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REVIEW

Recent Advances in Messenger Ribonucleic Acid (mRNA) Vaccines and Their Delivery Systems: A Review

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Abstract: Messenger ribonucleic acid (mRNA) was found as the intermediary that transfers genetic information from DNA to ribosomes for protein synthesis in 1961. The emergency use authorization of the two covid-19 mRNA vaccines, BNT162b2 and mRNA-1273, is a significant achievement in the history of vaccine development. Because they are generated in a cell-free environment using the in vitro transcription (IVT) process, mRNA vaccines are risk-free. Moreover, chemical modifications to the mRNA molecule, such as cap structures and changed nucleosides, have proved critical in overcoming immunogenicity concerns, achieving sustained stability, and achieving effective, accurate protein production in vivo. Several vaccine delivery strategies (including protamine, lipid nanoparticles (LNPs), polymers, nanoemulsions, and cell-based administration) were also optimized to load and transport RNA into the cytosol. LNPs, which are composed of a cationic or a pH-dependent ionizable lipid layer, a polyethylene glycol (PEG) component, phospholipids, and cholesterol, are the most advanced systems for delivering mRNA vaccines. Moreover, modifications of the four components that make up the LNPs showed to increase vaccine effectiveness and reduce side effects. Furthermore, the introduction of biodegradable lipids improved LNP biocompatibility. Furthermore, mRNA-based therapies are expected to be effective treatments for a variety of refractory conditions, including infectious diseases, metabolic genetic diseases, cancer, cardiovascular and cerebrovascular diseases. Therefore, the present review aims to provide the scientific community with up-to-date information on mRNA vaccines and their delivery systems.

Keywords: mRNA vaccines, in vitro transcription, lipid nanoparticles, transfection efficiency

Introduction

About This Review

Currently, there is considerable research being done on messenger ribonucleic acid (mRNA) vaccines to treat cancer, infectious diseases, gene therapy, and other disorders. The favorable safety and efficacy of the already developed and authorized BNT162b2 and mRNA-127 Covid-19 vaccines have boosted the promise for future vaccines to be based on mRNA. The high molecular weight and negative charge of mRNA vaccines, their susceptibility to ribonucleases, and the existence of intracellular and extracellular barriers are only a few of the hurdles they face despite their many benefits. To overcome these challenges, chemical modifications to the mRNA molecule, such as cap structures and modified nucleosides, and using novel drug delivery systems are crucial. Hence, this review provides updated information on mRNA vaccines overview, in vitro transcription method, the role of structural elements of mRNA vaccines, challenges and adverse effects of mRNA vaccines and methods how to overcome the challenges, mRNA vaccine delivery systems, entry, and endosomal escape of nanoparticle construct of mRNA, routes of mRNA vaccine administration and application of mRNA vaccines in different diseases (disorders).

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mRNA Vaccines Overview

Vaccines are classified as whole-pathogen, subunit, nucleic acid, or viral vector based on the antigen used in their synthesis.[1] In 1961, messenger ribonucleic acid (mRNA) has been identified as the mechanism by which genetic information is passed from DNA to ribosomes to produce proteins.[2] Moreover, mRNA vaccines can be synthesized in a laboratory using easily accessible components.[3,4] However, because of the extremely unstable nature of the mRNA molecule, the first protein was synthesized in vitro from isolated mRNA in 1969.[5,6]

In 1987, researchers devised a highly successful approach for in vitro mRNA production by encapsulating mRNA in cationic lipids and injecting it into eukaryotic cells.[4] Furthermore, activating anti-influenza cytotoxic T lymphocytes (CTLs) in the host after immunizing mice with liposome-encapsulated mRNA expressing the influenza-virus nucleoprotein (NP) marked a significant step forward in the development of the first mRNA vaccine.[7] Furthermore, Pfizer-BioNTech's (BNT162b2) and Moderna's (mRNA-1273)[8,9] mRNA vaccines have both been granted emergency use authorizations (EUA).[10] These vaccines have been a huge success, with excellent protective effectiveness of more than 90%.[11]

Why mRNA Vaccines are Preferable to DNA Vaccines?

In comparison to DNA, mRNA therapy has numerous benefits. While viral vectors are needed for high transfection efficiency in DNA treatments, non-viral vectors (such as lipids and polymers) can be used for mRNA delivery and still achieve extremely strong transfection efficiency.[12–18] Because they are generated in a cell-free environment via in vitro transcription, mRNA vaccines are also harmless. MRNA cannot induce vector- or carrier-specific immunogenicity, unlike viral vectors or virus-like particles (VLPs).[15,16,19]

Antigens encoded in mRNA vaccines can be expressed more expeditiously since mRNA can be functional in the cytoplasm whereas DNA must enter the nucleus and be transcribed before proteins can be created.[20] In cells, mRNA undergoes a series of molecular changes, such as deadenylation and decapping, before being hydrolyzed by RNase.[21] These processes ensure that exogenous mRNA treatments are only expressed briefly, which makes it to be safer.[22]

In vitro Transcription (IVT) Method and Role of Structural Elements of mRNA Vaccines

In vitro Transcription (IVT) Method

The in vitro transcription (IVT) technique is used to synthesize self-amplifying mRNA (saRNA) and conventional mRNA in a cell-free system. In this approach, the production of plasmid DNA (pDNA) carrying the sequence for a DNA-dependent RNA polymerase promoter (T7 or SP6), followed by the sequence matching to the mRNA construct is required.[14,23–28] T7 RNA polymerase can accurately integrate pseudouridine triphosphate and other modified nucleotides and make RNAs longer than 20,000 nucleotides.[29–31]

The pDNA can act as a template for mRNA transcription utilizing a DNA-dependent RNA polymerase after being linearized by an enzyme. After the transcription reaction is finished, the pDNA is treated with DNase to degrade it. Enzymatic post-transcription capping and simultaneous capping by an extra cap analog in the transcription mixture are the two main capping techniques used in IVT reactions.[32] Moreover, guanylyl transferase and 2′-O-methyltransferase can be employed to introduce a Cap 0 (N7MeGpppN) or Cap 1 (N7MeGpppN2′-OMe) structure, respectively.[14,23,24]

