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REVIEW

Advances and prospects of RNA delivery nanoplatforms for cancer therapy



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Abstract Modern oncology is rapidly evolving, driven by recent advances in RNA-based therapeutics. As new emerging cutting-edge technology, mRNA vaccines hold excellent promise for encoding immunostimulatory molecules, tumor-associated antigens, neoantigens, and chimeric antigen receptors for T-cell reprogramming. RNA interference tools enable highly effective post-transcriptional gene silencing that has rapidly progressed towards more tailored antitumor treatments targeting key molecular players in tumor progression and drug resistance. The inherent challenges and limitations of RNA-based tools, such as size, low stability and surface charges hindering direct cell entry, along with the short circulatory half-life and rapid clearance, call for new and improved RNA delivery systems enabling enhanced gene delivery. Nanoplatforms, particularly certain types of lipid, polymeric nanoparticles and inorganic nanoparticles, provide designed means to address the challenges of RNA delivery and cellular uptake. This paper explores the challenges and barriers while giving insight into the future perspective of RNA-based cancer therapeutics in the context of delivery nanoplatforms and the challenges during development.

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1. Introduction

The incidence of malignancies has grown steadily over the past few decades, contributing to significant mortality worldwide. Despite advancements in interventions like tumor resection, radiotherapy, and chemotherapy, cancer continues to claim many lives. Even though conventional cancer treatments primarily target the main malignant lesion, effectively curtailing metastatic dissemination to other organs remains a hurdle to successful treatment¹. Ongoing efforts aim to eradicate these circulating and metastatic tumor cells, among which innovative RNA-based therapeutics such as immunotherapeutic messenger RNA (mRNA) vaccines and antisense therapy are emerging promising avenues in this regard^{2,3}.

The first acknowledgement of the active role of the immune system can be traced back to around three millennia ago when the ancient Egyptians observed tumors vanishing following infections⁴. In 1868, Busch noted tumor regression in erysipelas-infected cancer patients⁵, and the hypothesis of bacterial infections for cancer treatment was further confirmed and advanced by William Coley^{6,7}. Morales et al.⁸, in 1976, further implemented Coley's concepts by applying Bacillus Calmette-Guérin (BCG) vaccine to treat bladder cancer. In 1909, Paul Ehrlich laid the foundation of the immunosurveillance hypothesis, describing the immune system as a critical defense mechanism to thwart the development of carcinomas by suppression and elimination of nascent cancer cells^{9,10}. Nowadays, immunotherapies are poised as key inspiring frontiers for the future of oncology¹¹, to restrict tumor growth and extend patient survival through the enhancement of the patient's antitumor immunity while surmounting the suppressive tumor microenvironment (TME)¹².

Immunosuppressive patients are facing a higher risk of developing cancers^{13,14}, frequently seen in transplant patients, which highlights a strong link between immunity and cancer. Cancer immunotherapies deploy the immune system as a tumor cell-killing tool, despite the risk of triggering undesirable immune-related adverse events (irAEs). Modern immunotherapies

have shown the ability to induce long-term remission with fewer side effects than standard chemotherapies¹⁵. Antibody-, protein-, and cell-based approaches have now been established as successful immunotherapies, with mRNA-based therapies emerging as an advancement of protein replacement strategies addressing some of the constraints associated with existing treatments¹⁶.

The journey of mRNA therapies in medical research arose in the late 1970s with liposomal *in vitro* delivery of globulin-encoding mRNA into human epithelial carcinoma cells (Fig. 1)¹⁷. Breakthroughs were made with the laboratory synthesis of biologically active mRNA *via* a bacteriophage-derived enzyme, SP6 RNA polymerase, in 1984¹⁸. Later in 1993, Transgene developed a liposomal formulation for mRNA that elicited an antiviral immune response in mice¹⁹. Vical and Merck attempted to develop an mRNA vaccine for influenza in 1991; however, the efforts were aborted due to the high manufacturing costs of mRNA²⁰. Although this era predominantly favored DNA vaccines, efforts to scale-up mRNA production laid the basis for future mRNA vaccine research.

Clinical trials demonstrated the promise of mRNA-based vaccines when introduced through safe and efficient transfection methods. Many ongoing preclinical and clinical developments explore mRNA-based vaccines and RNA interference technologies, offering a personalized approach to cancer treatment that leverages the body's immune response¹⁵. *In vitro* transcribed mRNAs as cancer vaccines present precision and versatility in controlled protein expression without the need for nuclear entry²¹. Direct intramuscular injection of mRNA-encoding viral, tumor-associated antigens and neoantigens has been demonstrated to express vaccine components effectively. In addition, *ex vivo* transfection of dendritic cells (DCs) with mRNA and reintroduction of mRNA DC as a vaccine presents an alternative approach to physical methods of vaccine delivery²². The recent development of COVID-19 vaccine technology during the pandemic has sparked interest in using this technology for cancer treatment. Despite the pre-established efficacy demonstrated by mRNA vaccines, their potential as cancer treatments is still under evaluation, with ongoing clinical trials for safety and efficacy assessment.

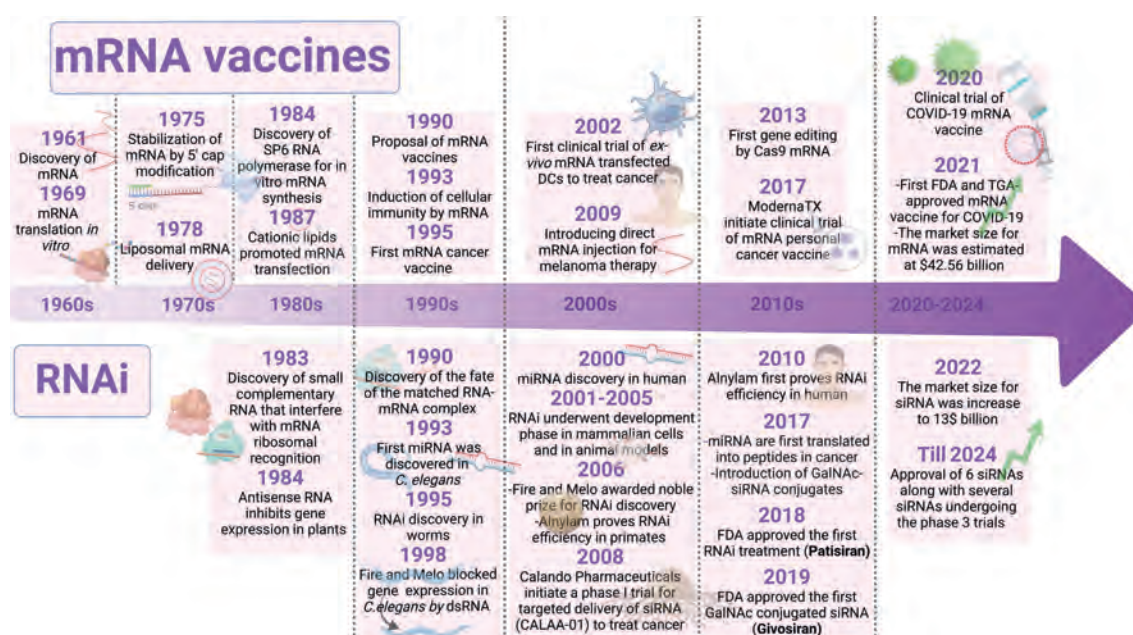


Figure 1 Timeline for the progress of mRNA and RNAi therapeutics. Figure was made by Biorender.com.

Since 1998, researchers have also exploited RNAi tools that comprise small interfering RNA (siRNA), circular RNA, short hairpin RNA (shRNA), and microRNA (miRNA) to identify cancer-related genes, elucidate complex regulatory pathways and validate potential therapeutic targets²³. RNAi has paved the way for tailored cancer treatments by targeting key drivers of cancer progression and drug resistance, which in turn inhibit tumor cell proliferation and are promising in treating cancer. Recent advances in the delivery of nucleic acids using lipoplexes, polyplexes, lipoplexes, or exosomes have overcome some of the challenges related to instability, innate immune responses, and ineffective transfection²⁴.

This paper reviews the new development of RNA therapeutics in cancer therapy along with the recent nanotechnological advances in RNA delivery. In addition to discussing the challenges and struggles impeding their efficacy, the article also presents a glimpse into these therapies' promise and prospects for cancer treatment.

2. The role of RNA therapeutics in cancer therapy

Recent reports demonstrated the potential of a wide variety of RNA-based therapies, with some approved by the US Food and Drug Administration (FDA). These include coding mRNAs and noncoding RNAs such as antisense oligonucleotides (ASOs), siRNAs, miRNAs, and RNA aptamers²⁵. Recently, mRNA therapeutics have been employed for protein replacement therapy, generating antigens that trigger immunity or expressing components of gene-editing tools (Fig. 2)²⁶.

The development of RNA-based therapeutics emphasizes two key strategies. The first relies on mRNA vaccines that encode specific antigens or defective proteins/peptides to transiently

initiate targeted immune responses. Presenting cancer antigens during immune stimulation, mRNA vaccines can induce tumor-specific T-cell responses, ultimately leading to tumor apoptosis. mRNA vaccines can potentially deliver tumor-specific antigens (TSA) or tumor-associated antigens (TAAs), along with activating innate immunity. Second, RNAi are designed to attach to the complementary sequences in endogenous RNA transcripts and alter their processing²⁷. The RNAi approach enables precise regulation of intracellular checkpoints, offering druggable targets for precisely controlling inflammatory and immune reactions. Table 1 presents the clinical translation of mRNA-based vaccines and RNAi approach, accompanied by details of the administration route, dosing, delivery vector, or method for each registered trial, along with the corresponding treated cancer type.

2.1. mRNA vaccines

Common vaccine strategies that include attenuated or inactivated viral vaccines have proven successful in defense against the progression of infections. However, this approach is not applicable to the evolution of tumor vaccines²⁸. A significant step forward has been made in cancer immunotherapy and infectious disease prevention with mRNA-based therapies. This has been seen by investigational vaccines for cancer, as well as influenza, HIV, and Zika viruses, and ultimately, the approved COVID-19 vaccines^{29,30}. A notable advantage over traditional vaccines is that mRNA development is fast and scalable, coupling with mRNA's dual activity: activating immune system response while translating immunostimulant antigens or molecules^{28,31}. Moreover, mRNA bears a low mutation risk since nuclear entry and transcription are

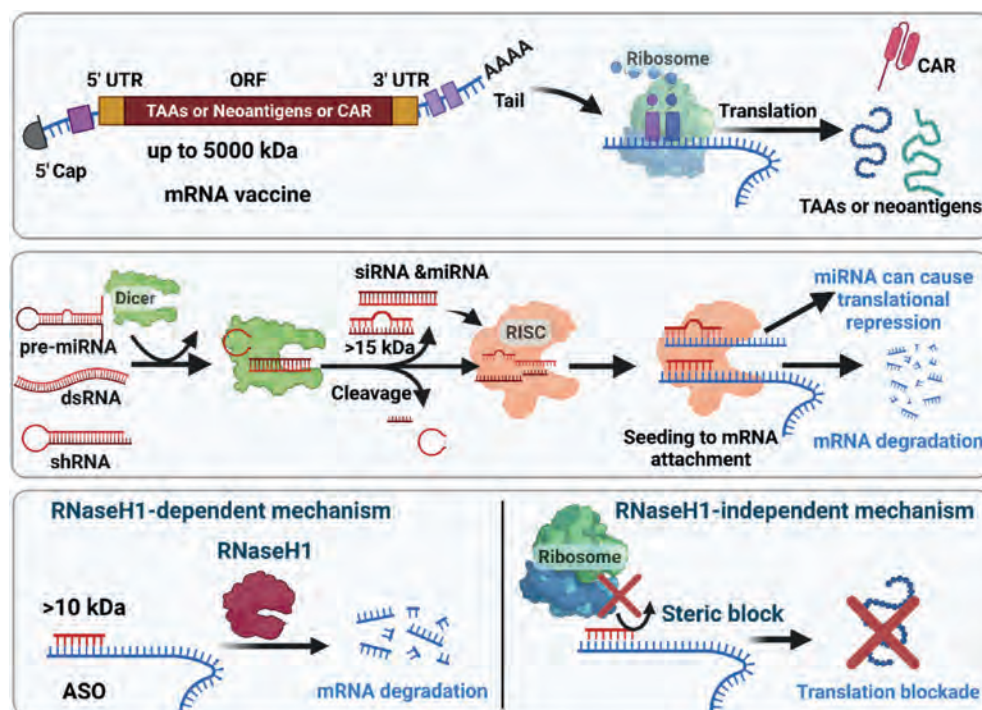


Figure 2 Mechanism of RNA therapeutics for cancer immunotherapy. mRNA vaccines function through transcription into antigens or specific immunostimulatory proteins. Similarly, siRNAs and miRNAs interfere with mRNA, causing degradation or translational repression. ASOs act through RNaseH1-dependent and independent mechanisms, resulting in the blocking of translation or mRNA degradation. Figure was made by Biorender.com.

Table 1 Completed and ongoing clinical trials using mRNA and RNAi-based therapeutics for cancer therapy.

Cancer type	Target protein expression/knockdown	Product name	Delivery system/method	Route of administration	Dosing	Phase	Trial start date	Completion date	National Clinical Trial (NCT) Identifier Numbers
MPM	mRNA	Anti-mesothelin	Electroporation	I.V.	Participants received one dose of 1×10^8 cells on Day 0, followed by one dose of 1×10^9 CAR-T cells on Day 7	I	2011	2015	NCT01355965
					Participants received three doses of 1×10^8 cells on Days 0, 2, and 4, followed by three doses of 1×10^9 T-cells on Days 7, 9, and 11				
					1 to 3×10^8 T-cells/m ² were given thrice weekly for three weeks				
					Participants received a single dose of two levels, 3×10^7 or 3×10^8 cells				
Metastatic PC			Electroporation	I.V.	In phase Ia: one dose of T-cells (0.5×10^6 /kg) was administered two days after rituximab (500 mg/m ²). If there is no toxicity and a positive response, a second dose (0.5×10^7 /kg) may be given 28 days later. IL-2 (1 million IU/m ² /day) was given subcutaneously for three days post-infusion	I	2013	2017	NCT01897415
Metastatic BC	Anti-c-Met	cMet redirected T-cells	Electroporation	I.T.		0	2013	2018	NCT01837602
B-CLL, and NHL	Anti-CD16V-BB- ζ	CD16V-BB- ζ redirected T-cells	Electroporation	I.V.		I/II	2014	Unknown	NCT02315118
Solid tumors Stage III/IV melanoma	Neoantigen Neoantigen	mRNA-4157 (V940) mRNA-4157 (V940)	LNP LNP	I.M. I.M.	Participants receive mRNA-4157 via I.M. every 21 days for up to 9 cycles	I IIb	2017 2019	Ongoing 2023	NCT03313778 NCT03897881
					RO7198457 was tested at 25, 38, 50, 75, and 100 μ g. Doses of 25, 38, and 50 μ g were combined with 1200 mg atezolizumab every three weeks. In the induction phase, RO7198457 was given weekly and biweekly for eight doses, with a boost in cycle seven and maintenance every eight cycles. Atezolizumab was administered on Day 1 of each 21-day cycle	I	2017	Ongoing	NCT03289962
					The dose amount was not stated. Administration of 8 weekly doses of Cevumeran starts at week 9 ± 2 post-tumor resection	I	2019	Ongoing	NCT04161755
Locally advanced tumors (melanoma, NSCLC, BC, CC, TNBC, RC, and HNC)	Neoantigen	Cevumeran or RO7198457	Lipoplex	I.V.					
PC									

(continued on next page)

Table 1 (continued)

Cancer type	Target protein expression/knockdown	Product name	Delivery system/method	Route of administration	Dosing	Phase	Trial start date	Completion date	National Clinical Trial (NCT) Identifier Numbers
Refractory LC	HBsAg	HBV mRNA vaccine	—	I.M.	Starting at 20 µg, doses increased per scheme. Each subject received one dose weekly for four weeks, with a 5th dose after one month	I	2023	Ongoing	NCT05738447
Refractory advanced solid tumors	CLDN6	BNT211	Lipoplex	I.V.	Patients received a single intravenous infusion of T-cells at a dose of 1×10^7 or 1×10^8 cells following lymphodepletion with cyclophosphamide (500 mg/m^2) and fludarabine (30 mg/m^2) on Days –5 to –3. CARVac is administered at 25 µg, with subsequent doses at 50 µg every 3 or 6 weeks	I/IIa	2020	Ongoing	NCT04503278
T-ALL and LBL	CD7	WU-CART-007	—	I.V.	Patients are administered four dose levels (ranging from 1 to 9×10^8 cells) of a single infusion of WU-CART-007 on Day 1, following lymphodepletion with fludarabine and cyclophosphamide from Days –5 to –3	I/II	2022	Ongoing	NCT04984356
Advanced solid tumor	siRNA Protein kinase N3	Atu027	Liposome	I.V.	Single and repeated infusions of Atu027 twice weekly over a 28-day cycle, with doses escalating from 0.001 to 0.336 mg/kg, were well tolerated, as was the maximum dose	I	2009	2012	NCT00938574
Solid tumors	VEGF and KSP	ALN-VSP02	LNP	I.V.	Patients were allocated to 7 dose levels (0.1–1.5 mg/kg)	I	2010	2012	NCT01158079
AAC and NETs	PLK1	TKM-080301	LNP	I.V.	Patients administered 0.6 or 0.75 mg/kg per week for up to 18 cycles	I/II	2010	2015	NCT01262235
Primary or secondary LC	PLK1	TKM-080301		H.I.A.	The starting dose received was 4 mg/m^2 , given every two weeks for up to 12 doses	I	2011	2012	NCT01437007
Advanced HC	PLK1	TKM-080301		I.V.	The starting dose was 0.3 mg/kg, increasing to 0.75 mg/kg, with a recommended MTD of 0.6 mg/kg. It was administered once weekly for three weeks and repeated every 28 days for up to 6 cycles	I/II	2014	2016	NCT02191878

Advanced PAAC	KRAS	siG12D LODER™	Biodegradable polymeric implant	I.T.	In three escalating cohorts, a single dose of siG12D-LODERs (0.025, 0.75 and 3.0 mg) was administered, followed by weekly gemcitabine	I/IIa	2010	2013	NCT01188785
PDAC and PC	KRAS	siG12D LODER™		I.T.	2.8 mg of siG12D-LODERs every 12 weeks alongside chemotherapy	II	2012	Unknown	NCT01676259
Advanced solid tumors	EphA2	EPHARNA	Liposome	I.V.	NS (dose-escalation study)	I	2012	2025	NCT01591356
PAAC	Protein kinase N3	Atu027	Liposome	I.V.	Atu027 was given at 0.253 mg/kg, and gemcitabine at 1000 mg/m ² was administered on Days 1, 8, and 15, followed by a one-week off	Ib/IIa	2013	2016	NCT01808638
GSM and recurrent GBM	BCL2L12	NU-0129	Spherical Nucleic Acid Gold Nanoparticle	I.V.	Patients received 1, 4, and 8 mg/kg of siRNA doses	0	2017	2022	NCT03020017
Metastatic PAAC, PDAC, and stage IV PC	KrasG12D mutation	KRAS G12D siRNA	MSCs-derived exosomes	I.V.	NS, dose-escalation study. Doses are given on Days 1, 4, and 10, with cycles repeating every 14 days for up to 3 cycles	I	2018	Ongoing	NCT03608631
MPM, and NSCLC	miRNA Mir 16	TargomiRs miRNA mimic	Nonliving bacterial nanocells (TargomiRs)	I.V.	All patients began on a microdose of 1 billion once a week and escalated to the full phase 1 dose on week 3	I	2015	2017	NCT02369198
Lymphomas and leukemia	Mir 155	MRG 106 (Cobomarsen) Anti-miR	Chemical modification (LNA)	I.T. I.V.	Patients received up to five I.T. injections of Cobomarsen over 15 days, starting at a maximum dose of 75 mg, with possible dose reductions to find the minimum effective dose. Otherwise, they received S.C. Cobomarsen (900 mg) on Days 1, 3, and 5, then weekly until intolerance, significant side effects, or disease progression	I	2015	2020	NCT02580552

AAC: adrenal cortical carcinoma; BC: bladder cancer; BC: breast cancer; B-CLL: B-cell chronic lymphocytic leukemia; CC: colorectal cancer; GSM: gliosarcoma; GBM: glioblastoma; HC: hepatocellular carcinoma; H.I.A.: hepatic intra-arterial; HNC: head and neck cancer; I.M.: intramuscular; I.T.: intratumoral; I.V.: intravenous; LC: liver cancer; LNA: locked nucleic acid; LBL: lymphoblastic lymphoma; MPM: malignant pleural mesothelioma; MSCs: mesenchymal stromal cells; MTD: maximum tolerated dose; NA: not applicable; NETs: neuroendocrine tumor; NHL: non-Hodgkin's lymphoma; NS: not stated; NSCLC: non-small cell lung cancer; PAAC: pancreatic adenocarcinoma; PC: pancreatic cancer; PDAC: pancreatic ductal adenocarcinoma; RC: renal cancer; S.C.: subcutaneous; TNBC: triple negative breast cancer; T-ALL: T-cell acute lymphoblastic leukemia.

not necessary for translation into amino acids, and mRNA is rapidly eliminated by cellular enzymes.

The large size of mRNA molecules and their high negative charges make them inaccessible to the body and must be delivered with reliance on delivery systems³². When handling mRNA, a significant concern is the maintenance of its stability³³, whether in clinical or preclinical studies. mRNA is highly susceptible to oxidative damage and enzyme degradation, both *in vitro* and *in vivo*, and therefore, must be handled under sterile and RNase-free conditions. Besides that, mRNAs are immunogenic molecules, and activating immunity against mRNA may compromise the expression of target proteins and thereby thwart their therapeutic efficiency³⁴. Considering sequence design is also essential to unleash the potential of mRNA. As a consequence, poor mRNA sequences can lead to unintended interactions leading to adverse events and possibly irAE³⁵, which may necessitate corrections to the therapeutic regimen. Finally, the overall success of the previous efforts to make mRNA deliverable is dependent on endosomal egress, as post-cellular uptake of mRNA molecules embeds a considerable amount within endosomes, rendering it non-functional³⁶. To successfully implement mRNA therapies in clinical practice, these limitations need to be tackled.

