Review Article

Nanomedicine-Based Approaches for mRNA Delivery

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Abstract:

Messenger RNA (mRNA) has immense potential for developing a wide range of therapies, including immunotherapy and protein replacement. As mRNA presents no risk of integration into the host genome and does not require nuclear entry for transfection, which allows protein production even in nondividing cells, mRNA-based approaches can be envisioned as safe and practical therapeutic strategies. Nevertheless, mRNA presents unfavorable characteristics, such as large size, immunogenicity, limited cellular uptake, and sensitivity to enzymatic degradation, which hinder its use as a therapeutic agent. While mRNA stability and immunogenicity have been ameliorated by direct modifications on the mRNA structure, further improvements in mRNA delivery are still needed for promoting its activity in biological settings. In this regard, nanomedicine has shown the ability for spatiotemporally controlling the function of a myriad of bioactive agents in vivo. Direct engineering of nanomedicine structures for loading, protecting, and releasing mRNA and navigating in biological environments can then be applied for promoting mRNA translation toward the development of effective treatments. Here, we review recent approaches aimed at enhancing mRNA function and its delivery through nanomedicines, with particular emphasis on their applications and eventual clinical translation.

Keyword: mRNA delivery, mRNA engineering, Immunology

Introduction:

Messenger RNA (mRNA) mediates the translation of genetic information from genes into proteins. Delivering exogenous mRNA into cells allows to transiently produce proteins in a precise manner. Such mRNA-mediated transfection offers an attractive alternative to plasmid DNA (p DNA)- based gene therapy by expressing proteins even in non-dividing and hard to transfect cells without the risks of genomic integration. (1) Moreover, while pDNA needs to be delivered inside the nucleus of targeted cells, the access of mRNA to the cytosol and the subsequent engagement with the translation machinery of the cells are sufficient to obtain the proteins of interest. The mRNA delivered inside the cells can also last for several days, which is convenient for developing efficient therapeutic strategies, as well as commercially viable approaches. On the other hand, mRNA presents inherent limitations for being used as a stand-alone drug, including fast degradation by nucleases, limited cellular uptake, and immunogenicity.

While the immunogenic signals triggered by mRNA could be exploited for vaccination or immunotherapy applications, (3) major efforts have been dedicated to reduce mRNA immunogenicity and improve the stability of the molecule by either chemical modification or by RNA architectonics, aiming at increasing the significance of mRNA as a therapeutic agent. Nevertheless, mRNA is still susceptible to degradation and the cellular uptake of naked mRNA should be improve for eliciting adequate amounts of proteins. Thus, the development of safe carrier systems capable of intracellular delivery of intact mRNA molecules is fundamental for progressing into effective treatments.

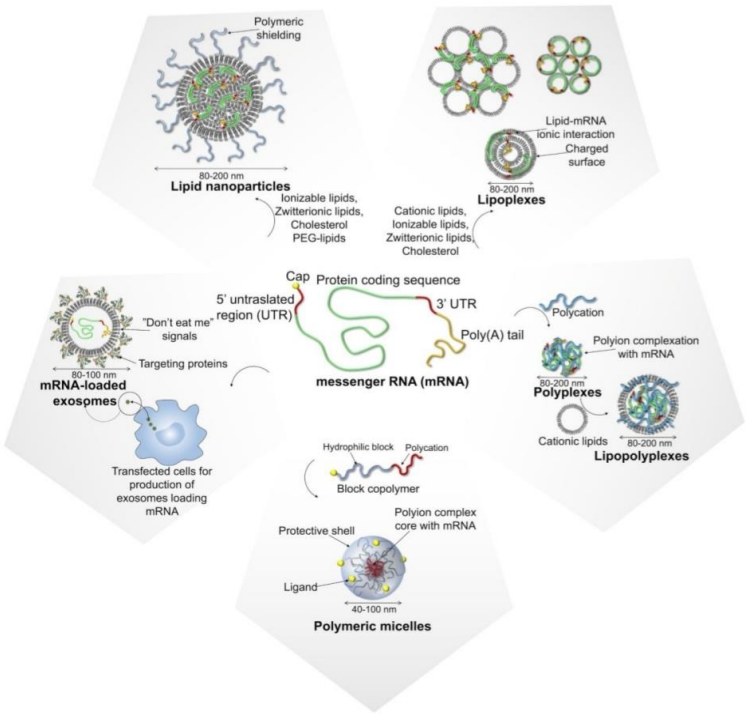

Figure 1. Nanomedicine approaches for mRNA delivery.

A wide range of nano-scaled carriers are under intense investigation for developing mRNA delivery systems.1 Viral vectors, which have been extremely useful for delivering other nucleic acids, have been among the first carriers to be considered for developing mRNA delivery systems.(4) Nevertheless, viral carriers present intrinsic limitations, such as small packing size, immunogenicity, cytotoxicity and complex production processes,(5) which have spurred the development of safe and effective non-viral vehicles. These non-viral vehicles can benefit from a myriad of biocompatible synthetic and natural materials for attaining specific physicochemical and functional features directed to develop mRNA-loaded nanomedicines with improved mRNA bioavailability, targeting to specific tissues and cells, and enhanced cellular uptake and intracellular release of mRNA molecules (Figure 1). Thus, various non-viral strategies have achieved major breakthroughs in the in vivo delivery of mRNA, as well as in the clinical translation of mRNA-based therapies.(1, 6, 7)Here, we present the recent progress in mRNA-loaded nanomedicine toward innovating vaccination, immunotherapy, treatment of genetic disorders and protein replacement approaches. We have focused on the different non-viral strategies with emphasis on the employed materials and the advantages offered by each approach. Moreover, the mRNA modification methods are also reviewed, highlighting opportunities for synergistically enhancing nanomedicine efficiency. Finally, the trends in the application of mRNA-loaded nanomedicines and their future perspectives are discussed.