Poly-A-tailed IVT mRNA is typically produced in two ways. One method involves attaching a poly-A tail to the 3' end of IVT mRNA after recombinant poly-A polymerase has synthesized the mRNAs. This method creates a varied length of poly-A and has less reliable batch controls, making it challenging to meet the standards.[33] The second technique involves utilizing a DNA template with poly-T nucleotides to co-transcribe the poly-A tail amid IVT mRNA production, producing homologous mRNA products. Producing fixed and repeatable poly(A) length is a benefit of DNA template-encoded poly(A).[34] However, because of its propensity for recombination, its poly-A tail's length is shortened.[35]

Role of Structural Elements of mRNA Vaccines

Mature eukaryotic mRNA is composed of the 5' cap structure (m7GpppN or m7Gp3N (N can be any nucleotide)), the 5' untranslated region (5'UTR), an open reading frame (ORF), the 3' untranslated region (3'UTR), and a poly(A) tail. These

fundamental structural domains influence the stability, immunogenicity, and translation efficacy of mRNA vaccines.[36,37] An mRNA ORF determines the target protein's basic sequence as well as higher-order RNA structures that influence translation efficiency. Unexpectedly, mRNA coding regions capable of generating secondary structures were found to be associated with highly expressed mRNAs.[38]

The 5' UTR is essential for ribosome binding and serves as the location of protein translation preinitiation complex formation.[39] Moreover, according to a scanning model of RNA translation, mRNA stability, and translation efficiency are influenced by the 5' UTR sequence and secondary structures.[39–43] Even a 5' cap-independent protein expression pathway is possible due to the presence of an internal ribosome entry site (IRES) for the encephalomyocarditis virus in the 5' UTR.[44,45] Additionally, the primary function of the 5′ UTR is to translate its downstream ORF sequence.[46,47] To boost translation efficiency, the Kozak sequence is usually inserted next to the 5′ UTR sequence.[24,48]

Likewise, the role of the 3′ UTR is to keep mRNA stable.[49,50] Most eukaryotic mRNAs have 3' UTR mRNA degradation signals that govern the stability of mRNA. The presence of AU-rich regions in the 3' UTR of mRNA has been shown to aid in the cleavage of the poly (A) tail during mRNA degradation.[51,52] As a result, the half-life of mRNAs could be enhanced by replacing their AU-rich regions with 3'UTR sequences.[53] Furthermore, the iron-responsive elements (IREs) are another essential mRNA stability-regulating segment within the 3'UTR and control mRNA translation.[54]

With a few exceptions (like histone), all cellular proteins that encode mRNAs consist of a poly(A) tail.[55] Most actively translated mRNAs in mammalian cells have a poly(A) tail containing 100–250 adenosine residues.[56] Additionally, the poly-A tail is necessary for the stability of the mRNA, translation, and recognition by the poly-A binding protein (PABP), which joins with the translation initiation complex (eIF4G) to form a loop-like conformation.[55,57] The cytoplasmic translocation of mature mRNA is mediated by the poly(A) tail.[58] In addition, the poly (A) tail modulates translation efficiency and mRNA breakdown.[59–61] Additionally, a poly(A) tail with the proper length can increase mRNA stability and translation efficiency[55,56] (Figure 1).

Types of mRNA Technologies

To develop mRNA vaccines, conventional (non-replicating) mRNA (nrRNA) and self-amplifying mRNA (saRNA) have been proposed.[62–64] Between its 5' UTR and ORF, saRNA has additional virus replication components than nrRNA. Furthermore, alphaviruses, flaviviruses, measles viruses, or rhabdoviruses could be the sources of the viral replicase of saRNA. As a result, saRNA might produce a lot of antigen protein while activating the immune system quickly and effectively.[14,63–65] While viral genes holding information for replication machinery proteins are intact in saRNA, genes encoding therapeutic proteins replace those for structural proteins.[14,66] Additionally, saRNA vaccines can carry genetic material encoding the desired antigen in addition to other genes, such as viral RNA polymerase, which enables mRNA to multiply on its own.[67,68] Safe trans-amplifying RNA (taRNA) vaccines have been optimized and produced based on saRNA technology.[65] However, because of its longer length, saRNA delivery is more difficult than nrRNA.[69]

Non-replicating mRNA vaccinations only provide genetic information that codes for the target antigen.[70] Also, the use of a simple structure and shorter-length RNA molecule is one of the advantages of nrRNA vaccines. Furthermore, a modified or tweaked mRNA can have significantly increased its efficacy.[71] Conventional mRNA has several advantages over saRNA, including its smaller size (2–3 kb vs 10 kb), lack of viral genes, which reduces the risk of showing unwanted immunogenicity, its easiness and scalable manufacturing techniques, as well as the ease with which its sequence can be altered to improve therapeutic efficacy and minimize any unwanted effects.[14,70]

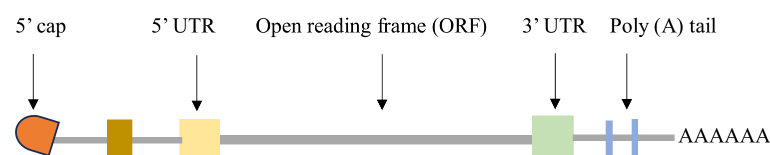

Figure 1 Structural elements of mRNA vaccines.

Challenges and Adverse Effects

Due to mRNA's physical properties such as high molecular weight, negative charge, vulnerability to ribonucleases (RNases),[15,21,70,72–76] and the presence of extracellular and intracellular barriers,[21,77] it is difficult for mRNA to be successfully uptaken into cells and translated to targeted antigens. Furthermore, after entry, enormous amounts of mRNA are ambushed in endosomes and cannot reach into the cytoplasm to perform its functions.[75,78,79] Furthermore, as indicated by a short half-life (5 min) in sera, IVT mRNA transcripts are unstable and extremely sensitive to nuclease destruction.[76,80]

Despite, immunogenicity is undesirable for several mRNA uses, such as protein replacement therapy and genome editing, it can be useful for vaccination techniques and may even replace the use of adjuvants.[37,81–84] Unmodified mRNA may activate innate immune systems through the endosomal recognition of pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs)[85–87] and the RIG-1- like receptor families, like RIG-1, MDA5, and LGP2, resulting in the production of pro-inflammatory cytokines and type I interferons.[15,88–90]