The mRNA structure significantly influences translation efficiency and subsequent immune effects³⁷. The inclusion of the 5' capping sequence protects mRNA from exonuclease damage and promotes effective translation, whereas the poly(A) tail promotes ribosome recruitment and stabilizes mRNA³⁸. Moreover, the secondary stem-loop or hairpin structures within coding sequences or unoptimized untranslated regions (UTRs) may interfere with the ribosome moving along the mRNA³⁸. The design of 5'UTR should avoid the start codons, and incorporating the 3' UTR of α - and β -globin usually enhances mRNA stability and translation^{38,39}. Still, mRNA's secondary structure is essential to confer protection from endo and exonuclease, which would prolong its half-life. Nevertheless, reports indicate that while high GC content can affect the secondary structure of mRNA, it also notably improves translation efficiency compared to the low GC counterparts⁴⁰. Hence, achieving optimal antigen expression may involve algorithmic strategies to maximize the content of highly expressed GC nucleotides⁴¹. Moreover, when optimizing codons, it is important to select codons corresponding to abundant tRNA species to accelerate translation and avoid ribosomal pauses. Also, altering the structure and stability of mRNA can be achieved by substituting nucleosides or adding chemical groups, such as N1-methyl-pseudouridine or pseudouridine^{42–44}. Further, nucleoside-modified mRNAs are less immunogenic, resulting in improved translation efficiency and protein synthesis.

Enhancing mRNA transfection efficiency by non-viral delivery tools mitigates the immunogenicity risks²⁸. Several mRNA-based vaccine immunotherapies are undergoing clinical trials for cancer treatment due to their unique capability to trigger immune responses through translating cancer antigens and other immunostimulants⁴⁵. One strategy is to use mRNA vaccines encoding TAAs, which induce reasonable immune responses and showed promise in pre-clinical studies and further progressed to clinical trials for oncology⁴⁶. The development of mRNA vaccines encoding TAAs was planned to primarily express antigens in malignant cells^{12,47}. Successful development of efficient TAA-encoded mRNA vaccines depends on antigen selection based on their expression levels and frequency, as well as tissue specificity in the target cancer⁴⁸.

In patients with advanced melanoma, the tetravalent BNT111 lipoplex encoding melanoma-associated antigens generated

durable T-cell responses against particular TAAs (NY-ESO-1, MAGE-A3, tyrosinase, and TPTE)⁴⁹. A further application of mRNA involves generating personalized vaccines targeting neo-antigens determined from tumor exosome analyses^{50,51}. Moreover, mRNA plays a role in cellular therapies, activating antigen-specific T-cells with patient-derived DCs or recognizing tumor antigens directly with T-cells encoded with chimeric antigen receptors⁵².

2.1.1. Immunostimulant mRNA vaccines

mRNA-encoding immunostimulants, typically cytokines or chemokines, are designated to mature and activate antigen-presenting cells, promote T-cell-mediated immunity, and counteract the immunosuppressive effects within the TME¹². Several mechanisms are involved in how cytokines contribute to cancer immunotherapy, including combating tumor growth and boosting anti-tumor immune responses. Cytokines such as interferon (IFN- α) can directly exert anti-proliferative effects on tumor cells by activating JAK-STAT pathways and upregulating pro-apoptotic proteins like TRAIL⁵³. Also, interleukins such as IL-2 and IL-12 are key players in modulating the TME by promoting Th1 differentiation and enhancing the cytotoxicity of immune cells against tumors⁵⁴. Moreover, cytokines can mitigate immunosuppressive signals and angiogenic processes that promote tumor growth and metastasis within the TME. On the other hand, the release of chemokines into the TME can trigger the infiltration of tumor-destroying immune cells, including cytotoxic T lymphocytes⁵⁵. Therefore, an effective antitumor immune response can be amplified by promoting specific chemokine expression that attracts immune cells to tumor sites. When chemokines are expressed in tumor cells, they become sensitive to chemotherapy, radiotherapy, and other immunotherapies, potentially enhancing their ability to fight tumors.

Immunostimulatory vaccines are preferably administered intratumorally⁵⁶ and intranodally^{57,58} for improved accumulation, along with alternative routes such as intradermal and intravenous routes⁵⁶. Clinical trials have shown great promise in combining mRNA vaccines, specifically those encoding immunostimulants and immune checkpoint inhibitors (ICIs), in treating melanoma⁵⁹. Additionally, the introduction of mRNA-protamine complexes enhanced immunostimulatory properties and triggered consistent immune responses⁶⁰.

In 2018, Uchida and colleagues⁶¹ proposed the introduction of safe and efficient dsRNA as an adjuvant to mRNA vaccine, leading to augmented synchronized antigen expression and immunostimulation within the same antigen-presenting cell. Uchida formulated mRNA hybrids with polyuridylic acid and full-length antisense RNA. The hybridized mRNA with poly A region successfully achieved efficient translation and robust immunostimulation. Notably, the immune response triggered by the polyuridylic mRNA hybrids was mediated through the Toll-like receptor (TLR3) and the retinoic acid-inducible gene. When TLR3 was activated, it recruited leukocytes into the TME and induced apoptosis by engaging natural killer (NK) cells and cytotoxic T-cells⁶². Compared to single-stranded mRNA, hybridized mRNA significantly improved specific cellular and humoral immune responses, as evident in DC activation.

A leading mRNA vaccine platform, RNAActive®, developed by CureVac AG, is currently undergoing clinical trials specifically tailored for oncological applications⁶³. RNAActive® technology maintains unmodified nucleotides to ensure optimal immunogenicity with some enhancements⁶⁴. These modifications include

highly translated untranslated regions, a poly-A tail, and algorithmic optimization of the open reading frame by including guanine and cytosine for superior protein expression⁴¹. Dual-component RNAActive® vaccines are self-adjuvanted, containing both “naked” mRNA and mRNA-protamine complexes, thereby enhancing the immunostimulatory properties and triggering potent and consistent immune responses⁶⁰. In the clinical context, CV9202, an RNAActive®, demonstrated robust translation efficiency, resulting in elevated antigen expression and triggering immune responses against a spectrum of non-small cell lung cancer when combined with local radiotherapy. The combination aims to initiate a broader immune response cascade by spreading antigens, inducing immunogenic cell death, and amplifying the overall antitumor immune response⁶³.

2.1.2. Personalized mRNA vaccines

There have been inherent challenges associated with TAAs originating from fetal genes or tissue-specific proteins⁶⁵. Restricted by thymic negative selection, T-cells generally fail to recognize TAAs due to their self-antigen status, inhibiting natural autoimmune responses and failing to initiate robust antitumor immunity⁵². Additionally, the induced immune response may also impact on-target noncancerous cells, as TAAs are not exclusive to tumor tissues. Moreover, the interpatient variability in TAAs expression, coupled with the tumor’s ability to employ various escape mechanisms *via* downregulation of TAAs expression, further complicate the pursuit of an effective mRNA vaccine^{52,66,67}.

Alternatively, advanced sequencing tools have opened the doors to new eras of cancer immunotherapy with the identification of TSA and neoantigens. Neoantigens are the product of tumor-specific mutations recognized as foreign proteins, allowing them to circumvent the thymic negative feedback loop. For these reasons, neoantigens can elicit a more robust antitumor-immune response⁶⁸. Neoantigens often emerge from mutations; therefore, new techniques that include leveraging personalized mutanome analysis and genomic comparison between healthy and cancerous tissues permit the recognition of neoantigens⁵¹. For instance, mRNA-4157 is an individualized neoantigen undergoing clinical evaluation for its efficacy in combination with Pembrolizumab in a phase 3 study (NCT05933577)⁶⁹.

Numerous challenges and technical hurdles need to be addressed to realize the potential of personalized cancer vaccines. The development process for personalized vaccines is particularly complicated, as seen in the identification and characterization of patient-specific neoantigens⁷⁰. In this procedure, predicting immunogenic mutations through sequencing each patient’s tumor genome is a prerequisite for formulating a tailor-made vaccine. Aside from that, another challenge lies in identifying reliable biomarkers to predict patient response. The search for specific biomarkers is hampered by the heterogeneous nature of the samples, limited sensitivity of the assays, and difficulty in validating the findings in a variety of patient populations⁷¹. Furthermore, regulatory and scalability hurdles impede the mass production of personalized mRNA cancer vaccines. The advancement of personalized vaccines from the bench to the clinical phase will require collaboration among researchers, clinicians, regulatory bodies, and industry stakeholders.

A primary tool for identifying neoantigens is Next-Generation Sequencing (NGS), which enables bioinformatics and MHC–peptide binding algorithms to predict neoantigen peptide (neopeptide) sequences. First, raw genomic data is acquired by whole exome sequencing (WES) of tumors and blood cells, which

focuses on the exome, a protein-encoding gene region⁷². Identifying neopeptide typically involves data preprocessing and mapping the processed data to a reference genome to identify genetic changes. Afterward, mutant peptide sequences are determined and ranked according to their potential to bind with MHC molecules and interact with T-cell receptors. Prioritizing neoantigen candidates can be achieved by RNA sequencing, which mirrors peptide expression and abundance on the cell surface through RNA levels. These sequencing data are further processed by bioinformatic pipelines to identify peptide production and MHC molecule localization⁷³. An example of such a pipeline is the Real-time Epitope Computation for Oncology (RECON) pipeline developed by Neon Therapeutics, which predicts therapeutically relevant neoantigen targets. This pipeline facilitated the generation of cancer vaccines targeting both unique neoantigens and those that are common among patients with similar cancer types⁷⁴.

Nevertheless, immunogenicity for the identified neopeptide-MHC complex is not guaranteed, and tools for recognizing the interactions with T-cell receptors are still in the early stages of development. Furthermore, reports from the Tumour Neoantigen Selection Alliance (TESLA) indicate that only a minority of predicted neoantigens are detectable by patients’ T-cells, contrary to what most models predict⁷⁵. Consequently, machine learning approaches have been employed to enhance prediction accuracy by analyzing and validating T-cell-stimulating neoantigens, as well as peptide features derived from immunopeptidomics. For instance, in 2021, Schmidt et al.⁷⁶ developed the Predictor of Immunogenic Epitopes (PRIME), integrating antigen presentation on HLA molecules and T-cell receptor recognition to rank neopeptides by immunogenicity. Nevertheless, computational algorithms are less reliable in predicting MHC class II bindings⁷⁷. Some algorithms enhance prediction accuracy by considering neopeptide foreignness and additional peptide features (such as cleavage and antigen transport). An example of the AI platform is EDGE, which analyzes tumor biopsy data to identify tumor-specific neoantigens and has been instrumental in developing personalized cancer vaccines like GRANITE-001^{78,79}. Given the traditional prediction models may not consistently predict neoantigen presentation efficiency, even when supported by data showing evident RNA expression levels and translation efficiency, an alternative and direct approach to identifying neoantigens is through immunopeptidomics techniques relying on mass spectrometry. Immunopeptidomics rapidly identifies MHC class I associated neopeptides on the tumor’s surface⁸⁰.

2.1.3. DC and T-cell-based vaccines

There are two approaches to mRNA-based cellular immunotherapy: one involves pulsing DCs with mRNA derived from autologous tumors, and the other relies on generating T-cells with chimeric antigen receptors (CAR-T cells) that directly target tumor antigens. The high antigen presentation potential of DCs and their immune-modulation abilities make them the optimal tool for immunotherapeutic interventions. This technique involves the procedure of *ex vivo* loading mRNA encoding TAA into patient’s DCs and re-infusing those cells to trigger antigen-specific T-cell responses (Fig. 3)⁸¹.

Immunotherapy using CAR-T cells with binding domains that target cancer cells expressing TAA or TSA has emerged as a promising approach for managing tumors⁸². CAR-T-cell modification by mRNA and electroporation was proved to be safer and cheaper than viral vectors since this strategy grants temporary CAR expression without inducing genomic alterations^{52,83}.

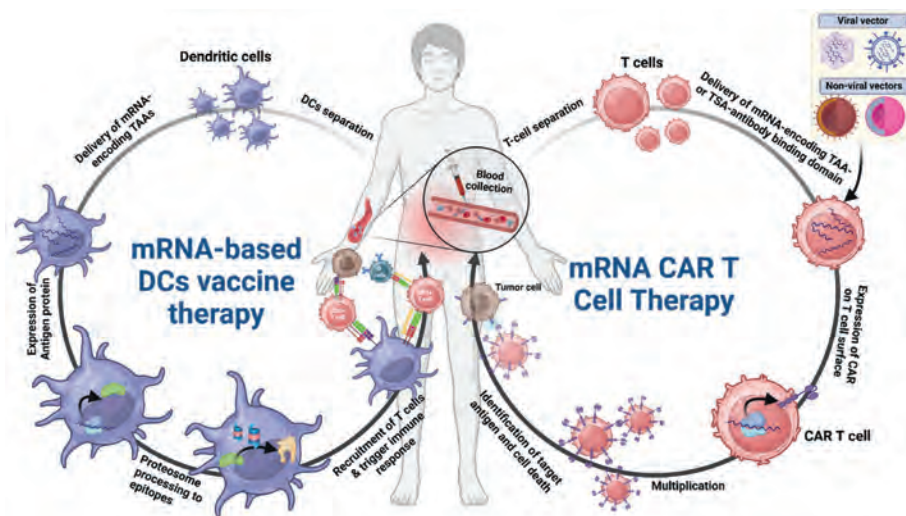


Figure 3 The development of mRNA-based CAR-Ts and DCs immunotherapies. (Right) CAR-T cell therapy has evolved by delivering mRNA-encoding antibody binding domain (CAR) to patient-isolated T-cells, which are then expressed, multiplied, and transferred back to the patient to recognize and destroy the malignant cells. (Left) DC vaccines have been developed by introducing mRNA-encoding TAAs into patient-isolated DCs, which are transferred to the patient after expression and epitope presentation for tumor recognition and triggering cytotoxic immune responses. Figure was made by Biorender.com.

Moreover, recently, in the BNT211-01 trial (NCT04503278), CAR-T cells expressing CLDN6 binding domains were investigated for tolerability and potential efficacy in solid tumors⁸⁴. With a 67% disease control rate, this therapy has shown promising efficacy in treating relapsed or refractory CLDN6-positive solid tumors. Despite its manageable toxicity, the vaccine caused the cytokine release syndrome in 46% of patients. However, an earlier phase 1 study of six patients with refractory metastatic pancreatic cancer treated with recurring infusion of electroporated mRNA CAR-T cells targeting mesothelin showed that the treatment was effective with no off-tumor side effects or cytokine syndrome (NCT01897415)⁸⁵. According to the trial, disease stabilization was observed in two patients, with 3.8 and 5.4-month progression-free survival. Also, another patient was reported to have his metabolic active volume declined by 69%, evidenced by the completely reduced uptake of fluorodeoxyglucose in all liver lesions following one month of treatment, even though primary pancreatic lesions were not affected.

Cancer therapy has advanced with the advent of CAR-T, but a few obstacles remain to optimize the treatment's efficiency. Post-CAR-T therapy, tumor cells may enter a state of antigen escape, where they lose the ability to express the target antigen⁸⁶. Thus, the proposal of the multi-antigen targeting approach could potentially resolve antigen escape; for instance, CD19 and CD22 were targeted in NCT03287804. However, the success of this approach is not guaranteed, since in this phase 1 trial, the proliferating ligand (APRIL) CAR showed only modest clinical responses despite manageable toxicity in people with refractory multiple myeloma⁸⁷. Nonetheless, cytokine release syndrome in five patients was grade 1 and there was no neurotoxicity reported. On the other hand, among eleven patients, only five showed clinical responses, including one who showed a very good partial response. There was observed mechanistic inadequacy of the APRIL CAR, including reduced IL-2 secretion, impaired IFN signaling, and lack of sustained tumor control. This suggests that T-cell-expressing APRIL had poor binding affinity for soluble B-cell maturation antigen, compromising the function of CAR.

In situ vaccination presents an opportunity to overcome the drawbacks of generating *ex vivo* CAR-T or DC vaccines using mRNA. This approach bypasses lengthy and labor-intensive *ex vivo* procedures by direct delivery of mRNA-encoding tumor antigens or CAR constructs into the patient. Rather, these constructs can be expressed *in vivo*, allowing them to be successfully targeted at DCs for antigen presentation or to be directly modified within T-cells⁸⁸. This method ensures sustained antigen expression, addressing the shortcomings such as limited T-cell response and resistance experienced with *ex vivo* treated DCs, as well as eliminating the necessity of lymphodepletion⁸⁹. Moreover, *in situ* development avoids the adverse events associated with *ex vivo* redirected CAR-T therapies, including cytokine storm and immune effector cell-associated neurotoxicity syndrome⁸⁹. However, safety concerns remain around mRNA expression within off-target cells⁹⁰. Another advantage of *in situ* vaccine development is the simplified treatment regimens and standardizable manufacturing at reduced production costs⁸⁹. Nevertheless, challenges remain in optimizing delivery systems to guarantee targeted and efficient delivery of mRNA while enhancing immunogenicity to improve antitumor responses.

2.2. Synthetic therapeutic oligonucleotides (STOs)

RNAi has proven a highly effective approach in cancer therapy with the advantages of precise and targeted gene silencing, cost-effectiveness, and diverse treatment options. Unlike traditional therapies such as chemotherapy, RNAi can concurrently inhibit multiple cellular pathways involved in tumor progression. Ongoing clinical research into RNAi-based cancer therapies has revealed their potential to revolutionize cancer treatment by targeting druggable and undruggable therapeutic targets, as presented in Fig. 2. The evolution of STOs, including Antisense oligonucleotides (ASOs), aptamers, and siRNAs, provides an avenue for RNA degradation and splicing modulation mechanisms (Fig. 2). Preclinical development of several STOs is underway, and more than a hundred STOs are currently experiencing the

clinical phase of development. Despite the promise of STOs, achieving optimal STO hinges on conquering a set of extracellular and intracellular constraints, including cellular uptake and escape from endolysosomal compartments, which call for the development of an effective platform for delivery⁹¹.

2.2.1. Small and short hairpin interfering RNAs (siRNAs & shRNA)

The concept of RNAi was first established by Fire and Melo in 1998 to degrade the target mRNA using dsRNA, which led to blocking gene expression unexpectedly⁹², which credited them with the Noble Prize in Physiology or Medicine in 2006. These dsRNAs are short noncoding RNA duplexes of 30 to more than 100 base pairs. As part of the RNAi process, Dicer enzyme cleaves dsRNA into ~ 20 nucleotides siRNAs. Once internalized into a cell, siRNAs can directly embed themselves in the RNA-induced silencing complex (RISC) without being subjected to any subsequent processing. Meanwhile, shRNAs exhibit distinct structural configurations and form stem-loop structures. Therefore, Dicer activation for shRNAs is necessary before participation in RNA interference. Next, shRNAs bind specifically to endonuclease argonaute 2 (Ago2) in the RISC assembly, converting it into a single antisense strand. shRNAs and siRNAs serve as guides to the target mRNA, leading to effective gene knockdown. This silencing strategy can suppress desired genes in a highly efficient and precise manner, as the antisense strand often fully complements the coding region of the target mRNA.

siRNAs have undergone several modifications to enhance their serum stability and deliverability. This includes tuning the sugar moiety, phosphate structure, RNA bases, and 3' overhangs. To enhance their stability in serum, modifications at the 2'-O position of the ribose are typically applied, such as adding methyl, methoxyethyl, and fluoride^{93,94}. A further modification to the 2' position involves introducing azido-modified cytidine or guanosine or introducing gemcitabine into siRNAs, which were reported to boost nuclease resistance and knockdown efficacy^{95,96}. It has also been reported that masking 2'-OH with acyloxymethyl facilitated cellular uptake and enhanced gene suppression⁹⁷. Moreover, modifying the 3' overhangs with 1-deoxy-D-ribofuranose increases nuclease resistance and knockdown efficiency. It was also reported that gene silencing activity could be augmented by replacing the traditional phosphate in the backbone structure with phosphorodithioate, boranophosphate⁹⁸, or triazole backbone linkages⁹⁹ has also been shown to augment siRNA gene silencing activity. According to Huang et al.¹⁰⁰, further modifications to L- and D-isonucleosides may affect thermal stability differently, with D-isonucleotides having a less pronounced effect than L-isonucleotides while increasing the serum stability of siRNA. Further, the presence of D- or L-isoNA at the 3'-terminus of the sense strand notably promotes gene knockdown.

Recently, modifying siRNAs with GalNAc presents a highly efficient delivery strategy to hepatocytes, exploiting the presence of highly expressed Asialoglycoprotein receptors to facilitate the uptake¹⁰¹. Following this breakthrough, multiple Phase III clinical trials have resulted in FDA approval for the first GalNAc-siRNA conjugate (Givosiran). Efforts to improve the stability of siRNA molecules include introducing phosphorothioates at the termini of siRNA molecules^{102,102}, forming small and readily synthesizable conjugates of siRNA and GalNAc that target asialoglycoprotein receptors in hepatocytes to reduce dosage and off-target effects¹⁰³. GalNAc linker arms can be naturally degraded by endosomal glycosidases, releasing siRNA into the cytoplasm. These

approaches are under clinical investigation and are also being developed by leading USA Biotech companies¹⁰⁴.

The clinical relevance of siRNAs gained momentum after the FDA approved Patisiran in 2018 when siRNA was accepted for treating hereditary polyneuropathy. A growing number of siRNA drugs have been approved over the past five years, showing their therapeutic potential¹⁰⁵. Even though there is growing evidence that sequence-specific antitumor siRNA could be designed for cancer treatment, several challenges remain, including concerns about their cost-effectiveness, deliverability, stability, off-target effects, poor biodistribution, and pharmacokinetics¹⁰⁶. siRNAs can be delivered into cells in several ways, whether naked, conjugated molecules or complexed with a vector.

In the quest to induce therapeutic antitumor immune responses, researchers strategically target specific molecular pathways, including anti-inflammatory cytokines, immune checkpoint proteins, and pivotal immune signaling molecules. Several studies show great promise for potential applications in TGF- β -targeting siRNA in both melanoma and glioblastoma^{107,108}. The delivery of siRNA in combination with the Trp 2 peptide and cytosine-phosphorous-guanine oligodeoxynucleotides (CPG ODN) has significantly enhanced vaccination efficacy in melanoma through reducing regulatory T-cell levels, increasing CD8⁺ T-cells infiltration¹⁰⁷. One reason to include CPG ODN as an adjuvant is to leverage bacterial DNA sequence patterns for TLR9 activation cytokine production, thereby initiating a sustained immune response^{109,110}. Additionally, modifying siRNAs with CpG ODNs was reported to allow them to be delivered to specific cells independently of a delivery agent¹¹¹. In glioblastoma, coadministration of temozolomide and TGF- β siRNA has achieved an effective antitumor immune response¹⁰⁸. Indirect methods have been used to suppress TGF by silencing β -catenin^{112,112}, or by knockdown of β -catenin to enhance CD8⁺ T-cell infiltration in hepatocellular carcinoma when used with anti-PD-1 treatment¹¹³.

