Molecular Therapy
Nucleic Acids
Review

Advancements and challenges in mRNA and ribonucleoprotein-based therapies: From delivery systems to clinical applications

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The use of mRNA and ribonucleoproteins (RNPs) as therapeutic agents is a promising strategy for treating diseases such as cancer and infectious diseases. This review provides recent advancements and challenges in mRNA- and RNP-based therapies, focusing on delivery systems such as lipid nanoparticles (LNPs), which ensure efficient delivery to target cells. Strategies such as microfluidic devices are employed to prepare LNPs loaded with mRNA and RNPs, demonstrating effective genome editing and protein expression *in vitro* and *in vivo*. These applications extend to cancer treatment and infectious disease management, with promising results in genome editing for cancer therapy using LNPs encapsulating Cas9 mRNA and single-guide RNA. In addition, tissue-specific targeting strategies offer potential for improved therapeutic outcomes and reduced off-target effects. Despite progress, challenges such as optimizing delivery efficiency and targeting remain. Future research should enhance delivery efficiency, explore tissue-specific targeting, investigate combination therapies, and advance clinical translation. In conclusion, mRNA- and RNP-based therapies offer a promising avenue for treating various diseases and have the potential to revolutionize medicine, providing new hope for patients worldwide.

INTRODUCTION

Background on mRNA and ribonucleoprotein therapies

In vitro transcription (IVT) of mRNA involves utilizing linearized plasmid DNA or PCR templates, which necessitate a promoter and the corresponding mRNA construct sequence.[1–3] Polymerases such as T7, T3, or SP6 are added to facilitate IVT, but additional capping is necessary to prevent rapid degradation of uncapped mRNA by RNase, which contains a 5′-ppp group causing heightened immune stimulation.[4,5] Capping can be achieved through two methods: co-transcriptional and post-transcriptional capping.[6] Co-transcriptional capping involves incorporating cap dinucleotide mixtures at the 5′ end of RNA during transcription, allowing coordinated transcription with mRNA capping.[7] However, this method encounters challenges such as the competitive incorporation of GTP nucleosides, which can impact capping efficiency. Initially, GTP binds to RNA chains through a 5′-5′ triphosphate bond and undergoes 7-methylation at the 5′ terminal guanosine during post-transcriptional capping.[8] Capping enzymes derived from vaccinia virus are efficient in capping mRNA, producing cap 0, while cap-specific 2′-O methyltransferase can further modify cap 0 to cap 1 or cap 2, reducing mRNA immunogenicity.[9,10] However, capping can lead to the formation of m7GpppGpG in a reversed linkage, hindering mRNA translation. Anti-reverse cap analogs are synthesized to enhance translation efficiency by modifying the m7G part of caps.[11,12] Poly(A) tails in IVT mRNAs can be encoded in the DNA template or added enzymatically, with the former method providing more precise control.[8,13,14] Linearization of plasmid templates using type II restriction enzymes can lead to overhangs at the 3′ end of poly(A) tails, affecting translational efficacy, necessitating the use of type IIS restriction enzymes to avoid this issue.[1,15]

In-vitro-transcribed mRNAs necessitate purification to eliminate immunostimulatory contaminants, free ribonucleotides, as well as short mRNA and DNA templates.[16–19] DNase is typically used to degrade excess DNA templates, followed by commercial purification kits and precipitation methods using ethanol or isopropanol to obtain high-purity mRNA. Chromatographic methods such as molecular exclusion chromatography, ion-exchange chromatography, or affinity chromatography can further purify mRNA, while reversed-phase HPLC is effective in removing dsRNA contaminants but may not be scalable for large-scale production.[18,19] Alternatively, RNase III has been proposed for the removal of dsRNA contaminants, and cellulose chromatography has shown promise in purifying IVT mRNAs efficiently and on a larger scale. Gel electrophoresis can also be employed to remove short RNAs and separate long RNAs. Ultimately, the choice of purification method depends on the specific purity requirements and scale of production, with stringent quality control being essential for maximizing the benefits of mRNA therapeutics.[18]

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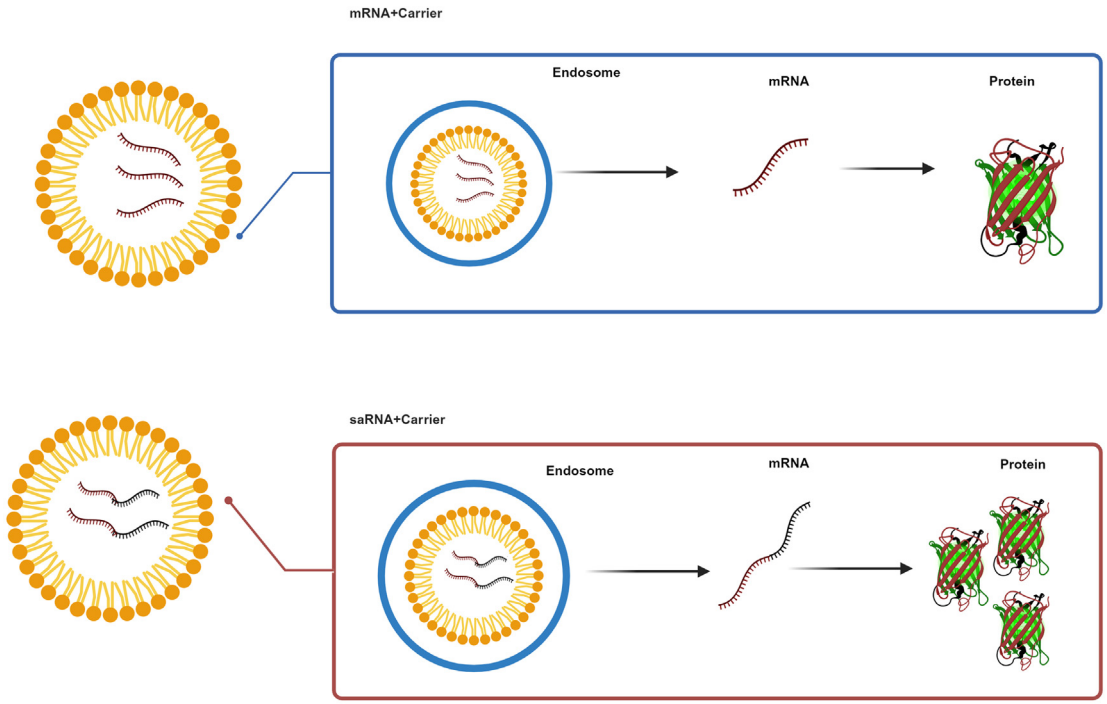

Figure 1. The differences between conventional mRNA and self-amplifying mRNA
saRNA, derived from alphaviruses, self amplifies for efficient protein expression, promising high antibody titers against pathogens.[32]

Different formats of mRNA, such as self-amplifying mRNA (saRNA),[20] unmodified mRNA with codon usage optimization,[21] nucleoside-modified mRNA,[22] and trans-amplifying mRNA (taRNA),[23] offer distinct advantages and challenges in therapeutic applications. saRNA, derived from alphaviruses, contains a replicase sequence enabling self-amplification and efficient protein expression[24] (Figure 1). Despite its longer sequence compared with conventional mRNA, saRNA shows promising results in inducing high antibody titers against pathogens.[25–28] Unmodified mRNA with codon optimization promotes immunogenicity by augmenting antigen presentation, acting as an adjuvant in mRNA vaccines.[29] Nucleoside-modified mRNA, incorporating modified nucleosides such as 2'-O methyl nucleoside, suppresses immune response by inhibiting TLR-mediated dendritic cell activation, potentially improving safety and efficacy.[30] taRNA, an advanced version of saRNA, separates the replicase from multiple target mRNAs, allowing simultaneous amplification of various proteins.[31] This flexibility simplifies transfection protocols and offers potential in infectious disease vaccines. Each mRNA format has its advantages and limitations, highlighting the importance of selecting the appropriate format based on specific therapeutic needs and challenges.

