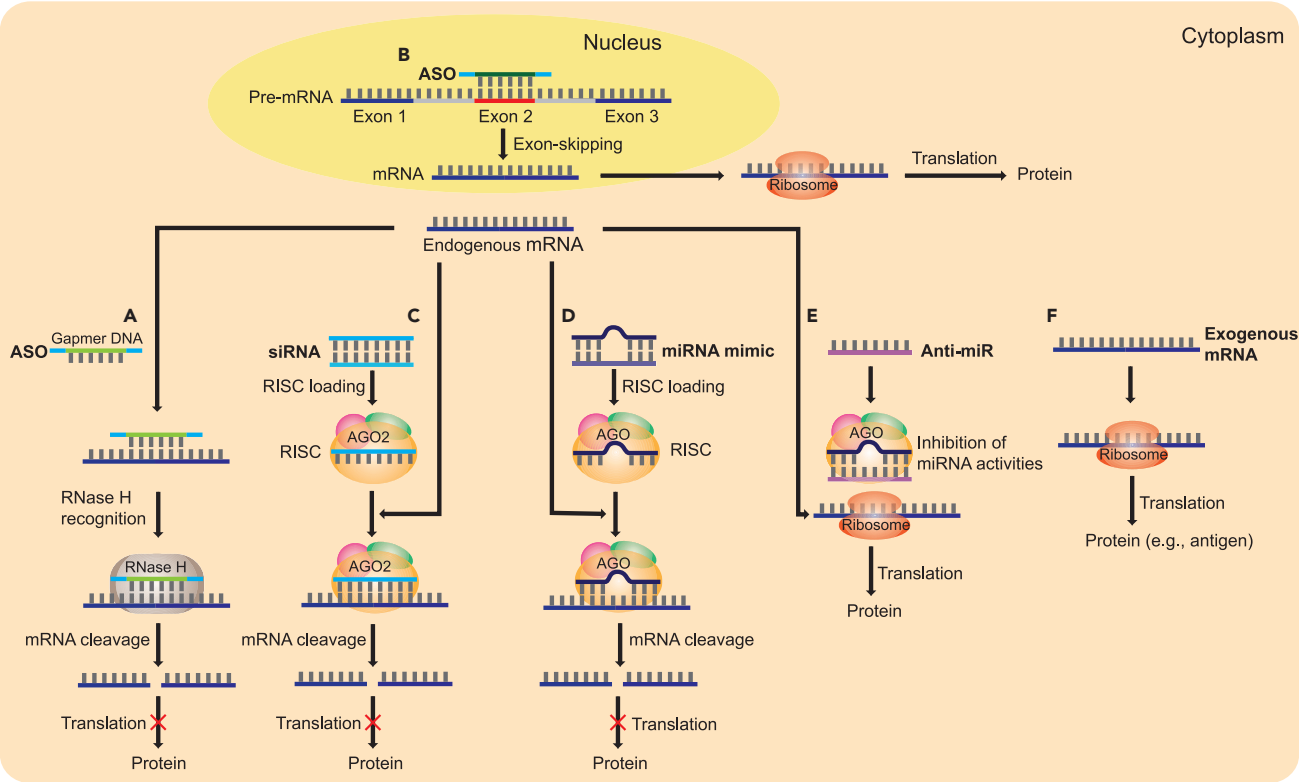

Figure 1. Mechanisms of RNA-Based Therapeutics

(A) RNase H-dependent ASO.
(B) Exon-skipping ASO.
(C) siRNA.
(D) miRNA mimic.
(E) Anti-miR.
(F) Exogenous mRNA.

Messenger RNAs

mRNAs, discovered in 1961,[19] are the middleman between DNA and proteins, which are able to be translated into proteins after their incorporation into protein synthesis machinery (Figure 1F). This mode of action without the risk of genomic integration has enabled two major applications of mRNA-based therapeutics, including induction of protein expression to replace faulty/missing protein (i.e., protein replacement therapy) or antigen expression for vaccination (i.e., mRNA vaccine therapy). As single-stranded RNAs, mRNAs are more susceptible to hydrolysis of the phosphate backbone through intramolecular attack of the 2′-hydroxyl group, due to their structural flexibility compared with double-stranded RNAs.[20] mRNAs are also much larger (700–7,000 kDa) than those short non-coding RNAs mentioned above (i.e., ASOs, siRNAs, and miRNAs),[21] which poses an additional challenge for delivery. Nevertheless, since the concept of using *in vitro*-transcribed (IVT) mRNAs as therapeutic agents took off in 1989,[22] mRNA-based cancer immunotherapies and infectious disease vaccines have been rapidly developed with the help of sophisticated delivery technologies. Currently, most mRNA drug candidates are in early-stage clinical trials.[14]

RNA-DELIVERY PLATFORMS IN THE CLINIC

Lipid-based nanoparticles (LNPs) are the most clinically advanced nanoplatform for RNA delivery. It is the only platform that has undergone clinical development for

delivering all four RNA therapeutics. For short RNA therapeutics, this platform enables their delivery to hepatocytes and tumor cells after systemic administration, which has led to the development of more than ten drug candidates for the treatment of liver-associated diseases and cancers (Table 1). The initial development of LNPs was focused on cationic lipids, which can electrostatically absorb polyanionic RNAs. However, many laboratories and pharmaceutical companies have shifted to use ionizable lipids that are positively charged only at acidic pH, due to the enhanced transfection efficacy and reduced toxicity compared with permanently charged lipids.[23] In particular, the FDA-approved patisiran is delivered by LNP composed of a proprietary ionizable lipid (DLin-MC3-DMA) and other helper lipids (see the next section).

While for mRNA therapeutics, LNP-mediated systemic or local delivery to somatic cells or antigen-presenting cells has spurred the clinical translation of more than 20 drug candidates for protein replacement therapy and vaccines (Table 2). Strikingly, Moderna's mRNA-1273, the first clinical batch of an mRNA vaccine against the coronavirus disease 2019 (COVID-19), is also delivered by LNP, making lipid-based platforms valuable in light of the ongoing pandemic.[8]

As an alternative to a nanoplatform, *N*-acetylgalactosamine (GalNAc) conjugates are the most clinically advanced molecular platform for liver delivery of short RNA therapeutics. GalNAc is a high-affinity ligand for the asialoglycoprotein receptor (ASGPR) present on hepatocytes, which mediates efficient uptake and transfection of its conjugated payload.[24] Due to its subcutaneous administration route, simplified chemical entity, specific liver targeting ability, improved knockdown potency and durability, and reduced toxicity, GalNAc-conjugated siRNAs have attracted tremendous interest. Based on their proprietary enhanced stabilization chemistry (ESC)-modified, trivalent GalNAc-conjugated siRNA (ESC-GalNAc-siRNA) technology (Figure 2), Alnylam is leading the clinical translation with one licensed drug and four drug candidates in late-stage clinical development. Other GalNAc conjugate platforms, including Dicerna's GalXC, Arrowhead's targeted RNAi molecule (TRiM), and Ionis/Akcea's ligand conjugated antisense (LICA), have also been adapted for liver delivery of siRNAs, anti-miRs, and ASOs.[25]

Several polymeric, biomimetic, and inorganic platforms have also been evaluated in the clinic for RNA delivery, which will be discussed below. It is worth mentioning that, based on protamine-complexed mRNA encoding tumor-associated antigens (TAAs), CureVac is developing a proprietary RNActive vaccine platform for cancer immunotherapy, which has triggered favorable immune responses in the treated patients.[26]

NANOMATERIAL-MEDIATED RNA DELIVERY

Naked RNAs are susceptible to nuclease degradation and they are too large, hydrophilic, and negatively charged to cross cellular membranes by themselves,[6] which requires the use of delivery vectors. Following administration, vector-formulated RNAs are confronted with a series of barriers posed by the host physiological system (Figure 3). Correspondingly, an ideal RNA-delivery vector ought to (1) protect RNAs from spontaneous hydrolysis, (2) protect RNAs from degradation by nucleases, (3) prevent non-specific absorption of proteins, (4) avoid toxicity and immune recognition, (5) evade renal filtration, (6) extravasate from blood vessels to target tissues, (7) promote cellular uptake, (8) escape endosomes, and (9) release RNAs to allow access to the cellular machinery.[27]

Table 1. Selected Clinical Trials of Delivery Platform-Enabled Non-coding RNA Therapeutics