mRNA design and manufacturing

The development of mRNA-based therapeutics encompasses several key steps, including mRNA design, synthesis, entrapment, pharmacodynamics, pharmacokinetics, safety evaluation in vivo and in vitro, manufacturing, and clinical trials. mRNA design and synthesis are critical stages in the development of mRNA-based medicines. mRNA consists of five functional regions: the 5′ cap, the 3′ poly(A) tail, the open reading frame (ORF) flanking, and the 3′ untranslated regions (UTRs). These regions play a role in mediating the translation efficacy and decay rate of mRNA. Achieving highly biologically active RNA relies heavily on reliable design and preparation. (8) In this section, we will focus on recent advancements and discuss the challenges associated with mRNA design and preparation. Additionally, we will review nucleoside modification and purification, which are commonly employed to meet the diverse demands for mRNA immune-stimulation in various therapies.

The structural elements of mRNA are produced through the transcription process. In eukaryotes, precursor mRNA is synthesized when RNA polymerase converts genes into primary mRNA transcripts in vivo. These primary transcripts typically contain noncoding sequence introns, which are subsequently removed through mRNA processing to yield mature mRNA. mRNA processing involves 5′ mRNA capping,

modifications, splicing, and A-to-I editing. (9) The preparation of mature mRNA for in vitro transcription (IVT) involves several steps, including obtaining a linear DNA template, IVT, 5′ capping, and adding a poly(A) tail. Once the mRNA is introduced into the cell, the poly(A)-binding protein (PABP) binds to the poly(A) tail and interacts with eukaryotic translation initiation factors (e IFs). The interaction between e IFs, the 5′ cap, UTRs, PABP, initiator methionyl transfer RNA (tRNA), and the 40S ribosomal subunit leads to mRNA circularization and the formation of an initiation complex. Following the scanning of the transcription initiation codon by the 40S ribosomal subunit, the 60S ribosomal subunits are recruited, and e IFs are released to initiate amino acid chain extension (10). Mature mRNA comprises the coding region, UTR, poly(A) tails, and the 5′ cap, which can be recognized by ribosomes and transported by tRNA to produce proteins. Similar to DNA, the genetic information in mRNA is contained in the sequence of nucleotides, arranged into codons consisting of three ribonucleotides each.(11) Consequently, IVT mRNA is performed to complete the transcription of RNA in vitro, mimicking the mechanism of eukaryotic mRNA synthesis to ensure mRNA expression in vivo. Hence, the optimization of mRNA is vital for the success of mRNA-based therapeutics.

The 5′ cap is a structure located at the 5′ terminus of mRNA and can have varying degrees of methylation. (12) In eukaryotes, the 5′ cap (m7Gppp) consists of a 7-methylguanosine (m7G) attached to the following nucleotide through a 5′-5′ triphosphate bridge. During translation initiation, the cap interacts with eIF4E through hydrophobic cation-π interactions of m7G and the negative electrostatic charge of the triphosphate bridge. The triphosphate bridge is the main target for cap removal by mRNA decamping enzymes in eukaryotic cells. Dcp1/2 cleave the α- and β-phosphates, while D cp S cleaves the β- and γ-phosphates. To optimize the structure of the mRNA cap, various strategies have been employed to enhance the affinity for eIF4E and reduce susceptibility to decamping enzymes. For example, Rydzik et al. increased cap resistance to decamping by substituting the oxygen atom of triphosphates with dialoging ethylene bisphosphonate. Additionally, modifying the m7G moiety is an important approach to improve mRNA translation. Previous studies have shown that replacing the 7-methylated guanosine (m7G) with 7-benzylated guanosine and attaching another m7G via tetraphosphate (m7Gppppm7G) significantly enhances translation efficiency. (13) These analogs have a higher affinity for eIF4E compared to natural eukaryotic 5′ caps.(14) The bridged oxygen atoms between α-β or β-γ phosphates were replaced with methylene to achieve this improvement.1. To avoid degradation of mRNA by Dcp1/2 or D cp S, modifications such as introducing dithiodiphosphate to the tri- or tetraphosphate bridge can be done. This modification reduces cap sensitivity to Dcp1/2 and improves mRNA translation. Additionally, the use of phosphonothioate cap analogs can increase the stability and translational efficiency of RNA vaccines in immature dendritic cells. It is important to note that the position of the phosphonothioate substitution is sensitive and may be associated with stereochemistry in catalysis (15).

mRNA Nanomedicine:

One of the major obstacles in using nucleic acid medicinal products in clinical settings is the need for delivery systems that are tailored to their unique characteristics and intended use. For mRNA therapy, the delivery vehicle must not only shield the nucleic acid and ensure it reaches the desired cell, but also facilitate its intracellular placement for effective translation, all while avoiding triggering an immune response (16) At present, the majority of clinical trials involving nucleic acids employ recombinant viruses as carriers. These carriers include retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses, among other options. (17) Nano-scaled carriers are essential for the successful application of mRNA molecules in vivo, as they enable the molecules to withstand harsh environments and facilitate their delivery into cells. Different materials have been utilized to construct nanomedicines loaded with mRNA, enabling precise control over their physicochemical properties and functions. Through careful engineering of the interaction between mRNA and the carrier components, nanomedicines can be developed to enhance mRNA stability in biological settings, achieve optimal translation in specific cells for therapeutic purposes, and minimize side effects and off-target translation (18).