Importance and potential impact on modern medicine

Efficient intracellular delivery of mRNA remains a significant challenge due to its large molecular weight, high negative charge density, and inherent instability. Various strategies, including microinjections, gene gun-based administration, and encapsulation in nanoparticles, have been explored to improve RNA delivery.[33–37] Formulating mRNA with delivery systems protects it against degradation and facilitates cellular uptake. Mechanisms for mRNA loading include electrostatic interactions, hydrogen bonds, or coordination interactions. Vectors such as lipid nanoparticles (LNPs), polymeric nanoparticles, and cationic nanoemulsions have been engineered to augment mRNA delivery.[38–40] Optimization of these delivery systems holds promise for enhancing mRNA transfection efficiency, thereby advancing mRNA therapeutics. Examples include LNPs modified with cationic peptides,[41] graphene oxide, and polyethylenimine (PEI) hydrogels carrying mRNA vaccines,[42] and mesoporous silica nanoparticles encapsulating mRNA and RNA-activated protein kinase inhibitors.[43]

OBJECTIVES OF THE REVIEW

This review provides a comprehensive overview of the advancements and challenges in mRNA and ribonucleoprotein (RNP)-based therapies, focusing on delivery systems, mechanisms of action, therapeutic applications, and future directions.

Overview of mRNA- and RNP-based therapies

mRNA therapies involve the delivery of mRNA molecules into cells, where they are translated into proteins that can perform therapeutic functions.[44] The mRNA is designed to encode specific proteins needed to treat or prevent diseases.[45] RNP therapies involve the use of RNPs, complexes of RNA and proteins, to achieve therapeutic effects.[46] These therapies often utilize CRISPR-Cas9 technology for

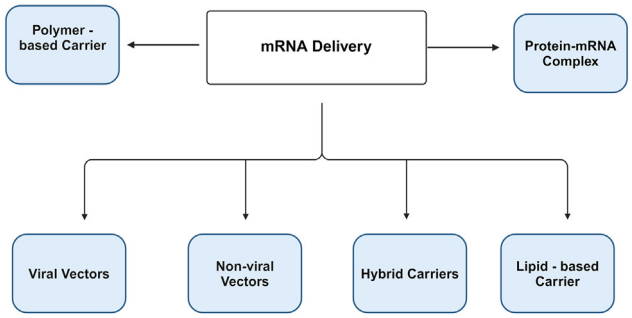

Figure 2. mRNA delivery systems
Efficient mRNA delivery is challenging due to its size and charge.[48]

genome editing, where RNPs can precisely target and modify specific genetic sequences.[47] While both mRNA and RNP therapies aim to treat diseases at the molecular level, mRNA therapies focus on protein production, whereas RNP therapies primarily involve gene editing. Both approaches offer unique advantages and face specific challenges in terms of delivery, efficiency, and safety.

Advancements in delivery systems

An overview of various delivery systems (different vectors and carriers employed to deliver mRNA effectively into target cells) used for mRNA-based therapies are shown in Figure 2. It includes sections on viral vectors, which utilize modified viruses to deliver genetic material; hybrid carriers, which combine multiple delivery mechanisms; polymer-based carriers, which use synthetic polymers to encapsulate and protect mRNA; lipid-based carriers, such as LNPs, which are commonly used for their efficiency in protecting and delivering mRNA into cells; protein-mRNA complexes, which involve the use of proteins to stabilize and transport mRNA; and non-viral vectors, which include a range of synthetic and natural materials designed to facilitate mRNA delivery without using viral components. These varied approaches are crucial for optimizing the stability, efficiency, and targeting of mRNA-based treatments.

LNPs

Lipid-based carriers, including LNPs and lipoplexes, are extensively utilized for delivering nucleic acids.[49] Proper engineering allows effective encapsulation of mRNA into LNPs and lipoplexes, protecting it from degradation and facilitating cellular uptake and endosomal escape. Components such as cationic or ionizable lipids, cholesterol, poly(ethylene) glycol (PEG)-lipid, and phospholipids are crucial for mRNA encapsulation and stability.[50] Precise molar ratios of these components generate LNPs with desired functionalities for mRNA delivery. Optimization studies have focused on factors such as lipid-to-mRNA weight ratio, phospholipid identity, and molar ratios of lipid components to enhance transfection efficiency. Novel cationic or ionizable lipids with modified head or tail groups have been explored to improve delivery efficacy.[50] In addition, various components such as proteins, vitamins, and aminoglycosides have been utilized to construct effective LNPs for mRNA delivery. For example, mechanism of action of mRNA-LNP vaccines are shown in Figure 3.

Zwitterionic phospholipids have also gained attention for their involvement in endosomal escape membrane via membrane fusion.[51,52] pH-switchable ionizable phospholipids with multi tails have shown promise in mRNA delivery, exhibiting organ selectivity *in vivo*.[53] However, concerns regarding their toxicity, especially those composed of polycationic and pegylated lipids, have been raised in several studies.[54] Polycationic lipids, known for their ability to encapsulate nucleic acids effectively, can also induce cytotoxic effects due to their positive charge, which may disrupt cellular membranes and lead to cell death.[55,56] This is particularly relevant in the context of therapeutic applications, where the balance between effective delivery and cellular safety is critical.[57,58] Pegylated lipids, while enhancing the stability and circulation time of LNPs in the bloodstream, can also elicit immune responses that may lead to adverse effects.[59] Studies have shown that pegylation can alter the pharmacokinetics of nanoparticles, potentially resulting in unexpected toxicity.[60] For instance, the formation of anti-PEG antibodies has been documented, which can lead to accelerated clearance of pegylated nanoparticles and reduced therapeutic efficacy.[60] Furthermore, the accumulation of these nanoparticles in various tissues can provoke inflammatory responses, highlighting the need for careful design and optimization of lipid formulations.[60]