Immune checkpoints such as PD-L1 (programmed death ligand-1) or NOTCH in DC and CTLA-4 in T-cells can also be targeted by siRNA. Silencing these checkpoints has increased infiltration of tumor-killing CD4⁺ and CD8⁺ T-cells and reduced levels of immunosuppressive cells^{114–116}. CD47 is another potential checkpoint protein target for siRNA and plays a vital role in regulating phagocytosis¹¹⁷. In a recent communication by Yu group, the antitumor cyclin-dependent kinases 4 and 6 (CDK4/6) inhibitors were shown to be compromised by PDL-1 overexpression. The researchers have demonstrated that siRNA-mediated PD-L1 knockdown in tumor cells has enhanced the effect of CDK4/6 inhibition and facilitated the infiltration of cytotoxic T lymphocytes, thus suppressing colorectal cancer growth efficiently *in vitro* and *in vivo*¹¹⁸.

There is an increasing interest in manipulating gene expression in DCs *via* RNAi approaches and using siRNA to minimize the immunosuppressive effects of TME in cancer therapy. This includes the release of inhibitory enzymes, interleukins, and other essential proteins and receptors¹¹⁹. A crucial immunosuppressive enzyme is Indoleamine 2,3-dioxygenase, which contributes to multiple negative immune responses, primarily suppressing T-cells and NK cells or activating Tregs^{120,121}. Therefore, NK cell infiltration was noted at the tumor site following the administration of shRNA to silence this dioxygenase in mice grafted with ovarian cancer¹²². Similarly, dioxygenase expression in splenocytes and lymphocytes was effectively blocked by siRNA, contributing to reduced apoptosis of CD8⁺ and CD4⁺ T-cells, along with enhanced response to melanoma antigen¹²³.

Additionally, siRNA targeting ubiquitin editing enzyme A20 or negative regulatory receptors like DlgR2 in DCs enhances antigen-specific T-cell reactions and suppresses regulatory T-cells. Similarly, dioxigenase expression reduced the apoptosis of CD8⁺ and CD4⁺ T-cells with improved response to melanoma antigen¹²³. Additionally, siRNA targeting ubiquitin s-has been applied to DCs triggers ubiquitin-editing enzyme (A20) or negative regulatory receptors like DlgR2 (dendritic cell-derived immunoglobulin receptor 2) in DCs enhances antigen-specific T-cell reactions and suppress regulatory T-cells^{124,125}. Bcl-2 siRNA has been applied to DCs triggers Rig-I (retinoic acid-induced protein I), ultimately resulting in the expression of IFN and tumor apoptosis in melanoma¹²⁶.

It is becoming evident that RNA can target negative regulators engaged with tumor progression. For example, selective shRNA-mediated knockdown of the negative regulator suppressor of cytokine signaling 1 (SOCS1) has improved antigen-specific antitumor responses¹²⁷. siRNA has the potential to eliminate the effects of TAMs on tumor progression and metastasis by targeting the M2-like receptor of colony-stimulating factor-1 and chemokine receptor-2 on monocytes^{128,129}. This further expands RNAi in cancer research.

2.2.2. MicroRNA

MiRNAs are non-coding RNAs of around 18–22 nucleotides that control mRNA stability and degradation through the RNAi machinery. Primary miRNAs are the initial product transcribed from genomic DNA, but they undergo cleavage by the Drosha subunit complex to form precursor miRNAs (pre-miRNAs). These pre-miRNAs are then transported to the cytoplasm, where DICER processes it to miRNA duplexes. Upon interaction of miRNAs with RISC, the duplexes are unraveled, forming miRISC that will then interfere with mRNAs, typically binding to the 3' untranslated region. Unlike siRNA, miRNAs may induce gene silencing through slicer-independent pathways.

MiRNAs notably foster the crosstalk between tumor and immune cells within TME, influencing immune cell functions and playing a demonstrated role in evading immune reactions. Additionally, these miRNAs can indirectly influence macrophage polarization by modulating signaling pathways like PI3K/AKT and PTEN/PI3K γ . For instance, miR-301a and miR-103a secreted in tumor exosomes were involved in regulating TAM polarization to M2, contributing to tumor progression and immunosuppression^{130,131}. This process creates a feedback loop where M2-polarized TAMs further enhance cancer progression and metastasis. In addition, miR-29a-3p, miR-21-5p, miR-29a, miR-21, miR-155-5p, miR-375, and miR-321a produced by M2 cells were implicated in modulating NF- κ B pathway and accordingly their cytokine production, and cancer cell invasion. Tumor-suppressing miRNAs, like miR-BART cluster, can directly regulate antitumor immune responses by simultaneously blocking key checkpoints like PD-1 and PD-L1 in the TME¹³². Also, several miRNAs, including miR-301a, miR-22, and miR-221, could affect cytokines produced from DCs. Moreover, MDSC was found to proliferate in response to STAT3 pathways activation by miR-155 and miR-21, thereby interfering with T-cell responses.

Emerging miRNA treatments, including INT-1B3 (miR-193a-3p), miR-10b, and MesomiR-1, show promise in treating advanced and metastatic tumors^{133–135}, as well as mesothelioma¹³⁶. Cobomarsen is another promising miRNA for treating cutaneous T-cell lymphoma by inhibiting miR-155, showing promising efficacy and safety in the phase 1 trial

(NCT02580552)¹³⁷. In this trial, those patients who received Cobomarsen intratumorally demonstrated a reduction in lesion index scores by more than 50%, which was maintained during the study, along with evidence from histological and gene expression analyses. The Cobomarsen injection was well tolerated and had no clinically significant side effects, except for one patient who experienced grade 3 pruritus.

2.2.3. Antisense oligonucleotides

Antisense oligonucleotides (ASOs) are short single-stranded synthetic oligonucleotides, highly modified to selectively bind *via* complementary base-pairing to RNA for treating specific disorders¹³⁸. Usually, ASOs are formed of either modified DNA or RNA bases or both. The development of ASOs took a significant step forward when Fritz Eckstein introduced phosphorothioate modifications in 1966¹³⁹. The procedure used sulfur to substitute one oxygen in the phosphate group, enhancing the hydrophobicity of ASO and increasing the resistance to phosphodiesterases. The modifications also enhanced the affinity for albumin binding and thereby improved stability and pharmacokinetics¹⁴⁰.

The selectivity of the ASO to the opposite strand permits its interference with mRNA processing¹⁴¹. When the ASO binds to complementary bases in pre-mRNA, it prevents splicing activities, hampers the attachment to ribosomal units, and blocks the protein translation because triggering RNase-H leads to mRNA cleavage¹⁴². Ultimately, this cleavage degrades the targeted RNA, resulting in an effective gene knockdown. Pre-mRNAs and long noncoding RNA are the targets of RNase H1-dependent ASOs, which are inaccessible to siRNA. However, there are also RNase H-independent ASOs, commonly known as steric blocking ASOs, which directly obstruct or enhance translation or splicing by their steric blocking ability¹⁴³.

These ASO-based therapeutic tools are highly promising for treating various genetic disorders and cancers by targeting specific mRNA molecules and modulating their expression. Mipomersen, Inotersen, and Volanesorsen are three FDA-approved ASO gapmers that are made up of a central DNA segment flanked by modified RNA segments that act selectively when coupled to RNA sequences of the target¹⁴⁴. In 2016, intrathecal injection of Nusinersen was the first 2'-methoxyethyl RNA-based ASO to gain FDA approval for spinal muscular atrophy¹⁴⁵. As yet, no approval has been granted for ASO for cancer treatment, though three ASOs containing DNA bases have been assigned orphan drug status, including oblimersen, Cobomarsen, and PNT2258¹⁴².

ASOs targeting specific vital cellular processes such as JAK2/STAT3 and PI3K/AKT axes and other potential immunosuppressive components might show promising effects in cancer treatment¹⁴⁶. Upregulation of Bcl-2 and Akt was correlated with human chronic lymphocytic leukemia¹⁴⁷. Additionally, targeting Bcl-2 with ASOs has demonstrated antitumor efficacy and longer survival time in lung cancer xenograft models¹⁴⁸. Also, targeting immune-inhibitory surface ectoenzyme, such as CD39, by ASOs reduced the production of immunosuppressive adenosine and ultimately decreased tumor growth¹⁴⁹. Moreover, the suppression of metadherin expression with locked nucleic acid-modified ASO is linked to the suppression of cytotoxic T-cell depletion and suppression of cancer growth¹⁵⁰.

2.3. Aptamers

Aptamers are opening up new possibilities for cancer therapy due to their unique intrinsic properties, such as small size and

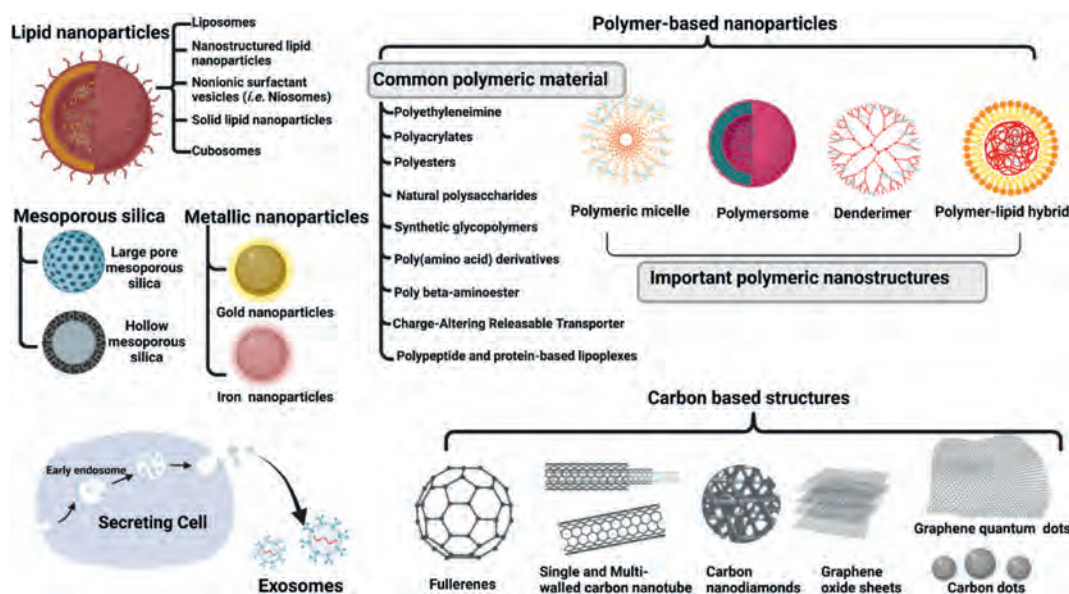


Figure 4 Nanoplatforams for RNA delivery. Figure was made by Biorender.com.

chemically adaptable nature, for better stability and specificity for one or more molecules^{151,152}. An aptamer is formed of a single strand of nucleic acid, either RNA or DNA, which folds into a 3D structure, being able to recognize and bind to a target molecule¹⁵². In addition, aptamers could be tailored to serve as dual-specific or multi-specific agents, capable of targeting tumor cells and enhancing antitumor immune responses¹⁵³.

There may be instances where standard immunotherapies cannot effectively fight resistant tumors. Aptamers have demonstrated remarkable potential to boost the immunotherapeutic response in various malignancies by modulating immune responses within TME¹⁵⁴. Aptamers may also target biomarkers associated with drug resistance, potentially exposing cancer cells to therapeutic intervention. They can also be used as delivery agents for cytotoxic drugs, bypassing multidrug resistance mechanisms, including P-glycoprotein transporters, and delivering drugs directly into cancer cells^{155,156}. Future perspectives include developing personalized aptamers that target specific molecular pathways with unique sequences specifically designed for individual patients, aiming to maximize therapeutic response and minimize adverse effects¹⁵⁷.

3. Nanoscale platforms for RNA delivery

Recent advances in nucleic acids and macromolecules-based therapies have demanded the development of diverse strategies to address delivery-related challenges. Still, designing effective delivery tools for cancer tissues entails overcoming robust biological barriers created under the effects of TME and loss due to interaction with serum proteins or extracellular enzymes¹⁵⁸. Cellular entry of these molecules is also prevented by electrostatic repulsion with the cancer cell membrane, and the escape from the formed endosomes is a major impediment to release at their target¹⁵⁹. Since the advent of mRNA lipoplexes, recent and future studies are pursuing promising non-viral components of the delivery systems. The quest for an optimal vector in RNA delivery has led to the exploration of various nanomaterials, such as lipid-based, polymeric, and inorganic excipients. Therefore, in this section, we highlight the significance of polymers-, lipids-, exosomes-, metal, and silica-based delivery

systems (Fig. 4). Each delivery system possesses its benefits and drawbacks, as outlined in Table 2¹⁶⁰⁻¹⁹⁶.

3.1. Lipid-based systems

3.1.1. Liposomes and liposome-like nanoparticles

Lipid-based nanovesicles (liposomes or LPS) are commonly explored for RNA delivery and are formed by encapsulating hydrophilic or lipophilic molecules within the lipid bilayer structure or entrapped in aqueous space. The lipoplex encapsulating materials involve cationic, ionizable, or zwitterionic lipids, as well as phospholipids and helper lipids, which are incorporated into the formulation to promote cellular uptake, membrane destabilization and, therefore, enhance nucleic acid delivery (Table 3)¹⁶²⁻¹⁹⁹. More recently, other lipid nanoparticles (LNPs), which can enclose molecules in the non-aqueous interior of liposome-like particles (LLPs) or micellar structures, have been found promising as a safe and reliable means of delivering RNA tools. Fig. 5 presents the differences between LPS and other lipid-based delivery systems.

Cationic lipids, like DOTAP, play a crucial role by forming electrostatic bonds with negatively charged RNAs, promoting binding to cell membranes and facilitating endosomal escape for cargo release into the cytosol. RNA payloads that are highly encapsulated in these lipoplexes are protected against breakdown by circulatory RNases. In clinical trials, cationic DOTAP-based LNPs are being investigated for their efficacy in delivering mRNA-DCs vaccine for recurrent glioblastoma cases¹⁹⁷.

Furthermore, targeting RNA lipoplexes to solid tumors relies on the enhanced permeability and retention (EPR) effect, which is not universal across all solid tumor types²¹¹. The short circulation half-life of lipoplexes is attributed to reticuloendothelial absorption, coupled with increased immunogenicity that results from phagocytosis. Moreover, cationic lipids may nonspecifically bind to other targets, causing off-target effects contributing to systemic toxicity. Therefore, balancing the positive charge for effective encapsulation with the neutral or nearly neutral surface charge under physiological conditions is critical to achieving circulatory

Table 2 The advantages and drawbacks of RNA delivery systems.

Delivery system	Advantage	Drawback	Ref.
Lipid-based systems	1. Functionality of certain lipids surfaces 2. PEGylated lipid nanoparticles are non-immunogenic and exhibit longer circulation time 3. Synergistic co-delivery of genes and pharmaceuticals 4. Protection against RNA degradation 5. Easily produced by microfluidics and can be scaled up 6. Protein corona could enable the targeting of specific organs	1. Cytotoxicity of cationic lipids 2. Limited transfection efficiency 3. Neutral lipid nanoparticles have low RNA loading capacity and limited stability 4. Serum proteins might impair targeted RNA delivery 5. Production costs and complexities 6. Inherent low biostability and short circulation time	160–167
Polymer-based systems	1. Low immunogenicity 2. Protection against RNA degradation 3. Size, shape, and surface are modifiable for drug or gene delivery 4. Improve intracellular uptake by interaction with the cellular membrane	1. Non-biodegradability of some polymers 2. Cytotoxicity and hemolytic activity of cationic polymers, especially PEI 3. Low loading capacity 4. Slow or incomplete cargo release	168–173
Exosomes	1. Biocompatibility and non-immunogenicity 2. Targeted delivery 3. Ability to cross biological barriers, such as the blood–brain barrier 4. Exosomes could camouflage the phagocytosis process	1. Lack of FDA approval 2. Heterogeneity in size and cargo content 3. Variability in encapsulation efficiency 4. Limited yield 5. Scalability and cost are challenging. 6. Storage instability	174–179
Organic-inorganic mesoporous silica nanohybrids	1. High surface area and pore volume, allowing for high loading capacity 2. Versatility in surface decoration 3. Able to co-load different active agents with targeting agents 4. Synergistic co-delivery of drugs and genes 5. Protection of loaded RNA from degradation 6. DMSOs show better biocompatibility and biodegradability	1. Traditional types are not suitable for RNA delivery 2. Low biocompatibility and potential toxicity 3. Complex and time-consuming preparation process. 4. Upon long-term storage, nanoparticles tend to aggregate 5. Clearance by the immune system	180–184
Carbon-based nanostructures	1. High surface-to-volume ratio 2. CNTs have a high aspect ratio 3. Tunability for enhancing solubility or targeted gene delivery 4. Protect genetic material and improve transfection efficiency 5. Biocompatibility 6. Temperature-responsive delivery 7. Magnetoporation can potentially enhance CNT delivery	1. Not biodegradable and toxicity concerns 2. Poor water dispersibility 3. Impaired pharmacokinetic properties 4. Instability of specific structures, such as quantum dots	185–191
Metallic nanoparticles	1. Biocompatibility 2. Easiness of surface decoration for targeted delivery 3. Inherent targeting properties of magnetic nanoparticles 4. Enhanced transfection could be obtained by magnetofection 5. Photoacoustic imaging and photothermal therapeutic properties of gold nanoparticles	1. Not biodegradable 2. Nanoparticle aggregation concerns upon long-term storage 3. Increased cost and production hurdles 4. Safety concerns with repeated administration	192–196

stability, which can be addressed by incorporating helper lipids into formulations¹⁶⁶. Fortunately, advances in smart ionizable lipids possessing weakly basic moieties may provide a potential solution to these challenges.

Ionizable lipids are pH-responsive and enhance transfection efficiency and target endosomes. They can protonate and deprotonate in response to pH changes. Under physiological pH, they retain a negative to neutral charge, thereby reducing the incidence of cytotoxic effects on blood cells and improving the system's biocompatibility. As soon as these lipids enter the endosomes, protonation occurs under acidic circumstances, creating a positive charge that facilitates their endosomal escape.

Moreover, ionizable lipids offer a set of remarkable features for RNA delivery. The lipid 1,2-dilinoyleoxy-*N,N*-dimethyl-3-aminopropane (DLin-DMA) with its linoleic acid chains increases gene knockdown efficiency²¹², as the less saturated fatty acid exhibits superior efficiency in intracellular nucleic acid delivery. Also, the gene-silencing efficiency is linked to the pK_a of ionizable lipids; hence, it is essential to maintain a pK_a range of 6.2 to 6.5^{213,214}. The ionizable lipid DLin-MC3-DMA has been successful in liver-targeted gene silencing²¹⁴. It is a major constituent of the FDA-approved formulation of Onpatro^{®215,216}.

Other types of lipids have been developed, such as zwitterionic and biodegradable lipids, aiming to boost potency,

Table 3 Roles of different lipid types in lipid-based nanoformulations.

LNPs component	Role	Ref.
Cationic lipids (<i>e.g.</i> , DOTMA, DOTAP, DODAC)	1. Encourage electrostatic interactions with negative phosphates in nucleic acid backbones, yielding higher encapsulation efficiency. 2. Cationic lipids are directly involved in interactions with cell membranes, facilitating endosomal uptake. 3. Interact with the anionic lipids in endosomal membranes to facilitate nucleic acid release to the cytosol.	162,165
Ionizable cationic lipids (<i>e.g.</i> , DODAP, DLin-MC3-DMA and DLin-KC2-DMA)	1. Their neutral charges at physiological pH reduce toxicity and immune responses. 2. Enhance the endosomal escape by responsive protonation under acidic environments. 3. Grant longer circulation times compared to cationic lipids. 4. Their apparent pK_a value is critical to attain efficient transfection of RNA molecules	160,161
PEG-lipids (<i>e.g.</i> , PEG-c-DMG, PEG-DSPE)	1. PEG positively affects LNP particle size and polydispersity, eliminating LNP aggregation. 2. Contribute to enhanced particle stability during preparation and storage. 3. Promote RNA encapsulation and transfection efficiency. 4. PEG could camouflage the immune responses, prolong circulation half-life, and promote <i>in vivo</i> distribution of the loaded RNA.	162–164
Helper lipids Cholesterol	1. Forms liquid-ordered phase in LNP membranes, leading to improved membrane integrity. 2. Contributes to LNP structural stability and prevents cargo leakage from the core by enhancing membrane rigidity.	162,165
Helper lipids Phospholipids (<i>e.g.</i> , DSPC, DOPE)	This lipid type helps improve encapsulation efficiency, stability, membrane fusion, and intracellular RNA delivery. For example: 1. DSPC stabilizes and improves the intracellular delivery of LNPs. 2. DOPE promotes hexagonal phase formation, facilitating transfection efficiency and enhancing expression of the delivered mRNA	162,198,199

biodegradability, and safety. Zwitterionic lipids with pH-sensitive properties have imparted potent protein expression *in vivo* as a consequence of bypassing endosomes. On the other hand, biodegradable lipids, such as SM-102, emerge as more effectively cleared with less toxicity alternatives, whereas 244cis offered several advantages for Cre recombinase enhanced potency, safety, and repeated administration, enabling substantial target protein expression in the lungs²¹⁷.

The lipid-like material (C12-200)-based lipidoid has significantly reduced the siRNA doses for liver multiple gene silencing by 100-fold in non-human primates²¹⁸. Among these lipidoids, cKK-12 demonstrated high potency in Factor VII knockdown by siRNA superior to DLin-MC3-DMA nanoparticles²¹⁹. Some lipids play a role in anti-cancer immunotherapy that goes beyond serving as carriers, having a synergistic therapeutic effect. Lipidoids, with cyclic amino head, triggered the STING (Stimulator of Interferon Genes) pathway that upregulates several immunostimulatory factors like IRF7, CXCL10, and IFN- β , necessary to potentiate T-cell reactions²²⁰.

To improve biodistribution patterns, LNPs can be surface decorated by apolipoprotein E (ApoE) that mimics physiological lipoproteins in their targeting strategy to hepatic tissue, which act naturally based on affinity to LDL (low-density lipoprotein) receptors on hepatocytes²²¹. ApoE improved cellular uptake²¹⁹, and enhanced the escape of siRNA confined within endosomal membranes, steering the delivery to the liver²²¹. It was therefore noted that the formation of a protein corona affects LNP recognition and immune cell interactions, as well as their delivery and biological fate^{222,223}. This has inspired Dilliard et al.²²⁴ to formulate targeted LNPs that are precise in delivering mRNA to target organs in mice by designing lipid structures with the selective organ targeting (SORT) molecule. SORT-based LNPs displayed unique and specific organ-targeting outcomes due to their tailored alkyl tail and head-group characteristics and their interactions with plasma proteins. For example, they showed vitronectin as a key protein interacted

favorably with 18:1 DOTAP as a SORT molecule and steered mRNA delivery from hepatic tissues to the lungs.