The incorporation of nanomedicines with charged surfaces into biological media such as systemic circulation, interstitial fluid, and lymph can lead to changes in their expression. This can result in alterations to their cellular uptake, pathway for attacking target sites, and other effects, which may either accelerate or retard their effectiveness.

Nanomedicine approaches for mRNA delivery:

Nano-scaled carriers are essential for the successful application of mRNA molecules in vivo, as they enable the molecules to withstand harsh environments and facilitate their delivery into cells. Different materials have been utilized to construct nanomedicines loaded with mRNA, enabling precise control over their physicochemical properties and functions. Through careful engineering of the interaction between mRNA and the carrier components, nanomedicines can be developed to enhance mRNA stability in biological settings, achieve optimal translation in specific target cells for therapeutic purposes, and minimize side effects and off-target translation. (20) The utilization of mRNA for therapeutic purposes in the absence of a delivery system has significant limitations. The administration of naked mRNA has primarily been carried out ex vivo through physical techniques such as electroporation, microinjection, and gene gun. These methods are capable of disrupting the cell membrane and facilitating the entry of mRNA into the cell (21).

Nowadays, pharmaceutical research for mRNA delivery is heavily focused on chemical nanocarriers. The progress of nanotechnology, material sciences, and nucleic acid chemistry has led to extensive research in developing new systems (22). However, it is important to note that a universal delivery system cannot be expected as mRNA carriers must be specifically designed for individual disease conditions. Chemical nanocarriers are composed of synthetic or natural biocompatible components that form complexes with the mRNA. They vary in composition, size, shape, and physico-chemical characteristics, and must protect the nucleic acid from degradation and denaturation while facilitating the transfection process. Additionally, they should be minimally toxic and avoid immunological responses.

1. Lipid-Based Delivery Systems

Cationic lipids are the primary component of lipid-based vectors, which are commonly used as non-viral carriers for nucleic acids. These lipids interact with mRNA through electrostatic interactions, resulting in the formation of a complex known as a lipoplex. The first synthetic cationic lipid used to complex IVT mRNA was DOTMA, which was able to deliver nucleic acid in vitro to human, rat, mouse, xenopus, and drosophila cells. DOTAP, a more cost-effective synthetic lipid derived from DOTMA, has also been frequently used in combination with DOPE to prepare colloidal systems that can bind nucleic acids. This mixture facilitates endosomal escape under acidic pH conditions, thanks to DOPE's ability to modify the lipoplex from a bilayer model to hexagonal phase II structures, which induce supramolecular arrangements resulting in the fusion of lipid bilayers. DOTAP alone or in combination with DOPE has been applied to mRNA delivery.(21) LNPs, which have been extensively, are considered to be highly advanced mRNA delivery systems. The preparation of LNPs involves the microfluidic mixing of lipids in ethanol and mRNA in an acidic buffer with a pH range of 3.0-4.0. These lipids consist of an ionizable lipid with a p Ka value below 7, which undergoes protonation at acidic pH levels to condense the mRNA and facilitate its release inside the cells. Additionally, cholesterol is included in the formulation to provide stabilization, while a helper lipid (typically DOPE) aids in the escape of LNPs from endosomes. Furthermore, a PEGylated lipid is incorporated to prevent the aggregation of LNPs. By utilizing an ionizable cationic lipid for mRNA complexation instead of a permanently charged one, the toxicity in vivo is minimized, and the half-life of the mRNA is increased. The rapid mixing process results in the formation of an electron dense core surrounded by a lipid monolayer. Subsequently, dialysis against a neutral buffer is performed to eliminate ethanol from the formulation. (20)

In recent years, alternative ionizable lipids with lower toxicity have been developed to maintain transfection capacity. Examples of these lipids include 1,2-dioleoyl-3-dimethylammonium propane (DODAP) and 1,2-dioleyloxy-N, N-dimethyl-3-aminopropane (DODMA) (6). Unlike conventional

cationic lipids, which have alkylated quaternary ammonium groups and remain cationic regardless of pH, ionizable lipids acquire positive charges through the protonation of free amines as pH decreases (24). These new lipids are neutral at physiological pH but become positively charged inside the endosome when pH values are below their pKa. The interaction between naturally occurring anionic lipids in endosomal membranes and ionizable cationic lipids is believed to be the underlying mechanism for nucleic acid release (25). These interactions can lead to the formation of membrane lytic nonplayer structures, such as the hexagonal HII phase, ultimately facilitating intracellular mRNA delivery (26). Currently, nanocarriers containing ionizable cationic lipids are considered promising delivery systems for siRNA and mRNA (27).