High frequencies of adverse reactions to vaccinations, including pericarditis, myocarditis, inflammation of the nervous system, and autoimmune hepatitis, had been associated with mRNA side effects.[91–94] Even though, studies suggested that these side effects were unrelated to the vaccine itself.[95] Moreover, in young adults and male adolescents who received BNT162b2 and mRNA-273 Coronavirus disease 2019 (COVID-19) vaccines, cardiomyopathy, myocarditis, and pericarditis were reported within a week of the second vaccination.[96,97] Furthermore, infarction, allergies, heart failure, and renal failure are a few possible side effects of mRNA vaccinations.[98] In addition, studies reported that the most common cause of acute myocarditis is a viral infection. For example, healthy people who have received smallpox or influenza vaccines have been known to manifest the adverse effect.[99]

According to studies, the bulk of adverse effects are caused by lipid nanoparticles (LNPs) ingredients like PEG and ionized lipids.[100,101] The necessity to improve the LNP delivery platform is highlighted by the reports of negative effects brought on by LNPs for mRNA-based COVID-19 vaccines.[102,103] PEG-lipids may cause allergic responses by activating the complement system.[75,104,105] Additionally, by hastening blood clearance, anti-PEG antibodies may cause fast systemic elimination of subsequently administered PEGylated nanoparticles.[104,105] In addition, rodents have been shown to suffer liver and lung damage as a result of LNP administration in vivo,[106,107] which could be explained by the delivery of LNP materials' cytotoxicity and the production of pro-inflammatory factors.[108,109]

How to Enhance the Stability and Suppress the Immunity of mRNA Vaccines?

The amount of mRNA that degrades can be considerably decreased by adding a 5'-cap, modifying nucleosides, adjusting 30-poly(A) tail length and structure, and optimizing nucleoside sequences.[36,110,111] To boost mRNA's stability and stop it from degrading, LNPs and alternative delivery systems like polymers, peptides, and cationic nano-emulsions (CNEs) can be used.[67,112] Moreover, mRNA-based vaccines can be lyophilized (freeze-dried), making them more stable and preserving their biological action.[113] Additionally, Kariko and colleagues were the first to change certain nucleosides (cytidine and uridine) with 5-methylcytidine and pseudouridine, respectively, to make the resulting mRNA molecules more stable intracellularly and less immunogenic.[37,114–116]

To overcome immunogenicity issues, achieve sustained stability, and achieve effective and precise protein production in vivo, chemical modifications to the mRNA molecule, such as cap structures and modified nucleosides, are essential.[70,117] In 2005, Karikó and colleagues revealed that mRNA generated with modified uridine might withstand immune system recognition and destruction, significantly improving mRNA stability and immunogenicity in vivo.[115] The uses of mRNA technology in the biomedical field are expanding because of improvements in delivery technology and the application of modified nucleosides to escape innate immune recognition.[21] By reducing the usage of uridine in the codons,[37,118–120] and altering the nucleotides used in IVT mRNA,[21,28,37,70,76,115,121,122] it is possible to block TLR recognition of mRNA. 5-methylcytosine (m5C), 5-methyluridine (m5U), 2-thiouridine (s2U), or pseudouridine (ψ) modifications such as 1-methyl pseudouridine (m1 ψ) are the most widely used alterations.[123] One of these changes, the substitution of pseudouridine for uridine, has been demonstrated to improve mRNA efficacy and decrease

immunogenicity.[39,124] In place of each uridine residue in the coding region and UTRs identified by the ribosome, the Pfizer/BioNTech BNT162b2 mRNA contains N1-methyl pseudouridine (m1ψ).[25,117,123]

By rigorously purifying mRNA using high-performance liquid chromatography (HPLC), which can eliminate the aberrant RNAs produced in the IVT reaction, immunogenicity could be further reduced.[37,125,126] The constituents of the LNPs were being altered by researchers to increase vaccine effectiveness and reduce side effects.[100,101,127] Furthermore, biodegradable lipids may be used to increase the biocompatibility of lipid nanoparticles.[128–132]

Purification of IVT mRNA

Abortive initiation products and double-stranded RNA produced by DNA-dependent RNA polymerases can trigger the production of type I IFN and inflammatory cytokines when PRRs bind to them.[133] DNase treatment can easily remove pDNA, however, several chromatographic methods are available to get rid of the remaining impurities.[134] While polyacrylamide gel electrophoresis can be used to remove short RNA moieties.[135] Chromatography, namely HPLC, is the sole method that can purge contaminants from longer mRNA preparations.[82] As a result, great translatability is made possible by mRNA purification via HPLC without inducing IFN1 and proinflammatory cytokine responses.[70,76,82,136] For large-scale mRNA production and Good Manufacturing Practice (GMP) procedures, purification using fast protein liquid chromatography (FPLC) or HPLC may be carried out.[82,137–140]

mRNA Vaccine Delivery

Viral and non-viral vector delivery techniques have been used to deliver mRNA vaccines.[141] To increase the safety and effectiveness of mRNA-based immunotherapy, delivery systems can be modified to offer tissue or cell specificity.[142] Bangham made the initial discovery of lipid-based systems in the 1960s when cationic LNPs (cLNPs), also known as liposomes, were seen to generate vesicles spontaneously in aqueous solutions.[143–146] Doxil®, a liposomal-formulated doxorubicin, was authorized in the US thirty years later. Since then, the FDA has approved several liposome[146] and LNP medications (comprising ionizable cationic lipids, iLNPs) for clinical use.[147,148]

To traverse membrane lipids and efficiently deliver RNA vaccines into the cytosol, a variety of vaccine delivery techniques (including protamine, LNPs, polymers, nanoemulsions, and cell-based administration) were optimized.[149] Therefore, both viral and non-viral delivery systems must be used to prevent RNases from degrading mRNA and to improve their intracellular effectiveness. Viral vector delivery system may result in the induction of immune response to the antibody, vector pre-existing immunity inhibiting transduction, potential safety issues with long-term expression, and risk of insertional mutagenesis.[15,150] Hence, in this review, non-viral delivery methods have been discussed in detail.