Lipopolyplexes exert different structural assemblies from the conventional LNPs to address challenges posed by lipopolyplexes. A redox-responsive lipopolyplex has been developed, using dendrimer (G0-C14), cysteine-based poly(disulfide amide), PEG-lipids (DSPE-PEG 2000 and DMPE-PEG 2000), to deliver p53 mRNA to treat liver and lung cancer²²⁵. The PEG lipids have relative stability with sustained degradation, providing longer circulating time and improved pharmacokinetic properties²²⁶. Meanwhile, DMPE-PEG is rapidly de-PEGylated, which makes it more prone to be absorbed by cells.

Despite the previously outlined benefits, lipopolyplexes pose various challenges, including complex synthesis, potential toxicity, and their large vesicular size, which interferes with diffusion and biodistribution to target cancer tissue. The quest to provide controlled particle size with a high polydispersity index and homogeneity opened an opportunity for applying microfluidic mixing devices. Microfluidic fabrication of LNPs enables an exceptionally high to full siRNA encapsulation, making the technique a potential technology for the biopharmaceutical industry¹⁶⁷.

3.1.2. Solid lipid nanoparticles and nanostructured lipid carriers

Solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) differ primarily in their interior lipidic core composition and loading capacity. In fact, SLN has limited capacity due to the presence of a solid crystalline matrix that limits the loading of molecules to the core structure. Meanwhile, the NLC, which is created by combining liquid and solid lipids, produces irregularities during its formation, which renders it easier for pharmaceuticals to be incorporated into the matrix^{207,227}.

Advances with DDAB-based cationic SLNs carrying siRNAs targeting EphA2 receptor tyrosine kinase (siEphA2) have yielded

Constituents	Lipid-based delivery systems					
	LPS	LLNPs	SLNs	NLCs	NSIVs	LLCs
Cationic or ionizable lipid	✗	+	+	+	+	+
Neutral lipid & phospholipids	+	+	+	+	✗	✗
Cholesterol	+	+	+	+	+	✗
Helper lipid	+	+	+	+	+	+
Stealth or PEG lipid	+	+	+	+	+	+
Liquid lipid	✗	✗	✗	+	✗	✗
Solid lipid	✗	✗	+	+	✗	+
Co-surfactant	✗	✗	+	+	✗	✗
Surfactant	+	+	+	+	+	+

+ Primary components
 + Supplementary components
 ✗ Not components

Figure 5 The constituents of different types of lipid-based delivery systems. Constituents were selected and sorted according to the literature^{148,162,166,198,200–210}. Figure was made by Biorender.com.

positive results against prostate cancer²²⁸. As well, cationic NLCs have been tested for miRNA delivery to treat cancer. Piao et al.²⁰⁸, for example, revealed that the delivery of miR-107 by cationic NLCs significantly inhibited the growth, migration, and invasion of head and neck squamous cell carcinoma. Nevertheless, the toxicity of cationic NLCs and SLNs limits their potential. Alternatively, neutral NLCs provide a safer alternative that is less toxic, although their encapsulation efficiency can be compromised.

3.1.3. Nonionic surfactant vesicles

Nonionic surfactant vesicles (NISVs) are categorized into cationic niosomes and SPANosomes that have shown potential for RNA delivery^{202,203}. The formation of nioplexes by complexing nucleic acids with cationic niosomes is crucial for delivering nucleic acids²²⁹. Span 20 and cholesterol have proven effective for creating NSIVs, suggesting a future direction for mRNA vaccine formulation in cancer immunotherapy. Nioplexes have a protective shield, ensuring that the RNA remains stable and intact while transported to the target site²³⁰. The condensation of RNA on the surface of cationic niosomes is a critical aspect of the nioplexes formulation process²³¹, enhancing their loading capacity and genetic material delivery to target cells.

A further advantage of nioplexes is controlled release, optimizing transfection efficiency and nucleic acid performance²⁰⁴. Moreover, nioplexes' compositions are versatile. This nanocarrier can be tailored to meet specific delivery requirements, including their entrapment efficiency, size, surface charge, and release properties^{232,233}. The development of advanced machine learning techniques has greatly aided the optimization process of niosomal drug delivery systems, reducing both laboratory time and cost²³⁴.

A novel synthesized spermine-based cationic lipid was incorporated to form cationic niosomes for the delivery of green fluorescent protein-encoding pDNA. Notably, these nioplexes demonstrated high transfection efficiency, serum stability, and safety *in vitro*, along with physical stability for at least 1 month at 4 °C²³¹. However, in a comparative study, the nioplex-based delivery system showed the lowest uptake by cells compared with lipoplexes and polyplexes²⁰³.

Microfluidic mixing was employed in 2017 to formulate NSIVs composed of Tween 85 and cationic charge from DDA, showcasing the ability of NISVs to transport and transfect GFP siRNA into cells. In 2012, Zhou and colleagues developed SPANosomes, constituted mainly of Span-80, DOTAP, and 1–5% D- α -tocopheryl PEG 1000 succinate. SPANosomes exhibited a vesicle size below 50 nm and high siRNA loading capacity, as well as colloidal stability. SPANosomes/siRNA complexes demonstrated efficient silencing activity compared to cationic lipoplexes²⁰². A significant finding was that SPANosomes were able to navigate the intracellular environment with superior efficiency, escaping endosomal compartments and ending in the cytoplasm. Microfluidic formulated NSIVs, composed of Tween 85 and cationic charged DDA, show significant efficiency of NISV to transport and transfect GFP siRNA into cells²³⁵.

3.1.4. Non-lamellar lyotropic liquid crystalline (LLC) lipid nanoparticles

Non-lamellar lyotropic liquid crystalline (LLC) lipid nanoparticles, including cubosomes and hexosomes, are in the potential for controlled drug release. Recently, cubosomes have demonstrated promise in drug delivery with unique abilities for

thermodynamic stabilization (Fig. 6A)^{205,206,209}. These cubosomes are in bicontinuous cubic structures and feature bilayers curved into gyroid, diamond, or primitive minimal surfaces, creating separate water channel arrays, enhancing colloidal stability and success of RNA delivery²³⁶. Cuboplexes encapsulating siRNA facilitated the release from endosomes by puncturing pores in endosomal membranes, providing an alternative to traditional proton sponge or electrostatic fusion mechanisms^{205,237}. Cuboplexes rely on elasticity energetics to precisely control particle–endosomal interactions, prompting their escape capability due to their unique structures (Fig. 6B)²³⁷.

The incorporation of cationic lipids in cubosome-based systems promotes siRNA encapsulation, stability, cellular uptake, and endosomal escape. Zhen et al.²³⁸ found the superior gene silencing efficiency of nonlamellar DOTAP lipid-functionalized cuboplexes with a low DOTAP percentage (7.5%, w/w), compared to their lamellar counterpart. Sarkar et al.²⁰⁶ doped cuboplexes with several cationic lipids and GFP siRNA, achieving successful delivery and gene silencing on GFP-expressing Chinese Hamster Ovary (CHO) cells (Fig. 6D). Introducing small amounts of DOTAP (0.1 mol/mol) has substantially enhanced siRNA encapsulation (75%) without a

notable increase in toxicity. The subtle enhancement in transfection efficacy within the first 24 h is attributed to the controlled release kinetics of cubosomes, highlighting their potential for sustained applications. DOTAP modified-cuboplexes demonstrated a 13% superior knockdown efficiency over 72 h compared to commercially available lipofectamine. The subtle enhancement in transfection efficacy over the first 24 h can be attributed to the controlled release kinetics of the cubosomes, highlighting their potential for sustained applications.

The PEGylated cuboplex offers biological stability for RNAi technology by forming a highly ordered internal structure and sterically stabilized design²⁰⁵. Encapsulation has achieved a promising gene knockdown efficiency of 75%, exceeding that achieved by traditional LPS-based systems and commercially available siRNA delivery products.

Moreover, conventional cubosome formulations usually possess particle sizes that are too large to meet the requirements for permeabilization of anticancer therapeutics. To address this concern, microfluidic nano-manufacturing approaches were introduced to formulate siRNA cuboplexes with efficient encapsulation and a size of around 75 nm (Fig. 6C)²¹⁰.

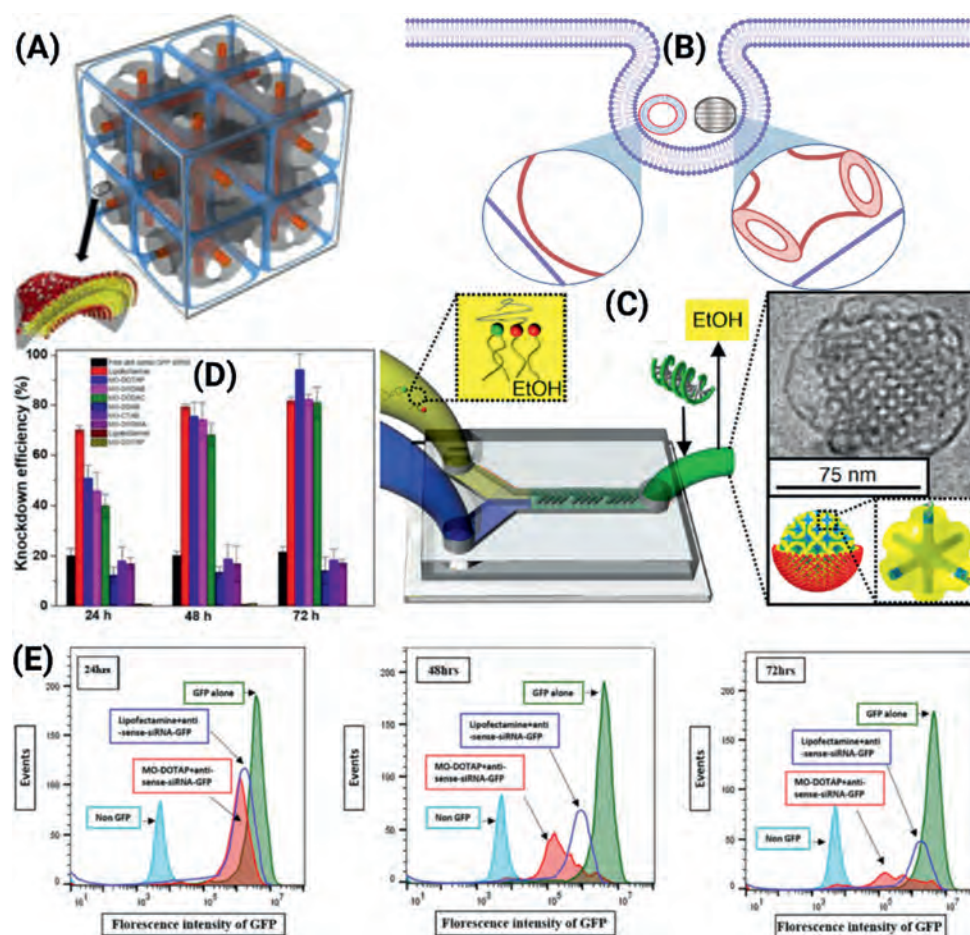


Figure 6 Cubosomal nanocarriers for enhanced RNA delivery. (A) Bicontinuous cubic Im3m phase structure of cubosomes. Reprinted with permission from Ref. 205. Copyright © 2015 American Chemical Society. (B) Schematic illustration for the cellular entry of lipid nanocarriers. (C) Preparation of cuboplexes by microfluidic staggered herringbone mixer. Reprinted with permission from Ref. 210. Copyright © 2018 American Chemical Society (D) Knockdown efficiency and (E) Fluorescence-activated cell sorting analysis following incubation of GFP-siRNA and different cationic cuboplexes with CHO-GFP cells over 24, 48, and 72 h. Reprinted with permission from Ref. 206. Copyright © 2021 American Chemical Society.

In clinical trials, LNPs continue to represent a beacon of hope and a leading tool in personalized medicine, thanks to their capacity to incorporate and protect nucleic acids while enabling targeted delivery and ultimately mitigating off-target effects. Besides, LNPs have a low incidence of adverse reactions, and they are known to be biocompatible and low immunogenic, making them promising and well-tolerated therapeutic vectors. The diversity of lipids' molecular structure allows for tailored delivery systems that efficiently address specific therapeutic needs in cancer treatment. Despite these strengths, challenges such as low biostability, low cell uptake, immune response modulation, and manufacturing scalability still remain for lipid-based nanosystems. Fabricating LNPs is often based on common bulk mixing or pipette mixing; however, they offer inadequate reproducibility. To address the manufacturing hurdles, microfluidics-based manufacture offers cost-effective, high-throughput, quick, and reproducible LNPs. Microfluidics leverages the principles of laminar flow and precise mixing at the microscale by parallelizing multiple mixing units on a single chip, obviating the demand for complicated fluid handling systems^{239,240}. As a first step in converting LNPs into clinically and commercially relevant products, the microfluidic device must be capable of generating high flow rates (>10 L/h)²⁴¹. In the SCALAR platform, 256 mixing units are integrated into a single microfluidic chip, which allows for high-throughput LNP fabrication²⁴². Recently, a microfluidic reactor with 128 staggered herringbone mixing channels has been able to generate mRNA/siRNA–LNPs with uniform quality²⁴³. Specifically, scaling up the microfluidic manufacture of mRNA–LNP vaccines remains challenged by the maintenance of purification and adherence to regulatory compliance with good manufacturing practice (GMP) standards. In addition, maintaining microchannels sterility presents a further concern for microfluidic-based technologies²⁴⁴. Aside from this, ethanol-containing manufacturing protocols require ethanol removal from the final formulation for its potential effect on nanoparticle integrity and encapsulation efficiency²⁴⁵.

3.2. Polyplexes

Polyplexes are generated through charge interactions between cationic polymers and negatively charged nucleic acids²⁴⁶. Besides being easy to prepare, purify, and chemically tunable, polyplexes ensure that the mRNA is effectively shielded from degradation by RNases through robust protective shields¹⁶⁸. As well, some polymers allow for efficient cellular uptake and endosomal escape, enhancing transfection efficacy¹⁶⁹. The merits of polymers extend to distributing biotherapeutics to lymph nodes, promoting robust immune responses²⁴⁷. New generations of polymeric nanoparticle delivery approaches, including micelles, polymersomes, nanoparticles, nanocapsules, nanogels, dendrimers, and nanocomposites, offer a promising avenue for the optimal delivery of RNA therapeutics. These nanostructures find applications in regenerative medicine, bioimaging, drug and gene therapy²⁴⁸. For instance, polymeric nanogels significantly improve drug loading, stability, and design versatility²⁴⁹. Their responsiveness to environmental changes and ability to form complexes with biomacromolecules make them ideal for a wide range of biomedical applications.

Advancements in self-assembling amphiphilic block copolymers have created different nanostructures, such as micelles and polymersomes with a core–shell structure²⁵⁰. Polymersomes, composed of amphiphilic triblock copolymers, resemble lipid

bilayers and aid the delivery of hydrophilic or hydrophobic molecules. Meanwhile, micelles serve as carriers, usually for hydrophobic agents²⁵¹. Polymersomes, due to their biocompatibility and efficient cellular uptake, surpass micelles in stability and versatility. They encapsulate larger or multiple agents, proving versatile in medical applications.

Synthetic charge-altering polymersomes have been developed to prevent degradation by ribonucleases. They facilitate cytoplasmic protein expression through escaping from endosomes, using the proton sponge effect under acidic endosome environments, guaranteeing structural protection against external threats¹⁷². A subset of polymeric materials applied in nucleic acid delivery will be discussed accordingly.

3.2.1. Polyethyleneimine

Polyethyleneimine (PEI) is well-acknowledged as a non-viral gene transfecting material as it exhibits robust nucleic acid encapsulation and inherent endosomolytic activity at the nano-sized level²⁵². The structure of PEI inhibits charge repulsion, promoting stability driven by the intrinsic pK_a of the polymer²⁵³. Linear or branched forms of PEI with high cationic charge density enhance interactions with extracellular matrix components and improve polyplex entry into cells¹⁷⁰. PEI complex achieves effective endosomal release through the “proton sponge effect”, causing osmotic expansion and rupture of endosomes^{254,255}.

A PEI–mRNA polyplex efficiently transfects multiple sets of cell lines, including bronchial epithelial cells, and nebulization does not adversely affect PEI–mRNA transfection efficiency, so the administration of aerosol is feasible *in vivo*^{256,257}. Cortez-Jugo et al.²⁵⁷ proposed employing the acoustomicrofluidic nebulization technique to facilitate aerosol delivery, demonstrating effective knockdown of lung carcinoma genes. However, linear PEI's cytotoxic effects limit its clinical application¹⁷³. High-molecular-weight PEI possesses notably lower apoptotic and necrotic effects, though it remains detrimental to DNA²⁵⁸. Modifications to the PEI structure to generate branches and chemical alterations to enhance biocompatibility paved the way for their therapeutic potential^{259,260}. Branched polyethyleneimines (BPEI) possess greater ionization diversity and an improved buffering capacity²⁶¹. Nevertheless, BPEI can form complexes with proteins in the body, resulting in probable immune responses and adverse events²⁶².

Modifications to BPEI structure, such as incorporating poly(ethylene glycols) (PEGs), generate more biocompatible and biodegradable polymers. These modifications enhance transfection efficiency without affecting PEI–DNA complexation, leading to prolonged circulation time²⁶³. Further modifications to BPEI, such as conjugation with β -cyclodextrin or the addition of lipoic acid moiety, have shown promising outcomes in terms of reduced cytotoxicity and improved transfectability^{264–266}. Bio-reducible disulfides within BPEI structure have shown promising outcomes regarding cytotoxicity and improved transfectability compared to standard BPEI²⁶⁴.

3.2.2. Poly(amino acid) derivatives

Biodegradable polymers derived from amino acids, such as *N*-substituted polyaspartamide (PAsp), have significant biomedical utility due to their good biocompatibility, biodegradability, and safety²⁶⁷. Poly[*N*–[*N*–(2-aminoethyl)–2-aminoethyl]aspartamide (PAsp(DET))] has pH-specific protonation, influencing the cationic charge density and facilitating endosomal²⁶⁸.

Aminoethylene repeats are pivotal in shaping the polymer characteristics affecting cationic charges, endosomolytic, cytotoxicity, and resistance to RNAase (Fig. 7A). The translational efficiency of polyplexes varies with the number of aminoethylene repeats²⁶⁹.

A further improvement in pH-specific protonation of aminoethylene within the endosomal environment was achieved by incorporating thiourea into the PAsp(TET) structure. Consequently, siRNAs were able to escape and distribute more efficiently within cells as well as exerted better knockdown efficiency (Fig. 7B)²⁷⁰. Cationic amphiphilic PAsp(DET) with alicyclic compounds, such as cyclohexyl ethyl, produced stable polyplexes with increased lung-specific mRNA expression. These advancements highlight the potential of poly(amino acid) derivatives for effective nucleic acid delivery²⁷¹.

3.2.3. Polyacrylates

Polyacrylates alone lack effective electrostatic interactions with nucleic acids, and to permit encapsulation, polyacrylate side chain modification is crucial for RNA delivery. At low pH, poly(2-dimethyl aminoethyl acrylate) (PDMAEA) demonstrated their cationic nature, resulting in gradual degradation to innocuous poly(acrylic acid) units²⁷². The incorporation of short alkyl methacrylate monomers into the polymeric backbone increased cationic density and improved mRNA entrapment and transfection efficiency with minimal cytotoxicity compared to PEI²⁷³.

PDMAEMA-based cationic star polymers, when coupled with positively ionized dimethacrylate crosslinkers, have been found to effectively transfer siRNA to murine myoblasts that retain lower cytotoxicity²⁷⁴. These nanomicelles exhibited a favorable surface charge with low inherent cytotoxicity, efficiently encapsulating and co-delivering siRNA and paclitaxel to cells²⁷⁵. Introducing disulfide bonds to the backbone of PDMAEMA consequently created a reductively responsive polymer with minimal cytotoxicity and improved transfectability in melanoma and pancreatic cancer cells²⁷⁶.

3.2.4. Polyesters

In an attempt to achieve improved clinical outcomes for polymer-based drug delivery systems, the focus is directed to biodegradable

polymers that are less toxic and produce harmless byproducts that can be easily eliminated by renal filtration²⁷⁸. Biodegradable polyesters, such as polylactic acid (PLA), polyglycolic acid (PGA), and poly(D,L-lactide-co-glycolide) (PLGA), have been broadly investigated for their potential as nanoplateforms for nucleic acid delivery. Lipopolyplexes, formed by modifying polymers with lipids such as DOTAP, DOTMA, and DOPE, improve nucleic acid entrapment and transfection efficiency²⁷⁹. Jensen et al.²⁷⁹ developed an inhalable powder formulation of enhanced green fluorescent protein (eGFP) siRNA using cationic PLGA-based lipopolyplex that shows potential for efficient gene silencing and stability. Pre-functionalization of PLGA or PLA with hydrophilic moieties and/or cationic polymer adjuvants, either PEI, polyarginine, chitosan, poly(β -amino esters) (PBAEs), has been found to enhance nucleic acid encapsulation efficiency and modulate their release^{280–283}. PLGA-PEI nanocomplexes coated with hyaluronic acid effectively co-deliver hypericin and HIF-1 α siRNA to hypoxic human nasopharyngeal carcinoma cells, demonstrating enhanced gene silencing and tumor accumulation²⁸¹.

3.2.5. Charge-altering releasable transporter

In 2017, the charge-altering releasable transporter (CART) emerged as a promising alternative to traditional cationic polymers¹⁷². At their core, CARTs are polycations that conveniently encapsulate mRNA due to the positive charges stemming from the oligo(α -amino ester)²⁸⁴, are explored to address the rate-limiting mRNA release from internally entrapped cargo for efficient transfection²⁸⁵. These carriers deliver polyanionic mRNA under physiological conditions (pH = 7.4) *via* a controlled self-degrading process, transforming into neutral molecules and facilitating the mRNA release from endosomes¹⁷².