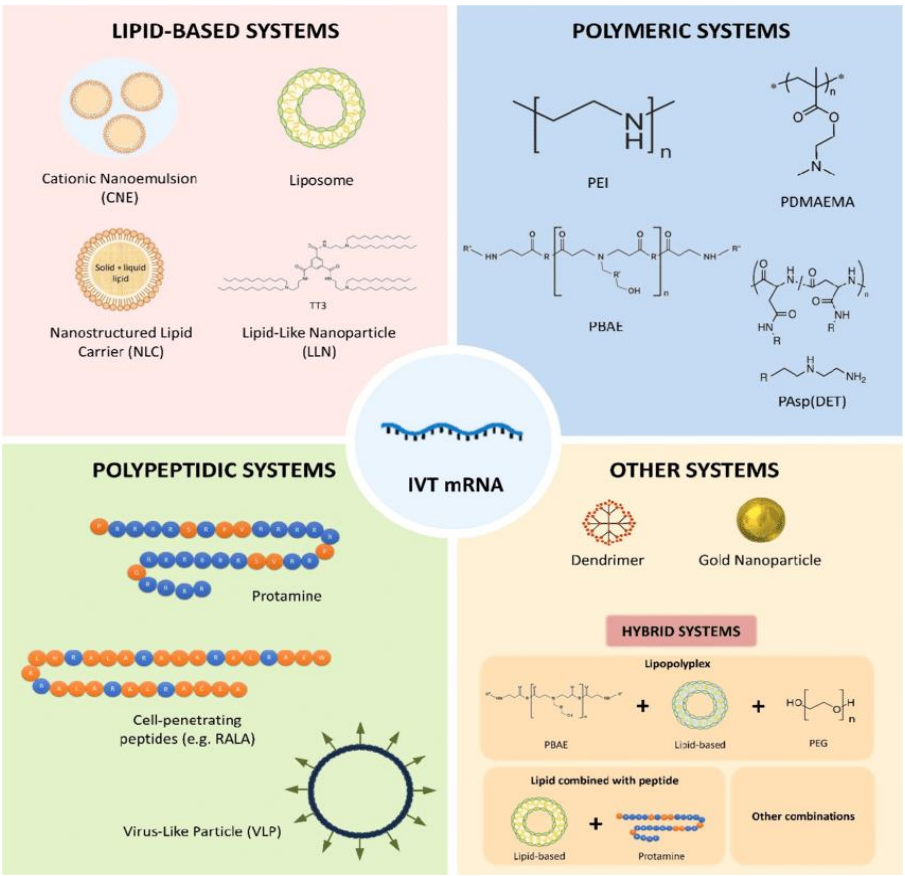

Figure 2. Representative scheme of chemical Nanocarriers for m RNA delivery.

Liposome-based formulations are spherical vesicles that are amphiphilic in nature. These vesicles are formed by one or more lipid bilayers that envelop an aqueous core. The size of these vesicles can range from 20 nm to a few microns. Typically, these formulations contain a cationic lipid along with a helper lipid that supports the bilayer structure and facilitates endocytosis. Additionally, cholesterol is added to stabilize the lipid bilayer of the LNP. A PEG-lipid is also included in the formulation, which provides the nanoparticle with a hydrating layer. This layer helps to improve colloidal stability, reduce protein adsorption and non-specific uptake, and prevent reticuloendothelial clearance. (28)

2. Polymeric system:

Studies have extensively explored the use of polymer nanoparticles for p DNA delivery, but only a few have investigated their potential for mRNA delivery. One of the main benefits of polymeric systems is their ability to modify their chemical properties to suit the active substance. The binding of cationic polymers and nucleic acids results in the formation of polyplexes (28). Various cationic polymers, such as PEI, polyacrylates, PBAEs, and P Asp, have been studied for mRNA complexation. However, despite the availability of numerous polymeric materials, lipidic systems are more clinically advanced for mRNA-based therapies. PEI, which contains a significant number of amine groups in its structure, was one of the first polymers used for nucleic acid delivery and can have both a linear and branched conformation. At physiological pH, Linear PEI has secondary amino groups that are partially protonated, while Branched PEI has primary and secondary groups, as well as a small number of tertiary amines. The presence of amino groups is what gives these polymers a strong affinity for nucleic acids, including mRNA. The cationic charges on the polymers also help them interact with the cell membrane and enter the target cell. Additionally, the amino groups allow for ionization and confer high buffering ability. This buffering capacity, which is still being debated, is due to the "proton sponge effect" and enables the swelling and rupture of endosomes by changing the osmolarity of acidic vesicles.(16))

However, the presence of a high concentration of positive charges is also associated with in vivo toxicity due to its interactions with proteins both inside and outside the cells, destabilization of lipid membranes, and activation of the immune system (29).To address this issue, new derivatives of PEI have been developed to enhance its biocompatibility and transfection efficiency. For instance, jet PEI®, a linear PEI that is commercially available as an in vivo transfection reagent for mice, was initially evaluated for transfecting p DNA and siRNA, and later for delivering mRNA (30).More recently, the direct myocardial injection of IVT mRNA with jet PEI® in mice demonstrated protein expression in the lungs (31).Polyacrylates have also been utilized for mRNA delivery, albeit with modifications in the side chain to enable electrostatic interaction with nucleic acids. One extensively studied polyacrylate is poly(2-dimethylaminoethyl methacrylate) (PDMAEMA), which exhibits lower affinity for mRNA compared to p DNA. However, its PEGylation enhances mRNA binding and transfection efficiency (32). The development of triblock copolymers, achieved through modifications in the PDMAEMA structure, has shown improved transfection efficiency in both siRNA and mRNA systems. These modifications include: (1) the incorporation of amphiphilic materials like PEG methacrylate (PEGMA) to enhance stability and biocompatibility; (2) the addition of a hydrophobic butyl methacrylate segment (BMA) to promote fusogenicity and (3) the inclusion of a pH-responsive diethyl aminoethyl methacrylate (DEAEMA) to disrupt endosomal membranes (16). Polymers have a wide range of applications in the pharmaceutical