Type	Delivery Platform	Drug Name	Target	Disease (Delivery Route)	Trial Number (Phase)	Status	Company
ASO	lipid-based nanoparticles	LErafAON-ETU	C-raf	advanced cancer (i.v.)	NCT00100672 (I)	completed	INSYS Therapeutics
		BP1001 (Prexigebersen)	Grb2	myeloid leukemia (i.v.)	NCT02781883 (II)	recruiting	Bio-Path Holding
		BP1002	Bcl-2	lymphoma (i.v.)	NCT04072458 (I)	not yet recruiting	
	GalNAc conjugates	AKCEA-APO(a)-L_{Rx} (TQJ230)	ApoA	hyperlipoproteinemia (s.c.)	NCT04023552 (III)	recruiting	Ionis/Akcea Therapeutics
		AKCEA-APOCIII-L_{Rx}	ApoCIII	hypertriglyceridemia (s.c.)	NCT03385239 (II)	active, not recruiting	
		AKCEA-ANGPTL3-L_{Rx}	ANGPTL3	hypercholesterolemia (s.c.)	NCT03360747 (II)	completed	
		AKCEA-TTR-L_{Rx}	TTR	hATTR with polyneuropathy (s.c.)	NCT04136184 (III)	recruiting	
				ATTR with cardiomyopathy (s.c.)	NCT04136171 (III)	recruiting	
siRNA	lipid-based nanoparticles	ALN-TTR02 (Patisiran, Onpattro)	TTR	hATTR with polyneuropathy (i.v.)	/	approved (2018)	Alnylam Pharmaceuticals
		ALN-TTR01	TTR	hATTR (i.v.)	NCT01148953 (I)	completed	
		ALN-VSP02	VEGF/KSP	liver cancer (i.v.)	NCT01158079 (I)	completed	
		ALN-PCS02	PCSK9	hypercholesterolemia (i.v.)	NCT01437059 (I)	completed	
		DCR-PH1	GO	primary hyperoxaluria type 1 (i.v.)	NCT02795325 (I)	terminated	Dicerna Pharmaceuticals
		DCR-MYC	MYC	solid cancer (i.v.)	NCT02314052 (I/II)	terminated	
		TKM-PLK11 (TKM-08030)	PLK1	liver cancer (i.v.)	NCT02191878 (I/II)	completed	Arbutus (Tekmira Pharmaceuticals)
		TKM-ApoB	ApoB	hypercholesterolemia (i.v.)	NCT 00927459 (I)	terminated	
		TKM-100201	VP24/VP35/ L-polymerase	Ebola virus infection (i.v.)	NCT 01518881 (I)	terminated	
		TKM-100802	VP24/VP35/ L-polymerase	Ebola virus infection (i.v.)	NCT 02041715 (I)	terminated	
		ARB-001467	HBsAg	HBV infection (i.v.)	NCT02631096 (II)	completed	
		siRNA-EphA2-DOPC	EphA2	solid cancer (i.v.)	NCT01591356 (I)	recruiting	M.D. Anderson Cancer Center
		ND-L02-s0201	HSP47	hepatic fibrosis (i.v.)	NCT02227459 (I)	completed	Nitto Denko
				idiopathic pulmonary fibrosis (i.v.)	NCT03538301 (II)	recruiting	
		Atu027	PKN3	solid cancer (i.v.)	NCT00938574 (I)	completed	

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Table 1. *Continued*

Type	Delivery Platform	Drug Name	Target	Disease (Delivery Route)	Trial Number (Phase)	Status	Company
	GalNAc conjugates	SLN124	TMPRSS6	β-thalassaemia/ myelodysplastic syndrome (s.c.)	NCT04176653 (I)	withdrawn	Silence Therapeutics
		ALN-AS1 (Givosiran, Givlaari)	ALAS-1	acute hepatic porphyria (s.c.)	/	approved (2019)	Alnylam Pharmaceuticals
		ALN-TTRsc02 (Vutrisiran)	TTR	ATTR with cardiomyopathy (s.c.)	NCT04153149 (III)	recruiting	
				hATTR with polyneuropathy (s.c.)	NCT03759379 (III)	active, not recruiting	
		ALN-PCSsc (Inclisiran)	PCSK9	hypercholesterolemia (s.c.)	NCT03705234 (III)	recruiting	
		ALN-GO1 (Lumasiran)	GO	primary hyperoxaluria type 1 (s.c.)	NCT04152200 (III)	recruiting	
		ALN-AT3sc (Fitusiran)	AT	hemophilia A and B (s.c.)	NCT03549871(III)	recruiting	
		ALN-CC5 (Cemdisiran)	CC5	paroxysmal nocturnal hemoglobinuria (s.c.)	NCT02352493 (I/II)	completed	
		ALA-AAT02	A1AT	A1AT deficiency-associated liver disease (s.c.)	NCT03767829 (I/II)	active, not recruiting	
		ALN-AGT01	AGT	hypertension (s.c.)	NCT03934307 (I)	recruiting	
		ALN-HBV	HBsAg	HBV infection (s.c.)	NCT02826018 (I)	terminated	
		ALN-HBV02 (VIR-2218)	HBsAg	HBV infection (s.c.)	NCT03672188 (I/II)	recruiting	
		DCR-PHXC (Nedosiran)	LDHA	primary hyperoxaluria (s.c.)	NCT04042402 (III)	enrolling by invitation	Dicerna Pharmaceuticals
		DCR-HBVS (RG6346)	HBsAg	HBV infection (s.c.)	NCT03772249 (I)	recruiting	
		DCR-A1AT	A1AT	A1AT deficiency-associated liver disease (s.c.)	NCT04174118 (I/II)	recruiting	
		ARO-AAT	A1AT	A1AT deficiency-associated liver disease (s.c.)	NCT03945292 (II/III)	recruiting	Arrowhead Pharmaceuticals
		ARO-APOC3	ApoCIII	hypertriglyceridemia (s.c.)	NCT03783377 (I)	recruiting	
		ARO-ANG3	ANGPTL3	hypertriglyceridemia (s.c.)	NCT03747224 (I)	recruiting	
		ARO-HBV (JNJ-3989)	HBsAg	HBV infection (s.c.)	NCT04129554 (II)	recruiting	
		ARO-LPA (AMG 890)	ApoA	hyperlipoproteinemia (s.c.)	NCT04270760 (II)	not yet recruiting	

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Table 1. ***Continued***

Type	Delivery Platform	Drug Name	Target	Disease (Delivery Route)	Trial Number (Phase)	Status	Company
	dynamic polyconjugates/ cholesterol conjugates	ARC-520	HBV transcripts	HBV infection (i.v.)	NCT02065336 (II)	terminated	
		ARC-521	HBV transcripts	HBV infection (i.v.)	NCT02797522 (I)	terminated	
		ARC-AAT	A1AT	A1AT deficiency (i.v.)	NCT02900183 (II)	withdrawn	
	polypeptide nanoparticles	STP705	TGF-1β/COX-2	hypertrophic scar (i.d.)	NCT02956317 (I/II)	recruiting	Sirnaomics
				cutaneous squamous cell carcinoma (i.t.)	NCT04293679 (I/II)	recruiting	
	LODER polymer matrix	siG12D-LODER	KRAS G12D	pancreatic cancer (local implantation)	NCT01676259 (II)	recruiting	Silenseed
	exosomes	iExosomes	KRAS G12D	pancreatic cancer (i.v.)	NCT03608631 (I)	recruiting	M.D. Anderson Cancer Center
	cyclodextrin polymer-based nanoparticles	CALAA-01	RRM2	solid cancer (i.v.)	NCT 00689065 (I)	terminated	Calando Pharmaceuticals
	spherical nucleic acid	NU-0129	BCL2L12	gliosarcoma/glioblastoma (i.v.)	NCT03020017 (I)	active, not recruiting	Northwestern University
miRNA mimic	lipid-based nanoparticles	MRX34	miR34	melanoma (i.v.)	NCT02862145 (I/II)	withdrawn	Mirna Therapeutics
	bacterially derived nanocells	TargomiRs	miR-16	mesothelioma/lung cancer (i.v.)	NCT02369198 (I)	completed	EnGenelC
Anti-miR	GalNAc conjugates	RG-101	miR-122	HCV infection (s.c.)	EudraCT2013-002978-49 (I)	completed	Regulus Therapeutics
		RG-125	miR-103/107	type 2 diabetes (s.c.)	NCT02826525 (I/II)	completed	

EudraCT, European Union Drug Regulating Authorities Clinical Trials; i.v., intravenous; s.c., subcutaneous; i.d., intradermal; i.t., intratumoral.

Table 2. Selected Clinical Trials of Delivery Platform-Enabled mRNA Therapeutics

Type	Delivery Platform	Drug Name	Target	Disease (Delivery Route)	Trial Number (Phase)	Status	Company
mRNA	lipid-based nanoparticles	W_ova1 vaccine	3 TAAs	ovarian cancer (i.v.)	NCT04163094 (I)	recruiting	University Medical Center Groningen
		NCI-4650	Neo-Ag	melanoma/epithelial cancer (i.m.)	NCT03480152 (I/II)	terminated	National Cancer Institute
		BNT162	spike protein of SARS-CoV-2 virus	COVID-19 (i.m.)	NCT04368728(II/III)	recruiting	BioNTech
		Lipo-MERIT	4 TAAs	advanced melanoma (i.v.)	NCT02410733 (I)	recruiting	
		TNBC-MERIT (IVAC_W_bre1_uID or IVAC_M_uID)	3 TAAs or Neo-Ag	triple-negative breast cancer (i.v.)	NCT02316457 (I)	active, not recruiting	
		RO7198457	TAAs	advanced melanoma (i.v.)	NCT03815058 (II)	recruiting	
		CV7202	RABV-G	rabies (i.m.)	NCT03713086 (I)	active, not recruiting	CureVac
		CVnCoV	spike protein of SARS-CoV-2 virus	COVID-19 (i.m.)	NCT04515147 (II)	recruiting	
		GSK 692342	fusion protein (M72) of *Mycobacterium tuberculosis*	tuberculosis (i.m.)	NCT01669096 (II)	completed	GlaxoSmithKline
		mRNA-1273	spike protein of SARS-CoV-2 virus	COVID-19 (i.m.)	NCT04470427 (III)	recruiting	Moderna
		mRNA-1325	Zika virus antigen	Zika virus (i.m.)	NCT03014089 (I)	completed	
		mRNA-1893	Zika virus antigen	Zika virus (i.m.)	NCT04064905 (I)	active, not recruiting	
		mRNA-1653	fusion proteins of hMPV and PIV3	hMPV and PIV3 (i.m.)	NCT03392389 (I)	completed	
		mRNA-1440 (VAL-506440)	H10N8 antigen	influenza H10N8 (i.m.)	NCT03076385 (I)	completed	
		mRNA-1851 (VAL-339851)	H7N9 antigen	influenza H7N9 (i.m.)	NCT03345043 (I)	active, not recruiting	
		mRNA-1647	6 CMV antigens	CMV infection (i.m.)	NCT04232280 (II)	active, not recruiting	
		mRNA-4157	Neo-Ag	melanoma (i.m.)	NCT03897881 (II)	recruiting	