Protamine

Cell-penetrating peptides (CPPs) showed a good safety profile and effective transfection capabilities.[151–153] Peptides have also been employed for mRNA administration because they include cationic or amphipathic amine groups, such as arginine, that can electrostatically attach to negatively charged mRNA and form nano complexes.[154] Moreover, a cationic peptide called protamine can stop lysosomal degradation while RNA is being delivered. It has been demonstrated that protamine-based delivery activates TLR 7 to cause a potent immunological response.[155,156]

Protamine, an arginine-rich cationic peptide, can attach to mRNA and efficiently transfer it into the cytosol.[157] Hence, it was utilized in the development of the self-adjuvanted RNActive vaccination platform, which has proven effective against several infectious illnesses and malignancies.[115,156,158,159] Furthermore, protamine protects mRNA from being degraded by serum RNases[151] and is found to guard against severe storage conditions for the mRNA rabies vaccine.[160]

To elicit an immunological response against the rabies virus, CureVac investigated the protamine-mRNA combination in 2016. In that investigation, the mRNA encoding the non-replicating rabies glycoprotein (RABVG) was tailored to induce strong virus-neutralization in mice and domestic pigs.[158] A brand-new lipid/protamine/mRNA nanoparticle technology was recently developed and widely used for systemic tumor administration. In this technique, 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) liposomes have been tested to enclose protamine-complexed mRNA before being coated with 1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol) (DSPE- PEG) and DSPE-PEG-anisamide.[161]

Lipid Nanoparticles (LNPs)

Due to their ability to preserve mRNA from enzymatic cleavage, efficiently carry mRNA into cell cytoplasm,[93,129,162] have minimal immunogenicity, are biocompatible, and have a high encapsulation rate,[163] lipids have been used for exogenous mRNA administration. In addition, LNPs are lipid-based spherical vesicles and can self-assemble into precise structures like cell membranes.[164] Furthermore, LNPs are lipid-based, and nanoscale carriers that can effectively transfer mRNA intracellularly and safeguard it from RNAase during systemic circulation.[165] Moreover, LNPs are now the most cutting-edge method of administering mRNA vaccines.[129,149,166–169] (Figure 2)

Modifying the lipid structure[130] and particle surface has been regarded as a strategy to increase the efficient delivery of mRNA into the cytosol.[14,170] It was discovered that LNPs having multilamellar, faceted, and lamellar lipid phases have better mRNA transfection effectiveness.[171,172] N-[1-(2,3-dioleyloxy) propyl]-N,N,N trimethylammonium chloride (DOTMA), for example, was a first-generation permanently charged lipid that was toxic,[173] subpar, and relied on non-scalable methods. At an acidic pH (where the amino lipids were positively charged), in the presence of ethanol, ionizable amino lipids were employed to create nucleic acids.[174,175]

Eminently, LNP delivery materials are employed in the existing mRNA vaccines approved by FDA.[57,176] The numerous advantages of lipid-nanoparticle-based mRNA delivery systems, such as their high stability, transfection efficiency, efficacy, safety, and low-cost production techniques, have facilitated the rapid development of mRNA vaccines and medicines, providing a powerful disease-fighting tool.[177]

Components of LNP

LNPs are made up of a cationic or a pH-dependent ionizable lipid layer, a PEG component, phospholipids, and cholesterol.[14,165,178–183] The lipid-anchored PEG ensures vial and storage stability, the ionizable lipid is essential for cellular absorption and endosomal escape, enabling mRNA to enter into the cytosol, and the phospholipid and sterol are essential for the stabilization of the LNP.[182,184,185] The ratio of the components can be changed depending on the target tissue, and the lipid content can also be changed to alter the physical characteristics of LNPs like particle size, shape, encapsulation effectiveness, and surface charge.[186,187] Numerous encapsulation devices can be created using lipids and lipid-based nanoparticles, including liposomes, LNPs, microbubbles, micelles, lipid implants, and emulsions.[71,165,188–193]

Cationic Lipids

Cationic lipids either have a quaternary nitrogen atom that permanently gives them a positive charge or a primary amine that gives them a positive charge at or below physiological pH. However, it was discovered that cationic lipids with such a long-lasting positive charge were more toxic, less effective, and non-biodegradable.[17,194–197] The addition of structural lipids like DOPE may decrease cytotoxicity while increasing endosomal release. Among the earliest LNP preparations that have been successful in the in vivo translation of mRNA is Lipofectin, which is made by combining DOTMA and DOPE.[198–200] The systemic toxicity of this combination, however, made it ineffective.[201]

Whereas net positively charged complexes have been demonstrated to improve mRNA stability in vitro, cationic complexes may interact with negatively charged serum proteins, resulting in clumps, clots, and rapid clearance.[202–204]

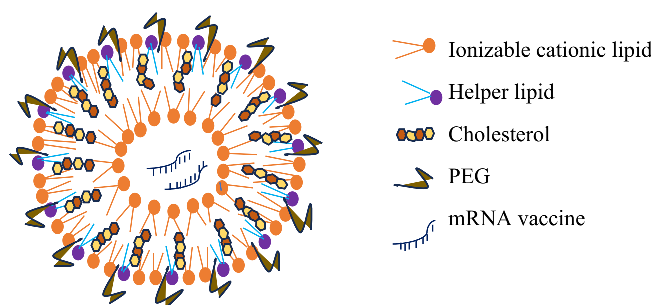

Figure 2 mRNA encapsulated in Lipid nanoparticle. PEG, Polyethylene glycol.