However, challenges exist with CARTs, including low transfection efficiency, biodegradability, and cytotoxicity. The limitations can be overcome by modifying lipid and cationic units to create novel CART analogs and incorporating hybrid polyplexes of CART analogs, which showed boosted cellular uptake over standalone CARTs²⁸⁶. Waymouth research group reported an amino acid-based CART in 2019, Ser-CARTs, with enhanced biocompatibility and charge-altering properties under different pH

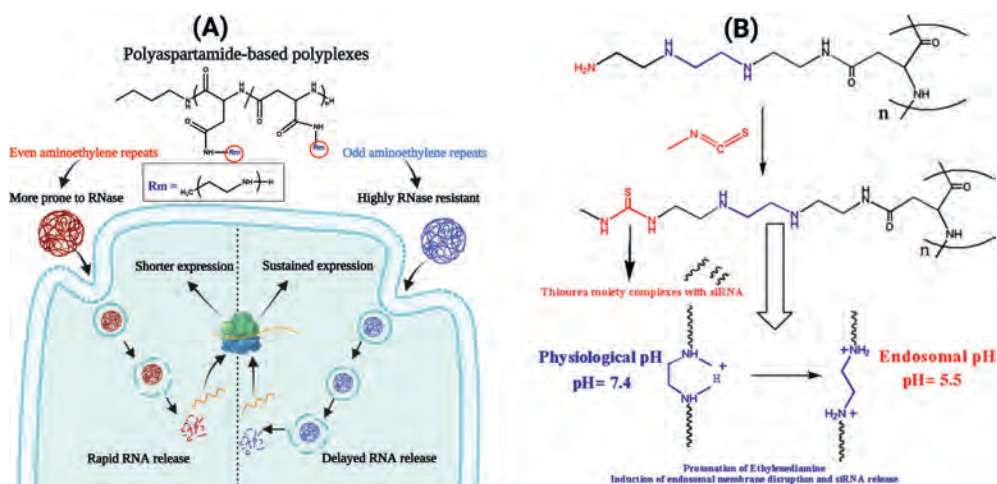


Figure 7 (A) Classes of *N*-substituted polyaspartamide derivatives and their endocytosis process²⁷⁷. (B) Protonation of polyaspartamide derivatives. Figure was made by Biorender.com.

conditions²⁸⁷. More recently, lysin-based CARTs were reported for efficient lung-targeted transfection for mRNA and siRNA, demonstrating their effectiveness in cancer vaccination and local immunomodulation in murine models²⁸⁸. The administration of Lys-CART/mRNA complex achieved selective protein expression in the lung with no supplementary targeting ligands. The ability to modulate protein expression selectively in the spleen or lung through simple modifications to the CART structure paves the way for innovative biotherapeutics delivery.

3.2.6. Polypeptide and protein-based lipoplexes

Polypeptide and protein delivery systems are made up of amino acid monomers that confer higher biocompatibility and biodegradability. Common polypeptide carriers include poly L-lysine polymers (PLL)²⁸⁹, poly oligo-D-arginine²⁹⁰, and poly L-histidine²⁹¹ for delivery of nucleic acids. Like PEI, PLL is cytotoxic to cells with increased cationic charge density, leading to disruption of cell membranes and cytotoxicity. This can be improved by incorporating other monomers, such as carbohydrates, PEGs, and phosphates, into PLL copolymers, reducing the overall charge density and mitigating toxicity concerns²⁹².

Protamine is a small peptide ~4000 Da, naturally occurring in sperm, often used as an anticoagulant. Protamine has a cationic charge and can form stable nanoparticle aggregates with mRNA, enhancing immune surveillance and cancer cell detection²⁹³. A protamine-based polylipoplex that incorporates a cationic lipid shell (DOTAP) can further enhance the overall efficiency of mRNA delivery²⁹⁴. GALA-complexed mRNA polyplexes, with approximately 350 nm size and slightly negative surface charge, achieved proper transfection in macrophages and DCs.

In the pursuit of overcoming the cellular plasma membrane barrier, cell-penetrating peptides (CPPs) have opened new avenues for the development of effective delivery approaches²⁹⁵. The CPPs, including PepFect14 and GALA (glutamic acid-alanine-leucine-alanine repeats), have been instrumental in proceeding with mRNA therapeutic delivery through potential mechanisms of glycosaminoglycan clustering and micropinocytosis²⁹⁶. PepFect14 has supported better mRNA delivery both *in vitro* for SKOV-3 cell lines and *in vivo* on the SKOV-3 xenografted mice²⁹⁷. GALA offers controlled permeabilization at an acidic pH²⁹⁸. GALA-complexed mRNA polyplexes, with a size of approximately 350 nm and a slightly negative surface charge, achieved proper transfection in macrophages and DCs (D1)²⁹⁹. GALA polyplexes enter DCs through sialic acid-mediated endo/phagocytosis, leading to an increase in cellular uptake.

3.2.7. Dendrimers

Dendrimers possess a unique highly branched structure bearing functional surface groups that are useful for drug encapsulation within the spherical structure³⁰⁰. Dendrimers, such as poly-amidoamine (PAMAM) and poly(propyleneimine) (PPI), have been explored for nucleic acid delivery due to their high cationic charge density and ability to form stable complexes with RNA^{301,302}. PAMAMs are effective for RNA delivery hinges on optimizing a set of the dendrimer functionalities, which enhance mRNA binding and contribute to improved stability and transfectability³⁰³.

However, dendrimers may face challenges related to cytotoxicity, immune response, and potential off-target effects, necessitating careful design and optimization for clinical applications. Although efficient in transfection, highly branched PAMAMs are restricted by their notable cytotoxicity due to their increased

charged cationic nature³⁰⁴. Asymmetric dendrimers composed of lower-generation peptides provide a less toxic alternative, improving cell transfection³⁰⁵. There are several approaches currently being considered to improve their biocompatibility, either through disulfide crosslinking³⁰⁶ or the formation of click chemistry-linked PAMAM-methacrylate polymeric scaffold³⁰⁷. Surface modifications with PEG enhance dendrimer biocompatibility, reducing cytotoxicity and immune response. PPI dendrimers modified with PEG and targeting ligands exhibit efficient siRNA delivery to specific cancer cells, resulting in enhanced gene silencing effects¹⁷¹. Fluorinated dendrimers have shown enhanced gene transfection efficiency for various genome engineering tools through the improvement of cell uptake, endosomal escape, and serum resistance³⁰⁸.

3.2.8. Poly beta-amino ester

The poly(beta-amino ester) (PbAE) was initially developed and demonstrated adequate transfection in human primary cells³⁰⁹. The biological attributes of PbAEs render them highly biodegradable and structurally tunable for nucleic acid delivery³¹⁰. Linear PbAE-based delivery systems are constrained by their homogenous backbones. To overcome these limitations, branched cationic polymers, such as highly branched PbAEs (hBPbAEs), were developed for the transfection of nucleic acids and show improved biocompatibility and transfection efficiency compared to linear PbAEs³¹¹.

Since naïve PbAE and nucleic acid complexes are inefficient at delivering mRNA to antigen-presenting cells (APCs), a call for modified PbAE that interact suitably with endosomal membranes, paving the way for cargo release, is necessary³¹². The introduction of poly-caprolactone (PCL) to PbAE has been proven effective in mRNA vaccine delivery for cancer immunotherapy. The success of PCL-grafted PbAEs-based polyplexes led to the development of ionizable polyplexes containing PbAE terpolymer that exhibited a high level of transfection³¹³.

The research team directed by Stephan has reported that antibody conjugation and polyglutamic acid linkers further improve PbAE-mRNA polyplexes for T-cell targeting, minimizing off-target binding^{314,315}. These nanocarriers reprogram circulating T-cells to target the cancerous tissues, avoiding the costly and complicated *ex vivo* T-cell reprogramming process.

Patel and colleagues introduced a method using inhalable BPbAEs-based polyplexes to deliver firefly luciferase mRNA³¹⁶. This approach achieved uniformly distributed mRNA throughout all lung lobes, maintaining stability for up to 90 days. Green and his team reported the delivery of the gene-editing tool CRISPR-Cas9 components with biodegradable PbAE nanoparticles, achieving up to 70% gene knockout efficiency and enabling a 45% gain-of-function expression³¹⁷.

3.2.9. Natural polysaccharides and synthetic glycopolymers

Polysaccharides such as chitosan, hyaluronic acid, dextran, and synthetic glycopolymers offer biocompatible, biodegradable, and less toxic scaffolds for drug and gene delivery^{318,319}.

Chitosan is a biocompatible, muco-adhesive, and versatile biopolymer that was previously applied for siRNA delivery^{320,321}. Modifications of chitosan make it easy to form stable polyplexes with siRNA³²². Hydrophobic modifications enable encapsulation of hydrophobic drugs within the core and enhance the codelivery capabilities of siRNA complexed in the hydrophilic shell and the hydrophobic drugs³²³. Due to the “proton sponge” effect as an

escape mechanism, conjugation of chitosan with cell-penetrating peptides aids in endosomal RNA escape³²⁴.

Another linear polysaccharide, hyaluronic acid (HA), naturally present in skin and joints, confers its biocompatibility and biodegradability³²⁵. HA is a primary binding ligand to cluster of differentiation 44 (CD44), a glycoprotein overexpressed in solid tumors. HA-functionalized lipoplexes are attractive for cancer targeting with overexpressed CD44, particularly when paired with cationic lipids^{326,327}. Furthermore, cyclodextrins have been found to enhance the delivery of liposomal RNA. A recent study showed that β -cyclodextrin forms hydrogen bonds with phosphates of siRNA molecules and DOPC LPS, leading to the avoidance of payload escape and improvement in serum stability³²⁸.

To summarize, the polymeric material shields RNA and acts similarly to LNPs in protecting against enzymatic degradation in the extracellular environment, thus prolonging the circulation time in the bloodstream and enhancing its bioavailability. Polymeric nanoparticles may also offer opportunities to customize their physicochemical properties and interactions with biological systems by modulating the composition, size, surface charge, and surface functionalization. Introducing targeting moieties on polymeric nanoparticles' surfaces can optimize their specificity for cell types, minimizing off-target effects. After internalization, nanoparticles must escape lysosomes to avoid degradation and reach the cytoplasm to deliver their RNA payload. The proton sponge effect arising from cationic polymers such as PEI and PAMAM is particularly effective in promoting endosomal escape, leading to osmotic swelling and rupture of endosomes, thereby releasing nanoparticles into the cytoplasm. Moreover, stimuli-responsive polymers have enabled the design of smart nanoparticles sensitive to specific cues within the TME. Anomalous conditions are often observed within the TME, including pH, temperature, redox potential, or enzyme levels. Thus, integrating stimuli-responsive moieties into the polymer matrix allows targeted and controlled release of RNA payloads³²⁹. Although polymeric nanoparticles show promise for RNA delivery, their clinical benefits are yet to be fully realized. Progress in improving tissue specificity and cellular uptake, biodegradability, biocompatibility and safety, and scaling up the manufacture is essential for clinical translation.

3.3. Exosomes

Exosomes are a class of extracellular vesicles with a nanoscale size ranging from 30 to 2000 nm. They are originally excreted from mammalian cells. These physiological nanovesicles have gained great attention as potential carriers for pharmaceuticals and biotherapeutics due to their inherent capacity to encapsulate naturally present biological cargo and cross biological barriers. For example, they have been implicated as natural carriers for miRNA³³⁰. Exosomes show preferential delivery to the brain by crossing the blood–brain barrier and are employed in treating neurodegenerative disorders³³¹. Additionally, exosomes could camouflage the phagocytosis and offer a safer platform for biotherapeutics delivery, especially for macromolecules¹⁷⁴. Zhao et al.¹⁷⁵ developed autologous breast cancer-derived exosomes for cationic bovine serum albumin conjugated siS100A4 for effective lung-targeted delivery. In *in vivo* experiments, loaded exosomes

displayed a superior lung affinity compared to LPS, indicating the possibility of integrin-mediated targeting.

Despite the advantages of exosomes like biocompatibility, to date, no FDA approval has been issued for exosomal products¹⁷⁹. Concerns about how a potentially preloaded cargo may affect patients non-specifically make approval difficult. Exosomes, therefore, require careful production processes to optimize exosome production, purification, and quality control processes to advance their therapeutic applicability¹⁷⁷. The heterogeneity in exosome size and loaded content poses significant weaknesses, affecting their encapsulation efficiency and yielding inconsistent therapeutic effects¹⁷⁶. Therefore, adopting GMP for standardized manufacturing processes in clinical trials becomes challenging, given difficulties in characterizing, controlling, and scaling up exosome production through maintaining batch-to-batch consistency³³².

Exosomes can be loaded with desired cargo through various methods, including incubation, transfection-based approach, and physical treatments such as electroporation or sonication³³³. Also, *in situ* assembly and synthesis offer a unique approach, enabling the loading of metal nanoparticles by reducing metal ions within exosomes. Nevertheless, exosomal transfection is highly efficient for small RNAs like siRNA and miRNA, but not for mRNA³³⁴. The restricted yield of exosomes impedes their use as a therapeutic tool due to limitations on the necessary doses for clinical trials³³⁵.

Scaling up exosome isolation to meet clinical demands presents challenges, given the high costs and limited production quantities. Moreover, the preservation of exosomes for clinical studies remains a major concern, which calls for further research into standard conditions. Current studies recommend storing exosomes at -80°C , though the storage effects on exosomes depend on their source^{336,337}. Storage at this temperature shows concerns about the availability of clinically approved treatments for patients. To address these problems, surface coating of exosomes shows promise to preserve longer shelf life, such as the nanofilm of supramolecular complexes of ferric ions and tannic acid³³⁸ or PEG modification³³⁹. These methods enhance stability at specific temperatures and biocompatibility, potentially advancing exosome-based nanoplatforms for more effective, safe, and stable therapeutic applications.

In closing, researchers are exploring the potential of these natural nanovesicles for RNA delivery due to their biocompatibility, minimal immunogenicity, and ability to permeate biological barriers. Another advantage of exosomes lies in their ability to display target-specific surface ligands, enabling them to precisely target specific cell types and tissues. Various loading methods have been implemented to successfully incorporate RNA into exosomes, including electroporation, incubation, sonication, and transfection with lipid-coated particles. Despite the potential of exosomes for RNA delivery, optimizing exosome manufacturing, purification, and loading remains challenging. Addressing the concerns associated with exosome yield, cargo loading, and tissue tropism would help advance exosomes into the clinical phase¹⁷⁸. Therefore, exploring innovative approaches, such as *in vivo* engineering of cells, 3D bioprinting, and microfluidics to produce exosomes for therapeutic purposes will pave the way to eliminate the cost and scalability challenges associated with exosome isolation³⁴⁰.

3.4. Organic-inorganic mesoporous silica nanohybrids

Standard mesoporous silica nanoparticles (MSNs), such as MCM-41 and SBA-15, are widely known for their significance in nanomedicine owing to their remarkable surface area and pore volume. MSNs have the potential for dual delivery of anticancer therapeutics, doxorubicin, and siRNA targeting P-glycoprotein through surface decoration with a PEI-PEG copolymer. This modification facilitated the protected delivery of both agents with improved EPR with reduced uptake in xenografted nude mice and led to synergistic inhibition of tumor growth¹⁸¹. The application of MSNs is restricted to small drug molecules due to nanopore size <5 nm^{341,342}. Large open pore MSNs with a pore diameter of >12 nm present a prospective siRNA delivery approach^{181–183}. Ultra-large MSNs protect loaded RNA from degradation and significantly enhance the transfection efficiency, cytotoxicity, and gene silencing *in vitro* and *in vivo* (Fig. 8)¹⁸².

Wang et al.³⁴³ generated dendritic mesoporous organosilica (DMSOs) with a small diameter (~ 50 nm) and large pore size (>20 nm). The DMSOs exhibited significantly improved *in vitro* mRNA transfection efficiency. The same group further studied mRNA delivery by combining DMSOs with zeolitic imidazolate framework-8³⁴⁴. In this system, Zeolite adds positive charges and large mesopores that are appealing for effective mRNA loading, and its imidazole ring eases the endosomal escape (Fig. 9). These merged effects culminated in enhanced mRNA translation and improved transfection efficiency, surpassing the performance of commercial products.

Hollow MSNs (HMSNs) with larger pores having diameter of ~ 24 nm allowed the encapsulation and controlled release of siRNA targeting P-glycoprotein and doxorubicin in a manner that reduced the tumor volume by 5.93-fold less than untreated doxorubicin³⁴². The increased efficiency of the doxorubicin is attributed to the synergistic impediment of multi-drug resistance in subcutaneous xenografted mice.

Xiong et al.¹⁸³ synthesized a novel magnetic core-shell MSN with a particle size of ~ 150 nm and radial mesopores of 12 nm, showing high siRNA loading (2%, w/w) and responsiveness to an external magnetic field. Although the siRNA-loaded MSNs showed a poor surface charge (5.4 mV), this problem was resolved by clogging the silica pores with a pH-responsive gatekeeper (tannic acid). Tannic acid is a dual role player in safeguarding the loaded siRNA cargo, enhancing the surface charge (-53.1 mV) and dispersion stability. With the assistance of the external magnetic field, the functional siRNA uptake into the cytoplasm of human osteosarcoma cancer cells was notably improved to 40% within 6 h. Furthermore, bowl-like MSNs with non-aggregated structures of ~ 180 nm and cavities of ~ 140 nm, along with amine functionalization, are suitable for pDNA delivery³⁴⁵. The loading efficiency of the nano-bowls was improved due to the accessibility of its crescent-shaped central cavity, which is absent in previously discussed silica-based nanoparticles.

3.5. Carbon-based nanostructures

A growing area of research for RNA delivery is carbon-based nanomaterials, thanks to their unique structure and electrical, thermal, optical, and chemo-mechanical properties¹⁸⁶. The application of carbon-based nanomaterials in RNA delivery encompasses a wide range of carbon allotropes, such as graphite, fullerenes, nanotubes, and diamonds with diverse nanostructures. Carbonaceous surfaces can be modified to bind with RNAs,

address poor water solubility, and avoid toxicity and structural problems.

Fullerenes belong to a particular category of carbon allotropes, capable of forming stable complexes with nucleic acids and carrying RNA cargo, facilitating cellular uptake and endosomal release. Tetra(piperazino)[60]fullerene epoxide (TPFE) is a cationic fullerene that was developed by the Nakamura group at the University of Tokyo for effective inhalable delivery of siRNA (Fig. 10A)³⁴⁶. Molecular dynamics simulations showed that this cationic fullerene has a strong tendency to embed into siRNA's major groove, forming a stable binding pattern with a high affinity for siRNA. Thus, fullerene guarantees stability against nucleases and pulmonary surfactants with comparatively higher RNAi activity (Fig. 10B).

Carbon nanodot is a zero-dimensional form having a size of ~ 10 nm and shows the potential to deliver siRNA when decorated with polycations³⁴⁸. Carbon dots may pose potential safety concerns. However, the passivation of nanodots with polycations such as PEI, alkyl PEI, PLL, and PAMAM has enhanced biocompatibility and transfectability³⁴⁹. PEI-modified nanodots and HER3 siRNA combined with HER2 targeting drug (trastuzumab) have improved their efficiency against breast cancer³⁵⁰. The amphiphilic characteristics of carbon dots facilitate their binding with RNA, and their fluorescent properties allow tracking the delivery process. The developed carbon nanodot, O12-Tta-CDs, have exhibited optimal OVA-mRNA transfection efficiency and can target the spleen for improved antigen presentation and T-cell infiltration (Fig. 11)³⁵¹.

Similarly, graphene nanodots can be conjugated to PEI for mRNA delivery. Graphene quantum dots demonstrate better biocompatibility than carbon allotrope due to residual metal impurities. Liu et al.¹⁸⁷ investigated the PEI-modified graphene/mRNA nanocomplex through electrostatic interactions, forming nanoparticles with sizes below 300 nm. These dots could deliver intact eGFP mRNA to hepatocarcinoma cells with dose-dependent expression, but the need for higher doses caused safety concerns that should be addressed.

Carbon nanotubes, including single-walled (SWCNTs) and multi-walled (MWCNTs), are potential materials for siRNA delivery. Due to the high aspect ratio of SWCNTs, they offer a promising nanoplatform for loading RNAs with high capability¹⁸⁸. Despite the potential endocytosis-independent cytoplasmic entry, CNT-PEI and CNT-pyridinium exhibited only modest silencing activity accompanied by moderate to high cytotoxicity¹⁸⁹. In 2017, Liu et al.³⁴⁷ applied modified SWCNTs with PEG-phospholipids to deliver siRNA (Fig. 10C). This modification effectively transported and released CXCR4 siRNA into human T-cells and peripheral blood mononuclear cells, exhibiting superiority compared to conventional liposomal formulation (Fig. 10D). SWCNT complexes demonstrated around 55% knockdown of CXCR4 receptors on T-cells within 24 h, which increased up to $\approx 90\%$ by the third day.

Siu et al.³⁵² have functionalized SWCNT with succinated PEI to make it water-soluble to deliver Cyanine3-labeled Braf-specific siRNA. These complexes exhibited sound gene silencing *in vitro* in B16-F10 cells. At the same time, *in vivo*, a significant siRNA uptake in a C57BL/6 mice melanoma model was observed over the course of 25 days through topical application, demonstrating a substantial decline in tumor growth. The paramagnetic MWCNTs were reported to disrupt cell membrane integrity due to self-rotating under a weak rotating magnetic field, suggesting these MWCNTs can be used for nucleic acid delivery through the non-

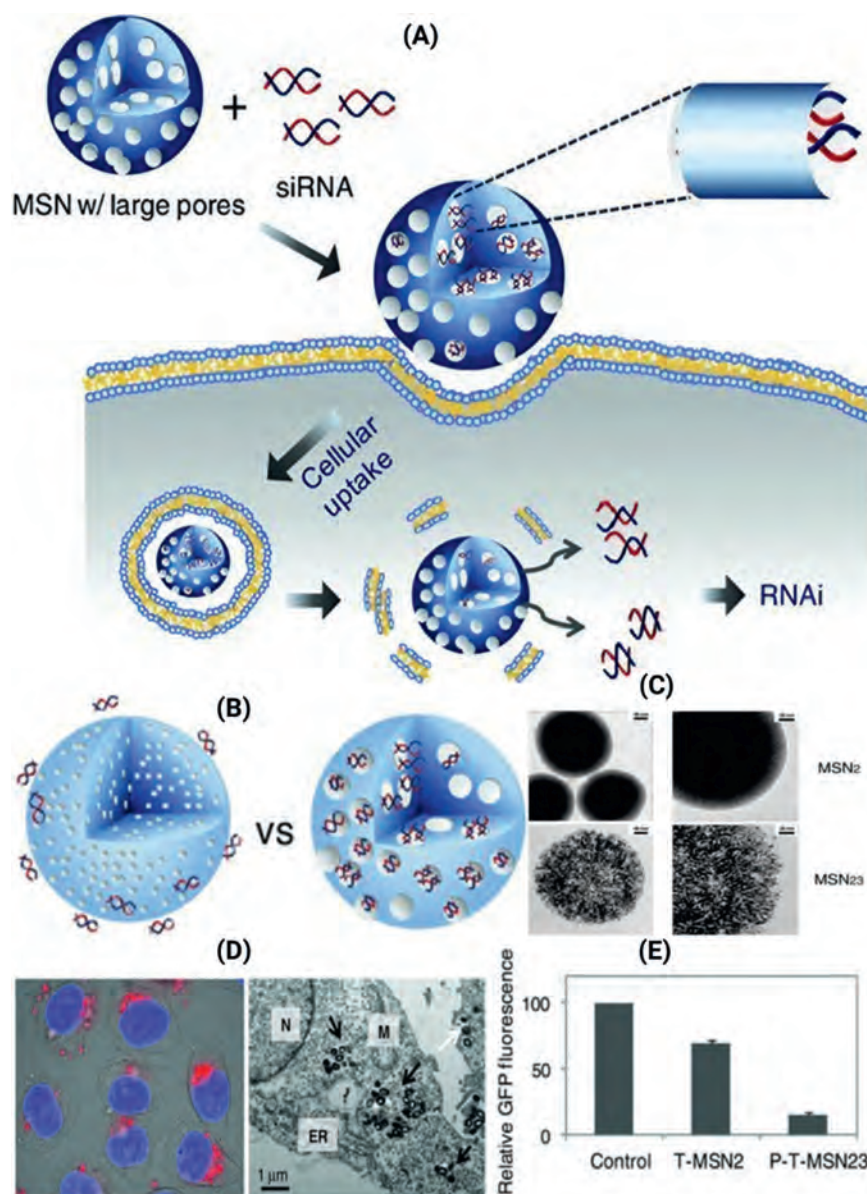


Figure 8 Ultra-large mesoporous silica for enhanced siRNA delivery. (A) Delivery of GFP siRNA using ultra-large pore MSNs. (B) siRNA loading to small pore and ultra-large pore MSNs. TEM images of (C) small pore (MSN2) and Ultra-large pore MSNs (MSN23). (D) Observation of MSNs after cellular uptake. (E) Relative GFP expression analysis showing that large pore MSNs (MSN23) were the most effective in promoting gene delivery in GFP-expressing Hela cells. Reprinted with permission from Ref. 182. Copyright © 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

lethal magnetoporation¹⁹⁰. However, magnetoporation can compromise cell viability, leading to leakage of cell components and magnetolysis, which can be beneficial for *in situ* tumor ablation. Both processes can be obtained but under different levels of controlled magnetic field flux density.