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Table 2. *Continued*

Type	Delivery Platform	Drug Name	Target	Disease (Delivery Route)	Trial Number (Phase)	Status	Company
		mRNA-5671 (Merck V941)	KRAS	KRAS mutant solid cancer (i.m.)	NCT03948763 (I)	recruiting	
		mRNA-1944[a]	CHKV-24 IgG	CHKV infection (i.v.)	NCT03829384 (I)	recruiting	
		mRNA-2416[a]	OX40L	solid cancer or lymphoma (i.t.)	NCT03323398 (I/II)	recruiting	
		mRNA-2752[a]	OX40L/IL-23/IL-36γ	solid cancer or lymphoma (i.t.)	NCT03739931 (I)	recruiting	
		MEDI1191[a]	IL-12	solid cancer (i.t.)	NCT03946800 (I)	active, not recruiting	
		mRNA-3704[a]	MUT	isolated methylmalonic acidemia (i.v.)	NCT03810690 (I/II)	active, not recruiting	
		mRNA-3927[a]	PCCA/PCCB	propionic academia (i.v.)	NCT04159103 (I/II)	active, not recruiting	
		MRT5005[a]	CFTR	cystic fibrosis (inhalation)	NCT03375047 (I/II)	recruiting	Translate Bio
		MRT5201[a]	ornithine transcarbamylase	ornithine transcarbamylase deficiency (i.v.)	NCT03767270 (I/II)	withdrawn	
	protamine	CV7201	RABV-G	rabies (i.d.)	NCT02241135 (I)	completed	CureVac
		CV9201	5 TAAs	non-small cell lung cancer (i.d.)	NCT00923312 (I/II)	completed	
		CV9202 (BI 1361849)	6 TAAs	non-small cell lung cancer (i.d.)	NCT03164772 (I/II)	recruiting	
		CV9103	4 TAAs	prostate cancer (i.d.)	NCT00831467 (I/II)	completed	
		CV9104	6 TAAs	prostate cancer (i.d.)	NCT02140138 (II)	terminated	
		/	6 TAAs	malignant melanoma (i.d.)	NCT00204607 (I/II)	completed	University Hospital Tuebingen

i.v., intravenous; i.d., intradermal; i.t., intratumoral; i.m., intramuscular.
[a]Protein replacement therapy.

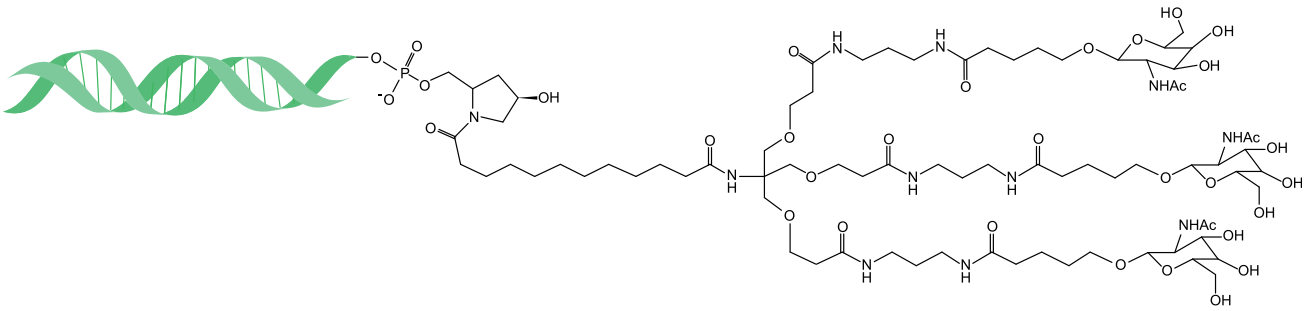

Figure 2. GalNAc-siRNA Conjugate
Structure of the trivalent GalNAc-conjugated siRNA.

Although it is not a simple task to achieve these goals, recent advances in the field of nanotechnology have demonstrated that NMs are promising vectors with the capability to overcome various barriers for successful RNA delivery. Here, we summarize five major NM-based delivery platforms, namely lipid, polymer, peptide, biomembrane, and inorganic NMs. Their advantages and disadvantages for RNA delivery are briefly summarized (Table 3), and their recent progress is discussed in detail below. For those interested in RNA conjugates (e.g., GalNAc, lipid, and aptamer) and nucleic acid-based delivery system, we refer readers to reviews focused on these topics.[25,28–30]

Lipid-Based Platform

LNPs have been the most popular non-viral vehicle for RNA delivery since the first introduction of a 1,2-di-*O*-octadecenyl-3-trimethylammonium propane (DOTMA)-formulated cationic liposome for therapeutic delivery of mRNA into mammalian cells in 1989.[22] Among various LNPs, two major categories are of great interest, i.e., liposome and lipid-like nanoparticles (NPs) (Figure 4A).[31] Liposome usually shows a unilamellar structure of a single lipid bilayer surrounding an aqueous core with a diameter of 100–200 nm. Early developments have used permanently charged cationic lipids to formulate liposomes (Figure 4B), which complex with RNAs and escort them to the site of action.[32] For example, Sato and colleagues prepared vitamin A-coupled liposomes composing a cationic lipid DC-6-14 for targeted delivery of heat-shock protein 47 (HSP47) siRNA to activated hepatic stellate cells to treat liver cirrhosis.[33] Their results showed that five treatments with this regimen successfully resolved liver fibrosis and prolonged survival of pathological rats. This formulation (namely ND-L02-s0201) is now under clinical development. However, cationic liposomes are usually associated with high toxicity and immunogenicity. When given intravenously, pulmonary deposition of NPs, hepatic damage and proinflammatory response were reported.[34–36] To avoid these, biocompatible anionic polymers can be coated on the surface.[37,38] Alternatively, to harness the proinflammatory effect, self-adjuvanting liposomes are being developed for mRNA vaccination.[39,40]

Recently, more advanced ionizable lipids have been developed to replace cationic lipids for reduced toxicity without compromising transfection efficacy. A typical ionizable lipid contains three moieties (a polar amine head, a linker region, and two nonpolar hydrocarbon tails) with an optimal pK_a value of 6.2–6.5,[43] which is designed to be positively charged at acidic pH to allow efficient encapsulation of RNAs into liposomes while maintaining neutrality at physiological pH, and further become protonated in endosomes to facilitate escape into the cytoplasm. Since the introduction of ionizable lipid in 2001,[44] great efforts have been made based on rational design approaches to optimize the molecular structure and properties such as the