Although there are certain disadvantages to utilizing cationic lipids, the positive charge traps nucleic acids very well. This approach was used to create pH-sensitive ionizable cationic LNPs for enhanced RNA delivery.[194]

Ionizable Lipids

Cullis developed the first pH-responsive cationic lipid (ionizable lipids (ILs)) in the early 1990s, which gained a net positive charge in an acidic pH while remaining neutral in a physiological pH. The reticuloendothelial system (RES) cannot break down ILs because of their pH-sensitive characteristics, which prolongs their half-life.[205–207] ILs have polar head groups that contain ionizable amines, a hydrophobic tail that promotes self-assembly, and linkers that join the head groups to the hydrophobic tail. After being carried into an endosome, they are assumed to be ionized negatively once more upon acidification, which helps to build hexagonal phase structures and, eventually, makes it easier for mRNA to escape from the endosome and enter the cytosol.[70,129,179,201,208–213]

Nanoparticles made with ILs have a low positive charge density in the bloodstream, resulting in better biocompatibility and less off-target accumulation.[214] In the COVID-19 vaccinations, the cationic lipids employed are SM-102 and ALC-0315 in the Moderna and Pfizer/BioNtech vaccines, respectively.[215] Ionizable lipids' enhanced capacity for biodegradation Due to quick metabolic breakdown and clearance, there is less exposure to adjacent tissues and a reduction in inflammation at the injection site.[216] Additionally, ILs have a better safety profile since they are less likely to stimulate the immune system or interact with serum proteins.[17,78]

Biodegradable functional groups were employed in the subsequent synthesis of lipids to speed up clearance. The incorporation of ester moieties is one method of increasing biodegradability.[217–219] The incorporation of disulfide bonds into the backbone of lipids is another method of imparting biodegradability. Disulfide bonds are bio-reduced in the cell by glutathione (GSH) or other disulfide-reductases.[220] Further modifications to ionizable lipid structures result in altered physicochemical properties that can influence the selective delivery of mRNA to different tissues.[221]

Cholesterol

Cholesterol can improve particle stability and affects the efficacy and biodistribution of in vivo mRNA injection. C-24 alkyl phytosterols, for example, improved the delivery effectiveness of LNP mRNA in vivo.[171] By preventing excessive amounts of endogenous cholesterol from being sequestered inside LNPs while they are in circulation, cholesterol helps maintain stability.[194,222] When compared to standard cholesterol, nanoparticles containing a -sitosterol substitute increased endosomal entry of mRNA in the cytoplasm by a factor of ten.[170,171,223] Therefore, cholesterol and its derivatives play a crucial role in the general stability of LNPs in circulation and may facilitate endosomal escape, improving mRNA entry into the cytoplasm.[224]

Furthermore, cholesterol is required for LNP to transition from the lamellar to the hexagonal phase. The hexagonal phase is required for mRNA to be released from LNPs and transported over the endosomal membrane into the cytosol.[225] The fluid phospholipid bilayer was strengthened by cholesterol and LNPs' content loss was reduced.[226] When employed in the optimum concentration, it may also aid in the fusing of the membrane for LNPs and gene transfer.[227]

PEG

It has been widely used to layer PEG on lipid carriers to slow down aggregation and lengthen blood circulation.[228–231] Additionally, lipid-anchored PEGs primarily form a barrier on the LNP surface, sterically stabilizing the LNP and decreasing specific protein binding.[179] Moreover, PEGylated nanoparticles are widely referred to as stealth nanoparticles due to their ability to avoid opsonization by serum proteins and detection by the reticuloendothelial system (RES).[172,232–235] PEG-lipids restrict LNP uptake while decreasing opsonization by serum proteins and reticuloendothelial clearance, extending LNP circulation lifetime.[236] Furthermore, By supplying a hydrophilic exterior coating, PEG regulates the lipid nanoparticle in many ways, including nanoparticle formation, inhibiting nanoparticle aggregation, and extending particle blood circulation.,[104,194,237–241] and by avoiding their physical aggregation in solution, may potentially improve the LNPs' storage stability.[201,208]

It's also crucial to take into account the so-called PEG dilemma, which is the decreased fusogenicity of PEG lipids and may prevent mRNA from being released from endosomes. A practical method for effective mRNA intracellular

delivery is cleavable PEGylation.[237,241–245] PEG on LNP surfaces has the potential to trigger anti-PEG IgM antibodies to be induced, especially after repeated doses.[246] PEG-lipid, unlike other components of LNP, is engineered to eventually dissociate and shed the PEG to avoid the potential generation of PEG-specific antibodies that would cause rapid systemic clearance of successive doses of PEGylated nanoparticles via the accelerated blood clearance (ABC) phenomenon.[105]

Helper Lipids

Phospholipids, also known as helper lipids, are frequently utilized to give LNP structure, which enhances formulation stability and may facilitate endosomal escape.181,[182,247,248] Phospholipids, such as DSPC and DPPC, are typically neutral and provide bilayer structural stability to LNPs. Additionally, phospholipids contribute to the fusogenicity and biodistribution of LNPs.[247] Moreover, LNPs, which were incorporated in the mRNA-1273 and BNT162b, might have their structural integrity stabilized by the DSPC.[249–251] In addition, DOPE was employed for mRNA and siRNA administration in vivo as an alternative to DSPC since it could destabilize the endosomal members and encourage mRNA entry into the cytosol.[182] Endosomal escape is made possible by phospholipids' induction of a transition from lamellar to hexagonal endosomal architecture by disrupting the lipid bilayer.[252]

Polymers

Early gene delivery attempts relied heavily on poly (ethylene imine) (PEI), poly(L-lysine) (PLL), and poly (amidoamine) (PAMAM). Though PEI was the only polymer employed for mRNA delivery.[253] Although optimized PEI structures have high cationic charge density, they are toxic.[254] In 1987, polylysine (PLL) was announced as the first non-viral cationic polymer vector to efficiently transfect plasmid DNA.[255] Because of their high net positive charges and inability to dissolve in physiological conditions, they could cause harmful levels of bioaccumulation, raising concerns about their limited efficacy and potential toxicity.[224]

Several polycationic systems were used to enhance the entry of mRNA into the cytosol, including DEAE (diethylamino-ethyl)-dextran, DOPE (1,2-dioleoyl-3-phosphoethanolamine), poly L-lysine, PEI (polyethylene imine), and DOTAP.[36] Self-amplifying -mRNA nanoparticles were also delivered using chitosan and PEI.[256] Chitosan has several advantages, such as biodegradability, biocompatibility, and cationic charge that allows nucleic acid binding, but it also has disadvantages, such as poor water solubility and limited target capability.[164]

A polyethyleneimine copolymer (PVES) treated with vitamin E succinate is used in the self-assembled polymeric micelle delivery technique. When VE binds to PEI, a conjugated polymer capable of self-assembling into stable micelles is formed.[257,258] Charge-altering releasable transporters are originally positively charged polymers that can efficiently load mRNA and improve physical characteristics by degradative, charge-neutralizing intramolecular rearrangement, releasing functional mRNA, and translating protein in cells.[259]