industry. They are used as adjuvants, suspending and emulsifying agents, flocculating agents, adhesives, packaging materials, and coating materials. In the past, only polymers of natural origin were used in pharmaceutical laboratories. However, with the development of synthetic drugs, there has been an increase in the availability of synthetic polymers suitable for use in pharmacy. Recent advancements in the field of pharmaceutics have led to the discovery of new compounds and techniques for preparing polymers. This account will focus on these developments and their applications in various pharmaceutical fields. The synthesis of custom polymers holds great potential, especially in the creation of new polymers for drug delivery devices, which are essential for the effective use of potent and toxic drugs. High polymers are characterized by their high molecular weight and unique properties, which are influenced by their size and asymmetry. The reactivity of polymers is determined by the chemistry of their monomer units, while their physical chemistry is largely influenced by the arrangement of these monomers. Synthetic polymers can have linear or branched chains, and these chains can be joined by cross-links. Extensive cross-linking results in a three-dimensional polymer network that is often insoluble. Copolymers, which involve more than one type of monomer, can have different arrangements of monomers A and B, leading to variations in their physical properties. Polymers with symmetrical chains and strong interchain forces can be drawn into fibers, while plastics, with lower degrees of crystallinity, can be molded. Rubbers and elastomers, on the other hand, have properties that make them flexible and elastic.

3. Polypeptide Systems:

Polypeptides are composed of one or multiple amino acids arranged in either block or random sequences. These polypeptides possess biocompatibility and physicochemical properties that contribute to the effectiveness of delivery systems. This is made possible by the presence of naturally occurring biodegradable monomeric units. Additionally, polypeptides have the ability to adjust their cationic and endosomatic properties due to their structural flexibility (16).

Protamine’s, which are small peptides derived from fish sperm, belong to a family of peptides with arginine-rich sequences. The presence of arginine’s imparts a positive charge to protamine’s, enabling electrostatic interactions with the negatively charged nucleic acids. In fact, protamine was discovered over 50 years ago as a facilitator of RNA uptake (35). The condensation of mRNA with protamine provides protection against ribonuclease degradation, and the resulting complex can activate TLRs, serving as danger signals that are beneficial for vaccination (36). Protamine-based formulations for intravenous transfusion (IVT) mRNA delivery are the second most commonly used chemical systems in clinical trials, although they are not as prevalent as lipidic systems. CureVac's RN Active® technology, which is a platform for mRNA vaccines based on

protamine/mRNA complexes, is currently undergoing clinical evaluation for its effectiveness against rabies(34),, as well as various types of cancer (37).Furthermore, the RN Active® platform has been tested in preclinical studies for its potential in combating influenza virus infection (38).

Cell-Penetrating Peptides (CPPs) have been utilized for delivering nucleic acids into cells, owing to their ability to traverse cell membranes. Although the precise mechanisms of cellular internalization are not yet fully understood, it is believed that CPPs may induce the clustering of negatively charged glycosaminoglycans on the cell surface, leading to micropinocytosis and lateral diffusion, or directly disrupting the lipid bilayer. An arginine-rich amphipathic RALA motif-containing cationic CPP was employed as an mRNA carrier for DCs. The nanocomplexes of RALA with Ψ and m5C modified IVT mRNA were found to elicit robust cytolytic T cell responses against the antigenic mRNA payload (39).

Virus-like particles (VLPs) composed of artificial viral coat proteins have been utilized as carriers for transfection purposes, primarily due to their ability to assemble and safeguard mRNA. In a study conducted by Li et al. monocytes(40), an mRNA vaccine was developed for prostate cancer treatment using recombinant bacteriophage MS2 VLPs. This vaccine successfully elicited robust humoral and cellular immune responses, effectively protecting mice against prostate cancer. Another research project involved the creation of an artificial viral coat protein, which consisted of an oligomycin (K12), a silk protein-like midblock S10, and a long hydrophilic random coil block C. This protein was then complexed with mRNA to form rod-shaped VLPs. Although this system was able to transfect cells with both EGFP and luciferase, its efficacy was comparatively lower than that of a lipoplex transfection reagent (41). More recently, VLPs were prepared by fusing protein G of Vesicular stomatitis virus (VSV-G) with a ribosomal protein L7Ae of Archeoglobus fulgidus. These VLPs demonstrated efficient delivery of EGFP in human induced pluripotent stem cells (iPSCs) and monocytes .

Dendrimers:

Dendrimers are macromolecules that have highly branched structures with uniform sizes and shapes, as well as adaptable surface functionalities. They consist of a central core, repetitive branching units, and terminal groups(43). Modified dendrimers, which are derived from polyamidation (PAMAM), have been extensively studied for their hydrophilic, biocompatible, and non-immunogenic properties, particularly in nucleic acid delivery. Chahal et a(44)l. developed a vaccine based on a PAMAM dendrimer formulated in nanoparticles, which generated protective immunity against lethal Ebola, H1N1 influenza, and Toxoplasma gondii challenges in mice after a single dose. In a later study, the same dendrimer-based nanoparticle was used to create a vaccine candidate that elicited Zika virus E protein-specific IgG responses and a CD8+ T cell response against a unique H-2Db-restricted epitope.