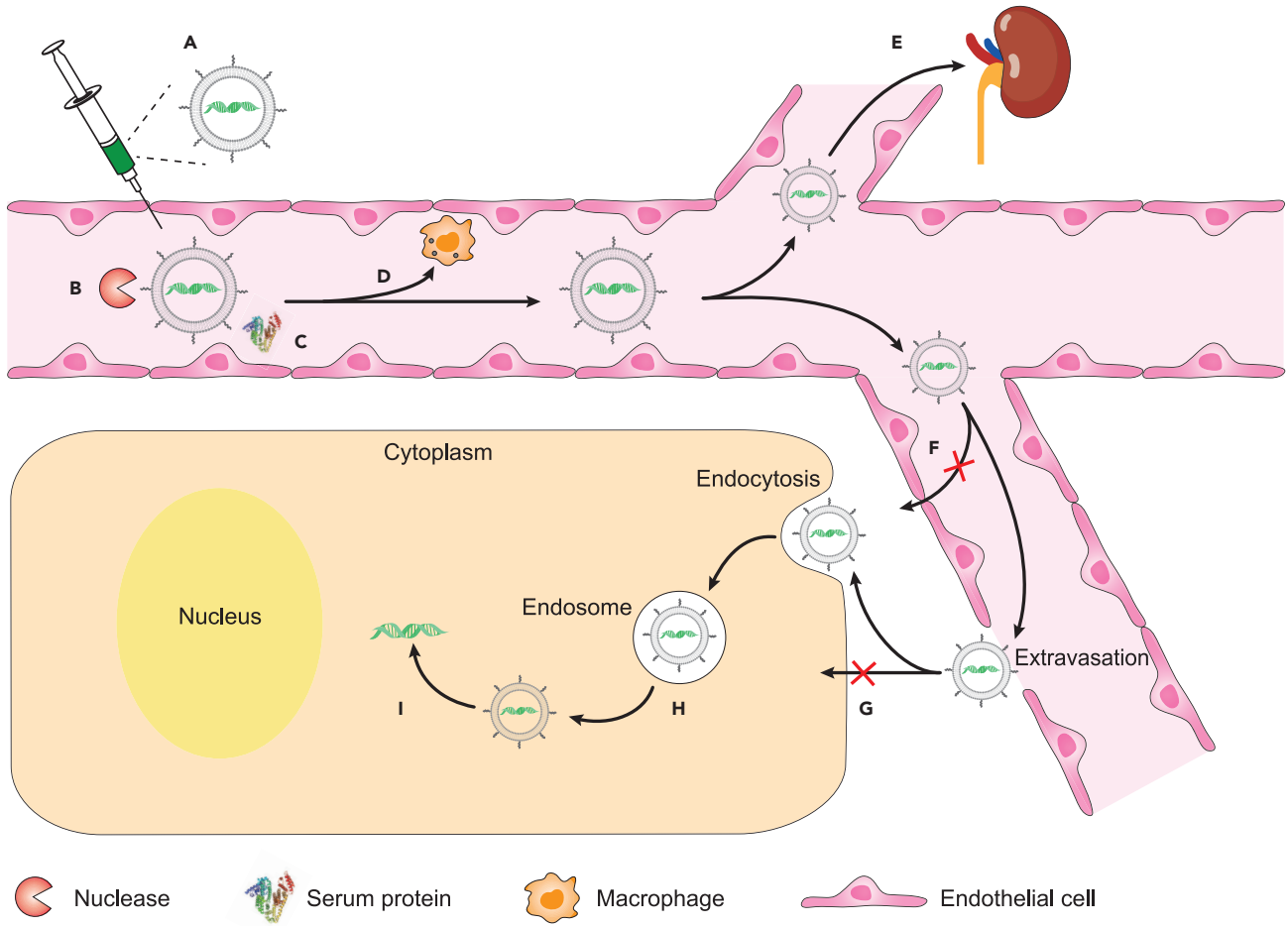

Figure 3. Obstacles Faced by *In Vivo* Delivery of RNA Therapeutics Using Non-viral Vectors

(A) Spontaneous hydrolysis of RNAs.
(B) Nuclease-mediated degradation.
(C) Protein adsorption.
(D) Immune recognition and clearance.
(E) Renal clearance.
(F) Endothelial barrier.
(G) Cell membrane barrier.
(H) Endosomal barrier.
(I) Insufficient cargo release.

tail saturation, the type of linker, the head group, and the pK_a,[43,45,46] leading to the discovery of best-in-class DLin-MC3-DMA (Figure 4C). Meanwhile, DLin-MC3-DMA or its analogs, together with phospholipid, polyethylene glycol (PEG) lipid, and cholesterol (Figures 4D–4F), were formulated into specialized liposomes (namely stable nucleic acid-lipid particle [SNALP]), which yielded potent and persistent *in vivo* gene-silencing activity after systemic delivery of siRNA therapeutics to the liver.[43,46,47] The absorption of endogenous apolipoprotein E onto the neutral surface is proposed to play an important role in SNALP-assisted RNA interference.[48]

Concurrently, combinatorial-chemistry synthesis and high-throughput screening was applied to identify novel ionizable lipid-like molecules (i.e., lipidoids) such as 98N_{12}-5.[49] This approach allows rapid generation of a diverse library of lipidoids, evaluation of their transfection abilities, and analysis of structure-function

Table 3. Advantages and Disadvantages of NM-Based RNA-Delivery Platforms

Delivery Platform	Advantages	Disadvantages
Lipid-based	FDA-approved, simple preparation, low immunogenicity, good biocompatibility, and high transfection efficiency	limited stability and rapid clearance
Polymer-based	chemical diversity, easy functionalization, synthetic scalability, good biocompatibility, and high transfection efficiency	low degradability and dose-limited toxicity for some cationic polymers, and high charge density
Peptide-based	straightforward design and synthesis, good biodegradability, and targeting capabilities	low stability and potential immunogenicity
Biomembrane-based	low immunogenicity and toxicity, long circulation half-life, self-targeting capabilities, and high transfection efficiency	difficult isolation, poor scalability, and limited stability
Inorganic NM-based	good uniformity and controllability, easy modification, and multifunctionality	complex synthesis, limited scalability, low degradability, and potential toxicity

relationships, which can increase the chance of finding an optimal structure or guide the next generation of lipidoids.[50] Using microfluidic mixing, lipidoids and other excipients are formulated into compact spheroid particles with either inverted micellar structures surrounding RNA molecules or multilamellar structures, which exhibit an average diameter of 50–120 nm.[51] In 2014, by screening 1,400 lipidoids generated from combinatorial reaction of alkylamines with alkylacrylate, Whitehead and coworkers summarized several empirical "efficacy criteria," including lipidoids comprising tertiary amines and at least three tails and a particle surface pK_a of 5.5–7.0, that could robustly predict the ability of LNPs to achieve potent *in vivo* gene silencing.[52] Recently, great efforts have been made to integrate extra functionalities into lipidoids and develop biodegradable,[53] bioreducible,[54] fusion-enhanced,[41] tumoricidal,[55] and vitamin-derived lipidoids.[56] Specifically, Miao and colleagues synthesized over 1,000 lipidoids using a one-step three-component reaction, and found that the top-performing lipidoid A18-Iso5-2DC18 with cyclic amino head group could activate the STING (intracellular stimulator of interferon genes) pathway, leading to superior mRNA delivery and vaccination efficacy.[57] Besides this, optimization of the constitution of LNP formulations has also been actively carried out to enhance RNA-delivery efficiency, such as optimizing molar composition and PEG length and using cholesterol analogs.[42,58,59] In a recent study, Cheng et al. introduced a supplemental selective organ-targeting (SORT) lipid into traditional LNPs and developed precisely controllable lung-, spleen-, or liver-targeted SORT LNPs. This technology holds great promise for protein replacement therapy and gene correction therapy in extrahepatic tissues.[60,61]

Polymer-Based Platform

Cationic polymers constitute another group of materials that are attractive for RNA delivery owing to their chemical diversity, facile functionalization, and synthetic scalability. They have long been used as non-viral vectors due to their ability to condense RNAs into nanosized polyplexes to deliver RNAs across cell membranes (Figure 5A).[6,7] Among various polymers (Figure 5B), polyethylenimine (PEI) is the most studied polycation due to its relatively high transfection efficiency. However, its broad application is hampered by substantial cytotoxicity, especially for the branched, high-molecular-weight PEI.[34] Chitosan, a natural cationic polysaccharide, and polyamidoamine, a dendrimer synthesized from methyl acrylate and ethylenediamine, have both been proposed as alternative polymeric materials with lower toxicity.[62] Since traditional polycations are usually associated with uncontrollable polyplex morphology, high charge density, non-specific interactions with blood components and non-target cells, tight binding with RNAs, and non-degradability, various functional modification strategies have been adopted to mitigate these issues (Figure 5C). Examples include ligand installation for targeted delivery,[63]

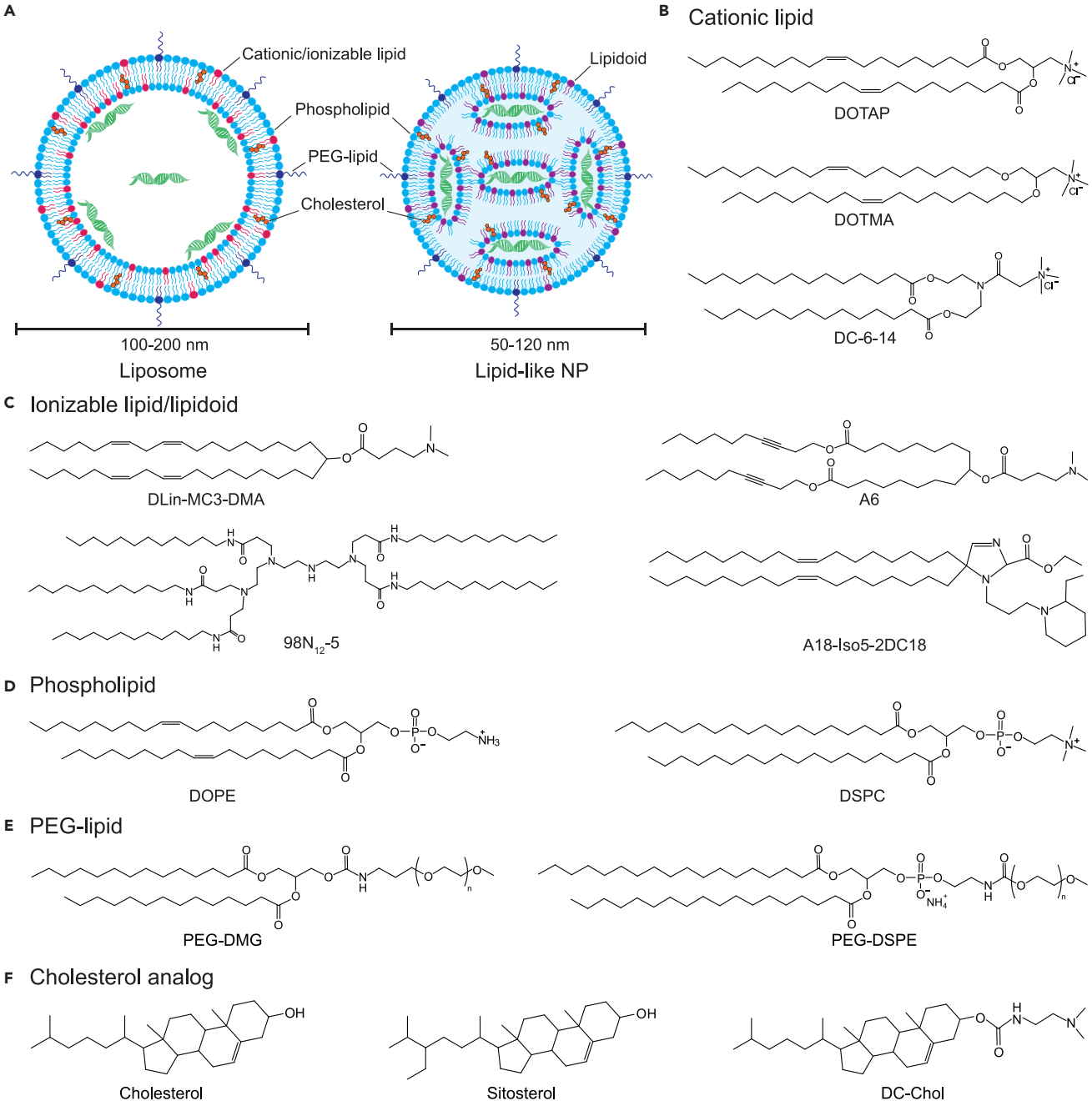

Figure 4. Synthetic Lipid and Lipid-like Materials for RNA Delivery

(A) Schemes of liposome and lipid-like NP. Liposome is presented as a unilamellar structure and lipid-like NP is presented as a spherical structure with inverted micelles surrounding RNAs.