PEI's toxicity and transfection effectiveness both rise as its molecular weight does. To address such limitations, different modifications to PEI have been researched, including ones that use polysaccharides and polyethylene glycol to boost biocompatibility and transfection effectiveness and wrap PEI in neutral or anionic liposomes to lessen non-specific adhesion.[260] Blakney et al created pABOL, a bioreducible, cationic polymer that improved transfection efficacy but not cytotoxicity at higher molecular weights.[261]

Nanoemulsions

The emulsions are often water-in-oil emulsions made of squalene, sorbitan trioleate, polysorbate 80, and DOTAP, much like the licensed MF59 adjuvant.[215] Above all, the main benefit of this platform is that MF59 is safe.[262,263] CNEs (cationic nanoemulsions) were proposed as a possible means of delivering nucleic acids in 1990.[264] Moreover, the presence of cationic lipids in the formulation is essential for nucleic acid complexation via electrostatic interactions, which also promotes nucleic acid stability and transfection efficiency while protecting them from nuclease degradation.[265] Anderluzzi et al discovered that CNE induced the highest number of antibodies against rabies when compared to DOTAP polymeric nanoparticles, DOTAP liposomes, and DDA liposomes.[266]

Moreover, Gennova Biopharmaceuticals Ltd in conjunction with HDT Biotech Corporation developed a lipid inorganic nanoparticle, which is called LION®, for the delivery of SARS-CoV2 vaccine candidate HGCO10 (self-

amplifying RNA). Genova reported LION ® is a very stable cationic lipid (DOTAP)-squalene emulsion akin to CNEs with 15 nm superparamagnetic iron oxide (Fe_3O_4) nanoparticles (SPIO) implanted in the hydrophobic oil phase (which offers therapeutic and imaging functions). When stored between 4° and 25 °C, this formulation was found to have colloidal stability for at least three months.[267,268]

Ex vivo Loading of mRNA to Dendritic Cells

This method can be accomplished using either mild electroporation[269] or lipid-derived carriers.[270] By reducing potential off-target effects, electroporation can boost mRNA transport to the target cells, resulting in a reduction in the amount of mRNA that is required.[166,271,272] Additionally, this strategy is generally employed for cancer immunotherapy since the majority of ex vivo loaded dendritic cells demonstrate cell-mediated immunity.[273] Nanoparticle formulations were required to improve dendritic cell targeting.[274]

Dendritic cells used in cancer immunotherapy may be transfected with total tumor RNA or tumor-associated antigens (TAAs) encoding mRNA.[272] Yet, the drawbacks of this strategy include a lack of known TAAs for various malignancies, and selecting TAAs may be difficult because not all recognized TAAs generate antitumor immunity. Furthermore, TAA mRNAs were found to induce antitumor immunity in experimental studies.[275]

Entry, and Endosomal Escape of Nanoparticle Construct of mRNA

The internalization of RNA-loaded lipid-based nanoparticles which involves endocytosis, micropinocytosis, macropinocytosis, and phagocytosis is mediated by caveolae and clathrin.[184,276–280] Because clathrin-mediated uptake is thought to be faster than caveolin-mediated uptake, targeting the caveolin pathway should result in more effective delivery and more time for the drug to escape endosomes than clathrin-mediated uptake, which can cause significant buildup in late endosomes and lysosomes.[281,282] With the aid of nanoparticles, several processes are required for mRNAs to enter the cytoplasm, including endocytosis, lysosomal escape, and mRNA release.[162] The availability of mRNA in the cytoplasm may be increased by stimulating endosomal escape and scavenger receptor activation to increase mRNA absorption.[73]

In the endosomes, nanoparticles undergo a pH gradient, beginning with neutral extracellular pH (7.4) and moving to gradual acidification in early endosomes (pH 6.3), late endosomes (pH 5.5), and finally lysosomes (pH 4.5).[283] Following cellular uptake, mRNA must escape endosomes to reach the cytosol (pH 7.2) for mRNA translation, which is a limiting step for productive mRNA delivery. For example, only 1–2.5% of mRNA was detected in the cytosol after transfection of human epithelial cells with mRNA, and this varies by cell type.[129,184,284,285] Additionally, the methods utilized to avoid this terminal degradation rely on units that are activated by acidic pH.[283,286]

Furthermore, ionizable units and/or fusogenic lipids in mRNA nanocarriers destabilize the endosomal membrane, enabling mRNA to enter into the cytosol.[285] As a result, endosomal escape, which has a high association with transfection effectiveness, is another crucial step in the delivery of mRNA to ribosomes.[287] Three scenarios are widely accepted among numerous techniques to induce nanocarrier endosomal escape: (1) destabilization of the endosomal membrane, (2) osmotic rupture of the endosomes via the "proton sponge" effect, and (3) endosome rupture via particle swelling. Moreover, distinct nano constructs use distinct pathways, such as pH-responsive endosomal escape and proton sponge effect.[288,289]

In addition, pH-responsive endosomal escape results from conformational changes brought on by protonation or the breakdown of a polymer link at endosomes.[290] Endosomal escape may be facilitated by interactions between cationic lipids and the negatively charged endosomal membrane. Following the protonation of its head group under acidic conditions, DOPE turns fusogenic, causing the formation of a hexagonal (HII) phase and momentarily destabilizing the endosomal membrane. The proton sponge effect, in which endocytosed polyplexes produce osmotic swelling of the endosome due to proton influx and eventually rupture the endosome, is hypothesized to be how cationic polyplexes undertake endosomal escape.[291] Flow cytometry appears to be the most efficient and informative tool for studying the cellular uptake and trafficking of nanoparticles.[292] Using nanoparticles that have been fluorescein-labeled, different information about particle localization on the cell surface, inside the cell, or into the acidic compartment, where the acidic pH quenches fluorescein's fluorescence, can be learned.[293,294]