Polymer-based delivery systems

Polymeric nanoparticles are a promising delivery system for mRNA-based therapeutics[62] (Figure 4). Cationic polymers can complex with mRNA to form nanoparticles called mRNA polyplexes.[63] While early materials such as PEI and poly(l-lysine) showed limited *in vivo* efficacy and toxicity, recent attention has turned to functional and biodegradable polymers for better outcomes.[64] Charge-altering releasable transport (CART) systems, capable of changing charge properties in different pH environments, aid in mRNA release in the cytoplasm, enhancing transfection efficacy.[65] Several chemical structures of CARTs have been explored successfully. In addition, poly(β-amino esters) (PBAEs) and their derivatives, such as polycaprolactone-based PBAEs and oligopeptide end-modified PBAEs, have shown promise in mRNA delivery by facilitating complex formation and enhancing endosomal escape.[66] Hyperbranched PBAEs, synthesized with a trifunctional amine, have demonstrated superior stability and transfection efficiency compared with linear PBAEs, particularly in delivering mRNA to the lung epithelium.[67] Libraries of biodegradable polymers such as poly(amine-co-ester)s have been developed to quantitate endosomal escape, with high encapsulation efficiency identified as a crucial step in mRNA transfection.[68] Ionizable amphiphilic Janus dendrimers (IAJDs) have emerged as a simple yet effective one-component system for mRNA delivery.[69] Various IAJDs have been synthesized and evaluated, with some showing high transfection efficacy, while the cation-π interaction has been identified as a potential avenue for further design optimization.[69]

Viral vectors and non-viral delivery methods

Delivery of mRNA can be accomplished using both viral and non-viral vectors.[71,72] Viral vectors, such as adeno-associated viruses and genetically modified viruses, offer the advantage of local

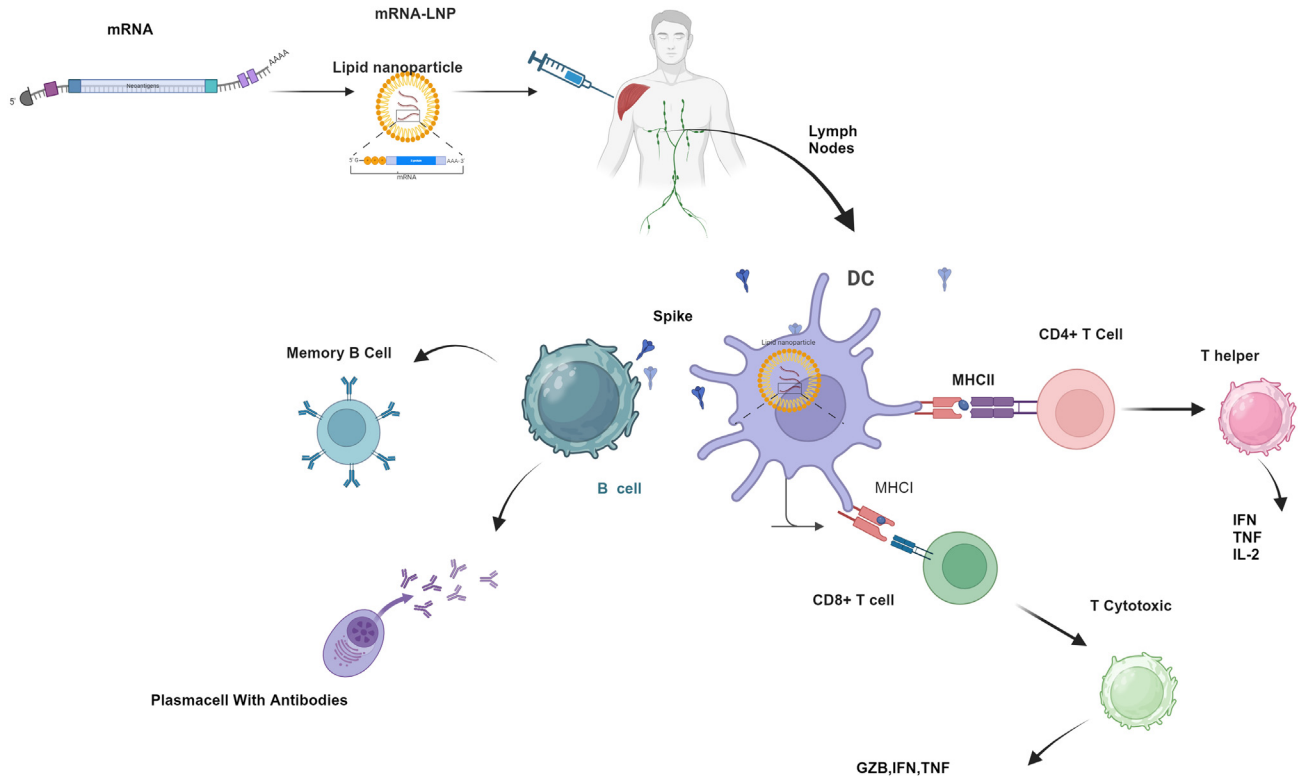

Figure 3. Mechanism of action of mRNA-LNP vaccines

This schematic diagram illustrates the process of how mRNA-LNP (messenger RNA-lipid nanoparticle) vaccines elicit an immune response. (1) mRNA packaging: mRNA encoding the pathogen's spike protein is encapsulated in LNPs to protect it from degradation and aid its delivery into human cells. (2) Delivery and translation: LNPs transport mRNA into dendritic cells (DCs), mainly in the lymph nodes, where it is translated into the spike protein. (3) Antigen presentation: the spike protein is displayed on DCs via MHC molecules, activating CD4+ and CD8+ T cells. (4) T cell activation: CD4+ T cells recognize MHCII-bound spike proteins, secreting cytokines to stimulate immune responses, while CD8+ T cells recognize MHCI-bound proteins and release cytotoxic molecules to kill infected cells. (5) B cell activation and memory: B cells recognize the spike protein, producing antibodies via plasma cells and forming memory B cells for long-term immunity against future infections.[61]

replication and expression in the cytoplasm.[73,74] However, cytotoxic effects and potential host rejection pose challenges for viral vectors. Non-viral vectors include naked mRNA, which can be administered intramuscularly, subcutaneously, or intradermally, bypassing obstacles associated with systemic administration.[71,75,76] Naked mRNA has demonstrated efficient translation and immune response induction, particularly when administered subcutaneously. Various physical and active methods have been employed to enhance skin penetration and mRNA delivery, including electroporation, microporation, and microneedle-based delivery.[77] These approaches offer advantages such as reduced cost and potential risk, although naked mRNA faces challenges such as short plasma half-life and susceptibility to degradation. Delivery systems have been developed to protect mRNA and promote cellular uptake, addressing these challenges.[78]

Protein-mRNA complex

Natural positively charged proteins can form complexes with negatively charged mRNA via electrostatic interactions, facilitating the self-assembly of protein-mRNA complexes.[79] Protamine, a positively charged protein abundant in arginine, has been utilized to complex with mRNA vaccines, enhancing immune stimulation by activating the TLR7 receptor.[80] Studies have demonstrated that protamine-complexed mRNA encoding tumor-associated antigens can induce a strong antitumor immune response in mice and metastatic melanoma patients with minimal toxicity.[81] In addition, mammalian retrovirus-like protein PEG10 has been reported as a promising vehicle for mRNA delivery, capable of binding, stabilizing, and delivering mRNA efficiently in human cells, including both single-guide RNA and *Streptococcus pyogenes* Cas9 (SpCas9).[82–84]

INNOVATIONS IN TARGETED DELIVERY AND TISSUE-SPECIFIC TARGETING

Advancements in targeted delivery, such as tissue-specific ligands and hybrid carriers, aim to improve the precision and efficacy of mRNA and RNP therapies.