Nanodiamonds with sp^3 carbon atoms and diameters ~ 20 nm are options for RNA delivery, with coatings for specific targets. Nanodiamonds can be coated with non-covalent cationic polymers or covalent interaction such as sialylation with a coupling agent ending with methacrylate that can further link to cationic polymers (Fig. 12)^{353,354}. Despite its successful use in inhibiting Ewing sarcomas and breast cancers^{355,356}, challenges like complex functionalization processes and biodegradability consequences still require attention. Nanodiamonds have been applied to deliver

siRNA targeting the Spalt-like transcription factor 4, abundant in hepatocellular carcinoma, and exhibited increased cellular uptake compared to Lipofectamine 3000 at a weight ratio of 50:1³⁵⁷. These complexes have shown remarkable knockdown efficiency in SNU398 spheroids ($P < 0.01$) and a significant decline in AKT, phosphorylated AKT, and Cyclin D2 expression downstream.

3.6. Metallic nanoparticles

The versatility of gold nanoparticles is reflected in their ability to be chemically resistant, enzymatically stable, less cytotoxic, and physiochemically tunable, along with the optical properties that render them valuable for photothermal therapy¹⁹². Thiol-based interactions enable siRNA to bind to gold nanoparticles for

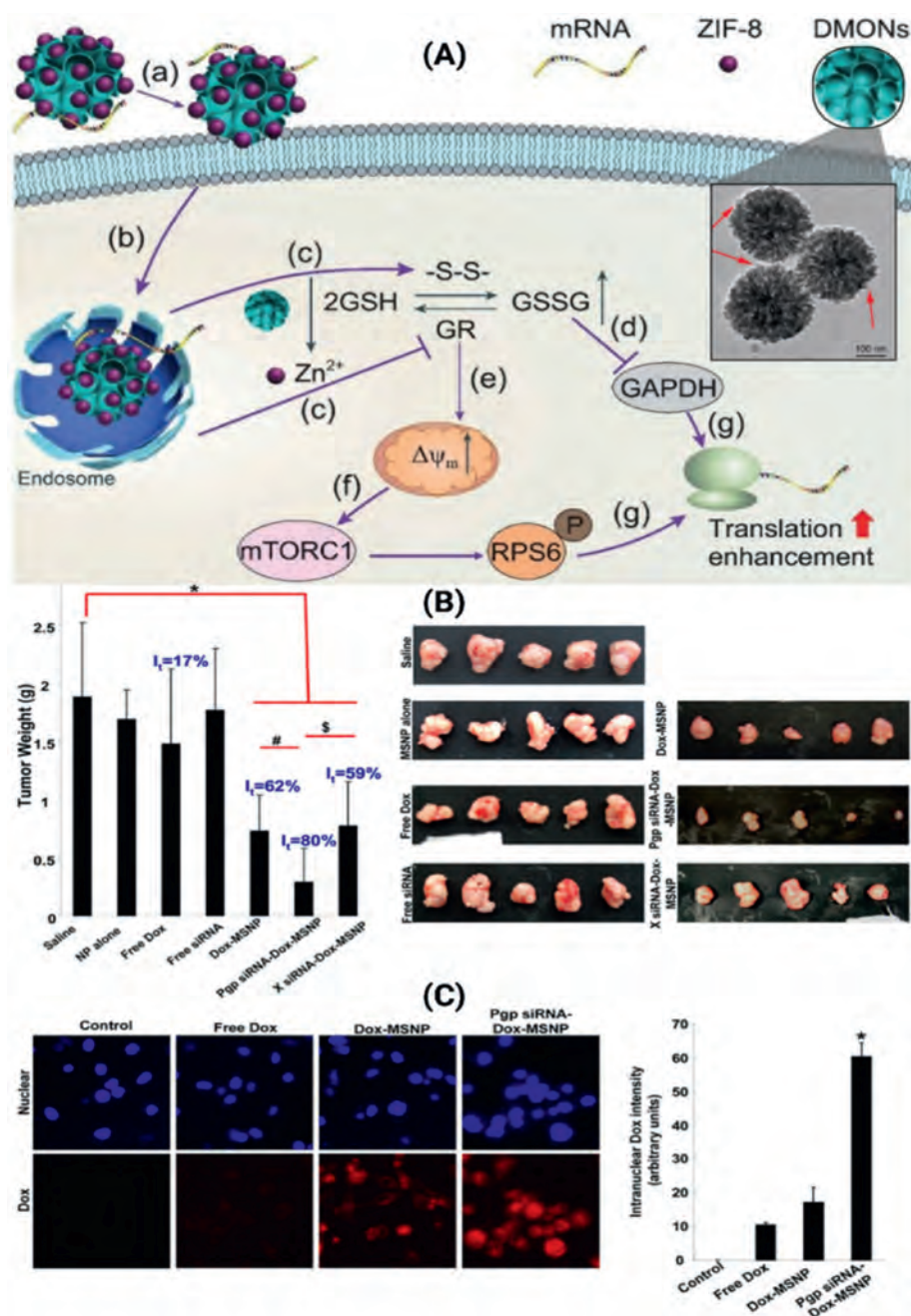


Figure 9 (A) Improvement of mRNA expression *via* DMSNs. Reproduced under Creative Commons from Ref. 181. Copyright © 2020, The Author(s) 2020. (B) Assessment of antitumor effect for Dox-loaded MSNP with and without P-glycoprotein siRNA versus controls. (C) The improved flux of Doxorubicin in MCF-7/MDR cells is presented in fluorescent images. Reprinted with permission from Ref. 344. Copyright © 2013, American Chemical Society.

enhanced cellular delivery. Further coated with PBAE, it allows multifunctional designs with adhesion and penetration-enhancing peptides³⁵⁸. Xue et al.¹⁹³ assembled gold nanoparticles with Y-shaped backbone-rigid triangle DNA bricks with sticky ends for siRNA delivery. This forms a protective covering layer surrounding siRNA. The release of siRNAs was responsive to cancer-specific endogenous miRNA, enabling targeted gene silencing within cancer cells with impressive *in vivo* therapeutic efficacy, showing complete inhibition of tumor growth.

Polymer-coated superparamagnetic iron oxide nanoparticles (SPIONs) are well suited for targeted delivery of nucleic acids by

leveraging their unique size and superparamagnetic properties. SPIONs can dually target the payload, employing ligands and magnetic guidance. A PEI-SPION formulation has been used for the delivery of ADAM10 siRNA to treat prostate cancer¹⁹⁴. In this formulation, the nanocomplex was conjugated to PEG to enhance the biocompatibility of the nanocomplex. The cellular uptake of SPIONs was evident by the confocal microscope, which had particle sizes of ~ 20 nm as detected by TEM.

In magnetofection, the magnetic nanomaterials are complexed with vectors to help them to be in proximity with target tissues, enhancing their uptake *via* endocytosis³⁵⁹. This approach involves

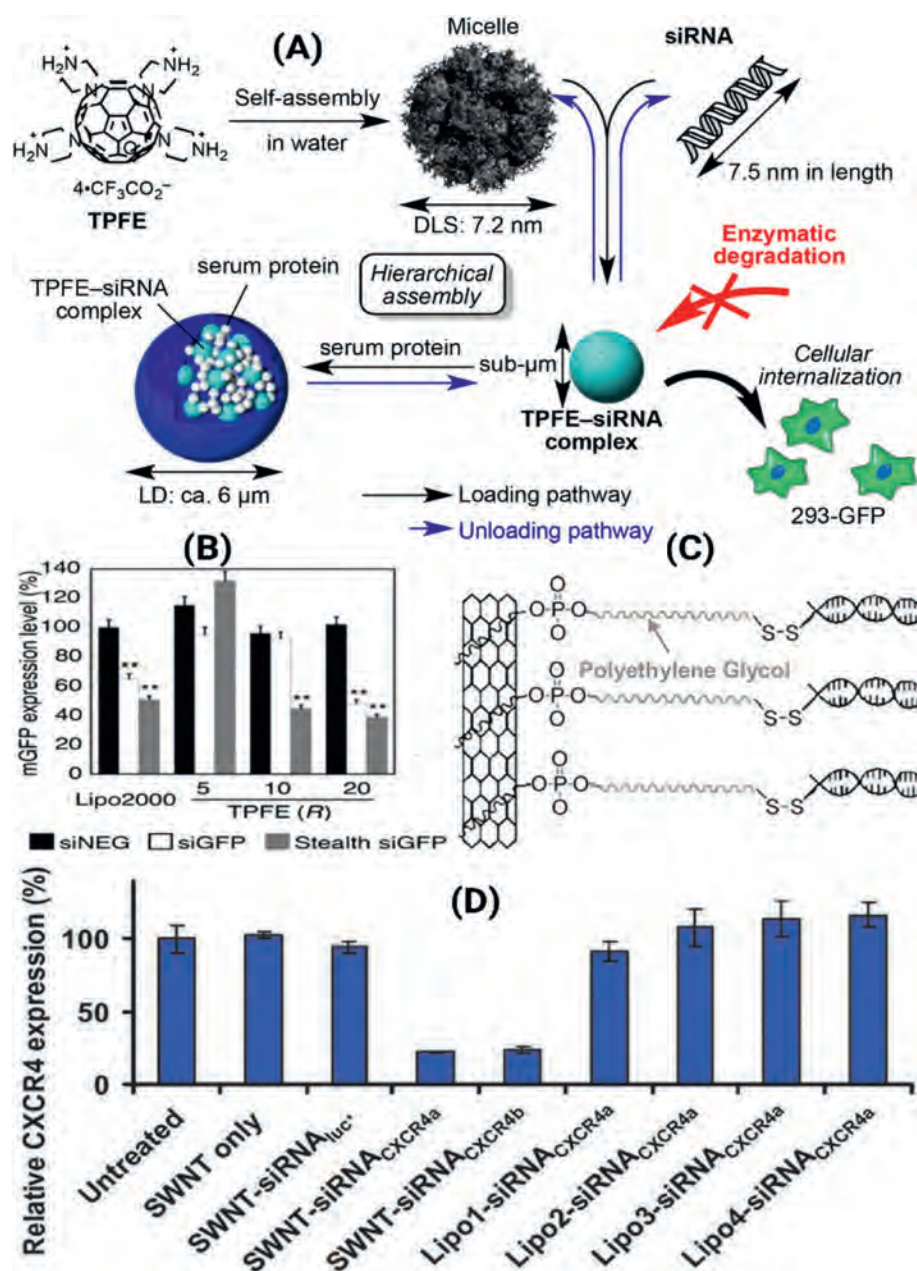


Figure 10 (A) Presentation of TPFE self-assembly. (B) TPFE-based siRNA RNAi efficiency *in vitro* using an eGFP non-specific siRNA (siNEG) and two eGFP-specific (siGFP and Stealth siGFP) at different TPFE/siRNA ratios (R: 5 to 20). Lipofectamine 2000 (Lipo2000) serves as a standard transfection agent. (** $P < 0.01$ vs. siNEG). Reprinted with permission from Ref. 346. Copyright © 2018 American Chemical Society. (C) SWCNTs surface decoration with NH_2 -terminated PEG-lipid for disulfide linkage with siRNA. (D) Assessment of CXCR4 expression on CEM cells post-treatment, including liposomes (Lipo1–4) and luciferase (Luc) or siRNA control. Reprinted with permission from Ref. 347. Copyright © 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

gene transfer using SPIONs coated with nucleic acids in the presence of a magnetic field¹⁹⁵. Nevertheless, there are persisting difficulties ahead of *in vivo* applications of magnetofection, including lower transfection efficiency, particle size requisites, rapid systemic clearance, and safety concerns with repeated administration¹⁹⁶.

4. Challenges and barriers in RNA delivery to tumors

As much as mRNA and RNAi technologies have appealed to Biotech companies, there are still several unresolved challenges

pertaining to their development. The challenges of RNA delivery include overcoming barriers related to stability, cellular uptake, immune response, and targeted delivery. RNA molecules are rapidly degradable by RNases and nucleases; therefore, they need to be incorporated into formulations³⁶⁰. Efficient cellular uptake is crucial for the therapeutic efficacy of RNA, and addressing endosomal escape is a common obstacle³⁶¹. Immune responses triggered by RNA can lead to unwanted reactions, requiring strategies to mitigate immunogenicity^{34,362}. Finally, achieving targeted delivery to specific tissues or cells while minimizing non-specific organ deposition and off-target effects poses a significant

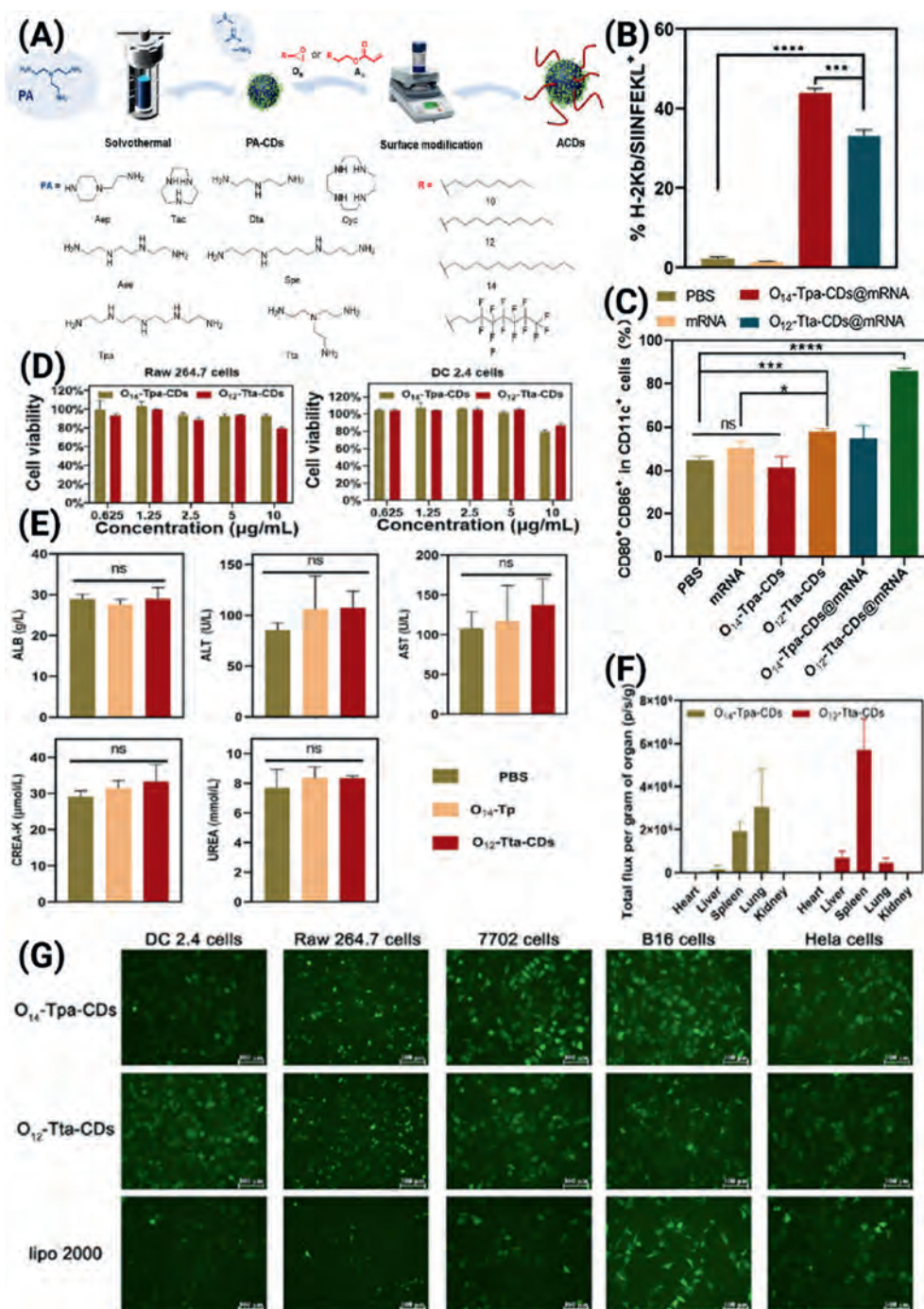


Figure 11 Amphiphilic carbon dots (ACDs)-based delivery system. (A) Preparation of ACDs. (B) Percentage of matured antigen (H-2Kb/SIINFEK⁺) presenting cells, and (C) Proliferation of CD80⁺ and CD86⁺ cells in bone marrow DCs following treatment of bone marrow DCs by ACDs. (D) Cell viability assessment of ACDs-treated DC2.4 and Raw264.7 cells, showing biocompatibility. (E) Measurement of serum toxicity markers (serum albumin, ALT, AST, Creatinine, and urea) following ACDs administration at doses of 3.6 mg/kg to mice. (F) Flux analysis of ACD flux in mice's excised organs. (G) Fluorescence of expressed GFP after incubation with ACDs compared to LPS. Reprinted with permission from Ref. 351. Copyright © 2023 American Chemical Society.

challenge in RNA delivery technologies^{363,364}. Furthermore, the co-delivery of gene therapies and chemotherapies requires a nanocarrier that maintains a synergistic effect with minimal side effects³⁶⁵. In the long run, once preclinical studies have been completed and all obstacles have been handled, gene therapy will

have to be approved by regulatory agencies to ensure the product is effective, reproducible, and scalable³⁶⁶. Stability issues, which often require demanding storage and stringent handling measures, could compromise the dispatch and distribution of RNA therapeutics.

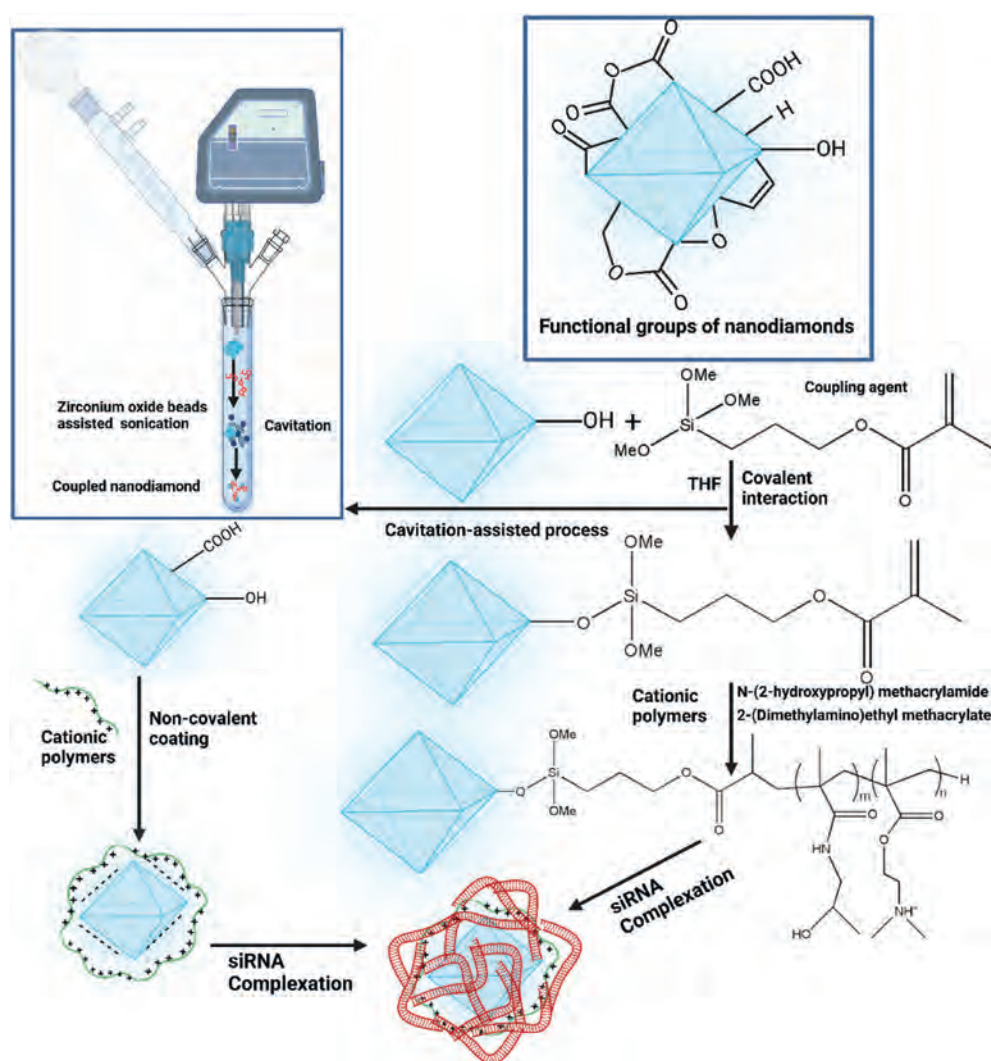


Figure 12 Nanodiamonds for RNA delivery. Nanodiamonds can bind to siRNA using two methods: a covalent interaction and a non-covalent binding approach. The covalent interaction involves surface modification with silylation through cavitation-assisted processing with zirconium oxide beads, followed by coupling with methacrylamide and a cationic polymer to form a complex with siRNA. In the non-covalent method, nanodiamonds are coated through electrostatic interaction with cationic polymers, which are then complexed with siRNA. Figure was made by Biorender.com.