Routes of Administration of mRNA Vaccines

The method of administration and formulation of mRNA vaccines have a crucial role in regulating the rate and amount of antigen expression as well as the effectiveness of the immune response.[34,291] The route of administration can have a considerable impact on the organ distribution, expression kinetics, and therapeutic effects of LNP-mRNA formulations.[165,295–298] The most popular routes of mRNA vaccines administration are intramuscular (IM), subcutaneous (SC), intradermal (ID), and intravenous (IV).[10,17,70,165,253,291,299–301]

The highest amount of encoded protein synthesis in the body can be achieved with IV injections of mRNA therapies. Moreover, the liver is typically the target of intravenous mRNA therapies, which effectively transfect endothelial, Kupffer, and hepatocyte cells.[17,75,302] It is possible to generate adaptive immune responses from IV–injected mRNA vaccines by transfecting the spleen as a site of transfection.[274,303] Some of the disadvantages of I, V administration include impediments to vaccine transport in the bloodstream caused by plasma proteins, enzymes, and mechanical forces.[304] Additionally, systemic adverse effects such as spleen damage and lymphocyte depletion may be brought on by the mRNA and its delivery vehicles.[21] LNP-mRNA vaccination IV injections are less frequent due to the possibility of systemic side effects. Infusing immunogenic material into the bloodstream may cause a cytokine storm, or the overwhelming synthesis of cytokines, which can result in shock and death.[305]

The most popular method of administering vaccines to patients is through intramuscular injection.[306,307] After IM injection, the LNPs are efficiently taken up by the myocytes before the cytoplasmic release of the mRNAs for S protein translation.[308] IM injection allows for a higher volume to be injected than the ID and SC routes, which may result in fewer unpleasant injection site reactions but increased systemic absorption.[130] In addition, SC injection-based mRNA vaccines allow for a relatively higher injection volume, which minimizes pressure and pain at the injection site.[309] However, one downside of SC injection is that the rate of absorption is slow, and inadvertent mRNA destruction may occur.[309,310] Furthermore, with mRNA-LNP vaccines, the intradermal (ID) method of delivery has been found to successfully produce a Th1-type immune response and cytotoxic T-cell activation.[311,312] According to certain studies, IM and ID delivery of LNP-mRNA vaccines led to longer-lasting protein expression than IV.[70,313,314]

Therapeutic protein augmentation in certain organs, such as heart,[315,316] eyes,[317–319] and brain, [320,321] is made possible by local injection of LNP-mRNA compositions. Additionally, immune stimulator-coding LNP-mRNA formulations can be injected directly into cancer tissue by intratumoral injection.[322–325] It has been noted that the intranodal (IN) injection of naked mRNA-encoding antigens causes a strong T-cell response.[326]

Utilizing the potential of mucosal immunity, intranasal (IN) vaccine delivery to the mucosal layers, like the nasal and pulmonary mucosa, is a practical, noninvasive method of vaccine administration.[253,289,327,328] Pathogen-specific antibodies that are produced in the mucus via mucosal vaccination can neutralize pathogens at the earliest stages of infection.[329] Furthermore, mucosal delivery of mRNA vaccines can result in the release of immunoglobulin A (IgA), which can neutralize bacterial toxins and viruses.[330] M cells move the LNPs from the nasal epithelium to the underlying nasal-associated lymphoid tissue, which is home to significant numbers of B cells, T cells, and DCs.[327]

Stability, and Storage of mRNA Vaccines

A cold chain is often required for vaccine storage and shipping, but the supply chain for mRNA vaccines may require an even colder cold chain[331] than the conventional vaccines which can commonly be stored at 4–8°C.[332] Spikevax and Comirnaty, two currently licenced COVID-19 mRNA vaccines, require storage temperatures of −20°C and between −80°C and −60°C, respectively.[333]

Inadequate mRNA storage can lead to chemical instability through reactions including oxidation and hydrolysis, changing the physical properties of the therapeutic product and perhaps its functionality.[334,335] Furthermore, pH, buffer composition and concentration, metal cation presence, non-viral vector formulation composition, and physiochemical properties all have a major impact on stability.[333] Currently, a cryoprotectant is used to store LNP-mRNA medications and vaccines for an extended time to avoid aggregation. Additionally, non-permeable cryoprotectants like sucrose and trehalose are used to permit vitrification of the surrounding aqueous solution.[336,337]

For mRNA-based vaccines, lyophilization, also known as freeze drying, is a common alternative storage technique that may support long-term stability at higher temperatures.[338,339] Lyophilized mRNA-LNPs were stable for 6 months at

4°C and 3 months at room temperature.[340] An interim ultra-cold chain storage device called Cryo-Vacc, created by the South African company Renergen, has been developed.[66] Because lyophilization is an expensive, time-consuming, and high-energy process, additional drying techniques including spray drying and supercritical drying should also be investigated.[201]

According to a study on the long-term storage conditions of mRNA-loaded lipid materials by Zhao et al, lipid-like nanoparticles (mRNA-LLNs) maintained in an aqueous solution undergo size changes and lose efficacy in vivo.[341] In comparison, freeze-dried mRNA LLNs kept their efficiency after being lyophilized with a 5% cryoprotectant solution and switched the preferential organ absorption from the liver to the spleen after being lyophilized with a 20% cryoprotectant solution.[289]

Application of mRNA Vaccines

mRNA-based medicines are projected to be effective treatments for a wide range of refractory disorders, including infectious diseases, metabolic genetic diseases, cancer, cardiovascular and cerebrovascular diseases, and others.[342] mRNA vaccines have been extensively researched over the last two decades for infectious disease prevention as well as cancer prophylaxis and therapy.[70,343]

The delivery of tumor-associated antigens (TAAs) expressing mRNA is the most fundamental application of mRNA vaccines in oncology.[343,344] In addition to being utilized in cellular therapies to ex vivo transfect patient-derived cells before reinserting transfected cells into patients, mRNAs may be employed therapeutically to immunize patients. The TAA of interest is expressed by patient-derived DCs after they have been transfected with the mRNA encoding it, and TAA-derived peptides are then presented to stimulate antigen-specific T cells in vivo.[345,346]

Prophylactic or therapeutic mRNA vaccines against infectious illnesses could be produced. mRNA vaccines that express an infectious pathogen's antigen elicit both strong and powerful T cell and humoral immune responses.[20,70,163,347] Vaccines are made from in vitro transcribed mRNAs encoding viral antigens, whereas immunotherapy is made from mRNAs encoding antibodies or immune modulators. Because of their interactions with cellular RNA sensors such as Toll-like receptors (TLRs), PKR, and RIG-I, some structural characteristics of mRNA have been identified as immunostimulatory.[348–350] The extraordinarily rapid development of mRNA vaccine candidates for the recent global COVID-19 pandemic highlights its clinical value.[351]

In pre-clinical and clinical investigations, IVT mRNAs are being examined to supplement missing or faulty proteins caused by hereditary diseases, or where the delivered protein could have a therapeutic effect. Among the studies are the use of IVT mRNA to cure hepatic disorders,[352] regenerate cardiac tissues,[353] and generate human stem cells[354] (Table 1).