Hybrid carriers

Hybrid carriers, combining both lipid and polymer components, offer advantages in mRNA delivery.[85] These carriers can enhance stability and pharmacokinetics, particularly when decorated with lipid to

prolong circulation time by evading uptake by the reticuloendothelial system.[85] Organic/inorganic hybrid nanoparticles, including metal-organic frameworks, gold nanoparticles, and graphene oxide-PEI complexes, have shown promise in mRNA delivery.[86] A study by Choi et al. demonstrated the efficacy of PEI-conjugated graphene oxide in mRNA delivery.[87] This hybrid nanoparticle increased loading capacity, protected mRNA against degradation, and significantly enhanced transfection efficacy compared with conventional materials.[87] Wang et al. utilized PEI-modified mesoporous organosilica for mRNA delivery, achieving high transfection efficacy by incorporating large-pore structures and tetrasulfide to activate the mTORC1 pathway.[88] Lipid/polymer hybrid nanoparticles are also promising mRNA carriers. Islam et al. combined cationic lipid, PLGA, and DSPE-PEG to construct robust hybrid nanoparticles, exhibiting superior transfection efficacy compared with conventional lipids.[89]

MECHANISMS OF ACTION AND THERAPEUTIC TARGETS

Current therapeutic targets (e.g., infectious diseases, cancer, genetic disorders)

mRNA-based therapeutics show great potential for treating a diverse range of challenging diseases, such as infectious diseases, metabolic genetic disorders, cancer, cardiovascular ailments, and others.[1] Multiple studies have illustrated mRNA's advantages over traditional protein and DNA drugs, including enhanced transfection efficiency, prolonged protein expression, and reduced risk of genomic integration.[90,91] Moreover, mRNA can be synthesized rapidly through IVT, facilitating quick adaptation to various therapies. Chemical modifications of specific nucleotides address concerns regarding immunogenicity and stability, further enhancing the appeal of mRNA therapy.[92] The burgeoning interest in mRNA has attracted substantial investment, contributing to the establishment of well-funded biotechnology companies such as Moderna, CureVac, BioNTech, and others. These companies are actively engaged in advancing mRNA-based drug technologies, underscoring the significant potential of mRNA in drug development.[93]

Hematologic diseases

Preclinical studies have investigated mRNA-based protein replacement therapy for hematologic diseases, particularly hemophilia.[94,95] Hemophilia, characterized by deficiencies in blood coagulation factors, such as factor VIII (hemophilia A) and factor IX (hemophilia B), has been targeted for correction using mRNA technology.[95] LNPs encapsulating mRNAs encoding different variants of factor VIII (F8) induced rapid and sustained expression of FVIII in hemophilia A mice.[95] In hemophilia B, mRNA encoding factor IX (FIX) was delivered using lipidoids called TTs, leading to restoration of FIX function in FIX-knockout mice.[96] In addition, lipid-enabled LUNAR LNPs encapsulating hFIX mRNA showed promising results in treating hemophilia B mice, with a rapid onset of FIX expression lasting up to several days.[96]

Metabolic diseases

mRNA-based therapies offer promise for treating metabolic diseases that currently lack effective treatments. Conditions such as hepatorenal tyrosinemia, acute intermittent porphyria, Fabry disease, glycogen storage disease type 1 A, Crigler-Najjar syndrome type 1, and ornithine transcarboxylase deficiency could potentially benefit from mRNA therapies.[97–103] For instance, in hepatorenal tyrosinemia, dendrimer LNPs loaded with mRNA encoding fumarylacetoacetate-hydrolase were designed to restore liver function in mice models.[104] In acute intermittent porphyria, LNP-encapsulated mRNA induced expression of porphobilinogen deaminase, normalizing urine porphyrin precursor excretion and mitigating porphyria attacks.[105] Methylmalonic acidemia, another metabolic disorder, showed reduction in plasma methylmalonic acid levels with systemic expression of functional mitochondrial methylmalonyl-CoA mutase delivered via LNPs.[106] Hybrid mRNA technology was utilized to deliver ornithine transcarboxylase mRNA, improving plasma ammonia levels and survival in deficient mice.[103,107] In addition, mRNA therapies have shown promise in treating diseases such as Fabry disease and alpha 1-antitrypsin deficiency.[108,109] Moreover, mRNA-based therapies have been explored for tumor treatment, with PTEN mRNA delivery inhibiting tumor growth in PTEN-null mice, and p53 mRNA delivery inducing growth inhibition and apoptosis in tumor cells.[110] Furthermore, mRNA encoding anti-angiogenic proteins has shown efficacy in inhibiting tumors.[111]

mRNA-based stem cell therapeutics

RNA-based genome editing has emerged as a potent tool for treating a variety of diseases, particularly in stem cell therapy.[112] Retroviral vectors have been utilized to deliver ZFN protein, mRNA, and DNA to disrupt targeted genes with high efficiency.[113] ZFN mRNA has demonstrated superior specificity compared with TALEN mRNA and CRISPR-Cas9 mRNA when delivered via electroporation into primary human hematopoietic stem and progenitor cells.[113] Plasmid-derived gRNA and Cas9 mRNA exhibited comparable acute cytotoxicity, emphasizing the need for optimization in CRISPR-Cas9 delivery to these cells. Innovative strategies involving macaque-specific CCR5 ZFN mRNA have enabled successful *ex vivo* modification of hematopoietic stem and progenitor cells in large animal models.[114–116]

mRNA-based monoclonal antibodies

Nucleic acid-encoded monoclonal antibodies (mAbs), particularly mRNA-based mAbs, hold promise for improving therapy efficacy and reducing production costs compared with traditional mAbs.[117] mRNA-mAbs are mainly applied in treating infections and tumors.[117] For instance, mRNA encoding the broadly neutralizing anti-HIV-1 antibody VRC01 successfully produced the antibody in mice and protected them from HIV-1 infection.[118] Similarly, mRNA encoding neutralizing antibodies against respiratory syncytial virus (RSV) and chikungunya virus (CHKV-24) demonstrated efficacy in inhibiting virus replication and protecting against disease in animal models.[119,120] In tumor treatment, mRNA-based antibodies induced rapid and sustained serum antibody levels, allowing mice to survive tumor challenges. Delivery methods such as LNPs have been employed to efficiently transfer mRNA-encoding antibodies. Moreover, mRNA-based bispecific T cell-engaging antibodies (bsAbs) showed promising results in inhibiting tumor growth.[121]