Gold nanoparticles:

Gold nanoparticles (AuNPs) possess unique characteristics that make them an ideal platform for delivering nucleic acids. They can be produced on a large scale with minimal size variation and can be easily modified by attaching multifunctional monolayers and incorporating various molecules and targeting agents. Additionally, the toxicity and distribution of AuNPs in living organisms can be controlled by optimizing their size and surface properties (46) In a study conducted by Yeom et al.(47), IVT mRNA encoding the Bcl-2-associated X (BAX) protein, a pro-apoptotic factor, was injected into xenograft tumors in mice using AuNP-DNA conjugates. The released mRNA successfully produced BAX protein, leading to the inhibition of tumor growth.

Hybrid system:

Hybrid systems consist of a combination of different materials, such as lipids, polymers, and peptides, among others. This allows the system to benefit from the unique advantages of each component, resulting in increased functionality and flexibility. This could potentially lead to easier clinical translation in the future. However, optimizing the combination of such diverse components poses a significant challenge in terms of scaling-up and clinical utility(16).

One commonly studied combination is cationic lipids and peptides for mRNA delivery. For instance, complexes formed by IVT mRNA, protamine, and liposomes have shown promising results in in vivo protein expression, activation of cytotoxic T lymphocytes, and production of IgG antibodies against the antigen. In a separate study, administering lipid/protamine/IVT mRNA to mice with human lung NCI-H460 carcinoma demonstrated better efficacy and toxicity results compared to the equivalent formulation with p DNA.(48)

Lip polyplexes, which involve the combination of cationic polymers and lipids with nucleic acids, were one of the earliest hybrids used for delivering DNA, siRNA, and later on, mRNA. Recently, biotinylated lip polyplexes have been synthesized by combining a PEGylated derivative of histidylated polyline with L-histidine-(N,N-di-n-hexadecyl amine) ethylamine liposomes, which incorporate a synthetic melanoma-associated antigen MART1 mRNA. These lip polyplexes were administered to mice as an mRNA cancer vaccine and were found to significantly protect against B16F10 melanoma tumor progression. The system was further modified by aminoxylation, which targeted the mRNA into the DCs by the mannose receptor.(49)

A platform based on nano micelles was created by combining IVT mRNA encoding an anti-angiogenic protein (sFlt-1) with PEG-polycation block copolymers. The cationic segment of the block copolymer was P Asp(TEP), and a cholesterol moiety was attached through hydrophobic interaction. The

nano systems of PEG-PAsp(TEP)-cholesterol showed effective protein expression in tumor tissue and significant inhibition of tumor growth (50).

Another instance of a multi-component delivery system involves poly(glycoamidoamine) (PGAAs) brush nanoparticles. These nanoparticles were utilized for intravenous administration of mRNA encoding erythropoietin (EPO) in mice (51). Initially, three different PGAA polymers were prepared using tartarated, galactarate, or glycerate sugars combined with three different amine-containing monomers. Polymer-brush materials were synthesized by reacting PGAAs with epoxides through ring opening reactions and then incorporated into LNPs. Cholesterol, DSPC, and mPEG2000-DMG (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000]) were added to the LNPs via a microfluidic-based mixing device to form the LNP polymer-brush nanoparticles.

In recent developments, DCs have been transfected with an mRNA delivery system that combines PLA-based micelles and the cationic CPP RALA. This hybrid nanoplatform offers the potential for additional multifunctionality through PLA core encapsulation.

Nanomedicine based approaches for mRNA delivery.

Category	Key component (s)	Core technologies	Size (nm)	Delivery route(s)	Gene(s)
	DLin-MC3-DMA[a)]	Clinically approved ionizable lipid	75-85	i.v., i.c.v. (mouse)	Luc, frataxin
	Lipid 5	Optimized ionizable lipids	86	in vitro, i.v. (mouse, rat, NHP)	Luc, EPO, IgG
	TT3[a)]	Orthogonal array optimization	110	in vitro, i.v. (mouse)	Luc, hFIX

Lipid-based nanoparticles	A18-Iso5-2DC18[a)]	cyclic head group in ionizable lipid for STING activation	96	in vitro, s.c.	Luc, Cre, OVA, tumor antigens
	C12-200[a)]	A large-scale *in vivo* screening of mixture ratio	152	i.v. (mouse)	Luc, EPO
	SORT lipid (DLin-MC3-DMA[a)], C12-200[a)], 5A2-SC8[a)], DOTAP[a)],18 PAa),etc.)	Addition of cationic, anionic and ionizable lipid to change tissue tropism	70-200	i.v. (mouse)	Cre, EPO, IL- 10, Cas9
	C-24 alkyl phytosterols[a)]	Optimized cholesterol	102	in vitro	Luc, GFP, Cas9
	5A2-SC8[a)]	Dendrimer LNP	100	in vitro, i.v. (mouse)	Luc, FAH
	PEtOx-PEI	Optimization of Mw and charge	80-450	in vitro	Luc

Polyplexes		density			
	hyperbranched PBAE	Hyperbranched polycation for nebulization.	150	inhalation (mouse)	Luc, GFP, Cre
	Di mannose-PGA/PBAE	Macrophage targeting	100	in vitro, i.p., i.v. (mouse)	IRF5, IKKβ
	PA sp (EDA), PA sp (TET)	Polyplex stabilization	82, 93	in vitro	Luc
	PA sp (TET), PA sp (TEP)	Efficient translation	62, 51	in vitro	Luc
	PA sp (DPT), PA sp (TET)	Pre-complexation with eIF4E to facilitate translation	100	in vitro, i.v. (mouse)	Luc
Lipopolyplexes	HpK-PEG + HDHE/chol	Protonable histidylated lipids	100	i.v. (mouse)	MART1
	PEI + Chol/DOPA/DOPE/DSPE-PEG/Man-lip	Neutral and mannosylated LPP	160-190	i.m., i.v. (mouse)	Luc, HA