Future Direction and Conclusions

Even though mRNA vaccines may be quickly made with commonly available materials and are relatively safer, there are still numerous difficulties. Bio-incompatibility, ineffective targeted delivery, poor transfection efficiency, immunogenicity, and instability are still a problem. Besides modification of the structure of the mRNA molecule, much emphasis has to be given to the delivery systems too.

Most of the adverse events observed from mRNA vaccines were also reported from RTS, S malaria vaccine, and other vaccines were also shared by other vaccines.[356] Therefore, as far as mRNA vaccines are the current choice of vaccine development, further research is required to optimize the in vitro transcribed RNA vaccine and delivery materials, notably lipid nanoparticles, to address the aforementioned difficulties. Use of biodegradable lipids, changing (optimization) of the four components of LNPs, use of effective purification techniques, chemical modifications to the mRNA molecule, such as cap structures and modified nucleosides, choice of appropriate delivery materials, use of cryoprotectants and lyophilization technique, and appropriate implementation of cold chain requirements of mRNA vaccines are among the strategies to increase the effectiveness of mRNA vaccines.

Abbreviations

PEG, Polyethylene-glycol; cLNPs, Cationic Lipid Nanoparticles; CNEs, Cationic nano-emulsions; CPPs, Cell-penetrating peptides; CTLs, Cytotoxic T lymphocytes; EUA, Emergency Use Authorization; HPLC, high-performance

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Table 1 Examples of mRNA vaccines (candidates) for infectious diseases, cancer, and other disorders currently in clinical trials.[355] (as of July 14, 2023)

Clinicaltrials.gov Identifier (NCT Number)	Name of the mRNA Vaccine	Condition	Delivery System (Route of Administration)	Phase	Status	Sponsor
NCT04847102	SARS-CoV-2 mRNA Vaccine/ ARCoV-005	SARS-CoV-2	LNP (IM)	III	Recruiting	Walvax Biotechnology Co., Ltd.
NCT03164772	BI 1361849 (formerly CV9202)	Metastatic Non-small Cell Lung Cancer	NI (ID)	I/II	Completed	Ludwig Institute for Cancer Research
NCT04978038	mRNA-COVID19-D3-2021	SARS-CoV2 Infection	NI (IM)	IV	Not yet recruiting	Mark Loeb
NCT05144139	COVID-19 mRNA vaccine (SWC002)	Covid-19	NI(IM)	I/II	Completed	Stemirna Therapeutics
NCT05639894	RSV mRNA LNP CL-0059 and RSV mRNA LNP CL-0137	Respiratory Syncytial Virus Infection	LNP (IM)	I/II	Active, not recruiting	Sanofi Pasteur, a Sanofi Company
NCT00833781	mRNA-transfected autologous dendritic cells	HIV-1 Infection	NI(ID)	I/II	Completed	Massachusetts General Hospital
NCT00204516	mRNA coding for melanoma associated antigens	Malignant Melanoma	NI(SC)	I/II	Completed	University Hospital Tuebingen
NCT05823974	GSK4382276A	Influenza, Human	NI(IM)	I/II	Recruiting	GlaxoSmithKline
NCT05526066	ARCT-810	Ornithine Transcarbamylase Deficiency (OTCD)	LNP (IV RECRUITING)	II	Recruiting	Arcturus Therapeutics, Inc.
NCT05650554	MRT5413	Influenza Immunization	NI(IM)	I/II	Active, not recruiting	Sanofi Pasteur, a Sanofi Company
NCT04852861	BNT162b2	Covid19	LNP (IM)	IV	Completed	Sciensano
NCT05079633	mRNA-1273	Covid19	LNP (IM)	IV	Active, not recruiting	National Taiwan University Hospital
NCT01446731	mRNA transfected DC	Prostatic Neoplasms	• (Direct injection of DCs)	II	Completed	Inge Marie Svane
NCT05939648	LVRNA021	SARS-CoV-2	NI (IM)	II	Not yet recruiting	AIM Vaccine Co., Ltd.
NCT04382898	BNT112	Prostate Cancer	LPX (IV bolus)	I/II	Recruiting	BioNTech SE
NCT00204607	Stabilized Tumor mRNA	Malignant Melanoma	NI(ID)	I/II	Completed	University Hospital Tuebingen
NCT05127434	mRNA-1345	Respiratory Syncytial Virus	NI(IM)	II/III	Recruiting	ModernaTX, Inc.
NCT04232280	mRNA-1647	Cytomegalovirus Infection	Lyophilized (IM)	II	Completed	ModernaTX, Inc.
NCT04159103	mRNA-3927	Propionic Acidemia	LNP(IV)	I/II	Recruiting	ModernaTX, Inc.

Abbreviations: NI, No Information; LNP, Lipid nanoparticle; LPX, Lipoplex, DC, Dendritic cell; IM, Intramuscular; ID, Intradermal; IV, Intravenous; SC, Subcutaneous.

liquid chromatography; IRES, Internal ribosome entry site; IREs, iron-responsive elements; IRES, Internal Ribosome Entry Site; IVT, In Vitro Transcription; LNPs, lipid nanoparticles; mRNA, Messenger Ribonucleic Acid; nrRNA, non-replicating mRNA; PABP, poly-A binding protein; pDNA, plasmid DNA; PRRs, Pattern-Recognition Receptors; saRNA, self-amplifying mRNA; TLRs, Toll-like receptors; VLPs, virus-like particles.

Disclosure

The authors declared that they have no competing interests in this work.

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