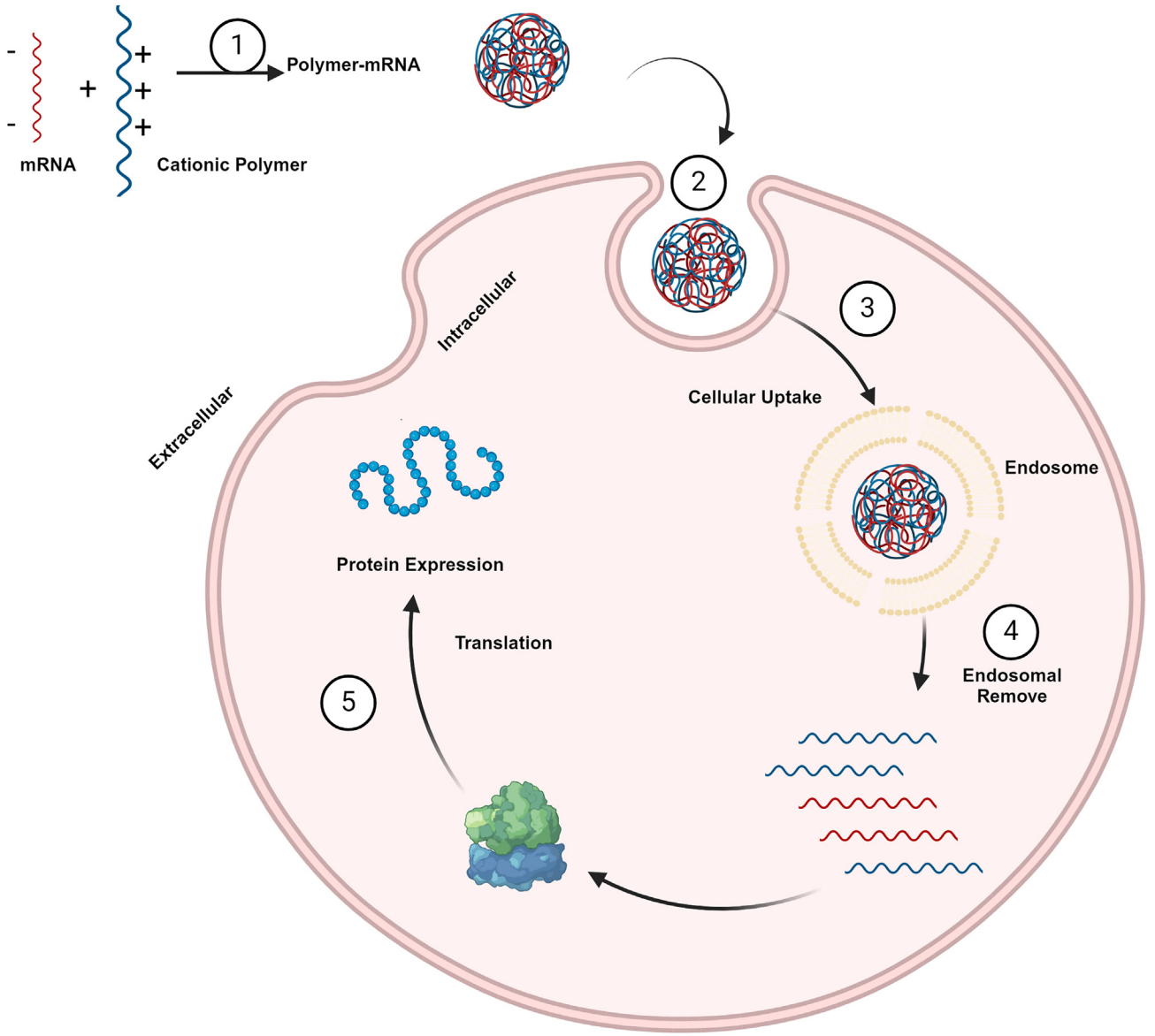

Figure 4. Polymer-mRNA delivery system for protein expression

This schematic diagram illustrates the process of mRNA delivery using a cationic polymer carrier, highlighting the key steps involved in cellular uptake and protein expression. (1) Complex formation: the mRNA (depicted as a red strand) is complexed with a cationic polymer (depicted as a blue strand) to form a polymer-mRNA complex. The cationic polymer protects the mRNA and facilitates its delivery into the cell. (2) Cellular uptake: the polymer-mRNA complex is taken up by the cell through endocytosis, a process where the cell membrane engulfs the complex and brings it into the intracellular environment. (3) Endosomal encapsulation: once inside the cell, the polymer-mRNA complex is encapsulated within an endosome, a membrane-bound vesicle. (4) Endosomal escape: the mRNA is released from the endosome into the cytoplasm. (5) Translation and protein expression: the released mRNA is translated by the cellular machinery to produce the target protein, completing the process of gene expression.[70]

However, some challenges remain, such as safety concerns with certain delivery vectors like those that viral vectors used for SARS-CoV-2 mRNA vaccine development.

Non-formulated mRNA vaccine

Non-formulated mRNA, administered intradermally or intranodally, has proven effective in initiating T cell responses in both mice and humans.[21,122–124] Intranodal injection specifically targets dendritic cells in the area of T cell activation, utilizing macropinocytosis for uptake.[124] Clinical trials with metastatic melanoma patients have demonstrated the safety and feasibility of naked mRNA vaccines, stimulating antigen-specific T cell responses.[123] For instance, in melanoma patients, intradermal injection of autologous tumor mRNA combined with GM-CSF enhanced T cell responses. Similarly, in

renal cell carcinoma (RCC) patients, intradermal administration of non-formulated mRNA encoding various antigens along with GM-CSF led to stable disease and partial responses, with a majority of patients showing antigen-specific T cell responses.[123]

CHALLENGES AND LIMITATIONS

Stability and degradation of mRNA

RNA is inherently unstable and can trigger immune responses, necessitating delivery vehicles for efficient transport to target cells.[125] Natural RNAs are prone to degradation by native nucleases, but stability can be significantly enhanced through synthetic modifications.[126] Developing effective carriers to protect RNA from the harsh physiological environment is crucial due to RNA's substantial negative charges and chemical alterations.[127] These challenges have impeded the clinical advancement of some RNA-based therapies, resulting in varied outcomes in trials. However, recent promising trial results indicate that these obstacles can be surmounted with improved synthetic delivery carriers and chemical modifications of RNA therapeutics. Encapsulating RNA within various carriers protects it from nuclease degradation after systemic administration, thereby enhancing its stability and longevity.[128–131]