4.1. TME-associated barriers to RNA delivery

The path lies ahead for RNA-based therapeutics before they can effectively reach their intended target within the intricate TME. Abnormal vascular networks, influenced by numerous essential components within the TME³⁶⁷, pose a major concern to their transport and release into target cells. The influential factors governing the eventual *in vivo* fate of RNA therapeutics are primarily TME, extracellular matrix (ECM), and increased interstitial fluid pressure (IFP).

Tumor hypoxia, arising from abnormal blood vessels, increased metabolic rate, dense and fibrotic TME, genetic mutations, and increased tumor size, creates barriers to oxygen diffusion and poses challenges for delivering therapeutic RNA molecules to target tissues³⁶⁸. Chronic hypoxia, marked by the formation of a necrotic core, further complicates the situation³⁶⁹.

Under normoxia, the constant degradation of the alpha subunit of hypoxia-inducible factor 1 (HIF-1) complex is mediated by

dioxygen-dependent mechanisms³⁷⁰. However, under hypoxia, HIF-1 α stabilizes, leading to metabolic reprogramming in cancer cells and impacting various cellular processes³⁷¹. The alpha subunit of HIF heterodimer complexes with the beta subunit, which is then translocated to the nucleus, where it triggers hypoxia response elements (HREs) on specific gene promoters, initiating transcription of HIF-1 genes and subsequent cellular adaptation to hypoxia (Fig. 13)³⁷². HIF-1 facilitates vasodilation by stimulating inducible nitric oxide synthase (iNOS), increasing oxygen supply to the tumor. This positive HIF-1 α -iNOS feedback loop influences the TME, and the ensuing hypoxia induces changes in cancer cell metabolism, including increased reliance on glycolysis and reductive glutamine flux.

Further changes to the TME occur as a counteracting response to tissue ischemia. HIF facilitates vasodilation by stimulating inducible nitric oxide synthase (iNOS), increasing oxygen supply to the tumor³⁷³. The positive HIF-1 α -iNOS feedback loop affects the TME³⁷⁴, and the resultant hypoxia induces changes in cancer

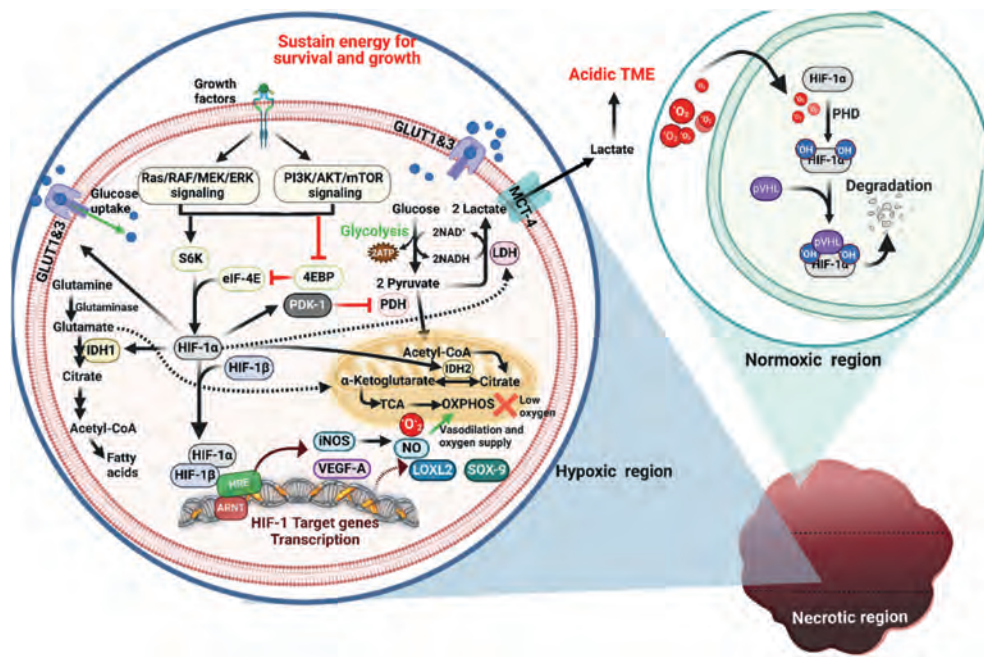


Figure 13 Effects of HIF-1 on the cellular adaptation mechanisms to hypoxia. HIF-1 is a major contributor to changes in TME as it plays a crucial role in adapting to tumor-associated hypoxia through the expression of VEGF-A, regulating angiogenesis, and improving oxygen supply via vasodilation triggered by iNOS. Furthermore, HIF-1 enhances glucose uptake and glycolysis, relying on the anaerobic pathway for pyruvate metabolism over the TCA pathway to generate sufficient energy under low oxygen conditions. The regulation of LOXL2 and SOX-9 expression is mediated by HIF-1, contributing to ECM stiffness and thereby hindering nanoparticle delivery. Figure was made by Biorender.com.

cell metabolism³⁷⁵, including increased reliance on glycolysis³⁷⁶ and reductive glutamine flux³⁷⁷.

Hypoxia also increases the stiffness of ECM within TME. The ECM comprises various proteins (*e.g.*, fibronectin and collagens), forming a dense matrix that creates a physical barrier to nanomedicine diffusion to the tumor core, such as nanoparticles or anticancer drugs (Fig. 14)³⁷⁸. Moreover, the negatively charged glycosaminoglycans in the ECM impede the deliverability of nanocomplexes-carrying nucleic acids, limiting the effective immunotherapy³⁷⁹. The gradient of escalating stiffness of ECM effectively steers T-cells and DCs away from direct interaction with the tumor cells and accumulates in the stroma, preventing them from reaching the tumor cells.

Within TME, the ECM influences the polarization of tumor-associated macrophages (TAMs) to the M2, leading to altered pro-inflammatory or anti-inflammatory responses³⁸⁰. M2 macrophages also contribute to the suppression of immune responses against tumors by impeding CD8⁺ T-cell proliferation, including the recruitment of Tregs via CCL22. They secrete inhibitory IL-10 and TGF- β , as well as iNOS-mediated free radical generation, etc³⁸¹.

Cancer-associated fibroblasts (CAFs) remodeled the ECM, resulting in high heterogeneity and lack of specific markers and creating challenges for identification. CAFs also cause the failure of immunotherapeutics by controlling matrix protein production and leukocyte infiltration^{382,383}. ECM primary components such as collagens I, II, XVII, and elastin can trigger immune inhibitory signaling in NK cells, converting it to tissue-resident NK and limiting its cytotoxicity^{384,385}. As well, ECM and the associated proteins exert other effects on TME through the regulatory rules in the escape mechanisms, including the expression of checkpoint molecules^{386,387}. The impact of ECM components on

immunotherapies has led to the emergence of strategies to address ECM-associated barriers, including enzymatic degradation of ECM components or co-delivery of drugs with ECM-modifying agents³⁸⁸.

As the tumor grows and exceeds the size of 2–3 mm³, angiogenesis occurs when oxygen and nutrients fail to meet the cells' needs to survive³⁷⁰. As a result, the formation of new blood vessels leads to the EPR³⁸⁹. However, the advantage of EPR in delivering nanoparticles to the tumor site is counteracted by increased IFP (Fig. 14)³⁹⁰. The increment of IFP in the tumor region impedes the penetration of therapeutic nanoparticles into the tumor mass³⁹¹. An organized blood supply within more advanced tumors allows for depositing therapeutic nanoparticles, although often limited to peripheral areas. The augmented IFP impedes the delivery of anticancer therapies, particularly to the core of the tumor, reducing treatment efficiency and leaving residual cells to initiate an environment, thus increasing the risk of tumor recurrence. A study has reported the positive effect of methyl-selenocysteine on angiogenesis and normalization of the vasculature, contributing to augmented tumor delivery³⁹². Methyl-selenocysteine acts by decreasing vascular leakiness while reducing microvessel density and increasing pericyte coverage. Therefore, vascular normalization mitigates the increased IFP, allowing drugs to be delivered and absorbed more efficiently within the tumor.

A thorough understanding of tumor vascular density, angiogenic factors, and nanomedicine delivery mechanisms is key to advancing more effective approaches to tumor targeting. Miscellaneous nanoplateforms harness the EPR effect to deliver drugs and genes. Besides EPR and IFP, there are other contributing factors that affect tumor uptake and accumulation, including vascular density and tumor doubling time^{393,394}. In TME, there is a

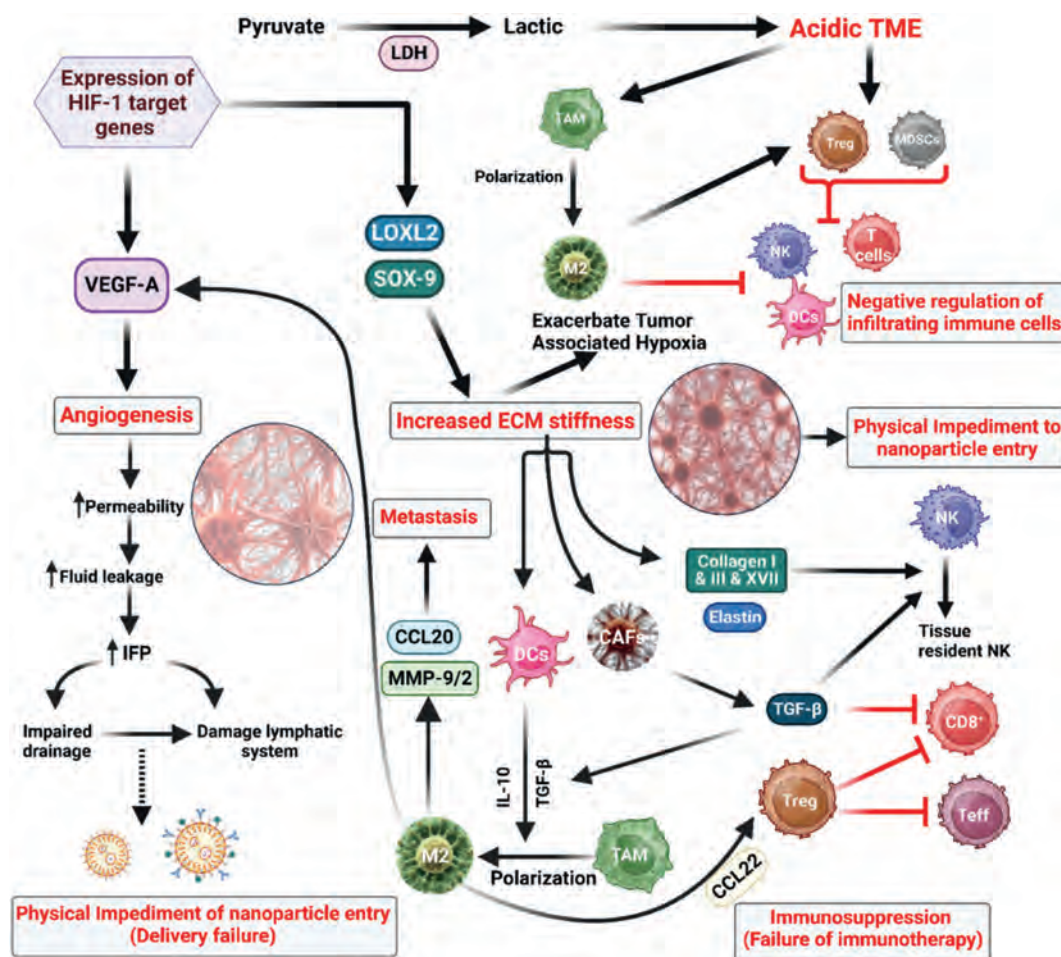


Figure 14 TME-associated barriers to immunotherapy delivery and action. Resistance to cancer immunotherapeutic formulations arises primarily from heightened IFP and the immunosuppressive nature of TME. Elevated IFP, a consequence of VEGF overexpression, leads to hyperpermeability, impairing lymphatic tissue drainage and posing obstacles for nanoparticle mobility. Conversely, components of the stiff ECM prompt cancer-associated fibroblasts (CAFs) and DCs to collaboratively hinder the function of cytotoxic immune cells, such as Natural Killer cells (NKs) and CD8⁺. Additionally, this environment promotes cancer development and metastasis through the expression of immunoinhibitory cytokines (IL-10 and TGF- β). Figure was made by Biorender.com.

noticeable difference in blood vessel density between tumor types and stages, which, in turn, influences tumor accumulation and treatment efficiency. For example, hepatic and renal carcinoma is characterized by its highly vascularized blood networks, which can facilitate tumor accumulation of therapeutics³⁹³. An analysis spanning ten years of nanoparticle delivery has shown that skin, pancreatic, brain, and liver cancer had a relatively higher accumulation of around 1.3%, 0.8%, 0.8%, and 0.7% of the total injected dose compared to other tumor types³⁹⁴. However, these dense networks may accelerate the progression to the metastatic state³⁹⁵. Meanwhile, ovarian carcinoma may manifest relatively less dense microvascular networks, compromising therapeutic agent accessibility^{393,394}.

Furthermore, researchers developing EPR-based nanoplatforms for tumor treatment should consider tumor doubling time (TDT) variance when designing gene therapy release rate for uptake by malignant tissue³⁹³. TDT varies based on the type, grade, and metastatic status of the tumor and can range from a few days to as long as a year in the case of pituitary adenomas, leading to heterogeneous outcomes³⁹⁶. Fast-releasing formulations may be necessary for treating quickly multiplying tumors³⁹³. With each

tumor doubling cycle, the amount of drug required to kill a given number of cells doubles, raising concerns about the safety of the RNA-based drug or vector. In other scenarios, sustained release and uptake formulations are preferable for tumors with long TDT.

4.2. Macromolecule-related challenges

4.2.1. Size and charge

The primary challenges in the delivery of RNA-based therapies stem from the large size and high charges of these macromolecules, which limits their bioavailability. This was particularly observed with mRNA vaccines due to their negative charges and large size (up to 5000 kDa), while the RNAi tools are usually around 4–15 kDa³². The increased size and charge present obstacles to systemic delivery, requiring the use of cationic, ionizable lipid, or polymeric nanoparticles³⁹⁷. Unlike ASO, which attaches to the mRNA target in an unaided fashion, other RNAi tools undergo several processing steps prior to loading into catalytic RISC/Ago2 complex and exerting silencing effects (Fig. 2). Therefore, siRNAs have specific biological prerequisites for maintaining activity. Several attempts have been unsuccessful in

enhancing their delivery through chemical modification or conjugation to other molecules. This requires careful replication of the A-form structure, minor groove contacts, and the charged phosphodiester backbone. Recent advancements in siRNA structural manipulation technologies aimed to enhance *in vivo* performance; the size and charged nature of this macromolecule dictate the presence of a carrier to work efficiently.

4.2.2. Stability

Achieving the desired clinical outcomes for RNA therapeutics relies on their stability *in vitro* and under physiological conditions until they are delivered to the target. RNA displays greater susceptibility to oxidation *in vitro* compared to DNA³⁹⁸. Meanwhile, *in vivo*, RNA undergoes enzymatic degradation primarily mediated by RNases, endonucleases, and 5' exonucleases³⁶⁰. Therefore, it is of utmost importance to ensure RNase-free conditions throughout the entire RNA preparation process, storage, handling, and administration. Similarly, the relatively short phosphodiester backbones of ASOs make them vulnerable to nuclease degradation because of their highly charged and hydrophilic characteristics. Nevertheless, second-generation ASOs showed superior resistance to nucleases, exhibiting improved stability and a longer half-life³⁹⁹.

Several strategies have emerged to protect RNA molecules; one general approach entails introducing them into lipid or polymeric nanoparticles to mitigate their sensitivity to RNases. Despite that, new hurdles have emerged since the RNA molecules are subjected to hydrolysis during preparation and storage within the hydrophilic core of the lipid nanovesicles⁴⁰⁰. Additionally, lyophilization was undertaken to minimize the effects of hydrolysis in the liquid form. However, RNA stability can still be potentially damaged by freezing and thawing cycles, which necessitates the addition of cryoprotectants⁴⁰¹. Further approaches that hinge on modifying nucleosides or optimizing the sequence are often adopted to increase the inherent stability of RNA molecules⁴⁰².

4.2.3. Endosomal escape

There should be careful engineering of RNA vectors to bypass the internal biological barriers, such as the epithelium and endothelium, that hinder RNA transfer from the blood to organs⁴⁰³. Immediately after RNA-nanoparticles are taken up by cells, endosomes are generated. Once the RNA payload reaches the target cells, it must egress the endosomal confinement to access the cytoplasm, where it operates on the expression machinery³⁶¹. The endosomal release poses a significant risk of almost total loss of the delivered RNA molecules such as siRNAs. Approximately 99% of free siRNA and ASOs remain confined within endosomal membranes, with the evasively released portion eliciting an RNAi response in the cytoplasm^{36,101,404,405}.

4.2.4. Non-specific immunogenicity

Applying mRNA vaccines in cancer immunotherapy emphasizes the immune—stimulatory activity of mRNA that can act as a self-adjuvant by inducing type I IFNs⁴⁰⁶. This immunogenicity is mainly mediated through the activation of TLRs. However, immunogenicity has a downside since it reduces the expression of the target protein by increasing mRNA clearance, which is against the desired clinical outcome³⁴. Earlier, short siRNA duplexes were assumed to be immunoevasive. Later, it was proven that introducing foreign RNA into the body can mediate immune responses, leading to the delivered RNA clearance or type III IFN-mediated

inflammation³⁶³. Administering siRNAs in non-conjugated form or without nanoparticle complexes brings in rapid kidney filtration within a few minutes with negligible circulation⁴⁰⁷. In this case, camouflaging techniques should be adopted to tackle the immunogenicity against RNA molecules^{408,409}.

In recent years, there has been evidence of a link between the structural properties of siRNAs (length, backbone, and sequence) and irAEs. A higher immunologic response can be observed for GU-rich sequences, ribose skeleton, and sequences ≥ 19 -nucleotide in length. Moreover, the therapeutic potential of siRNA is marred by the non-specific immune stimulation, rendering it challenging to discern the therapeutic outcomes. Recognizing and tackling these unintended immune responses is necessary for optimizing the safety and efficacy of siRNAs in cancer treatment.

4.2.5. Off-target effects

In addition to the common challenges for RNA therapies, precise and carefully designed sequences are crucial for siRNAs introduced to silence targeted genes, to avoid toxicity and IFN-mediated responses³⁶³. A defective siRNA design causes them to behave similarly to miRNAs, thereby interfering with or degrading mRNAs owing to the complementarity between the 3'UTR of unintended mRNAs and the 5' end guide strand of siRNA⁴¹⁰. These miRNA-like off-target effects potentially impair the on-target responses and, thereby, therapeutic efficiency, potentially giving rise to observed phenotypic changes. Non-specific adherence becomes a question when siRNA recognizes eleven nucleotide sequences in common between target and non-target genes⁴¹¹. Moreover, therapeutic siRNAs would likely compete with endogenous miRNAs for RISC, which may exacerbate the potential off-target effects⁴¹². Since these effects emerge in a dose-dependent manner, reducing siRNA doses. Also, optimizing siRNA design or siRNA pooling at lower concentrations might evade the adverse events of complementarity⁴¹³.

Similarly, virally engineered CAR-T-cell therapies pose off-target effects due to cross-reactivity to unintended antigens. As a result, serious neurotoxicity, cardiotoxicity, and anaphylactic reactions may occur, as well as complications related to immunosuppression³⁶⁴. While transfecting T-cells with mRNA may alleviate these effects, the potential risks should be closely monitored.

4.3. Vector-related challenges

4.3.1. Size, surface charge and tunability

An optimized nanocarrier design must consider characteristics such as size, shape, surface charges, decoration, and their influence on tumor uptake and accumulation. It is tricky to tune the optimal particle size for sustained circulation and effective uptake since very small particles may clear quickly, while larger particles may not deliver the payload effectively⁴¹⁴. Furthermore, particle morphology distribution in target organs is another fundamental factor associated with cellular uptake and biodistribution, particularly in the TME. Particles can be present in several forms, such as nanospheres, nanodiscoids, nanotubes, and nanorods^{415,416}. When it comes to elongated particles such as carbon nanotubes and gold nanorods, it is important to consider the aspect ratio, which should be reduced to enhance the efficiency of those particles¹⁹¹.

Multiple factors govern the nanoparticle transfer across the microvascular networks to malignant tissue. In addition to the EPR effect, particle size, morphology, and surface properties play

a role in vector accumulation and delivery efficiency, plus the dynamic nature of the tumor vasculature, as discussed earlier. The leaky nature of tumor vasculature offers passive uptake and retention opportunities for spherical nanoparticles with diameters near 100 nm⁴¹⁷. As for elongated particles, such as discoidal nanoparticles or nanorods, they may accumulate through adhesion to tumor vessels or binding to active ligands. Nevertheless, simulation data show the superiority of spherical and cuboidal nanoparticles over rod and discoidal forms⁴¹⁸. Meanwhile, filamentous particles tend to extravasate and accumulate within tumors despite their large size, thanks to their tiny radius⁴¹⁷.

Surface charges could exert an effect on particles' uptake, biodistribution, and interaction with immune cells. Negative-charged nanoparticles are difficult to absorb by cells and achieve adequate biodistribution as they are electrostatically repelled by cell membranes, whereas their cationic counterparts can induce cytotoxicity by compromising cell membrane integrity⁴¹⁹. Furthermore, a delicate balance must be achieved while modifying nanocarrier surfaces to enhance biocompatibility, mitigating interaction with immune cells, and maximizing cellular uptake while preserving the carriers' desired. Also, careful consideration of potential aggregation, change in nanoparticle fate, and pharmacokinetics.

4.3.2. Safety

Safety issues involve the assessment of potential unintended off-target effects or severe immune reactions to guarantee that both the payload and the vector do not pose a risk to patients. Non-viral vectors are safer options with larger capacity. Yet, concerns about toxicity arise due to the size, cationic charges, and complexity of preparing these nanocomplexes and the need for recurring exposure⁴²⁰. Treating chronic conditions will require prolonged administration of mRNA lipoplexes or polyplexes, where serious concerns remain regarding toxicities associated with cationic lipids or polymers²¹¹. Moreover, efficient delivery of negatively charged nucleic acid to cells requires cationic nanomaterial that can embrace this macromolecule and transfer it to the target. Positively charged nanomaterials may be toxic to the body since they exert nonspecific interactions with non-relevant cellular targets⁴²¹. Therefore, tuning RNA release in a controlled manner and sustaining the gene expression or knockdown levels is critical when a temporary effect is desired.