	HpK-PEG + KLN25/MM27	Mannosylated and histidylated lipids	100-140	i.v. (mouse)	MART1
	HpK-PEG +	Histidylated lipids and a tri-	100-	i.v. (mouse)	E7
	KLN25/MM27/TriMan-lip	mannosylated diether lipid	150		
	PBAE + EDOPC/DOPE/DSPE-PEG	Biodegradable polymer	n.d.	s.c. (mouse)	OVA
	PBAE + PEGylated DD90-C12-122	Alkyl chain installation to PBAE for anchoring PEG-lipid	190	i.v. (mouse)	Luc
	TT3[a)], PLGA	Optimal PLGA formulation for potentiate TT3LNP	100-250	in vitro	Luc, GFP
	PEG-PA sp (DET)	Alleviation of mRNA immunogenicity	50	in vitro, i.c., i.n., HTVi (mouse, rat)	Luc,GFP,BDNF,Bcl-2,EPO
	PEG-PA sp (TET)-Chol	Micelle stabilization using	56	in vitro, i.v. (mouse)	Luc, sFlt-1

Polymeric micelles		cholesterol			
	PEG-PA sp (DET)-Chol/Chol-mRNA	Introduction of cholesterol to mRNA for stabilization	60	in vitro, i.t. (mouse)	Luc
	C RGD-PEG /PNI PA M-Plys (SH)	Tumor targeting; hydrophobic layer and disulfide crosslinking for stabilization	59	in vitro, i.v. (mouse)	Luc, GFP
	PEG-Plys (AMP)	Disulfide crosslinking and polycation optimization for stabilization	53	in vitro	Luc
	PEG-PGBA	Flexible polycation for stabilization	56	in vitro, i.t., i.v. (mouse)	Luc
	HEK293T-derived exosome	miRNA-responsive translation system	70-220	i.v. (mouse)	Luc,PGCl α

Exosomes	Exosome possessing glioma-targeting peptides	microfluidic cellular nonoperation biochip with electrical pulses to increase exosome production.	30-170	i.v. (mouse)	PTEN

Therapeutic Application of mRNA

The expanding knowledge in the design and production of IVT mRNA, coupled with advancements in nanotechnology, has led to the broadening of potential therapeutic applications for mRNA-based medicines. Based on preclinical and clinical trials, there are four main applications for IVT mRNA: immunotherapy (targeting infectious diseases and cancer), protein replacement, gene editing, and regenerative medicine. Currently, ongoing clinical trials, utilizing both in vitro and in vivo approaches, are primarily in Phase I or II, with a focus on immunotherapy, particularly in cancer treatment. However, for the successful transition of mRNA from clinical studies to commercialization as medicinal products, there is still a crucial consideration regarding the manufacturing of large industrial batches. This involves optimizing the production and purification processes of IVT mRNA, with the aim of reducing costs (16).

Cancer Immunotherapy:

Cancer immunotherapy relies on the development of a host immune response against tumors, with cytotoxic T cells playing a crucial role due to their ability to identify and eliminate tumor cells. The initiation of a specific immune response through mRNA vaccines occurs when the antigen is expressed within antigen-presenting cells (APCs) such as dendritic cells (DCs) or macrophages in the cytosol. These antigens are then processed by proteasomes and presented on MHC class I molecules to activate CD8+ T cells, leading to a cellular immune response. Additionally, CD4+ T helper cell responses can be stimulated by incorporating trafficking signals from lysosomal proteins found in MHC class II processing compartments into the encoded antigen. Cancer mRNA vaccines offer significant advantages. Firstly, they allow for the amplification of patient-specific antigens by obtaining RNA from a tumor sample, resulting in large quantities of personalized antigens. Secondly, mRNA can act as an adjuvant by providing costimulatory signals, such as through TLR3, TLR7, and TLR8TLR8(21).

Immunotherapy involves the use of mRNA to induce an immune response, with mRNA vaccines being evaluated in clinical trials against infectious diseases and various types of cancer. A new application of mRNA-immunotherapy has emerged, demonstrating proof of concept for the prevention of

type 1 diabetes in mice by administering modified T cells redirected against diabetogenic CD8+ T cells. (53) Passive immunization through mRNA encoding monoclonal antibodies is also gaining interest in the biomedical field. This approach is considered a good alternative to therapeutic monoclonal antibodies due to its simpler, faster, and more cost-effective synthesis. Pre-clinical studies in small rodents have shown promising results, with antibody titters detected from the first day after mRNA intravenous administration. (54)

Protein Replacement therapy:

Protein replacement therapy, also known as mRNA delivery, has been extensively utilized alongside vaccines to supply therapeutic peptides or proteins. In 1992, the concept of in vivo protein replacement therapy was introduced when vasopressin mRNA was injected into the hypothalamus of model rats lacking vasopressin expression to treat diabetes insipidus. However, despite this early breakthrough, the focus of mRNA application shifted towards vaccines and immunotherapy, with only a few studies exploring its potential for protein replacement. One of the main challenges in mRNA-based protein replacement is managing mRNA immunogenicity, as inflammatory responses following mRNA delivery can hinder the therapeutic process and raise safety concerns. This stands in stark contrast to vaccines and immunotherapy, where the induction of innate immune responses is crucial for achieving preventive or therapeutic outcomes. To address this issue, various techniques have been developed to control mRNA immunogenicity.