Immunogenicity and immune response

RNA therapy, utilizing RNA-based molecules to influence biological pathways, represents a versatile and specific therapeutic approach with significant potential for treating a diverse array of diseases.[132] One of major hurdles to advancing RNA therapy is immunogenicity; injected or administered RNAs can be recognized by the immune system as foreign entities, triggering an innate immune response that might reduce the therapeutic efficacy, and possibly cause side effects.[133] There have been efforts to address this by modifying the structure or sequence of RNA nucleotides, coding sequence optimization, suppressing the immune system, and packaging RNA within a shielding delivery system.[134] In a study, researchers have identified a novel method to mitigate the immunogenicity problem. They used an acylating reagent to add acyl groups to the 2′-hydroxyl (OH) groups on RNAs.[135] Studies have shown that local delivery of naked small interfering RNAs (siRNAs) or aptamers, often preferred for lung, eye, and skin applications, can trigger a proinflammatory response due to the activation of TLRs and suffer from poor cellular uptake and nuclease sensitivity.[135] In some cases, these issues can be mitigated by encapsulating the nucleic acid with a synthetic carrier or introducing chemical modifications. These strategies are expected to enhance the specificity, stability, and immunoresistance of RNA-based drugs.[136] Modified ribonucleotides, such as N1-methylpseudouridine, are incorporated into therapeutic mRNAs primarily to reduce their innate immunogenicity.[137] The reduction of immune recognition is crucial for avoiding hyperinflammatory responses, ensuring that therapeutic mRNA reaches its target cells without being prematurely degraded by the body's innate immune system. These modifications have been essential to the success of mRNA vaccines, allowing them to deliver genetic instructions effectively and with fewer side effects.[137] However, incorporating N1-methylpseudouridine can lead to +1 ribosomal frameshifting during the translation of mRNA. These +1 frameshifted products may initiate immune responses, as demonstrated by cellular immunity to these abnormal proteins after vaccination with mRNA vaccines containing N1-methylpseudouridine. This response involves T cells and possibly B cells, indicating the potential for unintended immunogenic effects.[137,138] Frameshifting is mainly attributed to ribosomal stalling at particular slippery sequences, potentially leading to the synthesis of abnormal proteins. This phenomenon has been observed in both lab-based studies and cultured cells, possibly due to disruptions in aminoacyl-tRNA binding that slow down the translation process.[139–141] Despite evidence that N1-methylpseudouridine can induce frameshifting, it is important to note that no adverse reactions have been reported in humans who have received mRNA-based SARS-CoV-2 vaccines. The safety of these vaccines has been thoroughly assessed, and frameshifting has not been linked to any major clinical consequences.[142–144] To reduce the risks associated with frameshifting, researchers have pinpointed synonymous targeting of slippery sequences as an effective method. Optimizing mRNA sequences can reduce the occurrence of frameshifting events, thereby decreasing the production of aberrant proteins. This approach is crucial for enhancing the safety and efficacy of future mRNA-based therapies.[145] Further investigation into alternative ribonucleotide modifications is needed. For instance, using 5-methoxyU has shown to decrease translation efficiency, which could limit its clinical application.[146] Researching various modifications may lead to strategies that maintain low immunogenicity without affecting translation fidelity.[146]

Efficiency of delivery and cellular uptake

Recent advancements in nanotechnology and materials science offer promising solutions to the intricate challenges of delivering oligonucleotide drugs, especially for achieving effective intracellular penetration across biological barriers and membranes.[147] Nanoparticle-based drug delivery systems provide several advantages, including the ability to finely tune biophysical parameters such as size, shape, and chemical composition, alongside optimizing biological properties through targeted ligand functionalization.[148] Ensuring efficient RNA delivery into the cytoplasm is critical for successful RNA therapy, as RNA's large size, hydrophilicity, and negative charge hinder its passive diffusion across lipid bilayers.[149] Overcoming extracellular and intracellular barriers involves evading serum nucleases, bypassing macrophage scavenging in the reticuloendothelial system, and navigating through the extracellular matrix via receptor-mediated endocytosis.[150] Effective endosomal escape and non-toxic release of RNAs into the cytoplasm remain significant technical hurdles.[151] To address these challenges, researchers are exploring various chemical modifications and engineered delivery formulations to optimize pharmacodynamic and pharmacokinetic profiles. The complexity of delivering RNA-based drugs, due to their larger size compared with traditional therapeutics, underscores the need for precise targeting strategies within the body.

Off-target effects and specificity

Jackson and Linsley reported the first instances of off-target effects using genome-wide microarray profiling.[152] They observed modest alterations (1.5- to 3-fold changes) in the expression of numerous genes

upon transfecting individual siRNA molecules.[152] The degree of complementarity between the siRNA's sense or antisense strand and off-target genes varied widely, resulting in distinct off-target expression profiles for each siRNA sequence.[153] Off-target effects occur when siRNA is processed by the RNA-induced silencing complex, inadvertently suppressing unintended gene targets.[154] These unintended changes in gene expression can lead to observable phenotypic variations, such as false positives, underscoring the need to elucidate the underlying mechanisms of off-targeting.[155] Understanding these mechanisms is crucial for developing strategies to mitigate off-target effects. Similarly, ribozymes and aptamers encounter challenges related to delivery and off-target toxicity, akin to those faced by siRNAs.[156,157]

Risks associated with mRNA therapies

Continuous positive PCR tests for SARS-CoV-2 have been observed in patients long after recovery, raising questions about the cause.[158–160] While reinfection is possible, some studies suggest that these cases are not due to new infections, as no active virus has been isolated from such individuals.[161–163] One theory is that viral RNA might integrate into the host genome via a reverse transcription mechanism, leading to persistent RNA detection.[164] SARS-CoV-2, an RNA virus, replicates its RNA using an RNA-dependent RNA polymerase. However, nonretroviral RNA viruses such as SARS-CoV-2 could potentially be reverse-transcribed and integrated into host DNA by endogenous reverse transcriptase, such as those from LINE-1 elements.[165] These elements, prevalent in the human genome, can be activated by viral infections, including SARS-CoV-2, potentially explaining the persistent detection of viral RNA. This mechanism might also account for why some patients test positive long after recovery, as integrated viral DNA could lead to RNA expression, mimicking active infection.[166] Zhang et al. provide evidence that SARS-CoV-2 sequences can be reverse-transcribed and integrated into human cell DNA, primarily through endogenous LINE-1 elements. Such integration results in the expression of chimeric virus-host RNAs, possibly affecting clinical outcomes by continuously stimulating immune responses without producing infectious virus. While only a small fraction of cells may express viral sequences, this process could potentially influence the disease course or even trigger autoimmunity. This discovery also highlights potential challenges in using PCR tests to monitor antiviral treatment effectiveness, as they may detect integrated viral sequences rather than active infections.[166] The mRNA vaccines from Moderna and Pfizer-BioNTech use LNPs to deliver synthetic mRNA into human cells. This mRNA encodes the spike protein of SARS-CoV-2, facilitating the immune system's ability to recognize and fight the virus. Importantly, this mRNA does not enter the cell nucleus and thus does not interact with or integrate into human DNA.[167–169] The cellular machinery translates the mRNA into the spike protein in the cytoplasm, after which the mRNA is naturally degraded by normal cellular processes.[170]