(B–F) Chemical structures of representative cationic lipids (1,2-dioleoyl-3-trimethylammonium propane [DOTAP], DOTMA, and DC-6-14) (B), ionizable lipids (DLin-MC3-DMA and A6[41]) (C), lipidoids ($98N_{12}$-5 and A18-Iso5-2DC18) (C), phospholipids (dioleoylphosphatidylethanolamine [DOPE] and distearoylphosphatidylcholine [DSPC]) (D), polyethylene glycol (PEG) lipids (PEG-DMG [dimyristoyl glycerol] and PEG-DSPE [1,2- distearoyl-sn-glycero-3-phosphatidylethanolamine]) (E), and cholesterol analogs (cholesterol, sitosterol,[42] and DC-cholesterol [DC-Chol]) (F).

PEGylation for increasing stability and biocompatibility,[64] hydrophobic modification for improving assembly and enhancing potency,[65–67] disulfide crosslinking for bio-reducible degradation and accelerating RNA release,[68] and maleic acid amidation

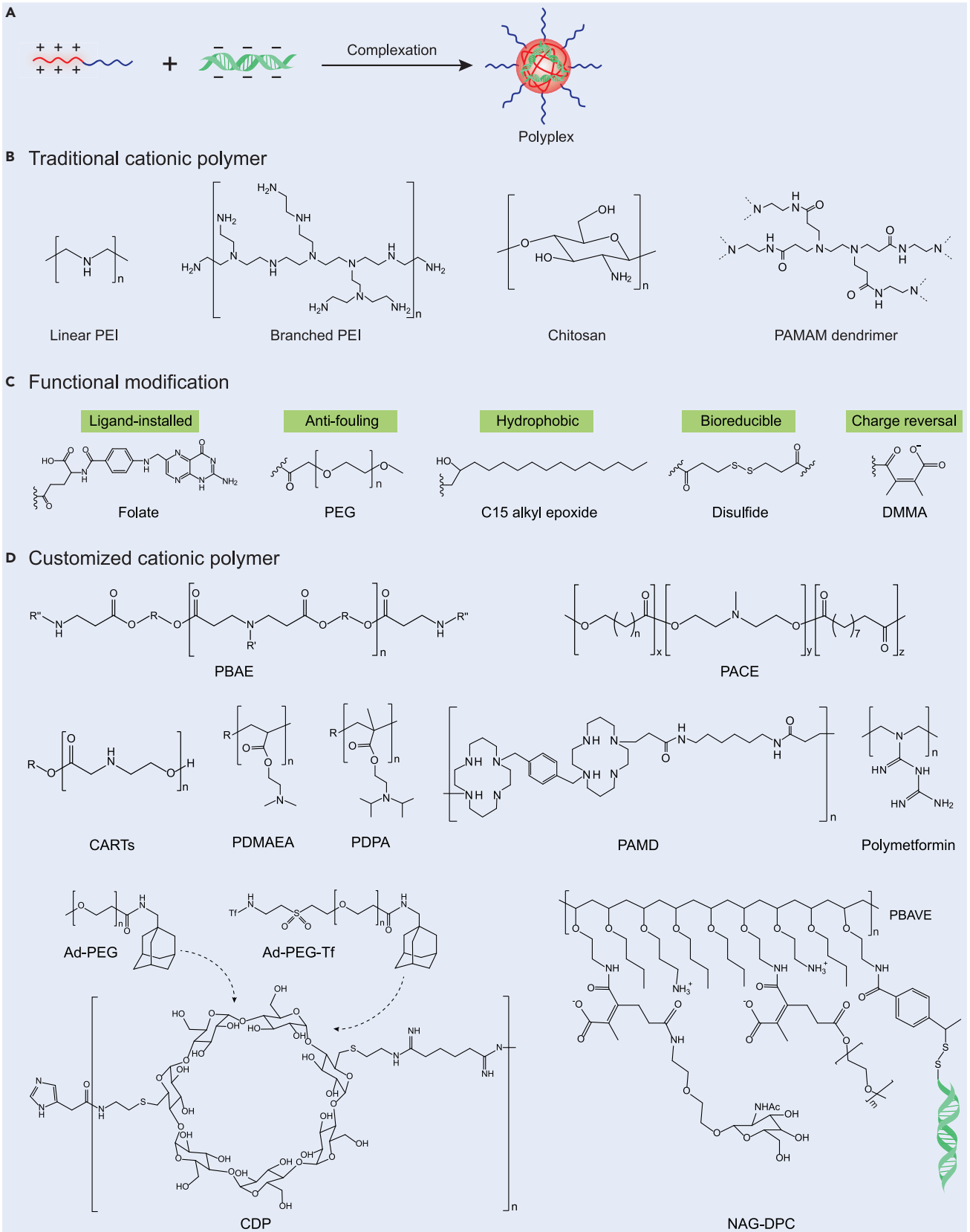
A
Complexation
Polyplex
B Traditional cationic polymer
Linear PEI
Branched PEI
Chitosan
PAMAM dendrimer
C Functional modification
Ligand-installed
Anti-fouling
Hydrophobic
Bioreducible
Charge reversal
Folate
PEG
C15 alkyl epoxide
Disulfide
DMMA
D Customized cationic polymer
PBAE
PACE
CARTs
PDMAEA
PDPA
PAMD
Polymetformin
Ad-PEG
Ad-PEG-Tf
PBAVE
CDP
NAG-DPC

Figure 5. Synthetic Polymeric Materials for RNA Delivery
(A) A scheme of polyplex formation between cationic polymers and RNAs.
(B and C) Traditional cationic polymers (B) and their functional modification (C).
(D) Chemical structures of customized cationic polymers. Ad-PEG, adamantane-PEG; Ad-PEG-Tf, adamantane-PEG-transferrin; PBAVE, poly(butyl amino vinyl ether).

for surface charge reversal and facilitating cell uptake.[69] Interestingly, modification of branched PEI or dendrimers with alkyl epoxides affords amphiphilic properties, promoting particle formation through hydrophobic aggregation and facilitating *in vivo* RNA delivery to endothelial cells with high specificity.[65,70,71] Additionally, reversible modification of polycations with maleic anhydride derivatives, such as 2,3-dimethylmaleic anhydride and carboxy dimethylmaleic anhydride, enables acidic pH-triggered charge reversal and PEG detachment, leading to enhanced tumor accumulation and gene silencing.[72,73]

In addition to the traditional polycations discussed above, custom-designed polymers with appealing features have also been developed (Figure 5D). Biodegradable polyesters bearing polymers such as poly(β-amino esters) (PBAE) and poly(amine-co-esters) (PACE) undergo enzymatic or non-enzymatic hydrolysis, which have been pursued as safe gene-delivery candidates. Although PBAE was initially developed for DNA delivery,[74] further optimization of the structure and composition has enabled efficient delivery of siRNAs and mRNAs, particularly to the lung.[75,76] Interestingly, PACE with low charge density achieves potent and safe RNA delivery as well, presumably due to the high molecular weight and increased hydrophobicity.[77,78] Recently, owing to the unique pH-triggered degradation mechanism, charge-altering releasable transporters (CARTs), an emerging polymer comprising a cationic oligo(carbonate-*b*-α-amino ester) domain and a hydrophobic block, have attracted great attention for mRNA vaccination therapy. At cytosolic pH, CARTs lose their positive charge through a controlled self-immolative degradation to a neutral, non-toxic small molecule, leading to rapid mRNA release into the cytoplasm for efficient translation.[79] Similarly, cationic poly(2-(dimethylamino)ethyl acrylate) (PDMAEA), which can degrade into benign poly(acrylic acid) and non-toxic 2-(dimethylamino)ethanol via a self-catalyzed hydrolysis mechanism, has been used for timed release of siRNAs.[80,81] A different strategy to accelerate RNA release is exploited in ultra-pH-sensitive copolymers that contain a hydrophilic PEG block and an ionizable poly(2-(diisopropylamino)ethyl methacrylate) (PDPA) domain.[82] Due to the protonation and reduced hydrophobicity,[83] the resulting micelles with a pK_a of ~6.2 undergo rapid disassembly in a narrow pH range corresponding to the endosomal pH, leading to early endosome escape, siRNA unloading, and efficient gene silencing.[82] Apart from bioresponsive polymers, great interest has been paid to synthetic polycations with intrinsic pharmacological activities. For example, PAMD, a polymer construction of a CXCR4 antagonist AMD300, can deliver genes and antagonize CXCR4 to inhibit tumor cell metastasis and tumor-stroma crosstalk, thereby improving the therapeutic outcome of gene oncotherapy.[84,85] Similarly, polymetformin, a polymeric derivative of the antidiabetic and anticancer drug metformin, is able to complex with siRNA while preserving pro-apoptotic activities, resulting in enhanced gene therapy against lung cancer.[86]