4.3.3. Biocompatibility and biodegradability

Creating effective nanocarriers for RNA entrapment and release hinges on finding the harmony between achieving effective transfection and biocompatibility with minimum unwanted cellular responses. Nanoparticle cytotoxicity is pertinent to their shape and charges. Cationic nanoparticles, including LNPs and PEIs-coated nanoparticles, impact cellular pathways as they exert proapoptotic and proinflammatory activities while arousing membrane protein destabilization¹⁷³. Even biocompatible polymers like PLGA or reducible polymers may not guarantee that RNA molecules will be released and interact successfully while achieving effective transfection efficiency⁴²². A further challenge is the cell-damaging effects attributable to nanoparticle shape, which could extend to the genotoxic effects as reported with SWCNTs by Kisin et al^{423,424}.

A downside of nanomaterials like traditional MSNs is their slow degradation rate, which raises concerns about accumulation and toxicity in the long term¹⁸⁰. If left unchecked, this persistence can accumulate silica nanoparticles in tissues and organs,

potentially resulting in health complications. Hence, to reduce potential risks associated with prolonged retention of common MSNs in the body, alternative silica-based nanostructures, such as DMSNs, have been developed for gene delivery. On the other hand, DMSNs offer enhanced biodegradability offered by dendritic structures, which reduces their bioaccumulation in the body⁴²⁵. In addition, it is pertinent to acknowledge the non-biodegradability of fullerenes and CNTs, even with their promise in gene delivery⁴²⁶. Although several attempts have been made to hydrophilize fullerenes, they remain prone to accumulation in the cytoplasm due to their hydrophobic structure. Consequently, fullerenes may build up in vital organs, whereas CNTs' intrinsic stability raises doubts concerning long-term safety. Use of these carbon structures necessitates modifications to develop biodegradable and biocompatible options with expedited clearance from tissues and organs where effective degradation pathways are absent.

4.4. Routes of administration

The administration route through which RNA formulations, whether systemically or locally, plays a critical role in determining their performance and efficacy. The administration of RNA therapeutics is facing several challenges, as summarized as follows: Intranodal or intratumoral injections are often used and hold promise for RNA cancer immunotherapeutics, but precision is crucial to avoid improper tissue infusion. Intratumoral administration faces challenges with limited reach to distant tumors, tumor variability, and immunosuppressive microenvironments. Intravenous delivery, while efficient, encounters rapid clearance, poor biodistribution, and the need for recurrent exposures. Intramuscular injections, advantageous for vaccines, may induce inflammation, risking patient compliance.

Intradermally and subcutaneously delivered RNA therapeutics leverage Langerhans cells present locally for effective antigen presentation. It is, however, complex to reach even immune responses across diverse racial and ethnic groups due to differences in skin thickness, vascularity, and tissue characteristics⁴²⁷. Also, it remains difficult to ensure consistency and targeting efficiency of RNA delivery by locally implantable hydrogels⁴²⁸. For successful intradermal administration, the dose should be precisely injected between the dermis and epidermis by skilled personnel for accuracy. However, the patient may experience discomfort due to local swelling and redness, affecting medication adherence.

The nasal delivery has been praised for the ease of self-administration, non-invasive nature, high vascularity, and escaping first-pass metabolism, along with the ability to circumvent biological barriers by directly accessing the brain through the olfactory pathway⁴²⁹. Nevertheless, optimizing brain delivery while keeping the lungs unexposed is not a simple task. Aside from that, this route is limited by limited dose volumes, RNA degradation by nasal enzymes, and removal *via* ciliary movement. This contributes to inadequate absorption due to brief dose retention, and in case repeated instillations would still produce uneven doses.

On the other hand, inhalable mRNA formulations present numerous technical difficulties, including physical and biological barriers, stability, accurate dosage, and clinical assessment. The lung mucosa is covered with negatively charged mucin, potentially exacerbating particle instability, aggregate formation, and confinement in this layer. In this regard, introducing formulations with negative outer-layer or PEG coatings confers their ability to repel mucin components, showing enhanced mucosal stability⁴³⁰.

It is also hard to develop a predictive model to characterize inhalation therapies through *in vitro* and *in vivo* correlations or computational modelling. A connection could be made to study the crucial airway-on-a-chip models, emphasize aerosol deposition in 3D cultures, and underline the need to enhance physiologically based pharmacokinetic models for formulation development⁴³¹. To evade the toxicity incurred by the inhalation route, direct instillation to bronchial proves an alternative rapid and efficient delivery approach⁴³².

Oral administration is the most patient-friendly, with great adherence and tolerability, yet this route is burdened with formidable limitations. When administered orally, nanoparticles, especially LNPs, are exposed to an unprecedented environment, including the acidic pH, digestive enzymes, and viscous mucin barrier layer. Gastric acidity, which reaches around pH 1.5, can negatively affect endosome-responsive systems such as ionizable lipids, CARTs, and other polymer substances. Additionally, pancreatic lipases quickly degrade lipids-based delivery systems, and a set of polymeric materials are also esterase-sensitive, leading to the premature release and loss of the RNA payload⁴³³.

The local administration route is renowned for sustained delivery capabilities, which can resolve the rapid clearance rate and bypass cellular barriers by creating alternative pathways and harnessing nanoparticle permeability to their intended destination⁴³⁴. Local administration requires non-immunogenic hydrogels for perpetual RNA release. Microneedles offer enhanced patient compliance for transdermal delivery but face safety concerns and limited clinical viability.

Hydrodynamic gene delivery uses high-pressure injection of DNA solution to permeabilize capillary endothelium, allowing nucleic acids to enter parenchymal cells⁴³⁵. Building on this, Dul et al.⁴³⁶ explored MicronJet600®, a microneedle device, for intradermal pDNA delivery in the human breast skin. MicronJet creates superficial microdisruptions, enabling gene expression in the epidermis and dermis with minimal damage, promising better results than conventional injections. The hydrodynamic delivery approach, while promising, raises safety concerns and lacks sufficient evidence in clinical cancer therapy.

Despite the promising results in preclinical studies, overcoming challenges in RNA therapeutic administration remains crucial for widespread clinical viability. Safety concerns around the large injection volumes that might lead to probable transient heart failure, with a risk of tissue damage, imprecise control of the hydrodynamic pressure, and interpatient variability. Addressing safety concerns, improving precision, and advancing delivery technologies are essential for realizing the full potential of RNA therapeutics in cancer treatment.

4.5. Combination therapy

mRNA cancer vaccines and other RNA therapies can be conjugated with conventional tumor therapies, including chemotherapy, radiotherapy, and immunotherapy. Monotherapy with chemotherapies, such as cytokine monotherapy, targets only a few of the mechanisms controlling tumor growth and metastasis, resulting in modest antitumor responses and high toxicity⁴³⁷. Cancer progression has proven to be challenging for some metastatic solid tumors, such as breast cancer, which calls for complementary interventions to address these complexities⁴³⁸. The integration of various adjuvants and therapeutic modalities forms the cornerstone of combination therapy in cancer immunotherapy, especially involving mRNA-based approaches. The strategy of co-delivery

leverages the synergistic effects of different pathways and mechanisms to enhance T-cell activation and overcome immune tolerance⁴³⁹. As well, combination mRNA vaccines, as multiple antigens generated from single or multiple strands, offer opportunities for enhancing therapeutic outcomes.

A rational combination approach would be to use adjuvant mRNA cancer vaccines with ICIs to jointly boost the immune response to fight malignancies. Inhibitory signals blockade by ICIs (e.g., CTLA-4 and PD-1/PD-L1 antagonists) promotes T-cell activation in response to mRNA vaccines⁴⁴⁰. TriMix, an adjuvant immunotherapy composed of three naked mRNAs, activates CD8⁺ and CD4⁺ T-cells by triggering the production of CD40 and CD40L, along with improving DC antigen presentation by generating constitutively active TLR4⁴⁴¹. Clinical trials have demonstrated the effectiveness of TriMix mRNA formulations in treating stage III/IV melanoma, either alone or in combination with CPIs. By overcoming immune ICIs, this combination enhances T-cell responses, thereby boosting immune activation pathways and sensitizing tumors to T-cell-mediated killing. This would ultimately aid in tumor control and prolong survival.

In the clinical context of local radiotherapy, the protamine-based mRNA (CV9202) has demonstrated robust translation efficiency, resulting in elevated expression of antigens, triggering immunity against a spectrum of non-small cell lung cancers⁴⁴². Radiation can induce immunogenic cell death, which promotes immune recognition and infiltration into the TME. Inversely, RNAi could potentiate the immune response of traditional cancer therapeutics hampered by certain ICIs, as was the case of CDK inhibitors and PDL-1 siRNA¹¹⁸. Furthermore, siRNA has been shown to increase the efficacy of photodynamic therapy in cancer immunotherapy. For instance, knocking down PD-L1 counteracts the immunosuppressive effects of the TME on T-cell antitumor immune responses^{114,443}.

Moreover, a synergistic therapeutic effect was observed when mRNA vaccines were co-administered with routine chemotherapy such as cisplatin and durvalumab. Infiltration of immunosuppressive cells such as Tregs and MDSCs into genital tract malignancies interferes with the ability of the E7-TriMix mRNA vaccine to stimulate immunity against the tumor. Bialkowski coupled mRNA vaccination with cisplatin, which turned out to be highly effective in fully eradicating neoplasms in 88% of cases⁴⁴⁴. Moreover, mRNA-2752, encoding OX40L, was administered in conjugation with durvalumab for improving T-cell response along with IL-23 and IL-36γ interleukins and has shown safety and tolerability, inducing sustained pro-inflammatory effects⁴⁴⁵.

Even though these combinations face several challenges that are worthy of careful consideration. To gain regulatory approval, these lot-consistent vaccines in a synergistic cocktail need to be clinically evaluated to ensure immunogenicity and tolerability through unbiased clinical trials with defined endpoints³⁶⁵. Besides, a potential increased immune response might arise from increased overall mRNA dose, or their co-delivery raises doubts regarding safety and tolerability. It is also difficult to coordinate the optimum administration sequence, timing, and dosing of mRNA cancer vaccines with radiotherapy since, at specific doses, radiotherapy may exert a potential immune suppressant effect⁴⁴⁶. Moreover, combination therapy is also plagued by inter-individual variability, as seen in the combination of GTI-2040, oxaliplatin, and capecitabine, which are used to treat metastatic solid malignancies⁴⁴⁷. Therefore, promising combination therapy has to be universally applicable to different populations. As chemotherapy can have immunosuppressive effects, it is critical to understand

how this might contradict mRNA vaccines' immune activation, as it might terminate with impairing the immune response. This was particularly evident when BNT162b2 was co-administered with chemotherapeutics for cancer patients. Also, the immunosuppressive action did not respond to adjustments in the timing of vaccine administration with on-cycle and off-cycle chemotherapeutics⁴⁴⁸. Hence, dose optimization is necessary to balance the subsequent immunostimulatory and immunosuppressive effects to reach high therapeutic efficacy while minimizing the undesired events.

4.6. Regulatory hurdles

Most RNA formulations are still in their infancy phase, and the uncertainty surrounding their regulatory approval for clinical applications is a stumbling block to their development. The application of gene therapy, initially pursued for genetic diseases, swiftly transitioned into a prominent approach for cancer treatment, with about two-thirds of gene therapy trials focusing on diverse cancer types^{449,450}. The advancement of cancer gene therapy (CGT) products with a scaling-up process requires addressing regulatory hurdles to ensure the effectiveness, safety, and tolerability of this innovative therapeutic approach.

During the development of CGT products, compliance with Chemistry, Manufacturing, and Controls (CMC) regulations is necessary to maintain safety, quality, and efficacy⁴⁵¹. Throughout the entire development process of CGT products, a comprehensive understanding and disclosure of all CGT product components, including vectors, cells, plasmids, and associated reagents, is necessary, along with rigorous quality control procedures. Stringent regulations for CGT call for a comprehensive description of vectors, cells, and reagents. Therefore, to ensure a safe and reliable final product, the quality control of research-grade reagents, and meeting the demand for RNase-free processing, adherence to current good manufacturing practice (cGMP) standards is imperative, necessitating the precise documentation and execution of the manufacturing process⁴⁵². To accomplish this, well-defined criteria for CGT product specifications and materials should be established, with certificates of analysis serving as checkpoints for acceptance. Identity, purity, and safety testing should be conducted, and accurate labeling guarantees a high-quality and impurity-free product. Further microbiological testing ensures sterility and excludes potential biological contamination.

Nevertheless, the regulatory approval process is involved and requires consent from multiple regulatory bodies, such as FDA and the National Institutes of Health's Office of Biotechnology Activities (NIH/OBA)⁴⁵³. To prevent delays associated with clinical investigation, it is crucial to communicate regularly with the FDA through pre-IND meetings and other formal engagements to align with regulatory expectations and address unforeseen issues⁴⁵². However, the lack of harmony between regulatory bodies further hampers establishing universally accepted standards. Also, mRNA vaccines and other RNAi therapeutics rely on new technologies, and the production processes are distinct from those of conventional ones; therefore, setting specific quality standards and guidelines is complex.

4.6.1. Challenges in pre-clinical development

Contrary to conventional drugs, there is no universal protocol for preclinical evaluation of CGT products. The variety of CGT products and vectors necessitates specific regulations for each class. Also, the nature of the cell source for cellular vaccines

necessitates a record of mobilization protocols, collection techniques, and eligibility of donors. To deal with this challenge, the FDA embraced flexibility in line with the CGT product's biology and intended use, along with the evaluation of safety and toxicity for raw materials and the final formulation³⁶⁶. Choosing an appropriate animal species and model for a pre-clinical study is problematic due to interspecies variability, which may make it difficult to generalize results to clinical use in humans. Additionally, RNA-nanoparticle biodistribution, ability to bypass biological barriers, and affinity to specific tissue vary according to size, outer surface characteristics, and interaction with plasma proteins⁴⁵⁴. This might inform their tissue-specific accumulation into affinity, which eventually affects the *in vivo* fate and side effects. The multidisciplinary nature of pre-clinical CGT studies emphasizes the need for coordinated efforts of formulation experts, biologists, toxicologists, and regulatory experts to address complicated tasks such as assessing off-target effects and irAEs.

4.6.2. Challenges in clinical development

Moving forward with the clinical evaluation of CGT, preclinical and clinical data should be bridged together to avoid unexpected toxicities. In the course of clinical development, CGT products will inevitably encounter scalability limitations. Moreover, any further changes to the CGT production process may require further preclinical studies. It is essential to know if critical quality attributes (CQAs) were altered when modifying processes or facilities during production. Performing *in vitro* and *in vivo* might be necessary to assure efficacy and safety.

In early-phase trials of CGT, efficacy and tolerability are more prominently considered. Careful monitoring of adverse events is required to mitigate potential toxicities emerging from CGT, vector, sustained biological effects, or irAEs. Aside from that, achieving effective dosing and using a highly adherable administration route that does not cause patient noncompliance is important for the developmental clinical phase. A dose-escalating approach might be adopted to determine the effective dose since there is a lack of knowledge of the conversion factor for CGT products. It is also imperative to conduct well-planned phase 2 trials to estimate CGT's activity and demonstrate therapeutic outcomes without rushing through this stage so as to obtain sufficient findings as proof for moving forward to the subsequent trial phase. It is also difficult to select the study population and decide on primary and secondary immunological endpoints for phase III trials. Alignment of these endpoints with improvements in patient survival or function is crucial at the final developmental stage³⁶⁶.

5. Future directions and conclusions

Innovations in RNA therapeutics are gaining traction and will be a great boon for future cancer treatment. Yet, the journey from preclinical to clinical translation in cancer treatment is laden with hurdles that extend far beyond the laboratory bench. Regarding RNA-based therapeutics, scientists are currently actively addressing the lacunae in understanding how to optimize RNA stability and enhance cellular uptake and endosomal egress while minimizing immunogenicity, aiming to reshape the landscape of therapeutic efficacy in cancer therapy. Thus, speeding up the transition of mRNA-based products to market requires extensive optimization effort, which goes beyond just the acquisition of the right technology. Also, achieving adequate tumor accumulation remains an obstacle to successful cancer treatment. Therefore, a key aspect of future endeavors lies in maximizing the potential of

nanoplatfroms to interact selectively with malignant tissues with precision, thereby refining cellular uptake mechanisms.

When developing mRNAs, careful design of promoter sequences, UTRs, and poly(A) tails, as well as optimizing coding sequences to enhance stability and translation efficiency, lead to considerable early-stage expenses. Therefore, addressing inherent cost constraints throughout the production phases of mRNA therapeutics is key to success. Optimizing mRNA sequences by LinearDesign, an algorithm to unlock highly stable coding and non-coding regions, provides a faster and more cost-effective alternative to traditional codon optimization, pointing to a promising future⁴⁰².

Scalability and cost-effectiveness are key issues that should be addressed to fully realize the potential of RNA therapeutics. Modification of the encoded genes has been introduced to reduce the costs of mRNA. Conventional and self-amplifying mRNA (saRNA) structures have been studied for vaccine development. Although conventional mRNA has a simple structure and is relatively safe, it may necessitate higher doses to achieve optimal results because of its short-lived nature and temporary effects. Meanwhile, by introducing the gene-encoding viral replicase, saRNA can produce high antigen expression levels at significantly lower doses with improved safety (Fig. 15). However, saRNAs still potentially pose safety issues that emerged with the generation of virus-like particles owing to the viral replicase⁴⁵⁵. Recent advances in saRNA technology have led to the creation of *trans*-amplifying mRNA (taRNA), which applies two RNAs: one encoding the replicating gene and the other encoding a non-replicating replicase (Fig. 15). By designing taRNAs with short RNA sequences, yield and quality can be improved.

Addressing both pre- and post-transcriptional challenges posed by the TME to intracellular nucleic acid delivery calls for strategic delivery approaches *via* well-designed nanoplatfroms. While PEI has been praised as a rising star polymer for gene delivery, cytotoxicity remains a concern. Recently, cyclic polymers are expected

to play an increasingly important role in gene delivery in the future since their distinctive topology allows them to be tunable and less toxic¹⁵⁸. For example, the sun-shaped PDMAEMA and cyclic PEI demonstrate a shift towards high-performance DNA vectors that open up new avenues of gene delivery⁴⁵⁶. Beyond that, the need for long-term formulation stability, especially for mRNA, is still unresolved, which may necessitate the reliance on DMSNs, and protamine-based delivery systems can provide a promising shelf-life for RNA therapeutics^{457,458}.

Moreover, there is a necessity for a forward-looking strategy that could enable improved treatment outcomes for extended periods and address the concerns encountered with extrahepatic organ-targeted lipid-based delivery. Introducing SORT molecules to develop LNPs with unique targetability represents a leap in delivery precision and gene transfection at the intended site. Nanomotors had been fabricated to deliver siRNA⁴⁵⁹; therefore, the future may witness the advancement with MSN-based nanobots for RNA delivery that can target hard-to-reach organs like bladder cancer⁴⁶⁰. Finally, the RNA delivery to the target is not the sole challenge; the route of administration also requires advancements. The search for a less invasive and more compliant administration route may be met by the dependence on micro-needle patches or inhalable formulations on account of the oral route due to the associated formidable obstacles.

In conclusion, it is clear that RNA-based therapy has the potential to be very effective at treating a wide range of malignancies. In the coming years, the adaptability of mRNA technology and the lessons learned from the widespread use of mRNA vaccines during the recent pandemic are expected to propel further advances in personalized cancer vaccines. As well, RNAi-mediated immunostimulation has been found effective in fighting cancer immune evasion, and several genetic tools can be used to achieve this goal. Thanks to advances in nanobiotechnology, it has become possible to meet the essential needs for gene delivery and targetability. In addition to looking at how

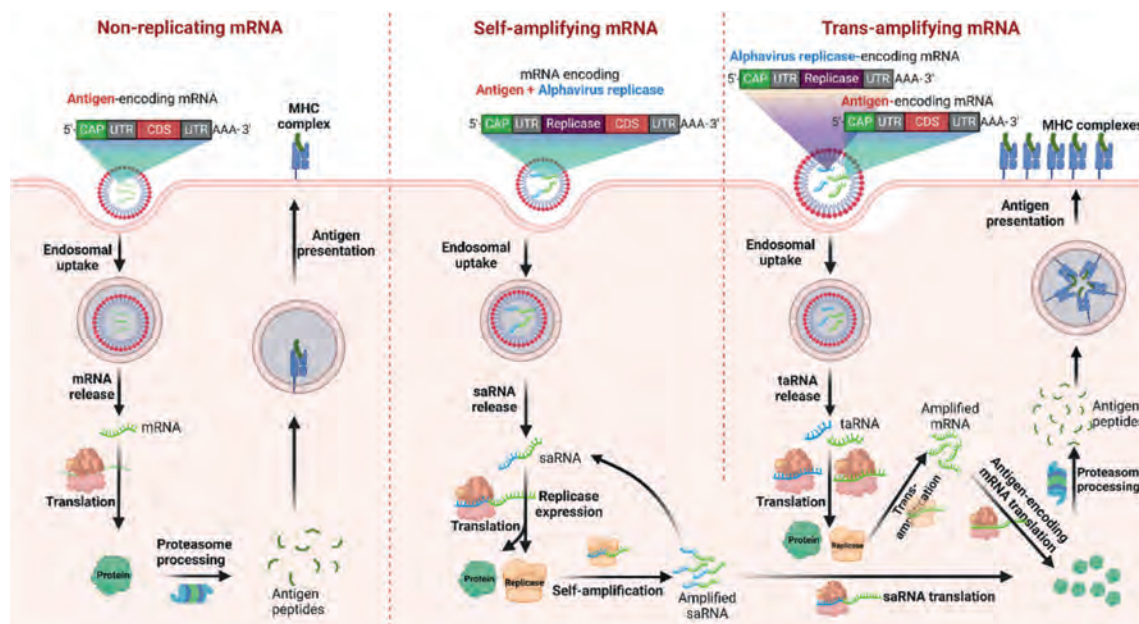


Figure 15 Protein expression and antigen presentation using mRNA, saRNA, and taRNA approaches. AAA: Poly A tail, CAP: Capping sequence, CDS: Coding sequence, MHC: Major histocompatibility complex, UTR: untranslated region. Figure was made by Biorender.com.

nanoplatforms are effective and safe, it is also important to consider how they break down and to consider how they are designed to be biodegradable and multifunctional. RNA immunotherapeutics have yet to realize their potential as they are confronted with unprecedented obstacles relating to either the molecules themselves or the nature of the malignant tumor. Furthermore, there are several regulatory issues to deal with until the product is marketed. Taking steps to overcome these obstacles could result in the approval of future RNA-based cancer treatments.

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Conflicts of interest

The authors declare no conflict of interest.

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