CLINICAL APPLICATIONS AND TRIALS

Various RNA-based strategies have been explored extensively in both experimental and clinical settings. There is significant interest in exploring the synergistic effects of combining mRNA- and RNP-based therapies with other treatments such as immunotherapy or chemotherapy, potentially enhancing patient outcomes. Moving forward from preclinical investigations to clinical trials is crucial to establish the safety and effectiveness of mRNA- and RNP-based therapies in human patients. This progression is essential to pave the way for broader adoption and application of these therapies in clinical practice.[171] RNA-based therapeutics have gained initial traction in addressing diseases with clear pathological mechanisms, such as oncology, neurological disorders, and infectious diseases (Table 1)[172–175]. These therapies are particularly aimed at conditions where conventional treatments have limited efficacy. Ongoing clinical studies are exploring RNA-based approaches for a wide range of incurable diseases. The specific RNA sequence plays a pivotal role in modulating the expression or activity of target molecules. Notably, a significant portion of phase I trials involving antisense oligonucleotide (ASO)-based therapies has progressed to phase II/III trials over the past 5 years, focusing on rare and common diseases, including orphan genetic disorders and cancer. The US FDA has approved several ASO drugs, such as mipomersen and inotersen (notably identified by the -rsen suffix), underscoring their clinical relevance and potential impact.[176] In 2013, the FDA-approved mipomersen as the second ASO drug, targeting homozygous familial hypercholesterolemia. Mipomersen functions by binding to the mRNA sequence of apolipoprotein B-100 (ApoB-100) and cleaving it to reduce cholesterol levels. Other FDA-approved ASO drugs, such as nusinersen, eteplirsen, and golodirsen, modulate target pre-mRNAs' splicing processes. In addition, the FDA has approved three siRNA-based drugs: patisiran, givosiran, and lumasiran, identified by the -siran suffix. Patisiran, approved in 2018, addresses hereditary transthyretin-mediated amyloidosis by targeting transthyretin mRNA to inhibit protein synthesis. Givosiran, the second approved siRNA-based drug, treats acute hepatic porphyria by reducing levels of aminolevulinic acid (ALA) and porphobilinogen, metabolic intermediates in heme biosynthesis pathway, thus alleviating symptoms associated with the disease.[177,178] Givosiran functions by targeting ALA synthase 1, thereby suppressing its expression and restoring normal heme biosynthesis.[179] Its delivery involves a trivalent N-acetylgalactosamine conjugate attached to the 3' end of its passenger strand, enabling subcutaneous administration and specific targeting of hepatocytes via the asialoglycoprotein receptor. This delivery approach, effective for liver-targeting siRNAs, is widely employed for similar therapeutics. In 2021, the US FDA-approved inclisiran for the treatment of primary hypercholesterolemia or mixed dyslipidemia.[171,180]

SAFETY AND REGULATORY CONSIDERATIONS

RNA therapy involves using RNA-based molecules to treat or prevent diseases. Unlike DNA therapy, RNA therapy does not pose significant genotoxic effects. In DNA-based therapies, the DNA molecule is introduced into cells using a viral vector, which can integrate into the genome and potentially cause mutations. This risk is mitigated with RNA therapy, as RNA is used instead of DNA. Despite its promise, RNA therapy faces challenges, including poor pharmacological properties, difficulties in intracellular delivery, and immune-related

toxicity. Issues such as off-target binding, sequence-induced toxicity, and oversaturation of the endogenous RNA processing pathway also impact the effectiveness of RNA-based approaches.[171,181,182]

Innovations in delivery systems and formulation

Effective delivery of RNA-based drugs poses a formidable hurdle in their therapeutic application. Current strategies include integrating targeting elements, encapsulating in lipid-based nanoparticles, or direct administration to specific organs with minimal alteration. Kim et al. emphasize the urgent need for advancing RNA drug delivery techniques as a cornerstone of future research efforts.[183] RNA therapeutics operate by modulating the expression and function of precise target molecules, offering a novel approach to treating diseases resistant to traditional pharmaceuticals. These therapies hold promise for customization across diverse RNA and protein formats, potentially revolutionizing personalized medicine and addressing unmet needs in rare disease treatments.[132] Stephenson and Zamecnik's pioneering work in 1978 marked the first therapeutic use of RNA base-pairing, employing an ASO to target the 35S RNA of the Rous sarcoma virus and inhibit viral replication.[184] Nearly two decades later, the US FDA approved the first ASO drug for treating cytomegalovirus retinitis, illustrating a significant milestone in RNA-based therapy.[185,186] RNA splicing, crucial for removing introns and joining exons in RNA transcripts, was first elucidated in 1977.[187] Variations in splicing are implicated in various human diseases, challenging conventional drug treatments but offering potential targets for RNA-based therapies such as ASOs.[188,189]

In contrast to the extended development timeline of ASO drugs, the progress from discovery to clinical application of siRNAs was notably swift. RNA interference (RNAi) was initially characterized in 1998, demonstrating potent and specific inhibition of targeted mRNAs in *Caenorhabditis elegans* embryos treated with sense and antisense RNAs.[190] The simplicity and effectiveness of RNAi quickly gained traction in scientific research and applications.[191] In 2002, RNAi was shown to inhibit hepatitis C virus replication in mice, prompting widespread exploration of its therapeutic potential.[192] Clinical trials employing RNAi technologies began in 2010, with a notable study using an siRNA targeting the M2 subunit of ribonucleotide reductase to treat melanoma, achieving successful mRNA cleavage via targeted nanoparticle delivery.[193] Subsequent evaluations led to the approval of the first siRNA-based drug for hereditary transthyretin-mediated amyloidosis in 2018, highlighting the transformative impact of RNA-based therapies in modern medicine.[132] Chemical modification represents a promising approach for enhancing the delivery of RNA-based drugs. By altering the nucleic acid backbone, ribose ring, and nucleobase, researchers can optimize these molecules to exhibit more favorable drug-like properties. For instance, extensive chemical modifications enable gapmer ASOs to reach various tissues effectively without requiring additional delivery agents. Currently, 8 out of the 10 approved oligonucleotide treatments are administered without the need for supplemental delivery vehicles. However, caution is warranted as certain synthetic nucleotides, such as LNA-modified nucleic acids, have been associated with significant hepatotoxicity risks. In response, bioengineered RNAi agents have emerged as a promising new class of *in vivo* RNA agents designed with minimal post-transcriptional modifications, offering exciting prospects for future applications.[132,171]

NEW THERAPEUTIC TARGETS AND APPLICATIONS

Targeting undruggable targets

RNA-based therapeutics offer a significant advantage in their ability to target virtually any genetic component within a cell, including those traditionally considered undruggable by small molecules and antibodies.[194,195] Noncoding RNAs, particularly small RNAs, are distinguished by their specific RNA sequences, enabling drugs such as antisense RNA and siRNA to bind selectively to their targets.[196] This sequence specificity suggests that these therapies can effectively target noncoding RNAs, which play crucial roles in disease pathogenesis.[197] Given the prevalence of noncoding RNAs in the human genome and their documented significance in various diseases, RNA-based treatments are increasingly recognized for their potential impact and therapeutic relevance.[198,199]

Less than one-third of human proteins are thought to be suitable targets for small-molecule drugs.[200–202] This limitation arises from the structural similarities shared among many proteins, which complicates the direct targeting of specific ones.[203] Membrane-integrated proteins pose an additional challenge because their interaction sites within the cytoplasm are limited, making them difficult targets for small molecules or antibodies.[204] In contrast, RNA-based drugs offer a different approach by targeting the biogenesis of these proteins. By inhibiting their production, RNA-based therapies have the potential to enhance therapeutic effectiveness in cases where direct protein targeting is challenging.[205,206] This strategy represents a promising avenue for developing treatments that can address conditions associated with membrane-integrated proteins and other challenging therapeutic targets.[205,206] Developing new small-molecule- or antibody-based drugs usually involves a lengthy timeline.[206] In contrast, once the chemical structure of RNA and its delivery method are determined, RNA-based drugs can be swiftly designed and synthesized for clinical trials.[202] For example, an siRNA drug designed to target a disease caused by excessive gene expression in a specific organ can easily be modified to treat other diseases affecting the same organ by adjusting the siRNA sequence.[207] This adaptability is evident in the extensive development of siRNA drugs using liver cell-specific conjugates to address various liver metabolism-related disorders.[207] This rapid adaptability underscores the potential of RNA-based therapies to quickly respond to emerging medical needs and expand treatment options within specific organs.[207]