Several pioneered polymeric vectors have been tested in early clinical trials. Cyclodextrin polymer (CDP)-based NPs (namely CALAA-01) is the first targeted siRNA delivery system to enter clinical trials for cancer.[87] CDP is composed of linear polycationic oligomers containing cyclodextrin, which not only can complex with

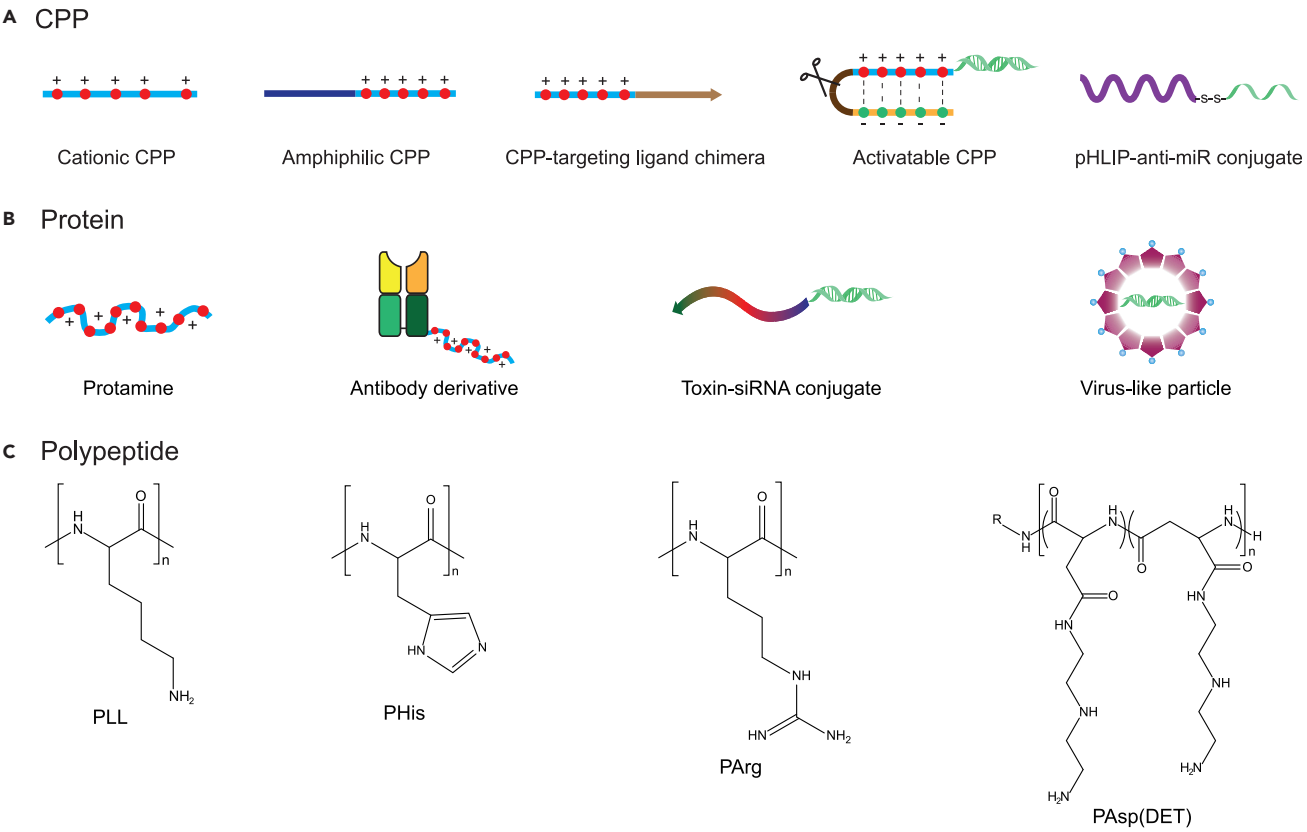

Figure 6. Peptide- and Protein-Based Materials for RNA Delivery

(A and B) Schemes of CPP-based (A) and protein-based (B) vectors.

(C) Chemical structures of representative polypeptides used for RNA delivery.

siRNAs but also can incorporate stabilizing (Ad-PEG) and targeting (Ad-PEG-Tf) moieties via host-guest complexation.[88] Another clinically advanced polymeric platform is Arrowhead's dynamic polyconjugate (DPC) system. The prototypical DPC (known as NAG-DPC) includes an endosomolytic poly(butyl amino vinyl ether) (PBAVE) polymer backbone that is reversibly linked by PEG, GalNAc, and siRNA.[73] Later, clinical tested new generation of DPC (known as NAG-MLP) is a two-molecule, coinjected system comprising a reversibly masked melittin-like peptide in place of the PBAVE polymer and a liver-tropic cholesterol-conjugated siRNA.[89] Unfortunately, despite some promising initial results,[90,91] clinical developments of CDP and DPC were both discontinued due to safety concerns.[21,92]

Peptide-Based Platform

Peptides are popular materials for RNA delivery due to their biocompatibility and structural flexibility.[93] Cell-penetrating peptides (CPPs) are a class of oligopeptides that can penetrate the cell membrane and translocate into the cytosol.[94] Various types of CPPs, including cationic CPPs, amphiphilic CPPs, CPP-targeting ligand chimeras, activatable CPPs and pH-(low)-insertion peptide (pHLIP), have been successfully used to deliver RNAs by either covalent conjugation or noncovalent complexation (Figure 6A).[94,95] For a more thorough discussion on CPP-mediated RNA delivery, we refer readers to reviews focused on this topic.[94,96] It is worth mentioning that modular design of tandem peptide containing different functional moieties enables cost-efficient screening of top-performing vectors with capabilities to overcome multiple barriers for systemic RNA delivery.[97,98]

Proteins and protein constructs also serve as promising carriers (Figure 6B). Protamines are a family of small arginine-rich proteins that can condense nucleic acids. Protamine/RNA complex and its liposomal formulation have been widely used for *in vivo* gene delivery.[99,100] Specifically, the complexation of mRNA with protamine not only enhances antigen production but also serves as a strong danger signal to activate toll-like receptors for enhanced immunostimulation.[101] This platform is under clinical development for cancer and virus vaccines by CureVac.[26] To achieve tissue and cell-specific delivery, RNAs can be either chemically conjugated or non-covalently complexed to modified antibody fragments,[102–104] engineered fusion protein assemblies,[105,106] and bacteria-derived toxins.[107] Of note, researchers have exploited the natural ability of viruses to deliver genes to host cells by developing artificial pseudovirus for RNA delivery. For example, virus-like particles (VLPs) assembled by viral coat proteins have been proved to efficiently package, protect, and deliver RNA payloads.[108,109] In one study, Li and coworkers developed recombinant bacteriophage MS2 VLPs that were able to completely package mRNAs, robustly prevent it from degradation for up to 18 h, and persistently transfect mammalian cells for 4 days.[108] Moreover, this prophylactic mRNA vaccine induced robust humoral and cellular immune responses and protected mice fully against prostate cancer challenge. Recently, Zheng and colleagues formulated PEGylated human papillomavirus (HPV) L1 protein-based VLPs with a high siRNA loading efficiency and long-term storage stability, which could stimulate innate immunity and further enhance PD-L1 knockdown-based immunotherapy.[109]

Synthetic polypeptides with polycationic properties represent another promising class of non-viral vectors (Figure 6C). Poly-L-lysine (PLL)-, polyhistidine (PHis)-, and polyarginine (PArg)-based homopolypeptides and block copolypeptides have been extensively studied for RNA delivery.[110] However, PLL has fairly poor transfection efficacy due to the lack of ionizable amino groups.[111] Since PHis bearing imidazole groups with a pK_a of ~6 has a strong proton buffering capacity at endosomal pH, grafting PHis segment into PLL can greatly improve its transfection activity,[112] supporting the clinical translation of polypeptide-based STP705.[113] Unlike PLL and PHis, PArg is suggested to shuttle siRNA into cells through transcytosis rather than endocytosis.[114] As a general strategy, polypeptides are also modified with PEG to form antifouling complexes with a long circulation half-life. Interestingly, PEG-PLL copolymer with a controlled degree of polymerization of PLL can spontaneously form a unit polyion complex (uPIC) with oligonucleotides through charge-matched polyionic complexation.[115] Owing to its longevity in the bloodstream and confined size, the uPIC achieved efficient gene delivery to poorly permeable tumors with remarkable antitumor activity. In addition to PLL, N-substituted polyaspartamide bearing aminoethylene repeats, a polypeptide derivative, has received great attention recently. Specifically, poly(*N*′-(*N*-(2-aminoethyl)-2-aminoethyl) aspartamide (PAsp(DET)) with a two-step protonation process at pH 9.5 and 6.0 demonstrates superior transfection efficacy compared with its analogs and has been extensively used for gene delivery.[110,116] However, for mRNA delivery, PAsp(DET) may not achieve sustained mRNA expression due to the unstable complexation, despite its rapid expression initially.[117]