The most commonly used method for reducing the immunogenicity of mRNA involves replacing mRNA nucleosides with chemically modified species like m5C and t2C. This approach inhibits the recognition of mRNA by innate immune receptor 54 and 110. Additionally, high performance liquid chromatography (HPLC) purification has been reported to effectively reduce mRNA immunogenicity by removing dsRNA contaminants. Fine-tuned designing of mRNA carriers can also inhibit mRNA recognition by immune receptors. Encapsulation of mRNA into PEGylated polymeric micelles (PMs) suppresses its recognition by TLR7 in endosomes, presumably through the steric repulsive effect of PEG. This leads to efficient in vivo delivery of mRNA with minimal inflammatory responses. It is worth noting that mRNA PMs have shown successful outcomes in tissue regenerative treatment for several diseases in model animals, such as osteoarthritis, intravertebral disc disease, and spinal cord injury, even without mRNA chemical modification and HPLC purification. This immune regulation approach is practically important because mRNA chemical modification often leads to impaired translational activity, compromising the therapeutic potential of mRNA (20).

Regenerative medicine and cell engineering:

The field of regenerative medicine is focused on restoring or replacing damaged or lost cells, tissues, or organs by promoting their normal function. This process relies on the activity of proteins like growth factors, cytokines, and transcription factors, which control cellular processes such as cell division, movement, and specialization. Reprogramming and trans differentiation of somatic cells into specific lineages are among the strategies used to repair tissue deficits in patients. However, the delivery of transcription factors to cells is limited by the plasma membrane, and the use of p DNA to express them carries the risk of mutagenesis. IVT mRNA encoding transcription factors has been explored as a promising alternative for somatic cell transfection, although research in this area is still in its early stages and limited to preclinical in vitro studies.

The first study on in vitro reprogramming of somatic cells using IVT mRNA was reported in 2010. Synthetic mRNA, encoding four transcription factors (Oct4, Sox2, Lin28, and Nanog), was delivered to human foreskin fibroblasts to generate iPSCs. Another study in the same year utilized IVT mRNA encoding a mix of transcription factors (Oct4, Sox2, Klf4, and c Myc), known as Yamanaka factors, to obtain iPSCs from fibroblasts. Additionally, in this study, the iPSCs were further differentiated into myofibers after transfection with myogenic differentiation factor (MyoD) IVT mRNA. (21)

Gene editing:

Gene editing has emerged as a novel therapeutic option for a wide range of clinical conditions in recent times. This cutting-edge technology utilizes programmable nucleases that are designed to create a DNA double stranded break (DSB) at a specific location in the genome. The mending of these DSBs can be achieved through two mechanisms: Homologous-dependent repair (HDR) and non-homologous end joining (NHEJ). In HDR, the nucleases function alongside a donor DNA template that contains a homologous sequence to be inserted into the DSB. This repair mechanism is valuable for rectifying genomic mutations or introducing new sequences that encode therapeutic proteins. On the other hand, NHEJ eradicates the target region by binding the DSBs, making it a useful tool for silencing or correcting abnormal genes. (55) There exist three primary categories of gene editing nucleases, consisting of a target-sequence-recognizing domain and a nuclease: ZFNs, TALENs, and CRISPR/Cas9.

Gene editing nucleases can be administered in different forms such as protein, pDNA, or mRNA. The use of mRNA as a delivery method holds significant potential for therapeutic purposes. When compared to pDNA, mRNA reduces the risk of genome insertion and offers a transient effect, thereby limiting the presence of the nuclease inside cells and the potential for off-target adverse effects. In contrast to protein delivery, the presence of nucleases within cells is more consistent when delivered through mRNA expression rather than directly administering the nuclease itself. Furthermore, the administration of

nucleases in protein form poses several limitations for in vivo therapy, including cellular and humoral immune responses, challenges in systemic administration due to its charge and large size, and complex protein purification procedures for large nucleases. (21)

Future perspective:

The use of mRNA therapeutics in clinical applications is steadily progressing. One area that has seen significant advancements is cancer vaccination, with the first clinical trials being reported in 2002.301 Targeting neoantigens, which are specific to cancer cells, offers a promising approach to enhance the effectiveness of vaccines by bypassing immune tolerance mechanisms. A recent clinical trial successfully demonstrated this concept by utilizing mRNA that expressed neoantigens identified through deep sequencing of cancer samples from individual patients. With ongoing advancements in mRNA vaccine technologies, such as RNA design, administration routes, and delivery systems, further enhancements in therapeutic outcomes can be anticipated. This personalized approach to therapy has the potential to revolutionize the methodology of cancer treatment (20).

Conclusion:

Considerable efforts have been dedicated to addressing the challenges associated with mRNA therapeutics, such as immunogenicity, short protein expression duration, rapid enzymatic degradation, and limited cellular uptake. These challenges have been tackled through two distinct approaches: mRNA designing and nanomedicines. By working together, these approaches enhance mRNA delivery and minimize safety concerns. These advancements in technology have paved the way for a wide range of therapeutic applications of mRNA, including vaccinations, protein replacement therapy, cancer treatment, and cell reprogramming. With further advancements in delivery systems and careful selection of target diseases, mRNA nanomedicines hold the potential to overcome various medical challenges that cannot be effectively addressed through conventional methods, while also promoting innovative therapeutic strategies.

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