LONG-TERM OUTLOOK AND FUTURE RESEARCH DIRECTIONS

Despite facing substantial hurdles in clinical testing, RNA-based therapeutics have gleaned invaluable insights from previous trials. These efforts have shown early potential in treating cancers, viral infections, and genetic disorders. However, fully realizing the capabilities of RNAi- and RNA-based therapies necessitates advanced delivery

Table 1. Approved and clinical trials for RNA therapeutics

Trade name	Approved and clinical trials	Disease type	Admiration route	Delivery system
mRNA: (elasomeran) *Moderna*	2021FDA approved	infectious	intramuscular	LNP
mRNA:Comirnaty(tozinameran) *Pfizer-BioNTech*	2021 FDA approved	infectious	intramuscular	LNP
siRNA:Givlaari(givosiran)Alnylam	2019 FDA approved	genetics	subcutaneous	conjugate (GalNAc)
siRNA:Onpattro(patisiran) *Alnylam*	2018 FDA approved	genetics	intravenous	LNP
siRNA:Oxlumo(lumasiran)*Alnylam*	2020 FDA approved	genetics	subcutaneous	conjugate (GalNAc)
siRNA:Leqvio (inclisiran)*Novartis*	2021 FDA approved	genetic and physiological	subcutaneous	conjugate (GalNAc)
ASO:Vitravene(fomivirsen) *IsisPharmaceuticals*–discontinued	1998 FDA approved	infectious	intravitreal	Mod/Sub (PS)
Spinraza (nusinersen) *Biogen*	2016 FDA approved	genetic	intrathecal	Mod/Subs (2′-MOE, PS, 5-methyl cytosine)
Kynamro (mipomersen) *Genzyme*–discontinued	2013 FDA approved	genetic	subcutaneous	Mod/Subs (2′-MOE, PS, 5-methyl cytosine)
Tegsedi (inotersen) *Akcea*	2018 FDA approved	genetic	subcutaneous	Mod/Subs (2′-MOE, PS)
Vyondys 53 (golodisen) *Sarepta*	2019 FDA approved	genetic	intravenous	Mod/Subs (PMO)
Exondys 51 (eteplirsen) *Sarepta*	2016 FDA approved	genetic	intravenous	Mod/Subs (PMO)
Milasen *Brammer Bio*	2018 FDA approved	genetic	intrathecal	Mod/Subs (2′-MOE, PS, 5-methyl cytosine)
Viltepso (viltolarsen) NS Pharma	2020 FDA approved	genetic	intravenous	Mod/Subs (PMO)
Waylivra (volanesorsen) *Akcea*	2019 EMA	genetic	subcutaneous	Mod/Subs (2′-MOE)
Aptamer: Macugen (pegaptanib) *Gilead*–discontinued	2004 FDA approved	physiological	intravitreal	conjugate (PEG)
NCT04573140	phase I	cancer	intravenous	liposome
NCT02316457	phase I	cancer	intravenous	liposome
NCT02872025	early phase I	cancer	intralesional	LNP
NCT03897881	phase II	cancer	intravenous	LNP
NCT03871348	phase I	cancer	intratumoral	LNP
NCT03164772	phase I/II	cancer	intradermal	LNP
NCT03948763	phase I	cancer	intradermal	LNP
NCT04163094	phase I	cancer	intravenous	liposome
NCT03313778	phase I	cancer	intramuscular	LNP
NCT04534205	phase II	cancer	intravenous	liposome
NCT05043181	phase I	genetic	intravenous	other (exosome)
NCT04990388	phase I/II	genetic	intravenous	LNP
NCT04442347	phase I	genetic	intravenous	LNP
NCT05130437	phase I/II	genetic	intravenous	LNP
NCT04652102	phase II/III	infectious	intramuscular	LNP
NCT05085366	phase III	infectious	intramuscular	LNP
NCT05001373	phase I	infectious	intramuscular	LNP
NCT04144348	phase I	infectious	intramuscular	LNP

strategies. Innovations such as aptamer-siRNA chimeras and transferrin-decorated nanoparticles are poised to significantly enhance the precision and efficacy of RNA drug delivery. These engineered designs represent crucial advancements in targeting specific tissues and cells, thereby paving the way for broader applications of RNA-based treatments in clinical settings.[171,172] The future of RNA-based drugs hinges on refining their biochemical properties to optimize potency while reducing off-target toxicity and immunogenicity. Specifically, siRNAs will require precise chemical modifications to mitigate nonspecific inflammatory responses, alongside the use of natural or synthetic carriers to achieve efficient and targeted delivery to tissues. These considerations have played a pivotal role in yielding promising clinical outcomes for various siRNA drugs such as CALAA-01, TD101, ALN-VSP02, and ALN-RSV01. While these successes highlight the potential of siRNA therapeutics, they also underscore the critical importance of developing tailored carriers that can selectively

target specific cells and tissues, thereby maximizing therapeutic efficacy while minimizing adverse effects. Continued advancements in carrier design and chemical modification strategies are essential for advancing RNA-based therapies into broader clinical applications.[172]

FUTURE DIRECTIONS AND EMERGING TRENDS

Continued research should focus on improving the efficiency of mRNA and RNP delivery systems, particularly addressing challenges such as off-target effects, RNP denaturation during production, and encapsulation efficiency. Developing strategies for precise tissue-specific targeting of mRNA- and RNP-loaded LNPs will be crucial for enhancing therapeutic outcomes while minimizing off-target effects.[208] The field of mRNA- and RNP-based therapies holds immense promise for revolutionizing disease treatment, ranging from cancer therapy to infectious diseases. Significant progress has been made in developing delivery systems, such as LNPs, to efficiently transport mRNA and RNPs into target cells, enabling precise genome editing and protein expression. Despite remaining challenges, including optimization of delivery efficiency and tissue-specific targeting, ongoing research efforts continue to drive innovation in this rapidly evolving field.[208] With continued advancements and translation into clinical practice, mRNA- and RNP-based therapies have the potential to significantly impact the landscape of modern medicine, offering new hope for patients with a wide range of diseases.[132]

CONCLUSION

RNA-based approaches encompass a diverse array of techniques applied in both experimental settings and clinical trials. Widely utilized methods include commoditized ASOs, siRNAs, antagomirs, and aptamers, which are instrumental in manipulating mRNA expression levels and inhibiting noncoding RNA functions through specific RNA targeting. Several ASOs, siRNAs, aptamers, and mRNA vaccines have received clinical approval, underscoring their therapeutic potential. Despite these advancements, the primary challenge hindering broader adoption of RNA-based therapies lies in effectively delivering these drugs to target organs and tissues beyond the liver. Issues such as off-target binding, sequence-induced toxicity, and saturation of endogenous RNA processing pathways can also impact treatment efficacy. To address these challenges, enhancing RNA drug delivery efficiency through chemical modifications and conjugation with nanocarrier systems holds promise. Continued research into RNA-based therapeutics, including the exploration of RNA molecules as therapeutic agents and their targeting with small molecules, will drive advancements toward more effective treatments for patients.

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AUTHOR CONTRIBUTIONS

Writing – review & editing, Z.E., H.Z., A.O., T.E., F.S.S., M.B., and F.K.-L.; conceptualization, F.K.-L.; supervision, F.K.-L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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