Biomembrane-Based Platform

Exosomes are cell-derived, nanosized membrane vesicles with a size of 40–150 nm,[118] which function as natural transporters of biomolecules including RNAs for intercellular communication and gene transfer.[119] Due to their low immunogenicity and intrinsic ability to traverse biological barriers for intracellular RNA delivery, exosomes have generated great interest for the treatment of neurologic disorders,

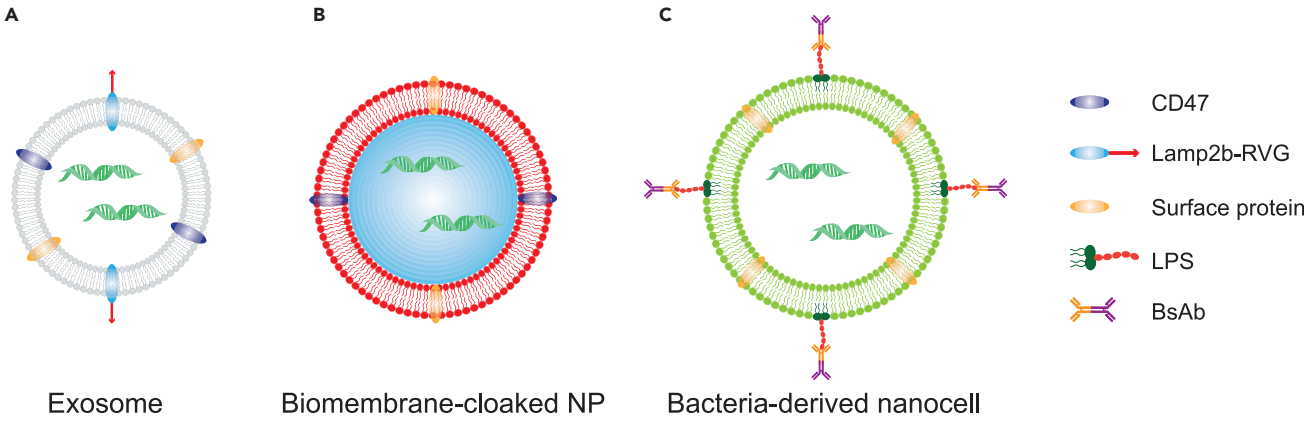

Figure 7. Biomembrane-Based Materials for RNA Delivery
(A) Neuron-targeting exosome.[120]
(B) Biomembrane-cloaked NP.
(C) Bacteria-derived nanocell.[124]

cancers, and other diseases.[120,121] An early example of exosome-mediated *in vivo* RNA delivery was demonstrated by Alvarez-Erviti et al., whereby they engineered dendritic cells to generate neuron-targeting exosomes with a rabies virus glycoprotein (RVG) peptide fused into its surface protein Lamp2b (Figure 7A).[120] The modified exosomes were able to cross the blood-brain barrier (BBB) and deliver siRNA specifically and safely into the brain, leading to 60% knockdown of a therapeutic target in Alzheimer's disease. In another study, Kamerkar and coworkers determined that exosomal protein CD47-mediated phagocytic prevention and Kras-stimulated macropinocytosis played important roles in enhancing specificity of exosomes to pancreatic cancer cells.[121] Therefore, exosomes carrying siRNA targeting oncogenic $Kras^{G12D}$ (iExosomes) demonstrated superior therapeutic efficacy compared with a liposomal counterpart in multiple mouse models of pancreatic cancer, which in turn has led to a clinical trial. Recently, to promote the encapsulation of exogenous RNAs and generate RNA-enriched exosomes, several universal techniques were developed, including using a cellular nanoporation biochip and integrating siRNA into pre-miR-452 hairpin.[122,123]

Despite their great promise discussed above, the isolation and large-scale production of exosomes remains technically challenging. As an alternative to exosomes, researchers have coated NPs with readily available cell membranes to generate biomimetic delivery platforms (Figure 7B). Membranes derived from blood cells can protect NPs from early clearance and immune activation due to their self-derived nature, and can endow lesion tropism for targeted delivery.[125] Recently, erythrocyte- and platelet-mimicking NPs have been shown to deliver therapeutic RNAs successfully.[126,127] For example, platelet membranes with specificity to CD24 expressed by cancer cells were used to camouflage NPs for targeted delivery of survivin siRNA for enhanced gene oncotherapy.[126]

Interestingly, exogenous bacteria-derived membrane vesicles (termed nanocells) produced by asymmetric cell division have displayed safe and efficient delivery of siRNAs and miRNAs in preclinical studies (Figure 7C).[128,129] Results showed that ~14,000 RNA duplexes could be packaged into a single nanocell (~400 nm). After functionalization with a bispecific antibody (BsAb) recognizing O-antigen of lipopolysaccharide and tumor surface antigen, ~30% of injected nanocells were localized in the tumor

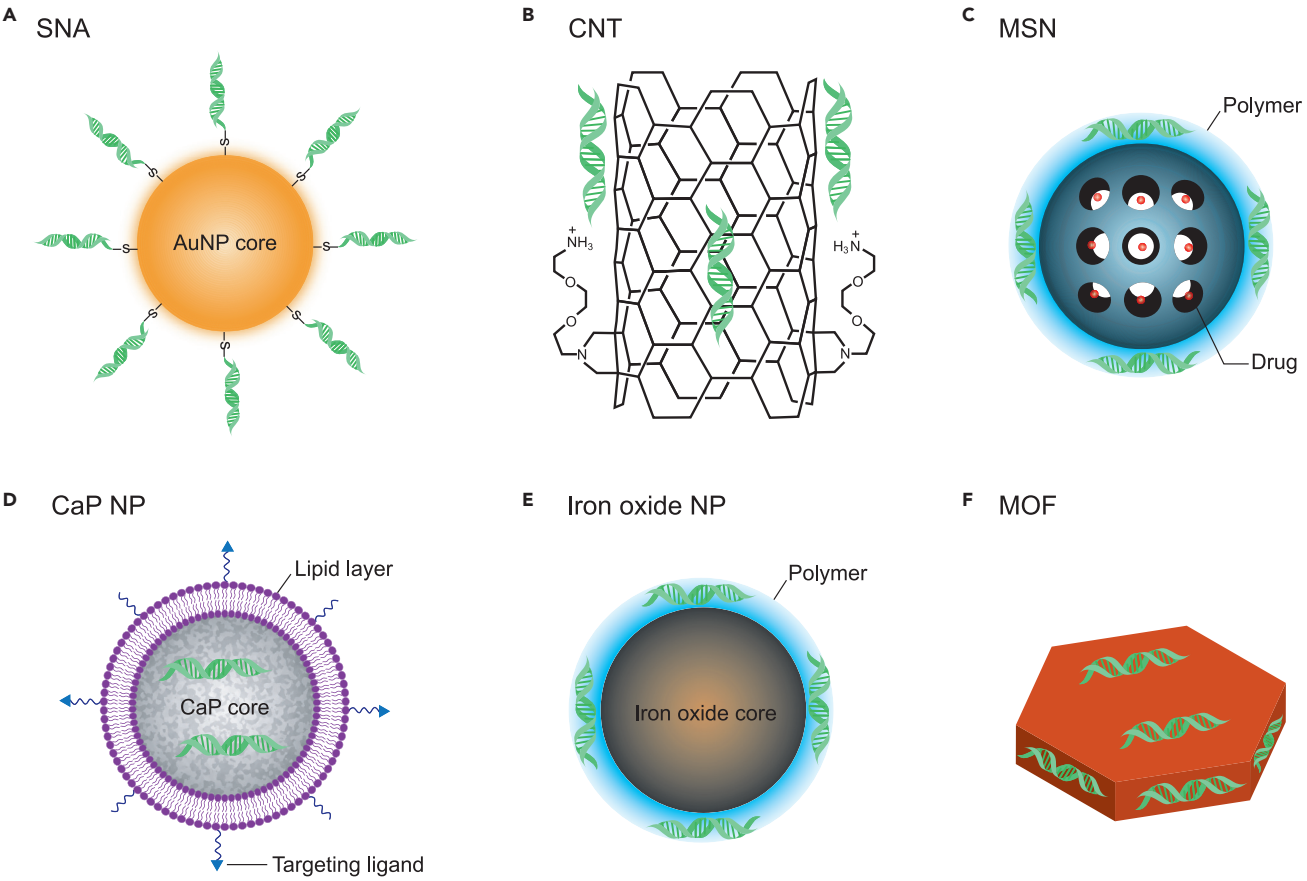

Figure 8. Representative Inorganic Nanomaterial-Based Platforms for RNA Delivery
(A) Spherical nucleic acid (SNA).
(B) Carbon nanotube (CNT).[140]
(C) Mesoporous silica nanoparticle (MSN).
(D) Calcium phosphate nanoparticle (CaP NP).[141]
(E) Iron oxide NP.
(F) Metal-organic framework (MOF).

following passive and active accumulation.[130] A phase I study revealed that miR-16 mimic-loaded nanocells (TargomiRs) were well tolerated except for elicited transitory inflammatory reactions,[124] which recently were proved to be beneficial due to the activation of innate and adaptive antitumor immune responses.[131]

Inorganic Nanomaterial-Based Platform

Inorganic NMs are popular vectors for RNA delivery due to their good controllability in morphology and multifunctionality such as imaging and stimulus-responsiveness.[132,133] It is noteworthy that inorganic NMs are typically decorated with polymers or lipids, and the resulting hybrid NMs are crucial for packaging, protecting, and delivering RNA payloads. Gold nanoparticles (AuNPs) are generally bioinert, simple to synthesize, and easy to functionalize via either thiol-gold bond or electrostatic absorption. Interestingly, thiolated siRNAs attached to AuNPs (Figure 8A), known as spherical nucleic acids (SNAs), show increased stability, high cellular uptake efficiency, and long therapeutic lifetime.[134] Moreover, SNAs were capable of effectively penetrating the BBB and blood-tumor barrier to silence oncogene Bcl2L12 and reducing tumor burden in xenografted mice without adverse side

effects.[135] This study laid the groundwork for the clinical translation of SNAs. Following the concept of SNAs, gold cores with different sizes, shapes, and modifications (e.g., drug and targeting molecules) have been developed to deliver thiolated RNAs for gene therapy and combination therapy.[136,137] In an alternative approach to gold-thiol bioconjugation, pristine RNAs are noncovalently absorbed onto the surface of polycation-capped AuNPs.[138] For example, PEG-PEI polymer-coated AuNPs have enabled potent siRNA delivery to pancreatic stellate cells,[139] which effectively silenced HSP47 and reduced pancreatic cancer-associated desmoplasia.

Other widely used bioinert carriers include carbon NMs and silica NPs. Functionalized carbon NMs have been reported to be viable vectors for delivering small RNAs *in vivo*, including carbon nanotubes (CNTs), graphene nanosheets, and nanodiamonds.[133,142] For example, ammonium-functionalized CNTs transported noncovalently bound siRNAs to renal proximal tubular cells after systemic administration (Figure 8B), which effectively halted the pathogenesis of acute kidney injury by silencing p53 and meprin-1β.[140] Among various types of silica NPs, studies have mainly focused on mesoporous silica nanoparticles (MSNs) due to their unique features including large surface area, tunable pore size, and the capability to accommodate guest molecules.[133] While MSNs are well recognized as an excellent drug reservoir, their surface functionalization with cationic polymers further facilitates electrostatic attachment of RNAs (Figure 8C), making them a promising platform for combination therapy.[143]

In addition to non-degradable NMs, there is a growing interest to use dissolvable NMs for a reduced risk of long-term accumulation in tissues. Calcium phosphate (CaP), which can form CaP-RNA complex between calcium ions and phosphate residues on the nucleic acid backbone, have long been used for chemical transfection of mammalian cells with little toxicity.[144] CaP can rapidly dissolve at acidic endosomal pH, causing osmotic swelling to release its cargo into the cytosol. A targeted lipid-coated CaP NP developed by Huang's group greatly facilitated systemic siRNA delivery to tumors compared with previous formulations (Figure 8D).[141] Iron oxide NPs, which have been approved for magnetic resonance imaging, are another acid-dissolvable carrier. The incorporation of siRNA into polymer- or lipid-coated iron oxide NPs generates multifunctional theranostic platforms that are suitable for simultaneous imaging and gene therapy (Figure 8E).[145,146] Since iron oxide NPs are capable of remotely controlled targeting and heating via the application of an external magnetic field, targeted transfection and combination therapy can be achieved.[147] Metal-organic frameworks (MOFs), formed by sensitive coordination bonds between inorganic metal ions and organic ligands, are another interesting vector due to the high surface area, porosity, and biodegradability (Figure 8F).[148] RNAs can be immobilized onto MOFs by coordination interaction,[149] physical encapsulation,[150] or *in situ* biomineralization,[126] and be released upon structural collapse in response to exogenous stimuli,[148] thereby enhancing delivery efficacy.

PERSPECTIVE AND OUTLOOK

The potential of RNA therapeutics (ASOs, siRNAs, miRNAs, and mRNAs) for the treatment of genetic diseases, metabolic disorders, infectious diseases, neurological disorders, and cancers is immense. For example, mRNAs with flexibility in the design and expression of antigens as well as large-scale manufacturing potential enable rapid vaccine development for infectious diseases. Currently, the global public health crisis of the COVID-19 pandemic has put mRNA vaccines under the

spotlight, as mRNA therapeutic companies started clinical testing of LNP-formulated mRNA vaccines within 2 months after identification of the virus sequence.[151] Recently, initial testing revealed positive clinical readout, whereby all vaccinated participants developed antivirus immune responses with no trial-limiting safety concerns.[8] Additionally, some RNA companies (e.g., Alnylam) are also developing RNAi therapeutics to interfere with the replication of SARS-CoV-2.

As mentioned above, NMs with the ability to enhance the stability and transfection efficacy of RNA therapeutics, while reducing unwanted side effects, show great promise for safe and effective gene delivery. However, there remain several challenges that must be addressed to achieve clinical translation of many of these NMs.

First, biosafety is a prerequisite for clinical translation of NMs. The recent successes and setbacks in the clinical development of non-viral RNA platforms emphasize the importance to minimize the complexity and toxicity of excipients in order to reduce the risk of adverse effects.[152] For example, clinical development of sophisticated NAG-MLP was recently halted due to safety issues of the excipient melittin, regardless of the promising therapeutic outcomes.[21,152] In contrast, patisiran, consisting of a generally biocompatible LNP, received FDA approval as the first siRNA drug. The maturity and success of the LNP platform has facilitated the clinical development of aforementioned COVID-19 mRNA vaccines. Biodegradable polymer-based NMs presumably have fewer regulatory hurdles. Nevertheless, the key hurdle for polymers is their high intrinsic complexity in parameters such as chemical composition, molecular weight and polydispersity, polymer impurities, size, architecture, and charge density.[153,154] PEGylation as a general strategy can increase the biocompatibility of synthetic NMs through shielding surface charge, avoiding immune recognition, and preventing non-specific interactions with blood components. However, PEG can induce anti-PEG antibodies and complement activation, which is associated with anaphylaxis, accelerated blood clearance and reduced efficacy of RNA therapeutics that require repeated dosing.[12,155] Therefore, developing a safer and more effective modification approach beyond PEGylation will benefit the RNA therapeutics field.[156] Additionally, self-derived exosomes are promising candidates due to their low toxicity and immunogenicity. While inorganic NMs generally face safety concerns, a clinical trial of a AuNP-formulated siRNA drug (SNAs) has been initiated.

Second, the preparation of NMs and their subsequent formulation with RNA must be scalable, reproducible, and stable for therapeutic applications. As for LNPs, microfluidic mixing and crossflow injection have emerged as alternative methods to conventional lipid-film hydration or ethanol injection for improved scalability, reproducibility, and encapsulation efficiency.[31] Some cationic polymers or peptides can be cost-effectively synthesized, but their electrostatic complexation with RNAs requires precise control of mixing to achieve well-controlled physicochemical properties and high-quality scale-up production.[132] While exosomes have long suffered from limited availability, recently group O blood-derived erythrocytes were used as universal donors for large-scale production of exosomes,[157] which could be scalable vectors for RNA delivery. Inorganic NMs can be manufactured with good uniformity and controllability, but are usually associated with complex synthesis, modification, and purification as well as low RNA-loading capacity.

Third, developing NMs for extrahepatic RNA delivery is highly desirable. Currently, GalNAc-small RNA conjugate is the only viable molecular platform that works specifically for hepatocyte targeting due to the highly expressed ASGPR (>10^6

copies/cell) and its rapid recycling (<15 min).[21] No other ligand-small RNA conjugates have been reported to achieve similar delivery potency to extrahepatic tissues. In contrast, NMs have the potential to deliver RNAs, including large mRNA payloads, to both hepatic and extrahepatic tissues. However, with so many factors that can affect the biodistribution of NPs, it is challenging to rationally design NPs for targeting specific tissues.[61] The aforementioned SORT technology, by simply introducing a supplemental lipid to tune the net surface charge of LNPs, can be a universal methodology that allows precise delivery of various RNAs to liver, lung, and spleen.[60] Additionally, genetic or chemically engineered exosomes are promising reservoirs for targeted transporting of RNAs to difficult-to-transfect tissues such as the brain and tumors.[120,121] Other antibody- or peptide-conjugated NPs can also achieve tissue- and cell-specific targeting, but the extra cost of production and regulatory hurdles should be considered.[158]

The approval of patisiran has inspired both academia and industry and has spurred a new wave of *in vivo* delivery of RNA therapeutics. The lessons learned from non-viral delivery of RNAi therapeutics will greatly promote the development of other RNA therapeutics. There is no doubt that NMs will play an increasing role in formulating RNA-based drugs and expanding accessible targets for the treatment of previously untreatable diseases. Notably, apart from delivering RNA therapeutics with transient activity, NMs also exhibit great potential for delivering CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated 9)-based therapeutics for genome editing to treat or even cure diseases. Several aforementioned NMs, including LNPs and AuNPs, have been proved to deliver therapeutic Cas9 mRNA/single guide RNA (sgRNA) or Cas9-sgRNA ribonucleoprotein complexes *in vivo* with high efficiency,[61,159–162] highlighting the compatibility of NMs with a variety of RNA payloads. Based on current advances, there is still plenty of room for innovation and optimization in the development of NMs, which in turn will provide enormous support for the broad application of RNA therapeutics in the near future.

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AUTHOR CONTRIBUTIONS

Conceptualization, X.H., M.J.M., and G.N.; Investigation, X.H.; Writing – Original Draft, X.H.; Writing – Review & Editing, X.H., M.J.M., and G.N.; Funding Acquisition, M.J.M. and G.N.; Supervision, M.J.M. and G.N.

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